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Identification of small peptides arising from hydrolysis of meat proteins in dry fermented sausages

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ABSTRACT

In this study, proteolysis and low molecular weight (LMW) peptides (<3 kDa) from commercial Argentinean fermented sausages were characterized by applying a peptidomic approach. Protein profiles and peptides obtained by Tricine-SDS-PAGE and RP-HPLC-MS, respectively, allowed distinguishing two different types of fermented sausages, although no specific biomarkers relating to commercial brands or quality were recognized. From electrophoresis, α-actin, myoglobin, creatine kinase M-type and L-lactate dehydrogenase were degraded at different intensities. In addition, a partial characterization of fermented sausage peptidome through the identification of 36 peptides, in the range of 1000–2100 Da, arising from sarcoplasmic (28) and myofibrillar (8) proteins was achieved. These peptides had been originated from α-actin, myoglobin, and creatine kinase M-type, but also from the hydrolysis of other proteins not previously reported. Although muscle enzymes exerted a major role on peptidogenesis, microbial contribution cannot be excluded as it was postulated herein. This work represents a first peptidomic approach for fermented sausages, thereby providing a baseline to define key peptides acting as potential biomarkers.

1. Introduction

There has been a renewed interest in traditional fermented meat products, mainly in Europe, where they have a great significance and economic impact (Vignolo, Fontana, & Fadda, 2010). A huge variety of dry fermented products can be found as a consequence of variations applied in raw materials, formulations and manufacturing processes, according to the habits of different countries and cultures.

The typical flavor of dry cured and fermented sausages is the result of a subtle balance between volatile (alcohol, ketones, aldehydes, furans) and non-volatile (amino acids, peptides, sugars, nucleotides) compounds. These molecules come from raw materials (meat, fat, spices, nitrites and other additives) and/or biochemical reactions during fermentation and ripening (Stahnke, 2002). Meat protein degradation is one of the main biochemical events which are catalyzed by enzymes that belong to meat (also called endogenous enzymes) or microorganisms. Meat proteins are known to undergo hydrolysis, first to polypeptides by endogenous muscle enzymes, such as cathepsins and calpains. Then, polypeptides release smaller peptides and amino acids by the action of peptidases and aminopeptidases from both muscle and bacteria. Low molecular weight peptides (<3 kDa) and free amino acids are major components of the non-protein nitrogen fraction in fermented meats. These compounds contribute, directly or indirectly, to the generation of volatile and non-volatile flavor compounds in dry and semi-dry sausages (Fadda, López, & Vignolo, 2010).

Prediction and/or characterization of the quality of fermented meat products is a difficult task since a high number of factors such as the type of raw materials, technologies and starter cultures are involved in the process. Analysis of proteins and peptides has a special interest because they play a major role in the end product quality. Recently, proteomic approaches have been applied to correlate proteolytic profiles with technological parameters in view to detect valuable biomarkers as meat quality predictors (Lametsch et al., 2003). Unlike proteomics, the novel concept of peptidomic aims at the systematic analysis of the small polypeptides content within an organism, tissue or cell (peptidome) in order to identify quantity, structure and function (Soloviev, 2010). Thus, this approach covers the mass range between proteomics and metabolomics. The pacemakers for the development of peptidomic technologies are modern mass spectrometry and bioinformatics. These tools are ideally suited for comprehensive peptide analysis, especially combined with the massive information available in today's genomic and transcriptomic databases. In contrast to proteomics, peptidomic has the potential to uncover cleavage sites of precursor proteins. But also, the peptide analysis should be performed on their native forms. Consequently, searching for peptides in databases of non-tryptic peptides is much less effective due to the lack of charge localization at the peptides N and C termini (Zürbig et al., 2006). Also, the poor fragmentation and the lack of specificity for intrinsic proteolytic enzymes are a problem for naturally occurring peptides analysis

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(Mischak, Julian, & Novak, 2007). However, global profiling approaches have been applied to the study and exploration of complex peptidomes in food science. For example, the identification and quantification of nutritionally relevant peptides (bioactive peptides) as well as the study of peptide fractions evolution, as in Parmigiano-Reggiano cheese samples (Lahrichi, Affolter, Zolezzi, & Panchaud, 2013; Panchaud, Affolter, & Kussmann, 2012; Picariello, Mamone, Addeo, & Ferranti, 2012).

Peptidomic analyses have a great potential for the determination of meat composition in processed foods due to the high stability of peptide material. Indeed, peptide fractions originated from meat have already been identified as biomarkers for meat tenderness, authenticity and sensory attributes (Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009; Ouali et al., 2013; Paredi et al., 2013; Sentandreu, Fraser, Halket, Patel, & Bramley, 2010; Sentandreu et al., 2007). To our knowledge, no substantial information about the characterization of peptides generated during fermentation of dry-cured sausages is available so far. The identification of the protein fragments naturally generated during sausage fermentation and ripening would be beneficial in order to better understand proteolysis and flavor development mechanisms that occur during the processing of fermented products. In this study, proteolysis of commercial Argentinean fermented sausages was characterized by a peptidomic approach in an attempt to evaluate their potential as biomarkers of technological and processing conditions. The role of bacterial enzymes responsible for the hydrolysis of meat proteins was also analyzed.

2. Materials and methods

2.1. Sausage sampling procedures

Ten fermented sausages (FS) of different commercial brands purchased from several local stores and supermarkets of Tucumán, Argentina were analyzed. The samples included three high comminuted salami (FS1, FS7 and FS8), five low comminuted salami (FS3, FS4, FS5, FS9 and FS10) and two fuet-type (FS2 and FS6) from industrial plants localized in northwestern and central regions of Argentina. Low and high comminuted salami refer to the degree of mincing of lean meat and fat used in the manufacture; fuet-type sausages are products with smaller diameter, less fermentation time and high pH. From the label, sausage formulation differed in meat composition; fuet-type were manufactured using pork meat while other samples included pork and beef meat (López, Bru, Vignolo, & Fadda, 2012) (Table 1). For sampling, at least three different batches from each commercial brand were analyzed. From each batch, 3 dry sausages without casings (approximately 500 g depending on the size of sausages) were pooled and thoroughly homogenized in order to obtain a representative sample. Subsequently, 20 g for SDS-PAGE analyses and 2.5 g for RP-HPLC analyses were taken from each batch of sausages, as described in items 2.2.1 and 2.3.1, respectively.

2.2. Protein hydrolysis

2.2.1. SDS-PAGE of sarcoplasmic and myofibrillar proteins

A portion of 20 g of three different batches of each sausage brand was selected for sarcoplasmic and myofibrillar protein extraction (Fadda et al., 1999). Bovine meat (muscle *Semimembranosus*) was used as a non-fermented control since SDS-PAGE profiles of meat proteins are not different among pork, beef and their mix at the post mortem time evaluated (Fadda et al., 1999; Fadda, Vignolo, Aristoy, Oliver & Toldrá, 2001, Sentandreu et al., 2010). Meat from Brangus animals with 9–12 months of age and half-carcass about 100–120 kg was obtained after 48 h post-mortem. Protein concentration of both sarcoplasmic and myofibrillar fractions was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) using bovine serum albumin as standard. It was further adjusted with deionized water to 0.5 μg/μl final concentration. Samples were diluted 1:1 with buffer (8 M urea; 2 M thiourea; 0.05 M Tris; 75 mM DTT; 3% SDS; 0.05% bromophenol blue; pH 6.80) and heated at 100 °C for 5 min prior to electrophoresis. Tricine-SDS-PAGE (17% acrylamide-bisacrylamide for the resolving gel and 3% acrylamide-bisacrylamide for the stacking gel) was performed using miniprotean II electrophoresis equipment (Bio-Rad, Hercules, CA) by loading 20 μl of each sample as well as Ultra Low and Wide Range molecular weight markers (Sigma-Aldrich, Buenos Aires, Argentina) (Schägger & Von Jagow, 1987). Electrophoresis was carried out at 50 and 70 V for stacking and resolving gel, respectively. Gels were fixed in 30% methanol, 10% acetic acid and stained with Sypro Ruby Gel Stain (Sigma-Aldrich, Buenos Aires, Argentina). Two technical repetitions for each batch were performed for Tricine-SDS-PAGE analyses. Digitalized images of gels were analyzed by Quanti Scan software (version 2.1) (Biosoft, Cambridge, UK) to visualize band intensities and to estimate molecular weight of bands by using low range (6.500 to 66.000 Da) and wide range (6.500 to 200.000 Da) markers (Sigma, MO, USA).

2.3. Peptide analysis

2.3.1. Peptide extraction

From the fermented sausages (10), five previously described as products with high (3) and low (2) consumer's acceptance (López et al., 2012) were selected for peptide analyses and bovine meat was used as non-fermented control. Portions of 2.5 g from each fermented sausage pool from each batch were homogenized in a stomacher (8 min, in ice) with 12.5 ml 0.01 N HCl and 0.1 N HCl for sausages and meat, respectively (Sentandreu et al., 2003). The meat slurries were then centrifuged (20,000 ×g at 4 °C for 20 min) and supernatants

Table 1
Information of fermented sausages as declared on the label.

	FS[a]1	FS2	FS3	FS4	FS5	FS6	FS7	FS8	FS9	FS10
Manufacture origin	Santa Fe	Buenos Aires	Córdoba	Tucumán	Santa Fe	Buenos Aires	Santa Fe	Santa Fe	Santa Fe	Santa Fe
Product type	Salami[b]	Fuet[c]	Salami	Salami	Salami	Fuet	Salami	Salami	Salami	Salami
Degree of mincing (lean meat and fat)	High	Low	Low	Low	Low	Low	High	High	Low	Low
Sodium (%)	1.69	1.48	ND	0.45	1.42	1.65	1.45	1.57	1.45	1.69
Total fat (%)	36.00	37.50	ND	27.50	27.50	40.00	32.50	32.50	27.5	36.00
Saturated fat (%)	13.00	13.75	ND	12.25	10.00	18.25	15.00	14.25	10.5	13.00
Proteins (%)	19.00	24.75	ND	15.50	20.25	25.00	18.00	17.00	19.25	19.00
Lean meat (%)	83.91	83.52	ND	88.54	87.58	82.35	85.55	85.43	87.55	83.91
Meat type	Beef/pork	Pork	Beef/pork	Beef/pork	Beef/pork	Pork	Beef/pork	Beef/pork	Beef/pork	Beef/pork
Colorant addition[d]	ND	D	ND	ND	ND	D	ND	ND	ND	ND

[a] Fermented sausage samples; ND: not declared; D: declared.
[b] Salami: fermented sausages: 4–5 cm diameter; ripening time, generally, more than 2 weeks.
[c] Fuet type fermented sausages having 2–3 cm diameter whose processing implies minor fermentation and ripening time (max 2 weeks), resulting in products less acidic than the traditional salami-type products.
[d] Cochineal carmine (INS120).

were submitted to ultra-filtration with a 3 kDa cut-off Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-3 membrane (Millipore, Billerica, USA).

2.3.2. RP-HPLC analyses of low molecular weight peptides

Peptide extracts (2 μg/μl protein) were injected in a Pursuit XRs C18 (250 × 4.6 mm, 5 μ particle size) reversed-phase column (Agilent, Palo Alto, CA). Separation was carried out in a Smartline Knauer HPLC system (Berlin, Germany) under the following conditions: isocratic 0.1% TFA in water (solution A) for 5 min, followed by a linear gradient from 0% to 100% of acetonitrile and 0.1% TFA (solution B) for 35 min. RP-HPLC was monitored at 214, 220 and 280 nm and the fractions were automatically collected at a flow rate of 1 ml/min. Different batches of fermented sausages and meat were evaluated by RP-HPLC at least in duplicate. Chromatograms were obtained by using Sigma Plot software (version 11.0). Fractions (1 ml) were collected and dried by centrifugation under vacuum. Then, the selected dried fractions were re-dissolved in bi-distilled water and subjected to MALDI-TOF-TOF analyses.

2.3.3. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-TOF)

Differential peaks between raw meat and fermented sausages were selected from the HPLC chromatograms obtained at 214 nm and subjected to identification using a MALDI-TOF-TOF spectrometer Ultraflex II (Bruker, Bremen, Germany) at CEQUIBIEM (Buenos Aires, Argentina) and further analyzed by MS-tagged software using Uniprot database. In order to know the exact position of peptides on the parental protein, BLASTP against *Bos taurus* data from non-redundant protein sequences (http://blast.ncbi.nlm.nih.gov) were used. Matches of MS/MS spectra against sequences of the database were manually verified. For a deep analysis of certain peptides and its parental protein, multiple alignments of protein sequences were conducted on line by ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2).

2.4. Statistical analysis

Data were statistically analyzed using the Infostat Statistical Software (Universidad Nacional de Córdoba, Argentina). SDS-PAGE results were analyzed by Quanti Scan software, band intensities were estimated by the Net Area parameter. Ratios of band intensity between fermented sausages and meat for each protein band were calculated. One-way analysis of variance (ANOVA) with the post-hoc Tukey test was used to evaluate significant differences among samples. Peak height values of RP-HPLC chromatograms were determined by the Sigma Plot software; results obtained from the same peak in different samples were compared by ANOVA and Tukey test (Infostat Statistical Software).

3. Results and discussion

3.1. Protein profiles of Argentinean fermented sausages by Tricine-SDS-PAGE analyses

Sarcoplasmic and myofibrillar protein profiles were analyzed in commercial fermented sausages (FS1-FS10) and beef meat as a non-fermented control. Tricine-SDS-PAGE was applied in order to establish patterns of protein degradation with an adequate resolution below 30 kDa. From these results, the roles played by microbial and/or muscle proteolytic enzymatic systems as well as potential influence of processing conditions were able to be drawn.

3.1.1. Sarcoplasmic proteins

The protein patterns obtained from the Argentinean FS are shown in Fig. 1a. When fermented sausages were compared to bovine meat (control), the most important change was observed in the 17 kDa band, assigned to myoglobin (Mb), which exhibited significant differences among samples ($p < 0.0001$). Mb degradation, attributed to muscle endogenous peptidases, was reported in meat during the post-mortem period (Hughes et al., 2002). However, the whole protein was highly represented in the sarcoplasmic fraction of meat, but not in fermented sausages samples, probably due to its degradation during ripening. This result agrees with the intense hydrolysis exhibited by it in pork meat inoculated with *Lactobacillus* strains, as well as in cured-fermented products (Basso et al., 2004; Candogan, Wardlaw, & Acton, 2009; Mora & Toldrá, 2012; Picariello et al., 2006). Hence, endogenous enzymes probably were activated by the prevailing acidic environment during meat fermentation (Fadda, Vildoza, & Vignolo, 2010). Concurrently, proteolysis of other large proteins may originate lighter bands in FS samples as those at 18 and 21 kDa (Fig. 1a). In addition, the 64 kDa band could be attributed to pyruvate kinase (PK) as reported by Hwang, Park, Kim, Cho, and Lee (2005). It is interesting to note that a greater intensity for PK band was observed in FS samples compared to non-fermented meat control (Fig. 1a), which was confirmed by ANOVA and Tukey test ($p < 0.0083$). This result can be explained by changes in proteins ratio during Mb and other proteins breakdown. Therefore, proteins which were not susceptible to proteolysis would be more represented at the end of ripening. In fact, a low degradation of PK was also reported in other meat products such as Naples-type salami, Coppa-type semi-dried sausage and cured ham (Picariello et al., 2006). Moreover, the 43 kDa and 36.6 kDa bands could be assigned to creatin kinase M-type (CK-M) and L-lactate dehydrogenase A (LDH-A), respectively. These proteins did not show evidence of degradation in fuet-type sausages (FS2 and FS6), whereas a pronounced hydrolysis in salami-type products was observed. From the statistical analysis, significant differences among samples were found with calculated $p < 0.0005$ and $p < 0.006$ for CK-M and LDH-A, respectively. Similarly, a remarkable proteolysis of CK-M and LDH-A at the end of ripening was reported in several cured fermented products (Di Luccia et al., 2005; Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009; Picariello et al., 2006). Some degradation of these proteins in fuet-type products cannot be discarded, although it could be masked by Mb hydrolysis. Based on differences from sarcoplasmic fraction, a characteristic degradation pattern from fuet-type sausages (FS2 and FS6) may be suggested, which clearly differed from that obtained from salami-type products. In addition, similar proteolytic profiles from fuet-type sausages and that from bovine meat were observed. This could be explained by the mild technological conditions applied during fuet-type sausage production such as low fermentation/ripening temperature which yield higher final pH values (López et al., 2012). Under this condition, acidic muscle cathepsins would not be fully active and a weak proteolysis occurred. In contrast a pronounced endogenous proteolysis at acidic pH experienced by sterile beef meat was reported (Fadda, Vildoza, & Vignolo, 2010). From this study, it could be inferred that technological conditions during fermented sausage manufacture may determine and/or regulate sarcoplasmic protein degradation, particularly at endogenous enzyme level. Accordingly, different proteolytic profiles during Iberian fermented sausages were obtained when processing time and temperature were modified (Casquete et al., 2011).

3.1.2. Myofibrillar proteins

Hydrolysis of the myofibrillar fraction was clearly observed in salami-type sausages at the 40.1 kDa band (Fig. 1b), exhibiting significant differences when bovine meat and fuet-type samples ($p < 0.0103$) were compared. This band was experimentally identified as α-actin by other authors (41.8 kDa, UniprotKB data base) (Hwang et al., 2005; Lametsch et al., 2003). Differences with the theoretical molecular weight of this protein may be assigned to somewhat degradation carried out during the post-mortem period. In this study, the 40.1 kDa band was differentially hydrolyzed in FS samples, which

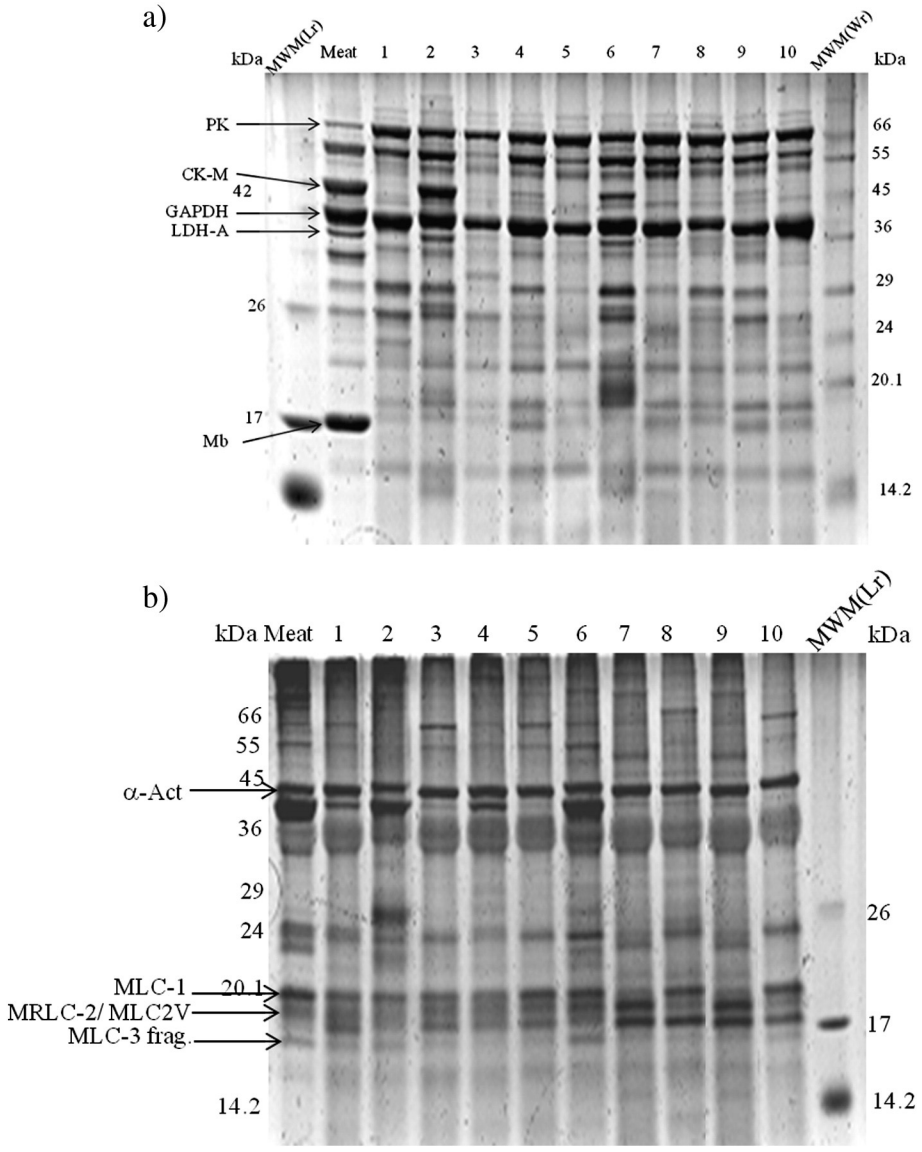

Fig. 1. Tricine-SDS-PAGE patterns of meat proteins obtained from commercial fermented sausages (FS). Lanes 1–10: FS1 to FS10 and bovine meat (Meat). Molecular weight markers in kDa (MWM) low range (Lr) and wide range (Wr). (a) sarcoplasmic proteins: PK: pyruvate kinase; CK-M: creatine kinase muscular type; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; LDH-A: L-lactate dehydrogenase, A chain; Mb: myoglobin and (b) myofibrillar proteins: α-Act: α actin; MLC-1: myosin light chain 1; MRLC-2: myosin light chain-2 regulatory unit; MLC2V: myosin light chain 2-V; MLC-3 frag: fragment of myosin light chain 3.

was also reported for fermented sausages by others (Aro Aro et al., 2010; García, Díez, & Zumalacárregui, 1997; Hughes et al., 2002). Furthermore, minor degree of hydrolysis was registered for 20, 18 and 16 kDa protein bands. The 20 and 16 kDa bands may be assigned to myosin light chain-1 (MLC-1) and -3 (MLC-3), respectively, while the 18 kDa band was associated to myosin light chain (MLC-2) as well as to myosin regulatory light chain-2 (MRLC-2) (Hwang et al., 2005; Sentandreu et al., 2010). From these results, myosin light chains were not as clearly degraded in these products as previously reported for sausages fermented with mixed starter cultures including Lactic Acid Bacteria (LAB) and Gram positive, catalase positive cocci (Casaburi et al., 2007; Hughes et al., 2002). Proteolytic profiles of myofibrillar proteins near 40 kDa allowed differentiating fuet-type from salami-type sausages. A reduced activity of muscle enzymes against meat proteins in the range of 14–66 kDa may be suggested for mild acidic sausages. As was discussed before for the sarcoplasmic protein fraction, an impact of processing conditions during sausage manufacture may be hypothesized. According to SDS-PAGE results, both proteolytic systems (endogenous and microbial) induced only slight changes in the analyzed fermented sausages. Nevertheless, during fermentation and ripening the modifications produced on the sarcoplasmic proteins were more pronounced than those exerted on the myofibrillar protein fraction, as was previously reported (Fadda et al., 1999; Spaziani, Torre, & Stecchini, 2009).

3.2. Low molecular weight (LMW) peptides analyzed by RP-HPLC

Peptides lower than 3 kDa were postulated to be responsible for flavor in meat and meat products (Henriksen & Stahnke, 1997; Nishimura, 2002; Sentandreu et al., 2003). Five out the ten FS products were selected based on previously reported consumer preference analysis (López et al., 2012) for LMW peptide analysis. The most accepted sausages namely FS2, FS5 and FS6 as well as the least accepted samples FS1 and FS4 were evaluated, bovine meat being included as non-fermented control. In general, peptide patterns from FS showed clear changes in peptide fractions; as a result

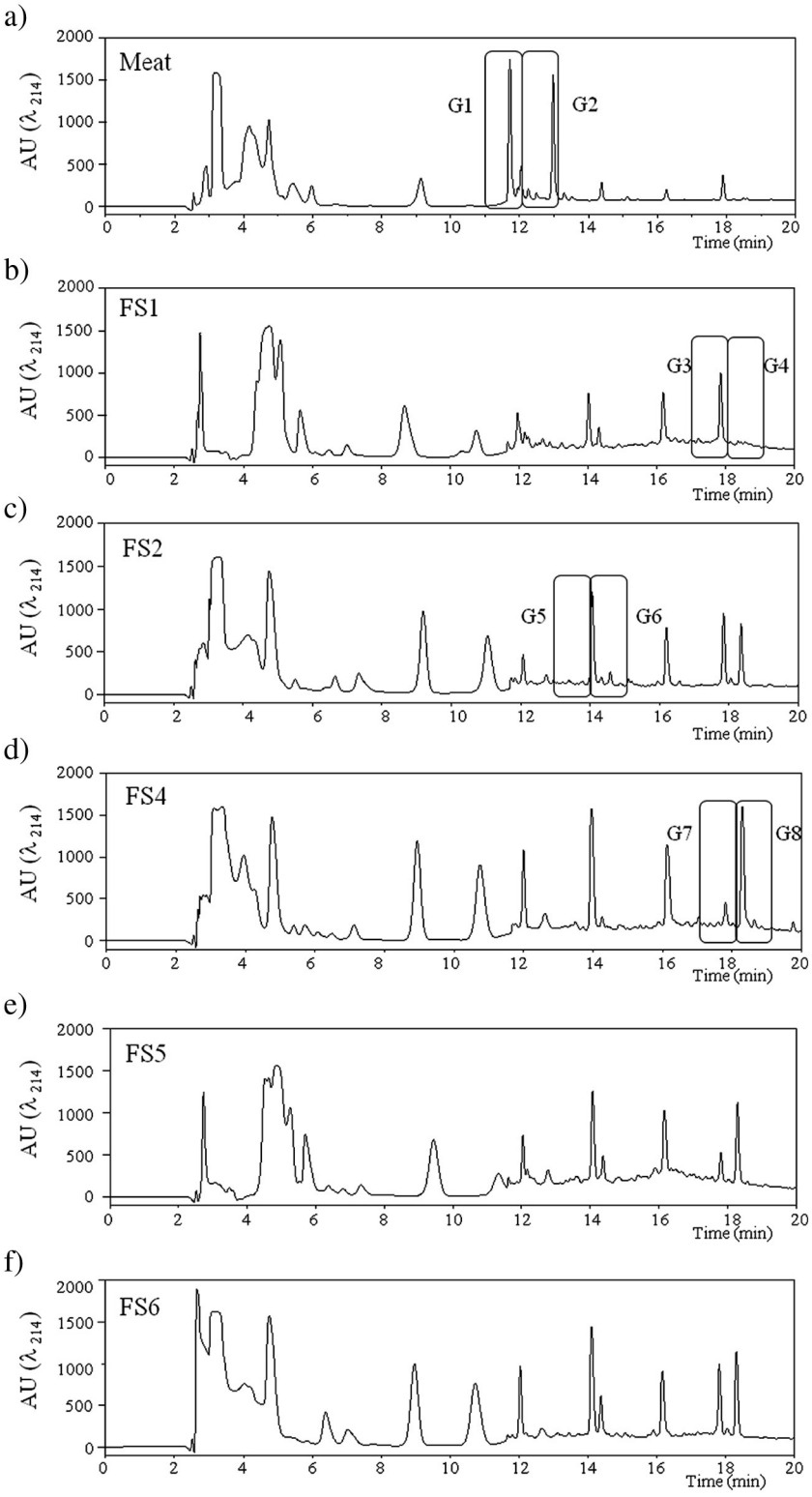

Fig. 2. RP-HPLC chromatograms of low molecular weight peptides (<3 kDa) from bovine meat and five selected Argentinean dry fermented sausages (FS). UA: arbitrary units. G1–G8: eluted fractions selected for MS analyses.

of fermentation and ripening, more enriched profiles were obtained when compared to meat. Chromatograms from meat sample (control) exhibited two peaks at 11.5 and 12.8 min, which were fully degraded in FS (Fig. 2). Moreover, six peaks from fermented sausage samples showed higher concentrations than non-fermented control ($p < 0.05$). RP-HPLC peaks may be classified according to their hydrophobic or hydrophilic nature (Hughes et al., 2002). Differential peptides eluted at 9, 10.5, 14, and 16 min were considered as relatively hydrophilic while those eluted at 17.8 and 18.2 min showed a relatively hydrophobic nature. Several peptide features such as hydrophobicity, size, primary sequence and volume were proposed to be contributors to food flavor (Maehashi & Huang, 2009; Wu &

Aluko, 2007). In contrast, pleasant flavors were correlated with hydrophilic peptides (Hughes et al., 2002; Ney, 1979; Nishimura & Kato, 1988). Since both, preferred and non-preferred sausages contained hydrophobic peptides; it may be assumed that they did not negatively affect the sensory quality of products, supporting the multi-factorial hypothesis of peptide influence on flavor. None of these differential peaks seemed to be associated with commercial FS brands or consumer preferences. However, the most interesting and significant changes observed in the chromatograms (selected RP-HPLC fractions) were analyzed by tandem mass spectrometry (MS) to get more information about their sequence, molecular weight and putative parental protein. Selected peaks (G1 to G8) were those with similar concentration eluting at 17.8 and 18.2 min in fuet-type sausages (FS2 and FS6), two peaks from bovine meat eluting at 11.5 and 12.8 min and the peak present in all FS samples that eluted at 14 min and exhibited the greatest significant difference when compared to meat ($p < 0.0001$) (Fig. 2).

3.3. Characterization of commercial fermented sausages by a peptidomic approach: MALDI-TOF-TOF analysis

Thirty six (36) different peptides from the selected RP-HPLC fractions were obtained by tandem mass spectrometric analysis (Table 2). These results showed a complex mixture of small peptides generated during fermentation and ripening due to the degradation of meat proteins. Molecular weight of the characterized peptides was in the range of 1000–2100 Da; consequently a potential role in flavor development may be assigned to them. Peptides numbered from 1 to 8 and 9 to 36 originated from myofibrillar and sarcoplasmic proteins, respectively. Hence, a larger variety of peptides derived from sarcoplasmic proteins were detected, indicating their main contribution to the diversity and richness of LMW fractions during ripening of FS. Peptide analysis demonstrated the sarcoplasmic fraction to be more susceptible to degradation than myofibrillar proteins, as was suggested by electrophoresis results from this and other previously reported studies (Di Luccia et al., 2005; Fadda et al., 1999; Mekchay, Teltathum, Nakasathien, & Pongpaichan, 2010; Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009; Mora et al., 2009; Sanz et al., 1999; Spaziani et al., 2009).

As shown in Table 2, myofibrillar proteins subjected to hydrolysis were α-actin skeletal muscle, myosin-1 (also named myosin heavy chain, MHC) and Capz-interacting protein. This result agrees with the proteome changes reported for post-mortem porcine muscle (Lametsch et al., 2003) and with the electrophoretic profiles (Fig. 1b). Consequently, the hydrolysis of the 40 kDa band, probably assigned to actin, is confirmed. Regarding sarcoplasmic parental proteins, CK and Mb were cleaved producing several peptides (peptide no. 16, 17, 20, 21 and 22, Table 2) as observed by SDS-PAGE analysis (Fig. 1a) and as reported by other authors (Di Luccia et al., 2005; Durá, Flores, & Toldrá, 2004; Picariello et al., 2006). In addition, sarcoplasmic proteins, not previously described as target for proteolytic enzymes, released peptides smaller than 3 kDa such as abhydrolase domain-containing protein 3, protein phosphatase-1A and transcription initiation factor TFIID subunit, among other (Table 2). The results obtained highlight the contribution of peptidomics for a better comprehension of the complete landscape of proteolysis during the ripening of fermented sausages. Tricine-SDS-PAGE combined with the peptidomics constituted a useful approach establishing a more complete framework to understand parental protein

Table 2
Low molecular weight peptides characterized in commercial fermented sausages (FS) identified by MALDI-TOF-TOF [a].

No.	Sequence	RP fraction	MH^+ calc.	Score	Parental protein; access number	Protein residues	Position
1	(G)FAGDDAPRAVFPS(I)	G8	1349.6484	23.4	Actin, alpha skeletal muscle; P68138	377	23–35
2	(A)VFPSIVGRPRHQG(V)	G7	1449.8073	19.1	Actin, alpha skeletal muscle; P68138	377	32–44
3	(M)EKIWHHTF(Y)	G7	1097.2400	39.3	Actin, alpha skeletal muscle; P68138	377	85–92
4	(L)RVAPEEHPTL(L)	G4 G7	1148.6058	38.0	Actin, alpha skeletal muscle; P68138	377	97–106
5	(G)AGQHPARASSSEAEDGCGSP(K)	G3	1970.8257	10.7	Capz-interacting protein; Q3ZBT0	381	253–272
6	(F)GEAAPYLRKaSEKERIEAQN(K)	G3	2189.1309	64.5	Myosin-1; Q9BE40	1938	11–29
7	(G)EAAPYLRKSEKERIEAQN(K)	G3	2132.1095	58.4	Myosin-1; Q9BE40	1938	12–29
8	(F)GEAAPYLRK(S)	G3	1004.5524	17.0	Myosin-1; Q9BE40	1938	11–18
9	(R)RLKSVGIPVL(C)	G5	1081.7092	22.30	Abhydrolase domain-containing protein 3; Q0VC00	411	330–339
10	(E)GALRWDLPRVQGGSQLSGLF(Q)	G7	2157.1563	17.90	AP-4 complex subunit mu-1; Q29RY8	452	359–378
11	(V)KAGASIVGVNCHFDPTIS(L)	G4	1872.9273	23.00	Betaine-homocysteine S-methyltransferase 1; Q5I597	407	207–224
12	(E)IIEQGIDLFT (K)	G8	1148.6198	17.10	Brefeldin A-inhibited guanine nucleotide-exchange protein 1; O46382	1849	701–710
13	(L)GASPTPGEAQRHLQTHR(I)	G3	1842.9288	20.90	Calmodulin-like protein 4; Q3T0E8	153	41–57
14	(R)GIQRAADIEQQ(A)	G8	1228.6280	16.80	Coagulation factor V precursor; Q28107	2211	534–544
15	(T)GAKNCLRDFIEKV(A)	G5	1549.8155	14.10	Complement factor B precursor; P81187	761	288–300
16	(D)DVIQTGVDNPGHPF(I)	G8	1495.7176	39.90	Creatine kinase M-type; Q9XSC6	381	55–68
17	(M)PFGNTHNKHKLNF(K)	G4	1553.7972	21.20	Creatine kinase M-type; Q9XSC6	381	02–14
18	(V)LGVTKDAGDEDL(K)	G7	1232.6005	13.90	DnaJ homolog subfamily B member 14; Q0IIE8	379	113–124
19	(Q)ERVGELMDQNA(F)	G7	1261.5841	12.10	General transcription factor IIH subunit 5; Q2T9Z5	71	55–65
20	(L)NAWGKVEADVAGHGQE(V)	G7	1667.7772	28.80	Myoglobin; P02192	154	13–28
21	(L)HAKHPSDFGADAQAAMSK(A)	G3	1868.8708	18.70	Myoglobin; P02192	154	117–134
22	(M)AAQYKVLGFHG(-)	G7	1190.6317	48.90	Myoglobin; P02192	154	144–154
23	(T)IGTDSALHRIMEVIDAITTT(A)	G4	2157.1220	20.60	6-phosphofructo-kinase, liver type; A1A4J1	780	176–195
24	(L)AGPRSDPAGPP(G)	G7	1021.5061	19.4	Phosphate carrier protein, mitochondrial precursor; P12234	362	27–37
25	(W)ASHHDINDASRGTLSS(Y)	G8	1667.7732	17.80	Poly(A) RNA polymerase GLD2; Q2HJ44	484	315–330
26	(L)DTAIVDRGKNVVS(G)	G4	1373.7383	18.30	Proteasomal ATPase-associated factor 1; Q148I1	392	181–193
27	(S)AGAPSVENVKNGIR(T)	G4	1411.7652	17.00	Protein phosphatase 1A; AO62829	382	89–102
28	(Q)TAAKLLHAGFKGRV(T)	G4	1468.8747	13.20	Prothrombin precursor; P00735	625	491–504
29	(Q)AGKATLFVSNNSRR(A)	G8	1520.8292	21.20	Pyridoxal phosphate phosphatase; Q3ZBF9	296	50–63
30	(T)LETAAGEALGQTL(T)	G5	1273.4562	18.30	Retinol dehydrogenase 8; Q9N126	312	48–60
31	(L)DSVNAQADRAF(Q)	G3	1193.5545	13.10	Testis-specific Y-en-coded-like protein 1; Q0P5N2	432	235–245
32	(N)SSTYWEGKSDMET(L)	G7	1520.6210	17.10	Threonyl-tRNA synthetase, cytoplasmic; Q0P5N2	723	281–293
33	(Y)SSHAKKATVD(A)	G8	1043.5480	15.20	Transcription initiation factor TFIID subunit 9; Q17QQ4	264	58–67
34	(D)PQGALSLEADGHPAAR(R)	G4	1589.8030	24.10	Uncharacterized protein KIAA1462 homolog; A2VE02	1305	1268–1283
35	(S)SSLIRHQRT(H)	G8	1097.6174	20.40	Zinc finger protein 572; Q32KN0	425	186–194
36	(S)AGPNSPTGGGGGGGSGGTR(M)	G3	1500.6786	13.80	Zinc finger SWIM domain-containing protein KIAA0913; A7E305	1413	49–67

[a] Peptides detected in FS originated from the breakdown of myofibrillar (no. 1–8) and sarcoplarmic (no. 9–36) proteins.

hydrolysis. In addition, the wide variety of cleavage sites observed on a range of diverse meat proteins (Table 2), suggests that both, muscle and bacterial proteolytic systems were involved in peptide generation. This complexity leads to a vast diversity within the FS peptidome, partially described in this study.

3.4. Contribution of muscle and microbial enzymes to LMW peptides production

The evolution of peptide patterns is the direct result of the changing enzymatic activities occurring in fermented sausages during fermentation and ripening which, in turn, are also related to the microbiological composition of the meat batter. The accumulation of a specific peptide depends on the delicate balance between its production and degradation as well as the availability of the parental substrate. Meat peptides generated by the action of endogenous proteases have been extensively reported, some of them related to sensory attributes (Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009; Mora, Sentandreu, & Toldrá, 2010; Mora, Valero, Sánchez del Pino, Sentandreu, & Toldrá, 2011; Sentandreu et al., 2003, 2007). Although muscle enzymes are greatly involved in meat protein degradation, the presence of microorganisms leads to a richer composition of small peptides and amino acids, contributing to the ripening process and to the generation of flavor compounds and/or precursors. As an attempt to elucidate the specific microbial contribution to this process, an in silico comparative study was carried out. Consequently, peptides identified from FS were contrasted with those reported by other authors, to hydrolytic products from muscle peptidases (cathepsins, calpains, exopeptidases) activities. This analysis was represented as peptide maps for each analyzed protein (Fig. 3) in which differential cleavage sites obtained in this work compared to those reported by others, might indicate the contribution produced by microorganisms during ripening.

Two peptides numbered as no. 16 and 17 (Table 2) were identified from degradation of creatine kinase M-type (CK-M) (Fig. 3a). To investigate the parental protein more precisely, a sequence alignment of both peptides with bovine (*Bos taurus*) and porcine (*Sus scrofa*) CK-M was performed by ClustalW2 (Fig. 4). Peptide no. 17 could be originated from the bovine CK-M, while peptide no. 16 may be derived from the bovine or porcine protein. Certain peptides were previously identified from CK during post-mortem storage of bovine *longissimus dorsi* muscle, as well as during ripening of Spanish cured ham (Fig. 3a, bold letters) (Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009; Stoeva, Byrne, Mullen, Troy, & Voelter, 2000). In fact, peptide no. 16, identified in this work is included in the sequence of previously reported peptides (Fig. 3a), whereas peptide no. 17 was firstly described herein. Cathepsins (B and L) and μ-calpain are involved on CK proteolysis in cured ham and are active even at acidic pH and under high salt concentrations (Mora, Sentandreu, Fraser, Toldrá, & Bramley, 2009); no microbial activity on this protein may be inferred from the present work.

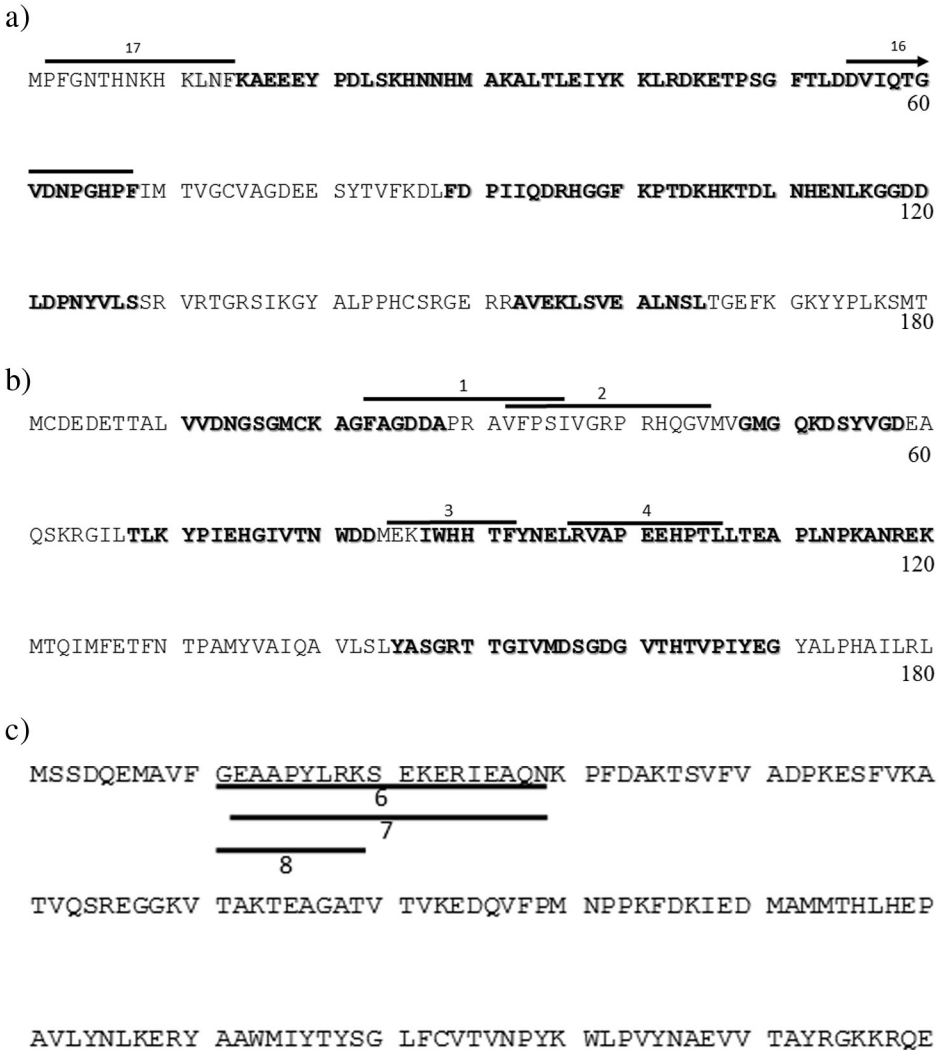

Fig. 3. Primary sequence of analyzed proteins. Peptides identified in the present work are marked with a line and a number according to Table 2; regions in bold letters correspond to already known peptides generated by muscle enzymatic hydrolysis, reported by other authors. (a) Creatine kinase; (b) alpha actin and (c) N-terminal region of myosin light chain 1 showing the position of identified peptides by MALDI-TOF-TOF marked with a line and numbered according to Table 2.

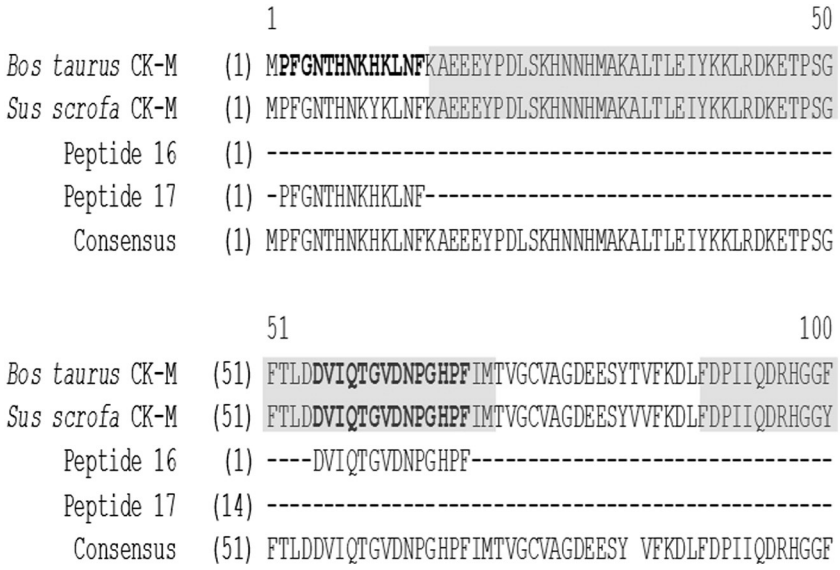

Fig. 4. Sequence alignment fragment of creatine kinase M-type (CK-M) from *Bos taurus* and *Sus scrofa*, also including the peptides no. 16 and 17 identified in this work (Table 2.). In CK-M sequences, fragments in bold indicate the perfect match with parental protein that allowed to hypothesize the origin of peptides. Shadowed regions correspond to already known peptides generated by muscle enzymatic hydrolysis and reported by other authors.

Four actin-derived peptides from the N-terminal region (peptides no. 1 and 2, Table 2) and from the central region of the protein (peptide no. 3 and 4, Table 2) were identified (Fig. 3b). Some peptides from this protein were previously reported from in vitro experiments and originated by cathepsins D and B (Hughes, Healy, McSweeney, & O'Neill, 2000; Hughes, O'Neill, McSweeney, & Healy, 1999). However, those here produced were different from the generated by cathepsin D in dry cured ham (Sentandreu et al., 2007). Regarding cleavage sites, the N-terminal end of peptide no. 1 (22G.F23), C-terminal end of peptide no. 3 and both corresponding to peptide no. 4 (Fig. 3b, Table 2) matched with those reported for cathepsins D and B (Hughes et al., 1999, 2000). However, the remaining cleavage sites (corresponding to C-terminal end of peptide no. 1, both cleavage sites of peptide no. 2 and N-terminal end of peptide no. 3) were not previously described for muscle enzymes (Fig. 3b). This analysis suggests that the microbiota is involved on actin degradation. In the FS, a wide microbial diversity was reported to be present (López et al., 2012) whereas LAB, as the dominant group, may have enhanced muscle endogenous activity or directly acted through their own peptidases. However, more studies should be performed to verify this hypothesis.

Three different peptides (no. 6, 7 and 8, Table 2) derived from myosin heavy chain 1 (MHC-1) had also been identified sharing the common sequence 12EAAPYLRK18 (consensus sequence) (Fig. 3c). It suggests endo and exopeptidase activity on the N-terminal region of MHC-1. Hydrolysis of this protein was not detected by Tricine-SDS-PAGE due to its large molecular size (222.99 kDa). Even when an intense hydrolysis of MHC by cathepsins L and D was observed, no identification of proteolytic products from MHC has been described in the scientific literature so far (Hughes et al., 1999; Zeece & Katoh, 1989). Consequently, the action of microbial enzymes by the technological microbiota of fermented sausages cannot be discarded. In fact, *Staphylococcus* species are recognized to have a strong proteolytic system and a remarkable activity against myofibrillar proteins (Casaburi et al., 2008; Mauriello, Casaburi, & Villani, 2002).

4. Conclusions

The changes in protein profiles from commercial Argentinean fermented sausages suggest that (i) the sarcoplasmic fraction was more susceptible to proteolysis than myofibrillar proteins when analyzed by Tricine-SDS-PAGE; (ii) major differences were determined between meat and fermented sausages, indicating that changes in meat proteins occurred during fermentation/ripening; (iii) protein and peptide profiles could be used to differentiate fuet-type from salami-type fermented sausages, although no specific biomarkers relating to commercial brands or quality could be established; (iv) muscle peptidases played a major role in proteolysis while some microbial contribution to meat protein degradation can be postulated; and (v) a relevant number of peptide sequences were identified by RP-HPLC off-line coupled to MS/MS. To our knowledge, this work represents the first peptidomic approach applied to fermented sausages allowing for a deeper insight into the modifications produced in LMW peptide fraction during fermentation/ripening by both, muscle and microbial enzymes. Although more research is needed for a better understanding of the role of microbiota on protein breakdown, this study provides an initial attempt to discover key peptides acting as biomarkers, as an innovative strategy to improve fermented sausage technology.

Abbreviations used

LMW	low molecular weight
Lr	low range
Wr	wide range
Tricine-SDS-PAGE	Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis
RP-HPLC	reverse phase high performance liquid chromatography
FS	fermented sausages
DTT	dithiothreitol
TFA	trifluor acetic acid
MALDI-TOF-MS	matrix assisted laser desorption/ionization time of flight mass spectrometry
Mb	myoglobin
PK	pyruvate kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
α-Act	alpha actin
CK	creatine kinase
CK-M	creatine kinase muscle type
LDH-A	L-lactate dehydrogenase A
MLC-1	myosin light chain 1
MLC-2	myosin light chain 2
MLC-3	myosin light chain 3
MRLC-2	myosin regulatory light chain 2

MLC2V	myosin light chain 2-V
MLC-3	fragment of myosin light chain 3
MHC	myosin heavy chain
A	alanine
R	arginine
N	asparragine
D	aspartic acid
C	cysteine
Q	glutamine
E	glutamic acid
G	glycine
H	histidine
I	isoleucine
L	leucine
K	lysine
M	methionine
F	phenylalanine
P	proline
S	serine
T	threonine
W	tryptophan
Y	tyrosine
V	valine

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