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MICROBIAL LOAD OF LUNCHEON MEAT AND BIOLOGICAL ACTIVITY OF SOME SELECTED BACTERIA AS AFFECTED BY NATURAL PRESERVATIVES

BY

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ABSTRACT

Different luncheon meat products (beef) and (chicken) were commercially collected and tested for microbiological load during storage at 4 or 25 ± 1°C for 8 weeks. *Salmonella sp*, *Shigella sp.*, total and faecal coliform group were not found in any tested samples. The microbiological load (counts of total bacteria, spore forming bacteria, thermophilic bacteria, psychrophilic bacteria, *Staphylococcus aureus*, *Bacillus cereus* and total fungi) of tested samples were differed according to the kind of samples and the conditions of storage. Storage temperature 4 ± 1°C was more effective in inhibiting growth of spore forming bacteria and thermophilic bacteria.

Chitosan 1% proved that it is necessary for inhibiting all pathogenic bacteria tested especially *Staph. aureus* giving zone of inhibition 47 mm. Chitosan showed bactericidal effects with *Staph. aureus*, *S. typhimurium*, *E. coli* and *Pseudomonas fluorescens*, and bacteriostatic effect on *B. cereus*. Sage and rosemary oils had no or slight antibacterial activity against the tested bacterial strains. By contrast clove and thyme oils had a very antibacterial activity for the same tested bacteria, with MIC 20 and 40 µL, respectively. The antibacterial activity of clove and thyme oils was bactericidal for all tested bacterial stains.

Key words: Luncheon, Microbiological load, Chitosan, Essential oils, Antimicrobial.

INTRODUCTION

Luncheon meats are one of the cooked meat products which are commonly vacuum-packaged and sold sliced. They are recontaminated during slicing and packing and as a result may have a starting count as high as 10^4–10^5 bacteria per g. Since the surface-to-volume ratio is comparatively high, bacterial spoilage may occur after only 2–3 weeks at 5°C. (Pamela *et al.*, 1987). The initial counts of mesophilic aerobes, *Staphylococcus* and *Salmonella* of luncheon meat were found in the ranges of 10^6-10^7, 10^4-10^5 and 10-100 cfu / g, respectively (Alur *et al.*, 1998). Most important fungi and yeasts contaminated the luncheon meat produced in Egypt were, *Aspergillus niger*, *A. flavus*, *Penicillium chrysogenum*, *Rhizopus stolonifer*, *Mucor circinelloides*. On the other hand, less common were *Cladosporium sphaerospermum*, *Alternaria alternate*, *Mycosphaerella tassiana*, *P. aurantio-griseum* and *P. oxalicum*. (Ismail and Zaky, 1999). Two major food-borne bacterial pathogens included *E. coli* O157: H7 and *Salmonella*, have been associated with the contamination of meat and meat products. Recently, *Listeria monocytogenes* has also been identified as a serious food-borne pathogen and has been demonstrated to be a contaminant of beef carcasses, (Bell, 2002).

For producing carcinogenic and mutagenic N-nitroso compounds, although nitrite was useful as a curing agent in curing meat products on the other hand, residual nitrite in the meat products poses a health risk to humans. ((Scanlan, 1983 and Blot *et al.*, 1999). For these reasons many of researches investigated the ability of using some agents beside or instead of nitrite during production of curing meat products. Potential use of red beet pigment and chitosan for the production of sausages with reduced nitrite content was investigated by Jong and Gang (2003). They found that, the red beet pigment had good nitrite scavenging ability, and the sausages produced had good water holding capacity, tenderness and colour development. Sausages produced using chitoson had good water holding capacity and tenderness, but the chitosan exhibited poor nitrite scavenging ability. Also they suggested that, the level of nitrite added during the manufacture of sausages could be reduced by half by using red beet pigment and chitosan.

Chitosan [poly (β (1→4)-2- amino-2-deoxy-D glucose)] is a natural non-toxic biopolymer derived by deacetylation of chitin, (Dutta *et al.*, 2002). Darmadji and Izumimoto (1994) reported that the antagonistic action of chitosan against microorganisms is important in foods, and its potential use as antimicrobial food preservative has also been reported. For example, total bacterial, *Pseudomonas*, Staphylococci, coliformes, gram-negative bacteria and *Micrococcus* reduced 1-2 log cycles in the presence of 1% chitosan. Recent studies on antibacterial activity of chitosan have revealed that, chitosan is more effective in inhibiting growth of bacteria, (Jeon *et al.*, 2001). Oh *et al* (2001) reported that, a slight inhibition (~1 log CFU g^{-1}) of total microbial growth in refrigerated beef patties was observed in the presence of 1% Chitosan. Chitosan inhibited *Lactobacillus frutivorans* and *Zygosaccharomyces bailii* that cause food spoilage and bactericidal activity against some Gram positive and Gram negative food-borne bacteria such as, *Staphylococcus aureus, Escherichia coli, Yersinia enterocolitica, Listeria monocytogenes* and *Salmonella typhimurium*. (Liu *et al.*, 2004). The minimum inhibitory concentrations (MIC) of chitosan were determined and varied widely from 0.01 to 1.0%. (Kim & Thomas, 2007).

The antimicrobial properties of 21 plant essential oils and two essences were investigated against five important food borne pathogens, *Campylobacter jejuni, Salmonella enteritidis, Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes*. The oils of bay, cinnamon, clove and thyme were the most inhibitors. In general, Gram positive bacteria were more sensitive to inhibition by plant essential oils than the Gram negative bacteria. (Smith-Palmer *et al.*, 1998). The greatest use of essential oils in the European Union (EU) is in food (as flavourings), perfumes (fragrances and aftershaves) and pharmaceuticals for their functional properties (Van de Braak and Leijten, 1999). Besides antibacterial properties, essential oils have been shown to exhibit antiviral, antimycotic, antitoxin, antiparasitic and insecticidal properties. These characteristics are possibly related to the function of these compounds in plants (Mahmoud and Croteau, 2002).

Therefore, the objective of this study was to determine the most common microorganisms contaminating the commercially prepared luncheon meat products during marketing, also the antimicrobial activity of chitosan and essential oils which included clove, thyme, rosemary and sage that was examined for its further application as presservatives in preparing luncheon meat instead of nitrite components.

MATERIALS AND METHODS

Local market luncheon products:

Different luncheon samples (beef and chicken meat) were collected from local market, samples were immediately transported to the laboratory using ice box and then stored at (4 and 25°C ± 1). Every

sample in three replicates were subjected to microbial, physical and chemical analysis initially and periodically during storage period every 2 weeks for 8 weeks. Samples were subjected for microbiological determinations by aseptic transfer of 10g of homogenized sub-sample into 90 ml of sterile diluents (0.1% peptone water). Serial dilutions were prepared the same above mentioned diluents and 1ml aliquots were used to enumerate the densities of different microbial groups using the plate count technique. For aerobic spore forming bacterial count, initial dilutions of different luncheon samples were pasteurized in water bath at 80° C for 15 min.

Media used:

Nutirent agar (Thiery and Francon, 1997) was used for enumeration of total aerobic bacteria, total aerobic spore forming bacteria, psychrophilic and thermophilic bacteria. Malt extract agar (Lowes *et al.*, 2000) was used for enumeration of total fungi. *Bacillus cereus* base agar (Oxoid, 2001) was used for enumeration of *Bacillus cereus*. Violet red bile agar (Difco, 2003) was used for enumeration of total and faecal coliform bacteria. Vogel Johnson agar (Difco, 2003) was used for enumeration of *Staphylococcus aureus*. *Salmonella Shigella* (SS) agar (Difco, 2003) was used to detect *Salmonella* sp. and *Shigella* sp.

Microorganisms:

Five strains of bacteria were tested for antimicrobial activity of chitosan and different essential oils. *Escherichia coli* ATCC 69373, *Bacillus cereus* DSMZ 345, *Staphylococcus aureus* DSMZ 20231 and *Salmonella typhimurium* ATCC 14028 as food poisoning and *Pseudomonas fluorescens* NRRL 800 as a spoilage strain. They were provided from Microbial culture collection center (Cairo MIRCEN), Fac. Agric., Ain Shams Uni., Cairo, Egypt.

Preparation of bacterial inocula:

Stock cultures were activated by loop of stock culture into 50 ml nutrient broth and incubated at 30 or 37°C for 24 h. They were counted using plate count method. One ml of each bacterial inoculum was contained 10^8 cfu / ml.

Determination of antimicrobial activity:

Paper disc diffusion method was used to determine the antimicrobial activity of chitosan and essential oils according to (Sleigh and Timburg, 1981). Sterilized filter paper discs (11 mm) were soaked with 110 µL of different concentrations (0.05, 0.1, 0.2, 0.4, 0.8, and 1%) of chitosan or (20, 40, 60, 80, 100, and 120µL) of each essential oil. The soaked discs were put in the middle of plates which were contained 1% bacterial inoculum and incubated at 37 or 30°C for 24 or 48 h. The diameter of the inhibition zones of microbial growth were measured in mm.

Chitosan:

Chitosan, was used as a natural preservative, crab shells with 85% degree of deacetylation [poly (β - (1, 4) – 2 – amino – 2 – deoxy – D -glucose)] was purchased from Sigma chemical company. Various amounts of chitosan used in this investigation were dissolved in 1% acetic acid and the pH was adjusted to 5.5 by using NaOH 1N.

Essential oils used:

The essential oils used in the present work, Clove (*Eugenia caryophylluta*), thyme (*Thymus vulgaris*), rosemary (*Rosemary officinalis*) and sage oils (*Salvia officinalis*) were obtained from CATO aromatic and Cairo ethans essential oils company.

Determination of the minimal inhibitory concentration (MIC):

MIC of chitosan and different essential oils was defined as the lowest concentration required for complete inhibition of test organism up to 48h incubation. (Canillac and Mourey, 2001).

Determination of bacteristatic and bactericidal:

Bactereristatic and bactericidal concentrations were determined for chitosan, clove and thyme oils. The appropriate volume of chitosan, clove and thyme oils were added to 100 ml of nutrient broth to give a final concentration of 0.4, 0.8, 1.0 and 0.2, 0.4, 0.8, 1.0%, respectively. The nutrient broth was

inoculated with 1 ml of a 24 h bacterial culture. Different flasks were incubated at 37° C (for *Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli*) and 30° C (for *Bacillus cereus* and *Pseudomonas fluorescens*) with regular shaking for 72 hr. Every 12 hr. growth was compared with the control broth visually and through the measurement of the number of colony forming units (cfu/ ml) by plate count procedure. Plates were incubated at 37 or 30°C for 24h and then counted for viable bacteria (Giannuzzi and Zaritzky, 1996). Bacteriostatic concentration was the lowest concentration at which bacteria fail to grow in broth, but can grow when broth is plated onto agar. Bactericidal concentration was the lowest concentration at which bacteria fail to grow in broth, and cannot grow when broth is plated onto agar, (Smith-Palmer *et al.*, 1998).

RESULTS AND DISCUSSION

Microbiological profiles of commercial luncheon products collected from local markets:

Figs (1 and 2), show the microbial load of collected commercial beef and chicken luncheon products, (counts of total bacteria, spore forming bacteria, thermophilic bacteria, psychrophilic bacteria, *Staphylococcus aureus*, *Bacillus cereus* and total fungi) stored at 4 and 25° C for 8 weeks. In the beginning, it could be reported that, *Salmonella sp.*, *Shigella sp.* and total faecal coliform group were not found in any tested sample at the beginning of storage period and during different storage periods, which revealed that, the collected products were in agreement with the Egyptian Standard of luncheon, (2005). From the same figures there are differences in microbial load of tested samples, this may be due to the differences of the microbiological load of raw materials which have been used in the manufacturing of these products, meanwhile, the processing operations had a great effect on the microbial load of final product. In the same time, the temperature of storage (4 and 25°C) had an effect on the microbial load of tested luncheon samples during storage period, where as the storage temperature increased the value of tested microbial parameters increased; this could be noticed at the beginning of storage time and through the storage period (8 weeks). For example, product ch1 had total bacterial count 1.2 and 12.9 log cfu / g at the end of storage period at 4 ° C and 25° C, respectively. The same trend was observed for other tested beef (B) and chicken (ch) luncheon samples included ch2, ch3, B1, B2 and B3. From the same data it could be noticed that, thermophilic bacteria were not detected in any tested samples stored at 4° C ± 1, this confirms the role played by chilling temperature on the growth inhibition of this group of bacteria where the minimum growth temperature of this bacteria was 35-45°C, (Hayes, 1992). In the same time, both of spore forming bacteria and *Bacillus cereus* had the trend closely to the trend observed for thermophilic bacteria, therefore, chilling temperature could be the more important factor in delaying the spoilage of meat products e.g. luncheon products. These results are in agreement with Giffel *et al.*, (1996) whose found that the counts of *B. cereus* and *B. subtilis* in meat product samples were ranged from $9x10^2$ to $9x10^4$ and from $2x\ 10^2$ to $2x10^4$ cfu / g, respectively. Generally it could be reported that, all estimated microbiological parameters were decreased almost with 3 log unit to below the detection level in all tested samples.

Antibacterial activity of chitosan:

As seen in Table (1), chitosan markedly inhibited growth of most bacteria tested including *Staphylococcus aureus, Salmonella typhimurium, Bacillus cereus, Escherichia coli* and *Pseudomonas fluorescens*. It could be noticed that, the inhibitory effects differed with regard to the type of bacteria. Chitosan generally showed stronger antibacterial effects for *Staphylococcus aureus* and *Salmonella typhimurium* than for *Pseudomonas fluorescens, Escherichia coli* and *Bacillus cereus* in the presence of 1% chitosan, where the inhibittion zones diameter were 47, 43, 26 20 and 19 mm, respectively. Higher antibacterial activity of chitosan was reported by several workers including Jeon *et al.*, 2001 and Uchida *et al.*, 1989 who observed that chitosan inhibited growth of *E. coli* and *Staphylococcus aureus* at level as high as 0.5 – 1%.

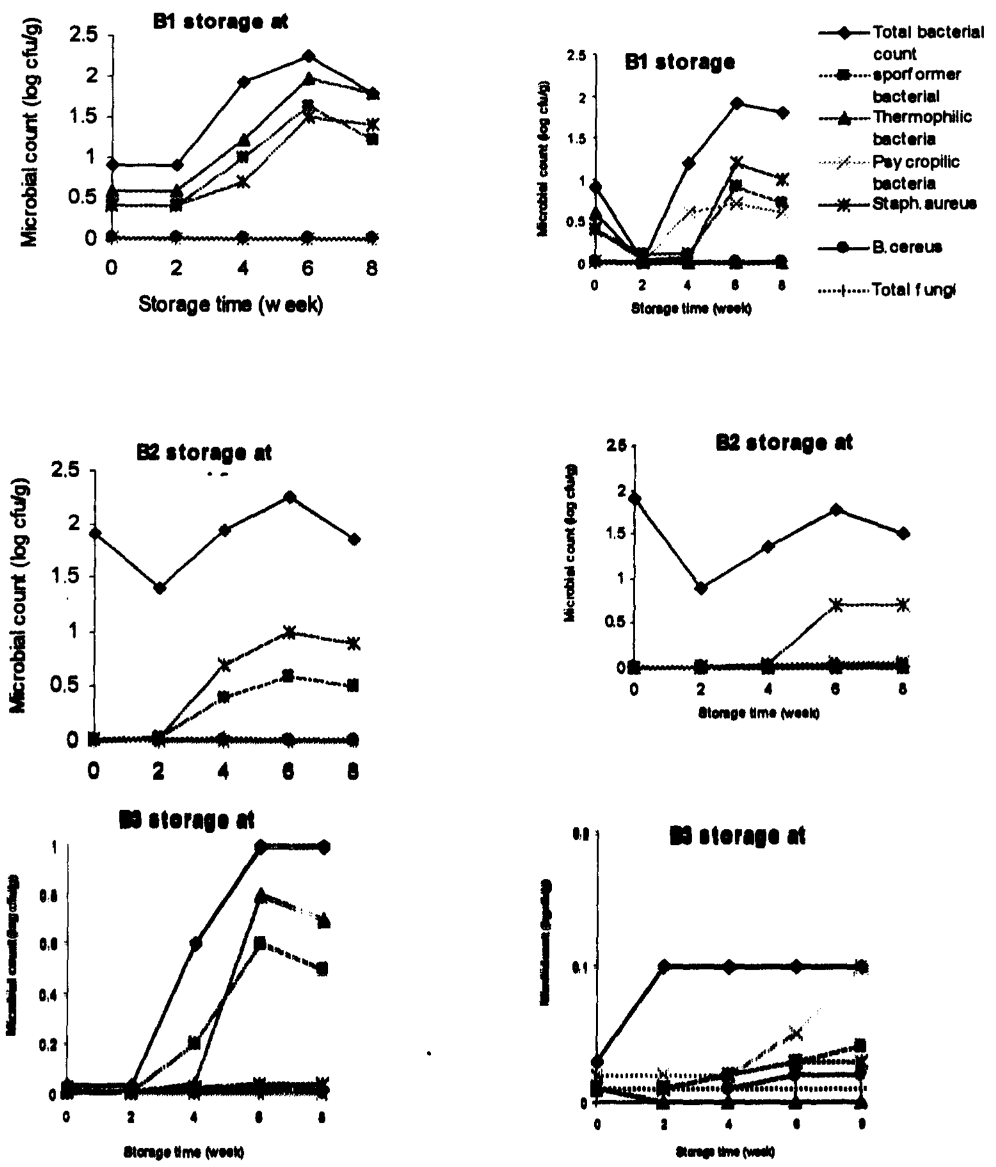

Fig (1): Microbial load of collected commercial beef luncheon products (B).

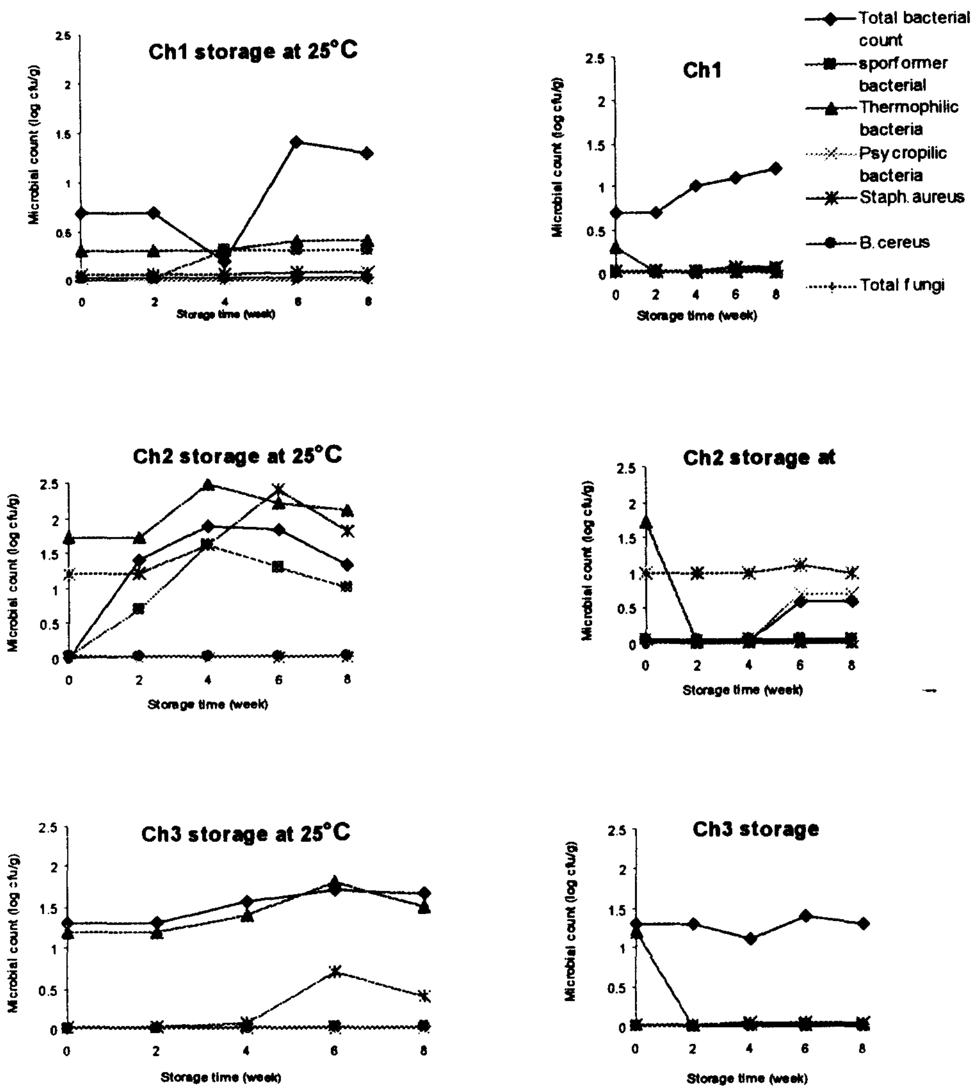

Fig (2): Microbial load of collected commercial chicken luncheon products (ch).

Table (1): Antibacterial activity of different concentrations of chitosan against some pathogenic and spoilage bacteria.

Bacterial strains	Inhibition zone diameter (mm)						
	Chitosan (%)	0.05	0.1	0.2	0.4	0.8	1.0
Staphylococcus aureus		20	22	33	34	37	47
Salmonella typhimurium		22	25	34	34	38	43
Bacillus cereus		0.0	0.0	15	15	19	19
E.coli		14	14	14	15	20	20
Pseudomonas fluorescens		13	19	21	23	24	26

Antibacterial activity of tested essential oils:

Results in Tables (2, 3, 4 and 5), showed the antibacterial activity of tested essential oils included sage, rosemary, clove and thyme against the tested bacterial strains. It could be reported that oils of sage and rosemary exhibited no or slight inhibition against the tested bacterial strains. Seydim and Sarikus,

(2006) reported that, the use of rosemary oil incorporated into whey protein based edible films did not exhibit antimicrobial activity against *Staphylococcus aureus, Salmonella enteritidis* and *Escherichia coli*. By contrast clove and thyme oils had a high antibacterial activity, where clove oil (120 µL) produced inhibition zones of 32, 17, 23, 27 and 23 mm for *Staphylococcus aureus, Salmonella typhimurium, Bacillus cereus, Escherichia coli* and *Pseudomonas fluorescens*, respectively. On the same time thyme oil (120 µL) produced inhibition zones 0.0, 28, 37, 40 and 29 mm for the same tested bacterial strains, respectively. Smith-Palmer *et al.*, (1998), showed that clove and thyme oils were the most inhibitors against *Salmonella enteritidis, Escherichia coli* and *Staphylococcus auerus*.

Table (2): Antibacterial activity of different volumes of sage oil against some pathogenic and spoilage bacteria.

Bacterial strains	Inhibition zone diameter (mm)					
Oil (µL)	20	40	60	80	100	120
Staphylococcus aureus	0.0	0.0	0.0	0.0	0.0	0.0
Salmonella typhimurium	0.0	0.0	0.0	0.0	0.0	0.0
Bacillus cereus	1.0	6.0	6.0	14	32	45
E.coli	0.0	0.0	0.0	0.0	13	13
Pseudomonas fluorescens	0.0	0.0	7.5	8.0	8.5	8.5

Table (3): Antibacterial activity of different volumes of rosemary oil against some pathogenic and spoilage bacteria.

Bacterial strains	Inhibition zone diameter (mm)					
Oil (µL)	20	40	60	80	100	120
Staphylococcus aureus	0.0	0.0	0.0	0.0	38	41
Salmonella typhimurium	0.0	0.0	0.0	0.0	0.0	0.0
Bacillus cereus	0.0	0.0	14	14	15	37
E.coli	[illegible]	[illegible]	[illegible]	20	23	31
Pseudomonas fluorescens	0.0	0.0	0.0	0.0	0.0	0.0

Table (4): Antibacterial activity of different volumes of clove oil against some pathogenic and spoilage bacteria.

Bacterial strains	Inhibition zone diameter (mm)					
Oil (µL)	20	40	60	80	100	120
Staphylococcus aureus	25	28	28	28	32	32
Salmonella typhimurium	13	13	15	16	17	17
Bacillus cereus	14	16	17	17	23	23
E.coli	16	17	19	19	27	27
Pseudomonas fluorescens	16	17	18	20	21	23

Table (5): Antibacterial activity of different volumes of thyme oil against some pathogenic and spoilage bacteria.

Bacterial strains	Inhibition zone diameter (mm)						
	Oil (µL)	20	40	60	80	100	120
Staphylococcus aureus		0.0	0.0	0.0	0.0	0.0	0.0
Salmonella typhimurium		0.0	22	23	28	28	28
Bacillus cereus		17	19	20	22	22	37
E.coli		0.0	31	31	36	38	40
Pseudomonas fluorescens		22	23	25	26	28	29

Minimum inhibitory concentrations of chitosan and tested essential oils:

The minimum inhibitory concentration (MIC) of chitosan and tested essential oils included clove, thyme, rosemary and sage were examined at concentrations of 0.05-1.0% for chitosan and 20-120 µL for tested essential oils. As shown in Table (6), the MIC values of chitosan ranged from 0.05 to 0.2%. *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli* and *Pseudomonas fluorescens* showed 0.05% MIC where as the value was 0.2% for *Bacillus cereus*. Hong *et al.*, (2002) reported the MIC of chitosan ranged from 0.05 to > 0.1% depending on the bacteria tested. Uchida *et al.* (1989) reported that MIC of chitosan for *Escherichia coli* and *Staphylococcus aureus* was found to be 0.025 and 0.05%, respectively. From Fig (3), it could be reported that, the antimicrobial activity of chitosan was bactericidal for *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli* and *Pseudomonas fluorescens*, on the centrary, it was bacteriostatic for *Bacillus cereus*. Table (6) also showed that MIC of clove oil was 20 µL with the aforementioned tested bacterial strains. On the other hand, thyme oil recorded MIC of 20 µL with *Bacillus cereus* and *Pseudomonas fluorescens*, where as it was 40 µL with *Salmonella typhimurium* and *Escherichia coli*. Rosemary and sage oils recorded 20 µL of MIC with *Staphylococcus aureus, Escherichia coli* and *Bacillus cereus*, where the same tested oils recorded MIC of 60 µL with *Bacillus cereus* and *Pseudomonas fluorescens*. According to the result in Figs (4 and 5), the antimicrobial activity of clove and thyme oils were bactericidal for all tested bacterial strains.

Table (6): Minimum inhibitory concentration or volume of tested antimicrobial agents.

Bacterial strains	Minimum inhibition concentration or volume					
	Tested agents	Chitosan (%)	Clove oil (µL)	Thyme oil (µL)	Rosemary oil (µL)	Sage oil (µL)
Staphylococcus aureus		0.05	20	0.0	20	0.0
Salmonella typhimurium		0.05	20	40	0.0	0.0
Bacillus cereus		0.2	20	20	60	20
E.coli		0.05	20	40	20	20
Pseudomonas fluorescens		0.05	20	20	0.0	60

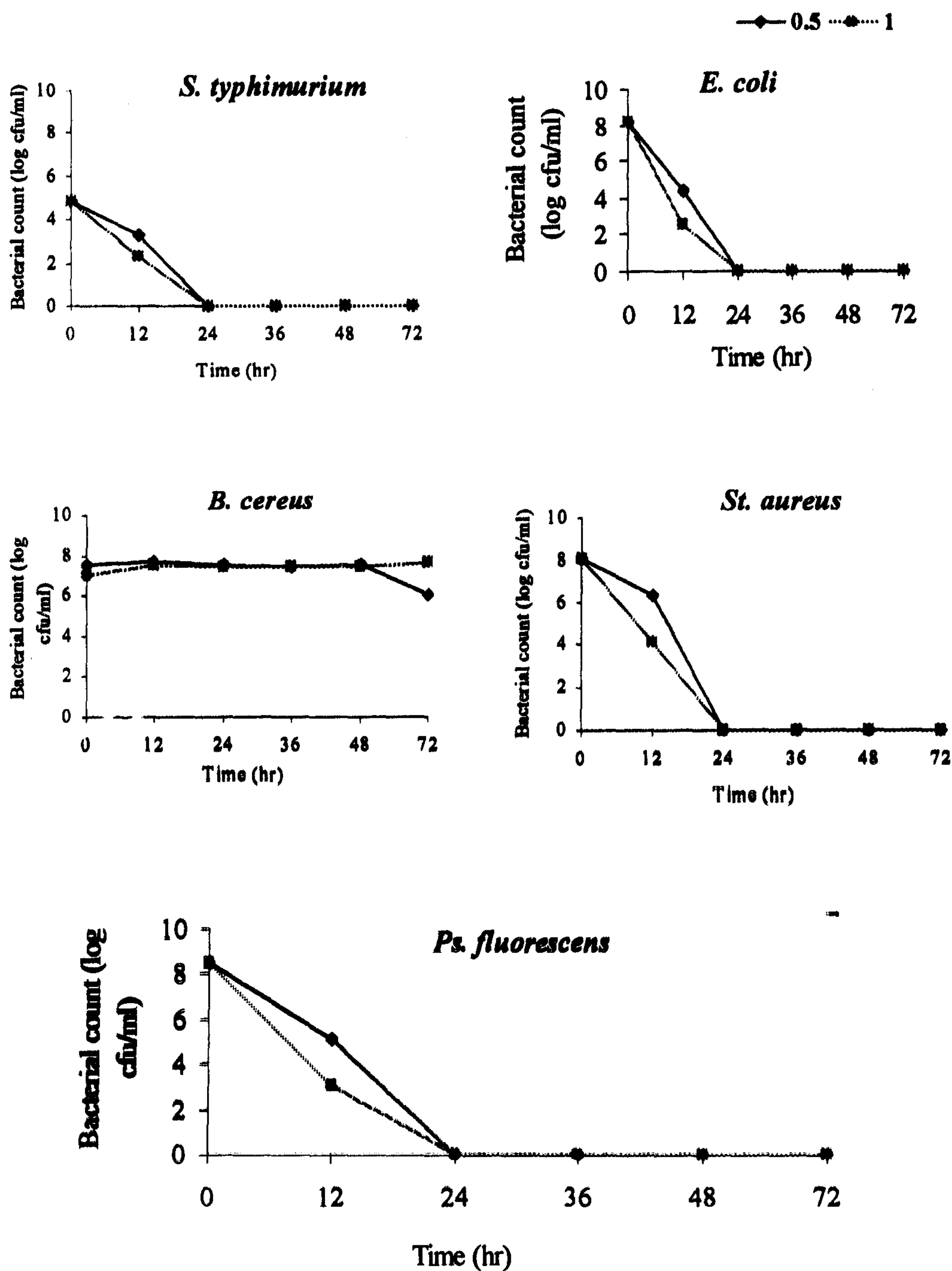

Fig (3): Effect of different chitosan concentrations on tested bacteria.

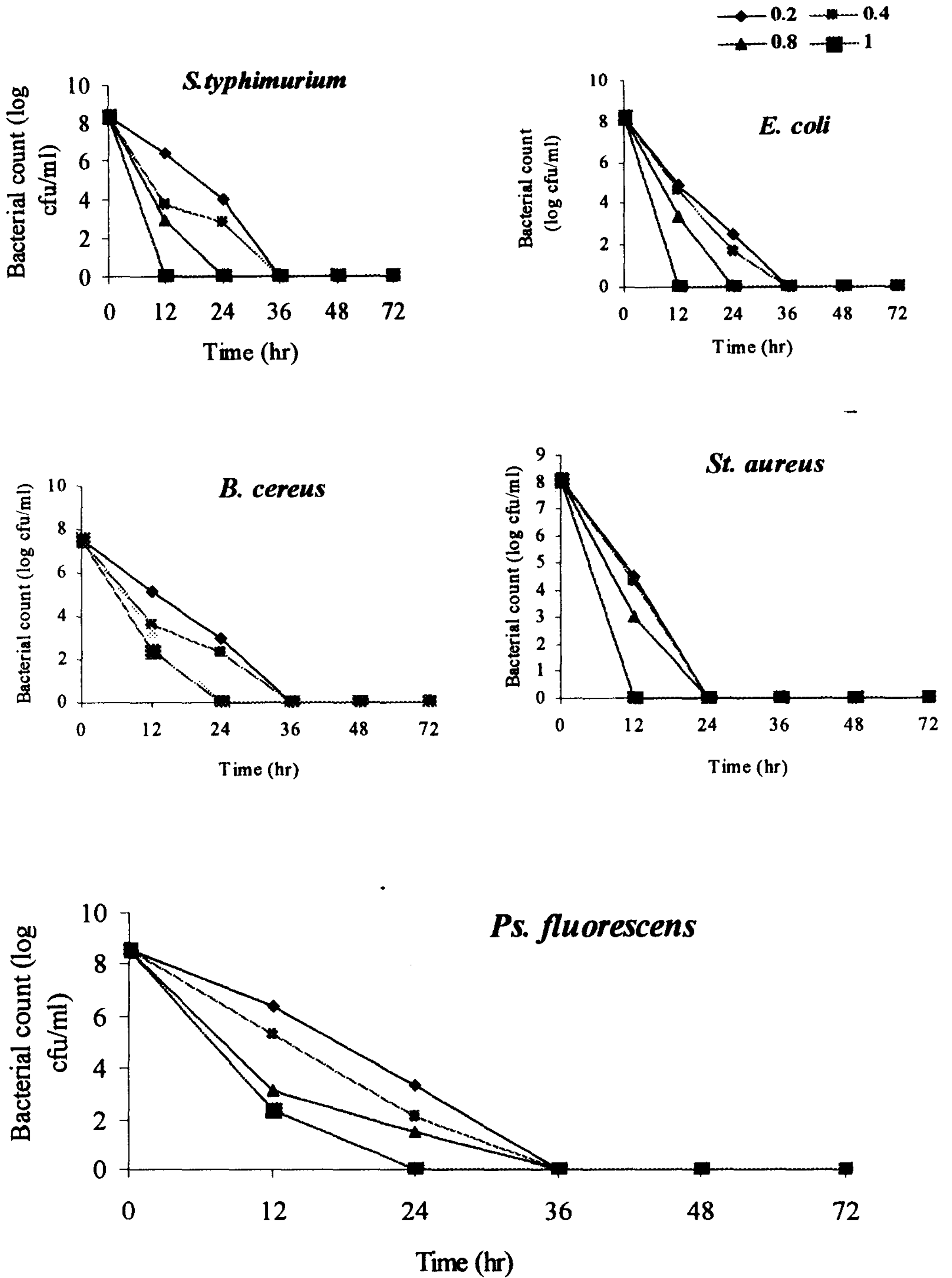

Fig (4): Effect of different clove oil concentrations on tested bacteria

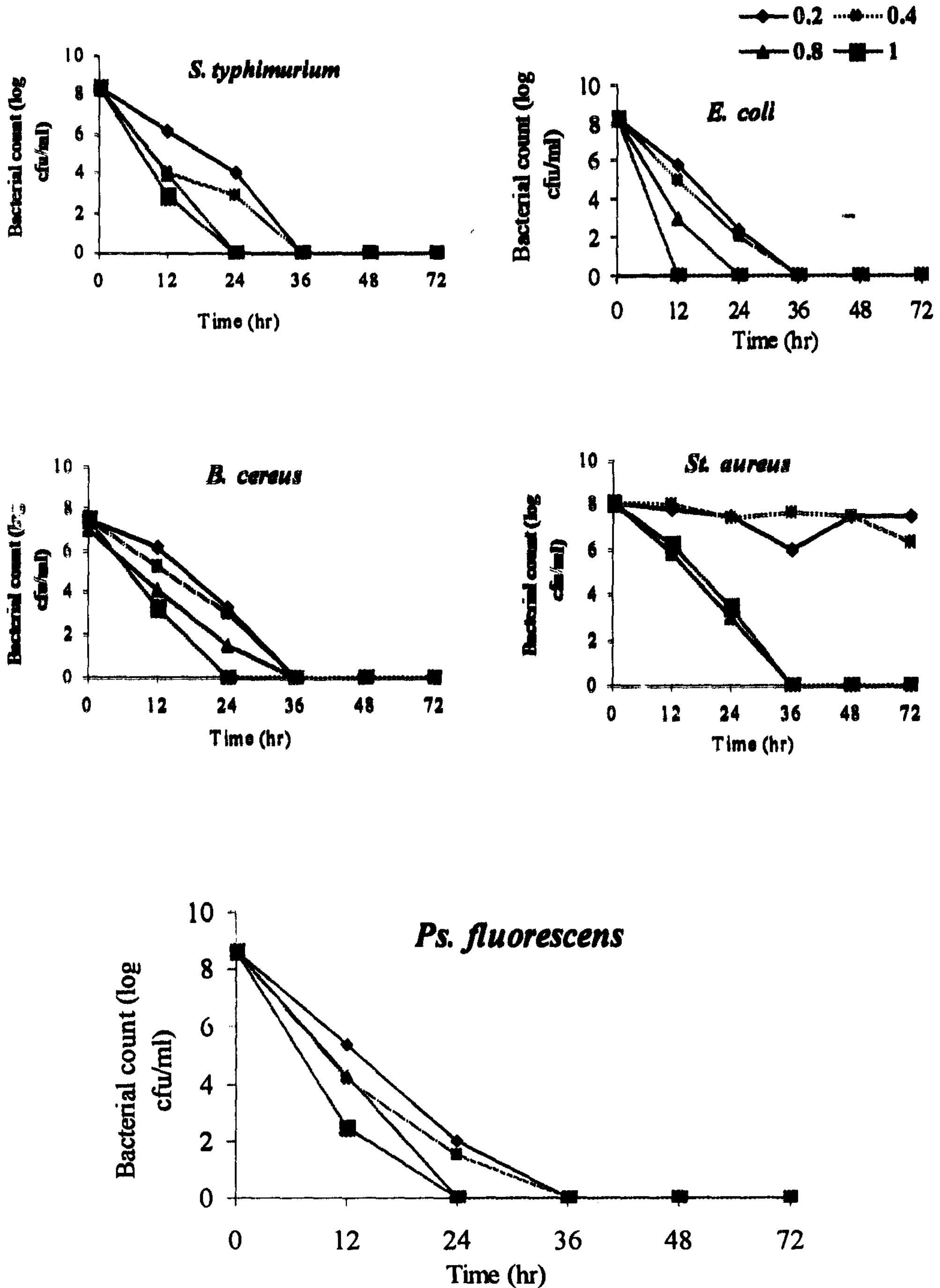

Fig (5): Effect of different thyme oil concentrations on tested bacteria

CONCLUSION

Recommendation for using 1% chitosan and also clove, thyme, rosemary and sage oils for inhibiting microbial load and as preservatives in producing or manufacturing luncheon meat.

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الحمل الميكروبى للحم اللانشون والنشاط البيولوجى لبعض السلالات البكتيريه المختارة تحت تأثير المواد الحافظة الطبيعيه

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تم تجميع عينات مختلفة من اللانشون البقرى ولانشون الدجاج المنتج محليا بالأسواق وتخزينهـا علـى درجات حرارة مختلفة (٤ و٢٥ °م) لمدة ٨ أسابيع لدراسة الحمل الميكروبى لها. ومن خلال التقديرات الميكروبيه لهذه العينات توصلنا الى أن كل من ميكروبات السالمونيلا والشيجلا ومجموعة القولون لم توجد فى جميع العينات المختبرة طوال فترة التخزين، بينما أعطى كل من العدد الكلى للبكتريا، البكتريا المتجرثمه، البكتريا المحبه للحرارة المرتفعة، البكتريا المحبه للحرارة المنخفضه، البكتريا العنقوديه، بكتريا *Bacillus cereus* والعدد الكلى للفطريات نتائج مختلفة أثناء فترة التخزين وذلك تبعا لنوع العينه وظروف تخزينها. وقد لوحظ خلال هذه الدراسه أن التخزين على درجة حرارة ٤°م كان له تأثير فعال فى انخفاض الحمل الميكروبى بالعينات خاصة على البكتريا المتجرثمـه والبكتريا المحبه للحرارة المرتفعة.

ولقد تم دراسة تأثير بعض المواد الحافظه الطبيعيه على تقليل الحمل الميكروبى للعينات مثل الشـيتوزان وبعض الزيوت العطريه مثل زيت القرنفل، الزعتر، الحصالبان والمرمريه ووجد أن الشيتوزان كان له تأثير قاتـل لكـل السـلالات البكتيريـه المختبـرة مثـل *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli* و *Pseudomonas fluorescens* فيما عدا بكتريا *Bacillus cereus* حيث كان موقف النمو فقط، وبتقدير اقل تركيز مثبط للشيتوزان (MIC) وجدنا أنه يتراوح بين ٠,٠٥ – ٢,٠٪ .
وبدراسة التأثير المثبط للزيوت العطريه وجد أن كل من زيت المرمريه والحصالبان لم يسجلا تأثيرا فعالا حيـث أنهما ذات تأثير ضعيف جدا على النشاط البكتيرى للسلالات المختبرة ، على العكس من زيت القرنفـل والزعتـر حيث أن لهما تأثير قاتل لكل السلالات البكتيريه المختبرة وكان اقل تركيز مثبط لزيت القرنفل ٢٠ميكروليتر ولزيت الزعتر ٤٠ميكروليتر.