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

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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 used as marker for *E. coli* O157:H7 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 between control (2.8 log CFU cm⁻²) and treated samples with MIC, 2 × MIC, 4 × MIC and 8 × 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 (Omarak 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

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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, *B. 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 *E. 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 *B. odorata* Barb. Rodr. extract, characterize it chemically, and apply the extract in sliced mozzarella cheese contaminated experimentally with *E. coli*.

Materials 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 *E. coli*

Escherichia coli was enumerated using 3 M 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 $35^{\circ} \pm 1^{\circ}\text{C}$ 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 5 M NaCl (Synth, Brazil) were added and, after centrifugation, the

pellet was washed with 70% (v/v) ethanol, dried at 37°C , and resuspended with sterile ultrapure water (Promega, USA) plus RNase (Invitrogen, USA).

The confirmation of the isolates at species level 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 *B. 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: $60^{\circ}\text{C}/3$ min, increased by $15^{\circ}\text{C}/\text{min}$ to 220°C , $220^{\circ}\text{C}/10$ min, increased by $15^{\circ}\text{C}/\text{min}$ 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°C were cultured on Tryptic Soy agar (TSA, Acumedia, USA) and incubated at 37°C for 24 h.

Table 1 Oligonucleotides used in this study

Target genes	Sequence (5′–3′)	Amplicon size (bp)	References
<i>uspA</i>	Fw: CCGATACGCTGCCAATCAGT Rv: ACGCAGACCGTAGGCCAGAT	884	Chen and Griffiths (1998)
<i>rfbE_{O157}</i>	Fw: GCGCGAATTCGTGCTTTTGA Rv: TATTTTCCGAGTACATTGG	239	Ateba and Mbewe (2011)
<i>fliC_{H7}</i>	Fw: GCTGCAACGGTAAGTGAT Rv: GGCAGCAAGCGGGTTGGT	984	Ateba and Mbewe (2011)
<i>stx1</i>	Fw: ATAAATCGCCATTCGTTGACTAC Rv: AGAACGCCCCACTGAGATCATC	180	Ateba and Mbewe (2011)
<i>stx2</i>	Fw: GGCAGTGTCTGAACTGCTCC Rv: TCGCCAGTTATCTGACATTCT	255	Ateba and Mbewe (2011)
<i>eae</i>	Fw: GACCCGGCACAAGCATAAGC Rv: CCACCTGCAGCAACAAGAGG	384	Ateba and Mbewe (2011)
<i>hlyA</i>	Fw: GCATCATCAAGCGTACGTTCC Rv: AATGAGCCAAGCTGGTTAAGCT	534	Ateba and Mbewe (2011)
<i>daaD</i>	Fw: TGAACGGGAGTATAAGGAAGATG Rv: GTCCGCCATCACATCAAAA	444	Guion et al. (2008)
<i>Elt</i>	Fw: GGCGACAGATTATACCGTGC Rv: CGGTCTCTATATCCCTGTT	330	Pass et al. (2000)
<i>Est</i>	Fw: TTTCCCCTCTTTTAGTCAGTCAACTG Rv: GGCAGGATTACAACAAAGTTCACA	160	Pass et al. (2000)
<i>ial</i>	Fw: CTGGTAGGTATGGTGAGG Rv: CCAGGCCAACAATTATTTCC	320	Nguyen et al. (2005)
<i>eagg</i>	Fw: AGACTCTGGCGAAAGACTGTATC Rv: ATGGCTGTCTGTAATAGATGAGAAC	194	Kong et al. (2002)

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 10⁶ 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 cm² and placed in Petri dishes. *Escherichia coli* was cultured on Tryptic Soy broth (TSB, Acumedia,

USA) and incubated at 37 °C for 24 h. Next, 20 µL of diluted bacterial suspension was put onto the surface of the cheese sample (about 10⁴ CFU cm⁻²). After 20 min at room temperature, 20 µL of *B. odorata* extract was added (MIC, 2 × MIC, 4 × MIC or 8 × 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 gene used as marker for *E. coli* O157:H7 (*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 Fig. 1. In the first 3 h of treatment there was one log reduction in *E. coli* counts utilizing 8 × 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 × MIC, 4 × MIC and 8 × MIC (1.3, 1.4, 1.6 and 0.5 log

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

Isolate ID	Antimicrobial resistance profile ^b	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
<i>Escherichia coli</i> O157:H7 NCTC 12900 ^a	–	15	15

^aReference strain

^bFOX cefoxitin, STR streptomycin, NAL nalidix acid, NIT nitrofurantoin, –: sensible, (I) intermediate

Fig. 1 Inhibition of *Escherichia coli* (E3 isolate) in sliced cheese treated with *B. odorata* extract

CFU cm⁻², respectively), being that with 8 × 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 °C.

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-Pentadecanol 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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