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Characterization of autochthonal *Hafnia* spp. strains isolated from Spanish soft raw ewe's milk PDO cheeses to be used as adjunct culture

Almudena V. Merchán [a,b], Santiago Ruiz-Moyano [a,b,*], María Vázquez Hernández [a,b], Alberto Martín [a,b], María Jesús Lorenzo [c], María José Benito [a,b]

[a] Departamento de Producción Animal y Ciencia de los Alimentos, Nutrición y Bromatología, Escuela de Ingenierías Agrarias, Universidad de Extremadura, Avda. Adolfo Suárez s/n, 06007 Badajoz, Spain

[b] Instituto Universitario de Investigación de Recursos Agrarios (INURA), Universidad de Extremadura, Avda. de la Investigación s/n, Campus Universitario, 06006 Badajoz, Spain

[c] Departamento de Bioquímica, Biología Molecular y Genética, Facultad de Medicina y Ciencias de la Salud, Universidad de Extremadura, Avda. de Elvas s/n, Campus Universitario, 06006 Badajoz, Spain

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ABSTRACT

The present work was performed to study the enterobacteria involved in the ripening of the artisanal raw ewe's milk PDO cheeses 'Torta del Casar' and 'Queso de la Serena' produced in Extremadura (Spain). These isolates were strain-typed, safety tested and characterized for some important technological properties. A total of 485 enterobacterial isolates were clustered by RAPD-PCR and subsequently identified by partial sequencing of the 16S rRNA gene. Among the 17 different species identified, *Hafnia paralvei* was the predominant species; *H. alvei* and *Lelliottia amnigena* were present to a lesser extent. Therefore, 55 *Hafnia* spp. strains, selected according to their genetic profile and dairy origin, were tested for the safe application. Overall, they were able to produce the biogenic amines putrescine and cadaverine under favourable conditions, presented α-haemolytic activity and did not produce cytolytic toxin active against HeLa cells or contain virulence genes. In addition, antibiotic susceptibility profiles showed that 17 *Hafnia* spp. strains were less resistant to the 33 antibiotics tested; subsequently, they were further technologically characterized. Although they showed differences, in general, they were well adapted to the stress conditions of cheese ripening. Among them, two strains, *H. alvei* 544 and 1142, are highlighted mainly due to their proteolytic activity at refrigeration temperatures and their low or null gas production. Although further studies are necessary before industrial application, these two strains are proposed for potential use as adjunct cultures to favour the homogeneity of these PDO cheeses, preserving their unique sensory characteristics.

1. Introduction

'Torta del Casar' and 'Queso de la Serena' are soft cheeses produced in Extremadura (Spain) under Protected Designation of Origin (PDO) using raw ewe's milk and an aqueous infusion of the dried flowers from the plant *Cynara cardunculus* L. as rennet. These traditional cheeses are made without the addition of a starter culture or thermal treatment during their processing, which lead to a diverse and heterogeneous microbiota during their ripening. This is mainly composed of lactic acid bacteria (LAB) and, to a lesser extent, Gram-positive catalase-positive bacteria, diverse Gram-negative bacteria (*Pseudomonas* spp., *Enterobacteriaceae*) and yeasts (Fernandez Del Pozo et al., 1988; Ordiales et al., 2013a; Sánchez-Juanes et al., 2020). The distinctive and complex flavour and spreadable texture of these types of cheeses are a consequence of biochemical events that take place during ripening, resulting from the high proteolytic activity from vegetable rennet and autochthonous microbiota (Delgado et al., 2010a, 2010b; Ordiales et al., 2013b, 2014). However, although the hygiene of the cheesemaking process for these traditional PDO cheeses has improved in the last decades, the use of raw milk does not guarantee the absence of spoilage and pathogenic microorganisms, such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus*, with the potential

* Corresponding author at: Departamento de Producción Animal y Ciencia de los Alimentos, Nutrición y Bromatología, Escuela de Ingenierías Agrarias, Universidad de Extremadura, Avda. Adolfo Suárez s/n, 06007 Badajoz, Spain.
E-mail address: srmsh@unex.es (S. Ruiz-Moyano).

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for safety risks and economic losses (Yoon et al., 2016). In addition to the lack of standardization of the vegetable rennet and raw milk, variations in the cheesemaking process between dairies have a great impact on sensory characteristics and lead to variability and heterogeneity of the final product (Montel et al., 2014).

Today, although the regulations for these PDO cheeses do not allow the use of commercial starter cultures, the implementation of autochthonous microorganisms as a starter culture may constitute a possible way to ensure adequate processing, reducing variability as well as keeping their unique sensory characteristics and minimizing safety risks (Araújo-Rodrigues et al., 2020; Montel et al., 2014). This approach has been demonstrated to be suitable for generating sensory characteristics in cheeses manufactured with pasteurized milk that are similar to those of raw milk cheeses (Centeno et al., 2017; Vázquez-Velázquez et al., 2018). In addition to guaranteeing the homogeneity and safety of these PDO cheeses and retaining their authenticity, the application of an autochthonous starter culture could also help to improve their competitiveness in national and international dairy markets, with the consequent economic benefit for the sector.

This autochthonous starter culture has to be composed of a mixture of microorganisms isolated from the original product, representative of the main microbial groups involved in the ripening of these PDO cheeses. In addition, it has to be safe for food application, not be able to cause spoilage in the product and possess desirable technological properties such as lipolytic and proteolytic activity associated with the development of the proper aroma and texture (Leroy and De Vuyst, 2004; Pereira et al., 2020).

LAB have been widely used as starter cultures in cheeses and many studies have been developed in order to select LAB for soft cheese production (Araújo-Rodrigues et al., 2021; Campagnollo et al., 2018). However, there are not many research studies available regarding the selection of secondary microbiota such as *Enterobacteriaceae*. Gram-negative bacteria are quite common in traditional cheeses and constitute a reservoir of enzymes that favour their maturation (Morales et al., 2003, 2005), contributing to the sensory characteristics of the final product.

Among the enterobacteria, *Hafnia* spp. have been described as tolerant to refrigeration conditions, consequently growing during cheese processing and storage (Ridell and Korkeala, 1997). In addition, it has been proven by many researchers that *Hafnia* spp. are predominant at the end of the ripening process of traditional cheeses (Gonçalves et al., 2018; Irlinger et al., 2012; Ordiales et al., 2013a). *Hafnia alvei* and *H. paralvei* are able to produce flavour compounds (Morales et al., 2004) but can also lead to stronger proteolysis, modifying the final texture of the cheese and improving its flavour by producing aromatic sulphur compounds (Irlinger et al., 2012; Morales et al., 2003). This species is the only Gram-negative bacterium used as a commercial ripening adjunct culture for cheesemaking (Bourdichon et al., 2012). According to Delbès-Paus et al. (2013), *H. alvei* appears to be a promising species for reducing the growth of *E. coli* O26:H11 and controlling the growth of antibiotic-resistant *Enterococcus faecalis* in cheese. More recently, D'Amico et al. (2021) have reported the capacity of this species to reduce the content of pathogenic microorganisms such as *Staphylococcus aureus* and *Salmonella enterica* in food materials. However, it is considered as an opportunistic pathogen of humans and an indicator of poor hygiene, being isolated from spoiled food (Tan et al., 2014), and there are even some sporadic cases in soft cheese of early blowing due to gas production (Tabla et al., 2016). Therefore, it is essential to develop serious identification and characterization studies to understand the real impact of *Hafnia* spp. on cheese quality. They would allow the selection of technologically suitable *Hafnia* spp. strains adapted to the ripening conditions, avoiding potential pathogenicity and spoilage due to gas production, amine production, antibiotic resistance and cytotoxicity (Coton et al., 2012). In this context, the aim of this study was to identify the autochthonous enterobacterial microbiota present in artisanal soft raw ewe's milk PDO cheeses produced in Extremadura (Spain) in order to study their safety and technological characteristics for their application in cheesemaking as a member of an autochthonal starter culture.

2. Materials and methods

2.1. Cheese sampling and bacterial counts

Samples were taken during cheese ripening at 0, 20, 40 and 60 days (final product) from three dairies belonging to PDO 'Queso de la Serena' and another three from PDO 'Torta del Casar' in two different seasons (winter and spring). Three cheese units from each dairy were randomly taken and transported under refrigerated conditions to the laboratory. Each microbiological analysis was performed in three different cheeses by dairy and sample time, each determination being made in duplicate.

Mesophilic aerobic bacteria and enterobacteria were counted on plate count agar (PCA; Condalab, Madrid, Spain) and on violet red bile glucose (VRBG; Condalab) agar, respectively, as previously described by Gonçalves et al. (2018). For isolating enterobacteria, ten colonies from each VRBG agar plate containing the highest dilutions were randomly selected and streaked onto brain heart infusion (BHI; Condalab) agar plates. Finally, pure isolates were grown in 5 mL of BHI broth (Condalab) under aerobic and shaking conditions at 30 °C for 24 h and stored at −80 °C in 25% glycerol.

2.2. Identification of enterobacteria

The enterobacterial colonies isolated were identified by randomly amplified polymorphic DNA–polymerase chain reaction fingerprint analysis (RAPD-PCR) and subsequent rRNA 16S gene sequencing analysis. To get the genomic DNA, 1 mL of each bacterial culture was collected by centrifugation at 10,000*g* for 5 min. The bacterial pellet was suspended in lysis buffer and disrupted with 400–600 μm silica grinding beads in a 1600 MiniG homogenizer (SPEX SamplePrep, Metuchen, NJ, USA) at 1500 rpm for 2 min. Next, the DNA was extracted using a GeneJET Genomic DNA Purification Kit following the manufacturer's instructions (Thermo Fisher Scientific, USA). The quality and concentration of DNA were determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific). The final DNA concentration was adjusted to 10 ng/μL for PCR reactions.

RAPD-PCR was carried out using the M13 minisatellite core sequence (5′-GAGGGTGGCGGCTCT-3′) as described by Huey and Hall (1989). The resulting fragments were separated on 1.5% agarose gels, and PCR product sizes were estimated by comparison with a GeneRuler 100 bp plus DNA Ladder (Thermo Fisher Scientific) using image analysis software (GeneTools, SynGene, Cambridge, United Kingdom). The fragment profiles obtained from isolates were grouped into operational taxonomic units (OTUs) using the unweighted pair group method with arithmetic average (UPGMA). The analysis was performed using the NTSYS.PC package version 2.0 (Rohlf, 1993).

Two representative isolates of each OTU were identified at species level by sequencing the 16S rRNA gene using 337F (5′-GACTCC-TACGGGAGGCWGCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers targeting V3–V9 regions. The PCR products were purified and sequenced as described by Gonçalves Dos Santos et al. (2017). To differentiate between *Hafnia* spp. isolates at species level, phylogenetic analysis based on 16S rRNA sequences from *Hafnia* spp. OTUs was performed using MEGA (Molecular Evolutionary Genetic Analysis) software version X. The 16S rRNA sequences from GenBank of *H. alvei* ATCC 13337 (accession number NR_044729.2) and *H. paralvei* ATCC 29927 (accession number NR_116898.1) were used as references.

2.3. Safety tests

H. alvei and *H. paralvei* isolates from different OTUs or belonging to the same OTU but originating from a different dairy were selected for safety characterization (Table S1). Prior to the safety and technological

assays, all selected *Hafnia* spp. isolates were subcultivated twice in BHI broth (Condalab) at 30 °C for 24 h, washed twice with phosphate-buffered saline (PBS) at pH 7.2 (Thermo Fisher Scientific) and their turbidity adjusted with PBS to a 0.5 McFarland standard. All experiments were carried out at least twice on separates days in triplicate.

2.3.1. Determination of antibiotic susceptibility and resistance, and virulence-associated genes

Antibiotic susceptibility testing was performed by disk diffusion assay in Mueller–Hinton (MH) agar (Condalab) against 33 antibiotics (BD, New Jersey, USA; Table S2) according to the guidelines for *Enterobacteriaceae* of the Clinical and Laboratory Standards Institute (CLSI) (2019) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2020). Sterile discs were placed on the surface of inoculated plates and incubated at 30 °C for 18 h. The diameters of the inhibition zones were measured and interpreted according to guidelines (CSLI, 2019). *E. coli* ATCC 25922 was used as a control. In addition, *Hafnia* spp. isolates were tested for the presence of $bla_{ACC\text{-}1}$ and $bla_{ACC\text{-}3}$ for β-lactamase genes. $bla_{ACC\text{-}1}$ was detected according to the primers and conditions proposed by Hasman et al. (2005), whereas the internal portion of $bla_{ACC\text{-}3}$ (~826 bp) was amplified using the primers acc-3F (5′-AGCCGCTGATGCAGAAGAAT-3′) and acc-3R (5′-ATCGCCA-TATCGTTGCCAGT-3′) designed in this study from the AMPC cephalosporinase precursor protein ACC-3 (acc-3) gene sequence of *H. alvei* available in the NCBI database (accession number AF180958.1). PCR amplification mixtures were subjected to 2 min of denaturation at 95 °C; 30 cycles at 94 °C for 45 s, 61 °C for 45 s and 72 °C for 1 min; followed by a final extension period of 10 min at 72 °C and refrigeration. To determine colistin resistance, a multiplex PCR for the detection of *mcr* genes (*mcr-1* to *mcr-5*) was carried out following the protocol described by Rebelo et al. (2018). Detection of additional *mcr* genes, *mcr-6.1*, *mcr-7.1* and *mcr-8*, was performed by the methods described by AbuOun et al. (2017), Yang et al. (2018) and Wang et al. (2018), respectively.

Related to toxin genes, the selected *Hafnia* spp. isolates were also tested for the presence of *eaeA* (attaching and effacing gene) by PCR using the primers C1E and C2E and conditions suggested by Ismaili et al. (1996). The reference strain *E. coli* CECT 4267 was used as a positive control and *Staphylococcus aureus* CECT 469 as a negative control. Cytolethal distending toxin (CDT) toxin was determined by primers for the *cdtB* subunit as previously described by Bang et al. (2001).

2.3.2. Biogenic amine production

The capacity of selected *Hafnia* spp. to produce biogenic amines was determined following the methodology described by Bover-Cid and Holzapfel (1999) with modifications. Selected *Hafnia* spp. isolates were grown in standard nutrient broth (Condalab, Spain) supplemented with 0.1% of the biogenic amine precursor amino acids and 0.005% pyridoxal-5-phosphate at 30 °C for 24 h, and subcultured five times. Subsequently, they were cultivated in the improved decarboxylase medium without agar containing 0.25% of each precursor amino acid. After incubation at 30 °C for 4 days, the amount and type of biogenic amines produced (tyramine, tryptamine, histamine, putrescine, phenylethylamine, spermine, spermidine and cadaverine; Sigma Chemical Co. St Louis, MO, USA) were measured by HLPC-ESI-MS-according to the method described by Ruiz-Moyano et al. (2019).

2.3.3. Haemolytic activity

Haemolysis was determined in blood agar (Condalab) supplemented with 5% defibrinated sheep blood (v/v) (Oxoid, Basingstoke, United Kingdom) plates incubated at 30 °C for 24 h after inoculation. The haemolytic reaction was recorded by observation of the partial hydrolysis of red blood cells and a green zone (α-haemolysis), a clear zone around bacterial growth (β-haemolysis) or no reaction (γ-haemolysis). *Listeria monocytogenes* CECT 911 and *Lactobacillus casei* HL233 (Ruiz-Moyano et al., 2009) were used as positive controls of β-haemolysis and α-haemolysis, respectively.

2.3.4. Cytotoxic activity of Hafnia isolates

2.3.4.1. Preparation of toxin extract. Toxin induction of selected *Hafnia* spp. isolates was conducted with mitomycin C (Thermo Fisher Scientific) as previously described by To and Bhunia (2019). *E. coli* CECT 4267 and CECT 4782, Shiga toxin-positive strains, and *E. coli* UEX 3193, a non-Shiga toxin strain, were used as positive and negative controls of activity, respectively. Briefly, all bacteria were grown in 10 mL of trypticase soy broth (TSB; Condalab) at 37 °C for 18 h in an orbital shaker set at 150 rpm. After that, the cultures were diluted to 1:50 in 10 mL of Luria Bertani broth (LB; Condalab) and incubated for 3 h at 37 °C with shaking at 150 rpm before the addition of mitomycin C (2 μg/mL), followed by incubation for 5 h at 37 °C with shaking at 150 rpm. The supernatants of all cultures were obtained by centrifugation at 10,000*g* for 5 min, sterilized by filtration through 0.22 μm filters (Thermo Fisher Scientific) and stored at −80 °C until used.

2.3.4.2. MTT assay. Human HeLa cervical epithelial cells were seeded at a density of 8×10^3 cells per well in a 96-well flat-bottomed microtitre plate in high glucose, pyruvate, no glutamine Dulbecco's Modified Eagle's Medium (DMEM; Gibco-Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-Thermo Fisher Scientific), 1% L-glutamine (200 mM; Gibco-Thermo Fisher Scientific) and antibiotics including 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco-Thermo Fisher Scientific). After incubation for 24 h at 37 °C in an atmosphere containing 5% CO_2, the medium was replaced with new medium without antibiotic and supplemented with toxin extracts at 1:4 dilution. Cell survival was determined by the microculture tetrazolium test (MTT) 24 h after addition of the supernatants according to Fernández et al. (2016). Cell viability was expressed as the mean percentage of treated cells with respect to the untreated positive control. Cells treated with LB broth served as a control for medium activity. MTT assays were carried out in triplicate.

2.4. Characterization of technological properties

H. alvei and *H. paralvei* isolates pre-selected based on their safety characteristics were used for screening their technological capacity. All assays were carried out at least twice on separate days in triplicate.

2.4.1. Evaluation of growth under cheese ripening conditions

A Box–Behnken experimental design (BBD) with three factors, temperature, salt concentration and pH, was applied for modelling their influence on the growth of the *Hafnia* spp. isolates. The factors were set up as shown in Table S3 in order to cover the conditions of the ripening process of the soft cheeses 'Torta del Casar' and 'Queso de la Serena' (Martínez et al., 2011). Yeast nitrogen base (YNB; Condalab) broth supplemented with 1% (w/v) glucose was used for the experiment. In 96-well plates, 200 μL of YNB for each pH and NaCl concentration condition was inoculated with a suspension of each *Hafnia* spp. strain (1% v/v) and incubated for 4 days at different temperatures (Table S3). The growth capacity of each strain was evaluated by turbidimetry, measuring the absorbance at 570 nm with a Fluostar Optima microplate reader (BMG LABTECH, Offenburg, Germany). The time to detection (TTD) of growth for $OD_{570nm} = 0.4$ for each well was calculated using a Microsoft Excel template (kindly provided by Dr. R. Lambert), which used linear interpolation between successive OD readings (Medina et al., 2012). The response variable considered in the model was the inverse of TTD to reach 0.4 OD_{570nm} for each strain.

2.4.2. Extracellular proteolytic and lipolytic activity

To determine proteolytic activity, 10 μL of each strain was spotted into 5% skimmed milk–agar medium and incubated at 8, 15 or 30 °C for 6 days. A result was considered positive when there was a clear zone around the colony. This clear area was measured to evaluate the intensity of the activity.

Lipolytic activity was evaluated by spotting 10 μL of each strain onto 5% pork fat–agar (pH 7) and incubated at 8, 15 or 30 °C for 6 days, assessing the activity every 2 days, as reported by Chaves-López et al. (2006). Lipolysis was evidenced by a green-blue halo around colonies, 15 min after pouring 10 mL of a saturated copper sulphate ($CuSO_4$) solution onto the plates. Also, lipase activity was determined by rhodamine B agar medium (Kouker and Jaeger, 1987) and esterase activity was determined by tributyrin agar (Ismail et al., 2000). Inoculated plates of both media with 10 μL of each isolate were incubated at 8, 15 or 30 °C and examined daily for 6 days.

2.4.3. Gas production

Gas production was tested by two methods described by Tabla et al. (2016) with some modifications. One hundred microlitres of each strain was inoculated into tubes containing 9 mL of reconstituted skimmed milk (10%) and sealed with 2 mL of sterile melted paraffin. Seal displacement was measured after 96 h at 30 °C. On the other hand, inverted Durham tubes were used to determine gas production from isolates, using lauryl sulphate broth inoculated with 100 μL of each culture. Gas trapped in Durham tubes was measured after 48 h at 30 °C. @@@

2.4.4. Alkalizing and acidifying activity

Alkalizing ability was determined by Simmons citrate agar (Condalab). The medium was spotted with 10 μL of each strain and incubated for 48 h at 30 °C. Alkalizing activity was observed when Simmons citrate agar turned blue around colonies. Acidifying activity was determined in sterile reconstituted skimmed milk (10%), inoculated with 100 μL of each strain and incubated at 30 °C for 48 h. The pH was measured using a Crison Basic 20 (2012) pH meter (Crison Instruments, Barcelona, Spain) at 0, 24 and 48 h. The data were expressed as variation of pH at 24 h or 48 h respect to initial pH (0 h).

2.4.5. Diacetyl/acetoin production

One hundred microlitres of each strain was inoculated in sterile reconstituted skimmed milk (10%) at 30 °C for 18 h. Acetoin production was detected by the Voges–Proskauer reaction. A volume of 2.5 mL of the bacterial suspension was added to 0.5 mL of α-naphthol (6%, w/v ethanol) and 0.2 mL of 40% sodium hydroxide. The resulting mixture was vortexed for 30 s and incubated at room temperature for 15 min. Acetoin production was detected as a red ring on the surface of the culture (VP+ phenotype). *Enterococcus cloacae* and *Proteus mirabilis* from the Department of Animal Production and Food Science (UEX) culture collection were used as positive and negative controls, respectively.

2.5. Statistical analysis

Counts of enterobacteria and mesophilic aerobic bacteria were subjected to two-way ANOVA, while cytotoxic activity and biogenic amines were evaluated by one-way ANOVA using the SPSS software package for Windows version 22.0 (SPSS, Inc., Chicago, IL, USA). In all determinations, Bonferroni adjustment was used for pairwise comparisons of group means. The statistical significance was set at $P \leq 0.05$.

On the other hand, as shown in Table S3, a three-factor, three-level BBD combined with surface response methodology (SRM) was applied to determine the effects of medium conditions on the growth of safety-selected *Hafnia* spp. isolates. SRM was performed employing StatGraphics Centurion XVI software version 8.0. The quadratic model was as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where Y is the response variable (inverse of the growth time) predicted by the model; β_0 is an offset value; β_1, β_2 and β_3 are the regression coefficients for the main (linear) terms; β_{11}, β_{22} and β_{33} are quadratic effects; β_{12}, β_{13} and β_{23} are interaction effects; and X_1, X_2 and X_3 are the independent variables. The models were used to estimate the growth time of each isolate under 'Torta del Casar' and 'Queso de la Serena' ripening conditions (Table S3). The software also generated ANOVA, establishing statistical significance at the 95% confidence level. The optimal growth time for each variable analysed was also obtained with the same statistical program.

In addition, hierarchical cluster analysis (HCA) was used to group *Hafnia* spp. according to antibiotic resistance, growth conditions and technological aptitude, using SPSS software for Windows 21.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Bacterial counts and identification

The aerobic mesophilic bacterial count in the cheese samples on day 0 was between 6.2 and 7.3 log cfu/g (Fig. 1A). Maximum levels were detected at 20 days of ripening, reaching around 9 log cfu/g, after which they decreased slightly until 60 days to final values between 8.5 and 8.8 log cfu/g. No significant differences between PDO and season were observed at the end of ripening (Fig. 1A).

Enterobacterial counts at the beginning of ripening were between 5.1 and 6 log cfu/g; maximum counts of around 7 log cfu/g were reached at 20 days, and then counts dropped significantly at the end of ripening to around 6 or 5 log cfu/g depending on the PDO and season (Fig. 1B). Interestingly, the winter season showed significantly lower counts at 40 days for both PDO; however, this difference was retained at 60 days only for the Serena PDO (Fig. 1B).

A total of 485 isolates of enterobacteria were obtained, 273 isolates from PDO 'Torta del Casar' and 212 isolates from PDO 'Queso de la Serena'. The isolates were clustered in 54 different profiles by the RAPD-PCR technique using the M13 minisatellite. Subsequent partial 16S rRNA sequencing of members from each cluster allowed the identification of seventeen different species in both PDO cheeses throughout ripening (Fig. 2). Fig. 2A shows the microbiological diversity of enterobacteria in 'Torta del Casar' cheeses at the beginning of the ripening process: there were 15 different species, dominated by the genus *Enterobacter* accounting for 54% of the identifications. After 20 days, the predominant species were *H. alvei* and *H. paralvei*, accounting for 90.9% of the isolates at 20 days, and then decreasing to 70.8% at 40 days and 69% at 60 days. Fig. 2B shows the results for the PDO 'Queso de la Serena'. Likewise, a great diversity in the enterobacterial population was observed at the beginning of the ripening process: there were 12 different species, with the genus *Enterobacter* dominating again, accounting for 37% of the identifications. After 20 days, *H. alvei* and *H. paralvei* were the dominant species, reaching 66.7% at 20 days, 100% at 40 days and 95.7% at 60 days.

Therefore, the predominance of the enterobacterial species *H. alvei* and *H. paralvei* in these PDO cheeses made it essential to perform a phylogenetic analysis to discriminate the isolates at the strain level for their technological characterization. Fig. 3 shows the genotyping carried out with the primer M13; a total of 11 different profiles were obtained, of which 6 are for *H. alvei* and 5 for *H. paralvei*. *H. alvei* profiles had between one and five bands, with the P1 profile having the highest number of bands. However, those of *H. paralvei* had between one and four bands, with the P10 and P11 profiles having the highest number of bands. The molecular weight of the bands ranged between 130 and 1341 bp for *H. alvei*, with the P5 profile having the biggest band size. For *H. paralvei*, the fragment sizes were between 310 and 1715 bp, and both were found in profile P9. Using RAPD-PCR with the M13 primer allowed us to discriminate between *Hafnia* species and also between different strains within the same species, which was essential for a proper selection of strains for further safety and technological analysis. A total of 55 *Hafnia* spp. strains were selected for these issues according to their genetic profile and dairy of origin (Table S1). A total of 19*H. alvei* strains

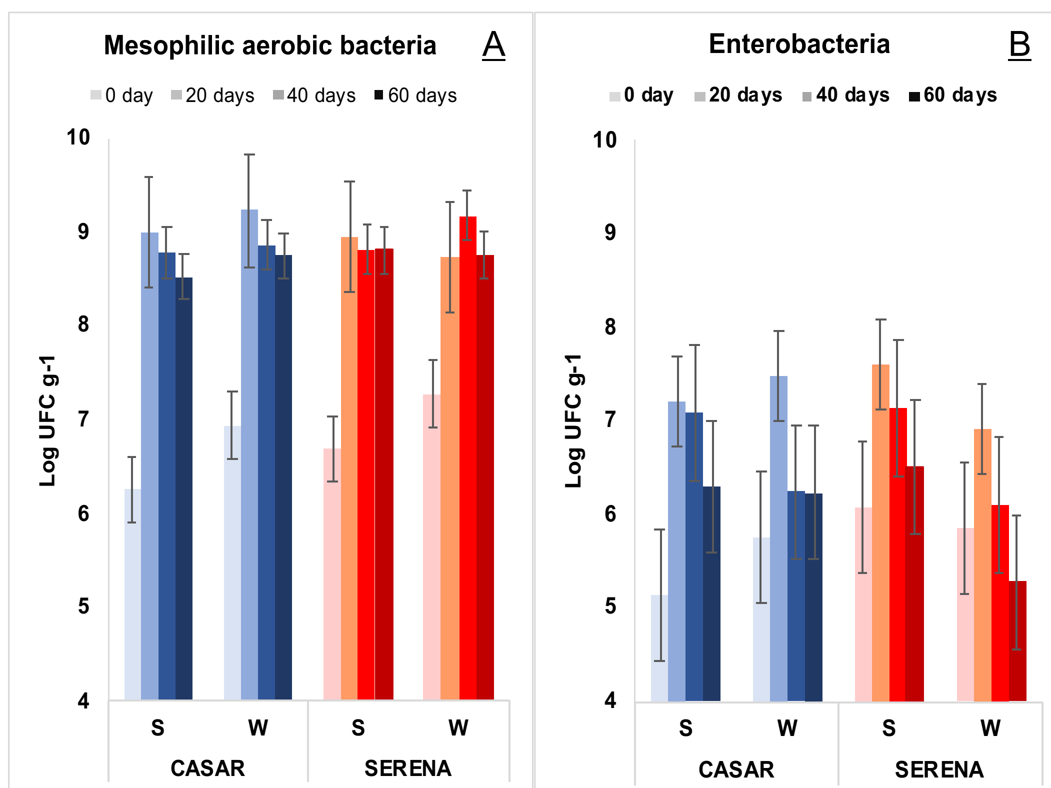

Fig. 1. Development of mesophilic aerobic bacterial (A) and enterobacterial (B) counts in PDO 'Torta del Casar' (Casar) and 'Queso de la Serena' (Serena) soft cheese from two seasons (winter (W) and spring (S)) throughout ripening (0, 20, 40 and 60 days). Results are expressed as mean of log cfu/g ± error bar. Error bars reflect the 95% confidence intervals of the differences, adjusted for multiple comparisons using Bonferroni correction.

and 36*H. paralvei* strains were selected, 26 from 'Torta del Casar' and 29 from 'Queso de la Serena' cheeses.

3.2. Safety tests

The selected *H. alvei* and *H. paralvei* strains were analysed for safety properties: antibiotic susceptibility, presence of virulence genes, biogenic amine production, haemolytic activity and cytotoxic activity.

The antibiotic susceptibility profile was performed by the disk diffusion method for 33 antibiotics belonging to 11 antibiotic families. Tables S4 and S5 show the profile of antibiotic susceptibility obtained for each *H. alvei* and *H. paralvei* strain, respectively. The results show that antibiotic resistance was strain-specific and there was a wide variability among the strains studied. The general susceptibility response of the *H. alvei* and *H. paralvei* species to the antibiotics tested is presented in Table 1. The results show that over 80% of the strains were resistant to the following six antibiotics, AMC (β-lactam), the cephalosporins CTX, CEP, CXM and CFZ (cephems) and sulphisoxazole (G). A high percentage of *H. paralvei* strains were also resistant to ampicillin (AMP), ampicillin-sulbactam (SAM) and doripenem (DOR) while in the case of *H. alvei* it was slightly lower at around 70% of the strains. In addition, between 50% and 80% of the strains were resistant to the antibiotics PTZ (β-lactam), ATM (monobactam), PIP (penicillin), CAZ (cephem), IMP, MER and ETP (penems) and AMK (aminoglycoside). On the contrary, for the remaining 16 antibiotics tested (β-lactam: TCL; cephems: FOX, FEP, CTT; aminoglycoside: GM, NN; quinolones: NA, CIP, NOR, LOM, LEV; tetracycline: TE; phenicol: C; nitrofuran: F; folate pathway inhibitors: SXT, W), less than 42% of strains were resistant. In particular, *Hafnia* strains were highly susceptible to seven antibiotics: CTT, NOR, LOM, LEV, F, SXT and W, with less than 11% of strains being resistant.

Table 2 shows the HCA based on the antibiotic susceptibility data of 55 *Hafnia* spp. strains. Cluster 1 and cluster 2 composed of 36 and 2 strains, respectively, comprised the most resistant strains. Overall, resistance to 20 and 18 antibiotics was recorded for strains from clusters 1 and 2, respectively. In addition, moderate susceptibility was detected for three and five antibiotics for the strains from clusters 1 and 2, respectively. In contrast, the 17 strains grouped in clusters 3 (16 strains) and 4 (1 strain) were more sensitive to the tested antibiotics. Members of cluster 3 were characterized by resistance to the five antibiotics of group 1 and moderate susceptibility to the nine antibiotics from groups 3 and 5. Cluster 4, with only one strain, *H. alvei* 130, presented a different antibiotic susceptibility profile characterized by resistance to 11 antibiotics from groups 1, 4, 5 and 7.

In addition, *Hafnia* spp. strains were tested for the presence of different genes involved in antibiotic resistance and virulence. None of the *Hafnia* spp. strains presented the *eaeA* gene, necessary to produce epithelial lesions in the intestine, or the *cdt* gene. Regarding the presence of antibiotic resistance genes, the presence of the β-lactamase *acc-1* gene was detected in 32 out of the 36 *H. paralvei* strains, whereas the β-lactamase *acc-3* gene was found in all *Hafnia* spp. isolates. In contrast, *mcr* genes were not found in any strains.

On the other hand, the results of the haemolytic capacity assays showed that all strains showed α-haemolysis, meaning partial lysis of erythrocytes on blood agar medium. In addition, cytotoxicity assays of the *Hafnia* spp. strains were performed on the HeLa cervical cancer cell line. The results evidenced that none of the strains, except *H. paralvei* 1498, affected the viability of the HeLa cells by more than 10% (Fig. 4). After 24 h of treatment, *H. paralvei* 1498 decreased cell viability at the same level as the two toxigenic *E. coli* strains used as positive controls.

Finally, due to the potential of enterobacteria to produce biogenic amines with a potential toxicological effect on consumers, this capacity was evaluated by HPLC. The analysis revealed that all *Hafnia* strains

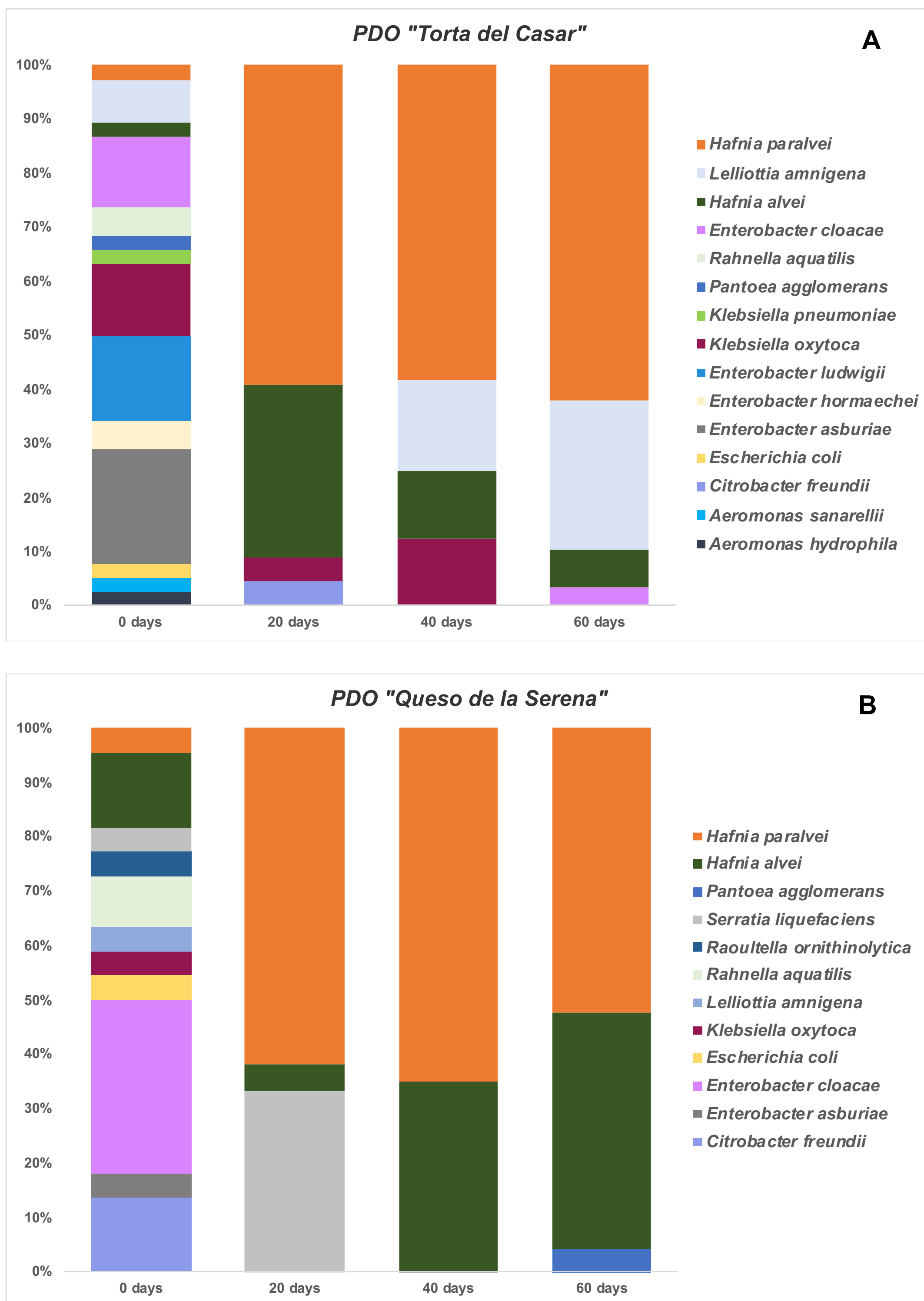

Fig. 2. Mean relative abundance of enterobacteria species in PDO 'Torta del Casar' (2A) and 'Queso de la Serena' (2B) soft cheese throughout ripening (0, 20, 40 and 60 days) using partial 16S rRNA gene sequencing of the isolates.

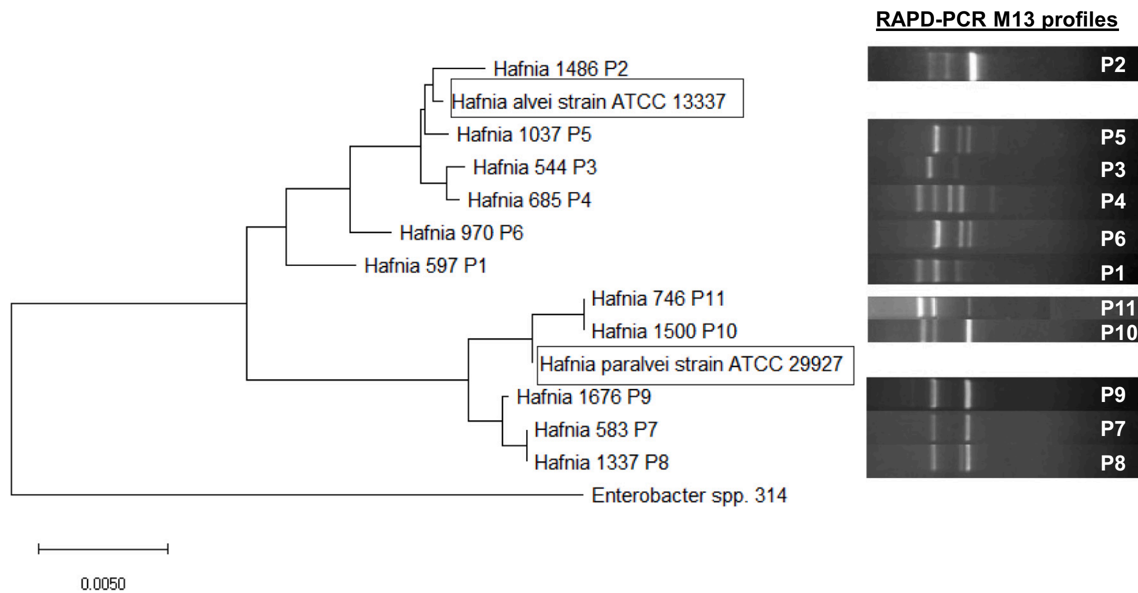

Fig. 3. Phylogenetic tree derived from comparison of 14 nucleotide sequences of the partial 16S rRNA gene. Phylogenetic analysis of a total of 822 nt of the 16S rRNA gene of 11 *Hafnia* strains, representative profiles obtained by RAPD-PCR with M13 primer and two type strains was performed using MEGA software version 10.0 applying the neighbour-joining method. The 16S rRNA gene of *Enterobacter* spp. 314 was used as an unrelated outgroup sequence. The optimal tree with the sum of branch length = 0.06368116 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are expressed as the number of base substitutions per site. The ME (minimum evolution) tree was searched using the close-neighbour-interchange (CNI) algorithm at a search level of 1.

Table 1
Percentage resistance of *Hafnia* spp. isolates to the 33 antibiotics tested. Antibiotics are grouped by class and referred to by their general agent abbreviation.

Family	Agent abbreviation	*H. alvei*	*H. paralvei*
Penicillins	AMP	68%	94%
	PIP	74%	69%
β-Lactam/β-lactamase inhibitor combinations	SAM	63%	86%
	AMC	95%	100%
	PTZ	68%	75%
	TCL	32%	22%
Monobactams	ATM	58%	69%
Cephems	CEP	100%	100%
	CFZ	100%	100%
	CXM	84%	97%
	CTX	95%	100%
	CAZ	79%	78%
	FEP	11%	17%
	CTT	0%	8%
	FOX	26%	11%
Penems	DOR	74%	86%
	IMP	68%	56%
	MER	68%	69%
	ETP	58%	56%
Aminoglycosides	AMK	63%	67%
	GM	42%	36%
	NN	32%	33%
Quinolones	NA	11%	28%
	CIP	32%	19%
	NOR	0%	0%
	LOM	0%	0%
	LEV	11%	8%
Tetracyclines	TE	32%	8%
Phenicols	C	21%	14%
Nitrofurans	F	11%	8%
Folate pathway inhibitors	G	100%	100%
	SXT	0%	0%
	W	0%	8%

cultured under favourable conditions displayed decarboxylase activity involving only the production of cadaverine and putrescine at different rates (Figs. S1 and S2). Overall, the cadaverine concentration ranged from undetectable for strain *H. alvei* 130 to 548.37 mg/L whereas putrescine concentrations ranged from 446.26 to 690.77 mg/L.

Therefore, 17 *Hafnia* spp. strains were then pre-selected based on their safety characteristics (Table S1; bold isolate code), mainly according to their antibiotic susceptibility profile and cytotoxicity capacity, for screening of their technological properties for application in cheesemaking.

3.3. Characterization of technological properties

Firstly, we studied the adaptability of the selected *Hafnia* strains to the soft cheese ripening conditions. The relatively high values for the adjusted R-squared (>75) obtained for all strains indicate that their growth patterns fitted well to the quadratic model designed. Table 3 shows the HCA based on the growth behaviour of the isolates under the cheese ripening conditions selected. Four clusters were established for the 17 strains considered. Overall, all strains displayed adequate growth under the different cheese ripening conditions; however, it was found that the five strains belonging to cluster 1 (4 *H. alvei* and 1 *H. paralvei*) had a longer growth time at stages S1, S4 and S5 of ripening. In contrast, the ten strains grouped in clusters 2 and 4 (3 *H. alvei* and 7 *H. paralvei*) presented great growth behaviour under the five cheese ripening conditions established, with a growth time of less than 2.1 days in all stages. Finally, the two strains grouped in cluster 3 (1 *H. alvei* and 1 *H. paralvei*) showed longer growth at stage 2 (8 °C; 2% NaCl; pH 4.5), characterized by acid pH, suggesting a certain sensitivity of these strains to this factor. In addition, optimal growth conditions for the three factors studied were established for selected strains. The 13 strains grouped in clusters A, B and D were characterized for optimal growth at 30 °C and pH 6, while they differed in their tolerance to salt concentration. Strain *H. alvei* 130 from cluster A showed optimal growth at a higher NaCl concentration (1.7%), followed by the strains from cluster B (3 *H. alvei* and 8 *H. paralvei*) at 1.1% NaCl and D (1 *H. paralvei*) at 1% NaCl. The four *H. alvei* strains grouped in cluster C were characterized by optimal

Table 2
Hierarchical cluster analysis performed on all antibiotic susceptibility data from 55 *Hafnia* strains. The dendrogram obtained made it possible to significantly classify the *Hafnia* strains into four groups.1, 2

Cluster[1]	Species	Strains	Antibiotic groups[2] G1	G2	G3	G4	G5	G6	G7	G8	G9
Cluster 1	*H. alvei*	595, 597, 602, 675, 1035, 1067, 1132, 1148, 1153, 1154, 1486	R	-	R	R	-	MS	-	-	R
	H. paralvei	583, 744, 746, 753, 837, 963, 965, 978, 990, 1049, 1061, 1140, 1146, 1337, 1339, 1344, 1369, 1380, 1418, 1478, 1488, 1492, 1494, 1498, 1676									
Cluster 2	*H. paralvei*	679, 718	R	-	R	R	R	MS	-	MS	-
Cluster 3	*H. alvei*	544, 683, 970, 1037, 1045, 1142, 1341	R	-	MS	-	MS	-	-	-	-
	H. paralvei	310, 561, 593, 677, 681, 1414, 1500, 1713, 1715									
Cluster 4	*H. alvei*	130	R	-	-	R	R	-	R	-	-
	H. paralvei										

Distance: 20 25

R: resistance to most antibiotics in the group; MS: moderate susceptibility to most antibiotics in the group; –: no resistance or susceptibility to most antibiotics in the group.

[1] Values in each cluster are the average of the data for the strains included in the cluster.

[2] Group 1 (G1): AMC, CFZ, CEP, G, DOR; Group 2 (G2): F, LEV, W, NOR, LOM, SXT; Group 3 (G3): CTX, CXM, AMP, PTZ, SAM, CAZ, IMP, NA; Group 4 (G4): MER, ETP, NN, ATM; Group 5 (G5): PIP; Group 6 (G6): AMK, TCL, C; Group 7 (G7): FOX; Group 8 (G8): GM, CIP; Group 9 (G9): FEP, CTT, TE.

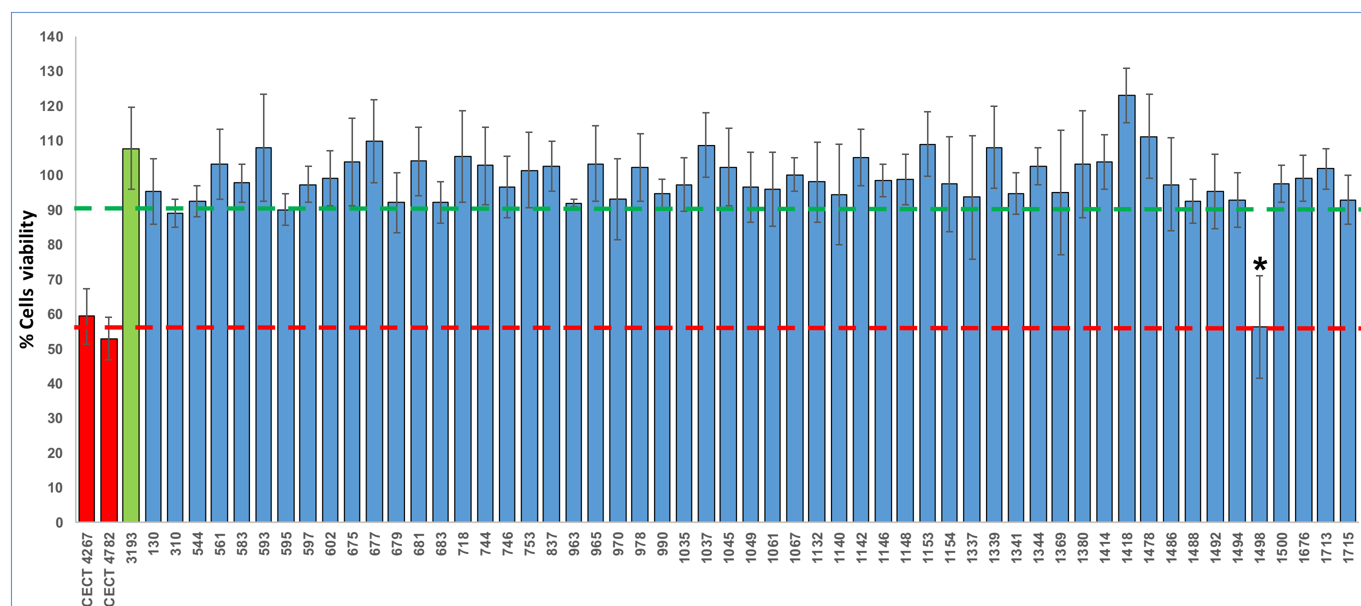

Fig. 4. Cytotoxicity activity of *Hafnia* spp. strains in Hela cells. Toxigenic *E. coli* CECT 4267 and CECT 4782 were used as positive controls (red) and non-toxigenic *E. coli* 3193 as a negative control (green). Results are expressed as the mean percentage of cell viability. Error bars indicate ±standard deviation and (*) not significantly different ($p > 0.050$) with respect to toxigenic *E. coli* positive controls.

growth at a lower temperature, 24.9 °C. The double HCA performed based on both the estimated growth of *Hafnia* spp. strains under cheese processing conditions and optimal growth conditions showed that cluster 2B comprised 7 of the 17 selected strains, while the remaining strains were widely distributed. Clusters 1C and 2C included two strains each while the remaining clusters with strains only contain one.

Subsequently, proteolytic and lipolytic activity, gas production, and the alkalinizing and acidifying capacity of the selected strains were determined. All strains were esterase- and lipase-negative at the temperatures assayed. Table 4 shows the HCA based on the rest of the technological characteristics evaluated. The strains were grouped in five clusters (P1–P5) according to the technological properties profile. Most strains, 8 out of 17 selected, were grouped in cluster P2. They were characterized by diacetyl production, alkalinizing capacity, pH-raising capacity, moderate proteolytic activity, mainly at 15 °C, and moderate gas production at 5 and 7 days (15.5 mm by Durham tube and 21.1 mm by paraffin). The three strains grouped in cluster P5 displayed similar technological characteristics, although they were characterized by better proteolytic capacity, with values ranging from 7.8 to 10.3 mm at all temperatures tested at 5 and 7 days. The strains grouped in clusters P1 and P4 did not display proteolytic capacity at 8 or 15 °C and were gas producers at different rates. The strain from cluster P1 was the greatest gas producer by paraffin assay. Finally, the strain grouped in cluster P3 showed alkalinizing capacity, pH-raising capacity and stable proteolytic capacity during the 7 days at temperatures of 15 and 30 °C, and no gas production. Therefore, four strains, *H. alvei* 544 from cluster P3 and *H. alvei* (970 and 1142) and *H. paralvei* 1414 belonging to cluster P5, showed the best characteristics, mainly due to their proteolytic activity at lower temperatures and gas production capacity.

Table 5 shows the specific data of technological characteristics corresponding to the strains grouped in clusters P3 and P5. The four strains presented relevant growth at the cheese processing stages, with a growth

Table 3
Clustering of selected *Hafnia* spp. strains based on the days of growth under cheese ripening conditions and at optimal growth conditions calculated by SRM model. Cheese ripening stages: S1 (8 °C; 1% NaCl; pH 6), S2 (8 °C; 2% NaCl; pH 4.5), S3 (10 °C; 3% NaCl; pH 5), S4 (12 °C; 3% NaCl; pH 5.5), S5 (12 °C; 3% NaCl; pH 5.9).

Cheese ripening stages (days of growth)[1]						*Hafnia* spp. optimal growth conditions[1]								Total
					Temp (°C)	30.0		30.0		24.9		30.0		
					NaCl (%)	1.7		1.1		1.0		1.0		
					pH	6.0		6.0		6.0		6.0		
					Clusters	Cluster_A		Cluster_B		Cluster_C		Cluster_D		
S1	S2	S3	S4	S5		Ha[2]	Hp[2]	Ha	Hp	Ha	Hp	Ha	Hp	
>7	0.2	2.0	>7	4.1	Cluster_1[3]	1		1	1	2				5
2.1	0.3	0.6	0.7	0.6	Cluster_2			1	6	2				9
0.4	6.9	1.3	0.5	0.5	Cluster_3			1	1					2
1.8	0.3	1.5	2.1	1.1	Cluster_4								1	1
					Total	1	0	3	8	4		0	1	17

[1] Values are the average of the data for the strains included in the cluster.
[2] Ha: *Hafnia alvei*; Hp: *Hafnia paralvei*.
[3] Strains included in each cluster: 1A (130); 1B (1037, 1715); 1C (544, 683); 2B (310, 561, 593, 677, 970, 1500, 1713); 2C (1045, 1142); 3B (1341, 1414); 4D (681).

Table 4
Clustering of selected *Hafnia* spp. strains based on technological properties assayed.

Cluster[1]	Cluster_P1[2]	Cluster_P2	Cluster_P3	Cluster_P4	Cluster_P5
N (17 strains)	1	8	1	4	3
Physico-chemical parameters					
Diacetyl production[3]	1	1	0	0.2	1
Acidifying (pH 24 h)	0.79	0.85	0.40	0.58	0.97
Alkalizing[3]	1	0.92	1	1	0.67
Proteolysis (mm)					
3 days					
8 °C	0	0	0	0	0
15 °C	0.0	1.2	10.5	0.0	8.7
30 °C	12.0	4.4	14.0	0.0	10.5
5 days					
8 °C	0.0	0.0	0.0	0.0	7.8
15 °C	0.0	9.5	11.0	0.0	10.2
30 °C	12.0	7.5	14.0	0.0	10.3
7 days					
8 °C	0.0	7.6	0.0	0.0	8.5
15 °C	0.0	9.5	11.0	0.0	10.2
30 °C	12.0	7.5	14.0	0.0	10.3
Gas production					
Durham tubes (mm)	13.0	15.5	0.0	5.7	16.5
Paraffin (mm)	48.0	21.1	0.0	6.3	16.7

[1] The values are the average of the data of the strains included in the cluster.
[2] Strains include in each cluster: P1 (130); P2 (310, 561, 593, 677, 681, 1500, 1713, 1715); P3 (544); P4 (683, 1037, 1045, 1341); P5 (970, 1142, 1414).
[3] Diacetyl production and alkalising: positive (1); negative (0).

time of less than 1.88 days in all conditions, except for strain *H. alvei* 1142 at S1 with 5.59 days. All strains raised the pH after 24 h and displayed relevant proteolytic activity at 15 and 30 °C, with values ranging from 8 to 14 mm. However, diacetyl production and proteolytic activity at 8 °C were restricted to the strains *H. alvei* 970 and 1142 and *H. paralvei* 1414, with a proteolysis halo of 8.5 mm at 7 days. Finally, gas production was variable between them. *H. alvei* 970 and *H. paralvei* 1414 displayed moderate production with values ranging from 17 to 25.5 mm, whereas *H. alvei* 544 and 1142, interestingly, showed null or low gas production.

4. Discussion

Soft raw ewe's milk cheeses present a diverse microbiota that actively participates in the biochemical events that take place during ripening, contributing to the sensory attributes of the final product (Ordiales et al., 2013b). A relevant contribution to the sensory properties of raw milk cheese is attributed to the secondary microbiota, where enterobacteria are involved (Montel et al., 2014; O'Sullivan and Cotter, 2017). In this study, the aerobic mesophilic bacteria and enterobacteria counts were similar to those described on this type of cheese in previous works by Ordiales et al. (2013a) and Gonçalves et al. (2018), but enterobacterial counts were higher than those reported in other raw ewe's milk cheeses with a different ripening process and physicochemical characteristics (Cardinali et al., 2021; Galán et al., 2012; Renes et al., 2019). These high enterobacterial levels, around 6–7 log cfu/g, during the whole ripening process suggest that this microbial group may have a great influence on the final characteristics of 'Torta del Casar' and 'Queso de la Serena' PDO cheeses. Commonly, enterobacteria are considered as an indicator of poor hygienic practices and associated with a negative role in cheese quality by causing sensory defects or decarboxylation of amino acids (Coton et al., 2012; Tabla et al., 2016; Westling et al., 2016). However, today this idea is changing and is not widespread to all species of the family *Enterobacteriaceae*. For example, dairy-related *Hafnia* spp. and *Proteus* spp. have been shown to contribute positively to flavour production in cheese (Deetae et al., 2009; Irlinger et al., 2012).

In this context, we investigated the enterobacterial community dynamics during cheese ripening in 'Torta del Casar' and 'Queso de la Serena' PDO cheeses. A wide diversity of species was found at the beginning of ripening in both PDO cheeses, but interestingly after 20

Table 5
Mean values of specific technological properties of *Hafnia* spp. strains from clusters P3 and P5 from Table 4.

Strain	Cheese ripening conditions (days of growth)					Diacetyl production[2]	Acidifying 24 h	Alkalizing[2]	Proteolytic activity (mm)									Gas production (mm)	
									3 days			5 days			7 days				
	S1[1]	S2	S3	S4	S5				8 °C	15 °C	30 °C	8 °C	15 °C	30 °C	8 °C	15 °C	30 °C	Durham	Paraffin
H. alvei 544	1.88	0.26	1.34	1.70	1.09	0	0.40	1	0.0	10.5	14.0	0.0	11.0	14.0	0	11	14	0	0
H. alvei 970	1.04	0.41	0.60	0.51	0.44	1	0.96	1	0.0	8.5	9.5	7.0	10.5	8.0	8.5	10.5	8	20.5	20.5
H. alvei 1142	5.59	0.28	1.42	1.54	0.86	1	0.94	0	0.0	8.5	9.5	7.5	8.5	10.0	8.5	8.5	10	12	4
H. paralvei 1414	0.92	0.23	0.55	0.70	0.51	1	1.01	1	0.0	9.0	12.5	9.0	11.5	13.0	8.5	11.5	13	17	25.5

[1] Cheese ripening stages: S1 (8 °C; 1% NaCl; pH 6), S2 (8 °C; 2% NaCl; pH 4.5), S3 (10 °C; 3% NaCl; pH 5), S4 (12 °C; 3% NaCl; pH 5.5), S5 (12 °C; 3% NaCl; pH 5.9).
[2] Diacetyl production and alkalizing capacity: positive (1); negative (0).

days of ripening *H. alvei* and *H. paralvei* were the dominant species, demonstrating their adaptability to the ripening conditions of these PDO cheeses. There is a vast literature describing the enterobacterial population in traditional raw milk cheese, which reports as the main dominant species those belonging to the genera *Klebsiella*, *Escherichia*, *Serratia*, *Hafnia*, *Citrobacter* and *Enterobacter*. However, in previous studies carried out on soft raw ewe's milk cheeses, the genus *Hafnia* was found to be predominant (Gonçalves et al., 2018; Ordiales et al., 2013a). Because of the frequency of the presence of *Hafnia* spp. in raw milk cheeses and previous scientific reports highlighting their potential for cheese flavouring, it is worth selecting *Hafnia* strains with technological properties to develop a mixed autochthonous starter culture for improving the cheesemaking process of this type of cheese. To address this issue, *Hafnia* spp. isolates were strain-typed by RAPD-PCR with the M13 primer and, based on the genetic profile and dairy of origin, 55 isolates were selected for further safety evaluation and technological characterization.

Concerning safety analysis, there is a major concern in world public health authorities about the dissemination of antibiotic resistance to human pathogenic bacteria. In microorganisms for food application, it is particularly relevant to know the antibiotic resistance profile since the genes encoding antibiotic resistance located in mobile elements may be potentially transferable. Plasmid-encoded antibiotic resistance is common among enterobacteria, with the consequent risk of dissemination of their resistance genes (Anjum et al., 2019). In this study, susceptibility to 33 antibiotics was tested. Although the results did not differ dramatically between strains, some differences were found, highlighting 17 of them for their better antibiotic susceptibility profile. There are few reports in which antibiotic susceptibility has been tested in many *Hafnia* spp. isolates from food matrices (Coton et al., 2012); they have been mainly focused on clinical isolates (Abbott et al., 2011; Günthard and Pennekamp, 1996; Stock et al., 2005). *Hafnia* spp. are naturally resistant to aminopenicillins and certain cephalosporins due to the presence of the chromosomal *ampC* gene, encoding for class C β-lactamase, while they are susceptible to the fourth generation of cephalosporins (Janda and Abbott, 2006; Stock et al., 2005). This evidence explains the general patterns of antibiotic resistance to these classes of antibiotics found in this work. A high percentage of strains, mainly associated with more resistant strains grouped in clusters 1 and 2 (Table 2), were also resistant to carbapenems. This resistance has been previously reported in clinical isolates (Abbott et al., 2011; Skurnik et al., 2010) and is of particular concern because they are almost the only treatment option for infections caused by enterobacteria resistant to third- and fourth-generation cephalosporins (Ramos-Vivas, 2020). In addition, we did not detect colistin resistance genes. Coton et al. (2012) found variable susceptible profiles for colistin resistance by disk diffusion test in *Hafnia* spp. strains isolated from cheese. However, this result does not agree with other studies that have revealed high colistin resistance of *Hafnia* spp. clinical isolates, suggesting that this genus may be naturally resistant to colistin, like *Proteus*, *Providencia*, *Morganella* and *Serratia* (Jayol et al., 2017; Zurfluh et al., 2017).

Regarding haemolytic activity, all strains showed α-haemolysis which, according to different authors is due to acquisition of the plasmid pIE 567, which is also found in *Enterobacter aerogenes* and *Salmonella typhimurium* (Padilla et al., 2005). On the other hand, the expression of cytolytic toxin is common among *Hafnia* spp. clinical isolates (Abbott et al., 2011). Our results showed cytotoxic activity only in the strain *H. paralvei* 1498. Likewise, Kim and Linton (2008) reported, for a strain of *H. alvei*, minimal or null cytotoxic activity in Ped-2E9 and Chinese hamster ovary cells. These results together with the absence of the virulence genes *eaeA* and *cdt* suggest that these *Hafnia* spp. isolates present low or no pathogenic capacity in comparison with clinical isolates associated with infections.

Additionally, due to the presence of the lysine and ornithine decarboxylase enzymes in this genus (Özogul and Özogul, 2007), all except one strain were cadaverine and putrescine producers under favourable

conditions. However, these biogenic amines do not cause significant toxicity by themselves (Wójcik et al., 2021). These results together with previous reports on *Hafnia* strains (Coton et al., 2012; Marino et al., 2000) suggest that this capacity may be widespread in *H. alvei* and *H. paralvei*. Biogenic amine producer strains are not the most suitable for food fermentation; however, during cheese ripening several factors such as the availability of free substrate amino acids, bacterial density and the synergistic effect between microorganisms, temperature, pH, a_w, redox potential, relative humidity and NaCl concentration affect decarboxylation activity and can severely restrict the accumulation of biogenic amines in the product (Linares et al., 2011). In fact, Delbès-Paus et al. (2012) showed in a cheese model that the production of biogenic amines by four high biogenic producer enterobacterial strains in vitro, including an *H. alvei* strain, was very low or even negligible.

Seventeen isolates of *Hafnia* spp. were pre-selected based on their safety characteristics for further technological characterization. Firstly, all selected strains displayed adequate growth under the stringent conditions of cheese ripening studied, although variable behaviour between them was observed. It has been shown that *Hafnia* spp. can grow at refrigeration temperatures, low pH and the concentration of salt present in this type of cheese (Mounier et al., 2008; O'Sullivan and Cotter, 2017; Ridell and Korkeala, 1997). These characteristics support their adaptability to the ripening process, enabling the count levels found at the end of ripening. With respect to biochemical characterization, proteolysis and lipolysis are the main mechanisms involved in the development of cheese flavour and texture. Proteolytic capacity, especially under refrigeration, is one of the most important technological characteristics for the manufacture of soft cheeses (Delgado et al., 2010b; Ordiales et al., 2013b). *Hafnia* spp. present low or negative lipolytic activity (Macedo and Malcata, 1997), as confirmed in this study. In contrast, it is well known that *Hafnia* spp. contribute to the liberation of free amino acids by degrading casein and peptides and, consequently, to the acceleration of cheese ripening and flavour development (Chaves-López et al., 2006; Irlinger et al., 2012; Morales et al., 2003). Our results show that proteolytic capacity was detected in 13 out of 17 selected strains, although its intensity was variable depending on the strain and temperature. This result is in accordance with previous scientific reports on *Hafnia* spp. (Decimo et al., 2014; Morales et al., 2003). In addition, most of the selected *Hafnia* spp. strains produced diacetyl and acetoin that are generally appreciated for their positive contribution to the aromatic profile of cheese (Curioni and Bosset, 2002) and related to the aromatic profile in 'Torta del Casar' cheeses (Ordiales et al., 2013b). Morales et al. (2004) reported high levels of this compound in model cheeses inoculated with *Hafnia* and *Serratia* strains.

Finally, microorganisms for technological application in food must not be responsible for spoilage. Enterobacteria, including *Hafnia* strains, are involved in the early blowing defect in soft raw ewe's milk cheese (Tabla et al., 2016). Among other factors, this defect is associated with the ability of certain enterobacterial species to ferment lactose and with the formation of gas. In this study, most of the strains had the capacity to produce gas, at different rates. In order to avoid this defect, we considered it appropriate to exclude those with higher gas production capacity.

In conclusion, this study reveals that *H. alvei* and *H. paralvei* are the dominant enterobacterial species during the whole ripening process of the PDO cheeses 'Torta del Casar' and 'Queso de la Serena' and may be involved in the development of their sensory characteristics. In this context, 55 *Hafnia* spp. isolates were selected for study of their suitability for cheesemaking application. Among them, *H. alvei* 544 and 1142, based on the safety and technological characteristics evaluated, are proposed as adjunct cultures alone or in combination with autochthonous starter bacteria. Both strains stood out by the absence of antibiotic acquired resistance, virulence genes, haemolytic and cytotoxic activity, as well as for having proteolytic activity at low temperature and a lower gas production capacity. The use of native starter cultures may contribute to overcome the heterogeneity associated with these traditional cheeses, preserving their unique sensory characteristics and improving their marketability at national and international levels. Therefore, advances in this direction would allow better process optimization and ripening control, promoting the quality and safety of these traditional PDO cheeses. Nevertheless, before their industrial application, it would be desirable to understand the real contribution of selected *H. alvei* strains in cheese ripening and to guarantee their safety. Therefore, in future studies, in addition to their whole-genome sequencing, it is necessary to determine in a cheese model system their ability to control pathogenic bacteria and their impact on biochemical changes, sensory characteristics and overall quality.

Declaration of competing 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.ijfoodmicro.2022.109703.

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