Technological and safety properties of lactic acid bacteria isolated from Spanish dry-cured sausages

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25 Abstract

26 Technological and safety-related properties were analyzed in lactic acid bacteria isolated from

27 Spanish dry-cured sausages in order to select them as starter cultures. In relation to

28 technological properties, all the strains showed significative nitrate reductase activity;

29 *Lactobacillus plantarum*, *Lactobacillus paracasei* and 52% of the *Enterococcus faecium*

30 strains showed lipolytic activity and only *Lactobacillus sakei* strains (43%) were able to form

31 biofilms. Related to safety aspects, *E. faecium* strains were the most resistant to antibiotics,

32 whereas, *L. sakei* strains were the most sensitive. In relation to virulence factors, in the *E.*

33 *faecium* strains analyzed, only the presence of *efaA* gene was detected. The analysis of

34 biogenic amine production showed that most *E. faecium* strains and *L. sakei* AI–142 produced

35 tyramine. In conclusion, *L. paracasei* AI–128 and *L. sakei* AI–143 strains possess the best

36 properties to be selected as adequate and safe meat starter cultures.

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51   Keywords

52   Lactic acid bacteria, starter culture, meat products, biogenic amines, biofilms, antibiotic

53   resistance

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1. Introduction

Elaboration of dry-cured sausages is a complex process characterized by deep changes on the main meat components, resulting in the production of specific taste and aroma. This process favours the growth of microorganisms which influence the sensorial and nutritional qualities, safety, and other characteristics of sausages. The fermentation of sausages involves the participation of mainly lactic acid bacteria (LAB), coagulase-negative staphylococci (CNS), and less importantly, yeast and moulds (Ruiz-Moyano, Martín, Benito, Aranda, Casquete & Córdoba 2009). The most frequent LAB species present in fermented sausage processes are *Lactobacillus sakei, Lactobacillus curvatus*, and *Lactobacillus plantarum.* However, in some instances, the contribution of enterococci seems to be also relevant (Comi, Urso, Iacumin, Rantsiou, Cattaneo, Cantoni, & Cocolin, 2005).

LAB play an important role in the formation of lactic acid by fermenting carbohydrates, and hence could contribute to the safety of the process. Moreover, the lactic acid production contributes to the formation of the texture and in the acid taste. As a consequence of this process, the muscle protein coagulates, resulting in the slice ability, firmness and cohesiveness found in the final product. The development of curing colour occurs also in acidic conditions when nitric oxide is produced from nitrite and can then react with myoglobin.

Today, modern meat industry has to ensure high quality, reduce variability and enhance organoleptic characteristics in sausages production, which is not feasible using spontaneous fermentation methods. Regarding such situations, the use of selected starter cultures is important to produce the desired flavour and aroma compounds and extend the shelf life of the final product. The indigenous LAB isolated from fermented meats are especially well adapted to the ecological conditions of specific meat fermentations, controlling the ripening processes and inhibiting the growth of spontaneous microorganisms. Autochthonous starter cultures are recommended to achieve the desired fermentation parameters specific for the product type. The identification of the autochthonous microbiota of traditional dry-fermented sausages is of

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82 great interest to standardize the fermentation process and for the selection of strains for their

83 use as starter culture. LAB from the genus *Lactobacillus* in Europe, mainly *L. sakei, L.*

84 *curvatus, L. plantarum,* and *Lactobacillus casei*, and from the genus *Pediococcus* in the USA

85 represent the most common starter cultures used in the production of fermented sausages

86 (Rantsiou, Drosinos, Gialitaki, Metaxopoulos, Comi, & Cocolin, 2006).

87 Generally, LAB from fermented sausages have been traditionally identified based on

88 simple physiological, biochemical, and chemotaxonomic methods. Although valuable from a

89 practical point of view, results obtained by these methods are not always sufficient to

90 characterize strains to species level, mainly within species from genera *Lactobacillus* (Ammor,

91 Rachman, Chaillou, Prévost, Dousset, Zagorec, Dufour, & Chevallier, 2005) and *Enterococcus*

92 (Velasco, Perez, Peña, Dominguez, Cartelle, Molina, Moure, Villanueva, & Bou, 2004).

93 Despite the main role of LAB in fermented meat products is related with lactic acid

94 production, LAB possess additional relevant characteristics that need to be taken into account

95 in order to select them as starter cultures. The aims of the present study were to taxonomically

96 identify a collection of LAB strains isolated from Spanish dry-cured sausages and analyze

97 activities relevant for their use as starter cultures.

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99 2. Materials and methods

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101 *2.1. Bacterial strains and growth conditions*

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103 Forty-six LAB strains were analyzed in this study. The strains were previously isolated

104 from dry-cured sausages, and have been molecularly identified in this study by sequencing

105 their 16S rDNA. Dry cured sausages were prepared in local meat factories using traditional

106 techniques and without using microbial starter cultures. They were made with 70–80% of lean

107 pork meat and 20–30% of pork back fat. Ten grams of each sausage sample were homogenized

108 with 90 mL of a sterile solution of tryptone (0.3%) and NaCl (0.85%), for 2 min, in a

109 Stomacher 400 Lab Blender (Seward Medical, London, UK). Ten fold dilutions were made in

110 the same diluent.

111   All the strains were grown in MRS medium (Pronadisa, Spain) at 30 ºC during 24 hours

112 under microaerobic conditions. The strains were grown also on MRS agar plates (1.5 %) at 30

113 ºC under microaerobic conditions.

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2.2. DNA extraction

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117   DNA extraction was carried out from overnight cultures as described by Sambrook,

118 Fritsch and Maniatis (1989). DNA precipitates were resuspended in an appropriate volume of

119 TE solution (10mM Tris–HCl, pH 8.0; 1 mM EDTA).

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2.3. Taxonomical identification of LAB strains

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123   LAB strains were identified by PCR amplification and DNA sequencing of their 16S

124 rDNA. The 16S rDNAs were PCR amplified using the eubacterial universal pair of primers 63f

125 and 1387r previosly described by Marchesi, Sato, Weghtman, Martin, Fry, Hion, & Wade

126 (1998) (Table 1). The 63f and 1387r primer combination generates an amplified product of 1.3

127 **kb. PCR was performed using AmpliTaq Gold DNA polymerase (Roche) in 25 μL**

128 amplification reaction mixture by using the following cycling parameters: 10 min at 94 ºC, 35

129 cycles of 1 min at 94 ºC, 1 min at 50 ºC and 1:30 min at 72 ºC. Amplified products were

130 resolved on 0.7% agarose gels. The amplification products were purified on QIAquick spin

131 Columns (Quiagen, Germany) for direct sequencing. DNA sequencing was carried out by

132 using an Abi Prism 377™ DNA sequencer (Applied Biosystems, USA). Sequence similarity

133 searches were carried out by comparing to sequences from type strains included on the

134 Ribosomal Database (http://rdp.cme.msu.edu).

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136 *2.4. Technological properties of the strains*

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138 *2.4.1. Nitrate reductase assay*

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140 Nitrate reductase activity was measured using a spectrophotometric method described

141 previously (Miralles, Flores, & Pérez-Martínez, 1996), using MRS medium for bacterial

142 growth. Briefly, an overnight culture (1.5 mL) was harvested by centrifugation and the pelleted

143 cells was resuspended in induction buffer [Bactotryptone (Difco), 10 g/L; KNO_3, 1 g/L;

144 cysteine, 1 g/L pH 7.0] to an $OD_{550}=1$. A fraction of the cell suspension was used for the

145 determination of the dry weight. Anaerobic induction of nitrate reductase activity was achieved

146 by incubating 1mL of cell suspension in Eppendorf tubes covered with a layer of sterile

147 mineral oil during 2 h at 30 ºC. As a control for the induction process, 1mL of the cell

148 suspension was kept on ice for 2 h. The cells were harvested by centrifugation and

149 permeabilized in 500 µL reaction buffer (50 mM KNO_3, 50 mM potassium phosphate, 100

150 mM NaCl, pH 7.0) by the addition of 30 µL of an acetone-toluene mixture (9:1, v/v). The

151 tubes were vigorously shaken in a vortex for 3 min and the reaction was allowed to proceed for

152 30 min at 30º C. A 100 µL aliquot of the samples was transferred to a new Eppendorf tube

153 containing 1 mL solution A [0.6 mg N-(1-Naphthyl)-ethylenediamine dihydrochloride in 100

154 mL 5 N acetic acid] and 1 mL solution B (0.8 g sulphanilic acid in 100 mL 5N acetic acid).

155 Absorbance at 540 nm was measured against a control tube. Relative activity was calculated as

156 the ratio: $OD_{540} \times mg^{-1}$ dry weight.

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158 *2.4.2. Catalase activity*

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160　　　　The catalase activity was determined as described previously (Essid, Ben Ismail, Bel

161　Hadj Ahmed, Ghedamsi, & Hassouna, 2007). The method was adapted to LAB by using MRS

162　medium for bacterial growth. The strains were incubated in MRS to an $OD_{600}=1$, and then 5

163　mL culture was centrifuged and the resulting pellet was mixed with 1.5 mL of 60 mM H_2O_2 in

164　20 mM phosphate buffer pH 7.0. A 200 µL aliquot of each culture was deposited in a 96-well

165　plate. Catalase activity was measured spectrophotometrically at 240 nm after 3 min of

166　incubation at room temperature in a microplate reader (Synergy^{TM}HT, Biotek, EEUU). Results

167　**were expressed in arbitrary units (µmoles of degraded H_2O_2/min/mL of cells with $OD_{600}=1.0$).**

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2.4.3 Proteolytic activity

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2.4.3.1 Gelatinase assay

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173　　　　Production of gelatinase was tested on MRS agar plates containing 10 g/L peptone

174　(Pronadisa, Spain) and 30 g/L gelatine (Pronadisa, Spain) as described previously (Cariolato,

175　Andrighetto, & Lombardi, 2008). After 16–18 h of incubation at 37 °C, the plates were placed

176　at 4°C for 5 h before examination for a zone of turbidity around the colonies indicating

177　gelatine hydrolysis.

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2.4.3.2. Calcium caseinate agar assay

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181　　　　The proteolytic activity was tested in calcium caseinate agar plates (Pronadisa, Spain)

182　as described previously (Martín, Hugas, Bover-Cid, Veciana-Nogués, & Aymerich, 2006). The

183　strains were grown in MRS broth during 18–20 h and then 10 µL of each culture was placed in

184 calcium caseinate agar at 37 ºC during 3 days. The diameter of the halos formed by the

185 proteolytic strains was measure in mm.

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187 *2.4.4. Lipolytic activity*

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189   *2.4.4.1. "Spirit Blue Agar" assay*

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191   The assay with Spirit Blue Agar plus Lipase Reagent (a mixture of tributyrin and

192 polysorbate 80) (Difco) was performed according to the recommendations of the supplier.

193 Lipolytic microorganisms metabolize the lipids present in the medium and halos around the

194 colonies appear.

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196   *2.4.4.2. Tween 80 / Tween 20 assay*

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198   In order to evaluate lipolytic activity a method previously described by Essid et al.

199 (2007) was also used. An overnight culture of each strain was plated on MRS agar

200 supplemented with 1% of Tween 80 or Tween 20, centrifuged and the pellet resuspended in 20

201 mM phosphate buffer, pH 7. Each cell suspension (10 μL) was inoculated on a spot at the

202 surface of the MRS plates containing Tween 80 or Tween 20 (1% v/v). After incubation at 30

203 ºC for 48h, the lipolytic activity was determined by the appearance of a clear halo surrounding

204 the spots which diameter was measured in mm.

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206 *2.4.5. Resistance to bile*

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208   *2.4.5.1. Oxgall method*

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210 Resistance to bile was tested according the method described previously by

211 Maragkoudakis, Zoumpopoulou, Miaris, Kalantzopoulos, Pot, and Tsakalidou (2006). LAB

212 cultures were grown in MRS broth supplemented with Oxgall (0.3%) (Difco, France) at 30 ºC

213 during 4h. The resistance was determined by the number of viable colonies after incubation

214 during 0 and 4 h, reflecting the time spent by the food in the small intestine.

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216 *2.4.5.2. LAPTG broth method*

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218 The bile resistance was evaluated on LAPTg broth as previously described (Saavedra,

219 Taranto, Sesma, & de Valdez, 2003). The LAB strains were grown in LAPTg broth (g/L:

220 peptone, 15; tryptone, 10; yeast extract, 10; glucose, 10; Tween 80, 0.1% v/v) and LAPTgO

221 broth (LAPTg containing 0.3% Oxgall) at 37ºC. Every hour, for the first 8 h, and after 24 h of

222 incubation, the A_{560} nm was determined using a microplate reader (Synergy^{TM}HT, Biotek.

223 EEUU). The time required for each of them to increase the A_{560} by 0.3 units was recorded. The

224 difference in time (min) between the LAPTg and LAPTgO cultures was considered as the

225 growth delay.

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227 *2.5. Presence of relevant safety traits in the strains*

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229 *2.5.1. Biofilm formation*

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231 *2.5.1.1. Spectrophotometric method*

232

233 Biofilm formation was determined according to the method described by Toledo–

234 Arana,Valle, Solano, Arrizubieta, Cucarella, Lamata, Amorena, Leira, Penadés, and Lasa

235 (2001). LAB cultures were grown overnight at 30 ºC in MRS broth containing 0.25% glucose.

236 The culture was diluted 1/20 in fresh MRS broth supplemented with 0.25 % glucose; 200 µL

237 of this suspension was used to inoculate sterile 96-well polystyrene microtitre plates. After 24

238 h incubation at 37ºC, wells were washed with PBS, dried in an inverted position and stained

239 with 1% crystal violet (Merk, Germany) for 15 min. The wells were rinsed with PBS once

240 more and the crystal violet was solubilised in methanol/acetone (80:20, v/v). The A_{595} was

241 determined using a microplate reader (Synergy^{TM}HT, Biotek. EEUU). Biofilm formation was

242 scored as follows: –, non-biofilm forming ($A_{595} \leq 1$); +, weak ($1 < A_{595} \leq 2$); ++, moderate ($2 <$

243 $A_{595} \leq 3$); +++, strong ($A_{595} > 3$). Each assay was performed in triplicate and repeated three

244 times.

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246 *2.5.1.2. Congo Red Agar method*

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248 The biofilm formation was evaluated using Congo Red Agar (CRA) (Freeman,

249 Falkiner, & Keane, 1989). The components of the CRA media were MRS broth (37g/L),

250 sucrose (0.8 g/L), agar-agar (10 g/L) and Congo red stain (0.8 g/L). Congo red stain was

251 prepared as a concentrated aqueous solution, autoclaved separately, and added to the media

252 when the agar had cooled to 55 ºC. Plates were incubated aerobically for 24 h at 37ºC. Biofilm

253 positive strains produced black colonies while biofilm negative strains were pink.

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255 *2.5.1.3. PCR method*

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257 In *Enterococcus* strains, the biofilm formation ability was also determined by PCR

258 amplification of the *ebpA* gene which is involved in biofilm-associated endocarditis

259 (Nallapareddy, Singh, Sillampaa, Garsin, Hook, Erlandsen, & Murray, 2006). For the PCR

260 reaction the primers Ef1091F and Ef1091R previously described were used (Cobo-Molinos,

261 Hikmate, Ben-Omar, Lucas-López, & Galvez, 2008) (Table 1). PCR was performed using

262 AmpliTaq Gold DNA polymerase (Roche) in 25 µL amplification reaction mixture by using

263 the following cycling parameters: 10 min at 94 ºC, 35 cycles of 1 min at 94ºC, 1 min at 50ºC

264 and 1:30 min at 72ºC. Amplified products were resolved on 0.7% agarose gels.

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266 *2.5.2. Antibiotic susceptibility testing*

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268 Susceptibility testing was assayed by the agar overlay disc diffusion test recommended

269 by the National Committee for Clinical Laboratory Standard (NCCLS, 2002). SensiDisc BBL

270 discs (Oxoid, England) were placed onto Mueller–Hinton agar (Difco, France) plates and

271 overlayed with 3 mL of MRS soft agar broth (0.7% agar, p/v) inoculated with 150 µL of the

272 cultures with a cell concentration corresponding to 0.5 MacFarland turbidity standard. After

273 incubation at 30 ºC for 24 h the diameter of inhibition halos around the colonies was measured.

274 Susceptibility or resistance was determined according to the recommendation of NCCLS. The

275 antibiotics discs used were: ampicillin (10 µg), penicillin G (10 U), vancomycin (30 µg),

276 amikacine (30 µg), gentamicin (10 µg), tetracycline (30 µg), erythromycin (15 µg),

277 clindamycin (2 µg), tobramicin (10µg), and rifampicin (5 µg). In the *Enterococcus* strains the

278 antibiotics tested were: ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg),

279 penicillin G (10 U), gentamicin (10 µg), tetracycline (30 µg), vancomycin (30 µg), teicoplanin

280 (30 µg), rifampicin (5 µg), nitrofurantoine (300µg), and ciprofloxacin (5 µg).

281 In tetracycline resistant strains the presence of the *tetM* gene, which codifies a protein

282 involved in tetracycline resistance, was determined. The primers used were tetM–F and tetM–R

283 (Gervers, Danielsen, Huys, Swings, J., 2003) (Table 1). PCR was performed using AmpliTaq

284 Gold DNA polymerase (Roche) in 25 µL amplification reaction mixture by using the following

285 cycling parameters: 10 min at 94ºC, 35 cycles of 1 min at 94ºC, 1 min at 50ºC and 1:30 min at

286 72ºC. Amplified products were resolved on 0.7% agarose gels.

287

288   *2.5.3. Presence of virulence genes in enterococci*

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290   In *Enterococcus faecium* strains, the presence of virulence factors previously described

291   was determined by PCR amplification (Table 1). The virulence genes detected were: *asa*1,

292   which encodes the aggregation substance Agg; *esp*, encodes the enterococcal surface protein

293   Esp; *cylA,* involved in cytolysin operon; *hyl*, encodes the hyaluronidase Hyl; *gelE*, the protease

294   GelE with gelatinase activity; *efaA,* the surface A antigen EfaA; *ace*, the collagen adhesine

295   Ace; and *hypR*, encodes HypR, a transcriptional regulator of the oxidative stress response and

296   intracellular survival within macrophages (Diarra, Rempel, Champagne, Masson, Pritchard, &

297   Topp , 2010). PCR was performed using AmpliTaq Gold **DNA polymerase (Roche) in 25 μL**

298   amplification reaction mixture by using the following cycling parameters: 10 min at 94 ºC, 35

299   cycles of 1 min at 94ºC, 1 min at 50ºC and 1:30 min at 72ºC. Amplified products were

300   resolved on 0.7% agarose gels.

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302   *2.5.4. Production of biogenic amines*

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304   *2.5.4.1. TLC method*

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306   Biogenic amine production was tested by a TLC method previously described (García–

307   Moruno, Carrascosa, & Muñoz, 2005). Briefly, the cultures were incubated at 30 ºC for 7 days

308   in MRS broth containing 0.5% of the corresponding amino acid precursor: L–histidine

309   monohydrochloride (Merk, Germany), L–tyrosine disodium salt (Merk, Germany), L–ornithine

310   hydrochloride (Sigma–Aldrich, Germany), L–lysine monohydrochloride (Merk, Germany).

311   After incubation, the supernatant containing the corresponding biogenic amine was collected.

312   As control, a stock standard solution of each amine (histamine, tyrosine, putrescine and

313   cadaverine) was made by preparing a 2% solution (5% in the case of histamine) in 40%

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314 ethanol. Amines were converted into their fluorescent dansyl derivatives and were incubated in

315 the dark at 55º C during 1h. Amine derivative extracts were applied to TLC plates (silica gel

316 60 F$_{24}$, Merk, Germany). The dansylated compounds were separated using a

317 chloroform:triethylamine (4:1) mobile phase. The fluorescent dansyl derivative spots were

318 visualized using a ChemiDoc XRS$^+$ (Bio Rad) under UV light exposure.

319

320 *2.5.4.2. PCR method*

321

322 PCR was used to detect the genes encoding the decarboxylase enzymes involved in the

323 production of biogenic amines. The primers used in the PCR reactions were described

324 previously: HIS1–F/HIS1–R to amplify a fragment of the histidine decarboxylase gene; TDC–

325 F/TDC–R a fragment of the gene encoding tyrosine decarboxylase; PUT1–F/PUT1–R and

326 PUT2–F/PUT2–R to amplify the two groups of ornithine decarboxylase genes, and finally

327 CAD2–F/CAD2–R to amplify a fragment of the lysine decarboxylase gene present in Gram–

328 positive bacteria (De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006) (Table 1). PCR was

329 performed using AmpliTaq Gold DNA polymerase (Roche) in 25 μL amplification reaction

330 mixture by using the following cycling parameters: 10 min at 94ºC, 35 cycles of 1 min at 94ºC,

331 1 min at 50ºC and 1:30 min at 72ºC. Amplified products were resolved on 0.7% agarose gels.

332

333 3. Results and discussion

334

335 *3.1. Identification of LAB strains*

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337 Identification of LAB species based on biochemical tests has proven to be difficult and

338 time–consuming. These phenotypic methods are limited in terms of both its discriminating

339 ability and accuracy. In this sense, Benito, Serradilla, Ruiz–Moyano, Martín, Perez–Nevado,

14

340 and Córdoba, (2008) reported that in LAB strains isolated from Iberian dry-fermented

341 sausages, biochemical identification by API 50 CHL showed different errors at genus and

342 species level, for instance, *Pediococcus acidilactici* was misidentified as *Pediococcus*

343 *pentosaceus* and *L. plantarum* as *Lactococcus lactis*. For this reason molecular methods have

344 been developed allowing the accurate identification of LAB from meat products (Rantsiou &

345 Cocolin, 2008).

346   Forty-six LAB strains previously isolated from Spanish dry-cured sausages were

347 analyzed in this study (Table 2). As the characterization of LAB using traditional methods may

348 sometimes be uncertain, these strains have been molecularly identified in this study by

349 sequencing their 16S rDNA. Among the LAB strains, the species most frequently identified

350 were *L. sakei* (20 strains) and *E. faecium* (19 strains), although strains from others LAB

351 species were also found, such as *L. plantarum* (4 strains), *Lactobacillus paracasei* (2 strains)

352 and *Lactobacillus coryniformis* (1 strain).

353   The isolation of a high number of *L. sakei* strains is in agreement with previous studies

354 on which the high incidence of this species in fermented sausages was reported (Bonomo,

355 Ricciardi, Zotta, Parente, & Salzano, 2008;; Ammor, et al., 2005). Leroy, Verluyten and De

356 Vuyst (2006) reported that, in spontaneously fermented European sausages, lactobacilli

357 constitute the predominant microbiota in fermented sausages. These authors concluded that *L.*

358 *sakei* and/or *L. curvatus* generally dominate the fermented process being *L. sakei* the most

359 competitive of both. Similarly the high prevalence of *E. faecium* strains in meat products have

360 been previously described (Ruiz-Moyano, Martín, Benito, Pérez-Nevado, Córdoba, 2008). In

361 this sense, Ammor et al. 2005 reported that the dominant species in sausages are usually

362 members of *Lactobacillus* genera, although in certain slightly acidified sausages *Lactobacillus*

363 and *Enterococcus* populations reach similar proportion in agreement with the results obtained

364 in this study. In the same manner, in spontaneously fermented Swiss meat product, a notable

365 presence of *Enterococcus* species (18%) was reported among the LAB population (Marty,

366 Buchs, Eugster-Meier, Lacroix & Meile, 2012).

367

368 *3.2. Technological properties*

369

370 In the present study forty-six LAB strains isolated from Spanish dry-cured sausages

371 were screened in order to select eligible strains as starter culture for fermented meat products.

372 In this sense, different technological and safety properties as nitrate reductase, catalase,

373 proteolytic, and lipolytic activity; bile resistance, biofilm formation, antibiotic susceptibility

374 and biogenic amine production were analyzed (Table 2).

375 Although CNS are the main bacteria responsible of the red colour in dry-cured

376 sausages, LAB participate in the formation of this typical colour through the spontaneous

377 reduction of nitrates to nitric oxide. Some meat LAB have been reported to possess

378 significative nitrate reductases and nitrite reductase enzymatic activity directly involved in the

379 mechanisms of nitrosomyoglobin formation, thus the screening of the nitrate reductase activity

380 is desirable, even though in LAB these activities are much lower than in CNS (Ammor &

381 Mayo, 2007). In our study, the LAB strains showed variable nitrate reductase activity (Table 2)

382 ranging from 4.14 (*L. plantarum* Al–110) to 35.04 (*L. sakei* Al–107) mM nitrate reduced to

383 nitrite per milligram of dry weight. This variability was observed also amongst strains even

384 belonging to the same species, e.g. in *E. faecium* strains nitrate reductase activity ranged from

385 4.92 to 15.77, and in *L. sakei* strains from 5.32 to 35.04. Overall, the highest reduction of

386 nitrates was observed in *L. sakei* strains. Bonomo et al. (2008) studied the nitrate activity

387 present in LAB strains isolated from fermented sausages and found that only the strains

388 belonging to *Leuconostoc* genera and *L. sakei* species showed nitrate reductase activity. On the

389 contrary, *P. pentosaceus, L. plantarum* and *Lactobacillus brevis* strains were not able to reduce

16

390 nitrate. Nevertheless, in our study, *L. plantarum* strains showed intermediate nitrate reductase

391 activity which ranged from 4.14 to 7.15 mM nitrate reduced (Table 2).

392 Most lactobacilli are able to form hydrogen peroxide by oxidizing lactate and thus

393 increasing rancidity and the discoloration of the final meat product. Some LAB involved in

394 meat fermentation, such as *L. sakei, L. plantarum, L. pentosus* and *P. acidilactici*, possess

395 weak catalase activity as compared to the constitutive catalase of CNS which is active in meat

396 products hydrolyzing the hydrogen peroxide produced (Ammor & Mayo, 2007). In this study,

397 catalase activity was analyzed using a spectrophotometric method (Table 2). All the analyzed

398 strains showed weak catalase activity in agreement with the reported by Ammor and Mayo

399 (2007).

400 Several aromatic substances and organic acids are released by protease and lipase

401 activity from microorganisms. Proteolysis and lipolysis influence both texture and flavour

402 development due to the formation of low molecular weight compounds (free fatty acids,

403 peptides, etc.). LAB usually do not posses strong proteolytic or lipolytic activities, although a

404 degree of peptidase and lipase activity has been observed for some LAB meat strains (Fadda,

405 Sanz, Vignolo, Aristoy, Oliver, & Toldrá, 1999). Exopeptidases from meat lactobacilli

406 contribute to the generation of free amino acids involved in the flavour. Lactobacilli and

407 pediococci display low catabolism of branched-chain amino acids, being this the reason they

408 do not play a major role in the formation of aroma compounds (Leroy et al., 2006). Moreover,

409 LAB have only weak proteolytic action on myofibrillar proteins (Sanz, Fadda, Vignolo,

410 Aristoy, Oliver, & Toldrá, 1999). Montel, Masson and Talon (1998) reported that despite

411 *Lactobacillus* species are weakly proteolytic in general, some *L. casei, L. plantarum, L.*

412 *curvatus,* and *L. sakei* strains actively contribute to the hydrolysis of the sarcoplasmic proteins,

413 and several peptidase activities have been reported in *L. sakei, L. curvatus* and *L. plantarum*

414 isolated from sausages (Fadda, et al.1999). In this sense, in this study none of the strains

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415 analyzed showed proteolytic activity using gelatine and calcium caseinate agar as substrates

416 (Table 2).

417   In order to analyze the lipolytic activity of LAB strains several assays were performed.

418 Despite the fact that none of the strains could hydrolyze Tween 20 or Tween 80, some of *E.*

419 *faecium* (52%) and all *L. plantarum* and *L. paracasei* strains analyzed showed lipolytic activity

420 in Blue Spirit. In addition, two *L. sakei* strains, AI–143 and AI–144, also showed lipolytic

421 activity in Blue Spirit (Table 2). In agreement with our results, Montel et al. (1998) reported

422 that *Lactobacillus* species are weakly lipolytic. Occasional descriptions of lipolytic activity

423 from LAB strains have been also reported. For example, Silva–Lopes, Cunha, Clemente,

424 Texeira–Carrondo, Barreto–Crespo (1999) described that *L. plantarum* DSMZ 12028, isolated

425 from fermented sausage, produces a lipase during the meat fermentation originating free fatty

426 acids.

427   Even though LAB from dairy products and from intestinal origin are considered to be

428 the main source of probiotics, strains of LAB species found in fermented sausages, such as *L.*

429 *brevis, L. plantarum, L. fermentum*, and *P. pentosaceus,* have also been characterized as

430 probiotic (Klingberg, Axelsson, Naterstad, Elsser, & Budde, 2005). The survival ability of

431 isolates in the presence of oxgall bile is an important characteristic for the selection of

432 potentially probiotic strains. Results obtained by the spectrophotometric method showed that,

433 in general, *L. sakei* strains were more sensitive to bile than *E. faecium* strains as their growth

434 retard was higher (Table 2). However, by the direct method which determines the number of

435 viable colonies after incubation in presence of Oxgall, only two *L. plantarum* strains, AI–122

436 and AI–148, were resistant to bile salt. Several studies described that some lactobacilli, like *L.*

437 *acidophilus, L. casei* and *L. plantarum,* possess hydrolase activity of bile salt (Gilliland &

438 Speck, 1977). When the functional properties of lactobacilli strains isolated from kimchi were

439 studied, only one *L. plantarum* strain survived at high rate in MRS supplemented with 0.5%

440 oxgall, although *L. sakei* strains showed different survival rates suggesting that survival ability

441 is a strain–specific property (Lee, Yoon, Ji, Kim, Park, Lee, Shin, Holzapfel, 2011).

442 Taking into account the activities analyzed so far, the strains possessing significative

443 lipolytic activity could be the ones that have the adequate technological characteristics to be

444 selected as starter cultures. Among these strains, *L. paracasei* Al–128, *L. plantarum* Al–122, *L.*

445 *sakei* Al–144, and the *E. faecium* strains Al–73, Al–74, Al–75, Al–76, Al–77, Al–78, Al–79, Al–

446 83, and Al–89, could be included.

447

3.3. Safety–related properties

449

450 Although LAB are considered GRAS (Generally Regarded As Safe) organisms, it must

451 be kept in mind their potential to transfer antibiotic resistances to pathogenic bacteria or the

452 capacity to produce toxic compounds as biogenic amines. In addition, enterococci are true

453 members of LAB community but they remain controversial because most species harbour a

454 series of virulence factors and have been associated with nosocomial infections, urinary tract

455 infections, bacteraemia, peritonitis, and endocarditis. For these reasons, it is necessary to do an

456 assessment in regards to toxicity and pathogenic determinants as well as the presence of

457 antibiotic resistances.

458 In nature, bacteria have developed a variety of mechanisms of resistance to

459 environmental stresses, such as the formation of biofilms. In food industry, biofilm formation

460 is undesirable for hygienic and safety reasons due to the possible attachment of food spoilage

461 or pathogenic microorganisms to food or food surfaces. The formation of biofilms by some

462 species of LAB has been reported and several studies have described genes responsible for

463 adhesion or biofilm formation (Lebeer, Keersmaecker, Verhoeven, Fadda, Marchal, &

464 Vanderleyden, 2007). In this study, 43% of *L. sakei* strains showed capacity for biofilm

465 formation. Although some authors consider this as a negative characteristic, others considered

466 that in food systems, colonization of surfaces by bacteria used as starters could be desirable to

467 inhibit the colonization by pathogenic or spoilage bacteria (Leriche & Carpentier, 2000).

468 Kubota, Senda, Nomura, Tokuda and Uchiyama (2008) when studied the biofilm formation in

469 *L. plantarum, L. brevis* and *L. fructivorans* strains, obtained that *L. plantarum* produced

470 biofilm better that the other studied species, and the biofilm produced showed a great

471 resistance to acid and ethanol. Similarly, in our study, *L. plantarum* AI–148 showed the

472 capacity to form biofilm, as well as *L. coryniformis* AI–127 and *L. paracasei* AI–120 strains.

473 The presence of *ebpA* gene (endocarditis– and biofilm–associated pilus) has been

474 studied in enterococcal isolates in relation with the capacity to form biofilm (Cobo–Molinos et

475 al., 2008). These authors reported the presence of *ebp* genes in enterococcal isolates using

476 specific primers and showed that a 68% of vegetable isolated strains and a 33% of clinical

477 isolates were PCR–positive. In our study using the spectrophotometric method, only two strains

478 were detected as biofilm–positive but were PCR–negative (*E. faecium* AI–79 and AI–89) (Table

479 2). These apparently contradictory results could be explained due to the variability in *ebp*

480 genes as reported by Cobo–Molinos et al. (2008) in which study the majority of clinical isolates

481 gave positive results using a labelled probe (94.59%) while PCR amplification of *ebp* gene was

482 only positive in 32.43% of clinical isolates. On the hand, other genes involved in biofilm

483 formation were described, for instance, the gene encoding the aggregation substance (Asc10)

484 (Chuang–Smith, Wells, Henry–Stanley, & Dunny, 2010)

485 Clinical use of antibiotics has achieved a significant reduction in the morbility and

486 mortality associated with infectious diseases. Their use has been extended to veterinary

487 medicine, where they are employed as therapeutic agents, prophylactics and animal growth

488 promoters, and to agriculture for the control of plant diseases. This has exerted a very strong

489 selective pressure for the appearance of resistant strains. Resistances are not virulence factors

490 by themselves, but infections with resistant microorganisms complicate the course of the

491 diseases and increase the price of their treatment (Ammor, Florez & Mayo, 2007). Antibiotic

492 resistance determinants contained in starter organisms or naturally occurring LAB may be

493 transferred to commensals or pathogenic bacteria as horizontal transfer of resistance genes

494 occurs in food (Teuber, Meile, & Schwarz, 1999). In this study, the susceptibility of the 46

495 LAB strains to different antibiotics was tested (Table 2). All non-enterococcal LAB strains

496 analyzed were penicillin G sensitive and only two *L. plantarum* strains were ampicillin

497 resistant. All strains were resistant to vancomycin and 43% resistant to rifampicin. In general,

498 the non-enterococcal strains analyzed were sensitive to erythromycin, tobramicin, gentamicin,

499 clindamycin (75%), and tetracycline (72%); and more resistant to amikacin (only 54%

500 sensitive). In agreement with our results, Ammor et al. (2007) reported that, in general,

501 lactobacilli are usually sensitive to penicillins, most species show a high level of resistance to

502 glycopeptides (e.g. vancomycin), are susceptible to antibiotics inhibiting the synthesis of

503 proteins (e.g. erythromycin, clindamycin and tetracycline), and are more resistant to

504 aminoglycosides (e.g. amikacin, and gentamicin). Nevertheless, resistant strains to these agents

505 have also been identified and the presence of several genes providing such resistances has been

506 studied (e.g., *tet* genes for tetracycline resistance). Furthermore, lactobacilli are usually

507 resistant to most inhibitors of nucleic acid synthesis (e.g. rifampicin) (Charteris, Kelly,

508 Morelli, Collins, 1998).

509   All the *E. faecium* strains analyzed in this study were sensitive to vancomycin,

510 gentamicin, chloramphenicol, teicoplanin, and ampicillin, but only 32% showed susceptibility

511 to penicillin G. Contrarily, all the strains were resistant to tetracycline, rifampicin, and

512 ciprofloxacin and showed intermedium resistance to erythromycin. Finally, only three strains

513 were resistant to nitrofurantoine (*E. faecium* AI-58, AI-59, and AI-93). In agreement with our

514 results, Diarra et al. (2010) reported that any of the *E. faecium* strains isolated from broiler

515 chickens was resistant to chloramphenicol, and vancomycin. In the present study, all the *E.*

516 *faecium* strains analyzed were resistant to at least three different classes of antibiotics tested.

517   Resistance to tetracycline was also evaluated by a molecular method based on the

518   amplification by PCR of the *tetM* gene. Resistance to tetracycline is one of the most common

519   acquired antibiotic resistances in *Enterococcus* strains isolated from food (Teuber, 1999).

520   Huys, D´Haene, Collard, and Swings (2004) reported that tetracycline resistance in

521   *Enterococcus* strains isolated from food is due to the presence of the genes already present in

522   clinical and veterinarian isolates, *tetM, tetL*, and *tetS*. In the present study, all *E. faecium*

523   strains analyzed amplified the *tetM* gene (Fig. 1), in agreement with the results obtained by

524   Gervers, et al. (2003) which reported that the *tetM* gene is the most widely distributed *tet* gene,

525   being detected in *Enterococcus, Streptococcus,* and *Bifidobacterium*. Nevertheless, in the non–

526   enterococcal tetracycline–resistant strains analyzed, only *L. sakei* AI–123 and AI–144 strains

527   were PCR–positive. However, *L. conyriformis, L. paracasei*, and *L. plantarum* tetracycline–

528   resistant strains were PCR–negative in agreement with the results of Ammor, Florez, van Hoek,

529   de los Reyes–Gavilán, Aarts, Margolles, Mayo (2008) who reported that *L. plantarum*

530   tetracycline–resistant strains were PCR–negative for the amplification of all *tet* genes. Gerver et

531   al. (2003) found that tetracycline–resistant *L. sakei* strains only amplified *tetM* gene while the

532   amplification of *tetO, tetS, tetK*, and *tetL* was negative. The high level of tetracycline

533   resistance observed in the present study was in agreement with a high detection frequency of

534   *tetM* gene.

535   Other important safety–related trait in enterococci is the presence of virulence factors,

536   as these have been associated with several important pathologies. Many factors determine the

537   virulence of *Enterococcus* species, for example, its ability to colonize the gastrointestinal tract,

538   to adhere to a range of extracellular matrix proteins, urinary tract epithelia, oral cavity, etc. For

539   this reason, in this study, the presence of genes encoding potential virulence factors was also

540   evaluated. The virulence of enterococci is associated with the presence of several genes,

541   including *asa*1 gene, which encodes the aggregation substance Agg, a pheromone–inducible

542   protein that increases the bacterial adherence to renal tubular cells and heart endocardial cells;

543 *esp*, encodes the enterococcal surface protein Esp associated with increased virulence,

544 colonization and persistence in the urinary tract, and biofilm formation; *cylA*, involved in

545 cytolysin production which significantly worsens the severity of endocarditis and

546 endophthalmitis; *hyl*, encodes the hyaluronidase Hyl, homologous to the hyaluronidases of

547 *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Staphylococcus aureus* which are

548 believed to contribute to the invasion of the nasopharinge; *gelE*, encodes the protease GelE

549 with gelatinase activity which has been shown to exacerbate endocarditis; *efaA*, encodes the

550 surface A antigen EfaA involved in cells adherence; *ace*, encodes the collagen adhesine Ace

551 involved in cell adherence and biofilm formation; *hypR*, encodes HypR, a transcriptional

552 regulator of the oxidative stress response and intracellular survival within macrophages. In this

553 study, all the strains were PCR–negative for all the virulence factors tested, however, in the

554 case of the *efaA* gene it was amplified in all the strains analyzed (with exception of AI–79

555 strain). Similarly, Ruiz–Moyano, et al. (2009) reported previously the presence of *efaA* gene in

556 all the starter *E. faecium* strains screened while the determinants for another enterococcal

557 adhesines (*esp* and *ace*) could not be detected in any strain. The absence of several virulence

558 genes in *E. faecium* isolated from food was also described by Fisher and Phillips (2009). These

559 authors reported that the gene encoding Agg was not present in *E. faecium* isolates, and the *esp*

560 gene was absent from dairy isolates but present in clinical isolates, suggesting that the *esp* gene

561 could be associated with pathogenicity. In the case of *cytA* gene, the same authors reported a

562 higher incidence of this gene in clinical isolates (33%) as compared to 6% in food isolates.

563 Moreover, any strain amplified the *gelE* gene, thought to provide nutrients to the bacteria by

564 degrading host tissue and involved in biofilm formation; therefore, the absence of *gelE* gene is

565 related to the low incidence of biofilm formation found in the strains analyzed in this study.

566 The presence of biogenic amines is a relevant food issue in meat products. In this study,

567 biogenic amine production was tested by using two different methods, TLC (Garcia–Moruno et

568 al., 2005) and PCR (de las Rivas et al., 2006). Results obtained are showed in Table 2. Most of

569 the *E. faecium* strains analyzed produced tyramine (79%) (Fig. 2) and all of them were PCR–

570 positive. The high incidence of tyramine production in these enterococci is in agreement with

571 the results reported by other authors (Komprda, Sládková, Petirová, Dohnal & Burdychová,

572 2010). In this study, *E. faceium* AI–74, AI–75, AI–79, and AI–87 were PCR–positive for the

573 presence of the tyrosine decarboxylase gene, but were TLC–negative for tyramine production.

574 In this sense, Muñoz–Atienza, Landeta, de las Rivas, Gómez–Sala, Muñoz, Hernández, Cintas

575 and Herranz (2011) similarly reported the non–production of tyramine in enterococci

576 possessing a tyrosine decarboxylase gene due to the absence of gene expression.

577 In the non–enterococcal LAB strains analyzed, only one *L. sakei* strain showed biogenic

578 amine production, *L. sakei* AI–142, which produced tyramine and was PCR–positive for the

579 presence of the tyrosine decarboxylase gene (Fig. 3). The low capacity for biogenic amine

580 production in the LAB strains analyzed in this study is in agreement with the results reported

581 by Ruiz–Moyano et al (2009). These results are in agreement with the ones reported by other

582 authors (Priyadarshani & Rakshit, 2011), which state that the presence, the activity and the

583 specificity of decarboxylases is strain–specific. Therefore, careful screening for amino acid

584 decarboxylase activity is recommended before selecting LAB as appropriate starter strains in

585 food industry.

586 In conclusion, in this study the technological and safety–related characterization of LAB

587 allowed to select strains to be used as meat starter. Attending to the safety–related properties, in

588 the final strain selection have been rejected the strains showing capacity to produce biogenic

589 amines (mainly tyramine), to produce biofilms, and to possess a high number of antibiotic

590 resistance determinants (3 or more resistances). As only the presence (or absence) of lipolytic

591 activity was clearly shown by the remaining strains, strains *L. paracasei* AI–128 and *L. sakei*

592 AI–143 strains were finally selected. Other technological properties such as catalase,

593 proteolytic, nitrate reductase, resistance to bile salts, etc. which did not shown a clear variation

594 among the strains analyzed were not considered.

595

4. References

Ammor, S., Rachman, C., Chaillou, S., Prévost, H., Dousset, X., Zagorec, M., Dufour, E., & Chevallier, I. (2005). Phenotypic and genotypic identification of lactic acid bacteria isolated from a small–scale facility producing traditional dry sausages. *Food Microbiology 22*, 373–382

Ammor, S. & Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Science 76*, 138–146

Ammor, S., Florez, A.B., & Mayo, B. (2007). Antibiotic resistance in non–enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology 24*, 559–570

Ammor, S., Florez, A.B., van Hoek, A.H.A.M., de los Reyes–Gavilán, C., Aarts, H.J.H., Margolles, A., & Mayo, B. (2008). Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. *Journal of Molecular Microbiology and Biotechnology 14*, 6–15

619

620 Benito , M. J., Serradilla, M. J., Ruiz–Moyano, S, Martín, A., Perez–Nevado, F., & Córdoba,

621 M. G. (2008). Rapid differentiation of lactic acid bacteria from autochthonous fermentation of

622 Iberian dry–fermented sausages. *Meat Science 80*, 656-661

623

624 Bonomo, M. G., Ricciardi, A., Zotta, T., Parente, E., & Salzano, G. (2008). Molecular and

625 technological characterization of lactic acid bacteria from traditional fermented sausages of

626 Basilicata region (Southern Italy). *Meat Science 80*, 1283–1248.

627

628 Cariolato, D., Andrighetto, C., & Lombardi, A. (2008). Ocurrence of virulence and antibiotic

629 resistances in *Enterococcus faecalis* and *Enterococcus faecium* collected from dairy and

630 human samples in North Italy. *Food Control 19*, 886–892

631

632 Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Antibiotic susceptibility of

633 potentially probiotic *Lactobacillus* species. *Journal of Food Protection 61*, 1636–1643.

634

635 Chuang–Smith, O. N. Wells, C. L., Henry–Stanley, M. J. & Dunny, G. M. (2010). Aceleration

636 of *Enterococcus* biofilm formation by aggregation substance expression in an Ex Vivo model

637 of cardiac valve colonization. PLoS ONE 5 (*12*) art. no e15798

638

639 Cobo–Molinos, A., Hikmate, A., Ben–Omar, N., Lucas–López, R., & Galvez, A. (2008).

640 Detection of *ebp* (endocarditis–and biofilm–associated pilus) genes in enterococcal isolates

641 from clinical and non–clinical origin. *International Journal of Food Microbiology 126*, 123–

642 126

643

644 Comi, G., Urso, R., Iacumin, L., Rantsiou, K., Cattaneo, P., Cantoni, C., & Cocolin, L. (2005).

645 Characterisation of naturally fermented sausages produced in the north coast of Italy. *Meat*

646 *Science 69*, 381–392.

647

648 Creti, R., Imperi, M., Bertuccini L., Fabretti, F., Orefici, G., Di Rosa, R., & Baldassarri, R.

649 (2004). Survey for virulence determinants among *Enterococcus faecalis* isolated from different

650 sources. *Journal of Medical Microbiology 53*, 13–20

651

652 De las Rivas, B., Marcobal, A., Carrascosa, A. V., & Muñoz, R. (2006). PCR detection of

653 foodborne bacteria producing the biogenic amines histamine, tyramine, putrescine, and

654 cadaverine. *Journal of Food Protection 69*, 2509–2514

655

656 Diarra, M. S., Rempel, H., Champagne, J., Masson, L., Pritchard, J., & Topp, E. (2010).

657 Distribution of antimicrobial resistance and virulence genes in *Enterococcus* spp. and

658 characterization of isolates from Broiler Chickens. *Applied and Environmental Microbiology*

659 *76*, 8033–8043

660

661 Essid, I., Ben Ismail, H., Bel Hadj Ahmed, S., Ghedamsi, R., & Hassouna, M. (2007).

662 Characterization and technological properties of *Staphylococcus xylosus* strains isolated from

663 a Tunisian traditional salted meat. *Meat Science 77*, 204–212

664

665 Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M., Oliver, G., & Toldrá, F. (1999). Hydrolisis of

666 pork muscle sarcoplasmic proteins by *Lactobacillus curvatus* and *Lactobacillus sakei. Applied*

667 *and Environmental Microbiology 65*, 578–584.

668

669 Fisher, K. & Phillips, C. (2009). The ecology, epidemiology and virulence of *Enterococcus*.

670 *Microbiology 155*, 1749–1757

671

672 Freeman, D. J., Falkiner, F. R., & Keane, C. T. (1989). New method for detecting slime

673 production by coagulase negative staphylococci. *Journal of Clinical Pathology 42*, 872–874

674

675 García–Moruno, E., Carrascosa, A. V. & Muñoz, R. (2005). A rapid and inexpensive method

676 for the determination of biogenic amines from bacterial cultures by thin–layer chromatography.

677 *Journal of Food Protection 68*, 625–629.

678

679 Gervers, D., Danielsen, M., Huys, G., & Swings, J. (2003). Molecular characterization of *tetM*

680 genes in *Lactobacillus* isolates from different types of fermented dry sausages. *Applied and*

681 *Environmental Microbiology 69*, 1270–1275

682

683 Gilliland, S. E., & Speck, M. L. (1977). Deconjugation of bile acids by intestinal lactobacilli.

684 *Applied and Environmental Microbiology 33*, 15–18

685

686 Huys, G., D´Haene, K., Collard, J. M., & Swings, J. (2004). Prevalence and molecular

687 characterization of tetracycline resistance in *Enterococcus* isolates from food. *Applied and*

688 *Environmental Microbiology 70*, 1555–1562

689

690 Klingberg, T. D., Axelsson, L., Naterstad, K., Elsser, D., & Budde, B. B. (2005). Identification

691 of potential probiotic starter cultures for Scandinavian–type fermented sausages. *International*

692 *Journal of Food Microbiology 105*, 419–431.

693

694 Komprda, T., Sládková, P., Petirová, E., Dohnal, V., & Burdychová, R. (2010). Tyrosine–and

695 histidine–decarboxylase positive lactic acid bacteria and enterococci in dry fermented sausages.

696 *Meat Science 86*, 870–877.

697

698 Kubota, H., Senda, S., Nomura, N., Tokuda, H., & Uchiyama, H. (2008). Biofilm formation by

699 acid lactic bacteria and resistance to environmental stress. *Journal of Bioscience and*

700 *Bioengineering 106*, 381–386

701

702 Lebeer, S., Keersmaecker, S. C. J., Verhoeven, T. L. A., Fadda, A. A., Marchal, K. &

703 Vanderleyden, J. (2007). Functional analysis of LuxS in the probiotic strain *Lactobacillus*

704 *rhamnosus GG* reveals a central metabolic role important for growth and biofilm formation.

705 *Journal Bacteriology 189*, 860–871.

706

707 Lee, H., Yoon, H., Ji, Y., Kim, H., Park, H., Lee, J., Shin, H., & Holzapfel, W. (2011)

708 Functional properties of *Lactobacillus* strains isolated from kimchi. *International Journal of*

709 *Food Microbiology 145*, 155–161

710

711 Leriche , V., & Carpentier, B. (2000). Limitation of adhesion and growth of *Listeria*

712 *monocytogenes* on stainless steel surfaces by *Staphylococcus sciuri* biofilms. *Journal of*

713 *Applied Microbiology 88*, 594–605

714

715 Leroy, F, Verluyten, J., & De Vuyst, L. (2006). Functional meat starter cultures for improved

716 sausage fermentation. *International Journal of Food Microbiology 106*, 270–285

717

718 Mannu, L., Paba, A., Caga, E., Comunian, R., Zanetti, S., Dupré, I., & Sechi, L. A. (2003).

719 Comparison of the incidence of virulence determinants and antibiotic resistance between

Enteroccus faecium strains of dairy, animal and clinical origin. *International Journal Food Microbiology 88*, 291–304

Marchesi, J. R., Sato, T.,Weghtman, A. J., Martin, T. A., Fry, J. C., Hion, S.J., & Wade, W. G. (1998). Design and evaluation of useful bacterium–specific DNA primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology 84*, 117–123

Martín, B., Hugas, M., Bover–Cid, S., Veciana–Nogués, M.T., & Aymerich, T. (2006). Molecular, technological and safety characterization of Gram–positive catalase–positive cocci from slightly fermented sausages. *International Journal of Food Microbiology 107*, 148–158

Marty, E., Buchs, J., Eugster–Meier, E., Lacroix, C., & Meile, L. (2012). Identification of staphylococci and dominant lactic acid bacteria in spontaneously fermented Swiss meat products using PCR–RFLP. *Food Microbiology 29*, 157–166

Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., & Tsakalidou, E. (2006). Probiotic potential of *Lactobacillus* strains isolated from dairy products. *International Dairy Journal 16*, 189–199

Miralles, M. C., Flores, J., & Pérez–Martínez, G. (1996). Biochemical test for the selection of *Staphylococcus* strains as potential meat starter cultures. *Food Microbiology 13*, 227–236

Montel, M.C., Masson, F., & Talon, R. (1998). Bacterial role in flavour development. *Meat Science 49* (Suppl. 1), S111–S123

745 Muñoz–Atienza, E., Landeta, G., de las Rivas, B., Gómez–Sala, B., Muñoz, R., Hernández, P.

746 E., Cintas, L. M., & Herranz, C. (2011). Phenotypic and genotypic evaluation of biogenic

747 amine production by lactic acid bacteria isolated from fish and fish products. *International*

748 *Journal of Food Microbioogyl 146*, 212–216.

749

750 Nallapareddy, S. R., Singh, K. V., Sillampaa, J., Garsin, D. A., Hook, M., Erlandsen, S. L., &

751 Murray, B. E. (2006). Endocarditis and biofilm–associated pili of Enterococcus faecalis.

752 *Journal of Clinical Investigation 116*, 2799–2807.

753

754 NCCLS. (2002). Performance standards for antimicrobial susceptibility testing; Twelfth

755 Informational Supplement M100–S–12 NCCLS. Wayne, Pennsylvania.

756

757 Priyadarshani, W. M. D., & Rakshit, S. K. (2011). Screning selected strains of probiotic lactic

758 acid bacteria for their ability to produce biogenic amines (histamine and tyramine).

759 *International Journal of Food Science and Technology 46*, 2062–2069

760

761 Rantsiou, K., Drosinos, E. H., Gialitaki, M., Metaxopoulos, I., Comi, G. & Cocolin, L. (2006).

762 Use of molecular tools to characterize Lactobacillus spp. Isolated from Greek traditional

763 fermented sausages. *International Journal of Food Microbiology 112*, 215–222

764

765 Rantsiou , K. & Cocolin, L. (2008) Fermented meat products. In L. Cocolin & D. Ercolini

766 (Eds.). Molecular techniques in the microbial ecology of fermented foods (pp. 91–118). New

767 York: Springer.

768

769 Ruiz–Moyano, S., Martín, A., Benito, M. J., Pérez–Nevado, F., & Córdoba, M. G. (2008).

770 Screening of lactic acid bacteria and bifidobacteria for potential probiotic use in Iberian dry

771 fermented sausages. *Meat Science. 80*, 715–721

772

773 Ruiz–Moyano, S., Martín, A., Benito, M. J., Aranda, E., Casquete, R., & Córdoba, M. G.

774 (2009). Safety and functional aspects of preselected enterococci for probiotic use in Iberian

775 dry–fermented sausages. *Journal of Food Science. 74*, 398–404

776

777 Saavedra, L., Taranto, M. P., Sesma, F., & de Valdez, G. F. (2003). Homemade traditional

778 cheeses for the isolation of probiotic *Enterococcus faecium* strains. *International Journal of*

779 *Food Microbiology 88*, 241–245

780

781 Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular Cloning: a laboratory manual,

782 second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

783

784 Sanz, Y., Fadda, S., Vignolo, G., Aristoy, M.C., Oliver, G., & Toldrá, F. (1999). Hydrolysis of

785 muscle myofibrillar proteins by *Lactobacillus curvatus* and *L. sakei*. *International Journal of*

786 *Food Microbiology 53*, 115–125

787

788 Silva Lopes, M. F., Cunha, A. E., Clemente, J. J., Texeira Carrondo, M. J. & Barreto Crespo,

789 M. T. (1999). Influence of environmental factors on lipase production by *Lactobacillus*

790 *plantarum. Applied Microbiology and Biotechnology 51*, 249–254

791

792 Teuber, M. (1999). Spread of antibiotic resistance with food–borne pathogens. *Cellular and*

793 *Molecular Life Sciences 56*, 755–763

794

795 Teuber, M., Meile, L., & Schwarz, F. (1999). Acquired antibiotic resistance in lactic acid

796 bacteria from food. *Antonie van Leeuwenhoek International Journal of General and Molecular*

797 *Microbiology 76*, 115–137

798

799 Toledo–Arana, A., Valle, J., Solano, C., Arrizubieta, M.J., Cucarella, C., Lamata, M.,

800 Amorena, B., Leira, J., Penadés, J.R. & Lasa, I. (2001). The enterococcal surface protein, Esp,

801 is involved in *Enterococcus faecalis* biofilm formation. *Applied Environmental Microbiology*

802 *67*, 4538-4545

803

804 Vankerckhoven, V, Van Autgaerden, T., Vael, C., Lammerns, C., Chapelle, S., Rossi, R.,

805 Jabes, D., & Goossens, H. *(2004). Development of a multiplex PCR for the detection of asa1,*

806 *gelE, cylA, esp,* and *hyl* genes in Enterococci and survey for virulence determinants among

807 European hospital isolates of *Enterococcus faecium. Journal of Clinical Microbiology 42*,

808 4473-4479

809

810 Velasco, D., Perez, S., Peña, F., Dominguez, M.A, Cartelle, M., Molina, F., Moure, R.,

811 Villanueva, R, & Bou, G. (2004). Lack of correlation between phenotypic techniques and

812 PCR–based genotypic methods for identification of *Enterococcus* spp. *Diagnostic*

813 *Microbiology and Infectious Disease 49*, 151–156

814

815 Verneuil, N., Sanguinettti, M., Breton, Y. L, Posteraro, B., Fadda, G., Auffray, Y., Hartke, A.,

816 & Giard, J. C. (2004). Effects of the *Enterococcus faecalis hypR* gene encoding a new

817 transcriptional regulator on oxidative stress response and intracellular survival within

818 macrophages. *Infection and Immunity 72*, 4424-4431

819

820 Figure captions

Fig. 1. PCR amplification of the *tetM* gene involved in tetracycline resistance by using primers

tetM–F and tetM–R that give an amplicon of 1513 pb. PCR was performed by using DNA from

E. faecium strains: AI–59 (lane 1), AI–73 (lane 2), AI–74(lane 3), AI–75 (lane 4), AI–76 (lane 5),

AI–77 (lane 6), AI–78 (lane 7), AI–79 (lane 8), AI–83 (lane 9), AI–85 (lane 10), AI–86 (lane 11),

AI–87 (lane 12), AI–88 (lane 13), AI–89 (lane 14). A DNA marker standard (EcoRI/HindIII

digested λ DNA) was included in the gel and some of their fragments indicated on the right of

the figure.

Fig. 2. Tyramine production by LAB strains using TLC. The strains were grown in MRS

containing tyrosine and the tyramine produced during the growth was converted into its

fluorescent dansyl derivative and detected. The analyzed strains were *E. faecium* AI–59 (lane

1), *E. faecium* AI–79 (lane 2), *E. faecium* AI–85 (lane 3), *E. faecium* AI–92 (lane 4), *L. sakei* AI–

142 (lane 5). Tyramine standard solution (lane 6).

Fig.3. PCR amplification of the tyrosine decarboxylase gene by using primers TDC–F and

TDC–R that give an amplicon of 825 pb. PCR was performed by using DNA from *L. sakei*

strains: AI–137 (lane 1), AI–139 (lane 2), AI–142 (lane 3), AI–143 (lane 4), AI–144 (lane 5), AI–

146 (lane 6), and AI–147 (lane 7). DNA from *E. faecium* AI–57 was used as positive control

(lane 8). A DNA marker standard (EcoRI/HindIII digested λ DNA) was included in the gel and

some of their fragments are indicated on the left of the figure.

Figure 1

Figure 1 (Landeta et al.)
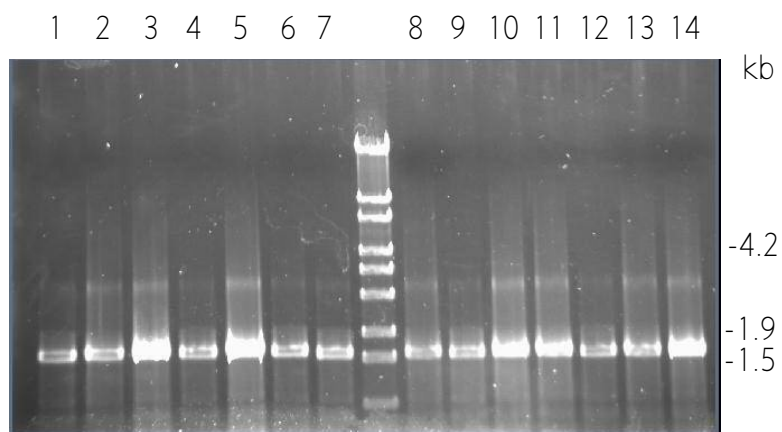
Figure 2

Figure 2 (Landeta el at.)

tyramine
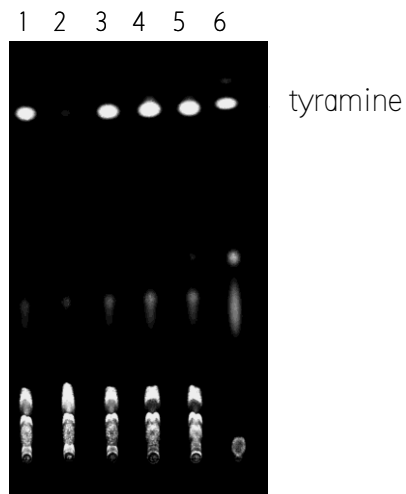
Figure 3

Figure 3 (Landeta et al.)
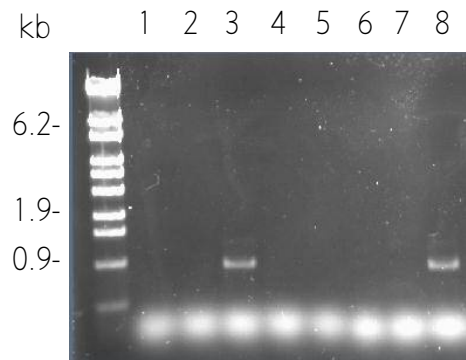
Table 1

TABLE 1. Primers used in this study

Gene	Primer	Sequence[a]	Amplicon size (bp)	references
16S	63f	CAGGCCTAACACATGCAAGTC	1324	Marchesi et al., 1998
	1387R	GGGCGGWGTGGTTACAAGGC		
ebpA	Ef1091F	CCGCTCGAGAACTAACAAAAATGATTCGGCTCCAG	1064	Cobo-Molinos et al., 2008
	Ef1091R	CCGCTCGAGCCATCTCACGCATTTTATCTTCAACT		
tetM	tetM-F	GAYACNCCNGGNCAYRTNGAYTT	1513	Gervers et al., 2003
	tetM-R	CACCGAGCAGGGATTTCTCCAC		
ace	Ace1	GGAATGACCGAGAACGATGGC	616	Creti et al. 2004
	Ace2	GCTTGATGTTGGCCTGCTTCCG		
gelE	GEL11	AGTTCATGTCTATTTTCTTCAC	213	Vankerckhoven et al. 2004
	GEL12	AGATGCACCCGAAATAATATA		
Asa1	ASA11	CACGCTATTACGAACTATGA	375	Vankerckhoven et al. 2004
	ASA12	TAAGAAAGAACATCACCACGA		
efaA	efaA1	CGTGAGAAAGAAATGGAGGA	499	Mannu et al. 2003
	efaA2	CTACTAACACGTCACGAATG		
esp	ESP 14F	AGATTTCATCTTTGATTCTTGG	510	Vankerckhoven et al 2004.
	ESP 12R	AATTGATTCTTTAGCATCTGG		
cylA	CYT I	ACTCGGGGATTGATAGGC	688	Vankerckhoven et al. 2004
	CYT I1b	GCTGCTAAAGCTGCGCTT		
hyl	HYL n1	ACAGAAGAGCTGCAGGAAATG	276	Vankerckhoven et al. 2004
	HYL n2	GACTGACGTCCAAGTTTCCAA		
hypR	hypR1	CGTGGCAAGAAGATTCCTTAC	465	Verneuil et al. 2004
	hypR2	GCTAAATATTCTCCTTCAGGTG		
hdc	HIS1-F	GGNATNGTNWSNTAYGAYMGNGCNGA	372	De las Rivas et al., 2006
	HIS1-R	ATNGCDATNGCNSWCCANACNCCRTA		

(Cont.)

tdc	TDC-F	TGGYTNGTNCCNCARACNAARCAYTA	825	De las Rivas et al., 2006
	TDC-R	ACRTARTCNACCATRTTRAARTCNGG		
odc	PUT1-F	TWYMAYGCNGAYAARACNTAYTTYGT	1440	De las Rivas et al., 2006
	PUT1-R	ACRCANAGNACNCCNGGNGGRTANGG		
	PUT2-F	ATHWGNTWYGGNAAYACNATHAARAA	624	De las Rivas et al., 2006
	PUT2-R	GCNARNCCNCCRAAYTTNCCDATRTC		
ldc	CAD2-R	CAYRTNCCNGGNCAYAA	1185	De las Rivas et al., 2006
	CAD2-F	GGDATNCCNGGNGGRTA		

[a] Y = C or T; R = A or G; W = A or T; S = C or G; M = A or C; D = A, G, or T; H = A, C, or T; B = C, G, ot T; N = A, C, G, or T.

Table 2

Table 2. Characteristics of analyzed strains.

Species	Strain	NR[a]	Catalase[b]	Proteolytic[c] Gel	CCA	Lipolytic[d] BS	T20/T80	Biofilm[e] RC	EM	ebpA	Bile salt[f] Rt	DM	Biogenic amines[g] TLC H	T	P	C	PCR H	T	P	C	Antibiotic[h] Am	Clo	Cli	Ery	Gen	PeG	Tet[i]	Van	Tei	Rif	Nit	Cip	Ai	To
E. faecium	AI-57	9.43	0.17	-	-	+	-	-	-	ND	67	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	I	R	ND	ND
	AI-58	6.74	0.12	-	-	-	-	-	-	ND	68	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	R	R	ND	ND
	AI-59	7.13	0.14	-	-	-	-	-	-	ND	70	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R+	S	S	R	R	R	ND	ND
	AI-73	8.39	0.12	-	-	+	-	-	-	ND	80	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	I	R	ND	ND
	AI-74	4.92	0.13	-	-	+	-	-	-	ND	36	S	-	-	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	I	R	ND	ND
	AI-75	11.84	0.14	-	-	+	-	-	-	ND	87	S	-	-	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	I	R	ND	ND
	AI-76	12.23	0.15	-	-	+	-	-	-	ND	68	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R+	S	S	R	I	R	ND	ND
	AI-77	15.16	0.17	-	-	+	-	-	-	ND	91	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R+	S	S	R	I	R	ND	ND
	AI-78	9.58	0.12	-	-	+	-	-	-	ND	103	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R+	S	S	R	I	R	ND	ND
	AI-79	6.00	0.13	-	-	+	-	-	+	-	79	S	-	-	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	I	R	ND	ND
	AI-83	15.77	0.15	-	-	+	-	-	-	ND	76	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	I	R	ND	ND
	AI-85	13.87	0.19	-	-	-	-	-	-	ND	54	I	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	I	R	ND	ND
	AI-86	10.20	0.14	-	-	-	-	-	-	ND	112	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	S	R	ND	ND
	AI-87	9.94	0.14	-	-	-	-	-	-	ND	88	S	-	-	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	I	R	ND	ND
	AI-88	7.95	0.16	-	-	-	-	-	-	ND	100	S	-	+	-	-	-	+	-	-	S	I	ND	I	S	R	R+	S	R	S	R	R	ND	ND
	AI-89	8.12	0.15	-	-	+	-	-	++	-	107	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	S	R	ND	ND
	AI-91	11.68	0.16	-	-	-	-	-	-	ND	92	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R+	S	S	R	S	R	ND	ND
	AI-92	6.15	0.10	-	-	-	-	-	-	ND	98	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	S	R+	S	S	R	S	R	ND	ND
	AI-93	15.36	0.16	-	-	-	-	-	-	ND	103	S	-	+	-	-	-	+	-	-	S	S	ND	I	S	R	R+	S	S	R	R	R	ND	ND
L. coryniformis	AI-127	7.61	0.05	-	-	-	-	+	+	ND	153	I	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	R-	R	ND	S	ND	ND	S	S
L. paracasei	AI-120	6.86	0.10	-	-	+	-	+	-	ND	109	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	R-	R	ND	S	ND	ND	R	S
	AI-128	15.49	0.10	-	-	+	-	-	-	ND	206	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	S	S
L. plantarum	AI-110	4.14	0.24	-	-	+	-	-	-	ND	113	S	-	-	-	-	-	-	-	-	S	ND	R	S	S	S	R-	R	ND	R	ND	ND	S	S
	AI-116	4.59	0.13	-	-	+	-	-	-	ND	90	S	-	-	-	-	-	-	-	-	R	ND	S	S	S	S	R-	R	ND	R	ND	ND	S	S
	AI-122	8.92	0.22	-	-	+	-	-	-	ND	87	R	-	-	-	-	-	-	-	-	R	ND	R	S	S	S	R-	R	ND	R	ND	ND	S	S
	AI-148	7.15	0.23	-	-	+	-	+	-	ND	74	R	-	-	-	-	-	-	-	-	S	ND	R	S	S	S	S	R	ND	R	ND	ND	S	S
L. sakei	AI-107	35.04	0.13	-	-	-	-	-	+	ND	136	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	S	S
	AI-109	11.31	0.12	-	-	-	-	+	+	ND	76	I	-	-	-	-	-	-	-	-	R	ND	S	I	S	S	S	R	ND	S	ND	ND	S	S
	AI-112	17.24	0.33	-	-	-	-	+	+	ND	9	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	R	ND	ND	R	S
	AI-113	18.62	0.10	-	-	-	-	+	+	ND	25	S	-	-	-	-	-	-	-	-	S	ND	S	I	S	S	S	R	ND	S	ND	ND	S	S
	AI-115	6.01	0.11	-	-	-	-	+	++	ND	43	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	I	S
	AI-121	5.32	0.21	-	-	-	-	+	++	ND	39	I	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	I	S
	AI-123	19.64	0.37	-	-	-	-	+	+	ND	48	I	-	-	-	-	-	-	-	-	S	ND	S	I	S	S	R+	R	ND	R	ND	ND	I	S
	AI-125	7.01	0.24	-	-	-	-	+	+	ND	53	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	I	S
	AI-129	11.64	0.13	-	-	-	-	-	-	ND	254	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	R-	R	ND	R	ND	ND	S	S
	AI-132	6.48	0.11	-	-	-	-	-	+	ND	203	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	I	S
	AI-134	24.85	0.15	-	-	-	-	+	-	ND	55	S	-	-	-	-	-	-	-	-	S	ND	R	S	S	S	S	R	ND	R	ND	ND	I	S
	AI-135	14.69	0.18	-	-	-	-	-	-	ND	526	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	S	S
	AI-136	18.61	0.12	-	-	-	-	+	-	ND	96	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	R	S
	AI-137	8.21	0.39	-	-	-	-	-	-	ND	1357	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	R	S
	AI-139	23.15	0.10	-	-	-	-	-	-	ND	75	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	S	S
	AI-142	9.04	0.33	-	-	-	-	-	-	ND	268	I	-	+	-	-	-	+	-	-	S	ND	R	S	S	S	S	R	ND	R	ND	ND	S	S
	AI-143	5.88	0.13	-	-	+	-	-	-	ND	469	S	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	R	S
	AI-144	16.57	0.27	-	-	+	-	-	-	ND	422	S	-	-	-	-	-	-	-	-	S	ND	R	S	S	S	R+	R	ND	R	ND	ND	I	I
	AI-146	6.72	0.45	-	-	-	-	-	-	ND	329	I	-	-	-	-	-	-	-	-	S	ND	S	S	S	S	S	R	ND	S	ND	ND	I	S
	AI-147	15.30	0.09	-	-	-	-	-	-	ND	111	S	-	-	-	-	-	-	-	-	S	ND	Cli	S	S	S	S	R	ND	S	ND	ND	S	S