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Analysis of biogenic amines in northern and southern European sausages and role of flora in amine production

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Abstract

The biogenic amine contents, 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 northern type 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 those studied. The two Belgian types were characterised by the highest putrescine contents, associated with 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 amines and only *Kocuria varians* and *Staphylococcus 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

1. Introduction

Many papers report results on the amine content in different types of meats and meat products, demonstrating a great variability in concentrations. In ripened meat products, it is known that the fermentation processes can increase the content of these types 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 accelerated using a proteinase (Bruna, Fernández, Hierro, Ordoñez, & 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 (Bauer, Potzelberger, Hellwig, & Paulsen, 1996; Edwards, Dainty, Hibbard, & Ramantanis, 1987). Regarding lactic acid bacteria, all strains of *Carnobacterium* found in meat products produced high amounts of tyramine and some strains of *Lactobacillus curvatus* and *Lactobacillus plantarum*, used as starter cultures, gave rise to tyramine production (Masson, Eclache, Compte, Talon, & Montel, 1996).

In Austrian dry sausages, highly significant differences were found in the histamine contents of products made by different manufacturers (Tschabrun, Sick, Bauer, & Kranner, 1990) and in long ripened products, its concentration reached up to 400 mg/kg DM (Paulsen & Bauer, 1999). Large variations in the amine content and composition were also found in retail Belgian sausages, possibly related to the method of manufacture and the specific flora of the sausages (Vandekerckhove, 1977). Furthermore, wide variations were observed in the

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amine content of different batches of the same commercial brand of fermented and cooked Spanish products (Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1997a) and also in cervelat sausages made with different amounts of starter culture (Treviño, Beil, & Steinhart, 1997). Considering the known toxicological effects of biogenic amines (Masson & Montel, 1995), it is important for the meat industry to produce fermented products with a high degree of safety with respect to biogenic amine content. The recommended upper limit for histamine in meat products proposed by the Netherlands Institute of Dairy Research is 100 mg/kg (Ten Brink et al., 1990) and in the Czech Republic it is 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 by the non-starter flora isolated from the different types of sausages was determined in order to understand the role of the flora in the final amine contents. Also, the influence of three different laboratories used in the analysis was evaluated.

2. Materials and methods

2.1. Preparation of sausages

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

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

2.2. 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 Cetrimide Fucidine Cephaloridine medium (CFC, Oxoid). The media were prepared, incubated and used according the manufacturer's instructions.

2.3. Amine determination in sausages

Three different laboratories carried out the quantitative separation of eight biogenic amines (tryptamine, phenylethylamine, histamine, putrescine, cadaverine, tryramine, spermine, spermidine) by reverse phase HPLC. Determinations were performed using different methods of analysis, all based on the derivatisation of amines to obtain dansyl derivatives which were detected by ultraviolet absorption at 254 nm. Two laboratories used perchloric acid (0.4M) as the extractant and 1,7-diaminoheptane as the internal standard (Eerola, Hinkkanen, Lindfors, & Hirvi, 1993). The derivatisation was carried out by incubation for 40 min in alkaline media and the sample was finally dissolved in acetonitrile. The other laboratory, used an extractant solvent of 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 (4 h). A further extraction of the amines with diethyl ether was included in this method. After the evaporation of the diethyl ether, the sample was dissolved in acetonitrile.

2.4. 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 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 seven amino acids (tyrosine, lysine, phenylalanine, arginine, tryptophan, histidine and ornithine) at a level of 2% for each one. The Maijala medium, was adjusted to pH 5.4 and incubated at 30 °C for 2 days. After centrifugation (3000 rpm, 3 min), supernatants were dansylated and amines were then quantified as described by Eerola et al. (1993).

2.5. 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 to estimate the contribution of the effect of laboratories (three), types of sausages (four), batches (five 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. Pearson's correlation coefficient was calculated between all amines. Calculations were performed using SPSS 9.0 for Windows.

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

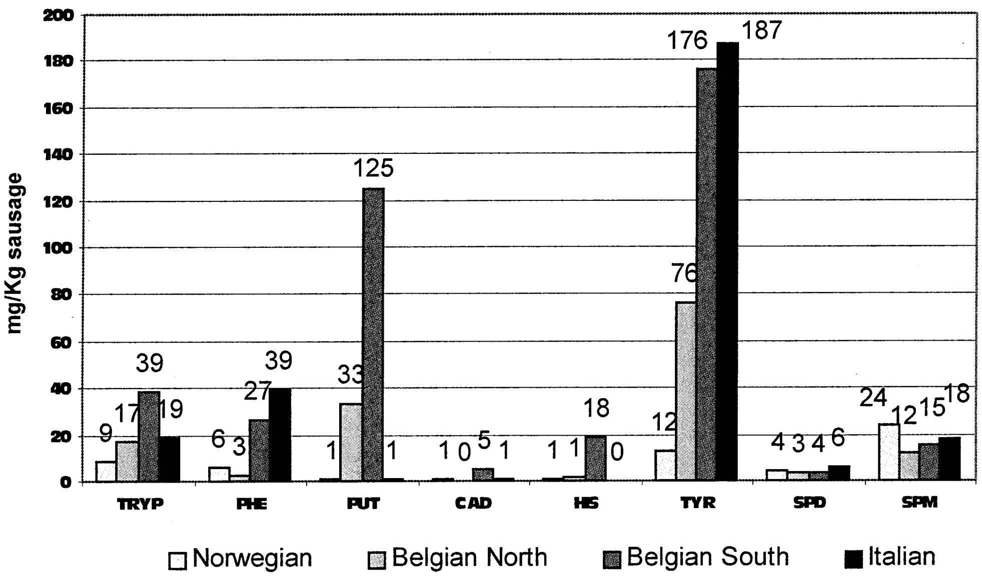
	Norwegian	Belgian North	Belgian South	Italian
Diameter (mm)	92	90	60	50–60
Weight	3 kg	1 kg	0.8 kg	0.5 kg
Meat species	Pork/beef (1/1)	Pork/beef (1/1)	Pork only	Pork only
Lean meat cuts	Miscellaneous	Pork shoulder + beef	Pork shoulder	Pork shoulder
Fat tissue	Pork back fat	Pork back fat	Pork back fat	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/h	27/55–96	20–26/62	5–24/113	21–22/12→17–18/96
ph after 24 h	5.45	5.70	5.8	5.75
ph after 3 days	4.79	4.68	5.2	5.95
%rH/h	90–96/55–96	50–90/62	10–90/89	95/12→90↓70–80/96
Drying				
°C/h	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] 2.10^6 each of *Pediococcus pentosaceus* and *Lactobacillus sakei* as lactic acid bacteria and *Staphylococcus xylosus, Kocuria varians* and *Staphylococcus carnosus* as flavour enhancers.
[c] 7.10^5 each of *P. pentosaceus* and *L. sakei* as lactic acid bacteria with 4.10^6 and 2.10^6 of *S. xylosus* and *K. varians* as flavour enhancers.
[d] A commercial powder containing a mixture of *Lactobacillus curvatus* and *K. varians* (2.10^6/g) was used (50 g/100 kg).
[e] Per kg meat and fat mince.
[f] g/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, type of sausage, and batch (within laboratory and type) on the amine content of the sausages[a]

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

[a] TRYP, Tryptamine; PHE, Phenylethylamine; PUT, Putrescine; CAD, Cadaverine; HIS, Histamine; TYR, Tyramine; SPD, Spermidine; SPM, Spermine. Figures show 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.



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

Fig. 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.

3. Results and discussion

The content of the eight biogenic amines was determined by the three different laboratories in four types of sausages. Five batches of each type were analysed and great variability was found for some of the amines, not only due to the different processing conditions, but also by the different laboratories and batches. A variance component estimation test was carried out on each amine to determine the main factors contributing to the total variability found in each case (Table 2). This test showed that the content of putrescine and tyramine was mainly determined by the type of sausage, where in both cases this factor explained around 53% of the total variability found for these two amines. Tyramine and putrescine are the main amines in ripened meat products (Hernández-Jover et al., 1997a) and the data for all amines in the four types of sausage are shown in Fig. 1. The analysis also revealed that the variability found in the concentrations of phenylethylamine, cadaverine and histamine was basically explained by the batch production, with the factor batch (labo*type) accounting for 55, 66 and 88% of the total variability in each case. This confirms the importance of the hygenic quality of the raw material on amine formulation during sausage ripening. Variability in results between laboratories was of little importance compared to the other factors studied, except for spermine and spermidine where this factor accounted for 43 and 83% of the total variance. For these two polyamines, little variability was found between the four types of sausages (Fig. 1). This can be explained by the fact that they are known to be naturally occurring amines in fresh pork and beef, and their formation is not associated with spoilage or fermentation (Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, & Vidal-Carou, 1996). An average value of 25 mg/kg was found for spermine during the ripening of mini-salami (Treviño et al., 1997) and 26.1 mg/kg was the mean found for 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 three of the four types of sausage was tyramine, in agreement with other authors (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2001; Eerola, Maijala, Roig-Sagués, Salminen, & Hirvi, 1996; Hernández-Jover et al., 1997a). Higher values were recorded for the southern type compared to the northern one (Fig. 1). The Mediterranean products clearly had over 100 mg/kg, which is the limit reported as unsafe by Stratton, Hutkins, and Taylor (1991). Also, ingestion of 10–40 mg of tyramine may be 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 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, no tyramine production was detected with the *Lactobacillus curvatus* and *Kocuria varians*, 2 strains used as starter

Table 3
Microbial flora associated with the four types of sausages[a]

	pH	Lactic acid bacteria	Cocci Gram +	*Pseudomonas*	*Enterobacteriaceae*	*Enterococcus*	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

[a] *Listeria* was not detected. Italian (I), Belgian North (BN), Belgian South (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[a]

	Flora	nt	TRYP		PHE		PUT		TYR	
			>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

[a] Norwegian (N), Belgian North (BN), Belgian South (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.

cultures in these sausages (Table 5). 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 these sausages. Consequently, further study of their evolution during processing should be done to determine their contribution to tyramine accumulation during ripening in Italian sausages. These results suggest that spontaneous non-starter lactic acid bacteria and cocci, were mainly responsible for 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 lesser 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 indicated as the two main groups responsible for the tyramine detected in these 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 also contained tyramine. The content of this amine could be explained by the presence of high levels of enterococci (Table 3) and by their capacity to produce high amounts of this amine in vitro (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 (Bacus, 1984; Bover-Cid, Hugas, Izquierdo-Pulido, & Vidal-Carou, 2000; Eitenmiller, Koehler, & Reagan, 1978; Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1997b). However, Maijala and Eerola (1993) concluded that

Table 5
Production of biogenic amines by the strains used as starter cultures in the different sausages[a]

	Starters	TRYP µg/ml	PHE µg/ml	PUT µg/ml	TYR µg/ml
Norwegian	*Lactobacillus species*	0	0	0	0
	Straphylococcus carnosus1	70	370	0	0
Belgium North	*Lactobacillus sakei*	0	0	0	0
	Pediococcus pentosaceus	0	0	0	0
	Kocuria varians 1	95	510	0	0
	Straphylococcus xylosus	0	0	0	0
	Straphylococcus carnosus2	115	585	0	0
Belgium South	*Lactobacillus sakei*	0	0	0	0
	Pediococcus pentosaceus	0	0	0	0
	Kocuria varians 1	95	510	0	0
	Straphylococcus xylosus	0	0	0	0
Italian	*Lactobacillus curvatus*	0	0	0	0
	Kocuria varians 2	0	65	0	0

[a] TRYP, tryptamine; PHE, phenylethylamine; PUT, putrescine, TYR, tyramin.

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), as 8–40 mg of this amine are necessary to produce slight skin disorders (Taylor, 1987).

Sausages with a high content in tyramine also had the highest content of phenylethylamine ($r = 0.64$). 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) since some strains are able to produce this amine (Table 4). For the Italian sausages, the starter *K. varians* 2, could also produce phenylethylamine (Table 5). In addition, micro-organisms isolated from AKEA, MRS, MSA and PDA produced this amine (Table 4).

In the Belgian North sausages, *K. varians* 1 and also *S. carnosus* 2, both produced high amounts of phenylethylamine in vitro (Table 5) and were included in the starter culture mix. However, in the manufacture of these products, very low concentrations of this amine were found in the final product (Fig. 1). The acidic conditions, pH = 4.68 on the third day of fermentation, may explain their low level. Furthermore, the importance of these two starters could be questionable as none of the isolates from MSA were able to produce phenylethylamine (Table 4).

The Norwegian sausages showed the lowest total amine content of the four. In these sausages, the presence of phenylethylamine could be largely 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 Maijala media (Table 5). Masson et al. (1996) found that *S. carnosus* strain 833 produced phenylethylamine (500 µg/ml) and to a lesser extent tryptamine (57 µg/ml).

A very high content of putrescine was detected in the Belgian South sausages (125 mg/kg) and to a lesser extent in the Belgian North ones in comparison with the Italian and Norwegian ones (Fig. 1). This could be linked to the high counts of *Enterococcus* (Table 3) and yeasts in the Belgian South sausages, since some of the strains isolated from AKEA (two strains of 13) and PDA (two of nine) produced more than 10 µg/ml of putrescine (Table 4). The production of putrescine could also be associated with endogenous flora not highlighted in this study. This amine has been reported not to be toxic by itself, but as potentiator, with cadaverine, for the toxic effect of histamine and tyramine. In fact, high 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 under laboratory conditions, and nor did strains of *Pediococcus pentosaceus* and *Staphylococcus xylosus*.

In summary, southern European sausages had higher contents of amines than the 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 safe. Spontaneous non-starter lactic acid bacteria and *Enterococci* could contribute to the content of amines in the sausages.

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