Essential Oils from Herbs against Foodborne Pathogens in Chicken Sausage

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Abstract: Consumption of chicken meat and its products, especially sausage, have increased in recent years. However, this product is susceptible to microbial contamination during manufacturing, which compromises its shelf life. The flavoring and preservative activities of essential oils (EO) have been recognized and the application of these antimicrobial agents as natural active compounds in food preservation has shown promise. The aim of this study was to evaluate the effect of Ocimum basilicum and Origanum vulgare EO on Listeria monocytogenes and Salmonella Enteritidis strains in artificially inoculated samples of fresh chicken sausage. First, the minimal inhibitory concentration (MIC) of EO in vitro was determined. The sausage was prepared and kept at ± 4°C; then, the inoculation of individual bacteria was carried out. EO were added at 0.3%, 1.0% and 1.5% v/w. After 0, 5, and 24 hours, the most probable number method (MPN) was performed. Transmission electron microscopy (TEM) was used to view the damage caused by these EO on bacterial morphology and/or structure. Only the 1.5% concentration was effective in reducing L. monocytogenes. 0.3% of O. vulgare EO was able to reduce the MPN/g of Salmonella Enteritidis (2 log) after 5 hours trials. O. basilicum EO showed no effect on Salmonella after 5 hours, but decreased by 2 log after 24 hours. O. vulgare EO at 1% gave a greater reduction of S. Enteritidis at 5 hours, increasing or maintaining this effect after 24 hours. The results confirmed the potential benefits of use EO in control of foodborne pathogens.

Key words: Ocimum basilicum, Origanum vulgare, Salmonella Enteritidis, Listeria monocytogenes, preservative

1 INTRODUCTION

Chicken meat and its products have increased in popularity and have become widespread throughout the world, with chicken sausage being one of the most popular categories among these products¹. Sausage manufacture involves a number of handling steps, which increase the chances of contamination by pathogens or spoilage². Fresh sausage does not undergo heat treatment and has a high water activity; giving this food a short shelf life and subjecting it directly to the action of the microorganisms presents⁰.

Application of agents with adequate antimicrobial and antioxidant activities has significant potential to extend the shelf life of chicken products and prevent economic losses⁴. Due to the negative perception of chemical preservatives, consumers’ attention is changing to natural alternatives and particular interest has been focused on the potential use of essential oils (EO) from aromatic plants⁵.

It is well known that most species, especially those belonging to the Lamiaceae family, have different biological and pharmacological activities, which has meant that for a long time they have been used for improving the taste and organoleptic properties of different foods⁶. Ocimum basilicum (basil) and its EO are used as flavoring in tomato-based products and those that are prone to deterioration by acid-tolerant microbiota⁷,⁸ and studies have revealed the potential use of the of Origanum vulgare (oregano) EO against several microorganisms⁹,¹⁰.

Foodborne diseases are a growing public health problem worldwide. Salmonella Enteritidis is considered the most important serovar of Salmonella, causing gastrointestinal disease of varying severity in humans¹¹. This pathogen is
commonly found in chicken, which is the primary vector for transmission of Salmonella to humans\textsuperscript{15,19}. Listeria monocytogenes, on the other hand, is common in dairy products and red meat, but it can also be found in chicken, adding to the health concerns of Salmonella and Campylobacter\textsuperscript{14}. Listeria is an opportunistic pathogen that mainly affects pregnant women, newborns, the elderly and immunocompromised individuals. This pathogen emerged in the late 20\textsuperscript{th} century and has caused many outbreaks with high mortality rates\textsuperscript{15,16}.

Thus the aim was to investigate the antimicrobial activities of O. basilicum and O. vulgare EO against Listeria monocytogenes and Salmonella Enteritidis in artificially inoculated fresh chicken sausage samples after different periods of contact between pathogen and EO.

2 EXPERIMENTAL
2.1 Essential oils
Fresh plant samples of O. basilicum and O. vulgare were purchased in the city of Botucatu, São Paulo, Brazil, and used in the preparation of EO by the steam distillation methodology in a Marconi device, Model M480. Dried specimens of plants were deposited in the Herbarium “Irina Delanova Gmetchujnicov” Department of Botany, Institute of Biosciences – IBB/ UNESP, whose numbers were: O. basilicum Botu 26037 and O. vulgare Botu 26287.

2.2 Chemical characterization
Chemical analysis of EO was performed by gas chromatography-mass spectrometry (GC-MS) in a Shimadzu device, model QP5050A, using a capillary column, CBP-5, 50 m in length, with an internal diameter of 0.25 mm and 0.25 μm film thickness. The carrier gas was He and the identification of EO compounds was made on the basis of the National Institute of Standards and Technology (NIST) library, analysis of the mass spectra, and also data in the literature\textsuperscript{17}.

2.3 Preparation of fresh chicken sausage samples
The formulation comprising 84.55% of boneless chicken breast, 10% lard, 3% water, 1.5% salt, 0.5% phosphosphate, 0.25% garlic, and 0.2% peppers\textsuperscript{18}. The mass was incorporated into in swine casings with a mean diameter of around 30 mm, and the samples produced were divided (buds), separated by lots, and stored in a refrigerator at 4°C.

2.4 Bacterial strains
Salmonella Enteritidis (ATCC-13076) and Listeria monocytogenes (ATCC-15313) strains were stored at −80°C until their use in microbiological essays.

2.5 Enumeration of L. monocytogenes and S. Enteritidis in chicken sausage assays
Susceptibility tests of the EO were performed with the inoculation of bacterial strains on chicken sausage samples (25 g) with suspensions standardized by a 0.5 MacFarland standard, aiming at a bacterial concentration of approximately 10\textsuperscript{7} colony forming unit/g (around 5 log CFU/g). After, volumes of O. vulgare and O. basilicum EO were added separately, to achieve concentrations of 0.3 (MIC obtained in previous microdilution in vitro assays – data not shown), 1.0, and 1.5% in inoculated sausage samples. All phases of assays were performed in sterile Petri plates, all procedures were carried out at laminar flow, and handling of the bacteria and EO homogenization were performed using sterile cutlery (knife and fork) made of stainless steel. Following homogenization, sausage samples were kept at 4°C (refrigerator temperature). After 0, 5, and 24 hours, quantification of the bacteria inoculated in the sausage samples was performed by the most probable number (MPN) method. Despite the inherent characteristics of the MPN technique (e.g., large volume of material required, workload, and the time necessary to complete identification), this method proved to have high sensitivity and high reproducibility\textsuperscript{19}. Different times were chosen to verify if the contact time influences the antibacterial action of essential oils. Control tests were also prepared using non-inoculated (negative control) and inoculated sausage samples (positive control) both without EO addition. Assays were performed in triplicate.

The detection of Listeria, 25 g were homogenized in stomacher with 225 ml of LEB broth (Listeria Enrichment Broth - Oxoid) and pre incubated at 30°C for 4 hours. The following were added selective agents (40 mg/L nalidixic acid 50 mg/L of cycloheximide and 15 mg/L of acriflavin) with reincubation under the same temperature for 48 hours. At 24 and 48 hours, aliquots were plated with the aid of a chromium nickel strap in Palcam agar (Oxoid) incubated at 35°C for 48 hours. After this period, up to 5 colonies (black with black halos, due to breakage Aesculin) characteristics were transferred to a tube with TSA-YE agar (TSA plus 0.6 % yeast extract), incubated at 35°C/24 hours. From this stock preliminary evidence of identification such as Gram stain (Gram positive rods are), the catalase (positive reaction) and plated on agar motility for observation of growth “like umbrella” were performed. Then, if necessary, suspected colonies are identified with the help of API\textsuperscript{20}. In tests with Salmonella, it is worth noting that although the methodology advocates the need for two means of enrichment, it was decided to use only Rappaport Vassiliadis Broth according to recent studies\textsuperscript{21,22}. For the determination of Salmonella also 25 grams of sausage diluted with 225 ml of buffered peptone, and homogenized in Stomacher water which six decimal dilutions were prepared in triplicate were used. After incubation at
37°C for 24 h, 0.1 ml was seeded into tubes containing 10 ml of Rappaport-Vassiliadis broth, incubated at 42°C for 24 h. After these periods of incubation in medium Rappaport-Vassiliadis, a heave was plated on agar Xylose lysine deoxycholate (XLD) agar and CHROmagar Salmonella. After 35°C/24 hours, the characteristic colonies of Salmonella were isolated and transplanted into tubes containing tryptic soy agar inclined (TSA) and maintained at 35°C/24 hours. This growth, biochemical tests were performed with triple sugar agar inclined iron (TSI)/agar and tilted phenylalanine, and incubated at 35°C/18-24. The positive strains were tested for Salmonella forward to somatic and flagellar serum according to Andrews et al. modified. MPN values were converted to log MPN/g.

### 2.6 Transmission electron microscopy (TEM)

The overnight cultures of S. Enteritidis and L. monocytogenes (Brain Heart Infusion at 37°C/24 h) received the O. vulgare or O. basilicum EO at 0.3%, the in vitro MIC found previously, and at three times this MIC value (1%). After 2 hours of contact with the EO, the bacteria were prepared for TEM, as recommended by Moosavi et al. Pre-fixation of the bacterial samples was performed by adding 3 mL of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 hours, followed by centrifugation (1500 g for 20 minutes). The supernatants were discarded and 3 mL of glutaraldehyde was added to the pellet, which was stored at 4°C for 24 hours. The post-fixation procedure was performed in an osmium tetroxide solution (1%) in 0.1 M phosphate buffer at pH 7.3 for 2 hours, followed by dehydration of the material in acetone and impregnation into blocks of Araldite® to obtain ultrathin sections. The sections were subsequently stained with uranyl acetate and lead citrate. The samples were analyzed and photographed with a transmission electron microscope (CM 100, Philips) operated at 80 kV. The images were analyzed with digital imaging software (Iten).

### 2.7 Statistical analysis

The results were analyzed by Kruskal-Wallis analysis of variance (ANOVA) and the Student-Newman-Keuls test (multiple comparisons), with the significance level of 5%.

### 3 RESULTS AND DISCUSSION

#### 3.1 Extraction and chemical characterization of EO

The plants showed a similar yield. Average of 0.20% for O. basilicum and 0.17% for O. vulgare EO. Chemical composition of O. basilicum and O. vulgare EO (Table 1) indicated that the terpenoids linalool (31.52%) and thymol (48.62%) as the major compounds of these EO respectively.

Linalool makes the membrane of the bacteria permeable; the MIC observed in some studies was 1000 μg/ml for Salmonella Typhimurium and ranged from 1000–2145 μg/ml for L. monocytogenes. In a study comprising the hydrophobic constituents of EO, linalool showed high bacteriostatic activities, with an MIC of ≤ 0.2 mg/ml against L. monocytogenes and E. coli O157:H7, while the minimal bactericidal concentrations (MBCs) were 0.2 mg/ml. The combination of mild heat (54°C/10 min) and 0.2 mg/ml of the antimicrobial showed a higher inactivation than the sum of the methods acting separately. The antimicrobial activity of thymol is due structural and functional damage of the cytoplasmic membrane that causes the release of lipopolysaccharides present in the outer membrane of Gram-negative bacteria and rupture of the outer membrane. Thymol at 0.4 and 0.2 mg/ml, as wash solution, reduced by 5 log and 2 log, respectively, the amount of Salmonella on the surface of contaminated grape tomatoes (Lu and Wu 2010).

Knowledge of the chemical composition of EO allows a better understanding of the sites of action in the bacterial cell and, at the same time, points to the use of these compounds alone or in combination with other preservation techniques for food safety.

#### 3.2 Enumeration of L. monocytogenes and S. Enteritidis in chicken sausage

It was verified that there was no contamination of sausage samples (negative control) with the bacterial strains studied, with values corresponding to < 3 MPN/g. This suggests to us that the bacteria recovered and identified during Listeria and Salmonella MPN assays were cert-
certainly the *Listeria* and *Salmonella* strains artificially inoculated in the sausage.

In research conducted in Brazil, *L. monocytogenes* was found in 25% of sausages produced industrially. Also, according to this research, the pathogen was detected in all samples of raw material used in the preparation of sausages, revealing that contamination by *L. monocytogenes* was observed during the production process through contact with the environment, equipment, and handlers. In another study conducted in Brazil, the percentage of *L. monocytogenes* isolated by the traditional method was 9.3% of sausages marketed in the city of Botucatu, São Paulo state. In chicken carcasses from the Brazilian state of Goiás, 52 samples, i.e., around 14.32% of total samples collected, were contaminated with *Salmonella* and 11 serovars were identified; *Salmonella* Enteritidis was the second most frequently found (13.5%). Overall, in general, the literature shows variability in results which were usually influenced by the technique used for the recovery of bacteria. As the sausage samples used in this study were produced in compliance with good manufacturing practices and using good quality raw material, there was no problem of contamination by these bacteria, which is usually common in fresh chicken sausages. The concentrations of 0.3 and 1% were ineffective for *L. monocytogenes* (*Table 2*). There was no reduction in MPN/g values immediately after the addition of EO and *L. monocytogenes* compared with the positive control. However, with 1.5% of *O. vulgare* EO reductions of 1.1 and 1.3 log MPN/g for *L. monocytogenes* were found after 5 and 24 hours of contact, respectively. In sausages treated with *O. basilicum* EO, there was a reduction of approximately 1.4 log MPN/g after 5 hours, which was maintained after 24 hours. All reductions were statistically significant compared with the control samples, sausage samples with bacteria and without EO, but there was no difference between the treatments with either EO.

For the *Salmonella* (*Table 3*) strain, a difference between negative control and treatments was found at 0 hours, with values of <3 MPN/g in all assays. Regarding the other contact times, it was found that after 5 hours of contact with 0.3% of *O. vulgare* EO, there was a reduction in the determination of *Salmonella* in 2 log MPN/g, and such a reduction was lower after 24 hours of storage (1.3 log MPN/g). On the other hand, *O. basilicum* EO had no effect after 5 hours of contact, but reduced in 2.1 log MPN/g after 24 hours of contact. At 1% of *O. vulgare* EO, it was found that 5 hours of contact between the bacteria and EO was sufficient to reduce the bacterial determination by 2 log MPN/g, and the determination was reduced by 5.1 log MPN/g after 24 hours of contact. For *O. basilicum* EO, there was a reduction of 0.6 and 3.1 log MPN/g in *Salmonella* Enteritidis after 5 and 24 hours, respectively. At 1.5%, it was found that *O. vulgare* EO reduced *Salmonella* Enteritidis by 1.6 log MPN/g after 5 hours and it remained significantly reduced at 24 hours. At 1.5%, *O. basilicum* EO significantly reduced the MPN/g of *L. monocytogenes*.

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**Table 2** Log of most probable number of bacteria per gram of sausage (negative control), for sausage inoculated with *Listeria monocytogenes* (positive control), sausage inoculated with *L. monocytogenes* and treated with *Origanum vulgare* EO. Readings were taken at 0, 5, and 24 hours.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Listeria monocytogenes (Log MPN/g)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Positive control</td>
<td>4.97 ± 0.00</td>
</tr>
<tr>
<td><em>O. vulgare</em> 0.3% EO</td>
<td>4.97 ± 0.00</td>
</tr>
<tr>
<td><em>O. basilicum</em> 0.3% EO</td>
<td>4.97 ± 0.00</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Positive control</td>
<td>4.80 ± 0.00</td>
</tr>
<tr>
<td><em>O. vulgare</em> 1.0% EO</td>
<td>4.80 ± 0.00</td>
</tr>
<tr>
<td><em>O. basilicum</em> 1.0% EO</td>
<td>4.80 ± 0.00</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Positive control</td>
<td>6.11 ± 0.85</td>
</tr>
<tr>
<td><em>O. vulgare</em> 1.5% EO</td>
<td>6.11 ± 0.00</td>
</tr>
<tr>
<td><em>O. basilicum</em> 1.5% EO</td>
<td>6.11 ± 0.00</td>
</tr>
</tbody>
</table>
Table 3  Log of most probable number of bacteria per gram of sausage (negative control), for sausage inoculated with *Salmonella* Enteritidis (positive control), sausage inoculated with *S*. Enteritidis and treated with *Origanum vulgare* EO. Readings were taken at 0, 5, and 24 hours.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Salmonella Enteritidis (Log MPN/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Positive control</td>
<td>7.08 ± 0.00</td>
</tr>
<tr>
<td><em>O. vulgare</em> 0.3% EO</td>
<td>7.08 ± 0.00</td>
</tr>
<tr>
<td><em>O. basilicum</em> 0.3% EO</td>
<td>7.08 ± 0.00</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Positive control</td>
<td>7.74 ± 0.00</td>
</tr>
<tr>
<td><em>O. vulgare</em> 1.0% EO</td>
<td>7.74 ± 0.00</td>
</tr>
<tr>
<td><em>O. basilicum</em> 1.0% EO</td>
<td>7.74 ± 0.00</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Positive control</td>
<td>7.38 ± 0.00</td>
</tr>
<tr>
<td><em>O. vulgare</em> 1.5% EO</td>
<td>7.38 ± 0.00</td>
</tr>
<tr>
<td><em>O. basilicum</em> 1.5% EO</td>
<td>7.38 ± 0.00</td>
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</table>

EO showed a significant reduction in *Salmonella* Enteritidis only after 24 hours of storage (1.4 log MPN/g).

The determination of *Listeria* was reduced by EO at the higher concentration, whereas *Salmonella* was inhibited at the lower concentrations (0.3%). Generally, Gram-negative bacteria are less sensitive to antimicrobial agents because of the bacterial wall and its outer membrane, which limit the diffusion of hydrophobic compounds. However, the literature has shown that this does not mean that the Gram-positive bacteria are always more susceptible\(^{37}\). Furthermore, this Gram-positive pathogen is able to adapt to several environmental conditions, such as refrigeration, acid foods, or foods with high salt contents, probably due the increased percentage of peptidoglycan in their wall\(^ {35, 36}\).

*L. monocytogenes* is psychrotrophic and can survive and multiply in refrigerated meat and ready to eat products. It is possible that, at a low concentration of EO plus refrigeration, *Listeria* shows better survival against the antimicrobial agent, which was not seen to occur with *Salmonella*. Another fact to consider is that the antimicrobial activity of the EO is influenced by several factors that are extrinsic and intrinsic to the food (e.g., fat, protein, and pH). With regard to the complex food matrix, the activity tends to be decreased compared with the results obtained with culture medium, since food can protect the bacteria from the action of EO. Smith-Palmer *et al.*\(^{35}\) studied cheeses with different fat contents and verified that the composition of the food influenced the efficiency of plant EO against *L. monocytogenes* and *Salmonella* Enteritidis. When considering the potential application of EO in foods, the authors found that *L. monocytogenes* was inhibited more readily with a low-fat content but that the composition was less influential with *S. Enteritidis*\(^ {37}\). Thus, the lard or lipid content of the sausage may also have negatively affected *L. monocytogenes* inhibition.

### 3.3 Transmission electron microscopy

The *L. monocytogenes* and *Salmonella* Enteritidis cells treated with 0.3% (MIC from *in vitro* assays) and 1.0% (around three times the MIC) and non-treated cells were observed by TEM. After the period of culture, untreated cells of *L. monocytogenes* and *Salmonella* Enteritidis showed a uniform cell structure with defined bacterial membranes and wall and cytoplasm with electron-dense material (Fig. 1. **A** and **B**). On the other hand, in cells treated with EO, morphological changes were observed, including irregularities in the shape and loss of structural integrity of the cell wall and intracellular matrix. Also, we observed the presence of cell debris around the damaged cells (Fig. 1. **A1–A4** and **B1–B4**).

The reduction in the enumeration of *Listeria* and *Salmonella* treated with the two EO corroborated by the results of TEM, which showed morphological changes in the bacteria after 2-hour exposure to the EO. These changes have been interpreted as being due to the effects...
of EO on the permeability of the membrane, and causing their lysis as well as damage of the bacterial cell wall and loss of intracellular contents, as shown by the accumulation of materials on the surface of treated cells. Structural changes of the cell wall of Gram-positive and Gram-negative bacteria may lead to different damage when exposed to antimicrobial compounds. Cellular damage in food-borne pathogens treated with natural products has also been shown by TEM by other authors. Wu et al. found that *L. monocytogenes* and *S. aureus* were not easily destroyed, even with injuries or channels in the cell wall, compared with *E. coli* O157 and *S. Typhimurium*. When using thyme EO, it was reported that in addition to all the degenerative changes in the cells of *L. monocytogenes* (e.g., loss of cytoplasm and uniform distribution of the agglomeration of intracellular material), with an increasing concentration of EO *Listeria* bacterial wall also lost its characteristics and uniformity.

### 4 CONCLUSION

*L. monocytogenes* was less sensitive to the action of EO than *Salmonella*. With respect to *Listeria* in fresh chicken sausage, a concentration of 1.5% was the most efficient for both EO. The best inhibitory effect on *Salmonella* was shown by 1.0% of *O. vulgare* EO, for which 5 hours of contact was sufficient to promote a reduction in the bacterial determination; in contrast, *O. basilicum* EO showed a significant reduction at the same concentration after 24 hours. Inhibitory effects of EO on bacterial strains were not immediate. The results showed an inhibitory effect at 5 hours, which remained constant or increased after 24 hours. Both EO have the potential to be explored by the food industry, and the combination of processes for food protection has shown a promising reduction of the factors limiting the use of these compounds, such as a negative impact on taste and high cost.

### ACKNOWLEDGMENTS

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