TITLE:

Analysis of biogenic amines in Northern and Southern European sausages and role of flora in amine production.

**AUTHORS: D. Ansorena ^{d*}, M.C.Montel ^b, M.Rokka ^c, R.Talon ^b, S.Eerola ^c, A.Rizzo ^c,
M. Raemaekers ^a and D.Demeyer ^a.**

^a *Department of Animal Production, Ghent University, Proefhoevestraat 10, 9090 Melle, Belgium*

^b *Station de Recherches sur la viande, INRA-Theix, 63122 Saint-Genès Champanelle, France*

^c *Department of Chemistry, National Veterinary and Food Research Institute, PO Box 368, 00231 Helsinki, Finland*

^d *Departamento de Bromatología, Tecnología de Alimentos y Toxicología, Universidad de Navarra, Irunlarrea s/n, 31008 Pamplona, Spain*

***Corresponding author:**

Diana Ansorena Artieda

Departamento de Bromatología, Tecnología de Alimentos y Toxicología

Universidad de Navarra

Irunlarrea s/n

31008 Pamplona

Spain

Tel: 34-948-42-56-00

Fax: 34-948-42-56-49

E-mail: dansorena@unav.es

ABSTRACT

The biogenic amines content, the microbial counts and flora producing amines were investigated in four types of fermented sausages. Southern type European sausages (Italian and Belgian) showed higher tyramine and phenylethylamine values than the Northern ones (Norwegian and Belgian). The spontaneous non-starter lactic acid bacteria could be responsible for the production of these amines in the Italian products, and the cocci Gram positive in the Belgian South ones. The Norwegian sausages showed the lowest total amine content of the four types. The two Belgian types were characterised by the highest putrescine content, associated to high counts of *Enterococcus*. The production of amines *in vitro* by the starter cultures used in the manufacture of the sausages revealed that none of the *Lactobacillus* species produced any amine and only *K.varians* and *S.carnosus* showed phenylethylamine and tryptamine production. High correlations were found between the content of putrescine, histamine and cadaverine.

Keywords: Dry fermented sausage, amines, food safety, microorganisms.

INTRODUCTION

Many papers report results on amine content in different types of meat and meat products, showing a great variability in concentration among them. Special mention deserve ripened meat products, thus it is known that fermentation processes can increase the presence of this type of compounds. Biogenic amines may be formed by the action of microbial decarboxylases on free amino acids resulting from the proteolytic process that normally takes place during the ripening of fermented sausages. An increase in the amine content was observed when proteolysis was deliberately accelerated with a proteinase (Bruna, Fernández, Hierro, Ordoñez, J.A. & de la Hoz, 2000). The micro-organisms responsible for the decarboxylation reactions may be introduced as starter culture in sausages (Ten Brink, Damink, Joosten & Huis in't Veld, 1990) or may constitute part of the natural population of the food. *Enterobacteriaceae* can be involved in the production of putrescine, histamine and cadaverine (Durlu-Ozkaya, Ayhan & Vural, 2001), and *Pseudomonas* in the production of putrescine (Edwards, Dainty, Hibbard & Ramantanis, 1987; Bauer, Potzelberger, Hellwig & Paulsen, 1996). Concerning lactic acid bacteria, all the strains of *Carnobacterium* found in meat products produced high amount of tyramine and some strains of *Lactobacillus curvatus* and *Lactobacillus plantarum* used as starter cultures, showed tyramine production (Masson, Eclache, Compte, Talon, & Montel, 1996).

On different Austrian dry sausages, highly significant differences were found between the histamine contents of dry sausages made by different manufacturers (Tschabrun, Sick, Bauer, & Kranner, 1990) and in the same type long ripened products, its concentration increased to up to 400 mg/kg DM (Paulsen and Bauer, 1999). A large variation in the amine content and composition was also found when retail Belgian sausages were analysed, possibly related to different methods of fabrication and to the specific flora of the sausages (Vandekerckhove, 1977). Furthermore, wide fluctuations were observed in the amine content of products from

different batches of the same commercial brand in fermented and cooked Spanish products (Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1997a) and also in cervelat sausages elaborated with different amounts of starter culture (Treviño, Beil & Steinhart, 1997). Considering the known relationship of biogenic amines with some toxicological effects (Masson & Montel, 1995) and the subsequent risk for the health of consumers, it is important for the meat industry to produce fermented meat products with a higher degree of safety with respect to the biogenic amine content. The recommended upper limit for histamine in meat products proposed by the Netherlands Institute of Dairy Research was 100 mg/kg (TEN BRINK et al., 1990) and in the Czech Republic it was 200 mg/kg (Standara, Pol, Kamen & Budig, 1994).

The objective of this work was to compare the amine content in four different European dry fermented sausages with different processing characteristics: two northern types (Norwegian and Belgian) and two Mediterranean types (Italian and Belgian). Furthermore, the production of amines by the strains inoculated as starter cultures and also by spontaneous-non starter flora isolated from the different types of sausages was determined in order to conclude about the role of the flora in the final content of amines. Also, the influence of three different laboratories of analysis was evaluated.

MATERIALS AND METHODS

Preparation of sausages

Four different types of sausages (Belgian North, Belgian South, Italian and Norwegian) were elaborated by three partners of the project in their plants, as described by Demeyer et al. (2000). Five batches per type were prepared with different lots of raw material and five sausages per batch were analysed by three laboratories involved.

Sausages were frozen at -20°C until microbial and amine analysis were carried out. The main production characteristics of the four types of sausages are described in table 1.

Microbial analysis

Lactic acid bacteria were numerated on Man Rogosa Sharp (MRS, Merck), Gram positive Cocci on Mannitol Salt Agar (MSA, Merck), *Enterococcus* on Agar Kanamycine Esculine Azide (AKEA, Merck), yeasts and moulds on Potato Dextrose Agar (PDA, Difco), *Listeria* on Palcam medium, *Enterobacteriaceae* on Crystal-violet neutral-red bile glucose (VRBD, Merck), *Pseudomonas* on Cefrimide Fucidine Cephaloridine medium (CFC, Oxoid). The media were prepared, incubated and read according the manufacturer's instructions.

Amine determination in sausages

Three different laboratories carried out the quantitative separation of 8 biogenic amines (tryptamine, phenylethylamine, histamine, putrescine, cadaverine, tyramine, spermine, spermidine) by Reverse Phase HPLC. Determinations were performed using different methods of analysis, all based in the derivatisation of amines to obtain dansyl derivatives detected by ultraviolet detector at 254 nm. Two laboratories used perchloric acid 0.4M as the extractant solvent and 1,7-diaminoheptane as the internal standard (Eerola, Hinkkanen, Lindfors & Hirvi, 1993). The derivatisation was carried out by incubation during 40minutes in alkaline media. Sample was finally solved in acetonitrile. In the method used by the other laboratory, the extractant solvents were acetone and trichloroacetic acid (5%), and the internal standard was 1,8-diaminooctane. The derivatisation was also carried out in alkaline media, but for a longer period of time (4 hours). A further extraction of the amines with diethyl ether was included as an additional step in this method. After the evaporation of the diethyl ether, the sample was solved in acetonitrile.

Production of amines by starter cultures and by strains isolated from different sausages

From the different selective media used to study the flora, 305 colonies were randomly picked up and inoculated in All Purpose Tween (APT) broth. After 24 h of growth, the strains were kept frozen at -20°C.

To determine their amine production, the strains isolated from the different media and the starter cultures used to manufacture the different sausages were grown in Maijala's medium (Maijala, 1993) supplemented with 7 amino acids (tyrosine, lysine, phenylalanine, arginine, tryptophan, histidine and ornithine) at a level of 2 % each one. The Maijala medium, was adjusted to pH 5.4 and incubated at 30°C during 2 days. After centrifugation (3000 rpm; 3 min), supernatants were dansylated and amines were then quantified as described by Eerola et al. (1993).

Statistical analysis

Mean values for data obtained from the three laboratories for the concentration of each amine and type of sausage were calculated. A Variance Components procedure was carried out in order to estimate the contribution of the effect of laboratories (3), types of sausages (4), batches (5 batches per type of sausage) to the total variability found for the amine content measured in the products. The Restricted Maximum Likelihood (REML) method was used for estimating the variance components. The Pearson's correlation coefficient was calculated among all amines. Calculations were performed using SPSS 9.0 for Windows.

RESULTS AND DISCUSSION

The content of 8 biogenic amines was determined by three different laboratories in four types of sausages. Five batches of each type were analysed and a great variability was found among the data for some of the amines, not only due to the different processing conditions of the sausages, but also because of the different laboratories of analysis and batches prepared. A variance component estimation test was carried out on each amine in order to elucidate the main factors that contribute for the total variability found in each case (Table 2). This test pointed out that the content of putrescine and tyramine was mainly determined by the type of sausage, where this factor in both cases explained around 53% of the total variability of these two amines. Thus, considering that both tyramine and putrescine are proved to be the prevailing amines in ripened meat products (Hernández-Jover et al., 1997a) and that one of the objectives of this work was to compare the four types of sausages analysed, the display of the data for the amine concentration in sausages was made showing the patterns for these four types (Fig 1). The test also revealed that the variability found for the concentration of phenylethylamine, cadaverine and histamine was basically explained by the batch production, accounting the factor batch(labo*type) for percentages of 55%, 66% and 88% of the total variability in each case. This fact contributes to corroborate the importance of the hygienic quality of the raw material on the amine accumulation during sausage ripening. Variability of results between laboratories of analysis could be considered of no importance with regard to the other factors studied in all amines except for spermine and spermidine where this factor accounted for 43% and 83% of the total variance. For these two polyamines, a low variability was found between the four types of sausages (fig 1). This could be explained by the fact that they are known to be natural occurring amines in fresh pork and beef meat, and their formation is not associated to food spoilage or fermentation process (Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués & Vidal-Carou, 1996). An average value of 25mg/kg was

found during the ripening of mini-salami for spermine (Treviño et al., 1997) and 26.1mg/kg was the mean detected among 20 different Spanish retail chorizos (Hernández-Jover et al., 1997a), both values being very close to those obtained in this work (Fig 1). A similar situation was observed for spermidine, with an average value of 4 mg/kg.

The most abundant amine found in 3 of the 4 types of sausages was tyramine, in agreement with other authors (Eerola, Maijala, Roig-Sagués, Salminen & Hirvi, 1996; Hernández-Jover et al., 1997a; Bover-Cid, Izquierdo-Pulido & Vidal-Carou, 2001). Higher values were recorded for the Southern type compared to the Northern one (Fig. 1). The Mediterranean products clearly surpassed the level of 100 mg/kg, which was the limit reported as unsafe by Stratton, Hutkins & Taylor (1991). Also, an ingestion of 10-40mg of tyramine has been suggested responsible for skin disorders (Askar & Treptow, 1986).

A concentration of 187 mg of tyramine/kg was found in the Italian sausages, where lactic acid bacteria and Gram positive cocci were the dominant flora (table 3). This tyramine content could be explained by the high percentage (64%) of lactic acid bacteria isolated from sausages producing more than 100 µg/ml and also by some strains of Gram positive cocci and enterococci (table 4). However, as observed in table 5, no tyramine production was detected in the *Lactobacillus curvatus* and *Kocuria varians* 2 strains used as starter cultures in these sausages. Although some of the strains isolated from AKEA medium were able to produce tyramine, low counts of enterococci were found at the end of the process in this type of sausages. Consequently, further study of their evolution during processing should be done to conclude about their contribution to tyramine accumulation during ripening in Italian sausages. These results lead to think that spontaneous non-starter lactic acid bacteria and cocci, were mainly responsible for the tyramine production in the Italian sausages.

The production of tyramine in the Belgian South sausages (176 mg/kg) was mainly due to the strains isolated from AKEA and to a less extent from MSA medium (table 4). These sausages

had a high count of *Enterococcus* (table 3). All the strains used as starter cultures to manufacture these sausages did not produce tyramine in laboratory media (table 5). Thus, enterococci and contaminant Gram positive cocci were pointed to be the two main bacterial groups responsible for the tyramine detected in the sausages. These results agreed with those obtained by Masson et al. (1996) when screening by *in vitro* tests microbial strains isolated from meat products.

Belgian North sausages contained also tyramine. The content of this amine could be explained by the presence of high level of enterococci (table 3) and by their capacity to produce high amount of this amine *in vitro* condition (table 4). Again no strains used as starter cultures produced this amine (table 5).

The use of bacterial starter cultures with limited tyrosine-decarboxylating activity may reduce tyramine formation (Eitenmiller, Koehler, & Reagan, 1978; Bacus, 1984; Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, Mariné-Font & Vidal-Carou, 1997b; Bover-Cid, Hugas, Izquierdo-Pulido & Vidal-Carou, 2000). However Maijala and Eerola (1993) had already concluded that contaminant LAB play an important role in tyramine and histamine formation during the ripening of dry sausages. In this study, values obtained for histamine were always low (the highest was 18 mg/kg in the Belgian South sausages), considering that, at least, amounts of 8-40 mg of this amine are necessary to produce slight skin disorders (Taylor, 1987).

Sausages with a high content in tyramine had also the highest content in phenylethylamine, showing a Pearson's correlation coefficient of $r=0.64$ between them. For the Belgian South sausages, the presence of phenylethylamine could be explained by the starter culture used: *K.varians* 1 that was able to produce 510 µg/ml (table 4) and by the high level of contaminant enterococci (table 3) in which some strains were able to produce this amine (table 4). For the Italian sausages, also the starter *K.varians* 2, could participate to the content of

phenylethylamine (table 5) but also micro-organisms isolated from AKEA, MRS, MSA and PDA produced this amine (table 4).

In the Belgian North sausages, *K.varians* 1 but also *S. carnosus* 2, both producing high amount of phenylethylamine *in vitro*(table 5), were included in the mixture of starter cultures. But, in the conditions of elaboration of these products, very low concentration of this amine was found in the final product (Fig 1). The acidic conditions, with a pH=4.68 at the third day of fermentation, may explain the low level of amines. Furthermore, the implantation of these two starters could be questionable as none of the isolates from MSA was able to produce phenylethylamine (table 4).

The Norwegian sausages showed the lowest total amine content of the four types. In these sausages, the presence of phenylethylamine could be mainly explained by the use of *S. carnosus* 1 as starter culture. This strain was able to produce 370 µg/ml of phenylethylamine when tested in the Majjala media (table 5). Masson et al. (1996) found that *S. carnosus* strain 833 produced phenylethylamine (500 µg/ml) and in less extent tryptamine (57 µg/ml).

A remarkable high content of putrescine was detected in the Belgian South sausages (125mg/kg) and to a less extent in the Belgian North in comparison with the Italian and Norwegian ones (Fig. 1). This could be linked to the high microbial counts of *Enterococcus* (table 3) and also yeasts for the Belgian South sausages, thus some of the strains isolated from AKEA (2 strains of 13) and PDA (2 of 9) produced more than 10 µg/ml of putrescine (table 4). The production of putrescine could also be associated to endogenous flora not highlighted in this study. This amine has been reported as not toxic by itself, but as potentiator, together with cadaverine, of the toxic effect of histamine and tyramine. In fact, high Pearson's correlation coefficients were found between the content of putrescine and cadaverine ($r=0.74$) and between cadaverine and histamine ($r=0.86$).

In this study, none of the *Lactobacillus* species used as starter cultures for the four types of sausages produced any amine in the laboratory conditions, and neither strains of *Pediococcus pentosaceus* or *Staphylococcus xylosus*.

In summary, Southern European sausages resulted in higher values of amines than Northern ones, due mainly to the presence of tyramine and phenylethylamine. From the analysis *in vitro* of the decarboxylase activity of the starters, it could be concluded that only the two strains *K.varians* and *S. carnosus* were able to decarboxylate phenylalanine. All the other starters were considered to be safe. Spontaneous non-starter lactic acid bacteria and enterococci could contribute to the presence of amines in sausages.

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Table 1. Characteristics of production parameters of the four types of dry fermented sausages studied.

	Norwegian	Belgian Northern	Belgian Southern	Italian
Diameter (mm)	92	90	60	50-60
Weight	3 kg	1 kg	0.8 kg	0.5 kg
Meat species	Pork/Beef (1/1)	Pork/Beef (1/1)	Pork only	Pork only
Lean meat cuts	Miscellaneous	Pork shoulder+Beef	Pork shoulder	Pork shoulder
Fat tissue	Pork back fat	Pork back fat	Pork back fat	Streaky bacon
Meat/Fat ratio	2/1	2/1	2/1	2/1
Starter bacteria (CFU)	+ 10 ⁶ /g ^a	+ 10 ⁷ /g ^b	+ 10 ⁷ /g ^c	+ 10 ⁷ /g ^d
Additives (g/Kg) ^e				
NaCl	39.79	30	30	25
Na-nitrite ^f	240	180	180	80
K-nitrate ^f	0	0	0	120
Saccharose	0	0	0	5
Dextrose	4.3	7	7	3
Na-ascorbate	0	0.9	0.9	0
Ascorbic acid	0	0	0	0.5
Skimmed milk	0	0	0	28
Caseinate	0	11.5	11.5	0
Spices ^f	540 ^g	700 ^h	700 ^h	1100 ⁱ
Fungi starters	-	-	+	+
Particle size	1-2 mm	1-2 mm	1-2 mm	3.5 mm
Ripening conditions				
Fermentation				
°C/hrs	27/55-96	20-26/62	5-24/113	21-22/12→17-18/96
Ph after 24h	5.45	5.70	5.8	5.75
Ph after 3 days	4.79	4.68	5.2	5.95
%rH/hrs	90-96/55-96	50-90/62	10-90/89	95/12→90↓70-80/96
Drying				
°C/hrs	14/80	14/78	14/78	12-13/83-87
Total ripening time	16-25 days	2 weeks	4 weeks	40 days

^a Unspecified *Lactobacillus*. ^b 2.10⁶ each of *Pediococcus pentosaceus* and *Lactobacillus sakei* as lactic acid bacteria and *Staphylococcus xylosus*, *Kocuria varians* and *Staphylococcus carnosus* as flavour enhancers. ^c 7.10⁵ each of *Pediococcus pentosaceus* and *Lactobacillus sakei* as lactic acid bacteria with 4.10⁶ and 2.10⁶ of *Staphylococcus xylosus* and *Kocuria varians* as flavour enhancers. ^d A commercial powder containing a mixture of *Lactobacillus curvatus* and *Kocuria varians* (2.10⁶ /g) was used (50 g/100kg). ^e per kg meat and fat mince. ^fg/kg. ^g oleoresines containing mainly black pepper, with coriander, paprika and cognac (140 mg/kg) and garlic (400 mg/kg). ^h pure black pepper only ⁱ cracked whole black pepper (700), white pepper (300) and garlic (100).

Table 2. REML Variance Component Estimation: effect of laboratory methods, type of sausages, and batches (whithin laboratory and type) on the amine content of the sausages.

Factors	TRYP	PHE	PUT	CAD	HIS	TYR	SPD	SPM
Laboratory	14.5	4.0	2.1	0.9	0.0	19.1	43.3	83.1
Type	17.3	38.3	52.5	28.5	12.0	53.3	1.5	0.0
Labo*Type	40.2	0.0	8.0	0.0	0.0	14.4	17.4	5.9
Batch(Labo*Type)	20.2	55.4	35.5	65.7	87.8	10.0	1.1	1.4
Error	7.8	2.3	1.9	4.9	1.0	3.2	36.8	9.6

TRYP: Tryptamine; PHE: Phenylethylamine; PUT: Putrescine; CAD: Cadaverine; HIS: Histamine; TYR: Tyramine; SPD: Spermidine; SPM: Spermine

*Figures showed the contribution of each factor (expressed as % of the total estimated variance) to the variability found for the results of each amine. In bold, the factor that accounts for the highest source of variability in each case.

*Factors; Laboratory: analyses have been done by three laboratories, Type: 4 types of sausages (Norwegian, Belgian North, Belgian South, Italian), Labo*Type: interaction between these two factors, Batch (Labo*Type): express variability associated to the batch within laboratory and type of sausage.

Table 3. Microbial flora of the four types of sausages.

	pH	Lactic Acid Bacteria	Cocci Gram+	<i>Pseudomonas</i>	<i>Enterobacteriaceae</i>	<i>Enterococcus</i>	Yeasts/Molds
N	4.78 ± 0.13	7.11 ± 0.76	2.23 ± 1.12	<2	<2	<2	<2
BN	4.66 ± 0.08	7.53 ± 0.33	6.07 ± 0.04	<2	<2	6.84 ± 0.50	<2
BS	5.23 ± 0.18	7.28 ± 0.74	5.97 ± 0.57	<2	<2	6.26 ± 1.09	4.49 ± 1.53
I	5.58 ± 0.07	7.92 ± 0.35	6.07 ± 0.55	<2	<2	<2	2.12 ± 1.23

Listeria was not detected.

Italian (I), Belgian Northern (BN), Belgian Southern (BS), Norwegian (N) sausages.

Results are means ± standard deviation of the analysis of five batches at 14 (BN), 16-25 (N) 28 (BS) or 40 (I) days of ripening and expressed in Log (N/g)

Table 4. Production of biogenic amines by strains isolated on selective media from the different sausages.

		nt	TRYP		PHE		PUT		TYR	
Flora			>10 µg/ml	>100 µg/ml	>10 µg/ml	>100 µg/ml	>10 µg/ml	>100 µg/ml	>10 µg/ml	>100 µg/ml
N	MSA	10	0	0	6	0	0	0	0	0
	MRS	15	0	0	4	0	0	0	1	0
	AKEA	2	0	0	0	0	0	0	0	0
	PDA	1	0	0	0	0	0	0	0	0
BN	MSA	26	17	8	0	0	0	0	0	2
	MRS	35	2	0	0	0	0	0	0	0
	AKEA	28	2	0	0	0	0	0	1	13
	PDA	11	1	1	0	0	0	0	0	0
BS	MSA	15	0	0	2	0	1	0	1	5
	MRS	14	0	0	1	0	0	0	1	1
	AKEA	13	0	0	4	0	2	0	3	7
	PDA	9	0	0	0	0	2	0	0	0
I	MSA	39	1	0	4	2	0	0	3	14
	MRS	39	0	0	14	0	0	0	1	25
	AKEA	28	1	0	2	5	0	0	0	12
	PDA	20	0	0	0	1	0	0	0	0

Norwegian (N), Belgian Northern (BN), Belgian Southern (BS), Italian (I) sausages

Flora: strains isolated from selective media, MRS=lactic acid bacteria, MSA=Gram positive cocci , AKEA=enterococci , PDA=yeasts/molds

nt : total number of strains studied, number of strains producing more than >10ug/ml and >100µg/ml.

TRYP: Tryptamine; PHE:Phenylethylamine; PUT:Putrescine ; TYR:Tyramine

Table 5. Production of biogenic amines by the strains used as starter cultures in the different types of sausages.

	Starters	TRYP μg/ml	PHE μg/ml	PUT μg/ml	TYR μg/ml
Norwegian	<i>L. species</i>	0	0	0	0
	<i>S. carnosus1</i>	70	370	0	0
Belgium North	<i>L. sakei</i>	0	0	0	0
	<i>P. pentosaceus</i>	0	0	0	0
	<i>K. varians 1</i>	95	510	0	0
	<i>S. xylosus</i>	0	0	0	0
	<i>S. carnosus2</i>	115	585	0	0
Belgium South	<i>L. sakei</i>	0	0	0	0
	<i>P. pentosaceus</i>	0	0	0	0
	<i>K. varians 1</i>	95	510	0	0
	<i>S. xylosus</i>	0	0	0	0
Italian	<i>L. curvatus</i>	0	0	0	0
	<i>K. varians 2</i>	0	65	0	0

TRYP: Tryptamine; PHE:Phenylethylamine; PUT:Putrescine ; TYR:Tyramin

Figure 1. Amine content in the four types of sausages. Results are the mean of the five batches analysed by the three laboratories (mg/kg sausage).

Note: TRYP: Tryptamine; PHE: Phenylethylamine; PUT: Putrescine; CAD: Cadaverine; HIS: Histamine; TYR: Tyramine; SPD: Spermidine; SPM: Spermine.