Formulation and evaluation of anti-MRSA nanoemulsion loaded with Achyrocline satureioides: a new sustainable strategy for the bovine mastitis

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Abstract
Methicillin-resistant Staphylococcus aureus (MRSA) causes mastitis in dairy cattle with serious economic and public health significance. This study developed nanoemulsions of Linum usitatissimum oil loaded with Achyrocline satureioides (macela) extract and investigated their in vitro antimicrobial activity against MRSA. Macela-nanoemulsions (NE-ML) were prepared using high-pressure homogenization (HPH) with different proportions of flaxseed oil, Tween 80 and crude extract. Four majoritarian flavonoids were identified in the macela extract: 3-O methylquercetin, achyrobichalcone, quer cetin and luteolin (187.3 ± 0.1, 155.4 ± 11.6, 76.3 ± 0.1 and 30.4 ± 0.0 μg ml⁻¹, respectively). NE-ML nanoemulsions were successfully obtained by the HPH method and showed a milky aspect with yellowish color. The mean particle size was around 200 nm with monodisperse distribution (PdI < 0.2), remaining stable for 160 days at room temperature. When analyzed on a LUMiSizer high-end dispersion analyzer, low values were found (≤0.5), indicating high stability index, mainly for NE-ML1:5 (0.2). The encapsulation efficiency of macela-nanoemulsions was greater than 94%, considering the four chemical compounds from extract. Minimum inhibitory concentration (MIC) against planktonic bacteria, inhibition of biofilm formation (MBIC), and eradication of MRSA biofilms (MBEC) were determined through in vitro tests on microplates. The MIC of NE-ML against planktonic MRSA showed values ranging from 1.2 to 10% (v/v), while blank-nanoemulsions (NE-B, without macela extract) showed values ranging from 6 to 50% (v/v). MBIC and MBEC of NE-ML were 25 and 80% (v/v), respectively. MBIC showed a mass reduction greater than 64%, and MBEC showed a mass reduction greater than 73%. Macela-nanoemulsions (NE-ML), mainly NE-ML1:5, showed high antimicrobial activity and appeared to represent a new alternative of sustainable antimicrobial product for the control of MRSA. Since this innovative nanoemulsion can impact animal health, future research should include in vitro and in vivo studies to evaluate intramammary therapy and control of MRSA infections in organic and agroecological milk production systems.

Introduction
Mastitis is a highly prevalent disease in dairy cattle (Abebe et al 2016, Acosta et al 2016, Busanello et al 2017), and it is responsible for significant economic losses (Guimarães et al 2017). Dairy production systems need more effective antimicrobials against bovine mastitis, in particular those that do not cause damage to human and animal health. In organic and agroecological milk production systems in most countries, the management of mastitis is potentially more difficult owing to the restrictions imposed by legislation in relation to the use of antimicrobials and the limited availability of effective treatments (Busato et al 2000, Pol and Ruegg 2007, Ruegg 2009). In organic production, which is on the rise, cases of mastitis are treated mostly with natural
products in popular use, but without scientific support, or even with conventional antimicrobials (Honorato et al 2014, Mushtaq et al 2018).

*Staphylococcus aureus* is a common cause of bovine mastitis, and its impact is associated with the ability of some strains to form biofilms and acquire mechanisms of resistance to multiple drugs. Methicillin-resistant *S. aureus* (MRSA) is composed of highly virulent bacteria that are difficult to control, making their dispersal a serious public health issue. MRSA is frequently found in samples from dairy cattle, with a prevalence of 1 to 60% (Spohr et al 2011, Locatelli et al 2017) of infections, and it is considered an important disease vector. Transmission of MRSA between animals within a herd and from animals to rural workers in contact with the herd has been reported and linked to cases of skin infection, endocarditis and pneumonia (Soavi et al 2010).

In bovine mastitis, the presence of biofilms has been associated with recurrent infections and unsuccessful treatment (Melchior et al 2006). Given this context, new strategies to control bovine mastitis are being developed, mainly for organic and agroecological dairy systems, including controlling the formation of and/or eradicating biofilm (Diaz et al 2010, Mubarak et al 2011 Gomes and Henriques 2016, Mushtaq et al 2018). As such, natural products and plant extracts with proven antimicrobial activity are potential candidates for use in new therapies (Fiodralisi et al 2016, Pinheiro Machado et al 2019). Moreover, for plant-derived drugs, the induction of microbial resistance after prolonged exposure is more difficult and represents an important advantage (Ohno et al 2003, Domadia et al 2007).

Macela (*Achyrocline satureioides*), a plant native to South America, has a broad spectrum of pharmacological properties, with some of its key biological properties aimed at reducing cytotoxic effect (Rivera et al 2004, Sabini et al 2013) and providing cytoprotection (Arredondo et al 2004, Blasina et al 2009), as well as having antimicrobial and anti-inflammatory potential (Mota et al 2011, Joray et al 2013, Vargas et al 2013). Several phenolic compounds have been identified in its composition, such as quercetin, luteolin, 3-O methylquercetin and achyrobichalcone (ACB), all of which have been attributed to the plant’s biological properties (Arredondo et al 2004, Polydoro et al 2004, Retta et al 2012). Despite the recognized bioactivity of macela, its bioactive compounds have little stability and limited solubility in aqueous solutions (Bidone et al 2014, 2015). However, the use of nanoemulsion systems for the release of drugs offers a tool that circumvents such limitations, while also supporting active compound permeation, thereby providing prolonged action and increased bioavailability (Harwansh et al 2019).

Nanoemulsions consist of a fine dispersion of oil in water stabilized by an interfacial film of surfactant molecules. The mean droplet size generally varies from 0.1 to 500 nm (Sutradhara and Amin 2013), up to 600 nm, depending on the mechanical energy, composition, and concentration of surfactants (Bouchema et al 2004, Jaiswal et al 2015). Nanoemulsions have advantages such as protection and stability on the active compounds through their encapsulation, while increasing the penetration power of the compounds of interest, resulting in more effective application (Mohanraj and Chen 2006, Riviere 2007, Troncarelli et al 2013, Harwansh et al 2019). In the present study, golden flaxseed oil (Linum usitatissimum) was selected for the oil phase of the developed nanoemulsions because it is a natural product with recognized antimicrobial potential, and its use has been previously tested against gram-positive and gram-negative pathogens in the treatment of bovine mastitis (Kaithwas et al 2011, Joshi et al 2014). Golden flaxseed has compounds such as alpha-linoleic acid (ALA, omega-3 fatty acid), flavonoids, glycosides, phenols and tannins, to which different biological activities have been attributed (Kaithwas et al 2011, Joshi et al 2014).

A macela-loaded nanoemulsion able to deliver the compounds of interest may, therefore, be a promising strategy to treat bovine mastitis caused by MRSA in various production systems after initial evaluation of its in vitro efficacy. Therefore, the present study aimed to develop and characterize a sustainable innovative delivery system of herbal bioactive compounds, i.e., flaxseed oil nanoemulsions loaded with macela extract, followed by an assessment of their antimicrobial and antibiofilm activities against MRSA.

**Material and methods**

**Macela samples and extract preparation**

Commercial samples were acquired from *Entre Ervas* aromatic and medicinal herbs in southern Brazil (30° 02’14.89’’S/51°12’39.08’’W). Macela extract was prepared by maceration of the inflorescences with 80% ethanol (1:60, w/v) for 10 min. The extracts were then vacuum-filtered, and the organic solvent was removed with a rotary evaporator (60 °C). Immediately after solvent removal, the aqueous phase of extract (20 ml) was used to prepare the nanoemulsions.

**Chemical characterization of macela extract via high-performance liquid chromatography (HPLC)**

Analysis of the phenolic composition of the macela extract was performed on a HPLC Thermo Scientific UltiMate 3000 RS Dual System (Thermo Fisher Scientific, San Jose, CA) using a Thermo Scientific C18 reverse-
phase column (4.6 × 150 mm; 5 μm; 120 Å; Acclaim™ 120, Thermo Scientific©) at 25 °C, operating at 240, 270, 320 and 375 nm. Mobile phase consisted of Milli-Q® water acidified to pH 2.3 (A) and methanol (B) eluted at 1.0 ml min⁻¹ flow using the following gradient program: 0–5 min, 90% A; 5–25 min, 30% A; 25–37 min, 90%A. The identification of quercetin, 3-O methylquercetin and luteolin was performed by comparison with retention times of the commercial standards (Sigma-Aldrich). The identification of acetylcholine (ACB) was performed at the Galenic Development Laboratory (UFRGS) by comparing retention time with the standard that was isolated and purified according to Carini et al (2013) and Bianchi et al (2019). Quantification was based on the integration of the peak areas through the quercetin calibration curve (Sigma-Aldrich; Q4951) (detection interval of 0.97–1000 μg ml⁻¹, R² = 1.0, y = 0.168x).

Chemical characterization of flaxseed oil via gas chromatography coupled to mass spectrometry (GC/MS)

The chemical composition of flaxseed oil was analyzed using gas chromatography coupled to a mass spectrometer (model GCMS-QP2010, Shimadzu, Japan), based on the methodology described by El-Deeb et al (2004). We used a silica capillary column RTX-5MS (60 m × 250 μm × 0.2 μm) and helium as a carrier gas at a flow rate of 25 ml min⁻¹. The temperature used varied from 60 to 240 °C, with an increase of 10 °C min⁻¹. The injection was performed in split mode: 1:40, containing 200 μl of sample (200 μl + 400 μl of hexane). Sample derivatization was performed by direct methylation of fatty acids (O’Fallon et al 2007). Compound identification was performed considering the results found in the mass libraries (GCMSolution software) at the Analysis Center, Department of Chemical Engineering, UFSC.

Formulation optimization study

Nanoemulsions were prepared by the high-pressure homogenization (HPH) method. To optimize the preparation conditions, blank-nanoemulsions (NE-B) were previously prepared using different concentrations of flaxseed oil and surfactant (Tween 80) (table 1). Details of the procedure used can not be provided at this moment, because the formulation developed in this study is matter of patent registration and industrial secret. Of the 11 NE-B developed, 4 formulations were selected to add macela extract at different concentrations (table 1).

Preparation of macela extract-loaded nanoemulsions (NE-ML)

Macela-nanoemulsions (NE-ML) were prepared by the high-pressure homogenization (HPH) method, using the same conditions selected in the formulation optimization study, as described above, with the addition of the extractive solutions (20%, v/v) to the oil phase, equivalent to 250 mg of extract. Of the 10 developed NE-ML formulations, 4 formulations that remained stable and presented the desired physicochemical characteristics were selected for further study (table 1).

Characterization of nanoemulsions

Mean particle size, polydispersity index (PdI), zeta potential and pH

The mean particle size, PdI, and zeta potential were determined by dynamic light scattering (DLS) and Laser Doppler Anemometry, respectively, using a Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, United Kingdom). Measurements were made at 25 °C. Particle size analyses were performed at a fixed 90° scattering angle. For zeta potential measurements, the samples were placed in electrophoretic cells where a potential of ±150 mV was established. The zeta potential values were calculated as the mean value of electrophoretic mobility using the Smoluchowski equation (Sze et al 2003). The pH of the formulations was measured using a pH meter (Hanna HI2221, São Paulo, Brazil).

Morphology

The morphology of the formulations previously diluted in ultrapure water was analyzed using transmission electron microscopy (TEM) (JEOL JEM 1011 TEM). Nanoemulsions, previously diluted in ultrapure water, were deposited on carbon-coated copper grids and negatively stained with 2% (w/v) uranyl acetate.

Accelerated physical stability

Analysis of the accelerated physical stability of the formulations was performed using a dispersion analyzer (LUMiSizer®, Berlin, Germany). Aliquots (1.0 ml) were placed in individual test tubes (optical path of 2.0 mm) and exposed to centrifugal force at 2300 g for 5 h at 25 °C. The analysis time interval was 20 s. This technique generates a numerical instability index, ranging from 0 to 1, with 0 indicating a very stable system and 1 indicating a complete separation of the dispersion (Detlof et al 2013, Fernandes et al 2017, Zielinska et al 2018). Instability index was calculated by the SEPView™ software.
Table 1. Composition of nanoemulsions with flaxseed oil (NE-B) and macela-loaded nanoemulsion (NE-ML) developed by high-pressure homogenization (HPH).

| Composition          | NE-B0.2:1 | NE-B1:5 | NE-B5:5 | NE-B5:10 | NE-ML0.2:1 | NE-ML1:5 | NE-ML5:5 | NE-ML5:10 |
|----------------------|-----------|---------|---------|----------|------------|----------|----------|----------|
| Tween 80 (%, w/v)   | 0.2       | 1       | 5       | 5        | 0.2        | 1        | 5        | 5        |
| Flaxseed Oil (%, w/v) | 1       | 5       | 5       | 10       | 1          | 5        | 5        | 10       |
| Extractive macela solution (%, v/v)* | —       | —       | —       | —        | 20         | 20       | 20       | 20       |
| Water (q.s. to) (ml) | 100      | 100     | 100     | 100      | 100        | 100      | 100      | 100      |

* In the final composition of the macela nanoemulsions (NE-ML), the extract content corresponded to 2.5 mg ml⁻¹. Proportions following NE-B and NE-ML refer to the concentration of Tween 80 and flaxseed oil in the formulations.
**Stability studies over time**

To evaluate the stability of nanoemulsions, samples were stored in amber flasks at room temperature for 160 days. Mean particle size, PDI, zeta potential and pH of samples were all determined after 0, 7, 14, 21, 30, 60, 90 and 160 days, as previously described.

**Encapsulation efficiency**

The encapsulation efficiency was determined using the ultrafiltration and centrifugation technique (Mazzarino *et al.* 2018, Pinheiro Machado *et al.* 2019), using Amicon Ultracel-100 membrane units (100 kDa, Millipore Corp., Billerica, MA). The encapsulation efficiency was determined to quercetin, 3-O methylquercetin, ACB and luteolin by HPLC, as described above.

**In vitro antimicrobial activity**

**Planktonic bacteria**

NE-B and NE-ML were tested against *S. aureus* ATCC 25923 and ATCC 33592, as well as six MRSA strains from mastitic milk, by the broth microdilution method (CLSI 2006). Initially, milk samples were collected from cows diagnosed with mastitis from pasture-based herds in southern Brazil. Mastitic milk (10 ml) was obtained aseptically from sick cows in sterile tubes. To assist in the diagnosis of mastitis, the California Mastitis Test and the physical examination of the udder were carried out. The samples were taken under refrigeration (4 °C–8 °C) and processed immediately for the phenotypic identification of *S. aureus* strains according to Quinn *et al.* (2011). The strains selected were those that showed resistance profile to Ampicillin, Gentamicin, Neomycin, Oxacillin, Penicillin, Sulfamethazine and Tetraycline by disc diffusion method performed according to Clinical and Laboratory Standards Institute (CLSI 2003). The inhibition halos for each tested antimicrobial were interpreted according to the Performance Standards for Antimicrobial Susceptibility Testing (CLSI 2006). For each formulation, eight different concentrations were tested against each strain (50 to 0.38% and 10 to 0.78% to NE-B and NE-ML, respectively). Dilutions of nanoemulsions were prepared in 100 μl of Muller Hinton (MH) broth. After dilution, 10 μl of the bacterial inoculum standard corresponding to 1.15 × 10⁵ CFU ml⁻¹ were added to microplate wells and incubated at 35 °C for 24 h. For comparison, the flaxseed oil (50 to 0.3 mg ml⁻¹) was dissolved in 1% (w/v) Tween 80 and macela extract (1.2 to 0.0009 mg ml⁻¹) was dissolved in 5% ethanol previously to perform the analysis. The vehicles (ethanol and Tween 80) were included as control. The minimum inhibitory concentrations MIC50 represents the MIC value at which 50% of the strains in a test population are inhibited, and MIC90 represents the MIC value at which 90% of the strains within a test population are inhibited (Schwarz *et al.* 2010). Both were determined as those concentrations that showed no visible growth, which was confirmed by the addition of 50 μl of resazurin dye (7-hydroxy-3H-phenoxazin-3-one 10-oxide) (100 μg ml⁻¹). Resazurin is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells (Sarker *et al.* 2007). Additionally, an aliquot of the contents of the wells was removed and placed in a sterile Petri dish containing BHI agar. Replating was performed from the wells containing the concentration that showed no bacterial growth with the addition of resazurin (MIC), as well as those with the two concentrations described above, whenever possible. Plates were incubated for 24 h at 35 ± 0.2 °C. After this period, the number of colonies in each plate was assessed, and the reduction in bacterial growth was calculated by comparing bacterial growth that indicated bacteriostatic activity with the initial inoculum control (CFU ml⁻¹) (Smith-Palmer *et al.* 1998).

**Antibiofilm activity**

We selected NE-ML:1.5 for use in *in vitro* antibiofilm tests based on results of the stability study, the physicochemical properties thereof, and the MIC.

**Evaluation of biofilm formation**

Initially, *S. aureus* ATCC 25923 and four MRSA strains (614, 534, 406 and 204 strains—Labinat/UFSC) were evaluated for biofilm formation in a 96-well microplate based on the methodology described in Stepanović *et al.* (2007) with modifications. For this, cultures were incubated in Tryptic Soy Broth (TSB) supplemented with 1% glucose at 35 °C ± 0.2. After 24 h, the culture was diluted in TSB medium to approximately 1.5 × 10⁶ CFU ml⁻¹, which is equivalent to 0.5 on the McFarland scale. A 200 μl aliquot of the culture was placed in quadruplicate in 96-well microplates. Wells containing only TSB were considered the negative control. Plates were then incubated for 48 h, washed three times with sterile phosphate-buffered saline (PBS; pH 7.2), and dried at room temperature. Methanol (200 μl) was added to the wells for 15 min for subsequent staining with 2% (w/v) violet crystal. After 20 min, the plates were washed once with PBS to remove excess dye, 200 μl of 33% acetic acid were added, and optical density was read at 570 nm (Babysystems, MultiSkan EX).
The strains were then classified as follows: optical density (OD): OD ≤ ODc = does not produce biofilm; ODc < OD ≤ 2xODc = weak biofilm producers; 2xODc < OD ≤ 4xODc = moderate biofilm producers; and 4xODc < OD = strong biofilm producers. To determine the antibiofilm activity of formulation, the strong and moderate biofilm-producing strains (n = 4) and ATCC 25923 were used.

**Prevention of biofilm formation**

Following the steps described above, the S. aureus culture cultivated for 48 h was diluted in fresh TSB at a concentration of 1.5 × 10^8 CFU ml⁻¹ (0.5 McFarland scale). An aliquot (200 μl) of diluted S. aureus culture was added to the microplate, and concentrations of NE-ML1:5 formulation (25, 10 and 5% v/v) were added to the wells with and without inoculum. Negative (TSB broth) and positive (inoculum without the addition of NE-ML1:5) controls were included in the tests. The effect of blank-nanoemulsion (NE-B 1:5 25, 10 and 5% v/v) and Tween 80 (2.5 to 0.5 mg ml⁻¹) on biofilm formation was tested earlier. The plates were incubated at 35 ± 0.2 °C for 48 h. Washing and staining were performed following the method described above. The percentage of inhibition of biofilm growth was determined by reading the absorbance at 570 nm with a microplate reader (SpectraMax® 190 Microplate Reader from Molecular Devices Corp.) and calculating according to equation (1), as

\[
IB(\%) = [1 - (AT/AI) \times 100],
\]  

where IB is the inhibition of biofilm growth, AT is the mean absorbance of the nanoemulsion concentrations tested with inoculum subtracted from the absorbance value of the same nanoemulsion concentrations without the addition of inoculum, and AI is the mean absorbance of biofilm growth control. The minimum biofilm inhibitory concentration (MBIC) was considered as the concentration in which the absorbance ≤ negative control (Adukwu et al. 2012). Simultaneously, on a second microplate, the results were confirmed by the addition of resazurin dye to enable a correlation between the biofilm biomass and the metabolic activity of viable bacterial cells.

**Eradication of formed biofilm**

Antibiofilm activity was evaluated using biofilm that had been preformed for 48 h. After this period, the culture medium in the wells containing biofilm was removed and immediately changed with new medium (control) or medium containing different concentrations of the NE-ML1:5 formulation (80, 50, 25, 12 and 5% v/v). A negative control containing only medium was also included in the tests. The plates were re-incubated for 24 h at 35 °C ± 0.2 °C, subsequently stained with violet crystal (2%), and the absorbance was measured as described above. After staining, the reduction of biofilms exposed to different concentrations of NE-ML1:5 was also analyzed using inverted optical microscopy (Olympus IX81). The concentration at which already established biofilms were removed from the bottom of treated wells was determined as the minimum biofilm eradication concentration (MBEC) (Adukwu et al. 2012). Separately, MBEC ≥70% was also confirmed by the addition of resazurin dye.

**Statistical analyses**

Statistical analyses were performed by analysis of variance (ANOVA) followed by Tukey’s test, using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). For the antimicrobial assays with planktonic bacteria, the data underwent logarithmic transformation. Values of P < 0.05 were considered significant. The data were presented as mean ± standard error of the mean (SEM).

**Results and discussion**

In the present work, we aimed to incorporate macela extract into a nanoemulsion formulation to develop an innovative, sustainable drug delivery system based on herbal bioactives. For each new nanoemulsion system developed, a systematic study of composition and water/oil/surfactant ratio was carried out in order to find the most stable conditions and the best performance against MRSA for use in bovine mastitis therapy. Macela extract and flaxseed oil were chosen for the composition of the nanoemulsions owing to their antimicrobial ability (Kaithwast et al. 2011, Joshi et al. 2013, Vargas et al. 2013, Joshi et al. 2014). Studies have reported on both to treat bovine mastitis, but never combined in a nanoformulation. Thus, we sought to bring this combined composition to a macela-nanoemulsion formulation, optimize it, and apply the resulting product as an antimicrobial agent against pathogens that cause bovine mastitis, in particular MRSA known to be highly pathogenic in mastitic milk. In doing so, we consider the potential of using this delivery system in organic and agroecological systems.
Chemical characterization of macela extract

Four majoritarian flavonoids were identified via HPLC in the macela extract: 3-O methylquercetin, ACB, quercetin and luteolin at concentrations of 187.3 ± 0.1, 155.4 ± 11.6, 76.3 ± 0.1 and 30.4 ± 0.0 μg ml⁻¹, respectively. These results are consistent with previous studies showing similar flavonoid compounds in macela (Retta et al 2012). ACB (4.2’,4’,2”-tetrahydroxy-6’,6”-dimethoxy-4’-O-4’-bichalcone) was recently isolated from samples of macela inflorescences from southern Brazil (Holzschuh et al 2010, Carini et al 2013, Bianchi et al 2019). In the present study, the high concentration of ACB is important, essentially because chalcones are precursors of flavonoid biosynthesis in plants (Yerragunta et al 2013). In addition, numerous biological properties have been attributed to flavonoid compounds in macela, such as antimicrobial activity (Joray et al 2013).

Chemical characterization of flaxseed oil

Analysis of the flaxseed oil sample revealed the presence of unsaturated fatty acids, principally linoleic (LOA), alpha-linolenic and oleic, at concentrations of 30.5% ± 0.1, 30.7% ± 0.1 and 25.7% ± 0.1, respectively, in relation to the total percentage of fatty acids in the sample. Other compounds identified were palmitic acid (7.2%) and stearic acid (4.2%). These results corroborate those of Kaithwas et al (2011) and Joshi et al (2014) who reported the presence of fatty acids, such as palmitic acid (5.5%), stearic acid (4.6%), oleic acid (19.0%), linoleic acid (13.6%) and alpha-linolenic acid (57.38%), in the composition of golden flaxseed oil. This oil has shown antimicrobial, anti-inflammatory, analgesic, expectorant, and diuretic activities (Kaithwas et al 2011, Khan et al 2017).

Formulation optimization study and characterization of nanoemulsions

Mean particle size, polydispersity index (Pdi), zeta potential, pH and morphology

Nowadays, a variety of methods for the preparation of nanoemulsions are described in the literature. Among high-energy emulsification techniques, the high-pressure homogenization method has some advantages, such as low polydispersity, better dispersion of formulations with high lipid concentration, avoidance, or low volumes, of organic solvents, quick preparation and feasibility of scale-up for large-scale production (Mistry et al 2012). In this study, nanoemulsions were successfully obtained by the HPH method.

Flaxseed oil nanoemulsions (NE-B) and macela-nanoemulsions (NE-ML) showed a milky aspect with white and yellowish color, respectively. They were homogeneous, without the formation of precipitation or phase separation during the period of analysis (figure 1).

Different particle sizes and ZP values were found among the NE-B formulations (table 2). The formulations with higher oil and surfactant content presented the smallest particle sizes and the smallest ZP in module (P < 0.05). Significant changes in mean particle size and pH were detected among the NE-ML formulations (table 2). Higher pH of NE-ML1:5 may have contributed to the production of larger droplet sizes (Sharma et al 2003). NE-ML1:5 presented the largest particle size (table 2), likely based on lower concentration of surfactant. NE-ML5:5, for example, had the same composition as NE-ML1:5, except for a higher concentration of Tween.
Table 2. Mean particle size, polydispersity index (PdI), zeta potential (ZP) and pH of the nanoemulsions with flaxseed oil (NE-B) and macela-loaded nanoemulsions (NE-ML) at day zero.

| Characteristic | NE-B0.2:1 | NE-B1:5 | NE-B5:5 | NE-B5:10 |
|---------------|-----------|---------|---------|----------|
| Size (nm)     | 251.4 ± 6.0a | 227.0 ± 3.6b | 212.8 ± 2.5b | 187.7 ± 3.5c |
| PdI           | 0.19 ± 0.0a  | 0.19 ± 0.0a  | 0.21 ± 0.0a  | 0.23 ± 0.0a  |
| ZP (mV)       | −37.6 ± 1.8a | −30.6 ± 0.9b | −27.1 ± 1.2b | −26.1 ± 1.7b |
| pH            | 4.7 ± 0.2a   | 4.9 ± 0.2a   | 5.1 ± 0.1a   | 5.2 ± 0.1a   |
| NE-ML0.2:10   | NE-ML1:5    | NE-ML5:5    | NE-ML5:10   |
| Size (nm)     | 229.8 ± 4.8a | 249.9 ± 2.6b | 222.3 ± 2.0a | 203.9 ± 2.7c |
| PdI           | 0.19 ± 0.0a  | 0.17 ± 0.0a  | 0.18 ± 0.0a  | 0.23 ± 0.0a  |
| ZP (mV)       | −40.3 ± 1.0a | −39.9 ± 2.7a | −38.6 ± 2.5a | −34.5 ± 0.9a |
| pH            | 4.8 ± 0.0a   | 5.1 ± 0.0a   | 4.6 ± 0.0a   | 4.7 ± 0.0a   |

Mean followed by different letters in the same row represents significant differences among NE-B or NE-ML nanoemulsions (Tukey, P < 0.05). The data are presented as mean ± SEM (n = 3). Proportions following NE-B and NE-ML refer to the concentration of Tween 80 and flaxseed oil in the formulations.

In this sense, it should be emphasized that surfactant concentration must be enough to stabilize the micro-droplets of a nanoemulsion (Jaiswal et al. 2015), contributing to the establishment of an adequate ZP and viscosity in order to confer optimal stability.

Nanoemulsions can be prepared with low concentrations of surfactant (Bouchemail et al. 2004). In the present study, a tendency toward decreased particle size arose with an increased amount of surfactant. A similar result was found in the development of jabuticaba nanoformulations, also prepared using HPH with different levels of surfactant (Mazzarino et al. 2018). In this case, when the concentration of Tween 80 increased from 1 to 10% (w/v), the particle size of the developed formulations decreased to about 100 nm, despite the same oil concentration and homogenization pressure (Mazzarino et al. 2018).

Comparing the ZP values between the NE-B and their respective NE-ML, we note that the addition of macela extract to the formulations increased the values in module (table 2). Similarly, a previous study (Bidone et al. 2014) identified a progressive increase of ZP after the addition of macela extract to coconut oil and Tween 80-based formulations. The authors suggest a possible adsorption of the extract compounds at the oil/water interface. It is important to highlight that the PdI values of all formulations developed were ≤0.2, indicative of their stability and monodispersity.

In the present study, transmission electron microscopy (TEM) of NE-B and NE-ML formulations showed particles with a spherical shape and size similar to that obtained by DLS (figure 2; table 2).

Characterization of nanoemulsions

**Accelerated physical stability**

In this study, LUMiSizer® was used to assess the stability of the developed nanoemulsions. In this analysis, the centrifugal separation, in which the variation of transmitted light is recorded, provides information on the kinetics of the process of separation and migration of the particles (Zielińska et al. 2018). The instability index creates a dimensionless number, ranging from 0 (more stable) to 1 (more unstable) (Fernandes et al. 2017). All developed formulations showed instability index between 0.2–0.6, indicating that the physical stability of the nanoemulsions was maintained (Detloff et al. 2013, Fernandes et al. 2017). The physical stability studies showed that the formulation NE-ML1:5 presented the best stability among all macela-loaded nanoemulsions (figure 3). NE-ML1:5 also showed the lowest PdI value among the developed nanoemulsions (table 2). Together, these characteristics show that optimization of this formulation was achieved through oil and surfactant concentrations. It should be noted that NE-B0.2:1 and NE-ML0.2:1, with lower flaxseed oil and surfactant content, showed higher instability values closer to 1.

**Stability studies over time**

Macroscopic analysis of nanoemulsions over a period of 160 days showed no visual alterations or signs of phase separation. Regarding the parameters analyzed, no significant changes were found for NE-B (table 3). On the other hand, minimal alterations were found only for NE-ML0.2:1 in relation to mean particle size (table 3). These results are consistent with those found in the physical stability analysis (figure 3), which showed a tendency toward destabilization with lower oil and surfactant content (NE-ML0.2:1).

These results showed the importance of performing studies on development, optimization and monitoring of the stability to ensure that only the most stable systems will attain the best biological responses.
Figure 2. Transmission electron micrographs of the nanoemulsions with flaxseed oil (NE-B) and macela-loaded nanoemulsion (NE-ML). Scale bar 200 nm. Proportions following NE-B and NE-ML refer to the concentration of Tween 80 and flaxseed oil in the formulations.

Figure 3. (A) Instability index for the nanoemulsions with flaxseed oil (NE-B) and macela-loaded nanoemulsion (NE-ML) after 5 h at 2300 g and at 25 °C. (B) TEM profiles obtained during centrifugation of NE-ML1:5. Proportions following NE-ML refer to the concentration of Tween 80 and flaxseed oil in the formulations.
the concentration of Tween 80 and

Table 3. Mean particle size, polydispersity index (PdI), zeta potential (ZP) and pH of the nanoemulsions with flaxseed oil (NE-B) and macela-loaded nanoemulsions (NE-ML) over 160 days of storage at room temperature.

| Characteristic | NE-B 0.2:1 | NE-B 1:5 | NE-B 5:5 | NE-B 5:10 |
|----------------|-----------|---------|---------|---------|
| Size (nm) Day0 | 251.4 ± 6.0* | 227.0 ± 3.6* | 212.8 ± 2.5* | 187.7 ± 3.5* |
| Day160         | 243.1 ± 8.4* | 229.8 ± 2.6* | 214.6 ± 4.1* | 187.0 ± 6.9* |
| PdI Day0       | 0.19 ± 0.0*  | 0.19 ± 0.0*  | 0.21 ± 0.0*  | 0.23 ± 0.0*  |
| Day160         | 0.23 ± 0.0*  | 0.18 ± 0.0*  | 0.19 ± 0.0*  | 0.23 ± 0.0*  |
| ZP (mV) Day0   | −37.6 ± 1.8* | −30.6 ± 1.9* | −27.1 ± 1.2* | 26.1 ± 2.4*  |
| Day160         | −36.0 ± 0.8* | −33.5 ± 2.3* | −28.1 ± 4.4* | 24.2 ± 2.3*  |
| pH Day0        | 4.7 ± 0.2*   | 4.9 ± 0.2*   | 5.1 ± 0.1*   | 5.2 ± 0.1*   |
| Day160         | 4.6 ± 0.3*   | 4.9 ± 0.1*   | 5.1 ± 1.1*   | 4.5 ± 0.3*   |

Mean followed by different letters in the same column and for the same characteristic represents significant differences between NE-B or NE-ML at 0 and 160 days (Tukey, P < 0.05). The data are presented as mean ± SEM (n = 3). Proportions following NE-B and NE-ML refer to the concentration of Tween 80 and flaxseed oil in the formulations.

Encapsulation efficiency

The encapsulation efficiency of macela compounds in nanoemulsions was greater than 94%. This parameter was calculated considering the four majoritarian chemical compounds found in macela inflorescences, i.e., 3-O methylquercetin, ACB, quercetin and luteolin. The only difference found among the formulations was lower 3-O methylquercetin encapsulation efficiency for NE-ML0.2:1 (94.2 ± 0.9). For the other compounds, the encapsulation efficiency was greater than 95.7%, with values close to 99%. High encapsulation efficiency values found in the present study suggest an affinity between the major flavonoids found in macela and either flaxseed oil or surfactant. High encapsulation of the extract compounds is relevant since nanoemulsions can offer protection from degradation. Protection of active compounds from hydrolysis and degradation is one of the most important functions of nanoemulsions (Jaiswal et al. 2015). Results similar to those of the present study were previously found with an association efficiency of almost 100% between macela flavonoids and the oil nucleus of nanoemulsions (Bidone et al. 2014).

In vitro antimicrobial activity

Planktonic bacteria

For the planktonic phase, through broth microdilution, it was possible to determine the minimum inhibitory concentration (MIC) of macela extract, flaxseed oil and the developed nanoemulsions NE-B and NE-ML (table 4).

Macela extract, flaxseed oil and nanoemulsions NE-B and NE-ML reduced microbial growth of MRSA strains from mastitic milk (table 4). However, the MIC of nanoemulsified flaxseed oil (NE-B) was about four times lower than that of pure oil (table 4). This result may be related to the reduction in particle size of flaxseed oil when nanoemulsified, which allows for greater penetration power and, consequently, greater antimicrobial potential. Similarly, previous study showed the greater antimicrobial activity for flaxseed oil nanoemulsions at the expense of pure oil (Hashim et al. 2019). It is important to note that the use of flaxseed oil in vivo and in vitro for the treatment of bovine mastitis has been previously reported, showing promising results in both evaluations (Kaithwas et al. 2011). However, to date, no studies have reported the use of flaxseed oil nanoemulsions in the context of mastitis. For NE-B, MIC50 was also influenced by oil concentration and mean particle size. In this case, a higher oil concentration was related to smaller particle sizes and lower MIC values. A previous study reports that the main parameter in determining the antimicrobial efficiency of oils appears to be their concentration in the aqueous phase, rather than the average droplet size of the systems (Donsi et al. 2012).

It is important to note that the MIC50 and MIC90 of NE-ML were lower than those of NE-B, showing that the macela extract in NE-ML nanoemulsions is responsible for the greater antimicrobial activity (table 4).

Among the NE-ML formulations, different MIC50 values were found, with particularly interesting results for NE-ML1:5. Although NE-ML formulations have the same concentration of macela extract, different MIC values may result from differences in flavonoid solubility in different media of each nanoemulsion. The greatest...
Table 4. Count \((\log_{10} \text{CFU ml}^{-1})\) of*S. aureus* ATCC 25923, ATCC 33592 and six MRSA strains from mastitic milk (mean ± SEM), using an initial inoculum population density of \(1.5 \times 10^4 \text{ CFU ml}^{-1}\), following exposure to macela extract, flaxseed oil, NE-ML and NE-B at MIC50° and MIC90° concentrations.

| Formulations | MIC Concentration | Control \((\log_{10} \text{CFU ml}^{-1})\) | Count \((\log_{10} \text{CFU ml}^{-1})\) |
|---------------|-------------------|--------------------------|--------------------------|
| Macela extract | MIC50 0.07 mg ml\(^{-1}\) | 5.2 ± 0.0 | 2.4 ± 0.0 |
|               | MIC90 0.31 mg ml\(^{-1}\) | 5.2 ± 0.0 | 2.3 ± 0.1 |
| Flaxseed oil  | MIC50 25 mg ml\(^{-1}\) | 5.3 ± 0.0 | 2.5 ± 0.0 |
|               | MIC90 50 mg ml\(^{-1}\) | 5.3 ± 0.0 | 2.5 ± 0.1 |
| NE-ML0.2:1    | MIC50 5% (v/v) | 5.3 ± 0.0 | 2.4 ± 0.1 |
|               | MIC90 5% (v/v) | 5.3 ± 0.0 | 2.5 ± 0.8 |
| NE-ML1:5      | MIC50 1.2% (v/v) | 5.3 ± 0.0 | 2.4 ± 0.0 |
|               | MIC90 5% (v/v) | 5.3 ± 0.0 | 2.0 ± 0.1 |
| NE-ML5:5      | MIC50 5% (v/v) | 5.3 ± 0.0 | 2.6 ± 0.0 |
|               | MIC90 5% (v/v) | 5.3 ± 0.0 | 2.6 ± 0.0 |
| NE-ML5:10     | MIC50 5% (v/v) | 5.2 ± 0.0 | 2.4 ± 0.1 |
|               | MIC90 10% (v/v) | 5.3 ± 0.0 | 2.6 ± 0.0 |
| NE-B0.2:1     | MIC50 50% (v/v) | 5.3 ± 0.0 | 2.3 ± 0.0 |
|               | MIC90 50% (v/v) | 5.3 ± 0.0 | 2.3 ± 0.0 |
| NE-B1:5       | MIC50 12% (v/v) | 5.3 ± 0.4 | 2.6 ± 0.1 |
|               | MIC90 25% (v/v) | 5.3 ± 0.0 | 2.7 ± 0.0 |
| NE-B5:5       | MIC50 6% (v/v) | 5.3 ± 0.0 | 2.7 ± 0.1 |
|               | MIC90 25% (v/v) | 5.3 ± 0.0 | 2.6 ± 0.1 |
| NE-B5:10      | MIC50 6% (v/v) | 5.2 ± 0.0 | 2.5 ± 0.2 |
|               | MIC90 25% (v/v) | 5.3 ± 0.0 | 2.6 ± 0.9 |

Proportions following NE-ML and NE-B refer to the concentration of Tween 80 and flaxseed oil in the formulations. ° MIC50 represents the MIC value at which 50% of the strains in a test population are inhibited, and MIC90 represents the MIC value at which 90% of the strains within a test population are inhibited.

...antimicrobial potential found for this nanoemulsion may be related to the fact that nanoemulsions are responsive to a blend of oil and surfactant. The right blend of flaxseed oil and surfactant (Tween 80) from NE-ML1:5 may have produced the best solubility of the macela extract and, consequently, the best antimicrobial response. In this context, for the new system herein presented, the optimum flaxseed oil and surfactant (Tween 80) ratio providing the most stable system (figure 3) and the best PdI value (table 2) was found for NE-ML1:5. This improved performance also provided the highest antimicrobial response of this formulation. In this context, MIC may also be influenced by the general characteristics of the formulation. For example, variations in the proportions of composites used in its development can alter its characteristics, particularly in terms of solubility of active compounds. Such changes can confer different characteristics on the formulations, in turn, resulting in different biological properties. Varying MIC values of the same active compound may be related to differences in solubility of the active compounds in the culture media (Wu et al. 2008). In this context, it is likely that the combination of oil and surfactant concentrations present in the composition of NE-ML1:5 provided the best solubilization of the active compounds and, consequently, the highest antimicrobial activity (Donsì et al. 2012).

The MIC of macela extract was higher than that of nanoemulsions (NE-ML), except for NE-ML5:10. NE-ML1:5 showed a MIC50 lower than the extract, i.e., 1.2% (v/v) of the formulation (equivalent to 0.03 mg ml\(^{-1}\) of extract; table 4). For NE-ML0.2:1 and 5:5, the MIC90 was 5% (v/v) (equivalent to 0.125 mg ml\(^{-1}\) of extract), while the MIC90 of the extract was 0.31 mg ml\(^{-1}\). These results showed the greater antimicrobial activity of these macela-nanoemulsions. However, NE-ML5:10 was the only formulation that did not show superior antimicrobial activity of the macela extract. It is important to note that the antimicrobial activity of NE-ML was assessed and maintained after the 160-day storage period (results not shown).

For nanoemulsions, it is important to remember that the encapsulation of active compounds has several advantages, including longer action time (because active compounds are gradually released), protection against degradation and better permeation and penetration (Harwansh et al. 2019, Troncarelli et al. 2013). The results of the antimicrobial activity were confirmed by addition of resazurin dye (figure 4). Resazurin dye (7-hydroxy-3H-phenoxazin-3-one 10-oxide) has been broadly used as an indicator of cell viability in several types of assays, including bacteria (Sarker et al. 2007). The reduction of resazurin to resorufin is the result of mitochondrial activity, which correlates with the number of live organisms.

The antimicrobial activity of macela-loaded nanoemulsion represents an important finding in the fight against MRSA-linked bovine mastitis. The problem of antimicrobial resistance is a global phenomenon that poses risks to humans and animals. Despite the recognized biological properties attributed to macela, few studies have investigated its use in the control of bovine mastitis caused by MRSA. Previous analyses have shown the...
antimicrobial potential of macela, showing the bactericidal and bacteriostatic potential of its aqueous extract against standard strains and *E. coli* and *S. aureus* strains from mastitic milk (Mota et al 2011). Avancini et al (2016) evaluated the antimicrobial/disinfectant action of the macela hydroalcoholic extract on a standard strain of *Candida albicans* (ATCC 14053) and strains from cases of bovine mastitis. The results showed its potential for use on sources of infection, in disinfection or antisepsis procedures. Studies on the action of macela flavonoids showed that quercetin is able to induce the aggregation of bacterial cells and cause morphological changes in these cells, being highly effective in controlling MRSA strains (Hirai et al 2010).

In view of the results presented herein, the combination of flaxseed oil and macela extract in the NE-ML1:5 formulation shows significant potential as an antimicrobial against planktonic MRSA. As such, the antibiofilm activity of this formulation was further investigated.

**Antibiofilm activity: prevention of biofilm formation**

NE-ML1:5 reduced biofilm formation of the *S. aureus* ATCC 25923 and four strains of MRSA from bovine mastitic milk (table 5). NE-ML1:5 prevented 100% of biofilm formation at a concentration of 10 and 25% (v/v) for strains 534 and 406. The formulation showed a reduction in the mass of biofilm greater than 64% for ATCC 25923 and strains 614 and 204 at a concentration of 25% (v/v). This reduction is proportional to the number of existing metabolically active cells, as confirmed by the addition of resazurin. Therefore, 25% (v/v) was considered the MBIC, which contains 0.6 mg ml⁻¹ of macela extract and 12 mg ml⁻¹ of flaxseed oil. It is important to highlight that the integrity of the biofilm structure in the present study was interrupted for all tested concentrations in three of the four strains and the ATCC. This was most evident at the highest concentrations

| Antibiofilm activity | NE-ML1:5 (%, v/v) | % of biofilm reduction |
|----------------------|------------------|------------------------|
|                      | ATCC 25923       | strain614  | strain34  | strain406 | strain 204 |
| Prevention of biofilm formation (MBIC) | 25 | 64  | 70  | 100 | 100 | 95 |
|                      | 10 | 42  | 67  | 100 | 75  | 96 |
|                      | 5 | 0   | 0   | 19  | 0   | 0 |
| Eradication of formed Biofilm (MBEC) | 80 | 86  | 90  | 93  | 96  | 73 |
|                      | 50 | 74  | 78  | 87  | 50  | 70 |
|                      | 25 | 47  | 60  | 65  | 38  | 16 |
|                      | 12 | 43  | 32  | 0   | 33  | 14 |
|                      | 5 | 40  | 29  | 0   | 30  | 11 |

**Table 5.** Effect of NE-ML1:5 on biofilm formation and eradication of preformed biofilm of *S. aureus* ATCC25923 and MRSA from bovine mastitic milk.

Figure 4. Microplate showing planktonic bacterial activity after exposure to different concentrations of NE-ML 1:5 formulation [pink colour indicates growth, and blue means inhibition of growth]; Petri dishes indicate the replating of wells containing (A) the concentration that showed no bacterial growth with the addition of resazurin, i.e., MIC (1.2%), and those (B) and (C) with the two concentrations described above (2.5 and 5%). Proportions following NE-ML refer to the proportion of Tween 80 and flaxseed oil in the formulations (1:5).
(10 and 25% v/v). However, NE-ML1:5 did not prevent the formation of biofilm (MBIC) for three of the four strains and for the ATCC 25923 at 5% (v/v).

**Antimicrobial activity: eradication of formed biofilm**

Similarly, this formulation was able to remove biofilm previously formed over 48 h (table 5).

Treatment with 80% (v/v) NE-ML1:5 (equivalent to 2 mg ml⁻¹ of macela extract) for 24 h showed a reduction of more than 73% of the preformed biofilm for all evaluated strains, and it was thus considered the MBEC. It is important to note that we only observed a reduction of about 50%, or more, with concentrations of at least 50% (v/v) for all analyzed strains. Figure 5 shows the effect of NE-ML1:5 on preformed biofilms using the violet crystal dye.
NE-ML1:5 reduced biofilm formation of *S. aureus* in the context of prevention and eradication, evidencing the potential of this nanoemulsion in the intramammary therapy of bovine mastitis. Previous studies have shown the antibiofilm effect of macela extract on anaerobic bacteria in the oral cavity, causing a significant reduction in these populations (Both and Petrovick 2016). Resistance to antimicrobial activity may be related to inefficient penetration in the biofilm matrix, which presents a barrier, or to the altered growth rate of biofilm organisms (Donlan and Costerton 2002). Cells associated with biofilm grow significantly slower than planktonic cells and, as a result, absorb antimicrobial agents more slowly (Donlan and Costerton 2002). This fact may be an advantage for the use of nanostructured antimicrobial. In addition to having a smaller particle size, which can favor penetration, nanoemulsions can also release the encapsulated compounds gradually over an extended period of time. In the context of bovine mastitis, *S. aureus* bacteria in mammary tissue are mainly located in clusters within alveoli and lactiferous ducts in association with epithelial cells and in interstitial tissue (Sandasi et al 2010). The invasion of *S. aureus* into interstitial tissue can help bacteria survive. Therefore, small-sized antimicrobial nanostructures may be excellent candidates for treatments that offer greater penetration power.

The results found in the present study support future *in vivo* assessments of the intramammary use of the developed macela-nanoemulsion. For future, applications *in vivo* studies should be performed in order to determine the effective doses. It is noteworthy that this innovative macela-nanoemulsion represents an alternative sustainable antimicrobial product for control of MRSA. It can also subsidize organic and agroecological milk production systems, which have limited availability of antimicrobial treatments for mastitis.

**Conclusion**

The developed macela-nanoemulsion (NE-ML) showed potential as an innovative antimicrobial product, in particular, NE-ML1:5 formulation. Future researches should include *in vitro* and *in vivo* studies to evaluate therapy and control of MRSA infections.

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**Conflict of interest**

The authors declare no conflict of interest. In addition, the authors declare the availability of the data for publication on Nano Express.

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