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Identification by means of molecular tools of the microbiota responsible for the formation of histamine accumulated in commercial cheeses in Spain

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ABSTRACT

Histamine intoxication is an important food safety and public health concern. Ripened cheeses are the most common dairy products in which histamine can accumulate. Histamine is formed by the microbiota present in cheese by decarboxylation of histidine, due to the action of the histidine decarboxylase. This study's objective was to identify the responsible for the formation of histamine accumulated in commercial cheeses. The content of histamine of 39 different types of cheeses marketed in Spain, of varying milk origin, was assessed. About one third of the cheeses analysed contained more than 200 mg/kg histamine; two cheeses exceeded 500 mg/kg histamine, the consumption of such cheeses can be harmful or even toxic for consumers. The five cheeses with the highest histamine concentrations were selected for in-depth molecular analysis. Firstly, bacterial and yeast isolates were obtained, and then the total genetic material from the cheeses was analysed, in order to verify the putative presence of the *hdc* histidine decarboxylase gene. In order to identify the histamine producing microorganisms, the nucleotide sequences of the histidine decarboxylase genes from the cheeses were amplified, and subjected them to Sanger sequencing. In four of the five selected cheeses, the main histamine producer was identified as *Lentilactobacillus parabuchneri*, whereas in the remaining cheese it was *Tetragenococcus halophilus*. The *hdc* gene was located in an unstable plasmid, only present in that cheese sample. Since all histamine producing microorganisms identified in this study are not part of the species used in cheese starter cultures, an improvement of hygienic manufacturing practices and/or thermal treatments for microbial inactivation in milk may be considered to prevent histamine accumulation in cheeses during ripening.

1. Introduction

Biogenic amines (BAs) are organic nitrogenous compounds frequently found in a number of foods, such as ripened cheese, red wine, fish or meat and their derivatives (Comas-Basté, Sánchez-Pérez, Veciana-Nogués, Latorre-Moratalla, & Vidal-Carou, 2020; Ruiz-Capillas & Herrero, 2019). Histamine is a heterocyclic BA formed from the precursor amino acid L-histidine via oxidative decarboxylation, catalysed by the L-histidine decarboxylase (HDC) enzyme (Ozogul & Ozogul, 2020). When accumulated, histamine has been shown to cause severe symptomatology such as skin rashes, headache, nausea, diarrhoea, and variations in blood pressure (Ladero, Calles-Enriquez, Fernandez, & Alvarez, 2010). Histamine in food is therefore an important issue concerning food safety (EFSA, 2011). Given its adverse health effects, it is essential to prevent its accumulation in food (Hrubisko, Danis, Huorka, & Wawruch, 2021). A potential no-observed-adverse-effect limit (NOAEL) of 50 mg histamine for headache and flushing has been suggested. However, no symptoms have been shown by some healthy individuals up to 300 mg histamine and even up to 2.4 times than the NOAEL did not affect some sensitive people (EFSA, 2011).

Whereas histamine limits for fish and fish products are established in legislation (200 mg/kg) according to the European Commission Regulation Nº 2073/2005 (European Parliament, 2005), no regulation on histamine content in dairy products has been imposed.

In fermented food, histamine is produced by some of the microbiota present as a consequence of the action of the HDC enzyme, from the free amino acid precursor histidine (Landete, Pardo, & Ferrer, 2008). Cheeses are a perfect environment for histamine accumulation, reaching levels of up to 2,500 mg/kg (Madejska, Michalski, Pawul-Gruba, & Osek,

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Abbreviations

BAs	Biogenic amines
FDA	Food and Drug Administration
HDC	L-histidine decarboxylase
HPLC	High-performance liquid chromatography
LAB	Lactic acid bacteria
MDA-H	Modified decarboxylating agar supplemented with histidine
NOAEL	No-observed-adverse-effect-level
NSLAB	Nonstarter lactic acid bacteria
OTU	Operational Taxonomic Units
PCR	Polymerase chain reaction
SLAB	Starter lactic acid bacteria
TCA	Trichloroacetic acid

2018). The formation of histamine in food by microorganisms depends on both microbiological and environmental factors (Moniente, García-Gonzalo, Ontañón, Pagán, & Botello-Morte, 2021). It is well known that proteolysis that occurs during cheese ripening contributes to an increase in the availability of different free amino acids, which serve as precursors for the formation of biogenic amines (Linares, Martin, Ladero, Alvarez, & Fernandez, 2011). Other important environmental factors determining the formation of histamine and other BAs are carbon source (0–3% glucose), salt content (up to 20% NaCl w/v), pH (5.0–6.5), water activity (0.90–1.00), and processing conditions such as ripening (10–20 °C) and storage (4–42 °C) temperatures/times among others (Moniente et al., 2021).

From a microbiological point of view, cheese microbiota is diverse, varies over time, and is composed of starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) as well as other desired and undesired microorganisms (Ercolini, 2020; Mayo, Rodriguez, Vazquez, & Florez, 2021; Nam, Cho, Rackerby, Goddik, & Park, 2021). Both gram-positive and gram-negative bacteria present in cheese are able to synthesize histamine due to the action of the HDC enzyme (Landete, de Las Rivas, Marcobal, & Muñoz, 2008). Spoilage bacteria or contaminants of milk, such as *Enterobacteriaceae* or *Pseudomonadaceae*, have been frequently reported as responsible bacteria for histamine production in cheese (Fernandez-Garcia, Tomillo, & Nunez, 2000; Roig-Sangüés, Molina, & Hernández-Herrero, 2002). Histamine forming gram-negative bacteria contain a copy of the *hdc* gene in the chromosome (Landete, de Las Rivas, Marcobal, & Munoz, 2007).

The main histamine producing bacteria in cheese are gram-positive LAB, due to their role in fermentation and maturation processes (Barbieri, Montanari, Gardini, & Tabanelli, 2019). NSLAB originate from raw milk and contribute to the development of flavour (Settanni & Moschetti, 2010). *Lentilactobacillus parabuchneri* and *L. buchneri* are the major histamine producers in cheese (Berthoud et al., 2017; Diaz et al., 2016; Díaz et al., 2018; Diaz, Del Rio et al., 2016; O'Sullivan et al., 2015; Wechsler et al., 2021; Wüthrich et al., 2017). Other NSLAB and microorganisms such as *Limosilactobacillus vaginalis* and *L. reuteri* (Diaz et al., 2015), *Latilactobacillus sakei/L. hilgardii* (Diaz et al., 2016), *Levilactobacillus brevis, Lacticaseibacillus casei* and *Lactiplantibacillus plantarum* (del Valle, Ginovart, Gordún, & Carbó, 2018), *Tetragenococcus halophilus* (Diaz et al., 2016), and *L. paracasei* (Møller, Ucok, & Rattray, 2020), have also been reported as histamine producers. In long-ripened cheeses, NSLAB need to be present, otherwise no desirable flavor will be developed. NSLAB can grow and survive in more adverse environmental conditions, such as pH as low as 5.0 or energy depletion (Blaya, Barzideh, & LaPointe, 2018).

However, certain SLAB, which are intentionally added to milk and participate in the fermentation of lactose in the initial ripening process (Blaya et al., 2018), can also contribute to histamine formation in cheese (Barbieri et al., 2019). For instance, histamine forming SLAB in cheese are *Streptococcus thermophilus* (Diaz et al., 2016; Gardini et al., 2012; Gezginc, Akyol, Kuley, & Ozogul, 2013; Ladero et al., 2015; Rossi et al., 2011), *Lactococcus lactis* (Roig-Sangüés et al., 2002), *L. delbrueckii* subsp. *lactis, L. curvatus* and *L. helveticus* (Burdychova & Komprda, 2007), or *Leuconostoc* spp. (del Valle et al., 2018).

L. parabuchneri, *L. vaginalis, Clostridium perfringens, Staphylococcus capitis*, among others, display the so-called histidine decarboxylase gene cluster in the bacterial chromosome (Landete, de Las Rivas, Marcobal, & Muñoz, 2008); however, in certain species such as *L. hilgardii* and *T. halophilus*, the *hdc* gene is located on a mobile plasmid (Lucas, Wolken, Claisse, Lolkema, & Lonvaud-Funel, 2005; Satomi, Furushita, Oikawa, Yoshikawa-Takahashi, & Yano, 2008; Wüthrich et al., 2017).

Furthermore, yeast contribute to the flavour, ripening and fermentation of cheeses, although some of them can also cause spoilage, producing undesired eyes, off-flavours or miscolouring of cheeses (Yeluri Jonnala, McSweeney, Sheehan, & Cotter, 2018). However, these microorganisms are usually hardly detectable inside the cheese because of their oxygen requirements; although some yeast able to ferment sugars such as *Kluyveromyces marxianus* or *Debaryomyces hansenii* have also been located in the cheese core (Frohlich-Wyder, Arias-Roth, & Jakob, 2019). The main histamine producing yeast in cheese is *D. hansenii* (Gardini et al., 2006; Helinck, Perello, Deetae, de Revel, & Spinnler, 2013), although it has also been shown to degrade histamine (Baumlisberger, Moellecken, Konig, & Claus, 2015). Nevertheless, *D. hansenii* was able to form little amounts of histamine: about 5 mg/L in a laboratory medium or about 17 mg/kg in a model cheese in a co-culture with *Psychrobacter* sp. (Helinck et al., 2013). *Geotrichum candidum* has likewise been reported as a histamine forming mould in Cabrales cheese (Roig-Sangüés et al., 2002). However, the gene coding for the enzyme responsible for the synthesis of histamine in moulds and yeasts has not yet been described.

In Spain, more than 100 cheese types are produced mainly from the milk of cow, sheep, or goat, or from blends of milks thereof. The aim of the present study was to detect and quantify histamine in a selection of cheeses marketed in Spain, and to identify histamine producing microorganisms. The ability to determine the relationship between histamine amounts and microbiota present in cheeses could contribute to propose preventive measures to control or avoid occurrence of the responsible microbiota, and thereby to address the serious public health and safety concern caused by commercial cheeses highly contaminated with histamine.

2. Materials and methods

2.1. Reagents

For histamine determination, all chemicals and reagents were HPLC or analytical grade. Histamine dihydrochloride and dansyl chloride were obtained from Sigma Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA) and $NaHCO_3$ solution were purchased from Panreac (Barcelona, Spain). Methanol and acetone were purchased from VWR (Barcelona, Spain). Deionised water was obtained by using a Milli-Q system (Millipore Sigma, USA). The microbiological culture media de Man, Rogosa and Sharpe (MRS), bacteriological agar, Tryptone Soya Agar-Yeast Extract (TSA-YE), Tryptone Soya Broth (TSB), Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) were purchased from Oxoid (Cheshire, England). Gram-Negative (GN) was purchased from Scharlab (Barcelona, Spain). M17 agar and broth were purchased from Sigma Aldrich and Buffered Peptone Water (BPW) were obtained from Merck. Histidine was purchased from Panreac. For DNA extraction, the DNeasy Blood and Tissue Kit (QIAGEN, Manchester, UK) was used. Trisodium citrate, EDTA were obtained from Panreac. Trizma Hydrochloride, Triton X-100, lysozyme and mutanolysin were purchased from Sigma Aldrich. Agarose and Tris Borate EDTA buffer used in PCR assays were purchased from Sigma Aldrich. Phusion High-fidelity DNA

polymerase and Sybr Safe-DNA were purchased from Thermofisher Scientific (Massachusetts, USA). DNA was quantified by using the Qubit 1x dsDNA HS Assay kit from Thermofisher Scientific.

2.2. Cheese samples

A total of 39 commercial cheeses from different types of milk (cow, sheep, goat, and blends), including both raw and pasteurized milk, were purchased from Spanish supermarkets from July to November 2019. Their main characteristics are summarized in Table 1.

2.3. Histamine quantification

Histamine determination was based on Saaid, Saad, Hashim, Mohamed Ali, and Saleh (2009). Briefly, 5 g of the central area of cheese were weighed and 20 g of 10% (w/v) TCA were added. Samples were homogenized for 5 min in a vertical Geno/Grinder® 2010 230 SPEX homogenizer at 800 rpm and centrifuged at 4,824 *g* for 10 min. The supernatants were filtered through Whatman No. 1 filters. For the derivatization process, 200 μL of extracts were mixed with 800 μL of 0.25 M $NaHCO_3$ solution, 600 μL acetone and 400 μL of dansyl chloride, and incubated in the dark in a water bath at 60 °C for 60 min. Samples were then cooled at room temperature in the dark, and filtered using 0.45 μm membrane filters. High performance liquid chromatography (HPLC) analyses were performed in an Agilent 1200 Series HPLC (Agilent, Santa Clara, CA, US) using a Tracer Extrasil ODS2 (Teknokroma, Barcelona, Spain) (200 mm × 4.6 mm internal diameter 5 μm) reversed-phase C18 chromatographic column, and monitored with a diode-array detector at 254 nm. The mobile phase was ultrapure water/methanol (25/75, v/v); the sample injection volume was 20 μL, and the flow-rate was 1 mL/min. Histamine stock standard solution (1, 000 mg/L) was prepared by dissolving 165.6 mg of histamine dihydrochloride in 100 mL of 10% TCA. Working standards solutions for the calibration curve were prepared by diluting stock solution with 10% TCA at different concentrations ranging 1–10 mg/L and 10–100 mg/L.

2.4. Isolation of histamine forming bacteria and yeast from cheese

10-g samples were homogenized with 90 mL of 0.1% (w/v) BPW in a stomacher for 5 min and serially diluted in sterile 0.1% (w/v) BPW. General microbial isolations were obtained on different common agar lab media: MRS, M17, TSA-YE, PDA, and GN, under aerobic conditions (except for MRS plates, which were incubated anaerobically) at 30 °C or 37 °C. Colonies were selected based on colony morphology. *Lactobacillus* strains were routinely cultured in MRS agar or broth in a DW Scientific MACS VA 500 anaerobic chamber (DW Whitley Scientific Don Whitley, Bingley, UK) (90% nitrogen, 5% hydrogen and 5% carbon dioxide) at 37 °C. *Streptococcus* and *Enterococcus* strains were grown on M17 Broth, or supplemented with 1.5% (w/v) bacteriological agar, aerobically at 37 °C. *Tetragenococcus* strains and *Morganella morganii* subsp. *sibonii* (NCIMB 865) were cultured in TSA or TSB aerobically at 37 °C. Yeasts were grown in PDA or PDB, aerobically at 30 °C. To detect histamine producing bacteria in cheese, microbial isolation was also performed in modified decarboxylating agar supplemented with histidine (MDA-H), as described by Maijala (1993).

2.5. Total DNA extraction from five selected cheeses and from microbial isolates

Total DNA was extracted from bacterial cultures grown to stationary phase using the DNeasy Blood and Tissue kit according to the manufacturer's instructions.

Isolation of DNA from cheeses was performed as described by Parayre et al. (2007), with some modifications. Duplicate 1-g cheese samples were homogenized in 9 mL of sterile 2% (w/v) trisodium citrate using Lab Blender 400 Stomacher (Seward, West Sussex, UK) at 300 rpm for 2 min. After centrifugation at 10,000 *g* for 10 min at room temperature, supernatants and fat layers were removed. Pellets were resuspended in 400 μL of enzymatic lysis buffer, composed of 20 mM Trizma Hydrochloride pH 8, 2 mM EDTA, 1.2% (v/v) Triton X-100, 20 mg/mL lysozyme, and 50 U/mL mutanolysin, and incubated at 37 °C and 300 rpm for 1 h. Then, 25 μL proteinase K and 200 μL buffer AL from the DNeasy Blood and Tissue kit were added, and incubated at 70 °C and 300 rpm for 30 min. Samples were then subjected to mechanical lysis by adding 0.1 mm glass beads (BioSpec Products, Bartlesville, USA) and using a mini bead beater (Biospec) at 2,500 rpm for 90 s. After centrifugation at 10,000 *g* for 10 min at room temperature, supernatants were transferred to a column of the DNeasy Blood and Tissue kit according to the manufacturer's instructions.

Isolated DNA was quantified by using Qubit 1x dsDNA HS Assay kit, following the manufacturer's instructions. This quantification method is based on a fluorescent dye specific for double-stranded DNA, to avoid measurements of contaminants such as RNA or impurities.

2.6. Detection of the hdc gene by polymerase chain reaction (PCR) amplification

PCR assays were carried out by implementing Phusion High-fidelity DNA polymerase, following the manufacturer's instructions, in a T100 thermal cycler (Bio-rad Laboratories, Madrid, Spain). PCR amplicons were analysed on a 1–1.2% (w/v) agarose gel in Tris Borate EDTA buffer, stained with Sybr Safe-DNA gel stain, and visualized using a GelDoc EZ (Bio-rad) documentation system.

Detection of the histidine decarboxylase (*hdc*) gene and the plasmid pHDC was performed by PCR with specific oligonucleotides for gram-positive and gram-negative bacteria, previously described in literature (Table 2). In PCR analyses, DNA from *L. parabuchneri* DSM 5987 and *M. morganii* subsp. *sibonii* (NCIMB 865) were used as histamine producing gram-positive and gram-negative bacterial controls, respectively.

For taxonomical studies and microbial identification, the universal oligonucleotides 27F/1492R for bacteria and NL1/NL4 for yeasts were used to amplify the genes coding for the 16S/28S rRNA genes, respectively (Table 2).

2.7. DNA sequencing and analysis

When necessary, PCR products were purified using Diffinity RapidTip (Sigma Aldrich, St. Louis, Missouri, USA) and sent to STABVIDA (Oeiras, Portugal) for Sanger sequencing. Sequences were analysed using the NCBI nucleotide database (BlastN: https://blast.ncbi.nlm.nih.gov/).

2.8. High-throughput sequencing of 16S RNA (V3–V4) regions

DNA isolated from the cheese samples was subjected to 16S rRNA gene (V3–V4 region) amplicon sequencing using the HiSeq2500 PE250 sequencing platform of Novogene Company Limited (Beijing, China).

2.9. Formation of histamine in broth media

To determine the production of histamine, a pure culture of *D. hansenii* was inoculated into 0.1% BPW or PDB, supplemented with 10 mM histidine and aerobically incubated at 30 °C for 24h. Histamine in the supernatant was quantified by HPLC. Furthermore, in order to evaluate histidine decarboxylase activity, a pure culture of *D. hansenii* was also plated on the chromogenic agar medium MDA-H (Maijala, 1993) and incubated for 24 h at 30 °C.

Table 1
Information regarding cheese samples including cheese variety, milk source, thermic treatment, geographic manufacturing area, appellation of origin, country, firmness, ripening time and histamine content quantified by HPLC.

Sample Code	Cheese variety	Milk source	Heat treatment of the milk	Geographic manufacturing area	Appellation of origin	Country	Firmness	Ripening time (days)	Histamine (mg/kg)[a]
Q1	Parmigiano Reggiano	cow	raw	Reggio Emilia	+	Italy	Hard	≥540	285
Q2	Gruyère	cow	raw	Vevy	+	France	Semi-hard	≥180	215
Q3	Grana Padano	cow	raw	Reggio Emilia	+	Italy	Hard	≥300	18
Q4	Ripened hard cow cheese	cow	pasteurized	Cavallermaggiore	–	Italy	Hard	≥360	57
Q5	Blue	cow	pasteurized	Bavaria	–	Germany	Semi-soft	≥90	<5
Q6	Blue	cow	pasteurized	Cantal	–	France	Semi-soft	≥90	<5
Q7	Camembert	cow	pasteurized	Normandia	–	France	Soft	≥21	<5
Q8	Camembert	cow	pasteurized	Normandia	–	France	Soft	≥21	<5
Q9	Camembert	cow	pasteurized	Normandia	–	France	Soft	≥21	<5
Q10	Brie	cow	pasteurized	Brie	–	France	Soft	≥21	<5
Q11	Brie	cow	pasteurized	Los Vosgos	–	France	Soft	≥21	<5
Q12	Tetilla	cow	pasteurized	Galicia	+	Spain	Semi-soft	7	<5
Q13	Cabrales	Min. 90% cow, 1% sheep, 1% goat	raw	Asturias	+	Spain	Semi-soft	≥60	**380**
Q14	Blue	Min. 80% cow, 10% goat	pasteurized	Valdeón	+	Spain	Semi-soft	≥60	<5
Q15	Ripened hard cheese	60% cow. 40% sheep	pasteurized	Castilla León	–	Spain	Hard	180	23
Q16	Ripened semi-soft goat cheese	Min. 50% cow, 30% goat, 5% sheep	pasteurized	Albacete	–	Spain	Semi-soft	7	<5
Q17	Aged hard cheese	Min. 45% cow. 20% sheep. 20% goat	raw	Zamora	–	Spain	Hard	≥360	213
Q18	Ripened hard cheese	Min. 40% cow. 45% sheep	pasteurized	Albacete	–	Spain	Hard	≥150	<5
Q19	Ripened hard goat cheese	goat	raw	Extremadura	+	Spain	Hard	90	172
Q20	Goat cheese with paprika	goat	pasteurized	Extremadura	+	Spain	Semi-hard	21	25
Q21	Ripened goat cheese	goat	pasteurized	Valencia	–	Spain	Semi-soft	180	<5
Q22	Camerano	goat	pasteurized	La Rioja	+	Spain	Semi-soft	≥30	<5
Q23	Idiazabal	sheep	raw	País Vasco	+	Spain	Hard	≥60	**571**
Q24	Ripened hard sheep cheese	sheep	raw	Zamora	–	Spain	Hard	≥240	**551**
Q25	Roncal	sheep	raw	Navarra	+	Spain	Hard	≥240	**353**
Q26	Roncal	sheep	raw	Navarra	+	Spain	Hard	≥240	**347**
Q27	Ripened hard sheep cheese	sheep	raw	Zamora	–	Spain	Hard	≥105	289
Q28	Aged hard sheep cheese	sheep	raw	Zamora	–	Spain	Hard	≥360	247
Q29	Ripened hard sheep cheese	sheep	raw	Navarra	–	Spain	Hard	≥60	236
Q30	Old hard sheep cheese	sheep	raw	Zamora	–	Spain	Hard	≥180	134
Q31	Idiazabal	sheep	raw	País Vasco	+	Spain	Hard	180	20
Q32	Long-ripened hard sheep cheese	sheep	raw	Castilla León	–	Spain	Hard	≥240	6
Q33	Ripened hard sheep cheese	sheep	raw	Zamora	–	Spain	Hard	≥240	5
Q34	Roquefort	sheep	raw	Cantal	+	France	Semi-soft	≥90	<5
Q35	Roquefort	sheep	raw	Roquefort-sur-Soulzon	+	France	Semi-soft	≥90	<5
Q36	Long-ripened hard sheep cheese	sheep	raw	Navarra	–	Spain	Hard	≥240	<5
Q37	Long-ripened hard sheep cheese	sheep	raw	Castilla León	–	Spain	Hard	≥210	<5
Q38	Long-ripened sheep cheese	sheep	pasteurized	Ciudad Real	–	Spain	Hard	≥270	<5
Q39	Manchego	sheep	pasteurized	Albacete	+	Spain	Hard	≥60	<5

[a] The relative standard deviations for reproducibility and the uncertainties of the method were 15 and 28% for 1–50 mg/kg, and 7 and 20% for 50–500 mg/kg, respectively. Histamine concentration above 300 mg/kg is highlighted in bold.

Table 2
List of oligonucleotides used in this study.

Primer name	Sequence	Target	Reference
27F	AGAGTTTGATCCTGGCTCAG	Prokaryotic 16S rRNA gene	Lane (1991)
1492R	GGTTACCTTGTTACGACTT	Prokaryotic 16S rRNA gene	Lane (1991)
NL1	GCATATCAATAAGCGGAGGAAAAG	Eukaryotic 28S rRNA gene	Kurtzman and Robnett (1998)
NL4	GGTCCGTGTTTCAAGACGG	Eukaryotic 28S rRNA gene	Kurtzman and Robnett (1998)
HIS2F	AAYTSNTTYGAYTTYGARAARGARGT	*hdc* gene from gram-negative bacteria	de Las Rivas, Marcobal, Carrascosa, and Munoz (2006)
HIS2R	TANGGNSANCCDATCATYTTRTGNCC	*hdc* gene from gram-negative bacteria	de Las Rivas et al. (2006)
JV17HC	AGACCATACACCATAACCTT	*hdc* gene from gram-positive bacteria	Le Jeune, Lonvaud-Funel, ten Brink, Hofstra, and van der Vossen (1995)
HDC3	GATGGTATTGTTTCKTATGA	*hdc* gene from gram-positive bacteria	Coton and Coton (2005)
pHDCF	CGCGGCAACAAAGGGTCC	Plasmid pHDC containing the *hdc* gene	This paper
pHDCR	CGCTGATTCAGAATGACTTGAC	Plasmid pHDC containing the *hdc* gene	This paper
HmF	TGTTTCGTATGACCGTGCGG	*hdc* gene from *Tetragenococcus*	Satomi et al. (2008)
HmR	CACCATTTTCGCCGGCAGTG	*hdc* gene from *Tetragenococcus*	Satomi et al. (2008)

3. Results and discussion

3.1. Histamine quantification in cheese

In a preliminary step of the in-depth study of the microbiota responsible for histamine accumulation, the selected 39 cheeses were analysed for their histamine content. To this aim, histamine present in cheese was quantified by HPLC (Table 1).

Twenty of the 39 cheeses (51.2%) showed a detectable level of histamine (>5 mg/kg), ranging from 5 to 571 mg/kg. The average histamine content in samples with a quantified level of histamine was 207.35 mg/kg. In almost one third (28.2%) of the 39 cheeses tested, histamine content exceeded 200 mg/kg. Notably in two cheeses, an Idiazabal (an aged hard cheese, made from raw sheep milk in Basque Country and Navarre regions, in the North of Spain) and a ripened hard sheep cheese, histamine levels were even higher than 500 mg/kg. The EFSA has considered such levels as potentially toxic to human health (EFSA, 2011). Such amounts of histamine have been reported to cause a severe symptomatology in histamine intolerants or sensitive people, who make up ca. 1% of the entire population (Maintz & Novak, 2007). However, considering a normal portion of cheese of 30 g, a total ingestion of about 15 mg histamine would result, which is below the NOAEL proposed by EFSA for healthy individuals (EFSA, 2011).

Up to 17 (85%) of 20 cheeses with a detectable level of histamine were made from raw milk, whereas cheeses made from pasteurized milk mostly had an unquantifiable histamine level (Table 1).

In previous studies, the highest histamine content had been detected in raw-milk cheeses: 1,042 mg/kg (Fernandez, Linares, Del Río, Ladero, & Alvarez, 2007), 391 mg/kg (Novella-Rodríguez, Veciana-Nogués, Izquierdo-Pulido, & Vidal-Carou, 2003), 43 mg/kg (Novella-Rodríguez, Veciana-Nogués, Roig-Sagués, Trujillo-Mesa, & Vidal-Carou, 2004) and 573 mg/kg (Fernandez, Linares, Del Río, Ladero, & Alvarez, 2007; Novella-Rodríguez et al., 2003; Novella-Rodríguez et al., 2004; Schneller, Good, & Jenny, 1997), probably due to higher microbial count in general and consequently, a possible higher amount of histamine producing bacteria in raw milk than in pasteurized milk (Benkerroum, 2016; Linares et al., 2012). However, it is noteworthy that raw milk cheeses made with high cooking temperatures or which are made from high-quality milk contain only traces of histamine (Wechsler et al., 2021).

It is remarkable to note that 11 (55%) of the 20 cheeses with detectable histamine levels were exclusively made from sheep's milk (Table 1). Conversely, cow milk cheeses did not tend to display histamine accumulation, a finding in line with other studies (Kandasamy et al., 2021; Linares et al., 2012). This is probably due to the fact that protocols and regulations regarding hygiene and quality are more relaxed in sheep and goat milk production than in the case of cow (Gonzalo, 2017; Kovacova et al., 2021). Furthermore, milk from small ruminants such as goats or sheep usually contains higher bacterial counts, since a higher number of animals must be milked for the same quantity of milk, compared to cow's milk. This also increases the risk of undesirable bacteria (e.g., histamine forming bacteria) entering the milk, due to insufficiently cleaned teats (van den Brom, de Jong, van Engelen, Heuvelink, & Vellema, 2020). The higher protein content of sheep milk (5.7%) versus cow milk (3.4%) could also allow for a greater degree of proteolysis and would thus entail a greater amount of free histidine as a histamine precursor than in cow milk (Moniente et al., 2021). However, when BAs-forming microorganisms grow during cheese ripening, the proteolytic activity of the starters used plays a certain role. Compared to mesophilic lactococci starters, proteolysis in depth is more pronounced when thermophilic lactobacilli are contained in a starter (Blaya et al., 2018).

Furthermore, hard or semi-hard cheeses such as Parmesan, Idiazabal, Roncal, etc. had more accumulated histamine in this study than soft cheeses such as Roquefort, Camembert or Brie varieties (Table 1), which correlated with a longer ripening time for the former (Linares et al., 2012). The differences in proteolysis between the different cheese types should also be considered. Soft cheeses are manufactured with mesophilic cultures. As a consequence, proteolysis in width is more pronounced than proteolysis in depth (De Pasquale, Di Cagno, Buchin, De Angelis, & Gobbetti, 2019).

According to our results, it would be advisable for histamine intolerant or sensitive individuals to remove certain raw milk cheese varieties and, ideally, hard and semi-hard cheeses (Idiazabal, Parmesan, Roncal, etc.) entirely from their diet.

Finally, the five cheeses with the highest histamine content (Q13, Q23, Q24, Q25 and Q26), all above 300 mg/kg, were selected for further analyses to obtain insight into the causes of their excessive histamine content. As mentioned above, all five of them were raw-milk cheeses: four of them were from sheep milk; and the fifth one was a blended-milk cheese (Table 1).

3.2. Isolation and identification of bacteria and yeast from cheese containing high concentrations of histamine

Cultured-based isolations of bacteria and yeasts present in selected cheeses were performed. Cheese homogenates were obtained and plated in different agar media (MRS, M17, GN, TSA-YE, Caseinate agar, PDA) to obtain microbial isolates. All the colonies with a distinctive morphology (shape, colour …) were subcultured to ensure their purity. Finally, 21 isolates were studied in MRS and M17, and 15 isolates in TSA-YE and caseinate agar, all of them belonging to the group of gram-positive bacteria, whereas 9 isolates were analysed in PDA, which corresponded to yeasts. All these isolates were subjected to DNA extraction and taxonomic identification based on 16S rRNA (Table 3). All the bacterial isolates identified had been previously detected in cheeses (Moniente et al., 2021). Notably, isolates belonging to the group of gram-negative bacteria were not obtained, in spite of plating in a specific laboratory medium, named Gram-Negative (GN) medium. The GN broth, also known as Hajna broth, is a liquid culture medium for selective enrichment of gram-negative bacteria (Hajna, 1955). A similar result was obtained in a study performed with Spanish cheeses where

Table 3
Identification of microbial isolates from cheeses by taxonomic studies, based on Sanger sequencing of the 16S/28S rRNA genes.

Cheese sample	Identification
Q13	*Lactiplantibacillus plantarum*
	Levilactobacillus brevis
	Enterococcus faecalis
	Tetragenococcus halophilus
	Debaryomyces hansenii
	Candida zeylanoides
Q23	*Lacticaseibacillus paracasei*
	Levilactobacillus brevis
	Enterococcus durans
	Enterococcus faecalis
Q24	*Lactiplantibacillus plantarum*
	Staphylococcus epidermidis
	Staphylococcus equorum
	Debaryomyces hansenii
Q25	*Lactiplantibacillus plantarum*
	Lacticaseibacillus paracasei
	Enterococcus faecalis
	Leuconostoc mesenteroides
	Staphylococcus equorum
	Debaryomyces hansenii
	Candida zeylanoides
Q26	*Levilactobacillus brevis*
	Lactiplantibacillus plantarum
	Lacticaseibacillus casei
	Lacticaseibacillus paracasei
	Enterococcus durans
	Enterococcus faecalis
	Enterococcus gilvus
	Candida parapsilosis
	Candida zeylanoides

enterobacteria were not detected in many cheese samples (Roig-Sangüés et al., 2002), probably due to the fact that enterobacteria are not favoured by ripening conditions. *Enterobacteriaceae* usually reach their maximal counts immediately after cheese production (day 1) and they continuously decrease and often disappear after ripening periods higher than 90 days (Vernozy-Rozand et al., 2005). Furthermore, some of the yeast isolates included *D. hansenii*, previously described as a histamine producer in literature (Gardini et al., 2006; Helinck et al., 2013). Microbial isolation was also performed directly in the chromogenic decarboxylation agar MDA-H, in which histamine producers can be detected by a distinct change in the colour of the medium as a result of an increase in pH (Maijala, 1993). However, no colour change was observed in any of the cases.

3.3. Searching for the hdc gene in the microbial isolates, and in total DNA from cheese

To assess whether the microbial isolates had the potential to produce histamine, a screening aimed to detect the *hdc* gene was performed. For this purpose, total DNA was obtained from the 45 isolates obtained from the five selected cheeses. Different pairs of previously described oligonucleotides were used to amplify via PCR the *hdc* gene from gram-positive bacteria. Although no gram-negative isolates were obtained from cheeses, other primers aimed to amplify the *hdc* gene from gram-negative bacteria were still used, since it was conceivable that certain gram-negative histamine producers could not be isolated by cultured-based methods used. Still, no bands appeared in the bacterial isolates from cheeses (data not shown). Instead of the analysis at molecular level of 45 isolates to detect the presence of the *hdc* gene, none of them resulted in a positive amplification. Later, template DNA was extracted directly from cheeses and not from microbiological isolates. In this case, no amplicons were generated in any cheese with primers HIS2F and HIS2R (for gram-negative bacteria) either (Fig. 1B), as observed by O'Sullivan et al. (2015). This is also consistent with the absence of gram-negative isolates from the cheeses. Nevertheless, as expected, a

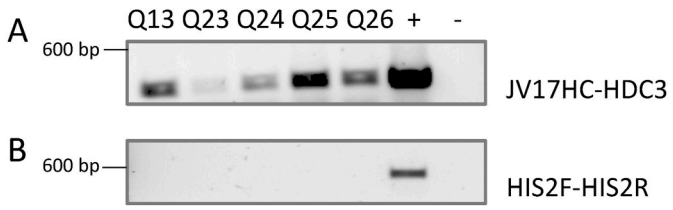

Fig. 1. PCR amplification of *hdc* gene, using total DNA obtained from cheeses as template, and specific oligonucleotides JV17HC and HDC3 for gram-positive bacteria (**A**) or HIS2F and HIS2R for gram-negative bacteria (**B**). *Lentilactobacillus parabuchneri* DSM 5789 and *Morganella morganii* subsp. *sibonii* were used as positive controls (+) of *hdc* in gram-positive and gram-negative bacteria, respectively.

band of about 370 bp corresponding to the *hdc* gene was detected in all the cheese samples with the primers JV17HC and HDC3 designed for gram-positive bacteria (Fig. 1A).

3.4. Sanger sequencing and identification of histamine producing bacteria

To identify the bacterial species responsible for the synthesis of histamine, the PCR amplicons were purified and subjected to Sanger sequencing. The results obtained are summarized in Table 4. In four out of five cheeses (samples Q23, Q24, Q25 and Q26), the *hdc* sequences corresponded to those of *L. parabuchneri* or *L. buchneri.* This species was persistent at the farm level, present in more than 97% of raw milk samples contaminated with histamine producing bacteria (Ascone et al., 2017). High histamine accumulation in ripened cheeses can result from even minimal contamination with *L. parabuchneri* in the raw milk from which it was made (Wechsler et al., 2021). Remarkably, *L. parabuchneri* is capable of growing and producing histamine even at refrigeration temperatures (Díaz et al., 2018). It is able to form biofilms on stainless steel (Diaz, Ladero, Del Rio et al., 2016). The colonization of the surfaces of cheese-making equipment such as milk containers, transport pipes and other accessories could result in bacterial proliferation and histamine production which causes a serious problem of safety and quality of milk for the cheese industry (Srey, Jahid, & Ha, 2013). *L. parabuchneri* should be carefully monitored throughout the entire cheese-making process, particularly in raw milk, to avoid histamine accumulation in the final product (Ascone et al., 2017; Wechsler et al., 2021).

In the remaining cheese Q13, however, the sequence obtained corresponded to the *hdc* gene from *T. halophilus*, a nucleotide sequence that is conserved across *L. sakei, T. halophilus, T. muriaticus, Oenococcus oeni* and *L. hilgardii*: the high level of similarity (>99%) among the *hdc* sequences impairs the capacity of distinguishing among the *hdc* of these species with this sequencing method (Wüthrich et al., 2017). It is worth highlighting that an isolate of *T. halophilus* was obtained from cheese sample Q13 (Table 3), hence it could be possible that the *hdc* sequence obtained would correspond to that bacterial species. However, it is more common that the genus *Tetragenococcus* is responsible for histamine

Table 4
Summary of the main BLAST analyses of the amplicons of the *hdc* genes obtained from cheeses.

Cheese sample	Main BLAST output	E-value	% identity	GenBank accession number
Q13	*Tetragenoccus halophilus*	6e-140	99.29%	AB670117.1
Q23	*Lentilactobacillus parabuchneri*	4e-152	99.67%	CP018796.1
Q24	*Lentilactobacillus parabuchneri*	8e-144	100%	CP018796.1
Q25	*Lentilactobacillus parabuchneri*	1e-147	99.66%	CP018796.1
Q26	*Lentilactobacillus parabuchneri*	1e-151	99.30%	CP018796.1

accumulation in fish and its derivatives (Kimura, Konagaya, & Fujii, 2001; Satomi et al., 2008). *T. muriaticus* as well as *T. halophilus* have been previously reported as histamine producers in Cabrales and Manchego-type cheeses (Diaz et al., 2016; Diaz et al., 2015). To confirm whether this species was the microorganism responsible for histamine formation in this cheese, it should be performed whole genome sequencing sample and investigated the HDC operon, as the approach applied by Martin, Fernandez, Linares, and Alvarez (2005) and Møller, Castro-Mejía, Krych, and Rattray (2021). This strategy will be addressed in future works.

The nucleotide sequence of *hdc* obtained by PCR amplification and Sanger sequencing is compared with those available in current databases. The lack of a specific decarboxylase gene database, as well as of the nucleotide sequences of many *hdc* genes in today's databases, clearly hinders the identification of histamine producers. Additionally, the selection of the specific primers to be used to amplify the *hdc* gene present in a sample is a critical step in the attempt to obtain positive results. Preliminary PCR experiments using other previously published pairs of primers did not render any *hdc* amplicon in positive samples (data not shown). The reason could be the target species used in the design of those traditional primers. The *hdc* sequences of some species in the samples could not be amplified with the primers we used, because of the lack of correct alignment. Additionally, the impossibility of detecting hitherto undescribed emerging histamine producing strains with traditional primers is also an important drawback. A recent example involved several *L. parabuchneri* strains (KUH4, KUH5, KUH6, KUH7) that had not been identified by PCR with JV16HC (with practically the same nucleotide sequence that HDC3 and aligning in the same area of the *hdc* gene) and JV17HC primers (Møller et al., 2020).

3.5. Confirmation of the presence of the pHDC plasmid containing the hdc gene

Since the *hdc* gene in *T. halophilus* is located in an unstable, mobile plasmid called pHDC (Satomi et al., 2008), the oligonucleotides pHDCF and pHDCR were designed to align in the plasmid sequence, with the purpose of assessing this plasmid's presence in the total DNA of Q13 (Fig. 2A). As shown in Fig. 2B, the Q13 sample was the only one which contained the pHDC plasmid.

Furthermore, by using the oligonucleotides HmF and HmR previously described in the literature (Satomi et al., 2008) as well as different combinations with the pair pHDCF and pHDCR, it could be concluded that cheese Q13 was the only one which contained the *hdc* gene from *T. halophilus*, and that it is located in the pHDC plasmid (Fig. 2C).

As mentioned above in Section 3.2, although a *T. halophilus* strain was isolated from cheese Q13 (Table 3), it contained neither the *hdc* gene nor the pHDC plasmid, as shown in Fig. 2, taking the 16S rRNA gene as a loading control (amplified by primers 27F and 1492R) in PCR (Fig. 2C).

Since it has been previously reported that plasmid curation containing the *hdc* gene strongly depends on bacterial culture conditions (Lucas et al., 2005), it was supposed that the pHDC plasmid was probably lost during the subcultures required for the isolation of the microorganisms from cheese. The plasmid's instability should be taken into account: it is thus essential to control culture conditions when attempting to isolate histamine producers in foods via culture-based methods. Nevertheless, the simultaneous detection of the *hdc* gene and the pHDC plasmid from the cheese sample allowed us to identify a *Tetragenococcus* species as the putative histamine producer in this sample. This approach is particularly useful for the detection of histamine producing *Tetragenococcus* strains in suspected cheese samples.

3.6. Analysis of cheese microbiota by metataxonomic analysis

In this study, in agreement with previous studies, *L. parabuchneri* was shown to be the main bacterium responsible for the synthesis of histamine in four out of the five investigated cheeses. However, it could not be isolated from cheeses using standard laboratory media such as MRS. Attempts to isolate *L. parabuchneri* in more specific media such as LAPTg (Diaz, Del Rio et al., 2016) and MDA-H (Berthoud et al., 2017) were not successful. It has been previously suggested that this bacterium might be in "a non-culturable state" (Randazzo, Pitino, Ribbera, & Caggia, 2010), or might be directly non-culturable (Van Hoorde, Verstraete, Vandamme, & Huys, 2008). Therefore, metataxonomic analyses were performed, to confirm the presence of *L. parabuchneri* in the cheeses, since it allows for the detection of uncultured bacteria. The occurrence of the DNA of both histamine producers proposed in this work, *L. parabuchneri* and *T. halophilus,* was confirmed in the cheese samples (Table 5). The rest of the relative abundance of the OTUs obtained at the specie level is shown in Supplementary Table S1.

3.7. Ability of D. hansenii isolated from cheese to produce histamine

Regarding yeast isolates from cheese, *D. hansenii* has been previously

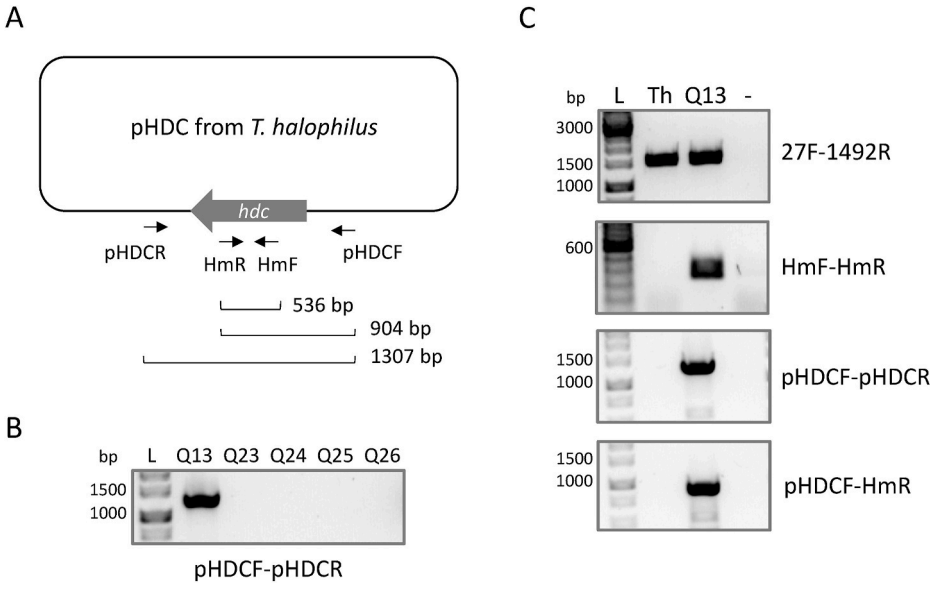

Fig. 2. **(A)** Schematic representation of the plasmid pHDC containing the *hdc* gene of *T. halophilus* and the position of the oligonucleotides pHDCF and pHDCR, used to amplify the plasmid sequence, and HmF and HmR, used to amplify the *hdc* gene. **(B)** PCR amplification of a fragment of 1,307 bp of the pHDC plasmid, using total DNA obtained from cheeses Q13, Q23 to Q26 as template, and specific oligonucleotides pHDCF and pHDCR. **(C)** PCR amplification using DNA from the *Tetragenococcus halophilus* isolate (Th) or total DNA obtained from cheese Q13 and specific oligonucleotides.

Table 5
Relative abundance of Operational Taxonomic Units (OTUs) at the species level of target microorganisms in selected cheeses.

Strain	Q13	Q23	Q24	Q25	Q26
Lentilactobacillus parabuchneri	0.0022	0.0032	0.0013	0.0006	0.0004
Tetragenococcus halophilus subsp. *halophilus*	0.0013	0.0001	0	0	0

described as histamine producer in cheese (Gardini et al., 2006; Helinck et al., 2013). However, the literature provides no genetic information regarding the histidine decarboxylase gene in yeast; therefore, histamine producing strains such as *D. hansenii* or *G. candidum* cannot be identified with this sequencing method. The need for new primer designs is a crucial challenge that needs to be addressed as soon as possible (Moniente et al., 2021).

For that reason, *in vivo* histamine production by the *D. hansenii* isolate was investigated by using the common decarboxylation medium MDA-H (Maijala, 1993), as well as HPLC quantification of the amount of histamine in the supernatant of the culture. Although a colour change was revealed in the chromogenic MDA-H medium (Fig. 3), similarly to what was observed by Gardini et al. (2006), no histamine could be quantified by HPLC after growth of *D. hansenii* for 24h either in a minimal or in a rich medium, both supplemented with 10 mM histidine. In the minimal medium (0.1% BPW), microbial counts remained constant throughout the experiment, indicating that the strain did not grow but also did not die. However, in the case of the rich medium (PDB), *D. hansenii* was able to grow by about 3-log orders in the course of the experiment (data not shown). The colour change in the decarboxylation agar could suggest that other amines could have been synthesized, since other free amino acids are also available in the media. In fact, false-positive results constitute a common drawback of this kind of culture-based method for the detection of histamine producing microbiota in food (Moniente et al., 2021).

Another possible reason that might explain the unquantifiable level of histamine in *D. hansenii* culture is that the culture conditions assayed were not optimal. It has been reported, for instance, that *S. thermophilus* strains containing the *hdc* gene were not able to produce histamine in culture (Ladero et al., 2015). Additionally, Helinck et al. (2013) evidenced a strain of *D. hansenii* that was only capable of producing ca. 5 mg/kg of histamine in a rich medium; thus it is also possible that the contribution of *D. hansenii* to histamine accumulation in cheese was very small or even insignificant. It is also conceivable that the strain would be synthesizing histamine as well as degrading it; a possible degradation of histamine by *D. hansenii,* as previously reported by Baumlisberger et al. (2015), was not investigated in the present study.

All in all, a possible contribution to the production of histamine on the part of *D. hansenii* in cheese matrix could not be discarded, but also not confirmed in this study.

3.8. Proposals of preventive measures

Overall, *L. parabuchneri* and *T. halophilus*, putative histamine producing species identified in this study, are classified as contaminant or environmental microorganisms, and not used as starter cultures for these cheeses. As explained above, NSLAB, which are able to grow under harsh conditions (Blaya et al., 2018), are required to the correct development of flavor in long-ripened cheeses. Monitoring of the pH, microbial composition of the NSLAB, use of microbial interaction, application of high salt content and low temperatures of ripening, between other strategies, should be included as preventive measures to cope with NSLAB (Moniente et al., 2021).

To prevent histamine formation in this case, it is important to identify the source of the histamine producing microbiota present in cheese, and to improve general hygiene conditions in order to impair the access of those histamine producers to the cheese-making process, either to raw milk or in a post-pasteurization step (Moniente et al., 2021). As mentioned above, pasteurization of milk for cheese production has been shown to be thoroughly effective in preventing the accumulation of histamine in cheeses. However, raw milk cheeses are a cultural heritage, which are usually chosen by consumers due to the exceptional sensory characteristics of many PDO raw milk cheeses. When a thermic treatment is applied as a hygienization preventive measure, the sensory characteristics may be altered. Pasteurized milk cheeses have been reported to exhibit a sweeter odour and taste, a creamier texture and a less characteristic taste than raw milk cheeses (Mendia, Ibáñez, Torre, & Barcina, 1999). In another study, pasteurization of milk resulted in cheeses with highest values of colour, sheep odour and bitter flavour (Rezaei et al., 2020).

4. Conclusions

Up to 51.2% of 39 commercial cheeses analysed showed a detectable histamine content, and almost one third (28.2%) contained more than 200 mg/kg. Notably, 85% cheeses with detectable histamine level were made from raw milk and 55% from sheep milk. The proposed histamine forming microorganisms identified in this study are the bacteria *L. parabuchneri* and *T. halophilus.* In *Tetragenococcus*, the *hdc* gene responsible for the synthesis of histamine is located in an unstable plasmid, which was probably curated in a *Tetragenococcus* isolate during subcultures under non-optimal conditions. For this reason, the use of

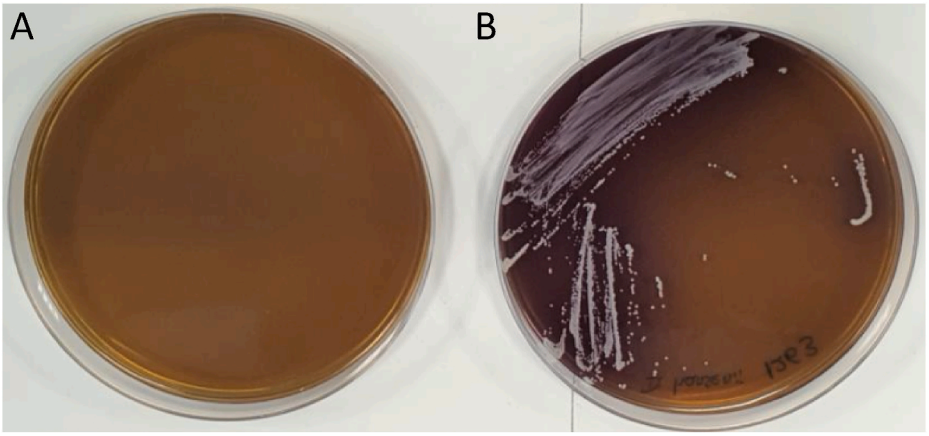

Fig. 3. Analysis of histamine production by the *Debaryomyces hansenii* isolate from cheese using decarboxylating MDA-H agar (A). The colour change of medium to purple after incubation indicates a pH change, putatively linked to histamine decarboxylation (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

total DNA from cheese and not from bacterial isolates could be proposed as a potentially more successful approach in the search for the *hdc* gene. Finally, in all cases, histamine producers were all nonstarter species; thus, strategies to obtain cheeses with lower histamine content should focus on controlling environmental or contaminant microorganisms that access raw milk and the cheese-making process, and on implementing good hygiene practices throughout the process.

CRediT authorship contribution statement

Laura Botello-Morte: Investigation, Supervision, Writing – original draft, Writing – review & editing. **Marta Moniente:** Investigation, Writing – original draft, Writing – review & editing. **Yolanda Gil-Ramírez:** Investigation, Writing – review & editing. **Raquel Virto:** Supervision, Project administration, Funding acquisition, Writing – review & editing. **Diego García-Gonzalo:** Supervision, Writing – review & editing. **Rafael Pagán:** Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.108595.

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