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Molecular characterization and aromatic potential of *Debaryomyces hansenii* strains isolated from naturally fermented sausages

Liliana Cano-García, Mónica Flores, Carmela Belloch *

Institute of Agrochemistry and Food Technology, (IATA-CSIC), Avda. Agustín Escardino 7, E-46980 Paterna, Valencia, Spain

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ABSTRACT

Twenty two *Debaryomyces hansenii* strains isolated from naturally fermented sausages were characterized by molecular methods and their contribution to sausage aroma measured by volatile compounds production on a defined culture media. The *D. hansenii* isolates were identified by RFLPs and sequencing of the ITS-5.8S rDNA region. Genetic characterization of strains was achieved by RFLPs of mitochondrial DNA and minisatellite M13 PCR amplification. The UPGMA dendrogram based on molecular patterns revealed an important genetic heterogeneity within the new sausage isolates of *D. hansenii*. Generation of ester compounds was tested on culture media supplemented with methanol or ethanol and 2-methyl butanoic acid. Only seven *D. hansenii* strains were able to produce differences in the aroma profiles detected by sensory and GC-MS analysis. The volatile compounds produced were mainly ester compounds, ethyl and methyl esters, sulfur, alcohols, aldehydes and ketones. The results of our study revealed no correspondence between genetic patterns and aroma profiles within *D. hansenii* strains.

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1. Introduction

Fermentation is an ancient technology used to extend the shelf life of raw meat. Essential chemical and physical changes take place during the process and the organoleptic and sensory characteristics of the final product are completely different from those of the starting materials. An important contribution to this transformation comes from the action of microorganisms present on the raw materials such as meat, salt, spices and casings. Together with the endogenous enzymes of the meat, they are responsible for the aroma formation (Lücke, 2000). The main microbial groups in fermented sausages are Lactobacilli and coagulase-negative cocci which are essential to achieve the sensory characteristics. However, other microbial groups such as yeast may also play a role in the final characteristics of fermented meat products (Lücke, 2000; Rantsiou & Cocolin, 2008).

Yeasts are considered to affect sausage colour and flavor due to their oxygen-scavenging and lipolytic activities. They may also delay rancidity and further catabolize products of fermentation, such as lactate produced by meat lactobacilli, to other by-products, thereby increasing the pH and contributing to the development of less tangy and more aromatic sausages (Hammes & Knauf, 1994).

Regarding yeast ecology in fermented sausages, several studies have documented the dominance of *Debaryomyces hansenii* (Baruzzi, Matarante, Caputo, & Morea, 2006; Cocolin, Urso, Rantsiou, Cantoni, & Comi, 2006). *D. hansenii* has been extensively studied because of its ability to hydrolyze pork muscle sarcoplasmic proteins, thereby influencing the aroma formation and sensory quality of dry-fermented sausages (Durá, Flores, & Toldrá, 2004a, 2004b; Flores, Durá, Marco, & Toldrá, 2004).

Identification and characterization of *D. hansenii* isolates using conventional morphological and physiological test is complex as many of the results obtained are variable. Recently, methods based on DNA have been applied on strains of this species to study its dynamics during natural fermentation of Italian sausages (Cocolin et al., 2006). Among other techniques, LTR fingerprinting, RAPD-PCR, DNA probes, IGS fingerprints and gene sequencing have demonstrated substantive strain heterogeneity in the strains of *D. hansenii* (Corredor, Davila, Gaillardin, & Casaregola, 2000; Nguyen et al., 2009; Prillinger, Molnar, Eliskases-Lechner, & Lopandic, 1999; Sohier et al., 2009). Previous studies had demonstrated that minisatellite M13 and RFLPs of mtDNA allowed good differentiation of genotypes within *D. hansenii* isolated from different environments (Andrade, Rodríguez, Sánchez, Aranda, & Córdoba, 2006; Groenewald, Daniel, Robert, Poot, & Smith, 2008).

The contribution of *D. hansenii* starter cultures to the aroma of meat products has been investigated by several authors. In 2000, Olesen and Stahnke reported a very low effect of *D. hansenii* on dry fermented sausage aroma attributed to a low survival of the yeast produced by the fungistatic effect of the garlic used as a spice in the formulation. Further studies done in our laboratory have proved the contribution of *D. hansenii* to sausage aroma by inhibiting the lipid oxidation phenomenon and promoting the generation of ethyl esters (Flores et al., 2004). Recently, Andrade, Rodriguez, Casado, Bermudez,

* Corresponding author. Tel.: +34 96 3900022; fax: +34 963636301.
E-mail address: belloch@iata.csic.es (C. Belloch).

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and Cordoba (2009a, 2010a) showed the contribution of *D. hansenii* to the aroma of meat products by the increase in methyl-branched aldehydes and few ester compounds. Moreover, *D. hansenii* has shown proteolytic and lipolytic activities which could have and important effect on flavor (Aquilanti et al., 2007; Baruzzi et al., 2006).

The development of the final sensory characteristics of fermented sausages depends on the different processing conditions, fermentation process and raw materials used in formulation, because they can also determine the selection of specific microbial populations (Rantsiou & Cocolin, 2008). In our study, naturally fermented sausages produced without the use of starter culture and at low fermentation temperatures produced a limited rate of acid formation turning out in low acid sausages. Naturally fermented sausages have been selected by consumers who appreciate products with singular attributes that distinguished them from others of the same category (Conter et al., 2008). Aroma analysis of naturally fermented sausages has revealed the presence of numerous esters, both ethyl and methyl esters contributing positively to the aroma (Olivares, Navarro, & Flores, 2010). It is generally accepted that coagulase-negative cocci are the most important group in the production of flavor compounds because of its capacity to generate high amounts of branched aldehydes, methyl ketones and ester compounds (Montel, Masson, & Talon, 1998). However, the origin of ester compounds is not well established as lactic acid bacteria, coagulase-negative cocci, yeast and molds are able to produce them (Tjener & Stanhke, 2007).

The aim of this study was to associate genetically diverse strains of *D. hansenii* with their different production of ethyl and methyl esters found in naturally fermented sausages.

2. Materials and methods

2.1. Sausage sampling and yeasts isolation

Naturally fermented sausages from eight different manufacturers (Requena, Valencia, Spain) were used to isolate *D. hanseni* yeasts. Fermented sausages were manufactured using lean pork, pork back fat, sodium chloride, nitrite, nitrate, sugar and species using traditional practices, without the addition of starter cultures and naturally ripened at 10–12 °C during 1 month. At the end of the process, a cylindrical sausage sample of 10 g was aseptically taken from each sausage. The sample was finely minced and homogenized (Ultra-Turax, Spain) in tubes containing 10 mL of saline solution (0.9% sodium chloride). A sample, 50 μL, of the homogenate was spread onto GYPA medium plates (glucose 2%, peptone 0.5%, yeast extract 0.5% and agar 2%, pH 6.0, chloramphenicol 100 mg/L) and incubated 4 days at 25 °C. Up to 6 colonies were selected from each plate, further streaked on GYPA medium and incubated for 2 days at 25 °C. The isolated yeasts were codified using the manufacture initials (M, P, T, V, EV, S, EN and I). Yeasts were conserved at −80 °C using 15% glycerol as cryoprotectant. In addition to the sausage isolates, three strains from fermented sausages and six strains from the CECT (Spanish Type Culture Collection) were used as reference strains (Table 1).

2.2. Identification of yeasts by molecular techniques

The isolated yeasts were cultured overnight on GPY medium (2% glucose, 0.5% peptone and 0.5% yeast extract) at 25 °C and shaking at 200 rpm. DNA was extracted as described in Querol, Barrio, and Ramón (1992). The ribosomal DNA region spanning the ITS1, 5.8S rDNA and ITS2 (ITS-5.8S rDNA) was amplified by PCR using the primer pairs its1 (5′-TCCGTAGGTGAACCTGCGG) and its4 (5′-TCCTCCGCTT ATTGATATGC) following the methodology of Esteve-Zarzoso, Belloch, Uruburu, and Querol (1999). The PCR product was digested with endonucleases *Hae*III, *Hin*fI and *Cfo*I according to the supplier's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Restriction fragments were separated by electrophoresis on 3% agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8), stained with RedSafe (INtRON Biotech., Spain) and visualized under UV light. DNA fragment sizes were determined using a 100-bp DNA ladder. Fragment sizes were compared with patterns in the Yeast-id database (http://www.yeast-id.com) and assigned to a known yeast species.

Table 1
List of *Debaryomyces hansenii* yeast investigated in this study.

Species	Strain	Isolation source
D. hansenii	CECT 11369[T]	Unknown
	CECT 10026	Salted cod, Spain
	CECT 10352	Tomato, Spain
	CECT 11363	Chilled beef, Australia
	CECT 11365	Dry white wine, South Africa
	C 1, C3, C7[a]	Fermented sausages, Spain
	M 1, M 2, M 3, M 4, M 5, M 6, P 2, P 3, T 3, T 4, V 1, V 2, EV 1, EV 2, S 1, S 2, EN 2, EN 3, EN 4, I 1, I 2, I 3	Natural fermented sausages, Spain
D. fabryii	CECT 11370[T]	Interdigital mycotic lesion, Germany

CECT, Spanish type culture collection, University of Valencia, Spain
[a] C1and C3 were isolated in a previous study by Bolumar, Sanz, Aristoy, and Toldrá (2003); Bolumar et al. (2006) and C7 was isolated by Durá et al. (2004b).

2.3. Characterization of yeast strains

Characterization of yeast strains was achieved using ITS-5.8S rRNA sequencing, RFLPs of mitochondrial DNA (mtDNA) and minisatellite PCR amplification using the M13 primer (Belloch, Barrio, Uruburu, García, & Querol, 1997; Fadda, Mossa, Pisano, Deplano, & Cosentino, 2004).

Sequences of ITS-5.8S rDNA were analyzed with MEGA 4 (Tamura et al., 2011). The sequences were compared with the sequences from the GenBank database using BlastN (NCBI).

The RFLPs of mtDNA were carried out as described elsewhere (Belloch et al., 1997). DNA was digested with the restriction enzyme *Hin*fI (Roche Molecular Biochemicals, Mannheim, Germany) according to the supplier's instruction. Restriction fragments were separated on 1% agarose gels in 1× TAE buffer, stained with ethidium bromide (100 μg/mL) and visualized under UV light. Band sizes were compared against fragment sizes of lambda phage DNA digested with *Hin*dIII and *Pst*I.

Minisatellite PCR amplification using the M13 primer (5′GAGGG TGGCGGTTCT3′) was performed as described in Fadda et al. (2004) using 50 μL reaction volume containing 0.3 μL rTaq (5U) DNA polymerase, 4 μL dNTP mix (2.5 mM), 5 μL buffer, 3 μL $MgCl_2$ (1.5 mM), 1 μL M13 minisatellite primer and 25 μL solution containing 80–100 ng of genomic DNA. PCR amplification conditions were as follow: 95 °C for 5 min followed by 40 cycles of 93 °C for 45 s, 44 °C for 1 min and 72 °C for 1 min with a final extension step at 72 °C for 6 min. The PCR products (10 μL) were resolved by electrophoresis on 2% agarose gel in 1× TAE buffer at 90 V for 3 h, stained with RedSafe (INtRON Biotech., Spain) and visualized under UV light. DNA fragment sizes were determined using a 100-bp DNA ladder.

2.4. Yeast growth on defined culture media

Yeasts were grown for 24 h in 5 mL GPY medium at 25 °C and 200 rpm shaking. Overnight cultures were adjusted to an absorbance of 0.3 at 655 nm (Biophotometer, Eppendorf) by dilution using fresh GPY medium and further incubated for 4 h at 25 °C and 200 rpm shaking. Cells were collected by centrifugation (3000 rpm for 10 min) and washed three times with 0.9% saline solution. Cell suspensions were adjusted to a concentration of 10^6 cell mL^{-1} using saline solution and 100 μL of each yeast suspension was used to inoculate the aroma producing media.

Two different aroma producing media containing 2-methyl-butanoic acid and methanol or ethanol were prepared. Media consisted of filter

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