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Microbial ecosystems of processing units and traditional products in France

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SUMMARY – A European project titled Tradisausage (QLK1-CT-2002-02240) was set up with the objective of evaluating and improving the safety of traditional dry sausages from producers to consumers while preserving their typical quality. French traditional processing units and sausages did not present sanitary risks as no pathogens or sporadic pathogens were found. Presence of biogenic amines was noticed in the majority of the products. Enterococci were present throughout the manufacturing period and seemed to be involved in the production of biogenic amines. Contamination by spoilage flora was sometimes high and could generate flavour defaults in sausages and thus be responsible for economic losses. The technological flora (staphylococci and lactic acid bacteria) were both in the environment and in the products, they could constitute a barrier flora.

Keywords: Dry sausages, technological flora, spoilage flora, pathogen, flora identification.

RESUME – *"Écosystèmes microbiens des unités de transformation et des produits traditionnels en France". Le but du Projet Européen Tradisausage (QLK1-CT-2002-02240) est de faire un bilan puis d'améliorer la sécurité des saucissons secs de production traditionnelle depuis le producteur jusqu'au consommateur, tout en préservant leur typicité au niveau des qualités sensorielles. Les ateliers traditionnels en France ne présentent pas de risques sanitaires car aucun pathogène ou quelques cas sporadiques ont été isolés. Des amines biogènes ont été mesurées dans la majorité des produits. Leur présence peut provenir des entérocoques, présents à toutes les étapes de fabrications et dans les produits. La flore d'altération était parfois à des niveaux élevés et peut produire des défauts d'odeurs entraînant ainsi des pertes économiques. La flore technologique (staphylocoques et bactéries lactiques) étai présente dans l'environnement et dans les produits, et pourrait constituer une flore de barrière.*

Mots-clés : *Saucissons secs traditionnels, flores technologiques, flore d'altération, pathogènes, identification des flores.*

Introduction

In the meat sector, different crisis, but also the recurring food poisoning cases, have undermined public confidence on intensive or industrial meat producing systems. Consumers are, therefore, turning to "traditional" products and the growth of the market of organic foods and farm products is a clear sign of that. Traditional fermented dry sausages account for a significant part in such a domain. Traditional dry sausages rely on natural contamination by environmental flora. This contamination occurs during slaughtering and increases during manufacturing. Each processing unit has a specific house flora, composed of useful micro-organisms for the fermentation and flavour of sausages, but also spoilage and pathogenic flora. Few sporadic studies have been conducted on traditional meat products and have shown that hygienic shortcomings can lead up to 25% of product loss with high economic consequences and may undermine consumer confidence for traditional products.

Few studies are available on the contamination during production, from raw materials to final products**.** The characterisation of typical house flora, therefore, is crucial because safety (pathogenic flora), acceptability (spoilage flora) and sensorial quality (technological flora) of the products depend totally on it. In this context, the objective of the European project Tradisausage (QLK1-CT-2002-02240) was to evaluate and improve safety of traditional dry sausages from the producers to the consumers while preserving their typical quality. To reach this objective we characterize the flora on the environment of traditional processing units and on the corresponding sausages. The study has been carried out in five Mediterranean and one East countries. Here, we reported only results from traditional French processing units.

Methodology

Samples

108 traditional processing units (PU) manufacturing dry fermented sausages and located in Massif Central in the centre of France were studied for their typology. From the study of Rason *et al.* (submitted), ten processing units (noted from F01 to F10) representative of the diversity of these processing units were selected. The house flora and the flora contaminating the sausages were studied in these ten processing units. Six environmental samples were collected per processing units: three for the machines: mincing (Mc), mixing (Mx), stuffing (St); one for the cutting table (T), one from the wall of cold room of storage (Cr) and one from deboning knives (K). Samples representing 500 cm^2 of the environment were transferred to sterile Buffered Peptone Water solution (BPW) and homogenised. Four meat samples per processing unit were collected: casings (Ca), stuffed batter (Z), after drying, fermentation and smoking period (M) and final product to be commercialised (F). 25 g of products were transferred to BPM and homogenised.

Analysis of the flora

The flora was numerated on selective media: (i) *Enterobacteriaceae* on Cristal Violat neutral Red Bile Glucose (VRBG, Merck) incubated at 37°C for 24 h; (ii) *Pseudomonas* spp. on cetrimide-fucidine-cephalodorine agar (CFC agar with selective supplement, Oxoïd) incubated at 25°C for 48 h; (iii) Lactic acid bacteria on MRS (Merck) incubated at 30°C for 48-72 h under anaerobic conditions; (iv) Coagulase negative *Staphylococcus* and *Kocuria* on Mannitol salt phenol red agar (MSA, Merck) incubated at 30°C for 48 h; (v) *Enterococcus* on M-*Enterococcus* selective agar (Merck) at 37°C for 48 h; (vi) Yeasts and moulds on yeast extract glucose chloramphenical agar (YGC, Merck) incubated at 25°C for 3 to 5 days; (vii) *S. aureus* and coagulase positive staphylococci were numerated on Baird Parker agar supplemented with tellurite egg yolk (BP, Merck), incubated at 37°C for 24 to 48 h; (viii) *Listeria monocytogenes* was numerated on *Listeria* agar (ALOA, AES Laboratory) incubated at 37°C for 24 to 48 h. PCR analysis was performed to confirm colonies; and (ix) *Salmonella* was isolated on modified Rappaport Vasiliadis Soft Agar at 42°C for 24 h and then on BPLS media at 37°C, 24 h if mobile bacteria were detected.

Characterisation of the meat samples

pH and water activity (a_w) measurements

a_w of the media was measured with an a_w-sprint TH500 (Novasina, Roucaire, France). A pHmeter MP230 (Mettler Toledo, Viroflay, France) was used with a pH probe (Inlab 427 penetration probe, Mettler Toledo).

Biogenic amines

Biogenic amines (tyramine, histamine, putrescine and cadaverine) were analysed on the meat samples, by ion-pair reverse phase high performance liquid chromatography. Briefly, the method involved the extraction of biogenic amines with 0.6 N perchloric acid from a homogenised aliquot (5-10 g). Then a chromatographic gradient allowed the separation of ion-pairs formed between biogenic amines and the octanesulphonic acid of the mobile phase. A post-column derivatization of separated amines with o-phthalaldehyde-2-mercaptoethanol was followed by spectrofluorometric detection. These analyses were performed by S. Bover-Cid *et al.* (University of Barcelona, Barcelona, Spain).

Identification of enterococci

A collection has been constituted from the ten processing units. The isolates of enterococci were identified by PCR according to Woodford *et al.* (1997). These analyses were performed by A. Laukova *et al.* (Institute of Animal Physiology Slovak Academy of Sciences, Košice, Slovakia).

Identification of Gram positive cocci

A collection has been constituted from the ten processing units (PU). The isolates of staphylococci and *Kocuria* were identified by a reverse line blot hybridisation method developed in our laboratory. The tool, called "Staph. Array", is based on reverse line blot hybridisation techniques. We designed oligonucleotide probes from the partial sequences of the *sodA* genes (super oxide dismutase) of 35 staphylococci species done by Poyart *et al*. (2001). These probes were fixed covalently on membranes. Colonies or lysates from isolated colonies were used as templates for a PCR using *sodA* primers. The Dig-labelled PCR products were hybridised with the immobilised probes.

Statistical analysis

Principal component analysis (PCA), analysis of variance (ANOVA) and Newman-Keuls tests were performed on the data using Statistica software (version 6.1, Statsoft inc., Maisons-Alfort, France).

Results and discussion

Environmental sampling results

60 samples were analysed. No *Salmonella* and no *S. aureus* were detected in the samples. *L. monocytogenes* was found in three samples. From the results of the technological and spoilage flora in the environment samples, an ANOVA and a Newman-Keuls test were performed. ANOVA showed that the processing units affected signicantly the level of lactic acid bacteria, staphylococci and enterococci, but did not affect significantly the population level of *Enterobacteriaceae*, *Pseudomonas*, and yeasts and moulds. For lactic acid bacteria, processing units were divided into two groups: one made of eight PU whose population was between 0.7 to 2.7 log CFU/100cm^2 and the second made of two PU (population level 4.0-5.0 log CFU/cm^2). For staphylococci, two similar groups were observed: the same eight PU had a population of 1.4 to 3.3 log CFU/cm^2, and the two other PU, a population of 5.0 log CFU/cm^2. For the enterococci, one group (nine PU) had a bacterial population of 0.7 – 2.6 3.3 log CFU/cm^2, and the second, made of one PU had a level of 4 log CFU/cm^2.

The ANOVA showed that the environmental samplings affected significantly the level of all flora. But, it was observed that cold room and mixing machine samples were the less contaminated (inferior to 2 log CFU/cm^2) while knives and table samples were the most contaminated (highest level of 4.0 log CFU/cm^2).

Meat sample results

pH and water activity (a_w)

The pH of the meat samples were all above pH 5. Great pH variations were observed between the processing units (Fig. 1). In some processing unit, glucose is added in the batter. When glucose is not added, an increase of pH occurs systematically as observed in PU F02, F07 and F08. When glucose was added, lactic acid bacteria can easily grow and then produce lactic acid that decreases the pH. The decrease can be observed between the fermentation and the final product steps as in PU F01 F04, or between the batter and fermentation steps as in PU F03, F05, F06 and F10. Increase in pH is sometimes observed after the fermentation step and is due to the production of ammonia by the other flora present in the product.

For all the samples, the a_w decreases during the process of the sausage (Fig. 2).

The time between the fermentation step (M) and the final product (F) is variable and depends on the manufacturers (Figs. 1 and 2).

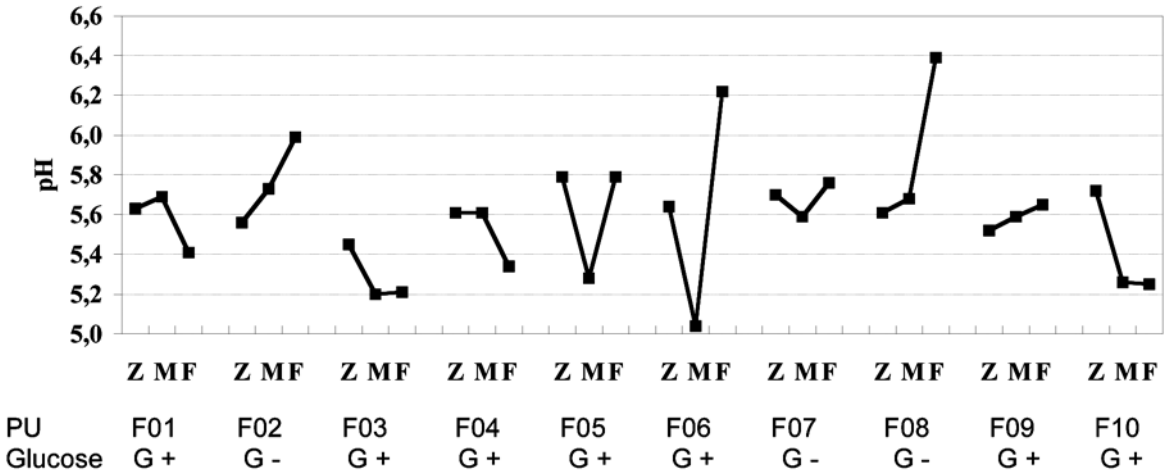

Fig. 1. pH of the meat samples at the three stages of production for the ten processing units (PU: F01 to F10). Stuffed batter (Z), after fermentation (M) and final product (F). Processing units with (G+) or without (G-) added glucose in the batter.

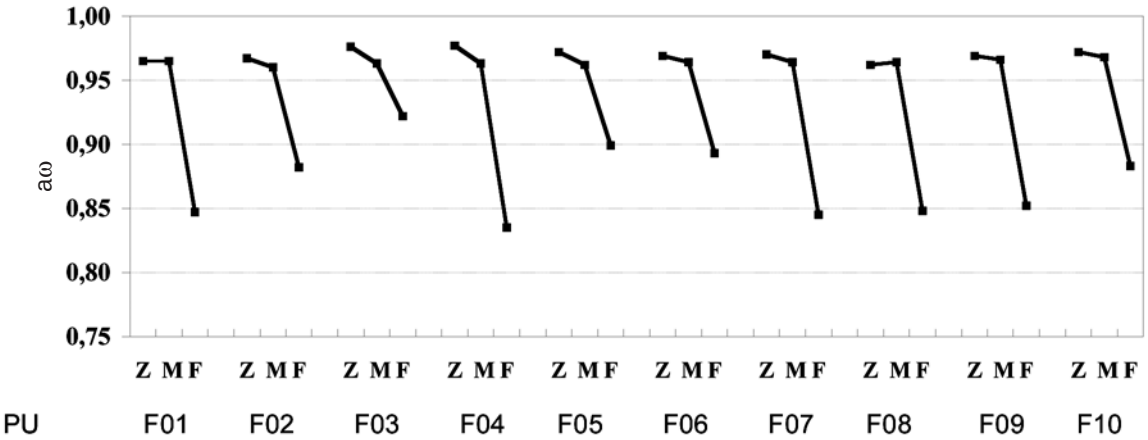

Fig. 2. Water activity (aω) of the meat samples at the three stages of production for the ten processing units (PU). Stuffed batter (Z), after fermentation (M) and final product (F).

Statement of the flora in meat

Of the 40 analysed samples, none were contaminated by *Salmonella.* Six samples were contaminated by *L. monocytogenes* but only one final product had a level slightly superior to 2 log/g. Two samples were contaminated by *S. aureus* with only one final product with a level superior to 2.7 log/g.

For the technological flora, lactic acid bacteria and staphylococci were present in all products (Fig. 3a). As a general rule, their population increased during the process and reached levels varying from 6.5 to 8 log/g in the final products. For the spoilage flora, *Pseudomonas* and enterobacteria were detected in most of the products (Fig. 3b) Their populations started at an average level of 4-5 log/g in the batter and remained constant during the fermentation. A decrease of 1.5 to 2.0 log/g was noticed at the end of ripening. Enterococci and yeasts/moulds contaminated the batter at level of 3 to 4 log/g in the batter, increased during fermentation (1 log) and then remained constant (Fig. 3c).

To our knowledge, no data concerned the environmental flora of traditional processing units manufacturing sausages. But many studies concerned traditional meat fermented products. In naturally fermented Italian sausages, the technological flora grew during the process and reached similar values we mentioned above. In these products *Enterobacteriaceae* and *Enterococcus* could

reach high level during fermentation and decreased during ripening. *Salmonella* and *S. aureus* were not found (Cocolin *et al*., 2001). In Greek salami, *L. monocytogenes* was detected in the batter but not in the final products (Samelis *et al*., 1998).

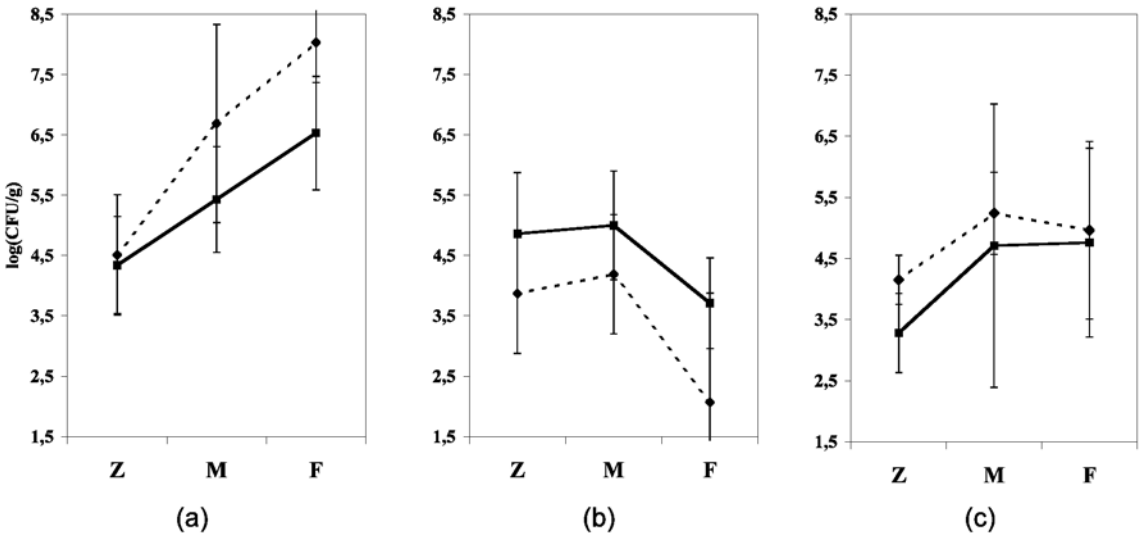

Fig. 3. Evolution of the different microbial population in the three products: stuffed batter (Z), after fermentation (M) and final product (F). (a) (—■—) Staphylococcus and Kocuria, (– – ♦ – –) Lactic acid bacteria; (b) (—■—) Pseudomonas, (– – ♦ – –) Enterobacteriaceae; (c) (—■—) Enteroccocus, (– – ♦ – –), Yeasts and moulds. Data were calculated from the average of the ten processing units (log(CFU/g). Vertical lines: standard deviation.

Origin of the contamination

For the environmental samplings, samples were collected after the cleaning and disinfection of the processing unit. Processing unit F04 (Fig. 4) is characterised by a high level for all flora at each environmental sampling. The flora level in the batter is also high. It could be assumed that the origin of the contamination came from the environment and from the raw material.

Processing unit F07 (Fig. 5) is characterised by low contamination in the environment and high levels of population in the casings and in the batter. Cleaning and disinfection were more efficient in PU F07 than in PU F04 and the assumption of a contamination of the product by the environment flora is not confirmed in this case. The origin of the contamination is mainly due to the flora of the raw material and sometimes of the casings.

Biogenic amines

The biogenic amine levels of initial meat samples were very low or not detected in all processing units, except one (F05). Histamine was the biogenic amine that appeared less frequently whatever the product (batter, after fermentation or final product). For tyramine, putrescine and cadaverine, concentrations increased throughout the fermentation and storage steps in all the PU (Fig. 6). Large variations of the three amines were observed between the PU: tyramine concentrations varied from not detected to 227 mg/kg, putrescine from not detected to 362 mg/kg and cadaverine from not detected to 390 mg/kg. Taking into account the biogenic amine total contents of the final products, three groups of sausages could be determined: those with extremely low contents with less than 10 mg/kg total amines (two PU: F02 and F10), those with intermediate amounts of total amines (between 150 and 450 mg/kg) and finally one PU in which sausages showed high contents of total amines (1021 mg/kg) in which cadaverine was the major amine.

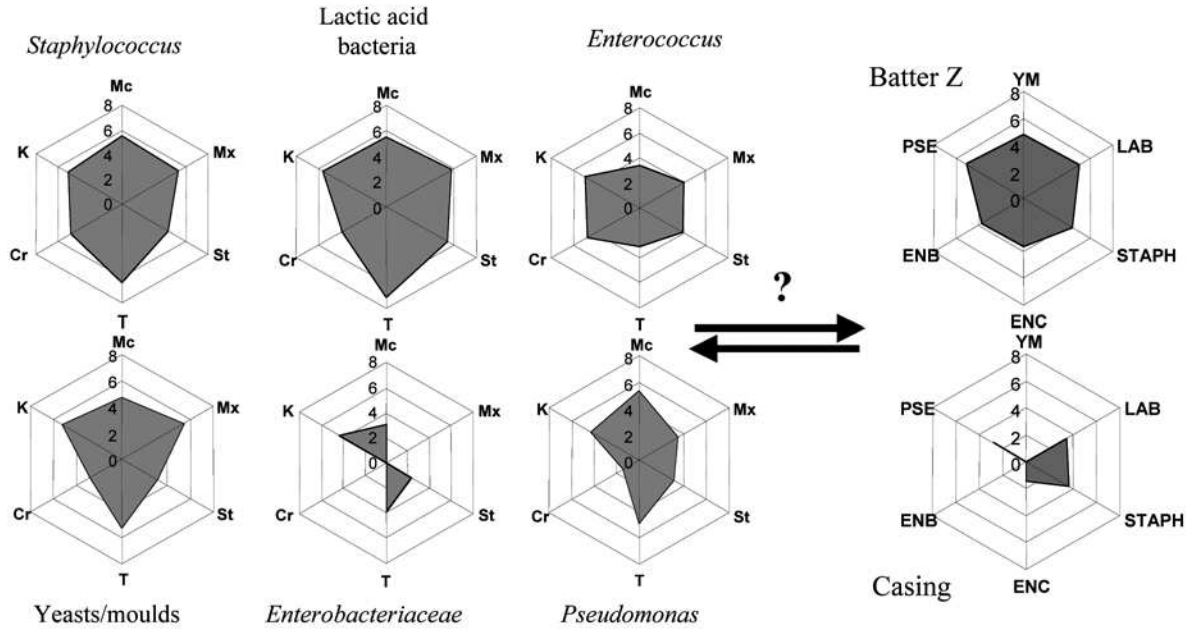

Fig. 4. Levels of the flora in the environment and in the products of processing unit F04. Environmental samples (log CFU/100cm^2): mincing machine (Mc), mixing machine (Mx), stuffing machine (St), table (T), cold room (Cr), knives (K). Analysed flora in the casings and batter (log CFU/g): lactic acid bacteria (LAB), *Staphylococcus* (STAPH), *Enterococcus* (ENT), *Enterobacteriaceae* (ENB), *Pseudomonas* (PSE), yeasts and moulds (YM).

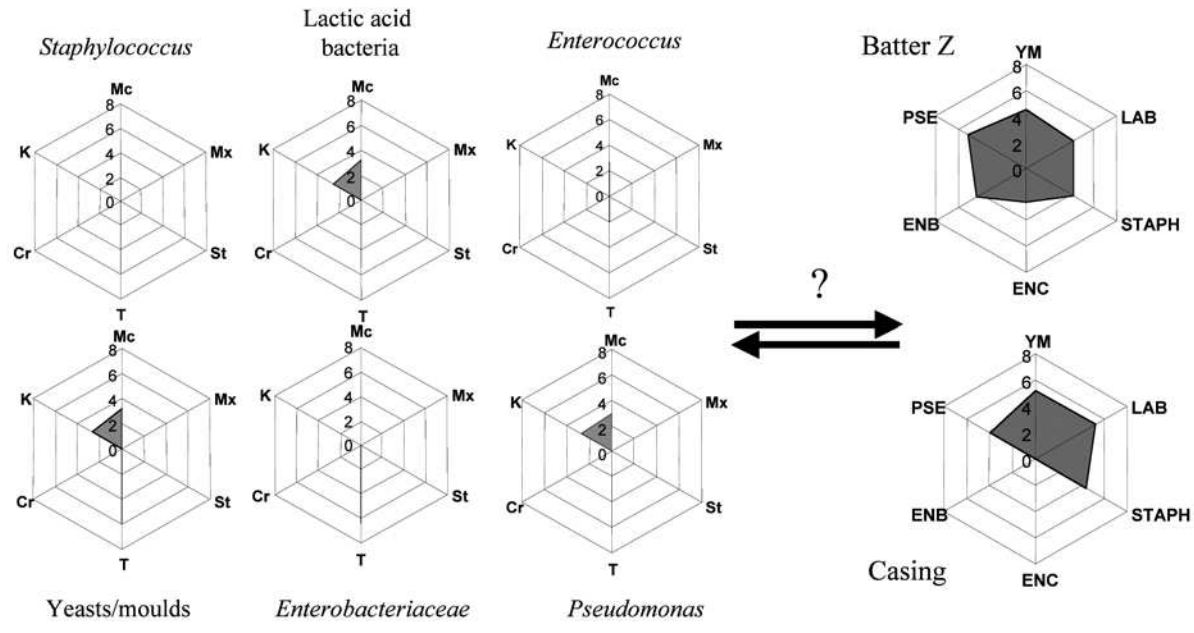

Fig. 5. Levels of the flora in the environment and in the products of processing unit F07. Environmental samples (log CFU/100cm^2): mincing machine (Mc), mixing machine (Mx), stuffing machine (St), table (T), cold room (Cr), knives (K). Analysed flora in the casings and batter (log CFU/g): lactic acid bacteria (LAB), *Staphylococcus* (STAPH), *Enterococcus* (ENT), *Enterobacteriaceae* (ENB), *Pseudomonas* (PSE), yeasts and moulds (YM).

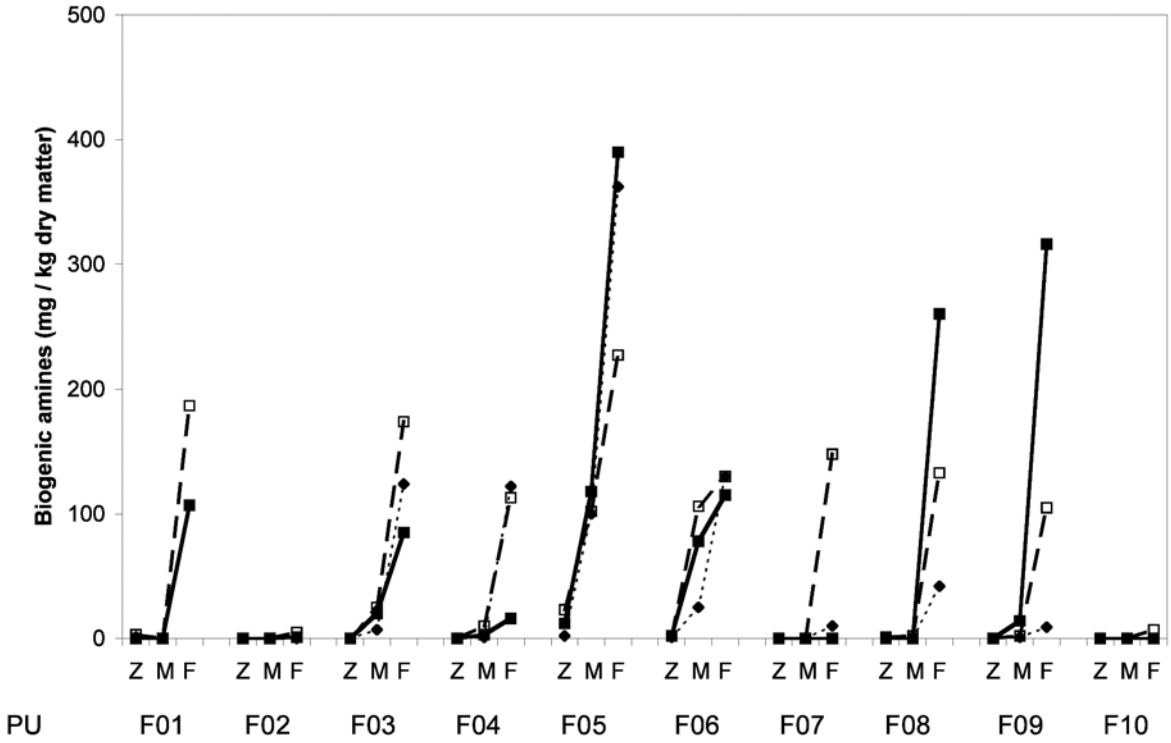

Fig. 6. Concentration in biogenic amines (mg/ kg dry matter) in the three meat samples (stuffed batter (Z), after fermentation (M) and final product (F)) of the ten processing units (PU).
(—○ —) tyramine, (– – ♦ – –) putrescine, (—■—) cadaverine.

In the principal component analysis, 64.8% of the information is presented by two axes 1 and 2 (Fig. 7). Three groups are observed and concerned the three meat products, Z, M and F. In the batter, products are mainly correlated to the presence of high levels of *Pseudomonas* and *Enterobacteriaceae*. Then in group M and F, these two flora decreased as shown in Fig. 3b. In the final products, some processing units are correlated to high levels of staphylococci and are characterised by low or intermediate levels in biogenic amines. Other processing units are correlated to lactic acid bacteria, enterococci and amines. One processing unit (F05) is associated to enteroccoci and amine and was shown to have high level of amines.

Biogenic amines are produced from amino acids by microbial decarboxylation. Some biogenic amines can be hazardous for health due to their vasoactive and/or psychoactive properties (Bover-Cid *et al*., 1999). Biogenic amines have repetitively been shown to be present in meat products (Montel *et al*., 1999). *Enterobacteriaceae* and *Pseudomonas* frequently isolated from raw materials but also from sausages, can produce biogenic amines, especially during the first days of fermentation. The hygienic quality of raw materials appears to be one of the main factors affecting biogenic amine formation in dry fermented sausages (Bover-Cid *et al*., 2001) Some strains of lactic acid bacteria, staphylococci and *Kocuria* are also able to produce amines. Biogenic amines have been also related to the presence of enterococci that can produce these amines.

Identification of enterococci

A total of 45 strains were identified from all the samples. It appears that 44 strains were identified as *E. feacium* and one as *E. feacalis*.

Enterococci form part of the lactic acid bacteria of importance in foods. They seem to be important for ripening and aroma development of traditional sausages. Many enterococci have the ability to produce enterocins harbouring antimicrobial activity against pathogens such as *L. monocytogenes* (Hugas *et al*., 2003). But in other hand, they are important nosocomial pathogens and some strains are resistant to many antibiotics. Some strains also produced biogenic amines. It appears that *E. feacium*, the dominant species of our study, harbours fewer virulence traits than *E. feacalis* (Franz *et al*., 2003).

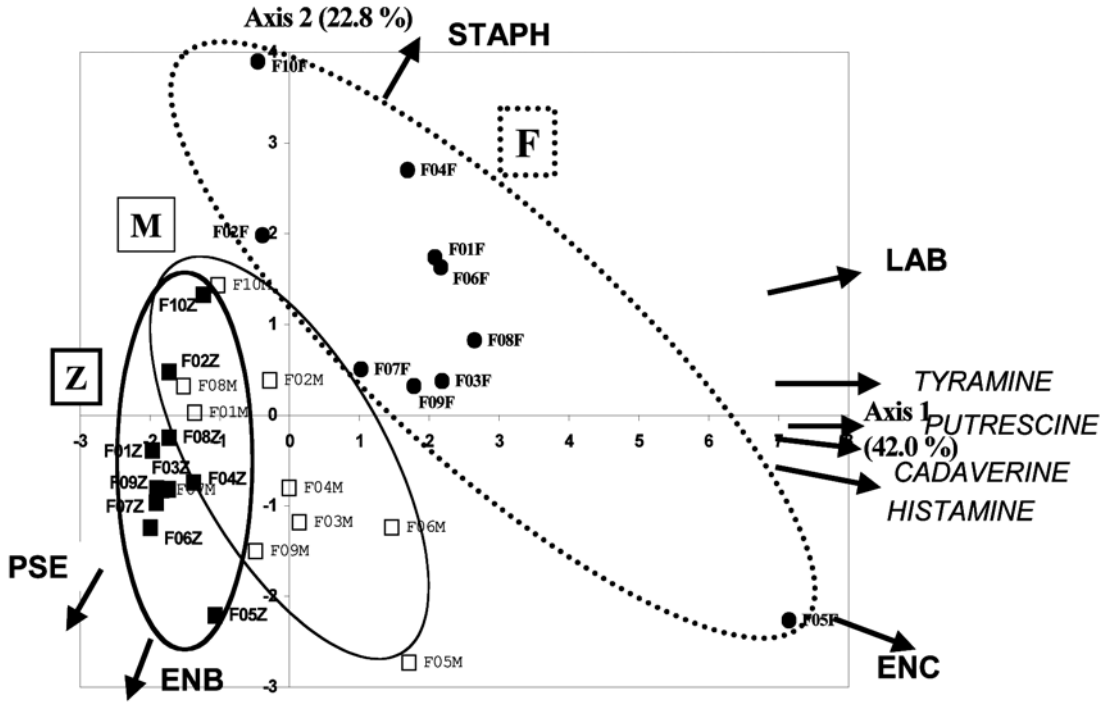

Fig. 7. Principal component analysis: links between variables that characterise the product (amines) and the six flora: lactic acid bacteria (LAB), *Staphylococcus* (STAPH), *Enterococus* (ENT), *Enterobacteriaceae* (ENB), *Pseudomonas* (PSE), yeasts and moulds (YM). Three groups: (■) stuffed batter (Z), (○) after fermentation (M) and (●) final product (F).

Identification of staphylococci

The screening of a collection of 755 strains showed that 666 strains belonged to the genus *Staphylococcus* and 90 could belong to the genus *Kocuria* (Table 1). With the method "Staph. Array", we design specific oligonucleotidic probes for 35 species of staphylococci. We check that all probes hybridised with their target but a few hybridised also with the partial *sodA* gene from close species. Even with these cross-hybridisations we were able to identify the 35 species since we obtained a unique and specific pattern for each species. Among the 666 strains of *Staphylococcus* of the collection, 410 were identified as *S. equorum*, 106 as *S. saprophyticus*, 62 as *S. xylosus* (Table 1). Great variations are observed between the PU. *S. equorum* is the dominant *Staphylococcus* and present everywhere, it colonises the environment and the products. When other staphylococci are present in initial stage of the product but their number decrease in favor of *S. equorum*.

Staphylococci are isolated from a wide range of foodstuff such as meat, cheese or milk and from environmental sources such as soil, sand, dust, air or natural water. In fermented meat products, *S. xylosus* and *S. saprophyticus* are frequently isolated (Blaiotta *et al*., 2003) but *S. equorum* we found as dominant species is not listed as dominant in other fermented meat products.

Conclusion

The French traditional processing units and the sausages did not present sanitary risk as no pathogens or sporadic pathogens were found but presence of biogenic amines was noticed in the majority of the products. Enterococci are present all along the manufacturing period and seemed to be involved in the production of biogenic amines that can be hazardous for the health. The contamination by spoilage flora was sometimes high and could generate flavour defaults in sausages and thus be responsible of economic looses. This spoilage flora could also synthesised biogenic amines. The technological flora (staphylococci and lactic acid bacteria) were both in the environment and in products, they could constitute a barrier flora.

Table 1. Diversity of *Staphylococcus* and *Kocuria* isolated the environment and the meat products in nine processing units. Identification made by "Staph array" method. Number of strains

Processing units	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	Total
Isolates	59	165	46	73	91	79	39	170	33	201	956
Kocuria	20	5	14	6	7	6	2	29	1	38	128
Staphylococcus	39	160	31	67	84	73	37	141	32	163	827
S. arletti					1						1
S. aureus		1									1
S. capitis			1								1
S. carnosus				3						13	16
S. epidermidis	6				2	2		1	1	1	13
S. equorum	19	92	24	41	50	30	12	116	26	10	420
S. fleurettii								1			1
S. hominis					1						1
S. pasteuri		5		3				1		2	11
S. saprophyticus	13	31	3	20	19	7	11	1	1	13	119
S. sciuri	1										1
S. succinus						1	12	21		2	36
S. vitulinus							2			1	3
S. warneri					11	8			1		20
S. xylosus		31	3			25			3	121	183

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