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Meat Science 54 (2000) 203–208

MEAT SCIENCE

www.elsevier.com/locate/meatsci

Yeast populations on Spanish fermented sausages

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Received 18 February 1999; received in revised form 21 May 1999; accepted 18 June 1999

Abstract

Yeast populations on 24 lots of Spanish fermented sausages, made by four factories (F1, F2 and F4, artisanal; F3, industrial) were investigated throughout manufacture and the influence of different variables evaluated. In addition, 41 yeast strains were identified at the species level using two miniaturised systems: ATB32C (API System) and Vitek Yeast Biochemical Card (Vitek YBC). Levels of yeasts found in the sausage mixture (mean counts around 4 log units/g) were similar to those described by other authors. In sausages from factories F1 and F2, a further increase was noted, reaching 5.5 log units/g after fermentation. Counts subsequently decreased to 3.6 and 5 log units/g, respectively. In sausages from factories F3 and F4, decreasing counts were observed from the beginning, particularly in sausages from F3, where yeasts were almost absent in the finished product. Type of manufacture and sausage diameter, were the variables most influencing yeast counts. *Debaryomyces hansenii* (teleomorph of *C. famata*) was the dominant species, being found at all stages of manufacture. *Trichosporon ovoides* (formerly *T. beigelii*), *Yarrowia lipolytica* (perfect form of *C. lipolytica*), *C. intermedia/curvata, C. parapsilosis, C. zeylanoides* and *Citeromyces matritensis* (teleomorph of *C. globosa*) were also present. Direct identification was possible only with 50% of the total of strains investigated, although a higher number of strains was identified using the API than the Vitek YBC system.

Keywords: Spanish fermented sausages; Chorizo; Yeasts

1. Introduction

Fermented sausages are considered good substrates for the growth of yeasts. In these products, competing microorganisms such as Gram-negative bacteria are inhibited by such properties as low pH, presence of lactic acid and nitrite, and low water activity (Dillon, 1998). Although the role of bacteria in the manufacture of these fermented foods is well known, the participation of yeasts is not clear (Dillon, 1998). However, studies carried out with different yeast starters (mainly *Debaryomyces hansenii*, teleomorph of *Candida famata*) have shown the contribution of yeasts to the development of colour (by removing the oxygen) and flavour (Jessen, 1995), due to their ability to degrade peroxides, lipolytic activity and, to a lesser degree, proteolytic activity (Leistner, 1986; Lücke, 1985). Furthermore, yeasts protect sausages from the adverse effects of light (Lücke & Hechelmann, 1987).

Spanish fermented sausages are appreciated for their organoleptic characteristics. "Chorizo" and "salchichón" are the most important products (over 120,000 tons/year). Both are made from coarsely cut pork meat (or a mixture of pork and beef) with pork fat, together with spices and seasoning (garlic, oregano, sodium chloride, paprika in "chorizo", black pepper in "salchichón", etc., Lois, Gutiérrez, Zumalacárregui & López, 1987). Over 50 different varieties of these fermented sausages, based on varying combinations of ingredients and processing, are produced by both large and small factories in Spain (Marcos, 1991).

Studies on the yeast flora of fermented sausages are limited, compared to those focused on bacteria. Difficulties in isolation and taxonomy of this group of microorganisms have contributed to this situation. Traditionally, identification of yeasts involved the use of numerous morphological and physiological tests (Barnett, Payne & Yarrow, 1990; Kreger van Rij, 1984), but simplified keys for food-borne yeasts (Deak & Beuchat, 1987) are now available, as well as several miniaturised methods (ATB32C, API Yeast Ident, Vitek YBC, etc.).

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0309-1740/99/$ - see front matter
PII: S0309-1740(99)00080-7

In this paper, the evolution of yeast counts throughout the manufacture of several Spanish fermented sausages is reported and the influence of various technological factors discussed. Also, part of the isolated yeast population has been identified and the usefulness of two miniaturised methods (ATB32C and Vitek Yeast Biochemical Card) evaluated.

2. Materials and methods

2.1. Sausages

Twenty four lots of different fermented sausages produced by four manufacturers were studied. Three of them (F1, F2 and F4) used traditional procedures (artisanal) and one (F3) used highly developed technological processes (industrial).

2.1.1. Factory 1 (F1)

Three batches of "chorizo picante" (seasoned with hot paprika) and three of "chorizo dulce" (seasoned with mild paprika) were made. The mixture (without starter culture) was kept at 10°C or lower for 48 h, stuffed into natural casing (30–40 mm diameter), hot-smoked and matured in chambers (relative humidity, 70–80%, and temperature, 10–14°C; normal process). One batch of each type of sausage was submitted to accelerated ripening, only 2 weeks at temperatures up to 18°C.

2.1.2. Factory 2 (F2)

Five batches of non-smoked "chorizo dulce" (with a diameter of 30–40 mm), processed by traditional procedures (without starter and air-dried during winter at room temperatures) were made. The lack of controlled temperature-humidity conditions allowed significant mould growth on the surface of some sausages.

2.1.3. Factory 3 (F3)

Five batches of "chorizo en barra" (80 mm diameter) and five batches of "chorizo en vela" (50 mm diameter) were made. Factory 3 is a large meat manufacturing plant in which a starter culture (lactic acid bacteria), a fermentable sugar and sorbates are added to the sausage mixture. High fermentation temperatures were used (24–26°C during 2–3 days; relative humidity, 80–90%) and drying conditions were controlled.

2.1.4. Factory 4 (F4)

Two batches of "longaniza" (a type of "chorizo" with 30 mm diameter) and one of "salchichón" (seasoned with black pepper instead of paprika, 50–60 mm diameter) were made. The manufacture of both sausages was artisanal. Conditions were uncontrolled for "longaniza" (the mixture was not always kept at 4°C, smoking was carried out using different kinds of wood, ripening was performed with no control of temperature or humidity, etc.). In the "salchichón" process, fermentation was carried out at 4°C for 72 h, oak was used for smoking and ripening lasted two months under controlled conditions.

2.2. Sampling and enumeration of yeasts

Duplicate samples for enumeration of yeasts and other analysis were taken at four stages of manufacture: sausage mixture (S1), sausage immediately after filling into casings (S2), sausage after fermentation and, optionally, smoking (S3, 1 or 2 weeks of manufacture) and finished product (S4, from 16th to the 32nd day of manufacture). After aseptically removing the casings from the sausages, each sample (100 g) was homogenised in a Stomacher 400 Lab Blender (Seward Medical, London) for 2 min using 400 ml of sterile peptone water (0.1% w/v) containing Tween 80 (1% w/v) as diluent. Further decimal dilutions were made with the same diluent and 0.1 ml portions were spread onto the surface of OGYE agar plates (Oxytetracycline Glucose Yeast Extract agar, Oxoid, code CM545). Plates were incubated at 25°C for 5–7 days.

2.3. Identification

Forty one yeast colonies were selected from the counting plates, picking representative colonies of each morphological type present (the relative occurrence of each type of colony isolated was estimated). After purification, strains were submitted to the following tests for identification. Growth at 5, 25, 30, 37 and 42°C for 72 h (10 days for 5°C incubations), and type of growth on solid medium (on Saboureaud Dextrose Agar -SDA-, Oxoid, code CM41) and the following characteristics noted (Barnett et al., 1990): presence of pseudohyphae, true hyphae, blastoconidia, chlamydospores, arthroconidia, ballistoconidia (by the Dalmau technique using Corn Meal Agar, Oxoid, code CM103), type of sexual reproduction, mode of asexual reproduction, shape and size of vegetative cells by direct microscopic examination, growth on liquid medium (Sabouraud broth, Oxoid, code CM147). In addition, yeasts were tested with two miniaturised methods: ATB32C (API System, Vercieu, France) and Vitek Yeast Biochemical Card (YBC, bioMérieux Vitek, Inc., Missouri, USA), this last in conjunction with the Vitek Junior database (Vitek Systems, version 1990). In both systems, the instructions of the manufacturer were followed and a bio-number of 10 (ATB32C) and 9 (YBC) digits was obtained for each strain. Identification criteria according to Kreger-van Rij (1984) and Barnett et al. (1990), were followed for final identification. Nomenclature used in this report is as described by Kurtzman and Fell (1998).

2.4. Statistical analysis

Statistical significance of differences between means of log counts was determined by a Student's test.

3. Results and discussion

Yeast count evolution during manufacture of fermented sausages, according to the factory, is shown in Table 1. Levels of yeasts in the initial sausage mixture were around 4 logarithmic units per gram, with no significant differences ($p < 0.05$) among the lots investigated. These values compare well with other studies on fermented sausages (Daporta, 1988, Sarasíbar, Sánchez-Monge & Bello, 1989; Samelis, Stavropoulos, Kakouri & Metaxopoulos, 1994). Afterwards, counts varied with the factory. In sausages made at factories F1 and F2, maximum counts (around 5.5 logarithmic units/g) were reached after fermentation, with similar values being found by Daporta (1988) and Samelis et al. (1994) In artisanal and non-smoked sausages (F2), counts decreased very slightly at the end. In naturally ripened Greek dry salami, Samelis et al. (1994) found increasing yeasts counts from the initial mixture to the end of the process. However, in sausages from factories F3 and F4, a continuous decrease in counts was found.

The yeasts counts found in our study help to explain the lactic acid and pH changes in our samples during ripening (Encinas, 1993). Lactic acid increased with time in all batches, but final values were clearly highest in sausages with the lowest yeast counts, which also had the lowest pH values (F3). Yeasts are potent consumers of lactic acid in foods which leads to an increase in the pH (Walker, 1977).

The differences in yeast counts between batches may be the result of several factors influencing the microflora of our samples. According to the data shown in Fig. 1, the type of manufacture (industrial or artisanal, Fig. 1a) and sausage diameter (Fig. 1c) were the most influential variables, the differences in yeasts counts being statistically significant ($p < 0.05$) from the second stage of manufacture (S2). The use of lactic acid starters and sorbate in the industrial process would explain the differences found between the artisanal (F1, F2 and F4) and industrial (F3) sausages. Sorbates, which were allowed by Spanish legislation until 1997 and were added to our industrial sausages at 1000 ppm, are known to control yeasts in foods and drinks (Fleet, 1990). The availability of oxygen would be higher in the smaller sausages (30–40 mm diameter), and thus explain the higher counts in these samples (Miller, 1979).

Table 1
Evolution of yeast counts[a] during manufacture and ripening of fermented sausages distributed by factory

Sausage factory[b]	Stage[c]			
	S1	S2	S3	S4
F1	3.98 ± 0.49[d]	4.68 ± 0.38	5.56 ± 0.47	3.63 ± 2.02
F2	4.11 ± 0.05	4.97 ± 0.11	5.59 ± 0.45	4.99 ± 0.56
F3	3.96 ± 0.64	3.89 ± 0.63	2.13 ± 1.96	1.19 ± 0.93
F4	4.64 ± 0.44	4.29 ± 0.71	3.31 ± 0.01	3.05 ± 2.33
All factories	4.08 ± 0.52	4.38 ± 0.65	4.29 ± 1.92	3.06 ± 2.01

[a] Counts on OGYE agar (oxytetracycline glucose yeast extract, Oxoid), at 25°C during 5–7 days.

[b] F, factories: F1, artisanal and smoked; F2, artisinal and non-smoked; F3, industrial and non-smoked; F4, artisanal and smoked.

[c] Stages: S1, initial sausage mixture; S2, after filling into casings; S3, after fermentation and smoking; S4, finished product.

[d] Log CFU/g (mean ± standard deviation).

Smoking, variety of spices and method of ripening had only limited influence on yeast counts in some stages of the manufacture (Fig. 1b, d, e). Differences were statistically significant ($p < 0.05$) only at the second stage of manufacture between smoked and non-smoked sausages and, from this stage onwards, mean counts in smoked sausages were lower than in the non-smoked ones (Fig. 1b). Yeasts are affected by smoking (Leistner, 1995), and factors such as time and temperature influence the effect. Studies on the influence of spices on yeast growth are limited, although Ghamnnoum (1990) and Asehraou, Mohieddine, Faid and Serhrouchini, (1997) found an inhibitory effect of garlic on yeasts. Ripening conditions (accelerated or normal, in sausages from F1) affected the yeast counts found in the final product, with counts being 1 log unit/g lower when an accelerated process was used (Fig. 1e).

The species found in our samples are shown in Table 2. *D. hansenii* (and its imperfect form, *C. famata*) was the dominant yeast. Its presence at high levels at all stages of manufacture, especially in the traditional sausages with the most desired organoleptic characteristics, suggests its possible contribution to the flavour of these products. Nowadays, this species, characterised by its tolerance to salt and nitrate, and high oxygen demands, is the main yeast used as starter in these meat products (Jessen, 1995).

D. hansenii, and other species found in our samples (*C. zeylanoides* and *Yarrowia lipolytica*, perfect form of *C. lipolytica*), are considered psychrotrophic (able to grow below 5°C, Eklund, Spinelli, Miyauchi & Groninger, 1965). They are commonly present in meat products (Deak, 1991) and are found among the dominant species in other Spanish fermented sausages (Iñigo, Arroyo & Somavilla, 1970; Sesma & Ramírez, 1976).

Candida intermedia/curvata, *Citeromyces matritensis* (perfect form of *C. globosa*), *C. zeylanoides* and *T. ovoides* (formerly *T. beigelii*) were detected only at the first stages of the sausage manufacture (sausage mixture, S1, and sausage after filling into casings, S2). A similar finding was reported by Fung and Liang (1990) in minced meat and fresh sausages. *C. parapsilosis* and *Y. lipolytica* have similar behaviour except in one batch (F2), made during the spring (humid period), which gave a slimy surface. The

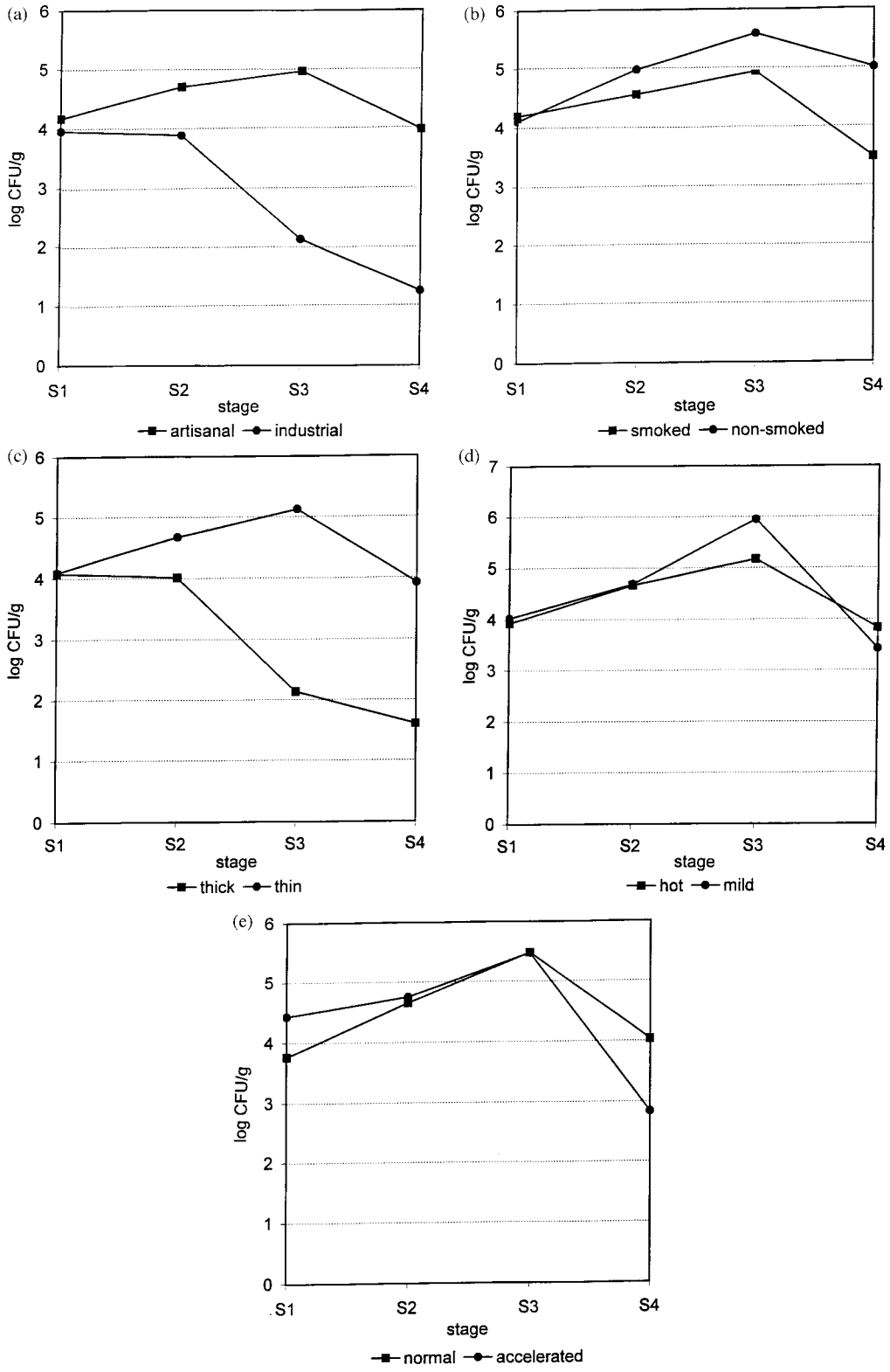

Fig 1. Influence of different manufacturing variables on the evolution of mean yeasts counts in fermented sausages (a) type of manufacture; (b) smoking; (c) sausage diameter (thin, 30–40 mm; thick 50–80 mm); (d) type of paprika; (e) type of ripening. Stages: S1, initial sausage mixture; S2, after filling into casings; S3, after fermentation and smoking; S4, finished product.

Table 2
Identification[a] of yeasts isolated from fermented sausages

Species (imperfect name)	No. strains
Dabaryomyces hansenii (Candida famata)	5(5)[b]
Trichosporon ovoides	10
Candida intermedia/curvata	6
Yarrowia lipolytica (C. lipolytica)	5(1)
Candida parapsilosis	5
Candida zeylanoides	3
Citeromyces matritensis (C. globosa)	1

[a] Following Kreger-van Rij (1984) and Barnett et al. (1990).
[b] Number of strains of the perfect form (in brackets, number of strains of the imperfect form).

importance of these two species in the final product ($>10^4$ CFU/g) and its presence in the initial mixture indicate excessive multiplication in the interior which could explain the surface spoilage of this sausage. *C. matritensis* is a species not previously reported in sausages, although it has been found in beef (Comi & Cantoni, 1985).

Comparing both systems of identification, the ATB32C was more satisfactory in our study. This system includes 4 more tests and the database is almost double (63 versus 36 species) that of the Vitek system.

Identification of most *D. hansenii* strains was problematic with both miniaturised systems, due to their inability to grow at 30°C (temperature recommended in both systems). Six strains could not be identified by the Vitek, but had similar profiles to *C. curvata* and *C. intermedia* when analysed with the other system. From the total of 36 strains identified by both methods, 33 were identified by the ATB32C, while 27 were identified by the Vitek system. In both methods, identification of more than 50% of the total of strains investigated, using exclusively the bionumber obtained, was not possible. Difficulties in the identification of yeasts isolated from foods using the ATB32C has been described by other authors (Rohm, Lechner & Lehner, 1990). The extension of the database to more foodborne yeasts (Rohm et al. 1990), as well as the possibility of lowering the incubation temperature (to 25 or 28°C), could help, in our opinion, to increase the efficiency of these systems.

In summary, although several factors may influence the flora of fermented sausages, the presence of yeasts at levels between 3 and 5 logarithmic units/g throughout manufacture of Spanish varieties seems to be constant and suggests the participation of this group of microorganisms, and particularly *D. hansenii* (*C. famata*), the dominant yeast, in the process.

Acknowledgements

This work was supported by the Spanish CICYT, Projects ALI91-515 and ALI95-132. "Junta de Castilla y León" also contributed.

References

Asehraou, A., Mohieddine, S., Faid, M., & Serhrouchni, M. (1997). Use of antifungal principles from garlic for the inhibition of yeasts and moulds in fermenting green olives. *Grasas y Aceites, 48*, 68–73.

Barnett, J. A., Payne, R. W., & Yarrow, D. (1990). *Yeasts: characteristics and identifications* (2nd ed.). New York: Academic Press.

Comi, G., & Cantoni, C. (1985). Lieviti e carni. *Industrie Alimentari, 24*, 683–687.

Daporta, P. M. (1988). *Microorganismos de interés tecnológico, con especial referencia a Micrococacceae, en chorizos de las variedades "Cantimpalos" y "Cerdo Ibérico" (Guijuelo)*. University of León, León: Tesina de Licenciatura, Veterinary Faculty.

Deak, T. (1991). Foodborne yeasts. In *Advances in applied microbiology*, (No. 36, pp. 179–278). New York: Academic Press.

Deak, T., & Beuchat, L. R. (1987). Identification of foodborne yeasts. *Journal of Food Protection, 50*, 243–264.

Dillon, V. M. (1998). Yeasts and moulds associated with meat and meat products. In *The microbiology of meat and poultry*. (pp. 85–117). London: Blackie Academic and Professional.

Eklund, M. W., Spinelli, J., Miyauchi, D., & Groninger, H. (1965). Characteristics of yeasts isolated from irradiated Pacific crab meat. *Applied Microbiology, 13*, 985–990.

Encinas, J. P. (1993). *Identificación de riesgos microbiológicos y puntos críticos durante la elaboración y maduración de embutidos crudos fermentados*. Ph.D. thesis. University of León, León, Veterinary Faculty.

Fleet, G. H. (1990). Yeast in dairy products. *Journal of Applied Bacteriology, 68*, 199–211.

Fung, D. Y. C., & Liang, C. (1990). Critical review of isolation, detection, and identification of yeasts from meat products. *Critical Reviews in Food Science and Nutrition, 29*, 341–379.

Ghamnnoum, M. A. (1990). Inhibition of *Candida* adhesion to buccal epithelial cells by an aqueous extract of *Allium sativum* (garlic). *Journal of Applied Bacteriology, 68*, 163–169.

Iñigo, B., Arroyo, V., & Somavilla, J. (1970). Estudio microbiológico del proceso de fabricación de embutidos I. Identificación de levaduras. *Revista de Agroquímica y Tecnología de Alimentos, 10*, 406–410.

Jessen, B. (1995). Starter cultures for meat fermentation. In G. Campbell-Platt, & P. E. Cook, *Fermented meats* (pp. 130–159). Glasgow: Blackie Academic and Professional.

Kreger-van Rij, N. J. W. (1984). *The yeasts: a taxonomic study* (3rd edn). Amsterdam: Elsevier.

Kurtzman, C. P., & Fell, J. W. (1998). *The yeasts: a taxonomic study*. (4th edn). Amsterdam: Elsevier.

Leistner, L. (1986). Mould-ripened foods. *Fleischwirtschaft, 66*, 1385–1388.

Leistner, L. (1995). Stable and safer fermented sausages word-wide. In G. Campbell-Platt, & P. E. Cook, *Fermented Meats* (pp. 160–175). Glasgow: Blackie Academic and Professional.

Lois, A. L., Gutiérrez, L. M., Zumalacárregui, J. M., & López, A. (1987). Changes in several constituents during the ripening of "chorizo". A Spanish dry sausage. *Meat Science, 19*, 169–177.

Lücke, F. K. (1985). Fermented sausages. In B. J. B. Wood *Microbiology of fermented foods*, (Vol. 2, pp. 41–81). Amsterdam: Elsevier.

Lücke, F-K., & Hechelmann, H. (1987). Starter cultures for dry sausages and raw ham. Composition and effect. *Fleischwirtschaft, 67*, 307–314.

Marcos, D. (1991). *Embutidos crudos curados españoles*. Madrid: Ayala.

Miller, M. W. (1979). Yeasts in food spoilage: an update. *Food Technology, 33*(1), 76–80.

Rohm, H., Lechner, F., & Lehner, M. (1990). Evaluation of API ATB 32C system for the rapid identification of foodborne yeasts. *International Journal of Food Microbiology, 11*, 215–224.

Samelis, J., Stavropoulos, S., Kakouri, A., & Metaxopoulos, J. (1994). Quantification and characterization of microbial populations asso-

ciated with naturally fermented Greek dry salami. *Food Microbiology, 11*, 447–460.

Sarasíbar, B., Sánchez-Monge, J. M., & Bello, J. (1989). Influencia de nitratos y nitritos sobre la estabilidad del pimentón (*Capsicum annuum* L.) y el desarrollo del color del chorizo de Pamplona. *Alimentaria, 26*(207), 19–23.

Sesma, B., & Ramírez, C. (1976). Estudio de la flora microbiana durante la maduración de embutidos. I. Características de las especies de la familia Micrococcaceae aisladas de salchichón de Pamplona. *Revista de Agroquímica y Tecnología de Alimentos, 16*, 539–545.

Walker, H. W. (1977). Spoilage of food by yeasts. *Food Technology, 31*(2), 57–61, 65.