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Tese

Atividade antibacteriana de extrato de butiá (*Butia odorata* Barb. Rodr.): mecanismo de ação, aplicação em sistema alimentar e em biofilme bacteriano

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Pelotas, 2019

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Resumo

Maia, Darla Silveira Volcan**. Atividade antibacteriana de extrato de butiá (*Butia odorata* Barb. Rodr.): mecanismo de ação, aplicação em sistema alimentar e em biofilme bacteriano.** 2019. 108f. Tese (Doutorado em Ciência e Tecnologia de Alimentos) – Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, 2019.

As bactérias transmitidas por alimentos constituem um perigo à saúde humana. Consequentemente, o interesse por novos antimicrobianos é crescente. Os objetivos deste estudo foram avaliar a atividade antibacteriana do extrato de *Butia odorata* (EBO), caracterizá-lo quimicamente, e estudar o seu mecanismo de ação contra *Staphylococcus aureus* e *Escherichia coli*, utilizados como modelos de bactérias Gram-positiva e Gram-negativa, respectivamente. Além disso, objetivou-se utilizar o EBO em queijo experimentalmente contaminado com *E. coli* e estudar o efeito do EBO nas células de *S. aureus* em biofilme. O teste de difusão em ágar demonstrou que todas as cepas testadas, tanto Gram-positivas como Gram-negativas (*Listeria monocytogenes*, *S. aureus*, *E. coli* O157:H7 e *Salmonella* Typhimurium) foram inibidas (zonas de inibição de 30±2,0 mm, 22±0,8 mm, 16±3,9 mm e 15±3,2 mm, respectivamente). A concentração inibitória mínima (CIM) variou de 4 a 8 $mg.mL^{-1}$ e a concentração bactericida mínima (CBM), de 16 a 33 $mg.mL^{-1}$. Quanto à caracterização química, os principais compostos presentes no EBO foram o Z-10-Pentadecenol (80,1%) e o ácido Palmítico (19,4%). Em relação ao mecanismo de ação, o EBO danificou a membrana celular bacteriana de *S. aureus* e de *E. coli*, como pode ser visualizado na microscopia eletrônica de varredura (MEV) e na microscopia confocal de varredura a laser (MCVL), resultando em saída de DNA para o ambiente extracelular. A análise de espectrofotometria de fluorescência demonstrou que o EBO causou danos ao DNA de *S. aureus* e de *E. coli*. No queijo contaminado experimentalmente com isolado de *E. coli*, houve uma diferença significativa ($p<0,05$) entre as amostras controle (2,8 log $UFC.cm^{-2}$) e as amostras tratadas com a CIM, 2 x CIM, 4 x CIM e 8 x CIM (1,3, 1,4, 1,6 e 0,5 log $UFC.cm^{-2}$, respectivamente). Em relação ao efeito do EBO nas células do isolado de *S. aureus* em biofilme, utilizando-se 4 x CIM houve uma diferença significativa ($p<0,05$) entre o controle e as amostras tratadas em todos os tempos avaliados (15, 30 e 60 min), sendo que com 30 e 60 min de contato houve redução de 99,9% no número de células do biofilme. Com 60 min de contato e adição de 2 x CIM houve um decréscimo de 4,21 log $UFC.cm^{-2}$ (99,99%) em relação ao controle, e adicionando a CIM a diferença foi de 1,1 log $UFC.cm^{-2}$ (90%). O EBO possui atividade antibacteriana contra micro-organismos patogênicos veiculados por alimentos, tendo potencial para ser utilizado como alternativa aos conservantes sintéticos. Além disso, este estudo demonstrou que a atividade antibacteriana do EBO contra *S. aureus* e *E. coli*, usados como modelos de bactérias Gram-positiva e Gram-negativa, ocorre por duplo mecanismo; causa danos na membrana bacteriana e no DNA genômico. O EBO, consistindo principalmente de Z-10-Pentadecenol e ácido palmítico, apresentou atividade antibacteriana no queijo mussarela fatiado contaminado experimentalmente com *E. coli*, indicando que pode ser uma alternativa para inibir o desenvolvimento deste micro-organismo nesse tipo de alimento. Além

disso, o EBO reduziu o número de células de *S. aureus* em biofilme, tendo potencial para ser utilizado como sanitizante.

Palavras-chave: conservante alimentício; DNA; integridade da membrana; planta; sanitizante

Abstract

Maia, Darla Silveira Volcan**. Antibacterial activity of butiá (*Butia odorata* Barb. Rodr.) extract: mechanism of the action, application in food system and on bacterial biofilm.** 2019. 108f. Tese (Doutorado em Ciência e Tecnologia de Alimentos) – Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, 2019.

Foodborne bacteria constitute a danger to human health. As a result, interest in new antimicrobials is increasing. The aim of this study was to evaluate the antibacterial activity of *Butia odorata* extract (BOE) against selected foodborne pathogens, characterize it chemically, investigate the mechanism of the action in *Staphylococcus aureus* and *Escherichia coli*, used as Gram-positive and Gram-negative bacterial models, as well as apply the BOE in sliced mozzarella cheese contaminated experimentally with *E. coli* and study the effect of BOE on *S. aureus* cells in biofilm. According to agar disc diffusion test all bacterial strains tested, both Gram-positive and Gram-negative bacteria (*Listeria monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *Salmonella* Typhimurium) are susceptible to the BOE (inhibition zone of 30±2.0 mm, 22±0.8 mm, 16±3.9 mm and 15±3.2 mm, respectively). The minimum inhibitory concentration (MIC) varied of 4 to 8 $mg.mL^{-1}$ and minimum bactericidal concentration (MBC) of 16 to 33 $mg.mL^{-1}$. Regarding to chemical characterize, the major compounds present in the extract were Z-10-Pentadecenol (80.1 %) and Palmitic acid (19.4 %). In relation to the mechanism of action, BOE caused cell membrane damage, which can be viewed in scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM), resulting in release of DNA to the extracellular environment. The fluorescence spectrophotometry analysis showed that BOE caused *S. aureus* and *E. coli* DNA damage. In cheese contaminated experimentally with *E. coli* isolate, there was significant difference ($p<0.05$) between control (2.8 log $CFU.cm^{-2}$) and treated samples with MIC, 2 x MIC, 4 x MIC and 8 x MIC (1.3, 1.4, 1.6 and 0.5 log $CFU.cm^{-2}$, respectively). Regarding the effect of EBO on *S. aureus* isolate cells in biofilm, when 4 x MIC was used, there was a significant difference ($p<0.05$) between control and treated samples at all evaluated times (15, 30 and 60 min), and at 30 and 60 min there was a reduction of 99.9% in the number of biofilm cells. At 60 min of contact and addition of 2 x MIC there was a decrease of 4.21 log $CFU.cm^{-2}$ (99.99%) in relation to the control, and with addition of MIC value the difference was 1.1 log $CFU.cm^{-2}$. BOE showed antibacterial activity against foodborne microorganisms, having potential to be used as alternative to synthetic preservatives. In addition, this study demonstrated that the mechanism of action of BOE against *S. aureus* and *E. coli*, used as models of Gram-positive and Gram-negative bacteria, occurs by dual mechanism, causing damage in the bacterial membrane and in the genomic DNA. BOE, consisting mainly of Z-10-Pentadecenol and Palmitic acid, showed antibacterial activity in sliced mozzarella cheese contaminated experimentally with *E. coli*, indicating that it can be an alternative for inhibit the growth of this microorganism in sliced cheese. In addition, BOE reduced the number of *S. aureus* biofilm cells, havi the potential to be used as a sanitizer.

Key-words: DNA; food preservative; membrane integrity; plant; sanitizer

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Sumário

1 Introdução

As doenças transmitidas por alimentos (DTA) resultam da ingestão de alimentos ou água contaminados com micro-organismos ou produtos químicos, sendo um problema de saúde pública crescente em todo o mundo. A contaminação dos alimentos pode ocorrer em qualquer fase do processo, desde a produção até o consumo (WHO, 2018). No Brasil, nos últimos dezessete anos, foram registrados mais de 12.000 surtos de DTA, sendo as bactérias responsáveis por 92,2% destes eventos. Dentre estas, *Salmonella* spp., *Escherichia coli* e *Staphylococcus aureus* são os principais agentes etiológicos envolvidos em surtos de DTA (BRASIL, 2018).

Considerando que as bactérias transmitidas por alimentos constituem um perigo à saúde humana, é crescente o interesse por novos antimicrobianos, bem como pelo mecanismo pelo qual essas substâncias agem (CLEMENTE et al., 2016). Além disso, há uma preocupação por parte dos consumidores sobre a segurança dos alimentos contendo conservantes sintéticos (ALZOREKY; NAKAHARA, 2003). Esse contexto impulsionou o desenvolvimento de pesquisas avaliando o potencial antimicrobiano de extratos de plantas (CHEN et al., 2018; LI et al., 2016; SUN et al., 2018).

Butia odorata Barb. Rodr. é uma fruta nativa da América Latina que vem sendo estudada quanto a sua composição, potencial tecnológico e bioatividade (BESKOW et al., 2015; CRUXEN et al., 2017; FERRÃO et al., 2013). Em pesquisas conduzidas recentemente, o extrato de *B. odorata* demonstrou atividade antibacteriana contra bactérias de importância em alimentos (HAUBERT et al., 2018; MAIA et al., 2017). Contudo, o mecanismo pelo qual ocorre a inibição, se esses resultados são reproduzidos *in situ* e, se o extrato apresenta atividade antibacteriana quando as células bacterianas estão organizadas na forma de biofilme, ainda não foram estudados.

Dessa forma, os objetivos deste estudo foram avaliar a atividade antibacteriana do extrato de *B. odorata* (EBO), caracterizá-lo quimicamente, e estudar o seu mecanismo de ação contra *S. aureus* e *E. coli*, utilizados como modelos de bactérias Gram-positiva e Gram-negativa, respectivamente. Além disso, objetivou-se utilizar o EBO em queijo experimentalmente contaminado com *E. coli* e estudar o efeito do EBO nas células de *S. aureus* em biofilme.

Artigo 1

Use of plant extracts to control bacterial foodborne pathogens

Capítulo publicado no livro *Antimicrobial research: Novel bioknowledge and educational programs (A. Méndez-Vilas, Ed.).*

Use of plant extracts to control bacterial foodborne pathogens

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Foodborne illness is a common public health problem, affecting the economic development of countries. In the United States of America (USA) it is estimated that 39% of foodborne illness are caused by bacteria, of which non-typhoid *Salmonella*, *Clostridium perfringens*, and *Campylobacter* are the main pathogens involved. In the year 2014 the European Union (EU) reported that 20% of foodborne outbreaks were caused by *Salmonella*, 16.1% by bacterial toxins, and 8.5% by *Campylobacter*. To ensure the production of safe food, synthetic chemical additives are traditionally used. However, natural compounds with preservative activity, known as green additives, have gained prominence in the field of food science as an alternative to traditional additives. Among these antimicrobials, plant extracts have demonstrated antimicrobial effect *in vitro* and *in situ* against foodborne pathogens. Several reports examine bioactive compounds with antimicrobial properties usually present in plants, such as phenolics, alkaloids, terpenoids, and saponins. In this chapter we will discuss the antimicrobial activity of plant extracts, the main subclass of active compounds responsible for the antibacterial effect and the application of these extracts in foods. We have analysed and critically discussed around 70 articles covering the latest improvements in the field.

Keywords: foodborne illness; synthetic preservatives; natural compounds

1. Introduction

Foodborne diseases are a serious global health problem. Each year, foodborne diseases cause about 600 million cases of illness, and 420,000 of these people die, including 125,000 children under 5 years of age [1]. This is not just a problem in the underdeveloped world. In the USA, 864 food-borne outbreaks were reported in 2014, resulting in 13,246 cases of illness, 712 hospitalizations, 21 deaths, and 21 food recalls [2]. In 2015, a total of 4,362 foodborne outbreaks were reported in the EU, resulting in 45,874 cases of illness, 3,892 hospitalizations and 17 deaths [3].

Microbial, chemical or physical agents can cause food-borne illness when ingested. Over recent years, bacterial foodborne agents have been the most thoroughly investigated and monitored causes of intestinal infectious disease. Throughout the last decade, *Escherichia coli, Salmonella* spp., *Listeria monocytogenes*, and *Campylobacter* spp. have persisted, commanding the most research and surveillance attention from government agencies and the most awareness from the food industry. These bacterial pathogens together constitute the greatest burden of foodborne illness for which the etiology is known. Examples of food-borne pathogens, clinical manifestations and associated foods are shown in Table 1 and will be addressed below.

Synthetic chemical additives are traditionally used to control the multiplication of foodborne pathogens [4]. Although these antimicrobials are approved for use in many countries, the recent trend has been the use of natural preservatives due to the adverse effects of chemical additives [5]. In addition, consumer awareness of the problems associated with synthetic additives has generated a profile of "green" consumers who require the absence of additives in foods [4]. In order to find alternatives to synthetic chemical additives, research studies on natural antimicrobials have appeared, and among these, in this chapter, plant extracts stand out. Compounds from plants are divided into two categories: the products of the primary metabolism, such as carbohydrates, proteins, and fats; and the secondary metabolites, such as phenolics, quinones, alkaloids, and terpenoids. Many of these metabolites are involved in plant defense mechanisms against insect and microorganism attack [6], and some secondary metabolites have shown antimicrobial activity [7].

2. Bacterial foodborne pathogens

2.1 *Escherichia coli*

There are several different categories of diarrheiagenic *E. coli,* and the most important or most studied are enterohemorrhagic (EHEC), enterotoxigenic (ETEC), and enteropathogenic (EPEC) *E. coli*. There are three other categories, including enteroinvasive (EIEC), enteroaggregative (EAEC), and diffusely adherent (DAEC) *E. coli* [8]. The enterohemorrhagic *E. coli* (EHEC) is capable of causing watery or bloody diarrhea, the latter termed hemorrhagic colitis (HC), and hemolytic-uremic syndrome (HUS). *Escherichia coli* serotype O157:H7 is the most well-known EHEC strain and can produce Shiga toxin (Stx), hence, it is known as a type of Shiga toxin-producing *E. coli* (STEC). *Escherichia coli* O157:H7 can be transmitted to humans through contaminated food and water, directly between persons, and through contact with animals or their environment. Cattle are the main reservoir of *E. coli* O157:H7, and ground beef is the most frequently identified vehicle of transmission to humans [9, 10, 11].

2.2 *Salmonella* spp.

The genus *Salmonella* belongs to the family *Enterobacteriaceae*, whose members are Gram-negative, facultative anaerobic, straight rods. Within the genus *Salmonella*, currently three species (*enterica*, *bongori*, and *subterranea*) and six subspecies (*arizonae*, *diarizonae*, *enterica*, *houtenae*, *indica*, and *salamae*) are differentiated [12, 13, 14]. There are more than 2600 serovars belong to *Salmonella enterica* subsp. *enterica* [15].

Bacteria of the genus *Salmonella* usually live in the intestinal tract of various warm- and even coldblooded animals and humans [16]. *Salmonella* infection usually occurs when a person eats food contaminated with the feces of animals or humans carrying the bacteria. Clinical salmonellosis caused by nontyphoidal salmonellae in humans is commonly accompanied by diarrhea, abdominal cramps and fever [17]. Illness has been linked to a wide range of food items including eggs, chicken, beef, pork, salad, vegetables, and dairy products, and other risk factors including overseas travel [18, 19].

Table 1 Food-borne disease-causing pathogens that frequently cause illness worldwide.

Organism	Onset time after ingesting	Signs and symptoms	Duration	Food sources
Escherichia coli producing toxin	1-3 days	Watery diarrhea, abdominal cramps, some vomiting	3-7 or more days	Water or food contaminated with human feces
Escherichia coli O157:H7	1-8 days	Severe (often bloody) diarrhea, abdominal pain and vomiting. Usually, little or no fever is present. More common in children 4 years or younger.	5-10 days	Undercooked beef (especially hamburger), unpasteurized milk and juice, raw fruits and vegetables, and contaminated water
Listeria monocytogenes	9-48 hours for gastro-intestinal symptoms, 2-6 weeks for invasive disease	Fever, muscle aches, and nausea or diarrhea. Pregnant women may have mild flu-like illness, and infection can lead to premature delivery or stillbirth. The elderly or immunocompromised patients may develop bacteremia or meningitis.	Variable	Unpasteurized milk, soft cheeses made with unpasteurized milk, ready-to-eat deli meats
Salmonella spp.	6-48 hours	Diarrhea, fever, abdominal cramps, vomiting	4-7 days	Eggs, poultry meat, unpasteurized milk or juice, cheese, contaminated raw fruits and vegetables
Campylobacter spp.	2-5 days	Diarrhea, cramps, fever, and vomiting; diarrhea may be	2-10 days	Raw and undercooked poultry, unpasteurized milk,

bloody contaminated water

2.3 *Listeria monocytogenes*

Listeria monocytogenes is a rod-shaped gram-positive and motile bacterium that is present in different environments. This bacterium is an important human foodborne pathogen and the third leading cause of foodborne deaths due to microbial causes in the USA [20]. *Listeria monocytogenes* is the etiologic agent of listeriosis, an opportunistic, invasive illness that occurs in immunocompromised individuals, such as HIV patients, elderly persons, infants, and pregnant women [21]. Symptoms vary from febrile gastroenteritis and flu-like symptoms to more severe clinical symptoms, such as encephalitis, meningitis, and bacteremia in immunocompromised individuals and spontaneous abortion in pregnant women [22]. It is believed that the main route of *L. monocytogenes* transmission occurs through the consumption of contaminated foods such as raw meats, raw vegetables, ready-to-eat seafood, raw seafood, unpasteurized milk, soft-service ice creams, and soft cheeses [23].

2.4 *Campylobacter* spp.

Campylobacter are of particular research interest as they consistently cause the greatest number of confirmed foodborne bacterial infections in developed countries. The most important species of *Campylobacter* are the thermophilic species: *C. jejuni*, *C. coli*, *C. lari,* and *C. upsaliensis*. The majority (over 90%) of the campylobacteriosis are caused by *C. jejuni* and to a lesser extent *C. coli* [24]. The intestinal tract of food-producing animals has been considered as one of the most important reservoirs for *Campylobacter* in the food supply chain. Poultry are the natural host for *Campylobacter* species and broilers are often colonized, especially with *C. jejuni* [25]. Human exposure can come through consumption of animal products, particularly in raw or undercooked broiler meat [3, 26].

3. Antimicrobial activity of plant extracts

Many studies report the antimicrobial activity of plant extracts, including activity against bacterial food-borne pathogens. These studies are with different types and parts of plants, such as herbs, spices, leaves, seeds and fruits. A study conducted by Shan et al. [27] evaluated the *in vitro* antibacterial activity of a total of 46 extracts from dietary spices and medicinal herbs against five food-borne pathogens (*Bacillus cereus*, *L. monocytogenes*, *Staphylococcus aureus*, *E. coli* and *Salmonella* serovar Anatum). Gram-positive bacteria were generally more sensitive to the tested extracts than Gram-negative; *S. aureus* was the most sensitive and *E. coli* the least sensitive. The authors reported a highly positive relationship between antibacterial activities and phenolic content of the tested extracts against each bacterium, suggesting that the antibacterial activity was closely associated with their phenolic constituents. On the other hand, Weerakkody et al. [28] studied the antibacterial activity of extracts of less used herbs and spices, and they reported a poor correlation between antimicrobial activity against food-borne pathogens (*L. monocytogenes*, *S. aureus*, *E. coli* and *S.* Typhimurium) and phenolic compounds, demonstrating that the antimicrobial activity was due to substances other than phenolic compounds.

Mhalla et al. [29] investigated the antibacterial activity of *Rumex tingitanus* leaf extracts and derived fraction and, from their results, they found that the ethyl acetate fraction showed the most potent antibacterial activity. This fraction eradicated the *L. monocytogenes* population in a concentration of 1.25 and 2.5 $mg.mL^{-1}$ after 20 and 10 minutes of contact time respectively. *Psidium guajava* L. (guava), especially the leaves, possesses useful pharmacological activities. Extracts from leaves of this species showed activity against Gram-positive bacteria (*S. aureus* MIC=100 $\mu g.mL^{-1}$) and Gram-negative bacteria (*E. coli* MIC=250 $\mu g.mL^{-1}$ and *Pseudomonas aeruginosa* MIC=500 $\mu g.mL^{-1}$) [30]. The polyphenols from muscadine grape seed (*Vitis rotundifolia* Michx.) (MIC=54.8-60.1 $\mu g.mL^{-1}$) showed stronger inhibition of *S. aureus* than polyphenols from muscadine grape skin (MIC=70.7-80.2 $\mu g.mL^{-1}$) [31]. The grape seed extract inhibited the growth of *E. coli* O157:H7 (MIC=4.0 $mg.mL^{-1}$), and with 0.25-2.0 $mg.mL^{-1}$ there was a reduction of Shiga toxin production (Stx) without inhibiting the development of the microorganism [32].

Basile et al. [33] assessed the antibacterial activity of the ethanol extract from *Paullinia cupana* Mart. seeds, commonly called guarana. The extract, at a concentration between 16 and 128 $\mu g.mL^{-1}$, showed a significant antibacterial effect against both Gram-negative and Gram-positive bacteria, in particular *P. aeruginosa* (MIC=16 $\mu g.mL^{-1}$), *E. coli*, *Proteus mirabilis* and *Proteus vulgaris* (MIC=32 $\mu g.mL^{-1}$), which were the most inhibited. According to the authors, the antibacterial activity of the extract was probably due to the polyphenols present. Medina et al. [34] evaluated the antibacterial activity of aqueous and

acetone extracts of red and yellow araçá (*Psidium cattleianum* Sabine) against *S*. Enteritidis. All araçá extracts showed antimicrobial activity with MIC of 5% except for the aqueous extract of red araçá (MIC=16%). Shen et al. [35] examined the antimicrobial effect of blueberry (*Vaccinium corymbosum* L.) extracts obtained from four cultivars on the growth of *L. monocytogenes* and *S.* Enteritidis. In general, the extracts at 900 $mg.mL^{-1}$ exhibited a growth-inhibitory effect against *L. monocytogenes*. The Elliott or Darrow extracts at 900 $mg.mL^{-1}$ reduced *S.* Enteritidis population to <1 $CFU.mL^{-1}$. Côté et al. [36] evaluated the antimicrobial effect of cranberry (*Vaccinium macrocarpon*) phenolic extract against seven food-borne pathogens. The authors found the MIC values ranged from 12.6 to 50.4 µg phenol/well among tested pathogens using methanol/water (85/15, v/v).

4. Compounds with antimicrobial activity

4.1 Alkaloids

Heterocyclic nitrogen compounds are called alkaloids [37]. These compounds are commonly found in the Solanaceae and Fabaceae families [38]. Erdemoglu et al. [39] evaluated the alkaloid profile of the aerial parts of *Lupinus angustifolius* L. and showed antibacterial activity against *Bacillus subtilis*, *S. aureus* and *P. aeruginosa* (MIC=62.5 $\mu g.mL^{-1}$). In that study, the capillary GC-MS determined the 13α-hydroxylupanine (50.78%) and lupanine (23.55%) as the two main alkaloids in the aerial parts of *L. angustifolius.* According to the authors, the presence of lupanine and 13α-hydroxylupanine explains the antibacterial activity of the extract, as described also by Tyski et al. [40] (Fig. 1). The ability of the some alkaloids to bind tightly to DNA and inhibit topoisomerase II [41] may explain its antimicrobial capacity.

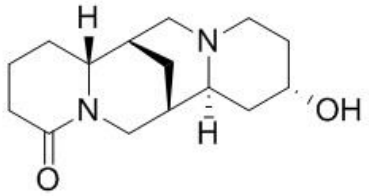


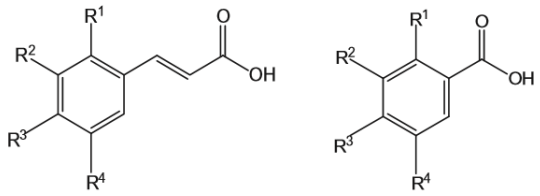
Fig. 1 13-Hydroxylupanine

4.2 Phenolics

Phenolics are one of the most abundant secondary metabolites of plants, with more than 8,000 phenolic structures currently known. Plant phenolics are generally involved in defense against ultraviolet radiation, aggression by pathogens, and contribute to plants color. The phenolic group includes mainly phenolic acids, flavonoids, and tannins [42].

4.2.1 Phenolic acids

Chemically, phenolic acids have at least one aromatic ring in which at least one hydrogen is substituted by a hydroxyl group. These compounds can be divided into two major groups, which are derived from non-phenolic molecules of benzoic acids (hydroxybenzoic acids) and from cinnamic acid (hydroxycinnamic acid) (Fig. 2) [43]. The antimicrobial activity of compounds from this group has been reported [44], and variations in the structure of the compounds influence the antimicrobial potential of phenolic acids. For example, caffeic acid is more effective than ρ-coumaric acid due to the number of OH groups in the phenolic ring [45]. A possible mechanism to explain the antimicrobial action of phenolic acids against pathogens is hyperacidification at the plasma membrane interphase, which alters cell membrane potential, making it more permeable, as well as affecting the sodium and potassium ATPase pump implicated in ATP synthesis [46, 47] . Lou et al. [48] demonstrated that ρ-coumaric acid has dual mechanisms of bactericidal activity: disrupting bacterial cell membranes and binding to bacterial genomic DNA to inhibit cellular functions, ultimately leading to cell death.

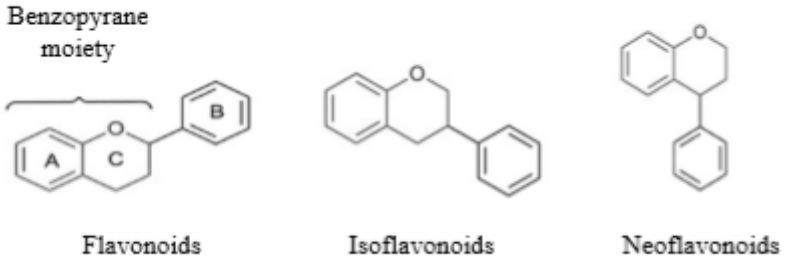


Cinnamic acid Benzoic acid derivatives

Fig. 2 Chemical structures of cinnamic and benzoic acid derivatives [43]

4.2.2 Flavonoids

Flavonoids are natural pigments abundantly present in the plant kingdom. The position of linkage of the B-ring to the C-ring is used to sort these molecules into three different classes: flavonoids, isoflavonoids and neoflavonoids (Fig. 3). Other plant secondary metabolites containing a similar carbon skeleton, such as chalcones, stilbenes, or aurones are considered minor flavonoids [49]. Many studies reported the antimicrobial activity of flavonoids [50, 51]. Cushnie and Lamb [52, 53] suggested that the antibacterial activity of flavonoids may be attributable to up to three mechanisms: cytoplasmic membrane damage causing perforation and/or reduction in membrane fluidity, inhibition of nucleic acid synthesis caused by topoisomerase inhibition, and inhibition of energy metabolism caused by NADH-cytochrome C reductase inhibition.

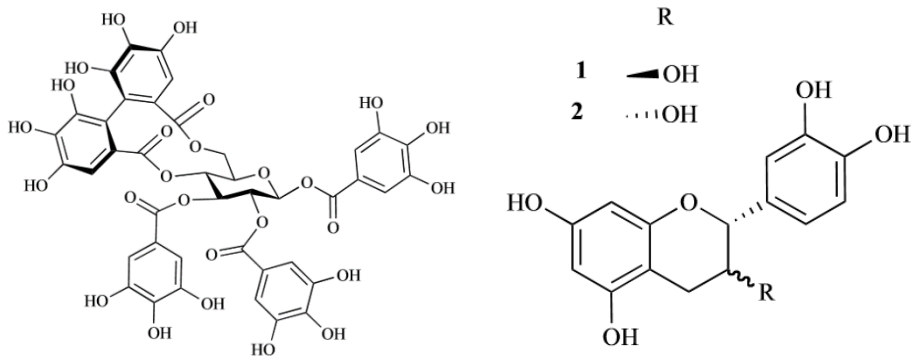


Flavonoids Isoflavonoids Neoflavonoids

Fig. 3 Chemical structures of flavonoids, isoflavonoids and neoflavonoids [49]

4.2.3 Tannins

Tannins are a complex group of natural polyphenols. Tannins are classified into two major groups on the basis of their structure: the hydrolysable and the condensed tannins. Hydrolysable tannins are compounds containing a central core of glucose or another polyol with gallic acid. Condensed tannins are oligomers or polymers composed of flavan-3-ol nuclei (Fig. 4) [54]. The biological activity of plant extracts containing tannins has been known for centuries, which has led to the isolation and characterization of many representatives of this class [55]. The ability of tannins to inhibit the development of pathogenic microorganisms has been recognized. The different mechanisms proposed so far to explain tannin antimicrobial activity include the inhibition of extracellular microbial enzymes caused by the typical astringent characteristic of tannins, direct action on microbial metabolism through inhibition of oxidative phosphorylation, and a mechanism involving iron deprivation [56].

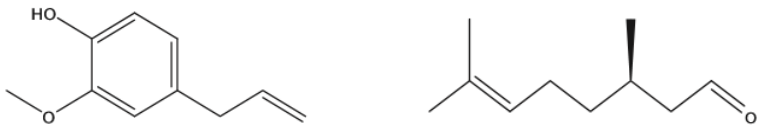


Hydrolysable tannin Condensed tannin

Fig. 4 Example of hydrolysable (tellimagradin II) and condensed (1: catechin; 2: epicatechin) tannin [54]

4.3 Terpenoids

Terpenoids are a diverse group of secondary metabolites, estimated to be around 40,000 compounds (Fig. 5) [57]. Terpenoids are important constituents of essential oils [4]. Several pieces of research reported the antimicrobial action of essential oils [58, 59, 60, 61] as well as its mechanism of action [62, 63]. Kurecki et al. [64] tested the antimicrobial potential of five terpenoid compounds (α-bisabolol, α-terpinene, cineole, nerolidol and terpinen-4-ol) and proved that terpinen-4-ol showed the highest activity against *Campylobacter* spp. and other reference strains.



Eugenol Citronellal

Fig. 5 Example of terpenoids [65]

4.4 Saponins

Saponins are molecules typically formed by an aglycone unit (sapogenin, hydrophobic) attached to a sugar (glycone, hydrophilic) [66]. Liu and Henkel [67] considered the polyphenols and saponins as the key ingredients in remedies used in traditional Chinese medicine responsible for most of the observed biological effects. Saponins have been described as antimicrobial compounds, as reported by Hassan et al. [68]. The authors evaluated the antimicrobial activity of saponin-rich extract from guar meal and showed antibacterial effect against *S. aureus*, *S.* Typhimurium and *E. coli,* although the mechanism of antibacterial action of saponins is unclear. However, increasing antibiotic activity was observed going from the saponin extracts to the sapogenin samples, suggesting that the sugar moiety is not important for the antimicrobial efficacy [69].

5. Potential food applications of plant extracts

Although many studies report the antimicrobial activity of plant extracts *in vitro*, there is some difficulty in replicating the results *in situ*. According to Hayek et al. [7], foods are not sterile, so a mixed microbial population can influence the results that are obtained by *in situ* assays. Miceli et al. [70] evaluated the antimicrobial activity of *Borago officinalis* and *Brassica juncea* aqueous extracts *in vitro* and *in situ* using different food model systems (meat broth, fish broth and vegetable broth). The final extract concentration that showed inhibition *in vitro* was not confirmed *in situ*; however, when added at a concentration 10-fold higher than that *in vitro* (100 and 31 $mg.mL^{-1}$ *B. officinalis* and *B. juncea*, respectively), they inhibited the growth of the sensitive strains. According to the authors, this was not surprising, since the activity of

inhibitory substances in foods can be influenced negatively by several factors, such as binding of the active compounds to additives of the food or inactivation by food inhibitors.

Zhang et al. [71] determined the effect of extracts from *Eugenia caryophyllata* and *Rosemarinus officinalis* alone and combined in raw chicken meat during storage for 15 days at 4 °C. The bacterial counts of the chicken samples with a combination of extracts were lower than those of control samples during storage. The antimicrobial effect of chestnut inner shell extract was characterized against *C. jejuni* strains on chicken meat [72]. The pathogen was not detected at 1 $mg.g^{-1}$ of extract with 3 log $CFU.g^{-1}$ of inoculum after 4 days at 4 °C.

Mariem et al. [73] applied the aqueous extract of the fruits of *Nitraria retusa* in beef patties. In general, there was a significant inhibition of the microbial growth in beef patties containing the fruit extract compared with control meat (without extract of *N. retusa*). The antimicrobial activity of the fruit extract against microbial proliferation was most effective on coliforms. By the end of the storage time (9 days at 4 °C), coliform populations in the control sample were 6.47 log $CFU.g^{-1}$ and significantly lower by 4.48, 4.13 and 3.81 log $CFU.g^{-1}$ when the sample was treated with 0.5%, 0.75% and 1% of *N. retusa* extract, respectively.

Plant extracts could be used as natural antimicrobials, but the amount required for microbial inhibition in foods would be considerably high, adversely affecting the sensory characteristics of the food. The use of natural compounds in combination with other natural antimicrobials or with other technologies could produce a synergistic effect against foodborne pathogens [45]. In this context, the application of plant extracts in films can be an alternative to increase the shelf life of foods. Choulitoudi et al. [74], for example, applied extracts and essential oil of *Satureja thymbra* (L.) in edible films to prolong the shelf life of fresh gilthead seabream (*Sparus aurata*) fillets. The ethyl acetate extract alone and combined with essential oil showed the best antimicrobial effect, resulting in 25 and 35% shelf life extension, respectively. Krasniewska et al. [75] determined the antimicrobial properties of pullulan coating enriched with extract from *Bergenia crassifolia* on pepper. After 14 days of storage, the reduction of the *S. aureus* population on pepper coated with pullulan and pullulan with extract was 0.74 and 1 log $CFU.g^{-1}$, respectively, compared to the uncoated samples.

6. Conclusions

The bioactive properties of plants have long been known. In order to find alternatives to synthetic chemical additives, studies on the antimicrobial effect of plant extracts against foodborne pathogens are being conducted with promising results. In recent years, advances have been made in the study of the action mechanisms of antibacterial compounds found in the extracts. Nowadays, studies are conducted on the application of extracts and/or compounds directly on foods or using antimicrobial films. In these studies there was a decrease in the microbial population of foods. Therefore, the application of plant extracts can be an alternative to synthetic preservatives to increase the shelf life of foods.

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3 Manuscrito 1

Butia odorata Barb. Rodr. extract has antibacterial activity by dual mechanism against *Staphylococcus aureus* and *Escherichia coli*

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Manuscrito submetido ao periódico *Journal of Functional Foods*

Abstract

This study aimed to evaluate the antibacterial activity and the mechanism of action of *B. odorata* extract (BOE), using *Staphylococcus aureus* and *Escherichia coli*, as Gram-positive and Gram-negative bacterial models. All bacterial strains tested, both Gram-positive and Gram-negative bacteria (*Listeria monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *Salmonella* Typhimurium), were susceptible to BOE. In relation to the mechanism of action, BOE caused cell membrane damage, resulting in release of DNA to the extracellular environment. Additionally, according to fluorescence spectrophotometry analysis, BOE caused *S. aureus* and *E. coli* DNA damage. BOE showed antibacterial activity against foodborne microorganisms, having the potential to be used as an alternative to synthetic preservatives. In addition, this study demonstrated that the mechanism of action of BOE against *S. aureus* and *E. coli*, used as models of Gram-positive and Gram-negative bacteria, occurs by dual mechanism, causing damage in the bacterial membrane and in the genomic DNA.

Keywords: food preservative; cell membrane; confocal laser scanning microscopic; DNA; fluorescence spectrophotometry

1 Introduction

Foodborne diseases are a growing public health problem worldwide. The most common clinical presentations of these diseases are gastrointestinal symptoms; however, complications can be triggered, culminating in deaths and considerable rate of disability (WHO, 2018). In the United States of America it is estimated that annually one in six Americans are affected by foodborne diseases, resulting in 128,000 hospitalizations and 3,000 deaths (CDC, 2018). In Brazil *Salmonella* spp., *Escherichia coli* and *Staphylococcus aureus* are the main bacteria responsible for outbreaks of foodborne diseases (Brasil, 2018).

Since foodborne bacteria can be dangerous to human health, interest in new antimicrobials and their mechanism of action is growing (Clemente, Aznar, Silva, & Nerín, 2016). Moreover, consumers are concerned about the safety of foods containing synthetic preservatives (Alzoreky & Nakahara, 2003), and it is important to develop novel and safe natural antibacterial agents (Shi et al., 2018). In this context, the development of plant-derived compounds with antibacterial activity, to be used as natural preservatives in food applications, is becoming increasingly attractive (Wu, Bai, Zhong, Huang, & Gao, 2017).

The antimicrobial activity of fruit extracts has been studied in recent years (Almulaiky et al., 2018; EL-Hefny, Mohamed, Salem, El-Kareem, & Ali, 2018; Taleb, Maddocks, Morris, & Kanekanian, 2016). In this context, *Butia odorata* Barb. Rodr., a native fruit of South America, showed antibacterial activity against microorganisms of importance in food (Haubert et al., 2019; Maia, Aranha, Chaves, & Silva, 2017), but the mechanism by which it acts is unknown. It is known that antimicrobial agents act mainly by altering the permeability of the cell membrane and/or causing damage to proteins and

nucleic acids (Tortora, Funke, & Case, 2012). Thus, the aim of this study was to evaluate the antibacterial activity of BOE against selected foodborne pathogens and investigate, for the first time, its mechanism of action in *S. aureus* and *E. coli*, used as Gram-positive and Gram-negative bacterial models.

2 Material and methods

2.1 Plant material

Butia odorata Barb. Rodr. fruits were harvested from a research orchard in a germplasm collection at the Centro Agropecuário da Palma, UFPel, Pelotas, Brazil (31º 52' 00'' S latitude, 52º 21' 24'' W Greenwich longitude and altitude of 13.24 m) in February 2018.

2.2 *Butia odorata* extract preparation

The extract was prepared according to Maia, Haubert, Soares, Würfel, & Silva (2019). In an Erlenmeyer flask, 30 g of lyophilized pulp *B. odorata* and 300 mL of acetone (Synth, Brazil) were added and placed in a SL 223 shaker (190 rpm) (Solab, Brazil) for 2 h. Afterwards, this was centrifuged (7500 rpm for 20 min) and the supernatant was filtered in filter paper and rotary-evaporated (30 °C) (Heidolph Rotary Evaporator, Laborota 4000, Sigma-Aldrich, USA) until constant weight. Next, the extract was stored at -80 °C in the absence of light.

2.3 Cultivation conditions of target microorganisms

Two Gram-positive (*Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC 7644) and two Gram-negative (*Salmonella* Typhimurium ATCC 14028 and *Escherichia coli* O157:H7 NCTC 12900) foodborne bacteria

were used as target microorganisms. The microorganisms stored at -80 °C were cultured on Tryptic Soy agar (Acumedia, USA) with 0.6% yeast extract (Himedia®) (TSA-YE), and incubated at 37 ºC for 24 h.

2.4 Agar disc diffusion method

Inoculum of each target microorganism was standardized at a concentration of 10^8 CFU.mL^{-1} and plated on Petri dishes containing Mueller-Hinton agar (MH, Kasvi, Brazil). After that, sterile paper filter discs (6 mm) impregnated with 20 µL of BOE were placed on the agar and the plates subsequently incubated at 37 ºC for 24 h. As control, streptomycin discs (10 µg) and discs impregnated with water were used. The results were expressed as mean ± standard deviation of the diameter of the zones of inhibition (DIZ).

2.5 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC was performed in a 96-well microplate. Firstly, BOE was diluted in Dimethyl sulfoxide (DMSO, Synth, Brazil) for 400 mg.mL^{-1}. After that, two-fold serial dilutions of BOE were prepared in Mueller Hinton broth (MHB, Kasvi, Brazil), varying from 130 to 1 mg.mL^{-1}, with 10^6 CFU.mL^{-1} of the bacterial inoculum added and incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration which showed no visible growth. For MBC, from the well where there was no visible growth, this was sown into plates containing TSA-YE, and incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration in which 99.9 % of cells initially inoculated were dead (Maia, et al., 2019).

2.6 Time-kill analysis

In tubes containing Brain Heart Infusion (BHI, Acumedia, USA) or Tryptic Soy broth (TSB, Acumedia, USA) (*S. aureus* and *E. coli,* respectively) BOE was added in the concentrations of MIC or MBC. After that, 20 µL of bacterial inoculum (10^8 UFC.mL^{-1}) was added and incubated at 37 °C with shaking (120 rpm) for determined times (0, 2, 4, 8, 12, 16 and 24 h). Then, aliquots were taken, serially diluted ten-fold and sown on plates containing TSA (37 °C/24 h). Tubes containing only broth and bacterial inoculum were used as control.

2.7 Cell membrane integrity evaluation

To assess the cell membrane integrity, the intracellular DNA concentration of *S. aureus* and *E. coli* was evaluated after exposure to BOE. For this, *S. aureus* and *E. coli* cells were cultivated in BHI or TSB, respectively, until the logarithmic phase. Next, BOE was added at concentrations of MIC and 2 x MIC, and incubated at 37 °C for 8 h. Samples without addition of the extract were used as control. The bacterial cells were then collected by centrifugation (13,000 *g* for 2 min), and genomic DNA was extracted using a Wizard® extraction and purification DNA kit (Promega, USA), following the manufacturer's instructions. The quality and quantity of DNA samples were measured in a Nanovue Plus spectrophotometer (GE Healthcare, USA) and subjected to electrophoresis at 80 V on a 1.0% (w/v) agarose gel (Invitrogen, USA).

2.8 Scanning electron microscopy (SEM) analysis

The SEM was performed according to Ning et al. (2017) with adaptations. Logarithmic growth phase cells in BHI broth or TSB (*S. aureus* and *E. coli,* respectively) were treated with BOE at the concentrations of MIC and 2 x MIC. Treated and non-treated (without addition of BOE) samples were incubated at 37 °C for 8 h (120 rpm). After incubation, cells were collected by centrifugation (6000 *g* for 10 min), washed three times with PBS buffer, and incubated with 2.5% glutaraldehyde in PBS (10 mM) overnight at 4 °C. After three washes with PBS, the samples were dehydrated with ascending concentrations of ethanol (30, 50, 70, 80, 90, and 100%), and then dried in an L 101 freeze dryer (Liotop, Brazil). Following that, the samples were fixed on SEM support, sputter-coated with gold under vacuum and after that analyzed in JSM-6610LV SEM (Jeol, Japan).

2.9 Confocal laser scanning microscopy (CLSM) analysis

Cells were grown to the logarithmic growth stage in BHI or TSB medium (for *S. aureus* and *E. coli*, respectively), treated with BOE at the concentration of MIC. and then incubated at 37 °C for 8 h. Non-treated cells were used as control. After centrifugation (5,000 *g*/5 min) and washing with PBS, cells were stained with SYTO 9 and propidium iodide according to the manufacturer's recommendations (LIVE/DEAD™ BacLight™ - Invitrogen, USA). Next, cells were washed twice with PBS and observed with a Leica TCS SP8 confocal laser scanning microscope (Germany) at 100 x magnification.

2.10 Effect of BOE on bacterial DNA

The effect of BOE on bacterial DNA was evaluated according to Ning et al. (2017), using an F-7000 fluorescence spectrophotometer (Hitachi, Japan). For this, the genomic DNA was extracted by Wizard® extraction and purification kit (Promega, USA), according to the manufacturer's instructions. The purity and concentration of DNA was verified in Nanovue Plus spectrophotometer (GE Healthcare, USA). DNA extracted was diluted with Tris-HCl 0.01M (pH 7.0) to 30 $\mu g.mL^{-1}$, and the same volumes of DNA and BOE in different concentrations (MIC, 2 X MIC, 4 X MIC and 8 X MIC) were mixed. Treated and non-treated (without addition of BOE) samples were incubated in the dark at 37 °C/10 min, and the fluorescence was measured at room temperature, using 280 nm excitation and 300-500 nm scanning emission with a slit width of 5 nm.

2.11 Influence of BOE in the binding capacity of the ethidium bromide

Bacterial genomic DNA was extracted and prepared according to item 2.10. The same volumes of DNA and BOE in different concentrations (MIC, 2 X MIC, 4 X MIC and 8 X MIC) were mixed, and incubated in the dark (37 °C/10 min). After that, ethidium bromide solution at 4 $\mu g.mL^{-1}$ was added to the mixture, and incubated again in the dark at 37 °C/10 min. Samples without addition of BOE were used as control. The fluorescence of the mixture was measured using 535 nm excitation, 570-645 nm scanning emission, with a slit width of 5 nm at room temperature.

2.12 Statistical analysis

Data were submitted to a one-way analysis of variance (ANOVA) followed by the Tukey test ($p \leq 0.05$) using STATISTICA software version 6.1 (StatSoft, France).

3 Results and discussion

3.1 Antibacterial activity of BOE

The antibacterial activity of BOE was assessed by agar disc diffusion test, MIC and MBC. According to the agar disc diffusion test, the BOE inhibited all bacterial strains tested, both Gram-positive and Gram-negative bacteria (Table 1). However, Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) were more susceptible to the extract (DIZ= 30±2.0 and 22±0.8 mm, respectively) than Gram-negative bacteria (*E. coli* O157:H7 and *S.* Typhimurium, DIZ=16±3.9 and 15±3.2 mm, respectively). The same behavior was observed in the MIC test, where the values were 4 $mg.mL^{-1}$ for Gram-positive bacteria and 8 $mg.mL^{-1}$ for Gram-negative bacteria. Regarding MBC, *S. aureus* and *E. coli* showed the lowest values, 16 $mg.mL^{-1}$, and for this reason they were selected to be used as models of Gram-positive and Gram-negative bacteria, respectively, in the evaluation of the mechanism of action of BOE.

Several studies reported that Gram-positive bacteria are more susceptible to plant extract, including fruit extracts, than Gram-negative bacteria (Morais-Braga et al., 2016; Shen et al., 2014; Yong, Dikes, Lee, & Choo, 2018). However, the results are variable according to fruit studied. Orange, yellow lemon and banana peel extracts, for example, showed better antibacterial activity against Gram-negative than Gram-positive bacteria (Saleem & Saeed,

2019). Additionally, the type of solvent used in extraction process also has an influence because it interferes in type of compound that will be extracted. In a previous study, for example, hexane extract of *B. odorata* showed better inhibitory activity against Gram-negative than Gram-positive bacteria (Maia et al., 2017).

3.2 Time-kill analysis

To further investigate the antibacterial activity of BOE against *S. aureus* and *E. coli*, a time-kill analysis was carried out based on the results of MIC and MBC assay. Figure 1 shows the effect of BOE on the viability of *S. aureus* and *E. coli* cells. As can be observed, the addition of MIC kept the bacteria in the lag phase in the first 8 h of contact in relation to control for both bacteria, demonstrating bacteriostatic effect. With MBC, the *S. aureus* cells were completely inactivated within 4 h, and *E. coli* after 12 h of contact.

3.3 Cell membrane integrity

To investigate the cell membrane damage, the intracellular concentration of *S. aureus* and *E. coli* DNA was evaluated after exposure to BOE for eight hours. Figure 2A shows that with addition of the extract the intracellular concentration of *S. aureus* DNA decreased, which was concentration-dependent. As can be seen in Figure 2C, the cells that were exposed to extract showed less intense DNA bands.

For *E. coli*, the addition of BOE in MIC increased the DNA concentration extracted in relation to control (Figure 2B). This is probably due to the increase in the susceptibility of the plasmatic membrane after treatment with the extract,

increasing the efficiency of DNA extraction. However, as can be seen in Figure 2C, the DNA was damaged. When *E. coli* cells were submitted to 2 X MIC, the DNA concentration decreased in relation to control, which is also observed by the decrease in the intensity of the band when compared to the control (Figure 2C).

When the cell membrane is damaged, large molecules, such as DNA, leak out (Sun et al., 2018). Recent studies indicated that BOE is mainly composed of Z-10-Pentadecenol (80.1%) (Maia et al., 2019). It is known that alcohols act by denaturing proteins. Thus, it is suggested that BOE caused damage to the bacterial cell membrane by denaturation of proteins, resulting in the release of DNA into the extracellular environment.

3.4 SEM analysis

The SEM images (Figure 3) showed morphological changes in *S. aureus* and *E. coli* cells after eight hours of exposure to BOE. Untreated *S. aureus* and *E. coli* cells displayed a smooth and regular surface (Figure 3A and 3D). However, when exposed to BOE at MIC, both *S. aureus* and *E. coli* showed changes in their surfaces compared to the control. The coccus form of *S. aureus* was clearly altered (Figure 3B), while *E. coli* demonstrated changes in cell integrity, with loss of cell constituents (Figure 3E).

When the bacterial cells were treated with 2 X MIC, morphological changes were even more evident. Figure 3C showed some *S. aureus* cells shrinking, and Figure 3F exhibited many *E. coli* cells with holes, with complete loss of cell membrane integrity. Disruption of membrane integrity results in the leakage of cell content and consequent bacterial death (Sun et al., 2018). The

results obtained in SEM are in agreement with what has been observed in cell membrane integrity test.

3.5 CLSM analysis

Damages to cell membrane were verified by CLSM, using SYTO 9 and propidium iodide fluorophores. SYTO 9 emits green fluorescence while propidium iodide emits red fluorescence. SYTO 9 usually stains all bacterial cells while propidium iodide penetrates only when membrane cells are damaged (Liu et al., 2017). According to what is shown in Figure 4, the majority of untreated cells emitted green fluorescence, whereas cells treated for 8 h with BOE emitted red fluorescence with a small fraction emitting green fluorescence. This result indicates that BOE caused damage to the cell membrane of *S. aureus* and *E. coli*, which corroborates the results of cell membrane integrity test and SEM, indicating that one of the antibacterial mechanisms of action of BOE is the alteration of cell membrane integrity.

3.6 Effect of BOE on *S. aureus* and *E. coli* DNA

Although DNA is a potential antimicrobial target in bacteria, it has only recently been addressed (Bolhuis & Aldrich-Wright, 2014). The effect of BOE on *S. aureus* and *E. coli* DNA was assessed by fluorescence spectrophotometer. As shown in Figure 5, the addition of BOE to *S. aureus* and *E. coli* DNA decreased the fluorescence intensity, and this was concentration-dependent. These results indicate that the extract may have triggered changes in the structure and conformation of DNA. In a study conducted by Ning et al. (2017),

the addition of phenyllactic acid to *L. monocytogenes* and *E. coli* DNA demonstrated a significant fluorescence quenching. The interactions of compounds with DNA likely interfere in transcription, replication, and cell repair, causing cell death (Bolhuis & Aldrich-Wright, 2014).

Moreover, the influence of BOE on the binding capacity of the ethidium bromide to *S. aureus* and *E. coli* DNA was evaluated. Ethidium bromide is a DNA intercalator, and could emit intense fluorescence by intercalation between the adjacent DNA base pairs (Ning et al., 2017). For *S. aureus* (Figure 6A), the addition of MIC decreased the fluorescence intensity in relation to control, and when higher concentrations were added (2 X MIC, 4 X MIC, and 8 X MIC), drastically decreased the fluorescence. In *E. coli* DNA (Figure 6B), the addition of 2 X MIC resulted in a significant decrease in the fluorescence. The same was observed using higher concentrations (4 X MIC and 8 X MIC).

The results obtained by fluorescence spectrophotometry suggest that BOE changed the structure of the DNA molecule of *S. aureus* and *E. coli* and, in this way, prevented the intercalation of ethidium bromide to DNA. Therefore, the damage to DNA is another antibacterial mechanism by which BOE acts.

4 Conclusion

BOE has antibacterial activity against foodborne microorganisms, having the potential to be used as an alternative to synthetic preservatives. In addition, it was observed that BOE has antibacterial activity against *S. aureus* and *E. coli*, which were used as Gram-positive and Gram-negative bacterial models, by dual mechanism, causing damage to the bacterial cell membrane and to the genomic DNA.

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Table 1. Inhibition zone, Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the *B. odorata* extract

Microorganism	Inhibition zone (mm)	MIC ($mg.mL^{-1}$)	MBC ($mg.mL^{-1}$)
L. monocytogenes	30±2.0[a]	4	33
S. aureus	22±0.8[b]	4	16
E. coli O157:H7	16±3.9[c]	8	16
S. Typhimurium	15±3.2[c]	8	33

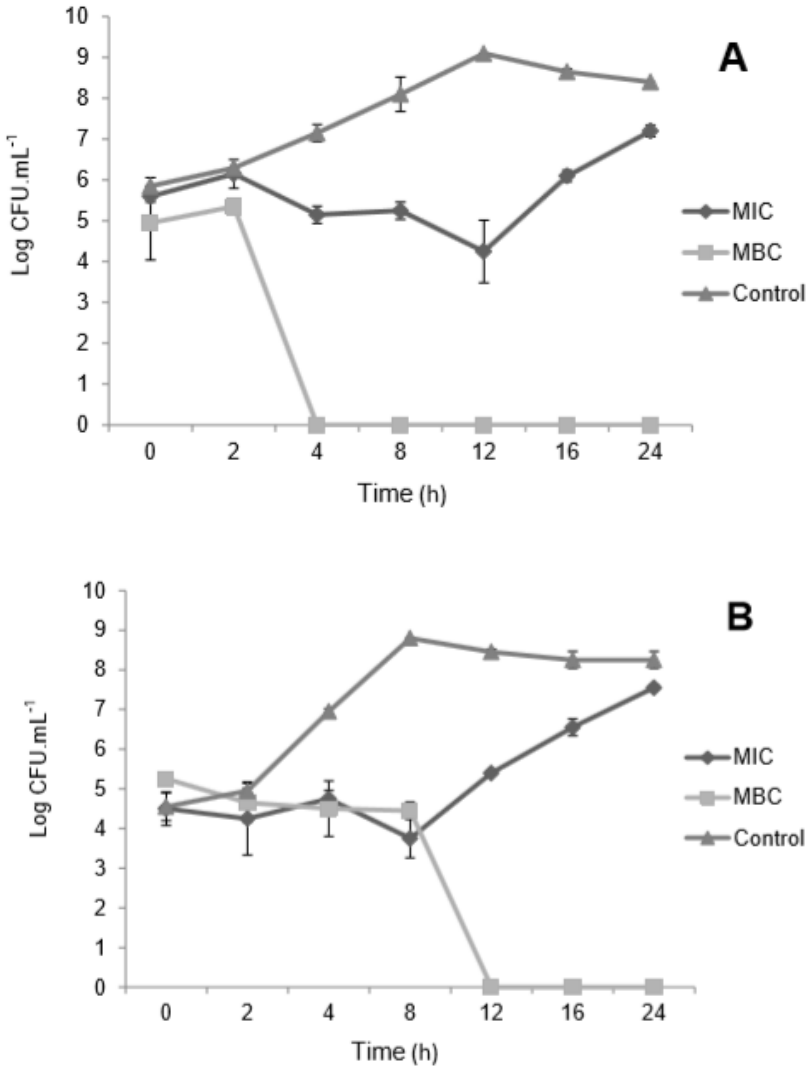

Figure 1 Time-kill curve of the *B. odorata* extract against *S. aureus* (A) and *E. coli* (B)

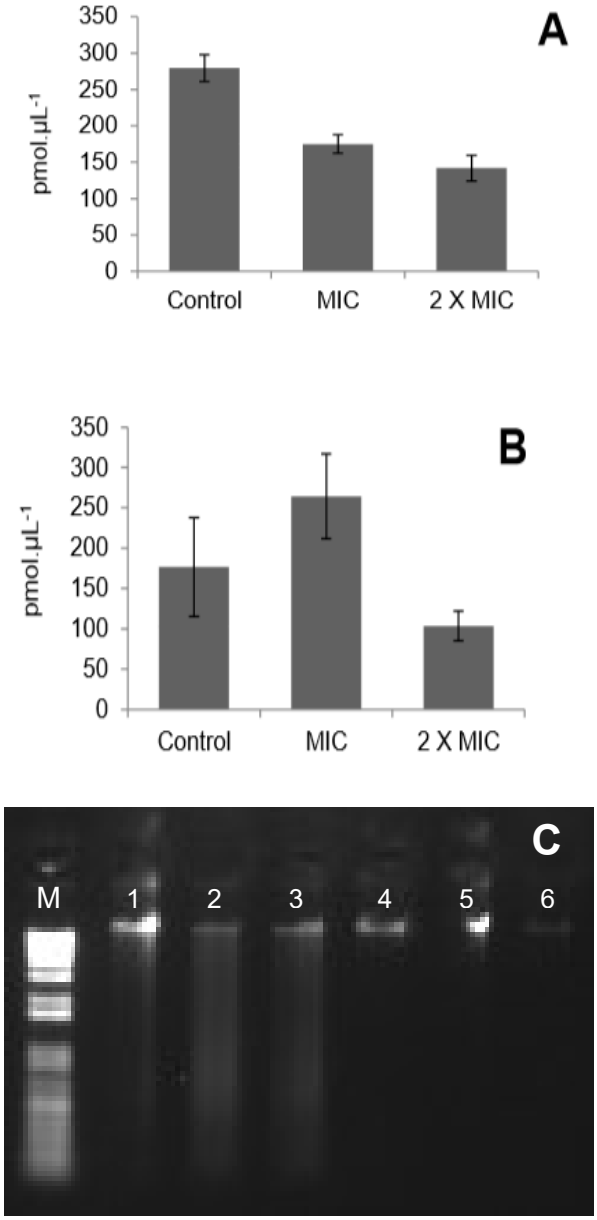

Figure 2 Intracellular concentration of *S. aureus* (A) and *E. coli* (B) DNA after exposure to *B. odorata* extract; and agarose gel eletrophoresis of DNA of *S. aureus* and *E. coli* (C) treated with different concentrations of *B. odorata* extract (M: marker DNA of 1 kb, 1 and 4: control, 2 and 5: MIC, 3 and 6: 2 X MIC).

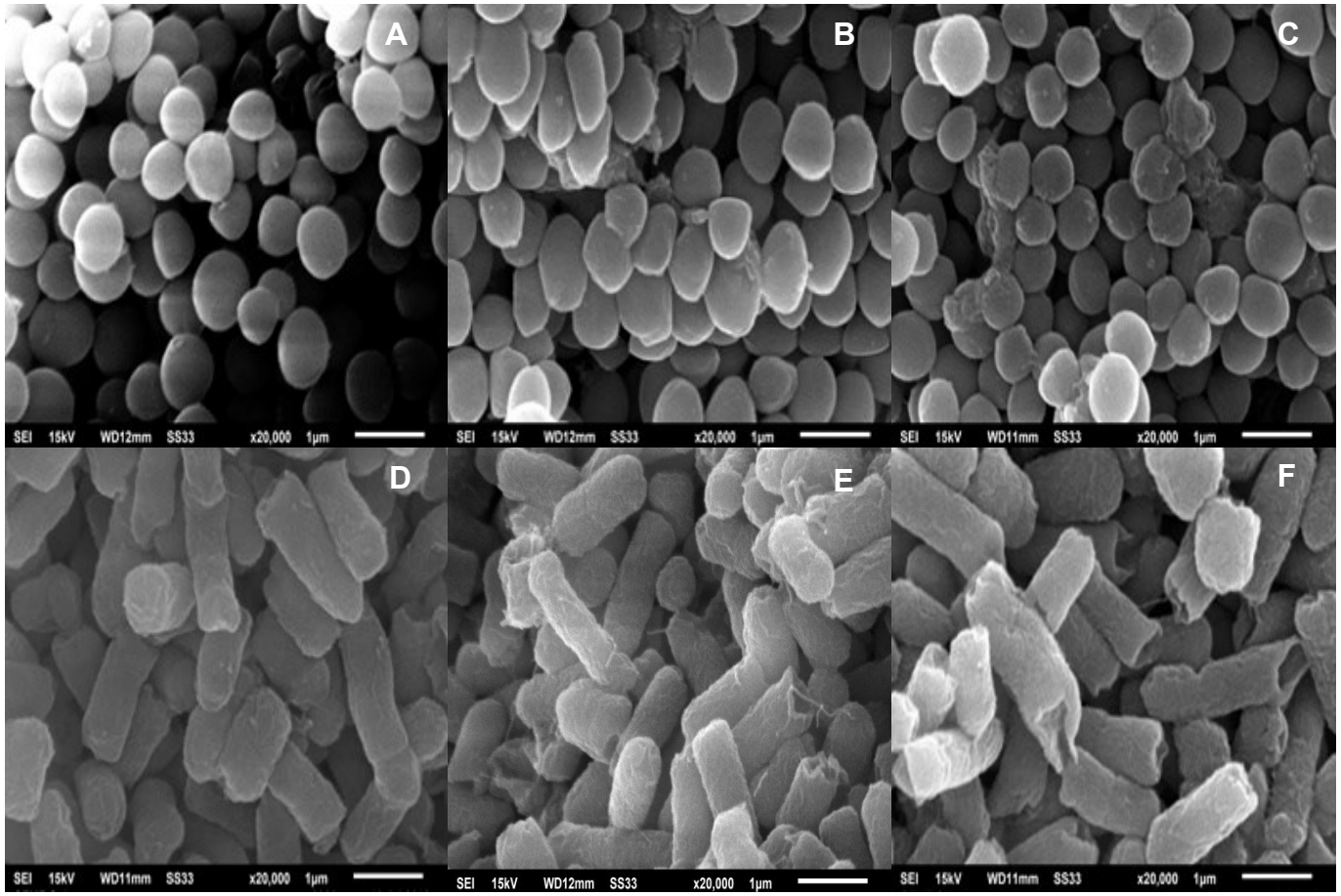

Figure 3 Scanning electron microscope of *S. aureus* (A, B, C) and *E. coli* (D, E, F). Control groups (A and D), *B. odorata* treatments for 8 h at MIC (B and E) and 2 X MIC (C and F).

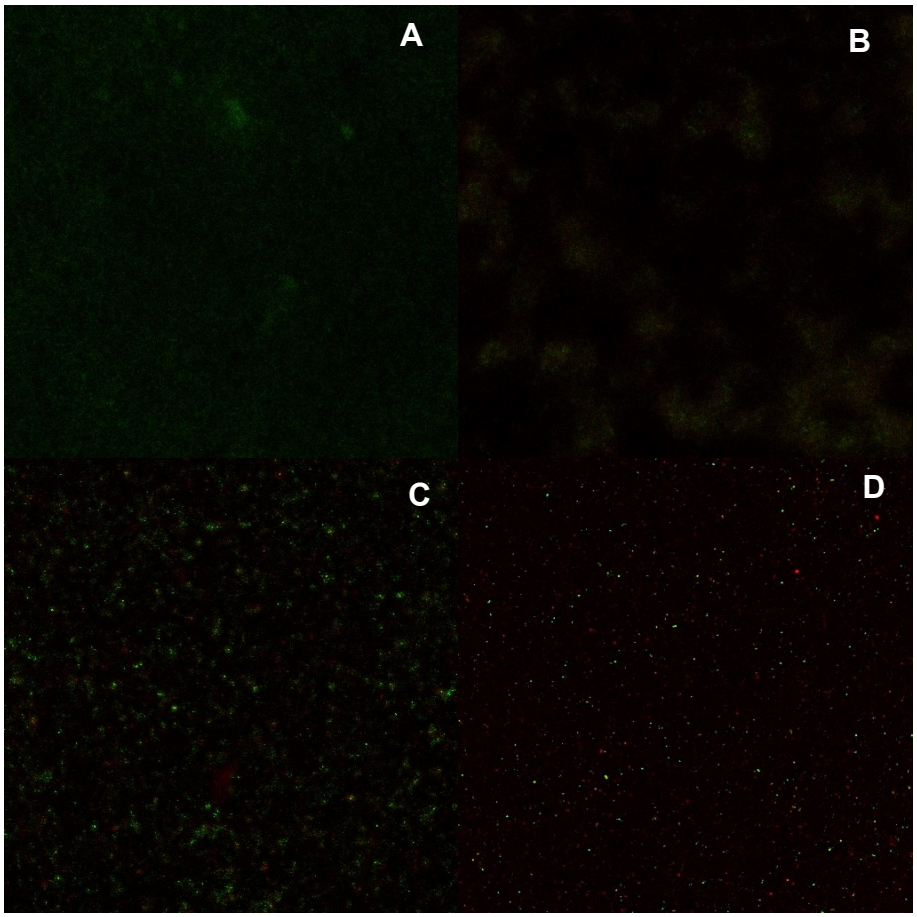

Figure 4 Confocal laser scanning microscopic of *S. aureus* (A, B) and *E. coli* (C, D). Control groups (A and C) and *B. odorata* treatments for 8 h at MIC (B and D)

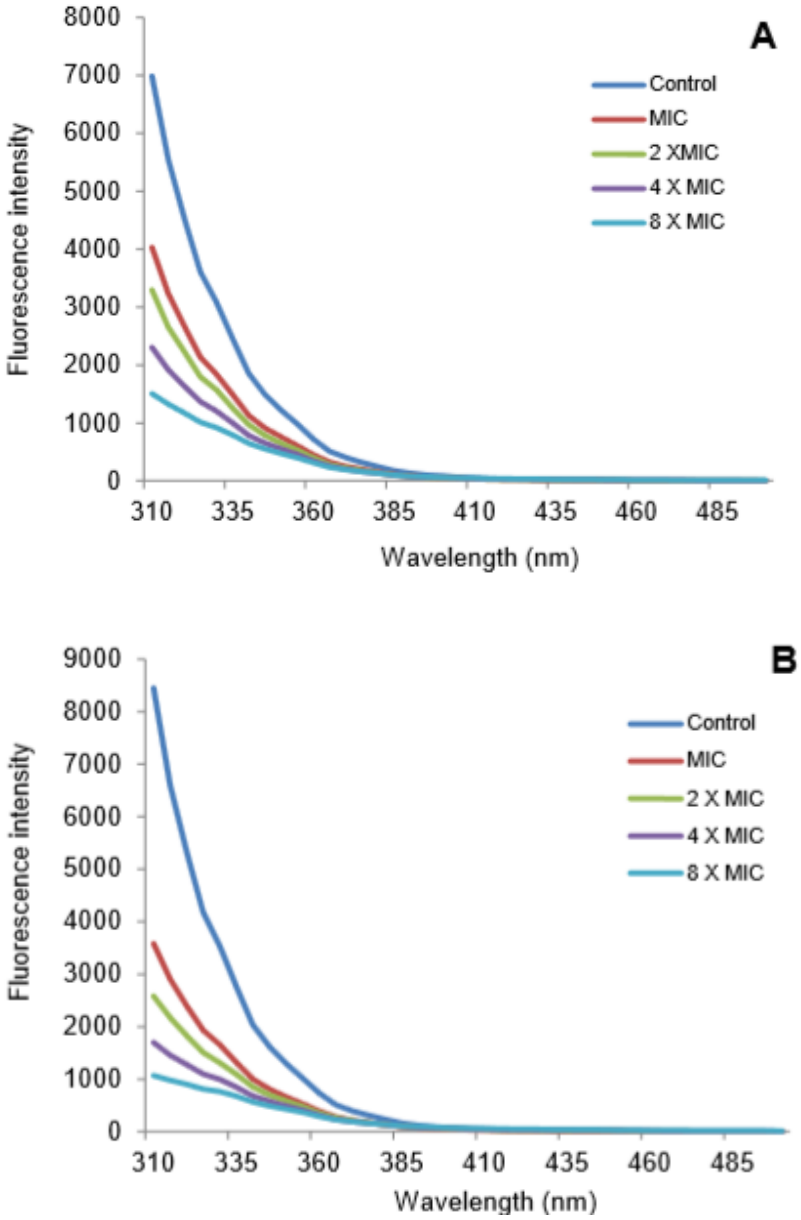

Figure 5 Fluorescence spectra of the effect of *B. odorata* extract on *S. aureus* (A) and *E. coli* (B) DNA.

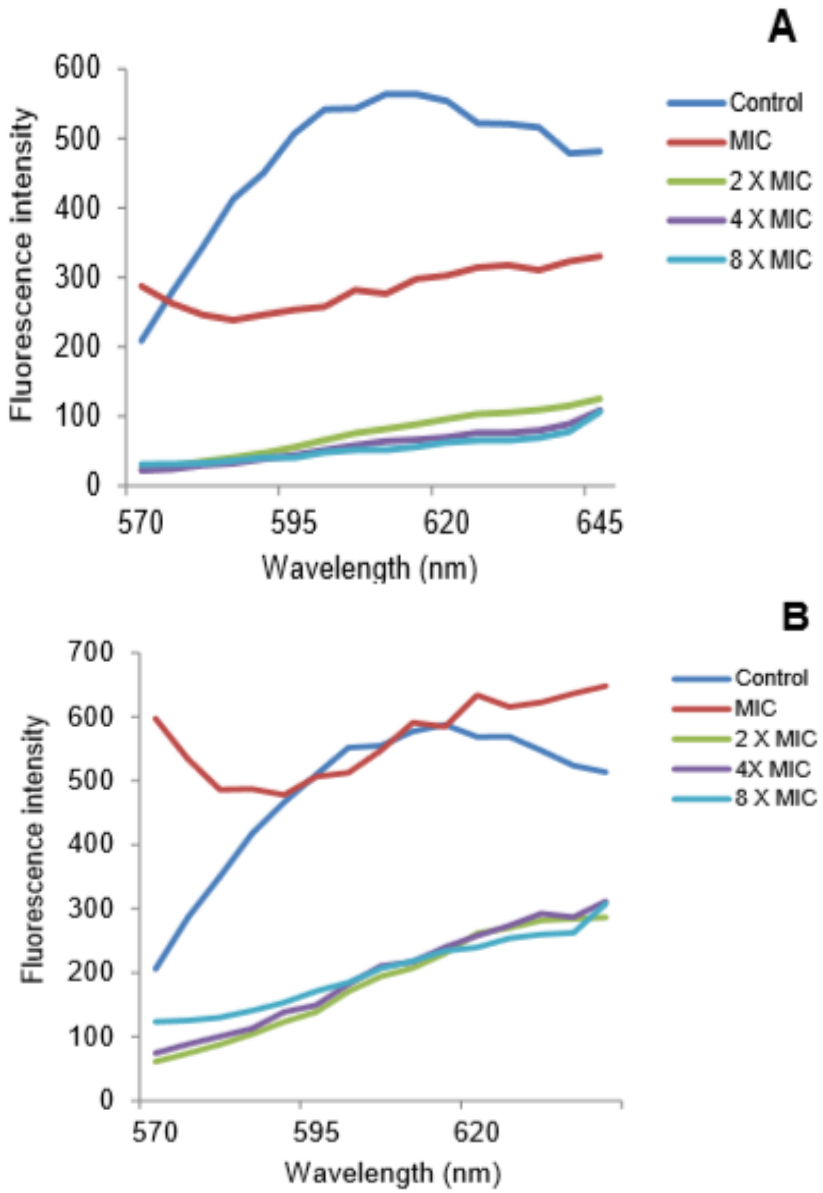

Figure 6 Fluorescence spectra of the effect on *S. aureus* (A) and *E. coli* (B) DNA treated with *B. odorata* extract and ethidium bromide.

4 Artigo 2

Butia odorata Barb.Rodr.extract inhibiting the growth of *Escherichia coli* in sliced mozzarella cheese

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Research highlights

1) *Escherichia coli* was isolated in 5% of cheese samples
2) *Escherichia coli* isolates were susceptible to *B. odorata* extract
3) *Butia odorata* extract inhibited the development of *E. coli* in sliced cheese

Abstract

The aims of this study were to verify the occurrence of *Escherichia coli* in sliced mozzarella cheese marketed in Pelotas city, Brazil and perform the phenotypic and genotypic characterization of the isolates. Besides that, evaluate the susceptibility of *E. coli* to *Butia odorata* extract, characterize it chemically, and apply the extract in sliced mozzarella cheese contaminated experimentally with *E. coli*. *Escherichia coli* was isolated in 5% (4/80) of cheese samples, but no gene of O157:H7 *E. coli* or virulence genes were detected. The isolates were susceptible to *B. odorata* extract (MIC 15 $mg.mL^{-1}$ and MBC 29-58 $mg.mL^{-1}$), and the major compounds present in the extract were Z-10-Pentadecenol (80.1 %) and Palmitic acid (19.4 %). In cheese, after 72 h there was a significant difference ($p<0.05$) between control (2.8 log $CFU.cm^{-2}$) and treated samples with MIC, 2 x MIC, 4 x MIC and 8 x MIC (1.3, 1.4, 1.6 and 0.5 log $CFU.cm^{-2}$, respectively). The isolation of *E. coli* in cheese indicates fecal contamination and poor hygienic practices. *Butia odorata* extract showed antimicrobial activity against *E. coli* both *in vitro* and *in situ*, indicating that it can be a good alternative for inhibiting the growth of this microorganism in sliced cheese.

Keywords: foodborne bacteria, PCR, natural antimicrobial, Z-10-Pentadecenol, palmitic acid

Introduction

Escherichia coli is a commensal member of the gut microbiota (Kaper et al. 2004), and its presence in foods, such as milk and milk products, is an important indicator of fecal contamination and hygienic practices (Ombarak et al. 2016). However, some *E. coli* groups are pathogenic to humans and may cause diarrheal diseases. Every year, 550 million people are affected by diarrheal diseases (WHO 2018). Diarrheagenic *E. coli* can be transmitted through contaminated food or water, or through contact with animals or people (CDC 2019), and these bacteria are classified according to their pathogenic features (Kaper et al. 2004; Nataro and Kaper 1998).

The pathotypes include the enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), and diffusely adherent *E. coli* (DAEC). Of these, EHEC triggers an infection with more severe symptoms, such as hemorrhagic colitis, which can progress to uremic hemolytic syndrome, of which the infectious dose is extremely low (around 100 cells) (Kaper et al. 2004). The O157:H7 serotype is the most important EHEC in North America (CDC 2019), the United Kingdom and Japan (Kaper et al. 2004). In Brazil, *E. coli* was the most common etiological agent involved in foodborne disease outbreaks between 2007 and 2017, responsible for more than 500 outbreaks (Brasil 2017).

Ready-to-eat (RTE) foods are consumed without further treatment that would eliminate or reduce the microbial load (Yang et al. 2016a), and so the contamination of these foods with pathogenic microorganisms, such as *E. coli*, represents a public health problem. For this reason, studies have been carried out in recent years, based on the application of plant extracts in RTE food with the purpose of inhibiting microbial growth (Nikmaram et al. 2018).

The consumption of cheese has increased significantly worldwide; consequently, the cheese industry has evolved, and research has an important role in increasing the product's quality and safety (Costa et al. 2018). In this context, *Butia odorata,* a native fruit of South America, showed antibacterial activity against foodborne bacteria (Maia et al. 2017), but no study on its application in foods was performed. For this reason, the aims of this study were to verify the occurrence of *Escherichia coli* in sliced mozzarella cheese marketed in Pelotas city, Brazil, and perform the phenotypic and genotypic characterization of the isolates. Besides that, to evaluate the susceptibility of *E. coli* to *Butia odorata* Barb. Rodr. extract, characterize it chemically, and apply the extract in sliced mozzarella cheese contaminated experimentally with *E. coli*.

Material and methods

Sampling

Samples of sliced mozzarella cheese (n=80) were collected in eight markets (A to H) in Pelotas city, Brazil, in 10 sampling events. The samples were kept in isothermal boxes with ice and immediately transported to the Laboratório de Microbiologia de Alimentos at the Universidade Federal de Pelotas.

Analysis of Escherichia coli

Escherichia coli was enumerated using 3M Petrifilm EC Plates according to AOAC 991.14 method (AOAC 2002). Briefly, 25 g of each sample was homogenized for 1 min with 225 mL buffered peptone water (BPW, Acumedia, USA). After that, serial dilutions were performed and 1 mL of each diluted sample was placed on Petrifilm plates with incubation at 35° ± 1°C for 24-48 h. Next, the characteristic colonies were selected according to the manufacturer's instructions.

Genotypic characterization of E. coli isolates

Genomic DNA was extracted according to Green and Sambrook (2012). Firstly, STES buffer, glass beads (Interprise USA Corporation, USA) and phenol-chloroform (1:1) (Synth, Brazil) were added to the pellet, followed by centrifugation. Next, absolute ethanol and 5M NaCl (Synth, Brazil) were added and, after centrifugation, the pellet was washed with 70% (v/v) ethanol, dried at 37 ºC, and resuspended with sterile ultrapure water (Promega, USA) plus RNAse (Invitrogen, USA).

The confirmation of the species of the isolates and the detection of virulence genes were performed by PCR assays. The *uspA* gene was used for confirmation of *E. coli* at species level; for identification of *E. coli* O157:H7, the genes $rfbE_{O157}$ and $fliC_{H7}$ were used; and the genes for ETEC (*Lt, St*), EHEC (*hlyA, stx1*, *stx2*), EIEC (*ial)*, EAEC (*eagg*), EPEC (*eae*), and DAEC (*daaD*) were evaluated (Table 1). Moreover, tests with antiserum *E. coli* O157, *E. coli* H7, EPEC and EIEC were performed.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by agar disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI 2018), evaluating ampicillin (10 µg), cefotaxime (30 µg), cefoxitin (30 µg), cephalothin (30 µg), gentamicin (10 µg), amikacin (10 µg), streptomycin (10 µg), tetracycline (30 µg), imipenem (10 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), sulfonamide (300 µg), trimethoprim (5 µg), and nitrofurantoin (300 µg) (Laborclin, Brazil).

Butia odorata extract preparation

The *Butia odorata* extract was prepared in a 500 mL Erlenmeyer flask, to which was added 30 g of lyophilized *B. odorata* fruit pulp and 300 mL of acetone. Then, it was placed in a shaker (190 rpm) for 2 h, filtered in filter paper and centrifuged for 20 min (6289 *g*), and the supernatant was rotary-evaporated at 30 °C to constant weight.

Chemical characterization of B. odorata extract by GC-MS

For chemical characterization of *B. odorata* extract the sample was diluted in ethyl acetate (2 $mg.mL^{-1}$) and injected by Split (1:50) in column RTx-5 with injection temperature of 280 °C. The analysis conditions used were: 60 °C/3 min, increased by 15 °C/min to 220 °C, 220 °C/10 min, increased by 15 °C/min up to 280 °C and kept at 280 °C for 5 min. The interface temperature was 250 °C and temperature of the ion source was 200 °C. For identification of the compounds library NIST-05 was used.

Antimicrobial activity of B. odorata extract

The isolates of *E. coli* and the standard strain *E. coli* NCTC 12900 stored at -80 ºC were cultured on Tryptic Soy agar (TSA, Acumedia, USA) and incubated at 37 ºC for 24 h. Minimum inhibitory concentration (MIC) was carried out in a 96-well microplate. For this, two-fold serial dilutions of *B. odorata* extract were prepared in Mueller Hinton broth (MH, Kasvi, Brazil) from 58 to 0.45 $mg.mL^{-1}$; then 10^6 $CFU.mL^{-1}$ of the bacterial inoculum was added and incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration which showed no visible growth or turbidity. For minimum bactericidal concentration (MBC), from the well where there was no visible growth, was sown into plates containing TSA, and incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration in which 99.9 % of cells initially inoculated were dead.

Application of B. odorata extract in sliced mozzarella cheese contaminated experimentally with E. coli

The *E. coli* isolate from sliced cheese that was most susceptible to *B. odorata* extract was selected for this experiment (lower MBC). Cheese (pH 5.3) was aseptically cut into pieces of 1 cm^2 and placed in Petri dishes. *Escherichia coli* was cultured on Tryptic Soy broth (TSB, Acumedia, USA) and incubated at 37 ºC for 24 h. Next, 20 µL of diluted bacterial suspension was put onto the surface of the cheese sample (about 10^4 $CFU.cm^{-2}$). After 20 min at room temperature, 20 µL of *B. odorata* extract was added (MIC, 2 x MIC, 4 x MIC or 8 x MIC) to the surface of the cheese and incubated at 4 °C. Cheese samples with the addition of 20 µL distilled water were used as negative control. The analyses were conducted at times 0, 3, 24, 48 and 72 h of incubation. For this, the samples were diluted in peptone water 0.1% (PW, Acumedia, USA), homogenized and serially diluted (1:9). An aliquot of 0.1 mL was seeded on Petri dishes containing Eosin Methylene Blue agar (EMB, Kasvi, Brazil) and incubated at 37 °C for 24 h. Three replications were performed.

Statistical analysis

Data from the *E. coli* counts were subjected to analysis of variance (ANOVA). Differences between treatments were assessed by Tukey test ($p < 0.05$).

Results and Discussion

Microbiological quality of mozzarella sliced cheese and characterization of E. coli isolates

Escherichia coli was isolated in 5% (4/80) of the cheese samples. The contaminated samples were obtained from market C (1/4), F (1/4), and H (2/4). Similar

results were observed by Wang et al. (2017), which found *E. coli* in 5.9% of the RTE food in Japan, while Zhang et al. (2016) found higher levels (39.2%) of *E. coli* in RTE products from China. It is noteworthy that different rates of *E. coli* contamination in foods can occur due to several factors, such as the type of sampling, number of samples, sanitary quality and geographical location (Zhang et al. 2016).

The four suspected *E. coli* isolates (E1-E4) were confirmed at species level by the presence of the *uspA* gene, although no genes from O157:H7 *E. coli* ($rfbE_{O157}$ and $fliC_{H7}$) or virulence genes (*St*, *Lt*, *ial*, *eagg*, *eae*, *stx1*, *stx2* and *hlyA*) were detected. Furthermore, the isolates were negative when tested with antiserum *E. coli* O157, *E. coli* H7, EPEC and EIEC, suggesting that they are non-pathogenic (Kaper et al. 2004). On the other hand, Yang et al. (2016b) reported diarrheagenic *E. coli* in 1.06% of the RTE products from China. Although in this study diarrheagenic *E. coli* was not isolated, the presence of generic *E. coli* in dairy products, such as cheese, is an important indicator of fecal contamination and poor hygienic practices (Ombarak et al. 2016).

Regarding antimicrobial susceptibility testing, isolates E2 and E4 showed intermediate resistance to streptomycin, and isolate E2 also showed intermediate resistance to cefoxitin. Isolate E3 was resistant to nitrofurantoin and nalidixic acid, and isolate E1 was susceptible to all antimicrobials evaluated (Table 2). Although the *E. coli* isolates were non-pathogenic, their resistance profile is of concern, since these isolates can harbor resistance genes that can be horizontally transferred to other bacteria in nature, in foods or even in the food processing environment (Haubert et al. 2018a).

Antimicrobial activity and chemical characterization of B. odorata extract

The *B. odorata* extract showed antimicrobial activity against all *E. coli* isolates, showing the MIC value of 15 $mg.mL^{-1}$. The MBC value was 58 $mg.mL^{-1}$ for most

isolates, except for isolate E3 (29 $mg.mL^{-1}$) (Table 2). Antibacterial activity of *B. odorata* was previously described by Maia et al. (2017) and Haubert et al. (2018b), but no study has been carried out with *E. coli* isolates.

The chemical characterization of *B. odorata* extract showed as major compounds Z-10-Pentadecenol (80.1%) and palmitic acid (19.4%). E-10-Pentadecenol was found in *Anthemis stiparum* subsp. *sabulicola*, a plant that presented good antimicrobial activity against *Staphylococcus aureus* and *Bacillus cereus* (Chemsa et al. 2018), and Z-10-Pentadecenol was found in *Hypericum adenotrichum*, a plant from Turkey used in folk medicine and that showed anti-growth effects on cancer cells lines (Sarimahmut et al. 2016). The second major compound present in *B. odorata* extract was palmitic acid. In a study made by Ivanova et al. (2017), palmitic acid nanostructures showed bactericidal activity against *Pseudomonas aeruginosa* and *S. aureus*.

Application of B. odorata extract in sliced mozzarella cheese experimentally contaminated with E. coli

The growth inhibition of *E. coli* in sliced cheese is showed in Figure 1. In the first 3 h of treatment there was one log reduction in *E. coli* counts utilizing 8 x MIC when compared to control. After 72 h there was a significant difference ($p<0.05$) in *E. coli* counts between control (2.8 log $CFU.cm^{-2}$) and treated samples with MIC, 2 x MIC, 4 x MIC and 8 x MIC (1.3, 1.4, 1.6 and 0.5 log $CFU.cm^{-2}$, respectively), being that with 8 x MIC there was a difference of 2.3 log $CFU.cm^{-2}$ in relation to control. This result is similar to that obtained by Harich et al. (2017), in a study that evaluated the antibacterial activity of cranberry juice concentrate in RTE foods, and observed a reduction of 2.5 log in *E. coli* after 7 days of storage at 4 °C. On the other hand, Palmeri

et al. (2018) found no significant difference in *Enterobacteriaceae* counts in sliced beef when applying prickly pear fruit extract after 8 days of storage at 4 ºC.

Many factors inherent to food can hinder the action of natural antibacterial compounds, including pH and food constituents, such as fat and proteins. For these reasons, the results obtained *in vitro* are often not reproduced *in situ*. In this study, however, even when the lower concentrations of extract were used, such as the MIC, inhibition of multiplication of *E. coli* in sliced cheese was obtained. These results are very interesting, since this bacterium is often isolated from cheese samples and some isolates can be pathogenic.

Conclusion

The isolation of *E. coli* in sliced mozzarella cheese in retail indicates fecal contamination and poor hygienic practices. *Butia odorata* extract, consisting mainly of Z-10-Pentadecenol and Palmitic acid, showed antimicrobial activity against *E. coli* both *in vitro* and *in situ*, indicating that it can be a good alternative for inhibiting the growth of this microorganism in sliced cheese.

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Table 1. Oligonucleotides used in this study

Target genes	Sequence (5' – 3')	Amplicon size (bp)	Reference
uspA	Fw: CCGATACGCTGCCAATCAGT Rv: ACGCAGACCGTAGGCCAGAT	884	Chen and Griffiths (1998)
rfbE$_{O157}$	Fw: GCGCGAATTCGTGCTTTTGA Rv: TATTTTTCCGAGTACATTGG	239	Ateba and Mbewe (2011)
fliC$_{H7}$	Fw: GCTGCAACGGTAAGTGAT Rv: GGCAGCAAGCGGGTTGGT	984	Ateba and Mbewe (2011)
stx1	Fw: ATAAATCGCCATTCGTTGACTAC Rv: AGAACGCCCACTGAGATCATC	180	Ateba and Mbewe (2011)
stx2	Fw: GGCACTGTCTGAAACTGCTCC Rv: TCGCCAGTTATCTGACATTCT	255	Ateba and Mbewe (2011)
Eae	Fw: GACCCGGCACAAGCATAAGC Rv: CCACCTGCAGCAACAAGAGG	384	Ateba and Mbewe (2011)
hlyA	Fw: GCATCATCAAGCGTACGTTCC Rv: AATGAGCCAAGCTGGTTAAGCT	534	Ateba and Mbewe (2011)
daaD	Fw: TGAACGGGAGTATAAGGAAG ATG Rv: GTCCGCCATCACATCAAAA	444	Guion et al. (2008)
Elt	Fw: GGCGACAGATTATACCGTGC Rv: CGGTCTCTATATTCCCTGTT	330	Pass et al. (2000)
Est	Fw: TTTCCCCTCTTTTAGTCAGTCAACTG Rv: GGCAGGATTACAACAAAGTTCACA	160	Pass et al. (2000)
Ial	Fw: CTGGTAGGTATGGTGAGG Rv: CCAGGCCAACAATTATTTCC	320	Nguyen et al. (2005)
Eagg	Fw: AGACTCTGGCGAAAGACTGTATC Rv: ATGGCTGTCTGTAATAGATGAGAAC	194	Kong et al. (2002)

Table 2. Antimicrobial resistance profile, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the *Escherichia coli* isolates for *Butia odorata* extract

Isolate ID	Antimicrobial resistance profile[a]	Minimum inhibitory concentration (MIC) ($mg.mL^{-1}$)	Minimum bactericidal concentration (MBC) ($mg.mL^{-1}$)
E1	-	15	58
E2	FOX (I); STR (I)	15	58
E3	NAL; NIT	15	29
E4	STR (I)	15	58
Escherichia coli O157:H7 NCTC 12900	-	15	15

[a]FOX: cefoxitin, STR: streptomycin, NAL: nalidix acid, NIT: nitrofurantoin.

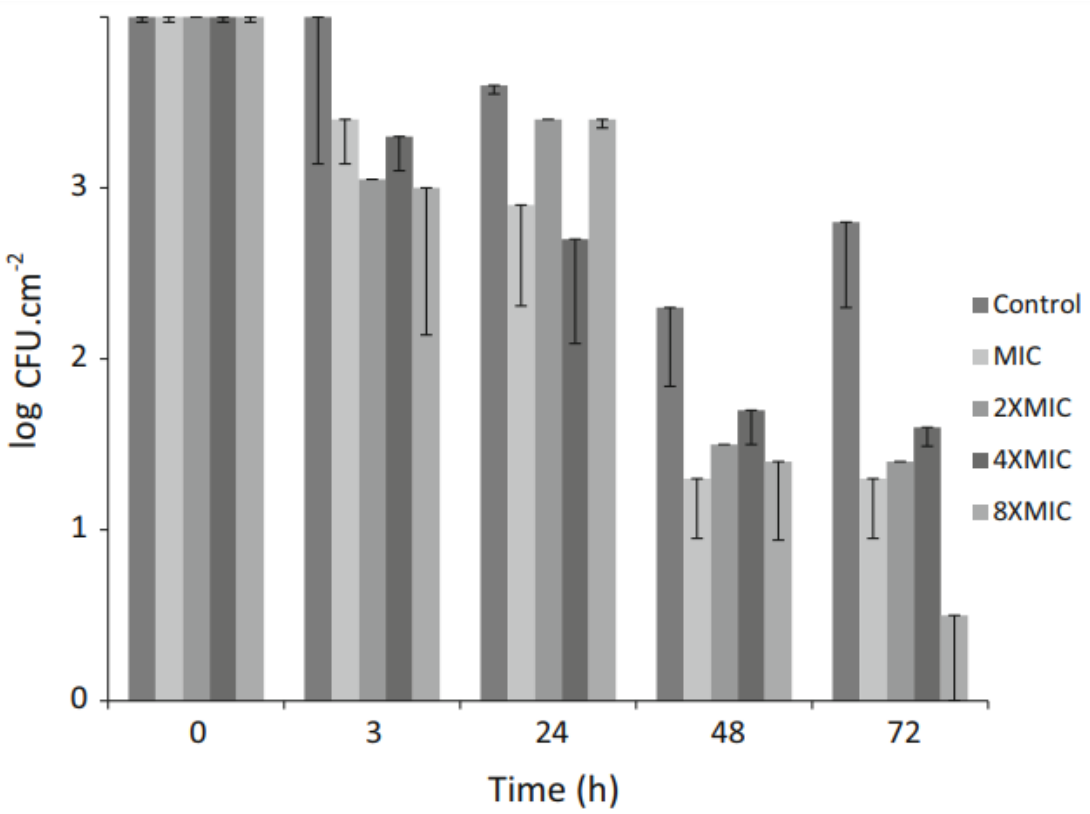

Figure 1 Inhibition of *Escherichia coli* in sliced cheese treated with *Butia odorata* extract

5 Manuscrito 2

Effect of *Butia odorata* Barb. Rodr. extract on biofilm of *Staphylococcus aureus* isolated from food poisoning outbreak

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Após as sugestões da banca o manuscrito será submetido ao periódico *Food Control*

Abstract

The aims of this study were to verify the ability of *S. aureus* isolates from food poisoning outbreaks to form biofilm, and evaluate the susceptibility of these isolates to *Butia odorata* extract (BOE) and its effect on *S. aureus* biofilm. Thirteen *S. aureus* isolates involved in food poisoning outbreaks in southern Brazil were selected. Biofilm formation was evaluated in 96-well microplates, and susceptibility to BOE was assessed by agar disk diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The effect of BOE on *S. aureus* biofilm cells was evaluated at 15, 30 and 60 min of contact. All 13 *S. aureus* isolates formed biofilm on polystyrene; and 84.6% (11/13) carried *icaA* and *icaB* genes, 69.2% (9/13) the *icaD* gene, and 61.5% (8/13) the *icaC* gene. All *S. aureus* isolates were susceptible to BOE, with inhibition zones varying from 13 to 32 mm, MIC values ranging from 2.8 to 11.4 $mg.mL^{-1}$, and MBC from 22.8 to 182 $mg.mL^{-1}$. The confocal laser scanning microscopy (CLSM) analysis showed that BOE damaged the cell membrane of *S. aureus*. The addition of MIC and 2 X MIC (60 min) decreased the number of biofilm cells by 99 and 99.99%, respectively; and using 4 X MIC (30 and 60 min) the reduction was 99.9%. All *S. aureus* isolates were biofilm formers on polystyrene, which causes concern because these isolates were involved in outbreaks. On the other hand, the isolates were susceptible to BOE, as the extract damaged the cell membrane of *S. aureus*. Besides that, BOE reduced the number of *S. aureus* biofilm cells, having the potential to be used as a sanitizer.

Keywords: Foodborne diseases; Plant extract; CLSM; *icaADBC*

1 Introduction

Foodborne diseases (FBD) encompass a wide spectrum of illness, constituting an increasing public health problem worldwide. Foods can be contaminated at any stage in the process from production to consumption (WHO, 2019). *Staphylococcus aureus* is a microorganism that contaminates foods and may cause FBD. In Brazil, the health authorities were notified of more than 12,000 FBD outbreaks between 2000 and 2017, with *S. aureus* among the main etiological agents (Brazil, 2018). It is noteworthy that in the United States of America, *S. aureus* is also among the main causes of FBD, causing around 241,000 illnesses per year (CDC, 2011). In the European Union, bacterial toxins, including toxins produced by *S. aureus*, were the second most frequently cause of FBD outbreaks in 2016 (EFSA/ ECDC, 2017).

Most of the pathogens that cause FBD, such as *S. aureus*, are able to form biofilm on most surfaces and under almost all the environmental conditions found in food industries (Bridier et al., 2015). A biofilm is an assemblage of microbial cells irreversibly associated with a surface and embedded in a matrix of primarily polysaccharide material (Donlan, 2002). In the process of biofilm formation, organic molecules from foods are deposited on the surfaces of equipment, and microorganisms are attracted to these surfaces. Next, some cells remain even after cleaning and sanitizing, and initiate growth. The attachment of pathogenic microorganisms to food-contact surfaces constitutes a problem, because biofilms provide a source of contamination (Shi & Zhu, 2009).

Different sanitizers are applied in the food industry in order to control biofilm formation and prevent food contamination (Silva, Camargo, Todorov, & Nero, 2016). However, several isolates have shown tolerance to these products (Haubert et al., 2018, Lee, Cappato, Corassin, Cruz, & Oliveira, 2016), and this can be one of the factors contributing to the involvement of microorganisms in FBD outbreaks (Lee et al., 2016). In this sense, many studies have emerged with the aim of proposing new antibacterial substances; and in view of the importance of biofilm formation by *S. aureus*, numerous studies have focused in its removal using plant extracts or individual constituents (Engel, Heckler, Tondo, Daroit, Malheiros, 2017; Oliveira, Fernandes Filho, Gomes, & Bergamasco, 2018; Vetas, Dimitropoulou, Mitropoulou, Kourkoutas, & Giaouris, 2017).

In this context, *Butia odorata* Barb. Rodr., a fruit native to South America, showed in vitro antibacterial activity against foodborne pathogens (Haubert et al., 2018), including *S. aureus* (Maia, Aranha, Chaves, & Silva, 2017). In a recent study, *B. odorata* extract inhibited *Escherichia coli* growth in sliced cheese (Maia, Haubert, Soares, Würfel, & Silva, 2019). However, until the present moment no research has been carried out to evaluate its potential to biofilm removal, with the aim of applying the extract on surfaces in food processing lines. Thus, the aims of this study were to verify the ability of *S. aureus* isolates from food poisoning outbreaks to form biofilm, to evaluate the susceptibility of these isolates to *B. odorata* extract and to assess the effect of the same extract on *S. aureus* biofilm.

2 Material and methods

2.1 Isolates and cultivation conditions

Thirteen *S. aureus* isolates involved in food poisoning outbreaks in southern Brazil and molecularly characterized were selected (Bastos, Bassani, Mata, Lopes, & Silva, 2017). *Staphylococcus aureus* strain FRI S6 (carrying the *sea* and *seb* genes) and *S. aureus* FRI 361 (carrying the *sed* gene) were used as reference strains. The microorganisms stored at -80 °C were cultured on Tryptic Soy agar (TSA, Acumedia, USA) and incubated at 37 ºC for 24 h.

2.2 Biofilm formation ability

The biofilm formation ability was tested in 96-well microplates according to Stepanovic et al. (2007), with adaptations. In Tryptic Soy broth (TSB, Acumedia, USA) supplemented with 1% (w/v) glucose (Synth, Brazil) the bacterial inoculum (10^6 CFU.mL^{-1}) was added and incubated at 25 °C for 24 h. After that, three washes with phosphate buffer solution (PBS, Laborclin, Brazil) were carried out and fixation with methanol (Synth, Brazil) for 20 min took place, followed by drying overnight at room temperature. Then, cells were stained with crystal violet (Laborclin, Brazil) (15 min), resolubilized in ethanol 95% (v/v) (Synth, Brazil) and incubated for 30 min at room temperature. The reading was performed in a microtiter-plate reader (Robonik, India) at 595 nm.

2.3 Detection of *S. aureus* biofilm-related genes

Genomic DNA was extracted following the protocol described by Green and Sambrook (2012) with minor modifications. Firstly, a cell suspension was centrifuged at 13,000 x *g* for 3 min, and the supernatant removed. Were added 100 µL STES buffer, 100 µL glass beads (Interprise USA Corporation, USA)

and 150 µL phenol-chloroform (1:1) (Synth, Brazil), vortexed for 1min, followed by centrifugation at 13,000 x *g* for 5 min. The supernatant was transferred to a new microtube, containing absolute ethanol (Synth, Brazil) (2 times the collected volume) and 5M NaCl (Synth, Brazil) (0.1 times the collected volume), maintained at -20 °C for 1h. It was then centrifuged at 13,000 x *g* for 20 min and the supernatant was removed. The obtained pellet was washed twice with 125 µL of 70% (v/v) ethanol and conditioned at 37 °C until complete drying. The pellet was then resuspended with 35µL sterile ultrapure water (Promega, USA) plus 1µL RNAse (Invitrogen, USA), and stored at -20 °C.

Next, the presence of genes involved in biofilm formation was evaluated by PCR, according to the referenced authors shown in Table 1. The reaction mixtures contained 12.5 µL of GoTaq® Green Master Mix 2x (Promega, USA), 1 µL of each primer at a concentration of 10 pmol.µL^{-1}, 2 µL of DNA (10 ng.µL^{-1}) and 8.5 µL of ultrapure water to a total volume of 25 µL. The mixtures were subjected to a thermocycler MJ Research® PTC 100. Afterwards, the PCR products were subjected to electrophoresis at 80 V for 70 min in a 1.5% (w/v) agarose gel (Invitrogen, USA) in 0.5 Tris/Acetate/EDTA buffer (TAE) using 1 kb molecular weight marker (Invitrogen, USA). The amplified products were visualized in an UV transilluminator (Loccus, Brazil).

2.4 *Butia odorata* extract preparation

The extract was prepared according to Maia, Haubert, Soares, Würfel, & Silva (2019). Firstly, 30 g of lyophilized *B. odorata* and 300 mL of acetone (Synth, Brazil) were added in an Erlenmeyer flask, and placed in a SL 223 shaker (190 rpm) (Solab, Brazil) for 2 h. Afterwards, it was centrifuged at 7500

rpm for 20 min, and the supernatant was filtered in filter paper and rotary-evaporated (Heidolph, Laborota 4000, Sigma-Aldrich, USA) to constant weight.

2.5 Agar disk diffusion method

The inoculum was standardized at a concentration of 10^8 CFU.mL^{-1} and plated on Petri dishes containing Mueller-Hinton agar (MH, Kasvi, Brazil). Sterile paper filter disks (6 mm) impregnated with 20 µL of BOE were placed on the agar, with subsequent incubation at 37 ºC for 24 h. As the control, streptomycin disks (10 µg) and disks impregnated with water were used. The results were expressed as mean ± standard deviation of the diameter of the zones of inhibition.

2.6 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of BOE

The MIC was performed in 96-well microplates. Two-fold serial dilutions of BOE were prepared in Mueller Hinton broth (MH, Kasvi, Brazil), varying from 182 to 1.4 mg.mL^{-1}. Next, 10^6 CFU.mL^{-1} of the bacterial inoculum was added, followed by incubation at 37 °C for 24 h. The MIC was defined as the lowest concentration with no visible growth. For MBC, from the well where there was no visible growth, this was sown into plates containing TSA, and incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration in which 99.9 % of cells initially inoculated were dead.

2.7 Confocal laser scanning microscopy (CLSM) analysis

For CLSM analysis, the isolate that showed most biofilm formation ability was selected (topic 2.2). *Staphylococcus aureus* cells were grown until the logarithmic growth stage in BHI (Kasvi, Brazil), treated with BOE at the concentrations of MIC, 2 x MIC and 4 x MIC, and then incubated at 37 °C for 8 h. Non-treated cells were used as control. After centrifugation (5,000 *g*/5 min) and washing with PBS, cells were stained with SYTO 9 and propidium iodide, according to the manufacturer's recommendations (LIVE/DEAD™ BacLight™ - Invitrogen, USA). Next, cells were washed twice with PBS and observed with a Leica TCS SP8 confocal laser scanning microscope (Germany) at 100 x magnification.

2.8 Effect of BOE on *S. aureus* biofilm

To evaluate the effect of BOE on *S. aureus* biofilm cells, the isolate that showed most biofilm formation ability (topic 2.2) was selected. To Petri dishes was added 9 mL TSB (Acumedia, USA) supplemented with 1% glucose, 1 mL bacterial inoculum standardized at 0.5 McFarland scale with incubation at 25 °C for 24 h. Next, two washes with PBS were done; 10 mL distilled water and BOE were added at concentrations of MIC, 2X MIC and 4X MIC, for incubation at 25 °C, for 15, 30 and 60 min. Petri dishes with only 10 mL distilled water added were used as control. Then, one wash with PBS was performed and attached cells were removed using a swab. After agitation for 1 min, serial dilutions were performed and samples were plated in TSA and incubated at 37 °C for 24 h.

2.9 Statistical analysis

Data were submitted to a one-way analysis of variance (ANOVA) ($p \leq 0.05$) using STATISTICA software version 6.1 (StatSoft, France).

3 Results and Discussion

3.1 Biofilm formation ability and presence of biofilm-related genes

All *S. aureus* isolates evaluated were biofilm-producers on polystyrene, being S7 isolate the most producer. These results were similar to those found by Rodrigues et al. (2017), where 97.2% of the *S. aureus* isolates from food-contact surfaces were biofilm-producers on a microtiter plate. Moreover, Torlak, Korkut, Uncu, & Sener (2017) found that 90.6% of *S. aureus* isolates had the ability to form biofilm on polystyrene. In another study, 57% of the *S. aureus* isolates formed biofilm on polystyrene (Naicker, Karayem, Hoek, Harvey, & Wasserman, 2016). Biofilm formation is important for the survival of staphylococci in a food-processing environment. The biofilm is a protection for bacteria, and in this condition it is generally more resistant to sanitizers used in industries than planktonic cells (Møretrø et al., 2003). In this study, the isolates tested for biofilm formation ability are enterotoxigenic and caused outbreaks in Brazil (Bastos et al., 2017). It is noteworthy that biofilm capacity is a virulence strategy, and this factor can contribute to the occurrence of FBD outbreaks.

Regarding the presence of *S. aureus* biofilm-related genes, 84.6% (11/13) of isolates carried *icaA* and *icaB* genes, 69.2% (9/13) the *icaD* gene, and 61.5% (8/13) the *icaC* gene. Besides that, 15.4% (2/13) of isolates did not carry genes of the *icaADBC* locus. Colonization and biofilm formation take place in a series of sequential stages; under experimental conditions, first the attachment to the surface occurs, followed by intercellular adhesion (Heilmann

et al., 1996). The importance of the *ica* operon is mediating the polysaccharide intercellular adhesion (PIA) production (Chaieb, Mahdouani, & Bakhrouf, 2005). Although the importance of *ica* genes locus is well known, some isolates did not carry genes of this locus and were biofilm formers. This happens because biofilm formation can be *ica*-dependent or *ica*-independent, where in this last case other genes are involved in the biofilm formation process (Doulgeraki, Ciccio, Ianieri, & Nychas, 2017).

3.2 Antibacterial activity of *B. odorata*

According to the agar disk diffusion test, all *S. aureus* isolates were susceptible to BOE, with inhibition zones varying from 13 to 32 mm. The MIC values ranged from 2.8 to 11.4 $mg.mL^{-1}$, and MBC from 22.8 to 182 $mg.mL^{-1}$ (Table 2). Higher MIC values were found by Zambrano et al. (2019), in a study that tested antimicrobial activity of grape, apple and pitahaya residue extracts with MIC values for *S. aureus* varying from 25 to >100 $mg.mL^{-1}$, while in another study young astringent persimmon tannin showed MIC of 1000 $\mu g.mL^{-1}$ against methicillin-resistant *S. aureus* (MRSA) strains (Liu et al., 2019).

Several studies reported the antibacterial activity of different types and parts of plants, such as herbs, spices, leaves, seeds and fruits, against foodborne bacteria (Maia, Lopes, & Silva, 2017). The extract of *Morinda citrifolia* Linneo (“noni”) seeds, for example, inhibited methicillin-resistant *Staphylococcus* spp. (MIC 16 $mg.mL^{-1}$) (Cruz-Sánchez et al., 2019), and *Psidium guajava* leaf extract inhibited MRSA growth (Chakraborty, Afaq, Singh, & Majumdar, 2018). The antibacterial activity of BOE against *S. aureus*

reference strains was also reported (Maia et al., 2017); however, its antibacterial activity against foodborne isolates was not evaluated.

With the purpose of evaluating whether BOE caused damage to the cell membrane of *S. aureus* (S7 isolate), CLSM analysis was performed, using two dyes, SYTO 9 and propidium iodide. SYTO 9 stains all bacterial cells, while propidium iodide penetrates only where the bacterial membrane is damaged. Then, bacterial cells with intact membranes are stained only by SYTO 9 and emit green fluorescence, and bacterial cells with damaged membrane are stained by propidium iodide, emitting red fluorescence (Liu et al., 2017). As showed in Figure 1, untreated cells emitted only green fluorescence. However, when BOE was added, the intensity of green fluorescence decreased, and the intensity of red fluorescence increased according to increased concentration of BOE. This result indicates that BOE caused damage to the cell membrane of *S. aureus,* and this was concentration-dependent. In a previous study, it was determined that BOE is composed of Z-10-Pentadecenol (80.1%) and palmitic acid (19.4%) (Maia et al., 2019). It is known that alcohols have antibacterial activity, and they act by denaturing proteins. Recently it was reported that palmitic acid nanostructures have bactericidal activity against *S. aureus* (Ivanova et al., 2017). This can explain the damage to the cell membrane of *S. aureus* induced by BOE observed in the present study.

3.3 Effect of BOE on *S. aureus* biofilm cells

The effect of BOE against *S. aureus* (S7 isolate) biofilm cells was evaluated at 15, 30, and 60 min of contact using MIC, 2 X MIC and 4 X MIC. When 4 X MIC was used, there was a significant difference ($p<0.05$) between

control and treated samples at all evaluated times, and at 30 and 60 min there was a reduction of 99.9% in the number of biofilm cells. At 60 min of contact and addition of 2 X MIC there was a decrease of 4.21 log $CFU.cm^{-2}$ (99.99%) in relation to the control, and with addition of MIC value the difference was 1.1 log $CFU.cm^{-2}$ (99%) (Figure 2).

According to Figure 2, although the utilization of BOE resulted in significant reductions in *S. aureus* biofilm cells, no treatment fully destroyed biofilm cells. The same behavior was demonstrated by Vetas et al. (2017), where sage and spearmint essential oils reduced *S. aureus* biofilm cells by 1.9-3.0 and 0.8-1.2 log $CFU.cm^{-2}$, respectively. In another study, *Moringa oleifera* extract (3.125 $\mu g.mL^{-1}$) reduced *S. aureus* biofilm by around 2 log $CFU.cm^{-2}$ (30 min contact) on stainless steel and polyvinyl chloride (PVC) (Oliveira, Fernandes Filho, Gomes, & Bergamasco, 2018). Similar to what was observed in this study, Vázquez-Sánchez, Galvão, Mazine, Gloria, & Oetterer (2018) tested an exposure of 30 min to *Lippia sidoides*, *Thymus vulgaris* and *Pimenta pseudochariophyllus* essential oils in 24 h-old *S. aureus* biofilm formed at 25 °C, and all essential oils were able to reduce the number of biofilm cells by 99.99%.

A study evaluating the effect on *S. aureus* biofilm of peracetic acid (30 $mg.L^{-1}$) and sodium hypochlorite (250 $mg.L^{-1}$) , sanitizers commonly used in food industries, demonstrated that these compounds did not fully eliminate biofilm cells (Meira, Barbosa, Athayde, Siqueira-Júnior, & Souza, 2012). This data shows the difficulty of eradicating *S. aureus* biofilm in the food industry environment and confirms that cells in sessile communities are more tolerant to sanitizers than in planktonic form. However, the present study demonstrated

a reduction in the number of biofilm cells, induced by BOE, indicating that it has the potential to be used as a sanitizer.

4 Conclusion

All *S. aureus* isolates were able to form biofilm on polystyrene, which is a hazard since these bacteria are enterotoxigenic and were involved in outbreaks that have occurred in Brazil. The isolates were susceptible to BOE, and the extract caused damage in the cell membrane of *S. aureus*. Besides that, BOE reduced the number of *S. aureus* biofilm cells, having the potential to be used as a sanitizer.

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Table 1 Oligonucleotides used in this study

Target genes	Sequence (5’ – 3’)	Amplicon size (bp)	Reference
icaA	Fw: GAGGTAAAGCCAACGCACTC Rv: CCTGTAACCGCACCAAGTTT	151	Atshan et al., 2013
icaD	Fw: ACCCAACGCTAAAATCATCG Rv: GCGAAAATGCCCATAGTTTC	211	Atshan et al., 2013
icaB	Fw: ATACCGGCGACTGGGTTTAT Rv: TTGCAAATCGTGGGTATGTGT	140	Atshan et al., 2013
icaC	Fw: CTTGGGTATTTGCACGCATT Rv: GCAATATCATGCCGACACCT	209	Atshan et al., 2013

Table 2 Inhibition zone, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *B. odorata* extract against *S. aureus* isolates causers food poisoning outbreaks

Isolate	Inhibition zone (mm)	MIC ($mg.mL^{-1}$)	MBC ($mg.mL^{-1}$)
S1	32±2.8	2.8	22.8
S2	32±0.0	5.7	91
S3	31±1.4	5.7	22.8
S4	30±1.4	5.7	91
S5	13±4.2	5.7	182
S6	21.5±2.1	2.8	91
S7	22.5±2.1	5.7	91
S8	24±0.0	5.7	91
S9	18.5±0.7	11.4	22.8
S10	24±0.0	11.4	22.8
S11	26.5±2.1	5.7	22.8
S12	17±4.2	5.7	91
S13	27±1.4	5.7	22.8
S. aureus FRI S6	27.5±0.7	5.7	5.7
S. aureus FRI 361	23.5±0.7	5.7	45.5

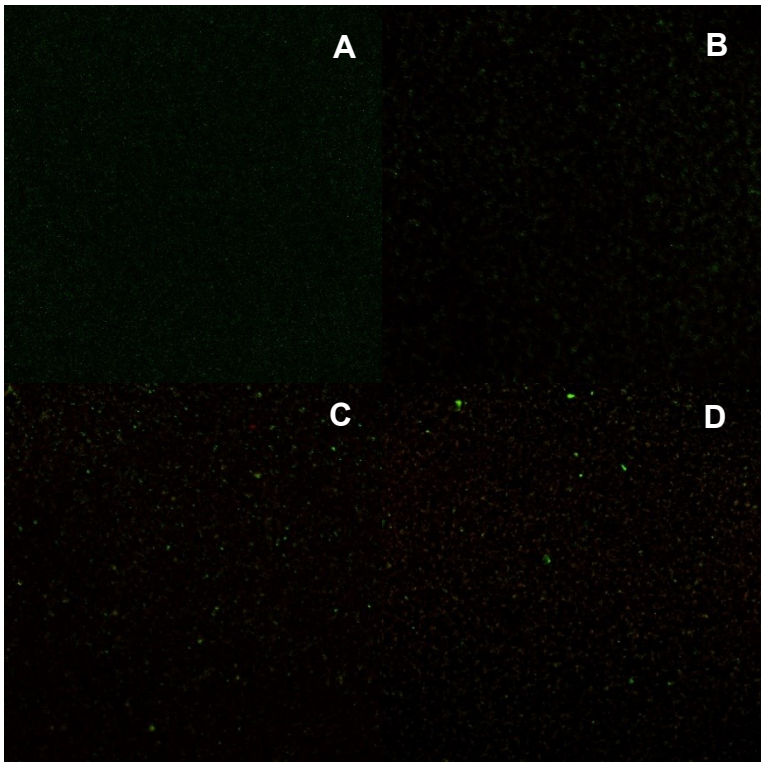

Figure 1 Confocal laser scanning microscopic of *S. aureus* (isolate S7). Control group (A) and *B. odorata* treatments for 8 h at MIC, 2 X MIC and 4 X MIC (B, C and D, respectively)

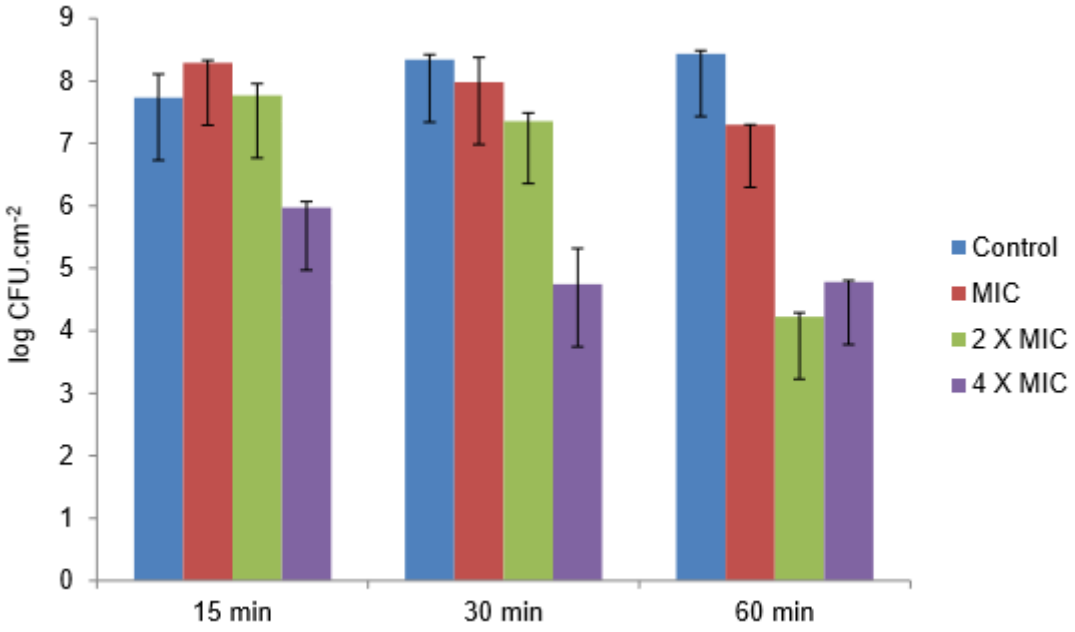

Figure 2 Effect of *B. odorata* on *S. aureus* (isolate S7) biofilm cells

6 Considerações finais

O EBO possui atividade antibacteriana contra micro-organismos patogênicos veiculados por alimentos, tendo potencial para ser utilizado como alternativa aos conservantes sintéticos. Além disso, este estudo demonstrou que a atividade antibacteriana do EBO contra *S. aureus* e *E. coli*, usados como modelos de bactérias Gram-positivas e Gram-negativas, ocorre por duplo mecanismo; causa danos na membrana bacteriana e no DNA genômico. O EBO, consistindo principalmente de Z-10-Pentadecenol e ácido palmítico, apresentou atividade antibacteriana em queijo mussarela fatiado, contaminado experimentalmente com *E. coli*, indicando que pode ser uma alternativa para inibir o desenvolvimento deste micro-organismo nesse tipo de alimento. Além disso, o EBO reduziu o número de células de *S. aureus* em biofilme, tendo potencial para ser utilizado como sanitizante.

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