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The Effect of Sodium Chloride and pH on the Antimicrobial Effectiveness of Essential Oils against Pathogenic and Food Spoilage Bacteria: Implications in Food Safety

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***Abstract*—**The purpose of this study was to elucidate the factors affecting antimicrobial effectiveness of essential oils against food spoilage and pathogenic bacteria. The minimum inhibition concentrations (MIC) of the essential oils, were determined by turbidimetric technique using Biocreen C, analyzer. The effects of pH ranging from 7.3 to 5.5 in absence and presence of essential oils and/or NaCl on the lag time and mean generation time of the bacteria at 37^0C, were carried out and results were determined showed that, combination of low pH and essential oil at 37^0C had additive effects against the test micro-organisms. The combination of 1.2 % (w/v) of NaCl and clove essential oil at 0.0325% (v/v) was effective against *E. coli*. The use of concentrations less than MIC in combination with low pH and or NaCl has the potential of being used as an alternative to "traditional food preservatives".

***Keywords*—**Antimicrobial, Bactria, Bioscreen C, essential oil.

I. Introduction

PLANT essential oils and their components are known to exhibit antimicrobial activities [1]-[2]-[3], and many applications for controlling the growth of foodborne pathogens and food spoilage bacteria have been developed using these essential oils as natural food preservatives [4]-[35]-[36]. It has been reported that the antibacterial activity derives from the terpenoid and phenolic compounds in the oils [5]-[6].

Most studies investigating the action of whole essential oils (EOs) against food spoilage organisms and food borne pathogens agree that, generally, EOs are slightly more active against gram-positive than gram-negative bacteria [7]-[8]-[9]-[10]-[11]-[12]-[13]-[24]. That gram-negative organisms are less susceptible to the action of antibacterials is perhaps to be expected, since they possess an outer membrane surrounding the cell wall [14], which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering [15]. In general, higher concentrations of essential oils are required in foods than in laboratory media [16] accordingly, the practical application of EOs is restricted, since effective antimicrobial doses most often exceed organoleptically acceptable levels. Thus the knowledge of the minimum inhibitory concentration (MIC) of an essential oil is important to enable striking a balance between sensory acceptability and antimicrobial efficacy [17]. Moreover a number of potential synergists have been suggested for use with EOs: low pH, low water activity, chelators, low oxygen tension, mild heat and raised pressure, although not all of these have been researched in foodstuffs [18].

However, sodium chloride (NaCl) 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. This study explored the possibility of inhibiting growth of microbes by combining NaCl with EOs of clove and oregano at varied pH with an aim of achieving synergy at reduced levels of NaCl and EO.

II. Materials and Methods

A. Bacterial strains and cultural methods

Test microorganisms 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). Each was stored for long term at 4^0C in the medium term on tryptone soya agar (TSA) on TSA plates. Active cultures for the experiments were prepared by transferring a loopful of cells from stock cultures to universal glass bottles containing 10ml Tryptone Soya Broth (TSB) which were incubated overnight at 37^0C without agitation in order to obtain cells in exponential phase. The overnight cell concentrations were approximately 10^9 cell ml^{-1} but these were diluted with sterile distilled water to achieve bacterial

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densities corresponding to 10^6 cell ml^{-1} prior to experiment.

B. Essential oils

Four different EOs that have been known to exhibit antimicrobial activity against *B. cerus*, *S. typhimurium*, Listeria sp. and *E. coli* at variable degrees of effectiveness were selected for the investigation. Pure grades which contained no synthetic chemicals or unnatural components essential oils of Oregano 70% (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. All essential oils were stored in transparent bottles covered with a brown tape and an aluminum foil at 4^0C protected from light and air. Serial dilutions of the oils in distilled water were done to obtain the required percentage concentrations prior to experiments.

C. Media

The growth media TSA and TSB were procured from International Diagnotics Group Plc, UK. TSA was used as growth medium for disc diffusion test, in determination of MICs of the oils. TSB was used as the growth medium in Bioscreen C experiments. The media were prepared according to the manufactures instruction.

D. Acid and Sodium chloride

Pure acetic acid at concentrations of 6.05 mg/ml was used throughout the experiment to adjust the pH of the growth medium while NaCl was used for adjusting the water activity levels of the growth media.

E. Disc diffusion method

The antibacterial activity of the four essential oils (oregano, clove, bay and cinnamon) was 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. Ten fold serial dilutions of essential oils were prepared and kept at room temperature (25^0C) prior to experiment. 40µl of the serially diluted essential oil suspensions were dropped into wells in the agent. Chlorophor, an antimicrobial agent, was filled into one of the wells which served as a positive control while for negative control; sterile distilled water was used. The treated plates were incubated at 37^0C for 48 hours but readings were taken after an interval of 24 hours. 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. Mean diameters of the inhibition zones were then determined.

F. Determination of MIC by turbidimetric technique using Bioscreen C, analyzer

The minimum inhibition concentrations of the antimicrobial activity of the essential oils against the four bacteria were determined precisely by turbidimetric technique using Bioscreen C analyser. Essential oils were diluted in distilled water by vortexing to obtain concentrations ranging between 0.03125 to 1%. 40µl of the diluted samples were then added to 300µl before finally adding 60µl of bacteria at concentrations of 10^7 cell ml^{-1} in the microtitre well to make up a maximum volume of 400µl. The concentration of the oil in the microtitre well was reduced accordingly to 10% of the original value, thus the range 0.03125 to 0.1% and that of the bacteria, 10^6 cell ml^{-1}. The microtitre wells were then incubated at 37^0C for 20 hours and growth curves obtained. The lowest concentrations of the oils that inhibited growth were obtained as MIC of the oil against the test bacteria.

G. Experimental procedure for Bioscreen C, turbidimetric technique

The effects of EO and pH and water activity on the population, lag phase, exponential phase (mean generation time) and maximum yield were studied. Triplicates of 300µl, TSB, 40µl, dilute essential oil and 60µl of 10^6 cell ml^{-1} bacterial concentrations were placed in the wells of the microtitre plate. Replicates of controls contained either TSB only, TSB and distilled water, TSB and oil only or TSB, water and bacteria were also placed in the microtitre plate and the plates were then incubated at 30^0C in the Labsystems Bioscreen C (Life Sciences International, Basingstoke, UK). The increase in turbidity at 600nm was monitored automatically every 15 minutes for 20hours. Plates were shaken for 10 seconds before OD readings were taken. Incubation temperatures were determined by the type of the technique used and the type of growth media. Hence the difference in the incubation temperatures in radial diffusion and the turbidimetric technique.

III. RESULTS

Results on the selection of test essential oils and bacteria as determined by radial diffusion test are shown in fig. 1. The zones of inhibition produced by oils diluted to 10^{-2} were negligible. Some oils did not produce any zone of inhibition at this dilution. Thus the results are not included in the figure. Further dilution to 10^{-3} was too weak to show any effect on the bacterial growth, therefore the results shown in Fig. 1 was taken as an estimate of MIC of the oils. 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 size of the zone of inhibition produced was also influenced by the type of the EO tested, thus indicating that the four test EOs differed in their antimicrobial effectiveness with clove being the most effective and cinnamon being the least effective among the four EOs. Table I shows MICs of oregano and clove against *B. cereus* and *E. coli* determined by Bioscreen C, technique. *B. cereus* and *E. coli* were selected from radial diffusion tests to represent the two categories of the test microorganisms. Table II shows the mean generation time and lag time of selected bacteria. The concentration of 0.0325% (v/v) of EO was used as it proved to combine well with NaCl against the test bacteria from a series of preliminary experiments. Statistical analysis of the experiment involving combination of NaCl with EO of clove on the growth of *E.*

coli showed that the effect was significant on the lag time of the bacteria. T-test showed that it was possible to achieve a greater inhibitory effect under conditions of 1.2% (w/v) NaCl and clove (0.0325% (v/v) on the growth of *E. coli*.

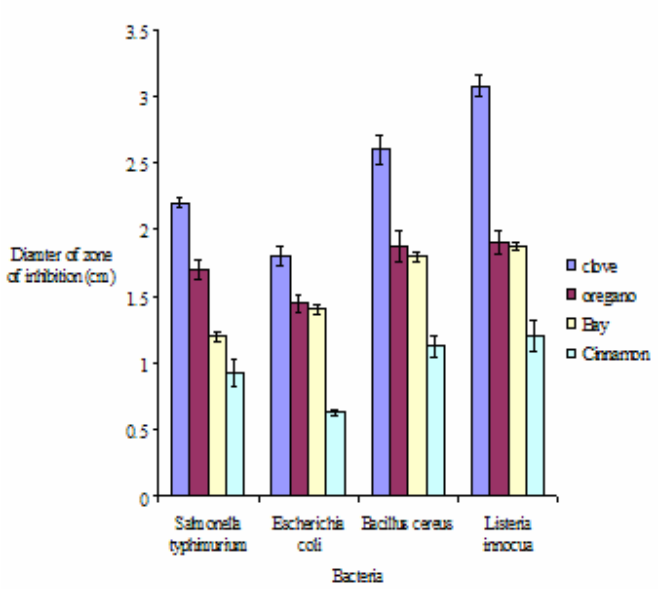

Fig. 3 Radial diffusion test of the four test essential oils diluted to 10^{-1} (v/v) against two gram negative and two gram positive bacteria incubated at 30^0C for 48 hours

IV. Discussion

It is now generally accepted that no single preservative will be effective against food poisoning and spoilage microorganisms and the use of MIC levels of EOs as a preservative method has shown to cause organoleptic effects in foods [19]. Therefore the use of EOs at concentrations below the MIC in combination with reduced pH and NaCl as described in this investigation has high potential of inhibiting growth or extending the lag time of *B. cereus* and *E. coli*. Since the oil is used at concentrations below the MIC, this combination of preservation systems may prove effective in overcoming the organoleptic effects on food associated with the use of EOs at higher concentrations. An organoleptic effect of oregano and clove essential oils in foods is influenced by the relative concentration of the oil used. 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 other preservative systems like NaCl and acetic acid that has a strong smell that would affect flavour of food [20].

The synergistic effects achieved by combining preservative methods where EOs and their components are used have been documented [9]. However this is a novel case where synergism has been achieved by combining sublethal

TABLE I
MINIMUM INHIBITION CONCENTRATION OF THE TWO TEST ESSENTIAL OIL OBTAINED BY BIOSCREEN C, TURBIDIMETRIC TECHNIQUE

EO	Bacteria	MIC (%)
Clove		
	Bacillus cereus	1.25
	Escherichia coli	2.5
Oregano		
	Bacillus cereus	1.5
	Escherichia coli	2.5

TABLE II
MINIMUM INHIBITION CONCENTRATION OF THE TWO TEST ESSENTIAL OIL OBTAINED BY BIOSCREEN C, TURBIDIMETRIC TECHNIQUE

	E. coli		*B. cereus*	
	Oregano		**Oregano**	
pH	Mgt	Lt	Mgt	Lt
5.5	1128.75±8.02	560±8.66	-	-
6	316.05±7.88	215±5.77	256.90±8.85	302.50±2.50
6.5	140.43+4.16	120±4.61	164.56±3.13	191.25±3.75
7.3	90.30+4.56	105±6.92	142.81±5.51	116.25±3.75
	clove		**clove**	
5.5	-	-	-	-
6	536.18±3.75	229.50±3.73	-	-
6.5	202.38±6.01	150.00±5.77	218.91±3.22	217.50±6.87
7.3	140.12±4.18	142.50±5.13	101.95±3.64	240.00±4.33
	Control		**Control**	
5.5	1003.33±7.38	530±6.35	677.25± 7.40	315±8.66
6	289.42±11.09	205.66±8.08	406.35±5.98	120±5.77
6.5	121.56±4.72	115±6.92	203.18±4.43	45±4.33
7.3	88.83±9.49	90±4.04	102.13±5.49	60±3.17

Mgt = mean generation time, Lt = lag time.

TABLE III
MEAN GENERATION TIME AND LAG TIME (IN MINUTES) OF *E. COLI* AND *B. CEREUS* AT PH 7..3 IN PRESENCE OF NACL AND EO (0.03125%), OG; OREGANO, C; CLOVE, CT; CONTROL

		E. coli		*B. cereus*	
	NaCl	Mgt	Lt	Mgt	Lt
Og	1	98.15±3.35	110±5.77	104.09±5.31	190±4.33
	1.2	102.97±3.30	135±6.63	106.60±2.90	225±1.68
	1.4	103.20±4.27	140±4.90	112.88±4.87	250±5.77
	1.6	106.23±4.66	150±4.33	131.69±2.23	285±2.88
	1.8	107.02±3.21	160±2.88	139.96±5.51	340±3.46
C	1	225.75±8.94	375.00±7.21	496.65±6.81	455.00±3.89
	1.2	307.84±4.81	735.00±8.66	180.60±4.33	240.00±5.48
	1.4	270.90±6.11	555.00±5.77	158.36±5.81	202.50±7.21
	1.6	209.03±7.23	210.00±4.33	146.73±8.51	150.00±5.05
	1.8	-	-	-	-
Ct	1	87.47±2.89	82.50±3.63	78.687±4.23	82.57±1.44
	1.2	90.30±2.03	90.00±2.88	83.54±4.04	75.00±1.44
	1.4	84.65±3.83	104.96±5.45	91.89±1.87	93.75±1.87
	1.6	82.34±3.06	75.00±7.21	87.49±3.10	105.00±2.88
	1.8	90.41±3.70	112.50±6.92	108.76±3.97	110.50±1.73

concentrations of EOs with reduced pH and NaCl against *B. cereus* and *E. coli*. Since plant EOs have more than one active component, it is unlikely that microorganism would mutate to produce resistant genes that will lead to production of resistant strains [19].

The antimicrobial activity of clove, oregano, bay and cinnamon against a broad spectrum of bacteria has been reported. These essential oils are effective against several bacteria at concentrations less than or equal to 0.5% (v/v). This study also showed that these EOs were effective against the tested bacteria at concentrations less than or equal to 0.5% (v/v). In agreement with previous reports, the strength and spectrum of activity of these essential oils varied between relative compositions, the active components and the type of the bacteria. *B. cereus* was generally more sensitive to the effect of the test oils than was *E. coli*. This report also agrees with [11] who reported the susceptibility of gram positive bacteria to EOs as compared to gram negative bacteria. MICs of clove were more inhibitory than the other three test EOs. The effectiveness of clove EO may have been attributed to its relative abundance of eugenol component which is approximated at 88.9% in 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.

The MICs of the clove and oregano essential oils were tested against both *B. cereus* and *E. coli* and were found to be lower at pH values below 7.3 and even much lower with addition of NaCl. This agreed with the previous reports where MICs and Minimum Bactericidal Concentration (MBCs) of oils have been observed to decrease with decreasing pH values and particularly with addition of NaCl [24].

It is thought that the potential of amino compounds which are less negatively charged, that protect the cell from direct contact with antimicrobial agents is weakened in the presence of NaCl thereby rendering the cells more susceptible to the action of the essential oil [19]. It has been reported 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 use EOs at concentrations of 0.0325% v/v in combination with NaCl in order to prolong the lag time of the bacteria [21].

MIC values were slightly different from other authors. This has been attributed to the design of the technique. Most studies test for MICs by synthetic growth medium with dilution or diffusion in solid medium using discs impregnated with antimicrobial agents. This investigation determined MIC concentrations by spreading bacteria on to the surface of the growth media [22]. Other factors that might have played a role leading to the difference in the MIC values of the EOs could have been the nature of the EO, its country of origin, altitude at which the source plant grew, harvest season, production process, level of purity and preservation all of which help to determine the presence of variable concentration of antimicrobials in the final product [23].

Experiments on the effects of pH on the growth of the bacteria showed that *E. coli* could grow at pH 5.0 in absence of essential loil and could also grow at pH 5.5 in presence of EO at concentrations below MIC levels but produced a longer lag time. However gram positive bacteria, *B. cereus*, could not survive under these conditions. Further experiments showed that gram-negative bacteria, *E. coli*, was less susceptible to the effects of the antimicrobial activities of the essential oils at levels below MICs in presence of NaCl compared to the gram positive bacteria *B. cereus*.

Experiments to determine 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 (results not shown). Previous work has shown that NaCl can lower the MIC of an antimicrobial agent at concentrations above 25g/l or 2.5 % (w/v) in presence of an antimicrobial agent even at sub lethal concentrations may inhibit growth of a microorganism. 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 [24].

The combination of 1.8 % (w/v) salt and oregano essential oil at 0.0325% (v/v) at pH 7.3 (Table 3) allowed the growth of both the *E. coli* and *B. cereus* but significantly prolonged their lag time. The lag time of *B. cereus* was longer than that of *E. coli*. However clove essential oil under similar inhibited the growth of the two bacteria within the test period. This emphasized that clove EO was more effective against the two bacteria than was oregano even at the same concentrations. This observation is attributed to the fact that clove contains eugenol as the major active component at 88.9% in composition while oregano contain carvacrol as the major component at 70% in composition [19].

The combination of 1.2% (w/v) of NaCl and clove essential oil at concentration of 0.0325% (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 [25]. This combination of essential oils at sub lethal concentrations with NaCl and at low pH conditions rnging from 7.3 to 6.0 have the potential of overcoming organoleptic problems associated with the use of EOs in foods which usually require higher concentrations in the growth media. The use of high levels of EO causes changes in flavor and taste. This investigation has shown that combination of these preservative systems in experimental conditions could be exploited to allow use of low levels of EOs in foods and further reduce heat treatment and use of synthetic chemicals in foods [34].

Studies have shown that carvacrol can be added to food at dose below MIC value, thereby reducing toxin production by *B. cereus*. Previous studies have also shown that levels of EOs and their components necessary to inhibit microbial growth are in many cases higher than the amount used as flavoring and is associated with adverse sensorial effects in food. The levels are usually higher in food than in culture medium. This is possibly due to interactions between phenolic compounds and food matrix [29]. 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 [19]. This investigation has shown that low pH acted synergistically with EOs of clove and oregano to improve their effectiveness against both gram-positive B. cereus and gram-negative *E. coli*.

Investigations on the effect of pH on the effectiveness of EOs involved the use of acetic acid to lower or adjust the pH of the growth medium. Acetic acid was preferred on the basis of it being an organic acid that can be obtained from natural sources. The acid dissociates into CH3 COO- and H+ ions. The hydrogen ions lowers the pH of the medium and also acts on the cell membranes of microbes rendering then permeable leading to leakage of the cell content and may result to death of the microbe [25].

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 6.5mg/ml. This allowed the use of sub-lethal concentrations of EOs or concentrations lower than MIC levels. This combined effect of low pH and EOs lower than their MICs levels thus acted synergistically to cause inhibition of *B. cereus* and *E. col*i as evidenced in the results of the experiment on the combined effect of pH and EO on the growth of *B. cereus* and *E. coli*. This observation is in agreement with previous investigations where a technology for fish preservation by combined treatment with electrolyzed NaCl solutions and essential oil compounds proved effective in controlling the proliferation of food spoilage micro-organisms [37].

Some studies have shown that the susceptibility of bacteria to antimicrobial effect of EOs may increase with decrease in temperature and available oxygen [28]. Investigative experiments carried out here were done at temperatures of 300C 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 30^0C. Nevertheless it would be interesting to establish the effect of lower temperatures on effectiveness of the tested EOs under these combined conditions of NaCL concentration and pH.

The medium in which the oil is mixed into may also have an influence on the survival of the microbes. For example, oil-in-watere emulsions has shown that that, depending on the mean droplet size of the emulsion, the bacteria can grow in films, in colonies or as planktonic cells [30]. 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 in the emulsions 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 or emulsions, thus 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.

The gram-negative bacteria (*E. coli* and *S. typhimurium*) tested here were in general slightly less susceptible than gram-positive bacteria (*B. cereus* and *L. innocua*). This difference 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 [31].

Studies have suggested that addition of acetate moiety to the molecule may increase their antimicrobial activity. Thus the use of acetic acid to lower pH conditions of the growth medium may have also acted to increase the effectiveness of carvacrol component of oregano EO and this could explain the synergy observed between the oil and the acid. Carvacrol is capable of disrupting the outer membrane of gram-negative bacteria, releasing polysaccharides and increasing the permeability of cytoplamic membrane to ATP. This could explain the effectiveness of oregano oil on *E. coli* [32].

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 [33]. Therefore this study agreed with the previous suggestion that gram-negative bacteria are less susceptible and that it is the major components of the oils that are responsible for the antimicrobial activity of the oil [29].

The hypothesis that the type of bacteria could influence its susceptibility or the antimicrobial effectiveness of the essential oil was realized but not conclusively given the paucity of the number of bacteria used in the investigations though generally it was observed that gram *E. coli* was less susceptible than *B. cerues* in some experiments *B. cereus* appeared less susceptible than *E. coli*. The investigation also showed that antimicrobial effectiveness of EOs can be affected by pH and preservation systems such as use of NaCl in combination with EOs can act synergistically to inhibit

growth or extend the lag time of bacteria under appropriate conditions and may lead to reduction in the level of EOs used in foods in overcoming organoleptic problems. In addition, the shelf life of food may be prolonged using combinations of EOs and NaCl since experimentally this has been proved to extend the lag time of the test bacteria as shown in the present study.

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