Screening of *Debaryomyces hansenii* strains for flavor production under reduced concentration of nitrifying preservatives used in meat products

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Abstract

Fifteen *D. hansenii* strains from different food origins were genetically characterised and tested on a culture medium resembling the composition of fermented sausages but different concentrations of nitrifying preservatives. Genetic typing of the *D. hansenii* strains revealed two levels of discrimination, isolation source or strain specific. Different ability to proliferate on culture media containing different concentrations of nitrate and nitrite, as sole nitrogen sources and in presence of amino acids, was observed within *D. hansenii* strains. Overall metabolism of amino acids and generation of aroma compounds was related to the strains origin of isolation. The best producers of branched aldehydes and ethyl ester compounds were strains isolated from pork sausages. Strains from cheese and llama sausages were good producers of ester compounds and branched alcohols, while vegetable strains produced mainly acid compounds. Nitrate and nitrite reduction affected in different ways the production of volatiles by *D. hansenii*.

Keywords: yeast; aroma; nitrite; nitrate; amino acid; *Debaryomyces hansenii*.

INTRODUCTION

In recent years, consumer preferences on a healthy diet demand healthier food. Despite the role of nitrites and nitrates on food safety as well as conferring desirable technological properties to meat products,¹ the actual trend is to reduce the use of nitrites and adjust their levels.²

Reduction of nitrifying agents in meat products poses two main problems: alteration in metabolism of microbiota favoring growth of undesirable microorganism, and variation in aroma.^{3, 4} During sausage fermentation, metabolism of lactic acid bacteria starters causes fermentation of carbohydrates which produces lactic acid and other aroma compounds such as diacetyl, acetaldehyde, ethanol, acetic, and propionic acids among others⁵. Other microbial groups added as starters, such as coagulase negative staphylococci (CNS), are essential for color and flavor development by degradation of branched chain amino acids and fatty acids, production of ester compounds and branched aldehydes, and production of methyl ketones through beta-oxidation pathways.⁶ Moreover, nitrate addition has been linked to an increase in the level of ester compounds derived from amino acid degradation and carbohydrate fermentation in fermented sausages.^{7,8}

Conversion of free amino acids, generated through proteolysis during sausage manufacturing⁹, into aroma compounds depends largely on microbial metabolism in which *D. hansenii* plays an important role.¹⁰ Lücke¹¹ reported that *D. hansenii* protected against the detrimental effect of oxygen during sausage processing and regulated the drying process. Other effects described of *D. hansenii* in meat fermentation were pH increase, lactate utilization and generation of aroma compounds.¹⁰ Moreover, inoculation of *D. hansenii* as starter culture has been used as strategy to improve aroma in fat and salt reduced sausages.¹² In this study, the authors confirmed the antioxidant effect of *D. hansenii* as well as an increase in lipolysis and generation of aroma compounds derived

from amino acid degradation. Several studies using meat models have shown the ability of particular *D. hansenii* strains to metabolize branched amino acids and produce volatile compounds (VOCs) (branched aldehydes, alcohols and acids).¹³⁻¹⁵

The aromatic characteristics of fermented sausages depend mainly on the processing factors (meat ingredients, preservatives, technological parameters, presence of starter cultures). Variations in these factors, such a decrease in the concentration of nitrifying agents, can affect the metabolic activity of *D. hansenii* and the potential of this yeast as contributor to flavor in meat products. Therefore, the selection of a *D. hansenii* strain which generation of volatile compounds is positively affected by a reduction of nitrates and nitrites in meat products must be investigated.

The objective of this study was the screening of *D. hansenii* strains for their potential as producers of volatile compounds in meat products with reduced concentrations of nitrate and nitrite. The selection of the best *D. hansenii* strain was carried out within a group of genetically well characterized strains isolated from several food fermentations. The strains were tested by their ability to grow on media containing different concentrations of nitrate and nitrite, metabolism of amino acids and production of volatile compounds.

MATERIAL AND METHODS

***D. hansenii* strains.** *D. hansenii* strains (L1 to L15) used in this study were isolated from different dry fermented pork and llama sausages, cheese and vegetables (Table 1).

DNA isolation and strain typing by PCR-M13 and inter-LTR fingerprinting of *D. hansenii* strains. *D. hansenii* strains were cultured overnight on GPY medium (2% w/v glucose, 0.5% w/v peptone and 0.5% w/v yeast extract) at 25 °C. DNA was extracted as

76 reported by Querol, Barrio and Ramon²¹ and dilutions containing about 10 ng/μL prepared
77 using a Nanodrop spectrophotometer ND-1000 (Thermo-Fisher Scientific, USA).

78 PCR-M13 amplification using the minisatellite M13 primer (5' GAGGGTGGCGGTTCT3')
79 was performed as described in Cano-García et al.¹⁸ PCR products were resolved by
80 electrophoresis on 2 % agarose gel in 1xTAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH
81 8) at 80 V for 3 h, stained with RedSafe (INTRON Biotech., Spain) and visualized under UV
82 light. Inter-LTR PCR was carried out as described by Sohier et al.²² using DH8 (5'-
83 CTCAATTTATTCTGACTTCGC-3') and DH9 (5'-GATTGTTGTTGAAGCTAT CATTGG-3')
84 primers. PCR products were separated on 2% (w/v) agarose gel in 1xTAE buffer at 60 V for
85 3 h, stained with RedSafe (INTRON Biotech., Korea) and visualized under UV light. DNA
86 fragment sizes were determined using a 100 bp DNA ladder (Life Technologies, USA).
87 Reproducibility of the techniques was verified using internal controls of few strains which
88 were included in all PCR DNA amplifications and electrophoresis.

89

90 **Screening of *D. hansenii* for nitrate and nitrite tolerance on minimum medium with**
91 **or without amino acids.** *D. hansenii* strains were cultured overnight on GPY medium
92 (glucose 2%, yeast extract 0.5%, peptone 0.5%) at 25 °C. After growth each culture was
93 three times washed in physiological saline solution. Cells were collected by centrifugation at
94 3220 g for 5 min at 25 °C and incubated 2 d at 25 °C on minimum medium (0.6 % YNB,
95 Yeast Nitrogen Base without amino acids, 3% sodium chloride and 1% glucose) to deplete
96 cellular storage of amino acids. After growth, cells were collected by centrifugation as
97 above, adjusted to an absorbance of 0.3 at 600 nm in a BioPhotometer (Eppendorf,
98 Germany) and 5-fold dilution series prepared in saline solution. Growth of *D. hansenii*
99 strains on nitrate and nitrite was tested on plates of minimum medium (YNB) or minimum
100 medium supplemented with amino acids (YNBa) simulating the composition of dry

101 fermented sausages.²³ Media were supplemented with 150 mg/L (C), 128 mg/L (RN15) or
102 113 mg/L (RN25) of nitrate, nitrite or a mixture of both (1:1) and sterilized by filtration (0.22
103 µm). Sterile agar was added to a final concentration of 2% and media poured into plates.
104 Five milliliters dilutions from each cell suspension dilution series was spotted on each
105 media. Plates were incubated aerobically at 25 °C and checked 1 to 5 consecutive d for
106 growth. Growth was evaluated as 0 (negative growth) or 1 (growth of not diluted cell
107 suspension) to 6 (growth of the 10⁻⁵ cell dilution). Experiments were carried out in triplicate.
108

109 **Growth of *D. hansenii* on media for volatile production.** *D. hansenii* cultures were
110 prepared as above. Cell suspensions were adjusted for inoculation at a final concentration
111 of 10⁶ cells/mL. Control medium (C) composition was 0.67 % YNB without amino acids, 150
112 mg/L NaNO₂ and 150 mg/L KNO₃, 30 g/L NaCl, 10 g/L glucose and amino acids in
113 concentration simulating the composition of dry fermented sausages reported by Corral et
114 al.²³ The European regulation allows the use of both, nitrite and nitrate, at a concentration
115 of 150 ppm each in the category of meat products ²⁴. This is the most usual concentration
116 used for the elaboration of meat products around Europe although specific regulations for
117 traditional European meat products are indicated. Composition of media with reduced
118 nitrifying agents was the same as the control medium except for concentration of nitrifying
119 agents (NaNO₂ and KNO₃) that was reduced to 15 % in RN15 (128 mg/L) and 25 % in
120 RN25 (113 mg/L). Media were sterilized using a vacuum-driven filtration system (0.22 µm).
121 A total of 17 experiments (50 mL in 100 mL Erlenmeyer flasks) were carried out using each
122 media C, RN15 and RN25 (51 in total). Fifteen flasks were inoculated with *D. hansenii*
123 strains and two flasks were not inoculated and used as controls before and after incubation.
124 Cultures were incubated at 20 °C during 16 d. Each experiment was carried out in triplicate.
125 After incubation, media were centrifuged at 3220 g for 2 min at 20 °C. Supernatant was

recovered and frozen at -20 °C until volatile analysis, amino acid content and nitrite and nitrate residual measurements. Pellet was washed in saline solution and used for OD measurement at 600 nm in a BioPhotometer (Eppendorf, Germany).

Amino acid content analysis. Free amino acids content in media supernatant was determined according to Aristoy & Toldrá²⁵ using norleucine (65.6 µg) as internal standard (IS). Phenylthiocarbamyl amino acids derivatives were analyzed by reversed-phase HPLC (1200 Series Agilent chromatograph; Agilent, Palo Alto, CA, USA) using a Waters Nova Pack C18 column (3.9 x 300 mm) (Waters Corporation, Milford, USA) and ultraviolet detection (254 nm) as described by Flores, Aristoy, Spanier, & Toldrá.²⁶ Quantification of amino acids was done relative to the IS and expressed as a percentage of concentration present in the control media before incubation. Amino acids β-alanine and taurine were excluded from the analysis.

Determination of Nitrite and nitrate concentration. Nitrate and nitrite content in the supernatant was determined using a colorimetric assay enzymatic kit (Nitrite/Nitrate Assay Kit, Sigma-Aldrich, MO, USA) used for plasma and culture medium.²⁷ Supernatants were diluted to ensure measurement within the linear range of the curve.

Profile and quantification of volatile compounds. Chemical compounds used for volatile compound identification and quantification were all obtained from Sigma-Aldrich (Steinheim, Germany). Analysis of volatile compounds was carried out using 7 mL aliquots of media supernatants into 20 mL headspace vials (Gerstel, Germany). Volatile compounds were extracted by SPME headspace (HS) analysis using an automatic injector Gerstel MPS2 multipurpose sampler (Gerstel, Germany) as described in Cano-García et al.¹⁵. Vial

151 was equilibrated at 37 °C for 15 min and then, 85 µm SPME fiber (CAR/PDMS) was
152 exposed to HS during 2 h at 37 °C while shaking at 250 rpm. Volatile compounds were
153 desorbed at the injection port of GC/MS (HP 7890A/5975C) (Hewlett Packard, Palo Alto,
154 CA) for 5 min at 240 °C (in splitless mode) and separated using a DB-624 capillary column
155 (30 m x 0.25 mm x 1.4 µm, J&W Scientific, Agilent Technologies, USA). Retention indices
156 (LRI) of volatile compounds were calculated using the series of n-alkanes (Aldrich,
157 Germany). Compounds were identified by comparison with mass spectra from the
158 NIST/EPA/NIH Mass Spectral Database, linear retention index²⁸ and by comparison with
159 authentic standards. Authentic samples of the compounds were obtained and analyzed
160 under the same GC-MS conditions to provide LRI values. Identified volatile compounds
161 were quantified in SCAN mode using either total or extracted ion chromatogram (TIC or
162 EIC) on an arbitrary scale. Abundance of volatile compounds (TIC x 10⁵) is expressed as
163 the increase respect to control media after incubation. Each media supernatant was
164 analyzed in triplicate.

165

166 **Statistical analysis.** Data were analyzed using Generalized Linear Model (GML)
167 procedure of statistical software XLSTAT 2011 v5.01 (Addinsoft, Barcelona, Spain). The
168 model includes the effect of yeast inoculation as fixed effect and replicates as random
169 effects. Additionally, the effect of the curing agent was also analyzed in each yeast
170 inoculated media. When significant effect of the treatment group was detected ($P < 0.05$),
171 least squares means (LSM) were compared using Tukey's test. In addition, principal
172 component analysis (PCA) was performed to evaluate the relationships among aroma
173 compounds and model inoculated media. Pearson correlation analysis was performed
174 between amino acids and derived volatile compounds. Heatmaps of volatile profiles and

amino acid degradation in the yeast inoculated media were calculated relative to the concentration in the control media before incubation.

RESULTS

Fifteen yeast strains from food origin pertaining to the species *D. hansenii* were genetically characterized, and screened for their ability to grow and produce volatiles on amino acid rich media containing nitrifying agents used as preservatives in meat products.

Genetic typing of *D. hansenii* strains isolated from food. Electrophoretic patterns of PCR amplification products of PCR-M13 or inter-LTR fingerprinting appear in Figure 1. Genetic typing of the *D. hansenii* strains revealed two levels of discrimination depending on the technique. PCR-M13 grouped the strains by isolation origin whereas inter-LTR fingerprinting generated strain-specific patterns. PCR-M13 patterns appeared to be very similar for *D. hansenii* strains isolated from sausages (pork and llama) and vegetables. Despite their similarity, few differences on the presence or absence of bands could be observed. Among the strains isolated from pork sausages the most different seemed to be L3. Strains isolated from llama sausages were very homogeneous, whereas within the strains isolated from vegetables strain L13 was different from L14 and L15. The most heterogeneous patterns overall seemed to be the ones of *D. hansenii* isolated from cheese. On the other hand, inter-RTL patterns revealed a higher level of genetic diversity among *D. hansenii* strains than PCR-M13, and no similarities could be observed between patterns of strains from the same food origin.

Screening of *D. hansenii* strains for growth on minimum medium with or without amino acids containing nitrate and nitrite- Large differences regarding growth of *D.*

200 *hansenii* strains on plates of minimum medium with or without amino acids and containing
201 nitrate or/and nitrite could be observed (Table 2). No strain was able to proliferate on
202 minimum medium without amino acids using nitrate as sole nitrogen source. However,
203 nitrate did not inhibit growth of *D. hansenii*, as all strains were able to grow at any
204 concentration of nitrate on minimum medium with amino acids. Nitrite utilization as sole
205 nitrogen source was variable in the *D. hansenii* strains investigated. Only five *D. hansenii*
206 strains were able to grow on minimum medium without amino acids containing nitrite or a
207 mixture of nitrate and nitrite. Four of them were isolated from meat sausages and one
208 isolated from Lupin beans. Nevertheless, nitrite or mixtures of both nitrifying agents did not
209 seem to inhibit growth of any *D. hansenii* strain, as all strains were able to grow on
210 minimum medium with amino acids at any of the concentrations tested.

211

212 **Growth of *D. hansenii* strains on media for volatile production.** Growth of *D.*
213 *hansenii* strains in control medium C and media with reduced nitrifying agents (nitrite and
214 nitrate) RN15 and RN25 is shown in Figure 2. After 16 d of incubation at 20°C, yeast counts
215 were above 10⁸ cells/mL. Most strains showed a tendency to aggregate or flocculate;
216 therefore, OD measuring was difficult and deviation between replicates was large.
217 Nevertheless, reduction of nitrate and nitrite curing agents as in media NR15 and NR25
218 seemed to favor growth of most *D. hansenii* strains except for L1, L3 and L11.

219

220 **Determination of amino acids and residual nitrate and nitrite content in media for**
221 **volatile production.** Concentration of each amino acid in media C, RN15 and RN25 after
222 16 d incubation of *D. hansenii* yeasts is shown in Figure 3. Methionine, tryptophan and
223 lysine seemed to be metabolized by the majority of yeasts and in larger quantities than
224 other amino acids. Similarly, valine, isoleucine, leucine and phenylalanine were partially

225 consumed by the majority of yeasts. On the contrary, glycine and tyrosine were hardly
226 metabolized by any yeast except L13. Comparisons between yeasts showed that strain
227 L13, isolated from Lupin beans, was the one with the highest metabolic activity as observed
228 by the nearly total consumption of most amino acids in all media. Moreover, other strains
229 isolated from vegetables (L14 and L15) displayed higher degree of amino acid metabolism
230 than strains from other food. Among the remaining yeasts, strains isolated from pork meat
231 products, displayed overall a higher degree of amino acid metabolism than strains isolated
232 from llama meat sausages. Excluding the already mentioned, some amino acids were
233 preferentially metabolized by individual yeasts. Among the strains isolated from pork
234 sausages L1, L2, L4 and L5 preferentially used serine, asparagine and glutamine. From the
235 strains isolated from cheese, strain L10 metabolized histidine whereas L11 metabolized
236 preferentially aspartic acid, glutamic acid and proline. Regarding the effect of nitrifying
237 agents' reduction on amino acid metabolism, a decrease in nitrifying agents increased
238 amino acid metabolism in some yeasts (L2), whereas in other yeasts (L1) the contrary was
239 observed.

240 Measurement of nitrate and nitrite concentration after incubation (data not shown)
241 showed that nitrate was not consumed by *D. hansenii*, whereas media were completely
242 depleted of nitrite.

243

244 **Generation of volatile compounds.** *D. hansenii* yeasts produced an important number
245 of flavor compounds resulting from catabolism of amino acids, sugar metabolism as well as
246 from other reactions on all media. Tables 1 and 2 included as Supplementary Material
247 report the identification and quantification of compounds. A summary of the most relevant
248 volatile compounds produced by *D. hansenii* strains on the three culture media tested (C,
249 RN15 and RN25) is shown in Figure 4. Volatiles appear grouped by their amino acid of

250 origin. Metabolism of valine, threonine, isoleucine, leucine, phenylalanine and methionine
251 produced most of the volatile compounds identified. Overall, few compounds were
252 produced by the majority of yeasts, but among them, 2-methyl propanol, 2-methyl-butanol
253 and 3-methyl-butanol were produced in large quantities. Other compounds such as ethanol,
254 propyl propanoate, 2-methyl butanoic acid, and benzaldehyde were also produced by most
255 yeasts but in minor abundance. A general relationship (Pearson Correlation Coefficient $r <$
256 0.05) between volatile compound production and metabolism of its corresponding amino
257 acid could not be found except in case of 3-methyl butanoic acid and leucine (Table 3,
258 Supplementary Material).

259 Specific differences on production of volatile compounds by individual yeasts or groups
260 of yeasts could be observed (Figures 4 & 5). In terms of volatile compounds production, *D.*
261 *hansenii* from cheese and llama sausages (right side in Figure 5) were characterized by the
262 production of ester compounds and branched alcohols. On the upper left side (Figure 5),
263 yeasts from vegetables produced branched and linear acid compounds. *D. hansenii* from
264 pork sausages (bottom left Figure 5), with the exceptions of L3 and L6, were the best
265 producers of branched aldehydes and ethyl ester compounds. The best producer of valine
266 derived 2-methylpropanal as well as isoleucine and leucine derived 2- and 3-methylbutanal
267 was strain L1 (Figure 4). Comparatively, strain L5 was the best producer of ethyl-2- and
268 ethyl-3-methylbutanoate. *D. hansenii* strains from llama sausages and cheese, with
269 exclusion of L11, produced the largest amounts of ethanol, ethyl acetate and propanol
270 derived compounds. Phenylethyl derived compounds were preferentially produced by
271 strains from cheese origin. Strains L10 and L12 were the highest producers of 2-
272 phenylethyl acetate in all media, whereas strain L11 was the best producer of phenylethyl
273 alcohol (Figure 4). Strains L3 and L6 outside the main group from pork sausages, were
274 characterized by generation of large amounts of acetic acid and ethanol (Figure 5). Strains

275 form vegetables produced large amounts of 2-methyl- and 3-methylbutanoic acids. Dimethyl
276 disulphide, derived from methionine, was mainly produced by yeasts of pork sausage origin.
277 Strains L11 and L13 produced less volatile compounds than any other *D. hansenii* strains
278 (Table 2, Supplementary Material).

279 The reduction in concentration of nitrifying agents in media RN15 and RN25 had a
280 significant impact on generation of volatile compounds in several strains. Strain L1 was
281 positively and significantly affected in production of branched aldehydes (2-methylpropanal
282 and 2- and 3-methylbutanal) by reduction of nitrifying agents RN15 and RN25 (Figure 4)
283 (upper left side Figure 6) respect to the C medium (center left side Figure 6). Moreover, this
284 strain produced also the highest amounts of 2-methylpropanol and 2-methylpropanoic acid
285 in media containing reduced concentration of nitrifying agents (Figure 4). Similarly, a
286 reduction on nitrifying agents increased the production of ethyl branched ester compounds
287 (ethyl 2-methyl- and ethyl-3-methylbutanoate) in strain L5 (bottom left side Figure 5). An
288 increase of acetic acid production was observed in several strains (L1 and L7) as nitrifying
289 agents' concentration in the medium decreased. On the right side (Figure 6) strains L7
290 showed a slight increase in production of acetic acid as concentration of nitrifying agents
291 decreased. Strain L10 generated lower abundance of ethyl propanoate and strain L7
292 generated more 3-methylbutanoic acid as concentration of nitrifying agents decreased
293 (Figure 4).

294

295 **DISCUSSION**

296 The effect of nitrite as growth inhibitor for undesirable bacteria in meat is well known;
297 however, few studies have focused on the ability of yeasts to tolerate this compound.^{29, 30}
298 Classical studies on nitrate and nitrite tolerance by yeast are usually done in absence of
299 amino acids. In those conditions, *D. hansenii* is not able to use nitrate, but some strains are

300 able to grow on nitrite.³¹ Our results regarding *D. hansenii* growth on media plates
301 containing nitrate as sole nitrogen source confirmed previous findings. Moreover, most of
302 the strains able to grow on nitrite were isolated from meat products.

303 In meat products, *D. hansenii* grows in presence of free amino acids and salt in addition
304 to nitrifying agents (nitrate and nitrite). Estimation of *D. hansenii* growth on the media
305 confirmed the ability of this yeast to proliferate on amino acid rich media containing nitrate
306 and nitrite. A general correspondence between nitrifying agents' reduction and yeast cell
307 growth was not observed in this study, although some yeasts experimented a significant
308 increase in population on media RN15 and RN25. Nitrate and nitrite efflux has been
309 elucidated in *Hansenula polymorpha*, a close relative of *D. hansenii*.²⁹ These studies have
310 shown a complex system in which the presence of nitrate reductase is not necessary for
311 nitrate uptake as well as several yeast permeases able to excrete nitrate and nitrite. On that
312 ground, further research seems necessary to elucidate the influence of a reduction in nitrate
313 and nitrite concentration on *D. hansenii* growth in meat products.

314 Generation of aroma compounds in yeasts depends largely on the amino acids present
315 which are assimilated and transformed by the Ehrlich pathway.³² In case of *S. cerevisiae*
316 wine yeasts, it has been demonstrated that different patterns of nitrogen (ammonium and
317 amino acids) consumption lead to different volatile compounds yields at the end of
318 fermentation.³³ Moreover, Gutierrez, Beltran, Warringer, and Guillamon³⁴ thoroughly
319 demonstrated that amino acid utilization varies between yeasts strains. In agreement with
320 these authors, our results on *D. hansenii* revealed that the majority of amino acids were
321 differentially metabolized by individual strains and only a few seemed to be partially or
322 thoroughly consumed by all yeasts (Figure 3). Among the amino acids partially consumed
323 by all *D. hansenii* strains, threonine, leucine, isoleucine, phenylalanine and valine are the
324 precursors of most volatile compounds generated in the media (Figure 4). A similar result

325 regarding consumption of these amino acids and volatile compounds generation was found
326 by Beltran et al³³ in conditions of nitrogen rich wine fermentations.

327 Comparisons between *D. hansenii* strains showed loose patterns of amino acid
328 consumption associated to groups of yeasts isolated from the same food. However,
329 exceptions such as strain L13, which metabolized exhaustively all amino acids and was
330 among the worst volatile producers, were also found. A similar level of disparity between
331 strains regarding amino acid use has not been observed in case of *S. cerevisiae* because
332 most studies are based on results from one or few strains.³³

333 The remarkable ability of *D. hansenii* to generate desirable volatile compounds in meat
334 products has been demonstrated.^{15,12,10} In terms of aroma contribution to dry-fermented
335 meat sausages, ester compounds have been identified as the source of fruity aromas for
336 masking rancid and vegetable cooked odours.³⁵ Likewise, the positive contribution of
337 branched aldehydes to the overall flavor of fermented meats has also been reported⁶. The
338 ability of *D. hansenii* to produce these volatiles was observed principally in strains isolated
339 from pork sausages (L1 and L5). Nevertheless, some of the strains situated on the right
340 side of Figure 5 (L7 to L9, L10 and L12) are also remarkable producers of ester compounds
341 and could be used to contribute specific flavor notes to meat fermentations. Among these
342 strains, the aroma generation potential of the ones isolated from llama sausages were, until
343 now, unknown. On the other hand, the ability for aroma production of cheese *D. hansenii*
344 strains has been already studied.^{36,37} Using a media emulating the composition of cheese,
345 Padilla et al³⁶ found differences in volatile production by *D. hansenii* strains isolated from
346 cheese. These results are in agreement with the ones found in the present study. Similar
347 differences in the ability to produce esters, branched aldehydes and sulphur compounds
348 was observed between *D. hansenii* strains isolated from pork sausages.^{15,18} Finally, very
349 little is known about *D. hansenii* isolated from fermented vegetables. Some studies have

350 linked the spoilage of fermented cucumbers during storage to a secondary fermentation by
351 yeast.³⁸ These yeasts are characterized by production of acetic, propionic and branched
352 acids, very similar to the volatile produced by strains L13 to L15.

353 Trends to reduce the use of nitrites and adjust their levels in meat products² has made
354 necessary a revaluation of the microbial impact to the meat fermentation process in terms
355 of volatile compounds generation. The catabolism of leucine and production of 3-
356 methylbutanoic acid by *Staphylococci* in an experimental model system was affected by the
357 presence of nitrate and nitrite.⁷ Moreover, the authors found that the influence of nitrate and
358 nitrite depended on the *Staphylococcus* strain analyzed. On the contrary, addition of nitrate
359 did not affect the ability of *Penicillium nalgiovensis* to produce volatile compounds when
360 assayed in sausage models.³⁹ Research from our group has shown the important
361 contribution of *D. hansenii* to the aromatic quality of fermented meat products.^{12,15} However,
362 previous studies indicated that nitrates and nitrites are an important stress factor for yeast
363 growth, even determining different fermentation patterns.^{30,40} The significance of a
364 reduction in nitrate and nitrite concentration in meat products for the ability of *D. hansenii* to
365 generate volatile compounds has not been previously investigated. Our results (Figure 4)
366 showed significant differences between volatile compounds generated in media RN15 and
367 RN25 respect to the control C. These differences were not observed in all *D. hansenii*
368 strains but only in a few (Figure 4 & 6). In these strains, a reduction of nitrifying agents in
369 the medium was not always accompanied by an increase in a volatile compound
370 concentration, but neutral or negative effects were also observed. The ability of strain L1 to
371 generate volatile compounds (2-methylpropanal, 2-methyl- and 3-methylbutanal) clearly
372 increased as nitrifying agents' concentration decreased in the medium (Figure 4). In case of
373 strain L5, a reduction of nitrifying agents' concentration in the medium did not cause any
374 significant effect on the ability of this strain to generate volatile compounds (Figure 4);

375 although a small positive effect on production of ethyl esters was observed (Figure 6).
376 Differences in volatile compounds production are not explained by differences in yeast
377 growth on RN15 and RN25 respect to medium C (Figure 2). In case on strain L1 the clear
378 increase in generation of some volatile compounds (Figure 6) did not correspond with an
379 increase in population (Figure 2). Similar comparisons for other yeasts also offered
380 contradictory correspondences.

381 The *D. hansenii* strains examined in this study have been isolated from fermentative
382 processes for food production. These fermentations are characterized by a high NaCl
383 content and abundance of free amino acids as well as by an initial step carried out by lactic
384 acid bacteria that causes a drop in pH by production of organic acids.⁴¹ The source of
385 isolation of these strains determined their different PCR-M13 patterns, as well as general
386 differences in metabolism of amino acids and production of volatiles. Nevertheless, the
387 uniqueness of some strains regarding volatile compounds generation was comparable to
388 the different patterns displayed by inter-LTR fingerprinting (Figure 1).

389 In summary, fifteen *D. hansenii* strains isolated from food fermentations were
390 investigated by their ability to metabolize amino acids and produce volatile compounds on
391 media resembling the composition of a dry fermented sausage in salt and amino acids but
392 differing in nitrate and nitrite content. On media containing regular amounts of nitrate and
393 nitrite the ability of the strains to metabolize amino acids and produce volatile compounds
394 was influenced by their origin of isolation. Generation of aroma compounds desirable in
395 fermented meat products, esters and branched aldehydes, were preferentially generated by
396 strains isolated from pork sausages. Among these strains, L1 and L5 appeared to be the
397 best producers of these compounds. Regarding *D. hansenii* strains from other origins, L7
398 (llama sausages), L10 and L12 (cheese) showed potential for production of branched ethyl
399 esters. The effect of nitrifying agents' reduction on generation of volatile compounds

400 depended largely on the yeast strain. Strains L1 and L5 seemed to be the best suited
401 strains for utilization as starters in meat fermented products with reduced content of these
402 additives however, this fact has to be confirmed in real fermented sausages. Genetic
403 differences between L1 and L5 strains were only uncovered by inter-LTR fingerprinting.
404 Finally, the metabolic pathways explaining the differences found between *D. hansenii*
405 strains on nitrate and nitrite tolerance, amino acid utilization and volatiles generation are the
406 subject of future investigations.

407

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411

412 **Supporting Information Available:**

413 **Tables 1 Supplementary Material.** Volatile compounds identified in yeast inoculated
414 control medium (C) and media with reduced nitrifying agents content after incubation at 20
415 °C for 16 d.

416 **Table 2 Supplementary Material.** Quantification of volatile compounds in yeast inoculated
417 control medium (C) and media with reduced nitrifying agents content after incubation at 20
418 °C for 16 d.

419 **Table 3 Supplementary Material.** Pearson Correlation Coefficients (r) of volatile
420 compounds and amino acids present in yeast inoculated control medium (C) and media
421 with reduced nitrifying agents content after incubation at 20 °C for 16 d.

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540 **Figure captions**

541 **Figure 1.** Electrophoretic patterns of PCR-M13 (A) and inter-RTL PCR fingerprints (B) of
542 *D. hansenii* strains used in this study.

543 **Figure 2.** Population of yeast strains (cells/mL x 10⁸) in control medium (C) containing
544 150 ppm sodium nitrite and 150 ppm potassium nitrate and media with nitrifying agents
545 (NaNO₂ and KNO₃) reduced to 15 % in RN15 (128 mg/L) and 25 % in RN25 (113 mg/L).

546 **Figure 3.** Heatmap showing the concentration of amino acids present in control medium
547 (C) containing 150 ppm sodium nitrite and 150 ppm potassium nitrate and media with
548 nitrifying agents (NaNO₂ and KNO₃) reduced to 15 % in RN15 (128 mg/L) and 25 % in
549 RN25 (113 mg/L), after incubation at 20 °C for 16 d. Data is relative to the internal standard
550 and expressed as a percentage of concentration present in control media before incubation
551 (100%).

552 **Figure 4.** Heatmap showing the concentration of volatile compounds generated by yeast
553 inoculated in control medium (C) containing 150 ppm sodium nitrite and 150 ppm potassium
554 nitrate and media with nitrifying agents (NaNO₂ and KNO₃) reduced to 15 % in RN15 (128
555 mg/L) and 25 % in RN25 (113 mg/L), after incubation at 20 °C for 16 d. Concentration is
556 expressed as abundance of compound (Area TIC x 10⁻⁵) in the media. * Indicate significant
557 differences (p<0.05) between media C, RN15 and RN25.

558 **Figure 5.** Loadings of the first two principal components (F1–F2) of control yeast
559 inoculated media (●), and volatile compounds derived from amino acid degradation and
560 yeast metabolism (♦).

561 **Figure 6.** Loadings of the first two principal components (F1–F2) of yeast inoculated
562 media (●) in control medium (C) containing 150 ppm sodium nitrite and 150 ppm potassium
563 nitrate and media with nitrifying agents (NaNO₂ and KNO₃) reduced to 15 % in RN15 (128

564 mg/L) and 25 % in RN25 (113 mg/L), and volatile compounds derived from amino acid
565 degradation and yeast metabolism (♦).

566

Table 1. List of *Debaryomyces hansenii* strains used in this study.

Yeast	Isolation Source	Reference
L1	Pork dry fermented sausage	Bolumar et al., ¹⁶
L2	Pork dry fermented sausage	Durá et al., ¹⁷
L3	Pork dry fermented sausage	Cano-García et al., ¹⁸
L4	Pork dry fermented sausage	Cano-García et al., ¹⁸
L5	Pork dry fermented sausage	Cano-García et al., ¹⁸
L6	Pork dry fermented sausage	Cano-García et al., ¹⁸
L7	Llama dry fermented sausage	Mendoza et al., ¹⁹
L8	Llama dry fermented sausage	Mendoza et al., ¹⁹
L9	Llama dry fermented sausage	Mendoza et al., ¹⁹
L10	Matured Cheese	Padilla et al., ²⁰
L11	Matured Cheese	Padilla et al., ²⁰
L12	Matured Cheese	Padilla et al., ²⁰
L13	Brine preserved Lupin beans	unpublished
L14	Vinegar preserved fermented cucumber	unpublished
L15	Vinegar preserved corn	unpublished

Table 2. Growth of *D. hansenii* strains (0 means negative growth; values from 1 to 6 means the log of diluted cell suspension growth up to 10⁻⁵ dilution) on minimum solid medium without (YNB) or with (YNBa) amino acids containing different concentrations of nitrate or/and nitrite.

Strains	YNB Nat ^a			YNB Nit ^a			YNB Nat-Nit ^a			YNBa Nat			YNBa Nit			YNBa Nat-Nit		
	C ^b	RN15	RN25	C	RN15	RN25	C	RN15	RN25	C	RN15	RN25	C	RN15	RN25	C	RN15	RN25
L1	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6	6	6
L2	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6	6	6
L3	0	0	0	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
L4	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6	6	6
L5	0	0	0	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6
L6	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6	6	6
L7	0	0	0	6	6	6	6	6	6	5	6	6	6	6	6	6	6	6
L8	0	0	0	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
L9	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6	6	6
L10	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6	6	6
L11	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6	6	6
L12	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6	6	6
L13	0	0	0	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
L14	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6	6	6
L15	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6	6	6

^a Nat, nitrate; Nit, nitrite; Nat-Nit, nitrate and nitrite.

^b Control medium (C) containing 150 ppm sodium nitrite and/or 150 ppm potassium nitrate, media with nitrifying agents (NaNO₂ and KNO₃) reduced to 15 % in RN15 (128 mg/L) and 25 % in RN25 (113 mg/L).

Figure 1 Flores et al. 2017

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

TOC Graphic