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Morphological alterations in sessile cells of *Listeria monocytogenes* after treatment with *Cymbopogon* sp. essential oils

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Abstract: *Listeria monocytogenes* is a pathogenic bacterium capable of forming biofilms on the surfaces of equipment and utensils, contaminating food products. Essential oils have been used as new alternatives to sanitizing solutions elaboration. The aim of this study was to evaluate, by Scanning Electron Microscopy (SEM), the structural effect of *Cymbopogon citratus* (D.C.) Stapf. (lemongrass) and *Cymbopogon nardus* (L.) Rendle (citronella) essential oils, applied isolated and in combinated, against *L. monocytogenes* sessile cells. The effect was assessed at different stages of biofilm formation on stainless steel surface (3 and 240 hours). For biofilm formation under stainless steel coupons, Tryptic Soy Broth (TSB) was used as substrate and the incubation was performed at 37 °C under agitation at 50 rpm. Sanitizing solutions based on essential oils and a control sanitizing solution were tested at two contact durations (15 and 60 minutes). Then, stainless steel coupons were analyzed by SEM. Several structural alterations were observed in the bacterial cells after the treatment with essential oil solutions: wrinkled surface aspects, acquisition of a curved appearance, reduction of intracellular content and occurrence of long cells. In contrast,scanning electron micrographs of the sessile cells that had been treated with the control sanitizing solution showed no alteration on the bacterial surface. *C. citratus* and *C. nardus* essential oils, isolated and in combination, caused morphological alterations in *L. monocytogenes* sessile cells. SEM is a valid methodology to evaluate the essential oils structural action against biofilm bacterial cells.
Key words: *Cymbopogon nardus, N*atural sanitizers, Structural alterations.

Alterações morfológicas em células sésseis de *Listeria monocytogenes* após tratamento com óleos essenciais de *Cymbopogon* sp.

Resumo: *Listeria monocytogenes* é uma bactéria patogênica capaz de formar biofilmes sobre a superfície de equipamentos e utensílios, contaminando produtos alimentícios. Óleos essenciais têm sido utilizados como novas alternativas para elaboração de soluções sanificantes. O objetivo deste estudo foi avaliar, por Microscopia Eletrônica de Varredura (MEV), o efeito estrutural dos óleos essenciais de *Cymbopogon citratus* (D.C.) Stapf. (capim-limão) e *Cymbopogon nardus* (L.) Rendle (citronela), aplicados isolados e em combinação, contra células sésseis de *L. monocytogenes*. O efeito foi avaliado em diferentes estágios da formação do biofilme sobre superfície de aço inoxidável (3 e 240 horas). Para formação do biofilme em cupons de aço inoxidável, foi utilizado Caldo Triptona de Soja (*Tryptic Soy Broth, TSB*) como substrato e incubação a 37 °C sob agitação de 50 rpm. Soluções sanificantes contendo óleos essenciais e uma solução sanificante controle foram testadas em dois tempos de contato (15 e 60 minutos). Cupons de aço inoxidável foram, então, analisados por MEV. Várias alterações estruturais foram observadas nas células bacterianas após tratamento com as soluções contendo óleos essenciais: aspecto superficial enrugado, aquisição de

aparência curva, redução do conteúdo intracelular e ocorrência de células muito alongadas. Por outro lado, as micrografias eletrônicas de varredura das células sésseis tratadas com a solução controle não mostraram alterações na superfície bacteriana. Óleos essenciais de *C. citratus* e *C. nardus*, isolados e em combinação, causaram alterações morfológicas nas células sésseis de *L. monocytogenes*. MEV é um método válido para avaliar a ação estrutural de óleos essenciais contra células bacterianas em biofilmes.

Palavras chave: *Cymbopogon nardus, S*anificantes naturais, Alterações estruturais.

Introduction

In food industries, several microorganisms are capable of participating in the adhesion processes and biofilm formation. Among the pathogenic microorganisms, *Listeria monocytogenes* is one of the most outstanding. This bacterium is an emergent pathogen of ubiquitous distribution in nature, surviving under adverse environmental conditions. Developing in different substrates, it is capable of colonizing biotic and abiotic surfaces through biofilm formation (GANDHI & CHIKINDAS, 2007; OLIVEIRA et al., 2010a, 2011).

Essential oils are potent natural antimicrobial agents (SIKKEMA et al., 1995; BAKKALI et al., 2008; TEIXEIRA et al., 2012, VIEIRA et al., 2012) that have been evaluated for their antibacterial activity against microbial biofilms, aiming at the possibility of using these secondary metabolites or their constituents as sanitizers in the food industry (CHORIANOPOULOS et al., 2008; OLIVEIRA et al., 2010b). The antibacterial activity of *Cymbopogon citratus* (D.C.) Stapf. (lemongrass) and *Cymbopogon nardus* (L.) Rendle (citronella) essential oils, for example, was determined *in vitro* against planktonic cells of *L. monocytogenes* (OLIVEIRA et al., 2011); and sanitizing solutions based on these essential oils reduced the number of viable cells of *L. monocytogenes* adhered on a stainless steel surface (OLIVEIRA et al., 2010b).

Various methodologies are employed in the study of bacterial biofilms, among them the Scanning Electron Microscopy (SEM), has been used to evaluate the biofilm formation on industrial surfaces, its structure and its susceptibility to chemical sanitizers, which can be observed in studies conducted by Marques et al. (2007), Oliveira et al. (2010a) and Caixeta et al. (2012). The antibacterial activity of essential oils is another parameter that has also been assessed by SEM (BAJPAI et al., 2009; AL-REZA et al., 2010).

However, these studies observed the performance of essential oils in planktonic bacterial cells, so that currently there is little knowledge about structural damage caused by the essential oils action against bacterial biofilms formed on surfaces used in food industries, such as stainless steel. This study was conducted in order to use SEM to evaluate the structural effect of *Cymbopogon citratus* and *Cymbopogon nardus* essential oils, applied isolated and in combination, against *Listeria monocytogenes* sessile cells. The effect was assessed at different stages of biofilm formation on stainless steel surface.

Material and methods

The strain used was *L. monocytogenes* ATCC 19117, acquired from the Culture Collection Section of the Medical Biology Division of the Adolfo Lutz Institute (São Paulo – SP, Brazil). The standardization, inoculum preparation and storage were conducted as previous described by Oliveira et al. (2010a, 2010b).

The biofilm formation experimental model used in this study was detailed described in the work of Oliveira et al. (2010a, 2010b). The biofilm was formed for 240 hours on the surface of stainless steel AISI 304 (number 4 finish) coupons (1 x 8 x 18 mm), using Tryptic Soy Broth (TSB) as substrate, incubation at 37 °C and 50 rpm agitation. The initial inoculum, in TSB, was standardized for 8 Log CFU/mL.

Fresh leaves of *C. citratus* and *C. nardus* were collected from Medicinal Plant Nursery of the Federal University of Lavras (Minas Gerais, Brazil) and the essential oils were extracted by hydrodistillation using a modified Clevenger apparatus, according the procedure detailed

described by Oliveira et al. (2011).

After 3 and 240 hours, stainless steel coupons were treated with solutions containing essential oils of *C. citratus* and *C. nardus* for 15 and 60 minutes. A control solution (without essential oils) was also used (OLIVEIRA et al., 2010b). For each disinfectant solution, the amount of essential oil used was the minimum inhibitory concentration previous determined by Oliveira et al. (2011). Saline solution (NaCl 0.9% w/v) and ethanol were used as diluents. The disinfectant action of each solution against the bacterial cells adhered to the stainless steel coupon surface was evaluated in two different biofilm formation phases: 3 and 240 h. Thus, the stainless steel coupons were previously immersed three times in saline solution to remove the planktonic cells, followed by immersion in 4 mL of the disinfectant solutions containing essential oils and in the control disinfectant solution for 15 and 60 min at 28 °C, under static conditions (OLIVEIRA et al., 2010b). The disinfectants solutions compositions are described in Table 1.

Table 1 - Compositions of the disinfectant solutions based on essential oils and control disinfectant solution according to Oliveira et al. (2010b).

Disinfectant solutions	**Composition (%)**		
	Essential oil	Ethanol	Saline solution with 0.5% (v/v) of Tween 80
C. nardus	3.12	16.88	80.00
C. citratus	1.56	18.44	80.00
Combination*	1.56	18.44	80.00
Control	0.00	20.00	80.00

*Combination of *C. citratus* and *C. nardus* essential oils at 1:1 ratio.

The stainless steel coupons were immersed three times in saline solution, to remove any essential oil residue, and prepared for viewing by SEM. The coupons were initially immersed in a fixing solution (modified Karnovsky's: glutaraldehyde 2.5%, formaldehyde 2.5% in sodium cacodylate buffer 0.05M, pH 7.2, $CaCl_2$ 0.001M) for a minimum of 24 hours, washed with sodium cacodylate buffer three times for 10 minutes, fixed in osmium tetroxide (1% in distilled water) for 1 hour at ambient temperature in an exhaust hood, washed three times in distilled water and dehydrated in a crescent acetone gradient (25%, 50%, 75%, 90% and 100%, three times). The coupons were later transferred to the critical point apparatus (Bal-tec CPD 030) to complete drying, mounted on stubs and sputter-coated with gold (Bal-tec CPD 050). At the end of this procedure, the coupons were examined in a scanning electron microscope (Leo Evo 040) to obtain the micrographs (OLIVEIRA et al., 2010a).

Results and discussion

Scanning electron micrographs of the *L. monocytogenes* sessile cells that had been treated with the control sanitizing solution showed no alteration on the bacterial surface. A typical structure of Gram-positive bacilli was observed, where the cells presenting a smooth surface (Fig. 1 and 2).

In contrast, structural alterations on the cellular surface were observed through the analyses of the scanning electron micrographs of the sessile cells that had been treated with essential oil solutions. All *L. monocytogenes* cells presented wrinkled surface aspects and some lost the normal straight shape, acquiring a curved appearance (see those marked with a circle in Fig. 3 and 4). In a set of cells we could clearly note that there was a reduction of intracellular content (see those marked with a square in Fig. 3 and 4). Very long cells could be observed as well (see those marked with a triangle in Fig. 3 and 4). All these structural changes compromised the common bacterial morphology and were noticed by comparison with the control treatments available in Fig. 1 and 2.

Figure 1 - Scanning electron micrographs of *Listeria monocytogenes* cells adhered to stainless steel surface after 3 hours of contact at 37 °C, using Tryptic Soy Broth (TSB) as substrate, submitted to control sanitizing solution (without essential oils). (A) 15 minute contact time and (B) 60 minute contact time. Arrows indicate the bacterial cells.

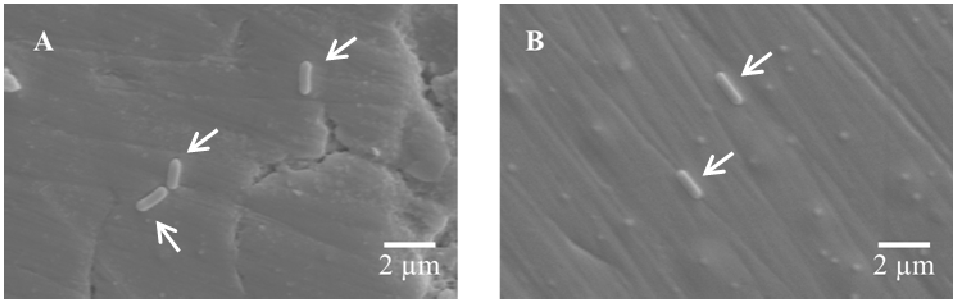
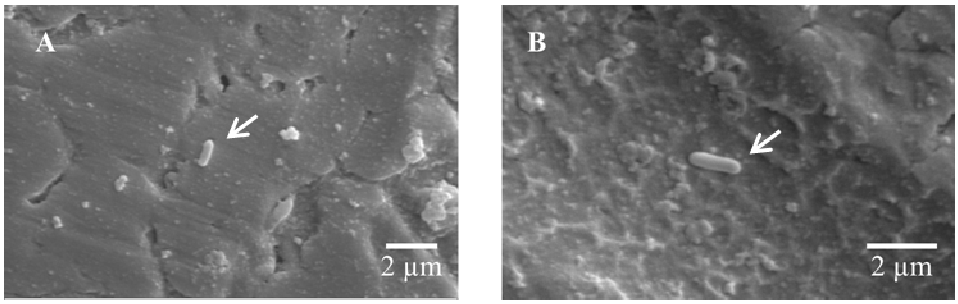


Figure 2 - Scanning electron micrographs of *Listeria monocytogenes* cells adhered to stainless steel surface after 240 hours of contact at 37 °C, using Tryptic Soy Broth (TSB) as substrate, submitted to control sanitizing solution (without essential oils). (A) 15 minute contact time and (B) 60 minute contact time. Arrows indicate the bacterial cells.



Despite the structural changes observed after contact between bacterial cells and essential oils solutions, by SEM methodology was not possible to detect differences in the antibacterial activity caused by the combined use of essential oils, such as synergistic or antagonistic effects. This result was expected, since SEM was used as a qualitative methodology, become impossible a precise comparison between treatments. In the same context, it was not possible to note a relationship between antibacterial activity and sanitizing solutions exposure time.

Regarding the SEM evaluation of the essential oil performances on biofilms formed by bacterial pathogens of food interest, few studies were found, especially using industrial surfaces, such as stainless steel. Recently, Millezi et al. (2013) observed, with scanning electron microscopy, that the biofilm of *Aeromonas hydrophila*, formed on stainless steel coupons, showed a dispersion of cells and a breakdown of the exopolysaccharide after treatment with detergent-sanitizer solutions constituted of thyme and lemongrass essential oils. Other interesting study was conducted by Kwieciński et al. (2009), which evaluated the effect of tea tree (*Melaleuca alternifolia)* oil on *S. aureus* biofilm formed in 96-well cell culture plates. SEM images showed that at 1% of tea tree oil destroyed the biofilm formed by *S. aureus* 8325-4. The biofilm eradication by tea tree oil was not only due to bacteria killing but also partly due to extracellular matrix damage and subsequent removal of biofilm from the surface, especially by higher concentrations. In contrast**,** Bajpai et al. (2009), working with planktonic cells, observed structural changes similar to those found in this study. The scanning electron microscope showed potential detrimental effect of the leaf essential oil of *Metasequioa glyptostroboides* Miki on the morphology of *S. aureus*. According to the authors these

morphological features in bacterial cells might be due to the lysis of outer membrane and the transformation by weak peptidoglycan followed by the loss of cellular electron dense material on surface of the treated cells, resulting in the release of inner cell material.

Figure 3 - Scanning electron micrographs of *Listeria monocytogenes* cells adhered to stainless steel surface after 3 hours of contact at 37 °C, using Tryptic Soy Broth (TSB) as substrate, submitted to sanitizing solutions based on essential oils, isolated and in combination. (A) *C. citratus*, after 15 minute contact time; (B) *C. citratus*, 60 minute contact time; (C) *C. nardus*, 15 minute contact time; (D) *C. nardus*, 60 minute contact time; (E) combination, 15 minute contact time; (F) combination, 60 minute contact time. Symbols: ● (cells with curved appearance), ■ (cells with reduction of intracellular content), ▲ (very long cells), ↑ (bacterial cells or a set of bacterial cells). In letter A the symbol represent the whole cell entirety.

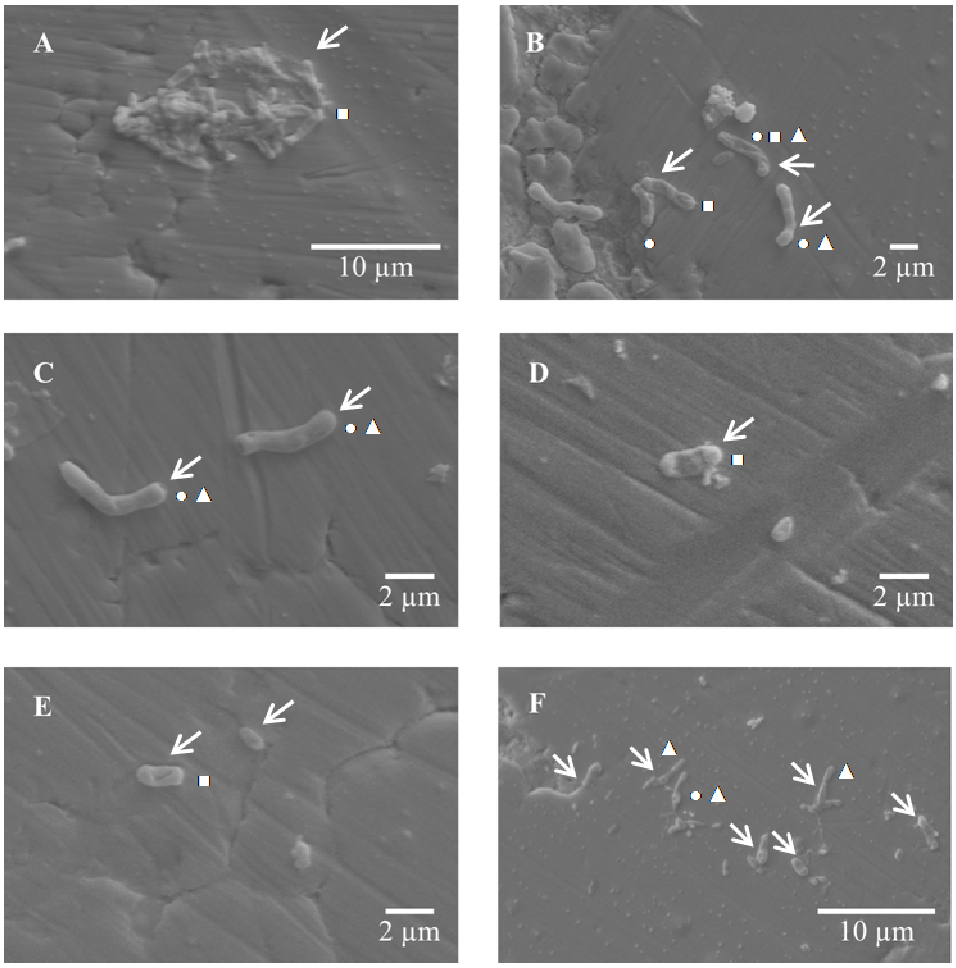
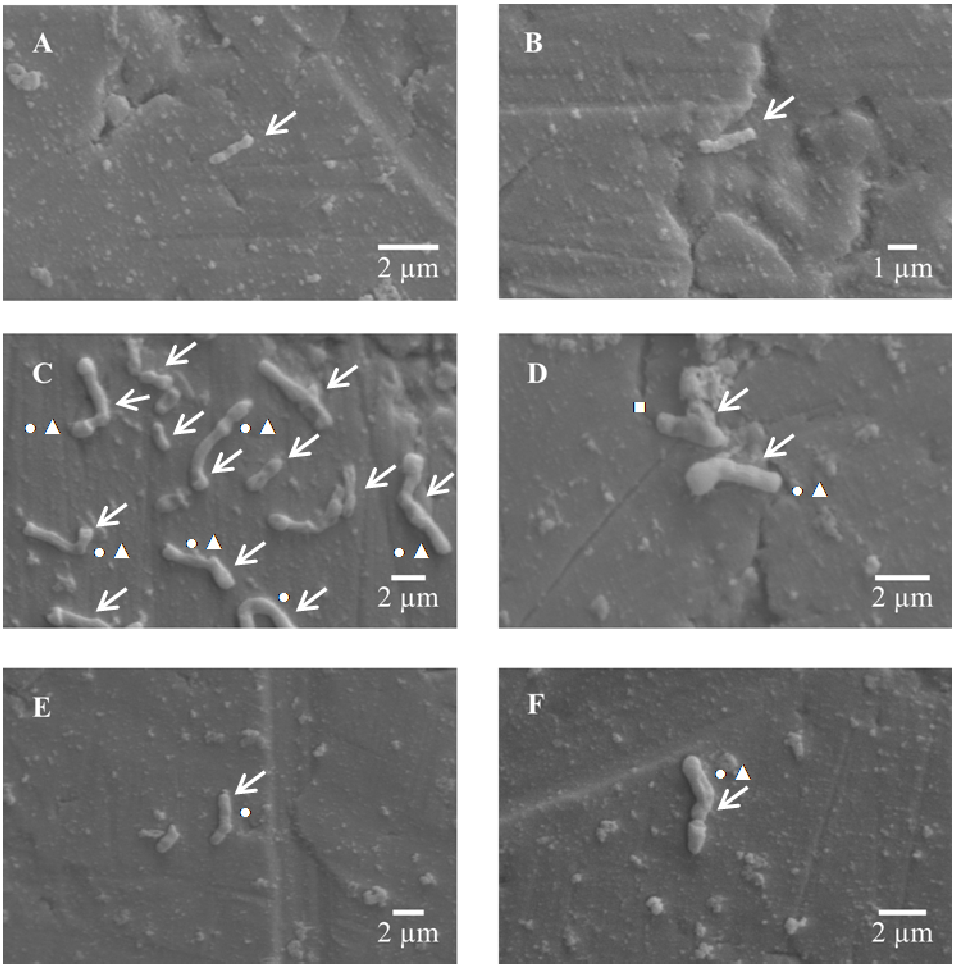


Figure 4 - Scanning electron micrographs of *Listeria monocytogenes* cells adhered to stainless steel surface after 240 hours of contact at 37 °C, using Tryptic Soy Broth (TSB) as substrate, submitted to sanitizing solutions based on essential oils, isolated and in combination. (A) *C. citratus*, after 15 minute contact time; (B) *C. citratus*, 60 minute contact time; (C) *C. nardus*, 15 minute contact time; (D) *C. nardus*, 60 minute contact time; (E) combination, 15 minute contact time; (F) combination, 60 minute contact time. Symbols: ● (cells with curved appearance), ■ (cells with reduction of intracellular content), ▲ (very long cells), ↑ (bacterial cells or a set of bacterial cells).



The complete chemical composition of the essential oils samples used in this study can be observed in the work conducted by Oliveira et al. (2011), who verified monoterpenes as major chemical constituents: geranial (42.92%) and neral (30.91%) were the major components of *C. citratus* essential oil, while citronellal (34.61%), geraniol (23.18%) and citronellol (12.10%) were predominant in *C. nardus* oil. According to Sikkema et al. (1995), the action mechanism of the monoterpenes involves mainly toxic effects on the structure and function of the cell membrane. As a result of their lipophilic character, the monoterpenes will preferably dislocate from the aqueous phase towards the membrane structures. However, other studies showed that the bacterial cell wall is also a target for the antibacterial action of essential oils. Carson et al. (2002) studied the action mechanism of tea tree essential oil against the Gram-positive bacterium *S. aureus* and verified that the cell lysis may also have been due to weakening of the cell wall and subsequent

rupture of the cytoplasmic membrane due to osmotic pressure (rather than a specific action on the cytoplasmic membrane). Based on these information, it appears that the wrinkled surface aspects observed by SEM on the bacterial surface have been caused by the action of the *C. citratus* and *C. nardus* essential oils sanitizing solutions in the cell wall and cytoplasmic membrane of *L. monocytogenes*. This structural damage probably occurred as consequence of intracellular material loss, such as cytoplasmic contents, due to an increase in permeability. In a group of cells, there was intracellular content reduction (see those marked with a square in Fig. 3 and 4). The curved appearance of the cells (see those marked with a circle in Fig. 3 and 4), in turn, was probably caused primarily by the action of essential oils in the cell wall, since it is a rigid structure that covers the bacterial cytoplasmic membrane and gives the cell its shape.

Another structural change observed in this study was the presence of cells longer than normal cells (see those marked with a triangle in Fig. 3 and 4). Previous studies reported that essential oils constituents were able to affect bacterial cell division, causing a phenomenon called filamentation (OGUNLANA et al., 1987; KWON et al., 2003). However, this phenomenon is complex and still poorly reported, so further studies using more detailed methods should be conducted to prove that the essential oils of *Cymbopogon* species used in this study are able to induce the process of filamentation in *L. monocytogenes*.

It is well diffused in the literature that essential oils act primarily in microbial cell membranes, causing increased permeability. However, further studies using appropriate methodologies should be conducted to elucidate the exact mechanisms of action that caused the morphological changes in biofilms cells observed by microscopy methods, such as SEM.

Conclusion

C. citratus and *C. nardus* essential oils, isolated and in combination, caused morphological alterations in *L. monocytogenes* sessile cells; and SEM is a valid method to evaluate the essential oils structural action against biofilm bacterial cells.

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