

## Effect of essential oils and major compound on *Clostridium botulinum* endospores inoculated in meat product

Efeito de óleos essenciais e composto majoritário sobre endósporos de *Clostridium botulinum* inoculados em produto cárneo

Efecto de aceites esenciales y compuesto mayoritario sobre endosporos de *Clostridium botulinum* inoculados en producto cárnico

Received: 05/02/2022 | Reviewed: 05/12/2022 | Accept: 05/24/2022 | Published: 05/28/2022

**Glécia de Cássia Aleixo**

ORCID: <https://orcid.org/0000-0002-3198-5995>  
Universidade Federal de Lavras, Brazil  
E-mail: [glectinhbio@yahoo.com.br](mailto:glectinhbio@yahoo.com.br)

**Monique Suela Silva**

ORCID: <https://orcid.org/0000-0002-6809-714X>  
Universidade Federal de Lavras, Brazil  
E-mail: [monique.silva3@estudante.ufla.br](mailto:monique.silva3@estudante.ufla.br)

**Heloísa Helena de Abreu Martins**

ORCID: <https://orcid.org/0000-0002-8071-6644>  
Instituto Federal de Educação, Ciência e Tecnologia Baiano, Brasil  
E-mail: [heloisa\\_amartins@hotmail.com](mailto:heloisa_amartins@hotmail.com)

**Raquel Martino Bemfeito Carvalho**

ORCID: <https://orcid.org/0000-0002-3937-8938>  
Universidade Federal de Lavras, Brazil  
E-mail: [raquel.bemfeito@estudante.ufla.br](mailto:raquel.bemfeito@estudante.ufla.br)

**Eduardo Mendes Ramos**

ORCID: <https://orcid.org/0000-0002-8240-8151>  
Universidade Federal de Lavras, Brazil  
E-mail: [emramos@ufla.br](mailto:emramos@ufla.br)

**Roberta Hilsdorf Piccoli**

ORCID: <https://orcid.org/0000-0003-2334-9400>  
Universidade Federal de Lavras, Brazil  
E-mail: [rpiccoli@ufla.br](mailto:rpiccoli@ufla.br)

### Abstract

Mortadella is a widely consumed product due to its sensory characteristics, however, nitrite is used as a preservative and this synthetic additive has been linked to some diseases. In this sense, this study aimed to evaluate the antimicrobial effect of oregano and clove essential oils, besides the major compound carvacrol on *Clostridium botulinum* endospores inoculated in mortadella and the physicochemical effect of them on the product. The minimum sporicidal concentration of the essential oils and the major compound was determined by the broth microdilution method and the best results used to prepare the mortadellas containing 0 and 150 ppm of nitrite. All the evaluated treatments showed decrease of nitrite levels over time and lower lipid oxidation. The combinations of essential oils and carvacrol did not affect the mortadella color in the final product. In the mortadellas added of combinations of essential oils and of major compound there was reduction in the population of *C. botulinum*, whereas in mortadella without essential oils the antimicrobial effect was attributed only to the nitrite. The results are promising and suggest the use of clove and oregano essential oils together with carvacrol compound in meat sausages as a natural preservative and action against foodborne pathogens.

**Keywords:** *Clostridium botulinum*; Endospore; Essential oil; Foodborne pathogen; Mortadella; Natural preservative.

### Resumo

Mortadela é um produto amplamente consumido devido suas características sensoriais, entretanto, o nitrito utilizado como conservante e os aditivos sintéticos têm sido associados a certas doenças. Sendo assim, este estudo objetivou avaliar o efeito antimicrobiano dos óleos essenciais de orégano e cravo, juntamente com o composto majoritário carvacrol, sobre endósporos de *Clostridium botulinum* inoculados em mortadela, bem como o efeito físico-químico deles sobre o produto. A concentração mínima esporicida dos óleos essenciais e do composto majoritário foi determinada pelo método de microdiluição em caldo e os melhores resultados utilizados para o preparo das mortadelas contendo 0 e 150 ppm de nitrito. Todos os tratamentos avaliados mostraram diminuição nos níveis de nitrito ao longo do tempo e redução da oxidação lipídica. A combinação de óleos essenciais e carvacrol não afetou a

cor da mortadela no produto final. Nas mortadelas adicionadas das combinações de óleos essenciais e do componente majoritário houveram redução da população de *C. botulinum*, no entanto em mortadelas sem óleos essenciais o efeito antimicrobiano foi atribuído apenas ao nitrito. Os resultados são promissores e sugerem o uso dos óleos essenciais de cravo e orégano juntamente com o carvacrol em embutidos cárneos como conservantes naturais e contra patógenos veiculados por alimentos.

**Palavras-chave:** *Clostridium botulinum*; Conservante natural; Endósporo; Mortadela; Óleo essencial; Patógeno alimentar.

### Resumen

La mortadela es un producto muy consumido por sus características sensoriales, sin embargo, el nitrito utilizado como conservante y los aditivos sintéticos se han asociado con ciertas enfermedades. Por lo tanto, este estudio tuvo como objetivo evaluar el efecto antimicrobiano de los aceites esenciales de orégano y clavo, juntamente con el compuesto mayoritario carvacrol, sobre endosporas de *Clostridium botulinum* inoculadas en mortadela, así como su efecto fisicoquímico sobre el producto. La concentración mínima esporicida de los aceites esenciales y del compuesto mayoritario se determinó por el método de microdilución en caldo y los mejores resultados se utilizaron para la elaboración de mortadelas con 0 y 150 ppm de nitrito. Todos los tratamientos evaluados mostraron una disminución en los niveles de nitrito con el tiempo y una reducción en la oxidación de lípidos. La combinación de aceites esenciales y carvacrol no afectó el color de la mortadela en el producto final. En la mortadela con la adición de combinaciones de aceites esenciales y el componente mayoritario hubo una reducción en la población de *C. botulinum*, sin embargo en la mortadela sin aceites esenciales el efecto antimicrobiano se atribuyó únicamente al nitrito. Los resultados son prometedores y sugieren el uso de aceites esenciales de clavo y orégano junto con carvacrol en embutidos cárnicos como conservantes naturales y contra patógenos alimentarios.

**Palabras clave:** Aceite esencial; *Clostridium botulinum*; Conservante natural; Endospora; Mortadela; Patógeno alimentario.

## 1. Introduction

*Clostridium botulinum* is a gram-positive, anaerobic and rod-shaped bacterium. It is an endospore-forming agent and shows high resistance to the thermal treatment applied to food, hence it is extremely important in the food industry, since in low acidity foods (pH >4.5) and under anaerobic conditions the endospores can germinate leading to the multiplication of vegetative cells and the production of toxins (Sharma et al., 2006). Ingestion of food contaminated with botulinum toxin causes botulism in humans, a disease with neurological symptoms that causes flaccid paralysis and high mortality rate (Peck et al., 2010).

Among the low acidity foods whose control of *C. botulinum* is highly relevant, the most important are cured meat sausages, such as mortadella. Although these products undergo heat treatment this is not enough to inactivate the endospores of the bacteria, that can germinate under favorable ambient conditions. Thus, nitrite and nitrate salts are widely used as preservatives in the meat industries because they are active against the germination of *C. botulinum* endospores (Cassens, 1997; Dutra, 2011). However, the nitrite in meat products is transformed into nitrous acid, which can react with amines and forming N-nitrous compounds, especially nitrosamines, which have toxic, mutagenic, neurogenic, nephrotoxic and carcinogenic effects (Oliveira et al., 2005). Therefore, total or partial substitution of this preservative is of great interest.

In this context the essential oils of condiments are promising proposals because they are generally recognized as safe (GRAS) and have antimicrobial, sporicidal, antioxidant and flavoring activities (Chaibi et al., 1997). There is a significant interest in the use of natural preservatives such as essential oils (Abdollahi et al., 2014, Fernandes et al., 2015) to replace the use of chemical preservatives due to evidence of toxicity of synthetic antimicrobials (Asensio et al., 2014). However, it is known that high concentrations of essential oils should be employed to achieve the desired antimicrobial effect in certain foods, and it can change the sensory characteristics of the final product.

As an alternative to essential oils can be used its major compounds that are GRAS and can be used in a lower concentration reducing the sensorial changes caused by essential oils (Bakkali et al., 2008). Hence, the aim of the present study was to evaluate the sporicidal effect of oregano and clove essential oils and the major compound carvacrol on *Clostridium*

botulinum endospores inoculated in mortadella, besides evaluating the effect of these combinations on lipid oxidation, residual nitrite, pH, texture and color development in the product.

## 2. Methodology

### Materials

Essential oils of oregano (*Origanum vulgare*) and clove (*Syzygium aromaticum*) were purchased from FERQUIMA Industria e Comércio Ltd., São Paulo, Brazil, and the major compound carvacrol from Sigma-Aldrich, São Paulo, Brazil.

### Culture of microorganism and standardization of endospore inoculum

The bacterium *C. botulinum* type D used in this study was gently provided by the National Laboratory for Agriculture (LANAGRO) of Pedro Leopoldo, MG, Brazil.

The strain was grown in DRCBB (Himedia®) medium supplemented with 0.5% filtered sodium sulfate and ferric citrate solution, and incubated in anaerobiosis at 37°C/48 h. The obtained inoculum was grown up to a population of 8 Log CFU/mL-1 and plated in AK No.2 (Himedia®) medium and incubated at 37°C/120 h in anaerobiosis to obtain the endospores. The plates were washed with 0.9% (w/v) saline solution in order to standardize the number of endospores through absorbance readings (D.O.600nm) and optical microscopy counts of endospores stained by the Wirtz-Conklin technique (Murray et al., 1999). The suspension was subjected to heat shock (75°C/15 min) and cooled rapidly in an ice bath. Serial dilutions were performed on endospore suspension and plating on isolation agar base of *C. botulinum*. The plates were incubated in anaerobiosis at 37°C/48 h and standardized suspension in 4 log CFU/mL-1 endospores.

### Determination of the minimum sporicidal concentration (MSC)

The minimum sporicidal concentrations (MSC) of the major compound and the essential oils were determined using the broth microdilution technique according to NCCLS (National Committee for Clinical Laboratory Standards, 2018), with modifications. The base broth for *Clostridium* containing 0.5% Tween 80 was added with the essential oils and carvacrol at concentrations 30; 25; 20; 18; 15; 13; 10; 8; 5 and 3 µL mL<sup>-1</sup> and inoculated with *Clostridium* endospores. The tubes were incubated in anaerobiosis at 37°C/48 h and plated to determine the MSC based on visual growth. The test was performed in triplicate and the base broth was used as positive control for *Clostridium* added with tween 80, and the negative control was added with chloramphenicol.

### Determination of the minimum sporicidal concentration of essential oil and carvacrol combinations

Oregano and clove essential oils and carvacrol were combined in their MSC, as shown in Table 1. The biocidal effect of the essential oil combinations and the major compound was evaluated using the broth microdilution methodology (National Committee for Clinical Laboratory Standards, 2018). The MSCs of the essential oils and carvacrol combinations were considered positive when plate growth was not observed. The analyzes were performed in triplicate.

### Preparation of mortadellas

The mortadellas were prepared according to Dutra (2011) with modifications and added the combinations of essential oils, carvacrol and nitrite, according to the best results of the MSC (Table 2). After preparation, the mortadellas were stored at 4°C/ 48h for technological analysis. Microbiological analyses were performed with 25 g aliquots of each treatment, cooked in plastic bags and inoculated with 4 log CFUg<sup>-1</sup> of *C. botulinum* type D endospores, being vacuum-sealed packages. Samples were stored at 10°C, simulating improper storage and analyzed after 24 h and after 30 days.

**Table 1.** Concentrations of oregano and clove essential oils and major compound carvacrol used in different combinations.

Test	Oregano $\mu\text{LmL}^{-1}$	Clove $\mu\text{LmL}^{-1}$	Carvacrol $\mu\text{LmL}^{-1}$
1	12.5	0	0
2	0	25	0
3	0	0	5
4	6.3	12.5	0
5	6.3	0	2.5
6	0	12.5	2.5
7	8.4	4.25	0.85
8	2	16.8	0.85
9	2	4.25	3.35
10	4.12	8.25	1.65

Source: Authors.

**Table 2.** Ratios of different combinations of essential oils, carvacrol and sodium nitrite concentrations added to mortadellas, based on the better msc results.

Samples	Nitrite (ppm)	Oregano ( $\mu\text{LmL}^{-1}$ )	Clove ( $\mu\text{LmL}^{-1}$ )	Carvacrol ( $\mu\text{LmL}^{-1}$ )
ONT	0	0	0	0
TCO1	0	8.4	4.25	0.85
TCO2	0	4.12	8.25	1.65
CONT	150	0	0	0
TCO1	150	8.4	4.25	0.85
TCO2	150	4.12	8.25	1.65

CONT, control; TCO1, treatment with essential oil and carvacrol (test 7); TCO2, treatment with essential oil and carvacrol (test 10). Source: Source: Authors.

### Vegetative cell count of *Clostridium botulinum*

10 g samples of each treatment were homogenized in 90 ml peptone water for 2 min in stomacher (490 strokes/min), diluted at 0.1% sterile peptone water and plated (in depth with over layer) in base medium for insolation of *C. botulinum* (Pinelli et al., 2021). Plates were incubated at 37°C/48 h for colony counting. The analyses were performed in triplicate.

### Technological analyses of mortadella

The physicochemical analyzes were performed in the mortadella to evaluate the changes during the storage time, and if there was interference of the addition of the essential oils.

### Concentration of residual nitrite

The residual nitrite content, expressed as sodium nitrite (ppm), was determined according to the official method N<sup>o</sup>. 973.31 of the Association of Official Analytical Chemists (1998). A total of 5 g of each treatment was homogenized with 40 mL of distilled water and heated at 80°C. The samples were washed successively with hot water until reaching the approximate volume of 300 mL and incubated at 80°C/2 h, being stirred occasionally. The solution was then cooled and the volume filled

with distilled water and filtered. The filtrate containing sodium nitrite ( $\text{NaNO}_2$ ) was added with 2,5 mL of sulfanilamide solution in acetic acid at a 50 mL flask and homogenized. After 5 min, 2.5 mL of the N-(1-Naphthyl) ethylenediamine (NED) dichloride reagent was added and the volume filled with distilled water. The solution was stirred and kept at rest 15 min for color development, being measured the absorbance (D.O. 540 nm). The concentration of sodium nitrite was obtained by analytical curve.

### ***Lipid oxidation***

The lipid oxidation was determined by the ratio of thiobarbituric acid reactive substances (TBARs), according to the methodology proposed by Raharjo et al. (1992), with adaptations. A total of 10 g of the samples were homogenized in 40 mL of 5% trichloroacetic acid (TCA) added with 1 mL of 0.15% butylhydroxytoluene (BHT) and centrifuged at 3000 g for 2 min. The supernatant was collected and filtered to a 50 mL volumetric flask and the volume was filled with 5% TCA. 5 mL aliquot of the solution was added with 5 mL of 0.08 M thiobarbituric acid at 50% acetic acid, incubated at approximately 100°C/5 min for absorbance reading at 531 nm. The values were expressed in milligrams of malondialdehyde (MDA) per kilogram of sample (mg MDA/kg), through the analytical curve using 1,1,3,3-tetraethoxypropane (TEP).

### ***Objective color***

Mortadellas were sliced in the middle and the internal surface evaluated by a CM-700d colorimeter-spectrophotometer (Konica Minolta Sensing Inc., Osaka, Japan). The color indices were luminosity ( $L^*$ ), red ( $a^*$ ) and yellow ( $b^*$ ) obtained using the illuminant D65, 10° observer angle and considering the average value of five readings performed at different points on the surface (Dutra et al., 2011).

### ***pH index***

The samples were homogenized in distilled water and the pH value was measured with a combined electrode (Ag/AgCl reference system) coupled to a DM20 potentiometer (Digimed, São Paulo, SP, Brazil).

### ***Texture***

The samples were evaluated by the texture profile analysis (TPA) test, according to the procedure described by Ramos and Gomide (2017) for cured products, using a TA.XT2i-plus Texture Analyser (Stable Micro System Ltd., Surrey, England), connected to a computer equipped with the Texture Expert® software. Mortadellas were cut into 1.0 cm edge cubes and compressed twice up to 50% of their size with a compression plate of 7.5 cm diameter. There was no sample rest time between the two compression cycles. The deformation curve over time was obtained at a compression speed of 180 mm/min, from which six texture parameters were generated according to Ramos and Gomide (2017): hardness (N), cohesiveness, adhesiveness (N.mm), flexibility (mm) and chewing (mm).

### ***Statistical analyses***

Data from the analyses of mortadellas prepared with the best MSC results were compared through the Statistical Analysis System (SAS), using a completely randomized design (CRD) in a split-plot: a plot in factorial design with two levels of nitrite x three levels of oil and two-stage subplot. Analysis of variance (ANOVA), Tukey test and F test were performed.

### 3. Results

#### Minor sporicidal concentration (MSC) and sporicidal effect of essential oil and carvacrol combinations

The MSC of oregano and clove essential oils was, respectively, 13 and 25  $\mu\text{L mL}^{-1}$ , and 5  $\mu\text{L mL}^{-1}$  for carvacrol. Regarding the combinations, all showed sporicidal action against *Clostridium botulinum*.

#### Preservative action against *C. botulinum* in mortadella

Once all tested combinations of essential oils and carvacrol were effective against *C. botulinum* endospores, the tests 7 and 10 were selected as preservatives added to mortadella. It was aimed not change the sensory characteristics of the product, since both oils are used as condiments.

The evaluation of treatments (nitrite levels and combination of essential oils) and storage time showed significant interaction ( $p < 0.05$ ). Except for the control, without addition of nitrite and essential oil, the other treatments involved a reduction in cell counts after 30 days of refrigerated storage (Table 3).

**Table 3.** Average ( $\pm$ standard deviation) of the effects of nitrite levels and the addition of essential oil blends in the count of vegetative cells of *Clostridium botulinum* ( $\text{Log CFUg}^{-1}$ ) with the storage time at  $10^\circ\text{C}$ .

NO2 (ppm)	Time (days)	CONT	TCO1	TCO2
0	0	3.69 $\pm$ 0.09 <sup>a</sup>	3.38 $\pm$ 0.22 <sup>a</sup>	3.43 $\pm$ 0.03 <sup>a</sup>
	30	4.92 $\pm$ 0.04 <sup>b</sup>	2.68 $\pm$ 0.23 <sup>b</sup>	2.61 $\pm$ 0.38 <sup>b</sup>
	$\Delta$	+1.23	-0.70	-0.81
150	0	3.22 $\pm$ 0.27 <sup>a</sup>	3.44 $\pm$ 0.16 <sup>a</sup>	3.18 $\pm$ 0.03 <sup>a</sup>
	30	2.62 $\pm$ 0.24 <sup>b</sup>	2.75 $\pm$ 0.04 <sup>b</sup>	2.76 $\pm$ 0.07 <sup>b</sup>
	$\Delta$	-0.60	-0.69	-0.42

CONT = control treatment without addition of oil;

TCO1 = treatment with addition of oil in combination 1;

TCO2 = treatment with addition of oil in combination 2.

$\Delta$  = Variation between the two times,

+ = increase in the count of vegetative cells,

- = decrease in the count of vegetative cells.

Averages followed by different letters in the column and within each nitrite level differed among themselves ( $p < 0.05$ ) by the F test.

Source: Authors.

Treatments, nitrite levels, essential oils and storage time did not show statistically significant differences for red (a\*) and yellow (b\*) indices. However, an isolated significant effect ( $p < 0.05$ ) for nitrite levels was verified by increasing the a\* values and reducing the b\* values. The value of parameter a \* was  $4.40 \pm 2.00$  for treatment without nitrite and  $10.14 \pm 2.7$  for the sample containing 150 ppm nitrite. The mean value b \* was  $11.98 \pm 1.80$  and  $10.01 \pm 1.48$  for 0 and 150 ppm of nitrite, respectively.

#### TBARS index

The highest results were found for the samples without addition of nitrite (0.51 mg MDA  $\text{kg}^{-1}$ ), whereas the mortadellas added with 150 ppm of nitrite showed a TBAR index of 0.36 mg MDA  $\text{kg}^{-1}$ .

#### Residual nitrite

Nitrite concentrations and storage time showed significant interaction in relation to the residual nitrite content found

in mortadella. Initially, mortadella formulated without sodium nitrite presented residual nitrite of 4.53 ppm, and at the end of storage (30 days), 3.35 ppm, with no significant change. In the sample containing 150 ppm nitrite, initially the value found was 32.51 ppm, and 19.76 ppm at the end of 30 days, with significant reduction of residual nitrite.

## pH

The CONT treatment presented pH from 6.28 to 6.54, while TCO1 presented pH between 6.28 and 6.50, and TCO2 showed pH from 6.28 to 6.73. There was a slight increase in pH during storage and this increase was slightly higher in samples without nitrite added. However, the pH variation was not significant.

## Texture

The evaluated texture parameters did not show a significant interaction between nitrite levels and the addition of essential oils. However, nitrite-containing samples showed values higher than the samples without this additive, demonstrating an isolated significant effect ( $p < 0.05$ ) (Table 4).

**Table 4.** Average ( $\pm$ standard deviation) of the effect of nitrite addition on the texture parameters of mortadellas.

NO <sub>2</sub>	HARD (N)	COHES	ADHES (N.mm)	FLEX (mm)	CHEW (N.mm)
0	18.68 $\pm$ 1.68 <sup>a</sup>	0.74 $\pm$ 0.01 <sup>a</sup>	8.64 $\pm$ 2.54 <sup>a</sup>	4.75 $\pm$ 0.16 <sup>a</sup>	67.93 $\pm$ 5.10 <sup>a</sup>
150	25.59 $\pm$ 3.08 <sup>b</sup>	0.76 $\pm$ 0.01 <sup>b</sup>	12.83 $\pm$ 3.05 <sup>b</sup>	5.51 $\pm$ 0.11 <sup>b</sup>	105.03 $\pm$ 11.49 <sup>b</sup>
Treatment	HARD (N)	COHES	ADHES (N.mm)	FLEX (mm)	CHEW (N.mm)
CONT	20.66 $\pm$ 2.05	0.74 $\pm$ 0.005	9.22 $\pm$ 1.78	4.78 $\pm$ 0.08 <sup>a</sup>	75.90 $\pm$ 8.01 <sup>a</sup>
TCO1	22.10 $\pm$ 1.63	0.75 $\pm$ 0.08	10.24 $\pm$ 3.26	5.12 $\pm$ 0.19 <sup>b</sup>	85.96 $\pm$ 4.45 <sup>ab</sup>
TCO2	23.65 $\pm$ 3.45	0.75 $\pm$ 0.007	12.75 $\pm$ 3.34	5.50 $\pm$ 0.14 <sup>c</sup>	97.57 $\pm$ 12.41 <sup>b</sup>

HARD = hardness; COHES = cohesiveness; ADHES = adhesiveness; FLEX = flexibility; CHEW = chewing; CONT = control treatment without addition of oil; TCO1 = treatment with addition of oil in combination 1; and TCO2 = treatment with addition of oil in combination 2. Averages followed by different letters in the column differ among themselves ( $p < 0.05$ ) by the Tukey. Source: Authors.

## 4. Discussion

Essential oils are a complex mixture of numerous molecules and their biological effect may be resulting from synergism with all molecules or only of the major compound, which is present in a greater amount (Bakkali et al., 2008). It is known that the chemical composition of the essential oils of a particular species of plant may vary according to the geographical origin and the period of harvest (Bassanetti et al., 2017). Many essential oils and their components have shown antimicrobial efficacy against pathogenic and deteriorating food microorganisms (Arsi et al., 2014; Pinheiro et al., 2015; Kim and Rhee, 2016). However, research have demonstrated that essential oils that have the major components carvacrol, thymol and eugenol are associated with greater antimicrobial activity than oils without these compounds (Ouattara et al., 1997).

The results found in the present study are in agreement with this statement, since all evaluated concentrations of the essential oils of oregano and clove, besides the major compound carvacrol, were efficient in inhibiting *C. botulinum* endospores. Similar results were also found by Marino, Bersani and Comi (2001) and Hoffmann et al. (1999) when evaluated the antimicrobial action of oregano and clove essential oils, respectively, under Gram-positive and Gram-negative bacteria. Asensio et al. (2014) demonstrated that gram-positive bacteria were strongly inhibited by oregano essential oil showing more

sensitivity than the Gram-negative bacteria evaluated. It was observed in a study by Bassanetti et al. (2017) that the minimum inhibitory concentrations and minimum bacterial concentrations of the tested oils against *Clostridium perfringens* were the same or lower than those observed against the gram-negative bacteria tested.

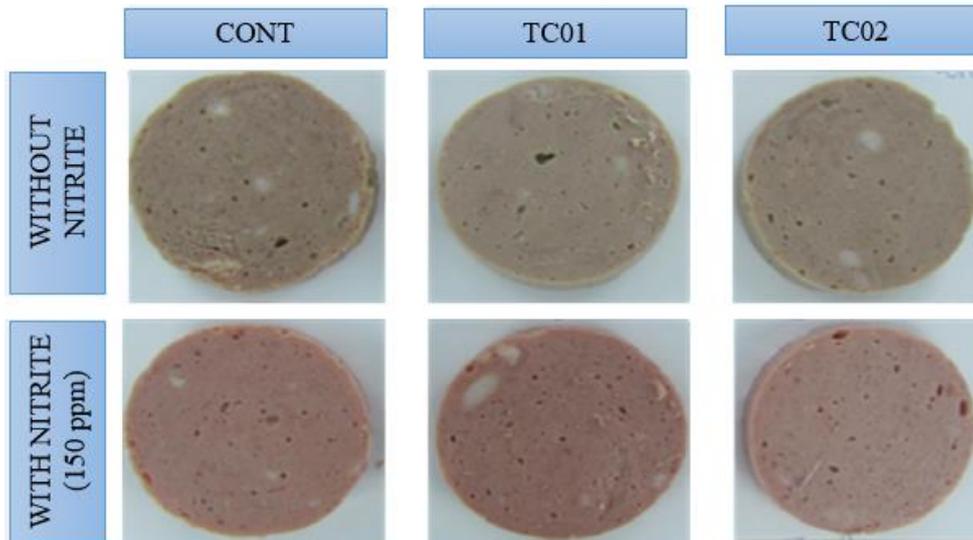
The synergistic effect of essential oils and major compounds is a source of research and has showed significant results, such as the present study. Different terpenoid components of essential oils may interact to amplify the antimicrobial effect (Delaquis et al., 2002). The interaction between EO chemical compounds can produce different effects, among them synergism, observed when the effect of the combined substances is greater than the sum of the individual effects while the absence of interaction is defined as indifference (Burt, 2004). Most studies in this regard deal with the mode of action of essential oil components with other preservatives or antibiotics (Ultee et al., 2000; Misaghi & Basti, 2007; Dimitrijevic et al., 2007). Few studies report the action between the essential oil components, however, there are some accepted mechanisms. Sequential inhibition of the biochemical pathway, inhibition of enzymes, and membrane porosity, which may increase uptake of other antimicrobials (Santiesteban-Lopez, 2007), are usually indicated as a mode of action. In Gram-negative bacteria Pei et al. (2009) suggested that the synergistic effects of eugenol/carvacrol and eugenol/thymol could be due to carvacrol and thymol disintegrating the outer mebrane of the cell, facilitating eugenol entry into the cytoplasm and causing enzyme inactivation of metabolism. The practical implications observed in this paper, which show that there was synergism between essential oils of clove and oregano and carvacrol are important when using essential oils in food matrices, as it shows that the use of the lowest concentration can produce antibacterial activity and this in a food entails a reduction in sensory impact.

In the absence of nitrite, the combination of oils showed satisfactory antimicrobial effect, similar to the conventional treatment in which the nitrite is added at a maximum concentration of 200 ppm. Synergism among oils and nitrite was not observed, although the lowest cell count after 30 days of refrigerated storage was observed in treatments with 150 ppm of nitrite and the TCO<sub>2</sub> combination. Dias et al. (2015) studied *C. perfringens* in mortadellas prepared with 75 ppm of nitrite in combination with essential oils of oregano (2.5%), cloves (0.6%) and thyme (0, 3%) and obtained a population reduction of the microorganism, demonstrating, similarly with the present study, the efficiency of essential oil combinations. Ghabraie et al. (2016) when studying the effect of combinations of cinnamon essential oil (0.025 and 0.05%), nisin (12.5 and 25 ppm), nitrite (100 and 200 ppm) and a mixture of potassium lactate and sodium acetate (1.55 and 3.1%) against *Clostridium sporogenes* endospores in the formulation of a meat product found that nitrite was the most important component in the control of *C. sporogenes*. However, there was a contribution of other components in the antibacterial activity, such as nisin, essential oils and salts of organic acids. Therefore, to ensure food safety antibacterial agents should be used together and under optimized conditions or of lowest possible concentration.

In this study, similarly as in Oliveira et al. (2012), higher values of a\* for mortadellas added with nitrite were found, indicating a more intense red hue. There was no significant interaction among the treatments (nitrite levels and combination of essential oils) and storage time for the objective color parameter. High values of the a\* index, referring to the red color of the product, are expected in meat products added with nitrite, since this provides the color characteristic of cured product (Ramos & Gomide, 2017). Similarly, Dutra (2011) evaluated color formation in mortadellas prepared with different nitrite levels (0.75 and 150 ppm) and subjected to irradiation, being found the lowest a\* values in the samples without nitrite addition. Blanco-Lizararo et al. (2017), when evaluating the effect of sodium nitrite (200 ppm), sodium lactate (1.5%) and thyme essential oil (100 ppm) added separately in the sausage formulation, found that the values of a \* were larger in the treatment containing sodium nitrite, from the second day to the 37th day of storage of the meat product. Oliveira et al. (2012) also reported a slight increase in L\* after 20 days of storage for mortadellas prepared with 31.25 µl of Sartureja montana essential oil and 200 ppm of nitrite. The same was observed by Dias et al., (2015) in mortadellas added with 150 ppm of nitrite after 20 days of refrigerated storage. According to Alahakoon et al. (2015), to preserve color generation during storage, it is necessary to

maintain the residual nitrite concentration between 10ppm and 15ppm. The addition of nitrite to mortadella, on other hand, slightly reduced the  $b^*$  values in the samples, parameter referring to the yellow coloration of the product. The addition of essential oil combinations in the mortadella generally did not affect the final product color, being the nitrite effect significant in the color change (Figure 1).

**Figure 1.** Mortadellas after 24 h of preparation. CONT = control without addition of oil; TCO1 = treatment with addition of oil in combination 1; and TCO2 = treatment with addition of oil in combination 2.



Source: Authors.

Regarding the TBARS index, the lowest values of lipid oxidation in mortadellas were found at higher concentrations of nitrite, as well as verified by Pinelli et al. (2021) and Dutra (2016). Oliveira et al. (2012), when evaluating the lipid oxidation of mortadellas added with essential oil of *Satureja montana* L. and sodium nitrite verified that in the treatments without addition of essential oil, lipid oxidation values were lower in all storage times (1, 10, 20 and 30 days) in samples with nitrite compared to the sample without nitrite. In the samples with essential oil and nitrite, they observed possible benefits of the combined use of essential oils and minimal amounts of sodium nitrite in cured meat products due to the reduced values of lipid oxidation in mortadella formulated with the lowest concentrations of essential oil without addition of nitrite and in samples with essential oil and reduced amounts of sodium nitrite. Although essential oils act as antioxidants, nitrite is still the most effective way to prevent or delay spoilage by lipid oxidation. Some compounds act as antioxidants whether added at low concentrations, such as eugenol. Otherwise, they can act as pro-oxidants whether present at high concentrations (Decker, 1997). Another fact refers to the composition of essential oils, which together with carvacrol are phenolic compounds and, when reacting with the peroxy radical, they form the phenoxyl radical that, although stable, can be affected by ultraviolet light and high temperatures, giving rise to new radicals, which can compromise the antioxidant efficiency (Angelo and Jorge, 2007).

The amount of residual nitrite in meat products is generally lower than that added one, since it is consumed throughout the storage time due to the ability to react with several mortadella compounds, such as myoglobin (Cassens et al., 1997). The residual nitrite values found in this study corroborate with those observed by Al-Shuibi and Al-Abdullah (2002) in mortadellas prepared with 120 ppm of nitrite. Under this condition, the residual nitrite concentration was 32 ppm, similar to the results obtained by Dutra (2011).

Nitrite is considered as one of the most relevant factors to inhibit the growth of *C. botulinum* in meat products, since it

is a mandatory anaerobic bacterium capable of being developed in vacuum packed products (Cassens, 1997). Residual nitrite, derived from the nitrite added in the mortadella formulation ensures the microbiological quality of the product preventing the development of microorganism. If concentrations below 150 ppm are added to the product the residual nitrite will be less than 10 ppm, so the risk of microorganisms growth cannot be ruled out. However, the use of different food preservation systems, such as thermal treatment, pH, refrigeration, water activity and the addition of natural antimicrobial agents may reduce or replace the use of nitrite in these foods and result in a safe, stable and loss-free sensory quality product. In this context, the combined use of oregano and clove essential oils, and the major compound carvacrol represent a safe alternative to the reduction of nitrite in this type of food, since they proved antimicrobial efficiency against *C. botulinum* endospores and the residual nitrite concentration agrees with the recommended safety parameter (19.76 ppm). Although the absence of reports in the literature on the action of essential oils or their compounds on *C. botulinum*, the results obtained in the present study corroborate those found in the literature for other microorganisms (Zhang et al., 2019; Šojić et al., 2019; Sharma et al., 2020; Tomović et al., 2020; Ajourloo et al., 2021; Pinelli et al., 2021).

Although, in this study, the pH variation was not significant, your determination in food provides the evaluation of possible predominant microbiota, and potential and probable nature of spoilage it may suffer and how it food should be processed (Toldrá, 2014). In general, the pH remained between 6.0 and 7.0, and are considered normal and within acceptable levels for good preservation of processed foods (Cheng et al., 2007). The values found do not indicate deterioration process by microorganisms. The variation found between mortadella treatments can be attributed to slight variations in ingredient quantities (antioxidant, acidulant and stabilizers) as well as different amounts of essential oils and carvacrol.

Texture is the result of the deformation of a food when bitten, pressed and cut. This change provides knowledge about hardness, adhesiveness, elasticity, cohesiveness, gomesity, chewability among others (Teixeira, 2009). Hardness is directly related to the maximum force applied in the first cycle of sample compression. Adhesiveness is a negative force resulting from work done to overcome the attraction between the food and the probe. Flexibility is the ability of the material to return to its original shape when subjected to deformity. Cohesiveness is the ratio of work done in the second cycle to work done in the first cycle. Chewiness is the work required to chew a sample (Chen & Opara, 2013).

The application of the essential oils implied in the increase of flexibility and chewing of mortadellas as observed at Dong et al. (2007) for increase in hardness and adhesiveness parameters in mortadellas added with nitrite at concentrations 0, 75 and 150 ppm. Similarly, found at Viuda-Martos et al. (2010) in the experiment with mortadella added with dietary fiber of orange and essential oil of rosemary.

## 5. Conclusion

The MSC of oregano and clove essential oils was, respectively, 13 and 25  $\mu\text{L mL}^{-1}$ , and 5  $\mu\text{L mL}^{-1}$  for carvacrol. There was synergism between the essential oils and carvacrol, reducing the concentration required for antimicrobial action. The population of *C. botulinum* in the mortadella was reduced with the addition of combinations of oils and the major compound both in the presence and absence of the nitrite. However, the antimicrobial effect was supported by nitrite in the absence of combinations. Therefore, the objective of microbiological analysis was achieved, the study showed the potential antimicrobial action of natural compounds when added to meat product. Regarding the physicochemical analysis, it was observed that nitrite has unquestionable technological action, however, the addition of essential oils did not cause significant changes. Clearly, further studies should be conducted in this regard also involving sensory analysis.

The results of this research are promising and suggest the use of clove and oregano essential oils together with the major compound (carvacrol) in meat sausages as a natural preservative and action against foodborne pathogens.

Furthermore, future research will be focus to investigate the antimicrobial potential of essential oils combination and major compound to films elaboration. Thereby mitigating sensory effects in the product while to act as natural preservative.

## Acknowledgments

The team thanks the Federal University of Lavras (UFLA), the National Council for Scientific and Technological Development (CNPq), the Foundation for Research of the State of Minas Gerais (FAPEMIG), and the Coordination of Improvement of Higher Education Personnel (CAPES).

## References

- Abdollahi, M., Rezaei, M. & Farzi, G. (2014). Influence of chitosan/ clay functional bionanocomposite activated with rosemary essential oil on the shelf life of fresh silver carp. *International Journal of Food Science and Technology*, 49 (3), 811–818. 10.1111/ijfs.12369
- Ajourloo, M., Khanjari, A., Misaghi, A., Basti, A. A., Kamkar, A., Yadegar, F., Gholami, F., Khansavar, F. & Fallah, f. (2021). Combined effects of Ziziphora clinopodioides essential oil and lysozyme to extend shelf life and control Listeria monocytogenes in Balkan-style fresh sausage. *Food Science and Nutrition*. 9 (3), 1665–1675. 10.1002/fsn3.2141
- Alahakoon, A. U., Jayasena, D. D., Ramachandra, S. & Jo, C. (2015). Alternatives to nitrite in processed meat: Up to date. *Trends in Food Science and Technology*, 45 (1), 37–49. 10.1016/j.tifs.2015.05.008
- Al-shuibi, A. M. & Al-abdullah, B. M. (2002). Substitution of nitrite by sorbate and the effect on properties of mortadella. *Meat Science*, 62 (4), 473-478. 10.1016/S0309-1740(02)00041-4
- Angelo, P. M. & Jorge, N. (2007). Phenolic compounds in food: a brief review. *Revista Instituto Adolfo Lutz* 66 (1), 232-240. <https://pesquisa.bvsalud.org/portal/resource/pt/vti-453047>
- Arsi, K., Donoghue, A. M., Venkitanarayanan, K., Kollanoor-johny, A., Fanatico, A. C., Blore, P. J & Donoghue, D. J. (2014). The efficacy of the natural plant extracts, thymol and carvacrol against Campylobacter colonization in broiler chickerns. *Journal of Food Safety*, 34 (4), 321-325. 10.1111/jfs.12129
- Asensio, C. M., Gallucci, N., Oliva, M. M., Demo, M. S. & Grosso, N.R. (2014). Sensory and bio-chemical preservation of ricotta cheese using natural products. *International Journal of Food Science and Technoogy*, 49 (12), 2692–2702. 10.1111/ijfs.12604
- AOAC. (1998). Official Methods of Analysis. Maryland, USA: Association of Official Analytical Chemists.
- Bakkali, F., Averbecka, S., Averbecka, D & Idaomarb, M. (2008). Biological effects of essential oils. *Food Chemistrt and Toxicological* 46 (2), 446–475. 10.1016/j.fct.2007.09.106
- Bassanetti, I., Carcelli, M., Buschini, A., Montalbano, S., Leonardi, G., Pelagatti, P., Tosi, G., Massi, P., Fiorentini, L. & Rogolino, D. (2017). Investigation of antibacterial activity of new classes of essential oils derivatives. *Food Control* 73, 606-612. 10.1016/j.foodcont.2016.09.010
- Blanco-lizarazo, C. M., Betancourt-cortés, R., Lombana, A., Carrillo-Castro, k. & Sotelo-díaz, I. (2017). Listeria monocytogenes behaviour and quality attributes during sausage storage affected by sodium nitrite, sodium lactate and thyme essential oil. *Food Science and Technology International*, 23 (3), 277–288. 10.1177/1082013216686464
- Burt, S. (2004). Essential oils: Their antimicrobial properties and potential applications in foods: A review. *International Journal of Food Microbiology*, 94 (3), 223–253. 10.1016/j.ijfoodmicro.2004.03.022
- Cassens, R. G. (1997). Composition and safety of cured meats in the USA. *Food Chemistry*, 59 (4), 561-566. 10.1016/S0308-8146(97)00007-1
- Chaibi, A., Ababoucha, I. H., Belasri, K., Boucetta, L. & Busta, F. F. (1997). Inhibition of germination and vegetative growth of *Bacillus cereus* T and *Clostridium botulinum* 62 A spores by essential oils. *Food Microbiology*, 14 (2), 161-174. 10.1006/fmic.1996.0075
- Chen, L. & Opara, U.L. (2013). Approaches to analysis and modeling texture in fresh and processed foods – A review. *Journal of food Engineering*, 119 (3), 497–507. 10.1016/j.jfoodeng.2013.06.028
- Cheng, J-H, Wang, S-T. & Wockerman, H. (2007). Lipid oxidation and color change of salted pork patties. *Meat Science*, 75 (1), 71-77. 10.1016/j.meatsci.2006.06.017
- Decker, E. A. (1997). Phenolics: prooxidants or antioxidants? *Nutrition Reviews*, 55 (11) 396-407. 10.1111/j.1753-4887.1997.tb01580.x
- Delaquis, P. J., Stanich, K., Girard, B & Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology*, 74 (1-2), 101–109. 10.1016/s0168-1605(01)00734-6
- Dias, N. A., Rodrigues, I. T. S., Palhares, P. C., Ramos, E. M. & Piccoli, R. H. (2015). Antimicrobial activity of essential oils on *Clostridium perfringens* type A inoculated in mortadella. *Journal of Food Safety*, 35(4), 466–472. 10.1111/jfs.12196

- Dimitrijević, S. I., Mihajlovski, K. R., Antonović, D. G., Milanović-Stevanović, M. R. & Mijin, D. Z. (2007). A study of the synergistic antilisterial effects of a sub-lethal dose of lactic acid and essential oils from *Thymus vulgaris* L., *Rosmarinus officinalis* L. and *Origanum vulgare* L. *Food Chemistry*, 104(2), 774–782. 10.1016/j.foodchem.2006.12.028
- Dong, Q-L., Tu, K., Guo, L-Y., Yang, J-L., Wang, H. & Chen, Y-Y. (2007). The effect of sodium nitrite on the textural properties of cooked sausage during cold storage. *Journal of Texture Studies*, 38 (5), 537–554. 10.1111/j.1745-4603.2007.00112.x
- Dutra, M. P., Ramos, E. M., Ramos, A. L. S., Fontes, P. R., Cardoso, G. P. & Leal, A. S. (2011). Gamma radiation and storage time on lipidic oxidation, color, heme pigments and residual nitrite of bologna-type sausages formulated with different nitrite additions. *Ciência Rural* 41(12), 2203-2209. 10.1590/S0103-84782011005000143
- Dutra, M. P., Aleixo, G. C., Ramos, A. L. S., Silva, M. H. L., Pereira, M. T., Piccoli, R. H & Ramos, E.M. (2016). Use of gamma radiation on control of *Clostridium botulinum* in mortadella formulated with different nitrite levels. *Radiation Physics and Chemistry*, 119, 125-129. 10.1016/j.radphyschem.2015.10.008
- Fernandes, R. V. B., Guimarães, I. C., Ferreira, C. L. R., Botrel, D. A., Borges, S. V. & Souza, A. U. (2015). Microencapsulated rosemary (*rosmarinusofficinalis*) essential oil as a biopreservative in minas frescal cheese. *Journal of Food Processing and Preservation*, 41 (1), 1-9. 10.1111/jfpp.12759
- Ghabraie, M., Vu, K. D., Tnani, S. & Lacroix, M. (2016). Antibacterial effects of 16 formulations and irradiation against *Clostridium sporogenes* in a sausage model. *Food Control*, 63, 21-27. 10.1016/j.foodcont.2015.11.019
- Hoffmann, F. L., Souza, S. J. F., Gracia-Cruz, C. H., Vinturim, T. M. & Dutra, A. L. (1999). Determinação da atividade antimicrobiana *in vitro* de quatro óleos essenciais de condimentos e especiarias. *Boletim do Centro de Pesquisa de Processamento de Alimentos*, 17 (1), 11-20. 10.5380/cep.v17i1.13794
- Hughes, E., Cofrades, S. & Troy, D. J. (1997). Effects of fat level, oat fibre and carrageenan on frankfurters formulated with 5, 12 and 30% Fat. *Meat Science*, 45, 273-281. 10.1016/S0309-1740(96)00109-X
- Juneja, V. K. & Friedman, M. (2007). Carvacrol, Cinnamaldehyde, Oregano Oil, and Thymol Inhibit *Clostridium perfringens* Spore Germination and Outgrowth in Ground Turkey during Chilling. *Journal of Food Protection*, 70 (1), 218-222. 10.4315/0362-028X-70.1.218
- Kim, S. A. & Rhee, M. S. (2016). Highly enhanced bactericidal effects of medium chain fatty acids (caprylic, capric and lauric acid) combined with edible plant essential thymol and vanillin against *Escherichia coli*. *Food Control*, 60, 447-454. 10.1016/j.foodcont.2015.08.022.
- Marino, M., Bersani, C. & Comi, G. (2001). Impedance measurements to study the antimicrobial activity of essential oils from *Lamiaceae* and *Compositae*. *International Journal of Food Microbiology*, 67, 187-195. 10.1016/S0168-1605(01)00447-0
- Misaghi, A. & Basti, A. A. (2007). Effects of *Zataria multiflora* Boiss. essential oil and nisin on *Bacillus cereus* ATCC 11778. *Food Control* 18 (9), 1043–1049. 10.1016/j.foodcont.2006.06.010
- Murray, P. R. (1999). *Manual of Clinical Microbiology*. Washington, USA: American Society for Microbiology.
- Oliveira, J. M., Araújo, W. M. C. & Borgo, L. A. (2005). Quantification of Nitrate and Nitrite in Fresh Sausage. *Journal of Food Science and Technology*, 25 (4), 736-742. 10.1590/S0101-20612005000400018
- Oliveira, T. L. C. (2012). Antioxidant effects of *Satureja montana* L. essential oil on TBARS and color of mortadella-type sausages formulated with different levels of sodium nitrite. *LWT Food Science and Technology*, 45(2), 204-212. 10.1016/j.lwt.2011.09.006
- Ouattara, B., Simard, R. E., Holley, R. A., Piette, G. J-P. & Bégin, A. (1997). Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. *International Journal of Food Microbiology*, 37 (2-3), 155-162. 10.1016/S0168-1605(97)00070-6
- Pei, R., Zhou, F., Ji, B. & Xu, J. (2009). Evaluation of Combined Antibacterial Effects of Eugenol, Cinnamaldehyde, Thymol, and Carvacrol against *E. coli* with an Improved Method. *Journal of Food Science*, 74 (7), 379–383. 10.1111/j.1750-3841.2009.01287.x
- Pinelli, J. J., Martins, H. H. A., Guimarães, A. S., Isidoro, S. R., Gonçalves, M. C., Moraes, T. S. J., Ramos, E. M. & Piccoli, R. R. (2021). Essential oil nanoemulsions for the control of *Clostridium sporogenes* in cooked meat product: An alternative? *LWT Journal of Food Science and Technology*, 143, 1-10. 10.1016/j.lwt.2021.111123
- Pinheiro, P. F., Costa, A. V., Alves, T. A., Galter, I. N., Pinheiro, C. A., Pereira, A. F., Oliveira, C. M. R. & Fontes, M. M. P. (2015). Phytotoxicity and cytotoxicity of essential oil from leaves of *Plectranthus amboinicus*, carvacrol, and thymol in plant bioassays. *J. Agric. Food Chem.* 63 (41), 8981-8990. 10.1021/acs.jafc.5b03049
- Peck, M. V., Plowman, J., Aldus, C. F., Wyatt, G. M., Izurieta, W. P., Stringer, S. C. & Barker, G. C. (2010). Development and application of a new method for specific and sensitive enumeration of spores of nonproteolytic *Clostridium botulinum* types B, E and F in foods and food materials. *Applied Environmental Microbiology*, 76(19), 6607-6614. 10.1128/AEM.01007-10
- Raharjo, S., Sofos, J. N. & Schmidt, G. R. (1992). Improved speed, specificity, and limit of determination of an aqueous acid extraction thiobarbituric acid-C18 method for measuring lipid peroxidation in beef. *Journal of Agricultural and Food Chemistry*, 40, 2182-2185. 10.1021/jf00023a027
- Ramos, E. M. & Gomide, L. A. M. (2017). *Avaliação da Qualidade de Carnes: Fundamentos e Metodologias*. Viçosa, BR: UFV.
- Santesteban-López, A., Palou, E. & López-Malo, A. (2007). Susceptibility of food-borne bacteria to binary combinations of antimicrobials at selected aw and pH. *Journal of Applied Microbiology*, 102 (2), 486–497. 10.1111/j.1365-2672.2006.03092.x
- Sharma, H., Mendiratta, S. K., Agarwal, R. K. & Gurunathan, K. (2020). Bio-preservative effect of blends of essential oils: natural antioxidant and antimicrobial agents for the shelf life enhancement of emulsion based chicken sausages. *Journal of Food Science and Technology* 57, 3040–3050. 10.1007/s13197-020-04337-1

- Sharma, S. K., Ferreira, J. L., Eblen, B. S. & Whiting, R. C. (2006). Detection of type A, B, E, and F *Clostridium botulinum* neurotoxins in foods by using an amplified enzyme-linked immunosorbent assay with digoxigenin-labeled antibodies. *Applied Environmental Microbiology*, 72 (2), 1231-1238. 10.1128/AEM.72.2.1231-1238.2006
- Šojića, B., Pavlića, B., Ikonić, P., Tomovića, V., Ikonića, B., Zekovića, Z., Kocić-Tanackova, K., Jakanovića, M., Skaljaca, S. & Ivić, M. (2019). Coriander essential oil as natural food additive improves quality and safety of cooked pork sausages with different nitrite levels. *Meat Science*, 157, 1-8. 10.1016/j.meatsci.2019.107879
- Teixeira, L. V. (2009). Sensory Analysis in the Food Industry. *Journal of Candido Tostes Dairy Institute*, 64 (366), 12-21. <https://www.revistadoilct.com.br/rilct/article/view/70/76>
- Terra, N. N. (2009). Use of liquid whey in the preparation of mortadella. *Ciência Rural* 39, 885-890. 10.1590/S0103-84782009000300038
- Tomovic, V., Sojic, B., Savanovic, J., Kocic-Tanackov, S., Pavlic, B., Jakanovic, M., Oordevic, V., Parunovic, N., Martinovic, A. & Vujadinovic, D. (2020). New Formulation towards Healthier Meat Products: *Juniperus communis* L. Essential Oil as Alternative for Sodium Nitrite in Dry Fermented Sausages. *Foods* 9 (8), 1-17. 10.3390/foods9081066
- Toldrá, F. (2014). *Handbook of Fermented Meat and Poultry*. United States, USA: John Wiley & Sons Inc.
- Ultee, A., Slump, R. A., Steging, G. & Smid, E. J. (2000). Antimicrobial Activity of Carvacrol toward *Bacillus cereus* on Rice. *Journal of Food Protection*, 63 (5), 620–624. 10.4315/0362-028x-63.5.620
- Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J. & Pérez-Álvarez, J. A. (2010). Effect of orange dietary fibre, oregano essential oil and packaging conditions on shelf-life of bologna sausages. *Food Control*, 21 (4), 436-443. 10.1016/j.foodcont.2009.07.004
- Zhang, X., Wang, H., Li, X., Sun, Y., Pan, D., Wang, Y & Cao, J. (2019). Effect of cinnamon essential oil on the microbiological and physicochemical characters of fresh Italian style sausage during storage. *Animal Science Journal*, 90 (3), 435–444. 10.1111/asj.13171