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Effect of starter culture on proteolytic changes during processing of fermented beef sausages

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ABSTRACT

Fermented beef sausages inoculated with four different starter cultures (*Pediococcus acidilactici, Lactobacillus curvatus, Lactobacillus sake*, or *Streptomyces griseus*) were evaluated for proteolysis during process stages (prefermentation, fermentation, drying and heating). Increases ($p \leqslant 0.05$) in the nonprotein nitrogen (NPN) fraction were found at sequential stages of processing, while starter cultures had no major effects on NPN content. Concentrations of most free amino acids increased ($p \leqslant 0.05$) during fermentation and drying, and culture effects were found for differences among concentrations of some individual free amino acids. From SDS–PAGE analysis of sarcoplasmic and myofibrillar protein fractions after fermentation and drying, myosin heavy and light chains, actin and troponin were degraded during processing. However, starter culture effects were absent from SDS–PAGE protein patterns.

1. Introduction

Many biochemical reactions involving proteins, carbohydrates and lipids occur during fermented sausage processing and determine the ultimate characteristics of the product (Hierro, de la Hoz, & Ordonez, 1999). Meat protein is one of the more important components in sausage due to its nutritional role and its functional and structural contributions to final product characteristics (Ibanez, Quintanilla, Cid, Astiasaran, & Bello, 1997). Protein breakdown evidently participates in the formation of desirable texture, flavour and availability of flavour precursors in all fermented dry sausage varieties (Diaz, Fernandez, Garcia de Fernando, de la Hoz, & Ordonez, 1993). Although alteration of meat proteins during meat conditioning has been extensively studied, due to the relation between protein degradation and meat tenderisation, knowledge of proteolysis occurring during fermented sausage processing is still limited (Sanz & Toldra, 1997).

Proteolysis in fermented sausages is one of the main biochemical reactions, catalysed by either endogenous enzymes present in the meat tissues or by those of microbial origin from added starter cultures. Meat proteins are known to undergo hydrolysis, first to polypeptides by endogenous muscle enzymes, such as cathepsins and calpains, and then further to smaller peptides by the action of peptidases. Free amino acid generation from peptides by aminopeptidases is the final step in proteolysis phenomena and has been attributed to bacterial enzymes as well as enzymes inherent in meat itself (Casaburi et al., 2007, 2008; Hughes et al., 2002; Verplaetse, 1994). Low molecular weight peptides and free amino acids are major components of the nonprotein nitrogen (NPN) fraction in fermented meats and contribute, directly or indirectly, to generation of volatile and nonvolatile flavour compounds in dry and semi-dry sausages (Demasi, Wardlaw, Dick, & Acton, 1990). Aldehydes and ketones with intense aroma characteristics, resulting from further catabolism of the amino acids, have an obvious role in development of flavour (Sanz & Toldra, 1997).

The most commonly used starter cultures among lactic acid bacteria are *Pediococcus acidilactici*, *Lactobacillus sake* and *Lactobacillus curvatus* with some additional strains of lactic acid bacteria reported to have a considerable proteolytic effect, due to their respective enzymatic activities (Montel, Seronie, Talon, & Hebraud, 1995; Sanz & Toldra, 1997). *Streptomyces griseus* has been introduced in Europe as a starter culture and produces a protease which has a possible role in proteolytic activity in fermented sausage processing (Diaz et al., 1993; Eilberg & Liepe, 1977).

The extent of proteolysis associated with starter cultures may be related to the particular processing technology utilised. In rapid sausage fermentation for quick acid production at elevated temperatures ($\geqslant 34$ °C) there may not be enough time for proteolytic activity expression by starter cultures. Rapid fermentations utilising *P. acidilactici* are commonly practised in the USA. European processors typically utilise lactobacilli and lower temperatures ($\geqslant 22$ °C) within traditional "ripening" processes (combining

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fermentation and ageing) over 3–5 days. This slower process allows starter cultures the ability to contribute to flavour development, *via* lipolytic or proteolytic activity, in addition to acidity development (Acton, 1977; Bacus, 1984).

In this study the typical USA rapid fermented sausage production process was modified by introducing a low temperature, pre-fermentation (ageing) period for sausages prior to temperature elevation for rapid fermentation. The effects at each process stage on resultant starter culture activities related to proteolysis were determined.

2. Materials and methods

2.1. Preparation of fermented sausages

Fresh boneless beef (approximately 92% lean) was purchased in three different months (for three replications) from a local market the day before sausage preparation and stored overnight at 4 °C. Four different batches of sausage mixes were prepared in separate 3.9 kg amounts, with each mix containing the following ingredients: 0.61 g $NaNO_2$, 1.83 g sodium erythorbate, 117 g NaCl, 39 g dextrose, a mixture of 9.75 g ground black pepper, 7.29 g white pepper and 2.42 g garlic powder, and 195 ml H_2O. Sausage mixes were inoculated with either 19.7 ml of a frozen concentrate of *P. acidilactici* (HP, Diversitech, Inc., Gainesville, FL) as a USA-type culture or with 1.72 g freeze-dried cultures of either *L. curvatus* (Rudolf Muller HJ7, Chr. Hansen, Inc., Stoughton, WI), *L. sake* (RML 63, Chr. Hansen, Inc., Stoughton, WI) or *S. griseus* (Rudolf Muller, Chr. Hansen, Inc., WI) as typical European-utilised cultures. Each starter culture was used according to the respective manufacturer's instructions.

After mixing ingredients in a Hobart A-200 Mixer (Hobart Co., Troy, OH), the prepared mixes (at 8–10 °C) were stuffed into 52 mm diameter dry sausage fibrous casings (Viskase Corp., Chicago, IL) using the vented Vogt 16 kg stuffer (Koch Supplies, Kansas City, MO), which was manually operated with a 36 mm diameter stuffing horn, and sectioned into chubs of approximately 300 g each. After stuffing, sausages from each mix were placed in a container, covered with a moist cloth and held in an Alkar Model 450-UA processing oven (Alkar, Lodi, WI) at 25 °C and 98% relative humidity (RH) for 72 h. This holding step at 25 °C resembles the slow early ripening period of European processes and was called "prefermentation" in this study.

After prefermentation, the sausages were hung on stainless steel sticks in the Alkar oven and fermented more rapidly, resembling a USA rapid process, at 96% RH through three holding temperatures, which were 32, 35, and 41 °C for 1, 2, and 4 h, respectively. After fermentation, the sausages were removed and placed in a 10 °C drying room having 15–20 air changes/h and a RH of 90–98%, and dried for 18 days. The chubs were weighed periodically during drying to determine percent weight loss. After drying, sausages from each treatment were heat processed in the Alkar oven using a programmed stepwise heating schedule, as follows: (1) 32 °C and 40% RH for 30 min, (2) 43 °C and 40% RH for 30 min (3) 54 °C and 40% RH for 30 min, (4) 66 °C and 30% RH for 30 min, (5) 77 °C and 30% RH for 30 min, (6) 82 °C and 30% RH until approximately 60 °C internal temperature was attained, (7) 61 °C internal temperature and 30% RH for 15 min. The chubs were then showered with cold water at 10–12 °C for 10 min, surface dried and then initially held at 3 °C, until sampled for various analyses.

2.2. Sampling procedures

Two randomly selected chubs were removed at each stage of processing, namely, after the initial step, after prefermentation, after fermentation, after drying and after heating. Samples of raw mixes collected for analysis did not require grinding prior to use. The two chubs were ground twice using a Rival Electric Food Grinder (Rival Manufacturing Co., Kansas City, MO) and immediately after grinding, 30-40 g of sample was vacuum-packaged and held at −20 °C until used for protein extraction for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis. The remainder of the ground samples was stored in sealed containers at 3 °C and analysed within 48 h for total nitrogen, nonprotein nitrogen (NPN), and free amino acids.

2.3. Total nitrogen and nonprotein nitrogen content

At each sampling interval previously noted, total nitrogen content of sausages from four batches was determined with AOAC Kjeldahl method (1995). For nonprotein nitrogen (NPN) analysis, a 20 g sausage sample was blended with 200 ml of distilled water for 1 min in an Oster blender (Sunbeam Oster, Inc., Boca Raton, FL). The slurry was transferred to a beaker, placed on a magnetic stirrer, slowly blended at 4 °C for 45 min and then filtered using Whatman #4 paper. To 100 ml of filtrate, 5.0 g of sulfosalicsylic acid, 0.455 g LiCl, and 0.450 g LiOH were added. The 5.0 g of sulfosalicylic acid addition yielded a solution of 5% acidity, which precipitated proteins present in the initial extraction. Following 15 min for precipitation, coagulated protein was removed by centrifugation at 6,000*g* for 20 min at 4 °C. Duplicate samples from each supernatant were analysed for nitrogen (as NPN), following the AOAC (1995) Kjeldahl method. The total nitrogen and NPN concentrations were expressed as mg N/100 g dry sample.

2.4. Amino acid analyses

The NPN fraction was further analysed for free amino acid contents using a Dionex Model D-300 amino acid analyzer (Dionex, Sunnyvale, CA). The NPN supernatants required no further treatment prior to amino acid analysis. A 1 ml sample of the sulfosalicylic acid supernatant was injected onto the column prepared with lithium form cation exchange resin (Transgenics, Omaha, NE) along with 0.1 ml of norleucine as internal standard. Amino acid concentrations determined after the initial step, after fermentation and after drying were expressed as mg/100 g dry sample.

2.5. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE)

Alteration of meat proteins in sarcoplasmic and myofibrillar protein fractions during processing was monitored with SDS–PAGE for the initial mix, fermented sausage, and dried product, according to the procedures reported by Toldra, Miralles, and Flores (1992), Toldra, Rico, and Flores (1993) (for protein extraction), and Laemmli (1970) (for SDS–PAGE), as described below in detail.

2.6. Protein extraction

Four grams of sample were blended with 40 ml of 0.03 M potassium phosphate buffer, pH = 7.4, (1:10 dilution) at 2 °C for 2 min in a Stomacher blender (Seward Model 400, Tekmar Co., Cincinnati, OH). After centrifugation at 10,000*g* at 4 °C for 20 min, the supernatant was collected by filtering through glass wool. The pellet was dispersed in 40 ml of phosphate buffer and the same procedure as above was repeated. Supernatants were combined as the sarcoplasmic protein fraction. The resulting pellet was dispersed in 40 ml of 8 M urea containing 1% β-mercaptoethanol and extracted for 2 min at 2 °C in a Stomacher blender. After centrifugation of the homogenate at 4 °C at 10,000*g* for 20 min, the supernatant was collected as the myofibrillar protein fraction (Toldra, Miralles, & Flores, 1992; Toldra, Rico, & Flores, 1993).

2.7. Protein assay

Protein concentations of both sarcoplasmic and myofibrillar protein extracts were determined using a standard assay with Bradford reagent (Sigma Chemical Co., St. Louis, MO) and bovine serum albumine (BSA) as the standard (Sigma). Absorbance at 595 nm was used for protein concentration, as described in the manufacturer's instructions (Sigma Product Information Manual, Sigma Chemical Co., St. Louis, MO).

2.8. SDS–PAGE electrophoresis

Protein concentration of each extract was adjusted to 1.0 mg/ml in a solution containing 2% sodium dodecyl sulfate (SDS) and 10 mM sodium phosphate buffer, pH = 7.0. Enough dithiothreitol (DTT) was added to give a concentration of 50 mM. The solution was boiled for 10 min. After cooling, samples were mixed with SDS-tracking dye solution (containing 6.5 ml of 50 mM DTT, 1.5 ml of 0.6% bromophenol blue, 8.2 ml of 13.1% SDS in 150 mM sodium phosphate buffer (pH = 7.0), and 4.5 ml glycerol) in a ratio of 2:1 (v/v) and boiled another 10 min.

SDS–PAGE electrophoresis was conducted, as described by Laemmli (1970), using 4–20% gradient polyacrylamide Precast Ready Gel (Bio-Rad Laboratories, Hercules, CA). Forty microlitres of the sample were run onto the gel. Molecular weight calibration standards were used for calculating sub unit molecular weights. The gel was run on a Bio-Rad Mini Protean II electrophoresis apparatus, with Bio-Rad Model 1000/500 power supply at 200 V constant voltage for about 1 hr. The gel was stained with Coomassie Brilliant Blue R 250 (1 g/l) containing 50% (v/v) methanol and 10% (v/v) acetic acid, and destained with the solution containing 10% (v/v) acetic acid and 10% (v/v) methanol in distilled water, until the background was clear. Standard proteins (high and low molecular weight) of See Blue Plus 2 pre-stained standard (Novex, San Diego, CA) were simultaneously run for protein identification. These standard proteins were myosin (250 kDa), phosphorylase B (148 kDa), bovine serum albumin (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (16 kDa), insulin B-chain (6 kDa).

Gel images were captured with a CCD (Charge-coupled device) camera and the acquired image was focused for records using FUJI FILM Science Lab 97 Image Gauge, Version 3.0, software (Fuji Film, Tokyo, Japan).

2.9. Statistical analyses

Data were analysed using general linear model (GLM) analysis of variance procedure (SAS, 1996) to determine the main effects from a factorial model of culture, process stage, and culture × process stage interaction. When culture effect was not significant (for total nitrogen and total free amino acids), then data were pulled together and analysed combining all culture treatments, to determine the effect of process stages. Least square means (LS means) and probability of difference were calculated for significant ($p \leqslant 0.05$) main effects and their interactions using PDIFF command.

3. Results and discussion

3.1. Changes in total nitrogen and nonprotein nitrogen content

No effect of starter cultures on total nitrogen content of the sausages was observed. However, total nitrogen concentration increased significantly ($p \leqslant 0.05$) throughout the process stages, as compared to respective initial mixes, in all starter culture groups (Table 1). While overall initial total nitrogen content was 9060 mg/100 g dry sample, it increased ($p \leqslant 0.05$) after prefermentation and reached 9590 mg/100 g dry sample after heating. Demasi et al. (1990) also noted a slight but statistically insignificant increase in total nitrogen in sausages fermented with *Pediococcus pentaceous* after fermentation, while heating and relatively short drying (12 days) resulted in higher ($p \leqslant 0.05$) total nitrogen content. Similarly, Johansson, Berdague, Larsson, Tran, and Borch (1994) showed an increase in total protein content (N × 6.25), from 14.8% initially to 22.4% after 21 days of processing, including maturation at 25 °C for 7 days and drying at refrigerated temperatures over 2 weeks.

Table 1
Total nitrogen (Total N) and nonprotein nitrogen (NPN) content (mg/100 g dry matter) at various processing stages for fermented beef sausages inoculated with different starter cultures.

Processing stages	Starter culture			
	PA	LS	LC	SG
Total N[c]				
Initial	9180 ± 537^{B}	8900 ± 400^{B}	9150 ± 367^{B}	9000 ± 504^{B}
Prefermentation	9600 ± 193^{A}	9800 ± 294^{A}	9540 ± 540^{A}	9680 ± 179^{A}
Fermentation	9570 ± 321^{A}	9580 ± 222^{A}	9480 ± 214^{AB}	9640 ± 252^{A}
Dried	9630 ± 246^{A}	9650 ± 282^{A}	9640 ± 261^{A}	9540 ± 229^{A}
Heated	9650 ± 149^{A}	9530 ± 195^{A}	9670 ± 230^{A}	9530 ± 195^{A}
NPN[d]				
Initial	940 ± 66.7^{F}	918 ± 56.4^{G}	954 ± 74.9^{G}	919 ± 68.3^{F}
Prefermentation	1310 ± 35.2^{abE}	1360 ± 44.2^{aF}	1320 ± 87.1^{abF}	1260 ± 56.8^{bE}
Fermentation	1380 ± 56.7^{abE}	1440 ± 88.3^{aE}	1410 ± 75.4^{abE}	1330 ± 118^{bE}
Dried	1520 ± 59.7^{D}	1560 ± 34.3^{D}	1540 ± 50.6^{D}	1550 ± 43.6^{D}
Heated	1610 ± 78.9^{C}	1660 ± 88.4^{C}	1630 ± 72.5^{C}	1650 ± 71.7^{C}

[a]Mean ± Standard deviation. PA: *P. acidilactici*, LS: *L. sake*, LC: *L. curvatus*, SG: *S. griseus*.
[b]LS means in a row (across starter cultures) not having a common superscript letter ($^{a-b}$) are different ($p \leqslant 0.05$). LS means without any superscripts were not different ($p > 0.05$).
[c]LS means in a column (for total N) not having a common superscript letter ($^{A-B}$) are different ($p \leqslant 0.05$).
[d]LS means in a column (for NPN) not having a common superscript letter ($^{C-G}$) are different ($p \leqslant 0.05$).

While sausages inoculated with *L. sake* had the highest NPN content during processing after initial mixing, it was only significantly different ($p \leqslant 0.05$) from the *S. griseus* containing sausages at the prefermentation and fermentation stages (Table 1). *P. acidilactici* and *L. curvatus* added sausages had a similar NPN content ($p > 0.05$) to the other starter culture groups throughout processing. Process stage was also a significant factor affecting NPN of fermented sausages. Significant increases ($p \leqslant 0.05$) in the nonprotein nitrogen (NPN) fraction were found after the prefermentation and fermentation stages and through the drying and heating processes (Table 1). While NPN increased ($p \leqslant 0.05$) after prefermentation in all sausage groups, only *L. curvatus* and *L. sake* containing sausages increased significantly ($p \leqslant 0.05$) in NPN concentrations after fermentation, as compared to their respective concentrations in the prefermentation stage. Flores, Marcus, Nieto, Navarro, and Lorenzo (1997) also reported a large increase in the NPN fraction during fermentation of dry sausages inoculated with *L. sake* and *S. carnosus* starter culture mix and they related it to a corresponding decrease in sausage pH value. Garcia de Fernando and Fox (1991), in fermented pork sausages, Astiasaran, Villanueva, and Bello (1990), in a variety of fermented sausages including chorizo, saucisson and salami, and Berian, Lizaso, and Chasco (2000) in salchichon independently noted significant increases in NPN concentration over various processing phases. In their studies, drying and heating in particular, caused significant NPN increases in all sausage mixes. In our study, when overall NPN values were compared between processing stages, regardless of culture treatment groups,

an approximate 75% increase in the NPN content of the sausages was observed from initial mixes, as compared to the sausages after heating. This significant effect of overall heating at the various stages (which includes fermentation) on NPN generation in fermented sausages was also reported by Klement, Cassens, and Fennema (1974), Klement, Cassens, Fennema, and Greaser (1975) and Demasi et al. (1990). Klement, Cassens, Fennema, and Greaser (1975) attributed it to possible decreases in myofibrillar protein solubility, which also occur upon heating.

3.2. Free amino acid content

Nonprotein nitrogen (NPN) extracts were used to determine the concentration of free amino acids initially, after fermentation, and after drying for sausages inoculated with starter cultures of *P. acidilactici*, *L. sake*, *L. curvatus*, and *S. griseus* (Table 2). Glutamine, taurine, alanine and glutamic acid appeared to be the predominant amino acids in all of the initial mixes, and they represented 32, 14, 10, and 6% of the total NPN amino acid concentration, respectively. Casaburi et al. (2007), Hierro et al. (1999), Garcia, Diez, and Zumalacarregui (1998) and Demasi et al. (1990) also reported high concentrations of these amino acids in their respective initial sausage mixes. In addition to these four amino acids, ornithine, lysine, phenylalanine, valine, isoleucine and leucine became the predominant amino acids overall after fermentation and drying. Most amino acid concentrations increased significantly during sausage fermentation and drying; however, taurine did not show a significant change during processing. Garcia et al. (1998) also noted that the taurine concentration remained unchanged during ripening of a dried Spanish beef product (cecina). Based on the highest to lowest concentration changes from the initial mixes through drying, dominant amino acids ranked from highest to lowest in the following order: leucine > methionine > isoleucine > valine > phenylalanine > lysine > ornithine > glutamic acid > alanine.

L. sake produced the highest glutamic acid concentration after fermentation, followed by *L. curvatus*, which was significantly different ($p \leqslant 0.05$) from *P. acidilactici* and *S. griseus* (Table 2). This higher concentration of glutamic acid in the sausages inoculated with *L. sake* can be explained by the significant decrease in glutamine concentration after fermentation, due to the conversion of glutamine to glutamic acid and NH_3 (Demasi et al., 1990). Fadda et al. (1999) also noted significant releases of glutamic acid by *L. sake* and *L. curvatus* in muscle sarcoplasmic protein extracts whereas Sanz et al. (1999) reported similar results in muscle myofibrillar protein extracts.

Although initial mixes contained an average arginine concentration of 26.7 mg/100 g dry sample, it appeared in measurable quantity only in sausages with *L. sake* and *S. griseus* after fermentation and only in those with *L. sake* after dehydration (Table 2). However, sausages inoculated with *L. sake* had the lowest ($p \leqslant 0.05$) ornithine concentration during fermentation and drying, as compared to sausages with the other three cultures. While all other cultures gave rise to increases ($p \leqslant 0.05$) in ornithine concentration during both fermentation and drying, the increase in orni-

Table 2
Free amino acid contents (mg/100 g dry sample) of the nonprotein nitrogen fractions at various processing stages for fermented beef sausages inoculated with different starter cultures.

Amino acid	Initial[b]	*Fermentation*				*Dried*			
		PA	LS	LC	SG	PA	LS	LC	SG
Acidics									
α-amino-n-butyric acid	Trace	44.2^{a}	8.24^{b}	5.28^{b}	trace	79.4^{a}	15.9^{b}	7.10^{b}	16.1^{b}
Aspartic	16.4 ± 6.88	40.0^{ab}	46.1^{a}	32.2^{bc}	26.6^{c}	85.5^{a}	57.5^{b}	55.3^{b}	61.8^{b}
Glutamic	53.5 ± 7.92	173^{b}	197^{a}	187^{ab}	145^{c}	217^{ab}	232^{a}	231^{a}	197^{b}
Basics									
Histidine	20.8 ± 5.32	31.3	32.1	36.0	41.3	85.0	74.8	64.6	70.0
Lysine	29.8 ± 5.76	70.9^{b}	71.9^{b}	92.3^{a}	73.1^{b}	172^{b}	196^{ab}	217^{a}	174 ^{b}
Arginine	26.7 ± 17.3	trace	16.2	trace	18.1	trace	30.8	trace	trace
Tryptophan	trace	trace	16.8	22.5	15.9	19.6^{b}	30.2^{a}	29.9^{a}	30.5^{a}
Ornithine	45.5 ± 14.7	131^{a}	60.0^{b}	110^{a}	99.0^{a}	228^{a}	165^{b}	222^{a}	245^{a}
Neutrals									
Threonine	10.5 ± 3.82	trace	45.9^{b}	57.9^{a}	44.6^{b}	3.22^{c}	61.5^{b}	93.8^{a}	78.5^{ab}
Serine	28.2 ± 8.64	16.5	trace	trace	13.7	2.95^{b}	68.1^{a}	80.5^{a}	53.0^{a}
Glycine	26.2 ± 4.00	49.3^{ab}	39.0^{b}	56.4^{a}	47.3^{b}	97.9	93.2	105	98.9
Alanine	84.3 ± 12.2	212^{a}	125^{b}	180^{ab}	121^{b}	279^{b}	255^{b}	378^{a}	287^{b}
Cystine	14.2 ± 5.62	25.4	17.7	14.9	13.5	37.0	26.8	30.0	37.2
Methionine	8.40 ± 2.04	43.8^{b}	50.0^{b}	64.7^{a}	47.9^{b}	92.9^{ab}	98.5^{a}	82.1^{b}	90.0^{ab}
Valine	15.0 ± 4.70	68.7	68.3	89.1	84.3	124	113	110	111
Isoleucine	13.9 ± 3.28	53.7^{b}	59.4^{b}	79.4^{a}	61.8^{ab}	104	124	113	109
Leucine	21.2 ± 2.53	154^{b}	169^{ab}	219^{a}	165^{b}	250^{b}	286^{a}	260^{ab}	252^{ab}
Tyrosine	15.4 ± 4.42	18.7^{c}	46.5^{b}	63.5^{a}	31.8^{bc}	49.8^{b}	91.2^{a}	90.5^{a}	37.9^{b}
Phenylalanine	18.6 ± 5.67	36.1^{c}	108^{a}	116^{a}	73.8^{b}	72.2^{b}	161^{a}	170^{a}	138^{a}
Glutamine	269 ± 33.9	199^{bc}	166^{c}	227^{ac}	261^{a}	198^{b}	221^{ab}	229^{ab}	264^{a}
Taurine	116 ± 26.7	143	128	110	94.7	122	126	126	135
Proline	trace	22.6	23.5	27.8	46.5	7.90^{b}	18.8^{a}	20.8^{a}	41.0^{a}
Other									
Carnosine[c]	743 ± 185	575	596	564	466	639	692	405	616
NH_3	19.0 ± 8.02	23.9	27.6	23.8	25.5	45.2	53.8	44.4	54.7
Total amino acids[d]	1600 ± 241	2130	2130	2380	2020	3090	3290	3080	3200

[a] PA: *P. acidilactici*, LS: *L. sake*, LC: *L. curvatus*, SG: *S. griseus*.
[b] Mean ± standard deviation of initial overall free amino acid concentrations (Given overall since there was no significant difference between culture treatments).
[c] Carnosine is a dipeptide.
[d] Sum of all free amino acids, carnosine and NH_3.
[e] LS means within a row and process stage (across starter cultures) not having a common superscript letter ($^{a–c}$) are different ($p \leqslant 0.05$). "Trace" indicates amino acid concentration lower than 5 mg/100 g dry sample.

thine concentration of sausages inoculated with *L. sake* was only significant after the drying stage of processing.

There was a large increase in lysine concentration after fermentation and drying in all culture groups. *L. curvatus* produced a higher ($p \leqslant 0.05$) lysine concentration than the other three cultures in the fermentation stage (Table 2). Fadda et al. (1999) also found generation of high amounts of lysine by *L. curvatus* from pork muscle sarcoplasmic proteins. *L. curvatus* also had the greatest increase ($p \leqslant 0.05$) in concentrations of methionine, isoleucine, leucine and tyrosine amino acids after fermentation. It also produced an increase ($p \leqslant 0.05$) in phenylalanine concentration together with *L. sake* after fermentation. The five amino acids, lysine, methionine, isoleucine, leucine and tyrosine, showed increases after drying with sausages inoculated with *L. curvatus* and *L. sake* having the highest ($p \leqslant 0.05$) tyrosine content and those with *P. acidilactici* having the lowest phenylalanine concentration, as compared to the other cultures.

Serine decreased significantly ($p \leqslant 0.05$) between the initial and post-fermentation concentrations, with *L. curvatus* and *L. sake* having a trace amount of this amino acid (Table 2). However, after fermentation, sausages with *L. curvatus*, *L. sake* and *S. griseus* had significant increases ($p \leqslant 0.05$) in serine concentration on drying. While *L. curvatus*, *L. sake* and *S. griseus* produced increases ($p \leqslant 0.05$) in threonine concentration, sausages fermented with *P. acidilactici* had only a very small amount of threonine at the end of the drying period. Demasi et al. (1990) also reported lower concentrations of threonine and serine in sausages fermented with *P. acidilactici*. Tryptophan, which did not appear in the initial mix, was detected at the end of fermentation in the sausages inoculated with *L. curvatus*, *L. sake* and *S. griseus*, but only in a trace amount for those with *P. acidilactici*. Although tryptophan was present in *P. acidilactici* containing sausages after drying, its concentration was the lowest ($p \leqslant 0.05$), as compared to that found for the other three cultures. Nevertheless, *P. acidilactici* gave rise to a significantly higher α-amino-*n*-butyric acid concentration than any other culture after fermentation and drying.

While the increase in aspartic acid concentration after fermentation was the highest with both *L. sake* and *P. acidilactici*, the concentration of this amino acid reached its highest value ($p \leqslant 0.05$) at the end of drying, in sausages fermented with *P. acidilactici*. Alanine was the other amino acid present in *P. acidilactici*-added sausages at high concentrations. The largest increase ($p \leqslant 0.05$) in the concentration of alanine occurred with *P. acidilactici* during fermentation and drying.

Type of starter culture was not a factor ($p > 0.05$) affecting total free amino acid concentration (Table 2), which agreed with the results of Casaburi et al. (2007). However, process stages did have a significant effect on overall total amino acid concentration (Fig. 1). While overall initial total amino acid concentration was 1600 mg/100 g dry sample, it increased ($p \leqslant 0.05$) with fermentation and drying, reaching 2160 mg/100 g dry sample and 3170 mg/100 g dry sample, respectively (Fig. 1). Astiasaran et al. (1990) also reported that fermentation and drying gave rise to significant increases in total free amino acid concentration in three different varieties of fermented sausage. Similarly, Casaburi et al. (2007) observed proteolysis over 38 day-ripening of Italian fermented sausages by the increase of total free amino acids.

Based on the concentration increases of nonprotein nitrogen and amino acids, modifying the traditional USA rapid fermentation by including a prefermentation or holding stage allowed development of culture activity similar to that previously reported in many of the European studies using the longer ripening process. *P. acidilactici* is not considered highly proteolytic, yet its activity did not substantially differ from the activity of the other starters in this study, using the modified process that resembled ripening processes.

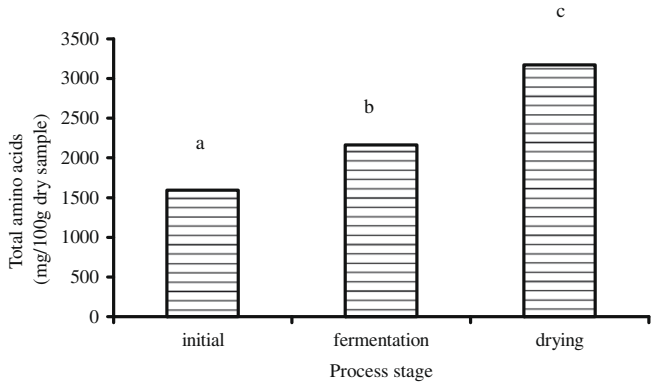

Fig. 1. Changes in overall total amino acid concentration (mg/100 g dry sample) in sausages, based on processing stages. Bars not having a common letter are different ($p \leqslant 0.05$). Given as sum of free amino acids, carnosine and NH_3.

3.3. SDS–PAGE electrophoresis

The protein profiles from hydrolysis of muscle sarcoplasmic and myofibrillar proteins by *L. curvatus*, *L. sake*, *P. acidilactici* and *S. griseus* are presented in Fig. 2. In general, no major change occurred between the SDS–PAGE patterns, due to culture treatments during processing. Bolumar et al. (2006) also reported that there was no significant difference in protein profile resulting from the proteolysis of sarcoplasmic and myofibrillar muscle proteins of three different batches of dry fermented sausages with the addition of cell-free extracts from *Debaryomyces hansenii* and *L. sakei*. This result can be explained by the fact that the first step in protein hydrolysis during fermented sausage ripening is some degradation of muscle proteins to polypeptides and is due to the activity of endogenous meat enzymes. Further degradation of polypeptides to smaller peptides and free amino acids is attributed to enzymes of microbial origin, as well as to remaining activity of the endogenous meat enzymes (Verplaetse, 1994). Thus, further analysis is needed to determine which changes in small peptides and free amino acids result from possible protein degradation by enzymes of the meat *versus* those from microbial origin.

At the processing stages of fermentation and drying, however, notable major changes in sarcoplasmic and myofibrillar protein patterns of all sausages inoculated with the four different starter cultures were evident. Hydrolysis of the sarcoplasmic proteins seemed to occur gradually during processing, with the major changes appearing after the fermentation stage, with resultant identical patterns at the end of drying. The bands at 105, 54, 46, and 31 kDa molecular weight disappeared and the intensity of the band at 18 kDa markedly decreased as a result of fermentation. The bands at 80, 62, 43, 40 and 13 kDa appeared to be more intense during fermentation, followed by slight decreases in the intensity of the bands at 40 kDa and 43 kDa throughout the drying period. Similar changes in the patterns of sarcoplasmic proteins were observed by Hughes et al. (2002) and Casaburi et al. (2007) during ripening of semi-dry fermented sausages and of an Italian traditional fermented sausage, respectively. Johansson et al. (1994) also reported that major changes in water soluble and salt soluble proteins of fermented sausage occurred in early stages of processing, e.g., between 0 and 3 days during ripening. They also indicated that water-soluble proteins with molecular weights between 20 and 30 kDa and salt-soluble protein with 50 kDa molecular weight disappeared almost entirely by the seventh day of processing. A smear of low molecular weight bands (<10 kDa) appeared after fermentation and drying, likely to be due to generation of low molecular weight peptides and free amino acids.

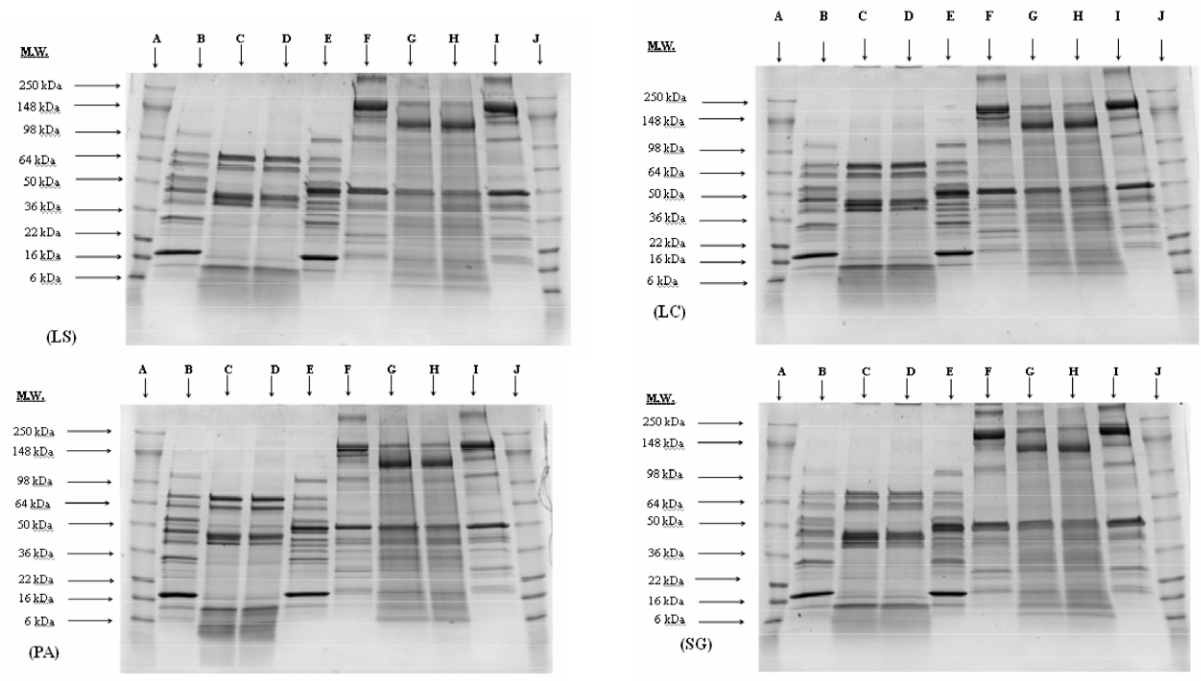

Fig. 2. SDS–Polyacrylamide Gel Electrophoretic patterns of sarcoplasmic (**B**–**E**) and myofibrillar (**F**–**I**) proteins for sausages inoculated with *L. sake* (LS), *L. curvatus* (LC), *P. acidilactici* (PA), and *S. griseus* (SG) during processing. **A** and **J**: molecular weight markers. **B** through **D** (for sarcoplasmic proteins) or **F** through **H** (for myofibrillar proteins): initial, fermented and dried samples, respectively. **E** and **I** were fresh beef muscle sarcoplasmic and myofibrillar extracts, respectively.

Changes in the myofibrillar protein profile were also observed over the processing stages. The band that appeared on the top of the gel with a calculated molecular weight greater than 250 kDa disappeared after fermentation with *L. curvatus*, *L. sake* and *P. acidilactici*. This band is presumed to be either nebulin or titin. In the *S. griseus*-added group, however, the intensity of the band decreased gradually after fermentation and drying. This was probably due to the higher pH in sausages inoculated with *S. griseus*. Lower pH is one of the factors favouring the hydrolysis of muscle proteins (Astiasaran et al., 1990).

The intensity of heavy myosin band (around 200–220 kDa) markedly decreased over fermentation and drying. The actin band (around 50 kDa) also decreased in intensity after fermentation, although there was a slight change in the intensity of the actin band after drying. Degradation of myosin heavy chain and actin during fermented sausage processing was also reported by Verplaetse, de Bosschere, and Demeyer (1989), Garcia de Fernando and Fox (1991), Hughes et al. (2002) and Casaburi et al. (2008).

During fermentation, the myofibrillar protein band in the region of 145 kDa disappeared while a sharp band with 120 kDa molecular weight appeared and remained unchanged after drying. This 120 kDa band could be generated from myosin degradation. Verplaetse et al. (1989) indicated that the heavy chain of myosin degraded to a polypeptide with a molecular weight in the range of 120–150 kDa during sausage processing.

Smears of bands in the regions of 25 and 36 kDa were observed in the myofibrillar protein fractions during fermentation and drying. The bands in the regions of 16 and 22 kDa involving troponins and myosin light chains disappeared, while smears of low molecular weight bands appeared over the fermentation and drying stages. In general, intensity of these bands increased after drying. An increase in the concentration of polypeptides with molecular weights varying from 14 to 36 kDa was reported as a result of hydrolysis of fermented sausage myofibrillar proteins during processing (Verplaetse et al., 1989).

4. Conclusions

With a few exceptions, there were only minor differences among sausages due to a starter culture effect. However, some individual amino acids as products of culture activities differed depending on the culture used. On the other hand, processing stages for each culture had a larger impact on changes in proteolytic characteristics, most likely due to the effects of lower pH or higher temperature applied during prolonged fermentation and in the heating process.

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