Mode of action of Lippia graveolens essential oil on Salmonellaenterica subsp. enterica serovar Typhimurium

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Research

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Abstract

The essential oils are a powerful natural resource with antibacterial activity. This work aims at the mode of action of Lippia graveolens Kunth essential oil of plants growing in Cuba on Salmonella enterica subsp. enterica serovar Typhimurium. The effects of this oil on cell integrity were determined by time-kill, bacteriolysis and loss of 260 and 280-nm-absorbing material assays and total proteins leakage. Also, depolarization of the membrane by essential oil was monitored and intracellular and extracellular ATP was measured. The transmission electron microscopy (TEM) was used for observed morphologic change. Minimum inhibitory concentration (MIC) of L. graveolens essential oil and minimum bactericide concentration (MBC) were 0.4 and 0.8 mg/mL respectively. This essential oil showed a bactericidal action against over S . Typhimurium in a few minutes. After treatment, the cell lysis was not occurred, but little intracellular material and total proteins leakage were observed. This essential oil depolarizes the cell membrane, disturb metabolic processes and changes the structure of cytoplasmic membrane. These results suggest as primary mode of action of L. graveolens essential oil over S . Typhimurium an increased permeability of the membrane and depolarization of inside membrane, inhibition of ATPase or disturbance in proton motive force that finally provokes death of cells. L. graveolens essential oil is a botanic resource can be used for the control of the salmonellosis, foodborne disease.

1. Introduction

Foodborne diseases have important repercussions on public health, food safety, productivity, and poverty. Every year, almost 600 million people get sick and 420,000 die from foodborne diseases, with a loss of 33 million of healthy life years. Low and middle-income countries are the most affected, with estimated annual costs of US $ 110 billion in productivity losses, commercial losses and costs of treatment of diseases due to the consumption of unhealthy food (FAO, WHO, & WTO, 2019). With the phenomenon of climate change, there has been a significant increase in the risk to public health, through its effects on microorganism. Besides, the influence of this phenomenon on antimicrobial resistance (RAM) and zoonotic diseases is known both directly related to food safety (WHO, 2019).

The genus *Salmonella* is among the main pathogens causing foodborne diseases worldwide (WHO, 2015). *Salmonella* is a zoonotic pathogen, a bacillus anaerobic facultative gram-negative bacteria, that belongs to the *Enterobacteriaceae* family (Jajere, 2019). There are more than 2,500 serovars of *Salmonella enterica* (Lamas et al., 2018), with a wide range of hosts that includes animals, human, and plants. It can be found in the intestines of many animals of economic and food importance, such as pigs and poultry (Ferrari et al., 2019). In plants, *S.* Typhimurium is not limited to the surface, it invades and reproduces in plant tissues, which makes common use practices, such as washing or sterilizing the surface of plants infected with *Salmonella*, not prevent infection in humans or animals (Chen et al., 2018; Schikora, Carreri, Charpentier, & Hirt, 2008). This species can acquire antibiotic resistance and form biofilms (Cardoen et al., 2009; Doyle et al., 2016; Morganti et al., 2018), elements that include it in the WHO priority pathogen list for research and development of new antibiotics (Tacconelli et al., 2017).

Essential oils are aromatic and volatile liquids, which have a complex composition and are obtained by different methods of extraction as hydrodistillation, steam distillation and cold-press techniques fom fresh and dry plant material, which includes flowers, roots, barks, leaves, seeds, husks, fruits, wood and whole plants (Aziz et al., 2018; Preedy, 2015). Essential oils stand out among natural products of plant origin for their versatility in biological properties. Antibacterial activity as broad-spectrum substances against Gram-positive and Gram-negative bacteria, including activity on antibiotic-resistant strains, is one of their main advantages (Guinoiseau et al., 2015; Leyva-López, Gutiérrez-Grijalva, Vazquez-Olivo, & Heredia, 2017).

Plants of the *Verbenaceae* family are known by the capacity for essential oils production with diversity uses. The *Lippia* genus includes more than 100 species of plants, which have been used in traditional Latin American medicine (Almeida et al., 2018). One of the major commercial species is *Lippia graveolens* Kunth known as Mexican oregano, an aromatic plant native of Southern North America, it is used in folk medicine mainly in dermatological, gastrointestinal and respiratory affections (Preedy, 2015) and as culinary seasoning (Pascual, Slowing, Carretero, Sánchez Mata, & Villar, 2001). The essential oil of this plant exhibits antibacterial activity against Gram-positive and Gram-negative bacteria, which is related to major components as thymol, carvacrol, *p*-cymene (Hernández et al., 2009).

Studies on the antimicrobial activity of essential oils are abundant throughout the world, however, in terms of understanding the mode of action of these natural products as antimicrobials, more depth is needed with an integrative vision. This article is aimed at the mode of action of the essential oil of *L. graveolens* of plants grown in Cuba on strain of *Salmonella enterica* subsp. *enterica* serovar Typhimurium.

2. Materials And Methods

2.1 Essential Oil

The essential oil of L. graveolens was supplied by the Chemical Ecology Laboratory of National Center of Planta and Animal Health of Cuba. The plant material was collected in the town of Jaruco, Mayabeque, Cuba, located at 23. 076573 and -81. 964266. The essential oil was obtained by the method of hydrodistillation for three hours, using a Clevenger equipment (Benachour, Ramdani, Lograda, Chalard, & Figueredo, 2020). This essential oil was characterized and analyzed its chemical composition analyzed by GC-MS in previous work. The majority compounds include thymol (42.7%), carvacrol (22.2%), p-cymene (6.5%).

2.2 Bacterial Strains and Growth Conditions

Salmonella enterica subsp. *enterica* serovar Typhimurium ATCC 14028 (CIP 104115) strain was purchased from the Collection of Institute Pasteur (CIP, Paris, France). Before each experiment, the strain was routinely grown at 37˚C on Mueller-Hinton 2 agar (MHA, Oxoid).

2.3 Antimicrobial susceptibility testing

2.3.1 Disc Diffusion Assays

The agar diffusion method was used for the determination of antibacterial activities (CLSI, 2018). Inoculum were prepared by diluting overnight cultures in Mueller-Hinton broth (MHB, Oxoid) medium to approximately 10^6 CFU/mL. Filter paper discs (6 mm diameter, Dominique Dutscher) were placed onto the inoculated Petri dishes containing Mueller-Hinton 2 agar (MHA, Oxoid) and was applied 15 µL of the tested products in the paper discs. After keeping at room temperature for 1 h, plates were incubated at 37˚C for 24 h. Diameters of inhibition zones were measured (mm) and recorded as the mean ± standard deviation (SD). Each test was performed in triplicate on at least three separate experiments. Ciprofloxacin discs (5 µg, Bio-Rad) was used as positive control.

2.3.2 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Assays

The minimum inhibitory concentration (MIC) assays were performed by a rapid INT (*p*-iodonitroterazolium chloride, Sigma-Aldrich) colorimetric assay (Guinoiseau et al., 2015). The *L. graveolens* oil was serially twofold diluted in dimethylsulfoxide (DMSO, Sigma-Aldrich). The DMSO was previously tested for antibacterial activity and no detrimental effect on bacterial growth has been observed at the concentration used. The solutions obtained were then added (10 µL) to a 96-well microplate containing 190 µL of MHB (1:20, v/v) inoculated with 10^6 CFU/mL. The microplates were incubated at 37˚C for 24 h. The MICs of the samples were then detected following addition (50 µL) of INT (0.2 µg/mL). Viable bacteria reduced the yellow dye to pink. The MIC is defined as the lowest sample concentration that prevents this change and results in the inhibition of bacterial growth. All determinations were performed in triplicate and a negative control, consisting of MHB with DMSO (5%, v/v), was systematically included. An inoculation loop was introduced in each well and seeded on a Muller Hinton agar plate, free of the antimicrobial agent to determine the minimum bactericidal concentration (MBC), which is defined as the lowest concentration of the oil that resulted in a negative subculture.

2.4 Time-Kill Studies

Time-kill procedure was performed according to the method described by Klepser et al (Klepser, Ernst, Lewis, Ernst, & Pfaller, 1998) and modified by Viljoen et al. (Viljoen et al., 2003). The antibacterial activity of *L. graveolens* essential oil used at their MIC were evaluated against *S.* Typhimurium by measuring the reduction in the number of CFU (Colony Forming Units) per milliliter at 0, 15, 30, 45, 60, 120, 180, 240 minutes and 24 h of incubation at 37°C with agitation. The tested product was applied at MHB with DMSO (0.1%) inoculated with 10^6 CFU/mL. The inoculated medium containing DMSO and without essential oil was used as control. At each evaluation time, aliquot of 100 µL was taken and serially diluted in MHB; from each serial dilution step, 100 µL were transferred to two MHA plates in numbered sections and incubated at 37°C for 24 h. Colony-forming units were counted after incubation. This assay was performed in triplicate.

2.5 Cell Integrity Studies

Bacteriolysis assay was carried out according to the standard method described by Carson et al. (Carson, Mee, & Riley, 2002). A bacterial suspension was prepared by inoculating two colonies of *S.* Typhimurium from overnight cultures on MHA into 40 ml of MHB, which was incubated at 37˚C for 24 h with shaking. After incubation, the bacteria were separated from the growth medium by centrifugation at 10,472 rad/s for 12 min at 4˚C, washed twice with phosphate-buffered saline (PBS, pH 7.4) and resuspended in PBS supplemented with 0.01% Tween 80 (PBS-T, v/v). The bacterial suspension was adjusted so that the optical density (OD) at 550 nm of a 1 in 100 dilution was 0.310 ($\sim 3 \times 10^8$ CFU/mL). *L. graveolens* essential oil was added to the bacterial suspension at the MIC. PBS-T was added to the control suspension. The suspensions obtained were mixed for 20 s with a Vortex mixer. Samples (1 mL) were taken in duplicate every 30 minutes from 0 h to 2 h. They were centrifuged and the pellet was resuspended in 1mL of PBS-T. The optical density at 620 nm was measured immediately (Jasco UVisco UV-1200 spectrophotometer). This assay was performed on three independent experiments. The results were expressed as a ratio (in percent) of the OD620 at each time point versus the OD620 at 0 min.

2.6 Loss of Cytoplasmic Material

The release of 260-nm and 280-nm absorbing materials from *S.* Typhimurium cells treated with *L. graveolens* essential oil at MIC, was performed on the bacterial suspension (10^8 CFU/mL) in PBS supplemented with 0.01% Tween 80 (PBS-T, v/v). Suspension without essential oil was used as a control. The samples were incubated at 37˚C with shaking. They were taken at time 0, 30, 60, 90 and 120 min, and centrifuged at 10,472 rad/s for 12 minutes. The absorbance of the obtained supernatant was measured at 260 and 280 nm using a spectrophotometer (Jasco UVisco UV-1200). Each test was performed on three independent experiments. The results were expressed as a difference of the OD260 or OD280 at each time point versus the OD260 or OD280 at time 0.

2.7 Determination of released proteins

Total protein losses released by the action of the *L. graveolens* essential oil over *S.* Typhimurium were determined by Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951). The preparation of the inoculum and the samples was performed equal to the loss of material. Also, 1% sodium lauryl sulfate (SDS) and bovine serum albumin (BSA) were used as a standard. After treatment with the MIC, the samples were taken at time 0, and 120 minutes, and centrifuged at 10,472 rad/s for 12 minutes. Suspension without essential oil was used as negative control. Lowry’s method was applied and the optical density was measured at 730 nm in the Jasco UVisco UV-1200 spectrophotometer. Each sample was prepared in triplicate and three independent experiments were performed. The results were expressed as mean ± standard error.

2.8 Measurement of Intra- and Extra-Cellular Adenosine 5’-Triphosphate (ATP) Concentrations

To determine the action of *L. graveolens* essential oil on energetic molecules, the intracellular and extracellular ATP concentrations were measured as described by Gill and Holley (Gill & Holley, 2006), with modifications of Turgis et al. (Turgis, Han, Caillet, & Lacroix, 2009). The overnight cultures of *S.* Typhimurium were centrifuged for 10 minutes at 10,472 rad/s and the supernatants were removed. The cell pellets were washed two times with 20 mM of phosphate potassium buffer (PPB, pH 7.0) and then cells were collected by centrifugation under the same conditions. A cell suspension (10^8 CFU/mL) was prepared in PPB (20 mM; pH 7.4) with glucose (50 mM) and DMSO (0,1%). The essential oil was applied at the MIC. 30 mM of polymyxin B (PMB) was used as positive control and the suspension without essential oil as the negative control. The treatments were incubated at 37°C for 7 min with agitation. Then the samples were centrifuged at 10,472 rad/s for 12 minutes and the supernatant was separated from the pellet.

For ATP extra-cellular, the supernatants were put quickly in ice, and 50 µL of the samples were added to 96 well black plate for duplicate. Then, 50 µL of MIXED kit for ATP (ATP-kit, Sigma) was applied in each well, and the plate was incubated for 30 min in ice in darkness. Fluorescence was measured using FP-83000 Jasco spectrofluorophotometer (excitation wavelength (λ_{ex}) = 535 nm and excitation wavelength (λ_{em}) = 587 nm). Each test was performed on three independent experiments. The results were expressed as a ratio (in percent) of the relative fluorescence unit (RFU) at each time point versus the RFU of the DMSO control.

For ATP intra-cellular, the pellets were resuspended in 1 mL of NaCl (0,85%) and centrifugated at the same previous conditions. The cells were resuspended in 200 µL of ATP buffer assays (ATP-kit, Sigma). After, 5 µL of 15% ammonium bromide (CTAB, Sigma) were added in each treatment and incubated at room temperature for 15 min. The samples were centrifuged and 50 µL of the supernatant was taken and applied to 96 well black plate for duplicate. Then this experiment was continued like for ATP extra-cellular assay.

2.9 Membrane depolarization assay

The cytoplasmic membrane depolarization activity of *L. graveolens* essential oil over *S.* Typhimurium was measured by using 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3$, Sigma) (Chehimi, Pons, Sablé, Hajlaoui, & Limam, 2010). A *S.* Typhimurium overnight culture in MHB was centrifuged at 10,472 rad/s for 12 minutes at 4˚C, washed twice with phosphate-buffered saline (PBS, pH 7.4), and resuspended in buffer solution (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with glucose 50 mM at pH 7.0, and DMSO 0.1%). The bacterial suspension was adjusted at 10^8 CFU/mL and 99 µL was applied to 96 well black plate for duplicate. The cells were incubated with 1.68 µM $DiSC_3$ at room temperature with shaking for 1h. Then KCl was added to a final concentration of 0.1 M to equilibrate the cytoplasmic and external K^+ and incubated for 5 min. An aliquot of 1µL of *L. graveolens* essential oil was applied at MIC and, as positive controls, 30 mM of polymyxin B (PMB) and 4 mM CTAB. Suspension without essential oil was used as the negative control. Fluorescence was monitored with an FP-83000 Jasco spectrofluorophotometer (excitation wavelength (λ_{ex}) = 622 nm and excitation wavelength (λ_{em}) =

670 nm). The results were expressed at relative fluorescence unit (RFU). This assay was performed on three independent experiments.

2.10 Transmission Electron Microscopy (TEM)

A suspension of *S.* Typhimurium in the exponential phase of growth was prepared by inoculating then incubating 80 mL of MHB at 37˚C for 24 h with shaking. The bacterial suspension was adjusted so that the optical density at 620 nm (OD620) of a 1 in 100 dilution in MHB was 0.200 (10^8 CFU/mL) with Tween 80 (0.1%, v/v). The cells of *S.* Typhimurium were treated with the MIC of *L. graveolens* essential oil for 7 min. The negative control was the suspension without essential oil. After centrifugation at 10,472 rad/s for 10 minutes, the pellets were first fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature and then post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature. The postfixed microbial pellets were processed in graded ethyl alcohol, propylene oxide, Spurr resin and cured for 24 h at 45˚C. Ultrathin sections were stained with uranyl acetate followed by lead citrate and then examined with a transmission electron microscope (HITACHI H-7650) at an accelerating voltage of 80 kV.

3. Results

3.1 Susceptibility of *Salmonella* Typhimurium to *Lippia graveolens* essential oil

The antibacterial activity of *L. graveolens* essential oil over *S.* Typhimurium strain is presented in Table 1. A great zone of inhibition was revealed by the action of this essential oil and It is correspondence with a strong inhibition zone (> 20.1 mm) (Mazzarrino et al., 2015). The results amount the essential oils and the antibiotic control showed significant differences, with larger value for the natural product. The MIC value was 0.4 mg/mL and double value of MIC was the MBC.

Table 1. Inhibition zone, Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Lippia graveolens* essential oil on *S.* Typhimurium strain.

Treatments	Inhibition zone (mm) ± STD	MIC (mg/mL)	MBC (mg/mL)
L. graveolens essential oil	56.67 ± 3.33 [a]	0.4	0.8
Ciprofloxacin	32.57 ± 0.33 [b]	-	-

Mean values ± standard deviation, in a column that are not followed by the same letter are significantly different ($p < 0.05$).

3.2 Time-Kill Studies

The effect of *L. graveolens* essential oil on the growth of *S.* Typhimurium demonstrated a negative kinetic with reduced viability at MIC (Fig. 1). This essential oil had a bactericidal end-point (99.9% or ≥ 2 log10

of inhibition) in only 15 minutes of treatment, time that was taken into account for the remainder of the experiments. The total inhibition was at 2 h of treatment and was remained for 24 h (data not shown). The control grew up in the time.

3.3 Cell Integrity Studies

The cells of *S.* Typhimurium not showed a change in OD at 620 nm after 2h of treatment with *L. graveolens* essential oil at MIC (Figure 2). In all times, the samples had more than 80% of cells integrity indicating that cell lysis did not occur.

3.4 Loss of Cytoplasmic Material

The *L. graveolens* essential oil at MIC induced the releasing of 260-nm and 280-nm absorbing materials from *S.* Typhimurium (Figure 3). The loss of absorbent material at both 260 and 280 nm was observed significantly ($p < 0.05$) after 60 min of exposure to the oil. The maximum proportion obtained at 2h of treatment; however, was less than 20 %.

3.5 Determination of released proteins

To corroborate the release of cellular components by the action of essential oil, total proteins were determined. This test was carried out at time 0 and 2 h, time of maximum release of cellular content demonstrated in the cytoplasmic material loss test. No significant changes were shown between oil treatment and the control at the time of exposure. Nevertheless, the results of total proteins leakage of *S.* Typhimurium for the action of the essential oil (0,35 ± 0,27 mg/mL) revealed larger concentration than the control (0,04 ± 0,02 mg/mL) ($p < 0.05$).

3.6 Membrane depolarization assay

After treatment with *L. graveolens* essential oil, *S.* Typhimurium cells have disturbances in the membrane (Figure.4). The change of value of the membrane potential was similar to the positive control, polymyxin B, which is known to induce the formation of pores in the membrane (Poirel, Jayol, & Nordmanna, 2017).

3.7 Measurement of Intra- and Extra-Cellular Adenosine 5'-Triphosphate (ATP) Concentration

Measurements of ATP intracellular and extracellular were determined in *S.* Typhimurium cells after 7 minutes treatment with *L. graveolens* essential oil at MIC (Figure 5). Intracellular ATP was reduced significantly ($p < 0.05$) by the essential oil (7.8% of RFU), in the same way as the antibiotic polymyxin B (8.6% of RFU). The concentration of extracellular ATP was very low (less than 10% of RFU) whatever the treatment but the highest value was observed in presence of the essential oil.

3.8 Transmission Electron Microscopy (TEM)

The essential oil effect over S. Typhimurium was observed by transmission electron microscopy (Figure 6). *L. graveolens* affected the cellular integrity of *Salmonella* cells after only 7 minutes of exposure. The

majority of cells conserved the external morphology. However, these cells presented a longer distance between the outside and plasmic membrane, which was observed as more periplasmic space. The plasmic material was conserved inside of the plasmic membrane, but a change in density was observed. The cells untreated were observed with the typical structure of gram-negative.

4. Discussion

The *Lippia graveolens* essential oil of plants cultivated in Cuba is a natural product with a phenolic group as majority compounds for 64% of total. Thymol, carvacrol and *p*-cimene have been reported as majoritarian compounds of essential oil of *L. graveolens* cultivated in Mexico (thymol 10.43%, carvacrol 43.7%, *p*-cimene 6,4) (Preedy, 2015) and Rio de la Virgen- Jutiapa in Guatemala (carvacrol 44.8%, *p*-cimene 21.8%, thymol 7.4%) (Salgueiro, Cavaleiro, Gonçalves, & Proença Da Cunha, 2003), but the Cuban cultivar has more quantities of this structurestogether. This composition assures a powerful activity over *Salmonella* Typhimurium. Results from disc diffusion assay and MIC (0.4 mg/mL) and MBC (0.8 mg/mL) values illustrated that it has a strong and consistent inhibitory effect against this foodborne pathogen.

Irreversible damage at viability cellular was confirmed in a short time (15 minutes) of treatment. Similar results were obtained by Kumar and Chul in 2014, using thymol over this strain (Chauhan & Kang, 2014). Thymol at 0.750 mg/mL reduced the number of cells after 20 min of exposition. The membrane cellular is vital to cell structure, because it intervenes in many processes such as energy conversion, nutrient processing, synthesis of structural macromolecules and secretion of growth regulators (Swamy, Sayeed Akhtar, Sinniah, Akhtar, & Sinniah, 2016). For this, plasmic membrane is considerate as an important site of action, and indeed, many essential oil constituents as thymol and carvacrol, have been described to possess activity on this particular structure (Hyldgaard, Mygind, & Meyer, 2012).

The hydrophobic nature of essential oils can interact with the lipid membrane of bacterial pathogens, resulting in the leakage of the inner cell components, damage in potassium ion reflux, and finally leading to cell death (Saad, Muller, & Lobstein, 2013). Membrane disrupts, affecting the structural stability of the membrane or change in the permeability are principal modifications over the membrane for action of essential oil (Hyldgaard et al., 2012). Generally, phenolic compounds are responsible of major bactericide effect against food-borne pathogenic bacteria. The phenolic compounds disrupt the cell membrane as well as effectively inhibit the functional properties of the cell, and eventually leaking the inner materials of the cell (Bajpai, Baek, & Kang, 2012).

However, *L. graveolens* essential oil did not induce abrupt cell lysis on *S*. Typhimurium within two hours of treatment, and was coherent with the outflow of little absorbent cytoplasmic matrix at 260 nm and 280 nm. The total proteins released showed a slow change in the permeability membrane. These results suggest that the initial action is not the total destruction of the membrane. Conversely, thymol severely disrupted the membrane of *S*. Typhimurium, resulting in the release of intracellular components such as deoxyribonucleic acid (DNA) in the cell supernatant (Chauhan & Kang, 2014). These differences in results

are consistent with the research approach in which has been worked and highlights the need to study the mode of action of essential oils as an integral product and not focus on the study of an only component.

On the other hand, effects in the cellular activity were monitored by messing the intracellular and extracellular ATP concentrations. Indeed, in cells treated with *L. graveolens* no ATPin, was detected. This drastic decrease is not correlated to the release of ATP in the extracellular medium. The depletion of the internal ATP pool was associated with a change in membrane potential. These results were similar to those demonstrated by Ultee et al in 2002 (Ultee, Bennik, & Moezelaar, 2002). More, those results are also associated to change in membrane potential. So, *L. graveolens* essential oil, like as carvacrol, does not enhance the membrane permeability for ATP and the depletion of the internal ATP pool results suggest the reduction of ATP synthesis and/or increase ATP hydrolysis. Also, this depletion of the ATP pool following the addition of lipophilic components was observed in other studies. The alcohol fraction extracts of *Cistus ladaniferus* essential oil have the capacity to vanish the ATPin on *Staphylococcus aureus* cells, without growing the ATP out (Guinoiseau et al., 2015). More, components like carvacrol, eugenol and cinnamaldehyde inhibit the ATPase activity of *Escherichia coli* and *Listeria monocytogenes* (Gill & Holley, 2006).

Adenosine triphosphate (ATP) is used in vital cell processes that require energy as respiration, survival, growth and replication. Other functions of ATP include signaling function, participation in storing and supplying energy in metabolism and enzymatic reactions. In Salmonella, is known that ATP regulate virulence gene mgtC (Mempin et al., 2013). Cellular respiration depends on the respiratory chain in the plasma membrane. This converts redox energy into an electrochemical gradient of protons (proton-motive force) which subsequently drives ATP formation from ADP and phosphate by ATP synthase. The membrane potential and the transmembrane proton gradient are the two parameters of respiratory chain in the plasma membrane. Nevertheless, ATP synthase in bacteria is dependent on the potential component (Dimroth, Kaim, & Matthey, 2000). So, *L. graveolens* essential oil change the potential of membrane of *S*. Typhimurium and it can affect the ATP synthase, with damage in the respiratory chain of cellular respiration.

TEM was used to observe the effects of *L. graveolens* essential oil inside of de *S*. Typhimurium cells. Outwardly, the cell remains morphologically intact changes occur inside of the bacteria: (i) outside membrane separates from the plasmic membrane; (ii) differences in the electron-dense structure of cytoplasmic material. This phenomenon can be related to the capacity of essential oil diffuse, penetrate and disorganize the lipid tail region of the membrane (Stevens, 2004). The total volume of the membrane is roughly constant, but membrane thinning results in lateral expansion, affecting the mechanical properties of the membrane. As the area per lipid grows the surface tension increases and the bending modulus decreases dramatically, implying membrane deformation (Stevens, 2004).

The expansion of the membrane also results in a reduction in the packing of the lipid molecules leading to the formation of a large number of cavities, significantly reducing the translocation free energy of water molecules across the lipid tail region. As a result, a large number of water translocations occur, and

the membrane becomes leaky with the colleapse of the transmembrane potential and additional membrane dysfunction, such as inhibition of ATP production and loss of proton motive force and rapid death of the bacterium (Dimroth et al., 2000; Li et al., 2017).

Diverse researches have already highlighted changes in the morphology external of *Salmonella* strains by the action of essential oils. Raybaudi-Massilia et al. (Raybaudi-Massilia, Mosqueda-Melgar, & Martín-Belloso, 2006) described damages to the cellular membrane of *Salmonella* Enteritidis by action of lemongrass essential oil at 5 µL/mL in 24 h, including its disruption of the same and leakage of cell content. *Zataria multiflora* Boiss. essential oil at 0.3 µg/mL in 1h trigger important morphological damages in *Salmonella* Typhimurium, such as the increase permeabilization and disruption of membranes which allowed to the dye and the contents of the cells appeared depleted and amorphous (Moosavy et al., 2008). Also, cinnamaldehyde at 0.4% in one hour of treatment causes severe damage in *Salmonella* MT 2195 (Yossa et al., 2014).

Studies on antimicrobial activity of essential oils are abundant worldwide. However, the subject of the mode of action of these natural products as antimicrobials needs more depth (Carson et al., 2002), and an integral comprehension. This work shows a mode of action of *L graveolens* essential oil on S. typhimurium, as antimicrobial alternative with an action over the membrane plasmatic and the metabolic process. Lipophilic and proteins studies can be realized in the future researches, for understand the molecular specific change in the membrane after treatment with the essential oil. Also, the action over metabolic process can be better comprehend with studies over particular enzymes, transcriptome, proteome or production of toxins.

The mechanisms of action are key to avoid antimicrobial resistance to antimicrobials. These studies create the basis for preventing the use of compounds with the same mode of action. The complexity of the chemical composition of essential oils allows different antimicrobial mode of action not only at a particular location but also at different cell sites. Synthetic antibiotic only has one mechanism of action over one target site: cell-wall biosynthesis, protein synthesis (subunit 30S or 50S of ribosome), DNA replication and repair (RNA polymerase, DNA gyrase), folic acid metabolism, membrane structure or Lipid A biosynthesis. Therefore, there is a reemerging interest on essential oils as antibiotic alternative.

5. Conclusion

This work shows a natural option for treatment *Salmonella* strains, without risk of antimicrobial resistance. *Lippia graveolens* essential oil increases permeability of the membrane, disturb the inside membrane, metabolic energy depletionand finally provokes the death of cells in a few minutes. These results suggest the action of the essential oil on the cell membrane and over metabolic energetic process. In depth studies are need to fully understand if another structure or specific metabolic pathway have been damaged by this essential oil. *Lippia graveolens* essential oil is a resource with several modes of action as antibacterial with possibility for the preservation of food safety.

Abbreviations

ATP: adenosine triphosphate; BSA: bovine serum albumin; CFU: Colony Forming Units; CTAB: ammonium bromide; $DiSC_3$: 3,3'-dipropylthiadicarbocyanine iodide; DMSO: dimethylsulfoxide; DNA: deoxyribonucleic acid; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; INT: *p*-iodonitroterazolium chloride; MBC: minimum bactericide concentration; MIC: minimum inhibitory concentration; MHA: Mueller-Hinton 2 agar; MHB: Mueller-Hinton broth; OD: optical density; PBS: phosphate-buffered saline; PBS-T: PBS supplemented with 0.01% Tween 80; PMB: polymyxin B; PPB: phosphate potassium buffer; RFU: relative fluorescence unit; SDS: sodium lauryl sulfate; TEM: transmission electron microscopy.

Declarations

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Authors' contributions

Annie Rubio- Ortega: designed and performed the experiments, processing of primary data, statistical analysis, prepared figures and/or tables, written the document. **Elodie Guinoiseau**: designed and performed the experiments, interpretation and integration of results. **Yann Quilichini**: designed and performed the experiments, review & editing. **Dominique de Rocca Serra**: supervision, interpretation and integration of results, review & editing. **Jean-Pierre Poli**: performed the experiments, processing of primary data and statistical analysis. **Maria del Carmen Travieso- Novelles:** supervision and interpretation and integration of results. **Ivette Espinosa- Castaño**: designed and performed the experiments and supervision, **Oriela Pino- Pérez**: interpretation and integration of results, review & editing and funding acquisition. **Liliane Berti**: review & editing, project administration and funding acquisition. **Vannina Lorenzi**: designed and performed the experiments, interpretation and integration of results, review & editing, project administration and funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

All authors have read and agreed to the ethics for publishing the manuscript.

Consent for publication

All authors approved the consent for publishing the manuscript to the Journal of Bioresources and Bioprocessing.

Competing interest

There are no conflicts of interest to be declared.

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Figures

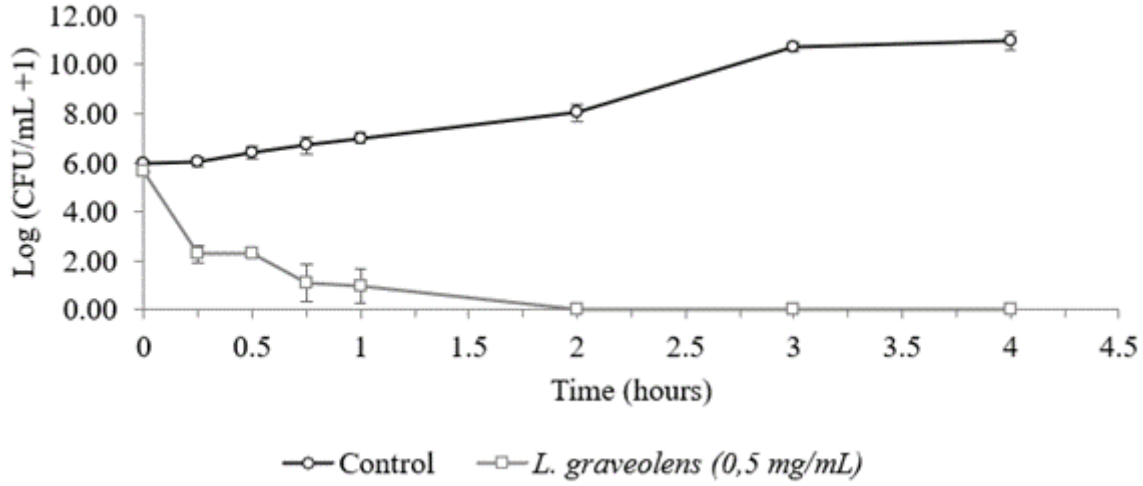

Figure 1

Time-kill curves of S. Typhimurium treated with Lippia graveolens essential oil (squares) at MIC and untreated cultures (circles). Mean values of triplicate independent experiments and standard deviation are shown (n=6).

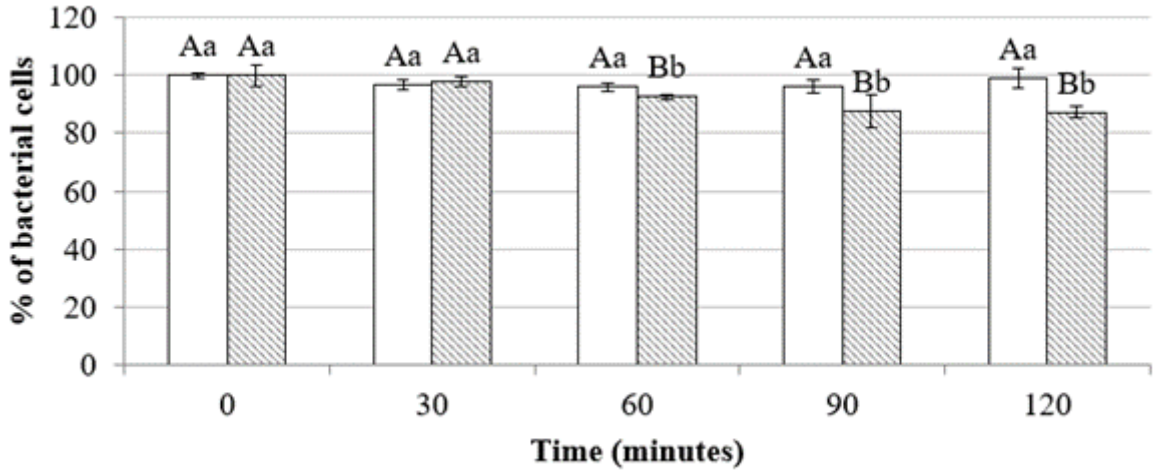

Figure 2

See manuscript for full figure caption.

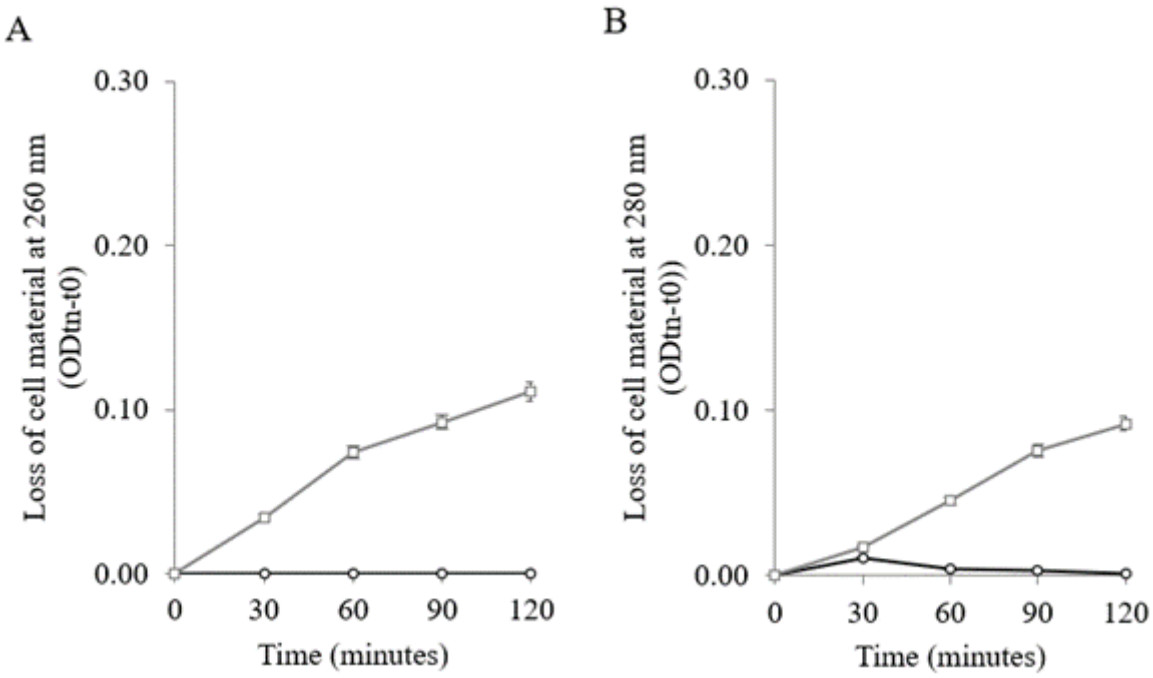

Figure 3

Absorbent material release to 260 nm (A) and 280 nm (B) in extracellular medium of S. Typhimurium cells untreated (circles) and treated (squares) with Lippia graveolens essential oil at MIC. Mean values of triplicate independent experiments and standard deviation are shown (n=6).

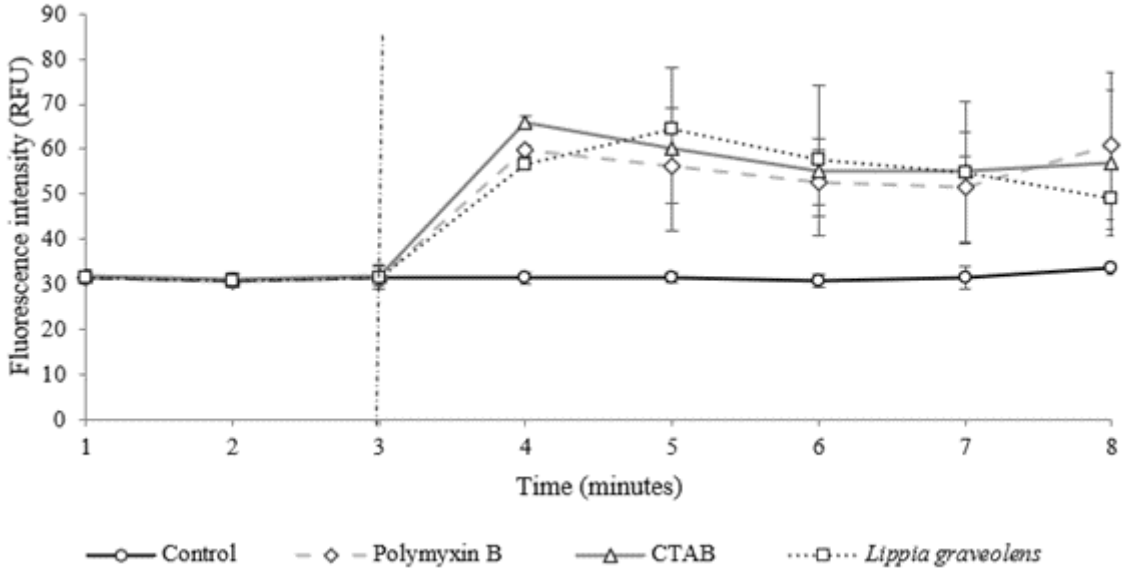

Figure 4

Depolarization of the cytoplasmic membrane of cells of S. Typhimurium treated with Lippia graveolens essential oil at MIC (squares), with 30 mM PMB (diamond) and 4 mM CTAB (triangle) monitored by fluorescence intensity change. Untreated cells used as control negative (circles). Mean values of triplicate independent experiments and standard deviation are shown (n=6). The treatment was added after two minutes of equilibration (dashed line).

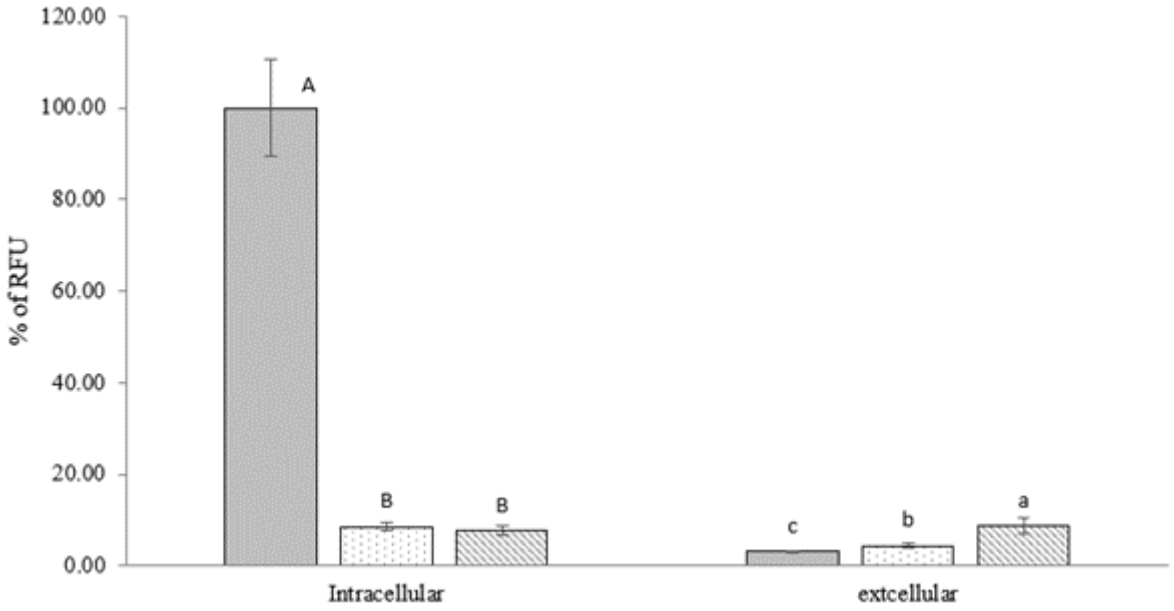

Figure 5

See manuscript for full figure caption.

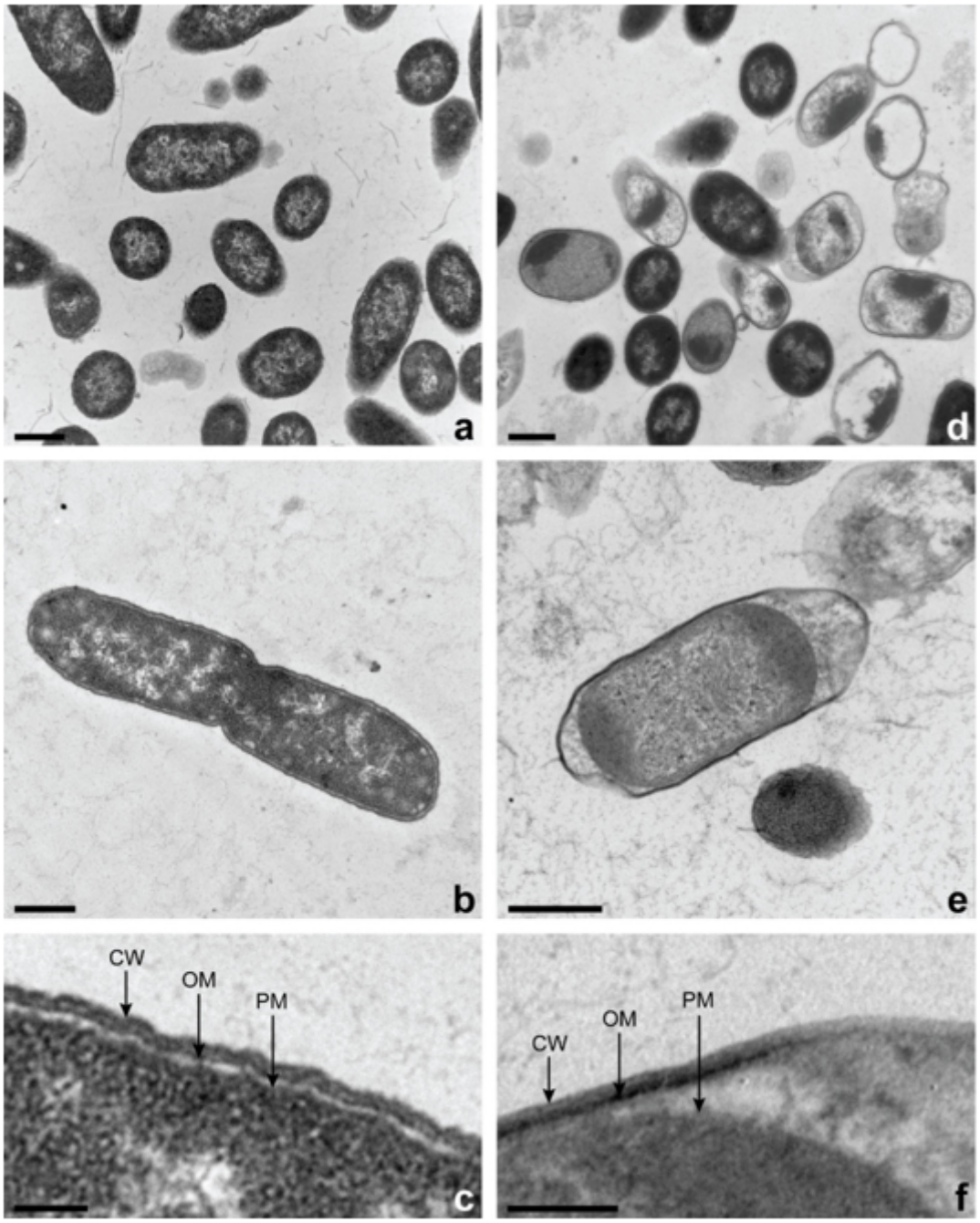

Figure 6

Transmission electron micrographs of S. Typhimurium cells stained. (a-b) control cells with 500 nm scale bar and (c) 100 nm scale bar. (d-e) S. Typhimurium cells treated with L. graveolens oil at the MIC with 500 nm scale bar and (f) 100 nm scale bar. OM: outer membrane; PL: peptidoglycan layer, PM: plasmic membrane.

Supplementary Files

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