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1 **A PCR-DGGE method for the identification of histamine-producing**
2 **bacteria in cheese**
3
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10 Running title: PCR-DGGE identification of histamine-producing bacteria
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20
21 **Abstract**

22

23 Histamine is the biogenic amine (BA) most frequently involved in food
24 poisoning. Cheese is among the foods in which it is most commonly found, and
25 in some of the highest concentrations. Its accumulation in cheese is mainly due
26 to the presence of lactic acid bacteria (LAB) that produce histidine
27 decarboxylase, an enzyme coded by the gene *hdcA*. This gene has been
28 sequenced in several histamine-producing LAB. This paper reports a new,
29 culture-independent method based on PCR-DGGE for detecting and identifying,
30 at the species level, the histaminogenic bacteria present in cheese. Primers
31 were designed based on the *hdcA* gene sequences available for Gram positive
32 bacteria, and PCR and DGGE optimized in order to differentiate between
33 amplicons corresponding to different histamine-producing species. The
34 proposed method provides a rapid and simple means of detecting and
35 identifying histamine-producing Gram positive bacteria in foods with complex
36 microbial communities, such as cheese.

37

38 **Keywords**

39

40 Biogenic amines, histamine, *hdcA*, PCR-DGGE, identification, cheese.

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43
44 ## 1. Introduction

45 Biogenic amines (BAs) are low molecular weight organic bases with biological
46 activity. Although they are naturally produced by most living organisms, the
47 consumption of foods containing large amounts of these amines can have
48 toxicological consequences (Ladero et al., 2010; Shalaby, 1996).
49 Histamine is one of the most toxic and most commonly found BAs in foods. The
50 intake of large amounts can trigger histamine intoxication (Ladero et al., 2010),
51 the symptoms of which may include a rash, headache and gastrointestinal and
52 respiratory problems (Maintz and Novak, 2007). It is formed by microorganisms
53 with histidine decarboxylase activity.
54 Fish and fish products, dairy products, and fermented meats and vegetables are
55 the foods that most frequently contain high concentrations of histamine (Halasz
56 et al., 1994; Linares et al., 2011; ten Brink et al., 1990). After fish, cheese is the
57 food in which the highest concentrations – sometimes >1000 mg kg^{-1} –
58 recorded (Fernandez et al., 2007). In raw fish products, histamine is mainly
59 produced by Gram-negative spoilage bacteria; its presence is therefore
60 indicative of undesired microbial activity (ten Brink et al., 1990). However, in
61 cheese and other fermented foods, the main histamine producers are lactic acid
62 bacteria (LAB) - the bacteria responsible for the fermentation process itself.
63 This, of course, hinders a solution being found to histamine accumulation
64 (Linares et al., 2011). Histamine-producing LAB may be present in the raw
65 material or in the starter cultures used, they may appear in the secondary
66 microbiota that develops over the fermentation period, or enter the food as
67 contaminants during manufacture and storage (Burdychova and Komprda,
68 2007; Ladero et al., 2009; Linares et al., 2011; Novella-Rodriguez et al., 2002).
69 In all cases, however, these histamine-producing LAB belong to species that
70 form part of the normal microbiota of milk and cheeses.
71 With the aim of improving the safety and quality of dairy foods, a number of
72 culture-dependent and culture-independent methods have been developed for
73 detecting histamine-producing microorganisms. The culture-dependent methods
74 are based on the use of differential media containing a pH indicator that
75 changes colour due to histamine-induced alkalinization (Bover-Cid and
76 Holzapfel, 1999; Maijala and Eerola, 1993). Unfortunately, these methods are

77 not always effective in the detection of histamine-producing LAB since the large
78 amount of lactate these produce can counteract this alkalinization (Ladero et al.,
79 2015). Culture-independent methods, however, avoid this inconvenience, are
80 more exhaustive in their detection possibilities, and are less-time consuming
81 (Jany and Barbier, 2008).
82 Different methods based on the PCR-amplification of the gene coding for
83 histidine decarboxylase, *hdcA*, have been developed for detecting both Gram-
84 positive (Coton and Coton, 2005; Le Jeune et al., 1995) and Gram-negative
85 histamine-producing bacteria (de Las Rivas et al., 2005). Real time PCR
86 methods allow for the quantification of such bacteria (Bjornsdottir-Butler et al.,
87 2011; Fernandez et al., 2006), but despite being rapid, specific and sensitive,
88 they cannot distinguish exactly which species are the histamine-producers in
89 complex microbial communities. Since *hdcA* has been identified in a number of
90 dairy LAB (Calles-Enriquez et al., 2010; Diaz et al., 2015a; Diaz et al., 2015b,
91 Martin et al., 2005), as well as in LAB of other origin (Lucas et al., 2005; Satomi
92 et al., 2008), it could be used to identify such histamine-producers; while the
93 gene remains quite conserved, those of different species show some variation.
94 PCR-denaturing gradient gel electrophoresis (PCR-DGGE), which can separate
95 amplicons of the same size but different sequence (Fischer and Lerman, 1979),
96 provides one means of distinguishing between variants of *hdcA*. PCR-DGGE
97 based on the 16S rDNA sequence is usually employed for determining the
98 genetic diversity of complex microbial populations, but functional genes
99 associated with metabolic activities of interest can also be used as molecular
100 markers (Cremonesi et al., 2001; Florez et al., 2014, Wawer and Muyzer, 1995).
101 Thus, PCR-DGGE could be used to identify the *hdcA* genes from different
102 species forming part of complex microbial communities, such as those that exist
103 in fermented food products.
104 The present study proposes a PCR-DGGE method for the detection and
105 identification of histamine-producing LAB, the use of which may allow for a
106 better understanding of the histamine-producing microbiota present in complex
107 substrates such as fermented foods. In the present work, it was optimized for
108 the testing of commercial cheese samples.
109

110 **2. Materials and Methods**

111

112 *2.1. Bacterial strains and culture conditions*

113

114 Table 1 shows the strains used as positive controls for generating markers of
115 the different *hdcA* gene sequences. Lactobacilli were grown in MRS broth
116 (Oxoid, Basingstoke, UK), while *Streptococcus thermophilus* was grown in M17
117 (Oxoid) supplemented with 2 g L^{-1} lactose. Both were incubated at 37 ºC without
118 aeration.

119

120 *2.2. Bacterial DNA: isolation from pure cultures and cheese samples*

121

122 Total DNA was isolated from 2 mL of bacterial pure cultures supplemented with
123 1% (w/v) glycine (USB Corporation, Cleveland, USA), using the GenElute™
124 Bacterial Genomic DNA Kit (Sigma-Aldrich, Steinheim, Germany) according to
125 the manufacturer's recommendations.
126 Thirty three commercially available (traditionally and industrially-produced)
127 Spanish cheeses were purchased at different supermarkets. Bacterial DNA was
128 extracted following the method described by Fernandez et al. (2006), which is
129 based on the method of Ogier et al. (2002).

130

131 *2.3. Quantification of histamine by ultra-high performance liquid*
132 *chromatography*

133

134 Histamine in the cheese samples was quantified by ultra-high performance
135 liquid chromatography (UPLC). For this, 1 g of cheese was mixed with 10 mL of
136 0.1 M HCl containing 0.2% (w/v) 3,3′thiodipropionic acid (TDPA) (Sigma-
137 Aldrich) using an Ultra Turrax T50 homogenizer (OMNI International,
138 Kennesaw, USA) for 2 min at 20,000 rpm. The samples were then disrupted for
139 30 min in an ultrasonic bath and centrifuged at 5,000 *g* for 30 min. After
140 removing the fat layer, the supernatant was filtered through 0.45 μm PTFE
141 filters (VWR, Barcelona, Spain). The filtrates were deproteinized by
142 centrifugation through Amicon Ultra-0.5 mL centrifugal filters (Merck Millipore
143 Ltd., Carrigtwohill, Ireland) at 3,500 *g* for 1 h (Herrero-Fresno et al., 2012).
144 Samples (100 μL) were then derivatized and the histamine quantified using an

145 H-Class AcquityUPLC™ UPLC system (Waters, Milford, USA) as previously
146 described (Redruello et al., 2013); separations were performed at 35 ºC using a
147 Waters AcquityUPLC™ BEHC18 1.7 μm column (2.1 x 100 mm). Data were
148 acquired and analyzed using Empower 2 software (Waters).
149
150 *2.4. PCR amplification*
151
152 PCR reactions were performed in 50 μL volumes using 5PRIME Taq DNA
153 polymerase (5 PRIME GmbH, Hilden, Germany), following the manufacturer's
154 instructions. All reactions were performed in an iCycler thermocycler (Bio-Rad,
155 Hercules, USA). All amplicons were analyzed on 1% agarose gels in TAE (40
156 mM Tris/acetate [pH 8.0], 1 mM EDTA) buffer; bands were visualized following
157 staining with ethidium bromide in a G-Box and using GeneSys image
158 acquisition software (Syngene, Cambridge, UK).
159
160 *2.5. DGGE analysis*
161
162 All PCR products were purified using the ATP™ Gel/PCR Extraction Kit (ATP
163 TM Biotech Inc., Taipei City, Taiwan). DGGE was then performed using a
164 DCode apparatus (Bio-Rad, Hercules, USA) at 65 ºC, employing 8% (w/v)
165 polyacrylamide gels with a denaturing gradient ranging from 25 to 45% (100%
166 corresponding to 7 M urea and 40% to deionized formamide). Electrophoresis
167 was performed at 75 V for 16 h. After staining the gel with ethidium bromide (0.5
168 μg mL^{-1}), the bands were visualized under UV light in a G-Box and using
169 GeneSys image acquisition software.
170
171 *2.6. Identification of DGGE bands*
172
173 The DGGE bands were identified by comparing their migration against markers
174 of known *hdcA* sequence. To confirm the results, and to identify those bands
175 that did not match any marker, all the bands were sequenced. For this, they
176 were excised from the gels and deposited in 20 μL sterile water overnight at 4
177 ºC to extract the DNA. This was then re-amplified using the primer pair hdcDG-
178 F/hdcDG-R (35 cycles of 94 ºC for 30 s, 55 ºC for 45 s and 68 ºC for 30 s, plus

179 a final extension step of 10 min at 68 ºC). All amplicons were purified using the
180 ATP™ Gel/PCR Extraction Kit (ATP TM Biotech Inc.) and sequenced at
181 Macrogen (Seoul, Korea). The resulting sequences were compared with the
182 *hdcA* gene sequences available in the GenBank database using the BLAST
183 program (Altschul et al., 1997).
184

185 **3. Results**

186

187 *3.1 Specific primer design*

188

189 The *hdcA* gene was chosen as a target for the detection and identification of
190 histamine-producing bacteria. Full-length *hdcA* sequences of the histamine-
191 producing Gram-positive strains present in databases, i.e., for *Staphylococcus*
192 *epidermidis* (AB583189), *Lactobacillus fructivorans* (NZ_JOJZ01000009),
193 *Lactobacillus reuteri* IPLA11078 (LN877767), *L. reuteri* DSM20016
194 (NC009513), *Streptococcus thermophilus* (FN686789), *Lactobacillus saerimneri*
195 30a (NZ_ANAG0000000), *Lactobacillus vaginalis* (LN828720),
196 *Tetragenococcus halophilus* (AB362339), *Tetragenococcus muriaticus*
197 (DQ132889), *Oenococcus oeni* (DQ132887), *Lactobacillus sakei* (DQ132888),
198 *Lactobacillus hilgardii* (AY651779), *Lactobacillus parabuchneri* (LN877764),
199 *Staphylococcus capitis* (AM283479) and *Clostridium perfringens* (BA000016),
200 were aligned using ClustalW software (Larkin et al., 2007) and visualized using
201 the Jalview v.2 programme (Waterhouse et al., 2009) (see Fig. 1 in Diaz et al.,
202 2015c). Conserved regions flanking the variable regions were examined and the
203 general primers hdcDG-F (5'-CCTGGTCAAGGCTATGGTGTATGGTC-3') and
204 hdcDG-R (5'-GGTTTCATCATTGCGTGTGCAAA-3') designed.
205

206 *3.2. Optimization of PCR amplification*

207 The efficacy of the above primers was tested using purified total DNA from
208 *hdcA*$^+$ bacteria of dairy origin as a template (Table 1). Amplifications were
209 performed over 35 cycles of 94 ºC for 30 s, 55 ºC for 45 s and 68 ºC for 30 s,
210 plus a final extension step of 10 min at 68 ºC. Positive amplification was
211 observed for all the *hdcA*$^+$ strains tested.

212 After testing the efficacy of the primers, a GC clamp (5'-
213 CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3') was
214 linked to both to obtain primers C-hdcDG-F (5'-
215 CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGCCTGGTCA
216 AGGCTATGGTGTATGGTC-3') and C-hdcDG-R (5'-
217 CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGTTTCAT
218 CATTGCGTGTGCAAA-3') respectively. PCR amplifications with the primer
219 pairs C-hdcDG-F/hdcDG-R and hdcDG-F/C-hdcDG-R were run at different
220 annealing temperatures ranging from 50 to 55 ºC, using DNA from *hdcA*$^+$
221 bacteria (Table 1) as a template. Positive amplification were observed for all the
222 *hdcA*$^+$ strains tested and using either primer combination. However, the best
223 amplification results were obtained with an annealing temperature of 50 ºC; this
224 was, therefore, used in all subsequent PCR amplifications.
225
226 *3.3. Optimisation of DGGE*
227
228 DNA from pure cultures of *hdcA*$^+$ LAB species of dairy origin (Table 1) was used
229 as a template in PCR reactions, employing primer pairs C-hdcDG-F/hdcDG-R
230 and hdcDG-F/C-hdcDG-R under optimized conditions. The amplicons obtained
231 were separated by DGGE using one of two different denaturing gradients: 33-
232 55% and 25-45%, in 8% polyacrylamide. Amplicons obtained with primer pair
233 hdcDG-F/C-hdcDG-R could not be separated under the tested conditions (data
234 not shown). Amplicons obtained with C-hdcDG-2F/hdcDG-R showed good
235 separation, with the best band separation obtained using the 25-45%
236 denaturing gradient (Fig. 1). Amplicons from pure cultures of *L. reuteri* IPLA
237 11078, *L. vaginalis* IPLA11060*, L. parabuchneri* IPLA11129 and *S.*
238 *thermophilus* CHCC1524 was used as markers in the subsequent
239 electrophoretic analysis of DNA from the cheese samples (Fig. 1).
240
241 *3.4. PCR-DGGE analysis of bacterial* hdcA *genes present in Cabrales cheese*
242 *samples*
243
244 The amount of histamine and the presence of bacterial *hdcA* genes in 18
245 Cabrales cheese samples were determined (Table 2 and Fig. 2). This traditional

246 blue cheese (made from raw milk) was chosen since, not only does it habitually
247 have high concentrations of BAs, including histamine (Fernandez et al., 2006;
248 Fernandez et al., 2007), it is also very diverse in terms of the microorganisms
249 present (Florez and Mayo, 2006). Histamine was found in all the samples
250 tested, ranging from 10 to 1271 mg kg^{-1} of cheese (Table 2). Bands on the
251 polyacrylamide gels were compared with those of the markers, but only those
252 matching *L. parabuchneri* could be identified (note band c, Fig. 2). Some of
253 these bands were excised from the acrylamide gel and the amplicons
254 sequenced and compared to sequences in GenBank; 100% similarity with the
255 *hdcA* gene of *L. parabuchneri* was observed. The bands that did not match any
256 of the markers were also excised from the gel, sequenced, and compared to
257 sequences in the above database. All those analyzed showed 99-100%
258 similarity with GenBank *hdcA* sequences. Bands i and j showed 99% similarity
259 with the *hdcA* gene of *L. parabuchneri*. Bands e and f were 100% identical to
260 the *hdcA* gene of *T. halophilus*. Bands g and h were 100% identical to the *hdcA*
261 genes of *L. hilgardii hdcA* and *L. sakei hdcA;* these two species could not,
262 therefore, be distinguished.
263
264 *3.5. PCR-DGGE analysis of bacterial* hdcA *genes present in samples of other*
265 *types of cheese*
266
267 The concentration of histamine and the presence of different bacterial *hdcA*
268 genes was analyzed in 10 Manchego-type cheeses (industrially-made semi-
269 hard cheeses) from different producers, three Gamoneu cheese samples (a
270 traditional smoked blue-veined cheese made from raw cow's, sheep's and
271 goat's milk), one Idiazabal cheese (a traditional cheese made from raw sheep's
272 milk), and one Casín cheese (a traditional, long-matured cheese made from raw
273 cow's milk) (Fig. 3). Histamine was present in 12 of these 15 samples (80%),
274 ranging from 17 to 421 mg kg^{-1} of cheese (Table 2).
275 After DGGE, the bands on the polyacrylamide gels were identified by
276 comparison with markers when possible. Bands that showed the same
277 migration pattern as that observed in the previous DGGE gels (Fig. 2) were
278 denoted with the same letter. Some representative bands that migrated in the
279 same fashion as that of the *L. parabuchneri* marker (band c, Fig. 3) were

280 sequenced and found 100% identical to the *hdcA* gene of the latter species. A
281 band that migrated in the same fashion as the *S. thermophilus* marker (band d,
282 Fig. 3) appeared in one of the samples. This band was also sequenced, and
283 was 100% identical to that of the *hdcA* gene of *S. thermophilus*.
284 The bands that matched none of the markers were sequenced and showed 99-
285 100% similarity with different *hdcA* sequences in the GenBank database. As in
286 the Cabrales cheeses, bands i and j showed 99% similarity to the *hdcA* gene
287 from *L. parabuchneri*, band e was 100% identical to the *hdcA* from *T.*
288 *halophilus*, and band h 100% identical to the *hdcA* from *L. hilgardii* and *L. sakei*.
289
290 *3.6. Diversity of histamine-producing species in the analyzed cheeses*
291
292 Taking all the analyzed samples as a whole, the diversity of histamine-
293 producing species detected was quite low (Fig. 4). *L. parabuchneri* was the
294 most common (present in all the analyzed samples), and the only species
295 present in the Cabrales samples with the highest concentrations of histamine. In
296 addition, it was the only histamine-producing species present in all the
297 Gamoneu and Casín samples.
298 The other histamine-producing species were relatively scarce. *T. halophilus*,
299 which was found in some Cabrales and Manchego cheeses, was the second
300 most common (present in six of the 33 samples tested). *L. hilgardii/L. sakei*
301 appeared in just two Cabrales samples. Histamine-producing *S. thermophilus*
302 was detected only in the Idiazabal cheese.
303 The maximum diversity of LAB histamine producers within a sample was two
304 species; this was only seen in the Cabrales and Manchego-type cheeses. This
305 presence of two species was not correlated with any greater histamine
306 concentration.
307
308 **4. Discussion**
309
310 Recent years have seen increasing efforts to produce safer and higher quality
311 dairy products, including products that contain no toxic BAs. Histamine, the only
312 BA for which, in some foods, a legal limit has been established, is one of the
313 most toxic and commonly encountered BAs in cheese (Linares et al., 2011). Its

314 accumulation in food depends on several environmental and technological
315 factors, although the presence of microorganisms with histamine-generating
316 capacity is essential (Linares et al., 2012). An in-depth knowledge of the
317 microbial species involved in its accumulation in cheese will be needed if we are
318 to prevent its build-up. However, classical microbiological methods cannot
319 always identify the BA-producing species present - the differential culture media
320 available are not sufficiently selective (Bover-Cid and Holzapfel, 1999; Maijala
321 and Eerola, 1993). Thus, when BA-producing microorganisms make up only a
322 small proportion of the full microbiota – as is the case in some cheeses (Ladero
323 et al., 2009) - it becomes virtually impossible to isolate them. Culture-
324 independent methods, mainly based on PCR, are also available, and these can
325 detect (Coton and Coton, 2005; de Las Rivas et al., 2005; Le Jeune et al.,
326 1995) and even quantify BA-producing bacteria (Bjornsdottir-Butler et al., 2011;
327 Fernandez et al., 2006), but they cannot always identify the species involved .
328 In dairy products, histamine is mainly produced by LAB with histidine
329 decarboxylase activity (Linares et al., 2012). In the present work, the alignment
330 of the *hdcA* sequences from different LAB and other Gram-positive bacteria
331 (allowing highly conserved regions to be detected) led to the design of a pair of
332 primers able to bind to the conserved regions of *hdcA*, but flanking a region that
333 varies between species, and of a size (approximately 250 bp) suitable for PCR-
334 DGGE analysis. The amplification of DNA from pure cultures of dairy *hdcA*$^+$
335 LAB with these primers allowed their efficacy to be confirmed and the optimal
336 conditions for further analysis by DGGE to be established. The optimization of
337 the DGGE gradient allowed for the production of good separation patterns when
338 DNA from cheese samples was used as a template. The reproducibility of the
339 PCR-DGGE profiles obtained was very good (results not shown).
340 The proposed method successfully detected and identified the *hdcA*$^+$ LAB
341 present in the tested cheeses, even in those with complex microbial
342 communities. Four species of histamine producing bacteria were identified in
343 the 33 cheeses tested. To our knowledge, this is the first time that *L. hilgardii/L.*
344 *sakei* and *T. halophilus* have been described as potential histamine producers
345 in cheese, underscoring the usefulness of the proposed method. Since the
346 sequence of the amplified region was identical in both species in *L. hilgardii* and
347 *L. sakei*, these species were indistinguishable. However, their common *hdcA*

348 sequence was only detected in two Cabrales cheese samples, both of which
349 had a relatively low histamine concentration, and in which *L. parabuchneri* was
350 also present. *L. hilgardii* is commonly present in wine (Sohier et al., 1999) and
351 *L. sakei* is involved in meat fermentation (Chaillou et al., 2013), and both have
352 previously been detected in cheese (Carafa et al., 2015; De Pasquale et al.,
353 2014), although neither have previously been associated with histamine
354 production.
355 *T. halophilus* was the second most common *hdcA*$^+$ species found in the present
356 work: it was detected in three Cabrales and three Manchego-type samples. This
357 species is usually found in salted and fermented foods such as soy sauce and
358 fish sauce (Kuda et al., 2014). It has also been isolated from cheese (Morales et
359 al., 2011), although never in large numbers, and detected in it by PCR-DGGE
360 (Alegria et al., 2012). It has been suggested that halophilic lactic acid bacteria
361 can come from marine environments via sea salt added to cheeses (Ishikawa,
362 2007). It has, however, never before been associated with histamine production
363 in this type of food.
364 The Idiazabal cheese, which contained no histamine, was the only one to return
365 a band corresponding to *S. thermophilus,* a species that includes strains able to
366 produce histamine (Calles-Enriquez et al., 2010; Rossi et al., 2011), although in
367 low amounts (Gezginc et al., 2013). These results suggest that this species is
368 not responsible for histamine accumulation in cheese. Although *S. thermophilus*
369 is usually present in this food (Montel et al., 2014), it has never before been
370 described in Idiazabal cheese. *S. thermophilus hdcA*$^+$ strains are little
371 mentioned in the literature, further highlighting the sensitivity of the proposed
372 PCR-DGGE method.
373 *L. vaginalis* and *L. reuteri* were included among the PCR-DGGE markers since
374 they are known histamine-producers that have previously been isolated from
375 cheese (Diaz et al., 2015a). However, they were not detected in any of the
376 samples analyzed.
377 *L. parabuchneri* was the most common species; it was present in all the
378 analyzed cheese samples. Indeed, the literature reports it to be one of the most
379 common obligate heterofermentative lactobacilli in cheese (Coton et al., 2008).
380 Further, most of the characterized *L. parabuchneri* dairy strains are histamine
381 producers (Carafa et al., 2015; Diaz et al., 2015a; Diaz et al., 2015b, Fröhlich-

382 Wyder et al., 2013; Sumner et al., 1985). The presence of other histamine-
383 producing species alongside *L. parabuchneri* was not associated with any
384 higher concentration of histamine. Indeed, in the samples with highest
385 histamine, *L. parabuchneri* was the sole histamine producer. Thus, *L.*
386 *parabuchneri* would seem to be the species most responsible for histamine
387 accumulation in the analyzed cheeses.
388 It is well known that the presence of BA producers is an essential condition that
389 must be met for BA to accumulate in food, but it is not the only one;
390 accumulation also depends on a number of environmental and technological
391 factors, e.g., the availability of amino acid substrates (Linares, Del Rio et al.
392 2012). This explains the presence of *L. parabuchneri* in the cheese samples
393 without histamine. It is important to note that the proposed method is based on
394 standard PCR, and as such can reveal the diversity of histamine-producers but
395 cannot determine the numbers of each type. Thus, no correlation can be
396 established between band intensity and the number of histamine-producing
397 LABs in the sample. Culture-independent quantitative methods have been
398 developed at our laboratory that would allow this (Fernandez et al., 2006), but
399 they cannot identify the histamine-producing species. Since knowing the
400 identity, the diversity, and the prevalence of histamine-producing bacteria in
401 cheese types is essential if measures to prevent their appearance in food are to
402 be taken, the proposed method and these culture-independent quantitative
403 methods should be used to complement one another.
404
405 In conclusion, the proposed PCR-DGGE method provides a useful and effective
406 means of identifying the species responsible for the accumulation of histamine
407 in foods with complex microbial communities, such as cheese. In the present
408 work it even identified species not previously known to be histamine producers.
409 The results reveal *L. parabuchneri* to be the species most likely responsible for
410 the accumulation of histamine; it was the only species present in all of the
411 samples tested and even on its own can produce large amounts of histamine.
412 Moreover, the proposed method could be applied along the whole cheese
413 production process to identify the entry points of histamine producers and
414 consequently, it may be of help in the design of strategies aimed at reducing the
415 numbers of histamine-producing bacteria in cheese.

416
417 ## 5. Acknowledgements
418
419 This work was performed with the financial support of the Spanish Ministry of
420 Economy and Competitiveness (AGL2013-45431-R) and the Plan for Science,
421 Technology and Innovation 2013-2017 of the Principality of Asturias, which is
422 co-funded by the European Regional Development Fund (GRUPIN14-137).
423 M.D. was a beneficiary of an FPI fellowship from the Spanish Ministry of
424 Economy and Competitiveness. The authors are grateful to Adrian Burton for
425 linguistic assistance.
426

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628 ## Figure Legends

629

630 Figure 1. DGGE analysis of amplicons for the internal region of *hdcA* from
631 histamine-producing LAB. Lane 1, markers consisting of amplicons from: a, *L.*
632 *reuteri*; b, *L. vaginalis*; c, *L. parabuchneri*; c, *S. thermophilus*; lane 2, *L. reuteri*;
633 lane 3, *L. vaginalis*; lane 4, *L. parabuchneri*; lane 5, *S. thermophilus.*

634

635 Figure 2. PCR-DGGE profiles and histamine concentrations of different
636 Cabrales cheese samples. Gel 1. M: marker. Lanes 1-9 represent samples from
637 Cabrales cheeses. Gel 2. M: marker. Lanes 10-18 represent samples from the
638 remaining Cabrales cheeses. Bands: a, *L. reuteri*; b, *L. vaginalis*; c, *L.*
639 *parabuchneri*; d, *S. thermophilus*. The bands indicated were identified by
640 sequencing: e and f, *T. halophilus*; g and h, *L. hilgardii*; i and j, *L. parabuchneri*.

641

642 Figure 3. PCR-DGGE profiles and histamine concentrations of different cheese
643 samples. Lane numbers correspond to sample numbers. Gel 3, lanes 19 and
644 20: Manchego-type cheese samples, lane 21: Idiazabal cheese. Gel 4, lanes 22
645 and 23: Manchego-type cheese samples, lane 24: Casín cheese, M: Marker.
646 Gel 5, M: Marker. lanes 25-30: Manchego-type cheeses 25-30. Gel 6, lanes 30-
647 33: Gamoneu cheese samples. Bands: a, *L. reuteri*; b, *L. vaginalis*; c, *L.*
648 *parabuchneri*; d, *S. thermophilus*. The bands indicated were identified by
649 sequencing: e, *T. halophilus*; h, *L. hilgardii*; k, *S. thermophilus*.

650

651 Figure 4. Diversity of histamine-producing species and frequency of each in the
652 different types of cheese. The abscissa represents the number of samples in
653 which each species is present. Black bars represent the Cabrales cheese
654 samples, grey bars the Manchego-type cheese samples, white bars the
655 Gamoneu cheese, striped bars the Casín cheese, and dotted bars the Idiazabal
656 cheese.

657

658

Table 1. Histamine-producing strains used in this study.

Specie	Strain	Origin	Reference
Lactobacillus vaginalis	IPLA11064	Cheese	Diaz et al., 2015a
Lactobacillus reuteri	IPLA11078	Cheese	Diaz et al., 2015a
Lactobacillus parabuchneri	IPLA11122	Cheese	Diaz et al., 2015 b
Streptococcus thermophilus	CHCC1524		CHCC

CHCC: Christian Hansen Culture Collection (Hørsholm, Denmark).

Table 2. Histamine content of cheese samples analyzed.

Sample number	Histamine content (mg kg-1)	Cheese type
1	10	Cabrales
2	22	
3	26	
4	48	
5	78	
6	92	
7	134	
8	137	
9	167	
10	348	
11	367	
12	439	
13	442	
14	566	
15	612	
16	805	

17	1066	
18	1272	
19	0	Manchego-type
20	0	
21	0	Idiazabal
22	122	Manchego-type
23	134	
24	421	Casín
25	28	
26	39	
27	50	
28	50	Manchego-type
29	56	
30	67	
31	17	
32	17	Gamoneu
33	352	

7

8

9

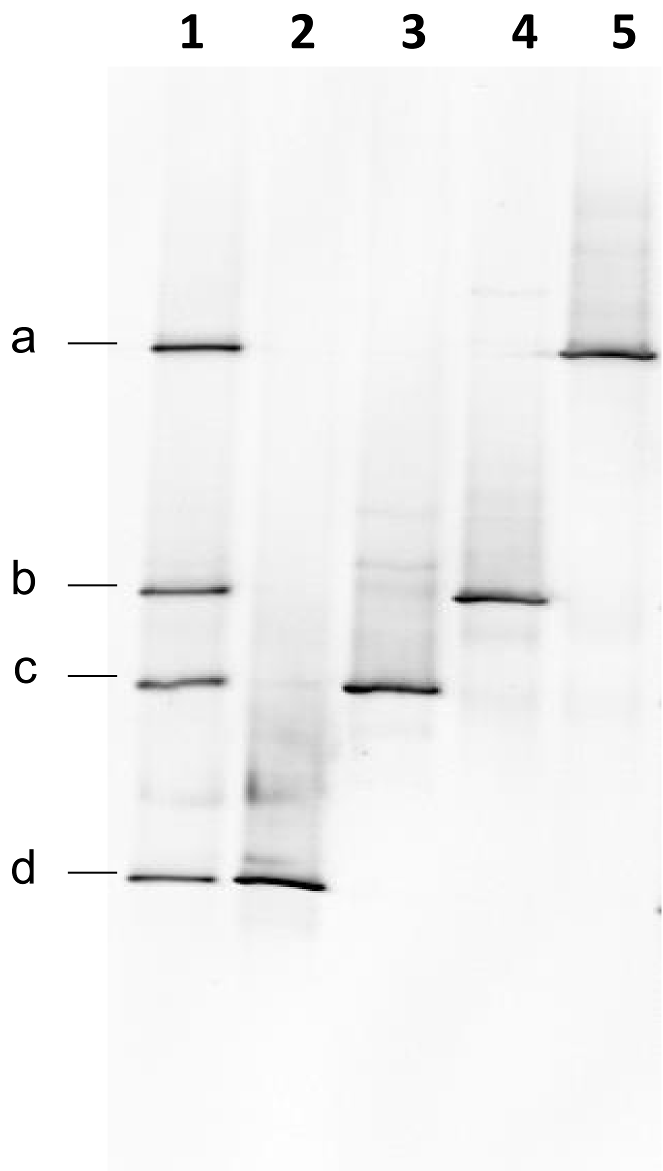
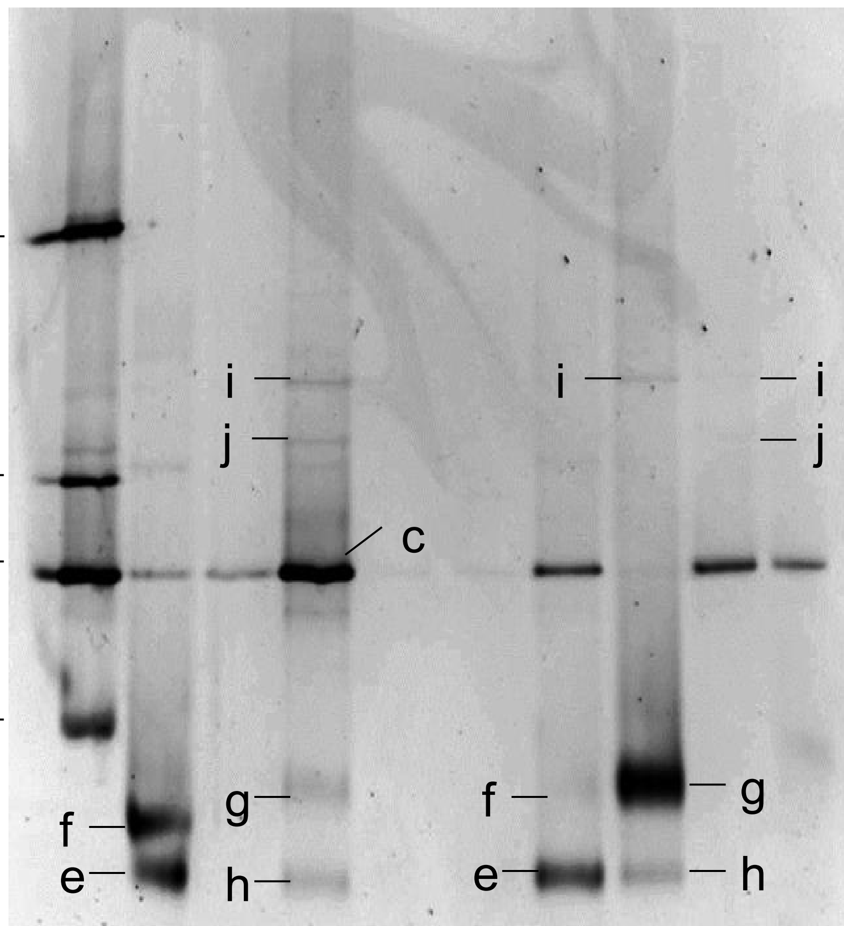


Figure 1

Gel 1

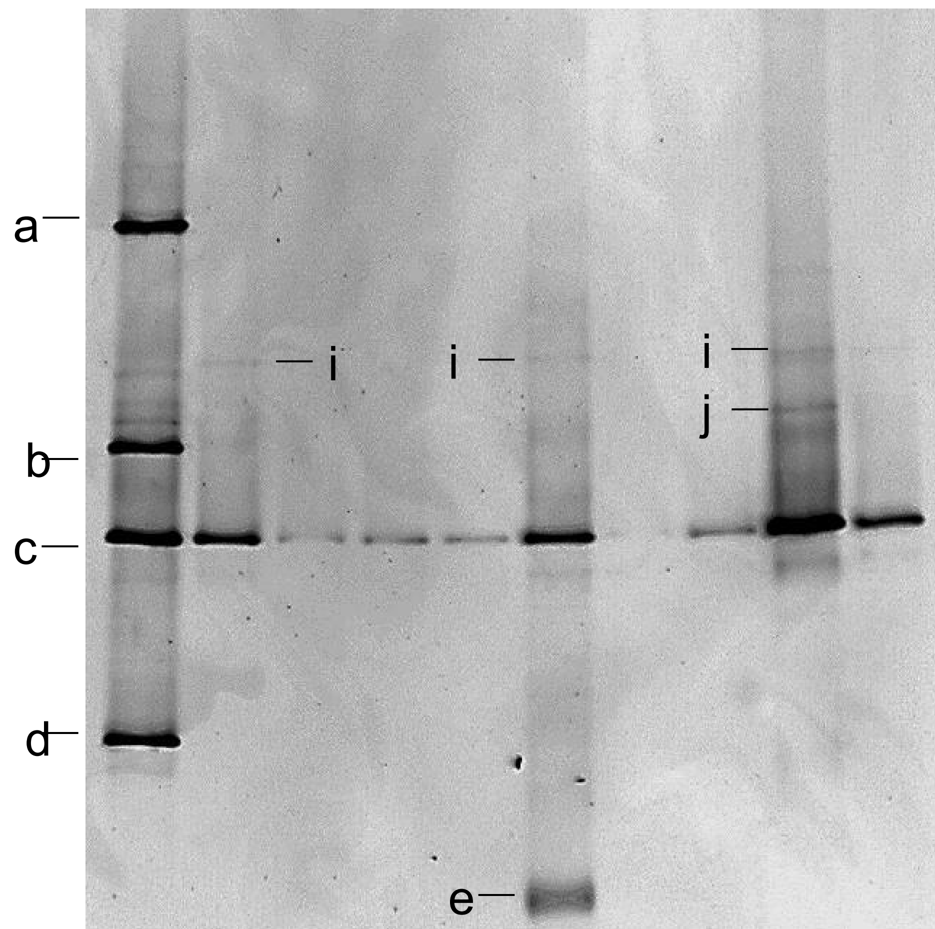
M 1 2 3 4 5 6 7 8 9



[Hma] mg/kg	10	22	26	48	78	92	134	137	167

Gel 2

M 10 11 12 13 14 15 16 17 18



[Hma] mg/kg	348	367	439	442	566	612	805	1066	1272

Figure 2

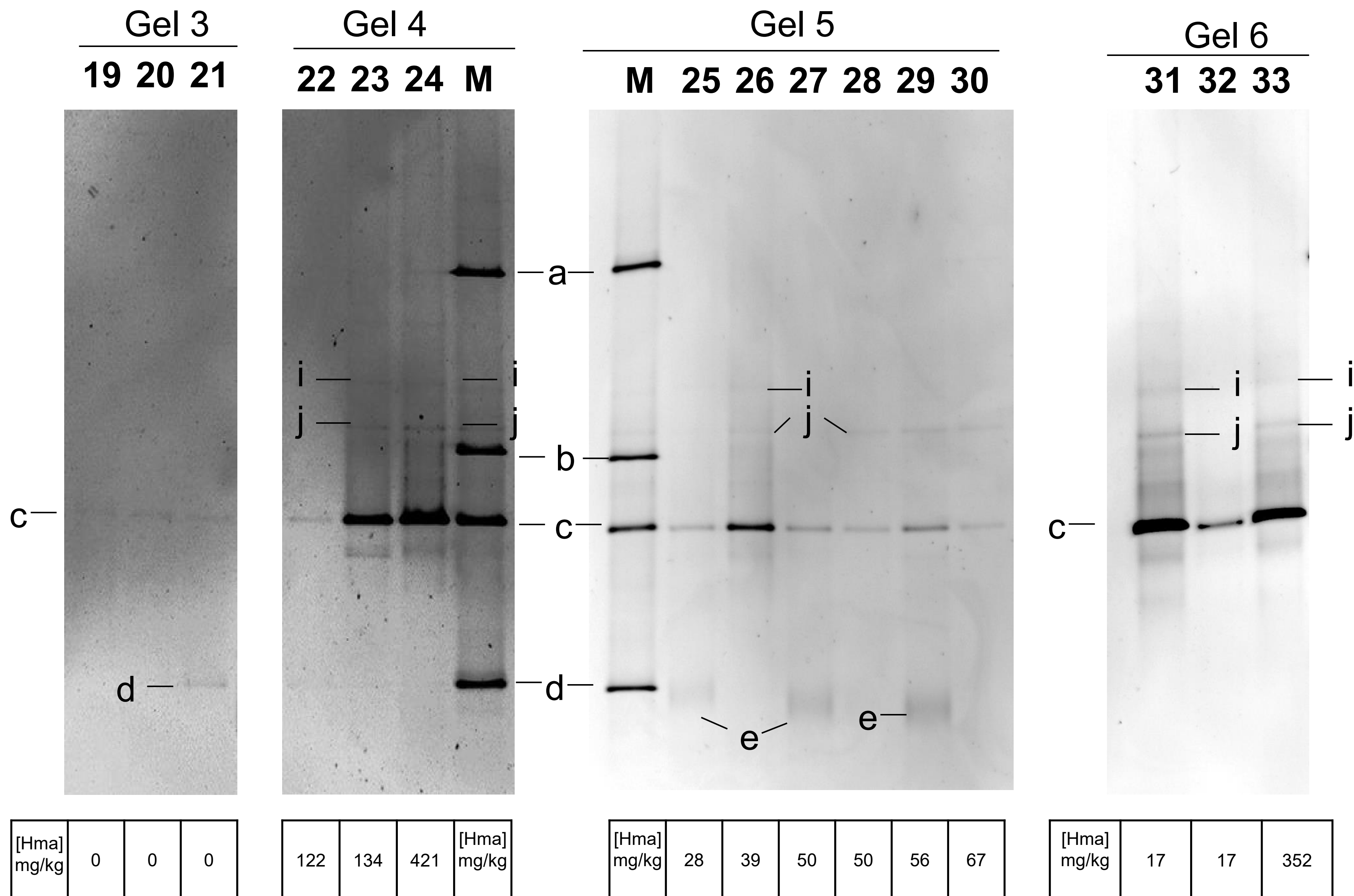


Figure 3

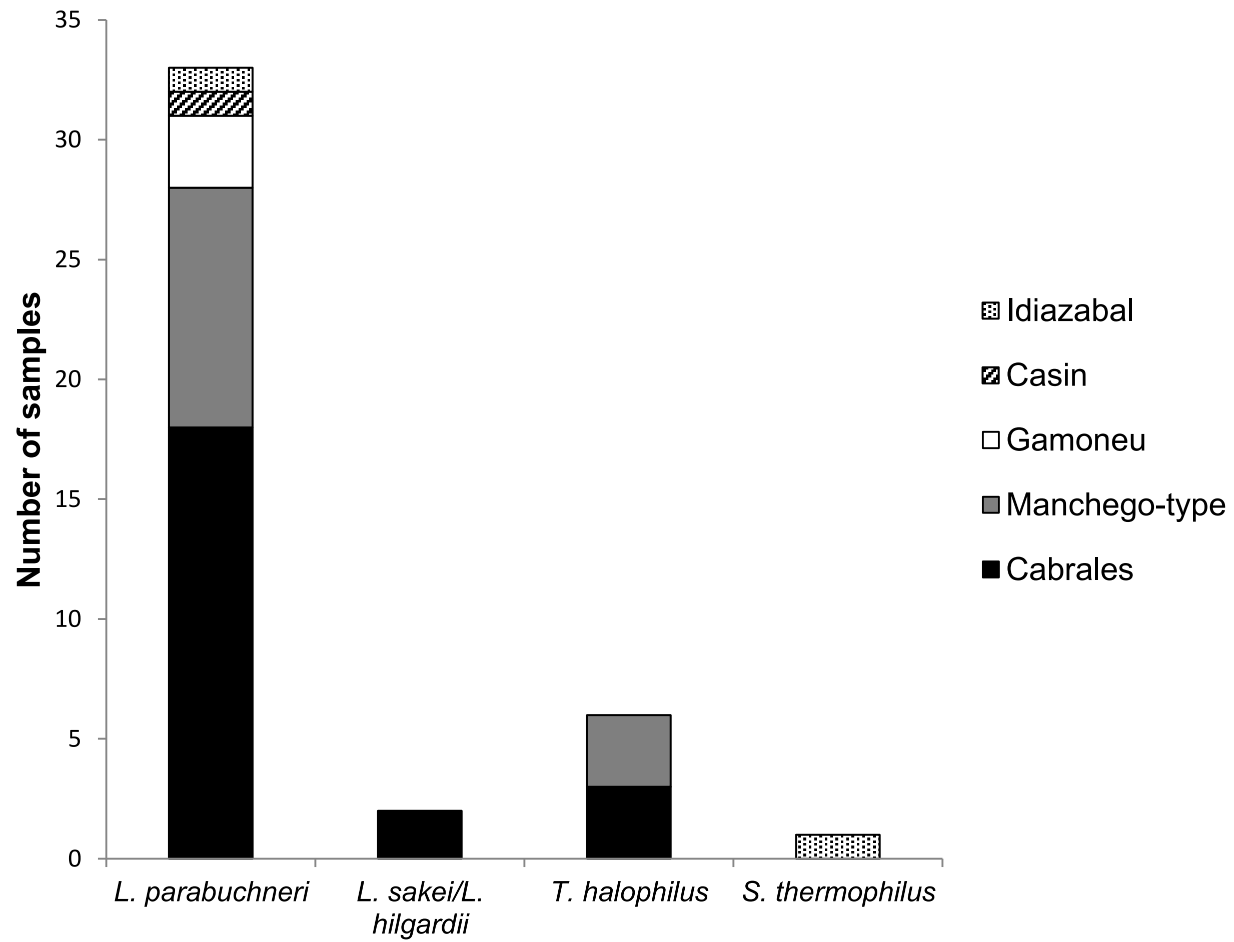


Figure 4