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Marta Moniente Aguilar

Determinación de histamina y de la microbiota responsable en productos lácteos: potenciales soluciones para reducir su acumulación en quesos madurados

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Tesis Doctoral

DETERMINACIÓN DE HISTAMINA Y DE LA MICROBIOTA RESPONSABLE EN PRODUCTOS LÁCTEOS: POTENCIALES SOLUCIONES PARA REDUCIR SU ACUMULACIÓN EN QUESOS MADURADOS

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Determinación de histamina y de la microbiota responsable en productos lácteos: potenciales soluciones para reducir su acumulación en quesos madurados

Memoria para optar al grado de Doctor por la Universidad de Zaragoza presentada por:

Marta Moniente Aguilar

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Dr. Rafael Pagán Tomás

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Tesis Doctoral como compendio de publicaciones

La Tesis Doctoral titulada "Determinación de histamina y de la microbiota responsable en productos lácteos: potenciales soluciones para reducir su acumulación en quesos madurados", de la que es autora Marta Moniente Aguilar, se presenta como un compendio de las siguientes publicaciones que se distribuyen en la introducción y resultados:

- **Moniente**, M.; Botello-Morte, L.; García-Gonzalo, D.; Pagán, R.; Ontañón, I. (2022). Analytical strategies for the determination of biogenic amines in dairy products. *Comprehensive Reviews in Food Science and Food Safety*, *21*(4), 3612-3646. https://doi.org/10.1111/1541-4337.12980
- **Moniente**, M.; García-Gonzalo, D.; Ontañón, I.; Pagán, R.; Botello-Morte, L. (2021). Histamine accumulation in dairy products: Microbial causes, techniques for the detection of histamine-producing microbiota, and potential solutions. *Comprehensive Reviews in Food Science and Food Safety*, *20*(2), 1481–1523. https://doi.org/10.1111/1541-4337.12704
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- **Moniente**, M.; García-Gonzalo, D.; Llamas-Arriba, M.G.; Virto, R.; Ontañón, I.; Pagán, R.; Botello-Morte, L. (2022). Potential of histamine-degrading microorganisms and diamine oxidase (DAO) for the reduction of histamine accumulation along the cheese ripening process. *Food Research International 160*, 111735. https://doi.org/10.1016/j.foodres.2022.111735.

Además, se incluye el siguiente trabajo que se encuentra en proceso de revisión en la revista *Food Research International*:

- **Moniente, M**.; García-Gonzalo; D. Botello-Morte; L. Ferreira, V.; Pagán; R. Ontañón; I. Combination of SPE and fluorescent detection of AQC-derivatives for the determination at sub-mg/L levels of biogenic amines in dairy products.

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Resumen

Resumen

Las aminas biógenas (ABs) son compuestos generados por la descarboxilación de su aminoácido precursor. Entre las ABs más importantes se encuentran la histamina, la tiramina, la cadaverina y la putrescina. Su ingesta a elevadas concentraciones por la población en general o su consumo a bajas concentraciones en personas sensibles puede suponer un problema importante para la salud pública. Es por ello que es necesario emplear métodos sensibles para la detección de estos metabolitos en alimentos. Los productos lácteos, y en especial los quesos madurados, son uno de los alimentos habitualmente implicados en intoxicaciones por histamina, generada por microorganismos con actividad descarboxilasa, lo que alumbra la necesidad de identificar estos microorganismos.

Los objetivos de esta Tesis Doctoral fueron evaluar la concentración de histamina en productos lácteos tras la puesta a punto de una técnica de análisis de ABs sensible; identificar los microorganismos responsables de la presencia de histamina en quesos comerciales; estudiar el patrón de distribución de la AB principal, la histamina, en diferentes áreas de quesos comerciales y evaluar su relación con la presencia de microorganismos productores y las características físicas y químicas y; ensayar posibles soluciones microbianas y enzimáticas que permitan reducir su acumulación en quesos de larga maduración.

Inicialmente, se desarrolló un método para la determinación de cuatro ABs (histamina, tiramina, putrescina y cadaverina) en diferentes productos lácteos (leche, yogur y kéfir) basado en cromatografía líquida de alta eficacia de fase reversa (RP-HPLC) acoplado a un sistema de fluorescencia. Las ABs se extrajeron selectivamente mediante extracción en fase sólida (SPE) y posteriormente se derivatizaron con 6-aminoquinolil-N-hidroxisuccinimidilo carbamato (AQC). Se obtuvo un método con una elevada sensibilidad, con límites de detección de entre 0,12 y 0,2 mg/L. Este método fue aplicado en 37 muestras de leche, 23 de yogur, y 14 de kéfir dónde se pudo comprobar la variabilidad de ABs entre las muestras. Si bien la mayoría de las muestras de leche no superaron el límite de detección (LD), la frecuencia de aparición y la concentración de estos compuestos fue mayor en yogur y kéfir alcanzando valores de hasta 79 mg/kg de ABs totales en muestras de kéfir.

Es conocido que los quesos madurados pueden contener cantidades elevadas de histamina. La microbiota presente en el queso es la responsable por la descarboxilación de su aminoácido precursor, la histidina, debido a la acción de la histidina descarboxilasa. Para conocer el origen de la histamina se llevó a cabo la identificación de los microorganismos

responsables de su formación en quesos. En primer lugar, se analizó la concentración de histamina de 39 tipos diferentes de quesos comerciales, para después identificar los microorganismos responsables de su aparición. Como resultado, una tercera parte de los quesos analizados contenían más de 200 mg/kg de histamina y dos de ellos superaron los 500 mg/kg histamina. Se obtuvieron aislados microbianos de los cinco quesos con las concentraciones más altas de histamina y se analizó el material genético total de los quesos, con el fin de verificar la presencia del gen de la histidina descarboxilasa (*hdc*). Tras comprobar la presencia del gen *hdc* en dichas muestras, se amplificó la secuencia de nucleótidos del gen *hdc*, y se sometió a secuenciación Sanger para identificar el microorganismo responsable de la formación de histamina en las muestras. En cuatro de los cinco quesos seleccionados, el gen *hdc* correspondió al microorganismo *Lentilactobacillus parabuchneri*, referido como principal productor de histamina en quesos, mientras que en uno de los quesos se identificó a *Tetragenococcus halophilus*, microorganismo que presenta el gen *hdc* ubicado en un plásmido inestable.

Una vez conocidos los microorganismos productores de histamina en quesos comerciales, se evaluó la distribución de histamina en cuatro zonas distales de una cuña (corteza periférica e interna y núcleo periférico e interior) de 12 quesos duros de larga maduración producidos con leche cruda de oveja o mezcla de leches, y se procedió a la identificación de los microorganismos que contenían el gen *hdc* mediante secuenciación Sanger. Se observó un patrón de distribución de histamina en las diferentes zonas acumulándose en el centro y disminuyendo en la corteza. Sin embargo, no se pudo establecer una correlación entre la distribución de la histamina y la microbiota presente, ya que la distribución de los microorganismos era similar en todas las partes del queso. En cuanto a la relación entre el análisis de las propiedades físicas y químicas de las diferentes áreas y la concentración de histamina se observó que la histamina tendía a acumularse en los lugares más salados, más húmedos y menos oxidados de la cuña.

Finalmente, se evaluaron medidas basadas en el uso de la enzima Diamino Oxidasa (DAO) y de microorganismos potencialmente degradadores de histamina (*Lacticaseibacillus casei* 4a y 18b, *Lactobacillus delbrueckii* subsp. *bulgaricus* Colección Española de Cultivos Tipo (CECT) 4005 y *Streptococcus salivarius* subsp. *thermophilus* CECT 7207, dos cultivos iniciadores comerciales de yogur (Abiasa y CHR Hansen), y la levadura *Debaryomyces hansenii*), para disminuir la acumulación en quesos de este metabolito tras 100 días de maduración. Se fabricaron un total de 8 lotes de queso de leche de vaca pasteurizada a los que se les añadieron los microorganismos mencionados anteriormente o la enzima DAO, todos ellos

conteniendo *L. parabuchneri* DSM 5987 (excepto el lote de queso de control negativo), responsable de la producción natural de histamina en el queso. Se analizaron las propiedades fisicoquímicas y sensoriales (olor), así como la concentración de histamina a lo largo de 100 días de maduración del queso. Como resultado, todas las estrategias aplicadas redujeron significativamente la concentración de histamina. Al final del periodo de maduración, se consiguió una reducción de más del 45% en los quesos preparados con *D. hansenii*, del 43% en aquellos preparados con las dos cepas de *L. casei*, del 42% en los quesos elaborados con *L. bulgaricus* y *S. thermophilus*, y del 23% en aquellos a los que se adicionó DAO. No se observaron cambios fisicoquímicos significativos (peso, pH, actividad del agua, color o textura) en los quesos como consecuencia de la adición de los cultivos microbianos o DAO. Sin embargo, la adición de microorganismos degradadores de histamina produjo quesos con un olor diferente al percibido en las muestras control, que no fue percibido como desagradable.

Como conclusión, cabe destacar la puesta a punto de un método sensible para la determinación de ABs en productos lácteos que permite detectar niveles bajos de estos metabolitos. Los microorganismos identificados como productores de histamina en quesos no proceden de los cultivos iniciadores empleados en su elaboración, sino que son microorganismos contaminantes. La concentración de histamina aumenta en las zonas interiores y disminuye en las zonas exteriores del queso lo que desvela la importancia de llevar a cabo una estrategia de muestreo cuando se pretende determinar la cantidad de histamina presente en un queso. La elaboración de quesos con bajos niveles de histamina se podría lograr mediante el empleo de técnicas sensibles para la determinación de este metabolito en productos lácteos que permitiera prevenir su entrada en el mercado, la utilización de leche libre de microorganismos productores de histamina, la limpieza y desinfección de los equipos para evitar la contaminación durante la obtención de la leche y su procesado en la industria láctea con microorganismos productores de histamina, o el uso de microorganismos degradadores o DAO que pudieran degradar la histamina a lo largo de la etapa de maduración del queso.

1. Introducción

1.1 Introducción

Los productos lácteos son uno de los alimentos más consumidos y apreciados por su riqueza nutricional en proteínas, carbohidratos, grasas, vitaminas y minerales (Pekcici et al., 2021). Sin embargo, esta composición proporciona un ambiente idóneo para la proliferación microbiana y la producción de metabolitos como las aminas biógenas (ABs) (Benkerroum, 2016).

Las ABs son compuestos orgánicos nitrogenados no volátiles de bajo peso molecular formadas principalmente por la descarboxilación enzimática de sus aminoácidos precursores, o por aminación y transaminación de aldehídos y cetonas (Alvarez & Moreno-Arribas, 2014; Benkerroum, 2016; Pluta-Kubica et al., 2020). La aparición de estos metabolitos en los alimentos requiere de la existencia de unas condiciones físicas y químicas (pH, actividad de agua, temperatura, etc.) que permitan el crecimiento microbiano y la actividad enzimática, de la disponibilidad de aminoácidos precursores y de la presencia de microorganismos con actividad descarboxilasa (Miranda et al., 2021; Moniente et al., 2021; Özogul & Özogul, 2020; Papageorgiou et al., 2018).

La histamina es una de las ABs más comúnmente presentes en alimentos (Linares et al., 2012) y se encuentra involucrada en numerosas actividades biológicas (Maintz & Novak, 2007). A nivel metabólico, la histamina es imprescindible para el buen funcionamiento del metabolismo humano incluyendo la neurotransmisión, la regulación del ritmo circadiano, la inmunomodulación, así como el crecimiento y la diferenciación celular (Ladero et al., 2010; Maintz & Novak, 2007). En las células de los mamíferos (mastocitos, basófilos, plaquetas, neuronas histaminérgicas y células enterocromafines) este metabolito es sintetizado intracelularmente por la L-histidina descarboxilasa (HDC) a partir del aminoácido histidina. Por el contrario, la histamina es catabolizada intracelularmente por la histamina N-metiltransferasa (HNMT) y extracelularmente por la Diamino Oxidasa (DAO) producida principalmente en los enterocitos (Ladero et al., 2010; Maintz & Novak, 2007; Moniente et al., 2021).

La presencia de histamina en alimentos en cantidades elevadas está implicada en la aparición de intoxicaciones alimentarias (Schirone et al., 2016) que pueden ocasionar serios problemas de salud a las personas que la ingieren. Se estima que el 1% de la población sufre una gran sensibilidad y es intolerante a la histamina, ya sea por causas genéticas o adquiridas, siendo en ellos más graves los incidentes toxicológicos (Comas-Basté et al., 2020). Además, el

consumo de alimentos ricos en histamina, de alcohol y de medicamentos que pueden liberar histamina o bloquear la actividad de DAO, pueden provocar problemas de salud como dolor de cabeza, asma, hipotensión, arritmia, urticaria, diarreas, sofocos y otras enfermedades tanto en personas sanas como en personas con intolerancia a la histamina (Maintz & Novak, 2007). Por estos motivos, su aparición en alimentos supone un problema para la salud pública que es necesario prevenir y controlar y, por tanto, se hace necesario el uso de métodos sensibles capaces de detectar niveles bajos de histamina que incluso permitan prescribir a este tipo de alimentos como de "bajo contenido en histamina" o "sin histamina".

En general, la cantidad de histamina presente en la leche y los productos lácteos fermentados es muy variable. En la leche, el yogur, el kéfir y los quesos frescos se han descrito valores que, aun siendo relativamente bajos, entre 1 y 10 mg/kg, podrían llegar a ocasionar síntomas a personas sensibles a la histamina (Linares et al., 2011; Spano et al., 2010). Por el contrario, en el queso madurado se pueden acumular cantidades muy elevadas de histamina como consecuencia de la actividad microbiana (Schirone et al., 2016) y el elevado contenido en proteínas y aminoácidos (Nuñez et al., 2016). Se han notificado cantidades de hasta 2.500 mg/kg de histamina (Bodmer et al., 1999; Moniente et al., 2021), pudiendo ocasionar graves problemas de salud o incluso la muerte a la persona que lo consume (Ladero et al., 2017; Ruiz-Capillas & Herrero, 2019). A pesar de ello, y a diferencia del pescado y productos pesqueros en los que la legislación establece un máximo de 200 mg/kg y 400 mg/kg, respectivamente (Reglamento Europeo, 2005), no existe un marco legal que regule la concentración máxima de histamina en productos lácteos. Algunos autores han estipulado que niveles de histamina superiores a 500 mg/kg se pueden considerar tóxicos para la salud humana (Nuñez et al., 2016). No obstante, existen recomendaciones que aconsejan no consumir productos lácteos con concentraciones de histamina superiores a 400 mg/kg (Rauscher-Gabernig et al., 2009).

Son muchos los microorganismos asociados a la formación de histamina en productos lácteos, entre ellos se incluyen principalmente las bacterias Gram positivas (algunas bacterias ácido lácticas (BAL)) (Barbieri et al., 2019), Gram negativas (algunos miembros de las familias *Enterobacteriaceae* o *Pseudomonadaceae* y *Aeromonas,* entre otras) (Linares et al.,2011; Andiç et al.,2013), levaduras (algunas cepas de *Kluyveromyces*, *Debaryomyces, Candida, Yarrowia* y *Pichia*) y mohos (*Geotrichum* y *Penicillium*) (Moniente et al., 2021; Nuñez et al., 2016; Roig-Sagués et al., 2002). Las BAL son las predominantes en este tipo de alimentos y poseen una gran importancia tecnológica debido a su uso como cultivos iniciadores en los procesos fermentativos (Settanni & Moschetti, 2010). Su aparición en la leche también puede suceder de

manera natural (*Enterococcus, Lentilactobacillus, Lactococcus, Leuconostoc, Pediococcus* y *Streptococcus*), por contaminación ambiental o por una mala higiene antes, durante o después del procesado (Moniente et al., 2021).

Estos microorganismos dan lugar a la síntesis de histamina gracias a la acción de la enzima HDC. El conocimiento de los mecanismos genéticos de producción de histamina es de gran utilidad para tratar de reducir los riesgos asociados con la intoxicación alimentaria por histamina. El gen *hdc* que codifica a la enzima HDC está organizado en clústeres con otros genes involucrados en el proceso de producción de histamina, como el transporte y la maduración de la enzima (Benkerroum, 2016; Linares et al., 2011). El gen *hdc* se puede encontrar en el cromosoma bacteriano o en un plásmido móvil. En las cepas de *Streptococcus thermophilus, Lentilactobacillus parabuchneri, Limosilactobacillus vaginalis, Clostridium perfringens,* entre otras, el gen *hdc* se encuentra en el cromosoma, mientras que en cepas de *Tetragenococcus halophilus* y *L. hilgardii,* el gen se recibe por transferencia horizontal de genes entre bacterias a través de un plásmido móvil llamado pHDC (Calles-Enríquez et al., 2010; Landete et al., 2008; Satomi et al., 2008). Esta capacidad de producción de histamina parece ser más un rasgo dependiente de la cepa bacteriana que específico de un género o especie microbiana (Diaz et al., 2018; Nuñez et al., 2016). No todos los microorganismos poseen esta capacidad de generar histamina, por ello, la selección de cultivos iniciadores en base a la ausencia de actividad descarboxilasa puede representar una estrategia para prevenir la presencia de histamina en productos lácteos.

En general, la aparición de histamina puede verse reducida al utilizar un buen sistema de limpieza y desinfección durante el proceso de obtención de la leche, unas materias primas de calidad, unas buenas prácticas higiénicas durante el procesado, y cultivos iniciadores que no contengan el gen *hdc*, limitando así el acceso de bacterias con capacidad de formación de histamina a los alimentos. A veces resulta complicado controlar todos estos factores, por la presencia entre otros de bacterias asociadas a la contaminación ambiental. Si estas medidas no fueran efectivas, una posible solución consiste en la degradación enzimática o microbiana de histamina mediante la adición de DAO o de cepas degradadoras de este metabolito, respectivamente (Benkerroum, 2016; Fausto Gardini et al., 2016).

Con objeto de profundizar en todos estos aspectos, la introducción de esta tesis doctoral incluye dos artículos de revisión bibliográfica:

- Moniente, M.; Botello-Morte, L.; García-Gonzalo, D.; Pagán, R.; Ontañón, I. (2022). Analytical strategies for the determination of biogenic amines in dairy products. *Comprehensive Reviews in Food Science and Food Safety*, *21*(4), 3612-3646. https://doi.org/10.1111/1541-4337.12980
- Moniente, M.; García-Gonzalo, D.; Ontañón, I.; Pagán, R.; Botello-Morte, L. (2021). Histamine accumulation in dairy products: Microbial causes, techniques for the detection of histamine-producing microbiota, and potential solutions. *Comprehensive Reviews in Food Science and Food Safety*, *20*(2), 1481–1523. https://doi.org/10.1111/1541-4337.12704

En el primer artículo se describen los diferentes métodos de análisis de ABs desarrollados para detectar, identificar y cuantificar estos metabolitos en productos lácteos con objeto de adquirir formación acerca de los distintos pretratamientos requeridos para la preparación de la muestra y de las características más destacables de las técnicas analíticas aplicadas. Cabe señalar el elevado número de investigaciones científicas a este respecto, lo que demuestra la preocupación por la presencia y concentración de ABs en este tipo de productos debido a los efectos nocivos para la salud que produce su ingestión, especialmente en pacientes intolerantes o sensibles a la histamina (Manuscrito I). La segunda revisión recoge una investigación exhaustiva sobre las causas microbianas que determinan la acumulación de histamina en productos lácteos, las técnicas de análisis para detectar e identificar la microbiota productora de histamina y las posibles soluciones descritas para limitar su aparición en leche y productos lácteos (Manuscrito II).

Mauscrito I. Analytical strategies for the determination of biogenic amines in dairy products

Moniente, M.; Botello-Morte, L.; García-Gonzalo, D.; Pagán, R.; Ontañón, I. (2022). *Comprehensive Reviews in Food Science and Food Safety*, *21*(4), 3612-3646. https://doi.org/10.1111/1541-4337.12980

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COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY

Analytical strategies for the determination of biogenic amines in dairy products

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Abstract

Biogenic amines (BA) are mainly produced by the decarboxylation of amino acids by enzymes from microorganisms that emerge during food fermentation or due to incorrectly applied preservation processes. The presence of these compounds in food can lead to a series of negative effects on human health. To prevent the ingestion of high amounts of BA, their concentration in certain foods needs to be controlled. Although maximum legal levels have not yet been established for dairy products, potential adverse effects have given rise to a substantial number of analytical and microbiological studies: they report concentrations ranging from a few mg/kg to several g/kg. This article provides an overview of the analytical methods for the determination of biogenic amines in

Abbreviations: AB, aminobutyric; AG, agmatine; ALA, β-alanine; AM, amylamine; AQC, 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate; BAs, biogenic amines; BCEC-CL, 2-(11H-benzo[a]-carbazol-11-yl) ethyl chloroformate; BNZ-Cl, benzoyl chloride; BU, butylamine; CAD, cadaverine; CCR, 4′-carbonyl chloride rosamine; CD, conductivity detection; CE, capillary electrophoresis; CO, colamine; CZE, capillary zone electrophoresis; C4D, capacitively coupled contactless conductivity detection; DAO, diamine oxidase; DBS-Cl, dabsyl chloride; DEEMM, ethoxymethyl malonate diethyl ester; DIM, dimethylamine; DLLME, dispersive liquid-liquid microextraction; DNS-Cl, Dansyl chloride; DOP, dopamine; d3-4-MBA-OSu, N-hydroxysuccinimidyl ester of d0/d4-4-methoxybenzoic acid; ELISA, enzyme-linked immunosorbent assay; ET, ethylamine; ETH, Ethanolamine; FIA, flow injection analysis; FITC, fluorescein; FL, fluorescence; FMOC-Cl, 9-fluorenylmethyl chloroformate; GA, glutaraldehyde; GC, gas chromatography; HE, Hexylamine; HCl, Hydrochloric acid; $HClO_4$, perchloric acid; HIM, histamine; H_2SO_4, sulfuric acid; IBCF, isobutyl chloroformate; IEC, ion exchange chromatography; IPAD, Integrated pulsed amperometric detection; IPR, isopropylamine; IS, isoamylamine; ISO, isopentylamine; LIF, laser radiation; LOD, limit of detection; LOQ, limit of quantification; MAE, microwave-assisted extraction; ME, methylamine; MIP, molecularly imprinted polymer; MS, mass spectrometry; MSA, methanesul-fonic acid; NBD-CL, 4-Chloro-7-nitrobenzofurazan; NDA, naphtalene-2,3-dicarboxyaldehyde; NMR, nuclear magnetic resonance spectroscopy; OCT, octopamine; OPA, o-phthalaldehyde; PAD, pulsed amperometric detection; PAO, plasmas amino oxidase; PBS, phosphate-buffered saline; PHE, phenylethylamine; PRO, propylamine; PUT, putrescine; SAMF, 6-Oxy-(N-succinimidyl acetate)−9-(2′-methoxycarbonyl) fluorescein; SPD, spermidine; TAO, trypanosome alternative oxidase; TCA, triclhoroacetic; TEA, triethylamine; TLC, Thin-layer chromatography; TMA, trimethylamine; TTMBB-Su, 1,3,5,7-tetramethyl-8-(N-hydroxysuccinimidyl butyric ester)-difluoroboradiaza-s-indacene; TRY, tryptamine; TYR, tyramine; 3-MBU, 3-methylbutylamine.

dairy products, with particular focus on the most recent and/or most promising advances in this field. We not only provide a summary of analytical techniques but also list the required sample pretreatments. Since high performance liquid chromatography with derivatization is the most widely used method, we describe it in greater detail, including a comparison of derivatizing agents. Further alternative techniques for the determination of BA are likewise described. The use of biosensors for BA in dairy products is emerging, and current results are promising; this paper thus also features a section on the subject. This review can serve as a helpful guideline for choosing the best option to determine BA in dairy products, especially for beginners in the field.

KEYWORDS
analytical methods, biogenic amines, biosensors, dairy products, liquid chromatography

1 | INTRODUCTION

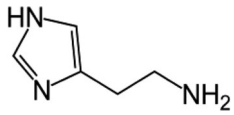
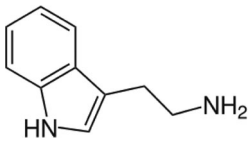
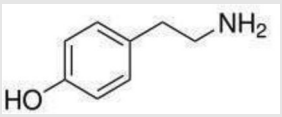
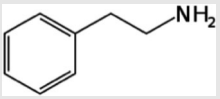
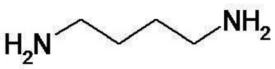
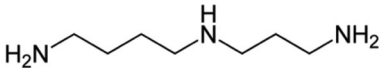
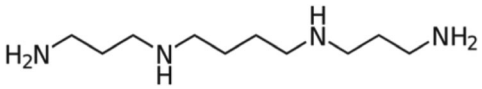
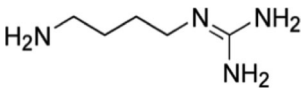
Biogenic amines (BA) are low-molecular weight nitrogenous compounds that emerge through enzymatic decarboxylation of amino acids (Linares et al., 2011; Zhang et al., 2019) or by amination and transamination of aldehydes and ketones (McCabe et al., 2003; Pluta-Kubica et al., 2020). Their chemical structures can be classified as: aliphatic, aromatic, or heterocyclic (Linares et al., 2011; McCabe et al., 2003; Papageorgiou et al., 2018; Spano et al., 2010). Furthermore, according to number of amino groups, they can be classified as monoamines, diamines, or polyamines (Ladero et al., 2017; Spano et al., 2010). An extensive variety of BA is usually present in plants, animals (Farooqui, 2013), and foods (Naila et al., 2010), but the most important BA in certain foods and beverages are histamine, tyramine, putrescine, cadaverine, tryptamine, spermidine, spermine, and phenylethylamine, which are produced by decarboxylation of their respective amino acids (Brito et al., 2014; del Rio et al., 2017; Palermo et al., 2013; Restuccia et al., 2011; Spano et al., 2010), as can be seen in Table 1.

BA act as precursors for the synthesis of hormones, alkaloids, nucleic acids, and proteins; some of them, such as histamine and tyramine, play an important role as neurotransmitters (del Rio et al., 2018). Despite their benefits, multiple negative effects on health derived from food intake with high amounts (ranged from 190 mg/kg to 500 mg/kg for histamine and from 301.8 mg/kg to 500 mg/kg for tyramine) of different amines have been described (del Rio et al., 2017, 2018; Fernández et al., 2006; Ladero et al., 2010; Lehane & Olley, 2000; Linares et al., 2016; McCabe et al., 2003; Ruiz-Capillas & Herrero, 2019; Spano et al., 2010). Synergistic effects among different BA present in food can increase these adverse effects, as is the case of tyramine, which has synergistic cytotoxic effects in combination with histamine (Palomino-Vasco et al., 2019). It should also be noted that BA are precursors to nitrosamines, which have been linked to carcinogenic and mutagenic activity (McCabe et al., 2003; Papageorgiou et al., 2018).

The toxicities of the various biogenic amines, alone or in combination, have for the most part not been established (Paulsen et al., 2017). Most regulations thus focus exclusively on the most dangerous ones, especially on histamine. While legal limits have been established to regulate histamine in certain foods such as fresh fish or enzyme-maturated fish product, allowing up 100 mg/kg and 200 mg/kg, respectively (Commission Regulation No. 2073/2005) (Ladero et al., 2017; UE, 2005), no regulation has been established for dairy products (Moniente et al., 2021). Only recommended maximum levels of histamine have been suggested for dairy products (Rauscher-Gabernig et al., 2009), despite the fact that high amounts of BA have been found in some of them. For example, 1000 to 2500 mg/kg of histamine have been detected in some varieties of cheese, as well as 1500 to 4000 mg/kg of tyramine in cheddar and Camembert cheese (Maintz & Novak, 2007).

BA have been also found in milk submitted to diverse treatments (raw, pasteurized and ultra-high temperature [UHT]) stemming from different animal species (sheep, cow, and goat), as well as in dairy products derived from fermented milk (Benkerroum, 2016; Costa et al., 2018; Ladero et al., 2017), although the levels of BA in the latter are much lower. It would nevertheless be important to control low concentrations of histamine in foods as well, since ingestion of small amounts of histamine can produce disorders in histamine-sensitive patients (Paulsen et al., 2017). Even their determination at very low levels could help to certify histamine-free products.

TABLE 1 Formation of the most important biogenic amines from their precursor amino acids

Precursor substrate	Enzyme	Biogenic amine	Chemical structure
Histidine	Histidine decarboxylase	Histamine 	Heterocyclic amines
Tryptophan	Tryptophan hydroxylase	Tryptamine 	
Tyrosine	Tyrosine descarboxylase	Tyramine 	Aromatic amines
Phenylalanine	Phenylalanine descarboxylase	Phenylethylamine 	
Ornithine	Ornithine descarboxylase	Putrescine 	Aliphatic amines
Lysine	Lysine descarboxylase	Cadaverine 	
Putrescine	Spermidine synthase	Spermidine 	
Spermidine	Spermine synthase	Spermine 	
Arginine	Arginine descarboxylase	Agmatine 	

BA detection requires sensitive, selective analytical methods (Henao-Escobar et al., 2013). In addition, most foods are complex matrices, and some of them have high percentages of proteins and fats that make it difficult to determine BA. To reduce matrix interferences and increase the sensitivity of analytes, sample preparation steps prior to analysis are usually necessary: deproteinization by acids (Andiç et al., 2010, 2011; Bunkova et al., 2013; Contreras et al., 2007; Custódio et al., 2007; Durlu-Özkaya, 2002; Ercan et al., 2019; Fiechter et al., 2013; Flasarová et al., 2016; Gobbi et al., 2019; Korös et al., 2008; Lanciotti et al., 2007; Marijan et al., 2014; J. L. Ordóñez et al., 2016; Samková et al., 2013; Sawilska-Rautenstrauch et al., 2010; Spizzirri et al., 2019; Tittarelli et al., 2019; Toro-Funes et al., 2015), for example, or solid phase extraction (SPE) (Calbiani et al., 2005; J. L. Ordóñez et al., 2016; Spizzirri et al., 2013, 2019).

Multiple analytical methods have been developed for the determination of BA in a wide variety of foods and beverages (Komprda et al., 2008; Marks & Anderson, 2005; Moracanin et al., 2015; Nalazek-Rudnicka & Wasik, 2017; Peña-Gallego et al., 2009; Spizzirri et al., 2013; Yigit & Ersoy, 2003). A series of analytical techniques such as gas chromatography (GC), thin layer chromatography

(TLC), and capillary electrophoresis (CE) have been used (Adımcılar et al., 2018; Draisci et al., 1998; Fernández-García et al., 1999, 2000; Gaya et al., 2005; Karovičová & Kohajdová, 2005; Kvasnička & Voldřich, 2006; Lange & Wittmann, 2002; Pham & Nguyen, 2016; Shalaby et al., 2016; Švarc-Gajic & Stojanovic, 2011) to analyze BA. Nevertheless, due to the low volatility and polarity of these analytes, most of the analytical methods used are based on analysis by liquid chromatography (LC) (Önal et al., 2013), since the derivatization of BAs is commonly applied to improve their determination. Other type of methods based on enzymatic procedures such as enzyme-linked immunosorbent assay (ELISA) have also been used (Aygün et al., 1999; Leszczyocha & Pytasz, 2018), and an increasing number of studies in recent years have determined amines using biosensors (Alonso-Lomillo et al., 2010; Calvo-Pérez et al., 2013; Carelli et al., 2007; Compagnone et al., 2001; Huang et al., 2011; Salleres et al., 2016; Telsnig et al., 2012).

Growing concern about the presence of BA in different foods is reflected in a growing quantity of published papers and reviews (Huang et al., 2011; Mayr & Schieberle, 2012; Papageorgiou et al., 2018; Paulo Vieira et al., 2020; Ramos et al., 2020). Given the variety of matrices in which BA can be found, recent reviews have covered the analytical methods developed for a variety of different foods and beverages (Biji et al., 2016; Guo et al., 2015; Önal, 2007; J. L. Ordóñez et al., 2016; Papageorgiou et al., 2018; Suzzi & Gardini, 2003; Zhang et al., 2019). Consumed in all cultures on a daily basis, dairy products encompass a wide range of products: milk, yogurt, cheese, kefir, butter, and cream, among others. This food group features considerable physicochemical variability with different characteristics of texture (liquid, solid, and gel), viscosity, or composition. Due to the global importance and variability of dairy products, we consider it necessary to review and compare the different strategies used to analyze BA in this group of foods, spotlighting the latest advances in the field.

2 | SAMPLE PRETREATMENT METHODS FOR DAIRY PRODUCTS

The decision of using sample pretreatments is based on the selectivity and sensitivity of the determination step and the physicochemical characteristics of the sample. So, two of the main objectives of sample pretreatment methods are cleaning the sample and preconcentrating the analytes. Sometimes, samples can be analyzed without any treatment and the easiest is applying the "dilute and shoot" method; however, in dairy products, perhaps due to their complexity and to the concentration of BA, it has not been used for the determination these analytes. The high protein content in dairy products implies that most sample pretreatments focus on protein removal; solvent pretreatments have thus been extensively applied for the precipitation of proteins (Gianotti et al., 2008; Gloria et al., 2011; He et al., 2016; Korös et al., 2008; Latorre-Moratalla et al., 2009; Madejska et al., 2018; Molaei et al., 2019; Redruello et al., 2013; Ubaldo et al., 2015).

The application of solid–liquid (S–L) or liquid–liquid (L–L) extraction methods depends on the dairy product's texture, seeking not only to precipitate proteins but also a maximum solubility of BA in the extracting phases. A wide variety of such methods (Andiç et al., 2010; Gaya et al., 2005; Innocente et al., 2007; Lange & Wittmann, 2002; Mayer et al., 2010; Rabie et al., 2011; Shalaby et al., 2016; Standarová et al., 2009; Yigit & Ersoy, 2003) have been used to extract BA from cheeses. Custódio et al. (2007) carried out a remarkable comparison, evaluating hydrochloric, trichloroacetic, perchloric, sulfosalicylic, and acetic acids, borate buffer, methanol, and ethanol as extracting agents of amines in grated Parmesan cheese. The level of amines present in the sample, the type of sample, the concentration and temperature of the extractor, as well as the method used, affected the degree of extraction efficiency. Each one of the studied BAs could be better recovered in a different way, although 1 M HCl was the most adequate solution for most of the amines.

Although solvent extractions are the most common methods for the analysis of BA in different dairy matrices such as milk, cheese, or yogurt (Mohammadi et al., 2017; Molaei et al., 2019; Wu et al., 2015) due to their high performance in removing undesirable compounds (Liu et al., 2018), they have several disadvantages, such as slowness, high amounts of harmful organic solvents, loss of objective analytes (Wu et al., 2015), low repeatability, complexity, laborious emulsion formation (Liu et al., 2018; Saaid et al., 2009), a large sample volume for trace analysis (Huang et al., 2011), and time-consuming procedures (J. L. Ordóñez et al., 2016). To overcome these disadvantages, modifications and improvements have been made based on liquid–liquid extraction (LLE) techniques by using liquid phase microextraction methods such as dispersive liquid–liquid microextraction (DLLME) and salting-out assisted liquid–liquid extraction (SALLE). Ultrasonic-assisted extraction (UAE) and microwave-assisted extraction (MAE) have also been used to avoid these drawbacks.

Some of the advantages of DLLME analysis are high enrichment capacity, simple technique, low consumption of organic solvents, time saving due to short extraction time, low cost, good repeatability, and high sensitivity (Kamankesh et al., 2013; Rezaee et al., 2006; Wu et al., 2015). Mohammadi et al. (2017) used this method in combination with the auxiliary application of MAE for the simultaneous determination of four BA in samples of Iranian Lighvan cheese, and demonstrated that

MAE–DLLME–GC coupled with mass spectrometry (MS) is effective in extracting and determining small amounts of BA in cheese with 20% fat. The analysis procedure was as follows: 1 g of sample was homogenized with 5 ml of 0.1 M HCl and microwaved at 500 MHz for 3 min; after the precipitation of the proteins, BA derivatization and microextraction were carried out simultaneously by adding 25 μl of isobutyl chloroformate (IBCF), 2 g of NaCl, 600 μl of acetonitrile (ACN) (dispersive solvent), and 60 μl of 1-octanol (extractive solvent). Solution was centrifuged at 4000 rpm for 5 min, and 2 μl of the floated phase of 1-octanol was injected directly into the GC–MS. DLLME has also been used a step prior to LC analysis. Wu et al. (2015) studied BA in cheese with UAE–DLLME, but added chloroform and acetone as extraction and dispersing solvents, respectively.

SALLE is used to prepare samples for organic extraction in chromatographic analysis (Tang & Weng, 2013). To increase the distribution ratio of a particular solute, the effect of salt is used by adding an electrolyte to an aqueous solution (Rice et al., 1993). This effect promotes the extraction of molecular species to an organic phase and allows for phase separation between the two solvents (Shishov et al., 2019).

Ramos et al. (2020) recently used SALLE for the determination of BA in seven different types of cheese, including derivatization with dansyl chloride (DNS-Cl) and HPLC coupled to a fluorescence detector (FLD). An extraction of soluble BA was carried out with 1 M HCl prior to the use of SALLE, simultaneously with the derivatization step, and the extract was injected into a liquid chromatography system.

UAE is used to improve extraction efficiency and reduce analysis times, thanks to the effects it produces such as vibration, cavitation, and agitation generated by ultrasonic waves that induce the effective components to enter the solvent (Khaled, 2014; Redruello et al., 2013; Švarc-Gajic & Stojanovic, 2011).

Several authors have used UAE to extract BA in cheese (Fernández et al., 2007; Herrero-Fresno et al., 2012; Krause et al., 1995; Redruello et al., 2013; Švarc-Gajic & Stojanovic, 2011; Zotou & Notou, 2012). Švarc-Gajic and Stojanovic (2011) compared the efficiency of different extraction procedures: they tested three different solvents and carried out a comparison among classical reflux extraction, UAE, and MAE. They concluded that methanol was the best solvent and that UAE was the most efficient procedure.

Further alternative pretreatment techniques have been developed, such as SPE (Calbiani et al., 2005; Gianotti et al., 2008; Gosetti et al., 2007; Restuccia et al., 2011; Spizzirri et al., 2019; Yang et al., 2016) and solid phase microextraction (SPME) (Ali Awan et al., 2008). Advantages provided by these methods include low consumption of extraction solvents, ease of use, and low cost, as well as the possibility of automation and of online coupling with analytical instruments (Pena-Pereira et al., 2012).

Gosetti et al. (2007) compared the recoveries obtained with LLE and SPE. In the SPE method, the BA were extracted from cheese with 0.1 M HCl and the solution was loaded onto the SPE cartridge (C18 sorbent and Strata X were compared and C18 was selected), which was washed with water and eluted with methanol. Better results were obtained with SPE than with LLE, with recovery yields greater than 90% for all amines through SPE. This procedure of extraction with 0.1 M HCl, loading onto C18 cartridges and cleaning with water, is the most common one when SPE is used to determine BA in cheeses (Gianotti et al., 2008; Restuccia et al., 2011); however, other kinds of sorbent material, such as cyanopropyl (CN) cartridges, and other washing steps have also been used (Calbiani et al., 2005).

One of the aforementioned advantages of SPE is the possibility of automatization. Yang et al. (2016) successfully developed a novel method based on online SPE coupled to capillary HPLC for the simultaneous separation and determination of 15 BA in cheese. Using the online SPE purification technique, it was possible to reduce the matrix effect of the samples while simplifying sample pretreatment, reducing manual error, and greatly improving analysis efficiency.

SPE has been also applied to determine BA in milk. Spizzirri et al. (2019) studied BA in reconstituted milk powder and ready-to-use liquid milk using SPE prior to derivatization and LC with ultraviolet (UV) analysis. The SPE cartridges (ENVI-18SpeTubes) were first preconditioned with a water/ammonia solution (70/30% v/v) and then with methanol. The sample was loaded, after which the washing step was performed with water and eluted with methanol.

Ali Awan et al. (2008) demonstrated the advantages of SPME as a technique to extract and derivatize BA for GC analysis. They used trifluoroacetylacetone (TFAA) as derivatizing agent; derivatization and extraction were carried out simultaneously into a SPME vial at 120°C during 20 min. This method was applied for the determination of putrescine and cadaverine in a cheese sample.

3 | ANALYTICAL METHODS FOR DAIRY PRODUCTS

One of the greatest challenges in food analysis remains BA determination, which is of considerable importance for two main reasons: on the one hand, the potential toxicity of BAs and, on the other hand, the possibility of using them as food quality markers.

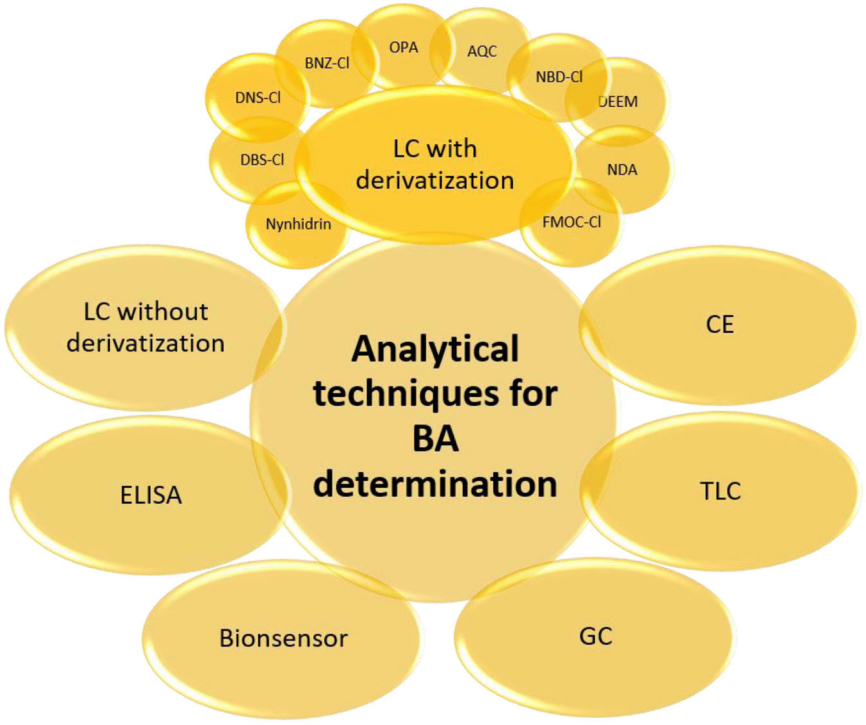


FIGURE 1 The most common analytical techniques used for the determination of biogenic amines.
Abbreviations: AQC, 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate; Bnz-Cl, benzoyl chloride; CE, capillary electrophoresis; Dbs-Cl, dabsyl chloride; DEEMM, ethoxymethyl malonate diethyl ester; Dns-Cl, dansyle chloride; ELISA, enzyme-linked immunosorbent assay; FMOC-Cl, 9-fluorenylmethyloxycarbonyl chloroformate; GC, gas chromatography; LC, liquid chromatography; NBD-CL, 4-Chloro-7-nitrobenzofurazan; NDA, naphtalene-2,3-dicarboxyaldehyde; OPA, o-phthalaldehyde; TLC, thin layer chromatography

One of the difficulties in BA determination is the polarity of these compounds, which makes them have a higher solubility in water than in the organic solvents used for analysis. Other difficulties are: the complexity of the sample; the presence of interfering compounds that cause the spread of chromatographic peaks and the appearance of shoulders; a limited concentration range within which analytes cannot be detected by the analytical detectors; and the absence of intrinsic properties of the BA for most of the usual detectors (Önal et al., 2013; Papageorgiou et al., 2018). To solve these analytical difficulties, analytical methods are generally combined with previous processes of extraction and derivatization prior to separation and detection.

A wide range of methods (Tables 2 and 3) have been developed for the analysis of BA in dairy products with different analytical techniques (Figure 1): TLC, GC, CE, ELISA, chronopotentiometry, and biosensors, whereby LC is the most widespread analytical technique.

3.1 | Analytical methods based on LC with derivatization

Many BA, for example putrescine, cadaverine, spermine, and spermidine, do not absorb in the UV region, while others, such as phenylethylamine, tyramine, and histamine, absorb in a shorter wavelength region (close to 200 nm) where matrix effects can appear (Jain & Verma, 2018; Zhang et al., 2019). Most of them require derivatization due to low volatility and lack of chromophores, and also in order to reduce their polarity with the purpose of improving chromatographic and detectability properties (Önal, 2007).

The derivatization process allows to improve the resolution of the analytes in reverse phase (RP) columns as well. Therefore, most analytical LC methods for the quantification of BA feature a pre- or postcolumn derivatization step (García-Villar et al., 2009). A broad array of derivatizing reagents have been used for the determination of BA in dairy products. The most common derivatization reactions are shown in Figure 2. Reproducibility, stability, and speed are the main reasons for choosing among the different options (Munir & Badri, 2020). The most common derivatizing reactions are the following:

3.1.1 | Derivatization by DNS-Cl reagent

Sulfonyl chlorides work to derivatize primary and secondary amines, and DNS-Cl is the one most widely used. This derivatizing reagent offers a high fluorescence efficiency, especially in organic solvents (Chen, 1967). Fluorescence detection is therefore recommended, although other types of detectors can also be used.

UV-Visible (UV-Vis) detection
In spite of the excellent fluorometric properties of dansylated compounds, most authors choose to use UV-Vis for their detection. The analytical method developed by

TABLE 2 Analytical methods based on separation techniques to determine biogenic amines in dairy products

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, TYR, PUT, CAD, PHE, SPM	RP-HPLC	0.1 M HCl	Dbs-Cl	UV (436 nm)	LOD: 0.12 and 0.52 pmol	Fernández et al. (2007)
Cheese	HIM, TYR, PUT, CAD, PHE, TRY	RP-HPLC	0.1 M HCl	Dbs-Cl	UV (436 nm)	–	Valsamaki et al. (2000)
Cheese	HIM, TYR, PUT, CAD, PHE, TRY	RP-HPLC	0.1 M HCl 40% TCA	Dbs-Cl	UV (436 nm)	LOD: 0.12 and 0.52 pmol	Krause, et al. (1995)
Cheese	HIM, TYR, PUT, CAD, PHE, TRY, SPD, ET	RP-HPLC	0.2 M $HClO_4$	Dbs-Cl	UV (436 nm)	LOD: 1.5 mg/L	Pinho et al. (2001)
Cheese	HIM, TYR, PUT, CAD, PHE, TRY	RP-HPLC	0.2 M $HClO_4$	Dbs-Cl	UV (436 nm)	–	Pinho et al. (2004)
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD, TRY	HPLC	0.1 M HCl	Dbs-Cl	UV (254 nm)	LOD: 0.25–0.76 pmol LOQ: 0.99–2.9 pmol	Bockhardt et al. (1996)
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD , TRY, TBA	RP-HPLC	0.4 M $HClO_4$	Dns-Cl	UV (254 nm)	–	Durlu-Ozkaya (2002)
Cheese	HIM, TYR, PUT, CAD, TRY, SPM, SPD	HPLC	0.4 M $HClO_4$	Dns-Cl	UV (254 nm)	LOD: 1–5 mg/kg	Contreras et al. (2007)
Cheese	HIM, TYR, PUT, CAD, PHE, TRY	HPLC	0.4 M $HClO_4$	Dns-Cl	UV (254 nm)	–	Andiç et al. (2010)
Cheese	HIM, TYR, PUT, CAD, TRY, SPM, SPD, PHE	HPLC	0.4 M $HClO_4$	Dns-Cl	UV (254 nm)	–	Marijan et al. (2014)
Cheese	HIM, TYR	HPLC	0.4 M $HClO_4$	Dns-Cl	UV (254 nm)	–	Tittarelli et al. (2019)
Cheese	HIM, TYR, PUT, CAD, PHE	HPLC	5% TCA	Dns-Cl	UV (254 nm)	–	Deabes et al. (2013)
Cheeses	HIM, TYR, PUT, CAD, TRY, PHE, SPD, SPM,	RP-HPLC	0.1 M HCl	Dns-Cl	UV (254 nm)	LOD: 0.002 μg/cm3 LOQ: 0.010 mg/kg	Bonczar et al. (2018)
Cheese	HIM, TYR, PUT, CAD, TRY, SPD, SPM, PHE	RP- HPLC	0.1 M HCl	Dns-Cl	UV (254 nm)	LOD: 0.002 μg/cm3 LOQ: 0.010 mg/kg	Pluta-Kubica et al. (2020)

(Continues)

TABLE 2 (Continued)

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, TYR, PUT, CAD, SPD, SPM, PHE, TRY	RP-HPLC	0.1 M HCl	Dns-Cl	UV (218 nm)	LOD: 1.3–3.1 mg/kg LOQ: 1.9–4.6 mg/kg	Combarros-Fuertes et al. (2016)
Cheese	HIM, TYR, PUT, CAD, TRY, SPM, SPD, PHE	RP-HPLC-DAD	0.1 M HCl	Dns-Cl	UV (254 nm) FL (ex. 330 nm, em. 500 nm)	LOD: 0.07 mg/kg–0.23 mg/kg LOQ: 0.20 mg/kg–0.70 mg/kg	Zazzu et al. (2019)
Cheese	HIM, TYR, PUT, CAD, SPM, SPD, TRY	RP-HPLC	10 % TCA	Dns-Cl	UV (436 nm)	–	Standarová et al. (2010)
Cheese	HIM	HPLC	TCA	Dns-Cl	DAD	LOD: 3 mg/kg	Gardini et al. (2012)
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD	HPLC	0.2 M $HClO_4$	Dns-Cl	DAD	LOD: 1-5 mg/kg	Lanciotti et al. (2007)
Cheese	HIM, TYR, PUT, CAD, PHE, TRY, SPD, SPM, ME, ET, AM, OCT	HPLC	Methanol 0.1 M HCl Online SPE	Dns-Cl	UV-VIS	LOD: 0.05—0.25 mg/L LOQ: 0.15—0.80 mg/L	Yang et al. (2016)
Cheese	HIM, TYR, PUT, CAD, SPM, SPD	HPLC	Acid extraction	Dns-Cl	UV (254 nm)	–	Mercogliano et al. (2010)
Cheese	HIM, TYR, PUT, CAD, TRY, SPM, SPD, PHE	HPLC	0.2 M $HClO_4$	Dns-Cl	UV (254 nm)	–	El Zahar (2014)
Cheese	HIM, TYR, PUT, CAD, TRY, SPM, SPD, PHE	HPLC	0.6 M $HClO_4$	Dns-Cl	UV (254 nm)	–	Flasarová et al. (2016)
Cheese	HIM, TYR, PUT, CAD, TRY, PHE	HPLC	0.4 M $HClO_4$	Dns-Cl	DAD (254 nm)	–	Andiç et al. (2011)
Cheese	HIM, TYR, CAD, PUT, TRY, PHE, SDP, SPM	HPLC	0.2 M $HClO_4$	Dns-Cl	DAD (254 nm)	–	Ordoñez et al. (1997)
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD, TRY	HPLC	0.1 M HCl	Dns-Cl	UV (254 nm)	LOD:1–5 mg/kg	Galgano et al. (2001)

(Continues)

TABLE 2 (Continued)

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD, TRY	HPLC	0.1 M HCl	Dns-Cl	UV (254 nm)		Moret and Conte (1996/)
Cheese	HIM, TYR, PUT, CAD, PHE	HPLC	0.1 M HCl	Dns-Cl	UV (254 nm)	LOD: 5 mg/kg for all the amines and 8 mg/kg only for PHE	Schirone et al. (2013)
Milk. cheese and yoghurt	HIM, TYR, PUT, CAD, PHE, SPD, SPM, TBA	HPLC	0.4 M $HClO_4$	Dns-Cl	DAD (254 nm)	–	Min et al. (2004)
Cheese	HIM, TYR, PUT, CAD	HPLC	0.1 M HCl	Dns-Cl	UV (254 nm)	LOD: 0.3 mg/kg of LOQ: 1 mg/kg	Forzale et al. (2011)
Cheese	ET, TRY, PHE, SPM, PUT	HPLC	0.1 M HCl	Dns-Cl	UV (254 nm)	–	Martuscelli et al. (2005)
Cheese	HIM, TYR, PUT, CAD	HPLC	0.1 M HCl	Dns-Cl	UV (254 nm)	–	Gennaro et al. (2003)
Cheese	HIM,TYR, CAD	HPLC	5% TCA	Dns-Cl	UV (254 nm)	–	Ibrahim and Amer (2010)
Cheese	HIM, TYR, CAD, TRY	HPLC	0.1 M HCl	Dns-Cl	UV (254 nm) $_1$ ESI-MS $_2$ APCI-MS $_3$	LOD_1: 14-67 μg/L LOQ_1: 45–229 μg/L LOD_2: 7–58 μg/L LOQ_2: 23–197 μg/L LOD_3:10–54 μg/L LOQ_3: 29–183 μg/L	Mazzucco et al. (2010)
Cheese	HIM, TYR, PUT, CAD, TRY	HPLC	0.1 M HCl	Dns-Cl	UV (254 nm)	–	Innocente and D'Agostin (2002)
Cheese	HIM, TYR, PUT, CAD, SPD, SPM, TRY	HPLC	0.1 M HCl	Dns-Cl	UV (254 nm)	–	Innocente et al. (2007)
Cheese	HIM, TYR, PUT, CAD, TRY, PHE	HPLC	0.4 M $HClO_4$	Dns-Cl	UV (254 nm)	–	Ercan et al. (2019)

(Continues)

TABLE 2 (Continued)

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, PUT, TYR, CAD, SPD, SPM, ME, DIM, ET	HPLC	SALLE 1 M HCl	Dns-Cl	FL (ex. 320 nm, em 523 nm) MS/MS	LOD: 0.015 and 1.77 mg/L LOQ: 0.050 and 5.9 mg/L	Ramos et al. (2020)
Cheese	HIM, TYR, PUT, CAD, SPM, SPD, PHE	HPLC	TCA	Dns-Cl	DAD	–	Mascaro et al. (2010)
Cheese	HIM, TYR, PUT, CAD	HPLC	0.1 M HCl	Dns-Cl	FL	LOD: 0.185–0.367 μg/g LOQ: 0.536–1.246 μg/g	Brito et al. (2014)
Cheese	HIM, TYR, PUT, CAD	RP-HPLC	0.2 M HClO4	Dns-Cl	FL (ex. 465 nm, em. 545 nm)	LOD: 0.7 mg/kg–1.3 mg/kg. LOQ: 1.4 mg/kg–2.6 mg/kg	Sawilska-Rautenstrauch et al. (2010)
Cheese	HIM, TYR, PUT, CAD, TRY, SPM, SPD, TBA	RP-HPLC	HCl	Dns-Cl	FL	LOD: 2–33 mg/kg	Standarová et al. (2009)
Cheese	HIM, TYR, CAD, SPD, SPM, TRY	HPLC	0.1 M HCl SPE	Dns-Cl	MS/MS	LOD: 5.1–35.0 μg/L LOQ: 14.2–101.2 μg/L	Gosetti et al. (2007)
Cheese	HIM, TYR, PUT, CAD, PHE, TRY, SPD, SPM, ETH	HPLC	0.1 M HCl	Dns-Cl	PDA (254 nm)	LOD: 1 μg/L LOQ: 2 μg/L	Esatbeyoglu et al. (2016)
Cheese	HIM, TYR, PUT, CAD, TRY, SPM, SPD, PHE	HPLC	0.1 M HCl	Dns-Cl	FL (ex. 340 nm, em. 520 nm)	–	Manca et al. (2015)
Cheese	HIM	HPLC	0.2 M TCA	Dns-Cl	UV–Vis (215 nm)	LOD: 3.52 mg/kg LOQ: 4.25 mg/kg	Madejska et al. (2018)
Cheese	HIM, TYR, PUT, CAD	HPLC	0.6 M $HClO_4$	Dns-cl	UV (254 nm)	0.24—1.39 mg/kg	Pachlova et al. (2016)
Cheese	HIM, TYR, PUT, CAD, TRY, SPM, SPD, PHE	HPLC	0.1 M HCl	Dns-Cl/OPA	UV (254 nm)	–	Renes et al. (2014)
Milk. yogurt and cheese	HIM, TYR, PUT, CAD, PHE, AG, SPM, SPD, TRY, OCT	HPLC	0.6 N $HClO_4$	OPA	FL (ex. 340 nm, em. 445 nm)	LOD: 0.10 mg/kg	Novella-Rodriguez et al. (2000)

(Continues)

TABLE 2 (Continued)

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD, AG, TRY	HPLC	0.6 N $HClO_4$	OPA	FL (ex. 340 nm, em, 445 nm)	LOD: 0.10 mg/kg	Novella-Rodríguez et al. (2002) b
Cheese. milk. rennet. curd and whey	HIM, TYR, PUT, CAD, PHE, SPM, SPD, AG, TRY	HPLC	0.6 N $HClO_4$	OPA	FL (ex. 340 nm; em. 445 nm)	LOD: 0.10 mg/kg	Novella-Rodríguez et al. (2002) a
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD, AG, TRY, TBA, OCT	HPLC	0.6 N $HClO_4$	OPA	FL (ex. 340 nm, em. 445 nm)	LOD: 0.10 mg/kg	Novella-Rodríguez et al. (2003)
Milk. rennet and brine. curd. whey and cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD, AG, TRY, TBA, OCT	HPLC	0.6 N $HClO_4$	OPA	FL (ex. 340 nm, em. 445 nm)	LOD: 0.10 mg/kg	Novella-Rodríguez et al. (2004)
Cheese	HIM, TYR, TRY, PHE	HPLC	5 % TCA	–	UV (215 nm)	LOD: < 10 mg/kg	Van Boekel and Arentsen (1987)
Cheese	HIM, TYR, TRY, PHE	HPLC	5% TCA	–	UV (215 nm)	LOD: < 10 mg/kg	Öner et al. (2006)
Cheese	HIM, TYR, PUT, CAD,PHE, SPM, SPD, AG, TRY	Ion-pair HPLC	0.1 and 1 M HCl Ethanol Methanol 12% sulfosalicylic acid	OPA	FL (ex. 340 nm, em. 445 nm)	LOD: 0.01-0.2 μg/ml	Custóordio et al. (2007)
Milk	HIM, TYR, PUT, CAD, SPD, SPM, TRY, PHE, AGM	HPLC	1.2 g of sulphos-alicylic acid	OPA	FL (ex. 340 nm; em. 445 nm)	LOD: < 0.010 mg/L	Gloria et al. (2011)
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD, AG, TRY	HPLC	1.5% sulfosalicylic acid	OPA	FL (ex. 340 nm, em. 450 nm)	LOD and LOQ: 0.5 mg/kg	Sampaio et al. (2015)
Milk. yogurt and cheese	PUT, CAD, SPD, SPM, AGM	RP-HPLC	5% TCA	OPA	FL (ex. 340 nm, em.455 nm)	–	Nishimura et al. (2006)
Cheese	HIM, TYR, TRY, AGM, PHE, SPD, SPM	HPLC	1 M $HClO_4$	OPA-ET-FMOC	DAD (190 and 400 nm) FL (ex. 334, ex. 262 nm)		Köros et al. (2008)

(Continues)

TABLE 2 (Continued)

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, TYR, PUT, CAD, SPM, SPD, AG	HPLC	5% TCA	OPA	MS	LOD: 0.5 mg/kg	Lange et al. (2002)
Fermented milk kefir	HIM, TYR, PUT, CAD, PHE, SPM, SPD , AG, TRY, ME	HPLC	0.2 M HCl	Bnz-Cl	UV (254 nm)	LOD: 0.2–2.5 mg/L Linearity: 133.6–1433 mg/L	Özdestan and Üren (2010)
Fermented milks	HIM, TYR, PUT, CAD, SPM, TBA	HPLC	5 % $HClO_4$	Bnz-Cl	UV (198 nm)	LOD: 0.03–1.30 mg/L LOQ: 0.20–5.00 mg/L	Costa et al. (2015)
Milk yogurt	PUT, CAD, SPD, SPM, TRY,	RP-HPLC	0.6 M $HClO_4$	Bnz-Cl	UV (254 nm)	LOD: 0.18—4.01 mg/L LOQ: 1–5 mg/L	Silva et al. (2019)
Dairy product	HIM, TYR, PUT, CAD	HPLC	5 % $HClO_4$	Bnz-Cl	UV (198 nm)	LOD: 0.05—150 mg/L LOQ: 0.05—50 mg/L	Molaei, et al. (2019)
Cheese	HIM, TYR, PUT, CAD	HPLC	0.013 N H_2SO_4	Bnz-Cl	UV (254 nm)	–	O'Sullivan et al. (2015)
Yogurt	TYR, PUT, CAD, SPD, SPM	RP-HPLC	0.6 M $HClO_4$	Bnz-Cl	UV (254 nm)	LOD: 0.18—4.01 mg/L. LOQ:1–5 mg/L	Vieira et al. (2020)
Cheese	HIM, TYR, PUT, CAD, PHE, TRY	HPLC	0.6 M TCA	Ninhydrin	UV (546 nm)	LOD: 0.02 mmol/kg	Joosten (1988)
Cheese	HIM, TYR, PUT, CAD, PHE, TRY	RP-HPLC	0.6 M TCA	Ninhydrin	UV (546 nm)	LOD: 2 mg/kg	Joosten and Olieman (1986)
Dairy beverage	HIM, TYR, PUT, CAD, TRY, PHE, SPD, SPM, DOP, OCT, AGM, AB, ALA	HPLC	0.2 % TFA	Ninhydrin	UV–Vis (570 nm) CAD CLND	LOD CLND: 0.1 μg/ml–0.4 μg/ml	J. Sun et al. (2011)
Cheese	HIM, TYR, PUT, CAD, SPD, SPM, TRY, PHE, ME, ET, PRO, IS	HPLC	Methanol and TCA	NDA	FL (ex. 424 nm, em. 494 nm)	LOD: 0.6–165 mg/kg	Zotou and Notou (2012)
Milk	HIM, TYR, PHE, TRY, PRO	ZF–HPLC	TCA	NDA	FL (ex. 424 nm, em. 494 nm)	LOD 2–2000 μg/L	Notou et al. (2014)

(Continues)

TABLE 2 (Continued)

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, TYR, PUT, CAD, SPM, SPD, PHE	HPLC	0.1 M HCl	FMOC	FL (ex. 265 nm, em. 315 nm)	–	Kirschbaum and Luckas (1994)
Cheese	HIM, TYR, PUT, CAD	RP-HPLC	PBS	FMOC	FL (ex. 265 nm, em. 315 nm)	LOD: 1 mg/kg	Aygün et al. (1999)
Cheese	HIM, TYR, PUT, CAD, TRY, PHE	HPLC	0.1 N HCl	DEEMM	PDA (269 nm and 280 nm)	LOD: 0.1 mg/kg	Poveda et al. (2016)
Cheese	HIM, TYR, PUT, CAD, TRY, PHE	HPLC	0.1 N HCl	DEEMM	PDA (269 nm and 280 nm)		Poveda et al. (2015)
Cheese	HIM, TYR, PUT, CAD, SPD, SPM, TRY, PHE	HPLC	0.1 M HCl	d3-4-MBA-OSu	Tandem mass spectrometry (MALDI MS and MS/MS)	LOD: 9.5–20.3 μg/kg LOQ: 15.6–25.3 μg/kg	Mazzotti et al. (2014)
Cheese	ETH, ME, ET, PRO, BU, PHE, HE	HPLC	0.1×10^{-3} M HCl	SAMF	FL (ex. 486 nm, em. 516 nm)	LOD: 2-320 fmol	Cao et al. (2005)
Milk and Yogurt	HIM, TYR, PUT, CAD, SPM, SPD, TRY, PHE	RP-HPLC	Acetonitrile	TMBB-Su	FL (ex. 490 nm; em. 510 nm)	LOD: 0.1–0.2 nM	Gao et al. (2011)
Yogurt and cheese	TYR, PUT, CAD, SPD, SPM	HPLC	5% TCA	BCEC-Cl	FL (ex. 279 nm, em. 380 nm)	LOD: 1.1—7.8 ng/ml LOQ: 3.5—26.1 ng/ml	Wu et al. (2015)
Cheese	TYR	HPLC	5% $HClO_4$	NBD-Cl	UV (458 nm)	LOD: 25 μg/g	Yigit and Ersoy (2003)
Cheese	HIM, TYR,PHE	HPLC	MSPD-SPE	–	Tandem mass spectrometry	LOD 0.05–0.25 mg/kg LOQ: 0.09-0.55 mg/kg	Calbiani et al. (2005)
Cheese	HIM, TYR, PUT, CAD, SPM, SPD, TRY	HILIC-HPLC	0.1 M HCl SPE	–	APCI MS/MS method	LOD: 1–3.5 μg/L LOQ: 3–10 μg/L	Gianotti et al. (2008)
Cheese	HIM, TYR, PHE, TRY	HPLC	5% TCA	–	UV and DAD (215 nm)	LOD: 0.006–0.146 μg	Arloriol et al. (1998)
Cheese	HIM, TYR, PUT, CAD, AG, TRY, SPD, SPM, ETH, ME, DIM, ET, OCT, IPR, PRO, BU, MBU	HPLC UPLC	0.6 N $HClO_4$	AQC	UV (254 nm) FL (ex: 250 nm, em: 395 nm)	LOD: 0.8-6.2 mg/kg LOQ: 2.9–60.9 mg/kg	Mayer et al. (2010)

(Continues)

TABLE 2 (Continued)

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, TYR, PUT, CAD, SPD, SPM, AG, PHE, TR, CO, DIM, ET, OCT, MBU	UHPLC	0.6 M $HClO_4$	AQC	UV (249 nm)	LOD: 0.05–0.44 mg/100 g LOQ: 0.16–1.45 mg/100 g	Fiechter, et al. (2013)
Cheese	HIM, TYR, PUT, CAD, SPD, SPM, TRY, PHE, ISO	UPLC	0.1 M $HClO_4$ in 50% acetonitrile.	Dns-Cl	UV (254 nm)	–	Ascone et al. (2017)
Cheese	HIM, TYR, PUT, CAD, TRY, SPM, SPD, PHE	UPLC	0.6 M $HClO_4$	Dns-Cl	–	LOQ: 1.2–3.7 mg/kg	Samková et al. (2013)
Cheese	HIM, TYR, PUT, CAD, AGM, PHE, SPD, SPM, TRY, OCT	UHPLC	0.6 M $HClO_4$	OPA	FL (ex. 340 nm, em. 445	LOD: 0.3 mg/kg	Latorre-Moratalla et al. (2009)
Cheese	HIM, TYR, PUT, CAD, PHE, TRY	UHPLC	0.1 M HCl	DEEMM	UV (280 nm)	LOD: 0.08—3.91 μM LOQ: < 13.02 μM	Redruello et al. (2013)
Cheese	HIM, TYR, PUT, CAD, SPM, SPD AG, PHE, TRY	UHPLC	In situ DUADLLME	CCR-acetonitrile	MS/MS	LOD: 0.9–6.0 μg/kg LOQ: 10-30 μg/kg	He et al. (2016)
Cheese and yoghurt	HIM, TYR, PUT, CAD, TRY, SPM, SPD, PHE, ETH, ALA	LC	10 % TCA	Bnz-Cl	Tandem mass spectrometry	LOQ: 0.05 μg/kg	Mayr and Schieberle (2012)
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD, AG, TRY	LC	0.1 N HCl	OPA	FL (ex. 340 nm, em. 445 nm)	LOD: 0.004 to 0.009 μg/20 μl LOQ: 0.066–0.149 mg/100 g	Vale and Gloria (1997)
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD, AG, TRY	LC	0.1 N HCl	OPA	FL (ex. 340 nm, em. 445 nm)	LOD: 1.98 mg/100 g	Vale and Gloria (1998)
Cheese	TYR	LC	0.1 M HCl	OPA	UV (254 nm)	–	Komprda et al. (2008)
Cheese	HIM, TYR, PUT, CAD, PHE, SPM, SPD, AG	LC	0.1 M HCl SPE	–	ELSD	LOD: 0.8-2.6 mg/kg LOQ: 2–6.4 mg/kg	Spizzirri et al. (2013)
Cheese	HIM, TYR, PUT, PHE, SPM, SPD	LC	0.1 M HCl SPE	–	ELSD	LOD: 1.4–3.6 mg/L LOQ: 3.6 —9.3 mg/L	Restuccia et al. (2011)

(Continues)

TABLE 2 (Continued)

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, TYR, PUT, PHE, SPM, SPD	LC	0.1 M HCl SPE	Dns-Cl	UV (254 nm)	LOD: 0.03–0.09 mg/L LOQ: 0.1–0.26 mg/L	Restuccia et al. (2011)
Reconstituted powdered milk and ready-to-use liquid milk	HIM, TYR, PUT, CAD,PHE, SPM, SPD	LC	Reconstituted powdered milk: 5 N HCl Liquid milk: SPE	Dns-Cl	UV (254 nm)	LOD Milk powder: 0.2–0.4 μg/g LOQ Milk powder: 0.5–1 μg/g LOD liquid milk:0.03–0.05 μg/ml LOQ liquid milk: 0.08–0.13 μg/ml	Spizzirri et al. (2019)
Acidified milk. Fermented milk. Fermented cream. Yogurt. Milk cheeses. Kefir. Fermented buttermilk	HIM, TYR, CAD, PUT, SPM	LC	0.6 M $HClO_4$	Dns-Cl	UV (254 nm)	Range: 0.5–29.4 mg/kg LOD: 0.24—1.39 mg/kg	Bunkova et al. (2013)
Yogurt and cheese	HIM, TYR, PUT, CAD, SPD	CE	6% TCA	–	Conductometry	LOD: 0.041 and 0.098 mg/L LOQ: 0.14–0.49 mg/L	Adımcılar et al. (2017)
Cheese	HIM, TYR, PHE, TRY	CE	5% TCA	–	UV (214 nm)	LOD: 2 mg/kg	Fernández-García (1999)
Cheese	HIM, TYR	CE	5% TCA	–	UV (214 nm)	–	Fernández-García (2000)
Cheese	HIM, TYR, TRY	CE	5% TCA	–	UV (214 nm)	LOD: 2 mg/kg	Gaya et al. (2005)

(Continues)

TABLE 2 (Continued)

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, TYR, PUT, CAD, TRY, PHE	CE	0.1 HCl	FITC	LIF (488 nm)	LOD: 0.5×10^{-10} M–1.5×10^{-10} M	Nouadje et al. (1995)
Milk	PUT, CAD, SPM, SPD	CE	5% $HClO_4$	–	PAD	LOD: 100–400 nM	X. Sun et al. (2003)
Cheese	HIM, TYR, PUT, CAD, AGM, TRY	CZE	0.1 M HCl	–	CD	LOD: 2–5 μmol/L LOQ: 5-15 μmol/L	Kvasnicka and Voldrich (2006)
Cheese	HIM, TYR, PUT, CAD	DLLME–GC	Acetonitrile/1-octanol	IBCF	MS	LOD: 5.9–14 ng/g LOQ: 19.7–46.2 ng/g	Mohammadi et al. (2016)
Cheese	PUT, CAD	GC	On-fiber derivatization (SPME)	TFAA	MS	–	Ali Awan et al. (2008)
Cheese	HIM, PUT, TYR, CAD	MAE-DLLME-GC	0.06 M HCl	IBCF	MS	LOD: 0.5–10,000 ng/g LOQ: 1.3-2.3 ng/g	Kamankesh et al. (2021)
Cheese	HIM	TLC	Methanol	–		LOD: 1.31 mg/L LOQ: 3.54 mg/L	Švarc-Gajic and Stojanovic (2011)
Cheese	HIM, TYR, PUT, CAD, TBA	TLC-densitometry method	5% TCA	Dns-cl	UV (254 nm)	–	Shalaby et al. (2016)
Cheese	HIM, TYR, PUT, CAD, TEA, TMA, AGM, SPM, SPD	LC (IEC)	MSA	–	CD	LOD: 23–65 μg/kg LOQ: 65–227 μg/kg	Palermo et al. (2012)
Cheese	HIM, TYR, PUT, CAD	LC (IEC)	Sodium citrate buffer	Ninhydrin	UV-Vis (570 nm)	0.24–1.39 mg/kg	Pachlova et al. (2012)

(Continues)

TABLE 2 (Continued)

Food	Biogenic amine	Analytical method	Sample treatment	Derivatizing reactive	Detection method	Detection limit	References
Cheese	HIM, TYR, PUT, CAD	LC (IEC)	Sodium citrate buffer	Ninhydrin	UV-Vis (570 nm)	LOD: 0.6-0.89 mg/kg	Bunková et al. (2010)
Cheese	HIM, TYR, PUT, CAD, SPM, TBA	LC (IEC)	10% TCA	Ninhydrin	Colorimetric (ex. 570 nm, em. 440 nm)	–	Rabie et al. (2011)
Cheese	HIM, TYR, PUT, CAD, SPM	LC (IEC)	0.375 M $HClO_4$	–	IPAD	LOD: 1.25–2.50 ng	Draisci et al. (1998)
Cheese	HIM	LC (IEC)	0.1 M HNO3	OPA	PCD-FL (ex. 360, em. 440 nm)	LOD: 0.15 mg/kg	Kouti et al. (2021)
Yoghurt and kefir	HIM	LC (IEC)	Methanol	OPA	FL (ex. 350 nm, em. 444 nm)	LOD: 0.175 mg/kg	Leszczyńska et al. (2004)

Abbreviations: 3-MBU, 3-methylbutylamine; AB, aminobutyric; AG, agmatine; ALA, β-alanine; AM, amylamine; AQC, 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate; BCEC-CL, 2-(11H-benzo[a]-carbazol-11-yl) ethyl chloroformate; Bnz-Cl, benzoyl chloride; BU, butylamine; C4D, capacitively coupled contactless conductivity detection; CAD, cadaverine; CCR, 4′-carbonyl chloride rosamine; CD, conductivity detection; CE, capillary electrophoresis; CO, colamine; CZE, capillary zone electrophoresis; d3-4-MBA-OSu, N-hydroxysuccinimidyl ester of d0/d4-4-methoxybenzoic acid; Dbs-Cl, dabsyl chloride; DEEMM, ethoxymethyl malonate diethyl ester; DIM, dimethylamine; Dns-Cl, dansyle chloride; DLLME, dispersive liquid–liquid microextraction; DOP, dopamine; FIA, flow injection analysis; ET, ethylamine; ETH, ethanolamine; FITC, fluorescein; FL, fluorescence; FMOC-Cl, 9-fluorenylmethyl chloroformate; GC, gas chromatography; HCl, hydrochloric acid; $HClO_4$, perchloric acid; HE, hexylamine; HIM, histamine; IBCF, isobutyl chloroformate; H_2SO_4, sulfuric acid; IPAD, integrated pulsed amperometric detection; IPR, isopropylamine; IEC, ion exchange chromatography; IS, isoamylamine; ISO, isopentylamine; LIF, laser radiation; LOD, limit of detection; LOQ: limit of quantitation; MAE, microwave-assisted extraction; ME, methylamine; MS, mass spectrometry; MSA, methanesul-fonic acid; NBD-CL, 4-Chloro-7-nitrobenzofurazan; NDA, naphtalene-2,3-dicarboxyaldehyde; OCT, octopamine; OPA, o-phthalaldehyde; PAD, pulsed amperometric detection; PBS, phosphate-buffered saline; PHE, phenylethylamine; PRO, propylamine; PUT, putrescine; SAMF, 6-Oxy-(N-succinimidyl acetate)—9-(2′-methoxycarbonyl) fluorescein; SPD, spermidine; SPM, spermine; TBA, total biogenic amines; TCA, triclhoroacetic; TEA, triethylamine; TMA, trimethylamine; TLC, thin-layer chromatography; TMBB-Su, 1,3,5,7-tetramethyl-8-(N-hydroxysuccinimidyl butyric ester)-difluoroboradiaza-s-indacene; TRY, tryptamine; TYR, tyramine.

TABLE 3 Other analytical methods based on non-separation techniques to determine biogenic amines in dairy products

Food	Biogenic amines	Analytical method	Sample treatment	Detection limit	Reference
Cheese	HIM	ELISA	PBS	LOD: 2 mg/kg	Aygün et al. (1999)
Yoghurt and kefir	HIM	ELISA	Methanol	LOD: 0.125 mg/100 g	Leszczyńska et al. (2004)
Cheese	TYR	Biosensor +260 mV Inmobilization: Crosslinking with GA and BSA Crosslinking Mediator: Ferrocene Electrode: SPCE Enzyme: PAO	5% $HClO_4$	LOD: 2.0 ± 0.18 μM	Calvo-Pérez et al. (2013)
Cheese	TYR	Biosensor Inmobilization: Covalent conjugating Electrode: Au NPs Enzyme: TAO		LOD: 2.9 μM	Navarro et al. (2020)
Cheese	HIM	Biosensor Inmobilization: Surface adsorption Electrode: ITO NPs Enzyme: DAO		LOD: 2.7 μM	Kaçar et al. (2020)
Cheese	TYR	Biosensor Inmobilization: Adsorption Electrode: PVF/GRO Enzyme: DAO MAO		LOD: 0.61 μM	Erden et al. (2019)
Cheese	TYR	Biosensor Colorimetric test strips Enzyme:TAO	5% TCA	LOD = $2.6{\cdot}10^{-6}$ M	Oliver et al. (2021)

(Continues)

TABLE 3 (Continued)

Food	Biogenic amines	Analytical method	Sample treatment	Detection limit	Reference
Cheese	HIM, PUT, CAD	FIA Biosensor +700 mV Inmobilization: Crosslinking (GA+Gel) Electrode: Pt Enzyme: DAO	PBS	LOD: 6–12 μM	Carelli et al. (2007)
Cheese	HIM, TYR, PUT	Biosensor +700 mV Inmobilization: Crosslinking (GA- transglutaminase) Electrode: Pt-SPE Enzyme: PAO. TAO. DAO	5% TCA	LOD: 5-10 mg/kg	Lange and Wittman (2002)
Cheese	HIM, TYR, PUT, CAD, PHE	Biosensor +200 mV Inmobilization: Crosslinking (membraneþGA) and electropolymerization Electrode: GC-Pt and GC-Rh/Ru Enzyme: DAO	0.1 M HCl	DAO-GC-Rh/Ru (1 μM PUT. CAD. PHE; 10 μM TRY. 5 μM HIM and TYR) DAO-GC-Pt (0.5 μM PUT. HIM. TYR. PHE; 1 μM CAD. 2 μM TRY)	Compagnone et al. (2001)
Cheese	HIM, TYR, PUT, CAD, SPD, TRY	Ion-pair-assisted extraction followed by H-NMR	0.1 M HCl	LOD: 2.25–6.25 μg/g LOQ: 6.75–18.7 μg/g	Chatzimitakos et al. (2016)
Yoghurt	TYR	MIP electrochemical sensor	–	LOD: 5.7×10–8 M	Huang et al. (2011)

Abbreviations: CAD, cadaverine; DAO, diamine oxidase; ELISA, enzyme-linked immunosorbent assay; GA, glutaraldehyde; GC, gas Chromatography; HCl, hydrochloric acid; $HClO_4$, perchloric acid; HIM, histamine; LOD, limit of detection; LOQ, limit of quantitation; MIP, molecularly imprinted polymer; PAO, plamas amino oxidase; PBS, phosphate buffered saline; PBS, phosphate buffered saline; NMR, nuclear magnetic resonance spectroscopy; PHE, phenylethylamine; PUT, putrescine; SPD, spermidine; TAO, trypanosome alternative oxidase; TCA, Triclhoroacetic; TRY, tryptamine; TYR, tyramine.

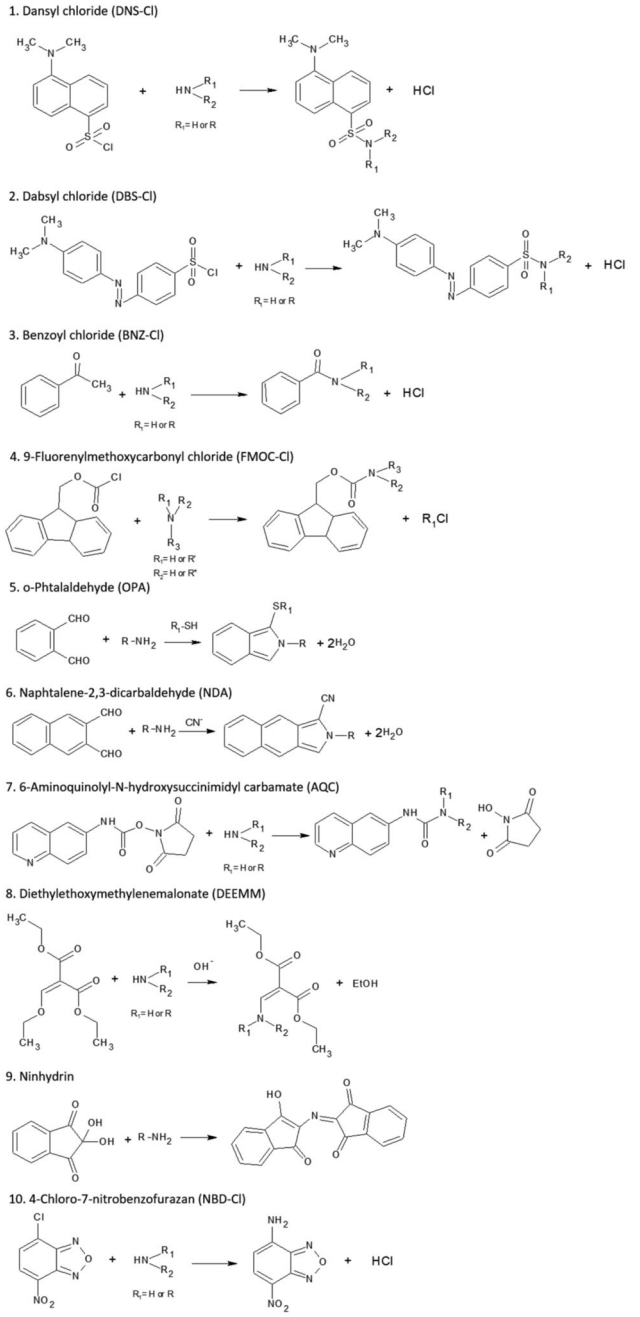


FIGURE 2 The most common derivatization reactions used for the determination of biogenic amines in dairy products with LC

Eerola et al. (1993) for the determination of BA in dry sausages has been modified and applied by several authors (Andiç et al., 2010, 2011; Contreras et al., 2007; Durlu-Özkaya, 2002; Ercan et al., 2019; Lanciotti et al., 2007; Marijan et al., 2014; Min et al., 2004; Tittarelli et al., 2019), in order to quantify BA in different cheeses. This method consists in a first step of acid extraction with 0.4 M $HClO_4$, followed by derivatization of the acid extract with DNS-Cl. Separation of the analytes is carried out by RP-HPLC with a C18 column, and the peaks are detected at 254 nm. The obtained limit of detection (LOD) varies between 1 mg/kg and 5 mg/kg for the different amines.

Another study which has been a reference for many authors (Bonczar et al., 2018; Esatbeyoglu et al., 2016; Forzale et al., 2011; Galgano et al., 2001; Gennaro et al., 2003; Martuscelli et al., 2005; Pluta-Kubica et al., 2020; Schirone et al., 2013; Zazzu et al., 2019) was published by Innocente et al. (2007). Improving upon a previous study by the same author (Innocente & D'Agostin, 2002), this method features a step of extraction of BA from cheese with 0.1 M HCl, after the derivatization of this extract with DNS-Cl. The addition of an L-proline solution is necessary to remove the excess of derivatizing reagent, after which the sample is extracted with diethyl ether, dried, and re-dissolved with ACN for injection. Forzale et al. (2011) used this method for the quantification of BA in several pecorino cheeses, obtaining better sensitivity than with methods based on the work of Eerola et al. (1993); they obtained a LOD of 0.3 mg/kg and a limit of quantitation (LOQ) of 1 mg/kg. A slight modification was proposed by Zazzu et al. (2019): detection was carried out at 218 nm instead of 254 nm, obtaining some improvement in terms of sensitivity (LOD 0.07 to 0.23 mg/kg).

Yang et al. (2016) developed an analytical method for the quantification of BA in cheese. It included a clean-up step using online SPE prior to separation. In spite of the fact that SPE is a cleaning and preconcentration tool, detection limits in that study ranged between 0.05 mg/kg and 0.25 mg/kg, which are not the best ones described in the literature for this derivatizing agent; still, the matrix effect of samples was reduced thanks to SPE. Some of the advantages of automatized analysis were the simplification of the sample pretreatment, as well as reduction of manual error, thereby resulting in a more efficient method.

BA have been also analyzed in commercial milk for infants and young children after derivatization with DNS-Cl and detection by UV-Vis (Spizzirri et al., 2019). Spizzirri used SPE (Supelclean ENVI-18Spe Tubes) for concentration and purification, but only for ready-to-use liquid milks, obtaining LOD one order of magnitude lower, in this case between 0.03 mg/L and 0.05 mg/L, than for powder milk samples.

Fluorescence detection

One example illustrating the better fluorometric properties of dansyl derivatives is the study by Standarová et al. (2010) carried out in 2010. They quantified seven different BA as dansyl derivatives in cheese, using FLD at 330 nm and 500 nm as excitation (ex.) and emission (em.) wavelengths, respectively, except for histamine, which was detected by UV. This method reached the lowest LOD (0.03 mg/kg for putrescine and cadaverine) published for these derivatives, to our knowledge. Excellent sensitivity was likewise

reported in the case of Ramos et al. (2020) who used SALLE as sample preparation step. In spite of good results with FLD, both studies showed that an UV detector is more appropriate for the determination of histamine. Several studies also reported LOD lower than 1 mg/kg for different BA in cheese using this system of detection (Brito et al., 2014; Sawilska-Rautenstrauch et al., 2010), thus achieving better sensitivity than most studies conducted with UV-Vis detection.

MS detection

Derivatization reactions are not only conducted with the purpose improving the detectability of analytes in terms of sensitivity and selectivity. Mazzucco et al. (2010) used a HPLC-MS method to validate a technique with UV detection for the determination of BA and amino acids in cheese and other foods. They found that dansyl derivatives decreased the matrix effect in the case of MS detection. As was expected, better LOD were achieved with this method compared with UV. The authors also included a SPE procedure for cleaning the dansylated sample extract.

That same year, La Torre et al. (2010) also combined dansyl derivatives with MS detection. They developed an excellent method with good linearity, free of matrix effects, and featuring high levels of sensitivity (ranging from 0.5 μg/L to 15 μg/L) for the determination of BA in donkey milk samples.

3.1.2 | Derivatization by dabsyl chloride

This derivatizing agent is a sulfonyl chloride analogous to DNS-Cl. It also acts on primary and secondary amino groups, obtaining compounds with good stability, although they are not detected by fluorescence (Toyo'oka, 1999).

UV-Vis detection

Several authors (Bockhardt et al., 1996; Calzada et al., 2013; Fernández et al., 2007; Valsamaki et al., 2000) have determined BA by extraction with 0.1 M HCl from dairy products, precolumn derivatization with DBS-Cl and UV-Vis detection (436 nm). Krause et al. (1995) pioneered this analytical method for the quantification of amino acids and BA in biological samples and food. LOD was between 0.12 pmol and 0.52 pmol, and repeatability was lower than 3.1%.

Also, Bockhardt et al. (1996) developed a method of derivatization with DBS-Cl in combination with a HPLC analysis for the determination of BA in cheese. Although they used a sample clean-up procedure by ultrafiltration, sensitivity was not improved as compared to the method of Krause et al. (1995) and the LOD was between 0.34 pmol and 0.76 pmol.

3.1.3 | Derivatization by benzoyl chloride

BNZ-Cl is another acyl chloride, similar to DNS-Cl or DBS-Cl, used for derivatization of amines, and obtaining shorter elution times than dansyl derivatives (Toyo'oka, 1999).

UV-Vis detection

Özdestan and Üren (2010) studied BA in kefir samples. They were extracted with 0.2 M HCl, and derivatization was subsequently carried out with BNZ-Cl. Benzamide derivatives were extracted with diethyl ether and redissolved with methanol, injected, and detected with UV at 254 nm. LOD lay between 0.2 mg/L and 2.5 mg/L, and linearity ranged between 133.6 mg/L and 1433 mg/L. Other authors used similar procedures or $HClO_4$ in the extraction step to quantify BA in yogurt (Paulo Vieira et al., 2020; Silva et al., 2019).

An important improvement with this derivatizing agent was the detection wavelength. Costa et al. (2015) quantified BA in fermented milks, and carried out detection at 198 nm. The detection limit thereby decreased to values between 0.03 mg/L and 1.30 mg/L, one order of magnitude lower than detection at 254 nm.

A remarkable analytical method was developed by Molaei et al. (2019) to quantify BA in Kashk, a sour yogurt, milk, and buttermilk used in Persian and Turkish cuisine. Histamine, cadaverine, putrescine, and tyramine were extracted and derivatized using SPE, based on magnetic mesoporous silica nanosorbent. Detection was carried out at 198 nm after chromatographic analysis. An excellent detection limit was reached (8 μg/L for Put), and reproducibility was lower than 4.14% of relative standard deviation (RSD).

MS detection

Using isotopically labeled BA as internal standards, Mayr and Schieberle (2012) developed an analytical method to quantify thirteen BA in several foods, including cheese and yogurt. Analysis of benzamide derivatives was carried out by LC-MS/MS. High sensitivity was reached (LOQ 0.05 μg/kg) and, thanks to the labeled BA, %RSD lower than 5.1% and a matrix effect-free method were obtained.

3.1.4 | Derivatization by 9-fluorenylmethoxycarbonyl chloride

This derivatizing agent has the considerable advantage of reacting not only with primary and secondary amines, but also with tertiary amines. The reaction product can be detected by FLD and UV (Toyo'oka, 1999).

Fluorescence detection

Kirschbaum et al. (1994) quantified BA in cheese after their extraction with 0.1 M HCl, precolumn derivatization with FMOC-Cl, and analysis by HPLC/FLD (ex. 265 nm, em. 315 nm). Only one step was required to remove the excess of reagent, thereby avoiding the interferences of hydrolysis products. Satisfactory detection limits were obtained for determination of BA in cheese, except for tryptamine and serotonine, for which the fluorescence quantum yield was too low.

3.1.5 | Derivatization by o-phthalaldehyde

As in the case of DNS-Cl, this reagent is preferably combined with FLD. Although it has been widely used (probably due to its versatility for pre-, on-, or postcolumn derivatization), it has the disadvantage of not reacting with secondary amines, and its products are less stable than dansyl or benzoyl derivatives (Peña-Gallego et al., 2012; Toyo'oka, 1999).

UV-Vis detection

Determination of BA in foods by o-phthalaldehyde (OPA) derivatization and UV detection is not very common in the literature: for dairy products, only studies of tyramine in cheese by Komprda et al. (2008) and Renes et al. (2014) were found. Komprda et al. (2008) extracted BA with 0.1 M HCl, and the extract was precolumn derivatized by OPA in borate buffer in the presence of 2-sulfonylethan-1-ol. Separation, after derivatization, was carried out with a C18 column, and tyramine was detected using a UV-Vis detector at 254 nm. Although the authors did not include figures of merit for this method, they reported concentrations of tyramine ranging from 5 mg/kg to 392 mg/kg; therefore, it can be supposed that LOD was lower than 5 mg/kg.

Fluorescence detection

Vale and Glória (1998) conducted the first determinations of BA in cheese using OPA as postcolumn derivatizing agent and FLD (340 nm for excitation and 445 nm for emission) (Gençcelep et al., 2008). They optimized the extraction of BA from cheese and the purification of the extracts, whereby 0.1 N HCl and diethyl ether were the best options, respectively. Good precision values, lower than 10% of RSD (except for tryptamine and agmantine) were obtained, and LOD was also acceptable (between 0.6 mg/kg and 1.4 mg/kg). Custódio et al. (2007) also tried out different extraction methods and analyzed the extracts according to the method described by Vale and Gloria (Gençcelep et al., 2008). They likewise concluded that HCl allowed for adequate extraction of BA from cheese; however, they stated that 1 M was more appropriate, resulting in better coefficients of variation.

Santos et al. (2003) conducted a study of formation of BA by *Lactococcus* in milk. BA were extracted with sulphosalicylic acid and analyzed by ion-pair HPLC/FLD after postcolumn derivatization with OPA. The same method was used by Gloria et al. (2011) for the determination of BA in milk, and they reported a highly satisfactory LOQ of < 0.010 mg/L.

3.1.6 | Derivatization by naphthalene-2,3-dicarboxaldehyde

Naphthalene-2,3-dicarboxaldehyde (NDA) has a structure analogous to OPA, although its derivatives are more stable: it can therefore be more properly used as a precolumn derivatizing agent (Toyo'oka, 1999). For analysis of BA in dairy products, FLD (ex 424 nm; em 494 nm) has mainly been used.

Fluorescence detection

Zotou and Notou (2012) developed an analytical method based on the derivatization of BA with NDA combined with analysis by HPLC–FLD for the quantification of BA in cheese. Several parameters of the reaction were studied: time of reaction, concentration of NDA and potassium cyanide (KCN) (used as nucleophile), and derivative stability (18 h at −4°C). The method's LOD lay between 0.6 mg/kg and 165 mg/kg. Some years later, the same authors developed an improvement based on zone-fluidics/HPLC (ZF-HPLC) for the simultaneous analyses of BA and online derivatization for the determination of BA in milk (Fernández et al., 2007). They managed to reduce the amount of NDA, the reaction time (3 instead of 30 min), and detection limits (0.6–6 μg/L in aqueous solutions).

3.1.7 | Derivatization by 6-aminoquinolyl-N-hydroxyuccinimidyl carbamate

6-Aminoquinolyl-N-hydroxyuccinimidyl carbamate (AQC) reacts rapidly with primary and secondary amines, obtaining highly stable derivatives that produce intense fluorescence signals. In spite of this, UV detection is mainly used for dairy products (Toyo'oka, 1999).

UV-Vis detection

A comparison between HPLC/FLD and UPLC/UV after derivatization of BA in cheese with AQC was carried out by Mayer et al. (2010). Results regarding differences in

sensitivity were not shown, and only detection limits with UV detection were supplied, ranging from 0.4 mg/kg to 16.2 mg/kg. In spite of the fact that the determination of AQC derivatives with UV has certain disadvantages, such as their lower signal intensity or a pronounced peak due to the byproduct of the AQC derivatization reaction, they were compensated by a much higher resolution due to the use of UPLC. It is remarkable that dopamine and tryptamine could only be detected by UV because they did not yield fluorescent-active AQC derivatives.

3.1.8 | Derivatization by diethylethoxymethylenemalonate

Diethylethoxymethylenemalonate (DEEMM) reacts with primary and secondary amines, and their derivatives remain stable for several weeks at room temperature. They can be detected by UV-Vis spectroscopy at 280 nm (Toyo'oka, 1999).

UV-Vis detection

Redruello et al. (2013) extracted BA and amino acids from cheese with 0.1 M HCl and quantified them after derivatization with DEEMM and analysis by UPLC. They validated the method, obtaining LOD lower than 0.15 mg/L and intraday repeatability much lower than 1% for BA (values estimated in standard mixtures). No significant effect of the cheese matrix was observed.

3.1.9 | Derivatization by ninhydrin

The reaction between this derivatizing agent and amines (NINHYDRIN only reacts with primary amines) yields purple, blue-violet-colored compounds (Toyo'oka, 1999). Ninhydrin is used for postcolumn derivatization, and only when high sensitivity is not required.

UV-Vis detection

Joosten and Olieman (1986) adapted the analytical method developed by LePage and Rocha (1983) for the determination of BA in cheese and other food products. BA were extracted from cheese using a trisodium citrate solution and 0.6 M trichloroacetic acid (TCA), separated by HPLC and detected after postcolumn derivatization at 546 nm. Although a significant disadvantage of this method lay in the extended amount of time required for it (1.5 h), good repeatability values (less than 10%) were nevertheless achieved. The detection limit for BA in cheeses was 2 mg/kg for each amine.

3.1.10 | Derivatization by 4-chloro-7-nitrobenzofurazan

4-Chloro-7-nitrobenzofurazan (NBD-Cl) reacts with primary and secondary amines, and their products are fluorescent. It is only used for precolumn derivatization (Toyo'oka, 1999).

UV-Vis detection

Despite the fluorescent properties of the NBD-Cl compound, in the case of dairy products it has only been used for UV detection. Yigit and Ersoy (2003) determined tyramine in cheese after extraction with 5% $HClO_4$ and derivatization with NBD-Cl. The reaction product was measured by UV at 458 nm after chromatographic separation on a C18 column. The LOD was 25 mg/kg, and the RSD was lower than 3%.

3.2 | Analytical methods based on LC without derivatization

Although BA are frequently determined after derivatization reactions in order to be analyzed by LC, some studies have quantified them directly. Some of the advantages of analyzing them without derivatization are shorter analysis times, as well as avoiding the cost of derivatizing agents (some of which are expensive), along with the waste products thereof and further solvents used during the derivatization process. Several detection modes have been used, but the development of MS has probably played the greatest role in the growth of the number of studies without derivatization.

One of the earliest detection modes studied was by Arlorio et al. (1998). They determined histamine, tyramine, 2-phenylethylamine, and tryptamine in cheese with ion-pair HPLC. The analytes were detected at 215 nm, and they compared sensitivity using an UV and a diode array detector (DAD), obtaining better results with DAD. One of their remarkable achievements was the addition of octylamine to the eluent in order to reduce peak asymmetry. Further authors (Andiç et al., 2011; Min et al., 2004; A. I. Ordóñez et al., 1997) have also used DAD as a detection method to analyze BA by HPLC.

One of the main reasons for using derivatization is the absence of chromophores, fluorophores or the very weak UV absorbance of some BA; however, the specificity of some detectors, such as a chemiluminescent nitrogen detector (CLND), could serve as a good solution, as was shown by J. Sun et al. (2011). They compared three different detection strategies: CLND, postcolumn derivatization

with ninhydrin, and a charged aerosol detector (CAD) after the separation of 14 BA by ion-pair HPLC and applied them in a variety of samples, including dairy beverages. Narrower peaks, better baselines, and excellent linearity were achieved with the CLND. In terms of sensitivity, detection limits ranged from 0.1 μg/ml to 0.4 μg/ml (estimated in standard solutions), which is lower than with ninhydrin, but not as good as other derivatizing agents.

The evaporative light scattering detector (ELSD) is also another possibility for the quantification of BA without derivatization, although it is not the most adequate one if a high sensitivity is required, as was shown by Restuccia et al. (2011). They compared a method based on LC-ELSD with a method based on derivatization by DNS-Cl for the determination of BA in cheese. Better repeatability was achieved with the derivatization method. As in the case of the CLND, good detection limits were estimated (lower than 3 mg/kg); however, they were not as good as those estimated with DNS-Cl.

In order to obtain very low detection limits without using derivatizing agents, MS needs to be used as a detector. Calbiani et al. (2005) obtained a LOD of 0.05 mg/kg (estimated in cheese samples) for histamine, with a method based on matrix solid phase dispersion (MSPD) followed by LC-MS. This method's figures of merit were highly satisfactory: excellent intraday repeatability (RSD lower than 5%) and linearity over two orders of magnitude.

The comparison carried out by Gosetti et al. (2007) between the analysis of native BA by LC-MS/MS and by LC-UV after derivatization with DNS-Cl showed that, in spite of the higher sensitivity of MS (LOD ranging from 5.1 μg/L to 35 μg/L in ricotta cheese, 1.7 to 22.5 μg/L in standard solutions), some parameters such as the linearity range are better when a derivatizing agent is used.

Draisci et al. (1998) integrated the pulsed amperometric detection method to IEC for the simultaneous determination of underivatized BA, obtaining recoveries ranging from 87.3% to 97.7% and a lower LOD of 1.25–2.50 ng in cheeses. Palermo et al. (2013) applied an analytical method based on IEC conductimetric detection for the determination of certain BA, and obtained recoveries of 82–103 % as well as good LOD, lying between 23 μg/kg and 65 μg/kg.

One of the difficulties of analyzing BA without derivatization is their bad chromatographic properties. Hydrophilic interaction chromatography (HILIC) offers a new possibility to avoid tedious derivatization processes. The hyphenation of HILIC and MS developed by Gianotti et al. (2008) to quantify seven BA in cheese is a perfect example of its possibilities (LOD ranging from 1.0 μg/L to 3.5 μg/L).

3.3 | Analytical methods based on capillary electrophoresis

CE is the second most commonly applied separation technique for the determination of BA in food. CE is sensitive, fast, uses small sample quantities, provides good precision, and does not require derivatization or sample cleaning (Karovičová & Kohajdová, 2005; Kvasnička & Voldřich, 2006). It is powered by high voltage electric fields and capillary separation. The separation is caused by the different mobility and distribution characteristics of each component in the sample (Shah et al., 2020). CE is efficient, but has a lower reproducibility of migration times than LC. Although it is a sensitive technique, its sensitivity is low compared to other methods (as results show below). Therefore, although derivatization is not compulsory, its use is generally recommended to solve the problem of determining BA in food samples. Detection can be carried out in two ways: either it is carried out directly with a UV detector or a DAD, or it is carried out after derivatization of BA with FLD (Etienne, 2006). Some of the reagents used are OPA, DNS-Cl, DBS-Cl, and β-naphthol (Karovičová & Kohajdová, 2005), with the same advantages and disadvantages previously explained for LC.

Nouadje et al. (1995) developed an analytical method based on CE and detection with a ball-lens laser-induced fluorescence detector for the determination of BA in cheeses, previously derivatizing them with fluorescein isothiocyanate (FITC). Detection limits ranged from 0.5 x 10^{-10} M to 1.5×10^{-10} M.

CE without derivatization has also been used for the quantification of BA in cheeses. Fernández-García et al. (1999, 2000) studied the influence of proteinases on the formation of biogenic amines. They were analyzed by CE and detected at 214 nm. The lowest amount of histamine they found was 2.84 mg/kg.

Pulsed amperometric detection (PAD) also offers a possibility for the detection of BA after separation by CE (X. Sun et al., 2003). Ten grams of fresh milk were homogenized with 6 ml of 5% $HClO_4$ for 30 min and centrifuged at 5000 rpm for 10 min. LOD ranged from 10^{-7} M to 4 10^{-7} M. Linearity was two orders of magnitude. Putrescine, cadaverine, spermidine, and spermine were searched for in six different samples, but only spermidine was found.

Kvasnička and Voldřich (2006) developed a capillary zone electrophoresis (CZE) method with conductometric detection for the determination of BA in different foods. For the analysis of two different cheeses, 5 g were extracted with 100 ml of 0.1 M HCl for 30 min in ultrasonic bath. The mixture was then filtered and diluted with demineralized

water before analysis. Linearity lay between 0 μmol/ml and 100 μmol/ml, and LOD ranged from 2 μmol/L to 5 μmol/L.

More recently, a faster method (less than 6 min) based on CE and conductivity detection was applied for the analysis of five different BA in yogurt, kefir, and cheese (Adımcılar et al., 2018). LOD ranged from 0.041 mg/L to 0.098 mg/L, and reproducibility was highly satisfactory (less than 4.25% RSD), as in the previous method.

3.4 | Analytical methods based on thin layer chromatography

TLC is used as a technique for the rapid separation and determination of small amounts of substances. In terms of advantages, TLC is a simple method, does not involve special equipment, and can analyze several samples at the same time (Hernández-Cassou & Saurina, 2011; Önal, 2007; Özdestan & Üren, 2009; Zhang et al., 2019). Nevertheless, in some cases, an excessive amount of time is required for analysis, and results are not sufficiently accurate (Bockhardt et al., 1996; Lázaro de la Torre & Conte-Júnior, 2014; Önal, 2007). The use of a variety of derivatizing agents to determine BA is a common practice.

The first TLC method for determining BA in cheese samples was the one developed by Shalaby (1999). They were extracted with TCA and then derivatized with DNS-Cl. Separation of the dansyl derivatives was achieved on silica gel TLC plates and quantified by densitometry at 254 nm. The limit of detection ranged from 5 ng to 10 ng, and the precision, expressed as %RSD, ranged from 0.15% to 4.41%.

Pham and Nguyen (2016) carried out a study of BA in milk using HPLC and TLC. They demonstrated the use of the TLC technique as a qualitative method and HPLC as a quantitative method to determine histamine. Ninhydrin was sprayed in TLC technique, and a purple spot appeared when histamine was present. The quantitative result was obtained by HPLC.

Švarc-Gajic and Stojanovic (2011) determined histamine concentration in cheese using the chronopotentiometric technique as a detection method after purification of the extract by TLC. LOD was 1.31 mg/L. This is a simple, rapid cheese histamine analysis technique, cheaper than other chromatographic techniques, although its sensitivity is not as good.

3.5 | Analytical methods based on gas chromatography

The volatility and polarity of BA is one of the reasons why the GC technique is not usually applied to determine them; derivatization is thus frequently used to improve those properties (Karovičová & Kohajdová, 2005; Lázaro de la Torre & Conte-Júnior, 2014; Papageorgiou et al., 2018). Frequently analyzed derivatives are trifluoroacetyl, trimethylsilyl or 2,4-dinitrophenyl (Kielwein et al., 1996). The most common detectors used to quantify them are flame ionization detectors (FID), electron capture detectors (ECD), and MS (Etienne, 2006; Kielwein et al., 1996; Silla Santos, 1996).

The number of studies that have analyzed BA in dairy products by GC is low. One featured technique is the analytical method developed by Ali Awan et al. (2008) for the determination of putrescine and cadaverine by SPME–GC–MS. This method is based on on-fiber derivatization using trifluoroacetylacetone (TFAA). Automatization is one of its main advantages, along with the fact that is matrix effect free. Although the authors generally applied the method by GC-MS, they applied it to cheese samples by GC-FID, whereby the concentration of Put and Cad was of 38 and 22 mg/kg, respectively.

Some authors have used GC-MS as a rapid and sensitive method for the analysis of BA in cheese samples (Kamankesh et al., 2021; Mohammadi et al., 2017). Mohammadi et al. (2017) also used simultaneous derivatization and microextraction to quantify BA in cheese samples. In this case, the derivatizing agent was isobutyl chloroformate (IBCF), and the microextraction technique was MAE–DLLME. Derivatization products were detected by GC-MS. The LOD for cheese samples ranged from 5.9 μg/kg to 14 μg/kg, which lies in the range of other works.

3.6 | Rapid methods

3.6.1 | ELISA

ELISA-based methods started to be developed in the 1970s, and they have been expanded since then, mainly due to their speed and low cost (Vaz et al., 2020). The first time that a competitive direct-ELISA (CD-ELISA) was applied to the determination of histamine in cheese was in Aygün et al. (1999). They compared their results with a HPLC method and found a good agreement, although some differences were found in the low concentration range from 2 mg/kg to 10 mg/kg. The LOD of the ELISA method (2 mg/kg) enabled detection of histamine at levels well below those that have been considered safe. They also showed one of the main advantages of ELISA methods: their speed. While more than 30 samples per day could be evaluated by CD-ELISA, the maximum daily evaluation achieved by the LC method was 10 samples of cheese.

Leszczyocha and Pytasz (2018) also compared an ELISA method with a spectrofluorimetric method, but in this case for the determination of histamine in yogurt and

kefir. Their comparison evidenced the possibilities of the immunoenzymatic method, which is faster and, in this case, more sensitive than the reference method, although more expensive and less precise, while adequate for higher concentrations.

3.6.2 | Biosensors

Development and use of sensors and biosensors have been growing exponentially in the last decades. Speed, low price, ease of use, and the minimum required amount of sample are some of the advantages that have contributed to their expansion in a wide number of sectors, including the food industry (Ahangari et al., 2021; Salleres et al., 2016).

Most biosensors used to quantify BA in dairy products are based on electrochemical responses (Compagnone et al., 2001; Rotariu et al., 2016) of the detection of hydrogen peroxide, generated as a byproduct in BA oxidation, catalyzed by amino oxidase enzymes (Calvo-Pérez et al., 2013; Oliver et al., 2021). Other systems also exist, such as the molecularly imprinted electrochemical sensor developed by Huang et al. (2011) for the determination of tyramine in yogurt samples. The LOD was 5.7×10^{-8} M, and the sensor exhibited an excellent selectivity and repeatability.

Compagnone et al. (2001) studied BA by amperometric detection in cheese using immobilized diamine oxidase (DAO). Two different systems were optimized to respond to six BA. The best system's sensitivity ranged from 5×10^{-7} to 2×10^{-6} M, with three orders of magnitude of linearity and good reproducibility (1–3% RSD). Operational lifetime was calculated to last for 300 samples.

The problem of using amine oxidases in biosensors to quantify BA is that most amine oxidases do not react to a single BA, but to several amines. Lange and Wittmann (2002) solved this problem for the determination of histamine, tyramine, and putrescine in different foods (two different cheeses were analyzed) by immobilizing three different amine oxidases and applying neural networks for pattern recognition. The LOD was 10 mg/kg for histamine and tyramine and 5 mg/kg for putrescine. This system was compared with a HPLC method, and good correlation between results was found. This comparison revealed one of the main drawbacks of some biosensors: false-positive and false-negative results, which reached 12% and 25%, respectively.

Selectivity problems were also shown by Carelli et al. (2007), who reported the total content of BA in cheese expressed as histamine equivalents, assuming that it was not possible to discriminate the contribution of each biogenic amine to the biosensor. In order to remove certain interferences, the authors developed a biosensor based on DAO, entrapped by glutaraldehyde, onto an electrosynthesized bilayer film, after having studied different electrodes and films. The sensitivity achieved with this system was high, with LOD between 6 μM and 12 μM.

Calvo-Pérez et al. (2013) developed an amperometric biosensor for the determination of tyramine in cheese, and they used a redox mediator (hydroxymethylferrocene) to reduce the applied potential and thereby decrease the possibility of interferences in the electrochemical measurement. In this case, the detection is based on the signal produced during the reduction of the mediator, instead of the electrochemical detection of H_2O_2 as in most of these systems. The enzyme used was plasma amine oxidase (PAO), and it was immobilized on a screen-printed carbon electrode. The LOD was 2 μM.

Recently, two different biosensors have been developed for the quantification of tyramine in cheese (Navarro et al., 2020; Oliver et al., 2021). One of them (Navarro et al., 2020) is based on the colorimetric determination (540 nm) of gold nanoparticles generated when Au (III) is reduced in the oxidase enzymatic reaction, using tyramine oxidase (TAO) as enzyme. A higher sensitivity is obtained after 30 min of reaction, although results can be achieved more rapidly (after 4 min) if reaction takes place at 50°C. Reproducibility was high (5.6% RSD), and the LOD was 2.9×10^{-6} M. Another biosensor (Oliver et al., 2021) (with similar analytical characteristics) has two important advantages: pretreatment of cheese is not necessary for determination, and measurements are conducted using a smartphone. This system is based on colorimetric test strips containing TAO, peroxidase, and 3,3′,5,5′-tetramethylbenzidine, which are added to the sample for 2 min for the reaction to take place, after which the RGB color coordinates are measured.

4 | CONCLUSION

The considerable number of studies that analyze BA in dairy products reflect general concern about the presence and concentration of BA in this kind of product, in view of the adverse health effects produced by their ingestion, especially in histamine-sensitive or allergic patients. The most common sample pretreatment previous to BA analysis is extraction with acids or solvents to precipitate the proteins and extract the analytes from the matrix. Some studies improve on cleanliness and sensitivity by using SPE; however, a greater number of steps is not always worth the effort. Without a doubt, the most widely used analytical technique for the determination of BA is HPLC with derivatization: sensitivity, the structure of the BA, and the detection technique are the three main reasons for choosing one of the numerous derivatizing agents. One of these techniques' most important drawbacks is their slowness; the development of rapid methods, such

as biosensors, is thus slightly on the increase (less than in other matrices), with good results in terms of sensitivity, although specificity still needs to be improved.

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AUTHOR CONTRIBUTIONS

Marta Moniente: conceptualization; data curation; writing – original draft; writing – review & editing. **Laura Botello-Morte**: conceptualization; writing – review & editing. **Diego García-Gonzalo**: Writing – review & editing. **Rafael Pagán**: conceptualization; funding acquisition; project administration; writing – review & editing. **Ignacio Ontañón**: conceptualization; data curation; supervision; writing – original draft; writing – review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Manuscrito II. Histamine accumulation in dairy products: Microbial causes, techniques for the detection of histamine-producing microbiota, and potential solutions

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COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY

Histamine accumulation in dairy products: Microbial causes, techniques for the detection of histamine-producing microbiota, and potential solutions

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Abstract

Histamine poisoning is a significant public health and safety concern. Intoxication from ingestion of food containing high amounts of histamine may cause mild or severe symptoms that can even culminate in cardiac arrest. Nonetheless, although histamine levels in dairy products are not subject to any regulation, important outbreaks and severe adverse health effects have been reported due to intake of dairy products with a high histamine content, especially ripened cheeses. Histamine, a biogenic amine, can accumulate in dairy products as a result of the metabolism of starter and nonstarter lactic acid bacteria, as well as yeasts that contribute to the ripening or flavoring of the final product, or even as a result of spoilage bacteria. The aim of this review is to describe the microbiological causes of the presence of histamine in fermented milk products, and to propose control measures and potential methods for obtaining histamine-free dairy products. Thus, this manuscript focuses on histamine-producing microbiota in dairy products, highlighting the detection of histamine-producing bacteria through traditional and novel techniques. In addition, this review aims to explore control measures to prevent the access of histamine-producing microbiota to raw materials, as well as the formation of histamine in dairy products, such as a careful selection of starter cultures lacking the ability to produce histamine, or even the implementation of effective food processing technologies

Nomenclature: BAs, biogenic amines; bp, base pairs; *C. farciminis*, *Companilactobacillus farciminis*; CFU/g, colony forming units per gram; CFU/mL, colony forming units by milliliter; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DAO, diamine oxidase enzyme; EFSA, European Food Safety Authority; FAO, Food and Agriculture Organization; GMO, genetically modified organisms; HDC, histidine decarboxylase enzyme; *hdc*, histidine decarboxylase gene; HNMT, histamine N-methyltransferase enzyme; HPB, histamine-producing bacteria; HPH, high-pressure homogenization; HPLC, high-performance liquid chromatography; *L. brevis*, *Levilactobacillus brevis*; *L. buchneri*, *Lentilactobacillus buchneri*; *L. casei*, *Lacticaseibacillus casei*; *L. curvatus*, *Latilactobacillus curvatus*; *L. fermentum*, *Limosilactobacillus fermentum*; *L. hilgardii*, *Lentilactobacillus hilgardii*; *L. kefiri*, *Lentilactobacillus kefiri*; *L. parabuchneri*, *Lentilactobacillus parabuchneri*; *L. paracasei*, *Lacticaseibacillus paracasei*; *L. pentosus*, *Lactiplantibacillus pentosus*; *L. plantarum*, *Lactiplantibacillus plantarum*; *L. reuteri*, *Limosilactobacillus reuteri*; *L. rhamnosus*, *Lacticaseibacillus rhamnosus*; *L. sakei*, *Latilactobacillus sakei*; LAB, lactic acid bacteria; *Lb.*, *Lactobacillus*; *Lb. acidophilus*, *Lactobacillus acidophilus*; *Lb. bulgaricus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*; *Lb. delbrueckii*, *Lactobacillus delbrueckii* subsp. *delbrueckii*; *Lb. delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *lactis*; *Lb. helveticus*, *Lactobacillus helveticus*; *Lc.*, *Lactococcus*; NSLAB, nonstarter LAB; PCR, polymerase chain reaction; PCR-DGGE, PCR-denaturing gradient gel electrophoresis; RT-qPCR, real-time quantitative PCR; SLAB, starter LAB; SNPs, single-nucleotide polymorphisms; WHO, World Health Organization.

to reduce histamine-producing microbiota. Finally, the removal of histamine already formed in dairy products through histamine-degrading microorganisms or by enzymatic degradation will also be explored.

KEYWORDS
biogenic amines, dairy products, cheese, histamine-producing microbiota, hdcA, DAO, NSLAB

1 | INTRODUCTION

As one of the most important biogenic amines (BAs), histamine is involved in immune system response, gastric acid secretion, and neurotransmission, among other processes. However, histamine is also associated with food intolerance and food poisoning. Strategies to prevent, detect, and overcome food safety problems caused by histamine accumulation will be presented in this review.

Histamine is an organic nitrogenous compound exclusively synthesized via oxidative decarboxylation of the amino acid L-histidine by L-histidine decarboxylase (HDC) enzyme. Fermentation in food (red wine, hard cheese, etc.) or improper preservation may result in the formation of high histamine concentrations. Histamine accumulated in food can cause symptoms such as nausea, headache, abdominal pain, diarrhea, and itching (Benkerroum, 2016; Gardini, Ozogul, Suzzi, Tabanelli, & Ozogul, 2016). An estimated 1% of the population could be histamine intolerant or hypersensitive: in such people, even lower intakes of histamine can lead to severe symptoms (Maintz & Novak, 2007). Fish and ripened cheese are the most common foods associated with histamine intoxication (EFSA, 2011). In fresh raw milk, histamine concentration is usually low; however, in fermented dairy products, such as yogurt and especially ripened cheese, variable concentrations of histamine can be detected. A high concentration of nutrients, marked biochemical changes during extended ripening periods, along with complex microbiota make ripened cheese an ideal matrix for histamine accumulation; it is becoming an increasing health concern (Costa, Rodrigues, Frasao, & Conte-Junior, 2018; Linares, Martin, Ladero, Alvarez, & Fernandez, 2011). Many different genera and species of microorganisms are responsible for histamine production in dairy products. This manuscript reviews histamine-producing microbiota, which can be present in dairy products as starter cultures, usually lactic acid bacteria (LAB), or as nonstarter cultures (naturally present in milk), as well as contaminant microorganisms (due to practices during dairy product manufacture or stemming from the processing environment), mainly members of the *Enterobacteriaceae* family. Some yeasts and molds have been reported as histamine producers in dairy products as well (Barbieri, Montanari, Gardini, & Tabanelli, 2019; Linares et al., 2012).

In the food industry, the determination of histamine is a key aspect for food safety, in spite of the fact that its levels in dairy products are not subject to any regulation. Histamine can be detected and quantified in food by several techniques reviewed by Gagic et al. (2019). However, the detection of potential histamine-producing microbiota, reviewed in the present study, may help to determine whether the starter cultures of dairy products are potential histamine producers. Consequently, histamine accumulation in dairy food might be prevented.

It is important to find solutions for obtaining histamine-free dairy products, and to control histamine production through a series of measures. First of all, good hygienic practices must be implemented during manufacturing processes. Ripening and storage temperatures, pH and salt concentration, among others, are important factors that may also exert an influence on histamine production. Additionally, heat or high-pressure homogenization (HPH) treatments applied to milk have been shown to prevent the production of histamine in dairy products (Benkerroum, 2016; Gardini et al., 2016; Linares et al., 2012).

Histamine degradation, on the other hand, is mainly performed by the diamine oxidase enzyme (DAO) or histamine N-methyltransferase (HNMT) (Maintz & Novak, 2007). Thus, chemical or biological histamine degradation by DAO or the addition of strains with the ability to degrade histamine could also be a preventive measure (Benkerroum, 2016; Gardini et al., 2016; Linares et al., 2012).

This review focuses on providing an overview of previous studies related to histamine production in dairy products, highlighting the implication of the present microbiota. In addition, we review potential solutions designed either to prevent the formation of histamine in manufactured products, or its removal. The increased prevalence of histamine intolerance and food allergies in the general population make this issue an emergent worldwide public health care concern.

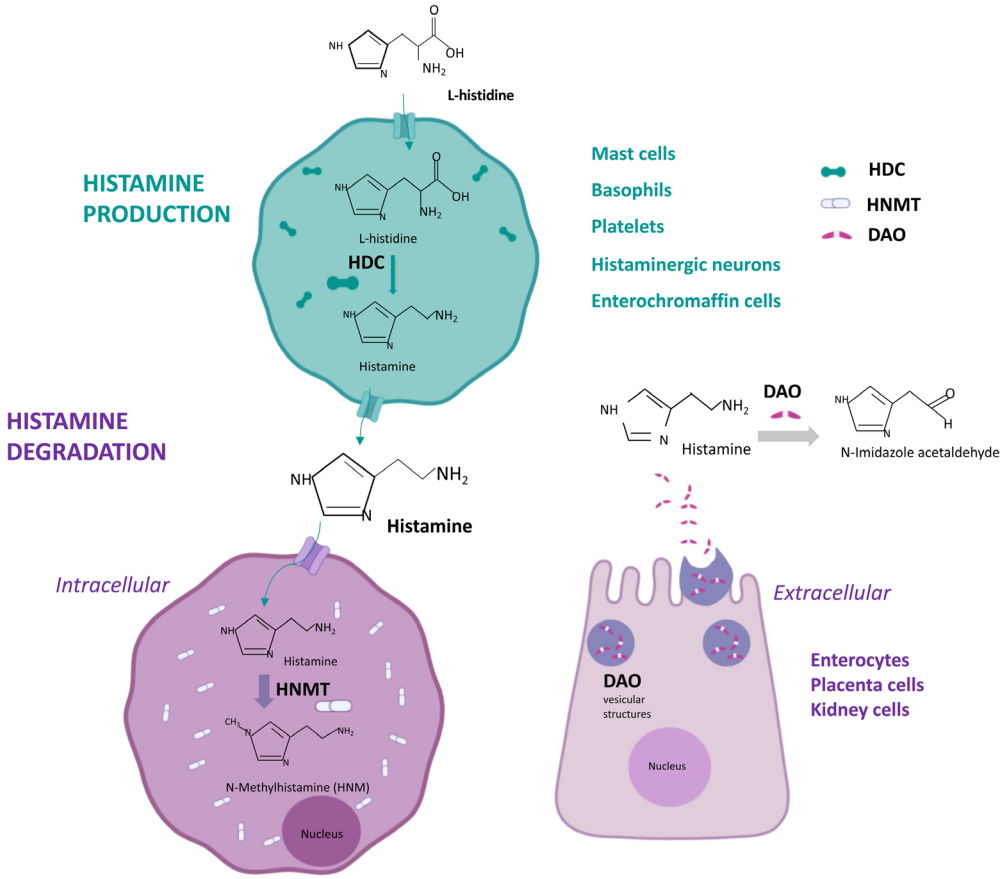

FIGURE 1 Overview of the main mechanisms of histamine production and degradation in the mammal cell. Histamine is intracellularly synthesized by L-histidine decarboxylase (HDC) from the amino acid histidine by certain mammal cells (mast cells, basophils, platelets, histaminergic neurons, and enterochromaffine cells). Conversely, histamine is intracellularly degraded by histamine N-methyltransferase (HNMT), ubiquitously expressed, and extracellularly by secreted diamine oxidase (DAO), mainly produced in enterocytes

2 | HISTAMINE AS A BIOGENIC AMINE: CONSEQUENCES OF ITS ACCUMULATION IN DAIRY FOOD

BAs are low-molecular-weight nitrogenous compounds synthesized by enzymatic decarboxylation of their precursor amino acids, or by amination and transamination of aldehydes and ketones (Benkerroum, 2016; Linares et al., 2011; Pluta-Kubica, Filipczak-Fiutak, Domagała, Duda, & Migdał, 2020). A great variety of BAs exist, with different chemical structures classified as aliphatic (agmantine, putrescine, cadaverine, ethylamine, methylamine, isoamylamine, ethanolamine, spermine, and spermidine), aromatic (tyramine, phenylamine, and phenylethylamine) or heterocyclic (histamine, tryptamine, and pyrrolidine), among others (Linares et al., 2011; Papageorgiou et al., 2018; Spano et al., 2010). From a physiological point of view, BAs are involved in the proper functioning of the human metabolism. On the other hand, histamine and other BAs can serve as indicators of quality and freshness of food and alcoholic beverages (Papageorgiou et al., 2018).

Despite the fact that putrescine and cadaverine have been recently reported as potentially cytotoxic (del Rio et al., 2019), it is well established that histamine and tyramine are the two most toxic BAs; they are the ones most frequently present in dairy products, and the ones which cause the most severe symptomatology. Notably, levels of histamine lower than tyramine appeared to cause typical symptoms in healthy people. This fact, together with the absence of detoxifying mechanisms for histamine in sensitive people who present intoxication symptoms even when exposed to small amounts thereof, makes this BA a major public health concern that needs to be addressed with the appropriate measures (Benkerroum, 2016).

Figure 1 provides an overview of histamine biosynthesis and degradation in the mammal cell. Histamine (2-[4-imidazolyl]ethylamine) is synthesized by oxidative decarboxylation of the amino acid L-histidine, catalyzed by the HDC enzyme. In humans, mast cells, basophils, platelets, histaminergic neurons, and enterochromaffin cells are responsible for synthesizing endogenous histamine, storing a heparin–histamine complex in secretory granules on

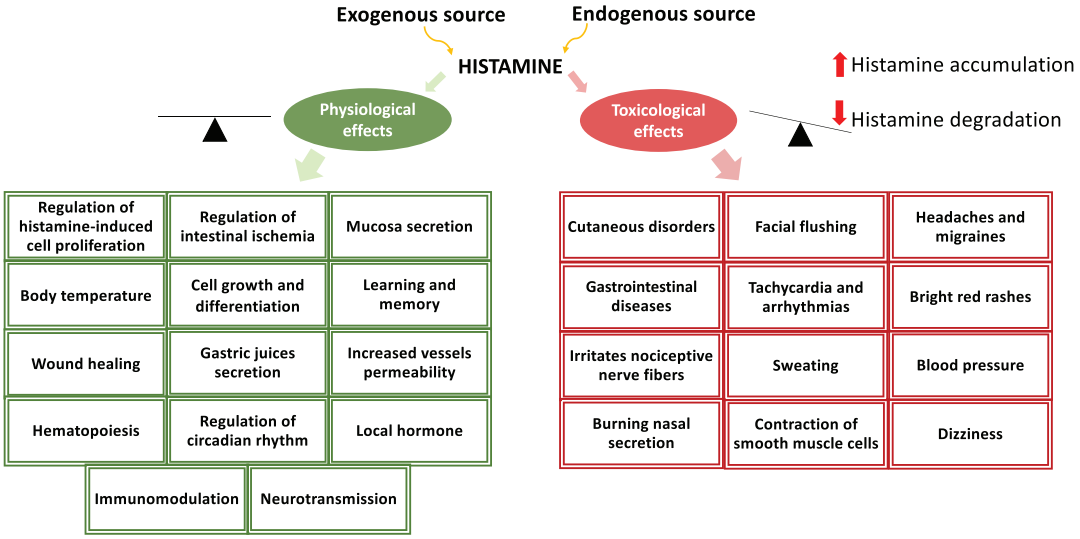

FIGURE 2 Physiological equilibrium between histamine synthesis/intake and degradation or the consequences of a misbalance due to an increase in histamine accumulation or a decrease in histamine degradation. The physiological and toxicological effects of histamine on the human metabolism are also shown

an intracellular level, and releasing it in response to various stimuli (Maintz & Novak, 2007). Other immune cells, such as T cells, dendritic cells, macrophages, and certain types of epidermal cells, have also been shown to synthesize lower amounts of histamine, which is released without having been stored (Huang, Li, Liang, & Finkelman, 2018).

Present in the brain, lungs, stomach, intestine, uterus, and ureters, histamine is an important mediator of a number of biological processes (Ladero, Calles-Enriquez, Fernandez, & Alvarez, 2010). Figure 2 shows that histamine fulfills important physiological functions, including neurotransmission, regulation of circadian rhythm, immunomodulation, hematopoiesis, gastric juice secretion, vessel permeability, wound healing, learning and memory, mucosa secretion, and regulation of temperature, as well as cell growth and differentiation (Ladero et al., 2010; Maintz & Novak, 2007; Schwelberger, Ahrens, Fogel, & Sánchez-Jiménez, 2014).

Once released, histamine binds one out of the 4 G-protein coupled receptors (H1, H2, H3, or H4) located in target cells, in order to produce those important physiological effects. Most of these effects are caused by the activation of H1 receptors, ubiquitously expressed, and they produce typical type 1 hypersensitivity reactions (allergic and asthma reactions). H2 receptors are involved in immunomodulation, gastric acid secretion, mucus secretion, or vascular permeability. H3 receptors, exclusively expressed in neurons, participate in blood–brain barrier function. H4 receptors are mainly involved in proinflammatory responses (Thangam et al., 2018).

As shown in Figure 1, intracellular histamine can be inactivated by methylation of the imidazole ring, catalyzed by HNMT, a widely distributed enzyme. Conversely, extracellular histamine can be metabolized by oxidative deamination of the primary amino group, catalyzed by DAO, a copper-dependent amino oxidase also called histaminase, which is mainly produced by enterocytes, but also by placenta and kidney cells (Comas-Basté, Sánchez-Pérez, Veciana-Nogués, Latorre-Moratalla, & Vidal-Carou, 2020; Ladero et al., 2010; Schwelberger et al., 2014).

Figure 2 depicts the physiological equilibrium between histamine synthesis/intake and degradation or the consequences of a misbalance. When increased availability of histamine or decreased histamine degradation occurs, histamine accumulation causes unspecific gastrointestinal symptoms as well as extra-intestinal symptoms, mainly immediately after (few min) or even during meals, for a period up to 24 hr (Comas-Basté et al., 2020; Tuck, Biesiekierski, Schmid-Grendelmeier, & Pohl, 2019). The toxicological effects of histamine include vascular disorders (dilation of arteries and increased capillary permeability producing headache, hypotension, edemas, urticaria, facial flushing, etc.), heart disorders (a stimulatory effect leading to palpitations, tachycardia, and arrhythmia), gastrointestinal disorders (contraction of smooth muscle cells causing cramps, diarrhea, and vomiting), and neurological disorders (stimulatory effects resulting in pain and itching) (FAO/WHO, 2013; Ladero et al., 2010; Maintz & Novak, 2007; Schnedl et al., 2019).

Histamine can be expected to be present in all foods containing free histidine or proteins that can suffer proteolysis (Tuck et al., 2019); foods rich in histamine are detailed in Comas-Basté et al. (2020). Histamine may be present in

fermented food as a consequence of the oxidative decarboxylation of L-histidine via the HDC enzymes from the microbiota of these products (Landete, Pardo, & Ferrer, 2008). A fairly efficient detoxification system, based on intestinal and liver amine oxidases, metabolizes the regular dietary intake of histamine (Schwelberger et al., 2014).

However, the presence of high amounts of this BA in food has been associated with histamine intolerance and intoxication (Maintz & Novak, 2007). Several studies of oral administration of histamine have shown that the same histamine dosage produces different effects and severity of symptomatology depending on each participant (EFSA, 2011). For that reason, it is well established that a percentage estimated in 1% of the population suffers from a great sensitivity to this compound, which is known as histamine intolerance (Comas-Basté et al., 2020). It is caused by the ingestion of moderate levels in food, and results from an imbalance between the amount of accumulated histamine and the capacity for its degradation, mainly linked to a DAO deficit. The enzymatic activity and detoxification efficiency of DAO vary significantly among individuals. In some cases related to DAO deficiency, it can lead to hypersensitivity to histamine and subsequent variable symptomatology (Comas-Basté et al., 2020; Ozogul & Ozogul, 2020).

In relation to the etiology of histamine intolerance, several single-nucleotide polymorphisms (SNPs) in the DAO-encoding gene result in decreased activity of the enzyme, whereas other SNPs in the promoter region of that gene produce a diminished transcription level and thus a decreased level of the enzyme. However, DAO deficiency is not only due to a genetic background. It could also be related to impaired DAO activity, caused by inflammatory bowel pathologies or certain functional intestinal disorders, such as carbohydrate malabsorption or nonceliac gluten sensitivity. Finally, temporary and reversible DAO inhibition could also result from the presence of other BAs, alcohol, or even certain drugs as chloroquine, clavulanic acid, metamizol, and so on (Comas-Basté et al., 2020). The diagnostic criteria of histamine intolerance include low serum DAO values, two or more of the typical symptoms exposed above, and clinical improvement as a consequence of histamine-free or reduced diet, or of the intake of antihistaminergic medication (Schnedl et al., 2019; Tuck et al., 2019). Scientific publications referring to histamine intolerance or histaminosis have exponentially increased over the last two decades, thereby indicating the importance of this disorder (Comas-Basté et al., 2020).

On the other hand, histamine intoxication, caused by the ingestion of food containing high levels of histamine (Bodmer, Imark, & Kneubühl, 1999), is an immune system response that usually appears in the course of a short period (up to 24 hr) after ingestion of contaminated food (Hungerford, 2010). The diagnosis is based on increased plasma histamine levels associated with the previous uptake of food with proved high histamine content (Comas-Basté et al., 2020).

Histamine is commonly found in dairy products, such as cheese and yogurt, or raw, pasteurized, and Ultra High Temperature (UHT) milks of different animal species, as well as reconstituted powdered milk (Benkerroum, 2016; Costa et al., 2018; Ladero et al., 2017; Linares et al., 2011; Spano et al., 2010). Amounts of BAs in milk, yogurt, cottage, and unripe cheeses can be expected to range from milligrams to tens of milligrams per kg (Linares et al., 2011; Spano et al., 2010). Histamine is present in higher amounts in fermented or ripened dairy products (Costa et al., 2018). In such products, variable amounts of histamine (7 mg/kg in sour cream, 13 to 21.2 mg/kg in yogurt, and 4 mg/kg in kefir) have been found (Bodmer et al., 1999; Özdestan & Üren, 2010). A drastic increase in histamine content often takes place in the course of cheese production, leading to histamine levels of up to 2,500 mg/kg in aged cheese, a highly toxic amount. Histamine concentration varies among different types of ripened cheese and may differ within the same type of cheese, even within parts thereof, also depending on ripening time, manufacturing process conditions, and the bacterial starter culture used (Madejska, Michalski, Pawul-Gruba, & Osek, 2018; Novella-Rodríguez, Veciana-Nogués, Izquierdo-Pulido, & Vidal-Carou, 2003).

The first outbreak of histamine poisoning related to cheese was reported in 1967, involving Gouda and Swiss cheeses containing 850 to 2,500 mg/kg, but other cheese varieties, including Gruyere, Parmesan, Emmental, Suisse, and Provolone, have also been involved in outbreaks (Fernandez-Garcia, Tomillo, & Nunez, 2000; Maintz & Novak, 2007). A study conducted by Rauscher-Gabernig, Grossgut, Bauer, and Paulsen (2009) concluded that tolerable limits for histamine in cheese would be 100 to 417 mg/kg on the basis of a supposed daily consumption of 60 g. Based on Austrian data for usual serving sizes and histamine concentration in foods, a proposed limit of 400 mg/kg is considered acceptable for cheeses (Rauscher-Gabernig et al., 2009). Given this threshold dose for histamine in cheese, Madejska et al. (2018) found that the amine content exceeded that value in Gorgonzola (400 and 730 mg/kg), and reached that level of toxicity in Camembert.

Maximum legal limits for histamine have been established for fresh fish (200 mg/kg) and cured fish products (up to 400 mg/kg) by European Commission Regulation No. 2073/2005 (European Parliament, 2005). Despite the existing legal limits for fish, the histamine content in dairy products is not regulated by any type of legislation; maximum recommended concentrations have only been

suggested. For instance, the Netherlands Institute of Dairy Research sets a limit of 100 to 200 mg/kg on histamine in foods. In order to guarantee food safety and consumer health, legal histamine limits for dairy products should be established in regulations and enforced.

Overall, ripening cheeses are the most common candidates among dairy products for the potential accumulation of high contents of histamine; they are thus prone to cause significant adverse health effects and thereby constitute a notable health risk for consumers. Further insights into the inherent characteristics of dairy products, including composition, biochemical changes, and above all present microbiota, should enable our health systems to prevent, detect, and overcome the formidable safety issue constituted by histamine in dairy products.

3 | INHERENT CHARACTERISTICS OF DAIRY PRODUCTS WITH POTENTIAL IMPACT ON HISTAMINE PRODUCTION

3.1 | Composition and biochemical changes in raw milk and fermented (cultured) dairy products

Milk is a secretion from mammary glands, which serves as the basic food for neonates. It contains multiple nutrients whose proportion varies among animal species, explained in Table 1, as well as in the course of the lactation period in order to meet the varying nutritional needs of neonates. Protein content in the milk of several different dairy animals might vary from 3.4% in cow milk to 5.7% in sheep milk (Table 1). This can be of particularly importance because proteins are the main source of histidine in milk as a precursor of histamine. Apart from mother's milk, humans consume milk from certain domestic animals, such as cows, goats, sheep, and buffalos, either in the form of fresh milk or as dairy products. Cow and buffalo milks are the most widely consumed milks in the world, although interest in goat and sheep milks has increased in recent years (OECD & FAO, 2020).

The proportions of chemical components in milk largely determine its nutritional, organoleptic, technological (i.e., chemical and physical reactions that can occur therein), and microbiological (i.e., microbiological species and microbial load) properties (Walstra, Wouters, & Geurts, 2006). Due to the importance of histidine in histamine formation, we will focus on milk proteins, as the main source of amino acids. In cow milk, two groups of proteins can be differentiated according to their pH stability. Caseins represent approximately 80% of total protein, while the remaining approximately 20% is composed of whey (serum) proteins. Caseins (a mixture of

TABLE 1 Average composition in % w/w and range (in parentheses) of milk from different species (Ballard & Morrow, 2013; Becskei et al., 2020; Jenness, 1980; Recio, de la Fuente, Juárez, & Ramos, 2009; Walstra et al., 2006)

	Human	Cow	Goat	Sheep	Buffalo
Fat	3.4 (3.2 to 3.6)	3.7 (2.5 to 5.5)	4.7 (3.0 to 7.8)	7.1 (5.1 to 8.7)	6.0 (4.3 to 7.2)
Protein	1.2 (0.6 to 1.4)	3.4 (2.3 to 4.4)	3.6 (2.9 to 5.0)	5.7 (4.8 to 6.6)	4.6 (4.1 to 5.6)
Lactose	7.2 (6.7 to 7.8)	4.8 (3.8 to 5.3)	4.9 (1.0 to 6.3)	4.6 (4.1 to 5.0)	5.4 (5.1 to 5.6)

four heat-stable proteins: α_{s1}-, α_{s2}-, β-, and κ-casein) are present in form of large colloidal particles, known as casein micelles (40 to 600 nm diameter with an average of 5,000 casein molecules/micelle). Casein micelles precipitate either at pH 4.6 or by action of rennet chymosin on κ-casein. Caseins are susceptible to proteolysis due to their open structure (Fox & Kelly, 2006). On the other hand, whey proteins (β-lactoglobulin, α-lactalbumin, blood serum albumin, and immunoglobulins) are globular, heat-sensitive, soluble at pH 4.6, and very resistant to chymosin and proteolysis. In addition, nonprotein nitrogenous compounds represent 5% of total nitrogen in fresh milk, comprising intermediate products of the animal's protein metabolism (e.g., ammonia, urea, creatine, creatinine, and uric acid), amino acids and their derivatives, as well as small peptides that may serve as essential nutrients for certain bacteria (Croguennec, Jeantet, & Shuck, 2016).

Milk additionally contains indigenous enzymes at trace levels, including proteinases, of which the trypsin-like endopeptidases plasmin (alkaline proteinase) and cathepsin D (acid proteinase) are the ones most relevant for this review. Plasmin is highly heat-resistant and contributes to proteolysis in cheese during ripening. Cathepsin D is less heat-resistant than plasmin; due to its low optimum pH (4.0), it displays a reduced activity in milk but causes proteolysis in cheese (Walstra et al., 2006).

In Europe and North America, the consumption of processed dairy products is greater than that of fresh dairy products. Furthermore, an increase of cheese consumption in those countries is expected for the next decade (OECD & FAO, 2020). Fermentation was a key process for food preservation in ancient times. Dairy products were central in Neolithic food cultures across much of the Old World, and it is likely that milk was often fermented to obtain a safer and more digestible product while avoiding seasonal or logistic fluctuations in the availability of fresh milk. Although it was previously assumed that food fermentation began with agriculture, it is now assumed that storage was and is widely practiced by nonsedentary foragers in order to have portable protein-rich foods at their disposal during travels (Sibbesson, 2019).

Due to its wide range of nutrients, which allow the growth of many spoilage and pathogenic microorganisms, milk is rarely consumed in its raw state. Microbial conversion of lactose is the basis for fermented milks. Microorganisms with lactase activity, such as LAB, metabolize lactose into glucose and galactose, which are degraded to lactic acid. LAB can produce 1% to 2% of lactic acid leading to milk acidification (pH 4.0 to 4.6) that destabilizes dispersed elements and controls bacterial growth (Kelly & Fox, 2012).

Yogurt is obtained from pasteurized milk inoculated with starter cultures containing *Streptococcus salivarius* spp. *thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) (Hill, Ross, Arendt, & Stanton, 2017). The Codex Alimentarius Commission (CODEX STAN 243–2003) has established the sum of the specific microorganisms constituting the starter culture in the final product at $\geq 10^7$ colony forming units per gram (CFU/g) (Commission, 2011). *Lb. bulgaricus* is required for acid production, whereas *S. thermophilus* is responsible for the flavor and texture of yogurt: the two bacteria have a synergistic relationship. After fermentation, yogurt is refrigerated to decelerate microbial metabolism and delay excessive microbial acidification or proteolysis (Walstra et al., 2006).

Kefir, on the other hand, is a creamy, aromatic, carbonated acid-alcohol milk beverage (0.7% to 1% lactic acid, pH 4.6) of Eastern European origin. It is prepared by adding "kefir grains" (composed of LAB, acetic acid bacteria, and yeast in a polysaccharide matrix of semihard granules) to milk and incubating for 24 hr at 25 ˚C (Guzel-Seydim, Kok-Tas, Greene, & Seydim, 2011). Volatile and nonvolatile compounds generated upon fermentation via lipolysis, glycolysis, and proteolysis provide its characteristic flavor. After fermentation, grains are separated and kefir is refrigerated to attain a shelf-life of 2 to 3 weeks (Farag, Jomaa, El-Wahed, & El-Seedi, 2020).

Cheese can be defined as the curd of milk that has been coagulated and separated from whey. Basically, in the cheese manufacturing process, water and whey are removed from milk, and casein and fat are concentrated. Figure 3 shows the basic process for cheese production (Walstra et al., 2006), including the microbiota associated with each step. In brief, the steps involved are the following:

1. The clotting of milk, consisting of the precipitation of casein micelles by acidification (acid coagulation) and/or enzymatically (rennet coagulation), leading to gel formation.
2. Removal of the whey: the separation of curd and whey is achieved by cutting and stirring, and is facilitated by the spontaneous syneresis of the formed gel. Soluble compounds, including whey proteins, small peptides, and most of the lactose, are squeezed out and excluded from cheese. However, certain proteases, such as plasmin and cathepsin D, tend to adsorb onto micelles, which are present during ripening, thereby facilitating amino acid availability.
3. Production of lactic acid by LAB before and/or after steps 1 and 2. After these three steps, a fresh cheese is obtained. For a typical ripened cheese, the following two additional steps are required.

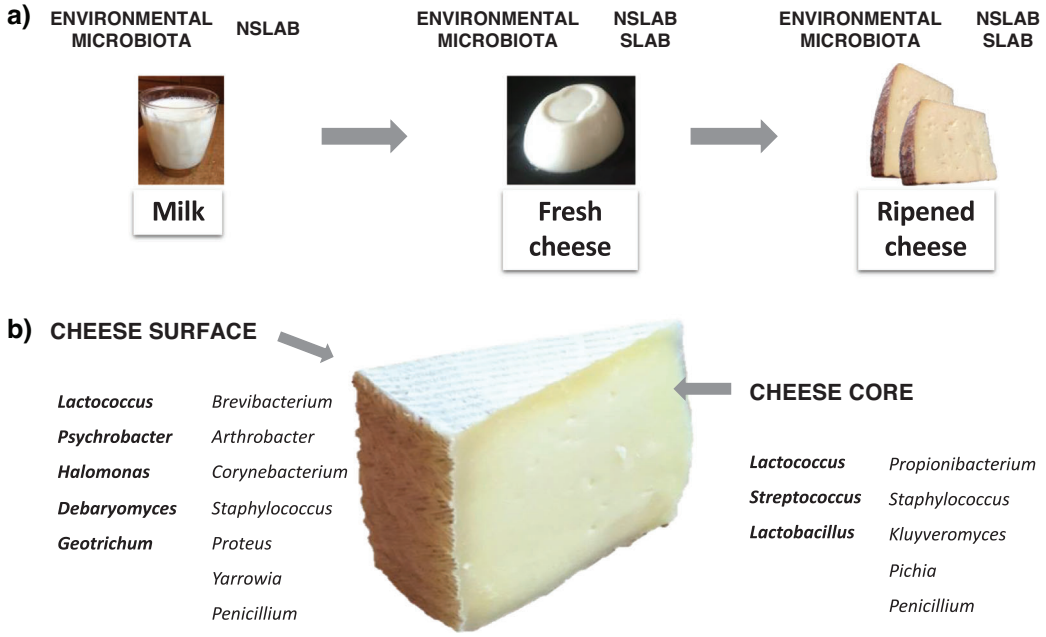

FIGURE 3 Cheese microbiota. (A) Source of histamine-producing microbiota in cheese making. (B) Microorganisms present in cheese surface versus core: those able to synthesize histamine are highlighted in bold

4. Curd fusion, assisted by pressing. A rind can be formed, shielding the interior of the cheese, which contributes to the limitation of oxygen and water transfer for microbial growth.
5. Ripening or curing: a biochemical process determined by a number of factors (Kelly & Fox, 2012), such as endogenous milk enzymes (e.g., plasmin or lipoprotein lipase), starter and nonstarter LAB, and their enzymes, thoroughly active secondary microbiota, which secrete proteases and lipases (e.g., *Penicillium roqueforti* in blue cheeses or *Leuconostoc* spp. in Dutch-type cheeses), and storage conditions (e.g., temperature, time, and humidity).

Salting (usually after step 2) is another key step designed to modify organoleptic characteristics and improve cheese preservation (by selecting growing microbiota). It involves the direct addition of salt crystals (in curd or rubbed onto surface) and/or immersion in a concentrated brine, in order to achieve a salt-in-water concentration ranging from 1% NaCl in cottage cheese up to 6% NaCl in Pecorino Romano cheese (Walstra et al., 2006). Further optional process steps can be mentioned, such as milk pasteurization (prior to step 1) with the purpose of inactivating pathogenic bacteria as well as microorganisms and enzymes that could be detrimental to ripening; and/or addition of microbial cultures (after steps 1 and/or 2), especially highly selected defined starters of LAB, and other microorganisms that are specific for certain cheese varieties.

Modifications in these steps allow for the achievement of more than 1,400 cheese varieties worldwide, with different shapes, flavors, and textures (Kelly & Fox, 2012). During ripening, which can take from 2 weeks up to more than 2 years, three major biochemical reactions take place (Croguennec et al., 2016): (1) fermentation of residual lactose and degradation of lactate to ethanol, acetaldehyde, CO_2, acetic acid, or propionic acid; (2) hydrolysis of lipids into fatty acids, and of proteins into peptides and amino acids, respectively; and (3) flavor: the production of aroma by the degradation of fatty acids to methyl ketones, esters or lactones, and of amino acids to aldehydes, alcohols, acids, amines, phenolic compounds, indole, or NH_3.

Cheese can be considered a solid-like system in which bacteria are immobilized and molecules do not diffuse easily (Floury, Jeanson, Aly, & Lortal, 2010; Walstra et al., 2006). Therefore, microbial growth conditions fluctuate and vary as a function of time and localization in cheese. After production of lactic acid, bacterial metabolism and proteolysis create NH_3, which increases pH (Kelly & Fox, 2012). Water evaporation decreases water activity of cheese and facilitates the formation of rind around the cheese, thereby preventing microbial contamination and limiting oxygen diffusion. Oxygen is rapidly used by starter bacteria, favoring the creation of anaerobic conditions inside the cheese. All these physicochemical changes modify the environmental conditions for bacterial development, thereby promoting a dynamic microbiota during cheese ripening.

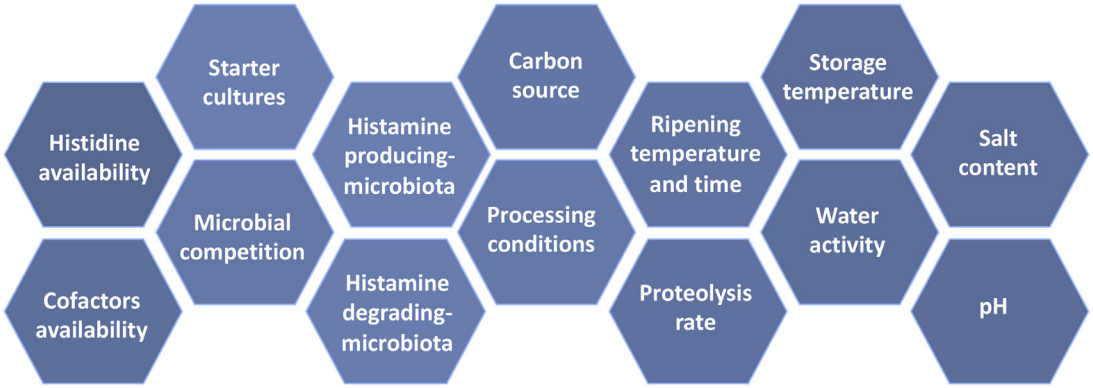

FIGURE 4 Factors related to histamine production in dairy products, including availability of precursors, environmental conditions, and microbiological factors. All these factors should be carefully controlled in dairy products to avoid histamine accumulation

3.2 | Microbiota in dairy food

The detection of the main agents responsible for histamine production should be regarded as an important objective for dairy industries in order to avoid harmful outbreaks. Deciphering the microbiota present in dairy food can be regarded as a first step to elucidate which particular microorganisms are responsible for histamine production. Figure 3 displays the main microbiota involved in the cheese-making process from raw milk to ripened cheese, highlighting the final histamine producers both in cheese surface and core. The formation of this BA by histamine-producing microbiota is modulated by a series of factors that are detailed in Figure 4. In cheese, factors, such as the type of starter cultures, salt content, ripening, and storage temperatures and times, among others, may influence the production of histamine and the amounts of this BA in cheese.

The microbiota of raw milk is mainly composed of LAB (starter and nonstarter), environmental microbiota or contaminants, putative spoilage bacteria, mostly stemming from the teat skin, but also from the farm environment, hygienic practices, or milking and storage equipment (Figure 3) (Jonnala, McSweeney, Sheehan, & Cotter, 2018; Irlinger, Layec, Helinck, & Dugat-Bony, 2015; Odeyemi, Alegbeley, Strateva, & Stratev, 2020). The composition of milk microbiota is diverse, with a high abundance of LAB, and differs depending on the milk's origin: cow, goat, sheep, or buffalo milk (Agrimonti, Bottari, Sardaro, & Marmiroli, 2019; Quigley et al., 2013; Tilocca et al., 2020). In Regulation (EC) No 853/2004, the European Union established the total bacterial plate count limit in raw cow's milk at $\leq 10^5$ colony forming units per milliliter (CFU/mL) at 30 °C, although this limit is allowed to increase to $\leq 1.5 \times 10^6$ CFU/mL for milk from other species (European Parliament, 2004). In general, bacteria present in cooled raw milk include gram-positive species, such as spore-forming bacteria (*Clostridium, Bacillus*), nonstarter LAB (*Lactobacillus* [*Lb.*], *Lactococcus* [*Lc.*], *Streptococcus, Leuconostoc*, and *Pediococcus*), and others (*Corynebacterium, Microbacterium*, and *Staphylococcus*). Gram-negative bacteria are also present in cooled raw milk, usually as environmental or contaminant microbiota: the *Enterobacteriaceae* family and others (*Pseudomonas, Aeromonas, Alcaligenes, Achromobacter, Acinetobacter, Flavobacterium*, and *Chryseobacterium*) (Odeyemi et al., 2020; Settanni & Moschetti, 2010). The *Pseudomonads* family has been reported to be the predominant spoilage bacteria found in cooled raw milk, reaching 70% to 90% of the total microbial load (Odeyemi et al., 2020). Pathogenic foodborne bacteria, such as *Listeria, Campylobacter, Yersinia, Mycobacterium, Escherichia, Salmonella*, *Coxiella*, and *Staphylococcus*, have also been found in raw milk (Agrimonti et al., 2019; Tilocca et al., 2020). The yeasts most commonly present in raw milk are *Kluyveromyces, Yarrowia, Geotrichum, Candida, Debaryomyces*, and *Pichia* (Frohlich-Wyder, Arias-Roth, & Jakob, 2019; Irlinger et al., 2015). Bacteriophages or phages are viruses capable of infecting bacteria, and they can achieve entry into dairy products through raw milk (Fernandez et al., 2017). *Lc. lactis, Lb. helveticus, Lb. delbrueckii, Lactiplantibacillus plantarum* (formerly *Lb. plantarum*), *Lb. acidophilus, Lacticaseibacillus casei* (formerly *Lb. casei*), *L. paracasei*, *S. thermophilus*, and *Leuconostoc* spp. can be infected by phages (del Rio et al., 2007; Marco, Moineau, & Quiberoni, 2012; Muhammed, Krych, Nielsen, & Vogensen, 2017).

Regarding yogurt, in addition to the aforementioned starter cultures *Lb. bulgaricus* and *S. thermophilus* used in adequate proportions to perform lactic fermentation, it can contain other beneficial or deleterious microorganisms. Probiotic bacteria, such as *Bifidobacterium* spp. and *Lactobacillus* spp., which are not part of the starter

cultures, can be found in probiotic fermented milks, namely bioyogurts (Aryana & Olson, 2017; Hill et al., 2017). Flavor can be improved by adding further cultures as *S. diacetylactis* or *Leuconostoc* spp. Phages active against *S. thermophilus* or *Lb. bulgaricus*, and yeast, such as *Torulopsis*, have also been reported for yogurt (Aryana & Olson, 2017). Additionally, viable *L. monocytogenes* and *S. enterica* cells have been detected in certain yogurts, as well as further pathogens, including *Y. enterocolitica, M. tuberculosis*, and *B. cereus*. However, the true hygienic state of yogurt has not been defined by the presence of pathogenic species, but has been suggested to be controlled by monitoring the *Enterobacteriaceae* family (Hervert, Martin, Boor, & Wiedmann, 2017). Other episodes of food poisoning involving yogurts have been caused by *E. coli* O157:H7, *C. botulinum*, and *S. typhimurium* (Aryana & Olson, 2017).

The microbiota of kefir and kefir grains comprises species of bacteria (*Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Acetobacter*, and *Enterococcus*) and yeasts (*Saccharomyces, Candida, Kluyveromyces, Zygosaccharomyces, Debaryomyces, Issatchenkia, Pichia*, and *Torulopsis*) (Guzel-Seydim et al., 2011; Singh & Shah, 2017; Tang et al., 2020). It should be noted that the Codex Alimentarius Commission (CODEX STAN 243–2003) specifically mentions the presence of *Lentilactobacillus kefiri* (formerly *Lb. kefiri*) and the yeasts *K. marxianus, S. unisporus, S. cerevisiae*, and *S. exiguous*. It also establishes at $\geq 10^7$ CFU/g the sum of the specific microorganisms constituting the starter culture in the final product, and the sum of yeasts at $\geq 10^4$ CFU/g (Commission, 2011). Some species of probiotics, such as *B. lactis, Lb. acidophilus*, or *L. rhamnosus* (formerly *Lb. rhamnosus*), can also be added to kefir (Aryana & Olson, 2017).

The microbiota present in cheese is key for its organoleptic and physicochemical properties. Cheese microbiota varies depending on starter and nonstarter cultures, and changes over time (Figure 3). Bacterial communities present in cheese display an immense diversity, greater than that of fungal communities, depending on cheese variety and manufacturing process (Afshari, Pillidge, Dias, Osborn, & Gill, 2020; Rezac, Kok, Heermann, & Hutkins, 2018). LAB are definitely the most important microorganisms present in cheese microbiota in view of their involvement in the fermentation and maturation processes (Settanni & Moschetti, 2010). Starter LAB (SLAB), including *Lactococcus*, *Streptococcus* and *Leuconostoc*, contribute to the initial ripening process, due to the fermentation of lactose. Thus, SLAB are involved in coagulation of milk and acid development. During cheese manufacture, the SLAB population comprises up to 10^8 to 10^9 CFU/g. The most common mesophilic SLAB is *Lc. lactis*, although strains of *Leuconostoc* spp. are also used; whereas thermophilic SLAB usually consist of strains of *S. thermophilus, Lb. delbrueckii, and Lb. helveticus* (Blaya, Barzideh, & LaPointe, 2018; Settanni & Moschetti, 2010). However, the stresses and harsh conditions (high salt, low pH, low sugar availability, low moisture…) that appear in the cheese matrix as a consequence of the cheese-making process lead to a reduction in the population of SLAB due to autolysis (Gatti, Bottari, Lazzi, Neviani, & Mucchetti, 2014; Møller, Christensen, & Rattray, 2021). Instead, adventitious nonstarter LAB (NSLAB), which mainly stem from raw milk, need to be present because they contribute to the development of desirable flavor. NSLAB can grow and survive in more adverse environmental conditions, such as pH as low as 5.0 or energy depletion (Barbieri et al., 2019). For that reason, an initial population of 10^2 to 10^3 CFU/g of NSLAB is found in cheese, but it can reach up to 10^9 CFU/g during the onset of ripening (Blaya et al., 2018; Gatti et al., 2014). Among the NSLAB *Lactobacillus* strains, the obligate homofermentative species *Companilactobacillus farciminis* (formerly *Lb. farciminis*), the facultative heterofermentative species *L. rhamnosus, L. paracasei, L. casei*, *L. plantarum, L. pentosus* (formerly *Lb. pentosus*), and *Latilactobacillus curvatus* (formerly *Lb. curvatus*), and the obligate heterofermentative species *Limosilactobacillus fermentum* (formerly *Lb. fermentum*), *L. buchneri* (formerly *Lb. buchneri*), *L. parabuchneri* (formerly *Lb. parabuchneri*), and *Levilactobacillus brevis* (formerly *Lb. brevis*), are considered to be the main NSLAB found in cheese. Other NSLAB found in cheese are *Pediococcus* species (*P. acidilactici* and *P. pentosaceus), Enterococcus* species (*E. durans, E. faecalis*, and *E. faecium*), and *Leuconostoc* spp. (Settanni & Moschetti, 2010). Other microorganisms as enterococci, micrococci, and yeasts are likewise important in cheese microbiota for maturation (Button & Dutton, 2012; Gardini et al., 2006; Gobbetti, Minervini, Pontonio, Di Cagno, & De Angelis, 2016). For instance, *B. linens* or *S. equorum* contribute to the development of flavor, aroma, and color in cheese; even *Propionibacterium freundenreichii* causes the typical holes in Swiss cheeses by producing CO_2 during fermentation (Button & Dutton, 2012; Jonnala, McSweeney, Sheehan, & Cotter, 2018). On the other hand, coliforms are considered indicative of nonhygienic conditions and thus regarded as undesirable contaminants; *Pseudomonas* spp., *Serratia* spp., and *Kluyvera* spp. can reduce the sensory quality of cheese (Coton et al., 2012). Foodborne pathogens, such as *L. monocytogenes, Salmonella* spp., *E. coli*, and *Campylobacter* spp., have been detected in soft cheese samples (Cremonesi et al., 2016). Cheese can also contain spoilage bacteria: in fact, the *Clostridium* spore might survive the entire cheese production process (Odeyemi et al., 2020). Bacteriophages active against *S. thermophilus* or *Lc. lactis*, for instance, are also present in cheese, thus helping to modulate the bacterial community (Gobbetti et al., 2016). Yeasts found in cheese

participate in the ripening process, and contribute to its texture and organoleptic properties. *Debaryomyces*, *Yarrowia, Candida, Geotrichum, Kluyveromyces, Saccharomyces*, and *Pichia* are the most commonly described genera (Gardini et al., 2006; van den Tempel & Jakobsen, 2000). Some of them, like *D. hansenii* and *Y. lipolytica*, can be used as starter cultures due to their capacity to grow under hostile conditions and to improve the flavor and quality of cheese (Ferreira & Viljoen, 2003). *Penicillium*, *Scopulariopsis*, and *Fusarium* are important filamentous fungi found in cheese (Irlinger et al., 2015). Opportunistic pathogenic yeasts, mainly the *Candida* species, can also be present in cheese (Frohlich-Wyder et al., 2019). With regard to cheese localization, Figure 3 shows that the microbiota in the cheese rind differs from the microorganisms present in the core. Ripening bacteria (*Brevibacterium, Arthrobacter*, and *Corynebacterium)* and psychrophilic and halophilic bacteria (*Psychrobacter*, *Halomonas*, and *Proteus)* are mostly present on the cheese surface because they cope with the deacidification process. However, LAB are usually found in the cheese core, as well as anaerobic bacteria, such as *Propionibacterium* that grow inside the wheel of cheese (Button & Dutton, 2012; Coton et al., 2012; Frohlich-Wyder et al., 2019). Only yeasts able to ferment carbohydrates, such as *K. marxianus*, *K. lactis*, and *P. fermentans*, can survive in the cheese core, while the yeasts predominant on the surface are acid and salt tolerant: the most abundant ones are *D. hansenii, Y lipolytica*, and *G. candidum* (Frohlich-Wyder et al., 2019). In relation to molds, spores of *P. camemberti* are inoculated into milk of Brie and Camembert cheeses to develop bloomy rind, while *P. roqueforti* grows in the core of blue cheese, producing its blue pigment during sporulation (Button & Dutton, 2012; Jonnala et al., 2018).

4 | ENVIRONMENTAL CONDITIONS APPLIED TO DAIRY FOODS MAY INFLUENCE HISTAMINE ACCUMULATION

The amount of histamine in dairy food, and even the presence or absence thereof, is determined by a number of factors, shown in Figure 4, which include available precursors or cofactors, environmental conditions, such as acidic pH, ripening and storage temperatures, water activity, and salt concentration (Costa et al., 2018). Furthermore, microbiological factors, such as microbial competition or the presence of microbiota capable of degrading histamine, could also contribute to modify the amount of histamine present in dairy food (Coton et al., 2012). All these factors should be carefully controlled in order to obtain histamine-free dairy products.

The availability of histidine, the precursor amino acid for the synthesis of histamine during the ripening of cheese, is a limiting factor on histamine formation (Linares et al., 2011). Although histidine can be naturally present in milk in a free state, the proteolysis of casein or other milk proteins is the main cause of the presence of this substrate amino acid in milk and dairy products (Benkerroum, 2016). Since the rate of proteolysis increases with ripening time, long-ripened cheeses present higher concentrations of histamine. Ripening time also contributes to the proteolysis rate, so that long-ripened cheeses have a higher proteolysis rate and thus a higher level of histamine (Fernandez, del Rio, Linares, Martin, & Alvarez, 2006). The addition of exogenous proteinases to milk with the aim of accelerating cheese ripening significantly increases the amount of histamine in a wide variety of cheeses (Linares et al., 2011).

NSLAB are known to survive and grow under very harsh conditions, such as an acidic pH. Since amino acid decarboxylases in bacteria are known to contribute to their adaptation to acidic environment (because the decarboxylation process results in an increase of environmental pH), an acidic pH in the final dairy product could also promote the synthesis of histamine (Barbieri et al., 2019; Linares et al., 2012). The HDC enzyme of *S. thermophilus* seems to be much more active at pH 4.5 than at pH 8 (Tabanelli, Torriani, Rossi, Rizzotti, & Gardini, 2012). It has also been reported that acidic pH may induce structural changes in the HDC from *Lactobacillus* sp. 30a (ATCC 33222) required for the protein to be active (Schelp, Worley, Monzingo, Ernst, & Robertus, 2001). At pH 8.0, however, histamine accumulation was also observed in a culture of *Tetragenococcus halophilus* (Satomi, Furushita, Oikawa, Yoshikawa-Takahashi, & Yano, 2008).

Sodium chloride concentrations higher than 5% (w/v) seem to notably decrease the amount of histamine, probably due to an inhibitory effect on the growth rate of histamine producers (Tabanelli et al., 2012). However, the halophilic bacterium *Tetragenococcus* can produce histamine even at up to 20% (w/v) NaCl (Kimura, Konagaya, & Fujii, 2001; Satomi et al., 2008).

The carbon source could also be a factor that influences bacterial histamine formation, depending on the histamine producer. High concentrations of glucose or lactose have been reported to inhibit the production of histamine, although a recent study showed no effect of the presence of up to 2% glucose on the synthesis of histamine for *L. parabuchneri* and *L. paracasei*, but completely inhibiting histamine formation by *P. pentosaceus* (Calles-Enriquez et al., 2010; Møller, Ucok, & Rattray, 2020).

High storage temperatures and prolonged ripening time increase the microbial production of histamine. For instance, the concentration of histamine was 10-fold higher at 42 °C than at 4 °C in a culture of *S. ther-*

mophilus grown in milk after 24 hr, due to the activity of the enzyme rather than to a variation in its gene expression (Calles-Enriquez et al., 2010). *L. parabuchneri*, isolated from cheese, has also been reported to grow and produce histamine at refrigeration temperatures (4 to 8 °C), but this characteristic seems to be strain-dependent (Díaz et al., 2018).

On the other hand, as mentioned above, the *hdc* genes in some bacteria, such as *T. muriaticus*, *T. halophilus*, *Oenococcus oeni*, and *L. hilgardii* (formerly *Lb. hilgardii*), are codified in unstable plasmids (Lucas, Claisse, & Lonvaud-Funel, 2008; Lucas, Wolken, Claisse, Lolkema, & Lonvaud-Funel, 2005; Satomi et al., 2008). In these cases, the instability of the plasmid depends on the bacterial culture conditions, since a poor and acidic medium seems to favor the maintenance of the plasmid and thus the expression of the gene.

5 | TECHNIQUES FOR THE DETECTION OF HISTAMINE-PRODUCING MICROBIOTA

A series of techniques for the study of microbial communities in food have been developed in recent years. High-throughput sequencing applications have provided detailed knowledge concerning food-associated microbiota and microbiomes. Not only metagenomics and metatranscriptomics, but also metaproteomics and metabolomics have been thoroughly exploited to decipher the composition and functionality of microbiota, thereby contributing to the improvement of food quality and safety. The expansion of our knowledge of food-associated microbiota by meta-omics technologies would allow us to control their main drivers along with the influence of environmental or technological factors over them. Monitoring food spoilage organisms or even pathogens could also help to improve hygienic practices in food production plants (De Filippis, Parente, & Ercolini, 2018). This multiomics approach applied to cheese has been recently called "Cheesomics", focusing on the ripening process and promoting the identification of biomarkers and bioactive metabolites to improve the attributes of cheese (Afshari et al., 2020). In addition, if we learn to consider the core microbiota of cheese as a superorganism comprising all microbial metabolisms and interactions among individual microbes, we can gain a better understanding of the complex metabolic network of dairy products on the whole (Gobbetti et al., 2016).

Techniques aimed at detecting a putative histamine intoxication in food are currently based on direct analysis of the metabolite, for example, on the detection and quantification of histamine. Nevertheless, it is interesting to highlight the interest in detecting and quantifying the microbiota responsible for synthesizing the metabolite, since putative outbreaks can thereby be prevented or detected even before they cause harmful effects to human health.

For that reason, this review focuses on describing techniques designed to detect histamine-producing bacteria (HPB), which can be classified into three types: culture-based, electroanalytical, and molecular methods. The advantages and disadvantages of these techniques are summarized in Figure 5.

5.1 | Culture-based methods

Techniques using chromogenic agar or broth media were implemented in the 80s and 90s as useful tools for the identification of HPB. Several methods were developed to detect histamine accumulation during the growth of bacteria, which is evident in a change of color in the growth medium as a consequence of change in pH. Møllert's group and, many years later, Niven and collaborators developed chromogenic agar media supplemented with L-histidine using bromocresol purple to reveal the change in pH during histamine production (Møller, 1954; Niven, Jeffrey, & Corlett, 1981). Nivent's agar medium was later modified to differentially support bacterial growth (Chen, Wei, Koburguer, & Marshall, 1989) and to be used with increased selectivity for the enumeration of HPB in fish products (Mavromatis & Quantick, 2002). That medium has also been used as a basis for the development of other media adapted to cheese (Joosten & Northolt, 1989) or meat (Maijala, 1993). A liquid decarboxylase medium using bromocresol green and chlorophenol red was also described by Yamani and Untermann (1985) for use in pure or mixed cultures, avoiding solid media that could prevent the growth of certain HPB. A leucocrystal violet detection method was also developed to detect high histamine-producing lactobacilli in cheese (Sumner & Taylor, 1989). A comparative analysis of the composition of some of these published decarboxylase media was reported in Bover-Cid and Holzapfel (1999). Also, an improved decarboxylase medium was proposed by these authors, which proved itself sensitive and suitable for screening the ability not only of LAB but also enterobacteria to produce different BAs. The main problem of these indicator media is the occurrence of false positives, caused by the simultaneous production of alkaline metabolites that lead to a pH-related color change (Bover-Cid & Holzapfel, 1999). For instance, a *P. pentosaceus* isolate from cheese was able to produce ornithine from arginine causing the release of ammonium ion to the medium, which raised the pH and rendered a false positive

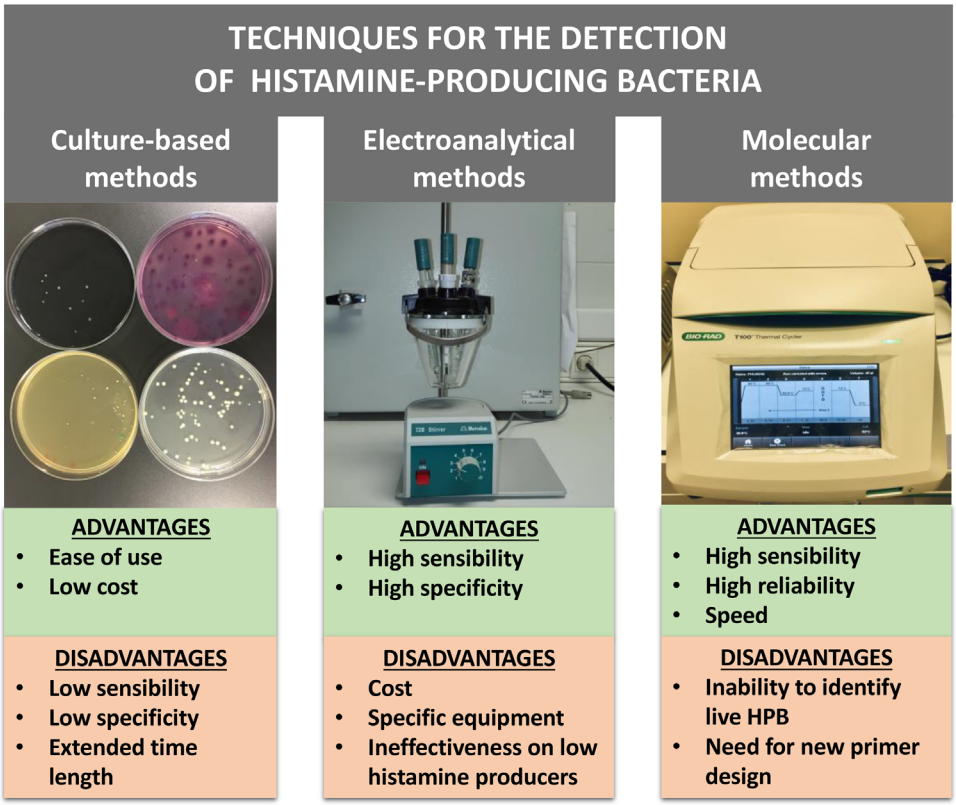

FIGURE 5 Techniques for the detection of histamine-producing bacteria in dairy products. Their main advantages and disadvantages are listed

result when tested in the indicator medium (Møller et al., 2020).

As shown in Figure 5, ease of use, availability, and low cost are some of the advantages of culture-based methods, whereas false positives, the great amount of time required, and the inability of growth of some HPB due to the conditions of the chromogenic medium are important disadvantages. Furthermore, such methods are not able to detect low histamine producers: thus, other methods might be required to confirm the detection of HPB (Bjornsdottir-Butler, Jones, Benner, & Burkhardt, 2011; Chen et al., 1989; Landete, de Las Rivas, Marcobal, & Munoz, 2007).

In order to solve the time length problem involved in the methods exposed above, a rapid technique has been recently described involving a two-layer membrane filtration assay and a subsequent bacterial culture on agar plates with histidine and bromothymol blue as pH indicator, requiring only 5 hr to analyze HBP in liquid samples as well as in seafood (Tao, Sato, Abe, Yamaguchi, & Nakano, 2009).

5.2 | Electroanalytical methods

Many methods based on measurements of potential (volts) and/or current (amperes) have been described in the literature to quantify histamine in food, as reviewed in Yadav, Nair, Sai, and Satija (2019). However, only few studies have applied electroanalytical techniques to reveal HPB, which are difficult to detect since they constitute a minority among the present microbiota. In the late 80s, Klausen and Huss (1987) developed a potentiometric method for the detection of HPB by measuring conductance produced by the histidine-decarboxylase activity of HPB using a histidine-decarboxylase medium: the method was validated in spoiled mackerel. It seems to be highly effective in the detection of high-histamine producers, but is ineffective with low HPB (Figure 5).

Recently, Trevisani et al. (2019) reported an enzyme-based amperometric biosensor designed to detect histamine and HPB in tuna, based on measurements of HDC activity in a histidine decarboxylase broth. However, to our knowledge, no electroanalytical methods for the detection of histamine-producing microbiota in dairy foods have yet been reported.

5.3 | Molecular methods

Culture-based as well as potentiometric techniques are nowadays being substituted by modern molecular meth-

ods that enhance sensibility and reliability, even involving the implementation of nucleic acid hybridization techniques. Molecular methods for the detection of BA-producing bacteria in food were reviewed some years ago (Landete et al., 2007), but, from our point of view, an update of that review, focusing on histamine, is required.

Molecular methods are based on the polymerase chain reaction (PCR), a useful and rapid technique that allows the exponential amplification (the increase of number of copies) of target DNA fragments or amplicons from a template by using a DNA polymerase enzyme and a series of cycles of different temperature. To perform this reaction, two short single-strand DNA fragments called oligonucleotides or primers are required. The primers are composed of the complementary sequence of the ends of target DNA (Erlich, 1989). These methods are rapid, specific, and sensitive, although they are unable to distinguish whether the HPB are dead, alive, or even viable but not cultivable (Figure 5) (Landete et al., 2007).

PCR methods to detect HPB are commonly based on the amplification of a fragment of the histidine decarboxylase (*hdc*) gene, sometimes named *hdcA* (Landete et al., 2007; Linares et al., 2011). Bacteria capable of producing histamine exhibit the *hdc* gene in the genome, which is mainly located in the chromosome, but can sometimes be found in an unstable plasmid (Landete et al., 2008). Figure 6 compiles the routes involved in the bacterial histamine metabolism, depicting *hdcA* and other genes involved in the production of histamine, such as *hdcC* (codifying for a histidine/histamine antiporter), *hdcB* (involved in HDC maturation), or *hisS* (codifying for a histidyl-t-RNA synthase like protein) that are usually present in gram-positive bacteria, constituting the typical so-called *hdc* cluster (Benkerroum, 2016; Linares et al., 2011). The genomic structure of the gene responsible for the synthesis of histamine in yeasts or molds has not yet been described.

Two HDC enzyme families have been identified with completely different sequential and biochemical characteristics: gram-positive bacteria, in which the enzyme requires a pyruvoyl moiety, and gram-negative bacteria, which contain pyridoxal phosphate-dependent HDC enzymes (Landete et al., 2008). Nucleotide sequences of enzymes from one or the other group share high similarity (Wuthrich et al., 2017); the nucleotide sequence alignment of the *hdc* gene in gram-positive bacteria was published some years ago (Diaz et al., 2016a). To our knowledge, no genomic studies regarding the putative *hdc* gene in yeasts have been published to date. Taking advantage of this high similarity of the nucleotide sequence of *hdc* genes among groups of bacteria, the design of primers that align in conserved regions within the *hdc* gene would allow for the amplification of the gene from whichever bacteria are present in food. Additionally, to better amplify the same gene from different microorganisms, degenerated primers (a mixture of similar but not identical oligonucleotides) could also be used.

For these reasons, different pairs of primers for the amplification of the *hdc* gene in food through a unique PCR reaction using only a pair of primers to detect each microorganism individually (uniplex PCR) are reported in the literature. However, only few of those studies refer to dairy products. Primers designed to amplify the *hdc* gene of bacteria from dairy products are detailed in Table 2. Specifically, STDEC-F and STDEC-R primers were designed to detect histamine-producing *S. thermophilus* (Rossi et al., 2011) and degenerated HIS1-F and HIS1-R primers were used in cheese to detect gram-positive bacteria (de Las Rivas, Marcobal, Carrascosa, & Munoz, 2006). Some authors adapted the pair of primers HDC3 and HDC4 to detect gram-positive HPB in cheese or homemade yogurt, which had been initially applied to smoked salmon by Burdychova and Komprda (2007) and Coton and Coton (2005). (Berthoud et al., 2017; Gezginc, Akyol, Kuley, & Ozogul, 2013; O'Sullivan et al., 2015) Primers CL1, CL2, JV16HC, and JV17HC, initially published by Le Jeune, Lonvaud-Funel, ten Brink, Hofstra, and van der Vossen (1995), were used by other authors to highlight LAB containing the *hdc* gene in ripened or artisan cheeses (del Valle, Ginovart, Gordún, & Carbó, 2018; Ladero et al., 2015; Møller et al., 2020). Primers HIS2-F and HIS2-R, initially described by de Las Rivas et al. (2006), were used to detect gram-negative HPB in cheese, although no amplification was obtained in any cheese sample (O'Sullivan et al., 2015). Figure 7 shows the regions of *hdc* genes from the alignment of different bacteria where the primers align. As observed, the high similarity among the *hdc* genes allows a good alignment. It is also noteworthy the great sequence similarity of most primers results in alignments in the same regions.

Several multiplex PCR methods (combining multiple pairs of primers in a single and optimized PCR reaction to detect several microorganisms simultaneously) have been reported to detect BAs in food. Coton and Coton (2005) described a PCR method for the simultaneous detection of histamine- and tyramine-producing gram-positive bacteria using HDC3-HDC4 and TD2-TD5 primers directly on bacterial colonies in a single reaction. Some years later, these authors incorporated other pairs of primers to additionally detect ornithine-producing bacteria from wine and cider (Coton et al., 2010). Another multiplex PCR was published for the simultaneous detection of LAB-producing histamine (primers JV16HC and JV17HC), tyramine (primers P1-rev and P2-for, first described by Lucas and Lonvaud-Funel 2002), and putrescine (primers 3 and 16) in food, specifically in wine and grape must (Marcobal, de las

TABLE 2 List and characteristics of primers aimed to amplify the *hdc* gene of bacteria from dairy products

Primer name	Primer sequence 5′→ 3′	Amplicon size	Microorganisms and references	Dairy product sources
STDEC- FSTDEC-R	GAATTACCGATCTATGATGC ACACCTTTGTTAGCACAAAC	121 bp	*Streptococcus thermophilus* (Rossi et al., 2011)	Grana-type and mozzarella cheeses Traditional yogurts
HIS1-FHIS1-R	GGNATNGTNWSNTAYGAYMGNGCNGA ATNGCDATNGCNSWCCANACNCCRTA	372 bp	*Lactobacillus* sp. 30a (ATCC 33222) and *Lentilactobacillus buchneri* StA2 (de Las Rivas et al., 2006) Other bacterial genera as *Micrococcus, Clostridium, Oenococcus* (de Las Rivas et al., 2006) *Streptococcus thermophilus* (Rossi et al., 2011)	Foodborne bacterial strains
HDC3 HDC4	GATGGTATTGTTTCKTATGA CAAACACCAGCATCTTC	435 to 440 bp	*Lentilactobacillus parabuchneri* (Berthoud et al., 2017), *Lentilactobacillus parabuchneri* DSM 5987 and *Lentilactobacillus parabuchneri* B301 (Diaz, Ladero et al., 2016), *Lentilactobacillus buchneri* DSM 5987, *Lactobacillus* sp. 30a (ATCC 33222), *Latilactobacillus sakei* LTH 2076 and *Lentilactobacillus hilgardii* IOEB 0006 **(E.** Coton & Coton, 2005**),** *Lentilactobacillus buchneri* and *Latilactibacillus* (O'Sullivan et al., 2015), *Latilactibacillus curvatus, Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis* (Burdychova & Komprda, 2007), *Limosilactobacillus vaginalis* (Diaz et al., 2015) *Tetragenococcus muriaticus* LMG 18498 **(E.** Coton & Coton, 2005) *Oenococcus oeni* IOEB 9204 **(E.** Coton & Coton, 2005) *Streptococcus thermophilus* (Gezginc et al., 2013)	Dutch-type semihard, Cabrales, Emmental, Reblochon, Irish Artisanal, Morbier, Pecorino Sardo, Ossau-Iraty, Emmental, Tête de Moine, Mont Soleil, Tilsit, Alpine, and Raclette cheeses Traditional home-made yogurts Foodborne bacterial strains
CL1 CL2	CCWGGWAAWATWGGWAATGGWTA GAWGCWGTWGTCATATTWATTTGWCC	150 bp	*Leuconostoc oenos* IOEB 9203 and *Leuconostoc oenos* IOEB 9204 (Le Jeune et al., 1995) *Lactobacillus* sp. 30a (ATCC 33222) (Le Jeune et al., 1995**),** *Lentilactobacillus buchneri, Levilactobacillus brevis, Lacticaseibacillus casei, Lactiplantibacillus plantarum*, and *Lactobacillus delbrueckii* subsp. *lactis* (del Valle et al., 2018) *Lactococcus* sp. (del Valle et al., 2018)	Ripened raw goat milk cheeses Foodborne bacterial strains

(Continues)

TABLE 2 (Continued)

Primer name	Primer sequence 5′→ 3′	Amplicon size	Microorganisms and references	Dairy product sources
HIS2-F HIS2-R	AAYTSNTTYGAYTTYGARAARGARGT TANGGNSANCCDATCATYTTRTGNCC	531 bp	*Morganella morganii* CECT 173^{T} (de Las Rivas et al., 2006) *Photobacterium phosphoreum* CECT 4192^{T} and *Photobacterium damselae* CECT 626^{T} (de Las Rivas et al., 2006) *Proteus vulgaris* CECT 484^{T} (de Las Rivas et al., 2006) Other bacterial genera as *Enterobacter, Pseudomonas* (de Las Rivas et al., 2006)	Foodborne bacterial strains
JV16HC JV17HC	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	367 bp	*Lactobacillus* sp. 30a (ATCC 33222), *Lentilactobacillus buchneri* StA2 and *Lentilactobacillus hilgardii* BIFI-87 (Marcobal et al., 2005), *Lentilactobacillus buchneri* B301 (Ladero et al., 2015), *Lentilactobacillus buchneri* StA2, *Lentilactobacillus buchneri* NZHD1, *Lentilactobacillus buchneri* NZHD2, *Lentilactobacillus buchneri* NZHD3, *Lentilactobacillus buchneri* NZHD4, *Lentilactobacillus buchneri* NZHD5 and *Lentilactobacillus buchneri* CIVO29 (Le Jeune et al., 1995), *Lentilactobacillus buchneri, Levilactobacillus brevis, Lacticaseibacillus casei, Lactiplantibacillus plantarum*, and *Lactobacillus delbrueckii* subsp. *lactis* (del Valle et al., 2018), *Lentilactobacillus parabuchneri* KUH1, *Lentilactobacillus parabuchneri* KUH2, *Lentilactobacillus parabuchneri* KUH8, and *Lacticaseibacillus paracasei* KUH3 (Møller, Ucok, & Rattray, 2020) *Clostridium perfringens* ATCC 13124 (Le Jeune et al., 1995) *Leuconostoc oenos* IOEB 9203 and *Leuconostoc oenos* IOEB 9204 (Le Jeune et al., 1995) *Staphylococcus* sp. (de Las Rivas et al., 2005) *Streptococcus thermophilus* (Ladero et al., 2015) *Lactococcus* sp. (del Valle et al., 2018)	Danish Gouda-type and artisanal cheeses Foodborne bacterial strains

(Continues)

TABLE 2 (Continued)

Primer name	Primer sequence 5′→ 3′	Amplicon size	Microorganisms and references	Dairy product sources
106 107	AAYTCNTTYGAYTTYGARAARGARG ATNGGNGANCCDATCATYTTRTGNCC	534 bp	*Morganella morganii* CECT 173^{T} (de Las Rivas et al., 2005) *Photobacterium phosphoreum* CECT 4192^{T} (de Las Rivas et al., 2005) *Proteus vulgaris* CECT 484^{T} (de Las Rivas et al., 2005) *Klebsiella planticola* CECT 843 (de Las Rivas et al., 2005)	Foodborne bacterial strains
hdcDG-F hdcDG-R	CCTGGTCAAGGCTATGGTGTATGGTC GGTTTCATCATTGCGTGTGCAAA -	250 bp	*Lentilactobacillus parabuchneri* and *Latilactobacillus sakei/Lentilactobacillus hilgardii* (Diaz, Ladero et al., 2016) *Tetragenococcus halophilus* (Diaz et al., 2016) *Streptococcus thermophilus* (Diaz et al., 2016)	Cabrales, Manchego-type, Idiazabal, Casín, and Gamoneu cheeses
hdc1 hdc2	TTGACCGTATCTCAGTGAGTCCAT ACGGTCATACGAAACAATACCATC	174 bp	*Lentilactobacillus parabuchneri* KUH1, *Lentilactobacillus parabuchneri* KUH2, *Lentilactobacillus parabuchneri* KUH8, and *Lacticaseibacillus paracasei* KUH3 (Møller et al., 2020), *Lentilactobacillus buchneri* B301, *Lentilactobacillus buchneri* B302, *Lentilactobacillus buchneri* B303, *Lentilactobacillus buchneri* DSM 5987, and *Lentilactobacillus hilgardii* 321 (Fernandez et al., 2006) *Enterococcus* 15A (Fernandez et al., 2006) *Oenococcus oeni* 206 and *Oenococcus oeni* 212 (Fernandez et al., 2006) *Pediococcus parvulus* 276 (Fernandez et al., 2006)	Danish Gouda-type and Cabrales cheeses

Y = C or T; K = G or T; R = A or G; S = C or G; W = A or T; M = A or C; D = G, A or T; N = A, C, G or T.
Reference highlighted in bold is the original manuscript that described the primers for the first time.

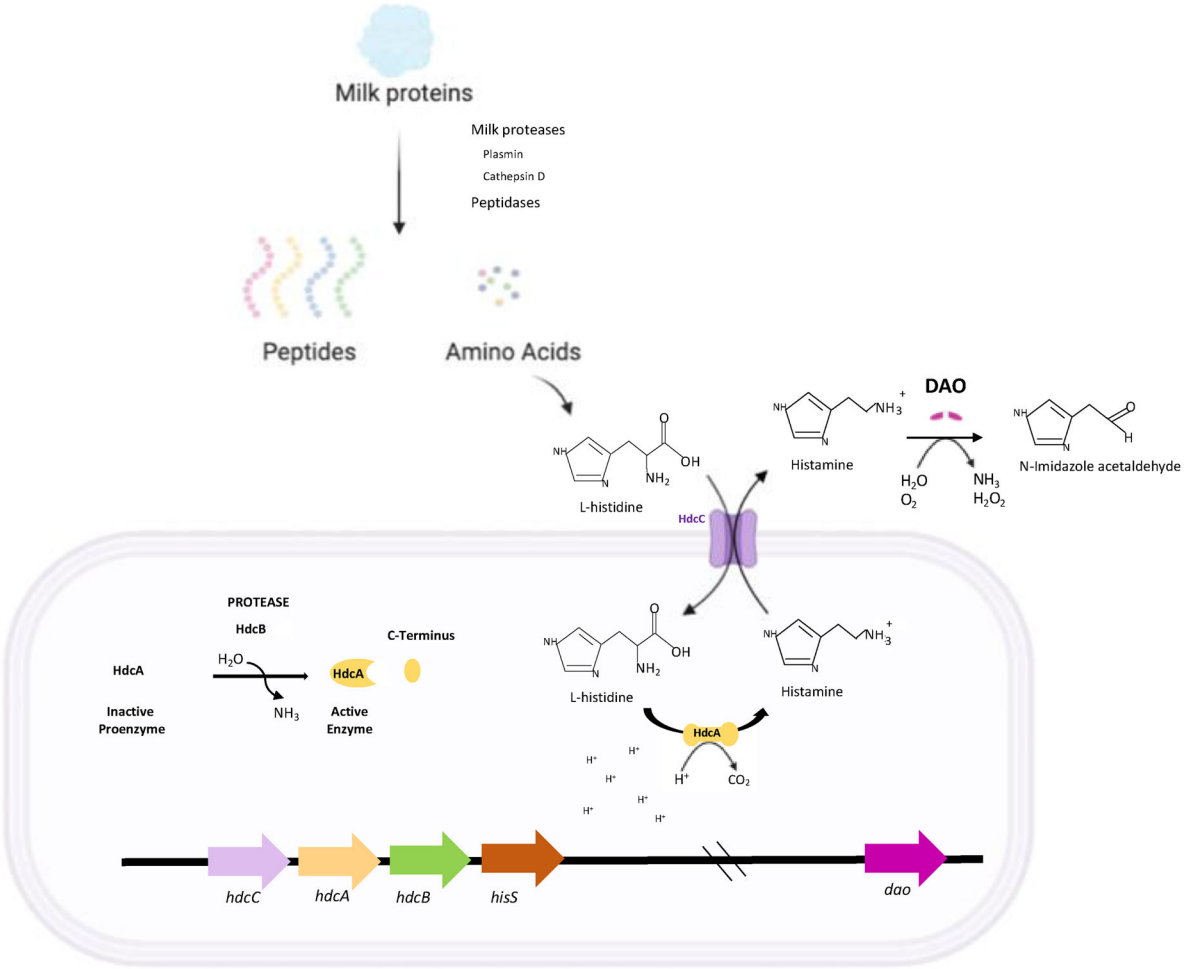

FIGURE 6 Overview of the mechanisms of histamine formation and degradation by microorganisms. The synthesis of histamine is mediated by the enzyme histidine decarboxylase (HDC or HdcA), codified by the *hdc* (or *hdcA*) gene. In some gram-positive bacteria, this gene takes part in the so-called *hdc* cluster, together with genes codifying for a histidine/histamine antiporter (*hdcC*), a histamine decarboxylase enzyme (*hdcA*), an enzyme involved in proenzyme HdcA cleavage and maturation (*hdcB*), and a protein similar to a histidyl tRNA synthetase (*hisS*). The HdcA enzyme is synthesized as a proenzyme, which requires the proteolysis of the C-terminus, mediated by HdcB, to be an active enzyme. In dairy products, breakdown of milk proteins by plasmin, cathepsin D, and other milk proteases and peptidases results in the formation of free peptides and amino acids, such as histidine, precursor of histamine. When the survival mechanism inducing histamine (no sugar available, low pH…) is activated in histamine-producing bacteria, the antiporter HdcC allows histidine to enter the cell in order to be decarboxylated by HdcA, to form histamine, with the consumption of a proton, that contributes to raise pH. When histamine needs to be metabolized, the same antiporter HdcC secretes this metabolite to be degraded by the enzyme DAO. Since a net positive charge is transported out of the cell by the electrogenic antiport, it results in the generation of proton motive force and energy generation (Molenaar, Bosscher, ten Brink, Driessen, & Konings, 1993)

Rivas, Moreno-Arribas, & Munoz, 2005). These pairs of primers, together with an extra pair (106 and 107 primers) aimed to detect harmful gram-negative HPB, were used in an improved multiplex PCR validated with DNA mixtures of several HPB (de Las Rivas, Marcobal, & Munoz, 2005). It is noteworthy that those multiplex PCR methods are mainly applied for the detection of BA-producing bacteria in wine and its derivatives, but not in dairy foods.

Methods that combine PCR with other techniques have also been used to determine HPB in food. For instance, a PCR-denaturing gradient gel electrophoresis (PCR-DGGE) method for the identification of HPB in cheese on the species level has been recently described. This is a useful and effective method that allows the separation of the *hdc* amplicons with the same size but different sequences, in order to distinguish among different *hdc* variants present in complex microbial communities. The pair of primers used in that study (hdcDG-F and hdcDG-R) aligns in the conserved regions of *hdc*, flanking a variable region, and renders a 250-base pair PCR products that are subsequently subjected to DGGE analysis (Diaz et al., 2016b).

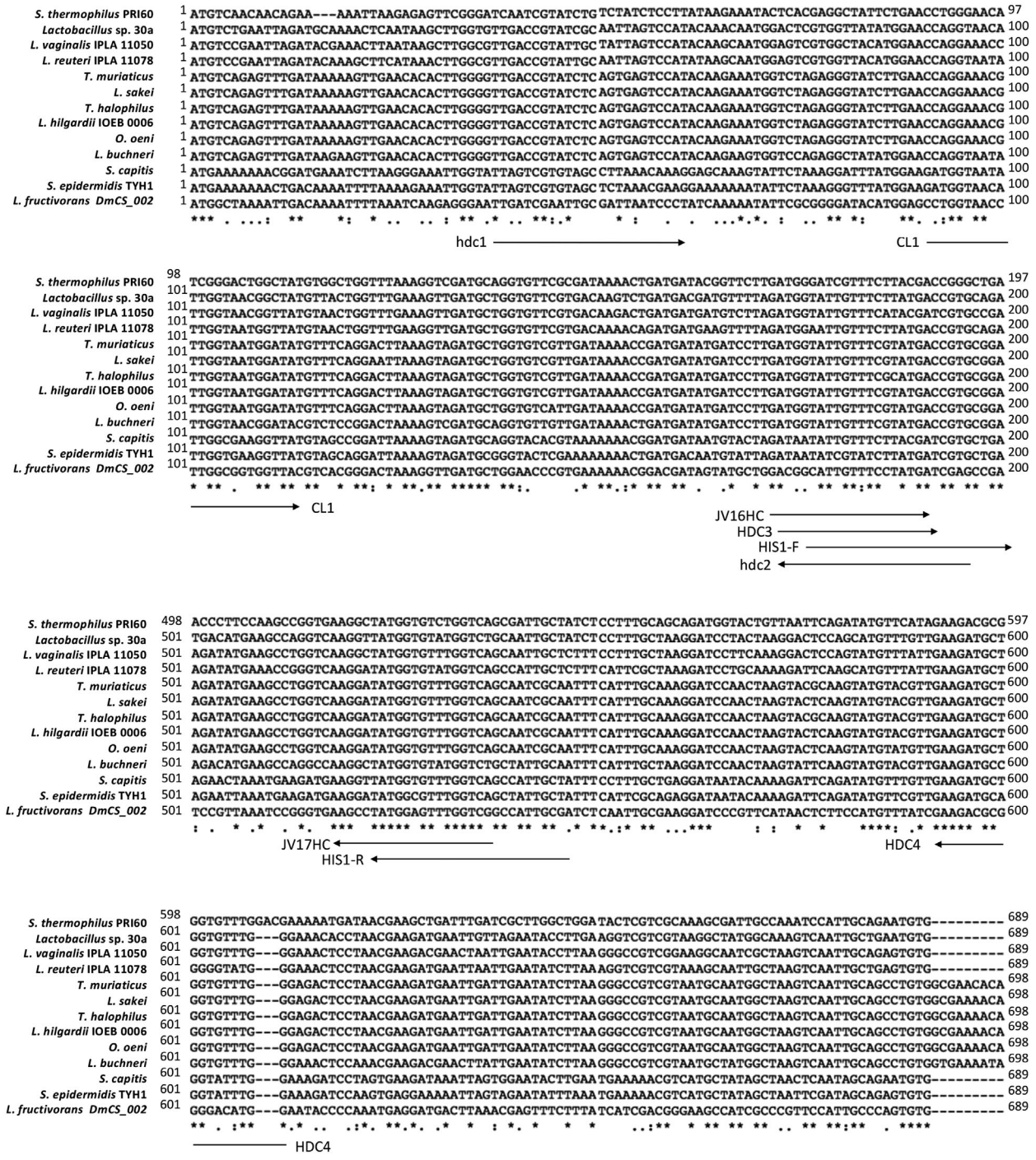

FIGURE 7 Partial nucleotide alignment of the *hdc* genes from representative gram-positive bacteria, using ClustalW software. The bacterial species, shown at the left of each sequence, as well as the GenBank accession numbers and the references they were taken from (in parentheses), are *S. thermophilus* strain PRI60 (FR693807.2, Rossi et al., 2011), *Lactobacillus* sp. 30a (AAB59151.1, Schelp et al., 2001), *L. vaginalis* strain IPLA11050 (LN828720.1, Diaz et al., 2015), *L. reuteri* strain IPLA11078 (LN877767.1, Diaz et al., 2016b), *T. muriaticus* (DQ132889.1, Kimura et al., 2001), *L. sakei* (DQ132888.1, Diaz et al., 2016b), *T. halophilus* (AB362339.1, Satomi et al., 2008), *L. hilgardii* strain IOEB 0006 (AY651779.1, P. M. Lucas et al., 2005), *O. oeni* (DQ132887.1, P. M. Lucas et al., 2008), *L. buchneri* (DQ132890.1, Diaz et al., 2016b), *S. capitis* (AM283479.1, de Las Rivas, Rodríguez, Carrascosa, & Muñoz, 2008), *S. epidermidis* strain TYH1 (AB583189.1, Yokoi et al., 2011), and *L. fructivorans* strain DmCS_002 (NZ_JOJZ01000009.1, Diaz et al., 2016a). Regions where the primers used in dairy products align are indicated by arrows. Numbers indicate the nucleotide position in the sequence of the *hdc* gene

The main disadvantage presented by end-point PCR methods is the impossibility of quantifying DNA template: thus, real-time quantitative PCR methods (RT-qPCR) have been developed to detect HPB in food, mainly in wine, fish, and cheese (Bjornsdottir-Butler et al., 2011; Lucas et al., 2008; Nannelli et al., 2008). Particularly, in cheese, primers hdc1 and hdc2 were used to detect and quantify gram-positive HPB (Fernandez et al., 2006; Ladero, Linares, Fernandez, & Alvarez, 2008; Møller et al., 2020; Tofalo et al., 2019). A RT-qPCR assay has also been developed in raw milk and cheese to detect and enumerate *L. parabuchneri*, one of the main histamine producers in dairy food, although this method is not based on the analysis of the *hdc* gene but on the unique locus *tmp*, not present in other species (Berthoud et al., 2017).

Finally, genomic-based tools for the rapid and accurate assessment of microbial communities have been developed in recent years. Target metagenomics is based on the sequencing of selected target genes: it provides variable information depending on the studied gene, for instance, 16S rRNA or BA synthetic genes (Ruiz & Alvarez-Ordoñez, 2019). As an example, high-throughput DNA sequencing has been implemented to assess the presence of bacterial histidine and tyrosine decarboxylases in cheeses. This method consists of amplifying the *hdc* and *tdc* genes with primers HIS2-F and HIS2-R or TD2 and TD5, and then cloning the PCR amplicons to subsequently perform high-throughput sequencing of the created amplicon libraries. Finally, the obtained *hdc* and *tdc* sequences are compared with a nucleotide database to identify bacteria with histaminogenic or tyraminogenic potential (O'Sullivan et al., 2015). Another example of the application of next-generation sequencing techniques combining sequencing and quantification of DNA has also been described in fish: the correlation of the histamine content with the presence of gram-negative harmful bacteria, based on the amplification of the 16S rRNA gene (de Lira et al., 2020; Tsironi et al., 2019). Unlike selected target gene sequencing which only targets 16S rRNA or another key gene, shotgun metagenomics sequences all given genomic DNA from a sample. As an example, several *L. parabuchneri* species isolated from cheese or raw milk were genomically characterized by sequencing their whole genomes to study the *hdc* cluster in profound detail and to conclude that it was gained by horizontal gene transfer among different lactobacilli species (Wuthrich et al., 2017).

In spite of the above-exposed advantages offered by modern molecular methods and summarized in Figure 5 (such as high sensitivity and reliability or rapidity), important disadvantages should be noted. One of the most important drawbacks is the impossibility of identifying *hdc* genes of novel strains with emerging ability of histamine formation by using traditional primers, as explained in Table 2. For instance, Møller et al. (2020) highlighted that, in cheese, the *hdc* genes of *P. pentosaceus* isolates capable of producing histamine could not be detected with the use of both JV16HC/JV17HC and Hdc1/Hdc2 primer pairs, described in the literature and useful for traditional histamine producers, such as *L. parabuchneri* and *L. paracasei*. Alternative methods should, therefore, be developed to allow the identification of all HPB in food. Among them, whole genome sequencing of emerging histamine producers and subsequent metagenomics annotation, or the search for new potential decarboxylase genes based on nucleotide sequencing or tridimensional protein similarity, could yield good results. Once all the putative histidine decarboxylase genes have been identified, the design of new matching primer sets is indispensable.

6 | HISTAMINE PRODUCERS IN DAIRY PRODUCTS

The use of the aforementioned techniques in dairy products has allowed the identification of a great variety of microorganisms with the ability to produce histamine (i.e., with histidine decarboxylase activity). Histamine-forming microbiota in dairy products could be classified according to different criteria. For instance, based on their origin and purpose, histamine producers could be divided into (1) NSLAB (naturally present in milk), (2) SLAB (intentionally added to dairy products), and (3) contaminants (due to practices during obtaining and handling the milk through dairy products manufacture, as well as from the processing environment––including insufficient cleaning-disinfection practices and biofilm formation). However,-the traditional classification of microorganisms allows to divide histamine-producing microbiota present in dairy products in gram-positive bacteria, gram-negative bacteria, or yeasts and molds. Specific genera, species, and strains of microorganisms capable of synthesize histamine are detailed in Table 3. Additionally, in bold type, Figure 3 highlights the species of histamine producers present on cheese surface and in cheese core among the total microbiota that can be found in cheese. It is key to consider that histamine formation is influenced by a series of factors, as exposed in Figure 4, which should be carefully controlled during the cheese-making process. Some of these factors directly focus on modulating the growth of histamine producers among total cheese microbiota; for instance, environmental conditions, such as salt content or water activity or even bacterial competition processes.

TABLE 3 Histamine-producing microbiota present in different dairy products

Microorganisms		References	Dairy product source	Techniques applied for identification (I) and confirming (C) histamine-forming ability
Gram-positive bacteria	*Lentilactobacillus buchneri* (formerly *Lb. buchneri*)	(O'Sullivan et al., 2015)	Reblochon, Irish artisanal, Morbier, Tête de Moine, and Pecorino Sardo cheeses	High-throughput DNA sequencing of total metagenomic DNA extracts (I) and HPLC quantification (C)
	Lentilactobacillus buchneri	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Lentilactobacillus parabuchneri (formerly *Lb. parabuchneri*) KUH8, KUH1, KUH2	(Møller et al., 2020)	Vintage Danish Gouda cheese	Histidine decarboxylase activity and PCR analysis (I) and UPLC quantification (C)
	Lentilactobacillus parabuchneri FAM21731, FAM21809, FAM21823, FAM21829, FAM21834, FAM23163, FAM23164, FAM23165, FAM23166, FAM23167, FAM23168, FAM23169	(Wuthrich et al., 2017)	Emmental, Tête de Moine, Mont Soleil, and Tilsit cheeses	Whole-genome sequencing and HPTLC quantification (C)
	Lentilactobacillus parabuchneri	(Berthoud et al., 2017)	Emmental, Tête de Moine, Mont Soleil, Tilsit, Alpine, and Raclette cheeses Raw milk	Histidine decarboxylase activity and qPCR analysis (I) and HPLC quantification (C)
	Lentilactobacillus parabuchneri	(Diaz et al., 2016)	Cabrales, Gamoneu, Manchego-type, Casín, and Idiazabal cheeses	PCR-DGGE analysis (I) and HPLC quantification (C)
	Lentilactobacillus parabuchneri IPLA 11118, IPLA 11119, IPLA 11120, IPLA 11121, IPLA 11122, IPLA 11123, IPLA 11124, IPLA 11125, IPLA 11126, IPLA 11127, IPLA 11128, IPLA 11129, IPLA 11130, IPLA 11131, IPLA 11132, IPLA 11133, IPLA 11134, IPLA 11135, IPLA 11136, IPLA 11137, IPLA 11138	(Diaz et al., 2016)	Emmental cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Lentilactobacillus parabuchneri IPLA 11122, IPLA 11117, IPLA 11150	(Díaz et al., 2018)	Different types of commercial cheeses	HPLC quantification (C)

(Continues)

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TABLE 3 (Continued)

Microorganisms		References	Dairy product source	Techniques applied for identification (I) and confirming (C) histamine-forming ability
	Lentilactobacillus parabuchneri	(Diaz et al., 2015)	Cabrales cheese	Histidine decarboxylase activity (I)
	Lactobacillus delbrueckii subsp. *lactis* (formerly *Lb. lactis*)	(Burdychova & Komprda, 2007)	Dutch-type semihard cheese	PCR analysis (I) and HPLC quantification (C)
	Lactobacillus helveticus	(Burdychova & Komprda, 2007)	Dutch-type semihard cheese	PCR analysis (I) and HPLC quantification (C)
	Lactobacillus delbrueckii	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Latilactobacillus sakei/Lentilactobacillus hilgardii (formerly *Lb. sakei* group)	(Diaz et al., 2016)	Cabrales cheeses	PCR-DGGE analysis (I) and HPLC quantification (C)
	Latilactobacillus gen.	(O'Sullivan et al., 2015)	Ossau-Iraty, Irish Artisanal, Morbier, and Pecorino Sardo cheeses	High-throughput DNA sequencing of total metagenomic DNA extracts (I) and HPLC quantification (C)
	Latilactobacillus curvatus (formerly *Lb. curvatus*)	(Burdychova & Komprda, 2007)	Dutch-type semihard cheese	PCR analysis (I) and HPLC quantification (C)
	Levilactobacillus brevis (formerly *Lb. brevis*)	(del Valle et al., 2018)	Raw goat milk cheese	PCR analysis (I) histamine formation assessment and HPLC quantification (C)
	Lacticaseibacillus casei (formerly *Lb. casei*)	(del Valle et al., 2018)	Raw goat milk cheeses	PCR analysis (I) histamine formation assessment and HPLC quantification (C)
	Lacticaseibacillus casei	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)

(Continues)

TABLE 3 (Continued)

Microorganisms		References	Dairy product source	Techniques applied for identification (I) and confirming (C) histamine-forming ability
	Lacticaseibacillus paracasei KUH3 (formerly *Lb. paracasei*)	(Møller et al., 2020)	Vintage Danish Gouda cheese	Histidine decarboxylase activity and PCR analysis (I) and UPLC quantification (C)
	Lactiplantibacillus plantarum (formerly *Lb. plantarum*)	(del Valle et al., 2018)	Raw goat milk cheese	PCR analysis (I) histamine formation assessment and HPLC quantification (C)
	Limosilactobacillus vaginalis (formerly *Lb. vaginalis*) IPLA11140, IPLA11141, IPLA11142, IPLA11143, IPLA11144, IPLA11145, IPLA11147, IPLA11050, IPLA11051, IPLA11052, IPLA11053, IPLA11054, IPLA11055, IPLA11056, IPLA11057, IPLA11058, IPLA11060, IPLA11062, IPLA11064, IPLA11065, IPLA11067, IPLA11068, IPLA11069, IPLA11070, and IPLA11075	(Diaz et al., 2015)	Cabrales cheese	Histidine decarboxylase activity and PCR analysis (I) and HPLC quantification (C)
	Limosilactobacillus reuteri (formerly *Lb. reuteri*)	(Diaz et al., 2015)	Cabrales cheese	Histidine decarboxylase activity (I)
	Lactococcus lactis subsp. *lactis*	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Streptococcus thermophilus PRI60	(Gardini et al., 2012)	Dairy products	HPLC quantification (C)
	Streptococcus thermophilus	(Gezginc et al., 2013)	Home-made natural yogurts	Histidine decarboxylase activity and PCR analysis (I) and HPLC quantification (C)
	Streptococcus thermophilus	(Ladero et al., 2015)	Artisanal raw milk cheeses	PCR analysis (I) and (U)HPLC quantification (no histamine) (C)

(Continues)

TABLE 3 (Continued)

Microorganisms		References	Dairy product source	Techniques applied for identification (I) and confirming (C) histamine-forming ability
	Streptococcus thermophilus PRI17, PRI18, PRI21, PRI60, PRI74	(Rossi et al., 2011)	Mozzarella and Grana-type cheeses. Traditional yogurts	PCR analysis (I) and HPLC quantification (C)
	Streptococcus thermophilus	(Diaz et al., 2016)	Idiazabal cheeses	PCR-DGGE analysis (I) and HPLC quantification (no histamine) (C)
	Pediococcus pentosaceus KUH5, KUH6, KUH7	(Møller et al., 2020)	Vintage Danish Gouda cheese	Histidine decarboxylase activity (I) and UPLC quantification (C)
	Tetragenococcus halophilus	(Diaz et al., 2016)	Cabrales and Manchego-type cheeses	PCR-DGGE analysis (I) and HPLC quantification (C)
	Leuconostoc sp.	(del Valle et al., 2018)	Raw goat milk cheese	PCR analysis (I) histamine formation assessment and HPLC quantification (C)
	Enterococcus faecium	(Tham et al., 1990)	Goat milk cheese	Fluorimetric histamine determination (C)
	Enterococcus faecalis	(Tham et al., 1990)	Goat milk cheese	Fluorimetric histamine determination (C)
	Enterococcus casseliflavus	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Microbacterium foliorum C45	(Helinck et al., 2013)	French cheeses	HPLC quantification (C)

(Continues)

TABLE 3 (Continued)

Microorganisms		References	Dairy product source	Techniques applied for identification (I) and confirming (C) histamine-forming ability
Gram-negative bacteria	*Citrobacter freundii*	(Marino et al., 2000)	Blue-veined cheeses	HPLC quantification (C)
	Citrobacter freundii	(Maifreni et al., 2013)	Montasio cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Citrobacter freundii UCMA 4217	(Coton et al., 2012)	Livarot cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Citrobacter braakii CtT 6, CtT 10, CtT 29, CtT 60, CtT 61	(Chaves-Lopez et al., 2006)	Pecorino Abruzzese cheeses	HPLC quantification (C)
	Hafnia alvei	(Marino et al., 2000)	Blue-veined cheeses	HPLC quantification (C)
	Hafnia alvei	(Maifreni et al., 2013)	Montasio cheeses	HPLC quantification (C)
	Hafnia alvei 1 B16	(Coton et al., 2012)	Livarot cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Hafnia alvei	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Hafnia paralvei 920	(Coton et al., 2012)	Munster cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Halomonas sp. nov. B39	(Coton et al., 2012)	Livarot cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Halomonas venusta 3D7M	(Coton et al., 2012)	Munster cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Halomonas venusta/alkaliphila/hydrothermalis 4C1A	(Coton et al., 2012)	Munster cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Morganella morganii 3A2A, 3A5A, 3D4A	(Coton et al., 2012)	Munster cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)

(Continues)

TABLE 3 (Continued)

Microorganisms		References	Dairy product source	Techniques applied for identification (I) and confirming (C) histamine-forming ability
	Providencia heimbachae GR4	(Coton et al., 2012)	Epoisses cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Serratia liquefaciens	(Marino et al., 2000)	Blue-veined cheeses	HPLC quantification (C)
	Serratia liquefaciens 1B4F	(Coton et al., 2012)	Munster cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Serratia liquefaciens	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Serratia marcescens 448	(Coton et al., 2012)	Munster cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Serratia proteomaculans 1C2F	(Coton et al., 2012)	Munster cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Serratia odorifera CtT 28, CtT 57, CtT 58, CtT 74	(Chaves-Lopez et al., 2006)	Pecorino Abruzzese cheeses	HPLC quantification (C)
	Serratia odorifera	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Serratia grimesii UCMA 3895	(Coton et al., 2012)	Livarot cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Serratia sp. (close *S. grimesii*) GB3	(Coton et al., 2012)	Epoisses cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Serratia spp.	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Chryseobacterium shigense PCA1 B2.3	(Coton et al., 2012)	Cow milk Salers cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)

(Continues)

TABLE 3 (Continued)

Microorganisms		References	Dairy product source	Techniques applied for identification (I) and confirming (C) histamine-forming ability
	Chryseobacterium sp. (close *C. bovis*) Pi 18	(Coton et al., 2012)	St. Nectaire cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Enterobacter hormaechei 380, 272, INRA 1439	(Coton et al., 2012)	Munster and Salers cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Enterobacter cloacae	(Marino et al., 2000)	Blue-veined cheeses	HPLC quantification (C)
	Enterobacter cloacae	(Maifreni et al., 2013)	Montasio cheeses	HPLC quantification (C)
	Enterobacter gergoviae	(Marino et al., 2000)	Blue-veined cheeses	HPLC quantification (C)
	Enterobacter aerogenes	(Marino et al., 2000)	Blue-veined cheeses	HPLC quantification (C)
	Enterobacter sakazaki CtT 9, CtT 23, CtT 29	(Chaves-Lopez et al., 2006)	Pecorino Abruzzese cheeses	HPLC quantification (C)
	Enterobacter sakazakii	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Enterobacter spp.	(Maifreni et al., 2013)	Montasio cheeses	HPLC quantification (C)
	Pseudomonas grp *putida* CV 30.6, VRBG 37.3, CFC25.4	(Coton et al., 2012)	Milk	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Pseudomonas lundensis PCAi D2.2	(Coton et al., 2012)	Cow milk Salers cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Pseudomonas stutzeri UCMA 3883	(Coton et al., 2012)	Livarot cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Psychrobacter celer 91	(Coton et al., 2012)	Camembert raw milk cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Psychrobacter sp. 580	(Helinck et al., 2013)	French cheeses	HPLC quantification (C)

(Continues)

TABLE 3 (Continued)

Microorganisms		References	Dairy product source	Techniques applied for identification (I) and confirming (C) histamine-forming ability
	Raoultella planticola 924	(Coton et al., 2012)	Munster cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Raoultella ornithinolytica	(Maifreni et al., 2013)	Montasio cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Sphingobacterium sp. (close *S. faecium*) PCAi F2.5	(Coton et al., 2012)	Cow milk Salers cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Acinetobacter sp. (close genospecies 3) PCA E6.10	(Coton et al., 2012)	Cow milk Salers cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Alcalingenes faecalis 1 904	(Coton et al., 2012)	Munster cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Proteus sp. (close *P. hauseri*) UCMA 3780	(Coton et al., 2012)	Livarot cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Proteus heimbachae 945	(Coton et al., 2012)	Munster cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Providencia sp. nov. GB1	(Coton et al., 2012)	Epoisses cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Escherichia coli	(Maifreni et al., 2013)	Montasio cheeses	HPLC quantification (C)
	Escherichia coli	(Marino et al., 2000)	Blue-veined cheeses	HPLC quantification (C)
	Escherichia coli CtT 1, CtT 24, CtT 43, CtT 75	(Chaves-Lopez et al., 2006)	Pecorino Abruzzese cheeses	HPLC quantification (C)

(Continues)

TABLE 3 (Continued)

Microorganisms		References	Dairy product source	Techniques applied for identification (I) and confirming (C) histamine-forming ability
	Escherichia coli	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Escherichia fergusonii	(Maifreni et al., 2013)	Montasio cheeses	HPLC quantification (C)
	Klebsiella oxytoca	(Maifreni et al., 2013)	Montasio cheeses	HPLC quantification (C)
	Klebsiella oxytoca	(Marino et al., 2000)	Blue-veined cheeses	HPLC quantification (C)
	Klebsiella pneumoniae	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Arizona spp.	(Marino et al., 2000)	Blue-veined cheeses	HPLC quantification (C)
	Salmonella enterica spp. *arizonae* CtT 31, CtT 33, CtT 37 CtT 50	(Chaves-Lopez et al., 2006)	Pecorino Abruzzese cheese	HPLC quantification (C)
	Kluyvera spp. CtT 3, CtT 26, CtT 49, CtT 53	(Chaves-Lopez et al., 2006)	Pecorino Abruzzese cheese	HPLC quantification (C)
	Cedecea spp.	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Edwarsiella spp.	(Roig-Sangüés et al., 2002)	Spanish traditional cheeses	Histidine decarboxylase activity (I) and HPLC quantification (C)
Yeasts and molds	*Geotrichum candidum*	(Roig-Sangüés et al., 2002)	Cabrales cheese	Histidine decarboxylase activity (I) and HPLC quantification (C)
	Debaryomyces hansenii LM21, LM24, LM26	(Gardini et al., 2006)	Pecorino Crotonese cheese	Histidine decarboxylase activity (I)
	Debaryomyces hansenii 304	(Helinck et al., 2013)	French cheeses	HPLC quantification (C)

6.1 | Gram-positive bacteria

LAB are the main histamine producers in dairy products; *Lactobacillus* species, such as *L. parabuchneri, L. buchneri, Lb. helveticus*, and *L. curvatus*, among others, seem to be responsible for histamine accumulation in cheese (Barbieri et al., 2019). Some of these species can be present in cheese because they were either already contained in milk (above all, NSLAB), or because they took part as contaminants or starter cultures in the course of the cheese production process (Linares et al., 2012). Notably, *L. buchneri* and *L. parabuchneri*, present as contaminants in fermented dairy products and closely related with one another phylogenetically, have been reported to be the major histamine producers in cheese, capable of synthesizing high amounts of histamine even at low temperatures (Berthoud et al., 2017; Díaz et al., 2018; O'Sullivan et al., 2015; Wuthrich et al., 2017). *L. parabuchneri* has been reported to produce histamine in a wide variety of cheese samples, even at low refrigeration temperatures (Diaz, Del Rio et al., 2016; Diaz, Ladero et al., 2016; Díaz et al., 2018; Møller et al., 2020). From several cheeses containing histamine, Berthoud et al. (2017) isolated certain *L. parabuchneri* strains with the *hdc* gene, and developed a molecular method to detect and enumerate *L. parabuchneri* in raw milk and cheese. Later on, the same authors investigated the genome variability of these strains and concluded that the *hdc* cluster is located in a genomic island that can be transferred within the *L. parabuchneri* species. Some strains have lost that island and thus the capacity to synthesize histamine (Wuthrich et al., 2017). Relative to *L. buchneri*, one isolate of a histamine-forming strain was detected in Spanish traditional cheeses, and was shown to be the predominant LAB with histaminogenic potential in 10 different cheese varieties, as evidenced by high-throughput DNA sequencing (O'Sullivan et al., 2015; Roig-Sangüés, Molina, & Hernández-Herrero, 2002). On the other hand, Diaz et al. (2015) isolated (for the first time from cheese) and typified several *L. vaginalis* (formerly *Lb. vaginalis*) strains capable of producing histamine, as well as a number of histamine-producing isolates identified as *L. reuteri* (formerly *Lb. reuteri*). Burdychova and Komprda (2007) also studied the histamine-producing potential displayed by certain bacterial communities in a Dutch-type semihard cheese. Among the histamine-producing strains isolated from the cheese, the authors found that *Lb. delbrueckii* subsp. *lactis* and *L. curvatus* played a role as contaminants, whereas *Lb. helveticus* originated from a starter culture used for cheese production. Other species, such as *L. brevis*, *L. casei*, and *L. plantarum*, were found to contain the *hdc* gene in cheeses prepared with raw milk, and some of those species had not been added as starter cultures (del Valle et al., 2018). The *Lb. delbrueckii* species was also reported as a histamine producer (Roig-Sangüés et al., 2002). *L. hilgardii*/*L. sakei* may present histaminogenic potential as well; the indistinguishable *hdc* genes of these species have been detected in two Cabrales cheeses and even highlighted by high-throughput DNA sequencing (Diaz, Ladero et al., 2016; O'Sullivan et al., 2015). An *L. paracasei* isolate from cheese was also shown to be a fast producer of high levels of histamine, together with several isolates of *L. parabuchneri* (Møller et al., 2020).

In addition to *Lactobacillus* species, the *Streptococcus* genus is also an important histamine producer in cheese, although the source of this microorganism in the product is unclear (O'Sullivan et al., 2015). However, not all of the strains actually contain the *hdc* gene. A recent study classifies most *S. thermophilus* strains into two major clusters: Cluster A and Cluster B. Strains belonging to Cluster A present larger genomes or complete histidine biosynthesis gene clusters, among other characteristics. The *hdc* cluster is also present in all *S. thermophilus* strains pertaining to Cluster A, supporting the hypothesis of acquisition by horizontal gene transfer from a satellite phage (Alexandraki et al., 2019). In fact, up to 6% of *S. thermophilus* strains isolated from natural sources contain the *hdc* gene, and some of them are able to produce histamine in milk under conditions relevant to cheese-making, or even at low temperatures (Calles-Enriquez et al., 2010; Gardini et al., 2012; Rossi et al., 2011). However, certain *S. thermophilus* strains isolated from cheeses or home-made natural yogurt were also shown to contain the *hdc* gene, although only a low amount or even no histamine at all was found in the supernatant in culture media (Diaz, Ladero et al., 2016; Gezginc et al., 2013; Ladero et al., 2015).

Apart from *Lactobacillus* and *Streptococcus*, other LAB genera have been shown to synthesize histamine in dairy products. Recently, Møller et al. (2020) reported *P. pentosaceus* for the first time as a histamine producer in cheese. *T. halophilus* has been previously described as a histamine producer in fish or soy sauces, although it was reported for the first time as a histamine-producing species in certain Cabrales and Manchego cheeses (Diaz, Ladero et al., 2016; Satomi et al., 2008). The *hdc* gene was also amplified in a *Leuconostoc* sp. strain isolated from raw goat milk cheese (del Valle et al., 2018). Potential histamine formation by *E. faecium* or *E. casseliflavus* in cheese has also been reported, but the contribution of enterococci to the level of histamine in cheese is probably irrelevant (Roig-Sangüés et al., 2002; Tham, Karp, & Danielsson-Tham, 1990).

6.2 | Gram-negative bacteria

On the other hand, common contaminants of milk or spoilage bacteria, such as the microbial families *Enterobacteriaceae* or *Pseudomonads*, could also be responsible for histamine production in food. Many members of the *Enterobacteriaceae* family can act as histamine producers in cheese, but they only produce low amounts thereof, usually in early steps of the cheese-making process (Barbieri et al., 2019; Costa et al., 2018).

Several studies have isolated gram-negative bacteria present in different cheese varieties, and some of them have also quantified the amount of histidine that every bacterial isolate was able to produce *in vitro* or even in cheese model. Coton et al. (2012) obtained gram-negative bacterial isolates from French cheeses or milk, and then evaluated their ability to produce histamine *in vitro*. Many of the isolates were able to produce histamine in a culture medium, but only few of them produced more than 1,000 mg/kg of histamine, namely *Morganella morganii* and *Serratia* sp. Additionally, *H. alvei, C. freundii, Halomonas* spp., *Raoultella planticola*, and *Providencia heimbachae* also produced more than 500 mg/kg of histamine (Coton et al., 2012). Many isolates of enterobacteria obtained from Montasio cheeses produced low amounts of histamine (<300 mg/kg), but only four isolates, two corresponding to *E. cloacae* and two more to *C. freundii*, produce more than 1,000 mg/kg (Maifreni et al., 2013). According to another study, more than 50% of the 104 bacterial isolates from blue-veined cheeses were able to form histamine; although the histamine production was very low (<20 mg/kg), isolates corresponding to *Enterobacter gorgoviae, S. liquefaciens, E. coli, H. alvei, E. cloacae, E. aerogenes, C. freundii, Arizona* spp., and *Klebsiella oxytoca* were confirmed to produce histamine (Marino, Maifreni, Moret, & Rondinini, 2000). The analysis of isolates of enterobacteria obtained from Pecorino cheese resulted in the production of very low amounts of histamine by all the strains (< 3 mg/kg), namely *E. coli, S. enterica* spp. *Arizonae, E. sakazakii, C. braakii, Kluyvera* spp., and *S. odorifera* (Chaves-Lopez et al., 2006).

Other studies have also analyzed the presence of histamine-producing microbiota but have failed to obtain quantitative results. For instance, Roig-Sangüés et al. (2002) isolated total microbiota from certain Spanish cheeses: most of the gram-negative isolates, identified as enterobacteria, displayed histamine-forming activity. The authors detected *H. alvei, E. coli, E. sakazakii, Edwarsiella* spp., and *Serratia* spp. as histamine producers in cheese. Additionally, one isolate of *Cedecea* spp., a genus genetically very close to *Serratia*, was reported for the first time to produce histamine (Roig-Sangüés et al., 2002).

On the other hand, *Psychrobacter* sp. was reported for the first time to produce histamine *in vitro* in a culture medium containing histidine, and even in a cheese model with the yeast *D. hansenii* as coculture (Helinck, Perello, Deetae, de Revel, & Spinnler, 2013).

6.3 | Yeasts and molds

Certain yeasts and molds can also produce histamine in food, although few studies have analyzed that production specifically in cheese. The major histamine producer in cheese belonging to this group is *D. hansenii*, but this seems to be a strain-specific characteristic (Gardini et al., 2006). In a cheese model, *D. hansenii* was able to produce histamine only in the presence of the bacterium *Psychrobacter* (Helinck et al., 2013). *G. candidum* was also mentioned as a histamine-forming mold in Cabrales cheese (Roig-Sangüés et al., 2002).

7 | POTENTIAL SOLUTIONS TO COUNTERACT HISTAMINE ACCUMULATION IN DAIRY FOOD: FROM PREVENTION TO HISTAMINE DEGRADATION

In order to avoid the release of dairy products with high levels of histamine to the market, the main measure the food industry could take would be the reduction of HPB in dairy products by (1) preventing their access to raw materials, (2) inactivating them, and/or (3) controlling environmental conditions. If those measures are not effective, (4) microbial or enzymatic degradation of histamine is the alternative. Figure 8 compiles the potential strategies for obtaining histamine-free dairy products, aimed at preventing histamine formation or promoting histamine degradation.

The promotion of hygienic conditions during milking and food processing could decrease and even inactivate histamine-producing microbiota. Additionally, the selection of suitable starter cultures unable to synthesize histamine is an appropriate alternative for the reduction of histamine production in dairy products, although it is necessary to assess whether the organoleptic characteristics of the final product are eventually thereby altered.

To obtain a safe product with an extended shelf-life, it is necessary to apply food preservation treatments designed to reduce the microbial load and guarantee milk safety in the cheese-making process (Quigley et al., 2013; Tilocca et al., 2020). Heat treatment (sterilization or pasteurization) is currently the most commonly applied process for

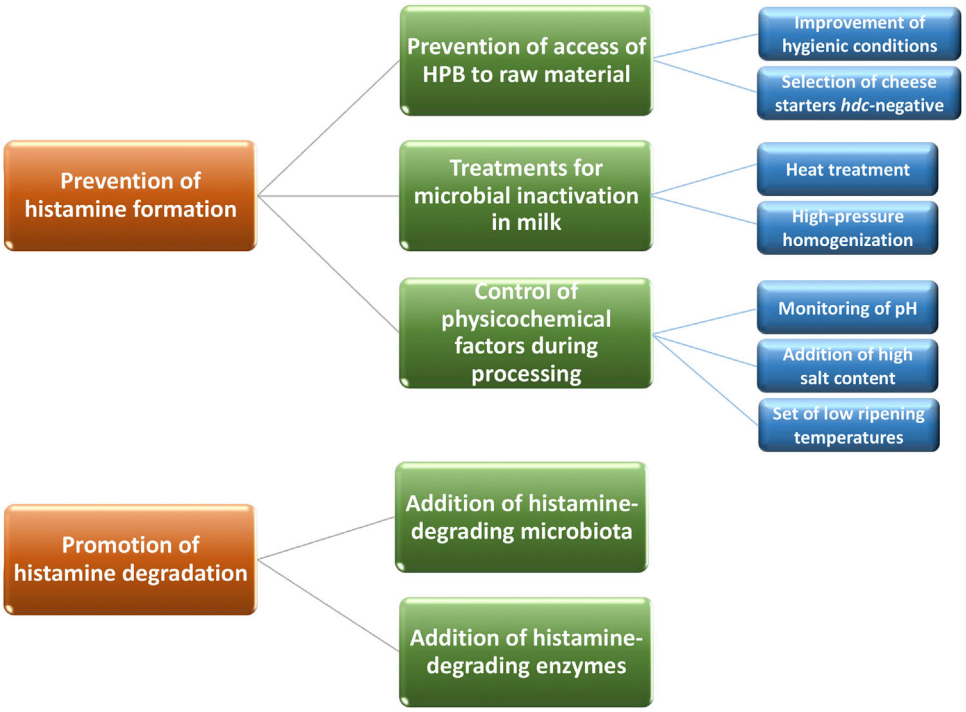

FIGURE 8 Strategies aimed at preventing histamine formation or promoting histamine degradation in dairy products

the preservation of liquid milk (Walstra et al., 2006). Nevertheless, nonthermal technologies, such as HPH, or irradiation, have also been proposed as alternative technologies to preservation of milk, although these methodologies are not currently being used industrially for this purpose (Ramaswamy, Ahn, Balasubramaniam, Rodriguez Saona, & Yousef, 2019).

As mentioned above, the production and quantity of histamine synthesized in dairy products, such as cheese, depends on a number of factors, such as histidine availability, ripening and storage temperatures, pH, sodium concentration, decarboxylation potential of the HPB, and carbon source (Benkerroum, 2016; Linares et al., 2012). These factors can be occasionally modified to prevent or reduce the rate of histamine production. In case the strategies for the prevention of histamine formation in dairy products fail, the degradation of histamine can be considered as a crucial alternative (Linares et al., 2012). Figure 8 summarizes the main strategies aimed at preventing or reducing histamine content in dairy products.

7.1 | Measures aimed to prevent histamine formation during processing of dairy products

One of the most important measures aiming to reduce histamine production is the overall improvement of hygiene during production and storage of dairy food. Other changes in food processing designed to inhibit or reduce HPB in dairy products include the selection of *hdc*-negative starters, pasteurization, HPH, and control of physicochemical factors during dairy processing (Linares et al., 2012; Naila, Flint, Fletcher, Bremer, & Meerdink, 2010).

7.1.1 | Preventing access of HPB to raw materials

Improving hygienic conditions along the dairy food chain

Hygienic conditions during milking are a very important factor for the dairy industry. The milk of healthy animals produced under hygienic conditions should contain less than 5×10^5 CFU/mL (Bereda, Yilma, & Nurfeta, 2012). The initial microbial load of milk varies between 10^3 and 10^5 CFU/mL, rising to 10^6 to 10^7 CFU/mL before processing (depending on its handling), and increasing during cheese ripening to up to 10^8 CFU/g in the final product (Benkerroum, 2016; Mlejnkova et al., 2016; Schirone, Tofalo, Visciano, Corsetti, & Suzzi, 2012). The microbiological quality of milk is clearly influenced by the way in which milk is handled from milking to consumption. The environment, handlers, equipment, and packaging materials can all be a reservoir for microbial contamination of milk and dairy products (Pal, Devrani, & Pinto, 2018). Lack of hygiene in the handling of milk, the misuse of milking equipment, and the lack of drinking water for cleaning purposes can contribute to the poor hygienic quality

of milk. Strict hygienic measures must be applied during preparation, storage, and delivery of a variety of dairy products for human consumption. It is thus necessary to educate food handlers regarding the basic principles of hygiene and manufacturing of dairy products, which ensure their quality and safety for consumption.

On the other hand, histamine-producing microorganisms are likely to appear in the food chain in the form of food contaminant microbiota or NSLAB contained in the raw material (Linares et al., 2012). Pintado et al. (2008) indicate that the production of BAs in cheese made from raw milk depends, among other variables, on the level of enterobacteria, enterococci, and lactobacilli present in raw milk, which can attain levels of 10^7 CFU/g. This level of contamination in raw milk appears to be frequently associated with a high histamine content in raw milk cheeses. The number and diversity of histamine-producing microorganisms increases as the total count in raw milk rises (Benkerroum, 2016). Ascone et al. (2017) reported repeated contamination of *L. parabuchneri* in milk from providers, capable of forming biofilms on stainless steel surfaces in dairy processing equipment, and thus constituting a reservoir and a source of contamination of postripening-processed cheeses (Diaz, Ladero et al., 2016). To reduce the histamine content in such cheeses, it would be necessary to perform routine screening of provided milks and to control the formation of biofilms containing HPB in the dairy food processing industry (Diaz, Ladero et al., 2016). This would allow the identification and exclusion of contaminated raw milk in order to prevent the production of contaminated raw milk cheeses (Ascone et al., 2017).

On the other hand, in the final histamine content, contamination stemming from food processing seems to be more important than contamination stemming from the raw material. Ladero, Fernández, and Álvarez (2009) studied the effect of postripening processing of different types of cheese on the presence of HPB and on the average histamine concentration of the final product. The highest concentrations of histamine (734 mg/kg) were reported in grated cheese samples in comparison with whole Emmental cheeses (115 mg/kg). In this case, the presence of HPB during cheese manufacturing was due to poor hygiene practices in product processing: the contact of the cheese with equipment surfaces increased the risk of microbiological contamination.

Thus, in sum, it is necessary to control and improve microbiological and hygienic conditions along the entire production chain (i.e., from farm to fork) in order to reduce the amounts of BAs or to avoid their presence altogether in dairy products (Benkerroum, 2016).

Selection of cheese starters unable to synthesize histamine

To guarantee the quality of dairy products and minimize the adverse health effects of histamine, starter cultures must be carefully selected on the basis of their inability to produce histamine and their capacity to degrade it (Naila et al., 2010; Spano et al., 2010).

Raw milk cheeses are particularly vulnerable to the formation of histamine, favored by high levels of secondary proteolysis as a consequence of the action of starter and nonstarter cultures, along with a higher microbial load and, in some cases, long ripening times (Guarcello et al., 2016; Linares et al., 2011; O'Sullivan et al., 2015; Schirone et al., 2013). The addition of proteinases to milk or curd has been widely used with the purpose of accelerating cheese ripening (Fernandez-Garcia et al., 2000). The effect of the selection of starter cultures on the proteolytic pattern and thus on histamine production in cheese was demonstrated by Gardini et al. (2012) by using a histaminogenic *S. thermophilus* strain (PRI60) and, alternatively, a nonhistamine-producing strain (PRI40) as starter cultures. Nieto-Arribas, Poveda, Seseña, Palop, and Cabezas (2009) suggested *L. plantarum* and *L. paracasei*, isolated from an artisan cheese, as possible starter cultures for cheese production due to their inability to produce BAs and, at the same time, because they do not alter the sensory characteristics of cheeses.

As a promising approach to the strain selection procedure, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas technique, commonly used for gene editing (Jiang, Bikard, Cox, Zhang, & Marraffini, 2013; Jinek et al., 2012), could also be applied either to inactivate the *hdc* gene and thus to obtain fermentative *hdc*-negative strains, or to ensure a greater phage resistance to starter LAB (Roberts & Barrangou, 2020). By generating these kinds of strains, fermented foods could be developed with similar sensory characteristics to those obtained with traditional strains, but with no histamine content or a greater phage resistance. CRISPR/Cas systems are present in many LAB, predominantly in *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*. However, to our knowledge, no approaches based on CRISPR/Cas techniques in dairy products have been published to date, since in the European Union, CRISPR/Cas methods are considered as genetically modified organisms (GMO) and thus regulatorily restricted. Consumers, and specifically those in the European Union, do not accept the use of GMOs. The United States, for instance, has recently allowed the use of CRISPR-Cas9 edited plants (Plavec & Berlec, 2020). Therefore, although the CRISPR/Cas technique is currently not approved for the production of starters in the European

market, it could serve as an alternative for other international markets.

7.1.2 | Treatments for microbial inactivation in milk

Heat

Heat treatment is an important step in the manufacturing of most dairy products, since high temperature can inactivate the bacterial species responsible for histamine formation (Naila et al., 2010).

-Sterilization virtually inactivates all present microbiota. Sterile milk is microbiologically stable, even at room temperature. Its shelf-life is usually limited by age-gelation (Deeth & Lewis, 2016), a progressive increase in viscosity leading to gel formation that can be associated with the action of heat-resistant proteases (e.g., plasmin or proteases of *Pseudomonas*) or other physicochemical factors (e.g., changes in micelles, availability of calcium ions, etc.).

-Pasteurization inactivates vegetative pathogenic microbiota. However, bacterial spores and vegetative spoilage microbiota (e.g., heat-resistant micrococci and thermophilic streptococci) might survive heat treatment, thus limiting shelf-life. Subsequent bacterial growth to 10^6 CFU/mL causes noticeable undesirable changes, such as acid production, protein breakdown, and lipolysis. Thus, it is necessary to refrigerate pasteurized milk in order to limit bacterial growth, allowing for up to 2 to 3 weeks of storage at 4 °C, depending on the milk's hygienic properties. As mentioned for sterile milk, heat-resistant proteases can also be active in pasteurized milk.

In artisanal dairies, a thermization process is applied to milk at 57 to 68 °C for 15 s or more, whereas in industrialized dairies, the milk is pasteurized at 72 °C for 15 s (Martuscelli et al., 2005).

In general, bacterial counts in cheeses made from pasteurized milk are lower than raw milk cheeses (Novella-Rodríguez et al., 2003). The decrease of the initial microbial load by pasteurization can lead to lower levels of BAs detected in dairy products obtained from pasteurized milk compared to those obtained from raw milk (Benkerroum, 2016). In this regard, Novella-Rodríguez, Veciana-Nogués, Roig-Sagués, Trujillo-Mesa, and Vidal-Carou (2004) reported lower levels of BAs in pasteurized milk cheeses in relation to raw milk cheeses. Tabanelli et al. (2012) determined that the inactivation of the HDC enzyme of *S. thermophilus* required a heat treatment of at least 75 °C for 2 min.

However, once histamine is formed, high-temperature treatment could not destroy it, since BAs appeared to be stable and difficult to degrade (McCabe, Frankel, & Wolfe, 2003).

Milk pasteurization thus contributes to reduce the risk of histamine content in the final cheese. However, survival of HPB or their HDC enzymes to the thermal treatment, and/or contamination with HPB in the subsequent steps of cheese formation (see Section 3.1), might be responsible for histamine outbreaks reported even in pasteurized cheeses (EFSA, 2011).

High-pressure homogenization

Currently, the food industry is particularly interested in nonthermal techniques for the inactivation of microorganisms, including foodborne pathogens. These techniques allow to increase shelf-life while achieving a "fresh-like" product presentation. HPH treatment is one of the most promising food preservation strategies that can help to inactivate microorganisms while likewise avoiding traditional thermal treatments (Lanciotti et al., 2007). In milk, for instance, an HPH treatment in pressure ranges between 100 and 1,200 MPa helps to maintain flavor, body, texture, and nutrients while improving rennet or acid coagulation. Pressure treatment also improves the preservation and rheological properties of yogurt (Chawla, Patil, & Singh, 2011).

HPH treatment can promote histamine synthesis because it produces a higher proteolysis rate than pasteurization, thereby leading to a higher availability of histamine precursors (Novella-Rodríguez, Veciana-Nogués, Saldo, & Vidal-Carou, 2002). Both aminopeptidase activity and free amino acid concentration of ripening cheeses are significantly increased by treatment at 400 or 600 MPa for 21 and 35 days. However, HPH can also inhibit BA formation in cheese depending on the level of pressure applied (Novella-Rodríguez et al., 2002). Total BA formation decreased by about 50% in cheeses treated at 600 MPa compared to untreated cheeses, thus suggesting that HPH exerts an antimicrobial effect (Calzada, Olmo, Picon, Gaya, & Nuñez, 2013). Lower doses of 100 MPa applied to milk before cheese-making also resulted in decreased microbial counts and a lower histamine concentration at the end of the ripening process (Lanciotti et al., 2007).

Therefore, HPH could be regarded by the dairy industry as a suitable treatment aiming to decrease the population of potentially histamine-producing microorganisms and, consequently, to inhibit BA production. This technique is also useful in the development of innovative dairy foods without harmful effects on safety and milk coagulation, as well as for the improvement of cheese yields (Lanciotti et al., 2007). Moreover, HPH can help to achieve improved nutritional and sensory quality combined with longer shelf-life, while maintaining a food's original texture (Chawla et al., 2011).

7.1.3 | Control of physicochemical factors during processing of dairy products

During the production of fermented dairy products, decarboxylase activities and the growth of BA-producing microorganisms are affected by a number of physicochemical factors, such as pH and salt concentration (see Section 4 and Figure 4) (Linares et al., 2012). If good hygiene conditions, controlled pH, and high salt content are achieved, the formation of BAs in cheese is decreased (Valsamaki, Michaelidou, & Polychroniadou, 2000).

Although some authors have proposed that low pH inhibits the accumulation of BAs in ripened cheese (Pintado et al., 2008; Valsamaki et al., 2000), most studies have suggested that acidic pH can encourage the formation of BAs in the course of cheese production (Coton, Rollan, & Lonvaud-Funel, 1998; Ladero et al., 2017; Landete et al., 2008; Marcobal, De Las Rivas, Moreno-Arribas, & Muñoz, 2006). The fermentation of lactose to lactic acid produces a low pH that is difficult to modify, since it is inherent to the milk fermentation process (Linares et al., 2012). In order to neutralize acid stress caused by dairy fermentation, it is assumed that specific amino acid decarboxylases produce BAs (Linares et al., 2012); in fact, the optimal pH for certain amino acid decarboxylases has been reported to be acid. Furthermore, histamine-producing NSLAB are able to survive and grow at low pH, and even produce high amounts of histamine at acidic pH (Barbieri et al., 2019; Frohlich-Wyder et al., 2015). Since formation of BAs raises pH (Barbieri et al., 2019), monitoring of pH could detect increases in pH which might be associated with histamine production. This change of pH could be used as a decision-making tool, for example, for determining the period allotted to the ripening of the monitored cheese.

On the other hand, high salt content seems to reduce BA-producing microbiota and amino acid decarboxylase activity (Linares et al., 2012; Pintado et al., 2008). Salt has been conventionally added to prevent spoilage and food poisoning, while indirectly inhibiting the production of histamine in the final product (Linares et al., 2012). Gardini et al. (2001) demonstrated that a concentration of 5% NaCl minimizes the production of BAs in culture medium and milk by inhibiting microbial growth. However, excessive addition of NaCl should be avoided (Dotsch-Klerk, Goossens, Meijer, & Van het Hof, 2015), since a limited intake of NaCl is recommended (less than 5 g per day) in order to avoid health issues.

Additional preventive measures that could be adopted during processing include low temperatures for ripening. It has been shown that refrigeration can help to reduce the final BA concentration (Calles-Enriquez et al., 2010). Thus, cheese ripening in cold storage and the freezing of cheese samples can reduce the rate of histamine production, probably due to a reduction or inhibition of microbial growth, as well as to a decrease in enzymatic activity of HDC at low temperatures (Martuscelli et al., 2005; Santos, Souza, Cerqueira, & Glória, 2003). However, it is noteworthy to mention that low temperatures could not always be an effective preventive measure, since it has been described that *L. parabuchneri* is capable of producing histamine even in refrigerated cheese (Díaz et al., 2018).

7.2 | Histamine degradation: addition of histamine-catabolizing strains or enzymatic degradation

The food preservation measures expounded above can be useful in preventing the production of histamine, but are in fact unable to eliminate accumulated histamine. As explained in Figure 6, histamine can be biologically catabolized by histamine-degrading microbiota through the activity of DAO enzyme (in the same or a different cell), which breaks down histamine to produce aldehyde, ammonia (which contributes to raise pH), and hydrogen peroxide (Pugin et al., 2017). Thus, to degrade histamine already formed in dairy food, the addition of histamine-degrading bacteria (biological degradation) or degrading enzymes, such as DAO (enzymatic histamine degradation), should also be considered (Naila et al., 2010).

7.2.1 | Addition of histamine-degrading microbiota

Histamine-degrading microbial strains can be used as starter cultures to reduce histamine content in dairy products (Benkerroum, 2016; Dapkevicius, Nout, Rombouts, Houben, & Wymenga, 2000). Guarcello et al. (2016) identified the enzymatic activities responsible for BA degradation in LAB isolated from Italian cheeses. They selected 431 isolates unable to synthesize histamine (*hdc*-negative); 94 of them were also able to degrade histamine during culture in chemically defined medium. Those isolates belonged to the *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus, Streptococcus, Enterococcus*, and *Weissella* genera. Among them, *L. paracasei* subsp. *paracasei* CB9CT exhibited the highest histamine-degrading activity. These results pointed toward a useful strategy to improve safety while maintaining the sensory characteristics of traditional cheeses. Tittarelli, Perpetuini, Di Gianvito, and Tofalo (2019) studied 24 isolates of a raw ewe's cheese unable to produce histamine and, at the same time, able to degrade it. The most interesting strains appeared to be

L. casei A422 and *E. casseliflavus* A143, with degradation rates higher than 50%; thus, they were proposed to be used as starter cultures to reduce the concentration of histamine in raw milk cheeses. Herrero-Fresno et al. (2012) also identified 17 histamine-degrading isolates of *L. casei* from cheese, among which two strains (*L. casei* 4a and 5b) with the highest histamine degradation rates (over 40%) were tested in a Cabrales-like mini-cheese manufacturing model. Due to their validated ability to degrade histamine during cheese ripening, those two *L. casei* strains are proposed as adjunct cultures for the reduction of histamine content in cheese. Leuschner and Hammes (1998) observed a degradation of 55% histamine content during a 4-week ripening period by the *B. linens* strains LTH456 and LTH3686 in a phosphate buffer. A reduction in histamine content was observed throughout the fermentation period of Munster cheese with both strains. Regarding yeasts, the strains of *D. hansenii* H525 and *Y. lipolytica* H446 were demonstrated to degrade several BAs, including histamine, when cultivated in red grape juice with each amine and in phosphate buffer (Baumlisberger, Moellecken, Konig, & Claus, 2015). It is interesting to once more point out the ability of *D. hansenii* to produce histamine as well, but in a strain-dependent manner (Gardini et al., 2006). Physicochemical and sensorial characteristics of dairy products should, nevertheless, be carefully assessed to guarantee their quality.

7.2.2 | Addition of histamine-degrading enzymes

Apart from histamine-degrading strains, the addition of the DAO enzyme represents another strategy for the degradation of preformed histamine (Naila et al., 2012). Although the ability of DAO to degrade histamine has not yet been studied in dairy products, Dapkevicius et al. (2000) and Naila et al. (2012) analyzed the use of DAO to degrade histamine in buffer and fish products. Dapkevicius et al. (2000) concluded that in fish slurry, the addition of DAO was more effective than histamine-degrading bacteria. Histamine degradation by DAO is pH- and temperature-dependent, whereas the addition of sucrose or NaCl does not affect histamine degradation. Naila et al. (2012) also evaluated the action of DAO in a tuna soup, corroborating that it is more efficient than histamine-degrading microorganisms in the removal of histamine from food. Enzymatic degradation of histamine by DAO might be considered a safe strategy in raw milk, since the enzyme would be inactivated by heat treatment before its consumption.

Although DAO is presented as an innovative and promising alternative for the degradation of histamine in food, important drawbacks are also associated with its use, especially in dairy products. First, as mentioned above, the enzymatic activity of DAO strongly depends on pH, temperature, and other environmental conditions. Thus, these parameters need to be adjusted and maintained within the enzyme's optimum ranges of activity, which can turn out to be extremely complicated in certain dairy products since yogurts, for instance, have a very acidic pH and must be stored in refrigerated condition. Second, DAO can be easily added to liquid or semiliquid dairy products, such as milk, yogurt, or kefir, without any inconvenience. It would be quite complicated, however, to add DAO to a complex and heterogeneous matrix as cheese, mainly because of putative problems and limitations of enzyme diffusion. The composition, heterogeneity, and microstructure of the cheese matrix would condition the diffusion pattern of the enzyme (Floury et al., 2010; Silva, Peixoto, Lortal, & Floury, 2013), and subsequently its ability to migrate and find the substrate histamine. Finally, although most dairy products are regarded as basic consumer goods, DAO is an expensive commercial product, and its addition would significantly increase retail prices. The production of greater amounts of DAO at a competitive price could represent an interesting challenge to help promote the implementation of this effective solution for the degradation of histamine from dairy products.

8 | CONCLUSION

Histamine in dairy products constitutes an important safety and health concern, specifically in fermented and ripened products. This BA is produced by present microbiota (gram-positive and gram-negative bacteria, as well as yeasts and molds) from the precursor amino acid histidine via oxidative decarboxylation by the HDC enzyme. It is important to detect and quantify histamine-producing microbiota, particularly through the *hdc* gene, which is responsible for the synthesis of histamine. The accumulation of histamine in dairy products can be additionally prevented by controlling specific environmental and microbiological conditions (pH, temperature, salt concentration, etc.) when preparing dairy products, and/or by applying milk treatments (pasteurization, HPH, etc.). The use of starter cultures unable to produce histamine is another strategy designed to prevent histamine from dairy products. Finally, once histamine is accumulated, it could be necessary to implement its biological or enzymatic degradation through the addition of histamine-degrading microbiota or DAO. Obtaining histamine-free dairy food is a formidable challenge: if met, it would improve the quality of life of histamine-intolerant individuals, but also of the rest of the population, since it would prevent histamine

outbreaks that cause significant harmful health effects on the public at large.

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AUTHOR CONTRIBUTIONS

M. Moniente collected test data and drafted the manuscript. D. García-Gonzalo designed the study, drafted and reviewed the manuscript. I. Ontañón reviewed the manuscript. R. Pagán designed the study, reviewed the manuscript, and carried out project administration and funding acquisition. L. Botello-Morte designed the study, collected test data, interpreted the results, and drafted, reviewed, and edited the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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1.2 Objetivos

El objetivo principal de la tesis doctoral es detectar y cuantificar la presencia de histamina en leche y productos lácteos, e identificar la microbiota responsable de su aparición, así como evaluar posibles soluciones para reducir su acumulación en quesos de larga maduración. Para la consecución de este objetivo general, se establecieron los siguientes objetivos parciales:

1. Puesta a punto de la metodología analítica para la determinación de ABs en leche y productos lácteos y evaluación del contenido en ABs en productos comerciales.
2. Identificación de la microbiota causante de la acumulación de histamina en quesos comerciales.
3. Estudio de la distribución espacial de la histamina y de la microbiota responsable de su acumulación en queso madurado.
4. Puesta a punto de herramientas que permitan reducir la concentración de histamina en queso de larga maduración.

1.3 Justificación de los trabajos

Durante la realización de la Tesis Doctoral se ha podido evidenciar que la histamina puede llegar a suponer un problema en productos lácteos fermentados, siendo relevante detectar y cuantificar los microorganismos que producen esta AB, así como las medidas para prevenir su acumulación mediante el control de parámetros físicos y químicos (pH, temperatura, sal, etc.), la aplicación de procesos de conservación (pasteurización por calor, altas presiones hidrostáticas, etc.), el uso de cultivos iniciadores que no producen histamina y la adición de DAO o cultivos microbianos degradadores de histamina.

Una vez realizada la revisión bibliográfica, se propuso el desarrollo de un nuevo método que permitiera analizar, con un bajo límite de detección, histamina y otras ABs, basado en RP-HPLC acoplado a un sistema de fluorescencia y derivatización previa mediante el uso del derivatizante carbamato de 6-aminoquinolil-N-hidroxisuccinimidilo (AQC). Este método permitió aplicarse en productos lácteos dónde existe una gran variabilidad en la concentración de ABs: leche, yogur, kéfir **(Manuscrito III)**. Este método fue adaptado y utilizado también en el análisis de histamina en queso (**Manuscrito VI**).

Después de la puesta a punto del método de análisis de histamina, se planteó la identificación de la microbiota responsable de su aparición en quesos. Para ello, se investigaron los microorganismos responsables de la producción de histamina en quesos curados comerciales. En aquellos quesos con mayor concentración de histamina se realizaron estudios moleculares para demostrar la presencia del gen *hdc*, responsable de la síntesis de histamina, e identificar la microbiota productora mediante secuenciación Sanger **(Manuscrito IV)**. Con el fin de conocer el patrón espacial de distribución de histamina en el queso, se realizó un análisis de la concentración de esta AB en cuatro zonas de una cuña de queso (zonas periférica e interna del núcleo y de la corteza del queso). Para tratar de explicar la distribución en las distintas áreas, se identificó a los microorganismos presentes en esas muestras mediante análisis de metataxonómica y a los microorganismos que contenían el gen *hdc* mediante secuenciación Sanger, y se relacionó con sus propiedades fisicoquímicas (actividad de agua (a_w), concentración de sal, pH, humedad, oxidación lipídica) **(Manuscrito V)**.

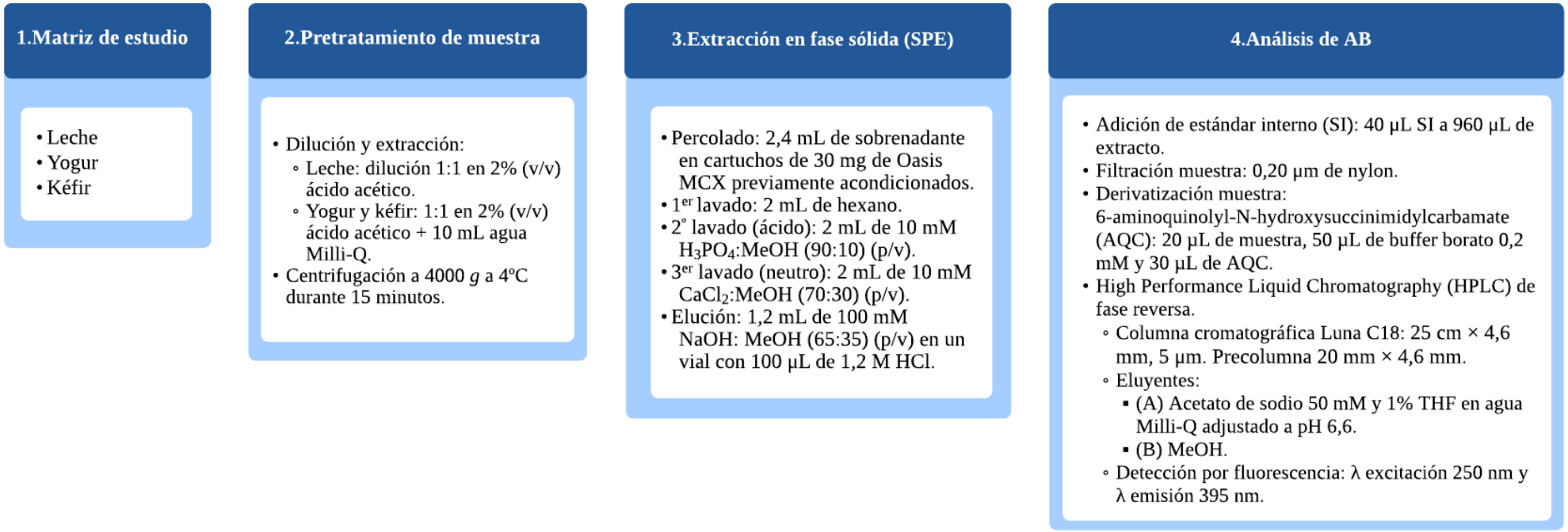
Por último, se aplicaron diferentes estrategias con el objetivo de reducir la concentración de histamina en quesos curados cuando es generada mediante uno de los mayores productores de esta AB, *L. parabuchneri*. Se inocularon diferentes cultivos microbianos con capacidad degradadora de histamina y la enzima DAO para disminuir la acumulación final de histamina en quesos tras 100 días de maduración. Además, se llevó a cabo una caracterización de las propiedades físicas y químicas, y un análisis sensorial de los quesos elaborados para determinar el efecto de la implantación de estas medidas sobre el olor del queso **(Manuscrito VI)**.

2. Materiales y métodos

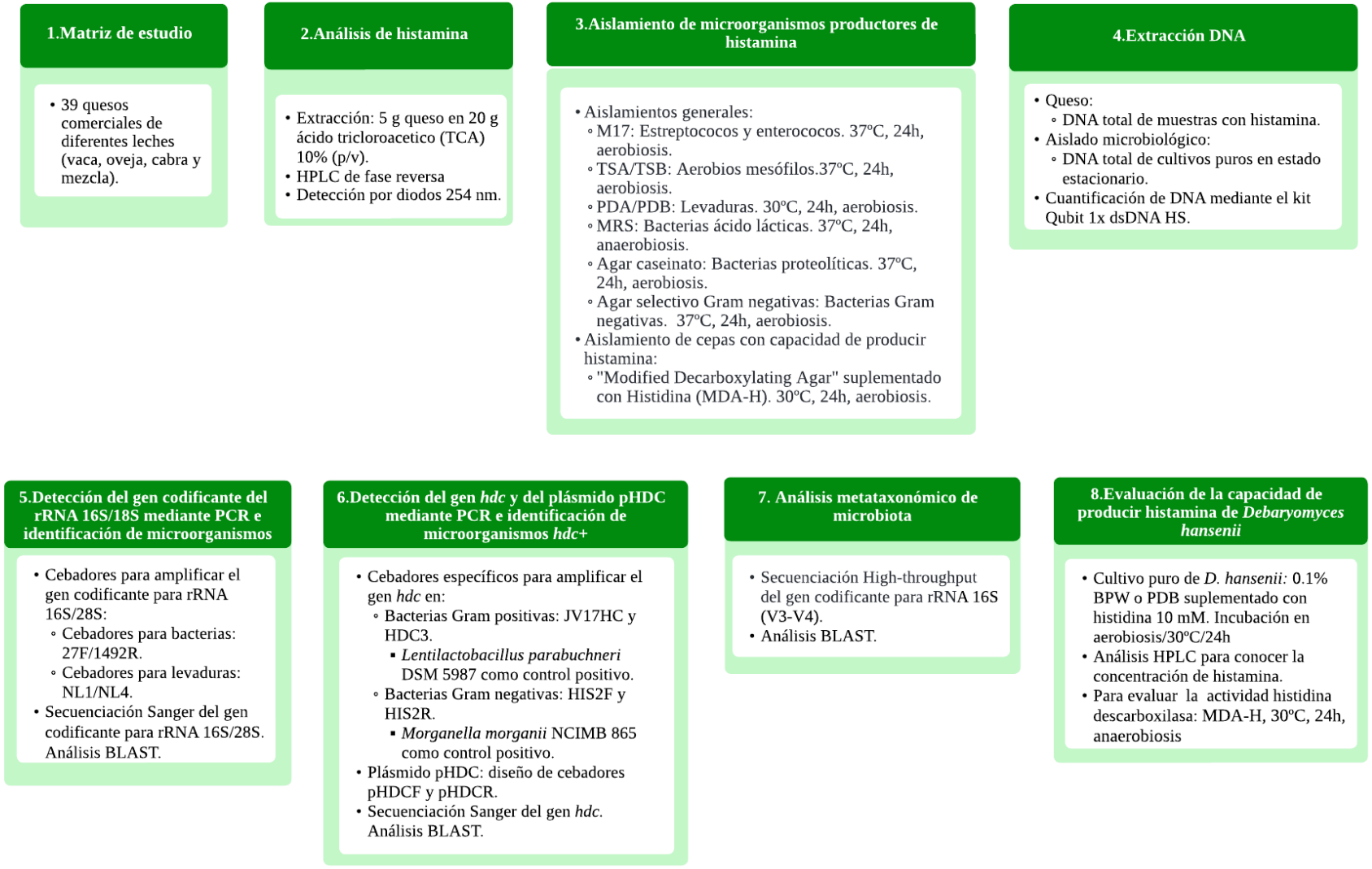
2. Materiales y métodos

Debido a que en los manuscritos publicados se han descrito ampliamente las distintas metodologías empleadas, en esta sección se expone, de manera esquemática, el diseño experimental llevado a cabo para lograr cada uno de los objetivos experimentales de la presente Tesis Doctoral.

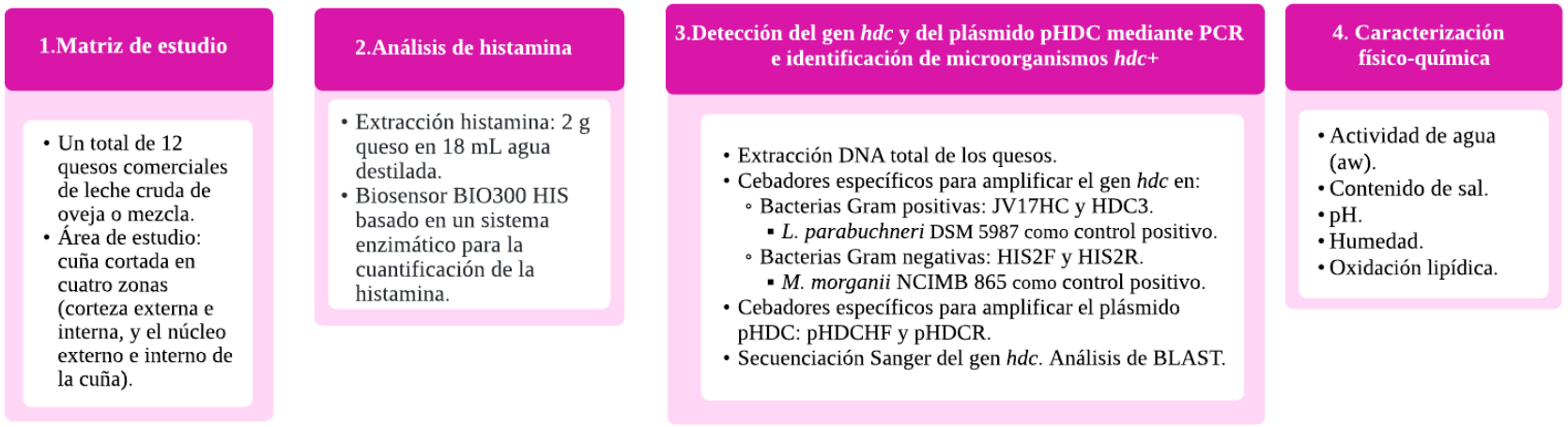
1. Desarrollo de un método de análisis de ABs en productos lácteos (Manuscrito III). Para ello se siguió la siguiente metodología:



2. Identificación de la microbiota responsable de la acumulación de histamina en quesos comerciales (Manuscrito IV), realizando el siguiente procedimiento:



3. Evaluación de la distribución espacial de la histamina y de la microbiota productora en cuñas de queso curados (Manuscrito V):



4. Estudio de diversas estrategias para disminuir la concentración de histamina en quesos de larga maduración (Manuscrito VI). En este sentido, se realizó el siguiente diseño experimental:

1.Selección y evaluación de cultivos microbianos y/o DAO

Evaluación de la capacidad de degradación de histamina *in vitro* de:
- Colección Española de Cultivos Tipo (CECT):
  - *Lactobacillus bulgaricus* CECT 4005.
  - *Streptococcus thermophilus* CECT 7207.
- Cultivo iniciador Yogur Tipo I (Abiasa).
- Cultivo iniciador YoFlex® Premium (CHR Hansen).
- Cepa *Debaryomyces hansenii* aislada de queso (Botello-Morte et al., 2022).
- Cepas de *Lacticaseibacillus casei* 4a y 18b (Herrero-Fresno et al., 2012).
- Diamino Oxidasa (DAO).

2.Confirmación de ausencia del gen *hdc* mediante PCR

- Búsqueda del gen *hdc* de las bacterias con potencial de degradación de histamina mediante el uso de cebadores JV17HC y HDC3.
- *L. parabuchneri* DSM 5987 como control positivo.

3. Preparación de lotes de queso con cepas potencialmente degradadoras de histamina

Se elaboraron 8 lotes de queso. Todos ellos contenían el cultivo iniciador F-DVS GRANA-102 (CHR Hansen) para la elaboración de queso. También se añadió *L. parabuchneri* DSM 5987 como microorganismo productor de histamina, excepto en el control negativo:
(I): Control negativo.
(II): Control positivo solo con $1{\cdot}10^6$ unidades formadoras de colonia (UFC)/mL de *L. parabuchneri* DSM 5987.
(III): $1{\cdot}10^6$ UFC/mL de *L. casei* 4a y *L. casei* 18b.
(IV): $1{\cdot}10^6$ UFC/mL de *Lb. bulgaricus* CECT 4005 y *S. thermophilus* CECT 7207.
(V): $1{\cdot}10^6$ UFC/mL de cultivo iniciador Yogur Tipo I (Abiasa).
(VI): $1{\cdot}10^6$ UFC/mL de cultivo iniciador YoFlex® Premium (CHR Hansen).
(VII): $1{\cdot}10^5$ UFC/mL de *D. hansenii* aislado de queso
(VIII): 71 U/L de DAO.

4.Análisis de histamina

- Siguiendo el método de análisis de aminas biógenas del punto 1:
  - Extracción:
    - Queso: 1:5 en 1M HCl.
  - Centrifugación a 4000 *g* a 4ºC durante 21 minutos.
  - SPE.
  - Derivatización con AQC.
  - RP-HPLC.
  - Detección por fluorescencia.

5.Análisis sensorial mediante Sorting Task

- Clasificación de los lotes de quesos por la similitud de olor a 100 días de maduración:
  - 7 g de muestra.
  - 42 catadores no entrenados.

6.Caracterización físico-química de los quesos madurados

- aw.
- pH.
- Color.
- Peso.
- Textura (dureza y adhesividad).

3. Resultados

Manuscrito III. Combination of SPE and fluorescent detection of AQC-derivatives for the determination at sub-mg/L levels of biogenic amines in dairy products

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Combination of SPE and fluorescent detection of AQC-derivatives for the determination at sub-mg/L levels of biogenic amines in dairy products

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Abstract

Biogenic amines (BAs) are compounds generated by decarboxylation of their amino acid precursors. Their intake, even at low concentrations, can lead to several types of health problems in sensitive individuals. As they can be easily formed in fermented dairy products, their quantitative determination is very relevant. In the present paper, a method for the quantitative determination of four biogenic amines in different dairy products has been developed, validated and applied to 37 samples of milk, 23 of yogurt, and 14 of kefir. Amines were selectively extracted using solid phase extraction, subsequently derivatizatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and further determined by High Performance Liquid Chromatography with fluorescence detection.

Abbreviations: AQC: 6-aminoquinolyl-N-hydroxyuccinimidyl carbamate; BAs: biogenic amines; BNZ-Cl: benzoyl chloride; CAD: cadaverine; DBS-Cl: dabsyl chloride; DNS-Cl: dansyl chloride; EDTA: Ethylenediaminetetraacetic acid; ELISA: enzyme-linked immunosorbent assay; HCl: hydrochloric acid; HIM: histamine; IS: internal standard; LC: liquid chromatography; LOD: limit of detection; LOQ: limit of quantification; MeOH: methanol; ND: non-detected; NSLAB: non-starter lactic acid bacteria; OPA: o-phthalaldehyde; PUT: putrescine; PVDF: polyvinylidene fluoride; RP-HPLC: Reverse Phase-High Performance Liquid Chromatography; RSD: relative standard deviation; SLAB, starter lactic acid bacteria; RSD: relative standard deviation; SPD: spermidine; SPE: solid phase extraction; SPM: spermine; TBA: total biogenic amines; THF: Tetrahydrofuran; TYM: tyramine; UHT: ultra-heat treated milk.

The method's sensitivity was highly satisfactory, with limits of detection lower than 0.2 mg/L. Optimal linearity and repeatability were also achieved. BAs were not detected in most of the milk samples, but they were found frequently at high levels in yogurt and kefir samples, reaching values of up to 79 mg/kg total BAs in kefir samples. Levels measured should not be a cause for concern for the population at large, but should be known by BAs-sensitive individuals.

Keywords: Histamine, tyramine, putrescine, cadaverine, dairy products, HPLC, AQC, biogenic amines

1. Introduction

Biogenic amines (BAs) are low-molecular-weight nitrogenous compounds formed by enzymatic decarboxylation of their precursor amino acids (Linares et al., 2011; Zhang et al., 2019) or by amination and transamination of aldehydes and ketones (McCabe et al., 2003; Pluta-Kubica et al., 2020). The presence of BAs in food and beverages is brought about by microbial enzymes inherent to the raw material, or by enzymes from spoilage microorganisms (Özogul & Özogul, 2020).

The presence of BA in food constitutes a public health concern due to their physiological and toxicological effects (Ruiz-Capillas & Herrero, 2019). Although these compounds are involved in beneficial metabolic functions in humans including the immune system, the regulation of body temperature, and the growth and renewal of organs (Papageorgiou et al., 2018), the presence of high amounts of certain BAs can lead to cases of food poisoning (Schirone et al., 2016). BAs such as histamine (HIM), tyramine (TYM), putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM) are involved in toxicological processes, since they can cause headache, accelerated heart rate, urticaria, nausea, allergies, and blood pressure alterations (Alvarez & Moreno-Arribas, 2014). TYM, PUT, and CAD produce synergistic cytotoxic effects together with HIM due to the inhibition of the detoxifying enzymes, which can increase the amines' cytotoxicity at their usual concentrations in food (del Rio et al., 2017; Palomino-Vasco et al., 2019).

Sensitive individuals may also be toxicologically affected by ingestion of low concentrations of BAs (Bodmer et al., 1999). The individual toxicological threshold can vary from a few mg/kg in a sensitive person to approximately one hundred mg/kg in a healthy person (Gardini et al., 2016).

HIM, TYM, PUT and CAD are the most common BAs present in dairy foods (Linares et al., 2012). Their occurrence is due to non-starter lactic acid bacteria (NSLAB) naturally occurring in milk, to bacteria added to the milk as a starter (SLAB), or to undesired bacteria caused by contamination from poor processing hygiene (Ladero et al., 2017; Moniente et al., 2021).

Fermented dairy products contain variable amounts of BAs. Ripened cheeses are the products most usually associated with high concentrations of BAs as a result of microbial activity (Schirone et al., 2016), reaching quantities of 1,844.5 mg/kg of CAD (Andiç et al., 2010; Papageorgiou et al., 2018) and 2,500 mg/kg of HIM (Bodmer et al., 1999; Moniente et al., 2021). Other dairy foods such as milk, yogurt, kefir, and unripened cheeses have lower concentrations of BAs, ranging from few milligrams to tens of milligrams per kg (Linares et al., 2011; Spano et al., 2010). In spite of these lower amounts, analytical controls to ensure safe levels and to improve manufacturing processes and practices are essential, particularly because of the expected notable increase of consumption rates of dairy products in the near future (Papageorgiou et al., 2018).

Due to the great variability of BAs concentration in dairy foods, as well as to the diversity of the toxicological threshold in healthy people as compared to those who are sensitive to BAs, it is important to have sensitive-enough analytical methods to supply consumers with safe dairy products – eventually certified as BAs-free.

Dairy products are considered as complex matrices with high percentages of proteins and fats that make it difficult to determine some analytes, for example BAs. Solid-liquid (S-L) or liquid-liquid (L-L) extraction methods depends on the dairy product´s texture, have been widely used for the determination of BAs in dairy products. However, these techniques have several disadvantages, such as slowness, high amounts of harmful organic solvents, low repeatability, a large sample volume…Solid phase extraction (SPE) is

a sample preparation technique which is free of these disadvantages. Moreover, different cleaning steps can be applied obtaining very clean extracts but, surprisingly it has been only occasionally used for the analysis of BAs in dairy products.

Several instrumental techniques have been used to quantify BAs in dairy products (thin layer chromatography, capillary electrophoresis, enzyme-linked immunosorbent assay (ELISA), biosensor), but liquid chromatography (LC) is undoubtedly the most widely used. Moreover, derivatization reactions tend to be applied prior to LC in order to improve separation and detection of BAs (Moniente et al., 2022).

Different chemical derivatizing reagents have been used for BAs determination in dairy products: dansyl chloride (DNS-Cl), o-phthalaldehyde (OPA), ninhydrin, benzoyl chloride (BNZ-Cl), fluorescamine, dabsyl chloride (DBS-Cl), fluorescein isothiocyanate, phenyl isothiocyanate, and fluorenyl 9-methylchloroformate (Korös et al., 2008; Moniente et al., 2022; Önal, 2007). A reagent seldom used for dairy products as an alternative to the most common chemicals is 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). It has nevertheless been used in pre-column derivatization for the analysis of primary and secondary amino acids and BAs (Korös et al., 2008). With AQC, highly stable derivatives are obtained, which produce intense fluorescent signals (Moniente et al., 2022).

The main objective of the present study was to develop and validate a sensitive (limit of detection lower than 1 mg/L) and reliable reverse phase-high performance liquid chromatography (RP-HPLC) method using a combination of SPE and AQC for the simultaneous determination of HIM, TYM, PUT, and CAD in dairy products. The developed method was validated and applied in the present study to commercial and non-commercial milk, yogurt, and kefir samples from Spanish sources in order to assess the occurrence and relevance of those analytes in these kinds of products.

2. Experiments

2.1 Chemicals, reagents, and standard solutions

Histamine (dihydrochloride) (≥99 %), tyramine (hydrochloride) (≥98 %), putrescine (dihydrochloride) (≥98 %), cadaverine (dihydrochloride) (≥98 %), the internal standard (IS) 1,7-diaminoheptane (≥98 %), ethylenediaminetetraacetic acid (EDTA) (≥98 %) and tetrahydrofuran (THF)

(≥99.9 %), were used and purchased from Sigma Chemicals (St. Louis, MO, USA).

Standard solutions of the four BAs (1,000 mg/L) were prepared by dissolving the pure compounds in Milli-Q water. For the IS, a solution of 3,000 mg/L of 1,7-diaminoheptane was prepared with Milli-Q water. These solutions were stored at 4º C until they were used. The AccQ Fluor reagent kit, consisting of AQC reagent, acetonitrile for dissolution of the reagent powder, and 0.2 mM sodium borate buffer (pH 8.8) for derivatization was supplied by Waters (Milford, MA, USA).

The reagents and HPLC solvents (methanol and hexane) used in this study were of chromatographic grade and were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from Milli-Q System (Millipore Corp., Milford, MA, USA). Acetic acid (CH_3COOH), phosphoric acid (H_3PO_4), calcium chloride ($CaCl_2$), sodium acetate ($C_2H_3NaO_2$), hydrochloric acid (HCl) and sodium hydroxide (NaOH) were obtained from Panreac (Barcelona, Spain).

2.2 Proposed method

A total of 5 mL of the milk sample or 10 mL of a 1:1 yogurt:water dilution are mixed with 5 mL of 2% acetic acid in a 15 mL plastic centrifuge tube and vortexed for 30 s. The tube is then centrifuged at 4000 *g* at 4 °C for 15 min to separate lipids and proteins from the aqueous phase. 2.4 mL of the clear supernatant are then taken and percolated through 30 mg of Oasis MCX SPE cartridges from Waters, previously conditioned by passing 2 mL of MeOH followed by 2 mL of Milli-Q water. The cartridges are then dried under vacuum, washed up with 2 mL of hexane to remove residual fats, dried again and washed first with 2 mL of 10 mM H_3PO_4:MeOH (90:10) solution followed by 1 mL of Milli-Q water, and then with 2 mL of a 10 mM $CaCl_2$:MeOH (70:30) solution also followed by 1 mL of Milli-Q water. The analytes are subsequently eluted with 1.2 mL of 100 mM NaOH: MeOH (65:35) in a vial already containing 100 µL of 1.2 M HCl. Next, 40 µL of IS was added to 960 µL of the extract and then they are filtered through 0.20 µm nylon filters (Sartorius, Goettingen, Germany).

For derivatization, 20 µL of standard or sample are first buffered with 50 µL of a

solution containing 0.2 M of sodium borate at pH 8.8 and 5 mM of disodium EDTA. Subsequently, 30 µL of the AQC solution are added to perform the derivatization reaction at 55 ºC for 10 min.

2.3 Chromatographic conditions

The separation of the AQC-derivatives of the amines was carried out in a reversed-phase Luna C_{18} chromatographic column (25cm×4.6mm, 5µm) (Phenomenex, Torrance, CA, USA) preceded by a 20 mm×4.6 mm precolumn and kept at 65 °C. The HPLC system was a 1220 Infinity LC device from Agilent Technologies (Santa Clara, CA, USA) coupled to a Prostar 363 fluorescence detector from Varian (Sunnyvale, CA, USA) set at excitation wavelength of 250 nm and emission wavelength of 395 nm. Data acquisition and processing were carried out with Chrom-Card software. The eluents were 50 mM sodium acetate in 1% THF in Milli-Q adjusted to pH 6.6 by the addition of acetic acid (A) and MeOH (B) according to the methodology described by Mayer et al. (2010), with some modifications. Injection volume was 5 µL. The elution program consisted of a gradient system with a flow rate of 1 mL/min using the following optimized gradient: from 15 to 80 % of eluent B linearly during 25 min, increasing to 100 % B within 1 min, and holding at 100% B for 5 min. Subsequently, decreasing to 15 % B over 1 min, and holding at 15 % B for 5 min.

2.4 Method validation

Method development and validation were carried out using whole cow's milk and Milli-Q water. Limit of detection (LOD), limit of quantitation (LOQ), linearity, repeatability, and stability were assessed. LOD and LOQ were estimated as the concentration that gave a signal-to-noise ratio of 3 and 10, respectively. They were calculated in water and milk spiked with low amounts of BA.

Calibration curves were prepared by adding increasing amounts (five points) of the stock solution containing the four analytes to milk and ultrapure water, in a range from 1 to 20 mg/L. t-tests were performed to analyze the effects of the matrix by comparing the slopes of the calibration curves for the different samples.

The method’s repeatability was studied by analyzing unspiked and spiked milk samples in triplicate.

2.5 Analysis of dairy samples

Seventy-four Spanish dairy samples (37 milk samples, 23 yogurt samples, and 14 kefir samples) were analyzed. Some of them were available in Spanish retail stores, whereas others were supplied by dairy product manufacturers. Information regarding milk, yogurt, and kefir samples is listed in Table 1. Samples were kept at -18º C until their analysis.

The quantification of BA was carried out by using a sample-dependent response factor estimated by the analysis of the own sample spiked with known amounts (5 mg/L) of HIM, TYM, PUT, and CAD. The increase in the area of the measured peak was used to calculate the response factor.

3. Results and discussion

In the present study, an analytical method for the quantification of BAs in dairy products has been optimized and validated. The SPE and the derivatization procedures are based on the method previously developed by Peña-Gallego et al., (2009) for the analysis of BAs in wine, with some modifications. As dairy products have physicochemical properties quite different from those of wine, the different steps of the method were reoptimized and further validated. The method was subsequently applied to the analysis of milk, yogurt, and kefir samples from Spain, in order to evaluate the presence of BAs in those kinds of products.

3.1 Optimization of the analytical method

Breakthrough volumes: The breakthrough volume is the maximum volume of sample which can be introduced into the SPE sorbent without appreciable losses of analytes (Bielicka-daszkiewicz & Voelkel, 2009). Breakthrough volumes were determined by percolating through the SPE cartridge a 6-mL milk sample spiked with 20 mg/L of each BAs. The percolate was collected in 10 consecutive 600-µL fractions, which were filtered, derivatized and analyzed. Results can be seen in Figure 1. The most retained BAs was TYM, which only could be detected in the 10th fraction, corresponding to the last 600 µL of the 6 mL percolated. HYM was also strongly retained and was only detected in the 9th and 10th fractions. By contrast PUT was the BAs most weakly retained in the SPE device, and was already detected in the 4th fraction, so

Table 1. Characterization of milk, yogurt, and kefir samples regarding type of dairy product, milk source, fat content, heat treatment, biogenic amine (histamine, tyramine, putrescine and cadaverine), contents quantified by HPLC, and total biogenic amines (TBA)

Sample Code	Type of Dairy Product	Milk Source	Fat content	Heat Treatment	Histamine[a]	Tyramine[a]	Putrescine[a]	Cadaverine[a]	TBA[a]
M1	Milk	Cow	Whole	UHT	<LOD	<LOD	<LOD	<LOD	-
M2	Milk	Cow	Whole	UHT	<LOD	<LOD	<LOD	<LOD	-
M3	Milk	Cow	Whole	UHT	<LOD	<LOD	<LOD	<LOD	-
M4	Milk	Cow	Whole	UHT	<LOD	<LOD	<LOD	<LOD	-
M5	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M6	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M7	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M8	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M9	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M10	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M11	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M12	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M13	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M14	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M15	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M16	Milk	Cow	Semi-skimmed	UHT	<LOQ	<LOD	<LOQ	<LOD	-
M17	Milk	Cow	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOD	-
M18	Milk	Cow	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOD	-
M19	Milk	Cow	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOQ	-
M20	Milk	Cow	Skimmed	UHT	<LOD	<LOD	<LOD	<LOQ	-
M21	Milk	Cow	Skimmed	UHT	<LOD	<LOD	<LOD	<LOD	-
M22	Milk	Cow	Skimmed	UHT	6.239	9.129	7.054	17.690	40.112
M23	Milk	Cow	Skimmed	UHT	<LOD	<LOD	<LOD	<LOD	-

M24	Milk	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
M25	Milk	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
M26	Milk	Sheep	Semi-skimmed	UHT	<LOD	<LOQ	<LOD	<LOQ	-
M27	Milk	Sheep	Semi-skimmed	UHT	<LOD	0.690	<LOD	1.060	1.750
M28	Milk	Sheep	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOD	-
M29	Milk	Sheep	-	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M30	Milk	Sheep	-	Pasteurization	<LOD	<LOD	<LOD	<LOD	-
M31	Milk	Sheep	-	-	<LOD	0.807	<LOD	<LOD	0.807
M32	Milk	Goat	Semi-skimmed	UHT	<LOD	<LOD	<LOD	1.303	1.303
M33	Milk	Goat	Semi-skimmed	UHT	<LOD	<LOD	<LOD	0.519	0.519
M34	Milk	Goat	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOQ	-
M35	Milk	Goat	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOD	-
M36	Milk	Goat	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOD	-
M37	Milk	Goat	-	-	<LOD	0.860	<LOD	<LOD	0.860
Y1	Yogurt	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
Y2	Yogurt	Cow	-	-	0.819	1.071	2.734	5.440	10.064
Y3	Yogurt	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
Y4	Yogurt	Cow	-	-	3.054	<LOD	0.418	<LOD	3.472
Y5	Yogurt	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
Y6	Yogurt	Cow	-	-	<LOD	<LOQ	<LOD	<LOD	-
Y7	Yogurt	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
Y8	Yogurt	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
Y9	Yogurt	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
Y10	Yogurt	Cow	-	-	<LOD	<LOD	1.704	<LOD	1.704
Y11	Yogurt	Cow	-	-	<LOD	<LOD	2.449	<LOQ	2.449

Y12	Yogurt	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
Y13	Yogurt	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
Y14	Yogurt	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
Y15	Yogurt	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
Y16	Yogurt	Cow	-	-	<LOD	<LOD	0.985	<LOD	0.985
Y17	Yogurt	Sheep	-	-	1.153	<LOD	<LOD	<LOD	1.153
Y18	Yogurt	Sheep	-	-	17.160	<LOD	8.955	<LOD	26.115
Y19	Yogurt	Sheep	-	-	8.064	<LOD	27.013	<LOD	35.077
Y20	Yogurt	Goat	-	-	<LOD	<LOD	<LOD	<LOD	-
Y21	Yogurt	Goat	-	-	<LOQ	2.108	0.891	<LOQ	3.266
Y22	Yogurt	Goat and Sheep	-	-	<LOQ	<LOQ	<LOD	<LOD	0.637
Y23	Yogurt	Goat and Sheep	-	-	<LOD	<LOD	<LOD	<LOD	-
K1	Kefir	Cow	-	-	<LOD	<LOQ	0.878	1.214	2.092
K2	Kefir	Cow	-	-	0.831	2.679	5.945	6.492	15.947
K3	Kefir	Cow	-	-	1.211	2.142	7.003	9.169	19.525
K4	Kefir	Cow	-	-	<LOD	1.579	14.055	64.032	79.666
K5	Kefir	Cow	-	-	<LOD	<LOD	0.452	<LOD	0.452
K6	Kefir	Cow	-	-	<LOD	2.598	10.970	9.420	22.988
K7	Kefir	Cow	-	-	<LOD	3.678	3.588	3.746	11.012
K8	Kefir	Cow	-	-	<LOD	<LOD	<LOD	<LOD	-
K9	Kefir	Cow	-	-	<LOD	<LOD	<LOD	<LOD	
K10	Kefir	Cow	-	-	<LOD	2.830	<LOD	<LOD	2.830
K11	Kefir	Goat	-	-	<LOD	<LOD	3.717	24.221	27.938
K12	Kefir	Goat	-	-	<LOD	0.850	2.799	<LOD	3.649
K13	Kefir	Goat	-	-	<LOD	<LOD	<LOD	<LOD	-
K14	Kefir	Goat	-	-	<LOD	<LOD	7.870	<LOD	7.870

LOD: Limit of detection; LOQ: Limit of quantification

[a] mg/L for milk and mg/kg for yogurt and kefir.

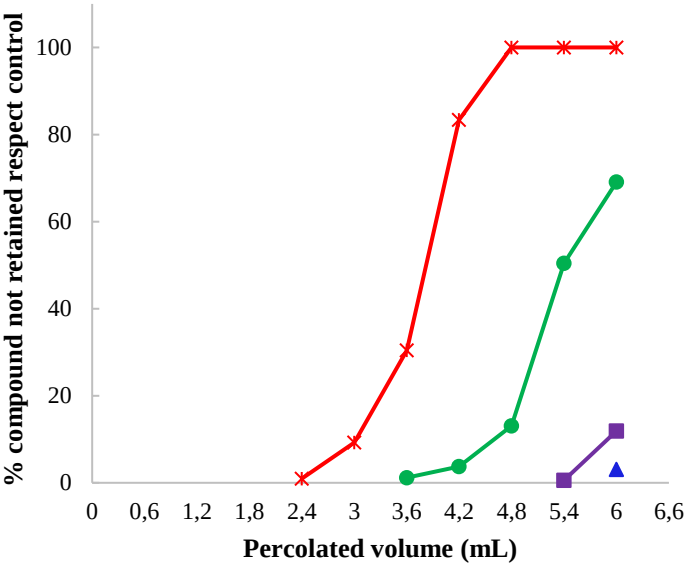

Figure 1. Breakthrough volume of biogenic amines (putrescine, histamine, tyramine, and cadaverine) present in whole milk in the Solid Phase Extraction (SPE) cartridges used in the method.

that its breakthrough volume is around 2,400 µL. Consequently, 2.4 mL was chosen as the maximum volume of sample to be loaded in the cartridge.

Washing steps: Although a large part of fat is removed in the initial centrifugation of the acidified sample, the elimination was not enough to avoid an evident turbidity of the final extracts. For this reason, a washing step with hexane was included in the procedure to completely eliminate fats and avoid possible interferences. For this experiment, 2.4 mL aliquots of whole milk samples spiked with 10 mg/L of BAs were loaded in the SPE cartridges. Increasing volumes of hexane (0, 2, 4, 6, and 8 mL) were then applied, and analytes were further eluted, derivatized and analyzed. These experiments were carried out in duplicate. Results revealed that a washing volume of 2 mL was sufficient to obtain clean extracts without turbidity.

As shown in Figure 2, the washing with hexane did not caused appreciable losses of analytes up to 6 mL, however, the imprecision increased significantly at volumes above 4 mL. Therefore, 2 mL of hexane was retained as the optimal volume.

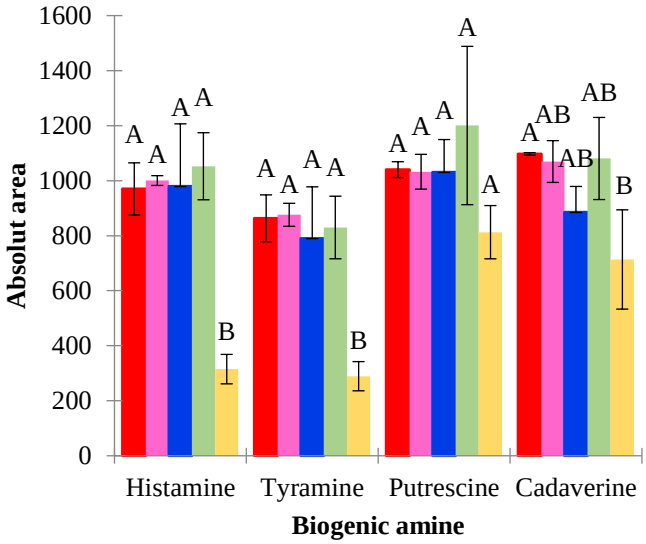

Figure 2. Effect of different hexane washing up volumes (0, 2, 4, 6 and 8 mL) for the removal of fats from the SPE cartridge on the chromatographic area of biogenic amines analyzed by AQC-HPLC-FLD. Capital letters indicate significant differences ($p<0.05$).

The three hydro-methanolic washing solutions proposed by Peña-Gallego et al. (2009) were also studied. The first is an acid solution (10 mM H_3PO4: MeOH [70:30]), the second is a neutral solution (10mM $CaCl_2$: MeOH [70:30]) and the third is an alkaline solution (10mM NaOH: MeOH [70:30]). For this, five aliquots of a 10 mg/L BAs spiked whole milk sample were loaded in the SPE cartridge and washed with hexane. In one of the aliquots no aqueous washing solutions (acid, neutral, and basic) were applied, in the second the acid washing was omitted, in the third, it was the neutral washing omitted, in the fourth the acid, and in the fifth, the three washings were included. Results are shown in Figure 3. As can be seen, only in the experiments in which the alkaline washing was omitted (blue bars in the figure), the levels of BAs recovered were not significantly smaller than those measured in the reference without any washing. Therefore, the basic washing solution was thus not applied to the final procedure.

Filtration: Four different filters (0.22 µm Polyvinylidene Fluoride (PVDF), 0.2 µm nylon and 10k and 3k ultrafiltration filters) were considered for the filtration of the eluted sample. The resulting chromatograms (available in

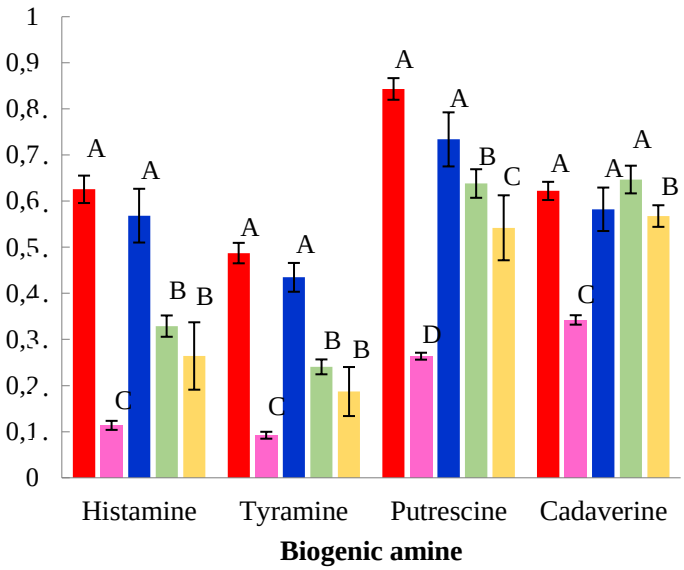

Figure 3. Effect of different polar washing up procedures on the content of BAs retained in the SPE cartridge. : reference (no washing); , neutral and alkaline washings, , acid and neutral washing; , acid and alkaline washings, , all three washings. Capital letters indicate significant differences ($p<0.05$).

Supplementary Material) revealed that the 10k and 3k ultrafiltration filters introduced a range of impurities interfering with the chromatographic peaks of the BAs, and that PVDF filters retained significant amounts of all the analytes. The best results were obtained with the 0.2 µm nylon filters, which were selected for the procedure.

Derivatization reaction: In order to obtain the best yield of the derivatization reaction, the influence of the pH of the extract previous to the reaction was optimized. For that, five standard

solutions containing 5 mg/L of each BAs, and pH in the range 1 to 12 (1, 5, 7, 10 and 12) were prepared, filtered and derivatized with the addition of 20 µL of the AQC reagent. Results are shown in Figure 4 and reveal that HIM and TYM were best derivatized at pH 10, while PUT and CAD, showed maxima signals at pH 12. Derivatization time was also studied by varying reaction times between 10 and 60 min, as can be seen in Figure 5. The result obtained for HIM showed a higher signal at 10 min compared to the other times we assessed (20, 30 and 60 min). Similar results occurred for the other three biogenic BAs (TYM, PUT and CAD): a greater signal appeared after 10 min of derivatization compared to other time intervals we studied (although these differences were not significant).

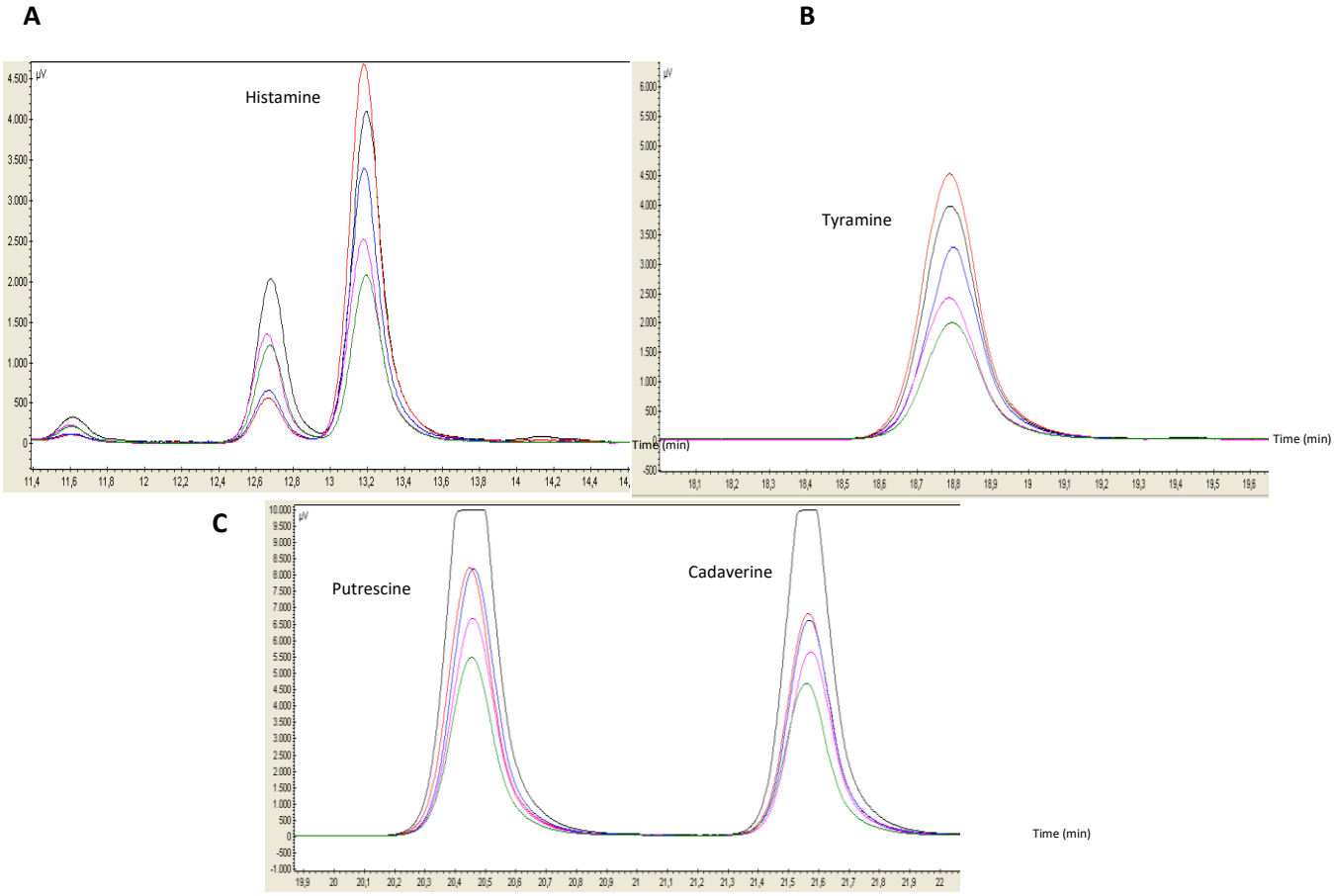

Figure 4. HPLC chromatograms of the influence of the pH variation (1■;5■;7■;10■;12■) on the signal of: (A) histamine, (B) tyramine, and (C) putrescine and cadaverine

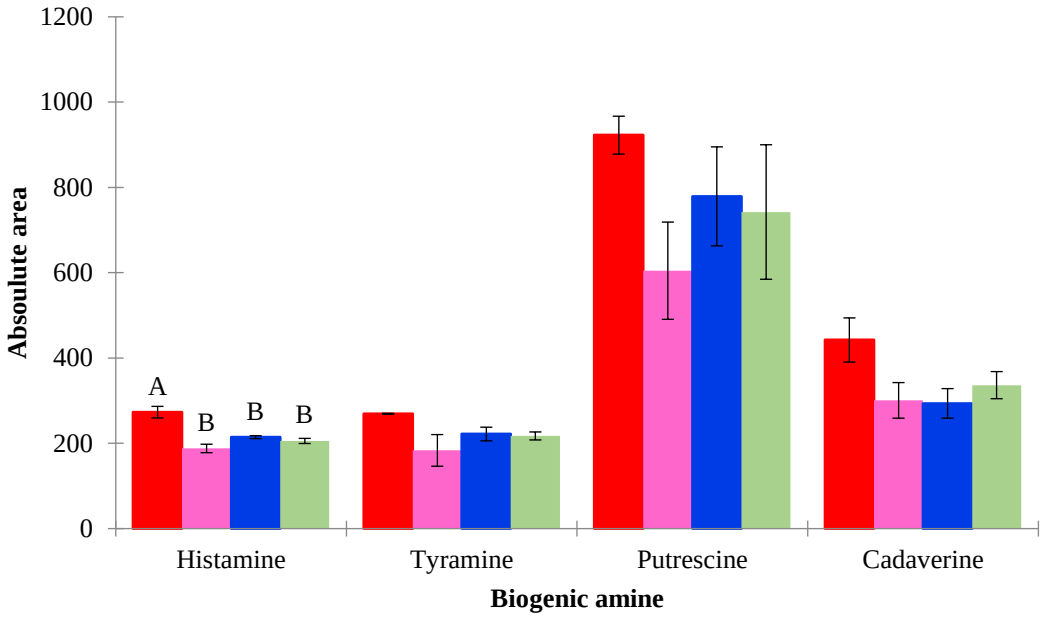

Figure 5. Effect of derivatization time (10 ■, 20 ■, 30 ■ and 60 ■ min) on the chromatographic area of biogenic amines in dairy products analysed by AQC-HPLC-FLD. Capital letters indicate significant differences ($p<0.05$).

Derivatization of BAs with AQC at 55 ºC for 10 min was the optimal condition, confirming the manufacturer (Waters) recommendation.

Some reproducibility problems were found with the signal of the derivatized samples; we therefore surmised that the temperature of the extracts after the derivatization process might be exerting an influence on their stability. To test this hypothesis, we stored extracts after derivatization at room temperature, refrigeration temperature (4 ºC), and freezing temperature (-18 ºC) for 5 min to decrease their temperature, after which they were analyzed. Based on absolute areas, the signals shown for all four BAs in the assay at refrigeration and freezing temperatures are higher than those observed at room temperature (Figure 6). Furthermore, results show better reproducibility at freezing temperature than at room temperature. The relative standard deviation (RSD) of samples stored at freezing temperature ranged from 2.19 % to 5 % for the different BAs, whereas the estimated values for samples stored at room temperature ranged from 3.23 % to 51.89 %. Samples were therefore kept at -18ºC for 5 min after the derivatization step.

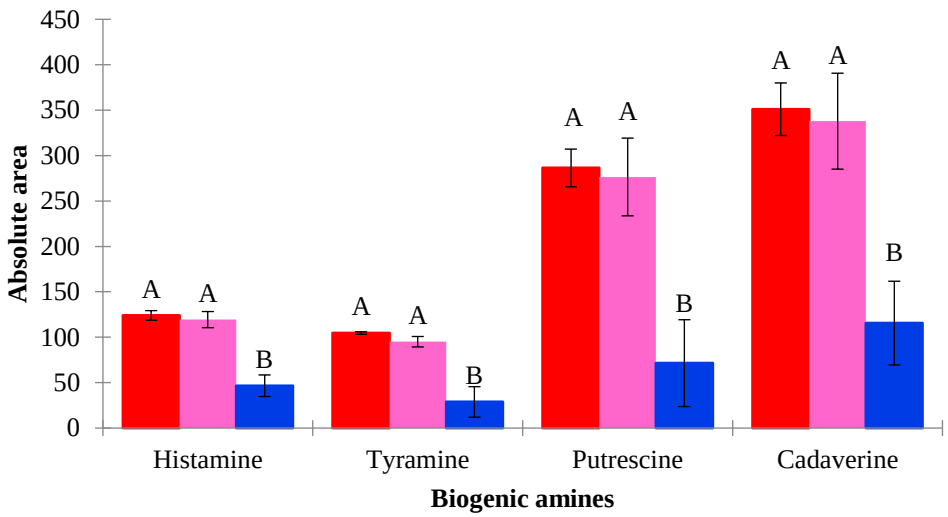

Figure 6. Effect of different temperatures (freezing ■, cooling ■, and room ■) on the chromatographic area of biogenic amines in dairy products analysed by AQC-HPLC-FLD. Capital letters indicate significant differences ($p<0.05$)

3.2 Method validation

The chro matograms of spiked milk, yogurt, and kefir samples are given in Figure 7. Repeatability of the experiment tested in milk samples is listed in Table 2. In the present study, mean repeatability of 6 milk samples was 9.34, 12.47, 2.68, and 6.36 % of HIM, TYM, PUT, and CAD, respectively. Good repeatability was also obtained in three samples of water spiked at 5 mg/L (0.27 %, 1.03 %, 7.84 %, 5.83 % of HIM, TYM, PUT, and CAD, respectively).

LODs and LOQs of the four BAs are also shown in Table 2. LODs ranged from 0.12 to 0.2 mg/L, with lowest values for HIM and PUT, while LOQs were 0.4 mg/L for HIM and PUT, 0.667 mg/L for TYM, and 0.5 mg/L for CAD. These LOQs are much lower than those obtained in fermented milk by Costa et al. (2015) (LOQ up to 5.00 mg/L) and are also better than those provided by other analytical fluorescence RP-HPLC methods (LOD: 0.7-1.3 mg/kg and LOQ: 1.4-2.6 mg/kg in Sawilska-Rautenstrauch et al. (2010)), and even better than the few other methods using AQC reagent (LOD: 0.8-6.2 mg/kg and LOQ: 2.9-60.9 mg/k in Mayer et al. (2010); LOD: 0.5-4.4 mg/kg and LOQ: 1.6-14.5 mg/kg in Fiechter et al. (2013)). The improved sensitivity can be attributed to the combination of the SPE procedure with the fluorescence detection of the AQC derivatives. This increase in sensitivity is crucial since it can be suggested that dairy products with

Table 2. Figures of merit of the method developed in this study

	Retention time (min)	**Linear range (mg/L)**	R^2	**LOD (mg/L)**	**LOQ (mg/L)**	**Repeatability (RSD%) in milk**	**Repeatability (RSD%) in water**
Histamine	13.67	1 – 20	0.998	0.12	0.400	9.34	0.27
Tyramine	19.23	1 – 20	0.999	0.2	0.667	12.47	1.03
Putrescine	20.91	1 – 20	0.962	0.12	0.400	2.68	7.84
Cadaverine	21.96	1 – 20	0.965	0.15	0.500	6.36	5.83

LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative Standard Deviation.

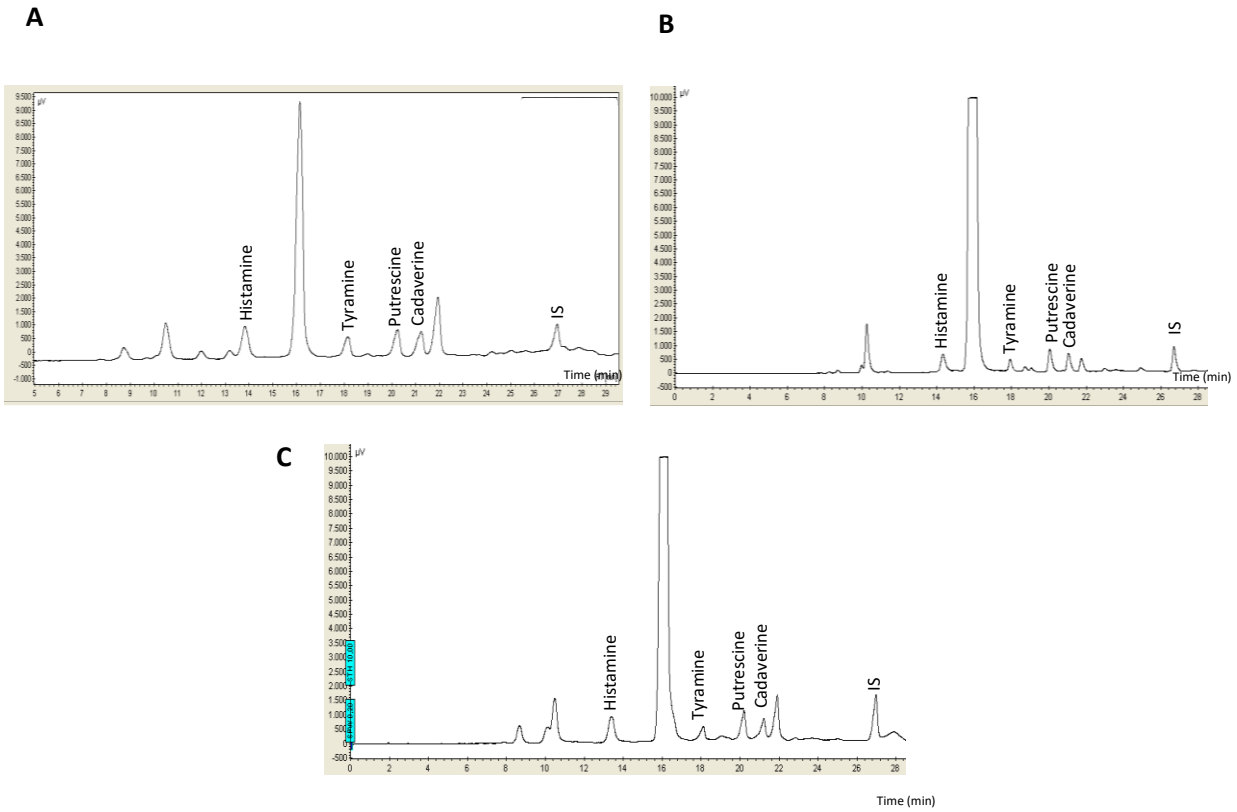

Figure 7. HPLC chromatogram of spiked (A) milk, (B) yogurt, and kefir (C) samples. Peak identification: Histamine, Tyramine, Putrescine, Cadaverine, 1,7-diaminoheptane (IS).

less than 1 mg/L of BA should be safe for consumers, including BAs-sensitive individuals (Rauscher-Gabernig et al., 2009).

The method's linearity was studied in water and in a milk sample spiked at different concentrations. For all BAs, our method showed adequate linearity in the range of concentration studied (1 to 20 mg/L) (Table 2). Determination coefficients (R^2) of calibration in milk samples ranged from 0.962 to 0.999.

Matrix effects were assessed by comparing the slopes of calibration curves obtained in water and in milk. Results revealed that the determination of HIM and TYM was free from matrix effects, but not the determination of CAD and PUT. Because of this, quantification was carried out by using a sample-dependent response factor. . For that, each sample was analyzed unspiked and spiked with known amounts of BAs. The difference of signal between these samples was used to estimate the concentration of BAs in unspiked sample.

3.3 Application of the method for detection of the presence of biogenic amines in dairy products

The method was applied to the analysis of four BA (HIM, TYM, PUT, and CAD) in several dairy products. Concentrations of BAs determined in 37 milk, 23 yogurt and 14 kefir samples are shown in Table 1 together with the samples' characteristics. All but five milk samples (provided by a cheese manufacturing company that used them for the production of ripened cheese) were commercial, and were from cows, sheep, and goats, subjected to pasteurization or UHT processes, and containing different percentages of fat content (whole, semi-skimmed, and skimmed). Concentrations of total BAs (TBA) are also included in Table 1. The use of TBA as a measure, in conjunction with specific BAs contents, has been proposed to define tolerable levels for food safety purposes (Benkerroum, 2016).

Remarkably, BAs were not present in most of the milk samples. TYM and CAD were detected in 5 and 8 samples, respectively, and could quantified just in 4 cases each. Maxima levels of TYM were 9.13 mg/L and those of CAD 17.7 mg/L. HIM and PUT were found only in two samples and could be quantified only in one, reaching 6.24 and 7.05 mg/L, respectively. The highest TBA corresponds to sample M22, which reached 40.11 mg/L. No obvious relationship was observed between concentration of BAs and characteristics of milk, such as technological process, animal species, or brand.

A literature comparison review of BAs results in dairy products is shown in Table 3. As can be seen, results reported here are consistent with those reported by other authors. In general, most authors report low or undetectable concentrations of BAs (Min et al., 2004; Novella-Rodríguez et al., 2000; Özdestan & Üren, 2010; Pekcici et al., 2021; Wu et al., 2015). In fact, previous reports on pasteurized milk showed levels generally not exceeding 1.07 mg/L HIM, 6.35 mg/L TYM, 1.4 mg/L PUT, and an almost unquantifiable value of 0.05 mg/L CAD (Pekcici et al., 2021), levels not raising a health concern with regard to BAs content (EFSA, 2011). However, Min et al. (2004) observed more than 18 mg/kg of CAD in commercial milk, which is a value very close to the one found in the Sample M22 of the present study.

A total of 23 yogurt samples were also analyzed. The incidence of BAs was

Table 3. Ranges of concentration of biogenic amines (histamine, tyramine, putrescine, and cadaverine) and of total biogenic amines (TBA) in dairy products (milk, yogurt and kefir) found in research literature.

	Histamine (mg/kg)	Tyramine (mg/kg)	Putrescine (mg/kg)	Cadaverine (mg/kg)	TBA (mg/kg)	Authors
Milk	ND-1.07	ND-6.35	ND-1.40	ND-18.52	ND-27.34	Bodmer et al., 1999; Min et al., 2004; Novella-Rodríguez et al., 2002; Pekcici et al., 2021
Yogurt	ND-65.18	ND-22.82	0.03-58.06	ND-34.53	ND-178.62	Bodmer et al., 1999; Min et al., 2004; Mayr & Schieberle, 2012; Bunkova et al., 2013; Adimcilar et al., 2017; Silva et al., 2019; Vieira et al., 2020; Pekcici et al., 2021
Kefir	ND-30.82	ND-12.8	0.3-47.69	ND -24.08	ND-115.69	Özdestan et al., 2010; Bunkova et al., 2013; Adimcilar et al., 2017; Pekcici et al., 2021

higher in yogurt samples than in milk samples, not only in terms of number of samples, but also in terms of concentration. PUT and HIM were the most frequently occurring BAs: they were found in 8 and 7 samples, respectively. Meanwhile, TYM and CAD were also detected and quantified in 4 and 3 samples, respectively. HIM was quantified in 5 samples and ranged from 0.82 to 17.16 mg/kg. The concentration of PUT and TYM was higher than LOQ in all samples. PUT ranged from 0.418 to 27.013 mg/kg, and the range of TYM was lower: from 0.539 to 2.108 mg/kg. CAD was detected in a lower number of samples (3) and could only be quantified in one sample at a concentration of 5.44 mg/kg.

PUT concentrations found by most authors in yogurt samples have been very low: 0.6 mg/L (Min et al., 2004), 0.47 mg/L (Pekcici et al., 2021) or even undetectable (Vieira et al., 2020). However, Adımcılar et al. (2017) quantified levels of up to 47 mg/kg in commercial yogurts and 58 mg/kg in homemade yogurts. In the present study up to 9 mg/kg of PUT in Sample Y18 have been observed.

The concentrations of HIM and TYM found in previous studies were higher than those of the other two BAs, reaching values from 21.2 to 65.2 mg/kg for HIM (Min et al., 2004) and up to 22.82 mg/kg for TYM (Vieira et al., 2020). Pekcici et al. (2021) observed maxima HIM values of 6.97 mg/kg, which is clearly smaller

than the concentration found in Y6 sample, 17 mg/kg HIM (Y6). This sample contained highest levels of HIM and TYM, which could indicate an instance of bacterial contamination. Maxima values of TYM in this study (2.108 mg/kg in Y7) are smaller than those previously reported. The highest TBA was obtained in Sample Y19 (35.07 mg/kg), due to a high content of HIM and PUT (8.064 and 27.013, respectively). It is noteworthy that the two yogurt samples with the highest TBA were made exclusively from sheep milk. Similar results occurred with the cured cheeses made with sheep's milk, where there was a higher concentration of HIM than in those made with cow's milk (Botello-Morte et al., 2022).

A total of 14 kefir samples were also analyzed. BAs were detected in 11 samples. Kefir samples showed the highest TBA levels, yielding up to 79.66 mg/kg, double of the highest concentration of TBA in milk (40.11 mg/kg) and yogurt (35.07 mg/kg). Although HIM was quantified only in two at 0.831 and 1.211 mg/kg, TIM, PUT, and CAD were found in a greater number of samples (7, 10 and 7, respectively) and in a higher concentration (between 0.850 - 3.678 mg/kg, 0.452 - 14.05 mg/kg and 1.21 - 64.032 mg/kg, respectively). PUT was the BA most found in kefir, and was detected in all but in four samples (K8, K9, K10 and K13).

It is well-known that fermentation processes can cause an increase in BAs in the final product (Ladero et al., 2017). A higher degree of formation of HIM is found in yogurt (65.18 mg/kg) and in kefir (30.82 mg/kg) (Adımcılar et al., 2017) than in milk (1.07 mg/kg) (Pekcici et al., 2021). In our case, a low amount of HIM (ND-1.211) was observed in kefir, while a considerable concentration of CAD (ND-64.032 mg/kg) was found, exceeding values described by other authors (24.08 mg/kg in Adımcılar et al. (2017) and 2.2 mg/kg in Özdestan & Üren (2010).

Milk samples had a lower concentration of BAs than was the case in yogurt or kefir. Several reasons could explain this difference. One reason could lie in the different capacity of certain fermentative lactic acid strains for producing BAs; a further reason might be found in the different kinds of environmental microbiota present in equipment used in industry (Moniente et al., 2021). The lower concentration of BAs in milk could also be attributed to the possible use of different starter cultures that may

also have different BAs production capacities (Mokhtar et al., 2012); moreover, the use of different milks with different concentrations of amino acid precursors during manufacture can likewise exert an influence on BAs content. Lastly, the type of processed food strongly affects the growth rate and the concentration of lactic acid microbiota present in the matrix during cold storage (Samelis et al., 2000).

Levels of BAs found in milk may be due to a lack of hygiene after the pasteurization process, which can lead to a bacterial proliferation capable of producing BAs. It is necessary to highlight the need to respect norms of good hygiene by properly cleaning and disinfecting food equipment throughout manufacturing. When such measures fail, high concentrations of BAs can emerge and cause a food risk for consumers. Moreover, if milk contaminated with BAs-producing microorganisms is used in the production of cheeses, BAs concentration can increase uncontrollably, even exceeding 2,500 mg/L of HIM in the case of ripened cheese (Maintz & Novak, 2007).

4. Conclusion

A new method for the determination of BAs in dairy products was validated in this study. The method is based on a SPE procedure followed by derivatization with AQC and analysis by HPLC with fluorescence detection. This methodology makes it possible to achieve detection limits lower than 0.2 mg/L, and quantification limits lower than 0.667 mg/L for the different amines, which is highly satisfactory. Mean repeatability is lower than 10 % of RSD, and the method is linear in an adequate range of concentrations.

We applied this method to the quantification of BAs in samples of Spanish milk, yogurt, and kefir. Our results show pronounced differences between milk and fermented products. While BA are just seldomly present in milk samples, the amount of BA in yogurt samples is greater, and is even greater and more frequent in kefir samples. Although these levels would not cause any harmful effect on most consumers, they should be taken into account by individuals who are sensitive to BAs.

Author statement

Marta Moniente: investigation, data curation, writing – original draft, writing – review & editing. Laura Botello-Morte: writing – review & editing. Diego García-Gonzalo: writing – review & editing. Raquel Virto: project administration, funding acquisition, writing – review & editing. Rafael Pagán: project administration, funding acquisition, writing – review & editing. Vicente Ferreira: funding acquisition, writing –review & editing. Ignacio Ontañón: supervision, investigation, data curation, writing – original draft; writing – review & editing

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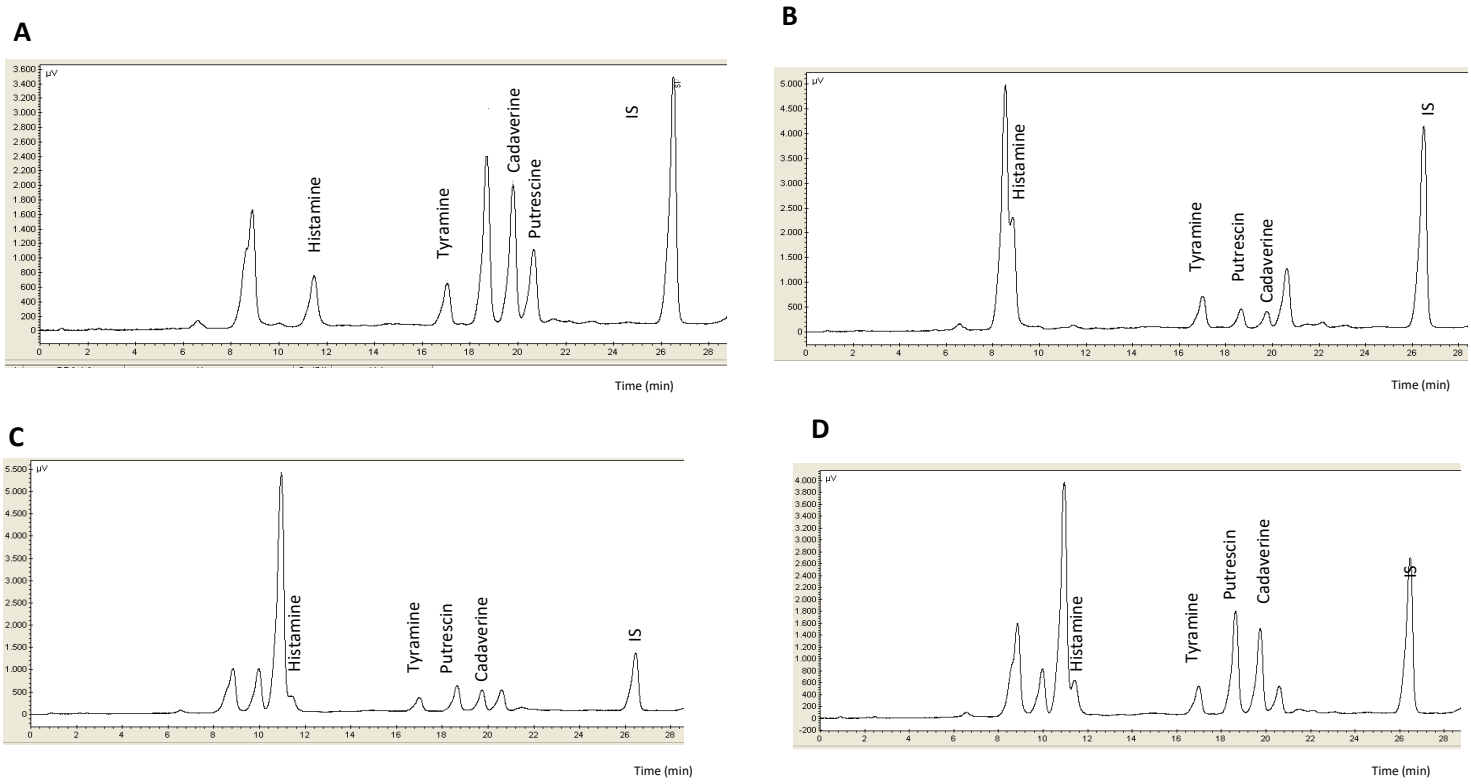
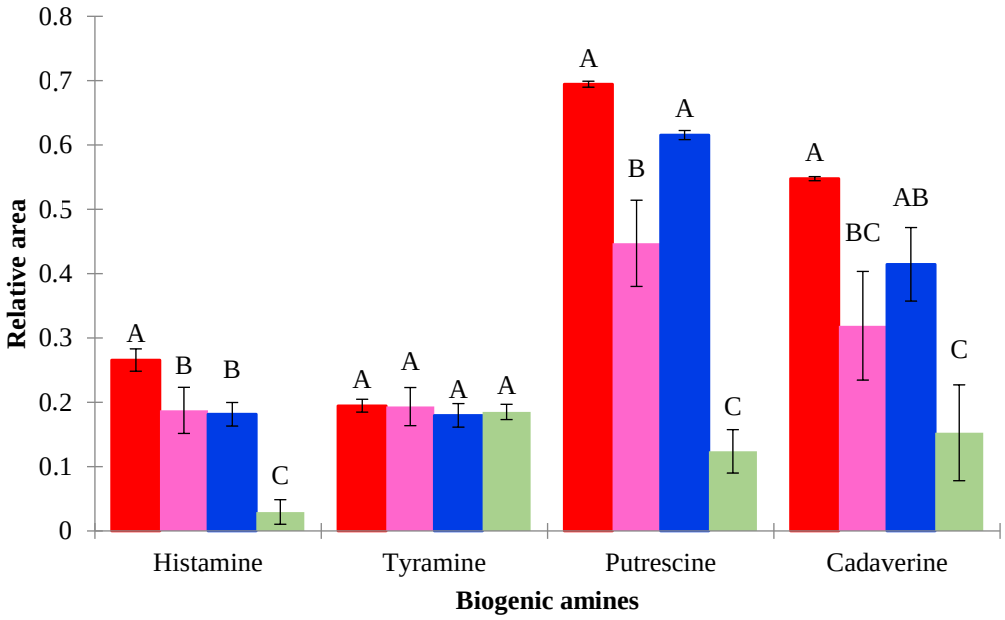
Supplementary tables



Supplementary Figure 1. HPLC chromatograms of the influence of the use of different filters on biogenic amine analysis by AQC-HPLC-FLD: (A) nylon, (B) 0.22 µM, (C) 3k and (D) 10k. Peak identification: Histamine, Tyramine, Putrescine, Cadaverine, 1,7-diaminoheptane (IS).



Supplementary Figure 2. Effect of different filters (Nylon , Polyvinylidene fluoride (PVDF) and 3k and 10k ultrafiltration (molecular weight cut-off of 3k and 10k) on the chromatographic area of biogenic amines in dairy products analysed by AQC-HPLC-FLD. Capital letters indicate significant differences ($p<0.05$).

Manuscrito IV. 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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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

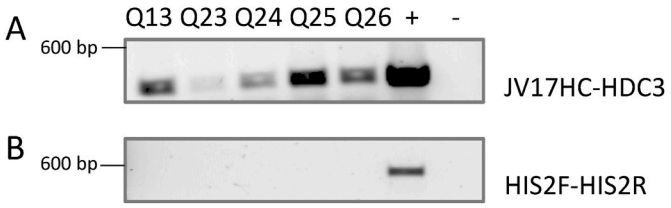

Fig. 1. PCR amplification of *hdc* gene, using total DNA obtained from cheeses as template, and specific oligonucleotides JV17HC and HDC3 for gram-positive bacteria (**A**) or HIS2F and HIS2R for gram-negative bacteria (**B**). *Lentilactobacillus parabuchneri* DSM 5789 and *Morganella morganii* subsp.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

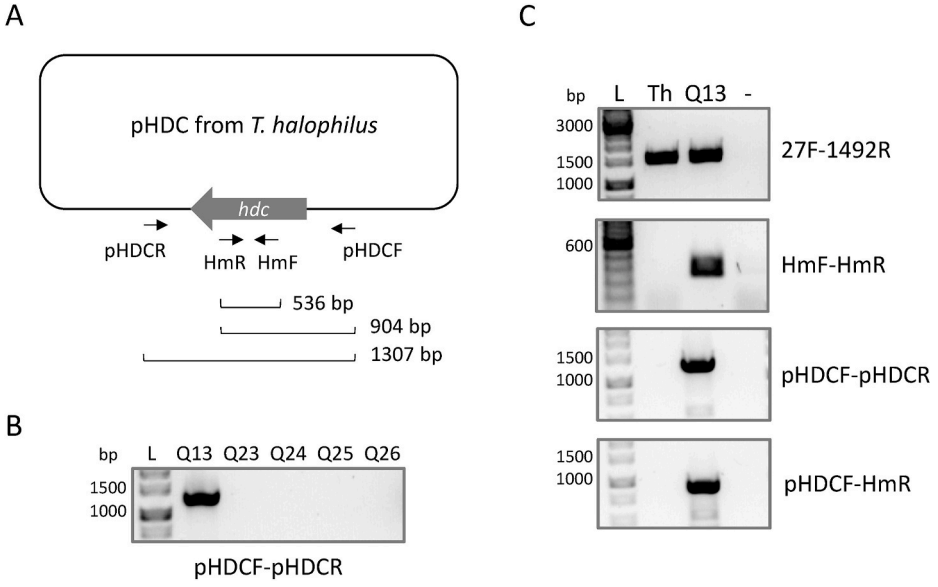

Fig. 2. **(A)** Schematic representation of the plasmid pHDC containing the *hdc* gene of *T. halophilus* and the position of the oligonucleotides pHDCF and pHDCR, used to amplify the plasmid sequence, and HmF and HmR, used to amplify the *hdc* gene. **(B)** PCR amplification of a fragment of 1,307 bp 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

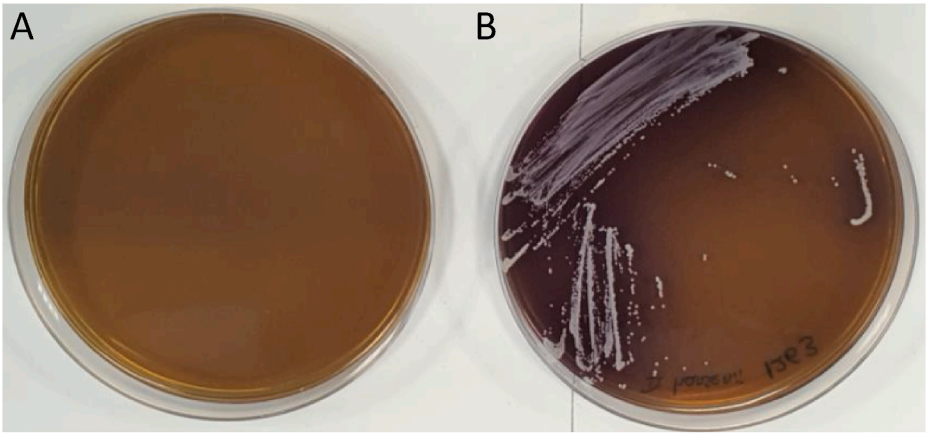

Fig. 3. Analysis of histamine production by the *Debaryomyces hansenii* isolate from cheese using decarboxylating MDA-H agar (A). The colour change of medium to purple after incubation indicates a pH change, putatively linked to histamine decarboxylation (B). (For interpretation of the references to 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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The significance of cheese sampling in the determination of histamine concentration: Distribution pattern of histamine in ripened cheeses

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ABSTRACT

Cheeses are becoming a major safety and public health concern: cheeses available in supermarkets occasionally contain high histamine concentrations that can have negative effects on consumer health. In this study, we have attempted to assess the histamine distribution pattern in ripened cheeses, with the purpose of establishing a correct cheese sampling strategy for the quantification of histamine. To this aim, histamine was determined in four distinct areas of twelve long-ripened hard cheeses: the external and internal rind, along with the outer and inner core of the wedge. The concentrations measured were remarkably different: histamine accumulated in the central core, whereas the lowest amount was found in the peripheral rind. To explain this heterogenous distribution, histamine producers were determined in the four areas by identifying the *hdc* sequences obtained from cheese samples. Non-starter bacteria were identified as main histamine producers; however, these microbiota were homogeneously distributed throughout the wedge. Nevertheless, the analysis of psychochemical properties of the different areas revealed an observable trend: histamine tended to accumulate in the saltier, more humid, and less oxidized areas in a wedge. Overall, this study highlights the significance of a correct sampling strategy when histamine is quantified in cheese.

1. Introduction

Histamine is a biogenic amine synthesized from the precursor amino acid histidine through an oxidative decarboxylation reaction. It is frequently present in fermented products as a consequence of the metabolism of the microbiota that are present therein (Benkerroum, 2016). When it accumulates in food, histamine intoxication can cause severe systemic symptomatology including headaches, itching and rashes, vomiting, tachycardia, sweating, diarrhoea, respiratory distress, and hypotension (Comas-Baste, Sanchez-Perez, Veciana-Nogues, Latorre-Moratalla, & Vidal-Carou, 2020). Long-ripened cheese is the dairy product that tends to accumulate the most histamine: the content of the latter in cheese can reach values of up to 2,500 mg/kg (Madejska, Michalski, Pawul-Gruba, & Osek, 2018). Although the limit for histamine is not legally regulated in cheese, Regulation (EC) No 2073/2005 regarding microbiological criteria for foodstuffs establishes a limit of 200 mg/kg histamine in fish (Parliament, 2005). Nevertheless, 400 mg/kg of histamine has been proposed as a justifiable limit in ripened

Abbreviations: a_w, water activity; DSM, DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen; EFSA, European Food Safety Authority; HDC, histidine decarboxylase enzyme; *hdc*, histidine decarboxylase gene; LAB, lactic acid bacteria; NCIMB, National Collection of Industrial and Marine Bacteria; PCR, polymerase chain reaction; PDO, Protected Designation of Origin; TBARS, 2-Thiobarbituric acid reactive substances.

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cheese (Rauscher-Gabernig, Grossgut, Bauer, & Paulsen, 2009).

Cheese presents a strategically favourable microenvironment for the synthesis of histamine due to its composition and physical and chemical properties. Cheese is obtained from milk, by removing water and whey, and concentrating casein and fat. The first step consists in the clotting of milk to produce the curd by an enzymatic process; subsequently, the whey is removed by cutting the curd and stirring. Then, microbiota which are either naturally present in milk or artificially added produce lactic acid to render fresh cheese. Pressing the curd leads to form a rind, which limits oxygen and water transfer in the core of cheese. Finally, curing the cheese allows to obtain the final ripened product. Milk pasteurization, addition of selected starter cultures, or salting are optional steps in the cheesemaking process.

Microbiological and environmental factors can influence the cheesemaking process (Moniente, García-Gonzalo, Ontañón, Pagán, & Botello-Morte, 2021). On the one hand, microbiota involved in the synthesis of histamine comprise Gram-negative and Gram-positive bacteria, as well as yeasts. Lactic acid bacteria (LAB) are the main microorganisms responsible for histamine production in cheese (Barbieri, Montanari, Gardini, & Tabanelli, 2019): *Lentilactobacillus parabuchneri* stands out as the main histamine producer (Berthoud et al., 2017; Díaz et al., 2018; Diaz, Del Rio et al., 2016; Diaz et al., 2016; O'Sullivan et al., 2015; Wechsler et al., 2021; Wuthrich et al., 2017), although other LAB such as *Lactobacillus* sp., *Tetragenococcus halophilus*, and *Streptococcus thermophilus* have also been proposed as histamine-producing bacteria in cheese (Botello-Morte et al., 2022; Diaz, Ladero, Redruello, et al., 2016; Roig-Sangüés, Molina, & Hernández-Herrero, 2002; Rossi et al., 2011). Gram-negative bacteria can also contribute to the accumulation of histamine in cheese, usually as a result of spoilage or contamination in early steps of the cheesemaking process (Costa, Rodrigues, Frasao, & Conte-Junior, 2018). *Morganella morganii*, *Hafnia alvei*, and *Serratia* sp. are examples of Gram-negative histamine producers (Fernandez-Garcia, Tomillo, & Nunez, 2000; Roig-Sangüés et al., 2002). Finally, yeasts, including *Debaryomyces hansenii* and *Geotrichum candidum*, are likewise involved in histamine production in cheese (Gardini et al., 2006; Helinck, Perello, Deetae, de Revel, & Spinnler, 2013; Roig-Sangüés et al., 2002). Bacteria capable of producing histamine contain the *hdc* gene, codifying for the histidine decarboxylase (HDC) enzyme (Landete, de Las Rivas, Marcobal, & Muñoz, 2008). The *hdc* gene is mainly present in the bacterial chromosome, although certain bacteria, such as *L. hilgardii* and *T. halophilus,* feature the gene encoded in an extrachromosomal element: a mobile plasmid called pHDC (Lucas, Wolken, Claisse, Lolkema, & Lonvaud-Funel, 2005; Satomi, Furushita, Oikawa, Yoshikawa-Takahashi, & Yano, 2008; Wuthrich et al., 2017).

Environmental and physicochemical factors also determine histamine production in cheese (Moniente et al., 2021). As expected, microorganisms tend to produce more histamine when the precursor free histidine is available, which occurs as a result of proteolysis (Fernandez, del Rio, Linares, Martin, & Alvarez, 2006). The presence of other bacteria can determine histamine accumulation, either due to microbial competition and/or due to degradation of histamine (Coton et al., 2012). Further factors such as ripening temperature and time, as well as storage conditions, can also affect the formation of histamine in cheese. Histamine production is promoted by long ripening times combined with high ripening and storage temperatures (Linares et al., 2012), although some strains of *L. parabuchneri* can even synthesize histamine at 4–8 °C (Díaz et al., 2018). During the cheese ripening process, physical and chemical properties of the product evolve as the final cheese product matures. Those parameters tend to vary somewhat between the cheese core and the rind (Choi et al., 2020; Mayo, Rodriguez, Vazquez, & Florez, 2021). Whereas the surface is mainly aerobic and exposed to environmental microorganisms (Irlinger, Layec, Helinck, & Dugat-Bony, 2015), the core is more anaerobic, and microbiota in that area are mainly composed of LAB (Yeluri Jonnala, McSweeney, Sheehan, & Cotter, 2018). Physical and chemical properties of cheese, in turn, may exert an influence on the accumulation of histamine, thereby representing a key factor that needs to be carefully controlled in cheese (Linares et al., 2012). For instance, salt content above 5% (w/v) will generally tend to diminish histamine accumulation in cheese (Tabanelli, Torriani, Rossi, Rizzotti, & Gardini, 2012). Nevertheless, the halophilic bacterium *Tetragenococcus* sp. has been shown to produce histamine even at 20% (w/v) sodium chloride (Kimura, Konagaya, & Fujii, 2001; Satomi et al., 2008).

It is well established that neither microbiota nor physical and chemical properties, such as water activity (a_w) or salt content, are homogeneously distributed in cheese. Since histamine production in cheese is carried out by microbiota, which in turn are influenced by environmental factors, it would be expected that the distribution of histamine concentration in a cheese wedge would also be heterogeneous. This aspect is crucial when sampling for purposes of determining histamine concentration. The sampling area is a key point for a correct analysis of histamine concentration in cheese to obtain accurate, reliable values. Thus, a well-defined cheese sampling strategy could have an impact on results in terms of histamine content. Similarly, a correct cheese sampling strategy proved to be key in revealing the pattern of microbiota distribution in a wedge (Tilocca et al., 2020).

The objective of this study was to analyse the pattern of histamine distribution in long-ripened cheeses in order to establish an appropriate general sampling strategy for the determination of histamine concentration in cheese. We quantified histamine in different areas of cheese wedges, identified histamine-producing microbiota, and determined the cheeses' physical and chemical properties.

2. Material and methods

2.1. Cheese samples

For the purposes of this study, 12 long-ripened hard cheeses produced and commercialized in Spain were purchased. All of them were made from raw milk, and most of them could be considered "aged cheeses" (with ripening periods longer than 9 months), since it has been previously shown that these are key factors that play a role in histamine accumulation in cheese (Madejska et al., 2018). A recently published study shows that cheeses with higher histamine content are mostly made from raw sheep's milk (Botello-Morte et al., 2022). In that study, a commercialized Protected Designation of Origin (PDO) Idiazabal cheese (a hard raw sheep's milk cheese produced in the regions of the Basque Country and Navarra in Spain) displayed the highest histamine content, exceeding 500 mg/kg, which can be harmful or toxic to consumer health according to the European Food Safety Authority (EFSA, 2011). For these reasons, we selected two PDO Idiazabal cheeses (cheeses 1 and 2), along with a series of sheep's and mixed-milk cheeses in order to assess the pattern of histamine distribution within them. In total, half of the cheeses we selected (cheeses 1 to 6) were manufactured from sheep's milk, and the other half (cheeses 7 to 12) were made from milk blends. The main characteristics of the cheeses selected are summarized in Table 1.

The cheeses were received in the laboratory of the Centro Nacional de Tecnología y Seguridad Alimentaria (CNTA) and they were cut into nine wedges (Fig. 1A) in order to analyse a series of parameters in three different laboratories in triplicate. Each wedge was divided, in turn, into four areas to delimit the core versus the rind, and the inner area versus the outer area. The four areas thereby obtained are represented in Fig. 1B. Area 1 corresponded to the central rind of the wedge, Area 2 to the central core, Area 3 to the peripheral core, and Area 4 to the peripheral rind. Then, a set of samples of the different areas of 3 distinct wedges (twelve samples in total) were sent for the quantification of histamine concentration to the laboratory at BIOLAN Microbiosensores S.L. A second set of samples were sent to the laboratory of the Faculty of Veterinary in the University of Zaragoza, to characterize the histamine-producing microbiota present in the samples. Finally, a third set of samples were subjected to analyses of the physical and chemical

Table 1
Main characteristics of the cheeses used in this study: cheese variety, milk source and ripening time. The cheeses were made from raw milk and commercialized in Spain.

Sample code	Cheese variety	Milk source	Ripening time (months)
Cheese 1	Idiazabal PDO	Sheep milk	≥2.0
Cheese 2	Idiazabal PDO	Sheep milk	≥2.0
Cheese 3	Aged sheep cheese	Sheep milk	13.5
Cheese 4	Aged sheep cheese	Sheep milk	18.0
Cheese 5	Aged sheep cheese	Sheep milk	21.9
Cheese 6	Aged sheep cheese	Sheep milk	10.4
Cheese 7	Aged cheese	Blended milk	9.9
Cheese 8	Aged cheese	Blended milk	10.0
Cheese 9	Aged hard cheese	Blended milk	13.6
Cheese 10	Aged cheese	Blended milk	13.0
Cheese 11	Aged cheese	Blended milk	9.0
Cheese 12	Aged cheese	Blended milk	9.8

PDO: Protected Designation of Origin.

properties of the cheeses in the CNTA laboratory. Then, samples of 2–10 g (depending on the assay performed) of each of the four delimited areas were obtained in each laboratory and homogenized for purposes of analysis.

2.2. Histamine quantification

In recent years, the food industry has started to use biosensors for the quantification of histamine due to their low cost, speed, ease of use, and minimum amount of sample required (Moniente, Botello-Morte, García-Gonzalo, Pagan, & Ontanon, 2022). Samples were analysed for histamine content with the novel enzymatic BIO300 HIS biosensor (BIOLAN, Zamudio, Spain), according to an adapted protocol for histamine determination in cheese samples (Salleres et al., 2016). The test method consists in the extraction of histamine in aqueous solution and subsequent quantification by the biosensor after previous calibration. The enzymatic biosensor is a system that incorporates a biochemical sensing element (based on a specific oxidative enzyme) placed in close contact with or in close proximity to a transducer system that relates the concentration of an analyte to a measurable signal. The addition of the analyte causes sequential redox reactions that involve a release of electrons proportional to the concentration of the analyte. This type of biosensor thus combines the specificity and the selectiveness of enzyme–analyte reactions with highly sensitive electrochemical transduction.

For histamine extraction, a sample of 2 g of grated cheese was blended with 18 mL of distilled water and mixed by vortex. The internal calibration of the biosensor was 0–50.0 mg/kg, performed prior to the analysis of samples by adding, at two different times, 300 μL of a histamine standard of 25 mg/mL to the measuring cuvette, containing 10 mL of measuring buffer at pH 6.5. The range of quantification for the sample, taking into account the dilution factor performed for histamine extraction, was 100.0–500.0 mg/kg. To proceed with sample analysis, 300 μL of the extract was added to the measuring cuvette at the required moment. Once the measuring process had concluded, the device displayed the result of the analysis on the screen.

2.3. Total DNA extraction from cheese

DNA was isolated from cheese and quantified as described in Botello-Morte et al. (2022). Briefly, samples of 5 g of cheese were homogenized in sterile 2% (w/v) trisodium citrate (Panreac, Barcelona, Spain) and centrifuged for 10 min at 10,000 *g*. After removing fat layers and supernatants, cellular pellets were digested for 1 h at 37 °C in 20 mmol/L Trizma Hydrochloride pH 8 (Sigma Aldrich, St. Louis, MO, USA), 2 mmol/L EDTA (Panreac), 1.2% (v/v) Triton X-100 (Sigma Aldrich) with 20 mg/mL lysozyme (Sigma Aldrich), and 50 U/mL mutanolysin (Sigma Aldrich). Subsequently, 25 μL proteinase K and 200 μL buffer AL from a QIAGEN DNeasy Blood and Tissue kit (QIAGEN, Manchester, UK) were added, and incubated at 70 °C for 30 min. Samples were then subjected to a mechanical lysis by using a Precellys 24 homogenizer (Bertin Instruments, Montigny le Bretonneux, France) and PowerBead Tubes, Glass 0.1 mm (QIAGEN). Finally, supernatants were transferred to a column of the DNeasy Blood and Tissue kit (QIAGEN) and processed following the manufacturer's instructions.

2.4. PCR amplification of bacterial histidine decarboxylase (hdc) gene

PCR reactions were carried out in a T-100 thermal cycler (Bio-rad Laboratories, Madrid, Spain) with the Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Massachussets, USA). The amplicons were visualized in a GelDoc EZ (Bio-rad Laboratories) documentation system, using Sybr-Safe DNA gel stain (Thermo Fisher Scientific). The *hdc* gene from Gram-positive bacteria was amplified by using the oligonucleotides JV17HC (AGACCATACACCATAACCTT) and HDC3 (GATGGTATTGTTTCKTATGA) according to Le Jeune, Lonvaud-Funel, ten Brink, Hofstra, and van der Vossen (1995) and Coton and Coton (2005), respectively. Genomic DNA from *L. parabuchneri* DSM 5987 was used as positive control in the PCR reaction. Oligonucleotides HIS2F (AAYTSNTTYGAYTTYGARAARGARGT)

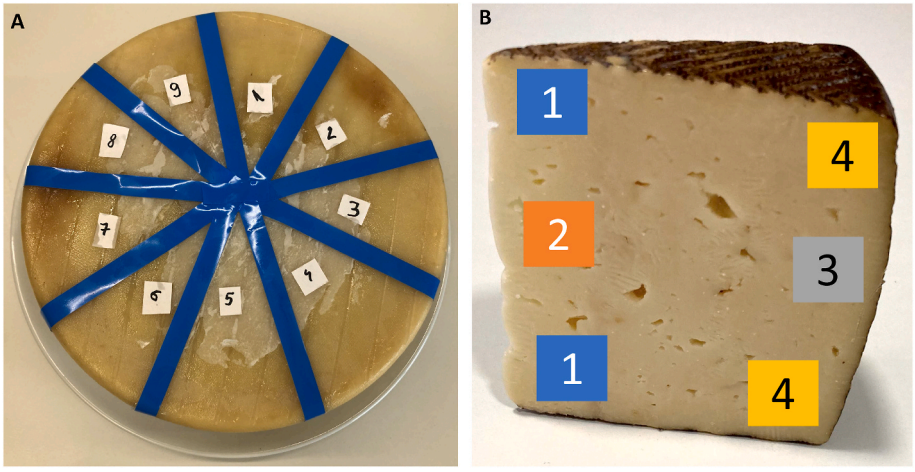

Fig. 1. Cheese sampling. A. The cheeses were cut into nine wedges. B. Each wedge was divided into four sampling areas. Area 1 (in blue) corresponded to the central rind of the wedge, Area 2 (in orange) to the central core, Area 3 (in grey) to the peripheral core, and Area 4 (in yellow) to the peripheral rind. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and HIS2R (TANGGNSANCCDATCATYTTRTGNCC), in accordance with de Las Rivas, Marcobal, and Munoz (2005), were used to assess the presence of the *hdc* gene amplified from Gram-negative bacteria, using total DNA of *M. morganii* subsp. *sibonii* NCIMB 865 as positive control. Since in certain histamine-producing bacteria the *hdc* gene can be codified in a mobile plasmid called pHDC, a sequence of the pHDC plasmid was amplified by using the pHDCF (CGCGGCAACAAAGGGTCC) and pHDCR (CGCTGATTCAGAATGACTTGAC) oligonucleotides from Botello-Morte et al. (2022).

2.5. DNA sequencing and BLAST analysis

Amplicons were purified with ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions, and sequenced by Macrogen Inc (Seoul, Korea). The analysis of nucleotide sequences was performed with the NCBI nucleotide database (BlastN: https://blast.ncbi.nlm.nih.gov/).

2.6. Analysis of physical and chemical properties of cheese

Several physical and chemical properties of the cheeses were analysed: humidity, salt content, and the oxidative state of the samples. Humidity was determined by drying the samples with a heating and drying oven (Memmert, Schwabach, Germany). Samples of 2 g of homogenized cheese measured in aluminium pans at 102 °C were used for this purpose. Salt content was determined by automatic potentiometric titration. Samples of 3 g of cheese were correctly homogenized, and then 0.1 mg of homogenate were subjected to salt content analysis, using a chloride-selective electrode and a 0.1 mol/L silver nitrate solution. The oxidative state of the four selected zones of the cheeses was determined by the 2-Thiobarbituric acid reactive substances (TBARS) assay (Pfalzgraf, Frigg, & Steinhart, 1995). For this purpose, samples of 4–10 mg of homogenized cheese were mixed with 35 mL of cold absolute ethanol (Sigma Aldrich). The mix was filtered, and distilled water was added up to the 50 mL mark. After that, 2 mL of 20% (v/v) trichloroacetic acid (Sigma Aldrich) and 1 mL of 0.67% (w/v) TBA were added to 2 mL of the extract previously obtained, and incubated for 15 min at 95 °C. Samples were then chilled for 5 min and centrifuged at 5000 *g* for 10 min. Finally, supernatants were subjected to spectrophotometric analysis at 440 nm and 532 nm wavelengths in a Jascov V-730 spectrophotometer (Jasco, Madrid, Spain). A blank sample was obtained using distilled water instead of the cheese sample. Calibration was obtained with different concentrations of malondialdehyde (Sigma Aldrich). Results were expressed as mg malonaldehyde/kg cheese. Water activity was measured in LabMASTER-a_w equipment (Novasina, Lachen, Switzerland) at 25 °C. This equipment continuously measures water activity and temperature, based on a dimensionless scale of 0–1.0.

2.7. Statistical analysis

Statistical analysis was performed with one-way analysis of variance and Tukey's test with the XLSTAT software (version 2022.2.1; Addinsoft, Paris, France). The correlation among physical and chemical parameters in cheese areas was carried out with Excel (2019 version), establishing a *p*-value of 0.05 for significance.

3. Results and discussion

3.1. Pattern of histamine distribution in cheese

Histamine was extracted from the cheese samples and subsequently quantified with the novel enzymatic BIO300 HIS biosensor. Obtained data are summarized in Table 2. In general, histamine accumulation in the central areas (Areas 1 and 2) was higher than in peripheral zones (Areas 3 and 4), and the core generally exhibited more histamine than the rind (Area 2 versus Area 1, and Area 3 versus Area 4). This pattern recurred in most of the cheeses analysed. The differences observed indicated that the highest values of histamine were generally obtained in Area 2, whereas the lowest values appeared in Area 4, most of them lower than 100.0 mg/kg of histamine. Two of the twelve cheeses (Cheeses 8 and 12) did not exhibit histamine values higher than the biosensor's optimized limit of quantification (100.0 mg/kg) in any area of sampling. For that reason, cheeses 8 and 12 were excluded from further analysis in this study.

Table 2
Histamine concentration in the different areas of the cheeses. The mean histamine concentration value and the relative standard deviation of three independent measurements using the BIO300 HIS Biosensor are indicated.

Sample code	Area 1	Area 2	Area 3	Area 4	Significance
Cheese 1	149.5 ± 11.0 ^{a}	178.3 ± 10.1 ^{a}	145.5 ± 34.8 ^{a}	≤100.0 ^{b}	**
Cheese 2	≤100.0 ^{b}	221.5 ± 20.5 ^{a}	115.0 ± 39.1 ^{b}	≤100.0 ^{b}	***
Cheese 3	295.8 ± 20.0 ^{a}	308.8 ± 15.8 ^{a}	237.3 ± 22.1 ^{b}	183.5 ± 10.2 ^{c}	***
Cheese 4	109.3 ± 6.6 ^{b}	154.0 ± 5.0 ^{a}	≤100.0 ^{c}	≤100.0 ^{c}	***
Cheese 5	202.3 ± 17.3 ^{a}	162.9 ± 29.6 ^{a}	≤100.0 ^{b}	≤100.0 ^{b}	**
Cheese 6	315.2 ± 28.2 ^{a}	325.1 ± 30.1 ^{a}	318.1 ± 19.2 ^{a}	298.6 ± 15.9 ^{a}	n.s.
Cheese 7	268.4 ± 54.7 ^{a}	282.7 + 47.3 ^{a}	160.7 + 44.8 ^{b}	≤100.0 ^{b}	**
Cheese 8	≤100.0 ^{a}	≤100.0 ^{a}	≤100.0 ^{a}	≤100.0 ^{a}	n.s.
Cheese 9	≤100.0 ^{a}	101.0 ± 3.5 ^{a}	≤100.0 ^{a}	≤100.0 ^{a}	n.s.
Cheese 10	≥500.0 ^{a}	≥500.0 ^{a}	479.5 ± 21.9 ^{ab}	450.0 ± 24.0 ^{b}	*
Cheese 11	214.6 ± 28.6 ^{b}	465.1 ± 34.9 ^{a}	111.3 ± 19.2 ^{c}	≤100.0 ^{c}	***
Cheese 12	≤100.0 ^{a}	≤100.0 ^{a}	≤100.0 ^{a}	≤100.0 ^{a}	n.s.

Area 1 corresponded to central rind of the wedge, Area 2 to the central core, Area 3 to the peripheral core, and Area 4 to the peripheral rind. The range of quantification optimized for the biosensor is 100.0–500.0 mg/kg: as a consequence, the histamine values lying outside of that range are mentioned as ≤ 100.0 mg/kg or ≥500.0 mg/kg n.s.: non-significant; *: *p*-value<0.05; **: *p*-value<0.01; ***: *p*-value<0.001. Lowercase letters indicate significant differences between Areas 1 to 4 in each cheese.

In order to highlight the heterogeneity of the distribution of histamine within a cheese wedge, we used box-and-whisker plots to reflect the trend observed in cheeses featuring significant differences among areas, regardless of whether the wedge belonged to a cheese with high or low histamine levels in general. This kind of plot allowed us to better differentiate the areas of the wedges that contained more histamine (with respect to the average value of histamine concentration in the cheese) from those that had less histamine. Fig. 2 shows the percentage of positive/negative deviation in each zone from the mean value of histamine concentration in the wedge, represented by 0% deviation. Our analyses of histamine content in cheese samples revealed that Areas 1 and 2 contained more histamine (17.8% and 54.4%, respectively) than the mean value of histamine content in the entire wedge, whereas the content of histamine in Areas 3 and 4 (−19.8% and −52.3%, respectively) was lower than the mean value of the wedge. Thus, histamine tended to have higher values in the central core (Area 2) of the wedge, which contained up to 115.4% more histamine than the mean value of the wedge's four areas. The peripheral rind (Area 4) displayed the lowest levels of histamine: its histamine content was always lower (up to −83.3%) than the mean value of the wedge's four areas. The central rind (Area 1) and the peripheral core (Area 3) of the wedge had mild histamine concentration values. In Area 1, histamine content tended to be higher than the mean value of the wedge: up to 73.3% higher, but it could also be lower, reaching −20.9%. Area 3 generally displayed values lower than the mean histamine content in the wedge: up to −50.3%

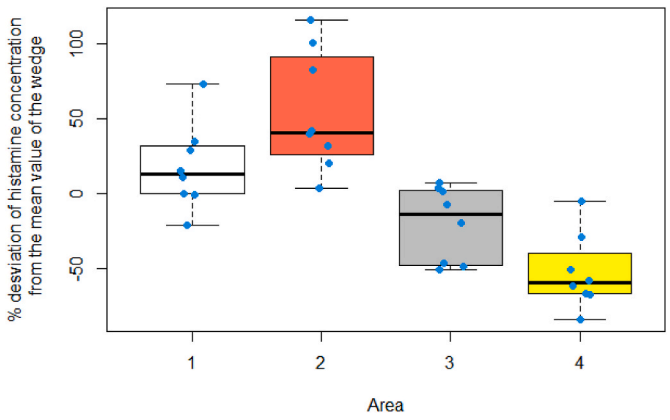

Fig. 2. The percentage of deviation of histamine concentration in each cheese area from the mean value of the entire wedge, represented in a box-and-whisker plot. Area 1 corresponds with the central rind of the wedge, Area 2 with the central core, Area 3 with the peripheral core, and Area 4 with the peripheral rind. Each blue point represents the deviation of histamine concentration from the mean value of the wedge. The median line of the box represents the medians of histamine concentration in each defined area of the wedge. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

lower, although it could likewise surpass 7.7% of the wedge's mean value (Fig. 2).

Previous studies performed on cheeses have suggested that biogenic amines might not be homogeneously distributed in cheese (Marijan et al., 2014; Novella-Rodríguez, Veciana-Nogués, Izquierdo-Pulido, & Vidal-Carou, 2003). In 1998, a team of authors suggested that biogenic amines could be more present in the central part of the wedge than close to the rind (Joosten & Stadhouders, 1987). Some years later, Novella-Rodríguez et al. (2003) and Marijan et al. (2014) confirmed this distribution in cheese for all the amines they tested, except for tyramine, which accumulates in the outer part of the cheese. However, Komprda et al. (2007) and Shehata and Rdwan (2017) found a higher content of biogenic amines in the cheese rinds. At any rate, the values of histamine quantified in all these studies were lower than 20 mg/kg in the two distribution zones; these values are thus not truly comparable with those of our study, where certain samples reached histamine concentrations higher than 500 mg/kg. Moreover, our study was performed in four accurately defined areas of each sample. However, it is worth noting that our proposal of a histamine distribution pattern could be more interesting when using ripened cheese with high histamine concentrations, even higher that the limit proposed by the authorities, since cheese sampling could thus represent a key point. It is possible that this pattern also occurs in cheeses with lower histamine amount, but in this case, it would be not as relevant.

Overall, histamine content was higher in the inner core of the wedge (Area 2), versus the other three areas in most cheeses (8 out of 12 cheeses). In 3 cheeses, the histamine values lay outside the range of quantification optimized for the biosensor: thus, in these cases, histamine content could not be accurately determined. Thus, the core of the wedge (Areas 1 and 2) generally exhibited a higher amount of histamine than the rind (Areas 3 and 4). This pattern of histamine distribution in four different areas highlights the importance of conducting a correct sampling procedure when determining histamine in cheese. Depending on the area of the wedge where the cheese sample is taken, the result in terms of histamine content can be extremely different. Taking different small-size samples from the four different areas of the entire wedge and homogenizing them can yield an intermediate value. However, to accurately determine the highest value of histamine present in the wedge, a sample from the cheese's inner core should be taken.

3.2. Molecular detection of the hdc gene and the pHDC plasmid

In order to search for a putative correlation between this pattern of distribution and histamine-producing microbiota in cheese, a molecular analysis of the presence of the *hdc* gene in the different areas of the wedge was performed. As evidenced in Botello-Morte et al. (2022), total DNA from cheese is the best starting material for this purpose. We therefore isolated total genomic DNA from each area in the cheese samples. Four cheeses were selected to perform molecular analysis: the two Idiazabal DPO cheeses (Cheeses 1 and 2), a further one made from sheep's milk (Cheese 3), and another one made from blended milk (Cheese 7). Bacterial *hdc* genes were PCR-amplified, using specific oligonucleotides previously described in the literature to amplify the gene from either Gram-positive or Gram-negative bacteria. Fig. 3 shows that *hdc* gene from Gram-positive bacteria was present in DNA isolated from all cheese samples, whereas the *hdc* gene from Gram-negative species was absent in all samples. Since the *hdc* genes of certain Gram-positive bacteria are occasionally located in the unstable pHDC plasmid, specific oligonucleotides that align within the plasmid sequence were also used in the PCR procedure to ascertain the presence of pHDC-containing bacteria. Certain areas, such as Area 1 in Cheese 3, also contained the pHDC plasmid, but not all of them did. Histamine producers with the *hdc* gene, belonging to the Gram-positive group, were present in all areas defined in cheese samples, and some of them contained this gene in an extrachromosomal, mobile plasmid.

3.3. Determination of histamine-producing microbiota in cheese

The *hdc* amplicons obtained by PCR were purified and subjected to Sanger sequencing to identify the histamine-producing microbiota. Two or more chromatographic peaks overlapped in each area, indicating that several *hdc* sequences, and, thus, histamine producers, were presumably present in the sample. The predominant species are listed in Table 3. The nucleotide sequences corresponded to *L. parabuchneri/buchneri* and the group *T. halophilus/T. muriaticus/Oenococcus oeni/Latilactobacillus sakei/L. hilgaldii,* whose *hdc* sequence is completely conserved and indistinguishable (Wuthrich et al., 2017). These histamine-producing bacteria are commonly found in cheese (Diaz et al., 2015; Diaz, Ladero, Redruello, et al., 2016; Møller, Ucok, & Rattray, 2020; O'Sullivan et al., 2015; Wechsler et al., 2021). We therefore presume that non-starter LAB were the main histamine producers in the ripened cheeses we analysed. The *hdc* gene of *L. parabuchneri/buchneri* is located in the chromosome, whereas the group *T. halophilus/T. muriaticus/O. oeni/L. sakei/L. hilgaldii* presumably contains the *hdc* genes in the pHDC plasmid (Lucas et al., 2005), which is in line with the results we obtained by PCR.

3.4. Analysis of physical and chemical properties of the different areas

The production of histamine is strongly influenced by microbiological and environmental factors present in food (Moniente et al., 2021). Since the same histamine producers were found in every cheese area subjected to analysis, the next step consisted in determining several of the cheeses' physical and chemical properties in order to elucidate their relationship with histamine accumulation in a particular pattern. Moisture (g/100 g cheese), a_W, salt content (g NaCl/100 g cheese), and oxidative state (measured by the TBARS assay as mg malonaldehyde/kg cheese) were determined in each area in order to correlate them with the amount of histamine in the samples. The values obtained for these measurements are summarized in Table 4. In order to globally represent the differences in each zone, the percentages of deviation of each parameter from the mean value of the wedge for each area are plotted in box-and-whisker plots in Fig. 4.

No relevant deviations of a_W were found in any area with respect to the mean value of the entire wedge. Moisture and salt content were significantly higher in the cores (with deviations of 15.1% and 22.4% in Area 2, and 8.0% and 9.0% in Area 3, respectively) with respect to the

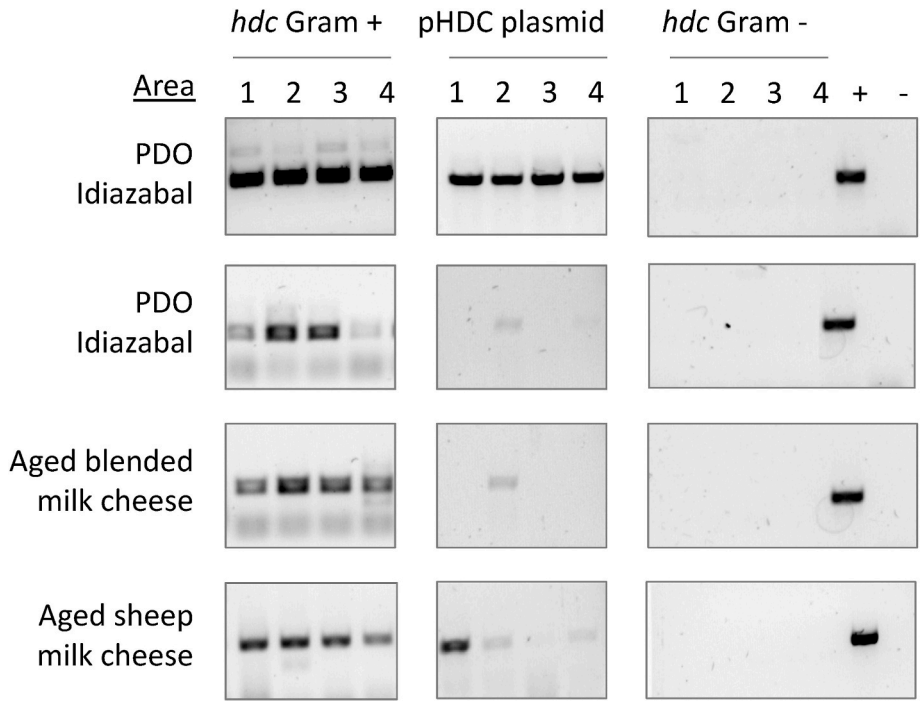

Fig. 3. PCR amplification of the *hdc* gene from Gram-positive and Gram-negative bacteria, as well as of the pHDC plasmid in the four areas of analysis established in the wedge of cheeses of several types of milk.

mean values in the entire wedge; whereas they were lower in the rind (moisture and salt content with deviations of −6.2% and −11.9% in Area 1, and -18.0% and −20.2% in Area 4, respectively). The same behavior was observed regarding histamine content distribution throughout the wedge, which was higher in the core (Areas 2 and 3) than in the outer parts.

Regarding oxidative state, despite the fact that non-significant differences have been found among areas, a slight tendency is observed: the core of the cheeses (Areas 1 and 4) was less oxidized (−29.6% and −40.3% in Areas 1 and 4, respectively) than the mean value of oxidation of the entire wedge, and also less oxidized than the outer portions. It is well known that lipid oxidation in cheese is associated with rancidity, flavor defects and oxidative deterioration, as well as loss of nutritional quality (Xiang et al., 2021). As a consequence of lipid oxidation, molecules such as aldehydes, ketones, alcohols, acids, and hydrocarbons are produced (Bergamo, Fedele, Balestrieri, Abrescia, & Ferrara, 1998). Additionally, the emergent trend of consumers towards low-fat dairy products results in an increase in the impact of protein oxidation in cheeses, which can also occur due to heat treatments applied during the cheesemaking process. Exposure to light during storage (frequently in packaging with transparent materials) can also lead to photo-oxidation of cheese (Kastrup Dalsgaard et al., 2010). The formation of sulphur compounds, such as dimethyl disulphide, and protein-bound carbonyls, is an indicator not only of the direct oxidation of aromatic and aliphatic amino acids, but also of secondary protein oxidation during glycation and lipoperoxidation (Rinaldi, Palocci, Di Giovanni, Iacurto, & Tripaldi, 2021). The influence of the glycation and the Maillard reaction during cheese manufacturing is worth highlighting (Fedele & Bergamo, 2001). Furfurals and advanced glycation end products are present in higher amounts in higher-fat and aged cheeses (Sharma, Kaur, Thind, Singh, & Raina, 2015) as well as in hard cheeses (Li et al., 2022). Since the cheeses used in this study were hard aged cheeses, it is possible that other TBARS products different than malonaldehyde could have been formed, leading to an overestimation of this product.

Despite the fact that the highest histamine content was found in the core of the cheeses (Areas 1 and 2), similarly to salt content and moisture, a positive, statistically significant correlation could not be established for any of these parameters (r −0.11, *p*-value 0.59 and r 0.33, *p*-value 0.082, respectively). Manca et al. (2020) were also unable to establish a relationship between histamine and water activity, moisture, or salt content in Fiore Sardo cheese. A significant correlation, negative in this case, between histamine content and oxidative state in the different areas of the cheese could not be determined either (r −0.37; *p*-value 0.056). However, a tendency to accumulate histamine in the wedge's saltier, more humid, and less oxidized areas was clearly notable. Other studies have indicated that high salt concentration could impair histamine production in food (Besas & Dizon, 2012; Møller, Castro-Mejía, Krych, & Rattray, 2021; Sumner, Roche, & Taylor, 1990; Tabanelli et al., 2012), probably due to the inhibitory effect of salt on microbial growth (Bansal & Mishra, 2020; Bisig, 2014). The same kind of microbial growth control can be exerted by a_w: the production of histamine can thus be inhibited when a_w has been sufficiently reduced to impair the proliferation of histamine producers (FAO/WHO, 2013). However, it has also been reported that the halophilic bacterium *Tetragenococcus* sp. (obtained in this study as histamine producer, as shown in Table 3) has been able to grow (Unno et al., 2020), and even to produce histamine, at 20% (w/v) NaCl (Kimura et al., 2001; Satomi et al., 2008). *Staphylococcus epidermidis* and *S. capitis* also produced notable histamine amounts at 10% (w/v) NaCl (Hernandez-Herrero, Roig-Sagues, Rodriguez-Jerez, & Mora-Ventura, 1999). Furthermore, *Tetragenococcus* sp. has been proposed as a producer of a histamine under O_2-limiting conditions (Kimura et al., 2001). The latter bacterium could be associated with higher histamine content in the cheese core, where oxidative state is lower, as suggested by our data. Taken together, the analyses of the physical and chemical properties of the different areas in cheese samples revealed an observable trend: histamine tended to accumulate in the saltier, more humid, and less oxidized areas in a wedge. Further studies are nevertheless required to elucidate the microbiological/physicochemical causes of the characteristic distribution pattern of histamine accumulation in long-ripened cheeses with high histamine content.

Table 3
Summary of the main BLAST analyses of the amplicons of the *hdc* genes as obtained from the different areas of the cheeses.

Cheese sample		Main BLAST output	E-value	% identity	GenBank accession number
Cheese 1	Area 1	*L. parabuchneri*	1E-151	99.67%	CP018796.1
		T. halophilus	1E-137	95.24%	AB670117.1
	Area 2	*T. halophilus*	5E-151	97.75%	AB670117.1
		L. parabuchneri	2E-139	95.83%	CP018796.1
	Area 3	*T. halophilus*	1E-152	99.67%	AB670117.1
		L. parabuchneri	8E-149	98.37%	CP018796.1
	Area 4	*T. halophilus*	1E-152	99.67%	AB670117.1
		L. parabuchneri	6E-145	97.11%	CP018796.1
Cheese 2	Area 1	*L. parabuchneri*	1E-156	99.00%	CP018796.1
	Area 2	*L. parabuchneri*	2E-150	99.01%	CP018796.1
	Area 3	*L. parabuchneri*	4E-152	98.40%	CP018796.1
	Area 4	*T. halophilus*	3E-74	86.13%	AB362339.1
		L. parabuchneri	1E-72	89.43%	CP018796.1
Cheese 3	Area 1	*T. halophilus*	2E-135	95.75%	AB670117.1
		L. parabuchneri	2E-125	93.79%	CP018796.1
	Area 2	*T. halophilus*	3E-179	98.89%	AB670117.1
	Area 3	*L. parabuchneri*	4E-173	99.13%	CP018796.1
	Area 4	*L. parabuchneri*	9E-175	99.42%	CP018796.1
Cheese 7	Area 1	*L. parabuchneri*	3E-158	99.68%	CP018796.1
		T. halophilus	4E-117	91.72%	AB670117.1
	Area 2	*L. parabuchneri*	5E-151	98.00%	CP018796.1
	Area 3	*T. halophilus*	2E-130	94.00%	AB670117.1
		L. parabuchneri	8E-144	100.00%	CP018796.1
	Area 4	*T. halophilus*	3E-115	92.86%	AB362339.1
		L. parabuchneri	1E-148	97.76%	CP018796.1

4. Conclusions

This study highlights the relevance of choosing appropriate cheese sampling areas for the accurate quantification of histamine in long-ripened cheeses with an elevated histamine concentration. A clear pattern of histamine distribution was inferred, since it accumulated in the core, whereas the peripheral rind exhibited the lowest concentration. Extracting the sample from the inner and the outer parts of the wedge can allow researchers to represent an average value of histamine content, whereas sampling the inner core of the wedge will tend to yield the highest value. A causative relationship with the distribution of the histamine-producing microbiota could not be established. On the whole, nevertheless, histamine tended to accumulate in the saltier, more humid, and less oxidized areas of the cheese wedge.

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Table 4
Analyses of physical and chemical properties (water activity [a_W], moisture [g/100 g cheese], salt content [g NaCl/100 g], oxidative state [measured by the 2-thiobarbituric acid reactive substances [TBARS] test in mg malonaldehyde/kg]) of the selected cheeses in the defined areas (Area 1 corresponded to the central rind of the wedge, Area 2 to the central core, Area 3 to the peripheral core, and Area 4 to the peripheral rind of the wedge).

Cheese sample		a_W	Moisture (g/100 g)	Salt content (g NaCl/100 g)	TBARS assay (mg malonaldehyde/kg)
Cheese 1	Area 1	0.910	25.4	1.57	2.83
	Area 2	0.917	29.6	1.88	2.32
	Area 3	0.917	29.3	1.79	2.33
	Area 4	0.903	22.2	1.47	3.28
Cheese 2	Area 1	0.929	26.9	1.30	2.60
	Area 2	0.933	33.2	1.83	4.67
	Area 3	0.930	29.8	1.60	1.75
	Area 4	0.929	23.7	1.12	6.38
Cheese 3	Area 1	0.916	27.8	1.38	15.07
	Area 2	0.910	30.4	1.48	1.94
	Area 3	0.915	29.8	1.38	2.48
	Area 4	0.919	25.7	1.16	5.04
Cheese 4	Area 1	0.915	26.0	1.39	5.87
	Area 2	0.914	29.6	1.63	1.73
	Area 3	0.917	28.6	1.55	1.79
	Area 4	0.917	24.1	1.22	5.56
Cheese 7	Area 1	0.862	20.9	1.23	4.64
	Area 2	0.840	28.1	2.36	2.84
	Area 3	0.872	24.0	1.90	2.50
	Area 4	0.850	13.8	1.15	11.09
Cheese 9	Area 1	0.870	20.5	1.62	14.12
	Area 2	0.882	27.4	2.74	7.32
	Area 3	0.876	25.9	2.37	5.99
	Area 4	0.870	17.6	1.54	16.87
Cheese 10	Area 1	0.912	26.1	1.29	1.34
	Area 2	0.916	31.2	1.80	0.93
	Area 3	0.927	29.7	1.59	1.02
	Area 4	0.903	24.3	1.18	0.97
Average	Area 1	0.902^a	24.8^a	1.40^a	6.64^a
	Area 2	0.902^a	29.9^b	1.96^b	3.11^a
	Area 3	0.908^b	28.2^b	1.74^b	2.55^a
	Area 4	0.902^a	21.6^a	1.26^a	7.03^a

Lowercase letters indicate significant differences in each cheese.

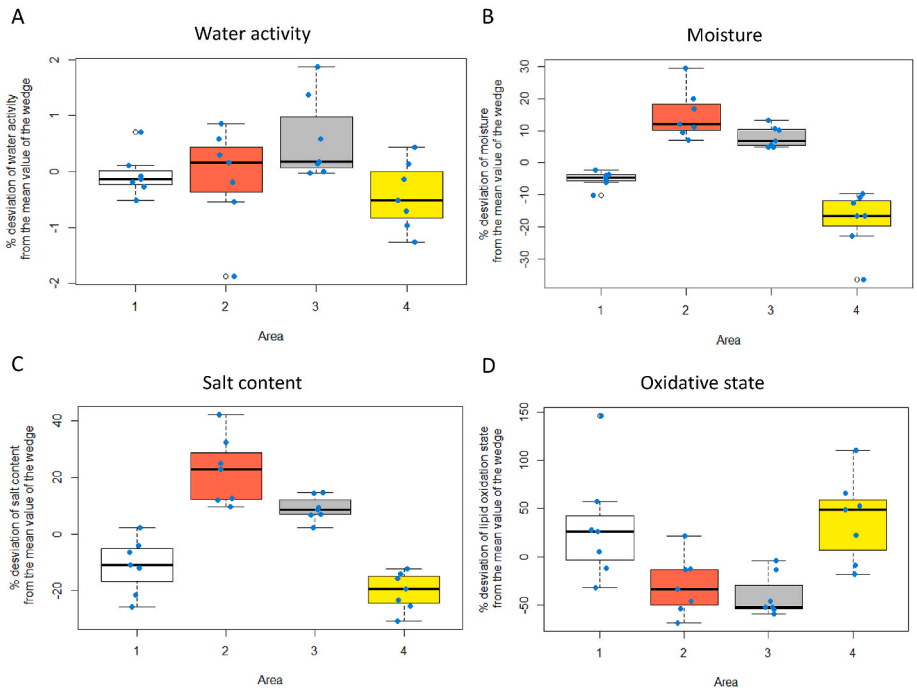

Fig. 4. The percentage of deviation of physical and chemical properties (water activity [A], moisture [B], salt content [C], and oxidative state [D]) measured in each cheese area from the mean value of the entire wedge, represented in a box-and-whisker plot. Area 1 corresponds with the central rind of the wedge, Area 2 with the central core, Area 3 with the peripheral core, and Area 4 with the peripheral rind. The median line of the box represents the medians of histamine concentration in each defined area of the wedge.

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Declaration of competing interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.114099.

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Manuscrito VI. Potential of histamine-degrading microorganisms and diamine oxidase (DAO) for the reduction of histamine accumulation along the cheese ripening process.

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Potential of histamine-degrading microorganisms and diamine oxidase (DAO) for the reduction of histamine accumulation along the cheese ripening process

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ABSTRACT

Lentilactobacillus parabuchneri is the main bacteria responsible for the accumulation of histamine in cheese. The goal of this study was to assess the efficiency of potential histamine-degrading microbial strains or, alternatively, the action of the diamine oxidase (DAO) enzyme in the reduction of histamine accumulation along the ripening process in cheese. A total of 8 cheese variants of cow milk cheese were manufactured, all of them containing *L. parabuchneri* Deutsche Sammlung von Mikroorganismen 5987 (except for the negative control cheese variant) along with histamine–degrading strains (*Lacticaseibacillus casei* 4a and 18b; *Lactobacillus delbrueckii* subsp. *bulgaricus* Colección Española de Cultivos Tipo (CECT) 4005 and *Streptococcus salivarius* subsp. *thermophilus* CECT 7207; two commercial yogurt starter cultures; or *Debaryomyces hansenii*), or DAO enzyme, tested in each cheese variant. Histamine was quantified along 100 days of cheese ripening. All the degrading measures tested significantly reduced the concentration of histamine. The highest degree of degradation was observed in the cheese variant containing *D. hansenii*, where the histamine content decreased up to 45.32 %. Cheese variants with *L. casei,* or *L. bulgaricus* and *S. thermophilus* strains, also decreased in terms of histamine content by 43.05 % and 42.31 %, respectively. No significant physicochemical changes (weight, pH, water activity, color, or texture) were observed as a consequence of the addition of potential histamine-degrading adjunct cultures or DAO in cheeses. However, the addition of histamine-degrading microorganisms was associated with a particular, not unpleasant aroma. Altogether, these results suggest that the use of certain histamine-degrading microorganisms could be proposed as a suitable measure in order to decrease the amount of histamine accumulated in cheeses.

1. Introduction

Biogenic amines (BAs) are non-volatile nitrogenous organic bases of low molecular weight mainly formed by enzymatic decarboxylation of their precursor amino acids, or by amination and transamination of aldehydes and ketones (Benkerroum, 2016; Linares et al., 2011). Histamine is one of the most important BAs in beverages and fermented foods, such as wine and cheese (Linares et al., 2011; Tittarelli et al., 2019). It is

Abbreviations: AQC, 6-aminoquinolyl-*N*-hydroxyuccinimidyl carbamate; a_w, water activity; BAs, biogenic amines; bp, base pairs; CECT, Colección Española de Cultivos Tipo; CFU, colony forming units; CIE, International Commission on Illumination; DAO, diamine oxidase enzyme; DSM, DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen; EFSA, European Food Safety Authority; HDC, histidine decarboxylase enzyme; *hdc*, histidine decarboxylase gene; HNMT, histamine *N*-methyltransferase enzyme; HPLC, high-performance liquid chromatography; IPLA, Instituto de Productos Lácteos de Asturias; LAB, lactic acid bacteria; MRS, De Man, Rogosa and Sharpe; NSLAB, non-starter LAB; PCA, Plate Count Agar; PCR, polymerase chain reaction; PDB, Potato Dextrose Broth; RP-HPLC, Reverse Phase – High Performance Liquid Chromatography; SPE, Solid Phase Extraction; subsp., subspecies; TPA, Texture Profile Analysis.

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synthesized by decarboxylation of the precursor amino acid L-histidine, catalyzed by the histidine decarboxylase (HDC) enzyme (Linares et al., 2011). Histamine is physiologically involved in numerous metabolic activities in the human body (Maintz & Novak, 2007; Yilmaz & Gökmen, 2019). It can be inactivated either extracellularly, mainly by the diamine oxidase (DAO) enzyme, or intracellularly, by the *N*-methyltransferase (HNMT) enzyme (Maintz & Novak, 2007).

Nevertheless, an imbalance caused by excessive oral ingestion, by misfunction in histamine catabolism, or by uncontrolled intake on the part of sensitive people can lead to human health disorders (Ladero et al., 2010; Schnedl et al., 2019). According to the European Food Safety Authority (EFSA), histamine can cause dangerous intoxication (EFSA, 2011): its accumulation can cause headache, bronchospasm, hypotension, edema, or even anaphylactic shock (Maintz & Novak, 2007).

Ripened cheese is one of the most widely studied fermented products commonly associated with histamine poisoning (Benkerroum, 2016; Muthukumar et al., 2020). Although maximum legal limits of histamine for fresh fish and fishery products (200 mg/kg) have been established by European Commission Regulation No. 2073/2005 (Commission Regulation, 2005), no legislation is in place regarding the content of histamine in dairy products (Ladero et al., 2017). According to the EFSA, concentrations over 500 mg/kg are harmful to health, and >1,000 mg/kg can be lethal for sensitive people (EFSA, 2011; Suzzi et al., 2022). Concentrations of histamine lying over 500 mg/kg have been detected in commercial cheeses (Botello-Morte et al., 2022) and concentrations of up to 2,500 mg/kg have even been reported (Maintz & Novak, 2007).

In food, the synthesis of histamine has a microbial origin (Herrero-Fresno et al., 2012). The main histamine-producing bacteria in cheese are lactic acid bacteria (LAB), which can be part of starter cultures, stem from milk, or contaminate food at some stage of manufacture due to poor hygienic practices (Linares et al., 2012; Yadav et al., 2019). Certain *Lactobacillus* species including *Lentilactobacillus parabuchneri, L. buchneri, Lactiplantibacillus plantarum, Latilactobacillus curvatus, L. helveticus* and *L. lactis* represent the main non-starter LAB (NSLAB) community responsible for the accumulation of histamine in cheese (Ascone et al., 2017; Barbieri et al., 2019; Burdychova & Komprda, 2007; Moniente et al., 2021). Among these, it has been reported that *L. parabuchneri* is the main responsible agent for the production of histamine in cheese (Botello-Morte et al., 2022; Diaz et al., 2018). Ascone et al., (2017) detected histamine-forming bacteria in almost 20 % of the raw milk samples they analysed, and *L. parabuchneri* was indeed present in 97.4 % of the positive samples.

It would be necessary to implement a series of measures designed for the prevention of histamine accumulation, such as 1) the use of starter cultures unable to produce histamine; 2) the removal of general microbiota and, thus, histamine-producing microorganisms from milk, through heat or high-pressure treatments; and 3) the modification of cheese ripening times (Herrero-Fresno et al., 2012; Jaguey-Hernández et al., 2021; Moniente et al., 2021). Proposed strategies to degrade histamine accumulated in cheese are based on the use of histamine-degrading strains (biological degradation) or enzymes such as DAO (enzymatic degradation) (Benkerroum, 2016; Moniente et al., 2021).

Leuschner & Hammes (1998) demonstrated the degrading ability of certain microorganisms isolated from food, such as *Brevibacterium linens*, *Geotrichum candidum, L. sakei*, *L. plantarum, L. pentosus*, *Arthrobacter* sp., *Rhodococcus* sp., *Pediococcus acidilactici*, and *Micrococcus* sp. Herrero-Fresno et al. (2012) isolated from cheese 17 strains of *Lacticaseibacillus casei* with histamine degradation capacity. Two strains (*L. casei* 4a and 18b) were associated with the highest degradation rates of histamine in a Cabrales-type mini cheese. Tittarelli et al. (2019) isolated strains of *L. casei* A422 and *Enterococcus casseliflavus* A143 from raw sheep cheese with histamine degradation rates higher than 50 %. Also, Guarcello et al. (2016) managed to isolate histamine-degrading strains from Italian cheeses: several strains of *L. casei*, *L. paracasei* subsp. *paracasei, L. paracasei* subsp. *tolerans*, *L. delbrueckii* subsp. *lactis, Leuconostoc mesenteroides* subsp. *mesenteroides, P. pentosaceus, Levilactobacillus brevis, Streptococcus gallolyticus* subsp. *macedonicus*, *S. thermophilus, L. lactis* subsp. *lactis*, *E. lactis*, and *Weissella paramesenteroides*. Calvo-Pérez et al. (2013) and Domingos Lopes et al. (2020) reported that strains of *L. mesenteroides, L. garvieae, L. lactis, L. plantarum, L. paracasei* and *L. otakiensis* isolated from cheese were capable of degrading over 50 % of histamine in culture media. In the area of yeasts, *Debaryomyces hansenii* H525 and *Yarrowia lipolytica* H446 have also been shown to degrade histamine (Bäumlisberger et al., 2015).

Regarding the use of enzymes capable of degrading histamine, the addition of DAO as a strategy for the degradation of preformed histamine has been studied in fish-based foods, but not in dairy products. In fish slurry and in tuna soup, enzymatic degradation was capable of achieving a greater degree of histamine degradation than biological degradation (Dapkevicius et al., 2000; Naila et al., 2012).

Our study's goal was to assess the effect of different strategies for the reduction of histamine accumulation along the ripening process of pasteurized cow milk cheeses contaminated with *L. parabuchneri*, the leading histamine producer. During the ripening of cheese, the prevention of accumulation/degradation efficiency of different adjunct starter cultures, comprised by certain LAB or the yeast *D. hansenii*, was compared with the action of the DAO enzyme.

2. Materials and methods

2.1. Microorganisms and culture conditions

The microbial cultures used in the present study are listed in Table 1. The laboratory media De Man Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, Hampshire, England), M17 broth (Oxoid), and Potato Dextrose Broth (PDB) (Oxoid) were used to culture the microorganisms under study. Yogurt Type I starter culture (Abiasa, Pontevedra, Spain) and YoFlex® Premium 1.0 starter culture (CHR Hansen, Hørsholm, Denmark) were composed of *L. bulgaricus* and *S. thermophilus*. Furthermore, F-DVS GRANA-102 cheesemaking starter culture (CHR Hansen) contained *S. thermophilus*, *L. helveticus, L. bulgaricus*, and *L. paracasei*.

Table 1
Microorganisms and starter cultures used in the cheesemaking process: sources and optimal culture conditions.

Strains and cultures	Culture conditions	Source
Lentilactobacillus parabuchneri DSM 5987	MRS, 37 °C, 24 h, anaerobiosis	DSMZ[1] collection
Lacticaseibacillus casei 4a	MRS, 37 °C, 24 h, anaerobiosis	IPLA[2] collection
L. casei 18b	MRS, 37 °C, 24 h, anaerobiosis	IPLA[2] collection
Lactobacillus delbrueckii subsp. *bulgaricus* CECT 4005	MRS, 37 °C, 24 h, anaerobiosis	CECT[3] collection
Streptococcus salivarius subsp. *thermophilus* CECT 7207	M17, 37 °C, 24 h, aerobiosis	CECT[3] collection
Yogurt Type I starter culture	Directly added to milk for cheesemaking	Abiasa[4]
YoFlex® Premium 1.0 starter culture	Directly added to milk for cheesemaking	CHR Hansen[5]
F-DVS GRANA-102 Starter culture	Directly added to milk for cheesemaking	CHR Hansen[5]
Debaryomyces hansenii	PDB, 30 °C, 24 h aerobiosis	Isolated from cheese (Botello-Morte et al., 2022)

1. Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh (DSMZ, Germany).
2. Instituto de Productos Lácteos (IPLA, Spain).
3. Colección Española de Cultivos Tipo (CECT, Spain).
4. Avances Bioquímicos Alimentación, S.L. (Abiasa, Spain).
5. Chr. Hansen Holding A/S (CHR Hansen, Denmark).

2.2. Evaluation of histamine degradation by microbial cultures

The degrading capacity of several microbial cultures was assessed by applying optimal culture conditions for each microorganism, summarized in Table 1. A total of 7 strains of *L. bulgaricus* and *S. thermophilus* from the Colección Española de Cultivos Tipo (CECT) were tested: *L. bulgaricus* CECT 4005, CECT 4006, CECT 5035, and CECT 5036; and *S. thermophilus* CECT 801, CECT 986, and CECT 7207. Additionally, two commercial yogurt starters were used for histamine degrading evaluation: Yogurt Type I starter culture (Abiasa) and YoFlex® Premium 1.0 starter culture (CHR Hansen). Histamine degradation was tested by adding 1 mM histamine dihydrochloride (Sigma, Munich, Germany). The histamine stock solution (1 M) was freshly prepared by dissolving 1,840 mg of histamine dihydrochloride in 10 mL of sterile deionized water, and the solution was passed through a 0.22-μm Millipore filter to be sterilized. Despite it was a 1000X stock solution, the addition of histamine (100 μL) did not change the pH of the medium (100 mL). Overnight cultures of bacteria in the appropriate media (from single colony picks) were refreshed (dilution 1/500) and histamine was then added. The experiments were incubated for 24 h at 37 °C in anaerobiosis in MRS broth for the growth of *L. bulgaricus* and/or M17 broth in aerobiosis for *S. thermophilus*. Samples were subsequently stored at −20 °C until histamine was quantified by Reverse Phase - High Performance Liquid Chromatography (RP-HPLC). Since the YoFlex® Premium 1.0 starter culture (CHR Hansen) was unable to degrade histamine in laboratory media, its histamine-degrading ability was also assessed in manufactured yogurt inoculated with 0.5, 1.0 or 1.5 mM histamine dihydrochloride (Sigma) and incubated at 41 ± 1 °C until reaching pH 4.32 ± 0.03. Samples were kept frozen (-20 °C) until analysis.

Together with the yogurt-producing microorganisms selected for our cheesemaking process, a review of prior relevant literature led us to identify potential histamine degraders, such as *L. casei* (Herrero-Fresno et al., 2012) and *D. hansenii* (Bäumlisberger et al., 2015). Thus, the 4a and 18b strains of *L. casei*, kindly provided by the Instituto de Productos Lácteos (IPLA), were selected for their proven ability to degrade over 40 % of histamine *in vitro*. *D. hansenii* strain isolated from cheese was also selected (Botello-Morte et al., 2022).

2.3. Analysis of the effect of DAO enzyme on milk at refrigeration temperature

Although the histamine–degrading activity of DAO is well known, our aim in this experiment was to evaluate whether that enzymatic activity was maintained in milk, the main matrix from which cheese is made. For that purpose, a preliminary study to assess the histamine–degrading capacity of DAO in milk at refrigeration temperature was carried out, based on Dapkevicius et al. (2000) and Naila et al. (2012) with some modifications. A total of 1 mM histamine dihydrochloride (Sigma), from a sterile 1 M stock solution prepared in deionized water (as described in section 2.2), and 108.7 U/L of DAO, from porcine kidney, 0.11 U/mg solid (Sigma, D7876, lot. Number 011 M7015), were added to commercial fat milk ultra-high-temperature processing (UHT)-treated, homogenized and aseptically packed in 1L Tetra-pack, and incubated under refrigeration for 0, 1, 8, and 24 h. Subsequently, milk samples were stored at −20 °C, until histamine was quantified by RP-HPLC.

2.4. Detection of bacterial histidine decarboxylase (hdc) gene by specific polymerase chain reaction (PCR) amplification

The presence of the histidine decarboxylase gene (*hdc*), responsible for the synthesis of histamine, was assessed in potential histamine-degrading bacteria by PCR, based on the method described in Botello-Morte et al. (2022) with the specific oligonucleotides JV17HC (AGACCATACACCATAACCTT) (Le Jeune et al., 1995) and HDC3 (GATGGTATTGTTTCKTATGA) (Coton & Coton, 2005). Total DNA from the histamine-producing bacteria *L. parabuchneri* DSM 5987 was used as positive control for *hdc* amplifications.

2.5. Experimental cheese manufacture model

During the cheesemaking process, *L. parabuchneri* DSM 5987, proposed as the major histamine producer in many types of cheese (Møller et al., 2021), was used as a natural producer of histamine. This microorganism is habitually present in cheese, where it produces large amounts of histamine (Diaz et al., 2018). Furthermore, it is usually used to inoculate cheese milk in studies in which cheeses contaminated with high amounts of histamine needs to be produced (Møller et al., 2019; Wechsler et al., 2021). A final concentration of $1{\cdot}10^6$ colony forming units (CFU)/mL was added to milk, based on recent studies by Diaz et al. (2018) and Wechsler et al. (2021), as well as on previous experiments carried out in our laboratory (data not shown). Several potentially histamine-degrading bacteria (Table 1) were also added to milk as microbial cultures at the same concentration. Yogurt Type I (Abiasa) and YoFlex® Premium 1.0 (CHR Hansen) starter cultures were added to milk following the manufactureŕs instructions. The yeast *D. hansenii* was added at a final concentration of $1{\cdot}10^5$ CFU/mL. Determinations of microbial counts were assessed by plating and by using a Thoma cell counting chamber. DAO enzyme from porcine kidney (Sigma, D7876, lot. Number 011 M7015 and 011 M7015V), at 0.11 U/mg solid, was used at a final concentration of 71 U/L.

Eight cheese variants were prepared in this experiment, with different potential histamine-degrading starters or DAO enzyme for each cheese variant (Table 2). Fig. 1 displays the cheese manufacturing flow diagram. All the cheese variants were inoculated using the F-DVS GRANA-102 cheese starter culture (CHR Hansen), following the manufactureŕs instructions. Every cheese variant of 18 cheeses, of approximately 200 g each, was made from 40 L of pasteurized whole-fat cow's milk obtained from a local farm (Zaragoza, Spain) and heated to 34 °C. Due to the cost of DAO enzyme, cheeses containing this enzyme were manufactured from 22.75 L of milk. Then, 0.025 % (v/v) calcium chloride was added and incubated during 30 min. After that, the cheese variants were inoculated with the cheese starter (8.3 g, except for cheese variant VIII, prepared from 22.75 L of milk, which was inoculated with 4.9 g of cheese starter) and with *L. parabuchneri* (except for control cheese variant I, only containing the cheese starter but not any adjunct cultures) as well as with specific adjunct cultures for each cheese variant. A total of 40 g Yogurt Type I Starter culture from Abiasa were added to 40 L of milk in cheese variant V, whereas 8 g of Yoflex Premium 1.0 from CHR Hansen were added in case of cheese variant VI. In case of the DAO enzyme, a total of 14.67 g DAO was dissolved in about 200 mL of milk and subsequently added to the vat. Milk was then kept at 34 °C for 45 min.

Table 2
Composition of the cheese variants during the cheesemaking process, indicating the use of diamine oxidase (DAO) or the addition of starter cultures, together with the F-DVS GRANA-102 starter culture (CHR Hansen), following the manufactureŕs instructions.

Cheese variant	Composition
I	Negative control (no adjunct cultures)
II	Positive control with *Lentilactobacillus parabuchneri* DSM 5987
III	*L. parabuchneri* DSM 5987 and *Lacticaseibacillus casei* 4a and *L. casei* 18b
IV	*L. parabuchneri* DSM 5987 and *Lactobacillus delbrueckii* subsp. *bulgaricus* CECT 4005 and *Streptococcus salivarius* subsp. *thermophilus* CECT 7207
V	*L. parabuchneri* DSM 5987 and Yogurt Type I starter culture (Abiasa)
VI	*L. parabuchneri* DSM 5987 and YoFlex® Premium 1.0 starter culture (CHR Hansen)
VII	*L. parabuchneri* DSM 5987 and *Debaryomyces hansenii*
VIII	*L. parabuchneri* DSM 5987 and DAO

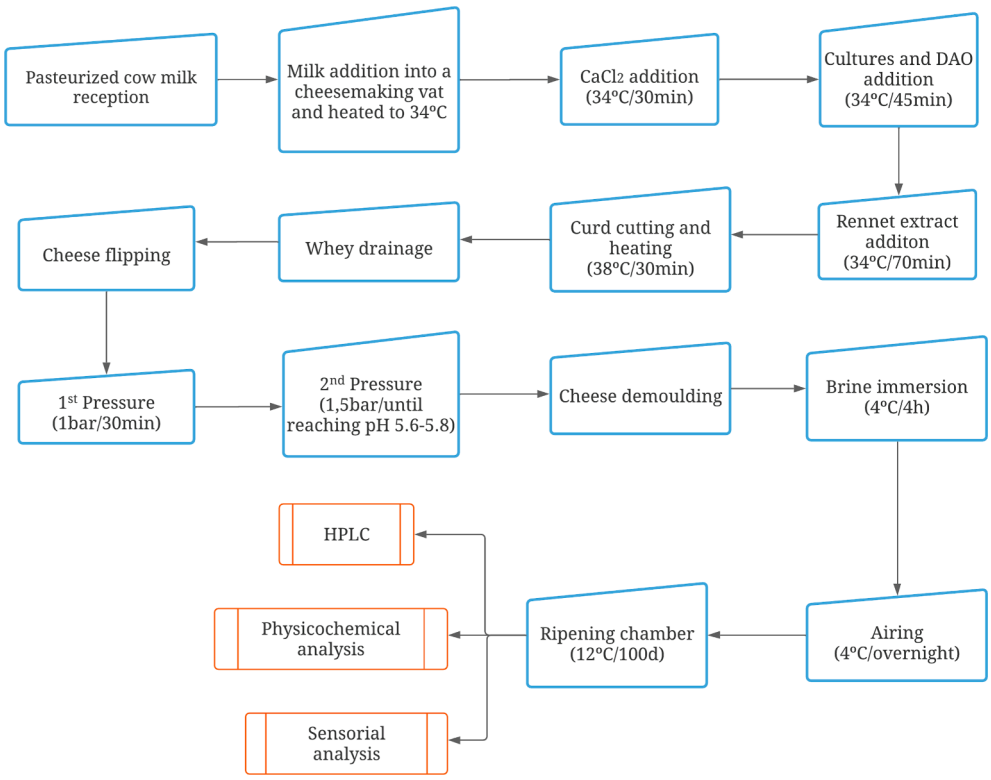

Fig. 1. Flow diagram of the different steps of the cheese manufacturing process (in blue) with the F-DVS-GRANA-102 cheese starter from CHR Hansen and *Lentilactobacillus parabuchneri* DSM 5987 as a histamine-producing microorganism, combined with DAO or histamine-degrading microorganisms. The analyses performed on the final cheese samples are shown in orange at the end of the diagram.

For coagulation, 6.5 mL of rennet extract (Carlina 1650, Danisco, Vinay, France) was diluted in 30 mL of mineral water, added to the milk, and incubated at 34 °C for 70 min. The curd was then cut into 1–cm cubes with knives equipped with vertical or horizontal wires. The tank was heated at 38 °C for 30 min. The whey was later removed, after which the curd grains were initially pressed into a pneumatic cheese press (Suministros Químicos Arroyo S.L., Santander, Spain) at 1 bar for 30 min and then at 1.5 bar for about 2 h, until the pH reached 5.6 – 5.8. They were then demolded and immersed in 18–20 % NaCl brine solution for 4 h, at 4 °C. The cheeses were subjected to overnight airing at 4 °C and finally stored for ripening for up to 100 days at 12 ± 1 °C with a relative humidity of 85 % in an Oscar Zarzosa J-700-Q ripening chamber (Oscar Zarzosa S.A., Villarcayo, Spain). From each cheese variant, 3 cheeses were collected at 0, 15, 30, 45, 60 and 100 days of ripening. According to Spanish legislation regarding cheese quality standards, these manufactured cheeses are to be classified as old cheeses (Real Decreto No 1113/2006).

2.6. Quantification of histamine by RP-HPLC

Different sample pretreatments were used for the determination of histamine in culture media, milk, yogurt, and cheese. To analyze histamine in a laboratory culture medium, a 1/20 dilution in Milli-Q water was carried out. In the case of milk, an aliquot of 5 mL of sample and 5 mL of 2 % (v/v) acetic acid (Panreac, Barcelona, Spain) was placed in a 15 mL plastic centrifuge tube and vortexed. The tube was then centrifuged at 4,000·*g* at 4 °C for 15 min to separate the lipid phase from the aqueous phase. Later, the supernatant was diluted 1/20 in Milli-Q water. The same analysis procedure was used for yogurt samples, except for the addition of 10 mL of Milli-Q water to the plastic tube before centrifugation (5 mL yogurt, 10 mL Milli-Q water, and 5 mL 2 % [v/v] acetic acid) and a 1/10 dilution in Milli-Q water of the supernatant. Finally, an aliquot of 5 g of cheese and 7.5 mL of 1 M HCl was placed in a 50 mL plastic centrifuge tube. The tube was capped and vortexed, after which it was centrifuged at 4,000·*g* at 4 °C for 21 min to separate the solution into two phases: lipids and proteins from the aqueous phase. The aqueous layer was collected and the sample extraction procedure was repeated 3 times. The final extracts were combined and their volume was brought up to 25 mL with 1 M HCl in a volumetric flask. After the pretreatment, samples were subjected to a solid-phase extraction procedure (SPE) with Oasis MCX cartridges (30 mg) (Waters Corporation, Milford, MA, USA). All samples were analyzed in triplicate. The analytes were eluted with 1.2 mL of 100 mM NaOH: MeOH (65:35) in a vial with 100 µL of 1.2 M HCl. A 20 µL aliquot of the neutralized eluate was obtained for derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (Waters AccQ, Milford, MA, USA). Samples were previously filtered through 0.20 µm Minisart NY nylon filters of 25 mm diameter (Sartorius, Göttingen, Germany). The separation, identification, and quantification of histamine was carried out by RP-HPLC 1220 Infinity LC (Agilent Technologies, Santa Clara, CA, USA) coupled with Fluorescence Detector Model 363 (Prostar, Varian, Sunnyvale, CA, USA) set at an excitation wavelength of 250 nm and an emission wavelength of 395 nm. For the separation of amines, a reverse-phase Luna C18 chromatography column (5 µm, 100 Å, 25 cm 4.6 mm) made by Analytica Phenomenex (Torrance, CA, USA) was used. The eluents were 50 mM sodium acetate and 1 % (v/v) tetrahydrofuran in milli-Q water adjusted to pH 6.6 by the addition of acetic acid (A) and HPLC-grade MeOH (B) according to Mayer et al. (2010). The elution program consisted of a gradient system with a flow-rate of 1 mL/min. Column temperature was 65 °C.

2.7. Analysis of physicochemical properties of cheese

To characterize the manufactured cheeses, weight, water activity (a_w), pH, color, and Texture Profile Analysis (TPA) were determined. Cheese samples were analyzed in triplicate at 0, 15, 30, 45, 60, and 100 days. An analytical balance (Sartorius A120S, Göttingen, Germany) was

used to assess weight evolution of the cheeses during ripening. The a_w was measured at 20 ± 1 °C by automatic water activity equipment (AquaLab 4 Tev, Decagon Devices Inc., Pullman, USA). A puncture pHmeter (Basic 20, Crison Instrument, Barcelona, Spain) was used to measure pH by introducing an electrode into the center of the cheese sample. To determine changes of cheese color during ripening, a Minolta ChromaMeters CR-400 colorimeter (Konika Minolta, Tokio, Japan) was used. International Commission on Illumination (CIE) standard illuminant D65 was performed with an angle vision of 10°. Color was expressed as CIElab coordinates (L*, a*, b*), which provide information regarding the product's luminosity (L *), varying from black = 0 to white = 100, with a deviation towards red (if a* > 0) or towards green (if a* < 0), and a deviation towards yellow if (b* > 0) or towards blue (if b* < 0) (Aktypis et al., 2018). TPA was performed on cheese samples using a 4 mm Ø Stainless Steel cylinder probe in a TA-XT2i Texture Analyser (Stable Micro Systems ltd, Godalming, UK). Cheeses were left at room temperature for 30 min until samples reached a specified temperature (20 ± 1 °C). Measures were performed on the cheeses' surface. Obtained data represent the mean of three measurements. Cheese hardness was measured as the maximum compression force required to compress the food during the first compression cycle, and represents the force exerted throughout that period (Katsiari et al., 2002). Hardness represents the force required to compress a cheese with the teeth until they penetrate (Szczesniak, 2002). Adhesiveness was measured according to the area of negative force after the first compression, and which is defined as the food's propensity to recover from a large deformation after removing the deformation stress. Adhesiveness corresponds with the degree to which the sample adheres to the teeth as chewing progresses (Szczesniak, 2002).

2.8. Sensory analyses of cheeses by sorting task

To evaluate eventual differences among sensory characteristics of manufactured cheeses, a sorting task was carried out (Rodrigues et al. 2020; Varela & Ares, 2012). A total of 42 untrained panelists accustomed to sensory analysis (19 male and 23 female, 23 to 60 years old) evaluated the cheese variants at the last point of ripening (100 days) and sorted them into groups based on odor similarities. Samples (n = 9; ca. 7 g) were presented in amber glass jars coded with three-digit numbers following a random order.

The sensory sheet contained a space to indicate each sample's code along with the descriptors or sensory attributes perceived by the panelists for each group of samples. In addition, a paragraph space for observations was provided at the bottom of the sensory sheet, so that panelists could specify their observations. The duration of sensory analysis was 20 min for each taster.

2.9. Statistical analyses

In order to compare the samples, Principal Component Analysis (PCA), one-way analysis of variance, two-way analysis of variance, and Tukey's test were performed with the XLSTAT software (version 2022.2.1; Addinssoft, Paris, France), establishing a *p* value of 0.05 for significance. Data analysis of the sorting task was carried out following the procedure described by Alegre et al. (2017).

3. Results and discussion

3.1. Selection of histamine-degrading microorganisms and DAO enzyme

3.1.1. Evaluation of histamine degradation by microbial cultures

A LAB strain related to yogurt production, specifically *S. thermophilus* PF3CT, has been identified as a histamine-degrading bacterium (Guarcello et al., 2016). Previous studies in our laboratory carried out with a wide collection of yogurt-producing LAB allowed to confirm the potential not only of *S. thermophilus*, but also of *L. bulgaricus* to degrade histamine (data not shown). Therefore, an in-depth study regarding the histamine-degrading capacity of certain yogurt-producing strains in their optimal *in vitro* culture conditions was required. To this aim, the histamine degradation ability of LAB strains was tested by adding 1 mM of histamine at exponential phase and incubating for 24 h at 37 °C in specific laboratory media. The histamine degradation rates of different strains of *L. bulgaricus* and *S. thermophilus* are shown in Table 3. In general, the *S. thermophilus* strains evaluated reached outstanding histamine degradation rates, namely higher than 36 %; although the specific strain *S. thermophilus* CECT 986 was unable to degrade histamine. Regarding the *L. bulgaricus* strains tested, they were not as effective, although the *L. bulgaricus* strain isolated from the commercial Yogurt Type I starter culture (Abiasa) was able to degrade up to 27 % of histamine under the experimental conditions assayed. To our knowledge, this is the first time that *L. bulgaricus* has been described as a microorganism capable of degrading histamine. Overall, the strains of *L. bulgaricus* and *S. thermophilus* with the highest histamine degradation rates (*L. bulgaricus* CECT 4005 and *S. thermophilus* CECT 7207, with 2.52 % ± 0.39 and 57.80 % ± 1.69, respectively) were selected to be used as adjunct cultures in cheesemaking. The synergistic relationship among these two bacteria during the production of yogurt (Aryana & Olson, 2017) led us to select the combination of *S. thermophilus* and *L. bulgaricus* in the cheesemaking process.

On the other hand, the strains isolated from the YoFlex® Premium 1.0 starter culture (CHR Hansen) were unable to degrade histamine in laboratory media (data not shown). A reduction of histamine concentration in yogurt manufactured with this starter culture, inoculated with 0.5, 1.0, or 1.5 mM of histamine and incubated at 41 ± 1 °C until reaching pH 4.32 ± 0.03 was nevertheless observed. Results showed a reduction of histamine to the order of 69.90 % ± 7.15; 36.99 % ± 5.14, and 11.92 % ± 7.74, depending on the initial concentration of histamine. It is well-known that the production of yogurt relies most often upon the success of the synergistic growth of *S. thermophilus* and *L. bulgaricus*. For that reason, it is possible that histamine degradation occurred in milk but not in synthetic medium. Due to the high degradation rates observed in a milk matrix, this commercial starter was also selected to be used as an adjunct culture in the subsequent *in situ* analysis of the cheesemaking process.

3.1.2. Histamine degradation by DAO enzyme

Since histamine is known to be a thermoresistant molecule, it is difficult to eliminate even by pasteurizing the milk. The occasional use of DAO could be proposed as a concrete alternative to reduce histamine levels in milk. According to the manufactureŕs specifications, the enzymatic activity of DAO is described as the amount of enzyme that

Table 3
Histamine degradation (%) in laboratory media under optimal culture conditions carried out by microbial strains tested.

Microorganisms	Histamine degradation (%)
Lactobacillus delbrueckii subsp. *bulgaricus* (*L. bulgaricus*) CECT 4005	2.52 % ± 0.39
L. bulgaricus CECT 4006	1.67 % ± 0.62
L. bulgaricus CECT 5035	− 1.05 % ± 0.08
L. bulgaricus CECT 5036	1.52 % ± 0.29
L. bulgaricus isolated from Yogurt Type I starter culture (Abiasa)	27.00 % ± 1.95
Streptococcus salivarius subsp. *thermophilus* (*S. thermophilus*) CECT 801	36.52 % ± 2.40
S. thermophilus CECT 986	− 0.94 % ± 0.93
S. thermophilus CECT 7207	57.80 % ± 1.69
S. thermophilus isolated from Yogurt Type I starter culture (Abiasa)	40.20 % ± 0.80

The results are presented as mean values with their standard deviation. The sign (-) does not denote a decrease, but an increase in the resulting final concentration of histamine.

will oxidize 1.0 μmol of substrate per hour at pH 7.2 at 37 °C. However, to produce manufactured cheese, a milk matrix is required, which has a pH of about 6.6. Furthermore, either the milk before cheese production or the final cheese product are usually stored at refrigeration temperatures before their use. In order to ascertain whether the DAO enzyme is able to maintain its enzymatic activity in a milk matrix under refrigeration temperatures, its histamine-degrading capacity in milk at 4 °C was analysed with the aim of assessing the potential of DAO either in histamine-contaminated milk or subsequently in cheese. DAO was able to degrade 22.76 % ± 1.46 of histamine after 1 h, 28.45 % ± 0.79 of histamine after 8 h, and 31.69 % ± 5.45 of histamine after 24 h of incubation. Therefore, a reduction of 0.21 mM of histamine was obtained in milk in one hour at 4 °C using 108.7 U/L DAO. Recent studies have shown that this enzyme is capable of degrading 0.54 mM substrate in buffer in one hour at 37 °C using 9.4 U/L DAO (Kettner et al., 2020). Thus, although the DAO enzyme had less overall degrading activity, it retained a remarkable degree of activity in a milk matrix at refrigeration temperatures. Dapkevicius et al. (2000) studied the effect of temperature on histamine degradation by DAO at suboptimal temperatures of 22 and 15 °C, reporting a considerable degradation activity. Accordingly, the addition of DAO to milk could be regarded as a useful measure to prevent histamine accumulation in case of milks subjected to inadequate hygiene practices or accidentally contaminated with histamine-producing bacteria.

3.2. Detection of bacterial histidine decarboxylase (hdc) gene by specific PCR amplification in histamine-degrading microorganisms used in cheeses

Some bacteria have been reported to be histamine-producing as well as histamine-degrading: for instance *L. delbrueckii* subsp. *lactis* (Burdychova & Komprda, 2007; Guarcello et al., 2016), *L. casei* (del Valle et al., 2018; Herrero-Fresno et al., 2012), and *E. casseliflavus* (Roig-Sagués et al., 2002; Tittarelli et al., 2019). The ability to produce or degrade histamine is a characteristic feature of a specific strain, and not attributable to all strains belonging to a bacterial species (Hrubisko et al., 2021). Consequently, a key factor for us to consider in the course of the cheese manufacturing experiment was the selection of potential histamine-degrading microorganisms lacking the *hdc* gene, in order to prevent an eventual simultaneous histamine production. Therefore, prior to the cheesemaking process, a *hdc* gene screening by PCR was carried out to evaluate whether the microorganisms used in the assay contained the *hdc* gene and consequently had the capacity to produce histamine. No bands appeared in any of the microorganisms selected for use in the cheesemaking process, except for the positive control corresponding to the DNA from *L. parabuchneri* DSM 5987 which, as expected, rendered a band of approximately 370 bp. The cheese starter used in this process did not contain the *hdc* gene (Supplementary Fig. 1).

3.3. Evolution of histamine concentration in manufactured cheeses

Histamine concentration varied significantly ($p < 0.05$) along ripening time (Fig. 2). At the onset of the experiment, histamine concentration in the cheeses was low and similar in all cheese variants, and likewise similar to that obtained elsewhere in milk, yogurt, and kefir (Özdestan & Üren, 2010; Pekcici et al., 2021), ranging, in our case, from 1.48 ± 0.39 (control cheese variant I) to 6.41 ± 0.04 (cheese variant VI) mg/kg. Furthermore, as expected, the maximum level of histamine was measured in all cheese variants after 100 days of ripening. Fresh cheeses usually have lower amounts of histamine than ripened cheeses, probably due to reduced growth of histamine–producing microbiota (Ercan et al., 2019), or due to lower availability of the precursor amino acid histidine because of less proteolysis (Moniente et al., 2021).

As expected, at the end of the 100-day ripening period, the amount of histamine in negative control cheese variant I was negligible (38.07 ± 13.90 mg/kg) with respect to the concentration obtained in the rest of the cheese variants, particularly taking into account the high histamine

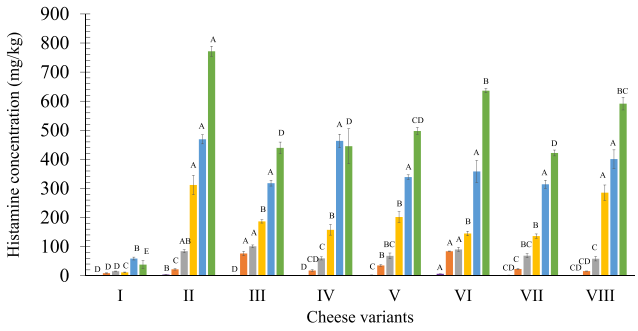

Fig. 2. Histamine concentration (mg/kg) in cheese variants (I to VIII) along the cheese ripening period: 0 days (purple), 15 days (orange), 30 days (grey), 45 days (yellow), 60 days (blue), and 100 days (green). Cheese variants: (I) control cheese, (II) *Lentilactobacillus parabuchneri* DSM 5987, (III) *L. parabuchneri* DSM 5987 and *Lacticaseibacillus casei* strains (4a and 18b), (IV) *L. parabuchneri* DSM 5987 and *Lactobacillus bulgaricus* CECT 4005 and *Streptococcus thermophilus* CECT 7207, (V) *L. parabuchneri* DSM 5987 and Yogurt Type I starter culture from Abiasa, (VI) *L. parabuchneri* DSM 5987 and YoFlex® Premium 1.0 starter culture from CHR Hansen, (VII) *L. parabuchneri* DSM 5987 and *Debaryomyces hansenii*, (VIII) *L. parabuchneri* DSM 5987 and DAO. The data shows the importance of ANOVA for the cheese variant factor. Capital letters (A, B, C, D, E) indicate significant differences for the cheese variant factor. See Supplementary Table 1 for the two-factor ANOVA data. Error bars show the mean error of data.

concentrations otherwise displayed by a large variety of commercial cheeses on the market (Botello-Morte et al., 2022). The little amount of histamine produced in control cheese variant I could be due to the presence of non-starter, environmental microbiota, colonizing the final product during the ripening period. The highest amount of histamine at this time was found in positive control cheese variant II with *L. parabuchneri* (771.55 ± 29.89 mg/kg). This microorganism is widely acknowledged as the major histamine producer in cheese, associated with a safety issue due to its formidable and rapid capacity to produce histamine under different conditions, such as refrigeration temperatures or even on stainless steel material (Diaz et al., 2016, 2018; Møller et al., 2019, 2021). The amount of histamine in cheese variant II was excessive and could lead to harmful consequences for consumer health according to EFSA determinations (EFSA, 2011). In that cheese variant, histamine accumulated progressively along the ripening period, with an increase in histamine concentration during the first 45 days; from that point on, it displayed a slower accumulation rate. For this reason, the difference in histamine concentration between cheese variant II and the other cheese variants was significantly greater at 45 days of ripening. An increase of histamine of around four times in cheese variant II was detected from 0 to 15 days (4.10 ± 0.44 to 21.85 ± 4.50 mg/kg), whereas similar increases were observed between 15 and 30 days (21.85 ± 4.50 to 84.21 ± 8.85 mg/kg) and between 30 and 45 days of ripening (84.21 ± 8.85 to 311.87 ± 56.86 mg/kg), respectively. From this point on, the rate of histamine accumulation slowed down from 45 to 60 days (311.87 ± 56.86 to 468.96 ± 27.06 mg/kg) and from 60 to 100 days of ripening (468.96 ± 27.06 to 771.55 ± 29.89 mg/kg), resulting in histamine increases of about 1.5 times. This gradual initial increase in histamine accumulation during the ripening period correlated with previous studies (Bunkova et al., 2013; Kebary et al., 1999; Novella-Rodríguez et al., 2002a; Novella-Rodríguez et al., 2004). The high histamine concentration observed in control cheese variant II allowed us to assess the potential of the measures implemented in the other cheese variants.

As shown in Fig. 3, at 100 days, the lowest histamine accumulation with respect to cheese variant II occurred in cheese variants VII (45.3 %), III (43.0 %), IV (42.3 %), and V (35.5 %), in which *D. hansenii*, *L. casei* 4a and 18b, *L. bulgaricus* in combination with *S. thermophilus* (both from the CECT), and the commercial yogurt starter culture from Abiasa, were used, respectively. It is worth highlighting that the two

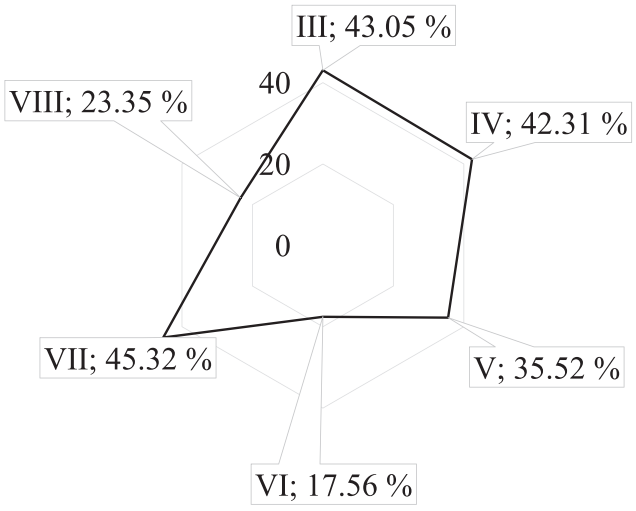

Fig. 3. Percentage (%) of histamine reduction by the effect of DAO or histamine-degrading strains on different cheese variants (III-VIII) with respect to control cheese variant II at 100 days of ripening time: (III) *Lentilactobacillus parabuchneri* DSM 5987 and *Lacticaseibacillus casei* strains (4a and 18b), (IV) *L. parabuchneri* DSM 5987 and *Lactobacillus bulgaricus* CECT 4005 and *Streptococcus thermophilus* CECT 7207, (V) *L. parabuchneri* DSM 5987 and Yogurt Type I starter culture from Abiasa, (VI) *L. parabuchneri* DSM 5987 and YoFlex® Premium 1.0 starter culture from CHR Hansen, (VII) *L. parabuchneri* DSM 5987 and *Debaryomyces hansenii*, and (VIII) *L. parabuchneri* DSM 5987 and DAO.

L. casei strains used in the cheesemaking process produced a decrease in histamine content (43.0 %) similar to that obtained *in vitro* by Herrero-Fresno et al. (2012). On the other hand, the commercial YoFlex® Premium 1.0 starter culture from CHR Hansen used in cheese variant VI turned out to be the least effective control measure applied, with a reduction of 17.56 % histamine with respect to control cheese variant II. Taking together, these results allowed to confirm that the biological measures applied produced a decrease in histamine content in ripened cheese. The main hypothesis relies on a putative histamine degradation mechanism, since the added strains have been demonstrated to degrade histamine, in this work or in other published studies (section 3.1). However, it could not either be discarded a putative mechanism of microbial competition between the cheese starter, *L. parabuchneri* and the adjunct cultures, that resulted in a reduction of the histamine amount produced during the cheese ripening period.

Regarding enzymatic histamine degradation, cheeses manufactured with DAO (cheese variant VIII) displayed a final histamine concentration of 591.41 ± 37.63 mg/kg at 100 days of ripening, which represented a reduction of 23 % of histamine with respect to the positive control cheese variant II (Fig. 3). Notably, DAO was one of the most effective agents in the control of histamine accumulation up to 30 days of ripening, resulting in a reduction of 30 % of histamine with respect to the positive control cheese variant II. The lower histamine reduction rate observed toward the end of the ripening period could be explained by a lower DAO activity. It has been reported that DAO displays a substrate inhibition at a histamine concentration of 56 mg/L, reaching 40 % of activity inhibition at 500 mg/L of histamine (Kettner et al., 2020). It is noteworthy that the final histamine concentration of cheeses from cheese variant VIII at 100 days of ripening reached 591.41 mg/kg. As explained below, the pH of those cheeses ranged from 5.79 to 5.0 along ripening in cheese variant VIII, which is far removed from the optimal pH for DAO (around pH 7.2). However, DAO can also be active at up to pH 5.0, as previously shown (Dapkevicius et al., 2000). It is also well established that the optimum temperature for DAO is 37 °C (Naila et al., 2012). Below that temperature, it can be less active, as also occurred with the histamine-degrading enzyme HNMT (Dapkevicius et al., 2000; Francis et al., 1977). However, it has been reported that DAO retains about 50 % of its maximum activity at 20 °C (Dapkevicius et al., 2000), and it is still active in milk at 4 °C (section 3.1.2.) as well as at 12 °C, as in our case (cheese ripening temperature). For these reasons, the addition of DAO to milk for the production of cheeses with a ripening period lower than one month could be proposed as an effective measure to prevent histamine accumulation in semi-hard cheeses.

To summarize, the histamine-degrading microbial cultures added to milk in cheesemaking significantly contributed to the reduction of final histamine content in ripened cheese, compared with over 700 mg/kg histamine in control cheese produced with *L. parabuchneri*. Although no current regulation applies to histamine content in dairy products, 400 mg/kg of histamine has been proposed as an acceptable limit for ripened cheese, assuming a daily cheese consumption of 60 g, based on a study conducted in Austria (Rauscher-Gabernig et al., 2009). Histamine concentrations of cheese samples from cheese variants III and VII were slightly higher than 400 mg/kg at the end of the ripening period, whereas cheese variants IV and V had a final concentration of histamine lower than 500 mg/kg, which is the limit set by the EFSA as potentially toxic for human health (EFSA, 2011). Overall, combined with improved hygiene practices and shorter ripening periods, the use of histamine-degrading microorganisms, mainly *L. casei* 4a and 18b or *D. hansenii,* could help to produce cheeses with histamine concentrations below that limit. Although these can indeed serve as effective measures for the reduction of histamine in long-ripened cheeses, it would be necessary to take into account the importance of using milk lacking *L. parabuchneri* or other histamine-producing microorganisms for the purpose of further reducing histamine content in cheese (Moniente et al., 2021).

3.4. Physicochemical properties of experimental model cheeses

In order to assess whether the addition of the proposed microorganisms and of DAO to cheese for the purpose of reducing histamine accumulation could affect the organoleptic properties of the final products, several physicochemical properties were evaluated. Results showed that the addition of histamine-degrading microorganisms and DAO enzyme did relevantly modify the main physicochemical characteristics of the cheeses in the course of the ripening period (Table 4).

Both a_w and pH are two factors that usually affect cheese preservation and contribute to prevent the growth of pathogenic microorganisms in cheese (Fox, 1999). Each cheese variety usually has a characteristic pH range (Lawrence et al., 1984). In the present study, the milk's initial pH was around 6.60 and, during the pressing stage, cheeses were kept until the pH had dropped to around 5.70 ± 0.10. The pH level at the onset was different in most cheese variants ($p < 0.05$), although evolution was similar: a slight pH decrease as cheeses aged, according to the expected evolution in fermented cheeses (Buffa et al., 2001; Novella-Rodríguez et al., 2002b; Valsamaki et al., 2000) followed by a slight pH increase at around 60 days, as observed by Novella-Rodríguez et al. (2002b), likely due to bacterial metabolism and proteolysis–forming NH_3 (Kelly & Fox, 2007). A slight pH decrease was then observed at final ripening time. The pH of cheeses at the end of ripening ranged between 5.32 ± 0.05 and 5.80 ± 0.18. Those final pH levels, which correlated with those of old cheeses, were similar to those observed in Cheddar, Emmental (Fox, 1999), Parma (Jaster et al., 2014; Marcos et al., 1981), and Chihuahua cheeses (Gutiérrez-Méndez et al., 2013).

The a_w was significantly reduced during ripening (from 0.977 ± 0.005 to 0.824 ± 0.010 at final ripening time), probably due to the loss of water and an increase in water-soluble proteolysis products (Hickey et al., 2013). Similar levels have been observed in Parma cheese (Jaster et al., 2014). According to EFSA (2011) regulations regarding cheeses made with raw milk and non-packaged cheeses made with heat-treated milk, they are considered safe when pH is <4.4 and the a_w is lower than 0.920. However, most cheeses usually have a pH that lies above 5.0, and their a_w is higher than 0.940. Thus, it is not feasible to attempt to obtain a combination of low pH and a_w in cheese (Trmčić et al., 2017).

No relevant colour changes were observed between the control

Table 4
Physicochemical analyses of samples from different cheese variants (CV) along the 100 days of ripening. Hardness (g), adhesiveness, color coordinates (L*, a* and b*), water activity, pH and weight (g) are shown in this table.

Physicochemical properties	Cheese variant (CV)	Days of ripening						2-way ANOVA		
		0	15	30	45	60	100	Ripening time (RT)	Cheese variant (CV)	RT × CV
Hardness (g)	CV I	163.63 ± 8.26 dAB	343.81 ± 41.95 dA	1084.01 ± 28.44 cA	1718.23 ± 445.63 bA	3727.29 ± 468.90 aA	3944.95 ± 272.87 aA			
	CV II	115.52 ± 5.54 cCD	157.60 ± 7.04 cB	612.74 ± 64.54 bC	942.19 ± 143.03 bBC	2101.72 ± 388.26 aB	2247.96 ± 229.47 aBC			
	CV III	196.68 ± 18.58 dA	343.43 ± 77.54 dA	756.72 ± 98.34 cB	1049.02 ± 165.09 cB	2174.65 ± 314.33 bB	3446.86 ± 419.09 aA			
	CV IV	147.83 ± 32.13 bBCD	165.78 ± 29.03 bB	616.07 ± 127.79 bBC	751.63 ± 334.27 bBCD	2036.31 ± 492.07 aB	2016.04 ± 621.11 aBC			
	CV V	177.25 ± 21.12 dAB	143.47 ± 18.65 dB	376.93 ± 72.49 cdDE	666.12 ± 221.74 cBCD	1937.59 ± 194.28 bB	2495.31 ± 365.60 aB	*	*	*
	CV VI	162.15 ± 35.77 cABC	114.33 ± 16.21 cB	411.44 ± 91.10 bcD	515.14 ± 223.34 bCD	1227.32 ± 138.27 aC	1543.64 ± 363.17 aC			
	CV VII	114.21 ± 30.73cD	132.99 ± 33.25 cB	314.07 ± 47.91 cDE	329.73 ± 33.93cD	1328.26 ± 48.67 bC	2475.69 ± 736.93 aB			
	CV VIII	174.59 ± 20.24 cAB	127.66 ± 25.41 cB	268.84 ± 83.39 cE	375.01 ± 193.36cD	1352.27 ± 152.65 bC	2265.30 ± 526.50 aBC			
Adhesiveness (g)	CV I	−39.47 ± 7.53 aAB	−123.45 ± 18.51 abC	−197.29 ± 36.43 bBC	−496.84 ± 75.67 cE	−656.54 ± 94.06 dB	−683.20 ± 173.26 dD			
	CV II	−30.08 ± 2.53 aA	−63.43 ± 7.34 aB	−224.40 ± 37.07 bC	−313.46 ± 108.93 bcCD	–332.50 ± 62.38 cB	−437.80 ± 61.84 dAB			
	CV III	−64.86 ± 0.25 aC	−142.07 ± 24.34 aC	−310.73 ± 55.50 bD	−391.48 ± 28.59 bDE	−486.94 ± 42.68 cA	−600.46 ± 79.20 dCD			
	CV IV	−34.29 ± 5.88 aA	−67.30 ± 10.78 aB	−264.16 ± 86.27 bCD	−244.95 ± 110.21 bBC	−330.06 ± 48.98 bcA	−389.01 ± 21.36 cAB			
	CV V	−43.02 ± 5.60 aAB	−50.99 ± 12.00 abAB	−120.44 ± 39.42 bAB	−199.04 ± 37.76 cABC	−281.92 ± 45.71 dA	−416.33 ± 71.47 eAB	*	*	*
	CV VI	−54.11 ± 17.80 aBC	−42.24 ± 7.73 aAB	−115.83 ± 24.47 aA	−118.41 ± 32.63 aA	−249.54 ± 125.06 bA	−327.00 ± 49.11 bA			
	CV VII	−30.60 ± 4.80 aA	−34.65 ± 1.93 aA	−101.15 ± 24.58 aA	−92.12 ± 27.70 aA	–222.31 ± 62.63 bA	−447.83 ± 102.83 cAB			
	CV VIII	−35.41 ± 3.57 aA	−35.93 ± 1.58 aA	−123.44 ± 36.85 aAB	−129.77 ± 53.32 aAB	−254.92 ± 47.94 bA	−489.17 ± 35.98 cBC			
Color coordinate L*	CV I	94.16 ± 0.40 aAB	83.23 ± 2.18 bB	62.67 ± 3.72cD	61.60 ± 1.78 cA	53.02 ± 2.24 dB	55.23 ± 3.70 dA			
	CV II	94.57 ± 0.36 aA	88.05 ± 1.25 bA	65.66 ± 1.15 cBCD	64.27 ± 2.29 cA	54.29 ± 6.51 dB	56.17 ± 2.32 dA			
	CV III	92.86 ± 0.55 aBC	82.29 ± 3.12 bB	67.97 ± 2.66 cBC	63.79 ± 7.68 cA	66.43 ± 3.22 cA	49.52 ± 7.86 dABC			
	CV IV	93.03 ± 0.84 aBC	84.17 ± 0.97 bB	64.39 ± 0.49 dCD	60.10 ± 0.93 eAB	68.91 ± 4.98 cA	52.98 ± 1.40 fAB			
	CV V	91.48 ± 0.75 aD	81.95 ± 1.22 bB	64.40 ± 3.24 dCD	54.19 ± 3.11 eB	72.34 ± 6.87 cA	48.17 ± 2.71 eABC	*	*	*
	CV VI	91.40 ± 0.88 aD	83.95 ± 1.42 bB	61.04 ± 2.75 dD	60.49 ± 0.99 dAB	71.76 ± 1.31 cA	43.22 ± 2.70 eC			
	CV VII	91.89 ± 1.08 aCD	90.54 ± 1.37 aA	73.73 ± 3.51 bA	65.00 ± 5.01 cA	71.35 ± 6.44 bcA	51.14 ± 4.01 dABC			
	CV VIII	92.43 ± 0.34 aBCD	89.82 ± 1.08 aA	69.98 ± 3.01 bAB	66.52 ± 3.24 bA	67.51 ± 3.49 bA	45.21 ± 8.95 cBC			
Color coordinate a*	CV I	−2.66 ± 0.02 cA	−2.25 ± 1.52 cC	0.01 ± 0.75 bC	1.51 ± 0.66 aC	−0.24 ± 0.50 bC	0.51 ± 0.56 abB			
	CV II	−2.65 ± 0.02 fA	−0.78 ± 0.12 eAB	2.34 ± 0.20 bAB	3.49 ± 0.47 aA	1.41 ± 0.84 cA	0.15 ± 0.34 dB			
	CV III	−2.59 ± 0.07 eA	−0.19 ± 0.14 dA	1.75 ± 0.33 bB	2.74 ± 0.41 aAB	0.28 ± 0.25 cdBC	0.70 ± 0.92 cB			
	CV IV	−2.60 ± 0.06 eA	−0.27 ± 0.09 dA	2.19 ± 0.52 bB	3.45 ± 0.18 aA	0.48 ± 0.30 cABC	2.15 ± 0.48 bA			
	CV V	−3.23 ± 0.35 fB	−0.36 ± 0.44 eA	1.79 ± 0.33 cB	3.63 ± 0.57 aA	0.66 ± 0.57 dABC	2.62 ± 0.40 bA	*	*	*
	CV VI	−3.42 ± 0.20 dB	−0.77 ± 0.08 cAB	3.07 ± 0.37 aA	3.29 ± 0.26 aA	1.22 ± 0.57 bAB	2.44 ± 1.13 aA			
	CV VII	−2.48 ± 0.08 dA	−1.45 ± 0.03 cBC	0.74 ± 0.41 bC	2.28 ± 0.65 aBC	0.23 ± 0.61 bBC	1.92 ± 0.65 aA			
	CV VIII	−2.64 ± 0.04 cA	−2.30 ± 0.02 cC	2.40 ± 0.64 aAB	1.42 ± 0.65 abC	0.21 ± 1.10 bBC	2.61 ± 0.64 aA			
Color coordinate	CV I	11.07 ± 0.35 cCD	22.14 ± 1.56 abA	23.90 ± 1.14 aBC	25.71 ± 2.94 aA	18.60 ± 1.74 bA	18.48 ± 3.82 bA			

(*continued on next page*)

Table 4 (*continued*)

Physicochemical properties	Cheese variant (CV)	Days of ripening						2-way ANOVA		
		0	15	30	45	60	100	Ripening time (RT)	Cheese variant (CV)	RT × CV
b*	CV II	10.93 ± 0.10 dCD	16.07 ± 0.37 cBC	25.64 ± 0.65 aAB	22.39 ± 3.24 bAB	15.91 ± 1.19 cA	18 0.02 ± 2.16 cA			
	CV III	11.58 ± 0.17 bCD	16.24 ± 2.68 bBC	25.59 ± 1.83 aAB	16.75 ± 5.74 bBC	10.03 ± 3.32 bB	15.41 ± 5.60 bA			
	CV IV	11.59 ± 0.45 dCD	17.84 ± 0.84 cB	23.90 ± 0.29 aBC	19.99 ± 0.55 bAB	11.36 ± 1.17 dB	18.69 ± 1.35 bcA			
	CV V	12.83 ± 1.24 cAB	21.35 ± 1.34 bA	26.87 ± 0.43 aA	22.99 ± 3.78 bAB	9.62 ± 1.11 cB	20.69 ± 2.10 bA	*	*	*
	CV VI	13.22 ± 0.91 bA	20.27 ± 0.31 aA	23.66 ± 3.06 aBC	21.58 ± 7.00 aAB	10.48 ± 1.01 bB	19.52 ± 2.68 aA			
	CV VII	10.48 ± 0.08cD	14.18 ± 0.86 bcC	21.14 ± 0.41 aC	18.13 ± 4.09 abBC	10.56 ± 2.65 cB	17.86 ± 2.38 abA			
	CV VIII	11.98 ± 0.23 bcBC	16.50 ± 0.58 abcBC	23.92 ± 2.57 aBC	11.56 ± 1.75 cC	11.92 ± 2.34 cB	19.04 ± 5.58 acA			
Water activity	CV I	0.977 ± 0.004 aAB	0.966 ± 0.006 aB	0.916 ± 0.010 bD	0.901 ± 0.008 bD	0.828 ± 0.024 cC	0.801 ± 0.013 dB			
	CV II	0.980 ± 0.009 aAB	0.968 ± 0.007 aAB	0.931 ± 0.010 bCD	0.880 ± 0.001 cE	0.811 ± 0.022 dC	0.819 ± 0.012 dB			
	CV III	0.977 ± 0.006 aAB	0.965 ± 0.003 aB	0.943 ± 0.010 bBC	0.919 ± 0.010 cC	0.874 ± 0.002 dB	0.830 ± 0.004 eAB			
	CV IV	0.974 ± 0.009 aAB	0.970 ± 0.009 aAB	0.940 ± 0.001 bC	0.915 ± 0.006 cCD	0.836 ± 0.012 dC	0.818 ± 0.013 eB			
	CV V	0.977 ± 0.006 abAB	0.975 ± 0.005 aAB	0.960 ± 0.007 aAB	0.931 ± 0.002 abBC	0.887 ± 0.003 bAB	0.826 ± 0.037 cB	*		*
	CV VI	0.972 ± 0.004 aAB	0.977 ± 0.005 aAB	0.960 ± 0.003 abA	0.944 ± 0.008 bAB	0.892 ± 0.005 cAB	0.859 ± 0.035 dA			
	CV VII	0.990 ± 0.001 aA	0.969 ± 0.001 abAB	0.963 ± 0.007 bA	0.957 ± 0.014 bA	0.881 ± 0.021 cAB	0.818 ± 0.013 dB			
	CV VIII	0.976 ± 0.007 aAB	0.980 ± 0.001 aA	0.973 ± 0.007 aA	0.956 ± 0.004 aA	0.906 ± 0.022 bA	0.824 ± 0.040 cB			
pH	CV I	6.18 ± 0.04 aA	5.77 ± 0.02 bA	5.51 ± 0.10 bcAB	5.45 ± 0.31 cA	5.58 ± 0.17 bcCD	5.46 ± 0.02 cBC			
	CV II	5.78 ± 0.04 abB	5.16 ± 0.05 cBC	5.64 ± 0.12 bAB	5.74 ± 0.19 bA	5.91 ± 0.15 aAB	5.68 ± 0.12 bAB			
	CV III	5.46 ± 0.07 aD	4.75 ± 0.21 bD	4.88 ± 0.02 bC	4.95 ± 0.03 bB	5.35 ± 0.16 aD	5.32 ± 0.05 aC			
	CV IV	5.71 ± 0.07 aB	4.82 ± 0.07 bD	5.62 ± 0.40 aAB	5.07 ± 0.26 bB	5.62 ± 0.20 aBCD	5.80 ± 0.18 aA			
	CV V	5.49 ± 0.03 aCD	5.30 ± 0.05 cB	5.44 ± 0.12 abAB	5.50 ± 0.04 aA	5.52 ± 0.05 aCD	5.33 ± 0.01 bcC	*	*	*
	CV VI	5.44 ± 0.03 aD	5.31 ± 0.21 aB	5.47 ± 0.41 aAB	5.48 ± 0.23 aA	5.72 ± 0.30 aBC	5.73 ± 0.23 aA			
	CV VII	5.76 ± 0.06 bB	5.21 ± 0.02 cBC	5.75 ± 0.20 bA	5.63 ± 0.07 bA	6.06 ± 0.20 aA	5.68 ± 0.07 bAB			
	CV VIII	5.55 ± 0.04 abC	5.00 ± 0.14 cCD	5.23 ± 0.17 bcBC	5.39 ± 0.01 bA	5.79 ± 0.13 aABC	5.65 ± 0.16 aAB			
Weight (g)		**a**	**b**	**c**	**d**	**e**	**e**	*	*	**ns**
	CV I	246.26 ± 20.52	192.33 ± 17.61	154.00 ± 11.35	164.74 ± 2.28	128.13 ± 15.50	124.04 ± 4.97	BC		
	CV II	214.06 ± 14.23	182.66 ± 8.32	131.33 ± 8.50	114.07 ± 7.11	108.10 ± 6.05	107.20 ± 4.41	E		
	CV III	213.93 ± 53.45	197.33 ± 10.59	164.66 ± 15.82	160.62 ± 13.36	127.84 ± 3.66	130.86 ± 11.65	BC		
	CV IV	228.06 ± 21.72	180.00 ± 32.04	152.33 ± 14.15	141.59 ± 22.88	119.36 ± 7.79	133.68 ± 6.31	CD		
	CV V	235.16 ± 16.93	203.33 ± 3.22	174.00 ± 18.19	158.64 ± 8.98	149.10 ± 17.25	140.92 ± 6.69	B		
	CV VI	244.77 ± 17.26	219.00 ± 13.11	194.66 ± 15.37	182.78 ± 7.66	164.22 ± 14.83	147.99 ± 4.34	A		
	CV VII	216.89 ± 18.10	201.66 ± 3.05	150.33 ± 11.84	146.80 ± 24.97	111.71 ± 9.04	119.25 ± 6.19	D		
	CV VIII	271.36 ± 16.90	218.00	210.00 ± 14.52	170.92 ± 10.41	167.15 ± 8.35	148.40 ± 2.26	A		

The results are presented as mean values with their standard deviation. * indicates significant differences for the 2-way ANOVA test. ns: non-significant. Cheese variant (CV), Ripening Time (RT) and interaction (RT × CV) The differences between groups were tested according to the Tukey's test, performed at the significant level of $p < 0.05$ (*). Lowercase letters indicate significant differences for the RT factor. Capital letters indicate significant differences for the cheese variant factor.

cheeses (cheese variant I) and the others. L*, a*, and b* values varied significantly during the 100-day ripening period. The L* decreased in all cheese variants by over 36 %, as was observed by Buffa et al., (2001). Likewise, the a* (red/green colour) and b* (yellow/blue colour) coordinates increased during ripening, whereby the greater increase could be observed in cheese variant V from -3.23 ± 0.35 to 2.62 ± 0.40, and from 12.83 ± 1.24 to 20.69 ± 2.1, respectively. These results indicate an evolution of shades of green to red (a*) and towards more intense yellow shades (b*). Cheeses with similar a* and b* values are present in Mont D'Or and Pont-L'Evêque PDO varieties, which have more yellow than red colour (Dufossé et al., 2005).

As expected, cheese hardness increased along the ripening period, whereby the most pronounced variations were $3,781.32 \pm 264.61$ g and an average of $2,398 \pm 423.67$ g with respect to the initial time. Adhesiveness decreased along the ripening period in all cheese variants, attaining a reduction of around -432.25 ± 68.39 g. Cheese texture varied over time, with the most pronounced changes occurring between 15 and 30 days, as well as between 45 and 60 days. Cheese textures were similar to those observed in samples of Parma, Chihuahua, Cheddar, and Emmental cheeses (Gutiérrez-Méndez et al., 2013; Jaster et al., 2014; Zheng et al., 2016). The change in texture is mainly due to the proteolysis produced by the residual coagulant and the present LAB, which break down casein structure (Lawrence et al., 2004; Perin & Nero, 2017).

3.5. Evaluation of sensorial characteristics of cheese by sorting task

A sorting task was carried out to evaluate the similarity of aromatic profile among the cheese variants. Panelists evaluated 9 samples (cheese variant III was replicated as an internal control) and sorted them into different groups according to aroma similarities. Fig. 4 shows the clustering of cheese samples derived from the sorting task. A higher dissimilarity value indicates a greater number of differences between samples. The two replicated samples of cheese variant III clustered in the same group, indicating that panelist results were globally reproducible.

The panelists noted three different groups. The first group consisted of cheese variants I and II (positive and negative control cheese variants), whose aroma was described as "lactic". The second group contained the two replicated samples from cheese variant III and the sample from cheese variant IV, represented by the "cold meat" attribute; these were the ones most similar to the control cheese variants. These two cheese variants were also those that had the lowest amount of histamine at 100 days of ripening. The third group consisted of cheese variants V, VI, VII and VIII, represented by the "fruity" and "wine" attributes. Since the physicochemical properties and the aroma of the final cheese products were notably different each other, so it could be inferred that the microorganisms present in each cheese variant have properly grown and evolved.

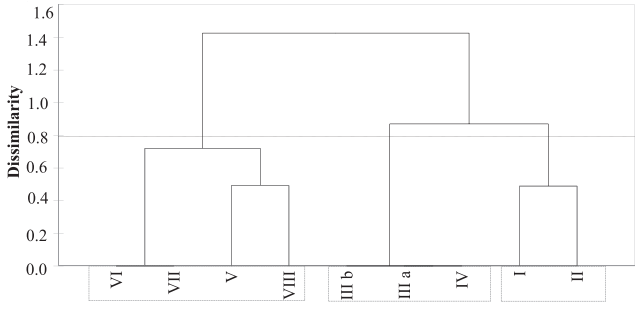

Fig. 4. Hierarchical Cluster Analysis (HCA) calculated on data from the sorting task performed on 100-day cheeses. Cheese variants: (I) control cheese, (II) *Lentilactobacillus parabuchneri* DSM 5987, (III) *L. parabuchneri* DSM 5987 and *Lacticaseibacillus casei* strains (4a and 18b), (IV) *L. parabuchneri* DSM 5987 and *Lactobacillus bulgaricus* CECT 4005 and *Streptococcus thermophilus* CECT 7207, (V) *L. parabuchneri* DSM 5987 and Yogurt Type I starter culture from Abiasa, (VI) *L. parabuchneri* DSM 5987 and YoFlex® Premium 1.0 starter culture from CHR Hansen, (VII) *L. parabuchneri* DSM 5987 and *Debaryomyces hansenii*, (VIII) *L. parabuchneri* DSM 5987 and DAO supplementation. Letters a and b indicate duplicates of the sample belonging to cheese variant III.

Results showed that the aromatic profile of cheese variant II (with *L. parabuchneri*) was not significantly different from cheese variant I. According to Rohn et al. (2005), histamine did not show any characteristic taste. However, another published study indicated that the growth of histamine-producing strains of *L. parabuchneri* has been associated with a burning taste (Fröhlich-Wyder et al., 2015). This specific taste could be related to an inflammatory effected caused by histamine in oral mucosa (Ascone et al., 2017). On the other hand, many studies have reported that histamine is an odorless compound (Lee et al., 2016; Lin et al., 2015; Zou & Hou, 2017). The sensory study performed in this experiment was based on the orthonasal perception of cheeses and the putative burning/itchy taste associated to the presence of *L. parabuchneri* that may could appear on the retronasal perception of cheese. Thus, the differences between both cheese variants I and II might not have been noticeable in the nose, but perceptible in the mouth. Hence it can be concluded that *L. parabuchneri* did not modify cheese aroma with respect to control cheese variant I, despite its production of histamine. On the contrary, the microorganisms used as adjunct cultures in cheesemaking produced sensory changes in the final products that need to be studied in depth in future assays, although the resulting aromas were not unpleasant. The use of instrumental techniques based for instance on gas chromatography/mass spectrometry would allow to detect the compound which were driving the sensory differences in cheese variants.

4. Conclusions

This study's main purpose was to evaluate whether the DAO enzyme and/or different microorganisms added as adjunct cultures were capable of reducing the accumulation of histamine naturally produced by *L. parabuchneri* in manufactured cheeses. The microorganisms tested for their putative histamine-degrading ability (*L. casei* 4a and 18b, *L. bulgaricus* CECT 4005 and *S. thermophilus* CECT 7207, two yogurt commercial starters, and *D. hansenii*) produced clear and significant reductions in histamine accumulation compared to the positive control along 100 days of ripening. The notable effect produced by the strains *L. casei* 4a and 18b, *L. bulgaricus* CECT 4005 and *S. thermophilus* CECT 7207 is worth highlighting, as well as that of *D. hansenii*. All of these were able to reduce histamine accumulation, reaching values lying considerably below 500 mg/kg in final cheese, which is the limit proposed by the EFSA as potentially toxic for human health. In the case of DAO, the highest histamine reduction was observed at 30 days of ripening; however, this measure did not result as effective in controlling histamine accumulation at the final ripening time. Additionally, no significant changes among the different cheese variants were observed in terms of main physicochemical parameters. Although *L. parabuchneri* did not modify cheese aroma compared to control, the aroma of the remaining cheese variants was affected, albeit not unpleasantly. The aroma of the cheese variant with *L. casei* 4a and 18b (one of the most effective measures) was the one most similar to that of control. Overall, the use of potential histamine-degrading microorganisms and DAO added directly to the milk matrix during the cheesemaking process can be regarded as an effective strategy to limit the production of histamine in long-ripened cheeses.

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CRediT authorship contribution statement

Marta Moniente: Investigation, Writing – original draft, Writing – review & editing. **Diego García-Gonzalo:** Supervision, Writing – review & editing. **Mª Goretti Llamas-Arriba:** Investigation, Writing – review & editing. **Raquel Virto:** Supervision, Project administration, Funding acquisition, Writing – review & editing. **Ignacio Ontañón:** Supervision, Writing – review & editing. **Rafael Pagán:** Supervision, Project administration, Funding acquisition, Writing – review & editing. **Laura Botello-Morte:** Investigation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111735.

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4. Discusión general

4. Discusión general

La discusión de los resultados se ha elaborado en profundidad en cada uno de los manuscritos presentados. En este apartado se recoge el enfoque general de toda la investigación realizada a lo largo de la Tesis Doctoral. La discusión se muestra dividida en 4 secciones que se corresponden con cada uno de los objetivos experimentales planteados: 1) Puesta a punto de la metodología analítica para la determinación de ABs en leche y productos lácteos y evaluación del contenido en ABs en productos comerciales; 2) Identificación de la microbiota causante de la acumulación de histamina en quesos comerciales; 3) Estudio de la distribución espacial de la histamina y de la microbiota responsable de su acumulación en queso madurado; y 4) Evaluación de herramientas que permitan reducir la concentración de histamina en queso de larga maduración.

4.1 Puesta a punto de la metodología analítica para la determinación de ABs en leche y productos lácteos y evaluación del contenido en ABs en productos comerciales (Manuscrito III)

Dada la importancia de la determinación de ABs, a lo largo de los años se han desarrollado multitud de métodos analíticos que permiten determinar estos metabolitos en productos lácteos. La cuantificación de ABs en estos alimentos puede ser complicada debido, entre otros factores, a la complejidad de la muestra (Papageorgiou et al., 2018). Los productos lácteos son muestras ricas en proteínas y en lípidos, cuya precipitación es necesaria para eliminar las sustancias macromoleculares que puedan interferir en el análisis. Por ello, se requiere de varias etapas de pretratamiento de muestra y de extracción previas a la cuantificación de ABs para garantizar la calidad de los resultados. El pretratamiento de muestra es utilizado para limpiar y concentrar la muestra. Habitualmente este proceso de desproteinización de la muestra se realiza mediante ácidos fuertes (Gobbi et al., 2019; Lanciotti et al., 2007; Marijan et al., 2014; Ordóñez et al., 2016; Spizzirri et al., 2019; Tittarelli et al., 2019; Toro-Funes et al., 2015).

A lo largo de los años se han utilizado diferentes técnicas analíticas (cromatografía de gases (GC), cromatografía en capa fina (TLC), electroforesis capilar (CE)…) para el análisis de ABs en productos lácteos, pero ciertas características fisicoquímicas de estos compuestos, como su polaridad o la baja volatilidad, hace de la cromatografía de líquidos (LC) la más idónea y, por tanto, la más utilizada. A pesar de su idoneidad es frecuente la derivatización de las ABs

para mejorar tanto sus características cromatográficas como su detectabilidad (Önal, 2007; Önal et al., 2013; Papageorgiou et al., 2018).

Un factor a tener en cuenta a la hora de seleccionar el método adecuado para analizar ABs es la concentración esperada en el producto lácteo, ya que si se trata de leche, queso fresco o productos de corta fermentación (yogur o kéfir), el nivel esperado suele ser habitualmente bajo (<10 mg/kg) (Linares et al., 2011; Spano et al., 2010). Es por ello que en algunos casos es necesario introducir etapas de limpieza y preconcentración anteriores a la etapa de análisis para conseguir métodos analíticos con una adecuada sensibilidad.

En las primeras etapas de esta tesis doctoral se estudió la posibilidad de llevar a cabo el análisis de las ABs en productos lácteos mediante derivatización y análisis por RP-HPLC aplicando únicamente un paso previo de desproteinización. Sin embargo, los resultados conseguidos no fueron satisfactorios, ya que los límites de detección estimados estaban alrededor de 2 mg/L y la determinación era muy irreproducible (más de un 30% de desviación estándar relativa). Debido a ello se decidió introducir una etapa de limpieza y preconcentración previa a la derivatización basada en extracción en fase sólida (SPE). Durante el desarrollo y validación del método se llevó a cabo una optimización de diferentes factores que pueden influir en las diferentes etapas y finalmente, el método validado se aplicó al análisis de ABs en muestras de leche, yogur y kéfir.

En primer lugar, quisimos conocer cuál era la cantidad máxima de muestra que era posible cargar en el cartucho de SPE sin que hubiese pérdida de los analitos, por lo que se hizo un estudio del volumen de ruptura (Figura 1, Manuscrito III). Para ello se analizaron los eluatos obtenidos tras haber percolado distintas cantidades de una muestra de leche dopada con 20 mg/L de histamina, tiramina, putrescina y cadaverina. La histamina y la tiramina empezaban a aparecer en el percolado a partir de 5,4 y 6 mL de muestra cargada, respectivamente. Sin embargo, la retención de las ABs, putrescina y cadaverina, fue menor, siendo detectadas en el percolado a partir de 2,4 y 3,6 mL respectivamente. Por tanto, el volumen de muestra máximo sin que haya pérdidas considerables (suele considerarse aceptable cuando las pérdidas son menos del 10% respecto de la concentración inicial) de ninguna de las ABs fue 2,4 mL.

Los extractos obtenidos tras la SPE, incluso aplicando diferentes lavados acuosos, eran de color blanquecino y turbios lo que podía ser origen de problemas de irreproducibilidad, interferencias y ciertos problemas instrumentales. Por esta razón, tras la adición de la muestra,

se decidió evaluar un lavado con hexano para eliminar por completo las grasas (posible responsable de la turbidez) y arrastrar otros compuestos apolares que impedían la obtención de un extracto totalmente transparente. El volumen de hexano ensayado fue de 2, 4, 6 y 8 mL comparado con una muestra control sin hexano. Los datos muestran como con el menor volumen de hexano (2 mL) no hay pérdidas de analito respecto del control, además de conseguir buena reproducibilidad. A medida que se aumentaba el volumen de lavado con hexano, la irreproducibilidad aumentaba y, además, se producía una disminución de las áreas para la histamina y la tiramina cuando se lavaban los cartuchos con 8 mL de hexano (Figura 2, Manuscrito III). El problema de la turbidez y del color de los extractos se solucionaba con el lavado de 2 mL. Por ello, y dado que no existían pérdidas de analitos y la reproducibilidad era óptima, se eligió este volumen de hexano.

Una vez optimizado el volumen de hexano, se estudiaron diferentes disoluciones de lavado para eliminar aminoácidos y otras especies que interfieren con el análisis de ABs. La primera era una disolución ácida (10 mM H_3PO_4:MeOH [70:30]), la segunda era una disolución neutra (10 mM $CaCl_2$:MeOH [70:30]) y la tercera era una disolución alcalina (10 mM NaOH:MeOH [70:30]). Para ello, se cargaron en cartuchos de SPE diferentes alícuotas de una muestra de leche entera enriquecida con 10 mg/L de ABs y se lavaron con hexano. En uno de los cartuchos no se aplicaron disoluciones de lavado acuosas (ácidas, neutras y básicas), en el segunda se omitió el lavado ácido, en la tercera se omitió el lavado neutro, en la cuarta el básico y en la quinta se incluyeron los tres lavados. En los casos en los que se utilizaba el lavado básico, el área absoluta de los analitos era menor que en ausencia de esta disolución, lo que era indicativo de pérdidas durante este lavado. Es por ello que se decidió no incorporar este lavado durante el pretratamiento de la muestra.

Después de la extracción, la muestra se eluye y se filtra. En esta fase de filtración se ensayaron varios filtros, tanto de diferentes materiales (polyvinylidene fluoride (PVDF) y nylon) como de tamaño de poro (ultrafiltración 10k y 3k, y filtración 0,2 μm). Se demostró que cuando se empleaban los filtros de PVDF existía una mayor pérdida de ABs obteniendo una menor señal y en el caso de los filtros de ultrafiltración existían interferencias con las ABs, en especial con la histamina. Sin embargo, las muestran filtradas con 0,2 μm de nylon presentaron una buena señal de áreas absolutas de las cuatro ABs, al igual que otros autores como Adımcılar et al. (2017) y Mayer et al. (2010) que también hicieron uso de este tipo de filtros para sus análisis.

Para mejorar las propiedades cromatográficas de los analitos, así como su detectabilidad, la mayoría de las técnicas analíticas cuentan con un paso de derivatización pre- o post-columna (García-Villar et al., 2009) antes de ser analizada por RP-HPLC. Existen multitud de derivatizantes para la determinación de ABs, uno de los utilizados para aminas primarias y secundarias, y con el cual se obtienen productos muy estables es el 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC). Este derivatizante se ha utilizado anteriormente en productos lácteos con detección ultravioleta (UV) (Moniente et al., 2022), sin embargo, los derivados producen señales muy intensas en fluorescencia, lo que puede mejorar la sensibilidad de los métodos analíticos. Es por ello que se optó por esta posibilidad para el desarrollo del método.

Con el objetivo de mejorar el rendimiento de la reacción de derivatización, se optimizó el pH de la reacción, el tiempo de derivatización y la temperatura de los extractos tras la derivatización. Para estudiar cómo influía el pH de la muestra en el proceso de derivatización, se realizó una disolución de 5 mg/L de cada AB y se ajustó el pH a 1, 5, 7, 10 y 12. Posteriormente cada disolución se filtró con los filtros de nylon 0,2 μm y se derivatizó siguiendo el protocolo descrito por el distribuidor. Los resultados mostraron como a los pH más bajos (pH 1, 5 y 7), la señal obtenida de las cuatro ABs fue menor que la obtenida a pH superiores (pH 10 y 12).

El tiempo de la reacción de derivatización de los analitos de la muestra es otro factor relevante para optimizar el método. La derivatización se llevó a cabo a 55ºC y se estudiaron diferentes tiempos: 10, 20, 30 y 60 min. Como resultado se comprobó que cuando se lleva a cabo el proceso durante 10 min se obtiene una mayor señal de las áreas absolutas obtenidas para las cuatro ABs en comparación con los otros tiempos de estudio evaluados (20, 30 y 60 min) lo que confirma que el valor de tiempo indicado por el fabricante del reactivo (Waters) también fue óptimo para este experimento.

Posteriormente, se estudió la influencia de la temperatura de los extractos después del proceso de derivatización para tratar de paliar algunos problemas de reproducibilidad con la señal de las muestras y detener la derivatización tras los 10 min de reacción. Las muestras se almacenaron a temperatura ambiente, a temperatura de refrigeración (4ºC) y a temperatura de congelación (-18ºC) durante 5 min. Los resultados mostraron cómo la señal de las áreas absolutas para las cuatro ABs fue más elevada a temperaturas de refrigeración y de congelación

que a temperatura ambiente y, además, los resultados muestran una mejor reproducibilidad a temperatura de congelación (entre 2,19-5%) que a temperatura ambiente (entre 3,23-51,89%). Por estas razones las muestras fueron almacenadas bajo congelación durante 5 min tras el paso de derivatización.

Tras optimizar todas las etapas del método se llevó a cabo la validación del método de análisis. Se llevaron a cabo estudios de linealidad, sensibilidad y precisión. La repetibilidad (%DSR) de seis muestras de leche se estudió analizando muestras dopadas y no dopadas por triplicado y fue de 9,34, 12,47, 2,68, y 6,36% para histamina, tiramina, putrescina y cadaverina, lo que demuestra que se ha conseguido un método reproducible. El método desarrollado se caracteriza por unos límites de detección y cuantificación (LD y LC) bajos (menores de 0,2 y 0,67 mg/L, respectivamente), siendo el valor más bajo el obtenido para histamina y putrescina (LD = 0,12 mg/L). Si se compara el método validado en esta tesis con otros existentes en la bibliografía con una configuración similar (derivatización y detección por UV o fluorescencia) pueden observarse mejores resultados que los reportados por otros autores para el estudio de ABs en lácteos, por ejemplo, Costa et al. (2015) obtuvieron un LD inferior a 1,3 mg/L y un LC inferior a 5,00 mg/L. Además, comparándolo con otros métodos de análisis de fluorescencia RP-HPLC, nuestro método también ofreció mejores resultados a los obtenidos por Sawilska-Rautenstrauch et al. 2010, quiénes consiguieron un LD inferior a 1,3 mg/kg y un LC inferior a 2,6 mg/kg. Cabe destacar nuestros resultados en relación a otros obtenidos con métodos en los que se utilizaba el mismo reactivo AQC. Mayer et al. (2010) logró un LD inferior a 16,2 mg/kg y un LC inferior a 60,9 mg/kg y; Fiechter et al. (2013) consiguieron un LD inferior a 4,4 mg/kg y un LC inferior a 14,5 mg/kg. Es destacable por tanto que la sensibilidad del método propuesto es altamente satisfactoria y bastante inferior a 1 mg/kg, un valor por debajo del cual los productos lácteos deberían de ser seguros para las personas que los consumen, incluidas las personas sensibles a las ABs (Rauscher-Gabernig et al., 2009). Teniendo en cuenta que el consumo habitual de leche son 200 mL diarios y conociendo que los límites de detección del método ensayado varían entre 0,12 y 0,2 mg/L, se estaría ingiriendo un máximo de entre 0,024 y 0,04 mg dependiendo de la AB. Estos valores son inferiores a los límites establecidos por la European Food Safety Authority (EFSA) como cantidades a partir de las cuales no se observa efecto adverso (NOAEL) en los que señala que una cantidad de 50 mg de histamina y de 600 mg de tiramina por comida sería segura en personas sanas que no toman medicamentos bloqueadores de monoamina oxidasa (MAO). Con el LD obtenido para la histamina en este método se necesitaría consumir más de 416 litros de leche para superar este valor, y con el LD

de la tiramina sería necesario consumir más de 2,6 litros de leche. Con estos valores podríamos decir que el LD obtenido es suficientemente bajo y por tanto tiene una elevada sensibilidad.

Aplicando este método se analizaron las cuatro ABs en 37 leches, 23 muestras de yogur y 14 muestras de kéfir. En el caso de la leche, en la mayor parte de las muestras la cantidad de ABs fue menor que el LD obtenido en el método desarrollado. La tiramina y la cadaverina fueron las ABs más frecuentes tanto en incidencia (5 y 8 muestras, respectivamente) como en cantidad (llegando a alcanzar hasta 9,13 y 17,70 mg/L, respectivamente). La histamina y la putrescina fueron detectadas únicamente en 2 muestras de leche, pero solo se pudo cuantificar en 1 muestra (6,24 mg/L y 7,05 mg/L, respectivamente). Tan sólo en una de las muestras se pudieron detectar las cuatro ABs y se obtuvieron niveles superiores a lo esperado en este tipo de producto (histamina 6,24 mg/L, tiramina 9,13 mg/L, putrescina 7,05 mg/L y cadaverina 17,70 mg/L) siendo en esta única muestra el total de aminas biógenas (TBA) de 40,11 mg/L debido probablemente a unas malas prácticas durante el procesado que se han visto repercutidas en una contaminación bacteriana productora de ABs. Estos resultados concuerdan con los obtenidos en otros estudios en los que en la mayoría de los casos muestran concentraciones bajas o indetectables de ABs (Min et al., 2004; Novella-Rodríguez et al., 2000; Özdestan & Üren, 2010; Pekcici et al., 2021; Wu et al., 2015).

En relación al yogur, tanto la frecuencia como la concentración general de ABs fue mayor que en las muestras de leche. Las ABs más frecuentes encontradas en yogur fueron la histamina y la putrescina (en 7 y 8 muestras, respectivamente) siendo la concentración de hasta 17,16 mg/kg y 27,01 mg/kg, respectivamente. Sin embargo, la cadaverina y la tiramina fueron detectadas en 3 y 4 muestras, respectivamente y su concentración fue menor, de hasta 2,11 mg/kg y 5,44 mg/kg, respectivamente.

Habitualmente, el yogur presenta bajas concentraciones de putrescina (0,6 mg/L, Min et al. (2004); 0,47 mg/L, Pekcici et al. (2021)) o incluso indetectables (Vieira et al., 2020). En nuestro estudio se encontró hasta 27 mg/kg de esta AB en yogur, probablemente debido o bien a la adición de un cultivo iniciador con capacidad de formar putrescina o bien por una contaminación ambiental por microorganismos productores. Esta cantidad concuerda con otras encontradas en yogures comerciales en los que se llegaron a cuantificar niveles de hasta 47 mg/kg (Adımcılar et al., 2017). La concentración de histamina y tiramina reportada en otros estudios es superior a la del resto de ABs, superando niveles de 65,2 mg/kg para histamina

(Adımcılar et al., 2017; Min et al., 2004) y 22,82 mg/kg para tiramina (Vieira et al., 2020). En comparación con los resultados obtenidos en este proyecto, los valores de histamina encontrados eran inferiores a los de estos autores (hasta 17 mg/kg), excepto si se comparan con el estudio de Pekcici et al. (2021) en el que los valores de histamina fueron inferiores a nuestros datos (6,97 mg/L). En una única muestra de yogur se obtuvo un TBA de 35,07 mg/kg producido por la alta cantidad de histamina y putrescina. Cabe destacar que, pese a que en la mayoría de las muestras de leche y de yogur no se llegaron a detectar ABs y, por tanto, no deberían de ocasionar un problema de intoxicación por estos metabolitos para las personas que los ingieren, se deberían de llevar a cabo análisis preventivos que permitan vigilar la ocurrencia de ABs.

Respecto al kéfir, se encontraron cantidades variables de ABs en 11 de las 14 muestras analizadas. La histamina fue la AB encontrada en un menor número de muestras (2 muestras) y en menor concentración (0,831 y 1,211 mg/kg). Sin embargo, la tiramina, la putrescina y la cadaverina se detectaron en un mayor número de muestras (7, 10 y 7, respectivamente) y en mayor concentración (entre 0,850 - 3,678 mg/kg, 0,452 - 14,05 mg/kg y 1,21 - 64,032 mg/kg, respectivamente). De estas ABs, la putrescina fue la más abundante y se detectó en todas las muestras excepto en dos. En estas muestras se alcanzó la mayor concentración de TBA logrando hasta los 79,66 mg/kg y superando el doble de la mayor concentración de TBA en leche (40,11 mg/kg) y yogur (35,07 mg/kg).

Las muestras de leche mostraron una concentración más baja de ABs que el yogur y el kéfir. Excepto en una muestra de leche, las cantidades de ABs no superaban los 1,3 mg/L, mientras que en el yogur se cuantificaron concentraciones de hasta 17 mg/kg de histamina y hasta 27 mg/kg putrescina, y en el kéfir hasta 65 mg/kg de cadaverina.

Los alimentos por debajo de 1 mg/L de ABs deberían ser seguros para los consumidores, incluidas las personas sensibles a las ABs (Rauscher-Gabernig et al., 2009). Los procesos fermentativos pueden provocar un aumento de ABs en el producto final (Ladero et al., 2017). La concentración de ABs encontrada en estos productos lácteos puede ser debida a varios factores como el uso de cepas de ácido láctico utilizadas como cultivos iniciadores (Mokhtar et al., 2012), la contaminación por la microbiota ambiental y/o la microbiota presente en equipos empleados en la industria con capacidad de producción de ABs (Moniente et al., 2021). También se puede atribuir al posible uso de diferentes leches con diferentes concentraciones de precursores de aminoácidos durante la fabricación; y finalmente, al hecho de que el tipo de alimento procesado afecta a la tasa de crecimiento microbiana y la concentración de las BAL

presentes en la matriz durante el almacenamiento en frío (Samelis et al., 2000). Las diferencias encontradas en el yogur y en el kéfir con respecto a la leche pueden deberse a una falta de higiene tras el proceso de pasteurización que puede verse reflejada en una proliferación bacteriana capaz de producir ABs durante el procesado del alimento. En la leche no suelen aparecer estos compuestos ya que hay menos puntos críticos en los que pueda contaminarse el alimento tras el proceso térmico. Suponiendo un consumo habitual de yogur y de kéfir de aproximadamente 125 g, en los alimentos con la concentración más elevada de ABs encontrada en este estudio, 27 mg/kg de putrescina en yogur y 64 mg/kg de cadaverina en kéfir, los consumidores estarían ingiriendo 3,37 mg de putrescina y 8 mg de cadaverina. Estas cantidades no deberían de suponer un riesgo para la población en general, sin embargo, estos alimentos podrían no ser seguros para las personas sensibles en las que se generan síntomas a partir de 1 mg/L de ingesta de ABs. Debido a estas razones, son necesarias medidas para controlar la presencia de estos compuestos para prevenir, controlar y poner a disposición de los consumidores alimentos seguros. También se precisa implantar unas buenas prácticas de higiene durante la fabricación y el almacenamiento de estos productos, así como unas buenas medidas de limpieza y desinfección de los equipos alimentarios utilizados en su procesado que impidan el acceso de microorganismos productos en la línea de procesado.

4.2 Identificación de la microbiota causante de la acumulación de histamina en quesos comerciales (Manucrito IV)

Las ABs generalmente se encuentran en productos fermentados como el queso en cantidades variables (5-4.500 mg/kg) (Papageorgiou et al., 2018). En este sentido, los quesos de larga maduración presentan una mayor concentración de estos metabolitos (Benkerroum, 2016; Muthukumar et al., 2020). Esta variabilidad podría ser debida en parte a la diversidad de microbiota del queso, que se compone de bacterias ácido lácticas utilizadas como cultivo iniciador (SBAL) y no iniciadoras (NSBAL), así como otras deseadas y microorganismos contaminantes no deseados con capacidad de producir ABs (Botello-Morte et al., 2022; Ercolini, 2020; Mayo et al., 2021).

De entre todas las ABs, la histamina es la biológicamente más activa y más dañina debido a los posibles efectos tóxicos, psicoactivos y cutáneos que produce su consumo en exceso, además de posibles daños en los vasos sanguíneos y afecciones gastrointestinales

(Palomino-Vasco et al., 2019). La histamina se genera a partir de L-histidina por la enzima HDC. En este sentido, existe una amplia gama de bacterias Gram positivas y Gram negativas que poseen genes que codifican la HDC (Botello-Morte et al., 2022; Landete et al., 2008). A este respecto, en esta tesis doctoral se pretendió investigar la acumulación de histamina en quesos de larga maduración a través del análisis del contenido de este metabolito y de la identificación de los microorganismos productores de histamina en este tipo de productos.

La concentración de histamina se estudió en 39 quesos españoles de diferentes tipos de leche cruda o pasterizada, de distintas especies (vaca, oveja, cabra y mezclas), mediante RP-HPLC. Cabe señalar que estas determinaciones no se realizaron mediante el método puesto a punto en esta tesis doctoral, sino que las llevaron a cabo técnicos del Centro Nacional de Tecnología y Seguridad Alimentaria (CNTA) (San Adrián, Navarra), responsables de esta tarea en el proyecto Histamilk. Se obtuvo como resultado que más de la mitad de los quesos analizados (51,2%) contenían un nivel de histamina detectable (LD = 5 mg/kg), llegándose a cuantificar hasta 571 mg/kg. En 11 de los 39 quesos analizados (28,2%) la cantidad de histamina superó los 200 mg/kg y 5 de ellos (45,45%) superaron los 345 mg/kg de histamina (Manuscrito IV, tabla 1). Los niveles de histamina superaron los 500 mg/kg en dos quesos curados elaborados con leche cruda (un queso Idiazabal y un queso duro de oveja). Estas cantidades de histamina podrían dar lugar a problemas severos en los consumidores y están considerados como potencialmente tóxicos para la salud humana según la EFSA (EFSA, 2011). En este sentido, este organismo estableció el límite de consumo de histamina NOAEL en 50 mg; considerando una porción diaria de consumo de 60 g, el nivel de histamina ingerido por el consumo del queso con mayor concentración sería de 34 mg aproximadamente. No obstante, un consumo puntual de 100 g de queso podría conducir a la ingesta de más de 50 mg, lo que probablemente pueda dar lugar a cierta sintomatología en función de la sensibilidad del consumidor a esta AB.

Alrededor del 85% de los quesos con niveles de histamina detectables, incluyendo los quesos con mayor concentración, habían sido elaborados con leche cruda. Este resultado concuerda con otros estudios previos en los que las concentraciones de histamina en quesos elaborados con leche cruda fueron superiores a las observadas en los elaborados con leche pasterizada. En este sentido, se ha descrito la presencia de 573 mg/kg (Fernández et al., 2007; Sonia Novella-Rodríguez et al., 2004b), 1.042 mg/kg (Fernández et al., 2007), y hasta 2.500 mg/kg de histamina en quesos madurados de leche cruda (Bodmer et al., 1999). Estas altas cantidades de histamina podrían deberse a la proliferación de la microbiota autóctona presente

en la leche cruda. Los quesos elaborados con leche cruda a partir de cultivos iniciadores naturales y microbiota autóctona representan un desafío tecnológico ya que se desconocen en gran medida las actividades metabólicas de estos microorganismos (Tittarelli et al., 2019). La pasteurización permite reducir significativamente esa microbiota inicial, evitando su proliferación y contribución a la generación de histamina.

Por otra parte, cabe destacar la diferente concentración de histamina detectada entre quesos de diferentes especies. Si bien los quesos elaborados con leche cruda de vaca no presentaron acumulación de histamina, el 55% de los quesos elaborados con leche de oveja presentaron niveles detectables de este compuesto. Estos datos concuerdan con otros previamente estudiados y probablemente su acumulación sea debida a unas medidas higiénicas más estrictas durante el proceso de obtención de la leche de vaca en comparación con leches de otras especies animales (oveja o cabra) (Gonzalo, 2017; Kovácová et al., 2021). Además, para obtener el mismo volumen de leche de oveja o de cabra es necesario ordeñar a un mayor número de animales por lo que las probabilidades de generar una contaminación en las ubres con microbiota productora de histamina son mayores que con el uso de leche de vaca (Van Den Brom et al., 2020). Otro factor que podría estar relacionado con este hecho es el contenido en proteínas de la leche. La leche de oveja contiene una mayor cantidad de proteínas (5,7%) que la leche de vaca (3,4%), lo que puede dar lugar a una mayor proteólisis. Se sabe que un mayor grado de proteólisis puede resultar en un aumento significativo de histamina durante la maduración del queso (Moniente et al., 2021). Finalmente, cabe destacar también que en nuestro estudio se observó una mayor concentración de histamina en quesos de pasta dura (Parmesano, Idiazabal…) con un mayor tiempo de maduración, en comparación con los quesos de pasta blanda (Camembert, Roquefort…).

Para proceder a la identificación de los microorganismos causantes de la producción de histamina en el queso, se seleccionaron cinco de los quesos analizados con mayor concentración de histamina, superior a 300 mg/kg. Cuatro de ellos fueron elaborados con leche cruda de oveja y el último se elaboró con leche cruda de mezcla. Inicialmente, se llevaron a cabo aislamientos microbianos de todos los quesos y se extrajo el DNA para su posterior identificación taxonómica basada en el rRNA 16S para identificar bacterias y rRNA 28S para identificar levaduras.

Como resultado, se obtuvieron 45 aislados microbianos de los cinco quesos analizados y se identificaron bacterias Gram positivas del género *Lactobacillus, Enterococcus, Tetragenococus, Staphylococcus* y *Leuconostoc.* Estas especies han sido identificadas previamente como microorganismos productores de histamina (Botello-Morte et al., 2022; Linares et al., 2011). Además, también se consiguieron identificar levaduras como *Debaryomyces,* también descrita con anterioridad como microorganismo potencialmente productor de histamina (Botello-Morte et al., 2022; Gardini et al., 2006; Suzzi & Gardini, 2003). Por el contrario, no se detectaron bacterias Gram negativas, lo que concuerda con otros estudios en los que tampoco se identificaron enterobacterias en la mayoría de sus quesos analizados, probablemente porque las condiciones de maduración no son óptimas para la proliferación de estos microorganismos (Roig-Sagués et al., 2002) que incluso pueden llegar a desaparecer a los 90 días de maduración (Vernozy-rozand et al., 2005).

A pesar de que se detectaron varios géneros de bacterias Gram positivas, la capacidad de los microorganismos para descarboxilar aminoácidos como la histidina es variable y depende no de la especie, sino de la cepa y de las condiciones ambientales. Por ello, para verificar si estos aislados microbianos eran capaces de producir histamina, se llevaron a cabo estudios para detectar el gen *hdc* mediante técnicas moleculares. La técnica de reacción en cadena de la polimerasa (PCR) se utiliza habitualmente para comprobar la capacidad de producción de histamina de cepas bacterianas en los alimentos debido a su rapidez, especificidad y fácil interpretación de los resultados. Para ello, se extrajo el DNA total de los aislados y se realizó una PCR con oligonucleótidos específicos para amplificar el gen *hdc* de las bacterias Gram positivas, y también de las Gram negativas (a pesar de su aparente ausencia), no observándose banda del gen *hdc* de ninguno de los aislados (Figura 1, Manuscrito IV). Por tanto, nos preguntamos si habíamos aislado correctamente los microorganismos responsables de la producción de histamina y también, si era posible que los microorganismos hubieran podido perder el gen a lo largo de los diferentes pases de cultivos por contenerse en un plásmido inestable. Por ello, se modificó la estrategia y en esta ocasión se decidió buscar el gen a partir del DNA total extraído directamente de los quesos. Se realizó la PCR con varias parejas de oligonucleótidos específicos para Gram positivas y también de Gram negativas. Como resultado, se obtuvo una banda correspondiente al gen presente en las bacterias Gram positivas de los cinco quesos analizados con los oligonucleótidos JV17HC y HDC3. Sin embargo, como era de esperar, no se obtuvieron amplicones para bacterias Gram negativas con los oligonucleótidos HIS2-F y HIS2-R, al igual que se describe en el trabajo de O'Sullivan et al.

(2015). Tras comprobar la presencia de bacterias Gram positivas productoras de histamina, decidimos conocer de qué especies bacterianas se trataba. Para ello, se realizó secuenciación Sanger de los amplicones de PCR y se obtuvo como resultado que las secuencias *hdc* correspondían a los microorganismos *L. parabuchneri o L. buchneri*, que no se habían podido aislar previamente en los cultivos, y *T. halophilus*, aislado previamente a partir de las muestras de queso.

En cuatro de los cinco quesos se identificó a *L. parabuchneri.* Esta bacteria aparece cuando no se han llevado a cabo unas buenas prácticas higiénicas durante el proceso de obtención de la leche y de fabricación del queso. Según Ascone et al. (2017), este microorganismo se encuentra en el 97% de leches crudas contaminadas con bacterias productoras de histamina. Este microorganismo también es capaz de crecer y producir histamina incluso a temperaturas de refrigeración (Diaz et al., 2018) por lo que, en este caso, la refrigeración no sería una medida preventiva para reducir la acumulación de histamina en quesos. Si la leche contaminada con *L. parabuchneri* es utilizada posteriormente para la elaboración de productos lácteos, es muy probable que el producto final contenga cantidades significativas de histamina. Además, si los planes de limpieza y desinfección en las instalaciones agroalimentarias no se elaboran y/o no se ejecutan con eficacia, es más que probable que este microorganismo aparezca en los equipos de producción dada su capacidad de formar biopelículas, incluso en acero inoxidable, lo que complica en gran medida el proceso de limpieza y desinfección (Diaz et al., 2016; Srey et al., 2013). Por ello, no sólo es necesario llevar a cabo un buen sistema Análisis de Peligros y Puntos de Control Críticos (APPCC) en la industria, sino también monitorizar a *L. parabuchneri* durante todo el proceso de elaboración del queso, especialmente aquellos quesos elaborados con la leche cruda, para evitar la acumulación de histamina en el producto final (Ascone et al., 2017; Wechsler et al., 2021).

Como se ha mencionado anteriormente, *L. parabuchneri* no fue aislada mediante los cultivos celulares y, por tanto, tampoco pudo amplificarse el gen *hdc* en la PCR con el DNA total de los aislados, lo que indica que este microorganismo podría estar en "un estado viable pero no cultivable" (Randazzo et al., 2010). Para evaluar su presencia en quesos se realizaron estudios de metataxonómica, ya que permite entre otras la detección de bacterias no cultivadas, y de este modo, pudo identificarse este microorganismo, confirmándose la presencia de *L. parabuchneri* como uno de los microorganismos productores de histamina en las muestras de queso.

Por otra parte, en uno de los cinco quesos analizados se obtuvo una secuencia del gen *hdc* que correspondía con un 99.29% de identidad al grupo *L. sakei, T. halophilus, T. muriaticus, Oenococcus oeni* y *L. hilgardii.* La falta de una base de datos específica del gen *hdc* y el alto nivel de similitud (>99%) entre las secuencias *hdc* del grupo bacteriano complica la distinción entre estas especies con este método de secuenciación Sanger (Wüthrich et al., 2017). Este microorganismo se pudo identificar en los aislados microbianos a partir de muestras de queso, pero no se observó la presencia del gen *hdc* con el DNA de dicho aislado. Por ello, se planteó que el gen *hdc* podía estar codificado en un plásmido que se había podido perder en los sucesivos cultivos para obtener los aislados microbiológicos, y para comprobar esta hipótesis se obtuvo el DNA total a partir de los quesos y se llevó a cabo la reacción de PCR para amplificar el gen *hdc* dentro del plásmido transmisible pHDC. De esta manera se pudo identificar a *T. halophilus* como microorganismo productor de histamina en la muestra de queso. Se diseñaron los oligonucleótidos pHDCF y pHDCR para alinearse en la secuencia del plásmido pHDC con el propósito de evaluar la presencia de este plásmido en el DNA total del queso en el que se identificó este microorganismo (Figura 2, Manuscrito IV) y se evaluó también para el resto de quesos estudiados. Como era de esperar, se obtuvo una banda específica para el queso con *T. halophilus* y no para el resto de quesos. Además de los oligonucleótidos diseñados que hibridan el plásmido (pHDCF y pHDCR), se utilizaron oligonucleótidos específicos que amplifican en el gen *hdc* de *T. halophilus* (HmF y HmR) previamente descritos en la literatura (Satomi et al., 2008), y oligonucleótidos que hibridan en ambas zonas, para confirmar efectivamente que se encontraba tanto en el gen como en el plásmido pHDC y que, por tanto, éste se había perdido durante los subcultivos. Estos resultados revelan la necesidad de tener en cuenta la inestabilidad del plásmido y controlar las condiciones de cultivo para evitar la pérdida del plásmido que contiene el gen *hdc* (Lucas et al., 2005) cuando se requiera confirmar la presencia del gen *hdc* en un aislado microbiano.

Como se ha comentado con anterioridad, *D. hansenii* también fue aislado en los cultivos microbianos iniciales e identificado como posible microorganismo productor de histamina en la bibliografía (Gardini et al., 2016). Sin embargo, la literatura no proporciona información genética sobre el gen *hdc* y, por tanto, no se pudo identificar esta levadura en los estudios de secuenciación realizados. Para verificar la producción de histamina por parte del aislado de *D. hansenii,* se llevó a cabo un estudio *in vivo* utilizando el medio de descarboxilación MDA-H (Maijala, 1993), y su posterior cuantificación por HPLC de la cantidad de histamina en el sobrenadante del cultivo. A pesar de que se detectó un cambio de color en el medio cromogénico

MDA-H, no se detectó la presencia de histamina. Este cambio de color podría sugerir la existencia de otras ABs generadas por la presencia de otros aminoácidos libres con actividad de descarboxilación disponibles en el medio o bien podría ser debido por una producción de histamina por debajo del límite de detección (LD) del método. Otra razón es la posible existencia de cepas de este microorganismo que simultáneamente fueran capaces de sintetizar histamina a la vez que degradarla (Bäumlisberger et al., 2015). En definitiva, aunque no se pudo identificar a *D. hansenii* como microorganismo productor de histamina en este estudio, no puede descartarse una posible contribución de este microorganismo a la producción de histamina en los quesos seleccionados.

Algunas NSBAL naturalmente presentes en la leche son necesarias para el correcto desarrollo del sabor en los quesos de larga maduración (Blaya et al., 2018). Sin embargo, tanto *L. parabuchneri* como *T. halophilus* son microorganismos contaminantes o ambientales formadores de histamina que pueden multiplicarse a baja temperatura o en condiciones extremas de sal, y no se utilizan como cultivos iniciadores para la elaboración de quesos y, por tanto, se consideran microorganismos indeseables en la producción de quesos.

Los microorganismos productores de histamina pueden aparecer en los productos lácteos si las condiciones higiénicas de fabricación y almacenamiento son deficientes, por lo tanto, el primer paso para prevenir la formación de estos compuestos sería llevar a cabo unas buenas prácticas de procesado. Para un mejor control y prevención de la aparición de histamina se requiere monitorizar el pH, ya que la formación de ABs incrementa el pH del producto, conocer la composición microbiana de las BAL no iniciadoras que podrían producir histamina, así como seleccionar aquellos cultivos iniciadores que carezcan de la capacidad de producir histamina, y que además sean capaces de interaccionar y competir con la microbiota autóctona en las condiciones de producción (Moniente et al., 2021). Por otra parte, el uso de tratamientos térmicos de la leche para la producción de queso, el alto contenido de sal y las temperaturas reducidas de maduración y almacenamiento, ayudarían a reducir el crecimiento microbiano, y por consiguiente a disminuir la producción de histamina en el producto final.

4.3 Estudio de la distribución espacial de la histamina y de la microbiota responsable de su acumulación en queso madurado (Manuscrito V).

Como se ha comentado anteriormente, la variabilidad de ABs en quesos es elevada y, además, su concentración puede diferir dentro de un mismo tipo de queso e incluso entre zonas distintas de un mismo queso (Novella-Rodríguez et al., 2003). Esta diversidad se atribuye a la microbiota productora de histamina en quesos que no se distribuye de manera homogénea en el queso. En la superficie se localizan las bacterias aerobias y levaduras, mientras que en el interior lo hacen las bacterias anaerobias, la mayoría BAL. También se ha observado cierta heterogeneidad en las propiedades físicas y químicas de los quesos (a_w, concentración de sal…) debido a los cambios bioquímicos que se producen en el alimento durante el proceso de maduración, lo que a su vez puede tener una gran influencia en el crecimiento microbiano (Mayo et al., 2021; Moniente et al., 2021).

Conociendo este hecho, debería tenerse en cuenta que un análisis adecuado de histamina en los quesos va a requerir la toma de muestras de diferentes porciones de queso, procedentes de diferentes áreas, acorde con la distribución de histamina que pudiera existir. Para comprobar esta hipótesis, en este estudio se utilizaron doce quesos españoles de larga maduración elaborados a partir de leche cruda. La mayoría de los quesos estaban hechos de leche de oveja y el resto de mezcla de diferentes tipos de leche. Los quesos se recibieron en el laboratorio del CNTA y se cortaron en nueve cuñas. Cada cuña se dividió en 4 áreas objeto de estudio (corteza delantera (zona 1), centro delantero (zona 2), centro trasero (zona 3) y corteza trasera (zona 4). Se enviaron tres de las nueve cuñas, divididas en las cuatro zonas de estudio cada una (doce muestras en total), a BIOLAN Microbiosensores S.L. que se encargó del análisis de concentración de histamina. Otras tres cuñas se enviaron a nuestro laboratorio en la Facultad de Veterinaria de la Universidad de Zaragoza donde se analizó la microbiota productora de histamina. Finalmente, un tercer grupo de doce muestras fueron sometidas al análisis de las propiedades físicas y químicas en el laboratorio del CNTA.

Para evaluar la distribución de histamina en el queso se llevó a cabo la cuantificación de esta AB en las diferentes zonas mediante un biosensor enzimático BIO300 HIS con un rango optimizado de cuantificación desde 100 a 500 mg/kg. Como resultado, la Figura 2 (Manuscrito V) muestra el porcentaje de desviación positiva/negativa en cada zona del valor medio de la concentración de histamina en la cuña, representada por 0% de desviación. El patrón observado fue que la acumulación de histamina, en general, se concentró en las áreas centrales 1 y 2

(17.8% y 54.4%, respectivamente) mientras que en las áreas de la periferia 3 y 4 la acumulación fue inferior con respecto a la cantidad total de histamina en la cuña (-19.8% y -52.3%, respectivamente). Además, el núcleo central (área 2) era la zona donde se acumuló una mayor cantidad de histamina (hasta 115,4% más que el valor medio de las cuatro zonas) y los valores más bajos de histamina se encontraron en la corteza exterior (área 4) (hasta 83,3% menos que el valor medio de las cuatro zonas). El orden de distribución de mayor a menor concentración de histamina encontrado fue: área 2 *versus* área 1 y área 3 *versus* área 4. No obstante, solo se observaron diferencias estadísticamente significativas en la mayoría de los quesos entre la zona 2 y la zona 4.

En estudios previos se había concluido que las ABs están más presentes en la parte central de la cuña que cerca de la corteza (Joosten & Stadhouders, 1987), mientras que otros investigadores determinaron que las ABs se acumulan en la parte exterior y en la corteza del queso (Komprda et al., 2007; Marijan et al., 2014; Novella-Rodríguez et al., 2003). Sin embargo, cabe señalar que estos estudios se realizaron en quesos cuyos valores de histamina fueron inferiores a 20 mg/kg en comparación con nuestro estudio en el que se alcanzaron niveles de histamina superiores a 500 mg/kg. Nuestros resultados revelan nuevamente la existencia de diferencias en el contenido de histamina en función de las zonas de muestreo de queso. Para conocer el valor más bajo de histamina presente en un queso, se debe tomar una muestra de la corteza trasera, mientras que para determinar con precisión el valor más alto, se debe tomar muestra del núcleo interno del queso. Así, si lo que se pretende es determinar el valor medio de concentración de histamina de un queso, debe tenerse en cuenta la necesidad de homogenizar porciones representativas de las diferentes partes del queso. Por el contrario, cabe destacar que el consumo de queso de su parte interna puede suponer un incremento en la concentración de histamina que puede superar el 115,4% respecto al valor promedio. Estos resultados denotan la importancia de establecer un procedimiento de muestreo adecuado cuando la finalidad del ensayo es estimar la concentración objetiva de histamina de un queso.

Conociendo la distribución de histamina en las diferentes zonas del queso, se realizaron ensayos para intentar establecer una correlación entre este patrón de distribución y la microbiota productora de histamina en el queso. Para ello, se llevó a cabo un estudio molecular de la presencia del gen *hdc* en las diferentes áreas a partir del DNA total de las cuatro zonas de queso. Para realizar este estudio se seleccionaron cuatro quesos: dos quesos Denominación de Origen Protegida (DOP) Idiazabal (Quesos 1 y 2), uno elaborado con leche de mezcla (Queso 3) y otro

elaborado con leche de oveja (Queso 5), y se realizó una PCR para amplificar el gen *hdc* usando oligonucleótidos específicos descritos previamente en la literatura para amplificar el gen de bacterias Gram positivas o Gram negativas. Como se muestra en la Figura 3 (Manuscrito V), aparecen bandas correspondientes al gen *hdc* de bacterias Gram positivas lo cual indica que este gen se encuentra en el DNA de todas las muestras de queso, mientras que no aparecieron bandas para el gen *hdc* de las especies Gram negativas por lo que se concluye que este grupo bacteriano estaba ausente en las muestras. Dado que los genes *hdc* de algunas bacterias Gram positivas pueden encontrarse en un plásmido pHDC inestable, también se usaron oligonucleótidos específicos que se alinean dentro de la secuencia del plásmido. Como resultado se obtuvo que no todas las áreas de todas las muestras de queso contenían DNA plasmídico, pero sí aparecieron bandas correspondientes al plásmido pHDC en algunas áreas, como el área 1 en el queso 5 o todas las áreas en el queso 1.

Tras conocer la presencia del gen *hdc* en el DNA genómico y plasmídico de las bacterias Gram positivas, los productos de la PCR fueron purificados y sometidos a secuenciación Sanger para identificar los microorganismos con capacidad de producir histamina. Los datos obtenidos mostraron que varios picos se superpusieron de manera generalizada en el cromatograma de los resultados de la secuenciación, lo que indica que hubo varias secuencias de *hdc* y, por lo tanto, hay varias especies microbianas productoras de histamina en la muestra. Las secuencias de nucleótidos correspondieron a *L. parabuchneri/buchneri* y al grupo *T. halophilus/T. muriaticus/O. oeni/L. sakei/L. hilgaldii*, cuya secuencia *hdc* está completamente conservada y es indistinguible entre estas bacterias (Wüthrich et al., 2017). No se obtuvo distinción de microorganismos en las distintas áreas y, por lo tanto, se concluye que los microorganismos productores de histamina estaban distribuidos de forma homogénea en todas las áreas de queso analizadas. Como se ha comentado anteriormente, estas bacterias no se utilizan como cultivo iniciador y son bacterias productoras de histamina que habitualmente contaminan el queso (Diaz et al., 2015a; Diaz et al., 2015b; O'Sullivan et al., 2015; Wechsler et al., 2021). El gen *hdc* de *L. parabuchneri/buchneri* se encuentra en el cromosoma, mientras que el grupo *T. halophilus/T. muriaticus/O. oeni/L. sakei/L. hilgaldii* presumiblemente contiene los genes *hdc* en el plásmido pHDC (Lucas et al., 2005), por lo que se entiende que los resultados obtenidos por PCR para el DNA plasmídico se corresponden a este grupo microbiano.

Debido a que se encontraron los mismos microorganismos en las diferentes zonas de queso se quiso conocer la relación de las propiedades físicas y químicas de los quesos con el patrón de acumulación de histamina. En cada área se determinó la humedad (g de agua/100 g

de queso), a_W, el contenido de sal (g NaCl/100 g de queso) y el estado oxidativo de los lípidos (medido por el ensayo TBA como mg de malonaldehído/kg de queso) para correlacionarlos con la cantidad de histamina en las muestras. No se encontraron diferencias significativas de a_W en ninguna zona. Se observó como el contenido de humedad y de sal fue significativamente mayor en los núcleos (con desviaciones de 15,1% y 22,4% en el área 2 y de 8,0% y 9,0% en el área 3, respectivamente) con respecto al valor medio en toda la cuña; mientras que fueron menores en la corteza del mismo modo que la distribución de histamina (con desviaciones de -6,2% y -11,9% en el área 1 y de -18,0% y -20,2% en el área 4, respectivamente). Para los valores de histamina se observó un comportamiento similar de acumulación de este metabolito. En cuanto al estado de oxidación lipídica, pese a no existir diferencias significativas, sí que se observa una tendencia: una mayor oxidación en el núcleo de los quesos respecto al valor medio (-29,6% y -40,3% en las zonas 1 y 4, respectivamente) y una menor oxidación en las zonas exteriores.

En cuanto a la relación entre las características físicas y químicas es necesario indicar que no se pudo establecer una correlación estadísticamente significativa para el contenido de histamina y el contenido de sal y la humedad, sin embargo, se muestra una tendencia positiva entre estos parámetros, al igual que la observada por otros autores (Manca et al., 2020). De la misma manera, no se obtuvo una correlación significativa entre el contenido de histamina y la oxidación lipídica en las distintas zonas del queso, aunque sí que se encontró una asociación negativa entre el contenido de histamina y la oxidación.

Como conclusión, cabe destacar la existencia de una tendencia a acumular histamina en las áreas más saladas, más húmedas y menos oxidadas de la cuña. Estos datos concuerdan con otros estudios que indican que la sal y la a_w podrían afectar la producción de histamina en los alimentos probablemente debido a su efecto inhibitorio sobre el crecimiento microbiano (Bansal & Mishra, 2020; Besas & Dizon, 2012; FAO/WHO, 2013; Møller et al., 2021; Tabanelli et al., 2012). También se sabe que *Tetragenococcus* sp., bacteria identificada como productora de histamina en estos quesos, es una bacteria halófila capaz de multiplicarse en cantidades elevadas de sal e incluso puede llegar a producir histamina a concentraciones superiores al 20% (p/v) de NaCl (Kimura et al., 2001; Satomi et al., 2008; Unno et al., 2020). Se conoce además que este microorganismo es capaz de producir histamina en condiciones limitantes de O_2 (Kimura et al., 2001), por lo que se podría suponer que esta bacteria está principalmente asociada a una mayor acumulación de histamina en el centro del queso donde el nivel de oxidación de lípidos es menor. Sin embargo, se requieren más estudios para dilucidar

las causas microbiológicas, físicas y químicas del patrón de distribución de histamina en los quesos de larga maduración.

4.4 Puesta a punto de herramientas que permitan reducir la concentración de histamina en queso de larga maduración (Manucrito VI).

Los quesos de larga maduración son el alimento fermentado que se asocia con mayor frecuencia con intoxicación alimentaria por ABs (Combarros-Fuertes et al., 2016). La presencia de estos compuestos depende en gran medida del tratamiento de la leche, de las condiciones higiénicas de procesado y del tiempo de maduración (Fernández et al., 2007), así como de los cultivos iniciadores utilizados para la elaboración del alimento (Moniente et al., 2021), entre otras. Durante la maduración de los quesos se producen aminoácidos libres como resultado de la proteólisis que, junto a la presencia de microorganismos descarboxilasa positivos y unas condiciones ambientales favorables (proceso tecnológico, pH, temperatura, disponibilidad de agua, etc.), permiten la formación de ABs (Linares et al., 2012).

Conscientes de estos hechos, en este último trabajo nos planteamos evaluar el uso de varias herramientas (microbiológicas y enzimáticas) que nos permitieran reducir la concentración de histamina en quesos de larga maduración. Concretamente, para realizar este estudio se evaluaron y se seleccionaron cultivos microbianos con capacidad de degradar histamina y que carecían de la actividad de producir histamina, así como la enzima capaz de degradar este compuesto.

Para empezar, la revisión bibliográfica nos mostró la capacidad de degradación de histamina de una cepa ácido láctica utilizada para la elaboración de yogur, *S. salivarius* subsp. *thermophilus* PF3CT (Guarcello et al., 2016). Al observar el potencial de este microorganismo, se realizó un estudio preliminar para confirmar la capacidad para degradar este metabolito, no solo de *S. thermophilus* sino también de *L. bulgaricus*, ya que ambos microorganismos son utilizados por su sinergia en la elaboración de yogur, de entre varias opciones existentes en el mercado. Se estudiaron un total de 7 cepas de *L. bulgaricus* y *S. thermophilus* de la Colección Española de Cultivos Tipo (CECT) (*L. bulgaricus* CECT 4005, CECT 4006, CECT 5035 y CECT 5036; y *S. thermophilus* CECT 801, CECT 986 y CECT 7207). Además, se ensayaron dos cultivos iniciadores comerciales para fabricar yogur para la evaluación de la degradación de histamina: Cultivo iniciador de yogur tipo I (Abiasa) y cultivo iniciador YoFlex® Premium 1.0 (CHR Hansen). Así, se seleccionaron como cultivos adjuntos en la elaboración de queso las cepas de *L. bulgaricus* y *S. thermophilus* con las tasas de degradación de histamina más altas

obtenidas en un medio de laboratorio pertenecientes a CECT (del 2,52% por *L. bulgaricus* CECT 4005 y hasta un 57,8% por *S. thermophilus* CECT 7207) y al cultivo iniciador Abiasa (del 27% por *L. bulgaricus* y hasta un 40,2% por *S. thermophilus*). El cultivo iniciador comercial utilizado en la elaboración de yogur (YoFlex® Premium 1.0, CHR Hansen) también fue seleccionado para adicionarlo durante la elaboración de queso debido a su tasa de degradación, de hasta casi un 70%, en medio lácteo (yogur). De este estudio previo, cabe destacar que *L. bulgaricus* ha sido descrito por primera vez en nuestro estudio como microorganismo con potencial de degradación de histamina por lo que se seleccionó junto a *S. thermophilus* dado su crecimiento sinérgico durante la producción de yogur (Aryana & Olson, 2017). Además de las bacterias productoras de yogur evaluadas, se realizó una revisión bibliográfica en búsqueda de otros posibles cultivos microbianos con capacidad de degradar este metabolito, y se identificaron a *L. casei* (Herrero-Fresno et al., 2012) y a *D. hansenii* (Bäumlisberger et al., 2015). Se seleccionaron las cepas 4a y 18b de *L. casei* con mayor actividad degradadora (más del 40% de histamina in vitro) (Herrero-Fresno et al., 2012) y una cepa de *D. hansenii* previamente aislada de queso en nuestro laboratorio (Botello-Morte et al., 2022) para evaluar su potencial en el posterior ensayo en quesos.

Un factor principal para seleccionar las bacterias utilizadas en la elaboración del queso fue que careciesen del gen *hdc*, para evitar la producción de histamina por su parte. La capacidad de producir o degradar histamina no es atribuible a todas las cepas que pertenecen a una especie bacteriana, sino que se trata de un rasgo característico nivel específico de cepa (Hrubisko et al., 2021). Teniendo esto en cuenta, se realizó una PCR para evaluar si las bacterias que iban a ser utilizadas en el ensayo contenían el gen *hdc* y, por tanto, tenían capacidad para producir histamina. No aparecieron bandas en ninguno de los microorganismos seleccionados para su uso en el proceso de elaboración del queso, excepto en el control positivo empleado en este estudio como microorganismo productor de histamina, correspondiente al DNA de *L. parabuchneri* DSM 5987 que, como era de esperar, generó una banda de aproximadamente 370 pb.

Como medida enzimática para reducir la concentración de histamina en queso de larga maduración se propuso el uso de DAO, añadido a la leche de modo previo a la elaboración de queso. Con el fin de comprobar si la enzima DAO era capaz de mantener su actividad enzimática en una matriz láctea a la temperatura de refrigeración utilizada para almacenar la leche, se analizó su capacidad degradadora de histamina en leche a 4ºC. Como resultado se

obtuvo que la enzima DAO ensayada (108,7 UD/L) fue capaz de degradar más de un 30% de histamina tras 24 h de incubación. Estudios anteriores habían demostrado que a la temperatura óptima de trabajo de la enzima de 37ºC en medio tamponado, era posible conseguir una degradación del doble de histamina (0,54 mM) utilizando 12 veces menos concentración de enzima (9,4 U/L) que en nuestro estudio (Kettner et al., 2020). Sin embargo, los resultados obtenidos revelaron que, pese a llevar a cabo el experimento en unas condiciones no óptimas para la DAO y verse reducida su actividad enzimática, esta enzima fue capaz de disminuir la histamina a temperaturas de refrigeración.

Tras haber comprobado la capacidad de degradación de la histamina de los microorganismos y la enzima mencionados, se procedió a realizar un ensayo en el que se reprodujo el proceso de elaboración de queso madurado a escala de planta piloto. Como paso previo, se añadió a la leche una concentración final de $1 \cdot 10^6$ unidades formadoras de colonias (UFC)/mL de *L. parabuchneri* DSM 5987 para producir la histamina de manera natural en el queso incluso a temperaturas de refrigeración, basándonos en estudios recientes de Diaz et al. (2018) y Wechsler et al. (2021), así como en ensayos anteriores realizados en nuestro laboratorio (datos no publicados).

Para llevar a cabo el experimento se fabricaron 8 lotes de quesos, cada uno de ellos con 18 unidades de queso de unos 200 g (tres réplicas para cada tiempo de estudio 0, 15, 30, 45, 60 y 100 días de maduración), a partir de leche pasterizada de vaca, todas ellas conteniendo *L. parabuchneri* DSM 5987 (excepto el lote de queso correspondiente al control negativo) y un cultivo iniciador de queso F-DVS GRANA-102 (CHR Hansen). Se estudió una condición por cada línea de elaboración de queso:

- Lote I: Lote control sin *L. parabuchneri* DSM 5987.
- Lote II: Lote control únicamente con *L. parabuchneri* DSM 5987.
- Lote III: *L. parabuchneri* DSM 5987 y *L. casei* 4a y 18b.
- Lote IV: *L. parabuchneri* DSM 5987 y *L. bulgaricus* CECT 4005 y *S. thermophilus* CECT 7207.
- Lote V: *L. parabuchneri* DSM 5987 y cultivo iniciador para fabricar yogur de la marca comercial Abiasa.
- Lote VI: *L. parabuchneri* DSM 5987 y cultivo iniciador para fabricar yogur de la marca comercial CHR Hansen.
- Lote VII: *L. parabuchneri* DSM 5987 y *D. hansenii* previamente aislado de queso.
- Lote VIII: *L. parabuchneri* DSM 5987 y la enzima DAO.

La histamina se cuantificó a lo largo de 100 días de maduración del queso mediante el método RP-HPLC puesto a punto para esta Tesis Doctoral a la que se sumó una etapa previa de extracción de ABs utilizando 5 g de queso con 7,5 mL de HCl 1 M. Se centrifugó a 4000·*g* a 4ºC durante 21 min y finalmente se recogió el sobrenadante, evitando la capa de grasa superior, y se repitió el procedimiento de extracción 3 veces. Finalmente, se llevó a un volumen de final de 25 mL con HCl 1 M en un matraz aforado.

Inicialmente, a tiempo 0, la concentración de histamina en los quesos era baja, similar en todos los lotes y oscilaba entre 1,48 a 6,41 mg/kg. Estas concentraciones son similares a las obtenidas en leche, en yogur, en kéfir (Özdestan & Üren, 2010; Pekcici et al., 2021), o como en este caso, en quesos frescos, probablemente debido a un menor crecimiento de la microbiota productora de histamina (Ercan et al., 2019) o a una menor disponibilidad del aminoácido precursor histidina debido a una menor proteólisis (Moniente et al., 2021). Al final del periodo de maduración (100 días) la cantidad de histamina en el lote de control negativo fue menor (38,07 ± 13,90 mg/kg) que la concentración obtenida en el resto de los lotes, y menor a la obtenida en una gran variedad de quesos comerciales (Botello-Morte et al., 2022). Como era de esperar, la mayor cantidad de histamina se encontró en el lote control positivo con *L. parabuchneri* (771,55 ± 29,89 mg/kg). La elevada concentración de histamina observada en el lote control positivo se utilizó como referencia para comparar y evaluar el potencial de las medidas degradadoras de los otros lotes. Como se ha mencionado anteriormente, este elevado nivel de histamina obtenido a los 100 días de maduración podría tener consecuencias nocivas para la salud del consumidor (EFSA, 2011). En un consumo de 60 g de queso se superarían los 46 mg de histamina y en un consumo puntual de 100 g de queso se alcanzarían hasta los 77 mg de histamina, muy superior a lo establecido por la EFSA como NOAEL para personas sanas. Como se observa en la Figura 2 (Manuscrito VI), la concentración de histamina fue aumentando progresivamente a lo largo del período de maduración; a partir de los 45 días se obtuvo una tasa de acumulación más lenta. Por este motivo, la diferencia de concentración de histamina entre el lote con *L. parabuchneri* y los demás lotes fue significativamente mayor a los 45 días de maduración. Estos resultados fueron similares a otros estudios previos en los que se mostraba un aumento inicial gradual en la acumulación de histamina durante el período de maduración (Bunkova et al., 2013; Kebary et al., 1999; Novella-Rodríguez et al., 2002; Novella-Rodríguez et al., 2004a).

Globalemente se puede decir que todas las medidas de degradación ensayadas redujeron significativamente la concentración de histamina (Figura 3, Manuscrito VI). Respecto a la eficacia de las medidas microbianas aplicadas, la mayor disminución de histamina se observó en el lote que contenía *D. hansenii* (45,32%). Los lotes con cepas de *L. casei* 4a y 18b, *L. bulgaricus* y *S. thermophilus* de la CECT y *L. bulgaricus* y *S. thermophilus* pertenecientes al cultivo iniciador de yogur de Abiasa también redujeron el contenido de histamina en un 43,05%, 42,31% y un 35,5%, respectivamente. Cabe destacar que las dos cepas de *L. casei* que se utilizaron en el proceso de elaboración del queso produjeron una disminución del contenido de histamina similar a la obtenida in vitro por Herrero-Fresno et al. (2012). El lote que contenía el cultivo iniciador comercial de yogur YoFlex® Premium 1.0 de CHR Hansen resultó ser la medida de control menos efectiva consiguiendo una reducción del 17,56% de histamina con respecto al lote control positivo con *L. parabuchneri*. Estos resultados demuestran que la adición de cultivos iniciadores con capacidad para degradar histamina podría plantearse en algunos casos como medida eficaz para controlar y prevenir la acumulación excesiva de este metabolito en queso.

En cuanto a la degradación enzimática de histamina, cabe destacar que la enzima DAO fue uno de los agentes más efectivos en el control de la acumulación de histamina hasta los 30 días de maduración, resultando en una reducción del 30% de histamina con respecto al control positivo. Sin embargo, a los 100 días de maduración se observó una reducción del 23% de histamina con respecto al control positivo. La menor tasa de reducción de histamina observada hacia el final del período de maduración podría explicarse por una menor actividad enzimática. Estudios recientes demuestran que existe una mayor actividad de DAO cuando la histamina se encuentra a una concentración de hasta 56 mg/L, pero, sin embargo, se alcanza una inhibición del 40% de la actividad de la enzima a una concentración de 500 mg/L de histamina (Kettner et al., 2020). En nuestro estudio la concentración final de histamina obtenida a los 100 días de maduración fue de 591,41 ± 37,63 mg/kg. Esta menor actividad podría estar relacionada con el bajo pH observado durante la maduración de los quesos (desde 5,00 hasta 5,79), alejado del pH óptimo para DAO (7,2) (Dapkevicius et al., 2000). Otro factor que pudo reducir la actividad enzimática fue la temperatura de maduración (12ºC), muy por debajo de la óptima para la DAO, de 37ºC, (Naila et al., 2012). Dado la escasa eliminación de histamina lograda en quesos con 100 días de maduración y los elevados precios que actualmente esta enzima alcanza en el mercado, ésta no parece ser la mejor solución de entre las testadas para evitar la acumulación de histamina en quesos madurados; no obstante, en las condiciones ensayadas en este estudio,

la adición de DAO a la leche se podría considerar para la elaboración de quesos con un periodo de maduración inferior a un mes.

En conclusión, cabe destacar que los microorganismos degradadores de histamina y la enzima DAO añadidos a la leche para la fabricación de queso redujeron significativamente la concentración de histamina en el queso al final de la maduración, en comparación con el queso control con *L. parabuchneri* que superaba los 700 mg/kg de histamina. Pese a no existir normativa que regule los niveles de histamina permitidos en lácteos, sí que existen recomendaciones de consumo de histamina de 400 mg/kg de histamina como nivel aceptable para el queso madurado, suponiendo un consumo diario de queso de 60 g (Rauscher-Gabernig et al., 2009). Las concentraciones de histamina en las muestras de los *L. casei* 4a y 18b y *D. hansenii* fueron ligeramente superiores a 400 mg/kg al final del período de maduración, mientras que el lote con *L. bulgaricus* CECT 4005 y *S. thermophilus* CECT 7207 y el lote con el cultivo iniciador Abiasa tenían una concentración final de histamina inferior a 500 mg/kg, que es el límite establecido por la EFSA como potencialmente tóxico para la salud humana (EFSA, 2011). Por tanto, se podrían obtener quesos con concentraciones de histamina por debajo de ese límite llevando a cabo unas buenas prácticas de higiene, tratando de evitar el uso de leches contaminadas con *L. parabuchneri,* períodos de maduración más cortos y empleando microorganismos degradadores de histamina, principalmente *L. casei* 4a y 18b o *D. hansenii*.

Por otra parte, se estudiaron las características físicas y químicas (peso, pH, a_w, color y textura) en cada uno de los tiempos estudiados (0, 15, 30, 60 y 100 días de maduración) para evaluar si la adición de los microorganismos propuestos y de DAO podrían afectar las propiedades organolépticas de los productos finales. Como resultado, se obtuvieron cambios significativos durante el proceso de maduración del queso en las propiedades físicas y químicas (peso, pH, a_w, color y textura) propios de la curación del queso, sin embargo, no hubo cambios fisicoquímicos significativos asociados a la adición de cultivos adjuntos potencialmente degradadores de histamina o de DAO en los quesos. El pH de los quesos al final de la maduración osciló entre 5,32 y 5,80, similar a los obtenidos en los quesos añejos, Cheddar, Emmental (Guinee & Fox, 1994), Parma (Jaster et al., 2014; Marcos et al., 1981) y Chihuahua (Gutiérrez-Méndez et al., 2013). La a_w se redujo significativamente durante la maduración (de 0,977 a 0,824 al final de la maduración), probablemente debido a la pérdida de agua y al aumento de los productos de proteólisis solubles en agua (Hickey et al., 2013). No se observaron cambios de color relevantes entre los quesos de control y el resto de lotes en el mismo tiempo

de estudio. Sin embargo, los valores de L*, a* y b* variaron significativamente durante el período de maduración de 100 días. Como era de esperar, la dureza del queso aumentó y la adhesividad del queso disminuyó a lo largo del período de maduración en todos los lotes. Estos resultados fueron similares a las observadas en muestras de quesos Parma, Chihuahua, Cheddar y Emmental (Gutiérrez-Méndez et al., 2013; Jaster et al., 2014; Zheng et al., 2016).

Finalmente, se llevó a cabo un ánalisis sensorial a través del *sorting task* basada en la tarea de clasificación para evaluar la similitud del perfil aromático entre los lotes de quesos elaborados. Un total de 42 panelistas no entrenados (19 hombres y 23 mujeres, de 23 a 60 años) consumidores de queso evaluaron el aroma de los ocho lotes de queso al final del periodo de maduración (100 días) y los clasificaron en grupos según sus similitudes. Se presentaron 9 muestras (una muestra por cada lote y una muestra duplicada de uno de los lotes utilizado como control del experimento), de aproximadamente 7 g cada una, en frascos de vidrio ámbar codificados con números de tres dígitos siguiendo un orden aleatorio. Como resultado (Figura 4, manuscrito VI), se apreció que en los quesos elaborados con los diferentes microorganismos potencialmente degradadores de histamina aparecía un olor particular, no desagradable, representado por el atributo sensorial "láctico" conformado por los lotes de control positivo y negativo; por el atributo "fiambre" formado por el lote con *L. casei* 4a y 18b y el lote con *L. bulgaricus* CECT 4005 y *S. thermophilus* CECT 7207; así como los descriptores "afrutado" y "vinoso" representado por el lote con el cultivo iniciador de yogur YoFlex® Premium 1.0 de CHR Hansen, el lote con *D. hansenii* aislado de queso, y el lote con DAO. Además, el perfil aromático del lote control positivo no fue significativamente diferente del lote control negativo, por lo que se puede concluir que *L. parabuchneri* DSM 5987 no modificó el aroma del queso a pesar de su producción de histamina y, por tanto, fueron los microorganismos degradadores utilizados como cultivos adjuntos en la elaboración del queso los que produjeron los cambios sensoriales en los productos finales. La olfatometría permitiría conocer de manera más exhaustiva y detallada cuáles son los compuestos más relevantes en el aroma de los quesos de estudio y en qué concentración se encuentran en cada muestra. Para valorar de forma adecuada las modificaciones sensoriales en boca generadas a causa de la adición de cultivos iniciadores en estos quesos madurados se requeriría una evaluación sensorial mediante un panel entrenado de catadores. No obstante, se decidió no realizar un análisis sensorial que incluyese la cata de los quesos dada la imposibilidad de garantizar las medidas higiénicas en la sala de procesado de la Planta Piloto de Ciencia y Tecnología de los Alimentos de la Universidad de Zaragoza.

5. Conclusiones

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En base a los objetivos planteados en la presente Tesis Doctoral, los resultados obtenidos respecto a la determinación de histamina y de la microbiota responsable en productos lácteos, así como al desarrollo de potenciales soluciones para reducir su acumulación en quesos madurados, han dado lugar a las siguientes conclusiones:

- Se ha puesto a punto un método para el análisis de ABs en productos lácteos basado en un procedimiento de extracción de SPE seguido de derivatización con AQC y análisis por HPLC con detección de fluorescencia. Esta metodología de análisis nos ha permitido alcanzar límites de detección inferiores a 0,2 mg/L y límites de cuantificación inferiores a 0,667 mg/L para las diferentes ABs.
- Se observó cierta variabilidad en la concentración de ABs entre la leche y los productos fermentados (yogur y kéfir). En pocas ocasiones se encontraron ABs en las muestras de leche, sin embargo, tanto la frecuencia como la cantidad de ABs fue más elevada en las muestras de yogur y de kéfir.
- Se detectó histamina en más del 50% de los 39 quesos comerciales analizados, y casi un tercio (28,2%) contenía más de 200 mg/kg. El 85% de los quesos con niveles detectables de histamina fueron elaborados con leche cruda y un 55% con leche de oveja.
- Los microorganismos identificados como productores de histamina en la mayoría de los quesos analizados fueron las bacterias *L. parabuchneri* y *T. halophilus*. El gen *hdc* responsable de la síntesis de histamina en el microorganismo *Tetragenococcus* está contenido en un plásmido inestable.
- En todos los casos, los microorganismos productores de histamina no se correspondieron con cultivos iniciadores sino con microorganismos contaminantes, lo que pone de manifiesto la importancia de mejorar las medidas higiénicas en el ordeño en las granjas, en el transporte de la leche y en la industria quesera.
- La concentración de histamina en los quesos de larga maduración no es homogénea, alcanzándose una mayor concentración en la parte central y menor en la parte periférica. Estos resultados ponen de manifiesto la importancia de realizar un muestreo adecuado cuando se pretende determinar la concentración de histamina en quesos.
- Las estrategias basadas en la adición de cultivos iniciadores o la enzima DAO han permitido reducir la concentración de histamina producida naturalmente por

L. parabuchneri en los quesos de larga maduración por debajo del límite establecido por la EFSA (500 mg/kg):

- Todos los microorganismos ensayados como cultivos adjuntos a la leche para la elaboración de queso (*L. casei* 4a y 18b, *L. bulgaricus* CECT 4005 *y S. thermophilus* CECT 7207, dos iniciadores comerciales de yogur y *D. hansenii*) consiguieron disminuir la concentración de histamina a lo largo de los 100 días de maduración del queso.
- La mayor reducción de histamina se consiguió mediante la adición de las cepas *L. bulgaricus* CECT 4005 y *S. thermophilus* CECT 7207, *L. casei* 4a y 18b, así como de *D. hansenii*, consiguiendo una reducción de aproximadamente un 42%, 43% y 45%, respectivamente.
- La mayor reducción de histamina causada por la adición de la enzima DAO se obtuvo a los 30 días de maduración, alcanzando hasta un 30% de disminución con respecto al lote control positivo. Sin embargo, esta medida no resultó tan efectiva para tiempos de maduración más largos.
- La adición de los cultivos microbianos modificó sensiblemente el olor de los quesos elaborados, aunque este olor no fue percibido por los catadores como desagradable. En este sentido el aroma del queso con *L. casei* 4a y 18b (una de las más medidas efectivas) era el más parecido al aroma del lote control.
- Por tanto, se considera que, de modo general, el uso de microorganismos potencialmente degradadores de histamina y DAO añadido directamente a la matriz de leche durante el proceso de elaboración del queso puede aceptarse como una estrategia eficaz para limitar la producción de histamina en quesos de maduración larga.

6. Bibliografía

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Anexo

Factor de impacto de las revistas y áreas temáticas

Manuscrito I. Moniente, M.; Botello-Morte, L.; García-Gonzalo, D.; Pagán, R.; Ontañón, I. (2022). Analytical strategies for the determination of biogenic amines in dairy products. *Comprehensive Reviews in Food Science and Food Safety*, *21*(4), 3612-3646. https://doi.org/10.1111/1541-4337.12980

Manuscrito II. Moniente, M.; García-Gonzalo, D.; Ontañón, I.; Pagán, R.; Botello-Morte, L. (2021). Histamine accumulation in dairy products: Microbial causes, techniques for the detection of histamine-producing microbiota, and potential solutions. *Comprehensive Reviews in Food Science and Food Safety*, *20*(2), 1481–1523. https://doi.org/10.1111/1541-4337.12704

Factor de impacto (2021): 15.786 (Food Science & Technology).

Manuscrito III. Moniente, M.; García-Gonzalo; D. Botello-Morte; L. Ferreira, V.; Pagán; R. Ontañón; I. Combination of SPE and fluorescent detection of AQC-derivatives for the determination at sub-mg/L levels of biogenic amines in dairy products. *Food Research International (en revisión).*

Factor de impacto (2021): 7.425 (Food Science & Technology).

Manuscrito IV. Botello-Morte, L.; Moniente, M.; Gil-Ramírez, Y.; Virto, R.; García-Gonzalo, D.; Pagán, R. (2021). Identification by means of molecular tools of the microbiota responsible for the formation of histamine accumulated in commercial cheeses in Spain. *Food Control 133*, 108595. https://doi.org/10.1016/j.foodcont.2021.108595

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