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TESIS DOCTORAL

AISLAMIENTO Y SELECCIÓN DE LEVADURAS Y ENTEROBACTERIAS PARA SU USO EN LA MEJORA DE LAS CARACTERÍSTICAS TECNOLÓGICAS Y FUNCIONALES EN QUESOS TRADICIONALES DE PASTA BLANDA.

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Aislamiento y selección de levaduras y enterobacterias para su uso en la mejora de las características tecnológicas y funcionales en quesos tradicionales de pasta blanda.

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"La conformidad del director/es de la tesis consta en el original en papel de esta Tesis Doctoral"

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"Estadísticamente todo se explica, personalmente todo se complica"

Daniel Pennac

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"*Draco Dormiens nunquam Titillandus"*

J.K. Rowling

La utopía está en el horizonte. Camino dos pasos, ella se aleja dos pasos. Camino diez pasos y el horizonte se corre diez pasos más allá. Por mucho que camine nunca la alcanzaré. ¿Entonces para qué sirve la utopía? Para eso, sirve para caminar.

Eduardo Galeano

A mis padres.

RESUMEN

Este trabajo estudia uno de los productos más característicos de Extremadura, el queso tipo "Torta", que presenta una corteza fina semidura y pasta blanda o semiblanda. Las características de este tipo de queso se obtienen debido a su elaboración tradicional sin la adición de cultivo iniciador utilizando leche cruda de oveja, cuajo vegetal obtenido de la maceración de las flores del cardo *Cynara cardunculus* L. y sal. Se trata de un queso con unas características sensoriales únicas, muy apreciado por los consumidores y producido exclusivamente en Extremadura, donde existen dos áreas de producción, la Comarca de la Serena y los llanos de Cáceres. Está protegido por dos Denominaciones de Origen Protegida, D.O.P. "Queso de la Serena" y "Torta del Casar", que agrupan más de 30 industrias entre las dos áreas de producción. Esto pone de relieve la importancia de la microbiota autóctona como responsable de las características sensoriales del queso, siendo uno de los factores más importantes en la calidad y la autenticidad. Además, esto también determina una gran heterogeneidad en las características del producto final, que son difíciles de controlar por el productor. Las características finales de aroma y textura de este tipo de quesos son producto de complejas reacciones de proteólisis y lipolisis que tienen lugar durante su maduración de al menos 60 días. La microbiota de este tipo de queso está dominada por bacterias acido lácticas, y en menor medida por otros microorganismos como bacterias gram-negativas y levaduras. En los últimos años ha despertado gran interés en la comunidad científica el papel de las levaduras y de algunas enterobacterias en la maduración debido a sus múltiples actividades biológicas. Además, son utilizadas en otras matrices alimentarias como agentes de biocontrol por su capacidad de inhibir a microorganismos alterantes o patógenos. Por tanto, el objetivo principal de este estudio es seleccionar cepas de levaduras y enterobacterias autóctonas con propiedades tecnológicas y funcionales para su posible uso como cultivo iniciador en quesos de pasta blanda tipo "Torta".

Para lograr los objetivos planteados en este estudio, se ha trabajado con muestras representativas de seis industrias de queso tipo "Torta", tres de ellas pertenecientes a la D.O.P "Queso de la Serena" (A, B y C) y otras tres de la D.O.P. "Torta del Casar" (D, E y F). Se realizó un muestreo a lo largo del proceso de maduración (0, 20, 40 y 60 días), en lotes en diferentes épocas del año (invierno y primavera).

En primer lugar, se realizó una caracterización de la microbiota de los quesos mediante técnicas de secuenciación masiva (HTS), empleando la plataforma de

secuenciación MiSeq (Illumina®). Este estudio se llevó a cabo mediante la secuenciación de la región 16S del rRNA para las bacterias y de los espaciadores ITS1-ITS2 del rRNA, para las levaduras y mohos. Los resultados mostraron la prevalencia de las bacterias ácido lácticas durante todo el proceso de maduración de los quesos, representando hasta un 90% del total de lecturas obtenidas. Las principales BAL identificadas fueron *Leuconostoc mesenteroides, Lactococcus lactis* y *Lactococcus raffinolactis*. Esta tecnología también permitió conocer la presencia de otros géneros pertenecientes a la microbiota secundaria, como *Psychrobacter, Serratia, Pseudomonas, Brochothrix, Staphylococcus* y *Hafnia*. Cabe destacar que no se detectaron secuencias asignadas a los principales patógenos alimentarios como *E. coli* enterohemorrágica, *L. monocytogenes* o *Salmonella* spp.

Las principales levaduras identificadas mediante la secuenciación de los espaciadores ITS del rRNA fueron *Yarrowia alimentaria, Y. lipolytica* y *Kluyveromyces lactis*, seguidas de *Debaryomyces* spp., *Pichia* spp., *Geotrichum candidum* y *Rhodotorula* spp. Los géneros *Candida, Cystobasidium, Torulaspora, Meyerozyma, Cyberlindnera, Aerobasidium* y *Penicillium* fueron detectados como minoritarios.

Los resultados de secuenciación masiva mostraron que la población bacteriana está altamente influenciada por la zona de producción de los quesos, mientras que la micobiota está determinada por las condiciones ambientales de la industria y la materia prima empleada.

El análisis de cultivo dependiente reveló que, de forma general, los recuentos de enterobacterias durante el proceso de maduración de los quesos fueron disminuyendo a niveles de 5-6 log ufc/g de queso. El análisis de RAPD-PCR con el cebador M13 reveló una amplia diversidad de especies de enterobacterias a lo largo de la maduración, siendo las especies *Hafnia alvei* y *Hafnia paralvei* las mayoritarias en casi todas las industrias al final del proceso de maduración. Considerando su prevalencia al final del proceso de maduración, se aislaron e identificaron cepas de *Hafnia* spp., a las cuales se les realizaron diversas pruebas para comprobar su seguridad como es la resistencia a antibióticos, la presencia de genes de resistencia a antibióticos, capacidad de producción de aminas biógenas y su capacidad hemolítica y citotóxica. De las 55 cepas aisladas, un total de 22 cepas (8 *H. alvei* y 14 *H. paralvei*) fueron seleccionadas para evaluar su capacidad tecnológica para su posible uso como cultivo iniciador. Los parámetros de proteólisis y producción de gas fueron determinantes, al igual que la evaluación de la capacidad de

crecimiento a diferentes condiciones de pH, % NaCl y temperatura, debido a que durante la maduración del queso se producen distintas condiciones de estrés para los microorganismos. Las cepas *H. alvei* 544, *H. alvei* 970, *H. alvei* 1142 y *H. paralvei* 1414 mostraron propiedades tecnológicas adecuadas para su posible utilización como cultivo iniciador en quesos de pasta blanda. Además, algunas de estas cepas fueron empleadas para la elaboración de quesos modelo tipo "torta" para así evaluar su contribución a las características sensoriales del producto final y su implantación en la matriz del queso.

Por otra parte, los recuentos de levaduras realizados mediante técnicas de microbiología clásica mostraron que están presentes durante todo el proceso de maduración de los quesos, si bien, estos recuentos no superaron los 4 log ufc/g de queso. Se encontraron diferencias significativas entre los productores de las DOP y los lotes elaborados. Se aislaron un total de 508 cepas de levaduras que fueron identificadas mediante ISSR-PCR, dando lugar a un total de 37 OTUS. Para la evaluación de las capacidades tecnológicas, se seleccionaron 157 levaduras en base a su origen y perfil genético. En base a su capacidad de crecimiento en las diferentes condiciones de maduración del queso y a sus propiedades tecnológicas como proteólisis, lipólisis, asimilación de azúcares y capacidad de acidificación y alcalinización, se seleccionaron un total de 9 cepas. Estas cepas, *Kluyveromyces lactis* 2287, 2725, 1507; *Pichia jadinii* 1731 y 433; *Yarrowia alimentaria* 1204 y 2150; *Y. lipolytica* 2495 y *P. kudriavzevii* 373, fueron utilizadas para elaborar quesos modelo tipo "torta" para evaluar su contribución a las características sensoriales del queso a los 15 días de maduración.

Finalmente, se llevó a cabo el estudio de las propiedades funcionales de 54 cepas de levaduras aisladas al final del proceso de maduración de los quesos elaborados en las seis industrias. Todas las cepas mostraron una buena tolerancia al tránsito gastrointestinal, aunque las especies *K. lactis* y *P. fermentans* mostraron una mejor capacidad para sobrevivir a esas condiciones restrictivas. Tras evaluar la capacidad antioxidante, de autoagregación e hidrofobicidad de las cepas, un total de quince cepas fueron seleccionadas por su potencial probiótico. Estas cepas, de las especies *K. marxianus* (3), *P. fermentans* (7), *P. kudriavzevii* (3), *D. hansenii* (1) y *Y. lipolytica* (1), han mostrado propiedades probióticas que, de usarse dentro de un cultivo iniciador, podrían aportar propiedades funcionales al queso tipo "torta".

ABSTRACT

This thesis studies one of the most characteristic products of Extremadura, the "Torta" type cheese, which has a thin semi-hard rind and soft or semi-soft paste. The characteristics of this type of cheese are obtained due to its traditional elaboration without the addition of a starter culture using raw sheep's milk, vegetable rennet obtained from the maceration of the flowers of the thistle *Cynara cardunculus* L. and salt. It is a cheese with unique sensory characteristics, highly appreciated by consumers and produced exclusively in Extremadura, where there are two production areas, the Serena region and the plains of Cáceres. It is protected by two Protected Designations of Origin, P.D.O. "Queso de la Serena" and "Torta del Casar", which group more than 30 industries between the two production areas. This highlights the importance of the autochthonous microbiota as responsible for the sensory characteristics of the cheese, being one of the most important factors in its quality and authenticity. Moreover, this also causes a great heterogeneity in the characteristics of the final product, which are difficult to control by the manufacture. The final aroma and texture characteristics of this type of cheese are the product of complex biochemical changes that take place during their maturation of at least 60 days. Lactic acid bacteria predominate in the microbiota of this type of cheese, and to a lesser extent, other microorganisms such as gram-negative bacteria and yeasts. In recent years, the role of yeasts and some enterobacteria in maturation has aroused great interest in the scientific community due to their multiple biological activities. In addition, they are used in other food matrices as biocontrol agents due to their ability to inhibit spoilage or pathogenic microorganisms. Therefore, this study aims to select autochthonous yeast and enterobacterial strains with technological and functional properties for their potential use as starter culture in "torta-type" soft paste cheeses.

To achieve the objectives set out in this study, representative samples of six cheese industries, three of them from PDO "Queso de la Serena" (A, B and C) and another three from PDO "Torta del Casar" (D, E and F), were selected. Sampling was carried out throughout the ripening process (0, 20, 40 and 60 days), in batches at different times of the year (winter and spring).

First, a characterization of the cheese microbiota was performed using High-Throughput Sequencing (HTS), with a MiSeq sequencing platform (Illumina®). This study was carried out by sequencing the 16S rRNA region for bacteria and the spacers ITS1-ITS2 rRNA for fungi. The results showed the prevalence of lactic acid bacteria

throughout the ripening process, representing up to 90% of the total reads. The main LAB identified were *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Lactococcus raffinolactis*. This technology enabled to know the presence of other genera belonging to the secondary microbiota, such as *Psychrobacter, Serratia*, *Pseudomonas*, *Brochothrix, Staphylococcus* and *Hafnia*. It is noteworthy that sequences assigned to the main food pathogens such as enterohemorrhagic *E. coli* O157:H7, *L. monocytogenes* or *Salmonella* spp. were not detected.

The yeasts species mainly identified by sequencing the ITS1-2 rRNA gene were *Yarrowia alimentaria*, *Y. lipolytica* and *Kluyveromyces lactis*, followed by *Debaryomyces* spp., *Pichia* spp., *Geotrichum candidum* and *Rhodotorula* spp. The genera *Candida, Cystobasidium, Torulaspora, Meyerozyma, Cyberlindnera, Aerobasidium* and *Penicillium* were detected as minorities.

Next Generation Sequencing results showed that the bacterial population is highly influenced by the cheese production area, while the mycobiota is determined by the environmental conditions of the industry and the raw material used.

The culture-dependent analysis revealed that, in general, the *Enterobacteriaceae* counts during the cheese maturation process decreased to levels of 5-6 log cfu/g of cheese. RAPD-PCR analysis with primer M13 revealed a wide diversity of *Enterobacteriaceae* species throughout maturation, with *Hafnia alvei* and *Hafnia paralvei* being the predominant in almost all industries at the end of the ripening process. Considering their prevalence at final stages of ripening, fifty-five strains of *Hafnia* spp. were isolated and identified, which were performed various tests to verify their safety such as antibiotic resistance, the presence of antibiotic resistance genes, their ability to production of biogenic amines and their hemolytic and cytotoxic capacity. A total of twenty-two strains (8 of *H. alvei* and 14 of *H. paralvei*) were selected to evaluate their technological capacity for possible use in a starter culture. The proteolysis and gas production parameters were decisive, as well as the assessment of the growth capacity at different conditions of pH, % NaCl and temperature, since different stress conditions for microorganisms occur during cheese maturation. The strains *H. alvei* 544, *H. alvei* 970, *H. alvei* 1142 and *H. paralvei* 1414 proved suitable technological properties for their possible use as a starter culture in soft cheeses. Furthermore, some of these strains were used to elaborate "torta"

type model cheeses in order to evaluate their implantation in the cheese matrix and their contribution to the sensory characteristics of the final product.

Yeast counts performed by culture-dependent techniques showed that yeasts are present throughout the cheese maturation process, although these counts did not exceed 4 log cfu/g of cheese. Significant differences were found between PDO producers and seasonal batches. A total of 508 yeast strains were isolated and identified by ISSR-PCR, resulting in a total of 37 OTUS. For the evaluation of technological capabilities, 157 yeasts were selected based on their origin and genetic profile. Based on their growth capacity in the different cheese maturation conditions and their technological properties such as proteolysis, lipolysis, substrate assimilation and acidification and alkalization capacity, a total of 9 strains were selected. These strains, *Kluyveromyces lactis* 2287, 2725, 1507; *Pichia jadinii* 1731 and 433; *P. kudriavzevii* 373; *Yarrowia alimentaria* 1204 and 2150 and *Y. lipolytica* 2495 were used to produce model "torta" type cheeses, to evaluate their contribution to the sensory characteristics of the model cheese at 15 days of ripening.

Finally, the study of the functional properties of 54 previously isolated yeast strains was carried out. All the strains showed adequate tolerance to gastrointestinal transit, although the species *K. lactis* and *P. fermentans* showed a better ability to survive these restrictive conditions. After evaluating the antioxidant, auto-aggregation and hydrophobicity capacity of the strains, a total of fifteen strains were selected for their probiotic potential. These strains, of the species *K. marxianus* (3), *P. fermentans* (7), *P. kudriavzevii* (3), *D. hansenii* (1) and *Y. lipolytica* (1), have shown probiotic properties that, if used in a starter culture, they could contribute functional properties to the "torta" type cheeses.

ÍNDICE

INTRODUCCIÓN

1. El queso.

1.1.Definición de queso.

Según el Codex Alimentarius, se entiende por queso el producto blando, semiduro, duro y extraduro, madurado o no madurado, y que puede estar recubierto, en el que la proporción entre las proteínas de suero y la caseína no sea superior a la de la leche, obtenido mediante:

- (a) coagulación total o parcial de la proteína de la leche, leche desnatada/descremada, leche parcialmente desnatada/descremada, nata (crema), nata (crema) de suero o leche de mantequilla/manteca, o de cualquier combinación de estos materiales, por acción del cuajo u otros coagulantes idóneos, y por escurrimiento parcial del suero que se desprende como consecuencia de dicha coagulación, respetando el principio de que la elaboración del queso resulta en una concentración de proteína láctea (especialmente la porción de caseína) y que por consiguiente, el contenido de proteína del queso deberá ser evidentemente más alto que el de la mezcla de los materiales lácteos ya mencionados en base a la cual se elaboró el queso; y/o
- (b) técnicas de elaboración que comportan la coagulación de la proteína de la leche y/o de productos obtenidos de la leche que dan un producto final que posee las mismas características físicas, químicas y organolépticas que el producto definido en el apartado (a).

1.2.Historia y origen del queso.

Se cree comúnmente que el queso evolucionó en una región conocida como la "Media Luna Fértil" o "Creciente Fértil", es decir, a partir de la Ríos Tigris y Éufrates, hasta el río Nilo. Esta región histórica ocupaba parte del levante mediterráneo, Persia y Mesopotamia hace alrededor de 8000 años. Actualmente, este territorio está conformado por la zona sur de Turquía, Israel, Siria, Palestina y Egipto. Fue en esta zona donde comenzó la llamada "Revolución Agrícola" con la domesticación de plantas y animales.

Las ovejas y las cabras fueron los primeros animales lecheros domesticados, según revelan los esqueletos encontrados en algunos restos arqueológicos la zona del suroeste asiático. Probablemente, los humanos pronto reconocieron el valor nutritivo de la leche producido por animales domesticados y creado para compartir la leche de la madre con su descendencia. Existen evidencias de la conservación de esta materia prima

en bolsas de piel transportada por los animales con el fin de cubrir las necesidades diarias de la población (Salque y col., 2013).

Al estar en ambientes cálidos y con balanceos continuos de los animales al andar, la leche terminaba separándose tanto en el suero como en la cuajada. La cuajada se producía por los enzimas presentes en los tejidos de piel en los que era transportada la leche, ya que procedían, en su gran mayoría, del sistema digestivo de los animales que consumían. La cuajada, a fin de preservarla, le añadían sal, dando lugar a los primeros quesos. De esta manera, observaron que a partir de la leche se podía elaborar diferentes alimentos, siendo un complemento o sustituto de la carne en épocas difíciles de caza.

La primera evidencia de la elaboración de queso con coagulante y a partir de él no aparece hasta la Edad de Bronce (Kindstedt, 2012). Esta sabiduría fue propagada a diversos lugares, aunque los primeros escritos en los que aparece la elaboración del queso no aparecen hasta la llegada de la cultura clásica griega. En la Antigua Grecia, el "queso fresco" como ellos le llamaban, era un alimento recurrente en el *opson*, también conocido como la salsa que acompaña a la comida principal que consistía en cereales y pan (Kindstedt, 2018). El queso fresco era, probablemente, un simple cuajado de leche de oveja o cabra (o mezcla de las dos) con coagulante procedente de estos animales, sin cocer ni prensar en su totalidad, con sal en la superficie o en salmuera, al igual que los quesos frescos producidos en las regiones del Mediterráneo Oriental (Kamber, 2007).

Marcus Terencio Varro (c. 116-27 A.C.) describió en su tratado, recogido en *De Agri Cultura*, que el empleo de coagulantes animales fue usado desde el inicio de la producción del queso de manera accidental, pero el uso de coagulantes vegetales, tales como el látex procedente del higo y el vinagre fueron implementándose en la elaboración del queso. Éste último aún sigue empleándose en la elaboración de algunos tipos de queso, como es el Ricotta (Modler, 1988). La cultura romana admiraba la cultura gastronómica griega, siendo cautivados por el queso *Pecorino* rallado, duro y seco. Esta admiración dio lugar al conocido actualmente como queso *Pecorino* siciliano con Denominación de Origen Protegida de la Unión Europea (DOP europea, Reglamento CE n. 1107/96).

Desde entonces, el queso ha sido un componente importante de la dieta humana en muchas partes del mundo debido al intenso tráfico de mercancías que se ha producido desde la época romana, llegando este nuevo tipo de alimento a todas las partes del mundo a lo largo de la historia. Por ello, se han desarrollado y aplicado muchos cambios

tecnológicos y métodos para su fabricación, permitiendo así la aparición de alrededor de 1400 variedades de quesos tradicionales en todo el mundo, mostrando una sorprendente diversidad de aromas, texturas y sabores (Dugat-Bony y col., 2016) (Tabla 1).

Tabla 1. Primeros datos de elaboración de las variedades más importantes de queso (Scott, 1986).

Tipo de queso (País)	**Año**	**Tipo de queso (País)**	**Año**
Gorgonzola (Italia)	897	Parmesano (Italia)	1579
Schabziger (Suiza)	1000	Gouda (Países Bajos)	1697
Roquefort (Francia)	1070	Gloucester (Reino Unido)	1783
Maroilles (Francia)	1174	Stilton (Reino Unido)	1785
Grana Padano (Italia)	1200	Camembert (Francia)	1791
Taleggio (Italia)	1282	St. Paulin (Francia)	1816
Cheddar (Reino Unido)	1500		

Los romanos habrían traído a España estas técnicas de producción de queso cuando la colonizaron en el siglo II a. C. Sin embargo, los quesos se producían incluso antes de su llegada por los Íberos y Celtas, y los arqueólogos descubrieron evidencia de que un queso muy similar al manchego se producía varios siglos antes de que los romanos llegasen a la Península Ibérica. Cada región de España habría desarrollado sus propios estilos de queso, de acuerdo con el terreno, el tipo de animal que prosperó allí y el clima. Una amplia gama de quesos de leche de oveja se desarrolló a lo largo de los años en el interior seco de España, mientras que los pastos más ricos y las montañas verdes de la costa norte y algunas de las islas de España, en la historia más reciente, comenzaron a producir quesos de leche de vaca cremosos. A lo largo de la historia, la cabra ha sido la fuente de lácteos del hombre pobre y el queso fresco de cabra para su consumo inmediato se habría hecho en toda España.

La industrialización de la producción de alimentos en los últimos dos siglos inicialmente tuvo poco efecto en los quesos españoles más conocidos, que continuaron siendo producidos por agricultores y artesanos, como lo habían sido durante cientos de años. Sin embargo, durante la dictadura después de la Guerra Civil española, se prohibió la producción de quesos artesanales en nombre de la modernización y las cuotas industriales. Algunos artesanos pasaron a la clandestinidad y continuaron haciendo sus quesos en los valles remotos de las montañas, pero otros quesos desaparecieron por completo. Fue a partir del comienzo de la democracia en España cuando se pudo volver a los métodos tradicionales de fabricación del queso artesanal.

En Extremadura, la tradición de fabricar quesos artesanales a partir de leche cruda ha estado vinculada a prácticas de transhumancia y pastoreo, obligando a las poblaciones asentadas a aprovechar al máximo cualquier recurso para así sustentar la economía. En la demarcación geográfica de la "*Torta del Casar*" ya existía en 1291 presencia de rebaños y fue por primera vez en 1791, en la obra "*Interrogatorios de la Real Audiencia. Extremadura al final de los tiempos modernos. Partido Judicial de Cáceres*" donde aparece la existencia del queso, que pagaba diezmos, como de las cabezas de ganado que producían leche y lana. También en 1791, en el libro *Viaje a La Serena* de D. Antonio Agúndez Fernández, sobre la comarca de La Serena, sacada de unos manuscritos del Magistrado Cubeles, aparece la siguiente alusión: "Famoso por el sabor y buena hechura es el queso que se hace con la leche de sus oveas, cuya arroba se vende a 60 reales". Además, en las ordenanzas del siglo XVI y XVII para Cabeza del Buey, se habla del diezmo del queso y de la primicia a la Iglesia Parroquial, ya que el dueño del ganado, la primera vez que ordeñaba para hacer queso, todos los que hicieran de ese ordeño, los debían de primicia, llevar a los curas a la Iglesia Parroquial de la villa.

1.3.Clasificación de los tipos de quesos.

La primera vez que se aprobó el Código Alimentario Español fue en 1967, amparado en el Decreto 2484/1967. Es en este documento donde aparece por primera vez regulada la calidad de los quesos producidos en España y su clasificación, dentro del capítulo XV "Leches y derivados". Este decreto se ha ido modificando con el paso de los años y la actualización tanto de las metodologías empleadas como de la seguridad alimentaria. Actualmente, la clasificación de los quesos se encuentra recogida en el Real Decreto 1113/2006, en el que se establece lo siguiente:

Según el origen de la leche:

Los quesos que no tengan una denominación concreta o aquellos que aun teniéndola no estén protegidos por una norma individual de composición y características específicas, que se fabriquen con leche distinta de la de vaca, deberán incluir en su denominación después de la palabra «queso» la indicación de la especie que corresponda. Los quesos elaborados con mezcla de leche de dos o más especies, deberán incluir en su denominación, después de la palabra queso, la indicación de las especies animales de las que proceda la leche en orden descendente de proporciones. Esta denominación podrá reemplazarse por la de «Queso de mezcla».

Atendiendo a su maduración, los quesos se denominarán de la siguiente forma:

- Queso fresco: es el que está dispuesto para el consumo al finalizar el proceso de fabricación.
- Queso blanco pasterizado: es aquel queso fresco en el que el coágulo obtenido se somete a un proceso de pasterización, quedando dispuesto para el consumo al finalizar su proceso de fabricación.
- Queso madurado: es el que, tras el proceso de fabricación, requiere mantenerse durante cierto tiempo a una temperatura y en condiciones tales que se produzcan los cambios físicos y químicos característicos del mismo.
- Queso madurado con mohos: es aquel en el que la maduración se produce, principalmente, como consecuencia del desarrollo característico de mohos en su interior, en la superficie o en ambas partes. Dicha denominación podrá sustituirse por la de «queso azul» o «queso de pasta azul», cuando corresponda.

La palabra madurado podrá sustituirse por los calificativos según el grado de maduración alcanzado por el producto a la salida de fábrica que figuran en la tabla 2.

Tabla 2. Denominación de los quesos en función del tiempo de maduración (RD 1113/2006).

Denominaciones facultativas	Peso > 1,5 kg	Peso ≤ 1,5 kg
	Maduración mínima en días	
Tierno	7	
Semicurado	35	20
Curado	105	45
Viejo	180	100
Añejo	270	

De acuerdo con su contenido en grasa, expresado en porcentaje masa/masa sobre el extracto seco total, los quesos se podrán denominar:

- Extragraso: el que contenga un mínimo de 60 por ciento.
- Graso: el que contenga un mínimo de 45 y menos de 60 por ciento.
- Semigraso: el que contenga un mínimo de 25 y menos de 45 por ciento.
- Semidesnatado: el que contenga un mínimo de 10 y menos de 25 por ciento.
- Desnatado: el que contenga menos de 10 por ciento.

En cuanto al tipo de coagulación que se emplea en la elaboración de este producto, puede ser ácida, debido a los microorganismos presentes en la leche o al cultivo iniciador que se añada a la leche pasterizada. Al consumir los azúcares presentes en la leche (lactosa) producen ácido láctico, disminuyendo el pH de las proteínas (micelas) y modificando su estructura para que se unan entre sí formando el coágulo. Otro tipo de coagulación es la enzimática, mediada por enzimas proteolíticas que pueden tener un origen animal (vaca, pollo y cerdo), de origen microbiano (*Mucor miehei*) o de origen vegetal como es el caso de los pistilos de la flor del cardo, *Cynara cardunculus* L. (cardosina A y B). Este tipo de coagulación no modifica la acidez de la leche, pero sí desestabiliza las micelas de caseínas de la leche, generando un gel láctico que da lugar al queso final (Ordiales y col., 2013a)

Según la región de origen, en España existen alrededor de 150 variedades de queso, de las cuáles 30 pertenecen a una Denominación de Origen Protegida (DOP) o Indicación Geográfica Protegida (IGP) (tabla 3).

Tabla 3. Quesos de España con D.O.P. e I.G.P. (MAPA, 2021)

Tipo de pasta	Nombre	Procedencia Leche	Comunidad Autónoma
Duro	Idiazábal	Oveja	Supra-Autonómica
Semiduro	Mahón-Menorca	Mezcla	Islas Baleares
	Queso Casín	Vaca	Principado de Asturias
	L'Alt Urgell y La Cerdanya	Vaca	Cataluña
	Queso de Acehúche	Cabra	Extremadura
	Queso de Murcia	Cabra	Región de Murcia
	Queso de Murcia al vino	Cabra	Región de Murcia
	Queso Ibores	Cabra	Extremadura
	Queso Majorero	Cabra	Canarias
	Queso Manchego	Oveja	Castilla La Mancha
	Queso Palmero	Cabra	Canarias
	Queso Roncal	Oveja	C. Foral de Navarra
	Queso Zamorano	Oveja	Castilla y León
	San Simón da Costa	Vaca	Galicia
Blanda	Afuega'l Pitu	Vaca	Principado de Asturias
	Arzúa-Ulloa	Vaca	Galicia
	Cabrales	Mezcla	Principado de Asturias
	Cebreiro	Vaca	Galicia
	Gamonedo	Mezcla	Principado de Asturias
	Picón-Bejes-Tresviso	Mezcla	Cantabria
	Queso Camerano	Cabra	La Rioja
	Queso de la Serena	Oveja	Extremadura
	Queso de Valdeón (IGP)	Mezcla	Castilla y León
	Queso Flor de Guía	Mezcla	Canarias
	Queso Los Beyos (IGP)	Mezcla	Supra-Autonómica
	Queso Nata de Cantabria	Vaca	Cantabria
	Queso Tetilla	Vaca	Galicia
	Quesucos de Liébana	Mezcla	Cantabria
	Torta del Casar	Oveja	Extremadura

Atendiendo a la procedencia de la leche, los quesos elaborados con leche de oveja y cabra presentan unas características organolépticas diferentes a las que se pueden percibir en los de leche de vaca. Estas diferencias sensoriales se deben a su composición físico-química, como es el caso del porcentaje de grasa. Actualmente en España existen 6 DOP en las que se elaboran quesos con leche de oveja como son *Idiazábal*, *Manchego*, *Roncal*, *Zamorano* y las 2 DOP de Extremadura, *Queso de la Serena* y *Torta del Casar*.

El Queso DOP *Idiazábal* está elaborado con leche cruda de oveja Latxa y/o Carranzana, teniendo su origen en el norte de España, más concretamente en la región del País Vasco y la Comunidad Foral de Navarra. Es un queso duro de pasta prensada y compacta, con un contenido mínimo de grasa y proteína de un 45%. Su aspecto exterior de forma cilíndrica y con una corteza dura y lisa. Tiene una maduración mínima de 60 días. En el caso de que la leche con la que se ha elaborado procede de la propia explotación, se le puede denominar "Queso de Pastor".

El Queso DOP *Manchego* está elaborado con leche de oveja manchega, pasterizada o cruda. En este último caso, adquiere el nombre de queso artesanal. Es un tipo de queso semiduro prensado con un contenido mínimo de grasa y proteína de 50% y 30%, respectivamente. Su forma es cilíndrica y de caras planas, presentando una corteza dura y de color amarillo pálido o verde-negruzco debido al tiempo de maduración. Su aspecto interior es firme, compacto y de color blanco a blanco amarillento dependiendo de su maduración. El tiempo de maduración puede varias desde los 30 días para quesos de <1.5 kg y de 60 días hasta dos años para quesos de formatos más grandes.

El Queso DOP *Roncal* está elaborado con leche cruda de oveja, principalmente de raza latxa. Se elabora principalmente en el Valle del Roncal, en la Comunidad Foral de Navarra durante los meses de diciembre a julio. Es un queso artesanal y tradicional con un tiempo de maduración mínimo de 4 meses. Es un queso graso y posee un aroma y sabor característicos, con un tono picante leve y de pasta dura.

El Queso DOP *Zamorano* se produce en todos los municipios de la provincia de Zamora, en la comunidad de Castilla y León. La leche procede de la mezcla de oveja Castellana y Churra, que puede emplearse tanto pasterizada como cruda. Es un tipo de queso semiduro, con una maduración mínima de 100 días.

En Extremadura se producen dos quesos de pasta blanda bajo DOP elaborados con leche de oveja, "*Torta del Casar"* y "*Queso de la Serena"*, reconocidos

internacionalmente por su singularidad organoléptica. Su elaboración se sitúa en diferentes zonas geográficas de la comunidad autónoma y están elaborados con leche cruda de oveja, coagulante vegetal procedente de la maceración de la flor del cardo *Cynara cardunculus* L. y sal. Se caracterizan por presentar una textura untuosa, en ocasiones casi líquida, lo que les confiere el nombre de "Torta".

1.3.1. Queso DOP "*Queso de la Serena*".

Es un producto de artesanía rural con un alto consumo a nivel regional, ya que es elaborado en un total de 21 municipios de la comarca de La Serena: Benquerencia de la Serena, Cabeza del Buey, Campanario, Capilla, Castuera, Esparragosa de Lares, Esparragosa de la Serena, El Risco, Garlitos, Higuera de la Serena, La Coronada, La Haba, Magacela, Malpartida de la Serena, Monterrubio de la Serena, Peñalsordo, Quintana de la Serena, Sancti-Spiritus, Valle de la Serena, Zalamea de la Serena y Zarza-Capilla.

Esta zona de producción está protegida por la DOP "*Queso de la Serena*" por Orden de 14 de abril de 1993 del Ministerio de Industria, Comercio y Turismo (BOE Nº 100 DE 27-04-93) y a nivel europeo por el Reglamento CE 1107/1996 de 12-07-96.

Para la fabricación de un kilogramo de este producto se necesita la producción de al menos 15 ovejas, ya que su producción diaria de leche no supera los 0,35 litros. Se emplea exclusivamente leche cruda de oveja de raza merina y su proceso de coagulación es llevado a cabo con coagulante vegetal procedente del cardo *Cynara cardunculus* L.

Presenta una forma discoidal con caras sensiblemente planas y de corteza semidura de color amarillo céreo a ocre. Su peso suele oscilar entre los 750 gramos hasta los 2 kg de peso, con una altura entre 4 y 8 centímetros y un diámetro entre 10 y 24 centímetros. Las piezas que presentan una pasta más dura se las denomina quesos, mientras que las que las que son blandas o semiblandas, se les llama "tortas". Es un queso graso o extragraso cuya maduración es llevada a cabo por los microorganismos presentes en la leche y en la infusión del cardo. En cuanto a sus características físico-químicas, este producto debe contener al menos un 50% de grasa mínima sobre el extracto seco, un mínimo de 50% de extracto seco, un pH entre 5,2 y 5,9 y un mínimo de 35% de proteína total sobre extracto seco.

Respecto a las características organolépticas, el Reglamento DOP (1993) establece que la textura al tacto debe ser untuosa en los quesos muy blandos y nada rugosa ni elástica. La textura en la boca debe ser de firmeza débil, blanda incluso líquida, cremosa y soluble. Su olor debe tener una intensidad media según la edad del queso y la época del año, que recuerda a la familia de los lácteos (yogur o nata dulce), a la familia animal (leche de oveja) y a la familia vegetal (heno, hierba mojada, etc.). En cuanto al sabor, debe ser algo ácido, nada salado y dependiendo de la edad del queso, de amargo a muy amargo.

Con respecto al etiquetado, el Pliego de Condiciones de la DOP establece que "Las etiquetas comerciales, propias de cada firma comercial inscrita, debe ser aprobadas por el Consejo Regulador. Figurará obligatoriamente en ellas la mención: Denominación de Origen "*Queso de la Serena*". El producto destinado al consumo irá provisto de contraetiquetas numeradas y expedidas por el Consejo Regulador, que serán colocadas en la industria inscrita y siempre que no permita una nueva utilización de las mismas" (Figura 1).

Figura 1. Queso de la Serena. Fuente: quesoserena.com

1.3.2. Queso DOP "*Torta del Casar*".

El término "Torta del Casar" se debe a su origen, ya que su elaboración comenzó a realizarse en Casar de Cáceres, a 10,6 km al noroeste de la ciudad de Cáceres. Actualmente la zona de producción se ha expandido hasta 36 municipios dentro de las comarcas de Los Llanos de Cáceres, Sierra de Fuentes y Montánchez: Albalá, Alcuéscar,

Aldea del Cano, Aliseda, Almoharín, Arroyo de la Luz, Arroyomolinos, Benquerencia, Botija, Brozas, Cáceres, Casar de Cáceres, Casas de Don Antonio, Garrovillas, Herreruela, Hinojal, La Cumbre, Malpartida de Cáceres, Monroy, Montánchez, Navas del Madroño, Plasenzuela, Ruanes, Salvatierra de Santiago, Santa Ana, Santa Marta de Magasca, Santiago del Campo, Sierra de Fuentes, Talaván, Torremocha, Torreorgaz, Torrequemada, Torre de Santa María, Valdefuentes, Valdemorales y Zarza de Montánchez.

La Denominación de Origen fue aprobada por Orden 9 de octubre de 2001 y modificado por Orden de 12 de marzo de 2002, de la Consejería de Economía, Industria y Comercio de la Junta de Extremadura. Posteriormente, el Reglamento fue aprobado por el Decreto 10/2017 de la Consejería de Medio Ambiente y Rural, Políticas Agrarias y Territorio. Además, ha sido ratificado a nivel estatal por la Orden APA/1144/2002 del 6 de mayo (BOE núm. 123 del 23 de mayo de 2002) y a nivel europeo europeo por el Reglamento (CE) 1491/2003 y Reglamento de Ejecución (UE) 2015/2196 por la Comisión Europea. Todos los quesos expedidos bajo el amparo de la DOP *Torta del Casar* llevarán las correspondientes etiquetas de certificación (Figura 2).

Se trata de un queso de pasta blanda elaborado con leche cruda de oveja de las razas merina y entrefina, con un tiempo de maduración mínimo de 60 días. Al igual que el "*Queso de la Serena*", su proceso de coagulación se realiza con la infusión de las flores desecadas del cardo *Cynara cardunculus* L. Al igual que en el proceso de elaboración del "*Queso de la Serena*", en la "*Torta del Casar*" no se añaden cultivos starter.

El producto final debe presentar una forma cilíndrica con caras sensiblemente planas y superficie perimetral plano-convexa y aristas redondeadas. En cuanto a las dimensiones, deberán presentar un diámetro mínimo de 7 cm, con una relación entre altura y diámetro máxima de un 50%. Con respecto al peso de las piezas, se establecen tres rangos:

i) Grande, de 801 – 1.100 grs.

ii) Mediano, de 501 – 800 grs.

iii) Pequeño, de 200 – 500 grs.

En cuanto a sus características físico-químicas, este producto debe presentar un mínimo de 50% de grasa sobre extracto seco, mínimo de 50% de extracto seco, pH mínimo de 5,20 y máximo de 5,90 y un contenido de sal no superior al 3%.

Con respecto a sus características organolépticas, el Decreto 10/2017 establece que la corteza debe ser semidura, definida y diferenciada de la pasta, con color uniforme de tonalidades ocres sin adición de colorantes, con presentación tradicional untada en aceite, pudiendo presentar pequeñas grietas en su superficie. La pasta debe tener una consistencia de blanda a muy blanda, de color blanco a amarillento. La estructura será uniforme, pudiendo presentar ojos redondeados propios de la maduración repartidos en el corte. Una cualidad fundamental y diferencial de este queso es la textura que presentará cremosidad moderada o alta, carácter graso, fundente, y granulosidad suave o nula. Por último, el olor será de intensidad media o baja de la familia láctica y/o vegetal, con sabor amargo medio o bajo, salado bajo, y acidez baja o nula.

Con respecto a la etiqueta, el Pliego de Condiciones de la DOP establece que "el etiquetado final estará compuesto por la etiqueta comercial de la Industria Quesera y la etiqueta de certificación, y se situará sobre cada queso calificado o fracción del mismo de forma inseparable e indeleble, siendo el fabricante responsable final de su buen uso" (Figura 2).

Figura 2. Torta del Casar, Virgen del Prado. Fuente: queseriadonafrancisca.com

1.3.3. Otros quesos de oveja de pasta blanda.

En la Península Ibérica se elaboran, además de los quesos de pasta blanda de Extremadura, quesos con procesos de elaboración tradicional similares a "*Torta del Casar*" y "*Queso de la Serena*", amparados bajo DOP en Portugal. Los quesos portugueses más conocidos son "*Serra da Estrela*", "*Queijo Serpa*" y "*Azeitao*".

El Queso "*Serra da Estrela"*, a menudo llamado queso Serra, es la variedad, tanto económica como organoléptica, mas importante de queso tradicional elaborado en Portugal. El área geográfica de producción y procesamiento de la leche, maduración, almacenaje, corte y embalaje de queso se limita a 10 municipios de la zona centro este de Portugal: Carregal do Sal, Celorico da Beira, Fornos de Algodres, Gouveia, Mangualde, Manteigas, Nelas, Oliveira do Hospital, Penalva do Castelo e Seia; así como a parroquias de otros 8 municipios: Aguiar da Beira, Arganil, Covilha, Guarda, Tábua, Tondela, Trancoso y Viseu. Este tipo de queso se elabora mediante la coagulación de leche de oveja de la raza Serra da Estrela Bordaleira y/o Churra Mondegueira, con extracto de flores de cardo *Cynara cardunculus* L. El tiempo mínimo de maduración es de 30 días, pero cuando el tiempo de maduración alcanza los 120 días, el queso pasa a denominarse "Velho" (curado).

Las características del Queso "*Serra da Estrela"* están reguladas por el Reglamento (CE) nº 510/2006. Este queso de define por tener una forma y consistencia cilíndrica regular y ligeramente abultado en la cara superior sin bordes definidos y semi blando. Sus dimensiones de peso están entre 500 y 1,700 gramos, con un diámetro entre 9 y 20 cm y altura de 4 a 6 centímetros. Su maduración mínima es de 30 días. En cuanto a la textura y color, es cerrado, de color mantequilla, medio corte, extremos deformables, cremoso y untuoso, con pocos o ningún ojos, blancos o ligeramente amarillentos. Presenta un aroma dulce, limpio y ligeramente acidulado. Por otra parte, el queso "*Serra da Estrela Velho*" presenta una forma y consistencia cilíndrica regular, corteza ligeramente arrugada y dura y ausencia de bordes. Su peso está comprendido entre 700 y 1.200 gramos, con un diámetro variable entre 11 y 20 cm y altura de 3 a 6 centímetros. Su maduración mínima de 120 días. En cuanto a su textura y color, cerrado o con unos ojos, masa ligeramente quebradiza y seca, graso, color amarillento a anaranjado acastañado, por el pimentón, que se desarrolla desde la periferia al centro. Aroma persistente y agradable, limpio, ligeramente fuerte o fuerte, y ligeramente picante y salado.

El "*Queijo Serpa"* es un queso originario del Alentejo (Portugal). Recibe su nombre de la comarca del Serpa, un área que incluye casi todo el distrito de Beja y cinco "freguesias" de tres "concelhos" del distrito de Setúbal. La zona de producción tradicional del "*Queijo Serpa"* estaba vinculada al desplazamiento de los rebaños al sur, hacia la Sierra de Serpa y hacia la región de Campo Branco (Castro Verde) (Lopes, 1985). La marca de certificación DOP "*Queijo Serpa*" sólo puede ser aplicada en quesos de lotes producidos con leche cruda de oveja de raza merina, obtenido en la región DOP, coagulado con un extracto de cardo (*Cynara cardunculus* L.). Este queso debe su nombre a un pueblo situado en el lado izquierdo del río Guadiana dado que su zona de producción está demarcada en el bajo Alentejo, al sur de Portugal entre los ríos Tajo y Guadiana (Araújo-Rodrigues y col., 2020; Roseiro y col, 2003). La zona de producción del queso coincide con las regiones de Mértola, Beja, Castro Verde, Almodôvar, Cuba, Ourique, Moura, Serpa, Vidigueira, Aljustrel, Ferreira do Alentejo y Alvito (Decreto Reg. Nº 39/87).

Las características de este queso están reguladas por el Decreto Reglamentario nº 39/87. Se define como un queso curado, de pasta semi-blanda, con un contenido de humedad del 61% al 69% y un contenido de grasa de 45% a 60%. Tiene forma abombada, regular con abultamiento lateral y un poco en la cara superior, sin bordes definidos. Tiene unas dimensiones variables con diámetros entre 10 y 30 cm, alturas entre 3 y 8 cm y pesos entre 200 y 2500 g, yendo del tamaño pequeño al gigante. La corteza presenta una consistencia suave, con una apariencia entera, bien formada, ligeramente rugosa y un color amarillo pajizo claro y uniforme. La pasta presenta una textura mantecosa, con zona de corte fácilmente deformable pudiéndose derramar, de aspecto untuosa, con pocos o ningún ojo y un color blanco amarillento o amarillo paja, oscureciéndose en contacto con el aire. Respecto al aroma y sabor, generalmente fuerte con dominancia del sabor picante. En cuanto a la maduración, el queso "*Serpa*" debe tener una maduración de un mínimo de 30 días a una temperatura entre 6 y 12ºC y una humedad relativa entre 85-90%. Aunque sea un queso madurado, por sus características después de su elaboración requiere condiciones especiales de conservación, en el almacén entre 0 y 5ºC, durante el transporte entre 0 y 10ºC y en el punto de venta entre 0 y 10ºC.

El "*Queijo de Azeitao*" está protegido por el Reglamento comunitario nº 1107/06. Según se describe en este Reglamento, el área geográfica definida para la producción de "*Queijo de Azeitão*" comprende condados de Palmela, Setúbal y Sesimbra, situados en la

Sierra de Arrabida. Es en esta sierra donde se recrean las condiciones necesarias para que las ovejas pasten a lo largo de las colinas y los valles de la península de Setúbal, que presenta una extraordinaria profusión floral. Es un queso curado, semi-blando, mantecoso, con pocos o ningún ojo, ligeramente amarillo, obtenido por el lento drenaje de la cuajada después de la coagulación de la leche cruda de oveja, del área geográfica de producción, por acción propia de una preparación de cardo (*Cynara cardunculus* L.) y sal añadida. Presenta una forma de cilindro bajo (placa), regular con abultamiento lateral y también en el lado superior, sin bordes perfectamente definidos, con una corteza de consistencia maleable y muy suave, lisa y delgada, con un color amarillo uniforme. En cuanto a la pasta, presenta una textura cerrada y mantecosa, con una zona de corte fácilmente deformable y cremosa, con pocos o ningún ojo. El color de la pasta es blanco o ligeramente amarillo. Este queso se caracteriza por tener un sabor ligeramente picante, mezclado con aroma acidificado y salado. El tiempo mínimo de maduración es de 16 días, ya que se elabora en dos fases que duran entre 8-12 días cada una.

2. Datos socioeconómicos.

2.1. Producción de queso a nivel mundial, nacional y regional.

En los últimos años, los mayores productores de queso de oveja a nivel mundial son los países de la cuenca del Mediterráneo: Grecia, Italia, España, Siria, Turquía y Francia (Figura 3). En 2018, Grecia lideró la producción de queso elaborado con leche de oveja con un 19,7% seguida de Italia (12,1%) y España (10,8%). En los últimos años, destaca China en cuanto a la producción de queso de oveja debido a la incipiente industria quesera en el país oriental.

En ese mismo año, en Europa se elaboraron 412.770 toneladas de queso de oveja, liderando la producción Grecia (34,7%), Italia (21,3%), España (19,1%) y Francia (9,6%). La producción estacional de leche de oveja genera grandes variaciones en la producción de queso. Los quesos elaborados con leche de oveja se producen mayoritariamente entre diciembre y junio, mientras que la producción de leche aumenta en primavera y desciende entre julio y noviembre.

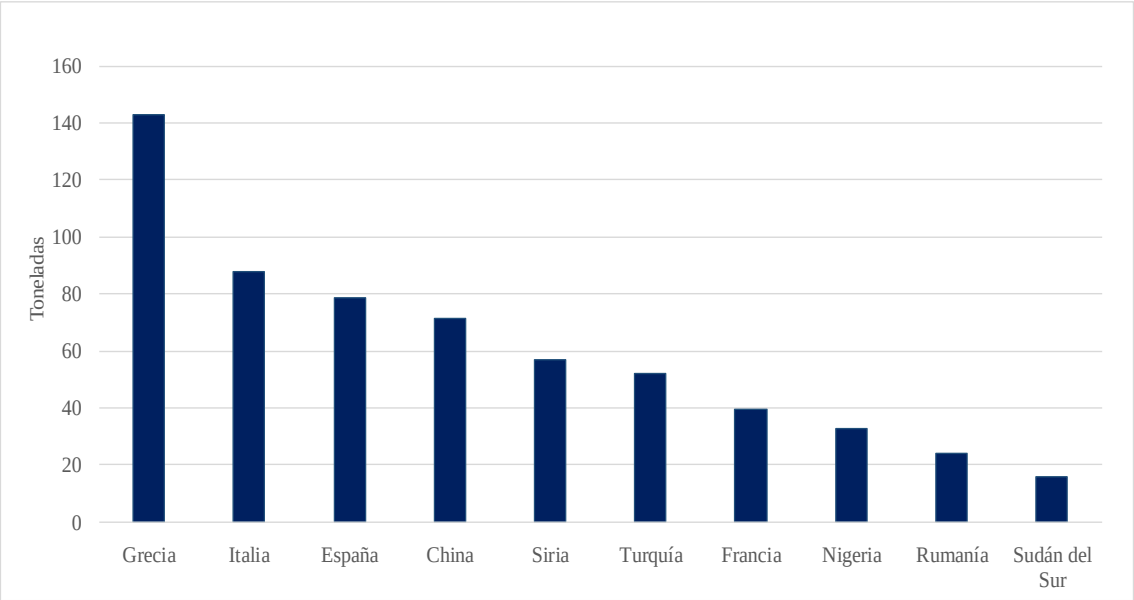


Figura 3. Principales productores de queso de oveja a nivel mundial en el año 2018 (FAOSTAT, 2021).

En 2018 en España, según los datos del Ministerio de Agricultura, Pesca y Alimentación (MAPA), la producción de leche para autoconsumo y producción de derivados lácteos alcanzó las 8.123,1 mil toneladas. En función del origen, la leche de oveja supuso un 6,7% de la producción anual con 544,6 mil toneladas. La producción de queso alcanzó las 474,7 mil toneladas, siendo un 1,33% inferior al año 2017, en el que se elaboraron 481,1 mil toneladas de queso. En función del origen de la leche, un 14,66% fue de queso puro de oveja frente al 37,67% de vaca, el 13,53% puro de cabra y un 34,13% de mezcla de los distintos tipos de leche. En cuanto a los tipos de queso, se elaboraron 54.600 toneladas de queso de pasta blanda (11,5%); 40.300 de pasta semiblanda (8,5%); 103.000 de pasta semidura (21,69%); 83500 de pasta dura (17,59%); 5.200 de pasta extradura (1,1%) y 188.100 toneladas de queso fresco (39,62%). La figura 4 muestra el incremento de la producción de queso de oveja en España en los últimos años.

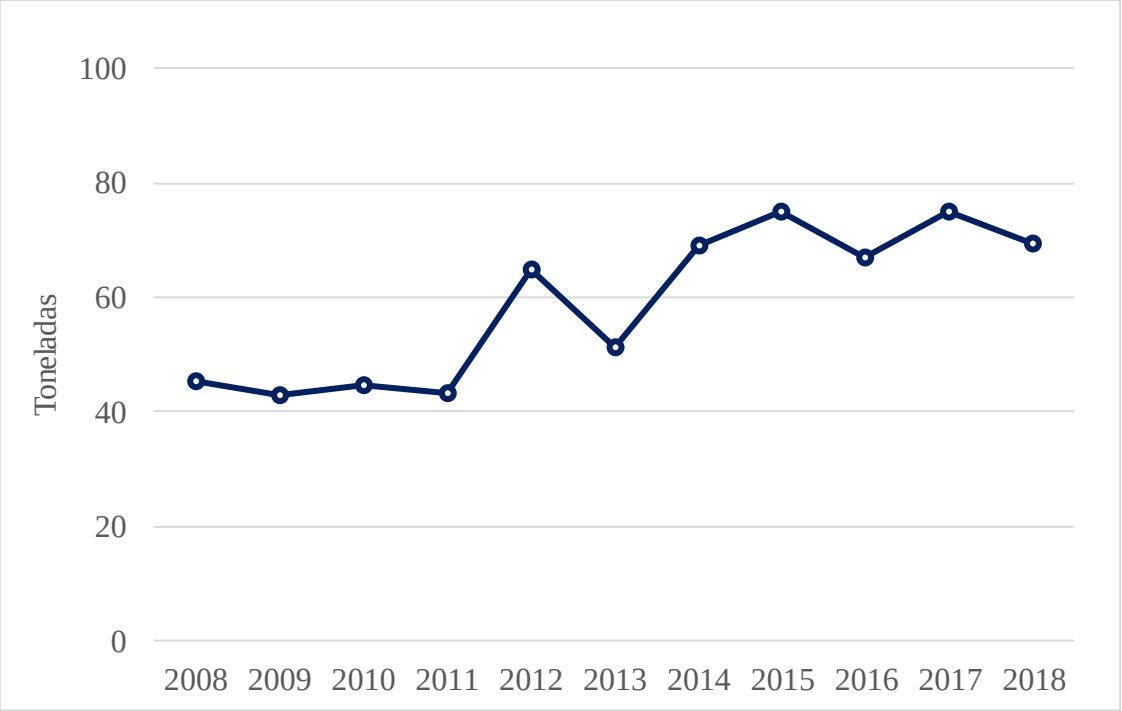


Figura 4. Producción de queso de oveja en España en el intervalo 2008-2018 (MAPA, 2021).

En cuanto a las Comunidades Autónomas, la producción de leche de oveja está liderada por Castilla y León (54,4%), seguida de Castilla-La Mancha (32%), Navarra (2,89%) y Extremadura (2,33%). Con respecto a la producción de quesos de oveja bajo DOP, la tabla 4 presenta los datos del año 2018 de queserías inscritas y producción de queso de oveja de cada una de las Denominaciones de Origen Protegida de España.

Tabla 4. Datos de producción de las DOP de queso de oveja en el año 2018 (MAPA, 2021).

DOP/IGP	Cabezas	Explotaciones	Queserías inscritas	Litros destinados a su elaboración	Volumen (toneladas)
Idiazábal	122.000	394	121	9.123.298	1.358
Queso de la Serena	100.000	115	14	420.367	93
Queso Manchego	561.519	706	67	77.927.289	15.425
Queso Zamorano	31.500	58	8	1.130.452	217
Roncal	38.673	136	5	2.451.376	385
Torta del Casar	18.900	29	7	1.942.569	350

En Extremadura, la DOP "*Torta del Casar*" consta de siete queserías certificadas para producir queso tipo "torta". A lo largo de los años, ha visto incrementada su producción de quesos certificados debido a la demanda del consumidor. En 2018, la DOP estaba formada por 29 explotaciones con 18900 cabezas de ganado, produciendo casi 2 millones de litros de leche para la elaboración de queso certificado. La evolución interanual desde el año 2015 hasta el año 2018 de la producción total de queso de oveja y la cantidad de quesos tipo "torta" certificados de esta DOP mostró una estabilidad en la producción total, sin embargo, la producción de queso certificado muestra una tendencia al alza, alcanzando el 92% de la producción en el año 2018 (Tabla 5). En el año 2018, casi la totalidad de la leche de oveja entregada a las industrias fue empleada para producir este tipo de producto.

Tabla 5. Evolución interanual de producción queso "Torta del Casar" (DOP "Torta del Casar").

	2015	**2016**	**2017**	**2018**
Producción Total	377.854	371.050	387.407	377.670
Total Kg queso certificados	291.191	294.371	303.467	350.128
% certificado / total producido	77,06%	79,33%	78,33%	92,71%

Debido al incremento de la demanda y el cambio en el gusto del consumidor, la DOP "*Torta del Casar*" ha aumentado la producción de quesos con formato mediano de un peso de 0,5 kg en detrimento del formato de 1 kg (Figura 5). Desde 2010, también elaboran quesos con un formato más pequeño (S) de 250 g.

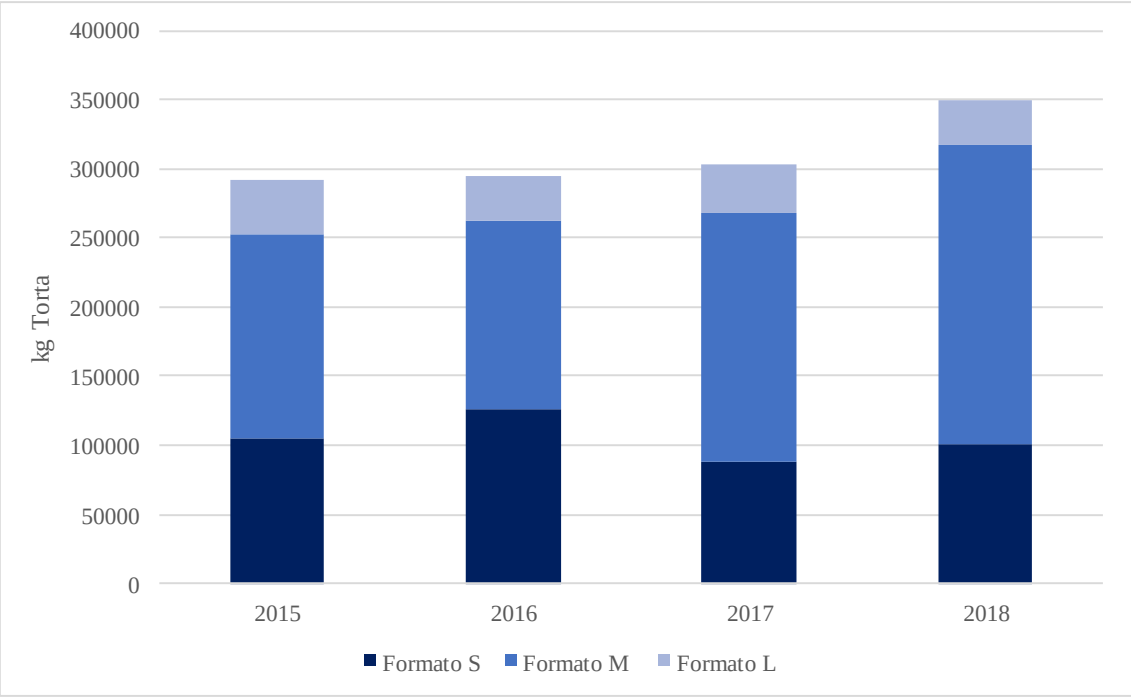


Figura 5. Cantidad de queso (kg) certificado por la DOP "Torta del Casar" según formato (DOP "Torta del Casar").

Las ventas de "*Torta del Casar*" en el mercado nacional ha ido aumentando de forma progresiva desde el año 2015, alcanzando el máximo en 2018 con 334.663 kg de queso vendido en España. Al igual que en el mercado nacional, las exportaciones y ventas de queso en el mercado internacional también han ido incrementando, llegando a 15.465 kg de queso vendido en el año 2018 (Tabla 6). Los países europeos absorbieron el 88,74% de las ventas internacionales, mientras que el continente americano adquirió un 5,29% y el resto de los países del mundo, un 5,96% (Figura 6).

Tabla 6. Datos de venta de queso "Torta del Casar", en kg, en el mercado nacional e internacional (MAPA, 2021).

Ventas (kg)	2015	2016	2017	2018
Mercado nacional	281.627	284.270	291.045	334.663
Mercado internacional	9.564	10.101	12.422	15.465
Total	291.191	294.371	303.467	350.128

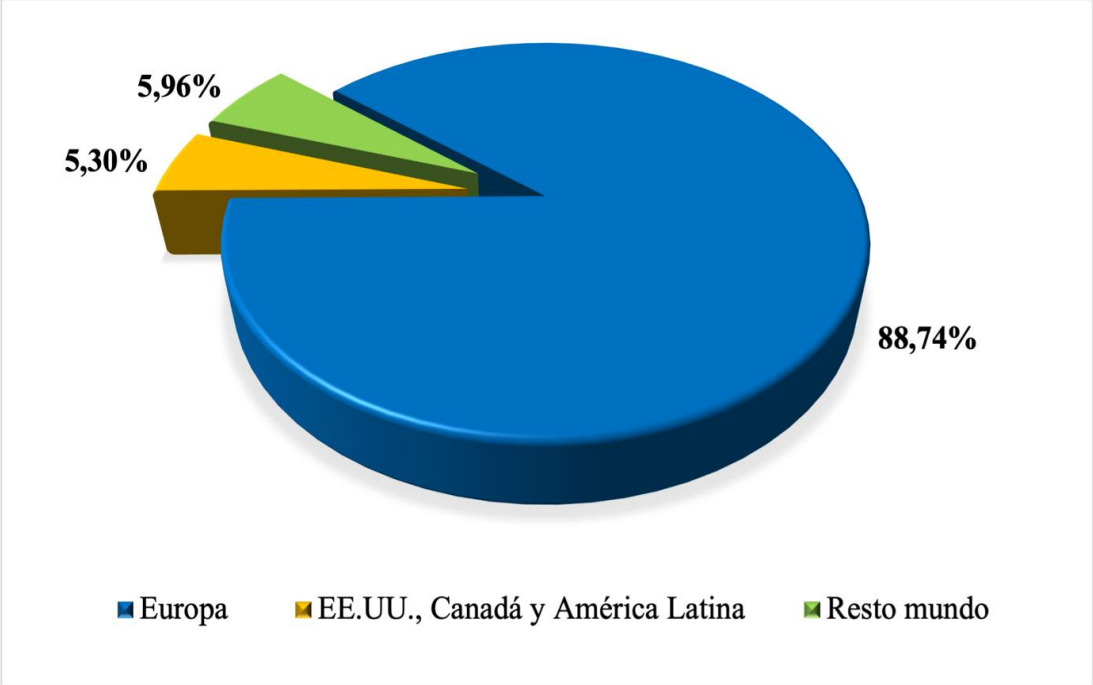


Figura 6. Distribución de ventas internacionales de "Torta del Casar" en el año 2018 (MAPA, 2021).

La DOP "*Queso de la Serena*" ha visto disminuido el número de queserías certificadas que producen queso de oveja en la Comarca de la Serena, pasando de diecisiete queserías en 2016 a catorce en 2018. En los últimos años su producción de queso ha disminuido, aunque en 2018 la producción de queso certificado se incrementó hasta 92711 kg (Figura 7).

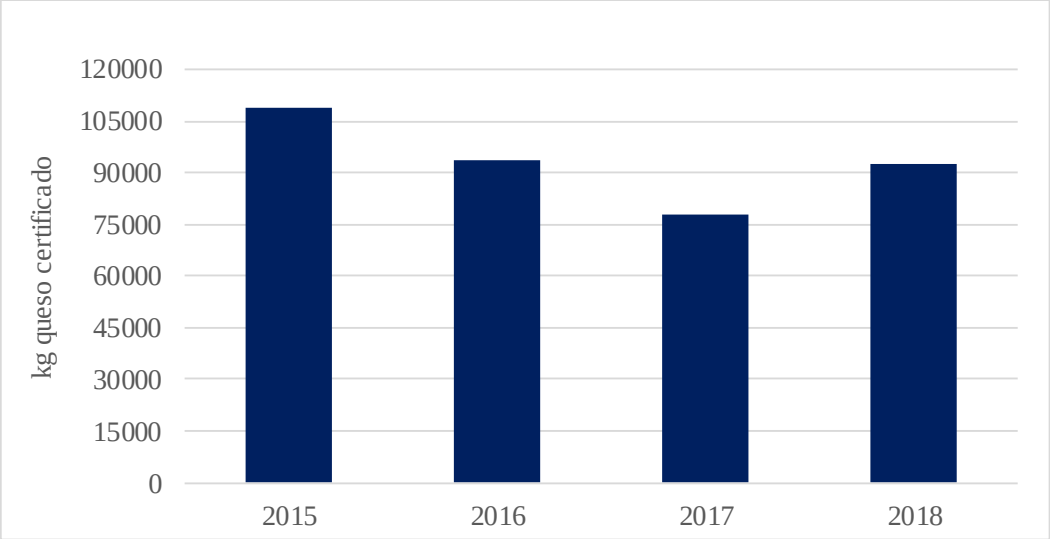


Figura 7. Evolución de la producción interanual de quesos DOP "Queso de la Serena" (MAPA, 2021).

Como se observa en la figura 8, el principal mercado para este queso es el nacional alcanzando en 2018 un 75% de las ventas frente a un 20% en Europa y un 5% en países terceros como EE.UU. En los últimos años, el año de mayor venta en el mercado nacional fue 2015 con 81.552 kg de queso, mientras que fue en el año 2016 cuando la venta de queso certificado en Europa alcanzó su mayor valor con 23.420 kg.

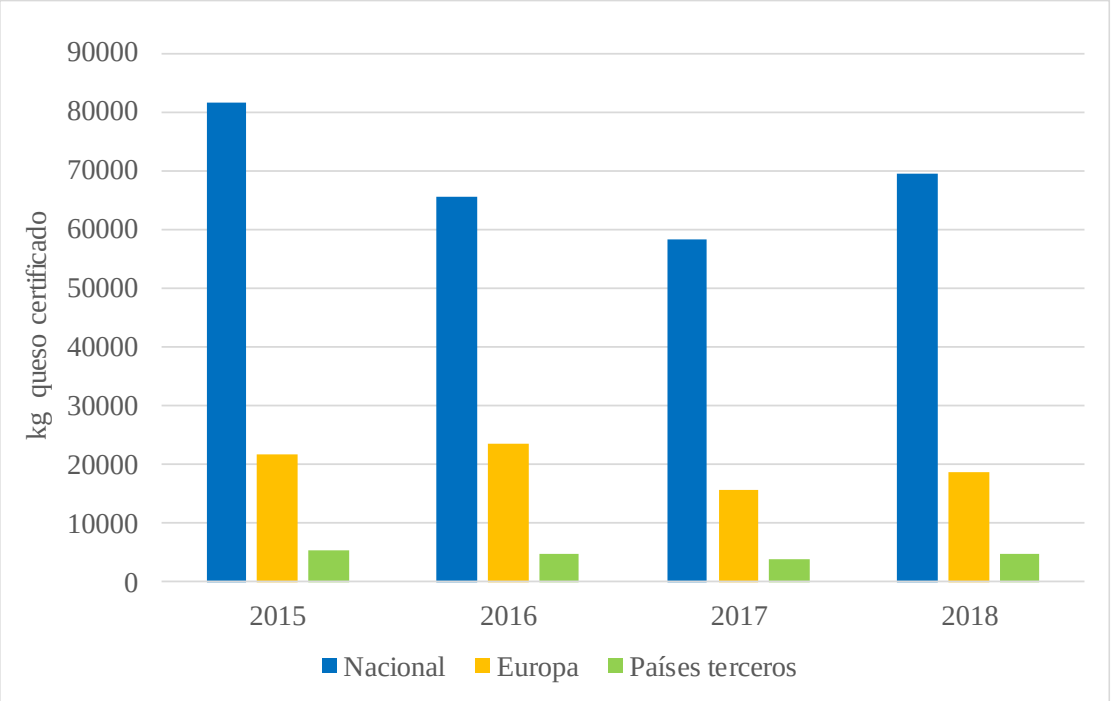


Figura 8. Distribución de ventas internacionales de "Queso de la Serena" en el período 2015-2018 (MAPA, 2021).

En el período 2015 a 2018, de forma comparativa la DOP "*Torta del Casar*" produjo un mayor número de quesos certificados que la DOP "*Queso de la Serena*" a pesar de que el número de queserías inscritas es superior en esta última. En el mercado nacional e internacional, ambas DOP están incrementando la distribución de piezas certificadas que dedican al mercado internacional, aunque el mercado nacional sigue en auge debido al interés del consumidor por los quesos tradicionales. Las características únicas de estos quesos de elaboración tradicional atraen al consumidor final, generando una estabilización y expansión de mercado de este tipo de producto, logrando afianzarse en los hábitos de compra a nivel tanto nacional como internacional.

3. Elaboración y maduración de la Torta.

3.1.Diagrama de flujo del proceso de elaboración.

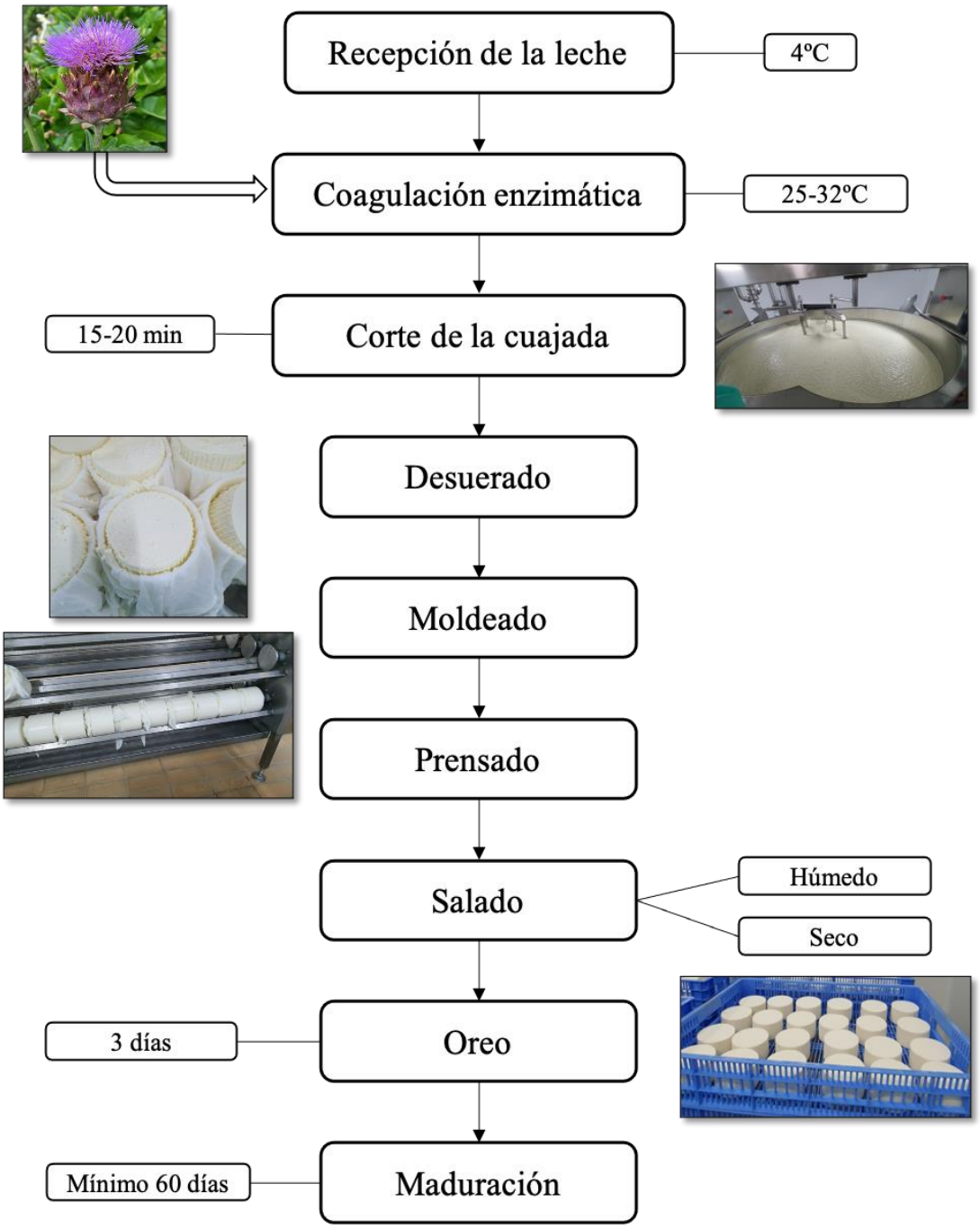


Figura 9. Diagrama de flujo del proceso de elaboración de quesos Torta del Casar y Queso de la Serena según sus Pliegos de Condiciones.

3.2. Proceso de elaboración.

Recepción de la leche

La leche empleada durante el proceso de elaboración del "*Queso de la Serena*" y "*Torta del Casar*" debe ser, según el Reglamento DOP (1993 y 2001, respectivamente), un producto natural íntegro obtenido del ordeño de ovejas sanas de las ganaderías inscritas. La leche debe ser de raza merino para la DOP "*Queso de la Serena*" y de raza merino y entrefino para la DOP "*Torta del Casar*". La leche debe estar limpia, sin impurezas y exenta de calostros. Además, debe estar exenta de inhibidores, antibióticos o productos medicamentosos, conservantes, etc., que puedan influir negativamente en la maduración y conservación del queso, así como en las condiciones higiénicas y sanitarias del mismo. La leche debe presentar como mínimo un 5% de proteína, 6,5-7% de materia grasa y 18% de extracto seco total. Además, la "*Torta del Casar*" incluye una acidez máxima de 22º Dornic, un extracto quesero mínimo de 11% y un pH mínimo de 6,60 y máximo de 6,90. El período máximo de conservación desde el ordeño hasta su transformación no podrá superar las 72 horas, debiendo permanecer la leche entre 1ºC y 6ºC, siempre en tanques de frío exclusivamente destinados a leche procedente de ganaderías inscritas, y situados en las instalaciones declaradas por los operadores.

Coagulación enzimática

El proceso de coagulación se llevará a cabo mediante coagulante vegetal procedente de la infusión de las flores desecadas del cardo *Cynara cardunculus* L. Este proceso se realizará a una temperatura entre 25 y 32ºC para el "*Queso de la Serena"* y 28 y 32ºC para "*Torta del Casar"* durante aproximadamente una hora (Reglamento CE 1491/2003). Aunque la duración de este proceso no está estandarizada y es decisión del maestro quesero, pero suele tener una duración entre 50 y 80 minutos.

Corte de la cuajada

Una vez realizada la coagulación de la leche, es necesario separar el suero de la cuajada. Para ello, se realizarán cortes sucesivos de la cuajada durante aproximadamente 15-20 minutos hasta obtener granos de tamaño fino de aproximadamente 10 a 20 milímetros de grosor. Este corte se realiza mediante una máquina con cuchillas que giran dentro del tanque de la cuajada. La cuajada se corta para aumentar la superficie de contacto y así, facilitar la separación del suero de la cuajada.

Desuerado, moldeado y prensado

La cuajada es introducida en un filtro para separar el suero de la misma, previamente a ser introducida en los moldes El moldeado se realizará introduciendo la cuajada en moldes cilíndricos con las dimensiones adecuadas para que los quesos, una vez madurados, presenten la forma, tamaño y peso que son peculiares en los mismos. Las paredes de estos moldes presentan ciertas perforaciones para que el suero residual difunda. La cuajada es sometida posteriormente a un prensado mediante equipos adecuados para este fin manteniendo una presión comprendida entre los 1 y 3 kg/cm^2 durante un máximo de 8 horas.

Salado

La salazón será húmeda o seca, utilizándose exclusivamente cloruro sódico. En los quesos "*Torta del* Casar" se suele utilizar una salazón húmeda, en la que el tiempo de permanencia estará entre 5-6 horas, en una solución salina de concentración aproximada de 16%. En cambio, en el "*Queso de la Serena"* está más extendido el uso de la salazón en seco.

Oreo

Una vez finalizado el proceso de salado, los quesos que han estado sumergidos en salmuera estarán completamente húmedos, mientras que los que han sido salados en seco presentarán una humedad más reducida. Las piezas se almacenarán en una cámara de oreo que tiene por objeto reducir la humedad de las piezas, y en la que permanecerán aproximadamente tres días.

Maduración

Este tipo de quesos, al estar elaborados con leche cruda, deben presentar una maduración mínima de 60 días, siempre que cumpla la normativa sanitaria vigente. Los quesos permanecerán en cámaras de maduración entre 4 y 12ºC para "*Torta del Casar*" y entre 4 y 15ºC para el "*Queso de la Serena*", con una humedad comprendida entre 75 y 90%. Además, podrán emplearse compuestos antifúngicos en la superficie para inhibir el crecimiento de mohos alterantes que puedan ocasionar desperfectos en las piezas.

Durante el proceso de maduración se producen cambios bioquímicos asociados a una bajada de pH por la acción de la microbiota endógena, además de otros procesos como la proteólisis inducida por las enzimas del coagulante vegetal.

3.3.Maduración: cambios bioquímicos y dinámica microbiana.

El queso es el resultado de procesos físicos, químicos y microbiológicos y su proceso de maduración engloba distintos cambios bioquímicos como proteólisis, lipolisis y glicolisis, responsables del flavor y textura del producto final (Gobetti y col., 2015; McSweeney y col., 2017, Sousa y col., 2001). Muchas de las características químicas y organolépticas de los quesos dependen de los procesos bioquímicos de los microorganismos presentes en la matriz. Las bacterias encontradas al final del proceso de maduración presentan mayor capacidad, que otros microorganismos, de utilizar substratos, adaptar su metabolismo a las variaciones físico-químicas del queso y, en consecuencia, competir con otros microorganismos durante el proceso de maduración (Ianni y col., 2020; Schirone y col., 2018).

3.3.1. Glicolisis de lactosa residual, catabolismo del lactato y metabolismo del citrato.

La fermentación de la lactosa residual se lleva a cabo principalmente por las BAL naturalmente presentes en la leche, aunque otros microorganismos como levaduras y coliformes pueden participar en este proceso. Durante las dos primeras semanas de maduración del queso, la lactosa es metabolizada a ácido D o L- láctico mediante fermentación homoláctica (por *Lactococcus* spp.) o heteroláctica (por *Leuconostoc* spp.). En la fermentación homoláctica, la lactosa se separa en galactosa y glucosa para finalmente obtenerse ácido L-láctico. En la heteroláctica, la glucosa y galactosa siguen diferentes rutas metabólicas para dar lugar a ácido láctico, acetato, etanol y CO_2 (Fox y col., 2017). El metabolismo del ácido láctico puede generar diferentes compuestos, como son acetato, etanol y CO_2 (Figura 10):

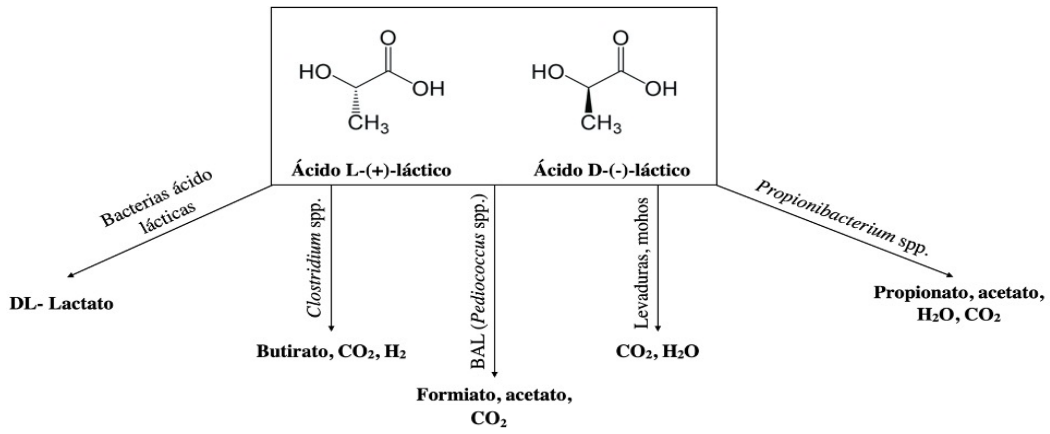


Figura 10. Rutas metabólicas del lactato durante la maduración del queso. (Adaptado de McSweeney y col., 2017).

La oxidación del lactato a acetato es producida principalmente por BAL y depende de la disponibilidad de oxígeno en el interior del queso. Generalmente, la concentración de acetato suele ser elevado en la mayoría de los quesos y contribuye a la formación de compuestos aromáticos que influyen en el flavor del producto, aunque en altas concentraciones puede generar aromas indeseados (McSweeney y col., 2017). Cuando se emplean cultivos iniciadores, las bacterias mesófilas empleadas producen ácido láctico en la cuajada, que es metabolizado muy rápido por microorganismos secundarios tales como *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Geotrichum candidum*, *Penicilium camemberti*, etc.). Estos microorganismos secundarios metabolizan el lactato a H_2O y CO_2 y aumentando el pH de la superficie del queso. Este cambio de pH es fundamental para el desarrollo de ciertos microorganismos responsables de algunas características relacionadas con el flavor, como es el caso de las bacterias corineformes en los quesos Camembert y Brie (Cholet y col., 2007; McSweeney y Sousa, 2000). Uno de los principales problemas de la oxidación de la lactosa es la formación de gas dentro del queso por la producción de CO_2, y los principales microorganismos involucrados son las levaduras, algunas BAL como *Leuconostoc mesenteroides*, *Lactobacillus casei* y *Lactobacillus brevis*, y *Clostridium* spp. (Tabla 7).

Tabla 7. Grupos microbianos responsables de la producción de gas en queso (Adaptado de Mullan, 2000).

Microorganismo	**Sustrato**	**Producto gaseoso**
Clostridium tyrobutiricum	Lactato	CO_2, H_2
Coliformes	Lactosa	CO_2, H_2
Lactobacillus brevis	Lactosa	CO_2
Lactobacillus casei	Citrato	CO_2
Lactococcus citrato (+)	Citrato	CO_2
Leuconostoc mesenteroides	Lactosa/citrato	CO_2
Levaduras	Lactosa	CO_2
Propionibacterium freudenreichii	Lactato	CO_2

Por otra parte, la concentración de citrato en el queso es muy baja, ya que la gran parte se pierde en el desuerado de la leche. Algunos microorganismos (lactococos citrato

positivo, *L. mesenteroides* sub. *cremoris*) metabolizan el citrato y producen diacetilo, acetato, acetoína y CO_2. No es usado como fuente de energía, pero lo metabolizan rápidamente en presencia de carbohidratos como la lactosa, generando compuestos que contribuyen al perfil aromático del queso (McSweeney y Sousa, 2000).

3.3.2. Proteólisis.

La proteólisis es fundamental para la maduración del queso y se produce debido a una serie de procesos bioquímicos que median la descomposición de las caseínas en péptidos y aminoácidos (Ärdo y col., 2017). Contribuye al desarrollo de la textura del queso a través de la hidrólisis de la matriz proteica y la disminución de la actividad de agua a través de la unión del agua con grupos carbonilo y grupos amino liberados. Además, influye en el flavor debido a la liberación de péptidos cortos y aminoácidos libres que en algunos casos pueden llegar a generar sabores extraños como el amargor (Andersen y col., 2010). Los aminoácidos libres son sustratos para una serie de reacciones catabólicas secundarias como la transaminación, deaminación, el catabolismo de los aminoácidos aromáticos como fenilalanina, tirosina y triptófano, descarboxilación y reacciones de aminoácidos con otros compuestos (McSweeney y Sousa, 2000; Sousa y col., 2001).

Durante la maduración, la proteólisis es catalizada por proteinasas y peptidasas procedentes de (i) leche (plasmina, catepsina D y otras proteinasas de células somáticas), coagulante animal o vegetal (quimosina, cardosinas A y B, pepsina y proteinasas ácidas de origen fúngico), BAL iniciadoras (proteinasas extacelulares de *Lactobacillus*, *Lactococcus* y *Streptococcus*), no iniciadoras o secundarias (*P. roqueforti* o *P. camemberti*) y proteinasas o peptidasas exógenas empleadas para acelerar la maduración (Ärdo y col., 2017; Fox y col., 2015) (Figura 11)

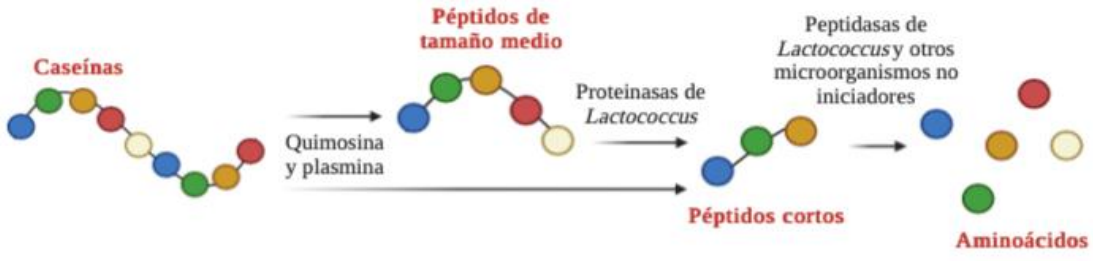


Figura 11. Representación esquemática de la proteólisis durante la maduración del queso (adaptado de McSweeney, 2004).

En la elaboración de quesos de pasta blanda, la primera proteólisis se produce principalmente por la acción de dos proteinasas aspárticas, cardosina A y B, naturalmente presentes en la flor de *Cynara cardunculus* L., y en menor medida, por las proteinasas de la leche (Alavi y Momen, 2020). En esta fase, la unión de las caseínas con las proteasas dará lugar a péptidos de tamaño grande (insolubles en agua) y medio (solubles en agua), que serán degradados posteriormente por las enzimas procedentes de la microbiota del queso. Esta fase es conocida como la segunda proteólisis, que dará como resultado la liberación de los aminoácidos que formaban las caseínas, que son sustrato para reacciones catabólicas de los microorganismos (Sousa y col., 2001). La segunda proteólisis está implicada en el flavor del producto final y por ello, es importante asegurar el equilibrio en la rotura de las caseínas para prevenir el desarrollo de atributos no deseados, como el amargor y la baja viscosidad (Visser, 1993).

Galán y col. (2008) estudiaron la actividad proteolítica del coagulante procedente de *C. cardunculus* L. en quesos "*Torta del Casar*" y "*Queso de la Serena*". Observaron que los quesos elaborados con coagulante vegetal dieron lugar a una textura más suave y cremosa que en los obtenidos con coagulante animal de ternero.

Ordiales y col. (2012, 2013, 2014) caracterizaron tecnológicamente las enzimas del cardo *C. cardunculus* L. y estudiaron su la influencia en la proteólisis de quesos "Torta del Casar". Concluyeron que la actividad de las cardosinas sobre la hidrólisis de las caseínas estaba correlacionada con las características sensoriales, principalmente el ratio de degradación de κ-caseína/$\boldsymbol{\beta}$-caseína; siendo la mejor elección aquellos coagulantes que presentasen una actividad proteolítca moderada sobre las $\boldsymbol{\beta}$-caseínas, responsables de la cremosidad del producto final.

En relación al ratio de degradación de las $\boldsymbol{\alpha}$ y $\boldsymbol{\beta}$-caseínas, Delgado y col. (2010) estudiaron el efecto de la proteólisis durante la maduración de quesos "*Torta del Casar"* y observaron que la degradación de las $\boldsymbol{\beta}$-caseínas ocurría de forma mucho más rápida al principio de la maduración, manteniéndose constante a partir de los 30 días, produciéndose a partir de ese momento una intensa proteólisis de las $\boldsymbol{\alpha}$s1-caseínas. Estos resultados sugieren una alta contribución de compuestos nitrogenados de bajo peso molecular derivados de la hidrólisis de las $\boldsymbol{\alpha}$s1-caseínas.

En quesos elaborados con leche cruda, la microbiota autóctona juega un papel importante en la segunda proteólisis. Numerosos autores han estudiado cómo influye la

actividad proteolítica de BAL (*Lactococcus* spp., *Lactobacillus* spp., *Streptococcus* spp.), levaduras (*D. hansenii* y *Y. lipolytica*) y mohos (*P. camemberti* y *P. roqueforti*) en quesos (revisado por Ärdo y col., 2017), dando lugar a péptidos bioactivos y aminoácidos libres como metionina, prolina y serina entre otros. Estos aminoácidos son precursores de reacciones catabólicas responsables del flavor y la textura (Atanassova y col., 2020; Chen y col., 2012; De Pasquale y col., 2014; Faion y col., 2020; Licitra y col., 2019; McSweeney, 2017).

3.3.3. Lipolisis.

Los principales lípidos de la leche son triglicéridos, que suponen más del 98% del total de los lípidos. Los triglicéridos son ésteres de glicerol compuestos por una columna de glicerol unida a tres ácidos grasos. Junto con los triglicéridos, la composición lipídica de la leche de oveja presenta otros lípidos simples (diacilglicéridos, monoacilglicéridos, ésteres de colesterol), lípidos complejos (fosfolípidos) y compuestos liposolubles (esteroles e hidrocarburos). En la fracción lipídica de la leche de oveja, los ácidos grasos más abundantes son el ácido palmítico (C16:0), oleico (C18:1), mirístico (C14:0) y esteárico (C18:0) (Goudjil y col., 2004; Park y col., 2007; Perea y col., 2000)

La lipolisis es una hidrólisis enzimática de triglicéridos que da lugar a ácidos grasos libres y glicerol, mono- y diacilglicéridos, y es considerada esencial para el desarrollo del flavor y compuestos aromáticos como metil-cetonas, ésteres, alcanos y alcoholes secundarios (Collins y col., 2003; McSweeney y Sousa, 2000) (Figura 12).

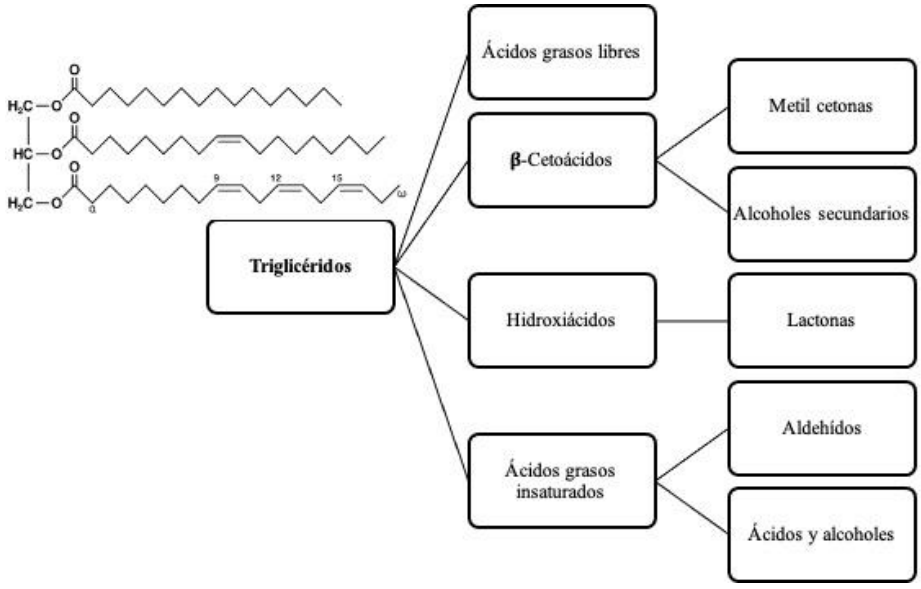


Figura 12. Productos de lipolisis (adaptado de McSweeney, 2017).

La acumulación de ácidos grasos libres durante la maduración se produce por la acción combinada de lipasas y esterasas presentes en el queso, que pueden tener diferentes orígenes: (i) lipoproteína lipasa (LPL) de la leche, (ii) lipasa / esterasa procedente del coagulante, (iii) lipasa / esterasa producida por los microorganismos iniciadores o no iniciadores y (iv) lipasa exógena añadida para acelerar la maduración del queso (Lomolino y col., 2015; McSweeney y Sousa, 2000; Thierry y col., 2017)

La lipasa de la leche (LPL) es una lipoproteína lipasa endógena que muestra preferencia por la hidrólisis de triglicéridos de cadena media y es específica en el lugar de corte liberando ácidos grasos de las posiciones *sn-1* y *sn-3* de mono-, di- y triglicéridos. Esta lipasa es importante en quesos elaborados con leche cruda ya que no es inactivada por tratamientos térmicos, ya que su actividad se reduce significativamente (Thierry y col., 2017).

Lomolino y col. (2015) caracterizaron la actividad lipolítica del coagulante procedente de *C. cardunculus* L., observando que exhibía actividad esterasa y no lipasa. La esterasa cataliza la escisión de los enlaces en los ésteres de cadena corta (C2-C10), presentando mayor afinidad por el ácido acético y propiónico. Estos ácidos grasos de cadena corta presentan un aroma más fuerte que los de cadena larga.

Las BAL poseen enzimas lipolíticas y esterolíticas capaces de hidrolizar ésteres de ácidos grasos libres y mono-, di- y triglicéridos. Sin embargo, estos microorganismos son considerados débilmente lipolíticos en comparación con otras especies (Collins y col., 2003). Las lipasas y esterasas de las BAL son intracelulares y, por tanto, deben ser liberadas en la matriz del queso para entrar en contacto con sus sustratos. Dentro de las BAL, los enterococos presentan mayor actividad lipolítica que otros géneros (Thierry y col., 2017). En cuanto a la microbiota secundaria, la levadura *Yarrowia lipolytica* ha sido ampliamente estudiada por su destacable actividad lipolítica (revisado por Fickers y col., 2011). Otras levaduras aisladas de queso como *K. lactis* y *K. marxianus* también han mostrado una alta actividad lipolítica (Binetti y col., 2013).

Diversos autores han estudiado la actividad lipolítica que se produce en quesos elaborados con leche cruda de oveja, como "*Serra da Estrela"* (Macedo y col., 1996; Carocho y col., 2015) y "*Torta del Casar"* (Delgado y col., 2009). Según estos autores, el queso *Serra da Estrela* presenta una baja actividad lipolítica debido a que la temperatura baja de maduración no favorece la lipolisis y tampoco se ve favorecida por

el corto tiempo de maduración de estos quesos (35-40 días). Además, los ácidos grasos mayoritarios encontrados en este queso fueron el ácido palmítico (C16:0) y ácido oleico (C18:1). En el queso "*Torta del Casar"* la actividad lipolítica es similar a otros quesos de pasta blanda, aunque se ve aumentada a lo largo del proceso de almacenamiento hasta los 90 días. Los ácidos grasos mayoritarios son similares al queso Serra da Estrela, aunque también se ha encontrado otros ácidos grasos mayoritarios como ácido butírico, que le confiere un aroma a rancio característico de estos quesos.

3.3.4. Dinámica microbiana.

La calidad de cualquier tipo de queso está sin duda ampliamente influenciada por la calidad de la materia prima con la que son elaborados, desde el punto de vista microbiológico, bioquímico y sensorial. La alta diversidad taxonómica que se encuentra en la leche cruda depende de muchos factores, entre los que se encuentran el período de lactación, la dieta, la salud, la higiene del ordeño y el ambiente en el que viven los animales, lo que determina las propiedades de coagulación de la leche y los rendimientos, composición y maduración de los quesos elaborados a partir de ella (Bokulich y Mills, 2013a; Montel y col., 2014).

Los microorganismos empleados en la elaboración de los quesos pueden provenir de la materia prima o bien, pueden ser añadidos como cultivos starter a la leche una vez pasteurizada. En Extremadura, los quesos tradicionales "*Torta del Casar*" y "*Queso de la Serena*" se elaboran sin la adición de ningún cultivo iniciador, es decir, que su proceso de maduración está mediado por los microorganismos presentes en la leche cruda de oveja y en las flores del cardo *Cynara cardunculus* L.

La fermentación del queso es llevada a cabo por una amplia diversidad de bacterias, levaduras y mohos (Montel y col., 2014; Irlinger y col., 2015). La microbiota autóctona de la la "Torta" o quesos similares está formada principalmente por Bacterias Ácido Lácticas (BAL). Diversos autores han encontrado que los géneros predominantes son *Lactobacillus, Lactococcus* y *Leuconostoc.* Las especies más representativas en estos quesos son *Lactobacillus casei, Lb. curvatus, Lb. diolivorans, Lb. paracasei, Lb. plantarum, Lb. rhamnosus, Lactococcus lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis* y *Leuconostoc mesenteroides* (Cogan y col., 1997; Gonçalves y col., 2018; Inácio y col., 2020; Ordiales y col., 2013a; Pereira y col., 2010; Sánchez-Juanes y col., 2020).

Poullet y col. (1991) y Ordiales y col. (2013a) estudiaron la microbiota bacteriana presente en quesos "*Torta del Casar*", encontrando recuentos de BAL en torno a 8,08 log ufc/g al inicio y de 9,13 log ufc/g al final del proceso de maduración. Resultados similares se han encontrado en estudios sobre la población de BAL en el "*Queso de la Serena*", situándose en torno a 8,66 log ufc/g a lo largo de todo el proceso de maduración de los quesos (Fernández del Pozo y col., 1988). Durante la maduración del queso juegan un papel fundamental debido a procesos tales como la fermentación de la lactosa, hidrólisis de caseínas y reacciones catabólicas que dan lugar a la producción de compuestos aromáticos (Dugat-Bony y col., 2015; Menéndez y col., 2000; Pereira y col., 2010). Además, actúan como agente inhibidor de la flora indeseable (Heredia-Castro y col., 2017).

Frecuentemente se han encontrado en quesos tradicionales otras especies de BAL, como son *Enterococcus faecalis*, *E. devriesei*, *E. durans* y *E. faecium* (Abriouel y col., 2008; Feutry y col., 2012; Ordiales y col., 2013a). Existe cierta controversia con estos microorganismos porque su hábitat natural es el tracto intestinal de los mamíferos. Su presencia en alimentos ha sido usualmente relacionada con pobres condiciones higiénicas durante la elaboración. Están asociados a posibles virulencias asociadas a infecciones humanas y a su resistencia a algunos antibióticos (Franz y col., 2003). En cambio, ejercen un papel importante en el proceso de elaboración de los quesos tradicionales durante la maduración, aportando sabores definidos y típicos (Foulquié Moreno y col., 2006). Tal y como indican Morandi y col. (2006) pueden influir en el sabor y en la textura gracias a sus actividades lipolíticas y proteolíticas, e incluso, algunas cepas son capaces de producir bacteriocinas que pueden actuar contra patógenos u otros microorganismos alterantes, mejorando así, la seguridad alimentaria (Giraffa, 2003).

La microbiota secundaria está constituida principalmente por otras bacterias, tanto gram-positivas como gram-negativas, levaduras y mohos. En estudios realizados por Cáceres y col. (1997), las estafilococáceas fueron detectadas en la mayoría de los quesos durante la maduración gracias a la resistencia a la sal y a la deshidratación. *Staphylococcus saprophyticus* y *Staphylococcus epidermidis* han sido aislados en este tipo de quesos (Alegría y col., 2009; Ordiales y col., 2013a).

Las bacterias gram-negativas, como la familia *Enterobacteriaceae*, están presentes en la leche y en productos lácteos. Diversos autores han encontrado recuentos

estables durante la maduración entre 6,8 y 7,33 log ufc/g (Chávez-López y col., 2006; Gonçalves y col., 2018; Morales y col., 2004; Ordiales y col., 2013a; Tabla y col., 2016). Su presencia está asociada a indicadores de calidad microbiológica, ya que son microorganismos indeseables a lo largo del proceso de elaboración. De ahí que puedan suponer un problema de salud pública por la presencia de especies patógenas (Irlinger y col., 2015). Por una parte, especies pertenecientes a los géneros *Escherichia*, *Enterobacter y Klebsiella* pueden provocar defectos en la textura y flavor, producir aminas biógenas y presentar resistencia a antibióticos (Tornadijo y col., 2001; Quigley y col., 2013). En cambio, algunas especies como *Hafnia alvei* (figura 13) y *Hafnia paralvei* se han encontrado de manera frecuente en leche cruda y productos derivados (Ercolini y col., 2009), siendo la especie predominante al final de proceso de maduración en quesos como "*Serra da Estrela*" (Tavaria y col., 1998), Queso "*Serpa*" (Gonçalves y col., 2018), "*Alberquilla*" (Abriouel y col., 2008) y "*Torta del Casar*" (Ordiales y col., 2013a; Tabla y col., 2016).

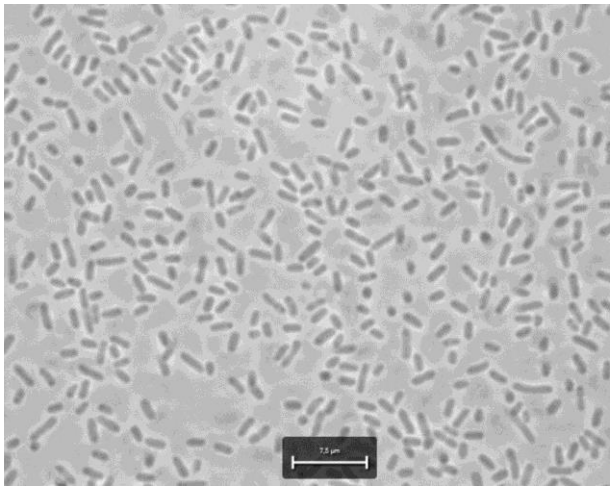


Figura 13. *Hafnia alvei*. Fuente: imagen propia obtenida con aumento 100x.

Dentro de la microbiota secundaria, las levaduras constituyen un grupo amplio y heterogéneo que desarrolla un papel fundamental tanto directa como indirectamente en el proceso de maduración de los quesos de pasta blanda (Alegría y col., 2009). Se han encontrado recuentos en el interior de los quesos entre 4 log ufc/g y 6 log ufc/g (Gonçalves y col., 2017; Pintado y col., 2008; Ordiales y col., 2013a; Atanassova y col., 2016), siendo normalmente superiores en el exterior. Sin embargo, en algunos quesos elaborados con leche cruda pueden llegar a valores de hasta 9 log ufc/g (Jacques y Casaregola, 2008; Padilla y col., 2014a). La presencia de levaduras en elevada concentración al final de la

maduración del queso no es sorprendente debido a que estos microorganismos encuentran un ambiente rico en nutrientes con altas concentraciones de sal, bajos pH, temperaturas y actividades de agua, al cual están bien adaptados (Viljoen, 2001).

Entre las especies mayoritarias, aunque existe una gran diversidad debido a la amplia gama de quesos elaborados a lo largo del mundo, destacan *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Geotrichum candidum*, *Saccharomyces cerevisiae* y miembros de los géneros *Candida* spp. y *Pichia* spp. (Banjara y col., 2015; Binetti y col., 2013; Ceugniez y col., 2015; Atanassova y col., 2016), aunque la prevalencia de ciertas especies puede verse influenciada por el tipo de queso (Dugat-Bony y col., 2016). En quesos tipo "Torta", Ordiales y col. (2013a) encontraron que las especies predominantes durante el proceso de maduración de la "Torta del Casar" fueron *Candida zeylanoides*, *Rhodotorula mucilaginosa*, *Candida parapsilosis* y *Yarrowia lipolytica*. Gonçalves y col. (2017) encontraron mayor diversidad en Queso Serpa, siendo mayoritarios los géneros *Debaryomyces* spp. y *Kluyveromyces* spp., y en menor medida, *Pichia* spp. (figura 14) y *Candida* spp.

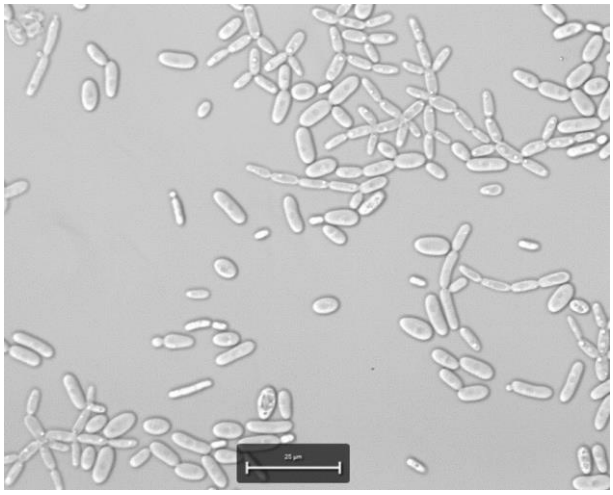


Figura 14. *Pichia jadinii.* Fuente: imagen propia obtenida con aumento 40x.

En el caso de los mohos, pueden provenir de la leche cruda o de contaminaciones ambientales durante el proceso de elaboración de los quesos. Delavenne y col. (2011) encontraron en muestras de leche de oveja la existencia de diversos mohos pertenecientes a los géneros *Aspergillus*, *Chrysosporium*, *Cladosporium*, *Fusarium* y *Penicillium*. Los mohos se encuentran principalmente en la parte externa, la cual está expuesta a las condiciones ambientales de la cámara de maduración (Bokulich y Mills, 2013a). En la

fabricación de los quesos se emplean algunos mohos que aportan las características necesarias para que el producto final sea el deseado, como es el caso del queso camembert (*P. camemberti*) y el queso roquefort (*P. roqueforti*). Aunque la presencia de los mohos en la corteza de los quesos puede ser positiva en algunos casos, la aparición de mohos alterantes puede suponer un problema. La adición de sal u otros compuestos antifúngicos como la natamicina pueden evitar el crecimiento de mohos que puedan modificar la apariencia y las características sensoriales del producto. Algunos de los mohos alterantes más comunes en quesos de pasta blanda son *Mucor plumbeus*, *Fusarium oxiosporum*, *F. verticilloides*, *Phoma* spp., *Penicillium commune* y *Cladosporium* spp. (Hymery y col., 2014; Hermet y col., 2012, 2014; Ozturkoglu-Budak y col., 2017).

***Microorganismos patógenos y alterantes*.**

El principal problema asociado a la elaboración de quesos a partir de leche cruda es la aparición de microorganismos patógenos y alterantes. Esto de debe a la ausencia de procesos térmicos en la leche y, además, a las condiciones higiénico-sanitarias de las granjas e industrias desde el ordeño hasta la elaboración del producto. Por ello, se produce una gran variabilidad que en algunos casos dan lugar a la pérdida de lotes debido a la presencia de microorganismos indeseables. Algunos de estos microorganismos desaparecen al final del proceso de maduración debido a un efecto combinado de varios parámetros como bajos valores de pH, concentración de sal, baja actividad de agua (a_w), producción de bacteriocinas por BAL (Elotmani y col., 2002), presencia en la leche de enzimas con actividad antimicrobiana como lactoperoxidasa, lisozima y lactoferrina (Fox y col., 2006) y competición por nutrientes (McSweeney y col., 2017)

En la elaboración de quesos de pasta blanda se utiliza leche a la que no se le aplica ningún tratamiento térmico para la eliminación de microorganismos que puedan llegar a ocasionar un peligro para la salud humana. Algunos de estos microorganismos patógenos son *Listeria monocytogenes*, *Escherichia coli* enterohemorrágica O157:H7, *Staphylococcus aureus* y *Salmonella* spp. Durante el proceso de maduración de los quesos, la incidencia de este tipo de microorganismos patógenos tiende a desaparecer al final del procesado. Diversos autores han estudiado la presencia de estos microorganismos durante el proceso de elaboración de queso tradicional español (Ordiales y col. 2013a) y portugués (Gonçalves y col., 2018) y no encontraron evidencias de patógenos tales como *L. monocytogenes*, *E. coli* O157:H7 o *Salmonella* spp. al final

del proceso de maduración de quesos "*Torta del Casar"* y "*Queso Serpa".* En cambio, se han reportado algunos casos de presencia de *E. coli* O157:H7 en quesos tipo "torta" (D'Amico y Donelly, 2011; Heuvelink y col., 1998; Honish y col., 2005), al igual que la presencia de *L. monocytogenes* (Almeida y col., 2007; Verraes y col., 2015) en quesos portugueses elaborados con leche cruda de oveja.

Staphylococcus aureus produce ciertas enterotoxinas (A, C y D) capaces de producir intoxicaciones alimentarias, encontrándose algunas de ellas asociadas a productos lácteos contaminados, aunque se necesitarían grandes cantidades de enterotoxinas para producir la enfermedad (Le Loir y col, 2003). Este microorganismo puede encontrarse a lo largo de todo el proceso de maduración de los quesos de pasta blanda, incluso al final del procesado (Montel y col, 2014; Ordiales y col., 2013a; Gonçalves y col., 2018; Sanchez-Rey y col., 1993).

Otros microorganismos, como *C. botulinum, C. perfringens o C. difficile* pueden producir toxinas capaces de provocar intoxicaciones en el ser humano tras su ingesta. En la industria quesera representan una de las causas responsables de pérdidas financieras, ya que pueden producir hinchazones en los quesos (Quigley y col., 2011). En quesos de pasta blanda no se ha detectado presencia de estos microorganismos al final de su procesado (Gonçalvez y col., 2018; Inácio y col., 2020; Ordiales y col., 2013a).

Por otra parte, algunos microorganismos pueden ejercer un efecto alterante en el producto, como pueden ser las *Pseudomonas* spp, enterobacterias y algunas levaduras. Con respecto a *Pseudomonas* spp., estas bacterias son Gram negativas y psicrotrofas, por lo que pueden crecer a temperaturas de refrigeración. Algunas especies como *P. fluorescens, P. aeruginosa* o *P. putida* producen ciertos pigmentos que pueden alterar el aspecto tanto interno como externo de los quesos (Carrascosa y col., 2015; del Olmo y col., 2018; Carminati y col., 2019). Las enterobacterias, a pesar de estar consideradas como indicadores de contaminación fecal, también pueden provocar diversas alteraciones en la pasta de los quesos. Algunas especies aisladas de quesos de leche cruda de oveja, como *Klebsiella oxytoca*, *Hafnia alvei* o *Citrobacter freundii* son capaces de producir gas de forma moderada, causando ojos característicos como los encontrados en el queso *Emmental* (Tabla y col., 2016). Por otra parte, el crecimiento excesivo de levaduras en el queso puede ocasionar sabores frutales, a «levadura» y otros sabores desagradables (Ledenbach y col., 2009); también gasificación, formación de limo, decoloración

(Fröhlich-Wyder y col., 2019), generación de pigmentos indeseados provocados principalmente por *Y. lipolytica* (Carreira y col., 2001; Bintsis y col., 2002) o la "piel de sapo" provocada por el exceso de crecimiento y actividad proteolítica de *G. candidum*, que se caracteriza por generar una corteza resbaladiza, arrugada, mucosa y viscosa en quesos de pasta blanda (Marcelino y col., 2001).

Finalmente, uno de los problemas asociados a la maduración de los quesos es la proliferación de mohos indeseables en la corteza. Como se ha comentado en el apartado anterior, algunos mohos son necesarios para la elaboración de ciertos tipos de quesos, pero suponen un problema cuando aparecen durante la maduración de quesos que no requieren de su actuación para conseguir el producto final. La presencia de estos microorganismos eucariotas no solo puede afectar al aspecto visual de los mismos, sino que pueden generar compuestos tóxicos para la salud del consumidor. Estos compuestos son metabolitos secundarios denominados micotoxinas, siendo la aflatoxina M1 y ocratoxina A las más peligrosas detectadas en quesos (Hymery y col., 2014), producidas principalmente por los géneros *Penicillium* y *Aspergillus*. La aflatoxina M1 suele aparecer en quesos que han sido fabricados a partir de leche de animales alimentados con piensos que contenían aflatoxinas o esporas de *Aspergillus* spp. (Ardic y col., 2009). Algunas de estas toxinas pueden llegar a difundir al interior de los quesos, como es el caso de la patulina y de roquefortina C producidas por *Penicillium* spp., en condiciones de almacenamiento en quesos azules y queso manchego (Hymery y col., 2014; Pattono y col., 2013). Otras micotoxinas, como el ácido ciclopiazónico producido por *P. camemberti* se ha encontrado en pequeñas cantidades en quesos italianos (Zambonin y col., 2001).

3.4.Características del producto final.

Composición

Los procesos bioquímicos producidos a lo largo del proceso de maduración influyen en las características físico-químicas finales de los quesos. En quesos "*Torta del Casar*", Ordiales y col. (2013c) encontraron que el pH desciende en las primeras etapas del proceso de maduración hasta alcanzar valores en torno a 5,6 al final del proceso. Este cambio de pH refleja la actividad metabólica de las BAL predominantes en quesos elaborados con leche cruda de oveja. Tanto la actividad de agua (a_w) como la humedad, disminuyen a lo largo del proceso de maduración debido a las condiciones del mismo,

presentando valores a 0,94 para la a_w y de ~40% de humedad. El descenso de la actividad de agua está influenciado por la presencia de sal, pérdida de humedad y la hidrólisis gradual de proteínas en compuestos solubles de bajo peso molecular. Con respecto al contenido total de proteínas y grasa, estos parámetros se mantienen estables a lo largo del proceso de maduración, siendo de ~20% y ~50% respectivamente, dentro de los valores establecidos en los pliegos de condiciones (Delgado y col., 2010; Ordiales y col., 2013c). En cuanto a la fracción proteica, el nivel de proteínas solubles en agua entre las que se encuentran las proteínas de suero en los quesos fue mayor a los 60 días de maduración que a los 2 días, debiéndose este aumento a la hidrólisis de las caseínas. Esta hidrólisis de las caseínas también generó un aumento de los valores de Nitrógeno No Proteico (NPN) de 2,05 a 3,54 mg/g de queso y de Nitrógeno aminoacídico (NA) de 0,6 a 2,49 mg/g al final del proceso. Estos resultados son similiares a los encontrados en "*Queso de la Serena"* por Núñez y col., (1991).

Propiedades sensoriales: aroma y textura.

Compuestos volátiles.

Los cambios bioquímicos producidos durante la maduración del queso dan lugar a numerosos compuestos aromáticos que contribuyen al favor del producto final. Estas moléculas volátiles pertenecen a diferentes familias químicas como ésteres, alcoholes, aldehídos, ácidos grasos libres, cetonas, lactonas, terpenos y pirazinas cuya producción está controlada por la glucólisis, lipólisis y proteólisis previamente descritas, desde el inicio hasta el final del proceso de maduración (Curioni y Bosset, 2002). La lactosa, grasa y proteínas se degradan en moléculas de bajo peso molecular que son, en consecuencia, precursoras de compuestos aromáticos volátiles (McSweeney y Sousa, 2000; Smit y col., 2005). Se han identificado más de seiscientos compuestos volátiles en diferentes variedades de queso y muchas de ellas corresponden a atributos de olor y aroma particulares. Sin embargo, solo una pequeña proporción de estos compuestos son responsables del flavor del queso (Curioni y Bosset, 2002).

El perfil volátil de quesos elaborados con leche cruda de oveja, pertenecientes a Denominaciones de Origen Protegida de España, ha sido ampliamente estudiado, como es el caso del queso Idiazábal (Barron y col., 2007; Zabaleta y col., 2017), Manchego (Fernández-García y col., 2002a, 2002b; Gómez-Ruiz y col., 2002), Roncal (Muñoz y col., 2003), Queso de la Serena (Carbonell y col., 2002a, 2002b), Torta del Casar

(Delgado y col., 2010; Ordiales y col., 2013c) y Zamorano (Fernández-García y col., 2004).

Según Ordiales y col. (2013c), los compuestos aromáticos encontrados al final del proceso de elaboración de "*Torta del Casar*" están relacionados con la población microbiana responsable de la maduración del queso. En este sentido, las rutas metabólicas en las que participan estos microorganismos influyen en el perfil aromático del producto final. Encontraron 55 compuestos volátiles agrupados en hidrocarburos, alcoholes, aldehídos, cetonas, ácidos carboxílicos y ésteres. Algunos de los alcoholes principales que aislaron fueron etanol, 3-metil 1- butanol, 1-butanol y 2,3 butanodiol. El etanol puede considerarse un producto directo de la fermentación de la lactosa, mientras que el 3-metil-1-butanol se origina por la reducción del aldehído de Strecker producido a partir de leucina y confiere un agradable aroma a queso fresco (Moio y col., 1993). Los ácidos más abundantes fueron el acético y butanoico, también descritos por Delgado y col. (2010) en "*Torta del Casar*". Demostraron también la correlación negativa entre las concentraciones de ácido acético, éster butílico de ácido acético y algunos alcoholes como 2-octanol, 4-octanol y bencenometanol, ligados a altos recuentos de lactobacilos, enterococococos, estreptococos, y en menor medida, levaduras. El ácido acético se ha asociado con un alto recuento de BAL en los quesos, lo que sugiere que el incremento incontrolado de estas bacterias podría tener un efecto negativo en el sabor de "*Torta del Casar*".

En el "*Queso de la Serena*", Carbonell y col. (2002a, 2002b) identificaron un total de 112 compuestos volátiles, siendo los alcoholes la familia química principal a lo largo del proceso de elaboración de este producto. Los ésteres etílicos de ácido acético, butanoico, hexanoico y octanoico y el acetato de 3-metil-1-butanol fueron encontrados en niveles elevados en el espacio de cabeza del "*Queso de la Serena*". Estos ésteres etílicos se correlacionaron positivamente con los atributos sensoriales lácticos, frutas-flores y sabor a queso limpio.

Textura

Los cambios bioquímicos y la composición físico-química durante la maduración de los quesos influyen directamente en la textura de estos al final del proceso de maduración (Fox y col., 2017). Entre todos los parámetros, destaca la pérdida de humedad

y el desarrollo de la proteólisis, relacionada directamente con la estructura de la red proteica de los quesos.

En quesos de pasta blanda, al presentar un período de maduración reducido, el descenso de la humedad no es tan notable como en quesos semicurados o curados y, por lo tanto, la matriz de proteínas presenta mayor elasticidad y es menos susceptible a la fractura (Fox y col., 2017). Delgado y col. (2010) sugieren que en quesos de pasta blanda la relación entre la textura y los parámetros proteolíticos podrían indicar que la degradación de la matriz de caseínas está relacionada con el proceso proteolítico. Ordiales y col. (2014) también observaron los cambios producidos en quesos "*Torta del Casar",* encontrando que a medida que aumenta el período de maduración, los parámetros de dureza y cohesividad disminuyen a la vez que se produce un incremento en la adhesividad del queso, pudiéndose atribuir estos cambios a la amplia proteólisis que se produce en quesos elaborados con coagulante vegetal.

Variabilidad en el producto final

La principal ventaja de la elaboración de quesos con leche cruda es la producción de quesos con características organolépticas distintivas aportadas por la microbiota autóctona, lo que confiere un valor diferenciado con respecto a otros productos. Diversos autores (Araújo-Rodrigues y col., 2020; Gonçalves y col., 2018; Inácio y col., 2020; Ordiales y col., 2013a, 2013c) han mostrado la heterogeneidad microbiológica que se produce en este tipo de quesos, lo cual conlleva a cambios en el perfil organoléptico y en la textura. Estos cambios están asociados a la microbiota presente en la flor del cardo y en la leche cruda, siendo muy importante el control de estos factores para que el producto final sea el esperado. El uso de leche sin pasterizar puede ocasionar riesgos higiénico-sanitarios, defectos en la fabricación de los quesos y pérdidas de lotes. En este sentido, incluir un tratamiento térmico en el proceso de elaboración garantizaría la calidad higiénico-sanitaria, reduciendo a su vez la pérdida de lotes. Esto supondría la pérdida de la microbiota autóctona de la leche, que es la que confiere esos atributos organolépticos propios de estos quesos tradicionales. Por ello, un método para conservar estas características sería emplear un cultivo iniciador a partir de microorganismos aislados de quesos tradicionales que presenten aptitud tecnológica y funcional, garantizando además su inocuidad. Esta selección de microorganismos posibilita la producción homogénea y

estandarizada, garantizando la calidad higiénico-sanitaria además de mantener las características sensoriales propias que definen la identidad de un queso artesanal.

4. Estrategias para garantizar la calidad del producto final mediante la inoculación de microorganismos.

4.1. Estudio de la dinámica microbiana mediante técnica dependiente del cultivo e independiente.

Las técnicas de identificación de microorganismos basadas en cultivos han permitido durante muchos años conocer la microbiota presente en los quesos. Sin embargo, este tipo de cultivo está limitado por la presencia de microorganismos difíciles de cultivar o aquellos que no están vivos en el momento de la toma de muestras. Algunos de los problemas con el uso del cultivo para el análisis de comunidades microbianas surgen del hecho de que un medio artificial homogéneo normalmente permite el crecimiento de solo una pequeña fracción de los microorganismos. Además, cuando se están investigando comunidades microbianas complejas, como es el caso del queso, la enumeración de su microbiota mediante técnicas tradicionales de cultivo puede producir resultados erróneos (Carraro y col., 2011). Además, durante la maduración del queso, las cepas pueden entrar en un estado viable pero no cultivable y, en consecuencia, el entorno del queso puede estar poblado con una variedad de tipos de células, incluidas las intactas, viables, no viables y parcial o totalmente desintegradas (Martín-Platero y col., 2008). En este sentido, las nuevas técnicas de secuenciación masiva permiten conocer este tipo de microorganismos sin la necesidad de realizar un cultivo microbiológico rutinario.

4.2. Nuevas técnicas de secuenciación masiva. Next Generation Sequencing o High Throughput Sequencing.

Durante varias décadas, la identificación de microorganismos presentes en alimentos se ha realizado mediante técnicas convencionales de cultivo combinados con herramientas moleculares basadas en la reacción en cadena de la polimerasa (PCR), tanto de las regiones ITS ribosómicas no codificantes para eucariotas como los genes 16S del ARN ribosomal para procariotas. Esta técnica se basaba en el aislamiento de microorganismos en cultivos puros y posteriormente, aislar su ADN para amplificarlo mediante PCR y finalmente identificarlos mediante secuenciación. Fue en 1977, cuando

Frederick Sanger desarrolló el método de secuenciación de ADN conocido como método de Sanger (Sanger y col., 1977).

Este tipo de secuenciación se basaba en sintetizar de forma secuencia una hebra de ADN complementaria a una hebra de cadena simple (que se usa como molde), en presencia de la enzima ADN polimerasa, un cebador (primer) para iniciar la síntesis de ADN, los cuatro 2'-deoxinucleótidos que componen la secuencia del ADN (dATP, dGTP, dCTP y dTTP) y dideoxinucleótidos (ddATP, ddCTP, ddTTP y ddGTP) marcados radioactivamente. Estos últimos interrumpen la síntesis de la nueva cadena debido a la ausencia de un grupo hidroxilo (3'-OH) que impide que se añada otra base, generando fragmentos de distinta longitud. Estos fragmentos se desnaturalizaban mediante electroforesis en geles de acrilamida, pudiendo observarse la secuencia en paralelo de un patrón de bandas y dando lugar a la secuencia, que alcanzaban un tamaño de hasta 200 pares de bases (pb).

Años después, en 1986, Hood y col. publicaron junto con la empresa Applied Biosystems, un avance en la técnica de secuenciación que se basaba en la introducción de terminadores fluoróforos en lugar de marcadores radioactivos (Smith y col., 1986). Esta variante de la secuenciación de Sanger destacaba por su automatización. Así, estos fragmentos marcados por fluorescencia se separaban mediante electroforesis capilar, donde se detectaba la fluorescencia emitida por el ddNTP terminal. El gráfico resultante se denomina electroferograma, en el que se observan picos con diferente color asociados a la posición de cada nucleótido en la molécula de ADN. Con esta mejora, la longitud de las secuencias era de entre 500 y 1000 pb (figura 15).

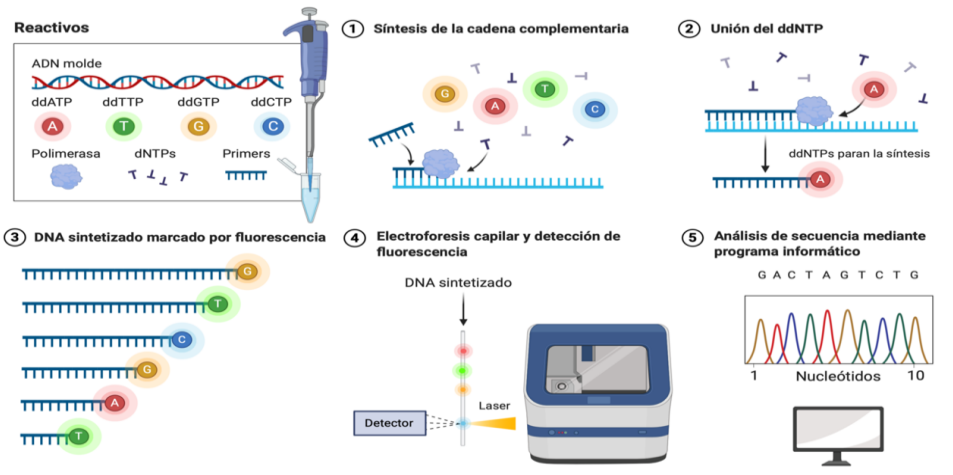


Figura 15. Secuenciación por terminadores fluoróforos. Creación propia.

Con la entrada al mercado de secuenciadores automatizados, se produjo una revolución en el campo de la genética. Debido a ello, la secuenciación del Genoma Humano se convirtió en un proyecto alcanzable, dando lugar a multitud de colaboraciones a nivel internacional en las que se secuenciaron genomas tanto de microorganismos, como *E. coli, S. cerevisiae* a nivel microbiano, como de ratón (*Mus domesticus*).

El paso de secuenciar fragmentos pequeños de ADN conservados quedó atrás para conseguir nuevas metodologías que permitiesen estudiar genomas completos, con un mayor rendimiento y sensibilidad. Esto dio lugar a lo que se conoce como segunda generación de secuenciación o Secuenciación de Alto Rendimiento (High Throughput Sequencing, HTS) mediante las denominadas plataformas Next Generation Sequencing (NGS), que surgieron en 2004 y se utilizan en multitud de campos de la ciencia, desde el estudio de las comunidades microbianas en alimentos hasta su aplicación en medicina genética para el estudio de enfermedades.

454 GS FLX de Life Science-Roche®.

Este método fue sacado al mercado en 2004 y se basa en la pirosecuenciación (Margulies y col, 2005). Es una metodología enzimática que emplea una PCR en emulsión (PCR sobre una superficie de perlas dentro de pequeñas burbujas de agua que flotan en una solución de aceite que contiene ADN polimerasa, cebadores y dNTPs). Estos dNTPs están formados por 3 fosfatos: **α**-fosfato (unido al azúcar), **β**-fosfato y ***γ***-fosfato. Durante la replicación, **α**-fosfato de cada nucleótido complementario se une enzimáticamente al grupo 3'-OH del último nucleótido de la hembra sintetizada, mientras que los fosfatos **β** y ***γ*** se escinden en una unidad llamada pirofosfato (PPi). Estos pirofosfatos reaccionan con ATP sulfurilasa, generando ATP, que reacciona con la luciferasa para producir una señal de luz que es registrada por una cámara (figura 16). Este proceso se repite hasta que se secuencian todas las bases. Este método genera tamaños de lectura largos (~700 pb) y rápidos tiempos de secuenciación, aunque uno de los principales problemas era la formación de homopolímeros (secuencias con el mismo nucleótido).

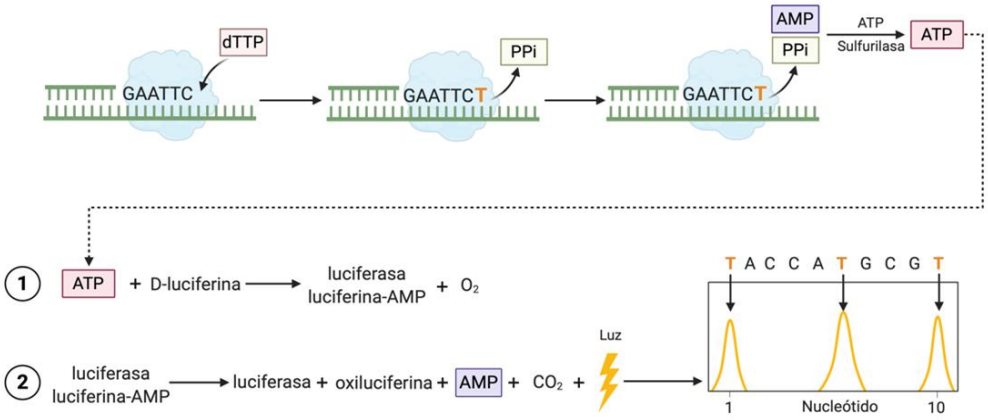


Figura 16. Pirosecuenciación. Creación propia.

Ion Torrent de Life Technologies™.

Life Technologies (posteriormente adquirida por ThermoFischer Scientific®) lanzó al mercado en 2011 el equipo *Ion Personal Genome Machine* (PGM). La metodología empleada en el secuenciador se basa en el uso de semiconductores y no emplea fluorescencia, sino en el cambio de pH cuando se libera un H^+ al incorporarse un nucleótido en la cadena de ADN que se está sintetizando. Utiliza chips que están formados por micropocillos, cuya base actúa como un pHmetro capturando las diferentes medidas de voltaje para detectar los cambios de pH. Esta tecnología no depende de ninguna cámara para captar imágenes, reduce los costos de secuenciación notablemente y genera longitudes de lectura de ~400 pb. Al igual que ocurre con la pirosecuenciación del 454 de Roche®, presenta ciertas dificultades para detectar regiones de homopolímeros (Kchouk y col., 2017).

Método Solexa de Illumina®.

Desarrollado originalmente por el Dr. Shankar Balasubramanian y Dr. David Klenerman en la Universidad de Cambridge, fundaron la compañía Solexa en 1998, siendo posteriormente adquirida por Illumina. Este método se basa en la secuenciación por síntesis con terminadores fluorescentes reversibles, al igual que la técnica de Sanger. Se divide en cuatro etapas: elaboración de la librería, generación de *clústers*, secuenciación y análisis de datos.

En la primera, los fragmentos de aleatorios de ADN o ADNc se unen a unos adaptadores 5’ y 3’ y posteriormente son amplificados mediante PCR. Estos fragmentos amplificados se unen a una secuencia conocida (índices) para ser identificados en el análisis de datos. En la segunda y tercera etapa, que ocurre en el equipo automatizado, se emplea una PCR puente (PCR-bridge) (Fedurco y col., 2006; Cao y col., 2017) en la que los fragmentos de ADN se amplifican a partir de unos adaptadores que están inmovilizados a una superficie sólida, denominada celda de flujo o flow-cell. Esta amplificación sucede gracias a una ADN polimerasa que agrega dNTPs marcados con fluoróforos a la cadena complementaria que se está generando, y estos son excitados por una fuente de luz siendo registrado el color que emite por un detector óptico. Una vez que el fluoróforo se incorpora a la hebra sintetizada, se escinde y desbloquea la incorporación del siguiente nucleótido. Una vez que la cadena de ADN sintetizada termina, se realiza el mismo proceso para la cadena inversa, denominándose este proceso como *paired-end.* (Figura 17).

Figura 17. Método Solexa (Illumina). Creación propia.

Esta metodología permite crear millones de copias idénticas de cada fragmento de ADN, siendo este proceso denominado generación de *clústers.* Por último, para el análisis de datos se basa en la lectura simultánea de millones de secuencias asegurando una alta precisión. A diferencia del equipo 454 de Roche®, la longitud de las lecturas son muchos más pequeñas (2 x 300 pb), pero la cantidad de lecturas que se obtienen alcanzan varían entre 1,2 y 6000 Gigabases (Gb) dependiendo de la escala del equipo. Este último paso se realiza mediante aplicaciones bioinformáticas que permiten, entre otras cosas, filtrar por calidad y alinear contra bases de datos específicas para identificar los taxones

microbianos de una muestra o ensamblar genes para descubrir mutaciones que generan enfermedades (Hernández y col., 2020).

4.3.Tercera Generación de Secuenciación.

La segunda generación de secuenciación revolucionó el análisis de ADN y ha sido ampliamente utilizada. Sin embargo, uno de los principales inconvenientes que presenta es su elevado costo debido al uso de PCR para amplificar los fragmentos de ADN y su incapacidad de generar longitudes de lecturas más largas, dificultando así el ensamblaje posterior del genoma (Kchouk y col., 2017). Debido a estas desventajas, se ha desarrollado una nueva generación de secuenciación denominada "Tercera Generación de Secuenciación", que no utiliza ninguna PCR de amplificación de ADN y, además, es capaz de generar longitudes de secuencias largas (> 10 kbp). Esta tecnología de secuenciación es conocida como SMRT (Single Molecule Long Read Sequencing) (Eid y col., 2009), y está disponible en los equipos de Pacific Bioscience (PacBio). Utiliza el mismo marcado fluorescente que la tecnología de Illumina®, pero en lugar de realizar ciclos de amplificación de nucleótidos, detecta señales en tiempo real, ya que se emiten cuando los dNTPs se incorporan a la cadena de ADN. Emplea una estructura tipo chip compuesta por muchas celdas SMRT en las cuales hay nanoestructuras denominadas "zero-mode waveguides" (ZMWs), que son pocillos de metal de decenas de nanómetros de diámetro apoyados sobre un vidrio y cada pocillo contiene una ADN polimerasa inmovilizada en su base. Una vez que la molécula de ADN, previamente ligada a un adaptador) entra en el pocillo, la ADN polimerasa se une a los adaptadores de la cadena de ADN e inicia la replicación. Los dNTPs marcados por fluorescencia se introducen en cada pocillo, y a medida que se incorpora cada base, se produce un impulso de luz que identifica el nucleótido y lo analiza para generar la secuencia de ADN. Las principales ventajas de esta tecnología son: la rapidez en la preparación de la muestra al no necesitar PCR, y las longitudes de lectura que pueden llegar a alcanzar ~30 kbp. En cambio, presentan una alta tasa de error alrededor del 13-15% debido a errores de inserción o eliminación (Ardui y col., 2018; Cao y col., 2017; Heather y col., 2016; Yang y col., 2020).

La tecnología SMRT también es empleada en los equipos de Oxford Nanopore Technologies, aunque la secuenciación se produce por nanoporos, que pueden ser biológicos (proteína) o sintéticos (grafeno). Una corriente iónica pasa a través del

nanoporo estableciendo un voltaje a través de esta membrana. Si una secuencia de ADN monocatenaria atraviesa el poro aparece una alteración característica en la corriente iónica. La medición de esta corriente permite identificar el nucleótido. Esta variación de corriente se registra progresivamente en un modelo gráfico y luego se interpreta para identificar la secuencia. Entre las ventajas que ofrece este tipo de secuenciación, son el bajo coste y el tamaño del equipo, ya que MiniION es de tamaño bolsillo. Además, al producirse la secuenciación en tiempo real, la secuencia aparece en el ordenador a medida que se está ejecutando. Este dispositivo puede generar lecturas muy largas hasta 300 kbp, con un rendimiento de aproximadamente 42 Gb. Sin. embargo, la tasa de error es mayor al 4% (Hernández y col., 2020; Jain y col., 2016; Kchouk y col, 2017).

4.4.Aplicación de la secuenciación masiva en microbiología de alimentos.

El desarrollo de las técnicas de secuenciación masiva ha permitido a los investigadores generar una gran cantidad de información genómica de forma rápida, permitiendo explorar en la medida de lo posible, los ecosistemas microbianos presentes en alimentos fermentados, ambientes y genomas completos de algunos microorganismos (Bokulich y Mills, 2013a; Choi y col., 2020; De Filippis y col, 2017a). El enfoque más utilizado para el estudio de la microbiota se basa en genes taxonómicos relevantes, como el gen 16S del ARNr (Caporaso y col., 2011; Quijada y col., 2020). Este gen está presente en cualquier bacteria y su secuencia consta de nueve regiones hipervariables (denominadas V1 - V9), que contienen información filogenética del gen, separadas por regiones altamente conservadas (Ercolini, 2013; Yang y col., 2016). Esto ofrece una oportunidad única para diseñar cebadores de PCR genéricos para amplificar y secuenciar estas regiones, para identificar la taxonomía bacteriana asociada a la matriz alimentaria (Cao y col., 2017).

Para la identificación de los microorganismos eucariotas se ha utilizado de forma más común la región ITS (Internal Transcribed Spacer), debido a la presencia de bases de datos curadas y actualizadas (Schoch y col., 2012; Bokulich y Mills, 2013b). Sin embargo, la longitud de la región ITS1-2 es muy variable entre diferentes géneros y especies, lo que ha promovido el uso de otros genes como los que codifican el ARNr 18S y 26S (De Filippis y col., 2017b).

La secuenciación metagenómica Shotgun permite obtener una alta resolución taxonómica de los microorganismos presentes en matrices alimentarias, llegando incluso

a identificar a nivel de especie. La metagenómica es una metodología que permite detectar e identificar no solo los microorganismos dominantes, sino también aquellos que tienen baja prevalencia (Escobar-Zepeda y col., 2016; Sogin y col., 2006). Debido a que la metagenómica cubre toda la información genética de la muestra, los datos pueden ser usados para análisis posteriores, como ensamblaje y agrupamiento metagenómico, perfil de función metabólica y perfil de genes de resistencia a antibióticos.

En la última década se han realizado numerosos estudios mediante secuenciación masiva para conocer la microbiota de los quesos industriales y artesanales. En los productos artesanales, el conocimiento profundo de la microbiota nativa no solo contribuye a su descripción y comprensión, sino que también podría servir para seleccionar microorganismos y establecer la dosis de cultivo iniciador para mejorar los pasos de transformación, aumentando las propiedades sensoriales del producto (Ruvalcaba-Gómez y col., 2021).

El primer estudio que se realizó con esta metodología fue realizado por Masoud y col., (2011), en el que estudió mediante pirosecuenciación la diversidad microbiana presente en leche cruda y quesos elaborados a partir de esta materia prima a diferentes estadios de maduración. Este estudio mostró que la diversidad microbiana disminuía a lo largo del proceso de maduración debido a los cambios de pH en la matriz del queso y a las condiciones de maduración.

Quigley y col. (2012) también estudiaron por pirosecuenciación la microbiota presente en 62 quesos artesanales irlandeses elaborados con leche cruda o pasterizada de vaca, oveja y cabra con diferentes tiempos de maduración. Encontraron la presencia de microorganismos pertenecientes a los géneros *Arthrobacter* y *Brachybacterium*, nunca antes aislados en quesos elaborados con leche de cabra.

Wolfe y col. (2014) estudiaron la composición microbiana de 137 quesos de 10 países diferentes e identificaron alrededor de 14 géneros de bacterias y 10 de hongos, y estudiando su potencial contribución al sabor típico de los quesos objeto de estudio.

Diversos autores (Dugat-Bony y col., 2015; De Filippis y col., 2016; Yang y col., 2020) estudiaron como cambia la microbiota de quesos a lo largo del proceso de maduración empleando análisis metagenómicos y metatranscriptómicos. Observaron cómo contribuyen las especies microbianas dominantes y las posibles interacciones que

se producen entre ellas para dar lugar a cambios bioquímicos que se generan durante el proceso de maduración.

En quesos de pasta blanda, Gonçalves y col. (2017, 2018) estudiaron la microbiota autóctona en queso "*Serpa*", de industrias pertenecientes a DOP y otras dos industrias no certificadas. Los estudios de la comunidad bacteriana y de levaduras mostraron una amplia diversidad de géneros y especies, así como también diferencias significativas entre la población microbiana de quesos DOP y no DOP. Ruvalcaba-Gómez y col. (2021) estudiaron la microbiota bacteriana en quesos Adobera (México) elaborados con leche cruda de vaca, encontrando *Streptococcus* spp., *Lactococcus* spp. y *Lactobacillus* spp. como los géneros más abundantes durante toda la maduración.

En este sentido, no existen estudios realizados sobre la microbiota de quesos de pasta blanda "*Torta del Casar*" y "*Queso de la Serena*" mediante técnicas de secuenciación masiva. Por ello, el interés de este trabajo es conocer los microorganismos involucrados en el proceso de maduración de los quesos de pasta blanda de la comunidad Autónoma de Extremadura. El principal objetivo es conocer cuáles son los principales microorganismos responsable de los cambios bioquímicos que otorgan a este tipo de queso características únicas.

4.5.Selección de microorganismos con propiedades tecnológicas y funcionales

4.5.1. Aspectos tecnológicos

En los últimos años, se ha incrementado el interés por conocer la implicación de los microorganismos durante el proceso de maduración de los quesos tradicionales. Diversos autores han estudiado algunos aspectos tecnológicos que son de interés en la maduración del queso, como puede ser la actividad proteolítica, lipolítica, actividad antimicrobiana y capacidad de inhibición de algunos mohos y bacterias que pueden alterar el aspecto externo de los quesos. Además, la capacidad de adaptación al medio es importante ya que la matriz del queso confiere un ambiente anaerobio, ligeramente ácido y con una concentración de sal elevada.

Aunque los microorganismos principales de la maduración de los quesos son las BAL, la microbiota secundaria presenta un gran interés debido a su implicación en los cambios que se producen durante el proceso de maduración.

Las enterobacterias juegan un papel importante en este proceso. No obstante, las enterobacterias están consideradas indicadores de contaminación, por lo que el estudio de su seguridad (resistencia a antibióticos, capacidad hemolítica y genes de resistencia a antibióticos) son imprescindibles. Algunos de los cambios bioquímicos, como la proteólisis, son producidos por la acción de estas bacterias. En los últimos años, el estudio de las propiedades tecnológicas de algunas especies mayoritarias en quesos, como son *Hafnia alvei* y *Hafnia paralvei*, ha aumentado. Tabla y col. (2016) estudiaron la capacidad de producción de gas de *H. alvei* en cepas aisladas de quesos "*Torta del Casar*", encontrando que la presencia de "ojos" en la pasta del queso está relacionada con la presencia de esta especie a lo largo del proceso de maduración. Las especies de *Hafnia* spp. utilizadas como cultivo iniciador en quesos de pasta blanda pueden mejorar el flavor del queso ya que generan compuestos volátiles aromáticos (Irlinger y col., 2012; Pham y col., 2019), además de disminuir los niveles de especies patógenas como *E. faecalis* y *E. coli* a la vez que estimulan el crecimiento de *L. plantarum* según lo descrito por Delbès-Paus y col. (2011, 2012, 2013).

Otros microorganismos que destacan de la microbiota secundaria son las levaduras. Estos microorganismos eucariotas contribuyen al aroma (Chen y col., 2012); a la asimilación/ fermentación de la lactosa (Pereira-Dias y col., 2000); presentan actividad antagonista frente a microorganismos patógenos o alterantes mediante la producción de compuestos antimicrobianos (Baeza y col., 2008; Bajaj y col., 2013; Saber y col., 2017); muestran adecuada tolerancia a diferentes estreses como alta concentración de NaCl (Tofalo y col., 2014; Fadda y col., 2004), tolerancia a bajos valores de actividad de agua (a_w) y pH (Capece y Romano, 2009), crecimiento a diferentes temperaturas (Cardoso y col., 2015; Padilla y col., 2014b) y efectos beneficiosos en la salud (Hatoum y col., 2012). Además, son capaces de metabolizar el ácido láctico y otros ácidos orgánicos producidos por las BAL (Jakobsen y Narvhus, 1996; Mounier y col., 2009). La actividad proteolítica y lipolítica de ciertas especies puede desempeñar también un papel importante en la formación de precursores del aroma, tales como aminoácidos, ácidos orgánicos y ésteres, además de contribuir con los cambios en la textura (Padilla y col., 2014a). Atanassova y col. (2016) estudiaron diferentes aspectos tecnológicos de aislados de levaduras de quesos elaborados con leche cruda de vaca y observaron que ciertas especies, como *Y. lipolytica, K. lactis, D. hansenii* y *P. guilliermondii* son capaces de crecer en ambientes similares a la matriz del queso, además de presentar actividad

proteolítica y lipolítica, y producir compuestos volátiles capaces de aportar atributos sensoriales característicos del queso.

4.5.2. Aspectos funcionales

El concepto de alimento funcional fue definido por investigadores por primera vez en Japón en 1984, quienes estudiaron las correlaciones entre la nutrición, la calidad sensorial y la modulación de los sistemas fisiológicos (Siro y col., 2008).

Los alimentos funcionales son definidos por la Organización de las Naciones Unidas para la Alimentación y la Agricultura (también conocida como FAO, por sus siglas en inglés: Food and Agriculture Organization) como aquellos alimentos que contienen componentes biológicos adicionales, con potencial de reducir el riesgo de enfermedad y favorecer la salud. Poseen efectos beneficiosos sobre una o más funciones en el organismo y se incluye alimentos que contienen minerales, vitaminas, ácidos grasos, fibra, alimentos con antioxidantes y probióticos.

Según Tufarelli y Laudadio (2016), las características de un alimento funcional deben ser:

- Ser un alimento convencional o cotidiano
- Consumirse como parte de la dieta normal/habitual
- Compuesto de componentes de origen natural (a diferencia de los sintéticos) en concentraciones superiores a las naturalmente presentes
- Tener un efecto positivo en las funciones objetivo más allá del valor nutritivo
- Poder mejorar el bienestar y la salud y/o reducir el riesgo de enfermedad o proporcionar beneficios para la salud a fin de mejorar la calidad de vida, incluido el rendimiento físico, psicológico y conducta
- Estar autorizado y tener base científica

En los últimos años, se ha incrementado la demanda de alimentos funcionales debido al interés de los consumidores en buscar mejoras para la salud (Balthazar y col., 2017). Estos alimentos brindan beneficios en el sistema inmunológico, tracto digestivo, respiratorio, etc.; por tanto, tendrían efecto en la disminución del riesgo de padecer ciertas enfermedades (Yerlikaya y Ozer, 2014).

En la lista de alimentos funcionales cabe destacar los aportados por la industria láctea, como son las leches enriquecidas y los productos lácteos fermentados. Estos últimos son los vehículos de probióticos más comercializados y además sus características físico-químicas favorecen la viabilidad de los microorganismos probióticos contribuyendo a que alcancen el tracto gastrointestinal (Abadía-García y col., 2013).

El queso, al ser un producto lácteo fermentado, es un alimento idóneo para la incorporación de nuevos ingredientes que contribuyan a mejorar la salud del consumidor, debido a su consumo habitual en los hogares. En los quesos tradicionales, la aplicación de microorganismos que pueda ejercer un beneficio para la salud, sin modificar los atributos sensoriales únicos de estos productos, puede aportar un valor funcional atractivo para el consumidor.

4.5.2.1.Probióticos.

El término probiótico proviene del latín (pro-) y del griego (-bios) y significa "a favor de la vida". Según la Organización Mundial de la Salud y la FAO los probióticos son "*microorganismos vivos que, cuando se consumen en cantidades apropiadas como parte de un alimento, confieren al huésped un beneficio para la salud*".

Algunos de los criterios más importantes para que un microorganismo sea considerado como probiótico son (Abatenh y col., 2018):

- Debe tener origen humano
- Debe ser capaz de crear un efecto beneficioso en el hospedador incrementando la resistencia a enfermedades
- Debe estar en concentraciones elevadas para poder colonizar el tracto gastrointestinal
- Ser capaz de sobrevivir a las condiciones del tracto gastrointestinal.
- Debe ser seguro, estable y efectivo
- No debe ser patógeno

Uno de los aspectos más importantes que debe tener un microorganismo para ser aplicado en alimentos tanto por sus propiedades tecnológicas como por su papel funcional

como probiótico es que sea seguro. Los alimentos fermentados actúan como reservorios de numerosas bacterias viables, incluidas las bacterias resistentes a los antibióticos, que pueden transferirse al intestino a través del consumo de alimentos. Estas bacterias pueden portar genes de resistencia a antibióticos transferibles que podrían transferirse a bacterias comensales o patógenas (Jeong y col., 2015). Las bacterias a menudo tienen dos tipos distintos de resistencia a los antibióticos, la intrínseca y la adquirida. La resistencia intrínseca es un rasgo que ocurre naturalmente en el organismo, mientras que la resistencia adquirida es la evolución de bacterias sensibles a bacterias resistentes. Las β-lactamasas, incluidas las β-lactamasas de espectro extendido (BLEE) y las carbapenemasas, son responsables de causar resistencia a múltiples antibióticos (Yuan y col., 2021).

Los microorganismos también pueden generar compuestos nitrogenados como las aminas biógenas, que suelen tener aromas fuertes y desagradables (Pagthinathan y col., 2017) y suponen un riesgo para la salud cuando están presentes en niveles significativos. La presencia de aminas biógenas en alimentos y bebidas ha suscitado gran interés debido a su capacidad de afectar directa o indirectamente a la salud de los consumidores y pueden tener efectos psicoactivos, vasoactivos e hipertensivos. Los géneros *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Streptococcus* y *Leuconostoc*, principales responsables de la maduración de los quesos están asociados a una alta producción de aminas biógenas debido a que presentan genes u operones responsables de rutas metabólicas relacionadas con el catabolismo o biosíntesis de estos compuestos. Además, algunos contaminantes lácteos como pueden ser las enterobacterias y *Pseudomonas* spp. también son productores de estos compuestos nitrogenados (Benkerroum, 2016; Bover-Cid y col., 1999). Las aminas psicoactivas (histamina, putrescina y cadaverina) pueden provocar dolor de cabeza, sarpullidos, palpitaciones (entre otros) y las aromáticas (tiramina, triptamina y **β**-feniletilamina) muestran actividad vasoconstrictora (Ekici y Omer, 2020). La tiramina es la principal amina biógena en quesos y es la causante del llamado "efecto queso" que se caracteriza por hipertensión, dolor de cabeza y migraña (Benkerroum, 2016; Ruiz-Capillas y col., 2019). Por lo tanto, evaluar la presencia de genes transmisibles de resistencia a antibióticos y la producción de aminas biógenas es una cuestión de seguridad importante que debe tenerse en cuenta durante la evaluación de las cepas que puedan ser empleadas como probióticos.

La mayoría de los microorganismos probióticos son bacterias gram-positivas, incluyendo bacterias no patógenas como *Streptococcus*, *Bacillus*, *Lactobacillus*, *Enterococcus* y *Pediococcus*. Los probióticos también pueden incluir especies de mohos y levaduras como *Aspergilus oryzae*, *Saccharomyces cerevisiae* var. *boulardii* y *Kluyveromyces marxianus*. Solo algunos microorganismos como *Lactobacillus* spp., *Bifidobacterium* spp. y *Lactococcus* spp. se conocen generalmente como seguros (GRAS-Generally Recognized As Safe) (Durazzo y col., 2020). Las principales especies utilizadas como probióticos están recogidas en la tabla 8

Tabla 8. Principales especies utilizadas como probióticos (Fijan, 2014; Kerry y col., 2018; Markowiak y Slizewska, 2017).

GÉNEROS	ESPECIES
Lactobacillus	*L. acidophilus, L. amylovorus, L. casei, L. crispatus, L. delbrueckii subespecie bulgaricus, L. fermentum, L. gallinarum, L. gasseri, L. helveticus, L. johnsonii, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus, L. brevis*
Lactococcus	*L. lactis, L. reuteri, L. rhamnosus, L. casei, L. acidophilus, L. curvatus, L. plantarum*
Bifidobacterium	*B. adolescentis, B. angulatum, B. animalis* subespecie *lactis, B. bifidum, B. breve, B. catenulatum, B. dentium, B. infantis, B. longum, B. thermophilum*
Otras BAL	*Enterococcus durans, Enterococcus faecium, Leuconostoc mesenteroides, Pediococcus acidilactici, Streptococcus salivarius* subespecie *thermophilus, Streptococcus diacetylactis*
Bacillus	*B. coagulans* BC30, *B. subtilis* R0179, *B. cereus* var. *toyoi, B. cereus* IP5832, *B. clausii*
Otros microorganismos	*Escherichia coli* Nissle 1917, *Propionibacterium freudenreichii, Saccharomyces boulardii*

Para que un alimento sea considerado como probiótico debe presentar microorganismos vivos y además estos deben estar en dosis altas (entre 10^6 - 10^8 ufc/g de producto) para obtener los efectos deseados. Algunos de estos efectos son: estimular el sistema inmune, disminuir la intolerancia a la lactosa, disminuir la incidencia de trastornos digestivos, reducir el colesterol en sangre, afectar positivamente la microflora intestinal, reducir el crecimiento de agentes patógenos, entre otros (Patrignani y col., 2019; Tufarelli y Laudadio, 2016). En la tabla 9 se muestran los posibles mecanismos de acción propuestos por el panel de consenso en 2014.

Tabla 9. Mecanismos de acción de probióticos propuesta por el panel de consenso en 2014 (Adaptado de Hill y col., 2014).

Ampliamente distribuidos	**Frecuentes** **Efectos a nivel de especie**	**Raros** **Efectos específicos de cepa**
Resistencia la colonización	Síntesis de vitaminas	Efectos neurológicos
Modulación de la microbiota intestinal	Antagonismo directo	Efectos inmunológicos
Producción de ácidos y ácidos grasos de cadena corta	Reforzamiento de la barrera intestinal	Efectos endocrinológicos
Aumento del recambio de enterocitos	Metabolismo de sales biliares	Producción de compuestos bioactivos específicos
Regulación del tránsito intestinal	Neutralización de carcinógenos	
Exclusión competitiva de patógenos		

El potencial probiótico de las BAL ha sido ampliamente estudiado (Angmo y col., 2016; de Alburquerque y col., 2018; Dilna y col., 2015; Hill y col., 2014; Jensen y col., 2012; Kimoto-Nira y col., 2007; Ruiz-Moyano y col., 2019; Sanders y col., 2018).

La mayoría de probióticos que se utilizan en la actualidad provienen de bacterias intestinales, aunque en los últimos años se está estudiando el potencial probiótico de BAL y levaduras procedentes de productos lácteos fermentados. En este sentido, las levaduras han supuesto un enorme interés, ya que solo *Saccharomyces cerevisiae* var. b*oulardii* es la única levadura reconocida hasta el momento como probiótico y utilizada como tratamiento y método preventivo de diversos trastornos del tracto gastrointestinal y síntomas ocasionados por enfermedades infecciosas como *Clostridium difficile* o *Helycobacter pylori* (Lukaszewicz, 2012; Zanello y col., 2009).

Cabe destacar la especie *Kluyveromyces marxianus*, recientemente reconocida como segura (GRAS) por la FDA (Administración de Alimentos y Medicamentos de los Estados Unidos) y calificada por la EFSA (Autoridad Europea de Seguridad Alimentaria) como "presunción cualificada de seguridad". Este microorganismo se ha aislado de muchos productos lácteos, como el kéfir (Gut y col., 2019; Cho y col., 2018) y distintas variedades de queso elaborado con leche cruda de oveja como el queso *Serpa*, *Serra da Estrela*, *Fiore Sardo* y *Peccorino*, entre otros (revisado por Fröhlich-Wyder y col., 2019).

Las levaduras, componentes de la microbiota secundaria de los quesos, están involucradas en numerosos cambios bioquímicos durante el proceso de maduración del producto y por ello, numerosos autores han estudiado el potencial probiótico de estos microorganismos (Banjara y col., 2016; Binetti y col., 2013; Ceugniez y col., 2015; Chen y col., 2010; Fadda y col., 2017; Gil-Rodríguez y col., 2015; Kumura y col., 2004; Psomas y col., 2001, 2003; Saber y col., 2017; Zivkovic y col., 2015).

5. Referencias bibliográficas.

Abadía-García, L., Cardador, A., Martín del Campo, S. T., Arvízu, S. M., Castaño-Tostado, E., Regalado-González, C., … Amaya-Llano, S. L. (2013). Influence of probiotic strains added to cottage cheese on generation of potentially antioxidant peptides, anti-listerial activity, and survival of probiotic microorganisms in simulated gastrointestinal conditions. *International Dairy Journal*, *33*(2), 191–197. https://doi.org/10.1016/j.idairyj.2013.04.005

Abatenh, E., Gizaw, B., Tsegay, Z., Tefera, G., & Aynalem, E. (2018). Health benefits of probiotics. *Journal of Bacteriology and Infection Diseases*, *2*(1), 17–27.

Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E., & Martínez-Bueno, M. (2008). Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology*, *127*(3), 200–208. https://doi.org/10.1016/j.ijfoodmicro.2008.07.004

Alavi, F., & Momen, S. (2020). Aspartic proteases from thistle flowers: Traditional coagulants used in the modern cheese industry. *International Dairy Journal*, *107*, 104709. https://doi.org/10.1016/j.idairyj.2020.104709

Alegría, Á., Álvarez-Martín, P., Sacristán, N., Fernández, E., Delgado, S., & Mayo, B. (2009). Diversity and evolution of the microbial populations during manufacture and ripening of Casín, a traditional Spanish, starter-free cheese made from cow's milk. *International Journal of Food Microbiology*, *136*(1), 44–51. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2009.09.023

Almeida, G., Figueiredo, A., Rôla, M., Barros, R. M., Gibbs, P., Hogg, T., & Teixeira, P. (2007). Microbiological characterization of randomly selected Portuguese raw milk cheeses with reference to food safety. *Journal of Food Protection*, *70*(7), 1710–1716. https://doi.org/10.4315/0362-028X-70.7.1710

Andersen, L. T., Ardö, Y., & Bredie, W. L. P. (2010). Study of taste-active compounds in the water-soluble extract of mature Cheddar cheese. *International Dairy Journal*, *20*(8), 528–536. https://doi.org/https://doi.org/10.1016/j.idairyj.2010.02.009

Angmo, K., Kumari, A., Savitri, & Bhalla, T. C. (2016). Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh. *LWT - Food Science and Technology*, *66*, 428–435. https://doi.org/10.1016/j.lwt.2015.10.057

Araújo-Rodrigues, H., Tavaria, F. K., dos Santos, M. T. P. G., Alvarenga, N., & Pintado, M. M. (2020). A review on microbiological and technological aspects of Serpa PDO cheese: An ovine raw milk cheese. *International Dairy Journal*, *100*, 104561. https://doi.org/10.1016/j.idairyj.2019.104561

Ardic, M., Karakaya, Y., Atasever, M., & Adiguzel, G. (2009). Aflatoxin M1 levels of Turkish white brined cheese. *Food Control*, *20*(3), 196–199. https://doi.org/10.1016/j.foodcont.2008.04.003

Ardö, Y., McSweeney, P. L. H., Magboul, A. A. A., Upadhyay, V. K., & Fox, P. F. (2017). Chapter 18 - Biochemistry of Cheese Ripening: Proteolysis. In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. B. T.-C. (Fourth E. Everett (Eds.) (pp. 445–482). San Diego: Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-417012-4.00018-1

Ardui, S., Ameur, A., Vermeesch, J. R., & Hestand, M. S. (2018). Single molecule real-time (SMRT) sequencing comes of age: Applications and utilities for medical diagnostics. *Nucleic Acids Research*, *46*(5), 2159–2168. https://doi.org/10.1093/nar/gky066

Atanasova, J., Dalgalarrondo, M., Iliev, I., Moncheva, P., Todorov, S. D., & Ivanova, I. V. (2020). Formation of Free Amino Acids and Bioactive Peptides During the Ripening of Bulgarian White Brined Cheeses. *Probiotics and Antimicrobial Proteins*. https://doi.org/10.1007/s12602-020-09669-0

Atanassova, M. R., Fernández-Otero, C., Rodríguez-Alonso, P., Fernández-No, I. C., Garabal, J. I., & Centeno, J. A. (2016). Characterization of yeasts isolated from artisanal short-ripened cows' cheeses produced in Galicia (NW Spain). *Food Microbiology*, *53*, 172–181. https://doi.org/10.1016/j.fm.2015.09.012

Baeza, M. E., & Sanhueza, M. A. (2008). Clinical Yeast Isolates. *Control*, 173–182.

Bajaj, B. K., Raina, S., & Singh, S. (2013). Killer toxin from a novel killer yeast *Pichia kudriavzevii* RY55 with idiosyncratic antibacterial activity. *Journal of Basic Microbiology*, *53*(8), 645–656. https://doi.org/10.1002/jobm.201200187

Balthazar, C. F., Pimentel, T. C., Ferrão, L. L., Almada, C. N., Santillo, A., Albenzio, M., … Cruz, A. G. (2017). Sheep Milk: Physicochemical Characteristics and Relevance for Functional Food Development. *Comprehensive Reviews in Food Science and Food Safety*, *16*(2), 247–262. https://doi.org/10.1111/1541-4337.12250

Banjara, N., Nickerson, K. W., Suhr, M. J., & Hallen-Adams, H. E. (2016). Killer toxin from several food-derived *Debaryomyces hansenii* strains effective against pathogenic Candida yeasts. *International Journal of Food Microbiology*, *222*, 23–29. https://doi.org/10.1016/j.ijfoodmicro.2016.01.016

Banjara, N., Suhr, M. J., & Hallen-Adams, H. E. (2015). Diversity of Yeast and Mold Species from a Variety of Cheese Types. *Current Microbiology*, *70*(6), 792–800. https://doi.org/10.1007/s00284-015-0790-1

Barron, L. J. R., Redondo, Y., Aramburu, M., Gil, P., Pérez-Elortondo, F. J., Albisu, M., … Fernández-García, E. (2007). Volatile composition and sensory properties of industrially produced Idiazabal cheese. *International Dairy Journal*, *17*(12), 1401–1414. https://doi.org/10.1016/j.idairyj.2007.04.001

Benkerroum, N. (2016). Biogenic Amines in Dairy Products: Origin, Incidence, and Control Means. *Comprehensive Reviews in Food Science and Food Safety*, *15*(4), 801–826. https://doi.org/10.1111/1541-4337.12212

Binetti, A., Carrasco, M., Reinheimer, J., & Suárez, V. (2013). Yeasts from autochthonal cheese starters: Technological and functional properties. *Journal of Applied Microbiology*, *115*(2), 434–444. https://doi.org/10.1111/jam.12228

Bintsis, T., & Papademas, P. (2002). Microbiological quality of white-brined cheeses: a review. *International Journal of Dairy Technology*, *55*(3), 113–120. https://doi.org/10.1046/j.1471-0307.2002.00054.x

Bokulich, N. A., & Mills, D. A. (2013). Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. *Applied and Environmental Microbiology*, *79*(17), 5214–5223. https://doi.org/10.1128/AEM.00934-13

Bokulich, N. A., & Mills, D. A. (2013). Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Applied and Environmental Microbiology*, *79*(8), 2519–2526. https://doi.org/10.1128/AEM.03870-12

Bover-Cid, S., & Holzapfel, W. H. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *International Journal of Food Microbiology*, *53*(1), 33–41. https://doi.org/10.1016/S0168-1605(99)00152-X

Cáceres, P., Castillo, D., & Pizarro, M. (1997). Secondary flora of Casar de Cáceres cheese: Characterization of Micrococcaceae. *International Dairy Journal*, *7*(8–9), 531–536. https://doi.org/10.1016/S0958-6946(97)00045-9

Cao, Y., Fanning, S., Proos, S., Jordan, K., & Srikumar, S. (2017). A Review on the Applications of Next Generation Sequencing Technologies as Applied to Food-Related Microbiome Studies. *Frontiers in Microbiology*, *8*(SEP), 1–16. https://doi.org/10.3389/fmicb.2017.01829

Capece, A., & Romano, P. (2009). "Pecorino di Filiano" cheese as a selective habitat for the yeast species, *Debaryomyces hansenii*. *International Journal of Food Microbiology*, *132*(2–3), 180–184. https://doi.org/10.1016/j.ijfoodmicro.2009.04.007

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., … Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, *108*(Supplement_1), 4516–4522. https://doi.org/10.1073/pnas.1000080107

Carbonell, M., Nuñez, M., & Fernández-García, E. (2002). Evolution of the volatile components of ewe raw milk La Serena cheese during ripening. Correlation with flavour characteristics. *Le Lait*, *82*(6), 683–698. https://doi.org/10.1051/lait:2002042

Carbonell, M., Nuñez, M., & Fernández-García, E. (2002). Seasonal variation of volatile compounds in ewe raw milk La Serena cheese. *Le Lait*, *82*(6), 699–711. https://doi.org/10.1051/lait:2002043

Cardoso, V. M., Borelli, B. M., Lara, C. A., Soares, M. A., Pataro, C., Bodevan, E. C., & Rosa, C. A. (2015). The influence of seasons and ripening time on yeast communities of a traditional Brazilian cheese. *Food Research International*, *69*, 331–340. https://doi.org/10.1016/j.foodres.2014.12.040

Carminati, D., Bonvini, B., Rossetti, L., Zago, M., Tidona, F., & Giraffa, G. (2019). Investigation on the presence of blue pigment-producing *Pseudomonas* strains along a production line of fresh mozzarella cheese. *Food Control*, *100*(February), 321–328. https://doi.org/10.1016/j.foodcont.2019.02.009

Carocho, M., Barreira, J. C. M., Antonio, A. L., Bento, A., Morales, P., & Ferreira, I. C. F. R. (2015). The incorporation of plant materials in "Serra da Estrela" cheese improves antioxidant activity without changing the fatty acid profile and visual appearance. *European Journal of Lipid Science and Technology*, *117*(10), 1607–1614. https://doi.org/10.1002/ejlt.201500018

Carraro, L., Maifreni, M., Bartolomeoli, I., Martino, M. E., Novelli, E., Frigo, F., … Cardazzo, B. (2011). Comparison of culture-dependent and -independent methods for bacterial community monitoring during Montasio cheese manufacturing. *Research in Microbiology*, *162*(3), 231–239. https://doi.org/10.1016/j.resmic.2011.01.002

Carrascosa, C., Millán, R., Jaber, J. R., Lupiola, P., del Rosario-Quintana, C., Mauricio, C., & Sanjuán, E. (2015). Blue pigment in fresh cheese produced by *Pseudomonas fluorescens*. *Food Control*, *54*, 95–102. https://doi.org/10.1016/j.foodcont.2014.12.039

Carreira, A., Ferreira, L. M., & Loureiro, V. (2001). Production of brown tyrosine pigments by the yeast *Yarrowia lipolytica*. *Journal of Applied Microbiology*, *90*(3), 372–379. https://doi.org/10.1046/j.1365-2672.2001.01256.x

Ceugniez, A., Drider, D., Jacques, P., & Coucheney, F. (2015). Yeast diversity in a traditional French cheese "Tomme d'orchies" reveals infrequent and frequent species with associated benefits. *Food Microbiology*, *52*, 177–184. https://doi.org/10.1016/j.fm.2015.08.001

Chaves-López, C., De Angelis, M., Martuscelli, M., Serio, A., Paparella, A., & Suzzi, G. (2006). Characterization of the *Enterobacteriaceae* isolated from an artisanal Italian ewe's cheese (Pecorino Abruzzese). *Journal of Applied Microbiology*, *101*(2), 353–360. https://doi.org/10.1111/j.1365-2672.2006.02941.x

Chávez-de la Peña, M. E., Higuera-Iglesias, A. L., Huertas-Jiménez, M. A., Báez-Martínez, R., Morales-de León, J., Arteaga-Cabello, F., … Ponce de León-Rosales, S. (2001). Brote por *Salmonella enteritidis* en trabajadores de un hospital. *Salud Pública de México*, *43*(3), 211–216. https://doi.org/10.1590/s0036-36342001000300006

Chen, L. S., Ma, Y., Maubois, J. L., He, S. H., Chen, L. J., & Li, H. M. (2010). Screening for the potential probiotic yeast strains from raw milk to assimilate cholesterol. *Dairy Science and Technology*, *90*(5), 537–548. https://doi.org/10.1051/dst/2010001

Chen, L. shui, Cui, J., Ding, Q. bo, Ma, Y., Chen, L. jun, Dong, J. ying, … Maubois, J. louis. (2012). The Effect of Yeast Species from Raw Milk in China on Proteolysis and Aroma Compound Formation in Camembert-Type Cheese. *Food and Bioprocess Technology*, *5*(6), 2548–2556. https://doi.org/10.1007/s11947-011-0589-4

Cho, Y. J., Kim, D. H., Jeong, D., Seo, K. H., Jeong, H. S., Lee, H. G., & Kim, H. (2018). Characterization of yeasts isolated from kefir as a probiotic and its synergic interaction with the wine by product grape seed flour/extract. *Lwt*, *90*(December 2017), 535–539. https://doi.org/10.1016/j.lwt.2018.01.010

Choi, J., In Lee, S., Rackerby, B., Frojen, R., Goddik, L., Ha, S. Do, & Park, S. H. (2020). Assessment of overall microbial community shift during Cheddar cheese production from raw milk to aging. *Applied Microbiology and Biotechnology*, *104*(14), 6249–6260. https://doi.org/10.1007/s00253-020-10651-7

Cholet, O., Hénaut, A., Casaregola, S., & Bonnarme, P. (2007). Gene expression and biochemical analysis of cheese-ripening yeasts: Focus on catabolism of L-methionine, lactate, and lactose. *Applied and Environmental Microbiology*, *73*(8), 2561–2570. https://doi.org/10.1128/AEM.02720-06

CODEX STAN 283-1978. (2013). Norma General Del Codex para el queso. *CODEX Alimentarius*, 1–4. Retrieved from http://www.fao.org/input/download/standards/175/CXS_283s.pdf

Cogan, T. M., Barbosa, M., Beuvier, E., Bianchi-Salvadori, B., Cocconcelli, P. S., Fernandes, I., … Rodriguez, E. (1997). Characterization of the lactic acid bacteria in artisanal dairy products. *Journal of Dairy Research*, *64*(3), 409–421. https://doi.org/10.1017/S0022029997002185

Collins, Y. F., McSweeney, P. L. H., & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: A review of current knowledge. *International Dairy Journal*, *13*(11), 841–866. https://doi.org/10.1016/S0958-6946(03)00109-2

Consejería de Economía, I. y C. J. de Ex. ORDEN de 9 de octubre de 2001, por la que se aprueba el Reglamento de la Denominación de Origen (D.O.P.) «Torta del Casar»., 119 § (2001).

Consejo de la union europea. (2006). Reglamento (CE) N 510/2006 del consejo de 20 de marzo de 2006 sobre la protección de las indicaciones geográficas y de las denominaciones de origen en los productos agrícolas y alimenticios. *Diario Oficial de La Union Europea*, (10), 12–25.

Coppa, M., Martin, B., Glasser, F., Chilliard, Y., Chassaing, C., Agabriel, C., … Ferlay, a. (2011). An European dataset to prediect milk fatty acid profile from production

conditions. *10th International Meeting on Mountain Cheese, 14-15 September 2011, Dronero, Italy*.

Costanzo, N., Ceniti, C., Santoro, A., Clausi, M. T., & Casalinuovo, F. (2020). Foodborne Pathogen Assessment in Raw Milk Cheeses. *International Journal of Food Science, 2020*. https://doi.org/10.1155/2020/3616713

Curioni, P. M. G., & Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography-olfactometry. *International Dairy Journal*, *12*(12), 959–984. https://doi.org/10.1016/S0958-6946(02)00124-3

D'Amico, D. J., & Donnelly, C. W. (2011). FDA's domestic and imported cheese compliance program results: January 1, 2004 - December 31, 2006. *Food Protection Trends*, *31*(4), 216–226.

de Albuquerque, T. M. R., Garcia, E. F., de Oliveira Araújo, A., Magnani, M., Saarela, M., & de Souza, E. L. (2018). In Vitro Characterization of *Lactobacillus* Strains Isolated from Fruit Processing By-Products as Potential Probiotics. *Probiotics and Antimicrobial Proteins*, *10*(4), 704–716. https://doi.org/10.1007/s12602-017-9318-2

De Filippis, F., Genovese, A., Ferranti, P., Gilbert, J. A., & Ercolini, D. (2016). Metatranscriptomics reveals temperature-driven functional changes in microbiome impacting cheese maturation rate. *Scientific Reports*, *6*(October 2015), 1–11. https://doi.org/10.1038/srep21871

De Filippis, F., Laiola, M., Blaiotta, G., & Ercolini, D. (2017). Different amplicon targets for sequencing-based studies of fungal diversity. *Applied and Environmental Microbiology*, *83*(17), 1–9. https://doi.org/10.1128/AEM.00905-17

De Filippis, F., Parente, E., & Ercolini, D. (2017). Metagenomics insights into food fermentations. *Microbial Biotechnology*, *10*(1), 91–102. https://doi.org/10.1111/1751-7915.12421

De Pasquale, I., Calasso, M., Mancini, L., Ercolini, D., La Storia, A., De Angelis, M., … Gobbetti, M. (2014). Causal relationship between microbial ecology dynamics and proteolysis during manufacture and ripening of Protected Designation of Origin (PDO) cheese Canestrato Pugliese. *Applied and Environmental Microbiology*, *80*(14), 4085–4094. https://doi.org/10.1128/AEM.00757-14

Decreo Regulamentar n.º 39/87 de 29 de Junho. (1987).

del Olmo, A., Calzada, J., & Nuñez, M. (2018). The blue discoloration of fresh cheeses: A worldwide defect associated to specific contamination by *Pseudomonas fluorescens*. *Food Control*, *86*, 359–366. https://doi.org/10.1016/j.foodcont.2017.12.001

Delavenne, E., Mounier, J., Asmani, K., Jany, J.-L., Barbier, G., & Le Blay, G. (2011). Fungal diversity in cow, goat and ewe milk. *International Journal of Food Microbiology*, *151*(2), 247–251. https://doi.org/10.1016/j.ijfoodmicro.2011.08.029

Delbès-Paus, C., Miszczycha, S., Ganet, S., Helinck, S., Veisseire, P., Pochet, S., … Montel, M. C. (2013). Behavior of *Escherichia coli* O26: H11 in the presence of *Hafnia alvei* in a model cheese ecosystem. *International Journal of Food Microbiology*, *160*(3), 212–218. https://doi.org/10.1016/j.ijfoodmicro.2012.10.019

Delbès-Paus, C., Pochet, S., Helinck, S., Veisseire, P., Bord, C., Lebecque, A., … Montel, M. C. (2012). Impact of Gram-negative bacteria in interaction with a complex microbial consortium on biogenic amine content and sensory characteristics of an uncooked pressed cheese. *Food Microbiology*, *30*(1), 74–82. https://doi.org/10.1016/j.fm.2011.12.008

Delgado Matínez, F. J., González-Crespo, J., Cava, R., García-Parra, J., & Ramírez, R. (2010). Characterisation by SPME–GC–MS of the volatile profile of a Spanish soft cheese P.D.O. Torta del Casar during ripening. *Food Chemistry*, *118*(1), 182–189. https://doi.org/10.1016/j.foodchem.2009.04.081

Diario Oficial de la Unión Europea. (2003). REGLAMENTO (CE) No 1491/2003 DE LA COMISIÓN de 25 de agosto de 2003 por el que se completa el Reglamento (CE) no 2400/96 (Ficodindia dell'Etna, Monte Etna, Colline di Romagna, Pretuziano delle Colline Teramane, Torta del Casar, Manzana de Girona o Poma de, *10*.

Diario Oficial de las Comunidades Europeas. (1996). REGLAMENTO (CE) N° 1107/96 DE LA COMISIÓN de 12 de junio de 1996 relativo al registro de las indicaciones geográficas y de las denominaciones de origen con arreglo al procedimiento establecido en el artículo 17 del Reglamento (CEE) n° 2081/92 del Consejo.

Dilna, S. V., Surya, H., Aswathy, R. G., Varsha, K. K., Sakthikumar, D. N., Pandey, A., & Nampoothiri, K. M. (2015). Characterization of an exopolysaccharide with potential health-benefit properties from a probiotic *Lactobacillus plantarum* RJF4. *LWT - Food Science and Technology*, *64*(2), 1179–1186. https://doi.org/10.1016/j.lwt.2015.07.040

Dugat-Bony, E., Garnier, L., Denonfoux, J., Ferreira, S., Sarthou, A. S., Bonnarme, P., & Irlinger, F. (2016). Highlighting the microbial diversity of 12 French cheese varieties. *International Journal of Food Microbiology*, *238*, 265–273. https://doi.org/10.1016/j.ijfoodmicro.2016.09.026

Dugat-Bony, E., Straub, C., Teissandier, A., Onésime, D., Loux, V., Monnet, C., … Bonnarme, P. (2015). Overview of a surface-ripened cheese community functioning by meta-omics analyses. *PLoS ONE*, *10*(4), 1–25. https://doi.org/10.1371/journal.pone.0124360

Durazzo, A., Nazhand, A., Lucarini, M., Atanasov, A. G., Souto, E. B., Novellino, E., … Santini, A. (2020). An updated overview on nanonutraceuticals: Focus on nanoprebiotics and nanoprobiotics. *International Journal of Molecular Sciences*, *21*(7), 1–24. https://doi.org/10.3390/ijms21072285

Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., … Turner, S. (2009). Real-time DNA sequencing from single polymerase molecules. *Science*, *323*(5910), 133–138. https://doi.org/10.1126/science.1162986

Ekici, K., & Omer, A. K. (2020). Biogenic amines formation and their importance in fermented foods. *BIO Web of Conferences*, *17*, 00232. https://doi.org/10.1051/bioconf/20201700232

Elotmani, F., Revol-Junelles, A.-M., Assobhei, O., & Millière, J.-B. (2002). Characterization of Anti- *Listeria monocytogenes* Bacteriocins from *Enterococcus faecalis, Enterococcus faecium,* and *Lactococcus lactis* Strains Isolated from Raïb, a Moroccan Traditional Fermented Milk. *Current Microbiology*, *44*(1), 10–17. https://doi.org/10.1007/s00284-001-0067-8

Ercolini, D. (2013). High-throughput sequencing and metagenomics: Moving forward in the culture-independent analysis of food microbial ecology. *Applied and Environmental Microbiology*, *79*(10), 3148–3155. https://doi.org/10.1128/AEM.00256-13

Ercolini, D., Russo, F., Ferrocino, I., & Villani, F. (2009). Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiology*, *26*(2), 228–231. https://doi.org/https://doi.org/10.1016/j.fm.2008.09.005

Fadda, M. E., Mossa, V., Deplano, M., Pisano, M. B., & Cosentino, S. (2017). In vitro screening of *Kluyveromyces* strains isolated from Fiore Sardo cheese for potential use as probiotics. *LWT - Food Science and Technology*, *75*, 100–106. https://doi.org/10.1016/j.lwt.2016.08.020

Fadda, M. E., Mossa, V., Pisano, M. B., Deplano, M., & Cosentino, S. (2004). Occurrence and characterization of yeasts isolated from artisanal Fiore Sardo cheese. *International Journal of Food Microbiology*, *95*(1), 51–59. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2004.02.001

Faion, A. M., Menegotto, A. L. L., Fernandes, I. A., Steffens, C., Steffens, J., & Valduga, E. (2020). Production of Serra da Estrela cheese from ultrafiltered sheep's milk. *Emirates Journal of Food and Agriculture*, *32*(5), 384–390. https://doi.org/10.9755/ejfa.2020.v32.i5.2109

FAO/WHO Working Group. (2014). Guidelines for the evaluation of probiotics in food: Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. London, Ontario, Canada: FAO/WHO.

Fedurco, M., Romieu, A., Williams, S., Lawrence, I., & Turcatti, G. (2006). BTA, a novel reagent for DNA attachment on glass and efficient generation of solid-phase amplified DNA colonies. *Nucleic Acids Research*, *34*(3), e22–e22. https://doi.org/10.1093/nar/gnj023

Fernandez Del Pozo, B., Gaya, P., Margarita, M., Medina, M., Rodriguez-Marin, M. A., & Nunez, M. (1988). Changes in the microflora of La Serena ewes' milk cheese during ripening. *Journal of Dairy Research*, *55*(3), 449–455. https://doi.org/10.1017/S0022029900028703

Fernandez Del Pozo, B., Gaya, P., Margarita, M., Medina, M., Rodriguez-Marin, M. A., & Nunez, M. (1988). Changes in chemical and rheological characteristics of La

Serena ewes' milk cheese during ripening. *Journal of Dairy Research*, *55*(3), 457–464. https://doi.org/10.1017/S0022029900028715

Fernández-García, E., Carbonell, M., & Nuñez, M. (2002). Volatile fraction and sensory characteristics of Manchego cheese. 1. Comparison of raw and pasteurized milk cheese. *Journal of Dairy Research*, *69*(4), 579–593. https://doi.org/10.1017/s0022029902005794

Fernández-García, E., Serrano, C., & Nuñez, M. (2002). Volatile fraction and sensory characteristics of Manchego cheese. 2. Seasonal variation. *Journal of Dairy Research*, *69*(4), 595–604. https://doi.org/10.1017/S0022029902005800

Fernández-García, E., Carbonell, M., Gaya, P., & Nuñez, M. (2004). Evolution of the volatile components of ewe´s raw milk Zamorano cheese. Seasonal variation. *International Dairy Journal*, *14*(8), 701–711. https://doi.org/10.1016/j.idairyj.2003.12.011

Fernández, M., Linares, D. M., Del Río, B., Ladero, V., & Alvarez, M. A. (2007). HPLC quantification of biogenic amines in cheeses: Correlation with PCR-detection of tyramine-producing microorganisms. *Journal of Dairy Research*, *74*(3), 276–282. https://doi.org/10.1017/S0022029907002488

Feutry, F., Oneca, M., Berthier, F., & Torre, P. (2012). Biodiversity and growth dynamics of lactic acid bacteria in artisanal PDO Ossau-Iraty cheeses made from raw ewe's milk with different starters. *Food Microbiology*, *29*(1), 33–42. https://doi.org/10.1016/j.fm.2011.08.011

Fickers, P., Marty, A., & Nicaud, J. M. (2011). The lipases from *Yarrowia lipolytica*: Genetics, production, regulation, biochemical characterization and biotechnological applications. *Biotechnology Advances*, *29*(6), 632–644. https://doi.org/10.1016/j.biotechadv.2011.04.005

Fijan, S. (2014). Microorganisms with claimed probiotic properties: An overview of recent literature. *International Journal of Environmental Research and Public Health*, *11*(5), 4745–4767. https://doi.org/10.3390/ijerph110504745

Foulquié Moreno, M. R., Sarantinopoulos, P., Tsakalidou, E., & De Vuyst, L. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology*, *106*(1), 1–24. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2005.06.026

Fox, P. F., & Kelly, A. L. (2006). Indigenous enzymes in milk: Overview and historical aspects—Part 1. *International Dairy Journal*, *16*(6), 500–516. https://doi.org/10.1016/j.idairyj.2005.09.013

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017). *Fundamentals of Cheese Science*. *Fundamentals of Cheese Science*. Boston, MA: Springer US. https://doi.org/10.1007/978-1-4899-7681-9

Fox, P. F., Uniacke-Lowe, T., McSweeney, P. L. H., & O'Mahony, J. A. (2015). *Dairy chemistry and biochemistry, second edition*. *Dairy Chemistry and Biochemistry, Second Edition*. https://doi.org/10.1007/978-3-319-14892-2

Franz, C. M. A. P., Stiles, M. E., Schleifer, K. H., & Holzapfel, W. H. (2003). *Enterococci* in foods—a conundrum for food safety. *International Journal of Food Microbiology*, *88*(2), 105–122. https://doi.org/https://doi.org/10.1016/S0168-1605(03)00174-0

Fröhlich-Wyder, M. T., Arias-Roth, E., & Jakob, E. (2019). Cheese yeasts. *Yeast*, *36*(3), 129–141. https://doi.org/10.1002/yea.3368

Galán, E., Prados, F., Pino, A., Tejada, L., & Fernández-Salguero, J. (2008). Influence of different amounts of vegetable coagulant from cardoon *Cynara cardunculus* and calf rennet on the proteolysis and sensory characteristics of cheeses made with sheep milk. *International Dairy Journal*, *18*(1), 93–98. https://doi.org/https://doi.org/10.1016/j.idairyj.2007.06.003

Gil-Rodríguez, A. M., Carrascosa, A. V., & Requena, T. (2015). Yeasts in foods and beverages: In vitro characterisation of probiotic traits. *LWT - Food Science and Technology*, *64*(2), 1156–1162. https://doi.org/10.1016/j.lwt.2015.07.042

Giraffa, G. (2003). Functionality of *enterococci* in dairy products. *International Journal of Food Microbiology*, *88*(2), 215–222. https://doi.org/https://doi.org/10.1016/S0168-1605(03)00183-1

Gobbetti, M., De Angelis, M., Di Cagno, R., Mancini, L., & Fox, P. F. (2015). Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. *Trends in Food Science and Technology*, *45*(2), 167–178. https://doi.org/10.1016/j.tifs.2015.07.016

Gómez-Ruiz, J. Á., Ballesteros, C., Miguel Ángel González Viñas, Cabezas, L., & Martínez-Castro, I. (2002). Relationships between volatile compounds and odour in Manchego cheese: comparison between artisanal and industrial cheeses at different ripening times. *Le Lait*, *82*(5), 613–628. https://doi.org/10.1051/lait:2002037

Gonçalves Dos Santos, M. T. P., Benito, M. J., Córdoba, M. de G., Alvarenga, N., & Ruiz-Moyano Seco de Herrera, S. (2017). Yeast community in traditional Portuguese Serpa cheese by culture-dependent and -independent DNA approaches. *International Journal of Food Microbiology*, *262*(September), 63–70. https://doi.org/10.1016/j.ijfoodmicro.2017.09.013

Gonçalves, M. T. P., Benito, M. J., Córdoba, M. de G., Egas, C., Merchán, A. V., Galván, A. I., & Ruiz-Moyano, S. (2018). Bacterial Communities in Serpa Cheese by Culture Dependent Techniques, 16S rRNA Gene Sequencing and High-throughput Sequencing Analysis. *Journal of Food Science*, *83*(5), 1333–1341. https://doi.org/10.1111/1750-3841.14141

Goudjil, H., Fontecha, J., Luna, P., Miguel Angel de la Fuente, Alonso, L., & Juárez, M. (2004). Quantitative characterization of unsaturated and trans fatty acids in ewe's milk fat. *Le Lait*, *84*(5), 473–482. https://doi.org/10.1051/lait:2004017

Gut, A. M., Vasiljevic, T., Yeager, T., & Donkor, O. N. (2019). Characterization of yeasts isolated from traditional kefir grains for potential probiotic properties. *Journal of Functional Foods*, *58*(April), 56–66. https://doi.org/10.1016/j.jff.2019.04.046

Hatoum, R., Labrie, S., & Fliss, I. (2012). Antimicrobial and probiotic properties of yeasts: From fundamental to novel applications. *Frontiers in Microbiology*, *3*(DEC), 1–12. https://doi.org/10.3389/fmicb.2012.00421

Heather, J. M., & Chain, B. (2016). The sequence of sequencers: The history of sequencing DNA. *Genomics*, *107*(1), 1–8. https://doi.org/10.1016/j.ygeno.2015.11.003

Heredia-Castro, P. Y., Hérnández-Mendoza, A., González-Córdova, A. F., & Vallejo-Cordoba, B. (2017). Bacteriocinas de bacterias ácido lácticas: Mecanismos de acción y actividad antimicrobiana contra patógenos en quesos. *Interciencia*, *42*(6), 340–346.

Hermet, A., Méheust, D., Mounier, J., Barbier, G., & Jany, J. L. (2012). Molecular systematics in the genus Mucor with special regards to species encountered in cheese. *Fungal Biology*, *116*(6), 692–705. https://doi.org/10.1016/j.funbio.2012.04.002

Hermet, A., Mounier, J., Keravec, M., Vasseur, V., Barbier, G., & Jany, J. L. (2014). Application of capillary electrophoresis single-stranded conformation polymorphism (CE-SSCP) analysis for identification of fungal communities in cheese. *Food Microbiology*, *41*, 82–90. https://doi.org/10.1016/j.fm.2014.01.013

Hernández, M., Quijada, N. M., Rodríguez-Lázaro, D., & Eiros, J. M. (2020). Aplicación de la secuenciación masiva y la bioinformática al diagnóstico microbiológico clínico. *Revista Argentina de Microbiologia*, *52*(2), 150–161. https://doi.org/10.1016/j.ram.2019.06.003

Herrero-Fresno, A., Martínez, N., Sánchez-Llana, E., Díaz, M., Fernández, M., Martin, M. C., … Alvarez, M. A. (2012). *Lactobacillus casei* strains isolated from cheese reduce biogenic amine accumulation in an experimental model. *International Journal of Food Microbiology*, *157*(2), 297–304. https://doi.org/10.1016/j.ijfoodmicro.2012.06.002

Heuvelink, A. E., Bleumink, B., Van Den Biggelaar, F. L. A. M., Te Giffel, M. C., Beumer, R. R., & De Boer, E. (1998). Occurrence and survival of verocytotoxin-producing *Escherichia coli* O157 in raw cow's milk in the Netherlands. *Journal of Food Protection*, *61*(12), 1597–1601. https://doi.org/10.4315/0362-028X-61.12.1597

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., … Sanders, M. E. (2014). Expert consensus document: The international scientific association for

probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology and Hepatology*, *11*(8), 506–514. https://doi.org/10.1038/nrgastro.2014.66

Honish, L., Predy, G., Hislop, N., Kowalewska-grochowska, K., Trottier, L., Kreplin, C., … Cphi, C. (2005). An Outbreak of *E. coli* O157:H7 Hemorrhagic Colitis Associated with Unpasteurized Gouda Cheese. *REVUE CANADIENNE DE SANTÉ PUBLIQUE*, *96*(3), 182–184.

Hymery, N., Vasseur, V., Coton, M., Mounier, J., Jany, J. L., Barbier, G., & Coton, E. (2014). Filamentous fungi and mycotoxins in Cheese: A review. *Comprehensive Reviews in Food Science and Food Safety*, *13*(4), 437–456. https://doi.org/10.1111/1541-4337.12069

Ianni, A., Di Domenico, M., Bennato, F., Peserico, A., Martino, C., Rinaldi, A., … Martino, G. (2020). Metagenomic and volatile profiles of ripened cheese obtained from dairy ewes fed a dietary hemp seed supplementation. *Journal of Dairy Science*. https://doi.org/10.3168/jds.2019-17954

Inácio, R. S., Gomes, A. M. P., & Saraiva, J. A. (2020). Serra da Estrela cheese: A review. *Journal of Food Processing and Preservation*, *44*(5), 1–10. https://doi.org/10.1111/jfpp.14412

Irlinger, F., In Yung, S. A. Y., Sarthou, A.-S., Delbès-Paus, C., Montel, M.-C., Coton, E., … Helinck, S. (2012). Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International Journal of Food Microbiology*, *153*(3), 332–338. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2011.11.022

Irlinger, F., Layec, S., Hélinck, S., & Dugat-Bony, E. (2015). Cheese rind microbial communities: Diversity, composition and origin. *FEMS Microbiology Letters*, *362*(2), 1–11. https://doi.org/10.1093/femsle/fnu015

Jacques, N., & Casaregola, S. (2008). Safety assessment of dairy microorganisms: The hemiascomycetous yeasts. *International Journal of Food Microbiology*, *126*(3), 321–326. https://doi.org/10.1016/j.ijfoodmicro.2007.08.020

Jain, M., Olsen, H. E., Paten, B., & Akeson, M. (2016). The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. *Genome Biology*, *17*(1), 1–11. https://doi.org/10.1186/s13059-016-1103-0

Jakobsen, M., & Narvhus, J. (1996). Yeasts and their possible beneficial and negative effects on the quality of dairy products. *International Dairy Journal*, *6*(8–9), 755–768. https://doi.org/10.1016/0958-6946(95)00071-2

Jefatura del Estado. (2006). Real Decreto 1113/2006, de 29 de septiembre, por el que se aprueban las normas de calidad para quesos y quesos fundidos. BOE-A-2006-17436. *Boletin Oficial Del Estado*, 34717–34720.

Jensen, H., Grimmer, S., Naterstad, K., & Axelsson, L. (2012). In vitro testing of commercial and potential probiotic lactic acid bacteria. *International Journal of Food Microbiology*, *153*(1–2), 216–222. https://doi.org/10.1016/j.ijfoodmicro.2011.11.020

Kamber, U. (2007). The Traditional Cheeses of Turkey: The Aegean Region. *Food Reviews International*, *24*(1), 39–61. https://doi.org/10.1080/87559120701762195

Kchouk, M., Gibrat, J. F., & Elloumi, M. (2017). Generations of Sequencing Technologies: From First to Next Generation. *Biology and Medicine*, *09*(03). https://doi.org/10.4172/0974-8369.1000395

Kimoto-Nira, H., Mizumachi, K., Nomura, M., Kobayashi, M., Fujita, Y., Okamoto, T., … Ohmomo, S. (2007). *Lactococcus* sp. as Potential Probiotic Lactic Acid Bacteria. *Japan Agricultural Research Quarterly: JARQ*, *41*(3), 181–189. https://doi.org/10.6090/jarq.41.181

Kindstedt, P. S. (2012). *Cheese and Culture. A History of Cheese and It Place in Western Civilization.* Chelsea Green Publishing.

Kindstedt, P. S. (2018). *The history of cheese*. *Global Cheesemaking Technology: Cheese Quality and Characteristics*.

Kongo, J. M., & Malcata, F. X. (2015). Cheese*:* Types of Cheeses - Soft. *Encyclopedia of Food and Health* (1st ed.). Elsevier Ltd. https://doi.org/10.1016/B978-0-12-384947-2.00132-X

Kumura, H., Tanoue, Y., Tsukahara, M., Tanaka, T., & Shimazaki, K. (2004). Screening of dairy yeast strains for probiotic applications. *Journal of Dairy Science*, *87*(12), 4050–4056. https://doi.org/10.3168/jds.S0022-0302(04)73546-8

Le Loir, Y., Baron, F., & Gautier, M. (2003). *Staphylococcus aureus* and food poisoning. *Genetics and Molecular Research*, *2*(1), 7–28. Retrieved from http://link.springer.com/10.1007/978-3-540-29676-8_1664

Ledenbach, L. H., & Marshall, R. T. (2009). Microbiological Spoilage of Dairy Products. In *Compendium of the Microbiological Spoilage of Foods and Beverages* (pp. 41–67). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4419-0826-1_2

Licitra, G., Caccamo, M., & Lortal, S. (2019). *Artisanal Products Made with Raw Milk. Raw Milk*. Elsevier Inc. https://doi.org/10.1016/b978-0-12-810530-6.00009-2

Lomolino, G., Zannoni, S., & Di Pierro, G. (2015). Characterization of crude esterase activity from two plants used in cheese making: *Cynara cardunculus* L. and *Ficus carica* L. *Food Biotechnology*, *29*(4), 297–316. https://doi.org/10.1080/08905436.2015.1091976

Lopes, J. F. P. (n.d.). O queijo de Serpa: subsídio para o seu estudo técnico-económico. Comissão de Coordenação da Região Alentejo.

Lukaszewicz, M. (2012). *Saccharomyces cerevisiae* var. *boulardii* – Probiotic Yeast. In *Probiotics*. InTech. https://doi.org/10.5772/50105

Macedo, A. C., Costa, M. L., & Malcata, F. X. (1996). Assessment of proteolysis and lipolysis in Serra cheese: Effects of axial cheese location, ripening time and lactation season. *Lait*, *76*(4), 363–370. https://doi.org/10.1051/lait:1996427

MAPA. (2019). Denominaciones de Origen e Indicaciones Geográficas Protegidas. Retrieved from https://www.mapa.gob.es/es/alimentacion/temas/calidad-agroalimentaria/calidad-diferenciada/dop/default.aspx

Marcellino, N., Beuvier, E., Grappin, R., Guéguen, M., & Benson, D. R. (2001). Diversity of *Geotrichum candidum* Strains Isolated from Traditional Cheesemaking Fabrications in France. *Applied and Environmental Microbiology*, *67*(10), 4752–4759. https://doi.org/10.1128/AEM.67.10.4752-4759.2001

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., … Rothberg, J. M. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature*, *437*(7057), 376–380. https://doi.org/10.1038/nature03959

Markowiak, P., & Ślizewska, K. (2017). Effects of probiotics, prebiotics, and synbiotics on human health. *Nutrients*, *9*(9). https://doi.org/10.3390/nu9091021

Martín-Platero, A. M., Valdivia, E., Maqueda, M., Martín-Sánchez, I., & Martínez-Bueno, M. (2008). Polyphasic approach to bacterial dynamics during the ripening of Spanish farmhouse cheese, using culture-dependent and -independent methods. *Applied and Environmental Microbiology*, *74*(18), 5662–5673. https://doi.org/10.1128/AEM.00418-08

Masoud, W., Takamiya, M., Vogensen, F. K., Lillevang, S., Al-Soud, W. A., Sørensen, S. J., & Jakobsen, M. (2011). Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis and pyrosequencing. *International Dairy Journal*, *21*(3), 142–148. https://doi.org/10.1016/j.idairyj.2010.10.007

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Lait*, *80*(3), 293–324. https://doi.org/10.1051/lait:2000127

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology*, *57*(2–3), 127–144. https://doi.org/10.1111/j.1471-0307.2004.00147.x

McSweeney, P. L. H. (2017). Biochemistry of cheese ripening: introduction and overview. In *Cheese* (pp. 379–387). Academic Press.

McSweeney, P. L. H., Fox, P. F., & Ciocia, F. (2017). Metabolism of Residual Lactose and of Lactate and Citrate. In *Cheese* (pp. 411–421). Elsevier. https://doi.org/10.1016/B978-0-12-417012-4.00016-8

McSweeney, P. L. H., Fox, P. F., Cotter, P. D., & Everettt, D. W. (2017). *Cheese. Chemistry, Physics and Microbiology*. (P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. Everettt, Eds.) (Fourth).

Menéndez, S., Centeno, J. A., Godínez, R., & Rodríguez-Otero, J. L. (2000). Effects of *Lactobacillus* strains on the ripening and organoleptic characteristics of Arzua-Ulloa cheese. *International Journal of Food Microbiology*, *59*(1–2), 37–46. https://doi.org/10.1016/S0168-1605(00)00286-5

Meng, J., LeJeune, J. T., Zhao, T., & Doyle, M. P. (2014). Enterohemorrhagic *Escherichia coli*. In *Food Microbiology* (pp. 287–309). John Wiley & Sons, Ltd. https://doi.org/10.1128/9781555818463.ch12

Ministerio de Agricultura, P. y A. (1993). ORDEN de 14 de abril de 1993 por la que se ratifica el Reglamento de la Denominación de Origen "Queso de la Serena", y su Consejo Regulador.

Ministerio De Agricultura, P. y. A. (2002). BOE num. 123. ORDEN APA/1144/2002, de 6 de mayo, por la que se ratifica el Reglamento de la Denominación de Origen «Torta del Casar», 18578–18586.

Modler, H. W. (1988). Development of a Continuous Process for the Production of Ricotta Cheese. *Journal of Dairy Science*, *71*(8), 2003–2009. https://doi.org/10.3168/jds.S0022-0302(88)79775-1

Moio, L., Dekimpe, J., Etievant, P., & Addeo, F. (1993). Neutral volatile compounds in the raw milks from different species. *Journal of Dairy Research*, *60*(2), 199–213. https://doi.org/10.1017/S0022029900027515

Monnet, C., Dugat-Bony, E., Swennen, D., Beckerich, J. M., Irlinger, F., Fraud, S., & Bonnarme, P. (2016). Investigation of the activity of the microorganisms in a reblochon-style cheese by metatranscriptomic analysis. *Frontiers in Microbiology*, *7*(APR). https://doi.org/10.3389/fmicb.2016.00536

Montel, M. C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D. A., Desmasures, N., & Berthier, F. (2014). Traditional cheeses: Rich and diverse microbiota with associated benefits. *International Journal of Food Microbiology*, *177*, 136–154. https://doi.org/10.1016/j.ijfoodmicro.2014.02.019

Morales, P., Feliu, I., Fernández-García, E., & Nuñez, M. (2004). Volatile Compounds Produced in Cheese by *Enterobacteriaceae* Strains of Dairy Origin. *Journal of Food Protection*, *67*(3), 567–573. https://doi.org/10.4315/0362-028X-67.3.567

Morandi, S., Brasca, M., Andrighetto, C., Lombardi, A., & Lodi, R. (2006). Technological and molecular characterisation of *enterococci* isolated from north–west Italian dairy products. *International Dairy Journal*, *16*(8), 867–875. https://doi.org/https://doi.org/10.1016/j.idairyj.2005.09.005

Mounier, J., Monnet, C., Jacques, N., Antoinette, A., & Irlinger, F. (2009). Assessment of the microbial diversity at the surface of Livarot cheese using culture-dependent

and independent approaches. *International Journal of Food Microbiology*, *133*(1–2), 31–37. https://doi.org/10.1016/j.ijfoodmicro.2009.04.020

Mullan, W. M. A. (2000). Causes and control of early gas production in cheddar cheese. *International Journal of Dairy Technology*, *53*(2), 63–68. https://doi.org/10.1111/j.1471-0307.2000.tb02660.x

Muñoz, N., Ortigosa, M., Torre, P., & Izco, J. M. (2003). Free amino acids and volatile compounds in an ewe's milk cheese as affected by seasonal and cheese-making plant variations. *Food Chemistry*, *83*(3), 329–338. https://doi.org/10.1016/S0308-8146(03)00133-X

Ordiales, E., Martín, A., Benito, M. J., Hernández, A., Ruiz-Moyano, S., & Córdoba, M. D. G. (2012). Technological characterisation by free zone capillary electrophoresis (FCZE) of the vegetable rennet (*Cynara cardunculus*) used in "torta del Casar" cheese-making. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2012.01.012

Ordiales, E., Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J., & de Guía Córdoba, M. (2013a). Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. *Food Control*, *33*(2), 448–454. https://doi.org/10.1016/j.foodcont.2013.03.027

Ordiales, E., Martín, A., Benito, M. J., Hernández, A., Ruiz-Moyano, S., & Córdoba, M. de G. (2013b). Role of the microbial population on the flavor of the soft-bodied cheese Torta del Casar. *Journal of Dairy Science*, *96*(9), 5477–5486. https://doi.org/10.3168/jds.2013-6587

Ordiales, E., Benito, M. J., Martin, A., Fernández, M., Hernández, A., & De Guia Córdoba, M. (2013c). Proteolytic effect of *Cynara cardunculus* rennet for use in the elaboration of "Torta del Casar" cheese. *Journal of Dairy Research*. https://doi.org/10.1017/S0022029913000411

Ordiales, E., Martín, A., Benito, M. J., Fernández, M., Casquete, R., & de Guía Córdoba, M. (2014). Influence of the technological properties of vegetable rennet (*Cynara cardunculus*) on the physicochemical, sensory and rheological characteristics of "Torta del Casar" cheese. *International Journal of Dairy Technology*, *67*(3), 402–409. https://doi.org/10.1111/1471-0307.12129

Ozturkoglu-Budak, S., & De Vries, R. P. (2017). Mold-ripened and raw milk cheeses: Production, risks, and benefits to human health. *Dairy in Human Health and Disease across the Lifespan*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-809868-4.00027-3

Padilla, B., Belloch, C., López-Díez, J. J., Flores, M., & Manzanares, P. (2014). Potential impact of dairy yeasts on the typical flavour of traditional ewes' and goats' cheeses. *International Dairy Journal*, *35*(2), 122–129. https://doi.org/10.1016/j.idairyj.2013.11.002

Padilla, B., Manzanares, P., & Belloch, C. (2014). Yeast species and genetic heterogeneity within *Debaryomyces hansenii* along the ripening process of

traditional ewes' and goats' cheeses. *Food Microbiology*, *38*, 160–166. https://doi.org/10.1016/j.fm.2013.09.002

Pagthinathan, M., & Nafees, M. S. M. (2017). Biochemistry of cheese ripening. *AGRIEAST: Journal of Agricultural Sciences*, *10*(0), 16. https://doi.org/10.4038/agrieast.v10i0.25

Park, Y. W., Juárez, M., Ramos, M., & Haenlein, G. F. W. (2007). Physico-chemical characteristics of goat and sheep milk. *Small Ruminant Research*, *68*(1–2), 88–113. https://doi.org/10.1016/j.smallrumres.2006.09.013

Patrignani, F., Siroli, L., Parolin, C., Serrazanetti, D. I., Vitali, B., & Lanciotti, R. (2019). Use of *Lactobacillus crispatus* to produce a probiotic cheese as potential gender food for preventing gynaecological infections. *PLOS ONE*, *14*(1), e0208906. https://doi.org/10.1371/journal.pone.0208906

Pattono, D., Grosso, A., Stocco, P. P., Pazzi, M., & Zeppa, G. (2013). Survey of the presence of patulin and ochratoxin A in traditional semi-hard cheeses. *Food Control*, *33*(1), 54–57. https://doi.org/10.1016/j.foodcont.2013.02.019

Perea, S., De Labastida, E. F., Nájera, A. I., Chávarri, F., Virto, M., De Renobales, M., & Barron, L. J. R. (2000). Seasonal changes in the fat composition of Lacha sheep's milk used for Idiazabal cheese manufacture. *European Food Research and Technology*, *210*(5), 318–323. https://doi.org/10.1007/s002170050557

Pereira-Dias, S., Potes, M. E., Marinho, A., Malfeito-Ferreira, M., & Loureiro, V. (2000). Characterisation of yeast flora isolated from an artisanal Portuguese ewes' cheese. *International Journal of Food Microbiology*, *60*(1), 55–63. https://doi.org/10.1016/S0168-1605(00)00323-8

Pereira, C. I., Graça, J. A., Ogando, N. S., Gomes, A. M. P., & Xavier Malcata, F. (2010). Influence of bacterial dynamics upon the final characteristics of model Portuguese traditional cheeses. *Food Microbiology*, *27*(3), 339–346. https://doi.org/10.1016/j.fm.2009.10.012

Pintado, A. I. E., Pinho, O., Ferreira, I. M. P. L. V. O., Pintado, M. M. E., Gomes, A. M. P., & Malcata, F. X. (2008). Microbiological, biochemical and biogenic amine profiles of Terrincho cheese manufactured in several dairy farms. *International Dairy Journal*, *18*(6), 631–640. https://doi.org/10.1016/j.idairyj.2007.11.021

Poullet, B., Huertas, M., SÁnchez, A., CÁceres, P., & Larriba, G. (1991). Microbial study of Casar de Cáceres cheese throughout ripening. *Journal of Dairy Research*, *58*(2), 231–238. https://doi.org/10.1017/S0022029900029770

Presidencia del Gobierno. (1967). Decreto 2484/1967, de 21 de septiembre, por el que se aprueba el texto del Código Alimentario Español. Consolidado. *Boletín Oficial Del Estado (BOE)*, 1–156.

Psomas, E., Andrighetto, C., Litopoulou-Tzanetaki, E., Lombardi, a., & Tzanetakis, N. (2001). Some probiotic properties of yeast isolates from infant faeces and Feta

cheese. *International Journal of Food Microbiology*, *69*(1–2), 125–133. https://doi.org/10.1016/S0168-1605(01)00580-3

Quesería Doña Francisca. (n.d.). Retrieved from queseriadonafrancisca.com

Queso de la Serena. (n.d.). Retrieved from quesoserena.com

Quigley, L., O'Sullivan, O., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2011). Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *International Journal of Food Microbiology*, *150*(2–3), 81–94. https://doi.org/10.1016/j.ijfoodmicro.2011.08.001

Quigley, L., O'Sullivan, O., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2012). High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. *Applied and Environmental Microbiology*, *78*(16), 5717–5723. https://doi.org/10.1128/AEM.00918-12

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Reviews*, *37*(5), 664–698. https://doi.org/10.1111/1574-6976.12030

Quijada, N. M., Hernández, M., & Rodríguez-Lázaro, D. (2020). High-throughput sequencing and food microbiology. *Advances in Food and Nutrition Research* (1st ed., Vol. 91). Elsevier Inc. https://doi.org/10.1016/bs.afnr.2019.10.003

Roseiro, L. B., Andrew Wilbey, R., & Barbosa, M. (2003). Serpa Cheese: Technological, biochemical and microbiological characterisation of a PDO ewe's milk cheese coagulated with *Cynara cardunculus* L. *Le Lait*, *83*(6), 469–481. https://doi.org/10.1051/lait:2003026

Ruiz-Capillas, C., & Herrero, A. M. (2019). Impact of biogenic amines on food quality and safety. *Foods*, *8*(2). https://doi.org/10.3390/foods8020062

Ruiz-Moyano, S., Gonçalves dos Santos, M. T. P., Galván, A. I., Merchán, A. V., González, E., Córdoba, M. de G., & Benito, M. J. (2019). Screening of autochthonous lactic acid bacteria strains from artisanal soft cheese: probiotic characteristics and prebiotic metabolism. *Lwt*, *114*(July), 108388. https://doi.org/10.1016/j.lwt.2019.108388

Ruzante, J. M., Lombard, J. E., Wagner, B., Fossler, C. P., Karns, J. S., Van Kessel, J. A. S., & Gardner, I. A. (2010). Factors associated with *Salmonella* presence in environmental samples and bulk tank milk from US dairies. *Zoonoses and Public Health*, *57*(7–8). https://doi.org/10.1111/j.1863-2378.2010.01333.x

Ryser, E. T. (2007). Incidence and behavior of *Listeria monocytogenes* in unfermented dairy products. In E. H. Marth & E. T. Ryser (Eds.), *Listeria, Listeriosis and Food Safety* (Second, pp. 358–367). New York, NY: Marcel Dekker, Inc.

Saber, A., Alipour, B., Faghfoori, Z., & Yari Khosroushahi, A. (2017). Secretion metabolites of dairy *Kluyveromyces marxianus* AS41 isolated as probiotic, induces

apoptosis in different human cancer cell lines and exhibit anti-pathogenic effects. *Journal of Functional Foods*, *34*, 408–421. https://doi.org/10.1016/j.jff.2017.05.007

Salque, M., Bogucki, P. I., Pyzel, J., Sobkowiak-Tabaka, I., Grygiel, R., Szmyt, M., & Evershed, R. P. (2013). Earliest evidence for cheese making in the sixth millennium bc in northern Europe. *Nature*, *493*(7433), 522–525. https://doi.org/10.1038/nature11698

Sánchez-Juanes, F., Teixeira-Martín, V., González-Buitrago, J. M., Velázquez, E., & Flores-Félix, J. D. (2020). Identification of species and subspecies of lactic acid bacteria present in Spanish cheeses type "Torta" by MALDI-TOF MS and pheS gene analyses. *Microorganisms*, *8*(2), 301. https://doi.org/10.3390/microorganisms8020301

Sanchez-Rey, R., Poullet, B., Caceres, P., & Larriba, G. (1993). Microbiological quality and incidence of some pathogenic microorganisms in La Serena cheese throughout ripening. *Journal of Food Protection*, *56*(10), 879–881. https://doi.org/10.4315/0362-028X-56.10.879

Sanders, M. E., Merenstein, D., Merrifield, C. A., & Hutkins, R. (2018). Probiotics for human use. *Nutrition Bulletin*, *43*(3), 212–225. https://doi.org/10.1111/nbu.12334

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(12), 5463–5467. https://doi.org/10.1073/pnas.74.12.5463

Schirone, M., Tofalo, R., Perpetuini, G., Manetta, A. C., Di Gianvito, P., Tittarelli, F., … Martino, G. (2018). Influence of iodine feeding on microbiological and physico-chemical characteristics and biogenic amines content in a raw ewes' milk cheese. *Foods*, *7*(7). https://doi.org/10.3390/foods7070108

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., & Chen, W. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences*, *109*(16), 6241 LP – 6246. https://doi.org/10.1073/pnas.1117018109

Scott, R. (1986). *Cheesemaking Practice*.

Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance—A review. *Appetite*, *51*(3), 456–467. https://doi.org/10.1016/j.appet.2008.05.060

Smit, G., Smit, B. A., & Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews*, *29*(3), 591–610. https://doi.org/10.1016/j.fmrre.2005.04.002

Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connell, C. R., … Hood, L. E. (1986). Fluorescence detection in automated DNA sequence analysis. *Nature*, *321*(6071), 674–679. https://doi.org/10.1038/321674a0

Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., … Herndl, G. J. (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences*, *103*(32), 12115 LP – 12120. https://doi.org/10.1073/pnas.0605127103

Sousa, M. J., Ardö, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, *11*(4–7), 327–345. https://doi.org/10.1016/S0958-6946(01)00062-0

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., … Lolkema, J. S. (2010). Biogenic amines in fermented foods. *European Journal of Clinical Nutrition*, *64*, S95–S100. https://doi.org/10.1038/ejcn.2010.218

Tabla, R., Gómez, A., Simancas, A., Rebollo, J. E., Molina, F., & Roa, I. (2016). *Enterobacteriaceae* species during manufacturing and ripening of semi–hard and soft raw ewe's milk cheese: Gas production capacity. *Small Ruminant Research*, *145*, 123–129. https://doi.org/10.1016/j.smallrumres.2016.11.008

Tanigawa, K., Kawabata, H., & Watanabe, K. (2010). Identification and typing of *Lactococcus lactis* by matrix-assisted laser desorption ionization - Time of flight mass spectrometry. *Applied and Environmental Microbiology*, *76*(12), 4055–4062. https://doi.org/10.1128/AEM.02698-09

Tavaria, F. K., & Malcata, F. X. (1998). Microbiological characterization of Serra da Estrela cheese throughout its Appellation d'Origine Protegee region. *Journal of Food Protection*, *61*(5), 601–607. https://doi.org/10.4315/0362-028X-61.5.601

Thierry, A., Collins, Y. F., Abeijón Mukdsi, M. C., McSweeney, P. L. H., Wilkinson, M. G., & Spinnler, H. E. (2017). Chapter 17 - Lipolysis and Metabolism of Fatty Acids in Cheese. In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. B. T.-C. (Fourth E. Everett (Eds.) (pp. 423–444). San Diego: Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-417012-4.00017-X

Tofalo, R., Fasoli, G., Schirone, M., Perpetuini, G., Pepe, A., Corsetti, A., & Suzzi, G. (2014). The predominance, biodiversity and biotechnological properties of *Kluyveromyces marxianus* in the production of Pecorino di Farindola cheese. *International Journal of Food Microbiology*, *187*, 41–49. https://doi.org/10.1016/j.ijfoodmicro.2014.06.029

Tornadijo, M. E., García, M. C., Fresno, J. M., & Carballo, J. (2001). Study of *Enterobacteriaceae* during the manufacture and ripening of San Simón cheese. *Food Microbiology*, *18*(5), 499–509. https://doi.org/10.1006/fmic.2001.0423

Triki, M., Herrero, A., Jiménez-Colmenero, F., & Ruiz-Capillas, C. (2018). Quality assessment of fresh meat from several species based on free amino acid and biogenic amine contents during chilled storage. *Foods*, *7*(9), 132. https://doi.org/10.3390/foods7090132

Tufarelli, V., & Laudadio, V. (2016). An overview on the functional food concept: prospectives and applied researches in probiotics, prebiotics and synbiotics. *Journal*

of Experimental Biology and Agricultural Sciences, *4*(3S), 273–278. https://doi.org/10.18006/2016.4(3S).273.278

van Cauteren, D., Silva, N. J., Weill, F. X., King, L., Brisabois, A., & Delmas, G. (2009). Outbreak of *Salmonella enterica* Serotype Muenster infections associated with goat's cheese, France, march 2008. *Eurosurveillance*, *14*(31), 14–16.

Verraes, C., Vlaemynck, G., Van Weyenberg, S., De Zutter, L., Daube, G., Sindic, M., … Herman, L. (2015). A review of the microbiological hazards of dairy products made from raw milk. *International Dairy Journal*, *50*, 32–44. https://doi.org/10.1016/j.idairyj.2015.05.011

Viljoen, B. C. (2001). The interaction between yeasts and bacteria in dairy environments. *International Journal of Food Microbiology*, *69*(1–2), 37–44. https://doi.org/10.1016/S0168-1605(01)00570-0

Visser, S. (1993). Proteolytic Enzymes and Their Relation to Cheese Ripening and Flavor: An Overview. *Journal of Dairy Science*, *76*(1), 329–350. https://doi.org/10.3168/jds.S0022-0302(93)77354-3

Wolfe, B. E., Button, J. E., Santarelli, M., & Dutton, R. J. (2014). Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell*, *158*(2), 422–433. https://doi.org/10.1016/j.cell.2014.05.041

Yang, B., Wang, Y., & Qian, P.-Y. (2016). Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinformatics*, *17*(1), 135. https://doi.org/10.1186/s12859-016-0992-y

Yang, C., Zhao, F., Hou, Q., Wang, J., Li, M., & Sun, Z. (2020). PacBio sequencing reveals bacterial community diversity in cheeses collected from different regions. *Journal of Dairy Science*, *103*(2), 1238–1249. https://doi.org/10.3168/jds.2019-17496

Yerlikaya, O., & Ozer, E. (2014). Production of probiotic fresh white cheese using co-culture with *Streptococcus thermophilus*. *Food Science and Technology (Campinas)*, *34*(3), 471–477. https://doi.org/10.1590/1678-457x.6365

Zabaleta, L., Albisu, M., & Barron, L. J. R. (2017). Volatile compounds associated with desirable flavour and off-flavour generation in ewe´s raw milk commercial cheeses. *European Food Research and Technology*, *243*(8), 1405–1414. https://doi.org/10.1007/s00217-017-2851-0

Zanello, G., Meurens, F., Berri, M., & Salmon, H. (2009). *Saccharomyces boulardii* effects on gastrointestinal diseases. *Current Issues in Molecular Biology*, *11*(33), 47–58. https://doi.org/10.21775/cimb.011.047

Zivkovic, M., Cadez, N., Uroic, K., Miljkovic, M., Tolinacki, M., Dousova, P., … Golic, N. (2015). Evaluation of probiotic potential of yeasts isolated from traditional cheeses manufactured in Serbia and Croatia. *Journal of Intercultural Ethnopharmacology*, *4*(1), 12. https://doi.org/10.5455/jice.20141128051842

JUSTIFICACIÓN Y OBJETIVOS

Justificación

Las D.O.P. "Queso de la Serena" y "Torta del Casar" están dedicadas a la elaboración de quesos de pasta blanda o semiblanda denominados "Torta" con unas características singulares que los hacen diferentes a otros quesos producidos en España. En este sector, debido a la falta de estandarización de la leche cruda de oveja y del cuajo vegetal obtenido de la maceración de las flores del cardo *Cynara cardunculus* L., ocasiona distintos problemas entre los que destacan la gran variabilidad en la calidad final obtenida, pérdidas por alteración del producto y riesgos higiénico-sanitarios por la potencial presencia de microorganismos patógenos. A pesar de cumplir un mínimo de 60 días de maduración, no están exentos de este tipo de peligros. Entre los microorganismos presentes en el queso, el papel de la microbiota dominante constituida por bacterias acido lácticas ha sido muy estudiado, sin embargo, estudios recientes en otro tipo de quesos han destacado la relevancia de la microbiota secundaria como bacterias gram-negativas y levaduras en el proceso de maduración. La caracterización de estos microorganismos por sus propiedades tecnológicas y funcionales y, además, estudiar su potencial aplicación como cultivo iniciador, contribuiría al sector industrial a mantener las características sensoriales distintivas de este producto garantizando su seguridad. Además, la aplicación de microorganismos con propiedades funcionales otorgaría a la "Torta" un valor añadido en el mercado.

Objetivos/Objectives

1. Caracterizar la microbiota de los quesos mediante metagenómica con técnicas de secuenciación masiva. Characterize the cheese microbiota by metagenomic with massive sequencing techniques.

2. Obtener un número elevado de cepas autóctonas de levaduras y enterobacterias de quesos tradicionales de diferentes industrias lácteas de nuestra región. Obtain a high number of native strains of yeasts and enterobacteria from traditional cheeses from different dairy industries in our region.

3. Caracterización y selección de las cepas por sus propiedades tecnológicas y funcionales para su utilización como cultivo iniciador en quesos tradicionales. Characterization and selection of the strains for their technological and functional properties for their use as a starter culture in traditional cheeses.

CAPÍTULO I

Metagenomics of artisanal torta-type cheeses using targeted 16S rRNA sequencing.

Capítulo I.

Metagenomics of artisanal torta-type cheeses using targeted 16S rRNA sequencing.

Abstract

"Torta del Casar" and "Queso de la Serena" are traditional soft body cheeses from Extremadura (Spain) with Protected Denomination of Origin (PDO). They are made with raw sheep milk and vegetable rennet from *Cynara cardunculus* L., matured over at least 60 days without the addition of a starter culture. This study investigated the bacterial community in six "torta-type" cheeses elaborated in two different production regions, Casar and Serena, using High Throughput Sequencing of 16S rRNA gene libraries. The dominant phyla in "torta-type" cheese were *Firmicutes* and *Proteobacteria*. Our data revealed 52 bacterial families (including *Streptococcaceae, Leuconostocaceae, Lactobacillaceae, Yersiniaceae*, and *Enterobacteriaceae*) and 91 bacterial genera (*Leuconostoc, Lactoccoccus, Lactobacillus, Serratia*, and *Hafnia*). When assessing the influence of the PDO region on the metagenomics of cheeses, it was found that the cheeses made in the Casar region are *Lactobacillus*-driven, while those made in the Serena region are *Leuconostoc*-driven. Undesirable microorganisms, including *Pseudomonas* spp., were identified in the early stages, not being detected at the end of ripening process. Secondary microbiota also has an important role during the maturation process, potentially contributing to the formation of the unique sensory characteristics presented these cheeses. The HTS methodology has allowed us to know the microbiota involved in the maturation process of these traditional soft body cheeses made with raw ewe's milk, highlighting the microbiological variability present in the four stages of maturation, mainly influenced by the raw material and the production region.

I.1. Introduction

Cheese microbiota has been studied in the last twenty years due to the importance of the microbial diversity along the ripening process (Afshari et al., 2018; Mayo et al., 2021). Cheese microbiome is based on the manufacturing conditions such as the use of raw milk (especially in traditional non-pasteurized cheeses), use of starter cultures, aging process, facilities environmental, equipment surfaces and geographical locations

(Bokulich and Mills, 2013; Calasso et al., 2016; De Filippis et al., 2016; Montel et al., 2014). The physical, chemical and microbiological processes throughout cheese maturation result in a complex matrix that encompasses different biochemical changes as proteolysis, lipolysis and glycolysis, responsible of flavor and texture of final product (Gobbetti et al., 2015; McSweeney et al., 2017, Sousa et al., 2001). Bacterial communities can adapt their metabolism through physic-chemical variations of cheese matrix and, consequently, compete against other microorganisms along the ripening process (Ianni et al., 2020; Schirone et al., 2018).

Artisanal soft cheeses made with raw sheep's milk such as "Serpa", "Serra da Estrela", "Torta del Casar" and "Queso de la Serena" show a high variability between bacterial communities due to the absence of the thermal process of the milk (Abriouel et al., 2008; Ercolini et al., 2009; Gonçalves et al., 2018; Ordiales et al., 2013; Tabla et al., 2016; Tavaria et al., 1998). This raises into public health risks due to the presence of pathogen species (Irlinger et al., 2015). This fact is responsible for product losses and therefore, economic losses to dairy industries because of the presence of undesirable microorganisms along maturation. "*Torta del Casar*" and "*Queso de la Serena*" are both Spanish PDO (Protected Designation of Origin) from Extremadura region, made with raw ewes' milk and vegetable rennet from *Cynara cardunculus* L., and matured over at least 60 days without starter cultures. Main microorganisms in soft ewe's cheese are lactic-acid bacteria (LAB), such as *Lactobacillus casei, Lb. curvatus*, *Lb. diolivorans*, *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus*, *Lactococcus lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis* and *Leuconostoc mesenteroides* (Gonçalves et al., 2018; Inácio et al., 2020; Ordiales et al., 2013; Pereira et al., 2010; Sánchez-Juanes et al., 2020). These LAB are responsible for the flavor and texture due to their proteolytic and lipolytic activities (Alessandria et al., 2016, Gatti et al., 2014), although the secondary microbiota, as Gram-negative bacteria, is of great interest due to its involvement in changes that occur during the maturation process (Chaves-López et al., 2006; Gonçalves et al., 2018; Morales et al., 2004; Ordiales et al., 2013; Tabla et al., 2016).

Molecular DNA-based approaches and culture-dependent methods can only detect 0,1% of a complex community, such as that found in the cheese microbiota (Cao et al., 2017). Although bacterial communities of traditional soft ewe' cheeses have been studied by numerous authors (Ordiales et al., 2013, Gonçalves et al., 2018, Sánchez-

Juanes et al., 2020), there are no studies carried out on the microbiota of soft paste cheeses "*Torta del Casar*" and "*Queso de la Serena*" using independent-culture methods. The development of massive sequencing techniques has allowed researchers to generate a large amount of genomic information quickly, allowing to explore, as far as possible, the microbial ecosystems present in fermented foods, environments and complete genomes of some microorganisms (Bokulich and Mills, 2013; Choi et al., 2020; De Filippis et al, 2017). The most widely used approach to the study of the microbiota is based on relevant taxonomic genes, such as the 16S rRNA gene (Caporaso et al., 2011; Quijada et al., 2020). In last decade, high-throughput sequencing (HTS) has been useful to understand the microbiota of industrial and artisanal cheeses (Castellanos-Rozo et al., 2020; Choi et al., 2020; De Filippis et al., 2016; De Pasquale et al., 2014; Dugat-Bony et al., 2015; Escobar-Zepeda et al., 2016; Salazar et al., 2018; Wolfe et al., 2014). Zago et al. (2021) studied the composition of the bacterial community of "Grana Padano" cheese by DNA metabarcoding. They found out that the major dominant taxa were LAB (32 taxa with more than 1% of total reads), finding wide diversity between the different cheese production regions. Yang et al. (2020) studied the bacterial communities of twelve cheeses (10 industrial and 2 traditional cheeses) using PacBio SMRT technology. They identified different bacterial species and their metabolic pathways, responsible for the distinctive flavor qualities of the cheese samples. Quigley et al. (2012) found the presence of microorganism belong to the genera *Arthrobacter* and *Brachybacterium*, never isolated in cheeses made with goat's milk. In artisanal soft cheeses, Gonçalves et al. (2018) studied the autochthonous microbiota in "Serpa" cheese, from industries belonging to PDO and two other non-certified industries, finding a wide diversity of genera and species, as well as significant differences between the microbial population of PDO and non-PDO cheeses.

In this study, we examined six different batches of torta-type cheese produced in two geographical locations (PDO "Torta del Casar" and "Queso del Serena") throughout ripening (0, 20, 40 and 60 days). The aim of this study is to analyze the microbial community along maturation process and their possible involvement in the biochemical changes that give this type of cheese unique characteristics.

I.2. Material and methods.

Cheese manufacture and sampling.

Cheeses were made by local formal producers from raw ewes' milk by enzymatic coagulation with rennet from *C. cardunculus* L., following the procedure described for the Protected Designation of Origin (PDO) of the cheeses. Cheese samples were provided by six local cheese producers (A, B, C, D, E, F) in winter 2018, in two different geographical locations. From PDO "*Queso de la Serena*", batches were named A, B and C and batches from PDO "*Torta del Casar*" were D, E and F. The samplings were done at 0, 20, 40 and 60 days of ripening. For each cheese batch, samples were collected at local industry and quickly transported to University of Extremadura for analysis. All samples were analyzed immediately or frozen at -80ºC.

Extraction of total genomic DNA.

Firstly, fifty grams of each cheese sample was pulverized with liquid nitrogen using a CryoMill equipment (Retsch, Germany) for 10 minutes. Total genomic DNA was extracted using Quick-DNA^{TM} Fecal/Soil Microbe MiniPrep Kit (Zymo Research, Irvine, CA, USA), following the instructions provided by the manufacturer. The quality and quantity of the extracted DNA was determined using a NanoDrop 2000 (ThermoFisher Scientific, USA).

Amplicon library preparation and sequencing.

Total DNA of each sample was subjected to NGS analysis at NUCLEUS (University of Salamanca, Spain). Bacterial diversity in the samples was analyzed by sequencing of amplified V1–V9 regions of the 16S rRNA gene with primers listed in table 1, using an Illumina MiSeq platform. A 2 × 300 bp of MiSeq amplicon library was prepared using the 16S Metagenomics Library Preparation workflow (Illumina Inc, California, USA).

Table 1. Primer sequences of V1-V9 regions of the 16S rRNA used in this study (Klindworth et al., 2013).

Hypervariable region of 16S	Primer sequence
V1_f	5'-GAGTTTGATCMTGGCTCAG-3'
V3_f	5'-CCTACGGGAGGCAGCAG-3'
V4_f	5'-GCCAGCAGCCGCGGTAA-3'
V4_r	5'-CTACCAGGGTATCTAATCC-3'
V5_r	5'-CCGTCAATTCMTTTGAGTTT-3'
V6_f	5'-ATGGCTGTCGTCAGCT-3'
V7_f	5'-GYAACGAGCGCAACCC-3'
V8_r	5'-GACGGGCGGTGTGTACAA-3'
V9_r	5'-TACCTTGTTACGACTT-3'

*f: forward, r: reverse

Bioinformatic analysis.

Both of the demultiplexed R1 and R2 raw sequences files as a fastq format were directly downloaded from MiSeq platform. The Quantitative Insights into Microbial Ecology 2 (QIIME 2) version 2020.8 (Bolyen et al. 2019) as an open-source pipeline was used for quality control of the sequence data. Demultiplexed sequences were joined and denoised based on quality scores through DADA2 scripts (Callahan et al., 2016) available in QIIME 2. DADA2 scripts were merged to 99% sequence homology to construct an amplicon sequence variant (ASV) table. The taxonomy of sequences was acquired from the SILVA reference database (version 138, https://www.arb-silva.de) using an ASV table at 99% sequence similarity with plugin classify-consensus-vsearch (Rognes et al., 2016). The **α**-diversity was carried out by QIIME package (q2-diversity-alpha) and the richness estimator Shannon index was consider. For prediction assay, a *k*-nearest neighbors (KNN) model was trained with the final ASV data (bacterial content of the samples) using the Classification Learner app from MATLAB. HeatMapper.ca (Babicki et al., 2016) was used to visualize the abundance of *Hafnia* genus throughout ripening process for samples of the six industries.

Statistical analysis.

Statistical analysis of the data was carried out using the program SPSS for Windows 21.0 (SPSS Inc Chicago, IL, USA). Two-way analysis of variance (ANOVA) was conducted, and the means were separated by Tukey's HSD test ($p \leq 0.05$).

I.3. Results.

Characterization of microbial communities in raw ewes' milk cheeses.

Microbial communities of cheeses made in six dairy industries belonging to PDO "Torta del Casar" and PDO "Queso de la Serena" were investigated by DNA metabarcoding and sequencing the V1-V9 hypervariable region of the 16S rRNA gene. A total of 11,083,150 raw reads were obtained for 24 duplicated cheese samples. The length of the raw reads was 301 bp. After trimming, reads per sample ranged from 16060 to 242267 (median 79684,5; mean 86296,625). Reads with a minimum length of 240 bases were kept for DADA2 analysis. Low-quality reads and chimeras were removed, and sequences were clustered into 12667 ASVs by 99% similarity against SILVA database (table 2).

Table 2. Metataxonomic data of "Queso de la Serena" and "Torta del Casar" artisanal cheeses (n=24) produced in Extremadura, Spain.

		Ripening (days)	Raw Sequences	Denoised	non-chimeric Total frequency of features	Total features (ASVs)	Families	Genera
PDO "QUESO DE LA SERENA"	**A**	0	195495	175583	39226	186	17	21
		20	455151	404117	81861	356	15	23
		40	337343	296733	64081	198	11	16
		60	87856	76224	17146	147	8	10
	B	0	289171	246799	42328	320	13	19
		20	383751	326269	69414	444	19	26
		40	495607	427657	80101	465	17	23
		60	598598	519457	105008	553	24	32
	C	0	197972	163089	32576	991	42	62
		20	775655	644262	117750	878	26	33
		40	788900	669860	130634	845	23	30
		60	224625	191345	35391	571	20	24
PDO "TORTA DEL CASAR"	**D**	0	105871	87824	22406	179	10	12
		20	462408	394863	94006	583	19	23
		40	999507	857582	242267	652	18	22
		60	947732	819409	222540	585	18	23
	E	0	475138	390373	78968	683	26	37
		20	633484	540361	112577	695	22	30
		40	633918	530006	112401	735	22	28
		60	474834	416219	93943	526	16	24
	F	0	106489	86447	16060	293	20	25
		20	407780	358824	79268	571	20	27
		40	387158	340364	70633	551	19	26
		60	618707	536936	110534	660	16	23
Total		n=24	11083150	9500603	2071119	12667	52	91

The taxonomic analysis allowed to identify 6 phyla, being *Firmicutes* with 60.06-99.72 % of relative abundance (RA) the most predominant phylum, followed by *Proteobacteria* (0.18-39.61% RA), *Actinobacteria* (0-2.84% RA), *Bacteroidetes* (0.0-0.32% RA), *Fusobacteriota* (0-0.01% RA) and *Desulfobacterota* (0-0.01% RA). A total of 52 families and 91 genera were identified as the microbiota representative of six batches of "torta-type" cheese through ripening process. Figure 1 represents the genera identified in 24 cheese samples clustered into cheese producers independent of the ripening process.

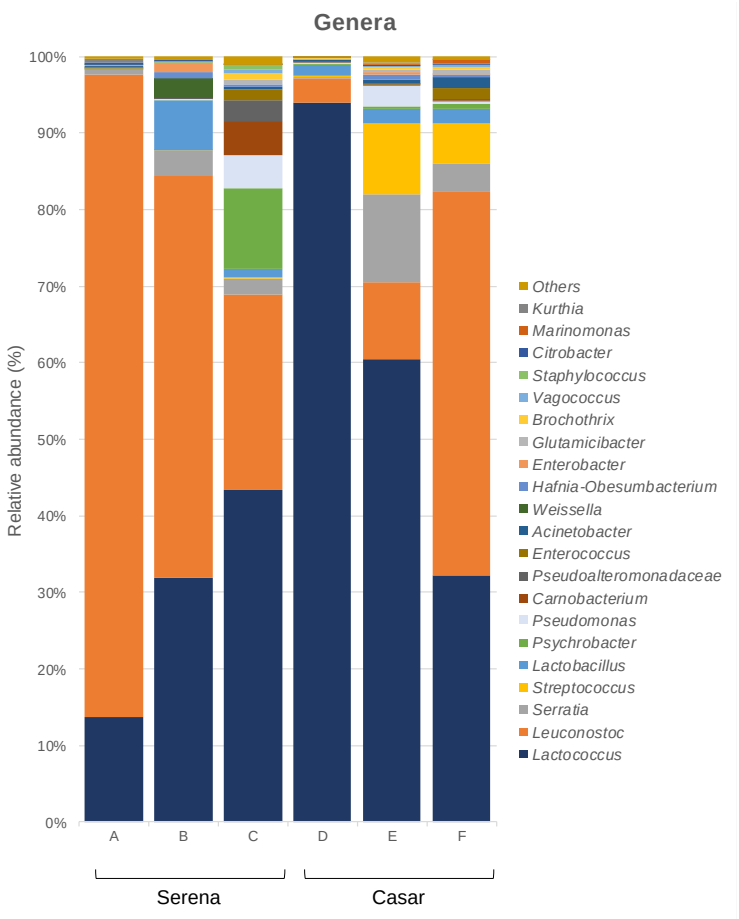


Figure 1. Assignment of cheese microbiota at the genus level according to SILVA database from Serena (A, B, C) and Casar (D, E, F) regions.

Lactic Acid Bacteria (LAB) are the main bacteria in torta-type cheeses. *Leuconostocaceae* and *Streptococcaceae* dominated the bacterial communities in all batches, being predominant *Leuconostocaceae* in batches A, B and F (44.70-92.61%) and *Streptococcaceae* in batches C, D and E (39.12-95.96%). Considering the prevalence of

Leuconostocaceace family, *L. mesenteroides* was the predominant specie in two of three cheese batches belonged to Serena region (A and B). On the other hand, *Lactococcus lactis* (58-94%) from *Streptococcaceae* family was the main LAB in cheese samples from Casar region (D and E). *Lactococcus raffinolactis* was present as a subdominant LAB species in all samples except those from industry A. It is worth highlighting the variability existing in industries C (Serena) and F (Casar) in terms of LAB. The cheeses belonging to the two industries do not have a single LAB as the majority, but rather the complex formed by *L. mesenteroides, Lc. raffinolactis* and *Carnobacterium maltaromaticum* in industry C and *L. mesenteroides* and *Lc. lactis* in industry F, are the main microorganisms throughout the ripening process. During the different days of maturation, it is observed that at 20 days *L. mesenteroides* increases in all samples. However, in cheese samples belonging to industry D, the presence of *L. mesenteroides* decreased after 20 days of ripening. Other LAB identified in cheese samples were *Streptococcus* sp. (1,4 -12,24 % RA) only present in cheese samples belong to industries E and F, increasing its presence after 20 days reaching values of 12,24 % RA at 60 days in industry E. Another *Lactobacillus* species were found in cheese samples in less percentage (0-9,36%) being identified as *Lb. plantarum, Lb. curvatus, Lb. coryniformis, Lb. paracasei, Lb. buchneri, Lb. parabuchneri, Lb. brevis, Lb, kefiri, Lb. kefiranofaciens, Lb. harbinensis, Lb. crustorum, Lb. sakei, Lb. parafarraginis, Lb. helveticus*. Belonging to *Lactococcus genera*, *Lc. piscium* and *Lc. garvieae* were identified with less than 0,8% of reads. Regarding the *Leuconostoccaceae* family, two genera were identified as *Leuconostoc* and *Weissella*. The species identified were *Leuconostoc lactis*, *L. citreum*, *L. suionicum*, *L. pseudomesenteroides*, *L. gelidum* and *L. carnosum*. Also, *Weissella confusa* was only identified in cheese samples from industry B.

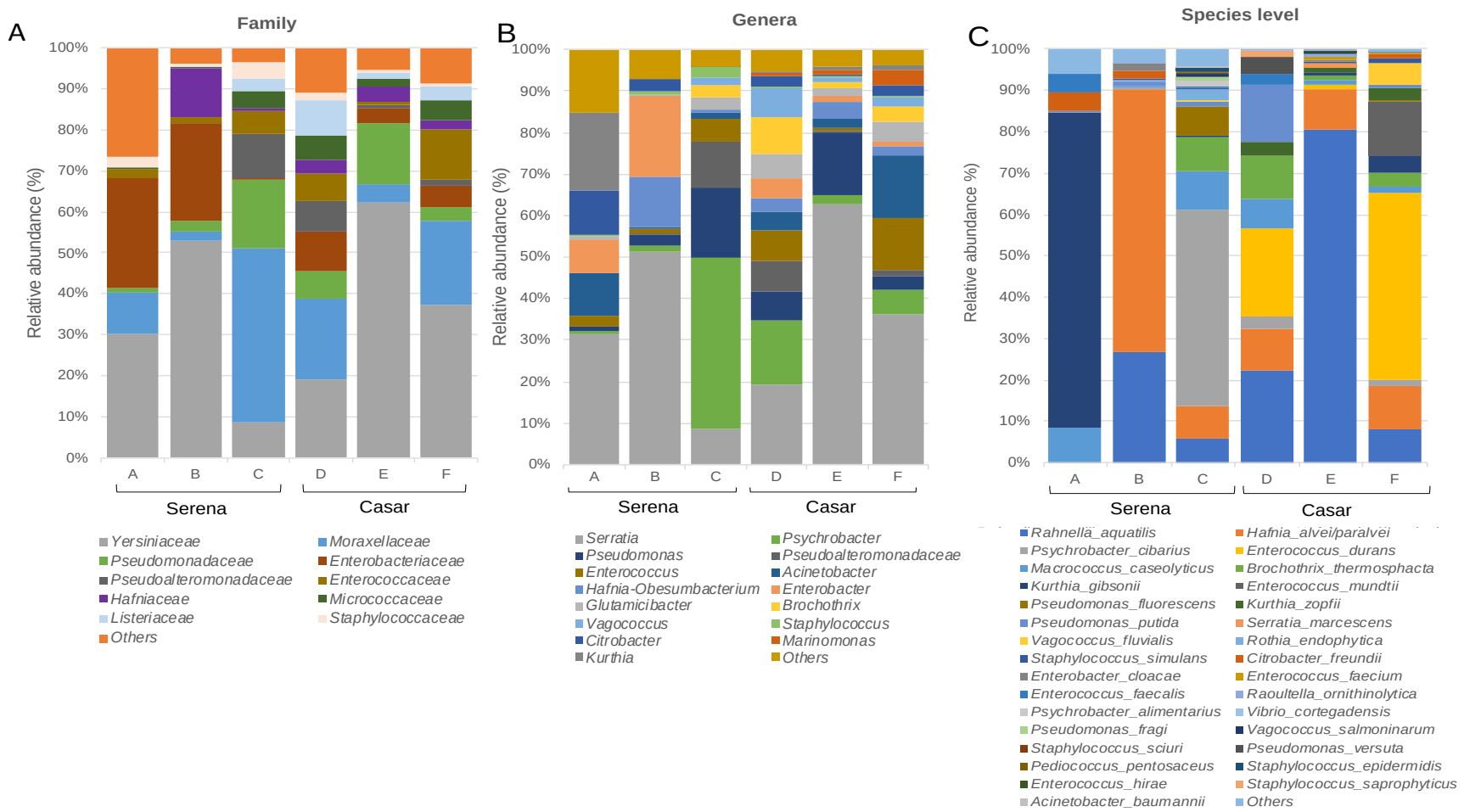


Figure 2. Bacterial communities apart from LAB identified in cheese samples clustered into producers. A) assignment to family level, B) assignment to genera level and C) assignment to species level of six cheese producers from different production regions.

Apart from the LAB, other families composed the main microbiota of the cheese samples (figure 2), such as *Yersiniaceae* (3.78%), *Moraxellaceae* (2.11%), *Pseudomonadaceae* (1.26%). Belonging to the *Yersiniaeae* family, *Serratia* sp. was present at all ripening times and at species level, only *Serratia marcenses* was identified in industry E and *Rahnella aquatilis* was found in all cheesemakers except in industry A. From *Moraxellaceae* family, *Psychrobacter* sp. and *Acinetobacter* sp. were identified in all cheese samples, being *Acinetobacter baumanii, A. rudis, P. alimentarius* and *P. immobilis* only detected in industry C. At 60 days of ripening, *Psychrobacter* spp. reached values up to 22,23% of RA in industry C. *Pseudomonadaceae* family was present at time 0 in all industries, decreasing their presence after 20 days. It should be noted that in industries C, E and F it was found in higher values between 7.93 and 25.64% at the beginning of maturation. At species level, *P. fluorescens, P. putida, P. fragi, P. versuta, P. aeruginosa, P. cannabina* and *P. oleovorans* were identified.

Regarding the secondary microbiota (1-0,1%, figure 2), the most abundant family at all ripening points were *Enterobacteriaceae* (0,53%), *Pseudoalteromonadaceae* (0,46%), *Enterococcaceae* (0,44%), *Hafniaceae* (0,33%), *Micrococcaceae* (0,31%), *Listeriaceae* (0,25%), *Staphylococcaceae* (0,19%), *Vagococcaceae* (0,15%), *Marinomonadaceae* (0,11%). At the genus level, the diversity and abundance of cheese microbiota were wide-ranging. Twenty-one genera were identified in cheese samples, although differences were found in cheese samples throughout ripening process. *Enterobacteriaceae* family was detected in all cheese samples, with relative abundances comprised between 0,03% and 4,48%. Some genera identified from this family were *Enterobacter* (*E. ludwigii, E. cloacae*), *Citrobacter* (*C. freundii*), *Klebsiella* spp., *Raoultella* (*R. ornithinolytica*) and *Yokenella* (*Y. regensburgei*). The relative abundance of *Hafniaceae* family was similar to other enterobacteria but was no detected any sequences in samples from industry A. *Hafnia alvei/paralvei* was identified at species level reaching 0,93% in one sample from industry B at 40 days of ripening (figure 3). Although *Hafnia* sp. belonged to *Enterobacteriaceae* family in the past, Adeolu et al. (2016) rearranged the order of *Enterobacterales* to place it as a different family from *Enterobacteriaceae*.

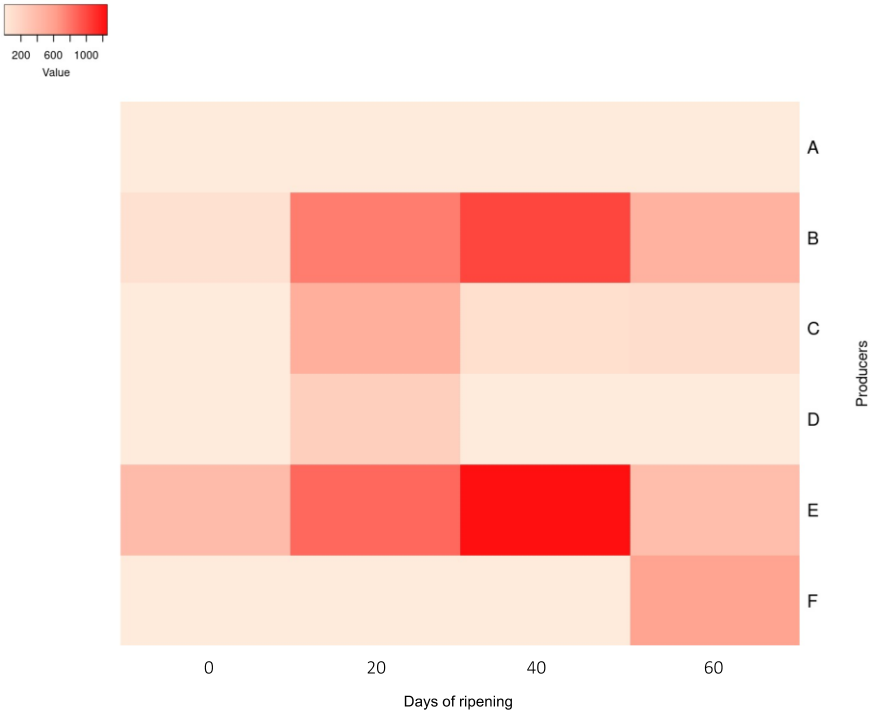


Figure 3. Heatmap showing the abundance of *Hafnia* spp. sequences found in the cheese samples from six industries at the four stages of maturation. The color scale indicates the Hafnia spp. abundance and the redder the color, more abundant is this genus.

Pseudoaltermonas spp. was found in all stages of maturation in industry C, increasing its relative abundance at 20 and 40 days of ripening to at least 3% of total reads. Other family identified was *Enterococcaceae*, predominated by genus *Enterococcus*. This genus was identified in all samples, although its presence was higher in samples from industries C and F with values greater than 1% of reads. Within the genus *Enterococcus*, some of the species identified were *E. durans*, *E. mundtii*, *E. faecium*, *E. faecalis*, *E. hirae*, *E. camelliae* and *E. italicus* in order of relative abundance. Only *E. faecalis* was found in all cheese samples, except from industry B and the other species identified were found only in industries from PDO "Torta del Casar". Sequences with relative abundances between 0,3% and 0,1% were assigned to the genera *Macrococcus*, *Staphylococcus*, *Jeotgalicoccus* (Fam. *Staphylococcaceae*), *Brochothrix* (Fam. *Listeriaceae*), *Glutamicibacter*, *Arthrobacter*, *Rothia* (Fam. *Micrococcaceae*), *Vagococcus* (Fam. *Vagococcaceae*), *Kurthia* (Fam. *Planococcaceae*), *Marinomonas* (Fam. *Marinomonadaceae*), *Corynebacterium* (Fam. *Corynebacteriaceae*), *Erwinia*, *Pantoea* (Fam. *Erwiniaceae*) and *Aeromonas* (Fam. *Aeromonadaceae*). At species level, the most relevant in terms of their relative abundances were *Macrococcus caseolyticus*,

Brochothrix thermosphacta and *Staphylococcus simulans.* Related to pathogenic bacteria, there were no sequences with annotation to *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* or *Staphylococcus aureus* in all batches throughout maturation days.

In addition, sequences that comprised between 0,1-0,00001% of total reads of each sample were identified at the genus level and are listed in table 3.

Table 3. Listed of genera identified in cheese samples with relative abundance between 0,1-0,00001%.

	A	**B**	**C**	**D**	**E**	**F**
FIRMICUTES						
Aerococcus	-	-	0,00001	-	-	-
Alloiococcus	-	-	0,00001	-	-	-
Marinilactibacillus	0,00001	-	-	-	-	-
Pediococcus	0,00004	-	-	-	-	-
PROTEOBACTERIA						
Alphaproteobacteria						
Enhydrobacter	-	-	0,00001	-	-	-
Betaproteobacteria						
Kerstersia	0,00001	-	-	-	-	-
Gammaproteobacteria						
Celerinatantimonas	0,00001	-	-	-	-	-
Klebsiella	-	-	-	0,00001	-	-
Morganella	0,00001	-	-	-	-	-
Proteus	0,00001	-	0,00004	-	-	-
Stenotrophomonas	0,00001	0,00001	-	-	-	-
Vibrio	0,00012	-	-	-	-	-
BACTEROIDETES						
Chryseobacterium	0,00003	-	-	0,00003	-	-
Fluviicola	0,00001	-	-	-	-	-
Myroides	0,00001	-	-	-	-	-
ACTINOBACTERIA						
Actinomyces	0,00001	-	-	-	-	-
Bifidobacterium	-	-	0,00001	-	-	-
Brevibacterium	0,00001	-	0,00009	-	-	-
Leucobacter	0,00005	-	-	0,00001	-	-
Microbacterium	0,00001	-	-	-	-	-
Mycetocola	0,00015	0,00001	0,00003	-	-	-
Corynebacterium	0,00008	-	0,00008	-	-	-

In this study, we have found that industries belonged to Serena region showed a higher variability related to minority microbiota, being cheese samples belonged to industry C the higher amount of ASVs showed after taxonomy assignment. It is remarkable that industry A showed less variability than other industries and it could be possible to distinguish this cheese samples from the other producers due to its bacterial communities. This result is represented in figure 4 by a train *k*-nearest neighbors' model.

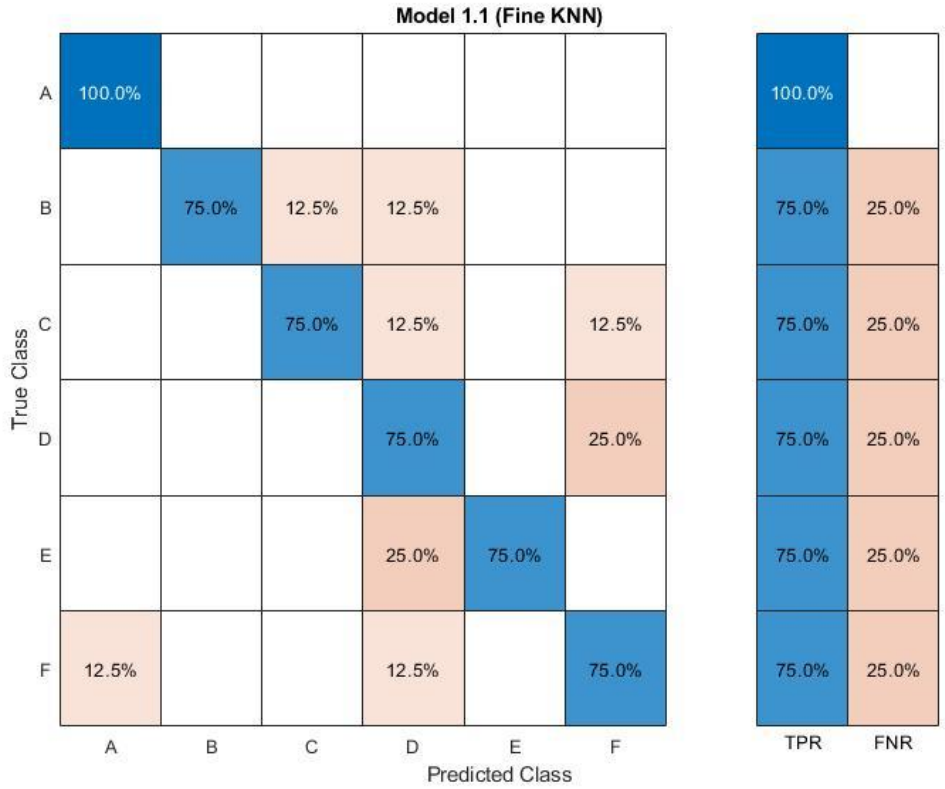


Figure 4. Prediction matrix of six dairy industries belonging to different PDO (Queso de la Serena and Torta del Casar) according to k-nearest neighbor (KNN) classifier.

Alpha diversity analysis.

There were significant differences (p<0.05) observed in alpha diversity analysis (Fig 5).

Figure 5. Boxplots describing **α**-diversity measure of cheese samples, represented by the Shannon index for the different industries (A) and at different stages of maturation (B). Different letters indicate statistically significant differences (P<0.05).

Shannon diversity showed differences between producers, finding significant differences only in industries D and E at different stages of ripening. Industry C showed the mayor index due to the highest variability found in its cheese samples, with ≥ 1% of relative abundance of reads in most microorganisms identified in this industry. Though, minor Shannon index was found in industries A and D, since in the sequencing data it has been found that these two industries had the lowest number of identified species (22 and 36 species, respectively).

I.4. Discussion.

There is a growing interest in the identification of microbiota present in artisanal soft cheeses elaborated with raw milk around the world. In the last decade, numerous studies have been carried out to evaluate the complex microbiota involved in cheese ripening, using novel technologies as high throughput sequencing. This technology allows researchers to learn more about how microorganisms interact in the biochemical processes produced during the process of making different dairy matrices, some as complex as cheese. The identification of bacteria in this study were carried out during four maturation stages, showing a diverse microbiota in the six industries, evidence that the microbiota of artisanal cheeses is prone to variability as a manufacturing effect and geographic location. As in another artisanal ewe's cheeses (De Pasquale et al., 2016; Fuka et al., 2013; Gonçalves et al., 2018; Inácio et al., 2020; Riquelme et al., 2015; Sánchez-Juanes et al., 2020), Lactic acid bacteria are the main microorganism responsible for cheese maturation. Gonçalves et al. (2018) found that *Lactococcus* and *Leuconostoc* were the main LAB in Serpa cheese, made with raw ewe's milk, in accordance with our results. Both LAB are usually found in traditional cheeses made with raw milk due to their due to their ability to grow competitively in environments as found in cheese. During ripening process, LAB play a fundamental role due to processes such as lactose fermentation, casein hydrolysis and catabolic reactions that lead to the production of aromatic compounds that influence in flavor and texture (Atanassova et al., 2020; Dugat-Bony et al., 2015; Chen et al., 2012; De Pasquale et al., 2014; Faion et al., 2020; Licitra et al., 2019; McSweeney, 2017; Pereira et al., 2010). In addition, they act as an inhibitor of the undesirable flora (Heredia-Castro et al., 2017). Lactococci are LAB that are important contributors to the production of fermented dairy products, and *Lc. lactis* is commonly found in raw milk, cheese, and other dairy products (Tanigawa et al., 2010).

The enterococci population was predominated by *E. durans* and *E. faecalis*, which are some common enterococci species found in raw ewe's milk cheeses (Abriouel et al. 2008; Feutry et al., 2012; Gonçalves et al., 2018; Mormile et. al, 2016; Ordiales et al., 2013). It is possible that raw milk may serve as a source due to their abundance (Dapkevicius et al. 2021). This genus raises some controversy because some members are linked to antibiotic resistance and virulence factors (Pimentel et. al, 2007). However, the metabolites produced by the enterococcal population impact the flavor and texture of

the cheese, thus contributing to the development of its unique sensory properties (Morandi et al., 2006).

In all samples analyzed, the population of Gram-negative bacteria was quite distributed, consisting mainly of the genera *Serratia* and *Psychrobacter* (main microbiota) and *Pseudomonas* spp., *Pseudoalteromonas* spp. and the *Enterobacteriaceae* family (secondary microbiota). Bokulich and Mills (2013) have shown that the γ-Proteobacteria (i.e., *Psychrobacter*, *Pseudomonas* and *Pseudoalteromonas*) present in the cheese core also dominated the surfaces of the ripening room, such as the aging grids or the brine tank. These genera are very common and have been reported in many dairy products, mainly in traditional cheeses (Irlinger et al., 2016; Mounier et al., 2009; Wolfe et al., 2014). The prevalence of psychrotrophic bacteria is linked to storage conditions of cheeses (temperature of 6-8 ºC for days or weeks). Regarding *Enterobacteriaceae*, their presence is associated with microbiological quality indicators since they are undesirable microorganisms throughout the production process. Hence, they can pose a public health problem due to the presence of pathogenic species such as *Klebsiella*, *Salmonella* or *E. coli* (Irlinger et al., 2015). Although large diversity of potentially pathogenic or spoilage species were found in raw sheep milk and artisanal ewe' cheeses (van den Brom et al., 2020), the bacteriological quality of the sixty days ripened torta-cheeses was satisfied such no major foodborne pathogens were detectable after 20 days of aging process. So, the absence of pathogens in the cheese indicates the safety quality of the final product and good cheesemaking' practices.

Most of the non LAB genera identified in this study were also frequently detected in other artisanal cheeses made from raw ewes' milk (Delcenserie et al., 2014; Gonçalves et al., 2018; Fuka et al., 2013; Quigley et al., 2012). *Serratia* spp. have been previously described in cheeses made with raw milk (Martín-Platero et al., 2009; Ordiales et al. 2013; Ruvalcaba-gómez et al., 2021) and have been linked to the production of hydrogen gas and the development of off-flavors (O'Sullivan and Cotter, 2017). Moreover, some species such as *Hafnia alvei* and *Hafnia paralvei* have been found frequently in raw milk and derived products (Ercolini et al., 2009), being the predominant species at the end of the ripening process in cheeses such as "Serra da Estrela" (Tavaria et al., 1998), Cheese "Serpa" (Gonçalves et al., 2018), "Alberquilla" (Abriouel et al., 2008) and "Torta del Casar" (Ordiales et al., 2013; Tabla et al., 2016). *Hafnia* spp. has a technological

relevance because is involved in the accumulation of volatile sulphur compounds (VSCs) (Fuka et al., 2013; Irlinger et al., 2015), proteolysis in the cheese core (Chaves-López et al., 2006; Morandi et al., 2021), its ability to reduce *E. coli* counts on cheese model (Delbès-Paul et al., 2013) and it is one of the few enterobacteria used as a commercial starter culture for cheese manufacturing processes (Mayo et al., 2021). In this study, *Staphylococcus* spp. has been found in all cheese samples, although no sequences have been assigned to *S. aureus* in any sample. According to Johler et al. (2018) and Castellanos-Rozo et al. (2020), *Staphylococcus* is part of natural raw milk microbiota and is also transmitted by producers' hands. This genus has been identified in almost all cheese varieties, due to their halotolerance to survive in complex environment found in cheeses. Some authors suggest that the minority microbiota can significantly influence the organoleptic characteristics of artisanal cheeses (Dalmasso et al., 2016) and this microbiota could be related to the geographic cheese production (Centi et al., 2017).

Overall, our study clearly indicates the benefits of using next generation sequencing to understand the cheese microbiota during ripening process. Detailed analysis of cheese microbiome can be related to biochemical changes that occur during maturation, such as proteolysis and the production of volatile compounds. In this regard, future research could address the study of how these changes can influence the texture and unique organoleptic characteristics of this type of cheese.

I.5. Conclusion

In conclusion, next-generation sequencing technology (Illumina) enabled an in-depth view of the microbiota in torta-type cheese, provided insight into the core microbiota of these artisanal Spanish products, allowed resolution between cheese-making units, ripening times, and provided information on the presence of bacterial genera of relevance for safety. The results obtained in this study showed great differences between the microorganisms identified from cheeses obtained from two torta-type cheese-producing regions. Our study showed that *Firmicutes* (*Leuconostoc, Lactococcus* and *Streptococcus*) predominate in these artisanal cheeses, leading us to conclude that PDO "Torta del Casar" cheese manufacture is a *Lactococcus*-driven process and PDO "Queso de la Serena" cheese manufacture is a *Leuconostoc*-driven process. Secondary microbiota also has an important role during the maturation process, as is the presence of

Hafnia spp. technological characteristics potentially contributing to the formation of the unique sensory characteristics presented these cheeses.

I.6. References.

Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E., & Martínez-Bueno, M. (2008). Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology*, *127*(3), 200–208. https://doi.org/10.1016/j.ijfoodmicro.2008.07.004

Adeolu, M., Alnajar, S., Naushad, S., & S. Gupta, R. (2016). Genome-based phylogeny and taxonomy of the 'Enterobacteriales': proposal for Enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morgane. *International Journal of Systematic and Evolutionary Microbiology*, *66*(12), 5575–5599. https://doi.org/10.1099/ijsem.0.001485

Afshari, R., Pillidge, C. J., Dias, D. A., Osborn, A. M., & Gill, H. (2018). Cheesomics: the future pathway to understanding cheese flavour and quality. *Critical Reviews in Food Science and Nutrition*, *60*(1), 33–47. https://doi.org/10.1080/10408398.2018.1512471

Alessandria, V., Ferrocino, I., De Filippis, F., Fontana, M., Rantsiou, K., Ercolini, D., & Cocolin, L. (2016). Microbiota of an Italian Grana-like cheese during manufacture and ripening, unraveled by 16S rRNA-based approaches. *Applied and Environmental Microbiology*, *82*(13), 3988–3995. https://doi.org/10.1128/AEM.00999-16

Atanassova, M. R., Fernández-Otero, C., Rodríguez-Alonso, P., Fernández-No, I. C., Garabal, J. I., & Centeno, J. A. (2016). Characterization of yeasts isolated from artisanal short-ripened cows' cheeses produced in Galicia (NW Spain). *Food Microbiology*, *53*, 172–181. https://doi.org/10.1016/j.fm.2015.09.012

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J. R., Maciejewski, A., & Wishart, D. S. (2016). Heatmapper: web-enabled heat mapping for all. Nucleic Acids Research, 44(W1), W147–W153. https://doi.org/10.1093/nar/gkw419

Bokulich, N. A., & Mills, D. A. (2013). Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. *Applied and Environmental Microbiology*, *79*(17), 5214–5223. https://doi.org/10.1128/AEM.00934-13

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Calasso, M., Ercolini, D., Mancini, L., Stellato, G., Minervini, F., Di Cagno, R., … Gobbetti, M. (2016). Relationships among house, rind and core microbiotas during

manufacture of traditional Italian cheeses at the same dairy plant. *Food Microbiology*, *54*, 115–126. https://doi.org/10.1016/j.fm.2015.10.008

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cao, Y., Fanning, S., Proos, S., Jordan, K., & Srikumar, S. (2017). A Review on the Applications of Next Generation Sequencing Technologies as Applied to Food-Related Microbiome Studies. *Frontiers in Microbiology*, *8*(SEP), 1–16. https://doi.org/10.3389/fmicb.2017.01829

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., … Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, *108*(Supplement_1), 4516–4522. https://doi.org/10.1073/pnas.1000080107

Castellanos-Rozo, J., Pulido, R. P., Grande, M. J., Lucas, R., & Gálvez, A. (2020). Analysis of the bacterial diversity of paipa cheese (A traditional raw cow's milk cheese from Colombia) by high-throughput sequencing. *Microorganisms*, *8*(2). https://doi.org/10.3390/microorganisms8020218

Centi, V., Matteucci, F., Lepidi, A., Gallo, M. Del, & Ercole, C. (2017). Microbiological and biochemical aspects of inland Pecorino Abruzzese cheese. *Heliyon*, *3*(2), e00258. https://doi.org/10.1016/j.heliyon.2017.e00258

Chaves-López, C., De Angelis, M., Martuscelli, M., Serio, A., Paparella, A., & Suzzi, G. (2006). Characterization of the Enterobacteriaceae isolated from an artisanal Italian ewe's cheese (Pecorino Abruzzese). *Journal of Applied Microbiology*, *101*(2), 353–360. https://doi.org/10.1111/j.1365-2672.2006.02941.x

Chen, L. shui, Cui, J., Ding, Q. bo, Ma, Y., Chen, L. jun, Dong, J. ying, … Maubois, J. louis. (2012). The Effect of Yeast Species from Raw Milk in China on Proteolysis and Aroma Compound Formation in Camembert-Type Cheese. *Food and Bioprocess Technology*, *5*(6), 2548–2556. https://doi.org/10.1007/s11947-011-0589-4

Choi, J., In Lee, S., Rackerby, B., Frojen, R., Goddik, L., Ha, S. Do, & Park, S. H. (2020). Assessment of overall microbial community shift during Cheddar cheese production from raw milk to aging. *Applied Microbiology and Biotechnology*, *104*(14), 6249–6260. https://doi.org/10.1007/s00253-020-10651-7

Dalmasso, A., Soto del Rio, M. de los D., Civera, T., Pattono, D., Cardazzo, B., & Bottero, M. T. (2016). Characterization of microbiota in Plaisentif cheese by high-throughput sequencing. *LWT - Food Science and Technology*, *69*, 490–496. https://doi.org/10.1016/j.lwt.2016.02.004

Dapkevicius, M. de L. E., Sgardioli, B., Câmara, S. P. A., Poeta, P., & Malcata, F. X. (2021). Current trends of enterococci in dairy products: A comprehensive review of their multiple roles. *Foods*, *10*(4), 1–30. https://doi.org/10.3390/foods10040821

De Filippis, F., Genovese, A., Ferranti, P., Gilbert, J. A., & Ercolini, D. (2016). Metatranscriptomics reveals temperature-driven functional changes in microbiome impacting cheese maturation rate. *Scientific Reports*, *6*(October 2015), 1–11. https://doi.org/10.1038/srep21871

De Filippis, F., Parente, E., & Ercolini, D. (2017). Metagenomics insights into food fermentations. *Microbial Biotechnology*, *10*(1), 91–102. https://doi.org/10.1111/1751-7915.12421

De Pasquale, I., Calasso, M., Mancini, L., Ercolini, D., La Storia, A., De Angelis, M., … Gobbetti, M. (2014). Causal relationship between microbial ecology dynamics and proteolysis during manufacture and ripening of Protected Designation of Origin (PDO) cheese Canestrato Pugliese. *Applied and Environmental Microbiology*, *80*(14), 4085–4094. https://doi.org/10.1128/AEM.00757-14

De Pasquale, I., Di Cagno, R., Buchin, S., De Angelis, M., & Gobbetti, M. (2016). Spatial distribution of the metabolically active microbiota within Italian PDO ewes' milk cheeses. *PLoS ONE*, *11*(4), 1–23. https://doi.org/10.1371/journal.pone.0153213

Delbès-Paus, C., Miszczycha, S., Ganet, S., Helinck, S., Veisseire, P., Pochet, S., … Montel, M. C. (2013). Behavior of Escherichia coli O26: H11 in the presence of Hafnia alvei in a model cheese ecosystem. *International Journal of Food Microbiology*, *160*(3), 212–218. https://doi.org/10.1016/j.ijfoodmicro.2012.10.019

Delcenserie, V., Taminiau, B., Delhalle, L., Nezer, C., Doyen, P., Crevecoeur, S., … Daube, G. (2014). Microbiota characterization of a Belgian protected designation of origin cheese, Herve cheese, using metagenomic analysis. *Journal of Dairy Science*, *97*(10), 6046–6056. https://doi.org/10.3168/jds.2014-8225

Dugat-Bony, E., Straub, C., Teissandier, A., Onésime, D., Loux, V., Monnet, C., … Bonnarme, P. (2015). Overview of a surface-ripened cheese community functioning by meta-omics analyses. *PLoS ONE*, *10*(4), 1–25. https://doi.org/10.1371/journal.pone.0124360

Ercolini, D., Russo, F., Ferrocino, I., & Villani, F. (2009). Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiology*, *26*(2), 228–231. https://doi.org/https://doi.org/10.1016/j.fm.2008.09.005

Escobar-Zepeda, A., Sanchez-Flores, A., & Quirasco Baruch, M. (2016). Metagenomic analysis of a Mexican ripened cheese reveals a unique complex microbiota. *Food Microbiology*, *57*, 116–127. https://doi.org/10.1016/j.fm.2016.02.004

Faion, A. M., Menegotto, A. L. L., Fernandes, I. A., Steffens, C., Steffens, J., & Valduga, E. (2020). Production of Serra da Estrela cheese from ultrafiltered sheep's milk. *Emirates Journal of Food and Agriculture*, *32*(5), 384–390. https://doi.org/10.9755/ejfa.2020.v32.i5.2109

Feutry, F., Oneca, M., Berthier, F., & Torre, P. (2012). Biodiversity and growth dynamics of lactic acid bacteria in artisanal PDO Ossau-Iraty cheeses made from raw ewe's milk with different starters. *Food Microbiology*, *29*(1), 33–42. https://doi.org/10.1016/j.fm.2011.08.011

Fuka, M. M., Wallisch, S., Engel, M., Welzl, G., Havranek, J., & Schloter, M. (2013). Dynamics of bacterial communities during the ripening process of different Croatian cheese types derived from raw ewe's milk cheeses. *PLoS ONE*, *8*(11), 1–10. https://doi.org/10.1371/journal.pone.0080734

Gatti, M., Bottari, B., Lazzi, C., Neviani, E., & Mucchetti, G. (2014). Invited review: Microbial evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters. *Journal of Dairy Science*, *97*(2), 573–591. https://doi.org/10.3168/jds.2013-7187

Gobbetti, M., De Angelis, M., Di Cagno, R., Mancini, L., & Fox, P. F. (2015). Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. *Trends in Food Science and Technology*, *45*(2), 167–178. https://doi.org/10.1016/j.tifs.2015.07.016

Gonçalves, M. T. P., Benito, M. J., Córdoba, M. de G., Egas, C., Merchán, A. V., Galván, A. I., & Ruiz-Moyano, S. (2018). Bacterial Communities in Serpa Cheese by Culture Dependent Techniques, 16S rRNA Gene Sequencing and High-throughput Sequencing Analysis. *Journal of Food Science*, *83*(5), 1333–1341. https://doi.org/10.1111/1750-3841.14141

Heredia-Castro, P. Y., Hérnández-Mendoza, A., González-Córdova, A. F., & Vallejo-Cordoba, B. (2017). Bacteriocinas de bacterias ácido lácticas: Mecanismos de acción y actividad antimicrobiana contra patógenos en quesos. *Interciencia*, *42*(6), 340–346.

Ianni, A., Di Domenico, M., Bennato, F., Peserico, A., Martino, C., Rinaldi, A., … Martino, G. (2020). Metagenomic and volatile profiles of ripened cheese obtained from dairy ewes fed a dietary hemp seed supplementation. *Journal of Dairy Science*. https://doi.org/10.3168/jds.2019-17954

Inácio, R. S., Gomes, A. M. P., & Saraiva, J. A. (2020). Serra da Estrela cheese: A review. *Journal of Food Processing and Preservation*, *44*(5), 1–10. https://doi.org/10.1111/jfpp.14412

Irlinger, F., Layec, S., Hélinck, S., & Dugat-Bony, E. (2015). Cheese rind microbial communities: Diversity, composition and origin. *FEMS Microbiology Letters*, *362*(2), 1–11. https://doi.org/10.1093/femsle/fnu015

Johler, S., Macori, G., Bellio, A., Acutis, P. L., Gallina, S., & Decastelli, L. (2018). Short communication: Characterization of Staphylococcus aureus isolated along the raw milk cheese production process in artisan dairies in Italy. *Journal of Dairy Science*, *101*(4), 2915–2920. https://doi.org/10.3168/jds.2017-13815

Klindworth, A., Pruesse, E. Schweer, T. Peplies, J., Quast,C., Horn, M., Glöckner, F.O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies, *Nucleic Acids Research, 41* (1). https://doi.org/10.1093/nar/gks808

Licitra, G., Caccamo, M., & Lortal, S. (2019). *Artisanal Products Made With Raw Milk*. *Raw Milk*. Elsevier Inc. https://doi.org/10.1016/b978-0-12-810530-6.00009-2

Martín-Platero, A. M., Valdivia, E., Maqueda, M., Martín-Sánchez, I., & Martínez-Bueno, M. (2008). Polyphasic approach to bacterial dynamics during the ripening of Spanish farmhouse cheese, using culture-dependent and -independent methods. *Applied and Environmental Microbiology*, *74*(18), 5662–5673. https://doi.org/10.1128/AEM.00418-08

Mayo, B., Rodríguez, J., Vázquez, L., & Flórez, A. B. (2021). Microbial Interactions within the Cheese Ecosystem and Their Application to Improve Quality and Safety. *Foods*, *10*(3), 602. https://doi.org/10.3390/foods10030602

McSweeney, P. L. H., Fox, P. F., & Ciocia, F. (2017). Metabolism of Residual Lactose and of Lactate and Citrate. In *Cheese* (pp. 411–421). Elsevier. https://doi.org/10.1016/B978-0-12-417012-4.00016-8

Montel, M. C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D. A., Desmasures, N., & Berthier, F. (2014). Traditional cheeses: Rich and diverse microbiota with associated benefits. *International Journal of Food Microbiology*, *177*, 136–154. https://doi.org/10.1016/j.ijfoodmicro.2014.02.019

Morales, P., Feliu, I., Fernández-García, E., & Nuñez, M. (2004). Volatile Compounds Produced in Cheese by Enterobacteriaceae Strains of Dairy Origin. *Journal of Food Protection*, *67*(3), 567–573. https://doi.org/10.4315/0362-028X-67.3.567

Morandi, S., Brasca, M., Andrighetto, C., Lombardi, A., & Lodi, R. (2006). Technological and molecular characterisation of enterococci isolated from north–west Italian dairy products. *International Dairy Journal*, *16*(8), 867–875. https://doi.org/https://doi.org/10.1016/j.idairyj.2005.09.005

Morandi, S., Pica, V., Masotti, F., Cattaneo, S., Brasca, M., De Noni, I., & Silvetti, T. (2021). Proteolytic traits of psychrotrophic bacteria potentially causative of sterilized milk instability: Genotypic, phenotypic and peptidomic insight. *Foods*, *10*(5). https://doi.org/10.3390/foods10050934

Mormile, A., Barile, M., Mercogliano, R., Johansson, P., Björkroth, K. J., Aponte, M., & Murru, N. (2016). Dynamics of lactic acid bacteria in “Pecorino di Tramonti”—a ewe’s milk cheese—with particular emphasis on enterococci: a preliminary study. *Annals of Microbiology*, *66*(1), 179–185. https://doi.org/10.1007/s13213-015-1094-1

Mormile, A., Barile, M., Mercogliano, R., Johansson, P., Björkroth, K. J., Aponte, M., & Murru, N. (2016). Dynamics of lactic acid bacteria in “Pecorino di Tramonti”—a ewe’s milk cheese—with particular emphasis on enterococci: a preliminary study.

Annals of Microbiology, *66*(1), 179–185. https://doi.org/10.1007/s13213-015-1094-1

O'Sullivan, O., & Cotter, P. D. (2017). Microbiota of Raw Milk and Raw Milk Cheeses. In *Cheese* (Fourth, pp. 301–316). Elsevier. https://doi.org/10.1016/B978-0-12-417012-4.00012-0

Ordiales, E., Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J., & de Guía Córdoba, M. (2013). Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. *Food Control*, *33*(2), 448–454. https://doi.org/10.1016/j.foodcont.2013.03.027

Pereira, C. I., Graça, J. A., Ogando, N. S., Gomes, A. M. P., & Xavier Malcata, F. (2010). Influence of bacterial dynamics upon the final characteristics of model Portuguese traditional cheeses. *Food Microbiology*, *27*(3), 339–346. https://doi.org/10.1016/j.fm.2009.10.012

Pimentel, L. L., Semedo, T., Tenreiro, R., Crespo, M. T. B., Pintado, M. M. E., & Malcata, F. X. (2007). Assessment of Safety of Enterococci Isolated throughout Traditional Terrincho Cheesemaking: Virulence Factors and Antibiotic Susceptibility. *Journal of Food Protection*, *70*(9), 2161–2167. https://doi.org/10.4315/0362-028X-70.9.2161

Quigley, L., O'Sullivan, O., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2012). High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. *Applied and Environmental Microbiology*, *78*(16), 5717–5723. https://doi.org/10.1128/AEM.00918-12

Quijada, N. M., Hernández, M., & Rodríguez-Lázaro, D. (2020). *High-throughput sequencing and food microbiology*. *Advances in Food and Nutrition Research* (1st ed., Vol. 91). Elsevier Inc. https://doi.org/10.1016/bs.afnr.2019.10.003

Riquelme, C., Câmara, S., Enes Dapkevicius, M. de L. N., Vinuesa, P., da Silva, C. C. G., Malcata, F. X., & Rego, O. A. (2015). Characterization of the bacterial biodiversity in Pico cheese (an artisanal Azorean food). *International Journal of Food Microbiology*, *192*, 86–94. https://doi.org/10.1016/j.ijfoodmicro.2014.09.031

Ruvalcaba-Gómez, J. M., Delgado-Macuil, R. J., Zelaya-Molina, L. X., Maya-Lucas, O., Ruesga-Gutiérrez, E., Anaya-Esparza, L. M., … Arteaga-Garibay, R. I. (2021). Bacterial succession through the artisanal process and seasonal effects defining bacterial communities of raw-milk adobera cheese revealed by high throughput dna sequencing. *Microorganisms*, *9*(1), 1–18. https://doi.org/10.3390/microorganisms9010024

Salazar, J. K., Carstens, C. K., Ramachandran, P., Shazer, A. G., Narula, S. S., Reed, E., … Schill, K. M. (2018). Metagenomics of pasteurized and unpasteurized gouda cheese using targeted 16S rDNA sequencing. *BMC Microbiology*, *18*(1), 1–13. https://doi.org/10.1186/s12866-018-1323-4

Sánchez-Juanes, F., Teixeira-Martín, V., González-Buitrago, J. M., Velázquez, E., & Flores-Félix, J. D. (2020). Identification of Species and Subspecies of Lactic Acid Bacteria Present in Spanish Cheeses Type "Torta" by MALDI-TOF MS and pheS gene Analyses. *Microorganisms*, *8*(2), 301. https://doi.org/10.3390/microorganisms8020301

Schirone, M., Tofalo, R., Perpetuini, G., Manetta, A. C., Di Gianvito, P., Tittarelli, F., … Martino, G. (2018). Influence of iodine feeding on microbiological and physico-chemical characteristics and biogenic amines content in a raw ewes' milk cheese. *Foods*, *7*(7). https://doi.org/10.3390/foods7070108

Sousa, M. J., Ardö, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, *11*(4–7), 327–345. https://doi.org/10.1016/S0958-6946(01)00062-0

Tabla, R., Gómez, A., Simancas, A., Rebollo, J. E., Molina, F., & Roa, I. (2016). Enterobacteriaceae species during manufacturing and ripening of semi–hard and soft raw ewe's milk cheese: Gas production capacity. *Small Ruminant Research*, *145*, 123–129. https://doi.org/10.1016/j.smallrumres.2016.11.008

Tanigawa, K., Kawabata, H., & Watanabe, K. (2010). Identification and typing of lactococcus lactis by matrix-assisted laser desorption ionization - Time of flight mass spectrometry. *Applied and Environmental Microbiology*, *76*(12), 4055–4062. https://doi.org/10.1128/AEM.02698-09

Tavaria, F. K., & Malcata, F. X. (1998). Microbiological characterization of Serra da Estrela cheese throughout its appellation d'Origine protegee region. *Journal of Food Protection*, *61*(5), 601–607. https://doi.org/10.4315/0362-028X-61.5.601

van den Brom, R., de Jong, A., van Engelen, E., Heuvelink, A., & Vellema, P. (2020). Zoonotic risks of pathogens from sheep and their milk borne transmission. *Small Ruminant Research*, *189*(April), 106123. https://doi.org/10.1016/j.smallrumres.2020.106123

Wolfe, B. E., Button, J. E., Santarelli, M., & Dutton, R. J. (2014). Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell*, *158*(2), 422–433. https://doi.org/10.1016/j.cell.2014.05.041

Yang, C., Zhao, F., Hou, Q., Wang, J., Li, M., & Sun, Z. (2020). PacBio sequencing reveals bacterial community diversity in cheeses collected from different regions. *Journal of Dairy Science*, *103*(2), 1238–1249. https://doi.org/10.3168/jds.2019-17496

Zago, M., Bardelli, T., Rossetti, L., Nazzicari, N., Carminati, D., Galli, A., & Giraffa, G. (2021). Evaluation of bacterial communities of Grana Padano cheese by DNA metabarcoding and DNA fingerprinting analysis. *Food Microbiology*, *93*, 103613. https://doi.org/10.1016/j.fm.2020.103613

CAPÍTULO II

Biodiversity of yeast community in artisanal soft cheeses "Torta del Casar" and "Queso de la Serena" by High-Throughput sequencing.

Capítulo II.

Biodiversity of yeast community in artisanal soft cheeses "Torta del Casar" and "Queso de la Serena" by High-Throughput sequencing.

Abstract

Traditional cheeses made with raw milk have been extensively studied to understand the microbiota involved in their ripening process by culture-dependent methods. In this study, the mycobiota profile of six Spanish PDO cheeses has been evaluated. High-Throughput DNA Sequencing was applied to elucidate the mycobiota occurring during torta-type cheese production, as an alternative to culture-dependent methods. Results showed that *Yarrowia alimentaria, Y. lipolytica* and *Kluyveromyces lactis* prevailed the fungal populations in all samples. Other species were identified, such as *Debaryomyces hansenii, Geotrichum candidum, Pichia fermentans, Candida zeylanoides* and *Trichosporon coremiiforme.* This study provides evidence of the diversity of yeast associated with traditional cheeses. In general, all cheese samples from the six industries showed the same predominant yeast genera, which suggests that the cheese-makers regions are not a determining factor in the mycobiota composition of soft cheeses, but rather the fungi composition of raw milk and the environmental conditions of the manufacture.

II.1. Introduction.

"*Torta del Casar*" and "*Queso de la Serena*" are both artisanal Spanish cheeses granted by the Protected Designation of Origin (PDO) label, in accordance with the Regulations of the European Commission (EC 1491/2003 and 1107/1996). These artisanal soft cheeses are made with raw ewes' milk, using an aqueous infusion of the dried flowers from *Cynara cardunculus* L. without the addition of a starter culture. Due to the lack of standardization or thermal process, the complex microbiota belonged to raw milk, vegetable rennet and dairy environment, conferring unique organoleptic characteristics highly appreciated by consumers (Bokulich and Mills, 2013b; Ordiales et al., 2013b; Gonçalves et al., 2017). Cheese fermentation is carried out by a wide diversity of bacteria, yeasts and molds (Irlinger et al., 2015; Montel et al., 2014). The autochthonous microbiota of torta-type cheeses is formed mainly by Lactic Acid Bacteria

(LAB). Various authors have found that predominant genera are *Lactobacillus*, *Lactococcus* and *Leuconostoc* (Gonçalves et al., 2018; Inácio et al., 2020; Ordiales et al., 2013a; Pereira et al., 2010; Sánchez-Juanes et al., 2020). Within the secondary microbiota, yeasts constitute a large and heterogeneous group that plays a fundamental role both directly and indirectly in the maturation process of soft paste cheeses (Alegría et al., 2009; Wolfe et al., 2014; Yeluri et al., 2018). In the core, the counts are normally between 4 – 6 log cfu/g (Atanassova et al., 2016; Pintado et al., 2008; Ordiales et al., 2013a). However, in some cheeses made with raw milk it can reach values of up to 9 log cfu/g (Jacques and Casaregola, 2008, Padilla et al., 2014). The presence of yeasts in high concentration at the end of cheese maturation is not surprising because these microorganisms find an environment rich in nutrients with high concentrations of salt, low pH, temperatures and water activities, to which they are well adapted (Gardini et al., 2006; Hatoum et al., 2012). Among the majority species, although there is great diversity due to the wide range of cheeses made throughout the world, stand out *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Geotrichum candidum*, *Saccharomyces cerevisiae* and members of the genera *Candida* spp. and *Pichia* spp. (Atanassova et al., 2016; Banjara et al., 2015; Binetti et al., 2013; Ceugniez et al., 2015), although the prevalence of certain species may be influenced by the type of cheese (Dugat-Bony et al., 2016).

On the other hand, overgrowth of some spoilage yeasts in cheese can lead to fruity, 'yeast' and other off-flavors (Ledenbach et al., 2009); also gasification, slime formation, discoloration (Fröhlich-Wyder et al., 2019), generation of unwanted pigments caused mainly by *Y. lipolytica* (Carreira et al., 2001; Bintsis et al., 2002) or the "toad skin" caused by the excess growth and proteolytic activity of *G. candidum*, which is characterized by generating a slippery, wrinkled, mucous and viscous rind in soft paste cheeses (Marcelino et al., 2001). In artisanal ewes' cheeses, Ordiales et al. (2013a) found that the predominant species during the maturation process of "*Torta del Casar*" were *Candida zeylanoides*, *Rhodotorula mucilaginosa*, *Candida parapsilosis* and *Yarrowia lipolytica*. Gonçalves et al. (2017) found greater diversity in Serpa cheese, with the most common genera *Debaryomyces* spp. and *Kluyveromyces* spp., and to a lesser extent, *Pichia* spp. and *Candida* spp. Furthermore, during cheese maturation, the strains may enter a viable but non-culturable state and consequently the cheese environment may be populated with a variety of cell types, including intact, viable, nonviable, and partial or totally disintegrated

(Martín-Platero et al., 2008). For several decades, cheese fungi had been investigated using culture-dependent methods combined with molecular tools based on polymerase chain reaction (PCR) of the Internal Transcribed Spacer amplicons (ITS) of the nuclear ribosomal RNA (rRNA) (De Filippis et al., 2016; Tofalo et al., 2014). The ITS region is considered a universal barcode for identification of fungi. This region includes the ITS1 and ITS2 regions, separated by the 5,8S gene (Bokulich and Mills, 2013a; Mota-Gutiérrez et al., 2019). In this sense, recent advances in High-throughput sequencing (HTS) technology have revolutionized the study of fungal communities in food fermentations. This technology allows to know this type of microorganisms without the need to carry out a routine microbiological culture (De Filippis et al., 2017) and their metabolic influence on the ripening and quality of cheeses (Fröhlich-Wyder et al., 2019). In artisanal soft cheeses, Gonçalves et al. (2017) studied the autochthonous microbiota in "Serpa" cheese, from industries belonging to PDO and two other non-certified industries, finding a complex community of yeast, as well as significant differences between the fungal population of PDO and non-PDO cheeses. So far, no studies have been carried out to determine the fungal community of "torta-type" cheeses.

In this study, we examined six different batches of torta-type cheese produced in two geographical locations (PDO "Torta del Casar" and "Queso del Serena") throughout ripening (0, 20, 40 and 60 days). The main focus of this study is to analyze the fungal community along their maturation process.

II.2. Material and methods.

Cheese manufacture and sampling.

Cheeses were made by local formal producers from raw ewes' milk by enzymatic coagulation with rennet from *C. cardunculus* L., following the procedure described for the Protected Designation of Origin (PDO) of the cheeses. Cheese samples were provided by six local cheese producers (A, B, C, D, E, F) in winter 2018, in two different geographical locations. From PDO "*Queso de la Serena*", batches were named A, B and C and batches from PDO "*Torta del Casar*" were D, E and F. The samplings were done at 0, 20, 40 and 60 days of ripening. For each cheese batch, samples were collected at local industry and quickly transported to University of Extremadura for analysis. All samples were analyzed immediately or frozen at -80ºC.

Extraction of total genomic DNA.

Firstly, fifty grams of each cheese sample was pulverized with liquid nitrogen using a CryoMill equipment (Retsch, Germany) for 10 minutes. Total genomic DNA was extracted using Quick-DNA^{TM} Fecal/Soil Microbe MiniPrep Kit (Zymo Research, Irvine, CA, USA), following the instructions provided by the manufacturer. The quality and quantity of the extracted DNA was determined using a NanoDrop 2000 (ThermoFisher Scientific, USA).

Amplicon library preparation and sequencing.

Total DNA of each sample was subjected to NGS analysis at NUCLEUS (University of Salamanca, Spain). Fungal diversity in the samples was analyzed by sequencing of amplified ITS1 and ITS2 regions of the ITS rRNA gene with primers listed in table 1, using an Illumina MiSeq platform. A 2 × 300 bp of MiSeq amplicon library was prepared using the Metagenomics Library Preparation workflow (Illumina Inc, California, USA).

Table 1. Primer sequences of ITS1-2 regions of the ITS rRNA used in this study (White et al., 1990; Mello et al., 2011).

ITS region	Primer sequence
ITS1f	5'-CTTGGTCATTTAGAGGAAGTAA-3'
ITS2r	5'-GCTGCGTTCTTCATCGATGC-3'
ITS3f	5'-GCATCGATGAAGAACGCAGC-3'
ITS4r	5'-TCCTCCGCTTATTGATATGC-3'

*f: forward, r: reverse

Bioinformatic analysis.

Raw FASTQ sequences longer than 221bp were converted into FASTA format using Seqtk software (version 1.3, https://github.com/lh3/seqtk, accessed on 7 March 2021). Afterwards, BLASTN command (*blastn -query sequence.fa -db fungidb -evalue 0.001 -outfmt 6 > sample.txt*) was used to detect the most similar ITS (E-value<10^{-3} and a minimum of 97% similarity over at least 80 base pairs) from a custom database to each read. A text file containing the FASTA sequences from ITS region of the most frequently fungi species found in traditional cheeses, previously downloaded from NCBI database (https://www.ncbi.nlm.nih.gov), was used to build a custom database with *makeblastdb* command (*makeblastbd -in fungidb.fa -dbtype nucl -parse_seqids -out fungidb*). Then, the tabular output for each sample was imported to MATLAB, where the species were

extracted, quantified and finally exported as a tabular ASV table compatible with other metagenomic software.

The **α**-diversity was carried out by QIIME package (q2-diversity-alpha) and the richness estimator Shannon index was considered. For prediction assay, a k-nearest neighbors (KNN) model was trained with the final ASV data (fungal content of the samples) using the Classification Learner app from MATLAB. The fungal relationship between production regions was visualized using Venny 2.1 for VennDiagrams (Oliveros, 2015). HeatMapper.ca (Babicki et al., 2016) was used to visualize the most abundance genera from the six industries.

Statistical analysis

Analysis of variance (ANOVA) followed by Tukey HDS post hoc test using the SPSS v21.0 software was carried out to assess the statistical differences between the cheese samples.

II.3. Results.

Characterization of fungal community in raw ewes' milk cheeses.

Fungal community composition of cheese samples was minor variable than bacterial communities observed in the culture independent study. A total of 11,083,150 raw reads were obtained for 24 duplicated cheese samples. The length of the raw reads was 301 bp. After trimming, reads per sample ranged from 165 to 34576 (median 2386; mean 7061,08). Reads with a minimum length of 221 bases were kept for BLASTn analysis. Sequences were clustered into 514 OTUs by 97% similarity against custom fungi database (table 2).

Table 2. Metataxonomic fungi data of “Queso de la Serena” and “Torta del Casar” artisanal cheeses (n=24) produced in Extremadura, Spain.

		Ripening (days)	Raw Sequences	Total ITS reads	Total frequency of features	Total features (OTUs)	Families	Genera
PDO "QUESO DE LA SERENA"	**A**	0	195495	3471	359	17	5	6
		20	455151	20896	4348	38	11	17
		40	337343	2427	182	14	7	10
		60	87856	1784	93	19	7	12
	B	0	289171	1058	117	19	8	12
		20	383751	1228	93	30	7	12
		40	495607	1002	100	21	7	10
		60	598598	1006	73	9	5	6
	C	0	197972	27568	4668	45	11	18
		20	775655	6372	686	27	6	9
		40	788900	2345	258	19	6	10
		60	224625	3200	156	25	8	12
PDO "TORTA DEL CASAR"	**D**	0	105871	836	36	11	5	8
		20	462408	1557	71	17	6	9
		40	999507	817	50	20	8	11
		60	947732	2429	104	21	7	11
	E	0	475138	13688	1093	25	9	13
		20	633484	198	18	9	4	5
		40	633918	407	37	18	5	6
		60	474834	165	6	3	2	2
	F	0	106489	4682	173	10	4	5
		20	407780	18906	2958	35	9	16
		40	387158	34576	6271	38	9	16
		60	618707	18848	778	24	8	12
Total		n=48	11083150	169466	22728	514	11	18

The Venn diagrams (Figure 1) were generated from the results of all ASVs to show the number of ASV shared among cheese production regions and the differences between them. It can be seen that the vast majority of ASVs are associated with the two production regions, although the Serena industries encompass a greater number of species that have not been identified in the Casar industries.

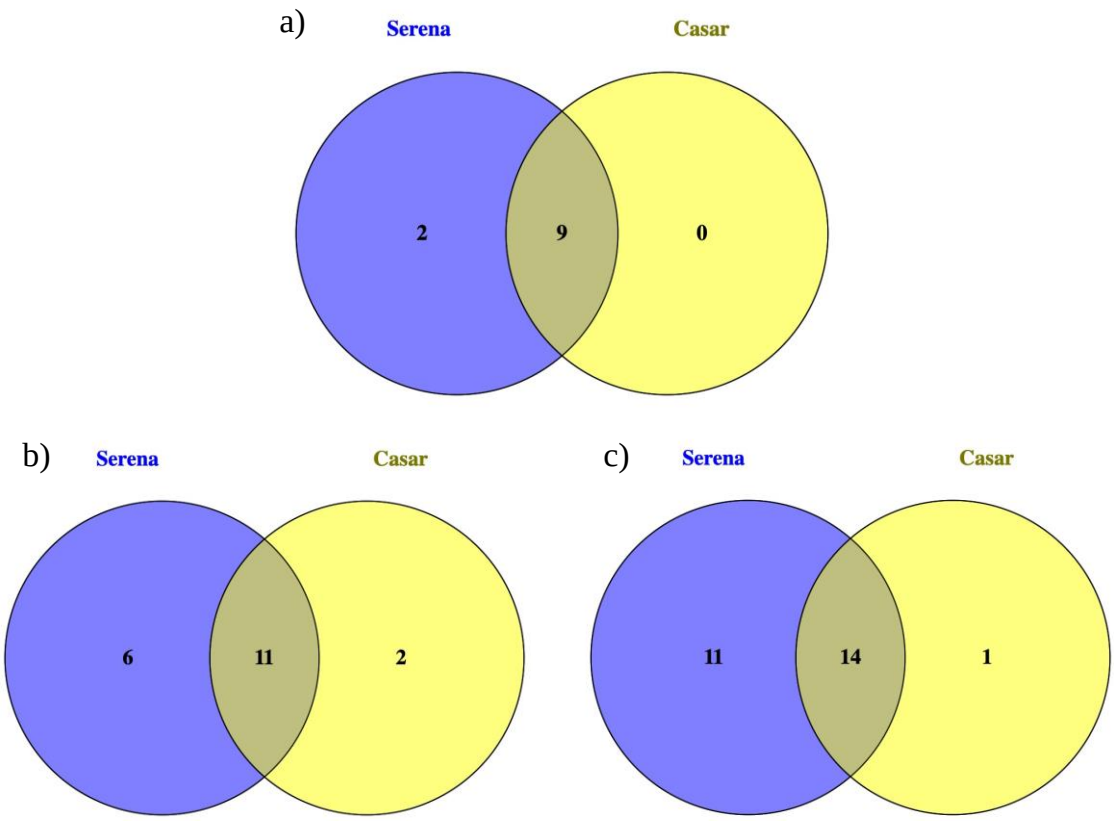


Figure 1. Venn diagrams showing the ASVs shared between Serena and Casar cheese industries at the family (a), genus (b) and species (c) level. Numbers indicate the number of shared taxa.

The taxonomic assignment allowed to identify two phyla, being *Ascomycota* (66,47% RA) and *Basidiomycota* (33,53% RA). A total of eleven families was identified as the microbiota representative of 24 cheese samples belonging to six different dairy industries. *Dipodascaceae* (0,55-83,3% RA, mean 45,99%), *Saccharomycetaceae* (0,67-26,92% RA, mean 15,14%), *Pichiaceae* (0,62-22,48% RA, mean 9,62%) and *Trichomonascaceae* (0,02-91,09% RA, mean 8,77%) were the main predominant families in all cheese samples at all time points of ripening. Figure 2(A) represent the wide-ranging variability related to genera presented in cheese samples. Sequencing the ITS rRNA region by HTS allowed identify to species level, as is showed in Figure 2(B). Twenty-six species were identified in cheese samples, although fourteen are present in all batches.

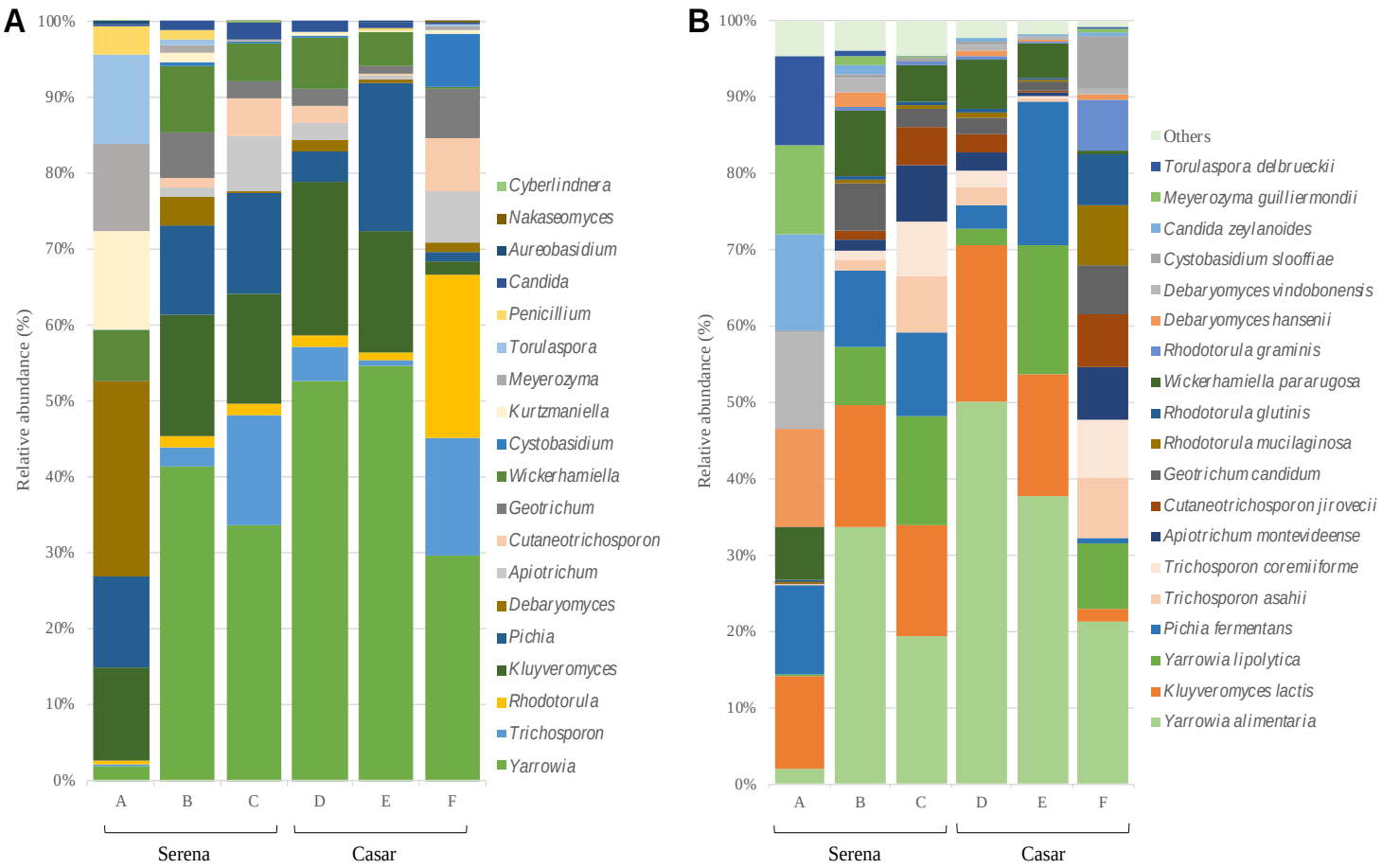


Figure 2. Fungal composition of six cheeses from two production regions. Vertical bars indicate the relative abundance of sequences corresponding to genera (A) and species (B) level.

Related to genus level of the *Dipodascaceae* family, *Yarrowia* (0,53-83,33% RA) was found at an important level in cheeses from all industries. Two species were identified as *Y. alimentaria* and *Y. lipolytica*, being the main yeasts throughout maturation, reaching up to 64,71 % of the total reads at 60 days of ripening. *Geotrichum candidum* (= *Galactomyces geotrichum*) was identified at all stages of maturation, being its majority presence at 40 days in cheese samples from industry F.

The second family with the highest number of assigned reads was *Saccharomycetaceae*, compound of *Kluyveromyces*, *Nakaseomyces* and *Torulaspora* genera. *Kluveromyces* spp. (0,43-26,92% RA) was the main genera of this family in all industries, followed by *Torulaspora* (0-13.1%) and *Nakaseomyces* (0-0.1%). At the species level, *Kluyveromyces lactis* (0.0-26.92% RA) was identified at all maturation points in all cheese samples, except at 0 days in industry A. On the other hand, *K. marxianus* (0.0-0.11% RA) was only detected in industries A, C and F in different maturation times (T20, T0 and T40, respectively). For *Torulaspora* genus, *T. delbrueckii* was identified in cheese samples all industries from PDO "Queso de la Serena" and only industry F from PDO "Torta del Casar". Mayor relative abundance was found in T20 (13,1%) of industry A. *Nakaseomyces* genus was identified at species level as *Candida glabrata* in industries C and F with less than 0,1% of reads. Inside *Pichiaceae* family, *Pichia fermentans*, *P. kudriavzevii*, *P. sporocuriosa* and *Candida cabralensis* were identified as species level, being *P. fermentans* the one that presented more abundance in all industries during the maturation process belonging to the genus *Pichia*. Within the *Trichomonascaceae* family, *Wickerhamiella pararugosa* presented a fairly high relative abundance (91,1%) at stage 0 in industry A, becoming part of the secondary mycobiota after 20 days in all samples.

In terms of relative abundance, the sequences of *Trichosporonaceae* and *Debaryomycetaceae* families, with 7,7% and 7,5% of raw reads respectively, were assigned to genus level as *Apriotrichum*, *Cutaneotrichosporon*, *Trichosporon* and *Debaryomyces*. The sequences assigned to *Trichosporon* genus were identified at species level as *T. asahii* and *T. coremiiforme*, being mainly detected in industries C and F. The same occurs with other species identified belonged to this family, *Apiotrichum montevideense* and *Cutaneotrichosporon jirovecii*. From *Debaryomycetaceae* family, the mayor relative abundance was related to *Debaryomyces hansenii* and *D. vindobonensis*

species, only detected in cheese samples belong to industry A and F at twenty days of ripening, decreasing their presence at the end of the process. *Meyerozyma guillermondii*, *Candida parapsilopsis* and *Candida zeylanoides* were also identified at all maturation points, finding a high number of reads assigned to *M. guillermondii* and *C. zeylanoides* in industry A in the first stages of ripening.

Subdominant fungi community consisted of 5 families that accounted for 14% of the total reads, being identified as *Sporidiobolaceae, Phaffomycetaceae, Cystobasidiaceae*, *Saccotheciaceae* and *Aspergillaceae.* A total of five genera were identified, being *Rhodotorula*, *Cyberlindnera*, *Cystobasidium, Aerobasidium* and *Penicillium*. *Rhodotorula* genus were found mainly in cheese samples belonging to industry F at T20 and T40, being *R. mucilaginosa* one of the predominant species at 40 days in cheese samples from this producer. Other species identified were *Cyberlindnera jadinii*, *Aureobasidium pullulans* and *Cystobasidium sloffiae*. This last specie was identified in industry F only after 20 days of maturation. Only one mould was identified in cheese samples, and it was identified as *Penicillium commune*. Its presence was ubiquitous in all stages of maturation of the cheeses made by industry A.

The results obtained in this study using HTS to know the autochthonous mycobiota of traditional cheeses has allowed to know the relationship between the different cheese production regions and the possibility of identifying the origin of a cheese sample based on its microbiology. This can be seen in figure 3, which represents the similarities between the industries and the possibility of knowing which sample of cheese has been made in any of them. As can be seen, no cheese sample could be predicted as unique.

Furthermore, the abundance of the five main genera identified throughout the maturation process is represented on a heatmap (figure 4), where the differences between the industries can be observed even if they are from the same cheese-making region. This indicates the microbiological variability present in raw milk and how it affects the mycobiota of the cheese during ripening.

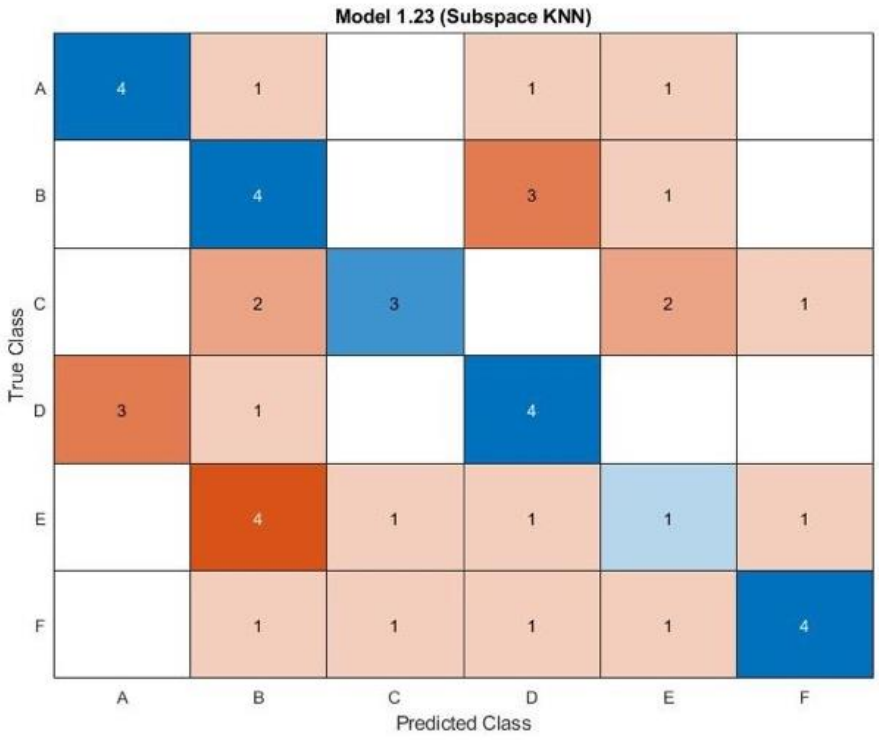


Figure 3. Prediction matrix of six dairy industries belonging to different PDO (Queso de la Serena and Torta del Casar) according to *k*-nearest neighbor (KNN) classifier.

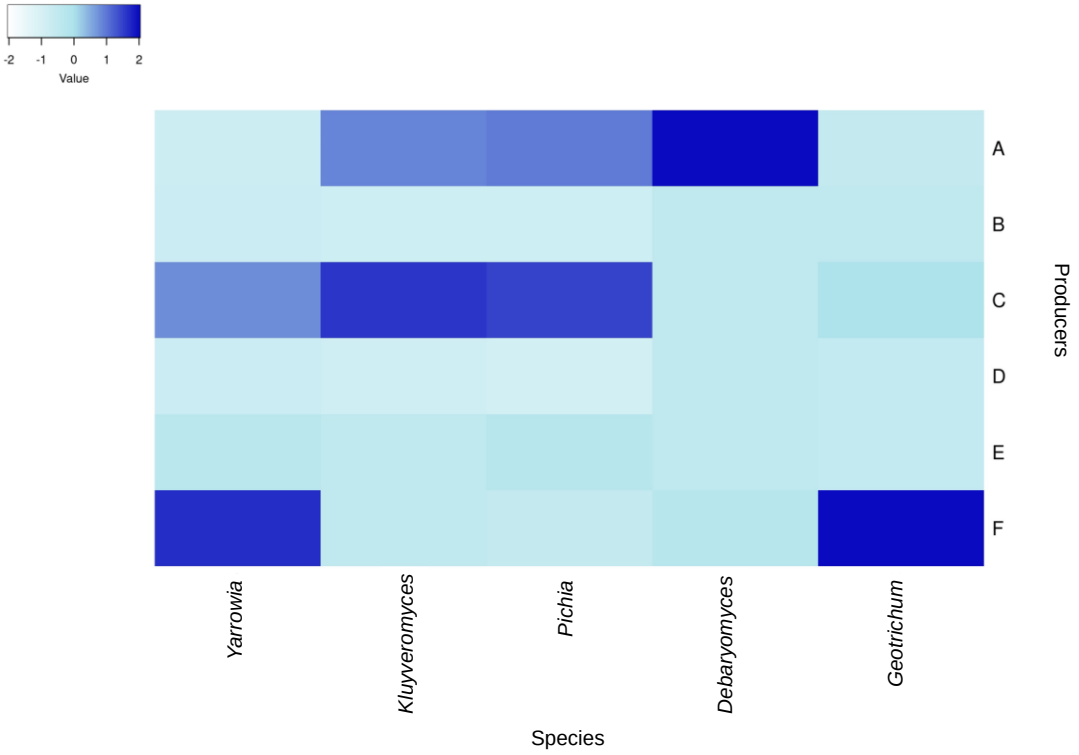


Figure 4. Heatmap showing the abundance of the five most representative genera found in cheese samples from six industries. The color scale indicates the abundance of the 5 genera and the bluer the color, the more abundant that genus is.

Alpha diversity analysis

The analysis of Shannon index takes into account mainly the species richness of cheese samples between industries and maturation stages. There were no significant differences (P>0,05) between the six industries, although significant differences (P<0.05) were found only in industry E throughout ripening process. Figure 5 shows Shannon diversity index of each cheese samples belonging to the Serena and Casar industries (A) and at the four maturation stages for each industry (B). As can be seen in fig. 5, the diversity of species is wide-ranging in all industries and in almost all maturation times, although there is a tendency for species to decreased as the maturation process progresses.

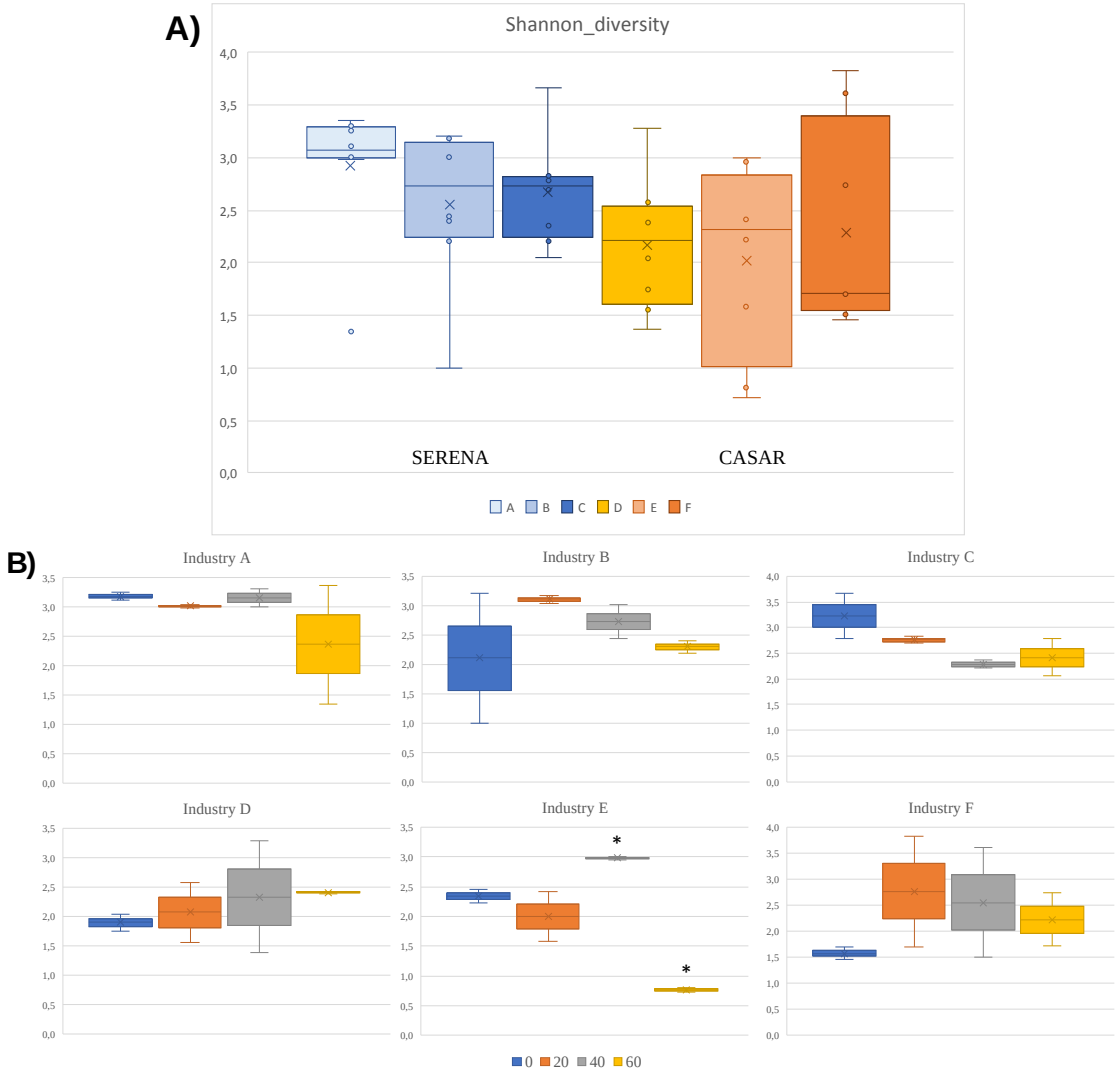


Figure 5. Boxplots describing **α**-diversity measures represented by the Shannon index for the different industries (A) and at different stages of maturation (B). Symbol * indicates significant differences (P<0.05 by Tukey's HSD post hoc test).

II.4. Discussion

In the last decade, most of the microbiome studies of traditional cheeses have focused on characterizing lactic acid bacteria and other bacteria involved in the ripening process (Alessandria et al., 2016; Castellanos-Rozo et al., 2020; Choi et al., 2020; Feutry et al., 2012; Quigley et al., 2012, Riquelme et al., 2015; Ruvalcaba-Gómez et al., 2021; Salazar et al., 2018; Zago et al., 2021). Also, fungi community of cheeses have been considered as spoilage but the origin and role of yeast in cheese matrix is not well stablished. Yeasts are widely dispersed in dairy environments and appear as frequent contaminants of raw milk and equipment surfaces of dairy facilities (Bokulich and Mills, 2013b). The presence of yeasts and molds is acceptable in certain cheeses and is considered essential in the development of the organoleptic characteristics of artisanal cheeses made from raw ewes' milk (Montel et al., 2014). The role of yeast in the cheese ripening process have been widely studied since they can ferment lactose, showing proteolytic and lipolytic activity, thus generating volatile compounds associated with the flavor of cheeses (Atanassova et al., 2016; Agarbati et al., 2021, Padilla et al., 2014). The cheese ecosystem is a complex habitat that supports the coexistence of bacteria, yeasts and molds with changes in pH, high salt concentration, lack of oxygen and low temperature in the ripening process. Numerous yeasts have been isolated from the surface and the core of various cheeses, such as *Yarrowia lipolytica*, *Kluyveromyces marxianus* and *Debaryomyces hansenii* (Gonçalves et al., 2017; Ordiales et al., 2013a; Wolfe et al., 2014). In this study, we have identified by HTS technology the fungal community of 24 cheese samples made with raw ewes' milk from two different production region of Extremadura, Spain. This technology has allowed us to know the predominance and the abundance of diverse yeast species in this type of artisan cheese, and their presence along maturation.

The predominant yeasts throughout the maturation process of the cheese samples from the Casar and Serena industries were those belonging to the *Yarrowia* and *Kluveromyces* genera. Although the species *Y. alimentaria* and *Y. lipolytica* are frequently identified in cheeses, they do not appear to be predominant during ripening (Ferreira and Viljoen, 2003; Gonçalves et al., 2017; Padilla et al., 2014). It should be noted that *Y. lipolytica* is useful in the industrial sector due to its technological properties for cheeses manufacture and other dairy products, because of its strong lipolytic and proteolytic activities (Nicaud, 2012). *Yarrowia* spp. improves the aromatic profile of cheeses due to

their amino acid catabolism and the production of high amounts of volatile sulfur compounds and short-chain ketones (Zheng et al., 2021). In contrast, certain strains have also been associated with the production of brown alterations as a consequence of pyomelanin pigment formation (Groenewald et al., 2014). As in other soft cheeses (Gonçalves et al., 2017; Ordiales et al., 2013a), *Kluyveromyces* spp. is generally involved in early steps of ripening, being also dominant in some varieties of Italian cheese (Fadda et al., 2017). This yeast is capable to ferment lactose through β-galactosidase activity (Biolcati et al., 2021), in addition to producing volatile compounds such as esters and alcohols related to the sensory characteristics of the final product (Binetti et al., 2013). Some species from *Kluyveromyces* genus, *K. lactis* and *K. marxianus* have been widely studied due to their probiotic properties, such as its resistance to gastrointestinal enzymes and tolerance to GI conditions, their coaggregation ability with pathogens and their antimicrobial activity (Andrade et al., 2021; Homayouni-Rad et al., 2020). Another yeast widely found on the surface of cheeses is *Geotrichum candidum* (*Galactomyces geotrichum*), which is also part of the raw milk mycobiota. This yeast has been previously identified by culture dependent and independent techniques in artisanal cheeses (Gonçalves et al., 2017), being predominant at the beginning of Serpa cheese ripening. It exhibits strain-specific proteolytic and lipolytic activity and it contributes to the texture and flavor of the cheese due to the production of secondary alcohols, esters and sulfur compounds (Mayo et al., 2021; Boutrou & Guéguen, 2005). In addition, this yeast contributes significantly to the cheese maturation by increasing the pH (Freitas et al., 2000), but its growth is inhibited with high concentrations of NaCl (Sacristán et al., 2012).

Debaryomyces hansenii is one of the more prevalence yeast species of cheeses (Fröhlich-Wyder et al., 2019) such as Cebreiro, Greek PDO cheeses, Gruyère, Brazilian Minas cheese, Pecorino di Filiano and Serpa, among others (Atanassova et al., 2016; Banjara et al., 2015; Capece et al., 2009; de Souza et al., 2021; Gonçalves et al., 2017; Michailidou et al., 2021). This yeast showed a greater resistant to low water activity, wide pH range, higher salt concentration and low temperature (Atanassova et al., 2016; Binetti et al., 2013; Montel et al., 2014). Species from *Debaryomyces* genus showed proteolytic and lipolytic activity, although this capacity is related to strain specific and in addition, has an important contribution to ripening and aroma profiles in different cheese types (Esen et al., 2021). In this way, some yeast strains, mainly from the *Debaryomyces, Geotrichum, Kluyveromyces* and *Yarrowia* genera are intentionally added as part of a

starter culture to improve the sensory properties of cheese (Hayaloglu, 2016; Fröhlich-Wyder et al., 2019).

It is noteworthy that the cheese ripening is highly dependent on microbial interactions and therefore, the flavor production of cheese is due to a mixture of various microorganisms (Chen et al., 2021; Carpino et al., 2017). The organoleptic characteristics of soft cheeses are often determined by the presence of some species of yeast that are part of the secondary mycobiota. Numerous authors have found a great variety within this mycobiota, made up by *Candida*, *Pichia*, *Rhodotorula*, *Torulaspora* and *Trichosporon* genera (Chen et al., 2010; Delavenne et al., 2011; Fadda et al., 2010; Larpin-Laborde et al., 2011; Lavoie et al., 2012; Montel et al., 2014). Species such as *Candida zeylanoides* and *Pichia kudriavzevii* give rise to volatile compounds such as ethanol, ethyl acetate, 3-methylbutanol and acetic acid (Zheng et al., 2018). It is worth highlighting the presence of *Aureobasidium pullulans* in this study, since it is not very common to find this species in cheeses but in vegetable matter (Rueda-Mejia et al., 2021). It may be related to the use of coagulant from the dried flowers of *Cynara cardunculus* L., used in the process of making "Torta del Casar" and "Queso de la Serena" cheeses.

In general, all cheese samples from the six industries showed the same predominant yeast genera, which suggests that the cheese-makers regions are not a determining factor in the mycobiota composition of soft cheeses, but rather the fungi composition of raw milk and the environmental conditions of the manufacture.

II.5. Conclusions

In the current study, the mycobiota profile of six Spanish PDO cheeses were analyzed using DNA metabarcoding analysis by MiSeq platform. HTS technology was applied to elucidate the mycobiota occurring during torta-type cheese production, as an alternative to culture-dependent methods. The most frequently identified fungal genera were *Yarrowia* spp., *Kluyveromyces* spp., *Pichia spp., Debaryomyces* spp. and *Geotrichum* spp. Most of the taxa identified were observed in all cheese samples, although the genus *Trichosporon* was mainly identified in the cheese samples from industries C and F. Therefore, this study provides evidence of the diversity of yeasts associated with traditional cheeses such as Queso de la Serena and Torta del Casar. Understanding the metagenomics of artisan raw ewes' milk cheeses can improve the quality and safety of

the cheeses, as well as knowing which microorganisms are involved in the biochemical changes that occur in this type of traditional products. Hence, we conclude that microbial populations within each cheese are strongly dependent on the raw material itself and affected by the microbiota present in cheese making industries. Our work takes the first step into the exploration of the microbial communities in artisan torta-type PDO cheeses by HTS technology and sets the base for the creation of a database used for meta-analysis, to create a unique identity of this type of cheese and contribute to product quality standardization and valorization.

II.6. References

Agarbati, A., Marini, E., Galli, E., Canonico, L., Ciani, M., & Comitini, F. (2021). Characterization of wild yeasts isolated from artisan dairies in the Marche region, Italy, for selection of promising functional starters. *LWT*, *139*, 110531. https://doi.org/10.1016/j.lwt.2020.110531

Alegría, Á., Álvarez-Martín, P., Sacristán, N., Fernández, E., Delgado, S., & Mayo, B. (2009). Diversity and evolution of the microbial populations during manufacture and ripening of Casín, a traditional Spanish, starter-free cheese made from cow's milk. *International Journal of Food Microbiology*, *136*(1), 44–51. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2009.09.023

Alessandria, V., Ferrocino, I., De Filippis, F., Fontana, M., Rantsiou, K., Ercolini, D., & Cocolin, L. (2016). Microbiota of an Italian Grana-like cheese during manufacture and ripening, unraveled by 16S rRNA-based approaches. *Applied and Environmental Microbiology*, *82*(13), 3988–3995. https://doi.org/10.1128/AEM.00999-16

Andrade, G. C., Andrade, R. P., Oliveira, D. R., Quintanilha, M. F., Martins, F. S., & Duarte, W. F. (2021). *Kluyveromyces lactis* and *Torulaspora delbrueckii*: Probiotic characterization, anti-*Salmonella* effect, and impact on cheese quality. *LWT*, *151*(July), 112240. https://doi.org/10.1016/j.lwt.2021.112240

Atanassova, M. R., Fernández-Otero, C., Rodríguez-Alonso, P., Fernández-No, I. C., Garabal, J. I., & Centeno, J. A. (2016). Characterization of yeasts isolated from artisanal short-ripened cows' cheeses produced in Galicia (NW Spain). *Food Microbiology*, *53*, 172–181. https://doi.org/10.1016/j.fm.2015.09.012

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J. R., Maciejewski, A., & Wishart, D. S. (2016). Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Research*, *44*(W1), W147–W153. https://doi.org/10.1093/nar/gkw419

Banjara, N., Suhr, M. J., & Hallen-Adams, H. E. (2015). Diversity of Yeast and Mold Species from a Variety of Cheese Types. *Current Microbiology*, *70*(6), 792–800. https://doi.org/10.1007/s00284-015-0790-1

Binetti, A., Carrasco, M., Reinheimer, J., & Suárez, V. (2013). Yeasts from autochthonal cheese starters: Technological and functional properties. *Journal of Applied Microbiology*, *115*(2), 434–444. https://doi.org/10.1111/jam.12228

Bintsis, T., & Papademas, P. (2002). Microbiological quality of white-brined cheeses: a review. *International Journal of Dairy Technology*, *55*(3), 113–120. https://doi.org/10.1046/j.1471-0307.2002.00054.x

Biolcati, F., Ferrocino, I., Bottero, M. T., & Dalmasso, A. (2021). Mycobiota Composition of Robiola di Roccaverano Cheese along the Production Chain.

Bokulich, N. A., & Mills, D. A. (2013a). Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Applied and Environmental Microbiology*, *79*(8), 2519–2526. https://doi.org/10.1128/AEM.03870-12

Bokulich, N. A., & Mills, D. A. (2013b). Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. *Applied and Environmental Microbiology*, *79*(17), 5214–5223. https://doi.org/10.1128/AEM.00934-13

Boutrou, R., & Guéguen, M. (2005). Interests in *Geotrichum candidum* for cheese technology. *International Journal of Food Microbiology*, *102*(1), 1–20. https://doi.org/10.1016/j.ijfoodmicro.2004.12.028

Capece, A., & Romano, P. (2009). "Pecorino di Filiano" cheese as a selective habitat for the yeast species, *Debaryomyces hansenii*. *International Journal of Food Microbiology*, *132*(2–3), 180–184. https://doi.org/10.1016/j.ijfoodmicro.2009.04.007

Carpino, S., Randazzo, C. L., Pino, A., Russo, N., Rapisarda, T., Belvedere, G., & Caggia, C. (2017). Influence of PDO Ragusano cheese biofilm microbiota on flavour compounds formation. *Food Microbiology*, *61*, 126–135. https://doi.org/10.1016/j.fm.2016.09.006

Carreira, A., Ferreira, L. M., & Loureiro, V. (2001). Production of brown tyrosine pigments by the yeast *Yarrowia lipolytica*. *Journal of Applied Microbiology*, *90*(3), 372–379. https://doi.org/10.1046/j.1365-2672.2001.01256.x

Castellanos-Rozo, J., Pulido, R. P., Grande, M. J., Lucas, R., & Gálvez, A. (2020). Analysis of the bacterial diversity of paipa cheese (A traditional raw cow's milk cheese from Colombia) by high-throughput sequencing. *Microorganisms*, *8*(2). https://doi.org/10.3390/microorganisms8020218

Ceugniez, A., Drider, D., Jacques, P., & Coucheney, F. (2015). Yeast diversity in a traditional French cheese "Tomme d'orchies" reveals infrequent and frequent species with associated benefits. *Food Microbiology*, *52*, 177–184. https://doi.org/10.1016/j.fm.2015.08.001

Chen, C., Zhou, W., Yu, H., Yuan, J., & Tian, H. (2021). Characterization of major odor-active compounds responsible for nutty flavor in Cheddar cheese according to

Chinese taste. *Flavour and Fragrance Journal*, *36*(2), 171–181. https://doi.org/10.1002/ffj.3627

Chen, L. S., Ma, Y., Maubois, J. L., Chen, L. J., Liu, Q. H., & Guo, J. P. (2010). Identifcation of yeasts from raw milk and selection for some specific antioxidant properties. *International Journal of Dairy Technology*, *63*(1), 47–54. https://doi.org/10.1111/j.1471-0307.2009.00548.x

Choi, J., In Lee, S., Rackerby, B., Frojen, R., Goddik, L., Ha, S. Do, & Park, S. H. (2020). Assessment of overall microbial community shift during Cheddar cheese production from raw milk to aging. *Applied Microbiology and Biotechnology*, *104*(14), 6249–6260. https://doi.org/10.1007/s00253-020-10651-7

De Filippis, F., Genovese, A., Ferranti, P., Gilbert, J. A., & Ercolini, D. (2016). Metatranscriptomics reveals temperature-driven functional changes in microbiome impacting cheese maturation rate. *Scientific Reports*, *6*(October 2015), 1–11. https://doi.org/10.1038/srep21871

De Filippis, F., Parente, E., & Ercolini, D. (2017). Metagenomics insights into food fermentations. *Microbial Biotechnology*, *10*(1), 91–102. https://doi.org/10.1111/1751-7915.12421

de Souza, T. P., Evangelista, S. R., Passamani, F. R. F., Bertechini, R., de Abreu, L. R., & Batista, L. R. (2021). Mycobiota of Minas artisanal cheese: Safety and quality. *International Dairy Journal*, *120*, 105085. https://doi.org/10.1016/j.idairyj.2021.105085

Delavenne, E., Mounier, J., Asmani, K., Jany, J.-L., Barbier, G., & Le Blay, G. (2011). Fungal diversity in cow, goat and ewe milk. *International Journal of Food Microbiology*, *151*(2), 247–251. https://doi.org/10.1016/j.ijfoodmicro.2011.08.029

Dugat-Bony, E., Garnier, L., Denonfoux, J., Ferreira, S., Sarthou, A. S., Bonnarme, P., & Irlinger, F. (2016). Highlighting the microbial diversity of 12 French cheese varieties. *International Journal of Food Microbiology*, *238*, 265–273. https://doi.org/10.1016/j.ijfoodmicro.2016.09.026

Esen, Y., & Çetin, B. (2021). Bacterial and yeast microbial diversity of the ripened traditional middle east surk cheese. *International Dairy Journal*, *117*, 105004. https://doi.org/10.1016/j.idairyj.2021.105004

Fadda, M. E., Viale, S., Deplano, M., Pisano, M. B., & Cosentino, S. (2010). Characterization of yeast population and molecular fingerprinting of *Candida zeylanoides* isolated from goat's milk collected in Sardinia. *International Journal of Food Microbiology*, *136*(3), 376–380. https://doi.org/10.1016/j.ijfoodmicro.2009.10.007

Fadda, M. E., Mossa, V., Deplano, M., Pisano, M. B., & Cosentino, S. (2017). In vitro screening of *Kluyveromyces* strains isolated from Fiore Sardo cheese for potential use as probiotics. *LWT - Food Science and Technology*, *75*, 100–106. https://doi.org/10.1016/j.lwt.2016.08.020

Ferreira, A. D., & Viljoen, B. C. (2003). Yeasts as adjunct starters in matured Cheddar cheese. *International Journal of Food Microbiology*, *86*(1–2), 131–140. https://doi.org/10.1016/S0168-1605(03)00252-6

Feutry, F., Oneca, M., Berthier, F., & Torre, P. (2012). Biodiversity and growth dynamics of lactic acid bacteria in artisanal PDO Ossau-Iraty cheeses made from raw ewe's milk with different starters. *Food Microbiology*, *29*(1), 33–42. https://doi.org/10.1016/j.fm.2011.08.011

Freitas, A. C., Macedo, A. C., & Malcata, F. X. (2000). Review: Technological and organoleptic issues pertaining to cheeses with denomination of origin manufactured in the Iberian Peninsula from ovine and caprine milks. *Food Science and Technology International*, *6*(5), 351–370. https://doi.org/10.1177/108201320000600502

Fröhlich-Wyder, M. T., Arias-Roth, E., & Jakob, E. (2019). Cheese yeasts. *Yeast*, *36*(3), 129–141. https://doi.org/10.1002/yea.3368

Gardini, F., Tofalo, R., Belletti, N., Iucci, L., Suzzi, G., Torriani, S., … Lanciotti, R. (2006). Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. *Food Microbiology*, *23*(7), 641–648. https://doi.org/10.1016/j.fm.2005.12.005

Gonçalves Dos Santos, M. T. P., Benito, M. J., Córdoba, M. de G., Alvarenga, N., & Ruiz-Moyano Seco de Herrera, S. (2017). Yeast community in traditional Portuguese Serpa cheese by culture-dependent and -independent DNA approaches. *International Journal of Food Microbiology*, *262*(September), 63–70. https://doi.org/10.1016/j.ijfoodmicro.2017.09.013

Gonçalves, M. T. P., Benito, M. J., Córdoba, M. de G., Egas, C., Merchán, A. V., Galván, A. I., & Ruiz-Moyano, S. (2018). Bacterial Communities in Serpa Cheese by Culture Dependent Techniques, 16S rRNA Gene Sequencing and High-throughput Sequencing Analysis. *Journal of Food Science*, *83*(5), 1333–1341. https://doi.org/10.1111/1750-3841.14141

Groenewald, M., Boekhout, T., Neuvéglise, C., Gaillardin, C., Van Dijck, P. W. M., & Wyss, M. (2014). *Yarrowia lipolytica*: Safety assessment of an oleaginous yeast with a great industrial potential. *Critical Reviews in Microbiology*, *40*(3), 187–206. https://doi.org/10.3109/1040841X.2013.770386

Hatoum, R., Labrie, S., & Fliss, I. (2012). Antimicrobial and probiotic properties of yeasts: From fundamental to novel applications. *Frontiers in Microbiology*, *3*(DEC), 1–12. https://doi.org/10.3389/fmicb.2012.00421

Hayaloglu, A. A. (2016). Cheese: Microbiology of Cheese. In *Reference Module in Food Science*. Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.00675-2

Homayouni-Rad, A., Azizi, A., Oroojzadeh, P., & Pourjafar, H. (2020). *Kluyveromyces marxianus* as a Probiotic Yeast: A Mini-review. *Current Nutrition & Food Science*, *16*(8), 1163–1169. https://doi.org/10.2174/1573401316666200217113230

Inácio, R. S., Gomes, A. M. P., & Saraiva, J. A. (2020). Serra da Estrela cheese: A review. *Journal of Food Processing and Preservation*, *44*(5), 1–10. https://doi.org/10.1111/jfpp.14412

Irlinger, F., Layec, S., Hélinck, S., & Dugat-Bony, E. (2015). Cheese rind microbial communities: Diversity, composition and origin. *FEMS Microbiology Letters*, *362*(2), 1–11. https://doi.org/10.1093/femsle/fnu015

Jacques, N., & Casaregola, S. (2008). Safety assessment of dairy microorganisms: The hemiascomycetous yeasts. *International Journal of Food Microbiology*, *126*(3), 321–326. https://doi.org/10.1016/j.ijfoodmicro.2007.08.020

Larpin-Laborde, S., Imran, M., Bonaïti, C., Bora, N., Gelsomino, R., Goerges, S., … Desmasures, N. (2011). Surface microbial consortia from Livarot, a French smear-ripened cheese. *Canadian Journal of Microbiology*, *57*(8), 651–660. https://doi.org/10.1139/w11-050

Lavoie, K., Touchette, M., St-Gelais, D., & Labrie, S. (2012). Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec. *Dairy Science & Technology*, *92*(5), 455–468. https://doi.org/10.1007/s13594-011-0051-4

Ledenbach, L. H., & Marshall, R. T. (2009). Microbiological Spoilage of Dairy Products. In *Compendium of the Microbiological Spoilage of Foods and Beverages* (pp. 41–67). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4419-0826-1_2

Marcellino, N., Beuvier, E., Grappin, R., Guéguen, M., & Benson, D. R. (2001). Diversity of Geotrichum candidum Strains Isolated from Traditional Cheesemaking Fabrications in France. *Applied and Environmental Microbiology*, *67*(10), 4752–4759. https://doi.org/10.1128/AEM.67.10.4752-4759.2001

Martín-Platero, A. M., Valdivia, E., Maqueda, M., Martín-Sánchez, I., & Martínez-Bueno, M. (2008). Polyphasic approach to bacterial dynamics during the ripening of Spanish farmhouse cheese, using culture-dependent and -independent methods. *Applied and Environmental Microbiology*, *74*(18), 5662–5673. https://doi.org/10.1128/AEM.00418-08

Mayo, B., Rodríguez, J., Vázquez, L., & Flórez, A. B. (2021). Microbial Interactions within the Cheese Ecosystem and Their Application to Improve Quality and Safety. *Foods*, *10*(3), 602. https://doi.org/10.3390/foods10030602

Mello, A., Napoli, C., Murat, C., Morin, E., Marceddu, G. & Bonfante, P. (2011) ITS-1 versus ITS-2 pyrosequencing: a comparison of fungal populations in truffle grounds, *Mycologia, 103*:6, 1184-1193, doi: 10.3852/11-027

Michailidou, S., Pavlou, E., Pasentsis, K., Rhoades, J., Likotrafiti, E., & Argiriou, A. (2021). Microbial profiles of Greek PDO cheeses assessed with amplicon metabarcoding. *Food Microbiology*, *99*(May), 103836. https://doi.org/10.1016/j.fm.2021.103836

Montel, M. C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D. A., Desmasures, N., & Berthier, F. (2014). Traditional cheeses: Rich and diverse microbiota with associated benefits. *International Journal of Food Microbiology*, *177*, 136–154. https://doi.org/10.1016/j.ijfoodmicro.2014.02.019

Mota-Gutierrez, J., Ferrocino, I., Rantsiou, K., & Cocolin, L. (2019). Metataxonomic comparison between internal transcribed spacer and 26S ribosomal large subunit (LSU) rDNA gene. *International Journal of Food Microbiology*, *290*(October 2018), 132–140. https://doi.org/10.1016/j.ijfoodmicro.2018.10.010

Nicaud, J.-M. (2012). Yarrowia lipolytica. *Yeast*, *29*(10), 409–418. https://doi.org/10.1002/yea.2921

Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. https://bioinfogp.cnb.csic.es/tools/venny/index.html)

Ordiales, E., Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J., & de Guía Córdoba, M. (2013a). Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. *Food Control*, *33*(2), 448–454. https://doi.org/10.1016/j.foodcont.2013.03.027

Ordiales, E., Martín, A., Benito, M. J., Hernández, A., Ruiz-Moyano, S., & Córdoba, M. de G. (2013b). Role of the microbial population on the flavor of the soft-bodied cheese Torta del Casar. *Journal of Dairy Science*, *96*(9), 5477–5486. https://doi.org/10.3168/jds.2013-6587

Padilla, B., Belloch, C., López-Díez, J. J., Flores, M., & Manzanares, P. (2014). Potential impact of dairy yeasts on the typical flavour of traditional ewes' and goats' cheeses. *International Dairy Journal*, *35*(2), 122–129. https://doi.org/10.1016/j.idairyj.2013.11.002

Pereira, C. I., Graça, J. A., Ogando, N. S., Gomes, A. M. P., & Xavier Malcata, F. (2010). Influence of bacterial dynamics upon the final characteristics of model Portuguese traditional cheeses. *Food Microbiology*, *27*(3), 339–346. https://doi.org/10.1016/j.fm.2009.10.012

Pintado, A. I. E., Pinho, O., Ferreira, I. M. P. L. V. O., Pintado, M. M. E., Gomes, A. M. P., & Malcata, F. X. (2008). Microbiological, biochemical and biogenic amine profiles of Terrincho cheese manufactured in several dairy farms. *International Dairy Journal*, *18*(6), 631–640. https://doi.org/10.1016/j.idairyj.2007.11.021

Quigley, L., O'Sullivan, O., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2012). High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. *Applied and Environmental Microbiology*, *78*(16), 5717–5723. https://doi.org/10.1128/AEM.00918-12

Regulation (CE) 1107/1996 of European Commission of 12 of June: Registration of the protected designation of origin "Queso de la Serena" in the register of protected designations of origin and the protected geographic indications.

Regulation (CE) 1491/2003 of European Commission of 25 of August: Registration of the protected designation of origin "Torta del Casar" in the register of protected designations of origin and the protected geographic indications.

Riquelme, C., Câmara, S., Enes Dapkevicius, M. de L. N., Vinuesa, P., da Silva, C. C. G., Malcata, F. X., & Rego, O. A. (2015). Characterization of the bacterial biodiversity in Pico cheese (an artisanal Azorean food). *International Journal of Food Microbiology*, *192*, 86–94. https://doi.org/10.1016/j.ijfoodmicro.2014.09.031

Rueda-Mejia, M. P., Nägeli, L., Lutz, S., Hayes, R. D., Varadarajan, A. R., Grigoriev, I. V., … Freimoser, F. M. (2021). Genome, transcriptome and secretome analyses of the antagonistic, yeast-like fungus Aureobasidium pullulans to identify potential biocontrol genes. *Microbial Cell*, *8*(8), 184–202. https://doi.org/10.15698/mic2021.08.757

Ruvalcaba-Gómez, J. M., Delgado-Macuil, R. J., Zelaya-Molina, L. X., Maya-Lucas, O., Ruesga-Gutiérrez, E., Anaya-Esparza, L. M., … Arteaga-Garibay, R. I. (2021). Bacterial succession through the artisanal process and seasonal effects defining bacterial communities of raw-milk adobera cheese revealed by high throughput dna sequencing. *Microorganisms*, *9*(1), 1–18. https://doi.org/10.3390/microorganisms9010024

Sacristán, N., González, L., Castro, J. M., Fresno, J. M., & Tornadijo, M. E. (2012). Technological characterization of *Geotrichum candidum* strains isolated from a traditional Spanish goats' milk cheese. *Food Microbiology*, *30*(1), 260–266. https://doi.org/10.1016/j.fm.2011.10.003

Salazar, J. K., Carstens, C. K., Ramachandran, P., Shazer, A. G., Narula, S. S., Reed, E., … Schill, K. M. (2018). Metagenomics of pasteurized and unpasteurized gouda cheese using targeted 16S rDNA sequencing. *BMC Microbiology*, *18*(1), 1–13. https://doi.org/10.1186/s12866-018-1323-4

Sánchez-Juanes, F., Teixeira-Martín, V., González-Buitrago, J. M., Velázquez, E., & Flores-Félix, J. D. (2020). Identification of Species and Subspecies of Lactic Acid Bacteria Present in Spanish Cheeses Type "Torta" by MALDI-TOF MS and pheS gene Analyses. *Microorganisms*, *8*(2), 301. https://doi.org/10.3390/microorganisms8020301

Tofalo, R., Fasoli, G., Schirone, M., Perpetuini, G., Pepe, A., Corsetti, A., & Suzzi, G. (2014). The predominance, biodiversity and biotechnological properties of *Kluyveromyces marxianus* in the production of Pecorino di Farindola cheese. *International Journal of Food Microbiology*, *187*, 41–49. https://doi.org/10.1016/j.ijfoodmicro.2014.06.029

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, 18(1), 315-322.

Wolfe, B. E., Button, J. E., Santarelli, M., & Dutton, R. J. (2014). Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell*, *158*(2), 422–433. https://doi.org/10.1016/j.cell.2014.05.041

Yeluri Jonnala, B. R., McSweeney, P. L. H., Sheehan, J. J., & Cotter, P. D. (2018). Sequencing of the Cheese Microbiome and Its Relevance to Industry. *Frontiers in Microbiology*, *9*(May), 1020. https://doi.org/10.3389/fmicb.2018.01020

Zago, M., Bardelli, T., Rossetti, L., Nazzicari, N., Carminati, D., Galli, A., & Giraffa, G. (2021). Evaluation of bacterial communities of Grana Padano cheese by DNA metabarcoding and DNA fingerprinting analysis. *Food Microbiology*, *93*, 103613. https://doi.org/10.1016/j.fm.2020.103613

Zheng, X., Shi, X., & Wang, B. (2021). A Review on the General Cheese Processing Technology, Flavor Biochemical Pathways and the Influence of Yeasts in Cheese. *Frontiers in Microbiology*, *12*(July). https://doi.org/10.3389/fmicb.2021.703284

CAPÍTULO III

Characterization of autochthonal *Enterobacteriaceae* isolated from Spanish soft raw ewe's milk PDO cheeses for technological application.

Capítulo III.

Characterization of autochthonal *Enterobacteriaceae* isolated from Spanish soft raw ewe's milk PDO cheeses for technological application.

Abstract

The enterobacteria involved in the ripening process of artisanal soft raw ewe's milk PDO cheeses produced in Extremadura (Spain), 'Torta del Casar' and 'Queso de la Serena', were isolated throughout their ripening process, strain-typing, security testing and characterized for some important technological properties. A total of 485 entobacteria isolates were obtained and identified by RAPD-PCR with primer M13 and subsequent sequencing of the 16S rRNA gene. Seventeen different species were detected in both PDO cheeses throughout the ripening. *Enterobacter and Hafnia* were the predominant genera, and *H. alvei* and *H. paralvei* were the predominant species at the end of the ripening process of both PDO cheeses. A total of 55 *Hafnia* spp. strains were selected for these issues according to their genetic profile and origin. After the evaluation of the safety properties involving antibiotic susceptibility and resistance, virulence genes, biogenic amine production, hemolytic activity and cytotoxic activity of *Hafnia* isolates, a total of 22 strains were selected to evaluate their technological characteristics. After the evaluation of the growth at the stress conditions of cheese ripening, their proteolytic and lipolytic activity, gas production, alkalinizing and acidifying capacity of the strains were determined. Consequently, four strains, 3 *H. alvei* 544, 970 and 1142, and 1 *H. paralvei* 1414, showed the best characteristics mainly due to their proteolytic activity at refrigeration temperatures. These strains presented considerably slower growth at the cheese processing condition corresponding to the first stage of ripening. The technological activities of these strains were considered mainly in terms of proteolytic activity at low temperatures. *H. alvei* 544 was also selected for not being a gas producer although this strain required more time to show proteolytic activity at low temperatures. Thus, *Hafnia alvei* strains (544, 970 and 1142) and *H. paralvei* (1414) were proposed due to their technological and safety characteristics for possible use as a culture in soft cheeses.

III.1. Introduction

Traditional cheeses under the Protected Designation of Origin (PDO) are defined as products made from raw milk with little or no prior processing and produced in a defined geographical area using specific knowledge and skills (Montel et al, 2014). 'Torta del Casar' and 'Queso de la Serena' are traditional soft cheeses produced in Extremadura (Spain) under the PDO using an aqueous infusion of the dried flowers from the plant *Cynara cardunculus* L. as rennet and without the addition of a starter culture and thermal treatment which lead to diverse and heterogeneous microbiota. This is mainly composed by lactic acid bacteria (LAB) and at lesser extend by Gram-positive catalase-positive, diverse Gram-negative bacteria (*Pseudomonas* spp., *Enterobacteriaceae*), and yeasts (Del pozo et al., 1988; Ordiales et al., 2013a; Sánchez-Juanes et al., 2020). Their distinctive and complex flavour and spreadable texture, which make them highly appreciated by consumers, are consequence of biochemical events that take place during ripening, resulting from the high proteolytic activity from vegetable rennet and enzymatic activity from autochthonous microbiota (Delgado et al. 2010a,b; Ordiales et al. 2013b, 2014). However, although the hygiene in cheesemaking process of these PDO cheeses have improved in the last decades, the use of raw milk does not guarantee the absence of foodborne pathogens such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*, with the potential safety risk, and spoilage microorganisms, which may cause significant important economic losses (Yoon et al., 2016). In addition, the lack of standardization of the vegetable rennet and raw milk as well as variations in the cheesemaking process between dairies impact on sensory characteristics and lead to variability and heterogeneity of the final product (Montel et al., 2014).

Today, although the legislation of these PDO cheeses do not allow the use of a starter culture, implementation of the use of an autochthonous starter culture in traditional cheese manufacture may constitute a possible way to ensure an adequate processing and promote a consistent quality in the final product, reducing variability and keeping the unique sensory characteristics as well as minimizing safety risks (Montel et al., 2014; Bassi, Puglisi, & Cocconcelli, 2015; Araújo-Rodrigues et al., 2020). This approach has demonstrated to be suitable for generating similar sensory characteristics to raw milk cheeses when they are manufactured with pasteurized milk (Centeno et al., 2017; Vázquez-Velázquez et al., 2018). In addition to guarantee the homogeneity and safety of these PDO cheeses retaining their authenticity, the application of an autochthonous starter

cultures 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 by a mixture of microorganisms isolated from the original product, representative of the main microbial groups involved in the cheese ripening of these PDO cheeses and to be well adapted to their cheesemaking process. In addition, they have 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 activities extremely relevant for the development of the aroma and texture (Leroy & De Vuyst, 2004; Hantsis-Zacharov & Halpern, 2007; 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 cheeses production (Garabal et al., 2008; Terzić-Vidojević et al., 2015; Campagnollo et al, 2018; Araújo-Rodrigues et al., 2021), however, there are not many research studies available regarding secondary microbiota selection such as *Enterobacteriaceae*. Gram-negative bacteria are quite common in traditional cheeses and constitute a reservoir of enzymes that favor the maturation of cheeses (Morales et al., 2003; 2005), contributing to the sensory characteristics of the final product.

Among the enterobacteria, *Hafnia* spp. has been proposed as tolerant to refrigeration conditions, consequently growing during cheese processing and storage (Martin et al., 2006). In addition, it has been proven for many researchers that *Hafnia* spp. is predominant at the end of the ripening process of traditional cheeses (Macedo et al. 1995; Tornadijo et al. 2001; Irlinger et al. 2012; Gonçalves et al., 2018). *Hafnia alvei* and *Hafnia paralvei* are able to produce flavor compounds (Morales et al., 2004), but can also lead to stronger proteolysis, thus modifying the final texture of the cheese and improving cheese flavor by producing sulfur aromatic compounds (Morales et al., 2003; Irlinger et al. 2012). Moreover, this species is the only Gram-negative bacterium used as a commercial ripening adjunct culture for cheesemaking (Bourdichon et al., 2012). Even, according to Delbes-Paus et al. (2013), *H. alvei* appears to be a promising strain for reducing the growth of *E. coli* O26:H11 and control the growth of antibiotic-resistant *Enterococcus faecalis* in cheese. However, they are considered as an opportunistic pathogen of humans and indicators of poor hygiene being isolated from spoiled food (Tan

et al., 2014), even there are some sporadic cases in soft cheese with early blowing due to gas production (Tabla et al., 2016). Therefore, it is essential to develop serious identification and characterization studies to understand their real impact on cheese quality, by selecting suitable *Hafnia* spp. strains adapted to the ripening conditions, with adequate technological properties such as proteolytic and lipolytic activities, avoiding the 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 enterobacteria microbiota present in artisanal soft raw ewe's milk PDO cheeses produced in Extremadura (Spain) in order to select and study their safety and technological characteristics for their cheesemaking application as member of an autochthonal starter culture.

III.2. Materials and methods

Cheese sampling and bacterial counts

Samples were taken during cheese ripening at 0, 20, 40 and 60 days from six different dairy industries located in the geographical area of production. Three industries belonged to PDO 'Queso de la Serena' and the other three from PDO 'Torta del Casar'. Three cheese units of each batch were randomly taken and transport under refrigerated conditions to the laboratory. Each microbiological analysis was performed in three different cheeses by industry and sample time, making each determination in duplicate

For microbial counts and enterobacteria isolates, 10 g of each cheese sample was taken aseptically from the core and placed into a sterile plastic pouch with 90 mL of 1% peptone water (Condalab, Madrid, Spain), and homogenized for 120 s in a Stomacher instrument (Lab-Blender 400, Seward, London, United Kingdom). Serial ten-fold dilutions were prepared from the cheese homogenates and inoculated onto agar plates. Mesophilic aerobic bacteria were counted on plate count agar (PCA; Condalab) after incubation at 30 °C for 48 h and enterobacteria on violet red bile glucose agar (VRBG; Condalab) incubated at 30 °C for 24 h. Ten enterobacteria 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) at 30 °C for 24 h and stored at −80 °C in 25% glycerol. Cheese samples were taken during the ripening period from six different dairy industries (Three

industries belonged to PDO ‘Queso de la Serena’ and the other three to PDO ‘Torta del Casar’) in Extremadura (Spain) at 0, 20, 40 days and final product, around 60 days. Samples consist in three cheeses randomly taken that were transported under refrigerated conditions to the facility of Agricultural Engineering School at University of Extremadura for analysis. Microbiological analyses were performed in three different cheeses by industry and sample time, making each determination in duplicate.

Enterobacteria identification

The enterobacteria colonies isolated were identified by Randomly amplified polymorphic DNA-polymerase chain reaction fingerprint analysis RAPD-PCR analysis 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 homogeniser (SPEX SamplePrep, Metuchen, NJ) at 1500 rpm for 2 min. Next, the DNA was extracted using a GeneJET Genomic DNA Purification Kit following manufacture instructions (Thermo Fisher Scientific, USA). The quality and concentration of DNA was determined by 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 the 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 region using 337F and 1492R primers targeting V3-V8 regions. The PCR products were purified with a GeneJET PCR Purification Kit (Thermo Fisher Scientific), sequenced by the Facility of Applied Bioscience Techniques (STAB) at the University of Extremadura (Badajoz, Spain) and edited with BioEdit software v7.2.5 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The sequences were compared with

the EMBL/GenBank database, using the BLAST algorithm. The taxonomic isolate identification was confirmed, based on the highest identity score (highest sequence homology). To differentiate between Hafnia spp. isolates at species level, a phylogenetic analysis based on 16S rRNA sequences from *Hafnia* spp. OTUs were performed using the Molecular Evolutionary Genetic Analysis (MEGA) software. The 16S rRNA sequences from GenBank of *H. alvei* ATCC 13337 (accession nº: NR_044729.2) and *H. paralvei* ATCC 29927 (accession nº: NR_116898.1) were used as references.

Security tests

Hafnia alvei and *H. paralvei* isolates from different OTUs or belong to the same OTU but from different industry origin were selected for safety characterization (Table S1). Prior to the security and technological assays, all selected *Hafnia* spp. isolates were subcultivated twice in brain heart infusion BHI broth (Condalab) at 30 °C for 24 h, washed twice with phosphate buffer saline (PBS) at pH 7.2 (Thermo Fisher Scientific) and turbidity adjusted with PBS to a 0.5 McFarland standard. All experiments were carried out at least twice in separates days in triplicates.

Antibiotic susceptibility and antibiotic resistance and virulence-associated genes determination

Antibiotic susceptibility testing was performed by disk diffusion assay in Mueller-Hinton (MH) agar (Condalab) against 33 antibiotics (BD, New Jersey, EEUU; Table S2) according to the guidelines for Enterobacteriaceae of Clinical and Laboratory Standards Institute (CSLI, 2019) and European Committee on Antimicrobial Susceptibility Testing (EUCAST). Sterile discs were placed on the surface of inoculated plates and incubated at 30ºC for 18 h. The diameters of the inhibition zones were measured and interpreted according to guidelines (CSLI, 2019). Escherichia coli ATCC 25922 was used as control. In addition, Hafnia spp. isolates were tested for the presence of different antibiotic resistance genes. AmpC cephalosporinase, blaACC-1, and blaOXA-48 genes were determined for β-lactamase presence according to the primers and conditions proposed by Abbott et al. (2011), Hasman et al. (2005) and Poriel et al. (2011), respectively. For colistin resistance, a multiplex PCR for detection mcr genes (mcr-1 to mcr-5) were determined followed the protocol described by Rebelo et al. (2018). Additional mcr

genes, mcr-6.1, mcr-7.1 and mcr-8 were 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 gene (attaching and effacing gene) by PCR using the primers C1E and C2E and conditions suggested by Ismaili et al. (1996). Reference strain *Escherichia coli* CECT 4267 was used as positive control and Staphylococcus aureus CECT 469 as negative control. Cytolethal distending toxin (CDT) toxin was determined by primers for cdtB subunit as previously described Bang et al. (2001).

Biogenic amine production

The capacity to produces biogenic amines (BA) of selected *Hafnia* spp. were carried out following the methodology described by Bover-Cid and Holzapfel (1999) with modifications. *Hafnia* spp. selected isolates were grown in Nutrient Standard Broth (Pronadisa, Spain) supplemented with 0.1% of the BA precursor amino acids and 0.005% of pyridoxal-5-phosphate at 30ºC for 24 h, subculturing them 5 times with the aim of inducing the amino acid decarboxylase activity. Subsequently, they were cultivated in the improved descarboxylase medium without agar containing 0.25% of each precursor amino acid. After incubating at 30ºC for 4 days, the amount and type of BA produced (tyramine, tryptamine, histamine, putrescine, phenylethylamine, spermine, spermidine and cadaverine; Sigma Chemical Co. St Louis, MO, USA) was measured by HLPC-ESI-MS-according to the method described by Ruiz-Moyano et al. (2019) on an Agilent series 1100 apparatus (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent series 6100 Series Single Quad LC/MS (Agilent Technologies) with a multimode source operating in electrospray ionisation mode.

Hemolytic activity

Blood agar (BA, VWR) supplemented with 5% of defibrinated sheep blood (v/v) (Oxoid, Basingstoke, United Kingdom) was used to α-, β- and γ- hemolytic activity test. Hemolysis was determined by incubate inoculated plates at 30ºC for 24 hours. The hemolytic reaction was recorded by observation of a partial hydrolysis of red blood cells and greening zone (α-hemolysis), clear zone around bacterial growth (β-hemolysis), and no reaction (γ-haemolysis). *Listeria monocytogenes* CECT 911 and *Lactobacillus casei*

HL233 (Ruiz-Moyano et al., 2009) were used as β-hemolysis and α-haemolysis positive controls, respectively.

Cytotoxic activity of Hafnia isolates

Toxin induction of Hafnia spp. selected isolates were conducted with mitomycin C (Thermo Fisher Scientific) as previously described To and Bhunia (2019). *E. coli* CECT 4267 and CECT 4782, shiga toxin positive strains, and *E. coli* UEX 3193, no 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 37ºC for 18 h in an orbital shaker (New Brunswick™ Excella ® E24/E24R; Eppendorf, Hamburg, Germany) set at 150 rpm. After, the cultures were diluted to 1:50 in 10 mL of Luria Bertani broth (LB; Condalab). All cultures were incubated for 3 h at 37 ºC with shaking at 150 rpm before the addition of mitomycin C (2 µg/mL), follow by incubation for 5 h at 37 ºC 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 -80ºC until used.

The cytotoxic activity of the supernatants was evaluated in Hela cervical cancer cell line. Cells were culture in Dulbecco's Modified Eagle's Medium high glucose, pyruvate, no glutamine (DMEM; Gibco-Thermo Fisher Scientific),supplemented with 10% Fetal Bovine Serum heat inactivated (FBS; Gibco-Thermo Fisher Scientific), 1% L-glutamine 200 mM (Gibco-Thermo Fisher Scientific) and antibiotics including Penicillin 100 IU/mL and Streptomycin 100 µg/mL (Gibco-Thermo Fisher Scientific). Cells were maintained in 75 cm2 tissue culture flasks at 37 °C in atmosphere containing 5% CO2. After trypsinization, cells were seeded into well (8x103 cells per well) of a 96-well flat-bottomed microtiter plate and grown for 24h. After, media was replaced with new media without antibiotic and supplemented at 1:4 dilution of supernatants. Cell survival was determined by the microculture tetrazolium test (MTT) at 24 h after addition the supernatants according to Fernández et al. (2016). Cells treated with LB broth served as control of medium activity.

Characterization of technological properties.

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

Evaluation of growth at cheese ripening conditions.

Box–Behnken experimental design (BBD) with 3 factors, temperature, salt concentration and pH, was applied for modelling the influence of their on the growth of the *Hafnia* spp. isolates. Factors were set-up at parameters showed in Table S3 in order to cover the conditions of the ripening process of soft cheeses 'Torta del Casar' and 'Queso de la Serena' (Martínez et al., 2011; Tabla et al., 2015). Yeast nitrogen base broth (YNB; Condalab) supplemented with 1% (w/v) of glucose was used for the experiment. The pH of the YNB medium was adjusted with lactic acid solution (10%) or NaOH 1 M. The YNB medium was sterilized by filtration through 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% (v/v) from a suspension of tested *Hafnia* spp. The microplates were incubated at different temperatures ranging from 10ºC to 30ºC (Table S3) for 4 days. The ability of each *Hafnia* spp. 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, Offenburg, Germany). The time to detection (TTD) of growth for OD_{570nm}=0.4 for each well was calculated obtained 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 isolate.

Extracellular proteolytic and lipolytic activity.

Proteolytic activity was determined by skim milk media composed of 5% reconstituted skim milk powder and 1.5% bacteriological agar (Scharlab, Barcelona-Spain). The medium was sterilized by autoclaving at 115ºC for 5 min and then poured onto Petri dishes. The medium was superficially spotted with 10 μL of each isolate. Plates were incubated at 8, 15 and 30ºC and examined daily for six days. 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 on pork fat-agar, as reported by Chaves-López et al. (2006). Media was prepared with 1% yeast extract, 1% peptone, 0.5% sodium chloride and 1.5% bacteriological agar. pH was adjusted to 7 with NaOH 2 M and sterilized at 121ºC for 15 min. Pork fat was pasteurized at 100ºC for 5 min and was added to the media to a final concentration of 5% after sterilization and poured onto Petri dishes. The medium was superficially spotted with 10 µL of each isolate and incubated at 8,15 and 30ºC for six days assessing the activity every 2 days. The lipolysis was evidenced by a green-blue colour around colonies, after pouring in the plates 10 mL of a saturated copper sulphate (CuSO4) solution for 15 min. Also, lipase activity was determined by Rhodamin 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 and 30ºC and examined daily for six days.

Gas production

Gas production was tested by two methods described by Tabla et al. (2016) with some modifications. One hundred microliters of each culture were 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 hours at 30ºC. On the other hand, inverted Durham tubes were used to check out gas production from isolates, using Lauryl sulphate broth inoculated with 100 µL of each culture. Gas trapped in Durham tubes was measured after 48 hours at 30ºC.

Alkalizing and acidifying activity.

The ability of alkalizing was determined by Simmons citrate agar (Condalab). Medium was spotted with 10 µL of each isolate and incubated for 48 h at 30ºC. Alkalizing activity was observed when Simmons citrate agar turned into blue color around colonies. Acidifying activity was determined in sterile reconstituted skimmed milk (10%), inoculated with 100 µL of each isolate and incubated at 30ºC for 48 h. The pH was measured using a Crison pH meter mod. Basic 20 (2012) (Crison Instruments, Barcelona, Spain) at 24 and 48 h.

Diacetyl-acetoin production.

Acetoin production was detected by the Voges-Proskauer reaction. One hundred microliters of each Isolate were inoculated in sterile reconstituted skimmed milk (10%) at 30ºC for 18 h. According to Speckman and Collins (1982), 2.5 mL of 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 vortex for 30 s and was incubated for 15 minutes. Acetoin production was detected as a red ring on the surface of the culture (VP+phenotype). *Enterococcus clocae* and *Proteus mirabilis* from the Department Animal Production and Food Science (UEX) culture collection were used as positive and negative control, respectively.

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 it is show in the Table S3, a three factor, three-level BBD combining with Surface Response Methodology (SRM) were applied to determine the effects of medium conditions on the *Hafnia* spp. safety selected isolates growth. SRM was performed employing the 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 models were used to estimate the growth time of each isolate at the ripening conditions of the 'Torta del Casar' and 'Queso de la Serena' (Table S3). The software also generated an analysis of variance (ANOVA), establishing the statistical significance at the 95% of confidence level. The optimal level

for growth time of each variable analyzed were also obtained with the same statistical program.

In addition, the growth of the safety selected *Hafnia* spp. isolates at cheese ripening conditions and technological characteristics were evaluated by hierarchical cluster analysis (HCA). SPSS software for Windows 21.0 (SPSS Inc., Chicago, IL, USA) was used.

III.3. Results

Bacterial counts and identification

As for the results of bacterial counts in the cheese samples, figure 1 shows the counts of aerobic mesophilic bacteria (A) and enterobacteria (B). The aerobic mesophilic bacteria started at counts between 6.2 and 7.3 log cfu/g at 0 days, reach in general maximum levels, around 9 log cfu/g, at 20 days and decrease slightly until 60 days with final values between 8.5 and 8.8 log cfu/g. No significant differences were observed at the end of ripening between PDO and seasons.

As for the count of enterobacteria, they started at counts between 5.1 and 6 Log cfu/g at 0 days to reach the maximum counts at 20 days of ripening, around 7 log cfu/g, and drop significantly at the end of ripening to value around 6 or 5 log cfu/g depending on the PDO and season. Interestingly, winter season showed significantly lower counts at 40 days in both PDO, however, this difference was only retained at 60 days in the Serena PDO.

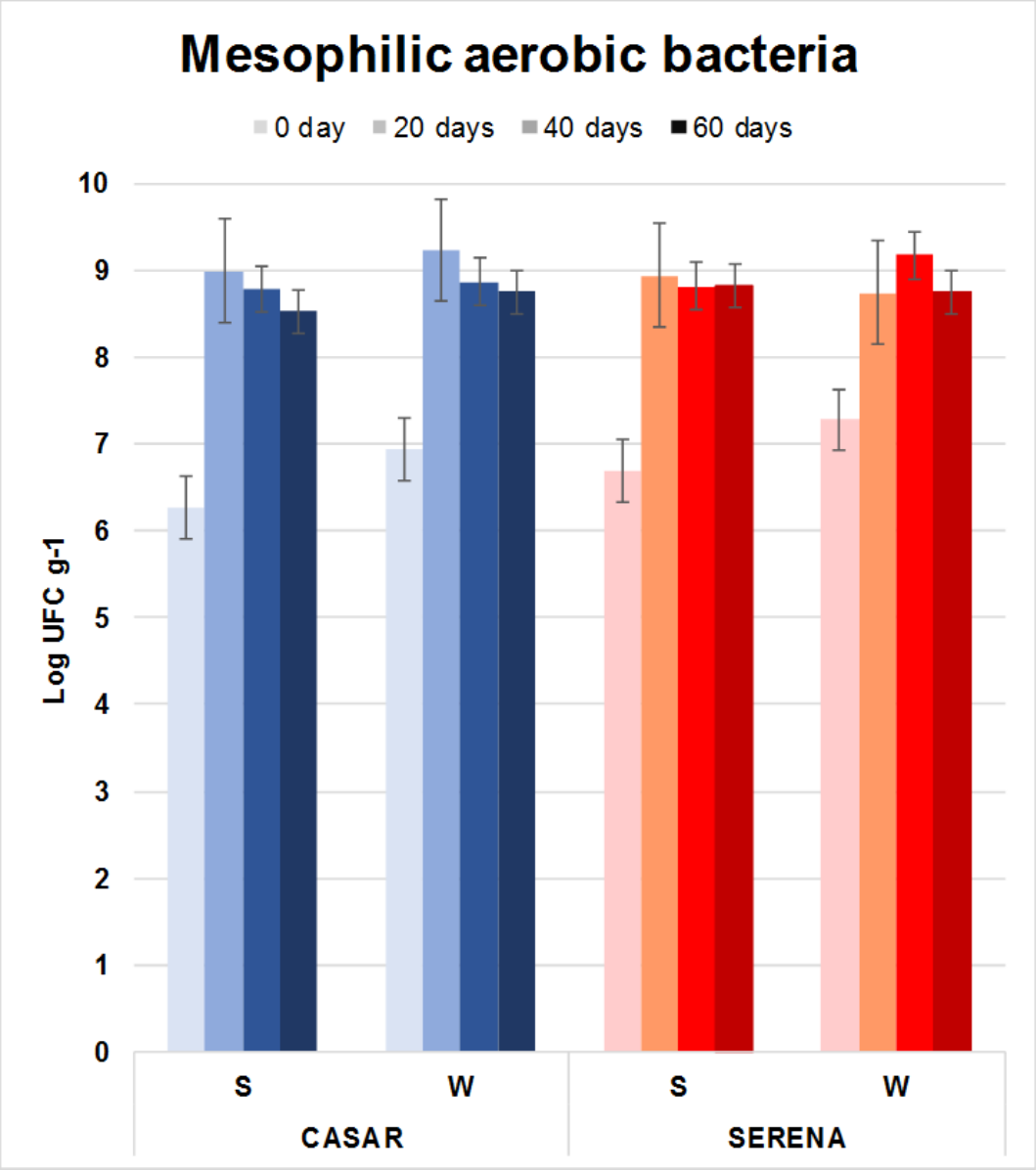


Figure 1A. Evolution of mesophilic aerobic bacteria counts in soft body cheese from PDO "Torta del Casar (Casar)" and "Queso de la Serena (Serena) from two seasons (Winter (W) and spring (S)) throughout ripening (0, 20, 40 and 60 days). Results are expressed as mean of log cfu/g ± error bar. Error bars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

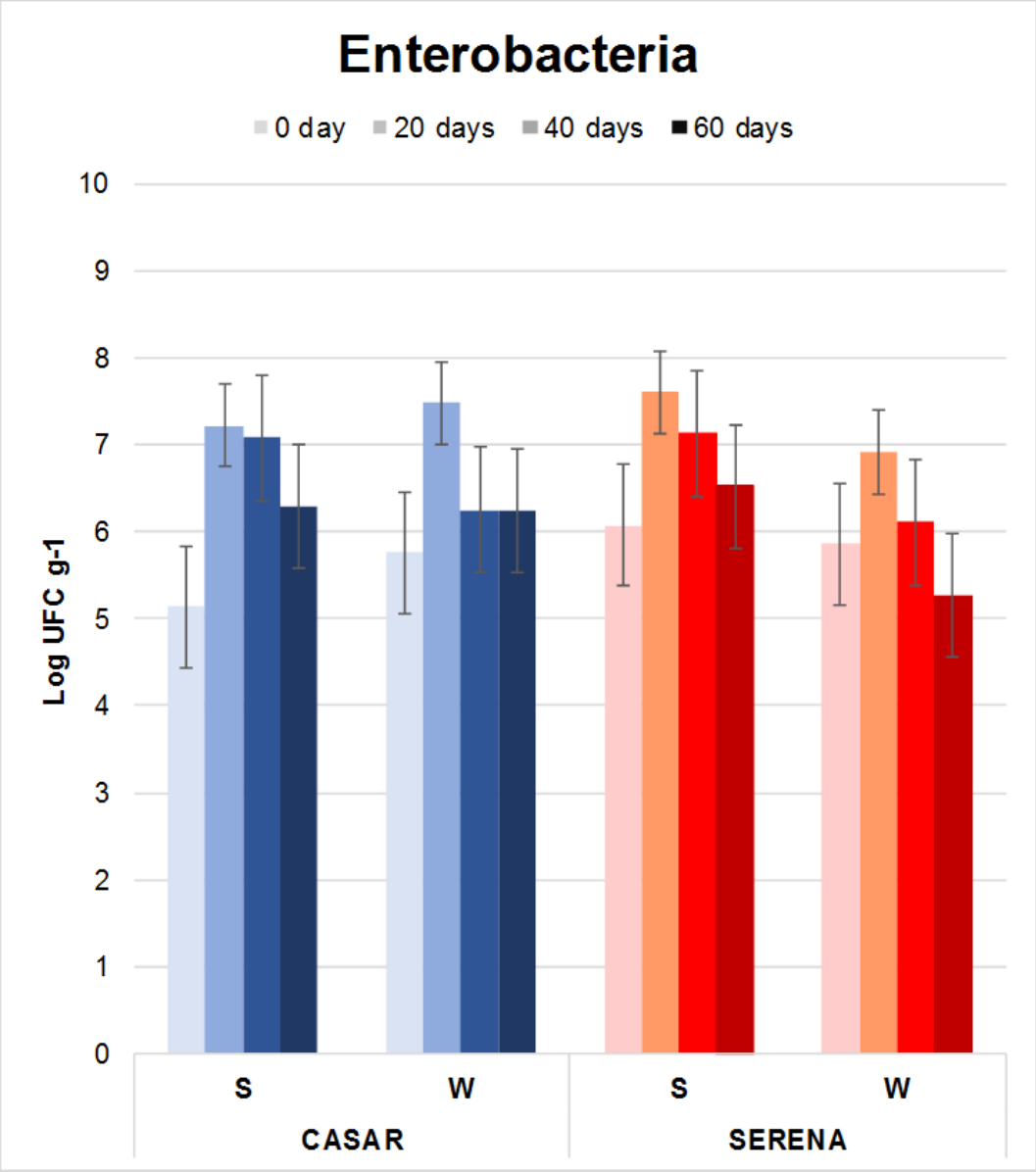


Figure 1B. Evolution of enterobacteria counts in soft body cheese from PDO "Torta del Casar (Casar)" and "Queso de la Serena (Serena) from two seasons (Winter (W) and spring (S)) throughout ripening (0, 20, 40 and 60 days). Results are expressed as mean of log cfu/g ± error bar. Error bars reflect the 95% confidence Intervals of the differences, adjusted for multiple comparisons using Bonferroni correction.

A total of 485 isolates of enterobacteria were obtained and identified by the RAPD PCR technique using the M13 minisatellite and subsequent 16S rRNA sequencing. Seventeen different species were detected in both PDO cheeses throughout the ripening (Figure 2). Figure 2A shows the microbiological diversity of enterobacteria in “Torta del Casar” cheeses at the beginning of the ripening process with 15 different species, dominated by the genus *Enterobacter* with 54% of the identifications. After 20 days, the predominant species were *Hafnia alvei* and *H. paralvei*, reaching 90.9% of the isolates at 20 days, and then decreasing to 70.8% at 40 days and 69% at 60 days. Figure 2B shows the results in the PDO "Queso de la Serena". Likewise, a great diversity in the enterobacterial population was observed at the beginning of the ripening process with 12 different species, with the genus *Enterobacter* also dominating with 37% of the identifications. After 20 days, *H. alvei* and *H. paralvei* were the dominant species, reached 66.7% at 20 days, 100% at 40 days and 95.7% at 60 days.

Therefore, the counts of enterobacteria in these PDO cheeses and dominance of these two genetically close species, *H. alvei* and *H. paralvei*, evidenced that they are involved in the cheese ripening, made it essential to perform a phylogenetic analysis to discriminate the isolates at the strain level for their technological characterization. Figure 3 shows the genotyping carried out with the primer M13, obtaining a total of 11 different profiles, of which 6 belong to *H. alvei* and 5 to *H. paralvei*. *H. alvei* profiles had between 1 and 5 bands, with the P1 profile having the highest number of bands. However, those of *H. paralvei* had between 1 and 4 bands with the P9 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 highest 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 origin (Table S3). A total of 19 strains were selected from *H. alvei* and 36 from *H. paralvei*. Based on the origin, 26 strains belonging to "Torta del Casar" and 29 to "Queso de la Serena".

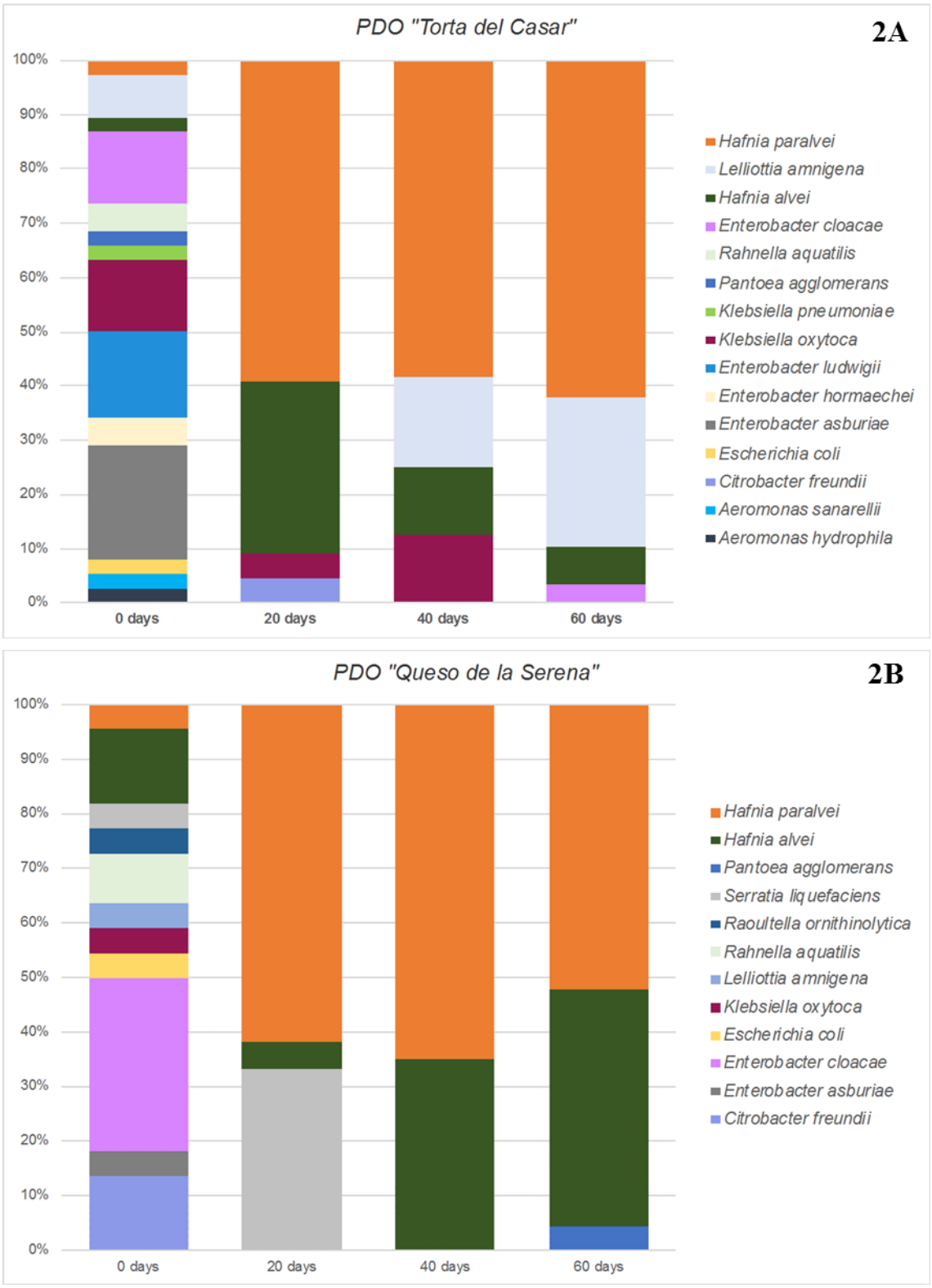
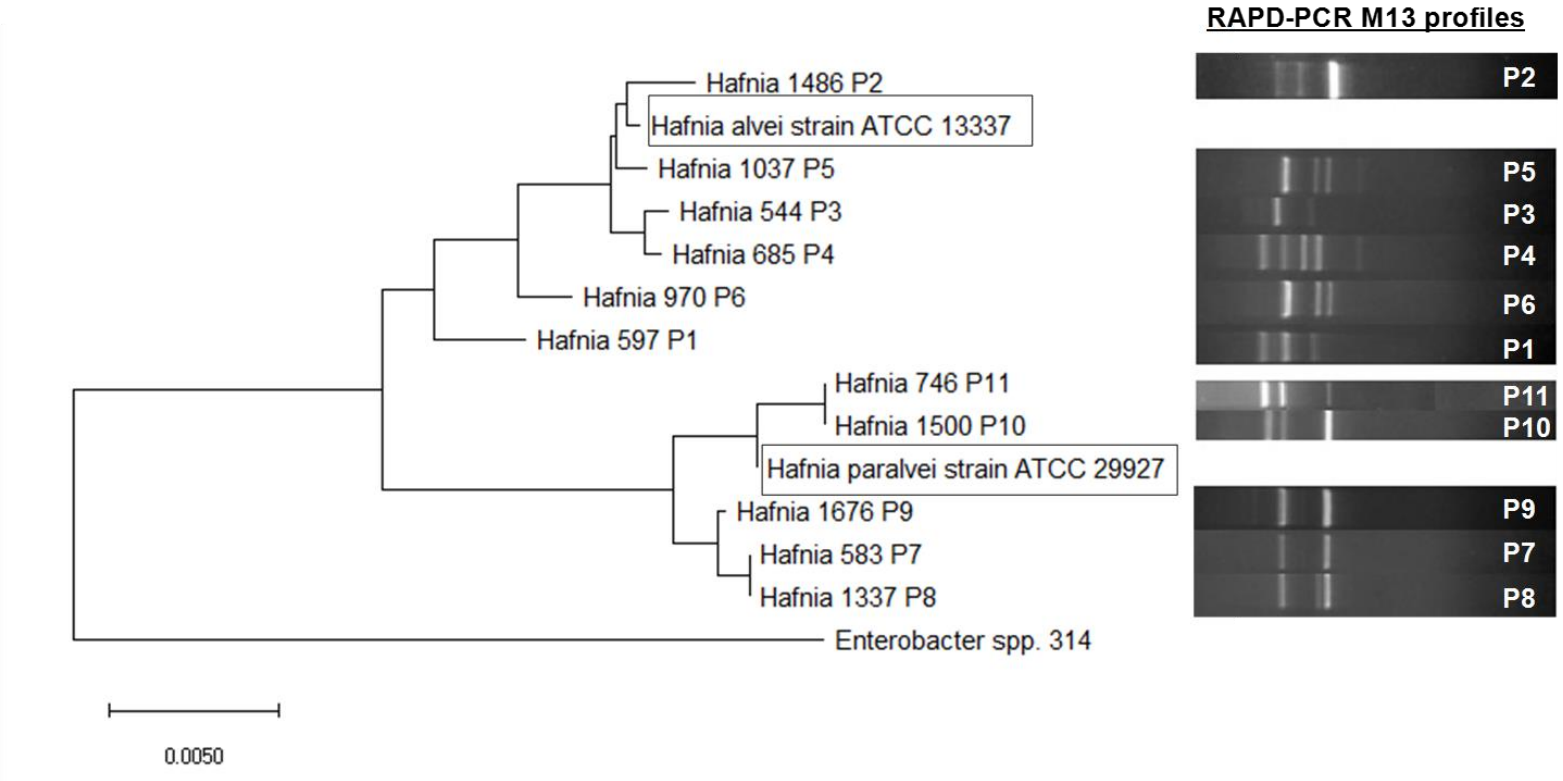


Figure 2. Mean relative abundance of enterobacteria species in soft body cheese from PDO "Torta del Casar (2A)" and "Queso de la Serena (2B) throughout ripening (0, 20, 40 and 60 days) using sequencing of the 16S rRNA gene.

Figure 3. Phylogenetic tree derived from comparison of 14 nucleotide sequences of 16S rRNA gene. The phylogenetic analysis of a total of 822 nt of the 16S rRNA gene of eleven *Hafnia* isolates, 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 in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbour-Interchange (CNI) algorithm at a search level of 1.



Security test

The *H. alvei* and *H. paralvei* selected strains were analysed for safety properties. Antibiotic susceptibility and resistance, virulence genes, biogenic amine production, haemolytic activity and cytotoxic activity of *Hafnia* isolates were performed. Susceptibility testing to 33 antibiotics was performed using *Escherichia coli* as a control. Tables S4 and S5 show the results obtained for the *H. alvei* and *H. paralvei* strains, respectively. The results showed that antibiotic resistance was strain-specific and there was a wide variability among the strains studied. Figure 4 shows the total percentage of antibiotic susceptibility of *H. alvei* (A) and *H. paralvei* strains (B). All strains showed 100% resistance to Sulfisoxazole (G). High resistance to β-lactams was also observed with more than 50%, to cephalosporins with more than 70%, except to FEP, FOX and CTT and to carbapenemics with more than 50% of the strains. The remaining antibiotics studied showed more susceptibility than resistance.

In addition, *Hafnia* spp. isolates were tested for the presence of genes involved in different antibiotic resistance and virulence genes. None of the *Hafnia* spp. isolates presented the *eae* "attaching and effacing" gene, necessary to produce epithelial lesions in the intestine. Regarding the presence of antibiotic resistance genes (carbapenemases, β-lactamases, AmpC and colistin), only the presence of β-lactamase resistance genes was detected, being a conserved gene in this species. Of the total of 55 isolates, 58% present the *acc-1* gene, corresponding to β-lactamases. These data agreed with the results obtained in the antibiotic susceptibility test presented above. A 95% of *H. alvei* and 100% of *H. paralvei* were resistant to CTX, a 3rd generation cephalosporin. To FEP, 4th generation cefepime, 89.47% of *H. alvei* and 83.3% of *H. paralvei* were susceptible. Therefore, considering the overall results, there was a clear acquired resistance to 3rd generation cephalosporins in both *H. alvei* and *H. paralvei*. In contrast, the intrinsic resistance to CTT, second generation cefotetan, in *H. alvei* was lost.

In terms of resistance to carbapenemics (imipinem, dorapenem, ertapenem and meropenem) there was variability because, although there was no amplification of the OXA-48 gene for this type of antibiotic, different strains showed resistance to all carbapenemics (Table S4 and S5), so it was considered acquired resistance . There was no amplification for genes coding for colistin resistance, and there are no studies that have tested this resistance in strains isolated from food matrices, only from clinical samples.

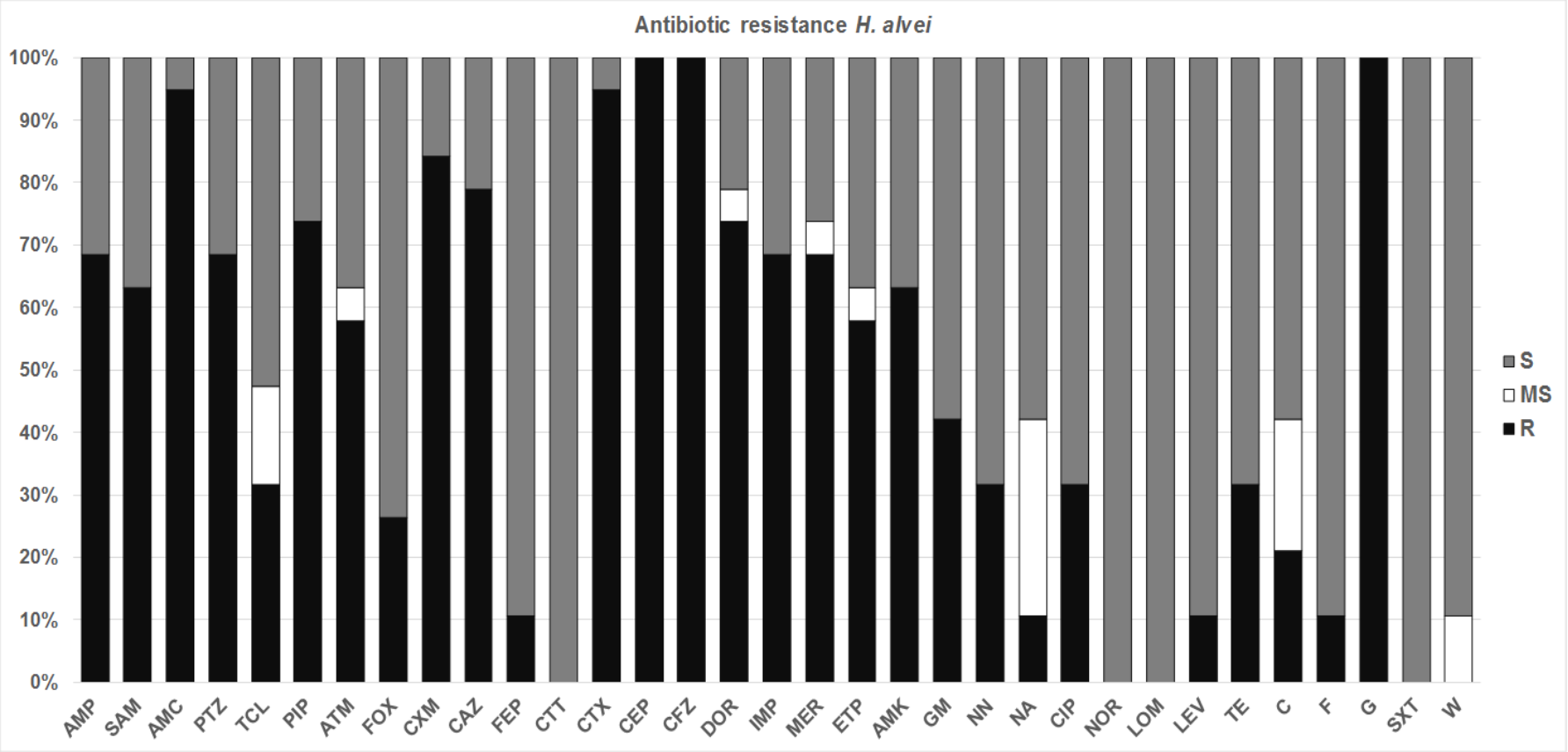


Figure 4. Total percentage of antibiotic susceptibility of *Hafnia alvei* strains (A) and *Hafnia paralvei* (B). R: resistant; MS: moderately susceptible; S: susceptible.

Figure 4. Total percentage of antibiotic susceptibility of *Hafnia alvei* strains (A) and *Hafnia paralvei* (B). R: resistant; MS: moderately susceptible; S: susceptible.

These results showed that only 4 strains of *H. alvei* and 2 strains of *H. paralvei* showed resistance to 5 or less than 5 antibiotics, while to 15 or less than 15 antibiotics for *H. alvei* were 13 strains and for *H. paralvei* were 31 strains.

On the other hand, the results of haemolytic capacity assays showed that all strains showed α -haemolysis, meaning partial lysis of erythrocytes on blood agar medium. In addition, the biogenic amine production analysis revealed that both species were producers of cadaverine and putrescine (500-600 mg/L) (Figures S1 and S2).

Finally, cytotoxicity assays of the *Hafnia* spp. strains were performed on the HeLa cervical cancer cell line. The results are shown in figure 5 where two toxigenic E. coli strains were used as positive control and one non-toxigenic E. coli strain as negative control. It was observed that none of the strains, except 1498, significantly affected the viability of the HeLa cells. Less than 10% of the HeLa cells were destroyed in the presence of 20 of the strains studied.

Therefore, 22 *Hafnia alvei* and *H. paralvei* isolates were then pre-selected based on their safety profile, among those with resistance to 10 or less antibiotics, for screening their technological capacity.

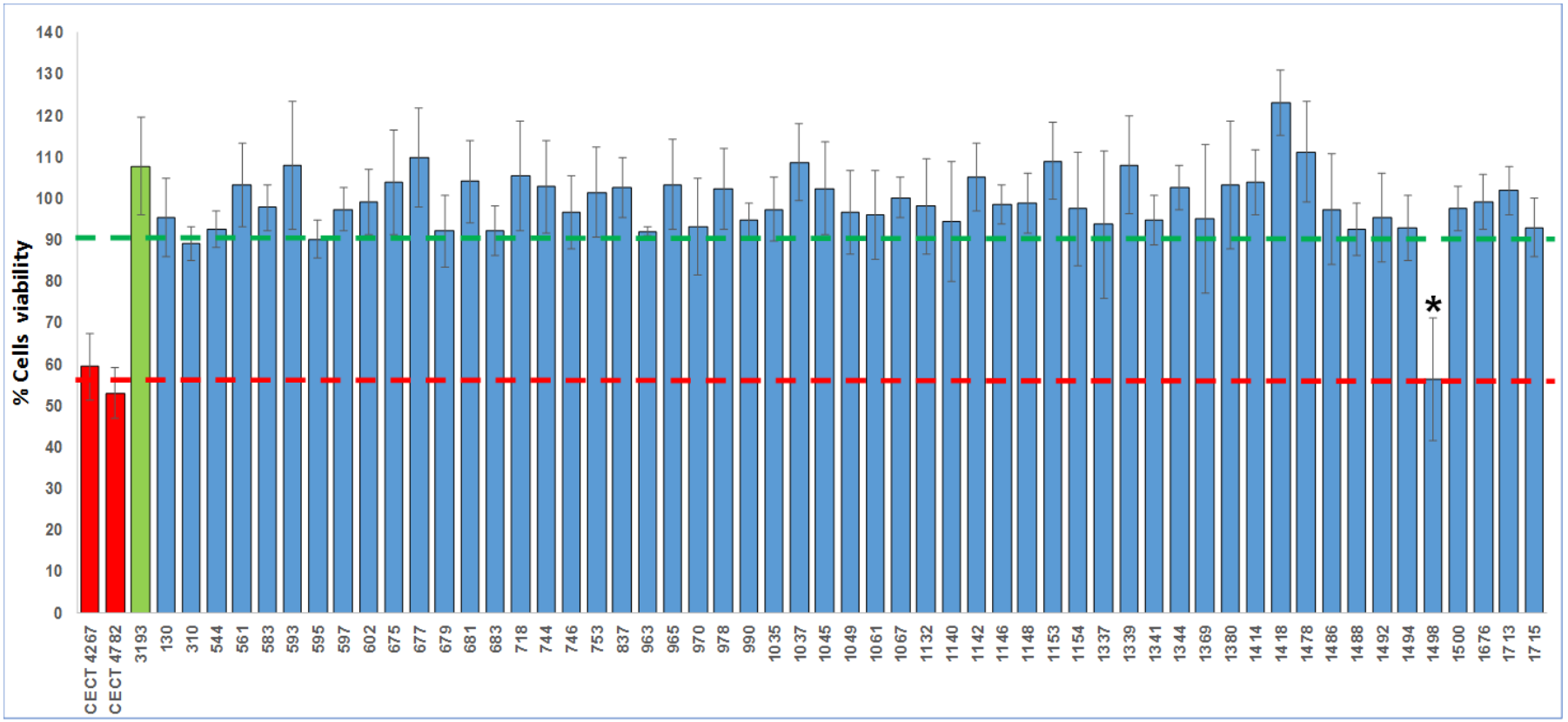


Figure 5. Cytotoxicity activity of *Hafnia* spp. strains on Hela cells. Toxigenic *E. coli* CECT 4267 and CECT 4782 were used as positive control (red colour) and non-toxigenic *E. coli* 3193 as negative control (green colour). Results are expressed as mean of percentage of cell viability. Error bars indicate ± standard deviation and (*) not significant different (P>0.050) respect to toxigenic *E. coli* positive controls.

Characterization of technological properties

Adaptability at cheese ripening conditions

Firstly, the growth of the selected strains was studied under the cheese ripening conditions, S1-S5 (Table 1). Based on the growth time (in days), 4 clusters were established for the 22 strains considered. Although all strains showed growth at the different conditions, it was found that strains belonging to cluster 1 showed a longer growth time than the rest of the clusters at the different stages of ripening. Data for days of growth were the mean of all strains showing the same behaviour grouped in each cluster. Based on the results obtained, 4 optimal growth conditions for the *Hafnia* spp. were selected, taking into account temperature, pH and NaCl concentration. It was possible to group the strains according to their optimal conditions and the results obtained for each of the maturation stages. It was observed that in cluster 1 with longer growth time in S1-S5, 4 strains (Cluster 1A and 1B) showed better growth at 30ºC with a salt concentration between 1.7 and 1.1 % (3 *H. alvei* and 1 *H. paralvei*), and 2 *H. alvei* (Cluster 1C) showed better growth at temperatures lower than 30ºC, specifically at 24.9 ºC (Table 1). Cluster 2 included the highest number of strains (14 in total, 3 *H. alvei* and 11 *H. paralvei*) and showed a shorter growth time (maximum 2.1 days in S1). Most strains were included in cluster 2B (9 strains; 30ºC, 1.1 % NaCl and pH 6) and the remaining strains were included in cluster 2C (24.9ºC, 1 % NaCl). Cluster 3 consisted of only 2 strains (1 *H. alvei* and 1 *H. paralvei*) with optimal cluster B growth conditions. Their growth times were longer, reaching 6.9 days at S2. Finally, cluster 4 was formed by 1 *H. paralvei* with optimal growth conditions of cluster D (30ºC, 1 % NaCl and pH6).

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

Cheese ripening stages (days of growth)					***Hafnia* spp. optimal growth conditions**									
					Temp (ºC)	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**		*Total*
S1	S2	S3	S4	S5		Ha[1]	Hp[1]	Ha	Hp	Ha	Hp	Ha	Hp	
<7	0.2	2.0	<7	4.1	**Cluster_1[2]**	1		1	1	2				*5*
2.1	0.3	0.6	0.7	0.6	**Cluster_2**			1	8	2	3			*14*
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*	*10*	*4*	*3*	*0*	*1*	***22***

[1]Ha: *Hafnia alvei*; Hp: *Hafnia paralvei*

[2]Clusters: 1A (130); 1B (1037, 1715); 1C (544, 683); 2B (310, 561, 593, 677, 970, 978, 1500, 1676, 1713); 2C (583, 679, 965, 1045, 1142); 3B (1341, 1414); 4D (681).

Subsequently, proteolytic and lipolytic activity, gas production, alkalinizing and acidifying capacity of the strains were determined. All strains were esterase and lipase negative at the temperatures assayed. Table 2 therefore shows the strains clusters based on the rest of performed assays, proteolytic, gas production, alkalinizing and acidifying activity. The strains were grouped by cluster according to these technological properties (P1-P5). Most strains were grouped in cluster P2, 12 strains, and were characterised mainly as diacetyl-producing and pH-raising. In addition, these strains were proteolytic at temperatures of 15 and 30 °C during the first 5 days, and at 7 days they were able to produce proteolysis at refrigeration. The strains were also found to be gas producers. The strain grouped in cluster P3 also showed stable proteolytic capacity during the 7 days at temperatures of 15 and 30°C and no gas production. In cluster P5 all 3 strains showed proteolytic capacity at refrigeration after 5 days of the assay. In clusters P1 and P4, strains with none or little proteolytic capacity were grouped.

Therefore, the 4 strains, 3 *H. alvei* 544, 970 and 1142, and 1 *H. paralvei* 1414, belonging to clusters P3 and P5 showed the best characteristics mainly due to their proteolytic activity in refrigeration. Therefore, table 3 shows the data corresponding to the strains selected for their technological characteristics from clusters P3 and P5. The 4 strains presented considerably slower growth at the cheese processing condition corresponding to the first stage (S1). That is due to the fact that the pH is that of the milk and is higher. However, with decreasing pH due to the LAB activity, the growth capacity increases, reducing the time needed for Hafnia spp. strains to grow. Increasing the NaCl percentage and pH also causes a reduction in their growth. The technological activities of these strains were taken into account mainly in terms of proteolytic activity at low temperatures. *H. alvei* 544 was also selected for not being a gas producer although this strain required more time to show proteolytic activity at low temperatures.

Thus, *Hafnia alvei* strains (544, 970 and 1142) and *H. paralvei* (1414) were proposed due to their technological and safety characteristics for possible use as a culture in soft cheeses.

Table 2. Clustering of selected *Hafnia* spp. strains based on technological properties assayed.

Cluster	Cluster P1[1]	Cluster P2	Cluster P3	Cluster P4	Cluster P5
N	1	12	1	5	3
Physic-chemical parameters					
Diacetyl production[2]	1	1	0	0.2	1
Acidifying (pH 24 h)	0.79	0.85	0.40	0.58	0.97
Alkalizing[2]	1	0.92	1	1	0.67
Proteolysis (mm)					
3 days					
8ºC	0	0	0	0	0
15ºC	0.0	1.2	10.5	0.0	8.7
30ºC	12.0	4.4	14.0	0.0	10.5
5 days					
8ºC	0.0	0.0	0.0	0.0	7.8
15ºC	0.0	9.5	11.0	0.0	10.2
30ºC	12.0	7.5	14.0	0.0	10.3
7 days					
8ºC	0.0	7.6	0.0	0.0	8.5
15ºC	0.0	9.5	11.0	0.0	10.2
30ºC	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
Paraffim (mm)	48.0	21.1	0.0	6.3	16.7

[1]Technological properties clusters: P1 (130); P2 (310, 561, 583, 593, 677, 681, 965, 978, 1500, 1676, 1713, 1715); P3 (544); P4 (679, 683, 1037, 1045, 1341); P5 (970, 1142, 1414).
[2]Diacetyl production and alkalizing: positive (1); negative (0).

Table 3. Specific technological properties of *Hafnia* spp. strains from clusters P3 and P5 from Tables 1 and 2.

Species	Strain	Cheese ripening conditions (Days of growth)					Diacetyl production[2]	Acidifying 24 h	Alkalising[2]	Proteolytic ativity (mm)									Gas production (mm)	
										3 days			5 days			7 days				
		S1[1]	S2	S3	S4	S5				8ºC	15ºC	30ºC	8ºC	15ºC	30ºC	8ºC	15ºC	30ºC	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

III.4. Discussion.

Bacterial counts in the cheese samples were similar to that reported by other authors in torta del Casar for aerobic mesophiles bacteria and enterobacteria (Ordiales et al. 2013a), but enterobacteria counts were higher than those reported by other authors in cheeses made from raw milk (Alegría et al., 2009; Galán et al., 2012). The influence of enterobacteria on the final characteristics of these products seems to be of great relevance in Torta del Casar as enterobacteria are present in high counts during the whole ripening process.

In this sense different genera within the Enterobacteriaceae family have been frequently found during cheese making (Mladenović et al., 2018; Chaves-Lopez et al., 2006; Morales et al., 2003). These authors have described species of *Citrobacter, Enterobacter, Escherichia, Hafnia, Klebsiella, Salmonella* and *Serratia* in traditional cheeses made from raw milk. In these studies, the dominant species belonged to the genera *Klebsiella, Escherichia, Serratia* and *Enterobacter*. However, in previous studies carried out on Torta del Casar type cheeses, the genus *Hafnia* was also found to be predominant (Ordiales et al., 2013a). *H. alvei* has also been described as dominant among Gram-negative bacteria in different cheeses made with raw milk (Lioliou et al., 2001, Abriouel et al., 2008, Coton et al., 2012). However, in these researches, no careful analysis was carried out to identify and separate the species of *H. alvei* from *H. paralvei*, so in these cases the microorganism could belong to any of the two species.

RAPD PCR with the M13 primer was used to differentiate between *Hafnia* species and also between different strains within the same species. It has been demonstrated by DNA-DNA hybridization and partial sequencing of the 16S rRNA gene that *Hafnia alvei* and *H. paralvei* are genotypically heterogeneous (Huys et al., 2010; Abbott et al., 2011).

After the *Hafnia* strains were accurately identified and differentiated, the following safety and technological analyses were performed.

Concerning safety analysis, susceptibility to 33 antibiotics was tested and the results showed that antibiotic resistance was very specific, and a high variability was found among the strains studied. The number of *H. paralvei* strains showed less antibiotic resistance was higher compared to the *H. alvei* strains studied. There are not many reports in which antibiotic susceptibility had been tested in many isolates at the same time. There are two studies in the literature in which a large number of strains and antibiotics were

used, but only for *H. alvei* from clinical samples. In the research conducted by Laupland et al. (2006), *H. alvei* strains were identified from samples from different patients. Most isolates were resistant to ampicillin, cephalothin, amoxicillin/clavulanate and cefazolin, but none to imipenem or ciprofloxacin. De Frutos et al. (2017) studied the resistance of 111 *H. alvei* strains, and all were resistant to ampicillin and amoxicillin/clavulanate, 84% were sensitive to cefuroxime, 99% sensitive to cefoxitin, and all sensitive to cefotaxime and imipenem. Ninety-eight per cent of isolates were sensitive to nalidixic acid and 100% to ciprofloxacin. In addition, 100% of isolates were sensitive to gentamicin and trimethoprim/sulfamethoxazole. Many of the reports described *H. alvei* as resistant to ampicillin, cephalothin, cefoxitin, cefuroxime, penicillin, piperacillin, amoxicillin-clavulanate and piperaziline-tazobactam combinations and, in other studies, the *H. alvei* strains tested showed variability in their sensitivity to tetracycline, cotrimoxazole, cefpodoxime, gentamicin and nitrofurantoin (Ramos-Vivas, 2020). In addition, other studies have revealed high colistin resistance of *Hafnia* spp., so it seems that this genus is naturally resistant to colistin, like *Proteus, Providencia, Morganella* and *Serratia* (Jayol et al., 2017; Zurfluh et al. 2017).

H. alvei possesses chromosomal genes that confer resistance to aminopenicillins and first generation cephalosporins, but not to third and fourth generation cephalosporins. However, Skurnik et al. (2010) isolated a strain of *Hafnia* spp. resistant to carbapenems (ertapenem, meropenem and imipenem). Plasmid-encoded antibiotic resistance is common among enterobacteria, with the consequent risk of dissemination of their resistance genes (Anjum et al., 2019).

Regarding haemolytic activity, all strains showed α-hemolysis, according to different authors who stated that it is due to the acquisition of the plasmid pIE 567, which is also found in *Enterobacter aerogenes* and *Salmonella typhimurium* (Padilla et al, 2005).

Additionally, due to the presence of the enzymes lysine and ornithine decarboxylase in this genus, all strains studied were cadaverine and putrescine producers (Özogul et al, 2007). However, different studies have been carried out to evaluate the concentration of biogenic amines in cheeses with *Hafnia* spp. as the predominant microbiota, and it has been observed that there is no presence or very low concentration of these amines, without reaching levels enough to produce a health risk (Ordiales et al.,

2013b). Marino et al. (2000) investigated the amino acid decarboxylating activity and biogenic amine production of 104 species of Enterobacteriaceae associated with cheese, seven belonging to the genus of *Hafnia* spp., observing that all of them were able to decarboxylate amino acids in the broth culture medium producing higher concentrations of biogenic amines mainly cadaverine than in cheese.

Finally, in the cytotoxicity assay performed on HeLa cells, our study confirmed that the Hafnia spp. strains studied did not exhibit toxicity to HeLa cells. These results confirm other studies performed for *H. alvei* with Hybrid B lymphocytes Ped-2E9 cells, highly susceptible to pathogenic bacteria, and CHO cells (Kim and Linton, 2008), who indicated minimal or null cytotoxic activity of *H. alvei* on these cells.

Twenty-two isolates of *Hafnia alvei* and *H. paralvei* were pre-selected based on their safety profile. All selected strains were among those with lower antibiotic resistance. Firstly, the growth of the selected strains under cheese ripening conditions was studied. All strains showed growth under the different conditions, although it was observed that the optimal conditions varied from one to another, although the majority grew better at 30°C, 1.1 % NaCl and pH 6. Decimo et al. (2014) isolated and identified five *H. alvei* strains isolated from milk to produce Grana Padano cheese. The growth capacity at different temperatures was studied and, although they were able to grow at 7 and 22 °C, the growth rate was higher at 30 °C.

Proteolytic and lipolytic activity, gas production, alkalinizing and acidifying capacity of the strains were also studied as important technological properties. None of the strains showed lipolytic activity but did show proteolytic activity and diacetyl production. Diacetyl production is a desirable characteristic of cheese production and was detected in Torta del Casar cheeses (Ordiales et al., 2013c) and in other types of cheese (Wolf et al., 2010, Fernández García et al., 2002; Peralta et al., 2020). It contributes to the cheese's flavour. Morales et al. (2004) reported high levels of this compound in model cheeses inoculated with *Hafnia* and *Serratia.*

Proteolytic capacity, especially under refrigeration, is one of the most important technological characteristics for the production of soft cheeses. This is because the cheese ripening process takes place at low temperature and for producing the desired flavour and texture characteristics, proteolysis is crucial. Decimo et al. (2014) studied the proteolytic

capacity and gas production of *H. alvei* strains and found that all were proteolytic at the temperatures studied of 7, 22 and 30ºC and also gas-producing. Morales et al. 2003 evaluated the proteolytic capacity of different strains of enterobacteria inoculated in model cheeses (*Hafnia, Serratia, Enterobacter* and *E. coli*) and observed that these strains were able to degrade caseins and release peptides, accelerating the ripening of the cheeses, as well as contributing to the release of amino acids. However, not all *Hafnia* spp. strains showed proteolytic activity in our assay, which is in agreement with the work of Morandi et al. (2021) who did not describe proteolytic capacity in *H. alvei* strains isolated from raw milk.

The behavior of the strains with respect to gas production is variable since, among other factors, it is due to the ability to ferment lactose. The lactose assimilation phenotype can be acquired and therefore strains capable of producing gas can be detected. Tabla et al., 2016, 2018 studied the gas-producing capacity of enterobacteria isolated from Torta type cheeses and found that of all *H. alvei* strains studied, 74.5% showed a high gas-producing capacity.

III.5. Conclusion.

In conclusion, four of the strains studied presented the best technological characteristics among the 22 strains selected for their safety characteristics. These four strains were affected by the changes in temperature, salt concentration and pH that were tested to simulate cheese processing conditions, however, they adapted properly to the different stages varying their growth rate. Proteolytic activity at low temperatures was crucial for the selection and, in addition, H. alvei 544 strain was non-gas producing.

Thus, *Hafnia alvei* strains (544, 970 and 1142) and *H. paralvei* (1414) were selected for their safety and technological characteristics for potential use as starter cultures in soft cheeses. Nevertheless, before their industrial application further studies are necessary in cheese model system to know their contribution to an adequate balanced of proteolysis, lipolysis and other reactions products that have a great influence on the texture, aromatic profile and general quality of the final product.

III.6. References

Abbott, S. L., Moler, S., Green, N., Tran, R. K., Wainwright, K., & Janda, J. M. (2011). Clinical and laboratory diagnostic characteristics and cytotoxigenic potential of *Hafnia alvei* and *Hafnia paralvei* strains. *Journal of clinical microbiology*, 49(9), 3122-3126.

Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E., & Martínez-Bueno, M. (2008). Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. International Journal of Food Microbiology, 127(3), 200-208.

AbuOun, M., Stubberfield, E. J., Duggett, N. A., Kirchner, M., Dormer, L., Nunez-Garcia, J., ... & Smith, R. P. (2017). mcr-1 and mcr-2 variant genes identified in *Moraxella* species isolated from pigs in Great Britain from 2014 to 2015. *Journal of Antimicrobial Chemotherapy*, 72(10), 2745-2749.

Alegría, A., Álvarez-Martín, P., Sacristán, N., Fernández, E., Delgado, S., & Mayo, B. (2009). Diversity and evolution of the microbial populations during manufac-ture and ripening of Casín, a traditional Spanish, starter-free cheese made fromcow's milk.International Journal of Food Microbiology, 136,44-51.

Anjum, M., Madsen, J. S., Nesme, J., Jana, B., Wiese, M., Jasinskytė, D., Nielsen, D. S., Sørensen S. J., Dalsgaard, A., Moodley, A., Bortolaia, V., & Guardabassi, L. (2019). Fate of CMY-2-encoding plasmids introduced into the human fecal microbiota by exogenous *Escherichia coli*. *Antimicrobial agents and chemotherapy*, *63*(5), e02528-18.

Araújo-Rodrigues, H., dos Santos, M. T. P., Ruiz-Moyano, S., Tavaria, F. K., Martins, A. P., Alvarenga, N., & Pintado, M. E. (2021). Technological and protective performance of LAB isolated from Serpa PDO cheese: Towards selection and development of an autochthonous starter culture. *LWT*, *150*, 112079.

Araújo-Rodrigues, H., Tavaria, F. K., dos Santos, M. T. P., Alvarenga, N., & Pintado, M. M. (2020). A review on microbiological and technological aspects of Serpa PDO cheese: An ovine raw milk cheese. *International Dairy Journal*, 100, 104561.

Bang, D. D., Scheutz, F., Ahrens, P., Pedersen, K., Blom, J., & Madsen, M. (2001). Prevalence of cytolethal distending toxin (cdt) genes and CDT production in *Campylobacter* spp. isolated from Danish broilers. *Journal of medical microbiology*, 50(12), 1087-1094.

Bassi, D., Puglisi, E., & Cocconcelli, P. S. (2015). Comparing natural and selected starter cultures in meat and cheese fermentations. *Current Opinion in Food Science*, 2, 118-122.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J.C.,Gerds,M.L., Hammes,W.P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I.B., Prajapati, J.B., Seto, Y., Ter Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S., & Hansen, E.B., (2012). Food fermentations: microorganisms with technological beneficial use. *International Journal of Food Microbiology*, 154 (3), 87–97.

Bover-Cid, S., & Holzapfel, W. H. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *International journal of food microbiology*, *53*(1), 33-41.

Campagnollo, F. B., Margalho, L. P., Kamimura, B. A., Feliciano, M. D., Freire, L., Lopes, L. S., Alvarenga, V. O., Cadavez V. A.P., Gonzales-Barron, U., Schaffner D. W., & Sant'Ana, A. S. (2018). Selection of indigenous lactic acid bacteria presenting anti-listerial activity, and their role in reducing the maturation period and assuring the safety of traditional Brazilian cheeses. *Food microbiology*, *73*, 288-297.

Centeno, J. A., Garabal, J. I., Docampo, F., Lorenzo, J. M., & Carballo, J. (2017). Recovering traditional raw-milk Tetilla cheese flavour and sensory attributes by using *Kocuria varians* and *Yarrowia lipolytica* adjunct cultures. *International journal of food microbiology*, *251*, 33-40.

Chaves-López, C., De Angelis, M., Martuscelli, M., Serio, A., Paparella, A., & Suzzi, G. (2006). Characterization of the *Enterobacteriaceae* isolated from an artisanal Italian ewe's cheese (Pecorino Abruzzese). *Journal of Applied Microbiology, 101*(2), 353–360. https://doi.org/10.1111/j.1365-2672.2006.02941.x

Clinical and Laboratory Standards Institute, CLSI (2019). Performance Standards for Antimicrobial Susceptibility Testing. 29th ed. CLSI supplement M100. Wayne, PA, USA.

Coton,M., DelbèsDelbes-Paus, C., Irlinger, F., Desmasures,N., LeflecheLe Fleche, A., Stahl, V.,Montel, M.C., Coton, E., 2012. Diversity and assessment of potential risk factors of Gram-negative isolates associated with French cheeses. *Food Microbiology 29* (1), 88–98.

de Frutos, M., López, E., Aragón, R., López-Urrutia, L., Ramos, C., Domínguez-Gil, M., ... & Eiros, J. M. (2017). Descriptivo de *Hafnia alvei* aisladas en coprocultivo: aproximación a su valoración en clínica. *Revista Española de Quimioterapia*, *30*(4).

Decimo, M., Morandi, S., Silvetti, T., & Brasca, M. (2014). Characterization of Gram-negative psychrotrophic bacteria isolated from Italian bulk tank milk. *Journal of Food Science, 79*(10), M2081–M2090. https://doi.org/10.1111/1750-3841.12645

Del Pozo, B. F., Gaya, P., Medina, M., Rodríguez-Marín, M. A., & Nuñez, M. (1988). Changes in the microflora of La Serena ewes' milk cheese during ripening. *Journal of Dairy Research*, *55*(3), 449-455.

Delbès-Paus, C., Miszczycha, S., Ganet, S., Hélinck, S., Veisseire, P., Pochet, S., ... & Montel, M. C. (2013). Behavior of *Escherichia coli* O26: H11 in the presence of *Hafnia alvei* in a model cheese ecosystem. *International Journal of Food Microbiology*, 160(3), 212-218.

Delgado, F. J., González-Crespo, J., Cava, R., García-Parra, J., & Ramírez, R. (2010a). Characterisation by SPME–GC–MS of the volatile profile of a Spanish soft cheese PDO Torta del Casar during ripening. *Food Chemistry*, *118*(1), 182-189.

Delgado, F. J., Rodríguez-Pinilla, J., González-Crespo, J., Ramírez, R., & Roa, I. (2010b). Proteolysis and texture changes of a Spanish soft cheese ('Torta del Casar') manufactured with raw ewe milk and vegetable rennet during ripening. International *Journal of Food Science and Technology*, 45(3), 512–519.

European Committee of antimicrobial susceptibility testing (EUCAST). URL: http://www.eucast.org/clinical_breakpoints/

Fernández, M., Ruiz-Moyano, S., Benito, M. J., Martín, A., Hernández, A., & de Guía Córdoba, M. (2016). Potential antimicrobial and antiproliferative activities of autochthonous starter cultures and protease EPg222 in dry-fermented sausages. *Food & Function*, 7(5), 2320-2330.

Fernández-García, E., Carbonell, M., & Nuñez, M. (2002). Volatile fraction and sensory characteristics of Manchego cheese. 1. Comparison of raw and pasteurized milk cheese. *Journal of Dairy Research, 69*(4), 579–593. https://doi.org/10.1017/s0022029902005794

Galán, E., Cabezas, L., & Fernández-Salguero, J. (2012). Proteolysis, microbiology and sensory properties of ewe's milk cheese produced with plant coagulant from cardoon *Cynara cardunculus*, calf rennet or a mixture thereof. *International Dairy Journal*, *25*,92-96.

Garabal, J. I., Rodríguez-Alonso, P., & Centeno, J. A. (2008). Characterization of lactic acid bacteria isolated from raw cows' milk cheeses currently produced in Galicia (NW Spain). *LWT-Food Science and Technology, 41*(8), 1452-1458.

Gonçalves, M. T. P., Benito, M. J., Córdoba, M. D. G., Egas, C., Merchán, A. V., Galván, A. I., & Ruiz-Moyano, S. (2018). Bacterial communities in Serpa cheese by culture dependent techniques, 16S rRNA gene sequencing and high-throughput sequencing analysis. *Journal of Food Science, 83*(5), 1333-1341.

Hantsis-Zacharov, E., Halpern, M., 2007. Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. *Applied and Environmental Microbiology 73*, 7162–7168.

Hasman, H., Mevius, D., Veldman, K., Olesen, I., & Aarestrup, F. M. (2005). β-Lactamases among extended-spectrum β-lactamase (ESBL)-resistant *Salmonella* from poultry, poultry products and human patients in The Netherlands. *Journal of Antimicrobial Chemotherapy*, *56*(1), 115-121.

Huey, B., & Hall, J. (1989). Hypervariable DNA fingerprinting in *Escherichia coli*: minisatellite probe from bacteriophage M13. *Journal of Bacteriology*, *171*(5), 2528 LP – 2532. https://doi.org/10.1128/jb.171.5.2528-2532.1989

Huys, G., Cnockaert, M., Abbott, S. L., Janda, J. M., & Vandamme, P. (2010). *Hafnia paralvei* sp. nov., formerly known as *Hafnia alvei* hybridization group 2. *International journal of systematic and evolutionary microbiology*, *60*(8), 1725-1728.

Irlinger, F., Yung, S. A. Y. I., Sarthou, A. S., Delbès-Paus, C., Montel, M. C., Coton, E., Coton M. & Helinck, S. (2012). Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International Journal of Food Microbiology*, *153*(3), 332-338.

Ismail, S. A. S., Deak, T., Abd El-Rahman, H. A., Yassien, M. A. M., & Beuchat, L. R. (2000). Presence and changes in populations of yeasts on raw and processed poultry products stored at refrigeration temperature. *International Journal of Food Microbiology*, *62*(1–2), 113–121. https://doi.org/10.1016/S0168-1605(00)00414-1

Ismaili, A., Bourke, B., De Azavedo, J. C., Ratnam, S., Karmali, M. A., & Sherman, P. M. (1996). Heterogeneity in phenotypic and genotypic characteristics among strains of *Hafnia alvei*. *Journal of Clinical Microbiology*, *34*(12), 2973-2979.

Jayol, A., Saly, M., Nordmann, P., Ménard, A., Poirel, L., & Dubois, V. (2017). *Hafnia*, an enterobacterial genus naturally resistant to colistin revealed by three susceptibility testing methods. *Journal of Antimicrobial Chemotherapy*, *72*(9), 2507-2511.

Kim, J. M., & Linton, R. H. (2008). Identification of a non-pathogenic surrogate organism for chlorine dioxide (ClO2) gas treatment. *Food Microbiology*, *25*(4), 597-606.

Kouker, G., & Jaeger, K. E. (1987). Specific and sensitive plate assay for bacterial lipases. *Applied and Environmental Microbiology*, *53*(1), 211–213.

Laupland, K. B., Church, D. L., Ross, T., & Pitout, J. D. (2006). Population-based laboratory surveillance of *Hafnia alvei* isolates in a large Canadian health region. *Annals of clinical microbiology and antimicrobials, 5(*1), 1-5.

Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology, 15*(2), 67–78. https://doi.org/10.1016/j.tifs.2003.09.004

Lioliou, K., Litopoulou-Tzanetaki, E., Tzanetakis, N., & Robinson, R. K. (2001). Changes in the microflora of manouri, a traditional Greek whey cheese, during storage. *International journal of dairy technology, 54*(3), 100-106.

Macedo, A. C., Malcata, F. X., & Hogg, T. A. (1995). Microbiological profile in Serra ewes' cheese during ripening. *Journal of Applied Bacteriology, 79*(1), 1-11.

Marino, M., Maifreni, M., Moret, S., & Rondinini, G. (2000). The capacity of *Enterobacteriaceae* species to produce biogenic amines in cheese. *Letters in Applied Microbiology, 31*(2), 169-173.

Martínez, S., Franco, I., & Carballo, J. (2011). Spanish goat and sheep milk cheeses. *Small Ruminant Research, 101*(1-3), 41-54.

Medina, A., Lambert, R. J., & Magan, N. (2012). Rapid throughput analysis of filamentous fungal growth using turbidimetric measurements with the Bioscreen C: a tool for screening antifungal compounds. *Fungal Biology, 116*(1), 161-169.

Mladenović, K. G., Muruzović, M. Ž., Žugić Petrović, T., Stefanović, O. D., & Čomić, L. R. (2018). Isolation and identification of *Enterobacteriaceae* from traditional Serbian cheese and their physiological characteristics. *Journal of Food Safety, 38*(1), e12387.

Montel, M. C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D. A., Desmasures, N., & Berthier, F. (2014). Traditional cheeses: rich and diverse microbiota with associated benefits. *International Journal of Food Microbiology, 177*, 136-154.

Morales, P., FELIU, I., Fernández-García, E., & Núñez, M. (2004). Volatile compounds produced in cheese by *Enterobacteriaceae* strains of dairy origin. *Journal of Food Protection, 67*(3), 567-573. https://doi.org/10.4315/0362-028X-67.3.567

Morales, P., Fernández-García, E., & Núñez, M. (2003). Caseinolysis in cheese by *Enterobacteriaceae* strains of dairy origin. *Letters in Applied Microbiology, 37*(5), 410-414. https://doi.org/10.1046/j.1472-765X.2003.01422.x

Morales, P., Fernández-García, E., & Nuñez, M. (2005). Volatile compounds produced in cheese by *Pseudomonas* strains of dairy origin belonging to six different species. *Journal of Agricultural and Food Chemistry, 53*(17), 6835-6843.

Morandi, S., Pica, V., Masotti, F., Cattaneo, S., Brasca, M., De Noni, I., & Silvetti, T. (2021). Proteolytic traits of psychrotrophic bacteria potentially causative of sterilized milk instability: Genotypic, phenotypic and peptidomic insight. *Foods, 10*(5). https://doi.org/10.3390/foods10050934

Ordiales, E., Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J., & de Guía Córdoba, M. (2013a). Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. *Food Control, 33*(2), 448-454.

Ordiales, E., Benito, M. J., Martin, A., Fernández, M., Hernández, A., & de Guia Córdoba, M. (2013b). Proteolytic effect of *Cynara cardunculus* rennet for use in the elaboration of 'Torta del Casar' cheese. *The Journal of dairy research, 80*(4), 429.

Ordiales, E., Martín, A., Benito, M. J., Hernández, A., Ruiz-Moyano, S., Córdoba, M. G. (2013c). Role of the microbial population on the flavor of the soft-bodied cheese Torta del Casar. *Journal of Dairy Science, 96(*9), 5477-5486.

Ordiales, E., Martín, A., Benito, M. J., Fernández, M., Casquete, R., & de Guía Córdoba, M. (2014). Influence of the technological properties of vegetable rennet (*Cynara cardunculus*) on the physicochemical, sensory and rheological characteristics of "Torta del Casar" cheese. *International Journal of Dairy Technology, 67*(3), 402–409. https://doi.org/10.1111/1471-0307.12129

Özogul, F., Özogul, Y., & Kuley, E. (2008). Nucleotide degradation and biogenic amine formation of wild white grouper (*Epinephelus aeneus*) stored in ice and at chill temperature (4 C). *Food Chemistry, 108*(3), 933-941.

Padilla, D., Real, F., Gómez, V., Sierra, E., Acosta, B., Deniz, S., & Acosta, F. (2005). Virulence factors and pathogenicity of *Hafnia alvei* for gilthead seabream, *Sparus aurata L. Journal of fish diseases, 28*(7), 411-417.

Peralta, G. H., Bergamini, C. V., Costabel, L., Audero, G., Ale, E. C., Binetti, A. G., … Hynes, E. R. (2020). Performance of *Lactobacillus paracasei 90* as an adjunct culture in soft cheese under cold chain interruption. *International Dairy Journal, 109*. https://doi.org/10.1016/j.idairyj.2020.104779

Pereira, G.V.D.M., Neto, D.P.D.C., Junqueira, A.C.D.O., Karp, S.G., Letti, L.A.J., Júnior, A.I.M., & Soccol, C.R.A (2020). A review of selection criteria for starter culture development in the food fermentation industry. *Food Reviews International, 36*(2), 135-167.

Poirel, L., Walsh, T. R., Cuvillier, V., & Nordmann, P. (2011). Multiplex PCR for detection of acquired carbapenemase genes. *Diagnostic microbiology and infectious disease, 70*(1), 119-123.

Ramos-Vivas, J. (2020). Microbiología de *Hafnia alvei. Enfermedades infecciosas y microbiologia clinica, 38*, 1-6.

Rebelo, A. R., Bortolaia, V., Kjeldgaard, J. S., Pedersen, S. K., Leekitcharoenphon, P., Hansen, I. M., ... & Battisti, A. (2018). Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5 for surveillance purposes. *Eurosurveillance, 23*(6).

Rohlf, F. J., (1993). NTSYS.PC: numerical taxonomy and multivariate analysis system, version 2.0. Applied Biostatistics Inc., New York, NY.

Ruiz-Moyano, S., Martín, A., Benito, M. J., Casquete, R., Serradilla, M. J., & de Guía Córdoba, M. (2009). Safety and functional aspects of pre-selected lactobacilli for probiotic use in Iberian dry-fermented sausages. *Meat science, 83*(3), 460-467.

Ruiz-Moyano, S., dos Santos, M. T. P. G., Galván, A. I., Merchán, A. V., González, E., de Guía Córdoba, M., & Benito, M. J. (2019). Screening of autochthonous lactic acid bacteria strains from artisanal soft cheese: probiotic characteristics and prebiotic metabolism. *LWT*, 114, 108388.

Sánchez-Juanes, F., Teixeira-Martín, V., González-Buitrago, J. M., Velázquez, E., & Flores-Félix, J. D. (2020). Identification of species and subspecies of lactic acid bacteria present in spanish cheeses type "Torta" by MALDI-TOF MS and pheS gene Analyses. *Microorganisms, 8*(2), 301.

Skurnik, D., Nucci, A., Ruimy, R., Lasocki, S., Muller-Serieys, C., Montravers, P., Andremont, A., & Courvalin, P. (2010). Emergence of carbapenem-resistant *Hafnia*: the fall of the last soldier. *Clinical Infectious Diseases, 50*(10), 1429-1431

Tabla, R., Gómez, A., Rebollo, J. E., & Roa, I. (2015). Salt influence on surface microorganisms and ripening of soft ewe cheese. *Journal of Dairy Research, 82*(2), 215-221.

Tabla, R., Gómez, A., Simancas, A., Rebollo, J. E., Molina, F., & Roa, I. (2016). *Enterobacteriaceae* species during manufacturing and ripening of semi–hard and soft raw ewe's milk cheese. Gas production capacity. *Small Ruminant Research, 145*, 123-129.

Tabla, R., Gómez, A., Simancas, A., Rebollo, J. E., Molina, F., & Roa, I. (2018). Early blowing in raw goats' milk cheese: Gas production capacity of *Enterobacteriaceae*

species present during manufacturing and ripening. *Journal of Dairy Research, 85*(3), 331–338. https://doi.org/10.1017/S0022029918000511

Tan, J. Y., Yin, W. F., and Chan, K. G. (2014). Quorum sensing activity of Hafnia alvei isolated from packed food. *Sensors 14,* 6788–6796. doi: 10.3390/s140406788

Terzić-Vidojević, A., Tonković, K., Pavunc, A. L., Beganović, J., Strahinić, I., Kojić, M., Veljović, K., Golić, N., Kos, B., Čadež N., Gregurek L., Šušković J., Raspor, P. & Topisirović, L. (2015). Evaluation of autochthonous lactic acid bacteria as starter cultures for production of white pickled and fresh soft cheeses. *LWT-Food Science and Technology, 63*(1), 298-306.

To, C. Z., & Bhunia, A. K. (2019). Three dimensional Vero cell-platform for rapid and sensitive screening of Shiga-toxin producing *Escherichia coli. Frontiers in microbiology, 10*, 949.

Tornadijo, M. E., Garcıa, M. C., Fresno, J. M., & Carballo, J. (2001). Study of Enterobacteriaceae during the manufacture and ripening of San Simón cheese. *Food Microbiology*, *18*(5), 499-509.

Vázquez-Velázquez, R., Salvador-Figueroa, M., Adriano-Anaya, L., DeGyves–Córdova, G., & Vázquez-Ovando, A. (2018). Use of starter culture of native lactic acid bacteria for producing an artisanal Mexican cheese safe and sensory acceptable. *CyTA-Journal of Food, 16*(1), 460-468.

Wang, X., Wang, Y., Zhou, Y., Li, J., Yin, W., Wang, S., ... & Wang, Y. (2018). Emergence of a novel mobile colistin resistance gene, mcr-8, in NDM-producing *Klebsiella pneumoniae. Emerging microbes & infections, 7*(1), 1-9.

Wolf, I. V., Perotti, M. C., Bernal, S. M., & Zalazar, C. A. (2010). Study of the chemical composition, proteolysis, lipolysis and volatile compounds profile of commercial Reggianito Argentino cheese: Characterization of Reggianito Argentino cheese. *Food Research International, 43*(4), 1204–1211. https://doi.org/10.1016/j.foodres.2010.02.018

Yang, Y. Q., Li, Y. X., Lei, C. W., Zhang, A. Y., & Wang, H. N. (2018). Novel plasmid-mediated colistin resistance gene mcr-7.1 in *Klebsiella pneumoniae. Journal of Antimicrobial Chemotherapy*, *73*(7), 1791-1795.

Yoon, Y., Lee, S., & Choi, K. H. (2016). Microbial benefits and risks of raw milk cheese. *Food Control, 63*, 201-215.

Zurfluh, K., Stephan, R., Widmer, A., Poirel, L., Nordmann, P., Nüesch, H. J., Hächler H. & Nüesch-Inderbinen, M. (2017). Screening for fecal carriage of MCR-producing

Enterobacteriaceae in healthy humans and primary care patients. *Antimicrobial Resistance & Infection Control*, *6*(1), 1-4.

Supplementary material

This chapter contains five supplementary Tables and two supplementary figures

Table S1. Antibiotic class, subclass, general agent name and abbreviation used in this study.

Antibiotic Class	Antibiotic Subclass	Agents Included, Generical Names	Agent Abbreviation
Penicillins	Aminopenicillin	Ampicillin	AMP
	Ureidopenicillin	Piperacillin	PIP
β-Lactam/β-lactamase inhibitor combinations		Ampicillin-sulbactam	SAM
		Amoxicillin-clavucanic acid	AMC
		Piperacillin-tazobactam	PTZ
		Tricarcillin-clavucanic acid	TCL
Monobactams		Aztreonam	ATM
Cephems	Cephalosporin I	Cephalotin	CEP
		Cefazolin	CFZ
	Cephalosporin II	Cefuroxime	CXM
	Cephalosporin III	Cefotaxime	CTX
		Ceftazidime	CAZ
	Cephalosporin IV	Cefepime	FEP
	Cephamycin	Cefotetan	CTT
		Cefoxitin	FOX
Penems	Carbapenems	Dorapenem	DOR
		Imipinem	IMP
		Meropenem	MER
		Ertapenem	ETP
Aminoglycosides		Amikacin	AMK
		Gentamicina	GM
		Tobramicina	NN
Quinolones	Quinolone	Nalidixic acid	NA
	Fluoroquinolone	Ciprofloxacin	CIP
		Norfloxacin	NOR
		Lomefloxacin	LOM
		Levofloxacino	LEV
Tetracyclines		Tetracycline	TE
Phenicols		Chloramphenicol	C
Nitrofurans		Nitrofurantoin	F
Folate Pathway Inhibitors		Sulfisoxazole	G
		Trimethoprim-sulfamethoxazole	SXT
		Trimethoprim	W

Table S2. Conditions of temperature, salt concentration and pH for modelling *Hafnia* spp. growth under cheese ripening process.

Temperature	pH	NaCl %
30	4.8	2
30	6	2
30	5.4	1
30	5.4	3
20	6	1
20	4.8	3
20	4.8	1
20	6	3
20	5.4	2
20	5.4	2
20	5.4	2
10	4.8	2
10	5.4	1
10	6	2
10	5.4	3

Table S3. *Hafnia alvei* and *Hafnia paralvei* selected strains, PDO origin and RAPD-PCR M13 profile.

IDENTIFICATION	ISOLATE CODE	*PDO*	PROFILE M13
H. alvei	597, 602, 1148, 1153, 1154	*"QUESO DE LA SERENA"*	P1
	1486	*"QUESO DE LA SERENA"*	P2
	130	*"TORTA DEL CASAR"*	P3
	544	*"QUESO DE LA SERENA"*	
	675, 683, 1035	*"TORTA DEL CASAR"*	P4
	595, 1067, 1142	*"QUESO DE LA SERENA"*	
	1037, 1045, 1341	*"TORTA DEL CASAR"*	P5
	970	*"TORTA DEL CASAR"*	P6
	1132	*"QUESO DE LA SERENA"*	
H. paralvei	679, 718, 837, 963, 978, 990, 1049, 1339, 1369, 1380, 1418, 1713	*"TORTA DEL CASAR"*	P7
	561, 583, 593, 744, 753, 1061, 1140, 1146, 1478, 1488, 1494, 1498	*"QUESO DE LA SERENA"*	
	677, 681, 965, 1337, 1414	*"TORTA DEL CASAR"*	P8
	310, 1676, 1715	*"TORTA DEL CASAR"*	P9
	1492, 1500	*"QUESO DE LA SERENA"*	P10
	1344	*"TORTA DEL CASAR"*	P11
	746	*"QUESO DE LA SERENA"*	

Tabla S4. Antibiotic susceptibility of *Hafnia alvei* strains. R: resistant; MS: moderately susceptible; S: susceptible.

CODE*,**	AMP	SAM	AMC	PTZ	TCL	PIP	ATM	FOX	CXM	CAZ	FEP	CTX	DOR	IMP	MER	ETP	AMK	GM	NN	NA	CIP	LEV	TE	C	F	W
130	S	S	R	S	S	R	MS	R	S	S	S	S	R	S	R	R	S	S	R	S	S	S	S	S	S	S
544	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	R	S	S	S
595	R	R	R	R	MS	R	R	S	R	R	S	R	R	S	R	R	R	R	S	S	S	S	R	R	S	S
597	R	R	R	R	R	R	R	S	R	R	S	R	R	R	R	R	R	S	R	MS	R	R	S	MS	S	MS
602	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	MS	R	S	S	MS	S	MS
675	R	R	R	R	MS	R	R	S	R	S	S	R	R	S	R	R	R	R	R	S	R	S	S	S	S	S
683	R	R	R	R	S	S	S	S	R	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S
970	S	S	R	S	S	R	S	S	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
1035	R	R	R	R	R	R	R	S	R	R	S	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S
1037	R	S	R	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S
1045	S	R	R	R	S	S	S	S	R	R	S	R	S	S	S	S	S	S	S	MS	S	S	S	S	S	S
1067	R	S	R	R	S	R	R	S	R	R	S	R	R	R	R	R	R	S	S	S	S	S	R	R	S	S
1132	R	R	R	R	S	R	R	S	R	R	S	R	R	R	MS	MS	R	R	S	S	S	S	S	R	S	S
1142	R	S	R	S	S	S	S	S	R	R	S	R	MS	R	S	S	R	S	S	S	S	S	R	S	S	S
1148	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	MS	R	S	S	R	S	S
1153	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	S	MS	R	R	S	MS	S	S
1154	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	S	R	S	R	MS	R	S
1341	S	R	R	S	S	R	S	S	R	R	S	R	R	R	R	S	S	S	S	R	S	S	S	S	S	S
1486	S	S	R	R	MS	R	R	R	R	R	R	R	R	R	R	R	R	R	S	MS	S	S	R	S	R	S

* All *H. alvei* are “R” to CFZ (cefazolin), CEP (cefalotin), G (sulfiaxole)

** All *H. alvei* are “S” to CTT (cefotetan), NOR (norfloxacin), LOM (lomefloxacin), SXT (Trimethoprimsulfamethoxazole)

Table S5. Antibiotic susceptibility of *Hafnia paralvei* strains. R: resistant; MS: moderately susceptible; S: susceptible.

CODE*,**	AMP	SAM	PTZ	TCL	PIP	ATM	FOX	CXM	CAZ	FEP	CTT	DOR	IMP	MER	ETP	AMK	GM	NN	NA	CIP	LEV	TE	C	F	W
310	R	R	R	S	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R
561	R	R	S	S	R	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R	S	S
583	R	R	S	S	S	S	S	R	R	S	S	R	S	R	S	R	R	R	MS	S	S	S	S	S	S
593	R	R	S	S	S	S	S	S	R	S	S	R	S	S	S	S	S	S	MS	S	S	R	S	S	S
677	S	R	S	S	S	S	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S
679	R	R	R	R	S	S	S	R	S	R	R	S	S	R	R	S	S	R	S	S	S	S	MS	S	S
681	R	R	S	S	R	S	S	R	R	R	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S
718	R	R	R	R	S	R	S	R	R	R	R	R	R	R	R	R	S	R	R	S	S	R	MS	S	S
744	R	R	R	R	S	R	S	R	R	S	R	R	R	R	R	R	R	R	S	R	R	S	MS	S	R
746	R	R	S	S	R	R	S	R	R	S	MS	R	S	R	R	S	S	R	R	R	S	S	MS	S	S
753	R	R	S	S	S	R	S	R	R	S	S	R	S	R	R	R	R	R	S	R	S	S	S	S	S
837	R	R	R	S	R	R	S	R	S	S	S	R	S	R	R	R	R	S	S	S	S	S	R	S	S
963	R	R	R	S	R	R	S	R	R	S	S	R	R	R	S	R	R	S	S	S	S	S	R	S	S
965	R	R	R	S	S	R	S	R	S	S	S	R	S	R	R	R	R	S	S	S	S	S	MS	S	S
978	R	R	R	S	R	R	S	R	S	S	S	R	S	R	S	R	S	R	S	S	S	S	MS	S	S
990	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	R	S	MS	S	R	S	S	MS	S	S
1049	R	R	R	S	R	S	S	R	R	S	S	R	R	R	S	R	R	R	R	S	S	S	MS	S	S
1061	R	S	R	S	R	R	R	R	R	S	S	R	R	R	R	R	R	R	MS	S	S	R	R	S	S
1140	R	R	R	S	R	R	S	R	R	S	S	R	R	R	R	S	S	S	MS	S	S	S	S	S	S
1146	R	R	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R	R	R	R	R	S	S	S	S
1337	R	R	R	S	R	R	R	R	R	S	S	R	R	R	R	R	S	S	S	R	S	MS	MS	S	S
1339	R	S	R	S	R	R	S	R	R	S	S	R	R	R	MS	S	R	S	MS	S	S	S	MS	S	S
1344	R	R	R	R	R	R	S	R	R	R	S	R	R	R	R	R	S	R	S	S	S	S	S	S	S

1369	R	R	R	S	R	R	S	R	R	S	S	R	R	R	R	R	S	S	S	S	S	S	S	R	S
1380	R	R	R	S	R	R	S	R	R	S	S	R	S	R	R	R	S	S	S	S	S	S	S	R	S
1414	S	S	R	S	S	R	S	R	R	S	S	R	R	S	S	R	S	S	MS	S	S	MS	S	S	S
1418	R	R	R	MS	R	R	S	R	R	R	S	R	S	R	R	R	R	S	MS	S	S	S	S	S	S
1478	R	R	R	MS	R	S	R	R	R	S	S	R	R	R	R	R	R	S	MS	S	S	MS	S	S	R
1488	R	R	R	R	R	R	S	R	R	S	S	R	R	R	R	R	S	S	MS	S	S	S	S	S	S
1492	R	S	R	S	R	S	S	R	R	S	S	R	R	S	R	R	S	S	R	S	S	S	S	S	S
1494	R	R	R	S	R	R	S	R	R	S	S	R	R	R	MS	R	R	S	R	S	S	S	S	S	S
1498	R	R	R	R	R	R	S	R	R	S	S	R	R	R	R	R	S	R	R	R	S	S	S	R	S
1500	R	S	S	S	R	R	S	R	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S
1676	R	R	R	S	R	R	S	R	R	S	S	R	R	S	S	S	S	S	R	S	S	S	S	S	S
1713	R	R	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
1715	R	R	S	S	S	S	S	R	R	S	S	R	S	S	S	S	S	S	R	S	S	S	S	S	S

* All *H.paralvei* are “R” to CFZ (cefazolin), CEP (cefalotin), G (sulfiaxole), AMC (amoxicilin/clavucanic acid), CTX (cefotaxime)
** All *H. paralvei* are “S” to CTT (cefotetan), NOR (norfloxacin), LOM (lomefloxacin), SXT (Trimethoprimsulfamethoxazole)

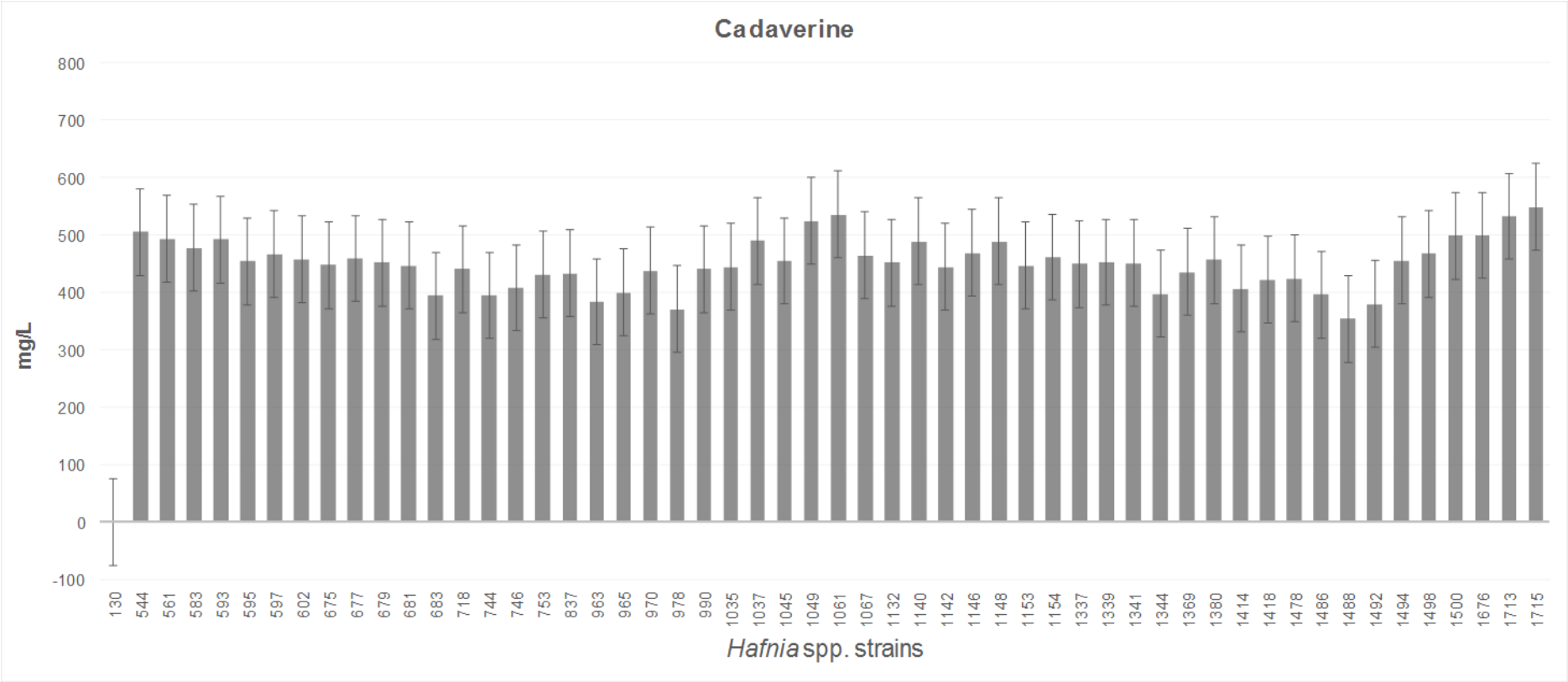


Figure S1. Cadaverine production of *Hafnia* spp. strains. Error bar reflects statistical significance difference calculated using Tukey's HSD test ($P \leq 0.05$).

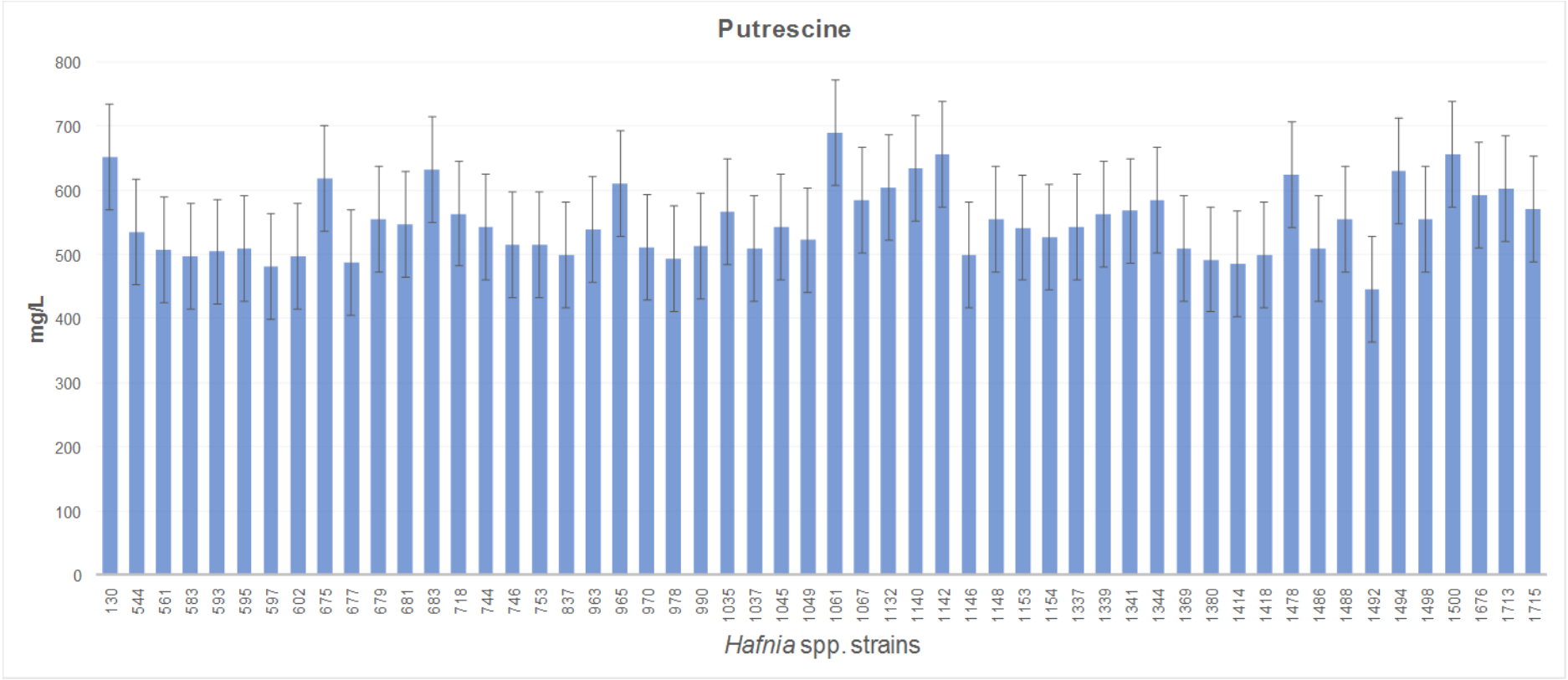


Figure S2. Putrescine production of *Hafnia* spp. strains. Error bar reflects statistical significance difference calculated using Tukey's HSD test ($P \leq 0.05$)

CAPÍTULO IV

Evaluation of the impact of enterobacteria with technological properties on the physico-chemical and sensory quality of raw sheep's milk cheeses

Capítulo IV.

Evaluation of the impact of enterobacteria with technological properties on the physico-chemical and sensory quality of raw sheep's milk cheeses

Abstract

Traditional soft-bodied cheeses such as "Torta del Casar" and "Queso de la Serena" are characterized by a lack of homogeneity in the final quality, which leads to a great problem. A solution to this problem would be the application of an autochthonous starter culture, which would contribute to the standardization of the final product. Lactic acid bacteria have been the most studied for this purpose, however, in recent years the role of other microorganisms such as *Enterobacteriaceae* has been highlighted. Studies for the selection and application of microorganisms belonging to the secondary microbiota are necessary to achieve a homogeneous product while maintaining their unique sensory characteristics. Thus, the aim of this work was to evaluate, in experimental cheeses, the contribution to the sensory properties of five *Enterobacteriaceae* strains previously selected for their technological properties in this thesis.

For this purpose, experimental cheeses with and without inoculation of autochthonous enterobacteria with technological properties were elaborated and their implantation and impact on the physical-chemical and sensory properties were evaluated. The results showed that the counts of the five *Enterobacteriaceae* strains increased significantly during ripening, adapting adequately to the cheese matrix. In relation to their impact on the properties of the cheese, two of the five strains inoculated in the experimental cheeses, *Hafnia paralvei* 593 and *Hafnia alvei* 1037, allowed to obtain a softer texture in cheese compared to the other strains of *Hafnia* spp. inoculated. In the sensory analysis, only significant differences were observed in the sensory parameter of firmness, while in the rest of the parameters (visual analysis of the pasta, intensity, persistence of aroma and global acceptance), no significant differences were observed between the inoculated cheeses and Controls batches. Finally, the analysis of principal components of the microbiological, physico-chemical and sensory parameters of the experimental cheese batches confirmed that the *Hafnia* spp. strains have a clear impact on the cheese ripening process. Although more studies are necessary, mainly based on the relationship with sensory parameters, four strains: *H. alvei* 544, *H. paralvei* 593, *H.*

alvei 1037 and *H. alvei* 1142 showed their suitability to form part of a mixed starter culture. to produce soft paste cheeses from raw sheep's milk.

IV.1. Introduction.

Raw milk cheeses have a very variable microbiota (bacteria, moulds and yeasts) that contribute positively to the final characteristics of the cheese, but also harmful because they cause alteration in the product or have a pathogenic character for humans. This great extensive and unpredictable variability is mainly associated with traditional cheeses. It is due to the use of raw milk, the absence of any thermal process of standardization and the non-application of starter cultures, along with different milking and handling protocols regarding milk (Pereira et al., 2010). Other factors that also have a significant influence on final quality are the non-standardized plant coagulant (Ordiales et al., 2014) and the industry microbiota (Bokulich & Mills, 2013).

As explained earlier in this thesis, microorganisms contribute directly to the biochemical changes that take place during cheese maturation. They are involved in the fermentation of lactose (glycolysis), the hydrolysis of proteins and other catabolic reactions during maturation (Menéndez et al., 2000; Kongo and Malcata, 2016), so they are essential and necessary in cheese maturation. In this sense, changes in the microbiota during cheese ripening translate into changes in biochemical processes, responsible for the final characteristics of the cheese. Consequently, the variability in the microbiota causes a lack of homogeneity in the final quality of the cheeses and distrust by the consumer.

Soft cheeses made with raw milk such as Torta del Casar and Queso de la Serena, are characterized by a very complex microbiota. This microbiota is dominated by several species of LAB, which reach the end of maturation at levels of 8-9 log cfu/g. Among the LAB, members of the genus *Lactococcus* and *Lactobacillus* stand out. The main species identified are *Lactobacillus casei, Lb. curvatus*, *Lb. diolivorans*, *Lb. paracasei*, *Lb. plantarum, Lb. rhamnosus*, *Lactococcus lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis* and *Leuconostoc mesenteroides* (Gonçalves et al., 2018; Ordiales et al., 2013; Sánchez-Juanes et al., 2020). Lactic acid bacteria have the ability to acidify due to lactose fermentation and the consequent production of lactic acid, which has an implication on the texture, taste, smell, aroma development and nutritional value in cheese (Dugat-Bony et al., 2015; Menéndez et al., 2000; Pereira et al., 2010). In addition, they also act as an

inhibiting agent of the undesirable flora (Heredia-Castro et al., 2017). To a lesser extent than LAB, we find mainly enterobacteria, gram-positive catalase positive cocci, *Pseudomonas* and yeasts (Ordiales et al., 2013), which contribute to the sensory properties of the final product (Montel et al., 2014). However, some of these microorganisms can cause product alteration or even be pathogenic. Among these microorganisms we can highlight different species of enterobacteria such as *Hafnia alvei, Hafnia paralvei, Escherichia coli, Salmonella* spp., species of the genus *Pseudomonas, Staphylococcus aureus, Listeria monocytogenes* and yeasts mainly of the *Kluyveromyces, Yarrowia, Geotrichum* and *Debaryomyces* genera. Some of these microorganisms, such as *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes,* pose a potential threat to public health (Almeida et al., 2007). These microorganisms may be present during maturation however, they tend to disappear at the end of it due to changes in pH, humidity, salt concentration, appearance of bacteriocins and nutrient competition (Elotmani et al., 2002; Fox et al., 2017).

Microorganisms in raw milk cheeses can cause different types of alterations. Among the most frequent is the appearance of pigmentations in the rind and core that cause the rejection of the product by the consumer. Various microorganisms have been associated with this alteration in raw milk cheeses, such as some species of *Pseudomonas (Pseudomonas fluorescens, Ps. putida, Ps. palleroni, Ps. gessardii*) produce fluorescent, green and blue pigmentations (del Olmo et al., 2018) and enterobacteria *Serratia marcenses* causes red pigmentation (Alberghini et al., 2010). In addition to bacteria, yeasts associated with raw milk cheeses such as *Yarrowia lipolytica* are responsible for brown pigmentations (Groenewald et al., 2014) and the development of molds on the surface causes colorations and damages the rind (Ledenbach and Marshall, 2010). On the other hand, some microorganisms that appear during maturation stages are gas producers, such as certain species of enterobacteria, *Clostridum* spp., heterofermentative LAB, propionibacteria and yeast species causing eyes or swelling (Fröhlich-Wyder et al., 2019; Lu and Wang, 2017; Tabla et al., 2016, 2018). Finally, the presence of certain microorganisms during maturation can cause undesirable aromas due to their metabolic activities, such as the formation of excessive amounts of volatile sulfur compounds (VSC) produced by *Brevibacterium linens* or another enterobacteria (Irlinger et al., 2012). Also, in the final organoleptic characteristics can be detected a bitterness or excessive formation of free fatty acids caused by lipolysis due to the action of heterofermentative LAB,

Pseudomonas, enterobacteria and yeasts (Quigley et al., 2013). All these alterations and potential presence of pathogens are minimal in the production of cheeses on an industrial scale, where heat treatment is applied to the milk followed by the inoculation of a starter culture. Therefore, milk pasteurization ensures safety and reduces variability in the final product. This entails changes with respect to the original sensory characteristics (Montel et al., 2014) giving rise to cheeses with flatter aromas than traditional raw milk cheeses.

Both PDO "Queso de la Serena" and "Torta del Casar" currently do not allow the application of a heat treatment to the milk or the application of a starter culture for the standardization of the final product. However, this route could be an alternative to overcome the lack of homogeneity of these traditional cheeses and thus obtain a consistent and safe quality product (Araújo-Rodrigues et al., 2020). However, to achieve this objective it would be necessary to develop a mixed autochthonous starter culture, with microorganisms adapted to cheese maturation process and that contribute positively to the biochemical changes that occur during maturation, in order to achieve a final product with an aromatic profile similar to a traditional raw milk cheese (Montel et al., 2014). This potential initiator culture should comprise different species of LAB and representatives of the secondary microbiota such as enterobacteria and yeasts, all of them with adequate technological properties and without causing alteration of the product.

Enterobacteria in cheeses: their use as starter culture

The microbiological control of milk is a very important factor to produce cheese and other dairy products. Therefore, it is advisable to comply with hygienic measures such as good practices during milking, cleaning and preventive maintenance of equipment and storage of milk in suitable aseptic containers, in order to avoid possible contamination, especially when the milk will not undergo any heat treatment as it happens with traditional raw milk cheeses. Enterobacteria are part of the secondary microbiota and are usually considered as indicators of fecal contamination. Mainly, they are present in milk in greater numbers when there are bad handling practices or a lack of hygiene. Several authors have found stable counts during cheese ripening process between 6.8 and 7.33 log cfu/g (Chaves-López et al., 2006; Gonçalves et al., 2018; Morales et al., 2004; Ordiales et al., 2013; Tabla et al., 2016).

Some enterobacteria can behave as spoilage, mainly causing eyes on cheese by the production of gas or pathogenic bacteria such as *E. coli* or *Salmonella* spp. Hence, they

can pose a public health problem due to the presence of pathogenic species (Irlinger et al., 2015). On the one hand, species belonging to *Escherichia*, *Enterobacter* and *Klebsiella* genera can cause defects in texture and flavor, produce biogenic amines and present resistance to antibiotics (Quigley et al., 2013; Tornadijo et al., 2001). On the other hand, some species such as *Hafnia alvei* and *Hafnia paralvei* have been frequently found in raw milk and derived products (Ercolini et al., 2009), being the predominant species at the end of the maturation process in cheeses such as "Serra da *Estrela*" (Tavaria et al., 1998), Cheese "*Serpa*" (Gonçalves et al., 2018), "*Alberquilla*" (Abriouel et al., 2008) and "*Torta del Casar*" (Ordiales et al., 2013; Tabla et al., 2016). Some strains of this genus have been selected for this study, as they could contribute to the final sensory properties of traditional raw sheep's milk cheeses. *Hafnia* spp. adapts positively to the ripening conditions of cheese, and, in fact, recent studies have proposed some strains of the species *Hafnia alvei* and *Hafnia paralvei* as suitable for use as starter cultures in cheese (Bourdichon et al., 2012; Ristagno et al., 2013). Even their positive contribution to the final taste has been demonstrated when applied to the product directly, as they can improve it by producing aromatic sulfur compounds and due to their high metabolic activity (Morales et al., 2003a, b). In addition, inoculation of *H. alvei* at 6 log cfu/mL to cheese prevents the growth of *E. coli* (Callon et al., 2015). The specie *Hafnia alvei* is capable of secreting enzymes such as L-methioninase with medical applications (Alshehri, 2020).

So, the aim of this work is to evaluate in a model cheese the impact of enterobacteria selected for their technological properties on the characteristics of the final product. To this end, the implantation of the strains of enterobacteria inoculated in the cheese and their impact on the physico-chemical, sensory parameters and the texture of the model cheeses will be validated.

IV.2. Material and Methods

For the development of this study, a set of *Hafnia* spp. strains isolated from soft cheeses of sheep's milk from the Extremadura region, previously selected for their technological properties in the previous chapter, was used. The *Hafnia* spp. strains used are listed in table 1.

Table 1. Lab code, isolate code, identification as species level and technological characteristics of strains of *Hafnia* spp. used in this study.

Lab code	Isolate code	Identification	Technological characterisitics
3	544	*H. alvei*	Proteolytic; Non-gas producer
6	593	*H. paralvei*	Proteolytic; modereate gas producer
26	1037	*H. alvei*	Non-Proteolytic; Non-gas producer
33	1142	*H. alvei*	Proteolytic; mild gas producer
54	1713	*H. paralvei*	Proteolytic; high gas producer

In addition, *Lactobacillus casei/ paracasei* UEX_Lc12 was used as a starter strain in the production of experimental cheeses, previously isolated from "Torta del Casar" cheeses (Ordiales et al., 2013). For the elaboration of the experimental cheeses, raw sheep milk belonging to the Merino and Entrefino trunk was used, ceded by the company COOPRADO. As a coagulating agent in the elaboration of the experimental cheese, the flower of the thistle *Cynara cardunculus* L. was used, given by the cheese industry El Castúo (PDO Torta del Casar).

Experimental cheese procedure

Starter culture and coagulant preparation.

Hafnia spp. strains and *Lb. casei*/*paracasei* UEX_Lc12 strain were subcultured twice prior to their incorporation into the milk. The enterobacteria were grown in VRBG agar at 30ºC for 24 h and the *Lb. casei/paracasei* strain in MRS broth at 30ºC for 48 h.

In order to prepare a concentrated solution of the microorganisms to be inoculated, from a VRBG agar plate each enterobacterium was grown in 20 mL of Trypticase Soy Broth (TSB) for 24 h at 30ºC. In the case of *Lb. casei/ paracasei* UEX_Lc12, from a plate of MRS agar it was grown in 10 mL of MRS broth at 30ºC for 24 h. Once grown, the cultures were centrifuged at 6000 *g* for 5 min, washed once with sterile water and resuspended in 10 mL of sterile water. After this, the solutions of microorganisms were stirred in vortex until a homogeneous solution was achieved. Then, because the solutions had a high concentration of cells, they were diluted 1000 times. A volume of 10 µL was then pipetted into Neubauer's chamber to be able to perform the cell count by visualization under the microscope. Three determinations were made for each solution of microorganism. Once the cell count has been performed and the arithmetic mean has been

calculated ($\bar{x}$), the appropriate Neubauer chamber formula was applied to calculate the concentration of the initial solution. The formula applied for the count is shown below: $[\bar{x} \times 2.5 \times 10^5] \times$ **dilution**.

Once calculated the concentration of the initial solution of each enterobacteria and *Lb. casei/paracasei* UEX_Lc12, a 50 mL solution was prepared with a concentration of each enterobacteria at 10^8 cfu/mL and 5 mL at 10^8 cfu/mL of *Lb. casei/paracasei* UEX_Lc12. The entire volume of the solutions to be inoculated was added to 5 L of sheep's milk with the aim of achieving a concentration of the enterobacteria under study of 10^6 cfu/mL and 10^5 cfu/mL of the starter culture of *Lb. casei/paracasei* UEX_Lc12.

On the other hand, for the extraction of the enzymatic coagulant from the thistle flower *Cynara cardunculus* L., it was based on homogenizing 25 g of the dried flowers in 500 mL of distilled water in an Erlenmeyer flask of 1 L and maceration under fixed conditions of agitation for 24 h at 15 ºC. Subsequently, a gross filtration was carried out using filter paper followed by a centrifugation in a centrifuge tube of 50 mL at 5000 *g* for 5 minutes, in order to eliminate most impurities. The liquid phase was carefully transferred into another new 50 mL sterile centrifuge tube removing impurities deposited at the bottom of the tube. In the elaboration of the cheese, 10 mL of flower extract per liter of milk was inoculated.

Making and maturation of experimental cheese in laboratory.

To study the impact of the enterobacteria selected in the previous chapter for their technological properties on the sensory properties of soft sheep cheeses, a total of 7 batches inoculated were elaborated using the combinations of microorganisms exposed in Table 2. Each batch was composed of 3 cheeses.

Table 2. Summary table of the batches made with the selected strains.

Batch code	**Inoculated cultures**
Control (C)	No inoculated
LAB	*Lb. casei* UEX_LC12
H3	*H. alvei* 544 + *Lb. casei* UEX_LC12
H6	*H. paralvei* 593 + *Lb. casei* UEX_LC12
H26	*H. alvei* 1037 + *Lb. casei* UEX_LC12
H33	*H. alvei* 1142 + *Lb. casei* UEX_LC12
H54	*H. paralvei* 1713 + *Lb. casei* UEX_LC12

Figure 1 shows the flowchart of the manufacturing process.

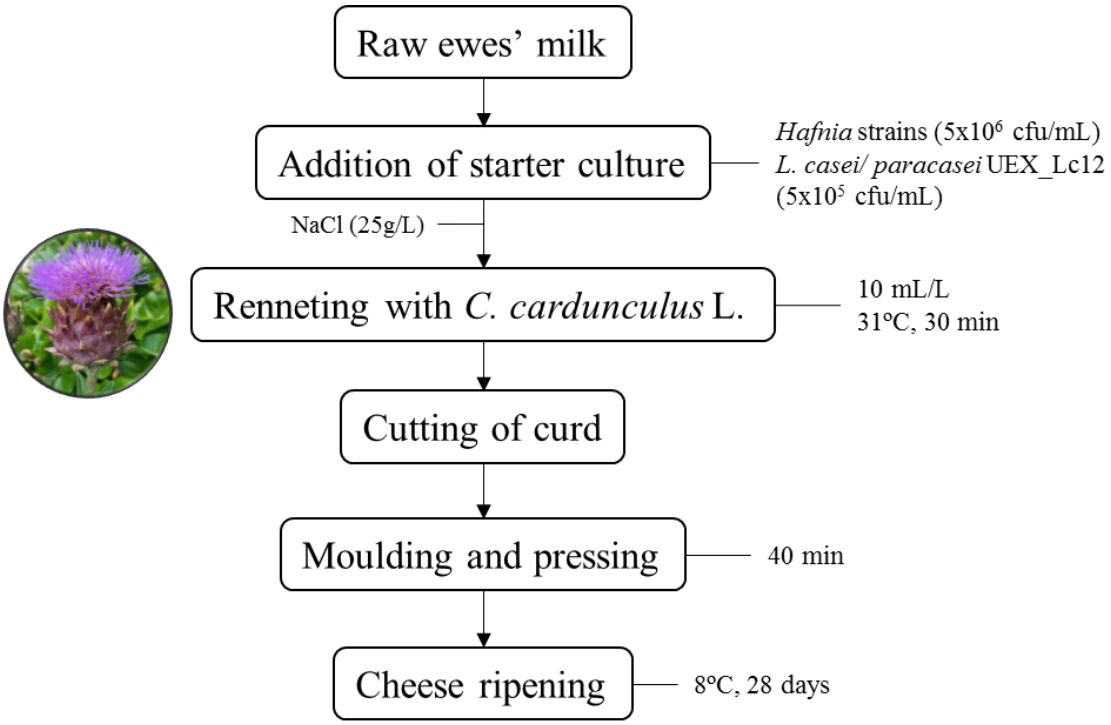


Figure 1. Flowchart of the experimental cheese-making process.

In the first place, prior to the elaboration of each cheese, the inoculums corresponding to the batch to be elaborated were prepared, in their corresponding volumes and quantities. In addition, the flower extract of *Cynara cardunculus* L. was prepared and 10 mL/L of milk was added for a time of 30 min. Finally, for each liter of milk, 25 g NaCl/L were added based on the salt content found in soft-paste sheep cheeses studied in this thesis. The elaboration of the cheeses was carried out in the pilot plant of the School of Agricultural Engineering using cheesemaking material, vat, automatic press and liras for the curd cutting.

Once all the ingredients were prepared, 5 liters of raw sheep's milk from the Entrefino and Merino trunk was used for each batch, to obtain three cheeses of approximately 300 gr. The milk was added to a 25 L container and proceeded to the inoculation of the respective enterobacteria inoculums and LAB, according to the batch and salt. After a proper mixing of all the components (milk, vegetable coagulant and starter culture) added with a sterile spatula, the container was deposited in a thermostatic bath at 31ºC for 20 minutes for the coagulation of the milk (figure 2A).

A  B 

Figure 2. Cheese curd (A) and cutting of curd (B)

After the coagulation time and after checking the correct state of the curd, the container was extracted from the thermostatic bath and then proceeded to cut the curd with lyre until a granulated appearance was achieved, with a size similar to the grain of rice (Figure 2B). Then, the molds were filled manually, eliminating as much whey as possible. Once the mold was full, it was placed in an automatic press (figure 3) in which it was subjected to constant pressure for 40 minutes at 100-120 psi. During this period of time, a large volume of serum was removed, and the curd was compacted acquiring the shape.

Figure 3. Press used to make experimental cheeses.

Finally, the cheeses were carefully extracted from the mold and placed on a refrigerated stove (JP Selecta mod. HOTCOLD A-B-C) at a ripening temperature of 8ºC for 28 days (figure 4).

Figure 4. Experimental cheeses with 5 days of ripening.

Microbial counts.

Microbiological analyses were carried out at 0 days and at the end of ripening process (28 days). Ten grams of the core of each cheese sample were taken aseptically into sterile plastic bags and diluted with 90 mL of sterile 1% peptone water, homogenized for 60 s using a laboratory stomacher 400 Circulator Seward (Worthing, West Sussex, UK). Serial 10-dilutions were prepared and inoculated onto agar plates. Plate count agar medium (PCA, Condalab, Spain) was used for mesophilic aerobic bacteria counts, incubating at 30 ºC for 48 h. Lactic acid bacteria (LAB) were cultured on Man, Rogosa and Sharpe agar (MRS, Condalab) supplemented with acetic acid at 10% (v/v) at 30 ºC for 2 days. For enterobacteria count, Violet Red Bile Glucose (VRBG, Condalab) agar was used, incubating at 30 ºC for 24 h.

For the routine growth of the enterobacteria prior to the elaborations of the cheeses, Trypticase Soy Broth (TSB) was used. The microbiological analysis was performed in triplicate for each batch produced. The results obtained were expressed in logarithm of colony-forming units per gram of sample (log cfu/g).

Hafnia spp. strains implantation in the cheese matrix

The implantation of the inoculated *Hafnia* spp. strains was verified by RAPD-PCR of the colonies isolated at 28 days from the VRBG plates of the highest dilutions. Ten colonies per batch were randomly isolated and the DNA of each one was obtained using the commercial kit "GeneJET Genomic DNA Purification Kit" following the manufacturer's indications. To assess the quality and quantify the quantity of DNA, a NanoDrop 2000 visible UV spectrophotometer (Thermo Scientific™) was used. The DNA of each isolate was amplified using the RAPD-PCR technique and compared with the band profile of the inoculated strain obtained by the same PCR conditions. To do this, a PCR reaction was performed with M13 primer (5'-GAGGGTGGCGGCTCT-3', Huey & Hall, 1989). Each of the amplifications was carried out using 50 µL of a reaction mixture containing: 5 µL of DreamTaq Green Buffer 10X (KCl, $(NH_4)_2SO_4$ y $MgCl_2$ 20 mM), 1 µL of dNTP Mix (10 mM), 2,5 µl of M13 primer (10 µM), 0,25 µL of DreamTaq DNA Polymerase (5 U/ µL), 40,25 µL of sterile deionized water y 1 µl of DNA target (10 ng/µL). The amplification program that was carried out in the thermocycler (Eppendorf Mastercycler Nexus Gradient) consisted of an initial denaturation at 94ºC for 4 min, followed by 40 cycles of denaturation at 94ºC for 60 s, annealing at 40ºC for 20 s and amplification at 72ºC for 120 s, with a final extension at 72ºC for 10 min.

The amplification products were separated by electrophoresis in 1.5% agarose gels and detected by staining with "Midori Green Advanced DNA stain". To prepare the agarose gels, the TAE 1X buffer was used. This was made by dissolving 20 mL of TAE 50X pH 8.5 at 20ºC (EDTA · Na2 · 2H2O: 18.6 g/L (0,05 M), Acetic acid glacial: 60.05 g/L (1 M), Tris: 242.30 g/L (2 M); Applichem) in 980 mL of deionized water. The agarose used and dye for the visualization of the DNA bands were obtained from the commercial house Nippon Genetics. In addition, a molecular weight marker "Generuler 100 bp plus ladder" was used to determine the size of the RAPD-PCR products. The visualization of the RADP-PCR reaction was carried out using an Azure Biosystems c200 transilluminator. The analysis of the sizes of the molecular weights of the different bands obtained by RAPD-PCR in each isolate was carried out with the Gene Tools program, version 3.06 (Syngene, England).

Physico-chemical analyses.

pH determination.

The pH measurement of the cheeses produced was carried out using the Model Crison pH20. The pH was determined in each batch in triplicate at the end of the maturation process by measuring the pH to stability of a homogenized 2 gr of cheese in 20 mL of distilled water.

Water activity (a_w) determination.

The water activity (a_w) was determined by using a Novasina equipment with temperature control. The determinations were made starting from 3 or 4 g of each sample at a temperature of 25 ºC and with a balancing time of 30 min.

Texture analysis.

The evaluation of the rheological characteristics of the experimental cheeses was carried out through the study of the analysis of the texture profile in a texture analyzer model TA.XTPlus (STABLE MICRO SYSTEM). From each cheese made at the end of maturation, 6 samples were taken with a punch per batch obtaining cheese cylinders of approximately 1.5 cm in diameter and 2 cm in height. The cheeses were tempered to 20ºC before proceeding to the texture measurement. Once tempered, the texture was determined in triplicate in each portion by a compression test (TCA) using a 4 mm cylindrical probe and a 30 kg cell. The parameters of work were: Pre-test speed 2 mm/s; Test speed 1 mm/s; Post-test speed 1 mm/s and Distance 5 mm. The cheese firmness values (g) were calculated with the software version 5.0.4.0 (Stable Micro System).

Lipolysis index determination.

The determination of total acidity was performed following the method described by Dugat-Bony et al. (2015). For this, an acid-base titration was carried out, using KOH 0.025N as a titrating agent until reaching a color shift, phenolphthalein 1%, as a pH indicator added prior to the sample. For the realization of the acid-base titration, approximately 2.5 grams of sample were weighed in a beaker to which 50 mL of a neutralized ethanol-diethyl ether mixture (1:1 v/v) and 3-4 drops of phenolphthalein 1% were added. Subsequently, with the help of a burette containing the solution of KOH

0.025N, this solution was added drop by drop to the sample that was in constant movement in a magnetic stirrer. The titration was carried out until the color of the sample was turned. Each sample was performed in triplicate. The result was expressed in mg KOH/100g of cheese.

Protein content determination.

First, the total nitrogen content was determined using the Kjeldhal method (IDF 20; ISO 8968-1:2014) using a Kjeldahl digester/distiller (SELECTA PRO NITRO S) and converted into protein percentage using the 6.38 conversion factor. For this, the proteins and other organic components of the cheese were digested in a mixture with sulfuric acid in the presence of catalysts. The tubes with the samples were put into the digester with its proper smoke extraction system. The digestion was carried out in 3 steps, 150 ºC for 15 minutes, then the temperature was increased to 300ºC for 30 minutes and to finish 400ºC for 60 minutes. The total organic nitrogen was converted by this digestion into ammonium sulfate. Once the digestion was complete, the digestion tubes were allowed to cool to room temperature and then 100 mL of distilled water was added to each tube very slowly and moving the sample slightly without letting the sample solidify. The digested mixture was neutralized with a base and subsequently distilled. To do this, a semi-automatic distillation was made using 100 mL of 40% NaOH, collecting approximately 150 mL of distillate over 50 mL 4% boric acid in an Erlenmeyer flask of 250 mL. The borate anions thus formed were titled with standardized HCl 0.1 N using as an indicator 3-4 drops of the mixed indicator (methyl red 41 mg and bromocresol green 60 mg in 100 mL of ethanol) to determine the nitrogen contained in the sample. All samples were made in triplicate.

Secondly, Non-protein nitrogen (NPN) was determined by the Nessler method (Benito et al., 2005). Each sample (4 g) was homogenized in 0.6 N perchloric acid using an Omni Mixer Homogenizer. The homogenate was centrifuged for 10 min at 4000 rpm. The soluble fraction located between the upper layer and the precipitate was filtered through Whatman no. 54 filter paper. The pH was neutralized to pH 6 with 30% KOH. To eliminate the potassium perchlorate formed during neutralization, extracts were filtered again. Subsequently, 200 µL of the deproteinized solution was evaporated in a sand bath at 100ºC and then digested with 200 µL of 95% sulfuric acid in a sand bath at 120ºC. Then, 4.8 mL of distilled water, 3 mL of NaOH 4 N and 2 mL of Nessler reagent

were added successively. Finally, after incubation for 10 min in darkness, the samples were stirred in the vortex and the absorbance in the spectrophotometer (Shimadzu UV-1800) at 490 nm was determined. To determine the concentration of N in the reaction, a regression pattern line was made with known amounts of nitrogen using ammonium sulfate solutions at different concentrations as a reference. All determinations were made in triplicate. Finally, the proteolysis index in the experimental cheese batches was determined using the following equation:

Proteolysis index= ((Total Nitrogen mg/g cheese)/(NNP mg /g cheese))*100

Sensory analysis.

The evaluation of the sensory quality of the final product was carried out through a descriptive analysis carried out by a panel of 15 specialized panelists. The members of the panel were previously selected and trained under the standards of the International Organization for Standardization (ISO, 2006; UNE-ISO 4121:2006) with samples of commercial "Torta" cheese in the IB16038 project. All sessions were held in a sensory panel room conditioned at 20-22 °C and 60%-70% relative humidity in cabinets equipped with white light (6000 °C). Before the analysis, the cheeses were balanced to the temperature of the sensory analysis room. The sensory tasting was based primarily on the evaluation of olfactory and visual parameters since they are experimental cheeses with a maturation period of only 15 days, so cheeses do not have the maturation time of 60 days required for raw milk cheeses to make a safe tasting. Cheese samples approximately 0.5 cm thick were distributed to each panelist in closed 20 mL tubes.

The organoleptic parameters that were evaluated by the panelists are shown in the following questionnaire model (figure 5). In addition, the panelists made a hedonic assessment of the overall acceptability of the cheese samples.

ANÁLISIS SENSORIAL QUESO

*Obligatorio

1. Código Muestra *

TEST DESCRIPTIVO

ANÁLISIS VISUAL

2. Análisis visual de la pasta *

Marca solo un óvalo.

	1	2	3	4	5	6	7	8	9	
Poco color amarillo	◯	◯	◯	◯	◯	◯	◯	◯	◯	Mucho color amarillo

TEXTURA (SIN PROBAR)

3. Firmeza *

Marca solo un óvalo.

	1	2	3	4	5	6	7	8	9	
Poco	◯	◯	◯	◯	◯	◯	◯	◯	◯	Mucho

AROMA

4. Intensidad *

Marca solo un óvalo.

	1	2	3	4	5	6	7	8	9	
Poco	◯	◯	◯	◯	◯	◯	◯	◯	◯	Mucho

5. Persistencia *

Marca solo un óvalo.

	1	2	3	4	5	6	7	8	9	
Poco	◯	◯	◯	◯	◯	◯	◯	◯	◯	Mucho

6. En caso de detectar algún aroma, indicar cuál y su intensidad *

TEST HEDÓNICO

7. Valoración Global del queso. Aceptabilidad *

Marca solo un óvalo.

	1	2	3	4	5	6	7	8	9	
Muy mala	◯	◯	◯	◯	◯	◯	◯	◯	◯	Muy buena

Figure 5. Descriptive and hedonic test.

Statistical data analysis.

The data obtained were analyzed with the SPSS program for Windows version 21.0 (SPSS Inc Chicago, IL, USA). Significant differences and homogeneous groups of means were established by analysis of variance (ANOVA) following one-way procedures. When the effect of the interaction is significant($P \leq 0.05$) we proceeded to perform a mean comparison analysis by the TUKEY method that determines the minimum difference between the means of each group so that it is statistically significant. Through principal component analysis (PCA), the effect of the inoculated enterobacteria strains on the microbiological, physico-chemical and sensory parameters was determined.

IV.3. Results and discussion.

Microbial counts of experimental cheeses.

The results of mesophilic aerobic bacteria count grown in the PCA agar medium at the beginning and end of the maturation of the different batches are shown in Figure 6. These results showed significant differences*(P*<0.05) in all batches between the beginning and end of maturation (0-28 days). These show a variation in the initial microbial load, being the lowest counts in the control batch, with 4.35 log cfu/g, since it was not inoculated with any bacteria and has the microbiota of the raw milk used. On the other hand, the LAB batch inoculated with the starter LAB showed 4.85 log cfu/g, which were also significantly lower than the rest of the batches inoculated with the selected enterobacteria, and the LAB. Batches inoculated with enterobacteria showed values ranging from 6.65 log cfu/g for H26 batch to 7.06 log cfu/g in H6 batch. This variation in the initial microbial load between batches is logical, as the control batch was not inoculated with any microorganisms and the BAL batch was inoculated with *Lb. casei/paracasei* UEX_Lc12 at a dose of 10^5 cfu/mL of milk, while the enterobacteria batches were inoculated with a 10 times higher number of cells.

At 28 days of ripening, a significant increase in total counts was observed in all batches compared to the beginning of maturation (Figure 6). Counts ranged from 8.24 log cfu/g obtained in H54 batch to a maximum of 9.27 log cfu/g in H6 batch. Control batch, with 9.03 log cfu/g showed counts similar to the LAB batch (9.06 log cfu/g). These results indicate that both the microbiota of the raw milk and the bacteria inoculated in the different batches adapted adequately to the maturation process. The final counts obtained from total bacteria are similar to those obtained in raw sheep's milk cheeses with a similar maturation time (Araújo-Rodrigues et al., 2020; Gonçalves et al., 2018). Instead, almost all enterobacteria batches showed total bacterial counts equal to or greater than the Control batch. The evolution of the microbiota during maturation depends on several factors, such as initial microbiology, added starter culture, ripening conditions, equipment microbiology, among others (Bokulich and Mills, 2013; Hayaloglu, 2016).

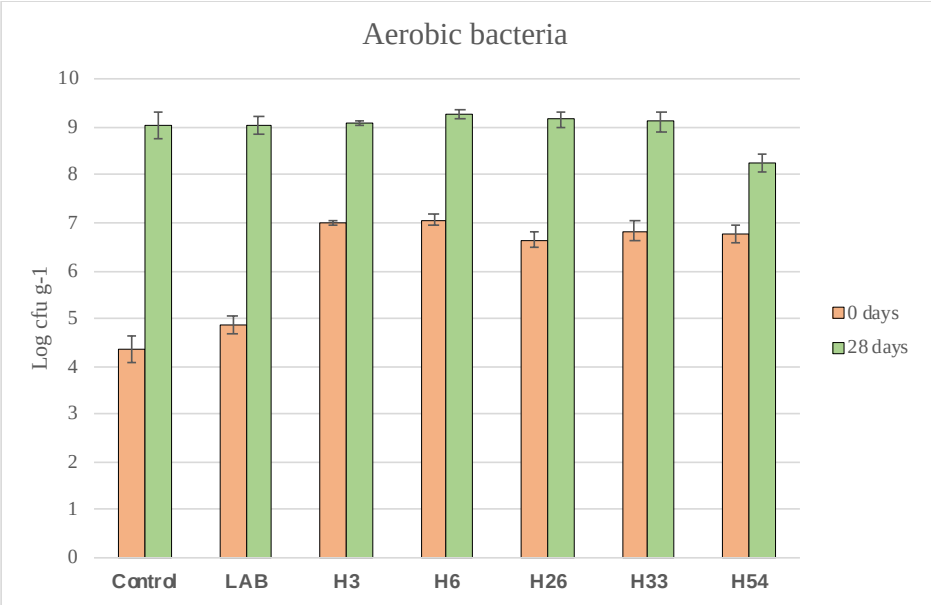


Figure 6. Mean values of mesophilic aerobic bacteria (PCA) at 0 and 28 days of maturation, expressed in log cfu/g. Error bars represent the standard deviation.

Figure 7 shows the bacterial counts at 28 days of cheese ripening and the statistical differences between the batches processed. In this way, it is shown how all batches showed no difference in total bacteria counts at the end of maturation, except for H54 batch which showed significantly lower counts.

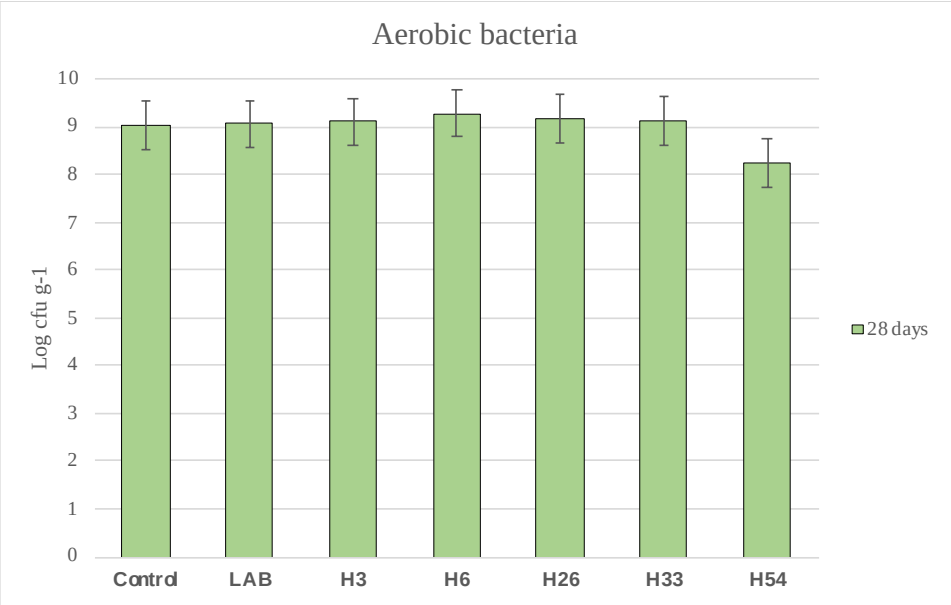


Figure 7. Mean values of mesophilic aerobic bacteria (PCA) at 28 days of maturation, expressed in log cfu/g. The error bars represent the 95% confidence interval of significant differences obtained by Tukey's HDS test.

Figure 8 shows the results of the LAB counts obtained in acidified MRS agar of the experimental cheeses at 0 and 28 days of maturation. The LAB counts in the Control batch at the beginning of maturation were significantly lower than the rest of the batches, which had been inoculated with a strain of *Lb. casei/paracasei* as starter culture. Regarding the enterobacteria batches, all presented average counts higher than the LAB batch at the beginning of maturation, although all the batches inoculated with the starter LAB showed counts of 5 log cfu/g due to the inoculation of this microorganism.

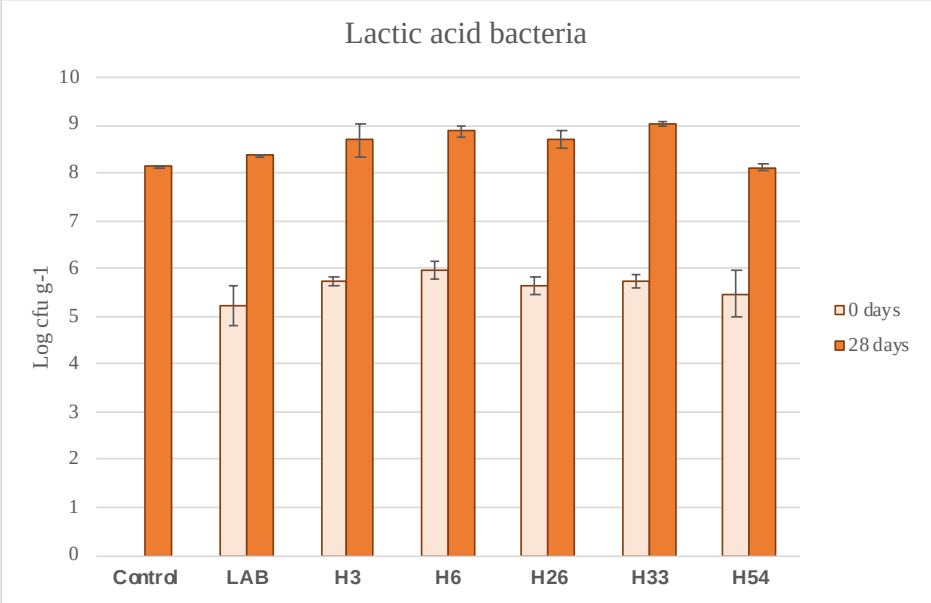


Figure 8. Mean values of lactic acid bacteria (MRS) at 0 and 28 days of maturation, expressed in log cfu/g. Error bars represent the standard deviation.

Figure 9 shows LAB counts at the end of the ripening process and statistical differences between batches. LAB counts increased significantly in all batches during maturation, between 0 and 28 days of maturation ($P<0.05$). The lowest values were detected in the Control batch with 8.13 log cfu/g and the highest in H6 batch with 8.87 log cfu/g. Therefore, the values obtained in MRS agar at 28 days of maturation are similar to those obtained in PCA agar for mesophilic aerobic bacteria. LAB are mainly responsible for the cheese ripening process, being the dominant group in traditional raw milk cheeses and in pasteurized milk cheeses with starter culture (Kongo and Malcata, 2016). In this sense, in previous studies of torta-type cheeses, it has been shown that they are part of the main microbiota and their values were 8-9 log cfu/g at the end of the cheese ripening in previous studies carried out (Gonçalves et al., 2018; Ordiales et al., 2013),

similar to that found in the experimental cheeses of this study. They contribute to the biochemical changes that occur along cheese ripening such as glycolysis, proteolysis and lipolysis that determine the final characteristics of the cheese. Among them, they mainly stand out for the consumption of lactose and lactic acid production, that causes a decrease in pH and limits the growth of altering and pathogenic microorganisms in the cheese matrix (Hayaloglu, 2016; Kongo and Malcata, 2016).

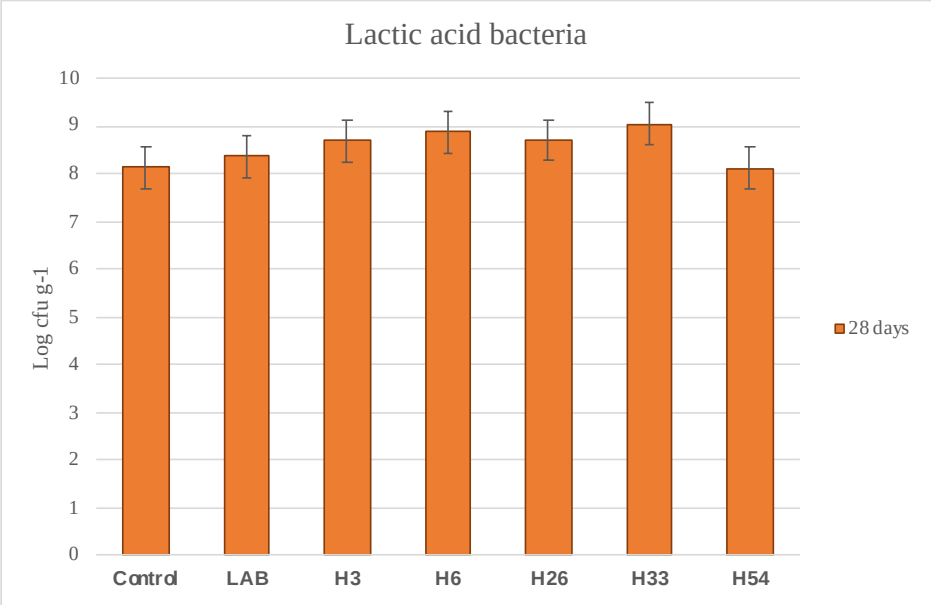


Figure 9. Mean values of lactic acid bacteria (MRS) at 28 days of maturation, expressed in log cfu/g. The error bars represent the 95% confidence interval of significant differences obtained by Tukey's HDS test.

Figure 10 shows enterobacterial counts in VRBG agar at 0 and 28 days of maturation. At the beginning of maturation, in the batches not inoculated with enterobacteria (Control and LAB), the counts were significantly lower, with values of 3.20 log cfu/g in Control batch and 3.79 log cfu/g in LAB batch. In batches inoculated with different enterobacteria, initial counts ranged from 6.61 log cfu/g in H26 batch to 7.23 log cfu/g in H54 batch. In this sense, all enterobacteria were inoculated at similar levels of approximately 10^6-10^7 bacteria per gram of cheese, in order to see their impact on the sensory properties of cheese and its ability to be used as starter culture in "torta" cheese.

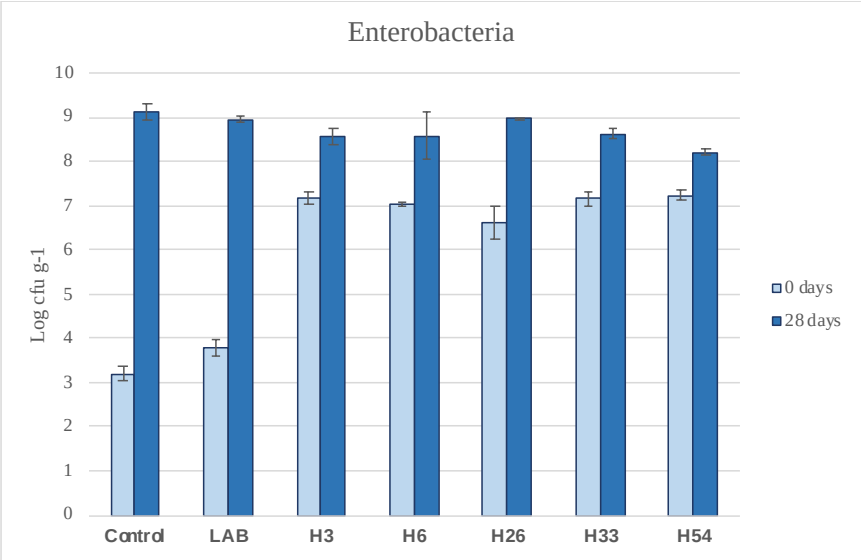


Figure 10. Mean values of enterobacteria (VRBG) at 0 and 28 days of maturation, expressed in log cfu/g. Error bars represent the standard deviation.

Figure 11 shows the enterobacteria counts and significant differences between the batches made of enterobacteria at 28 days of maturation. No significant differences were observed between batches except for H54 which showed values lower than the Control, LAB and H26. In all batches, both inoculated with enterobacteria, Control and LAB, mean enterobacterial counts increased significantly *(P*< 0.05) at the end of the ripening process of experimental cheeses.

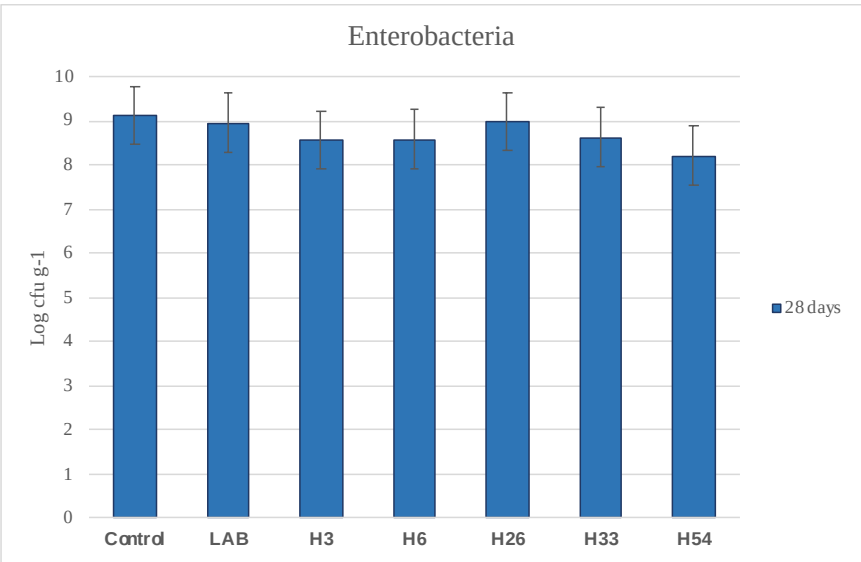


Figure 11. Mean values of enterobacteria (VRBG) at 28 days of maturation, expressed in log cfu/g. The error bars represent the 95% confidence interval of significant differences obtained by Tukey's HDS test.

Enterobacteria, and specifically the species of the genus *Hafnia* spp., have been shown to be adapted to different stresses, pH, % NaCl and temperature found during ripening process of soft-paste cheeses made with raw ewes' milk. Several studies have found that they are part of the secondary microbiota, being the predominant enterobacterial species at the end of maturation (Abriouel et al., 2008; Gonçalves et al., 2018; Morandi et al., 2021; Ordiales et al., 2013).

Hafnia spp. strains implantation in the cheese matrix.

To confirm the presence of *Hafnia* spp. strains inoculated at high levels at the end of ripening process, the detection of the strains was carried out using RAPD-PCR techniques with M13 primer. Banding pattern of the inoculated strains was compared with enterobacteria isolated from the highest dilutions. The results obtained showed that all inoculated strains were present in the highest dilutions at 28 days of maturation. Figure 12 shows an example of agarose gel with band pattern of the inoculated strain (standards) and enterobacteria isolates of batches H26 and H6. RAPD-PCR profiles of isolated showed identical pattern to standards from each batch. With these results, the implantation of the strains can be confirmed due to their ability to resist maturation conditions, as previously studied in this thesis.

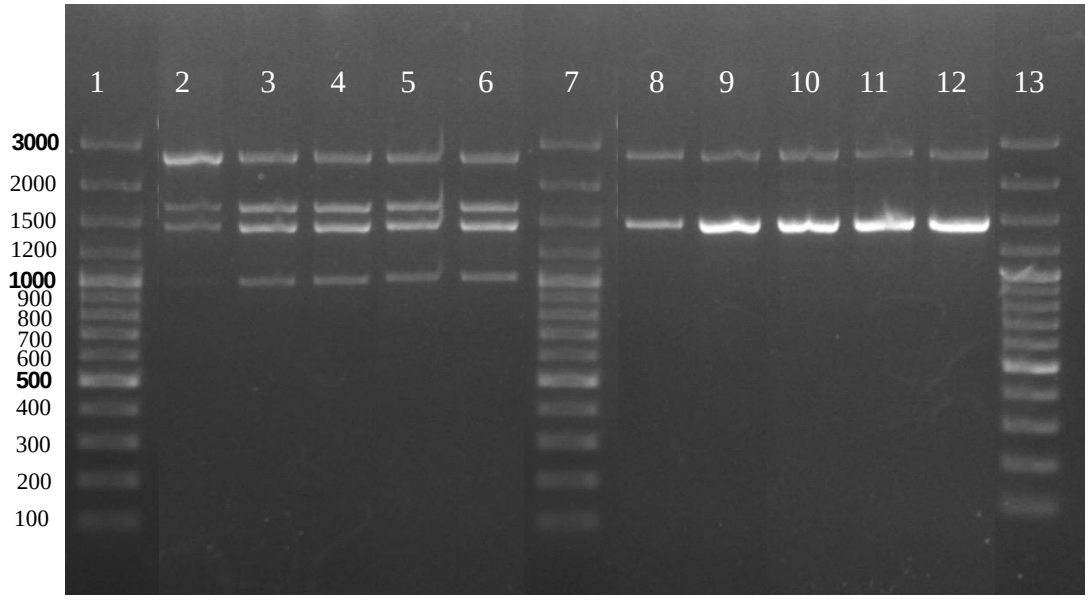


Figure 12. RAPD-PCR profile of *Hafnia* spp. strains isolated from 7th decimal dilution of the cheeses inoculated with *Hafnia* 26 and *Hafnia* 6. Lines 1, 7 and 13, molecular size marker. Banding pattern from H26 batch found in lines 2 to 6 (line 2, standard H26); banding pattern from H6 batch found in lines 8 to 12 (line 8, standard H6).

Evaluation of physico-chemical analysis in experimental cheese.

Table 3 shows the mean values of the physico-chemical parameters analyzed at 28 days of maturation in the different experimental cheese batches. These parameters were: pH, $a_{w,}$ acidity (mg KOH/g cheese), proteolysis index and firmness.

The mean values of a_w was 0.958, with the lowest value being 0.948 of H3 batch and the highest value being 0.968 of H6 batch. In general, the a_w values obtained are in accordance with what is obtained in cheeses made with raw sheep's milk and similar ripening conditions (Gonçalves et al., 2018) and that allow the growth of enterobacteria as species of the genus *Hafnia.* As for the pH, the average of the values of all the batches was 5.83, being the lowest value 5.63 belonging to the H26 batch and the highest value 6.20 of the Control batch. In general, torta cheeses are characterized by intense proteolysis due to the action of the vegetable coagulant and the microbiota, that generates nitrogenous compounds that buffer the pH. Thus, these cheeses usually present high values (pH 5.5-6) at the end of maturation (Gonçalves et al., 2018; Ordiales et al., 2013). Apart from the H26 strain, the rest of the strains showed an intense *in-vitro* proteolysis that can cause nitrogenous compounds that buffer the low pH by the production of lactic acid from the LAB. This may explain the lower pH values in H26 batch, which also had a lower mean proteolysis index value. Conversely, the Control batch was the one with the highest pH value and proteolysis index. This batch, although not inoculated and the final microbiota is due to the microbiota of the raw milk used, was the one that presented the highest counts of mesophilic aerobic bacteria and enterobacteria (figures 6 and 10). The *Enterobacteriaceae* species associated with cheese are characterized by having a high proteolytic capacity that generates nitrogen compounds that raise the pH (Morales et al., 2003b).

Lipolysis and proteolysis are the main biochemical changes that determine the final sensory characteristics of cheese (Kongo and Malcata, 2016). In this study, lipolysis was assessed by determining acidity. The mean value of all batches was 3.9, while the results ranged from 1.61 for batch H33 to 5.37 for batch H6, with the value of the Control batch being 4.28. However, the lipolytic activity of *Hafnia* spp. it is null or limited (Irlinger et al., 2012). Therefore, the differences between the batches in acidity may be due to the development of the indigenous microbiota in relation to the inoculated bacteria. On the contrary, the species *H. alvei* and *H. paraalvei* are characterized by having an

intense proteolytic capacity (Morales et al., 2003b). This activity has a great impact on the final characteristics of the cheese such as pH, aroma and texture. The mean value of proteolysis in the batches was 7.23, with 4.62 being the lowest value, batch H26 (non-proteolytic strain *in-vitro*), and the highest value was 11.13 in the Control batch. The proteolytic activity together with other factors, such as pH, has a strong impact on the texture (Montel et al., 2014). Cheeses with high pH generally have a softer texture than acid cheeses (McSweeney, 2004). However, the firmness data were significantly lower in batch H26, contrary to expectations, since it presented less proteolysis and more acidic pH. In general, a relationship between proteolysis, pH and firmness values of the processed batches was not observed. Texture is one of the most appreciated characteristics of Torta-type cheeses (Ordiales et al., 2014). In the experimental cheeses made, the mean value was 286.73 g, observing significant differences (P <0.05) between batches. The lowest value was 86.64 from batch H26 and the highest value was 585.95 g from batch H54. Two of the batches inoculated with *Hafnia* spp. strains, H6 and H26, showed lower mean values than the LAB and Control batch. Therefore, strains H6 and H26 contributed positively to the final texture of the cheese.

Table 3. Mean values of the physico-chemical parameters analyzed after 28 days of maturation in the different cheese batches. Different letters note significant differences obtained by Tukey's HDS test.

Parameters		**pH**			**a_w**			**Acidity (mg KOH/g)**			**Proteolysis index**			**Firmness (g)**		
Batches	N	Mean		SD*	Mean		SD	Mean		SD	Mean		SD	Mean		SD
Control	3	$6,20^{c**}$	±	0,12	$0,960^{d}$	±	0,002	$4,28^{b,c}$	±	0,66	$11,13^{d}$	±	0,98	$210,34^{b}$	±	59,26
LAB	3	$5,98^{b}$	±	0,02	$0,967^{e}$	±	0,001	$3,90^{b,c}$	±	0,40	$5,79^{a,b}$	±	1,08	$317,08^{c,d}$	±	79,32
H3	3	$5,66^{a}$	±	0,02	$0,948^{a}$	±	0,001	$4,91^{b,c}$	±	0,64	$8,06^{b,c}$	±	1,29	$232,08^{b,c}$	±	91,34
H6	3	$5,68^{a}$	±	0,02	$0,968^{e}$	±	0,001	$5,37^{c}$	±	0,64	$8,79^{c,d}$	±	2,04	$163,96^{a,b}$	±	57,23
H26	3	$5,63^{a}$	±	0,03	$0,955^{c}$	±	0,001	$3,88^{b,c}$	±	0,60	$4,62^{a}$	±	1,29	$86,64^{a}$	±	21,30
H33	3	$5,67^{a}$	±	0,05	$0,958^{d}$	±	0,001	$1,61^{a}$	±	0,81	$5,47^{a,b}$	±	1,79	$406,52^{d}$	±	111,15
H54	3	$5,97^{b}$	±	0,08	$0,951^{b}$	±	0,001	$3,36^{b}$	±	0,27	$6,75^{a,b,c}$	±	1,57	$585,95^{e}$	±	172,27
Total	21	$5,83^{b}$	±	0,22	0,958	±	0,007	3,90	±	1,26	7,23	±	2,52	286,73	±	181,31
Significance (*P*)		0,000			0,000			0,000			0,000			0,000		

*SD: standard deviation

** For each batch the data in the same column with different letters is significantly different ($P \leq 0,05$)

Evaluation of sensory parameters in experimental cheese.

Table 4 shows the mean values of the sensory parameters analyzed at 28 days of ripening in the different cheese batches. The species of the genus *Hafnia* spp., although they are members of the secondary microbiota in traditional cheeses (Gonçalves et al., 2018) can contribute significantly to their sensory characteristics. The presence of these microorganisms can be positive or negative in the characteristics of the cheese according to its metabolic activities (Irlinger et al., 2012; Tabla et al., 2018). This makes it necessary to know their impact on the sensory properties for their use as members of a starter culture.

In relation to the mean values obtained in each batch for the parameters evaluated in the sensory analysis on a scale from 0 to 9, the scores showed some variability. Except for firmness, no significant differences were observed in the rest of the parameters studied and in the overall acceptability between batches. As in the instrumental analysis of firmness, the lowest mean value was in H26 batch and the highest in H54 batch. Except for H54 batch, none of the batches inoculated with *Hafnia* spp. showed significant differences in sensory firmness. In the visual analysis of the paste, the mean scores were in the range of 4.46 to 6, being the lowest value in the Control batch and the highest value in the H3 batch. In the intensity and persistence parameters, the mean values obtained were 6.05 and 5.27, respectively. In both parameters, the LAB batch was the one with the lowest mean values and the H3 batch, the highest. Finally, in the global acceptance, the mean was 6.22, the lowest value being 5.86, belonging to LAB batch and the highest value was 7, belonging to H26 batch with less firmness. Apart from batch H33, all batches inoculated with *Hafnia* spp. showed an average acceptability superior to the Control and LAB batches.

In general, the mean values of the parameters analyzed show that the batches inoculated with the strains of *Hafnia* spp. showed a greater intensity of color of the pasta and higher average values in intensity and persistence of the aroma and overall acceptability. These bacteria are characterized by an intense proteolytic activity that has a great influence on the final aroma of the cheese (Irlinger et al., 2012; Morales et al., 2003a, b). This fact means that they can be used for inoculation as a cheese starter culture in order to improve the characteristics of the processing and the final product (Mounier et al., 2005, 2009).

Table 4. Mean values of the sensory parameters analyzed at 28 days of maturation in the different batches of cheese. Different letters mean significant differences obtained by Tukey's HDS test.

Parameters		**Paste visual analysis**			**Firmness**			**Intensity**			**Persistence**			**Global acceptance**		
Batches	N	Mean		SD*	Mean		SD	Mean		SD	Mean		SD	Mean		SD
Control	13	4,46	±	1,61	5,00^{a,b**}	±	1,63	5,85	±	1,52	4,92	±	1,75	6,08	±	1,44
LAB	14	4,71	±	1,38	6,21^{b,c}	±	1,37	5,43	±	1,09	4,50	±	1,40	5,86	±	1,03
H3	14	6,00	±	1,47	5,71^{a,b,c}	±	1,73	6,71	±	1,33	5,79	±	1,48	6,36	±	1,50
H6	14	4,93	±	1,73	5,64^{a,b,c}	±	1,74	6,21	±	1,25	5,50	±	1,40	6,29	±	1,07
H26	13	5,85	±	1,57	4,15^{a}	±	1,77	6,39	±	0,87	5,54	±	0,97	7,00	±	0,82
H33	15	5,73	±	1,71	6,60^{b,c}	±	1,30	6,13	±	1,73	5,47	±	1,25	5,93	±	0,88
H54	14	5,43	±	1,55	7,29^{c}	±	1,20	5,64	±	1,28	5,14	±	1,29	6,07	±	1,21
Total	97	5,31	±	1,63	5,84	±	1,77	6,05	±	1,35	5,27	±	1,40	6,22	±	1,17
Significance (*P*)		0,77			0,000			0,171			0,219			0,195		

*SD: standard deviation.

** For each batch the data in the same column with different letters is significantly different ($P \leq 0,05$).

Analysis of the principal components of the different parameters analyzed in the cheese batches.

The analysis of principal components of the microbiological, physico-chemical and sensory parameters is shown in figure 13. In the projection, it can be observed how the principal component 1 (PC1) explains a variability of 25.97% and the principal component 2 (PC2) of 45.84%, therefore, both principal components collect 71.81% of the variability of the data. As can be seen in figure 13, the VRBG (*Enterobacteriaceae*) variable, physico-chemical parameters (a_w, acidity and proteolysis index) and all the sensory parameters have more weight on PC 1, therefore, PC 1 defines better the difference between them. While the other variables studied, microbiological parameters (MRS and PCA) and physico-chemical parameters (pH and instrumental firmness) are explained by both principal components.

In relation to the parameters studied, the analysis of principal components clearly shows how the Control and LAB batches differ from the other batches inoculated with *Hafnia* spp. The Control and LAB batches are positively correlated with the physico-chemical parameters (water activity, pH and proteolysis index) and microbiological (VRBG and PCA), while it was negatively correlated with the sensory parameters. In relation to the five batches of *Hafnia* spp. inoculated, the PC analysis showed three different behaviors. Batches H3 and H33 were the ones that best correlated with the sensory attributes: persistence, intensity and global assessment (acceptability) and LAB counts in MRS, showing a clear influence on the sensory characteristics of the experimental cheeses made. On the other hand, batches H6 and H26 showed similar results, presenting an intermediate behavior in relation to microbiological, physico-chemical and sensory parameters between the two controls (Control and LAB) and batches H3 and H33. Finally, batch H54 was the most divergent, being positively correlated with both instrumental and sensory firmness, and negatively with the rest of the parameters studied. Therefore, the analysis of principal component shows that strains *H. alvei* 3 (544), *H. paralvei* 6 (593), *H. alvei* 26 (1037) and *H. alvei* 33 (1142), individually or in a mixed culture, could be used as part of a starter culture due to their contribution to aroma and creamier firmness.

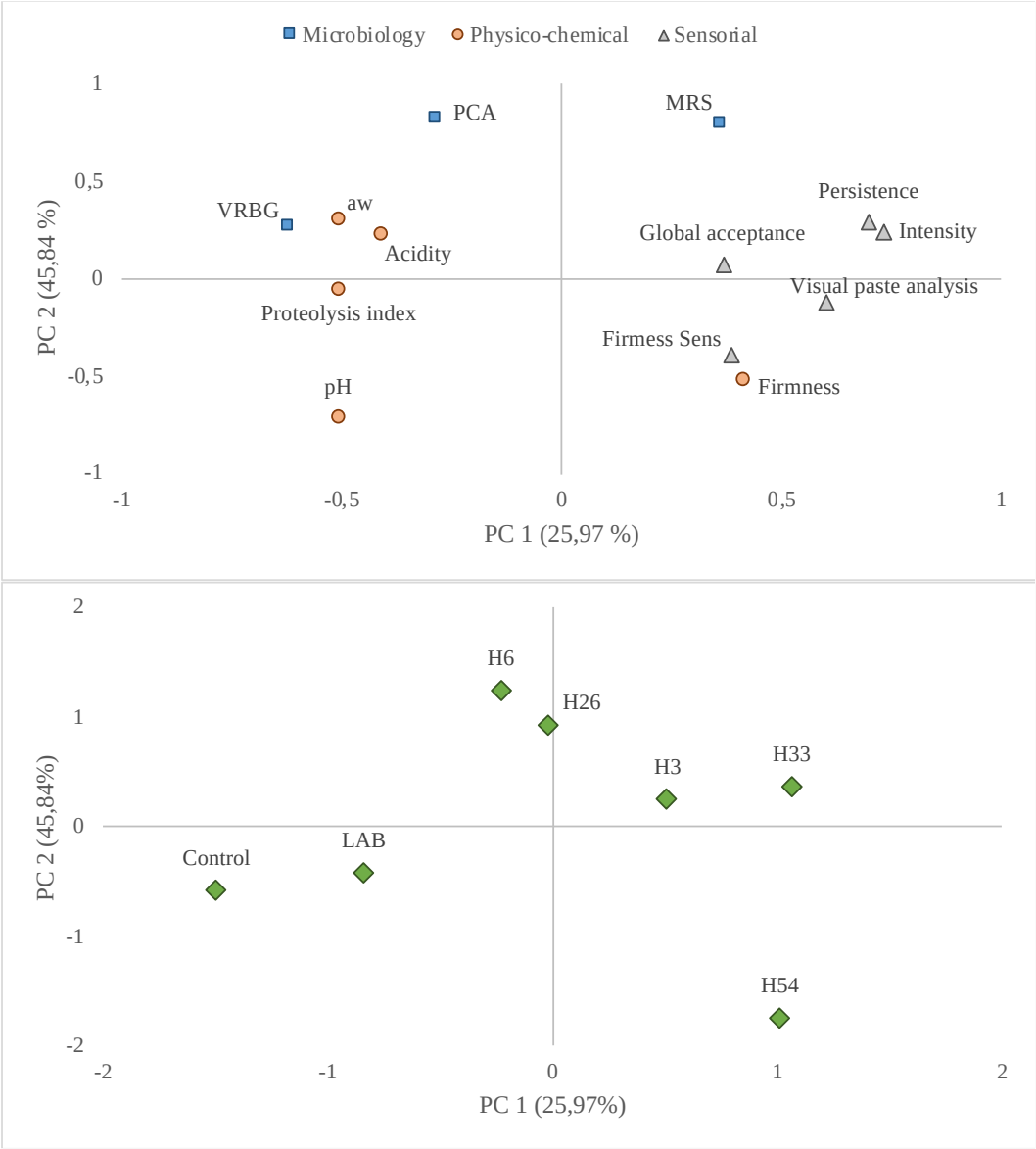


Figure 13. Analysis of principal components of different cheese batches made and microbiology parameters (PCA: mesophilic aerobic bacteria; MRS: lactic acid bacteria; VRBG: enterobacteria), physico-chemical (pH, a_w, firmness, proteolysis index and acidity) and sensorial (visual paste analysis, persistence, intensity, firmness (Firmness Sens) and global acceptance).

IV.4. Conclusion.

In conclusion, the five *Hafnia* spp. strains selected in the previous chapter, based on their technological properties were correctly implanted in the cheese matrix. All strains significantly increased the counts at the end of maturation of the experimental cheeses made. Two of the five strains inoculated in the experimental cheeses, *Hafnia paralvei* 6 (593) and *Hafnia alvei* 26 (1037) showed a softer instrumental texture than the other

batches. In addition, *Hafnia alvei* 3 (544) showed average values higher than Control batch and lower than LAB batch. The sensory analysis showed significant differences in firmness parameter, meanwhile the other parameters (visual paste analysis, intensity, aroma persistence and global acceptance) did not showed significant differences between inoculated batches and Control and LAB batches. However, an impact on the evaluated sensory characteristics could be appreciated by the effect of the enterobacteria. The mean values of the sensory analysis were higher in inoculated batches than in control batch.

The analysis of principal components of the physico-chemical, microbiological and sensory parameters of the cheese batches show that *Hafnia* spp. have a clear impact on the cheese ripening process. In addition, although more studies are necessary, four of these five *Enterobacteriaceae* strains, *Hafnia alvei* 3 (544), *Hafnia paralvei* 6 (593), *Hafnia alvei* 26 (1037) and *Hafnia alvei* 33 (1142), based on their relationship with the sensory parameters (intensity and persistence) and with sensory and instrumental firmness, and physico-chemical parameters showed their suitability to form part of a mixed starter culture to produce soft body cheeses from raw sheep's milk.

IV.5. References

Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E., & Martínez-Bueno, M. (2008). Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology*, *127*(3), 200–208. https://doi.org/10.1016/j.ijfoodmicro.2008.07.004

Alberghini, L., Tallone, G., & Giaccone, V. (2010). A new discoloration of Ricotta cheese. *Italian Journal of Food Safety*, 8, 7-10.

Almeida, G., Figueiredo, A., Rôla, M., Barros, R. M., Gibbs, P., Hogg, T., & Teixeira, P. (2007). Microbiological characterization of randomly selected Portuguese raw milk cheeses with reference to food safety. *Journal of Food Protection*, *70*(7), 1710-1716.

Alshehri, W. A. (2020). Bacterium *Hafnia alvei* secretes L-methioninase enzyme: Optimization of the enzyme secretion conditions. *Saudi journal of biological sciences*, 27(5), 1222-1227.

Araújo-Rodrigues, H., Tavaria, F. K., dos Santos, M. T. P., Alvarenga, N., & Pintado, M. M. (2020). A review on microbiological and technological aspects of Serpa PDO cheese: An ovine raw milk cheese. *International Dairy Journal*, 100, 104561.

Benito, M. J., Núñez, F., Córdoba, M. G., Martín, A., & Córdoba, J. J. (2005). Generation of non-protein nitrogen and volatile compounds by *Penicillium chrysogenum* Pg222 activity on pork myofibrillar proteins. *Food microbiology*, *22*(6), 513-519.

Bokulich, N. A., & Mills, D. A. (2013). Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. *Applied and environmental microbiology*, 79(17), 5214-5223.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., ... & Hansen, E. B. (2012). Food fermentations: microorganisms with technological beneficial use. *International journal of food microbiology*, 154(3), 87-97.

Callon, C., Arliguie, C., Montel, M. (2015). Control of Shigatoxin-producing *Escherichia coli* in cheese by dairy bacterial strains. *Food Microbiology* 53 (2016) 63-70.

Chaves-López, C., De Angelis, M., Martuscelli, M., Serio, A., Paparella, A., & Suzzi, G. (2006). Characterization of the *Enterobacteriaceae* isolated from an artisanal Italian ewe's cheese (Pecorino Abruzzese). *Journal of Applied Microbiology*, *101*(2), 353–360. https://doi.org/10.1111/j.1365-2672.2006.02941.x

Del Olmo, A., Calzada, J., & Nuñez, M. (2018). The blue discoloration of fresh cheeses: A worldwide defect associated to specific contamination by *Pseudomonas fluorescens*. *Food Control*, 86, 359-366.

Dugat-Bony, E., Straub, C., Teissandier, A., Onésime, D., Loux, V., Monnet, C., … Bonnarme, P. (2015). Overview of a surface-ripened cheese community functioning by meta-omics analyses. *PLoS ONE*, *10*(4), 1–25. https://doi.org/10.1371/journal.pone.0124360

Elotmani, F., Revol-Junelles, A.-M., Assobhei, O., & Millière, J.-B. (2002). Characterization of Anti- Listeria monocytogenes Bacteriocins from *Enterococcus faecalis* , *Enterococcus faecium* , and *Lactococcus lactis* Strains Isolated from Raïb, a Moroccan Traditional Fermented Milk. *Current Microbiology*, *44*(1), 10–17. https://doi.org/10.1007/s00284-001-0067-8

Ercolini, D., Russo, F., Ferrocino, I., & Villani, F. (2009). Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiology*, *26*(2), 228–231. https://doi.org/https://doi.org/10.1016/j.fm.2008.09.005

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017). *Fundamentals of Cheese Science*. *Fundamentals of Cheese Science*. Boston, MA: Springer US. https://doi.org/10.1007/978-1-4899-7681-9

Fröhlich-Wyder, M. T., Arias-Roth, E., & Jakob, E. (2019). Cheese yeasts. *Yeast*, *36*(3), 129–141. https://doi.org/10.1002/yea.3368

Gonçalves, M. T. P., Benito, M. J., Córdoba, M. D. G., Egas, C., Merchán, A. V., Galván, A. I., & Ruiz-Moyano, S. (2018). Bacterial communities in Serpa cheese by culture

dependent techniques, 16S rRNA gene sequencing and high-throughput sequencing analysis. *Journal of food science*, 83(5), 1333-1341.

Groenewald, M., Boekhout, T., Neuvéglise, C., Gaillardin, C., Van Dijck, P. W. M., & Wyss, M. (2014). *Yarrowia lipolytica*: Safety assessment of an oleaginous yeast with a great industrial potential. *Critical Reviews in Microbiology*, *40*(3), 187–206. https://doi.org/10.3109/1040841X.2013.770386

Hayaloglu, A. A. (2016). Cheese: Microbiology of Cheese. In *Reference Module in Food Science*. Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.00675-2

Heredia-Castro, P. Y., Hérnández-Mendoza, A., González-Córdova, A. F., & Vallejo-Cordoba, B. (2017). Bacteriocinas de bacterias ácido lácticas: Mecanismos de acción y actividad antimicrobiana contra patógenos en quesos. *Interciencia*, *42*(6), 340–346.

Huey, B. & Hall, J. (1989). Hypervariable DNA fingerprinting in *E. coli* minisatellite probe from bacteriophage M13. *Journal of bacteriology*, 171(5), 2528 – 2532.

International Organization for Standardization. (2006). Sensory Analysis–Guidelines for the Use of Quantitative Response Scales. (UNE-ISO Standard No. 4121-2006). ISO: Geneva, Switzerland.

International Organization for Standardization. (2014). Milk and milk products-Determination of nitrogen content–Part 1: Kjeldahl principle and crude protein calculation. (UNE-ISO Standard No. 8968-1:2014). ISO: Geneva, Switzerland.

Irlinger, F., Yung, S. A. Y. I., Sarthou, A. S., Delbès-Paus, C., Montel, M. C., Coton, E., ... & Helinck, S. (2012). Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International journal of food microbiology*, *153*(3), 332-338.

Irlinger, F., Layec, S., Hélinck, S., & Dugat-Bony, E. (2015). Cheese rind microbial communities: Diversity, composition and origin. *FEMS Microbiology Letters*, *362*(2), 1–11. https://doi.org/10.1093/femsle/fnu015

Kongo, J. M., & Malcata, F. X. (2016). Cheese: Types of Cheeses–Soft. *Encyclopedia of Food and Health*, 768-773.

Ledenbach, L.H., Marshall, R.T., (2010). Microbiological spoilage of dairy products. In: Sperber, W.H., Doyle, M.P. (Eds.), *Compendium of the Microbiological Spoilage of Foods and Beverages, Food Microbiology and Food Safety*. Springer, New York, pp. 41–67.

Lu, M., & Wang, N. S. (2017). Spoilage of milk and dairy products. In *The microbiological quality of food* (pp. 151-178). Woodhead Publishing.

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology*, *57*(2–3), 127–144. https://doi.org/10.1111/j.1471-0307.2004.00147.x

Menéndez, S., Centeno, J. A., Godínez, R., & Rodríguez e Otero, J. L. (2000). Effects of various *Lactobacillus* strains on the ripening and organoleptic characteristics of Arzua-Ulloa cheese. *International Journal of Food Microbiology, 59*, 37-46.

Montel, M. C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D. A., Desmasures, N., & Berthier, F. (2014). Traditional cheeses: rich and diverse microbiota with associated benefits. *International journal of food microbiology*, *177*, 136-154.

Morales, P., Feliu, I., Fernández-García, E., & Núñez, M. (2003a). Volatile Compounds Produced in Cheese by *Enterobacteriaceae* Strains of Dairy Origin. *Journal of Food Protection, Vol. 67, No. 3, 2004,* Pages 567–573.

Morales, P., Fernández-García, E., & Núñez, M. (2003b). Caseinolysis in cheese by *Enterobacteriaceae* strains of dairy origin. *Letters in Applied Microbiology*, *37*(5), 410-414.

Morales, P., Feliu, I., Fernández-García, E., & Nuñez, M. (2004). Volatile Compounds Produced in Cheese by *Enterobacteriaceae* Strains of Dairy Origin. *Journal of Food Protection*, *67*(3), 567–573. https://doi.org/10.4315/0362-028X-67.3.567

Morandi, S., Pica, V., Masotti, F., Cattaneo, S., Brasca, M., De Noni, I., & Silvetti, T. (2021). Proteolytic traits of psychrotrophic bacteria potentially causative of sterilized milk instability: Genotypic, phenotypic and peptidomic insight. *Foods*, *10*(5). https://doi.org/10.3390/foods10050934

Mounier, J., Gelsomino, R., Goerges, S., Vancanneyt, M., Vandemeulebroecke, K., Hoste, B., ... & Cogan, T. M. (2005). Surface microflora of four smear-ripened cheeses. *Applied and environmental microbiology*, *71*(11), 6489-6500.

Mounier, J., Monnet, C., Jacques, N., Antoinette, A., & Irlinger, F. (2009). Assessment of the microbial diversity at the surface of Livarot cheese using culture-dependent and independent approaches. *International journal of food microbiology*, *133*(1-2), 31-37.

Ordiales, E., Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J., & de Guía Córdoba, M. (2013). Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. *Food Control*, 33(2), 448-454.

Ordiales, E., Martín, A., Benito, M. J., Fernández, M., Casquete, R., & de Guía Córdoba, M. (2014). Influence of the technological properties of vegetable rennet (*Cynara cardunculus*) on the physicochemical, sensory and rheological characteristics of 'Torta del Casar'cheese. *International Journal of Dairy Technology*, 67(3), 402-409.

Pereira, C. I., Graça, J. A., Ogando, N. S., Gomes, A. M., & Malcata, F. X. (2010). Influence of bacterial dynamics upon the final characteristics of model Portuguese traditional cheeses. *Food Microbiology*, *27*(3), 339-346.

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Reviews*, *37*(5), 664–698. https://doi.org/10.1111/1574-6976.12030

Ristagno, D., Bunka, F., & McSweeney, P. L. H. (2013). 5. Effect of *Hafnia alvei* on Cheddar cheese ripening. *Evaluation of microbial adjuncts and their effect on the ripening of*, 124.

Sánchez-Juanes, F., Teixeira-Martín, V., González-Buitrago, J. M., Velázquez, E., & Flores-Félix, J. D. (2020). Identification of Species and Subspecies of Lactic Acid Bacteria Present in Spanish Cheeses Type "Torta" by MALDI-TOF MS and pheS gene Analyses. *Microorganisms*, 8(2), 301.

Tabla, R., Gómez, A., Simancas, A., Rebollo, J. E., Molina, F., & Roa, I. (2016). *Enterobacteriaceae* species during manufacturing and ripening of semi–hard and soft raw ewe's milk cheese: Gas production capacity. *Small Ruminant Research*, *145*, 123-129.

Tabla, R., Gómez, A., Simancas, A., Rebollo, J. E., Molina, F., & Roa, I. (2018). Early blowing in raw goats' milk cheese: Gas production capacity of *Enterobacteriaceae* species present during manufacturing and ripening. *Journal of Dairy Research*, *85*(3), 331–338. https://doi.org/10.1017/S0022029918000511

Tavaria, F. K., & Malcata, F. X. (1998). Microbiological characterization of Serra da Estrela cheese throughout its appellation d'Origine protegee region. *Journal of Food Protection*, *61*(5), 601–607. https://doi.org/10.4315/0362-028X-61.5.601

Tornadijo, M. E., García, M. C., Fresno, J. M., & Carballo, J. (2001). Study of Enterobacteriaceae during the manufacture and ripening of San Simón cheese. *Food Microbiology*, *18*(5), 499–509. https://doi.org/10.1006/fmic.2001.0423

CAPÍTULO V

Characterization of autochthonal yeasts isolated from Spanish soft raw ewe's milk PDO cheeses for technological application.

Capítulo V.

Characterization of autochthonal yeasts isolated from Spanish soft raw ewe's milk PDO cheeses for technological application.

Abstract

The yeasts involved in the ripening process of artisanal soft raw ewe's milk PDO cheeses produced in Extremadura (Spain), 'Torta del Casar' and 'Queso de la Serena', were isolated throughout their ripening process, strain-typing and characterized for some important technological properties. A total of 508 yeast isolates were obtained and identified by ISSR-PCR with primer $(GTG)_5$ and subsequent sequencing of the internal transcribed spacer ITS1/ITS2-5.8S rRNA. Nineteen yeast species representing eight genera were detected. *Debaryomyces hansenii, Pichia kudriavzevii, Kluyveromyces lactis* and *Yarrowia lipolytica* were the predominant species. One hundred and fifty-seven isolates were selected by genotyping and dairy origin for their technological characterization. The evaluation of the growth at the stress conditions of cheese ripening showed that 87 presented better adaption. Among them, 71 isolates were not able to catabolize tyrosine producing brown pigment. The principal component analysis of the biochemical features of these isolates showed that nine strains, three *K. lactis* strains (2287, 2725 and 1507), two *P. jadinii* (1731 and 433), two *Y. alimentaria* (1204 and 2150), *Y. lipolytica* 2495 and *P. kudriavzevii* 373, stood out. All 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 in *P. kudriavzevii* 373 and *K. lactis* 2725. Although the enzymatic characterization by API-ZYM 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 concentration higher than 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 alkalinizing process relevant for biochemical processes that take place in the last part of ripening. Contrary, *K. lactis* strains showed acidifying capacity and β-galactosidase activity and may participate at

initial stages of ripening, together with lactic acid bacteria. Thus, considering the technological characteristics studied these nine strains presented great biochemical features for their potential use as adjunct cultures alone or in combination with autochthonous starter bacteria in cheesemaking process to overcome the heterogeneity of these PDO cheeses preserving their unique sensory characteristics.

IV.1. 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 Regulation (CE) 1491/2003 and Regulation (CE) 1107/1996 of the European Commission, respectively. These cheeses are produced in Extremadura region, in the south-west of Spain, from raw ewes' milk using aqueous maceration of the dried flowers from *Cynara cardunculus* L. plant as coagulant. They are characterized by a light and thin semi-hard rind, spreadable texture, sometime almost liquid, and unique aroma (Carbonell et al., 2002; Delgado et al., 2010), which make them highly appreciated by consumers. In these artisanal cheeses characterized by the absence of thermal treatment and starter microorganisms, the product quality depends mainly of spontaneous fermentation developed by the natural microbiota present in the raw materials, milk and vegetable coagulant, and 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 by cocci gram-positive catalase-positive, enterobacteria, and yeasts (Freitas and Malcata, 2000; Ordiales et al., 2013a, b).

LAB cause a 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 associated also to the metabolic activities of the diverse endogenous microbiota other than LAB. Volatile compound and sensory analysis in traditional cheeses have showed that the secondary microbiota contributes greatly to the development of the final flavor and texture (Montel et al., 2014; Irlinger et al., 2017). Recent advances in the study of 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 elevate salt

concentration and low temperature, water activity and pH (Ferreira and Viljoen, 2003). In addition, yeast ability to metabolize lactose and galactose, or the assimilation of succinic, lactic and citric acids favor its occurrence in cheese. On cheese surface, yeast growth is favor by the aerobic environment, reaching high level around 6-8 log cfu/cm^2 in few days of ripening. There, yeast can consume lactate and produce NH_3 from amino acids, leading to increase of pH that helps to establish salt-tolerant and acid sensitive Gram-positive bacteria (Cogan et al., 2014; Monnet et al., 2015). However, in the cheese core, the availability of oxygen change and the conditions for yeast growth are more restrictive. Only yeast species capable of fermenting residual lactose or galactose or both are more competitive (Fröhlich-Wyder et al., 2019). Nevertheless, in the core of soft or semi-soft cheese raw ewe's artisanal cheese, counts around 4-5 log cfu/g has been reported during cheese ripening (Tavaria and Malcata, 2000; Ordiales et al., 2013a, Gonçalves et al., 2017). Yeast species associated to cheese environment display relevant proteolytic and lipolytic activities that may contribute positively to 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 enhances the formation of volatile aroma compounds. At similar level, *Yarrowia lipolytica* modify the flavour profile and contribute to the quality of "Tetilla" cheese (Centeno et al., 2017). Likewise, the use of adjunct cultures of the yeast *Geotrichum candidum* in pasteurized soft and semi-hard cheeses result in taste and aroma closer to those obtained for raw milk cheeses (Boutrou and Guéguen, 2005). These evidences confirm that yeasts even at lower counts than dominant bacteria contribute to the final organoleptic characteristics of cheese. In this context, today there are *Debaryomyces hansenii*, *K. lactis*, *K. marxianus*, and *G. candidum* strains commercially available for this issue (Irlinger et al., 2017).

On the other hand, yeasts may also cause spoilage in dairy products (Garnier et al., 2017). Several defects like yeasty off-flavours, eye formation, early blowing early, toad skin defect, and brown pigmentation have been linked to yeast proliferation and may have a detrimental effect on appearance and sensorial characteristics of cheese (Carreira et al., 1998; Yalcin & Ucar, 2009; Garnier et al., 2017; Fröhlich-Wyder et al., 2019).

Although 'Torta del Casar' and 'Queso de la Serena' are highly value PDO cheeses, the lack of standardization in their cheesemaking process implies that microbial

diversity and abundance can vary over ripening, resulting in heterogeneous organoleptic characteristics according to the batch or the dairy industry. Implementation of the use of autochthonous adjunct cultures adapted to the cheesemaking process may contribute to overcome these problems, reducing variability in the final product as well as preserving their typical sensory profile (Araújo-Rodrigues et al., 2020). Most efforts, in this type of cheeses, have been conducted in 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 secondary microbiota. In this context, the present study aimed to identify the predominant yeast microbiota present in artisanal soft raw ewe's milk PDO cheeses produced in Extremadura; and to characterize the yeast isolates for technological relevant properties.

III.2. Materials and methods

Cheese sampling and yeast counts

Cheese samples were taken during the ripening period from six different dairy industries (Three industries belonged to PDO 'Queso de la Serena' and the other three to PDO 'Torta del Casar') in Extremadura (Spain) at 0, 20, 40 days and final product, around 60 days. Samples consist in three cheeses randomly taken that were transported under refrigerated conditions to the facility of Agricultural Engineering School at University of Extremadura for analysis. Microbiological analyses were performed in three different cheeses by industry and sample time, making each determination in duplicate.

For yeast counts and isolation, 10 g of each cheese was taken aseptically from the core of the cheese and placed into a sterile plastic pouch with 90 mL of 1% peptone water (Condalab, Madrid-Spain), and homogenised for 120 s in a Stomacher instrument (Lab-Blender 400, Seward, London, United Kingdom). The homogenates were ten-fold serial diluted, and aliquots from each dilution were inoculated onto acidified potato dextrose agar plates (PDA, Pronadisa) with 1% (v/v) sterilised solution of tartaric acid at 10% (w/v) and incubated at 25 ºC for 72 h. Ten yeast colonies from each PDA agar plate containing the highest dilutions were randomly selected and streaked onto acidified PDA 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% glycerol. Prior to the assays, all yeast isolates were sub-cultured twice in YPD broth at 25ºC for 24 h.

Yeast typing by molecular techniques

To get the genomic DNA, 1 mL of each yeast culture in YPD broth was collected by centrifugation at 10,000 *g* for 5 min. The yeast pellet was suspended in lysis buffer and disrupted with 400−600 µm silica grinding beads in a 1600 MiniG® homogeniser (SPEX SamplePrep, Metuchen, NJ) at 1500 rpm for 5 min. Genomic DNA was extracted using a GeneJET Genomic DNA Purification Kit following manufacture instructions (Thermo Fisher Scientific, USA). The amount of DNA was determined by using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific) and concentration was adjusted to 10 ng/µL for PCR reactions.

Inter-single sequence repeat anchored polymerase chain reaction amplification analysis (ISSR-PCR) was carried out using the 5'-anchored $(GTG)_5$ primer (5'-GTGGTGGTGGTGGTG-3') as described by Mahmoud et al. (2014). The ISSR-PCR fragments were separated by electrophoresis on a 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, Cambridge, United Kingdom). 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 (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 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 belong to the same OTU but from different dairy industry origin were selected for screening their technological capacity (Table S1). Prior to the assays, all yeast isolates were sub-cultured twice in YPD broth at 25°C for 24 h. After, isolates were washed twice with phosphate buffer saline (PBS) at pH 7.2 (Thermo Fisher Scientific) and yeast concentration adjusted to $1x10^6$ cells/mL using a Neubauer chamber. All experiments were carried out in triplicate.

Evaluation of yeast growth at cheese ripening conditions.

Box–Behnken experimental design (BBD) with 3 factors was applied for modeling the influence of the variables temperature, salt concentration and pH, on the growth of the yeast isolates. The variable conditions are shown in Table S2 and were stablished according to the ripening process of soft cheeses "Torta del Casar" and "Queso de la Serena" (Martínez et al., 2011; Tabla et al., 2015). Yeast nitrogen base broth (YNB; Pronadisa) supplemented with 1% (w/v) of lactose was used for the experiment. pH and NaCl concentration of YNB medium were set up at conditions showed in Table S2. The pH of the YNB medium was adjusted with lactic acid solution (10%) or NaOH 1 M. The YNB medium was sterilized by filtration through 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% (v/v) from a suspension of tested yeasts at 10^6 cells/mL. The microplates were incubated at different temperatures ranging from 8ºC to 30ºC (Table S2) for 15 days. 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, Offenburg, Germany). The time to detection (TTD) of growth for OD_{570nm}=0.4 for each well was calculated obtained 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 isolate.

Pigmentation capacity

The pigmentation production by yeast isolates were evaluated in cheese agar medium with 1% (w/v) L-tyrosine elaborated according to Carreira et al. (1998) using soft cheese "'Torta del Casar'. Cheese agar medium without 1% (w/v) L-tyrosine was used as negative control. Ten μL of each isolate was spotted on the surface of the medium and incubated at 8 and 25 ºC for 20 days. The pigment presence was evaluated every two days. Results were considered positive when the medium turned brown. The intensity of the pigmentation was classified in four levels according to the colour 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. Media, composed of reconstituted skim milk powder at 5% and bacteriological agar at 1.5% (Scharlab, Barcelona), was sterilized by autoclaving 5 min at 115ºC and then poured onto petri dishes. The medium was superficially spotted with 10 µL of each yeast isolate and incubated at 15, 20 and 25ºC. The proteolytic activity was examined daily for six days. Positive activity was considered when there was clear zone around the colony. This area was also measured to evaluate the intensity of the activity.

Lipase activity was determined by Rhodamin B agar medium (Kouker and Jaeger, 1987) and esterase activity was determined by tributyrin agar (Ismail et al., 2000). Inoculated plates with 10 µL of each yeast isolate were incubated at 15, 20 and 25ºC and examined daily for six days. Pink coloured colony on Rhodamine B agar plate were considered as positive. In the case of tributyrin agar, 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). Medium was spotted with 10 µL of each isolate and incubated for 48 h at 30ºC. Alkalizing activity was observed when Simmons citrate agar turned into blue color around colonies. Acidifying activity was determined in sterile reconstituted skimmed milk (10%), inoculated with 100 µL of each isolate and incubated at 30ºC for 48 h. The pH was measured using a Crison pH meter mod. Basic 20 (2012) (Crison Instruments, Barcelona, Spain) at 24 and 48 h.

Diacetyl-acetoin production.

Acetoin production was detected by the Voges-Proskauer reaction. One hundred microliters of each isolate were inoculated in sterile reconstituted skimmed milk at 25ºC 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%, w/v ethanol) and 0.2 mL of 40% sodium hydroxide. The resulting mixture was vortex for 30 s and was incubated for 15 min. Acetoin production was detected as a red ring on the surface of the culture (VP^+ phenotype).

Enterococcus clocae and *Proteus mirabilis* from the Department Animal Production and Food Science (UEX) culture collection were used as positive and negative control, respectively.

Assimilation of lactose, galactose, lactate and citrate.

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

Enzymatic activity.

The API-ZYM system (bioMérieux SA) was used to evaluate the enzymatic activities from nine 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 5-6 McFarland were inoculated into each cupule and incubated for 4 ½ hours at 37ºC. The metabolic end products produced during the incubation period were detected through colored 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-chymotripsin, phosphatase acid, naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucoronidase, 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 (SPSS Inc., Chicago, IL, USA), and the differences between groups were studied by two-way analysis of variance (ANOVA) and separated by Tukey's honest significant differences test ($P \leq 0.05$). To determine the effects of medium conditions on the yeast isolates growth, a three factor, three-level BBD combining with Surface Response Methodology (SRM) were applied to determine the effects of medium conditions on the yeast isolates growth (Table S2). SRM was performed employing the 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 models were 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 (Table S2). The software also generated an analysis of variance (ANOVA), establishing the statistical significance at the 95% of confidence level. Optimal level for growth time of each variable analyzed were also obtained with the same statistical program.

In addition, the growth of the yeast isolates at cheese ripening conditions, pigmentation capacity, and biochemical characteristics (proteolysis and lipolysis) were evaluated by hierarchical cluster analysis (HCA). Finally, the relationships among technological features and selected yeast strains were established by Pearson correlation coefficients and principal component analysis (PCA). SPSS software for Windows 21.0 (SPSS Inc., Chicago, IL, USA) was used.

III.3. Results

Yeast counts and identification

Yeast counts through ripening ranged between 3.94-4.52 log cfu/g in "Queso de la Serena" and between 3.42-3.97 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 through ripening process. "Queso de la Serena" showed significantly ($P < 0.05$) higher counts at different sampling time points through ripening. However, similar tendency in yeast counts was observed in both PDO cheese. Yeast increased at the beginning of the ripening, reaching maximum counts after 20 d ($P < 0.05$), and then decreased slightly until the end (60 d).

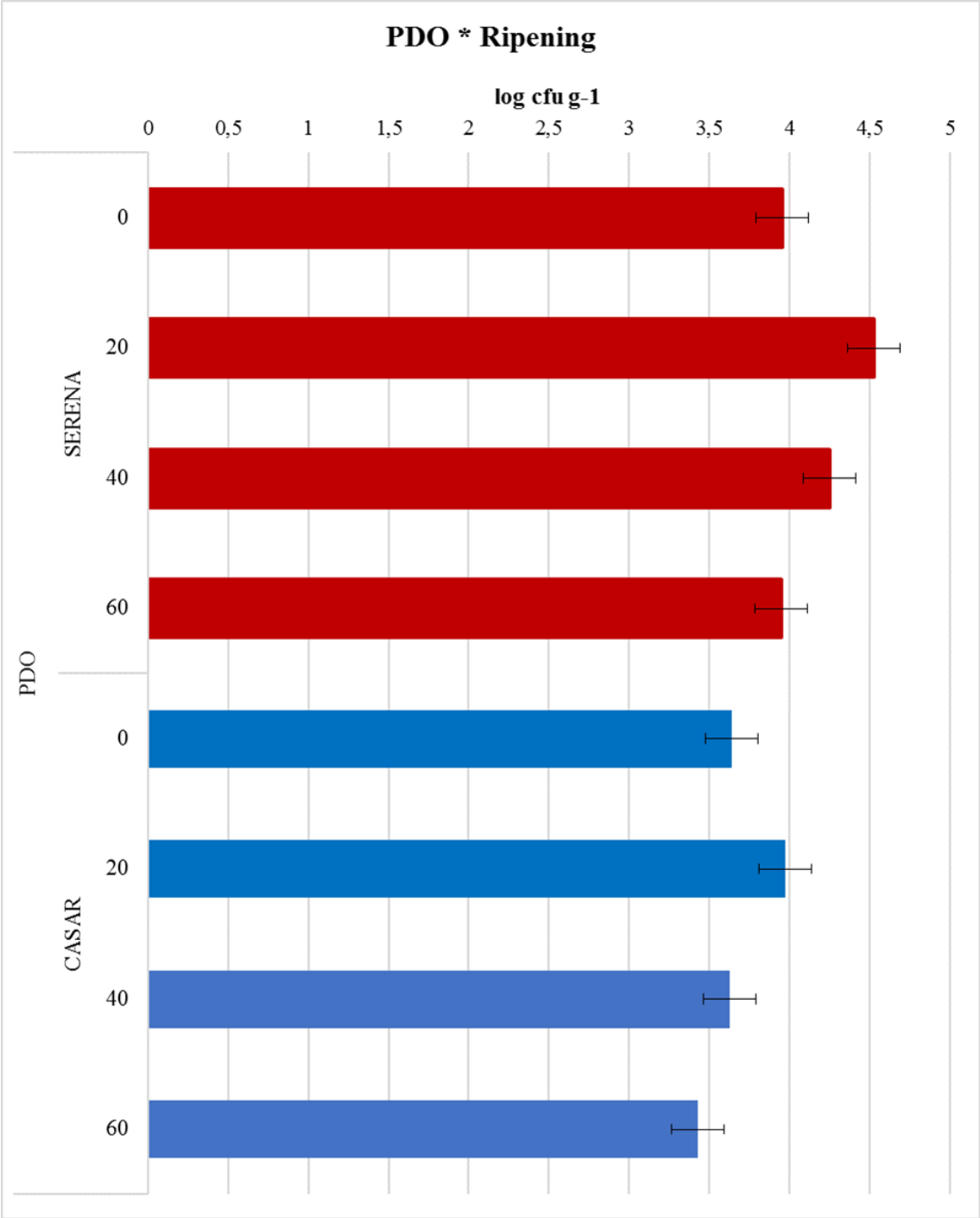


Figure 1. Yeast counts expressed in log cfu g^{-1} by PDO cheese type throughout ripening process. Red colour represent yeast counts in Serena PDO cheese and blue colour in Casar PDO cheese. Error bars correspond to the 95% confidence interval from Tukey HSD test.

A total of 508 yeast isolates were obtained from the highest dilutions of cheese core samples collected from six dairies in two seasons. Table S1 shows the molecular identification by ISSR analysis combined with the sequencing of ITS1/ITS2-5.8S rRNA

region. Based on ISSR analysis, the yeast isolates were clustering into 37 OTU. The sequencing of ITS region of two representative isolates by OTU allows their identification at species level. Nineteen different yeast species representing eight genera were identified. The predominant yeast genera were *Kluyveromyces*, *Debaryomyces*, *Pichia* and *Yarrowia* that were detected in all dairies. At species level, *D. hansenii* and *Pichia kudriavzevii* were the most frequent, being detected in all dairies, followed by *K. lactis* and *Y. lipolytica*, which were not detected in one of the six dairies. Among the 508 isolates identified, 157 isolates were selected for the study of technological properties based on genetic profile and dairy origin (Table S1). The most frequent species comprised approximately 60% percent (90 isolates) of the strains selected while the rest (67 isolates) were distributed across 14 species. In addition, isolates identified as *Candida parapsilopsis* were not selected as this species is an opportunistic yeast pathogen.

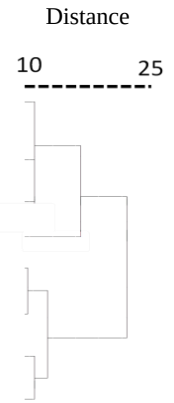
Technological properties

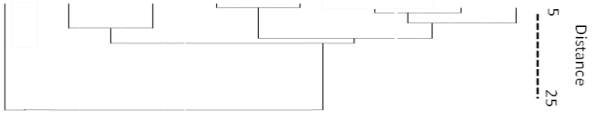
Adaptability at cheese ripening conditions

Table 1 shows the clustering of isolates base on their ability to growth under soft cheese ripening conditions set by Box–Behnken experimental design applying three-factor (Temperature, pH, and %NaCl). The relative high values for the adjusted R-squared (>50) obtained for most yeast isolates and the random patter of the residuals indicate that their growths 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 conditions set for the BBD. Concretely, 8 isolates of *Candida zeylanoides*, 7 of *Y. lipolytica*. 5 of *G. candidum*, 4 of *P. kudriavzevii*, 3 of *D. hansenii*, 2 of *P. fermentans*, and 3 belonging to three different species (*Candida pararugosa*, *K. lactis* and *Y. alimentaria*) were discarded on this basis (Figure 2). For all other isolates tested (a total 125), their optimal growth conditions were also estimated by fitting the data to the quadratic model developed for each isolate (Table 1).

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

Days of growth Cheese ripening stages					**Optimal growth conditions**									
					Temp (ºC)	30	8	23.2	8	18.5	30	30	30	
					Salt (%)	8	8	8	1	1.1	1	8	1	
					pH	6.5	5.3	6.5	5	4.8	3.5	3.5	3.5	
					(Days)	(8.5)	(1.8)	(1.2)	(3)	(3.2)	(0.7)	(0.3)	(4.9)	
S1	**S2**	**S3**	**S4**	**S5**	**Clusters**	**C_H**	**C_D**	**C_B**	**C_F**	**C_C**	**C_A**	**C_G**	**C_E**	**N.G**	**Total**
<25	<25	<25	<25	<25	**C_4**	1					4		2		7
3.7	<25	<25	<25	<25	**C_6**						11	4	1		16
<25	<25	17.5	17.3	<25	**C_7**						1				1
0.5	2.2	3.2	<25	<25	**C_8**							2			2
<25	12.7	7.2	6.5	5.9	**C_2**			1		0	10				11
8.1	8.8	6.2	5.5	5.2	**C_1**		1	5	1	8	51	6	2		74
<25	<25	<25	23.9	6.4	**C_3**						2				2
9.1	<25	<25	11.4	9.3	**C_5**						11		1		12
					N.G									32	32
					Total	1	1	6	1	8	90	12	6	32	157





The hierarchal cluster analysis based on estimated growth of yeast isolates showed a good adaptation of a relevant number of them at cheese processing conditions. Concretely, 74 isolates grouped in the cluster 1 (C_1) presented an adequate growth (5.2-8.8 days) at the five cheese processing conditions stablished; 2 isolates grouped in cluster 8 (C_8) at the three firsts cheese processing conditions and 11 isolates grouped in the cluster 2 (C_2) at the three ends cheese processing conditions (Table 1; Figure 2). The rest of the isolates grouped in other clusters, 38, showed a limited ability to grow (>25 days) at two or more of the set conditions, and were therefore excluded for subsequent technological characterisation (Table 1). Between these isolates, can be found all isolates identified as *Aureobasidium pullulans, Candida glabrata, C. humilis, C. intermedia, Kazachstania bulderi, Kz. humilis,* and *P. 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. Then, a total of 87 isolates from clusters 1, 2 and, 8, mostly belonging to *D. hansenii*, *Y. lipolytica*, *Y. alimentaria*, *K. lactis*, *K. marxianus*, *P. kudriavzevii*, *P. fermentans*, and *P. jadinii*, were selected to evaluate their technological capacity (Figure 2).

The double hierarchal cluster analysis performed based on both estimated growth of yeast isolates at cheese processing conditions and optimal growth conditions showed that the cluster 1A (51 isolates) and 2A (10 isolates) comprised most of the selected isolates (Table 1; Figure 3). These isolates exhibited excellent growth at 30ºC, 1% of salt and pH values of 3.5 (Table 1). The growths of the most of isolates belonging to cluster A (1A and 2A) were positively affected by the increase of temperature and decrease salt concentration, showing also interaction between both factors for a substantial number of them (Table 2). On the contrary, the effect of pH on the growth of yeast isolates grouped into cluster A and other clusters was minimal compared with temperature and salt concentration (Table 2).

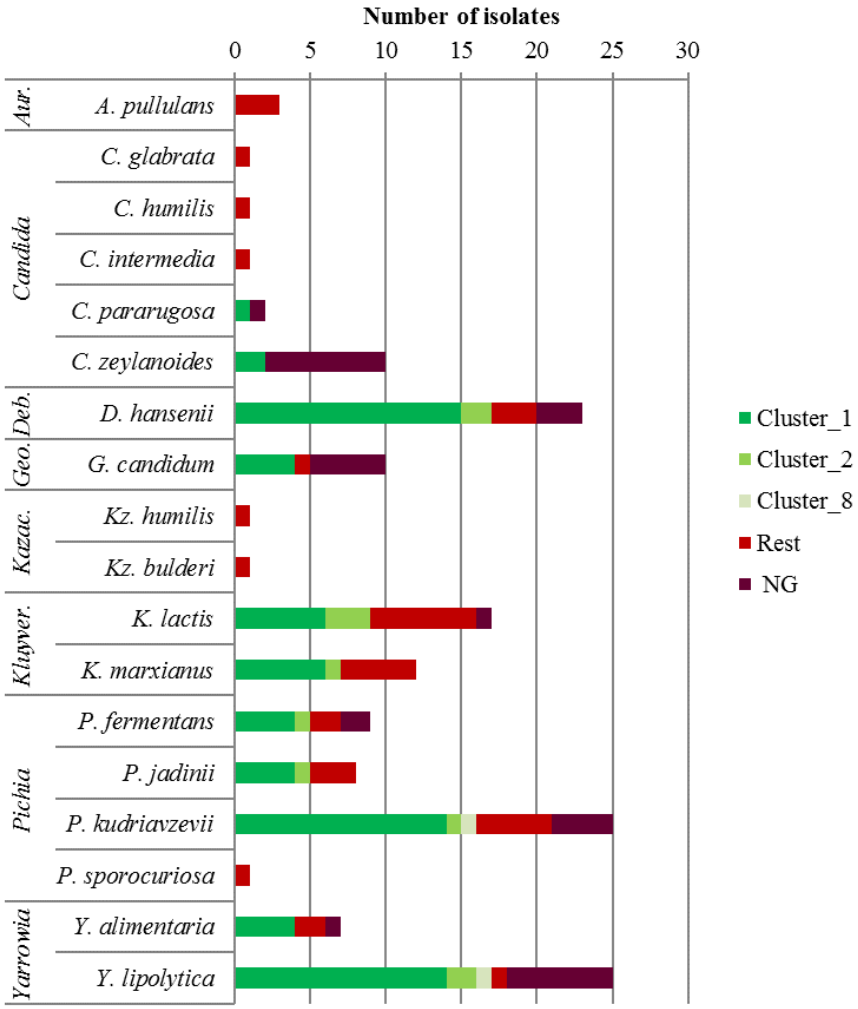


Figure 2. Number of isolates by yeast specie clustered based on their ability to grow at the soft cheese ("Torta") ripening conditions obtained by three-factor (Temperature, pH, and NaCl%) modelling using Box–Behnken experimental design. Cluster purple (NG): no growth under conditions assays. Cluster red (Rest): yeast isolates classified in clusters 3, 4, 5, 6 and 7 associated to no optimal growth under cheese ripening conditions assays.

The identical optimal growth conditions of the cluster A were estimated for the 2 isolates included into cluster E1 that were identified as *G. candidum*, and *P. fermentans*. However, the growth of these isolates was rather slower and strongly affected by salt concentration (Table 2). The isolates grouped into the cluster B (1B and 2B) showed as optimal conditions for growth 23.2 ºC, 8% of salt and neutral values of pH, being identified all of them (6 isolates) as *D. hansenii* (Table 1; Figure 3). In this case, their growth was mainly influenced by temperature (main and quadratic effects), with salt concentration not significantly affecting it (Table 2). On this way, the selected isolates belong to clusters G (1G and 8G) and D1 also showed halotolerant behaviour with the

temperature as the most influencer parameter. Most of the isolates integrated into the cluster G were identified as *D. hansenii* and *P. kudriavzevii*. These isolates presented optimal growth conditions at 30 ºC, 8% of salt and pH of 3.5, whereas for the isolate from cluster D, identified as *Y. alimentaria*, the optimal conditions were 8 ºC, 8% of salt and pH of 5.3 (Table 1; Figure 3).

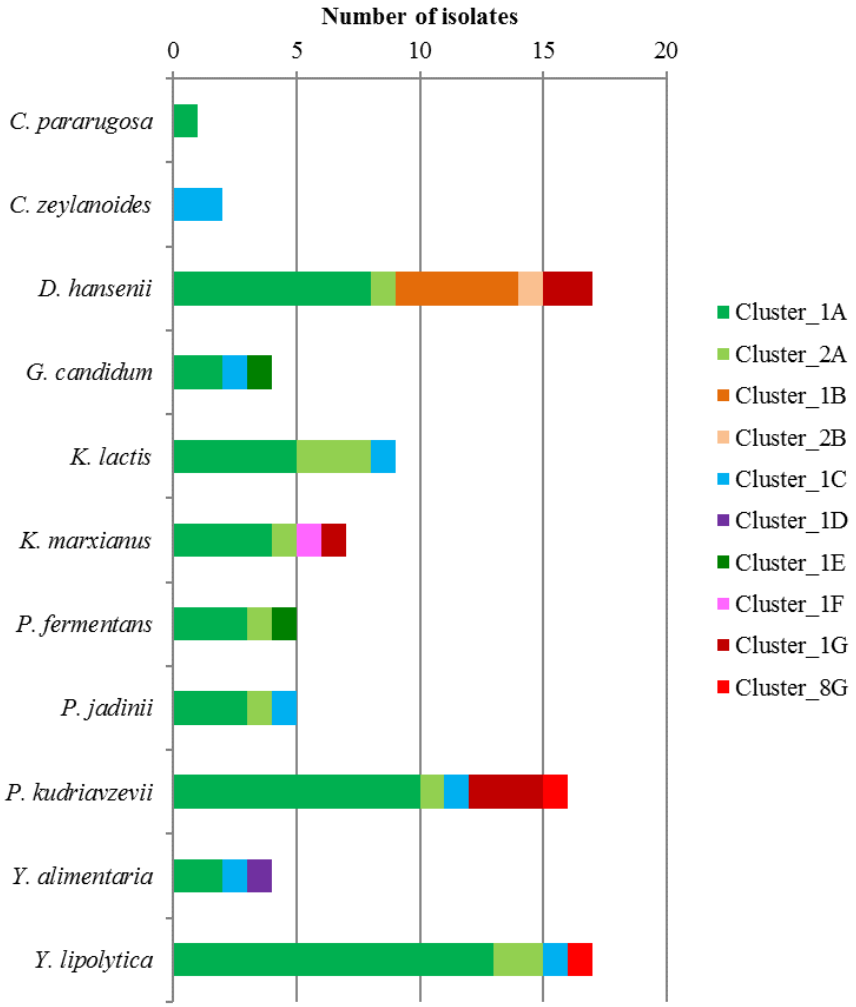


Figure 3. Number of isolates by yeast specie from clusters 1, 2 and 8 classified by double dendrogram analysis based on the days of growth at cheese ripening conditions and optimal conditions of growth.

On the contrary, the cluster 1C grouped 8 isolates with optimal growth at 18.5 ºC, 1.1 % of salt, and pH of 4.8 (Table 1; Figure 3). Their growth was adversely affected by the salt concentration (main effect) and also influenced by temperature as quadratic effect (Table 2). Those were identified as *C. zeylanoides*, *Yarrowia* spp., *Pichia*, spp., *G. candidum*, and *K. lactis*.

Table 2. Contingency table of the significant effects for the regression models developed for the select isolates based on their growth.

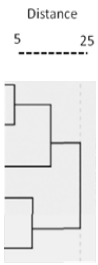
			ANOVA (*p*-values < 0.1)																	
			Main effects						Quadratic effects						Interactions					
			P_TEMP		P_NACL		P_PH		P_AA		P_BB		P_CC		P_AB		P_AC		P_BC	
			N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Cluster A	Cluster_1A	+	45	88.2			3	5.9	11	21.6	1	2.0	2	3.9			5	9.8	1	2.0
		-			37	72.5	2	3.9	8	15.7	9	17.6	3	5.9	19	37.3	7	13.7		
	Cluster_2A	+	9	90.0	1	10.0									1	10.0				
		-			7	70.0	1	10.0			2	20.0	1	10.0	6	60.0				
Cluster B	Cluster_1B	+	5	100			1	20.0											2	40.0
		-							4	80.0										
	Cluster_2B	+																		
		-							1	100										
Cluster C	Cluster_1C	+							0				1	12.5						
		-			7	87.5	1	12.5	6	75.0			1	12.5			1	12.50		
Cluster D	Cluster_1D	+																		
		-	1	100											1	100				
Cluster E	Cluster_1E	+																		
		-			3	100											2	66.7		
Cluster F	Cluster_1F	+	1	100	1	100														
		-															1	100		
Cluster G	Cluster_1G	+	6	100					1	16.7	1	16.7			1	16.7	2	33.3		
		-					2	33.3	1	16.7			1	16.7			1	16.7		
	Cluster_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	3	3.37
		-	1	1.1	55	61.80	6	6.74	21	23.60	11	12.36	6	6.74	26	29.21	12	13.48		

Pigmentation capacity

Among the 87 yeast isolates selected based on their adaptation to cheese processing condition, only yeast isolates belonging to *Yarrowia* genus were able to produce pigment on cheese agar supplemented with tyrosine at different rates. Table 3 shows a dendrogram based on pigmentation intensity of the isolates identified as *Yarrowia* spp. under different temperatures. Eleven of twenty-one *Yarrowia* spp. isolates, identified as *Y. lipolytica*, displayed medium to high pigmentation capacity (Cluster_P1, Cluster_P2 and Cluster P3) under the different conditions tested. Different pigmentation profile was observed in other 5 isolates of this species grouped in Cluster_P4. They developed a progressive and moderate pigmentation capacity when grew at 8ºC, whereas this activity was lower at 25ºC. In contrast, 4 *Y. alimentaria* isolates together with one *Y. lipolytica* isolate were grouped together into the cluster_P5 due to their null or weak pigmentation ability under all conditions tested. On this basis, *Yarrowia* spp. from cluster_P5 together with non-pigmenting isolates, in total 71 isolates were selected to evaluate further technological characteristics.

Table 3. Dendrogram based on pigmentation intensity of the isolates identified as *Yarrowia* spp. after growth at different temperatures during different days (d) indicating the number of isolates of each pigmentation profile within each yeast specie. The intensity values are the average of the data of the isolates included in the cluster.

Pigmentation intensity after growth[1]						**Number of isolates**		**Clusters**
25ºC			8ºC					
3 d	5 d	7 d	10 d	15 d	20 d	*Y. lipolytica*	*Y. alimentaria*	
1,0	1,0	1,0	2,0	3,0	3,0	1	0	Cluster_P1
1,4	2,6	2,7	2,7	3,0	3,0	7	0	Cluster_P2
1,0	1,7	2,0	1,3	2,0	2,0	3	0	Cluster_P3
0,0	0,6	0,8	0,4	1,4	1,6	5	0	Cluster_P4
0,0	0,0	0,2	0,0	0,0	0,2	1	4	Cluster_P5



[1]Pigmentation intensity: 0 (no pigmentation); <1 (low); 1-2 (moderate); 2-3 (high).

Technological characterization

Table 4 shows the hierarchical cluster analysis based on lipolytic (lipase and esterase) and proteolytic capacity of selected isolates. Regarding lipolytic activity, 59 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 isolates were grouped in

cluster_R^+ as they developed mainly lipase activity. Isolates displaying solely esterase activity (3 isolates) were classified in cluster_Tf (higher activity at lower temperatures), and cluster T^+ (higher activity at 25ºC). The last three isolates developed both lipolytic activities and were classified two in cluster T^+R^+, with activity over a wide temperature range, and one in $T20R^+$, with esterase activity in a narrow temperature range. In respect to proteolytic activity, likewise, a high number of isolates, 60, show null or limited activity (Cluster_P^-). Contrary, eleven isolates display 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 two isolates were grouped in cluster Pf, linked to proteolytic activity at lower temperature, and cluster P25_6, associated to activity at 25ºC.

Table 4. Hierarchical cluster analysis of preselect isolates based on lipolytic and proteolytic activities. The values are the average of the data of the isolates included in the cluster.

	Clusters[1]					
Lipolytic activities	T-R-	Tf	R+	T+R+	T20R+	T+
N	59	2	6	2	1	1
Esterase (15ºC)[3]	0,6	9,0	0,9	6,3	0,0	7,0
Esterase (20ºC)	1,3	7,5	3,0	10,3	18,0	4,0
Esterase (25ºC)	0,0	3,5	0,0	10,0	0,0	16,0
Lipase (15ºC)[4]	0,0	0,0	0,7	1,0	1,0	0,0
Lipase (20ºC)	0,0	0,0	1,0	1,0	1,0	0,0
Lipase (25ºC)	0,0	0,0	0,9	1,0	1,0	0,0
	Clusters[2]					
Proteolytic[3] **activity**	P-	P++	P+	P25_6	P+++	Pf
N	60	4	2	1	3	1
15 ºC (3 days)	0,3	10,0	5,0	0,0	14,2	13,0
20 ºC (3 days)	0,0	10,5	6,0	0,0	16,4	0,0
25 ºC (3 days)	0,0	10,8	6,5	0,0	14,9	0,0
15 ºC (6 days)	0,6	15,0	10,5	0,0	23,0	17,0
20 ºC (6 days)	0,1	14,3	9,5	0,0	24,8	13,0
25 ºC (6 days)	0,3	15,5	3,5	27,0	22,6	0,0

[1]Lipolytic clusters: T-R- (Esterase and Lipase negative); Tf (Esterase at low temperature); R+ (Lipase positive); T+R+ (Esterase and lipase positive); T20R+ (Esterase at 20ºC and lipase positive); T+ (Esterase positive).
[2]Proteolytic clusters: P- (proteolytic negative); P+(low proteolytic); P++ (moderate proteolytic); P++ + (high proteolytic); Pf (proteolytic at low temperature); P25_6 (Proteolytic at 25ºC on day 6).
[3]The intensity of esterase and proteolytic activities are expressed in mm.
[4]Lipase activity: negative (0); positive (1).

The combined analysis of lipolytic and proteolytic activity allowed to classify the 71 selected isolates into 13 clusters (Table 5). The cluster $T^{-}R^{-}\_P^{-}$ included the 74.6 % of the isolates (53 isolates) that were associated to scarce o negative proteolytic and lipolytic capacity. The remaining 18 isolates displayed one or both activities in different ranges and were distributed in the remaining twelve clusters. The most interesting clusters were "$T^{+}R^{+}\_\ P^{++}$" and "$T20R^{+}\_\ P^{+++}$" that included the isolates *Y. alimentaria* 1204 and *Y. lipolytica* 2495, respectively, and displayed all activities tested at different rates. Also, noteworthy, some isolates showed some lipolytic activity, esterase or lipase, together with proteolytic activity. They were grouped in clusters "R^{+}_Pf" (Lipase activity and proteolytic activity at low temperature: *Y. alimentaria* 2150) and "Tf_P^{++}" (Esterase activity at low temperature and moderate proteolytic activity: *P. kudriavzevii* 373). Finally, some isolates stood out because they displayed lipolytic or proteolytic activity. *G. candidum* isolates developed lipase activity and *C. pararugosa* esterase activity, whereas four isolates of *K. lactis* and two *P. jadinii* developed only proteolytic activity at different rates.

Table 5. Distribution of clusters from Table 4 of the preselect yeast isolates according to their lipolytic and proteolytic activities.

Cluster 2D		*Candida*				*Kluyveromyces*		*Pichia*			*Yarrowia*		**Total**	
TR	**P**	[1]C. par.	C. zeyl.	D. han.	G. can.	K. lact.	K. mar.	P. fer.	P. jad.	P. kud.	Y. ali.	Y. lip.	**N**	**%**
T-R-	P-		2	17		4	7	5	2	15	1		53	74,6
T-R-	P++					2							2	2,8
T-R-	P+					1			1				2	2,8
T-R-	P+++					1			1				2	2,8
Tf	P-	1											1	1,4
Tf	P++									1			1	1,4
R+	P-				2				1		1		4	5,6
R+	P25_6				1								1	1,4
R+	Pf										1		1	1,4
T+R+	P-				1								1	1,4
T+R+	P++										1		1	1,4
T20R+	P+++											1	1	1,4
T+	P-					1							1	1,4
	Total	1	2	17	4	9	7	5	5	16	4	1	71	100

[1]Yeast species: C. par. (*C. pararugosa*); C. zeyl. (*C. zaylanoides*); D. han. (*D. hansenii*); G. can. (*G. candidum*); K. lact. (*K. lactis*); K. mar. (*K. marxianus*); P. fer (*P. fermentans*); P. jad. (*P. jadinii*); P. kud. (*P. kudriavzevii*); Y. ali. (*Y. alimentaria*): Y. lip. (*Y. lipolytica*).

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 methodology (Figure 4). The first axis accounted for 20.34% of the variance (PC1) and was mainly defined by Proteolytic and to a lesser extent, by 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 concentration and alkalizing capacity. Finally, 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. As we can see in Figure 4, *Candida* spp. isolates were not correlated with any of the technological properties studied. Respect to other species, *D. hansenii* isolates were associated with lactose assimilation, *K. marxianus* with glucose and galactose assimilation and most of *P. kudriavzevii*, *P. fermentans*, *P. jadinii*, *G. candidum* and two *Y. alimentaria* were associated with lactate and citrate assimilation.

On the other side, *K. lactis* isolates showed better acidification capacity and diacetyl production. In addition, some *K. lactis* strains (2287, 2725 and 1507) together with two *P. jadinii* (1731 and 433), two *Y. alimentaria* (1204 and 2150), *Y. lipolytica* 2495 and *P. kudriavzevii* 373 were the ones with better proteolytic capacity. Finally, lipolytic activity was mainly associated to *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 ewes' milk cheese manufacture. Specifically, nine strains, three *K. lactis* strains (2287, 2725 and 1507), two *P. jadinii* (1731 and 433), two *Y. alimentaria* (1204 and 2150), *Y. lipolytica* 2495 and *P. kudriavzevii* 373, stand out among the 71 selected yeast, due to the higher proteolytic capacity at lower temperatures and substrate assimilation, but also lipolytic activity, diacetyl production and acidifying capacity of some of them (Figure 4; Table 6).

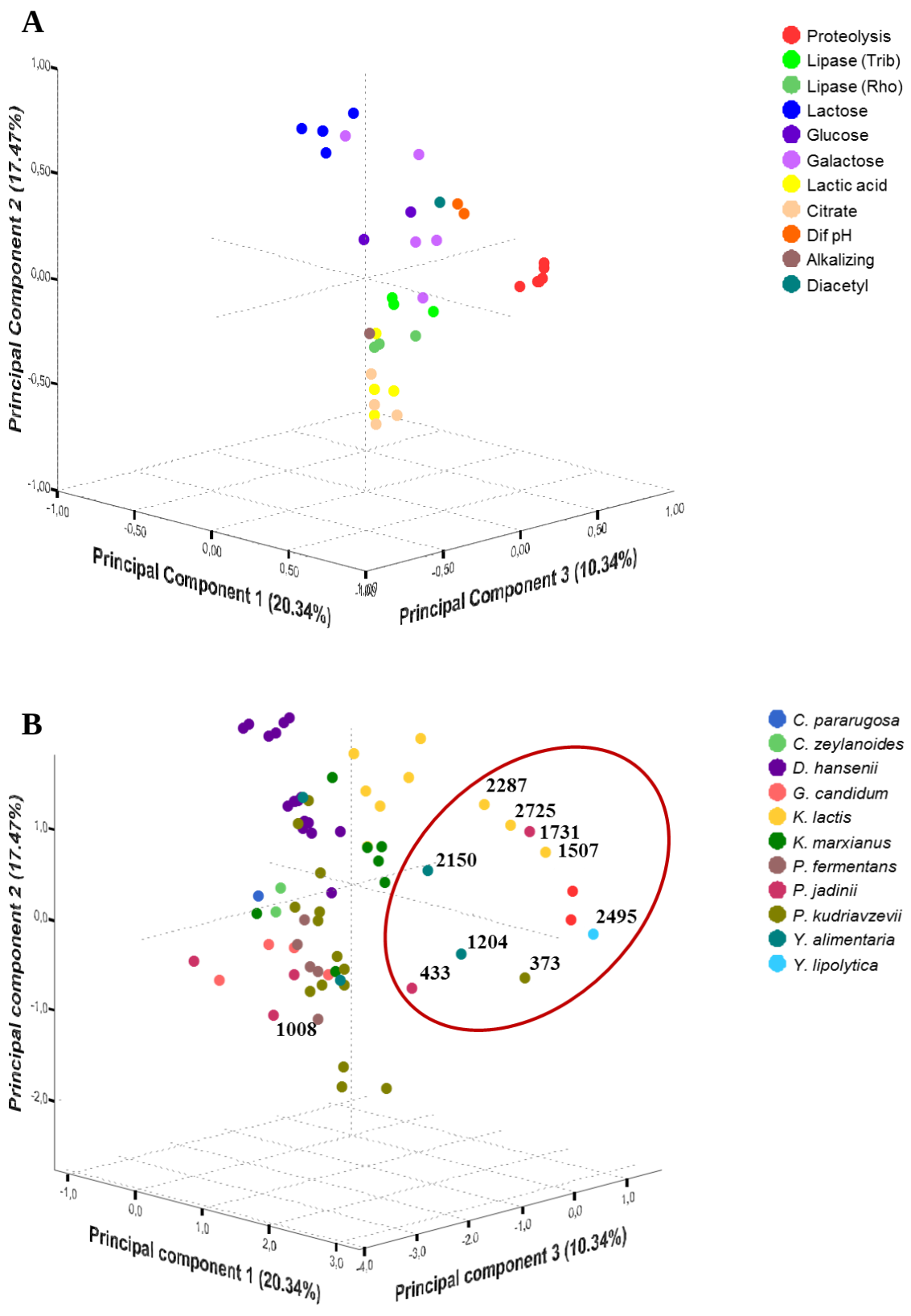


Figure 4. Principal component analysis for technological characterization of selected yeast isolates. A: Technological parameters; B: selected yeast strains species.

Table 6. Specific properties of the nine selected yeasts by PCA analysis of technological characteristics. *P. kudriavzevii (P. kudr)* 373, *P. jadinii* (1731 and 433), *K. lactis* (2287, 2725 and 1507), *Y. alimentaria* (1204 and 2150), and *Y. lipolytica (Y. lipol)* 2495).

Genus	*Yarrowia*			*Kluyveromyces*			*Pichia*		
Species	*Y. lipol.*	*Y. alimentaria*		*K. lactis*			*P. jadinii*		*P. kudr*
Strains	2495	1204	2150	1507	2287	2725	433	1731	373
Proteolytic activity at 15ºC 6 days (mm)	24	17	17	25	11	15	13	26	17
Lipolytic activity (15 ºC)									
Lipase	+	+	+	-	-	-	-	-	-
Esterase (mm)	18[1]	12	4[1]	0	0	4	0	0	6
Alkalizing	-	-	-	-	-	-	+	-	+
Acidifying activity (ΔpH)[2]									
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	-	-	+	+	+	+	-	-	-
Substrate assimilation									
Lactose	7.5[3]	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 activities (API-ZYM system)[4]									
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-Chymotripsin	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
Naptol-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

[1]Esterase activity at 20ºC. (0 mm at 15ºC).
[2]Results expressed as a variation from the initial pH.
[3]Maximum NaCl concentration allowing assimilation.
[4] Enzyme activities (API-ZYM system): values range from 0 (negative activity) to 5 (highest activity). **β**-glucoronidase **α**-glucosidase, α-galactosidase, N-acetyl-β-glucosaminidase, α-fucosidase and trypsin were negative (0) in the nine strains.

Finally, the enzymatic activity profiles of the nine selected strains evaluated by API-ZYM system were given in Table 6. It was found that 6 of the 19 activities assayed, **β**-glucoronidase **α**-glucosidase, α-galactosidase, N-acetyl-β-glucoaminidase, α-fucosidase and trypsin, were not detected in any of the strains. In addition, other three activities, **α**-mannosidase, Lipase (C14), and α-chymotrypsin Lipase (C14) were only detected at low level (Level 1) in some strains. For all other enzyme, different profiles of activities were found. All strains presented string esterase (C4), leucine arylamidase, and valine arylamidase activities (>level 3). Likewise, esterase Lipase (C8), cystine arylamidase, phosphatase acid and naphtol-AS-BI-phosphohydrolase were reported in all strains but at highly variable levels. Alkaline phosphatase and **β**-glucosidase were detected for most of the strains at different levels, while only the three *K. lactis* strains presented **β**-galactosidase activity (Level 5).

III.4. Discussion

Raw milk cheese ripening is a complex process where occurs a cascade of biochemical events mediated by the metabolic flux of the autochthonous microbiota which define the sensory characteristics of the final product. An important contribution is attributed to the secondary microbiota, mainly composed by different bacterial groups and yeasts where the interactions between them and principal microbiota are not yet fully understood (Fox et al., 2015). Yeasts, in particular, have 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 levels of yeast around 4 log cfu/g in soft cheeses during the whole ripening process. Similar values have been reported by other authors in the interior of this type of cheese (Ordiales et al., 2013b; Gonçalves et al., 2017). Specially, we detected the presence of 19 different species mainly associated to *Kluyveromyces*, *Debaryomyces*, *Pichia* and *Yarrowia* genera. These genera are common inhabitants of cheese environment and some strains of them have been proposed as potential adjunct cultures for cheesemaking application (Irlinger et al., 2017). In this context, these PDO cheeses may be a rich source of technological relevant yeasts to develop a mix autochthonous starter culture for improving the cheesemaking process and ensuring their authenticity and quality. For this contribution, they must first of all be well adapted to the stringent conditions of cheesemaking and ripening. Cheese environment change from the surface to the interior and along ripening process shaping the yeast

species composition (Padilla et al., 2014a; Dugat-Bony et al., 2015). Our results show that a high percentage of yeasts showed a null or low adaptability to the cheese growth conditions evaluated. Most of yeast species grow well at wide range of pH, temperature and high NaCl concentration, however, they differ in substrate and oxygen requirements which conditions their adaptability to the interior of cheese (Fröhlich-Wyder et al., 2019). In this work, the isolates with better growth ability were restricted to species belong to *Kluyveromyces*, *Debaryomyces*, *Pichia*, *Yarrowia*, *Candida* and *Geotrichum* genera that are well documented their capacity to tolerate cheese ripening conditions (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 increase of salt concentration. Most growth cheese tolerant isolates showed optimal growth in the range of 20–30°C, temperatures commonly reported for adequate yeast growth in the literature. However, notably some isolates, belonging to clusters 1D, 1F and 1C (Table 1; Figure 3), display optimal growth at lower temperature. Many yeast species grow well at low temperature (Fleet, 2011), which can positively influence the development of their biochemical properties under cold cheese ripening conditions. Regarding to 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*.

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

The results of technological characterization obtained of cheese ripening growth adapted strains were variable depending on the species studied, as well as between strains of the same species. In this investigation 9 yeasts isolates belonging to 5 different species highlighted by their biochemical features. An important role on cheese flavor and texture development is associated to 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 nine selected strains display relevant extracellular proteolytic activity at low temperature and also great arylamidases (aminopeptidase) activity. Aminopeptidases are involved in the hydrolysis of N-terminal amino acids from peptides (Gatti et al., 2008) and contribute to prevent a 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 impact on proteolysis. It is well known that yeast populations frequently decrease during cheese ripening but as far as we know, no data are reported in the literature about if yeast death is accompanied by cell lysis during cheese ripening. Moreover, it has been suggested that acid phosphatases, present at different level in the 9 selected strains, act synergistically with proteolytic enzymes on phosphorylated proteins/peptides allowing a higher production of small peptides and free aminoacids and thus contributing actively to the aroma formation in cheese (Akuzawa and Fox, 2004). The high peptidase activity and phosphatases together with the absence or weak protease activity (Trypsin and chymotrypsin) of these strains has been reported desirable for aroma and texture development in dairy fermentations (Mathara et al., 2004).

Contrary, extracellular lipolytic activity was mainly restricted to *Yarrowia* spp. isolates and 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 level 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. As consequence, various studies have tried to exploit its great potential for 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 been also reported as good lipase producers (Atanassova et al. 2016; Zheng et al., 2018), whereas no report, to our knowledge, has yet documented this activity in *Y. alimentaria*.

Regarding to other technological properties evaluated, substrate assimilation of most relevant yeast isolates at different NaCl concentration showed that these strains were able to assimilate lactose, galactose and glucose at NaCl concentration higher than 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 three strains, *Y. lipolytica* 2495, *P. kudriavzevii* 373 and *P. jadinii* 433, used both substrates. In addition, two of these isolates presented alkalizing activity. These properties have strong influence in the pH, favoring its increase and consequently changes in the cheese microbiota that impact in the texture and biochemical processes that take place during ripening (Irlinger and Mounier, 2009). Contrary, determined yeast species have the ability to acidify the cheese environment. This capacity was mainly restricted to *K. lactis* and at lesser extend to *Y. alimentaria* 1204. In this work, *K. lactis* strains showed β-galactosidase activity that is a key enzyme in the transformation of lactose to lactic acid. This is a relevant feature that allow them to contribute at initial stages of ripening, together with LAB, to the lactose depletion (Fadda et al., 2004). Moreover, these isolates also produced diacetyl-acetoin in skim milk that is a generally appreciated for its positive contribution to the aromatic profile of cheese (Curioni and Bosset, 2002).

IV.5. Conclusion.

In conclusion, this study reveals that a high diversity of yeasts species is involved in the ripening of soft raw ewe's milk PDO cheeses 'Torta del Casar' and 'Queso de la Serena'. Among the 157 isolates selected based on their genetic profile, 87 showed good adaptation to the stress conditions of cheese ripening process. Their technological characterization evidenced 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 great biochemical features for their potential use as adjunct cultures alone or in combination with autochthonous starter bacteria in 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 system to know their contribution to an adequate balanced of proteolysis, lipolysis and other reactions products that have a great influence on the texture, aromatic profile and general quality of the final product.

IV.6. References

Akuzawa, R., and P.F. Fox. 2004. Acid phosphatase in cheese. Anim. Sci. J. 75:385–391. doi:10.1111/j.1740-0929.2004.00202.x.

Araújo-Rodrigues, H., F.K. Tavaria, M.T.P.G. dos Santos, N. Alvarenga, and M.M. Pintado. 2020. A review on microbiological and technological aspects of Serpa PDO cheese: An ovine raw milk cheese. Int. Dairy J. 100:104561. doi:10.1016/j.idairyj.2019.104561.

Atanassova, M.R., C. Fernández-Otero, P. Rodríguez-Alonso, I.C. Fernández-No, J.I. Garabal, and J.A. Centeno. 2016. Characterization of yeasts isolated from artisanal short-ripened cows' cheeses produced in Galicia (NW Spain). Food Microbiol. 53:172–181. doi:10.1016/j.fm.2015.09.012.

Barth, G., and C. Gaillardin. 1997. Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. FEMS Microbiol. Rev. 19:219–237. doi:10.1111/j.1574-6976.1997.tb00299.x.

Binetti, A., M. Carrasco, J. Reinheimer, and V. Suárez. 2013. Yeasts from autochthonal cheese starters: Technological and functional properties. J. Appl. Microbiol. 115:434–444. doi:10.1111/jam.12228.

Bockelmann, W., M. Gareis, and K.J. Heller. 2010. Can acid curd cheese be manufactured safely?. DMZ, Leb. und Milchwirtschaft 131:26–28.

Boutrou, R., and M. Guéguen. 2005. Interests in *Geotrichum candidum* for cheese technology. Int. J. Food Microbiol. 102:1–20. doi:10.1016/j.ijfoodmicro.2004.12.028.

Carbonell, M., M. Nuñez, and E. Fernández-García. 2002. Evolution of the volatile components of ewe raw milk La Serena cheese during ripening. Correlation with flavour characteristics. Lait 82:683–698. doi:10.1051/lait:2002042.

Carreira, A., L. Paloma, and V. Loureiro. 1998. Pigment producing yeasts involved in the brown surface discoloration of ewes' cheese. Int. J. Food Microbiol. 41:223–230. doi:10.1016/S0168-1605(98)00054-3.

Centeno, J.A., J.I. Garabal, F. Docampo, J.M. Lorenzo, and J. Carballo. 2017. Recovering traditional raw-milk Tetilla cheese flavour and sensory attributes by using *Kocuria varians* and *Yarrowia lipolytica* adjunct cultures. Int. J. Food Microbiol. 251:33–40. doi:10.1016/j.ijfoodmicro.2017.03.014.

Chen, L. shui, J. Cui, Q. bo Ding, Y. Ma, L. jun Chen, J. ying Dong, T. ming Jiang, and J. louis Maubois. 2012. The effect of yeast species from raw milk in china on

proteolysis and aroma compound formation in Camembert-type cheese. Food Bioprocess Technol. 5:2548–2556. doi:10.1007/s11947-011-0589-4.

Cogan, T.M., S. Goerges, R. Gelsomino, S. Larpin, M. Hohenegger, N. Bora, E. Jamet, M.C. Rea, J. Mounier, M. Vancanneyt, M. Guéguen, N. Desmasures, J. Swings, M. Goodfellow, A.C. Ward, H. Sebastiani, F. Irlinger, J.-F. Chamba, R. Beduhn, and S. Scherer. 2014. Biodiversity of the surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit, and Gubbeen cheeses. Microbiol. Spectr. 2. doi:10.1128/microbiolspec.CM-0010-2012.

Curioni, P.M.G., and J.O. Bosset. 2002. Key odorants in various cheese types as determined by gas chromatography-olfactometry. Int. Dairy J. 12:959–984. doi:10.1016/S0958-6946(02)00124-3.

De Freitas, I., N. Pinon, J. Maubois, S. Lortal, and A. Thierry. 2009. The addition of a cocktail of yeast species to Cantalet cheese changes bacterial survival and enhances aroma compound formation. Int. J. Food Microbiol. 129:37–42. doi:10.1016/j.ijfoodmicro.2008.10.026.

Delgado, F.J., J. González-Crespo, R. Cava, J. García-Parra, and R. Ramírez. 2010. Characterisation by SPME-GC-MS of the volatile profile of a Spanish soft cheese P.D.O. Torta del Casar during ripening. Food Chem. 118:182–189. doi:10.1016/j.foodchem.2009.04.081.

Dugat-Bony, E., C. Straub, A. Teissandier, D. Onésime, V. Loux, C. Monnet, F. Irlinger, S. Landaud, M.N. Leclercq-Perlat, P. Bento, S. Fraud, J.F. Gibrat, J. Aubert, F. Fer, E. Guédon, N. Pons, S. Kennedy, J.M. Beckerich, D. Swennen, and P. Bonnarme. 2015. Overview of a surface-ripened cheese community functioning by meta-omics analyses. PLoS One 10:1–25. doi:10.1371/journal.pone.0124360.

Fadda, M.E., V. Mossa, M.B. Pisano, M. Deplano, and S. Cosentino. 2004. Occurrence and characterization of yeasts isolated from artisanal Fiore Sardo cheese. Int. J. Food Microbiol. 95:51–59. doi:10.1016/j.ijfoodmicro.2004.02.001.

Ferreira, A.D., and B.C. Viljoen. 2003. Yeasts as adjunct starters in matured Cheddar cheese. Int. J. Food Microbiol. 86:131–140. doi:10.1016/S0168-1605(03)00252-6.

Fleet, G.H. 2011. Yeast spoilage of foods and beverages. Pages 53-63 in The Yeasts, a Taxonomic Study. C. P. K. C. J. W. Fell, & T. Boekhout, eds. Elsevier. Amsterdam. doi: 10.1016/B978-0-444-52149-1.00005-7

Fox, P.F., T. Uniacke-Lowe, P.L.H. McSweeney, and J.A. O'Mahony. 2015. Dairy Chemistry and Biochemistry, Second Edition. doi: 10.1007/978-3-319-14892-2.

Freitas, C., and F. Xavier Malcata. 2000. Microbiology and biochemistry of cheeses with Appélation d'Origine Protegée and manufactured in the Iberian Peninsula from ovine

and caprine milks. J. Dairy Sci. 83:584–602. doi:10.3168/jds.S0022-0302(00)74918-6.

Fröhlich-Wyder, M.T., E. Arias-Roth, and E. Jakob. 2019. Cheese yeasts. Yeast 36:129–141. doi:10.1002/yea.3368.

Gardini, F., R. Tofalo, N. Belletti, L. Iucci, G. Suzzi, S. Torriani, M.E. Guerzoni, and R. Lanciotti. 2006. Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. Food Microbiol. 23:641–648. doi:10.1016/j.fm.2005.12.005.

Garnier, L., F. Valence, and J. Mounier. 2017. Diversity and control of spoilage fungi in dairy products: an update. Microorganisms 5:42. doi:10.3390/microorganisms5030042.

Gatti, M., J. De Dea Lindner, F. Gardini, G. Mucchetti, D. Bevacqua, M.E. Fornasari, and E. Neviani. 2008. A model to assess lactic acid bacteria aminopeptidase activities in Parmigiano Reggiano cheese during ripening. J. Dairy Sci. 91:4129–4137. doi:10.3168/jds.2008-1069.

Gkatzionis, K., L. Hewson, T. Hollowood, J. Hort, C.E.R. Dodd, and R.S.T. Linforth. 2013. Effect of *Yarrowia lipolytica* on blue cheese odour development: Flash profile sensory evaluation of microbiological models and cheeses. Int. Dairy J. 30:8–13. doi:10.1016/j.idairyj.2012.11.010.

Gonçalves Dos Santos, M.T.P., M.J. Benito, M. de G. Córdoba, N. Alvarenga, and S. Ruiz-Moyano Seco de Herrera. 2017. Yeast community in traditional Portuguese Serpa cheese by culture-dependent and -independent DNA approaches. Int. J. Food Microbiol. 262:63–70. doi:10.1016/j.ijfoodmicro.2017.09.013.

Irlinger, F., and J. Mounier. 2009. Microbial interactions in cheese: implications for cheese quality and safety. Curr. Opin. Biotechnol. 20:142–148. doi:10.1016/j.copbio.2009.02.016.

Irlinger, F., S. Helinck, and J.L. Jany. 2017. Secondary and Adjunct Cultures. Pages 273-300 in Cheese: Chemistry, physics and microbiology, volume 1, general aspects. P. L. H. McSweeney, P. F. Fox, P. D. Cotter, and D. W. Everett, eds. Elsevier Academic Press. London. doi: 10.1016/B978-0-12-417012-4.00011-9

Ismail, S.A.S., T. Deak, H.A. Abd El-Rahman, M.A.M. Yassien, and L.R. Beuchat. 2000. Presence and changes in populations of yeasts on raw and processed poultry products stored at refrigeration temperature. Int. J. Food Microbiol. 62:113–121. doi:10.1016/S0168-1605(00)00414-1.

Kongo, J.M., and F.X. Malcata. 2016a. Cheese: Processing and Sensory Properties. Pages 748-754 in Encyclopedia of Food and Health. 1st ed. Elsevier. Netherlands. doi: 10.1016/B978-0-12-384947-2.00136-7

Kongo, J.M., and F.X. Malcata. 2016b. Cheese: Chemistry and Microbiology. Pages 735-740 in Encyclopedia of Food and Health. 1st ed. Elsevier. Netherlands. doi: 10.1016/B978-0-12-384947-2.00135-5

Kouker, G., and K.E. Jaeger. 1987. Specific and sensitive plate assay for bacterial lipases. Appl. Environ. Microbiol. 53:211–213.

Kurtzmann, C.P., and J.W. Fell. 1998. The Yeast-A Taxonomic Study. Pages 420-422. C.P. Kurtzmann and J.W. Fell, ed. Elsevier Science, B.V., Amsterdam, The Netherlands.

Mahmoud, M. a., H.M. Ali, a. R.M. El-Aziz, M.R. Al-Othman, and a. S. Al-Wadai. 2014. Molecular characterization of aflatoxigenic and non-aflatoxigenic *Aspergillus flavus* isolates collected from corn grains. Genet. Mol. Res. 13:9352–9370. doi:10.4238/2014.November.11.2.

Martínez, S., I. Franco, and J. Carballo. 2011. Spanish goat and sheep milk cheeses. Small Rumin. Res. 101:41–54. doi:10.1016/j.smallrumres.2011.09.024.

Mathara, J.M., U. Schillinger, P.M. Kutima, S.K. Mbugua, and W.H. Holzapfel. 2004. Isolation, identification and characterisation of the dominant microorganisms of kule naoto: the Maasai traditional fermented milk in Kenya. Int. J. Food Microbiol. 94:269–278. doi:10.1016/j.ijfoodmicro.2004.01.008.

Medina, A., R.J.W. Lambert, and N. Magan. 2012. Rapid throughput analysis of filamentous fungal growth using turbidimetric measurements with the Bioscreen C: a tool for screening antifungal compounds. Fungal Biol. 116:161–169. doi:10.1016/j.funbio.2011.11.001.

Merchán, A. V., M.J. Benito, A.I. Galván, and S. Ruiz-Moyano Seco de Herrera. 2020. Identification and selection of yeast with functional properties for future application in soft paste cheese. LWT 124:109173. doi:10.1016/j.lwt.2020.109173.

Monnet, C., S. Landaud, P. Bonnarme, and D. Swennen. 2015. Growth and adaptation of microorganisms on the cheese surface. FEMS Microbiol. Lett. 362:1–9. doi:10.1093/femsle/fnu025.

Montel, M.C., S. Buchin, A. Mallet, C. Delbes-Paus, D.A. Vuitton, N. Desmasures, and F. Berthier. 2014. Traditional cheeses: Rich and diverse microbiota with associated benefits. Int. J. Food Microbiol. 177:136–154. doi:10.1016/j.ijfoodmicro.2014.02.019.

Ordiales, E., A. Martín, M.J. Benito, A. Hernández, S. Ruiz-Moyano, and M. de G. Córdoba. 2013a. Role of the microbial population on the flavor of the soft-bodied cheese Torta del Casar. J. Dairy Sci. doi:10.3168/jds.2013-6587.

Ordiales, E., M.J. Benito, A. Martín, R. Casquete, M.J. Serradilla, and M. de Guía Córdoba. 2013b. Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. Food Control 33:448–454. doi:10.1016/j.foodcont.2013.03.027.

Padilla, B., C. Belloch, J.J. López-Díez, M. Flores, and P. Manzanares. 2014a. Potential impact of dairy yeasts on the typical flavour of traditional ewes' and goats' cheeses. Int. Dairy J. 35:122–129. doi:10.1016/j.idairyj.2013.11.002.

Padilla, B., P. Manzanares, and C. Belloch. 2014b. Yeast species and genetic heterogeneity within *Debaryomyces hansenii* along the ripening process of traditional ewes' and goats' cheeses. Food Microbiol. 38:160–166. doi:10.1016/j.fm.2013.09.002.

Price, E.J., R.S.T. Linforth, C.E.R. Dodd, C.A. Phillips, L. Hewson, J. Hort, and K. Gkatzionis. 2014. Study of the influence of yeast inoculum concentration (*Yarrowia lipolytica* and *Kluyveromyces lactis*) on blue cheese aroma development using microbiological models. Food Chem. 145:464–472. doi:10.1016/j.foodchem.2013.08.081.

Prista, C., M. Loureiro-Dias, V. Montiel, and J. Ramos. 2005. Mechanisms underlying the halotolerant way of *Debaryomyces hansenii*. FEMS Yeast Res. 5:693–701. doi:10.1016/j.femsyr.2004.12.009.

Regulation (CE) 1107/1996 of European commission of 12 of June: Registration of the protected designation of origin "Queso de la Serena" in the register of protected designations of origin and the protected geographic indications.

Regulation (CE) 1491/2003 of European commission of 25 of August: Registration of the protected designation of origin "Torta del Casar" in the register of protected designations of origin and the protected geographic indications.

Rohlf, F.J. 1993. NTSYS.PC: numerical taxonomy and multivariate analysis system, version 2.0. Appl. Biostat. Inc.

Speckman, R.A., and E.B. Collins. 1982. Microbial production of 2,3-Butylene Glycol from cheese whey. Appl. Environ. Microbiol. 43:1216–1218. doi:10.1128/aem.43.5.1216-1218.1982.

Tabla, R., A. Gómez, J.E. Rebollo, and I. Roa. 2015. Salt influence on surface microorganisms and ripening of soft ewe cheese. J. Dairy Res. 82:215–221. doi:10.1017/S0022029915000023.

Tavaria, F.K., and F.X. Malcata. 2000. On the microbiology of Serra da Estrela cheese: geographical and chronological considerations. Food Microbiol. 17:293–304. doi:10.1006/fmic.1999.0315.

Yalcin, H.T., and F.B. Ucar. 2009. Isolation and characterization of cheese spoiler yeast isolated from Turkish white cheeses. Ann. Microbiol. 59:477–483. doi:10.1007/BF03175134.

Zheng, X., K. Li, X. Shi, Y. Ni, B. Li, and B. Zhuge. 2018. Potential characterization of yeasts isolated from Kazak artisanal cheese to produce flavoring compounds. Microbiologyopen 7:1–12. doi:10.1002/mbo3.533

Supplementary material

This chapter contains two supplementary Tables

Table S1: Identification and strain typing of the selected yeasts isolated throughout ripening process from PDO "Torta del Casar" and "Queso de la Serena" cheeses for their technological characterization.

Genus	Specie	Nº of isolates selected	Code strain	ISSR-GTG$_5$ profile (OTU)	Nº industries
Aureobasidium	*A. pullulans*	3	383, 2182, 2271	2	3
Candida	*C. glabrata*	1	908	1	1
	C. pararugosa	2	649, 2710	1	2
	C. humilis	1	2285	1	1
	C. intermedia	1	2290	1	1
	C. parapsilopsis	0	oportunistic pathogen	1	4
	C. zeylanoides	10	17, 330, 350, 665, 882, 896, 921, 925, 1214, 1459	3	3
Debaryomyces	*D. hansenii*	23	329, 353, 898, 910, 923, 933, 954, 1088, 1092, 1100, 1206, 1232, 1238, 1249, 1251, 1522, 1575, 1600, 1615, 1630, 1760, 2190, 2214	3	6
Geotrichum	*G. candidum*	10	369, 663, 902, 1006, 1461, 1466, 1538, 2171, 2184, 2550	2	4
Kazachstania	*K. humilis*	1	2169	1	1
Kazachstania	*K. bulderi*	1	2481	1	1
Kluyveromyces	*K. lactis*	17	371, 874, 890, 904, 1098, 1351, 1507, 1528, 1542, 2141, 2146, 2267, 2287, 2478, 2554, 2561, 2725	1	5
	K. marxianus	12	364, 1070, 1463, 1518, 1540, 1730, 2144, 2154, 2459, 2469, 2484, 2744	1	3
Pichia	*P. fermentans*	9	170, 1076, 1364, 1438, 1520, 2143, 2472, 2497, 2720	3	3
	P. jadinii	8	173, 433, 659, 1008, 1468, 1544, 1731, 2489	2	3
	P. kudriavzevii	25	2, 165, 373, 435, 645, 871, 939, 1074, 1094, 1193, 1224, 1241, 1360, 1368, 1436, 1509, 1536, 1548, 1732, 1756, 2188, 2281, 2298, 2457, 2578	6	6
	P. sporocuriosa	1	1443	1	1
Yarrowia	*Y. alimentaria*	7	929, 1204, 1221, 1476, 1584, 1729, 2150	2	3
	Y. lipolytica	25	387, 498, 502, 510, 522, 667, 888, 912, 918, 947, 996, 1090, 1160, 1202, 1362, 1604, 1738, 2156, 2181, 2186, 2283, 2454, 2495, 2556, 2714	4	5
Total: 8	*Total:* 19	*Total:* 157		*Total:* 37	*Total:* 6

Table S2. Conditions of temperature, salt concentration and pH for modelling yeast growth under cheese ripening process.

Block	Temperature (ºC)	%NaCl	pH
1	30	8	5
2	30	4.5	3.5
3	30	4.5	6.5
4	30	1	5
5	19	1	3.5
6	19	8	3.5
7	19	1	6.5
8	19	8	6.5
9	19	4.5	5
10	8	1	5
11	8	4.5	6.5
12	8	8	5
13	8	4.5	3.5
14	19	4.5	5
15	19	4.5	5

Factor combinations of ripening process

Stage	Temperature (ºC)	%NaCl	pH
Initial ripening	8	1	6
Early ripening	8	3	4.5
Mid ripening	10	3	5
Late ripening	12	4	5.5
End ripening	15	5	5.9

CAPÍTULO VI

Evaluation of the contribution of autochthonous yeasts with technological properties to the sensory properties of experimental soft cheeses.

Capítulo VI

Evaluation of the contribution of autochthonous yeasts with technological properties to the sensory properties of experimental soft cheeses.

Abstract

The lack of homogeneity in the final quality of traditional cheeses is one of its main problems. This group of traditional cheeses includes soft cheeses made with raw ewe's milk in Extremadura. The application of an indigenous starter culture could contribute to the standardization of the product. Lactic acid bacteria have been the most studied for this purpose; however, in recent years the role of other microorganisms such as yeasts has been highlighted. Therefore, studies for the selection and application of yeasts in these cheeses are necessary to achieve a homogeneous product with a unique sensory quality. Thus, the aim of this work was to evaluate in an experimental cheese the contribution to the sensory properties of nine yeasts selected for their technological properties previously studied.

To this end, experimental cheeses were made in the laboratory with and without inoculation of native yeasts with technological properties and their implantation and impact on sensory properties at the end of maturation were studied. The results showed that the nine yeast strains used increased significantly during maturation, adapting adequately to the cheese matrix. In relation to its impact on the properties of the cheese, two of the nine strains inoculated in the experimental cheeses, *Pichia kudriavzevii* L373 and *Yarrowia lipolytica* L2495 allowed to obtain a softer texture than the rest of the elaborated batches. While in the sensory analysis no significant differences were observed between the parameters: visual analysis of the paste, sensory firmness, intensity, persistence of aroma and overall valuation between the batches of cheese inoculated with yeasts and the Control and LAB batches. However, the average values of the sensory parameters in the batches with yeasts compared to the control were generally higher in color of the paste, intensity and persistence of the aroma and lower in firmness and overall assessment. Which indicates that the yeasts partly modified the sensory characteristics evaluated. Finally, the analysis of main components of the physic-chemical, microbiological and sensory parameters of the batches of cheese made corroborated that yeasts have a clear impact on the maturation process of cheese. In addition, although more studies are needed, three yeasts *Y. alimentaria* L2150, *Y. lipolytica* L2495 and *K. lactis*

L1507 based on their relationship with the sensory parameters of intensity and persistence and with sensory and instrumental firmness showed to be the best candidates to be part of a mixed starter culture to produce soft cheeses from sheep's milk.

VI.1. Introduction.

Cheese microbiome variability.

The quality of any type of cheese is influenced by the quality of the milk with which it has been made, both by its microbiological, biochemical, sensory and other parameters. Raw milk is the main variable starting point, as its composition varies according to some factors such as the state of lactation, the diet and the state of health of the producing animal. In addition, the microbiological quality of milk will influence the microbiological quality of derived dairy products. In cheeses made with raw milk and, therefore, the absence of any thermal standardization process, together with different milking and handling protocols and therefore, the variation in the hygiene conditions prevailing on farms, leads to extensive and unpredictable variability in the milk microbiota and therefore, in that of cheese made from raw milk during maturation (Pereira et al., 2010). The set of microorganisms that make up the milk microbiota is composed of a great diversity of bacteria, molds and yeasts. These microorganisms can be categorized into three large groups: non-pathogens, spoilage and beneficial. Within the group of pathogenic microorganisms most found can be highlighted *Listeria monocytogenes,* Shiga toxin-producing *Escherichia coli* (STEC), *Staphylococcus aureus,* some spore-forming bacteria (*Bacillus* spp. and *Clostridia), Salmonella* spp. and others of the genus *Mycobacteria (Mycobacterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis)* and *Brucella* spp. The current regulation stipulates that all cheeses made from raw milk from bovine, caprine and ovine species must remain in the ripening stage for a minimum period of 60 days to eliminate the presence of pathogenic microorganisms, therefore, it is essential to analyze the presence of pathogens in the products after this time.

In the last two decades, cheese industries have improved the hygienic conditions of production, and this has resulted in better hygienic quality of milk and dairy products. Thus, recent works, in soft-paste sheep cheeses such as "Torta del Casar" and "Serpa" no pathogenic microorganisms have been detected in the final product (Ordiales et al., 2013a; Gonçalves et al., 2018). However, in cheeses made with raw milk, although in

Europe at present the incidence is lower, it is necessary to develop strategies for its control and thus ensure the safety of the product (Gonzales-Barron et al., 2017). Among the spoilage microorganisms, responsible for significant economic losses in the sector, the most frequently detected are sporulated and non-sporulated bacteria such as *Clostridium botulinum* and *Bacillus anthracis*, some strains of *B. cereus*, psychrotrophs (grow at <10ºC, some are thermoduric and survive the pasteurization), enterobacteria and *Pseudomonas* (O'Sullivan and Cotter,2017). In this way, various defects can be related to microorganisms that are presented in milk or even in the vegetable coagulant without standardization and that in turn are transferred to the cheese. Certain strains of *Pseudomonas* spp. are responsible for pigmentations in cheese (del Olmo et al., 2018). Other microorganisms, such as species of the *Enterobacteriaceae* family are present in the maturation of this type of cheeses (Ordiales et al., 2013a), characterized by an important enzymatic activity composed of proteolytic and lipolytic enzymes that modify the organoleptic properties of cheeses during maturation (Irlinger et al., 2012). Also, they can produce biogenic amines (Montel et al., 2014) and certain enterobacteria can produce gas (Tabla et al., 2016). In this regard, the gas production capacity of many species of enterobacteria and coliforms can lead to early swelling and eyes on the cheese. Heterofermentative LAB also contribute to this fact due to its gas production to eye formation and has been shown to contribute negatively to the taste of this type of cheese (Ordiales et al., 2013b). Therefore, the microbial variability associated with this type of cheeses can trigger different types of alterations of the final product.

Finally, during maturation process, there are many species of beneficial bacteria present in raw milk among which stand out the lactic acid bacteria (LAB) whose main function is lactic fermentation, transforming lactose into other products such as lactic acid, triggering in turn a drop in pH that inhibits the development of other pathogenic microorganisms (Kongo and Malcata, 2016a). Among other functions, they have proteolytic capacity so they can metabolize proteins and peptides giving rise to other simpler nitrogen compounds. This group remains dominant throughout the maturation process being the most frequently found some LAB species such as *Lactobacillus delbrueckii, Lb. casei, Lb. paracasei, Lb. plantarum, Lc. lactis, Leuconostoc mesenteroides, Streptococcus thermophilus, Enterococcus faecalis* and *E. faecium* among others (Ordiales et al., 2013a; Sánchez-Juanes et al., 2020). Within the secondary microbiota of these cheeses, it can be highlighted mainly with enterobacteria and yeasts

(Cáceres et al., 1997; Gonçalves et al., 2017, 2018). In recent years, special attention has been paid to the role of yeasts in cheeses. The population of molds and yeasts present in raw milk comes mainly from external contamination of the industry environment such as the walls, shelves of the ripening room or the equipment surfaces (Bokulich and Mills, 2013). Yeasts can metabolize lactic acid in milk causing a pH elevation and, resulting in secondary compounds such as short-chain fatty acids that contribute to aroma formation (Montel et al., 2014). It is usually found species of the genera *Candida, Debaryomyces, Pichia, Kluyveromyces, Saccharomyces* and *Yarrowia* in artisanal soft cheeses. In turn, cheeses matured with molds have very specific sensory characteristics due to the high proteolytic and lipolytic capacity of these microorganisms, which release fatty acids and peptides thus contributing to the formation of very distinctive aromas and flavors. Such species of the genera *Aspergillus, Geotrichum, Mucor, Fusarium* and *Penicillium* are the most common (Irlinger et al., 2017).

Standardization of the final product: application of starter cultures

Microbiological control of milk is a very important factor in the production of cheese and other dairy products. The factors that can alter the microbiological quality of the milk and that require control are the state of health of the producing animal and the cross-contamination produced during the extraction of the milk itself through the udders. Therefore, it is advisable to comply with hygienic measures in their due degree of availability such as good practices during milking, cleaning and preventive maintenance of equipment and storage of milk in suitable aseptic containers. Some steps that can be applied to reduce the microbiological load of milk are heat treatments such as pasteurization or sterilization, centrifugation and microfiltration, that eliminate up to 99% of the microorganisms present. It is necessary to apply starter cultures after a heat treatment for the elaboration of different dairy products from which a series of sensory characteristics are expected (Fernández García, 2019; O'Sullivan and Cotter, 2017). On the other hand, in raw ewes' milk cheese with a variable microbiota, the use of indigenous starter cultures could be a possible way to overcome the homogeneity problems of traditional soft-paste sheep's milk cheeses and thus obtain a consistent quality product reducing variability and minimizing safety risks (Araújo-Rodrigues et al., 2020). The application of these starter cultures could contribute positively to the biochemical changes that occur during maturation and favor the development of desirable microorganisms in

this process avoiding the proliferation of spoilage and pathogen microorganisms. However, one of the challenges in the development of a starter culture is the selection of one or a group of strains with relevant and safe technological properties for its use. Therefore, these must be well adapted to the process of making the "Torta" cheese, improve or optimize the maturation process and ensure the uniqueness of the sensorial properties of the final product (Montel et al., 2014; Silvetti et al., 2017). Although the main microorganisms studied as starter culture are LAB, it is important to understand the evolution of the microbiota throughout the cheese maturation to identify the role of the different microbial groups and understand their technological importance to select the most relevant autochthonous strains (Silvetti et al., 2017). Therefore, in traditional raw milk cheeses, such as "Torta", with a very diverse microbial population (Ordiales et al., 2013a), the development of an indigenous starter culture must include various technologically relevant microbial groups related to its maturation. In addition to LAB, other microorganisms representing the secondary microbiota such as cocci gram-positive catalase positive, enterobacteria and yeasts should be included in an autochthonous starter culture. In this sense, in the last decade various works on yeasts isolated from different varieties of cheeses have shown their potential to contribute to the sensory properties of cheese (Atanassova et al., 2016; Chen et al., 2012; Gkatzionis et al., 2014; Padilla et al., 2014a,b).

Yeasts in traditional cheeses have a fundamental role in their maturation. They develop mainly on the surface, but also to a lesser extent inside the core and causes a pH gradient from the surface to the center due to the consumption of lactic acid. When the lactic acid is depleted, they catabolize the amino acids producing NH_3 that diffuses inwards, further increasing the pH (Fröhlich-Wyder et al., 2019). The increase in pH is crucial as it allows the development of other salt-tolerant and low pH-sensitive microorganisms that contribute to the final characteristics of the cheese. Hence, the role of yeasts in the maturation of cheese is recognized. In addition to these complex interactions between yeasts and bacteria, yeasts are characterized by significant metabolic activity due to their important proteolytic and lipolytic enzymes that contribute to the biochemical changes that take place during maturation and, therefore, to the final characteristics of the cheese. In fact, recent studies have proposed some strains of *Debaryomyces hansenii, Yarrowia lipolytica* and *Kluyveromyces marxianus* as suitable for use as starter cultures in cheese (Binetti et al., 2013; Lanciotti et al., 2005; Padilla et

al., 2014a,b), even its positive contribution to the final aroma has been demonstrated by applying it to the product directly (Chen et al., 2012; Lanciotti et al., 2005).

Therefore, the aim of this study is to evaluate the contribution to the texture and sensorial properties of yeasts selected for their technological properties in experimental cheeses.

VI.2. Material and methods.

For the development of this study, a set of yeast strains isolated from soft cheeses of sheep's milk from the region of Extremadura previously selected for their technological properties in the previous chapter was used. The yeast strains used are listed in table 1.

Table 1. Yeast strains selected for their technological properties for their use as starter culture in experimental cheeses.

Yeast specie	**Code**
Kluyveromyces lactis	L1507, L2287, L2725
Pichia kudriavzevii	L373
Pichia jadinii	L1731, L433
Yarrowia alimentaria	L1204, L2150
Yarrowia lipolytica	L2495

In addition, *Lactobacillus casei/ paracasei* UEX_Lc12 was used as a starter strain in the production of experimental cheeses, previously isolated from "Torta del Casar" cheeses (Ordiales et al., 2013). For the elaboration of the experimental cheeses, raw sheep milk belonging to the Merino and Entrefino trunk was used, ceded by the company COOPRADO. As a coagulating agent in the elaboration of the experimental cheese, the flower of the thistle *Cynara cardunculus* L. was used, given by the cheese industry El Castúo (PDO Torta del Casar).

Experimental cheese procedure

Generic cheese material was used to make the cheeses. For the heat treatment of milk, the 20000 GASTROVAC equipment (International Cooking Conceps) was used to pasteurize

raw sheep's milk at 62 °C for 15 minutes at a constant pressure between -0.15 and -0.20 bar. For the coagulation of the milk, a thermostatic bath (Selecta mod. Precisdig) was used at 31ºC. During the curd molding and pressing stage, 250 gr cheese molds, cloths to drain the whey and manual pressing were used. The cheeses remained in the ripening stage for 15 days at 12 °C inside a refrigeration stove of the JP Selecta model Hotcold A-B-C.

Optimization of cheese procedure

Coagulant from C. cardunculus L.

For this purpose, two types of thistle flower collected in different geographical areas and ceded by "El Castúo" cheese factory were used. In this way, it was essential to verify which was more suitable to be used in the elaboration of the experimental cheeses. In addition, to control and optimize the coagulation stage of milk, a trial was carried out in which the parameters "coagulation time" and "volume of coagulant incorporated in milk" were analyzed.

In traditional dairy industries, they use amounts relative to 50 grams of flower of the thistle *Cynara cardunculus* L. to dilute in 1 liter of distilled water, allowing macerate at temperatures 5-20°C for 2-24 hours. Therefore, this preparation is highly variable across industries and varies according to the experience of the cheese master.

For optimization, the extraction of the enzymatic coagulant from the thistle flower *Cynara cardunculus* L. was firstly performed in relation to the general rule. It was based on homogenizing 2.5 g of the dried flowers in 50 mL of distilled water in an Erlenmeyer flask of 250 mL and maceration in fixed conditions of agitation for 24 h at 15 °C. Subsequently, a crude filtration was carried out using filter paper followed by a centrifugation in a centrifugation tube of 50 mL at 5000*g* for 5 minutes, in order to eliminate most of the impurities. The liquid phase was carefully transferred to another 50 mL sterile centrifuge tube removing the impurities deposited at the bottom of the tube. Once the coagulant was extracted from both types of thistle flower, the letters C (Flowers collected in Cáceres area) and E (Flowers collected in La Morera area) were identified. Then, we proceeded to the inoculation of different volumes (0.6, 0.9, 1.2 and 1.5 mL) of the enzymatic coagulants extracted in 8 sterile vials of 100 mL with 60 mL of pasteurized sheep's milk at 62ºC for 15 minutes in each one. After being properly mixed with a

spatula, the bottles were deposited in a thermostatic bath at 31ºC, checking the coagulation status at 20-minute intervals. The essay was repeated 3 times under the same conditions for each type of thistle to standardize the process.

Starter culture preparation.

The yeasts and the strain *Lb. casei/paracasei* UEX_Lc12 were subcultured twice prior to incorporation into the milk for the cheese elaboration. The yeasts were grown in YPD agar at 25ºC for 48 h and *Lb. casei/paracasei* strain in MRS broth at 30ºC for 48 h.

In order to prepare a concentrated solution of the microorganisms to be inoculated, from a plate of YPD agar with the selected yeast was taken with a disposable planting handle an inoculum with a high number of cells that was resuspended in 1 mL of sterile water. In the case of *Lb. casei/paracasei* UEX_Lc12, 1 mL of the grown culture was taken and centrifuged at 6000 *g* for 5 min, washed once with sterile water and resuspended in 0.5 mL of sterile water. After this, the solutions of microorganisms were stirred in vortex until a homogeneous solution was obtained. Then, serial dilutions were performed up to 1:1,000 due to the higher concentration of initial solutions. Next, a volume of 10 µL was pipetted in Neubauer's chamber to be able to perform the cell count by visualization under the microscope (figure 1). Three determinations were made for each solution of microorganism.

Figure 1. *Lb. casei/paracasei* UEX_Lc12 cell count in Neubauer's chamber.

Once the cell count had been carried out and the arithmetic mean **($\bar{X}$)** had been calculated, the appropriate formula was applied to calculate the concentration of the initial

solution. The formula applied for the count is shown below: [$\bar{\mathbf{X}} \times \mathbf{2.5 \times 10^5}$] × **dilution**. After this calculation, the concentration of the initial solution was obtained ranging between 10^8 and 10^{10} cfu/mL. Once the concentration of the initial yeast solution and *Lb. casei/paracasei* UEX_Lc12 have been calculated, it was prepared a solution of 10 mL with a concentration of yeast 5×10^8 cfu/mL and 1 mL of 5×10^8 cfu/mL of *Lb. casei*/*paracasei* UEX_Lc12. To calculate the volume that was required to be taken from the initial solution to prepare the solution to be inoculated, the following formula was used: $Concentration_1 \times Volumen_1 = Concentration_2 \times Volumen_2$. The exact volume of the initial solution was then taken and homogenized into the differential volume of sterile distilled water. The entire volume of the solutions to be inoculated was added to 1 L of pasteurized sheep's milk with the aim of achieving a concentration of the yeast under study of 5×10^6 cfu/mL and 5×10^5 cfu/mL of the starter culture of *Lb. casei/paracasei* UEX_Lc12.

Elaboration and maturation of experimental cheese in laboratory.

To study the impact of the yeasts selected for their technological properties on the sensory properties of soft sheep paste cheeses, a total of 11 batches inoculated using the combinations of microorganisms shown in table 2 were produced. Each batch was composed of 3 cheeses.

Table 2. Summary table of the batches made with the selected strains.

Batch code	**Inoculated cultures**		
Control (C)	Non-inoculated		
LAB	*Lb. casei* UEX_Lc12		
L1731	*P. jadinii* 1731	+	*Lb. casei* UEX_Lc12
L433	*P. jadinii* 433	+	*Lb. casei* UEX_Lc12
L2725	*K. lactis* 2725	+	*Lb. casei* UEX_Lc12
L2287	*K. lactis* 2287	+	*Lb. casei* UEX_Lc12
L1507	*K. lactis* 1507	+	*Lb. casei* UEX_Lc12
L2150	*Y. alimentaria* 2150	+	*Lb. casei* UEX_Lc12
L1204	*Y. alimentaria* 1204	+	*Lb. casei* UEX_Lc12
L2495	*Y. lipolytica* 2495	+	*Lb. casei* UEX_Lc12
L373	*P. kudriavzevii* 373	+	*Lb. casei* UEX_Lc12

Figure 2 shows the flowchart of the manufacturing process.

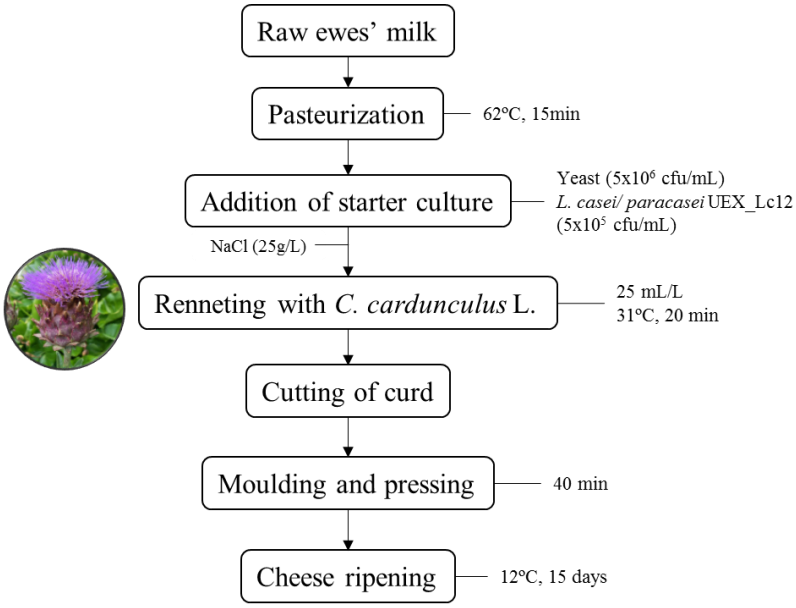


Figure 2. Flowchart of the experimental cheese-making process.

Firstly, prior to the preparation of each cheese, the inoculums corresponding to the batch to be produced were prepared, in their corresponding volumes and quantities, as described above. In addition, in the coagulation optimization test described before, the type of *Cynara cardunculus* L. flower, the amount of coagulant (25 mL/L of milk) and the coagulation time (20 min) to be used in the production of the experimental cheese were selected. Finally, for each liter of milk, 25 g NaCl/L were added based on the salt content found in soft-paste sheep cheeses studied in this thesis.

Once all the ingredients were prepared, for each cheese 1 liter of raw sheep's milk from was used in order to obtain a cheese of approximately 200 gr, obtaining a yield of 20%. The milk was pasteurized in a vacuum cooking equipment (Gastrovac) in sterile tubes of 100 mL using as parameters in the equipment 62ºC, -0.15 and -0.20 bar and 15 min. Thus, by means of the vacuum heat treatment, the microbial load of raw milk is reduced and that the characteristics of the final cheese are due to the microorganisms under study. After 15 min of heat treatment, the milk was tempered before adding the vegetable coagulant, starter culture and salt. Subsequently, the milk was added to a 2 L beaker and proceeded to the inoculation of the respective yeast inoculums and LAB, according to the batch and salt. The preparation and concentration of the inoculums was

carried out, obtaining a final concentration in the milk of the culture *Lactobacillus casei/ paracasei* UEX_Lc12 $5x10^5$ cfu/mL and the corresponding yeast $5x10^6$ cfu/mL. After a proper mixing of all the components added with a sterile spatula, the container was deposited in a thermostatic bath at 31ºC for 20 minutes for the coagulation of the milk (Figure 3).

Figure 3. Thermostatic bath JP SELECTA mod. PRECISDIG used during the coagulation phase of milk (31 ºC, 20 min).

After the coagulation time and after checking the correct condition of the curd, the container was extracted from the thermostatic bath and then proceeded to cut the curd with a spatula until a grainy appearance, with a size similar to the grain of rice. After cutting the curd, the filling of the molds was carried out manually eliminating the largest possible volume of whey using, for this, some vessels with grooves that facilitate the evacuation of the whey. Once the mold was filled, it was placed in a manual press in which it was subjected to constant pressure for 40 minutes (figure 4). During this period, a large volume of whey was removed, and the curd was compacted and took shape.

Figure 4. Moulds and presses used to make cheeses**.**

Finally, the cheese was carefully extracted from the mold and placed on a Petri dish inside a refrigerated stove at a ripening temperature of 12ºC for 15 days (figure 5).

Figure 5. Experimental cheeses matured in a stove refrigerated at 12 °C for 15 days during the maturation stage.

Microbial counts

Microbiological analyses were carried out at 0 days and at the end of ripening process. Ten grams of the core of each cheese sample were taken aseptically into sterile plastic bags and diluted with 90 mL of sterile 1% peptone water, homogenized for 60 s using a laboratory stomacher 400 Circulator Seward (Worthing, West Sussex, UK). Serial 10-dilutions were prepared and inoculated onto agar plates. Plate count agar medium (PCA, Condalab, Spain) was used for mesophilic aerobic bacteria counts, incubating at 30 ºC for 48 h. Lactic acid bacteria (LAB) were cultured on Man, Rogosa and Sharpe agar (MRS, Condalab) supplemented with acetic acid at 10% (v/v) and 15 mL/L of cycloheximide (Oxoid) at 1% to prevent the growth of yeast and incubating at 30 ºC for 2 days. For yeast count, dextrose potato agar (PDA, Condalab) acidified at pH 3.5 with a solution of 10% tartaric acid (w/v) was used. For the routine growth of the yeasts prior to the elaborations of the cheeses, yeast extract peptone dextrose (YPD) agar was used. The microbiological analysis was performed in triplicate for each batch produced. The results obtained were expressed in logarithm of colony-forming units per gram of sample (log cfu/g).

Physico-chemical analysis.

The pH measurement of experimental cheeses was carried out using a pHmeter model Crison PH 20. The pH was determined in each batch in triplicate at the end of the maturation process measuring the pH to stability of a homogenized 2 g of cheese in 20 mL of distilled water. The texture analysis was performed using a texture analyzer model TA.XTPlus (Stable Microsystems; figure 6). From each cheese made at the end of maturation, 6 samples were taken with a punch per batch obtaining cheese cylinders of approximately 1.5 cm in diameter and 2 cm in height. The cheeses were tempered to 20ºC before proceeding to the measurement of the texture. Once tempered, the texture was determined in triplicate in each portion by a compression test (TCA) using a 4 mm cylindrical probe and a 30 kg cell. The parameters of the equipment were: Pre-test speed 2 mm/s, Test speed 1 mm/s, Post-test speed 1 mm/s and Distance 5 mm. The firmness values of the cheese (g) were calculated with the software version 5.0.4.0 (Stable Micro System).

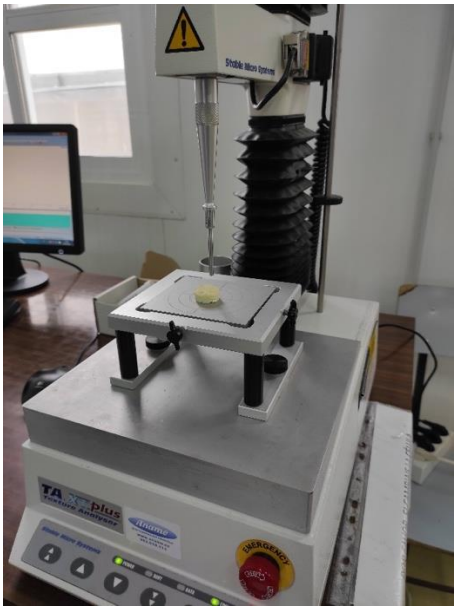


Figure 6. STABLE MICRO SYSTEM texture analyzer mod. TA. XT plus.

Sensory analysis

The evaluation of the sensory quality of the final product was carried out through a descriptive analysis carried out by a panel of 15 specialized panelists. The members of the panel were previously selected and trained under the standards of the International Organization for Standardization (ISO, 2006; UNE-ISO 4121:2006) with samples of commercial "Torta" cheese in the IB16038 project. All sessions were held in a sensory

panel room conditioned at 20-22 °C and 60%-70% relative humidity in cabinets equipped with white light (6000 °C). Before the analysis, the cheeses were balanced to the temperature of the sensory analysis room. The sensory tasting was based primarily on the evaluation of olfactory and visual parameters since they are experimental cheeses with a maturation period of only 15 days, so cheeses do not have the maturation time of 60 days required for raw milk cheeses to make a safe tasting. Cheese samples approximately 0.5 cm thick were distributed to each panelist in closed 20 mL tubes.

The organoleptic parameters that were evaluated by the panelists are shown in the following questionnaire model (figure 7). In addition, the panelists made a hedonic assessment of the overall acceptability of the cheese samples.

ANÁLISIS SENSORIAL QUESO

*Obligatorio

1. Código Muestra *

_____

TEST DESCRIPTIVO

ANÁLISIS VISUAL

2. Análisis visual de la pasta *

Marca solo un óvalo.

	1	2	3	4	5	6	7	8	9	
Poco color amarillo	◯	◯	◯	◯	◯	◯	◯	◯	◯	Mucho color amarillo

TEXTURA (SIN PROBAR)

3. Firmeza *

Marca solo un óvalo.

	1	2	3	4	5	6	7	8	9	
Poco	◯	◯	◯	◯	◯	◯	◯	◯	◯	Mucho

AROMA

4. Intensidad *

Marca solo un óvalo.

	1	2	3	4	5	6	7	8	9	
Poco	◯	◯	◯	◯	◯	◯	◯	◯	◯	Mucho

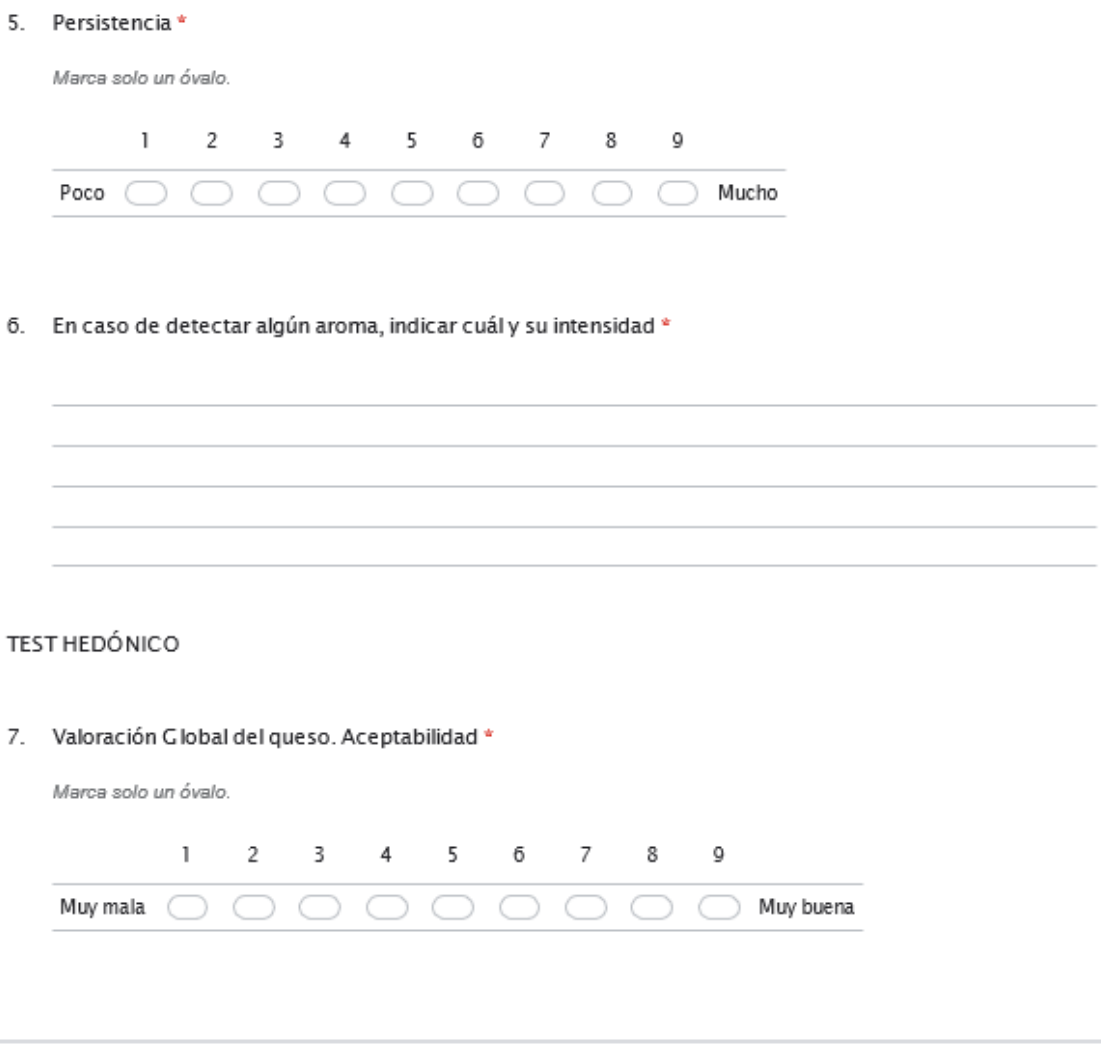


Figure 7. Questionnaire model used in the sensory analysis of the cheeses made.

Statistical analysis.

The data obtained were analyzed with the SPSS program for Windows version 21.0 (SPSS Inc Chicago, IL, USA). Significant differences and homogeneous groups of means were established using an analysis of variance (ANOVA) following one-way procedures. When the effect of the interaction is significant ($P \leq 0.05$) we proceeded to perform a comparison analysis of means by the TUKEY method that determines the minimum difference between the means of each group so that it is statistically significant. By means of the analysis of principal components (PCA) the effect of the inoculated yeast strains on the microbiological, physico-chemical and sensorial properties analyzed (sensory analysis, aromatic profile and texture) was determined.

VI.3. Results and discussion.

Coagulation phase optimization.

The specie *Cynara cardunculus* L. commonly known as thistle, specifically its flower, is collected from different rural areas for use in the cheese industries as a raw material for obtaining vegetable coagulant. It is a non-standardized plant product, so considerable differences can be found in the enzymatic activity of the different ecotypes of this species, which translates into a very variable proteolytic capacity (Ordiales 2013c; Gomes et al., 2019). The vegetable coagulant has been associated with one of the causes of variability in the final quality of the "Torta" (Ordiales et al., 2014). Therefore, the objective of this study was to select one of the two types of thistle flower provided by "El Castúo" industry in relation to the efficiency in the coagulation of sheep's milk.

The results on the coagulant activity of the two types of enzymatic coagulant that were used are shown in table 3. The table shows the coagulation capacity of the infusion of the thistle flowers of each ecotype at different doses. Coagulation was considered **positive** (+) when the curd formed presented an adequate and consistent texture. In contrast, when the state of the curd still had a liquid appearance was categorized as **negative** (-). Finally, the intermediate state referring to a curd with a weak texture and not yet adequate was considered at an intermediate point of coagulation and indicated by the symbol (±).

Table 3. Evolution of enzymatic coagulation by applying two ecotypes of vegetable coagulant at different doses and using different coagulation times.

	Elapsed clotting time (minutes)											
	20'		**40'**		**60'**		**80'**		**100'**		**120'**	
Volume of coagulant (mL)	**C[1]**	**E[2]**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**
0,6	-	-	±	-	+	-	+	-	+	-	+	-
0,9	±	-	+	-	+	-	+	-	+	-	+	±
1,2	+	-	+	-	+	-	+	-	+	±	+	+
1,5	+	-	+	-	+	-	+	±	+	+	+	+

[1]Coagulant type C from thistle flowers collected in Cáceres (C).
[2]Coagulant type E from flowers collected in La Morera, Badajoz (E).

As can be seen in table 3, the enzymatic coagulant extracted from the thistle flower type **C** showed a greater ability to coagulate sheep's milk. At the highest doses, of 1.2 and 1.5 mL in 60 mL of milk, since at 20 minutes of incubation a solid and compact textured curd had already been formed in all the inoculated samples. At lower doses of 0.9 and 0.6

mL, 40 and 60 minutes were necessary, respectively. In contrast, with 1.5 mL of coagulant type **E**, it is not possible to form an ideal curd until 100 minutes from the beginning of the trial, even after 120 minutes of incubation at doses of 0.6 and 1.2 mL a consistent curd was not formed. These results show a clearly differentiated enzymatic activity between both plant coagulants. Confirming the variability shown by other authors in the enzymatic activity of flowers of different ecotypes of *Cynara cardunculus* L. and its influence on the coagulation process and final characteristics of cheese (Gomes et al., 2019; Ordiales et al., 2013c, 2014).

With the obtained results, the necessary time, and the optimal dose in both ecotypes of thistle flower were determined. Thus, in this way to be able to select the ecotype to be used and conditions of application in the elaboration of the experimental cheese for the development of the present study with the aim of applying it homogeneously in all batches and that it was not a factor of variability. Therefore, since coagulant type C proved to be the most effective in the coagulation of sheep's milk in the shortest time established by its outstanding enzymatic activity, it was selected for the development of the process of making experimental cheeses. Establishing the application of 2.5 mL of infusion of the flowers of coagulant C in 100 mL of sheep's milk and a coagulation time of 20 minutes for the formation of the curd.

Application of yeasts in experimental cheese

Microbiological analysis

The results of the analysis of the counts of mesophilic aerobic bacteria grown in the PCA agar medium at the beginning and end of the maturation of the different batches elaborated are shown in figure 8. The results show a variation in the initial microbial load, with the lowest counts in the control batch, with 4.2 log cfu/g, followed by the LAB batch with 4.66 log cfu/g, which were significantly lower than the rest of the batches inoculated with the selected yeasts and the LAB. The batches inoculated with the yeasts showed values ranging from 4.88 log cfu/g for batch L2150 to 6.4 log cfu/g in batch L433. This variation in the initial microbial load between the batches is logical, since the Control batch was not inoculated with any microorganisms and the LAB batch was inoculated with a load of *Lb. casei/paracasei* UEX_Lc12 $5x10^5$ cfu/mL. The yeast batches were inoculated with a number of cells ten times higher, so it is logical to expect counts at the beginning of maturation of a higher logarithmic unit in the yeast batches. On the other

hand, although the milk used in the test was previously pasteurized to reduce the initial microbial load of the same, various factors can influence the initial microbiological quality of the raw milk used as the manipulation in the obtaining, transport, conservation and microbiota of the surfaces in the elaboration of the cheese (D'amico and Donnelly, 2010; Reguillo et al., 2018). Therefore, the initial microbial load of the raw milk used may also influence the initial counts obtained from different processed batches.

At 15 days of maturation, a significant increase in total counts was observed in all batches with respect to the beginning of maturation (figure 8). The counts ranged from 7.47 log cfu/g obtained in batch LAB to a maximum of 8.89 log cfu/g in batch L1507. The control batch, with 8 log cfu/g showed higher counts than the LAB batch, indicating that the LAB used was not adequately adapted to the maturation process, despite showing adequate technological parameters *in-vitro*. On the other hand, all batches of yeasts showed total counts equal to or greater than the control batch. The evolution of the microbiota throughout ripening will depend on several factors, such as initial microbiology, maturation conditions, industry microbiology, among others (Beresford and Williams, 2004; Hayaloglu, 2016). In general, counts of approximately 8 log cfu/g are logical in sheep's milk cheeses with 15 days of maturation (Inácio et al., 2020; Ordiales et al., 2013a).

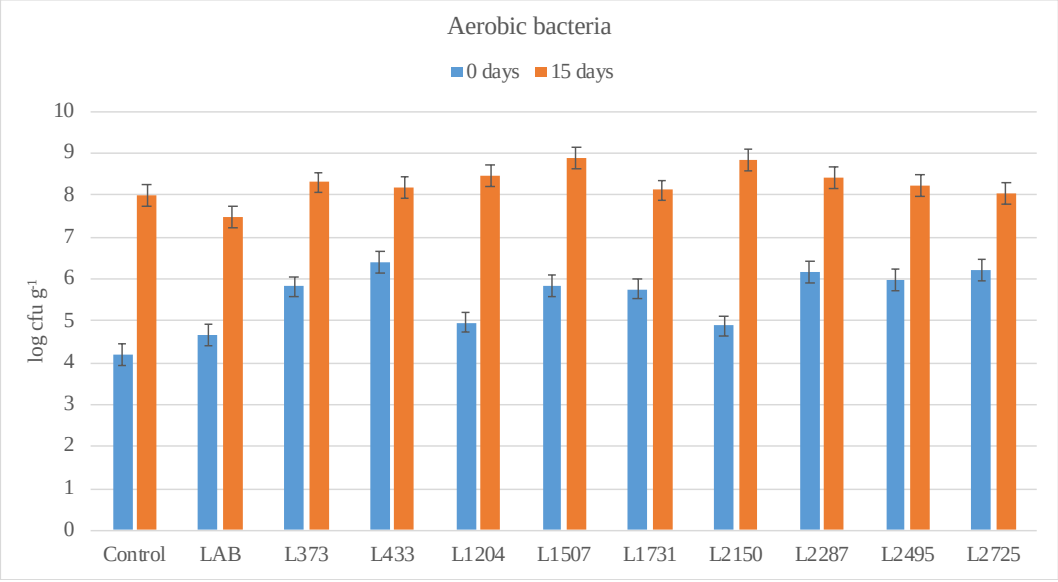


Figure 8. Mean values of mesophilic aerobic bacteria (PCA) at days 0 and 15 of maturation, expressed in log cfu/g. The error bars represent the confidence interval at 95% of significant difference obtained by Tukey's HDS test.

The results of LAB counts obtained in MRS agar at 0 and 15 days of maturation are represented in figure 9. The LAB counts in the control at the beginning of maturation were significantly lower than the rest of the batches, which had been inoculated with a strain of *Lb. casei/paracasei* as starter culture. Regarding the yeast batches, 7 of the 9 batches, except L1204 and L1250, showed LAB counts higher than the LAB batch. This can be explained either by the presence of LAB present in raw milk resistant to pasteurization treatment or due to the resistance of yeast strains to the added doses of cycloheximide, allowing its growth in MRS agar supplemented with fungicide. In fact, some of the colonies of MRS agar plates were observed under a microscope and presented a yeast morphology (data not shown). LAB increased significantly in all batches during maturation, reaching the lowest values in the control batch with 7.38 log cfu/g and the highest in batch L1507 with 8.83 log cfu/g. Therefore, the values obtained in MRS agar at 15 days of maturation are similar to those obtained in PCA agar for mesophilic aerobic bacteria, being this microbial group the dominant in experimental cheeses made. In the literature, LABs are the main responsible for the cheese maturation process, being the dominant group in both traditional raw milk cheeses and pasteurized milk cheeses with starter culture (Kongo and Malcata, 2016b). LAB during maturation contribute to the biochemical changes that occur in cheese such as glycolysis, proteolysis and lipolysis that determine the final characteristics of the cheese. Among them, they mainly stand out for the consumption of lactose and production of lactic acid that causes a decrease in pH and limits the growth of altering microorganisms and pathogens in the cheese matrix (Hayaloglu, 2016; Kongo and Malcata, 2016b).

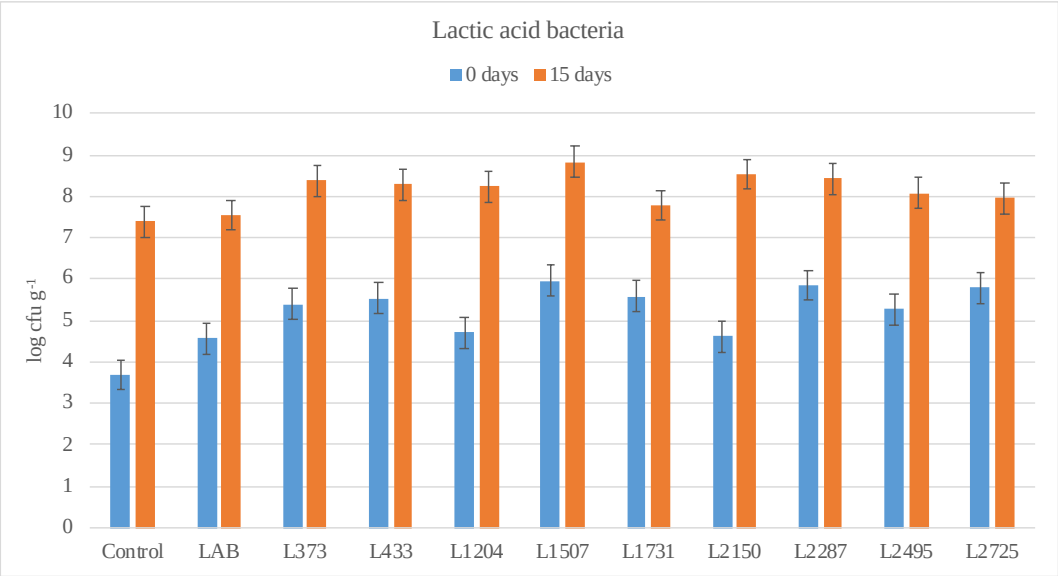


Figure 9. Mean values of lactic acid bacteria (MRS) at days 0 and 15 days of maturation, expressed in log cfu/g. The error bars represent the 95% confidence interval of significant difference obtained by Tukey's HDS test.

Figure 10 shows the yeast counts in acidified PDA agar at 0 and 15 days of ripening. At the beginning of maturation, in Control and LAB batches, the counts were significantly lower, with values of 2.83 log cfu/g in the control batch and not detected in the LAB batch (<2 log cfu/g). In general, raw milk usually has yeast values in the range of 1-3 log cfu/mL (Lavoie et al, 2012). Therefore, these results are in accordance with the initial values of yeasts in cheeses without the addition of a yeast starter culture (Fröhlich-Wyder et al., 2019). In the batches inoculated with different yeasts, the initial counts varied from 5.02 log cfu/g in batch L2150 to 5.85 log cfu/g in batch L1507. Therefore, all yeasts were inoculated at significant levels of approximately 10^5-10^6 cells per gram of cheese in order to see their impact on the sensory properties of the cheese and its suitability to be used as a starter culture.

At 15 days of maturation, the counts of the Control and LAB batches showed no significant differences with the beginning of maturation, while in the inoculated batches the mean values of the yeast counts increased significantly ($P \leq 0.05$) in all batches. Therefore, all inoculated yeast strains were properly adapted to the cheese matrix and properly implanted in the experimental cheese. Species of the genera *Kluyveromyces*, *Yarrowia* and *Pichia* are commonly identified in traditional cheeses and best adapted to the cheese matrix (Gonçalves et al., 2017; Montel et al., 2014). The strains of *Y. lipolytica* L2495 and *K. lactis* L1507 and L2725, were the ones that showed the highest levels at 15 days, with counts slightly higher than 7 log cfu/g, while the rest of the yeasts were

approximately 6 log cfu/g. These three yeast strains plus *Y. alimentaria* L1204, were the ones that showed the greatest progression, with increases in counts during maturation in the range of 0.7-1.09 log cfu/g. In contrast, the species *Pichia kudriavzevii* L373 and *Pichia jadinii* L1731, with an increase of approximately 0.3 log cfu/g, were the ones that showed a smaller increase during maturation.

Although yeasts in cheese are generally considered spoilage, in the last decade, numerous studies in yeasts isolated from cheese have shown their ability to contribute to the aroma and sensory properties of cheese (Atanassova et al., 2016; Binetti et al., 2013; Chen et al., 2012; Padilla et al., 2014a). In this way, some yeast species, mainly of the genera *Kluyveromyces, Yarrowia, Debaryomyces* and *Geotrichum* are intentionally added as part of a starter culture to improve the sensory properties of cheese (Fröhlich-Wyder et al., 2019; Hayaloglu, 2016).

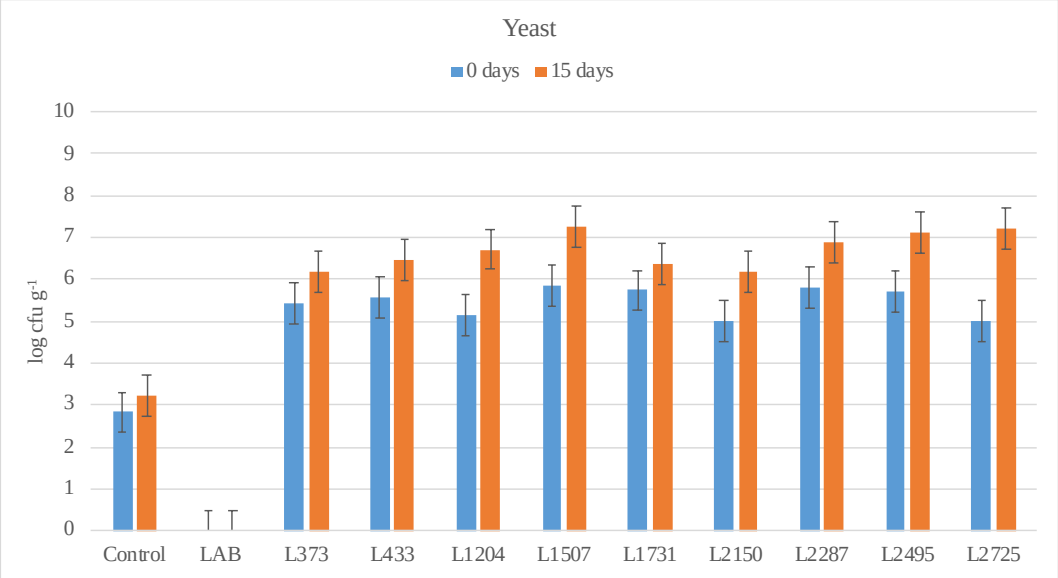


Figure 10. Mean values of yeasts (PDA) at days 0 and 15 of maturation expressed in log cfu/g. The error bars represent the 95% confidence interval of significant difference obtained by Tukey's HDS test.

Physico-chemical analysis.

pH determination.

The pH is a very important parameter to take into account in the production of cheeses since it directly influences the metabolic activity of microorganisms and, therefore, affects the biochemical changes that occur in the maturation of cheeses, mainly in proteolysis, which has a great influence on texture and aroma (McSweeney, 2004). The

results of the pH parameter obtained from the cheese samples taken at the end of the maturation of the experimental cheese (15 days) are shown in figure 11. The pH values obtained were very variable depending on the batch. The lowest pH corresponded to lot L1204 with a value of 5.5 and the highest value to lot LAB with a pH of 6.41. The control batch, without the addition of any microorganisms, showed an intermediate value of 6.1.

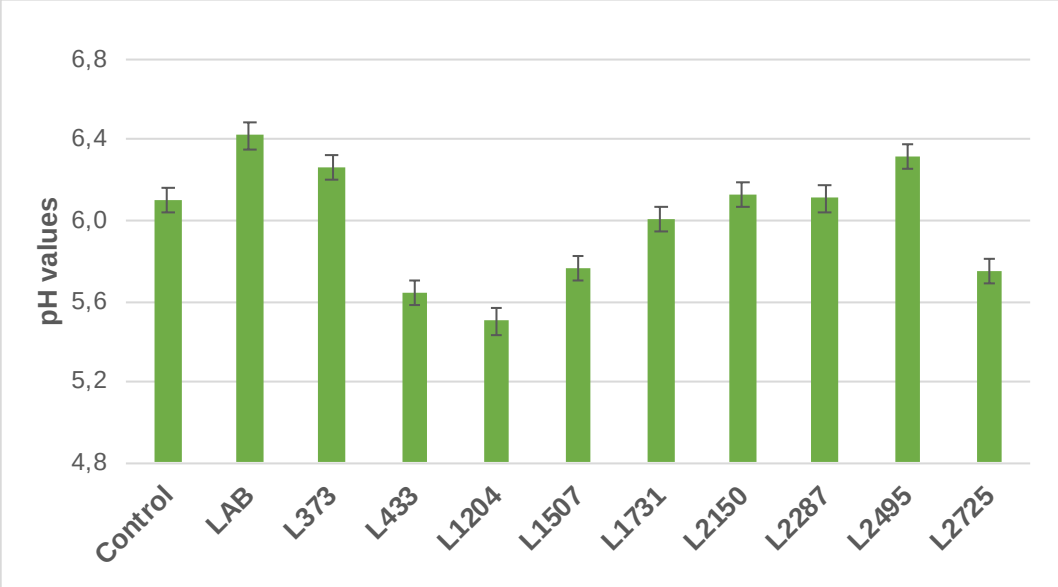


Figure 11. Mean pH values at 15 days of maturation of the different batches. The error bars represent the 95% confidence interval of significant difference obtained by Tukey's HDS test.

It can be observed that the cheeses made in the Control batch had a significantly higher pH than the cheeses belonging to the batches inoculated with the yeasts L1204, L1507, L1731, L2725 and L433. Whereas the batches L2150 and L2287 showed no significant differences from the control batch and, the batches L373 and L2495, were significantly higher. It should be noted that the LAB batch had a pH significantly higher than all batches, which indicates that the starter culture of *Lb. casei/paracasei* UEX_Lc12 was not adequately adapted to the ripening conditions of the experimental cheese produced. This is in accordance with the LAB results of this batch (fig. 9).

In general, in soft cheeses made with raw ewe's milk, the pH decreases at the beginning of maturation to pH below 5 by the action of the LAB and then progressively increase reaching at the end of maturation (60 days) values around pH 5.5 (Ordiales et al., 2013b; Inácio et al., 2020). However, the evolution of pH is variable depending on the type of cheese and ripening conditions. In this study, the final pH of the cheeses indicates that the evolution of the pH was different. In addition, it must be considered that

it is an experimental cheese with a short maturation and using heat-treated milk, so most of the microbiota of raw milk was eliminated, and the metabolic changes that occur in the cheese will depend on the inoculated microorganisms. The pH of 6.41 in the LAB batch indicates that the LAB starter culture was not implanted correctly, which can cause a slower decrease in pH in the batches where it was used and be responsible for slightly high pH values. Another relevant aspect is that in the cheese maturation, the yeasts metabolize the lactic acid in the cheese, causing the increase in pH and leading to the formation of a characteristic yeast or fruity flavor (Kongo and Malcata, 2016b). However, seven of the nine batches inoculated with yeast showed a pH equal to or lower than the Control batch. It indicates they did not have a great impact on the high pH values, even contributing to their decrease compared to the control. The data from the *in-vitro* study of the technological properties of the yeasts selected in the previous chapter showed that the strains of *K. lactis* and *P. jadinii* have some acidification capacity.

Texture analysis.

Texture is one of the main differentiating features of soft cheeses. So, the selection of microorganisms as a starter culture that contribute to obtaining a final product with a creamy or unctuous texture is crucial. The average firmness values obtained in the cheese batches at the end of ripening are shown in figure 12.

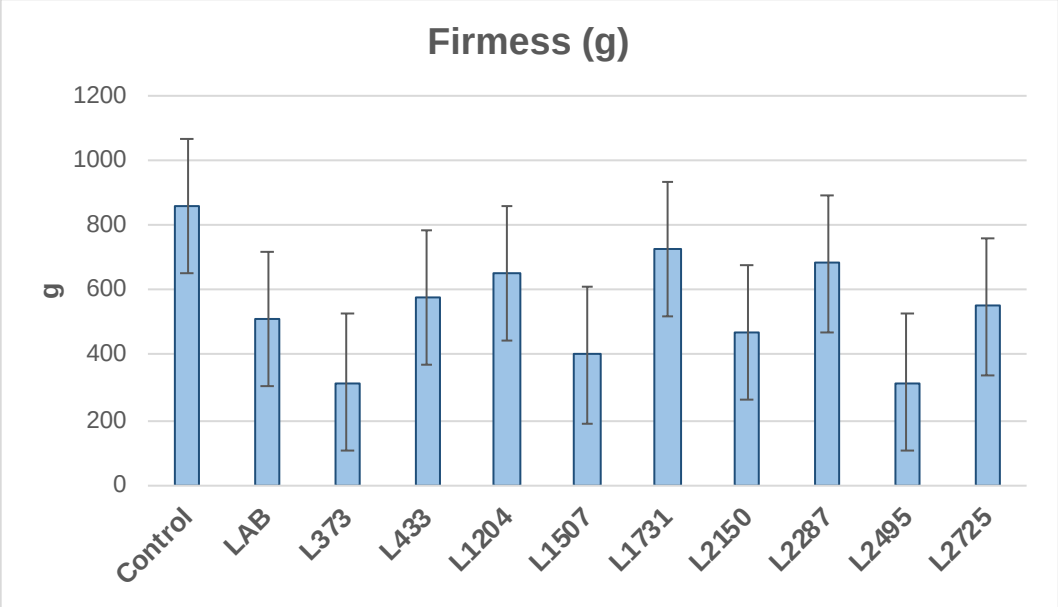


Figure 12. Average firmness values at 15 days of maturation of the batches of cheese produced. The error bars represent the 95% confidence interval of significant difference obtained by the HSD Tukey test.

The highest firmness value achieved was obtained from samples taken from the cheeses made in the Control batch with an average value of 859 (g). As for cheeses inoculated with LAB, the mean value was 573 (g). Among the batches inoculated with the combination of LAB and yeasts, two batches L2495 and L373, showed values statistically lower than the LAB batch ($P<0.05$), while two other batches L1507 and L2150 showed lower mean values although the differences were not significant ($P>0.05$). Thus, batches L2495, L373, L1507 and L2150 showed the lowest firmness results with mean values of 315, 315 400 and 470 (g), respectively. In addition to these four batches, L433 and L2725 batches also showed significantly lower firmness compared to the Control batch. Therefore, six of the nine inoculated yeasts allowed to obtain a softer texture compared to the control.

One of the factors that mainly influences the texture is the pH. Cheeses with high pH generally have a softer texture than acidic cheeses (McSweeney, 2004). In this study, among the batches inoculated with yeasts, L373 and L2495 batches were those with the highest pH values and the lowest firmness values (Figures 11 and 12). In addition, the pH of cheese will influence the activities of enzymes involved in the biochemical changes of cheese and those responsible for proteolysis, which is the main process involved in the final texture (Inácio et al., 2020). Enzymes with proteolytic capacity or proteases in the maturation of cheese have their origin in the coagulant, are endogenous to milk or in the microbiota that acts during cheese ripening. In this study, both coagulant and milk were of the same origin, while the differentiating factor was the inoculated microorganisms. The proteolytic activity of microorganisms has a great impact on this process, especially in secondary proteolysis, affecting the texture and aroma of cheese (McSweeney, 2004). In the scientific literature, the yeast species used in this study stand out for their proteolytic activity. In fact, it was one of the parameters used in the previous chapter for its selection. The results show that the lowest value of firmness was in the batch inoculated with *Y. lipolytica* L2495. This species is characterized by a strong proteolytic activity contributing to the sensory properties of cheese (Fröhlich-Wyder et al., 2019), and was the one that presented a greater proteolytic activity in the *in-vitro* studies carried out for its selection. Therefore, the use of the strains *Y. lipolytica* L2495 or *P. kudriavzevii* L373 as microorganisms of a mixed starter culture could contribute to ensuring a creamy texture in soft cheeses.

Sensory analysis.

Table 4 presents the average values of the sensory parameters evaluated in the different experimental cheeses at the end of maturation (15 days) and the global acceptance of each batch. Yeasts, although they are members of the secondary microbiota in traditional cheeses (Gonçalves et al., 2017) can contribute significantly to their sensory characteristics. The presence of these microorganisms can be positive or negative in the characteristics of cheese depending on its metabolic activities (Fröhlich-Wyder et al., 2019). This makes it necessary to know their impact on sensory properties for use as members of a starter culture. In relation to the average values obtained in each batch, scores showed some variability for the parameters evaluated in sensory analysis on a scale of 0 to 9. In the visual analysis of the paste, the mean scores were in the range of 3.8 to 5.7. The maximum colour value was in *Y. alimentaria* L1204 batch (5.7 ± 1.8) and the lowest in *K. lactis* L1507 batch (3.8 ± 1.6), followed by the Control batch with 3.9 ± 1.1 and LAB batch with 4.2 ± 1.8. Different studies have associated yeast strains as responsible for brown pigmentations in cheeses (Carreira et al., 2001; Groenewald et al., 2014), yellow paste or even very wrinkled crust (Fröhlich-Wyder et al., 2019). The results obtained in this study show in general a greater intensity of color of the paste in the yeast batches. However, the differences were not significant ($P>0.05$) between the control and the batches inoculated with yeasts, and the panelists did not detect alterations associated with color or pigmentations in the additional comments. Therefore, none of the yeasts negatively affected the color, even being inoculated at levels higher than the average counts associated with traditional soft cheeses (Gonçalves et al., 2017; Ordiales et al., 2013a). The intensity and persistence of the aroma evaluated are parameters that largely determine the sensory quality of the cheeses and that directly influence the acceptance. In reference to aroma intensity, the average values ranged from 5.2 ± 1.8 for the Control batch to 6.5 ± 1 for *P. jadinii* L1731 batch. Regarding the persistence of the aroma, the lowest mean score was also in the Control batch, 3.8 ± 1.4; and the maximum in *Y. lipolytica* L2495 batch (5.5 ± 1.3). Although the results in both parameters did not show significant differences between the batches ($P>0.05$), in general the mean values obtained show that the batches inoculated with yeasts presented higher scores in intensity and persistence of aroma in relation to the control and LAB batches.

Table 4. Average values of the sensory parameters analyzed at 15 days of maturation in the different cheese batches. Different letters mean significant differences obtained using Tukey's HDS test.

		Paste visual analysis			**Firmness**			**Intensity**			**Persistence**			**Global acceptance**		
Batches	**N**	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD
LAB	15	4,2	±	1,8	6,5^{a,b}	±	1,1	5,6	±	1,0	3,8	±	1,5	5,9	±	1,3
Control	15	3,9	±	1,1	7,4^{a,b}	±	0,9	5,2	±	1,8	3,8	±	1,4	6,3	±	1,7
L373	15	4,5	±	2,0	6,6^{a,b}	±	1,7	6,0	±	1,4	4,8	±	1,9	5,8	±	1,4
L433	15	4,7	±	1,9	5,8^{a}	±	1,4	5,8	±	1,1	5,0	±	1,7	6,0	±	1,4
L1204	15	5,7	±	1,8	6,8^{a,b}	±	1,2	6,4	±	1,0	5,2	±	1,5	6,1	±	0,9
L1507	15	3,8	±	1,6	5,9^{a,b}	±	1,4	6,3	±	1,1	4,8	±	1,6	5,5	±	1,8
L1731	15	4,2	±	1,9	6,5^{a,b}	±	1,3	6,5	±	1,0	4,7	±	1,4	6,2	±	0,9
L2150	15	4,6	±	1,9	6,6^{a,b}	±	1,1	6,4	±	1,5	5,1	±	2,0	6,1	±	1,4
L2287	15	4,9	±	1,8	7,5^{b}	±	0,8	5,9	±	1,0	4,9	±	1,7	5,5	±	1,6
L2495	15	4,7	±	1,4	6,1^{a,b}	±	1,4	6,3	±	1,5	5,5	±	1,3	5,9	±	1,3
L2725	15	4,5	±	2,0	6,6^{a,b}	±	1,7	6,0	±	1,4	4,8	±	1,9	5,8	±	1,4
Sig.		0,309			0,008			0,198			0,192			0,898		

^{a,b} For each batch the data in the same column with different letters is significantly different ($P \leq 0,05$) .

Yeasts contribute to the aromatic profile of cheese due to its proteolytic and lipolytic capacity, although it depends on the species and strain of yeast (Fröhlich-Wyder et al., 2019). Among the inoculated yeasts, *Y. lipolytica* L2495 presented the highest mean value of aroma persistence and among the highest intensity values. This species is characterized by a strong metabolic activity, which results in the generation of a wide variety of volatile compounds positively related to the sensory properties of traditional cheeses. In fact, several authors have demonstrated the significant contribution of strains of this species to the aromatic profile of cheese (Atanassova et al., 2016; Sørensen et al., 2011; Zheng et al., 2021). The continuation of this study through the analysis of the aromatic profile of each batch using instrumental techniques such as gas chromatography coupled to a mass detector will allow to know more specifically the contribution of each yeast to the aromatic profile.

Another of the most important sensory parameters in cheese is firmness, especially in soft cheeses which are very appreciated for their unctuous texture. The mean values obtained were in the range of 5.8 ± 1.4 obtained in *P. jadinii* L433 batch to a maximum of 7,5 ± 0,8 for *K. lactis* L2278 batch. In this parameter, significant differences ($P \leq 0.05$) were obtained between batches. The sensory firmness of batch L433 was significantly lower than batch L2287, while it showed no significant differences with other batches. In relation to the results obtained in the instrumental texture (figure 12)**,** there is a similar trend in certain batches, showing the batches L2495 and L1507 low values and the control a high firmness in both cases. However, in other batches there is a greater discrepancy between both firmness analyses, probably associated with the subjective nature of the sensory analysis.

Finally, the overall assessment of acceptability involves the collection and analysis of all the afore mentioned data to assess the suitability of the products made with the selected strains with respect to control. As shown in table 4**,** the Control batch with 6.3 ± 1.7 showed the highest mean value, while the lowest mean score was obtained in the batches of *K. lactis* L1507 and L2287 with 5.5 ± 1.8 and 5.5 ± 1, 6, respectively. Yeasts, in addition to contributing positively to the aroma of cheese, can also be responsible for alterations in cheese. They can affect both the appearance and the presence of inappropriate aromas, such as fermented or fruity aromas, because of the combination of short-chain fatty acids and ethanol (Garnier et al., 2017; Ledenbach and Marshall,

2009). However, the results of the statistical study do not reflect significant differences between any of the batches (P>0.05) and all were scored with a mean score higher than 5.5. Therefore, despite the high levels of inoculated yeasts, the panelists did not detect serious abnormalities in the aroma of the batches inoculated with the different yeasts tested in this study compared to the control.

Analysis of principal components of the different parameters analyzed in the cheese batches.

The analysis of principal components of microbiological, physico-chemical and sensory parameters is shown in figure 13. In the projection, it can be seen how the principal component 1 (PC1) explains a variability of 47.05% and the main component 2 (PC2) of 18.77%, so that both principal components collect 65.82% of the variability of the data. As can be seen in figure 13 the variables PCA (aerobic mesophilic bacteria), PDA (yeasts), MRS (LAB), and sensory parameters (persistence and intensity) have more weight on PC 1, so PC 1 better defines the difference between them. While the other variables, pH and sensory variables: sensory firmness, firmness, visual analysis of the paste and acceptability are explained by both main components.

In relation to the parameters studied, the analysis of principal components clearly shows how the batches inoculated with yeasts differ from the control and LAB batch. The control batch is correlated with higher values in both firmness and better acceptability, while the LAB batch correlates positively with the pH and negatively with the rest of the parameters, corroborating the non-implantation of the LAB starter culture used in the study. In relation to the nine batches with inoculated yeasts, in general they were positively correlated with the microbiological parameters, demonstrating their implantation in the cheese matrix and with the sensory parameters of intensity, persistence and visual analysis of the paste, while they showed a negative correlation with sensory and instrumental firmness and acceptability. Among the yeast batches, the batch *Y. alimentaria* L2014 was the most divergent, while the batch *P. jadinii* L1731 was the most similar to the control batch. However, among them stand out the batches *Y. alimentaria* L2150, *Y. lipolytica* L2495 and *K. lactis* L1507 for their positive correlation with the sensory parameters of intensity and persistence and negative with both firmness. Therefore, the analysis of principal components shows that these last three yeasts could

be used as part of a mixed starter culture by contribution to aroma and creamy firmness in the production of soft cheeses.

Figure 12. Analysis of principal components of microbiology parameters (PCA: aerobic mesophilic bacteria; MRS, lactic acid bacteria; PDA: yeasts), pH, firmness and sensory (paste visual analysis, persistence, intensity, firmness and global acceptance).

VI.4. Conclusion

In conclusion, the nine yeast strains selected in the previous chapter, based on their technological properties were correctly implanted in the cheese matrix. All yeasts significantly increased the counts at the end of maturation of the experimental cheeses made. Two of the nine strains inoculated in the experimental cheeses, *Pichia kudriavzevii* L373 and *Yarrowia lipolytica* L2495 allowed to obtain a significantly softer texture than the rest of the batches made. In addition, two other yeasts *Yarrowia alimentaria* L2150 and *Kluyveromyces lactis* L1507 showed values statistically lower than the control batch and lower mean values than the LAB batch. In terms of sensory parameters, no significant differences were observed between the cheese batches inoculated with yeasts and the control and LAB batches. However, sensory analysis shows an impact of yeasts on the sensory characteristics assessed. The mean values of the sensory parameters in the batches with yeasts compared to the control were in general higher in color of the paste, intensity and persistence of the aroma and lower in firmness and global valuation. Finally, the analysis of principal components of the physico-chemical, microbiological and sensory parameters of the experimental cheese batches shows that yeasts have a clear impact on the maturation process of the cheese. In addition, although more studies are needed, three yeasts, *Y. alimentaria* L2150, *Y. lipolytica* L2495 and *K. lactis* L1507 based on their relationship with the sensory parameters of intensity and persistence and with sensory and instrumental firmness showed to be the best candidates to be part of a mixed starter culture to produce soft cheeses from sheep's milk.

VI.5. References

Araújo-Rodrigues, H., Tavaria, F. K., dos Santos, M. T. P., Alvarenga, N., & Pintado, M. M. (2020). A review on microbiological and technological aspects of Serpa PDO cheese: An ovine raw milk cheese. *International Dairy Journal*, 100, 104561.

Atanassova, M. R., Fernández-Otero, C., Rodríguez-Alonso, P., Fernández-No, I. C., Garabal, J. I., & Centeno, J. A. (2016). Characterization of yeasts isolated from artisanal short-ripened cows' cheeses produced in Galicia (NW Spain). *Food Microbiology*, 53, 172-181.

Banjara, N., Suhr, M. J., & Hallen-Adams, H. E. (2015). Diversity of yeast and mold species from a variety of cheese types. *Current Microbiology*, 70(6), 792-800.

Beresford, T., & Williams, A. (2004). The Microbiology of Cheese Ripening–Cheese: *Chemistry, Physics and Microbiology, Volume 1: General Aspects*, Ed. Fox P., McSweeney P., Cogan T., Guinee T., pp, 287-318.

Binetti, A., Carrasco, M., Reinheimer, J., & Suárez, V. (2013). Yeasts from autochthonal cheese starters: technological and functional properties. *Journal of Applied Microbiology*, 115(2), 434-444.

Bokulich, N. A., & Mills, D. A. (2013). Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. *Applied and Environmental Microbiology*, 79(17), 5214-5223.

Cáceres, P., Castillo, D., & Pizarro, M. (1997). Secondary flora of Casar de Cáceres cheese: characterization of *Micrococcaceae*. *International Dairy Journal*, 7(8-9), 531-536.

Carreira, A., Ferreira, L. M., & Loureiro, V. (2001). Brown pigments produced by *Yarrowia lipolytica* result from extracellular accumulation of homogentisic acid. *Applied and environmental microbiology*, 67(8), 3463-3468.

Chen, L. S., Cui, J., Ding, Q. B., Ma, Y., Chen, L. J., Dong, J. Y., Tie-Ming, J. & Maubois, J. L. (2012). The effect of yeast species from raw milk in China on proteolysis and aroma compound formation in Camembert-type cheese. *Food and Bioprocess Technology*, 5(6), 2548-2556.

D'amico, D. J., & Donnelly, C. W. (2010). Microbiological quality of raw milk used for small-scale artisan cheese production in Vermont: effect of farm characteristics and practices. *Journal of Dairy Science*, 93(1), 134-147.

del Olmo, A., Calzada, J., & Nuñez, M. (2018). The blue discoloration of fresh cheeses: A worldwide defect associated to specific contamination by *Pseudomonas fluorescens. Food Control*, 86, 359-366.

Fernández García, D. (2019). Tesis Doctoral. Influencia de la raza, tratamiento térmico, cultivo iniciador y tiempo de maduración en las características del queso Zamorano. (1.7) 43-45.

Fröhlich-Wyder, M. T., Arias-Roth, E., & Jakob, E. (2019). Cheese yeasts. *Yeast*, 36(3), 129-141.

Garnier, L., Valence, F., & Mounier, J. (2017). Diversity and control of spoilage fungi in dairy products: An update. *Microorganisms*, 5, 1– 33.

Gkatzionis, K., Yunita, D., Linforth, R. S., Dickinson, M., & Dodd, C. E. (2014). Diversity and activities of yeasts from different parts of a Stilton cheese. *International Journal of Food Microbiology*, 177, 109-116.

Gomes, S., Belo, A. T., Alvarenga, N., Dias, J., Lage, P., Pinheiro, C., ... & Martins, A. P. (2019). Characterization of *Cynara cardunculus* L. flower from Alentejo as a coagulant agent for cheesemaking. *International Dairy Journal*, 91, 178-184.

Gonçalves, M. T. P. G., Benito, M. J., de Guía Córdoba, M., Alvarenga, N., & Ruiz-Moyano, S. (2017). Yeast community in traditional Portuguese Serpa cheese by culture-dependent and-independent DNA approaches. *International Journal of Food Microbiology*, 262, 63-70.

Gonçalves, M. T. P., Benito, M. J., Córdoba, M. D. G., Egas, C., Merchán, A. V., Galván, A. I., & Ruiz-Moyano, S. (2018). Bacterial communities in Serpa cheese by culture dependent techniques, 16S rRNA gene sequencing and high-throughput sequencing analysis. *Journal of Food Science*, 83(5), 1333-1341.

Gonzales-Barron, U., Gonçalves-Tenório, A., Rodrigues, V., & Cadavez, V. (2017). Foodborne pathogens in raw milk and cheese of sheep and goat origin: a meta-analysis approach. *Current Opinion in Food Science*, 18, 7-13.

Groenewald, M., Boekhout, T., Neuvéglise, C., Gaillardin, C., Van Dijck, P. W. M., & Wyss, M. (2014). *Yarrowia lipolytica*: Safety assessment of an oleaginous yeast with a great industrial potential. *Critical Reviews in Microbiology*, 40(3), 187–206. https://doi.org/10.3109/1040841X.2013.770386

Hayaloglu, A. A. (2016). Cheese: Microbiology of cheese. *Reference Module in Food Science*, 1, 1-11.

Inácio, R. S., Gomes, A. M., & Saraiva, J. A. (2020). Serra da Estrela cheese: A review. *Journal of Food Processing and Preservation*, 44(5), e14412.

Irlinger, F., Helinck, S., & Jany, J. L. (2017). Secondary and adjunct cultures. In *Cheese* (pp. 273-300). Academic Press.

Irlinger, F., Yung, S. A. Y. I., Sarthou, A. S., Delbès-Paus, C., Montel, M. C., Coton, E., Coton, M. & Helinck, S. (2012). Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International Journal of Food Microbiology*, 153(3), 332-338.

Kongo, J. M., & Malcata, F. X. (2016a). Cheese: processing and sensory properties. *Encyclopedia of Food and Health* 748-754.

Kongo, J. M. & Malcata, F. X., (2016b). Cheese: Chemistry and Microbiology. *Encyclopedia of Food and Health* 735-740.

Lanciotti, R., Vannini, L., Lopez, C. C., Gobbetti, M., & Guerzoni, M. E. (2005). Evaluation of the ability of *Yarrowia lipolytica* to impart strain-dependent characteristics to cheese when used as a ripening adjunct. *International Journal of Dairy Technology*, 58(2), 89-99.

Lavoie, K., Touchette, M., St-Gelais, D., & Labrie, S. (2012). Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec. *Dairy Science & Technology*, 92(5), 455-468.

Ledenbach, L. H., & Marshall, R. T. (2009). Microbial spoilage of dairy products. In M. P. Doyle, & W. H. Sperber (Eds.), *Compendium of the microbiological spoilage of foods and beverages* (pp. 41– 67). Heidelberg: Springer

McSweeney, P. L. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology*, 57(2-3), 127-144.

Montel, M.C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D.A., Desmasures, N., & Berthier, F., (2014). Traditional cheeses: Rich and diverse microbiota with associated benefits. *International Journal Food Microbiology*, 177, 136–154.

Ordiales, E., Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J., & de Guía Córdoba, M. (2013a). Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. *Food Control*, 33(2), 448-454.

Ordiales, E., Martín, A., Benito, M. J., Hernández, A., Ruiz-Moyano, S., & de Guía Córdoba, M. (2013b). Role of the microbial population on the flavor of the soft-bodied cheese Torta del Casar. *Journal of dairy science*, 96(9), 5477-5486.

Ordiales, E., Benito, M. J., Martin, A., Fernández, M., Hernández, A., & de Guia Córdoba, M. (2013c). Proteolytic effect of *Cynara cardunculus* rennet for use in the elaboration of 'Torta del Casar' cheese. *Journal of Dairy Research*, 80(4), 429.

Ordiales, E., Martín, A., Benito, M. J., Fernández, M., Casquete, R., & de Guía Córdoba, M. (2014). Influence of the technological properties of vegetable rennet (*Cynara cardunculus*) on the physicochemical, sensory and rheological characteristics of 'Torta del Casar' cheese. *International Journal of Dairy Technology*, 67(3), 402-409.

O'Sullivan, O., & Cotter, P. D. (2017). Microbiota of raw milk and raw milk cheeses. In *Cheese: Chemistry, Physics and Microbiology*, Eds McSweeney, P. L., Fox, P. F., Cotter, P. D., & Everett, D. W, pp. 301-316. Academic Press.

Padilla, B., Manzanares, P., & Belloch, C. (2014a). Yeast species and genetic heterogeneity within *Debaryomyces hansenii* along the ripening process of traditional ewes' and goats' cheeses. *Food Microbiology*, 38, 160-166.

Padilla, B., Belloch, C., López-Díez, J. J., Flores, M., & Manzanares, P. (2014b). Potential impact of dairy yeasts on the typical flavour of traditional ewes' and goats' cheeses. *International Dairy Journal*, 35(2), 122–129. https://doi.org/10.1016/j.idairyj.2013.11.002

Pereira, C. I., Graça, J. A., Ogando, N. S., Gomes, A. M., & Malcata, F. X. (2010). Influence of bacterial dynamics upon the final characteristics of model Portuguese traditional cheeses. *Food Microbiology*, 27(3), 339-346.

Reguillo, L., Hernández, M., Barrientos, E., Perez-Rodriguez, F., & Valero, A. (2018). Evaluation of the influence of frequency of milk collection and milking dayshift on the microbiological quality of raw milk. *Journal of Food Quality*, Article ID 1306107.

Sánchez-Juanes, F., Teixeira-Martín, V., González-Buitrago, J. M., Velázquez, E., & Flores-Félix, J. D. (2020). Identification of Species and Subspecies of Lactic Acid Bacteria Present in Spanish Cheeses Type "Torta" by MALDI-TOF MS and pheS gene Analyses. *Microorganisms*, 8(2), 301.

Silvetti, T., Capra, E., Morandi, S., Cremonesi, P., Decimo, M., Gavazzi, F., ... & Brasca, M. (2017). Microbial population profile during ripening of Protected Designation of Origin (PDO) Silter cheese, produced with and without autochthonous starter culture. *LWT*, 84, 821-831.

Sørensen, L. M., Gori, K., Petersen, M. A., Jespersen, L., & Arneborg, N. (2011). Flavour compound production by *Yarrowia lipolytica, Saccharomyces cerevisiae* and *Debaryomyces hansenii* in a cheese-surface model. *International Dairy Journal*, 21, 970– 978.

Tabla, R., Gómez, A., Simancas, A., Rebollo, J. E., Molina, F., & Roa, I. (2016). *Enterobacteriaceae* species during manufacturing and ripening of semi–hard and soft raw ewe's milk cheese: Gas production capacity. *Small ruminant research*, 145, 123-129.

Zheng, X., Shi, X., & Wang, B. (2021). A Review on the General Cheese Processing Technology, Flavor Biochemical Pathways and the Influence of Yeasts in Cheese. *Frontiers in Microbiology*, 12(July). https://doi.org/10.3389/fmicb.2021.703284

CAPÍTULO VII

Identification and selection of yeast with functional properties for future application in soft paste cheese.

Capítulo VII.

Identification and selection of yeast with functional properties for future application in soft paste cheese.

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Abstract

This study investigated the yeast community in traditional soft cheese from the Extremadura region to be able to establish possible beneficial properties for human health. A total of 149 yeast isolates were characterised at strain level using the ISSR-PCR technique with the primer $(GTG)_5$. Forty-three different genetic profiles were obtained, and the yeasts were grouped into 12 species allowing their typification at strain level. Regarding probiotic tests, 54 strains were selected for the study of probiotic properties based on genetic profile and origin. All of them showed adequate tolerance to the most restrictive stomach and bile salts conditions, although strains of *Kluyveromyces lactis* and *Pichia fermentans* presented better ability to survive to these stresses. *P. fermentans* was also the species that showed better antioxidant, auto-aggregation and hydrophobicity capacity, at the same time as antimicrobial activity, followed by *Kluyveromyces* sp. strains. Thus, considering the probiotic characteristics studied, 15 strains, *P. fermentans* 1826, 1865, 1859, 1911,1913, 1916 and 1938; *P. kudriavzevii* 1801,1809 and 1832; *K. marxianus* 1886, 1917 and 1919; *D. hansenii* 1860; and *Y. lipolytica* 1961, are promising potential probiotic candidates for developing a functional cheese.

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Identification and selection of yeast with functional properties for future application in soft paste cheese

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ABSTRACT

This study investigated the yeast community in traditional soft cheese from the Extremadura region to be able to establish possible beneficial properties for human health. A total of 149 yeast isolates were characterised at strain level using the ISSR-PCR technique with the primer $(GTG)_5$. Forty-three different genetic profiles were obtained, and the yeasts were grouped into 12 species allowing their typification at strain level. Regarding probiotic tests, 54 strains were selected for the study of probiotic properties based on genetic profile and origin. All of them showed adequate tolerance to the most restrictive stomach and bile salts conditions, although strains of *Kluyveromyces lactis* and *Pichia fermentans* presented better ability to survive to these stresses. *P. fermentans* was also the species that showed better antioxidant, auto-aggregation and hydrophobicity capacity, at the same time as antimicrobial activity, followed by *Kluyveromyces* sp. strains. Thus, considering the probiotic characteristics studied, 15 strains, *P. fermentans* 1826, 1865, 1859, 1911,1913, 1916 and 1938; *P. kudriavzevii* 1801, 1809 and 1832; *K. marxianus* 1886, 1917 and 1919; *D. hansenii* 1860; and *Y. lipolytica* 1961, are promising potential probiotic candidates for developing a functional cheese.

1. Introduction

In the last decade there has been special interest from the scientific community in exploring the probiotic properties of yeast species of food origin and recent studies have shown the probiotic capacity of yeast strains mainly belonging to the genera *Debaryomyces*, *Torulaspora*, *Kluyveromyces*, *Pichia* and *Candida* (Binetti, Carrasco, Reinheimer, & Suárez, 2013; Chen et al., 2010; Gil-Rodríguez, Carrascosa, & Requena, 2015).

A microorganism to be considered as a potential probiotic candidate has to survive the restrictive conditions of the gastrointestinal tract (GIT), adhere to the intestinal mucosa and colonise the colon, at least temporarily, and exert potential health benefits on the host (FAO/WHO, 2014). Dairy products, especially, artisanal fermented products are interesting because they contain a wide diversity of unexplored microbes that may have desirable properties, even though they have been proposed as part of dietary recommendations (Ebner, Smug, Kneifel, Salminen, & Sanders, 2014). The soft raw ewe's milk cheeses 'Torta del Casar' and 'Queso de la Serena' produced in Extremadura (Spain) under the Protected Designation of Origin (PDO) status follow a traditional cheese-making process using an aqueous infusion of the dried flowers from the plant *Cynara cardunculus* L. as rennet and without the addition of a starter culture and thermal treatment which lead to richly diverse microbiota (Ordiales et al., 2013). Most of the microbial communities present in raw milk cheese are lactic acid bacteria (LAB), but yeasts are also present at an important level and play an essential role during maturation. The most important yeasts isolated from raw milk cheese are *Debaryomyces hansenii*, *Galactomyces* spp., *K. marxianus*, *K. lactis*, *Pichia* spp., *Candida* spp. and *Yarrowia lipolytica* (Banjara, Suhr, & Hallen-Adams, 2015; Binetti et al., 2013; Ceugniez, Drider, Jacques, & Coucheney, 2015; Gonçalves Dos Santos, Benito, Córdoba, Alvarenga & Ruiz-Moyano Seco; de Herrera, 2017; Ordiales et al., 2013). Despite these traditional soft cheeses being highly appreciated for their sensorial characteristics, little is known about their yeast diversity and functional properties. Since the relevance of yeast in cheese is well known, and representative strains from the predominant species in dairy products have been reported to survive to the GIT, adhere to intestinal mucosa and have the capacity to inhibit pathogenic microorganisms (Živković et al., 2015; Gil-Rodríguez et al., 2015; Kumura, Tanoue, Tsukahara, Tanaka, & Shimazaki, 2004), studies

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focusing on selecting new probiotic yeast strains could be of interest for the dairy industry. So, the aim of the present work was to evaluate the probiotic potential of 154 yeast strains isolated from Extremadura traditional soft raw ewe's milk cheeses; in particular, the capacity of the strains to survive gastrointestinal (GI) transit was assessed, as well as the antioxidant, auto-aggregation, hydrophobicity and antimicrobial ability, for their potential application during the cheese manufacturing process.

2. Materials and methods

2.1. Cheese sampling and yeast isolation

Eighteen samples were collected at the end of processing (60 days) from six different industries belonging to PDO 'Torta del Casar' and PDO 'Queso de la Serena'. Each cheese sample was represented by one whole cheese. For yeast isolation, acidified potato dextrose agar plates (PDA, Pronadisa) with 1% (v/v) sterilised solution of tartaric acid at 10% (w/v) were incubated at 25 °C for 72 h. Yeast colonies from the highest dilutions were randomly selected and streaked onto acidified PDA agar plates. Finally, pure isolates were grown in 5 mL of yeast extract peptone dextrose broth (YPD, Pronadisa) at 25 °C for 24 h and stored at −80 °C in 25% glycerol. Prior to the assays, all yeast isolates were subcultured twice in YPD broth at 25 °C for 24 h.

2.2. Yeast genotypic identification

2.2.1. ISSR analysis and identification by sequencing analysis of ITS rRNA region

Yeast DNA was extracted using a GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA). Inter-single sequence repeat anchored polymerase chain reaction amplification analysis (ISSR-PCR) was carried out using the 5'-anchored $(GTG)_5$ primer (5'-GTGGTGGTGGTGGTG-3') as described by Mahmoud, Ali, E-Aziz, Al-Othman & Al-Wadai (2014). The fragment profiles obtained were grouped into operational taxonomic units (OTUs). Cluster analysis of the bands from each isolate was carried out using the unweighted pair group method with arithmetic average (UPGMA), from which a dendrogram showing the relationships between yeasts isolates was obtained. The analysis was performed using the NTSYS.PC package, version 2.0.

Two representative isolates of each OTU were identified at species level by sequencing the internal transcribed spacer ITS1/ITS2-5.8 S rRNA region using ITS1 and ITS4 primers (Gallardo et al., 2014). The taxonomic isolate identification was confirmed, based on the highest identity score (highest sequence homology).

2.3. Determination of probiotic properties

Yeast isolates from different OTUs or belong to the same OTU but of different industry origin were selected for screening their potential probiotic capacity. After yeast culture, cells from each strain were harvested by centrifugation at 5000g for 5 min at room temperature, washed twice with phosphate-buffered saline (PBS; Thermo Fisher Scientific) at pH 7.2 and the cell concentration adjusted to around 10^6–10^7 cfu/mL. For all strains, three biological replicates of the cultures were performed, and the assays for each strain were conducted in duplicate.

2.3.1. Survival at low pH

The tolerance of each strain to the pH usually found in the stomach was evaluated in PBS supplemented with 3 g/L (w/v) of pepsin and the pH adjusted to 2, 2.5, 3 or 5 with HCl 2.5 mol/L (Thermo Fisher Scientific, USA) following the method described by Ruiz-Moyano, Martín, Benito, Nevado, and Córdoba (2008) with modifications. PBS at pH 5 was used as the control. Strains with viable counts lower than 2 log cfu/mL respective to the control were considered not resistant. Viable cells were monitored by decimal dilutions in YPD broth at 0, 1, 2 and 3 h, incubating the microtitre plates at 25 °C for 48 h. Those yeast strains that at pH 2.5 after 3 h of assay decreased by more than 2 log cfu/mL with respect to the counts obtained in the control were considered not acid-tolerant.

2.3.2. Survival in bile salts

Each yeast strain was inoculated in PBS at pH 8 supplemented with different concentrations of bovine bile salts (Sigma, USA), previously sterilised by filtration with 0.22 μm pore size filter, until achieving final concentrations of 0%, 0.3%, 0.6% and 1% (w/v) (Ruiz-Moyano et al., 2008). PBS without bile salts at pH 8 was used as the control. Viable cells were monitored as above for tolerance to low pH for 0, 2, and 4 h of bile salt exposure. Those yeast strains that at different bile salt concentrations after 4 h of assay decreased by more than 2 log cfu/mL with respect to the control (0% bile salt concentration) were considered not bile salt-tolerant.

2.3.3. Survival in simulated gastrointestinal transit

Briefly, yeast strains were inoculated in simulated gastric juice (3 g/L of pepsin (Sigma, USA) and 2 g/L of NaCl (Thermo Fisher Scientific, USA)) at pH 2.5 for 2 h, followed by 6 h in simulated intestinal juice (1 g/L of trypsin (Sigma, USA), 5 g/L of bile salts (Sigma, USA), 2 g/L of pancreatin (Sigma, USA), 11 g/L of sodium bicarbonate and 2 g/L of NaCl) at pH 8 to simulate intestinal tract conditions (Bao et al., 2010). The viable yeasts were monitored as above at 0, 2, 4, 6 and 8 h of exposure to GI transit. Strains with viable counts lower than 2 log cfu/mL respective to time 0 h were considered not resistant.

2.3.4. Growth at low pH and in bile salts

Capacity to resist the GI conditions was further investigated by growing the yeast strains in semisolid yeast nitrogen base broth (YNB, Pronadisa) containing 0.125% (w/v) of bacteriological agar (Pronadisa) at 37 °C, and the pH of the medium was adjusted to pH 2.5 or supplemented with 0.3% (w/v) bile salts (Sigma). The experiment was performed in a 200 μL volume in 100-microwell honeycomb sterile plates as previously described by Pedersen, Owusu-Kwarteng, Thorsen, and Jespersen (2012). Semisolid YNB without inoculation was used as a negative control and inoculated standard semisolid YNB was used as a positive control. An automated turbidimeter (Bioscreen C Microbiology Reader (ThermoLab Systems, Finland)) was used to monitor the growth of strains by reading the optical density (OD) at 600 nm at regular intervals for 24 h without shaking. The growth rate of the yeasts at pH 2.5 or in 0.3% (w/v) bile salts was calculated relative to their growth rate in positive controls in YNB.

2.3.5. Auto-aggregation capacity

Specific cell–cell interactions were determined using the auto-aggregating method described by Fadda, Mossa, Deplano, Pisano, and Cosentino (2017). Yeast cell suspensions were vortexed for 10 s and incubated at 37 °C for 2 h. The upper zone was carefully removed and measured at OD_{560nm} before and after incubation. The percentage of auto-aggregation was expressed as $[1 - (OD_t/OD_0)] \times 100$, where OD_0 and OD_t are the optical density before and after incubation, respectively.

2.3.6. Hydrophobicity

The hydrophobic capacity of the yeasts was evaluated as described by Binetti et al. (2013). Yeast cell suspensions were adjusted with PBS to an OD_{560nm} of approximately 0.4 and 0.6. Three millilitres of these suspensions were added to a 0.2 mL/mL suspension of n-hexadecane (Sigma) and vortexed for 60 s. The two phases were allowed to separate for 30 min at 37 °C. The aqueous phase was carefully removed and the OD_{560nm} was measured. The percentage hydrophobicity was evaluated as the percentage of reduction in the OD of the aqueous phase and

Table 1
Identification and strain typing of the yeasts isolated from cheeses of six industries.

Identification by ITS sequencing	ISSR profiles	Number of isolates	Strain codes	Number of profile bands	Range of molecular weight (bp) From - To	
Debaryomyces hansenii	1	3	1791, 1802, 1803	11	405	2890
(32 isolates)	2	18	1821, 1822, 1824, 1828, 1829, 1830, 1835, 1836, 1837, 1838, 1841, 1842, 1843, 1844, 1845, 1846, 1848, 1849	10	405	2890
	3	1	1823	11	405	2890
	4	9	1839, 1840, 1853, 1855, 1856, 1860, 1862, 1863, 1864	7	405	2890
	5	1	1933	10	535	2890
Debaryomyces vindobonensis	6	1	1790	12	405	2445
(5 isolates)	7	1	1796	11	535	2445
	8	1	1825	13	535	2445
	9	1	1834	15	535	2445
	10	1	1921	11	405	2445
Kluyveromyces lactis	11	4	1794, 1922, 1923, 1924	8	420	1900
(27 isolates)	12	7	1875, 1876, 1885, 1910, 1915, 1939, 1941	10	405	1900
	13	16	1882, 1892, 1893, 1894, 1899, 1912, 1926, 1927, 1928, 1929, 1930, 1932, 1934, 1935, 1936, 1937	11	405	1900
Kluyveromyces marxianus	14	15	1868, 1869, 1870, 1871, 1872, 1873, 1878, 1879, 1884, 1886, 1887, 1891, 1918, 1919, 1924	9	535	1900
(25 isolates)	15	2	1881, 1883	10	405	1900
	16	8	1880, 1888, 1891, 1896, 1897, 1898, 1900, 1917	9	405	1900
Candida zeylanoides	17	2	1798, 1940	15	405	2200
(18 isolates)	18	2	1847, 1851	12	405	2200
	19	7	1890, 1895, 1901, 1902, 1903, 1904, 1905	11	405	2200
	20	3	1906, 1907, 1962	16	405	2200
	21	4	1945, 1947, 1950, 1951	13	380	2200
Candida cabralensis	22	2	1858, 1866	10	405	1900
(2 isolates)						
Candida pararugosa	23	2	1807, 1808	6	860	3520
(4 isolates)	24	1	1815	11	560	3520
	25	1	1819	11	405	1900
Yarrowia lipolytica	26	1	1908	14	630	1620
(5 isolates)	27	3	1857, 1861, 1961	11	630	1840
	28	1	1946	13	630	1620
Pichia cactophila	29	1	1806	10	560	3120
(5 isolates)	30	2	1811, 1812	10	560	3120
	31	2	1814, 1818	9	560	1310
Pichia kudriazevii	32	1	1801	7	590	2320
(6 isolates)	33	5	1809, 1810, 1816, 1817, 1832	6	590	2320
Pichia fermentans	34	2	1804, 1805	13	505	2820
(14 isolates)	35	1	1813	13	505	2820
	36	2	1826, 1827	16	505	3350
	37	1	1909	17	505	3350
	38	1	1831	14	505	2820
	39	1	1911	18	535	3350
	40	5	1865, 1913, 1914, 1916, 1938	19	505	3350
	41	1	1859	14	505	3270
Pichia jadinii	42	1	1877	9	645	2545
(1 isolate)						

calculated with the formula $[(OD_0 - OD)/OD_0] \times 100$, where OD_0 and OD are the optical density before and after extraction with n-hexadecane, respectively.

2.3.7. Assessment of antioxidant activity

The DPPH scavenging activity (Sigma Chemical Co., St. Louis, MO, USA) of yeasts was analysed by the method of Shimada, Fujikawa, Yahara, and Nakamura (1992) with modifications. Eight hundred microlitres of each yeast strain suspension and 1 mL of 0.2 mmol/L 1,1-diphenyl-2-picrylhydrazyl solution in methanol were mixed. This mixture was stirred and then incubated for 30 min in darkness at room temperature. The reaction tubes were centrifuged (2000 *g*, 2 min) to collect the supernatant. To measure the percentage antioxidant capacity, the absorbance was measured at 517 nm, using sterile PBS solution as the target. The scavenging ability was defined as $[1 - A_{517\ (sample)}/A_{517\ (blank)}] \times 100$.

2.3.8. Antimicrobial activity

Inhibitory effects of yeast strains were evaluated on selected pathogenic microorganisms, four bacteria (*Salmonella choleraesuis* CECT 4395, *Escherichia coli* CECT 4267, *Staphylococcus aureus* CECT 976 and *Listeria monocytogenes* CECT 911), two yeasts (*Candida albicans* CECT 1394 and *C. parapsilosis* 1953 (Fungi Culture Collection of the Agricultural Engineering School, Extremadura University, Badajoz, Spain)) and three mycotoxigenic moulds (*Penicillium commune* CBS 311, *P. camemberti* CBS 273 and *Aspergillus flavus* CQ103 (Fungi Culture Collection of the Agricultural Engineering School, Extremadura University, Badajoz, Spain).

To evaluate the antibacterial activity, 5 μL of a 10^6 cells/mL yeast culture was spotted onto the surface of YPD agar and incubated at 25 °C for 48 h. Then, the plates were overlaid with brain heart infusion soft agar (0.7% w/v) inoculated with pathogenic bacteria at a final concentration of 10^6 cfu/mL and incubated at 37 °C for 48 h. After incubation, the plates were examined for zones of inhibition around yeast spots.

The antifungal activity was determined as described by Pérez-Nevado, Albergaria, Hogg, and Girio (2006) with modifications. Prior to the assay, moulds were grown on PDA (Pronadisa, Madrid, Spain) at pH 3.5 ± 0.1 at 25 °C for 7–10 days until sporulation. The spores were harvested by adding distilled water with 0.05% (v/v) Tween 80 (Scharlab), and the suspension formed was filtered through two layers of cheesecloth; the concentration was determined in a Neubauer chamber. For the experiment, plates of YEPD-MB medium (0.5% yeast extract, 1% peptone, 2% glucose, 2% agar, 0.003% methylene blue, 0.1 MOL/L sodium citrate) at pH 5.0 were inoculated with 100 μL of a spore suspension at 10^5 spores/mL or pathogenic yeast culture at 10^5 cells/mL and spread out. After drying, 5 μL of each yeast suspension at 10^5 cells/mL was spotted onto agar plates and incubated at 25 °C for 7 days. Yeast strains exhibiting clear inhibition halos were considered positive.

2.4. Statistical analysis

Statistical analysis of the data was carried out using the program SPSS for Windows 21.0 (SPSS Inc Chicago, IL, USA). One-way analysis of variance (ANOVA) was conducted and the means were separated by Tukey's honestly significant difference (HSD) test ($p \leq 0.05$). The relationships among probiotic assays and yeast strains were established by Pearson correlation coefficients and principal component analysis (PCA). SPSS for Windows 21.0 (SPSS Inc., Chicago, IL, USA) was used.

3. Results and discussion

3.1. Identification of yeast by ISSR-PCR analysis and sequencing

The isolates from the PDA plates were further investigated to identify them at the species level. The 149 yeast isolates from cheeses were grouped by ISSR analysis and identified by subsequent sequencing of ITS1/ITS2-5.8 S rRNA region domains.

On the basis of ISSR analysis, 43 different profiles were obtained which corresponded to 12 different species (Table 1). The species identified in this study were: *D. hansenii*, *D. vindobonensis*, *K. marxianus*, *K. lactis*, *C. cabralensis*, *C. pararugosa*, *C. zeylanoides*, *Y. lipolytica*, *P. fermentans*, *P. cactophila*, *P. kudriazevii* and *P. jadinii* which were also differentiated at the strain level using the ISSR technique. The predominant yeasts belong to the genus *Kluyveromyces* (25 identifications of *K. marxianus* and 27 of *K. lactis*) followed by the species *D. hansenii* and *P. fermentans*. Similar results were found by Binetti el al. (2013) who identified yeast strains isolated from autochthonal cheeses.

PCR products obtained by ISSR ranged from 380 to 3545 bp. The strains belonging to *P. fermentans* were those that presented a profile with the highest number of bands, profile 40 being the one that had the highest number with a total of 19 bands. On the contrary, strains belonging to the species *C. pararugosa* were the ones that presented the lowest number of bands in profile number 23 (six bands).

Fig. 1 shows the dendrogram of the different profiles obtained through a cluster analysis of the ISSR-PCR. In this study, with 44% similarity, the isolates were separated into clusters. Seven totally defined groups were obtained. Cluster 1 groups the isolates of the species *P. cactophila*; Cluster 2 the isolates of the species *C. pararugosa*; Cluster 3 is formed by isolates belonging to several species, *K. marxianus*, *K. lactis*, *C. cabralensis*, *C. zeylanoides*, *D. hansenii* and *D. vindobonensis*; Cluster 4 contains isolates of *Y. lipolytica*; Cluster 5 of *P. jadinii*; Cluster 6 of *P. fermentans*; and finally Cluster 7 of *P. kudriazevii*. Species from Cluster 3 were differentiated with between 57% and 67% similarity, which has been shown to present adequate results for species differentiation (Tofalo, Perpetuini, Schirone, Suzzi, & Corsetti, 2013). Finally, isolates with a similarity greater than 90% were considered to be the same strains (Tofalo et al., 2013).

Among the 149 isolates identified, 54 strains were selected for the study of probiotic properties based on genetic profile and origin (Table 2). Strains belonging to the 12 yeast species identified in cheese in this work have presented characteristics that allow them to survive the conditions of GI transit (Maccaferri, Klinder, Brigidi, Cavina, & Costabile, 2012; Psomas, Fletouris, Litopoulou-Tzanetaki, & Tzanetakis, 2003). That indicates that they could be an object of study as potential probiotics.

3.2. Probiotic properties of yeast

3.2.1. Tolerance to gastrointestinal tract conditions

Results for the tolerance to acid and bile salts at 37 °C indicated that all strains tested were tolerant to these stresses. However, in order to further investigate in more detail their ability to survive the stresses found in the GIT, their growth capacity was studied through the Bioscreen C assay. Table 2 shows the growth ability of yeast strains at pH 2.5 and 0.3% bile salt concentration, comparing them with their growth in regular YNB media. The 54 strains were able to survive simulated gastric and intestinal conditions; however, not all exhibited the same ability to grow at pH 2.5 or in the presence of 0.3% bile salts.

In general, the effect of pH on the growth of strains was more restrictive than that of bile salts. Seventeen percent of the strains were able to grow at pH 2.5 with a growth rate of 60% with respect to the control. Nevertheless, 81% of the yeast strains were able to grow in bile salts with growth rate values greater than 60%. Although most yeasts show survival at pH 2.5, growth at this pH condition is usually more restricted. Van der Aa Kühle, Skovgaard, and Jespersen (2005) found that only 44% of the *S. cerevisiae* strains assayed were able to grow at pH 2.5; however, those same strains showed adequate growth in bile salts (0.3%).

Some *K. lactis* strains, 1794, 1910 and 1912, presented outstanding growth in bile salts (above 100%) but their growth at pH 2.5 was very

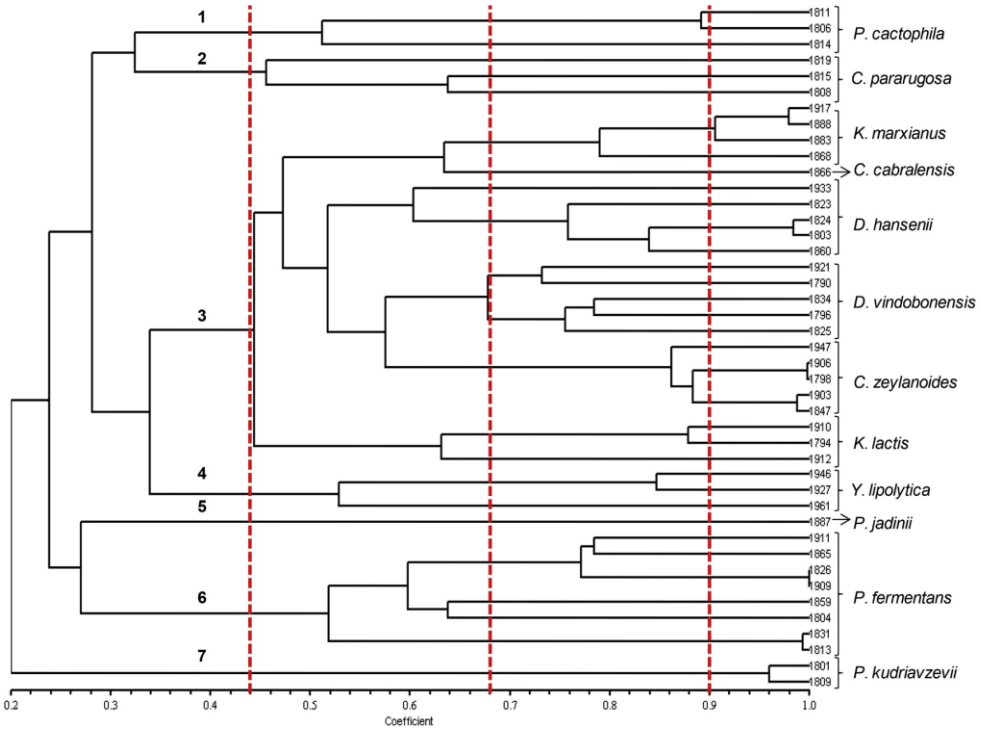

Fig. 1. Dendrogram of different profiles obtained by cluster analysis of ISSR-PCR $(GTG)_5$ bands obtained for yeasts isolated from cheeses. The dendrogram is based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic average clustering algorithm (UPGMA). Identity is by sequencing of the ITS rRNA gene as described in the text.

low. The strains of the species *P. fermentans* and *P. kudriavzevii* grew successfully in the presence of bile salts, even above 140% (strains 1859 and 1911). The rest of the species grew very little with respect to the control; there were even some species like *D. vindobonensis* that did not present detectable growth at pH 2.5. Yeasts tolerate acidic conditions, but some did not grow at pH 2.5. The main barrier for microorganisms in the stomach is the gastric acid, but bile resistance is also an important characteristic to consider in the selection of a probiotic culture allowing a microorganism to grow in the intestinal tract (Suscovic, Brkic, Matosic, & Maric, 1997). These results showed high differences between strains of the same species so probiotic capacities are strain-dependent as reported by other authors (Binetti et al., 2013; Fadda et al., 2017). The results of our study suggest that a significant number of yeast strains may survive GI transit, and then possibly affect the microbial balance in the human body.

3.2.2. Auto-aggregation, hydrophobicity and antioxidant capacity of the selected yeasts

The results for auto-aggregation and hydrophobicity activity are presented in Table 3. In general, the results showed interspecies and intraspecies differences in both cell-surface properties. *P. fermentans* was the species that showed the highest auto-aggregation capacity, particularly strain 1859, and strains of this species also showed noteworthy hydrophobicity capacity. However, *C. zeylanoides*, strain 1847, presented the highest percentage of hydrophobicity but low auto-aggregation capacity. Regarding other species, *P. cactophila* presented the lowest auto-aggregation capacity and this species next to *D. vindobonensis* also had low hydrophobicity capacity.

The cell-surface properties of auto-aggregation and hydrophobicity play a key role in host epithelial adhesion and are generally correlated (Burns, Reinheimer, & Vinderola, 2011). However, in this study although most of the strains displayed moderate or high auto-aggregation values, hydrophobicity was highly variable among strains and the same trend was not always kept between both properties. Specifically, it was observed that the genus *Debaryomyces* presented medium-high auto-aggregation values while hydrophobicity values were low, mainly for *D. vindobonensis* strains, without reaching 22%. Likewise, *P. fermentans* 1859 with 90.35% auto-aggregation presented scarce hydrophobicity (34.83%). Cell–cell interactions at the molecular level use complex mechanisms under different conditions, and they do not always show a positive correlation in the literature.

Concerning antioxidant activity, the strains tested exhibited antioxidant activity at different levels (Table 3). Among the 54 yeasts analysed, only two strains, *P. cactophila* 1806 and 1814, showed low antioxidant activity, with a DPPH scavenging percentage under 20%. On the other hand, four strains displayed excellent activity with a percentage of DPPH reduction exceeding 50% (three *P. fermentans* strains, 1826, 1865 and 1913; and one *D. hansenii* strain, 1860). *P. fermentans* was the species that showed the highest antioxidant capacity, particularly strain 1865. These results are similar to those found by Gil-Rodríguez et al. (2015) when studying the health-beneficial potential of 130 yeast strains isolated from food. The antioxidant activity of yeasts seems higher than that of LAB; in this study, the antioxidant activity was generally higher than that reported by other authors for different LAB strains (Amaretti et al., 2013).

3.2.3. Antimicrobial activity

Table 4 shows the inhibition of yeasts against four important food-borne pathogenic bacteria. Only 16 of the 54 strains selected showed activity against pathogenic bacteria. None of them showed growth

Table 2
Growth at pH 2.5 adjusted with HCl and in bile salts (0.3% bile salts) of 54 selected yeast strains measured using a Bioscreen C.

Isolate	Code	Growth at pH 2.5	Growth in 0.3% bile salts
Debaryomyces vindobonensis	1790	–	+ +
	1796	–	+ +
	1825	–	+ +
	1834	–	+ +
	1921	±	+ +
Debaryomyces hansenii	1802	±	+ +
	1823	±	+
	1842	–	+
	1860	+ +	+ +
	1864	±	+
	1933	±	+
Kluyveromyces lactis	1794	+	+ + +
	1910	+	+ + +
	1912	+	+ + +
	1915	±	+ +
	1922	+	+ +
	1934	+	+ +
Kluyveromyces marxianus	1869	+ +	+ +
	1883	+ +	+ +
	1886	+ +	+ +
	1888	+	+ +
	1900	+	+ +
	1917	+ +	+ +
	1919	+ +	+ +
Pichia fermentans	1804	+	+ + +
	1813	+	+ +
	1826	+	+ + +
	1859	+ + +	+ + + +
	1865	+ +	+ + +
	1911	+ +	+ + + +
	1913	+	+ +
	1916	+	+ +
	1938	+	+ + +
Pichia kudriavzevii	1801	+ +	+ +
	1809	+ +	+ +
	1816	+ +	+ +
	1817	+ +	+ + +
	1832	+ +	+ + +
Pichia cactophila	1806	+	+ +
	1811	+	+ +
	1812	+	+ +
	1814	+	+ +
Pichia jadinii	1877	+ +	+ +
Yarrowia lipolytica	1927	+	+ +
	1946	+	+
	1961	+ +	+
Candida zeylanoides	1847	±	+
	1903	±	+ +
	1906	±	+
	1947	±	+ +
Candida pararugosa	1808	+	+
	1815	+	+ +
	1819	+ +	+
Candida cabralensis	1858	+	+ +

+ + + +: 140% or more of the growth rate in YNB; + + +: 100–139% of the growth rate in YNB; + +: 60–99% of the growth rate in YNB; +: 20–59% of the growth rate in YNB; ±: 1–19% of the growth rate in YNB; –: 0% or less of the growth rate in YNB.

Table 3
Ability of selected yeast strains to scavenge DPPH radical, auto-aggregation and hydrophobicity capacity expressed as a percentage. SSB: statistical significance bar using Tukey's HSD test.

	Strain	Auto-aggregation	Hydrophobicity	DPPH
Debaryomyces vindobonensis	1790	58.01	18.27	39.03
	1796	58.83	22.33	35.22
	1825	78.47	21.08	35.54
	1834	75.02	37.02	29.81
	1921	78.15	47.47	32.35
Debaryomyces hansenii	1802	71.33	82.33	25.69
	1823	82.74	73.83	25.38
	1842	80.20	49.73	32.45
	1860	60.49	82.10	56.29
	1864	73.72	86.48	48.44
	1933	82.73	4.17	36.19
Kluyveromyces lactis	1794	81.04	41.48	24.32
	1910	83.04	61.55	38.58
	1912	77.31	85.30	32.61
	1915	51.31	66.13	40.19
	1922	84.96	64.43	38.50
	1934	58.78	41.78	27.60
Kluyveromyces marxianus	1869	80.88	70.34	31.45
	1883	84.50	45.07	44.91
	1886	82.97	69.12	20.27
	1888	82.51	75.80	27.84
	1900	71.66	70.50	37.38
	1917	79.13	84.11	47.04
	1919	78.92	84.87	44.51
Pichia fermentans	1804	68.66	65.01	29.25
	1813	83.84	66.97	30.56
	1826	87.95	51.68	56.17
	1859	90.35	34.83	49.40
	1865	79.02	89.99	59.66
	1911	80.98	74.32	43.07
	1913	86.47	81.46	54.65
	1916	89.48	56.43	47.53
	1938	87.18	62.79	49.78
Pichia kudriavzevii	1801	82.06	70.23	48.92
	1809	81.63	68.74	38.58
	1816	84.73	67.85	34.57
	1817	80.76	67.69	26.89
	1832	87.32	55.96	48.00
Pichia cactophila	1806	40.05	33.07	11.82
	1811	50.83	55.11	27.87
	1812	38.75	43.70	16.29
	1814	30.16	38.23	15.98
Pichia jadinii	1877	88.02	53.99	22.70
Yarrowia lipolytica	1927	65.82	63.88	47.94
	1946	60.24	48.96	39.21
	1961	79.49	69.05	47.72
Candida zeylanoides	1847	49.43	92.72	25.04
	1903	83.64	77.57	42.90
	1906	40.52	71.74	22.72
	1947	58.81	87.94	42.57
Candida pararugosa	1808	60.61	82.01	24.53
	1815	41.71	82.09	15.02
	1819	88.98	55.35	34.98
Candida cabralensis	1858	78.84	35.62	36.78
HSD Tukey SSB		± 7.08	± 20.57	± 13.18

inhibition of *L. monocytogenes* CECT 911. It is to be highlighted that three strains belonging to the species *Pichia fermentans* presented inhibition of *S. choleraesuis* and *E. coli* at the same time. These results are very valuable because other authors (Binetti et al., 2013) found no activity against *E. coli*, *Salmonella enteritidis* and *S. aureus* by yeast strains isolated from cheeses belong to the species *K. marxianus*, *K. lactis*, *C. lusitaniae*, *P. kudriavzevii*, *S. cerevisiae* and *Galactomyces* spp. However, Bajaj, Raina, and Singh (2013) and Saber, Alipour, Faghfoori, and Khosroushahi (2017) found that *P. kudriavzevii* strain RY55 and *K. marxianus* AS41, respectively, exhibited antibacterial activity against *E. coli*, *Salmonella* spp. and *S. aureus*. Antibacterial products secreted by yeasts are not common. Antagonistic action of killer yeasts/toxins against other yeasts/fungi is well reported (Baeza, Sanhueza, & Cifuentes, 2008), but scarce reports exist on antibacterial activity by yeasts.

On the other hand, among the 54 strains analysed, 34 showed inhibition of the growth of the human pathogens yeasts and moulds used in this study (Table 5). The results show that strains of the genus *Kluyveromyces* and *Pichia* are the ones that present growth inhibition of several species at the same time. Strains 1922, 1934 and 1939 of *K. lactis*, and 1816 of *P. kudriavzevii* that inhibited at the same time four of

Table 4
Selected yeast inhibition of pathogenic bacteria.

Isolate	Code	*S. choleraesuis* CECT 4395	*S. aureus* CECT 976	*E. coli* CECT 4267
Kluyveromyces lactis	1934	–	–	A2
	1939	–	A2	A1
	1922	–	–	A2
Kluyveromyces marxianus	1919	–	–	A1
Pichia fermentans	1804	–	–	A3
	1813	–	–	A2
	1859	–	–	A5
	1865	A1	–	A3
	1911	–	–	A3
	1916	A2	–	A3
	1938	A2	–	A2
Pichia kudriavzevii	1801	–	–	A4
	1816	A4	–	–
	1832	–	A5	–
Pichia cactophila	1811	–	–	A2
Yarrowia lipolytica	1927	A4	A2	–

(–): Negative inhibition; A: halo of inhibition. Range between 1 and 5, radius of inhibition zone (millimetres).

the five species tested should be highlighted. Different *Kluyveromyces* strains also exhibited the largest inhibition halos, with a radius of 3 mm. Other authors have found similar results with *Pichia anomala* and *K. wickerhamii* that showed killer toxins against *Dekkera/Brettanomyces* wine spoilage yeasts (Comitini, De Ingeniis, Pepe, Mannazzu, & Ciani, 2004). The antifungal activity of yeasts has been associated with the production of killer toxins or 'mycocins'. Killer systems have been already reported in *K. lactis* (Gunge, Tamaru, Ozawa, & Sakaguchi, 1981) and *Pichia* species (Magliani, Conti, Gerloni, Bertolotti, & Polonelli, 1997). Antimicrobial activity is an interesting characteristic especially considered to preserve against spoilage and pathogen microorganisms, so our study could be used as a basis for selecting yeasts to be used in biocontrol during cheese making.

3.3. Correlations between probiotic properties and yeast strains

Fig. 2 shows the results of PCA analysis including the probiotic properties assayed with the selected strains identified. The pH growth, bile salts growth, ability to scavenge DPPH radical and auto-aggregation were located on the positive axis of principal component 1 explaining 43.65% of the total variance; hydrophobicity capacity was located on the positive axis of principal component 2 which describes an additional 21.01% of the original variability of the data (Fig. 2). The parameters of pH growth, bile salts growth, ability to scavenge DPPH radical and auto-aggregation were significantly correlated with each other ($P < 0.01$). However, hydrophobicity was only positively correlated with pH growth and the ability to scavenge DPPH radical ($P < 0.05$). As stated above, this statistical analysis confirms that in our study auto-aggregation and hydrophobicity capacity did not follow the same trend.

In Fig. 2 of the PCA, within the different circles, the most relevant strains related to the parameters studied were selected. The PCA showed a clear relationship between the probiotic properties assayed and 15 yeast strains: seven of *P. fermentans*, three of *P. kudriavzevii*, three of *K. marxianus*, one of *D. hansenii* and one of *Y. lipolytica* (Fig. 2). The strains in the discontinuous circle correlated more strongly with the parameters of hydrophobicity, pH growth and ability to scavenge DPPH radical and less with auto-aggregation and bile salts growth. The strains in the continuous circle correlated less with the hydrophobicity

Table 5
Selected yeast inhibition of pathogen yeasts and mycotoxigenic moulds in cheese.

Isolate	Code	*C. albicans* CECT 1394	*C. parapsilosis* 1953	*P. camemberti* CBS 273	*A. flavus* CQ103	*P. commune* CBS 311
D. vindobonensis	1790	–	–	–	–	B2
	1825	–	–	B1.5	–	–
	1834	–	B2.5	–	B2	–
D. hansenii	1842	–	B2	–	–	–
	1860	–	B2	–	–	–
K. lactis	1794	–	–	–	–	B1.5
	1882	A2	–	–	A2	B2
	1885	A1	A2.5	–	–	A1
	1910	–	B2.5	–	A2	–
	1912	–	A2.5	–	B2	–
	1915	–	B2.5	–	B2	–
	1922	–	B3	B2	A3	B2
	1934	A2	B2	–	A1.5	B2
	1939	A3	–	B2	A1.5	B2
K. marxianus	1879	–	A3	–	–	–
	1883	A1.5	B3	–	A1	–
	1886	–	B2.5	–	–	–
	1888	–	–	–	–	B1.5
	1900	–	B3	B3	–	B2
	1917	–	B2.5	–	B2	–
	1919	–	B2.5	–	–	–
P. fermentans	1804	–	–	B2	–	–
	1813	–	B2	B2	–	–
	1859	–	–	–	B1.5	–
	1865	–	–	A1.5	A1.5	–
	1911	–	–	B1.5	–	–
	1913	–	B2.5	–	A1	–
	1916	–	B2.5	–	–	–
	1938	–	B2.5	–	A2	–
P. kudriavzevii	1816	A1	A2.5	B2	B1.5	–
	1817	–	B2.5	–	–	–
	1832	–	B2.5	–	–	–
Y. lipolytica	1927	A2.5	–	–	A1.5	B1.5
C. cabralensis	1858	–	–	–	B2	–

(–): Negative inhibition; A: halo of inhibition; B: impaired development of growth. Range between 1 and 5, radius of inhibition zone (millimetres).

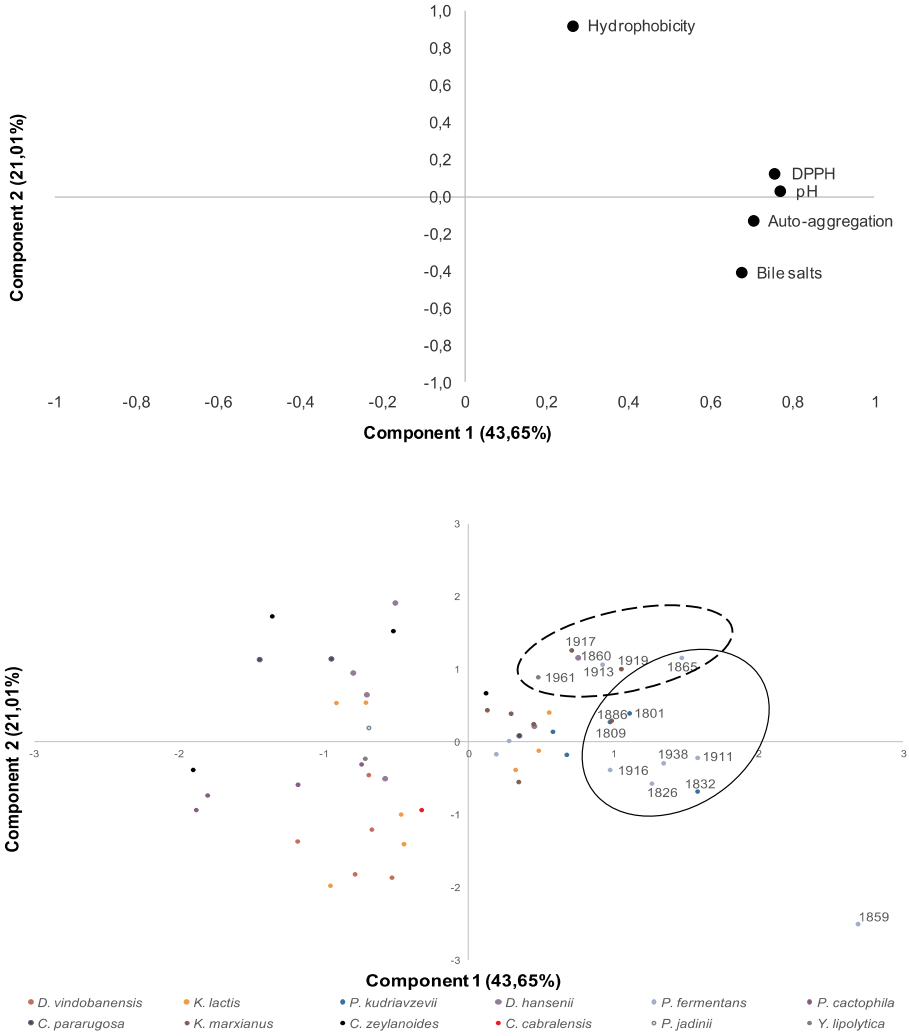

Fig. 2. Principal component analysis (PCA) of the probiotic properties pH growth, bile salts growth, ability to scavenge DPPH radical, auto-aggregation and hydrophobicity capacity (a) and the selected yeast strain species (b).

parameter, with *P. fermentans* 1859, although negatively correlated with hydrophobicity, showing the best correlation with the rest of the parameters assayed. On the other hand, *P. fermentans* 1865, included at the junction of the two circles, was the strain that showed the best correlation with all of these probiotic activities studied, and also showed antimicrobial activity (Tables 4 and 5).

4. Conclusion

The microorganisms identified mainly corresponded to *D. hansenii* and *K. marxianus*, and sequencing of the ribosomal ITS gene combined with ISSR-PCR analysis allowed differentiation of isolates at species and strain level. The 54 strains tested showed adequate tolerance to the most restrictive stomach and bile salts conditions, however, strains of *K. lactis* and *P. fermentans* presented better ability to survive to these stresses. *P. fermentans* was also the species that showed better antioxidant, auto-aggregation and hydrophobicity capacity, at the same time as antimicrobial activity, followed by *Kluyveromyces* sp. strains. Probiotics properties have showed to be strain-dependent, thus, considering the characteristics studied, 15 strains, *P. fermentans* 1826, 1865, 1859, 1911, 1913, 1916 and 1938; *P. kudriavzevii* 1801, 1809 and 1832; *K. marxianus* 1886, 1917 and 1919; *D. hansenii* 1860; and *Y. lipolytica* 1961, are promising probiotic candidates for developing a cheese with probiotic characteristics.

CRediT authorship contribution statement

Almudena V. Merchán: Conceptualization, Methodology, Investigation. **María José Benito:** Writing - original draft, Investigation, Writing - review & editing, Supervision. **Ana Isabel Galván:** Investigation. **Santiago Ruiz-Moyano Seco de Herrera:** Methodology, Investigation, Supervision, Funding acquisition, Writing - review & editing.

Declaration of competing interest

No known.

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References

Amaretti, A., di Nunzio, M., Pompei, A., Raimondi, S., Rossi, M., & Bordoni, A. (2013). Antioxidant properties of potentially probiotic bacteria: In vitro and in vivo activities. *Applied Microbiology and Biotechnology, 97*, 809–817. https://doi.org/10.1007/s00253-012-4241-7.

Baeza, M. E., Sanhueza, M. A., & Cifuentes, V. H. (2008). Occurrence of killer yeast strains in industrial and clinical yeast isolates. *Biological Research, 41*(2), 173–182. https://doi.org/10.4067/S0716-97602008000200007.

Bajaj, B. K., Raina, S., & Singh, S. (2013). Killer toxin from a novel killer yeast *Pichia kudriavzevii* RY55 with idiosyncratic antibacterial activity. *Journal of Basic Microbiology, 53*(8), 645–656. https://doi.org/10.1002/jobm.201200187.

Banjara, N., Suhr, M. J., & Hallen-Adams, H. E. (2015). Diversity of yeast and mold species from a variety of cheese types. *Current Microbiology, 70*, 792–800. https://doi.org/10.1007/s00284-015-0790-1.

Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X., et al. (2010). Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. *Food Control, 21*(5), 695–701. https://doi.org/10.1016/j.foodcont.2009.10.010.

Binetti, A., Carrasco, M., Reinheimer, J., & Suárez, V. (2013). Yeasts from autochthonal cheese starters: Technological and functional properties. *Journal of Applied Microbiology, 115*(2), 434–444. https://doi.org/10.1111/jam.12228.

Burns, P., Reinheimer, J., & Vinderola, G. (2011). Impact of bile salt adaptation of *Lactobacillus delbrueckii* subsp. *lactis* 200 on its interaction capacity with the gut. *Research in Microbiology, 162*, 782–790. https://doi.org/10.1016/j.resmic.2011.07.005.

Ceugniez, A., Drider, D., Jacques, P., & Coucheney, F. (2015). Yeast diversity in a traditional French cheese "Tomme d'orchies" reveals infrequent and frequent species with associated benefits. *Food Microbiology, 52*, 177–184. https://doi.org/10.1016/j.fm.2015.08.001.

Chen, L. S., Ma, Y., Maubois, J. L., Chen, L. J., Liu, Q. H., & Guo, J. P. (2010). Identification of yeasts from raw milk and selection for some specific antioxidant properties. *International Journal of Dairy Technology, 63*, 47–54. https://doi.org/10.1111/j.1471-0307.2009.00548.x.

Comitini, F., De Ingeniis, J., Pepe, L., Mannazzu, I., & Ciani, M. (2004). *Pichia anomala* and *Kluyveromyces wickerhamii* killer toxins as new tools against *Dekkera/Brettanomyces* spoilage yeasts. *FEMS Microbiology Letters, 238*(1), 235–240. https://doi.org/10.1111/j.1574-6968.2004.tb09761.x.

Ebner, S., Smug, L. N., Kneifel, W., Salminen, S. J., & Sanders, M. E. (2014). Probiotics in dietary guidelines and clinical recommendations outside the European Union. *World Journal of Gastroenterology, 20*(43), 16095. https://doi.org/10.3748/wjg.v20.i43.16095.

Fadda, M. E., Mossa, V., Deplano, M., Pisano, M. B., & Cosentino, S. (2017). In vitro screening of *Kluyveromyces* strains isolated from Fiore Sardo cheese for potential use as probiotics. *LWT- Food Science and Technology, 75*, 100–106. https://doi.org/10.1016/j.lwt.2016.08.020.

FAO/WHO Working Group (2014). *Guidelines for the evaluation of probiotics in food: Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food*London, Ontario, Canada: FAO/WHO.

Gallardo, G., Ruiz-Moyano, S., Hernández, A., Benito, M. J., Córdoba, M. G., Pérez-Nevado, F., et al. (2014). Application of ISSR-PCR for rapid strain typing of *Debaryomyces hansenii* isolated from dry-cured Iberian ham. *Food Microbiology, 42*, 205–211. https://doi.org/10.1016/j.fm.2014.03.022.

Gil-Rodríguez, A. M., Carrascosa, A. V., & Requena, T. (2015). Yeasts in foods and beverages: In vitro characterisation of probiotic traits. *LWT- Food Science and Technology, 64*(2), 1156–1162. https://doi.org/10.1016/j.lwt.2015.07.042.

Gonçalves Dos Santos, M. T. P., Benito, M. J., Córdoba, M. D. G., Alvarenga, N., & Ruiz-Moyano Seco de Herrera, S. (2017). Yeast community in traditional Portuguese Serpa cheese by culture-dependent and -independent DNA approaches. *International Journal of Food Microbiology, 262*, 63–70. https://doi.org/10.1016/j.ijfoodmicro.2017.09.013.

Gunge, N., Tamaru, A., Ozawa, F., & Sakaguchi, K. (1981). Isolation and characterization of linear deoxyribonucleic acid plasmids from *Kluyveromyces lactis* and the plasmid-associated killer character. *Journal of Bacteriology, 145*, 382–390.

Kumura, H., Tanoue, Y., Tsukahara, M., Tanaka, T., & Shimazaki, K. (2004). Screening of dairy yeast strains for probiotic applications. *Journal of Dairy Science, 87*(12), 4050–4056. https://doi.org/10.3168/jds.S0022-0302(04)73546-8.

Maccaferri, S., Klinder, A., Brigidi, P., Cavina, P., & Costabile, A. (2012). Potential probiotic *Kluyveromyces marxianus* B0399 modulates the immune response in Caco-2 cells and peripheral blood mononuclear cells and impacts the human gut microbiota in an in vitro colonic model system. *Applied and Environmental Microbiology, 78*(4), 956–964. https://doi.org/10.1128/AEM.06385-11.

Magliani, W., Conti, S., Gerloni, M., Bertolotti, D., & Polonelli, L. (1997). Yeast killer systems. *Clinical Microbiology Reviews, 10*(3), 369–400. https://doi.org/10.1128/cmr.10.3.369-400.1997.

Mahmoud, M. A., Ali, H. M., El-Aziz, A. R. M., Al-Othman, M. R., & Al-Wadai, A. S. (2014). Molecular characterization of aflatoxigenic and non-aflatoxigenic *Aspergillus flavus* isolates collected from corn grains. *Genetics and Molecular Research, 13*, 9352–9370. https://doi.org/10.4238/2014.November.11.2.

Ordiales, E., Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J., & Córdoba, M. G. (2013). Bacterial communities of the traditional raw Ewe's milk cheese "Torta del Casar" made without the addition of a starter. *Food Control, 33*, 448–454. https://doi.org/10.1016/j.foodcont.2013.03.027.

Pedersen, L. L., Owusu-Kwarteng, J., Thorsen, L., & Jespersen, L. (2012). Biodiversity and probiotic potential of yeasts isolated from Fura, a West African spontaneously fermented cereal. *International Journal of Food Microbiology, 159*(2), 144–151. https://doi.org/10.1016/j.ijfoodmicro.2012.08.016.

Pérez-Nevado, F., Albergaria, H., Hogg, T., & Girio, F. (2006). Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology, 108*(3), 336–345. https://doi.org/10.1016/j.ijfoodmicro.2005.12.012.

Psomas, E. I., Fletouris, D. J., Litopoulou-Tzanetaki, E., & Tzanetakis, N. (2003). Assimilation of cholesterol by yeast strains isolated from infant feces and Feta cheese. *Journal of Dairy Science, 86*(11), 3416–3422. https://doi.org/10.3168/jds.S0022-0302(03)73945-9.

Ruiz-Moyano, S., Martín, A., Benito, M. J., Nevado, F. P., & Córdoba, M. G. (2008). Screening of lactic acid bacteria and bifidobacteria for potential probiotic use in Iberian dry fermented sausages. *Meat Science, 80*(3), 715–721. https://doi.org/10.1016/j.meatsci.2008.03.011.

Saber, A., Alipour, B., Faghfoori, Z., & Khosroushahi, A. Y. (2017). Secretion metabolites of dairy *Kluyveromyces marxianus* AS41 isolated as probiotic, induces apoptosis in different human cancer cell lines and exhibit anti-pathogenic effects. *Journal of Functional Foods, 34*, 408–421. https://doi.org/10.1016/j.jff.2017.05.007.

Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry, 40*(6), 945–948. https://doi.org/10.1021/jf00018a005.

Suscovic, J., Brkic, B., Matosic, S., & Maric, V. (1997). *Lactobacillus acidophilus* M92 as potential probiotic strain. *Milchwissenschaft, 52*, 430–435.

Tofalo, R., Perpetuini, G., Schirone, M., Suzzi, G., & Corsetti, A. (2013). Yeast biota associated to naturally fermented table olives from different Italian cultivars. *International Journal of Food Microbiology, 161*(3), 203–208. https://doi.org/10.1016/j.ijfoodmicro.2012.12.011.

Van der Aa Kühle, A., Skovgaard, K., & Jespersen, L. (2005). In vitro screening of probiotic properties of *Saccharomyces cerevisiae* var. *boulardii* and food-borne *Saccharomyces cerevisiae* strains. *International Journal of Food Microbiology, 101*(1), 29–39. https://doi.org/10.1016/j.ijfoodmicro.2004.10.039.

Živković, M., Čadež, N., Uroić, K., Miljković, M., Tolinački, M., Doušova, P., ... Golić, N. (2015). Evaluation of probiotic potential of yeasts isolated from traditional cheeses manufactured in Serbia and Croatia. *Journal of Intercultural Ethnopharmacology, 4*(1), 12–18. https://doi.org/10.5455/jice.20141128051842.

CONCLUSIONES

Conclusiones/Conclusions.

1. Los resultados obtenidos mediante secuenciación masiva (HTS) mostraron que las bacterias ácido-lácticas son los microorganismos predominantes a lo largo del proceso de maduración de los quesos de pasta blanda tipo "torta". El género *Leuconostoc* representó aproximadamente del 44 al 92% de la microbiota del queso en las industrias pertenecientes a la DOP "Queso de la Serena", mientras que el género *Lactobacillus* fue el mayoritario en las industrias de la DOP "Torta del Casar" con 58-94% del total de lecturas. Esta tecnología también permitió conocer la presencia de otros géneros pertenecientes a la microbiota secundaria, como *Psychrobacter*, *Serratia*, *Pseudomonas*, *Brochothrix*, *Staphylococcus* y *Hafnia*. Cabe destacar que no se detectaron secuencias asignadas a los principales patógenos alimentarios como *E. coli* enterohemorrágica O157:H7, *L. monocytogenes* o *Salmonella* spp.

The results obtained by High-Throughput Sequencing (HTS) showed that lactic acid bacteria are the predominant microorganisms throughout the ripening process of "torta-type" soft cheeses. The genus *Leuconostoc* represented approximately 44 to 92% of the cheese microbiota in the industries belonging to the PDO "Queso de la Serena", while the genus *Lactobacillus* was the majority in the industries of the PDO "Torta del Casar" with 58 -94% of total reads. This technology also allowed us to know the presence of other genera belonging to the secondary microbiota, such as *Psychrobacter*, *Serratia*, *Pseudomonas*, *Brochothrix*, *Staphylococcus* and *Hafnia*. It should be noted that sequences assigned to the main food pathogens such as enterohemorrhagic *E. coli* O157: H7, *L. monocytogenes* or *Salmonella* spp. were not detected.

2. Con respecto a la micobiota, los resultados obtenidos con la secuenciación masiva han revelado una alta diversidad de levaduras en todos los tiempos de maduración, siendo los géneros *Yarrowia* spp., *Kluyveromyces* spp., *Geotrichum* spp., *Pichia* spp., *Debaryomyces* spp., y *Rhodotorula* spp. los más comunes. Las especies mayoritarias al final del proceso de maduración han sido identificadas como *Yarrowia alimentaria* (39-83%), *Kluyveromyces lactis* (5-27%), *Pichia fermentans* (3-14%) y *Yarrowia lipolytica* (1-15%). Otros géneros identificados como

minoritarios fueron *Candida*, *Cystobasidium*, *Torulaspora*, *Meyerozyma*, *Cyberlindnera*, *Aerobasidium* y *Penicillium.*

Regarding the mycobiota, the results obtained by High-Throughput Sequencing (HTS) revealed a high diversity of yeasts species at all ripening stages, being *Yarrowia* spp., *Kluyveromyces* spp., *Geotrichum* spp., *Pichia* spp., *Debaryomyces* spp. and *Rhodotorula* spp. the most common genera. The majority species at the end of the ripening process have been identified as *Yarrowia alimentaria* (39-83%), *Kluyveromyces lactis* (5-27%), *Pichia fermentans* (3-14%) and *Yarrowia lipolytica* (1-15%). Other genera such as *Candida*, *Cystobasidium*, *Torulaspora*, *Meyerozyma*, *Cyberlindnera*, *Aerobasidium* and *Penicillium* were identified as minority.

3. El análisis de cultivo dependiente reveló que, de forma general, los recuentos de enterobacterias durante el proceso de maduración de los quesos fueron disminuyendo a niveles de 5-6 log ufc/g de queso. El análisis de RAPD-PCR reveló una amplia diversidad de especies de enterobacterias a lo largo de la maduración, siendo las especies *Hafnia alvei* y *Hafnia paralvei* las mayoritarias en casi todas las industrias al final del proceso de maduración. Considerando su prevalencia al final del proceso de maduración, se aislaron e identificaron cepas de *Hafnia* spp., a las cuales se les realizaron diversas pruebas para comprobar su seguridad como es la resistencia a antibióticos, la presencia de genes de resistencia a antibióticos, capacidad de producción de aminas biógenas y su capacidad hemolítica y citotóxica. De las 55 cepas aisladas, un total de 22 cepas (8 *H. alvei* y 14 *H. paralvei*) fueron seleccionadas para evaluar su capacidad tecnológica para su posible uso como cultivo iniciador. Los parámetros de proteólisis y producción de gas fueron determinantes, al igual que la evaluación de la capacidad de crecimiento a diferentes condiciones de pH, % NaCl y temperatura, debido a las diferentes condiciones de estrés encontradas durante el proceso de maduración. Las *cepas H. alvei* 544, *H. alvei* 970, *H. alvei* 1142 y *H. paralvei* 1414 mostraron propiedades tecnológicas adecuadas para su posible utilización como cultivo iniciador en quesos de pasta blanda.

The culture-dependent analysis revealed that, in general, the *Enterobacteriaceae* counts during the cheese maturation process decreased to levels of 5-6 log cfu/g of cheese. The RAPD-PCR analysis revealed a wide diversity of *Enterobacteriaceae* species throughout maturation, with the *Hafnia alvei* and *Hafnia paralvei* species being the majority in almost all industries at the end of the maturation process. Considering their prevalence at the end of the maturation process, strains of *Hafnia* spp. were isolated and identified, to which various tests were performed to verify their safety such as antibiotic resistance, the presence of antibiotic resistance genes, ability to production of biogenic amines and their hemolytic and cytotoxic capacity. Of the 55 isolated strains, a total of 22 strains (8 *H. alvei* and 14 *H. paralvei*) were selected to evaluate their technological capacity for possible use as a starter culture. The proteolysis and gas production parameters were decisive, as was the evaluation of the growth capacity at different conditions of pH, % NaCl and temperature, due to the different stress conditions found during the maturation process. The strains *H. alvei* 544, *H. alvei* 970, *H. alvei* 1142 and *H. paralvei* 1414 showed suitable technological properties for their possible use as a starter culture in soft cheeses.

4. En los quesos modelo tipo “torta” inoculados con cepas de *Hafnia* spp., se observó que todas las cepas seleccionadas se implantaron de forma correcta en la matriz del queso a los 28 días de maduración. Los quesos inoculados con las cepas, *Hafnia paralvei* 593 y *Hafnia alvei* 1037 mostraron una textura instrumental más blanda que el resto de los lotes, mientras que el lote inoculado con la cepa *Hafnia alvei* 544 mostró valores más elevados que el lote Control. El análisis sensorial solo reveló diferencias significativas en el parámetro de firmeza entre los lotes inoculados y los lotes control, aunque los valores obtenidos en este análisis fueron mayores en los lotes inoculados con las enterobacterias. En base a la relación entre los parámetros sensoriales (intensidad y persistencia), la firmeza sensorial e instrumental y los parámetros físico-químicos, cuatro de las cinco cepas de *Hafnia* spp., *Hafnia alvei* 544, *Hafnia paralvei* 593, *Hafnia alvei* 1037 y *Hafnia alvei* 1142, mostraron idoneidad de formar parte de un cultivo iniciador para elaborar quesos de pasta blanda con leche cruda de oveja.

In the "torta" type model cheeses inoculated with *Hafnia* spp. strains, it was observed that all the selected strains were correctly implanted in the cheese matrix

at 28 days of maturation. The cheeses inoculated with the strains, *Hafnia paralvei* 593 and *Hafnia alvei* 1037 showed a softer instrumental texture than the rest of the batches, while the batch inoculated with the *Hafnia alvei* 544 strain showed higher values than the Control batches. The sensory analysis only revealed significant differences in the firmness parameter between the inoculated batches and the control batches, although the values obtained in this analysis were higher in the batches inoculated with *Enterobacteriaceae*. Based on the relationship between the sensory parameters (intensity and persistence), the sensory and instrumental firmness and the physical-chemical parameters, four of the five strains of *Hafnia* spp., *Hafnia alvei* 544, *Hafnia paralvei* 593, *Hafnia alvei* 1037 and *Hafnia alvei* 1142, showed a great suitability of being part of a starter culture to produce soft cheeses with raw sheep's milk.

5. El estudio sobre la micobiota de los quesos tradicionales reveló la existencia de una alta diversidad de especies de levaduras involucradas en el proceso de maduración de quesos elaborados con leche cruda de oveja pertenecientes a las DOP "Torta del Casar" y "Queso de la Serena". De los 157 aislados seleccionados en base a su perfil genético, 87 de ellos mostraron una buena adecuación a las condiciones de estrés encontradas en el proceso de maduración de los quesos. Su caracterización tecnológica evidenció que 9 cepas, *K. lactis* 2287, 2725 y 1507, *P. jadinii* 1731 y 433; *Y. alimentaria* 1204 y 2150; *Y. lipolytica* 2495; y *P. kudriavzevii* 373, no producían pigmentación en los quesos y presentaban adecuadas reacciones bioquímicas para su potencial uso como parte de un cultivo iniciador solas o en combinación con microorganismos autóctonos en el proceso de elaboración del queso para disminuir la heterogeneidad de estos quesos tradicionales conservando sus características sensoriales únicas. No obstante, antes de su aplicación industrial sería necesario realizar más estudios en quesos modelo para conocer su contribución en la proteólisis, lipólisis y producción de compuestos que estén implicados en la textura, perfil aromático y calidad final del producto.

The study of the mycobiota of traditional cheeses revealed that a high diversity of yeasts species is involved in the ripening of soft raw ewe's milk PDO cheeses 'Torta del Casar' and 'Queso de la Serena'. Among the 157 isolates selected based on their genetic profile, 87 showed good adaptation to the stress conditions of cheese

ripening process. Their technological characterization evidenced 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 great biochemical features for their potential use as adjunct cultures alone or in combination with autochthonous starter bacteria in 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 system to know their contribution to an adequate balanced of proteolysis, lipolysis and other reactions products that have a great influence on the texture, aromatic profile and general quality of the final product.

6. Con respecto a los quesos modelo inoculados con las nueve levaduras seleccionadas, todos los lotes mostraron un incremento de los recuentos de levaduras a los 15 días de maduración. Los quesos inoculados con *Pichia kudriavzevii* L373 y *Yarrowia lipolytica* L2495 presentaron una textura instrumental más blanda que el resto de los lotes. Los resultados de análisis sensorial mostraron que los quesos inoculados con levaduras presentaban valores más elevados en los parámetros de color de la pasta, intensidad y persistencia del aroma. En base a los resultados obtenidos tanto en el análisis sensorial (intensidad y persistencia del aroma) y en la textura instrumental y sensorial, tres cepas: *Y. alimentaria* L2150, *Y. lipolytica* L2495 y *K. lactis* L1507 mostraron ser las mejores candidatas para formar parte de un cultivo iniciador para su uso en la elaboración de quesos de pasta blanda tipo “torta”.

Regarding the model cheeses inoculated with the nine selected yeast strains, all the batches showed an increase in the yeast counts at 15 days of maturation. Two of the nine strains inoculated in the experimental cheeses, *Pichia kudriavzevii* L373 and *Yarrowia lipolytica* L2495 allowed to obtain a significantly softer texture than the rest of the batches made. In terms of sensory parameters, mean values in the batches with yeasts compared to the control were in general higher in color of the paste, intensity and persistence of the aroma and lower in firmness and global valuation. Based on their relationship with the sensory parameters of intensity and persistence and with sensory and instrumental firmness, although more studies are needed,

three yeasts, *Y. alimentaria* L2150, *Y. lipolytica* L2495 and *K. lactis* L1507 showed to be the best candidates to be part of a mixed starter culture to produce soft cheeses from sheep's milk.

7. Tras realizar los ensayos *in vitro* para evaluar las características probióticas de 54 levaduras autóctonas aisladas, todas presentaron una adecuada tolerancia a las condiciones más restrictivas del estómago y las sales biliares. Sin embargo, las especies *Kluyveromyces lactis* y *Pichia fermentans* mostraron una mejor capacidad de sobrevivir a esas condiciones. *P. fermentans* fue, además, la especie que mostró mayor capacidad antioxidante, de autoagregación e hidrofobicidad y actividad antimicrobiana, seguida de las cepas pertenecientes al género *Kluyveromyces* spp. Las propiedades probióticas mostraron ser dependientes de cepa, siendo seleccionadas en base a las características estudiadas quince (15) cepas: *P. fermentans* 1826, 1865, 1859, 1911, 1913, 1916 y 1938; *P. kudriavzevii* 1801, 1809 y 1832; *K. marxianus* 1886, 1917 y 1919; *D. hansenii* 1860; y *Y. lipolytica* 1961. Estas cepas han mostrado propiedades probióticas que, de usarse dentro de un cultivo iniciador, podrían aportar propiedades funcionales al queso tipo "torta".

After conducting *in vitro* tests to evaluate the probiotic characteristics of 54 isolated autochthonous yeasts, all of them showed adequate tolerance to the most restrictive stomach and bile salts conditions, however, strains of *K. lactis* and *P. fermentans* presented better ability to survive to these stresses. *P. fermentans* was also the species that showed better antioxidant, auto-aggregation and hydrophobicity capacity, at the same time as antimicrobial activity, followed by *Kluyveromyces* spp. strains. Probiotics properties have showed to be strain-dependent, thus, considering the characteristics studied, 15 strains, *P. fermentans* 1826, 1865, 1859, 1911, 1913, 1916 and 1938; *P. kudriavzevii* 1801, 1809 and 1832; *K. marxianus* 1886, 1917 and 1919; *D. hansenii* 1860; and *Y. lipolytica* 1961, are promising probiotic candidates for developing a cheese with probiotic characteristics.