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Effect of Eugenol on Growth and Listeriolysin O Production by *Listeria monocytogenes*

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ABSTRACT

The inhibitory effect of eugenol, a naturally occurring compound mainly present in the essential oil fraction of cloves, was studied on the growth and listeriolysin O (LLO) production by Listeria monocytogenes. Potassium efflux from cells promoted by eugenol was also determined after 24 h incubation in phosphate buffered saline. Eugenol promoted a delay on the growth of L. monocytogenes at concentrations of 100, 300 and 500 μg mL^{-1}and above 800 μg mL^{-1} the effect was bactericidal. Production of LLO by L. monocytogenes in the presence of eugenol was reduced 80-100%. An accumulation of external K^+ was observed above 300 μg mL^{-1} of eugenol which indicated that the cell membrane was affected. The results showed the effectiveness of eugenol in controlling growth and LLO production of L. monocytogenes cells.

Key words: Eugenol, *Listeria monocytogenes*, antilisteric, LLO

INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative anaerobic rod and psychrotrophic bacterium, which is associated with foodborne disease. This pathogen produces several characterized virulence factors. Listeriolysin O (LLO) is considered the major of them and is produced by all pathogenic strains of *L. monocytogenes* (Dimmig et al., 1994; Kim et al., 1994; Giammarini et al., 2004). The environmental conditions in which L. *monocytogenes* can grow affect its virulence and LLO production (McKellar, 1993; Dimmig et al., 1994; Kim et al., 1994).

The ubiquitous distribution of this pathogen in nature, its ability to proliferate at refrigeration temperature and its tolerance to certain preservatives have resulted in an extensive effort to develop processes to control its growth in foods. Mild preservation technologies are becoming more important in modern food industries, and essential oils should be an alternative to combine mild process to obtain safe products (Ultee et al., 1999). The antilisterial properties of these components have been described. Essential oils of cinnamon, clove, oregano, pimento and thyme showed antilisteric effectiveness in tryptone soy broth (Aureli et al., 1992). In minced pork meat added of thyme oil, the population of *L. monocytogenes* was reduced approximately 100 fold over the first week of storage (Aureli et al., 1992). Rosemary oil, at 10 μLL^{-1} in brain heart infusion was listeriostatic after 24 h of incubation (Pandit and Shelef, 1994). Clove and its essential oil had been

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considered by Smith-Palmer et al. (1998) as the most applicable spices to control of *L. monocytogenes* in foods. Clove oil at 1% reduced the number of *L. monocytogenes* of 10^6 to < 1 $\log_{10}$ CFU (colony forming unit) g^{-1} in low and full fat cheese (Smith-Palmer et al., 2001). The antilisteric activity of clove oil was confirmed in cheese and meat when added at concentration of 0.5% and 1% (Menon and Garg, 2001). Other pathogens and foodborne microorganisms also showed sensitivity to this spice or its essential oil (Stecchini et al., 1993; Bara and Vanetti, 1995; Smith-Palmer et al., 1998; Suhr and Nielsen, 2003).

The mechanism of action of the antimicrobial activity of botanical biopreservatives is not fully understood (Draughon, 2004). The antimicrobial effect of clove is attributed to eugenol, which is the major active constituent of its essential oil (Pruthi, 1980). Although the exact inhibitory action of eugenol on microorganisms has not yet been established, it is widely believed that its action would be similar to the other phenolic compounds exhibiting antimicrobial activity. The microbial inhibition of eugenol might be related to the membrane disruption or, according to Wendakoon and Sakaguchi (1993), by inactivation of enzymes and genetic materials. A better knowledge of the mode of action of eugenol on microbial cells is important regarding its application in food systems.

This study was conducted to determine the activity of eugenol on liquid media on *L. monocytogenes* cells and its effect on LLO production.

MATERIALS AND METHODS

Strain and culture conditions

L. monocytogenes strain Scott A was obtained from the Department of Food Science and Nutrition at São Paulo University (SP, Brazil). Stock cultures were maintained on tryptone soy agar (TSA Oxoid, Basingstoke, HA) slants at 5 ^oC and transferred monthly. Cells were activated in tryptone soy broth - TSB at 37 $^oC \pm 2$ ^oC for 18 h. A suspension of 10^8 CFUmL^{-1} in 0.85% saline solution was prepared and used as inoculum.

Determination of effect of eugenol against *L. monocytogenes*

Eugenol (2-methoxy-4-[2proenyl]phenol) was obtained from Sigma Chemical Co. (St Louis, MO). A stock solution was prepared by dissolving 100 mg of eugenol in 10 mL of 95% ethanol. Aliquots were added to sterilized and cooled proteose peptone broth - PPG (Geoffroy et al., 1989) to give the final concentrations of 100, 300, 500, 800 and 1000 μg mL^{-1}. In order to detect the absence of antibacterial activity of ethanol, the experiments were performed with a control, with the solvent added to the medium.

The bacterial suspension was diluted to 10^7 CFU mL^{-1} in saline solution and 100 μL were used to inoculate 5 mL of broth. Cultures were incubated at 37 ^oC and growth was determined at 600 nm (OD_{600}) using a Spectronic 20 (Milton Roy, Rochester, NY) spectrophotometer. The viability of the cells treated with eugenol was evaluated by colony counts on the surface of TSA plates.

At 24 h incubation, 1.5 mL of cultures were centrifuged at 10,000 *g* for 5 min to remove cells. The supernatants were filter sterilized and used as the source of LLO.

LLO assay

LLO activity was determined using sheep red blood cells as described by McKellar (1992). The percentage of haemolysis was determined by comparison with a control without eugenol.

Determination of extracellular K^+

The efflux of K^+ ions from *L. monocytogenes* treated with eugenol was determined by estimating potassium concentration in the external cell-buffer system. Overnight cells grown on PPG were harvested by centrifugation (16,000 *g*, 10 min) and washed in 0.85% saline solution. The cell mass obtained was resuspended in 5 mL of phosphate buffered saline (PBS) (McKellar, 1992), pH 7.4 containing 100, 300, 500, 800 and 1000 μg mL^{-1} of eugenol. After 24 h incubation at 37 ^oC cells were removed by filtration and K^+ ions determined by atomic absorption spectrophotometer (Perkin Elmer, Brookfield, CT). A standard calibration curve was made with KCl solutions.

All the experiments were performed in two sets, in duplicate and the data expressed as the average of the results.

RESULTS

Concentrations up to 300 μg mL^{-1} eugenol extended the lag phase but did not cause changes in the final OD_{600} of cultures of *L. monocytogenes* (Fig. 1). No growth was detected in PPG broth with 800 or 1000 μg mL^{-1} eugenol over 48 h incubation and some decreased in OD_{600} indicating cell lysis was found at highest eugenol concentration (Fig. 1). The influence of ethanol used as eugenol diluent on the growth of *L. monocytogenes* was examined and it was found that the addition of up 1000 μg mL^{-1} ethanol to the PPG broth did not cause any changes in the growth curve of *L. monocytogenes* (data not shown). In order to verify if eugenol was bacteriostatic or bactericidal, the viable cell number was determined.

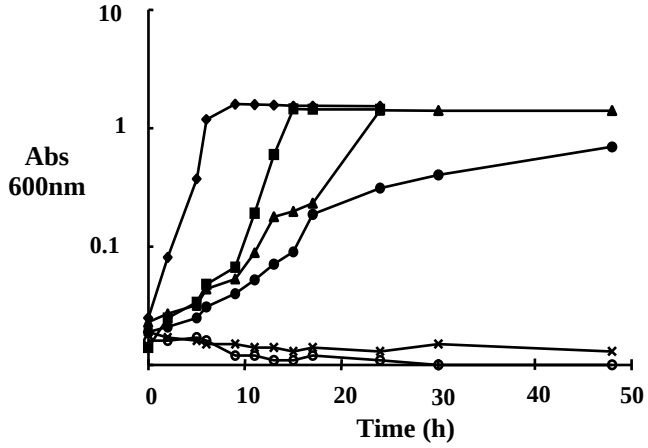

Figure 1 - Growth of *Listeria monocytogenes* Scott A at 37 °C in proteose pepton broth containing eugenol. (♦) Control; (■) 100 μg mL^{-1}; (▲) 300 μgmL^{-1} (●) 500 μg mL^{-1}; (**x**) 800 μg mL^{-1}; (○) 1000 μg mL^{-1}.

The bactericidal effect of eugenol was evidenced at 800 μg mL^{-1} when a reduction of viable counts of *L. monocytogenes* was about 6 log cycles after 10 h incubation (data not shown). At the same time, no viable cells were detected in the presence of 1000 μg mL^{-1}. When cells were maintained for 24 h at 37 °C in PBS buffer containing 100 μg mL^{-1} eugenol, no K^+ was lost. However, in the presence of over 300 μg mL^{-1} eugenol, an accumulation of external K^+ was observed (Fig. 2). Eugenol increased the K^+ ion permeability of *L. monocytogenes* cells in a concentration-dependent way and the addition of a bactericidal concentration of eugenol (800 and 1000 μg mL^{-1}) greatly increased the released of K^+ in PBS buffer.

The influence of eugenol on LLO activity was also examined and it was observed that the production of LLO was remarkably reduced after 24 h incubation in the presence of eugenol and complete inhibition was found with 500 μg mL^{-1} (Fig. 2).

DISCUSSION

Previous studies demonstrated that clove has listericidal effect and our results confirmed that eugenol, a major constituent of essential oil of clove, could respond to this effect. The bactericidal activity of clove against foodborne pathogens, like *L.monocytogenes* was reported in TSB by Ting and Deibel (1992) and in saline solution by Aureli et al. (1992). Stecchini et al. (1993) sugested a marked reduction in the number of *Aeromonas hydrophila* in meat samples treated with clove oil. In combination with NaCl, clove showed a bactericidal effect upon *Enterobacter aerogenes* in mackerel

muscle broth (Wendakoon and Sakaguchi, 1993). Clove extract at 2000 μg mL^{-1} showed bactericidal activity towards *Yersinia enterocolitica* in TSB (Bara and Vanetti, 1995). Eugenol was effective at reducing the growth of *L. monocytogenes* on cooked beef stored at 5 or 15 °C (Hao et al., 1998). Results presented by Smith-Palmer et al. (1998) established that the essential oil of clove was among the most applicable oil for the control of *L. monocytogenes* as it retained their low bacteriostatic and bactericidal concentrations even at 4 °C. Bactericidal concentrations of clove essential oil against *L. monocytogenes* was 400 μg mL^{-1} at 4 °C and 500 μg mL^{-1} at 35 °C in TSB broth. In full-fat cheese, clove oil was the only oil, among four oils tested, which reduced *L. monocytogenes* number of 10^6 CFU mL^{-1} to < 1.0 CFU mL^{-1} (Smith-Palmer et al., 2001).

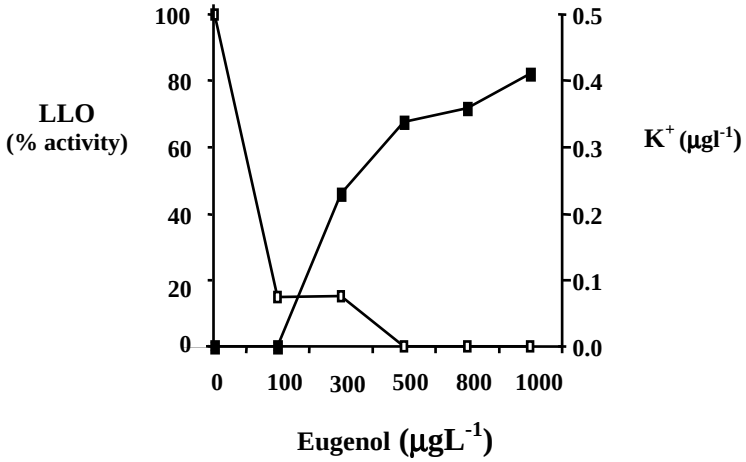

Figure 2 - Effect of eugenol on LLO activity during growth of *L. monocytogenes* in TSB (□) and on potassium ions efflux in PBS buffer (■).

Potassium efflux could be used as an indicator of the membrane damage caused by chemical and physical agents. It has been suggested that the cytoplasmic membrane is also a target for eugenol action and results evidencing the K^+ efflux corroborated this hypothesis. This result was in agreement with Degré and Sylvestre (1983) who considered that the probable antimicrobial activity of eugenol was on cellular lipids resulting in the loss of intracellular contents. Eugenol was more effective in inhibiting LLO secretion than cellular growth. Other inhibitory agents like sorbate and NaCl were also more effective to inhibit LLO secretion while having little effect on growth (McKellar, 1993).

Results from the present study indicated that *L. monocytogenes* growth and LLO production were sensitive to eugenol. Although it did not provide support for establishing a link between eugenol and virulence, it contributed to the evaluation of the potential of eugenol in controlling *L. monocytogenes* in foods.

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RESUMO

O efeito inibitório do eugenol, o principal constituinte do óleo essencial de cravo, foi avaliado sobre o crescimento e produção de listeriolisina O (LLO) por *Listeria monocytogenes*. O efluxo de íons potássio das células também foi

determinado após 24 h de incubação em solução tampão, contendo eugenol. Concentrações de 100, 300 e 500 μg mL^{-1} de eugenol promoveram a inibição do crescimento de *L. monocytogenes* e, em concentrações acima de 800 μg mL^{-1}, constatou-se um efeito bactericida. O crescimento de *L. monocytogenes* na presença de eugenol resultou na inibição de 80 a 100% da produção de LLO. O efluxo de K^+ promovido pelo eugenol indicou que a membrana celular foi afetada. Estes resultados indicam a efetividade do eugenol para o controle do crescimento e da produção de LLO por *L. monocytogenes*.

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