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Full Length Research Paper

Potential application of plant essential oils at sub-lethal concentrations under extrinsic conditions that enhance their antimicrobial effectiveness against pathogenic bacteria

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The aim of the present study was to assess the effect of extrinsic factors on the antimicrobial effectiveness of essential oils (EOs) against medically important gram positive and gram negative bacteria. Clove, oregano, bay and cinnamon essential oils were tested against *Salmonella typhimurium*, *Escherichia coli*, *Bacillus cereus* and *Listeria innocua*. Agar diffusion test was used to estimate the strength of the essential oil. Clove was most effective against all groups of bacteria. The minimum inhibition concentrations (MIC) of the four essential oils ranged from 1.25 to 3.50% (v/v) as determined by turbidimetric technique using Biocreen C, analyzer. In order to test the combined effect of pH, sodium chloride, temperature and EOs on the growth of bacteria, the concentrations of the EOs were set at 0.03125% (v/v) below MICs. A synergistic effect against the test bacteria was achieved at the concentration 1.2% (w/v) NaCl and either oregano or clove oil (P < 0.01), however clove was bactericidal on both classes of bacteria at 37°C, pH 5.5 and in combination with NaCl at 1.8% (w/v) pH (7.3), suggesting that EOs can be combined with NaCl at low concentrations overcome organoleptic effects in food and inhibit growth of pathogenic bacteria.

Key words: Antimicrobial, bacteria, essential oils, extrinsic factors.

INTRODUCTION

Essential oils (EOs) are aromatic oil liquids obtained mostly from plant material. They exhibit antiviral, antibacterial, antimycotic, antitoxigenic, antiparasitic and insecticidal properties and their use is allowed in food (Burt, 2004; Vukovic et al., 2007). Antimicrobial activities of EOs and their components have been exploited in controlling pathogenic and food spoilage bacteria (Azzouz and Bullerman, 1982; Deans and Ritchie, 1987; Akgul and Kivanc, 1989; Hao et al., 1998; Hseih et al., 2001; Grande et al., 2007; Sinigaglia et al., 2008). Essential oil comprise more than 60 unique components, the major component constitutes up to 85% of the EO, whereas minor components are present only as a trace; minor components could have a critical role in the antibacterial activity, possibly by producing a combined effect (Burt, 2004). Terpenoid and phenolic (thymol, carvacrol, eugenol) components are chiefly responsible for the antibacterial properties of several EOs (Dorman and Deans, 2000; Ultee et al., 2000).

Steam distillation is the most commonly used method for producing EOs, but other methods can be employed (expression, fermentation, enfleurage or extraction) and their selection can influence chemical composition and therefore organoleptic properties and antimicrobial activity (Corbo et al., 2009). The main advantage of natural agents is that they do not enhance the ‘‘antibiotic resistance’’, a phenomenon commonly encountered with the long-term use of synthetic antibiotics (Vukovic et al., 2007).

The essential oils and their components are known to be active against a wide variety of microorganisms,

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including gram-negative and gram-positive bacteria (Ouattara et al., 1997; Smith-Palmer et al., 2001; Marino et al., 2001; Lambert et al., 2001; Delaquis et al., 2002; Pintore et al., 2002; Harpaz et al., 2003). Gram-negative bacteria were shown to be generally more resistant than gram-positive ones to the antagonistic effects of essential oils because of the lipopolysaccharide present in the outer membrane which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Ratledge and Wilkinson, 1988; Vaara, 1992) but this was not always true (Vukovic et al., 2007).

In general, higher concentrations of essential oils are required in foods than in laboratory media (Viuda-Martos et al., 2008), accordingly, the practical application of essential oils is restricted, since effective antimicrobial doses most often exceed organoleptically acceptable levels. However, if essential oils were to be more widely applied as antibacterials in foods, it must be taken into account that the antibacterial efficiency is diminished when they are added to more complex materials (such as food products) and the organoleptic impact would be important and also that issues of safety and toxicity will need to be addressed (Viuda-Martos et al., 2008)

Therefore, study on the minimum inhibitory concentration (MIC) of essential oil and factors that would enhance minimal use of EOs in foods is timely to enable striking a balance between sensory acceptability and antimicrobial efficacy (Bhurinder et al., 2001; Burt, 2004; Viuda-Martos et al., 2008). Moreover a number of potential synergists have been suggested for use with EOs: pH and temperature (Valero et al., 2003; Calsamiglia et al., 2007), low water activity (Guynot et al., 2005), chelators and low oxygen tension (Tsigarida et al., 2000), mild heat (Karatzas et al., 2000) and raised pressure (Karatzas et al., 2001), although not all of these have been researched in foodstuffs.

However, sodium chloride has been shown to work as a synergist and an antagonist under different circumstances with EOs and/or their components. It has also been suggested that the effectiveness of EOs could be improved in combination with mild preservation methods. Salt has traditionally been used in preservation of food but there is little evidence of its combination with EOs to inhibit the growth of microbes in experimental conditions and in foods. The aim of the present study was to (i) determine the effect of pH and sodium chloride on the antimicrobial effectiveness of essential oils of oregano, clove, bay and cinnamon on both gram positive and negative bacteria and (ii) explore the potential of the use of minimal concentrations of essentials in combination with salt to overcome the adverse effects of essential oils in foods under different pH conditions.

MATERIALS AND METHODS

The bacteria used in the present study were *Bacillus cereus* WU10 obtained from the National Collection of Industrial Marine Biology (NCIMB no. 3329), *Escherichia coli* WU40 W1485 K12 obtained from Cardiff University, *Salmonella typhimurium* WU73 obtained from Cardiff University and *Listeria innocua* WU 507 obtained from Nation Collection of Type Cultures (NCTC no. 11288). All the bacteria were stored at 4°C in the medium term on tryptone soya agar (TSA). Active cultures for the experiments were prepared by transferring a loopful of cells from stock cultures to universal glass bottles containing 10 ml Tryptone Soya Broth (TSB) which were incubated overnight at 37°C without agitation in order to obtain cells in exponential phase. The overnight cell concentrations were approximately 10^9 cell ml^{-1}. Dilutions were made with sterile distilled water to achieve bacterial densities of 10^6 cell ml^{-1} prior to experiment.

Essential oils of oregano (Code no. 001128, Batch no. PD 40208), cinnamon (Code no. 000788, PD 042893), bay (Batch no. Q404T) and clove (Code no. 016309, Batch no. 029095) were used in the study. The oils contained no synthetic chemicals or unnatural components. All the essential oils were stored in brown bottles covered with aluminium foil at 4°C protected from light and air. The oils were serially diluted in distilled water by vortexing to obtain homogeneous mixtures prior to experiments.

The growth media TSA and TSB were procured from International Diagnotics Group Plc, UK. TSA was used as growth medium for agar diffusion test. TSB was used as the growth medium in bioscreen C experiments. The media were prepared according to the manufactures instruction. Acetic acid at concentrations of 6.05 mg/ml was used throughout the experiment to adjust the pH of the growth medium while sodium chloride was used for adjusting the water activity levels of the growth media.

Minimum inhibition concentrations (MIC) of the antibacterial activity of the four essential oils (oregano, clove, bay and cinnamon) were estimated by disc diffusion method. Culture plates were prepared with TSA and bacterial culture (10^6 cell ml^{-1}) spread uniformly on the surface of the media. 10 fold serial dilutions of essential oils were prepared and kept at room temperature (25°C) prior to experiment. 40 µl of the serially diluted essential oils were dropped into the wells. One of the wells was filled with chlorophor, an antimicrobial agent, which served as a positive control while for negative control; sterile distilled water was used. The treated plates were incubated at 37°C for 48 h and readings taken at intervals of 24 h. The plates were visually inspected for any zone of inhibition around the wells and the diameter of the zone of inhibition measured. Duplicate plates were prepared for each test.

The MICs of the antimicrobial activity of the essential oils were also determined precisely by turbidimetric technique using bioscreen C, analyser. Essential oils were serially diluted to obtain concentrations ranging between 0.03125 to 1%. 40 µl of the diluted samples were then added to 300 µl TSB before finally adding 60 µl of bacteria at concentrations of 10^7 cell ml^{-1} in the microtitre well to make up a maximum volume of 400 µl. The concentration of the oil in the microtitre well was reduced accordingly to 10% of the original value, thus the range become 0.03125 to 0.1 and that of the bacteria to 10^6 cell ml^{-1}. The microtitre wells were then incubated at 37°C for 20 h and growth curves is obtained. The lowest concentration of the oil that inhibited growth was obtained as the MIC.

The effects of essential oil, pH and water activity on the lag phase, exponential phase and maximum yield were studied. Each experimental well on the microtitre plate contained 300 µl of TSB, 40 µl of dilute essential oil and 60 µl of 10^6 cell ml^{-1} bacterial concentrations. For controls, the wells contained either TSB only, TSB, distilled water and bacteria, or TSB and oil only. Incubated was done at 37°C in the Labsystems Bioscreen C (Life Sciences International, Basingstoke, UK). The increase in turbidity at 600 nm was monitored automatically every 15 min for 20 h.

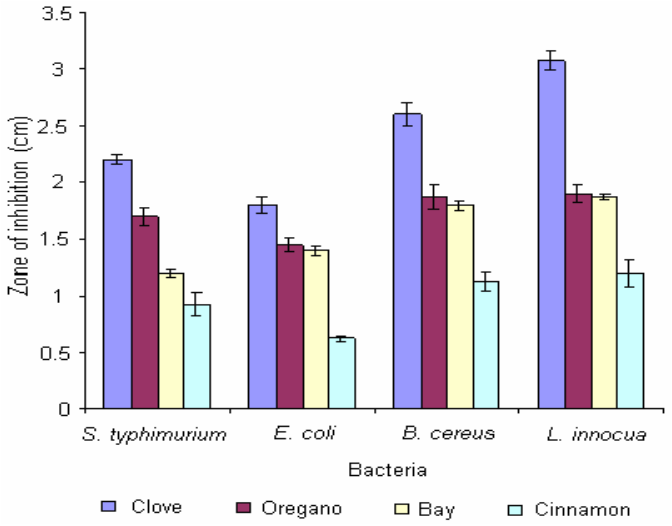

Figure 1. Antimicrobial sensibility pattern of clove, oregano, bay and cinnamon 10^{-1} (v/v) against *S. typhimurium*, *E. coli*, *B. cereus* and *L. innocua* obtained by agar diffusion method.

RESULTS

Determination of the effectiveness of the EOs and estimation of MICs against the test bacteria were done by agar diffusion test (Figure 1). Clove EO oil was the most effective against both gram negative and positive bacteria followed by oregano, bay and cinnamon, respectively. Gram negative bacteria (*E. coli* and *S. typhimurium*) were generally less susceptible than the gram positive bacteria (*B. cereus* and *L. innocua*). The zones of inhibition produced by oils diluted to 10^{-2} were insignificant. Bay and cinnamon did not produce any zone of inhibition at this dilution. The results suggested that the type of the bacteria may influence the effectiveness of the antimicrobial activity of the EO as gram negatives showed significantly smaller zones of inhibition compared to gram positives. The four EOs had varying degree of effectiveness within and between the classes of the test bacteria, suggesting that the oils contained active components that were either missing or present at varying levels from each other. An accurate measure of the MIC was determined by turbidimetric technique using bioscreen C analyser. The MIC levels of the four essential oils ranged from 1.25 to 3.5%. Clove and oregano had much lower MICs than bay and cinnamom (Table 1).

In order to test the influence of sodium chloride (water activity) and pH on the effectiveness of the EO, the amount of the EO was lowered to 0.03125% (v/v) below the MIC levels. This concentration of the EO was determined from a series of preliminary experiments. It proved effective at variable pH conditions (Table 2) against the four test bacteria. The results on the antimicrobial effectiveness of the EOs against the test bacteria (Table 2) showed insignificant difference within the classes of the bacteria. Therefore the subsequent experiments where done with *E. coli* and *B. cereus* as representatives of the two classes of the bacteria using the two most effective EOs clove and oregano (Table 3).

The effect of NaCl on the lag time and mean generation time of the test bacteria showed that the effectiveness of the oil was enhanced at concentrations of 1.0 - 1.6% (w/v) of NaCl. Concentrations of NaCl above this range proved bactericidal in presence of EO. However combining the EO with NaCl at concentrations below 1.0% (w/v) did not show any significant effects on the lag time and mean generation time of the tested bacteria that could be attributed to the effect of the NaCl. Therefore the range 1.0 to 1.6% (w/v) of NaCl in presence of EO was realistic to produce a long lag time but not to inhibit growth completely. Statistical analysis (two way analysis of variance) of the results of experiments involving combination of sodium chloride with EO of clove and oregano on the growth rate of both classes of bacteria showed that the effect was highly significant ($P < 0.01$). The results showed that it was possible to achieve a greater inhibitory effect against all classes of bacteria under conditions of 1.8% (w/v) NaCl and clove (0.03125%) (v/v).

DISCUSSION

The present study has shown that plant essential oils of oregano, clove, bay and cinnamon are active against both gram positive and gram negative bacteria but at varying degrees of effectiveness depending on extrinsic factors. The type of bacteria also has an influence on the effectiveness of the EO Gram negative bacteria were generally less susceptible than gram positive. This was in agreement with Delaqiuis et al. (2002) who reported the susceptibility of gram positive bacteria to EOs as compared to gram negative bacteria.

The difference in susceptibility of the bacteria to an EO is thought to arise as a result of the differences in their cell membrane structure. The cell envelopes of gram-negative bacteria are more complex than the cell wall of gram-positive bacteria. Gram-negative bacteria are composed of two layers that protect the cell and provide rigidity. Gram-positive bacteria lack the outer membrane thus the reason why they would be more susceptible to action of phenolic components of EOs. EOs and their components are hydrophobic, a characteristic that enables them to partition in the lipids of the bacterial cell membrane and mitochondria, distorting the structure and rendering them more susceptible to antimicrobial action leading to leakage of the cell content. Extensive loss of cell content would eventually lead to death (Sikkema et al., 1994).

That clove essential oil was the most effect is perhaps to be expected since the relative abundance of its active component (eugenol) is approximated 88.9% in

Table 1. MIC of the four test essential oils and bacteria obtained by Bioscreen C, turbidimetric technique. Tests were done in three replicates and the concentrations of the oil are given as percentage.

		Essential oil MIC (%)			
	Bacteria	**Clove**	**Oregano**	**Bay**	**Cinnamon**
Gram negative	*S. typhimurium*	2.50 ± 0.05	2.55 ± 0.05	2.80 ± 0.05	3.50 ± 0.10
	E. coli	2.50 ± 0.03	2.50 ± 0.05	2.75 ± 0.05	3.05 ± 0.05
Gram positive	*B. cereus*	1.25 ± 0.05	1.50 ± 0.05	1.75 ±0.10	2.05 ± 0.05
	L. innocua	1.30 ± 0.05	1.55 ± 0.05	1.80 ± 0.05	2.05 ± 0.05

composition. The second most effective oil was oregano with cavarcrol as its major component at 70% in composition and the least effective being cinnamon EO. Carvacrol is capable of disrupting the outer membrane of gram-negative bacteria, releasing polysaccharides and increasing the permeability of cytoplamic membrane to ATP (Helander et al., 1998). Eugenol, the major component of clove has been shown to inhibit production of amylase and proteases by *B. cereus* at sub-lethal concentrations. The hydroxyl group of eugenol is thought to bind to proteins preventing enzyme action (WendaKoon and Sakaguchi, 1995; Ultee et al., 2002).

It is now generally accepted that no single preservative will be effective against pathogenic bacteria and the use of high concentrations of EOs as a preservative method has shown to cause organoleptic effects in foods (Burt, 2004; Viuda-Martos et al., 2008). The present study has shown that combination of EO and sodium chloride at near neutral pH has worked synergistically to inhibit growth of bacteria. Since the oil was used at very low concentrations in this preservation system, it has the potential of overcoming the organoleptic effects associated with high use of EOs in food and food products. Usually MIC levels have no impact on the flavour of foods but the impact may vary from mild to severe with increasing concentrations. Other factors that may influence this phenomenon include temperature and pH (Tsigarida et al., 2000). Therefore the use of very low concentrations of EOs in combination with sodium chloride (a preservative) at neutral pH as described in the present study has the potential of overcoming organoleptic effects in food and inhibits bacterial growth.

The synergistic effects achieved by combining preservative methods where EOs and their components are used have been documented (Marino et al. 2001). However this is a novel case where synergism has been achieved by combining sub-lethal concentrations of EOs with sodium chloride at near neutral pH against both gram negative and positive bacteria. Sodium chloride works synergistically with EOs by rendering the cells more susceptible to the action of the essential oil. It has been shown that with a higher saline concentration, a greater bacterial surface hydrophobicity may facilitate EO penetration or contact with microorganism. This could explain why it was possible to inhibit bacterial growth by combining EOs at concentrations of 0.03125% v/v with NaCl (1.8%).

The combination of 1.2% (w/v) of NaCl and clove essential oil at concentration of 0.03125% (v/v) proved very effective in delaying the growth of *E. coli*. The concentration of 1.2% (w/v) of NaCl seemed to work best with the clove oil to produce a synergistic effect that slowed down the growth of the bacteria. This concentration of NaCl appeared to have been enough to overcome the effect of amino compounds of the cell wall proteins that act to protect the cell and to allow the penetration of the oil (Juven et al., 1994). The levels are usually higher in food than in culture medium. This is possibly due to interactions between phenolic compounds and food matrix (Ultee and Smid, 2000). In addition this study has also shown a potential of overcoming this hurdle by use of combination of preservation systems.

It has been suggested that physical conditions like pH, temperature and low oxygen levels could improve the action of EOs (Burt, 2004). This study has shown that low pH facilitated the action of EOs of clove and oregano to improve their effectiveness against both gram-positive and gram-negative bacteria. To ensure that the inhibitory effect of the acid did not overshadow that of the EO, very low concentrations of the acid were used, as low as 6.5 mg/ml. This allowed the use of sub-lethal concentrations of EOs. This combined effect of pH and EOs thus acted additively to cause inhibition of the test bacteria.

Susceptibility of bacteria to antimicrobial effect of EOs may increase with decrease in temperature and available oxygen (Nychas, 1995). Investigative experiments carried out here were done at temperatures of 37°C and under aerobic conditions. Given the paucity of the data, it was not possible to ascertain the effectiveness of the EOs under temperatures lower than 37°C. Nevertheless it would be interesting to establish the effect of lower temperatures on effectiveness of the tested EOs under these combined conditions of EOs and NaCl under varied pH conditions.

The medium in which the oil is mixed into may influence the survival of the microbe. For example, it has been shown that, depending on the mean droplet size of the mixture of oil and water, bacteria can grow in films, colonies or as planktonic cells (Brocklehurst et al., 1995).

Table 2. Mean generation time (Mgt) and lag time (Lt) in minutes at pH 5.5 to 7.3 in presence oregano or clove essential oil at 0.03125% (v/v).

Bacteria			Oregano		Clove		Control	
		pH	Mgt	Lt	Mgt	Lt	Mgt	Lt
Gram negative	*S. typhimurium*	5.5	1130.65 ± 7.12*	548 ± 5.16	-	-	1007.13 ± 4.02	534 ± 2.16
		6.0	319.15 ± 4.68	205 ± 4.14	538.20 ± 1.65	234.10 ± 1.03	294.22 ± 6.11	210.5 ± 8.01
		6.5	144.28 ± 3.86	117 ± 2.11	206.17 ± 4.05	156.05 ± 4.05	126.15 ± 5.14	118 ± 8.43
		7.3	92.14 ± 2.16	103 ± 4.71	144.08 ± 2.01	143.55 ± 6.10	98.71 ± 7.41	92 ± 3.09
	E. coli	5.5	1128.75 ± 8.02*	560 ± 8.66	-	-	1003.33 ± 7.38	530 ± 6.35
		6.0	316.05 ± 7.88	215 ± 5.77	536.18 ± 3.75*	229.50 ± 3.73	289.42 ± 11.09	205.66 ± 8.08
		6.5	140.43 ± 4.16	120 ± 4.61	202.38 ± 6.01	150.00 ± 5.77	121.56 ± 4.72	115 ± 6.92
		7.3	90.30 ± 4.56	105 ± 6.92	140.12 ± 4.18	142.50 ± 5.13	88.83 ± 9.49	90 ± 4.04
Gram positive	*B. cereus*	5.5	-	-	-	-	677.25 ± 7.40	315 ± 8.66
		6.0	256.90 ± 8.85	302.50 ± 2.50*	-	-	406.35 ± 5.98	120 ± 5.77
		6.5	164.56 ± 3.13	191.25 ± 3.75	218.91 ± 3.22	217.50 ± 6.87	203.18 ± 4.43	45 ± 4.33
		7.3	142.81 ± 5.51	116.25 ± 3.75	101.95 ± 3.64	240.00 ± 4.33	102.13 ± 5.49	60 ± 3.17
	L. innocua	5.5	-	-	-	-	689.14 ± 4.20	317 ± 5.16
		6.0	260.10 ± 5.15	308.31 ± 2.10*	-	-	411.55 ± 8.13	122 ± 3.05
		6.5	168.22 ± 6.10	195.16 ± 5.51	222.11 ± 4.05	211.41 ± 2.14	221.73 ± 2.41	47 ± 5.11
		7.3	146.13 ± 5.48	119.55 ± 4.05	107.17 ± 1.22	234.12 ± 2.11	108.21 ± 5.33	65 ± 2.15

Bactericidal, * P < 0.01.

Table 3. Mean generation time (Mgt) and lag time (Lt) in minutes of *E. coli* and *B. cereus* at pH 7.3 in presence of NaCl (1.0 – 1.8 % w/v) and oregano or clove oil at concentrations of 0.03125% (v/v).

Bacteria	NaCl	Oregano		Clove		Control	
		Mgt	Lt	Mgt	Lt	Mgt	Lt
E. coli	1	98.15 ± 3.35	110 ± 5.77	225.75 ± 8.94	375.00 ± 7.21	87.47 ± 2.89	82.50 ± 3.63
	1.2	102.97 ± 3.30	135 ± 6.63	307.84 ± 4.81*	735.00 ± 8.66*	90.30 ± 2.03	90.00 ± 2.88
pH 7.3	1.4	103.20 ± 4.27	140 ± 4.90	270.90 ± 6.11	555.00 ± 5.77*	84.65 ± 3.83	104.96 ± 5.45
	1.6	106.23 ± 4.66	150 ± 4.33	209.03 ± 7.23	210.00 ± 4.33	82.34 ± 3.06	75.00 ± 7.21
	1.8	107.02 ± 3.21	160 ± 2.88	-	-	90.41 ± 3.70	112.50 ± 6.92

Table 3. Continued.

Bacteria	NaCl	Oregano		Clove		Control	
		Mgt	Lt	Mgt	Lt	Mgt	Lt
B. cereus	1	104.09 ± 5.31	190 ± 4.33	496.65 ± 6.81*	455.00 ± 3.89*	78.687 ± 4.23	82.57 ± 1.44
	1.2	106.60 ± 2.90	225 ± 1.68	180.60 ± 4.33	240.00 ± 5.48	83.54 ± 4.04	75.00 ± 1.44
	1.4	112.88 ± 4.87	250 ± 5.77	158.36 ± 5.81	202.50 ± 7.21	91.89 ± 1.87	93.75 ± 1.87
pH 7.3	1.6	131.69 ± 2.23	285 ± 2.88	146.73 ± 8.51	150.00 ± 5.05	87.49 ± 3.10	105.00 ± 2.88
	1.8	139.96 ± 5.51	340 ± 3.46	-	-	108.76 ± 3.97	110.50 ± 1.73

Bactericidal, * $P < 0.01$.

It has been suggested that colonial growth may restrict diffusion of oxygen and cells situated within a colony may be shielded to a certain extent by the outer cells from the substrates, thus it could be possible for bacteria growing within colonies to be protected from the action of EOs in this way. In this investigation, oils were mixed in sterile distilled water forming droplets. Therefore it is possible that the bacteria growing within colonies survived the effect of EOs and therefore the lag time and mean generation time of the bacteria observed here may not be a true reflection of the effect of the oil on growth of bacteria. However, formation of droplets was minimised by ensuring thorough vortexing of the mixture.

A number of factors such the nature of the EO, country of origin, altitude at which the source plant grew, harvest season, production process, level of purity and preservation may affect the MIC of EO, all of which help to determine the presence of variable concentration of antimicrobials in the final product (Valero et al., 2003). However, despite the variations, EOs are still preferred over synthetic preservatives since they have more than one active component and therefore it is unlikely that microorganism would mutate to produce resistant genes that will lead to production of resistant strains.

In conclusion, the present study has shown that combination of these preservative systems (EOs, pH and sodium chloride) in experimental conditions could be exploited to allow use of very low levels of EOs to overcome organoleptic effects in food and food products and further reduce heat treatment and use of synthetic chemicals in foods (Valero and Salmeron, 2003).

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