BACTERIAL CONTRIBUTION TO PROTEOLYSIS DURING FERMENTATION AND RIPENING OF DRY SAUSAGES

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Abstract. **Argentine has a well established reputation as a high quality beef supplier around the world, as well as possesses one of the world's highest levels of beef consumption per capita. Although this well known globally perception, quality objective measure for processed meat products seldom reach such a high quality standards. To enhance hygienic and sensory features of fermented meat products, attention on the development of starter cultures with adequate fermentation characteristics must be provided. Meat proteins degradation that takes place during ripening of dry fermented sausages leads to an increase in peptides and amino acids concentration as a result of the proteolytic activities of both endogenous and microbial enzymes. In this regards, lactic acid bacteria are essential agents mainly through their metabolic activities on carbohydrates and proteins resulting in pH reduction and the generation of flavour compounds. In this context, this study aims to determine the contribution of Argentinean sausage microbiota in the generation of typical flavour compounds to standardize quality while preserving product typicity to be awarded a Protected Designation of Origin (PDO).**

Index terms- **Fermented sausages, flavour, meat, protein degradation, starter cultures**

I. INTRODUCTION

Fermented sausages are defined as meat products consisting of meat and fat particles, salt, curing agents and spices which have been stuffed into a casing, fermented (ripening) and dried. Nowadays, their manufacture is a very important part of the meat industry, large-scale industrial processes relying on selected starter cultures to get high quality final products. Lactic acid bacteria (LAB) and Coagulase negative Gram positive Cocci (CGC) such as *Staphylococcus* and *Kocuria*, are the two main groups involved in fermentation of dry cured sausages (Fontana, Cocconcelli & Vignolo, 2005; Vignolo, Fontana & Fadda, 2010). Although, it is still unknown which bacterial group play the major role in the desirable aroma development, two interdependently reactions occur: pH decrease by LAB acid production and nitrates reduction by CGC. The characteristic taste and aroma of fermented sausage are due to many different non-volatile and volatile compounds, some of them originated from added spices while others are products of metabolic or chemical reactions derived from carbohydrates, proteins and lipids during ripening. Meat proteolysis has been widely studied and the contribution of some peptide fractions from the degradation of meat proteins to flavour was generally accepted (Sentandreu, Armenteros, Calvete, Ouali, Aristoy & Toldrá, 2007). The reduction in the concentration of peptides from 2700 to 4500 Da during dry cured ham process was reported by Toldrá, Flores and Sanz (1997) while those between 1000 and 10000 Da from cooked beef have also been related with desirable flavor (Nishimura, 2002). In this study, peptide fractions lower than 3000 Da from protein breakdown potentially related with taste will be focused. Knowledge about the identity of peptides generated by the microbiota present in Argentinean fermented sausages will be of value as a tool to improve product quality and to enhance competitiveness.

II. MATERIALS AND METHODS

A. Samples

In this study, five different commercial dry sausages purchased in Argentinean supermarkets as well as raw bovine meat were analyzed.

B. Peptides extraction

A 2.5 g sample from each dry sausage and row meat were homogenized in stomacher (8 min, in ice) with 12.5 ml 0.01N HCl and 0.1N HCl, respectively as has been described by Sentandreu, Stoeva, Aristoy, Laib, Voelter and Toldrá (2003). The slurries were then centrifuged (13500 rpm at 4ºC for 20 min) and supernatants submitted to ultra-filtration with a 3 kDa cut-off Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-3 membrane (Millipore, Billerica, USA).

C. RP-HPLC

Peptide extracts (2µg/µl protein) were injected in a Pursuit XRs C18 (250_4.6 mm, 5µ particle size) reversed-phase column (Palo Alto, CA, USA). Separation was monitored at 214, 220 and 280 nm and fractions were automatically collected using a Smartline Knauer HPLC system (Berlin, Germany) at a flow rate of 1 mL/min and using the following conditions: isocratic 0.1% TFA in water (solution A) for 5min, followed by a linear gradient from 0% to 100% of acetonitrile and 0.1% TFA (solution B) for 35 min.

D. Mass spectrometry (MS)

Differential peaks between raw meat and fermented sausages were selected from the chromatograms obtained at 214 nm and subjected to identification using a MALDI-TOF-TOF spectrometer Ultraflex II (Bruker) from 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 employed.

III. RESULTS AND DISCUSSION

In order to identify peptides generated during fermentation and to determine those putatively assigned to microbial proteolysis, five commercial dry sausages and raw meat as pre-fermentation control were analyzed. Peptide fractions with a cut-off less than 3000 Da from fermented sausages analyzed by RP-HPLC exhibited differential peaks when compared to raw meat (Fig. 1). Thirty six (36) different peptides deriving from fractions G3, G4, G5, G7 and G8 were obtained from mass spectrometric analysis. The results showed a complex mixture of small peptides generated from meat during fermentation and ripening due to degradation of myofibrillar and sarcoplasmic proteins (Table 1). A large variety of peptides derived from sarcoplasmic proteins were detected, indicating that this fraction mainly contribute to the peptide diversity. On the other hand, peptides from myofibrillar proteins such as actin alpha skeletal muscle, myosin-1 and capz-interacting protein were detected, these findings being in accordance with those of Lametsch, Karlsson, Rosenvold, Andersen, Roepstorff and Bendixen (2003) in which the post-mortem process of pig muscle was studied.
The wide range of cleavage sites obtained clearly exhibits the activity of both, muscle and bacterial proteolyt ic systems. Molecular weight of characterized peptides was observed to range between 1000 and 2100 Da suggesting that they may have a potential role in flavor development. When meat proteins breakdown by muscle proteases such as cathepsin B, D, L and calpains (Dransfiel, 1992; Molly, Demeyer, Johansson, Raemaekers, Ghistelinck & Geenen, 1997; Hughes, Healy, McSweeney & O'Neill, 2000; Mora, Sentandreu, Fraser, Toldrá & Bramley, 2009a;b) were compared with proteolysis occurred during ripening of fermented sausages, it may be highlighted that some of the obtained peptides in this study may have been originated by microbial proteases since they were absent in muscle enzymatic breakdown. Figure 2 shows the primary sequence of peptides identified as follows: ^{32}VFPSIVGRPRHQG^{44} and ^{85}EKIWHHTF^{92} derived from actin (A) and ^{2}PFGNTHNKHKLNF^{14} derived from creatine kinase (B), all of them belonging to the N terminal region. Since the three peptides originated from myosin contained the common sequence ^{12}EAAPYLRK^{18} (C), the activity of endopeptidases against N-terminal region and their further degradation by exopeptidases may be suggested.

IV. CONCLUSION

From this study, the bacterial contribution in the generation of peptides during ripening of dry cured sausages was demonstrated. An adequate selection of starter culture is essential to achieve high quality PDO products.

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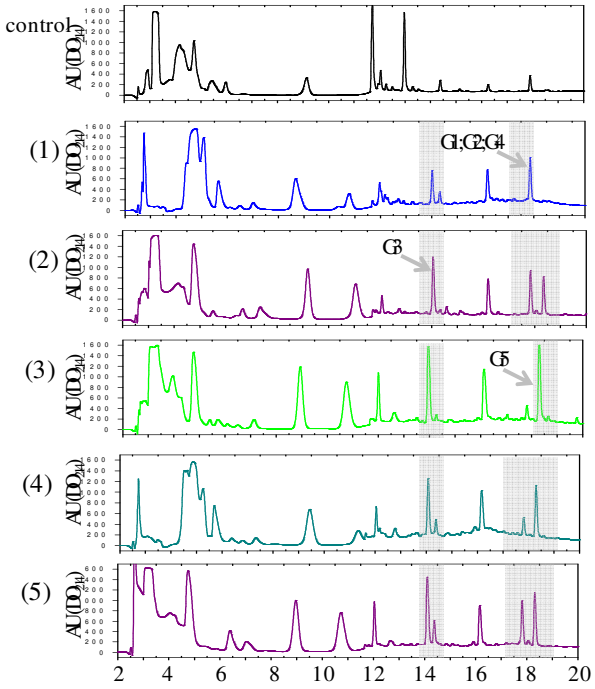

Figure 1:RP-HPLC chromatograms of less than 3 kDa peptide fractions from fermented sausages and meat. Sausage samples: (1) high comminuted sausage; (2) and (5) fuet type salami; (3) and (4) low comminuted sausage. Industrial manufacture: (1), (2) and (5); artisanal manufacture: (3) and (4). Differential peaks between meat and dry sausages are highlighted in gray. Arrows indicate peaks selected for MS analysis.

A)

MDDDIAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQS
KRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPTLLTEAPLNPKANREKMT
QIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYALPHAILRLDL
AGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKSY
ELPDGQVITIGNERFRCPEALFQPSFLGMESCGIHETTFNSIMKCDVDIRKDLYANTVLS
GGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQ
EYDESGPSIVHRKCF

B)

MPFGNTHNKHKLNFKAEEEYPDLSKHNNHMAKALTLEIYKKLRDKETPSGFTLDDVIQTG
VDNPGHPFIMTVGCVAGDEESYTVFKDLFDPIIQDRHGGFKPTDKHKTDLNHENLKGGDD
LDPNYVLSSRVRTGRSIKGYALPPHCSRGERPAVEKLSVEALNSLTGEFKGKYYPLKSMT
EQEQQQLIDDHFLFDKPVSPLLLASGMARDWPDARGIWHNDNKSFLVWVNEEDHLRVISM
EKGGNMKEVFRRFCVGLQKIEEIFKKAGHPFMWNEHLGYVLTCPSNLGTGLRGGVHVKLA
HLSKHPKFEEILTRLRLQKRGTGGVDTAAVGSVFDVSNADRLGSSEVEQVQLVVDGVKLM
VEMEKKLEKGQSIDDMIPAQK

C)

MSSDQEMAVFGEAAPYLRKSEKERIEAQNKPFDAKTSVFVADPKESFVKATVQSREGGKV
TAKTEAGATVTVKEDQVFPMNPPKFDKIEDMAMMTHLHEPAVLYNLKERYAAWMIYTYSG
LFCVTVNPYKWLPVYNAEVVTAYRGKKRQEAPPHIFSISDNAYQFMLTDRENQSILITGE

Figure 2: Peptide map of (A) actin, alpha skeletal muscle; (B) cretine kinase M-type; (C) N terminal region of myosin-1. In red: bacterial probably originated peptides. In green: cathepsin B cleavage sites.

Nº	Identified Sequence	Z calc.	Score	Original Protein	Protein aa.	Position
1	FAGDDAPRAVFPS	1349.6484	23.4	Actin, alpha skeletal muscle; P68138	377	23 - 35
2	VFPSIVGRPRHQG	1449.8073	19.1	Actin, alpha skeletal muscle; P68138	377	32 - 44
3	EKIWHHTF	1097.24	39.3	Actin, alpha skeletal muscle; P68138	377	85 - 92
4	RVAPEEHPTL	1148.6058	38.0	Actin, alpha skeletal muscle; P68138	377	97 - 106
5	AGQHPARASSSEAEDGCGSP	1970.8257	10.7	Capz-interacting protein; Q3ZBT0	381	253 - 272
6	GEAAPYLRKSEKERIEAQN	2189.1309	64.5	Capz-interacting protein; Q3ZBT0	1938	11 - 29
7	EAAPYLRKSEKERIEAQN	2132.1095	58.4	Myosin-1; Q9BE40	1938	12 - 29
8	GEAAPYLRK	1004.5524	17.0	Myosin-1; Q9BE40	1938	11- 18
9	RLKSVGIPVL	1081.7092	22.30	Abhydrolase domain-containing protein 3; Q0VC00	411	330 - 339
10	GALRWDLPRVQGGSQLSGLF	2157.1563	17.90	AP-4 complex subunit mu-1; Q29RY8	452	359 - 378
11	KAGASIVGVNCHFDPTIS	1872.9273	23.00	Betaine--homocysteine S-methyltransferase 1; Q5I597	407	207 - 224
12	IIEQGIDLFT	1148.6198	17.10	BrefeldinA-inhibited guanine nucleotide-exchange protein 1; O46382	1849	701 - 710
13	GASPTPGEAQRHLQTHR	1842.9288	20.90	Calmodulin-like protein 4; Q3T0E8	153	41 - 57
14	GIQRAADIEQQ	1228.6280	16.80	Coagulation factor V precursor; Q28107	2211	534 - 544
15	GAKNCLRDFIEKV	1549.8155	14.10	Complement factor B precursor; P81187	761	288 - 300
16	DVIQTGVDNPGHPF	1495.7176	39.90	Creatine kinase M-type- Q9XSC6	381	55 - 68
17	PFGNTHNKHKLNF	1553.7972	21.20	Creatine kinase M-type- Q9XSC6	381	02 - 14
18	LGVTKDAGDEDL	1232.6005	13.90	DnaJ homolog sub- familyB member 14; Q0IIE8	379	113 - 124
19	ERVGELMDQNA	1261.5841	12.10	General transcription factor IIH subunit 5;Q2T9Z5	71	55 - 65
20	NAWGKVEADVAGHGQE	1667.7772	28.80	Myoglobin-P02192	154	13 - 28
21	HAKHPSDFGADAQAAMSK	1868.8708	18.70	Myoglobin-P02192	154	117 - 134
22	AAQYKVLGFHG	1190.6317	48.90	Myoglobin-P02192	154	144 - 154
23	IGTDSALHRIMEVIDAITTT	2157.1220	20.60	6-phosphofructo-kinase. liver type;A1A4J1	780	176 –195
24	AGPRSDPAGPP	1021.5061	19.4	Phosphate carrier protein, mitochondrial precursor; P12234	362	27- 37
25	ASHHDINDASRGTLSS	1667.7732	17.80	Poly(A) RNA polymerase GLD2; Q2HJ44	484	315 - 330
26	DTAIVDRGKNVVS	1373.7383	18.30	Proteasomal ATPase-associated factor 1; Q148I1	392	181 - 193
27	AGAPSVENVKNGIR	1411.7652	17.00	Protein phosphatase 1; AO62829	382	89 - 102
28	TAAKLLHAGFKGRV	1468.8747	13.20	Prothrombin precursor; P00735	625	491 - 504
29	AGKATLFVSN*N*SRR	1520.8292	21.20	Pyridoxal phosphate phosphatase; Q3ZBF9	296	50 - 63
30	LETAAGEALGQTL	1273.4562	18.30	Retinol dehydrogenase 8; Q9N126	312	48 - 60
31	DSVNAQADRAF	1193.5545	13.10	Testis-specificY-en-coded-like protein 1; Q0P5N2	432	235 - 245
32	SSTYWEGKSDMET	1520.6210	17.10	Threonyl-tRNAsyn- thetase, cytoplasmic; Q0P5N2	723	281 - 293
33	SSHAKKATVD	1043.5480	15.20	Transcription initiation factor TFIID subunit 9; Q17QQ4	264	58 - 67
34	PQGALSLEADGHPAAR	1589.8030	24.10	Uncharacterized protein KIAA1462 homolog; A2VE02	1305	1268-1283
35	SSLIRHQRT	1097.6174	20.40	Zinc finger protein 572; Q32KN0	425	186 - 194
36	AGPNSPTGGGGGGGSGGTR	1500.6786	13.80	Zinc finger SWIM domain-containing protein KIAA0913; 7E305	1413	49 - 67

Table 1: Characterized peptides present en fermented dry sausages originated from breakdown of sarcoplasmic proteins (1-9) and myofibrillar (9-36). It also shows Molecular weight calculated (Z calc.), mascot score, parental protein and accession number in UniProtKB database (original protein), amino acids number of whole protein (Protein aa.) and localization of peptide into protein (Position).