Detection of *Listeria innocua* in the dairy processing chain: resistance to antibiotics and essential oils

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Abstract

*Listeria monocytogenes* is a food pathogen responsible for many foodborne disease outbreaks. *Listeria innocua* is similar to *L. monocytogenes*, could be considered biologically similar to *L. monocytogenes* and could be used to predict its behavior. The aims of this work were to isolate *Listeria* spp. in dairy processing plants, perform genotypic characterization of virulence, evaluate the antimicrobial resistance profile, and verify the sensitivity of the strains to essential oils. Sampling was held in six dairy processing plants in the region of Campinas, Brazil. Seventeen *Listeria innocua* strains were isolated, all negative for virulence genes *inlA*, *inlB*, *inlC*, *inlF*, *actA*, *plcA*, and *plcB*. The strains were resistant to kanamycin (5.9%), penicillin (11.8%), ampicillin (11.8%), cefoxitin (58.8%), clindamycin (76.50%) and oxacillin (100%). The susceptibility of the strains to commercial essential oils (*Rosmarinus officinalis*, *Cymbopogon citratus*, *Eucalyptus citirodora*, *Mentha piperita*, *Piper nigrum*, *Vetiveria zizanioides*, *Cymbopogon nardus*, *Cymbopogon martini* and *Pogostemon cablin*) was evaluated by Minimum Inhibitory Concentration (MIC) test. The results showed *Cymbopogon citratus*, *Cymbopogon martini*, *Cymbopogon nardus* and *Mentha piperita* oils were most efficient. The resistance of *Listeria innocua* isolated in dairy processing plants is worrisome, and the use of essential oils could be an alternative for development of new drugs and products.

Keywords: food safety; *Listeria* infection; resistance; food processing; dairy.

Practical Application: It could be an alternative to the use of chemical products in the sanitizing processes of surfaces in contact with food.

1 Introduction

Foodborne illnesses are a public health problem and contamination with microorganisms can occur at any stage in the process of food production. It is estimated that one in ten people worldwide get sick and that 420,000 die every year after ingesting contaminated food (World Health Organization, 2016).

In the dairy industry, different pathogens are frequently isolated from milk and dairy products, mainly gram positive and negative bacteria. A gram-positive bacteria usually isolated in dairy products is *Listeria monocytogenes*, is a major food pathogen responsible for many foodborne illness outbreaks, including from dairy products (Centers for Disease Control and Prevention, 2015, 2016, 2017). The pathogen was responsible for the most cases of hospitalization and death in Europe (European Food Safety Authority, 2012).

*L. innocua* is similar to *L. monocytogenes*, but is non-pathogenic for humans (Picart et al., 2002) and has a similar response when compared to *L. monocytogenes* in chemical and physical stresses and in biofilm formation, and both species share the same natural environments (Friedly et al., 2008; Silva-Angulo et al., 2015; Costa et al., 2018). These similarities mean that *L. innocua* could be considered as biologically similar for *L. monocytogenes* and can be used in experiment to assess its susceptibility to essential oil. In addition, if some strains of *L. innocua* are susceptible or resistant to antibiotics that automatically *L. monocytogenes* are similar. Since *L. innocua* is non-pathogenic, it is preferred to study over *L. monocytogenes* to predict its behavior (Costa et al., 2018).

Phenotypic antimicrobial resistance profile is relevant for public health. Although *Listeria* sp. are generally susceptible to a wide variety of antimicrobial agents Zhang et al. (2007), strains of *Listeria* spp. resistant to antibiotics have already been reported (Zhang et al., 2007; Moreno et al., 2014; Obaidat & Stringer (2019), which can affect the treatment.

*L. monocytogenes* is considered a microbiological hazard in food production, which justifies the monitoring of this microorganism or its possible indicator within the food production chain.
Additionally, research on agents that may have an inhibitory action over this species, such as essential oils, once the demand for new natural products is growing.

2 Materials and methods

2.1 Sampling and microbiological analysis

A total of two samplings were performed in six dairy processing plants in Campinas region, São Paulo State, Brazil from raw milk, pasteurized milk, processing environment surfaces (floor, cold chamber, drains, pasteurization and coagulation tank, table, molds, boxes, shovels) and Minas frescal cheese, totaling 164 samples. Sampling from the environment and equipment were conducted using swabs moistened in peptone (0.1%) with neutralizing sanitizers, when needed, in areas delimited by sterile molds. Drains and tools were sampled without a delimited area. Immediately after collection, the swabs were placed in tubes containing 15 mL of buffered *Listeria* enrichment broth (BLEB). The collected samples were refrigerated and transported in isothermal boxes to Higiene e laticínios laboratory (Escola Superior de Agricultura "Luiz de Queiroz", São Paulo University) for analysis.

The milk and cheeses were homogenized (25 g or 25 mL of sample) with 225 mL of BLEB in a Stomacher and then incubated (48 h/30 °C). After 4 hours of incubation, antimicrobials acriflavine (10 mg/L); nalidixic acid (40 mg/L); cycloheximide (50 mg/L) were added to the enrichment broth (BLEB) containing the samples. The method described by the FDA in the Bacteriological Analytical Manual (BAM) (Hitchins et al., 2017) was used for the analysis. Each sample incubated in BLEB, were placed on to Oxford and ALOA agars, followed by incubation at 35 °C/24-48 h and 37 °C/24 h, respectively.

Typical *Listeria* colonies were selected and hemolysis test were performed following Gram staining, catalase and motility test. The isolates were subjected to carbohydrate fermentation tests with alpha-methyl-d-mannoside, D-xylose, D-rhamnose, D-mannitol and D-glucose. In addition, three to five colonies were taken to perform the detection test.

2.2 Molecular detection of virulence factors

The bacterial DNA was extracted using InstaGene™ DNA Purification Matrix (Bio-Rad, Hercules, CA, USA). The DNA samples were stored at -20 °C until further analysis. Polymerase chain reaction (PCR) was performed with primers for the 23S rRNA and for the *hly* gene specific for the *Listeria* genus and *L. monocytogenes* species, respectively, according as previously reported to Hudson et al. (2001).

Positive isolates for 23S rDNA and/or *hly* were tested for *inlA*, *inlB*, *inlC*, *inlJ*, *actA*, *plcA*, *plcB*, *prfA* genes, as described in Table 1.

2.3 Phenotypic antimicrobial resistance

The antibiotics susceptibility of strains was tested by disk diffusion technique according to Clinical Laboratory Standards Institute (CLSI) guidelines (Clinical & Laboratory Standards Institute, 2015), Mueller–Hinton agar (Difco) was used for analysis. Tests and results were performed following recommendations for *Staphylococcus* sp (Obaidat et al., 2015). The antibiotics

### Table 1. Primer and their properties used for the confirmation of the *L. monocytogenes* species and for the detection of the regulatory gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of primers (5’→3’)</th>
<th>Annealing temperature (°C)</th>
<th>bp</th>
<th>Reference</th>
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<tr>
<td>inlA</td>
<td>F – AATCTAGCACCACGTTCGGG&lt;br&gt;R - TGTGACCTTCTTCTTACGGGC</td>
<td>50</td>
<td>733</td>
<td>Rousseaux et al. (2004)</td>
</tr>
<tr>
<td>inlB</td>
<td>F - TGGGAGAGTAACCACCAACAC&lt;br&gt;R - GGTGACCTTCGATGGTTGCT</td>
<td>40</td>
<td>884</td>
<td>Liu et al. (2007)</td>
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<tr>
<td>inlC</td>
<td>F - AATTCCCACAGGACACAC&lt;br&gt;R - CGGGAATGCACATTTTTTCAC TA</td>
<td>40</td>
<td>517</td>
<td>Liu et al. (2007)</td>
</tr>
<tr>
<td>inlJ</td>
<td>F - TGTAACCCCGGCTTACACAGTT&lt;br&gt;R - AGGGGCTTGGACTACAA TA</td>
<td>40</td>
<td>238</td>
<td>Liu et al. (2007)</td>
</tr>
<tr>
<td>hlyA</td>
<td>F – GGACTTGCAAAGCCTTGGAGTGA&lt;br&gt;R - GCAAAGATCTTCCAGAGTGATCG</td>
<td>60</td>
<td>456</td>
<td>Paziak-Domańska et al. (1999)</td>
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<td>actA</td>
<td>F – CGCGCAGGAAATTAAAAAGA&lt;br&gt;R - ACAAGGAAACCGGCGCTGCTAG</td>
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<td>839</td>
<td>Schoder et al. (2003)</td>
</tr>
<tr>
<td>iap</td>
<td>F - ACAAGCTGCACGTTGTCG&lt;br&gt;R - TGACAGGCTGTGATGACCA</td>
<td>60</td>
<td>131</td>
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</tr>
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<td>plcA</td>
<td>F - CTTGTTAGCTTGGTATAC&lt;br&gt;R - GATTGGCGTCCTGGACTGCG</td>
<td>55</td>
<td>1155</td>
<td>Roche et al. (2005)</td>
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<tr>
<td>plcB</td>
<td>F - ATTTGCGGTGGTTCTGGTAC&lt;br&gt;R - TTAATACGGACATAAACGC</td>
<td>55</td>
<td>1103</td>
<td>Roche et al. (2005)</td>
</tr>
<tr>
<td>prfA</td>
<td>F - GGGAACCCTGTGCACACTTAAACAC&lt;br&gt;R - GCTAGGGAAACTCCACTGCGCTTGCC</td>
<td>33</td>
<td>1285</td>
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</tr>
<tr>
<td>luxS</td>
<td>F – TCCGAGCCACGCTTCTTGCGGCT&lt;br&gt;R - TGTTTTAGGCAACGCTTACGATCG</td>
<td>57,5</td>
<td>117</td>
<td>Bonsaglia et al. (2014)</td>
</tr>
</tbody>
</table>

bp: base pair; F: Forward; R: Reverse.
tested were clindamycin (2 µg), oxacillin (1 µg), sulfazotrim (25 µg), cefoxitin (30 µg), gentamicin (10 µg), ampicillin (10 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), rifampicin (5 µg), vancomycin (30 µg), kanamycin (30 µg), tetracycline (30 µg), ampicillin (10 µg), cephalothin (30 µg) and erythromycin (15 µg), all antibiotics were acquired by LABORCLIN.

2.4 Strains susceptibility to essential oils

The susceptibility of the isolates to commercial essential oils (Rosemarinus officinalis, Cymbopogon citratus, Eucalyptus citriodora, Mentha piperita, Piper nigrum, Vetiveria zizanioides, Cymbopogon nardus, Cymbopogon martini and Pogostemon cablin) was evaluated by Minimum Inhibitory Concentration (MIC) test.

For the MIC evaluated, the assays were conducted according to the methodology described by Iacuzio (2019), the isolates were grown in Triptica Soy Agar with 0.6% Yeast Extract (TSA-YE; Difco) at 35 °C/24 h. Bacterial colonies from fresh pure culture were mixed with sterile saline solution (0.85%) to prepare the turbidity of each inoculum, which was adjusted to McFarland (0.5; ~108 CFU/mL) standards, using a densitometer (Densichek, BioMérieux, Durham, NC). The bacterial suspension was further diluted to ~105CFU/mL concentration. The MIC was determined by serial dilution using 96-well plates and the bacterial suspension incubated with essential oils at concentrations of 10 to 0.0049%, in final volumes of 100 µL of Trip Soy Broth (TSB- Diﬁco) containing up to 2.5% of Tween 80. Sterility controls, positive growth and oils controls were included.

After the determination of the MIC, the minimum bactericidal concentration (MBC) was determined (Hafidh et al., 2011). The conversion of the values obtained in the Minimum Inhibitory Concentration test from percentage to mg.ml⁻¹ was performed according to Silva et al. (2011).

2.5 Statistical analyses

For the MIC was used a factorial ANOVA (8x4). Where 8= essential oils and 4= strains used. Significant factors (p ≤ 0.05) were then subjected to multiple comparisons using the Duncan test. Statistical analyses were performed using XLSTAT 2018 software for Microsoft Excel® (Microsoft®, WA, USA).

3 Results and discussion

3.1 Isolation of Listeria sp. and virulence factors

Seventeen isolates were obtained from 5 samples (3.1% of the samples) with biochemical profile characteristic of L. innocua: Gram-positive rods, catalase positive, characteristic motility, positive for alpha-methyl-d-mannoside and D-glucose fermentation, negative for D-xylose, D-rhamnose, D-mannitol fermentation and without hemolysis in blood agar. These isolates derived from floor (from different processing plants), from drain (of the same plant, but different samplings); and one from a storage box. In these isolates, a PCR was performed for the 23S rDNA region and hly gene, with all isolates being positive for the 23S rDNA and negative for the hly gene. Also, the isolates were submitted to PCR for virulence genes dltA, dltB, dltC, dltD, actA, plcA, prfA, found mostly in L. monocytogenes, but that may be present in other species of the genus (Gouin et al., 1994; Johnson et al., 2004). These genes are associated with the mechanism of pathogenicity and multiplication of this microorganism in the host (Silva et al., 2015; Iglesias, 2014). All isolates were negative for the virulence genes analyzed.

3.2 Phenotypic antibiotic resistance

Table 2 show the results obtained in this study regarding the phenotypic profiles found for antibiotic resistance. It was possible to observe that isolates from the same sample, showed different resistance profile.

Table 2. Resistance profile of the isolates of L. innocua.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dairy</th>
<th>Sampling</th>
<th>Origin</th>
<th>Species</th>
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</table>

S = Sensitive; R = Resistant; I = Intermediate; VAN: vancomycin; TET: tetracycline; ERI: erythromycin; PEN: penicillin; AMP: ampicillin; CLO: chloramphenicol; CIP: ciprofloxacin; SUT: sulfazotrim; CFO: cefoxitin; CLI: clindamycin; RIF: rifampicin; OXA: oxacillin; GEN: gentamicin; KAN: kanamycin; CFL: cephalothin.
Considering the antibiotics tested, resistance was observed for kanamycin (5.9%), penicillin (11.8%), ampicillin (11.8%), cefoxitin (58.8%), clindamycin (76.5%) and oxacillin (100%).

According Troxler et al. (2000), the species of Listeria were naturally sensitive or intermediate to tetracyclines, aminoglycosides, penicillins (except oxacillin), carbapenems, macrolides, lincosamides, glycopeptides, chloramphenicol and rifampicin. This work presents the resistance of 100% of strains to oxacillin. The resistance observed to other antibiotics could be a result of horizontal gene transfer, once is growing the bacterial resistance. Other studies also observed that all L. innocua isolates were resistant to oxacillin and a high percent (75%) of them were resistant to clindamycin (Davis & Jackson, 2009; Osman et al., 2016).

Prates (2010) evaluated the antibiotic-resistance profile of Listeria spp. in dairy processing plants, and 20 isolates from equipment, processing environment and cheese have been tested for 15 antimicrobials. In that study, it was observed that all isolates were resistant to at least one antimicrobial and it also showed resistance to oxacillin, corroborating the present study. According to this same author, antibiotic resistance may occur via plasmids and transposons, which confer resistance to antimicrobials and can also be transferred from one bacterium to the other.

Other studies observed resistance or intermediate resistance of L. monocytogenes isolated from food (Iglesias, 2014; Moreno et al., 2014) which is a concern for consumers and public health.

### 3.3 Susceptibility of the isolates to essential oils

Figure 1 show the average means of the MIC obtained for each oil (Rosemarinus officinalis, Cymbopogon citratus, Eucalyptus citriodora, Mentha piperita, Piper nigrum, Vetiveria zizanioides, Cymbopogon nardus, Cymbopogon martini and Pogostemon cablin) used in relation to susceptibility of strains evaluated.

According to ANOVA showed in Figure 1: Cymbopogon citratus, Cymbopogon martini, Cymbopogon nardus and Mentha piperita oils had the lowest MIC and they did not present a significant difference, i.e., it requires the least amount of active compound, which means it was more efficient in inhibiting the microorganisms tested (MIC ranged 0.6 to 1.8 mg.ml$^{-1}$). Piper nigrum oil was the one that needed the highest quantity of the active compound (above 25 mg.mL$^{-1}$) and statistically different with Rosemarinus officinalis and Vetiveria zizanioides. For the remaining oils, the concentrations of the active compounds necessary for inactivation of the bacteria tested ranged from 3 to 6 mg.mL$^{-1}$.

The action of the essential oils on bacteria is related to the disruption of the cytoplasmic membrane, damage to the protein in the membrane, coagulation of the cytoplasm, change in the flow of electrons, interruption of the proton-motive force and change in the active transport and reduction of the intracellular ATP pool (Nazzaro et al., 2013). The biological activity of essential oils depends on their composition, as well as on the microorganism's characteristics (Caixeta, 2010; Caputo et al., 2020).

Citral is the major component of Cymbopogon sp. essential oils. The mechanism of action of citral are not totally clear, but there is evidence that this component can disrupt the cell membrane integrity causing effects on the homeostasis of the microbial cell (Somolinos et al., 2010; Caixeta, 2010; Sharma et al., 2019).

Oliveira et al. (2010) analyzed the composition of C. citratus and C. nardus essential oils, finding that monoterpenes as major chemical constituents. For C. citratus essential oil, the major constituents observed were: geranial and neral, which isomerically form citral. Minor components were 2-undecanone, linalol,
myrcene, geraniol, (E)-β-ocimene and (Z)-β-ocimene. For C. nardus essential oil, citronellal, geraniol and citronellol were the dominant components followed by β-elemene, γ-cadinene, δ-cadinene, citronellyl acetate, germacrene D, α-murolene, limonene, geranial, linalool and neo-isopulegol. The author’s results suggest that C. citratus and C. nardus essential oils could be alternatives to sanitize industrial stainless steel surfaces contaminated by L. monocytogenes (Oliveira et al., 2010).

Many studies reported the antimicrobial activity of Cymbopogon spp. against bacteria (Bassolé et al., 2011; Boeira et al., 2020), which reveal for these genera an important alternative for antibiotics and food preservatives.

While, Igarashi (2010) evaluated the antimicrobial activity of 12 essential oils in relation to 12 strains of Listeria monocytogenes, among other microorganisms isolated from chicken carcasses, a MIC of 0.8% could be observed for the essential oil from Mentha piperita in relation to the microorganism of interest. From the results obtained in the present study, it can be affirmed that the essential oil extracted from Mentha piperita has greater effectiveness as antimicrobial agent against strains of Listeria spp. isolated from the processing environment of Minas frescal cheese, since the MIC ranged from 0.078% and 0.312% (0.7 to 2.8 mg/mL), depending on the strain. This fact can be observed due to variations in the composition of the essential oils used in the studies, which can be caused by numerous factors, such as growing conditions, external factors and methodology used in the harvesting and extraction of the essential oils, as well as due to factors inherent to the strains identified and analyzed themselves, such as species variability, expression or suppression of resistance genes, among other factors.

From the results obtained in the present study, it can be affirmed that the essential oil extracted from Mentha piperita has greater effectiveness as antimicrobial agent against the isolates tested, while the oils from Piper nigrum and Eucalyptus citriodora were less efficient.

According to Singh et al. (2015) and Valeriano et al. (2012), antibacterial activity maybe associated with the compounds present in higher amounts, such as menthol, menthone, methyl acetate, isomenthene. The high antimicrobial activity of the essential oil of the Mentha spp. genus is also associated with the mechanisms of action in bacterial cells and their approach to multiple targets, acting on and causing damages to the cytoplasmic membrane’s functions, to respiratory activity and to the efflux pump (Guedes & Souza, 2017).

In general, essential oils exhibit antimicrobial properties which makes them appropriate alternatives to conventional medicines (Chaves et al., 2008). Besides that, there is a demand for natural food additive options which highlight the use of EOs as potential alternative antimicrobials (Smaoui et al., 2016).

The use of Mentha piperita as biopreservation has been studied, its essential oil used alone limited the microbial deterioration of minced meat (Smaoui et al., 2016). This shows the antimicrobial action of the oil and its prospective use in food, which values the current work, since Listeria sp. is an important foodborne pathogen (Centers for Disease Control and Prevention, 2017).

Based on the results obtained, and with the prospect of an expansion in the market of minimally processed products with less additives such as preservatives, the use of essential oils stands out as an alternative to conventional antimicrobials or as an additional safety barrier in ready-to-eat foods. Depending on the product, these compounds can be inserted into the formulation itself (Pesavento et al., 2015; Guedes & Souza, 2017) or in the packaging, with the goal of reducing contamination levels mainly in the product’s most shallow layer, as well as the gradual release of these compounds during its shelf life (Atarés & Chiralt, 2016).

4 Conclusion

The isolation of Listeria spp. from the environment and utensils of dairy processing plants implies that these are suitable niches for the development of other dangerous species, such as L. monocytogenes. As we observed resistance of isolates, the monitoring of the antimicrobial resistance of these microorganisms is of paramount importance. The tests performed and the results obtained from essential oils, especially Cymbopogon citratus, Cymbopogon martini, Cymbopogon nardus and Mentha piperita, indicate their great potential for being employed as an alternative to chemical additives already used by the food industry, improving the safety value of the products when added in low quantities or with different approaches.

Conflict of Interest

No conflict of interest declared.

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