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Repeated sub-inhibitory doses of cassia essential oil do not increase the tolerance pattern in *Listeria monocytogenes* cells

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ABSTRACT

Antimicrobial resistance in *Listeria monocytogenes* biofilms is considered a risk. When using disinfectants, the minimum inhibitory concentration (MIC) must be contemplated to avoid resistance. The objective of the present study was to determine the MIC of four essential oils in four *L. monocytogenes* strains. Once the MICs were obtained, the effect of subinhibitory doses of the most effective oil was determined. Strains were subsequently subjected to increasing doses of cassia and the MICs were determined again to evaluate differences. The effect of subinhibitory doses for biofilm formation was evaluated using quantitative and observational methodologies. Last, it was studied whether the strains were more sensitive to antibiotics after being in contact with the oil. After continuous exposure to 1/2 MIC, a decrease in the initial MIC ($P = 0.013$) was observed, specifically for strains belonging to serotype 1/2a ($P = 0.041$). In contrast, the formation of biofilms did not show differences between the control and exposed groups ($P > 0.05$). The qualitative study showed that there were no differences in the structure of the biofilms before and after contact with cassia, except for the CECT 935 strain, indicating a strain-dependent trend. Moreover, species-dependent differences were observed in the conformation of the extracellular matrix.

1. Introduction

Cross contamination has been identified as one of the main vehicles of contamination of food by *Listeria monocytogenes* due to its ability to form biofilms (Mazaheri et al., 2021), which are structured communities of microbial cells adhered to a surface and embedded in a matrix of extracellular polymeric substances (EPS), characterized by presenting an altered phenotype and gene expression (Colagiorgi et al., 2017). *L. monocytogenes* can form biofilms on the surface of different materials used in the food industry, representing a serious concern in terms of food safety because of their possible relation with consequent sources of contamination (Colagiorgi et al., 2017; González-Rivas et al., 2018).

The presence of biofilms on industrial surfaces is an important factor in the persistence of pathogens in food processing environments. In addition, biofilms have a significant impact on public health since they can reach food as a result of cross contamination (Mazaheri et al., 2021). This contamination can occur in any phase of food processing through handlers, equipment, or contact surfaces (Abdallah et al., 2014; Winkelstroter, 2015). In addition to the impact at the safety level, biofilms increase the economic losses of these industries because not only can the products be withdrawn from the market but biofilms damage the heat transfer processes and increase the corrosion of surfaces (Winkelstroter, 2015). Furthermore, the settlement and persistence of pathogens in the food industry is closely related to their response to factors such as humidity, temperature, pH, and the type of adhesion surface, among others (Abdallah et al., 2014; Bridier et al., 2015; Simões et al., 2010; Winkelstroter, 2015).

The cells that make up a biofilm are more resistant to antimicrobial compounds than cells in a planktonic state (González-Rivas et al., 2018). This fact is directly related to the architecture and physiology in the biofilm environment, which confers greater cell survival due to reduced diffusion of compounds, anaerobic growth, physiological changes due to reduced growth rates, or production of enzymes that degrade antimicrobial substances. (González-Rivas et al., 2018; Simões et al., 2010; Winkelstroter, 2015). Due to the greater cellular resistance in these biofilms, their complete elimination is more complex. The strategies to combat biofilms are basically divided into two aspects: the inhibition of their formation and the application of a treatment to eliminate them once they have formed. In this context, repeated efforts have been made to prevent and control their formation, with elimination strategies

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becoming particularly prominent over time. This is why in the food industry and after cleaning a wide variety of chemical disinfectants are used, such as acid compounds, biocides based on aldehydes, caustic products such as chlorine, hydrogen peroxide, iodine, isothiazolinones, ozone, peracetic acid, phenolics, biguanides, halogen surfactants, and quaternary ammonium compounds. However, factors such as interference from inorganic substances, water hardness, chemical inhibitors, concentration and contact time, among others, generally affect the effectiveness of disinfectants and may make them insufficiently effective to completely eliminate biofilms (Simões et al., 2010; Winkelstroter, 2015).

Likewise, the persistence of *L. monocytogenes* is related to the resistance of strains to disinfectants, with studies showing that adaptation to disinfectants can occur when microorganisms are exposed to sublethal or sub-inhibitory concentrations (Carpentier & Cerf, 2011; González-Rivas et al., 2018). Therefore, it is important to consider the Minimum Inhibitory Concentration (MIC) in the use of disinfectants for the elimination of biofilms because their improper use can induce resistance. In recent times it has also been suggested that there is a direct relationship between the phenomena of tolerance and adaptation of biocides and resistance to antibiotics because the mechanisms that favor the two phenomena are similar. In this regard, it has been shown that exposure to sublethal concentrations of antimicrobials facilitates the selection of bacterial strains resistant to antibiotics, mainly through mechanisms of cross-resistance or co-resistance (Capita & Alonso-Calleja, 2013; Molina-González et al., 2014). Resistance to antibiotics thus becomes a complex problem that transcends international borders, particularly if the global rates of their constant use continue to increase. By 2050, antibiotic resistance is forecasted to cause around 10 million deaths worldwide annually, costing the global economy 100 billion dollars (McCullough et al., 2016). Therefore, effective actions are urgently required by all sectors, including governments, researchers, doctors, different types of industries and the public, to minimize antibiotic resistance by reducing the use of antibiotics or compounds that favor the emergence of resistance (McCullough et al., 2016). In this context, essential oils are gaining interest in the food industry for their antimicrobial properties (Dumas et al., 2021). Their use against different pathogenic microorganisms is therefore being investigated (Mutlu-Ingok et al., 2020; Tariq et al., 2019). These substances' ability to induce resistance must also be evaluated in the context of the strategy against pathogens such as *L. monocytogenes*.

This explains why the need to develop solutions and tools for the control of biofilms, such as new disinfectants that do not induce the generation of resistance to the biocide itself or cross-resistance to antibiotics, is becoming more urgent. To this end, the objectives of the present study were: (1) To determine the MIC of four essential oils (cassia, clove, oregano and cinnamon) in four different strains of *L. monocytogenes* (CECT 935, CECT 5672, S2-bac, EDG-e); (2) To determine the effect of exposure to sub-inhibitory doses on the MIC of the most effective oil, cassia oil, subjecting the 4 mentioned strains to increasing doses of the essential oil; (3) To evaluate the effect of the application of these subinhibitory doses on the formation of biofilms of *L. monocytogenes*; (4) To determine the effect of increasing subinhibitory concentrations applied on the pattern of susceptibility to various antibiotics.

2. Material and methods

2.1. Essential oils

A total of four essential oils were used: cassia, clove, oregano, and cinnamon (Merck, Darmstadt, Germany) (Table 1). All the oils, except the oregano, were kept at room temperature in a cool, dry place protected from sunlight. The oregano oil was preserved at refrigeration temperature (4 °C) as per the product's technical data sheet.

Table 1
Essential oils used and their corresponding active compounds (Bagheri et al., 2020).

ESSENTIAL OIL	SCIENTIFIC NAME	ACTIVE COMPOUND
CASSIA	*Cinnamomum cassia* Blume	*Trans*-cinnamaldehyde, cinnamyl acetate
CLOVE	*Eugenia* spp.	Eugenol, eugnyl acetate, caryophyllene
OREGANO	*Thymus capitatus*	Carvacrol, p-cimeno
CINNAMON	*Cinnamomum zeylanicum*	Eugenol, cinnamaldehyde, cinnamyl acetate, β-phelandrene

2.2. Bacterial strains

In this study, four strains of *Listeria monocytogenes* of different origins were used, two of them from the Spanish Collection of Type Cultures (CECT, University of Valencia, Valencia, Spain) and belonging to serotype 4b: CECT 935 and CECT 5672; and the other two, considered wild, were isolated from surfaces of Iberian pig processing plants, with serotype 1/2a: S2-bac and EDG-e (Ripolles-Avila, Cervantes-Huaman, Hascoët, Yuste, & Rodríguez-Jerez, 2019). The strains started in a lyophilized state, so were rehydrated in Soy Tryptone Broth (TSB; Oxoid, Madrid, Spain) by incubation at 30 °C for 40–48 h. After this initial recovery period, the strains were streaked onto plates with Tryptone Soy Agar (TSA; Oxoid, Madrid, Spain) and incubated at 37 °C for 16–24 h. Colonies were isolated from the culture and re-seeded on TSA plates, and again incubated at 37 °C for 16–24 h. Last, the re-seeded TSA plates were stored as stock cultures in refrigeration (4 °C) for a maximum period of one month.

2.3. Determination of minimum inhibitory concentration (MIC) before exposure

Bacterial inoculum was obtained from stock cultures kept in refrigeration. For each strain, a colony was recovered from the seeded TSA plates and inoculated into a TSB tube, which was incubated at 37 °C for 24 h. After the incubation time, the inoculum was prepared at a concentration of 10^5 CFU/mL in TSB.

The MIC of the four essential oils was determined starting from a concentration prepared in a previous screening carried out by the same research group (data not shown). For this, 96-well microtiter plates (Sudelab SL, Barcelona, Spain) were used, into which 20 μL of the required dilution of essential oil were first inoculated in 3% Tween 80 (Sigma-Aldrich, Madrid, Spain) together with 180 μL of the bacterial inoculum (10^5 CFU/mL). At the same time, negative and positive controls were included for each test performed. Once the wells were inoculated, in the case of cassia, the plates were incubated at 37 °C for 48 h. For the essential oils clove, oregano, and cinnamon, and due to the impossibility of reading the wells due to the opacity derived from the color of the oil itself, an incubation was carried out at 37 °C for 24 h, followed by a dilution with 180 μL of TSB in new plates. These new plates were incubated at 37 °C for 24 h. After the incubation time, the microtiter plates were read and the concentration in the wells from which there was no longer any growth determined as the MIC.

2.4. Exposure to increasing sub-inhibitory concentrations of cassia essential oil

Once the MIC of the four essential oils was set, the most effective one was chosen (*i.e.* the one that presented a lower MIC for the four strains in common), this being cassia. It was subsequently decided to use a MIC/2 of this oil as a subinhibitory base dose (*i.e.* 206 ppm; see section 3.1.) to adapt the *L. monocytogenes* strains to increasing concentrations of the oil (Molina-González et al., 2014). For this, 20 μL of the MIC/2 dilution of the essential oil with 3% Tween 80 and 180 μL of the previously prepared bacterial inoculum at 10^5 CFU/mL were inoculated into 96-well

microtiter plates. Positive and negative controls were included in each experimental test. After inoculation, the plates were incubated at 37 °C for 48 h. Once the incubation time had elapsed, 100 μL from these plates were transferred to new microtiter plates into which 80 μL of TSB were also added as a nutrient to promote bacterial growth, as well as 20 μL of the MIC/2 concentration of the cassia essential oil. The plates were then incubated again at 37 °C for 48 h, and this last step was repeated on new plates, making a total of two transfers at increasing doses of essential oil. Last, 100 μL from random wells were transferred to 9.9 mL TSB tubes and incubated at 37 °C for 24 h. These tubes with the exposed strains were the ones that were subsequently used as starting inoculum in the evaluations determined in section 2.5. and 2.6.

2.5. Biofilm formation

To carry out the tests on the biofilm formation ability, the strains were used once they had been subjected to the increasing concentrations of cassia essential oil. In this case, 100 μL were removed from different wells at random where there was growth after adaptation to the essential oil and transferred to TSB tubes, which were incubated at 37 °C for 16–24 h. After this time, serial dilutions were made in $TSYEB_{gluc1\%+NaCl2\%}$ (TSB supplemented with 0.3% yeast extract (BD, Madrid, Spain), 1% glucose (BioLife, Barcelona, Spain) and 2% chloride sodium (Panreac, Castellar del Vallès, Spain)), an enriched culture medium specific for the growth of *L. monocytogenes* (Pan et al., 2010; Ripolles-Avila, 2018), until an inoculum concentration of 10^6 CFU/mL was obtained. These inoculums were used for the formation of biofilms. For the formation of biofilms from the controls (*i.e.* strains not exposed to the compound), the same procedure was followed with the only difference being the inoculation of between 3 and 5 colonies of each of the stock cultures of *L. monocytogenes* in tubes of $TSYEB_{gluc1\%+NaCl2\%}$

Afterwards, the biofilms were formed following the model established by Ripolles-Avila et al. (2018). For this, AISI 316 grade 2B stainless steel surfaces 2 cm in diameter and 1 mm thick were used, prior to which they were cleaned, disinfected and sterilized in accordance with the European standard UNE-EN 13697:2015, related to non-porous materials (AENOR, 2015). To form the biofilms, 30 μL of the suspension described above were inoculated into the center of the stainless-steel discs, resulting in a surface concentration of approximately 4 log (CFU/cm^2). Subsequently, the discs were placed in sterile Petri plates and these, in turn, were placed in a humid chamber with saturated relative humidity for incubation at 30 °C for 72 h to improve the growth of adhered cells and promote biofilm formation.

2.6. Evaluation of the cassia oil subinhibitory concentrations effect

2.6.1. Determination of MICs after adaptation

Once the experimental part described in section 2.4. was completed, the inoculum made from the strains exposed to the increasing doses of cassia oil was used to again determine the MIC of the compound, and thus be able to evaluate whether this subjection of the strains at increasing sub-inhibitory concentrations of the oil had induced changes in the level of resistance/susceptibility. For this, the same procedure established in section 2.3. was carried out, with the difference that this time it was based on the inoculum of the exposed bacterial strains.

2.6.2. Biofilms quantitative analysis

The quantitative analysis was carried out using the TEMPO system. First, the discs where the biofilms were formed were washed in duplicate with 3 mL of sterile distilled water to discard the non-adhered cells. The discs were then placed in a sterile bottle with 3.5 g of sterile glass beads, 10 mL of neutralizer [0.1% tryptone (BD, Madrid, Spain), 0.85% sodium chloride (Panreac, Castellar del Vallés, Spain), and 3% of Tween 80]. Once the samples were introduced into the flasks, they were vortexed for 90 s at 40 Hz to detach the adhered cells from the surface and thus be able to quantify them (Ripolles-Avila, Cervantes-Huaman et al., 2019). From here on, serial dilutions were made in diluent (0.1% tryptone, 0.85% sodium chloride) and were quantified by transferring 1 mL to the TEMPO vials, which contained the lyophilized nutrient medium previously rehydrated with 3 mL of sterile distilled water. The vials were vortexed for homogenization before their contents were transferred to a card using the TEMPO filler station. The barcodes on each card were recorded and encoded using the TEMPO prep station. Last, the cards were incubated at 30 °C for 48 h and the reading was carried out using the TEMPO reader station. The necessary mathematical adjustments were made to express the results in log (CFU/cm^2).

2.6.3. Qualitative assessment of the biofilms structure and matrix

Biofilm formation was also qualitatively evaluated to observe two different parameters, the first of which was the structure. For this purpose, the discs were washed in duplicate with 3 mL of sterile distilled water to remove any cells that had not adhered to the surface and which, therefore, were not part of the biofilm generated. The discs were then placed on slides and 5 μL of the vital Live/Dead BacLight stain (Molecular Probes, Oregon, USA) added before covering with coverslips. Due to the sensitivity of the reagent, the procedure was carried out keeping the discs protected from sunlight. They were then incubated at room temperature for 15 min (Ripolles-Avila et al., 2018).

Second, the matrix produced was evaluated. For this, the FilmTracer SYPRO Ruby Biofilm Matrix Stain (Thermo Fisher Scientific, Barcelona, Spain) was used. Like the procedure for the previous evaluation, the discs were washed with 3 mL of sterile distilled water in duplicate and then placed on slides. They were stained with 200 μL of the stain and incubated for 30 min at room temperature, protected from sunlight. After this time, the reagent was removed and a coverslip placed on them. This type of staining stains biofilm matrices, marking most protein classes including glycoproteins, phosphoproteins, lipoproteins, calcium-binding proteins, and fibrillar proteins (Ripolles-Avila et al., 2018).

The stained surfaces were evaluated by DEM, using an Olympus BX51/BX52 direct epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a 100 W mercury lamp (USH-103OL, Olympus) and a double-pass filter (U- M51004 F/R–V2, Olympus), and attached to a digital camera (DP73, Olympus). The discs were observed at 20X to examine the structure of the biofilm formed, to be able to evaluate the vital status of the bacterial cells and the production of matrix. During the observational study, three photographs were taken at random of each disk from three different fields to later be able to carry out a qualitative evaluation in comparison with the controls of the strains that had not been exposed.

2.6.4. Antibiotic susceptibility

To determine the susceptibility to antibiotics of the strains after having been in contact with cassia, antibiograms were performed using 15 different antibiotic discs (Oxoid, Madrid, Spain) (Table 2) on Mueller-Hinton agar (Oxoid, Madrid, Spain) using the disk diffusion method described by Clinical and Laboratory Standards Institute (CLSI). Based on a bibliographic review, the 15 antibiotics of greatest interest to the study were selected (Baquero et al., 2020; Chow et al., 2021; Noll et al., 2018; Olaimat et al., 2018; Park et al., 2021; Wai et al., 2015). Zones of inhibition were measured and rated as sensitive (S), intermediate susceptibility (I), and resistant (R) according to the CLSI (2018) and EUCAST (2021) guidelines. A total of 720 tests were carried out considering all the combinations of strains and antibiotics, plus the comparative controls.

2.7. Statistical analysis

In this study, all trials were performed in duplicate in three separate experiments (n = 6). Regarding the quantitative evaluation of the biofilms, the bacterial counts obtained were converted into decimal logarithmic values to coincide with the assumption of a normal distribution. The IBM SPSS Statistics version 28 program was used for the statistical

Table 2
Antibiotic disks used for susceptibility screening, with their respective concentrations and pharmacological families (Obando Pacheco et al., 2020).

ANTIBIOTICS	CONCENTRATION (MG)	FAMILY
AMPICILLIN (AMP)	10	Beta-lactams
GENTAMICIN (CN)	10	Aminoglycosides
TRIMETHOPRIM-SULFAMETHOXAZOLE (SXT)	25	Sulfonamides
TETRACYCLINE (TE)	30	Tetracyclines
CHLORAMPHENICOL (C)	30	Amphenicols
CIPROFLOXACIN (CIP)	5	Quinolones
CEFOXITIN (FOX)	30	Cephalosporins
CEFOTAXIME (CTX)	30	Cephalosporins
OXACILLIN (OX)	1	Beta-lactams
ERYTHROMYCIN (E)	15	Macrolides
VANCOMYCIN (VA)	30	Glycopeptides
RIFAMPIN (RD)	5	Rifamycins
ENROFLOXACIN (ENR)	5	Quinolones
CEFEPIME (FEP)	30	Cephalosporins
NITROFURANTOIN (F)	300	Nitrofurans

analysis of the data. The "One Way ANOVA" test was used for the comparison of the mean of the four oils, with a posteriori contrast using the "Tukey" test to observe whether there were statistically significant differences between the MIC concentrations obtained for each oil. In addition, the data obtained from the study of exposure to increasing sub-inhibitory concentrations of essential oil and the quantitative analysis of biofilms was analyzed using a general univariate linear model with a posteriori contrast using the "Tukey" test. In all cases, a significance level of $P < 0.05$ was set.

3. Results and discussion

3.1. MIC determination in 4 essential oils

Table 3 shows the MIC values of each essential oil studied for the different strains of *L. monocytogenes*. Cassia was the oil with the highest effectiveness as it obtained a lower MIC value (*i.e.* 412.5 ppm) and presented significant differences ($P < 0.05$) from the rest of the oils. In turn, it was shown that all the oils presented significant differences between them ($P < 0.05$) in relation to their inhibitory capacity. These results agree with those reported by Oussalah et al. (2007) and Mith et al. (2014) in relation to the maximum effectiveness found for the essential oil cassia. Cinnamon-based oils, such as cassia and cinnamon itself, have been shown to have a very effective antimicrobial spectrum against *L. monocytogenes* (Bagheri et al., 2020; Barbosa et al., 2021).

Table 3
MIC values of the evaluated essential oils obtained for four strains of *Listeria monocytogenes* (n = 6) together with the corresponding standard error of the mean (SEM).

Essential oils	MIC (ppm)				Average MIC (ppm)
	CECT 5672	CECT 935	S2-bac	EDG-e	Species level
Cassia	400 ± 00 ^{Aa}	350 ± 50 ^{Aa}	450 ± 50 ^{Aa}	450 ± 50 ^{Aa}	412.5 ± 22.66 ^{A}
Clove	4000 ± 500 ^{Ba}	3000 ± 00 ^{Ba}	3000 ± 500 ^{Ba}	3500 ± 00 ^{Ba}	3375 ± 205.94 ^{B}
Oregano	3250 ± 250^{Ca}	2500 ± 00 ^{Cb}	2000 ± 00 ^{Cb}	2500 ± 00 ^{Cb}	2562.5 ± 175.19 ^{C}
Cinnamon	5250 ± 250 ^{Da}	4500 ± 500 ^{Da}	4000 ± 00 ^{Da}	4500 ± 00 ^{Da}	4562.5 ± 199.05 ^{D}

$^{a-b}$ Values in a row that lack a common lowercase superscript differ significantly ($P < 0.05$).
$^{A-D}$ Values in a column lacking a common uppercase superscript differ significantly ($P < 0.05$).

Likewise, Chahbi et al., (2020), Kim et al. (2021), and Mutlu-Ingok et al. (2020) point out that oils that contain cinnamaldehyde among their composition, such as oregano in whose composition carvacrol stands out, are more effective compared to other essential oils. However, in the present study, the results obtained for cinnamon oil showed a lower antibacterial activity. In this case, it is important to highlight that the composition of the oil is a fundamental factor that directly influences its inhibitory capacity (de Oliveira et al., 2012). Cinnamaldehyde is one of the main components in the essential oils of cassia and cinnamon (Bagheri et al., 2020). In this regard, cinnamon oil obtained a significantly higher MIC value ($P < 0.001$) (*i.e.* 4562.5 ppm) than cassia oil (*i.e.* 412.5 ppm), which could be related to a different concentration of its active components or a different steric conformation of the molecules because cassia oil contains cinnamaldehyde in *trans*-form. Regarding this last point, Hill et al. (2013) indicated that the steric conformation of the molecules of a compound can influence the solubility of the oil, which would improve the distribution of the compound in the solution obtained with Tween 80. In the study mentioned, MIC values around 500 ppm were obtained for *Listeria innocua*, consistent with those obtained in the present study for *L. monocytogenes*. In parallel, the main active compound in cinnamon oil is usually eugenol (Bagheri et al., 2020). This compound has been shown to have a lower MIC than carvacrol, the majority compound in oregano oil (Walsh et al., 2019).

However, it was questioned whether the degree of sensitivity could vary between strains. In this case, for the cassia, clove and cinnamon oils, no significant differences were observed between the strains ($P > 0.05$). However, oregano oil did present statistically significant differences between the CECT 5672 strain and the CECT 935 ($P = 0.044$), S2-bac ($P = 0.007$), and EDG-e ($P = 0.044$) strains, showing a lower susceptibility of the CECT 5672 strain by requiring more concentration of the compound to obtain an effect. This strain in particular is known as Scott A and tends to have greater resistance to different types of treatments (Bucur et al., 2018). It has been recognized that the ability of *L. monocytogenes* to survive stress conditions may be strain-dependent, the variations being more related to the genetic lineage than to the origin of the strain (Dumas et al., 2021). Kawacka et al. (2021) also show differences in MICs of distinct strains when in contact with essential oils, unlike the results obtained in the present study, apart from those for oregano oil. Last, in the study carried out by Dumas et al. (2021) and in the present study, no significant differences were observed between *L. monocytogenes* strains when faced with the essential oil of cassia. As there were no differences, the average MIC value between strains was used as a reference value for the continuation of the study.

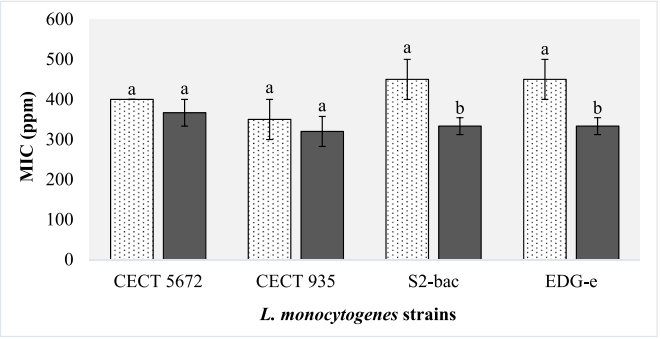

Fig. 1. MIC determination for *L. monocyotgenes* strains before (Control ☐) and after (Exposed ■) exposure to increasing concentrations of cassia. Each value corresponds to the mean of two replications carried out on three different days (n = 6). The error bars represent the standard error of the mean. $^{a-b}$ indicate the significant differences ($P < 0.05$) between the two groups.

3.2. MIC comparison after exposure to subinhibitory concentrations of cassia

Fig. 1 shows the MIC values obtained before and after exposure to increasing concentrations of cassia oil, previously described as the most effective. Significant differences were observed at the species level between the values obtained in the control and the values obtained after exposure ($P = 0.013$). More specifically, the strains that showed significant differences between the MIC required in the control and the MIC manifested after adaptation were the S2-bac and EDG-e strains belonging to serotype 1/2a ($P = 0.041$), as opposed to the strains of serotype 4b ($P = 0.605$ and $P = 0.195$, respectively for CECT 5672 and CECT 935). In this case, after adaptation the serotype 1/2a strains showed a lower MIC than the serotype 4b strains. To reinforce this argument, significant differences were observed between serotypes ($P < 0.05$). The results obtained differ from those reported for other active compounds such as eugenol and carvacrol, for which previous exposure of *L. monocytogenes* to sublethal doses conditioned an increase in its resistance (Souza et al., 2015).

Thus, a trend is observed where the difference in MIC between the two serotypes is evident. To this effect, after adaptation, serotype 1/2a shows a proportionally greater decrease in MIC. In addition, a trend was also observed for the CECT 935 strain, which showed a lower MIC compared to the other strains in both the control group and the exposed group. According to Álvarez-Ordóñez et al. (2008), exposure to antimicrobials in sublethal concentrations can result in the development of greater tolerance to the same factors, an adaptation called homologous. This tolerance was not demonstrated in the results of the present study, but rather a sensitization of the population or a lack of influence. It could be that the decrease in MIC occurs for two reasons: (1) That the sensitized strains are inhibited and have difficulty growing at the same initial concentrations of MIC; (2) or that this second MIC has had a lethal effect on them and therefore they do not show growth. In the second case, MIC would be considered what is known as Minimum Bactericidal Concentration (MBC), which is defined as a concentration that eliminates 99.9% or more of the initial inoculums (Tariq et al., 2019). Nonetheless, further studies are needed to expand the number of strains evaluated to be able to confirm the trend described here, since in most experimental studies no observations have been made between strains of the same species.

3.3. Effect of cassia subinhibitory doses on biofilm formation

Biofilm counts of *L. monocytogenes* obtained from subjection to subinhibitory doses of cassia can be observed in Fig. 2. The ability of the strains of *L. monocytogenes* to form biofilms on stainless steel was evaluated during a 72-h incubation period. This process was established to ensure optimal conditions for growth and to favor the formation of biofilms of the pathogen (Kadam et al., 2013). Statistically, the counts obtained in the biofilms of the exposed group did not present significant differences from those of the control group ($P = 0.222$), and there were also no significant differences between strains ($P = 0.240$). The count of the biofilms obtained in this study agrees with the findings of other investigations in which quantitative methods were used to evaluate the biofilm formation (Koo et al., 2014; Ripolles-Avila et al., 2018).

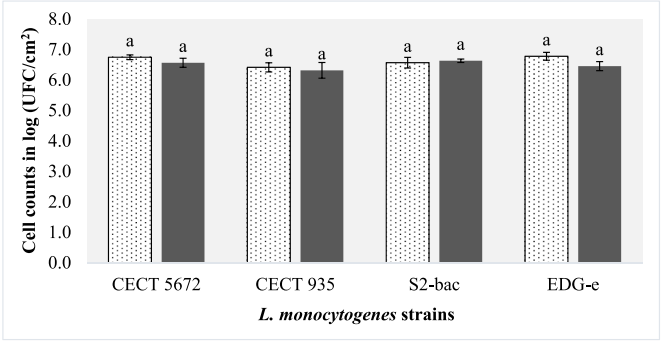

Fig. 2. Count of cells that make up the biofilms of *L. monocytogenes* strains before (Control ▯) and after (Exposed ■) exposure to increasing subinhibitory doses of cassia essential oil. Each value corresponds to the mean of two replications carried out on three different days (n = 6). The error bars represent the standard error of the mean. [a] indicates the absence of significant differences ($P < 0.05$) between the two groups.

Since there were no significant differences between the biofilm counts before and after exposure to increasing doses of cassia, but significant differences were obtained between the MICs of the control and species-exposed groups, this suggests that *L. monocytogenes* can form biofilms as a protection against the stress agent. Thus, although the planktonic cells were damaged by the effect of the oil, the moment they came into contact with the study surface they began to structure themselves as biofilm, forming microcolonies and producing extracellular substances that act as a protective polymeric matrix (Fuster-Valls et al., 2008).

Furthermore, it is known that there is variability regarding the ability to form biofilms between strains that belong to the same species (Borges et al., 2018; Ripolles-Avila, 2018), but in this study biofilm resistance did not show a strain dependent trend, but rather a species dependent one. Likewise, the results obtained do not allow us to conclude whether there is a relationship between strain and biofilm formation capacity. There were also no significant differences between the counts obtained by the serotype 1/2a strains and the serotype 4b strains (P = 0.381). Therefore, although a differentiation between the two serotypes has been previously observed, the same is not shown in the formation of biofilms. However, several researchers have associated serotypes with various attributes of L. monocytogenes, including the formation of biofilms (Orsi et al., 2011; Wang et al., 2017; Zoz et al., 2017). However, the relationship between L. monocytogenes serotype and biofilm formation remains unresolved (Kadam et al., 2013; Ripolles-Avila et al., 2019)

3.4. Subinhibitory doses impact on the structure and matrix of biofilms produced by L. monocytogenes

Fig. 3 shows the biofilms generated by the four *L. monocytogenes* strains under study before and after exposure to subinhibitory doses of cassia essential oil. In both study groups (*i.e.* control and exposed), the shape of the cells in terms of the distribution of the colonies on the surface can be observed, in some cases establishing themselves in geometric shapes, and in other cases being more dispersed. In this regard, it has been indicated that the formation of biofilms can be determined based on the organization of the cells that comprise it, through observation by the DEM method (Ripolles-Avila et al., 2018). One characteristic that shows that a biofilm has reached its state of maturity is the presence of water channels (Abdallah et al., 2014). These formations promote the constant circulation of nutrients and the elimination of waste within the biofilm (Ripolles-Avila et al., 2018). Taking this into account, it can be confirmed that the CECT 5672, S2-bac, and EDG-e strains used in the study formed biofilms in a 72-h incubation period in the same way before and after being subjected to increasing doses of cassia oil, although with a different organization level. Following this line, the images obtained are similar to those presented in the studies by Marsh et al. (2003) and Ripolles-Avila et al. (2018).

In contrast, a disaggregated cell arrangement indicates that the biofilm has not formed (Chmielewski & Frank, 2003). Following these indications, in the images it can be seen that the control strain CECT 935 (Fig. 3-B1) does not follow the same organization, but rather shows a greater dispersion. This behavior was also observed in the study by Marsh et al. (2003), at 72 h of incubation by a *L. monocytogenes* strain that also corresponded to serotype 4b. Thus, at the count level this strain does not present differences, but differences are observed at an observational structural level. In this aspect, the CECT 935 strain shows an

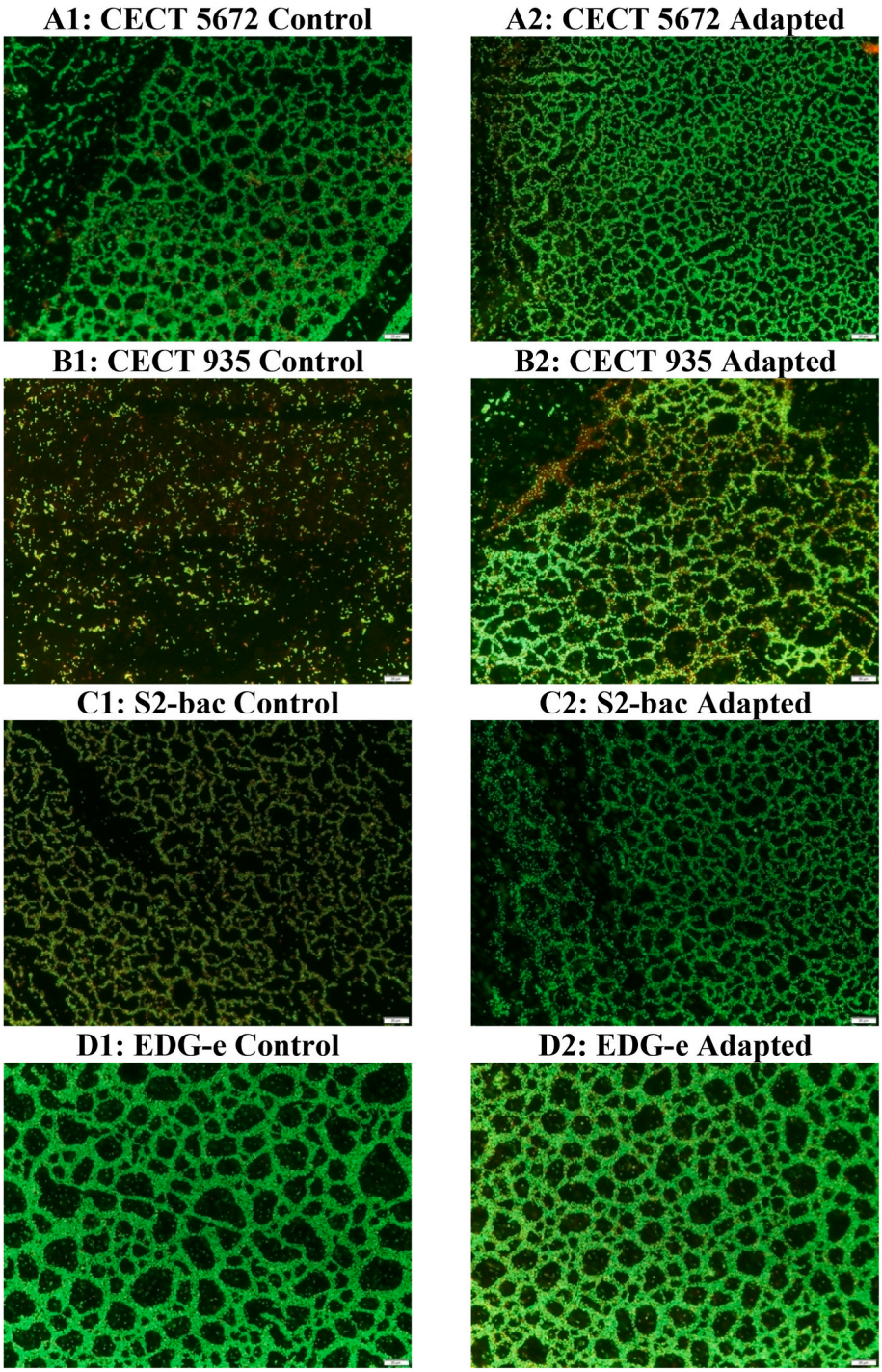

Fig. 3. Images obtained by DEM of *L. monocytogenes* biofilms: (A) CECT 5672, (B) CECT 935, (C) S2-bac and (D) EDG-e, before (1) and after (2) exposure to subinhibitory does of cassia essential oil, stained with Live/Dead Baclight. 20× magnification.

adaptation after being in contact with cassia oil.

Biofilms, as mentioned above, are described as microbial aggregations that grow on surfaces and excrete different substances such as polysaccharides, proteins, and eDNA among others, all embedded in the extracellular matrix (ECM) (Colagiorgi et al., 2017). Thus, the ECM generated to form the biofilms was studied using DEM. Fig. 4 shows the difference in ECM production of the four study strains, before and after exposure to cassia oil subinhibitory doses (*i.e.* control and exposed, respectively). This qualitative study, which has been used in various investigations (Dapa et al., 2013; Frank & Patel, 2007; Sanchez et al., 2013), was carried out to be able to observe the biofilm matrices by staining most of the protein classes (Ripolles-Avila et al., 2018). Furthermore, this parameter also indicates the maturity of the biofilm and contributes greatly to the final architecture of the community (Branda et al., 2005). The results obtained show that after subjection to increasing doses of cassia all the strains generated a greater production of protein substances that gave the matrix consistency. Based on these results, it can be deduced that a considerably greater amount of extracellular protein is generated. On the other hand, the images obtained from the control group agree and present similarities with those presented in the study by Ripolles-Avila et al. (2018).

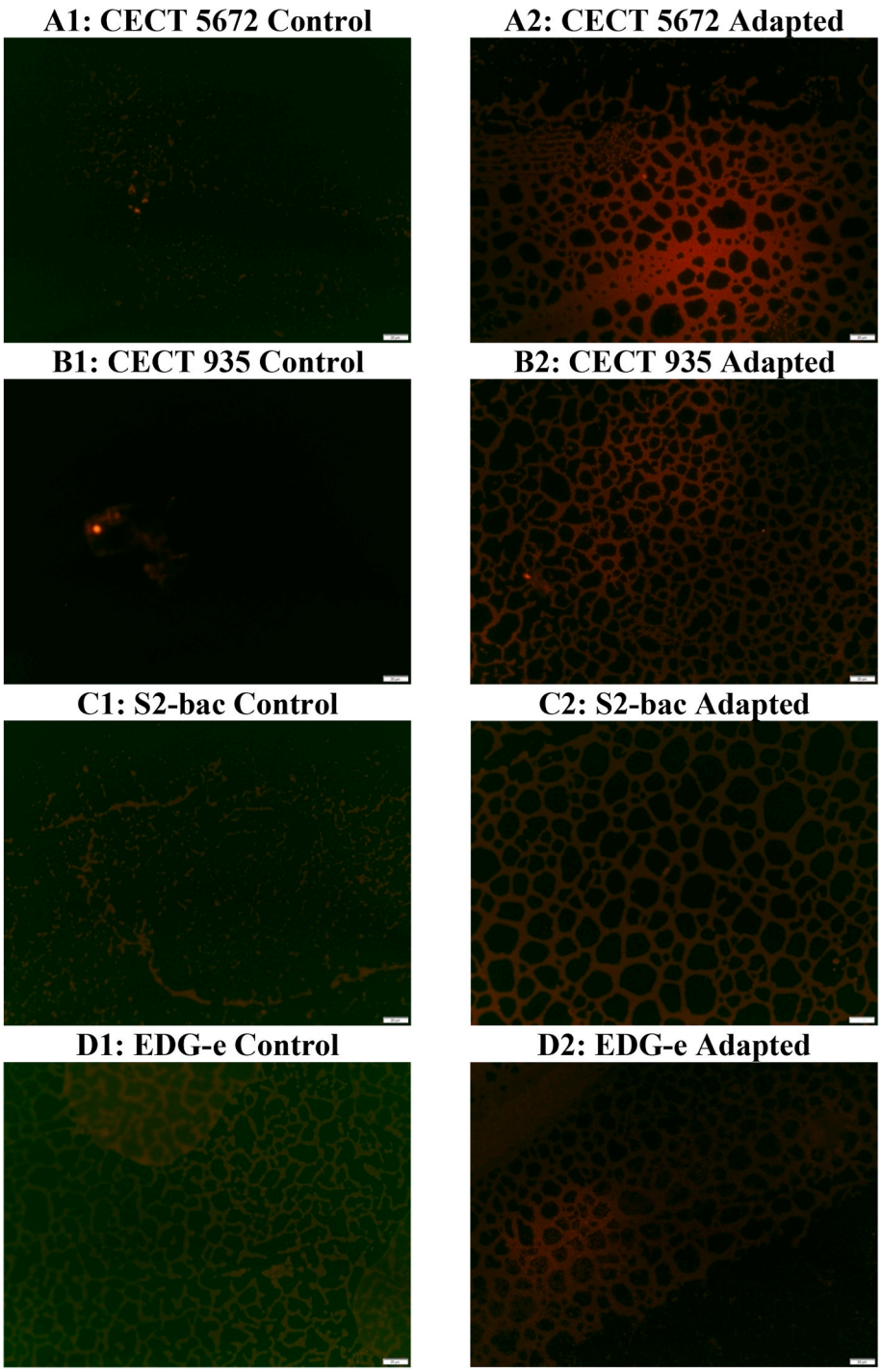

Fig. 4. Images obtained by DEM of *L. monocytogenes* biofilms: (A) CECT 5672, (B) CECT 935, (C) S2-bac, and (D) EDG-e, before (1) and after (2) exposure to subinhibitory doses of cassia essential oil, stained with FilmTracer SYPRO Ruby Biofilm Matrix Stain. 20× magnification.

3.5. Susceptibility profiles derived from continuous exposure to antibiotics

Table 4 shows the susceptibility of the studied strains to 15 different antibiotics, before and after exposure to increasing doses of cassia oil. For most antibiotics, no changes were observed in the susceptibility/resistance pattern after this exposure. To this effect, there were only five changes: (1) from R to I for FEP in strain CECT 5672; (2) from R to S for CIP in strain S2-bac; (3) changes from I to R for CTX in the EDG-e strain; (4) from I to S for ENR; and (5) from S to I for FEP. Of these five changes, three show an increase in sensitivity and, conversely, the other two show an increase in resistance to antibiotics.

As seen in Table 2, the FEP and CTX antibiotics belong to the cephalosporin family, and two of their three induced changes in the susceptibility pattern are related to an increase in resistance. In contrast, CIP and ENR, which belong to the quinolone family, induced an increase in sensitivity. These differences in the susceptibility pattern can therefore be explained by the different mechanism of action of the different families (Reygaert, 2018). On the one hand, cephalosporins are bactericidal antibiotics that act by inhibiting the synthesis of the bacterial cell wall. They inhibit transpeptidation in the final stages of peptidoglycan synthesis, an essential polymer for the bacterial wall (Georgopapadakou & Bertasso, 1993).

Table 4
Susceptibility of *L. monocytogenes* against 15 antibiotics, before (CNT) and after (EXP) contact with cassia essential oil. Susceptibility was designated as Sensitive (S), Resistant (R), or Intermediate Susceptibility (I), according to the criteria of CLSI (2018) and EUCAST (2021). Each value corresponds to the mean of two replications carried out on three different days (n = 6).

Antibiotic	*Listeria monocytogenes* strains							
	CECT 5672		CECT 935		S2-bac		EDG-e	
	CNT	EXP	CNT	EXP	CNT	EXP	CNT	EXP
AMP	S	S	S	S	S	S	S	S
CN	S	S	S	S	S	S	S	S
SXT	S	S	S	S	S	S	S	S
TE	S	S	S	S	S	S	S	S
C	S	S	S	S	S	S	S	S
CIP	S	S	S	S	R	S	S	S
FOX	R	R	R	R	R	R	R	R
CTX	R	R	R	R	R	R	I	R
OX	R	R	R	R	R	R	R	R
E	S	S	S	S	S	S	S	S
VA	S	S	S	S	S	S	S	S
RD	S	S	S	S	S	S	S	S
ENR	S	S	I	I	S	S	I	S
FEP	R	I	R	R	I	I	S	I
F	S	S	S	S	S	S	S	S

4. Conclusions

The development of new strategies for the elimination of microorganisms that do not induce the generation of resistance is currently crucial, highlighting the use of essential oils as an alternative. In the present study, it was shown that cassia essential oil is the most effective among the oils evaluated. At the same time, when *L. monocytogenes* strains were subjected to increasing subinhibitory doses of cassia, a decrease in MIC or no influence was observed depending on the strain under study, which implies that in no case is there an increase in the resistance of the pathogen towards cassia.

From the quantitative and qualitative studies of the biofilms formed after contact with cassia oil, it was determined that the strains could be developing some protection by showing greater structural and matrix conformation after being subjected to cassia oil. However, the cellular sensitivity of *L. monocytogenes* derived from exposure to subinhibitory doses within the biofilm could remain. Thus, when aggressive cleaning processes are applied to disintegrate the structure, the disinfection result could be more effective as the cells become sensitive, although more studies are required to verify this. Last, differences were observed in the susceptibility profile of antibiotics from different pharmacological families (*i.e.* cephalosporins and quinolones) after contact with cassia oil. These results indicate that different patterns of susceptibility can be induced depending on the family or mechanism of action of the antimicrobial. However, more studies are required to understand the mechanisms of action responsible for this effect.

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Declaration of competing interest

The authors declare no conflict of interest.

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