Impact of *Debaryomyces hansenii* strains inoculation on the quality of slow dry-cured fermented sausages

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Abstract

Debaryomyces hansenii strains, M4 and P2, isolated from natural fermented sausages were inoculated in slow fermented sausages to study their effect on processing parameters, microbial population, volatile compound and sensory characteristics. The inoculation of *D. hansenii* strains, M4 and P2, did not affect the ripening process as no differences in pH and Aw were detected. The dominance of the inoculated yeast strains along the process was followed by RAPDs of M13 minisatellite. The inoculated yeasts, P2 and M4, were recovered at the end of the ripening process although P2 appeared in higher counts than M4. The sausages inoculated with P2 resulted in a decrease in lipid oxidation values (TBARS) and a reduction of lipid-oxidation derived aldehydes in addition to a highest acid compound abundance. M4 inoculated sausages resulted in highest sulphur containing compounds abundance. However, no differences in consumer acceptance were detected. Moreover, both yeast strains were responsible for the generation of ethyl methyl-branched ester compounds in the dry-cured sausages.

Keywords: yeast, *D.hansenii*, dry-cured sausages, flavour, aroma and volatile compound.

1. Introduction

Slow fermentation of large diameter fermented sausages is a process that requires long ripening times (Marco, Navarro, & Flores, 2008). These traditional low-acid **dry-cured** fermented sausages are much appreciated in Southern European countries due to their moderate pH decrease produced by microbial activity and by the development of specific sensory characteristics (Ravyts, De Vuyst & Leroy, 2012).

The improvement and diversification of fermented flavour by selecting microorganism with unique flavour-developing capabilities is an opportunity to increase the competitiveness of the final **dry-cured** fermented sausage. In this case, the contribution of yeast strains to the sensory characteristics of **dry-cured** fermented sausages has been mainly attributed to the stabilization of the reddening reaction and development of a characteristic yeast flavour, due to their ability to degrade peroxides and the presence of lipolytic and proteolytic activities (Olesen & Stahnke, 2000, Dura, Flores & Toldrá, 2004). Among spontaneous fermented sausages, *Debaryomyces hansenii* is often the dominated yeast strain in fermented sausages (Cocolin , Urso, Rantsiou, Cantoni & Comi, 2006; Mendonça, Gouvêa, Hungaro, Sodré, & Querol-Simón, 2013).

The selection of yeast strains to improve sausage fermentation with respect to flavour may be affected due to yeast growth inhibition by ingredients, antifungal compounds or technological parameters (Olesen & Stahnke, 2000). Also, yeast metabolic capabilities to produce flavour compounds may differ among strains. The addition of *D. hansenii* yeast strains in **dry-cured** fermented sausages has been related to the increase in esters and acids compounds (Flores, Durá, Marco, & Toldrá, 2004; Andrade, Córdoba, Casado, Córdoba, & Rodríguez, 2010; Purriños, Carballo, & Lorenzo, 2013) an increase in branched alcohols and aldehydes

(Andrade et al., 2010) a decrease in linear aldehydes (Flores et al., 2004; Purriños et al., 2013) although Olesen & Stahnke, (2000) did not find any volatile compound effect.

The differences in flavour development in dry-cured fermented sausages associated with a particular yeast strain have been followed throughout the ripening process using yeast media (Olesen & Stahnke, 2000; Flores et al., 2004), morphological and biochemical yeast characteristics (Flores et al., 2004; Lucci, Patrignani, Belletti, Ndagijimana, Guerzoni, Gardini, & Lanciotti, 2007) but few studies have demonstrated the presence of the inoculated yeast strains by molecular characterization (Andrade *et al.*, 2010; Mendonça et al., 2013). Also, the implantation of the inoculated yeast strains throughout the ripening process has not been followed.

The molecular characterization of the different yeast strains of *D. hansenii* isolated from traditional fermented sausages have demonstrated that there is a large variability within *D. hansenii* (Cano-García, Flores, & Belloch, 2013; Mendonça *et al.*, 2013). Also, the aromatic potential is different among selected *D. hansenii* strains as it was shown by Cano-García, Rivera-Jiménez, Belloch, & Flores, (2014) in a meat model system. In this study, two *D. hansenii* strains, M4 and P2, showed a remarkable aroma potential due to their ester and sulphur production capabilities. However, the metabolic activity of these yeasts might be affected by meat ingredients, technological parameters and also microorganism present since competition between microbial populations is occurring throughout the ripening process (Ravyts et al., 2012).

Therefore, our aim was to evaluate the effect of *D. hansenii* yeast strains, M4 and P2, on the sensory quality of slow dry-cured fermented sausages. In addition,

the implantation of the inoculated *D.hansenii* strains was checked by molecular techniques throughout the whole ripening process.

2. Materials and methods

2.1. Preparation of yeast strains starter

Two *Debaryomyces hansenii* strains, M4 and P2, previously isolated from naturally fermented sausages “salchichón de Requena” (Requena, Valencia, Spain) were selected by their aromatic potential (Cano-García *et al.*, 2013a, b) to be inoculated in slow dry-cured fermented sausages. The yeasts were grown on GPY medium (glucose 2%, peptone 0.5%, yeast extract 0.5%, pH 6.0) to a concentration of 10^8 cfu mL⁻¹. The cells were separated by centrifugation (7000 rpm for 10 min at 4 °C) and washed with sterile saline solution (0.9 % salt). The collected cells were concentrated in 1 mL of saline solution per gram of the cells (wet weight) and the suspensions stored at – 80 °C until further use.

2.2. Preparation of dry-cured fermented sausages

Three different batches of dry-cured fermented sausages were manufactured; a control batch without yeasts (batch C) and two batches inoculated with M4 (batch M4) and P2 (batch P2) yeast strains, respectively. Twenty two sausages were manufactured in each batch.

Sausage batches were prepared with lean pork (75%) and pork back fat (25%) and the following additives (g/kg): NaCl (27), lactose (20), dextrin (20), sodium caseinate (20), glucose (7), sodium ascorbate (0.5), sodium nitrite (0.15), potassium nitrate (0.15). The meat was ground through a 10 mm hole diameter plate, vacuum minced with the remaining ingredients and inoculated with a starter culture C-P-77 S bactoform (Chr. Inc., Hansen, Denmark) containing *Lactobacillus pentosus* and

Staphylococcus carnosus. For the yeast inoculated batches, appropriate volumes of P2 or M4 yeast strains suspension were added to the meat batter at final concentration of 10^6 cfu g⁻¹ of yeast strain. The meat mixture was stuffed into collagen casings of 9.5 cm diameter (FIBRAN, S.A., Girona, Spain) making sausages of approximately 0.7 kg as the final weight. All sausage batches were ripened in the same drying chamber and subjected to an initial stage at 15-20 °C and 75-85% HR for 22 h, followed by drying at 9 °C and 75-85% HR for 43 days. In order to control the ripening process, weight losses and pH were measured during processing (Olivares, Navarro, & Flores, 2010).

From each batch, a 500 g portion of meat mixture (0 days) and three sausages at 10, 27 and 43 days were randomly collected to study the effect of inoculated yeast strains and ripening time. 150 g portion of each sausage was minced and used for moisture, water activity and pH tests and a 10 g portion was taken for microbiological analysis. In addition, the remaining minced sausage was vacuum packaged and frozen at -20 °C for subsequent analyses (TBARS) after measuring sausage colour. Also, several sausage slices were wrapped in aluminium foil, vacuum packaged and stored at -80 °C for volatile compounds analysis. Finally, sensory analysis was carried out at 43 d of the drying process. Results were expressed as the mean of three replicates per 100 g of dry matter at each processing time and batch.

2.3. Chemical analysis

The measurement of pH, water activity, colour evaluation (CIE L*, a*, b*), and moisture was performed as described Olivares *et al.* (2010). Thiobarbituric acid reactive substances (TBARS) were quantified to determine the degree of lipid oxidation (Olivares, Navarro, & Flores 2011) using trichloroacetic acid as solvent instead of perchloric acid. The results were expressed as mg malonaldehyde (MDA)/kg in dry matter.

2.4. Microbial analysis

For microbial analysis, minced sausage samples were homogenized with sterile saline solution (1/10) in a Stomacher (IUL Instruments, Barcelona, Spain) for 1 min. Further decimal dilutions of the homogenate were made in sterile saline solution and 0.1 mL of each dilution were spread onto the surface of appropriate agar media. Rose Bengal Agar with chloramphenicol (RBA) (Conda SA, Madrid, Spain) incubated at 28 °C for 72 h was used for yeast counts. Lactobacilli counts were done on (MRS) Man Rogosa Sharpe Agar (Scharlau Chemie SA, Barcelona, Spain) in anaerobic jars while *Staphylococci* were counted by surface plating on (MSA) Mannitol Salt Agar (Scharlau Chemie SA, Barcelona, Spain) and both were incubated at 37 °C for 48 h.

2.5. Strains characterization by molecular methods

Ten yeast strains were isolated from each inoculated batch at the different ripening times and then subjected to molecular characterization by minisatellite PCR amplification using the M13 primer. DNA was extracted as described in Querol, Barrio, & Ramón, (1992) and M13 minisatellite PCR amplification was carried out as described elsewhere (Cano-García *et al.*, 2013a). PCR products (10 µL) were resolved by electrophoresis on 2 % agarose gel in 1xTAE buffer at 90 V for 3 h, stained with Red Safe nucleic acid staining solution 20,000x (Intron biotechnology, Kyunggi-do, Korea) and visualized under UV light. DNA fragment sizes were determined using 100-bp DNA ladder. Minisatellite PCR patterns were compared with those previously obtained for these strains (Cano-García *et al.*, 2013a).

2.6. Analysis of volatile compounds.

Frozen sausage slices were minced with 0.75 mg of BHT and 5 g weighted into 20 mL headspace vial (Gerstel, Germany). The vial was maintained at 37 °C during 30 min to equilibrate its headspace. Then, volatile compounds in samples were extracted by a Solid Phase Microextraction (SPME) device using an automatic

injector Gerstel MPS2 (Gerstel, Germany). The extraction was done using a 85 μm CAR/PDMS sf fiber (Supelco, Bellefonte, PA, USA) by headspace exposure during 3 h at 37°C and a previous vial equilibration for 30 min at 37°C.

Identification and quantification of volatile compounds 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 with a linear velocity of 34.3 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 Cano-García et al., (2013a). 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) calculated using the series of n-alkanes (Aldrich, Germany). 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.

2.7. Sensory analysis

A consumer sensory analysis was performed at the end of the ripening process (43 d). Testing was carried out in a sensory laboratory equipped with individual booths (Olivares et al., 2011). Data acquisition was performed using Compusense *five* release 5.0 software (Compusense Inc., Guelph, Ontario, Canada). The casing was removed and sausages cut into slices of 4 mm thickness. Sausages from each batch (C, M4, P2 batches) were labelled with random three-digit codes and presented on a plate at room temperature with water and unsalted toasts to cleanse the palate among samples. An acceptability test was carried out using 9-box hedonic scale (1 extremely dislike – 9 extremely like). A panel of 80 untrained consumers

was used. The consumers were asked to evaluate each sausage based on appearance (evaluated inside a camera with D65 illuminant), flavour, taste, hardness, juiciness and overall quality.

2.8. Statistical analysis

The effect of inoculated yeast strains and processing time on chemical, microbial and volatile compounds was tested by two-factor analysis of variance (ANOVA) using the statistic software XLSTAT 2009.4.03 (Addinsoft, Barcelona, Spain). Differences between samples means were analyzed according to Fisher's Least Significant Difference (LSD) test. The effect of inoculated yeast strain on sensory parameters was done by one factor ANOVA analysis.

3. Results and discussion

3.1. Microbial analysis

Figure 1 shows the results concerning the viable counts of lactic acid bacteria, *Staphylococci* and yeasts during ripening of control and inoculated *dry-cured* sausages (M4 and P2 batches). The population of lactic acid bacteria and *Staphylococci* was within the range expected in *dry-cured* fermented sausages (figure 1A and 1B) (Durá *et al.*, 2004). A slight decrease in the number of *Staphylococci* was observed during processing in all batches although a highest *Staphylococci* population was detected in M4 sausages ($p < 0.001$) (Figure 1b). The presence of a highest *Staphylococci* population may modulate the *dry-cured* sausage aroma through the conversion of branched chain amino acids and free fatty acids (Leroy, Verluyten & De Vuyst, 2006).

Yeast population during *dry-cured* sausage processing is reflected in figure 1C. After 10 days of processing M4 and P2 batches showed an increase in the yeast population up to 6 and 8 logarithmic cycles, respectively, which were maintained

along the ripening process. This is in contrast to the slight reduction observed in other yeast inoculated fermented sausages (Dura et al., 2004, Andrade et al., 2010) or the completely disappearance reported by Olesen & Stahnke (2000). Nevertheless, control sausages showed a low yeast population similar to those reported in other non yeast inoculated sausages (Dura et al., 2004, Andrade et al., 2010).

The implantation of the inoculated yeast strains was followed by comparison of the M13 minisatellite PCR amplification patterns with those obtained from the original yeast strains (Cano-García *et al.*, 2013a). At the beginning of the process, all yeasts isolated from batches M4 and P2 displayed their original M13 minisatellite PCR amplification pattern (Figure 2A). In case of P2 batch all isolated yeasts (100%) along the ripening process showed the same pattern as the originally inoculated (Figures 2B, 2C and 2D). However, in case of batch M4 approximately 70 % and 50 % of the isolated yeasts at 10 (Figure 2B) and 27 (Figure 2C) days respectively, were ascribed to the original pattern. Nevertheless, at the end of the process dominance of M4 was 100% (Figure 2D).

3.2. Chemical parameters

The evolution of the pH, Aw and TBARS values during ripening time are shown in figure 3. The pH was very similar in all batches (figure 3A) but for M4 sausages the pH was slightly highest. These results are in agreement with previous studies, as few changes in pH have been reported in yeast inoculated **dry-cured** fermented sausages (Selgas, Ros & García, 2003, Dura et al., 2004, Andrade et al., 2010, Patrignani, Iucci, Vallicelli, Guerzoni, Gardini & Lanciotti, 2007). About water activity Aw, there were not relevant differences among **dry-cured** sausages (Figure 3B). In addition, moisture decreased significantly ($p<0.05$) in all the samples, from initial values of about 65% to 44% at the end of ripening. Sausage colour was also

measured along the process in the three batches, but not differences in colour parameters L* (Lightness), a* (redness) and b* (yellowness) were detected among them (data not shown). The lipid oxidation (TBARS values) in dry-cured fermented sausages was measured along ripening time (Figure 3C). It was significant the increase in TBARS values during the drying process in the three batches especially after 30 days of processing. However, at the end of the process TBARS values were significantly highest in control sausages followed by M4 sausages and finally, the lowest TBARS values were detected in P2 sausages. This inhibition of the lipid oxidation was previously reported by Flores et al., (2004) when they detected a reduction in the generation of volatile compounds derived from lipid oxidation reactions in fermented sausages inoculated with *Debaryomyces* spp.

3.3. Volatile compound analysis.

Table 1 shows the quantification of volatile compounds by SPME-GC-MS in control and inoculated dry-cured sausages throughout the processing time (0, 10, 27 and 43 days). A total of 53 volatile compounds were identified in the dry-cured fermented sausages, all of them were confirmed using authentic standards except 4-hexen-1-ol that was tentatively identified (Table 1). The volatile compounds were grouped in chemical families including aldehydes (8), ketones (6), alcohols (12), acids (7), esters (11), sulfur (5), and alkanes (4) compounds. All of them have been previously reported in fermented sausages using the same extraction technique (Marco, Navarro, & Flores, 2007; Olivares et al., 2011; Corral, Salvador, & Flores, 2013). The effect of inoculated yeasts and ripening time was significant in all volatile compounds analyzed except for acetaldehyde, 4-hexen-1-ol and 1-octanol that were not affected by the ripening time and 2-methylpropanal, 3-methylbutanal, methanethiol, dimethyl disulfide, 2-butanone, 3-methylbutanol, ethyl acetate, butanoic acid and octanoic acids that were not affected by the inoculated yeast.

Moreover, hexane was not significantly affected by inoculated yeast and ripening time (Table 1).

Figure 4 shows the abundance of volatile compounds classified by chemical groups at the end of the ripening process in all batches (C, M4 and P2). The inoculation with P2 yeast produced a significant decrease in the abundance of aldehydes, sulphur containing compounds, alcohols, esters and alkanes. However, the M4 inoculated sausages did not show this decrease indicating different behaviour between P2 and M4 *D. hansenii* strains.

The generation of aldehydes during ripening showed an increase during the whole process except for the branched aldehydes (2-methylpropanal and 3-methylbutanal) which presented a decrease along time in all dry-cured sausages studied (Table 1). This fact could be due to the conversion of these aldehydes to their corresponding alcohols (Table 1) as this has been favoured by the presence of high levels of yeast in inoculated fermented sausages (Andrade et al., 2010). However, the main differences were detected at the end of the process in hexanal, octanal and nonanal, these compounds derived from lipid oxidation reactions were significantly highest in M4 sausages followed by control sausages and they were the lowest in P2 sausages. The generation of these compounds can be related to the TBARS values measured and therefore, Pearson correlation coefficients were calculated (Table 2). In fact, positive and significant correlations were obtained between aldehydes (nonanal) and TBARS values in the three batches. However, hexanal, the aldehyde significantly related to the lipid oxidation phenomenon (Gandemer, 2002, Olivares et al., 2011), was only significantly correlated in control and M4 batches probably due to the low hexanal concentration detected in P2 batch (Table 1). As seen in table 1, the two inoculated yeast showed a different behaviour; P2 inhibited the lipid oxidation process while M4 was not able to do it. This fact

agrees with other studies who have indicated an inhibition of the lipid oxidation (Flores et al., 2004) while other did not find it (Andrade et al., 2010).

Ketone abundance was the highest in yeast inoculated dry-cured fermented sausages (Figure 4). 2,3-butanedione was the ketone compound with the highest abundance in both inoculated sausages and acetone was highest in P2 inoculated sausages. Both compounds are generated from carbohydrate fermentation and contribute to the aroma in dry-cured fermented sausages (Marco et al., 2007).

As observed in figure 4, sausages inoculated with P2 strain generated the lowest alcohol abundance. In addition, ethanol was the compound detected in highest abundance among the alcohols reported however, it was generated in lowest abundance in P2 sausages (Table 1). Although, ethanol is not an essential aroma contributor (Marco et al., 2007) it is a precursor of ester compounds (Molimard & Spinnler, 1996) that are responsible of fruity odours in fermented sausages (Marco et al., 2007, Olivares, Navarro & Flores, 2013). Other alcohols derived from the lipid oxidation reactions, 1-pentanol and 1-hexanol (Shahidi, Rubin, & D'Souza, 1986), were also in lowest abundance in P2 sausages indicating an inhibition of the lipid oxidation process in this P2 inoculated sausage as seen previously (Table 1). In addition, the effect of the inoculated yeast on the production of alcohols derived from the amino acid catabolism, 2-methylpropanol, 3-methyl and 2-methylbutanol, was not clear (Table 1).

Acid compound generation increased during ripening process and it was the highest in P2 inoculated sausages (Figure 4). As seen in table 1, acetic acid was the compound present in the highest abundance and it was responsible for the differences among sausages. Not only acetic acid was increased in P2 inoculated sausages but also 2-methyl-propanoic, propanoic and butanoic acids were significantly produced in these P2 sausages. In contrast, 3-methyl butanoic and

hexanoic acids were mainly produced in M4 inoculated sausages (Table 1). This highest production of acid compounds has been reported in other *Debaryomyces* spp. inoculated fermented sausages (Flores et al., 2004, Andrade et al., 2010).

Ester compounds increased along the ripening time in all batches although the highest amounts were detected in control and M4 sausage (Figure 4) due to the highest abundance of ethyl acetate (Table 1). However, other differences among batches were detected such as the highest production of ethyl 2-methylpropanoate and ethyl 2-methyl- and 3-methyl butanoate in the two M4 and P2 inoculated batches (Table 1), confirming the presence of ester activity in the inoculated *Debaryomyces* yeasts (Olesen & Stahnke, 2000, Flores et al., 2004 and Andrade et al., 2010). These ester compounds have a high impact on sausage aroma contributing to fruity notes and masking rancid odours (Schmidt & Berger, 1998, Olivares et al., 2013). Moreover, the abundance of these ester compounds in inoculated dry-cured sausages confirms our previous results obtained in a meat model system that reported a high ester production capability of M4 yeast strain followed by P2 yeast strain (Cano-García et al, 2013b).

The abundance of sulphur containing compounds in the headspace of sausages increased during the ripening time (Table 1). At the end of the process, control and M4 batches showed a higher abundance of sulphur containing compounds than P2 inoculated sausage (Figure 4). The main difference in the production of sulphur containing compounds was due to the highest abundance of methionol, dimethyl disulfide and dimethyl trisulfide in M4 inoculated sausages. On the contrary, P2 inoculated sausages did not show an increase in these sulphur containing compounds (Table 1). In our previous studies, a similar sulphur capability of M4 and P2 yeast strains was detected in a defined culture media (Cano-García et al., 2013a) although in a meat model system, P2 showed a higher sulphur capability

than M4 yeast strain (Cano-García *et al.*, 2013b). However, in a real dry-cured fermented sausage opposite results have been obtained being M4 the highest sulphur containing compounds producer, even though M4 yeast counts were lower than those found in P2 inoculated sausages (Figure 1c). These results indicate that yeast strains are strongly influenced by the composition of the media (meat, fat, salt, and additives) and environmental processing conditions (temperature and relative humidity). Probably, this is the reason for low sulphur containing compounds production detected in other dry-cured fermented sausages inoculated with *Debaryomyces spp* strains (Andrade *et al.*, 2010; Flores *et al.*, 2004; Olesen & Sthanke, 2000). Furthermore, Andrade *et al.*, (2010) indicated a low sulphur containing compounds production in fermented sausages inoculated with *D. hansenii* individually but an absence of sulphur production when three different strains were combined and inoculated.

Respect to alkanes, its abundance increased during the ripening time (Table 1). Significant differences were detected at the end of the process ($p < 0.05$) among dry-cured sausages obtaining the highest levels in M4 sausage while P2 sausage was the lowest producer of these volatile compounds (Table 1 and Figure 4). However, the contribution of linear hydrocarbons to the aroma is low due to their high odour threshold (Shahidi *et al.*, 1986).

3.4. Sensory analysis.

The sensory attributes evaluated in the dry-cured fermented sausages were appearance, aroma, taste, tenderness, juiciness and overall quality (Table 3). The consumer sensory panel only detected significant differences in appearance ($p < 0.001$) among sausages being the P2 inoculated sausages preferred by the panel. A higher taste preference of the P2 inoculated sausages was obtained but the results were not significant. Therefore, under the ripening conditions used, the

inoculated yeast strains P2 and M4, did not affect the sensory characteristic. Few authors have reported a significant effect on the sensory characteristics of fermented sausages inoculated with *Debaryomyces* spp (Flores et al., 2004, Iucci et al., 2007) while other studies reported absence of sensory effects (Olesen & Stahnke, 2000, Selgas et al., 2003). Nevertheless, these studies did not confirm the implantation of the inoculated yeasts during the ripening process. Moreover, it has been reported that the effect on the sensory characteristics depends on the selected *Debaryomyces* strain (Olesen & Stahnke, 2000) and their inoculated quantity (Flores et al., 2004). In addition, the presence of other starters may affect their growth (Flores et al., 2004) and results in different sensory characteristics such as the presence of Staphylococci which can modulate the sausage aroma through the conversion of branched chain amino acids and free fatty acids (Leroy et al., 2006). In the present study, the starter used was a combination of *Lactobacillus pentosus* and *Staphylococcus carnosus* and a significant highest concentration of Staphylococci was detected in M4 inoculated sausages (Figure 1b) that could have affected the final sensory characteristic perceived. However, further studies with more samples should be done to evaluate if those differences found in volatile compounds are really detected in sensory analysis.

4. Conclusion

The inoculation of selected *Debaryomyces hansenii* strains, M4 and P2, in slow dry-cured fermented sausages did not affect the ripening process as no changes in pH and Aw were detected. The inoculated yeast strains were recovered at the end of the process although a greatest implantation degree was observed for P2 than M4. The inoculated *D. hansenii* strains affected the lipid oxidation process and the generation of volatile compounds. However, the changes depended on the yeast

strain inoculated as a decrease in lipid oxidation values (TBARS) and a reduction of lipid-oxidation derived aldehydes were observed in P2 inoculated sausages in addition to a highest acid compound abundance. On the other hand, M4 inoculated sausages resulted in highest sulphur containing compounds abundance while both yeast strains were responsible for the generation of ethyl methyl-branched ester compounds in the dry-cured fermented sausages. Although, significant results were obtained for production of volatile compounds, differences between the inoculated dry-cured fermented sausages were not sensory appreciated by the consumer panel probably due to the interference of the bacterial starter used in the ripening process. Nevertheless, the aroma potential shown by M4 and P2 *D.hansenii* strains in dry-cured fermented sausages may be used to improve and diversify the flavour of low aroma fermented sausages.

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Figure legends

Figure 1.- Evolution of microbial counts during ripening of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage). a) Lactic acid bacteria, b) **Staphylococci** and c) Yeasts.

Figure 2.- Electrophoretic patterns of minisatellite M13 PCR amplification of strains isolated at 0 d (A), 10 d (B), 27 d (C) and 43 d (D). The arrows indicate the bands present in the strains. In each photograph: first lane represents “M” 100 pb ladder (Invitrogen, Carlsbad, CA, USA) followed by the original inoculated strain M4 or P2 yeast and the 10 isolated strains in M4 or P2 inoculated batches, respectively.

Figure 3.- Evolution of pH, Aw and lipid oxidation values (TBARS) during ripening of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage).

Figure 4.- Total volatile compounds abundance (expressed as AU x 10⁴) in the headspace of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage) at the end of the ripening process (43 d). Different letters in the same chemical group indicate significant differences (p<0.05) among sausages.

Highlights

Two *D. hansenii* strains, M4 and P2, were inoculated in fermented sausages.

Implantation was followed by M13 minisatellite PCR amplification patterns

Inoculated yeast strains were recovered at the end of the process

D. hansenii strains affected lipid oxidation process and volatile compound generation.

D. hansenii did not affect consumer acceptance.

Table 1.- Volatile compounds identified and quantified (expressed as AU x 10⁵ extracted by HS-SPME) by GC-MS in the headspace of dry fermented sausages inoculated with D. hansenii strains during ripening (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage).

VOLATILE COMPOUNDS	KI ¹	0 Days			10 Days			27 Days			43 Days			P ⁴			
		C	M4	P2	C	M4	P2	C	M4	P2	C	M4	P2	SEM ³	Pt	Pc	Ptxc
Aldehydes																	
Acetaldehyde	466				289 abcd	385 ab	56 cd	396 abc	405 a	67 de	266 bcd	432 a	140 de	50	n.s.	***	n.s.
2-Methylpropanal	592				97 ab	164 a			90 ab			48 b		24	**	n.s.	n.s.
3-Methylbutanal	689				1306 a	693 ab	1142 a	295 b	272 b	717 ab	232 b	340 b	360 b	122	***	n.s.	n.s.
Pentanal	737						148 d	214 d	810 bc	274 d	1588 a	1001 b	272 d	253	***	***	***
Hexanal	840				187 d			2498 d	7091 c	1256 d	12799 b	17236 a	2550 d	2458	***	***	**
Heptanal	940							193 d	462 cd	285 d	833 bc	1431 a	911 b	211	***	*	n.s.
Octanal	1047						133 c	119 c	216 bc	296 bc	354 b	555 a	180 c	78	**	*	n.s.
Nonanal	1149				109 e	84 e	129 e	258 cd	314 c	156 d	444 b	563 a	324 c	55	***	**	**
Sulfur compounds																	
Methanethiol (47) ²	472	5 de	5 de	6 de	5 e	6 de	4 e	13 bc	9 cde	18 ab	22 a	17 ab	14 bc	2	***	n.s.	n.s.
Carbon disulfide	537	297 a	225 abc		233 ab	205 abc	28 bc	261 ab	195 abc	31 bc	192 abc	61 c	70 c	29	**	*	n.s.
Dimethyl disulfide	771	3 c	4 c	2 c	2 c	1 c	2 c	5 c	1 c	7 c	8 bc	24 a	15 b	2	***	n.s.	**
Dimethyl trisulfide (126)	1002	2 b			3 b		1 c	2 b		1 c		93 a	2 b	13	**	***	***
Methionol	1062				15 d		24 d	50 cd	71 bc	20 d	65 bc	260 a	59 bc	28	***	***	***
Ketones																	
Acetone	528	242 bc	199 cd	207 cd	259 bc	98 de	229 bc	764 a	93 de	403 ab	232 bc	138 de	352 bc	55	***	***	***
2,3-Butanedione (43)	625		364 a		164 bc	224 b	294 ab	118 c	195 b	218 b		106 c	97 c	35	***	**	n.s.
2-Butanone (72)	631	286 a	211 b	197 b	87 d		103 d	92 d	126 bc	61 d	114 bc	205 b	168 bc	24	***	n.s.	***
2-pentanone (86)	732				196 c	1140 a	62 c	185 c	508 b	68 c	90 c	224 bc	155 c	115	***	***	***
3-Hidroxy-2-butanone	780		3480 a	1445 bc	2120 b	4114 a	2056 b	1515 bc	2006 b	1438 bc	817 c	1067 bc	1407 bc	375	***	***	*
2-Heptanone	933		126 e		862 b	1025 a	42 ef	462 c	765 b	41 ef	193 de	44 cd	103 e	116	***	**	n.s.
Alcohols																	
Ethanol	509				4645 cd	5934 abc	4169 cd	5441 abc	6242 abc	4152 cd	5406 abc	5262 bc	3495 e	629	***	**	**
1-Propanol	613				122 a	142 a	49 c	93 a	118 a	82 b	79 b	111 a	119 a	11	***	***	**
2-Methylpropanol	682				399 a	489 a	185 d	386 a	360 b	188 d	267 c	253 c	457 a	37	***	***	***
1-Butanol	725				117 c			251 a	138 bc		171 b	133 bc		24	***	***	*
3-Methylbutanol	795			4383 e	9263 a		9191 a	8224 b	7144 c	5961 d	5258 d	3979 ef	4241 e	1012	***	n.s.	n.s.
2-Methylbutanol	797			351 e	950 b	1364 a	1843 a	914 b	823 b	1118 a	621 c	587 d	725 c	113	***	*	**
1-Pentanol	827				112 c			291 c	1115 ab		680 b	1662 a	190 c	249	*	*	*
1-Hexanol	922				945 c	676 cd	103 d	1784 b	3843 a	138 d	1820 b	4051 a	262 d	507	***	***	***
4-Hexen-1-ol	917TI				4142 a	246 c	869 bc	2570 a	181 c	341 bc	1604 b		59 c	545	n.s.	***	n.s.
2-heptanol	947							575 ab	517 bc	191 e	580 ab	504 cd	454 de	64	***	***	n.s.
1-Octanol	1123				126 ab			135 a	88 c		120 ab	83 c		10	n.s.	*	n.s.
Phenylethyl alcohol (91)	1192				71 cd	176 ab	106 b	59 d	70 cd	202 a	60 d	72 cd	94 b	14	***	***	**
Ester compounds																	
Ethyl acetate	635				247 c	775 c	1288 c	2441 ab	3144 a	1241 b	2923 ab	3018 ab	3159 a	380	***	n.s.	n.s.
Ethyl 2-methylpropanoate	788					108 e	217 de	289 de	297 de	594 b	363 d	460 c	731 a	63	***	***	*
Ethyl butanoate	830				322 d	526 cd	366 d	934 bc	1204 ab	541 cd	1538 a	1367 ab	667 cd	164	***	***	**
Ethyl 2-hydroxypropanoate	865				95 e	128 de	114 de	385 c	410 c	256 c	858 a	651 b	433 c	87	***	***	**
Ethyl 2-methylbutanoate	877					118 d		121 d	257 ab	214 ab	166 cd	293 a	257 ab	30	***	***	n.s.
Ethyl 3-methylbutanoate	881					394 d		311 d	618 b	488 c	448 c	813 a	652 b	76	***	***	**
3-Methylbutyl acetate	906				87 f	184 de	83 f	283 c	369 b	156 de	469 a	486 a	243 cd	55	***	***	**
Ethyl hexanoate (99)	1028				135 d	179 d	156 d	386 c	610 b	136 d	956 a	1057 a	379 c	119	***	***	***
Ethyl 2,4-hexadienoate	1144				194 b	287 a		74 c	206 b		72 c	97 c		36	***	***	n.s.
Ethyl octanoate (127)	1229				87 de	114 de	112 de	229 c	323 b	135 d	397 a	416 a	124 d	49	***	***	***
Ethyl decanoate (101)	1425							41 d	56 c		76 b	91 a	28 d	10	***	***	n.s.
Acids																	
Acetic acid	737				662 e		2618 bc	2392 cd	2192 cd	2405 bc	3061 b	2990 b	6392 a	571	***	***	**
Propanoic acid	806						89 b			116 ab			198 a	40	**	**	*
2-Methylpropanoic acid	864						271 b	79 c	71 c	469 ab	115 c	113 c	659 a	79	***	***	***
Butanoic acid	891	192 d	177 d	178 d	402 b	425 b	390 c			692 ab			949 a	93	***	n.s.	n.s.
3-Methylbutanoic acid	949					146 b			178 b		124 b	225 a		22	***	*	**
Hexanoic acid	1074				110 c	92 c	80 c	157 bc	161 bc	211 b	294 ab	350 a	288 ab	33	**	***	n.s.
Octanoic acid	1264				70 c	58 cd	68 c	97 ab	90 b	118 a	107 a	108 a	109 a	9	***	n.s.	n.s.
Alkanes																	
Pentane	500							122 c	416 b	106 c	474 b	848 a	201 c	116	***	***	n.s.
Hexane	600								72 b	20 d	72 b	93 a	53 c	12	n.s.	n.s.	n.s.
Heptane (71)	700				186 e	317 cd	99 f	273 d	569 c	96 f	705 b	899 a	242 d	94	***	**	**
Octane	800				103 d		107 d	360 c	699 b	293 cd	1084 ab	1956 a	393 c	225	**	*	n.s.

¹ KI: Kovats Index calculated for DB-624 capillary column (J & W Scientific: 30 m, 0.25 mm i.d., 1.4 µm film thickness) installed on a gas chromatograph equipped with a mass-selective detector. Identification of compounds was confirmed by mass spectrum and retention time with an authentic standard except marked with (TI) that was tentatively identify by mass spectrum.

² Target ion used to quantify the compound when the peak was not completely resolved.

³ SEM: Standard error of the mean.

⁴ P value; Pt: p value of ripening time effect; Pc: p value of inoculated yeast effect; Ptxc: p value of interaction between ripening time and inoculated yeast effect.

***: p<0.001, **: p<0.01, *: p<0.05, n.s.: p>0.05. a-f: Identical letters in each volatile compound indicate that there is no significant difference at p>0.05 (Fisher's test).

Table 2.- Pearson correlation coefficients of aldehydes obtained by SPME-GC-MS and TBARS values in dry fermented sausages inoculated with *D. hansenii* strains; (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage)

	CONTROL		M4		P2	
	r	P	r	P	r	P
Acetaldehyde	0.468	0.290	0.267	0.488	0.771	0.439
2-methyl-propanal	0.635	0.249	0.699	0.036	-*	-
3-methyl-propanal	0.473	0.141	0.465	0.176	0.650	0.550
Pentanal	0.956	0.003	0.593	0.292	0.254	0.836
Hexanal	0.940	0.000	0.850	0.007	0.412	0.730
Heptanal	0.890	0.017	0.725	0.166	0.232	0.851
Octanal	0.923	0.009	0.718	0.108	0.999	0.035
Nonanal	0.939	0.000	0.920	0.000	1.000	0.000

*(-): not detected.

Table 3.- Sensory acceptability of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage)

	CONTROL			M4			P2		P ¹	
	Mean	SD		Mean	SD		Mean	SD		
Appearance	5.86	1.58	a ²	5.69	1.80	a	6.71	1.46	b	***
Aroma	6.71	1.67		6.86	1.31		6.74	1.35		n.s.
Taste	6.21	1.78		6.28	1.49		6.58	1.40		n.s.
Tenderness	6.59	1.46		6.51	1.41		6.38	1.45		n.s.
Juiciness	6.66	1.34		6.53	1.38		6.48	1.38		n.s.
Overall quality	6.39	1.65		6.24	1.57		6.53	1.35		n.s.

¹P value of inoculated yeast effect: *** p<0.001, ** p<0.01, * p<0.05, n.s.: p>0.05.

²Different letters in each row indicate differences at p<0.05 (Fisher test). The values represent the mean and standard deviation (SD).

Figure 1
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Figure 2

Figure 3
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Figure 4
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