Characterization of autochthonal yeasts isolated from Spanish soft raw ewe milk protected designation of origin cheeses for technological application

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ABSTRACT

The yeasts involved in the ripening process of artisanal soft raw ewe milk Protected Designation of Origin (PDO) Torta del Casar and Queso de la Serena cheeses produced in Extremadura, Spain, were isolated throughout their ripening process, strain typed, and characterized for some important technological properties. A total of 508 yeast isolates were obtained and identified by inter-single sequence repeat anchored PCR amplification analysis and subsequent sequencing of the internal transcribed spacer ITS1/ITS2 5.8S rRNA. A total of 19 yeast species representing 8 genera were identified. *Debaryomyces hansenii*, *Pichia kudriavzevii*, *Kluyveromyces lactis*, and *Yarrowia lipolytica* were the predominant species. We selected 157 isolates, by genotyping and origin, for technological characterization. The evaluation of yeast isolates' growth under stress conditions of cheese ripening showed that 87 presented better performance. Among them, 71 isolates were not able to catabolize tyrosine to produce a brown pigment. Principal component analysis of the biochemical features of these isolates showed that 9 strains stood out, 3 *K. lactis* strains (2287, 2725, and 1507), 2 *Pichia jadinii* (1731 and 433), 2 *Yarrowia alimentaria* (1204 and 2150), *Y. lipolytica* 2495 and *P. kudriavzevii* 373. These strains displayed strong extracellular proteolytic activity on skim milk agar as well as an adequate enzymatic profile (strong aminopeptidase and weak protease activity), suggesting their great potential for cheese proteolysis. Extracellular lipolytic activity was mainly restricted to *Yarrowia* spp. isolates and weakly present in *P. kudriavzevii* 373 and *K. lactis* 2725, although enzymatic characterization by API-ZYM (bioMérieux SA) evidenced that all may contribute, at least in part, to the lipolysis process. Moreover, these strains were able to assimilate lactose, galactose, and glucose at

NaCl concentrations higher than that usually found in cheese. However, lactate and citrate assimilation were limited to *Y. lipolytica* 2495, *P. kudriavzevii* 373, and *P. jadinii* 433, and may contribute to the alkalizing process relevant to biochemical processes that take place in the last stages of ripening. By contrast, *K. lactis* strains showed acidifying capacity and β -galactosidase activity and may take part in the initial stages of ripening, together with lactic acid bacteria. Thus, considering the technological characteristics studied, the 9 selected strains presented biochemical features well suited to their potential use as adjunct cultures, alone or in combination with autochthonous starter bacteria in the cheesemaking process, to overcome the heterogeneity of these PDO cheeses, preserving their unique sensory characteristics.

Key words: yeast, optimal growth, technological characterization, soft raw milk cheese, cheese ripening

INTRODUCTION

Torta del Casar and Queso de la Serena are high-quality Spanish cheeses marketed under the Registry of the Protected Designation of Origin (PDO) in accordance with regulations 1491/2003 and 1107/96 of the European Commission, respectively (European Commission, 1996, 2003). These cheeses are produced in the Extremadura region of southwestern Spain from raw ewe milk, using an aqueous maceration of dried flowers from the *Cynara cardunculus* L. (cardoon) as coagulant, and are ripened for at least 60 d at 4 to 12°C and relative humidity of 75 to 95%. In these artisanal cheeses, which are characterized by the absence of thermal treatment and starter microorganisms in the manufacture, product quality depends mainly on spontaneous fermentation developed by the natural microbiota present in the raw materials, the milk and the vegetable coagulant, and the environment (Ordiales et al., 2013a; Kongo and Malcata, 2016a). They present a complex microbiota mainly dominated by several species of lactic acid bacteria (LAB) and to a lesser extent

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by gram-positive, catalase-positive cocci, enterobacteria, and yeasts (Freitas and Malcata, 2000; Ordiales et al., 2013a,b).

Lactic acid bacteria cause rapid acidification through lactose fermentation and are involved in the biochemical processes occurring during cheese ripening, playing an important role in the final organoleptic characteristics (Kongo and Malcata, 2016a,b). However, raw milk cheeses are characterized by a complex aromatic profile also associated with the metabolic activities of the diverse endogenous microbiota other than LAB (Montel et al., 2014; Irlinger et al., 2017). Recent advances in the study of the entire microbial population have highlighted the importance of yeasts in cheese ripening (Fröhlich-Wyder et al., 2019). Cheeses provide ideal physicochemical conditions for their growth, owing to their high salt concentration and low temperature, water activity, and pH (Ferreira and Viljoen, 2003). In addition, yeast's ability to metabolize lactose and galactose, or the assimilation of succinic, lactic, and citric acids, favors its occurrence in cheese. In fact, it has been reported that on cheese's surface, yeast growth is favored by the aerobic environment, reaching high levels around 6 to 8 log cfu/cm² after a few days of ripening (Cogan et al., 2014; Fröhlich-Wyder et al., 2019). There, yeast can consume lactate and produce NH₃ from amino acids, leading to an increase in pH that helps to establish salt-tolerant and acid-sensitive gram-positive bacteria (Cogan et al., 2014; Monnet et al., 2015). However, in cheese's core, the availability of oxygen decreases, and the conditions for yeast growth are more restrictive. Only yeast species capable of fermenting residual lactose or galactose or both have a competitive advantage (Fröhlich-Wyder et al., 2019). Species associated with the cheese environment display relevant proteolytic and lipolytic activities that may contribute positively to the development of cheese aroma (Chen et al., 2012; Padilla et al., 2014a,b; Atanassova et al., 2016). In fact, De Freitas et al. (2009) reported that yeast adjuncts of *Kluyveromyces lactis* at about 5 log cfu/g enhance the formation of volatile aroma compounds. At a similar level, *Yarrowia lipolytica* modifies the flavor profile and contributes to the quality of Tetilla cheese (Centeno et al., 2017). Likewise, the use of the adjunct culture *Geotrichum candidum* during the manufacture of pasteurized soft and semi-hard cheeses results in taste and aroma closer to those obtained from raw milk cheeses (Boutroun and Guéguen, 2005). In this context, *Debaryomyces hansenii*, *K. lactis*, *Kluyveromyces marxianus*, and *G. candidum* strains are now commercially available for this purpose (Irlinger et al., 2017).

However, yeasts may also cause spoilage in dairy products (Garnier et al., 2017). Several defects, such as yeasty off-flavors, eye formation, early blowing, toad

skin, and brown pigmentation, have been linked to yeast proliferation and may have detrimental effects on the appearance and sensorial characteristics of cheese (Carreira et al., 1998; Yalcin and Ucar, 2009; Garnier et al., 2017; Fröhlich-Wyder et al., 2019).

Although Torta del Casar and Queso de la Serena are high-value PDO cheeses, the lack of standardization in their cheesemaking processes implies that microbial diversity and abundance can vary during ripening, resulting in heterogeneous organoleptic characteristics according to the batch or the dairy industry. Use of autochthonous adjunct cultures adapted to the cheesemaking process may contribute to overcoming these problems, reducing variability in the final products as well as preserving their typical sensory profiles (Araújo-Rodrigues et al., 2020). Most efforts in this type of cheeses have been conducted via the selection of autochthonous LAB cultures with technological capacity. However, to the best of our knowledge, little is known about the features of members of the secondary microbiota. In this context, the present study aimed to identify the predominant yeast microbiota present in artisanal soft raw ewe milk PDO cheeses produced in Extremadura and to characterize the yeast isolates for technologically relevant properties.

MATERIALS AND METHODS

Cheese Sampling and Yeast Counts

Cheese samples were taken during their ripening period at 0, 20, 40, and 60 d (final product) from 6 different dairies (3 dairies belonging to PDO Queso de la Serena and the other 3 to PDO Torta del Casar) in 2 different seasons (winter and spring). Samples consisted of 3 randomly selected cheeses taken from each dairy, which were transported under refrigerated conditions to the facility located at the Agricultural Engineering School at University of Extremadura, Badajoz, Spain, for analysis. Microbiological analyses were performed on 3 different cheeses by dairy and sample time, and each determination was performed in duplicate.

Yeast counts were carried out on acidified potato dextrose agar plates (Condalab) with 1% (vol/vol) sterilized solution of tartaric acid at 10% (wt/vol) as previously described by Gonçalves Dos Santos et al. (2017). For isolation, 10 yeast colonies from each potato dextrose agar plate containing the highest dilutions were randomly selected and streaked onto acidified potato dextrose agar plates. Finally, pure isolates were grown in 5 mL of yeast extract peptone dextrose broth (YPD, Condalab) at 25°C for 48 h and stored at -80°C in 25% (vol/vol) glycerol. Before the assays, all yeast isolates were subcultured twice in YPD broth at 25°C for 24 h.

Yeast Typing by Molecular Techniques

To obtain genomic DNA, 1 mL of each yeast culture in YPD broth was collected by centrifugation at $10,000 \times g$ for 5 min at 4°C. The yeast pellet was suspended in lysis buffer and disrupted with 400- to 600- μm silica grinding beads in a 1600 MiniG homogenizer (SPEX SamplePrep) at 1,500 rpm for 5 min. Genomic DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The amount of DNA was determined by using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and the concentration was adjusted to 10 ng/ μL for PCR reactions.

Inter-single sequence repeat anchored PCR amplification analysis (**ISSR-PCR**) was carried out using the 5'-anchored (GTG)₅ primer (5'-GTGGTGGTGGTGGTGG-3') as described by Mahmoud et al. (2014). The ISSR-PCR fragments were separated by electrophoresis on 1.5% agarose gels, and amplified band sizes estimated by comparison with the GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific) using Gene Tools software (SynGene). The clustering of the ISSR-PCR profiles from yeast isolates into operational taxonomic units (**OTU**) was carried out using the unweighted pair group method with arithmetic average. The analysis was performed using the NTSYS.PC package, version 2.0 (Rohlf, 1993). Two representative isolates of each OTU were identified at the species level by sequencing the internal transcribed spacer ITS1/ITS2 5.8S rRNA region, as previously described Merchán et al. (2020).

Characterization of Technological Properties

Yeast isolates from different OTU or belonging to the same OTU but originating from a different dairy were selected for screening of their technological capacities (Supplemental Table S1; <https://data.mendeley.com/datasets/6rst447xpn/1>). Before the assays, all yeast isolates were subcultured twice in YPD broth at 25°C for 24 h. Subsequently, isolates were washed twice with PBS at pH 7.2 (Thermo Fisher Scientific) and the yeast concentration adjusted to 1×10^6 cells/mL using a Neubauer chamber. All experiments were carried out in triplicate.

Evaluation of Yeast Growth in Cheese Ripening Conditions. A Box-Behnken experimental design with 3 factors was applied to model the influence of the variables temperature, salt concentration, and pH on the growth of the yeast isolates. The variable factor conditions are shown in Supplemental Table S2 (<https://data.mendeley.com/datasets/6rst447xpn/1>) and were established according to the ripening process of soft cheeses Torta del Casar and Queso de la Serena (Mar-

tínez et al., 2011; Tabla et al., 2015). Yeast nitrogen base broth (**YNB**; Pronadisa) supplemented with 1% (wt/vol) lactose was used for the experiment. The pH and NaCl concentrations of the YNB medium were set up at the conditions shown in Supplemental Table S2. The pH of the YNB medium was adjusted with lactic acid solution (10%) or 1 M NaOH. The YNB medium was sterilized by filtration through a 0.22- μm filter. Sterile 200- μL aliquots from the different YNB broth conditions were placed in 96-well sterile microplates and inoculated at 1% (vol/vol) from a suspension of tested yeasts at 10^6 cells/mL. The microplates were incubated at different temperatures ranging from 8°C to 30°C (Supplemental Table S2) for 15 d. The ability of each yeast isolate to grow under the set conditions was periodically evaluated by measuring the absorbance at 570 nm with a Fluostar Optima microplate reader (BMG Labtech). The time to detection of growth to optical density at 570 nm of 0.4 for each well was calculated using a Microsoft Excel template (kindly provided by Dr. R. Lambert, Imperial College London, London, UK), which used linear interpolation between successive optical density readings (Medina et al., 2012). The response variable considered in the model was the inverse of time to detection to reach optical density at 570 nm of 0.4 for each isolate.

Pigmentation Capacity. The pigmentation production by yeast isolates was evaluated in cheese-based medium with 1% (wt/vol) L-tyrosine elaborated according to Carreira et al. (1998) using the soft cheese Torta del Casar. Cheese-based medium without 1% (wt/vol) L-tyrosine was used as a negative control. Ten microliters of each isolate was spotted on the surface of the cheese-based medium and incubated at 8 and 25°C for 20 d. The presence of pigment was evaluated every 2 d. Results were considered positive when the medium turned brown. The intensity of the pigmentation was classified in 4 levels according to the color classification proposed by Carreira et al. (1998).

Extracellular Proteolytic and Lipolytic Activity. Proteolytic activity was determined by the method described by Gardini et al. (2006), with some modifications. A medium composed of reconstituted skim milk powder at 5% and bacteriological agar at 1.5% (Scharlab) was sterilized by autoclaving for 5 min at 115°C and then poured onto petri dishes. The medium was superficially spotted with 10 μL of each yeast isolate and incubated at 15, 20, and 25°C. The cultures were examined for proteolytic activity daily for 6 d. A clear zone around the colony was considered positive for activity. This area was also measured to evaluate the intensity of the activity.

Lipase activity was determined by using Rhodamin B agar medium (Kouker and Jaeger, 1987), and ester-

ase activity was determined by using tributyrin agar (Ismail et al., 2000). Inoculated plates with 10 μ L of each yeast isolate were incubated at 15, 20, and 25°C and examined daily for 6 d. Pink-colored colonies on Rhodamine B agar plates were considered as positive. In the case of tributyrin agar, a clear zone around the colony was measured to determined esterase activity.

Alkalizing and Acidifying Activity. The alkalizing capacity was evaluated on Simmons citrate agar (Condalab). The medium was spotted with 10 μ L of each isolate and incubated for 48 h at 30°C. Alkalizing activity was observed when Simmons citrate agar produced a blue color around the colonies. The acidifying activity was determined in sterile reconstituted skim milk (10%), inoculated with 100 μ L of each isolate, and incubated at 30°C for 48 h. The pH was measured using a Crison pH meter, model Basic 20 (2012; Crison Instruments) at 24 and 48 h.

Diacetyl-Acetoin Production. Acetoin production was detected by the Voges-Proskauer reaction. One hundred microliters of each isolate was inoculated in sterile reconstituted skim milk at 25°C for 48 h. According to Speckman and Collins (1982), 2.5 mL of yeast suspension was added to 0.5 mL of α -naphthol (6%, wt/vol ethanol) and 0.2 mL of 40% sodium hydroxide. The resulting mixture was vortexed for 30 s and then incubated 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 (University of Extremadura) culture collection were used as positive and negative controls, respectively.

Assimilation of Lactose, Galactose, Lactate, and Citrate. All selected yeast isolates were tested for their assimilation of lactose, galactose, lactate, and citrate in the presence of different concentrations of NaCl (0, 2.5, 5, 7.5, and 10%) according to Yarrow (1998) and Gardini et al. (2006).

Enzymatic Activity. The API-ZYM system (bioMérieux SA) was used to evaluate the enzymatic activities from 9 selected yeast isolates, considering their technological properties. The API-ZYM strip was composed of 20 cupules containing synthetic substrates. Suspensions of each yeast with a turbidity of 5 to 6 McFarland were inoculated into each cupule and incubated for 4.5 h at 37°C. The metabolic end products produced during the incubation period were detected through color reactions revealed by the addition of reagents. A total of 19 enzymatic activities were studied: alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, phosphatase acid, naphthol-AS-BI-

phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase.

Statistical Analysis

Descriptive statistics of the yeast counts were performed using SPSS for Windows, version 21.0 (IBM Corp.), and the differences between groups were studied by 2-way ANOVA and separated by Tukey's honest significant differences test ($P \leq 0.05$). A 3-factor, 3-level Box-Behnken design combined with surface response methodology was applied to determine the effects of medium conditions on the growth of yeast isolates (Supplemental Table S2). Surface response methodology was performed employing StatGraphics Centurion XVI version 8.0 software. 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 model was used to estimate the growth time of each isolate at the ripening conditions of the Torta del Casar and Queso de la Serena PDO cheeses (Supplemental Table S2). The software also generated an ANOVA, establishing statistical significance at the 95% confidence level. Optimal levels for the growth time of each variable analyzed were also obtained with the same statistical program.

In addition, the growth of the yeast isolates at the cheese ripening conditions, their pigmentation capacity, and their biochemical characteristics (proteolysis and lipolysis) were evaluated by hierarchical cluster analysis. Finally, the relationships among technological features and selected yeast strains were established by Pearson correlation coefficients and principal component analysis (PCA). Analyses were performed using SPSS software for Windows 21.0 (IBM Corp.).

RESULTS

Yeast Counts and Identification

Yeast counts through ripening ranged between 3.94 and 4.52 log cfu/g in Queso de la Serena and between 3.42 and 3.97 log cfu/g in Torta del Casar (Figure 1). No significant differences were found between seasons

($P > 0.05$); however, significant differences were detected between cheese types and throughout the ripening process. Queso de la Serena showed significantly ($P < 0.05$) higher counts at different sampling time points throughout ripening. For both types of PDO cheeses, yeast levels increased at the beginning of ripening, reaching maximum counts after 20 d ($P < 0.05$), and then decreased slightly until the end (60 d).

A total of 508 yeast isolates were obtained from the highest dilutions of cheese core samples collected from 6 dairies in 2 seasons. Supplemental Table S1 shows the molecular identification by ISSR analysis combined with sequencing of the ITS1/ITS2 5.8S rRNA region. Based on ISSR analysis, the yeast isolates clustered into 37 OTU. Sequencing of the ITS region of 2 representative isolates by OTU allows their identification at the species level. Nineteen different yeast species representing 8 genera were identified. The predominant yeast genera detected in all dairies were *Kluyveromyces*, *Debaryomyces*, *Pichia*, and *Yarrowia*. At the species level, *D. hansenii* and *Pichia kudriavzevii* were the most frequently found, being detected in all dairies, followed by *K. lactis* and *Y. lipolytica*, which were not detected in 1 of the 6 dairies. Among the 508 isolates identified, 157 were selected for the study of technological properties based on genetic profile and origin (Supplemental Table S1). The most frequent species comprised approximately 60% (90 isolates) of the selected strains, and the remaining ones (67 isolates) were distributed across 14 species. In addition, isolates identified as *Candida parapsilosis* were not selected, as this species is an opportunistic yeast pathogen.

Technological Properties

Adaptability Under Cheese Ripening Conditions. Figure 2 shows the clustering of isolates based on their ability to grow under the soft cheese ripening conditions set by the Box-Behnken experimental design applying 3 factors (temperature, pH, and NaCl concentration). The relatively high values for the adjusted R^2 (>50) obtained for most yeast isolates and the random pattern of the residuals indicate that their growth patterns fitted well to the quadratic model designed. However, for a total of 32 isolates, the model did not represent a good approximation, due to their poor growth under various set conditions by the Box-Behnken design. Concretely, 8 isolates of *Candida zeylanoides*, 7 of *Y. lipolytica*, 5 of *G. candidum*, 4 of *P. kudriavzevii*, 3 of *D. hansenii*, 2 of *Pichia fermentans*, and 3 isolates belonging to 3 different species (*Candida parapsilosis*, *K. lactis*, and *Yarrowia alimentaria*) were discarded on this basis (Figure 3). For the remaining isolates tested (a total of 125), their optimal growth

Figure 1. Yeast counts, expressed in log cfu/g, by protected designation of origin (PDO) cheese type throughout the ripening process (0 d, 20 d, 40 d, and 60 d). Red color represents yeast counts in Queso de la Serena PDO cheese and blue color those in Torta del Casar PDO cheese. Error bars correspond to the 95% confidence interval from Tukey's honestly significant difference test.

conditions were also estimated by fitting the data to the quadratic model developed for each isolate (Figure 2).

Hierarchical cluster analysis based on the estimated growth of yeast isolates showed good adaptation of a relevant number of them under the cheese processing conditions. Specifically, 74 isolates grouped in cluster 1 (C_1) presented adequate growth (5.2 to 8.8 d) under the 5 cheese processing conditions established; 2 isolates grouped in cluster 8 (C_8) under the first 3 cheese processing conditions checked; and 11 isolates grouped in cluster 2 (C_2) under the last 4 cheese processing conditions (Figures 2 and 3). The rest of the isolates grouped in other clusters: 38 showed a limited ability to grow (>25 d) under 2 or more of the set conditions and were therefore excluded from subsequent technological characterization (Figure 2). All the isolates in this group were identified as *Aureobasidium pullulans*, *Candida glabrata*, *Candida humilis*, *Candida intermedia*, *Kazachstania bulderi*, *Kazachstania humilis*, and

Pichia sporocuriosa. In addition, isolates of the most frequent species belonging to the genera *Debaryomyces*, *Kluyveromyces*, *Pichia*, *Yarrowia*, and *Geotrichum* were also assigned to these clusters and excluded for further analysis. Then, a total of 87 isolates from C_1, C_2, and C_8, mostly belonging to *D. hansenii*, *Y. lipolytica*, *Y. alimentaria*, *K. lactis*, *K. marxianus*, *P. kudriavzevii*, *P. fermentans*, and *Pichia jadinii*, were selected to evaluate their technological capacity (Figure 3).

The double hierarchical cluster analysis performed based on both the estimated growth of yeast isolates under cheese processing conditions and optimal growth conditions showed that C1_A (51 isolates) and C2_A (10 isolates) comprised most of the selected isolates (Figure 2; Figure 4). These isolates exhibited excellent growth at 30°C, 1% NaCl, and pH values of 3.5 (Figure 2). The growth of most of the isolates belonging to cluster A (C1_A and C2_A) were positively affected by the increase in temperature and decrease in salt concentration, also showing an interaction between both factors for a substantial number of them (Table 1). By contrast, the effect of pH on the growth of yeast isolates grouped into cluster A and other clusters was negligible compared with temperature and salt concentration (Table 1). The identical optimal growth conditions of

cluster A were estimated for the 2 isolates included in cluster E1 that were identified as *G. candidum*, and *P. fermentans*. However, the growth of these isolates was rather slow and was strongly affected by salt concentration (Table 1).

The isolates grouped into cluster B (C1_B and C2_B) showed as optimal conditions for growth 23.2°C, 8% NaCl, and neutral values of pH (6.5), all of them (6 isolates) being ascribed to *D. hansenii* (Figure 2; Figure 4). In this case, their growth was mainly influenced by temperature (main and quadratic effects), with NaCl concentration not significantly affecting it (Table 1). In this way, selected isolates belonging to clusters G (C1_G and C8_G) and C1_D also showed halotolerant behavior, with temperature as the most influential parameter. Most of the isolates integrated into cluster G were identified as *D. hansenii* and *P. kudriavzevii*. These isolates presented optimal growth conditions at 30°C, 8% NaCl, and pH 3.5, whereas for the isolate from cluster D, identified as *Y. alimentaria*, the optimal conditions were 8°C, 8% NaCl, and pH 5.3 (Figure 2; Figure 4).

By contrast, cluster C (C1_C) grouped 8 isolates with optimal growth at 18.5°C, 1.1% NaCl, and pH 4.8 (Figure 2; Figure 4). Their growth was adversely af-

Figure 2. Double dendrogram based on the days of growth under cheese ripening conditions and optimal growth conditions of the yeast isolates indicating the number of isolates per cheese ripening condition cluster within each optimal growth condition cluster. Cheese ripening stages: S1 (8°C; 1% NaCl; pH 6), S2 (8°C; 3% NaCl; pH 4.5), S3 (10°C; 3% NaCl; pH 5), S4 (12°C; 4% NaCl; pH 5.5), S5 (15°C; 5% NaCl; pH 5.9). N.G. = no growth.

fect by the NaCl concentration (main effect) and also influenced by temperature as a quadratic effect (Table 1). These were identified as *C. zeylanoides*, *Yarrowia* spp., *Pichia* spp., *G. candidum*, and *K. lactis*.

Pigmentation Capacity. Among the 87 yeast isolates selected on the basis of adaptation to cheese processing conditions, only yeast isolates belonging to the genus *Yarrowia* were able to produce pigment on

cheese-based agar supplemented with tyrosine at different concentrations. Figure 5 shows a dendrogram based on the pigmentation intensity of the isolates identified as *Yarrowia* spp. under different temperatures. Out of 21 *Yarrowia* spp. isolates, 11 identified as *Y. lipolytica* displayed medium to high pigmentation capacity (cluster_P1, cluster_P2, and cluster_P3) under the different conditions tested. Different pigmentation profiles were

Figure 3. Number of isolates by yeast species clustered based on their ability to grow under the soft cheese (Torta del Casar and Queso de la Serena) ripening conditions obtained by 3-factor (temperature, pH, and NaCl concentration) modeling using Box-Behnken experimental design. Green clusters = yeast isolates classified in clusters 1, 2, and 8, associated with adequate growth. Purple clusters (NG) = no growth under assay conditions. Red clusters (Others) = yeast isolates classified in clusters 3, 4, 5, 6, and 7, associated with no optimal growth. Aur. = *Aureobasidium*; Deb. = *Debaryomyces*; Geo. = *Geotrichum*; Kazac = *Kazachstania*; Kluyver. = *Kluyveromyces*.

observed in 5 other isolates of this species, grouped in cluster_P4. These developed a progressive and moderate pigmentation capacity when grown at 8°C, whereas this activity was lower at 25°C. In contrast, 4 *Y. alimentaria* isolates together with 1 *Y. lipolytica* isolate were grouped together into cluster_P5 due to their null or weak pigmentation ability under all conditions tested. Based on these findings, *Yarrowia* spp. from cluster_P5, together with nonpigmenting isolates—in

total, 71 isolates—were selected for evaluation of further technological characteristics.

Technological Characterization. Table 2 shows the hierarchical cluster analysis based on the lipolytic (lipase and esterase) and proteolytic capacities of the selected isolates. Regarding lipolytic activity, 59 out of 71 selected isolates displayed limited or no activity under all conditions tested (cluster_T[−]R[−]). The other 12 isolates showed different activity profiles. Six iso-

Figure 4. Number of isolates by yeast species from clusters 1, 2, and 8 classified by double dendrogram analysis based on the days of growth under cheese ripening conditions and optimal conditions of growth. Species: *Candida pararugosa*, *Candida zeylanoides*, *Debaryomyces hansenii*, *Geotrichum candidum*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Pichia fermentans*, *Pichia jadinii*, *Pichia kudriavzevii*, *Yarrowia alimentaria*, and *Yarrowia lipolytica*.

Table 1. Contingency table of the significant effects for the regression models developed for the selected isolates based on their growth under the cheese ripening conditions assayed^{1,2}

Analysis of variance ($P < 0.1$)																		
Cluster	Main effects						Quadratic effects						Interactions					
	P_TEMP		P_NACL		P_PH		P_AA		P_BB		P_OC		P_AB		P_AC		P_BC	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
A	1A	+	45	88.2			3	5.9	11	21.6	1	2.0	2	3.9			5	9.8
		-			2	3.9	37	72.5	8	15.7	9	17.6	3	5.9	19	37.3	7	13.7
	2A	+	9	90.0			1	10.0							1	10.0		
B	1B	+	5	100			1	10.0			2	20.0	1	10.0	6	60.0		
		-			1	20.0	7	70.0										
	2B	+							4	80.0							2	40.0
C	1C	+							1	100								
		-							0				1	12.5				
	1D	+			1	12.5	7	87.5	6	75.0			1	12.5			1	12.50%
D	1D	+																
		-	1	100									1	100				
	1E	+																
F	1F	+					3	100									2	66.7
		-	1	100			1	100										
	1G	+	6	100					1	16.7	1	16.7			1	16.7	1	100
G	1G	+							1	16.7							2	33.3
		-			2	33.3			1	16.7			1	16.7			1	16.7
	8G	+	2	100					1	50.0					2	100		
Total		+	68	76.4	2	2.25	4	4.49	13	14.61	2	2.25	3	3.37	4	4.49	7	7.87
		-	1	1.1	55	61.80	6	6.74	21	23.60	11	12.36	6	6.74	26	29.21	12	13.48
		-															3	3.37

¹P_TEMP = P_Temperature; P_AA = Temperature × Temperature; P_BB = NaCl × NaCl; P_CC = pH × pH; P_AB = Temperature × NaCl; P_AC = Temperature × pH; P_BC = NaCl × pH.

²N = number of isolates from the cluster (Figure 2) with this behavior. % = percentage of isolates from the cluster (Figure 2) with this behavior.

Table 2. Hierarchical cluster analysis of preselected isolates based on lipolytic and proteolytic activities¹

Lipolytic activity	Cluster ²					
	T ⁻ R ⁻	T ⁺ R ⁺	R ⁺	T ⁺	T20R ⁺	Tc
Number of samples	59	2	6	1	1	2
Esterase (15°C) ³	0.6	6.3	0.9	7.0	0.0	9.0
Esterase (20°C)	1.3	10.3	3.0	4.0	18.0	7.5
Esterase (25°C)	0.0	10.0	0.0	16.0	0.0	3.5
Lipase (15°C) ⁴	0.0	1.0	0.7	0.0	1.0	0.0
Lipase (20°C)	0.0	1.0	1.0	0.0	1.0	0.0
Lipase (25°C)	0.0	1.0	0.9	0.0	1.0	0.0

Proteolytic activity ³	Cluster ⁵					
	P ⁻	P ⁺⁺⁺	P ⁺⁺	P ⁺	P25_6	Pc
Number of samples	60	3	4	2	1	1
15°C (3 d)	0.3	14.2	10.0	5.0	0.0	13.0
20°C (3 d)	0.0	16.4	10.5	6.0	0.0	0.0
25°C (3 d)	0.0	14.9	10.8	6.5	0.0	0.0
15°C (6 d)	0.6	23.0	15.0	10.5	0.0	17.0
20°C (6 d)	0.1	24.8	14.3	9.5	0.0	13.0
25°C (6 d)	0.3	22.6	15.5	3.5	27.0	0.0

¹Values are the average of the data of the isolates included in the cluster.²Lipolytic clusters: T⁻R⁻ = esterase and lipase negative; Tc = esterase at low temperature; R⁺ = lipase positive; T⁺R⁺ = esterase and lipase positive; T20R⁺ = esterase at 20°C and lipase positive; T⁺ = esterase positive.³Intensities of esterase and proteolytic activities are expressed in mm.⁴Lipase activity: negative = 0; positive = 1.⁵Proteolytic clusters: P⁻ = proteolytic negative; P⁺ low proteolytic; P⁺⁺ = moderate proteolytic; P⁺⁺⁺ = high proteolytic; Pc = proteolytic at low temperature; P25_6 = proteolytic at 25°C on d 6.

lates were grouped in cluster_R⁺, as they developed mainly lipase activity. Isolates displaying solely esterase activity (3 isolates) were classified in cluster_Tc (higher activity at lower temperatures) and cluster T⁺ (higher activity at 25°C). The remaining 3 isolates developed both lipolytic activities and were classified 2 in cluster_T⁺R⁺, with activity over a wide temperature range, and 1 in T20R⁺, with esterase activity over a narrow temperature range. With respect to proteolytic activity, likewise, a high number of isolates, 60, show null or limited activity (cluster_P⁻). By contrast, 11 isolates

displayed proteolytic activity at different rates. Nine isolates exhibited activity under all conditions and were classified in clusters P⁺, P⁺⁺, and P⁺⁺⁺ based on the intensity. The last 2 isolates were grouped in cluster Pc, linked to proteolytic activity at a lower temperature, and cluster P25_6, associated with activity at 25°C.

The combined analysis of lipolytic and proteolytic activities allowed us to classify the 71 selected isolates into 13 clusters (Table 3). The cluster T⁻R⁻_P⁻ included 74.6% of the isolates (53 isolates), which were associated with scarce or negative proteolytic and lipolytic

Figure 5. Dendrogram based on pigmentation intensity of the isolates identified as *Yarrowia* spp. after growth at different temperatures during different days indicating the number of isolates with each pigmentation profile within each yeast species. Pigmentation intensity: 0 (no pigmentation); <1 (low); 1–2 (moderate); 2–3 (high). The intensity values are the average of the data of the isolates included in the cluster.

capacity. The remaining 18 isolates displayed one or both activities in different ranges and were distributed in the remaining 12 clusters. The most interesting clusters were $T^+R^+_P^{++}$ and $T20R^+_P^{+++}$, which included the isolates *Y. alimentaria* 1204 and *Y. lipolytica* 2495, respectively, and displayed all the activities tested at different rates. Also of note, some isolates showed some lipolytic activity, esterase or lipase, together with proteolytic activity. They were grouped in clusters R^+_Pc (lipase activity and proteolytic activity at low temperature: *Y. alimentaria* 2150) and Tc_P^{++} (esterase activity at low temperature and moderate proteolytic activity: *P. kudriavzevii* 373). Finally, some isolates displayed lipolytic or proteolytic activity. *Geotrichum candidum* isolates developed lipase activity and *C. pararugosa* esterase activity, whereas 4 isolates of *K. lactis* and 2 *P. jadinii* developed only proteolytic activity at different rates.

The overall relationship between the technological properties studied (proteolytic and lipolytic activity, substrate assimilation, alkalizing and acidifying capacity, and diacetyl production) of the 71 selected isolates was evaluated by PCA (Figure 6). The first axis accounted for 20.34% of the variance (PC1) and was mainly defined by proteolytic and, to a lesser extent, lipolytic activity. The second axis (PC2; 17.47% of the variance) was defined mainly by the assimilation of glucose, galactose, lactose, citrate, and lactate under different NaCl concentrations and alkalizing capacities. Finally, the third axis (PC3; 10.34% of the variance) was mainly defined by diacetyl production, acidifying capacity, and proteolytic activity. In general, isolates belonging to the same species were grouped close to each other in the PCA. Figure 6B shows that *Candida* spp. isolates were not correlated with any of the technological properties studied. With respect to other species, *D. hansenii* isolates were linked with lactose assimilation; *K. marxianus* with glucose and galactose assimilation; and most *P. kudriavzevii*, *P. fermentans*, *P. jadinii*, *G. candidum*, and 2 *Y. alimentaria* with lactate and citrate assimilation. By contrast, *K. lactis* isolates showed a higher acidification capacity and greater diacetyl production. In addition, some *K. lactis* strains (2287, 2725, and 1507), together with 2 *P. jadinii* (1731 and 433), 2 *Y. alimentaria* (1204 and 2150), *Y. lipolytica* 2495, and *P. kudriavzevii* 373, showed higher proteolytic capacity. Finally, lipolytic activity was mainly associated with *Y. alimentaria* 1204. On this basis, the circle in the PCA plot encompasses the yeast strains with the most suitable technological properties for soft raw ewe cheese manufacture. Specifically, 9 strains, 3 *K. lactis* strains (2287, 2725, and 1507), 2 *P. jadinii* (1731 and 433), 2 *Y. alimentaria* (1204 and 2150), *Y. lipolytica* 2495, and *P. kudriavzevii* 373, stand out among the 71

Table 3. Distribution of clusters from Table 2 of the preselected yeast isolates according to their lipolytic and proteolytic activities

Cluster 2D	<i>Candida</i>				<i>Kluyveromyces</i>				<i>Pichia</i>				<i>Yarrowia</i>				Total
	<i>C. pararugosa</i>	<i>C. zaglanoides</i>	<i>C. hansenii</i>	<i>C. marxianus</i>	<i>G. candidum</i>	<i>K. lactis</i>	<i>K. marxianus</i>	<i>P. fermentans</i>	<i>P. jadinii</i>	<i>P. kudriavzevii</i>	<i>Y. alimentaria</i>	<i>Y. lipolytica</i>	<i>Y. alimentaria</i>	<i>Y. lipolytica</i>	<i>Y. alimentaria</i>	<i>Y. lipolytica</i>	
T^+R^-																	53
T^-R^-																	2
T^+R^+																	2
T^-R^+																	2
Tc																	2
Tc																	1
Tc																	1
R^+																	4
R^+																	1
R^+																	1
T^+R^+																	1
T^+R^+																	1
$T20R^+$																	1
T^+																	1
Total	1	2	17	7	4	9	7	5	5	16	4	1	4	1	1	1	100

¹Lipolytic clusters: T^-R^- = esterase and lipase negative; Tc = esterase at low temperature; R^+ = lipase positive; T^+R^+ = esterase and lipase positive; $T20R^+$ = esterase at 20°C and lipase positive; T^+ = esterase positive.

²Proteolytic clusters: P^- = proteolytic negative; P^+ low proteolytic; P^{++} = moderate proteolytic; P^{+++} = high proteolytic; Pc = proteolytic at low temperature; $P25_6$ = proteolytic at 25°C on d 6.

selected yeasts due to their higher proteolytic capacity at lower temperatures and substrate assimilation, as well as the lipolytic activity, diacetyl production, and acidifying capacity of some of them (Figure 6; Table 4).

Finally, the enzymatic activity profiles of the 9 selected strains evaluated by the API-ZYM system are presented in Table 4. It was found that 6 of the 19 activities assayed (β -glucuronidase, α -glucosidase, α -galactosidase,

Figure 6. Principal component analysis for technological characterization of selected yeast isolates. (A) technological parameters; (B) selected yeast species. Species: *Candida parugosa*, *Candida zeylanoides*, *Debaryomyces hansenii*, *Geotrichum candidum*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Pichia fermentans*, *Pichia jadinii*, *Pichia kudriavzevii*, *Yarrowia alimentaria*, and *Yarrowia lipolytica*.

Table 4. Specific properties of the 9 selected strains of yeasts by principal component analysis of technological characteristics

Item	<i>Yarrowia</i>			<i>Kluveromyces</i>			<i>Pichia</i>		
	<i>Y. lipolytica</i>	<i>Y. alimentaria</i>		<i>K. lactis</i>			<i>P. jadinii</i>	<i>P. kudriavzevii</i>	
	2495	1204	2150	1507	2287	2725	433	1731	373
Proteolytic activity at 15°C 6 d (mm)	24	17	17	25	11	15	13	26	17
Lipolytic activity (15°C)									
Lipase ¹	+	+	+	–	–	–	–	–	–
Esterase (mm)	18 ²	12	4 ²	0	0	4	0	0	6
Alkalizing	–	–	–	–	–	–	+	–	+
Acidifying activity ³ (ΔpH)									
24 h	–0.17	–0.26	–0.13	–0.90	–0.52	–0.72	0.17	–0.1	0.05
48 h	–0.14	–0.25	–0.92	–1.12	–0.85	–0.91	0.08	–0.15	–0.01
Diacetyl production ¹	–	–	+	+	+	+	–	–	–
Substrate assimilation ⁴									
Lactose	7.5 ⁵	5	7.5	7.5	7.5	7.5	5	7.5	7.5
Glucose	10	5	10	5	10	10	10	10	10
Galactose	10	5	7.5	7.5	10	7.5	7.5	7.5	7.5
Citrate	2.5	–	–	–	–	–	5	–	5
Lactate	2.5	–	–	–	0	0	2.5	–	5
Enzyme activity ⁶ (API-ZYM system)									
Leucine arylamidase	5	5	5	5	5	5	5	5	5
Valine arylamidase	3	4	3	5	5	4	5	4	4
Cystine arylamidase	3	2	1	3	3	3	5	3	3
α-Chymotrypsin	0	1	1	0	0	0	0	0	0
Esterase (C4)	5	4	4	3	3	5	5	3	4
Esterase lipase (C8)	4	4	1	2	3	4	3	2	3
Lipase (C14)	1	0	0	0	0	1	1	0	1
Phosphatase acid	2	5	5	1	3	4	1	4	5
Alkaline phosphatase	1	5	5	0	0	1	1	1	2
Naphthol-AS-BI-phosphohydrolase	2	4	4	2	2	1	1	3	1
β-Glucosidase	5	4	3	1	1	1	1	1	0
β-Galactosidase	0	0	0	5	5	5	0	0	0
α-Mannosidase	0	0	1	0	0	0	0	1	0

¹Lipase and diacetyl production: positive activity (+); negative activity (–).²Esterase activity at 20°C (0 mm at 15°C).³Results expressed as variation from the initial pH.⁴Substrate assimilation: – = non-assimilation.⁵Maximum NaCl concentration allowing assimilation.⁶Enzyme activities (API-ZYM system, bioMérieux SA): values range from 0 (negative activity) to 5 (highest activity). β-glucuronidase, α-glucosidase, α-galactosidase, N-acetyl-β-glucosaminidase, α-fucosidase, and trypsin were negative (0) in the 9 strains.

N-acetyl- β -glucoaminidase, α -fucosidase, and trypsin) were not detected in any of the strains. In addition, 3 other activities, α -mannosidase, lipase (C14), and α -chymotrypsin were found only at low levels (level 1) in some strains. For all other enzymes, different profiles of activities were encountered. All strains presented strong esterase (C4), leucine arylamidase, and valine arylamidase activities (>level 3). Likewise, esterase lipase (C8), cystine arylamidase, phosphatase acid, and naphthol-AS-BI-phosphohydrolase were reported in all strains, albeit at highly variable levels. Alkaline phosphatase and β -glucosidase were detected for most of the strains at different levels, whereas only the 3 *K. lactis* strains presented β -galactosidase activity (level 5).

DISCUSSION

Raw milk cheese ripening is a complex process, in which a cascade of biochemical events occurs, mediated by the metabolic flux of the autochthonous microbiota that define the sensory characteristics of the final product. An important contribution to sensory properties of cheese is attributed to the secondary microbiota, mainly composed of different bacterial groups and yeasts, but the interactions between them and the principal microbiota are not yet fully understood (Fox et al., 2015). Yeasts in particular have been demonstrated to contribute significantly to the sensory characteristics of traditional ripened cheeses (Padilla et al., 2014a; Price et al., 2014). In this study, we found values of yeast counts similar to those reported by other authors in this type of cheese (Ordiales et al., 2013b; Gonçalves Dos Santos et al., 2017). In addition, yeast species were identified, and the presence of 19 different species was detected, mainly associated with *Kluyveromyces*, *Debaryomyces*, *Pichia*, and *Yarrowia* genera. These genera are common inhabitants of the cheese environment, and some strains of them have been proposed as potential adjunct cultures for application in cheesemaking (Irlinger et al., 2017). In this context, these PDO cheeses may be a rich source of technologically relevant yeasts to develop a starter culture including selected autochthonous strains for improving the cheesemaking process and ensuring cheese authenticity and quality. For this contribution, they must first be well adapted to the stringent conditions of cheesemaking and ripening. The cheese environment changes from the surface to the interior and throughout the ripening process, shaping yeast species composition (Padilla et al., 2014a; Dugat-Bony et al., 2015). Our results show that a high percentage of yeasts showed null or low adaptability to the cheese growth conditions evaluated. Most yeast species grow well over a wide pH and temperature range and at a high NaCl concentra-

tion; however, they differ in substrate and oxygen requirements, which conditions their adaptability to the interior of the cheese (Fröhlich-Wyder et al., 2019). In this work, the isolates with better growth ability were ascribed to species belonging to the genera *Kluyveromyces*, *Debaryomyces*, *Pichia*, *Yarrowia*, *Candida*, and *Geotrichum*, whose capacity to tolerate cheese ripening conditions are well documented (Gardini et al., 2006; Binetti et al., 2013; Atanassova et al., 2016). In general, we observed that their growth was mainly affected by low temperature and increased NaCl concentration. Most cheese-tolerant isolates showed optimal growth in the range of 20 to 30°C, temperatures commonly reported in the literature for adequate yeast development. However, notably, some isolates belonging to clusters 1D, 1F, and 1C (Figure 2; Figure 4) displayed optimal growth at lower temperatures. Many yeast species grow well at low temperatures (Fleet, 2011), which can positively influence the development of their biochemical properties under cold cheese ripening conditions. Regarding NaCl concentration, as expected, the increase in salt concentration limited the growth of most of the isolates, except for some isolates mainly belonging to the well-known halotolerant species *D. hansenii* (Prista et al., 2005) and *P. kudriavzevii* (Kitagawa et al., 2010).

Microorganisms for technological application in food must not be responsible for spoilage. Brown pigment formation in this type of cheese has been associated with oxidation of tyrosine to pyomelanin by yeasts (Carreira et al., 1998). As previous literature reports, screening for pigment formation evidenced that this capacity was widespread among *Y. lipolytica* isolates (Carreira et al., 1998; Gardini et al., 2006) but not for *Y. alimentaria*. In this work, we have reported, for the first time, this undesirable ability of *Y. alimentaria*.

The results of technological characterization obtained for cheese ripening growth-adapted strains varied depending on the species studied, as well as between strains of the same species. This investigation highlighted 9 yeast isolates belonging to 5 different species according to their biochemical features. An important role in cheese flavor and texture development is associated with microbial proteolysis and lipolysis (Kongo and Malcata, 2016a,b). Mainly strains with extracellular proteolytic and lipolytic activities could effectively contribute to cheese ripening (Barth and Gaillardin, 1997; Gardini et al., 2006). Our results showed that these 9 selected strains displayed relevant extracellular proteolytic activity at low temperature, as well as high arylamidase (aminopeptidase) activity. Aminopeptidases are involved in the hydrolysis of N-terminal amino acids from peptides (Gatti et al., 2008) and help to prevent bitter taste (Bockelmann et al., 2010). However, the intracellular localization of

aminopeptidases raises the question of whether they are released into the cheese matrix through cell lysis and have a significant effect on proteolysis. In general, yeast populations decrease during cheese ripening, but, as far as we know, no data have been reported in the literature on whether yeast death is followed by cell lysis during cheese ripening. Moreover, it has been suggested that acid phosphatases, present at different levels in the 9 selected strains, act synergistically with proteolytic enzymes on phosphorylated proteins and peptides, allowing a higher production of small peptides and free amino acids, thus actively contributing to aroma formation in cheese (Akuzawa and Fox, 2004). The high peptidase activity and phosphatases, together with the absent or weak protease activity (trypsin and chymotrypsin) of these strains, have been reported as desirable for aroma and texture development in dairy fermentations (Mathara et al., 2004).

By contrast, extracellular lipolytic activity was mainly restricted to *Yarrowia* spp. isolates and found weakly in *P. kudriavzevii* 373 and *K. lactis* 2725. Enzymatic characterization by API-ZYM confirmed these strains as the most lipolytic, together with *P. jadinii* 433; however, esterase (C8) and esterase lipase (C4) were detected at different levels in the 9 selected strains, suggesting that all may contribute, at least in part, to the lipolysis process during cheese ripening. Isolates of *Y. lipolytica* from cheese have generally been reported to display strong lipolytic activity. Consequently, various studies have tried to exploit its great potential in cheese ripening to generate unique sensory characteristics (Gkatzionis et al., 2013; Centeno et al., 2017). Similarly, although more limited, dairy isolates from *P. kudriavzevii* and *K. lactis* have also been reported as good lipase producers (Atanassova et al., 2016; Zheng et al., 2018), whereas no report, to the best of our knowledge, has yet documented this activity in *Y. alimentaria*.

Regarding other technological properties evaluated, substrate assimilation of the most relevant yeast isolates at different NaCl concentrations showed that these strains were able to assimilate lactose, galactose, and glucose at NaCl concentrations higher than those usually found in cheese. This feature could help them to compete with the cheese microbiota and support their presence during cheese ripening. However, lactate and citrate assimilation were more limited, and only 3 strains (*Y. lipolytica* 2495, *P. kudriavzevii* 373, and *P. jadinii* 433) used both substrates. In addition, 2 of these isolates presented alkalizing activity. These properties have a strong influence on the pH, favoring its increase, and, consequently, changes in the cheese microbiota that affect the texture and biochemical processes that

take place during ripening (Irlinger and Mounier, 2009). By contrast, the identified yeast species have the ability to acidify the cheese environment. This capacity was mainly restricted to *K. lactis* and, to a lesser extent, to *Y. alimentaria* 1204. In this work, *K. lactis* strains showed activity of β -galactosidase, a key enzyme in the transformation of lactose to lactic acid. This is a relevant feature that allows them to contribute at the initial stages of ripening, together with LAB, to lactose depletion (Fadda et al., 2004). Moreover, these isolates also produced diacetyl-acetoin in skim milk, which is generally appreciated for its positive contribution to the aromatic profile of cheese (Curioni and Bosset, 2002).

In conclusion, although for the application a starter culture including selected autochthonous strains the regulations of Torta del Casar and Queso de la Serena PDO cheeses have to be modified, the use of such a culture may contribute to guarantee the quality, homogeneity, and unique sensory characteristics of the final products. This study reveals that a high diversity of yeast species is involved in the ripening of the soft raw ewe milk PDO cheeses Torta del Casar and Queso de la Serena. Among the 157 isolates selected on the basis of their genetic profiles, 87 showed good adaptation to the stress conditions of the cheese ripening process. Their technological characterization demonstrated that 9 strains—*K. lactis* 2287, 2725, and 1507; *P. jadinii* 1731 and 433; *Y. alimentaria* 1204 and 2150; *Y. lipolytica* 2495; and *P. kudriavzevii* 373—were not able to cause brown pigmentation and presented biochemical features highly suitable for their potential use as adjunct cultures, alone or in combination with autochthonous starter bacteria in the cheesemaking process, to overcome the heterogeneity of these PDO cheeses, preserving their unique sensory characteristics. Nevertheless, before their industrial application, further studies are necessary in cheese model systems to determine their contribution to an adequate balance of proteolysis, lipolysis, and other reaction products that have a great influence on the texture, aromatic profile, and general quality of the final products.

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