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Biogenic amine production by bacteria isolated from ice-preserved sardine and mackerel

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ABSTRACT

The occurrence of *in vitro* production of biogenic amines in bacteria isolated from ice–preserved sardine and mackerel was studied. Biogenic amine production was investigated by means of amino acid decarboxylation by growth on decarboxylase differential medium, biogenic amine detection by thin–layer chromatography (TLC) and decarboxylase gene detection by PCR. Decarboxylase medium overestimate the number of biogenic amine–producer strains, as the production of amine was confirmed by TLC in only five out the 17 presumptive strains. On the producer strains, PCR was used to confirm the presence of the genes encoding the amino acid decarboxylase responsible for the synthesis of these amines. Moreover, biogenic amine–producer bacteria were molecularly identified by sequencing their 16S rRNA. Form sardine, enterobacteria producing simultaneously several biogenic amines were isolated. A *Kluyvera intermedia* strain producing histamine, putrescine and cadaverine, and an *Enterobacter asburiae* strain producing only the diamines cadaverine and putrescine were identified. From mackerel, lactic acid bacteria from the *Enterococcus durans* species producing tyramine were isolated. This study constitutes the first description of the presence on these putatively harmful species on ice–preserved sardine and mackerel.

Keywords: *Sardina pilchardus*; *Scomber scombrus*; *Enterobacteriaceae*; Histamine; Tyramine; Putrescine; Cadaverine.

42   1. Introduction

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44   The presence of biogenic amines in fish is of concern to researchers, consumers,

45   food companies, and health authorities due to their toxicological effects. Biogenic amines

46   have been classified regarded as potentially hazardous compounds of food that may cause

47   disorders to consumers (Halász, Baráth, Simon–Sarkadi, & Holzapfel, 1994; Silla, 1996).

48   Moreover, these amines serve as chemical indicators of fish spoilage as these amines are

49   essentially absent or occur at very low levels in fresh fish (Al Bulushi, Poole, Deeth, &

50   Dykes, 2009). Despite a widely reported association between histamine and scombroid

51   food poisoning, histamine alone appears to be insufficient to cause food toxicity.

52   Putrescine and cadaverine have been suggested to potentiate histamine toxicity. With

53   respect to spoilage on the other hand, only cadaverine has been found to be a useful index

54   of the initial stage of fish decomposition (Al Bulushi, Poole, Deeth, & Dykes, 2009). In

55   most early studies on biogenic amine formation in fish, researchers have focused on

56   histamine poisoning and concluded that the families *Scombridae* and *Scomberesocideae*

57   are commonly implicated in incidents of histamine poisoning as they contained high levels

58   of free histidine in the muscle. Histamine is produced by bacteria that decarboxylate

59   histidine to histamine as the fish decomposes. Indeed, various scombroids, including

60   mackerel, tunas, saury, bonito, seerfish and butterfly kingfish, have been implicated in

61   cases of histamine poisoning. However, non–scombroid fish which also contained high

62   levels of free histidine in the muscle, such as sardine, pilchards, anchovies, herring and

63   marline, has also been implicated in cases of histamine poisoning (Taylor, 1986).

64   Biogenic amines accumulation usually results from the decarboxylation of amino

65   acids by enzymes of bacterial origin, which is associated with food hygiene and

66   technology. Therefore, poor hygiene is probably the main factor involved in the formation

67 of these compounds. Bacterial contamination could be derived from postcatching

68 contamination on board fishing vessels, at the processing plant or in the distribution

69 system. The formation of biogenic amines in fish requires the presence of decarboxylase–

70 producing microoganisms, which may be introduced by contamination. Adequate

71 concentrations of the precursor free amino acids and environmental factors supporting

72 bacterial growth and favouring the synthesis of decarboxylase enzymes are also of critical

73 significance (Halász, Baráth, Simon–Sarkadi, & Holzapfel, 1994).

74   The presence of bacteria able to produce biogenic amines can be detected by a

75 variety of techniques including HPLC, TLC, GC/MS and enzymatic test (Marcobal, de las

76 Rivas, & Muñoz, 2006). Molecular methods for detection and identification of food–borne

77 bacteria are becoming more widely accepted as an alternative to traditional culture

78 methods. The use of molecular tools for early and rapid detection of biogenic amine

79 bacteria is important for preventing the accumulation of these biogenic amines in fish and

80 other food products. Genetic procedures accelerate getting results and allow the

81 introduction of early control measures to avoid the development of these bacteria. Several

82 studies describing PCR techniques targeting bacterial amino acid decarboxylase genes

83 have been reported (De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006; Marcobal, de

84 las Rivas, & Muñoz, 2006; Landete, de las Rivas, Marcobal, & Muñoz, 2007)

85   Histamine producer strains (*Morganella morganii*, *Proteus vulgaris*, *Pantoea*

86 *agglomerans*, *Enterobacter cloacae*) have been identified from mackerel, a scombroid fish

87 (Kim et al., 2001; Tsai et al., 2005). From sardine, a non–scrombroid fish which possess a

88 high level of free histidine on the muscle, in spite that microbiological analysis have been

89 done, none specific bacterial species has been related to biogenic amine production

90 (Ababouch, Afilal, Benabdeljelil, & Busta, 1991; Erkan & Özden, 2008). Moreover, none

91 of the molecular methods available for bacterial identification and for the detection of

92   biogenic amine producer bacteria had never been applied in mackerel and sardines

93   samples. Therefore, the present study deals, for the first time, with the molecular

94   characterization of the *in vitro* biogenic amine–producer microbiota present on ice–

95   preserved sardine and mackerel.

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98   2. Materials and methods

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100   *2.1. Sampling procedure*

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102   The freshwater fishes, sardine (*Sardina pilchardus*) and mackerel (*Scomber*

103   *scombrus*) were caught in Mediterranean sea (the coast of Tunisia), iced immediately in an

104   ice box and delivered to the laboratory approximately 2 h later. Upon arrival at the lab,

105   sardines and mackerel were placed individually. Whole ungutted fish were stored at 4 ºC

106   for 7 days. Samples of dorsal muscle were aseptically homogenised with a pestle in sterile

107   saline solution.

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109   *2.2. Evaluation of amino acid–decarboxylase activity*

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111   *2.2.1. Growth in decarboxylase Niven´s medium*

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113   For detection of amino acid decarboxylating bacteria in fish homogenates, samples

114   were serially diluted in saline and spread plated on modified Niven´s agar [0.5% tryptone,

115   0.5%yeast extract, 0.5% NaCl, 0.1% $CaCO_3$, 3% agar, and 0.006% bromocresol purple,

116   pH 5.3] (Mavromatis, & Quantick, 2002) containing L–histidine monohydrochloride, L–

117   ornithine monohydrochloride, and L–lysine, at 0.25%, and tyrosine disodium salt at 0.2%

118   due to its low solubility. The inoculated plates were incubated at 30 ºC during 1–3 days.

119   After the incubation time, the colour around the colonies was reported. Presumptively, the

120   appearance of a purple or slight–purple colour indicated biogenic amine production.

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123   *2.2.2. Biogenic amine analysis from bacterial cultures by ion–exchange chromatography*

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125   Purple or slight–purple colonies from the Niven´s medium plates were picked out

126   from an appropriate dilution plate. Production of biogenic amines was confirmed by

127   inoculating these individual colonies from Niven´s medium plates directly into tubes

128   containing 5 ml of LB medium containing the same precursor amino acids. After

129   incubation, the broth media was centrifuged and the supernatant was analysed by thin

130   layer chromatography (TLC) for biogenic amine content as described previously (García–

131   Moruno, Carrascosa, & Muñoz, 2005). Briefly, amines were converted to their fluorescent

132   dansyl derivatives by adding to one volume of the supernatant, one volume of 250 mM

133   disodium phosphate (pH 9.0), 0.1 volume of 4 N sodium hydroxide solution, and two

134   volumes of dansyl chloride solution (5 mg/ml of dansyl chloride in acetone). The reaction

135   mixture was thoroughly mixed and incubated in the dark at 55 ºC for 1 h. Then the

136   samples were cooled and stored at 4 ºC until use.

137   The amines were fractionated on precoated silica gel 60 F_{254} TLC plates. The

138   dansylated compounds were separated in chloroform:thriethylamine (4:1), and revealed

139   with isopropanol:triethanolamine (4:1). The fluorescent dansyl derivative spots were

140   visualized with the aid of a transiluminator with a suitable UV–light source (312 nm).

141   A standard solution of amines (5 g/l of histamine and 250 mg/l of tyramine,

142   putrescine, and cadaverine) was prepared similarly and used as control.

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145   *2.2.3. Presence of amino acid decarboxylase genes in the biogenic amine–producer strains*

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147   For DNA extraction, bacteria were routinely growth in LB broth at 30 ºC during 24

148   h. Bacterial chromosomal DNA was isolated directly from the cultures by using the

149   Wizard genomic DNA purification kit (Promega) according to the manufacturer´s

150   instructions. The DNA concentration was determined by using the Nano–drop

151   Spectrophotometer. Chromosomal DNAs from the biogenic amine–producer strains were

152   subjected to PCR amplification to detect the presence of the corresponding amino acid

153   decarboxylase encoding genes (Table 1) (De las Rivas, Marcobal, Carrascosa, & Muñoz,

154   2006; Landete, de las Rivas, Marcobal, & Muñoz, 2007). These primers were previously

155   described in a complete method for the PCR detection of foodborne bacteria producing

156   biogenic amines (histamine, tyramine, putrescine, and cadaverine) in Gram–positive as

157   well as in Gram–negative bacteria (De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006).

158   PCR reactions were performed in 0.2 ml microcentrifuge tubes in a total volume of 25 µl

159   containing 1µl of template DNA (aprox. 10 ng), 20 mM Tris–HCl, pH 8.0, 50 mM KCl,

160   2.5 mM MgCl$_2$, 200 µM of each dNTP, 1µM of each primer, and 1 U of Ampli*Taq* Gold

161   DNA polymerase. The reactions were performed in a Mastercycler® Personnal

162   (Epperdorf) using the following cycling parameters: 10 min for enzyme activation at 95 ºC

163   followed by 30 cycles of 30 s at 95 ºC, 30 s at 53 ºC, and 2 min at 72 ºC, and a final

164   extension step of 20 min at 72 ºC. PCR products were resolved on a 0.7% agarose gel

165   (Pronadisa, Spain) and stained with ethidium bromide.

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167 *2.2.4. Analysis of the amino acid decarboxylase genes from the biogenic amine-producer*

168 *strains*

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170   The amplification products were gel purified on QUIA-quick spin columns

171 (QUIAGEN) for sequencing with the same PCR primers used for the amplification. DNA

172 sequencing was carried out by using an Abi Prism 377 DNA^{TM} sequencer (Applied

173 Biosystems). Sequence similarity searches were carried out using Basic Local Alignment

174 Search (BLAST) on the EMBL/GenBank databases (Altschul, Madden, Schaffer, Zhang,

175 Zhang, Miller, & Lipman, 1997). Multiple alignments were done using CLUSTAL W on

176 the EBI site (http://www.ebi.ac.uk) after retrieval of sequences from GenBank and Swiss–

177 Prot.

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180 *2.3. Taxonomical identification of the biogenic amine-producer strains.*

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182   Biogenic amine-producer strains were identified by PCR amplification and DNA

183 sequencing of their 16S rDNA. The 16S rDNAs were PCR amplified using the eubacterial

184 universal pair of primers 63f (5´-CAGGCCTAACACATGCAAGTC) and 1387r (5´–

185 GGGCGGWGTGTACAAGGC) previously described (Marchesi, Sato, Weghtman,

186 Martin, Fry, Hion, & Wade, 1998). The 63f and 1387r primer combination generates an

187 amplified product of 1.3 kb. PCR was performed in **25 µl** amplification reaction mixture

188 as described above. The reaction was performed by using the following cycling

189 parameters: initial 10 min for enzyme activation at 95 ºC followed by 35 cycles of 1 min at

190 94 ºC, 1 min at 50 ºC and 1:30 min at 72 ºC. Amplified products were resolved on a 0.7%

191 agarose gel. The amplifications products were purified on QIAquick spin Columns

192 (Quiagen, Germany) for direct sequencing. DNA sequencing was carried out by using an

193 Abi Prism 377[TM] DNA sequencer (Applied Biosystems, USA). Sequence similarity

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

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

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198 3. Results and discussion

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200   Biogenic amines are compounds implicated in food poisoning incidents. Biogenic

201 amines in fish products are mainly produced by bacterial decarboxylation of amino acids.

202 Several amino acids can be decarboxylated and, as a result biogenic amines are usually

203 found, with histamine, tyramine, putrescine, and cadaverine being the most frequent.

204   Many procedures have been proposed to evaluate the decarboxylase activity of

205 microorganisms isolated from foods. Rapid screening methods can have some limitations

206 in terms of sensitivity in detecting biogenic amine production leading to contradictory

207 results. The presence of false–positive and false–negative strains is not negligible. For

208 these reasons, biogenic amine production has to be confirmed by analytical methods such

209 as HPLC. Most of the rapid screening procedures generally involve the use of a

210 differential medium containing a pH indicator. The pH change is dependent on the

211 production of the more alkaline amine form the amino acids initially included in the

212 medium. In order to identify biogenic amine–producer strains, dilutions of sardine and

213 mackerel homogenates were spread on modified Niven´s agar containing histidine,

214 ornitine, lysine and tyrosine. The production of at least one biogenic amine will be

215 recorded by the appearance of a purple or slight–purple colour of the media around the

216   colony. A total of 17 strains appeared as presumptively biogenic amine–producer in the

217   differential Niven´s medium. In order to confirm the production of biogenic amines by

218   these strains, presumptively positive strains were inoculated in liquid media containing the

219   same amino acids and the production of the amines was confirmed by a chromatographic

220   assay (García–Moruno, Carrascosa, & Muñoz, 2005).

221   From the 17 strains analyzed, only five strains were found to be strong amine

222   producers (Fig. 1, Table 2). From the sardine homogenate two biogenic amine producer

223   strains were isolated, a putrescine and cadaverine–producer strain (strain 5) (Fig. 1A, Table

224   2), and a histamine, putrescine and cadaverine–producer strain (strain 9) (Fig. 1B, Table

225   2). From the mackerel homogenate tyramine–producer strains were only isolated (strains

226   11, 12 and 13) (Fig. 1C, Table 2). On this study the decarboxylase medium overestimates

227   the number of biogenic amine–producer strains, giving false–positive results. These false

228   results could be due to the production of a substance able to alkalinize the media since

229   when these cultures were analyzed for the presence of biogenic amines by thin–layer

230   chromatography none of them showed amine production. The results obtained in this work

231   confirmed previous results describing that false–positive results could be obtained in

232   decarboxylase growth media (Marcobal, de las Rivas, & Muñoz, 2006). In addition, it has

233   been described in ice–preserved anchovies that ice–storage could hinder the growth of

234   biogenic amine–producer bacteria, since the microorganisms showing this ability are

235   mainly mesophilic bacteria (Pons–Sánchez–Cascado, Bover–Cid, Veciana–Nogués, &

236   Vidal–Carou, 2005). Enteric microorganisms with amine–forming ability constitute a

237   minor proportion of the fish microbiota and are difficult to isolate in high yields.

238   In order to correlate the production of these amines with the presence of the

239   corresponding decarboxylase genes, we performed PCR assays for the detection of the

240   *hdc*, *tdc*, *odc* and *ldc* genes, involved in the production of histamine, tyramine, putrescine

241 and cadaverine, respectively. Since a complete molecular method has been described to

242 detect biogenic amine producer bacteria, we checked the presence of the corresponding

243 genes by PCR (De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006). By this method

244 seven bacterial decarboxylase encoding genes could be detected (Table 1). The PCR assay

245 for the seven genes was applied to the biogenic amine–producer strains independently of

246 the amine produced. As showed in Fig. 2A and Table 2, strain 5, a putrescine and

247 cadaverine producer strain, amplified a 1440 pb DNA fragment from the ornithine

248 decarboxylase with PUT1–F and PUT1–R oligonucleotides, and a 1098 pb DNA with

249 CAD1–F and CAD1–R primers corresponding to lysine decarboxylases from Gram–

250 negative bacteria. Similarly, strain 9, a histamine–, putrescine–and cadaverine–producer

251 strain, amplified the same two DNA fragments (1440 and 1098 pb) and one additional 531

252 bp histidine decarboxylase fragment amplified with oligonucelotides HIS2–F and HIS2–R

253 (Fig. 2B, Table 2). The tyramine–producer strains (strains 11, 12, and 13) gave only an

254 amplicon corresponding to the expected size of the tyrosine decarboxylase internal

255 fragment (825 bp) (Fig. 2C, Table 2).

256   In order to confirm that these DNA fragments really correspond to internal fragments

257 of amino acid decarboxylase encoding genes, they were sequenced and similarity searches

258 were performed. Unfortunately, the sequence of both 1440 bp ornithine decarboxylase

259 fragments, from strains 5 and 9, could not be obtained as they seem to contain a mix of

260 two different sequences. This is not an unexpected result as it have been described that

261 enterobacteria sometimes are able to synthesize two sets of ornithine decarboxylases. One

262 set, produced during growth on neutral minimal medium, is referred to as the constitutive

263 (or biosynthetic) ornithine decarboxylase; when cultures are growth at low pH in the

264 presence of ornithine, an inducible (or degradative) ornithine decarboxylase is induced

265 (Morris, & Boecker, 1983). In *E. coli*, the constitutive ornithine decarboxylase bears quite

266 a striking resemblance to the inducible one, suggesting that they probably share a common

267 evolutionary ancestor (Applebaum, Dunlap, & Morris, 1977). Similarly, *Morganella*

268 *morganii* possess two closely related ornithine decarboxylases which their encoding genes

269 could be amplified by the same degenerate oligonucleotides (De las Rivas, González,

270 Landete, & Muñoz, 2008). Therefore, strains 5 and 9 could possess two ornithine

271 decarboxylases, constitutive and inducible, being the PCR amplicon a mixture of the two

272 DNA sequences.

273 As expected, BLAST databases searches of the translated DNA fragments identified

274 high-scoring similarities with amino acid decarboxylases that act on histidine, lysine, and

275 tyrosine. The predicted sequence of the histidine decarboxylase fragment from strain 9

276 was aligned with some histidine decarboxylases that had the highest overall identity with

277 that of strain 9 (Fig. 3A). The highest sequence identity (99%) was between *Morganella*

278 *morganii* and strain 9, followed by *Morganella psycrotolerans* (94%), *Enterobacter*

279 *aerogenes* and *Photobacterium phosphoreum* (84%), and finally, *Photobacterium*

280 *damselae* and *Raoultella planticola* (82%). These data confirm that strain 9 possess a

281 histidine decarboxylase enzyme, and probably is an enterobacteria.

282 Similarly, the predicted sequences from the lysine decarboxylase fragments were

283 aligned with lysine decarboxylase proteins included in the databases (Fig. 3B). Lysine

284 decarboxylase from strain 5 showed high identity to lysine decarboxylases from

285 *Citrobacter rodentium* (97%), *Citrobacter koseri* (96%), *Enterobacter cloacae* (94%),

286 *Salmonella enterica* (94%), *Cronobacter sakazaki* (92%) and *Escherichia coli* (92%).

287 Similarly, lysine decarboxylase from strain 9 showed 96 % identity to the lysine

288 decarboxylase from *Enterobacter cloacae*, 91% to *Salmonella enterica* and *Citrobacter*

289 *koseri*, and 90% to the protein from *Citrobacter koseri*. The lysine decarboxylases from

290 strain 5 and 9 were 91% identical among them. From the similarity showed by their lysine

291 decarboxylase fragments it could be assumed that both strains are enterobacteria.

292   Finally, strains 11, 12, and 13 shared an identical sequence on the tyrosine

293 decarboxylase fragment. This fragment was identical to the tyrosine decarboxylase from

294 *Enterococcus faecium*, and showed 96% identity to the corresponding protein from *E.*

295 *durans* and 94% to the *E. hirae* protein (Fig. 3C). These results indicated that strain 11 is a

296 lactic acid bacteria, possibly belonging to the *Enterococcus* genera. Pons–Sánchez–

297 Cascado, Bover–Cid, Veciana–Nogués, & Vidal–Carou (2005) indicated that, as a general

298 rule, lactic acid bacteria were mainly tyramine producers.

299   These results indicated that detection of the production of a biogenic amine in the

300 culture media by TLC is confirmed by the presence of the gene encoding the

301 decarboxylase enzyme by PCR. Therefore, and contrarily to the results obtained in

302 decarboxylase medium, a close relation was observed among the results obtained by TLC

303 and PCR methods.

304   Since the production of biogenic amines was confirmed by chromatographic and

305 molecular methods, we decided to taxonomically identify the bacteria producing amines in

306 this study. Sometimes the phenotypic identification of microbiota is time–consuming and

307 often problematic due to ambiguous biochemical or physiological traits. The development

308 of molecular methods has offered the possibility of accelerating a great deal of bacterial

309 identification. Therefore, the taxonomical identity of the biogenic amine–producer strains

310 was assessed by the amplification of the DNA fragment coding the 16S rDNA. The

311 bacterial isolates were then identified using sequence data from the first 500 bp of the 16S

312 rRNA genes. The sequences obtained were compared to sequences from type strains

313 included on the Ribosomal Database. This molecular analysis identified strain 5 as

314   belonging to the *Kluyvera intermedia* species, strain 9 to the *Enterobacter asburiae*, and

315   finally, strains 11, 12 and 13 as *Enterococcus durans* strains.

316   Fish spoilage is mainly caused by bacterial activity and some compounds, such as

317   biogenic amines, can be formed by bacterial action. In fact, biogenic amines should be

318   absent or found at very low levels in fresh fish and their formation is usually associated

319   with spoilage. Therefore, bacterial contamination is probably the main factor involved in

320   the formation of these compounds. Fresh fish can be contaminated by a mixed bacterial

321   population consisting of psychrotrophic Gram-negative bacteria like enterobacteria, and

322   Gram-positive bacteria like lactic acid bacteria. The type of bacteria present in food

323   determines the type and amount of biogenic amine formed. As demonstrated in this study,

324   enterobacteria and enterococci are particularly active. Indeed, enterobacteria have been

325   mostly described as strong producers of histamine and the diamines putrescine and

326   cadaverine in fish. However, lactic acid bacteria have been mainly associated with the

327   formation of tyramine in other fermented food products (Silla, 1996).

328   Enterobacteriaceae are generally considered as microorganisms with a high

329   decarboxylase activity. Møller (1954) studied that the distribution of the decarboxylases of

330   lysine and ornithine differs from the different species of enterobacteria. Diamines,

331   putrescine and cadaverine, are usually common amines often related to the activity of

332   enterobacteria. According to the Bergey´s manual, strains from the *Kluyvera* genera (only

333   the *K. ascorbata* and *K. cryocrescens* species were studied) are putrescine-and

334   cadaverine-producers (Brenner, 1984). As far as we known, this is the first report

335   describing the presence of *Kluyvera* strains in sardine samples. Contrarily, the presence of

336   *Enterobacter* strains had been extensively reported in fish or fish products such as in fresh

337   albacore (Kim, Field, Morrisey, Price, wei, & An, 2001), tuna fish (López Sabater,

338   Rodríguez-Jérez, Roig-Sagués, & Mora-Ventura, 1994), salt-ripened anchovies (Pons

339   Sanchez–Cascado, Veciana–Nogués, Bover–Cid, Mariné–Font, & Vidal Carou, 2005), ice–

340   preserved anchovies (Pons–Sanchez Casado, Bover–Cid, Veciana–Nogués, & Vidal–Carou,

341   2005), salted mackerel (Tsai. Lin, Chang, Chen, Kung, Wei, & Hwang, 2005), among

342   others. It had been also described that strains of *E. cloacae* produced simultaneously

343   putrescine and cadaverine, but hot histamine (Pons–Sánchez–Cascado, Bover–Cid,

344   Veciana–Nogués, & Vidal–Carou, 2005). Bjornsdóttir–Butler, Bolton, Jaykus, McClellan–

345   Green, & Green (2010) described that while that *E. cloacae* strains did not produce

346   histamine and did not posses the corresponding gene, strains from the *E. aerogenes* and *E.*

347   *gergoviae* species produced histamine and contained the gene. Likewise, *E. intermedium*

348   (López–Sabater, Rodríguez–Jérez, Hernández–Herrero, & Mora–Ventura, 1996) and *E.*

349   *amnigenus* (Takahashi, Kimura, Yoshikawa, & Fujii, 2003) strains were reported as

350   histamine–producers. In this study, a histamine–, putrescine– and cadaverine–producer *E.*

351   *asburiae* strain, strain 9, had been described.

352   Among lactic acid bacteria, *Enterococcus* sp. has been frequently isolated from swabs

353   taken from the skin of fish in various processing phases and from work surfaces (Zivkovic,

354   Miokovic, & Sosa, 2001). In this study *E. durans* strains able to produce tyramine were

355   isolated from the mackerel samples. Pons–Sánchez–Cascado, Bover–Cid, Veciana–Nogués,

356   & Vidal–Carou (2005) identified *Enterococcus* strains among the tyrosine decarboxylase

357   positive bacteria isolated from ice–preserved anchovies. In their study, enterococci were

358   found only at the last sampling point, at the end of the ice–preserver storage.

359   In conclusion, the present study provides information about the bacterial species

360   producing biogenic amines in ice–preserved sardine and mackerel. The results of the study

361   indicated that only a low proportion of strains decarboxylated amino acids in vitro.

362   Enterobacteria were histamine and diamine producers, being strains of *Kluyvera*

363   *intermedia* and *Enterobacter asburiae* isolated from the first time from sardine.

364 Enterococci produced tyramine and *E. durans* strains were isolated from ice-preserved

365 mackerel. However, biogenic amine production by decarboxylase positive strains in

366 screening media do not necessarily imply a similar behaviour in fish products. Regardless

367 of strain variation and the effects of environmental parameters, bacterial biogenic amine

368 formation in fish or fish products could represent a hazard for sensitive individuals.

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372 Acknowledgements

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474   Figure captions

475

476   Fig. 1. Thin layer chromatographic (TLC) analysis of biogenic amine producer strains

477   isolated from ice-preserved sardine and mackerel. Supernatants of bacteria grown in LB

478   media containing 0.25% histidine (1), 0.2% tyrosine (2), 0.25% lysine (3) or 0.25%

479   ornithine (4), were dansylated and separated on a precoated silica gel F_{254} plate. The

480   strains analyzed were the putrescine and cadaverine-producer strain 5 (A), the histamine,

481   putrescine and cadaverine-producer strain 9 (B), and the tyramine-producer strain 11.

482   Cadaverine (C), putrescine (P), and tiramine (T) standard solutions in LB medium are also

483   indicated.

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485

486   Fig. 2. PCR amplifications of amino acid decarboxylase encoding genes in bacteria

487   isolated from sardine and mackerel and identified as biogenic amine producer by TLC.

488   DNA extracted from strain 5 (A), strain 9 (B) and strain 11 (C) were used to amplify an

489   internal fragments of amino acid decarboxylase genes. Oligonucleotides HIS1–F and

490   HIS1–R (1) were used to amplify a 372 internal fragment of histidine–decarboxylase from

491   Gram–positive bacteria; oligonucleotides HIS2–F and HIS2–R (2) to amplify a 531 bp

492   fragment of histidine decarboxylases from Gram–negative bacteria; primers TDC–F and

493   TDC–R (3) to amplify an 825 bp fragment of tyrosine decarboxylase encoding genes;

494   primers PUT1–F and PUT1–R (4) and PUT2–F and PUT2–R (5) to amplify DNA

495   fragments of 1440 and 624 bp, respectively, coding for ornithine decarboxylases;

496   oligonucleotides CAD1–F and CAD1–R (6) to amplify a 1098 bp DNA fragment of lysine

497   decarboxylases from Gram–negative bacteria; and, primers CAD2–F and CAD2–R (7) to

498   amplify 1185 bp fragments of lysine decarboxylases from Gram–positive bacteria (Table

499   1). A molecular size standard (*Eco*RI digested λ DNA) is included in the left of the

500   agarose gels.

501

502

503   Fig. 3. Alignments of amino acid decarboxylase protein fragments. Clustal W program

504   was used to compare predicted sequences. Dashes on the alignment indicate identical

505   residues. At the botton of the alignment, residues that are identical (*), conserved (:), or

506   semiconserved (.) in all sequences of the alignments are also indicated. (A) Histidine

507   decarboxylases from strain 9 (9), *Morganella morganii* NCIMB 865 (MMO), *Morganella*

508   *psychrotolerans* F39–1 (MPS), *Enterobacter aerogenes* ATCC 13048 (EAE),

509   *Photobacterium phosphoreum* mb36 (PHP), *Photobacterium damselae* ATCC 33539

510   (PDA), and *Raoultella planticola* Y1–1 (RPL). (B) Lysine decarboxylases from strain 5

511   (5), strain 9 (9), *Citrobacter koseri* ATCC BAA–895 (CKO), *Salmonella enterica* serovar

512   Heidelberg str SL 476 (SEN), *Escherichia coli* SE15 (ECO), *Citrobacter rodentium* ICC

513   168 (CRO), and *Enterobacter cloacae* SCF1 (ECL), *Cronobacter sakazakii* ATCC BAA–

514   894 (CSA). (C) Tyrosine decarboxylase from strain 11 (11), *E. faecium* (EFA), *E. durans*

515   (EDR), and *E. hirae* (EHI).

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Table 1

TABLE 1. Primers used for the detection of amino acid decarboxylating bacteria (described by De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006)

Gene[a]	Primer	Sequence[b]	Amplicon size (bp)	Bacteria
hdc	HIS1-F	GGNATNGTNWSNTAYGAYMGNGCNGA	372	Gram-positive
	HIS1-R	ATNGCDATNGCNSWCCANACNCCRTA		
	HIS2-F	AAYTSNTTYGAYTTYGARAARGARGT	531	Gram-negative
	HIS2-R	TANGGNSANCCDATCATYTTRTGNCC		
tdc	TDC-F	TGGYTNGTNCCNCARACNAARCAYTA	825	Gram-positive
	TDC-R	ACRTARTCNACCATRTTRAARTCNGG		
odc	PUT1-F	TWYMAYGCNGAYAARACNTAYTTYGT	1440	Gram-positive/ Gram-negative
	PUT1-R	ACRCANAGNACNCCNGGNGGRTANGG		
	PUT2-F	ATHWGNTWYGGNAAYACNATHAARAA	624	Gram-positive/ Gram-negative
	PUT2-R	GCNARNCCNCCRAAYTTNCCDATRTC		
ldc	CAD1-F	TTYGAYWCNGCNTGGGTNCCNTAYAC	1098	Gram-negative
	CAD1-R	CCRTGDATRTCNGTYTCRAANCCNGG		
	CAD2-R	CAYRTNCCNGGNCAYAA	1185	Gram-positive
	CAD2-F	GGDATNCCNGGNGGRTA		

[a] *hdc*, histidine decarboxylase; *tdc*, tyrosine decarboxylase; *odc*, ornithine decarboxylase; *ldc*, lysine decarboxylase.
[b] 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. Biogenic amine production by bacteria isolated from ice-preserved sardine (strains 1 to 10) and mackerel (strains 11 to 17).

No.	Histamine[a]			Tyramine[b]		Putrescine[c]			Cadaverine[d]		
		hdc			*tdc*		*odc*			*ldc*	
	HIS	1	2	TYR		PUT	1	2	CAD	1	2
1	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	+	+	-	+	+	-
6	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-
9	+	-	+	-	-	+	+	-	+	+	-
10	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	+	+	-	-	-	-	-	-
12	-	-	-	+	+	-	-	-	-	-	-
13	-	-	-	+	+	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-

[a] Histamine production detected by TLC (HIS) or by PCR (*hdc*) using oligonucelotides HIS1-F/R (1) or HIS2-F/R (2); [b] Tyramine production detected by TLC (TYR) or by PCR (*tdc*) using TDC-F/R primers; [c] Putrescine production detected by TLC (PUT) or by PCR (*odc*) using PUT1-F/R (1) or PUT2-F/R (2) oligonuelotides; [d] Cadaverine production detected by TLC (CAD) or by PCR (*ldc*) using CAD1-F/R (1) or CAD2-F/R (2) primers.
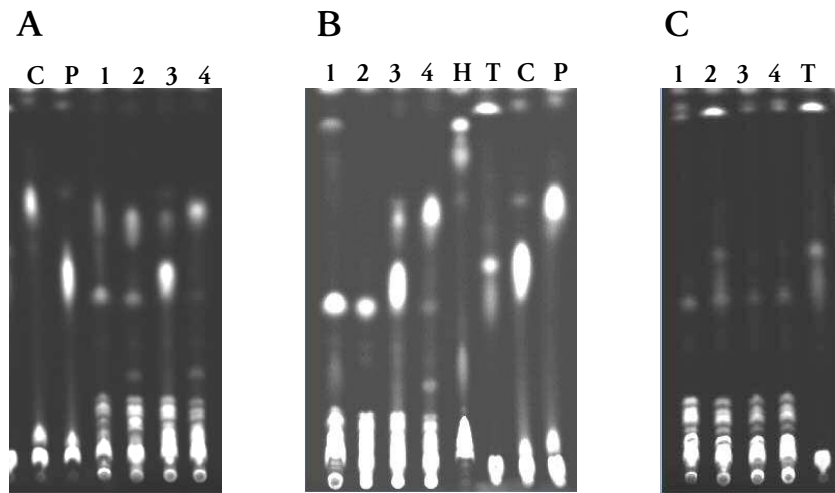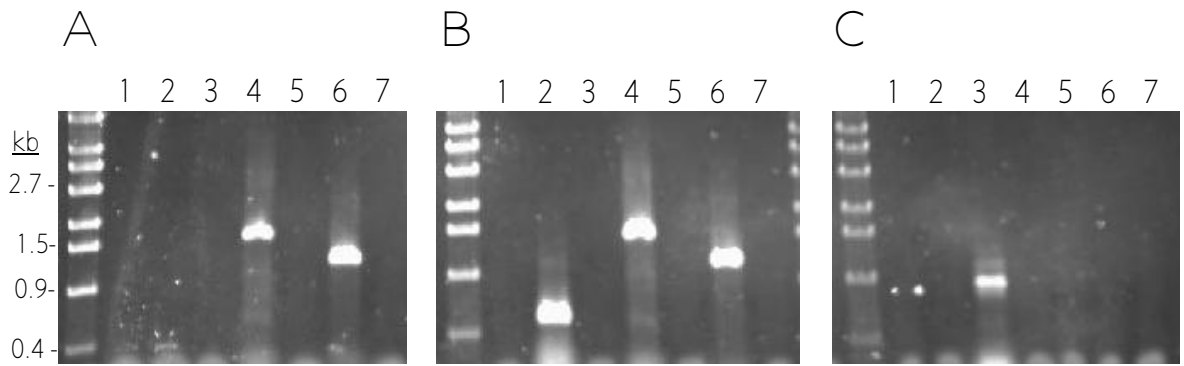
Figure 1

Figure 2

Figure 3

A

```
  9  NGGTEGNMFGCYLGREIFPDGTLYYSKDTHYSVAKIVKLLRIKSQVVESLPNGEIDYDDL 60
MMO  -----------------------------------------------------Q---------- 60
MPS  -----------------------------------------------------AQ---------- 60
EAE  -----S-----------L—E-------------------------L---Q-D—M------ 60
PPH  -----S-----------L----------------------------L------------- 60
PDA  -------------A—L----S---------------------------------VN---- 60
RPL  ------------------N-----------------------TL---Q----M--A-- 60
     *****.*******.**:**:.*********************** :**: *:**::* **

  9  MKKIADDKEAHPIIFANIGTTVRGAIDDIAEIQKRLKAAGIKREDYYLHADAALSGMILP 120
MMO  ----------------------------------L----------------------- 120
MPS  -----A-----------------------T-----M----------------------- 120
EAE  IN—RTSG-R----------------V-N--------A-L-P------------------- 120
PPH  IA—KQ-D-K--------------------SK--AM-G-L--------I----------- 120
PDA  I---EQ-G-K-----------K----N-F---D—A-L-A-------I----------- 120
RPL  I---KR-N-K-----------------N-I---QS-S-L—E—K---------------- 120
     : **  . * *************:**:*:*  **  :   ** *:***:*********** 
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B

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  5  SGMSGERVPGKVIYETQSTHKMLAALSQASLIHIKGDYDEDTFNEAFMMHTSTSPSYPIV 60
  9  R-----------F-----------F----------E---------------T------L- 60
CKO  ------------F--------------------------------------------- 60
SEN  -----D-------F----------------------E---E------------------ 60
ECO  --------A----F----------------------E---EA---------T-------- 60
CRO  ------------F----------------------E------------------- 60
ECL  -----G------FF----------------------E------------------ 60
CSA  -----------FF----------------------E-----Y----T------L- 60
     **** *:.***::**********:**********:***::****:****:******:* 

  5  ASIETAAAMLRGNAGKRLINRSVERALHFRKEVQRLREESDSW 103
  9  -------------P---------------------KD-A-G- 103
CKO  -------------P----------------------G- 103
SEN  -------------P--------------------------- 103
ECO  --V----------P--------------------------- 103
CRO  -------------P--------------------------- 103
ECL  -------------P----------------------G- 103
CSA  -------------P-R------------------------- 103
     **:**********.*:***:**:****.********::*::.* 
```

C

```
 11  GIGLDQVIPVPVDHNYRMDINELEKIVRGLAAEKTPILGVVGVVGSTEEGAIDGIDKIVA 60
EFA  --------------------------------------------------------- 60
EDR  -V------------------------------------------------------E 60
EHI  --------------------------Q------------------------------ 60
     *:*************************  ***************************** 

 11  LRRVLEKDGIYFYLHVDAAYGGYGRAIFLDEDNNFIPFEDLKDVHYKYNVFTENKDYILE 120
EFA  --------------------------------------------------------- 120
EDR  ------------------------------------E-----F-H-------N---- 120
EHI  -----------------------S-----E-------E-----A-HH------N---- 120
     ***********************:*****:*******:***** *:;*******:**** 

 11  EVHSAYKAIEEAESVTIDPHKMGYVPYSAGGIVIKDIRMRDVISYFATYVFEKGADIPAL 180
EFA  --------------------------------------------------------- 180
EDR  -----------------------------------V---------------------- 180
EHI  D----F-----------------------------V---------------------- 180
     :****:************************************:**************** 
```