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Research Article

Effect of lactic acid bacteria in improving microbial properties of fermented sausage under refrigeration storage

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Abstract
The fermented sausage product was prepared from beef and belly fat by a fermentation process and inoculated using separate concentrations 3 and 5% of *Lactobacillus plantarum* and *L. delbrueckii* subsp. *bulgaricus* at 37°C for 48 hours after adding salt, sugar, spices and garlic and packed in artificial casings. Sausages are matured at 7°C with 75-80% relative humidity for 4 weeks. The changes in the microbial quality of the fermented sausage were examined during this period. Based on the results, the sausage samples inoculated with 5% *L. plantarum* bacterium showed the highest numbers of starter bacteria reaching a logarithm of 7.63 colony units/ g, and reducing not accepted microorganisms such as coliform bacteria, psychrotrophic bacteria, molds, and yeasts, and *Staphylococcus aureus*. *Salmonella* has not been found in all treatments The pollinated coefficients with the same concentration of *L. delbrueckii* subsp. *bulgaricu*s was registered second, as the logarithm in the fourth week was 7.48 colony units/g compared with other sausages treatments.

Keywords: Lactic acid bacteria, Fermented sausage, *Lactobacillus*, Storage.

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Introduction

Meat and meat products are important sources of protein, fats, essential amino acids, minerals, vitamins and other nutrients (Reddy et al. 2013). The high content of saturated fats in these products leads to restriction of consumption for those prone to cardiovascular diseases and those who were overweight. However, fat was an important component of human nutrition and contributes to giving flavor, tenderness, juiciness, texture, and extending the shelf life of meat products. Thus, the challenge for the meat industry is to develop low-fat meat products without compromising their organoleptic properties (Furlán et al. 2014). Meat fermentation is a bio-acidification process using precursors with the least amount of energy. It was a method of preservation and results in unique and distinctive properties such as color, flavor, microbial safety, tenderness, and palatability, which makes meat bear all the desired characteristics of the consumer (Toldra & Hui 2015).

Lactic acid bacteria (LAB) play an important role in various food fermentation processes (Liu et al. 2021). When carbohydrates of foods are fermented, their main product would be lactic acid. Lactic acid bacteria were used as starters in the fermentation of many food products such as cheese, yogurt, meat, fish, fruits, vegetables, and grain products through their contribution to imparting the desired flavors and improving the texture as well as the nutritional value of fermented foods (Laranjo et al. 2019). The ability of LAB to produce bacteriocins and anti-fungal compounds contributes to extending the life span of food preservation (Bintsis 2018). One of the

processes associated with fermentation is proteolysis, which is an important reaction that affects the texture and flavor of meat products such as fermented sausages (Candogan & Acton 2001). Although the activity of LAB in proteolytic degradation is somewhat weak, some types of LAB show a high activity of protease enzyme production such as *Lactobacillus plantarum*, especially during the fermentation of sausages (Fadda et al. 2002; Nie & Zhang 2014).

This work aimed to study the effect of proteolysis by different starter bacteria viz. *Lactobacillus plantarum* and *L. delbrueckii* subsp. *bulgaricus* to produce fermented meat and prolong its shelf life by reducing the unwanted microbial load using lactic acid bacteria and improving the quality and organoleptic characteristics of the product in terms of taste, flavor and general acceptance.

Materials and methods

Sausages were prepared from beef by adding belly fat as 25% that had been obtained from the local markets of Basrah governorate. The study includes the use of two *Lactobacillus* bacteria viz. *L. plantarum* (SWANSON Corporation of America) and *L. delbrueckii* subsp. *bulgaricus* (Danisco French Company) in the form of freeze-dried bacterial cultures and supplied in the form of tablets. After being activated in MRS liquid medium and incubated at a temperature of 37°C for 24 hours in the medium of skimmed milk, it was inoculated with the lactic acid bacteria at a rate of 10% and incubated at a temperature of 37°C until coagulation for three consecutive times (Robinson 1990). The weights of pure beef meat and fat were prepared in a ratio of 3: 1, respectively, for all experimental treatments. The weight of the processed portion for each treatment was 2 kg.

Mincing pure meat and fat was done using an electric chopping machine, for the first time using a disc with a whole diameter of 1.5cm and the second time using a disc with a whole diameter of 0.8cm. Then salt and sugar are added to the mixture of the pure meat and fat at a rate of 1.5 and 0.75%, respectively, for all experimental treatments. The mixture was manually mixed to obtain homogeneity of the materials. The activated starter was added to the minced mixture at a rate of 3 and 5% for the two types of bacteria separately and incubated at a temperature of 37°C for 48 hours. Both spices and fresh chopped garlic were added to the mixture at a ratio of 0.5 and 1%, respectively.

The prepared mixture of chopped sausages was filled using industrial casings using an electric mincing machine from which both the knife and the disc are removed. A sterile plastic tube of 15cm in length and 2cm in diameter is attached to its opening to obtain a regular shape without voids. The samples were hung in the refrigerator using cotton threads.

Microbiological analysis: The total count of lactic acid bacteria was estimated by taking 25g of the sausage sample and adding 225ml of 0.1% sterilized peptone water. The ingredients were mixed well and serial decimal dilutions were prepared. The pour-plate method (Speak 1984) was followed using a Solid MRS culture medium. The dishes are incubated at 37°C for 48 hours in anaerobic conditions. After the incubation, the number of developing colonies was calculated using a colony counter. The total count of bacteria was estimated using the pour plate method (APHA 1978) and using a Nutrient Agar, incubating it at 32°C for 48 hours. The method of pour plate APHA (1978) was followed to estimate the total count of Coliform bacteria using the medium of Eosin Methylene Blue Agar (EMB) and incubated at 37°C for 48 hours.

The total count of molds and yeasts was estimated using the pour-plate method using a solid potato-dextrose medium (PDA) and incubating at 22°C for 4 days. The pour-plate method was followed using a Nutrient Agar solid medium to estimate the total count of Psychrotrophic bacteria by incubation at 7°C for 10 days. The method mentioned in Speak (1984) was followed using mannitol salt agar to determine *Staphylococcus aureus,* and it was incubated at 37°C for 48 hours. The possibility of the presence of

Table 1. Numbers of starter bacterial count in fermented sausages during refrigeration (Aging time).

Aging time (day)	starter ratio %))	Starter bacteria	numbers of starter bacteria count (Log cfu/gm)
2	3	*L. bulgaricus*	8.41
		L. plantarum	8.63
	5	*L. bulgaricus*	8.68
		L. plantarum	8.87
7	3	*L. bulgaricus*	8.23
		L. plantarum	8.53
	5	*L. bulgaricus*	8.61
		L. plantarum	8.78
14	3	*L. bulgaricus*	7.64
		L. plantarum	7.72
	5	*L. bulgaricus*	7.94
		L. plantarum	7.98
21	3	*L. bulgaricus*	7.42
		L. plantarum	7.51
	5	*L. bulgaricus*	7.64
		L. plantarum	7.80
28	3	*L. bulgaricus*	7.21
		L. plantarum	7.36
	5	*L. bulgaricus*	7.48
		L. plantarum	7.63

*Each number represents an average of three replicates. LSD $_{0.05}$ values for the effect of periods, concentration and their interaction are 0.011, 0.008 and 0.018 for *L. bulgaricus*, and 0.007, 0.006 and 0.013 for *L. plantarum*, respectively.

Salmonella bacteria was examined according to Andrews (1997) using Salmonella Shigella Agar (SSA) medium and incubated at 37°C for 24 hours.

Results and Discussion

Starter bacteria: Table 1 shows the numbers of starter bacteria under refrigeration in the sausage inoculated with the two starters with two different concentrations. There was a linear decrease in the count of bacteria during the storage period. The treatments with 5% starter were maintained a higher count than that of 3%. The treatments inoculated with *L. plantarum* at a concentration of 5% had a higher number of starter bacteria than that of *L. bulgaricus*. The results were in agreement with the findings of (Minor-Perez et al. 2004).

The count of starter bacteria of *L. plantarum* has decreased from 5×10^8 cfu/g after maturation and then reduced to $9\text{x}10^7$ CFU/g after cooling at 4°C. The results are in agreement with findings of Klingberg et al. (2005) who has pointed out a decrease in the numbers of *L. plantarum* in fermented sausage from 3×10^8 CFU/g after 28 days of maturation to $4.7\text{x}10^7$ cfu/g and after the second week of cooling at 5°C. The reason for the low numbers of lactic acid bacteria may be due to the loss of moisture during refrigeration (Drosinos et al. 2006) or the low temperature which was not suitable for the growth of this type of bacteria (Talon et al. 2007). The results revealed a significant effect ($P\leq0.05$) of the time, and concentration and their interactions on the numbers of lactic acid bacteria.

Coliform bacteria: Table 2 shows the number of coliform bacteria in sausage inoculated with the two starters after cooling. There was a decrease in the number of bacteria after the second week of cooling, and this decrease continued with the increasing cooling time in the third week. The logarithm of their preparation was inversely proportional to the concentration of the starter and the bacteria did not appear after the fourth week of storage in both bacterial types and different concentrations. The results were in agreement with findings of Porto-Fett et al. (2008). There was a linear decrease in the

Table 2. Numbers of Coliform bacteria count in fermented sausages during refrigeration (Aging time).

Aging time (day)	Starter ratio (%)	Starter bacteria	numbers of Coliform bacteria count (Log cfu/gm)
2	3	*L. bulgaricus*	2.42
		L. plantarum	2.33
	5	*L. bulgaricus*	2.19
		L. plantarum	2.02
	Control		2.62
7	3	*L. bulgaricus*	2.26
		L. plantarum	2.16
	5	*L. bulgaricus*	2.02
		L. plantarum	1.77
	Control		3.11
14	3	*L. bulgaricus*	1.92
		L. plantarum	1.87
	5	*L. bulgaricus*	1.65
		L. plantarum	1.60
	Control		3.34
21	3	*L. bulgaricus*	1.65
		L. plantarum	1.69
	5	*L. bulgaricus*	1.60
		L. plantarum	1.54
	Control		**
28	3	*L. bulgaricus*	0
		L. plantarum	0
	5	*L. bulgaricus*	0
		L. plantarum	0
	Control		**

*Each number represents an average of three replicates. LSD $_{0.05}$ values for the effect of periods, concentration and their interaction are 0.036, 0.028 and 0.063 for *L. bulgaricus*, and 0.045, 0.015 and 0.078 for *L. plantarum*, respectively.

number of these bacteria after storing at 4 and 10°C for 22 days in semi-dry sausages. The reason was attributed to the ability of lactic acid bacteria to reduce the coliform bacteria to three logarithmic cycles (Aksu et al. 2008), and the ability of lactic acid bacteria to produce anti-compounds such as bacteriocins, hydrogen peroxide and organic acids in the medium and making it unsuitable for their growth.

An increase in the number of coliform bacteria was found with increasing in the maturation period in the control treatment (not inoculated with the starter bacteria) (Table 2). However, it has remained within the standard limits of the specification in the second week of maturation. In the third week, the control treatment was excluded due to the appearance of unpleasant odors and unacceptable colors. The results of Al-Halfy et al. (2017) showed no significant effect of adding metabolites of *B. bifidum* bacteria at concentrations of 0.75, 1.5, 2.25, and 3% during cold storage on total coliform bacteria. The highest inhibition was at 3% concentration for all bacteria, respectively, for ground meat patties. The reason for this was that the number of coliform bacteria exceeded the standard limits of the specification, and the number of coliform bacteria in the sausage product does not exceed 10^3 CFU/g (Al-Faydhi 1996). The results revealed a significant effect ($P \leq 0.05$) by the time and concentration and their interactions on the numbers of Coliform bacteria.

Molds and yeasts: Table 3 shows a decrease of molds and yeasts in the sausage inoculated with the two starters and in the two different concentrations after fermentation for 48 hours. In the maturation stage, there was a decrease in the molds and yeasts in

Table 3. Numbers of Molds and Yeasts in fermented sausages during refrigeration (Aging time).

Aging time (day)	starter ratio (%)	Starter bacteria	Molds and Yeasts count (Log cfu/gm)
2	3	*L. bulgaricus*	2.40
		L. plantarum	2.24
	5	*L. bulgaricus*	1.92
		L. plantarum	1.74
Control			2.56
7	3	*L. bulgaricus*	2.19
		L. plantarum	1.87
	5	*L. bulgaricus*	1.54
		L. plantarum	1.39
Control			3.33
14	3	*L. bulgaricus*	0
		L. plantarum	0
	5	*L. bulgaricus*	0
		L. plantarum	0
Control			3.42
21	3	*L. bulgaricus*	0
		L. plantarum	0
	5	*L. bulgaricus*	0
		L. plantarum	0
Control			**
28	3	*L. bulgaricus*	0
		L. plantarum	0
	5	*L. bulgaricus*	0
		L. plantarum	0
Control			**

*Each number represents an average of three replicates. $LSD_{0.05}$ values for the effect of periods, concentration and their interaction are 0.017, 0.013 and 0.029 for *L. bulgaricus*, and 0.026, 0.020 and 0.045 for *L. plantarum*, respectively.

the first week of cooling. The decline continued to zero in treatments inoculated with starter bacteria until the end of the maturation period. The reason for this was due to the ability of lactic acid bacteria to produce anti-compounds such as bacteriocins, hydrogen peroxide, and organic acids in the medium, making it unsuitable for the growth of fungi, in addition to the low water activity which inhibits its growth (Soyer et al. 2005). These results agreed with Roseiro et al. (2010), that reported a decrease in the number of molds and yeasts to zero during the maturation stage in Portuguese fermented sausage. The numbers of molds and yeasts increased with the increasing maturation time in the control treatment but remained within the standard limits until the second week of maturation. As for the third week of maturation, the control treatment was excluded due to the appearance of unpleasant odors and unacceptable colors. The reason for this was because the numbers of molds and yeasts have exceeded the permissible limits in the sausage product, which the number of yeasts and molds do not exceed 10^4 CFU/g (Al-Zobaie 2010). The results showed a significant effect ($P \leq 0.05$) in time and concentration and their interactions on a number of molds and yeasts.

Psychrotrophic bacteria: There was a decrease in the number of psychrotrophic bacteria in sausage inoculated with two types of starter bacteria for both concentrations and stored under refrigeration after 48 hours of fermentation (Table 4). There was a decrease in the count of these bacteria after the second week of cooling, and the decrease in the number of these bacteria continued with the passage of cooling time in the third week. These bacteria do not appear after the fourth week of storage, for both types and different concentrations. The reason for

Table 4. Numbers of Psychrotrophic bacteria count in fermented sausages during refrigeration (Aging time).

Aging time (day)	starter ratio (%)	Starter bacteria	Psychrotrophic bacteria count (Log cfu/gm)
2	3	*L. bulgaricus*	2.45
		L. plantarum	2.38
	5	*L. bulgaricus*	2.36
		L. plantarum	2.31
	Control		2.62
7	3	*L. bulgaricus*	2.32
		L. plantarum	2.30
	5	*L. bulgaricus*	2.26
		L. plantarum	2.20
	Control		3.13
14	3	*L. bulgaricus*	2.21
		L. plantarum	2.19
	5	*L. bulgaricus*	2.09
		L. plantarum	2.02
	Control		3.41
21	3	*L. bulgaricus*	1.97
		L. plantarum	1.90
	5	*L. bulgaricus*	1.81
		L. plantarum	1.60
	Control		**
28	3	*L. bulgaricus*	0
		L. plantarum	0
	5	*L. bulgaricus*	0
		L. plantarum	0
	Control		**

*Each number represents an average of three replicates. $LSD_{0.05}$ values for the effect of periods, concentration and their interaction are 0.018, 0.014 and 0.032 for *L. bulgaricus*, and 0.033, 0.025 and 0.057 for *L. plantarum*, respectively.

this was due to the ability of lactic acid bacteria to produce anti-compounds such as bacteriocins, hydrogen peroxide, and organic acids in the medium, making it unsuitable for growth. In addition, the decrease in water activity prevents their growth (Soyer et al. 2005; Drosinos et al. (2005). The results showed an increase in the number of Psychrotrophic bacteria with an increase in the maturation period in control but remained within the standard limits of the specification until the second week of maturation. The reason for this was because psychrotrophic bacteria have exceeded the standard limits of the specification, in which the number of psychrotrophic bacteria in the sausage product does not exceed 10^3 CFU/g (Faydi 1996). These findings agreed with what was indicated by Nassif & Mirza (2012) that the use of the metabolites of lactic acid bacteria with different concentrations in the biological preservation of minced meat led to a decrease in the numbers of cryophilic bacteria in the samples to which the metabolites were added compared with the control sample when the samples were cooled for ten days.

These results are identical to those of Majeed et al. (2007) that was added the metabolites of *L. acidophilus* at concentrations of 1 and 2% to minced meat tablets stored in refrigeration for 0, 3, and 5 days, which led to a decrease in the Psychrotrophic bacteria in manufactured tablets with an average of 0.80% due to inhibition of lipolytic enzymes. It also agrees with the study of Majeed et al. (2002) on the pastrami product. The results showed a significant effect ($P \leq 0.05$) in time and concentration and their interactions on the numbers of Psychrotrophic bacteria count.

***Staphylococcus aureus* bacteria:** Table 5 shows the numbers of *S. aureus* in the sausage inoculated with the two starters and in two concentrations at cold

Table 5. Numbers of *Staphylococcus aureus* count in fermented sausages during refrigeration (Aging time).

Aging time (day)	starter ratio (%)	starter bacteria	*S. aureus* Count (Log cfu/gm)
2	3	*L. bulgaricus*	2.40
		L. plantarum	2.37
	5	*L. bulgaricus*	2.31
		L. plantarum	2.23
	Control		2.48
7	3	*L. bulgaricus*	2.26
		L. plantarum	2.16
	5	*L. bulgaricus*	2.20
		L. plantarum	2.06
	Control		3.07
14	3	*L. bulgaricus*	2.06
		L. plantarum	1.87
	5	*L. bulgaricus*	1.92
		L. plantarum	1.65
	Control		3.32
21	3	*L. bulgaricus*	0
		L. plantarum	0
	5	*L. bulgaricus*	0
		L. plantarum	0
	Control		**
28	3	*L. bulgaricus*	0
		L. plantarum	0
	5	*L. bulgaricus*	0
		L. plantarum	0
	Control		**

*Each number represents an average of three replicates. LSD $_{0.05}$ values for the effect of periods, concentration and their interaction are 0.014, 0.011 and 0.025 for *L. bulgaricus*, and 0.028, 0.022 and 0.049 for *L. plantarum*, respectively.

storage. There was a decrease in the numbers of bacteria after the second week of cold storage in a linear proportion with the concentration of the starters, and the numbers reached zero after the third and fourth weeks of cold storage for all treatments under study. The results agree with the findings of Castillejo-Rodríguez et al. (2002), as no growth of bacteria was reported in chicken meat products chilled at 10°C.

The results indicate that the numbers of *S. aureus* increase by the maturation period in the control treatment of the sausage. However, it has remained within the standard limits of the specification until the second week of maturation. The number of *S. aureus* in the sausage product exceeds 10^3 CFU/g (Al-Faydhi 1996). The reason for the presence of *S. aureus* bacteria in meat products fermented under refrigeration conditions may be due to the ability of this bacteria to grow in refrigeration temperatures and at a pH of 4-10 with a concentration of sodium chloride up to 25%. Moreover, these bacteria can survive in a wide range of compelling circumstances such as hard surfaces for a long time (Koutsoumanis et al. 2004; Skandamis et al. 2007). Studies have indicated the presence of *S. aureus* in edible and perishable foods such as soft meat, fish, and prepared and refrigerated foods. It can also be found in cooked meat products, cheese and fermented foods (Pepe et al. 2006; Simon & Sanjeev 2007). The results showed a significant effect ($P \leq 0.05$) in time and concentration and interaction on the numbers of *S. aureus* count. Liu et al. (2021) and Charlier et al. (2009) reported that lactic acid bacteria inhibit the growth of *S. aureus* through several factors, including the production of inhibitory compounds such as hydrogen peroxide and bacteriocins and lowering the pH, and the consumption of nutrients necessary for its growth such as amino acids,

vitamins, minerals, and sugars. The results showed a lack of *Salmonella* bacteria in the samples of our study showing the safety of the product for human consumption.

The bacteria that make up starter cultures may limit or restrict the establishment of spoilage and/or hazardous populations through mechanisms such as the synthesis of particular metabolites or competitive exclusion. As a result, substituting starter cultures for chemical additives such as nitrites and nitrates may reduce demand. Furthermore, because starter cultures can metabolize nitrates and nitrites, the residual quantities of those compounds in fermented meat products inoculated with starter cultures are reduced. In addition to their positive influence on safety, which should be the major argument for using starters in fermented meat products, they may also improve the reproducibility of product attributes.

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