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Effect of *Lactobacillus plantarum* and *Lactobacillus fermentum* on Nitrite Concentration and Bacterial Load in Fermented Sausage During Fermentation

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Abstract: Nitrite,and N-nitroso compoundsare recognized as carcinogenicsubstancesin foods.Therefore, the detection and control of the concentrations of these substances is important in terms of food safety. In the present study investigated *Lactobacillus plantarum* PTCC 1058, *Lactobacillus fermentum*PTTC 1638and their mixtures (as starter culture) ability to reducethe residual nitrite concentration, pH, acidity, mesoplilic aerobic bacteria,lactic acid bacteriaand Enterobacteriaceae counts have been identifiedduring fermentation in fermented sausages. *L. plantarum*, *L. fermentum* and mixed starter cultures with cell density of approximately 8.5×10^8cfu/ml inoculated to sausagesbattercontained 120 $^{mg}/_{kg}$ sodium nitrite. Residual nitrite concentration was reducedto 86.6%, 76.1%, 81.9% in starter culture added sausages respectively (P<0.05) whereas sausage undergoing spontaneous fermentation (control) decreased 29%of initial nitrite concentration present in sausage. pH and acidity decreased (P<0.05) during fermentation period. At the same time,Enterobacteriaceaeand mesoplilic aerobic counts decreased (P<0.05) in sausages and LAB counts increased (P<0.05) in all of the sausages. The rapid abilities of LAB to reduce residual nitrite concentration inhibited the growth of Enterobacteriaceae and bacterial load of sausages could be the reasons to produce of fermented sausage with LAB starter cultures in Iran.

Key words: Nitrite · Lactic acid bacteria · Fermented sausage · Fermentation period · Food safety

INTRODUCTION

Fermented sausage is a very popular meat product in the world. Theymay be manufactured either traditionally or commercially [1] andare 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 [3]. Gram- negative proteolitic such as*Psudomonas*, *Acinetobacter*, *Moraxella* and lipolitic bacteria such as *Psudomonas* and *Entrobacteriacea* case several spoilages also biogenic amines can be formed by *Porteus*and *murganella*in sausages [4,5]. LAB have a positive effect on the hygienic properties of the product, inhibiting pathogenic and spoilage flora by acidification or production of antimicrobials [6]. 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 [7,8]. 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 [9]. Thus, use of Lactic acid bacteria (LAB) as starter culture can improve properties offermented sausages.However, starter culture mixturesare addedin formulation [10]. The microorganisms

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of importance during fermentation and maturation of fermented sausages are Gram-positive and rod shaped belonging to the genera *Lactobacillus*, *Micrococcus* and *Staphylococcus* [2,3]. 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 [6,11,12]. On the contrary, it recognized as tratogenic and carcinogenic substance because of extreme reductive and oxidative activities [2,12]. Moreover, consumption of nitrite has been linked to methemoglobinemia and incidence of cancers. Methemoglobinemia is a condition where reduced iron(Fe^{2+})in haemoglobin is oxidized by nitrite to it's maximum oxidized state (Fe^{3+}),thus reducing the total oxygen carrying capacity of blood [13]. Moreover, extensive experimental and epidemiological data suggest that human are susceptible to carcinogenesis N-nitroso compounds resulted from endogenous nitrosatoin reaction of nitrite [14]. 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 foods [15-17]. 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 [3,17-19]. 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.

MATERIAL AND METHOD

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 [20]. The accuracy of the McFarland standard was verified by adjusting bacterial suspensions, preparing serial 10-fold dilutions, then performing plate counts [21,23]. Optical Density of microbial suspension cultures were read in 600 nm with Jenway spectrophotometer (JenwayUV 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 samplesof fermented sausages were prepared as fellows: A control sample produced without adding starter culture (spontaneous fermentation). Three more samples were produced with starter cultures containing one strain each of *L. plantarum*, *L. fermentum* and mixedculture 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 sample 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 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 [1]. The sausages were fermented at 30°C, RH=90-95% for 4 days as fermentation period in microaerophilic condition (Figure 1).

Sampling: Sampling performed using 4 batches. The samples were sent by chilled air freight under aseptic condition to Institute of standard and industrial researches (Sanandaj, Iran). The residual nitrite, pH, acidity were determined on 0,1,2,… and 4th day of fermentation. The numbers ofEnterobacteriaceae, mesophilic aerobic bacteria and lactic acid bacteria counted during fermentation.

Sample Preparation for Microbiological Analysis: A sample of 25 g was removedfrom each sausage batch aseptically, transferred to sterile plastic pouchesand homogenized using-Stomacher Lab-Blender 400 (Seward, UK) containing 225 ml 0.1% pepton water [23].

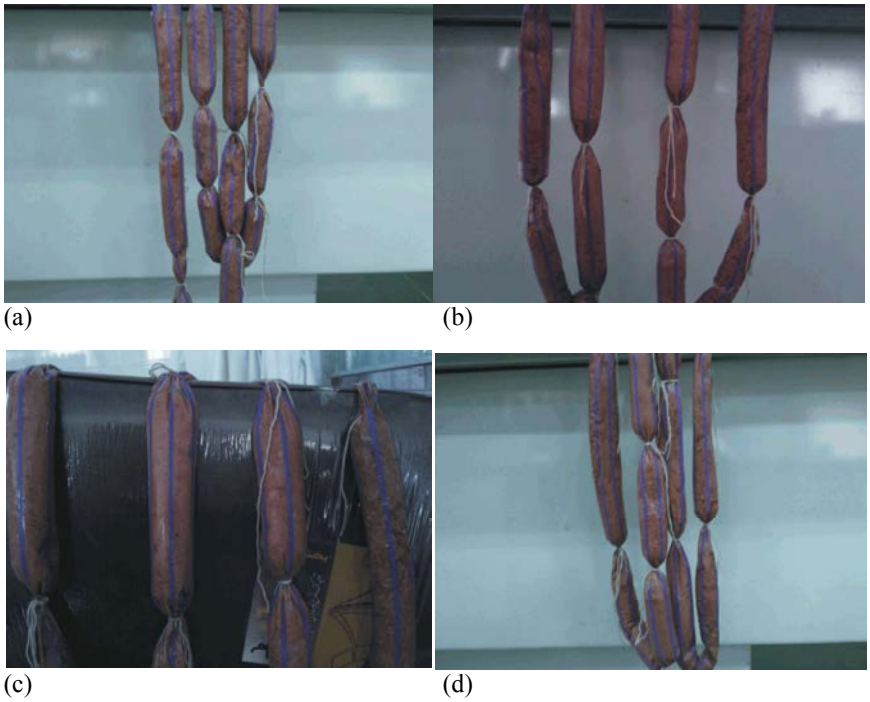

Fig. 1: Fermented sausages. (a):Sausages inoculated with L. fermentum, (b): Sausages inoculated with L. plantarum,(c): Sausages inoculated with mixed culture,(d): Sausages with spontaneous fermentation

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
Special spices	1

Sample Preparation for Chemical Analysis: A sample of 200 g wascut into small spices (5×5×5 mm) and homogenizedusing a Tefal blender from each sausage batch.Twenty grams of the homogenized samples wereused for the determination of pH and acidity, the remainder of homogenized samples used for residual nitrite concentration determination [1].

Chemical Analysis

Residualnitrite Concentration of Fermented Sausage: Nitrite concentration was determinedin triplicate on sausages according to the colorimetric method ofISIRI[1] [24]. The nitrite analysis was based on water, protein participation withdisodium tetra borate decahydrate (Merck, Germany), potassium ferrocyanidetrihydrate (Merck, Germany) in presence of zinc acetate dehydrate (Merck, German)and photometric determination (JenwayUV 1653, spectrophotometer, LTD, UK) of nitrite after derivatisation withsulfanilamide 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 nmagainst the reagent blank. The calibration curve was linear for 0-3000 mg/kg of nitrite.

Ttitratable Acidityandph: A sample of 10 g was homogenized in 90 ml of distilled water [25] and pH was determined by pH meter(WTW 720, Germany). Titratableacidity was determined bytitrating the sample filtrate with 0.1 N NaOH(Merck, Germany)with phenolphethalein (0.1% m/v in 95% ethanol) as the indicator [26].

Enumeration of Lactic Acid Bacteria, Enterobacteriace, Mesophilic Aerobic Bacteria: Plate counts of mesophilic aerobic bacteria 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 [27]. Plate counts of lactic acid bacteria were counted using Pure Plate Method on de Man, Rogosa and Sharpemedium(MRS, Merck, Germany) were anaerobically incubated at 30°Cfor 48-72 h [28]. Enterobacteriaceae numbers were counted usingMost Numbering Probability (MNP) method on PeptonWater 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 [29].

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

Residual Nitrite Concentrations: It has been known for years that Nitrite and N-nitroso compounds are recognized as carcinogenic substance in foods. The residual nitrite concentrations are shownin Figure 2. Nitrite concentration of sausages inoculated with starter cultures were significantly lower than that of spontaneous fermentation during the fermentation period (P<0.05). The nitritewas addedinto the batter in all samplesi.e 120 mg/Kg.The nitrite concentration was reduced to a mean content of62 mg/Kgafter5 hours. This very rapid reduction in the nitrite content of meat mixtures is the most likely that they have high reactivity [3,30]. 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 sample was approximately 48 mg/Kg and that of sausage with*L. fermentum, L. plantarum*andmixed culture were approximately 41.0, 29.5 and 38.0mg/Kg respectively. LAB reduced nitrite concentration from 1000 to 200 mg/Kg in pork [31]. Nitrite concentration was reduced from 150 to 2 mg/Kg in Sucuk during fermentation [2]. Other researchers demonstrated that *L. plantarum* and *L. lactis* reduced nitrite concentration in curd and minced meat [32]. Tow mechanisms namely chemical depletion and enzymatic reduction during LAB growth lead to depletion of nitrite [34]. Some *lactobacilli* isolated from cured meat products have capable ofenzymatically reducing of nitrite [33] also some strains of meat-borne *lactobacilli* exhibit the essential activities like nitrate and nitratereductase, catalase, lipase and protease [34]. 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 [35,36]. LAB areidentified 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 [3]. A 0.2 pH unite reduction results at the doubling of the depletion rate of nitrites [37]. Reduction of residual nitrite concentration in samples inoculated 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, about29% 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 [33].

Acidity of Sausage: Changes in pH and acidity of sausages are shown in Figures 3 and 4 respectively. The initial pH value was approximately 6.15.pH of inoculated samples was quickly reduced in the first 3 days of fermentation in due to fermentation to organic acids of carbohydrates occurred in sausages by LAB.pH of starters cultures added sausages were lower in the beginning of fermentation than the pH of sausage undergoing spontaneous fermentation (P<0.05). At the end offermentation, pH of sausage inoculated with mixed starter culture was approximately 3.5. There is no significantdifference (P>0.05) betweenpH of the samples inoculated with *L. plantarum* and *L. fermentum*, they decreased to 4.5 and 4.2 respectivelywhile control sample was approximately 5.4 at the end of fermentation period. The results were in agreement with the literature that pH values of fermented sausages decreased sharply at the first 3 days of fermentation [1,3,23].The lowering pH at the beginning of fermentation is an essential requirement because ofit's contribution in the inhibition of the undesirable microorganisms and accelerate proliferation of LAB in batter.Asignificant negative correlationwas

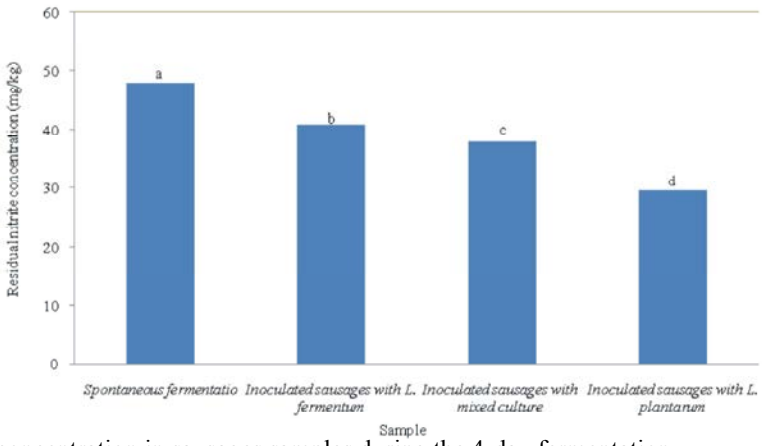

Fig. 2: Residual nitrite concentration in sausages samples during the 4-day fermentation

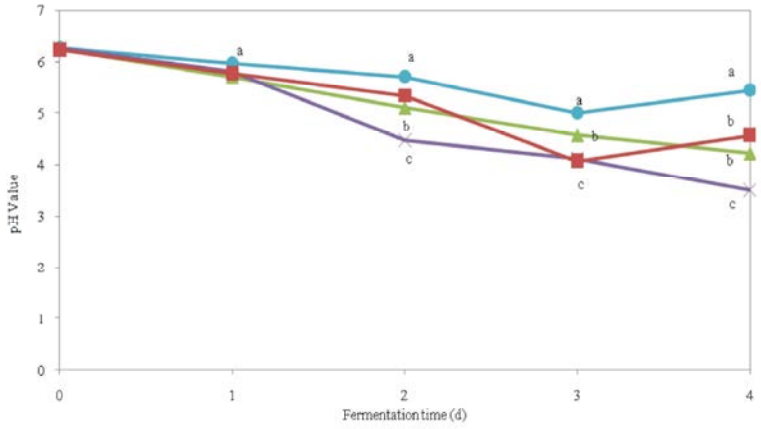

Fig. 3: Changing pH of sausages during fermentation.
Symbols: (?):Spontaneous fermentation,(¦):Inoculated sausages with L. plantarum,(?):Inoculated sausages with L. fermentum ,(×):Inoculated with mixed culture .a-c: Means in the same fermentation time with unlike letters are significant different. (P<0.05).

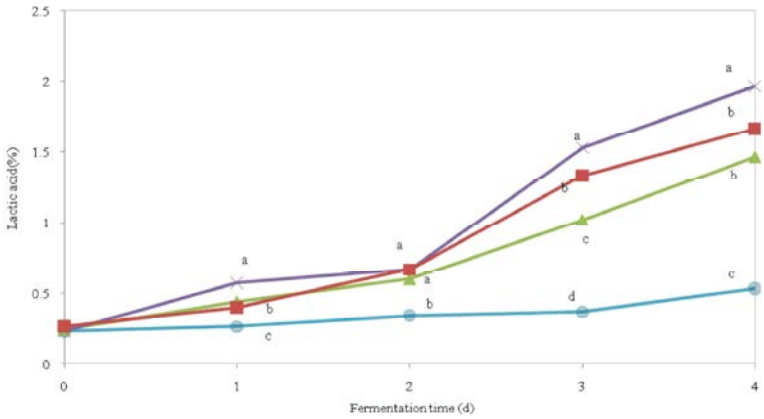

Fig. 4: Changing pH of sausages during fermentation.
Symbols: (?):Spontaneous fermentation,(|):Inoculated sausages with *L. plantarum*,(?):Inoculated sausages with*L. fermentum*,(×): Inoculated sausages with mixed culture. a-c: Means in the same fermentation time with unlike letters are significant different (*P<0.05*)

Table 2:Lactic acid bacterial, Mesophilic aerobic bacterial and Enterobacteriacea counts of sausages during fermentation.

		Fermentation (d)				
	Treatment	0	1	2	3	4
Lactic acid bacterial	Control					
(Log cfu/gr)	(Spontaneous fermentation)	4.84±0.02a	4.93±0.01a	5.22±0.03a	5.37±0.03a	5.91±0.07a
	L. plantarum	6.80±0.02b	7.03±0.01b	7.22±0.01b	7.86±0.02b	9.06±0.04b
	L. fermentum	6.44±0.02b	6.90±0.06c	7.02±0.04b	7.71±0.06b	8.92±0.06b
	Mixed culture(1/1)	6.83±0.01b	7.73±0.01d	7.93±0.01c	8.13±0.08c	9.53±0.01c
Mesophilic aerobic	Control					
bacteria (Log cfu/gr)	(Spontaneous fermentation)	5.80±0.11a	6.03±0.13a	6.20±0.06a	6.95±0.05a	6.97±0.06a
	L. plantarum	6.89±0.01b	7.26±0.01b	7.64±0.01b	6.66±0.06b	6.20±0.08b
	L. fermentum	6.82± 0.02b	7.76±0.06c	7.80±0.04c	7.24±0.12c	6.53±0.07c
	Mixed culture(1/1)	7.16±0.01c	7.83±0.3c	7.93±0.01c	5.96±0.02b	6.13±0.02b
Enterobacteriaceae	Control					
(Log cfu/gr)	(Spontaneous fermentation)	3.76±0.11a	3.70±0.13a	3.66±0.06a	3.50±0.05a	3.22±0.06a
	L. plantarum	3.86±0.01b	3.60±0.07b	3.41±0.07b	2.96±0.1b	2.26±0.04b
	L. fermentum	3.86± 0.02b	3.73±0.05c	3.45±0.14c	2.50±0.11c	1.59±0.03c
	Mixed culture(1/1)	3.66±0.51c	3.47±0.3c	1.94±0.08c	1.63±0.02b	0

a-d: Means in the same fermentation time with unlike letters are significant different (P<0.05).

found between pH values of fermented sausages and the LAB count throughout the fermentation time [3]. As indicated in Table 1, the LAB number of control sausage was significantly lower than the number of the samples with starter cultures so decreasing of pH value was slightly. Changeof pH value of fermented sausages reflected the amount of lactic acid produced during fermentations. Lactic acid increased more rapidly in sausages inoculated with mixed starter culture (Figure 4) and lactic acid concentration in sausages fermented with starter cultures was higher than that of spontaneous fermentation during the whole fermentation. Acidity of treatmented samples with starter cultures increased sharply after 2 days of fermentation. Pure *L. plantarum* and *L. fermentum* showed the same fermentation activity, *L. plantarum* belong to facultative heterofermentativesand *L.fermantum* belong to obligativeheterofermentatives. The *lactobacillus* strains showed synergistic effective obviously in mixed starter culture added fermented sausages(Figures 3,4).

Numbers of Lactic Acid Bacterial, Mesophilic Aerobic Bacterial and Enterobacteriaceae: The number of LAB, mesophilic aerobic and Enterobacteriaceae of fermenting sausages are shown in Table 2. The dominance of LAB is a basic requirement for the successful production of fermented sausages. The number of LAB in sausages increased(P<0.05) during fermentation and drastic increments were found in sausages fermented with starter cultures. LAB number of samples with *L. fermentum*slower increased than that of other inoculated samples during the fermentation process and reached the same level with the *L. plantarum* added sample at the end of fermentation($P>0.05$).However, LAB numbers in inoculated samples were higher than control samplesat all times of fermentation($P<0.05$). In conclusion, the LAB cultures had a rapid growth and dominated the population of LAB duringfermentation process as they were well adopted to the fermentation process. Similar results have been reported by other researchers [2,3,38]. The result showed that the numbers of LAB and mesophilic aerobic bacteria are approximately the same in the beginning of fermentation whereas the proliferation of mesophilic aerobic bacteria was similar to that of LAB by the second day (Table 2). Starter culture application resulted in with an immediate increasing of the microbial population then it reduced by 1-2 log cycle. On the contrary,the number of both mesophilic aerobic bacteria and LAB continuously increasedand slowlyincreasedin control samplesas shown in Table2. Natural LAB strains in meat have probably no enough fermentation activity due to reduction of microbial load in sausage. At the end of fermentationthe number of mesopilic aerobicbacteria decreased ($P<0.05$)in sausages inoculated with starter cultures but there is no significant difference ($P>0.05$) between inoculated samples with *L.plantarum* and mixed starter cultures. Asshown in Table 2 all of the fermented sausages showed decreases ($P<0.05$) in number of Enterobacteriaceae during fermentation. After 2 days of fermentation the total Enterobacteriaceae count of sausage control samplewas approximately 5,10,24 fold higher than those in the inoculated sausage samples with *L. plantarum*, *L. fermentum* and mixed starter culture respectively. When compared with the others, inoculated sausage samples

with *L.fermentum* and mixed culture showed more drastic decreasingin Enterobacteriaceaecount. At the end of fermentation, Enterobacteriaceae was not detected in sausage was I noculated with mixed starter culture. Similar results have been reported by other researchers [3,4,10,39,40] also lactic acid fermentation inhibited the proliferation of Gram-negative pathogenic bacteria including toxicogenic *Eschrichia coli*, *Comylobacter jejuni* and *Shiglla flexneri* [41]. 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.

CONCLUSION

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 childspecially. However, this study showed that LAB strainsare able to reduce the concentration of nitrite and they can be used asstarter cultures in some fermented foods. Fermented sausages with LAB cultures can more rapidly reduce the residual nitrite concentration than spontaneous fermentation. *L. plantarum* has the highest potential to reduce of residual nitrite concentration and microbial load in fermented sausagewhereas fermented sausageswith mixed LAB culture could reduce microbial load too.

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