Promising nanostructured lipid carriers-based essential oils with activity against strains of *Campylobacter* ssp. isolated from chicken carcasses

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Abstract

Campylobacter is a virulent Gram-negative bacteria genus present mainly in poultry and broilers intestines. The indiscriminate use of traditional antibiotics, such as ciprofloxacin and other fluoroquinolones, led to drug resistance of these virulent pathogens remaining a public health issue. Thus, it is necessary to develop new therapies that are more efficient and less toxic, which is mandatory for use in biological applications. Essential oils (EO) are lipids arising from the secondary metabolism of vegetables, besides showing complex and biologically active structures, its clinical use is limited. Nanotechnology seems to be an alternative to increase bioavailability, stability and biocompatibility, decreasing the photodegradation and toxicity of EO. In this work, 14 EO were evaluated using the diffusion disk test, and it was selected 5 EO with the best anti-campylobacter activity. Then, these liquid lipids were used as active and structural compounds in the composition of nanostructured lipid carriers (NLC). Such resultant formulations were tested through the minimum inhibitory concentration (MIC), in order to find the lowest concentration able to inhibit the *Campylobacter* ssp. growth. These systems were also monitored in terms of particle size (nm), polydispersity index (PDI) and Zeta potential (mV), confirming its physicochemical stability for 210 days at 25 °C. Morphological analyses through FE-SEM elucidated spherical shape with well-delimited contours of nanoparticles. Then, the best NLC was tested regarding the nanotoxicity, through the chicken embryo model. In relevance to the above results, NLC-based geranium EO was the most promising and safe system for the control and treatment of multidrug-resistant strains of *Campylobacter*.

Keywords: geranial, cinnamon, nanobiotechnology, bioavailability, toxicity

1. Introduction

Campylobacter is a virulent Gram-negative bacteria genus mainly present in poultry, broilers (Hakeem, Fathima, Shanmugasundaram, Selvaraj, 2022), dogs and cats intestines (Montgomery, 2018). These pathogens can cause bloody diarrhea, abdominal cramps, nausea and vomits, and even in more complex cases, can cause Guillain-Barré syndrome and even deaths (CDC, 2022). After the first recognition, a large number of pathogenic species of *Campylobacter* that cause human campylobacteriosis have been cataloged by phylogenetic tools (Kaakoush, 2009). The *Campylobacter jejuni* is the main specie that results in foodborne infections in the United States, with 1.5 million people affected each year (CDC, 2021)

The indiscriminate use of traditional antibiotics, such as ciprofloxacin and other fluoroquinolones, led to a drug resistance of these virulent pathogens remaining a public health issue (EFSA, 2019). The *Campylobacter* drug mechanism resistance is usually interposed by mutations in the region of DNA gyrase of the microorganism. Then, a single mutation in this enzyme is capable of reducing the susceptibility of several drugs against different strains of *Campylobacter* ssp. (Alfredson, Korolik, 2007;). Therefore, the discovering of new antimicrobial compounds against strains of *Campylobacter* ssp. is urgent, in order to mitigate the cases of campylobacteriosis in worldwide.

Natural lipids from vegetables or animals, such as waxes, oils and butters, have been used for thousands of years as popular medicine, processed as infusions, syrups, poultices and ointments (Carbone, 2018). In this sense, essential oils (EO) are lipids arising from the secondary metabolism of vegetables, showing complex structure with biological activity, used as fungicide, antibiotics, antiviral, biopesticide, and antioxidant (Ribeiro, 2021; Ćavar Zeljković, 2022; Assadpour, 2023; Hou, 2022; Pinto, 2023). EO are mainly composed of terpenes and phenols that present recognized antibiotic activity (Mohammadi-Cheraghabadi, Hazrati, 2023), being widely used as candidate treatment against several species, such as *Salmonella ssp*. (Thanissery, Kathariou, Smith, 2014), and *Campylobacter jejuni* (Ribeiro, 2021). However, it presents certain limitations regarding its hydrophobicity, photosensitivity, high volatility, strongly basic pH, marked organoleptic properties, and hydrolysis ability (Guidotti-Takeuchi, 2022). Thus, nanoencapsulation becomes an alternative to prevent photodegradation, changes in physicochemical properties and even increase the bioavailability, optimizing the efficacy of EO (Chaudhari, 2021).

In this sense, nanotechnology is an innovative tool in the development of various nanostructured formulations for different applications (Chawla, Sivakumar, Kaur, 2021). Nanostructured lipid carriers (NLC) are composed of a blend of two or more solid and liquid lipids at room temperature, stabilized by surfactants. These systems present high efficiency in the encapsulation of water insoluble molecules, prolonged release of actives and excellent physicochemical stability (Ribeiro, 2016, Ribeiro, 2017). Thus, it is possible to be produced on a large scale, being a promising alternative for the delivery of antimicrobials (Liu, 2018). Therefore, when NLC have natural-based lipid matrices, through the use of butters, waxes and EO with strong antimicrobial activity, these excipients can play a dual role in the system: structural, once it is a component of nanoparticle, and bioactive, as antimicrobials agent (Ribeiro, 2021). This

strategy aims to increase bioavailability, decrease toxicity and prevent degradation of the EO, which are the main factors that limit its uses.

This work described the development of novel NLC formulations composed of natural lipids with antimicrobial activity against strains of *Campylobacter* ssp. EO were previously selected from a screening performed by the diffusion disk test. The resulting formulations were evaluated in terms of physicochemical stability for 210 days at 25 °C (n=3). The *vitro* antimicrobial activity of them was determined through the microdilution test to obtain the minimum inhibitory concentration (MIC) of the systems. Finally, structural characterization was performed by scanning electron microscopy (FE-SEM), which had confirmed the spherical morphology of NLC and nanotoxicity assays in embryo chicken model was performed, evidencing that the NLC-based geranium EO and beeswax was the most promising NLC formulation.

2. Material and Methods

2.1. *Bacterial strains inoculation*

The total of 3 *Campylobacter jejuni* strains (64/5, 30/1, 34763/3) and 3 *Campylobacter coli* strains (131/5, 131/6, 131/7) were selected from chicken carcasses. For all microbial susceptibility tests, bacterial strains were inoculated onto blood agar plates fortified with Ca^{2+} and Mg^{2+} and 5 % sheep's blood (Laborclin®, Brazil) and incubated at 42 ° C ± 1 °C in microaerophilic conditions for 48 h. After 2 days, isolated colonies of 3 to 5 species of the same morphological type and species were collected and dispersed in 1 mL of sterile saline solution (0.9 %) until reach the final concentration at 1.5 x 10^8 colony forming units (CFU) mL^{-1}, with the inoculum corresponding to 0.5 MacFarland scale turbidity.

2.2. *Screening of EO*

Lemongrass EO, cinnamon EO, geranium EO, clove EO, oregano EO, tea tree EO, sandalwood EO, citronella EO, thyme EO, copaiba EO, garlic EO, lavender EO and 2 vegetable oils, avocado and aloe vera oils (Engenharia das Essências®, Brazil) were submitted to *in vitro* antimicrobial susceptibility testing by diffusion disk test. Then, plates containing blood agar were fortified with Ca^{2+} and Mg^{2+} and 5 % sheep blood (Laborclin®, Brazil) and its sterility were ensured. With a sterile swab, the inoculum of *Campylobacter ssp.* strains was evenly distributed on the agar surface and allowed to stand at room temperature for approximately 5 min. After, disks were placed under the agar and 35 uL of each EO were added. The plates were incubated in an oven at 42 ± 1 °C for 48 h in microaerophilic conditions (Duarte, 2016). After this, the growth inhibition zone (mm) was measured, in duplicate.

2.3. *Preparation of nanostructured lipid carrier (NLC) formulations*

The preparation of the different formulations was done by the hot emulsification-ultrasonication method. The lipid phase of the formulations was composed of different natural lipids (Table 1). All lipid phases were heated in a water bath, 10 °C above the melting temperature of each solid lipid. Synchronously, the aqueous phase, which was the same for all formulations, was composed of the poloxamer solution (5 %, w/v,

Sigma Aldrich®, USA), which was heated at the same temperature to the lipid phase. For pre-emulsion formation, the aqueous phase was dropwise to each lipid phase under stirring at 10000 rpm for 2 min in an Ultra-Turrax homogenizer (Ultra-Turrax® T18, Germany). The pre-emulsions formed were immediately subjected to sonication for 15 min. At the end of this step, the formed nanoemulsions were cooled in an ice bath until reaching 25 °C, in order to solidify the formed nanoparticles (Ribeiro, 2016).

Table 1 – Composition of nanostructured lipid carriers

NLC	Solid Lipid (%)	Essential Oil (%)	Surfactant (%)
F1	Murumuru butter, (8 %)	Lemongrass, (5 %)	Poloxamer, (5 %)
F2	Cocoa Butter, (8 %)	Cinnamon, (5 %)	Poloxamer, (5 %)
F3	Beeswax, (8 %)	Geranium, (5 %)	Poloxamer, (5 %)
F4	Cocoa Butter, (8 %)	Clove, (5 %)	Poloxamer, (5 %)
F5	Beeswax, (8 %)	Oregano, (5 %)	Poloxamer, (5 %)
F6	Murumuru butter, (8 %)	-	Poloxamer, (5 %)
F7	Cocoa Butter, (8 %)	-	Poloxamer, (5 %)
F8	Beeswax, (8 %)	-	Poloxamer, (5 %)

2.4. *Physicochemical Stability Study*

Particle size (nm), polydispersity index (PDI) and Zeta potential (mV) measurements of NLC and NLC-based EO formulations were determined by dynamic light scattering technique, diluting the formulations (1:1000 v/v) in deionized water and analyzed by LiteSizer 500 equipment (Anton Paar, Berlin, Germany). The same parameters were followed for 210 days (25° C) in triplicate (Ribeiro, 2017). One-way ANOVA/Tukey post hoc statistical tests were used to determine intragroup statistical differences or not over time ($p<0.05$).

2.5. *Determination of the minimum inhibitory concentration of nanostructured lipid carriers*

NLC formulations were evaluated through the minimum inhibitory concentration (MIC) determination of different strains of *Campylobacter ssp.* The experiment was performed in 96-well plates in triplicate and the bacterial suspension was diluted in each well of the plate to reach a final concentration of 1×10^5 CFU·mL^{-1} per well. Then, the different concentrations of the formulations were added into 96-well plates up to a final volume of 0.1 mL. The positive control (Mueller Hilton Broth (Biolog®, Brazil) fortified with Ca^{2+} and Mg^{2+} and with 5 % sheep blood (Laborclin®, Brazil) with the inoculum was prepared to a final volume of 0.1 mL with 1×10^5 CFU·mL^{-1} of bacteria. The negative control was also prepared, without bacteria. The 96-well plate was incubated at 42 °C for 48 h in microaerophilic conditions (Duarte, 2016). MIC values was determined for

each NLC formulation. The t-test was employed to evaluate intergroup statistical differences ($p<0.05$).

2.6. *Characterization of the formulations by Scanning Electron Microscopy (FE-SEM)*

The elucidation of nanoparticle morphology of NLC and NLC-based EO samples was performed by FE-SEM technique. Thus, a drop of each sample was added onto a glass coverslip previously nailed to an aluminum stub. After complete evaporation of the solvent, the stubs were subjected to the *sputtering* process for 120 s at 30 kV and stored in a dissector until the analysis. The samples were observed on a Tescan VEJA 3 LMU FE-SEM with secondary and backscattered electron detectors, operating in high vacuum under a voltage of 20 kV.

2.7. *Nanotoxicity assay in vivo in chicken embryo model*

The nanotoxicity of NLC formulations and their respective emulsified EO (as controls) was evaluated by the *in vivo* chicken embryo model, according to the following parameters: viability (%), embryo (g) weight changes.

A total of 68 eggs of *Gallus gallus*, lineage W-36 were used. Before the analyses, the eggs were subjected to ovoscopy to ensure that embryos with 7 days of development were alive. Eggs were weighted and divided into 9 groups (n = 7): negative control (NC) composed of 0.85 % saline solution; NLC control (no EO addition); EO emulsion (3 %, w/v) and NLC with 3 % (w/v) EO. After this, all eggs were incubated for 72 h. Embryo mortality was daily analyzed to determine the viability (%). At 14 days of embryonic development, the eggs were weighted. After its death, the embryo was weighted. The changes in the weight of the embryo were calculated through the difference between the weight of the eggs before and after the treatments, according to the equation below:

$$aW = (ce.ysW \times 50) \div ieW \qquad \text{(Equation 1)}$$

where: aW: egg weight adjusted to 50 g; ce.ysW: embryo weight; ieW: initial egg weight.

ANOVA/Tukey statistical tests were used to assess intergroup statistical differences regarding embryo weight changes considering $p < 0.05$. For the embryonic viability test, the chi-square test was used followed by the test of the difference between two proportions, considering the NC and all other groups. GraphPad Prism 8 was used for statistical analyses.

193 # 3. Results

194 ## 3.1. *Screening of Essential Oils*

195 A screening was conducted to select the EO with the best antimicrobial activity against
196 *Campylobacter* ssp. strains, to be further used as active and structural excipient in the
197 preparation of NLC. Among the 12 EO tested, 5 of them have obtained the most
198 promising results against different strains of *Campylobacter* ssp as follows: cinnamon
199 EO, lemongrass EO, clove EO, geranium EO and oregano EO, that presented the
200 average inhibition halos of 95.00, 93.50, 47.50, 51.00 and 92.00 mm, respectively
201 (Table 2). Thus, these EO were used in the composition of NLC as liquid lipids, besides
202 the different solid lipids (murumuru butter, cocoa butter and beeswax) and surfactant.

203

Table 2 – Disk diffusion test of pure EO in terms of inhibition zone diameter (mm) against *Campylobacter ssp.* strains (n=3)

Strains Samples EO	131/5 *C.coli*	131/6 *C.coli*	131/7 *C.coli*	30/1 *C.jejuni*	64/5 *C.jejuni*	34763/3 *C.jejuni*
Cinnamon	95.00±0.00	69.00±0.00	82.00±0.00	86.00±0.00	33.00±0.00	95.00±2.82
Lemongrass	93.50±0.71	85.50±0.71	80.00±0.00	84.00±0.00	24.00±0.00	106.0±1.41
Clove	47.50±0.71	53.00±1.41	80.50±0.71	45.00±0.00	15.50±0.71	64.00±0.00
Geranium	51.00±0.00	43.00±0.00	64.00±0.00	65.50±0.71	*	60.00±0.00
Oregano	92.00±0.00	89.00±0.00	95.50±0.71	85.00±0.00	26.00±1.41	83.50±0.71
Avocado	*	*	*	*	*	*
Tea tree	07.50±0.71	*	*	*	*	*
Sandalwood	*	*	*	*	10.00±0.00	12.00±0.00
Citronella	*	*	14.00±0.00	08.00±0.00	*	*
Copaiba	*	12.00±0.00	*	07.50±0.71	*	*
Lavender	15.00±1.41	*	*	*	*	*
Aloe Vera	*	*	*	*	*	*
Garlic	*	*	*	*	08.00±0.00	07.50±0.71

204 *Note: *there was not any inhibition; One-way ANOVA plus Tukey post hoc tests were used to analyze*
205 *intragroup statistically significant differences over time; $p < 0.05$.*

206

207 ## 3.2. *Evaluation of the in vitro antimicrobial activity of nanostructured lipid carriers.*

208 The 5 EO selected in the screening step were encapsulated in NLC, resulting in the F1
209 (lemongrass EO and murumuru butter), F2 (cinnamon EO and cocoa butter), F3
210 (geranium EO and beeswax), F4 (clove OE and cocoa butter), F5 (oregano EO and
211 beeswax) formulations. Then, the MIC was determined for each sample against 3 strains
212 of *C.coli* and 3 strains of *C.jejuni* (Table 3). All formulations showed intragroup
213 statistically significant differences, by One-way ANOVA plus Tukey post hoc tests ($p <$
214 0.05).

215 It can be elucidated that the formulations containing lemongrass EO (F1), cinnamon EO
216 (F2), geranium EO (F3), clove EO (F4) and oregano EO (F5), were able to inhibit the
217 growth of the most strains of *Campylobacter* at low concentrations, around 0.2 to 4.0

mg/mL^{-1}. Some NLC containing EO, such as lemongrass EO and geranium EO, were able to inhibit *C.jejuni* strain 64/5 at highest concentrations, around 24.51 mg/mL^{-1} and 39.47 mg/mL^{-1}, respectively. Moreover, the control formulations (F7 - cocoa butter and F8 - beeswax), had also showed antimicrobial effect against *C.coli* strains 131/5 and 131/6 at highest concentrations, on average of 39.21 mg/mL^{-1} and 13.16 mg/mL^{-1}, respectively. On the other hand, the formulation that only contained murumuru butter (F6), was able to inhibit bacterial growth of strain 131/5 at a concentration of 3.33 mg/mL^{-1} and the strain 131/6 at a concentration of 0.32 mg/mL^{-1}. In short, NLC composed of lemongrass EO, cinnamon EO and geranium EO, were able to inhibit the most of multidrug-resistant *Campylobacter* strains at the lowest concentrations (~ 0.2 mg/mL^{-1}).

Table 3 – Determination of the minimum inhibitory concentration (MIC, mg/mL^{-1}) of NLC formulations against *Campylobacter ssp*. strains (n=3)

Strains	131/5	131/6	131/7	30/1	64/5	34763/3
Samples	*C.coli*	*C.coli*	*C.coli*	*C.jejuni*	*C.jejuni*	*C.jejuni*
F1	00.23±0.00	00.23±0.00	00.23±0.00	00.23±0.00	24.51±8.49	00.23±0.00
F2	00.23±0.00	00.23±0.00	00.23±0.00	00.23±0.00	01.53±0.53	00.23±0.00
F3	00.20±0.00	00.20±0.00	00.20±0.00	00.23±0.00	39.47±22.79	00.20±0.00
F4	00.20±0.00	00.20±0.00	13.16±0.00	00.47±3.31	*	00.20±0.00
F5	00.26±0.11	00.19±0.00	00.78±0.00	00.19±0.00	04.16±1.81	00.19±0.00
F6	03.33±3.97	00.32±0.14	>62.50±0.00	52.08±18.04	*	10.66±17.8
F7	39.21±16.98	29.41±0.00	39.21±16.98	*	*	29.41±0.00
F8	13.16±0.00	13.16±0.00	>52.63±0.00	*	*	26.32±0.00

*NOTE: F1: lemongrass EO and mumururu butter, F2: cinnamon EO and cocoa butter, F3: geranium EO and beeswax, F4: clove EO and cocoa butter, F5: oregano EO and beeswax, F6: mururumu butter, F7: cocoa butter and F7: beeswax. All of the formulations have employed poloxamer as surfactant. *no inhibition. $p < 0.05$.*

3.4. *Physicochemical Stability Study*

Figure 1 elucidates the physicochemical stability of all NLC formulations. In general, the particle size of the NLC-based EO were around 148.18 to 284.21 nm. F1 and F3 were the formulations that did not show statistical significance differences over time, exhibiting initial and final particles sizes around 151.81 nm to 179.16 nm and 208.4 nm to 219.73 nm, respectively. Other formulations have showed particle sizes fluctuations during the analysis, without any evidence on instability process during the analysis, as expect for nanocolloids (Ribeiro, 2018). However, the control formulation, containing only the solid lipid, had the highest initial and final sizes during the monitoring, with F6 (NLC-based murumuru butter) reaching a size of 307.5 nm, F7 (NLC-based cocoa butter) reaching a size of 512.3 nm and F8 (NLC-based beeswax) reaching a size of 431.8 nm, at the end of the experiment ($p < 0.05$).

Regarding the PDI, the most of the formulations have remained such values constant, with minor variations. F1 showed initial and final values around 0.18 and 0.22, respectively; F3 showed 0.13 and 0.23 respectively, F6 and F8 control formulations also exhibited initial and final values around 0.19-0.24 and 0.17-0.26, respectively. Finally, F7 showed final PDI values of 0.521 ($p < 0.05$).

Zeta potential values showed some variations during the analysis. F1 showed initial values of -40.03 mV and -46.80 mV after 210 days; F3 exhibited initial and final values of -42.5 and -28.83 mV, respectively. In relation to NLC controls, F6 and F8 had initial and final values of -43.13 and -43.00 mV, -41.8 and -33.41 mV, respectively. In contrast, F7 showed the highest Zeta potential values, with initial of -41.07 and final values of -54.83 mV.

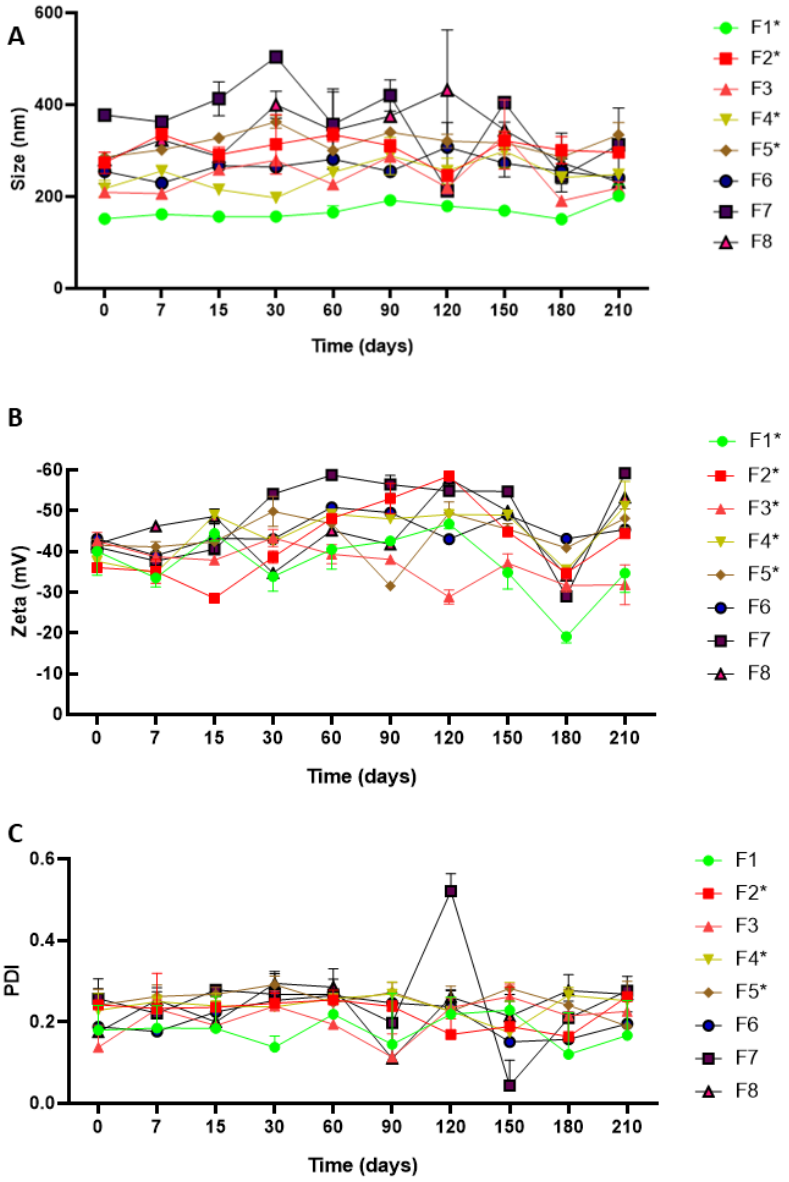

*Figure 1 - Long-term physicochemical stability of NLC formulations, in terms of size (A), PDI (B), and Zeta potential (C) values, monitored by DLS for 210 days (25 °CD); n = 3. One-way ANOVA plus Tukey post hoc tests were used to analyze intragroup statistically significant differences over time; *p < 0.05.*

3.5. *Structural Characterization*

The morphological features of all formulations were elucidated by FE-SEM (Figure 2). All NLC formulations evidenced spherical shapes with visible contour, as expected for this system (Ribeiro, 2017).

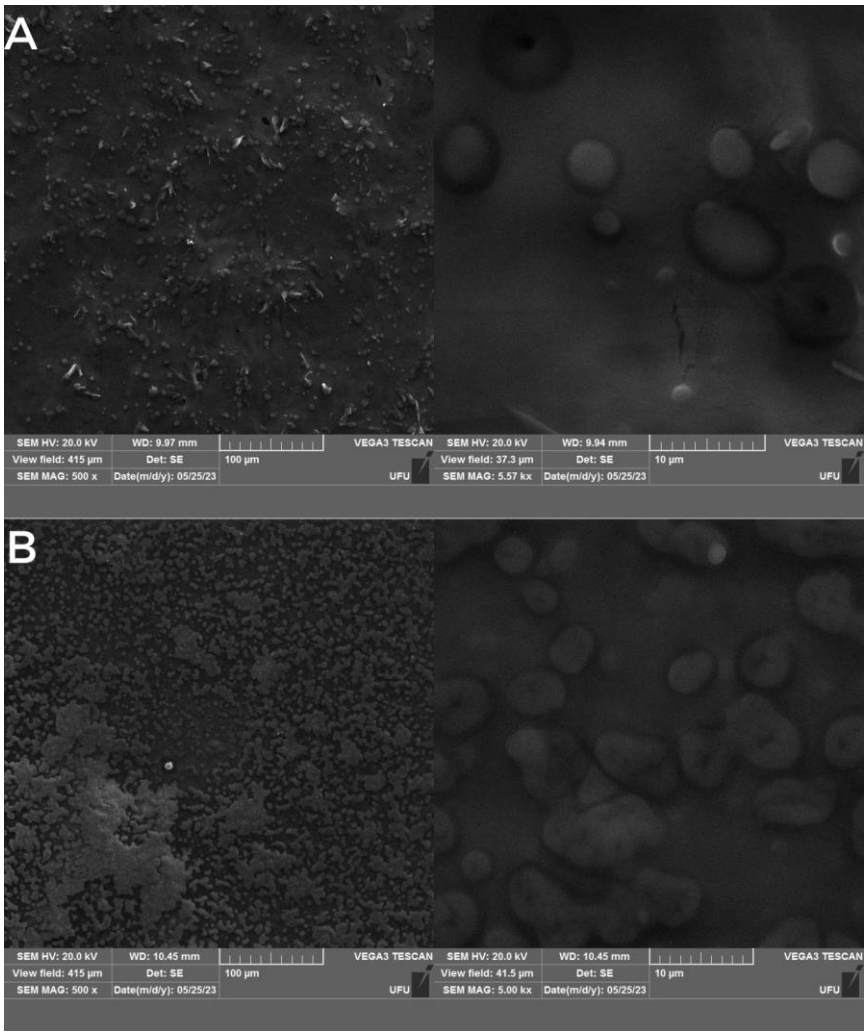

Figure 2 -FE-SEM images of NLC) (A) and respective NLC – Control (B) with different magnifications: 500x (left) and 5000x (right)

3.6. Nanotoxicity assay *in vivo* through chicken embryo model

To elucidate the safety of NLC-based EO, the nanotoxicity test was carried out through the chicken embryo model. The emulsified EO, as positive control, was prepared with both EO and poloxamer at the same concentration of the NLC formulations.

The formulation containing geranium-EO (F3) with 1 % EO and its emulsified form (GE-EM) with 3 % EO were the safest systems, once they did not show any mortality in the analysis (Table 4). On the other hand, emulsified cinnamon EO (CIN-EM) and lemongrass EO (LEM-EM) showed a mortality rate of 28.57 % e 42.85 %, respectively, after treatment inoculation, Regarding the formulations that only contained solid lipids (F6, F7, F8), F6 and F7 did not induce embryos deaths.

Table 4 – Mortality rate of chicken embryo after different treatments

Samples	Mortality (%)
GE-EM	0.00 %
F3	0.00 %
F6	0.00 %
CIN-EM	28.57 %
F2	28.57 %
F7	14.28 %
LEM-EM	42.85 %
F1	28.57 %
F8	0.00 %
NC	25.00 %

NOTE: GE-EM: emulsified geranium EO, F1: lemongrass EO and mumururu butter, CIN-EM: emulsified EO cinnamon, F2: cinnamon EO and cocoa butter, LEM-EM: emulsified lemongrass EO emulsified, F3: geranium EO and beeswax, NC: saline solution as negative control, F6: mururumu butter, F7: cocoa butter and F8: beeswax. All of the formulations have employed poloxamer as surfactant None of the formulations presented statistically significant by chisquare test ($p<0.05$).

It can be observed in Figure 3 that all formulations had no statistically significant difference ($p > 0.05$) regarding the embryo weight. It can be observed that the geranium EO in both in emulsified (GE-EM) and nanoencapsulated (F3) forms were the safest treatment once both have exhibited 0 % mortality rates.

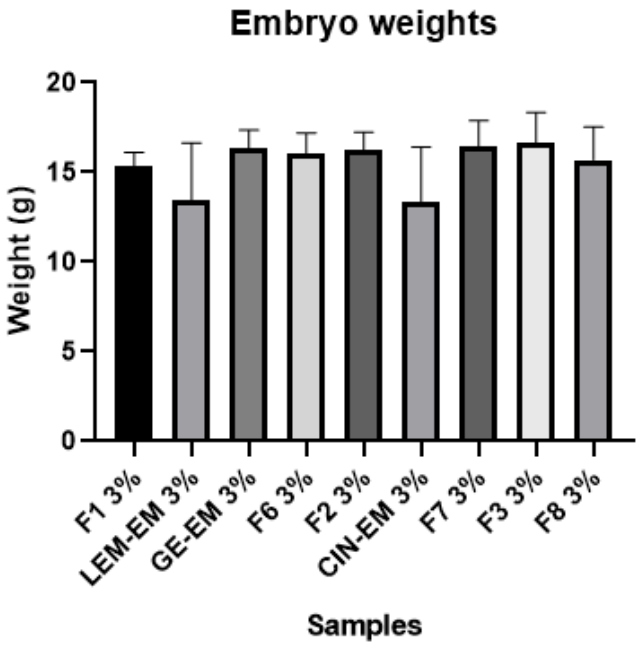

Figure 3 – Embryo weight changes after treatment with F1, F6, F7, and F8 formulations and their respective emulsified EO, called as LEM-EM, CIN-EM and GE-EM. None of the formulations presented statistically significant by ANOVA/TUKEY test ($p < 0.05$).

Discussion

The nanoencapsulation is a strategic approach to act as physical protection of EO, being able to decrease its photodegradation, changes in physicochemical properties and even increase the bioavailability of EO (Chaudari, 2021). The NLC formulations was based on the EO with the best in vitro anti-Campylobacter activity. The choice of cocoa and murumuru butters and beeswax as solid lipids of NLC were based on their thermal stability, melting point higher than physiological temperature and ability to encapsulate hydrophobic molecules successfully (Salminen, 2020).

Regarding the *in* vitro antimicrobial tests, it was observed a strictly inverse relation between the results, once the EO that showed the higher halo inhibition, also exhibited the lower MIC values against *Campylobacter* strains, as observed for F1, F2 and F3. In addition, Gram-negative bacteria, as *Campylobacter* are considered to be more resistant to EO than Gram-positive bacteria, due to the differences in its cell wall. In Gram-positive bacteria, the structure of the cell wall makes it easy for hydrophobic molecules to pass through the cells and act both in the cytoplasm and on the cell wall. In contrast, in the wall of Gram-negative bacteria, there is the 2-3 nm thick peptidoglycan layer, which is thinner than in the cell wall of Gram-positive bacteria. This peptidoglycan layer is intrinsically linked to the outer membrane (OM) with various lipopolysaccharides (LPS) and functions as an effective natural barrier. This OM has abundant porins that act as hydrophilic transmembrane channels, and this is one of the reasons that Gram-negative bacteria are essentially resistant to EO, which are hydrophobics (Nazarro, 2013).

Generally, EO have composed of terpenes, polyphenols, terpenoids, phenylpropenes, among other minor compounds (Nazarro, 2013). Lemongrass EO is majorly composed of geranial and neral stereoisomer pair of citral terpenes, conferring marked and less intense lemon aroma to the plant, respectively (Mukarram, 2022). There is evidence that citral has antimicrobial properties against various bacteria, such as: *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* typhimurium (Mukarram, 2022; Fisher, 2016). Citral mechanism of action against bacteria is commonly explained by intracellular ATP concentration decrease, inducing a hyperpolarization of the microbial cell membrane together with a reduction of bacterial cytoplasmic pH, causing bacterial death (Shi, 2017).

Oregano EO is mainly based on carvacrol (Béjaoui, 2013), a monoterpene phenol which has antimicrobial activity against different bacteria, as *Bacillus subtilis*, *Pseudomonas aeruginosa*, and group A streptococcus resistant to erythromycin (Magi, Marini, Facinelli, 2005). On the other hand, geranium EO is based on citronellol terpene and geranial isomer of the citral. These compounds have showed moderate antimicrobial effect against *Staphylococcus aureus* and *Escherichia coli* bacteria (Mangalagiri, Panditi, Jeevigunta, 2021).

Regarding cinnamon EO, that is mainly composed of cinnamaldehyde, a phenylpropene (Alizadeh Behbahani, 2020) that had proven inhibitory effects against *Escherichia coli*

through cell membrane disruption and oxidative damage (He, 2018). Finally, eugenol is the highest component present in clove EO (Haro-González, 2021). The mechanism of action of eugenol is due to the presence of the free hydroxyl group in its molecule that causes destabilization of cellular membrane (Nazarro, 2013).

Moreover, the fatty acids are the main constituents of the used vegetable butters (cocoa and murumuru) as solid lipids of NLC in this work. The ability of fatty acids to lysis the bacterial membrane is related to its amphipathic structure, which leads to microbial membrane destabilization, increasing cell permeability and lysis, presenting both bacteriostatic and bactericidal activity (Yoon, 2018). Some works have evidenced that lauric acid, palmitic acid and oleic acid possess antimicrobial activity against different bacteria, such as *Clostridium perfringens*, *Staphylococcus aureus* (Hovorková, Laloučková, Skřivanová, 2018). On the other hand, beeswax consists of a mixture fatty acid, esters, diesters and hydrocarbons. This lipid has shown antimicrobial activity against Gram-positive bacteria, especially *Spreptococcus epidermitis* and *Spretococcus* pyogenes (Ghanem, 2011). Besides possessing antimicrobial activities, processing solid and liquid lipids as structural and bioactive matrices of NLC have fundamental advantages, as the mask of organoleptic properties, optimization of their solubility and stability, decreasing photodegradation and volatility, which allows its further uses as campylobacteriosis treatment. In here, cinnamon, lemongrass, clove, geranium and oregano EO have showed excellent antimicrobial activity.

The control quality is required for all pharmaceutical formulations. It is determined by the evaluation of the long-term physicochemical stability, in terms of particle size (nm), polydispersity index (PDI), and zeta potential, elucidating the shelf life of systems (Carvalho, 2022;). Currently, it is expected some biophysical properties of long-term stable nanocolloids, such as particle size < 250 nm (for the administration of invasive routes), PDI values < 0.2 and Zeta potential >± 25 mV (Vedanti; Pawar, 2019), All these parameters were observed for all NLC formulations claimed in here, even after 210 days and stored at room temperature. The suitable stability of NLC is related to the desirable biological activity (Souto, 2022). In addition, the structural characterization confirmed the compatibility of the excipients used in the formulations, being F1, F2 and F3 the most promising formulations.

Therefore, such NLC formulations were evaluated regarding the *in vivo* nanotoxicity assay on chicken embryo. This alternative model allows the evaluation of drug toxicity in different embryo incubation times and also simulating several administration routes, being widely used to determine the safety of other antimicrobial nanostructured formulations (Silva, 2023). In here, F3 (NLC-based geranium EO) was the safest system, once did not show any toxicity in all the analyzed parameters. Such formulation is able to be further tested in the *in vivo* efficacy assays against *Campylobacter jejuni* and *Campylobacter coli*.

Conclusions

The use of new therapies to mitigate the cases of campylobacteriosis is urgent. Thus, nanostructured natural lipids are a versatile alternative for the treatment and control of multidrug resistant Campylobacter strains. In this work, 8 NLC formulations containing natural lipids were prepared and shown to have physicochemical stability for 210 days. The formulation F3 composed of geranium EO and beeswax, was chosen as promising agent against *Campylobacte*r. Finally, such system did not show any nanotoxicity in all the parameters evaluated on chicken embryo models.

Data availability

The data used during the current study are available from the corresponding author on reasonable request.

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Author contributions

H.M.P, R.J.O.J, B.B.F., L.M.B., L.N.M.R conceptualized the research. H.M.P, S.S. performed the experiment. H.M.P., S.S., B.B.F, L.N.M.R. analyzed the data. H.M.P. and L.N.M.R. wrote the manuscript. All the authors contributed to the discussion and revision of this manuscript.

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Competing interests

The authors declare no competing interests.