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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

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ABSTRACT

This research evaluated the antimicrobial effect of the winter savory (*Satureja montana* L.) essential oil (EO) against *Clostridium perfringens* type A (ATCC 3624) inoculated in mortadella-type sausages formulated with different levels of sodium nitrite ($NaNO_2$: 0 ppm, 100 ppm and 200 ppm) in addition to EO at concentrations of 0.0%, 0.78%, 1.56% and 3.125% stored at 25 °C for 30 days. The EO extracted by hydrodistillation and analyzed by gas chromatography–mass spectrometry (CG–MS) was tested *in vitro* using an agar well diffusion method for determination of minimum inhibitory concentration (MIC) on *C. perfringens*. According to compositional analysis of the winter savory EO, 26 chemical compounds were identified, and the major constituents were thymol (28.99%), *p*-cymene (12.00%), linalool (11.00%) and carvacrol (10.71%). The results obtained showed that EO applied at a concentration of 1.56%, which was defined as the MIC, exhibited antimicrobial activity against *C. perfringens* in the *in vitro* assays, and the transmission electron microscopy (TEM) revealed structural damage and cell lysis of *C. perfringens* caused by EO treatment. A synergistic effect between $NaNO_2$ and EO was observed. In mortadella-type sausages formulated with 100 ppm of $NaNO_2$ and EO at all concentrations tested, the population of target microorganisms was reduced ($p \leq 0.05$) compared to control samples during all storage period. This data suggests the potential combined use of savory EO and minimal amounts of the synthetic additive, $NaNO_2$ to control *C. perfringens* in mortadella, which goes according to current market trends, where consumers are requesting natural products.

1. Introduction

Clostridium perfringens is an anaerobic, Gram-positive, spore-forming, rod-shaped and non-motile bacterium widely found in soil, water, air, and in the gastrointestinal tract of humans and animals, which can contaminate raw and processed foods, particularly meat, meat products and poultry. This bacterium produces over 13 different toxins and is commonly classified into five types (A, B, C, D and E) depending on the production of four major lethal toxins including alpha, beta, epsilon and iota (Juneja et al., 2003; Carman et al., 2008). Foodborne illness occurs after the ingestion of food contaminated with a large number (10^6–10^7 cells/g) of type A viable vegetative cells carrying the *cpe* gene encoding the *C. perfringens* enterotoxin (CPE). Foodborne illness is the result of CPEs that are produced during *in vivo* sporulation, which usually occurs in the small intestine and is stimulated by acid conditions. Approximately 8 h to 12 h after eating contaminated food, the symptoms start with acute abdominal pain, nausea and diarrhea. The contaminated food is almost always heat-treated, which kills competing flora, while the *C. perfringens* spores survive and germinate (McClane and Rood, 2001; Brynestad and Granum, 2002; Byrne, et al., 2008; Juneja et al., 2009). *C. perfringens* lacks the genetic machinery to produce 13 essential amino acids, and outbreaks related with this organism are associated with protein-rich foods. Approximately 75% of cases of illness due to *C. perfringens* are attributed to meat, meat products and poultry (Johnson and Gerding, 1997).

Meat products are widely consumed foodstuffs. In addition to appreciable sensory aspects, meat products have a relatively low price when compared to traditional *in natura* meat cuts. Mortadella is a cured, emulsified and stuffed meat product that provides lower social classes access to animal proteins, making the minimal recommended protein intake possible (Feiner, 2006). Cured meat products have nitrite in their composition, a key ingredient in the curing process, which performs the following functions: first, it contributes to the

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development of the typical cured meat flavor and prevents lipid oxidation, inhibiting the development of rancid off-flavors; second, it reacts with myoglobin producing nitrosylhaemochrome, which gives the characteristic pink color of cured meat; third, it allows growth inhibition of spoilage and pathogenic bacteria, specially *Clostridium* sp. (Cammack et al., 1999; Marco et al., 2006). However, a high intake of nitrite presents a risk to human health due to possible allergenic effects, vasodilator effects and metamyoglobin production *in vivo* (Cammack et al., 1999). In addition, nitrous acid from the hydration of nitrite oxide produced from the reduction of sodium nitrite ($NaNO_2$) may react with secondary amines and amino acids naturally present in muscle foods and meat products to form *N*-nitroso compounds, especially nitrosamines, which are chemical substances with strong toxic, mutagenic, neurotoxic and nephrotoxic and carcinogenic effects (Rywotycki, 2002; Karl-Otto, 2008). Due to the potential risk of nitrite addition in foods, the reduction or elimination is desirable. Cassens (1997) suggested two alternatives to control the problem: use of agents that partially or completely replace nitrite or agents that block formation of nitrosamines in products containing conventional concentrations of nitrite. According to Brazilian legislation for additives and preservatives in meat products, the maximal concentration of sodium or potassium nitrite, with or without nitrate should not exceed 150 ppm or 0.015% in the product ready for consumption (Brazil, 2009).

Consumers increasingly demand natural antimicrobials as alternative preservatives in foods because the safety of additives has been questioned in the last few years. Alternative preservation techniques with such naturally derived ingredients are under investigation for their application in food products. Due to negative consumer perceptions of chemical preservatives, attention is shifting toward alternatives that consumers perceive as natural, especially plant extracts, including the essential oils (EOs) and essences of plant extracts. In this context, plant EOs are gaining significance for their potential as preservative, since they have classification of "generally recognized as safe (GRAS)" and a wide acceptance from consumers (Smith-Palmer et al., 1998; Burt, 2004; Gutierrez et al., 2009).

EOs are volatile, natural and complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites. In addition to being used as flavoring agents in foods, EOs exhibit antibacterial, antifungal and antioxidant properties (Bakkali et al., 2008). *Satureja montana* L., commonly known as winter savory or mountain savory, belongs to the *Lamiaceae* family, *Nepetoideae* subfamily and *Mentheae* tribe and is a perennial semi-shrub (20 cm–30 cm) that inhabits arid, sunny and rocky regions. *S. montana* L. is native to the Mediterranean and is found throughout Europe, Russia and Turkey. *S. montana* L. is a strong aromatic herb and has been used for centuries as a spice for food and teas; is used in Mediterranean cooking, mainly as a seasoning for meats and fish and in flavoring agents for soups, sausages, canned meats and spicy sauces (Slavkovska et al., 2001; Mastelić and Jerković, 2003; Bezbradica et al., 2005; Ćetković et al., 2007; Silva et al., 2009). *S. montana* L. has biological properties that are related to the presence of its major EO chemical compounds thymol and carvacrol (Radonic and Milos, 2003; Mirjana and Nada, 2004).

This research was aimed to evaluate the antimicrobial effect of winter savory (*S. montana* L.) EO (0.0%, 0.78%, 1.56% and 3.125%) on *C. perfringens* type A (ATCC 3624) added in mortadella-type sausages formulated with different levels of $NaNO_2$ (0 ppm, 100 ppm and 200 ppm) stored at 25 °C for 30 days. This study was also aimed to determine the feasibility of reducing the levels of nitrite used in the product formulation through the combined use of savory EO to control *C. perfringens*.

2. Materials and methods

2.1. Bacterial strain, standardization, inoculum preparation and storage

The bacterium used in this research was *C. perfringens* type A ATCC 3624 (history I.C. Hall: L.S. Mc Clung 1997; A.J. Wildson type A, cep26; INCQS 00053), which was kindly provided by the National Institute of Quality Control in Health (INCQS) of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). The bacterial strain was reactivated in a specific medium semisolid Clostridium Broth (Biolife Italiana Srl, Italy) under anaerobic conditions at 37 °C for 24 h. After the strain grew, the bacterial cells were pelleted by centrifugation (5000 *g* for 5 min at 24 °C), covered by freezing culture medium (15% glycerol Vetec, Brazil; 0.5% bacteriological peptone and 0.3% of yeast extract, Biolife Italiana Srl, Italy; and 0.5% of NaCl, final pH of 7.2 ± 0,2) and maintained under a freezing temperature (−20 °C) throughout the experiment. For bacterial reactivation and use, an aliquot of the freezing culture medium was transferred to test tubes containing the Clostridium Broth medium and grown with two subcultures (last in Brain Heart Infusion broth) at 37 °C for 24 h under anaerobic conditions. The standardization of cell counts was carried out by the growth curve. Bacterial populations in the inoculum were determined with a spectrophotometer (CARY Varian Inc.) by optical density (periodic absorbance readings) at 620 nm in culture media Brain Heart Infusion broth (BHI, HiMedia, India). Throughout the growth curve, cell counts were determined as log CFU/ml by serial dilution in peptone water 0.1% (w/v) and subsequent enumeration on Brain Heart Infusion agar (BHI agar, HiMedia, India) by a spread plate methodology. *C. perfringens* spores were quantified during the growth curve by a Most Probable Number (MPN) method previously described by Scott et al. (2001).

2.2. Essential oil (EO)

2.2.1. Plant material and EO extraction

Dried aerial parts of winter savory spice (*S. monatana* L.) originating from Albania (Mediterranean climate country and mountainous region located in Southeastern Europe on the Balkan peninsula 41°21′N and 19°59′ W), were acquired from a spice store (Mr. Josef Herbs and Spices) at the local market city of São Paulo (SP, Brazil). The EO was extracted by hydrodistillation using a modified Clevenger apparatus. Dry plant material was placed with water in a 6000 ml volumetric distillation flask. The flask was coupled to the modified Clevenger apparatus, and the extraction was performed for 3 h with the temperature maintained at 100 ± 5 °C. The obtained hydrolate (water/oil fraction) was centrifuged at 321.8 *g* for 10 min at 25 °C. The EO was collected with a Pasteur pipette, and the water traces were removed with anhydrous sodium sulfate (Vetec, Brazil). The oil was stored under refrigeration temperature (5 ± 2 °C) in glass flasks wrapped in aluminum foil (Guimarães et al., 2008).

2.2.2. Determination of moisture content and yield

Aerial parts of the winter savory (5 g) were placed with 80 ml cyclohexane (Vetec, Brazil) in a 250 ml volumetric distillation flask. The flask was coupled to a condenser with a graduated volumetric collector and heated at 100 ± 5 °C for 2 h. After the distillation process, the volume of water in the collector was measured and expressed as the moisture content contained per 100 g sample. For the yield calculation, 350 g of dry spice was subjected to extraction by hydrodistillation, and the EO obtained was quantified. In parallel to the moisture content measurement, the EO yield for dried plants was obtained (% w/w) as the moisture free basis (MFB) (Pimentel et al., 2006).

2.2.3. Identification and quantification of chemical constituents

The EO chemical components were identified by gas chromatography coupled to mass spectrometry (GC–MS). A Shimadzu gas chromatograph (model GC 17A) equipped with a mass selective detector (model QP 5000) was operated under the following conditions: fused silica capillary column (30 m × 0.25 mm) coated with a DB-5 MS stationary phase; ion source temperature of 220 °C; column temperature programmed at an initial temperature of 40 °C,

and increased by 3 °C/min up to 240 °C; helium carrier gas (1 ml/min); initial column pressure of 100.2 kPa; split ratio of 1:10 and volume injected of 1 μl (1% solution in dichloromethane). The following conditions were used for the mass spectrometer (MS): impact energy of 70 eV; decomposition velocity of 1000, decomposition interval of 0.50 and fragments of 45 Da and 450 Da decomposed. A mixture of linear hydrocarbons (C_9H_{20}; $C_{10}H_{22}$; $C_{11}H_{24}$;...$C_{24}H_{50}$; $C_{25}H_{52}$; $C_{26}H_{54}$) was injected under identical conditions. The mass spectra obtained were compared to those of the database (Wiley 229), and the Kovats retention index (KI) calculated for each peak was compared to the values according to Adams (2007).

Quantification of the EO constituents was carried out using a Shimadzu gas chromatograph (model GC 17A) equipped with a flame ionization detector (FID) under the following conditions: DB5 capillary column; column temperature programmed from an initial temperature of 40 °C finalizing at a temperature of 240 °C; injector temperature of 220 °C; detector temperature of 240 °C; nitrogen carrier gas (2.2 ml/min); split ratio of 1:10; volume injected of 1 μl (1% solution in dichloromethane) and column pressure of 115 kPa. Quantification of each constituent was obtained by means of area normalization (%).

2.3. In vitro antibacterial activity — minimum inhibitory concentration (MIC)

The agar well diffusion method proposed by Deans and Ritchie (1987) was used with slight modifications to evaluate the inhibitory activity of EO and to determine the MIC concentration. Ten sterilized glass spheres (volume of 10 mm^3) were distributed on a previously solidified layer of BHI agar that was poured in 150 mm plates followed by another layer of the same molten culture medium at 45 ± 2 °C, inoculated with revealing culture of *C. perfringens* at concentrations of 10^8 CFU/ml (OD_{620nm} = 1,2972). After solidification the glass spheres were removed to microwells formation, where 10 μl of EO diluted in dimethylsulfoxide DMSO ($(CH_3)_2SO$; Vetec, Brazil) were dispensed, at concentrations of 50.0; 25.0; 12.5; 6.25; 3.125; 1.56; 0.78; 0.39% and 0.0% with the latter being the negative control. A positive control was prepared with a 1000 mg/l chloramphenicol solution. The plates were incubated at 37 °C for 24 h under anaerobic conditions (anaerobic jars BBL GasPak system; anaerobic atmosphere generator Anaerobac PROBAC, Brazil) and inhibition zones were measured (mm) with a digital caliper (Digimess, Brazil). The MIC was defined as the lowest EO concentration applied able to inhibit the visible growth of the tested microorganism (Delaquis et al., 2002).

2.3.1. Transmission electron microscopy of EO-treated bacteria

The visualization of structural damage caused by EO contact on the *C. perfringens* cells was carried out by transmission electron microscopy (TEM). All procedures of sample preparation for visualization were performed according to methods described by Bozzola and Russell (1998), and all chemicals, solutions and accessories used were acquired from supplier Electron Microscopy Sciences (EMS, Hatfield, England). After incubation (18 h at 37 °C in BHI broth), aliquots of bacterial suspension were centrifuged (5000 *g* for 5 min at 24 °C). The pelleted bacterial cells were then exposed to 2 ml of EO solution diluted in BHI broth and Tween-80 (solvent) at the MIC determined by *in vitro* tests. The control cells were treated with only solvent and media broth. After different contact periods (15, 30, 45 min, 1, 2, 4, 6, 8, 10 and 12 h), the treated bacterial suspensions were centrifuged (5000 *g* for 5 min at 24 °C). The supernatant was then discarded, and the pelleted cellular content was washed with a saline solution (0.85%). Subsequently, a 1.0% agarose solution at 45 ± 2 °C was added to the cellular content in block formation. The block was cut, immersed in a Karnovsky fixative solution (2.5% glutaraldehyde, 2.5% formaldehyde in 0.05 M cacodylate buffer and $CaCl_2$ buffer at pH 7.2) and stored overnight protected from light and under refrigeration. After fixation, the samples were washed three times (10 min each) with a 0.05 M cacodylate buffer. Four droplets of osmium tetroxide (O_4Os) were added at final wash, and the samples were stored for 4 h at 25 °C. The O_4Os was removed, and the sample was washed three times with distilled water. A 2% solution of uranyl acetate was added to the samples, and the samples were then stored under refrigeration (5 ± 2 °C) for 12 h.

The samples were dehydrated in ethyl alcohol gradients of 25%, 50%, 75%, 90% and 95% for 10 min each. A final dehydration step was carried out for 3 h in 100% ethyl alcohol with changes every 30 min. The samples were then suspended in SPURR resin epoxy (Electron Microscopy Sciences) in a 30% gradient for 8 h, 70% gradient for 12 h and 100% resin for 12 h. The specimens were mounted in molds, and embedded in pure Spurr resin polymerized in an oven at 70 °C for 48 h.

The resin blocks were subjected to hand-trimmed with a razor blade to remove the excess resin. Soon afterwards, thick (0.85 μm) and thin (<100 nm) sections were cut using a Reichert-jung (ultracut) ultramicrotome equipped with a diamond knife. The thick sections were collected with a gold ring, placed on glass slides, stained with toluidine blue (1.0 g toluidine blue, 1.0 g sodium borate and 100 ml of water), filtered with a 0.22 μm cellulose membrane and permanently mounted in Permalt medium. The thin sections were picked up on gold slot grids and allowed to dry on Formvar-coated aluminum racks (Rowley and Moran, 1975). The sections were post-stained with 2% uranyl acetate for 3 min followed by 1% lead citrate for 3 min. Visualization and acquisition of micrographs were done with a Zeiss EM 109 transmission electron microscope (Zeiss, West Germany).

2.4. Sausage formulation and manufacture

Batches of mortadella-type sausages were formulated with different concentrations of $NaNO_2$ (0 ppm, 100 ppm and 200 ppm) and EO from winter savory (0.0%, 0.78%, 1.56% and 3.125%). Refrigerated, vacuum packaged lean beef and frozen pork backfat were obtained within 48 h of slaughtering from a local meat packer. Each batch was prepared using a Brazilian typical formula as follows: ground meat (58%), pork backfat (14%), NaCl (1.9%), ice water (20%), cassava starch (5%), polyphosphate Fosmax (0.3%, New Max Industrial, Brazil), ascorbic acid (0.05%), spice mix for Mortadella 913 (0.5%, New Max Industrial, Brazil) and $NaNO_2$ (0 mg/kg, 100 mg/kg and 200 mg/kg: Vetec, Brazil). The sausages samples were packed with a weigh of 200 ± 5 g, and showed a pH = (6.29 ± 0.11) and water activity Aw = (0.941 ± 0.008). The mortadella-type sausages were made in a pilot plant in the Products of Animal Origin Laboratory at the Federal University of Lavras (Brazil).

Lean beef, salt, phosphate and $NaNO_2$ were placed in a cutter (Sire, Filizola S.A., Brazil) and mixed for approximately 1 min. Fifty percent of the ice and spices were then added and mixed at a high speed. After complete homogenization, the speed of the cutter was reduced. Ground pork backfat was then added and mixed until the temperature of the mixture reached 10 °C. The remaining 50% of the ice, cassava starch, ascorbic acid and EO were added and mixed until the temperature of the mixture reached 13 °C. The total emulsification time was approximately 10 min, and the processing room temperature was approximately 20 °C. The batters were stuffed into nylon bags (Unipac Darlon, Brazil, 50 μm thickness) and were cooked by immersion in water according to the following program: 55 °C for 30 min, 65 °C for 30 min, 75 °C for 30 min, and 85 °C until the temperature of the mass reached 73 °C (measured by a thermopar inserted into the center of the packed sausage batter). The cooked sausage was cooled in a water bath for 10 min and stored in a controlled chamber (Thermostat cabinets *LS* Logen Scientific) at 25 °C before analysis at 1, 10, 20 and 30 days.

2.4.1. Preparation of mortadella samples

The EO concentrations used in manufacturing of the sausages were based on the following factors: *in vitro* antimicrobial activity results;

possible reductions in activity when applied to the food model (reported in literature); and combined effect of different nitrite levels used in the product manufacturing. The mortadella samples were inoculated with a microorganism culture (*C. perfringens*) to obtain an initial level of 10^7 CFU/g viable cells. Silicone was deposited on different points on the surface of the product package. After drying, the grown culture of the target microorganism was injected with a sterile needle and syringe in a laminar flow biosafety cabinet.

2.5. Enumeration of C. perfringens in mortadella

For the enumeration of *C. perfringens*, 10 g of the mortadella samples were weighed, transferred into sterile stomaching bags, combined with 90 ml of sterile peptone water (0.1% w/v) and homogenized in a Stomacher (Metroterm, Brazil) with 490 strokes/min for 2 min at room temperature. Stomached slurries were decimal serially diluted in peptone water (0.1% w/v), and aliquots (100 μl) of the sample dilutions were spread on Tryptose Sulphite Cycloserine differential selective agar (TSC, HiMedia, Mumbai, India) supplemented with 200 mg of D-cycloserine (inhibition of microbial anaerobic companion) and egg yolk emulsion (12.5 ml of yolk and 12.5 ml of 0.85% saline solution) to verify the phospholipase activity of α-toxin (lecithinase). The plates were incubated at 37 °C for 24 to 48 h under anaerobic conditions (anaerobic jars BBL GasPak system; anaerobic atmosphere generator Anaerobac PROBAC, Brazil) and the colonies were counted. Typical colonies were subjected to biochemical tests for confirmation according to the Compendium of Methods for the Microbiological Examination of Foods from the American Public Health Association (Labbé, 2001).

2.5.1. Spore counts in mortadella-type sausages

After homogenization and dilution in peptone water 0.1% w/v, the slurries diluted mortadella samples were subjected to heat treatment at 75 °C for 20 min to inactivate the viable cells and activate the dormant spores. Subsequently, 1 ml aliquots of appropriate dilutions were inoculated in a series of three tubes containing culture medium Reinforced Clostridial Medium (RCM, Oxoid Ltd., England, UK) and covered with a thioglycollate agar seal (2% agar and 0.1% sodium thioglycollate) for generation of anaerobic atmosphere. The tube's series were incubated at 37 °C for 7 days with periodic evaluations every 24 h. Tubes with characteristic growth (turbidity and gas production) were considered positive and interpreted in the appropriate MPN tables (Most Probable Number). The results are expressed in MPN of spores per gram of sample (MPN/g) (Scott et al., 2001).

2.5.2. Microbiological control

C. perfringens counts were taken in mortadella (control samples) produced without inoculum of the target organism to verify the contamination of samples, which may result in interference of the observed results. Total plate count (Plate Count Agar PCA, HiMedia, India) 37 °C for 24 to 48 h, was estimated.

2.6. Experimental design and data statistical analysis

Treatments were arranged in split plot factorial designs with different EO concentrations (0.0%, 0.78%, 1.56% and 3.125%) and nitrite levels (0 ppm, 100 ppm and 200 ppm) for the plots and times of storage (1, 10, 20 and 30 days) for the subplot. The data were obtained from three independent experiments and the means were from triplicate results. The data obtained were subjected to analysis of variance (ANOVA), and the comparison between means was determined by Scott–Knott test adopting a 5% significance level. The statistical analyses of data were carried out using statistical R software (2010).

3. Results

3.1. EO chemical characterization

The EO of winter savory (*S. montana* L.) was subjected to a detailed GC–MS analysis to determine its chemical composition. As shown in Table 1, 26 compounds were identified representing 99.48% of the total EO. The average extraction yield of the *S. montana* EO was 0.47% (4.7 ml/kg of spice dried aerial parts) in a MFB. The major groups of the compounds were monoterpene hydrocarbons and phenolic compounds. Thymol (28.99%), *p*-cymene (12.00%), linalool (11.00%) and carvacrol (10.71%) were found to be the major chemical constituents of the investigated EO.

3.2. Minimum inhibitory concentration (MIC)

The observed values for the diameter of inhibition zones in determining the MIC of EO on *C. perfringens* are shown in Fig. 1. The evaluated variable (concentration) was significant ($p = 7.25e^{-06}$), with larger inhibition zones at higher concentrations of the savory EO. We observed formation of inhibition zones at concentrations higher than 1.56% with an average diameter of 4.67 ± 0.67 mm, which was determined as the MIC. The largest inhibition zone (11.67 ± 0.33 mm) was observed at the highest concentration of oil applied (50%; 500 μl/ml), which was probably due to the higher concentration of active chemical components in the EO fraction. In the positive control (microwell filled with a 1000 mg/l chloramphenicol solution), an average inhibition zone of 16.67 mm was observed. In the negative control (microwell filled with DMSO without EO), no inhibition zones were observed, suggesting that there was no interference from the diluents used in the tests.

Table 1
Chemical constituents of *Satureja montana* L. essential oil identified by GC–MS and their contents.

Rt	Compound	(%)	IRRexp	IRRlit
6.580	α-Thujene	0.23	924	930
6.801	α-Pinene	1.37	932	939
7.314	Canphene	0.55	948	954
8.297	1-Octen-3-ol	1.32	979	979
8.607	Myrcene	0.68	989	990
9.549	α-Terpinene	1.13	1016	1017
9.826	ρ-Cymene	12.00	1024	1024
9.985	Limonene	0.62	1028	1029
10.094	1,8-Cineole	1.26	1031	1031
11.030	γ-Terpineno	2.91	1057	1059
11.480	*cis*-Sabinene	1.34	1069	1070
12.586	Linalool	11.00	1100	1096
12.815	*cis*-Thujene	1.62	1106	1102
14.307	Camphor	1.59	1146	1146
15.241	Borneol	3.43	1171	1169
15.558	Terpinen-4-ol	3.96	1180	1177
16.100	α-Terpineol	1.33	1194	1188
17.344	Eter methyl thymol	0.45	1229	1235
17.672	Eter methyl carvacrol	2.16	1238	1244
19.356	Isobornyl acetate	0.35	1284	1285
19.576	Thymol	28.99	1290	1290
19.845	Carvacrol	10.71	1298	1299
24.007	(E)-caryophyllene	4.54	1418	1419
26.502	NI*	0.52	1494	–
26.909	β-Bisabolene	1.86	1507	1505
29.071	Spathulenol	1.00	1576	1578
29.236	Caryophyllene oxide	3.08	1581	1583
Total identified		99.48%		
Moisture (dry spice)		9.9561% (±1.9586)		
yield (MFB**)		0.4721% (±0.0006)		

Rt = retention time (min). IRRexp — experimental index. IRRlit — literature index. NI* not identified compound. MFB** moisture free basis.

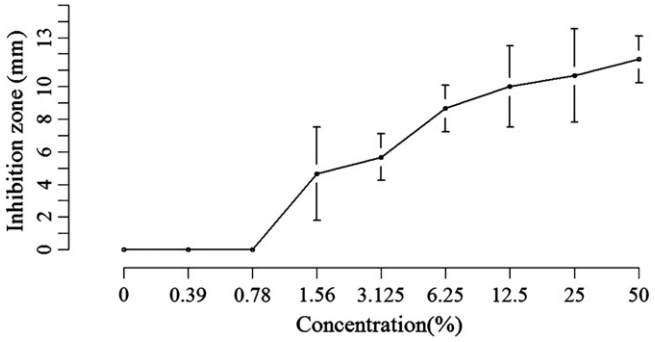

Fig. 1. Inhibition zones diameters (mm) by different concentrations of *Satureja montana* L. essential oil on *Clostridium perfringens* Type A ATCC 3624. Mean values ± Standard Error (bars), without considering the well diameter.

3.3. Ultrastructural effects on C. perfringens

The morphological cell damage of *C. perfringens* caused by treatment with *S. montana* EO at a MIC concentration are shown in the transmission electron micrographs in Fig. 2. The micrographs of untreated cell culture (A and B) without exposure to EO showed continuous thin, smooth cell walls and other defined cellular structures. The *C. perfringens* cells treated with EO (C, D, E and F) had adulterated morphology, where cell walls had irregularities, less smoothness, less uniformity and degenerative changes leading to wall ruptures and subsequent cellular lysis in some cases. An unequal cytoplasm distribution caused by the clumping and agglomeration of intracellular material was observed in the treated cells (indicated by arrows). Furthermore, the cells lacked cytoplasm in certain regions due to the loss of membrane functionality, which was characteristic of the mechanism of action of the major chemical components of *S. montana* EO. The primary events of the sporulation process were not observed due to contact of the EO with viable cells from the microorganism studied.

3.4. Inhibitory effects of the EO on C. perfringens inoculated in mortadella

The population variations of *C. perfringens* type A viable cells in mortadella-type sausages formulated with different concentrations of *S. montana* EO and levels of $NaNO_2$ during storage at 25 °C for 30 days are shown in Table 2. In mortadella elaborated without the addition of EO and nitrite (control samples), the *C. perfringens* populations increased reaching 8.95 $\log_{10}$ CFU/g at the first day of storage. After 10 days of storage, the counts were decreased in the control samples, showing a population of 4.83 $\log_{10}$ CFU/g at the end of the storage period. The samples formulated without nitrite and with 0.78% EO had populations that were not significantly different ($p>0.05$) than the initial inoculum at the first day of storage. However, their growth was restricted ($p\leq0.05$) when compared to the control (bacteriostatic effect) at the first day. In the mortadella with 1.56% EO (MIC concentration) without nitrite, we observed a decrease of 1.02 $\log_{10}$ CFU/g on the first day of analysis showing a antimicrobial effect of the EO evaluated at concentrations higher than 1.56% added in sausages. The most drastic effect was observed in samples elaborated with 3.125% EO without $NaNO_2$ where the bacterial population was reduced to 4.65 $\log_{10}$ CFU/g after 24 h of storage. However, the use of savory EO at high concentrations produces undesirable flavors in the meat product. In samples formulated without $NaNO_2$, in all EO concentrations tested, the *C. perfringens* population was less ($p\leq0.05$) than the control throughout the entire storage period. However, the antimicrobial effect of the EO-containing sausages was visibly reduced when compared to *in vitro* tests where the growth was totally restricted at 1.56%.

In mortadella samples manufactured with $NaNO_2$ at 100 ppm and 200 ppm without EO, the *C. perfringens* counts were significantly lower ($p\leq0.05$) than the control after the first day of storage. These populations were maintained lower ($p\leq0.05$) than the control during the entire storage period. The antimicrobial effect was significantly better in samples elaborated with 200 ppm when compared to samples elaborated with 100 ppm throughout the storage time with a population of 1.78 and 2.08 $\log_{10}$ CFU/g, respectively, at the 30 day of storage.

A combined effect of $NaNO_2$ and savory EO on *C. perfringens* in mortadella sausages was observed. In samples formulated with 100 ppm and 200 ppm of $NaNO_2$ with EO at concentrations of 0.78% and 1.56%, more pronounced reductions ($p\leq0.05$) were observed when compared to samples with the same concentrations of EO without the addition of $NaNO_2$ after the first day of storage. The antimicrobial effect was higher in these treatments when compared to treatments with only $NaNO_2$. The use of $NaNO_2$ at 100 ppm combined with 1.56% EO showed a similar effect to the effect found in samples with 200 ppm of $NaNO_2$ without EO, suggesting the use of reduced amounts of nitrite combined with EO. Among the treatments evaluated the use of nitrite at 100 ppm and EO at 0.78% or 1.56% appears to be a feasible alternative.

Under all EO concentrations evaluated for sausages formulated with 100 ppm of $NaNO_2$, the populations were less ($p\leq0.05$) than control at the end of the storage period. The greater population reduction among all treatments evaluated was observed in sausages formulated with 3.125% EO and either 100 ppm or 200 ppm of $NaNO_2$, where the bacterial population was reduced to 4.71 and 4.30 $\log_{10}$ CFU/g, respectively, after the first day of storage. In samples treated with combinations of 200 ppm of $NaNO_2$ and EO at 0.78%, 1.56% or 3.125% the *C. perfringens* counts were not detected at the end of storage period, which was probably due to the higher concentration of active chemical components of nitrite additive.

In all treatments evaluated, the populations of *C. perfringens* showed an increase after the 10 day of storage. At the end of the storage period (day 30) we observed a pronounced decrease in viable cell counts in all of treatments evaluated including the control samples.

The *C. perfringens* spore counts in mortadella-type sausages formulated with different concentration of *S. montana* EO and varying levels of $NaNO_2$, during storage at 25 °C for 30 days, are presented in Table 3. Throughout the storage period, the conditions were unfavorable for viable cell growth in the food model favoring the sporulation process. This event was observed in all treatments evaluated.

In the microbiological control evaluated (mortadella without target microorganism), *C. perfringens* counts were not detected during all the storage time, showing non-interference in the observed results.

4. Discussion

The extraction yield value of *S. montana* EO was similar to that found by Ćavar et al. (2008). However the yield found in our study was lower than the yield reported by the following groups: Bezbradica et al. (2005); Mastelić and Jerković (2003) and Radonic and Milos (2003). The phytochemical profile found for the winter savory EO in this study was in agreement with the results observed by several authors who have also evaluated this vegetal specie (Radonic and Milos, 2003; Skočibušić and Bezić, 2003; Mastelić and Jerković, 2003; Silva et al., 2009). In contrast, the savory EO evaluated by Ćavar et al. (2008) was characterized by a high content of alcohols, such as geraniol and terpinen-4-ol. The final composition of EO is genetically influenced with specificity to the following factors: each organ and its stage of development; climatic conditions of the plant collection site; degree of terrain hydration; level of macronutrients and micronutrients; and drying conditions to which the plant material is exposed

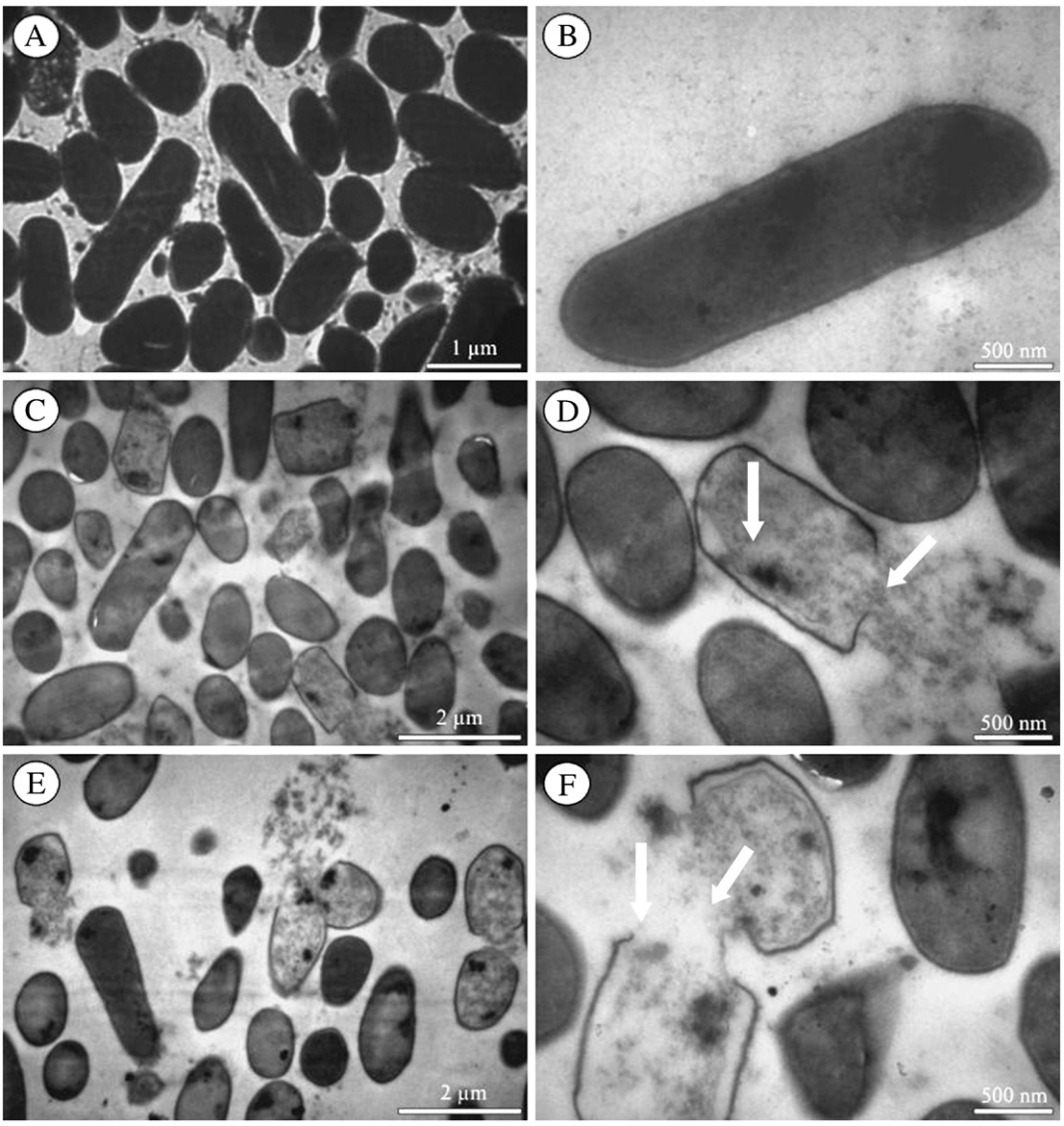

Fig. 2. Transmission electron micrographs (TEM). Magnifications A ×7000; B ×12,000; C ×4400; D ×12,000; E x4400; F ×12,000. Figures A and B show *Clostridium perfringens* type A ATCC 3624 cells untreated (control). Figures C and D show cells treated with *Satureja montana* L. essential oil at Minimum Inhibitory Concentration (MIC) after 15 min contact; figures E and F cells treated after 45 min contact. The arrows indicate damages caused by treatment with essential oil. Approximately 18% injured cells at MIC concentration treatment.

to (Burt, 2004; Bakkali et al., 2008). Slavkovska et al. (2001) and Mirjana and Nada (2004) reported the chemical variability of *S. montana* EO according to factors like plant stage of development and different geographic locations. The antimicrobial properties of winter savory EO are related to the presence of its major chemical compounds, such as thymol and cravacrol in the EO fraction (Mirjana and Nada, 2004; Radonic and Milos, 2003).

The formation of growth inhibition zones on the tested growth bacterial cultures showed the antimicrobial effect of *S. montana* EO. The MIC is cited by most researchers as the measure of performance of antibacterial EOs (Burt, 2004). Considering the large number of different groups of chemical compounds present in EOs, it is likely that their antibacterial activity is not attributable to one specific mechanism but to several targets in the cell. An important characteristic of EOs is their hydrophobicity, which allows the accumulation and partition of the lipids in bacterial cell membranes modifying their structure, distorting the lipid/protein interactions and disturbing their function (Juven et al., 1994; Sikkema et al., 1994, 1995). The loss of differential permeability of the cytoplasmatic membrane is considered the cause of cell death. Other events that may lead to dysfunction of the membrane and subsequent disruption include the following events: dissipation of the two components of the proton motive force (pH gradient and electrical potential); interference with the system of generating energy (ATP) in the cell; inhibition of enzymes; and prevention of substrate utilization for energy production (Helander et al., 1998; Cox et al., 2000; Lambert et al., 2001; Ultee et al., 2002). In this study, we identified thymol and carvacrol as the major chemical compounds of *S. montana* EO. These components are able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmatic membrane to ATP (Helander et al., 1998). Juven et al. (1994) evaluated the effect of thymol on *Salmonella typhimurium* and *Staphylococcus aureus*, and they hypothesized that the component binds to membrane proteins hydrophobically and through hydrogen bonds altering the characteristics of membrane permeability. In studies with *Bacillus cereus*, Ultee et al. (2002) have shown that carvacrol interacts with the cell membrane and dissolves the phospholipid bilayer, which is assumed to be aligned between the fatty acid chains. This distortion of the physical structure causes expansion and destabilization of the membrane increasing its fluidity, which in turn increases the passive permeability. *p*-cymene was found in significant concentrations in the studied EO; *p*-cymene is the

Table 2
Populations of *Clostridium perfringens* type A ATCC 3624 (log_{10} CFU/g) in mortadella-type sausages formulated with different levels of sodium nitrite (0, 100 and 200 ppm) and *Satureja montana* L. essential oil at concentrations of 0.0%, 0.78%, 1.56% and 3.125% during storage at 25 °C for 30 days.

Treatment		log_{10} CFU/g									
Sodium nitrite (ppm)	OE (%)	Day 0		Day 1		Day 10		Day 20		Day 30	
0	0.00	7.16 (±0.17)	Ab	8.95 (±0.03)	Aa	7.65 (±0.21)	Ab	7.38 (±0.04)	Ab	4.83 (±0.24)	Ac
0	0.78	7.34 (±0.20)	Aa	7.04 (±0.07)	Ba	5.96 (±0.10)	Cb	6.17 (±0.07)	Bb	2.96 (±0.09)	Bc
0	1.56	7.22 (±0.34)	Aa	6.20 (±0.02)	Cc	6.60 (±0.06)	Bb	6.93 (±0.04)	Ab	2.79 (±0.05)	Bd
0	3.125	7.70 (±0.35)	Aa	4.65 (±0.07)	Ec	5.74 (±0.12)	Cb	6.07 (±0.08)	Bb	2.78 (±0.19)	Bd
100	0.00	7.01 (±0.06)	Aa	7.32 (±0.18)	Ba	6.45 (±0.29)	Ba	6.94 (±0.15)	Aa	2.08 (±0.25)	Cb
100	0.78	7.33 (±0.33)	Aa	6.73 (±0.30)	Ba	6.81 (±0.06)	Ba	5.50 (±0.29)	Bb	1.86 (±0.09)	Dc
100	1.56	7.71 (±0.71)	Aa	5.79 (±0.08)	Db	6.06 (±0.13)	Cb	5.86 (±0.13)	Bb	2.08 (±0.25)	Cc
100	3.125	7.05 (±0.03)	Aa	4.71 (±0.15)	Ec	6.10 (±0.02)	Cb	5.94 (±0.11)	Bb	1.69 (±0.00)	Dd
200	0.00	6.93 (±0.04)	Aa	6.46 (±0.33)	Ca	6.47 (±0.36)	Ba	5.99 (±0.04)	Ba	1.78 (±0.09)	Db
200	0.78	7.03 (±0.03)	Aa	6.15 (±0.07)	Cb	6.11 (±0.11)	Cb	5.86 (±0.04)	Bc	0.00 (±0.00)	Ed
200	1.56	7.56 (±0.34)	Aa	5.66 (±0.22)	Dc	6.53 (±0.07)	Bb	4.71 (±0.09)	Cd	0.00 (±0.00)	Ee
200	3.125	6.04 (±0.04)	Aa	4.30 (±0.18)	Eb	5.98 (±0.08)	Ca	5.80 (±0.05)	Ba	0.00 (±0.00)	Ec

Means values ± Standard Error.
Values followed by the different small letter within the same line, and by the different capital letter within the same column, are significantly different ($P \leq 0.05$) according to Scott–Knott test.
OE Essential oil.

biological precursor of carvacrol and causes swelling of the cytoplasmic membrane making it more extensive than the carvacrol molecule. Has no effect if acting alone. In combination with cravacrol, however, it has a synergistic effect acting on *B. cereus in vitro* and in rice (Ultee et al., 2002). Randrianarivelo et al. (2009) and Oussalah et al. (2007) reported a pronounced antimicrobial effect of linalool, which is an important compound of the EO studied in this research.

Several authors have reported the antimicrobial effect of *S. montana* EO *in vitro*. Mirjana and Nada (2004) observed the antimicrobial activity of savory EO on Gram-negative and Gram-positive bacteria, filamentous fungi and yeasts using the agar dilution method. Bezbradica et al. (2005) found that *S. montana* EO in a 5% ethanol solution has wide antimicrobial activities against several microorganisms using the same methodology used in this study. Ćavar et al. (2008) reported the antimicrobial effect of *S. montana* EO obtained by hydrodistillation using the disc diffusion method. Si et al. (2009) studied the inhibition potential of 66 EOs and several of their components on *C. perfringens* type A, and they found an inhibition of over 80% in 33 of the tested components. The reported MIC values ranged between 167 and 425 μg/ml, with thymol and carvacrol as the most efficient inhibitors among the tested by the authors. The MIC values for *S. montana* EO against *C. perfringens* were not reported, so further comparisons were not made.

The transmission electron micrographs revealed morphological damages caused by EO treatment in *C. perfringens* cell structure. The *C. perfringens* cell damage observed in this study was also detected by Si et al. (2009). They evaluated the effect of different EOs on bacterial cell morphology by scanning electron microscopy, observing formation of holes on cell walls in cultures treated with EO at low concentrations. At MIC concentration, the pathogen cells were lysed.

Table 3
Spore counts of *Clostridium perfringens* Type A ATCC 3624 in mortadella-type sausages formulated with different levels of sodium nitrite (0, 100 and 200 ppm) and *Satureja montana* L. essential oil at concentrations of 0.0, 0.78, 1.56 and 3.125% during storage at 25 °C for 30 days.

Nitrite (ppm)	Time (days)	Essential oil (%)			
		0	0.78	1.56	3.125
0	1	<3.0	<3.0	<3.0	<3.0
	10	2.3×10^{2}	<3.0	<3.0	<3.0
	20	3.1×10^{2}	3.0×10^{1}	1.33×10^{2}	3.3×10^{2}
	30	2.4×10^{4}	2.4×10^{4}	7.8×10^{3}	2.4×10^{3}
100	1	<3.0	<3.0	<3.0	<3.0
	10	<3.0	<3.0	<3.0	<3.0
	20	4.3×10^{2}	1.8×10^{2}	2.1×10^{2}	3.6×10^{1}
	30	7.8×10^{3}	6.7×10^{3}	2.4×10^{3}	2.4×10^{3}
200	1	<3.0	<3.0	<3.0	<3.0
	10	<3.0	<3.0	<3.0	<3.0
	20	3.3×10^{2}	2.3×10^{2}	1.85×10^{2}	3.3×10^{2}
	30	4.6×10^{3}	2.4×10^{3}	2.4×10^{3}	7.8×10^{3}

Mean values (MPN/g spores).

The *C. perfringens* cell counts were higher in the control samples during the entire storage period. The mortadella food model used in experiment was an excellent medium for growth of *C. perfringens* because it presented a wide nutritional spectrum, with considerable amounts of carbohydrates, minimal required moisture (A_w) and protein content providing all the essential amino acids necessary for growth. In addition, factors including storage temperature and atmosphere packaging (reduced oxygen tensions) contributed to the population growth in the control samples.

The effect of the EO on the target microorganism was considerably reduced when applied in the food model (compared to *in vitro* studies). The application of EOs for the control of pathogens and spoilage bacteria requires the evaluation of their effectiveness in food products or models that roughly simulate the composition of foods. Generally, the efficiency of some additives and natural antimicrobial agents can be reduced by certain components of foods. If higher concentrations of EO are generally required when added to food to maintain product safety, undesirable flavor and sensory changes may occur (Gutierrez et al., 2008, 2009). Researchers who have evaluated the effect of EO added to meat reported undesirable sensory changes caused by EO treatment in food samples (Skandamis and Nychas, 2001; Hayouni et al., 2008; Govaris et al., 2010).

Mortadella has a large amount of fat and protein in its proximate composition, and it is assumed that high levels of fat or protein in food may protect the bacteria from the action of EOs. The EO dissolved in the lipid phase of the food is relatively less available for action on bacteria present in the aqueous phase (Mejlholm and Dalgaard, 2002). The antimicrobial activity of EOs was found to be more pronounced in low-fat foods when compared to high-fat products (Tassou et al., 1995; Singh et al., 2003; Cava et al., 2007). The protein content appears to inhibit the antimicrobial effect of EO's (Smith-Palmer et al., 2001; Vrinda Menon and Garg, 2001; Hao et al., 1998; Pol et al., 2001). Food carbohydrates protect bacteria from the EO action to a lesser extent than fat and protein (Skandamis and Nychas, 2000). Gutierrez et al. (2008) evaluated the interference of food constituents (lipids, carbohydrates and proteins) on antibacterial activity of thyme and oregano EOs on *L. monocytogenes*, and they suggest that potato starch and lipid oils at concentrations higher than 5% reduce the antilisterial effect. The physical structure of the food model may affect the EO antimicrobial effect. After the cooking process, mortadella has a

semisolid physical structure that may impact the distribution of oil and impair its antimicrobial effect. Skandamis et al. (2000) studied the performance of oregano oil on *S. typhimurium* in liquid (broth) and gelatin gel revealing that the gel matrix drastically reduced the inhibitory effect of the oil, possibly due to the limitation of diffusion by the structure of the gel matrix.

Our study demonstrated that $NaNO_2$ had activity against *C. perfringens* inoculated in mortadella-type sausages. Jafari and Eman-Djomeh (2007) reported the effect of nitrite on *C. perfringens* in hot dog sausages, and they suggest that the antimicrobial activity is more pronounced in sausages made with higher levels of nitrite, similar to the activity observed in this research. Several mechanisms for the inhibitory effect of nitrite on microorganisms have been reported. Riha and Solberg (1975) proposed that the inhibition of nitrite on *C. perfringens* is by the reaction of nitrite and nitrous acid with SH-constituents of bacterial cells. The reaction of nitrous acid with tiols produces nitrosotiols, which may interfere with the action of enzymes, such as glyceraldehyde-3-phosphate dehydrogenase. In *C. botulinum* nitrite reacts with several iron/sulfur links of certain proteins, such as ferredoxin, to form iron/nitrous oxide complexes, inhibiting the phosphoroclastic system, which involves the conversion of pyruvate to acetyl-phosphate, electron transfer and ATP synthesis (Cammack et al., 1999). Furthermore, they reported the effect of nitrite on DNA, gene expression, membrane damage and cell wall damage. O'Leary and Solberg (1976) reported that *C. perfringens* cells inhibited by 14 mM of nitrite had a dark gray or brown color. The authors postulated that this pigment is associated with cell walls and membranes, suggesting that damage to these structures is the primary event in the activity of nitrite on this microorganism.

Samples elaborated with $NaNO_2$ and EO had significantly reduced populations, suggesting that a combined effect may allow the nitrite reduction and control of *C. perfringens*. However, it is important to emphasize that nitrite has an important role in the formation of sensory attributes typical of cured products, and their reduction should not affect its organoleptic parameters of color, flavor and aroma. The addition of 50 ppm of nitrite to meat products is sufficient for the development of characteristic sensory attributes, nevertheless higher amounts are necessary for microbiological safety (Feiner, 2006). Cui et al. (2010) evaluated the antimicrobial effects of plants extracts combined with $NaNO_2$ against *C. botulinum* and found a synergistic effect between the components suggesting their combined use in *C. botulinum* control. This positive interaction (EO with nitrite) was observed by Ismaiel and Pierson (1990) on *C. botulinum* in laboratory media and ground pork with oregano EO.

In all treatments evaluated, an initial population decrease (day 1), and an increase of *C. perfringens* cell counts between the 10th and 20th days of storage were observed. This pattern of EO inhibition on pathogens inoculated in fresh meat and meat products stored at temperatures higher than cooling (>4 °C) was also reported by Govaris et al. (2010) and Lemay et al. (2002). These results may be explained by the rich nutritional contents that are favorable for growth of target microorganism and temperature of samples storage (25 °C). According to Labbé (2000), *C. perfringens* grows at temperatures between 15 and 50 °C; in addition the spores germination, favored by elevated storage temperature, may lead to a population growth. A possible alternative to control the *C. perfringens* population growth during the shelf-life of the product is the use of combined preservation methods called the "hurdle technology". This technology combines low temperatures, radiation, modified atmosphere packages (MAP) and vacuum packaging (VP), high-pressure techniques and heat processing among others, always aiming to maintain the natural sensory properties of food (Tsigarida et al., 2000; Tassou et al., 1995; Ouattara et al., 2001).

At the end of the storage period (day 30) a pronounced decrease of viable cell counts in all of the treatments was observed, which may have been a result of a decrease in pH (data not shown) in the mortadella samples at the end of the storage period. According to Scott et al. (2001), *C. perfringens* is a spoilage saccharolytic bacterium that ferments carbohydrates, generating final products including butyric and acetic acids, which are able to reduce pH medium. Mortadella has a considerable amount of fermentable carbohydrates (starch), which were metabolized by a large numbers of *C. perfringens* cells inoculated in the food model, resulting in acid production and thereby decreasing the pH. In addition, the possible growth of thermotolerant microbial companion, remnants after the cooking process, which are naturally present in meat and meat products, produces antibacterial factors such as bacteriocins, antimicrobial peptides, ethanol, peroxide and organic acids leading to a reduction in *C. perfringens* counts. High levels of indigenous, nonpathogenic microorganisms may have a protective effect on meat and meat products by out-competing pathogens.

The spore counts increased at the end of the storage period. According to Mitchell (2001), the sporulation is initiated in response to a lack of nutrients, and is affected by pH, oxygen tension and temperature. In general sporulation process is favored by conditions that result in a reduced growth rate.

In conclusion, the synergistic effect between EO and $NaNO_2$ on *C. perfringens* type A inoculated in mortadella-type sausages was observed. The results suggest the combined use of EO and reduced amounts of $NaNO_2$ to control *C. perfringens*, going according current market trends, for natural products. However, EOs alone cannot provide complete protection against *C. perfringens* in mortadella. EOs can be used to increase the bacterial "hurdle" in products that have a strong flavor, thereby reducing the resultant organoleptic properties. The most promising application is the use of EO in conjunction with other preservation techniques to develop a synergistic alternative to current methods. The application of EOs to control pathogenic and spoilage microorganisms in food requires an evaluation of the following aspects: sensory, concentration required for activity, chemical composition of food and interference to the antimicrobial action and the characteristics of the microorganism.

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