DEBARYOMYCES HANSENII METABOLISM OF SULFUR AMINO ACIDS AS PRECURSORS OF VOLATILE SULFUR COMPOUNDS OF INTEREST IN MEAT PRODUCTS

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1 **ABSTRACT**

2 The ability of *Debaryomyces (D.) hansenii* to produce volatile sulfur compounds

3 from sulfur amino acids and the metabolic pathway involved has been studied in

4 seven strains from different food origins. Our results proved that L-methionine is

5 the main precursor for sulfur compounds generation. Crucial differences in the

6 sulfur compound profile and amino acid consumption among *D. hansenii* strains

7 isolated from different food sources were observed. Strains isolated from dry

8 pork sausages displayed the most complex sulfur compound profiles. Sulfur

9 compounds production, such as methional, could result from chemical reactions

10 or yeast metabolism, while, according to this study, thioester methyl thioacetate

11 appeared to be generated by yeast metabolism. No relationship between sulfur

12 compounds production by *D. hansenii* strains and the expression of genes

13 involved in sulfur amino acids metabolism was found, except for the *ATF2* gene

14 in L1 strain for production of methyl thioacetate. Our results suggest a complex

15 scenario during sulfur compounds production by *D. hansenii*.

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INTRODUCTION

A wide range of volatile compounds are responsible for meat product aromas, among which volatile sulfur compounds contribute to the characteristic meaty notes.[1] The main source of sulfur compounds in fermented meat is the transformation from sulfur amino acids. During meat processing for manufacturing dry fermented sausages, sulfur amino acids transformation is mainly conducted by microorganisms and, to a lesser extent, chemical reactions like Maillard and Strecker degradation[2]. L-methionine, L-cysteine, and L-cystine are the main sulfur amino acids present in dry fermented sausages, L-methionine being the most abundant followed by L-cysteine.[3] Microbial transformation of sulfur amino acids into volatile sulfur compounds is conducted by bacteria (lactic acid bacteria and staphylococci) and yeasts present in the meat product.[4]

The generation of volatile sulfur compounds from sulfur amino acids by yeasts metabolism has been thoroughly investigated in cheese.[5-7] The main sulfur compound precursor in yeasts seems to be L-methionine.[8-10] Methanethiol is believed to result from direct L-methionine demethiolation by β/γ-lyases activity (demethiolation pathway) or by a two-step transformation carried out by an aminotransferase, giving α-keto-γ-methylthio-oxobutyric acid (KMBA), followed by a demethiolase activity in the Ehrlich pathway. Further transformation of methanethiol leads to generation of other sulfur compounds such as, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide generated by an oxidation reaction; while thioester methyl thioacetate is generated by a chemical or enzymatic reaction. KMBA can be transformed directly to methional through decarboxylation and methional can be subsequently reduced to methionol.

46　Among the yeasts isolated from dry fermented sausages, *Debaryomyces (D.)*

47　*hansenii* is the dominant yeast species.[11] Addition of a *D. hansenii* starter has

48　potential functionalities in the manufacture of dry fermented sausages

49　contributing to the proteolytic activity[12] and producing free amino acids which

50　act as precursors of volatile compounds.[3,13] The ability of *D. hansenii* to

51　generate ester and sulfur compounds among other volatiles and reduce the

52　production of oxidation derived compounds in dry fermented sausages has

53　been demonstrated.[14,15] Moreover, addition of *D. hansenii* improves the sensory

54　quality of the final dry fermented sausages product.[16,17] However, the metabolic

55　pathways involved in sulfur compounds generation in dry fermented sausages

56　have not yet been elucidated. The aim of this study was to compare the ability

57　of several *D. hansenii* strains to produce volatile sulfur compounds from sulfur

58　amino acids and get an inside into the metabolic pathway involved in the

59　generation of L-methionine derived volatile compounds.

60

61　**MATERIALS AND METHODS**

62　**Chemicals**

63　The following compounds were commercially purchased from Sigma-Aldrich

64　(Missouri, USA): dimethyl sulfide, methyl thioacetate, dimethyl disulfide, ethyl

65　thioacetate, methional, dimethyl trisulfide, methionol, L-methionine, L-cysteine

66　and L-cystine.

67　**Yeast strains**

68　*D. hansenii* strains used in this study (Table 1) were isolated from different dry

69　fermented pork and Llama sausages, cheese, and vegetables.

Growth of D. hansenii on sulfur amino acids supplemented media

D. hansenii strains were pre-cultured during 48 h on a GPY liquid medium (2% glucose, 0.5% peptone, and 0.5% yeast extract) at 25 °C. After growth, each culture was centrifuged and washed three times with saline solution (0.9% NaCl). Cell suspensions were adjusted for inoculation in culture media at a final concentration of 10^6 cells/mL. A control medium (C) composition was: 0.5% yeast extract (amino acids content in Table S1), 10 g/L glucose, 30 g/L NaCl, 127.5 mg/L $NaNO_2$, and 127.5 mg/L $NaNO_3$. Composition of the sulfur amino acids supplemented media were the same as the control medium, except for the addition of 100 mg/L L-methionine (M), 250 mg/L L-cysteine (Cy), and 50 mg/L L-cystine (Ci), respectively. The media were adjusted to pH 6.5 and sterilized using a vacuum-driven filtration system of 0.22 μm. Experiments were conducted in 100 mL flasks containing 50 mL of C, M, Cy, and Ci media. Seven flasks were inoculated with each *D. hansenii* strain while a non-inoculated (NI) flask of each medium were the control. Each experiment was conducted in triplicate (96 experiments in total). Flasks were incubated at 25 °C for 15 days for sulfur compounds analysis. After incubation, optical density (OD) was measured at 600 nm in a BioPhotometer (Eppendorf, Germany). The supernatant was recovered by centrifugation at 4000 rpm for 5 min at 20 °C, cell-free filtered (0.22 μm) and frozen at -20 °C until the volatiles and amino acid analyses. Additional experiments in media C and M inoculated with yeasts L1 and L74 were carried out in triplicate (12 in total) for studying gene expression. After 2 days of incubation, cells were collected by centrifugation at 4000 rpm for 5 min at 20 °C. The resulting yeast pellet was resuspended in sterile milliQ water and frozen at -80 °C until RNA isolation.

Volatile sulfur compounds analysis

The sulfur compounds analyzed (Figure 1) were methanethiol (**1**), dimethyl sulfide (**2**), dimethyl disulfide (**3**), dimethyl trisulfide (**4**), methional (**5**), methionol (**6**), methyl thioacetate (**7**), and ethyl thioacetate (**8**). Analysis was carried out by headspace (HS) solid-phase microextraction (SPME) with an 85 µm Carboxen/Polydimethylsiloxane (CAR/PDMS) fiber (Supelco, Bellefonte, PA) using a gas chromatograph (Agilent HP 7890 series II (Hewlett-Packard, Palo Alto, CA)) with a quadrupole mass detector (HP 5975C (Hewlett-Packard)) and equipped with an autosampler (MPS2 multipurpose sampler (Gerstel, Germany)). Seven mL of supernatants were placed into 20 mL headspace vials containing 2.37 g of NaCl to produce a salting out effect. To prevent oxidation, the vials were purged with nitrogen gas for 5 s before sealing. The internal standard 2-methyl-3-heptanone (256 ng) was added, and the vials were incubated at 37 °C for 30 min at 250 rpm for equilibration. Then, the fibre was exposed to the headspace for 30 min at 250 rpm while maintaining the sample at 37 °C, after it was desorbed in the injection port of the GC-MS for 5 min at 240 °C in splitless mode. The volatile compounds were separated using a DB-624 capillary column (30 m, 0.25mm i.d., film thickness 1.4 µm, (J&W Scientific, Agilent Technologies, USA)). Helium was used as a carrier gas with a linear velocity of 34.3 cm/s. The GC oven temperature was held at 38 °C for 13 min, ramped to 100 °C at 3 °C/min, held at 100 °C for 5 min, ramped to 150 °C at 4 °C/min and to 210 °C at 10 °C/min, and held at 210 °C for 5 min. The MS interface temperature was set to 240 °C. Sulfur compounds were identified in full scan mode and by their retention time according to authentic standards. The identified volatile compounds were quantified in SIM mode using specific *m/z*

ions: 48 for (**1**), 62 for (**2**), 94 for (**3**), 126 for (**4**), 104 for (**5**), 106 for (**6**), 90 for (**7**), and 104 for (**8**). Calibration curves for each sulfur compound (except for methanethiol (**1**), relative to the internal standard, were obtained using the same SPME conditions in water. The peak areas of the compounds were compared to their respective standard and expressed as ng per ml of supernatant. Sulfur compounds were counted based on the normalized area and using the response factors shown in Table 2. The calibration curve for methanethiol (**1**) was not obtained and it was expressed as ng equivalents of dimethyl disulfide (**3**) per ml of supernatant. Experimental triplicates were analyzed in duplicate.

Amino acid analysis

The analysis of free amino acids was done using the EZ-Faast™ kit bought from Phenomenex (Torrance, CA, USA). Media supernatants were diluted 1:5 (v/v) with distilled water and analyzed using the kit. The derived amino acids were analyzed using GC-FID. A gas chromatograph (Agilent Technologies 7890B) with a flame ionization detector (FID) equipped with an autosampler G4513A and a ZB-AAA 10 m x 0.25 mm GC column (Phenomenex) was used. The injection volume was 2.5 µL at 250 °C in split mode (15:1). Helium was used as a carrier gas at a constant flow of 27 mL/min during the run, and the column head pressure was 8.78 psi. The GC oven temperature was initially held at 110 °C and then raised to 320 °C at 32 °C/min; the inlet temperature was 250 °C; the detector was set at 320 °C. Identification and quantitation was based on retention times and peak area integration of the reference amino acids (Phenomenex). Norleucine was the internal standard. Calibration curves for each amino acid were obtained with the standard amino acids solutions (Phenomenex). Results were expressed in mg/100 mL of supernatant.

145　Consumption of amino acids was analyzed for each yeast strain in each

146　medium, subtracting the concentration measured in the respective NI medium,

147　at the end of incubation time. The method used did not allow for detection of L-

148　cysteine in liquid media.

149　**RNA extraction and cDNA copy**

150　About 10^8 yeast cells were used for RNA isolation. Total RNA was extracted as

151　described by Sanvisens et al.[21] with modifications. Cell pellets were suspended

152　in 400 µL of LETS buffer (2 M LiCl, 1 M EDTA, 1 M Tris-HCl pH 7.4, SDS 10%)

153　and 400 µL of phenol, chloroform, and isoamyl alcohol (120:24:1), glass beads

154　were added and cells lysed using a Millmix 20 Bead Beater (Tehtnica,

155　Slovenia). The supernatant was collected by centrifugation and RNA was

156　precipitated in two steps: with 5 M LiCl and 96% ethanol at -20 °C overnight;

157　with 3 M sodium acetate and 96% ethanol at -80 °C for 2 - 3 h. RNA was

158　isolated by centrifugation and washed with 70% ethanol. Isolated RNA was

159　dried and suspended in 200 µL of RNase-free MilliQ water (VWR, USA). RNA

160　concentration and purity were determined using a NanoDrop (Thermo Scientific,

161　USA) and DNA was removed using the DNA-free Kit (DNase Treatment &

162　Removal, Invitrogen, USA). Reverse transcription of cDNA was made with

163　Reverse Transcriptase SuperScript III (Invitrogen) with the Oligo(dT)$_{12\text{-}18}$ primer,

164　a using Protector RNase inhibitor kit (Roche, Switzerland) according to the

165　manufacturer's recommendations. DNA-free RNA and cDNA concentration and

166　purity were determined using a NanoDrop (Thermo Scientific).

167　**Real-time quantitative reverse transcription polymerase chain reaction**

168　**(RT-qPCR) experiments**

169   Expression of *S. cerevisiae* orthologous genes in L1 and L74 *D. hansenii*

170   strains: *ARO8*, *CYS3*, *AAT2*, *BNA3*, *ADH1*, *ILV6*, *ATF1*, *ATF2*, *PDC1*, *PDC6*,

171   *STR3*, *PDB1,* and *BAT2* related to sulfur compounds generation[7,22] were

172   studied. Primers, listed in Table 3, were designed using OligoAnalyzer® tool on

173   the IDT webpage (https://eu.idtdna.com/pages/tools/oligoanalyzer). We

174   searched, close to the 3' end of the orthologous gene sequences for conserved

175   regions using available *D. hansenii* genome sequences: NRRLY-7426T (syn.

176   CBS767), MTCC234, and J6. Our PCR and Sanger-sequencing of genes *PDB1*

177   and *STR3* helped redesign the pairs of primers which failed in our first

178   approach.

179   The RT-qPCR was performed using a LightCycler (Roche) and LightCycler 480

180   SYBR Green I Master Kit (Roche), according to the manufacturer's

181   recommendations. The *ACT1* gene was used as the reference gene. The

182   quantity of cDNA for each gene was normalized to the quantity of the *ACT1*

183   cDNA in each sample. The E-method (LightCycler 480, Roche) was used for

184   accurate relative quantitation data analysis. The amplification efficiency of the

185   reference and target genes was calculated using LinRegPCR 2017.[23] The

186   relative change in the expression of each gene was described as the ratio of

187   normalized quantity of cDNA for each gene studied under different conditions

188   low L-methionine content in control medium (C) and high L-methionine content

189   in (M) medium. A gene was considered overexpressed when the ratio of its

190   transcriptomic response in treatment M and C conditions (M/C) was > 2.

191   **Statistical analysis**

192   Data was analyzed using the Generalised Linear Model (GLM) procedure in

193   XLSTAT 2018.4 (Addinsoft, Paris, France). Sulfur compounds and amino acids

194 consumption data was analyzed using the linear mixed model including the

195 yeast strain as the fixed effect and replicates as the random effect. Differences

196 in gene expression between L1 and L74 strains growing on C and M media

197 were analyzed using the same model including replicates as the random effect.

198 In case an effect because of yeast inoculation or media was detected ($P <$

199 0.05), least squares mean (LSM) were compared using Tukey test. Heatmaps

200 of amino acid consumption in each medium were calculated, relative to the

201 concentration in the control medium without inoculation (XLSTAT).

202

203 **RESULTS**

204 **Volatile sulfur compounds analysis.**

205 The sulfur compounds methanethiol (**1**), dimethyl sulfide (**2**), dimethyl disulfide

206 (3), dimethyl trisulfide (**4**), methional (**5**), methionol (**6**), methyl thioacetate (**7**),

207 and ethyl thioacetate (**8**) were identified and quantified in all experiments. The

208 highest generation of sulfur compounds was seen on the M medium (Figure

209 2b). Comparisons between culture media show that the concentration of volatile

210 sulfur compounds in the sulfur amino acids supplemented media inoculated with

211 yeasts (Figures 2b - d) was higher than in their respective C flasks (Figure 2a).

212 The amount of sulfur compounds produced in sulfur amino acids rich media (Ci,

213 Cy and M) inoculated with yeasts were similar except for medium M. Regarding

214 the sulfur compounds profile, dimethyl trisulfide (**4**), methionol (**6**), and methyl

215 thioacetate (**7**), were not produced in media Ci or Cy (Tables 4 and S2 - S4).

216 Generation of sulfur compounds occurred in NI flasks of all media. Methional (**5**)

217 was exceptionally abundant in all NI flasks while methyl thioacetate (**7**) was

218 absent in NI flasks of sulfur amino acids supplemented media. The compound

10

219 dimethyl trisulfide (**4**) appeared only in NI flasks from Ci and Cy media.

220 Significant differences were observed between *D. hansenii* strains regarding

221 sulfur compounds generation on all media (Table 4 and S2 - S4). However,

222 differences between replicates were not significant ($P > 0.10$). In medium M

223 (Table 4), strain L74 was the highest producer of sulfur compounds in contrast

224 to strain L25, the lowest. The most abundant sulfur compounds in medium M

225 were methyl thioacetate (**7**) by strain L1, methionol (**6**) by strain L12, dimethyl

226 disulfide (**3**) by strain L74, and methional (**5**) by strain L21. It is worth note, that

227 methyl thioacetate (**7**) was only produced by L1, L5, and L74. The volatile

228 profile between strains was also different. Strains isolated from pork sausages,

229 L1 and L5, produced the same sulfur compounds but in different amounts.

230 Between strains isolated from Llama sausages we found larger differences in

231 the production of methionol (**6**) and methional (**5**) in strains L12 and L21. Strain

232 L25 differed from the other strains from Llama sausage, regarding its sulfur

233 compound profile. Cheese strain L66 showed a sulfur compound profile like the

234 strains isolated from meat. The most different strain regarding sulfur

235 compounds profile was L74, isolated from lupine. Media Cy and Ci, inoculation

236 of strain L66 had a remarkable effect on the generation of methionol (**6**) which

237 was not produced in C (Tables S3 and S4).

238 **Amino acid analysis.**

239 Amino acids asparagine (ASN), glutamine (GLN), and ornithine (ORN) were

240 chemically produced after 15 days incubation in NI media (Tables S5 - S8).

241 Data on consumption of amino acids by each yeast strain was represented in a

242 heatmap with hierarchical clustering (Figure 3) and Table 5 for sulfur amino

243 acids. Concentration of the remaining amino acids in C, M, Cy, and Ci media

244 are shown in Tables S5 - S8. In the heatmap, consumption of each amino acid

245 is relative to the total consumption of amino acids. The highest consumption is

246 given a red color, while the lowest consumption is given a yellow color.

247 Hierarchical clustering is done on the rows of the data matrix and is represented

248 in the left part of the figure. Clustering in Figure 3 divided *D. hansenii* strains

249 into two groups of yeasts, the low amino acid consumption group (top) and the

250 high amino acid consumption group (bottom). The principal differences between

251 both groups was consumption of tyrosine. Strains L66 and L12 showed the

252 lowest amino acid consumption, while strains L21 and L25 displayed an

253 intermediate amino acid consumption. The main differences between those two

254 groups was the consumption of alanine, glycine, proline, hydroxyproline,

255 glutamic acid, phenylalanine, and histidine. Strains L1, L5, and L74 were in the

256 highest amino acid consumers group, being the consumption of ornithine the

257 main difference between L1 and the other strains.

258 Yeast consumption of individual amino acids was different depending on the

259 culture media (Tables S5 - S8). The most consumed amino acids in all media

260 were glutamic acid and threonine, and the least were valine and tyrosine.

261 Biogenic amine production was not observed in any of the strains assayed in

262 the amino acid medium following the method of Aslankoohi et al.[24] In individual

263 sulfur amino acids, L1, L5, and L74 consumed the highest amount of L-

264 methionine in all media (Table 5). L-Cystine was only detected in media

265 supplemented with L-cystine or L-cysteine (Ci and Cy) and L1, L5, L21 and, L74

266 were the highest consumers of this sulfur amino acid.

267 **Gene expression**

268   Expression of genes related to generation of sulfur compounds derived from L-

269   methionine in media containing low L-methionine concentration (C) and high L-

270   methionine concentration (M) was analyzed in RNA extracted from yeasts

271   cultivated for 2 days (Table 6). Differential expression of few genes between M

272   and C culture media (M/C ratio) were significant in strains L1 and L74.

273   Expression of gene *DhATF2* (alcohol acetyltransferase) was overexpressed in

274   L1 on media supplemented with L-methionine ($p<0.01$). Expression of genes

275   *DhAAT2* (cytosolic aspartate aminotransferase) and *DhPDC1* (pyruvate

276   decarboxylase) was significantly different ($p < 0.05$) in C and M media in L74.

277   However none of these genes could be considered overexpressed or

278   repressed.

279

280   **DISCUSSION**

281   *D. hansenii* is added as a starter in the production of many Mediterranean meat

282   products contributing to the generation of volatile compounds and overall quality

283   of the products.[17] During the dry curing process many amino acids are released

284   by different proteolytic activities.[25] The amounts of amino acids in the media

285   used in this study are like the quantities found in dry fermented meat products.[3]

286   The free amino acids are potential sulfur compounds precursors and,

287   especially, L-methionine catabolism has been investigated as the source of

288   methanethiol (**1**). Methanethiol (**1**) is the first-step degradation product of L-

289   methionine and is the main precursor for many sulfur compounds[9] and

290   appeared in medium M in very small amounts. The highest production of sulfur

291   compounds by yeast was observed in the medium supplemented with L-

292   methionine (M) confirming that methionine is the main precursor of sulfur

293   compounds in *D. hansenii*, as observed by other authors in cheese yeasts. [5, 26]

294   *D. hansenii* strain L1 consumed the highest amount of L-methionine and

295   produced the most complex sulfur compound profile. Overexpression of genes

296   involved in sulfur compounds generation from L-methionine has been revealed

297   in several cheese yeast species including: *Geotrichum candidum, Yarrowia*

298   *lipolytica, Kluyveromyces lactis* and *Saccharomyces cerevisiae*.[27-29] Among the

299   genes involved in conversion of L-methionine to methanethiol (**1**), we tested the

300   expression of *DhAAT2* (cytosolic aspartate aminotransferase), *DhARO8*

301   (aromatic amino acid transferase), *DhBAT2* (cytosolic branched-chain amino

302   acid transferase), *DhBNA3* (arylformamidase), *DhCYS3* (cystathionine gamma-

303   lyase), *DhSTR3* (cystathionine beta-lyase), and *DhILV6* (acetolactate

304   synthase). However, we did not find any of these genes overexpressed in the

305   strains and media evaluated, although *DhARO8* M/C ratios in L1 indicate high

306   levels of expression of this gene in medium M respective to medium C.

307   Alternatively, chemical oxidation plays a key role in generation of sulfur

308   compounds. Dimethyl sulfide (**2**), dimethyl disulfide (**3**), dimethyl trisulfide (**4**)

309   are considered the result of chemical oxidation of methanethiol (**1**).[9] These

310   sulfur compounds appear in lesser amounts in media inoculated with *D.*

311   *hansenii*, except for dimethyl disulfide (**3**) by L74. Strong chemical

312   transformations of methional (**5**) into methanethiol (**1**) do not occur in NI M

313   medium, therefore enzymatic production of (**1**) is the most probable explanation

314   for their presence in inoculated M media. Comparison between inoculated M

315   media revealed that L25 M medium was like the NI M medium, except for the

316   number of compounds; indicating that strain L25 is preventing oxidation of L-

317   methionine. The large amount of methionol (**6**) and methyl thioacetate (**7**) in L1

318   and L5 M media indicates that the L-methionine catabolic pathway plays an

319   important role in their generation. The large amount of dimethyl disulfide (**3**),

320   produced by oxidation indicates that strain L74 is not preventing oxidation of L-

321   methionine or any other sulfur compound. Taking into account that the

322   precursor of dimethyl disulfide (**3**) is methanethiol (**1**), and the presence of

323   methyl thioacetate, whose precursor is also methanethiol (**1**). The most

324   probable explanation is that methanethiol (**1**) is being produced by catabolism of

325   L-methionine and rapidly oxidized to dimethyl disulfide (**3**).

326   Chemical oxidation of amino acids and non-biological generation of sulfur

327   compounds take place in NI medium.[2] In addition, conversion from L-cysteine to

328   L-methionine in the medium could have happened.[30] Methional (**5**) was the only

329   sulfur compound whose generation in NI was always higher than in media

330   inoculated with yeasts. The most probable explanation could be that methional

331   (5) is the consequence of an oxidative chemical reaction from L-methionine

332   (Strecker reaction).[31,32] Escudero et al.[31] established the relationship between

333   oxidised wines and the generation of methional (**5**), but this compound was not

334   found in not oxidized wines. Similarly, our results show that in NI, methional (**5**)

335   generation is the consequence of chemical oxidation, while in the inoculated

336   media, chemical oxidation was not prevalent. This would agree with earlier

337   studies reporting the role of *D. hansenii* in prevention of lipid oxidation in meat

338   products.[14] However, our study did not reveal changes in the expression of

339   pyruvate decarboxylase genes (*PDC*) responsible for the conversion of KMBA

340   into methional (**5**). On the contrary, Cholet et al.[22] found *PDC* expression of

341   *DhPDC1* and *DhPDC6* genes in *Yarrowia lipolytica*, although the authors did

342   not detect generation of methional (**5**). This might indicate that methional (**5**) is

343   not the result of pyruvate decarboxylase activity from KMBA. Another

344   explanation could be the rapid conversion of methional (**5**) into methionol (**6**),

345   which would prevent accumulation of the former in inoculated media. This

346   conversion occurred in some of our *D. hansenii* yeasts in M medium.

347   Conversion of methionol (**6**) from methional (**5**) is conducted by alcohol

348   dehydrogenases (ADH), however these genes (*DhADH1*) were also not

349   overexpressed in our *D. hansenii* strains. Moreover, the lower amounts of

350   methional (**5**) found in the inoculated media could result from methional

351   oxidation and generation of methyl thiopropionic acid.[32] Nevertheless, this

352   compound was not analyzed in our study. Thioesters generated from alcohol

353   acetyltransferases activity (ATF), methyl thioacetate (**7**), and ethyl thioacetate

354   (8) were detected in media inoculated with *D. hansenii* strains, although in very

355   low amounts among the sulfur compounds analyzed. Generation of ethyl

356   thioacetate (**8**) is absent in sulfur compounds produced by cheese yeasts.[6,8,27-

357   29] Although, in most studies, including ours, methyl thioacetate (**7**) is the most

358   produced thioester from L-methionine. *D. hansenii* strain L1 produced the

359   highest methyl thioacetate (**7**) amount in medium M, and the *DhATF2* gene was

360   overexpressed in this yeast strain.

361   Gene expression was similar in *D. hansenii* strains growing on M medium

362   respective to C medium at 48 hours except for *DhATF2*. Comparable

363   experiments of gene expression were also conducted at 4 and 15 days (data

364   not shown); however, expression of all genes evaluated did not change

365   between media M and C. Moreover, sulfur compounds were analyzed at 15

366   days because previous studies show a measurable concentration at this time.[15]

367   However, after 15 days incubation yeasts are in stationary phase of growth and

368 none of the selected genes must be overexpressed at this late time in the

369 growth curve. Most authors used 48 and 96 hours incubation to test

370 overexpression of genes and its relationship with sulfur compounds production

371 in other yeast species.[8,27-29] *D. hansenii* strains L1 and L74 were selected for

372 gene expression analysis because of their great ability to generate sulfur

373 compounds from amino acids, plus their different volatile production pattern.

374 Medium M was selected for gene expression analysis because L-methionine is

375 the precursor of most sulfur compounds. Other genes tested in previous studies

376 dealing with sulfur compounds production by Ehrlich or demethylation pathways

377 such as *ARO9*, *ARO10*, and *BAT1* were overexpressed in *S. cerevisiae* and *Y.*

378 *lipolytica*[22,28,33] and assayed in high L-methionine medium. Liu et al.[28] found

379 overexpression of the *STR3* gene (demethiolase activity) and *ADH4* and *ADH5*

380 genes (alcohol dehydrogenase activity) in *S. cerevisiae.* Although, other studies

381 did not find these genes overexpressed in *K. lactis* and *Y. lipolytica.*[27,34]

382 Our results showed that C, Cy, and Ci media contained all necessary amino

383 acids but generated low amounts of sulfur compounds, in agreement with

384 previous studies.[6,8,26,27] One explanation could be that L-cysteine is preferably

385 consumed by yeasts producing H_2S. However, H_2S is highly volatile and

386 reactive, preventing the production of its derived sulfur compounds and is

387 difficult to detect by GC-MS.[26]

388 Regarding the relationship between amino acid consumption and sulfur

389 compounds generation in the *D. hansenii* strains tested, a clear link between

390 these variables was found in strains L1 and L5, isolated from pork dry

391 fermented sausages, and L74 isolated from lupine. Moreover, the high

392 consumption of amino acids, besides L-methionine, by these yeast strains

393   indicate the probable production of other volatile compounds plus other sulfur

394   compounds, which can provide an interesting flavor profile to the product.[13]

395   Nevertheless, the performance of *D. hansenii* strains in amino acid

396   supplemented media for production of sulfur compounds could change with the

397   composition of the media and environmental conditions in production of dry

398   cured meat products.[14]

399   In summary, L-methionine supplemented media (M) was the most efficient for

400   production of sulfur compounds by yeasts. Methional (**5**) was preferably

401   generated by chemical oxidation of L-methionine, while methyl thioacetate (**7**)

402   was solely produced by yeasts. Profiles of sulfur compounds generated by

403   yeast were different and strains from pork meat origin presented the most

404   complex sulfur compound profiles. Expression of genes in the metabolic

405   pathway of L-methionine, for generation of sulfur compounds, could not be

406   directly related to sulfur compound production in *D. hansenii*, except in case of

407   methyl thioacetate (**7**), strain L1 and overexpression of gene *DhATF2*. Sulfur

408   compounds produced by yeast were detected long after genes involved in the

409   metabolic pathway of sulfur compounds generation were expressed. This could

410   explain the absence of overexpression of most genes in M media.

411   Nevertheless, this study has deeply investigated the metabolic pathway

412   involved in the generation of L-methionine derived volatile compounds in *D.*

413   *hansenii* isolated from meat products. It has subsequently shown their

414   relevance as a producer of volatile sulfur compounds with aroma impact in meat

415   products. The results have shown that different metabolic pathways are

416   expressed in *D. hansenii* from fermented pork and Llama sausages and their

417   impact on the final meat product aroma is different. However, the expression of

418 the metabolic pathways depends on many processing conditions that affect the

419 production of sulfur compounds from their precursor sulfur amino acids.

420 Therefore, it is necessary to continue to elucidate in real conditions (sausage

421 manufacture) that expression of genes impact the produced sulfur compounds

422 and subsequently, meat product aroma.

423

Abbreviations

Alcohol acetyltransferases activity (ATF), α-keto-γ-methylthio-oxobutyric acid (KMBA), headspace (HS), solid-phase microextraction (SPME), Carboxen/Polydimethylsiloxane (CAR/PDMS).

Funding sources

Supporting Information

Table S1. Initial amino acids concentration in control medium (C).

Table S2. Concentration of sulfur compounds (µg/L) in the control medium (C) inoculated with different *D. hansenii* strains.

Table S3. Concentration of sulfur compounds (µg/L) in the L-cysteine medium (Cy) inoculated with different *D. hansenii* strains.

Table S4. Concentration of sulfur compounds (µg/L) in the L-cystine medium (Ci) inoculated with different *D. hansenii* strains.

Table S5. Concentration of amino acids (mg/100 ml) in the control media (C) inoculated with different *D. hansenii* strains.

Table S6. Concentration of amino acids (mg/100 ml) in the L-methionine media (M) inoculated with different *D. hansenii* strains.

Table S7. Concentration of amino acids (mg/100 ml) in the L-cysteine media (Cy) inoculated with different *D. hansenii* strains.

Table S8. Concentration of amino acids (mg/100 ml) in the L-cystine media (Ci) inoculated with different *D. hansenii* strains.

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571

Figure Captions

Figure 1. Chemical structures of the volatile sulfur compounds studied.

Figure 2. Volatile sulfur compounds generated in sulfur amino acid supplemented media non-inoculated (NI) and inoculated with *D. hansenii* (Table 1) in different media: a, control (C); b, L-methionine supplemented medium (M); c, L-cysteine supplemented medium (Cy); and d, L-cystine supplemented medium (Ci).

Figure 3. Heatmap showing the consumption of non sulfur amino acids by the metabolic activity of *D. hansenii* strains (Table 1) in all media. C (control medium), M (L-methionine supplemented medium), Cy (L-cystine supplemented medium), and Ci (L-cystine supplemented medium). Red color indicates relative high amino acid consumption (> 1) while yellow color indicates low amino acid consumption (> -1) and orange color indicates no difference.

Tables

Table 1. *D. hansenii* strains used in this study

Yeast strain	Source	Reference
L1	Pork dry fermented sausage	Bolumar et al.[18]
L5	Pork dry fermented sausage	Cano-García et al.[14]
L12	Llama dry fermented sausage	Mendoza et al.[19]
L21	Llama dry fermented sausage	Mendoza et al.[19]
L25	Llama dry fermented sausage	Mendoza et al.[19]
L66	Ewe's cheese	Padilla et al.[20]
L74	Lupine	Flores et al.[13]

Table 2: Response factors for the analysis of sulfur compounds in Selected-Ion-Monitoring (SIM) mode and using 2-methyl-3-heptanone as the internal standard.

Compound	Monitored ion (*m/z*) used for quantitation	Response factor[a]
Dimethyl sulfide	62	0.0941
Methyl thioacetate	90	0.0589
Dimethyl disulfide	94	1.2547
Ethyl thioacetate	104	0.0723
Methional	104	0.0370
Dimethyl trisulfide	126	1.0625
Methionol	106	0.0603

Table 3. Primers used in RT-qPCR.

D. hansenii open reading frame	D. hansenii gene name	Enzyme	Direction	Sequence
DEHA2D05412g	DhACT1	Actine	F	GGTAACATTGTTATGTCTGGTG
			R	TACTTTCTTTCTGGAGGAGC
DEHA2C05236g	DhAAT2	Cytosolic aspartate aminotransferase	F	AACACCGTCAGAACCAAG
			R	CAATTCAATAACTTGTTCAGC
DEHA2A06886g	DhARO8	Methionine aminotransferase	F	CAA GGT TGT TTG ATG ATC YCC
			R	AACGGCAGCATATGTACCTC
DEHA2C09724g	DhATF1	Alcohol acetyltransferase	F	CTGGTGCAGCATTAGGAC
			R	AAA TGG CTT YAA TCT GTC TC
DEHA2D14762g	DhATF2	Alcohol acetyltransferase	F	GCCTCAACTTGCTGRC
			R	GTTCCAAGAGTTTTGTAGTAAAC
DEHA2D06952g	DhBAT2	Cytosolic branched-chain amino acid transferase	F	TTTAGAAGGTGTCACCAGAG
			R	CYCTTTCTTCAATTTCGTGG
DEHA2A04818g	DhBNA3	Arylformamidase	F	CCYTATACATCTGCTCAAGG
			R	TCCGATTTCYTTTATCAACCAG
DEHA2C15686g	DhCYS3	Cystathionine gamma-lyase	F	CACGGTGGTATTCCAAAAG
			R	AAGCTTGTCTGACATCTTCG
DEHA2E21604g	DhILV6	Acetolactate synthase	F	GTT GAY ATT GCT GAT AGA AAC G
			R	CTTGGTAATGCCATCATACC
DEHA2C09152g	DhPDB1	E1beta subunit of pyruvate dehydrogenase complex	F	ACGGTGTTAAGGCTGAAGTTAT
			R	ATGATTTGGGCACAGATTTC
DEHA2B03872g	DhPDC1	Pyruvate decarboxylase	F	GGTACATCAGCATTTGGRATTG
			R	CCA TKA CTG CTC CTA ATG TAG
DEHA2G18348g	DhPDC6	Pyruvate decarboxylase	F	GATTAATTCATGGTGAAAATGCC
			R	AGC ATC GTA ATC CTC AGC AC
DEHA2A06798g	DhSTR3	Cystathionine beta-lyase	F	TATCTTTGGAATTGCCGTTTC
			R	TTCTTCTCTGGTCTTGGCATC
DEHA2G21032g	DhADH1	Alcohol dehydrogenase	F	GGGCACATGGAGTWATTAATG
			R	CACKACAGTACCACGAGATC

Table 4. Concentration of sulfur compounds (µg/L) in L-methionine medium (M) after 15 d incubation with *D. hansenii* strains.

	NI[1]	L1	L5	L12	L21	L25	L66	L74	RMSE[2]	*P*[3]
Methanethiol	0.349 ab	0.402 ab	0.453 a	0.346 ab	0.382 ab	0.161 bc	0.005 c	0.546 a	0.078	***
Dimethyl sulfide	0.069 b	0.216 b	0.792 a	0.072 b	0.180 b	0.074 bc	0.034 b	0.175 b	0.066	***
Methyl thioacetate	n.d.[5]	2.116 a	0.366 b	n.d.	n.d.	n.d.	n.d.	0.051 b	0.309	*
Dimethyl disulfide	0.284 bc	1.391 b	1.621 b	0.556 bc	0.539 bc	0.253 c	0.268 c	7.935 a	0.370	***
Ethyl thioacetate	0.009 b	0.029 a	0.026 a	0.027 a	0.025 ab	0.007 b	0.006 b	0.012 b	0.002	***
Methional	7.585 a	1.505 bc	0.620 c	1.428 bc	2.438 b	0.550 c	0.781 bc	2.079 bc	0.617	***
Dimethyl trisulfide	n.d.	0.204 ab	0.330 a	0.072 c	0.090 c	0.059 c	0.051 c	0.094 bc	0.025	**
Methionol	0.004 b	1.680 ab	0.711 ab	3.447 a	0.707 ab	0.004 b	2.149 ab	0.153 b	0.849	*
Total	8.300	7.543	4.919	5.948	4.361	1.108	3.294	11.045		

[1]NI: medium non-inoculated. [2]RMSE: root mean square error. [3]*P*: P value of inoculation effect of different yeasts (Table 1). Different letters in the same row indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. ns: $p > 0.05$. [4]n.d.: not detected.

Table 5. L-methionine and L-cystine consumption (mg/100ml) conducted by the *D. hansenii* yeasts strains in each medium.

Medium	L1	L5	L12	L21	L25	L66	L74	RMSE[1]	*P*[2]
L-methionine consumption									
C[3]	3.27 a	3.11 a	1.14 bc	1.57 b	1.13 bc	0.84 c	3.27 a	0.24	***
M	9.24 a	6.04 ab	1.13 c	3.55 bc	3.24 bc	0.82 c	5.5 b	1.81	***
Cy	2.99 a	2.86 a	0.92 b	1.4 b	0.92 b	1.12 b	2.66 a	0.39	***
Ci	3.4 a	3.23 a	1.08 bc	1.49 bc	2 b	0.96 c	3.18 a	0.54	***
L-cystine consumption									
Cy	2.81 bc	2.05 c	-0.28 d	3.89 ab	-0.7 d	-1.4 d	4.83 a	0.87	***
Ci	4.93 ab	3.4 bc	1.22 c	6.53 a	3.44 bc	0.93 c	6.53 a	1.7	**

[1]RMSE: root mean square error. [2]P: P value of inoculation effect of different yeasts (see table 1). Different letters in each row indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. ns: $p > 0.05$. [3]Control medium (C), supplemented medium with 100 mg/L L-methionine (M), 250 mg/L L-cysteine (Cy) and 50 mg/L L-cystine (Ci).

Table 6. Transcriptomic response of *D. hansenii* L1 and L74 to L-methionine

D. hansenii gene name	L1		L74	
	Ratio M/C	p^1	Ratio M/C	p
DhARO8	1.73	ns	1.25	ns
DhCYS3	1.13	ns	0.89	ns
DhAAT2	0.95	ns	0.71	*
DhBNA3	0.71	ns	1.27	ns
DhADH1	1.59	ns	0.97	ns
DhILV6	1.56	ns	0.91	ns
DhATF1	0.76	ns	1.36	ns
DhATF2	*2.33*	**	1.51	ns
DhPDC1	1.57	ns	1.73	*
DhPDC6	1.49	ns	1.06	ns
DhBAT2	1.56	ns	0.80	ns
DhSTR3	0.79	ns	1.32	ns
DhPDB1	1.52	ns	0.94	ns

[1]p significant differences between transcriptomic response in C and M treatment at *** $p<0.001$, ** $p<0.01$, * $p<0.05$. ns: $p>0.05$.

Abbreviations are indicated in Table 3. Ratio above 2 means overexpressed; A ratio below 0.5 means repressed.

Figures

Figure 1.
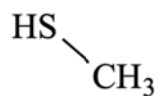
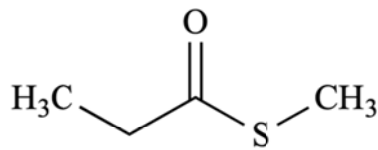Methanethiol (**1**)   Dimethyl sulfide (**2**)   Dimethyl disulfide (**3**)   Dimethyl trisulfide (**4**)
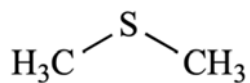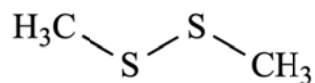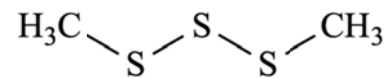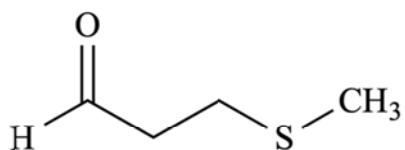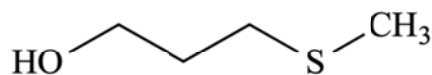
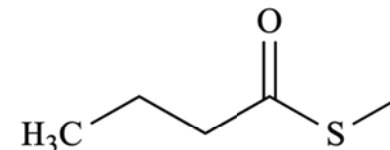Methional (**5**)   Methionol (**6**)   Methyl thioacetate (**7**)   Ethyl thioacetate (**8**)

Figure 2.

Figure 3.

TOC Graphic