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Impact of a selected *Debaryomyces hansenii* strain's inoculation on the quality of Sardinian fermented sausages

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Abstract

Dominant yeast species in Salsiccia Sarda, a traditional fermented sausage produced in Sardinia (Italy), were evaluated through the monitoring three typical production processes. Six different species were identified by molecular techniques, but *Debaryomyces* (*D.*) *hansenii* proved to be dominant. A *D. hansenii* strain was selected according to its technological features and used in three experimental sausage productions at farm scale with the aim to evaluate its antifungal effect. In all cases, two batches were inoculated with a previously selected autochthonous starter cultures (*Lactobacillus plantarum* and *Staphylococcus xylosus*), whereas two batches were left to spontaneous fermentation. *D. hansenii* was inoculated on the sausages surface by brushing after the sausages drying, by immersion in a yeast suspension after the stuffing, or, alternatively, casings were dipped in a yeast suspension before the dough stuffing. Microbial counts in the sausages core did not appear to be affected by *D. hansenii* application, while outcomes obtained for casings appeared soundly diversified. Brushing on the sausages surface at the onset of fermentation proved to be the best approach to treat sausages. Yeast inoculation exerted a noteworthy anti-mould effect, independently of the mode of application and, on the other hand, did not affect the overall quality and typical features of the product.

Keywords: Meat products; *Debaryomyces hansenii*; Lipolytic activity; Moulds; Sensory analysis; Sardinian sausage

1. Introduction

Although it is well known that the main microbial groups in fermented meat products are lactic acid bacteria (LAB) and staphylococci, several studies have been focused on the potential role

of yeasts in the maturation of salami with specific reference to both flavour and aroma development as consequence of proteolytic and lipolytic activities (Mendonça, Gouvêa, Hungaro, Sodré, & Querol-Simon, 2013) and on the colour stabilization as a result of oxygen depletion (Encinas, Lopez-Diaz, Garcia-Lopez, Otero, & Moreno, 2000). Yeasts are usually found in high numbers, up to 10^6 CFU g^{-1}, in dry-cured meat products, such as fermented sausages (Cocolin, Urso, Rantsiou, Cantoni, & Comi, 2006), even though they are not usually added as starter cultures. The yeasts source in fermented meat products is mainly represented by raw material and processing environment, (Nielsen, Jacobsen, Jespersen, Koch, & Arneborg, 2008). *Candida*, *Cryptococus*, *Debaryomyces*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Trichosporon* and *Yarrowia* spp. are the most commonly isolated genera (Cano-García, Rivera-Jiménez, Belloch, & Flores, 2014a). Amongst them, *Debaryomyces* (*D.*) *hansenii* is undoubtedly the predominant yeast species (Andrade, Córdoba, Casado, Córdoba, & Rodríguez, 2010). *D. hansenii* has attracted scientific interest mostly because of its halotolerance, and many studies deal with the mechanisms of conferring salt tolerance to this yeast, and with the genome analysis (Bolumar, Sanz, Aristoy, & Toldrá, 2008). *D. hansenii* is often found in protein rich fermented products other than sausages (Aponte, Pepe, & Blaiotta, 2010; Mangia, Garau, Murgia, Bennani, & Deiana, 2014). *D. hansenii* can positively contribute to the stabilization of the reddening effect by its ability to degrade peroxides. Moreover, this yeast may favour the development of flavour in dry fermented sausages due to its lipolytic and proteolytic activities (Andrade et al., 2010; Cano-García, Belloch, & Flores, 2014b). As matter of fact, the role of yeasts as potential aroma enhancers in dry fermented sausages has been recently reviewed (Flores Corral, Cano-García, Salvador, & Belloch, 2015).

Several yeast species, including *D. hansenii* have been proved to be able to control the development of ochratoxigenic moulds in speck and dry cured ham (Andrade, Thorsen,

Rodríguez, Córdoba, & Jespersen, 2014; Iacumin, Manzano, Andyanto, & Comi, 2017; Meftah, Abid, Dias, & Rodrigues, 2018; Nuñez et al., 2015; Virgili et al., 2012).

Salsiccia Sarda is a traditional fermented dry cured sausage (National list of traditional products) produced exclusively in Sardinia (Italy) in small factories according to a traditional technology previously detailed (Mangia et al., 2013). Briefly, pork ham and tenderloin are trimmed and mixed with pork fat, salt (2.5–3 %), and spices (pepper, garlic, fennel, or anise seeds). Sometimes, vinegar or white wine is added. Dough is stuffed into natural casing obtained by ovine or pig gut. Ripening starts with drying and lasts about 20–25 days at 10–13°C and 80 % RH; during this period, sausages are lightly smoked. Current literature on the microbial ecology of Sardinian fermented sausages mostly refers to bacterial microflora (Greco, Mazzette, De Santis, Corona, & Cosseddu, 2005; Mangia et al., 2013). However, to authors' knowledge the yeast overall diversity in such food environment has not yet been described. In such light, in the present study, typical Sardinian sausage productions were followed and the yeast microflora was characterized. Then a *D. hansenii* strain, selected according to its technological features, was used in three experimental sausage productions at farm scale with the aim of evaluating its effect on mould development during fermentation. In every single production, the approach to inoculate the yeast starter was changed in order to find the best inoculum strategy to pursue the goal of controlling fungal growth. The effects of different treatments, to the core and surface microflora, as well as the impact on the final product at sensory level, were evaluated.

2. Material and methods

2.1. Chemicals and culture media.

Except YPD, which was Difco BD (Milan, Italy), all media and supplements (MRS, BHI, Mannitol Salt agar, Rose Bengal agar, *Pseudomonas* agar base plus CFC supplement, tryptone, and yeast extract) were purchased from Oxoid (Basingstoke, UK). Except Petroleum ether (Merck & Co. Inc., NJ, USA), used reagents (sodium citrate, NaCl, NaOH, chloramphenicol, tetracycline, glycerol, casein, gelatine, Griess reagent, tributyrin, tween 80, sodium nitrate, sodium nitrite, phenolphthalein, and bromocresol purple) were all purchased from Sigma (St. Louis, MO, USA).

2.2. Sampling, yeast count and pH determination

Meat and sausages samples were collected during a typical production of Sardinian fermented Salsiccia in a factory located at Ploaghe (Sassari province, Sardinia Region, Italy). The technological process has been previously detailed (Mangia et al., 2013). Three different productions were monitored. In detail, minced meat samples (Mm) were collected before salt and spice addition; sausages (S) were analysed at 1, 3, 7, 12, and 21 days of ripening; casings (Ca) from sausages at 21 days were aseptically removed and analysed.

For microbiological analysis, 10 g of samples were re-suspended in 90 mL of sterile 2% (w/v) sodium citrate solution and homogenized in Stomacher (PBI, Milan Italy) for 30 s at normal speed. Decimal dilutions were spread-plated in duplicate onto YPD agar plates supplemented with tetracycline (2 mg mL^{-1}) and incubated at 25°C for 5 days.

Potentiometric measurements of the pH were carried out by using a pin electrode of a pH-meter (KNICK Portamess 911) directly inserted into the sample.

2.3. Yeast cultures isolation and molecular identification

Fifty-seven yeast cultures were selected from the counting plates by picking colonies representative of the different morphological types. After purification by streaking onto YPD agar plates, strains were stored at -80°C in the same medium added of glycerol (20%).

Yeasts were identified by sequencing the Internal Transcribed Spacer within rDNA. For genomic DNA isolation, yeast cultures were overnight grown in YPD broth at 28°C under stirring. DNA was extracted according to Comi, Maifreni, Manzano, Lagazio, and Cocolin (2000). rDNA ITS region PCR amplification was performed according to Esteve-Zarzoso, Belloch, Uruburu, and Querol (1999). Sequencing was carried out by BMR-Genomics Ltd (http://www.bmr-genomics.it/). Results were firstly analysed by Sequence Scanner software and then submitted to BLAST comparison with the nucleotide database of the NCBI.

2.4. Evaluation of technological features of Debaryomyces hansenii strains

For all tests *D. hansenii* strains were first grown in YPD broth at 25°C for 24 h, and cell densities were spectrophotometrically (OD_{600}, Optical Density at 600 nm) assessed. Cultures were washed with sterile water, suspended at concentration of one OD_{600} and then used for all tests.

The ability to grow at two salt concentrations (10 and 15% NaCl) was evaluated in YPD broth after inoculation at 1% (v/v). Growth was spectrophotometrically assessed after incubation at 25°C for 48 h. Data obtained from the O.D. readings were the average value of three replicates (subtracted of the blank) performed in the same experimental conditions.

Nitrate reductase activity was evaluated according to the method described by Baruzzi, Matarante, Caputo, and Morea (2006) with minor modifications. Briefly, activated yeast cultures were used to inoculate (1%) YT (tryptone 1.0%, yeast extract 0.5%, pH 7.0) broth supplemented with 1 g L^{-1} KNO_3 and incubated at 25 C for 48 h. Griess reagent containing sulphanilic acid (1% w/v 0.5 M HCl) and N1-1-napthylethylendiamine hydrochloride (0.1%

w/v) was added to the tubes. The development of red colour indicated partial conversion of nitrates into nitrites.

Proteolityc activity was ascertained by spot-test method onto casein and gelatine media. Specifically, for casein test, YT agar plates containing 2% casein at pH 6.5 were spot-inoculated with 50 µL of YPD yeast cultures. After incubation at 25°C per 5 days, the diameter of the opaque halo around each colony was measured. For gelatine hydrolysis, yeasts were inoculated in YT tubes added of 12% gelatine. After incubation at 25°C for 21 days, protease activity was highlighted by the liquefaction of the medium, due to the gelatine hydrolysis.

Lipolytic activity was initially tested against tributyrin or Tween 80 by plate assay on YT agar medium containing 5% of one or other. Before pouring, both media were emulsified by stirring for 1 min with an Ultra-Turrax T25 homogenizer. After cooling, wells (7 mm diameter and 4 mm depth) were realized into the agar and aseptically filled with yeast suspensions in saline solution. After incubation at 25°C for 5 days, the diameter of the clear areas was measured. Lipolytic activity was even assessed against pork fat by means of the titration method described by Casaburi, Blaiotta, Mauriello, Pepe, and Villani (2005). Overnight cultures of yeast strains were used to inoculate (1% v/v) YTF broth (tryptone 1%, yeast extract 0.5%, NaCl 3%, pH 7.0) supplemented with pork fat (4% w/v). After incubation at 5°C for 7 days, lipids were extracted by adding petroleum ether and shaking for 1 min. Free fatty acids (FFAs) of the upper phase (lipid extract) were titrated with NaOH 0.1 N in ethanol, using 1% phenolphthalein ethanol solution as indicator. The percentage of palmitic acid, instead of oleic acid, was used to quantify the lipolytic activity.

Modified ECM-M medium (Blaiotta, Murru, Cerbo, Romano, & Aponte, 2018), containing pork muscle instead of bovine meat, was used to assess the growth rate of five selected *D. hansenii* strains. The medium was prepared by boiling for 20 min, minced fresh meat in deionized water (500 g/1 L); after paper filtering, dextrose (1.5 g L^{-1}), NaCl (30 g L^{-1}), Na

nitrate (0.2 g L^{-1}), Na nitrite (0.05 g L^{-1}), and 2.8 mL of an hydro-alcoholic solution of bromocresol purple (17 g L^{-1}) were added (pH 6.20-6.30). After inoculum (1%), at time 0 up to 24 hours of incubation at 22°C, cells were regularly counted on YPD agar.

2.5. Sardinian sausages experimental productions

Sausages were prepared by using pork meat and swine lard in ratio 5:1, according to the traditional recipe previously detailed (Mangia et al., 2013). Three independent productions (200 Kg each) were performed in May, September and January - across the biennium 2016-2017 - in the same farm where monitoring was carried out. The dough was divided into four batches of 50 Kg. Two batches were added of the starter culture (Trials S_1 and S_2), and two were naturally fermented (Trials N_1 and N_2). Specifically, starter culture was prepared by mixing at ratio 1:1 selected cultures of *Lactobacillus plantarum* PC23, and *Staphylococcus xylosus* SA23 (Mangia et al., 2013), which were cultured twice in MRS and BHI, respectively, before use. Both strains were inoculated as cell suspensions in saline solution at an initial level of about 6 Log CFU g^{-1} of dough.

Doughs were then stuffed into swine casings and kept for 24 hours at 19-20°C (RH 90-92%). Hereafter, the sausages were transferred for further 20 days at 12-13°C (RH 75-80%). One batch added of the starter (Trial S_2), plus one in spontaneous fermentation (Trial N_2), were superficially inoculated with *D. hansenii* strain Ca3. In detail, cell suspensions were distributed on the sausages surface according the following scheme: manufacture A) by brushing on the surface (about 6 Log CFU cm^{-1}), 24 h after the sausages drying, in May 2016 (Trials A-S_2 and A-N_2); manufacture B) by sausages immersion in a yeast suspension (about 7 Log CFU mL^{-1}), immediately after the stuffing, in September 2016 (Trials B-S_2 and B-N_2); manufacture C) by casings' immersion in a *D. hansenii* suspension (8 Log CFU mL^{-1}) before dough stuffing in January 2017 (Trials C-S_2 and C-N_2).

2.6. Microbial monitoring of Sardinian sausages

Two sausages were analysed for each sampling point for each single trial. Microbial loads were determined after 7, 15 and 21 days of ripening. The doughs immediately before stuffing were analysed as well. Casings were weighed and analysed too. After decimal dilutions, samples were plated on: Plate Count Agar (30°C, 72 h) for total aerobic microflora; MRS agar at pH 5.50 for LAB (30°C, 72 h, anaerobiosis); Mannitol Salt Agar for Micro-staphylococci (37°C, 48 h); Pseudomonas agar base with CFC supplement for *Pseudomonas* spp. (25°C, 48 h); Rose Bengal agar with chloramphenicol (100 mg mL^{-1}) for yeasts and moulds (28°C, 72 h).

2.7. Chemical analysis

For pH and titratable acidity determination, 10-g sample was homogenized in 90 mL of sterile saline for 10 min in a Stomacher Lab Blender 80 (PBI, Milan, IT). The pH was measured with a pH-meter (Crison Instruments SA, Barcelona, Spain) while Titratable acidity was determined by titrating with 0.1 N NaOH, phenolphthalein was used as indicator and acidity was expressed as percentage of lactic acid.

2.8. Statistical analysis

Significant differences among the different microbial groups (total viable microflora, lactic acid bacteria, *Staphylococcus* spp.; *Pseudomonas* spp.; yeasts; moulds) between samples (S1, S2, N1 and N2), at the same fermentation time within the same manufacture, were computed by using ANOVA and Tukey t-test ($p<0.05$) (XLStat 2012.6.02 statistical pocket, Addinsoft Corp., Paris, France).

2.9. Sensory analysis of experimental products

In order to evaluate the impact of *D. hansenii* on the sensory profile of sausages, analyses were carried out exclusively on products manufactured without the starter, namely just on batches N. In detail, sensory analysis was performed on 2.5 mm thick slices of Sardinian sausages after 21 days of ripening according to a Paired comparison test or alternative forced choice (ISO 5495:2005) by a descriptive panel of 50 assessors. Such procedure allows determining whether exists a perceptible sensory difference between samples of two products concerning the intensity of a sensory attribute and is electively applicable if the products are quite homogeneous. Assessors were asked to indicate only which samples they preferred. The taste tests were organized by comparing the control (N_1) with A-N_2 (yeast cell suspensions brushed on sausages surface 24 h after drying); B-N_2 (sausages' immersion in a yeast suspension after stuffing) and C-N_2 (casings' immersion in yeast solution before stuffing).

2.10. Sensory data analysis

Results are compared with the statistical tables of the significance of the bilateral test p = ½, α = 0.05 (ISO 5495:2005).

3. Results and Discussion

3.1. Isolation and identification of dominant yeast species occurring in Sardinian sausages

The yeast dynamics throughout sausages ripening are reported in Table 1. In the dough before salting, yeasts were around 3 Log CFU g^{-1} and started to increase after stuffing. Population levels on YPD medium slowly increased during the first week of monitoring, but then remained relatively stationary at nearly 5 Log CFU g^{-1} for up to 21 days of ripening, while on the casings yeast counts were higher. Yeast counts seem to be unaffected by the increase in acidity produced by LAB in the first part of the ripening process in both long- and short-ripened

sausage (Baruzzi et al., 2006; Coppola, Mauriello, Aponte, Moschetti, & Villani, 2000). pH values varied from 5.80±0.02 in minced meat to 5.05±0.02 at the end of fermentation (Data not shown).

Fifty-seven cultures were randomly isolated from counting plates seeded with the highest dilutions to analyse dominant yeast microflora. All cultures were identified by ITS sequencing. PCR amplification provided amplicons sized between 320 and 650 bp (Table 1).

The yeast species identified throughout the production process are reported in Table 1. As expected, the highest yeast diversity was detected at the onset of fermentation, whereas at the end of the ripening, isolates could be mainly reported to *D. hansenii* and, at less extent, to *C. zeylanoides* (Table 1). Obviously, the dominance of *D. hansenii* at the end of ripening and on the casing has to be considered undeniably positive with respect to the quality and the safety of the products (Asefa et al., 2009).

Strains belonging to the genus *Candida* were also isolated. In details, *C. zeylanoides* was detected in all ripening phases, except on 12 days. In fact, the only species retrieved on day 12 was *Y. lipolitica*, teleomorph form of *Candida* spp. (Table 1).

Overall, the yeast species isolated from the Sardinian sausages tended to be those found in other fermented meat products (Encinas et al., 2000; Mendoza, Padilla, & Vignolo, 2014) for which *D. hansenii* and *C. zeylanoides* are often reported as the dominant species.

T. cutaneum has been proved to be the most common yeast species in meat processing environments (Samelis & Sofos, 2003). Most of the species currently reported to this genus are widely distributed in the environment (soil and air) and as an integral part of both human and pig skin (Carregaro et al., 2010; Zhang, Sugita, Tsuboi, Yamazaki, & Makimura, 2011). As matter of fact, the isolation of such species is usually associated to little attention in the application of good manufacturing practice during slaughtering and/or dough preparation (Suzzi and Gardini, 2003). *Trichosporon* sp. is also often isolated from meat and fermented

meat products (Coppola et al., 2000; Mendoza et al., 2014), but to authors' knowledge the species *T. domesticum* has never been previously retrieved from fermented sausages. *Y. lipolitica*, a species with a recognized technological potential due to its lipolytic and proteolytic activities (Patrignani et al., 2007), appears to be dominant after 12 days of ripening. Since Sardinian sausages production is characterized by short-term ripening, a key role of this specie in the sensory definition of the product cannot be excluded.

3.2. Technological characterization of D. hansenii strains

D. hansenii is considered safe for use in foods (BIOHAZ, 2012), and according to the current EU legislation in force, it is the only species allowed as a starter culture. For this reason, strains within this species were further analysed for features of technological interest (Table 2). *D. hansenii* is characterized by several key traits, including high levels of salt tolerance (Breuer and Harms, 2006). Moreover, such species is characterized by a high oxygen demand (Encinas et al., 2000) and this could explain its dominance on the surface of the Sardinian sausages. In the present survey, none of the isolated *D. hansenii* strains were able to reduce nitrates, whereas all were able to grow in YPD supplemented with 10% NaCl (Data not shown). When the NaCl concentration was raised to 15%, all cultures isolated from the sausages were able to grow, however this was not the case for the one isolated from the minced meat, and 6 of the 10 strains isolated from the casings (Table 2). No strain exhibited proteolytic activity versus casein components, and just one, isolated from the sausages after 21 days of ripening (S_{21}-8), showed proteolytic activity on gelatine agar (data not shown). Regarding lipolytic activity, 5 strains were able to hydrolyse tributyrin and 2 were able to hydrolyse Tween 80. Only two *D. hansenii* strains (S_{21}-8 and Ca_8) showed activity in both tributyrin and Tween 80-based media (Table 2). Results of lipolytic activity on pork fat, obtained by titration, highlighted a high variability within *D. hansenii* cultures. The strain Mm_0-1 isolated from the minced meat proved to be by

far the most active, responsible for producing almost 8% of free fatty acid, expressed as palmitic acid. Controversial results on the proteolytic and lipolytic abilities of *D. hansenii* strains have been previously reported. *D. hansenii* can hydrolyze the sarcoplasmic and myofibrillar proteins of pig muscle (Durá, Flores, Toldrá, 2004; Santos et al., 2001) as well as α- and ß-casein (Breuer & Harms, 2006).

Supplementary Figure 1 reports the growth performances of five strains (S1-5, S21-8, S21-10, Ca2 and Ca3), selected on the basis of their salt tolerance and intermediate lipolytic activities on pork fat in ECM-M broth, a medium that mimic the nutritional conditions prevailing in sausages at the beginning of fermentation. As the figure shows, the best performances were exhibited by *D. hansenii* strain Ca3, which was used for further trials. Competition for nutrients and space has been suggested to be the major mechanism of action of yeasts isolated from foods, including dry-cured ham (Andrade et al., 2014).

3.3. Experimental manufacture of Sardinian sausages

Sausage productions were labelled A, B and C depending on the *D. hansenii* application. It has been well established that the inoculation with *D. hansenii* cells should be made at the beginning of processing, or more specifically at the end of the post-salting stage, when the a_w of the product is still high, that is even close to the optimum value for OTA (Ochratoxin A) production by moulds (Andrade et al., 2014). Moreover, in all cases, *D. hansenii* cells were superficially applied since, OTA produced by moulds occurring on casing, does not generally migrate into the sausage dry meat (Iacumin et al., 2009).

The outcomes related to microbial loads in sausages are collectively summarized in Table 3. First of all, the data clearly confirm the good adaptation of the starter, with LAB populations constantly higher in trials S_1 and S_2. In all case, LAB population increased during ripening

(Table 3). As expected, pH and TTA showed the same trend, with lower values in the trials in which the starter was added (Suppl. Fig. 2).

Addition of the starter also affected the development of pseudomonas, which were progressively less prevalent in trials S1 and S2. Staphylococci on day zero were notably higher in sausages in which the starter was added, but members of this group increased in all trials, and no difference could be noticed between samples at the end of monitoring period (Table 3). As general consideration, microbial loads in the sausages core did not seem to be significantly affected by the mode of *D. hansenii* application. The values detected in sausages A, B and C rarely showed any significant difference. In some cases, however, differences in the microbial counts were revealed between productions A, B and C in trials S_1 and N_1, where no differences should have occurred. On the other hand, it should be stressed that the three independent productions (May, September and January) may be hardly compared.

Yeast counts appeared different after one week, as result of the *D. hansenii* application in trials S_2 and N_2 (Table 3). In the following sampling points, yeast levels increased even in samples of the trials S_1 and N1 in which starter was used alone. The presence of moulds increased at the end of ripening, likely as a result of the reduced metabolic activity of LAB (Dalié, Deschamps & Richard-Forget, 2010). Nonetheless, moulds appeared earlier and attained higher populations levels in the sausages of trial N_1 obtained exclusively by natural fermentation (Table 3). These outcomes did not match those reported by Meftah et al. (2018). According to authors, the growth of *Aspergillus westerdijkiae* and *Penicillium nordicum* (in *in vitro* trials) was slightly stimulated by the use of the commercial starter culture Texel®ELCE Br (Danisco) composed of *Pediococcus pentosaceus*, *Lactobacillus sakei*, *Staphylococcus carnosus*, and *Staphylococcus xylosus* besides *D. hansenii*.

When the yeasts and moulds were counted on the casings, the outcomes appeared soundly diversified as result of the mode of yeast application (Table 4). First of all, yeast loads were

always more than a Log higher in trials S_2 and N_2. Moreover, the best way to treat sausages with yeast appeared to be by surface brushing at the onset of fermentation, i.e., according to manufacture A. Loads were significantly higher when compared with productions coded as B, i.e. by sausages dipping, and C, i.e. by casings treatment before stuffing (Table 4). As matter of fact, the brushing of sausages is even supposed to be the easier approach for yeast inoculation in the production process. Worthy of comment is the anti-mould effect exerted by *D. hansenii* inoculation: independently of the mode of application, moulds were consistently lower in trials S_2 and N_2. As expected, when brushed on the sausages surface (A), the antagonistic effect against moulds was noticed earlier along the ripening (Table 4). In fact, in the case of manufacture A, comparing the results of S_1 and S_2 and N_1 and N_2 at 21 days, an approximate 4 logs reduction in moulds occurred (Table 4). Whereas in the case of productions B and C less than 2 log moulds reduction was recorded (Table 4). Albeit with some differences, mainly referable the food source of isolation (Flores et al., 2017), *D. hansenii* cultures are supposed to be potential aroma enhancers in dry fermented sausages (Flores et al., 2015). As matter of fact, several surveys have focused on the application of different strains within such species for improving volatile compound generation, as well as the quality and sensory properties of several dry fermented sausages (Andrade et al., 2010; Cano-García et al., 2014a, b; Corral, Belloch, López-Díez, Salvador, & Flores, 2017). On the other hand, very few studies have approached this yeast species as a biocontrol agent against fungal and mycotoxin contamination, although processed meat products are frequently affected by OTA contamination. Recently, Iacumin and co-workers (2017) proved the inhibition of ochratoxigenic moulds by selected cultures of *D. hansenii* and *Saccharomycopsis fibuligera* in Speck, a meat product obtained from the deboned leg of pork that is salted, smoked and seasoned for four to six months. By contrast, according to Meftah et al. (2018) the use of *D. hansenii* in meat products seemed to exert varying responses on different ochratoxigenic fungi.

According to the outcomes presented here, the application of *D. hansenii* appeared to be a promising strategy for controlling moulds development in dry Sardinian sausages. Such conclusion was even derived by Simoncini, Virgili, Spadola, and Battilani (2014). In their survey, *D. hansenii* showed the greatest antagonistic activity against *P. nordicum* and proved to be effective in the conditions commonly set in ripening plants. Nevertheless, the authors performed their experiments using a dry-cured pork meat model system, whereas in the present work the impact of the yeast application was performed at the pre-competitive level.

3.4. Sensory analysis

Results obtained by the Paired comparison test were analysed according to the reference tables proposed by the ISO 5495:2005 for p = ½ e α = 0.05 for which a sample may be regarded as preferred if it is chosen by at least 29 assessors out of 50. Results, clearly indicated that none of the samples could be significantly discriminated as, in the first set of tasting (N_1 against A-N_2), 27 out of 50 assessors preferred N_1; in the second (N_1 against B-N_2) 26 out of 50 assessors preferred N_1, and in the third set of tasting (N_1 against C-N_2) 24 out of 50 assessors preferred N_1 (Data not shown). Therefore, in spite of the anti-mould effect, the application of the yeast did not affect the overall quality and typical features of the specific product.

4. Conclusions

This research conducted on fermented Sardinian sausages indicated that the inoculation of an autochthonous *D. hansenii* strain, selected according to its technological features, may provide a positive contribution to control the development of moulds on the sausages' surface. The application of *D. hansenii* at the onset of seasoning improved the safety and quality of sausages and did not affect the typical flavour development.

Moreover, the anti-mould effect exerted by the strain proved to be effective in the conditions commonly set in ripening plants, but unaffected by the way adopted for its application, even if the brushing of cell suspensions on the sausages surface at the beginning of ripening, demonstrated to be the best approach to treat sausages.

As matter of fact, sausages casings are complex matrices able of supporting the growth of moulds that may contribute to flavour, but also of mycotoxins-releasing species. In such light, outcomes here presented may provide a tool to prevent economic losses during dry fermented sausages ripening. Moreover, the convenience and the cost-effectiveness make the approach suitable in both artisan and industrial food industries.

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Authors' contributions

NPM, PD and GB conceived the experimental design. MAM and AM performed all microbial counts and the strains technological characterization. MAM, AM and GB carried out DNA extractions and PCR analyses. NPM and PD followed the sausages' experimental productions. GB managed the data statistical analysis. MA and NPM drafted the manuscript. All authors are individually mentioned, and all authors read and approved the final manuscript.

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Supplementary Figures legend

Suppl. Figure 1

Growth rate in ECM-M medium of five *D. hansenii* selected strains (S1-5, S21-8, S21-10, Ca2 and Ca3). Results reported as mean Log CFU mL^{-1}.

Suppl. Figure 2

Evolution of pH and Titratable Acidity (TTA) during Sardinian sausages ripening.

For trials code refer to Suppl. Figure 1 caption.

Table 1

Yeast loads (Log CFU g^{-1}) and species in minced meat and in sausages during ripening on three batches of Sardinian sausages

	Counts Log CFU g^{-1}	ITS size	320	650	550	620	380	64
		Taxon[b]	*T. domesticum*	*D. hansenii*	*C. parapsilosis*	*C. zeylanoides*	*Y. lipolitica*	*R. mucilla*
d meat	2.70±0.37		3	1	-	-	-	-
S_1[a]	3.10±0.31		2	2	1	2	-	-
S_3	3.95±0.09		-	1	4	2	-	-
S_7	2.76±0.11		-	-	1	4		4
S_{12}	4.77±0.14		-	1	-	-	9	-
S_{21}	4.66±0.11		-	8	-	2	-	-
s	5.27±0.28		-	10	-	-	-	-
		Total of isolates	5	23	6	10	9	4
		Frequency	9%	40%	10%	18%	16%	7%

[a] S_1, S_3, S_7, S_{12} and S_{21}: Sausages after 1, 3, 7, 12 and 21 days of ripening.

[b] Similarity percentages with ITS rDNA sequences available on NCBI GenBank were at least 98%.

Table 2

Technological characterization of the *D. hansenii* strains.

Strain	Growth with 15% NaCl	Lipolytic activity		
		Tributyrin	Tween 80	Pork fat[a]
Mm_0-1	-	-	-	7.95
S_1-2	+	-	-	0.71
S_1-5	+	-	5[b]	1.18
S_3-1	+	-	-	2.53
S_{12}-4	+	-	-	1.11
S_{21}-3	+	-	-	0.00
S_{21}-4	+	-	-	3.55
S_{21}-5	+	-	-	0.00
S_{21}-6	+	-	5	0.84
S_{21}-7	+	-	-	0.00
S_{21}-8	+	5	4	1.50
S_{21}-9	+	-	-	1.33
S_{21}-10	+	-	5	1.63
Ca1	-	-	-	1.34
Ca2	+	-	-	1.25
Ca3	+	-	-	1.20
Ca4	-	-	-	0.94
Ca5	+	-	-	1.01
Ca6	+	-	-	1.17
Ca7	-	-	-	0.21
Ca8	-	4	4	1.22
Ca9	-	-	-	0.92
Ca10	-	-	-	1.18

[a] Data are expressed as % palmitic acid

[b] Halo diameters in mm

Table 3

Evolution of different microbial groups in the core of the product of experimental Sardinian sausages during 21 days of ripening. Standard deviations were lower than 0.7 CFU g^{-1}

		Manufacture A				Manufacture B				Manufacture C			
MG[a]	Samples	Fermentation days				Fermentation days				Fermentation days			
		0	7	15	21	0	7	15	21	0	7	15	21
TVM	S_1[b]	6.5a[c]	7.6b	7.7bc	8.0a	6.7a	8.2a	8.0ab	8.1a	7.0a	8.1a	8.7a	6.2b
	S_2	6.8a	8.0ab	8.2ab	8.0a	6.8a	7.8ab	8.3a	8.0a	7.2a	7.9a	8.8a	8.0a
	N_1	5.1b	8.3a	8.7a	8.0a	4.8b	7.8ab	8.1ab	8.5a	5.8c	8.3a	7.9b	6.0b
	N_2	4.6b	6.2c	7.3c	8.2a	4.9b	7.2b	7.8b	8.2a	6.5ab	6.4b	8.3ab	8.1a
LAB	S_1	6.42a	7.5a	7.9a	8.0ab	6.5a	7.7a	8.0a	8.1a	6.2a	7.7b	8.3a	8.7a
	S_2	6.0ab	7.7a	8.0a	8.2a	6.6a	7.7a	8.0a	8.1a	6.5a	8.3a	8.9a	8.7a
	N_1	4.8c	5.1b	5.4b	6.8ab	4.5b	4.8b	4.9b	6.2b	4.0b	4.4c	5.1b	5.7b
	N_2	5.2bc	5.1b	5.4b	6.5b	4.6b	4.7b	5.0b	6.3b	4.2b	4.5c	5.0b	6.3b
ST	S_1	6.9a	7.2a	6.3a	7.4a	6.6a	7.5a	7.6a	7.7a	7.3a	7.5a	8.1b	7.0a
	S_2	6.8a	7.6a	6.6a	7.5a	6.9a	7.6a	7.9a	7.4a	7.4a	7.6a	8.7a	7.6a
	N_1	4.8b	6.5a	6.4a	7.3a	4.7b	6.4b	7.4a	7.2a	5.0b	6.6b	7.3c	6.8a
	N_2	4.7b	6.1a	6.6a	7.3a	4.6b	6.8ab	7.3a	7.2a	4.8b	6.7b	7.2c	7.4a
Y	S_1	2.2a	3.9b	4.3a	5.3a	2.0a	4.9ab	3.6b	3.4b	2.1a	3.7b	4.8a	3.1b
	S_2	2.3a	5.7a	4.7a	5.2a	2.1a	5.4a	4.5a	5.1a	2.3a	5.7a	4.7ab	3.0b
	N_1	2.4a	4.2b	3.9a	3.7b	2.7a	4.1b	3.8b	2.3b	2.5a	3.3b	4.0b	4.9a

	N_2	2.3a	4.9a	4.4a	4.0a	2.5a	5.2ab	5.1a	5.3a	2.8a	5.1a	3.2c	5.0a
PS	S_1	2.4a	0.7b	0.8a	0.0c	2.1a	0.0b	0.3b	0.0b	2.4a	0.8c	0.0c	0.0b
	S_2	2.3a	0.0b	0.0a	0.0c	2.0a	0.6b	0.3b	0.2b	2.4a	0.3c	0.4c	0.0b
	N_1	2.6a	2.2a	2.9b	1.8b	2.3a	2.3a	3.0a	1.7a	2.4a	2.5b	2.9a	1.8a
	N_2	2.4a	3.7a	3.1b	2.4a	2.9a	4.1a	3.2a	1.7a	2.5a	4.0a	2.5ab	2.1a
M	S_1	0.0a	0.0a	0.0b	1.4bc	0.0a	0.0a	0.0b	2.2ab	0.0a	0.0a	0.0b	0.6c
	S_2	0.0a	0.0a	0.0b	0.9c	0.0a	0.0a	0.0b	1.4ab	0.0a	0.0a	0.0b	1.6b
	N_1	0.0a	0.0a	1.1a	3.1a	0.0a	0.0a	0.8a	2.5a	0.0a	0.0a	0.5a	2.5a
	N_2	0.0a	0.0a	0.0b	1.8b	0.0a	0.0a	0.0b	0.9b	0.0a	0.0a	0.0b	1.6b

[a] MG, microbial groups; TVM, total viable microflora; LAB, Lactic acid Bacteria; ST *Staphylococcus* spp.; PS, *Pseudomonas* spp.; Y, yeasts; M, moulds.

[b] N_1: Natural fermentation; S_1: Starter added; N_2: N_1 plus *D. hansenii* added; S_2: S_1 plus *D. hansenii* added. Productions: A, *D. hansenii* cell suspensions brushed on sausages surface 24 h after drying; B, sausages immersion in a *D. hansenii* suspension after stuffing; C, casings immersion in a *D. hansenii* suspension before stuffing.

[c] Significance using ANOVA and Tukey t-test ($p<0.05$): different letters indicate significant differences, at each time for each microbial group, among samples (S1, S2, N1 and N2) within the same manufacture.

Table 4

Evolution of yeasts (Y) and moulds (M) during Sardinian sausages ripening on the casings surface. Standard deviations were lower than 0.8 CFU g^{-1}

		Manufacture A				Manufacture B				Manufacture C			
	Samples[a]	Fermentation days				Fermentation days				Fermentation days			
		0	7	15	21	0	7	15	21	0	7	15	21
Y	S_1	4.0b[b]	5.2b	6.5b	6.7b	4.0b	5.1b	6.3b	6.5c	3.9b	5.8b	6.3b	5.9b
	S_2	8.3a	9.2a	9.5a	9.5a	7.1a	8.4a	8.3a	8.1ab	5.9a	7.9a	8.1a	8.4a
	N_1	4.0b	5.7b	6.3b	6.7b	3.8b	6.0b	6.3b	6.7bc	4.2b	6.0b	6.3b	6.1b
	N_2	8.3a	9.2a	9.3a	8.9a	7.0a	8.3a	8.1a	8.4a	6.1a	7.6a	7.7a	7.8a
M	S_1	3.1a	6.4a	6.1a	6.3a	3.2a	7.0a	7.2a	7.3a	3.1a	7.2a	7.6a	7.2a
	S_2	2.0a	2.5b	2.2b	2.6b	3.1a	2.9b	3.3b	5.5b	3.1a	3.9b	3.8b	5.3b
	N_1	3.5a	6.4a	6.2a	6.7a	2.8a	6.9a	6.5a	6.7a	3.3a	7.4a	7.5a	7.0a
	N_2	1.8a	2.2b	2.0b	2.9b	3.4a	3.8b	3.7b	5.7b	3.6a	4.2b	3.8b	5.2b

[a] For samples code see Table 3.

[b] Significance using ANOVA and Tukey t-test ($p<0.05$): different letters indicate significant differences, at each time for each microbial group, among samples (S1, S2, N1 and N2) within the same manufacture.

Highlights

- *Debaryomyces hansenii* proved to be the dominant yeast species in Salsiccia Sarda
- Isolated yeast cultures were characterized for features of technological interest
- Strain Ca3 was selected and used in 3 experimental sausages productions at farm scale
- The yeast exerted an anti-mould effect, independently by the mode of application
- The yeast application did not affect the overall quality and features of the product