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Title: Molecular characterization and aromatic potential of *D. hansenii* strains isolated from naturally fermented sausages

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Abstract

Twenty two *D. hansenii* strains isolated from naturally fermented sausages were characterised 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.

Keywords: *D. hansenii*, sausages, genetic diversity, aroma, volatile compounds.

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 and Cocolin, 2008).

Yeasts are considered to affect sausage colour and flavour 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 and Knauf, 1994).

Regarding yeast ecology in fermented sausages, several studies have documented the dominance of *Debaryomyces hansenii* (Baruzzi et al., 2006, Cocolin et al., 2006). *Debaryomyces 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 et al., 2004a,b; Flores et al., 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* (Prillinger et al., 1999; Corredor et al., 2000; Nguyen et al., 2009; Sohler 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 et al., 2006; Groenewald et al., 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 et al. (2009; 2010) 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 an important effect on flavour (Baruzzi et al., 2006; Aquilanti et al., 2007).

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 and 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 et al., 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 et al., 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 and 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. hansenii* 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 GYP 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 GYP 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 *et al.* (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'-TCCTCCGCTTATTGATATGC) following the methodology of Esteve-Zarzoso *et al.* (1999). The PCR product was digested with endonucleases *HaeIII*, *HinfI* and *CfoI* according to the supplier's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Restriction fragments were separated by electrophoresis on 3 % agarose gels in 1xTAE 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.

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 *et al.*, 1997; Fadda *et al.*, 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 *HinfI* (Roche Molecular Biochemicals, Mannheim, Germany) according to the supplier's instruction. Restriction fragments were separated on 1 % agarose gels in 1xTAE 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 *HindIII* and *PstI*.

Minisatellite PCR amplification using the M13 primer (5' GAGGGTGGCGGTTCT3') 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 MgCl₂ (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 sec, 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 1xTAE 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 sterilized YNB with amino acids (histidine, methionine, tryptophan) (Difco-BD, Spain) and supplemented with 100 ppm 2-methyl-butanoic acid and 20,000 ppm ethanol (YNB+B+E) or methanol (YNB+B+M). Yeasts suspensions were inoculated in 10 mL of each media and cultured at 24°C and 200 rpm shaking for 15 days. Control tubes of each media without yeast were incubated under the same conditions. Growth experiments were done in triplicate.

After 15 days of incubation, eight expert panelists evaluated yeast growth and aroma of each tube using the non inoculated control tubes as reference. The method was used to estimate the aromatic impact of each yeast in the media. A total of eight assessments were done per inoculated yeast. The aroma descriptors were recorded and the results expressed as the number of times a descriptor was detected by the assessors (Pollien et al., 1997).

2.5. Analysis of the volatile compounds produced in the culture media.

Only those inoculated tubes showing different aroma than the control tube were selected for volatile compound analysis. Selected culture tubes were centrifuged at 4000 rpm for 10 min and supernatant separated. Seven ml of supernatant were added to a 15 mL headspace vial and stored at – 20 °C until analysis. Volatile compounds in the headspace of supernatant samples (YNB+B+E or YNB+B+M media) were extracted by Solid Phase Microextraction (SPME) device. The

extraction was done using a 85 μm CAR/PDMS sf fiber (Supelco, Bellefonte, PA, USA) by headspace exposure during 2.5 h at 37°C in a water bath while stirring (Durá et al., 2004b).

The identification and quantification of volatile compounds extracted by SPME from the supernatants was done using a gas chromatograph (GC HP 7890 series II) equipped with an HP 5975C mass selective detector (MS Hewlett Packard, Palo Alto, CA, USA). The compounds absorbed by the fiber were desorbed in the injection port of the GC-MS for 15 min at 220 °C with the purge valve off (splitless mode) using He as carrier gas at a flow rate of 34 cm sec^{-1} . Compounds were separated on a DB-624 capillary column (J&W Scientific, 30 m x 0.25 mm x 1.4 μm) and analyzed as described Flores *et al.* (2004). Volatile compounds were identified by comparison with mass spectra from the library database (Nist'05) and with Kovats retention indices of authentic standards (Kovats, 1965). Quantification of each volatile compound was based on the abundance of total extracted area (TIC) or the area of a target ion when different compounds coeluted. The results were expressed in percentage as the relation between area in inoculated and control tubes.

2.6. Statistical analysis

In order to estimate the similarity between electrophoretic patterns, DNA markers were scored for the presence (1) and absence (0) of homologous bands. A dendrogram was constructed using the UPGMA (unweighted pair-group method using arithmetic averages) method using Jaccard Similarity Index in the NTSYS package version 2.21p (Numerical Taxonomy and Multivariate Analysis System, Exeter Publishing Ltd.).

The effect of yeasts inoculation on the generation of volatile compounds in the defined culture media was tested by one-way analysis of variance (ANOVA) using

the statistic software XLSTAT, 2009.4.03 (Addinsoft, Barcelona, Spain). Differences between sample means were analyzed according to Fisher's least significant difference (LSD) test.

3. Results and Discussion

3.1. Genetic characterization of yeast isolates

D. hansenii isolates were identified using RFLPs of PCR amplified rDNA region ITS1-5.8S-ITS2 and presence of different genetic groups within isolates revealed by RFLPs of mitochondrial DNA, PCR amplification of minisatellite M13 and sequencing of the ITS-5.8S rDNA region.

A total of 22 yeasts were isolated from eight naturally fermented sausages (Table 1). PCR amplification and restriction of the ITS-5.8S rDNA region resulted in a pattern (PCR product: 650 bp; *CfoI*: 300 + 300 + 50; *HaeIII* 420 + 150 + 90; *HinfI* 325 + 325) (Figure 1) matching with the yeast species *D. hansenii* as deduced by comparison with www.yeast-id.com database and collection strains used as references in this study.

RFLPs of mtDNA and minisatellite PCR amplification allowed detection of DNA polymorphisms among 31 strains of *D. hansenii* (Figures 2 and 3). RFLPs of mtDNA revealed 17 bands distributed in 9 different restriction patterns, whereas a total of 23 bands distributed in 8 different profiles were obtained using minisatellite M13 PCR amplification. Band presence or absence was recorded in a 0/1 matrix (Table 1 Supplementary Material) and used to construct an UPGMA dendrogram based on RFLP mtDNA and minisatellite M13 combined patterns (Figure 4). The primary trend observed in the dendrogram was separation of most *D. hansenii* new isolates and reference strains CECT 11369^T, CECT 10026 and CECT 10352 into group G1 at

approximately 85% similarity. The remaining new *D. hansenii* isolates and CECT 11363 appear in groups G2 and G3 at 65% and 60% similarity respectively. Reference strains CECT 11365 *D. hansenii* and CECT 11370^T *D. fabryi* appear in a separated branch of the dendrogram.

Alignment of ITS1-5.8S-ITS2 rDNA sequences from the new sausage isolates and reference strains showed only one nucleotide substitution in position 430 within 639 bp (GenBank ACCN JX982234) respect to the sequence of the type strain of *D. hansenii* CECT 11369^T (GenBank ACCN AJ586526). The strains of *D. hansenii* showing this variation in the ITS1-5.8S-ITS2 are labeled as ITS-5.8S II on the UPGMA dendrogram. Sequence type ITS-5.8S II is also present in *D. fabryi* CECT 11370. An additional nucleotide substitution in position 448 (GenBank ACCN JX982235), labeled ITS-5.8S III, appeared in reference strain CECT 11363.

Among the yeast found in fermented meat products *D. hansenii* has been recognized as the predominant species by culture and culture-independent-DNA analyses (Rantsiou et al., 2005; Cocolin et al., 2006; Aquilanti et al., 2007). This yeast species displays significant metabolic properties including, hydrolysis of sarcoplasmic proteins and generation of several polar and nonpolar peptides and free amino acids, lipolysis of pork fat, as well as production of flavour volatiles such as alcohols, acids, esters and various carboxyls (Sorensen, 1997).

The species *D. hansenii* displays a high intraspecific physiological variability (Suzuki et al., 2011), therefore recent studies have focused on genetic differentiation of the strains. Molecular analyses using inter-LTR fingerprinting, RAPD-PCR, RFLP of mtDNA and DNA probes have demonstrated substantive strain heterogeneity in the strains of *D. hansenii* (Prillinger et al., 1999; Sohler et al., 2009; Corredor et al., 2000). A similar level of strain heterogeneity within *D. hansenii* strains isolated from

naturally fermented sausages produced in the same locality can be also deduced from our study. This is plainly observed in case of *D. hansenii* new isolates within groups G2 and G3 on the UPGMA dendrogram. This is in agreement with similar studies using RAPDs and minisatellite M13 where comparable levels of heterogeneity between strains of *D. hansenii* were found (Andrade et al., 2006; Groenewald et al., 2008).

The large genetic heterogeneity observed within *D. hansenii* difficults its separation from the type strain of *D. fabryi* isolated from human interdigital mycosis. As can be observed in Figure 4, taxonomic affiliation of strain *D. hansenii* CECT 11365 could not be accurately resolved. The lowest similarity value between CECT 11365 and other *D. hansenii* strains (around 20%) confirms the complex population structure within the species pair *D. hansenii* – *D. fabryi*. Previous taxonomic studies demonstrated that PCR based techniques allowed good differentiation of genotypes (Prillinger et al., 1999; Petersen et al., 2001; Andrade et al., 2006; Groenewald et al., 2008; Sohler et al., 2009). Moreover, Andrade et al. (2009, 2010b) revealed the presence of different groups within *D. hansenii* strains isolated from dry-cured Iberian hams on dendrograms based on RFLPs of mitochondrial DNA.

The UPGMA dendrogram in this study (Figure 4) based on the combined patterns of minisatellite and RFLPs of mtDNA indicates the separation of *D. fabryi* from *D. hansenii* although leaving strain CECT 11365 in a middle position. The large genetic variability found within the strains analyzed could be interpreted as intraspecific variation or presence of cryptic and hybrid strains within *D. hansenii* as already described (Jacques et al., 2009, 2010). The lowest similarity values found in the Jaccard Similarity Matrix (data not shown) for strains in UPGMA groups G2 and G3 would point out to new varieties or species within *D. hansenii*.

Evaluation of the genetic diversity within *D. hansenii* strains using ITS-5.8S rDNA sequences demonstrated that the only difference between *D. hansenii* and *D. fabryi* was at position 430 due to a replacement of a thymine by a cytosine (Petersen et al., 2001). Our study shows that several *D. hansenii* sausage isolates present the same ITS-5.8S rDNA sequence than *D. fabryi* (ITS-5.8S II). Moreover, reference strain CECT 11363 presents an additional nucleotide substitution (ITS-5.8S III) which is present in several ITS-5.8S rDNA sequences of *D. hansenii* sausage isolates found in GenBank (data not shown).

3.2. Aroma production of isolated yeast in “in vitro” media

All yeasts were able to grow at 25°C on both defined media (YNB+B+E and YNB+B+M) containing 2-methyl butanoic acid and ethanol or methanol. After 15 days of incubation, the supernatant was sensory evaluated and compared to the non inoculated media. For most of the 30 strains assayed, the panelist did not observe differences in aroma between inoculated and non inoculated tubes. However, the panelist detected different aroma descriptors in seven isolates as shown in table 2.

D. hansenii yeasts M4, M6 and P2 produced fruity and floral aroma notes, whereas T3, V2 and EV2 strains produced sulfur, feet and cooked vegetables notes (table 2). On the other hand, I2 strain displays an intermediate behavior regarding aroma descriptors as contribute to fruity and sulfur notes in both media.

Only those isolates that were able to produce different aroma than the control tube on both media (table 2) were analyzed by SPME-GC-MS analysis. Table 3 shows the volatile compounds identified in both inoculated media, YNB+B+E and YNB+B+M. The compounds ethanol or methanol and 2-methyl butanoic acid were detected in the control tubes as they were added. Also in the YNB+B+M control media several volatile compounds were detected although they represented less

than 2 % of the total extracted area. The inoculated tubes contained different volatile compounds, such as aldehydes, ketones, alcohols, esters and sulfur compounds, in addition to the supplemented compounds (Table 3). YNB+B+E medium was characterized by the presence of ethanol and ethyl esters while YNB+B+M medium presented methanol and methyl esters (Table 3).

The generation of volatile compounds by the seven inoculated yeasts on both media was expressed as a percentage relative to the abundance found in the control tube as shown in Table 4 and 5, respectively. Significant differences were found among yeasts and control tubes in the generation of volatile compounds in YNB+B+E medium (Table 4). The volatile compounds produced by the yeasts in highest abundance were ethanol, ethyl acetate, ethyl 2-methylbutanoate, dimethyl disulfide, dimethyl trisulfide, 2-methyl and 3-methyl butanol, 2-ethyl 1-hexanol and in less abundance aldehydes and ketones (Table 4). However, there were significant differences in the volatile production among yeasts. M4 was the yeasts that produce the highest significant proportion of ester compounds and it produced the highest abundance of ethyl acetate and ethyl 2-methyl butanoate. M4 strain could be responsible for the fruity and floral notes detected in the sensory odor analysis (Stahnke, 1994). M4 was the only yeast able to consume ethanol and the lowest producer of 2-methyl butanoic acid. In addition, M4 yeast also produced sulfur compounds (dimethyl disulfide and dimethyl trisulfide) but in significant lower proportion than V2 yeast. This V2 yeast was the main significant producer of sulfur compounds, it showed the highest significant abundance in comparison to control and other yeasts in the three sulfur compounds detected (methanethiol, dimethyl disulfide and dimethyl trisulfide). V2 also showed significant abundance of ester compounds (ethyl acetate and ethyl 2-methyl butanoate) in comparison to control

tubes but in significant less proportion than M4 yeast. A similar yeast that was able to produce sulfur compounds was EV2 strain as it showed a highest significant abundance of dimethyl disulfide and dimethyl trisulfide but in this case, the production of methanethiol was not different to the control tube. These sulfur compounds have been identified as essential contributors to sausage aroma due to their low detection threshold and their sensory notes (Schmidt and Berger, 1998, Söllner and Schieberle, 2009).

The remaining yeasts displayed an intermediate behavior in the production of ethyl esters and sulfur compounds respect to M4, V2 and EV2. M6, P2, T3 and I2 yeasts produced significant proportions of ester compounds (ethyl acetate and ethyl 2-methyl butanoate) and dimethyl disulfide but they did not show significant differences in the production of dimethyl trisulfide in comparison to control tubes. Moreover, V2, EV2 and T3 yeasts that showed unpleasant aromas (cooked vegetables, mustier, sulfur) in the sensory analysis produced the highest proportion of sulfur compounds. Finally, T3 yeasts showed a significant different generation of 2-butanone, ethyl hexanoate, ethyl octanoate in comparison to control and other yeasts. Nevertheless, all the yeasts showed a significant generation of methyl-branched alcohols but few significant differences were detected among them.

The incubation of yeasts in the culture media containing 2-methyl butanoic acid and methanol (YNB+B+M) showed also significant differences in the generation of volatile compounds (Table 5). The volatile compounds produced in highest abundance by the yeasts were methyl acetate, methyl 2-methylbutanoate, methyl 2-methyl 2-butenate, dimethyl disulfide, dimethyl trisulfide, 2-ethyl 1-hexanol, styrene and in less abundance aldehydes and ketones (table 5). As observed previously, there were also significant differences in the generation of volatile compounds

among yeasts. M4 yeast was the strain with the highest significant production of volatile compounds and it showed the highest significant production of nonanal, 2-pentanone, dimethyl trisulfide, 3-methyl and 2-methyl butanol, methyl butanoate, methyl 2-methyl butenoate, methyl dodecanoate and styrene. M6 yeast produced a significant increase of methyl 2-methyl butanoate and methyl 2-methyl 2-butenate esters, P2 was the yeast that produced the highest significant 2-nonanone abundance. T3 significantly produced decanal and dimethyl disulfide and V2 was the main producer of methyl 2-methyl-butanoate and methanol. I2 yeast showed a significant highest production of methyl decanoate and methyl 2-methyl-butanoate whereas EV2 yeast generated the highest abundance of 2-ethyl 1-hexanol and it was the only one that produced methyl 2-pentenoate.

As observed in both media, the generation of volatile compounds by yeasts depends on the culture media. In the inoculated media three amino acids (histidine, methionine, tryptophan) were present as a nitrogen source, and their degradation generates histamine, methional and methanethiol and indol compounds, respectively (Yvon and Rijnen, 2001). Several of these compounds were detected in the inoculated media such as methanethiol although the yeasts were capable to produce many other volatile compounds.

In addition, the present study has shown the ability of the isolated yeasts to generate ester compounds apart from other volatile compounds. Until now it was not possible to attribute the ester compound generation in fermented sausages to a specific microbial group (Tjener and Stanhke, 2007). The inoculated media were supplemented with 2-methyl butanoic acid and an alcohol (ethanol or methanol) and it was confirmed the generation of the corresponding ethyl or methyl 2-methyl butanoate ester in high abundance respect to the control tube. Therefore, this study

has confirmed the esterase activity in the species *D. hansenii* and the ability to produce ethyl and methyl esters from simple media containing an alcohol and an organic acid (Besançon et al., 1995).

Previous studies done by Andrade et al., (2009b) also reported the generation of similar volatile compounds from *D. hansenii* yeasts isolated from dry cured ham and inoculated in a culture media resembling the conditions of the process. They found a high generation of branched alcohols, aldehydes, ketones and also sulfur compounds as we have observed under our experimental conditions. However, we detected important differences such as an absence of branched aldehydes and a high generation of ester compounds in our culture media. In addition, in 2004, Flores et al., applied a *D. hansenii* strain to a fermented sausage and the increase in ester compounds in the inoculated batches was small, in contrast to the results reported by Andrade et al., (2010) who observed a higher generation of ester compounds in *D. hansenii* inoculated batches. Therefore, it is essential to select the appropriate *D. hansenii* strain with the ability to form ester compounds that can provide fruity notes to the fermented sausages increasing the sensory quality (Stahnke, 1994).

This study confirms the large genetic variability within strains of *D. hansenii* isolated from naturally fermented sausages. Our results indicate that depletion or production of alcohols and production of esters and sulfur compounds seems to be a strain trait not corresponding with M13 and mtDNA profiles. Therefore selection of a unique representative strain from each *D. hansenii* UPGMA group for testing of aroma production on methanol and ethanol media might have lead to different conclusions. Finally, the differences detected in the aroma profiles produced by the selected *D. hansenii* isolates have to be confirmed in real fermented sausages.

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Figure legends

Figure 1.- Electrophoretic patterns showing PCR amplification of the rDNA region ITS1-5.8S-ITS2 (lane 2) and restriction using enzymes *CfoI* (lane 3), *HaeIII* (lane 4) and *HinfI* (lane 5).

Figure 2.- Patterns of mtDNA RFLPS after DNA digestion with enzyme *HinfI*. Lanes are labeled with the strains names or with an “M” showing DNA markers (lambda DNA digested with *HindIII* and *PstI*).

Figure 3.- Electrophoretic patterns of minisatellite M13 PCR amplification. Lanes are labeled with the strains names or with an “M” showing the 100 pb ladder (Invitrogen, Carlsbad, CA, USA).

Figure 4.- UPGMA dendrogram analysis showing the relationships among *D. hansenii* strains isolated from fermented sausages and CECT reference strains based on mtDNA restriction and M13 patterns analysis. Three different ITS1-5.8S-ITS2 rDNA region sequence types are indicated as ITS-5.8S I, ITS-5.8S II and ITS-5.8S III.

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

Species	strain	isolation source
<i>D. hansenii</i>	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,	natural fermented sausages,
	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	
<i>D. fabryii</i>	CECT 11370 ^T	interdigital mycotic lesion,
Germany		

CECT, Spanish Type Culture Collection, University of Valencia, Spain

^a C1 and C3 were isolated in a previous study by Bolumar *et al.* (2003, 2006) and C7 was isolated by Durá *et al.* (2004).

Table 2. Aroma descriptors detected in YNB media supplemented with 2-methyl butanoic acid and ethanol (YNB+B+E) or methanol (YNB+B+M) after incubation with *D. hansenii* yeasts.

Yeast strains	Media	Aroma descriptors*				
M4	YNB+B+E	Fruity ⁽⁶⁾	Floral ⁽¹⁾	Sweet ⁽¹⁾		
	YNB+B+M	Fruity ⁽⁴⁾	Floral ⁽²⁾	Cheese ⁽²⁾		
M6	YNB+B+E	Fruity ⁽⁵⁾	Floral ⁽²⁾	Sweet ⁽¹⁾		
	YNB+B+M	Fruity ⁽⁴⁾	Feet ⁽²⁾	Cheese ⁽¹⁾		
P2	YNB+B+E	Fruity ⁽⁵⁾	Leather ⁽²⁾	Roasted nuts ⁽¹⁾		
	YNB+B+M	Roasted nuts ⁽³⁾	Feet ⁽¹⁾	Leather ⁽¹⁾	Mustier ⁽¹⁾	
T3	YNB+B+E	Roasted nuts ⁽²⁾	Cheese ⁽²⁾	Cooked vegetables ⁽²⁾	Fruity ⁽¹⁾	Feet ⁽¹⁾
	YNB+B+M	Feet ⁽³⁾	Mustier ⁽²⁾	Cooked vegetables ⁽¹⁾	Cheese ⁽¹⁾	
V2	YNB+B+E	Cooked vegetables ⁽⁵⁾	Cheese ⁽²⁾			
	YNB+B+M	Cheese ⁽³⁾	Mustier ⁽³⁾	Asparagus ⁽¹⁾	Fruity ⁽¹⁾	
EV2	YNB+B+E	Sulphur ⁽³⁾	Mustier ⁽²⁾	Bitter almond ⁽¹⁾	Cheese ⁽¹⁾	Fruity ⁽¹⁾
	YNB+B+M	Mustier ⁽³⁾	Cooked vegetables ⁽²⁾	Herbaceous ⁽¹⁾	Cheese ⁽¹⁾	
I2	YNB+B+E	Fruity ⁽³⁾	Sulphur ⁽³⁾	Feet ⁽¹⁾	Cheese ⁽¹⁾	
	YNB+B+M	Mustier ⁽⁶⁾	Sulphur ⁽¹⁾	Feet ⁽¹⁾		

* Numbers in parentheses indicate the number of times a descriptor was detected by assessors.

Table 3. Volatile compounds identified in YNB media supplemented with 2-methyl butanoic acid and ethanol (YNB+B+E) or methanol (YNB+B+M) after incubation with *D. hansenii* yeasts.

KI *	R **	Compounds	YNB+B+E		YNB+B+M	
			Control	Inoculated	Control	Inoculated
462	a	Acetaldehyde		+		
472	a	Methanethiol (47) ^c		+		
473	a	Methanol			+	+
509	a	Ethanol	+	+		
552	a	Methyl acetate				+
633	a	2-Butanone (72)		+	+	+
634	a	Ethyl acetate		+		
734	a	2-Pentanone (86)		+	+	+
741	a	3-Pentanone				+
745	a	Ethyl propanoate		+		
755	a	Methyl butanoate (74)			+	+
772	a	Dimethyl disulfide		+	+	+
794	a	3-methyl 1-butanol		+		+
797	a	2-methyl 1-butanol		+		+
805	b	Methyl 2-methyl butanoate			+	+
831	a	Ethyl butanoate		+		
833	b	Methyl 2-Pentenoate (83)				+
878	a	Ethyl 2-methyl butanoate		+		+
882	a	Ethyl 3-methyl butanoate		+		+
899	b	Methyl 2-methyl 2-butenate (55)				+
918	a	Styrene		+		+
934	a	2-Heptanone		+		
949	a	2-methyl butanoic acid	+	+	+	+
971	b	Ethyl 2-methyl 2-butenate		+		
1002	a	Dimethyl trisulfide (126)		+		+
1015	a	Benzaldehyde (77)		+		+
1029	a	Ethyl hexanoate (99)		+		
1071	b	4-Nonanone (71)		+	+	+
1082	a	2-ethyl 1-hexanol,		+		+
1127	a	Ethyl heptanoate (88)		+		
1149	a	Nonanal (95)		+		+
1229	a	Ethyl octanoate (127)		+		
1256	a	Decanal		+		+
1260	a	Methyl nonanoate (141)				+
1327	a	Ethyl nonanoate		+		
1358	a	Methyl decanoate				+
1425	a	Ethyl decanoate (101)		+		
1553	b	Methyl dodecanoate (143)				+

KI*: Kovats Index calculated for DB-624 capillary column (J & W Scientific: 30 m, 0.25 mm i.d., 1.4 μ m film thickness) installed on a gas chromatograph equipped with a mass-selective detector.

R*: Reliability of identification a: mass spectrum and retention time identical with an authentic standard, b: tentative identification by mass spectrum.

^c: Target ion used to quantify the compound when the peak was not completely resolved.

Table 4. Abundance of volatile compounds (expressed in percentage as the relation between area in inoculated and control tubes) in YNB media supplemented with 2-methyl butanoic acid and ethanol (YNB+B+E) after incubation with *D. hansenii* yeasts.

Volatile compound	CONTROL		M4		M6		P2		T3		V2		EV2		I2		SEM ²	P -Value ¹
	Mean		Mean		Mean		Mean		Mean		Mean		Mean		Mean			
Aldehydes																		
Acetaldehyde	100	a ³	2363	b	2637	b	2921	b	100	a	1434	ab	1537	ab	1784	ab	507	*
Benzaldehyde	100	a	2802	c	354	ab	100	a	100	a	3030	c	373	ab	687	b	147	**
Nonanal	100	b	44	a	100	b	100	b	100	b	100	b	100	b	45	a	7	**
Ketones																		
2-Butanone	100	a	100	a	100	a	100	a	1313	b	284	a	434	a	424	a	196	**
2-Pentanone	100	a	2887	c	2304	b	405	a	2597	bc	2245	b	2476	bc	2199	b	148	**
4-Nonanone	100		100		100		100		100		430		100		306		91	ns
Sulfur compounds																		
Methanethiol	100	a	100	a	100	a	100	a	147	b	224	c	100	a	100	a	9	**
Dimethyl disulfide	100	a	14219	bc	10259	b	9493	b	7737	ab	20241	c	19631	c	12311	bc	2923	**
Dimethyl trisulfide	100	a	6721	b	494	a	466	a	410	a	8184	c	6350	b	1077	a	435	**
Alcohols																		
Ethanol	100	a	2	a	953625	c	518298	bc	510021	b	577879	bc	594057	bc	724193	bc	145987	**
3-Methyl1-Butanol	100	a	7757	b	8651	bc	9904	bc	11604	c	8706	bc	9832	bc	7274	b	1055	**
2-Methyl1-Butanol	100	a	3175	b	3187	b	4698	c	4625	c	3130	b	3519	bc	3523	bc	438	**
2-Ethyl 1-Hexanol	100	a	17954	c	10901	bc	8786	b	13143	bc	7581	b	14758	bc	10140	bc	2685	*
Ester compounds																		
Ethyl acetate	100	a	21473	d	10711	bc	19670	cd	8060	ab	11210	bc	11065	bc	15708	bcd	3347	**
Ethyl 2-methyl butanoate	100	a	32545	d	19111	c	7323	b	17194	c	17474	c	17443	c	13806	bc	2250	**
Ethyl hexanoate	100	a	436	a	413	a	254	a	4495	b	399	a	100	a	301	a	900	*

Ethyl heptanoate	100	a	225	b	100	a	100	a	207	ab	100	a	100	a	261	b	139	*
Ethyl octanoate	100	a	2483	b	58	a	53	a	3310	b	65	a	100	a	809	a	346	**
Ethyl nonanoate	100	a	1648	b	100	a	100	a	100	a	100	a	100	a	100	a	10	**
Ethyl decanoate	100	a	100	a	100	a	100	a	100	a	100	a	100	a	134	b	4	**
Other compounds																		
2-Methyl butanoic acid	100	a	440	a	8261	b	126292	c	70337	b	94448	bc	83880	b	56127	b	11641	**
Styrene	100		100		100		510		459		751		596		992		227	ns

¹, ns = $p > 0.05$, * = $p < 0.05$; ** = $p < 0.01$; ² SEM: standard error of the mean. ³ Identical letters in each row indicate the absence of significant differences at $p > 0.05$.

Table 5. Abundance of volatile compounds (expressed in percentage as the relation between area in inoculated and control tubes) in YNB media supplemented with 2-methyl butanoic acid and methanol (YNB+B+ME) after incubation with *D. hansenii* yeasts.

COMPOUND	CONTROL		M4		M6		P2		T3		V2		EV2		I2		SEM ²	P –Value ¹
	Mean		Mean		Mean		Mean		Mean		Mean		Mean		Mean			
Aldehydes																		
Benzaldehyde	100		100		100		100		100		100		147		100		23	ns
Nonanal	100	a ³	12320	b	100	a	100	a	100	a	100	a	100	a	36	a	895	**
Decanal	100	a	100	a	140	b	142	b	1220	c	100	a	100	a	100	a	42	**
Ketones																		
2-Butanone	100	a	1404	b	1143	b	1090	b	195	a	1480	b	1144	b	1341	b	156	**
2-Pentanone	100	a	3939	c	1333	b	1360	b	1122	b	1376	b	1336	b	1437	b	113	**
3-pentanone	100	a	1533	d	1177	bc	1379	bcd	1080	b	1216	bc	1405	cd	1588	d	105	**
2-Nonanone	100	a	100	a	100	a	399	b	100	a	100	a	100	a	100	a	9	**
4-Nonanone	100		100		100		332		100		100		100		100		82	ns
Sulfur compounds																		
Dimethyl disulfide	100	a	1376	a	4027	b	7717	c	14507	d	5283	b	6039	bc	4589	b	700	*
Dimethyl trisulfide	100	a	10832	b	259	a	875	b	246	a	172	a	100	a	100	a	643	**
Alcohols																		
Methanol	100	a	1067	b	897	b	742	b	1037	b	2611	c	1246	b	1008	b	199	**
3-Methyl 1-butanol	100	a	323	b	119	a	124	a	89	a	84	a	119	a	127	a	34	**
2-Methyl 1-butanol	100	a	1792	c	1169	b	1135	b	815	b	815	b	901	b	1186	b	161	**
2-Ethyl 1-hexanol	100	a	9779	b	15398	bcd	13633	bcd	12334	bc	14565	bcd	21412	d	17297	cd	2678	**
Ester compounds																		
Methyl acetate	100	a	1930	d	1521	bcd	911	b	1736	cd	947	b	1193	bc	1330	bcd	247	**
Methyl butanoate	100	abc	438	d	56	a	176	c	89	ab	100	abc	170	bc	100	abc	32	**
Methyl 2-methyl butanoate	100	a	2386	a	15097	c	7891	b	8644	b	16422	c	8433	b	14554	c	1590	**
Methyl 2-Pentenoate	100	a	100	a	100	a	100	a	100	a	100	a	349	b	100	a	27	**
Methyl 2-methyl 2-butenate	100	a	49673	d	7913	c	408	a	487	a	5861	bc	100	a	3645	b	701	**

Methyl nonanoate	100	a	100	a	100	a	100	a	100	a	100	a	133	b	100	a	9	ns
Methyl decanoate	100	a	100	a	96	a	409	a	100	a	77	a	169	a	1077	b	133	**
Methyl dodecanoate	100	a	1144	c	89	a	51	a	100	a	234	b	45	a	68	a	52	**
Other compounds																		
2-Methyl butanoic acid	100	a	53	a	100	a	847	b	418	ab	1043	b	757	ab	1006	b	120	**
Styrene	100	a	25197	d	3181	c	2982	c	770	b	773	b	2357	c	3282	c	489	**

¹, ns = $p > 0.05$; ** = $p < 0.01$; * = $p < 0.05$. ² SEM: standard error of the mean. ³ Identical letters in each row indicate the absence of significant differences at $p > 0.05$.

Figure 1

ACCEPTED

Figure 2

Figure 3

Figure 4

HIGHLIGHTS

Diversity in *D. hansenii* sausage isolates was explored using M13 PCR and mtDNA RFLPs

Large genetic diversity within *D. hansenii* isolates was analyzed by UPGMA

D. hansenii strains produced different aroma profiles in a defined culture media

D. hansenii strains were able to produce ester and sulfur compounds

No correspondence between genetic groups and aroma profiles was found.