Modelling the growth and death of *Staphylococcus aureus* against *Melaleuca armillaris* essential oil at different pH conditions

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Abstract: Essential oils (EO) are a great antimicrobial resource against bacterial resistance in public health. Math models are useful describing the growth, survival, and inactivation of microorganisms against antimicrobials. We evaluated the antimicrobial activity of *M. armillaris* EO obtained from plants placed in the province of Buenos Aires (Argentina) against *Staphylococcus aureus*. Minimum Inhibitory and Bactericidal Concentrations were close and decreased slightly acidifying the medium from pH 7.4 to 6.5 and 5.0. This result was also evidenced by applying a sigmoid model, where the time and EO concentration necessary to achieve 50% of the maximum effect decreased when medium was acidified. Moreover, at pH 7.4, applying the Gompertz model, we found that subinhibitory concentrations of EO decreased the growth rate and the maximum population density, and increased the latency period in comparison to the control. Additionally, we established physicochemical parameters for quality control and standardization of *M. armillaris* EO. Mathematical modelling allowed us to estimate key parameters in the behavior of *S. aureus* and *Melaleuca armillaris* EO at different pHs. This is interesting in situations where the pH changes are relevant, such as the control of intracellular infections in public health or the development of preservatives for food industry.

Keywords: *Staphylococcus aureus*; *Melaleuca armillaris*; essential oil; Gompertz model; Sigmoid model; antibacterial

1. Introduction

Antimicrobial resistance is a critical problem with high prevalence in both human and animal medicine [1]. Essential oils (EO) are a great resource as an alternative therapy, providing a lot of antimicrobials (ATMs) compounds produced by aromatic plants. In addition to their usefulness in medicine, essential oils are of great importance in the food industry to guarantee food preservation and safety [2]. These can act as bacteriostatics or bactericides in several ways responding to different action mechanism, having a wide variety of target sites, which generally lead to destabilization of the phospholipid bilayer, destruction of the function and composition of the plasma membrane, loss of vital intracellular components and inactivation of enzyme mechanisms [3]. The genus *Melaleuca* belongs to the Myrtaceae family which contains a lot of species of plants producing EO.
Among the species of *Melaleuca* genus, *Melaleuca armillaris* Sm. is one of the most widely cultivated. It is commonly known as Honey bracelet myrtle and grows as a small tree or as large bush. Investigations by GC-MS (gas chromatography coupled to mass spectrometry) of its essential oil revealed the presence of 1.8-cineole as the main component [4-7]. Several authors evaluated biological activities for this essential oil. For instance, Rizk et al. (2012), obtained good results in vivo using it for the treatment of the parasite *Schistosoma mansoni*, responding to the oxidative activity generated by this pathogen [8]. *In vitro* inhibitory activity was also found against *Staphylococcus aureus* [4] and other bacterial species such as *Bacillus subtilis*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* [9]. Reports about this plant EO are scarce, but these have potential as antimicrobial and more studies must be conducted to exploit it. Antibacterial agents from plants can act as important sources of new antibiotics, efflux pump inhibitors, compounds that target bacterial virulence, or can be used in combination with existing drugs [10].

*S. aureus* is recognized worldwide as a causative pathogen of different types of infections in humans and animals. Moreover, is commonly found in animals that are intended for food production such as dairy cows, sheep, goats, particularly when they are affected by subclinical mastitis [11] and represent a high risk for human consumption [12]. This microorganism can express a wide spectrum of pathogenic factors used to colonize, invade, and infect the host [13]. This pathogen can survive intracellularly contributing to the recurrence of infections like mastitis in cows. Despite several ATMs shows good in vitro activity, cure rates are low, because bacteria do not face to adequate concentrations and exposure time sufficient to eradicate it [14]. This may be related to the low intracellular penetration of some ATMs or their loss of activity at the acidic pH of lysosomes, the low diffusion of acidic ATMs through the lysosomal membrane due to their high ionization at neutral extracellular or cytoplasmic pH, and the poor retention of ATMs inside that enter freely [15]. Ideally, the ATMs needed to treat these infections should penetrate the phagocytic cells in adequate concentrations and time, not being metabolized in the cells and being active at acidic pH [16].

The change of pH can influence the antimicrobial activity of the different molecules. For example, β-lactams increase their potency by acidifying the media [17], while macrolides lose antibacterial activity with a decrease in pH [18]. This is interesting for the treatment of intracellular pathogens causing infection such as *S. aureus*, which can internalize in the phagolysosome where the pH is close to 5 [19]. The susceptibility of microorganisms to EO seemed to be higher at lower pH, the hydrophobicity of EO is higher at low pH and this favor its dissolution in the lipids of the cell membrane [20].

There are different methods to evaluate antimicrobial activity. Microdilution in broth technique is the most common technique, standardized by CLSI. This provides very useful parameters like minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The problem with these parameters is that they give us static information. On the other hand, time kill assay is a very useful method which describes the antimicrobial activity of a compound in a dynamic way, allowing the analysis of the bacterial behavior in presence of the antimicrobial along the time [21]. The curves (bacterial concentrations vs. time) obtained by this last kind of technic can be analyzed by mathematical models. Kinetic models can explain the behavior of a bacterial inoculum in the time because of the presence of an antimicrobial or the change of environmental variables [22]. There exist several kinetic models like Gompertz and Square Root models which can provide the growth rate (μ), lag period duration (LPD) and the maximum population density (MPD) [23]. There also exist models to describe the survival or the destruction of bacteria over the time like Sigmoidal Minus Base model [24], which is similar to Emax models [25].

Modelling antimicrobial activity of natural products, like EO is mainly associated to the research of food preservatives [22]. There are no studies on models applied to natural products with antimicrobial activity in veterinary medicine. This is a powerful tool to
understand the behavior of bacteria against new therapeutic alternatives molecules to control infections.

The aim of our work was to describe by mathematical models the behavior of S. aureus against M. armillaris EO at different pH conditions.

2. Results

We obtained 550 mL of EO representing a yield of 1.22% v/w (volume/100 g of fresh material). In Table 1 is shown the composition of the EO extracted and in Table 2 are mentioned the physicochemical parameters analyzed.

Table 1. Composition of M. armillaris essential oil analyzed by GC-MS-FID.

| COMPOUND          | AREA % | COMPOUND         | AREA % |
|-------------------|--------|------------------|--------|
| 1,8-Cineole       | 72.3   | β-Caryophyllene  | 0.5    |
| Limonene          | 7.8    | α-Terpinene      | 0.2    |
| α-Pinene          | 6.0    | trans-β-Ocimene  | 0.2    |
| Myrcene           | 2.2    | Geranyl acetate  | 0.2    |
| β-Pinene          | 2.2    | α-Phellandrene   | 0.1    |
| α-Thujene         | 1.5    | Terpinolene      | 0.1    |
| p-Cymene          | 1.4    | δ-Terpinol       | 0.1    |
| Terpinen-4-ol     | 1.4    | Aromandendrene   | 0.1    |
| α-Terpinol        | 1.4    | Geranyl isobutyrate | 0.1 |
| Sabinene          | 1.0    | cis-Calamenene   | 0.1    |
| γ-Terpinene       | 0.5    | Oxi-Caryophyllene| 0.1    |

Table 2. Physicochemical parameters of M. armillaris essential oil.

| Parameter                  | Value obtained                     |
|----------------------------|------------------------------------|
| Appearance at 20°C         | Oily and limpid liquid             |
| Smell                      | Penetrating, very fresh            |
| Taste                      | Bitter, astringent                 |
| Color                      | Pale yellow                        |
| Density (using pycnometer) | 0.89197-0.93013 g/mL               |
| Solubility in mineral oil  | Soluble                            |
| Solubility in water        | Partially soluble                  |
| Solubility in ethanol 70%  | Soluble                            |
| Refractive index at 20 °C  | 1.4698-1.4703                      |
| Acidity index              | 0.7824                             |
| Esterification index       | 32.8526                            |

For the MIC determination assay, erythromycin was used for the quality check of microdilution method. The MIC of this antibiotic was 0.5 μg/mL for S. aureus ATCC 29213 at pH 7.4 (it must range between 0.25 and 1 μg/mL according to CLSI (2013) [26]). Inhibitory and bactericidal concentrations of EO against the reference strain and the 6 wild type strains are shown in Table 3.

Table 3. MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) in μL/mL of each strain analyzed at pH 7.4; 6.5 and 5.0.
MICs values mentioned before (Table 3) were used to perform time kill assay. Each strain was exposed to different concentrations of EO (0.5 MIC, 1 MIC, 2 MIC, 4 MIC, 8 MIC, and a control without EO). Figure 1 shows the time kill assay for the reference strain and Figure 2 for the wild types. In both cases we evaluated S. aureus behavior at pH 7.4; 6.5 and 5.0 in presence and absence of EO.

| Strain   | pH 7.4 MIC | pH 7.4 MBC | pH 6.5 MIC | pH 6.5 MBC | pH 5.0 MIC | pH 5.0 MBC |
|----------|------------|------------|------------|------------|------------|------------|
| ATCC 29213 | 25         | 50         | 25         | 50         | 12.5       | 25         |
| SA 13    | 12.5       | 25         | 12.5       | 25         | 6.25       | 25         |
| SA 96    | 12.5       | 50         | 12.5       | 25         | 6.25       | 12.5       |
| SA 139   | 12.5       | 25         | 12.5       | 25         | 6.25       | 12.5       |
| SA 78A   | 12.5       | 12.5       | 6.25       | 6.25       | 3.1        | 3.1        |
| SA 79A   | 12.5       | 12.5       | 6.25       | 6.25       | 3.1        | 3.1        |
| SA 86B   | 12.5       | 12.5       | 6.25       | 6.25       | 3.1        | 3.1        |

The bacterial growth curves were modelled with the Gompertz equation. The data obtained are presented in Tables 4 (reference strain) and 6 (wild type strains). At pH 6.5 and 5.0 there was a decrease in the bacterial count for 0.5 MIC, therefore it was not possible to analyze them with this model.

Table 4. Parameters estimated by the Gompertz model for the ATCC 29213 strain at pH 7.4; 6.5 and 5.0.

| Parameter | pH 7.4 | pH 6.5 | pH 5.0 |
|-----------|--------|--------|--------|
| Control   | 0.5 MIC| Control 0.5 MIC | Control 0.5 MIC |
Those curves where the bacterial inoculum decreased were analyzed with the sigmoidal model minus base and the parameters obtained are observed in Tables 5 (reference strain) and 7 (wild type strains).

Table 5. Parameters estimated by the sigmoidal model minus base for the ATCC 29213 strain at pH 7.4; 6.5 and 5.0.

| Parameter                      | 0.5 MIC | 1 MIC | 2 MIC | 4 MIC | 8 MIC |
|--------------------------------|---------|-------|-------|-------|-------|
| **pH 7.4**                     |         |       |       |       |       |
| \( R^2 \)                      | 0.925   | 0.996 | 1.000 | 1.000 |       |
| \( \mu (\text{Log}_{10} \text{CFU/mL} \times \text{hours}) \) | 0.74    | 0.70  | 0.67  | -     | -     |
| LPD (hours)                    | 1.80    | 2.48  | 2.54  | -     | -     |
| MPD (\( \text{Log}_{10} \text{CFU/mL} \) | 12.20   | 8.66  | 10.84 | 10.40 |       |

| **pH 6.5**                     |         |       |       |       |       |
| \( R^2 \)                      | 0.949   | 0.998 | 0.998 | 0.998 | 1.000 |
| \( \text{Nmax (CFU/mL)} \)     | 2.97E+05| 2.97E+05| 3.71E+05| 4.20E+05|       |
| \( \gamma \)                   | 29.33   | 1.91  | 1.70  | 1.92  |       |
| \( T_{50} \) (hours)           | 1.96    | 1.53  | 1.36  | 1.36  |       |
| \( N_0 \) (CFU/mL)             | 4.10E+05| 3.70E+05| 3.65E+05| 4.15E+05|       |

| **pH 5.0**                     |         |       |       |       |       |
| \( R^2 \)                      | 0.776   | 0.998 | 1.000 | 1.000 | 1.000 |
| \( \text{Nmax (CFU/mL)} \)     | 2.97E+05| 2.97E+05| 3.71E+05| 4.20E+05|       |
| \( \gamma \)                   | 5.62    | 1.48  | 1.49  | 1.85  | 2.68  |
| \( T_{50} \) (hours)           | 11.53   | 2.30  | 1.94  | 1.54  | 1.16  |
| \( N_0 \) (CFU/mL)             | 5.40E+05| 6.49E+05| 6.79E+05| 7.70E+05| 5.90E+04|

\( N_0 \) is the initial inoculum concentration; \( \text{Nmax} \) is the maximum drop in bacterial count; \( T_{50} \) is the time necessary to reach the 50% of maximum bacterial inhibition; \( \gamma \) is the sigmoidicity coefficient.

Table 6. Mean of parameters estimated by the Gompertz model for the wild type strains at pH 7.4; 6.5 and 5.0.

| Parameter                      | Control | 0.5 MIC* | 0.5 MIC* | Control | 0.5 MIC* | 0.5 MIC* | Control | 0.5 MIC* | 0.5 MIC* |
|--------------------------------|---------|----------|----------|---------|----------|----------|---------|----------|----------|
| **pH 7.4**                     |         |         |         |         |         |         |         |         |         |
| \( R^2 \)                      | 0.990   | 0.972   | 0.994   | -       | 0.992   | -       | -       |         |         |
| \( \mu (\text{Log}_{10} \text{CFU/mL} \times \text{hours}) \) | 0.72    | 0.52    | 0.71    | -       | 0.49    | -       | -       |         |         |
| LPD (hours)                    | 1.86    | 6.81    | 1.93    | -       | 2.92    | -       | -       |         |         |
| MPD (\( \text{Log}_{10} \text{CFU/mL} \) | 12.06   | 6.86    | 10.91   | -       | 9.27    | -       | -       |         |         |

\( \mu \): Growth rate; LPD: Latency Period Duration; MPD: Maximum Population Density.

0.5 MIC* strain 78A was excluded because it could not be analyzed by Gompertz.

Table 7. Mean of parameters estimated by the sigmoidal minus base model for the wild type strains at pH 7.4; 6.5 and 5.0.

| Parameter                      | 0.5 MIC | 1 MIC | 2 MIC | 4 MIC | 8 MIC |
|--------------------------------|---------|-------|-------|-------|-------|
| **pH 7.4**                     |         |       |       |       |       |
| \( R^2 \)                      | -       | 0.968 | 0.994 | 0.998 | 1.000 |
N\text{max} \text{ (CFU/mL)} & - & 7.14E+05 & 6.49E+05 & 6.57E+05 & 6.46E+05 \\
\text{Y} & - & 17.16 & 3.93 & 4.02 & 4.32 \\
T_{1/2} \text{ (hours)} & - & 7.62 & 2.38 & 2.23 & 1.87 \\
N_0 \text{ (CFU/mL)} & - & 6.73E+05 & 7.02E+05 & 6.54E+05 & 6.40E+05 \\

\text{pH 6.5} \\
R^2 & 0.943 & 0.996 & 1.000 & 1.000 & 1.000 \\
N\text{max} \text{ (CFU/mL)} & 3.22E+05 & 6.33E+05 & 6.40E+05 & 6.51E+05 & 5.95E+05 \\
\text{Y} & 23.58 & 9.67 & 6.02 & 4.94 & 6469.42 \\
T_{1/2} \text{ (hours)} & 5.62 & 2.83 & 2.28 & 1.95 & 1.65 \\
N_0 \text{ (CFU/mL)} & 6.71E+05 & 6.04E+05 & 6.43E+05 & 6.53E+05 & 5.95E+05 \\

\text{pH 5.0} \\
R^2 & 0.962 & 0.998 & 1.000 & 1.000 & 1.000 \\
N\text{max} \text{ (CFU/mL)} & 4.90E+05 & 6.67E+05 & 5.64E+05 & 6.46E+05 & 6.34E+05 \\
\text{Y} & 6.85 & 3.44 & 4.46 & 4.52 & 4.50 \\
T_{1/2} \text{ (hours)} & 3.25 & 2.37 & 1.71 & 1.34 & 1.28 \\
N_0 \text{ (CFU/mL)} & 6.31E+05 & 6.69E+05 & 5.63E+05 & 6.45E+05 & 6.33E+05 \\

N_0 \text{ is the initial inoculum concentration; N\text{max} is the maximum drop in bacterial count; T}_{1/2} \text{ is the time necessary to reach the 50\% of maximum bacterial inhibition; } \text{Y is the sigmoidicity coefficient.} \\

The data obtained in the time kill assay allowed us to obtain the index E of antibacterial activity. In Figure 3 the index E \text{ vs. EO concentration is plotted at the 3 different pHs evaluated. In this way it is possible to observe the incidence of pH in the drop of the bacterial count added to the effect of EO. Table 8 shows the parameters obtained after modelling this data with the sigmoid model.

Table 8. Parameters obtained by the sigmoidal model applied to curves Index E (CFU/mL) \text{ vs. EO concentration (μL/mL) at pH 7.4, 6.5 and 5.0.}

| Strain    | Parameter | pH 7.4 | pH 6.5 | pH 5.0 |
|-----------|-----------|--------|--------|--------|
| ATCC 29213| $R^2$     | 0.996  | 0.998  | 0.998  |
|           | E_{\text{max}} (Log_{10} CFU/mL) | 10.20  | 8.85   | 8.63   |

Figure 3. Graphic representation of the antibacterial effect (E: ΔLog CFU/ml 24–0 h) of EO against S. \text{aureus} ATCC 29213 (A), wild type (n = 3, SA13, SA96, SA139, using the mean of triplicates for each strain) (B), wild type (n = 3, SA78A, SA79A, SA86B, using the mean of triplicates for each strain) (C). In the three cases are plotted E \text{ vs. EO concentration at pH 7.4, 6.5, and 5.0.}
| Y       | 2.42 | 2.14 | 1.12 |
|---------|------|------|------|
| C₅₀ (μL/mL) | 17.56 | 11.13 | 4.56 |
| E₀ (Log₁₀ CFU/mL) | 6.36 | 4.90 | 4.50 |

| R²     | 0.998 | 1.000 | 0.998 |
|---------|-------|-------|-------|
| Emax (Log₁₀ CFU/mL) | 9.11 | 8.46 | 8.85 |
| WT (13-96-139) | Y | 2.71 | 2.51 | 1.01 |
| C₅₀ (μL/mL) | 6.52 | 5.15 | 1.50 |
| E₀ (Log₁₀ CFU/mL) | 5.73 | 5.10 | 4.90 |

| R²     | 0.996 | 0.994 | 0.978 |
|---------|-------|-------|-------|
| Emax (Log₁₀ CFU/mL) | 9.35 | 8.56 | 5.48 |
| WT (78A-79A-86B) | Y | 0.75 | 1.41 | 1.68 |
| C₅₀ (μL/mL) | 3.13 | 2.28 | 1.86 |
| E₀ (Log₁₀ CFU/mL) | 4.70 | 4.63 | 1.92 |

E₀ is the index E (antibacterial index) in absence of antimicrobial; Emax is the maximum reduction in Log₁₀ of E₀; C₅₀ is the concentration that causes the 50% of the reduction of Emax; Y is the coefficient of sigmoidicity.

3. Discussion

Physicochemical characterization is important to assess the quality of essential oils. It is very important for standardization and design of commercial products, especially if they are destined for food and healthcare in both animal and human medicine. The M. armillaris EO isolated for this work presented a liquid consistency with a pale-yellow color and a penetrating and fresh odor. Other parameters analyzed were refractive index; density; pH; solubility in mineral oil, ethanol 70%; and water; acid and esterification indexes (Table 3). There is no information in the literature to compare these values. These represent a starting point for the standardization of M. armillaris EO and consider them for quality control. Chromatographic analysis of this EO revealed the presence of 1.8 cineole as main component (72.3%), limonene (7.8%) and α-pinene (6.0%). These are commonly present in essential oils with high antimicrobial activity. The 1.8 cineole is a monocyclic monoterpenic with an important antimicrobial activity and was found as the main component in M. armillaris EO in other works [5-7]. This compound is, in general, the major compound in the essential oil of Eucalyptus species too [27].

The MIC of M. armillaris EO necessary to inhibit S. aureus ATCC 29213 was 25 μL/mL at pH 7.4 and 6.5 but decreased twofold at pH 5.0. Concerning wild type strains, the MIC was 12.5 μL/mL at pH 7.4 for all strains, without change at pH 6.5 for 3 strains (SA13, SA96, SA139) and decreasing by half for the other strain at pH 6.5 (SA78A, SA79A and SA86B). Variations may be due to the existence of bacterial subpopulations with different sensitivities. At pH 5.0, the MIC decreased by half from that obtained at pH 6.5 (6.25 and 3.1 μL/mL, respectively). Something similar occurs when evaluating the MBC, since this parameter decreased between 2 and 4 times depending on the strain, comparing what happens at pH 7.4 and 5.0.

Falci et al. (2015) studied the composition and the antimicrobial activity of the essential oil of a Melaleuca species (not specified) cultivated in Brazil [28]. This essential oil had 70.8% of 1.8 cineole, 8.95% of terpineol and 8.25% of limonene. The amount of 1.8 cineole, limonene, and myrcene (1.99%) was similar to the essential oil of M. armillaris obtained in this work. Although the species of Melaleuca is not specified, a parallelism can be made with the composition of the mentioned essential oil. These authors demonstrated an important antimicrobial activity against S. aureus strains with MIC values between 1 and 2 μL/mL, and MBC between 2 and 4 μL/mL. Li et al. (2014) found that the MIC of 1.8 cineol against S. aureus ATCC 25923 was 6.25 μL/mL [29].

The high content of 1.8 cineole may be one of the factors that contribute to the antibacterial activity of the EO, to which the permeabilization of the membranes of microorganisms such as S. aureus has been attributed as an antimicrobial action mechanism.
due to its great hydrophobicity [30, 31]. This compound is usually the most abundant in *Eucalyptus globulus* essential oil. Yáñez Rueda and Cuadro Mogollón (2012) found an important activity for this species against *S. aureus* ATCC 29213 (MIC of 12.4 μg/mL), in which its composition was similar to *M. armillaris* EO evaluated in this work: 1.8 Cineol (82.27%), Limonene (3.70%), α-Pinene (3.16%), Terpinen-4-ol (1.4%), α-Terpinol (1.2%), β-Myrcene (1.12%) and α-Terpinene (1.1%), among others [32]. This could indicate a synergism between these components particularly effective against strains of *S. aureus*.

According to the ratio MIC/MBC an antimicrobial may be considered bactericidal or bacteriostatic. A compound is bacteriostatic if the MBC/MIC ratio is greater than 4 [33]. Analyzing the MIC and MBC of the EO, we found that for strains SA13, SA96 and SA139 these parameters are the same, and this coincidence is maintained even when pH conditions were modified. For the other strains, the MBC/MIC ratio was between 2 and 4, maintaining the ratio when acidifying the culture medium. Therefore, it could be considered that the EO of *M. armillaris* has bactericidal activity against *S. aureus*, which is independent of the pH.

The *M. armillaris* EO mechanism of action has not yet been investigated against *S. aureus*. Hayouni et al. (2008) studied the antimicrobial activity of this species against different *Lactobacillus* species [6]. As 1.8 cineole was the main component found (68.92%), these authors hypothesized that this compound could have destabilized the cytoplasmatic membrane of these bacteria, as was demonstrated by Li et al. (2014) [29]. However, the way of action postulated for *M. armillaris* by Hayouni et al. (2008) also involves the minority components found (α-Pinene Terpinen-4-ol, sabine, β-Myrcene and α-Terpinene, among others) [6]. According to these authors, these molecules interact with the cell membrane, where they dissolve in the phospholipid bilayer, aligning themselves between the fatty acid chains. This distortion of the physical structure would cause expansion and destabilization of the membrane, increasing the fluidity of the membrane, which in turn would increase the passive permeability.

The MIC and MBC are the parameters most used to quantify the antibacterial activity of a drug against an infectious pathogen. However, the temporal evaluation of different concentrations of the antimicrobial against a microorganism allows a better description of the magnitude of its antibacterial effect [34]. For this reason, it is also important to analyze what occurs over time through the construction of bacterial death curves. In the time kill assay for the EO against *S. aureus* (Figures 1 and 2), is possible to observe a decrease in the bacterial count after being exposed to EO. In general, a slight drop in the slope of the curve was observed with a concentration equivalent to the MIC of the essential oil against each strain and isolate. In turn, for concentrations of 0.5 MIC, a relevant rate of growth was not perceived, and in many cases, there was a decrease in the initial inoculum. For concentrations of 2, 4 and 8 times the MIC, a drop in the bacterial cell count was evidenced at 2 hours, continuing the decrease exponentially until 8-12 hours, and then maintaining the bacterial count until 24 hours after assay started. This pattern was generally maintained for all strains even changing the pH of the medium.

In the case of the reference strain (ATCC 29213) at pH 7.4 it was possible to achieve a decrease of 2.6 Log_{10} of the initial inoculum for concentrations of 2, 4 and 8 times the MIC. At pH 6.5 the decrease for these concentrations was 2.8 Log_{10}. At pH 5.0 the decrease in inoculum was 2.8 for 2 CIM, 3.6 for 4 CIM and 3.9 for 8 MIC. With these results, we can observe that, at higher concentration and higher acidity, the antibacterial activity of the essential oil is higher, being similar for concentrations of 4 and 8 CIM. For strains SA13, SA96 and SA139, the decrease in the initial inoculum for 2 CIM at 24 h is 2.7-3.0 Log_{10} at pH 7.4, 3.0-3.2 at pH 6.5 and 2.9-3.2 at pH 5.0. As for 4 and 8 CIM, the decrease is 2.5-3.5 Log_{10} at pH 7.4, from 3.1-3.7 at pH 6.5 and 3.2-3.9 at pH 5.0. Respect to strains SA78A, SA79A and SA86B, a fall of 3.1-3.8 was observed for the 3 pH values at concentrations of 4 and 8 CIM. In the case of the 2 CIM concentration for these strains, the drop in inoculum was between 2.5 and 3.4 Log_{10}. The strains analyzed had slight differences in the susceptibility against the EO but was common the antimicrobial activity improvement by increasing the acidity and EO concentration.
The mathematical modelling of a microorganism response at different conditions or with an inhibitor compound is very useful to understand its behavior, and to predict the effectiveness of a treatment under controlled conditions. To assess the validation of the model applied, it is important that the model has a good fit to experimental data, in terms of $R^2$, which must range between 0 and 1, the adjustment is better if this parameter is near to 1 [22].

The application of Gompertz model to data obtained from time kill assay allow us to know parameters like growth rate ($\mu$), lag period duration (LPD) and the maximum population density (MPD). We used this model for bacterial growth, being applied for control condition and 0.5 MIC at pH 7.4 (Tables 4 and 6). With these results we could observe that the presence of EO diminished $\mu$, extended the LPD and reduced the MPD. Something similar occurred in other study using *M. armillaris* EO against lactic acid bacteria [6]. These parameters also changed in a same way because of the pH decrease, highlighting the lower growth capacity of *S. aureus* under acidic conditions. Weinrick et al. (2004) have found that *S. aureus* in acidic conditions modifies its gene expression to promote defense mechanisms against acidity, which can lead to a lower growth rate [35].

At pH 6.5 and 5.0 only the control increased the bacterial count, so Gompertz model was not applied for 0.5 MIC. In this case and for curves obtained using 1 MIC, 2 MIC, 4 MIC and 8 MIC (where it was observed bacterial death) we applied the sigmoidal minus base model (Tables 5 and 7). With this model we obtained the $T_{50}$ (time in which was reach the 50% of the maximum drop in bacterial count, Nmax). This parameter is lower while the EO concentration increases, and the reduction is independent of the pH. On the other hand, Nmax is much closer to N0 with a higher concentration of EO, indicating that there is a much greater bacterial effect since it is possible to eliminate all the initial inoculum. Navarro-Cruz et. (2018) found that when modelling the antibacterial effect of the essential oil of *Lippia berlandieri* against *S. aureus*, the time needed to decrease the initial inoculum by 50% was shorter when modifying the pH from 7 to 5 [36].

The antibacterial effect (Index E) of EO is shown in Figure 3. The EO improves its antimicrobial activity at lower pH, lower concentrations are required to achieve the same effect. This behavior is similar for both reference and wild type strains. Modelling this data applying a sigmoid model let us to obtain different valuable parameters (Table 8) such as the concentration necessary to reach the 50% of the maximum effect ($C_{50}$). This parameter was smaller at lower pH for all the strains evaluated. Other important parameter is Emax, this decrease at acid pH, but we must consider that E0 is smaller too, this is because *S. aureus* is slightly susceptible at acid pH. The lower maximum antibacterial effect at a lower pH may be influenced by a lower bacterial growth capacity. At high concentrations, the antibacterial effect is similar (and close to virtual eradication) for the 3 pHs, while at acidic pH the E0 is lower, which had incidence in the value of Emax.

4. Materials and Methods

4.1. *M. armillaris* essential oil extraction

Leaves and herbaceous branches collection was carried out in Coronel Brandsen, Buenos Aires, Argentina (latitude 35°06’18.9” S and longitude 58°10’57.0” W). A sample portion was reserved for identification and further storage at the LPAG herbarium at the Faculty of Agrarian and Forestry Sciences, UNLP [37]. EO was obtained by steam distillation of the whole collected fresh biomass (44.85 kg). Subsequently, the EO was dried with sodium sulphate anhydrous at room temperature, filtered with a cotton funnel and stored at 4°C in amber glass bottle.

4.2. Essential oil characterization
The EO composition was analyzed by GC–FID–MS, as we previously described [4]. We performed assays to established parameters of quality control commonly used for essential oils [38] to characterize the EO of *M. armillaris*, since it is not described in the literature. In this way, physicochemical characteristics such as appearance at 20 °C, odor, flavor, color, refractive index, density (using a pycnometer) and pH were analyzed. Also, the solubility in different solvents was verified: mineral oil (1: 1); water (1:10) and ethanol 70% (1: 1). On the other hand, the acidity and esterification indices were determined following the recommendations of Argentinian Pharmacopeia VII Ed (2013) [39]:

- **Acidity index** (content of free fatty acids, defined as the amount, in mg, of potassium hydroxide necessary to neutralize the free acids present in 1.0 g of sample): 10.0 g of sample, exactly weighed and previously neutralized against phenolphthalein with 0.1 N sodium hydroxide, were dissolved in 50 mL of alcohol contained in an Erlenmeyer flask. 1 mL of phenolphthalein (prepared at 1% in alcohol) was added, and it was titrated with 0.1 N potassium hydroxide until persistent pink coloration for 30 seconds. The acid number was calculated as the mg of KOH necessary to neutralize the free fatty acids in one gram of sample. All reagents were purchased from Sigma Aldrich, MO, USA.

- **Esterification index** (defined as the amount, in mg, of potassium hydroxide necessary to saponify the esters present in 1.0 g of sample): 2 g of sample, exactly weighed, was transferred to a 250 mL Erlenmeyer, previously weighed and 25 mL of neutralized alcohol were added while stirring and 1 mL of phenolphthalein (prepared in a 1% ethanol solution) was added. It was titrated with 0.5N alcoholic potassium hydroxide until completely neutralizing the free fatty acids. Then 25.0 mL of 0.5 N alcoholic potassium hydroxide was added. It was heated in a water bath, with an appropriate coolant to maintain reflux for 30 minutes, stirring frequently, and excess potassium was titrated with 0.5 hydrochloric acid. N. A determination was made with a blank. The difference between the volumes, in mL, of 0.5N hydrochloric acid consumed by the sample and the blank, multiplied by 28.05 and divided by the weight, in g, of the sample taken, is the esterification index.

4.3. **Inhibitory and bactericidal activity of M. armillaris essential oil against S. aureus**

Six wild type (n = 6) *S. aureus* isolated, according National Mastitis Council procedure [40] from subclinical mastitis Holstein cows were used. The protocol was following the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies –FASS) and was approved by the Institutional Committee (CICUAL) of the Faculty of Veterinary Sciences, National University of La Plata (47.3.15)). The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of EO were established by broth microdilution method in 96-wells polystyrene microplates. Mueller Hinton Broth (MHB) culture medium was used with the addition of 0.5% of Tween 80. This surfactant enhances the dissolution of the EO in the aqueous culture medium. The MICs and MBCs were evaluated at 3 different pH conditions (7.4; 6.5 and 5.0). The MIC of the essential oil was also evaluated at pH 7.4, 6.5 and 5.0. This pH adjustment of the medium was carried out with the addition of HCl to the broth. The range of essential oil concentrations evaluated was 50 to 0.09 µL/mL and each plate was inoculated with a final bacterial concentration of 5 x 10⁵ UFC/mL and incubated at 35 °C for 18–24 h. MIC was established as the lowest concentration inhibiting bacterial growth. Positive and negative controls with MHB containing 0.5% Tween 80 were performed. Every determination for each strain was evaluated at the different 3 pHs conditions by triplicate. For quality control *S. aureus* ATCC 29213™ was used and the susceptibility to erythromycin was checked for this strain as control procedure [26].
Once MIC was established, 25 μL were taken from each well showing no evident bacterial growth. Then, inoculated individually in nutritive agar plates for colony counting after incubation at 35 °C for 18–24 h. MBC was the lowest antimicrobial concentration in which the initial inoculum fell 99.9%.

4.4. Time kill assay and antibacterial activity index of the EO

Once the MICs of EO and its combinations were identified, data were used to perform time-kill assays to evaluate the antibacterial activity index (E). Each S. aureus strains were faced to different concentrations (0.5 MIC, 1 MIC, 2 MIC, 4 MIC and 8 MIC) of EO, including the quality control strain S. aureus ATCC 29213®.

We prepared 7 tubes, one for each concentration, a positive (without antimicrobials) and a negative (without antimicrobials and inoculums) controls. Each one contained a final volume of 1 mL including MHB with 0.5% Tween 80 (pH 7.4, 6.5 and 5.0), antimicrobials and a final inoculum of 5x10⁵ CFU/mL. Incubations were carried out at 35 °C. Bacterial plate count was carried out at the initial time, 2, 4, 8, 12 and 24 h incubating at 35 °C by 24 h. The assay was carried out in triplicate for each strain.

Data obtained in time kill assay was used to create CFU/mL vs. time graphs and to evaluate the antibacterial activity index (E). Once E indexes were obtained, we graphed the E index vs. EO concentration (Log10) to compare what happens at the 3 pHs evaluated. The wild strains were grouped according to the MIC, obtaining two groups of three strains each one (using the mean of triplicates for each strain). Graphics were plotted using the GraphPad Prism 6 program.

E index was defined as the difference (in Log10 between the bacterial count (CFU/mL) at the initial time (nt-0) and at the end of the test (nt-24): E = (nt-24) - (nt-0). We considered 3 theoretical breakpoints to establish Bacteriostatic effect (E = 0), Bactericidal effect (E = −3), and Effect of virtual eradication of bacteria (E = −4) [41].

4.5. Modelling bacterial growth and death for S. aureus in presence of EO

With data obtained in the time-kill assay we carried out the mathematical modelling to describe the growth and death of S. aureus in presence of EO. For growth we applied Gompertz model obtaining the specific growth rate (μ), the lag phase duration (LPD) and the maximum population density (MPD) [23]. The mathematical expression of this model is:

\[ \text{Log } N = a + c \cdot \exp (-\exp (-b \cdot (t-m))) \]  

Where Log N is the decimal logarithm of the microbial counts (Log10 CFU/mL) at time t (hours); a is the Log of the asymptotic bacterial counts when the time decreases indefinitely (Log10 CFU/mL); c is the Log10 of the asymptotic counts when the time increases indefinitely (Log10 CFU/mL); m is the time required to reach the maximum growth rate (hours); b is the growth rate relative to time m (hours⁻¹). So, we can obtain: \( \mu = b \cdot c/e \) (Log10 CFU/mL*hours), (where e = 2.7182); LPD = m - 1/b (hours); MPD = a + c (Log10 UFC/mL). The equation was fitted to the microbial development data by means of a non-linear regression with the Sigma Plot program (Sigma Plot 12.0, 2011), since the parameters of the Gompertz model are nonlinear.

In the case of the curves where bacterial death was observed, the experimental data of CFU/mL vs. time were fitted with a sigmoid model minus base:

\[ N = N_0 - (N_{max} \cdot T)/(T_{iso} + T) \]
Where N is the bacterial count (CFU/mL) at time T (hours), N₀ is the initial inoculum concentration (CFU/mL), N_max is the maximum drop in bacterial count (CFU/mL), T_{I_{50}} is the time necessary to reach the 50% of maximum bacterial inhibition (hours), ϒ is the sigmoidicity coefficient. Experimental data was fitted with the non-linear least squares regression models using the software Sigma Plot 12.0 as mentioned before.

Finally, we applied the sigmoid model, which is analogous to the maximum response or Hill [25, 42], to the values of index E vs. EO concentration, in order to understand the mechanics of the relationship between the concentration of these and their bactericidal effect, and thus be able to obtain more information about the behavior of S. aureus under the different conditions evaluated. Redefining the previous equation, we have:

\[ E = E_0 - \frac{E_{\text{max}} \cdot C}{C_{50} + C} \]  

(3)

Where E is the index E (Log_{10} CFU/mL) for a concentration C (μL/mL), E₀ is the index E in the absence of antimicrobial (Log_{10} CFU/mL), E_{max} is the maximum reduction in Log_{10} of E₀, C_{50} (μL/mL) is the concentration that causes the 50% of the reduction of E_{max}, ϒ is the coefficient of sigmoidicity. The experimental data were fitted with the non-linear least squares regression models using the Sigma Plot software (Sigma Plot 12.0, 2011).

5. Conclusions

The essential oil of M. armillaris has good antimicrobial activity against S. aureus. This improves slightly with the acidification of the culture medium and presents a bactericidal activity where the MBC is close to the MIC. The analysis of biological systems using mathematical models allows obtaining more information that simplify collecting data from the observation of the results of an in vitro test. We highlight the antimicrobial potential of M. armillaris EO against S. aureus under acidic conditions, resulting in an interesting factor for the control of S. aureus infections and food contaminations.

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