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Synergistic Effect of Ascorbic Acid and Soybean Bioactive Compounds in Extending Shelf Life of Refrigerated Burger.

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ABSTRACT

Preservation of meat products can be done using wide range of preservatives alone or in combination with other techniques disregarding health hazards. Therefore, effective and safe natural preservatives are actively in demand. Burger was prepared with soybean additives (fermented Natto & protein hydrolysate) as natural food preservatives containing bioactive compounds (antioxidant and antimicrobial) plus ascorbic acid to produce functional meat products. Oxidative stability and microbial growth as well as total phenols and flavonoids were determined. Results showed that, lipid and protein oxidation were significantly decreased in samples with soybean additives. Ascorbic acid showed synergistic effect with soybean bioactive compounds (phenolics & bioactive peptides) resulting in increasing inhibition of lipid and protein oxidation of burger sample from 1.5 to 2 times. The protein oxidation results were synchronous with lipid oxidation, correlations ($R^2 = 0.89$; $p < 0.05$). Also, significant reversed correlations were found between either protein oxidation or lipid oxidation and total phenol. Microbiological profile was significantly affected by soybean additives, especially protein hydrolysate. *Staphylococcus aureus* was the most affected microorganism by soybean additives. Finally, shelf life of burger had been extended from 5 to 8 days by soybean additives (Natto & protein hydrolysate) and/or ascorbic acid comparing to control.

Keywords: Antioxidant, antimicrobial, bioactive compounds, total phenol, protein oxidation

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INTRODUCTION

Consumption of foods containing significant amounts of natural antioxidants may help the human body to reduce oxidative damage related to ageing and diseases, such as atherosclerosis, cancer and liver cirrhosis. It is worthy of attention that natural antioxidants in addition to the important impact on human health, it can also have a role in food preservation rather than synthetic antioxidants. The use of synthetic antioxidants for food preservation, such as butylated hydroxytoluene (BHT) and butylated hydroxyl anisole (BHA) have a safety concerns. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Moreover, these synthetic antioxidants also show low solubility and moderate antioxidant activity (Barlow, 1990). Therefore, attention has been focused on the use of natural antioxidants for inhibition and/or protection from oxidative damage in food system as well as in human body.

Incorporation of bioactive compounds such as phenolics, vitamins, probiotics, bioactive peptides, and antioxidants into food systems provide a simple way to develop novel functional foods that may have physiological benefits or reduce the risks of diseases. Many researches provided that, a number of food protein hydrolysates, specific peptides and phenolics effectively inhibit lipid peroxidation in different food products (Kong and Xiong, 2006), suggesting that phenolics and specific food-derived peptides can be utilized as natural antioxidants adding into food products to improve quality and stability. Also, wide range of activities has been described, including antimicrobial properties, blood pressure-lowering (ACE inhibitory) effects, cholesterol-lowering ability, antithrombotic and antioxidant activities, enhancement of mineral absorption & bioavailability and cytoor immune modulatory effects (Hartmann and Hans Meisel, 2007). On the other hand, nutrition with specific compounds of soy —acidic peptides together with phenolic compound (genistein) might protect against coronary atherosclerosis by attenuating platelet activity (Borgwardt et al., 2008). Thus, the significant occurrence of bioactive peptides & phenolic compounds, the relevant antioxidant capacities along with the interesting functional properties of dehydrated bean flours, specially soybean, make them to be considered functional ingredients for food formulation including processed meat.

The aim of this study, burger was prepared with soybean additives (fermented soybean Natto & soybean protein hydrolysate) in order to determine their activity with or without ascorbic acid addition. Also, synergism effect of additives against inhibition of meat protein and peroxidation of lipid and protein as well as antimicrobial activity were measured as a result of its bioactive compounds activity in order to extend shelf life of burger to produce functional meat products.

MATERIAL AND METHODS

Material:

Soybean (*Glycine max*) was obtained from The Agriculture Research Center-Giza-Egypt. The source of applied enzymes was crude Papain obtained from Technolab, Chemical-Scientific Equipments. The strain of *Bacillus natto* (NBRC 13169) which was obtained from National Institute of Technology and Evaluation Biotechnology Center (NITE) Japan. All other reagents were of highest analytical grade available.

Soy Protein isolates hydrolysate (SPIH):

SPI was prepared in our laboratory as described by Wang et al., (1998) and the enzymatic hydrolysis was carried out according to Chen et al., (1995).

Natto Preparation:

It was prepared according to the method of Wei et al., (2001).

Burger manufacture:

Independent replicates of burger formula were processed on the same day containing additives as following: formula 1 contains natto + ascorbic acid; formula 2 contains soy protein hydrolysate + ascorbic acid; formula 3 contains only natto; formula 4 contains soy protein hydrolysate; formula 5 contains ascorbic acid as

positive control; formula 6 contains no additives as negative control. Products were prepared in a pilot unit according to the commercial processing of meat factory.

Determination of total phenolic content:

Total phenolic content (TPC) of soybean and soy products were determined by Folin–Ciocalteu assay using gallic acid as standard (Kim et al., 2003).

Determination of total flavonoid content:

Total flavonoid content (TFC) was determined using a colorimetric method described previously (Heimler et al., 2005). Briefly, 0.25 ml of the sample extract or (+)-quercetin standard solution was mixed with 1.25 ml of distilled water followed by 75 µl of 5% NaNO₂ solution. After 6 min, 150 µl of 10% AlCl₃.6H₂O solution were added and allowed to stand for another 5 min before adding 0.5 ml of 1 M NaOH. The mixture was brought to 2.5 ml with distilled water, mixed well then absorbance was measured immediately at 510 nm. The results were calculated and expressed as (mg of QE/g of sample) using the calibration curve of quercetin.

Microbiological analysis:

To determine the microbial counts of pathogenic flora, analysis of the total viable bacterial count as well as *Salmonella* sp., *Clostridium perfringens*, *Bacillus cereus*, *Staphylococcus aureus*, Coliform bacteria, and yeast & fungi count. 10 g burger or minced meat samples were aseptically taken and transferred into sterile plastic bags containing 90 ml peptone water (Oxoid CM 9,UK), homogenized for 1–2 min, 10-fold serial dilutions were made in sterile peptone–salt water up to 10⁷. Finally, there were inoculated onto specific culture media for total aerobic plate count (nutrient agar), coagulase positive staphylococci (Baird Parker Medium, Oxoid CM 275,UK), coliforms (VL), sulphite-reducing anaerobic bacterial counts (*perfringens* agar), *Salmonella*, (*salmonella-shigella* agar), *B. cereus* (CSM) and moulds/yeasts (potato dextrose agar). For the isolation of coagulase positive staphylococci, up to five typical colonies (black or grey colonies) grown on BP agar were selected and transferred into tubes containing Brain Heart Infusion Broth (BHI-Oxoid CM 225,UK). The tubes were incubated at 37°C for 24 h. After the incubation, coagulase tests were done.

Determination of TBARS (2-thiobarbituric acid reactive substances):

TBARS of samples were determined by the spectro photometric method (Bozkurt, 2006).

Protein oxidation measurement:

Protein oxidation was assessed by the estimation of carbonyl groups formed for the duration of the experiment according to the method described by (Mercier et al., 2004). Each sample of homogenate was divided into two equal aliquots of 0.5 ml. Proteins were precipitated in both aliquots by 10% trichloroacetic acid and centrifuged at 2000 g for 10 min. One pellet was treated with 1ml of 2N HCl and the other with 1ml of 0.2% (w/v) 2,4-dinitrophenylhydrazine (DNPH) in 2N HCl. Both samples were incubated for 1 h at room temperature and stirred regularly, then precipitated with 10% TCA and centrifuged at 2000 g for 10 min. The pellets were then washed twice with 1 ml of ethanol: ethyl acetate mix (1:1). Finally, they were dissolved in 2ml of 6 M guanidine HCl with 20 mM sodium phosphate buffer pH 6.5, centrifuged 10 min at 2000 g. Protein concentration was measured at 280 nm against HCl as blank and BSA in 6M guanidine was used as standard. Carbonyl concentration was measured at 370 nm for protein hydrazones. The results were expressed as nanomoles of DNPH fixed per milligram of protein.

Statistical analysis:

Data were subjected to statistical analysis using the General Linear Models Procedure of the Statistical Analysis System (SAS, 2000). The significance of the differences among treatment group was determined by Waller-Duncan k-ratio (Waller and Duncan). All statements of significance were based on probability of P < 0.05. The correlation calculation was carried out using ToolPack to determine whether two ranges of data move together.

RESULTS AND DISCUSSION

Total phenols and flavonoids of burger:

Total phenols and flavonoids of burger samples with ascorbic acid and/or soy additives (Natto & protein hydrolysate) were measured to discover the relation between them and oxidative stability of burger. Fig. (1) showed the flavonoid content where the highest content of flavonoids was in burger with soy Natto additive, 622.5 mg/100g, while protein hydrolysate samples contained less amounts of flavonoids, 447.5 mg/100g, compared to flavonoid content of the control which was 182.5 mg/100g sample. On the other hand, addition of ascorbic acid decreased the content of flavonoids of burger samples with soy Natto and protein hydrolysate additives; 552 and 277.5 mg/100g respectively.

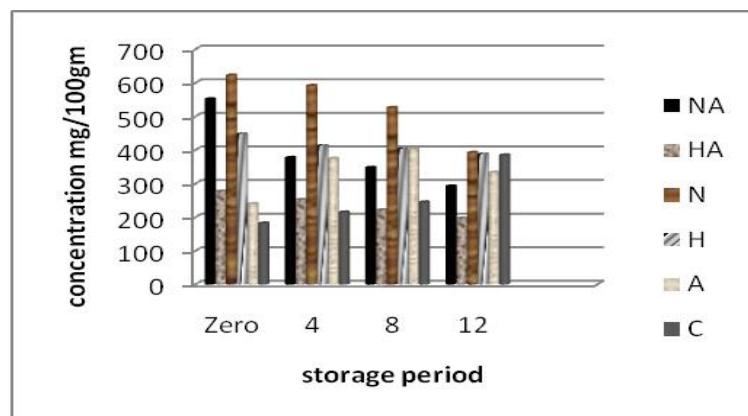


Fig 1: Effect of storage period on Flavonoids content of burger.

Total phenols was also decreased with ascorbic acid addition whereas Natto and Natto + ascorbic acid had total phenols higher than other sample which was 447.5 and 363 mg/100g respectively compared to the control samples 290.56 mg/100g, Fig. 2. These results may be due to acidic conversion of phenolic and flavonoid to another compound. With the absence of ascorbic acid, storage period impacted significantly ($p<0.05$) on both of flavonoid and phenolic compound content, thereby there was a significant increase in phenolic content and a decrease in flavonoid content with time.

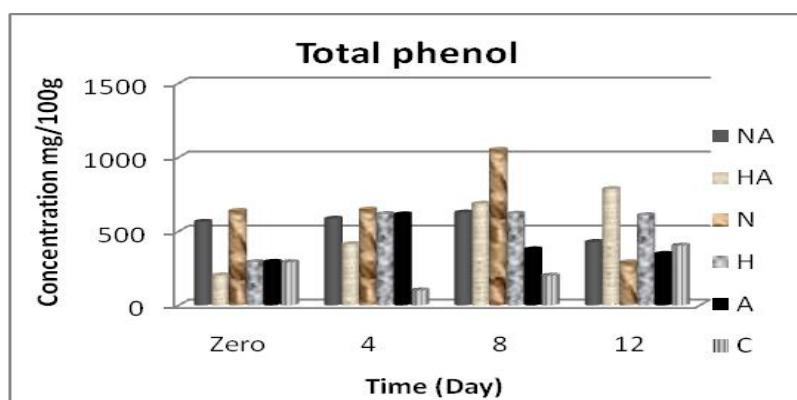


Fig 2: Effect of storage period on total phenols of burger.

Lipid oxidation TBA:

TBA values were evaluated to recognize the effect of soy bioactive compounds (phenolics) with synergistic effect of ascorbic acid on lipid oxidation. The analysis of variance for the TBARS data indicated that the TBA values were significantly affected ($P < 0.05$) by both the storage period and the treatments (soy additives & ascorbic acid). Our results clearly showed that the meat lipid oxidation was gradually preceded against the storage time as reflected by the dramatic increase of TBARS values (Fig.3). Initial (zero time) TBA

values for all samples significantly lower than those for the control ($P < 0.05$), suggesting that soy antioxidants retarded lipid oxidation immediately after formulation of burger. These were agreeing with that reported by Fernández-López et al., (2005) for natural antioxidants applied to meatballs. For all samples, there was a significant decrease in TBARS values between treatments by the addition of soybean Natto and protein hydrolysate with or without ascorbic acid. With regard to the total phenolic content of samples Fig. 2, burger samples with Natto additive, which had the highest phenolic content showed the highest capacity of inhibiting lipid oxidation. D'Souza and Skonberg, (2011) found that soybean whole meal had more antioxidative effects as compared to SPI (protein without phenolics). Also, the antioxidative peptides existing in fermented Natto and protein hydrolysate may shear in lipid oxidation inhibition of burger samples which gave enhancement effect with the other antioxidants, such as phenolic compounds and/or ascorbic acid. Therefore, antioxidant activity did not depend only on total phenol concentration, but also on their polarity and molecular structure (Hernandez-Hernandez et al., 2009).

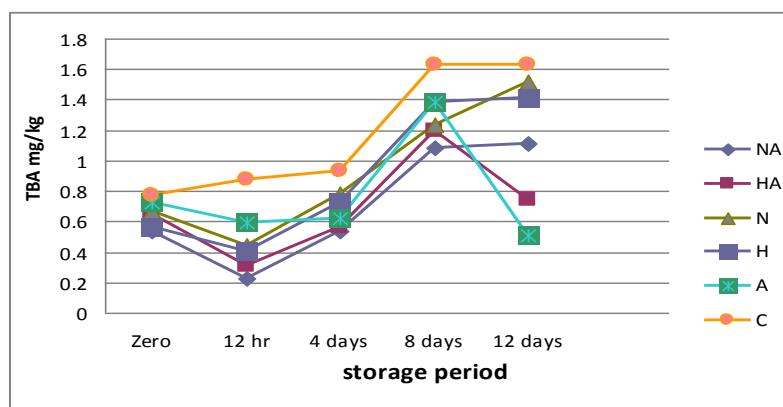


Fig 3: Effect of bioactive compounds on the TBA of refrigerated burger

On the other hand, ascorbic acid played an important role in the inhibition of lipid oxidation. The addition of Natto + ascorbic acid to the burger samples gave the lowest TBARS values followed by protein hydrolysate + ascorbic acid burger samples, comparing to other samples. This proved that ascorbic acid has synergistic effect with soybean bioactive compounds (phenolic compounds & bioactive peptides) resulting in increasing inhibition of the lipid oxidation of burger samples from 1.5 to 2 times. Hatano et al., (2008) found that ascorbic acid enhanced the antioxidant activity of epigallocatechin gallat EGCG (phenolics). Also, there was no significant difference between samples with Natto and protein hydrolysate additives. At the end of storage period (after 12 days) all treatments showed in significantly lower ($P < 0.05$) TBA values compared to the control sample.

Protein oxidation:

Determination of protein carbonyl content is generally used to describe the extent of protein oxidation in biological samples. Oxidation of protein is associated with the alteration of protein structure, peptide chain scission, formation of amino acid derivatives and polymers, decrease in solubility, and changes in the functional properties (Aewsiri et al., 2013).

Results showed that the addition of Natto and protein hydrolysate decreased the protein oxidation at zero time, which mean that soybean bioactive compounds bounded to carbonyl groups resulting in immediate decrease in TBARs. Fig.(4) demonstrated the difference between treatments in which, Natto + ascorbic acid gave the lowest amount of protein oxidation followed by protein hydrolysate (11.15 and 11.38 nmol DNPH/gm protein, respectively), while protein hydrolysate + ascorbic acid was 12.74 nmol DNPH/gm protein. This mean that ascorbic acid has a synergistic effect with Natto, but protein hydrolysate + ascorbic acid gave a reversed effect. The accumulation of protein oxidation products was noted in refrigerated stored burger (Fig. 4). The amount of carbonyls significantly increased ($p < 0.05$) from 11.15 to 14.73 nmoles carbonyls/mg protein, from 12.74 to 15.12 nmoles carbonyls/mg protein, from 12.91 to 16.43 nmoles carbonyls/mg protein and 11.38 to 15.72 nmoles carbonyls/mg protein in Natto + ascorbic acid, protein hydrolysate + ascorbic acid, Natto and protein hydrolysate burger samples, respectively compared to control which was from 14.81 to 18.87 nmoles

carbonyls/mg protein. Ganhão et al., 2010 have reported the antioxidant activities of phenolic compounds from fruit extracts against protein carbonyl formation in porcine burgers.

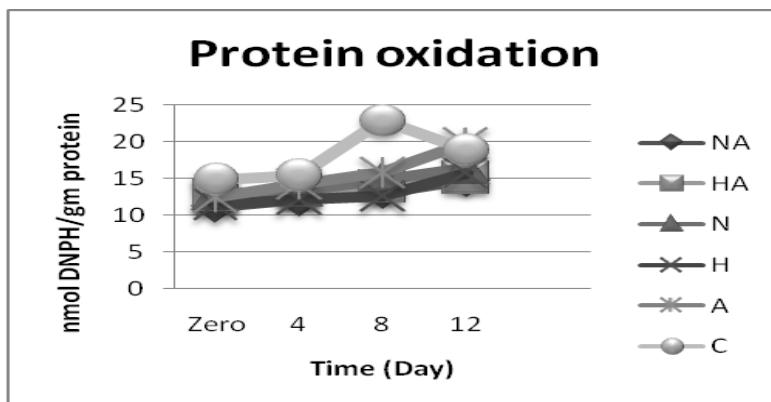


Fig 4: Effect of storage on the protein oxidation of refrigerated burger

Relationship between total phenols and protein oxidation & TBA:

The protein oxidation results were synchronous with lipid oxidation, suggesting relationship between lipid and protein oxidation with statistically high significant correlations between protein oxidation and TBARS ($R^2 = 0.89$; $p < 0.05$). Este'vez et al., 2007 have recently reported statistically significant correlations between TBARS values and carbonyls contents in refrigerated-stored frankfurters. They suggested interactions between such processes. A causal relationship between lipid and protein oxidation is probable since primary and secondary oxidation products can interact with proteins leading to protein radicals. Also, significant reversed correlations were found between either protein oxidation or TBA and total phenol Fig. (5). In addition of that, protein oxidation as well as lipid oxidation affect on sensory attributes especially, color and flavor resulting in deteriorating burger quality. The impact of protein oxidation reactions on meat quality (i.e. warmed-over flavor) has been extensively studied whereas research on the onset and extent of protein oxidation in processed meat products is limited (Ganhão et al., 2010).

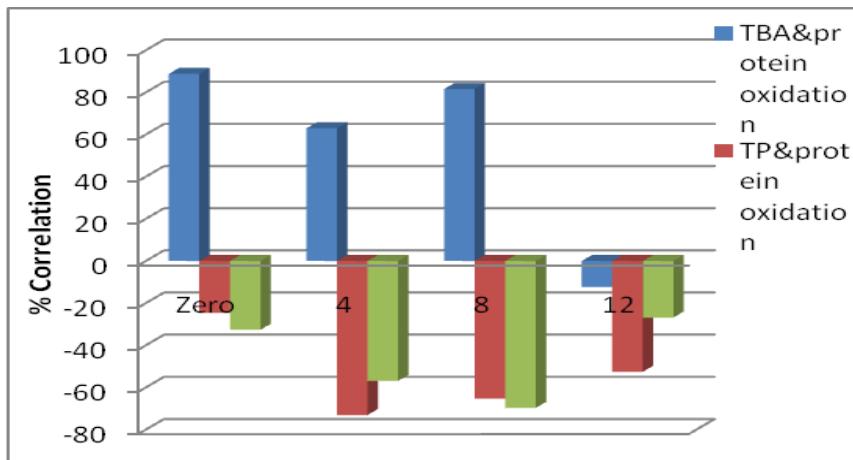


Fig 5: Relationship between total phenols and protein oxidation & TBA.

Microbiological profile:

Since some natural polyphenols have antibacterial effects, therefore, we examined the enhancement or prolongation of the effects of soybean phenolics and bioactive peptides in the presence of food-additives such as ascorbic acid. Results of the burger microbiological analyses during the storage period are presented in Fig (6, 7, 8 and 9). There was no growth count of pathogenic flora (*Clostridium perfringens*, *Salmonella* sp. and *Bacillus cereus*) in all burger samples. The counts of all determined microbiological indicators were significantly affected by the addition of the natural soybean bioactive compounds ($P < 0.05$) and especially protein

hydrolysate. All microbial groups increased in the control burger but in samples containing Natto, total count was increased as a result of Natto additives (containing *Bacillus subtilis*.). Increasing trends of different extents were also observed in samples of the remaining treatments for total viable counts, coli form, *Staphylococcus aureus*, yeasts and moulds. Storage period was significantly ($P < 0.05$) affected on microbial count in all samples. *Staphylococcus aureus* was the lowest increased microbial count followed by *Coli form* group in samples containing soybean additives (protein hydrolysate and Natto), while mold and yeast counts showed an increasing trend ($P < 0.05$) until the end of the storage period.

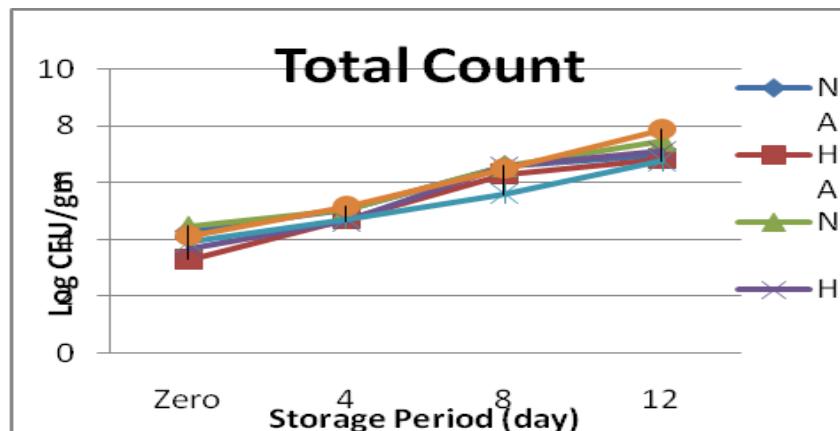


Fig 6: Effect of refrigerated storage on the total count of burger with different bioactive compound additives

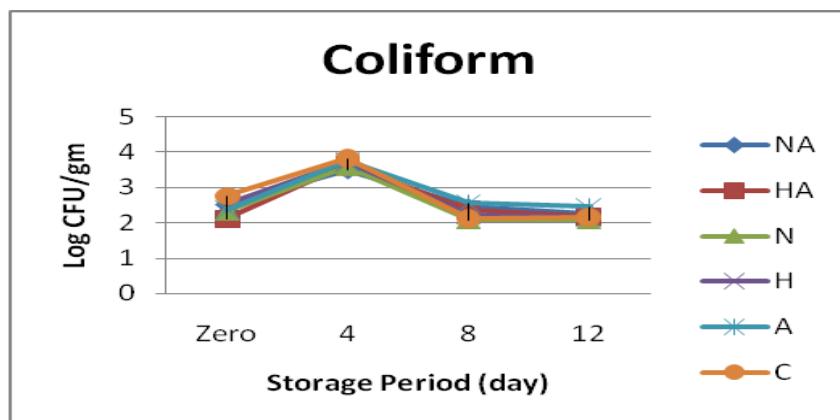


Fig 7: Effect of refrigerated storage on the Colifom of burger with different bioactive compound additives

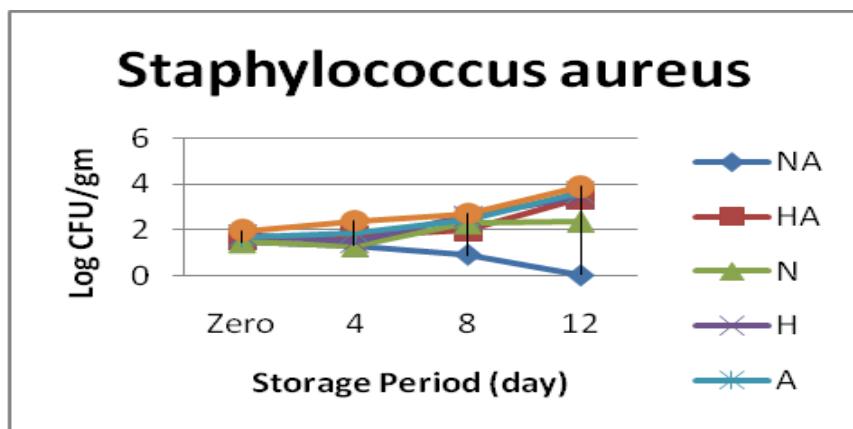


Fig 8: Effect of refrigerated storage on the staph of burger with different bioactive compound additives

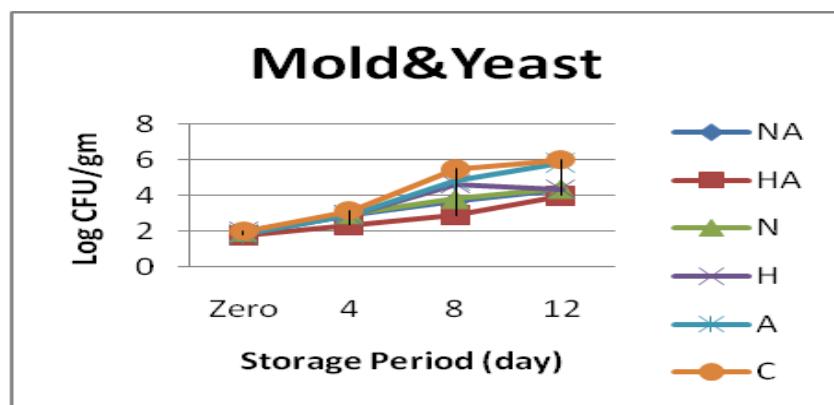


Fig 9: Effect of refrigerated storage on the mold&yeast of burger with different bioactive compound additives

Vallejo-Cordoba et al., 1987 found the same effect in frankfurters and fish frankfurter analogs, in which soy protein hydrolysates have reduced bacterial counts and extended their shelf-life stored at 25 °C without influencing the flavor and texture properties of the products.

The results of the present study as regards to microbial inhibition in meat products are in agreement with a number of other studies. Zambuchini et al., 2008 suggested that, addition of EA (0.03%) alone or in combination with L-AA (1.71%)/SA (1.98%) significantly delayed the proliferation of aerobic plate counts, psychrotrophic counts and Pseudomonas bacteria extending the product shelf life up to 10 days, versus 8 days for control. Hatano et al., (2008) found a strong enhancement effect of ascorbic acid with EGCG in inhibition of MRSA strains. Addition of polyphenols (as antioxidant) has been reported to produce significant reduction in growth of APC in fish meat (Jiang and Zhou, 2003), in frozen fish (Shishkanova et al., 1963), refrigerated buffalo meat (Ahmad et al., 2005) and beef meat (Nerín et al., 2006) as well as in salted cooked ground fish (Ramanathan and Das, 1993).

The possible mechanisms for antimicrobial effect of phenolic compounds include: altering microbial cell permeability (Bajpai et al., 2008) interfering with membrane function including electron transport, nutrient uptake, protein and nucleic acid synthesis, and enzyme activity (Bajpai et al., 2008) interacting with membrane proteins causing deformation in structure and functionality (Rico-Munoz et al., 1987) and substituting alkyl groups into phenol nucleus (Dorman and Deans, 2000). Phenolic diterpenes are organic molecules with high molecular weight bulky substituents, which could reduce their ability to reach the bacterial cell membrane of Gram-negative bacteria. However, growth inhibition of Gram-negative bacteria has been reported, especially in combination with factors that can disturb cell membrane integrity and/or permeability, such as low pH values and increased NaCl concentrations (Del Campo et al., 2000). A similar effect could arise from the combination of soybean Natto and protein hydrolysate with ascorbic acid, since the latter is able to interact with membranes and cell wall components.

CONCLUSION

Shelf life of meat and meat products is defined as the storage time until spoilage. The point of spoilage may be defined by a certain maximum acceptable level of microbial group and/or chemical indicators (lipid oxidation) as well as by an unacceptable off-odour/off-flavour or appearance. In our study, shelf life of burger had been extended by the addition of soybean products (Natto& protein hydrolysate) comparing to control sample (8 days in sample with soybean additives versus 5 days in control sample. Thus, because of the potentiality of soybean bioactive compounds (proteins hydrolysate and fermented soybean Natto) as obtained from the current results, they may be used as effective natural antioxidants and antimicrobial to improve quality and shelf-life of meat products.

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