SMALL PEPTIDES PRODUCED BY AUTOCHTHONOUS LACTIC ACID BACTERIA IN A LOW SODIUM BEAKER SAUSAGE MODEL

Almeida, M.A. [1], Contreras-Castillo, C. J.[1] Sentandreu, M. A.[2] and Fadda, S. G[3]

[1]Department of Agroindustry, Food and Nutrition of Escola Superior de Agricultura "Luiz de Queiroz", University of São Paulo, Av. Pádua Dias 11, 13418-900 Piracicaba, São Paulo, Brazil
[2] Instituto de Agroquímica y Tecnología de Alimentos, CSIC, C/ Agustín Escardino 46980 Paterna (Valencia), Spain.
[3]Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina.

Abstract –The typical flavor of fermented sausages is produced in part by compounds coming from meat protein degradation, such as small peptides and free amino acids. In order to improve and standardize salami production, the use of starter cultures has been extended during the last decades. Lactic acid bacteria (LAB) and Coagulase negative Gram positive Cocci are the most common groups of bacteria constituting starter cultures. In this study Beaker Sausage (BS) models were used to simulate the production of salami with low-sodium contents to evaluate the effect of three different autochthonous starter cultures on meat proteolysis, by a peptidomic approach. The BS containing *Enterococcus mundtii* CRL35 showed the highest variety of small peptides followed by the BS containing *L. curvatus* CRL1862. However, the meat model inoculated with *Lactobacillus plantarum* CRL681 produced the highest number of peptides derived from sarcoplasmic proteins. The considerable amount of small peptide produced in the inoculated BS allows inferring that lower sodium contents did not negatively affect the action of microbial peptidases.

Key Words – fermentated sausages, proteolysis starter culture, peptidomics.

I. INTRODUCTION

Salami production and consumption have a long tradition in Europe, initiated in Mediterranean countries and expanded to other nations including Brazil, United States, Argentina and Australia [1]. 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 [2]. 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 [3]. As well as, a current tendency in all food industries is lowering sodium contents in view to obtain healthier meat products [4].

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 derived from meat proteins to flavor, was generally accepted [5]. The non-volatile components, such as small peptides and amino acids providing basic tastes such as sweet, salty, sour and bitter are produced during the hydrolysis of meat proteins and contribute to the sensory characteristics of final products [6]. The objective of this study was to evaluate the effect of selected autochthonous starter cultures on protein hydrolysis during fermentation of a low sodium beaker sausage model by analyzing the small peptide production. The analysis of low molecular weight peptides (< 3 kDa) derived from meat proteins was assessed by Liquid Chromatography coupled to Electrospray Ionisation tandem Mass Spectrometry (LC-ESI-MS/MS).

II. MATERIALS AND METHODS

Strains and culture conditions: *Lactobacillus (L.) plantarum* CRL681; *L. curvatus* CRL1862

and *Staphylococcus (S.) vitulinus* GV318, were isolated from artisanal fermented sausage (Argentina); *Enterococcus (E.) mundtii* CRL35 from Argentinean artisanal cheese. They were routinely grown in De Mann Rogosa & Sharpe (MRS) or Brain Heart Infusion (BHI) broth (Britania, Argentina), for LAB or *Staphylococcus* respectively.

Beaker sausage models (BS): Beaker sausage (BS) models were prepared as follows meat from *Longissimus dorsi* (70%) and bovine *Semimembranosus* (30%) were aseptically sampled, according to Vignolo [7]. The additives were sterilized in autoclave (121°C, 15 minutes) or by filtration (0.22 µm) (nitrate, nitirite and erythorbate solutions). Additives were added to chopped meat to a final concentration of 1.0% NaCl, 0.25% KCl and 0.25% $CaCl_2$, replacing the usual amount of NaCl, 0.75% glucose; 0.75% sucrose, 0.015% sodium nitrite, 0.015% sodium nitrate and 0.5% sodium erythorbate. Four batches were prepared: BS control added with antibiotics (20.000 UI/Kg penicillin, 20 mg/Kg streptomycin, 50 mg/Kg amphotericin B and 0.01% sodium azide) and BS inoculated with approximately 7-8 log CFU/g with three different starter cultures: BS-Lps681 containing *L. plantarum* CRL681 + *S. vitulinus* GV318; BS-Lss1862, inoculated with *L. sakei* CRL1862 + *S. vitulinus* GV318 and BS-Ems35, inoculated with *Enterococcus mundtii* CRL35 + *Staphylococcus vitulinus* GV318. Portions of 30 g were included on sterile tubes and incubated at 22ºC. Three replicates were assayed and samples were collected at 0, 3, 6 and 10 days of ripening.

Peptide extraction and sequence identification by LC-ESI-MS/MS: Ten grams from each BS sample were homogenized with 0.1N HCl (1:5 w/v). The meat slurries were centrifuged (13500 rpm at 4 ºC for 20 min) and supernatants submitted to ultra-filtration in Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-3 membrane (Millipore, USA). The obtained filtrate was freeze-dried until LC-ESI-MS/MS analyses.

Samples were re-dissolved, injected and separated as reported by Sentandreu [5]. A Surveyor LC system directly coupled to a LCQ Advantage Ion trap MS instrument (Thermo Scientific, USA) and a Jupiter Proteo reverse phase column (150 x 0.5 mm; Phenomenex, USA) were used for this study. Data acquisition was done using the Xcalibur v2.0 software. Peptide identification was obtained from the MS/MS spectral data using an in-house version of the Mascot search engine v2.3 and Uniprot KB protein database. Only top ranking significant peptides were considered, taking a reference peptide score threshold of 25. Selected identifications were manually checked with respect to the assignation of the identified masses to *b* and *y* series ions.

III. RESULTS AND DISCUSSION

In this work a total of 75 peptides were accurately identified. However **Table 1** summarizes only the 50 peptides originated exclusively on each system due to the action of assayed starter cultures. In the non-inoculated batch (BS-control) only two peptides could be identified, these coming from actin and frutose-1,6- bi-phosphatase. They were surely produced by the action of endogenous enzymes. In contrast, a higher diversity of peptides was observed in the batches inoculated with the different starter cultures. Regarding the peptides originated exclusively by the presence of starter cultures, BS-Ems35 produced the 66% of identified peptides, BS-Lps681 the 20%, BS-Lss1862 the 10% while the non-inoculated control (BS control), as expected, produced only the 4% of the total identified peptides. The combination of strains containing *E. mundtii* and *S. vitulinus* GV318 (BS-Ems35), produced 33 original peptides; 24.3% derived from myosin and 66.6% from actin, and a small percentage derived from sarcoplasmic proteins. The other studied strains produced, in general, smaller amount of unique peptides when compared with *E. mundtii* CRL35, although we can highlight that the batch containing *L. plantarum* CRL681 produced the highest number of peptides arising from sarcoplasmic proteins (80%).

The considerable amount of small peptides produced allows inferring that lower sodium contents did not negatively affect the action of microbial enzymes. Moreover, sodium chloride has the important function of exposing proteins by denaturation to the action of peptidases.

Table 1 – Identification of small peptides produced after 10 days of Beaker Sausage incubation at 22°C.

No	Sequence identified (BS control)	Parental protein	I	F
1	M.WITKQEYDEAGPSIVH.R	Actina, Alpha skeletalmuscle	Trp_{358}	His_{373}
2	T.CVLVSEEDEHAIIVEPE.K	Frutose - 1,6- biphosphatase	Cys_{93}	Glu_{109}
No	**Sequence identified (BS-Ems35)**	**Parental protein**	**Initial**	**Final**
1	G.RLNVKNEELDAMMKEASGPIN.F	Myosin regulatory light chain 2 skeletal muscle isoform	Arg_{60}	Asn_{80}
2	R.LNVKNEELDAMMKEASGPIN.F	Myosin regulatory light chain 2 skeletal muscle isoform	Leu_{61}	Asn_{80}
3	V.KNEELDAMMKEASGPIN.F	Myosin regulatory light chain 2 skeletal muscle isoform	Lys_{64}	Asn_{80}
4	N.EELDAMMKEASGPIN.F	Myosin regulatory light chain 2 skeletal muscle isoform	Glu_{66}	Asn_{80}
5	G.DVLRALGTNPTNAEVKKVLGNPSN.E	Myosin light chain 1/3 skeletal muscle	Asp_{75}	Asn_{98}
6	T.LTVKEDQVFPMNPP.K	Myosin - 2 OS	Leu_{70}	Pro_{83}
7	L.TVKEDQVFPMNPP.K	Myosin - 2 OS	Thr_{71}	Pro_{83}
8	F.AGDDAPRAVFPSIVGRPR.H	Actin, Alpha skeletal muscle	Ala_{24}	Arg_{41}
9	A.GDDAPRAVFPSIVGRPR.H	Actin, Alpha skeletal muscle	Gly_{25}	Arg_{41}
10	G.DDAPRAVFPSIVGRPR.H	Actin, Alpha skeletal muscle	Asp_{26}	Arg_{41}
11	A.PSIVGRPR.H	Actin, Alpha skeletal muscle	Pro_{32}	Arg_{41}
12	L.DFENEMATAASSSSLEKS.Y	Actin, Alpha skeletal muscle	Asp_{224}	Ser_{241}
13	A.ASSSSLEKSYELPDGQVIT.I	Actin, Alpha skeletal muscle	Ala_{233}	Thr_{251}
14	A.TAASSSSLEKSYELPDGQVIT.I	Actin, Alpha skeletal muscle	Thr_{231}	Thr_{251}
15	A.TAASSSSLEKSYELPDGQVITGNER.F	Actin, Alpha skeletal muscle	Thr_{231}	Arg_{256}
16	S.SSSLEKSYELPDGQVIT.I	Actin, Alpha skeletal muscle	Ser_{236}	Thr_{251}
17	S.SSSLEKSYELPDGQVITIGN.E	Actin, Alpha skeletal muscle	Ser_{236}	Asn_{254}
18	S.SSSLEKSYELPDGQVITIGNER.F	Actin, Alpha skeletal muscle	Ser_{236}	Arg_{256}
19	S.SSSSLEKSYELPDGQVIT.I	Actin, Alpha skeletal muscle	Ser_{235}	Thr_{251}
20	S.LEKSYELPDGQVITIGN.E	Actin, Alpha skeletal muscle	Leu_{238}	Asn_{254}
21	S.LEKSYELPDGQVIT.I	Actin, Alpha skeletal muscle	Leu_{238}	Thr_{251}
22	L.EKSYELPDGQVITIGN.E	Actin, Alpha skeletal muscle	Glu_{239}	Asn_{254}
23	E.KSYELPDGQVITIGN.E	Actin, Alpha skeletal muscle	Lys_{240}	Asn_{254}
24	S.YELPDGQVIT.I	Actin, Alpha skeletal muscle	Tyr_{242}	Thr_{251}
25	S.YELPDGQVITIGNER.F	Actin, Alpha skeletal muscle	Tyr_{242}	Arg_{256}
26	I.TNWDDMEKIWHH.T	Actin, Alpha skeletal muscle	Thr_{79}	His_{90}
27	T.NWDDMEKIWHH.T	Actin, Alpha skeletal muscle	Asn_{80}	His_{90}
28	N.WDDMEKIWHH.T	Actin, Alpha skeletal muscle	Trp_{81}	His_{90}
29	L.DSGDGVTHNVPIYEGYALPHA.I	Actin, Alpha skeletal muscle	Asp_{156}	Ala_{176}
30	E.RNVKMQRQEGAKVCLMSPEQLQKKFP.W	FAD Dependent oxidoredutase domain containing protein 1	Arg_{173}	Pro_{198}
31	N.KMIRKGVFKDQHFDPNLNFMYIEVDK.V	Serine/threonine protein kinase PRP4	Trp_{911}	Lys_{936}
32	F.DPNLNFMYIEVDK.V	Serine/threonine protein kinase PRP4	D_{924}	Lys_{936}
33	M.WAAFPPDVGGNVDYK.N	Myosin regulatory light chain 2 skeletal muscle isoform	Trp_{141}	Lys_{155}
No	**Sequence identified (BS-Lss1862)**	**Parental protein**	**Initial**	**Final**
1	N.EMATAASSSSLEKSYELPDGQVITIGNER.F	Actin, Alpha skeletal muscle	Glu_{228}	Arg_{256}
2	A.AAPAPAPAPAPAPAPAPP.K	Myosin light chain 1/3 skeletal muscle	Ala_{14}	Pro_{31}
3	D.DAPRAVFPSIVG.R	Actin, Alpha skeletal muscle	Asp_{27}	Gly_{38}
4	V.FPSIVG.R	Actin, Alpha skeletal muscle	Phe33	Gly_{38}
5	I.TNWDDMEKIWH.H	Actin, Alpha skeletal muscle	Thr_{79}	His_{89}
No	**Sequence identified (BS-Lps681)**	**Parental protein**	**Initial**	**Final**
1	A.FPPDVG.G	Miosina regulatory light chain 2 skeletal muscle isoform	Phe_{144}	Gly_{149}
2	A.FPPDVGGNVD.Y	Miosina regulatory light chain 2 skeletal muscle isoform	Phe_{144}	Asp_{153}
3	L.DDVIQTGVDNPGHPFI.M	Creatine Kinase M - Type	Asp_{54}	Ile_{69}
4	R.DWPDARGIWH.N	Creatine Kinase M - Type	Asp_{210}	His_{219}
5	T.APPIQSPLPVIPH.Q	LIM dominan binding protein 3	Ala_{93}	His_{105}
6	T.APPIQSPLPVIPHQ.K	LIM dominan binding protein 3	Ala_{93}	Gln_{106}
7	Q.SPLPVIP.H	LIM dominan binding protein 3	Ser_{98}	Pro_{104}
8	Q.SPLPVIPH.Q	LIM dominan binding protein 3	Ser_{98}	His_{105}
9	V.MQRDIAAGDFIEHAEFSGNIYG.T	Guanylate Kinase	Met_{61}	Gly_{82}
10	K.LRNKMTPSGYTLDQCIQTGVDNPGHPF.I	Creatine Kinase S - Type - mitochondrial	Leu_{76}	Phe_{102}

Glycine (Gly); Alanine (Ala); Leucine (Leu); Valine (Val); Isoleucine (Ile); Proline (Pro); Phenylalanine (Phe); Serine (Ser); Threonine (Thr); Cysteine (Cys); Tyrosine (Tyr); Asparagine (Asn); Glutamine (Gln); Aspartate (Asp); Glutamate or Glutamate acid (Glu); Arginine (Arg); Lysine (Lys); Histidine (His); Tryptophan (Trp) and methionine (Met). I - the initial cleavage site (N-terminal) F - final cleavage site (C-terminal)

Some authors reported that endogenous enzymes are involved in the production of small peptides, specially derived from actin and myosin [8]. In this work, a low action of muscle enzymes can be inferred by the low number of peptides identified in the non-inoculated control, although muscle enzymes could be activated due to the acid produced by LAB metabolism in the inoculated batches. The presence of each LAB strain in the BS could generate a unique profile that could serve as a distinctive biochemical trait to differentiate specific Salami production. Results showed that *E. mundtii* CRL35 has the highest peptidogenic ability. Lu [9] reported that the primary structure and amino acid sequence are related to taste properties of peptides. Thus we can assume that each bacterial combination assayed herein could influence the global taste of salami when applied as starter culture. Due to the optimal peptidogenic ability of *E. mundtii*, it could be combined with other strain such as *L. sakei* CRL1862 with an optimal proteolytic activity and a high free amino acid production [10]. This combination could constitute a new starter culture for low sodium fermented products.

IV. CONCLUSION

The peptidogenic ability of three LAB strains in combination of *Staphylococcus vitulinus* was demonstrated in a low sodium fermented sausage model using a peptidomic approach. Unique profiles of small peptides coming from sarcoplasmic and myofibrillar proteins are produced by each system containing different LAB. Finally we can conclude that the use of lower concentrations of sodium in the preparation of fermented sausages could have a further positive effect to the known action on the cardiovascular system, related to the production of small peptides, during ripening, beneficial to the flavor development in these products.

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