Survival of *Listeria monocytogenes* in Sucuk during Manufacturing and Storage Periods at Different Modified Atmosphere

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Abstract

The effect of manufacturing steps and storage in different modified atmosphere (MA) on L. monocytogenes in sucuk were studied. Sucuk dough was fermented for 3 days and ripened and dried with a stepwise RH and temperature reduction. The sucuks were packaged at different MA and stored at 4°C. After 3 days of fermentation, L. monocytogenes was decreased by 0.34 and 0.32 log cfu g^{-1} in sucuks made with and without starter culture respectively. The decrease was continued during ripening and storage periods at different MA. MA had significant ($p<0.05$) effect on the survival of L. monocytogenes during storage. 100 % CO_2 and vacuum packaging provided significant ($p<0.05$) decrease when compared to the storage in the presence of O_2 (40 % and air). After 20 days of storage in 100 % CO_2 package, all added L. monocytogenes (6.06 log cfu g^{-1}) was inactivated while 1.27 and 1.87 cfu g^{-1} survived in packages containing 40 % O_2 and air respectively. LAB were also reduced during 20 days of storage. pH values of sucuks were decreased during 3 days of fermentation and increased during storage periods. CO_2 in combination with low pH assists in reducing the numbers of the organism in the sucuk.

Keywords: Sucuk; Modified atmosphere; *Listeria monocytogenes*

Introduction

Sucuk (Turkish dry-fermented sausage) have traditionally been produced by fermentation combined with drying without heat treatment. Sucuk is a mixture of meat, spices, salt and sodium nitrite/nitrate. Sausages are commonly eaten in sandwiches in many countries. According to the Centers for Disease Control (CDC) estimates collected through 1997, listeriosis, the food borne illness resulting from ingestion of *Listeria monocytogenes*, affects approximately 2,500 people annually with approximately 500 deaths (CFSPH, 2005). Although vulnerable groups typically include those with developing and compromised immune systems such as children, critically ill individuals, transplant patients, and the elderly, the affliction of pregnant women generates the largest regulatory concern due to the high percentage of maternal susceptibility to this disease and infant mortality. Consequently, most recent *L. monocytogenes* outbreaks have been attributed to refrigerated, ready-to-eat foods such as deli meats, including sliced ham and turkey, Mexican-style soft cheeses, and frankfurters [1, 2, 3, 4].

Food safety has become an increasingly important issue for the meat industry, and improved control of bacterial pathogens, including *L. monocytogenes*, has become a high priority for regulatory agencies [5, 6]. Meat and meat products (such as minced meat, sausage) have frequently been found to be contaminated with *L. monocytogenes* and could serve as vehicles of this pathogenic bacteria [7, 8] and the outbreak of *L. monocytogenes* from contaminated

meat was reported [9, 10]. Its ability to grow at refrigerated temperatures helps the organism to evolve from a low initial level to an infective dose level during the storage of refrigerated foods, that include those that originally harbored the pathogen and those that were post-process contaminated. It is important, therefore, to develop new procedures that destroy or limit the growth of this harmful microbe in meat products. Modified atmosphere (MA) packaging is a technology in which foods are packaged in high barrier packages in which air has been replaced with an artificial (modified) atmosphere. The composition (e.g. O_2, N_2 and CO_2) of MA systems can be an effective means to restrict and/or inhibit growth of aerobic spoilage organisms of perishable foods such as meat, fish and their products, as well as to sustain the visual quality of red meat to prevent food borne disease and to maintain the color of products [11, 12]. CO_2 shows an inhibitory effect on food microflora, which is dependent on several factors: CO_2 concentration, partial pressure of CO_2, temperature, pH, water activity (a_w), type of microorganism and microbial growth phase [13].

Due to the low pH and a_w of sausages, growth of most pathogenic bacteria is inhibited. The bacteria may, however, not be eliminated and several pathogens have been found in fermented sausages [14, 15]. In dry-cured sausages analyzed by Agriculture Canada, 10 out of 42 samples were positive for *L. monocytogenes* before fermentation and with 5 out of 10 still remaining positive after the maturation [16]. *L. monocytogenes* has been found to survive the initial fermentation in beaker sausages and storage of pepperoni [15]. *L. monocytogenes* was found in 22.7 % of Italian salami samples (GIANFRANCESCHI et al. 2006). About 63 (21 %) and 35 (11.6 %) of sucuk samples obtained from İstanbul province in Turkey were positive for *Listeria* spp. and *L. monocytogenes*, respectively, [17]. *Listeria* spp. were detected in 9 % of sucuks collected from Afyon province in Turkey [4]. *Listeria* spp. were isolated in 73 % of minced meats, in 74 % of meats, in 16 % of salamis, in 32 % of dried meats and in 76 % of sucuk collected from Van province in Turkey [17].

The objective of the present work was to investigate the survival of *L. monocytogenes* and lactic acid bacteria in sucuk during manufacturing and storage periods at different MA.

Materials and methods

Preparation of inoculum

Listeria monocytogenes serotype 4a and ATCC 13932 was obtained from University of İstanbul, Faculty of Medicine, Microorganism's Culture Collection Research and Applied Center, İstanbul, Turkey and LGC Standards GmbH Mercatorstr, 51 46485 Wesel Germany respectively. The cultures were prepared by inoculating 10 ml of sterile brain heard infusion broth (BHIB, Disfco) with one strain of *L. monocytogenes*, and incubating for 48 h at 35ºC. This procedure was done for each of the two strains being used. One loop of this culture was then streaked onto sterile listeria selective agar (LSA, Difco) to ensure for the confirmation of pure *L. monocytogenes* and incubated for 48 h at 35ºC. A confirmed colony of *L. monocytogenes* from the LSA culture was transferred back into 100 ml of sterile BHIB and incubated for 48 h at 35ºC. The samples were centrifuged at 9,000 x G at room temperature for 10 min and the pellet was resuspended into sterile 0.1 % peptone (Difco) water (g 100 ml^{-1}). A cocktail of *L. monocytogenes* was prepared by mixing equal proportions of each of the two strains into a sterile container and diluting with sterile 0.1 % peptone water to obtain the desired level for inoculation into sucuk dough.

A commercial dry mixed starter culture (*Lactobacillus plantarum*, *Pediococcus acidilactici* and *Staphylococcus carnosus*) (Bactoferm, Chr.Hansen, Pohlheim, Germany) was suspended in sterile 0.1 % peptone water to obtain the desired level for inoculation into sucuk dough.

Processing of sucuk

Sucuk was prepared from basic formula per kg: 900 g beef meat (containing 18 % fat), 100 g tail fat, 5.5 g cumin, 1.1 g cinnamon, 11.4 g allspice, 0.5 g clove, 5.5 g red pepper, 11 g black pepper, 20.6 g garlic, 0.4 g ginger, 2.0 g sugar, 18 g NaCl, 0.3 g $NaNO_3$, 0.05 g $NaNO_2$ and 2.1 g olive oil. Starter culture is added into sucuk dough with a level of approximately 10^5 cfu g^{-1}. The olive oil was added to soften the mixture and to help in peeling the casing from the sucuk.

The general outline processing of sucuk was given in Figure 1. The meat was minced in a meat grinder (Tefal Prep'Line 1600, France) to about 1.3-2.5 mm, and spices were added and mixed with the minced meat in the cutter. Dough was held for 24 h at 4°C (conditioning) and then refrigerated tail fat was added, mixed and minced to about 3 mm in the meat grinder. Sucuk dough was used to prepare three different batches: (i) dough to which the starter culture was added and (ii) dough to which the starter culture and *L. monocytogenes* were added.

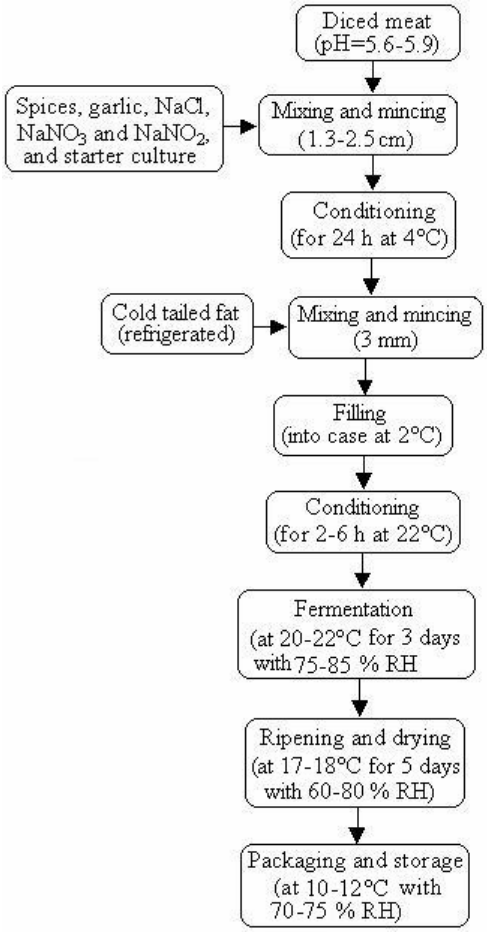

Figure 1. General production flow-chart of sucuk

After that, each dough was filled (about 100 g) into natural casings (25 mm diameter) under aseptic conditions using a filling machine (Tefal, Prep'Line 1600, France) at 2°C. The filled casings were allowed to remaining for 2-6 h at 22°C (conditioning) with RH <60 %.

Fermentation: After conditioning, sucuk dough was fermented at 22°C for 1st and 2nd days with 90 % RH and for 3rd days at 20°C with 90 % RH.
Ripening: After 3 days fermentation, the sucuks are ripened and dried with a stepwise RH and temperature reduction: Sucuk is ripened at 18°C for 2 days at 85 % RH, at 17°C for 3 days at 75 % RH and at 17°C for 2 days at 70 % RH. After 7 days of ripening, sucuks are packaged at different atmospheres.

Packaging and storage of sucuk
Two pack of sucuk in natural casing (about 100 g) was placed in polyethylene/polyamide film (PE/PE; Polinas Plastik Sanayii ve Ticareti A.S., Manisa, Turkey) packages (30x20 cm) under aseptic condition. According to the manufacturer's data, PE/PA film has a permeability at 25°C; oxygen: 160 $cm^3m^{-2}day^{-1}$ and water vapor: 8.5 g $m^{-2}day^{-1}$. The packages (in casings) containing the sucuk (made with starter culture and *L. monocytogenes* addition) were packaged and double sealed with heat in vacuum packing machine (La Minerra, D.V.P. Vacuum Technology, s.r.l., Italy) in one of five different atmospheres: (1) with air, (2) with vacuum, (3) with 100%CO_2, (4) with 77%CO_2+11%O_2+40%N_2 mixture and (5) with 30%CO_2+40%O_2+30%N_2 mixture. Sucuks (without *L. monocytogenes* addition) made with and without starter culture were also packed with only air. The CO_2, O_2 and N_2 were mixed in the various combinations using Witt-Gas mixer (GmbH and Co Kg, Deutschland) and the gases were flushed into vacuum machine during packaging. After packaging, sucuks were stored at 4±1°C in a cold room (< 60 % RH) equipped with a temperature controller until the sampling day. The seal was visually inspected to ensure appropriate closure before storage.

Sampling
Two packages were taken at the following sampling time for microbiological and pH analysis: before and after LAB and *L. monocytogenes* addition; during fermentation (after 1 and 3 days), after 7 days of ripening, and during storage (after 4, 8, 14 and 20 days).
Each sucuk was removed from the package and were cut into small pieces (about 0.3x1 cm) under aseptic condition. For microbiological and pH analysis, 25 g of sucuk sample was homogenized for 2 min in a sterile Warring blender (Torrington, CT, US) containing 225 ml of sterile 0.1 % peptone water. Further decimal dilutions were prepared with sterile 0.1 % peptone water.

Microbiological analysis
Lactic acid bacteria (LAB) counts were determined by spreading one ml of diluted samples on duplicate plates of de Man Rogosa Sharpe (MRS) agar (Merck, Darmstadt, Germany) and the plates were incubated at 30°C for 2 days [18]. *L. monocytogenes* was counted by spreading one ml of diluted samples on duplicate plates of Listeria selective agar (LSA; Disco). LSA plates were incubated at 35°C for 48 h, after which all the characteristic visible black-blue colonies with black zone were counted [18]. The minced meat and additives were also tested to ensure that the sucuk formulation were free from *Listeria* by enriching samples in buffered Listeria enrichment broth (BLEB; Difco) and incubating at 35°C from 24 to 48 h. Enriched culture was streak plated onto LSA and incubated at 35°C for 24 h [18].
Presumptive *L. monocytogenes* colonies exhibiting black-blue colonies with black zone from plate counts on LSA were streaked onto trypticase soya agar (TSA, Difco) and incubated for 24 h at 35°C for confirmation tests. Confirmation of *L. monocytogenes* from TSA plates was performed randomly on both typical and atypical colonies. Catalase-positive, Gram-positive rods that exhibited β-hemolysis on TSA plates containing 5 % sheep blood and umbrella-like growth at room temperature using 7-day motility test medium (MTM, Difco) were presumed

L. monocytogenes [18]. These tests were done randomly throughout the experiments or when any atypical colonies were discovered.
The whole experimental procedure was performed twice. The average value of microbial counts was recorded for each time point from four data (two from duplicate experiments of sucuk and two sample analyses at each sampling time). The number of survivors was expressed as log colony forming unit (cfu) g^{-1} of sucuk.
The initial numbers of *L. monocytogenes* and LAB in sucuk dough prepared with starter culture were approximately 6.06 and 5.56 cfu g^{-1}, respectively, and the initial numbers of LAB were approx. 4.83 cfu g^{-1} in sucuk prepared without addition of starter culture and *L. monocytogenes*.

pH analysis
The pH of each sucuk from every treatment was taken immediately after being homogenized using a WTW 720 pH meter with a combination electrode (WTW pH/mV/Temperature Meters 3150 Commercial Ave Northbrook, IL 60062 , USA).

Statistical analysis
The statistical analyses were performed using the SPSS 11.0 (SPSS Inc., Chicago, IL, USA) software for windows. The one-way analysis of variance (ANOVA test) was performed on the changes of pH and microbial counts as a function of storage periods and modification of atmospheres to determine significant differences ($P < 0.05$) by using the Duncan's multiple range test.

Results and Discussion

The minced meat and ingredients before mixing to prepare sucuk dough had harbored *L. monocytogenes*. The sucuks (made without *L. monocytogenes* addition) at sampling points had also harbored *L. monocytogenes*, which confirming that the samples were not cross-contaminated.
The changes of pH values during manufacturing and storage of sucuks made with and without starter culture are given in Figure 2. The initial pH of sucuk dough used for sucuk manufacturing tested was approximately 5.69. Within the 3 days of fermentation, the pH decreased ($p<0.05$) rapidly to 5.43 and 5.04 in sucuks made without and with starter culture respectively. The pH of sucuks was constant during 7 days of ripening. The pH were raised during storage periods in all sucuks but the rises were higher ($p<0.05$) in sucuks packed without (air vacuum) or with 30 % CO_2. The decrease is a consequence of the activity of added or naturally occurring LAB to produce organic acids and the subsequent rise is caused by accumulation of basic compounds as a result of proteolysis and lipolysis by naturally occurring microorganisms [19, 20, 21]. The decline in the pH value during the fermentation period is very important due to the formation of desired quality and safety point in sucuk such as the inhibition of undesired bacteria.
The viable cell counts of LAB during manufacturing and storage of sucuks made with starter culture and stored at different atmospheres are shown in Figure 3. Before fermentation, the number of endogenous LAB in the control sucuk dough (made without starter culture addition) was 4.83 log cfu g^{-1} and in the sucuk dough prepared with starter culture was 5.56 log cfu g^{-1}. During fermentation, the counts of LAB grew at a faster rate in the sucuks. After 3 days of fermentation, the counts of LAB were increased by approx. 2.03 and 1.43 log units in sucuks made with and without starter culture respectively. During the ripening and storage

period, LAB were reduced between 1.09 and 0.57 log units in sucuks made with starter culture. The LAB were increased during 3 days of fermentation at 22-20°C and decreased during ripening and storage period at 17-18°C and 4°C respectively. NISSEN and HOLCK [15] reported that LAB counts of Norwegian dry-fermented sausage showed desirable increase during fermentation period and than decreased at all storage periods (4°C). SOYER et al. [22] reported that LAB count of sucuk showed a desirable increase during 2 days of fermentation and remained constant during 8 days of ripening periods. The number of surviving LAB was higher ($p<0.05$) in packaged sucuks without or with lower amount of CO_2 (vacuum, air and 30 % CO_2) than higher amount of CO_2 (100 and 77 %).

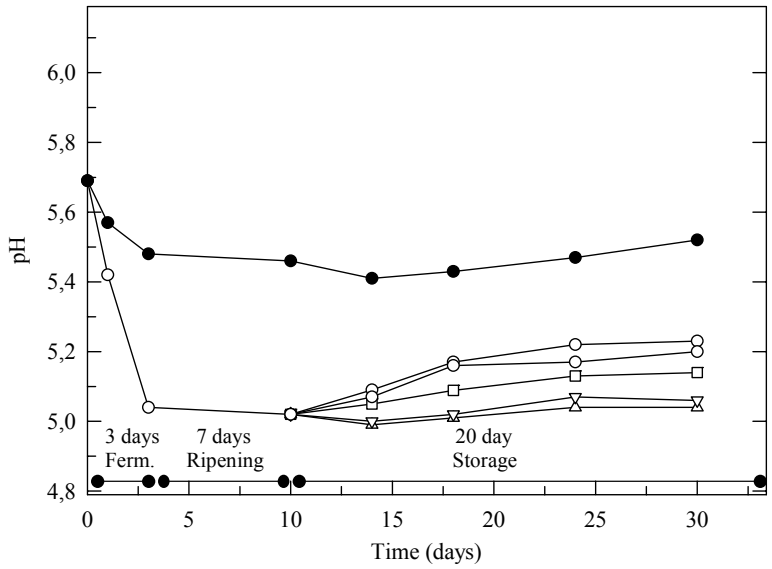

Figure 2. The changes of pH values during manufacturing and storage periods of sucuks made with starter culture: (○) packaged with air, (□) packaged with vacuum, (△) packaged with 100%CO_2, (●) packaging with 77%CO_2+11%O_2+12%N_2 and (■) packaged with 30%CO_2+40%O_2+30%N_2; sucuk made without starter culture: (▲) packaged with air

The survival of *L. monocytogenes* in sucuks made with starter culture and stored at different MA is given in Figure 4. During the first one day of fermentation there was little reduction in the number of *L. monocytogenes* in sucuks made with starter culture. *L. monocytogenes* were reduced by 0.25 and 1.36 log units during next 2 days of fermentation and 7 days of ripening respectively. This was due to lowering pH and drying of a_w of sucuk. On the other hand the number *L. monocytogenes* was increased (by 0.32 log units) in sucuk made without starter culture during 3 days of fermentation and decreased 7 days of ripening by 0.52 log units. The survival of *L. monocytogenes* was 0.97, 1.27 and 0.68 log cfu g^{-1} in sucuks made with starter culture and packaged with 77 % CO_2, 30 % CO_2 and vacuum, respectively, after 20 days of storage while 1.87 log cfu g^{-1} survivors observed in air packaged sucuks. There was no survival of *L. monocytogenes* in sucuk packaged with 100 % CO_2 after 20 days of storage. Inactivation of *L. monocytogenes* was due to the formation of carbonic acid with carbonation of CO_2 in water phase beside low pH resulted from activities of LAB and drying of sucuks. The survival of *L. monocytogenes* was 2.92 log cfu g^{-1} in air packed sucuk made with starter culture after 20 days of storage. There were significant differences ($p<0.05$) in air-packaged sucuks made with and without starter culture and this would be due to high pH values of sucuks made without starter culture. There were significant ($p<0.05$) differences in the

number of *L. monocytogenes* between sucuks (made with starter culture) packaged in the presence of high (100 or 77 %) and low (30 and 11 %) concentrations of CO_2, and between sucuks packaged in the presence of CO_2 and air. These indicated that additional positive antimicrobial effect was obtained by using CO_2 in packaging atmosphere.

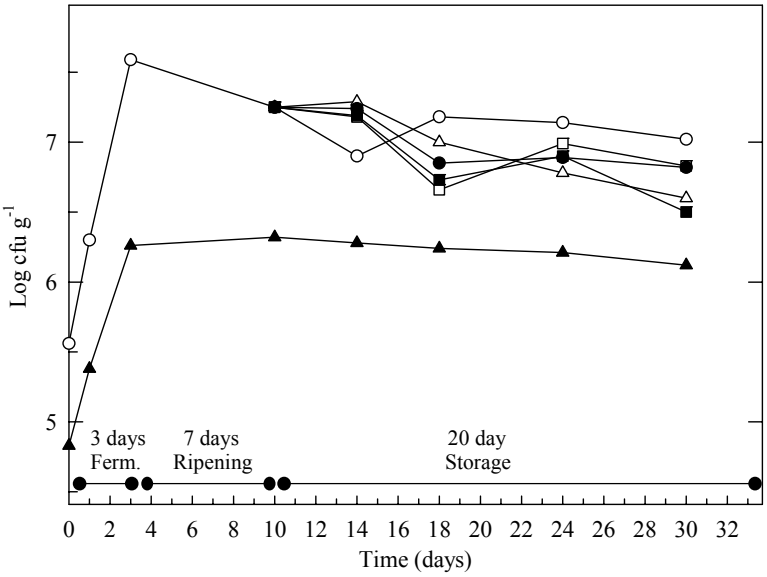

Figure 3. Survival of lactic acid bacteria during manufacturing and storage periods of sucuks made with starter culture: (○) packaged with air, (□) packaged with vacuum, (△) packaged with 100%CO_2, (●) packaging with 77%CO_2+11%O_2+12%N_2 and (■) packaged with 30%CO_2+40%O_2+30%N_2; sucuk made without starter culture: (▲) packaged with air

There were significant ($p<0.05$) differences in pH between sucuks made with and without starter culture. Packaging atmosphere had an effect ($p<0.05$) on pH during storage period; the pH values of sucuks made with starter culture were ranged from 5.04 to 5.23 during 20 days of storage. The higher amount of CO_2 (100 or 77 %) in package allowed slight increase in the pH values ($p>0.05$) during 20 days of storage than sucuk packaged with 30 % CO_2, air and vacuum ($p<0.05$). According to the Turkish Standard Institute (Anonymous, 2000 and 2002) pH for high quality sucuk should be in the range of 4.7-5.4. The sucuks prepared with starter culture were found to be close to range 5.04-5.23. So, it can be concluded that pH results were found to be in the range of the Turkish standard values. But the sucuks made without starter culture were ranged from 5.41 to 5.52 during 20 days of storage.

CO_2 dissolves in water to form carbonic acid that reduces pH of foods. The antimicrobial effects of the MA on microorganisms in sucuks seems to be responsible for the data obtained, together with a probable contribution from pH which in turns is likely to be influenced by the gas atmosphere [23, 24]. At the beginning of fermentation of sucuk made with starter culture, the numbers of inoculated LAB increased and this increase was approx. 2.03 and 1.98 log units g^{-1} after 3 days of fermentation and slightly decreased during 20 days of storage at different MA. The fermentation of carbohydrates to lactic acid decreases the pH value of the sucuk. The lower external pH disturbs the homeostasis of the bacterial cell [14], including pathogenic and spoilage bacteria, and therefore restricts their growth. Furthermore, the decrease in pH causes a decrease in the water binding capacity of the sucuk, which accelerates the drying process of sucuk and results with a low a_w of the end product [25, 26].

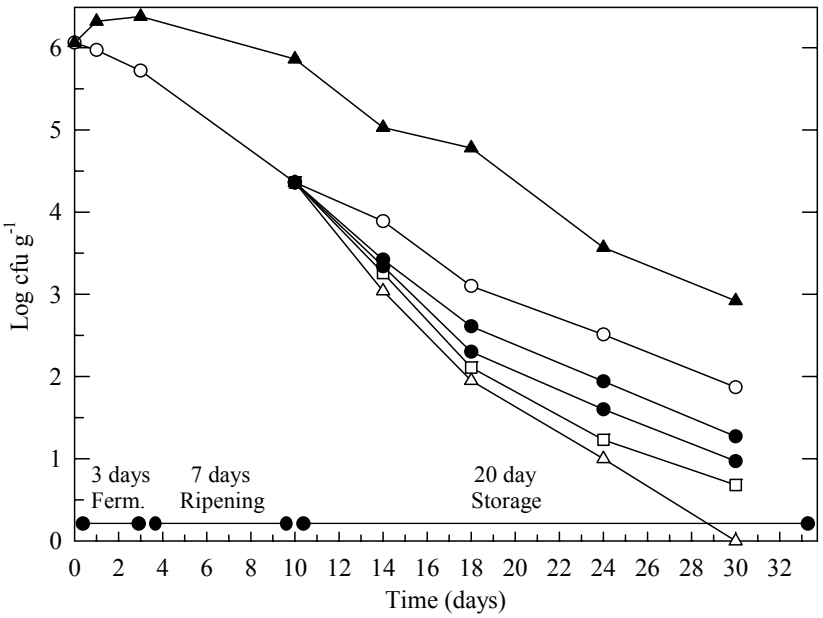

Figure 4. Survival of *L. monocytogenes* in sucuks during manufacturing and storage periods of sucuks made with starter culture: (○) packaged with air, (□) packaged with vacuum, (△) packaged with 100%CO_2, (●) packaging with 77%CO_2+11%O_2+12%N_2 and (■) packaged with 30%CO_2+40%O_2+30%N_2; sucuk made without starter culture: (▲) packaged with air

Bacterial counts in the vacuum and CO_2 (100 and 77 %) packaged sucuks were more reduced than those for the air packaging. The inactivation of *L. monocytogenes* in sucuks stored by packaging in the presence of gas mixture and vacuum was attained mainly with the combination of removal of oxygen and addition of CO_2. On the other hand during the first day of fermentation, microbes use up the oxygen mixed into the sucuk material during chopping. This results in lowering of the redox potential making nitrite more effective and inhibiting the growth of aerobic spoilage bacteria in sausages [27].

In the specific case of sucuk, fermentation refers to the breakdown of carbohydrates ("sugars") present in meat mixtures, mainly to lactic acid. Processors of sucuk relied on the action of fermentation bacteria, added to sucuk dough together with naturally present in the meat-contaminating flora. Relatively low temperatures (about 21°C) are instrumental in stimulating the growth of the desired fermentation flora, while the growth of the spoilage bacteria is suppressed. Under moderate climatic conditions and storage (e.g. 4°C and 50% RH with MA in the presence of higher amount of CO_2), the products have a prolonged shelf life.

The combined use of several preservative methods is known as "hurdle" or "inhibitory factor" concept. This concept may also be applied to MAP of sucuk, as the change in gaseous atmosphere combined with other hurdles, such as, preservatives, low pH, low relative humidity, low temperature, fermentation, etc., can exert a strong pressure on the developing microflora in sucuk. The chances of a heavy contamination corresponding to the amount of high inoculum in this study are probably small, but it should be noted that *L. monocytogenes* does survive to greater than 20 days during storage in sucuk with this level of contamination. Even though sucuk is generally regarded as safe and stable products, our results emphasize the necessary for good manufacturing practices during production to assure product safety. These safe practices are especially important for all pathogens have been reported to cause disease, in some cases even when ingested in very low numbers. This research also

emphasizes the prevention of *L. monocytogenes* infection from cattle and other carrier species from farms and hygienic processing conditions in slaughterhouse and sucuk manufacturing plants.

Acknowledgements

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