Food Research International 160 (2022) 111735

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

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

Marta Moniente [a], Diego García-Gonzalo [a], Mª Goretti Llamas-Arriba [c], Raquel Virto [c], Ignacio Ontañón [b], Rafael Pagán [a], Laura Botello-Morte [a,*]

[a] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain
[b] Laboratorio de Análisis del Aroma y Enología. Química Analítica. Facultad de Ciencias. Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain
[c] CNTA, Centro Nacional de Tecnología y Seguridad Alimentaria, San Adrián, Spain

ARTICLE INFO

Keywords:
Histamine
Histamine degradation
Long-ripening cheese
HPLC
DAO
Starter culture

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.

* Corresponding autor at: Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), C/ Miguel Servet 177, 50013 Zaragoza, Spain.
E-mail address: lbotello@unizar.es (L. Botello-Morte).

https://doi.org/10.1016/j.foodres.2022.111735
Received 22 February 2022; Received in revised form 12 July 2022; Accepted 20 July 2022
Available online 22 July 2022

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

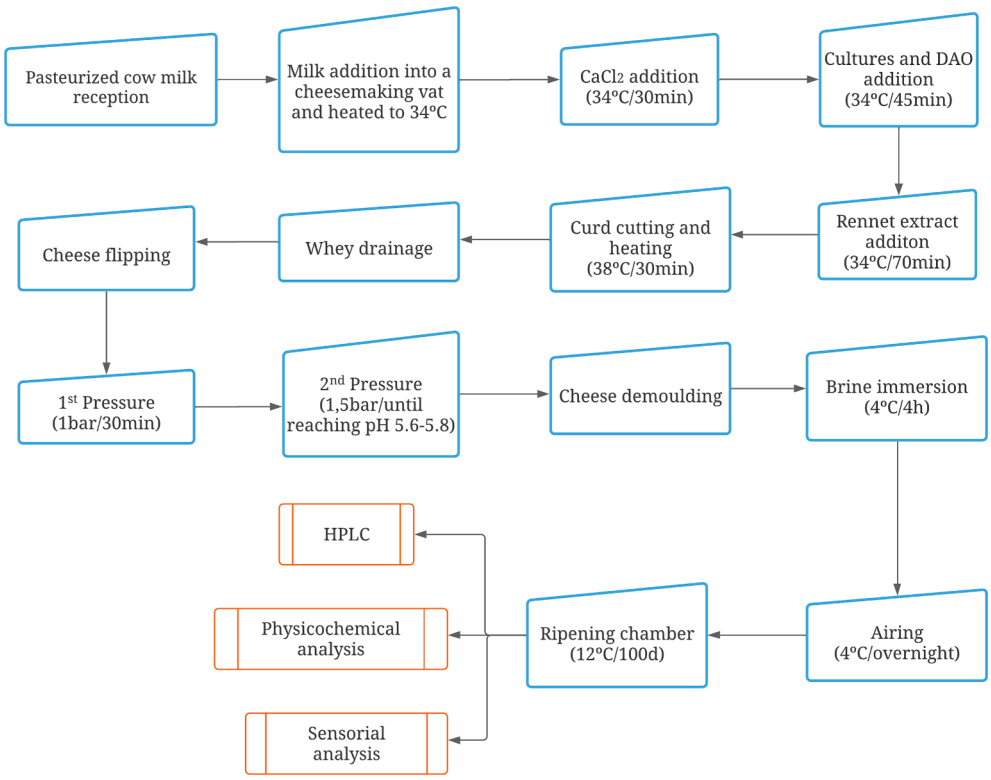

Fig. 1. Flow diagram of the different steps of the cheese manufacturing process (in blue) with the F-DVS-GRANA-102 cheese starter from CHR Hansen and *Lentilactobacillus parabuchneri* DSM 5987 as a histamine-producing microorganism, combined with DAO or histamine-degrading microorganisms. The analyses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

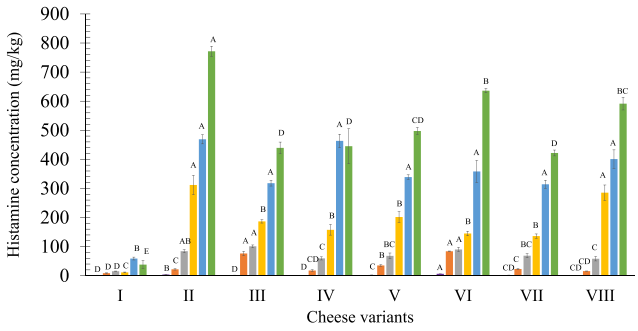

Fig. 2. Histamine concentration (mg/kg) in cheese variants (I to VIII) along the cheese ripening period: 0 days (purple), 15 days (orange), 30 days (grey), 45 days (yellow), 60 days (blue), and 100 days (green). Cheese variants: (I) control cheese, (II) *Lentilactobacillus parabuchneri* DSM 5987, (III) *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

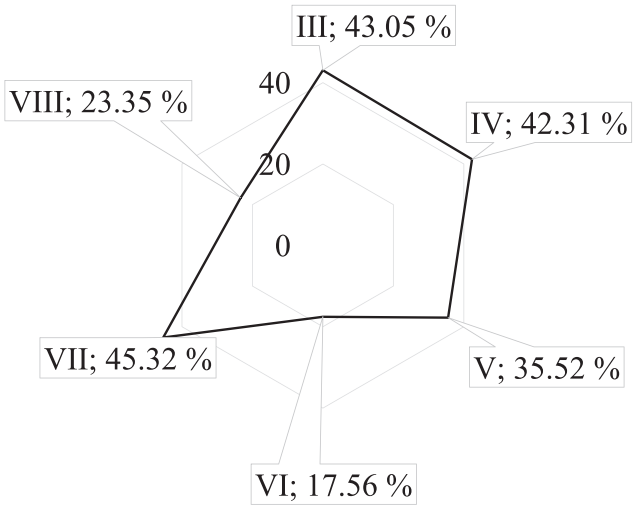

Fig. 3. Percentage (%) of histamine reduction by the effect of DAO or histamine-degrading strains on different cheese variants (III-VIII) with respect to control cheese variant II at 100 days of ripening time: (III) *Lentilactobacillus parabuchneri* DSM 5987 and *Lacticaseibacillus casei* strains (4a 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.

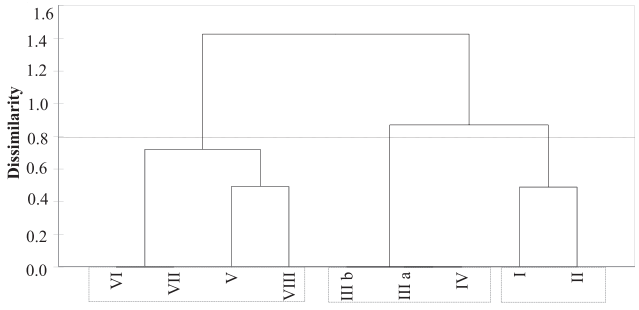

Fig. 4. Hierarchical Cluster Analysis (HCA) calculated on data from the sorting task performed on 100-day cheeses. Cheese variants: (I) control cheese, (II) *Lentilactobacillus parabuchneri* DSM 5987, (III) *L. parabuchneri* DSM 5987 and *Lacticaseibacillus casei* strains (4a and 18b), (IV) *L. parabuchneri* 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.

Funding.

This study was financially supported by "FEDER/Ministerio de Ciencia, Innovación y Universidades – Agencia Estatal de Investigación/ _ Proyecto RTC-2017–6275-2", and by the Aragonese Office of Science, Technology and University Research.

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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