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Effect of starter cultures on microbial and physicochemical parameters of a dry fermented poultry meat sausage

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The aim of this work was to study the effect of *Staphylococcus xylosus* and *Lactobacillus plantarum* on microbial and physicochemical characteristics of a dry fermented poultry meat sausage. The microbial results show that lactic acid bacteria (LAB) and coagulase negative staphylococci (CNS) represent the two predominant microflora in all stages of ripening. The number of enterobacteria decreased significantly in sausages previously inoculated respectively with *L. plantarum* and with a mixed starter cultures, due to the acidifying and antibacterial activities of lactic acid bacteria. The moisture content of control and inoculated sausages decreased in all stages of ripening which allows a good preservation and consequently, improved their shelf life. Moreover, total free amino acids content increased during ripening of dry fermented sausages. Their concentrations were higher in sausages previously inoculated with starter cultures than in control ones. The main amino acids present in all stages of ripening were aspartic and glutamic acids, arginine, glycine, threonine, alanine, tyrosine, phenylalaline, leucine and isoleucine.

Key words: *Staphylococcus xylosus*, *Lactobacillus plantarum*, ripening, dry fermented sausage.

INTRODUCTION

The demand for meat products with lower fat contents or healthier fatty acid compositions has increased in recent years due to new guidelines recommending reduced saturated fat intake and consumers' desire to lose weight (Akesowan, 2008; Archer et al., 2004; Colmenero, 2000). Several alternative strategies have been used in the manufacture of these products, such as the substitution of red meat by skinless poultry meat. Skinless poultry

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Abbreviations: LAB, Lactic acid bacteria; **CNS,** coagulase negative staphylococci

meat contains more protein, less fat, and less cholesterol than red meat (Hu, 2005; Ressurreccion, 2004). The protein of poultry meat is of excellent nutritional quality, and it contains all of the essential amino acids for human consumption (Varnam and Sutherland, 1995). Furthermore, the manufacture of poultry meat products usually costs less than that of similar beef and pork products (Barbut, 2002). An added benefit is that poultry meat is not restricted by most cultural and religious laws, and it is consumed by both Jews and Muslims (Deumier and Collignan, 2003).

Processed poultry meat products offered a favorable environment for the growth of pathogenic bacteria. Different strategies have been used such as fermentation to guarantee food safety and to standardize product properties. Spontaneous fermentation has been applied for thousands years mainly as method of preservation of excess foodstuff and secondarily as a way to obtain spicy and flavorful foods (Drosinos et al., 2007). In fact, during this stage, the major physical, biochemical and microbiological transformations take place such as: acidification, reduction of nitrates to nitrites, formation of nitrosomyoglobin, solubilization and gelification of myofibrillar and sacroplasmic proteins, proteolytic, lipolytic and oxidative phenomen and dehydratation (Casaburi et al., 2008). Nowadays, the use of selected starter cultures for sausage production is becoming increasingly necessary to guarantee food safety, reduce the fermentation times, extend the shelf life and enhance the organoleptic characteristics of the final product (Parente et al., 2001; Drosinos et al., 2005). Most dry fermented sausages are produced with starter cultures combination of lactic acid bacteria (LAB) and coagulase negative staphylococci (CNS) (Ammor and Mayo, 2007).

Lactic acid bacteria (LAB) play an important role in meat preservation and fermentation processes because they affect both the technological properties and the microbial stability of the final product. In fact, the use of LAB is essential for carbohydrate fermentation and lactic acid generation with subsequent pH drop which contribute to the flavor of the final product through the formation of noticeable acidic and vinegary (acetic acid) tastes. At isoelectric pH, muscle proteins coagulate, lose their water-holding capacity and result in the sliceability, firmness and cohesiveness of the final product. Besides, at this acidic environment ripening is favored as well as color development (Drosinos et al., 2007).

Moreover, LAB inhibits growth of spoilage and pathogenic microorganisms through the production of lactic acid and antimicrobial compounds such as bacteriocins, acetic acid, ethanol, acetoin, carbon dioxide and pyruvic acid (Albano et al., 2009). The species most used as commercial meat LAB starter cultures are *Lactobacillus plantarum, Lactobacillus sakei, Lacobacillus curvatus, Lactobacillus fermentum, Lactobacillus brevis, Pediococcus pentoseaceus* and *Pediococcus acidilactici* (Hammes, 1990; Rovira et al., 1994; Schillinger and

Lücke, 1987). Whereas, LAB are mainly responsible for acidification, CNS are associated with color and flavor development (Olesen et al., 2004; Leroy et al., 2006). CNS are Gram-positive, catalase positive cocci with antioxidant activities, preventing the formation of off-flavors and rancidity and their nitrate reductase activity is important for color formation (Ravyts et al., 2010). Their proteolytic and lipolytic activities contribute to the sensory quality of fermented sausages through the generation of flavor-active compounds and their precursors, such as amino acids, free fatty acids, aldehydes, amines, ketones and alcohols (Montel et al., 1996; Leroy et al., 2006). Unfortunately, CNS are generally poorly competitive in a fermented sausage environment, resulting in poor growth and even a decrease in viability during manufacturing (Di Maria et al., 2002; Tjener et al., 2004; Casaburi et al., 2008). The species most used for the production of dry fermented sausages are *Staphylococcus carnosus* and *Staphylococcus xylosus* (Laukova et al., 2010).

In Tunisia, sausages are mainly produced from red meat. The aim of this work is to produce a skinless poultry meat sausage previously inoculated with selected starters of *L. plantarum* and *S. xylosus* and to study the microbial and physicochemical quality during ripening.

MATERIALS AND METHODS

Bacterial strains and culture condition

The *S. xylosus* and the *L. plantarum* strains used in this study were both isolated from a Tunisian traditional salted meat "kadid" as previously reported by Essid et al. (2007) and Essid et al. (2009). These strains were selected for having the best attributes for use as starter meat cultures (Essid et al., 2007; Essid et al., 2009). Strains of *S. xylosus* were cultivated in Trypton Soy Broth (Biolife, Italy) at 37°C, whereas strains of *L. plantarum* were cultivated in Man Rogosa and Sharp Broth (MRS broth) (Biolife, Italy) at 30°C. After 24 h of incubation, the bacterial suspensions were centrifuged at 12 000 *g* for 15 min (Universal, 320) and the pellets were washed with sterile distilled water and resuspended in 10 ml of the nutrient broth (Pronadisa, Spain). Cells were harvested by centrifugation (12 000 *g* for 15 min) and resuspended in 5 ml of distilled water. Finally, the number of bacterial cells in each suspension was adjusted to reach the range of 6 log CFU/ml (λ= 600 nm, OD= 0.5 for *L. plantarum*, OD = 0.6 for *S. xylosus*) by using a spectrophotometer (Jenway 6305).

Preparation of dry fermented sausages

The sausage formulation included 7.5 kg of poultry meat (75%), 2.5 kg of poultry fat (25%), 400 g of salt (4%), 20 g of black pepper (0.2%), 20 g of paprika (0.2%), 100 g glucose (1%) and 1 g of potassium nitrate (0.01%). After chopping and mixing the ingredients, the mixture was divided into four batches (2.5 kg for each batch): batch 1, inoculated with *S. xylosus* alone; batch 2, inoculated with *L. plantarum* alone; batch 3, inoculated with *L. plantarum* and *S. xylosus* and batch 4, control without inoculation. All the samples were inoculated with about 10^6 viable cells per g of sausages. The mixture of each batch was stuffed into artificial casings, giving approximately 500 g as the final mass of each sausage and then placed in a fermentation chamber (BCR, CF 1 B,

France). The sausages were fermented for 5 days at 24°C and (80%) RH. After five days of processing, the temperature was decreased to 14°C for 23 days. For sampling, three sausages of each batch at 0 day (mix before stuffing) and after 7, 14, 21 and 28 days of ripening were taken for microbiological and physico-chemical analyzes and each analysis was carried out in triplicate.

Microbiological analysis

Sausage samples (10 g) of each batch were homogenised with 90 ml of sterile peptone water (Biolife, Italy) and decimal dilutions were prepared. Mesophilic lactic acid bacteria (LAB) were enumerated on MRS Agar (Biolife, Italy) after 48 h incubation at 30°C. The number of staphylococci was determined on Mannitol Salt Agar (Biolife, Italy) after incubation at 37°C for 48 h. Yeasts and molds were enumerated on Sabouraud Dextrose Agar (Biokar, France) at 28°C for 4 days.

Total viable counts were determined on Standard Plate Count Agar (PCA) (Biolife, Italy) at 30°C for 48 h. *Enterobacteriaceae* were determined on Violet Red Bile Glucose (VRBG) (Biokar, France) at 37°C for 24 h.

Physicochemical analysis

pH, moisture and weight loss

The pH values were measured in homogenates prepared by blending 10 g of sausage (Moulinex DPA141, France) with 50 ml of distilled water for 2 min. Measurements were taken with a pH meter (Microprocessor pH meter BT-500, BoecoGermany). The moisture percentage was calculated by weight loss experimented by the sample (5 g) maintained in an oven (Memmert, UL 60) at 105°C, until constant weight according to the ISO recommended method (ISO, 1973). Weight loss was expressed as a percentage of the initial weight (Liaros et al., 2009).

Color measurement

Color measurements were carried out using a CR-300 colorimeter (Minolta Chroma Meter CR-300, Japan). Each sausage was cut and the color of the slices was measured three times for each analytical point L*, a* and b* scale coordinates were obtained: L* (lightness), a* (redness) and b* (yellowness). Before each series of measurements, the instrument was calibrated using a white ceramic tile.

Free amino acids content (FAA)

The content of FAA was determined by reverse phase HPLC. The amino acids were extracted after hydrolysis of meat proteins in the presence of concentrated hydrochloric acid. Thus, 5 g of dry fermented sausages were chopped and added to 4 ml of HCl 37% (6 M). The mixture was homogenized and then placed in an oven at 105°C for 24 h. Hydrolysis was stopped by adding approximately 6 ml of NaOH (6 N). Then, the mixture was filtered through a syringe filter and the filtrate was stored at 4°C until injection. The separation of the protein fraction of sausages was performed using HPLC Agilent L 100 system, on a column C18 (250 mm x 4.6 mm dimensions of the column, 5 μm porosity). This system contains: high pressure pump, an automatic injector FLD, FLD detector and control software and acquisition of data Chemstation. The separation was carried out for 30 min, the flow rate of the mobile phase is 1 ml / min. The excitation was at 340 nm and the emission was at 440 nm. The injection solution is composed of 2.5 μl of the

borate, buffer supplemented with 0.5 μl of the sample, 0.5 μl of H_2O and 0.5 μl of *o*-phthaldialdehyde solution. The whole is mixed with 3.5 μl of air. Then, everything was injected. The injection of reference amino acids allowed determining their retention times. To determine the concentration of free amino acids in different samples, straight standards have been established relating the concentration of each reference amino acid to the area of the peak obtained.

Statistical analysis

Data were statistically analyzed using one-way ANOVA procedure of SPSS® 17.0. Duncan's multiple range test were used to determine any significant difference between mean values and evaluations were based on a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Microbial results

Figure 1 shows the evolution of lactic acid bacteria (LAB), staphylococci, yeasts and molds, *Enterobacteriaceae* and total viable counts during fermentation of starter inoculated and control sausages. Our results show that the addition of selected starter cultures did not affect significantly ($p > 0.05$) the quantitative evolution of different microbial groups with the exception of staphylococci ($p < 0.05$). The growth of microorganisms has been affected significantly ($p < 0.05$) by the time parameter with the exception of staphylococci ($p > 0.05$).

The concentrations of total viable counts in the first day of fermentation were 5.17, 6.53, 6.71 and 6.82 log CFU/g, respectively for control sausages and sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture. During ripening, these numbers increased to reach respectively values of 8.75, 8.92, 8.89 and 9.78 log CFU/g (Figure 1). The numbers of total viable counts remain significantly higher in sausages inoculated compared to those measured on control samples and this at all stages of ripening due to the prior inoculation of sausages by *S. xylosus* and *L. plantarum*. Our results are in agreement with those obtained by Drosinos et al. (2005).

Lactic acid bacteria were the dominant microflora in both control and starter inoculated sausages. This result is in agreement with other studies reporting that LAB are the dominant microflora of dry fermented sausages (Casaburi et al., 2008; Albano et al., 2009; Papamanoli et al., 2003; Bolumar et al., 2006; Ambrosiadis et al., 2004). This predominance is explained by the suitability of LAB to survive in meat environment and their ability to multiply rapidly during ripening (Drosinos et al., 2005). The LAB count in the first day of fermentation was 5.89 log CFU/g for control sausages against 6.14, 7.59 and 7.35 log CFU/g, respectively for sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture. During the first 14th days of ripening, the number of mesophilic LAB increased significantly to reach values of 8.34, 8.27,
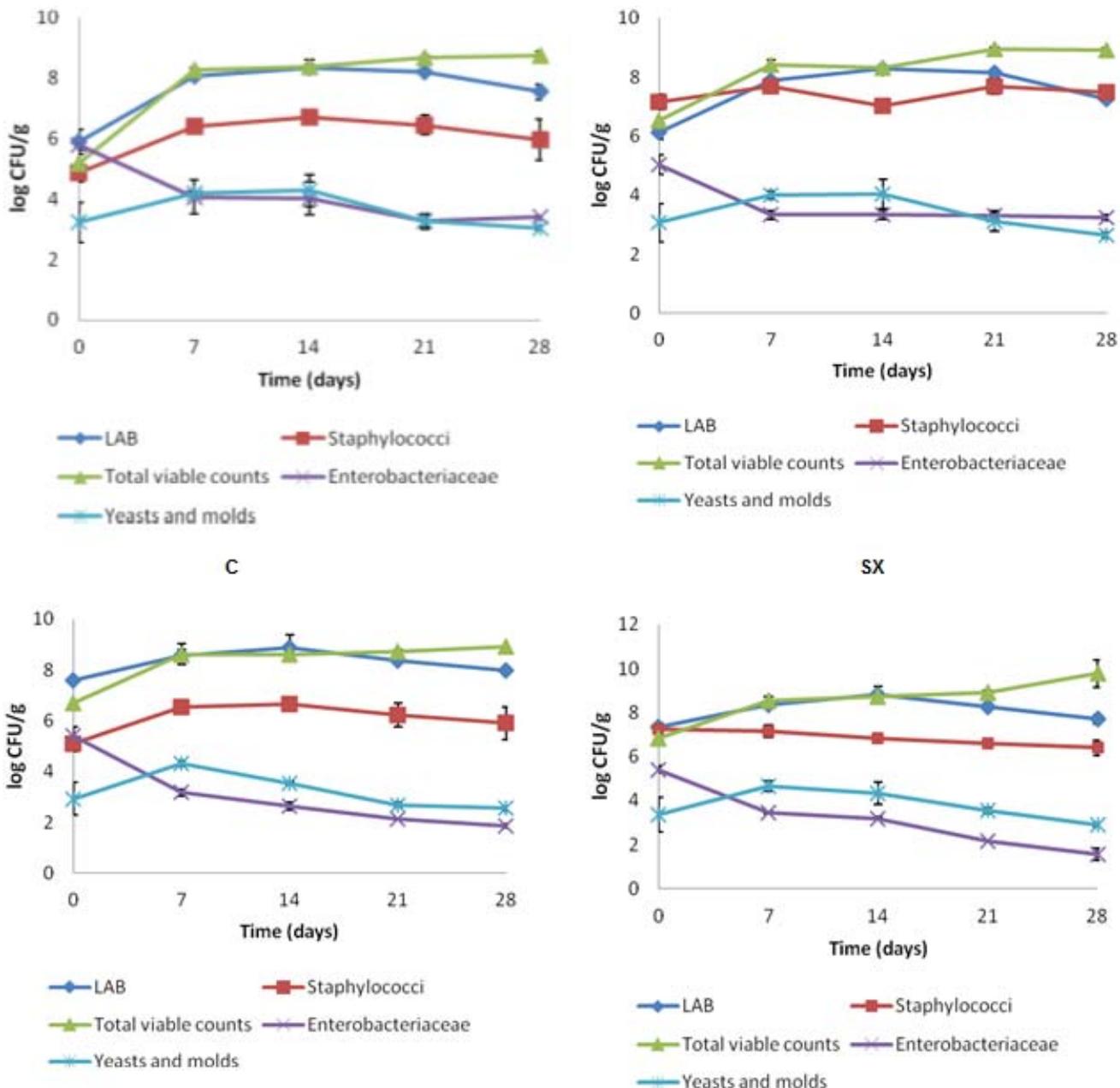
Figure 1. Evolution of microbial population during the ripening of control and inoculated dry fermented sausages: **C** (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

8.88 and 8.82 log CFU/g, respectively for control sausages and sausages inoculated with *S. xylosus, L. plantarum* and mixed starter culture. Beyond the 14th day, the number of LAB decreased slightly (Figure 1) due to the exhaustion of the sugar (Fernandez-Lopez et al., 2008).

Staphylococci are the dominant microflora next LAB in both control and inoculated sausages. This result is in agreement with many other studies reporting that LAB

and staphylococci are the dominant microflora of dry fermented sausages (Casaburi et al., 2008; Rubio et al., 2007). The numbers of staphylococci remain significantly higher in sausages respectively inoculated with *S. xylosus* and mixed starter culture compared to those measured on control samples and samples inoculated with only *L. plantarum* strains. The number of staphylococci increased in control samples and sausages inoculated with *L. plantarum* during the two first weeks of
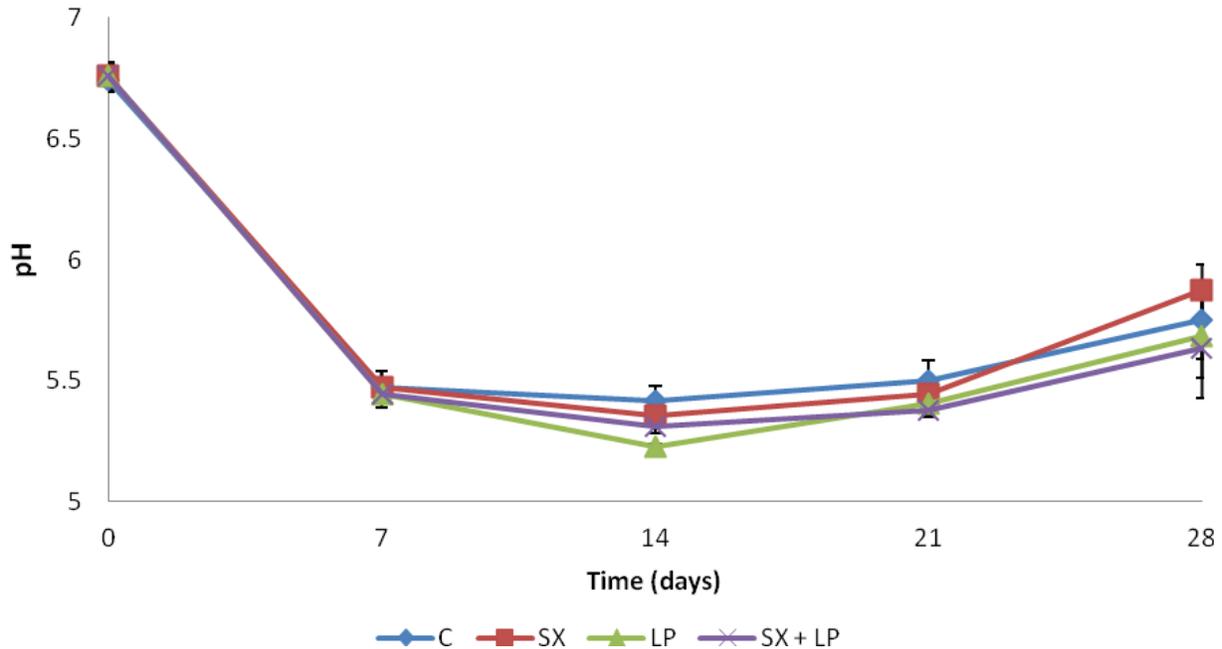
Figure 2. Evolution of pH during the ripening of control and inoculated dry fermented sausages: C (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

ripening to reach, respectively at 14th day 6.72 and 6.65 log CFU/g against 7.02 and 6.82 log CFU/g, values measured on samples inoculated respectively with *S. xylosus* and mixed starter culture (Figure 1). The number of staphylococci decreased steadily at the end of ripening and this for all samples of sausages. This inhibition of staphylococci is due to the decrease of pH caused by lactobacilli as reported by other authors (Johansson et al., 1994; Lizaso et al., 1999; Samelis et al., 1998).

The number of *Enterobacteriaceae* decreased during the ripening phase of control and inoculated dry fermented sausages (Figure 1). In fact, during the ripening of sausages inoculated with *L. plantarum* and mixed starter culture, this group showed a strong decrease (99.9%) and reached the level of 1.87 and 1.57 log CFU/g, while, in the control samples and samples inoculated with *S. xylosus,* this group reached the level of 3.39 and 3.24 log CFU/g at the end of ripening (p < 0.05). Our results are in agreement with other studies reporting that the sausages inoculated with starter cultures have the lowest number of *Enterobacteriaceae* than in control ones (Casaburi et al., 2008; Papamanoli et al., 2003; Rubio et al., 2007). The reduction of the number of viable cells of *Enterobacteriaceae* is attributed first of all, to the acidification activity of LAB that plays an important role in the inhibition of spoilage and pathogenic microorganisms (Bronomo et al., 2008; Deumier and Collignan, 2003) and secondly, to the antimicrobial compounds excreted by LAB (Ammor and Mayo, 2007; Deumier and Collignan, 2003).

Finally, the number of yeasts and molds increased steadily during the first seven days of maturation and this for all samples of sausages. Then, their concentrations decreased especially in the last two weeks of ripening to reach at 28th day values of 3.03, 2.65, 2.55 and 2.91 log CFU/g, respectively for control samples and sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture (Figure 1). Our results are in agreement with those obtained by Casaburi et al. (2008) who found that the number of molds and yeasts in control and inoculated sausages increased during the first days of ripening and then underwent a reduction to reach at the end of maturation values between 10^2 and 10^3 CFU/g.

pH, moisture and weight loss

The pH values underwent a rapid reduction in control and inoculated sausages from 6.74 ± 0.05 and 6.76 ± 0.05 to reach after 14 days of ripening 5.42 ± 0,06, 5.36 ± 0.05, 5.23 ± 0.01 and 5.31 ± 0.03 respectively for control samples and sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture (Figure 2). This drop in pH is due to the development of lactic acid bacteria that convert sugar added into lactic acid. During the last two weeks of maturation, pH of control and inoculated samples increased gradually. This increase in pH is explained fist of all, by the reduction of the number of LAB due to the exhaustion of the sugar and secondly, to proteolytic activity generated by microorganisms.
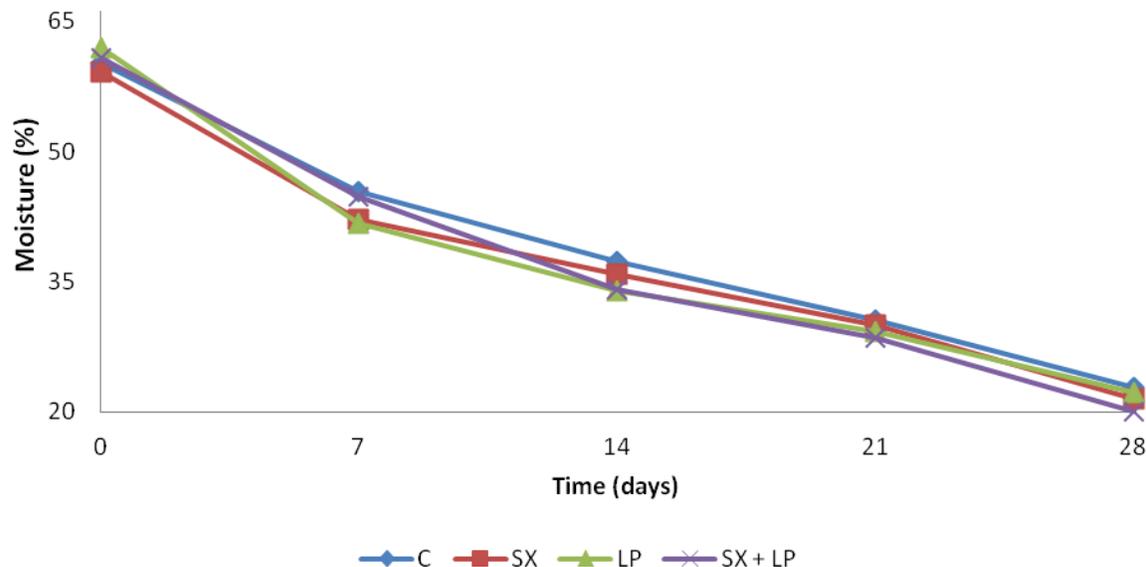
Figure 3. Evolution of moisture during the ripening of control and inoculated dry fermented sausages: C (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

Bacterial proteases induce proteolytic degradation, generating peptides, amino acids and amines which have a buffering effect on the organic acids produced by lactic acid bacteria during fermentation (Benito et al., 2007; Ruiz-Moyano et al., 2011).

Our results show that the sausages respectively inoculated with *L. plantarum* and mixture starter culture have a lower pH than in control sausages and sausages inoculate with *S. xylosus*. These results are in agreement with many other studies (Casaburi et al., 2008; Drosinos et al., 2005; Bozkurt et al., 2002) reporting that the pH of dry fermented sausage inoculated with lactic acid bacteria is lower than the pH of control sausage. The moisture content of control and inoculated dry fermented sausages decreased during the ripening phase (Figure 3). This water loss especially during the first two weeks of ripening is due first of all, to the elevated temperature of fermentation (24°C) that accelerates the drying of the product and secondly, to the decrease of pH of sausages to their isoelectric pH which causes protein denaturation and thus a decrease in water retention capacity of myofibrillar proteins (Solignat, 1999).

Our results are in agreement with those of other studies (Jin et al., 2010; Casaburi et al., 2008) reporting that the moisture content of a dry fermented sausages undergoes a decrease during the ripening period. The weight of control and inoculated sausages decreased during the ripening period (Figure 4).

This loss in weight is due to the temperature of fermentation (24°C) that accelerates the dehydration of the product. Our results are in agreement with other studies (Jin et al., 2010; Liaros et al., 2009) reporting that

the weight loss of dry fermented sausage increases during the ripening period.

Color measurement

The color development was significantly affected by the ripening time of sausages ($p < 0.05$) and not by the addition of starters ($p > 0.05$). The quality parameters L*, a* and b* values underwent a decrease through ripening period of different samples studied (Figure 5). Our results are in agreement with those found by Casaburi et al. (2007) and Olivars et al. (2010).

In relation to L* values, a decrease was observed during ripening, since sausage became darker due to weight loss (Olivares et al., 2010). With respect to a* values, a decrease was observed during the first two weeks of maturation, followed by a slight increase. The variation of the parameter color a* during ripening of dry fermented sausages is linked to the formation of a small amount of nitrosomyoglobin pigment (pink-red). Indeed chicken muscle has lower myoglobin content (Mielnik et al., 2002; Yilmaz et al., 2002).

Free amino acids content

To evaluate the effect of starter of *S. xylosus* and *L. plantarum* on proteolysis, free amino acids were determined through ripening using a chromatographic approach. The concentrations of total free amino acids during ripening are shown in Table 1. Total free amino
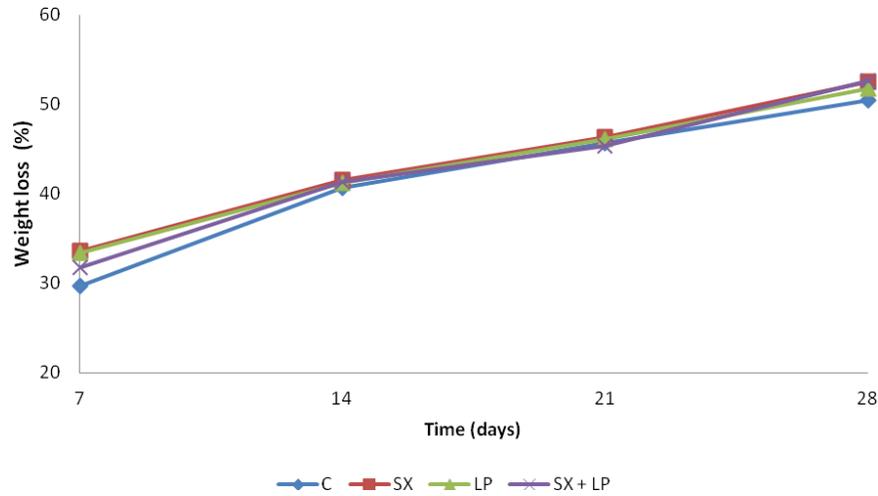
Figure 4. Evolution of weight loss during the ripening of control and inoculated dry fermented sausages: C (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).
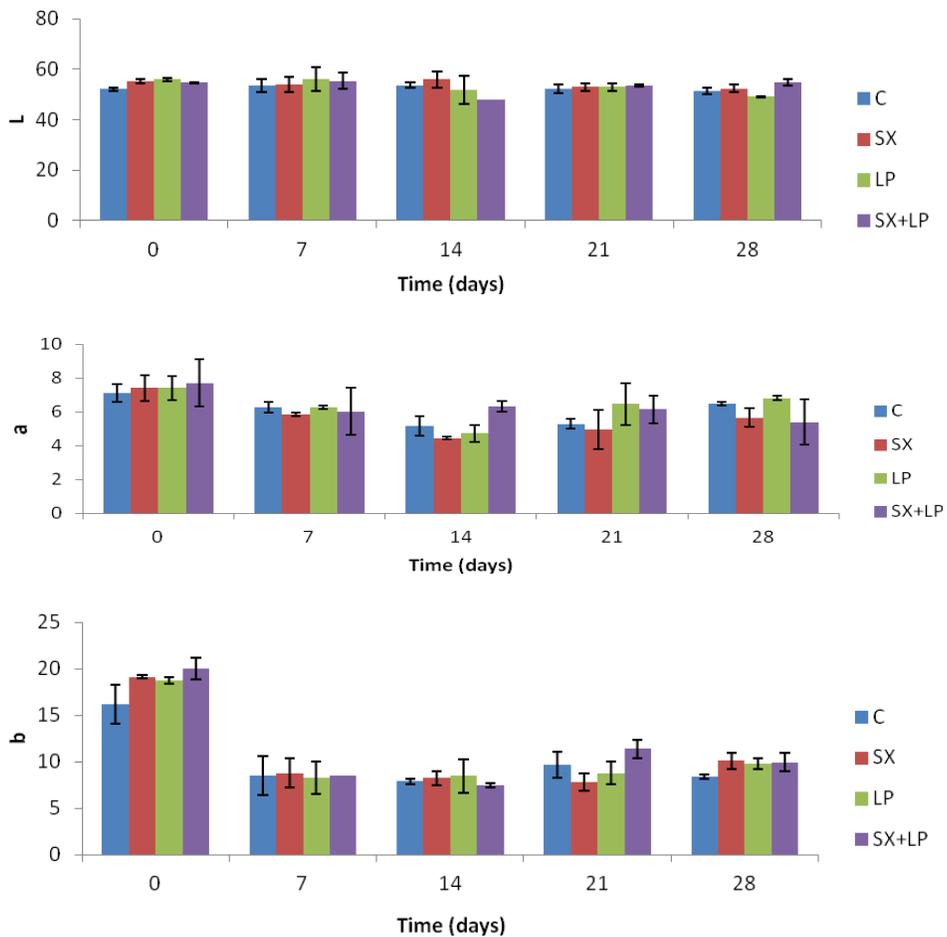

Figure 5. Evolution of L*, a*and b* values during the ripening of control and inoculated dry fermented sausages: C (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

Table 1. Free amino acids content (mg/kg) during the ripening of control and inoculated dry fermented sausages: C (control sausage), SX (sausage inoculated with *Staphylococcus xylosus*), LP (sausage inoculated with *Lactobacillus plantarum*), SX + LP (sausage inoculated with mixed starter culture *Staphylococcus xylosus* and *Lactobacillus plantarum*).

FAA (mg/kg)	Time (days)				
	0	28			
	C	C	SX	LP	SX+LP
Aspartic acid	109,343	75,104	106,299	132,328	99,522
Glutamic acid	4,787	109,082	131,731	58,884	129,499
Serine + histidine+glutamine	13,792	117,549	139,175	82,373	165,283
Arginine + glycine + threonine	130,150	409,206	454,428	386,513	593,840
Alanine	44,862	81,723	93,342	70,875	94,280
Valine + mèthionine + tryptophan	62,041	18,546	45,443	84,399	52,675
Lysine	19,936	3,039	0,482	19,839	8,296
Asparagine	83,860	56,140	0,000	0,000	94,035
Tyrosine	1238,607	1330,547	1603,881	1512,139	1417,612
Phenylalaline	100,284	180,758	187,488	275,640	213,081
Leucine	123,695	313,096	312,350	233,776	314,943
Isoleucine	128,465	251,535	258,091	420,664	278,008
Total	2059,822	2946,325	3332,71	3277,43	3461,074

acids content increased significantly during the ripening phase from 2059.82 mg/kg on day 0 to 2946.32, 3332.71, 3277.43 and 3461.07 mg/kg, respectively for control sausages and sausages inoculated with *S. xylosus*, *L. plantarum* and mixed starter culture. Our results are in agreement with those of many other studies (AroAro et al., 2010; Candogan et al., 2009; Casaburi et al., 2008; Lorenzo and Franco, 2012) reporting an increase in total free amino acids content during ripening of sausages. The hydrolysis of meat proteins generates polypeptides that can be further degraded to smaller peptides and free amino acids, this degradation can be produced by endogenous and microbial enzymes as reported by different authors (AroAro et al., 2010; Hughes et al., 2002). The concentrations of total free amino acids during ripening are higher in sausages inoculated than in control samples. This difference in the evolution of total free amino acids are related to the proteolytic activities of endogeneous and microbial enzymes activated by the decrease in pH of the medium (pH_{op} = 4.5 - 5.5) and by the decrease in temperature (T_{op} = 15 - 20°C) (Casaburi et al., 2008).

At the end of the ripening phase, sausages inoculated with *L. plantarum* had the highest concentrations of aspartic acid, valine, methionine, tryptophan, lysine, phenylalanine and isoleucine. While, the highest levels of serine, histidine, glutamine, arginine, glycine, threonine, alanine and leucine were observed in the samples inoculated with the mixed culture. Sausages inoculated with only *S. xylosus* had the highest concentrations of glutamic acid and tyrosine. Moreover, aspartic acid, glutamic acid, glycine, threonine, alanine, phenylalanine, serine, histidine, glutamine, tyrosine, leucine and isoleucine were the dominant amino acids in the four dry

fermented sausages. These amino acids play an important role in development of characteristic taste and flavor of the final product (Lorenzo and Franco, 2012; Casaburi et al., 2008).

Conclusion

The use of starter cultures can improve the safety of the dry fermented sausages by the inhibition of spoilage and pathogenic microorganisms. The number of enterobacteria decreased significantly in sausages previously inoculated respectively with *L. plantarum* and with a mixed starter cultures, due to the acidifying and antibacterial activities of lactic acid bacteria. The moisture content of control and inoculated sausages decreased in all stages of ripening which allows a good preservation and consequently, improved their shelf life.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGMENTS

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