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Proteomic and peptidomic insights on myofibrillar protein hydrolysis in a sausage model during fermentation with autochthonous starter cultures

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ABSTRACT

The hydrolysis of myofibrillar proteins during fermentation of sausage models by an autochthonous starter culture was investigated. In order to provide a whole map of the generated products, proteomic and peptidomic were used and complemented with the amino acid profile. Beaker sausages (BS) were used as models which were inoculated or not with *Lactobacillus curvatus* CRL705 and *Staphylococcus vitulinus* GV318 as starter cultures. The hydrolysis of actin, myosin light chain 1/3 (MLC 1/3), myosin regulatory light chain-2 (MRLC-2) and myosin heavy chain (MHC) was evidenced by two-dimensional gel electrophoresis (2-DE). In addition, a total of 33 peptides arisen from troponin T, MRLC-2 and particularly from actin were identified by LC–MS/MS. These results showed that the starter culture significantly enhanced the proteolysis of the proteins named above, even when the endogenous enzymes induced a clear breakdown. *L. curvatus* CRL705 highly enriched both peptide pattern and amino acid concentrations. When the autochthonous starter culture was inoculated, although proteolysis was remarkably reinforced, a reduction in peptide and amino acid composition was observed. Regarding actin primary structure, three regions of this protein were highly susceptible to degradation by the starter culture. Additionally, the essential role of exopeptidases – from meat and bacteria – in diversity of actin peptides during fermentation was shown. This study improved the knowledge of the proteolysis of myofibrillar proteins and the involved enzymes, as well as, completed the previously reported degradation of sarcoplasmic proteins by the same autochthonous starter culture. The singular peptides and amino acids pattern generated might contribute to the uniqueness of produced fermented sausages while they may be used as quality markers.

1. Introduction

Although many fermented sausages are commonly produced in industrial plants, there has been an increasing interest in traditional naturally fermented meats as outlined by the recent abundant literature. Traditional fermented sausages are manufactured without the addition of starter cultures in small-scale processing units. In this case, microorganisms of technological interest are originated from the meat itself or from the environment, representing a part of the so-called "house-microbiota" (Ammor, Dufour, Zagorec, Chaillou, & Chevallier, 2005; Talon, Leroy, & Lebert, 2007). In fact, autochthonous meat starter cultures are a valuable tool to improve safety and flavor quality of fermented sausages while preserving their typicity. Therefore, the knowledge and control of these autochthonous bacteria are essential not only to keep the global quality of the final fermented product but also for traceability purposes.

This is mainly because two microbial reactions occur simultaneously and interdependently during sausage fermentation: acidification by Lactic Acid Bacteria (LAB) and the production of nitric oxide by nitrate- and nitrite-reducing bacteria as Coagulase Negative Cocci (CNC) involving *Staphylococcus* and/or *Micrococcus*. The two microbial groups, including *Lactobacillus* among LAB and CNC, have been widely adapted to the stringent conditions existing during meat fermentation while are actively involved in the development of texture, color and flavor (Fadda, López & Vignolo, 2010; Fontana, Fadda, Cocconcelli, & Vignolo, 2011). Sensory characteristics largely depend on proteolysis products such as peptides and amino acids. Low molecular weight (LMW) peptides have been also related to biological activities (Castellano, Aristoy, Sentandreu, Vignolo, & Toldrá, 2013; Sentandreu et al., 2003) and proposed as biomarkers for a specific applied technology (Sentandreu & Sentandreu, 2011). Meat major proteins involve sarcoplasmic and myofibrillar fractions which may be differentiated based on their solubility in low and high ionic strength buffers, respectively. As a first peptidomic approach in commercial fermented sausages, several LMW peptides (1000–2100 kDa) arising from both fractions were identified (López, Bru, Vignolo & Fadda, 2015). Most of these peptides were originated from sarcoplasmic proteins as creatine kinase and a wide variety of other enzymes not previously detected as cleaved. Recently, the identification of small peptides originated from sarcoplasmic and myofibrillar fractions during fermentation of dry-sausages inoculated with a commercial starter culture (*Lactobacillus pentosus* + *Staphylococcus carnosus*) was carried out (Mora et al., 2015).

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Concerning myofibrillar proteins, although they have been reported to be less degraded than the sarcoplasmic fraction (Fadda, López, et al., 2010; Spaziani, Torre, & Stecchini, 2009), several studies reported the contribution of LAB to proteolysis during sausages fermentation (Casaburi et al., 2007, 2008; Fadda et al., 1999). Results indicated that meat hydrolysis was the result of the combined action of muscle and bacterial peptidases, except for di- and tri-peptides breakdown, mostly due to microbial di- and tri-peptidases (Mora et al., 2015).

In a previous study, the autochthonous starter culture involving *Lactobacillus curvatus* CRL705 and *Staphylococcus vitulinus* GV318, both strains formerly isolated from traditional Argentinean fermented sausages, was designed based on its technological and safety features (Castellano, Holzapfel, & Vignolo, 2004; Hebert et al., 2012; López, Sentandreu, Vignolo & Fadda, 2015; Vignolo, Fadda, De Kairuz, de Ruiz Holgado, & Oliver, 1996). Then, its role in the proteolysis of sarcoplasmic proteins in beaker sausage models was evaluated, and consequently, a broad diversity of both new peptides and protein targets was identified (López, Sentandreu, et al., 2015). These findings increased the knowledge about the effect of microorganisms in meat proteolysis and allowed proposing peptide biomarkers, but only based in the analysis of the sarcoplasmic fraction. In the present study, the hydrolysis of myofibrillar proteins by *L. curvatus* CRL705 and *S. vitulinus* GV318 was undertaken to complete the whole proteolytic map and to unravel the involvement of this promising culture. For this purpose, two-dimensional electrophoresis (2-DE) and Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) and RP-HPLC were applied. In addition, the possible technological applications of identified peptides and the muscle and bacterial peptidases involved in their generation are also discussed.

2. Materials and methods

2.1. Strains and culture conditions

L. curvatus CRL705 and *S. vitulinus* GV318, isolated from artisanal fermented sausage (Tucumán, Argentina) were selected based on previous studies (Castellano et al., 2004; López, Sentandreu, et al., 2015; Vignolo et al., 1996; Vignolo, Suriani, Holgado, & Oliver, 1993) and routinely cultivated in appropriate conditions.

2.2. Beaker sausage model (BS)

Beaker sausages (BS) models were prepared and monitored as described Lopez et al. (López, Sentandreu, et al., 2015): (i) BS-control containing antibiotics (20,000 UI/Kg penicillin, 20 mg/Kg streptomycin and 50 mg/Kg amphotericin B) (Gibco, Grand Island, USA); (ii) BS-Lc inoculated with *L. curvatus* CRL705 (7–8 log CFU/g) and (iii) BS-Mx inoculated with mixed strains (7 and 6 log CFU/g for *L. curvatus* and *S. vitulinus*, respectively). Flasks containing the different BS models were incubated at 22 °C and samples were collected, for analyses, at 0, 3 and 10 days of fermentation and ripening. Three independent replicates were performed for each BS model. The experimental workflow used to evaluate the proteolysis of myofibrillar proteins can be observed in Fig. 1.

2.3. Two-dimensional electrophoresis (2-DE)

2-DE was performed in a pool of all three BS at initial time (BS t_0) as well as in BS-control, BS-Lc and BS-Mx at 10 days of incubation (t_{10}). The extraction of myofibrillar proteins was adapted from Théron et al. (2011) and carried out before 2-DE run as reported by Fadda, Anglade, et al. (2010). Differential spots were identified by applying MALDI-ToF–ToF mass spectrometry using an Ultraflex II device (Brunker, Germany) at CEQUIBIEM Institute (Universidad Nacional de Buenos Aires, Argentina). Results were analysed by MS-Tagged software using UniProt database and matches were manually verified. These studies were carried out in two technical replicates on protein extracts from two biological replicates of each sample counting for a total of 16 gels.

2.4. Peptide extraction and sequence identification by LC–ESI–MS/MS

Ten grams from each BS sample (BS t_0, BS-control t_{10}, BS-Lc t_{10} and BS-Mx t_{10}) were analysed in three independent replicates for each condition and processed according to Sentandreu et al. (2003).

Freeze-dried samples containing peptides lower that 3 kDa were redissolved in 1 ml of 0.1% trifluoroacetic acid (TFA). Twenty-five microlitres of this solution were injected into a Surveyor LC system directly coupled to a LCQ Advantage Ion trap MS instrument (Thermo Scientific, San Jose, CA, USA) as described by López, Sentandreu, et al. (2015). The potential biological activity or sensory properties for identified peptides was investigated by using the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

2.5. Free amino acids composition

Concentrations of the released amino acids were determined by RP-HPLC with previous derivatization with o-phthaldialdehyde (OPA) reagent (OPA 2% p/V, methanol 90% v/v, borate buffer 10% v/v, β-mercaptoethanol 1,6% v/v). A C18 Gemini column (Phenomenex, Torrace, CA, USA) was used. The separation was carried out in a Smartline Knauer HPLC system (Berlin, Germany) and the elution system comprised a gradient of the phase A (NaH2PO4 40 mM, pH = 6) and the phase B (CH3CN/MeOH/H2O at 45:45:10). Data were expressed as g/100 g of sample. The samples BS t_0 (pool of BS at initial time) as well as BS-control, BS-Lc and BS-Mx after 3 (t_3) and 10 (t_{10}) days of incubation were analysed, which allowed determining the compositions of 16 amino acids. Different batches of each model were evaluated by RP-HPLC at least in duplicate.

2.6. In silico analysis of the genes encoding L. curvatus CRL705 peptidases

Analysis of LAB proteolytic system components in the draft genome sequence of *L. curvatus* CRL705 was carried out in order to complete the discussion on proteolysis contribution of this strain in the assayed conditions. The complete genome sequence of this strain was obtained from the NCBI microbial genome database (accession number AGBU01000000). Traditionally, described members of LAB proteolytic system (two cell- wall bound proteinases and 17 peptidases) were selected based on the comparative genomic study of this microbial group reported by Liu, Bayjanov, Renckens, Nauta, and Siezen (2010). Nucleotide sequences of the hydrolytic enzymes were obtained from the European Nucleotide Archive (ENA) database (www.ebi.ac.uk/ena). Then, a local alignment of these sequences against the *L. curvatus* CRL705 genome was performed by using the BLAST tool and Nucleotide database (www.ncbi.nlm.nih.gov). Finally, these results were compared with those reported for the meat born strain *Lactobacillus sakei* 23 K (Chaillou et al., 2005; Liu et al., 2010).

2.7. Statistical analyses

2-DE gel results were analysed by using Prodigy Same Spot software (Progenesis, Non linear) submitted to a one-way analysis of variance (ANOVA) with $p < 0.05$. From RP-HPLC, the amino acid concentrations were obtained by PeakSimple software based on the concentration of standards for each amino acid and compared by ANOVA ($p < 0.05$) and Tukey test using Infostat Statistical Software (Universidad Nacional de Córdoba, Argentina).

3. Results

As summarized in Fig. 1, the hydrolysis of myofibrillar proteins by the autochthonous starter culture was studied using proteomic, peptidomic and free amino acid profiles. The contribution of starter culture was achieved by comparing the different models.

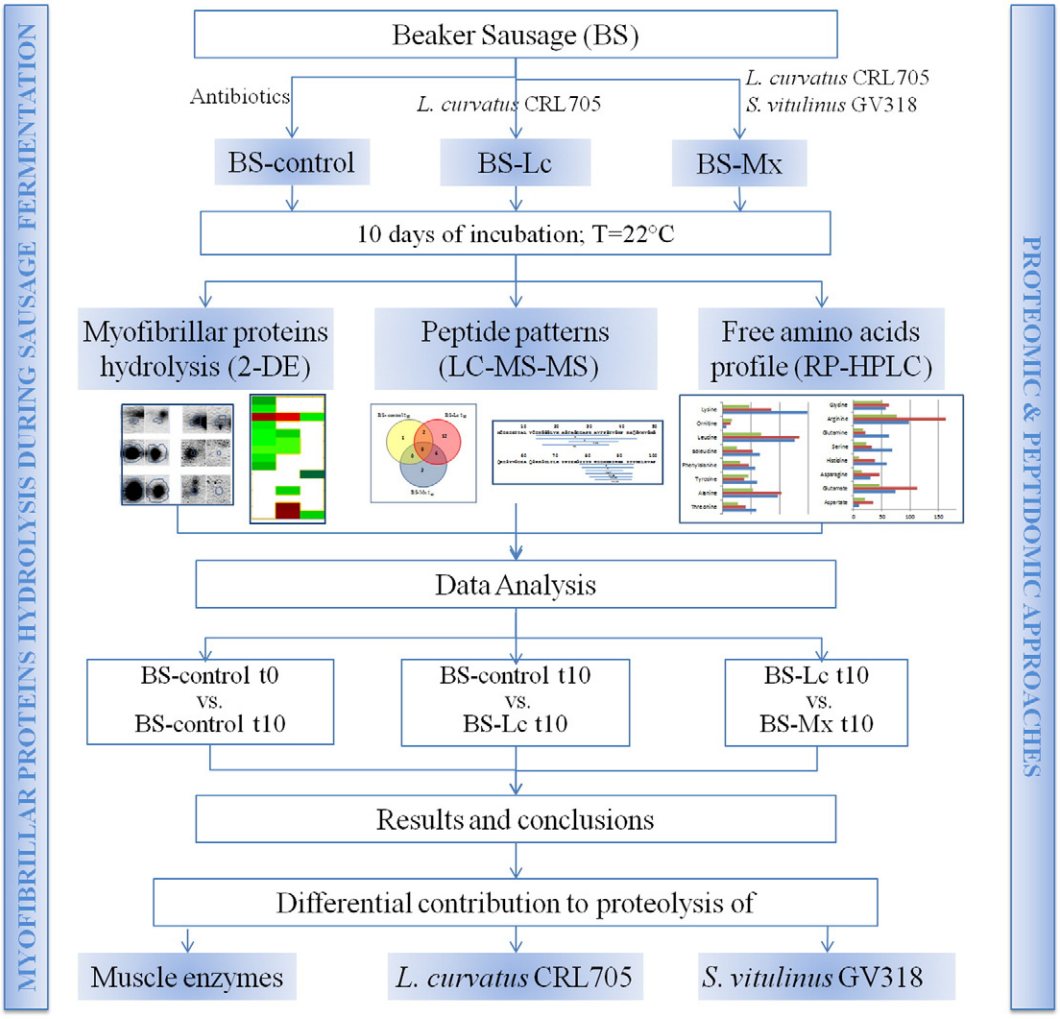

Fig. 1. Experimental workflow used to evaluate the proteolysis of myofibrillar proteins and contribution of the autochthonous starter culture.

3.1. Hydrolysis of myofibrillar proteins by 2-DE

The pool of the three BS at initial time (BS t_0) as well as BS-control, BS-Lc and BS-Mx at 10 days of incubation (t_{10}) were analysed by 2-DE (Fig. 1 Supplemental Material). Results showed that approximately 20 spots on each gel were detected, 15 of them exhibited significant differences when ANOVA ($p < 0.05$) was applied. From them, a total of 8 spots were successfully identified by MALDI-ToF–ToF mass spectrometry (Table 1). This set of polypeptides was originated from 3 proteins: actin, myosin heavy chain (MHC) and myosin light chain (MLC). Although spot 10 was not identified by mass spectrometry, it could be probably assigned to tropomyosin according to Hwang, Park, Kim, Cho, and Lee (2005). Besides, the comparison of statistically significant spot abundances among models (Fig. 1) allowed inferring the role of (i) muscle peptidases (BS-control t_0 vs. BS-control t_{10}), (ii) *L. curvatus* CRL705 (BS-control t_{10} vs. BS-Lc t_{10}) and (iii) *S. vitulinus* GV318 in the mixed culture (BS-Lc t_{10} vs. BS-Mx t_{10}) after 10 days of incubation. Indeed, the evolution of myofibrillar protein hydrolysis could be followed by the reduction of spot abundance (Fig. 2, green scale), but also by the increase in abundance of several protein fragments (Fig. 2, red scale). The greater the intensity of colour in each scale, the more change in the abundance of each spot. It could be observed that endogenous

Table 1
Spots identified from 2-DE gels by MALDI-ToF–ToF mass spectrometry.

Spots n°	MW (kDa); pI estimated	Score	Expected mass (Da)	Protein name	Sample
1	40; 5.2	138	42,340	α actin	BS t_0; BS-control t_{10}; BS-Lc t_{10}; BS-Mx t_{10}
2	17; 4.5	143	19,721	MLC-1	BS t_0; BS-control t_{10}; BS-Lc t_{10}; BS-Mx t_{10}
3	116; 5.2	82	223,180	MHC	BS-control t_{10}; BS-Lc t_{10}; BS-Mx t_{10}
5	40; 5	162	42,340	α actin	BS t_0; BS-control t_{10}; BS-Lc t_{10}; BS-Mx t_{10}
6	23; 5	106	17,057	MLC 1/3	BS t_0; BS-control t_{10}; BS-Lc t_{10}; BS-Mx t_{10}
11	45;5	124	42,451	α actin	BS t_0; BS-control t_{10}; BS-Lc t_{10}; BS-Mx t_{10}
12	40; 4.9	170	42,338	α actin	BS t_0; BS-control t_{10}; BS-Lc t_{10}; BS-Mx t_{10}
14	36; 5.5	83	42,451	α actin	BS-Lc t_{10}; BS-Mx t_{10}

MW (molecular weight) and pI (isoelectric point) are estimated from spots positions in the gel; MLC: myosin light chain; MHC: myosin heavy chain. Spots are numbered in relation with the text and with Fig. 1 Suppl. Mat.

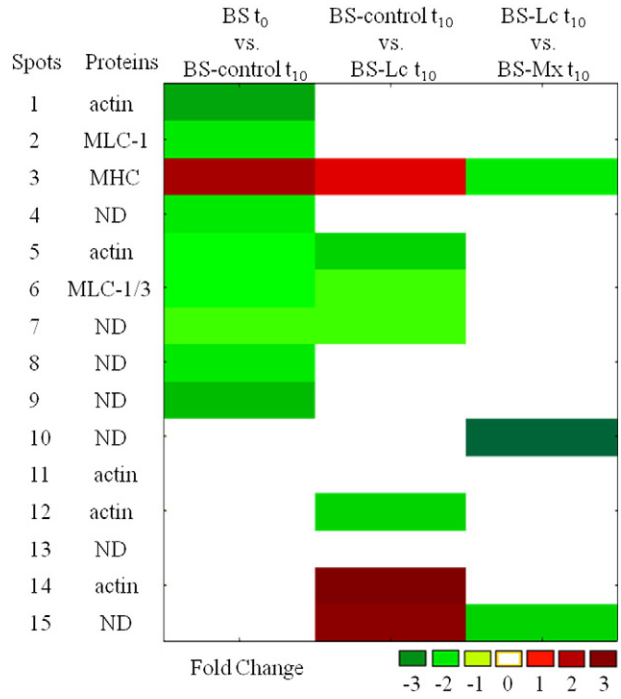

Fig. 2. Heat map of abundance levels comparison based on proteomic data. Changes in abundance of statistically significant ($p < 0.05$) spots among BS models were analysed. ND: non-determined. Fold change: negative values (decreasing abundance); 0 (no differences); positive values (increasing abundance).

enzymes (BS t_0 compared to BS-control t_{10}) produced reduction in the abundance of most spots [2 (MLC-1), 4 (non determined, ND), 5 (actin), 6 (MLC-1/3), 7 (ND) and 8 (ND)] with fold changes from 1.8 to 2.1 and particularly, in spots 1 (actin) and 9 (ND) that showed a fold change of 2.9 and 2.7, respectively. These changes highlighted the hydrolysis of MLC 1/3 and mainly of actin after 10 days of incubation by the action of muscle peptidases. Furthermore, spot 3 (MHC), attributed to a proteolysis fragment of MHC, increased its intensity 2.2 folds after 10 days. Beyond the action of muscle enzymes, the presence of *L. curvatus* CRL705 reduced even more the abundance of spots 5 (actin), 6 (MLC-1/3), 7 (ND) with fold changes between 1.5 and 2.3. In addition, spot 12 (actin), with no changes in the non-inoculated model, exhibited a remarkable drop (2.4 fold change) of its abundance when CRL705 was present. Spots 14 (actin) and 15 (ND) showed an increase of 3.3 and 2.7 folds, respectively also assigned to the presence of *L. curvatus* CRL705. Moreover, spot 3 (MHC), which already evidenced an increasing abundance by the endogenous action, followed the same trend in BS-Lc t_{10} model, with an increment of 1.5 fold change. Hence, *L. curvatus* CRL705 increased the degradation already started by endogenous enzymes, leading to a more noticeable hydrolysis of actin, MHC and MLC-1/3. On the other hand, *S. vitulinus* GV318 combined with the selected LAB induced a clear decrease of spot 10 (ND) (3.9 folds). It could also be noticed decreasing intensities for spots 3 (MHC) and 15 (ND) with 2.0 and 2.3 fold changes, respectively; this is opposite to its increasing abundance described above. Therefore, the autochthonous starter culture involving *L. curvatus* CRL705 and *S. vitulinus* GV318 promoted the breakdown of the MHC fragment and two non-identified spots, which were not registered in the other comparisons of models.

3.2. LC-ESI-MS/MS analysis of small peptides (<3 kDa) coming from myofibrillar proteins

In order to identify peptides smaller than 3 kDa originated from myofibrillar proteins in the studied models, LC–MS/MS was applied to BS t_0 (pool of all BS at initial time), BS-control, BS-Lc and BS-Mx at

Table 2
Identified peptides from the analysed BS models.

N°	Sample	Sequence of peptides	Parental protein	Position[a]
1	BS-t0	E.APPPPAEVPEVHEEVH.E	Troponin T, fast skeletal muscle	23–38
2	BS-control t10	G.IVLDSGDGVTHNVPIYEG.Y	Alpha actin, skeletal muscle	153–170
3	BS-control t10	F.TVIDQNRDGIIDKEDLRDTF.A	Myosin regulatory light chain-2	36–55
4	BS-control t10	L.NVKNEELDAMMKEASGPIN.F	Myosin regulatory light chain-2	62–80
5	BS-Lc t10	C.DNGSGLVKAGFAGDDAPRAVFPS.I	Alpha actin, skeletal muscle	13–35
6	BS-Lc t10	C.DNGSGLVKAGFAGDDAPRAVFPSIVGRPRHQG.V	Alpha actin, skeletal muscle	13–44
7	BS-Lc t10	G.FAGDDAPRAVFPSIVG.R	Alpha actin, skeletal muscle	23–38
8	BS-Lc t10	G.IITNWDDMEKIWHHTF.Y	Alpha actin, skeletal muscle	77–92
9	BS-Lc t10	I.ITNWDDMEKIWHHTF.Y	Alpha actin, skeletal muscle	78–92
10	BS-Lc t10	I.TNWDDMEKIWHH.T	Alpha actin, skeletal muscle	79–90
11	BS-Lc t10	I.TNWDDMEKIWHHTF.Y	Alpha actin, skeletal muscle	79–92
12	BS-Lc t10	T.NWDDMEKIWHHTF.Y	Alpha actin, skeletal muscle	80–92
13	BS-Lc t10	N.WDDMEKIWHHTF.Y	Alpha actin, skeletal muscle	81–92
14	BS-Lc t10	G.IVLDSGDGVTHNVPIYEG.Y	Alpha actin, skeletal muscle	153–170
15	BS-Lc t10	L.EKSYELPDGQVITIGN.E	Alpha actin, skeletal muscle	239–254
16	BS-Lc t10	S.YELPDGQVIT.I	Alpha actin, skeletal muscle	242–251
17	BS-Lc t10	S.YELPDGQVITIGN.E	Alpha actin, skeletal muscle	242–254
18	BS-Lc t10	S.YELPDGQVITIGNER.F	Alpha actin, skeletal muscle	242–256
19	BS-Lc t10	S.YELPDGQVITIGNERF.R	Alpha actin, skeletal muscle	242–257
20	BS-Lc t10	L.NVKNEELDAMMKEASGPIN.F	Myosin regulatory light chain-2	62–80
21	BS-Lc t10	L.NVKNEELDAMMKEASGPINF.T	Myosin regulatory light chain-2	62–81
22	BS-Lc t10	K.NEELDAMMKEASGPIN.F	Myosin regulatory light chain-2	65–80
23	BS-Lc t10	D.PEDVITGAFKVLD.P	Myosin regulatory light chain-2	97–109
24	BS-Lc t10	A.FPPDVGGNVDY.K	Myosin regulatory light chain-2	144–154
25	BS-Mx t10	N.GSGLVKAGFAGDDAPRAVFPS.I	Alpha actin, skeletal muscle	15–35
26	BS-Mx t10	G.FAGDDAPRAVFPSIVG.R	Alpha actin, skeletal muscle	23–38
27	BS-Mx t10	I.TNWDDMEKIWHH.T	Alpha actin, skeletal muscle	79–90
28	BS-Mx t10	I.TNWDDMEKIWHHTF.Y	Alpha actin, skeletal muscle	79–92
29	BS-Mx t10	T.NWDDMEKIWHHTF.Y	Alpha actin, skeletal muscle	80–92
30	BS-Mx t10	N.WDDMEKIWHHTF.Y	Alpha actin, skeletal muscle	81–92
31	BS-Mx t10	L.DFENEMATAASSSSLEKS.Y	Alpha actin, skeletal muscle	224–241
32	BS-Mx t10	S.LEKSYELPDGQVITIGN.E	Alpha actin, skeletal muscle	238–254
33	BS-Mx t10	S.YELPDGQVITIGNERF.R	Alpha actin, skeletal muscle	242–257

[a] Position in the parental protein.

10 days of incubation (t_{10}). The identified sequences, their position within the complete protein sequence as well as the model where they had been detected are shown in Table 2. A total of 33 peptides arising from the myofibrillar fraction were identified in all samples, most of them (25) originated from actin. The other fragments derived from Regulatory Myosin Light Chain-2 (MRLC-2) (7) and from troponin T (1). From BS models comparison, BS-Lc after incubation (t_{10}) exhibited the highest number of identified peptides (20). In fact, in BS-control t_{10} only three peptides from myofibrillar proteins were detected, suggesting that endogenous peptidases only exerted a slight effect on the extensive hydrolysis on these proteins, similarly as in 2-DE results. Hence, it could be hypothesized that *L. curvatus* CRL705 notably enhanced the myofibrillar proteolysis and enriched the peptide composition. However, when *S. vitulinus* GV318 was also present in the BS (BS-Mx t_{10}), differences in the peptide pattern with a reduction in peptides diversity were observed, as only 9 peptides were identified. These results and those from 2-DE studies might indicate that LMW peptides (<3 kDa) were massively degraded to smaller peptides and free amino acids and eventually, consumed by the microorganisms.

With regards to peptides released from α actin (Fig. 3), many of them came from three main regions as defined by amino acid positions 12–43, 75–92 and 238–255. Consequently, many peptides from the same region partially share the amino acid sequence. For example, peptides 8, 9, 10, 11, 12 and 13, released in BS-Lc t_{10}, have in common the sequence fragment ^{81}WDDMEKIWHH^{90} (Fig. 3; Table 2). This suggests that once released, some large fragments were sequentially cleaved by exoproteases from both carboxy (C) and amino (N) termini. Indeed, peptide diversity may be attributed to the activity of these enzymes

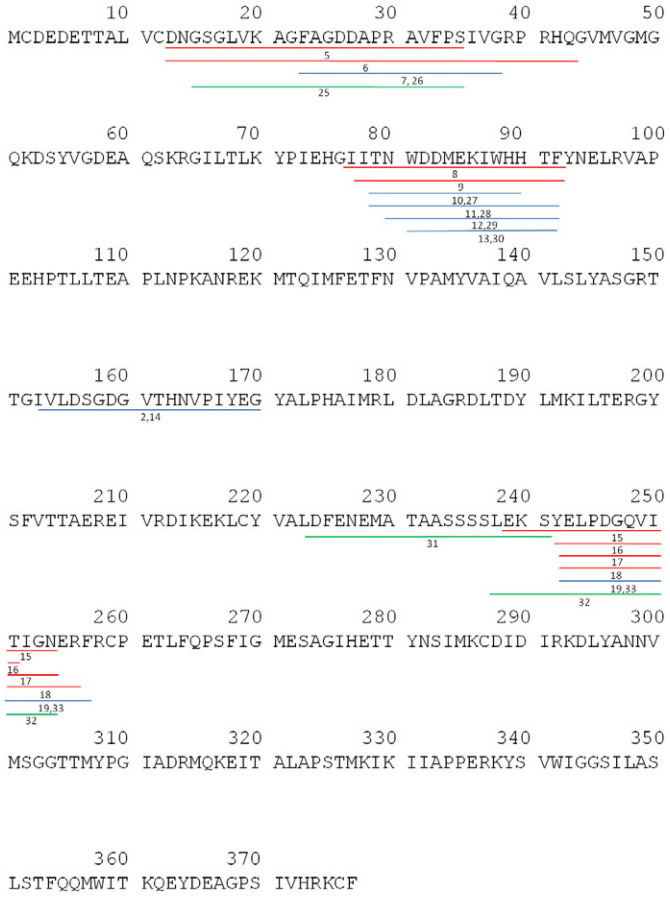

Fig. 3. Map of peptides originated from α actin. Identified peptides are represented by lines and its numbers are in coincidence with those shown in Table 2. Blue lines: peptides present in more than one BS model, red lines: peptides derived from BS-Lc t_{10} and green lines: peptides derived from BS-Mx t_{10} model.

that could be from muscle or bacterial origin. In addition, several peptides were common in the different BS models (Fig. 3, indicated with blue lines). For example, peptides 2 and 14 were detected in both BS-control t_{10} and BS-Lc t_{10}, respectively and similarly, peptides 10, 27; 11, 28; 12, 29; 13, 30 and 19, 33. On the contrary, peptides 5, 6, 8, 9, 15, 16, 17 and 18 were exclusively from BS-Lc t_{10}, while peptides 25, 31 and 32 were only present in BS-Mx t_{10} model. When the originated peptides after incubation were graphical grouped in a Venn diagram (Fig. 4), it may be noticed that two peptides were common to BS-control t_{10} and BS-Lc t_{10}, while six were present in both BS-Lc t_{10} and BS-Mx t_{10}. As well, several peptides were exclusively produced in BS-control t_{10} (1), BS-Lc t_{10} (12) and BS-Mxt_{10} (3). As a whole, from Figs. 3 and 4, two hypotheses could be postulated. At first, the selected strains produced qualitative differences in the peptide patters depending on models. Secondly, inoculated BS models exhibited more similarities between them than with the non-inoculated BS.

On the other hand, the identified peptides were examined in the BIOPEP database. Currently, this database counts with 3211 bioactive peptides, from which 14 were encrypted into the actin or myosin (MLC or MHC) sequences, and 327 sensory peptides and amino acids. This search showed that peptides reported herein did not match with those previously registered in the BIOPEP database neither as bioactive nor as sensory peptides since they were not previously identified. Nevertheless, these peptides or shorter encrypted sequences have to be tested by experimental assays to evaluate their bioactive/sensory potential. In fact, peptide DSGVT, reported in the used database for antioxidant activity and originated from pork actin, is quite similar to the fragment ^{156}DSGDG^{160} encrypted in peptides 2 and 14 identified in the present study.

3.3. Free amino acids profile

Free amino acids contents in BS t_0 (pool) and BS-control, BS-Lc and BS-Mx at 3 and 10 days of incubation were analysed by RP-HPLC (Table S1. Supplemental Material). As expected, total amino acid composition clearly increased during incubation due to the proteolysis and peptidolysis occurred (Table 3), particularly in BS-control and BS-Lc. At the third day of incubation, a net increase of 747.5 mg/100 g and 593.2 mg/100 g in BS-control and BS-Lc, respectively, was registered; in contrast to BS-Mx t_3, which exhibited a smaller amino acids increment (46.7 mg/100 g). Likewise, the total amino acid concentration at 10 days exhibited slight differences between BS-control t_{10} (775.9 mg/100 g) and BS-Lct$_{10}$ (724.3 mg/100 g) but a significant lower value was registered for BS-Mxt$_{10}$ (270 mg/100 g). When the amino acids profiles of BS-control, BS-Lc and BS-Mx at both t_3 and t_{10} were compared (Fig. 5), it could be observed that *L. curvatus* CRL705 caused specific increases in the concentration of

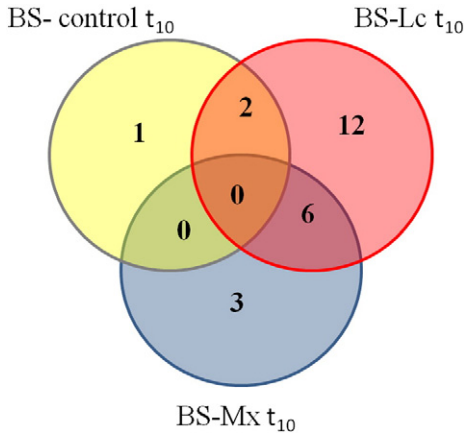

Fig. 4. Peptidenumbers from different BS models after 10 days of fermentation shown as a Venn diagram. Non-inoculated BS (BS-control); BS inoculated with *L. curvatus* CRL705 (BS-Lc) and BS inoculated with the mixed autochthonous starter culture (BS-Mx).

Table 3
Total amino acid concentrations (mg/100 g) in the BS models analysed during incubation.

Amino acids	BS-control t_0	BS-control t_3	BS-Lc t_3	BS-Mx t_3	BS-control t_{10}	BS-Lc t_{10}	BS-Mx t_{10}
Total content	305.2 ± 48.6	1052.7 ± 154.1	898.4 ± 192.9	351.9 ± 41.9	1081.1 ± 142.9	1026.5 ± 205.3	575.2 ± 83.9
Net increase*	–	747.5	593.2	46.7	775.9	724.3	270.0

Results are represented by means and standard deviation. BS-control: non inoculated model; BS-Lc: model inoculated with *L. curvatus* CRL705; BS-Mx: model inoculated with *L. curvatus* CRL705 and *S. vitulinus* GV318. Initial time (t_0); 3 days (t_3); 10 days (t_{10}).
Net increase = (BS-Xt_n − BS-control t_0); BS-X = BS-control, BS-Lc, BS-Mx; $t_n = t_3, t_{10}$.

certain amino acids different to the non-inoculated model. For instance, after 10 days of incubation BS-Lc showed increasing compositions compared to BS-control t_{10} in the concentrations of aspartate, glutamate, asparagine, arginine, alanine, leucine and ornithine (Fig. 5). On the other hand, the other amino acids such as histidine, serine, glutamine, glycine, lysine, threonine, tyrosine, phenylalanine and isoleucine exhibited a drop in their concentrations in BS-Lc t_{10} model. However, in the presence of the mixed starter culture, amino acids composition greatly varied with a significant decrease for all amino acids. It must be noticed that even though these amino acids had been generated not only from myofibrillar proteins but also from the sarcoplasmic fraction, results showed a general trend of amino acid releasing during incubation of the studied BS models.

3.4. Genes codifying for peptidases in *L. curvatus* CRL705

From the *in silico* analysis of the draft genome sequence of *L. curvatus* CRL705, the presence of genes involved in its proteolytic system was evaluated and compared to those of *L. sakei* 23 K, a microorganism intensively studied as model of meat adaption (Table 4). Results showed that both meat borne strains might lack the cell-wall bound proteinases PrtP type, which indicated that these microorganisms may be unable to breakdown proteins from the extracellular environment. However, genes encoding for intracellular peptidases as the endopeptidases PepO and PepF, the aminopeptidases PepC and PepN as well as the dipeptidases, tripeptidases and proline peptidases PepV, PepT, PepX, PepR and PepQ, were detected in both lactobacilli. Since, *L. sakei* 23K also codify for other endo and exopeptisases as PepE, PepG, PepD and PepL, *L. curvatus* CRL705 may exhibit a more reduced proteolytic system than the 23K strain.

4. Discussion

In this study, a global investigation of the proteolytic process during meat fermentation with an autochthonous starter culture was developed, particularly focusing on myofibrillar proteins by proteomic and peptidomic approaches. The beaker sausage models allowed discriminating the action of muscle peptidases and of bacterial strains. Results indicated that muscle peptidases exerted a slight myofibrillar proteolysis during incubation in non-inoculated model (BS-control). Although

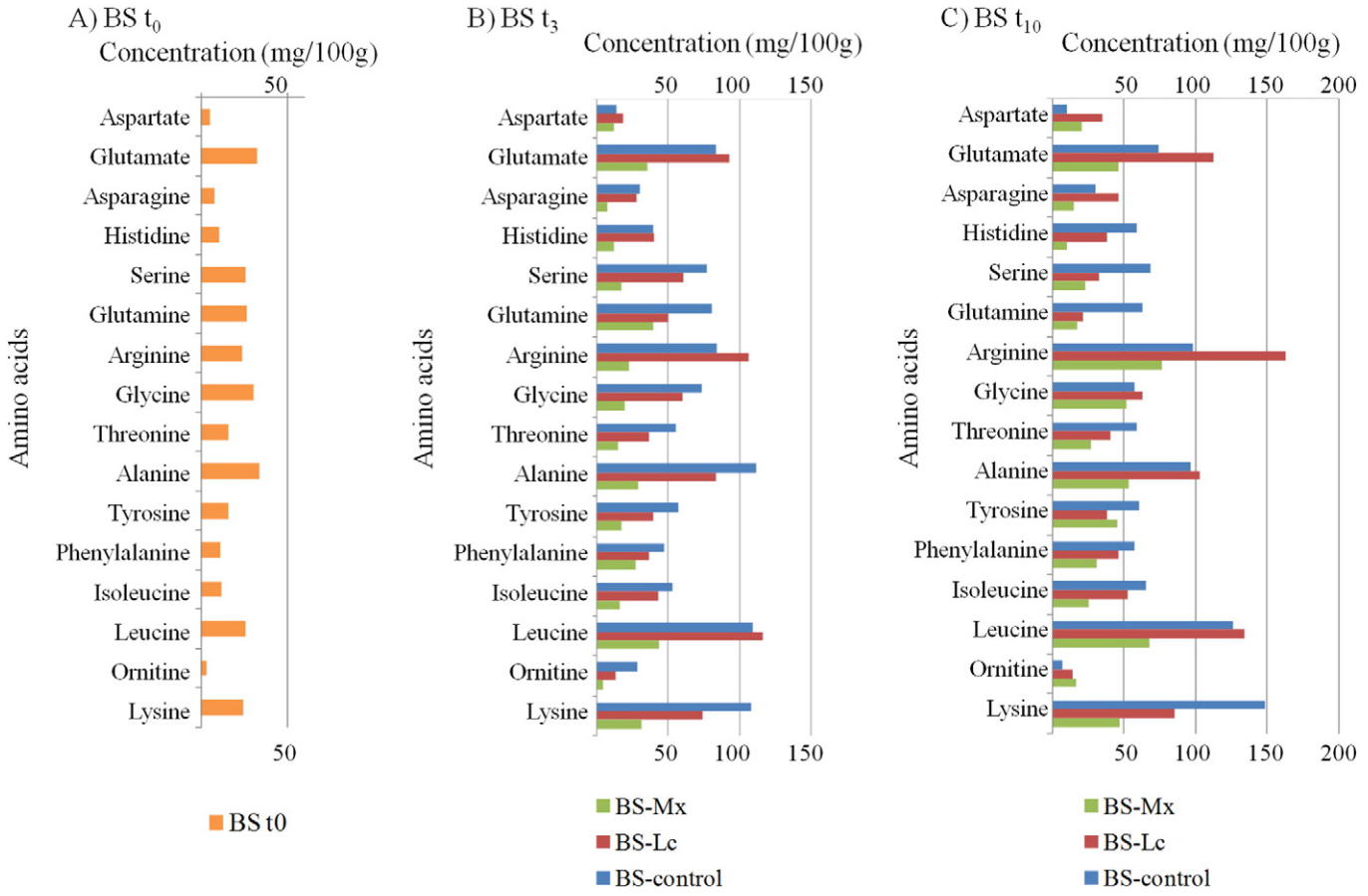

Fig. 5. Amino acid analysis. Concentration of amino acids identified from the different BS models at initial time (t_0) (A), after 3 (t_3) (B)and 10 days (t_{10}) (C) of incubation.

Table 4
In silico comparison between the proteolytic system of *L. curvatus* CRL705 and *L. sakei* 23K according to annotated genome sequences.

Proteolytic system members	Gene (access number in lactobacilli)	Substrate specificity of peptidases	*L. curvatus* CRL705 (gene sequence access number)	*L. sakei* 23K (Liu et al., 2010)
Cell-wall bound proteinases				
	PrtP(CAR88169)	–	–	–
	PrtM(CAR91105)	–	–	–
Peptidases				
Aminopeptidases	*PepC*(CAI55995)	X↓(X)n	✓ (NZ_AGBU0100001)	✓
	PepN(CAI54526)	X↓(X)n	✓ (NZ_AGBU0100075)	✓
Endopeptidases	*PepE*(CAX66570)	(X)n-X↓X-(X)n	–	✓
	PepG(CAX66140)	(X)n-X↓X-(X)n	–	✓
	PepO(CAI54353)	(X)n-X↓X-(X)n	✓ (NZ_AGBU0100049)	✓
	PepF(CAI54587)	X)n-X-X↓X-(X)n	✓ (NZ_AGBU0100001)	✓
Dipeptidases	*PepD*(CAI54622)	X↓X	–	✓
	PepV(CAI54725)	X↓X	✓ (NZ_AGBU0100022)	✓
Tripeptidase	*PepT*(BAG27201)	X↓X-X	✓ (NZ_AGBU0100035)	✓
Proline peptidases	*PepX*(EHE86008)	X-Pro↓ (X)n	✓ (NZ_AGBU0100016)	✓
	PepI(AGE37840)	Pro↓X-(X)n	–	–
	PepR(CAI54430)	Pro↓X	✓ (NZ_AGBU0100066)	✓
	PepL[a] (AAV43751)	Leu↓X o Leu↓X-X	–	✓
	PepP(CAX66584)	X↓Pro-(X)n	–	–
	PepQ(EHE85816)	X↓Pro	✓ (NZ_AGBU0100046)	✓

(✓): presence; (–) absence.
[a] PepL is included even when this peptidase do not break down a proline bound but belongs to the S33 protein family with PepI and PepR.

actin and MLC were hydrolyzed and the released amino acids increased in some degree, a reduced number of peptides were identified. Similarly, in previous studies using sterilized meat models, some endogenous proteolysis after four days was also evidenced (Fadda et al., 1999; Sanz et al., 1999). The main muscle enzymes involved in *post-mortem* muscle proteolysis and the precise role of each one is not yet fully understood, but some specific groups such as cathepsins and calpains have been deeply studied by meat scientists during decades (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010). While cathepsins remain active after months, the second ones are inactivated by salting (Armenteros, Aristoy, & Toldrá, 2009; Mora, Fraser, & Toldrá, 2013). Therefore, as sodium chloride was included in BS models, cathepsins might be responsible for the observed protein breakdown in the absence of microorganisms together with, most probably, other less studied proteolytic systems such as caspases, matrix metallopeptidases, etc. (Sentandreu, Coulis, & Ouali, 2002). In addition, the key contribution of *L. curvatus* CRL705 to myofibrillar proteolysis was brought to light in the present study. Specifically, the proteolysis of the myofibrillar fraction was intensified. As proposed in previous studies, *L. curvatus* CRL705 exerts a double role during protein breakdown (Fadda, López, et al., 2010; Fadda, Oliver, & Vignolo, 2002; Paredi et al., 2013). Indirectly and due to its acidogenic metabolism, the activity of acidic cathepsins is enhanced during fermentation, and although limited, a direct role of its own peptidases may be proposed. According to *L. curvatus* CRL705 genome data here analysed, this bacterium would lack cell-wall proteases, as a probable consequence of its adaptation to a nutritional rich environment as meat (Hebert et al., 2012). However, this strain contains genes encoding a wide set of intracellular peptidases such as endopeptidases, aminopeptidases, dipeptidases and tripeptidases. These enzymes, released by cell lysis during fermentation, probably allowed reaching the observed proteolysis in accordance with the aminopeptidase activities previously reported for this strain (Sanz et al., 1999). In conclusion, both effects of *L. curvatus* CRL705 (acidogenic + proteolytic) generated the unique proteolysis pattern: peptide and amino acids profiles. On the other hand, *S. vitulinus* GV318 in the presence of *L. curvatus* CRL705 also contributed to myofibrillar proteolysis, but in a different way. Actually, *S. vitulinus* GV318 might promote the degradation of several large peptides. In addition, the number of identified peptides from actin was lower than that generated by *L. curvatus* CRL705 suggesting that small peptides released from this protein may be further massively degraded. Concurrently, the mixed starter culture originated a remarkable lower amino acid composition than the LAB strain. This can be explained by the active bacterial metabolism and the concomitant amino acid consumption during fermentation in the inoculated models (López, Sentandreu, et al., 2015). Indeed, the enzymatic activities of *S. vitulinus* could be probably implicated in aroma compounds generation as a consequence of amino acids catabolism (Beck, 2005). Although it is a strain-dependent property, CNS had demonstrated a relevant protein hydrolytic ability even higher than LAB (Casaburi et al., 2008) whereas a few *Staphylococcus* isolated from meat products were able to degrade myofibrillar proteins (Drosinos, Paramithiotis, Kolovos, Tsikouras, & Metaxopoulos, 2007; Mauriello, Casaburi, Blaiotta, & Villani, 2004; Mauriello, Casaburi, & Villani, 2002). Other authors also reported an intense effect on meat proteins during sausage fermentation inoculated by a mixed starter culture formulated by *Lactobacillus* and *Staphylococcus* (Casaburi et al., 2008; Hughes et al., 2002). On the contrary, when *Pediococcus* and *Staphylococcus* were assayed as a mixed starter culture in a traditional dry fermented sausage, only scarce meat degradation was reported (Casquete et al., 2011). These facts pointed out the particular symbiotic interaction between *L. curvatus* CRL705 and *S. vitulinus* GV318 that allowed the generation of a specific proteolysis pattern involving proteins, peptides and amino acids under the conditions applied herein.

In addition, when the autochthonous starter culture used in this study had been evaluated in a similar beaker sausage model, the set of small peptides derived from hydrolysis of sarcoplasmic fraction was investigated. The described catalogue of peptides <3 kDa after a 10-day incubation (López, Sentandreu, et al., 2015) accounted for a total of 144 peptides, from which 44 arisen from de BS-control, 41 from the BS-Lc and 59 from the BS-Mx. Comparing these data with the number of peptides identified here after 10 days of incubation (3 peptides from BS-control, 20 from BS-Lc and 9 from BS-Mx), a minor contribution to the diversity of the whole peptidome could be assigned to the myofibrillar proteins. Similarly, when MS-MS analysis was applied to dry-cured ham after nine months of ripening, 69 peptides (11 from myoglobin and 58 from creatine kinase) were identified from sarcoplasmic proteins (Mora, Sentandreu, Fraser, Toldrá & Bramley, 2009; Mora & Toldrá, 2012), while only 14 peptides (9 from MLC-1 and 5 from titin) were detected from myofibrillar proteins (Mora, Sentandreu, Fraser, et al., 2009; Mora, Sentandreu, Koistinen, Fraser, Toldrá and Bramley, 2009). These results are in coincidence with the greater susceptibility to proteolysis of

sarcoplasmic proteins compared to myofibrillar fraction, supporting other studies (Fadda, López, et al., 2010).

Moreover, among the hydrolyzed myofibrillar proteins, the observed actin breakdown has to be underlined. In a previous study, actin degradation in commercial fermented sausages was related to the acidification of the meat matrix and explained by the known enhancement of cathepsins activity in acidic environments (López, Bru, et al., 2015). Moreover, the protein regions susceptible to hydrolysis reported herein are similar to those obtained when muscle cathepsins B and D were monitored by *in vitro* studies (Hughes, Healy, McSweeney, & O'Neill, 2000; Hughes, O'Neill, McSweeney, & Healy, 1999). Indeed, it could be proposed that, *L. curvatus* CRL705 command the broad degradation of actin by an indirect mechanism, as discussed before. In addition, the starter culture here evaluated was able to generate a particular set of LWM peptides from actin by the high contribution of exopeptidases. Particularly, peptides detected solely in the inoculated models are excellent candidates to be further proposed as reliable biomarkers for the distinctively presence of this specific autochthonous starter culture.

5. Conclusion

These studies allowed gaining insight on the proteolysis of myofibrillar proteins during meat fermentation emphasizing the role the starter cultures in the development of final product characteristics. A sequential enzymatic action might occur, involving firstly endopeptidases and then highly active exopeptidases, from both muscle and microbial origin. The model containing *L. curvatus* CRL 705 produced the highest number of LMW peptides and a net increase in amino acid content. In the light of these results, *L. curvatus* CRL705 and the mixed starter culture of this LAB with *S. vitulinus* GV318 greatly contributed to the proteolysis that is a key process for final product quality. In fact, these strains generated distinctive patterns of peptides and amino acids during proteolysis, which might contribute to the unique characteristics of produced fermented sausages. However, further studies must be conducted to determine the reproducibility in real fermented sausages and whether the identified peptides may be used as specific biomarkers of the starter culture inoculation. In addition, this study complete the whole peptidic (<3 kDa) map generated during 10 days of fermentation by *L. curvatus* CRL705 and *S. vitulinus* GV318 as that corresponding to the sarcoplasmic fraction has just been recently published.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2015.11.009.

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