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A PCR-DGGE method for the identification of histamine-producing bacteria in cheese

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Running title: PCR-DGGE identification of histamine-producing bacteria

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Abstract

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

Keywords

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

1. Introduction

Biogenic amines (BAs) are low molecular weight organic bases with biological activity. Although they are naturally produced by most living organisms, the consumption of foods containing large amounts of these amines can have toxicological consequences (Ladero et al., 2010; Shalaby, 1996).

Histamine is one of the most toxic and most commonly found BAs in foods. The intake of large amounts can trigger histamine intoxication (Ladero et al., 2010), the symptoms of which may include a rash, headache and gastrointestinal and respiratory problems (Maintz and Novak, 2007). It is formed by microorganisms with histidine decarboxylase activity.

Fish and fish products, dairy products, and fermented meats and vegetables are the foods that most frequently contain high concentrations of histamine (Halasz et al., 1994; Linares et al., 2011; ten Brink et al., 1990). After fish, cheese is the food in which the highest concentrations – sometimes >1000 mg kg^{-1} – recorded (Fernandez et al., 2007). In raw fish products, histamine is mainly produced by Gram-negative spoilage bacteria; its presence is therefore indicative of undesired microbial activity (ten Brink et al., 1990). However, in cheese and other fermented foods, the main histamine producers are lactic acid bacteria (LAB) - the bacteria responsible for the fermentation process itself. This, of course, hinders a solution being found to histamine accumulation (Linares et al., 2011). Histamine-producing LAB may be present in the raw material or in the starter cultures used, they may appear in the secondary microbiota that develops over the fermentation period, or enter the food as contaminants during manufacture and storage (Burdychova and Komprda, 2007; Ladero et al., 2009; Linares et al., 2011; Novella-Rodriguez et al., 2002). In all cases, however, these histamine-producing LAB belong to species that form part of the normal microbiota of milk and cheeses.

With the aim of improving the safety and quality of dairy foods, a number of culture-dependent and culture-independent methods have been developed for detecting histamine-producing microorganisms. The culture-dependent methods are based on the use of differential media containing a pH indicator that changes colour due to histamine-induced alkalinization (Bover-Cid and Holzapfel, 1999; Maijala and Eerola, 1993). Unfortunately, these methods are

not always effective in the detection of histamine-producing LAB since the large amount of lactate these produce can counteract this alkalinization (Ladero et al., 2015). Culture-independent methods, however, avoid this inconvenience, are more exhaustive in their detection possibilities, and are less-time consuming (Jany and Barbier, 2008).

Different methods based on the PCR-amplification of the gene coding for histidine decarboxylase, *hdcA*, have been developed for detecting both Gram-positive (Coton and Coton, 2005; Le Jeune et al., 1995) and Gram-negative histamine-producing bacteria (de Las Rivas et al., 2005). Real time PCR methods allow for the quantification of such bacteria (Bjornsdottir-Butler et al., 2011; Fernandez et al., 2006), but despite being rapid, specific and sensitive, they cannot distinguish exactly which species are the histamine-producers in complex microbial communities. Since *hdcA* has been identified in a number of dairy LAB (Calles-Enriquez et al., 2010; Diaz et al., 2015a; Diaz et al., 2015b, Martin et al., 2005), as well as in LAB of other origin (Lucas et al., 2005; Satomi et al., 2008), it could be used to identify such histamine-producers; while the gene remains quite conserved, those of different species show some variation. PCR-denaturing gradient gel electrophoresis (PCR-DGGE), which can separate amplicons of the same size but different sequence (Fischer and Lerman, 1979), provides one means of distinguishing between variants of *hdcA*. PCR-DGGE based on the 16S rDNA sequence is usually employed for determining the genetic diversity of complex microbial populations, but functional genes associated with metabolic activities of interest can also be used as molecular markers (Cremonesi et al., 2001; Florez et al., 2014, Wawer and Muyzer, 1995). Thus, PCR-DGGE could be used to identify the *hdcA* genes from different species forming part of complex microbial communities, such as those that exist in fermented food products.

The present study proposes a PCR-DGGE method for the detection and identification of histamine-producing LAB, the use of which may allow for a better understanding of the histamine-producing microbiota present in complex substrates such as fermented foods. In the present work, it was optimized for the testing of commercial cheese samples.

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

H-Class AcquityUPLC™ UPLC system (Waters, Milford, USA) as previously described (Redruello et al., 2013); separations were performed at 35 ºC using a Waters AcquityUPLC™ BEHC18 1.7 µm column (2.1 x 100 mm). Data were acquired and analyzed using Empower 2 software (Waters).

2.4. PCR amplification

PCR reactions were performed in 50 µL volumes using 5PRIME Taq DNA polymerase (5 PRIME GmbH, Hilden, Germany), following the manufacturer's instructions. All reactions were performed in an iCycler thermocycler (Bio-Rad, Hercules, USA). All amplicons were analyzed on 1% agarose gels in TAE (40 mM Tris/acetate [pH 8.0], 1 mM EDTA) buffer; bands were visualized following staining with ethidium bromide in a G-Box and using GeneSys image acquisition software (Syngene, Cambridge, UK).

2.5. DGGE analysis

All PCR products were purified using the ATP™ Gel/PCR Extraction Kit (ATP TM Biotech Inc., Taipei City, Taiwan). DGGE was then performed using a DCode apparatus (Bio-Rad, Hercules, USA) at 65 ºC, employing 8% (w/v) polyacrylamide gels with a denaturing gradient ranging from 25 to 45% (100% corresponding to 7 M urea and 40% to deionized formamide). Electrophoresis was performed at 75 V for 16 h. After staining the gel with ethidium bromide (0.5 µg mL^{-1}), the bands were visualized under UV light in a G-Box and using GeneSys image acquisition software.

2.6. Identification of DGGE bands

The DGGE bands were identified by comparing their migration against markers of known *hdcA* sequence. To confirm the results, and to identify those bands that did not match any marker, all the bands were sequenced. For this, they were excised from the gels and deposited in 20 µL sterile water overnight at 4 ºC to extract the DNA. This was then re-amplified using the primer pair hdcDG-F/hdcDG-R (35 cycles of 94 ºC for 30 s, 55 ºC for 45 s and 68 ºC for 30 s, plus

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

3. Results

3.1 Specific primer design

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

3.2. Optimization of PCR amplification

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

After testing the efficacy of the primers, a GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3') was linked to both to obtain primers C-hdcDG-F (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGCCTGGTCAAGGCTATGGTGTATGGTC-3') and C-hdcDG-R (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGTTTCATCATTGCGTGTGCAAA-3') respectively. PCR amplifications with the primer pairs C-hdcDG-F/hdcDG-R and hdcDG-F/C-hdcDG-R were run at different annealing temperatures ranging from 50 to 55 ºC, using DNA from *hdcA*$^+$ bacteria (Table 1) as a template. Positive amplification were observed for all the *hdcA*$^+$ strains tested and using either primer combination. However, the best amplification results were obtained with an annealing temperature of 50 ºC; this was, therefore, used in all subsequent PCR amplifications.

3.3. Optimisation of DGGE

DNA from pure cultures of *hdcA*$^+$ LAB species of dairy origin (Table 1) was used as a template in PCR reactions, employing primer pairs C-hdcDG-F/hdcDG-R and hdcDG-F/C-hdcDG-R under optimized conditions. The amplicons obtained were separated by DGGE using one of two different denaturing gradients: 33-55% and 25-45%, in 8% polyacrylamide. Amplicons obtained with primer pair hdcDG-F/C-hdcDG-R could not be separated under the tested conditions (data not shown). Amplicons obtained with C-hdcDG-2F/hdcDG-R showed good separation, with the best band separation obtained using the 25-45% denaturing gradient (Fig. 1). Amplicons from pure cultures of *L. reuteri* IPLA 11078, *L. vaginalis* IPLA11060*, L. parabuchneri* IPLA11129 and *S. thermophilus* CHCC1524 was used as markers in the subsequent electrophoretic analysis of DNA from the cheese samples (Fig. 1).

3.4. PCR-DGGE analysis of bacterial hdcA *genes present in Cabrales cheese samples*

The amount of histamine and the presence of bacterial *hdcA* genes in 18 Cabrales cheese samples were determined (Table 2 and Fig. 2). This traditional

blue cheese (made from raw milk) was chosen since, not only does it habitually have high concentrations of BAs, including histamine (Fernandez et al., 2006; Fernandez et al., 2007), it is also very diverse in terms of the microorganisms present (Florez and Mayo, 2006). Histamine was found in all the samples tested, ranging from 10 to 1271 mg kg^{-1} of cheese (Table 2). Bands on the polyacrylamide gels were compared with those of the markers, but only those matching *L. parabuchneri* could be identified (note band c, Fig. 2). Some of these bands were excised from the acrylamide gel and the amplicons sequenced and compared to sequences in GenBank; 100% similarity with the *hdcA* gene of *L. parabuchneri* was observed. The bands that did not match any of the markers were also excised from the gel, sequenced, and compared to sequences in the above database. All those analyzed showed 99-100% similarity with GenBank *hdcA* sequences. Bands i and j showed 99% similarity with the *hdcA* gene of *L. parabuchneri*. Bands e and f were 100% identical to the *hdcA* gene of *T. halophilus*. Bands g and h were 100% identical to the *hdcA* genes of *L. hilgardii hdcA* and *L. sakei hdcA;* these two species could not, therefore, be distinguished.

3.5. PCR-DGGE analysis of bacterial hdcA *genes present in samples of other types of cheese*

The concentration of histamine and the presence of different bacterial *hdcA* genes was analyzed in 10 Manchego-type cheeses (industrially-made semi-hard cheeses) from different producers, three Gamoneu cheese samples (a traditional smoked blue-veined cheese made from raw cow's, sheep's and goat's milk), one Idiazabal cheese (a traditional cheese made from raw sheep's milk), and one Casín cheese (a traditional, long-matured cheese made from raw cow's milk) (Fig. 3). Histamine was present in 12 of these 15 samples (80%), ranging from 17 to 421 mg kg^{-1} of cheese (Table 2).

After DGGE, the bands on the polyacrylamide gels were identified by comparison with markers when possible. Bands that showed the same migration pattern as that observed in the previous DGGE gels (Fig. 2) were denoted with the same letter. Some representative bands that migrated in the same fashion as that of the *L. parabuchneri* marker (band c, Fig. 3) were

sequenced and found 100% identical to the *hdcA* gene of the latter species. A band that migrated in the same fashion as the *S. thermophilus* marker (band d, Fig. 3) appeared in one of the samples. This band was also sequenced, and was 100% identical to that of the *hdcA* gene of *S. thermophilus*.

The bands that matched none of the markers were sequenced and showed 99-100% similarity with different *hdcA* sequences in the GenBank database. As in the Cabrales cheeses, bands i and j showed 99% similarity to the *hdcA* gene from *L. parabuchneri*, band e was 100% identical to the *hdcA* from *T. halophilus*, and band h 100% identical to the *hdcA* from *L. hilgardii* and *L. sakei*.

3.6. Diversity of histamine-producing species in the analyzed cheeses

Taking all the analyzed samples as a whole, the diversity of histamine-producing species detected was quite low (Fig. 4). *L. parabuchneri* was the most common (present in all the analyzed samples), and the only species present in the Cabrales samples with the highest concentrations of histamine. In addition, it was the only histamine-producing species present in all the Gamoneu and Casín samples.

The other histamine-producing species were relatively scarce. *T. halophilus*, which was found in some Cabrales and Manchego cheeses, was the second most common (present in six of the 33 samples tested). *L. hilgardii/L. sakei* appeared in just two Cabrales samples. Histamine-producing *S. thermophilus* was detected only in the Idiazabal cheese.

The maximum diversity of LAB histamine producers within a sample was two species; this was only seen in the Cabrales and Manchego-type cheeses. This presence of two species was not correlated with any greater histamine concentration.

4. Discussion

Recent years have seen increasing efforts to produce safer and higher quality dairy products, including products that contain no toxic BAs. Histamine, the only BA for which, in some foods, a legal limit has been established, is one of the most toxic and commonly encountered BAs in cheese (Linares et al., 2011). Its

accumulation in food depends on several environmental and technological factors, although the presence of microorganisms with histamine-generating capacity is essential (Linares et al., 2012). An in-depth knowledge of the microbial species involved in its accumulation in cheese will be needed if we are to prevent its build-up. However, classical microbiological methods cannot always identify the BA-producing species present - the differential culture media available are not sufficiently selective (Bover-Cid and Holzapfel, 1999; Maijala and Eerola, 1993). Thus, when BA-producing microorganisms make up only a small proportion of the full microbiota – as is the case in some cheeses (Ladero et al., 2009) - it becomes virtually impossible to isolate them. Culture-independent methods, mainly based on PCR, are also available, and these can detect (Coton and Coton, 2005; de Las Rivas et al., 2005; Le Jeune et al., 1995) and even quantify BA-producing bacteria (Bjornsdottir-Butler et al., 2011; Fernandez et al., 2006), but they cannot always identify the species involved .

In dairy products, histamine is mainly produced by LAB with histidine decarboxylase activity (Linares et al., 2012). In the present work, the alignment of the *hdcA* sequences from different LAB and other Gram-positive bacteria (allowing highly conserved regions to be detected) led to the design of a pair of primers able to bind to the conserved regions of *hdcA*, but flanking a region that varies between species, and of a size (approximately 250 bp) suitable for PCR-DGGE analysis. The amplification of DNA from pure cultures of dairy *hdcA*$^+$ LAB with these primers allowed their efficacy to be confirmed and the optimal conditions for further analysis by DGGE to be established. The optimization of the DGGE gradient allowed for the production of good separation patterns when DNA from cheese samples was used as a template. The reproducibility of the PCR-DGGE profiles obtained was very good (results not shown).

The proposed method successfully detected and identified the *hdcA*$^+$ LAB present in the tested cheeses, even in those with complex microbial communities. Four species of histamine producing bacteria were identified in the 33 cheeses tested. To our knowledge, this is the first time that *L. hilgardii/L. sakei* and *T. halophilus* have been described as potential histamine producers in cheese, underscoring the usefulness of the proposed method. Since the sequence of the amplified region was identical in both species in *L. hilgardii* and *L. sakei*, these species were indistinguishable. However, their common *hdcA*

sequence was only detected in two Cabrales cheese samples, both of which had a relatively low histamine concentration, and in which *L. parabuchneri* was also present. *L. hilgardii* is commonly present in wine (Sohier et al., 1999) and *L. sakei* is involved in meat fermentation (Chaillou et al., 2013), and both have previously been detected in cheese (Carafa et al., 2015; De Pasquale et al., 2014), although neither have previously been associated with histamine production.

T. halophilus was the second most common *hdcA*$^+$ species found in the present work: it was detected in three Cabrales and three Manchego-type samples. This species is usually found in salted and fermented foods such as soy sauce and fish sauce (Kuda et al., 2014). It has also been isolated from cheese (Morales et al., 2011), although never in large numbers, and detected in it by PCR-DGGE (Alegria et al., 2012). It has been suggested that halophilic lactic acid bacteria can come from marine environments via sea salt added to cheeses (Ishikawa, 2007). It has, however, never before been associated with histamine production in this type of food.

The Idiazabal cheese, which contained no histamine, was the only one to return a band corresponding to *S. thermophilus,* a species that includes strains able to produce histamine (Calles-Enriquez et al., 2010; Rossi et al., 2011), although in low amounts (Gezginc et al., 2013). These results suggest that this species is not responsible for histamine accumulation in cheese. Although *S. thermophilus* is usually present in this food (Montel et al., 2014), it has never before been described in Idiazabal cheese. *S. thermophilus hdcA*$^+$ strains are little mentioned in the literature, further highlighting the sensitivity of the proposed PCR-DGGE method.

L. vaginalis and *L. reuteri* were included among the PCR-DGGE markers since they are known histamine-producers that have previously been isolated from cheese (Diaz et al., 2015a). However, they were not detected in any of the samples analyzed.

L. parabuchneri was the most common species; it was present in all the analyzed cheese samples. Indeed, the literature reports it to be one of the most common obligate heterofermentative lactobacilli in cheese (Coton et al., 2008). Further, most of the characterized *L. parabuchneri* dairy strains are histamine producers (Carafa et al., 2015; Diaz et al., 2015a; Diaz et al., 2015b, Fröhlich-

Wyder et al., 2013; Sumner et al., 1985). The presence of other histamine-producing species alongside *L. parabuchneri* was not associated with any higher concentration of histamine. Indeed, in the samples with highest histamine, *L. parabuchneri* was the sole histamine producer. Thus, *L. parabuchneri* would seem to be the species most responsible for histamine accumulation in the analyzed cheeses.

It is well known that the presence of BA producers is an essential condition that must be met for BA to accumulate in food, but it is not the only one; accumulation also depends on a number of environmental and technological factors, e.g., the availability of amino acid substrates (Linares, Del Rio et al. 2012). This explains the presence of *L. parabuchneri* in the cheese samples without histamine. It is important to note that the proposed method is based on standard PCR, and as such can reveal the diversity of histamine-producers but cannot determine the numbers of each type. Thus, no correlation can be established between band intensity and the number of histamine-producing LABs in the sample. Culture-independent quantitative methods have been developed at our laboratory that would allow this (Fernandez et al., 2006), but they cannot identify the histamine-producing species. Since knowing the identity, the diversity, and the prevalence of histamine-producing bacteria in cheese types is essential if measures to prevent their appearance in food are to be taken, the proposed method and these culture-independent quantitative methods should be used to complement one another.

In conclusion, the proposed PCR-DGGE method provides a useful and effective means of identifying the species responsible for the accumulation of histamine in foods with complex microbial communities, such as cheese. In the present work it even identified species not previously known to be histamine producers. The results reveal *L. parabuchneri* to be the species most likely responsible for the accumulation of histamine; it was the only species present in all of the samples tested and even on its own can produce large amounts of histamine. Moreover, the proposed method could be applied along the whole cheese production process to identify the entry points of histamine producers and consequently, it may be of help in the design of strategies aimed at reducing the numbers of histamine-producing bacteria in cheese.

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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	Manchego-type
26	39	
27	50	
28	50	
29	56	
30	67	
31	17	Gamoneu
32	17	
33	352	

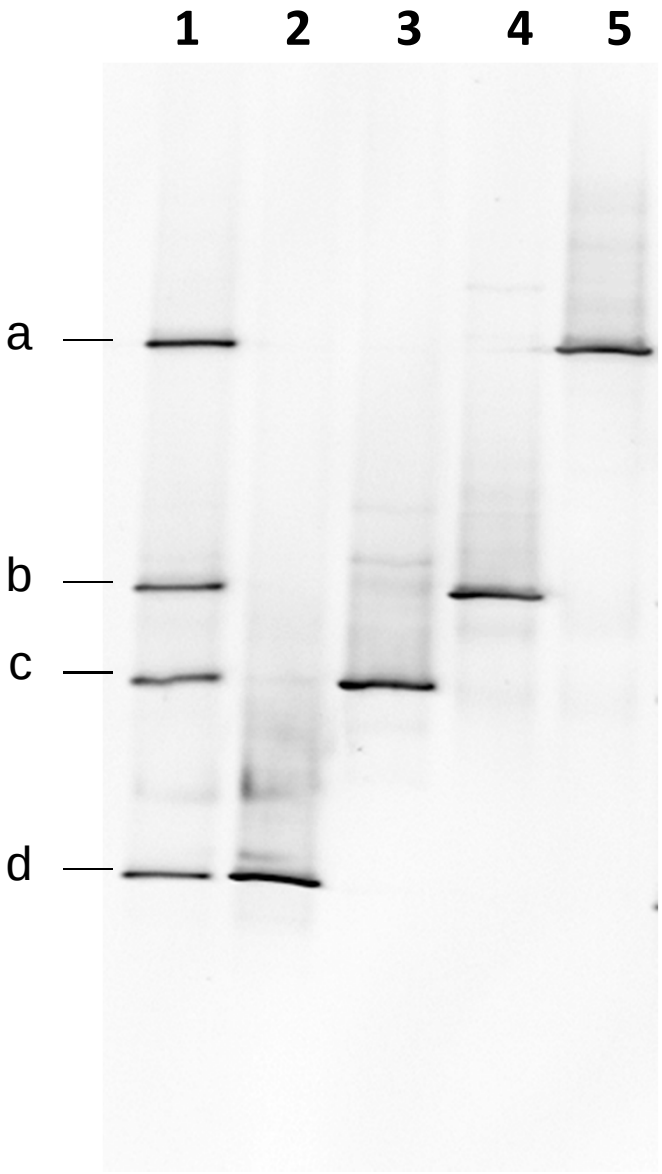
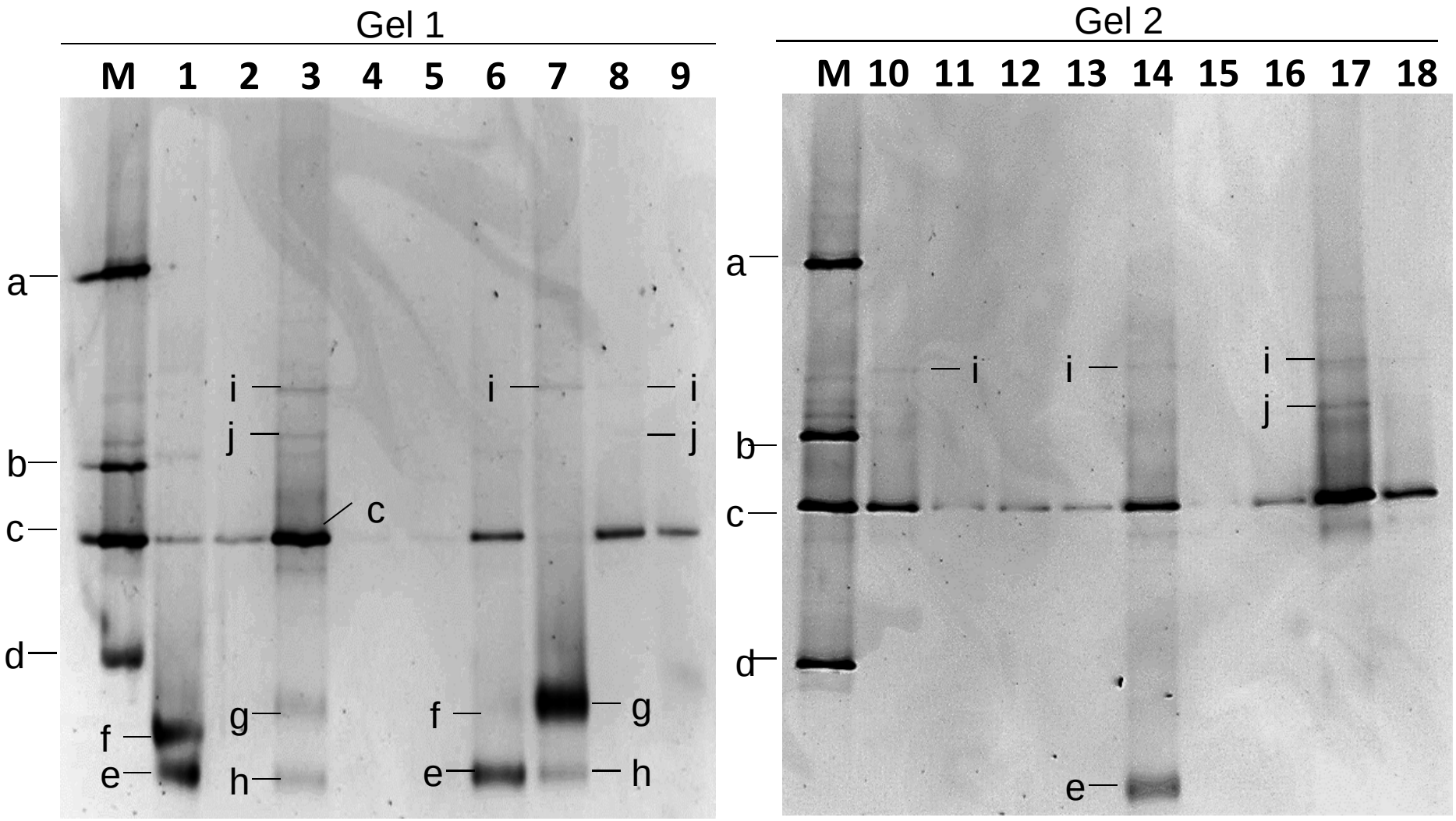


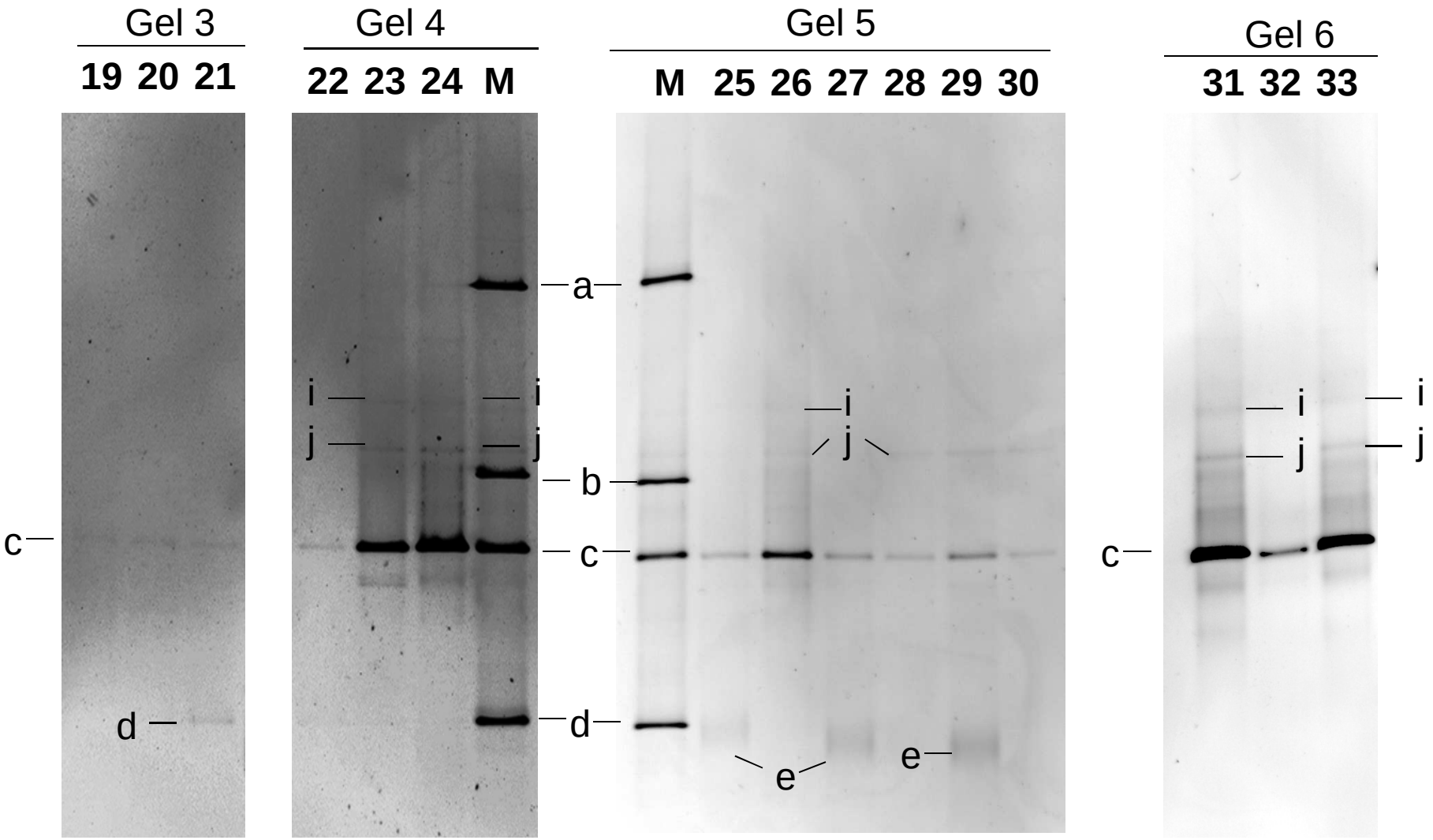
Figure 1



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

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

Figure 2



[Hma] mg/kg	0	0	0

122	134	421	[Hma] mg/kg

[Hma] mg/kg	28	39	50	50	56	67

[Hma] mg/kg	17	17	352

Figure 3

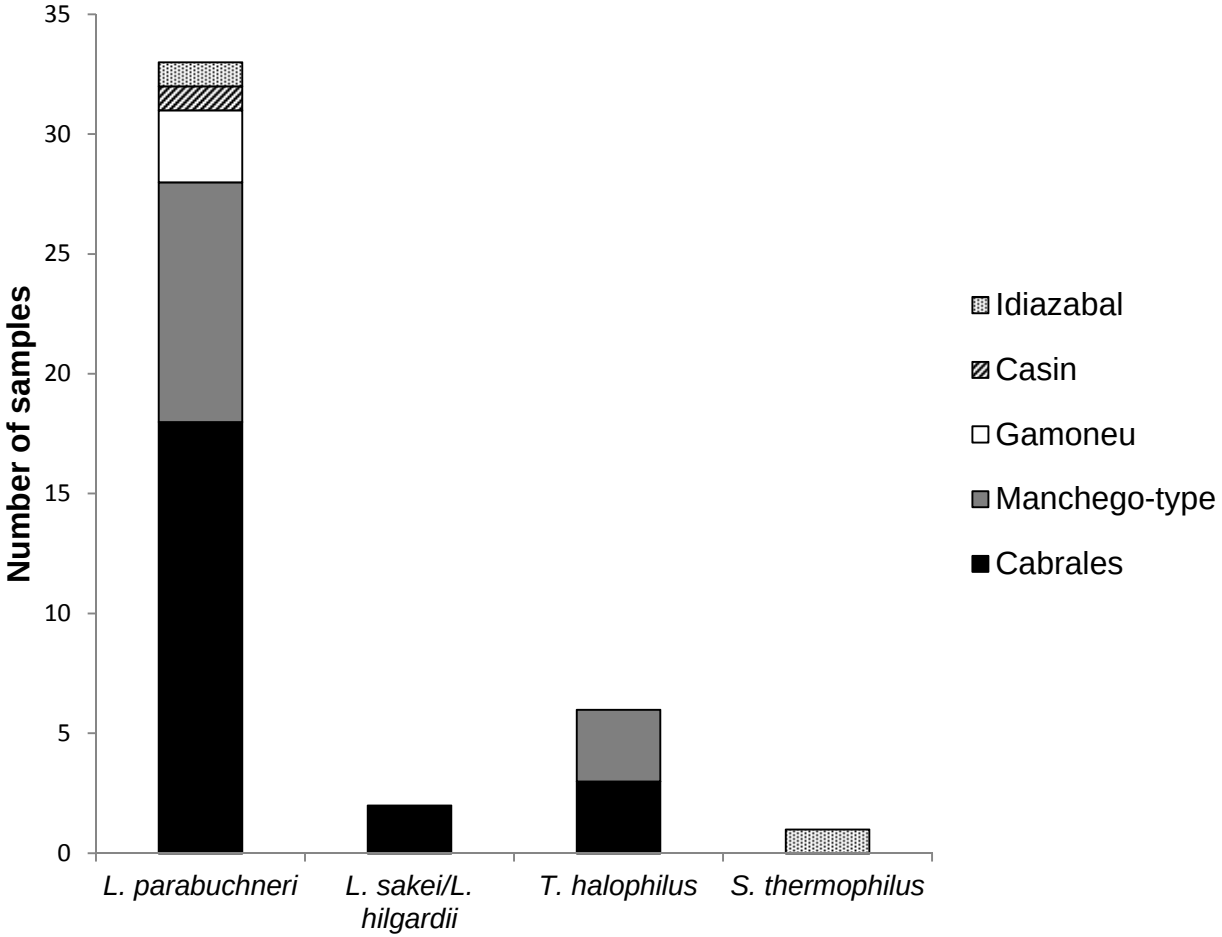

35
30
25
20
15
10
5
0
Number of samples
L. parabuchneri
L. sakei/L. hilgardii
T. halophilus
S. thermophilus
Idiazabal
Casin
Gamoneu
Manchego-type
Cabrales

Figure 4

Highlights

- New PCR-DGGE method identifies histamine-producing bacteria at species level
- The method was validated using samples of different cheese types
- New species were identified as potential histamine producers in cheese
- The dominant histamine-producing species in cheese is *L. parabuchneri*