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Food Microbiology 25 (2008) 335–347

FOOD MICROBIOLOGY

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Proteolytic and lipolytic starter cultures and their effect on traditional fermented sausages ripening and sensory traits

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Received 25 July 2007; received in revised form 14 September 2007; accepted 1 October 2007
Available online 5 October 2007

Abstract

In this study, three starter formulations including *Lactobacillus curvatus* and *Staphylococcus xylosus* strains selected *in vitro* on the basis of their lipolytic and proteolytic activities were employed for the manufacture of traditional fermented sausages of southern Italy.

Microbial population, proteolysis, lipolysis, changes in free amino acids (FAA) and free fatty acids (FFA) and development of characteristic taste and flavor of the final product were investigated. Proteolysis and lipolysis were observed in sausages inoculated with proteolytic and lipolytic *S. xylosus* coupled with *L. curvatus*, while the sausage started with only *S. xylosus* without lactobacilli was identical to the non-inoculated control, indicating that the proteolysis could be due to both microbial activity and endogenous proteases activated by the decrease in pH. The statistical analysis applied to the instrumental and sensory data showed that there was an effect of the starter used on the characteristics of the sausage obtained. In particular, the control samples showed very close features different from the sausages obtained by adding starter cultures. Finally, analyzing the sensory parameters the sausages ripened without starter addition and those started without the *L. curvatus* AVL3 showed similar features indicating an influence of the presence of the lactobacilli on the final organoleptic quality of the sausages. An appropriate choice of a combination of strains in a starter formulation is fundamental to obtain products of the expected quality.

Keywords: Fermented sausage; Starter cultures; Proteolysis; Lipolysis; Sensory traits

1. Introduction

In Italy, there are many different types of natural fermented sausages and almost all of them are only known at local or regional level. In the Campania region (southern Italy), a traditional fermented sausage called "*salsiccia* of Vallo di Diano" is produced. This kind of dry cured sausages is obtained from fresh pork meat and lard mixed with sugar, NaCl, black pepper, red wine, fennel seeds and sweet and hot chili pepper powder. Its formulation is characterized by the absence of additives such as nitrate and nitrite and their safety and ripening are assessed by fermentation. The fermentation is a crucial phase of the curing process of sausages, since at this stage the major physical, biochemical and microbiological transformations take place (Lizaso et al., 1999; Villani et al., 2007). These changes can be summarized as follows: decrease in pH, changes in the initial microflora, reduction of nitrates to nitrites and the latter to nitric oxide, formation of nitrosomyoglobin, solubilization and gelification of myofibrillar and sarcoplasmic proteins, proteolytic, lipolytic and oxidative phenomena and dehydration (Casaburi et al., 2007). All these transformations are influenced by ripening conditions, raw meat and ingredients and have a considerable effect on the organoleptic quality of fermented meat products. Breakdown products of lipolysis and proteolysis, i.e. peptides, amino acids, carbonyls and volatile flavor

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0740-0020/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2007.10.006

compounds contribute to the characteristic flavor and texture of fermented meats (Diaz et al., 1997; Demeyer et al., 1995; Fadda et al., 1999a). The pattern of the proteolysis in fermented sausages is influenced by several variables such as product formulation, processing condition and starter culture (Hughes et al., 2002). The respective roles of indigenous and bacterial enzymes in protein degradation during sausages ripening have been a source of controversy (Verplaetse et al., 1989). Proteolysis in muscle meats is due to the action of the cathepsins, particularly cathepsin D, that is responsible for the breakdown of sarcoplasmic and myofibrillar proteins, while microbial enzymes are more important during the latter stages of ripening (Molly et al., 1997; Hugas and Monfort, 1997; Lizaso et al., 1999; Sanz et al., 1999; Hughes et al., 2002). Proteolytic activity on meat proteins has also been described *in vitro* for strains of lactic acid bacteria (LAB) and staphylococci (Fadda et al., 1999a, b; Rodríguez et al., 1998; Mauriello et al., 2002, 2004; Casaburi et al., 2005; Drosinos et al., 2007) leading to the hypothesis that both endogenous and bacterial peptidases are required for complete hydrolysis of oligopeptides. Therefore, the activity of these enzymes could be strongly involved in the quality of the final product.

Lipolysis, together with proteolysis, is believed to play a central role in aroma formation. This phenomena is only the first step in the process and is followed by further oxidative degradation of fatty acids into alkanes, alkenes, alcohols, aldehydes and ketones (Viallon et al., 1996; Chizzolini et al., 1998) that enhances the development of the flavor. In fact, medium and long-chain fatty acids act as precursors of aroma compounds whereas the short-chain fatty acids ($C<6$) lead to strong cheesy odors (Ansorena et al., 2001). Although some authors (Molly et al., 1997; Kenneally et al., 1998; Galgano et al., 2003) have concluded that tissue lipases are primarily responsible for lipolysis during the fermentation, numerous studies over the last decade described lipolytic bacteria, especially staphylococci (Hugas and Monfort, 1997; Montel et al., 1998; Mauriello et al., 2004). Hugas and Monfort (1997) highlighted the need to use selected strains of gram-positive, catalase-positive cocci to ensure sensory quality of fermented sausages. Moreover, Stahnke et al. (2002), Becke et al. (2004) and Olesen et al. (2004) described the capability of *Staphylococcus xylosus* and *Staphylococcus carnosus* strains to modulate the aroma through the conversion of amino acids and free fatty acids (FFA). Strains of *S. xylosus* have been recommended for the production of very aromatic sausages of southern Europe (Samelis et al., 1998). Therefore, the use of well-selected strains with lipolytic and/or proteolytic activity, able to generate high amounts of aroma components, could allow achieving improved sensory quality.

In this study, three starter formulations were employed: (i) the proteolytic against myofibrillar proteins but low lipolytic (prt S^-M^+, lip^+) *S. xylosus* FVS23 and the *Lactobacillus curvatus* AVL3 (starter S3), (ii) the *S. xylosus* CVS14 (prt S^+M^-, lip^{++}) and the same strain of *L. curvatus* (starter S4) and (iii) the *S. xylosus* DVS4 (prt S^+M^-, lip^-) alone (starter S5). The aim of this work was to investigate the effect of the three different starter formulations on the proteolysis, lipolysis, changes in free amino acids (FAA) and FFA and on the development of characteristic taste and flavor of the final product.

2. Methods

2.1. Bacterial strains and culture conditions

The *S. xylosus* and *L. curvatus* strains used in this study are listed in Table 1. *S. xylosus* strains were isolated and identified as previously reported (Blaiotta et al., 2003; Casaburi et al., 2005). Working cultures were grown overnight at 37 °C in Trypton Soy Broth (Oxoid) supplemented with 0.5% yeast extract and maintained on P-agar, Phillips and Nash (1985) slants stored at 4 °C. The strain of *L. curvatus* AVL3, was cultivated in MRS broth and maintained at −80 °C in 15% (v/v) glycerol. The strains were studied *in vitro* to evaluate the proteolytic and lipolytic activities as previously described (Casaburi et al., 2005).

2.2. Sausages manufacture

Dry fermented sausages were manufactured at a meat processor in Vallo di Diano. The sausage formulation

Table 1
Strains used in this study and their proteolytic and lipolytic activities

Species	Strains	Origin[a]	Lipolytic activity[b]	Proteolytic activity on sarcoplasmic/myofibrillar proteins[c]
S. xylosus	FVS23	DSA	6.0	0/10
	CVS14	DSA	23.3	12/0
	DVS4	DSA	0	12/0
L. curvatus	AVL3	DSA	0	12/0

[a]DSA, Dipartimento di Scienze degli Alimenti, Università degli studi di Napoli "Federico II".
[b]Percentage of oleic acid.
[c]Proteolytic activity was checked by the measurement of the clear zone (mm) surrounding the wells, (Casaburi et al., 2005). The values are the mean of three independent assays.

(500 g of meat for each sausage) included 73% lean pork, 23.8% fat, 2.6% NaCl, 0.02% black pepper, 0.2% glucose, 0.1% red wine, 0.03% fennel seeds, 0.25% sweet and hot chilli pepper powder. After chopping and mixing the ingredients, the mixture was divided into following four batches: batch CG2, control without inoculation; batch S3, inoculated with proteolytic and low lipolytic strains of *S. xylosus* FVS23 and *L. curvatus* AVL3; batch S4, inoculated with proteolytic and lipolytic strains of *S. xylosus* CVS14 and *L. curvatus* AVL3 and batch S5, inoculated with the proteolytic strain of *S. xylosus* DVS4 (Table 1). All the samples were inoculated with about 10^7 viable cells per gram of sausages.

Subsequently, the sausage mixture was stuffed into a natural casing and placed in a fermentation chamber. The sausages were kept at 23 °C and 95% relative humidity (RH) for 4 h and 50% RH for 6 h. After a pause at 15–23 °C and 85–90% RH for 4 h, the sausages were dried at 23 °C and 90–95% RH for 3 h, at 50% RH another 3 h and subjected to a further pause step. They were then dried at 20–18 °C and 65–60% RH for 3 days and finally ripened at 14 °C and 76% RH for 28–66 days up to moisture content of 23–34%.

Two sausages in each trial were taken during the ripening (after 0, 6, 10, 20 days and at the end of ripening) to analyze proteins, amino acids, total lipid content, FFAs, pH, water activity, moisture, color and microbial population. All the analyzes were carried out in triplicate.

2.3. Microbial analyzes

Sausage samples (duplicates of 10 g) of each batch were collected aseptically, transferred to sterile plastic pouches, 10-fold diluted with sterile quarter-strength Ringer's Solution (Oxoid) and homogenized for 90 s using a Stomacher 400 (Seward, London UK). Serial 10-fold dilutions were prepared in sterile quarter-strength Ringer's Solution (Oxoid) and inoculated (in triplicate) in appropriate growth media. Mesophilic LAB were enumerated on MRS Agar (Oxoid) in anaerobic conditions after 48 h at 30 °C; staphylococci on Mannitol Salt Agar (MSA, Oxoid) after 48 h at 30 °C; yeasts and molds on Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Oxoid) after 4 days at 25 °C, *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBG, Oxoid) after 24 h at 30 °C and Enterococci on Slanetz and Bartley Medium (SBA; Biolife, Milan, Italy) after 48 h at 37 °C.

2.4. Physical and chemical analyzes

Measurement of pH was performed with a Mettler Toledo GmbH pH meter (mod. MP200) in a homogenate prepared with an aliquot of sausage (10 g) 10-fold diluted with sterile quarter-strength Ringer's Solution. Water activity (a_w) was measured using a Hygropalm Rotronic mod. HW3 (International PBI Milano, Italy) at 25 °C. To determine moisture, drying of a homogenous mixture of the samples with sand and ethanol at 103 ± 2 °C was done until constant weight (ISO, 1973). The measurements were performed in triplicate. In order to evaluate the sausage color, the surface color of sausage slices (CIE L^*, a^* and b^* values) was measured using a tristimulus colorimeter (Minolta Chroma Meter, model CR-300, Osaka 541, Japan) having a circular measurement area (D) of 8 mm. The colorimeter was calibrated using a white standard plate ($L = 100$). Six readings were carried out on each slice. Only the lean color was measured, and care was taken to avoid any fat.

Sarcoplasmic and myofibrillar proteins were extracted as described by Mauriello et al. (2002) and the protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad) using Bovine serum albumin as standard. Proteins were analyzed by SDS PAGE according to Laemmli (1970) using the Mini Protean III (Bio-Rad) electrophoresis equipment at the same conditions described by Casaburi et al. (2007).

Free amino acids were extracted as previously described (Casaburi et al., 2007) and analyzed by reverse phase HPLC in a Water Novapak C18 column (300 × 3.9 mm), as described by Toldrá et al. (2000).

The lipids were extracted according to Bligh and Dyer (1959) and the FFA composition was determined as reported by Navarro et al. (1996) by means of gas chromatography of their corresponding methyl esters.

2.5. Sensory analysis

The sausages were submitted to sensory evaluation in order to determine if differences exist between control sample (CG2) and samples inoculated with starter cultures (S3, S4 and S5). Eight judges participated in the panel for the evaluation of the quantitative descriptive profile of the samples. They were selected for their sensory ability and their previous experience in performing sensory profiling on other sausages inoculated with starter cultures (Casaburi et al., 2007).

Samples, consisting of three thin slices of sausages made 2 mm thick by means of a slicing electric machine and equilibrated for 1 h at room temperature, were served monadically and in white plastic plates, identified by three random digit codes. During each session, all the four samples were evaluated; tasted in a randomized design with three replications. Two appearance, two taste, three flavor and four texture attributes were evaluated, by using continuous unstructured scales (10 cm) without references. All sessions were conducted at 20–22 °C in an eight-booth sensory panel room equipped with white fluorescent lighting. Sensory data were collected by means of "FIZZ Acquisition" software (Biosystèmes, Couternon, France).

2.6. Data analysis

Microbiological data were shown as mean values ± standard deviation. Chemical, physical and sensory data

were analyzed by means of one-way ANOVA and Duncan's test ($p \leqslant 0.05$) (SPSS v.13.0).

In order to analyze chemical, physical and sensory data by means of the principal component analysis, the samples (indicated as CG1, S1 and S2) analyzed in a previous work (Casaburi et al., 2007) were also added to our samples (CG2, S3, S4 and S5). Chemical, physical and sensory data were correlated by using partial least squares regression analysis. PCA and PLS were carried out by using SIMCA-P 10 statistical software (Umetrics, Sweden).

3. Results

3.1. Moisture, pH and a_w

In both started and control samples, the final product reached moisture values of 23–34% while the a_w ranged between 0.80 and 0.83.

The pH underwent a rapid reduction in sausages inoculated with starters S3 and S4 while in control samples and in sausages started with starter S5, the pH remained constant during the ripening.

At the end of ripening, samples S3 and S4 showed pH values (5.42 and 5.64, respectively) that were not significantly different, and the same happened for pH values of samples CG2 and S5 (5.93 and 5.96, respectively). However, statistical analysis showed that all the samples were significantly different ($p = 0.001$). In addition, in samples CG2 and S5 the pH never decreased under values of 5.5 during the whole fermentation (data not shown).

3.2. Microbial population

In order to evaluate the capability of the different starter strains to compete with the authochtonous microbial flora of the fermented sausages of Vallo di Diano, viable counts were performed during fermentation of started and control sausages. The results of viable counts of staphylococci *Enterobacteriaceae*, lactobacilli, enterococci, yeast and molds, are reported in Fig. 1.

In samples inoculated with starters S3 (*S. xylosus* FVS23 and *L. curvatus* AVL3) and S4 (*S. xylosus* CVS14 and *L. curvatus* AVL3), the number of LAB increased from values of 10^7 CFU/g at time zero, to about 10^9 CFU/g at the end of ripening. By contrast, in sausages inoculated with the starter S5 containing only *S. xylosus* DVS4, the initial LAB concentration is about 10^4 CFU/g, reaching levels of 10^8 CFU/g at the end of ripening (Fig. 1). The staphylococci were shown to increase in number in control samples CG2 and in sausages inoculated with the starter S5 while in sausages samples started with S3 and S4, the viable counts of staphylococci was lower than the control even though the initial value was about 10^6 CFU/g (Fig. 1).

The number of *Enterobacteriaceae* reached 10^5 CFU/g at the end of ripening in control samples and in sausages inoculated with the starter S5 containing only *S. xylosus* DVS4. However, in sample started with starter S3 and S4 the enterobacteria were kept between 10 and 10^3 CFU/g and decreased of 2 log cycles compared to the initial value in samples started with S4 (Fig. 1).

The final number of yeast and molds was about 10^2 CFU/g in control samples and in sausages started with S3 and S5 while their load reached values higher than 10^3 CFU/g in samples inoculated with the formulation S4.

Enterococci increased during the first 3 days of ripening in all the samples and maintained their load constant for the rest of the process till the end of ripening, reaching values between 10^4 and 10^5 CFU/g in sausages inoculated with starters S3 and S4 while they reached higher values in samples CG2 and S5 with a final load of almost 10^8 CFU/g in S5 (Fig. 1).

3.3. Proteolysis

Myofibrillar and sarcoplasmic protein degradation by the different starter formulations was assessed by SDS-PAGE. The electrophoretic profiles of sarcoplasmic proteins extracted during ripening of the sausages are shown in Fig. 2. A complete degradation of the protein fraction of 40 kDa could be noted after 10 days of ripening of the control sample CG2 while the band at 97 kDa was present until the end of the ripening. The electrophoretic profiles of the sarcoplasmic proteins extracted from the sausages inoculated with the starter S5 were identical to the control while the profiles of the samples started with S3 and S4 are identical and different from the control (Fig. 2). After 6 days of ripening (data not shown), sarcoplasmic profiles of sausages inoculated with starters S3 and S4 displayed a lack of the band of 97 kDa and a reduction of intensity of the bands in the range of 37 and 50 kDa. The latter fractions disappeared after 10 days of ripening and the profiles kept constant until the end of the ripening.

The electrophoretic profiles of myofibrillar proteins extracted during the ripening of the different samples are shown in Fig. 3 where a significant degradation of the actin and myosin fraction can be observed. The intensity of the protein band corresponding to the myosin heavy chain (MHC) decreased during ripening and the band disappeared completely after 10 days in samples started with S3 and S4 while in samples started with S5 and in the control sausages, the complete degradation of the MHC took place only at the end of the ripening (Fig. 3). The actin fraction was also affected during ripening especially in samples started with S4 where it decreased in intensity within the first 20 days of ripening and increased afterwards probably due to the release of the peptides arising from MHC degradation. By contrast, in control samples the actin fraction remained constant during the whole ripening (Fig. 3). The α-actinin (94 kDa) was completely degraded in all the samples, while the started sample S3 after 10 days of ripening also showed a reduction of intensity of the troponin T and the appearance of a fragment of about 32 kDa.

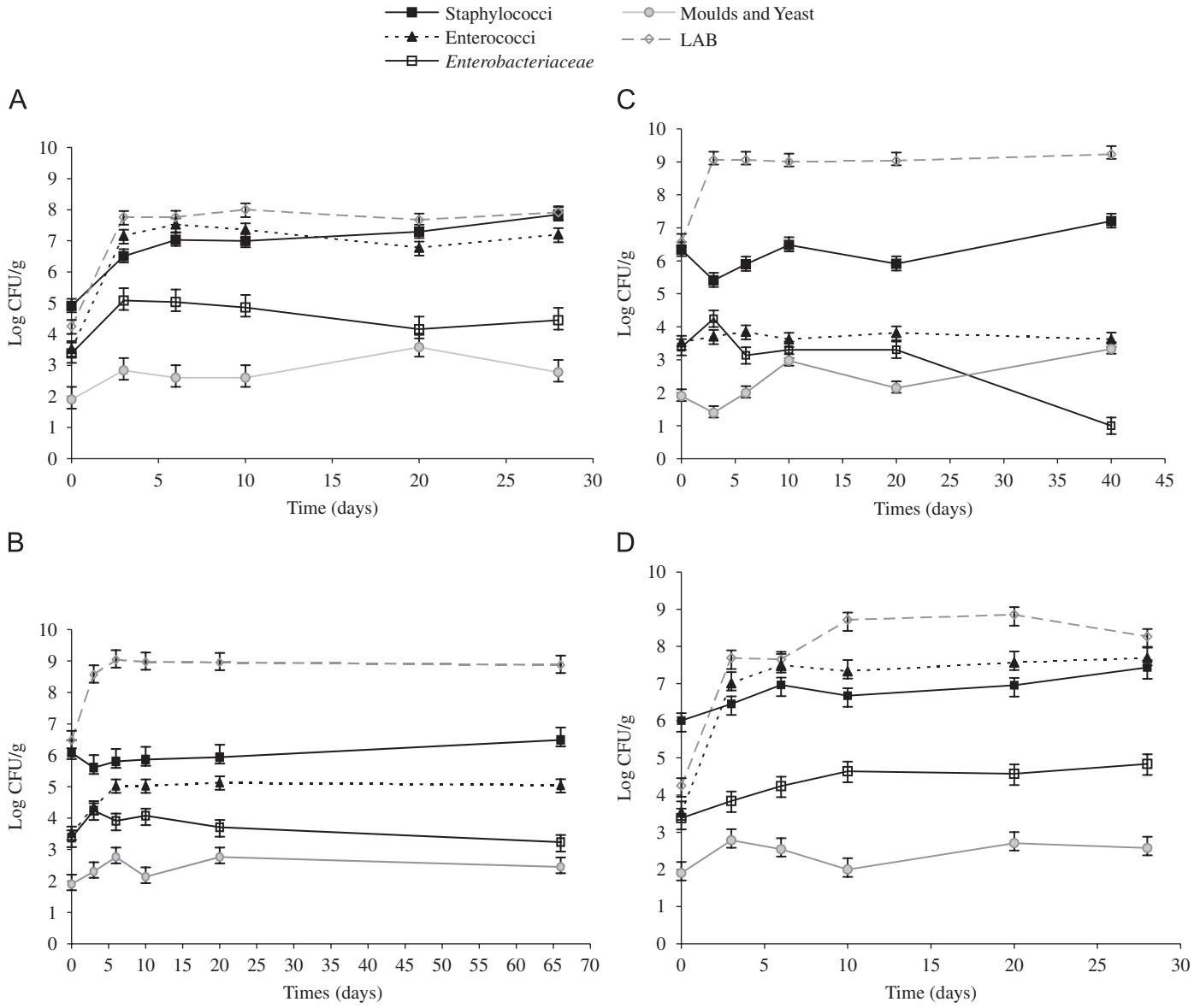

Fig. 1. Evolution of microbial population during the ripening of fermented sausages of Campania region (Vallo di Diano) with and without starter addition: (A) control sausage CG2 without starter cultures, (B) sausages inoculated with starter S3 (*S. xylosus* FVS23 and *Lactobacillus curvatus* AVL3), (C) sausages inoculated with starter S4 (*S. xylosus* CVS14 and *Lactobacillus curvatus* AVL3) and (D) sausages inoculated with starter S5 (*S. xylosus* DVS4).

3.4. Free amino acids

The chromatographic method used in this study allowed the detection of the following amino acids: aspartic acid, glutamic acid, serine, threonine, tyrosine, glutamine, asparragine, glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, histidine, arginine lysine, ornitine, β-alanine, taurine and two natural dipeptides, carnosine and anserine.

The identified amino acids and their concentrations determined during ripening of the fermented sausages are reported in Table 2. The main amino acids present in the initial mixture were glutamine, alanine and taurine with a concentration higher than 68.74 mg/100 g of dry matter (Table 2). At the end of the ripening, the S3 and S4 started samples showed an increase in total amino acids concentration with values ranging between 651.94 and 961.36 mg/100 g dry matter. In all the samples analyzed, the amino acids that determined the increase were mainly valine, leucine, isoleucine, phenylalanine, proline and alanine. The S3 started sausages showed a significant increase of all the above amino acids while the other samples showed differences only for some amino acids compared to the control. Total amino acids concentration during ripening of control CG2 and of the sausage inoculated with only *S. xylosus* DVS4 (S5) was constant even though differences in the single amino acids concentrations could be observed (Table 2).

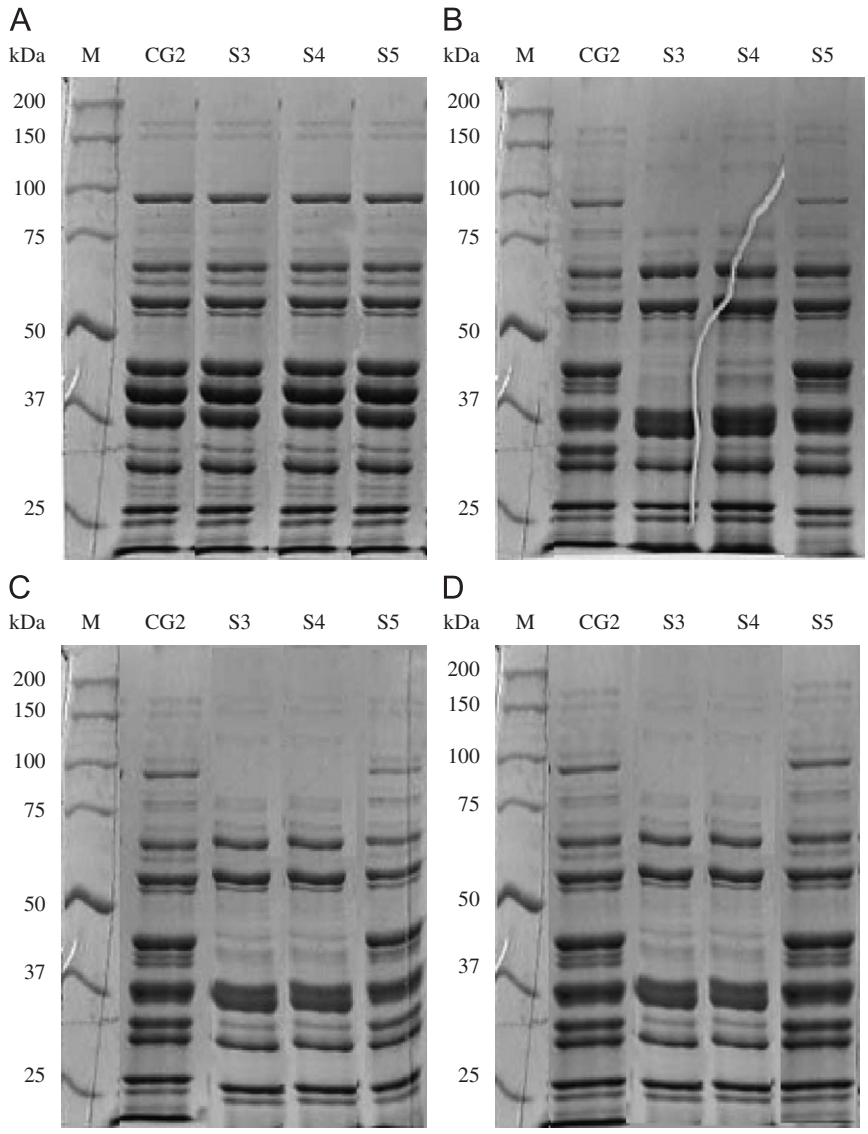

Fig. 2. SDS PAGE profile of sarcoplasmic proteins throughout the ripening of fermented sausages: (A) at time zero, (B) after 10 days of ripening, (C) after 20 days of ripening and (D) at the end of ripening. Lane M, Precision Plus Protein Standards; lane CG2, uninoculated sausages; lane S3, sausages inoculated with starter S3 (*S. xylosus* FVS23 and *L. curvatus* AVL3); lane S4, sausages inoculated with starter S4 (*S. xylosus* CVS14 and *L. curvatus* AVL3) and lane S5, sausages inoculated with starter S5 (*S. xylosus* DVS4).

3.5. Free fatty acids

Composition and quantity of saturated, unsaturated and polyunsaturated fatty acids determined during ripening of the different sausage samples are reported in Table 3. All the samples regardless of the starter addition showed an increase of FFA during ripening; oleic and linoleic acids showed concentrations of 622.8–1392.4 mg/100 g ($p \ll 0.001$) and 232.7–510.5 mg/100 g of dry matter ($p \ll 0.001$), respectively, at the end of the ripening. The monounsaturated fatty acids displayed concentrations higher than polyunsaturated and saturated in all the samples analyzed during ripening. At the end of the ripening, the samples started with S3 and S4 comprising *S. xylosus* strains with low and medium lipolytic activity, respectively, showed an increase of FFA compared to the control. By contrast, the sausages inoculated with only *S. xylosus* DVS4 displayed an FFA concentration lower than the control (Table 3). The sample started with S4 containing the *S. xylosus* CVS14, selected as capable to release the 20.6% of oleic acid *in vitro*, produced the highest percent of mono and the lowest of polyunsaturated fatty acids after 6 days of ripening while at the end of the ripening, it showed the same value found in sample S3 (data not shown). The total increase of FFA in the started samples S3 and S4 was due to oleic, linoleic and palmitic acids, although the evaluation of the single fatty acid percent value compared to the total content, confirmed an increase of the oleic acid only.

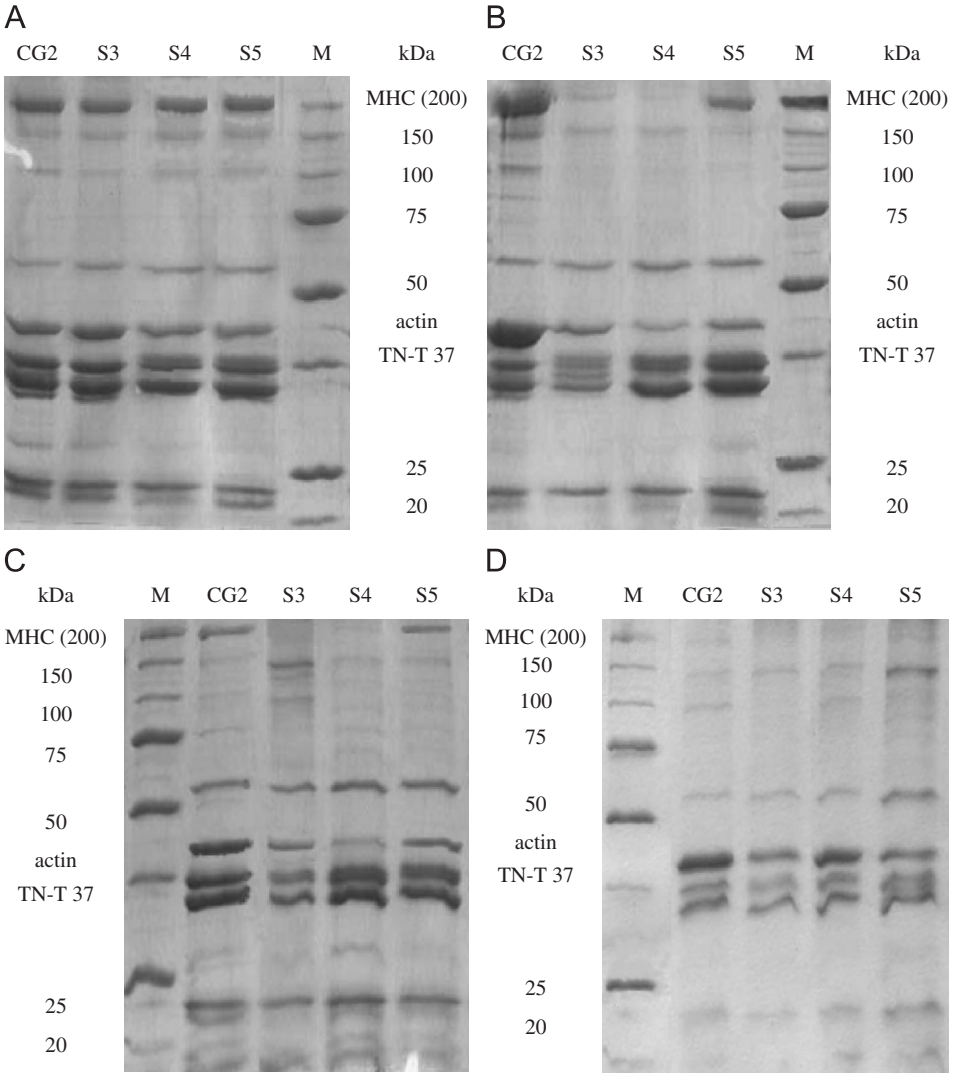

Fig. 3. SDS PAGE profile of myofibrillar proteins throughout the ripening of fermented sausages: (A) after 0 days of ripening, (B) after 10 days of ripening, (C) after 20 days of ripening and (D) at the end of ripening. Lane M, Precision Plus Protein Standards; lane CG2, uninoculated sausages; lane S3, sausages inoculated with starter S3 (*S. xylosus* FVS23 and *L. curvatus* AVL3); lane S4, sausages inoculated with starter S4 (*S. xylosus* CVS14 and *L. curvatus* AVL3) and lane S5, sausages inoculated with starter S5 (*S. xylosus* DVS4).

3.6. Principal component analysis of instrumental data

Free fatty acids, free amino acids, colorimetric coordinates, pH, water activity and RH were analyzed by means of PCA. Figs. 4a and b are the representations on the plane PC_1–PC_2 of the samples and their instrumental variables, respectively. The explained variance by the first two principal components was 72%.

As clear from Fig. 4, the samples showed different instrumental variables. In particular, four subgroups of sausages were found. One of them groups the control samples, CG1 and CG2, which were characterized by a high percentage of polyunsaturated FFA and showed a high concentration of the FAA ornitine and β-alanine. The sample S1 and the sample S2 were completely different from the other samples, they were characterized by the most part of FAA and have the highest RH. The sample S3 exhibited a high concentration of the FAAs tryptophan and taurine. The sample S4 and the sample S5 have opposite behavior compared to S1 and S2.

3.7. Sensory analysis

ANOVA results showed that all the sensory attributes discriminate among the samples CG2, S3, S4 and S5 except for the spices odor ($p > 0.05$) (Table 4) that was the same in all samples.

Figs. 5a and b are the representations of the samples and their sensory attributes, respectively, on the plane PC_1–PC_2. The descriptors evaluated by the panel were able to discriminate among the sausages and the first two principal components explain the 84% of the variance among the samples. Four subgroups of samples can be found in the plane PC_1–PC_2 of Fig. 5. In particular, control

Table 2
Changes in free amino acid (FAA) and natural dipeptides content during the ripening of different dry fermented sausages of Campania region (Vallo di Diano) with and without starter addition

Time (days)																	
FAA	0[a]	6				10				20				28	66	40	28
	CG2	CG2	S3	S4	S5	CG2	S3	S4	S5	CG2	S3	S4	S5	CG2	S3	S4	S5
Non essential FAA																	
Asp	1.73±0.55	0.57a	8.41b	5.28c	N.D.	0.93a	8.97b	4.84c	N.D.	1.72a	14.39b	3.79a	1.35a	0.53a	5.17b	4.20c	1.76a
Glu	25.99±1.4	14.78a	42.17b	29.22c	N.D.	14.27a	38.15b	23.51c	N.D.	21.42a	51.30b	18.16c	14.08d	27.45a	24.98a	23.10a	17.73b
Ser	17.25±0.8	18.67a	11.67b	9.94c	N.D.	15.10a	11.96b	11.06b	N.D.	14.67a	19.09 b	13.55c	12.22d	18.76a	26.88b	16.31c	16.28c
Asn	9.81±0.8	9.74a	13.65a	10.04b	N.D.	3.72a	13.25b	9.83c	N.D.	5.44a	17.31b	10.33c	7.36d	4.92a	12.19b	7.15c	8.03d
Gly	21.06±1.4	20.57a	30.93b	24.57c	N.D.	18.83a	29.98b	24.63c	N.D.	17.28a	39.59b	25.72c	16.65a	21.69a	42.68b	24.85c	27.0d
Gln	86.80±7.2	60.58a	58.49a	56.12a	N.D.	43.62a	49.00b	48.72b	N.D.	33.95a	40.76b	41.38b	39.29b	12.79a	6.11b	8.41c	12.35a
Ala	68.74±3.3	72.00a	85.32b	73.10a	N.D.	65.17a	84.95b	77.90c	N.D.	66.11a	106.3b	79.71c	65.90a	81.59a	127.46b	80.21a	85.22c
Arg	32.31±0.6	21.39a	30.92b	24.76a	N.D.	0.00a	33.69b	25.24c	N.D.	0.00a	42.11b	20.51c	0.00a	0.00a	29.33b	28.85b	22.91c
Pro	33.71±2.0	38.71a	41.84b	38.76a	N.D.	36.30a	39.08a	37.49a	N.D.	34.78a	46.93b	39.92c	37.05c	51.02a	73.51b	46.51c	47.11c
Tyr	15.64±1.7	2.90a	1.32b	1.00c	N.D.	1.29a	1.08a	0.94a	N.D.	1.27a	1.15a	0.84b	1.55c	1.95a	1.14b	0.60c	0.62c
Orn	1.05±0.1	6.35a	5.34b	1.56c	N.D.	1.88a	4.75b	1.79a	N.D.	7.53a	8.39b	1.58c	7.31a	5.26a	4.57b	1.36c	1.64d
Essential FAA																	
His	10.31±2.4	10.32a	14.08b	11.32a	N.D.	9.28a	13.60b	11.37c	N.D.	9.15a	18.88b	11.19c	10.40c	11.39a	19.28b	12.49c	11.26a
Thr	16.47±0.5	18.74a	27.02b	21.44c	N.D.	16.19a	26.66b	22.04c	N.D.	16.49a	36.08b	23.41c	17.71d	19.60a	41.17b	23.52c	24.08c
Val	16.48±1.1	23.00a	32.80b	27.72c	N.D.	25.66a	41.09b	33.55c	N.D.	25.99a	54.30b	36.62c	27.42d	38.94a	82.51b	47.36c	44.24ac
Met	11.65±1.5	13.16a	19.81b	16.81c	N.D.	13.49a	23.72b	18.77c	N.D.	12.62a	31.6b	20.05c	13.53d	13.90a	35.81b	23.69c	19.14d
Ile	15.63±1.5	22.39a	30.38b	25.25a	N.D.	24.25a	37.34b	31.26c	N.D.	24.29a	50.29b	35.21c	26.26a	26.97a	69.05b	43.87c	41.84d
Leu	26.28±3.0	38.10a	57.18b	48.43c	N.D.	42.17a	71.78b	61.73c	N.D.	42.71a	97.36b	68.10c	47.22d	48.41a	134.44b	85.94c	86.05c
Phe	17.34±3.1	23.25a	36.37b	30.31c	N.D.	27.41a	45.71b	36.31c	N.D.	27.50a	56.55b	38.01c	52.63d	27.39a	55.21b	34.72c	37.88c
Trp	4.49±1.0	5.82a	7.16b	6.72b	N.D.	6.98a	8.68a	7.53a	N.D.	6.88a	10.84b	8.59c	28.21d	8.02a	18.63b	13.68c	10.43c
Lys	22.74±5.2	15.7a	26.98b	19.16c	N.D.	13.18a	26.98b	22.95c	N.D.	12.44a	37.67b	23.07c	7.73d	20.65a	42.09b	27.54c	21.86a
Other FAA																	
β-Ala	6.66±1.7	4.78a	5.94b	5.00a	N.D.	4.07a	5.29b	4.97c	N.D.	3.65a	5.59b	4.53c	4.44c	3.76a	4.29b	3.38c	3.69a
Tau	120.56±2.6	111.36a	135.03b	130.05b	N.D.	95.15a	124.60b	123.19b	N.D.	87.40a	129.68b	119.58c	94.43d	87.37a	102.77b	94.20c	105.92d
Total[b]	582.7±10.1	552.9a	722.8b	605.24c	N.D.	480.3a	740.3b	639.6c	N.D.	475.34a	922.5b	643.87c	532.68a	533.27a	961.36b	651.94c	647.04c
Natural dipeptides																	
Car	766.59±47.0	454.5a	534.54b	430.01a	N.D.	322.25a	486.74b	382.34c	N.D.	367.72a	552.34b	280.40c	287.09c	370.06a	373.68a	325.39ab	296.74b
Ans	51.90±12.9	32.37a	42.58b	37.27c	N.D.	25.51a	37.35b	32.97b	N.D.	26.50a	40.34b	28.62c	29.11b	16.76a	16.62a	13.25b	16.25a

N.D., not detected.
For each time of ripening, values (a–d) within rows followed by different letters are significantly different (Duncan's test, $p \leqslant 0.05$).
[a]The results are expressed as mg/100 g dry matter and are means of three replicates ± standard deviations.
[b]Total FAA does not include natural dipeptides.

samples CG1 and CG2 were grouped together with the sample S5 that is the only one inoculated without *L. curvatus* AVL3; they resulted to be the greasiest samples and exhibited the most intense red color. S1 and S2 were the chewiest and sour samples and showed a less intensity of ripened flavor. S3 was perceived as the most rancid sample and was characterized by lower intensity of cohesiveness. S4 was the sweetest sample and showed the other sensory attributes with an intermediate intensity.

3.8. Relationships between instrumental parameters and sensory attributes

Instrumental parameters were used as predictive variables (X block) of sensory attributes (response variables, Y block) in a PLS model. Total variance explained by the first three extracted components was 93%, whereas predicted variance accounted for 81%. Table 5 shows the individual explained and predicted variance by the first three components of PLS analysis. Chewiness, cohesiveness of the slice and greasiness were the best-predicted sensory attributes, followed by red color and color uniformity. Rancidity was not well predicted by the model, whereas spices odor was not predicted at all.

Loadings plot and scores plot for the first two PLS components are shown in Figs. 6a and b.

The examination of Fig. 6b allows the finding of four subgroups of samples: the first one grouped the control samples CG1 and CG2, the second one was formed by S1 and S2, S4 and S5 made the third group, whereas S3 represented the last group.

The most influential instrumental parameters were identified for each sensory attribute (Fig. 6a). In particular, chewiness and sourness were positively related to the presence in the sample of the most part of FAA; chewiness and easy peeling capability were positively related to the RH of the samples and negatively to the pH and to the polyunsaturated FFAs. Cohesiveness is opposed to chewiness

Table 3
Changes in free fatty acid (FFA) during the ripening of different dry fermented sausages of Campania region (Vallo di Diano) with and without starter addition

Time (days)																	
FFA	0[a]	6				10				20				28	66	40	28
	CG2	CG2	S3	S4	S5	CG2	S3	S4	S5	CG2	S3	S4	S5	CG2	S3	S4	S5
C14:0	1.1±0.1	2.5a	3.7b	2.2a	N.D.	3.9a,b	3.2a	4.4b	N.D.	3.6a	7.0b	4.7c	5.0c	17.9a	35.4b	29.0c	13.1d
C16:0	32.4±1.2	44.9a	68.3b	54.3c	N.D.	67.0a	62.0b	71.1c	N.D.	63.3a	99.7b	82.5c	82.6c	243.1a	353.2b	366.2b	201.6c
C18:0	19.4±0.2	25.7a	35.0b	29.7a	N.D.	35.8a	34.9a	40.9b	N.D.	32.3a	58.0b	50.6b,c	42.8c	96.8a	119.8a,b	147.9b	98.7a
C16:1	3.2±0.2	6.3a	8.9b	7.5a,b	N.D.	9.6a	7.9b	8.6b	N.D.	8.5a	16.3b	11.6c	14.2b,c	44.6a	81.7b	70.2c	34.7d
C18:1	76.3±0.2	124.6a	183.3b	215.3c	N.D.	202.1a	173.0b	211.2c	N.D.	231.0a	310.4b	229.8a	248.5a	794.7a	1392.4b	1229.5c	622.8d
C20:1	1.3±0.2	1.9a	3.1a	5.7b	N.D.	3.5a,b	2.1a	5.3b	N.D.	3.1a,c	5.0b	4.6b,c	1.6a	15.1a	23.1b	22.8b	9.7a
C18:2	50.0±1.8	64.2a	86.9b	80.9c	N.D.	99.6a	80.9b	110.0c	N.D.	98.2a	122.2b	97.9a	113.0c	414.5a	510.5b	503.4b	232.7c
C18:3	2.9±0.04	3.3a	3.6a	3.8a	N.D.	4.8a	3.3b	5.2c	N.D.	4.8a	5.1a	3.9b	5.1a	19.9a	21.1a	22.1a	8.7b
C20:2	1.1±0.02	1.5a	2.8b	2.3b	N.D.	2.7a	3.1a	4.4b	N.D.	2.51a	5.6b	3.6c	2.6c	11.6a	20.8b	20.6b	7.2c
C20:3	0.8±0.1	0.9a	1.2a	1.1a	N.D.	1.2a	1.0a	1.6b	N.D.	0.9a	1.3b	1.1c	1.0a,c	4.9a	4.4a	4.1a	2.6b
C20:4	6.7±0.5	6.6a	9.9b	9.3b	N.D.	9.8a	9.0b	11.1c	N.D.	7.1a	8.9b	8.7b	8.3b	40.6a	38.6a	38.8a	20.7a
C22:4	0.8±0.1	1.0a	1.3a	1.1a	N.D.	1.3a,b	1.1a	1.6b	N.D.	0.8a	1.7b	1.0a	1.0a	6.1a	5.4a	3.9b	2.2c
SFA	52.9±1.3	73.2a	107.0b	86.2c	N.D.	106.7a	100.0b	116.5c	N.D.	99.1a	164.7b	137.7c	130.4c	357.8a	508.5b	543.1b	313.4a
MUFA	80.8±0.2	132.8a	195.3b	228.6c	N.D.	215.3a	182.9b	225.1c	N.D.	242.5a	331.8b	246.0a	264.2a	854.5a	1497.1b	1322.5c	667.2d
PUFA	62.1±2.5	77.6a	105.7b	98.4c	N.D.	119.5a	98.4b	133.9c	N.D.	114.3a	144.8b	116.2a	131.0c	497.6a	600.9b	593.1b	274.1c
Total FFA	195.8±5.7	283.6a	408.0b	413.2b	N.D.	441.5a	381.4a	475.4b	N.D.	455.9a	641.3b	499.9a,c	525.7c	1709.8a	2606.6b	2458.7b	1254.8c
PUFA/sat	1.17±0.03	1.06a	0.984a	1.14a	N.D.	1.12a	0.98b	1.15c	N.D.	1.15a	0.88b	0.84b	1.0c	1.39a	1.18a,b	1.09b,c	0.87c

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; N.D., not detected.
For each time of ripening, values (a–d) within rows followed by different letters are significantly different (Duncan's test, $p \leqslant 0.05$).
[a]The results are expressed as mg/100 g dry matter and are means of three replicates ± standard deviations.

and easy peeling capability. Greasiness was positively related to the pH and negatively to the RH, a_w and to the FAA content. The most predictive parameters of red color were the *a* and *b* chromatic coordinates and the polyunsaturated FFA content. In particular, samples that showed high values of *a* and *b*, were perceived by sensory judges as less red intense whereas when the polyunsaturated FFA content was high, an intense red color was noticed. Color uniformity was positively related to the monounsaturated FFA C16:1 and C18:1 content and negatively to the *L* coordinate. Sweetness and ripened flavor were related one to each other, negatively related to FAA content and to the saturated FFA content.

4. Discussion

In the Mediterranean countries, more efforts have been dedicated to the study of technological properties of LAB and staphylococci isolated from traditional fermented sausages (Garcia-Varona et al., 2000; Mauriello et al., 2004; Casaburi et al., 2005, Drosinos et al., 2007). However, recent literature data are lacking on the effect of starter cultures on the sensory characteristics of fermented sausages.

In this study, three different starter cultures were used to produce typical fermented sausages of Vallo di Diano. Moisture, pH, a_w and microbiological analyzes were evaluated on the raw meat and during the ripening of the sausages.

The constant pH values in the control sample CG2 and in the sample S5 started with the single strain *S. xylosus* DVS4 may be due to the presence of autochthnous LAB with low acidifying capability, while the decrease in pH of the other started samples can be explained by the use of the strongly acidifying *L. curvatus* AVL3 in the starter formulation. This is in agreement with our previous studies where the *L. curvatus* AVL3 was used as starter for fermented sausages (Casaburi et al., 2007).

The different starter cultures used influenced the ripening duration: the control and S5 samples reached the end of ripening after 28 days, while samples started with S3 and S4 could be considered as completely ripened after 66 and 40 days, respectively. This result is not in line with the study by Stahnke et al. (2002) who found that the addition of *S. carnosus* starter culture showed to decrease the maturation time of Italian dry sausages by more than 2 weeks. The viable counts showed an exponential growth of LAB in all samples and an inhibition of the staphylococci especially in the samples started with combination of lactobacilli and staphylococci. This inhibition 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). Therefore, the growth of staphylococci in the control sample CG2 and in started sample S5 was favored by the pH values that were never lower than 5.5. Moreover, the acidification by the *L. curvatus* AVL3 also determined a load reduction of the *Enterobacteriaceae* that are rarely found in long ripened fermented sausages because of their sensitivity to pH and water activity (Gonzalez and Diez, 2002; Zuber and Horvat, 2007). The viable counts of yeast and molds ranged between 10^2 and 10^3 CFU/g in agreement with other studies (Lizaso et al., 1999) while the number of the enterococci was comparable to the one reported in a previous investigation (Casaburi et al., 2007).

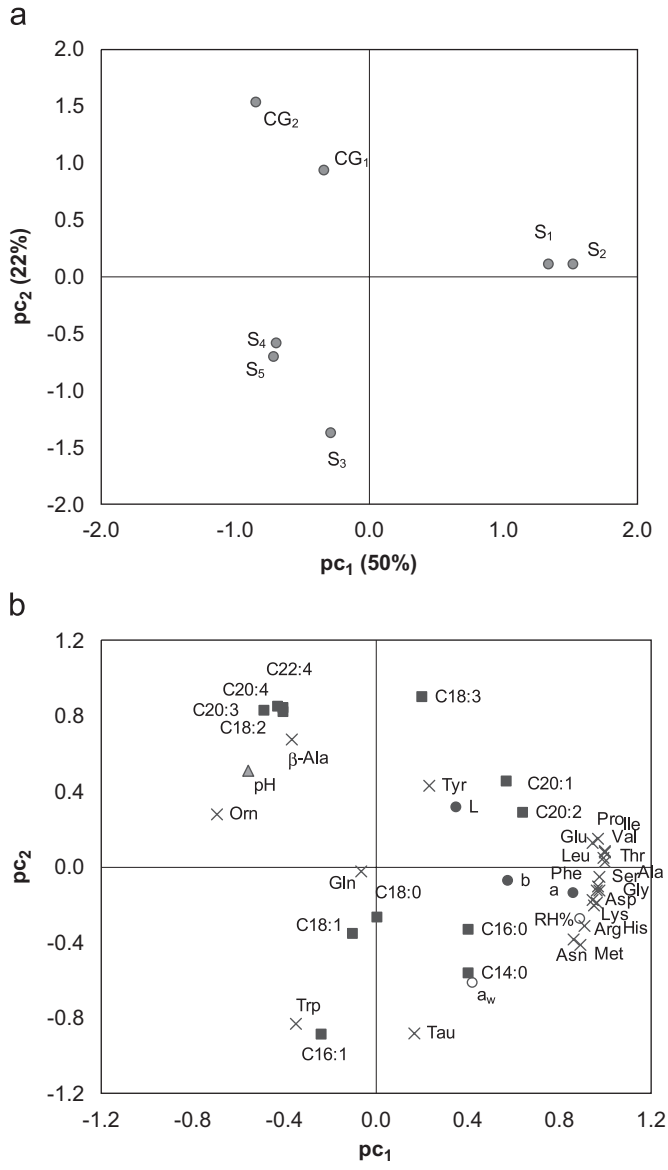

Fig. 4. Principal component analysis of instrumental data: (a) scores plot and (b) loadings plot.

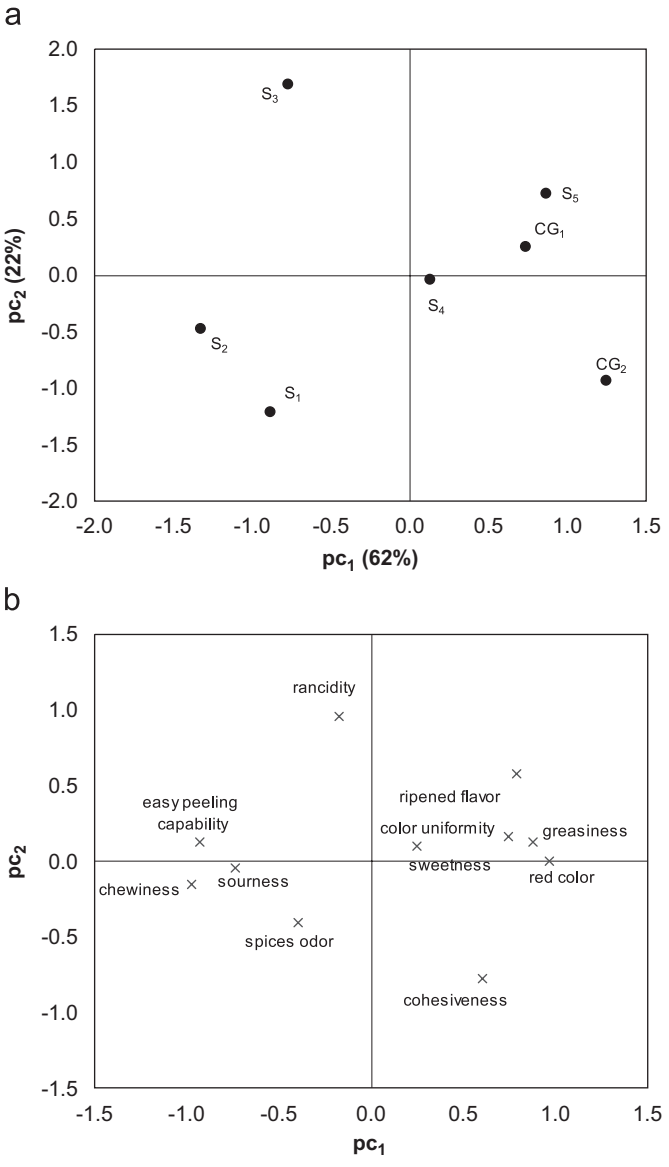

Fig. 5. Principal component analysis of sensory data: (a) scores plot and (b) loadings plot.

Table 4
Effect of starter cultures on sensory attributes evaluated by ANOVA

Sensory attribute	*p*-Values
Red color	⩽0.001
Color uniformity	0.002
Cohesiveness of the slice	<0.001
Greasiness	<0.001
Easy peeling capability	<0.001
Chewiness	0.001
Spices odor	0.053
Sweetness	0.001
Sourness	0.039
Ripened flavor	0.002
Rancidity	<0.001

Table 5
Partial least squares regression model overview

Sensory attribute	Explained variance (%)	Predicted variance (%)
Red color	93	81
Color uniformity	92	80
Cohesiveness of the slice	98	88
Greasiness	95	88
Easy peeling capability	95	79
Chewiness	98	90
Spices odor	54	−14
Sweetness	90	77
Sourness	89	71
Ripened flavor	80	30
Rancidity	82	62

The results of the proteolysis indicate that the protein degradation occurred during the first 2 weeks of ripening as reported by other authors (Beriain et al., 2000; Hughes

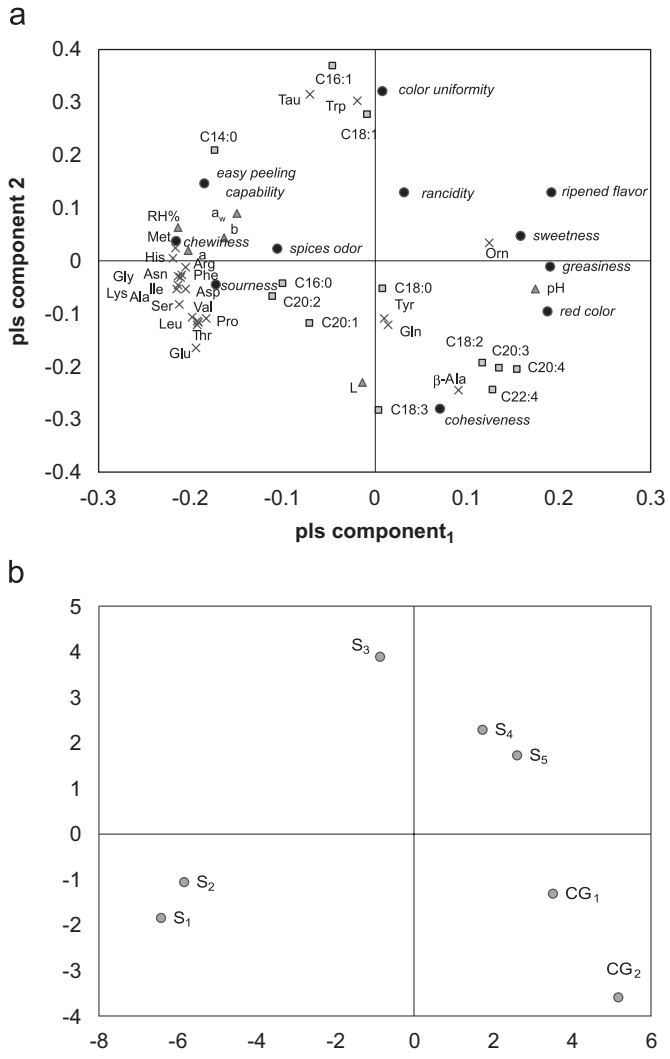

Fig. 6. (a) PLS loadings-plot of instrumental data (X-block): amino acids (×); free fatty acids (■); physical parameters (▲) and sensory attributes (Y-block) (●); (b) PLS scatter-plot.

et al., 2002). In fact, the protein profiles obtained after 10 days of ripening did not change further and were identical after 20 days of ripening. The sarcoplasmic protein profiles showed that the proteolysis occurring in samples S3 and S4 could be due to both microbial activity and endogenous proteases activated by the decrease in pH. This would also explain the lack of proteolysis in the control sample as previously described (Casaburi et al., 2007), where the higher pH values negatively affected the endogenous enzymatic activity (Zanardi et al., 2004).

The results of the myofibrillar protein degradation confirmed what reported for the sarcoplasmic proteins except for the sausages started with S3. The severe degradation of the MHC during ripening of all the samples is in agreement with other studies (Diaz et al., 1997; Toldrá, 1998; Hughes et al., 2002; Casaburi et al., 2007) describing the degradation of MHC during the ripening of fermented sausages with or without starter addition. The actin band confirmed to be a stable protein during the ripening of fermented sausages in agreement with previous results (Diaz et al., 1997; Toldrá, 1998). The analysis of myofibrillar proteins also confirmed that the strain *L. curvatus* AVL3 could not hydrolyze these proteins and that the proteolytic activity found in a previous work could be due to acidification and promotion of the endogenous proteases (Casaburi et al., 2007).

The lack of increase of total amino acids in the control sample CG2 is in disagreement with the results reported by other authors (Beriain et al., 2000; Bolumar et al., 2001) and could be due to the inactivity of the endogenous enzymes in the complex system as already observed (Casaburi et al., 2007). Moreover, Verplaetse et al. (1989) indicated a range of pH 4.5–5.5 and temperature of 15–20 °C as optimal conditions for the activity of endogenous enzymes. The increase of essential amino acids found in this study is in agreement with previous reports (Hughes et al., 2002; Casaburi et al., 2007) describing an increase of alanine, valine, leucine, isoleucine, proline and phenylalanine during the ripening of fermented sausages prepared with or without starter addition. The evolution of amino acid content in sample S3 was very different from the other samples highlighting a role of the bacterial rather than endogenous aminopeptidases in this effect, as already pointed out in other studies (Molly et al., 1997; Hughes et al., 2002; Bolumar et al., 2006). In fact, the pH of the sausages started with mixed cultures (S3 and S4) was always around 5.0 and thus the activity of the endogenous proteases would have led to aminoacidic profiles identical in the two fermented sausages S3 and S4.

The increase in FFA during ripening is consistent with the results reported by other authors who examined both started and non-started fermented sausages (Hernández et al., 1999; Galgano et al., 2003; Zuber and Hovart, 2007; Casaburi et al., 2007). This increase, according to Bolumar et al. (2001), is due to the monounsaturated fatty acids. Finally, the control samples showed the highest content of polyunsaturated FFA and clustered in a single group as shown by PCA analysis reported in Fig. 4.

The strains used in the starter formulations were selected and used for their proteolytic and lipolytic activities assayed *in vitro*. Overall, the biochemical analyzes showed that there was no correlation between the technological activities of the starter cultures and their effect on the biochemical characteristics of the fermented sausages. This is likely due to the discrepancy between the proteolytic and lipolytic activities assayed *in vitro* and *in situ* during ripening of fermented sausages as we recently demonstrated (Villani et al., 2007).

For the statistical analysis of the sensory and instrumental data, results obtained in a previous work were also considered (Casaburi et al., 2007). Therefore, the analysis included the following starter formulations: (i) *S. xylosus* CVS11 (prt S^+M^-, lip^-) with the *L. curvatus* AVL3 (starter S1) and (ii) the *S. xylosus* FVS21 (prt S^-M^-, lip^+) with the same strain of *L. curvatus* (starter S2). The

statistical analysis applied to the instrumental data showed that there was an effect of the starter used on the characteristics of the sausage obtained. Particularly, the control samples showed very close features different from the sausages obtained by adding starter cultures. The latter could be clustered in two groups (Fig. 4a), which differed for the types of FFA and FAA released (Fig. 4b); a higher amino acids content was found in sausages started with S1 and S2. The *Lactobacillus* strain used in S1, S2, S3 and S4 was the same while the starter cultures differed for the *S. xylosus* used; this is indicative of the fact that the *S. xylosus* strains were able to influence the ripening and the biochemical characteristics of the fermented sausages. Analyzing the sensory parameters, the sausages ripened without starter addition and those started without the *L. curvatus* AVL3 showed similar features indicating an influence of the presence of the lactobacilli on the final organoleptic quality of the sausages (Fig. 5). Indeed, the load of staphylococci was higher in controls and in sausages started without the *L. curvatus* AVL3 (Fig. 1) probably causing a more intense red color. A similar distribution was found in PLS plot where control and started samples were totally different; the S3 started sample was alone in one group and this was related to the rancidity; the sample was perceived as the most rancid (Fig. 6). This is in agreement with the reduced polyunsaturated FFA content in S3 started samples as can be observed in the plot of Fig. 6 where the S3 sample is opposed to the polyunsaturated FFA. This reduction could be due to oxidative phenomena that can lead to the release of aldehydes and other compounds that can cause a greasy, oily and rancid off-flavor (Gandemer, 2002).

In conclusion, the use of starter cultures can improve the safety of the traditional fermented sausages prepared without antimicrobial additives. An appropriate choice of a combination of strains in a starter formulation is fundamental to obtain products of the expected quality.

Acknowledgments

This work was supported by the Ministry of University, Rome, Italy; action PRIN 2004. M.I. Nadal and M.A. Aristoy are gratefully acknowledged for technical help with FFA and FAA analysis, respectively. We are grateful to the producer Santo Jacopo for collaboration.

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