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Behaviour of *Listeria monocytogenes* in raw sausages (merguez) in presence of a bacteriocin-producing lactococcal strain as a protective culture

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Abstract

The effectiveness of a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* M in reducing population level and growth of *Listeria monocytogenes* ATCC 7644 in fermented merguez sausage was examined. Two different formulas (with or without added nitrites) were assayed and predetermined numbers of *Listeria* (ca 10^6 cfu g^{-1}) were added to sausage mixture. The effect of in situ production of the bacteriocin by *Lactococcus lactis* M on *Listeria monocytogenes* ATCC 7644 during fermentation and storage of merguez sausages at room (ca 22 °C) or at refrigeration (ca 7 °C) temperature was tested. Results indicated that counts of *Listeria monocytogenes* were decreased during fermentation of merguez samples fermented with either the bacteriocin-producing *Lactococcus lactis* M (Bac^+) or a nonbacteriocin-producing *Lactococcus lactis* J (Bac^-). However, reduction in *Listeria* cfu's was greater in samples fermented with the Bac^+ than in those fermented with the Bac^- starter. In merguez sausage made without nitrites addition, the Bac^+ starter induced further decrease in *Listeria* counts by 1.5 log cycles compared with that induced by the Bac^- starter. While in merguez samples with added nitrites (0.4%), the effect of the bacteriocin produced in situ was less important than in those made without nitrites addition.

Keywords: *Lactococcus lactis*; Merguez; Sausage; In situ bacteriocin production, *Listeria monocytogenes*.

1. Introduction

Listeria monocytogenes is widely distributed in nature and the association of this pathogen with meat and slaughter environment is well established (Autio, Säteri, Fredriksson-Ahomaa, Rahkio, Lundén, & Korkeala, 2000; Fenlon, Wilson, & Donachie, 1996; Johnson, Doyle, & Cassens, 1988). There is therefore a concern that such meat when used in sausage making may be responsible for transmission of *Listeria monocytogenes* thereby causing listeriosis outbreaks. In fact, cases of listeriosis due to consumption of meat products were reported in Norway (Nesbaken, 1995), in France (Goulet, Lepoutre, Rocourt, Coutieu, Dehaumont, & Veit, 1993) and elsewhere (Bredholt, Nesbakken, & Holck, 1999).

In recent years, many bacteriocins have been described to have anti-listerial activity in vitro and several studies have been conducted to assay the potential of bacteriocin-producing strains in meat systems (Fœgeding, Thomas, Pilkington, & Klaenhammer, 1992; Hugas & Monfort, 1997; Nielson, Dickson, & Crouse, 1990; Schillinger, Kaya, & Lücke, 1991). The subclass IIa bacteriocins alone or in combination with the lantibiotics appear to have the best potential in controlling *Listeria monocytogenes* in meat systems (Lüke, 2000).

Most studies on the in situ effect of bacteriocins against *Listeria monocytogenes* in meat and meat products have been carried out using bacteriocinogenic strains of *Pediococcus*, *Lactobacillus* and *Carnobacterium* genera. Although, inhibition of *Listeria* spp. by bacteriocins from *Lactococcus* genus has been extensively reported, few studies have been done, to our knowledge, with Bac^+ lactococcal strains as starter cultures for fermented sausages to provide added control against the pathogen.

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Merguez is a raw sausage of North Africa origin and widely consumed in different countries of the world including Europe. It is generally made of lean and fat beef mixed with condiments and has a short shelf life even when stored at refrigeration temperature. In Morocco, the product is usually made in poor hygienic conditions and exposed for sale at ambient temperature usually around 20 °C. Therefore, it is a potential vehicle for serious pathogens including *Listeria monocytogenes*.

The present study was undertaken to investigate the possibility of developing a bacteriocin-producing starter culture that inhibits intentionally introduced *Listeria monocytogenes* ATCC 7644 throughout fermentation and storage of fermented merguez sausage at refrigeration (ca 7 °C) or at room temperatures (ca 22 °C).

2. Materials and methods

2.1. Bacterial cultures and media

Listeria monocytogenes ATCC 7644, used for the challenge experiments was grown in Trypticase Soya Broth (TSB; Biokar, France) at 37 °C. *Lactococcus lactis* subsp. *lactis* M (Benkerroum, Oubel, Zahar, Dlia, & Filali-Maltouf, 2000), previously isolated and confirmed to produce an anti-*Listeria* bacteriocin different from nisin was used to ferment merguez. A Bac$^-$ *Lactococcus lactis* subsp. *lactis* J isolated from fresh cheese (Benkerroum et al., 2000) was used in bacteriocin-negative samples. Lactic cultures were grown in M17 broth (Difco, USA) at 30 °C.

Cultures were stored at −20 °C in the presence of 25% glycerol. Before experimental use, cells were subcultured twice in appropriate media (1% inoculum).

2.2. Starter preparation

The starters (Bac$^+$ or Bac$^-$) were activated by inoculation of reconstituted milk (11% w/v) with *Lactococcus lactis* M or *Lactococcus lactis* J (3%) and an overnight incubation at 30 °C.

2.3. Sausage manufacture

Two independent sausage trials (1 and 2) of 2 kg each were conducted. Each trial consisted of four simultaneous fermentations: (1) a non-inoculated control; (2) no starter culture and contaminated with *Listeria monocytogenes* ATCC 7644 (ca 10^6 cfu g^{-1}); (3) Added Bac$^+$ (*Lactococcus lactis* M) starter culture (3%) and *Listeria monocytogenes* ATCC 7644 (ca 10^6 cfu g^{-1}) and (4) Added Bac$^-$ (*Lactococcus lactis* J) starter culture (3%) and *Listeria monocytogenes* ATCC 7644 (ca 10^6 cfu g^{-1}). To achieve the initial contamination level of approximately 10^6 cfu g^{-1}, an overnight *Listeria* culture was added to sausage mixture to the level of 1 ml kg^{-1}.

In trial 1, the sausage mixture contained: lean beef, 80%; fat beef, 20%; sugar, 5 (g kg^{-1}); salt, 18 (g kg^{-1}); red pepper, 10 (g kg^{-1}); cumin, 5 (g kg^{-1}); black pepper, 5 (g kg^{-1}); ginger, 5 (g kg^{-1}); olive oil (10 ml). Composition of trial 2 mixture differed from that of the first trial by nitrites (0.4%) addition. *Listeria monocytogenes* ATCC 7644 or active starter cultures were added where appropriate together with the sausage mixture to ensure good homogeneity.

For each trial, the batter was prepared first with the negative control (non-inoculated), followed by the positive control (inoculated with the Bac$^-$ starter) and then, the test samples (inoculated with Bac$^+$ starter) to ovoid carryover of the bacteriocin-producing strain into the batter intended to contain the Bac$^-$ culture.

The meat lean and fat was cut up with a Kilia (Germany) cutter then mixed with the additives. The prepared sausage mixture was stuffed into natural casing and allowed to ferment at 30 °C for 24 h. The final products were split into two parts. The first was stored in the refrigerator (ca 7 °C) and the second was held at ambient temperature (ca 22 °C).

2.4. Sausage sampling and analysis

Sausage was sampled at selected intervals to perform microbial counts by aseptically removing 10 g. Samples were mixed with saline solution (0.85% NaCl) with an Ultra-thurrax©. *Listeria* was enumerated after incubation for 48–72 h at 37 °C on Alzoreky®–Sandine-listeria medium (ALSM; Al-Zoreky & Sandine, 1990). Lactic acid bacteria were enumerated on M17 agar (Difco, USA) after 24–48 h of incubation at 30 °C.

The pH of merguez sausage was determined at selected intervals during fermentation and storage with a pH meter (Crisson, micropH 2000, Germany). A sample of 10 g was mixed with 10 ml of distilled water prior to pH measurement.

2.5. Statistical analysis

Each trial was repeated twice and each determination was done in duplicates. Analysis of variance ($\alpha = 0.05$) and student *t*-test were performed for comparison of means.

3. Results and discussion

3.1. Sausage fermentation and storage

Variations in the pH in Bac$^+$ and in Bac$^-$ samples of merguez sausage without nitrites added were recorded during fermentation and storage at refrigeration (ca

7 °C) and at room temperature (ca 22 °C). The results showed that the pH decreased in control batch (without added starter culture) to a mean value of 5.4 at the end of the fermentation period suggesting that the raw material contains naturally acidifying bacteria responsible of spontaneous acidification of merguez sausage. Similar pH evolution was observed in batches inoculated with the Bac^+ or the Bac^- starter culture where respective values of 5.3 and 5.2 were reached after 24 h (Table 1). No significant difference (>0.05) in pH value attained after 24 h was observed between all trials including the non-inoculated control. Such results indicate that deliberate addition of the lactococcal strain for fermentation would not acidify significantly merguez sausage and thus will not alter its gustatory quality. Nonetheless, the pH reached is not low enough to inhibit *Listeria monocytogenes* which was demonstrated to grow at pH 5.1 in a laboratory media (Benkerroum & Sandine, 1988) and to survive at pH 4.9 in dry fermented sausages (Fœgeding et al. 1992; Schillinger et al. 1991). Therefore, from the technological standpoint, the bacteriocin-producing *Lactococcus lactis* M may be recommended as a "protective" culture in merguez sausage or other mildly acid sausages to control *Listeria* by means of in situ bacteriocin production.

The evolution of pH in samples stored at refrigeration (ca 7 °C) or at room (ca 22 °C) temperature is illustrated in Table 2. At both temperatures, the pH increased in all samples of trial 1 starting from the first day indicating the onset of a proteolytic activity ultimately leading to the putrification of the product. Samples stored at room temperature spoiled 2 days earlier than those stored at 7 °C regardless of the bacteriocin-production phenotype. The spoilage was judged by the development of a bad smell, changes in the physical appearance or growth of moulds on the surface.

In merguez sausage with added nitrites (trial 2), the pH has decreased during fermentation in a similar way as in samples not containing nitrites to reach a final value of 5.2 (Table 1). It has continued to decrease during the first day of storage at both temperatures and then it has increased until the alteration was evident (Table 2). The alteration was perceivable in samples stored at room temperature (ca 22 °C) after the fifth day, while in samples kept at the refrigeration temperature (ca 7 °C) no marks of alteration (odours, appearance or

Table 1
pH measurements during fermentation of merguez sausage with a bacteriocin producing *Lactococcus lactis* subsp. *lactis* M (Bac^+) or with a non-bacteriocin-producing *Lactococcus lactis* subsp. *lactis* J (Bac^-)

Hours	Bac^+		Bac^-	
	No nitrites added	+ 0.4% nitrites	No nitrites added	+ 0.4% nitrites
0	6.5 (±0.1)[a]	6.6 (±0.3)	6.5 (±0.3)	6.6 (±0.4)
2	6.4 (±0.3)	6.1 (±0.2)	6.5 (±0.4)	6.4 (±0.3)
4	6.4 (±0.3)	6.0 (±0.4)	6.3 (±0.2)	6.2 (±0.1)
6	6.2 (±0.3)	6.0 (±0.3)	6.2 (±0.3)	6.1 (±0.3)
8	6.0 (±0.4)	5.9 (±0.4)	6.1 (±0.4)	5.8 (±0.3)
10	5.7 (±0.2)	5.8 (±0.3)	5.7 (±0.4)	5.8 (±0.4)
24	5.3 (±0.3)	5.2 (±0.2)	5.2 (±0.1)	5.0 (±0.1)

Results are means of two repetitions.
[a] Standard deviation.

Table 2
pH measurements during storage at 7 or at 22 °C of merguez sausage fermented with a bacteriocin-producing *Lactococcus lactis* subsp. *lactis* M (Bac^+) or with a nonbacteriocin-producing *Lactococcus lactis* subsp. *lactis* J (Bac^-) in presence or absence of nitrites

Days	Bac^+				Bac^-			
	No nitrites		+ 0.4% nitrites		No nitrites		+ 0.4% nitrites	
	7 °C	22 °C	7 °C	22 °C	7 °C	22 °C	7 °C	22 °C
1	5.3 (±0.5)[a]	5.7 (±0.2)	4.8 (±0.2)	4.8 (±0.4)	5.5 (±0.1)	5.5 (±0.3)	4.5 (±0.3)	4.7 (±0.5)
3	5.4 (±0.2)	5.8 (±0.2)	5.3 (±0.4)	5.5 (±0.2)	5.5 (±0.1)	6.4 (±0.5)	5.4 (±0.2)	6.0 (±0.2)
5	5.7 (±0.3)	S	5.2 (±0.1)	5.1 (±0.3)	5.6 (±0.4)	S	5.2 (±0.4)	5.1 (±0.2)
7	S		5.0 (±0.2)	S	S		5.4 (±0.2)	S
9			5.7 (±0.3)				5.6 (±0.4)	
11			5.8 (±0.2)				6.0 (±0.1)	

Results are means of two repetitions. S, spoiled samples as judged by the smell and the physical appearance.
[a] Standard deviation.

growth of moulds) were noticed after 11 days. It appears, therefore, that the bacteriocin production phenotype had no effect on the shelf-life of the product. Temperature of storage and nitrites addition were more involved in the shelf-life extension of merguez sausage.

3.2. *Behaviour of* Listeria monocytogenes *in fermented merguez sausage*

The evolution of cell counts of *Listeria monocytogenes* ATCC 7644 in merguez sausage fermented with the Bac^+ or with the Bac^- starter was carried out during the manufacture and storage until perceivable alteration. Fig. 1 shows that *Listeria* grew well in control samples to reach 8.1 log cfu g^{-1} after 24 h of incubation at 30 °C. Such results suggest that the pathogen has a potential to grow in a merguez sausage when no starter culture is added. Therefore, deliberate addition of bacteriocin-producing LAB culture seems appropriate as protective culture to prevent growth of *Listeria* in sausages when a mild souring activity is desired.

During the fermentation period, the counts of *Listeria monocytogenes* were decreased in Bac^- and in Bac^+ merguez samples by 1.6 and 2.7 log cfu g^{-1} units, respectively compared with initial level of contamination. Meanwhile, LAB grew well in all samples to exceed 9 log cfu g^{-1} after 1 day of fermentation. According to Lüke (2000), the in situ effect of bacteriocins against *Listeria monocytogenes* in meat systems has resulted, in most reported cases, in the reduction in *Listeria monocytogenes* counts by 1–2 log units compared with a bacteriocin-negative control. Our results are in agreement with such findings since counts of *Listeria monocytogenes* ATCC 7644 were further reduced by 1.52 log cycles in the Bac^+ samples compared with the Bac^- control samples (Fig. 1).

During storage at refrigeration (ca 4 °C) or at room (ca 22 °C) temperature, *Listeria* counts increased by approximately 1 log cfu g^{-1} during the first day of storage and were maintained constant thereafter (Fig. 2).

In order to assess the influence of technological formulas on the performance of bacteriocin-producing starter culture, two different mixtures were assayed, one of which contained 0.4% of nitrites (trial 2). In the nitrites added trial, the effect on in situ bacteriocin production on growth of *Listeria monocytogenes* ATCC 7644 was determined in Bac^+ and in Bac^- batches during fermentation, and storage at ambient (ca 22 °C) or at refrigeration (ca 7 °C) temperatures. Populations of LAB were monitored simultaneously. The results of microbial cell-counts in merguez sausage during fermentation are summarized in Fig. 3. In Bac^+ batch samples, a 2.4 log units reduction in *Listeria* populations was

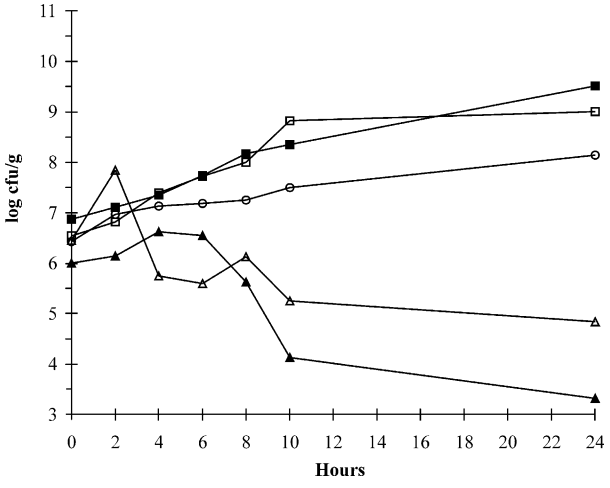

Fig. 1. Growth of lactic acid bacteria (—■—, —□—) and *Listeria monocytogenes* ATCC 7644 (—▲—, —△— , —○—) during fermentation of merguez sausage with a Bac^+ (—■—, —△—), a Bac^- (—□—, —△—) starter culture in absence of nitrites. Growth of *Listeria monocytogenes* ATCC 7644 in a control (—○—) without starter added was also monitored.

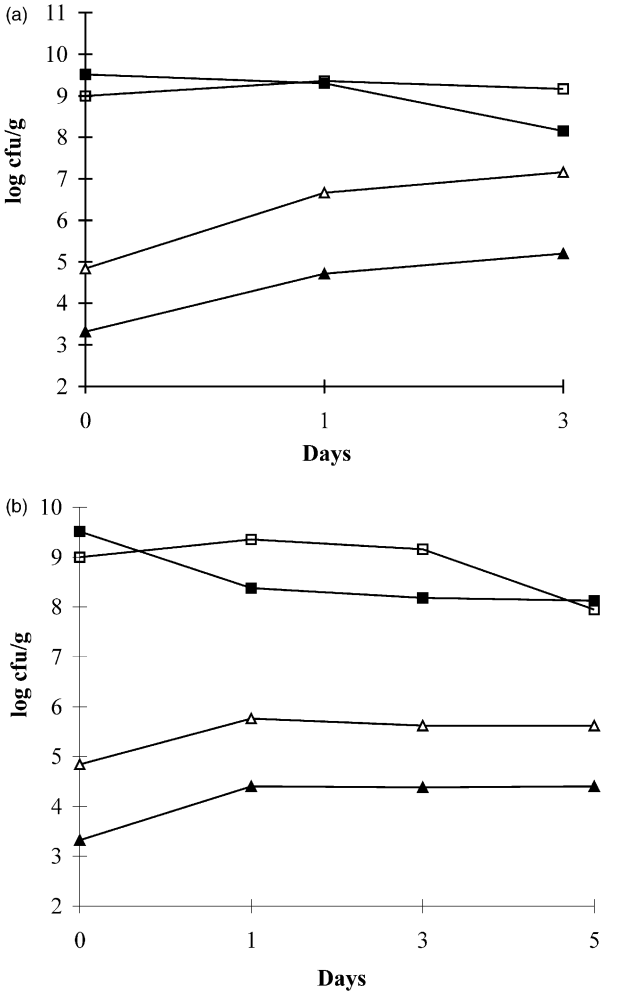

Fig. 2. Growth of lactic acid bacteria (—□—, —■—) and *Listeria monocytogenes* ATCC 7644 (—△—, —▲—) in merguez sausage fermented with a Bac^+ (—■—, —▲—) or with a Bac^- (—□—, —△—) starter culture during conservation at (a) room (ca 22 °C) or at (b) refrigeration (ca 7 °C) temperatures.

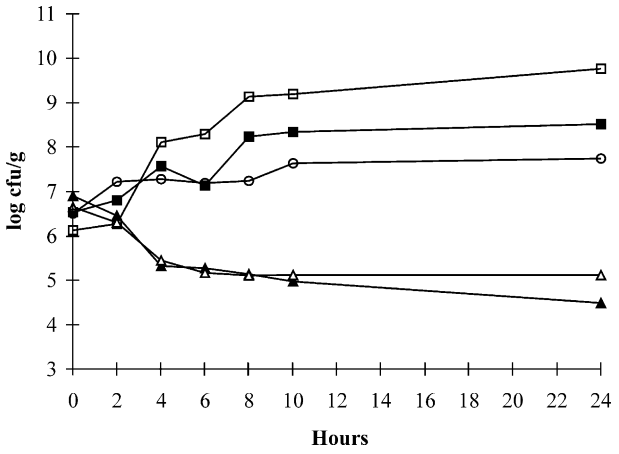

Fig. 3. Growth of lactic acid bacteria (—■— , —□—) and *Listeria monocytogenes* ATCC 7644 (—▲—, —△—, —○—) in merguez sausage during fermentation with a Bac^+ (—■—, —▲—) or with a Bac^- (—□—, —△—) starter culture in presence of nitrites (0.4%). Growth of *Listeria monocytogenes* ATCC 7466 was also monitored in merguez samples without added starter culture (—○—).

noted, while in samples of the Bac^- batch, the viable count of *Listeria monocytogenes* was reduced by 1.5 log units compared with the initial inoculum. The Bac^+ starter induced only 0.7 log units compared with the nonbacteriocin-producing starter. Concurrently, LAB counts has increased in all samples. The results of *Listeria* cell-counts in both trials indicate that in situ bacteriocin production is more efficient in controlling *Listeria monocytogenes* ATCC 7644 in absence (Fig. 1) than in presence (Fig. 3) of nitrites suggesting that the curing agent would delay or antagonize with the bacteriocin effect. In this regard, conflicting data are available in the literature. Hugas, Veujmeyer, Pagés, Garriga, and Hammes (1996) have shown that nitrates and nitrites enhance the bacteriocin activity against *Listeria monocytogenes* in sausage, while Leroy and De Vuyst (1999) found that the curing agent antagonises with the bacteriocins activity in meat systems by reducing in situ bacteriocin production. In fact, interference between nitrites and bacteriocins may depend on the composition of the product, the producer organism and the pathogen considered.

Fig. 4 summarizes results of growth of surviving cells in merguez sausage during storage at room (ca 22 °C) or at refrigeration (ca 7 °C) temperatures. The figure shows that *Listeria* counts levelled-off after 3 days of storage at 7 °C (Fig. 4B) and after 1 day at 22 °C (Fig. 4A) in Bac^+ samples, while in Bac^- samples, cell counts of *Listeria* remained practically constant in both cases. Thereafter, re-growth of *Listeria monocytogenes* has occurred in all samples throughout the storage period at both storage temperatures. Similar behaviour was observed in samples without added nitrites. Such rebound phenomenon has been extensively reported and

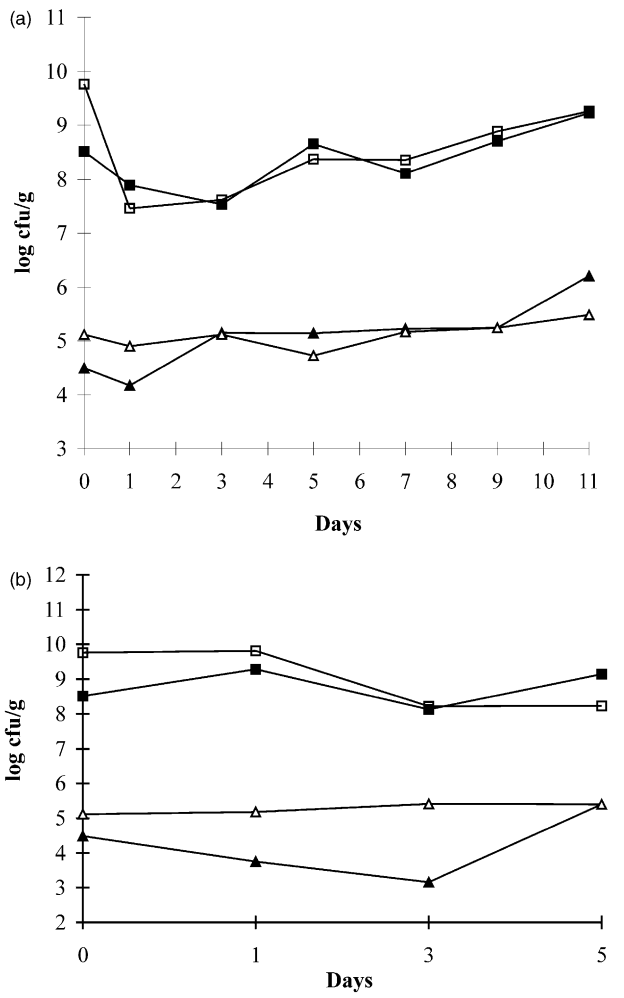

Fig. 4. Growth of lactic acid bacteria (—□—, —■—) and *Listeria monocytogenes* ATCC 7644 (—△—, —▲—) in merguez sausage fermented with a Bac^+ (—■—, —▲—) or with a Bac^- (—△—, —□—) starter culture in presence of nitrites (0.4%) during conservation at (a) room (ca 22 °C) or at (b) refrigeration (ca 7 °C) temperatures.

could be explained by the emergence of bacteriocin-resistant strains of *Listeria monocytogenes* or by the increase of pH to an unfavourable value to the action of the bacteriocin. It should be mentioned that the activity of the bacteriocin produced by *Lactococcus lactis* M is optimal at acid pH (data not shown). The increase in pH that has been observed during storage (Table 2) may be attributed to the onset of a proteolytic activity which may also inactivate partially the bacteriocin. Proteolytic activity may be due to proteases naturally occurring in meat or to those produced by microbial contaminants such as *Pseudomonas*, *Proteus* and other genera of the Enterobacteriaceae family generally recognized as highly proteolytic and as the main responsible of meat products putrification. Other factors such as adsorption of bacteriocins to meat and fat particles were also reported to interfere with bacteriocin activity (Schilinger et al. 1991; Stiles, 1996).

4. Conclusion

This study demonstrated that the use of bacteriocin-producing starter culture in merguez sausage contribute to reduce the risks for transmission of listeriosis to consumers. Therefore, the bacteriocin-producing *Lactococcus lactis* M studied herein may be considered as a potential biological adjunct to protect mildly acid fermented sausages from contamination and growth of *Listeria monocytogenes*. It may also be used in combination with an appropriate acidifying lactic acid bacterium in a mixed starter for the same purpose in low acid sausages.

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