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Evaluation of a Three-Component Plant Derived Extract as Preservative for Fresh Retail Meat

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ABSTRACT

The aim of this study was to evaluate the potential of a three-component plant derived extract as a preservative of fresh retail meat. The metabolomic fingerprint of the extract was obtained by High Performance Thin Layer Chromatography. The antibacterial activity of the extract against the spoilage bacteria: *Carnobacterium maltaromaticum*, *Brochothrix thermosphacta*, *Escherichia coli*, *Pseudomonas fluorescens*, *Lactobacillus curvatus* and *L. sakei* was assessed in a broth model meat system. The bacterial growth inhibition zone (mm) ranged from 27.33 ± 0.68 to 30.00 ± 1.00, as was found by disc diffusion test with 100 µL extract, 100 µL ciprofloxacin (wt/v) as positive control and 100 µL sterile distilled water as negative control. The bacterial percent growth reduction ranged from 86.31 ± 1.15 to 90.51 ± 1.15; from 78.79 ± 1.00 to 91.53 ± 2.08; from 61.18 ±1.30 to 69.21 ± 0.50; from 36.56 ± 1.10 to 62.83 ± 1.33 in the broth microdilution method at different extract dilutions (1:10 to 1:100,000). Viable bacterial cells were detected in experimentally-contaminated minced meat up to the second day after treatment (100 µL extract per 10 g meat), except for *C. maltaromaticum* and *P. fluorescens*, which were detected up to the 4th day, by PCR and nested PCR with propidium monoazide (PMA™) dye. On the basis of the reported results the tri-component plant derived extract should be considered as a potential preservative for fresh retail meat.

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE

1,8-cineol (PubChem CID: 12031); lauric acid (PubChem CID:3893); linalool (PubChem CID: 6549) were considered as markers for the commercial product which compounds are from a hydro-alcoholic extract (phytocomplex) of Bay Tree (*Laurus nobilis* L.) berries, Marshmallow (*Althea officinalis L.*) root and English Lavender (*Lavanda angustifolia* Mill) flowers.

INTRODUCTION

Preservatives are used in processed meats for food safety, shelf life and food technology reasons. Their use besides inhibit the growth of micro-organisms while retaining the fresh colour and appearance of red meat. Preservative use is under governmental regulations because of some preservatives can have adverse effects on human health. The levels of nitrates and nitrites in meat are restricted because they can be converted chemicals recognised to cause cancer. Sulphur dioxide exposure bring on breathing difficulties in some people. In addition they can also be regulated to prevent use which is incompatible with other manufacturing processes.

Nowadays, zoonotic food- and water-borne pathogens began resistant to antimicrobials. These resistant strains have been isolated from food and could be entering the human gastrointestinal tract on an almost daily basis. The increasing incidence of food-borne diseases, coupled with the resultant social and economic implications, causes a constant striving to produce safer feed and food, as to develop new natural antimicrobial agents.

Plants and their agro-industrial waste and by-products constituents could be sources of biologically-active substances compared to the current antimicrobials.

The exploration of plant derived extracts as antimicrobial preservatives is an innovative way to find new alternative substances for meat preservation [1,2]. The use of plant derived extracts as preservatives is important since they represent a lower perceived risk to the consumer as well consumer's demand for minimally processed, preservative free products increases. To be suitable the antimicrobial plant derived extracts should be: low cost, eco-friendly, target tailored, besides being effective [3,4].

The International Life Sciences Institute-Europe has developed a comprehensive document on the use of plant materials in food products [5], which stresses that the ingredient for use in food products must be well identified and characterized. The starting material must be accurately identified in order to ensure that the plant materials for food use are consistent with respect to quality and quantity of active ingredient and the method of preparation must meet good manufacturing practices.

Limitations of the use of antimicrobials for meat preservation include inactivation of compounds on contact with the meat surface or dispersion of compounds from the surface into the meat mass. Incorporation of bactericidal compounds into meat products may result in their partial alteration by muscle components knew to significantly affect the efficacy of the antimicrobial substances and their release. So, physical and chemical characteristics of muscle could alter the activity of antimicrobials. In addition, the antimicrobial activity and chemical stability of incorporated active substances could be influenced also by water activity of the meat [6].

The evaluation of biological activity is necessary for screening new antimicrobials from plants.

It is here reported the antibacterial activity of a three-component plant derived extracts against meat spoilage bacteria assessed by two innovative methodologies, High Performance Thin Layer Chromatography and the Broth Meat Model System, for its potential as candidates for fresh retail meat preservation. The High Performance Thin Layer Chromatography allows obtaining the metabolomic fingerprint of the extracts. It is desirable to standardize products and to establish the scientific evidences of their biological activity. In fact the metabolomics approach allows to obtain the widest possible coverage, in terms of the type and number of compounds analyzed. The fingerprint by High Performance Thin Layer Chromatography method was used to determine the herbal composition of the studied product [7-9]. In addition the Broth Meat Model System [10] was used to evaluate directly on meat the potential of the three-component plant derived extract as preservative for fresh retail meat. It also allows to study several spoilage agents at the same time.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The meat spoilage bacteria, namely *Carnobacterium maltaromaticum* (ATCC® 43224™), *Brochothrix themosphacta, Escherichia coli, Pseudomonas fluorescens, Lactobacillus curvatus* (ATCC® 25601™) and *Lactobacillus sakei* were considered in the experiment [11-14].

B. thermosphacta, E. coli, P. fluorescens and *L. sakei* were previously isolated and characterized [10,15] and then maintained in Microbank™ vials at -70 °C.

All of the Lactic Acid Bacteria examined in the experiment did not produce bacteriocins. *Escherichia coli* presence is considered an indicator of the quality of packed meat. A high presence of *E. coli* (higher than 100 per g) on stored meat could indicate temperature abuse, because it does not grow below 7 °C. *E. coli* presence may also indicate a food safety issue. Bacteria growth media and conditions are reported in **Table 1**. The reference strains from American Type Culture Collection were grown on media and at the growth conditions as reported on products sheets.

For antibacterial activity assay, 1mL of each culture was diluted to 10^5-10^6 CFU/mL.

Plant Derived Extract

The three-component plant derived extract considered is a commercial product by Caira Laboratorio Erboristico. It is a hydro-alcoholic extract of Bay Tree (*Laurus nobili*s L.) berries, Marshmallow (*Althea officinali*s L.) root and English Lavender (*Lavanda angustifolia* Mill) flowers.

Extracts utilized as mono-ingredient standards were hydro alcoholic extracts (10 g/10 mL) obtained from the market or by a lab extraction of identified herbal raw materials, the last also used as reference to confirm the identities of the marketed ones. Detailed information, i.e. producers, production conditions, storage method, etc. can be obtained by directly asking the authors.

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Table 1. Bacteria growth media and growth conditions used in the experiment.

Bacteria	Growth media	Growth conditions
Carnobacterium maltaromaticum ATCC® 43224™	Brain Heart Infusion Agar/Broth (Becton, Dickinson Italia, Milan, Italy)	at 26 °C for 24 h
Brochothrix themosphacta	Tryptic Soy (Oxoid SpA, Milan, Italy)	at 22 °C for 48 h
Escherichia coli	70722 HiCrome™ *E. coli* Agar/Broth B (Fluka Sigma-Aldrich, Milan, Italy)	at 37 °C for 48 h
Pseudomonas fluorescens	Nutrient Agar/Broth (Fluka Sigma-Aldrich, Milan, Italy)	at 35 °C for 24 h
Lactobacillus curvatus ATCC® 25601™	Lactobacilli MRS Agar/Broth (Becton, Dickinson Italia, Milan, Italy)	at 37 °C under the atmosphere of 5% CO_2 for 24-48 h
Lactobacillus sakei	Lactobacilli MRS Agar/Broth (Becton, Dickinson Italia, Milan, Italy)	at 30 °C for 48 to 72 h in an atmosphere of 5% CO_2 .

Metabolomic Fingerprint

Equipment For Chemical Analysis

Total composition of the three-component plant derived extract was obtained by High Performance Thin Layer Chromatography. The High Performance Thin Layer Chromatography system consisted of sample applicator using 100 µL syringes and connected to a nitrogen tank; automatic developing chamber containing twin trough chamber 20x10 cm; Immersion device III; TLC Plate Heater III; TLC visualize linked to winCATS software. Glass plates 20 cmx10 cm with glass-backed layers silica gel 60 (2 µm thickness). Before use, plates were prewashed with methanol and dried for 3 min. at 100°C.

Development and Derivatization

The dried extracts of the analysed samples were weighted and dissolved in methanol (30 mg/mL). Filtered solutions were applied with nitrogen flow. Then the High Performance Thin Layer Chromatography plates were developed in the automatic developing chamber ADC 2, saturated with the same mobile phase, EtOAc/CH2Cl2/CH3COOH/HCOOH/H2O (100:25:10:10:11; v/v) for 20 min. at room temperature. The length of the chromatogram run was 70 mm from the point of application. The developed layers were allowed to dry on plate heater for 5 min at 120°C and then derivatized with a selected solution, including anisaldehyde-sulfuric acid (1 ml anisaldehyde, 10 ml sulfuric acid, 20 ml Acetic acid in 170 ml methanol) and NPR (1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate). Finally, the plates were warmed for 5 min at 120°C before inspection. All treated plates were then inspected under UV light at 254 or 366 nm or under reflectance and transmission white light (WRT), respectively, at a CAMAG TLC visualizer, before and after derivatization.

Validation

Sample solutions of the extracts were found to be stable at 4°C for at least 1 month and for at least 3 days on the High Performance Thin Layer Chromatography plates. Repeatability was determined by running a minimum of three analyses. Ratio between the migration distance of substance and the migration distance of solvent front (Rf) for main selected compounds varied ± 0.02%. The effects of small changes in the mobile phase composition, mobile phase volume, duration of saturation were minute and reduced by the direct comparison. On the contrary, the results were critically dependent on pre-washing of High Performance Thin Layer Chromatography plates with methanol.

Assessment of Antibacterial Activity

The antibacterial activity of the three-component plant derived extract was assessed by the Broth Meat Method System as previously reported [16]. It consists of three steps: i) growth inhibition on solid medium using the standardized disc diffusion method (100 mL three-component plant derived extract 1:1 v/v in sterile distilled water); ii) percent growth reduction using broth microdilution method in conventional sterile polystyrene flat bottom microplates of 96 wells (Corning, Sigma Aldrich, Milan Italy), each well filled with 100 µL of sterile suitable liquid media for each microorganism considered, 50 µL of inoculum and amount of extract at lower dilutions 1:10 to 1:10,000; bacterial growth was determined by OD reading at 630 nm/10 mm path-length with an ELISA microplate reader then bacterial cell concentration was transformed to cells/mL using the reference curve equation. iii) experimentally minced vacuum-packed meat (10 g) inoculation with each bacterium (ca. 10^6 CFU/mL) and treatment with three-component plant derived extract (100 µL) at 10 °C, to simulate an abusive refrigeration, for 12 days.

Detection and identification of bacteria from experimentally treated samples with the extract were carried out using molecular biology and microbiological techniques at two day intervals up to the 12th day of refrigerated storage.

DNA extraction was performed using ChargeSwitch® gDNA Mini Bacteria Kit (Life Technologies Italia, Monza, MB, Italy) following manufacturer's instructions. The molecular identification and characterization was made using specific primer pairs for each bacterium as reported in **Table 2**. Mixture and reaction conditions were those reported in literature as shown in **Table 2**. PMA™, a photo-reactive dye with high affinity for DNA that intercalates into DNA and forms a covalent linkage upon exposure to intense visible light, allows selective detection of the sole live cell (PMA™ Biotium Inc., Hayward, CA, USA, www.biotium.com) [17].

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Table 2. Primer pairs used for detection of the bacteria considered in the experiment. They amplify unique species-specific genomic sequences of each bacteria.

Bacteria	Primer pairs sequences	Reference
Carnobacterium maltaromaticum ATCC® 43224™	Cb1 5′-CCGTCAGGGGATGAGCAGTTAC-3′ Cb2r 5′-ACATTCGGAAACGGATGCTAAT-3′	Yost et al., 2000
Brochothrix themosphacta	pA 5′-AGAGTTGATCCTGCCTCAG-3′ pE 5′-CCGTCAATTCCTTTGAGTTT-3′	Xu et al., 2010
Escherichia coli	Ec1 5′-CCGATACGCTGCCAATCAGT-3′ Ec2 5′-ACGCAGACCGTAAGGGCCAGAT-3′	Osek, 2001
Pseudomonas fluorescens	16SPSEfluF 5′-TGCATTCAAAACTGACTG-3′ 16SPSER 5′-ATCACACCGTGGTAACCG-3′	Scarpellini et al., 2006
Lactobacillus curvatus ATCC® 25601™	Y1 5′-TGGCTCAGAACGAACGCTGGCCCG-3′ Y2 5′-CCCACTGCTGCCTCCCGTAGGAGT-3′ and 16S 5′-GCTGGATCACCTCCTTTC-3′ Lc 5′-TTGGTACTATTTAATTCTTAG-3′	Yost et al., 2008
Lactobacillus sakei	Y1 5′-TGGCTCAGAACGAACGCTGGCCCG-3′ Y2 5′-CCCACTGCTGCCTCCCGTAGGAGT-3′ and 16S 5′-GCTGGATCACCTTTC-3′ Ls 5′-ATGAAACTATTAAATTGGTAC-3′	Yost et al., 2008

Amplified products (7 µL) were analyzed by electrophoresis in 2% or 3% agarose gels buffered in 0.5× TBE (TBE buffer: 90 mM tris(hydroxymethyl)aminomethane, 90 mM boric acid, 3 mM ethylediaminetetraacetate Na salt, pH 8,3) against a 50 bp, 100 bp and 1 Kb ladder used as size marker (Invitrogen, Milano, Italia) and visualized by UV light at 260 nm after staining with a fluorescent dye, Gel Green dye The results were recorded as the means ± SD of the duplicate experiment considering three repetitions for each experiment. Differences between the means of data were compared by LSD calculated using the SAS.

RESULTS

Metabolomic Fingerprint

Fingerprint of three-component plant derived extract was in good accordance with the mixture of three extracts obtained in the laboratory from identified raw materials. It was necessary to perform several derivations and revelations in order to evidence the constituents, as many as possible. Most relevant chromatographic results are reported in **Figure 1**, but other chromatographic analyses can be obtained under request.

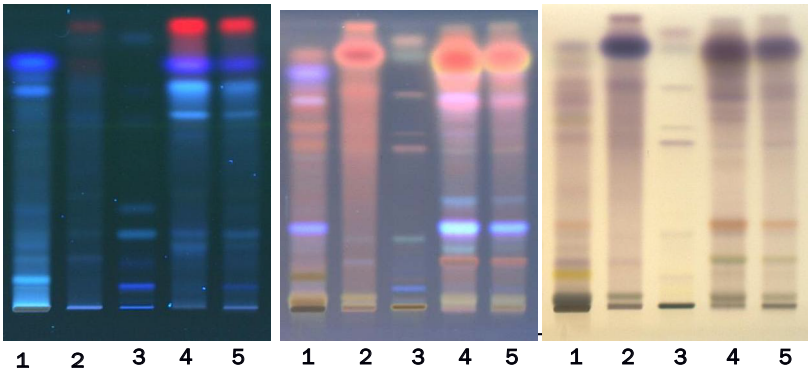

Figure 1. High Performance Thin Layer Chromatography analysis on the three-component plant derived extract containing Bay Tree (*Laurus nobilis* L.), Marshmallow (*Althea officinalis* L.) and English Lavander (*Lavandula angustifolia* L.). A: Visualization: 366 nm; Derivatization: None. B: Visualization: 366 nm; Derivatization: NP reagent and Anhysaldeide; C: Derivatization: NP reagent and Anhysaldeide; Visualization: white light, upper and lower;. Tracks: 1, marketed analysed product; 2, Bay Tree (*Laurus nobilis* L.) extract standard fingerprint; 3, Marshmallow (*Althea officinalis* L.); 4, English Lavander (*Lavandula angustifolia* L.) extract standard fingerprint; 5. Mixture of extracts of tracks 2-4.

Assessment Of Antibacterial Activity

The obtained results show that the extract is effective against all the spoilage bacterial agents considered. The antibacterial activity was evaluated based on the diameters of the clear growth inhibition zone surrounding the paper disks soaked with 100 µL of the extract. As presented in **Table 1**, the average of the growth inhibition zone (mm) ranged from 27.33 ± 0.68 to 30.00 ± 1.00. There was no significant difference between the growth inhibition zone of the extract and ciprofloxacin with the exception of *C. maltaromaticum. E. coli* and *C. maltaromaticum* resulted the least susceptible to the extract and the antibiotic among the tested bacteria (**Table 3**).

The highest bacterial growth reductions (%) were observed with 100 µL and 10 µL of extract. They ranged from 86.31 ± 1.15 to 90.51 ± 1.15 and from 78.79 ± 1.00 to 91.53 ± 2.08, respectively. There was significant differences in the percent of the bacterial

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growth reduction revealed at 100 µL and 10 µL of the extract among the considered bacteria. *E. coli* and *L. curvatus* were the most susceptible at 100 µL while Lactic Acid Bacteria resulted more susceptible at 10 µL **(Table 4)**.

Table 3. Antibacterial activity of tri-component plant derived extract against spoilage bacteria detected by the disc diffusion method as growth inhibition zone.

	GIZ (mm)*		
Bacteria	**Treatment**		
	TC-PDE	**WTR**	**CFX**
Carnobacterium maltaromaticum	27.33 ± 0.68 a	-	29.00 ± 1.00 b
Brochothrix thermosphacta	29.53 ± 1.05 a	-	29.12 ± 1.00 a
Escherichia coli	28.83 ± 1.10 a	-	27.81 ± 1.00 a
Pseudomonas fluorescens	30.00 ± 1.00 a	-	29.31 ± 1.73 a
Lactobacillus curvatus	28.53 ± 0.48 a	-	29.11 ± 1.00 a
Lactobacillus sakei	27.80 ± 0.30 a	-	28.33 ± 2.08 a

* Diameter of inhibition zones, including the diameter of the disc (6 mm). TC-PDE, tri-component plant derived extract; WTR, sterile distilled water; CFX, ciprofloxacin (1 mg/mL). Three disks papers per plate and three plates for each bacterium were considered. The experiment was repeated twice. - absence of inhibition zone. Values expressed are as the mean ± standard deviation of two experiments. Mean values with a different letter in the row are significantly different ($p \leq 0.05$).

Table 4. Bacterial growth reduction (GR%) at 24 h in liquid medium with differences in the percentage of bacterial growth reduction at different concentrations of extract using the control treatment as reference (without extract).

	GR (%)			
Bacteria	**(100 µL)**	**(10 µL)**	**(1 µL)**	**(0.1 µL)**
Carnobacterium maltaromaticum	89.25 ± 1.53 c	88.21 ± 1.00 b	67.56 ± 1.13 b	39.41 ± 1.08 a
Brochothrix thermosphacta	86.31 ± 1.15 d	81.20 ± 1.00 d	61.18 ± 1.30 d	36.56 ± 1.10 a
Escherichia coli	90.51 ± 1.15 b	85.70 ± 1.00 b	62.48 ± 1.00 c	60.16 ± 1.14 c
Pseudomonas fluorescens	86.86 ± 1.00 d	78.79 ± 1.00 e	69.21 ± 0.50 a	61.88 ± 1.00 c
Lactobacillus curvatus	94.50 ± 1.53 a	91.53 ± 2.08 a	68.39 ± 1.80 b	62.83 ± 1.33 c
Lactobacillus sakei	89.31 ± 1.00 c	89.41 ± 0.58 a	69.17 ± 0.00 a	57.58 ± 0.89 b

Values expressed as the mean ± standard deviation of two experiments (three repetitions for each experiment). Mean values with different letters in the column are significantly different ($p \leq 0.05$).

Amplicons of the expected sizes were detected directly in experimentally-inoculated minced vacuum-packed meat up to the second day after treatment with the exception of Lactic Acid Bacteria, which were detected up to 4th day after treatment. Bacteria were always detected in the control samples (water) at the 2nd, 4th, 6th, 8th, 10th and 12th storage days, but never in samples treated with ciprofloxacin collected on the same storage days **(Table 5)**. The microbiological detections of microorganisms by official methodologies were in agreement with the molecular biology detections carried out as previously described at each interval to reveal the antibacterial activity in experimentally contaminated meat. The numbers of viable bacterial cells were significantly ($p \leq 0.05$) lower respect the inocula used to experimentally contaminate meat at each interval considered.

Table 5. Detection and identification by PCR and nested PCR of the tested bacterial strain viable cells in vacuum-packed minced beef meat stored at 10°C at 2, 4, 6, 8, 10 and 12 days after treatment with extract, water and ciprofloxacin.

	Detection of viable cells after treatments (100 µL for each treatment)																	
	2 days			4 days			6 days			8 days			10 days			12 days		
Bacteria	W	C	T	W	C	T	W	C	T	W	C	T	W	C	T	W	C	T
C. maltaromaticum	+	+	+	+	+	–	+	–	–	+	–	–	+	–	–	+	–	+
B. thermosphacta	+	+	–	+	–	–	+	–	–	+	–	–	+	–	–	+	–	+
E. coli	+	-	–	+	+	–	+	–	–	+	–	–	+	–	–	+	–	+
P. fluorescens	+	+	+	+	–	–	+	–	–	+	–	–	+	–	–	+	–	+
L. curvatus	+	–	–	+	–	–	+	–	–	+	–	–	+	–	–	+	–	+
L. sakei	+	–	+	+	–	–	+	–	–	+	–	–	+	–	–	+	–	+

T- tri-componenet plant derived extract; W- sterile bi-distilled water; C- ciprofloxacin.
+:detected; – :not detected

DISCUSSION AND CONCLUSIONS

The contamination of food and spoilage by microorganisms is a major concern to consumers, government authorities and food industries. The technologies, used to increase the storage time and to ensure the safe consumption of highly perishable products, such as meat, have undergone a continuous evolution over the time, in response to the needs of consumers and industry [18-20]. The new sustainable solutions in meat packaging have to ensure the safety and quality of food and to reduce food losses and

environmental impact. Food packaging plays a crucial role in preserving the quality and safety of food during distribution and storage from farm to fork. The need to use materials more sustainable and more compatible with food, represents a new market and leads to an intense activity in the study of natural substances for the production of biodegradable wrapping and edible coatings. Beside the diffusion of active packaging, systems capable of interacting dynamically with the food, and/or with the atmosphere, in order to save the healthiness of the product and to extend its shelf life, are increasing [21]. The effectiveness of these systems has been improved with the use of film activated by antibacterial substances and chemical or natural preservatives slow release [22]. An antimicrobial packaging, active against spoilage microorganisms and/or pathogens, can prolong the shelf life and improve the safety for all types of foods, especially those processed [23,24] . In addition, interest in the use of active as well as intelligent packaging systems for meat and meat products has increased in recent years [25,26]. In recent, the incorporation of natural antimicrobial substances into edible films has attracted great interest, as alternative to control or reduce the growth of foodborne and spoilage microorganisms [27].

Traditional Medicine experienced for thousands of years the properties of herbs. Plants were selected on the experience on high numbers of individuals and results are recorded in a large quantities of books. However, the enormous quantity of information needs to be revised and adapted to the current marketed forms. The compounds of the plants composing the three-component plant derived extract studied show inter alia antimicrobial activity [28-33]. These plants were evaluated as herbal medicines for human use by European Medicine Agency [34-36].

The obtained results showed the antibacterial activity of the studied phytocomplex against the main spoilage agent of muscle, confirming its potential use as preservative for fresh retail meat. Accordingly with literature and High Performance Thin Layer Chromatography analysis, the activity should be related to the predominance of essential oils and polyphenols in the extracts.

Quantitative variety in composition of plant extracts is normality. Several factors pre- and post-harvesting can contribute to change the percentage of several constituents, in particular in case of secondary metabolites [37-39]. Analytical control of quality is even more complicated in case of a multi-ingredient product. An application of the High Performance Thin Layer Chromatography fingerprint method is here reported, as specific application to determine the identification of utilized plants and quali- and quantitative pattern, in order to maintain the correspondence between composition and activity.

In consideration of the multi-resistance of micro-organisms to the preservatives in use, exploration of substances with antibacterial activity is necessary and natural products from plants can give important advances. The search for other natural additives continues as well before a preservative is used in a food, it is necessarily checked to ensure it does not alter taste or colour and can be easily incorporated [40,41].

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AUTHOR'S CONTRIBUTIONS

Research concept and design: Paola Del Serrone. Collection and/or assembly of microbiological data: Paola Del Serrone. Collection and/or assembly of chemical data: Chiara Toniolo and Marcello Nicoletti. Statistical analysis: Paola Del Serrone. Data analysis and interpretation: Paola Del Serrone, Chiara Toniolo and Marcello Nicoletti. Writing the article: Paola Del Serrone, Marcello Nicoletti. Critical revision of the article: Paola Del Serrone, Chiara Toniolo and Marcello Nicoletti. Final approval of article: Paola Del Serrone, Chiara Toniolo and Marcello Nicoletti.

CONFLICTS OF INTEREST

The authors declare no conflict of interest

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