Research, Society and Development, v. 11, n. 9, e22111931106, 2022
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i9.31106

Viability of *Clostridium difficile* in mortadella with added essential oils and reduced sodium nitrite content

Viabilidade de *Clostridium difficile* em mortadella adicionada de óleos essenciais e conteúdo reduzido de nitrito de sódio

Viabilidad de *Clostridium difficile* en mortadela acrescida de aceites esenciales y reducido contenido de nitrito de sodio

Received: 05/29/2022 | Reviewed: 06/07/2022 | Accept: 06/28/2022 | Published: 07/07/2022

Nayane Aparecida Araújo Dias
ORCID: https://orcid.org/0000-0003-2259-5570
Universidade Federal de Lavras, Brazil
E-mail: nayaneadias@hotmail.com
Juliana Junqueira Pinelli
ORCID: https://orcid.org/0000-0003-0332-9680
Universidade Federal de Lavras, Brazil
E-mail: jujjpinelli@gmail.com
Monique Suela Silva
ORCID: https://orcid.org/0000-0002-6809-714X
Universidade Federal de Lavras, Brazil
E-mail: monique.silva3@estudante.ufla.br
Silas Rodrigo Isidoro
ORCID: https://orcid.org/0000-0002-7285-3673
Universidade Federal de Lavras, Brazil
E-mail:silasrodrigoisidoro@hotmail.com
Heloísa Helena de Abreu Martins
ORCID: https://orcid.org/0000-0002-8071-6644
Instituto Federal de Educação, Ciência e Tecnologia, Brazil
E-mail: heloisa_amartins@hotmail.com
Michelle Carlota Gonçalves
ORCID: https://orcid.org/0000-0002-9567-6905
Universidade Federal de Lavras, Brazil
E-mail: mychellinhagoncalves@hotmail.com
Eduardo Mendes Ramos
ORCID: https://orcid.org/0000-0002-8240-8151
Universidade Federal de Lavras, Brazil
E-mail: emramos@ufla.b
Roberta Hilsdorf Piccoli
ORCID: https://orcid.org/0000-0003-2334-9400
Universidade Federal de Lavras, Brazil
E-mail: rhpiccoli@ufla.br

Abstract
The effects of additioning essential oil mixtures and reducing the sodium nitrite content (75 ppm) on *Clostridium difficile* inoculated in mortadellas and on the product's technological characteristics were evaluated. 15 essential oils were tested preliminarily, but only four were selected. The minimum bactericidal concentrations found were 1.2 % for *Ocimum basilicum*, 0.3 % for *Origanum vulgare* and for *Thymus vulgaris* and 0.15 % for *Litsea cubeba*. There wasn`t significant difference in viable cell growth between treatments and the control; however, there was an increase in viable cells of approximately 2.5 $\log_{10}$ MPN/g after the fifth day. Combinations of oils didn`t prevent sporulation, however a synergistic effect was observed in vitro. There was a reduction in pH during storage and a greater amount of residual nitrite for the F1 treatment (*Origanum vulgare* (0.2 %); *Thymus vulgaris* (0.05 %) and *Litsea cubeba* (0.025 %), with 10.23 ppm, and reduction of residual nitrite during the storage period from 18.75 ppm on the first day of storage to 5.11 ppm on the last. No significant changes were observed in the technological characteristics of the product with the analyzed treatments.
Keywords: *Clostridium difficile*; Essential oils; Food safety; Meat product; Natural additives.

Resumo

Foram avaliados os efeitos da adição de misturas de óleos essenciais e a redução do teor de nitrito de sódio (75 ppm) em mortadelas inoculadas com *Clostridium difficile*, bem como as características tecnológicas do produto. 15 óleos essenciais foram testados anteriormente, mas apenas quatro foram selecionados. As concentrações bactericidas mínimas encontradas foram de 1,2% para *Ocimum basilicum*, 0,3% para *Origanum vulgare* e *Thymus vulgaris* e 0,15% para *Litsea cubeba*. Não houve diferença significativa no crescimento de células viáveis entre os tratamentos e o controle; contudo, houve um aumento nas células viáveis de aproximadamente 2.5 $\log_{10}$ NMP/g após o quinto dia. As combinações de óleos não impediram a esporulação, no entanto efeito sinergístico foi observado nas análises *in vitro*. Houve uma redução no pH durante o armazenamento e maior quantidade de nitrito residual no tratamento F1 (*Origanum vulgare* (0,2%); *Thymus vulgaris* (0,05%) e *Litsea cubeba* (0,025%), com 10,23 ppm, e redução de nitrito residual durante o período de armazenamento de 18,75 ppm no primeiro dia de armazenamento para 5,11 ppm no último. Nenhuma mudança significativa foi observada nas características tecnológicas do produto para os tratamentos analisados.

Palavras-chave: Aditivos naturais; *Clostridium difficile*; Óleos essenciais; Produto cárneo; Segurança alimentar.

Resumen

Se evaluaron los efectos de la adición de mezclas de aceites esenciales y la reducción del contenido de nitrito de sodio (75 ppm) sobre Clostridium difficile inoculado en mortadelas y sobre las características tecnológicas del producto. De los 15 aceites esenciales testados previamente, solamente cuatro fueron seleccionados. Las concentraciones bactericidas mínimas encontradas fueron de 1,2 % para *Ocimum basilicum*, 0,3 % para *Origanum vulgare* y para *Thymus vulgaris* y 0,15 % para *Litsea cubeba*. No hubo diferencias significativas en el crecimiento de células viables entre los tratamientos y el control; sin embargo, hubo un aumento de células viables de aproximadamente 2,5 $\log_{10}$ NMP/g después del quinto día. Las combinaciones de aceites no impidieron la esporulación, pero efecto sinergico fue observado en analises in vitro. Hubo una reducción del pH durante el almacenamiento y una mayor cantidad de nitrito residual para el tratamiento F1 (*Origanum vulgare* (0,2 %); *Thymus vulgaris* (0,05 %) y *Litsea cubeba* (0,025 %), con 10,23 ppm, y una reducción del nitrito residual durante el período de almacenamiento de 18,75 ppm en el primer día de almacenamiento a 5,11 ppm en el último. No se observó ningún cambio significativo en las características tecnológicas del producto para los tratamientos analizados.

Palabras clave: Aceites esenciales; Aditivos naturales; *Clostridium difficile*; Producto cárnico; Seguridad alimentaria.

1. Introduction

In recent decades, there have been constant changes in the food consumption patterns of the population worldwide, leading to increased demand for fresh and minimally processed foods without added chemical preservatives in search of greater healthfulness. Consequently, new food preservation strategies have been promoted to partially or completely replace the additives commonly available on the market, especially for processed meat products. Research on essential oils (EOs) has been receiving great attention because these oils are natural and safe antimicrobial agents (Abdollahi et al., 2014, Fernandes et al., 2015) and can replace traditionally used preservatives such as nitrite and nitrate salts, which can be harmful to consumers' health when consumed in excess, (Asensio et al., 2014) although they are essential in conferring some characteristics to cured meat products, such as mortadella.

Curing is a process of preserving food products, such as meats, by adding sodium nitrite/nitrate and other ingredients, such as sugar and phosphates (Sebranek & Bacus, 2007). Sodium or potassium nitrite is one of the main ingredients of this process; it has the basic purposes of conferring the characteristic pink color and flavor of cured products, preventing unpleasant changes that arise from the oxidative rancidity of lipids and acting as a preservative, mainly against the growth and proliferation of *Clostridium* sp. (Cassens, 1997; Feiner, 2006). However, nitrous acid can react with amines in cured meat products to form N-nitrous compounds, especially nitrosamines, which have toxic, mutagenic, neurotoxic, nephrotoxic and carcinogenic effects (Rywotycki, 2002).

Clostridium difficile, a mesophilic, Gram-positive, strictly anaerobic and spore-forming bacterium, is among the main human pathogens of great concern for the meat industry (Weese, 2010; Hoover & Rodriguez-Palacios, 2013). The infection occurs as a result of the ingestion of vegetative cells or endospores that resist the action of gastric acid, germinate in the small intestine and colonize the colon, producing toxins that initiate a series of phenomena ranging from watery diarrhea to more

severe cases of pseudomembranous colitis, toxic megacolon or bowel perforation (Sunenshine & McDonald, 2006; Rodríguez-Pardo et al., 2013).

As a result, food preservation has become a challenge for the food industry, as increasingly effective preservatives are required that do not pose risks to consumer health, especially due to the growing trend of refusal to use chemical preservatives and synthetic antimicrobials to inhibit the growth of pathogenic microorganisms (Tajkarimi, Ibrahim & Cliver, 2010). Several studies have worked towards fully or partially replacing sodium nitrite (Dutra et al., 2011; Oliveira et al., 2012; Martins et al., 2014; Dias et al., 2015; Pinelli et al., 2021; Aleixo et al., 2022). EOs may be a viable alternative for this purpose and have attracted the attention of researchers due to their antioxidant, antimicrobial, flavoring, aromatic, antiseptic, carminative, antispasmodic and expectorant potential (Burt, 2004; Oussalah et al., 2007; Bakkali et al., 2008; Oliveira et al., 2012; Pereira et al., 2014).

Therefore, in search of an alternative to the use of high concentrations of sodium nitrite in mortadella (on average 250 mg/kg), the objective of this study was to evaluate the antimicrobial activity of different EOs and their combinations on *C. difficile* by determining *in vitro* the minimum bactericidal concentration of each oil and its combinations; identifying the chemical components present in the EOs of *Origanum vulgare*, *Thymus vulgaris* and *Litsea cubeba*; evaluating the action of these oils in mortadellas prepared with reduced sodium nitrite content against this microorganism; and determining the influence of these EOs on the technological characteristics of the product (residual nitrite, pH and water activity).

2. Methodology

Essential oil identification and chemical characterization by gas chromatography-flame ionization detection (GC-FID)

The EOs of *Cinnamomum camphora* (ho wood), *Cinnamomum zeylanicum* (cinnamon), *Citrus limon* (lemon), *Citrus nobilis* (Mandarin orange), *Foeniculum vulgare dulce* (sweet fennel), *Litsea cubeba* (mountain pepper), *Mentha piperita* (peppermint), *Myristica fragrans* (nutmeg), *Ocimum basilicum* (basil), *Origanum vulgare* (oregano), *Pimpinella anisum* (star anise), *Piper nigrum* (black pepper), *Rosmarinus officinalis* (rosemary), *Syzygium aromaticum* (clove) and *Thymus vulgaris* (thyme) were acquired from the company FERQUIMA Indústria e Comércio Ltda. (Vargem Grande, São Paulo, Brazil).

The chemical analyses of the EOs were performed at the Phytochemistry Laboratory, Department of Agriculture, Federal University of Lavras. The chemical components of the best EOs were characterized by gas phase chromatography on an Agilent® 7890A gas chromatograph operated with the HP GC Chemstation Ver. A.01.14 data processing system and equipped with a CombiPAL autosampler system (CTC Analytics AG, Switzerland) and a flame ionization detector (GC-FID). The samples were prepared by diluting the EO in ethyl acetate (1 %, v/v). The injection volume was 1.0 µL in split mode at an injection ratio of 50:1. An HP-5MS-fused silica capillary column (30 m length x 250 µm internal diameter x 0.25 µm film thickness) was used (California, USA). Helium was used as the carrier gas, with a flow rate of 1.0 mL/min; the injector and detector temperatures were kept at 240 °C. A temperature increase of 3 °C/min from 60 °C to 200 °C was used, followed by an increase of 10 °C/min to 270 °C, followed by isothermal holding for 1 min. The concentrations of the constituents are expressed as the mean percent area under the chromatographic peaks ± the standard deviation of three analyzed samples. The results of the analysis are presented for the five constituents with the largest area under the peak. The qualitative analyses were performed in an Agilent® 7890A chromatograph coupled to an Agilent® MSD 5975C mass selective detector (Agilent Technologies, California, USA) operated with electron impact ionization at 70 eV in scanning mode at a speed of 1.0 scan/s, with a scan mass range of 40-400 m/z. The operating conditions were the same as those used in the GC-FID analyses. The mass spectra obtained were compared with the Wiley 8.0 MS library, the compound retention indices were calculated after the

previous analysis of a homologous series of alkanes, and the data obtained were compared with those available in the literature (Adams, 2007).

Obtaining the Clostridium difficile inoculum

Clostridium difficile ATCC 9689 (INCQS 00214) was used. The strain was reactivated in *Clostridium* broth medium (CB, HiMedia, India) supplemented with 0.5 % glucose (CB-Glu) at 37 °C/72 h under anaerobiosis generated by the addition of sterile mineral oil (NCCLS, 2019). The inoculum was standardized at 10^5 CFU/mL using a growth curve in CB-Glu, absorbance monitoring (OD 600 nm) and a plate count using SPS medium (sulfite-polymyxin-sulfadiazine; HiMedia, India) and the pour plate method. The plates were incubated at 37 °C for 72 h under anaerobic conditions using a PROBAC® anaerobic atmosphere generator. After each new strain reactivation, the purity of the culture was verified in SPS agar and by Gram staining.

Evaluation of in vitro antimicrobial activity

To evaluate the minimum bactericidal concentration (MBC), screening was performed using the broth dilution technique (NCCLS-National Committee for Clinical Laboratory Standards, 2019) with modifications. Aliquots of the standardized culture (OD 600 nm = 0.100) were transferred to test tubes containing 5 mL of CB-Glu plus 0.5 % (v/v) Tween 80 and EO at the following concentrations: 10.0, 8.0, 7.0, 6.0, 5.0, 4.0, 3.5, 3.0, 2.5, 2.0, 1.0, 0.5, 0.25, 0.12, 0.06, 0.03, 0.01 % and 0% (negative control). The positive control was prepared with 1000 mg/L chloramphenicol solution. After homogenization, the tubes were incubated at 37 °C for 24 h under anaerobic conditions. After this period, aliquots of 0.1 mL were transferred to tubes containing CB-Glu and incubated again under anaerobic conditions at 37 °C for 24-48 h. The analyses were performed in triplicate. The lowest EO concentrations at which there was no turbidity in the tubes were considered the MBC.

After this first stage, four EOs were selected from the 15 evaluated for the *in vitro* test – vacuum tubes were used to guarantee the obtained results, given the sensitivity of the strain to oxygen – for possible use in mortadella given their potential bactericidal action and lower sensory impact on the meat product. The method use was the same as that described above, but without the use of mineral oil.

Essential oil combinations

After the tests, the EOs with the lowest MBC were selected to be evaluated in combinations using an experimental design based on the broth dilution technique. Aliquots of the culture standardized at 10^5 CFU/mL were transferred to vacuum tubes containing 5 mL of CB-Glu plus 0.5 % (v/v) Tween 80 and the EO combinations at the ratios shown in Table 1. The tubes were incubated at 37 °C for 24 h under anaerobic conditions; after this period, 0.1-mL aliquots were transferred to a vacuum tube containing CB-Glu and incubated again at 37 °C for 24-48 h. The analyses were performed in triplicate. Growth was determined according to the turbidity of the medium and the absorbance reading.

Considering the *in vitro* evaluations of these EOs, two assays from the experimental design (Table 1) were chosen for the preparation of mortadella: assay 7, containing 67 % MBC of *O. vulgare* EO and 17 % MBC of the other two EOs, and assay 10, containing 33 % MBC of all tested oils.

Table1. Experimental design used to evaluate the combination of three essential oils, considering the ratio corresponding to the minimum bactericidal concentration (MBC)* of each oil.

Assay	Oil A (%)	Oil B (%)	Oil C (%)
1	100	0	0
2	0	100	0
3	0	0	100
4	50	50	0
5	50	0	50
6	0	50	50
7	67	17	17
8	17	67	17
9	17	17	67
10	33	33	33

* The MBC of each oil studied was considered 100 %, and the other numbers represent the ratios of the oils used based on the minimum inhibitory concentration. Source: Authors.

Mortadella preparation

The typical Brazilian mortadella formulation was used, according to Dutra et al. (2011). Ground beef (58.5 %) was added to the cutter (model KJ-10, Industrias Jamar Ltda.; Tupa, SP, Brazil), followed by sodium chloride (1.9 %), Fosmax polyphosphate (0.3 %, New Max Industrial, Brazil), sodium nitrite (0.0075 %), ascorbic acid (0.05 %), water/ice (20 %), cassava starch (5 %) and lard (14 %). The EOs were added at the end of the process to avoid evaporative losses because they are highly volatile. $NaNO_2$ (75 ppm) was used in all treatments in quantities representing half of the maximum amount allowed by law (150 ppm) (ANVISA Ordinance No. 1,004 of December 11, 1998). The mass obtained was stuffed in artificial casings (CALEBRE, 67 mm, Shur company) and cooked by immersion in water until it reached 73 °C (in the cold spot). After cooking, the mortadellas were kept in an ice bath for 20 min and stored under refrigeration (4 °C).

For the microbiological analyses, the mortadellas were sliced into 25 g portions, ground, inoculated with 10^5 CFU/g of the *C. difficile* inoculum, vacuum-packed (TM300 sealer, Tecmaq, São Paulo, SP, Brazil) and stored in a biochemical oxygen demand incubator at 25 °C. The samples were removed for analysis at 1, 5, 10 and 15 days. For the technological analyses, the vacuum-packed portions weighed 100 g, without addition of the inoculum, and the storage temperature was 25 °C ± 1 °C. All analyses were performed in triplicate.

Quantification of *C. difficile* in mortadella

To quantify the vegetative cells and endospores of *C. difficile* in the meat product, the methods proposed by Dutra et al. (2016) and de Oliveira et al. (2011) were followed, with modifications. Twenty-five-gram packages of the product were added to 225 mL of 0.1 % peptone water (w/v) and homogenized in a Metroterm® stomacher (490 strokes/min) for 2 min. After this procedure, serial dilutions were made with peptone water (0.1 % w/v), and 1 mL aliquots were added to a sequence of three test tubes with screw caps containing CB-Glu and incubated at 37 °C for 24-48 h under anaerobic conditions created using mineral oil. The tubes showing growth characteristics (turbidity and gas production) were considered positive and were interpreted using the most probable number (MPN) table. The results are expressed as the MPN of viable cells per gram in the mortadella samples (MPN/g), a method described by Scott, Anderson & Wang (2001).

Endospores were also quantified by the MPN technique using a series of three tubes. After homogenization, the samples were subjected to thermal shock (75 °C for 15 min, followed by ice bath) to inactivate viable cells. Subsequently,

serial dilutions were performed with peptone water (0.1 % w/v), and 1 mL of sample was added sequentially to three test tubes with screw caps containing CB-Glu medium and incubated at 37 °C for 24-48 h under anaerobic conditions created by the addition of mineral oil. The results are expressed as the MPN of endospores per gram in the mortadella sample (MPN/g).

Chemical and technological analyses

The pH values of the mortadellas were measured by inserting a combined penetration electrode (Ag/AgCl reference system) coupled to a DM20 potentiometer (Digimed, São Paulo, SP, Brazil) into the product at three different points.

The water activity (Aw) of the mortadella was determined with an AquaLab water activity meter (model CX2, Decagon Devices, Inc.), which uses the dew point determination technique in an encapsulated mirror to measure the Aw of a product (AOAC method, 1995).

The residual nitrite content of the prepared products was quantified according to AOAC method 973.31 (1995). The residual nitrite values are expressed as parts per million (ppm) using the standard curve of sodium nitrite.

Scanning electron microscopy (SEM)

To visualize the effect of the EOs on *C. difficile*, 0.1 mL of standardized culture was inoculated into mortadella slices, which were vacuum-packed and incubated at 37 °C. After 2 h of exposure, 0.5-cm-diameter cylinders were removed from the mortadellas. The samples were immersed in modified Karnovsky's fixative, pH 7.2, for 24 h. After this period, they were washed with cacodylate buffer (0.05 M) three times for 10 min and postfixed in 1 % osmium tetroxide in water for 1 h. After this period, the samples were washed three times in distilled water and then dehydrated in an acetone gradient (25 %, 50 %, 75 % and 90 % for 10 min; 100 % three times for 10 min). The material was then placed in a critical point dryer (Bal-Tec CPD 030) to replace the acetone with CO_2 and complement the drying process. Subsequently, the samples were coated with gold (Bal-Tec SCD 050 sputter coater), using stubs as support. Electron micrographs of the microorganisms adhering to the surface of the mortadella slices were obtained using an LEO EVO 040 scanning electron microscope (Alves, 2004).

Statistical analysis

Principal component analysis (PCA) using Chemoface software (version 1.5) was used to evaluate the MBC of the oils and combinations. The data were preprocessed and centered on the mean for a better fit of the data before the analyses.

The experiment had a completely randomized design (CRD) with a 3 (treatments) x 4 (storage times) factorial scheme with three replicates. Time regression analyses were performed for each treatment. The results were subjected to analysis of variance (ANOVA), and the means were compared using Tukey's test, with a significance level of 5 %. Statistical analyses were performed using the Statistica® software version 5.0 from STATSOFT.

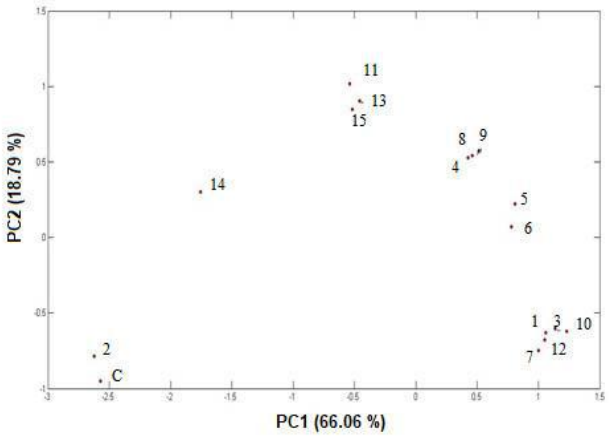
3. Results and Discussion

In vitro antimicrobial activity of the essential oils and their selection

The antimicrobial effects of different EOs on *C. difficile* were evaluated by testing the MBC of each (Fig. 1). The first and second dimensions of the PCA plot (Fig. 1) represented 84.85 % of the variability of the experimental data, accounting for 66.06 % and 18.79 %, respectively. *P. nigrum* EO affected microbial growth in a manner similar to the control treatment and differed from the other oils as it was not effective at 10 % (the highest concentration evaluated). The EOs of *R. officinalis*, *C. camphora*, and *F. vulgare* had MBCs with inhibition at 1 %, showing similar behavior. The EOs of *M. fragans*, *C. limon* and *M. piperita* also showed similar effects, with MBCs of 0.12 %. *T. vulgaris* and *C. nobilis* were inhibitory at 0.06 %, and *O. vulgare*, *C. zeylanicum*, *O. basilicum*, *L. cubeba* and *P. anisum* had MBCs of 0.015 %; these were the EOs with the lowest

MBC and thus were the most effective. EO of clove showed an MBC of 4 %, differing from the other evaluated oils and from the control treatment.

Figure 1. Principal component analysis (PCA) of the MBC of the essential oils of *O. basilicum*/basil (1), *P. nigrum*/black pepper (2), *P. anisum*/star anise (3), *M. fragans*/nutmeg (4), *T. vulgaris*/thyme (5), *C. nobilis*/mandarin orange (6), *L. cubeba*/mountain pepper (7), *M. piperita*/peppermint (8), *C. limon*/lemon (9), *O. vulgare*/oregano (10), *R. officinalis*/rosemary (11), *C. zeylanicum*/cinnamon (12), *C. camphora*/ho wood (13), *S. aromaticum*/clove (14), *F. vulgare*/sweet fennel (15) and control (C) on *C. difficile*.



Source: Authors.

According to the chromatographic report made available by FERQUIMA, the main components of the evaluated EOs are as follows: *P. nigrum* (limonene) had the highest analyzed MBC, followed by *S. aromaticum* (eugenol); *R. officinalis* (cineol), *C. camphora* (linalool), *F. vulgare* (anethole); *M. fragans* (α,β-pinene), *C. limon* (limonene), and *M. piperita* (menthol). The most effective EOs were *O. vulgare* (carvacrol), *C. zeylanicum* (cinnamic aldehyde), *O. basilicum* (linalool), *L. cubeba* (citral) and *P. anisum* (anethole), followed by the EOs of *T. vulgaris* (thymol) and *C. nobilis* (limonene).

Studies have shown that the high antimicrobial activity of EOs against pathogens is most likely due to high percentages of one or more compounds known as major components, especially phenolic compounds such as carvacrol, eugenol and thymol, as shown by some of the analyzed EOs (Nazzaro et al., 2013; Lambert et al., 2001), although some studies also mention synergy with minor or trace components (Burt, 2004; Tajkarimi, Bakkali et al., 2008; Ibrahim & Cliver, 2010).

Of the 15 EOs evaluated, *O. vulgare*, *O. basilicum*, *T. vulgaris and L. cubeba* were selected for possible use in the meat product because they were likely to have a lower sensory impact on the product and greater bactericidal activity potential. The MBC of EO of *O. basilicum* was 1.2 %, while EO of *O. vulgare* and *T. vulgaris* showed a bactericidal effect at 0.30 %, and EO of *L. cubeba* showed a bactericidal effect at 0.15 %, demonstrating a small increase in the MBC for all of the EOs tested. As the four EOs had similar MBC values, three EOs were selected for the analysis of their synergistic effect in mortadella: *O. vulgare*, *T. vulgaris* and *L. cubeba*. All the mixtures tested were effective and did not allow the growth of *C. difficile*.

The MBC of the EOs of *O. vulgare*, *T. vulgaris* and *L. cubeba* may have differed when analyzed in test tubes using mineral oil and in vacuum tubes given the trace amount of oxygen present in the medium, because *C. difficile* is sensitive to oxygen (7,8).

A wide range of components was observed in the studied oils (Table 2). Carvacrol is the major component of *O. vulgare*, representing 73.10 % of its constituents, whereas thymol represents 50.90 % of the chemical compounds contained in

the EO of *T. vulgaris*, followed by σ-cymene, γ-terpinene, linalool and carvacrol, and citral (geranial and neral) corresponds to 71.83 % of the compounds of the EO of *L. cubeba.*

Table 2. Chemical constituents identified in the essential oils of *O. vulgare*, *T. vulgaris* and *L. cubeba* by gas chromatography.

Essential Oils	Major components (%)	Amount (%)
	Carvacrol	73.11
	β- caryophyllene	4.32
Origanum vulgare	γ-terpinene	3.93
	ρ-cymene	3.92
	Thymol	2.97
	Total	88.25
	Thymol	50.89
	σ-cymene	24.97
Thymus vulgaris	γ-terpinene	5.91
	Linalool	4.46
	Carvacrol	2.93
	Total	89.16
	Geraniol	40.50
	Neral	31.33
Litsea cubeba	Limonene	13.21
	α-pinene	1.34
	Sabinene	1.28
	Total	**87.66**

Source: Authors.

The antimicrobial activity of the EO of *O. vulgare* is mainly attributable to its major components, carvacrol and thymol. However, some precursors, such as γ-terpinene and ρ-cymene, in addition to β-caryophyllene, may also be present and may affect antimicrobial activity via a synergistic effect with the other components (Burt, 2004). Stefanakis et al. (2013) evaluated seven samples of *O. vulgare* and observed that the antibacterial activity depends on the type of herb and its composition; however, the phenolic compound content of the oils is not the only factor that contributes to its activity.

Similar to the EO of *O. vulgare*, the EO of *T. vulgaris* has high antimicrobial activity mainly due to the presence of the phenolic compounds thymol and carvacrol, which are considered important in the food industry because they show strong bacterial inhibition (Rota et al., 2008). Grosso et al. (2008) reports that the major components of EOs can constitute up to 85 % of their composition, and other components are usually present at residual levels.

The major components of the EO of *L. cubeba* are geraniol and neral (two stereoisomers of citral), which have a strong antimicrobial action and act mainly on the cell membranes of some microorganisms. In addition, geraniol has high antifungal activity, which can be attributed to two combined effects: an increase in the lipid bilayer and ion leakage; these effects alter the osmotic balance of the cell, making the membrane-associated proteins inefficient and leading to cell death (Chen & Viljoen, 2010; Liao et al., 2015).

Some studies report that mixtures of different EOs may produce an additive or synergistic effect between their constituents, considerably increasing the antimicrobial activity (Dias, 2011; Oliveira et al 2012; Jayasena & Jo, 2013). This phenomenon was observed in this study, in which combinations of the EOs of *O. vulgare*, *T. vulgaris* and *L. cubeba* inhibited

the growth of *C. difficile* at all concentrations analyzed *in vitro*, which allowed lower oil concentrations to be used and thus achieved the dual objective of reducing undesirable impacts on sensory quality and controlling microbial growth.

Considering the variability in the chemical compounds present in EOs, their antibacterial activity is not attributable to a specific mechanism; rather, there are different targets in the microbial cell (Carson, 2002). EOs act by causing different types of structural and functional damage to the bacterial cell membrane (Goñi et al., 2009), such as affecting the permeability of the cytoplasmic membrane and acting mainly on membrane proteins, causing the loss of cellular constituents; harming enzymatic systems; inactivating or destroying the genetic material of bacteria; directly affecting the proton-motive force; and causing cell wall lysis (Ultee &Smid, 2001; Burt, 2004; Tajkarimi, Ibrahim & Cliver, 2010; Dutra et al., 2016). Some studies also suggest that gram-positive bacteria are more sensitive to EOs than gram-negative bacteria due to differences in the cell envelopes of these bacterial groups, since the outer membrane surrounding the cell wall in gram-negative bacteria restricts the diffusion of hydrophobic compounds through the lipopolysaccharide layer, hindering EOs' access to the cell membrane (Delaquis et al., 2002; Vergis et al., 2015). However, this does not mean that gram-positive bacteria are always more susceptible, as shown in some studies in which gram-positive bacteria were as sensitive or less sensitive to EOs than gram-negative bacteria were (Burt, 2004; Oussalah et al., 2007; Bakkali et al., 2008; Pereira et al., 2014).

The combinations that were selected for use as preservatives in mortadella in the form of emulsions were F1, containing the EOs of *O. vulgare* (0.20 %), *T. vulgaris* (0.05 %) and *L. cubeba* (0.025 %), and F2, containing the EOs of *O. vulgare* (0.10 %), *T. vulgaris* (0.10 %) and *L. cubeba* (0.05 %).

Effects of EOs on *C. difficile* inoculated in mortadella

Some studies have demonstrated the effectiveness of EOs as natural antimicrobials, and they are also considered potential additives for the food industry, including for meat and meat products (Burt, 2004; Bakkali et al., 2008; Gutierrez et al., 2008; Jayasena & Jo, 2013; Oliveira et al., 2013). The mortadella food model used in various experiments is an excellent medium for the growth of anaerobic microorganisms since it contains a large amount of nutrients and a considerable amount of carbohydrates and proteins, which provide all the essential amino acids necessary for growth, and the minimal Aw required. In addition, factors such as storage temperature and modified atmosphere packaging (reduced oxygen tensions) contributed to population growth in the control samples (Oliveira et al., 2011), justifying the choice of mortadella for the *in situ* analysis of this study. The antimicrobial activity of the combined EOs was evaluated against *C. difficile* inoculated in mortadella during storage for 15 days at 25 °C, a temperature at which many mortadellas are sold (Figure 2).

The count of vegetative *C. difficile* cells in the mortadellas was not affected ($P > 0.05$) by the treatments containing EO or the control condition. However, the effect differed significantly ($P < 0.05$) during the storage of the product (Figure 2A). When the *C. difficile* spore count was analyzed, a significant interaction ($P < 0.05$) between the treatments was observed during the storage period of 15 days at 25 °C (Figure 2B).

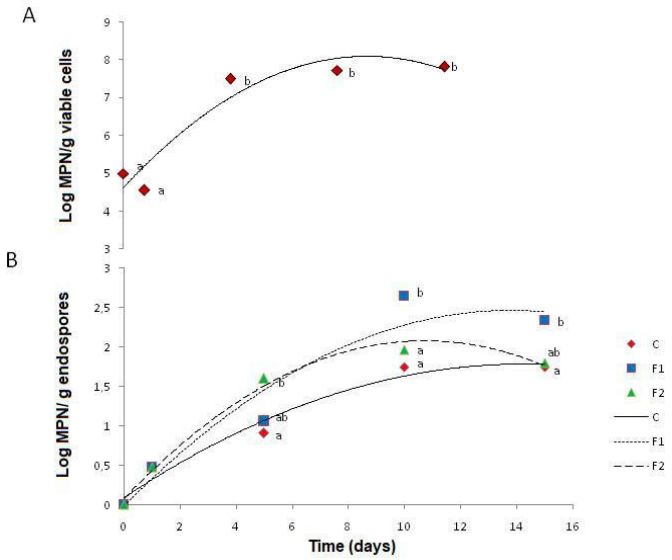
There was no growth ($P < 0.05$) of *C. difficile* on the first day of storage; however, there was an increase of approximately 2.5 $\log_{10}$ MPN/g in viable cells after the fifth day of storage, with no significant growth in the subsequent storage periods (Figure 2A).

There are differences between *in vitro* and *in situ* assays of antimicrobials of plant origin (Tajkarimi, Ibrahim & Cliver, 2010). The effectiveness of EO applications in food is usually reduced by certain components, such as proteins and/or fats, that, at high levels, can protect bacteria against the action of EOs, thus reducing the availability of the EOs (Mejlholm & Dalgaard, 2002; Glass & Johnson, 2004; Jayasena & Jo, 2013).

In addition, high nutrient availability in food compared to culture media prepared in laboratories can cause bacteria to reach their maximum replication rate and give them access to available nutrients, which allows them to repair cellular damage

more quickly. These cells may also exhibit increased resistance under stress conditions (Gill et al., 2002), which may explain the results found in this study, since the antimicrobial effects of EOs on *C. difficile* were observed in *in vitro* tests; however, these effects were not observed when the EOs were applied to mortadellas, and *C. difficile* growth occurred during storage. Burt (2004) states that to obtain similar results in a food matrix, higher EO concentrations than those used in *in vitro* tests are usually necessary.

Figure 2. Growth (A) and sporulation (B) of *C. difficile* (Log_{10} MPN/g) in mortadellas formulated with reduced sodium nitrite content and added essential oils and stored at 25 °C for 15 days.



Legend: C: 75 ppm sodium nitrite; F1: 75 ppm sodium nitrite and EO of *O. vulgare* (0.20 %), *T. vulgaris* (0.05 %) and *L. cubeba* (0.025 %); F2: 75 ppm sodium nitrite and EO of *O. vulgare* (0.10 %), *T. vulgaris* (0.10 %) and *L. cubeba* (0.05 %). Equations for the log_{10} MPN/g of viable cells: $y = -0.026x^2 + 0.608x + 4.623$ ($R^2 = 0.91$); log_{10} MPN/g endospores: C: $y = -0.008x^2 + 0.237x + 0.085$ ($R^2 = 0.97$); F1: $y = -0.013x^2 + 0.359x - 0.011$ ($R^2 = 0.94$); F2: $y = -0.017x^2 + 0.373x + 0.073$ ($R^2 = 0.99$). Source: Authors.

On the day of inoculation, no endospores were observed, which confirms that only viable cells were used in the study. No differences were observed between treatments on the first day of storage, when 0.48 log_{10} MPN/g of endospores was observed. However, there was increased sporulation during the other days of storage, especially on the 10th day; a greater MPN of endospores was observed at the end of storage in the mortadellas treated with EOs, and the F1-treated mortadella in particular contained 2.3 log_{10} MPN/g of endospores (Figure 2B).

According to Mitchell (2001), sporulation is initiated mainly in response to nutrient scarcity; however, other factors, such as pH, oxygen and temperature, can alter sporulation, and it is generally favored by stress conditions that result in a reduced growth rate. This phenomenon was observed in the present study given the increase in *C. difficile* sporulation throughout the storage time and the higher spore count in the mortadellas supplemented with EO compared to the control.

The evaluated EOs (*O. vulgare*, *T. vulgaris* and *L. cubeba*) have considerable antimicrobial properties against foodborne pathogens when used alone or in combination and are considered potential natural additives for the food industry (Guitierrez et al., 2008; Rota et al., 2008).

Ismaiel and Pierson (2006) tested the inhibitory effects of EO on *C. botulinum* strains, classifying the tested oils into three categories - very active (cinnamon, oregano and clove), active (mountain pepper and thyme) and less active (garlic, onion and black pepper) - and confirming the high antimicrobial activity of the EOs evaluated in the study against *Clostridium* spp. However, different analyses of the application of antimicrobials show notable differences (Goñi et al., 2009).

One of the limitations of the use of EOs as natural additives in foods is their concentration in the products. Deans and Ritche (1987) note that the replacement of synthetic additives with natural additives will fundamentally depend on the determination of ideal concentrations. According to Shelef (1984). , the concentrations normally used to enhance aroma and flavor vary from 0.5 to 1 %; however, for the inhibition of microbial development, concentrations greater than 1 % are necessary (Gutierrez, Barry-Ryan & Bourke, 2008). Therefore, in this study, the final EO concentration used in F1 was 2.75 %, and in F2, it was 2.50 %. The great challenge is to determine the ideal concentration that simultaneously exerts antimicrobial effects and enhances flavor and aroma in order to replace the synthetic additives traditionally used in foods with EOs.

Technological analyses

There were no significant isolated or interaction effects ($P > 0.05$) of the treatments and storage times on Aw values, which remained at approximately 0.97 ± 0.005 for all treatments and storage times. These values are consistent with those found in the literature for mortadellas and stuffed meat products in general (Fiorda & Siqueira, 2009; Giarratana et al., 2016).

Regarding pH, no significant effects ($P > 0.05$) of the treatments were observed in the mortadellas; however, a slight linear reduction ($P < 0.05$) was observed during storage.

When the residual nitrite concentrations in the mortadellas were analyzed, there was no interaction between treatment and storage time ($P > 0.05$). However, there was a slight reduction in the residual nitrite level ($P > 0.05$) throughout storage (Table 3).

A slight decrease in pH values was observed during the storage of the mortadellas, from 6.17 on the first day to 5.93 on the 15th day of storage. This slight decrease in pH can be explained by the presence of lactic acid bacteria in the meat, which are able to grow in anaerobic environments and acidify the medium, leading to defects such as off odors and off flavors (Borch et al., 1996).

There was a decrease in the residual nitrite content on the first day of analysis: the mortadellas were formulated with 75 ppm nitrite, and on the first day, they contained 18.75 ppm nitrite. The nitrite level decreased significantly after five days of storage then continued to decrease slightly throughout the storage (total decrease of 13.64 ppm) (Table 3).

As the pH of the meat decreases to values close to 6.0 or lower, there is a reduction in the added nitrite as it is transformed into nitrous acid, a phenomenon that was observed in the present study. The latter can then react with substances that are endogenous (such as cysteine or quinones) or exogenous (ascorbic acid and its salts, for example) to the meat and be transformed into nitric oxide (Viuda-Martos et al., 2010).

Table 3. Residual nitrite during the storage of mortadellas prepared with reduced sodium nitrite and different essential oil combinations.

Time (days)	Residual nitrite (ppm)
1	18.75 ±2.15 [a]
5	6.62 ±2.53 [b]
10	5.14 ±1.64 [b]
15	5.11 ±0.87 [b]

Legend: C: 75 ppm sodium nitrite; F1: 75 ppm sodium nitrite and EO of *O. vulgare* (0.20 %), *T. vulgaris* (0.05 %) and *L. cubeba* (0.025 %); F2: 75 ppm sodium nitrite and EO of *O. vulgare* (0.10 %), *T. vulgaris* (0.10 %) and *L. cubeba* (0.05 %). Means ± standard deviation. Means followed by different letters in the column differ significantly ($P < 0.05$) according Tukey's test. Source: Authors.

Honikel (2008) reported that the greatest nitrite loss is observed during manufacturing before the end of the heating process (approximately 65 %); after 20 days of storage, there is a one-third decrease in the post-heating concentration. A

similar effect was observed in this study, with a 75 % decrease in the initial nitrite concentration until the first day of storage, with a greater decrease after the fifth day, followed by a small decrease until the end of the storage period. According to Cassens (1997), after nitrite is added to the meat system, between 1 % and 10 % is oxidized to nitrate; 5 % to 10 % reacts with myoglobin, 5 % to 15 % with the sulfhydryl groups of proteins, 1 % to 5 % with fat, 20 % to 30 % with protein, and approximately 1 % to 5 % is transformed into gas and released from the product. These complex nitrite reactions may contribute to the variation in the residual nitrite content of meat products; as a result, only 10 % to 20 % of the added nitrite can be detected after processing cured products, and this level gradually decreases with storage. The reduction in the residual nitrite content of meat products represents the possibility of reduced nitrite intake and the consequent formation of carcinogenic compounds known as N-nitrosamines (Parthasarathy & Bryan, 2012; Karolyi, 2003).

When the residual nitrite concentrations in the mortadellas were analyzed, there was no interaction between the treatments and the storage time ($P > 0.05$); however, a greater amount ($P < 0.05$) of residual nitrite was observed in the F1 treatment, followed by F2, which was not different from the former or from the control (Table 4).

Table 4. Residual nitrite in mortadella elaborated with reduced sodium nitrite and different combinations of essential oils stored at 25 ° C for 15 days.

Treatments	Residual nitrite (ppm)
C	7.63 ± 6.20^{a}
F1	10.23 ± 6.41^{b}
F2	8.86 ± 5.85^{ab}

Legend: C: 75 ppm sodium nitrite; F1: 75 ppm sodium nitrite and EO of *O. vulgare* (0.20 %), *T. vulgaris* (0.05 %) and *L. cubeba* (0.025 %); F2: 75 ppm sodium nitrite and EO of *O. vulgare* (0.10 %), *T. vulgaris* (0.10 %) and *L. cubeba* (0.05 %). Means ± standard deviation. Means followed by different letters in the column differ significantly ($P < 0.05$) according Tukey's test. Source: Authors.

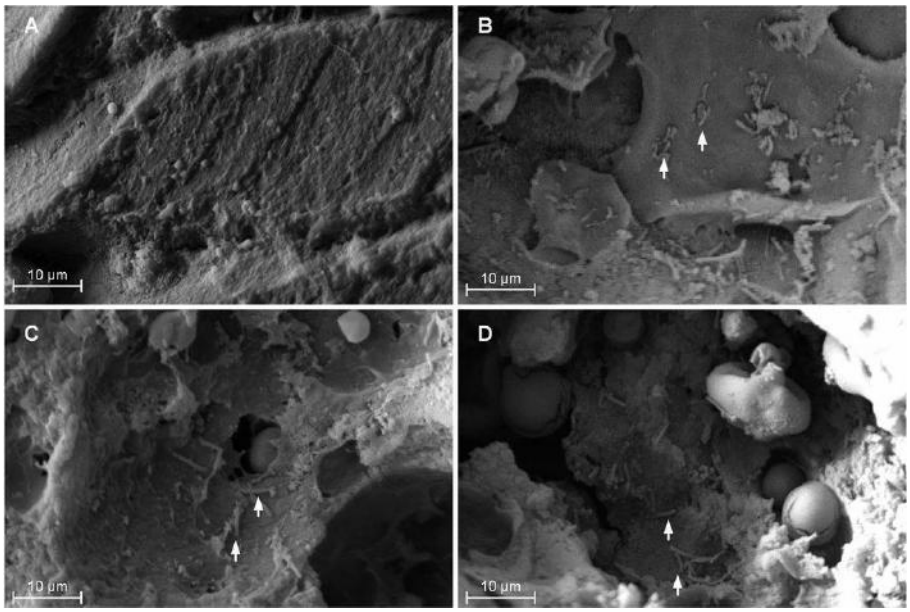
For effective control over several bacteria, including *C. botulinum*, some authors consider that approximately a residual nitrite concentration of 10 ppm is needed in the final product and claim that added values lower than 150 ppm are insufficient to reach this residual level and therefore do not prevent the development of this microorganism (Cassens, 1997); this phenomenon was observed in this study since at the end of the 15th day, there was 5.11 ppm of residual nitrite. However, treatment F1, which contained the highest amount of oregano EO, had a final residual nitrite concentration of 10.23 ppm, which was higher than that observed in the control sample. In a study conducted by Dias (2011), it was observed that mortadellas containing EOs had a higher concentration of residual nitrite than those that did not contain EOs and, consequently, had a better antibacterial effect during 20 days of storage.

Ultrastructural analysis

The ultrastructure of the mortadella surface and the morphology of *C. difficile* after 2 h of exposure to the treatments are shown with SEM (Fig. 3).

It was possible to observe fat and/or EO structures in the negative control, whereas in the positive control, there was considerable growth of *C. difficile*. In the F1 treatment, it was possible to identify intact cells without morphological changes, and the same was observed in the F2 treatment. However, there was greater growth of these cells in treatment F2 than in F1, which showed isolated cells. The results obtained are consistent with those of the literature, which indicate the influence of food composition, such as fats and proteins, on the antimicrobial activity of EOs against target cells (Nazer et al., 2005; Aloui & Khwaldia, 2016).

Figure 3. Scanning electron micrographs of mortadellas formulated with reduced nitrite and essential oils and inoculated with *C. difficile*. (A) Negative control; (B) Positive control; (C) F1: EO *O. vulgare* (0.20 %), *T. vulgaris* (0.05 %) and *L. cubeba* (0.025 %); (D) F2: EO of *O. vulgare* (0.10 %), *T. vulgaris* (0.10 %) and *L. cubeba* (0.05 %). Arrows indicate *C. difficile* cells.



Source: Authors.

4. Conclusion

A synergistic effect of the combination of the EOs of *O. vulgare*, *T. vulgaris* and *L. cubeba* was observed *in vitro*; however, their combination was not effective for inhibiting the growth of *C. difficile* in mortadella. Nevertheless, the F1 treatment, composed of the combination of the EOs of *O. vulgare* (0.20 %), *T. vulgaris* (0.05 %) and *L. cubeba* (0.025 %), showed a higher concentration of residual nitrite at the end of storage, in addition to presenting isolated cells in micrographs and greater sporulation than control. No significant changes were observed in the technological characteristics of the product with the analyzed treatments. However, nanoemulsified essential oils, as well as combinations of these, are known to have biotechnological applications as natural preservatives in meat products extending shelf life. Thus, new analyses will be carried out, aiming at the application of nanoemulsified essential oils.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.The authors thank to CAPES, Foundation for Research of the State of Minas Gerais (FAPEMIG) and the Federal University of Lavras (UFLA) for their financial support.

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. *Inernational Journal of Food Science and Technology*, 49 (3), 811–818. 10.1111/ijfs.12369

Adams, R. P. (2007). Identification of essential oils components by gás chromatog- raphy/quadrupole mass spectroscopy (4th ed.). Carol Stream: Allured.

Aleixo, G. C., Silva, M. S., Martins, H. H, A., Carvalho, R. M. B., Ramos, E. M. & Piccoli, R. H. (2022). Effect of essential oils and major compound on Clostridium botulinum endospores inoculated in meat product. *Research, Society and Development*, 11(1), 1-13. http://dx.doi.org/10.33448/rsd-v11i1.XXXXX

Aloui, H. & Khwaldia, K. (2016). Natural antimicrobial edible coatings for microbial safety and food quality enhancement. *Comprehensive Reviews in Food Science and Food Safety*, 15 (6), 1080-1103. https://doi.org/10.1111/1541-4337.12226

Alves, E. (2004). Introdução à microscopia eletrônica de varredura e de transmissão. FAEPE-UFLA.

AOAC. (1995). Association of Official Analytical Chemists. Official methods of analysis of AOAC international. Virginia, 1995. v.16.

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

Bakkali, F., Averbeck, S., Averbeck, D. & Idaomar, M. (2008). Biological effects of essential oils – A review. *Food and Chemical Toxicology*, 46 (2), 446–475. https://doi.org/10.1016/j.fct.2007.09.106

Borch, E., Kant-Muermans, M. L & Blixt, Y. (1996). Bacterial spoilage of meat and cured meat products. *International Journal of Food Microbiology*, 33 (1), 103-120. https://doi.org/10.1016/0168-1605(96)01135-X

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. *International Journal of Food Microbiology*, 94 (3), 223–253. https://doi.org/10.1016/j.ijfoodmicro.2004.03.022

Calo, J. R., Crandall, P. G., O' Bryan, C. A. & Ricke, S. C. (2015). Essential oils as antimicrobials in food systems – A review. *Food Control*, 54, 111-119. https://doi.org/10.1016/j.foodcont.2014.12.040

Carson, C. F., Mee, B. J. & Riley, T. V. (2002). Mechanism of action of *Melaleuca alternifolia* (Tea Tree) Oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrobial Agents and Chemotherapy*, 46 (6), 1914–1920. https://doi.org/10.1128/AAC.46.6.1914-1920.2002

Cassens, R. G. (1997). Residual nitrite in cured meat. *Food Technology*, 51(2), 53–55.

Chen, W. & Viljoen, A. M. (2010). Geraniol—a review of a commercially important fragrance material. *South African Journal of Botany*, 76 (4), 643-651. https://doi.org/10.1016/j.sajb.2010.05.008

Deans, S. G. & Ritche, G. (1987). Antibacterial properties of plant essential oils. *International Journal of Food Microbiology*, 5 (2), 165–180. https://doi.org/10.1016/0168-1605(87)90034-1

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. https://doi.org/10.1016/S0168-1605(01)00734-6

de Oliveira, T. L. C., de Carvalho, S. M., Soares, R. A., Andrade, M. A., Cardoso, M. D. G., Ramos, E. M. & Piccoli, R. H. (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. https://doi.org/10.1016/j.lwt.2011.09.006

Dias, N. A. A., Rodrigues, L. T. S., Palhares, P., 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. https://doi.org/10.1111/jfs.12196

Dutra, M. P., Aleixo, G. C., Ramos, A. D. 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. https://doi.org/10.1016/j.radphyschem.2015.10.008

Dutra, M. P., Ramos, E. M., Cardoso, G. P. & Leal, A. S. (2011). Radiação gama e tempo de armazenamento sobre a oxidação lipídica , cor objetiva , pigmentos heme e nitrito residual de mortadelas elaboradas com diferentes níveis de nitrito. *Ciência Rural*, 41(12), 2203-2209. https://doi.org/10.1590/S0103-84782011005000143

Feiner, G. (2006). Meat products handbook practical science and technology. Boca Raton: CRC, p. 627.

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 biopreservativein minas frescal cheese. *Journal of Food Processing and Preservation*, 41 (1), 1-9. 10.1111/jfpp.12759

Fiorda, F. A. & de Siqueira, M. I. D. (2009). Avaliação do pH e atividade de água em produtos cárneos. *EVS- Revista de Ciências Ambientais e Saúde*, 36 (4), 817-826. http://dx.doi.org/10.18224/est.v36i4.1132

Giarratana, F., Muscolino, D., Ragonese, C., Beninati, C., Sciarrone, D., Ziino, G. & Panebianco, A. (2016). Antimicrobial activity of combined thyme and rosemary essential oils against *Listeria monocytogens* in Italian mortadella packaged in modified atmosphere: Thyme & Rosemary EOs vs *L. monocytogenes*. *Journal of Essential Oil Research*, 28 (6), 467-474. https://doi.org/10.1080/10412905.2016.1165744

Gill, A. O., Delaquis, P., Russo, P. & Holley, R. A. (2002). Evaluation of antilisterial action of cilantro oil on vacuum packed ham. *International Journal of Food Microbiology*, 73 (1), 83-92. https://doi.org/10.1016/S0168-1605(01)00712-7

Glass, K. A. & Johnson, E. A. (2004). Antagonistic effect of fat on the antibotulinal activity of food preservatives and fatty acids. *Food Microbiology*, 21 (6), 675–682. https://doi.org/10.1016/j.fm.2004.03.002

Goñi, P., López, P., Sánchez, C., Gómez-Lus, R., Becerril, R. & Nerín, C. (2009). Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. *Food Chemistry*, 116 (4), 982–989. https://doi.org/10.1016/j.foodchem.2009.03.058

Grosso, C., Ferraro, V., Figueiredo, A. C., Barroso, J. G., Coelho, J. A. & Palavra, A. M. (2008). Supercritical carbon dioxide extraction of volatile oil from Italian coriander seeds. *Food Chemistry*, 111 (1), 197–203. https://doi.org/10.1016/j.foodchem.2008.03.031

Gutierrez, J., Barry-Ryan, C. & Bourke, P. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*, 124 (1), 91-97. https://doi.org/10.1016/j.ijfoodmicro.2008.02.028

Honikel, K. O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science*, 78 (1-2), 68–76. https://doi.org/10.1016/j.meatsci.2007.05.030

Hoover, D. G. & Rodriguez-Palacios, A. (2013). Transmission of *Clostridium difficile* in foods. *Infectious Disease Clinics*, 27(3), 675–685. https://doi.org/10.1016/j.idc.2013.05.004

Ismaiel, A. A. & Pierson, M. D. (2006). Inhibition of growth and germination of *C. botulinum* 33 A, 40 B e 1623E by essential oil spices. *Journal of Food Science*, 55(6), 1676–1678. https://doi.org/10.1111/j.1365-2621.1990.tb03598.x

Jayasena, D. D. & Jo, C. (2013). Essential oils as potential antimicrobial agents in meat and meat products: A review. *Trends in Food Science and Technology*, 34 (2), 96–108. https://doi.org/10.1016/j.tifs.2013.09.002

Karolyi, D. (2003). Cured meat and consumer health. *Meso*, 5 (5), 16-18.

Lambert, R. J. W., Skandamis, P.N., Coote, P. J. & Nychas, G. J. E. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology*, 91 (3), 453–462. https://doi.org/10.1046/j.1365-2672.2001.01428.x

Liao, P. C., Yang, T. S., Chou, J. C., Chen, J., Lee, S. C., Kuo, Y. H. & Chao, L. K. P. (2015). Anti-inflammatory activity of neral and geranial isolated from fruits of *Litsea cubeba* Lour. *Journal of Functional Foods*, 19, 248-25. https://doi.org/10.1016/j.jff.2015.09.034

Martins, H. H. A., Simões, L. A., Isidoro, S. R., Nascimento, S. S., Alcântara, J. P. Ramos, E. M. & Piccoli, R. H. (2021). Preservative of essencial oil blends: Control of *Clostidium perfringens* Type a in mortadella. Brazilian Archives of Bioloby, 64, 1-9. https://doi.org/10.1590/1678-4324-2021200106

Mejlholm, O. & Dalgaard, P. (2002).Antimicrobial effect of essential oils on the seafood spoilage micro-organism *Photobacterium phosphoreum* in liquid media and fish products. *Letters in Applied Microbiology*, 34 (1), 27–31. https://doi.org/10.1046/j.1472-765x.2002.01033.x

Mitchell, W. J. (2001). General biology and physiology. In: Bahl, H., Dürre, P. Clostridia: biotechnology and medical applications. New York: J. Wiley.

Nazer, A. I., Kobilinsky, A., Tholozan, J. L. & Dubois-Brissonnet, F. (2005). Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* sv. Typhimurium: a synergistic effect? *Food Microbiology*, 22 (5), 391-398. https://doi.org/10.1016/j.fm.2004.10.003

Nazzaro, F., Fratianni, F., de Martino, L., Coppola, R. & de Feo, V. (2013). Effect of essential oils on pathogenic bacteria. *Pharmaceuticals* (Basel), 6 (12), 1451–1474. https://doi.org/10.3390/ph6121451

NCCLS (National Committee for Clinical Laboratory Standards) (2019). Performance standards for antimicrobial susceptibility testing, 29th ed. Clinical and Laboratory Standards Institute, Wayne, PA.

Oliveira, T. L. C., Soares, R. A., Ramos, E. M., Cardoso, M. G., Alves, E. & Piccoli, R. H. (2011). Antimicrobial activity of *Satureja montana* L. essential oil against *Clostridium perfringens* type A inoculated in mortadella-type sausages formulated with different levels of sodium nitrite. *International Journal of Food Microbiology*, 144 (3), 546–555. https://doi.org/10.1016/j.ijfoodmicro.2010.11.022

Oliveira, T. L. C., Soares, R. D. A. & Piccoli, R. H. (2013). A Weibull model to describe antimicrobial kinetics of oregano and lemongrass essential oils against *Salmonella* Enteritidis in ground beef during refrigerated storage. *Meat Science*, 93 (3), 645–651. https://doi.org/10.1016/j.meatsci.2012.11.004

Oussalah, M., Caillet, S., Saucier, L. & Lacroix, M. (2007). Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157 : H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes. Food Control*, 18 (5), 414–420. https://doi.org/10.1016/j.foodcont.2005.11.009

Parthasarathy, D. K. & Bryan, N. S. (2012). Sodium nitrite: The "cure" for nitric oxide insufficiency. *Meat Science*, 92 (3), 274-279. https://doi.org/10.1016/j.meatsci.2012.03.001

Pereira, A. D., Piccoli, R. H., Batista, N. N., Camargos, N. G. & Oliveira, M. M. M. (2014). Thermochemical inactivation of *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* enterica Enteritidis by essencial oils. *Ciência Rural*, 44 (11), 2022–2028. https://doi.org/10.1590/0103-8478cr20140092

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

Rodríguez-Pardo, D., Mirelis, B. & Navarro, F. (2013). Infecciones producidas por *Clostridium difficile*. *Enfermedades Infecciosas y Microbiologia Clinica*, 31 (4), 254-263. https://doi.org/10.1016/j.eimc.2012.12.010

Rota, M. C., Herrera, A., Martínez, R. M., Sotomayor, J. A. & Jordán, M. J. (2008). Antimicrobial activity and chemical composition of *Thymus vulgaris*, *Thymus zygis* and *Thymus hyemalis* essential oils. *Food Control*, 19(7), 681–687. https://doi.org/10.1016/j.foodcont.2007.07.007

Rywotycki, R. (2002). The effect of selected functional additives and heat treatment on nitrosamine content in pasteurized pork ham. *Meat Science*, 60 (4), 335–339.https://doi.org/10.1016/S0309-1740(01)00138-3

Sebranek, J. G. & Bacus, J. N. (2007). Cured meat products without direct addition of nitrate or nitrite: what are the issues? *Meat science*, 77(1), 136-147. https://doi.org/10.1016/j.meatsci.2007.03.025

Scott, V. N., Anderson, J. E. & Wang, G. (2001). Mesophilic anaerobic sporeformers, In: Downes, F.P., Ito, K. (Eds.), Compendium of Methods for the Microbiological Examination of Foods, 4 ed. American Public Health Association, Washington, pp. 325–330.

Shelef, L.A. (1984). Antimicrobial effects of spices. *Journal of Food Safety*, 6 (1), 29-44. https://doi.org/10.1111/j.1745-4565.1984.tb00477.x

Stefanakis, M. K., Touloupakis, E., Anastasopoulos, E., Ghanotakis, D., Katerinopoulos, H. E. & Makridis, P. (2013). Antibacterial activity of essential oils

from plants of the genus *Origanum*. Food Control, 34 (2), 539–546. https://doi.org/10.1016/j.foodcont.2013.05.024

Sunenshine, R. H. & McDonald, L. C. (2006). *Clostridium difficile*-associated disease: new challenges from an established pathogen. *Cleveland Clinic Journal of Medicine*, 73 (2), 187-197. Retrieved from https://www.sociedad-iih.cl/doc_biblioteca/consejos_guias/6-Clostridiumdifficile.pdf

Tajkarimi, M. M., Ibrahim, S. A. & Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control*, 21 (9), 1199–1218. https://doi.org/10.1016/j.foodcont.2010.02.003

Ultee, A. &Smid, E. J. (2001). Influence of carvacrol on growth and toxin production by *Bacillus cereus*. *International Journal of Food Microbiology*, 64 (3), 373–378. https://doi.org/10.1016/S0168-1605(00)00480-3

Vergis, J., Gokulakrishnan, P., Agarwal, R. K. & Kumar, A. (2015). Essential oils as natural food antimicrobial agents: a review. *Critical Reviews in Food Science and Nutrition*, 55 (10), 1320-1323. https://doi.org/10.1080/10408398.2012.692127

Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J. & Pérez-Álvarez, J. A. (2010). Effect of added citrus fibre and spice essential oils on quality characteristics and shelf-life of mortadella. *Meat Science*, 85 (3), 568–576. https://doi.org/10.1016/j.meatsci.2010.03.007

Weese, J. S. (2010). *Clostridium difficile* in food-innocent bystander or serious threat? *Clinical Microbiology and Infection*, 16 (1), 3–10. https://doi.org/10.1111/j.1469-0691.2009.03108.x