Production of biogenic amines by lactic acid bacteria and enterobacteria isolated from fresh pork sausages packaged in different atmospheres and kept under refrigeration

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Abstract

The occurrence of *in vitro* amino acid activity in bacterial strains associated with fresh pork sausages packaged in different atmospheres and kept in refrigeration was studied. The presence of biogenic amines in decarboxylase broth was confirmed by ion–exchange chromatography and by the presence of the corresponding decarboxylase genes by PCR. From the 93 lactic acid bacteria and 100 enterobacteria strains analyzed, the decarboxylase medium underestimates the number of biogenic amine–producer strains. 28% of the lactic acid bacteria produced tyramine and presented the *tdc* gene. All the tyramine–producer strains were molecularly identified as *Carnobacterium divergens*. Differences on the relative abundance of *C. divergens* were observed among the different packaging atmospheres assayed. After 28 days of storage, the presence of argon seems to inhibit *C. divergens* growth, while packing under vacuum seems to favour it. Among enterobacteria, putrescine was the amine more frequently produced (87%), followed by cadaverine (85%); agmatine and tyramine were only produced by 13 and 1%, respectively, of the strains analyzed. Packing under vacuum or in an atmosphere containing nitrogen seems to inhibit the growth of enterobacteria which produce simultaneously putrescine, cadaverine, and agmatine. Contrarily, over–wraping or packing in an atmosphere containing argon seems to favour the growth of agmatine producer–enterobacteria. The production of putrescine and cadaverine was associated with the presence of the corresponding amino acid decarboxylase genes. The biogenic amine–producer strains were included in a wide range of enterobacterial species, including *Kluyvera intermedia*, *Enterobacter aerogenes*, *Yersinia kristensenii*, *Serratia grimesii*, *Serratia ficaria*, *Yersinia rodhei*, *Providencia vermicola* and *Obesumbacterium proteus*.

Keyworks: Fresh pork sausage; Packaging atmosphere; Refrigeration storage; Lactic acid bacteria; Enterobacteria; Amino acid decarboxylase; Tyramine; Putrescine; Cadaverine.

1. Introduction

Today, society is increasingly aware of the importance of diet for health, and hence, any issue relating to food safety has a considerable impact on consumer behaviour and official policy. At the same time, consumers increasingly prefer high–quality products that are safe and minimally processed, with less additives and ingredients, with a long shelf–life and easy to prepare. The meat industry is, therefore, looking for emerging technologies that can achieve this in processing and storage. Protective atmospheres are one of the preservation systems which are becoming increasingly significant (Nadon, Ismond, & Holley, 2001; Ruiz–Capillas & Moral, 2001a; Ruiz–Capillas & Moral, 2005; Ruiz–Capillas & Jiménez–Colmenero, 2004a). Although protective atmospheres could be applied in a variety of ways, traditionally meat products have been packaged for the retail trade in packaging containing a modified atmosphere denominated "modified atmosphere packaging" (MAP) (Church & Parsons, 1995; Ruiz–Capillas & Jiménez–Colmenero, 2004a). Usually the gases used for meat and meat products storage employing MAP is CO_2 with O_2 or N_2 mixes in different proportions (Farber, 1991; Church & Parson, 1995); however, there is an increasing interest in the potential benefits of argon and other noble gases in MAP applications (Fraqueza, Ferreira, & Barreto, 2008; Mostardini & Piergiovanni, 2002).

The conditions in which traditional or emerging technologies are applied affect the characteristics of the products, and such modifications may produce changes in the formation of different compounds some of which may be toxic and/or mutagenic, with implications for consumer health. Biogenic amines have been classified regarded as potentially hazardous compounds of food that may cause disorders to consumers (Halász, Baráth, Simon–Sarkadi, & Holzapfel, 1994; Silla, 1996). The level of biogenic

77 amines in sterile meat are very low and their levels increase with microbial spoilage
78 (Ruiz–Capillas & Jiménez–Colmenero, 2004b). Biogenic amines accumulation usually
79 results from the decarboxylation of amino acids by enzymes of bacterial origin, which is
80 associated with food hygiene and technology. The formation of biogenic amines in
81 meats requires the presence of decarboxylase–producing microoganisms, which may be
82 introduced by contamination before, during or after meat processing. Adequate
83 concentrations of the precursor free amino acids and environmental factors supporting
84 bacterial growth and favouring the synthesis of decarboxylase enzymes are also of
85 critical significance (Halász et al., 1994). There is, therefore, a clear interest in the study
86 of biogenic amines and the factors determining their formation in the context of food
87 processing conditions and preservation.
88 Recently the effects of a packaging atmosphere with non–conventional gas
89 mixtures containing CO_2 and argon, CO_2 and nitrogen on the maintenance of microbial
90 and physico–chemical characteristics of fresh pork sausages during refrigerated storage
91 was studied and compared with vacuum and normal atmosphere packaging (Ruiz–
92 Capillas & Jiménez–Colmenero, 2010). The biogenic amine levels remained low during
93 storage in all the samples. In addition, the presence of argon in the mixture of gases did
94 not affect the growth of lactic acid bacteria (LAB) and only seems to affect the growth
95 of enterobacteria. Despite the available knowledge on the nature of the spoilage
96 microbiota present in all the fresh pork sausages samples under different MAP, there is
97 not information on the taxonomy of the spoilage bacteria and the putative effect of the
98 different MAP conditions on the selection towards the final microbiota.
99 The present study deals with the characterization of *in vitro* biogenic amine–
100 producer microbiota (LAB and enterobacteria) present during the storage of fresh pork
101 sausages packaged in different atmospheres and kept in refrigeration.

2. Materials and methods

2.1. Sampling procedure, strain isolation and growth conditions

Fresh pork sausages ("longaniza" 59% of lean meat and 25 % of pork backfat) were produced and packaged under commercial conditions in a Spanish meat factory. Immediately after production, 40 kg of sausages (from production batch of 118 kg), were randomly allocated in four batches (10 kg per each), packed under different conditions and kept in refrigeration at 1 ± 1 ºC, as described previously (Ruiz-Capillas & Jiménez-Colmenero, 2010). Briefly, four different packaging atmospheres were assayed. Batch "**N**" was over-wrapped with oxygen-permeable cling film (LINPAC Plastics, Pontivy, France) in the tray without injecting any mixed gas inside (normal atmospheric conditions); batch "**V**" was kept in vacuum packaging; batch "**A**" **was** packaged in modified atmosphere containing 30% CO_2 and 70% argon, and was over-wrapped with film (CRYOVAC®LID2050); and finally, batch "**C**" **was packaged in an** atmosphere containing 20% CO_2 and 80% N_2, and over-wrapped with the latter film. At initial time and after 28 days of storage at 1 ± 1 ºC, the sausages from each batch were homogenized. Ten grams of each sample were placed in a sterile plastic bag with 90 ml of 0.1% peptone water and 0.85% NaCl. Appropriate decimal dilutions of the homogenized samples were placed on MRS agar (Merck, Germany) for LAB (30 ºC for 3-5 days) and on VRBG agar (Merck, Germany) for enterobacteria (37 ºC for 24 h) counting.

127 *2.2. Examination of bacterial amine–forming ability*

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129 *2.2.1. Growth in differential media for amino acid decarboxylase activity*

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131 A total of 193 isolates were picked out from each different selective agar (MRS for
132 LAB and VRBG for enterobacteria). Generally, twenty representative strains of each
133 bacterial group (LAB and enterobacteria) were selected from each different packaging
134 batch (N, V, C, and A) at 28 days of refrigerated storage, and 20 strains were also
135 selected from the initial sample. Isolates were picked out randomly from an appropriate
136 dilution plate. Production of biogenic amines was tested by inoculating individual
137 colonies from MRS or VRBG plates directly into tubes containing 5 ml of differential
138 amino acid decarboxylase media. The medium described by Maijala (1993) was used
139 for LAB, whereas for enterobacteria was used the Bacto decarboxylase Møller base
140 medium (Difco) (Møller, 1954). Pyridoxal–5–phosphate was included in both media (at
141 0.005%) since its a cofactor for the decarboxylation reactions. The media were
142 supplemented with the corresponding precursor amino acids (L–histidine
143 monohydrochloride, L–ornithine monohydrochloride, L–lysine and L–arginine
144 monohydrocloride at 0.25% final concentration, and tyrosine disodium salt at 0.2% due
145 to its low solubility). The precursor amino acids were purchased from Sigma (St. Louis,
146 MO, USA). Both media included purple bromocresol as pH indicator. The pH was
147 adjusted to 5.3 in Maijala medium, and to 6.7 in Møller medium. Later, the media were
148 autoclaved. The inoculated tubes were incubated at 30 ºC during 4 days for
149 enterobacteria and 7 days for LAB. After the incubation time, the colour media was
150 reported. Presumptively, a purple colour indicated biogenic amine production.

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153 *2.2.2. Biogenic amine analysis from bacterial cultures by ion–exchange*

154 *chromatography*

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156 Bacterial strains were grown in differential amino acid decarboxylase media as

157 described in section 2.2.1. After incubation, 1 ml of the broth media was centrifuged

158 (12,000 × *g*, 5 min) (Microspim 24S, Sorvall), then 0.5 ml of supernatant was extracted

159 with 0.5 ml of 0.1 N HCl, centrifuged again (12,000 × *g*, 5 min), and filtered through

160 0.22 nm. The extract was analysed by ion exchange chromatography for BA content.

161 Tyramine, histamine, putrescine, cadaverine, and agmatine were determined following

162 the methodology of Ruiz–Capillas and Moral (2001b) in a HPLC model 1022 (Perkin

163 Elmer, Spain), with a Pickering PCX 3100 post–column system (Pickering

164 Laboratories, Mountain View, CA, USA).

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167 *2.2.3. Presence of amino acid decarboxylase genes in the biogenic amine–producer*

168 *strains*

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170 Bacterial chromosomal DNA was isolated directly from the cultures in

171 differential amino acid decarboxylase media by using a protocol previously described

172 (Vaquero, Marcobal, & Muñoz, 2004). Chromosomal DNAs from the biogenic amine–

173 producer strains were subjected to PCR amplification to detect the presence of the

174 corresponding amino acid decarboxylase encoding genes (De las Rivas, Marcobal,

175 Carrascosa, & Muñoz, 2006; Landete, de las Rivas, Marcobal, & Muñoz, 2007). In

176 LAB we used the oligonucleotides TDC–F (5´–

177 TGGYTNGTNCCNCARACNAARCAYTA) and TDC–R (5´–
178 ACRTARTCNACCATRTTRAARTCNGG) previously described, that amplified an
179 825–pb *tdc* DNA fragment in the tyramine–producer LAB strains. In enterobacteria, we
180 used oligonucleotides PUT1–F (5´–TWYMAYGCNGAYAARACNTAYYYTGT) and
181 PUT1–R (5´–ACRCANAGNACNCCNGGNGGRTANGG) which amplified a 1,440–pb
182 internal *odc* fragment in the putrescine–producer enterobacteria strains; and
183 oligonucleotides CAD1–F (5´–TTYGAYWCNGCNTGGGTNCCNTAYAC) and
184 CAD1–R (5´–CCRTGDATRTCNGTYTCRAANCCNGG) wich amplified a 1,098–pb
185 fragment of the lysine decarboxylase encoding gene (*ldc*) in the cadaverine–producer
186 enterobacteria strains (being Y = C or T; R = A or C, W = A or T; M = A or C; and N =
187 A, C, G, or T). These primers were previously described in a complete method for the
188 PCR detection of foodborne bacteria producing biogenic amines (histamine, tyramine,
189 putrescine, and cadaverine) in Gram–positive as well as in Gram–negative bacteria (De
190 las Rivas et al., 2006). PCR reactions were performed in 0.2 ml microcentrifuge tubes in
191 a total volume **of 25 µl containing 1µl of template DNA (aprox. 10 ng), 20 mM Tris–**
192 HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, **200 µM of each dNTP, 1µM of each**
193 primer, and 1 U of Ampli*Taq* Gold DNA polymerase. The reactions were performed in
194 a Mastercycler® Gradient (Epperdorf) using the following cycling parameters: 10 min
195 for enzyme activation at 95 ºC followed by 30 cycles of 30 s at 95 ºC, 30 s at 53 ºC, and
196 2 min at 72 ºC, and a final extension step of 20 min at 72 ºC. PCR products were
197 resolved on a 0.7% agarose gel (Pronadisa, Spain) and stained with ethidium bromide.
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200 *2.3. Taxonomical identification of the biogenic amine–producer strains.*
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202 Biogenic amine–producer strains were identified by PCR amplification and DNA
203 sequencing of their 16S rDNA. The 16S rDNAs were PCR amplified using the
204 eubacterial universal pair of primers 63f (5´–CAGGCCTAACACATGCAAGTC) and
205 1387r (5´–GGGCGGWGTGTACAAGGC) previously described (Marchesi et al., 1998).
206 The 63f and 1387r primer combination generates an amplified product of 1.3 kb. PCR
207 was performed in 25 µl amplification reaction mixture as described above. The reaction
208 was performed by using the following cycling parameters: initial 10 min for enzyme
209 activation at 95 ºC followed by 35 cycles of 1 min at 94 ºC, 1 min at 50 ºC and 1:30 min
210 at 72 ºC. Amplified products were resolved on a 0.7% agarose gel. The amplifications
211 products were purified on QIAquick spin Columns (Quiagen, Germany) for direct
212 sequencing. DNA sequencing was carried out by using an Abi Prism 377^{TM} DNA
213 sequencer (Applied Biosystems, USA). Sequence similarity searches were carried out
214 by comparing to sequences from type strains included on the Ribosomal Database
215 (http://rdp.cme.msu.edu).
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218 3. Results and discussion
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220 *3.1. Lactic acid bacteria producing–biogenic amines*
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222 Many procedures have been proposed to evaluate the decarboxylase activity of
223 microorganisms isolated from foods. Rapid screening methods can have some
224 limitations in terms of sensitivity in detecting biogenic amine production leading to
225 contradictory results. The presence of false–positive and false–negative strains is not
226 negligible. For these reasons, biogenic amine production has to be confirmed by

227 analytical methods such as HPLC. Most of the rapid screening procedures generally
228 involve the use of a differential medium containing a pH indicator. The pH change is
229 dependent on the production of the more alkaline amine form the amino acids initially
230 included in the medium. In order to facilitate the growth of meat LAB, Maijala (1993)
231 developed a modified decarboxylase media. A total of 93 strains picked out from MRS
232 plates were tested for biogenic amine–production in the Maijala´s decarboxylase liquid
233 medium, containing the amino acid precursors for the production of histidine, tyramine,
234 putrescine, cadaverine, and agmatine. The production of at least one biogenic amine
235 will be recorded by the formation of a purple colour in the decarboxylase broth. From
236 the 93 strains tested, only tubes from 13 strains (14%) showed a faint purple colour
237 (Table 1). The positive strains were mainly found among those isolated from the initial
238 sample and from the batch only over–wrapped and stored refrigerated during 28 days
239 (N).
240 When the same liquid media was analyzed by a chromatographic assay, a relation
241 was not found between purple positive tubes and the presence of biogenic amines
242 (Table 1). By ion–exchange chromatography, 28 out 93 strains (30%) produced at least
243 one biogenic amine. The decarboxylase medium underestimates the number of biogenic
244 amine–producer strains, giving false–negative results which could be produced by an
245 insufficient growth of the strains. On the other hand, false–positive results were obtained
246 in three strains from the initial sample at time of packaging (strains 30, 33 and 37). This
247 could be due to the production of a substance able to alkalinize the media since when
248 these cultures were analyzed for the presence of biogenic amines by ion–exchange
249 chromatography none of them showed amine production. The results obtained in this
250 work confirmed previous results describing that false–positive and false–negative results

could be obtained in decarboxylase growth media (Marcobal, de las Rivas, & Muñoz, 2006).

From the bacteria isolated from the initial sample at time of packaging (t_0), one strain produced tyramine (strain 6) and two strains were able to produce putrescine and cadaverine simultaneously (strains 1 and 2). In order to correlate the production of these amines with the presence of the corresponding decarboxylase genes, we performed PCR assays for the detection of the *tdc*, *odc* and *ldc* genes, involved in the production of tyramine, putrescine and cadaverine, respectively. Since a complete molecular method has been described to detect biogenic amine producer bacteria, we checked the presence of the corresponding genes by PCR (De las Rivas et al., 2006). The tyramine–producer strain (strain 6) did not give an amplicon of the expected size, so, it seems that a known tyrosine decarboxylase gene was not present on it. However, the putrescine–and cadaverine–producer strains (strains 1 and 2), with PUT1–F + PUT1–R and CAD1–F + CAD1–R primers produced amplicons of the expected sizes, 1440 and 1098 bp, respectively (Figure 1A).

The biogenic amine–producer strains (strains 1, 2 and 6) were taxonomically identified by the amplification of the DNA fragment coding the 16S rDNA. The bacteria isolated and identified as positive for biogenic amine–production were then identified using sequence data from the first 500 bp of the 16S rRNA genes. The sequences obtained were compared to sequences from type strains included on the Ribosomal Database Project. The tyramine–producer strain (strain 6) was identified as belonging to the *Staphylococcus carnosus* species, and the putrescine and cadaverine–producer strains (strains 1 and 2), as *Serratia grimesi* strains. Surprisingly, none of these strains were lactic acid bacteria, in spite that they were isolated from MRS plates.

All the pork sausages samples packaged in different atmospheres (N, V, C, and A batches) and kept 28 days in refrigeration showed the presence of tyramine–producer bacteria from MRS plates. A high number of tyramine–producing strains (11 out 13 strains) were found in the sample kept in vacuum. Contrarily, only 3 out 20 strains were found to be tyramine producer when argon was included in the gas mixture of packaging (Table 1). By comparing to the modified atmospheres used, this could indicate that argon seems to selectively inhibit the growth of tyramine–producer lactic acid bacteria. On the isolated strains, the presence of the corresponding *tdc* gene was demonstrated by PCR amplification with TDC1–F and TDC1–R primers which amplifies a 825 bp DNA fragment (Figure 1B).

After storage, all the different samples presented tyramine–producer lactic acid bacteria which were molecularly identified as *Carnobacterium divergens* strains. It constituted an interesting observation the limited number of dominant LAB species that cohabitated this product under different atmospheres. In spite of that only *C. divergens* strains were isolated from all atmospheres, differences on their relative abundance could be observed indicating that the presence of argon seems to inhibit *C. divergens* growth, while vacuum seems to favour it. Similarly, it was previously described that *Carnobacterium* spp. represents up to the 71 or 85% of the bacteria isolated from fresh vacuum–packed pork held at –1.5 ± 0.5 ºC for 25 0r 45 days, respectively (Holley, Peirson, Lam, & Tan, 2004). *C. divergens* is frequently isolated from natural environments and foods. This species is able to grow in meat products at temperatures as low as 2 to –1.5 ºC, and they are frequently predominant members of the microbial community of raw meat (beef, pork, lamb, and poultry) (Leisner, Laursen, Prévost, Drider, & Dalgaard, 2007). The data obtained in this study confirmed previous results indicating that this species is found in atmospheres with different gas compositions

300 (Leisner et al., 2007). However, little information is available explaining the typical
301 dominance of this LAB species; specific functionalities such as bacteriocin production,
302 are well-described by carnobacteria, and might contribute to the successful
303 establishment of this species.
304 In spite of the presence of *C. divergens* strains able to produce tyramine, the content
305 on this biogenic amine was low in all the pork sausage samples packaged in different
306 atmospheres and kept in refrigeration (Ruiz-Capillas & Jiménez-Colmenero, 2010). The
307 effects of several physico-chemical factors influencing tyramine production by *C.*
308 *divergens* was studied previously (Masson, Lebert, Talon, & Montel, 1997; Masson,
309 Johansson, & Montel, 1999). These studies demonstrated that maximal tyramine
310 production occurred during the stationary phase in acidic conditions. Production was
311 slower at 5 ºC than at 23 ºC, but temperature slows down rather inhibits the tyramine
312 production (Masson et al., 1997). Nevertheless, temperature, influencing the
313 relationship among the activities of the different microorganisms present in sausage, can
314 have opposite effect on amine accumulation. In fact, this variable has different
315 influences on many phenomena related to amine production, such as growth kinetics,
316 cell yields and enzymatic activity. In addition, its effects on the activity of proteolytic
317 and decarboxylating enzymes and the relationships between the microbial population
318 have an important role on the total amount of amines. Higher temperature can favour
319 proteolytic and decarboxylating reactions, resulting in increased amine concentration
320 after storage. On the other hand, the spoilage of the product kept in modified
321 atmosphere is higher as the temperature increases. The MAP is not effective if the
322 temperature of storage is higher (Farber 1991).
323 Tyramine production by *C. divergens* strains in screening media do not necessarily
324 imply a similar behaviour in meat products. Regardless of strain variation and the

325 effects of environmental parameters, tyramine production by *C. divergens* has been
326 reported in a range of foods, including meat. Consequently tyramine formation by
327 carnobacteria in specific foods can represent a hazard for sensitive individuals who
328 might suffer migraine headaches. However, in all the samples analyzed in this study,
329 carnobacteria is not known to spoil the stored fresh pork sausages (Ruíz–Capillas &
330 Jiménez–Colmenero, 2010); thus, the large numbers of carnobacteria present in these
331 samples probably has little significance for product storage life.
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334 *3.1. Enterobacteria producing–biogenic amines*
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336 *Enterobacteriaceae* are generally considered as microorganisms with a high
337 decarboxylase activity. Møller (1954) studied that the distribution of the decarboxylases
338 of lysine, arginine, and ornithine differs for the different species of enterobacteria. By
339 using the same decarboxylase medium described by Møller (1954), 71 out 100 strains
340 (71%) were presumptively detected as biogenic amine–producer. However, 87 out 100
341 strains analyzed (87%) were confirmed by ion–exchange chromatography to be able to
342 produce amines (Table 1). Similarly to previously described for LAB, although with a
343 lower incidence, the decarboxylase medium used underestimates the number of
344 biogenic amine–producer strains, giving false–negative results. In this case this
345 disagreement could not be produced by an insufficient growth of the strains, as all the
346 enterobacteria growth well in the decarboxylase medium used. The production of
347 acidifying compounds by these strains could explain the false–negative results observed.
348 Putrescine was the amine more frequently produced (86%), followed by cadaverine
349 (85%). Agmatine and tyramine were only produced by the 14 and 1%, respectively, of

350 the strains analyzed (Table 1). Putrescine is produced by 86 strains by the action of an
351 ornithine decarboxylase on the amino acid ornithine. Cadaverine is produced by a
352 similar number of strains, 85 strains. This amine is produced by the decarboxylation of
353 lysine by action of the lysine decarboxylase enzyme. Agmatine was produced by 14
354 strains and is produced from arginine by the action of the arginine decarboxylase.
355 Finally, tyramine was produced only by one strain by the action of the tyrosine
356 decarboxylase on the amino acid tyrosine. It is interesting to note that all the biogenic
357 amine producer enterobacteria, but one putrescine–producer strain, synthesized
358 simultaneously more than one biogenic amine. Moreover, from Table 1 it could be
359 observed that the packing under vacuum or in an atmosphere containing a gas mixture
360 of 20% CO_2 and 80% N_2 had a similar effect on the growth of enterobacteria producing
361 simultaneously putrescine, cadaverine, and agmatine. The growth of these
362 enterobacteria seems to be inhibited by both packed conditions. Contrarily, over–
363 wrapping or packing in an atmosphere containing argon seems to favour the growth of
364 agmatine producer–bacteria. These evidences need to be further corroborated by the
365 direct inoculation of these amine producer–bacteria on the fresh pork sausage before
366 packing.
367 In order to correlate the production of these amines with the presence of the
368 corresponding decarboxylase genes, we performed the PCR assay for the detection of
369 the *tdc*, *odc* and *ldc* genes, involved in the production of tyramine, putrescine and
370 cadaverine, respectively (De las Rivas et al., 2006) on selected strains. No such a similar
371 method has been described for the detection of the agmatine producer strains. The
372 putrescine, agmatine and tyramine–producer strain (strain 178), isolated from the
373 sausage packed in the argon–containing atmosphere), gave the corresponding amplicons
374 from the *odc* and *ldc* genes by using PUT1–F + PUT1–R and CAD1–F + CAD1–R

primers, respectively. However, this strain did not give a *tdc* amplicon of the expected size by using TDC–F and TDC–R oligonucleotides, so, it seems that a known tyrosine decarboxylase gene was not present on it. This is an expected result, as both primers were based on *tdc* genes from lactic acid bacteria, based on the only unambiguously described tyrosine decarboxylase proteins. All the selected putrescine and cadaverine–producer strains produced amplicons of the expected sized, 1440 and 1098 bp, by using with PUT1–F + PUT1–R and CAD1–F + CAD1–R primers, respectively (Figure 2A and 2B).

Since the production of biogenic amines was confirmed by chromatographic and molecular methods, we decided to taxonomically identify the bacteria producing amines in this study. The taxonomical identity of the amine–producer strains was assessed by the amplification and sequencing of the DNA fragment coding the 16S rDNA. The strain which only produce putrescine, strain 158, was identified as belonging to the *Aeromonas salmonicida* species. This bacterial species has been also isolated from meat in Nigeria (Amadi, Obumwenre, & Akani, 2005), and *Aeromonas* spp. was a consistent part of the meat microbiota of vacuum packaged fresh pork (Holley et al., 2004) and in poultry skin (Bunková, Bunka, Klcovska, Mrkvicka, Dolezalová, & Kracmar, 2010). The only strain which produce simultaneously putrescine, agmatine and tyramine, strain 178, was molecularly identified as *Providencia vermicola*. Previously, strains from this species have never been described from meat products; it was firstly isolated in 2006 from a soil nematode (Somvanshi et al., 2006). *Shigella flexneri* and *Yersinia rohdei* strains (strain 98, 101, 107, and 168, among others) were identified as putrescine, cadaverine and agmatine–producer strains. According to the Bergey´s manual, *Shigella flexneri* strains are described to be lysine decarboxylase, arginine dihydrolase and ornithine decarboxylase negative by the differential Møller media (Brenner, 1984);

400 however, the complete genome sequence of the *Shigella flexneri* 2a str. 2457T strain
401 (accession AE014073.1) contains genes annotated as putatively coding for lysine
402 decarboxylase, arginine decarboxylases, and ornithine decarboxylase, which are in
403 agreement with the results obtained in this study. In spite that *Yersinia rohdei* was not
404 included in the Brenner study (Brenner, 1984), the unfinished genome from the type
405 strain of this species also reveals the presence of putative ornithine and arginine
406 decarboxylase enzymes (accession NZ_ACCD00000000).
407 Diamines, putrescine and cadaverine, are usually common amines often related to
408 the activity of enterobacteria. Putrescine and cadaverine–producer strains were included
409 in a wide range of enterobacterial species, including *Serratia grimesii* (such as strains
410 105, 113, 133, 142 or 148), *Serratia ficaria* (strains 125 and 139), *Kluyvera intermedia*
411 (strains 103 and 163), *Enterobacter aerogenes* (strain 174), *Yersinia kristensenii* (strain
412 168), and *Obesumbacterium proteus* (strains 115, 120, 173, 181, and 187). Some of this
413 species have been already described as putrescine and cadaverine–producer species by
414 Brenner (1984) in the Møller media, e.g., *E. aerogenes* and *O. proteus* biotype 2.
415 However, bacterial species, such as *S. ficaria* have been previously described as lysine
416 decarboxylase and ornithine decarboxylase negative by the differential Møller media
417 (Brenner, 1984; Grimont, Grimont, & Starr, 1979). Further biochemical and genetic
418 studies are needed to solve these discrepancies.
419 Some of these enterobacteria species have been previously isolated from meat
420 products. *Serratia* spp. and *Kluyvera* spp., are among the enterobacteria commonly
421 encountered before of after the thermal processing of cooked ham. Their presence has
422 been attributed to inadequate hygiene techniques, cross–contamination incidents, and the
423 psychrotrophic traits of these bacterial species (Vasilopoulos, De Maere, De Mey,
424 Paelinck, De Vuyst, & Leroy, 2010). Studies carried out *in vitro* indicated that *Serratia*

425 species were high putrescine and cadaverine producers during ripening and storage of
426 dry sausages (Bover–Cid, Izquierdo–Pulido, and Vidal–Carou, 2001). Strains from the
427 species *Serratia grimesii* showed a high putrescine production in ground meat and
428 processed meat products (Durlu–Özkaya, Ayhan, & Vural, 2001). Strains from the
429 *Yersinia kristensenii* have been previously isolated from raw meat (pork and chicken)
430 and precooked meat in Mexico city (Ramirez, Vázquez–Salinas, Rodas–Suárez, &
431 Pedroche, 2000) and from pork **sausages in Brazil (Falcão, 1991).**
432 However, due that only biochemical identification of the strains have been generally
433 made or that new enterobacteria species are recently described, some of the putrescine
434 and cadaverine–producer enterobacteria species have never been described in meat
435 products. As far as we known, this study represents the first description of *Providencia*
436 *vermicola* (Somvanshi et al., 2006), *Yersinia rodhei* (Aleksic, Steigerwalt, Bockemuhl,
437 Huntley–Carter, & Brenner, 1987), *Serratia ficaria* (Grimont et al., 1979), and
438 *Obesumbacterium proteus* (Priest, Somerville, Cole, & Hough, 1973) strains isolated
439 from meat products.
440 These results indicated that there is a great diversity of the enterobacteria species
441 present during the storage of fresh pork sausages packaged in different atmospheres and
442 kept in refrigeration. Contrarily, it was previously described the homogeneous presence
443 of tyramine–producer *C. divergens* strains in all these samples.
444
445 4. Conclusions
446
447 Microbial growth and metabolism contribute to the limitation of the shelf–life of
448 meat products. MAP in combination with refrigeration, is one of the most widespread
449 methods to delay spoilage in meat products. Not only does packaging act as a barrier

against contaminants, it also plays a crucial role in the selection of spoilage microorganisms due to its effect on oxygen availability. From the large group of microorganisms that initially colonise the raw ecosystem, some psychrotrophic LAB and enterobacteria are favoured. Despite this biodiversity, the MAP end-products are dominated by only a few bacterial groups that are highly competitive and are able to grow out and outcompete bacteria. In this work, *in vitro* tyramine-producer *C. divergens* strains were predominant in all the fresh pork sausages samples packaged in different atmospheres, in spite that the presence of argon seems to inhibit *C. divergens* growth, while packed under vacuum seems to favour it. However, a high number of enterobacteria species were found to be mainly putrescine and cadaverine-producer in the *in vitro* assays used in this study. Different packaging atmospheres seem to influence enterobacterial growth. Inhibition of enterobacteria producing simultaneously putrescine, cadaverine, and agmatine was observed under vacuum or in an atmosphere containing nitrogen. However, agmatine producer-enterobacteria were favoured by a packing atmosphere containing argon or over-wraped.

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584

585 Figure captions

586

587 Fig. 1. Amplification by PCR of amino acid decarboxylase genes in bacteria isolated
588 from MRS plates obtained from fresh pork sausages at the time of packaging or after 28
589 days of refrigeration storage of sausages packed in different atmospheres. (A)
590 Amplification of the *odc* or *ldc* genes from the putrescine–and cadaverine–producer
591 strains. Primer set PUT1–F + PUT1–R that amplified a 1440–bp *odc* fragment from
592 *Serratia grimesii* 1 (1) or *S. grimesii* 2 (2) strains, and primers CAD1–F + CAD1–R
593 which amplified a 1098–bp *ldc* fragment from the same strains, *S. grimesii* 1 (3) and 2
594 (4). (B) Amplification of the *tdc* gene from the tyramine–producer strains. TDC–F +
595 TDC–R primers amplified a 825–bp *tdc* fragment from *C. divergens* 21 (1), 31 (2), 32
596 (3), 37 (4), 38 (5), 40 (6), 59 (7), and 69 (8) strains. A molecular size standard
597 (*Eco*RI/*Hin*dIII–digested λ DNA) is included in the left of both agarose gels.

598

599 Fig. 2. Amplification by PCR of amino acid decarboxylase genes in bacteria isolated
600 from VRBG plates obtained from fresh pork sausages at the time of packaging or after
601 28 days of refrigeration storage of sausages packed in different atmospheres.
602 Amplification from the putrescine–and cadaverine–producer strains of an *odc* gene
603 fragment (primer PUT1–F + PUT1–R which amplified a 1440–bp) (A) or a *ldc* gene
604 fragment (primers CAD1–F + CAD1–R which amplified a 1098–bp) (B) from the
605 following strains *Yersinia rohdei* 98 (1), *Shigella flexneri* 101 (2), *Kluyvera intermedia*
606 103 (3), *Serratia grimesii* 105 (4), *Shigella flexneri* 107 (5), *S. grimesii* 113 (6),
607 *Obesumbacterium proteus* 115 (7), *O. proteus* 120 (8), *Serratia ficaria* 125 (9), *S.*
608 *grimesii* 133 (10), *S. ficaria* 139 (11), *S. grimesii* 142 (12), *S. grimesii* 148 (13), *K.*

609 *intermedia* 163 (14), *Yersinia kristensenii* 168 (15), *O. proteus* 173 (16), *Enterobacter*
610 *aerogenes* 174 (17), *O. proteus* 181 (18), *O. proteus* 187 (19), and *K. intermedia* 193
611 (20) strains. A molecular size standard (EcoRI/HindIII-digested λ DNA) is included in
612 the left of both agarose gels.
613

Figure 1

Figure 1 (Curiel et al.)

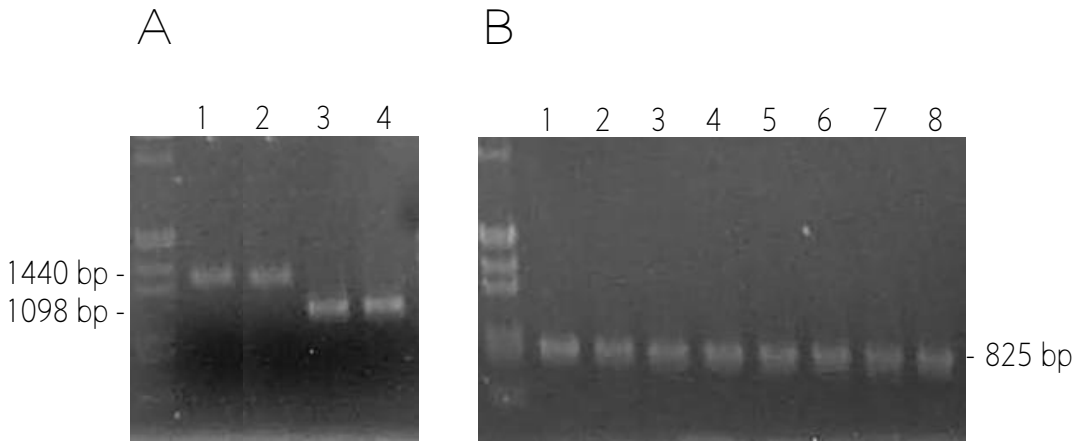

Figure 2

Figure 2 (Curiel et al.)

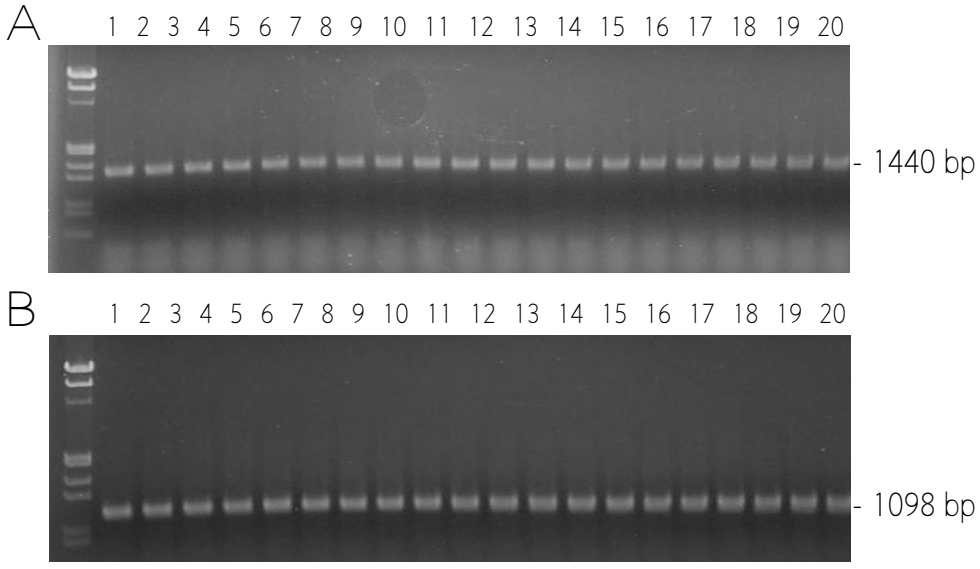

Table 1

Table 1. Biogenic amines produced by bacteria isolated from fresh pork sausages affected by over-wrap packaging (N) and modified atmosphere in vacuum (V) and with a gas mixture of 20% CO_2 and 80% N_2 (C) and 30% CO_2 and 70% argon (A) during refrigeration

				Biogenic amine[d]					
		(n)[a]	DM[b]	None	T	P	P-C	P-C-A	P-A-T
Lactic acid bacteria									
t_0[c]		1-20 (20)	6	17	1	0	2	0	0
t_{28}	N	21-40 (20)	6	14	6	0	0	0	0
	V	41-53 (13)	0	2	11	0	0	0	0
	C	54-73 (20)	0	15	5	0	0	0	0
	A	74-93 (20)	1	17	3	0	0	0	0
Enterobacteriaceae									
t_0		94-113 (20)	14	2	0	0	15	3	0
t_{28}	N	114-133 (20)	13	5	0	0	10	6	0
	V	134-153 (20)	19	1	0	0	19	0	0
	C	154-173 (20)	13	3	0	1	16	0	0
	A	174-193 (20)	12	3	0	0	12	4	1

[a] n, strain number and number of strains (in parenthesis)
[b] DM, number of positive strains, recorded as a purple colour, in the differential growth media for biogenic amine production
[c] 0 and 28, days of chilled storage
[d] biogenic amine produced and detected by ion-exchange chromatography: T (tyramine), P (putrescine), C (cadaverine), A (agmatine)