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Chitosan and mint mixture: A new preservative for meat and meat products

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Abstract

Meat is prone to both microbial and oxidative spoilage and therefore it is desirable to use a preservative with both antioxidant and antimicrobial properties. Mint extract alone had good antioxidant activity but poor antimicrobial activity, while chitosan alone showed poor antioxidant activity with excellent antimicrobial properties. Therefore, the potential of chitosan and mint mixture (CM), as a preservative for meat and meat products, was investigated. Addition of chitosan to mint extract did not interfere with the antioxidant activity of mint. In the case of 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, the IC_{50} value for CM (17.8 μg/ml) was significantly ($p \leqslant 0.05$) lower than that for mint extract (23.6 μg/ml). CM efficiently scavenged superoxide and hydroxyl radicals. The antimicrobial activities of CM and chitosan were comparable against the common food spoilage and pathogenic bacteria, the minimum inhibitory concentration being 0.05%. CM was more effective against Gram-positive bacteria. The shelf life of pork cocktail salami, as determined by total bacterial count and oxidative rancidity, was enhanced in CM-treated samples stored at 0–3 °C.

Keywords: Chitosan mint mixture; Antioxidant; Antimicrobial; Meat products

1. Introduction

Chitosan is a versatile biopolymer, having a broad range of applications in the food industry (Rudrapatnam & Farooqahmed, 2003). It exhibits antimicrobial activity against a range of food-borne microorganisms and consequently has attracted attention as a potential natural food preservative (Chen, Lian, & Isai, 1998; El Ghaonth, Arul, Asselin, & Benhamon, 1992; Shahidi, Arachchi, & Jeon, 1999). Binding of trace metals and effect on membrane permeability have been postulated to be the main mechanisms for its antibacterial action (Helander, 2001; Zheng & Zhu, 2003). Muscle foods have low oxidative stability and are very susceptible to rancidity during production and storage. Numerous studies have indicated that lipid oxidation in meat and meat products may be controlled or minimized through the use of antioxidants (Gray, Gomaa, & Buckley, 1996; Nissen, Byrne, Bertelsen, & Skibsted, 2004; Rhee, 1987). However, chitosan does not have significant antioxidant activity.

Flavour is an important consideration that may limit the use of some antioxidants in meat and meat products. Spices and herbs have been used in many cuisines to impart flavour, aroma and piquancy to food. Several studies have shown the antimicrobial and antioxidant potential of spices and herbs, such as basil, thyme, rosemary, garlic, clove, coriander, ginger, mustard and pepper (Sebranek, Sewalt, Robbins, & Houser, 2005; Tipsrisukond, Fernando, & Clarke, 1998). Mint (*Mentha spicata*) is an herb used extensively in Indian cuisine and also for curing several common ailments (Choudhury, Kumar, & Garg, 2006). Earlier studies in our laboratory showed that mint extract had very good antioxidant potential, which was comparable to that of the synthetic antioxidant, butylated hydroxy toluene (BHT) (Kanatt, Chander, & Sharma, 2007). Mint extract did not show any antibacterial activity, though essential

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oils of some *Mentha* species have been reported to have antibacterial activity (Marino, Bersani, & Comi, 2001; Moreira, Ponce, del Valle, & Roura, 2005).

Development of natural preservatives with both antioxidant and antibacterial activities, that prolong the shelf life of meat and prevent food-borne illness, is desirable. The objective of this study was to determine the potential of chitosan and mint extract mixture as an antioxidant and antibacterial agent in preservation of meat and meat products.

2. Materials and methods

2.1. Chemicals

Chitosan, in powder form, was obtained from Mahatani Chitosan Pvt. Ltd. (Veraval, India). Moisture content of chitosan was less than 10% and it had a deacetylation range of 78–82% (Manufacturer's data). BHT, 2,2-diphenyl-1-picrylhydrazyl (DPPH·) and nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Co. (St. Louis, MO). Phenazine methosulphate (PMS), deoxyribose and nicotinamide adenine dinucleotide (NADH) disodium salt were purchased from HiMedia (India). All other reagents used were of analytical grade and procured from Qualigens Fine Chemicals (Mumbai, India) and Sisco Research Lab (Mumbai, India).

2.2. Bacterial cultures

Escherichia coli JM109, *Pseudomonas fluorescens* ost5 (16s rRNA gene sequence submitted to Genbank, Accession no. DQ439976) a laboratory isolate, *Staphylococcus aureus* ATCC 6538P, and *Bacillus cereus* MTCC 470, *Salmonella typhimurium* from the Central Research Institute, Kasauli, India, were stored in 20% glycerol (v/v) at −20 °C. Before the start of the experiment, the cultures were grown on nutrient agar. The isolates were subcultured twice before inoculation.

2.3. Preparation of chitosan and mint extract mixture (CM)

Mint extract was prepared as reported by Kanatt et al. (2007). It was then dissolved in distilled water to make the required concentration. Chitosan solution was made in 1% glacial acetic acid. CM (1%) was prepared by mixing the two solutions and autoclaved and used for all the assays.

2.4. DPPH radical-scavenging activity

The DPPH radical-scavenging activity of the test samples was estimated by the method of Yamaguchi, Takamura, Matoba, and Terao (1998). The diluted sample (200 µl) was mixed with 800 µl of tris-HCl buffer (100 mM, pH 7.4). To this was added 1 ml of 500 µM DPPH· in ethanol and the mixture was vortexed and absorbance measured at 517 nm after 20 min incubation in the dark. Percent DPPH·-scavenging activity was calculated as

$$[(\text{Control absorbance} - \text{Extract absorbance})/(\text{Control absorbance})] \times 100$$

2.5. Hydroxyl radical-scavenging activity

The hydroxyl radical-scavenging abilities of CM, mint extract and chitosan were determined according to the deoxyribose method of Halliwell, Gutteridge, and Aruoma (1987). To 1 ml of appropriately diluted sample, 1 ml of phosphate buffer (0.1 M pH 7.4) containing 1 mM ferric chloride, 1 mM EDTA, 1 mM ascorbic acid, 30 mM deoxyribose and 20 mM hydrogen peroxide, were added and incubated at 37 °C for 90 min. To this, 2 ml of 2% (w/v) TCA and 2 ml of 1% (w/v) TBA were added and the mixture then heated in a boiling water bath for 15 min. The absorbance of pink colour developed was measured at 532 nm. The reaction mixture not containing the test sample was used as control. Site-specific hydroxyl radical-scavenging activity of sample was determined as described above except that EDTA was absent in the reaction system. The percent inhibition of hydroxyl radical was calculated as above for the DPPH assay.

2.6. Superoxide anion radical-scavenging activity

The superoxide anion radical-scavenging ability of the test samples was assessed as described by Liu, Ooi, and Chang (1997) with some modifications. To 1 ml of NBT (156 µM in 0.1 M potassium phosphate buffer pH 7.4), 1.0 ml of NADH (468 µM in 0.1 M potassium phosphate buffer pH 7.4) and 0.5 ml of an appropriately diluted sample were added. To initiate the reaction, 100 µl of PMS (60 µM in 0.1 M potassium phosphate buffer pH 7.4) were added to the mixture. The absorbance was measured at 560 nm after incubation at ambient temperature for 5 min. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\%\ \text{Inhibition} = [(A_o - A_s)/A_o] \times 100$$

where A_o is absorbance of the control and A_s is absorbance of the sample.

2.7. Measurement of reducing power

The reducing power of the samples was determined according to the method of Oyaizu (1986). An aliquot of sample was mixed with 200 mM sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide and the mixture was incubated at 50 °C for 20 min. 10% TCA (2.5 ml) was added and the mixture was centrifuged at 650 g for 10 min. The upper layer (5 ml) was mixed with D/W (5 ml) and 0.1% ferric chloride (1 ml) and the absorbance was measured at 700 nm.

2.8. Determination of antibacterial activity

The antibacterial activities of CM, mint extract and chitosan were tested against *E. coli*, *P. fluorescens*, *S. typhimurium*, *S. aureus* and *B. cereus*. Stock cultures of all tested bacteria were grown in nutrient broth for 18 h. CM/chitosan were added to nutrient broth tubes in order to get final concentrations of 0.01%, 0.05% and 0.1%. In the case of mint extract, only 0.1% concentration was taken. The test culture (0.1%) was then inoculated into the nutrient broth tubes containing CM/chitosan/mint extract. Control tubes had only the test culture. At the initial point (0 h) sample was withdrawn, serial dilutions carried out, plated on plate count agar (by spread plate method) and counted after incubation at 37 °C for 18 h. This gave the initial number of the test organisms. All the tubes were then incubated for 4 h and 24 h at 37 °C, the aliquots were again taken and the surviving population was determined. The antibacterial activity of CM/chitosan/mint extract was assessed by decrease in log cfu/ml of the test culture in 24 h.

2.9. Effect of CM on survival of bacteria inoculated in minced lamb meat during chilled storage

Minced lamb meat (leg portion) was sterilised by irradiation at 10 kGy in Gamma Cell 5000 at a dose rate of 8.5 kGy h^{-1}. Stock cultures of all test bacteria were grown in nutrient broth for 18 h. Meat (25 g) was aseptically packed in sterile polythene bags to which CM (0.1%) and test culture were added. The control samples did not contain CM. All the packets were then stored at 0–3 °C. The total viable counts of the control and treated samples were determined immediately and at intervals of one week.

2.10. Effect of CM on the shelf life of pork cocktail salami

CM (0.1%) was incorporated in the meat formulation for the commercial preparation of pork cocktail salami in Hygienic Meat Products, Mumbai. The salamis were then stored at 0–3 °C and the total counts determined weekly. Thiobarbituric acid-reactive substances (TBARS), produced due to lipid peroxidation, were determined using the method of Alasnier, Meynier, Viau, and Gandmer (2000). Meat sample (4 g) was blended with 5% trichloroacetic acid (TCA) (16 ml) and BHT (10 μg BHT/g of lipids). It was then filtered through a Whatman filter paper (No. 4). Equal amount of filtrate and 0.02 M TBA were heated in a boiling water bath for 30 min, cooled, and the absorbance measured at 532 nm. TBARS were expressed as mg of malonaldehyde per kg of meat.

2.11. Sensory analysis of CM-treated pork cocktail salami

The sensory attributes evaluated were appearance, flavour, texture and overall acceptability of the product, using a 10-point numerical scale, where 10 corresponded to "components characteristic of the highest quality". Scores from 10–6 were considered acceptable. The panel consisted of 15–20 experienced members of the staff who were familiar with meat characteristics. The salamis were shallow-fried before serving.

2.12. Statistical analysis

All experiments were carried out in triplicate and average values with standard errors are reported. Analysis of variance was conducted and differences between variables were tested for significance by one-way ANOVA with Tukey's post test using GraphPad InStat version 3.05 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com. A statistical difference at $p \leqslant 0.05$ were considered significant.

3. Results and discussion

3.1. Antioxidant potential

3.1.1. DPPH activity

The capacities of various extracts to scavenge the stable DPPH free radical (by donation of a hydrogen atom) were investigated as shown in Fig. 1. CM, mint extract and BHT exhibited very good radical-scavenging activities and there was a strong correlation between antioxidant activity and concentration ($r^2 = 0.95$, $p \leqslant 0.002$). Chitosan alone did not show any DPPH radical-scavenging activity. CM showed the highest antioxidant activity and it was better than mint alone. The IC_{50} value for CM was 17.8 μg/ml, which was significantly ($p \leqslant 0.05$) lower than the 23.6 μg/ml for mint extract. Earlier studies in our laboratory have shown that mint extract is a potent antioxidant and its radical-scavenging activity was comparable to that of the standard synthetic, BHT (Kanatt et al., 2007). Other

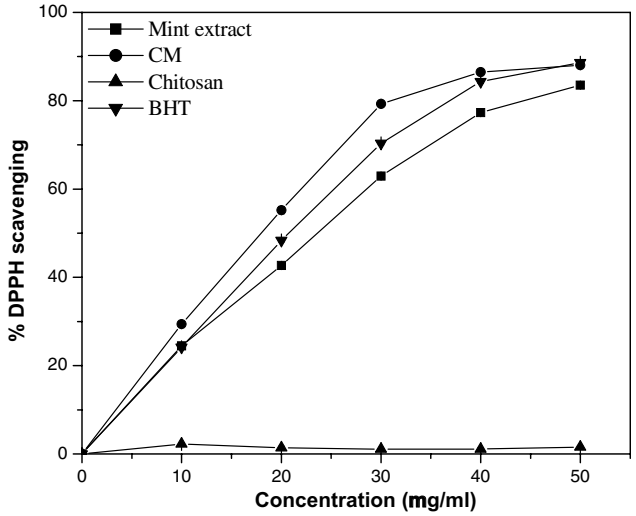

Fig. 1. Antioxidant activity of CM, measured as percent scavenging of DPPH radical. Each value represents the mean ± standard deviation of three experiments.

researchers have also shown the antioxidant potential of mint extract (Caillet et al., 2007; Choudhury et al., 2006; Mata et al., 2007). A noteworthy observation of the present study was that the inclusion of chitosan in the mint extract did not affect its antioxidant activity. Georgantelis, Ambrosiadis, Katikou, Blekas, and Georgakis (2007a) reported that the antioxidative effect of chitosan was greatly enhanced by the addition of chitosan to rosemary extract.

3.1.2. Superoxide radical-scavenging activity

Superoxide anion is a reduced form of molecular oxygen and plays an important role in the formation of other reactive species, such as hydrogen peroxide, hydroxyl radical and singlet oxygen. The efficacy of the extracts in scavenging superoxide radical generated in the PMS–NADH system was studied. The superoxide scavenging abilities of CM and mint extract were similar while chitosan alone did not show any activity (Fig. 2). Several leafy herbs and spices, especially those belonging to the Labiatae family, such as sage, rosemary, oregano and thyme, also show radical-scavenging activity (Zheng & Wang, 2001).

3.1.3. Hydroxyl radical-scavenging activity

Hydroxyl radical is the most reactive free radical and it can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions. The scavenging abilities of CM, mint extract and chitosan are shown in Fig. 3. The hydroxyl-scavenging activity of CM was found to be significantly ($p \leqslant 0.05$) higher than that of mint extract. Chitosan alone did not scavenge the hydroxyl radical. Addition of chitosan to mint extract therefore probably had a synergistic effect. The effectiveness of CM in inhibiting deoxyribose degradation, due to hydroxyl radical damage, was greater in the non-site-specific assay than in the site-specific assay. In most herbs, the antioxidant activity is due to the flavonoids containing multiple hydroxyl groups. The number of hydroxyl groups and substitution with electron-donating alkyl or methoxy groups of flavonoid increases their antioxidant potential (Kahkonen et al., 1999).

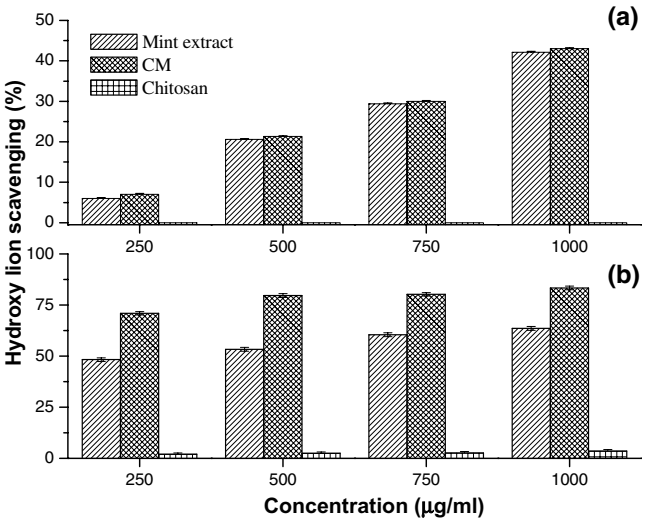

Fig. 3. Hydroxyl ion-scavenging activity, [a] site-specific [b] non-site-specific, of CM. Values are the means ± standard deviation of three replicate experiments.

3.1.4. Reducing power

Antioxidant activity has been reported to be concomitant with reducing power. In this study it was seen that, over the entire concentration range tested, CM and mint extract had similar reducing powers (Fig. 4). Chitosan showed almost negligible reducing power. In the case of most herbal extracts, their antioxidant activity has been attributed to their ability to break the free radical chain by donating a hydrogen atom (Pin-Der-Duh, 1998). The reducing properties are generally associated with the

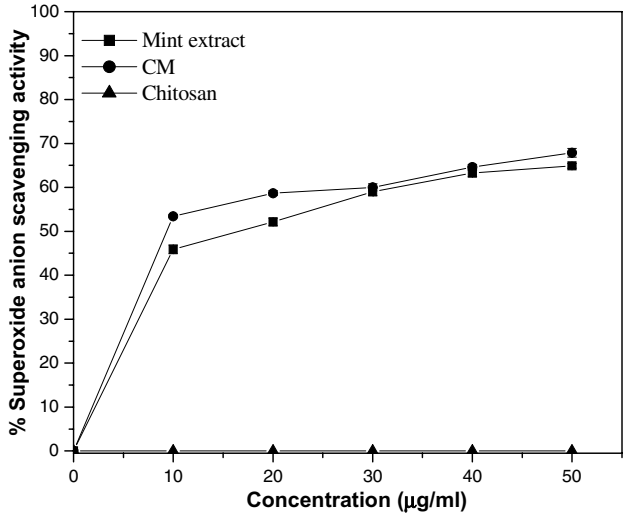

Fig. 2. Superoxide anion-scavenging activities of CM. Values are the means ± standard deviation of three replicate experiments.

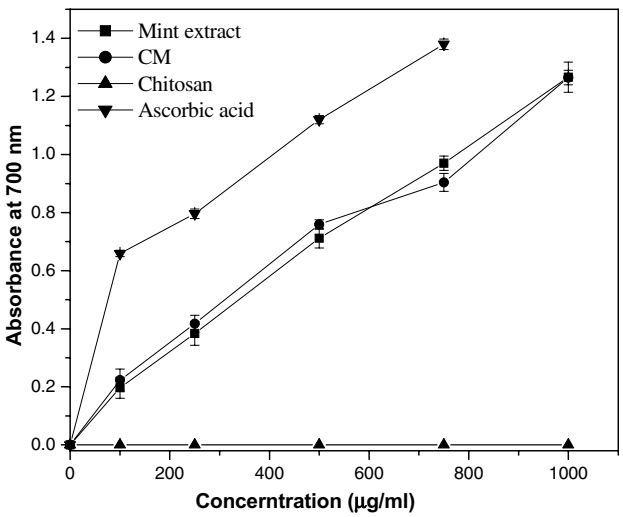

Fig. 4. Reducing power of CM. Values are the means ± standard deviation of three replicate experiments.

presence of reductones, which also react with certain precursors of peroxide, thus preventing peroxide formation.

3.2. Antibacterial activity

The survival of common spoilage and pathogenic bacteria normally encountered in meat and meat products was investigated in the presence of CM, chitosan and mint extract in nutrient broth. The counts of all the test organisms studied were significantly ($p \leqslant 0.05$) affected by the addition of CM and chitosan (Figs. 5–9). The effect of CM and chitosan was found to be concentration-dependent. At a concentration of 0.01%, neither CM nor chitosan was effective against any of the test cultures. At higher concentrations (0.05%, 0.1%), the antibacterial effect was enhanced and 100% kills of *B. cereus*, *S. aureus*, *E. coli* and *P. fluorescens* were achieved. However, in the case of *S. typhimurium* at a concentration of 0.1% CM or chitosan, a 4 log cycle kill was seen but growth was not completely suppressed (Fig. 9). The antimicrobial activity of chitosan is well documented against a number of food spoilage and pathogenic microorganisms with MIC varying from 0.01% to 1% (Sagoo, Board, & Roller, 2002). In this study, it was observed that the antimicrobial action of CM was similar to that of chitosan. Mint (0.1%) did not have any antibacterial activity, as growths of all test organisms in nutrient broth containing it were similar to the control in which no preservative was added (Figs. 5–9). Many spices and herbs exert antimicrobial activities due to their essential oil fractions (Moreira et al., 2005). Skandamis, Koutsoumanis, Fasseas, and Nychas (2001) found that oregano essential oils and EDTA inhibited the growth of *E. coli* by inducing morphological changes. Chitosan, in combination with sulphites, reduced lactic acid bacteria by 1–2 log cfu/g in fresh pork sausages after 24 h of refrigerated storage (Roller et al., 2002). Lopez-Caballero, Gomez-Guillen, Perez-Mateos, and Montero (2005) reported that a coating consisting of a blend of chitosan dissolved in acetic acid and gelatin exerted an inhibitory effect on the Gram-negative flora of fish patties.

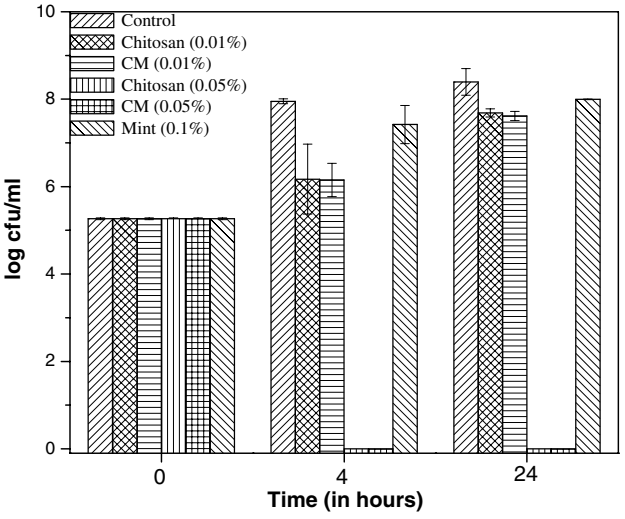

Fig. 5. Antimicrobial activity of CM/chitosan/mint extract against *E. coli*, measured in nutrient broth. At concentrations above 0.01%, CM/chitosan completely inhibited the growth of the test culture. Results are mean values of three independent experiments.

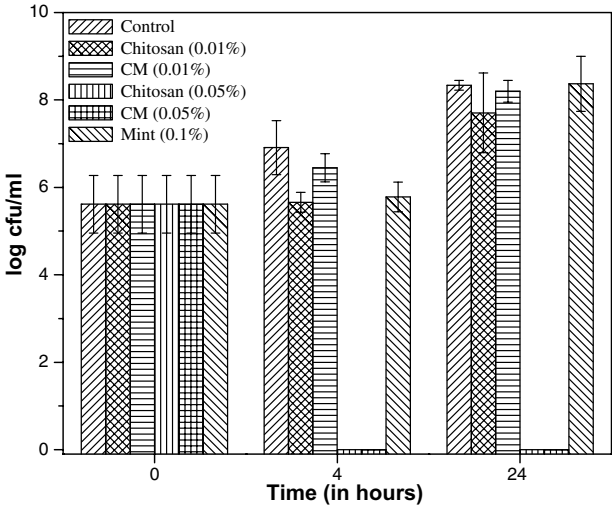

Fig. 6. Antimicrobial activity of CM/chitosan/mint extract against *Pseudomonas* measured in nutrient broth. At concentrations above 0.01%, CM/chitosan completely inhibited the growth of the test culture. Results are mean values of three independent experiments.

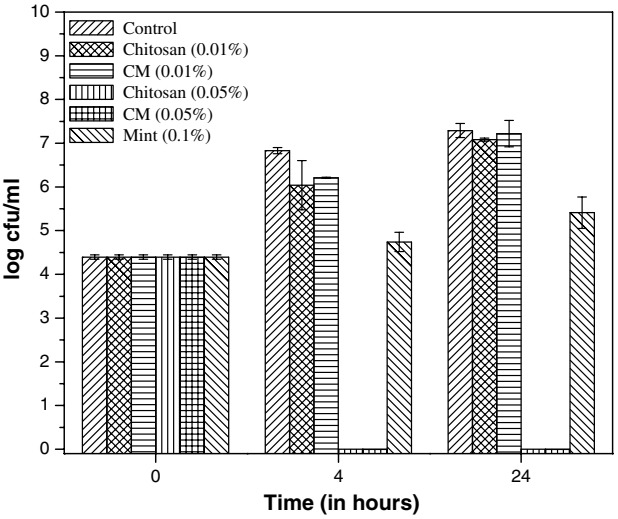

Fig. 7. Antimicrobial activity of CM/chitosan/mint extract against *Bacillus cereus* measured in nutrient broth. At concentrations above 0.01%, CGC/chitosan completely inhibited the growth of the test culture. Results are mean values of three independent experiments.

3.3. Effect of CM on survival of bacteria inoculated in minced lamb meat

The survival of test cultures inoculated into sterile minced lamb meat containing CM (0.1%) was also studied. In the case of the Gram-negative bacteria, it was seen that, in samples containing CM, there was one log cycle reduc-

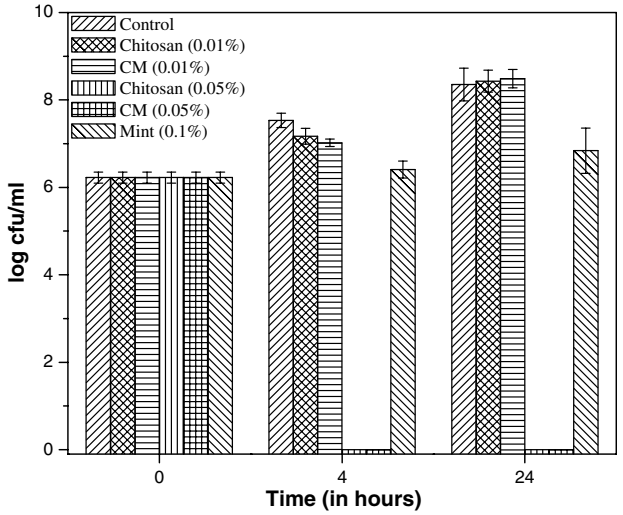

Fig. 8. Antimicrobial activity of CM/chitosan/mint extract against *S. aureus* measured in nutrient broth. At concentrations above 0.01%, CM/chitosan completely inhibited the growth of the test culture. Results are mean values of three independent experiments.

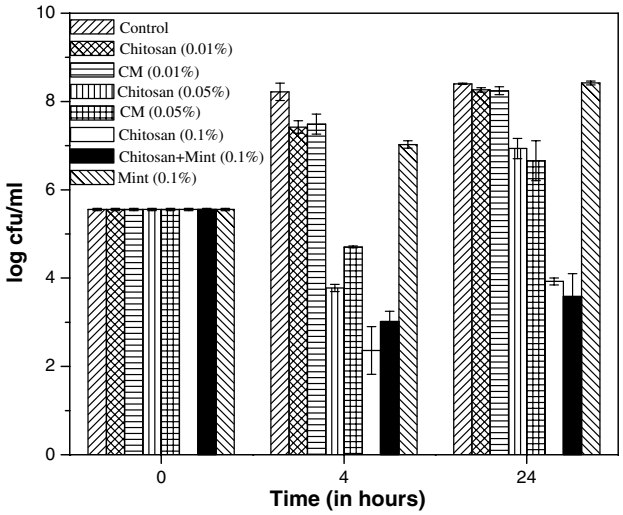

Fig. 9. Antimicrobial activity of CM/chitosan/mint extract against *Salmonella typhimurium,* measured in nutrient broth. Results are mean values of three independent experiments.

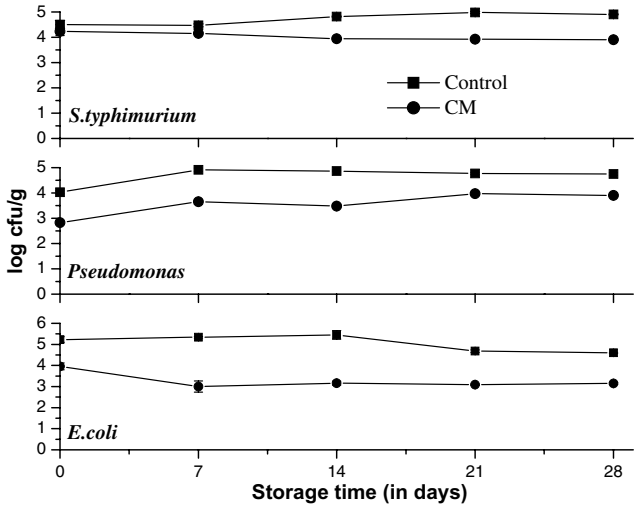

Fig. 10. Antimicrobial activity of CM against spoilage/pathogenic Gram-negative bacteria were inoculated into lamb meat during storage at 0–3 °C. Results are mean values of three independent experiments.

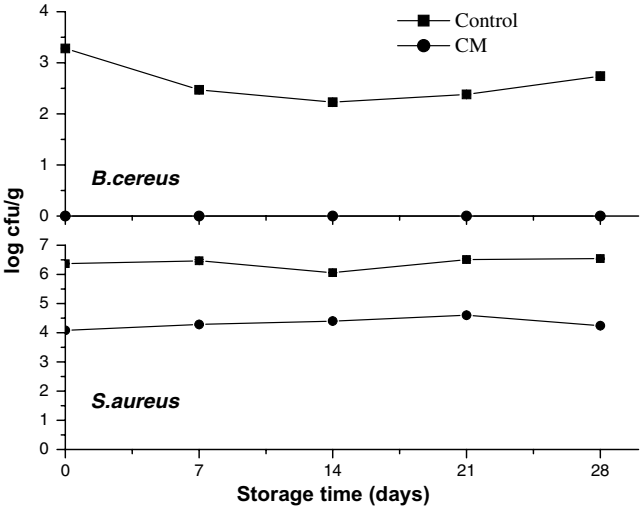

Fig. 11. Antimicrobial activity of CM against spoilage/pathogenic Gram-positive bacteria were inoculated into lamb meat during storage at 0–3 °C. Results are mean values of three independent experiments.

tion in growth as compared to the control during 4 weeks of chilled storage (Fig. 10). However, in the case of Gram-positive bacteria *S. aureus* and *B. cereus,* the effect was more pronounced. There was 2–3 log cycle reduction in growth of these organisms in lamb meat containing CM (Fig. 11). In the case of other extracts, Gram-positive bacteria were reported to be more sensitive than were Gram-negative bacteria (Negi & Jayapraksha, 2003; Smith-Palmer, Stewart, & Fyfe, 1998). This has been reported to be due to the cell wall lipopolysacchride in Gram-negative bacteria (Russel, 1991). Georgantelis, Blekas, Katikou, Ambrosiadis, and Fletouris (2007b) also reported that, in pork sausages, the lowest microbial counts were obtained in samples containing chitosan and rosemary, indicating a possible synergistic effect.

3.4. Effect of CM on the shelf life of pork cocktail salami

In the commercial preparation of pork cocktail sausages, CM was incorporated in the normal formulation that is added during its preparation. Initial sensory analysis of the salamis showed that sensorily there was no significant ($p \leqslant 0.05$) difference between the treated and untreated samples. With respect to colour, flavour, taste and texture, the CM-treated and control samples were similar. The total bacterial counts of both the samples increased on chilled storage. Control salamis spoiled in less than two weeks, while salamis containing CM displayed a shelf life of three weeks (Fig. 12) at chilled temperature (0–3 °C). In urban Indian markets, ready-to-eat products are marketed only in the frozen state, but freezing facilities

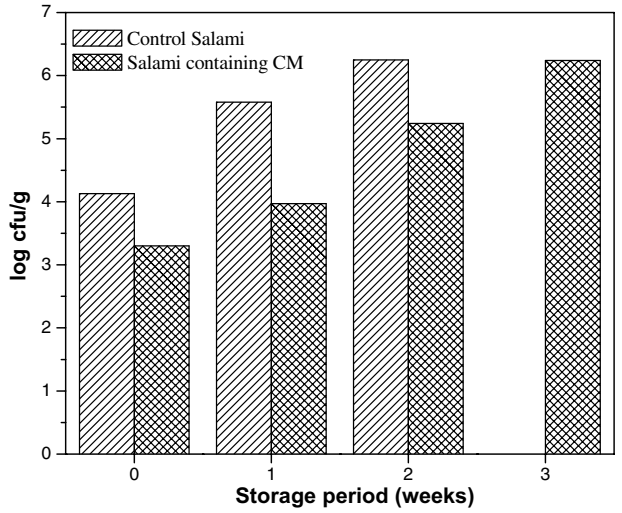

Fig. 12. Total bacterial count of pork cocktail salami containing CM during chilled storage. Values are the means ± standard deviation of three experiments.

are expensive and inadequate. Therefore, CM-treated salamis that can be stored at chilled temperatures would be of advantage.

Lipid oxidation, measured in terms of TBARS of pork cocktail salami containing CM during chilled storage, is shown in Fig. 13. The TBARS data indicated that the TBA values were significantly affected ($p \leqslant 0.05$) by both storage period and the CM treatment. Salamis with CM incorporated were relatively resistant to lipid oxidation throughout the storage period. Shelf life of meat and meat products is greatly influenced by oxidative changes. Pork, because of its relatively high content of unsaturated fatty acids, oxidizes more rapidly than do either beef or lamb (Pearson, Love, & Shorland, 1977). Cooking of meat makes it more sensitive to lipid oxidation than raw meat, due to protein denaturation and structural damages in membranes caused by heat (Gray et al., 1996). Addition of antioxidants before the cooking process therefore minimizes oxidative rancidity in meat products.

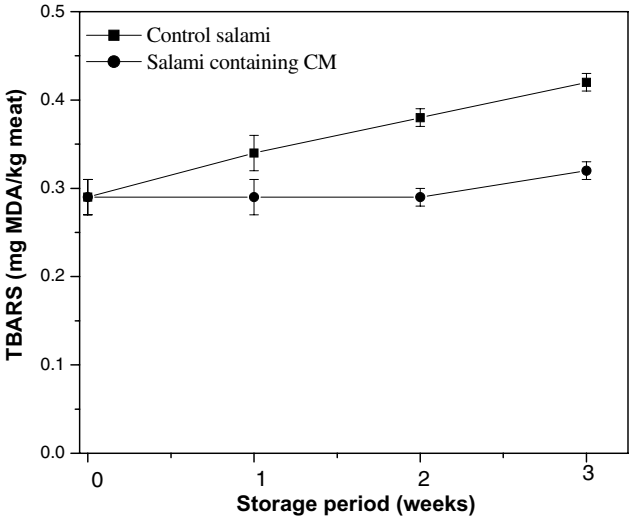

Fig. 13. TBARS of pork cocktail salami containing CM during storage at 0–3 °C. Values are the means ± standard deviation of three experiments.

4. Conclusion

One of the solutions to the problem of preservation of meat and meat products may lie in the development of effective antioxidant and antimicrobial blends. The present study demonstrates the efficacy of chitosan and mint mixture as a potent antibacterial and antioxidant agent that can be used for the preservation and shelf life extension of meat and meat products. Use of mint and chitosan in meat products is well suited to improving shelf life and safety of meat and other flesh foods.

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