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Effect of Lactic Acid Bacteria Inoculation on Nitrite Concentration of Fermented Sausage in Fermentation and Ripening Periods

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Abstract: Selected starter cultures i.e. *Lactobacillus fermentum* PTCC 1638, *Lactobacillus plantarum* PTCC 1058 and their mixtures were used as starter cultures in addition to a control treatment (spontaneous fermentation) in the production of fermented sausages due to consideration of nitrite reduction ability. The starter cultures had a rapid growth and dominated the fortuitous population of LAB during the fermentation and ripening process improving some sensory attributes like: flavor, color and cutting. All of starter cultures reduced nitrite concentration to values lower than 29 mg/kg. Total aerobic count (Mesophilic aerobic bacteria) increased ($p<0.05$) during fermentation during the first 2 days of fermentation and they decreased ($p>0.05$) at the end of ripening. Enterobactericeae count increased ($p<0.05$) during process time. They not detected in all of the treatments apart from the control treatment. The results indicated that the mixed starter culture is the best for chemical and microbiological safety of sausages whereas that one produced with *L. plantarum* had the highest score in flavor ($p<0.05$) but there was no significant difference between overall sensory quality in all treatments with the exception of sausages produced with LAB mixed culture.

Key world: Lactic acid bacteria · Nitrite · Food safety · Fermented sausage

INTRODUCTION

Sausages-fermented or heated-are very popular meaty products in the world, they can be manufactured either traditionally or commercially [1]. Consumption of these products as fast foods is expanding in Iran like other progressive countries. A critical point is nitrite as additives used to sausages formulation. Nitrite causes some special properties such as color, flavor, prevention of oxidation, helping to domination of gram-positive bacteria, stability and hygienic safety [2, 3, 4]. Lack of fermented foods or functional foods such as fermented sausages in Iranian markets, is a major problem. In the other hand progressive consumption of heated sausages is a problem too, by child specially. Fermented sausage is a very popular meat product in the world. They may be manufactured either traditionally or commercially [3] and are made from sheep/beef -meat containing 18% fat-pork [2]. These products are manufactured without addition of starter cultures in small processing units, but it is not possible to insure that the population and variety of microorganisms present in the raw material will always be the same or behave the same way [5]. Gram- negative proteolitic such as Psudomonas, Acinetobacter, Moraxella and lipolitic bacteria such as Pseudomonas and Entrobacteriacea case several spoilages also biogenic

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amines can be formed by Porteus and murganella in sausages [6, 7]. LAB has a positive effect on the hygienic properties of the product, inhibiting pathogenic and spoilage flora by acidification or production of antimicrobials [8]. A pH of 3.5-4 and other anti microbial substances produced by the dominating LAB in addition to lactic acid have been reported to inhibit Enterobacteriaceae and other Gram-negative bacteria [9, 10]. Other factors influence the survival of pathogen and spoilage microflora are amount of NaCl, nitrite water activity, choice of starter culture and addition of antimicrobial compounds, in other hand process variables like fermentation temperature and storage time play important roles [11]. Thus, use of Lactic acid bacteria (LAB) as starter culture can improve properties of fermented sausages. However, starter culture mixtures are added in formulation [2]. The microorganisms of importance during fermentation and maturation of fermented sausages are Gram-positive and rod shaped belonging to the genera Lactobacillus, Micrococcus and Staphylococcus [2, 5]. Nitrite causes some special properties in both of heated or fermenting sausages such as: color, flavor, prevention of oxidation, help to the domination of gram-positive bacteria [8, 12, 13]. On the contrary, it recognized as tratogenic and carcinogenic substance because of extreme reductive and oxidative activities [2, 13]. Moreover, consumption of nitrite has been linked to methemoglobinemia and incidence of cancers. Methemoglobinemia is a condition where reduced iron (Fe2+) in haemoglobin is oxidized by nitrite to it's maximum oxidized state (Fe3+), thus reducing the total oxygen carrying capacity of blood [14]. Moreover, extensive experimental and epidemiological data suggest that human are susceptible to carcinogenesis N-nitroso compounds resulted from endogenous nitrosatoin reaction of nitrite [15]. There have been many attempts to reduce the N-nitroso compound concentration in fermented foods. Lactic acid bacteria were found to contribute to the depletion nitrite in many fermented food [16, 17, 18]. Moreover, researchers demonstrated that vegetable and sausage fermentation based on inoculation of starter cultures are more effective in lowering nitrite concentration and biogenic amins compared to spontaneous fermentation [5, 18-20]. Unfortunately, not lack of fermented sausage but over usage of nitrite in heated sausages, quickly selling and consumption of sausages by consumers is a problem in Iran. However, in the present study we introduce novel fermented sausages with LAB starter cultures in order to reduce nitrite risks.

Table 1: Raw material and common ingredients used in fermented sausages formulation

Ingredient	Quantity (g)
Beef meat	85
Fat	1
Isolated Soy Protein	5
Sodium chloride	2
Sodium nitrite	0.25
Sodium deltagloconolacto	0.37
Sugar powder	1
Sodium ascorbat	0.88
Black pepper powder	2
Poly phosphtate	1
Garlic	0.5

MATERIAL AND METHODS

Material: Formulation was based on Iranian appetite and Halal meat production. Fresh boneless beef meat and fat was purchased from industrial slaughterhouse. Raw material and common ingredients (Table 1) was obtained from "Tanita" factory. The lactic acid bacteria species were selected according to occurrence in fermented sausage and availability in Iran. *L. fermentum* PTTC 1638, *L. plantarum* PTCC 1058 were obtained from Persian Type Culture Collection (PTCC).

Starter Culture Preparation: MacFarland standards were used to visually approximate the concentration of cells in a suspension. The 0.5 McFarland was prepared as described by Sutton [21]. The accuracy of the McFarland standard was verified by adjusting bacterial suspensions, preparing serial 10-fold dilutions, then performing plate counts [22, 23]. Optical Density of microbial suspension cultures were read in 600 nm with Jenway spectrophotometer (Jenway UV 1653, LTD, UK). Starter cultures were fixed in 8.5×10^8cfu/ml. Pure *L. plantarum*, *L. fermentum* and mixtures of those cultures (1:1 ratio) were used as starter cultures.

Sausage Preparation: Four treatments of fermented sausages were prepared as fellows: A control treatment produced without adding starter culture (spontaneous fermentation). Three more treatments were produced with starter cultures containing one strain each of *L. plantarum*, *L. fermentum* and mixed culture inoculated into the meat mixture (batter) of sausage as wet inoculums (8.5×10^8cfu/g). The raw materials and common ingredients were used per Kg of meat mixture for the production of fermented sausages as showed in Table 1. The respective starter culture was added to each treatment as a 5 ml wet inoculums per Kg of batter. In control sample, 5 ml sterile saline water was added per Kg of batter. The meat was

Table 2: Fermentation and ripening condition of sausages

Period	Days	Temperature (°C)	Relative humidity (%)
Fermentation	1-2	30	95
	3-4	30	90
Ripening	5-6	25	85
	7-8	20	80
	9-10	20	75

minced in meat grinder to about 1.5-2 cm. A Naturin Cutter (Naturin, Germany) was used for preparation of batter, the cutter was sterilized before the preparation of meat mixture for each treatment. The spice mixture including starter culture was added and mixed with minced meat in a cutter for about 20 min at 5°C. The batter had been held for 12 h at 4°C then were filled into artificial collagen casings (Germany) of 28 mm diameter, under aseptic conditions using a filling machine (Naturin, Germany) at 2°C [4]. The sausages were fermented and maturated at RH=95-75% relative humidity and from 30 to 20°C during 10 days as outline in Table 2.

Sampling: Sampling performed using 4 batches. The samples were sent by chilled air freight under aseptic condition to Institute of Standard and Industrial Researches of Iran (ISIRI, Sanandaj, Iran). The residual nitrite, pH, acidity were determined on 0,1,2,… and 10th day of processing time. The numbers of Enterobacteriaceae, Mesophilic aerobic bacteria and lactic acid bacteria counted during fermentation and ripening.

Sample Preparation for Microbiological Analysis: A sample of 25 g was removed from each sausage batch aseptically, transferred to sterile plastic pouches and homogenized using-Stomacher Lab-Blender 400 (Seward, UK) containing 225 ml 0.1% pepton water [24].

Sample Preparation for Chemical Analysis: A sample of 200 g was cut into small spices (5×5×5 mm) and homogenized using a Tefal blender from each sausage batch. Twenty grams of the homogenized samples were used for the determination of pH and acidity, the remainder of homogenized samples used for residual nitrite concentration determination [2].

Chemical Analysis

Residual Nitrite Concentration: Nitrite concentration was determined in triplicate on sausages according to the colorimetric method of ISIRI [25]. The nitrite analysis was based on water, protein participation with disodium tetra borate decahydrate (Merck, Germany), potassium ferrocyanide trihydrate (Merck, Germany) in presence of zinc acetate dehydrate (Merck, German) and photometric determination (Jenway UV 1653, spectrophotometer, LTD, UK) of nitrite after derivatisation with sulfanilamide and N-(1-nephtyl)ethylene diamine in a hydrochloric acid medium (Merck, Germany). The detection limit is 0.5-1 mg/kg. The absorbance of colored mixtures was red at 538 nm against the reagent blank. The calibration curve was linear for 0-3000 mg/kg of nitrite.

Ttitratable Acidity and pH: A sample of 10 g was homogenized in 90 ml of distilled water [26] and pH was determined by pH meter (WTW 720, Germany). Titratable acidity was determined by titrating the sample filtrate with 0.1 N NaOH (Merck, Germany) with phenolphethalein (0.1% m/v in 95% ethanol) as the indicator [27].

Enumeration of Lactic Acid Bacteria, Enterobacteriaceae, Mesophilic Aerobic Bacteria: Plate counts of Mesophilic aerobic bacteria (MAB) were determined using the Spread Plate Method on Aerobic Plate Count Agar (PCA, Merck, Germany) were incubated at 30°C for 24-72 h, as described by ISIRI [28]. Plate counts of lactic acid bacteria (LAB) were counted using Pure Plate Method on de Man, Rogosa and Sharpe medium (MRS, Merck, Germany) were anaerobically incubated at 30°C for 48-72 h [29]. Enterobacteriaceae numbers were counted using Most Numbering Probability (MNP) method on Pepton Water Broth (PWB, Merck, Germany) medium then EE broth and Violet Red Bile Glucose Agar (VRBG, Merck, Germany), finally incubated at 37°C for 24 h [30].

Sensory Analysis: Sensory attributes (flavor, color, cutting) of 25 g of sausage were evaluated, twice for each sample by a ten panel trained panelists. Panelists gave scores for each sample with respect to their perceptions of flavor and color as 1 (worth) to 10 (best) biased on Hedonic test [3]. Cutting score evaluated by panelists by assessing whether the sausage was easily cut or stuck to the knife, on a scale of 1 (worth) to 10 (best). The Overall Sensory Quality (OSQ) was determined from equation [3]. Iqaliberuim 1

$$OSQ = (flavor \times 0.50) + (color \times 0.25) + (cutting \times 0.25)$$

Statistical Analysis: An ANOVA was performed for both chemical and microbial data as a function of fermentation time to determine significant differences ($P<0.05$) using SPSS version 9.0 software. Means were compared using the Duncan's multiple test range.

RESULTS AND DISCUSSION

Effect on Microbial Counts: The evolution of LAB, MAB and Enterobacteriaceae counts during the fermentation and ripening of fermented sausages are shown in Figures 1, 2 and 3 respectively. In all samples LAB dominated the microflora during fermentation and ripening. At the beginning of production process the LAB count of the treatments with the starter cultures (6.3 to 7.9 CFU/g) was higher ($p<0.05$) than that of control (5× CFU/g). The ALB count of the treatment inoculated with the strain of *L. plantarum* was increased and reached at 4th day of fermentation up to 9.73 log CFU/g. It was decreased little and remained almost constant during ripening as at the end of ripening to range of 8.8 log CFU/g. However, the LAB count of treatment produced with mixed starter culture was higher at the initial samples, it was increased rapidly during the fermentation period and reached at the end of ripening at the same level ($p>0.05$) with the treatments produced with other starter cultures. The LAB count of treatment produced with *L. fermentum* was increased slowly during fermentation and reduced little after 4 days, remained almost constant during ripening. The ALB count of the control was steadily increased but with significantly ($p<0.05$) lower rate during the fermentation and ripening than all the treatments produced with starter cultures and reached at the end of ripening to 6.6 log cfu/g. In conclusion, the starter cultures had a rapid growth and dominated the fortuitous population of LAB during the fermentation and ripening process, as they were well adapted to the fermentation process. Similar result have been reported by Casaburi *et al*. [31], Talon *et al*. [32] and Baka *et al*. [5]. The dominance of LAB is a basic requirement for the successful production of fermented sausages. LAB belong to the desirable microflora of fermented sausages. They contribute to development of texture, Color and flavor and have positive effect on the hygienic properties of the product, inhibit the pathogenic and spoilage flora by acidification or by production of antimicrobioals [5].

The initial MAB of batter of fermented treatments with starter cultures was 7.6 to 6.3 CFU/g. A significant reduction was observed between the 2th and 5th day of fermentation to the MAB count of the treatment produced with mixed starter culture as well as to the treatment produced with *L. fermentum.* The reduction was continued gradually in ripening period and reached to 4.8

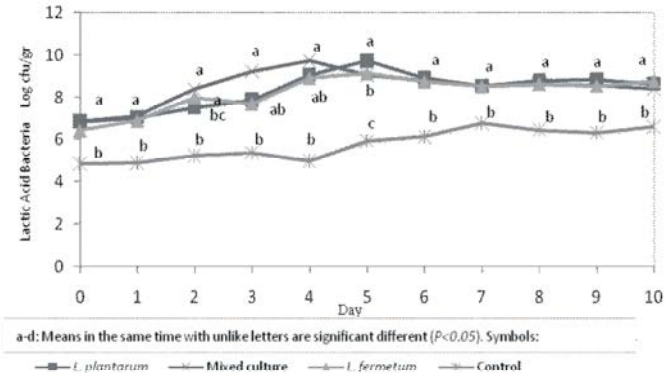

Fig. 1: Evolution of Lactic Acid Bacteria during the fermentation and ripening of fermented sausage

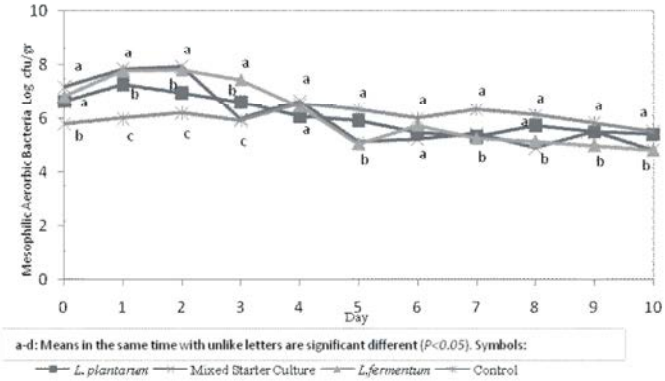

Fig. 2: Evolution of Mesophilic Aerobic Bacteria during the fermentation and ripening of fermented sausage

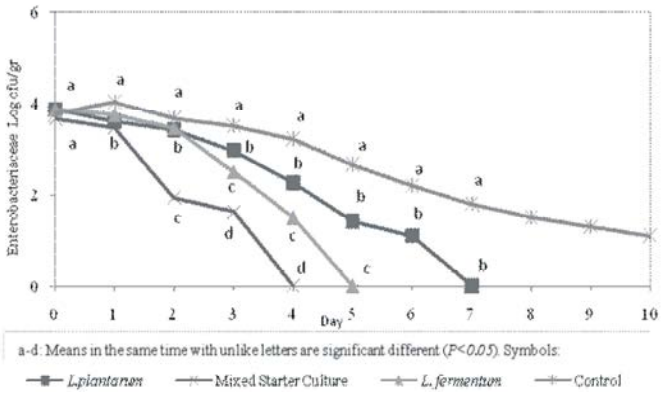

Fig. 3: Evolution ofEnterobacteriacea during the fermentation and dripening of fermented sausage

log CFU/g in the same level (*p>0.05*) whereas the MAB count of the treatment produced with *L. plantarum* was reduced after 24 hours and continued gradually in fermentation period so that was almost constant in ripening period and reach to the same level with control treatment (*p>0.05*) to 5.5 log CFU/g. MAB count of the control treatment was increased (*p<0.05*) slowly in fermentation period by 4th day then was almost constant. We found that LAB cultures resulted in a reduction of mesophilic count in fermentation of fermented sausages. As indicated in Figures 1,2 and 3, the high LAB count the low MAB and Enterobacteriacea count. The Entrobacteriaceae count was about 6 CFU/g in initial samples of batter in all treatments. The Enterobacteriaceae count reduced (*p<0.05*) during the fermentation and not detected in days of 4,5 and 7 of processing time in treatments produced with mixed, *L. fermentum* and *L. plantarum* respectively whereas it reduced to 10 CFU/g in control treatment at the end of ripening period. Similar results have been reported by other researchers [1, 2, 5, 33, 34]. The domination of LAB and the inhibition of Gram negative bacteria in fermented sausages during fermentation and ripening are necessary for successfully production of fermented sausages [5, 35]. Enterobacteriacea and gram negative bacteria, in general, are considered as undesirable microflora in fermented sausages. These reduction of Enterobacteriaceae and MAB counts are probably due to the rapid reduction of pH, acid production and other antimicrbioal substances like bacteriocins. As indicated, the results clearly showed that LAB colonized in the sausages more efficiently than LAB in spontaneous fermentation. Furthermore, results showed that the used bacteria act as protecting agent in all the inoculated sausages. Natural LAB strains in meat have probably no enough fermentation activity due to reduction of microbial load in control sausage that produced with spontaneous fermentation (without LAB starter culture).

Effect on Physicochemical Parameters: The evaluation of pH and acidity during fermentation and ripening of fermented sausages are given in Figures 4 and 5. The initial mean pH value of batter was 6.15. No differences (*p>0.05*) were found between the treatments. At the first 3 days of fermentation a very rapid reduction of pH was observed and continued by 5th day in all treatments due to fermentation to organic acids of carbohydrates occurred in sausages by LAB. The results were in agreement with the literature that pH values of fermented sausages decreased sharply at the first 3 days of fermentation [3, 5]. The pH of treatments produced with LAB starters cultures were lower in the beginning of fermentation than the pH of sausage undergoing spontaneous fermentation (*P<0.05*). There was no significant difference between pH of the samples inoculated with *L. plantarum* and *L. fermentum* at the end of fermentation and ripening period. It was decreased slowly in all treatments in ripening period. Mean pH of sausage inoculated with mixed, *L. plantarum* and *L. fermentum* starter cultures were 3.5, 4.7 and 4.5 respectively while control treatment was approximately 5.5 at the end of ripening period. The lowering pH at the beginning of fermentation is an essential requirement because of it's contribution in the inhibition of the undesirable microorganisms, proliferation of LAB, accelerate the development of red color of fermented sausages affected their taste and reduces the water binding capacity of proteins ensuring texture [36]. A significant negative correlation was found between pH values of fermented sausages and the LAB count

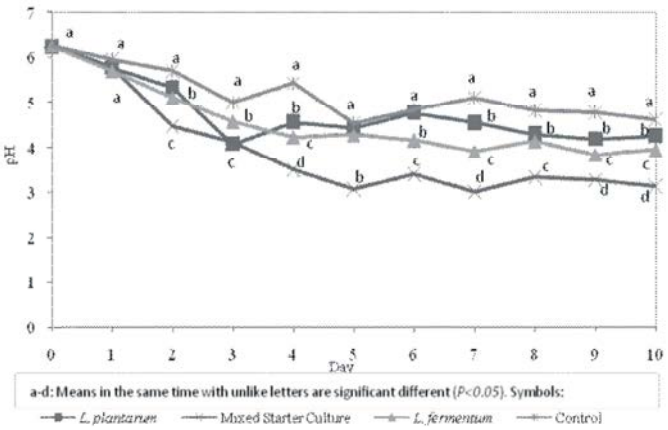

Fig. 4: Evolution of pH during the fermentation and ripending of fermented sausage.

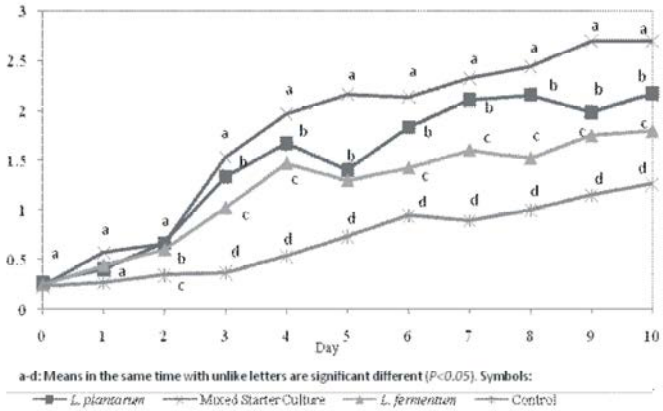

Fig. 5: Evolution of lactic acid concentration during the fermentation and ripening of fermented sausage.

throughout the fermentation time [5]. As indicated in Figure 1 the LAB number of control sausage was significantly lower than the number of the treatments with starter cultures so decreasing of pH value was slightly. Although the LAB count of inoculated treatments were the same, pH mean of treatment inoculated with mixed culture was lower ($p<0.05$) than the other treatments due to synergistic activity of LAB starters. This high reduction pH (3.5) may be the reason for no increasing of their population more (Figure 1). The same pH mean of the treatments inoculated with *L. platarum* and *L. fermentum* may be due to *L. plantarum* belonging to the facultative heterofermentative. A small increase in pH was observed during initial ripening period to the all treatments then the reduction was continued slowly. This may be due to the formation of N-non protein basic compounds and ammonium ions, as well as to the buffering action of the proteins.

Change of pH value of fermented sausages reflected the amount of lactic acid produced during fermentations. Acidity of treatments produced with starter cultures increased sharply after 2 days of fermentation. Lactic acid increased more rapidly in sausages inoculated with mixed starter culture (Figure 5) also lactic acid concentration in all sausages of fermenting with starter cultures was higher ($p<0.05$) than that of spontaneous fermentation during processing time. Pure *L. plantarum* and *L. fermentum* showed the same ($p>0.05$) acidity formation activity at the end of fermentation period but not in ripening time ($p<0.05$). The lactobacillus strains showed synergistic effective obviously in mixed starter culture added fermented sausages (Figures 3, 4).

It has been known for years that Nitrite and N-nitroso compounds are recognized as carcinogenic substance in foods. The residual nitrite concentrations are shown in Figure 6. Nitrite concentration of sausages inoculated with starter cultures were significantly lower than that of spontaneous fermentation during the fermentation period ($P<0.05$). The nitrite was added into the batter in all samples i.e 120 mg/Kg. The nitrite concentration was reduced to a mean content of 62 mg/Kg after 5 hours. This very rapid reduction in the nitrite content of meat

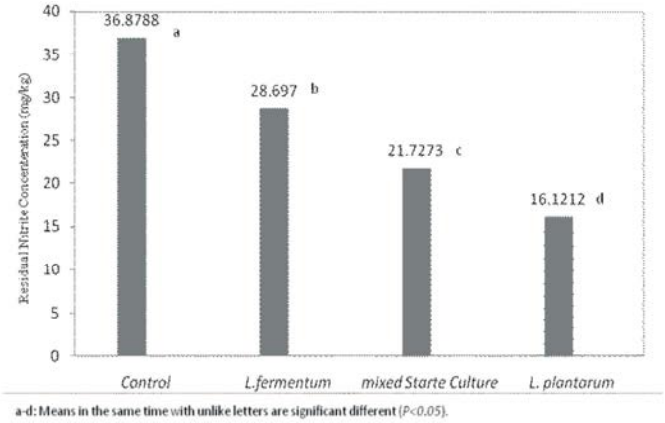

Fig. 6: Evolution of nitrite concentration during the fermentation and ripening of fermented sausage.

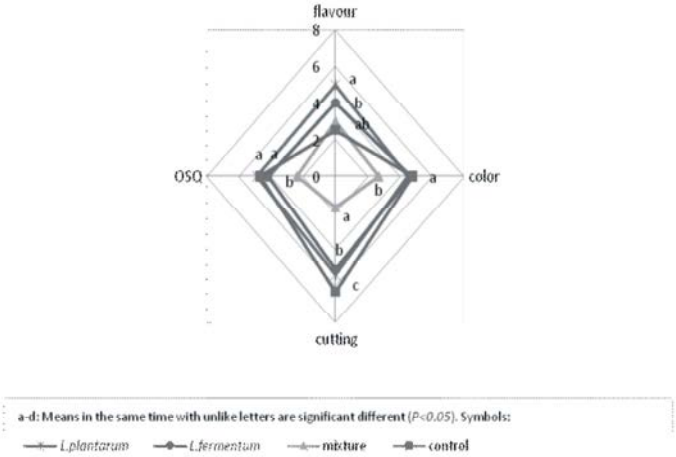

Fig. 7: Effect of lactic acid starter cultures on the sensory attributes of the fermented sausages.

mixtures is the most likely that they have high reactivity [5, 34]. The nitrite content of inoculated sausages were reduced ($P<0.05$) on 3th day. At the end of fermentation period, the residual nitrite concentration of control treatment was approximately 37 mg/Kg and that of sausage with *L. fermentum*, *L. plantarum* and mixed culture were approximately 16.1, 28.7 and 21.7 mg/Kg respectively. LAB reduced nitrite concentration from 1000 to 200 mg/Kg in pork [37]. Nitrite concentration was reduced from 150 to 2 mg/Kg in Sucuk during fermentation [4]. Other researchers demonstrated that *L. plantarum* and *L. lactis* reduced nitrite concentration in curd and minced meat [38]. Tow mechanisms namely chemical depletion and enzymatic reduction during LAB growth lead to depletion of nitrite [18]. Some lactobacilli isolated from cured meat products have capable of enzymatically reducing of nitrite [39] also some strains of meat-borne lactobacilli exhibit the essential activities like nitrate and nitrate reductase, catalase, lipase and protease [40]. In the present study, sausages inoculated with *L. plantarum* included the lowest residual nitrite concentration. That was found in previous studies *L. plantarum* has nitritereductas activity in both aerobic and anaerobic conditions [4, 41]. LAB are identified as nature microbial flora in meat normally, besides some nitrite/nitrate reducing bacteria like: Enterobacteriacea may be in meat mixture. But when LAB starter cultures were inoculated into the sausages, they quickly proliferated, produced acid, became the dominant spices and inhibited the growth of nitrite/nitrate reducing bacteria and at the same time, as population of LAB grew present nitrite on the sausages could be reduced more rapidly. High correlation between the nitrite content of fermented sausages and their pH values during fermentation [5]. A 0.2 pH unite reduction results at the doubling of the depletion rate of nitrites [42]. Reduction of residual nitrite concentration in treatment produced with *L. plantarum*, *L. fermentum* and mixed culture were 86.6%, 76.1% and 81.9% of initial nitrite concentration respectively. On the other hand, about 69.3% of initial nitrite was reduced as a result of

spontaneous fermentation. Other researchers found that some LAB reduced nitrite concentration and suggested that the LAB were responsible for 30% of nitrite loss in Bologna [39].

Effect on Sensory Attributes: The treatment inoculated with *L. plantarum* had the highest scores for flavor. All treatments with LAB starter culture had the same ($p>0.05$) color, easy cutting, Overall Sensory Quality and except of the treatment with mixed starter culture, lower ($p<0.05$) scores for color, easy cutting, Overall Sensory Quality than the control. The control treatment had the highest scores for easy cutting. LAB starter culture contribute to the flavor, color and texture development providing new sensory properties [5, 43]. The desired red color is formed when myoglobin reacts with nitric oxide to produce nitrosomyoglobin. Micrococaccea belong to the desirable microflora of fermented sausages that contribute to the development of color and participate in the development of flavor of fermented sausages [5]. The main texture of defects in some sausage like susuk come from low quality raw material, none or wrong fermentation, high water and fat content, conditions of fermentation and pH values, etc [3].

CONCLUSIONS

The nitrite concentration of sausages produced with starter culture reduced rapidly and *L. plantarum* starter culture had the most effective for reduction nitrite. The LAB count of sausages produced with pure and mixed strains increased among of fermentation and remained almost constant in ripening period by the end of processing. All of the starter cultures reduced Mesophilic bacteria count and Enterobacteriaceae not detected in sausages. Sausages were prepared with *L.plantarum* and *L. fermentum* and without inoculation had the highest scores for Overall Sensory Quality. In present study, we focused on consideration of chemical and microbiological safety potential of certain LAB. So using single LAB strains as starter cultures without the other strains affected on sensory attributes like: pediococcoce, Micrococcus resulted in no acceptable sensory attributes. Finally, *L. plantarum* and *L. fermentum* could be suitable for more considerations for fermented sausage productions or starter cultures designation using in fermented foods.

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