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Characterization of yeasts associated with Portuguese pork-based products

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Abstract

Yeasts from pork-based products, such as country-cured hams and several types of bacon, were studied. Contamination levels ranged from 10^3–10^9 CFU/g of fat. Identification using a simplified method (SIM) showed that the predominant species were *Debaryomyces hansenii*, *Cryptococcus laurentii*, *Cryptococcus humicolus*, *Debaryomyces polymorphus* and *Pichia guilliermondii*. Lipolytic activity was observed in 24 out of 29 of the isolated strains and was not a determinant for attaining high levels of contamination. The analysis of yeast long-chain fatty acid composition for most isolated strains showed a predominance of the acid C18:1 followed by decreasing proportions of the acids C16:0, C18:2, C18:3, C18:0 and C16:1. The fatty acid profiles were roughly similar for all yeasts analyzed suggesting that in these types of pork-based products the yeast lipid composition may have an ecological significance. © 1997 Elsevier Science B.V.

Keywords: Contamination yeasts; Pork meat; Yeast fatty acids; Lipolysis

1. Introduction

The typical contaminating flora in fresh meat and meat products is made up of bacteria (Jay, 1996). In comparison with products of plant origin, reports referring to the isolation and characterization of yeasts from meat products are relatively few (Deák, 1991; Fleet, 1992). Nevertheless, the occasional role of yeasts in meat has been described (Dalton et al., 1984; Banks and Board, 1987; Viljoen et al., 1993). The spoilage potential of the yeasts is not considered to be of great importance (Deák, 1991), and appears to be related to their proteolytic or lipolytic activity which may even include desirable effects (Fleet, 1992). For dry-cured meats, with reduced product a_w by the addition of salt and drying, several reports refer to the isolation of yeasts (Comi et al., 1982; Monte et al., 1986; Huerta et al., 1988; Molina et al., 1990; Giménez, 1992).

The scope of this work was to quantify and characterize the yeast flora of Portuguese dry-cured hams and other traditional Portuguese pork-based products. The characterization included the assessment of lipolytic properties and a comparative evaluation between two different approaches for the

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differentiation of contaminating yeasts. One of them, known as SIM (Simplified Identification Method) followed the methodology described by Deák and Beuchat (1987) and Deák (1991), (1992) which is based on a simplification of the conventional identification procedures. The other uses the yeast long-chain fatty acid composition as a biomarker and follows the technique developed in our laboratory (Moreira-da-Silva et al., 1994; Noronha-da-Costa et al., 1996).

2. Materials and methods

2.1. Yeast isolation and maintenance

The yeast strains investigated were either isolated from pork products randomly selected at retail outlets or obtained from culture collections (Table 1). All samples were vacuum-packed, except fresh meat, and kept at refrigeration in retail stores. The samples investigated comprised 1 g of the fatty portion of the meat. The diluent used was peptone water (peptone (Difco Laboratories, Detroit, USA) 10 g/l, NaCl (Merck, Darmstadt, Germany) 5 g/l, $NaH_2PO_4.12H_2O$ (Merck) 9 g/l, KH_2PO_4 (Merck) 1.5 g/l) with 1% of Tween 80 (Sigma Chemical Co., St. Louis, USA) added. Initial homogenization was carried out with a vortex mixer and 0.1 ml from appropriate dilutions was spread onto GYP agar (glucose (Merck) 20 g/l, yeast extract (Difco) 5 g/l, peptone 10 g/l and agar 20 g/l, pH 6.0) containing 100 ppm of chloramphenicol (Sigma). The plates, in triplicate, were incubated at 25°C for 72 h. Yeasts were counted and purified by subcultivations onto GYP plates and were maintained at 4°C on slants of the same medium.

2.2. Assessment of lipolytic activity

Pure cultures of yeasts were streaked onto a culture medium composed of 40 g/l gelatine (Difco), 24 g/l tryptone glucose extract agar (Oxoid, Unipath Ltd., Basingstoke, UK), 5 g/l tributyrin (Sigma), 5 g/l Tween 80 (Sigma), and 10 ml/l of a solution of Nilus blue sulfate (Sigma) obtained by dissolving 66 mg of this compound in 100 ml of water. After autoclaving, the pH of this medium was 7.0±0.2. For lipolytic yeasts, colonies were surrounded by a transparent ring on a blue background as a result of tributyrin hydrolysis. This medium permits the simultaneous assessment of the proteolytic activity by gelatine hydrolysis and was used by Elias (1993), who reported the absence of this activity in yeasts isolated from Portuguese hams.

Table 1
Origin of the yeast strains analyzed

Strains	Origin	Reference
ISA 1491, ISA 1493	Vacuum-packed artisanal dry-cured ham	Elias (1993)
ISA 1492, ISA 1494, ISA 1495, ISA 1496	Vacuum-packed industrial dry-cured ham	Elias (1993)
ISA 1559, ISA 1560, ISA 1561, ISA 1562, ISA 1563, ISA 1564, ISA 1565, ISA 1566, ISA 1567, ISA 1568	Vacuum-packed industrial dry-cured ham	This work
ISA 1569, ISA 1570	Vacuum-packed salted bacon	This work
ISA 1571	Fresh pork fat	This work
ISA 1572, ISA 1573, ISA 1574, ISA 1575, ISA 1576, ISA 1577, ISA 1578, ISA 1579, ISA 1580, ISA 1581	Vacuum-packed smoked pork fat	This work
Reference strains		
Cryptococcus humicolus ISA 1588	IGC 3387, CBS 571, soil	
Cryptococcus laurentii ISA 1590	IGC 3966, palm wine	
Debaryomyces hansenii ISA 1507	IGC 2968, CBS 767	
Debaryomyces polymorphus ISA 1587	IGC 2606, CBS 186, soil	
Pichia guilliermondii ISA 1589	IGC 3440, *Ulmus americana*	

2.3. Yeast identification

Identifications were carried out using the simplified identification method (SIM) proposed by Deák and Beuchat (1987) and Deák (1991), (1992). The results of the biochemical and morphological tests described by these authors were used in the dicotomic keys given by Deák (1991), (1992). Classical identification was carried out at the Gulbenkian Institute of Science, Oeiras, Portugal, according to Barnett et al. (1983) and Kreger-van Rij (1984).

2.4. Determination of yeast long-chain fatty acid compositions

The fatty acid yeast profiles were determined as described by Moreira-da-Silva et al. (1994). However, instead of GYP agar plates for yeast biomass production, yeasts were grown on slants of the same medium in test tubes (24 mm diameter) with cotton plugs because previous results had shown higher reproducibility for this procedure (Noronha-da-Costa et al., 1996). Fatty acid separation was achieved in a gas chromatograph (Fisons Instruments, model 8130, Rodano, Italy) by using a DB-Wax column, 30 m in length, 0.53 mm internal diameter and 1 μm film thickness (JW Scientific, Folsom, USA). Oven temperature was programmed as follows: hold 2.6 min at 140°C, 140°C × 5°C/min to 190°C, hold 5 min at 190°C, 190°C × 5°C/min to 210°C and hold 15 min at 210°C. Injector and detector temperatures were 240°C and 260°C, respectively.

Multivariate statistical analysis of fatty acid compositions by Principal Component Analysis (PCA) used the SPAD.N program (Systéme Portable d'Analyse des Donnés Numériques, Centre International de Statistique et d'Informatique Appliquées, Saint-Mandé, France, 1993), version 2.5 for PC compatible processors. This analysis separates the yeast strains in clusters according to the similarity in their fatty acid compositions (Lebart et al., 1984).

3. Results and discussion

Yeast counts ranged from 10^3–10^9 CFU/g of fat (Table 2). The higher values indicate that the fat portion of meats is a suitable environment for yeast multiplication and activity. In other investigations of dry-cured ham and bacon, yeast counts from 10^3–10^5 CFU/g have been demonstrated (Cantoni et al., 1985; Monte et al., 1986; Banks and Board, 1987; Huerta et al., 1988; Molina et al., 1990; Giménez, 1992).

Since the strains were isolated from the fat portion of the products it was not surprising to find that most strains showed lipolytic activity (Table 2). All type strains and those from Elias (1993) were also shown to be lipolytic (data not included). As seen from Table 2 lipolytic activity was strain-dependent and strains without lipolytic activity may reach high levels of contamination. However, when strains were present, with and without this characteristic, the former was dominant (Table 2). The low number of samples of salted bacon does not allow any significance to be given the fact that only strains without lipolytic activity were isolated from these products. Huerta et al. (1988) also found lipolytic activity for most yeasts isolated from cured hams. This activity seems to contribute to the curing process but there is no evidence that yeast lipolytic activity is implicated in cured meat spoilage (Fleet, 1992).

The identification of the isolates performed using the SIM dicotomic keys is shown in Table 2. The strains isolated in this work were tentatively identified as *Debaryomyces hansenii* (13 out of 23), *Cryptococcus laurentii* (2 out of 23), *Cryptococcus humicolus* (6 out of 23), *Pichia guilliermondii* (1 out of 23) and *Debaryomyces polymorphus* (1 out of 23). The six strains isolated by Elias (1993) were identified as *C. laurentii* (ISA 1491, ISA 1492, ISA 1493, ISA 1494 and ISA 1495) and *D. hansenii* (ISA 1496). As indicated in Table 2 the identifications of six isolates were checked by the classical methods. For all except one, the SIM identification was confirmed. The reason for this discrepancy was probably due to the absence of the species *Candida zeylanoides* in the dicotomic key of Deák (1991).

The species diversity was not dependent on the type of product and the predominance of *Debaryomyces* spp. is in accordance with other works (Comi et al., 1982; Monte et al., 1986; Huerta et al., 1988). This is an ascomycetous species while the second more frequent isolated species *C. laurentii* is a basidiomycete. The identification of these species by SIM only differs in the result of the urease reaction test, which was positive for *C. laurentii*. In

Table 2
Enumeration of yeast contaminants in the fat portion of pork meat products, identification[a] and lipolytic activity of selected isolates (enumeration results are the average of three replicates)

Product	CFU/g of fat	Relative percentage[b]	Strains ISA	Identification	Lipolytic activity
Ham 1	$1.9\pm1.6\times10^{6}$	100	1559	*Debaryomyces hansenii*	+
Ham 2	$7.0\pm2.1\times10^{7}$	88.8	1560	*Debaryomyces hansenii*[c]	+
		11.2	1561	*Debaryomyces polymorphus*	+
Ham 3	$2.4\pm0.4\times10^{8}$	84.2	1562	*Cryptococcus laurentii*	+
		15.8	1563	*Debaryomyces hansenii*	+
Ham 4	$1.6\pm0.03\times10^{6}$	100	1564	*Debaryomyces hansenii*[c]	+
Ham 5	$3.4\pm0.34\times10^{6}$	79.4	1565	*Debaryomyces hansenii*	+
		20.6	1566	*Debaryomyces hansenii*[c]	+
Ham 6	$1.9\pm1.6\times10^{6}$	19.4	1567	*Cryptococcus humicolus*	−
		80.6	1568	*Cryptococcus humicolus*	+
Salted bacon 1	$2.2\pm0.1\times10^{6}$	100	1569	*Cryptococcus humicolus*	−
Salted bacon 2	$3.2\pm0.95\times10^{8}$	100	1570	*Debaryomyces hansenii*	−
Fresh fat	$1.8\pm0.36\times10^{5}$	100	1571	*Pichia guilliermondii*[d]	+
Smoked fat 1	$1.7\pm0.22\times10^{8}$	40.6	1572	*Debaryomyces hansenii*	+
		59.4	1573	*Debaryomyces hansenii*	+
Smoked fat 2	$1.1\pm0.21\times10^{9}$	30	1574	*Cryptococcus humicolus*	+
		13.8	1575	*Cryptococcus humicolus*	+
		56.2	1576	*Debaryomyces hansenii*	+
Smoked fat 3	$2.4\pm0.48\times10^{3}$	100	1577	*Debaryomyces hansenii*[c]	+
Smoked fat 4	$5.5\pm0.99\times10^{4}$	100	1578	*Debaryomyces hansenii*[c]	+
Smoked fat 5	$7.5\pm1.8\times10^{5}$	100	1579	*Debaryomyces hansenii*	−
Smoked fat 6	$3.5\pm2.0\times10^{3}$	100	1580	*Cryptococcus humicolus*	+
Smoked fat 7	$2.4\pm0.04\times10^{5}$	100	1581	*Cryptococcus laurentii*	−

[a]Yeast identification was performed by SIM.
[b]Determined by the ratio between the number of colonies with a distinct morphology and the total number of colonies on agar plates.
[c]SIM identification was confirmed by conventional methodology.
[d]Classical identification identified this strain as *Candida zeylanoides*.

mixed populations, *D. hansenii*, when present, showed higher counts than the other yeasts in two samples and smaller counts in one sample, where *C. laurentii* dominated (Table 2). *D. hansenii* is a species known for its high salt tolerance (Deák, 1991; Fleet, 1992), and so its isolation from products with high salt content was to be expected. The refrigerated storage of the analyzed products may also partially explain the high level of *D. hansenii* and *Cryptococcus* spp. given their psychrotrophic character (Deák, 1991; Fleet, 1992).

The mean fatty acid compositions of the strains analyzed are shown in Fig. 1. Fatty acids from C12 to C18 with a variable degree of unsaturation were detected, those with 16 or 18 carbon atoms predominating. Although differences between the profiles were seen, the profile was similar for all species (Fig. 1). Basically, for the most dominating fatty acids, the profiles roughly showed the following

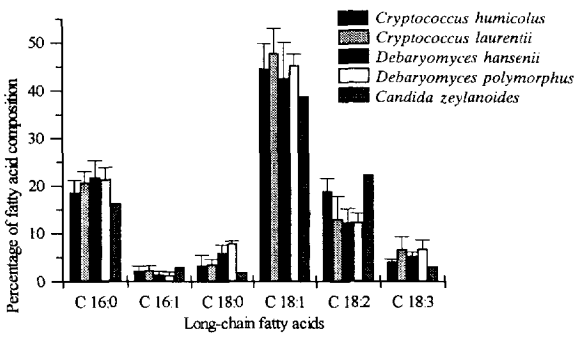

Fig. 1. Main fatty acid compositions for the analyzed yeast species. The percentages were calculated by the ratio betwen the amount of each fatty acid and the total amount of fatty acids determined for each strain. The columns represent the average percentage of the fatty acids indicated for 15 strains of *Debaryomyces hansenii*, eight strains of *Cryptococcus laurentii*, seven strains of *Cryptococcus humicolus*, two strains of *De-*

C18:0 > C16:1. In some cases the fatty acid composition variability within the species was higher than between strains of different species (e.g. strains of *C. humicolus* and *C. laurentii*). The tested type strains showed profiles similar to other isolates of the same species. The major difference was observed for the type strain of *C. humicolus* where the acids C16:1, C18:2 and C18:3 had percentages lower than the strains of this species whereas C18:0 and C18:1 acids presented the highest values for this species. This homogeneity in the yeast fatty acid composition is quite unique as our experience in other food products showed a considerable variability in the profiles of contaminating yeasts (Malfeito-Ferreira et al., 1989; Moreira-da-Silva et al., 1994, unpublished data on fruit juice industry). The ecological meaning of this observation may be related to a high microbial selectivity based on the chemical composition of the product together with low storage temperatures. In fact the isolated species share a psychrotrophic character (Fleet, 1992), which is associated with high percentages of unsaturated long-chain fatty acids in cellular biomass (Rose, 1989).

Although fatty acid compositions are dependent on strain growth conditions (e.g. temperature and composition of growth medium) a rough comparison between our profiles and others from the literature is possible. Both *C. laurentii* and *D. hansenii* are considered to be heterogenous species (Smit et al., 1988; Tredoux et al., 1987). For *C. laurentii* our results show an average proportion of 6.6% for C18:3 while the values mentioned in Rattray (1988) and Smit et al. (1988) are lower than 0.5%. However the profiles presented by Smit et al. (1988) for other species of *Cryptococcus* spp. presented profiles similar to those of *C. laurentii* determined in our work. In the case of *D. hansenii* the resulting profiles are comparable with those mentioned by Tredoux et al. (1987) and Rattray (1988). For *D. polymorphus* Rattray (1988) presents profiles similar to those in our work. Viljoen et al. (1988) also refer to comparable fatty acid compositions for *Candida zeylanoides*. As to *C. humicolus* no values for comparison were found in the literature.

The execution of the principal component analysis (PCA) allowed the grouping of strains according to the similarity in their profiles (Fig. 2). Two analyses were performed, one splitting the strains into five clusters and the other obtaining six clusters. The following five clusters were obtained: I, with eight strains of *D. hansenii* and two strains of *D. polymorphus*; II, with one strain of *C. humicolus*, five strains of *C. laurentii* and three strains of *D. hansenii*; III, with four strains of *C. humicolus*, two strains of *C. laurentii*, one strain of *D. hansenii* and one strain of *P. guilliermondii*; IV, with two strains of *C. humicolus*, one strain of *C. laurentii*, one strain of *D. hansenii* and one strain of *Candida zeylanoides* and V, with two strains of *D. hansenii*. The inertia relation (Lebart et al., 1984) of this partition was 0.78. An increase in the number of clusters to six increased this to 0.84 by splitting the cluster III, resulting in two strains of *C. humicolus* and one strain of *C. laurentii* being grouped in cluster IIIa

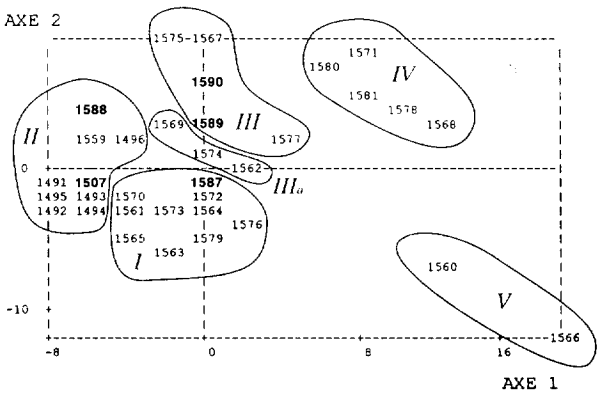

Fig. 2. Principal component analysis of fatty acid yeast compositions. Strains are represented by the respective ISA number and type strains are written in bold. See Table 1 for strain assignment to the represented clusters.

(Fig. 2). Further increases in the number of clusters did not significantly increase the value of the inertia relation (results not shown) reflecting the stability of the partition in six clusters. The grouping of different species in the same cluster and the separation of strains of the same species in different clusters was observed in both analyses.

Guerzoni et al. (1993) found a relation between the fatty acid composition and the physiological features or origin of the strain. In this study it was not possible to separate the strains according to their lipolytic character or to their original product sample because the clusters obtained by PCA included strains with different origins or lipolytic ability. However the strains isolated from dry-cured hams by Elias (1993) were all in the cluster II even though five strains were *C. laurentii* and one strain *D. hansenii*.

Most *D. hansenii* strains were not within the cluster of type strain (cluster II in Fig. 2). This may be due to the inadequate ability of conventional techniques in identifying the strains of this species as it was observed for four other culture collection strains assigned to *D. hansenii*, with fatty acid profiles distinct from others of this species, that the DNA did not hybridize with the type chain DNA (unpublished results). Results of this kind have also been observed in *Pichia membranaefaciens* (Noronha-da-Costa et al., 1996), *Zygosaccharomyces rouxii*, *Zygosaccharomyces bailii*, *Kluyveromyces marxianus*, *Dekkera bruxellensis* and *Torulaspora delbrueckii* (unpublished results). These observations highlight the importance of previous confirmation of identifications to evaluate the characteristic fatty acid profile of a particular species.

In spite of five different yeast species from pork-based products having been isolated this kind of food appears to be a favourable habitat for a selected group of yeasts characterized by similar fatty acid composition.

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