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CARACTERIZACIÓN DE LA MICROBIOTA DE QUESO SERPA Y SELECCIÓN DE CEPAS NATIVAS CON APTITUD PROBIÓTICA

MARIA TERESA PEREIRA GONÇALVES DOS SANTOS

PROGRAMA DE DOCTORADO EN CIENCIA DE LOS ALIMENTOS

(R013)

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COMFORMIDAD DE LOS DIRECTORES

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2020

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A mis, Zés e João

Abstract

Research around Serpa cheese, a traditional Portuguese cheese registered as a Protected Designation of Origin (PDO), has focused essentially on its physicochemical, sensory and technological characteristics, but the study on its microbiota and specific benefits that can be achieved with this knowledge is limited. Thus, this study aims to characterize the indigenous microbial community of this cheese from a quantitative and qualitative point of view through innovative technologies, as well as to evaluate the bioactive potential of representative indigenous strains.

With the acquired knowledge it is intended to contribute to the improvement of the quality and safety of this cheese, in order to consolidate its position in the market and, ultimately, for the preservation of a product that is part of the Portuguese gastronomic and socio-cultural heritage. Additionally, the foundations for innovative proposals can be found, which allow the profitability of the raw material and the diversification of supply.

To achieve the objectives, a representative sample obtained from three certified Serpa cheese industries (G, C, A) and two non-certified cheese industries (B, V) was selected. Sampling was performed at the end of the ripening process (30 days), from two batches per season (winter and spring) in each of the PDO producers and, equally, but only in winter in non-PDO industries. In each sample unit a physicochemical (pH, a_w, moisture) and quantitative microbiological characterization were performed.

The characterization of the microbial community under culture dependent methodology was carried out in bacteria by 16S rRNA gene sequencing analysis and by PCR-RFLP analysis followed by 26S rRNA sequencing for fungi isolates. In parallel, microbiological characterization was carried out under the culture independent approach. For such, the extracted DNA from each cheese unit was used for the characterization of the microbial community by high-throughput sequencing - HTS (Illumina® MiSeq® sequencer) of the 16S rRNA gene in the case of bacteria and of the internal transcribed spacers - ITS rRNA gene in the case of yeasts.

In a third phase of the study, 116 identified lactic acid bacteria (LAB) isolated from Serpa cheese were selected to be evaluated for probiotic potential with respect to tolerance to the gastrointestinal tract conditions (tolerance to low pH and to bile salt, survival on

complete gastrointestinal transit), ability to colonize the intestine (aggregation capacity, cell surface hydrophobicity), safety assays (antibiotic susceptibility, biogenic amine production), growth on prebiotic and short chain fatty acid production.

Overall, the results obtained in the physicochemical characterization of the sample from the PDO-industries agreed with the findings of other studies on Serpa cheese. The cheese samples presented pH values between 4,95-5,49, a_w from 0,90 to 0,98, while moisture content ranged from 45,02 to 48,76% for the PDO industries and 39,10-51,90% for non-PDO industries. Significant differences in moisture and/or a_w were observed among non-PDO industries and between these and other industries. Considering the physicochemical characterization the sample used appeared to be representative and adequate given the objectives of this work.

Through quantitative microbiological characterization the total amount of mesophilic bacteria at the end of ripening is on average 8,5 log cfu/g. LAB predominate, with some significant differences among cheeses from different industries and seasons. Among the microbial groups present in all sample units, presumed lactobacilli ranged from 7,33 to 9,54 log cfu/g, followed by enterococci (6,32 to 7,65), enterobacteria (5,20 to 7,28), yeasts (4,24−5,81) and *E. coli* (0,86-3,93). *Leuconostoc* spp and staphylococci were isolated from all spring units (7,01 to 7,82 and 2,18 to 6,01, respectively), but have a shifting presence in winter cheeses units (not detected to 7,96 or 3,04, respectively). *Leuconostoc* spp. presented the largest quantitative variation among the industries.

As regards bacterial characterization, qualitative results confirmed LAB as the main microbial group followed by enterobacteria. Data obtained following culture-dependent methods identify *Lactobacillus paracasei/Lactobacillus casei* as the main species in cheese from PDO registered industries, whereas in non-PDO registered industries *Lb. brevis* was highlighted, among other LAB. Other LAB identified were *Lb. plantarum, Lb. brevis, Lb.pentosus, Lb. curvatus, Leuconostoc mesenteroides, E. faecalis, E. faecium and E. hirae. Enterobacteriaceae* were represented essentially by *Hafnia alvei.* The genus *Staphylococcus*, isolated only in spring, was discriminated as *S.epidermidis, S. warneri* and *S. cohnii*.

However, the results obtained by high-throughput sequencing reveal the *Lactococcus* genus contributing to approximately 40% to 60% of the population, followed by the

Leuconostoc and *Lactobacillus* genres. This technology allows for a more detailed community assessment also identifying the presence of less abundant (0-24%) genera as *Streptococcus* and *Brochothrix* and trace genres (0-<5%), like *Pseudomonas*, *Brochothrix*, *Macrococcus*, and *Carnobacterium*.

It is noteworthy that although high levels of enterobacteria were found and *E. coli* was quantified in all units, the main bacterial pathogens of food origin, *L. monocytogenes*, *Salmonella* spp. and enterohemorrhagic *E. coli* were not detected in cheese samples at the end of maturation, which guarantee the microbiological safety of the final product.

The yeast species identified mainly corresponded to *Debaryomyces hansen*ii and *Kluyveromyces marxianus*, with *Candida spp*. and *Pichia spp*. present to a lesser extent. The culture-independent results confirmed those prevalences, but add *Galactomyces spp*. with a relevant presence in three producers. The other species are minor contributors and include *Cryptococcus oeirensis*; *Yarrowia lipolytica*; *Cyberlindnera jadinii*; *Moniliella suaveolens*; *Magnusiomyces capitatus*. Three other non-dominant genera detected by HTS were, *Metschnikowia, Saccharomyces* and *Hanseniaspora*.

The differences between microbial communities from PDO and non-PDO registered industries, either with fungi or bacteria, suggest that the lack of regulation of the cheese-making practices may unfavorably influence cheese quality and safety.

Considering the probiotic characteristics studied three potential probiotic strains (PPS) namely, *Lb. brevis* C1Lb21, *Lb. plantarum* G1Lb5 and *Lb. pentosus* G4Lb7 were selected as they were safe, showed good tolerance to stress conditions found in the gastrointestinal tract (GIT) and the ability to colonize the intestine. Especially *Lb. plantarum* G1Lb5 and *Lb. pentosus* G4Lb7 revealed the production capacity of short-chain fatty acids (SCFA) and lactic acid, respectively, by lactulose fermentation, which are health beneficial compounds. Following further validation by *in-vitro* and *in-vivo* investigations, these PPS are potential candidates along with lactulose, for use as an inoculum in the production of a functional probiotic cheese, analogous to Serpa cheese. These strains also have the advantage of being native strains well adapted to the technological process of producing this type of cheese.

Resumen

La presente tesis estudia el queso Serpa, un queso portugués tradicional con Denominación de Origen Protegida (DOP). La investigación sobre este tipo de queso se ha centrado básicamente en el estudio de sus características fisicoquímicas, sensoriales y tecnológicas, pero poco se conoce sobre su microbiota y los beneficios específicos que produce. Por tanto, el objetivo principal de este estudio es caracterizar la flora microbiana autóctona del queso Serpa desde un punto de vista cuantitativo y cualitativo, a través de tecnologías innovadoras, así como evaluar el potencial bioactivo de la flora autóctona más representativa en este tipo de queso.

Con los conocimientos adquiridos, se pretende contribuir a la mejora de la calidad y seguridad de este tipo de queso, a fin de consolidar su posición en el mercado y, en última instancia, para la preservación de un producto que forma parte del patrimonio cultural y gastronómico portugués. Además, se pueden encontrar las bases para nuevas propuestas innovadoras, que permitan la rentabilidad de la materia prima y la diversificación de la oferta del queso Serpa.

Para lograr los objetivos planteados, se trabajó con muestras representativas obtenidas de tres industrias de queso Serpa certificadas (G, C, A) y dos industrias de queso no certificadas (B, V). El muestreo se realizó al final del proceso de maduración (30 días), en lotes de diferentes epocas del año (invierno y primavera) para cada una de las industrias con DOP e, igualmente, pero solo en invierno, en los productores sin DOP. A cada una de las muestras se les realizó una caracterización fisicoquímica (pH, a_w, humedad) y microbiológica.

La caracterización de la flora microbiana aislada se llevó a cabo, para bactérias, mediante análisis de secuenciación del gen 16S rRNA y, para levaduras, mediante análisis de PCR-RFLP seguido de secuenciación del gen 26S rRNA. Paralelamente, la caracterización microbiológica se realizó bajo un enfoque independiente de cultivo, mediante secuenciación masiva. Para ello, el ADN extraído de cada muestra de queso se utilizó para la caracterización de la comunidad microbiana mediante secuenciación de alto rendimiento - HTS (secuenciador Illumina® MiSeq®) del gen 16S rRNA en el caso de bactérias y de los espaciadores ITS rRNA, en el caso de las levaduras.

En una tercera fase del estudio, se seleccionaron 116 bacterias ácido lácticas (BAL) aisladas del queso Serpa, previamente identificadas, para evaluar su potencial probiótico con respecto a la tolerancia a las condiciones del tracto gastrointestinal (tolerancia al pH bajo y a la sal biliar, supervivencia al tránsito gastrointestinal), capacidad de colonizar el intestino (capacidad de agregación, hidrofobicidad de la superficie celular), ensayos de seguridad (susceptibilidad a antibióticos, producción de aminas biogénicas), crecimiento en la producción de ácidos grasos prebióticos y de cadena corta.

En general, los resultados obtenidos en la caracterización fisicoquímica de las muestras de las industrias con DOP coincidieron con los hallazgos de otros estudios sobre el queso Serpa. Las muestras de queso presentaron valores de pH entre 4,95-5,49, a_w de 0,90 a 0,98, mientras que el contenido de humedad varió de 45,02 a 48,76% para las industrias con DOP y 39,10-51,90 % para industrias no DOP. Se observaron diferencias significativas en la humedad y/o a_w entre las industrias sin DOP, y entre estas y las otras industrias. En base a la caracterización fisicoquímica, las muestras estudiadas fueron representativas y adecuadas para los objetivos de este trabajo.

Segun los resultados de los recuentos microbianos se observó que el nivel de bacterias aerobias mesofílicas al final de la maduración fue de aproximadamente 8,5 log ufc/g. Se apreción que predominaban las BAL, con diferencias significativas entre los quesos de diferentes industrias y estaciones del año. Entre los grupos microbianos presentes en todos los lotes estudiados se comprobó que los lactobacilos variaron de 7,33 a 9,54 log ufc/g, seguidos de los enterococos (6,32 a 7,65 log ufc/g), enterobacterias (5,20 a 7,28 log ufc/g), levaduras (4,24−5,81 log ufc/g) y *Echerichia coli* (0,86-3,93 log ufc/g). Por su parte, *Leuconostoc* spp y estafilococos se aislaron de todas las unidades de primavera en niveles que oscilaron entre 7,01 a 7,82 log ufc/g y 2,18 a 6,01 log ufc/g, respectivamente, aunque mostraron presencia variable en las unidades de quesos de invierno (no detectado a 7,96 o 3,04 log ufc/g, respectivamente). *Leuconostoc* spp. presentó la mayor variación cuantitativa entre las industrias.

En cuanto a la caracterización bacteriana mediante técnicas de biologia molecular de las cepas aisladas de los recuentos microbianos, confirmaron que las BAL eran el principal grupo microbiano seguido de las enterobacterias. La especie mayoritaria en quesos de industrias registradas con DOP fue *Lactobacillus paracasei*/*casei*, mientras que en industrias sin DOP destacó *Lb. brevis*, entre otras BAL. Así mismo, otras BAL

identificadas fueron *Lb. plantarum, Lb.pentosus, Lb. curvatus, Leuconostoc mesenteroides, Enterococcus faecalis, E. faecium* y *E. hirae*. Dentro de la familia de la enterobacterias, las cepas aislas se identificaron esencialmente como *Hafnia alvei*. El género S*taphylococcus,* aislado solo en primavera, fue identificado como *S.epidermidis, S. warneri* y *S. cohnii*

Sin embargo, los resultados obtenidos por secuenciación masiva revelan que el género *Lactococcus* contribuye a aproximadamente el 40% al 60% de la población, seguido por los géneros *Leuconostoc* y *Lactobacillus*. Esta tecnología permite una evaluación comunitaria más detallada que también identifica la presencia de géneros menos abundantes (0-24%) como *Streptococcus* y *Brochothrix* y trazas de géneros (0-<5%), como *Pseudomonas*, *Brochothrix*, *Macrococcus* y *Carnobacterium*.

Es importante destacar que, aunque se encontraron elevados recuentos de enterobacterias y de *E. coli* en todas las muestras analizadas, los principales patógenos bacterianos de origen alimentario como, *L. monocytogenes, Salmonella spp*. y *E. coli* enterohemorrágica no se detectaron en los quesos al final de la maduración, lo que garantiza la seguridad microbiológica del producto.

Las especies de levaduras identificadas correspondieron principalmente a *Debaryomyces hansenii* y *Kluyveromyces marxianus*, seguidas de *Candida* spp. y *Pichia* spp. Por su parte, los resultados de secuenciación masiva confirmaron la prevalencia de estas especies, pero además identificaron *Galactomyces* spp. con presencia relevante en tres productores. Otras especies encontradas fueron *Cryptococcus oeirensis*; *Yarrowia lipolytica*; *Cyberlindnera jadinii*; *Moniliella suaveolens*; *Magnusiomyces capitatus*. Así mismo, los generos *Metschnikowia*, *Saccharomyces* y *Hanseniaspora* fueron detectados mediante HTS como minoritarios.

Las diferencias entre las comunidades microbianas de las industrias con DOP y sin DOP, ya sean levaduras o bacterias, sugieren que la falta de regulación en las prácticas de elaboración del queso Serpa puede influir desfavorablemente en la calidad y seguridad del queso.

En base al estudio de sus características probióticas, se seleccionaron tres cepas potencialmente probióticas (CPP), *Lb. brevis* C1Lb21, *Lb. plantarum* G1Lb5 y *Lb.*

pentosus G4Lb7, por sus seguridad, buena tolerancia a las condiciones de estrés del tracto gastrointestinal (GIT) y la capacidad de colonizar el intestino. Especialmente *Lb. plantarum* G1Lb5 y *Lb. pentosus* G4Lb7 revelaron una buena capacidad de producción de ácidos grasos de cadena corta (SCFA) y ácido láctico, respectivamente, por fermentación de lactulosa, que son compuestos beneficiosos para la salud. En este sentido, después de una validación adicional mediante estudios in vitro e in vivo, estos CPP son candidatos potenciales junto con lactulosa, para su uso como inóculo simbiótico en la producción de un queso funcional probiótico, análogo al queso Serpa. Además, estas cepas también tienen la ventaja de ser cepas autóctonas bien adaptadas al proceso tecnológico de producción de este tipo de queso.

TABLES OF CONTENTS

Table of Contents

Abstract ix

Resumen xii

TABLES OF CONTENTS xvi

Table of Contents xviii

List of Figures xxiv

List of Tables xxvi

List of Appendices xxviii

List of abbreviations and acronyms xxix

I – INTRODUCTION 1

I.1. Cheeses with geographical indication schemes in Portugal 3

I.1.1. Portuguese PDO/PGI cheese 3

I.1.2. Portuguese PDO/PGI cheese production data 6

I.2. Serpa cheese 11

I.2.1. Serpa cheese historical review 11

I.2.2. Serpa PDO cheese recognition process 16

I.2.3. Serpa cheese specifications and applicable legal aspects 19

I.2.4. Previous work on the microbiological characterization of Serpa cheese 23

I.2.5. Serpa cheese manufacture 29

I.2.5.1. Milk handling 29

I.2.5.2. Milk coagulation 31

I.2.5.3. Cutting and curd work and whey drainage 31
I.2.5.4. Ripening..... 32
I.3. Raw milk cheese microbiology..... 34
I.3.1. Raw milk microbiota 34
I.3.2. Microbiology of raw milk cheese 36
I.3.2.1. Cheese core..... 37
I.3.2.1.1. Firmicutes 37
I.3.2.1.2. Proteobacteria 39
I.3.2.1.3. Other bacteria..... 40
I.3.2.1.4. Fungi 40
I.3.2.2. Cheese rind 42
I.3.3. Raw milk cheeses microbiological safety..... 43
I.3.4. High throughput sequencing in the study of dairy microbial ecology..... 49
I.4. Functional foods concept..... 59
I.4.1. Concept of functional food and general aspects 59
I.4.2. European regulatory systems governing of functional foods..... 63
I.4.3. Concept of probiotic, probiotic food, dairy probiotics and general aspects..... 65
I.4.4. The use of health claims in probiotic foods – legal issues..... 68
I.4.5. Mechanisms underlying probiotic effects on health 74
I.4.6. Resistance and safety aspects in the selection of probiotic microorganisms..... 81
I.4.6.1. Tolerance to the conditions of the stomach and small intestine 83
I.4.6.2. Adherence to the intestinal mucosa and persistence in the intestinal tract 86
I.4.6.3. Safety Aspects - Origin and identification of strain..... 88

I.4.6.4. Transmission of antibiotic resistance genes and safety 90
I.4.7. Functional probiotics market trends 92
I.5. Cheese potencial as functional food 94
I.5.1. Dairy as functional food 94
I.5.2. Cheese as a matrix for probiotics 97
I.5.3. Cheese as a source of probiotics 103
I.5.4. Most common cheese probiotics 105
I.5.4.1. *Bifidobacterium* genus 106
I.5.4.2. *Lactobacillus* genus 108
I.5.4.4. *Enterococcus* genus 109
I.5.4.5 *Propionibacterium* genus 111
I.5.4.6. Yeasts 113
I.5.5. Cheese as matrix for the synthesis of bioactive compounds 114
I.5.5.1. Organic acids (lactate) 114
I.5.5.2 Bioactive peptides 116
I.5.5.3. Fatty acids 117
I.5.4.4. Gamma-aminobutyric acid (GABA) 119
I.5.6. Cheese as a source of harmful compounds and mitigating factors 120
I.5.6.1. Saturated fatty acids (SFA) 120
I.5.6.2. Biogenic amines (BA) 122
II – OBJETIVES / OBJETIVOS 128
II.1 Justification - The Serpa cheese problem 130
II.2. Partial objetives/objetivos 133

III - MATERIAL AND METHODS 135
III.1. Biological material 137
III.2. Physicochemical analysis 137
III.3. Microbiological analysis 137
III.3.1. Bacterial counts 137
III.3.2. Fungus counts 138
III.3.3. Pathogen detection and confirmation 138
III.3.4. Bacteria identification by DNA sequencing analysis 139
III.3.5. Fungi identification by PCR-RFLP analysis of ITS region and sequencing 139
III.3.6. Identification of bacterial community by HTS of the 16S rRNA gene 140
III.3.7. Identification of yeast community by HTS of the 16S rRNA gene 141
III.4. Selection of autochthonous lactic acid bacteria strains for symbiotic cheese production 142
III.4.1. Latic acid bacteria isolates for study 142
III.4.2. Tolerance to the gastrointestinal tract conditions 142
III.4.2.1. Tolerance to low pH 142
III.4.2.2. Bile salt tolerance 143
III.4.2.3. Survival on complete gastrointestinal transit 143
III.4.3. Capacity to colonise the intestine 143
III.4.3.1. Aggregation capacity 143
III.4.3.2. Cell surface hydrophobicity 144
III.4.4. Safety assays 144
III.4.4.1. Antibiotic susceptibility 144
III.4.4.2. Biogenic amine production 144

III.4.5. Growth on prebiotic 145

III.4.6. Short-chain fatty acid production 145

IV – RESULTS 147

IV.1. Bacterial Communities in Serpa Cheese by Culture Dependent Techniques, 16S rRNA Gene Sequencing and High-throughput Sequencing Analysis 149

IV.2. Yeast community in traditional Portuguese Serpa cheese by culture dependent and independent DNA approaches 159

IV.3. Suitability of autochthonous lactic acid bacteria strains and prebiotics for the development of a symbiotic artisanal soft cheese 168

V - GENERAL DISCUSSION 195

V.1. Sample physicochemical characterization 197

V.2. Serpa cheese microbiota quantitative characterization 197

V.2.1. Bacterial counts 197

V.2.2. Yeast counts 198

V.3. Serpa cheese microbiota qualitative characterization 199

V.3.1. Bacterial microbiota 199

V.3.2. Fungal microbiota 202

V.4. Safety assessment results 205

V.5. Selection of autochthonous lactic acid bacteria strains for symbiotic cheese production . 206

V.5.1. Lactic acid bacteria autochthonous isolates for study 206

V.5.2. Tolerance to the gastrointestinal tract conditions 207

V.5.2.1. Tolerance to low pH 207

V.5.2.2. Bile salt tolerance 209

V.5.2.3. Survival on complete gastrointestinal transit 209
V.5.3. Capacity to colonise the intestine 209
V.5.3.1. Aggregation ability 209
V.5.3.2. Cell surface hydrophobicity 210
V.5.4. Safety assays 211
V.5.4.1. Antibiotic susceptibility 211
V.5.4.2. Biogenic amine production 214
V.5.5. Growth on prebiotic 215
V.5.6. Short-chain fatty acid production 216
VI – CONCLUSIONS / CONCLUSIONES 218
BIBLIOGRAPHY 227
APPENDICES 295

List of Figures

Figure 1 - Portuguese PDO/PGI sheep and/or goat cheeses and production data in 2015, 2016 and 2017 (Sources: DGADR, 2016a, 2017, 2018). 11

Figure 2 - Map of Portugal with location of the Serpa cheese production area (orange), and detail showing the municipalities and parishes that compose it. Also shown, the PDO brand label (Source: DGADR, 2016b; EC, 2014). 17

Figure 3 - Serpa cheese certification labels from the beginning of its recognition as a PDO product, according to the private control and certification body involved. A-ACOS (outdated); B-Certialentejo (outdated); C-Certis (currently in use) (Sources: DR, 1991, 2005). 19

Figure 4 - Flowchart of PDO Serpa cheesemaking procedure. 30

Figure 5 - Overview of microbiological (blue), chemical (purple), and physical (green) hazards (most important in bold) reported in scientific literature for dairy products processed at the dairy farm. Data available at European Food Safety Authority (EFSA), Listeria monocytogenes Raiid Alert System for Food and Feed (RASFF) for data notifications of food safety hazards in milk and milk products within the EU during 2009 to 2014, and the Dutch monitoring program on chemical hazards (2009 to 2013) as well as expert information (Adapted from Asselt et al., 2017). 45

Figure 6 - Proportion of L. monocytogenes positive sampling units in cheeses in 2017 (red) and in 2016 (blue) across all sampling stages (overall), retail and processing plant levels (Source: EFSA/ECDC, 2018). 46

Figure 7 - Frequency distribution of causative agents associated with strong evidence food borne and waterborne outbreaks, by food vehicle. (Milk and milk products include cheese, dairy products other than cheeses and milk) (Source: EFSA/ECDC, 2018). 47

Figure 8 - Summary of potential NGS use by the food industry (Source: Jagadeesan et al., 2019). 55

Figure 9 - Regulation on probiotics in different countries outside the European Union (Sources: Hill et al., 2014; IPA, 2017; WGO, 2017). 72

Figure 10 - Classification of the mechanisms underlying probiotic effects as Widespread, Frequent and Rare as they can be observed, respectively, in several studied probiotic species, or only in some specific species or strains (Source: Hill et al., 2014)..... 82

Figure 11 - Screening approaches used for characterization of potential probiotic strains (PPS) according to WHO/FAO (2006) and alternative screening for beneficial health effects and industrial requirements and the possibility of using OMICS technologies in identification and characterization of strains (Source: Melo Pereira et al., 2018). 84

Figure 12 - Main factors that may affect the viability of probiotic bacteria in cheese (Adapted from Karimi et al., 2011 and de Prisco & Mauriello, 2016). 99

Figure 13 - Role of microorganisms during the cheese production process as precursors of bioactive compounds beneficial to health from milk components. Microorganisms, especially LAB and yeast act as precursors of bioactive compounds such as organic acids, peptides, fatty acids, γ-aminobutyric acid (GABA), exopolysaccharides and vitamins (Adapted from Santiago-López et al., 2018)..... 115

List of Tables

Table 1 - Portuguese cheeses with PDO/PGI by region of production with registration and production data and main characteristics (Sources: DGADR, 2018; EC, 2019). 5

Table 2 - Data on the production and marketing of PDO cheeses, non-PDO sheep cheese and Serpa cheese (Sources: DGADR, 2016a, 2017, 2018; GPP, 2014a, 2014b, s/d-a, s/d-b; IDRHa, 2003; INE, 1998-2018; Oliveira, 2001, 2002, 2005a, 2005b, 2006, 2007, 2014). 9

Table 3 - Specifications for Serpa cheese (Sources: DR, 1987, 1994; Vieira, 1994). 20

Table 4 - Microbiological criteria for raw milk (Source: EC, 2006a). 21

Table 5 - Microbiological safety and hygiene criteria (EC, 2005a) applicable to Serpa cheese. 22

Table 6 – Quantitative microbiological characterization of Serpa cheese laid down in different works. Most represent the characterization of cheese paste, but also of the rind. The results are also presented under different production conditions, such as spring and winter, artisanal and semi industrial and natural and artificial curing system (Sources: Amaral, 1996; Barbosa, 2000; Canada, 2001; Dias, 1998; Roseiro & Barbosa, 1996; Roseiro, Wilbey et al., 2003). 25

Table 7 - Microbiological characterization of milk for Serpa cheese realized in different works. The results are also presented under different production conditions, such as spring and winter, artisanal and semi industrial and manual and mechanical milking (Sources: Canada, 2001; Dias, 1998; Roseiro, Wilbey et al., 2003). 28

Table 8 - Microbial counts and types of microorganisms in cow, goat and sheep milk (Source: Montel et al. (2014) and references therein). 35

Table 9 - Yeast counts (log ufc/g) during cheese ripening period or in the final product and identification of the isolated yeasts in raw ewes milk cheeses whit vegetal renet from the Iberian Peninsula. 41

Table 10 - Characterization of the three generations of sequencing technologies most commonly used in the context of the characterization of microbial profiles (Sources: Bokulich & Mills, 2012; Ercolini, 2017; O'Flaherty & Klaenhammer, 2011; Scholz, Lo, & Chain, 2012). 53

Table 11 - Main classes of functional or bioactive food ingredients, their characterization, the health benefits normally attributed to it and examples of each type of ingredients and the

respective health claim approved under applicable European legislation (EC, 2012a) as it is considered scientifically proven (Source: Abdel-Salam, 2010; EC/DGRI, 2010; Gibson et al., 2017; Martins et al., 2004; Serafini et al., 2012)..... 61

Table 12 - Main reasons given by the EFSA panels for rejecting the proposed health claims on probiotics (Source: EC, 2018)..... 70

Table 13 - Human GIT conditions affecting microbial growth, quantitative and qualitative GIT microbiota, description in eubiosis and diseases associated with dysbiodis (Sources: Binns, 2013; Bäckhed et al., 2012; D'Argenio & Salvatore, 2015; Zoumpopoulou et al., 2017)..... 76

Table 14 - Biological functionality of probiotics and mechanisms of action (Binns, 2013; Bron et al., 2017; D. Granato et al., 2010; Landete et al., 2016; Markowiak & Śliżewska, 2017; Vandenplas et al., 2015; WGO, 2017). 78

Table 15 - The most relevant bacterial and yeast species/subspecies, with potential for use as probiotics in cheese (Source: Castro et al., 2015; Fadda et al., 2017; García-Tejedor, Sánchez-Rivera, Recio, Salom, & Manzanares, 2015; Karimi et al., 2011)..... 106

Table 16 - Culture media and incubation conditions (temperature / time and atmosphere), used in the quantification of different bacteria or bacterial groups within the scope of this work..... 138

Table 17 - Selected isolates of Serpa cheese LAB to study and strains selected following the application of the gastrointestinal tract (GIT) resistance and safety tests. 207

Table 18 - Antibiotic susceptibility and biogenic amines (BA) production of acid-tolerant LAB strains. 213

List of Appendices

Appendix 1 - European and national legislation applicable to the production of Serpa cheese. 299

Appendix 2 - Dairy nutrients, substances or foods for which the authorization request to use the health claim referred, submitted to EFSA under Regulation (EC) Nº 1924/2006 , was not accepted (EC, 2018). 303

Appendix 3 - Health claims authorized for nutrient, substance or food, under Article 13º of the Regulation (EC) Nº 1924/2006 (EC, 2006c), which can be applied to dairy products. Source: (EC, 2012a, 2018; Hess et al., 2016). 309

Appendix 4 - Main biologically active components of milk and dairy products and some of their potential beneficial effects on health fects. 313

Appendix 5 - Results of the bibliographic search performed regarding the use of sheep cheese as a matrix for different probiotics. The type of cheese and the strain (s) tested are given, as well as a summary of the results obtained as regards the viability of the strains concerned in this matrix and the consequences on the characteristics of the cheese. 317

List of abbreviations and acronyms

AAD – Antibiotic Associated Diarrea

ACE – Angiotensin Converting Enzyme

ACOS - Associação de Agricultores do Sul

AP - Alkaline Phosphatase

ATCC - American Type Culture Collection

BA - Biogenic Amines

BC – Before Christ

BSH - Bile Salt Hydrolase Enzymes

C - Cytosine

CAP - Common Agricultural Policy

CB - Control Body

CFU - Colony Forming Units

CLA - Conjugated Linoleic Acid

DGADR - Directorate General for Agriculture and Rural Development

DGGE - Denaturing Gradient Gel Electrophoresis

DNA - Deoxyribonucleic Acid

EC – European Commission

EEC - European Economic Community

EFSA - European Food Safety Authority

ESA – IPBeja - Agrarian School of the Beja Polytechnic Institute (ESA-IPBeja)

EU - European Union

FA - Fatty Acids

FAA - Free Amino Acids

FAO - Food and Agriculture Organization of the United Nations

FBO – Food Borne Outbreaks

FDA – Food Drug Administration

FDF - Functional Dairy Foods

FDM - Fat in dry matter

FF - Functional Food

FIC - Food Information to Consumers

FNFC - Foods with Nutrient Function Claims

FOS - Shorter Fructooligosaccharide

FOSHU - Foods for Specified Health Use

GABA - Gamma-aminobutyric Acid

GI - Geographical Indication

GIT - Gastrointestinal Tract

GRAS - Generally Regarded as Safe

HACCP - Hazard Analysis and Critical Control Point

HCR - Health Claims Regulation

HTS – High Throughput Sequencing

HVR - Hypervariable Regions

IBD - Inflammatory Bowel Disease

IBS - Irritable Bowel Syndrome

ICTF - International Commission on the Taxonomy of Fungi

IMAIAA - Agricultural Markets and Food Industry Institute

INPI - National Institute of Industrial Property

IPA - International Probiotic Association

ISAPP - International Scientific Association for Probiotics and Prebiotics

ITS - Internal Transcribed Spacer

LAB – Lactic Acid Bacteria

LAS - Sensory Analysis Laboratory

LHT – Low Heat Treated

LPS - Lipopolysaccharide

MFFB - Moisture on a fat free basis

MHLY - Ministry of Health, Labour and Welfare

MS - member state

nd - Not done

NDA - Panel on Nutrition, Novel Foods and Food Allergens

NGS - Next Generation Sequencing

NHP – Natural Health Products

NK – Natural Killer

NSLAB - Non-Starter Lactic Acid Bacteria

ONT - Oxford Nanopore Technologies

OUT - Operational Taxonomic Unit

PA – Polyamines

PAH – Polycyclic aromatic Hydrocarbon

PCR - Polymerase Chain Reaction

PDO - Protected Designation of Origin

PGI - Protected Geographical Indication

PPS - Probiotic Potentials Strains

QC - Quality Checking

QPA - Quantitative Descriptive Analysis

QPS - Qualified Presumption of Safety

RAE – Retinol Activity Equivalents

RASFF - Raiid Alert System for Food and Feed

RFLP - Restriction Fragment Length Polymorphism

RNA - Ribonucleic Acid

RTE - Ready to Eat

SCFA – Short Chain Fatty Acids

SEPyP - International Spanish Society of Probiotics and Prebiotics

Seq - Sequencing

SFA - Saturated Fatty Acids

SLAB- Starter Lactic Acid Bacteria

SMS - Single Molecule Sequencing

STEC – Shiga Toxin *Escherichia coli*

TBEV – Tick-borne encephalitis virus

TGGE - Temperature Gradient Gel Electrophoresis

TGS - Third Generation Technologies

TLR – Toll-like receptor

TRFLP - Terminal Restriction Fragment Length Polymorphism

TSG - Traditional Speciality Guaranteed

US FDA - United States Food and Drug Administration

WGS - Whole Genome Sequencing

I – INTRODUCTION

I.1. Cheeses with geographical indication schemes in Portugal

I.1.1. Portuguese PDO/PGI cheese

In recent decades, consumers have been increasingly turning their attention to the quality and origin of food products. To enable consumers to make an informed choice and to protect producers from unfair competition, the European Union (EU) has adopted schemes where quality labels can be awarded to products that represent an important part of the culture, history, identity, heritage and local economy of a region or country, and comprise key elements of the dietary patterns of each country (Dias & Mendes, 2018; Fernández-Ferrín, Calvo-Turrientes, Bande, Artaraz-Miñón, & Galán-Ladero, 2018). Since food products with quality labels are often perceived as products of higher quality and with unique properties, they are among the choices of demanding consumers (Albuquerque, Oliveira, & Costa, 2018; Fernández-Ferrín et al., 2018; Guerrero et al., 2012).

Thus, since 1992 with Regulation (EEC) Nº 2081/92 (ECC, 1992a) the EU has been implementing specific regulation on quality schemes whose application ultimately leads to the official registration of the name of a product under the applicable quality label. The latest regulation, which repeals that and other previous regulations, came into force on 3 January 2013 – Regulation (EU) N° 1151/2012 (EC, 2012b), and promotes four types of quality schemes for agricultural products and foodstuffs. Applications for registration in any of these schemes are managed by producers or producers groups, through the national food authority's communication with the European Commission. The Commission analyses applications, in order to guarantee the right to use the respective label (EC, 2012b, 2014).

According to this regulation, product names can be granted a 'geographical indication' (GI) quality scheme if they have a specific link to the place where they are made and follow a particular traditional production process. The GI includes the quality labels “Protected Designation of Origin” (PDO) and “Protected Geographical Indication” (PGI). PDO covers agricultural products or foodstuffs that are produced, processed and prepared in a specific geographical area, using recognized know-how. PGI covers agricultural products or foodstuffs closely linked to a geographical area; at least one of the stages of

production, processing or preparation occurs in that area, while the raw material used in production can come from another region (EC, 2012b).

Other quality schemes emphasise the traditional production process or its composition, without being linked to a specific geographical area, which allows the use of the quality label “Traditional Speciality Guaranteed” (TSG). The two most recent quality schemes have been introduced only in this latest regulation, and include the application of quality labels to products made in remote natural areas such as mountains (Mountain Products) or islands (Product of Island Farming) (EC, 2012b).

According to the DOOR database (EC, 2019) , Southern European countries present the greatest number of PDO, PGI and TSG registrations. Italy and France stand out, having a long tradition of protecting and promoting products nationally, followed by Spain, Portugal and Greece, respectively. The reasons usually given for these results are, on the side of production, the bureaucratic support for candidates and a greater ease in understanding and implementing the concept of collective quality marks and, for consumers, higher levels of awareness of the EU quality labels, relative to other countries (Albuquerque et al., 2018; Grunert & Aachmann, 2016). It is interesting to note that, in each country, levels of brand awareness are usually higher in the regions where protected products have their origin (Grunert & Aachmann, 2016).

The most used EU quality label is PGI (51,8%), followed by PDO (44,0%) and, with a much lower number of registrations, TSG (4.2%). The fruit, vegetables and cereals (fresh or processed) category is the product type with most registrations (26,9%), followed by cheeses (16,3%) and meat products (cooked, salted, smoked) (12,3%) categories (DOOR database) (EC, 2019) .

According to the same database (EC, 2019), Portugal follows the general trend regarding the type of products registered, with PGI taking up 53,2%, PDO 46.1% and TSG only 0,7%. As regards the type of food products, meat products (smoked, cooked, salted, among others) are the most represented group (29,5%), followed by fresh meat and offal (22,3%). The group of fruits, vegetables and cereals, fresh or processed, take the third place with about 20,1% of the products. Cheeses and other products of animal origin each account for around 8,6% of registrations, bread and pastry products 5,0% and finally spices with about 0,7%.

Table 1 - Portuguese cheeses with PDO/PGI by region of production with registration and production data and main characteristics (Sources: DGADR, 2018; EC, 2019).

CHEESE	Geographical Indication (GI)	Date of Registration	Region of Portugal	Raw Milk	Rennet	Ripening time (DAYS)	Paste Consistency	Distribution of Cheese Production in 2017 (%)	Production Value in 2017 (€)
Azeitão	PDO	21/06/1996	Lisbon (Setúbal peninsula)	Sheep	Vegetable (Cardoon)	20	Semi-soft	12.0	4.353.240
Nisa	PDO	21/06/1996	Alentejo	Sheep (Merina Branca)	Vegetable (Cardoon)	45	Semi-hard	4.0	963.115
Serpa	PDO	21/06/1996	Alentejo	Sheep	Vegetable (Cardoon)	30	Semi-soft	4.0	830.875
Évora	PDO	21/06/1996	Alentejo	Sheep (Merina Branca)	Vegetable (Cardoon)	30-90	Hard or semi-hard	1.2	_
Mestiço de Tolosa	PGI	06/06/2000	Alentejo	Sheep and goat	Animal or Vegetable (Cardoon)	20-30	Semi-soft	0.2	47.670
Serra da Estrela	PDO	21/06/1996	Center	Sheep	Vegetable (Cardoon)	30	Semi-soft (Serra da Estrela) or semi-hard to extra-hard (Serra da Estrela Velho)	6.7	1.837.635
Castelo Branco (Beira Baixa)	PDO	21/06/1996	Center	Sheep	Vegetable (Cardoon)	45-90	Semi hard or semi soft	3.6	788.473
Amarelo da Beira Baixa	PDO	21/06/1996	Center	Sheep and goat	Vegetable (Cardoon)	45	Semi hard or semi soft	6.3	1.281.364
Picante da Beira Baixa	PDO	21/06/1996	Center	Sheep and goat	Vegetable (Cardoon)	120	Semi hard to hard	2.7	589.685
Rabaçal	PDO	21/06/1996	Center	Sheep and goat	Animal	20	Semi-hard to hard consistency	0.4	_
Cabra Transmontano	PDO	02/07/1996	North	Goat (Serrana)	Animal	60	Extra-hard	0.7	_
Terrincho	PDO	21/06/1996	North	Sheep (Churra da Terra Quente)	Vegetable (Cardoon)	30	Semi-hard to hard (Queijo Terrincho Velho)	0.5	129.442
São Jorge	PDO	13/11/1996	Autonomous Region Azores	Cow	Animal	90	Hard or semi-hard	57.6	6.453.830
Pico	PDO	19/06/1998	Autonomous Region Azores	Cow	Animal	17-30	Semi-soft	0	0

In the category of cheeses, the number of registered products in Portugal is 12, one of which (Beira Baixa) includes three types of cheeses (Castelo Branco, Amarelo and Picante). Thus, 14 types of cheese enjoy a quality label (PDO or PGI). Table 1 presents these cheeses with some registration and production data and the main product specifications.

All cheeses are produced with raw milk, although of different kinds. Only one was registered as PGI, the Tolosa Mestiço cheese, made with a mixture of sheep and goat milks, and the others are PDOs. Most PDOs are sheep cheeses, represented by Azeitão, Nisa, Serpa, Évora, Terrincho, Serra da Estrela, and Castelo Branco, from the Beira Baixa cheese group. The other two Beira Baixa, Amarelo and Picante cheeses, are made from the mixture of sheep and goat milk, like Rabaçal. Cabra transmontano is made with goat milk. Finally, those made of cow milk, São Jorge cheese and Pico cheese, the former makes up more than half of the production of PDO/PGI cheese in Portugal, while the latter has stopped production a few years ago (Table 1).

All names of Portuguese PDO cheeses were registered until the late 1990s, between 1996 and 1998; and PGI cheese, as early as 2000 in the current millennium (Table 1). Since then, no more Portuguese cheeses have been registered under these quality schemes. However, new registrations of the PDOs Azeitão and Beira Baixa have recently been requested (April 2019 and February 2018, respectively). These applications are in the DOOR database (EC, 2019) with the status "applied", awaiting resolution.

I.1.2. Portuguese PDO/PGI cheese production data

Quality schemes are multifunctional instruments since, while promoting local values such as environmental stewardship, culture and tradition, should foster rural development, generating significant commercial and economic value even for small businesses (Albuquerque et al., 2018). For rural areas, these schemes are expected to provide part of the physical and conceptual structure for affirming and valuing the unique sociocultural and agro-ecological characteristics of a particular place and are credited with generating significant economic value, especially in certain countries (EC, 2012b; Maye, Kirwan, Schmitt, Keech, & Barjolle, 2016). They also tend to have positive effects in terms of improving the reputation of a region, influencing other products in the region and

fostering tourism (Albuquerque et al., 2018; Alderighi, Bianchi, & Lorenzini, 2016; Pellin, Ribeiro, & Mantovaneli Jr, 2016).

The more established European experiences in GI status production suggest that products add value, increase sales in the markets in which they already operate, and are better placed to enter new markets, that is, they become more competitive (Pellin et al., 2016).

A international study, conducted for the European Commission on the value premium of PDO and PGI products, estimated that for agricultural products and foodstuffs the premium rate was 1.55 (Chever, Renault, Renault, & Romieu, 2012). This does, however, not mean that GI producers' margins are as much higher, since GI producers often face additional costs due to compliance with the GI specification (Chever et al., 2012; Hajdukiewicz, 2014).

Aware of these advantages for rural development, the EU started implementing measures to defend traditional products through the reform of the Common Agricultural Policy (CAP), in the 1980s and with the various editions of the LEADER programme on the second half of the 1990s (Pellin et al., 2016). At the same time, Community quality schemes for agricultural products and foodstuffs were implemented in accordance with Regulation (EEC) Nº 2081/92 (ECC, 1992a), with the proliferation of the registration of PDO and PGI products and concomitant increase in production due to financial incentives (Pirisi, Comunian, Urgeghe, & Scintu, 2011).

Portugal joins the EU (at the time European Community) in 1985 and benefits from these measures, something which is reflected in the dairy sector. In fact, in the post-accession period the national dairy sector recorded a remarkable performance which translated into an increasing supply of milk and milk products and an overall improvement in the quality of raw materials and processed products (MADRP/GPP, 2007). According to (Canada, 1998) total cheese production in Portugal increased by 31,2% in the 10 years following accession and, in the same period, there was an increase of about 18,8% of traditional sheep and goat cheeses production, following the European trend.

As already mentioned, most Portuguese PDO/PGI cheese names were registered in 1996. Between 1997 and 2001, the production of cheese with protected names increased by 43%, reaching around 1.5 thousand tons (Table 2), representing about 2% of the total

production of ripened cheese in the country, which in turn decreased by 21% in this period (Oliveira, 2014). In 2001/02 and at current prices, production values reached EUR 13 million, mainly due to the substantial increase in quantities sold, as prices decreased slightly over this period (Oliveira, 2014).

These values remain in this order of magnitude, although with some oscillations (EUR 11-14 million), almost until the end of the first decade of this millennium (Table 2). The production of PDO/PGI cheeses shows a similar evolution with values that, after the peak of 2001, vary between 1.2 and 1.4 thousand tons until the end of this decade. Serpa cheese production follows these trends until 2007, from when there is an apparent decrease in production (Table 2).

The MADRP report (2007) points out the significant proliferation of medium/small enterprises in this period, many of which are related to the production of small ruminant cheese with PDO status, whose production represented, as of 2007, about 8% of small ruminant cheese production.

According to data from the Portuguese official statistics (INE, 1998-2018), unlike GI production, the total production of sheep cheese began to decline since the beginning of the millennium, suffering a sharp reduction between 2000 and 2014 (from 17.3 to 11.4 thousand tons), worsening from 2008 (Table 2). Probably this was due to difficulties in the dairy sector related to the economic crisis (since 2008), together with community (e.g. dismantling of the dairy produce quota system) and national (e.g. difficulties in implementing support programs) policy decisions (MADRP/GPP, 2007). As of 2008, the production in Portugal of PDO/PGI cheeses and Serpa cheese, in particular, shows a decrease similar to that observed for sheep cheese in general (Table 2).

The prolonged economic crisis lead to a rise in consumer prices, leading in turn to a decrease or even a drop in the consumption of this type of product and its replacement by products with lower values (e.g. white-label products).

Table 2 - Data on the production and marketing of PDO cheeses, non-PDO sheep cheese and Serpa cheese (Sources: DGADR, 2016a, 2017, 2018; GPP, 2014a, 2014b, s/d-a, s/d-b; IDRHa, 2003; INE, 1998-2018; Oliveira, 2001, 2002, 2005a, 2005b, 2006, 2007, 2014).

YEAR	Total number of GI cheeses Portugal (PDO + PGI)	Nº of PDO/PGI cheeses with production	Total sheep cheese production in Portugal (t)	Total PDO/PGI production in Portugal (t)	Serpa Cheese Production (Kg)	Production of Serpa cheese relative to total PDO/PGI (%)	Total number of dairies in the demarcated region of Serpa cheese	Total number of certified dairies for the production of Serpa cheese	Total number of units supplying sheep milk	Price/Kg sheep cheese without PDO (€)	Price/Kg Serpa PDO cheese (€)	Production values -total PDO cheeses in Portugal (X1000 €)	Production values - Serpa cheese (X1000 €)
2017	14	13	11800	1812,3	72250	4,0	-	7	-	-	-	16444,45	830,88
2016	14	13	12100	1429,4	76990	5,0	22	7	-	-	-	11776,21	-
2015	14	13	11500	1422,9	50300	3,5	22	7	-	-	-	11668,14	-
2014	14	13	11400	1370,2	52230	3,8	-	5	-	-	-	11373,39	-
2013	14	13	11600	1485,9	64070	4,3	-	6	20	-	-	9611,16	-
2012	14	10	12000	1323,7	-	_	-	6	-	-	-	11576,87	-
2011	14	13	12000	1353,5	47584	3,5	-	7	-	-	-	11590,59	-
2010	14	12	13011	1314,9	-	_	-	6	-	-	-	11427,06	-
2009	14	12	13679	1397,2	-	_	-	8	-	-	11,5	13765,51	118,43
2008	14	12	14752	1454,2	-	_	-	7	-	-	11,5	14144,72	133,02
2007	14	13	-	1403,6	89541	6,3	-	7	-	-	11,5	12954,65	1029,72
2006	14	12	-	1306,8	65011	-	-	6	-	-	11,5	12515,95	747,63
2005	14	13	16000	1297,5	50000	3,9	-	8	30	9,5	11,5 (10,5 - 13,0)	11065,56	575,00
2004	14	13	-	1449,6	67257	4,6	-	8	25	-	12,5	13631,62	840,71
2003	14	13	-	1286,8	81600	6,3	-	8	35	10	12,0 (11,0 - 13,5)	12390,67	979,20
2002	14	14	-	1458,3	60000	4,1	-	7	28	-	11,50 (11,0- 12,5)	13371,61	690,00
2001	14	14	-	1526,0	62000	4,1	-	4	19	-	10,47 (9,98-11,22)	12757,00	649.43
2000	14††	14	17322	1365,1	20000	1,5	-	4	20	-	10,97 (9,98-13,47)	11495,00	649.43
1999	13	13	-	1256,1	33000	3,0	-	4	15	-	11,47 (10,97-12,22)	10725,00	378.59
1998	13†	11	-	1120,7	30000	-	-	-	-	-	10.47	8014,00	314.24
1997	12	12	_	1064,1	35000	3,3	38	12	-	-	10.97	7634,00	384,10

† (+Pico); †† (+ Tolosa IGP)

On the other hand, with the crisis, there is an increase in production spending, mainly on animal feed, labor and energy, which cancels the hypothesis of valuing the producers' incomes. In this context, the number of holdings and the number of sheep also declined during the crisis, which affected the availability of raw materials. In fact, since 2001 there has been a stabilization of sheep and goat milk production, but with a decrease of 2,5% and 30%, respectively, compared to the average figures at the end of the 1990s. This production represents less than 7% of total national milk production (MADRP/GPP, 2007).

Since 2015, however, there seems to be a trend towards stabilization and even a slight increase in production, both for total sheep cheese and for most PDO, and in particular for Serpa (Table 2; Figure 1). In Figure 1, a graph showing the evolution in production (kg) of PDO/PGI cheeses of small ruminants between 2015 and 2017, it can be observed that, except for 4 types of cheese (Mestiço de Tolosa, Terrincho, Cabra Transmontano and Nisa) there has been an increase in production since 2015 (Azeitão, Serra da Estrela, Serpa, Castelo Branco, Évora, Rabaçal) or at least a recovery of production in 2017, practically to 2015 levels (Amarelo and Picante da Beira Baixa). In the case of Serpa, the production decreases a little in 2017 but it remains above 70,000kg and much higher than 2015. São Jorge cheese (raw cow milk) is the PDO cheese that has always yelded the highest production, which has been increasing since 2015 (around 700,000 kg), having exceeded one million kilos produced in 2017.

This development is probably due to the emergence from the economic crisis and, on the other hand, to conclusion of the implementation of measures within the framework of community legal requirements, in terms of animal and food production. Indeed, the global cheese market appears to be on a growth trend since 2015, with a projected global annual growth of 2,70% and of 2,89% in Europe, by 2023 (Allied Market Research, 2018). At the same time, an annual growth of 3.78% is expected for the unprocessed cheese market, which includes traditional unprocessed products of controlled origin such as PDOs and PGIs (Euromonitor International, 2018), due to health concerns from consumers. With regard to raw materials, worldwide sheep and goat milk production is also expected to increase by approximately 26% and 53% respectively, by 2030 (Pulina et al., 2018).

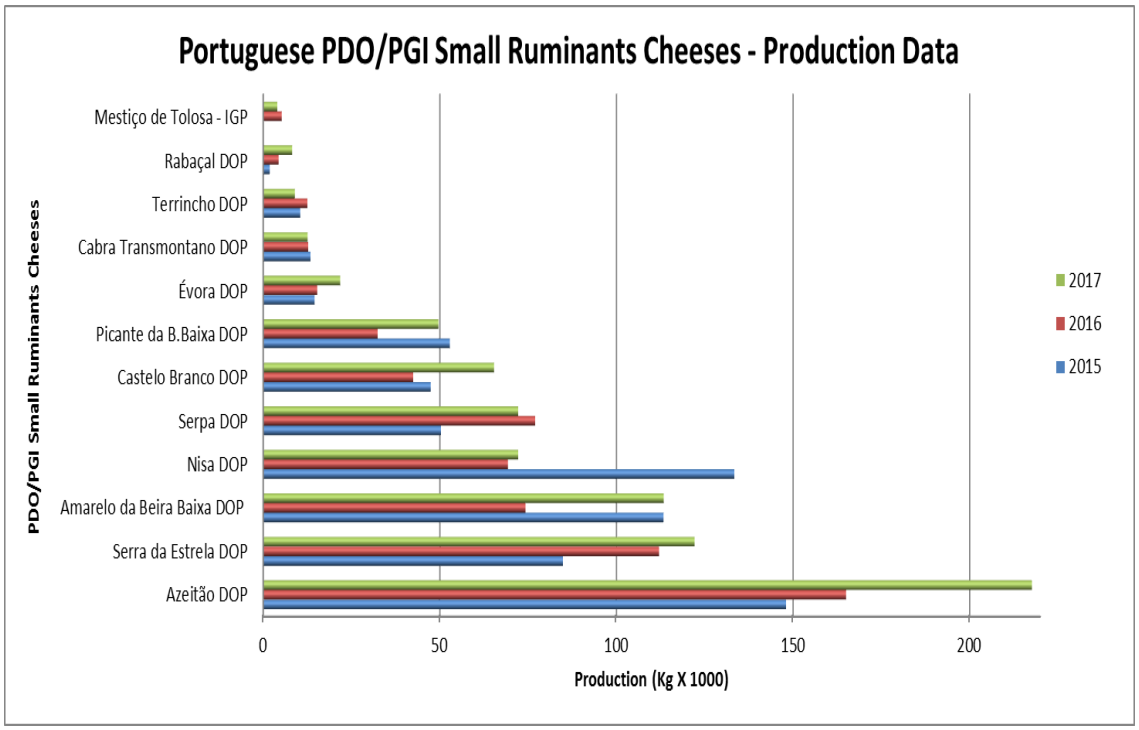

Figure 1 - Portuguese PDO/PGI sheep and/or goat cheeses and production data in 2015, 2016 and 2017 (Sources: DGADR, 2016a, 2017, 2018).

Predictions seem to be encouraging; however, authors are unanimous in considering that in order to fully benefit from the system and thus improve stability and profitability of production, producers must constantly develop their own production and marketing skills, within the framework of strong producers associations. If well managed, special quality product names can deliver many benefits, both to producers and consumers as well as to the whole region (Gangjee, 2017; Hajdukiewicz, 2014).

I.2. Serpa cheese

I.2.1. Serpa cheese historical review

The manufacture of cheese is an activity whose origin is difficult to find, but it is consensual that it is one of the farthest and most useful achievements of man, since for millennia it was used as the only way to preserve milk while maintaining its nutritional richness (Alves, de Medeiros, & Dias, 2016; Beresford, Fitzsimons, Brennan, & Cogan, 2001).

In the region where Serpa cheese is produced, this practice seems to have been introduced by semi-nomadic communities linked to livestock production, which settled in the

Guadiana river valley (Dias, 1998). Among the archaeological remains of this region, objects were found that were used for the manufacture of cheese, dating from 5000 BC, which testifies to this age-long activity in the region (Bettencourt, Pinheiro, & Carrasco, 2008). It is natural that sheep cheese was introduced into the region even before Roman occupation, by the Lusitanians or, especially, by the Carthaginians, due to their great tradition in sheep and goat grazing (Dias, 1998).

The presence of the Romans from the 3rd century BC onwards brought great progress in technical terms, being attributed to them the effective implementation of cheese production in the region that later became Portugal, however they seemed to prefer cow cheese (Dias, 1998; Rebelo, 1994). Just from the beginning of the eighth century, with the arrival of Islamic peoples (mainly the Berbers), grazing and the manufacture of sheep and goat cheese become widespread in the region. The nomadic nature of this people is related to the need to provide the cattle with the necessary food escaping the harsh climate of the region, which gives this activity a seasonal nature. The seasonality of this activity was common until the mid-nineteenth century. The Arabs also brought with them the sheep breed Merina Branca, much more efficient in milk production than the Campaniça sheep breed, predominant until then (Dias, 1998).

In order to encourage the repopulation of the hills adjacent to Serpa, after the Christian reconquest (12th and 13th centuries) that led to the exodus of the population due to conflicts in that area, tax benefits are established which aim to encourage pastoral activity and even the production of cheese (Dias, 1998). Coelho (2003) writes that Alentejo cheeses were already mentioned in a description of the Kingdom of Portugal dating back from the 16th century, being defined as the best in the world in terms of finesse and flavor.

After the Christian reconquest (14th and 18th centuries) there was a large increase in the number of sheep, supported by the increase of the rural population and the flourishing wool trade. These conditions led to a large expansion of transhumance (Ferreira, 2008). The long-distance transhumant flow (involving trips taking from several weeks to months, of about 400km) took place between Serra da Estrela, where cattle stayed in the hottest weather, and the Alentejo fields (Ourique, Castro Verde, Beja), where they stayed during the winter. This activity was extinguished in the first quarter of the twentieth century, among other reasons due to agricultural modernization and wheat campaigns that

led to the end of the common pastures, making it difficult for cattle to graze and to pass through the fields (Ferreira, 2008).

These movements have certainly facilitated the exchange of experiences on cheese making, and because of that some authors consider that Serpa cheese was derived from the equally famous Serra da Estrela cheese, due to the similar manufacture method and ingredients used (Canada, 1998; Roseiro, 1991; Roseiro, Wilbey & Barbosa, 2003). However, the milk coming from different sheep breeds, pastures, milking areas and cheese making contribute to a diverse cheese microbiota, resulting in a substantially different flavor and texture end product (Roseiro, Wilbey et al., 2003).

Thanks to the data collected by Dias (1998) in the Beja District Archives, it is possible to see that, at the end of the 17th century, the production of cheese in Serpa was around 11 thousand units per year, with a slight increase during the 18th century to around 12 thousand units. During the 19th century, production declined slightly, with production below 10 thousand units per year. According to the same author, the number of cheese producers had a similar evolution, estimating between 35 and 40 dairies in the 17th and 18th centuries, with only 20 to 25 reaching the 19th century. At around that time, the cheese trade was practically limited to the region where it was produced (Coelho, 2003).

In the late 19th and early 20th centuries, Alentejo cheese produced in the region of Beja, possibly what today is called Serpa, is then disseminated in the famous Universal Exhibitions, in national exhibitions, in fairs, exhibitions and agricultural or industrial congresses, which encourages their commercialization beyond the region where it is produced (ACOS, 1995; Coelho, 2003). In these events, this cheese is referred to as one of the best known and as distinguished as the Serra da Estrela, Castelo Branco or Rabaçal, being sold in large quantities in cities such as Évora and Lisbon, directly by the farmer or the cheesemaker or by intermediaries, who kept it in storage until favorable sale conditions (Coelho, 2003).

Since this time, there has been a large increase in the consumption of milk and dairy products worldwide, associated with the industrial evolution of the sector. This was mainly due to technical advances in milking methods, product conservation, animal feeding, species selection and, simultaneously, to studies related to the nutritional value of these products (Johnson, 2017; Reis & Malcata, 2011; Wilbey, 2017). The major

challenges were focused on improving hygiene, therefore the safety and quality of cheese, and on automation (Johnson, 2017; Wilbey, 2017).

In Portugal, this industrialization process was slower compared to other countries, with traditional practices persisting. Alves et al. (2016), citing Rasteiro, states that around 1908 milk production in Portugal would be around 55 million liters of cow milk, 21 million sheep milk and 24 million goat milk, being the second almost fully processed into cheese and the third in more than half of the production. The same source states that only in the Azores could it be considered that there was already an industrial-level (cow) cheese production. It also insists that in mainland Portugal, although small cow and goat cheese industries were starting to emerge, homemade sheep cheese (Alentejo, Serra da Estrela) was predominant, followed by goat cheese, where the owners processed only some of their milk, however creating magnificent cheeses within its type, citing the cases of Serra da Estrela, Beja, Castelo Branco and Azeitão.

In contrast, the industrial cheese that is just beginning to be produced, which is mainly an imitation of Dutch ("Flamengo") and English cheese, is generally of poor quality. These results were attributed to the lack of technical and scientific knowledge and hygiene standards in both cheese and milk production. There was talk of great indiscipline in the dairy sector at this time (Alves et al., 2016). Only in the thirties was the production of "Flamengo" cheese definitively established in Portugal, marking the beginning of a new era in the Portuguese dairy economy, with the progressive development of the cheese industry (Alves et al., 2016; DG, 1939). However, only from 1950 on did the milk sector undergo a strong state intervention, with laws directed at the discipline of the sector, in articulation with the active and growing presence of cooperatives (Alves et al., 2016; DG, 1953, 1961).

The quality problems observed in industrial cheeses at that time would certainly extend to traditional ones. In Government Order Nº 18186/1961 (DG, 1961), in which a commission is created for studying a new reorganization of the sector, one of the justifications used mentions problems in the production of serra cheese (now Serra da Estrela PDO). They state that the co-operative organization, as already applied in France, would be advantageous, since the cheese is manufactured in many dispersed installations, without technical uniformity or type, offering exceptional and other manifestly bad

specimens, which devalues the product in commercial terms. The problem would certainly be the same with Alentejo cheese.

On the other hand, technical innovations that were emerging were also used, whenever applicable, by traditional cheese factories in order to minimize some problems. In a review article (100 years) on cheese production and quality, Johnson (2017) argued that these innovations were applied to all types of cheese, and this made the sector more robust. In the same article, it is argued that a cheese can be made exactly the same way as many years ago, even using innovative automated means.

The emergence of industrial cheese would eventually lead to the appreciation of traditional cheese. Wilbey (2017), and references therein, notes that in the UK the automation and concentration of cheesemaking into fewer larger-scale units offered opportunities for smaller cheesemaking units to specialise in the less demanded cheeses and for the regrowth of artisanal cheesemaking. Indeed, this type of cheese has a distinctive quality that is a factor of competitiveness, as it meets the growing appetite of consumers for products with intrinsic qualities from specific productions (Pellin et al., 2016). Thereof arises the need to certify, to guarantee and to defend this differentiation.

In Portugal, the first step to certify traditional products was the 1966 ratification of the "Lisbon Agreement on the Protection of Designations of Origin and their International Registration" by Decree-Law Nº 46852/1966 (DG, 1966). Only in 1984 did the Ministry of Agriculture, Fisheries and Food, through Decree-Law Nº 146/84 (DR, 1984), allow the creation of demarcated regions for traditional cheeses, authorizing the use of PDO in cheeses produced in these regions, and whose characteristics met legally established quality requirements. Thus, from 1985 onwards, the first "demarcated regions" for cheese began to be delimited, the first being the Serra da Estrela cheese. The same procedure was followed for other traditional cheeses, including Serpa cheese with Decree-Law Nº 39/87 of June 29 (DR, 1987).

The certification procedures succeeded one another, in accordance with the implementation in Portugal of the applicable European legislation on Quality Schemes, as explained in the following sections. Towards the end of the 1980s, several Member States demanded stronger legal protection for their traditional products, in line with pre-existing national legislation. The EU starts implementing measures to defend traditional

products through the reform of the Common Agricultural Policy (CAP), in the 1980s, and with the various editions of the LEADER programme on the second half of the 1990s (Pellin et al., 2016). In 1992 it promulgates Regulations (EEC) 2081/92 (ECC, 1992a) and 2082/92 (ECC, 1992b), respectively on geographical indications and protected designation of origin and on certificates of specific character for agricultural product and foodstuffs. In the case of Serpa cheese, the certification process led to the acceptance of its registration as a PDO product under this legislation, made official in 1996 by Regulation (EC) Nº 1107/96 (EC, 1996).

In the 21st century, consumers in the European Union and around the world are more and more demanding about food quality, expecting more than just meeting the dietary, health and hygiene standards of the products they buy. They are increasingly looking for provenance, taste, respect for the environment and other individual product characteristics, often attributed to the specific origin or production method (Hajdukiewicz, 2014). This points to a shift in the concept of quality from an 'industrial world', with its highly standardized quality conventions and the logic of mass production of goods, to a 'domestic world', where quality conventions are built on trust, tradition and local support, with differentiated, localized and environmentally friendly products (Goodman, 2003). Serpa cheese is adjusted to these conditions and, taking into account market trends, production must also be adjusteded to meet the demand.

I.2.2. Serpa PDO cheese recognition process

The first Portuguese governmental initiative specifically around Serpa Cheese comes with Regulatory Decree Nº 39/87 of June 29 (DR, 1987), where measures are adopted to defend this product. This document defines the respective geographical area of production (Figure 2), sets the parameters that guarantee its genuineness and quality (conditions of ripeness and conservation, shape, dimensions and weight, crust and paste of cheese). It also establishes conditions for the process of setting up a certification entity for Serpa cheese. Thus, under Article 3 of this law (DR, 1987), Ordinance Nº 252/91 (DR, 1991) assigns the private control and certification functions to the ACOS (Associação de Criadores de Ovinos do Sul/Southern Sheep Breeders Association).

The Demarcated Region of Serpa cheese production (Figure 2) is thus defined and includes the municipalities of Mértola, Beja, Castro Verde, Cuba, Ourique, Moura, Serpa,

Vidigueira, Aljustrel, Ferreira do Alentejo and Alvito, as well as the civil parishes of Colos and Vale de Santiago belonging to the Odemira municipality, as well as São Domingos, Alvalade and Abela of the Santiago do Cacém municipality, Azinheira de Barros, in the Grândola municipality, and Torrão, in the Alcácer do Sal municipality. It extends over a very large area totaling about 17000 Km^2.

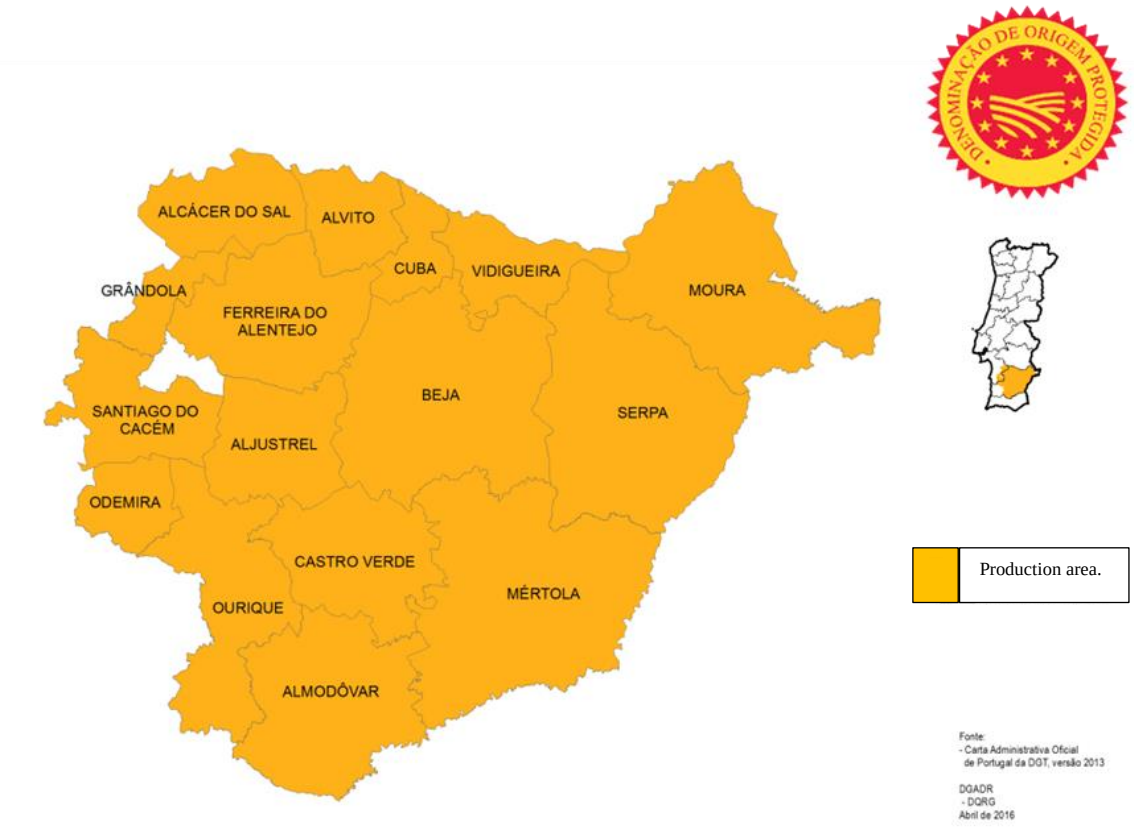

Figure 2 - Map of Portugal with location of the Serpa cheese production area (orange), and detail showing the municipalities and parishes that compose it. Also shown, the PDO brand label (Source: DGADR, 2016b; EC, 2014).

Ministerial Dispatch Nº 52/94 (DR, 1994) recognizes the protected designation of origin (PDO) of this cheese, establishes the characteristics it must have and recognizes the legal entity Production and Marketing of Agricultural Products, lda. (SULPAR) as the producer group responsible for its registration in the National Institute of Industrial Property (INPI) and for the application for registration as PDO in the European Economic Community (EEC), on behalf of the Agricultural Markets and Food Industry Institute (IMAIAA), as the responsible department in the Member State. That legal entity is later replaced in its functions by Queijo Serpa–Producer Group - Ministerial Dispatch 5/97 (DR, 1997)

The application for registration in the European Community under Articles 5 and 17 of ECC (1992a), is accepted (Case EEC Nº: VIB14/P0/0260/24.1.94) and the registration of Serpa cheese as a PDO is published in Regulation (EC) Nº 1107/96 (ECC, 1996). This status reveals a quality-differentiated product that must be identified by the European PDO product label (EC, 2014) shown in Figure 2. Cheese conforming to the corresponding product specifications should bear this symbol on the labeling in the same field of view as the registered name of the product. The indication "Protected Designation of Origin" or its abbreviation 'PDO' may also appear on the labeling (EC, 2012b).

In October 2005, the recognition of ACOS as the private inspection and certification body of Serpa-PDO cheese is annulled (DR, 2004). This is replaced in those functions by CERTIALENTEJO - Certification of Agricultural Products, Lda (DR, 2005) currently CERTIS-Control and Certification, Ltd. Since February 5, 2010, CERTIS is accredited by IPAC (C0015 Accreditation), first according to NP EN 45011:2001 (IPQ, 1990), and now according to ISO/IEC 17065/2012 (ISO, 2012), for the certification and control of, among other products, Geographical Indications (GI), including Serpa cheese. Thus, it is recognized as a Control Body (CB) according Regulation (EC) Nº 882/2004 (EC, 2004c) by the Directorate General for Agriculture and Rural Development (DGADR), the competent authority for this recognition, according to Legislative Order Nº 11/2018 (DR, 2018). This Directorate should publicize the name and address of the authorities and bodies, and update that information periodically (EC, 2012b). CB means an independent third party to which the competent authority may delegate certain control tasks (EC, 2004c), in this case in the context of certification.

Registered Serpa cheese producers require authorization from the certification and control body to use the PDO quality label. That legal entity is responsible for the verification of compliance with the product specifications before marketing the product by carrying out appropriate controls. If the product meets the specifications, it receives a label or certification seal that indicates with sufficient confidence that the product conforms to a specific regulatory document. These labels are issued by the certifying entity duly numbered, according to a pre-established model, approved and published in the corresponding legislation. They are to be affixed to each cheese unit upon leaving production (Vieira, 1994). The dimensions of the seal may vary according to the size of

the cheese but always keeping the relative dimensions. It must be affixed in such a way that it is not mistaken for the producer's label (DR, 1991).

The certification label of Serpa cheese has undergone changes related to the change of private inspection and certification body. Figure 3 shows the three labels used since the beginning of the process in 1994 by ACOS (Figure 3A), through CERTIALENTEJO (Figure 3B) and currently by CERTIS (Figure 3C).

Figure 3 - Serpa cheese certification labels from the beginning of its recognition as a PDO product, according to the private control and certification body involved. A-ACOS (outdated); B-Certialentejo (outdated); C-Certis (currently in use) (Sources: DR, 1991, 2005).

I.2.3. Serpa cheese specifications and applicable legal aspects

Serpa cheese, as laid down in Annex II of Regulatory Decree Nº 39/87 (DR, 1987) and in Ministerial Dispatch Nº 52/94 (DR, 1994), is defined as a cured cheese, of buttery, semi-soft consistency with few or no holes, obtained by slowly draining the curds, after coagulation of the pure raw ewe's milk originating in the said geographical area, through the action of a cardoon (*Cynara cardunculus L.*) infusion. According to the same document, this cheese must still meet the conditions given in Table 3.

In the document attached to the registration process, "Serpa Cheese Production Rules" (Vieira, 1994), in addition to the above, other conditions are set out, namely the conditions to be followed in the production of milk, manufacture, ripening and preservation of Serpa cheese. The rules of control and certification of Serpa cheese are also established. According to these rules, it is established that the control actions to be carried out by the certifying entity shall focus on the following areas: (a) herd health, management and hygiene; (b) conditions for milking, collection, packaging, transport and preservation of

milk; (c) qualitative characteristics of the raw material; (d) technical and hygienic rules observed in the manufacture and ripening of the cheese.

Table 3 - Specifications for Serpa cheese (Sources: DR, 1987, 1994; Vieira, 1994).

Parameter	Specification
Moisture on a fat free basis (MFFB) (%)	61% a 69%
Fat in dry matter (FDM) (%) (NP-2105)	45% - <60%
Shape	Low cylinder (plate), regular with lateral bulging and on the upper face (slight).
Dimension and weight	**Merendeira:** diameter from 10 cm to 12 cm, height from 3 cm to 4 cm and weight between 200g and 250g; **Cunca**: diameter 15 cm to 18 cm, height 4 cm to 5 cm and weight between 800g to 900g; **Normal**: diameter from 18 cm to 20 cm, height from 4 cm to 6 cm and weight from 1000g to 1500g; **Gigantes**: diameter from 25 cm to 30 cm, height from 6 cm to 8 cm and weight between 2000g to 2500g.
Rind	**Consistency:** malleable, allowing some fluctuation; **Appearance**: Whole, well formed, slightly rough and thin; **Color**: Light straw yellow, uniform.
Paste	**Texture:** closed, buttery, with easily deformable cutting zones, which may spill; **Appearance:** unctuous, with few or no eyes; **Color:** yellowish white or straw yellow, darkening on contact with air.
Aroma and taste	Generally strong and dominantly spicy.
Ripening	**Ambient Conditions:** -Temperature between 6 ° C and 12 ° C; -Relative humidity between 85% and 90%; **Minimum cure time**: 30 days; **Maturation index:** 45 (minimum) (method not specified).
Conservation (Temperature)	**In the warehouse**: between 0 ° C and 5 ° C **In transport:** between 0 ° C and 10 ° C **In the retailer**: between 0 ° C and 10 ° C

Regarding milk, that document (Vieira, 1994) lays down the conditions for milk-supply herds with regard to animal health status, withdrawal periods for vaccinations, medication and feeding type. The same document also contains hygiene and milk preservation procedures. The milk-producing sheep breed is not specified, it is only stated that it must be pure raw sheep milk, coming exclusively from the defined geographical area.

Traditionally, Serpa cheese was made from milk derived exclusively from sheep of the "Merina" and "Campaniça" native breeds. For some years now, many producers have opted to explore exotic breeds, mainly the French “Lacaune” sheep breed, which are more

specialized in milk production, mixed herds being common (Bettencourt et al., 2008; Canada, 2001).

Bettencourt *et al.* (2008) justifies that the native breeds originally used are more oriented to meat production, presenting reduced individual levels of milk production. This low production would be offset by large herds milked using extraordinary labor, which would not be profitable in the current scenario. Research carried out in the context of the characterization of milk of the different species mentioned and of the cheese obtained from it does not seem to show differences in the quality of the cheese (Bettencourt, Matos, Batista, Canada, & Fialho, 1996; Bettencourt et al., 2008), despite the differences in the physicochemical characteristics of the milk (Amaral, 1996; Bettencourt et al., 1996; Bettencourt et al., 2008; Roseiro, Barbosa, Ames & Wilbey, 2003).

The quality of milk is a very important factor in cheese making, especially when made with raw milk, however, the document (Vieira, 1994) does not indicate parameters or limits, so current legislation should be followed. Table 4 presents the microbiological criteria to be complied with raw sheep's milk for the production of raw milk products based on current legislation (EC, 2006a).

Table 4 - Microbiological criteria for raw milk (Source: EC, 2006a).

Type of Milk	Total Count at 30 ºC (cfu/ml)
Raw milk from species other than the cow to be used in the manufacture of products made from raw milk by a process that does not include any heat treatment.	$\leq 5{,}0 \times 10^5$ (5,70 log ufc/ml) *

* Geometric average over a two-month period, with at least two monthly milk sample collections.

The same document (Vieira, 1994) establishes the following additional analyzes to be carried out on cheeses: investigation of the presence of foreign milks, coliform bacteria, *E. coli*, *Staphylococcus aureus*, *Salmonella spp*. and *Listeria monocytogenes*. Later, an article drawn up by the certifying entity at the time (ACOS, 1996), adds the determination of dry matter and protein and replaces the search for *E. coli* and *Staphylococcus aureus*, by their count, stating that all determinations should yield satisfactory results. There are no limits on microbiological parameters and it is therefore assumed that the limits established by current legislation should be met, at that time Ordinance Nº 533/93 (DR, 1993) and its amendments according to the Council Directive 94/71/EC (EC, 1994), currently repealed.

As regards hygiene and safety, Serpa cheese production is currently subject to European Union law. Accordingly, the requirements set out in Regulation (EC) Nº 852/2004 (EC, 2004a) and Regulation (EC) Nº 853/2004 (EC, 2004b), both of 29 April, and their amendments should be met, always taking into account the adaptations deriving from the fact that it is a traditional product as considered in Regulation (EC) Nº 2074/2005 (EC, 2005b). In Appendix 1, the applicable European and national legislation in force is presented. The microbiological criteria for cheese made from raw milk are those laid down in Regulation (EC) Nº 2073/2005 (EC, 2005a) microbiological criteria for foodstuffs (Table 5).

Table 5 - Microbiological safety and hygiene criteria (EC, 2005a) applicable to Serpa cheese.

Criteria	Microorganisms	Limits / Sampling-plan
Security Criteria	**Search for *Salmonella***	Absence in 25g n=5; c=0
	Search for *Listeria monocytogenes*	
Hygiene Criteria	**Coagulase Positive *Staphylococci* Count (cfu/g)**	m = 10^4 ufc/g (4 log); M = 10^5 ufc/g (5 log) n=5; c=2
m and M - Limits; n - number of units constituting the sample; c = number of sample units above or below m and M allowed for interpretation of results.		

Sensory control for certification is also provided for in the production rules (Vieira, 1994). These will be performed by a panel of tasters, consisting of a minimum of five trained and selected tasters, and will always be performed in the morning. The cheeses to be evaluated (maximum 8 per session) must respect the minimum curing time (30 days) and, until evaluation, will be kept at 10ºC, being placed at room temperature 2 hours before the test. The cheese is first observed whole, then cut in half and finally cut into slices of about 15g for the remainder.

Cheese will be graded on the basis of the sequential assessment of the following parameters: - Cheese rind, appreciated for its consistency, appearance and color (score between 0 and 4); - Form and Consistency, for its bulging, edges, consistency and sound (score between 0 and 4); - Paste Texture and Color (score between 0 and 6); and -Taste and Smell (score between 0 and 6). To pass this test, cheeses must have a minimum total score of 14 points, with a minimum of 4 points for the taste and smell features.

Currently this test is performed at the Sensory Analysis Laboratory (LAS) of the Polytechnic Institute of Beja, accredited for this purpose according to the standard ISO/IEC 17025 (ISO, 2017) by IPAC (Accreditation LO685), in a test room with standard specifications (IPQ, 2001) and using a quantitative descriptive analysis (QPA) method, based on what are considered to be the specific sensory characteristics of Serpa cheese and carried out by a group of selected and trained Serpa cheese tasters (IPQ, 2001) for this purpose.

For certification purposes, the responsible entity must periodically obtain milk and cheese samples from the various producers for physical, chemical, microbiological and sensory testing. The certifying entity must draw up reports with the results obtained to keep the producer group informed. Cheeses coming from registered Serpa cheese producers that meet these specifications must display a casein seal containing the identification of the cheese and the batch number and they will be awarded the certification label according to the rules presented above. Such cheeses may be placed on the market under the name Serpa cheese and bear the PDO brand symbol (Vieira, 1994).

I.2.4. Previous work on the microbiological characterization of Serpa cheese

Despite the importance of Serpa cheese, especially in terms of local economy, scientific knowledge gained from research on Serpa cheese, as it happens with other traditional Portuguese cheeses, trails behind other countries (Reis & Malcata, 2011), especially regarding its qualitative microbiological characterization.

However, the Agrarian School of the Beja Polytechnic Institute (ESA-IPBeja) has maintained a line of research for the past 25 years associated with Serpa cheese, where we can highlight the participation in some research projects, as well as some publications (Alvarenga, J. Canada, & I. Sousa, 2011; Alvarenga, Silva, Garcia, & Sousa, 2008; Bettencourt et al., 1996) and master (Alvarenga, 2000; Amaral, 1996; Canada, 1998) and doctoral theses (Alvarenga, 2008; Canada, 2001). These works have mainly focused on technological aspects and on the physicochemical and sensorial characterization, although some (Amaral, 1996; Canada, 1998, 2001) resort to the microbiological characterization of the sample.

In those and other works (Barbosa, 2000; Dias, 1998; Roseiro & Barbosa, 1996; Roseiro, Wilbey et al., 2003), different microbial groups were quantified under specific manufacturing conditions, or from the perspective of product hygiene and safety assessment, both in milk or cheese. Indeed, despite the importance of microbiology in the quality of this cheese, research on this subject has been limited, including only a classical microbiological characterization through the use of culture techniques where microorganisms are differentiated by the use of selective culture media and eventually by morphological and biochemical characterization (Jany & Barbier, 2008; Quigley et al., 2011). Table 6 presents a summary of results of the microbiological characterization of Serpa cheese obtained in the aforementioned studies. Most include the characterization of cheese paste, but also of the rind (Dias, 1998). Results are also presented for different production conditions such as spring and winter, artisanal and semi industrial and natural and artificial cure system.

According to Roseiro & Barbosa (1996), the microbial communities of Serpa cheese are qualitatively similar to those of the milk from which it is produced, being dominated by lactobacilli. Throughout the maturation period, a decrease in coliforms and streptococci bacteria was reported, together with an increase in proteolytic bacteria. In another study (Barbosa, 2000), it is concluded that mesophilic LAB (8,0 log cfu/g) and enterococci (7,0 log cfu/g) are predominant groups (Table 6), which agrees with other works researched, regarding the two parameters (Canada, 2001) or regarding the LAB count (Table 6), (Canada, 2001; Dias, 1998; Roseiro & Barbosa, 1996; Roseiro, Wilbey et al., 2003). Barbosa (2000) presents the genres *Leuconostoc* and *Lactococcus* as the most abundant.

In turn, Amaral (1996) studied several microbiological characteristics of Serpa cheese manufactured from three different ovine breeds (Merina, Serra da Estrela, and Lacaune) and the effect of longer ripening (40 to 55 d) on cheese quality. No significant differences were found between breeds in terms of total viable microflora, which ranged from 8,0 to 9,0 log cfu/g, as in the other works analyzed (Table 6), (Barbosa, 2000; Canada, 2001; Dias, 1998; Roseiro & Barbosa, 1996; Roseiro, Wilbey et al., 2003).

Table 6 – Quantitative microbiological characterization of Serpa cheese laid down in different works. Most represent the characterization of cheese paste, but also of the rind. The results are also presented under different production conditions, such as spring and winter, artisanal and semi industrial and natural and artificial curing system (Sources: Amaral, 1996; Barbosa, 2000; Canada, 2001; Dias, 1998; Roseiro & Barbosa, 1996; Roseiro, Wilbey et al., 2003).

MICROORGANISM / MICROBIAL GROUP	**Paste**	**Paste**	**Paste**	**Rind**	**Paste (Winter)**	**Paste (Spring)**	**Paste (Natural ripening)**	**Paste (Controlled ripening)**	**Paste (Industrial manufacture)**	**Paste (Artisanal manufacture)**	**Paste (Semi-industrial manufacture)**	**Paste (Artisanal manufacture)**
Total viable count at 30 °C (log ufc/g)	8,0-9-0	8,0-9-0	9,00	8,05	8,46*	8,13*	8,34	8,26	8,24	8,34	8,74	8,89
Psychrotrophic microorganisms (log ufc/g)	nd	nd	7,68	7,52	3,54*	2,54*	1,55[2]	4,35[2]	2,11[3]	3,70[3]	nd	nd
Lactic acid bacteria (log ufc/g)	8,00	nd	8,50	8,09	7,65	7,60	7,68	7,58	7,42	7,77	8,40	8,18
Enterococci (log ufc/g)	7,00	nd	nd	nd	6,82	7,32	7,08	7,06	6,80	7,26	nd	nd
Coliforms (log ufc/g)	3,00-5,00	6,4-7,4	6,17	7,05	9,57*	7,21*	10,04[2]	6,98[2]	6,78	9,54	5,17	5,83
***E.coli* (log ufc/g)**	nd	3,2-4,9	nd	nd	4,06*	3,33*	4,73[2]	2,80[2]	2,50	4,54	3,14	4,49
***S.aureus* (log ufc/g)**	<1,00	<2->3,3	nd	nd	<2,00	<2,00	<2,00	<2,00	<2,00	<2,00	<4,00	<1,00
Total Staphylococci (log ufc/g)	nd	nd	7,04	7,51	nd	nd	nd	nd	nd	nd	nd	nd
Yeasts (log ufc/g)	3,00-5,00	nd	5,08	6,53	3,95*	3,28*	3,78	3,48	2,60[3]	4,33[3]	3,36	5,11
Molds (log ufc/g)	nd	nd	nd	nd	2,12*	0,75*	1,83	1,10	1,27	1,56	Undetected	Undetected
Bibliographic source	(Barbosa, 2000; Roseiro & Barbosa, 1996)	(Amaral, 1996)	(Dias, 1998)					(Canada, 2001)			(Roseiro, Wilbey, et al., 2003)	

nd - Not done; *; [2]; [3] - significant differences between opposite conditions under study.

Other results of the microbiological characterization performed by the author are presented in Table 6. It warns that 34% of the cheeses analyzed contained *Staphylococcus aureus* above 3 log cfu/g (the limit at that time) and *Escherichia coli* is also said to be high in some samples, proof of hygiene problems (Table 6). Roseiro, Wilbey, et al. (2003) warns of the same situation with *S. aureus* in cheeses of semi-industrial origin. *E. coli* counts averaging over 4.0 log cfu/g (limit at that time) are also shown for artisanal cheese (Canada, 2001; Roseiro, Wilbey et al., 2003), as well as winter cheese and uncontrolled ripening cheese (Canada, 2001) (Table 6).

Dias (1998) carries out the characterization of Serpa cheese and cheese made from refrigerated milk in two distinct seasons (April-May and June-July) and in three cheeses axial positions (smear, surface and paste). This study tries to identify the quality problems of cheese made with chilled milk. The main microbial groups isolated were LAB (LAB), total staphylococci, coliforms and yeasts, without relevant differences between the two cheese types and the two seasons at the end of cheese ripening (28 days) (Table 6). It identifies *Debaryomyces hansenii* and *Yarrowia lipolytica* as the main yeasts among 51 isolated species.

In the same year, Canada (1998) studied the evolution of Serpa cheese production as PDO and concluded that all samples submitted for certification within five years met the microbiological criteria applicable at that time. Later, in his work on the physicochemical and sensorial characterization of Serpa cheese (Canada, 2001), he analyzes cheeses with a ripening time of 30 days or more, but variable since they are collected only when the producer considers them ready for consumption (finished cheese, in the cheesemaker's language). Higher counts were detected in winter cheeses, in artisanal production and for natural ripening conditions (also associated with the artisanal process). In the first case the results are justified by the longer cure times due to the lowest ambient temperature in winter, and in the second case by the use of less efficient (manual) whey drainage processes in artisanal production. In natural curing the temperature is higher, which promotes the development of microorganisms, with the exception of psychrotrophic, which are higher in controlled cured cheeses. Results are presented in Table 6 with the identification of parameters with significant differences between conditions under study.

In another study (Roseiro, Wilbey et al., 2003), the microbiological characterization of raw sheep milk and Serpa cheese from two dairies, one representative of traditional

production (turnout of 100 L of Merino ewe milk per day) and the other of semi-industrial (turnout of 1000 L of Lacaune ewe milk per day), was performed. Overall, the authors report significant differences between physicochemical parameters of cheeses from the two dairies, what they consider naturally related to differences in both the milks and cheesemaking practices. As regards microbiological characterization, despite the differences in the microbiology between curds from the two dairies, in cheese aged 30 days or more, no differences are found, for yeasts, which are higher in artisanal cheese (Table 6), The study emphasizes the considerable presence of *S. aureus* in milk and throughout cheese ripening in semi-industrial dairy products, which is associated with machine milking, which leads to a higher incidence of mastitis in the herd (Table 6) (Roseiro, Wilbey et al., 2003).

Reference should be made to the absence of *Listeria monocytogenes* and *Salmonella spp.* in 25g of cheese (Amaral, 1996; Canada, 1998, 2001; Roseiro, Wilbey et al., 2003) or 25ml of milk (Roseiro, Wilbey, et al., 2003), the aforementioned works in which these researches were carried out.

Some of the works cited also assess the quality of milk used in Serpa cheese making (Canada, 2001; Dias, 1998; Roseiro, Wilbey et al., 2003), with the results being presented in Table 7. The results point to high mean values in the total count, often exceeding the legal limit of 5.70 log cfu/g (Table 4). There seems to be a trend towards higher values of microbial counts in milks obtained in spring (Canada, 2001) and by semi-industrial production /mechanical milking (Canada, 2001). Spring results are related to higher temperatures (Canada, 2001). In the case of semi-industrial production, the results are justified with the longest interval between milking and milk use (Canada, 2001). On the other hand, in the mechanical milking used in semi-industrial production, the results are generally higher than in manual milking (Dias, 1998), probably due to problems with the hygiene of more complex equipment. Results obtained by Roseiro, Wilbey et al. (2003) contradict this trend, and these authors only emphasize the high concentration of *S. aureus* in semi-industrial production, for the reasons already mentioned (Roseiro, Wilbey et al., 2003).

Table 7 - Microbiological characterization of milk for Serpa cheese realized in different works. The results are also presented under different production conditions, such as spring and winter, artisanal and semi industrial and manual and mechanical milking (Sources: Canada, 2001; Dias, 1998; Roseiro, Wilbey et al., 2003).

MICROORGANISM / MICROBIAL GROUP	Manual milking	Mechanic milking	Winter	Spring	Semi-industrial manufacture	Artisanal manufacture	Semi-industrial manufacture	Artisanal manufacture
Total viable count at 30 °C (log ufc/g)	5,67	5,83	5,92	6,21	6,67	4,85	5,15	6,93
Psychrotrophic microorganisms (log ufc/g)	5,02	6,35	4,20	3,77	4,67	3,31	nd	nd
Lactic acid bacteria (log ufc/g)	nd	nd	5,15	4,06	4,93	3,97	4,82	3,41
Enterococci (log ufc/g)	nd	nd	1,82	5,36	2,13	3,86	nd	nd
Coliforms (log ufc/g)	4,88	5,02	nd	nd	nd	nd	2,04	2,81
Enterobacteria (log ufc/g)	nd	nd	4,47	5,60	5,05	3,49	nd	nd
***E. coli* (log ufc/g)**	nd	nd	nd	nd	nd	nd	<1,00	2,75
***S. aureus* (log ufc/g)**	nd	nd	nd	nd	nd	nd	<4,00	<1,00
Yeasts (log ufc/g)	nd	nd	3,06	2,26	3,49	1,83	3,95	2,32
Molds (log ufc/g)	nd	nd	3,05	1,78	2,68	2,15	nd	nd
Bibliographic source	(Dias, 1998)				(Canada, 2001)		(Roseiro, Wilbey, et al., 2003)	

nd - Not done

The results presented point to a raw material and a final product of high microbial content, where LAB usually predominate among other microbial groups such as enterobacteria (coliforms), staphylococci, yeasts and molds. The results are also indicators of some hygiene problems. Indeed, the conditions of production of Serpa cheese, such as the use of raw milk, and thus the absence of any standardizing thermal process, coupled with different milking and handling protocols, and thus variation in hygienic conditions prevailing in the farmhouses, lead to an extensive and unpredictable variability in the present microflora and therefore in the quality and safety of the final product, like in other traditional Portuguese cheeses (Pereira, Graca, Ogando, Gomes, & Malcata, 2010).

Notwithstanding the importance of these works as a basis for the qualitative characterization of Serpa cheese, the great dependence on indigenous microflora for the development of the final characteristics of cheese highlights the importance of further studying the microbiology of this cheese, by identifying and characterizing the specific

cheese flora. This characterization may prove an important factor in correcting those aspects that lead to the heterogeneity of the final product (Canada, 2001; Roseiro, Wilbey et al., 2003).

I.2.5. Serpa cheese manufacture

Serpa cheese production was traditionally a family-owned business with few workers, in facilities known as "rouparias" (Bettencourt et al., 2008). This designation, currently underused, arose in the 16th century associated with the use of various types of cloths in different essential stages of the manufacture of this cheese (Bettencourt et al., 2008). The manufacture of this cheese continued to develop mainly at a farm scale; however, in recent years they have gradually been modernizing to respond to market demands, improving processing and hygiene conditions, as with other traditional cheeses (Reis & Malcata, 2011; Roseiro, Wilbey, et al., 2003). Nevertheless, they must comply with the certification recommendations to be considered a PDO cheese (Vieira, 1994).

In the production of Serpa cheese, artisanal technology continues to be used as a basis, but with some improvements introduced over time, especially in terms of mechanical processes (Canada, 2001;Dias, 1998; Roseiro, Wilbey et al., 2003). The major operations associated with the manufacture of this type of cheese are milk handling, coagulation, cutting, working of the curd and draining of the whey, pressing, salting and ripening. Figure 4 shows the flowchart of this production.

I.2.5.1. Milk handling

The milking process can be performed twice a day (Vieira, 1994), once in the morning and once in the afternoon (Bettencourt et al., 2008). Roseiro, Wilbey et al. (2003) states that in the artisanal manufacture studied milk was collected twice a day by the cheesemaker himself, from nearby local breeders, and immediately transported to the dairy and made into cheese. At present the milk is normally obtained by machine milking. In this case, it passes through a closed circuit, to a cooling tank, where it remains until it is sent to the dairy in stainless steel containers (Bettencourt et al., 2008; Roseiro, Wilbey et al., 2003).

Once in the dairy, the first step of Serpa cheese manufacture is milk filtration, which aims to remove particulate impurities, typically performed with cloths (Bettencourt et al., 2008;

Canada, 2001; Dias, 1998; Roseiro, Wilbey et al., 2003). Cheesemakers consider filtration to be one of the most important operations of cheese making because, if not carried out under appropriate conditions, it produces lower quality cheese, which needs longer ripening and other defects such as hard centers, excess holes, or fractures in the paste (Bettencourt et al., 2008; Canada, 1998).

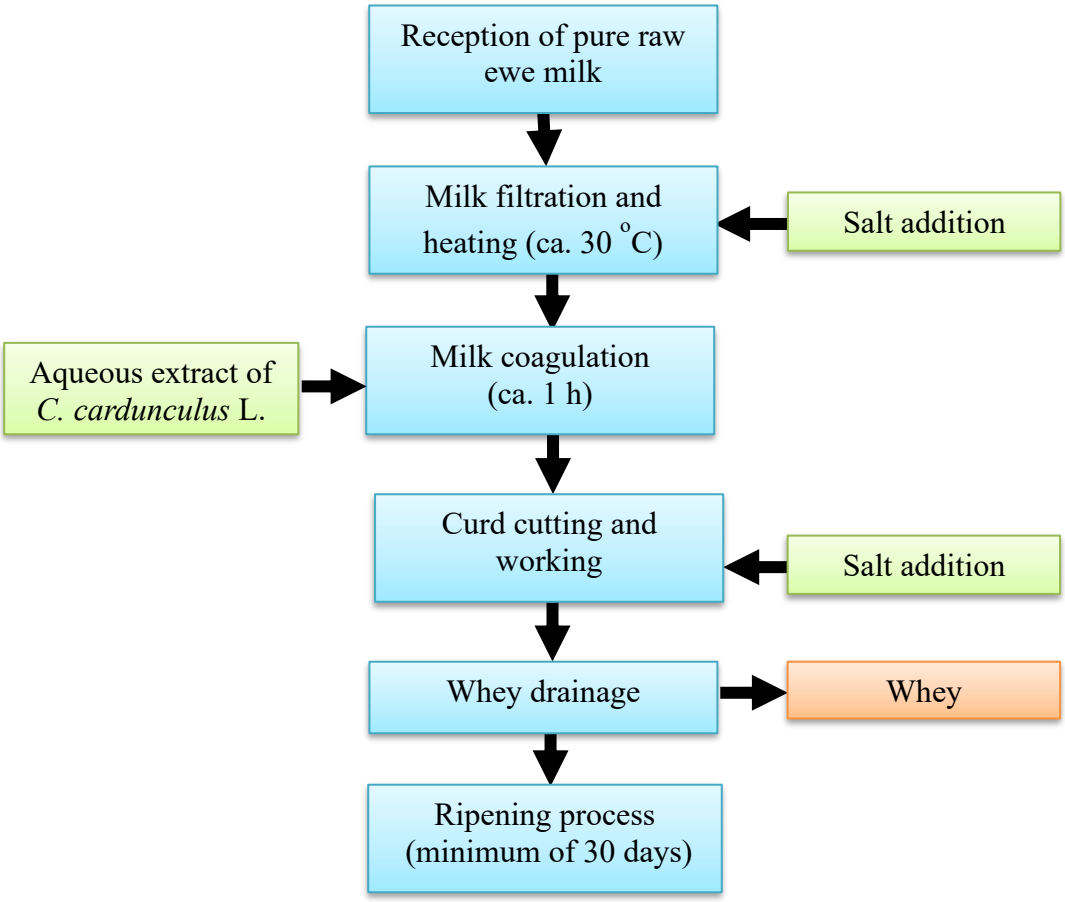

Figure 4 - Flowchart of PDO Serpa cheesemaking procedure.

For this purpose, cotton cloths, folded white wool blankets, or cellulose filters are used (Bettencourt et al., 2008; Canada, 2001; Dias, 1998; Roseiro, Wilbey et al., 2003). One study specifies the use of between 10 and 20 cotton cloths, 1 to 5 wool blankets or about 15 cellulose filters (Dias, 1998), if the milking is manual. In the case of machine milking, filtration may be less stringent since the milking machine has a built-in filter, usually using 1 to 5 wool blankets for filtration (Dias, 1998; Roseiro, Wilbe yet al., 2003). Canada (2001) mentions the use of the most recent filtration processes, such as the use of purifying centrifuges or suitable plastic or stainless metal filters associated with porous cotton elements.

When salt is added during filtration, it is placed between the filter cloths. Salt may also be added immediately prior to the coagulation process or at the same time as the coagulant addition. The amount of salt to add at this stage varies with the authors. Canada (2001) mentions a concentration of 500g of salt per 100L of milk, while Roseiro, Wilbey et al. (2003) mentions 900g/50L, and an amount of 13g per L of milk corresponding to 2/3 of the total amount to be added is still mentioned (Bettencourt et al., 2008). After filtration, the milk undergoes slight heating, only until it reaches the ideal coagulation temperature (28°C - 30°C). It is then taken to the coagulation vat, where the curds are made (Dias, 1998; Roseiro, Wilbey et al., 2003).

I.2.5.2. Milk coagulation

The addition of the thistle flower (*Cynara cardunculus* L.) aqueous extract is made when the milk reaches the desired temperature, about 29°C-30ºC, and the coagulation process takes 45-60 minutes (Alvarenga, 2008;Dias, 1998; Roseiro, Wilbey et al., 2003).

The vegetable coagulant preparation may vary between producers with respect to the preparation technique used and the quantity of dried flowers added. The aqueous extract of *C. cardunculus* L. is done on the day prior to the cheese production. Basically, the thistle flowers and some salt are crushed in a mortar or, most commonly today, in an electric blender, followed by maceration until the following day (Bettencourt et al., 2008; Canada, 2001). Salt improves extraction of the proteinases (Conceição et al., 2018). The filtrate, of purple or brown color, is used as a coagulant (Dias, 1998; Roseiro, Wilbey et al., 2003).

The amount of thistle used per volume of milk depends largely on its coagulant power, milk quality and the amount of salt used (Bettencourt et al., 2008; Canada, 2001). For a suitable coagulant activity, the amount of cardoon extract necessary per liter of ovine milk frequently ranges between 0.2 and 0.6 g (Conceição et al., 2018). Other authors report approximately the same values (Dias, 1998; Roseiro, Wilbey et al., 2003).

I.2.5.3. Cutting and curd work and whey drainage

After the time required for coagulation, the casein gel is destroyed in order to release the serum and facilitate subsequent manufacturing operations. This process begins with cutting the curd through the use of a wooden stick (palheto or fataca), a glass, a metal

strip or even the hands, until it breaks down completely into a smooth grainy mass (Dias, 1998; Roseiro, Wilbey et al., 2003), followed by a 10 to 15 minute rest (Bettencourt et al., 2008).

After the preliminary cutting, that mass is then poured into special perforated cheese molds (cinchos) and pressed with both open hands so as to release as much whey as possible. These operations (work of the curd and drainage) are performed on the top of a sloped table (francela), which facilitates the drainage of the whey into an open vessel where the whey is collected prior to undergoing heat treatment to precipitate the soluble proteins and to produce curd cheese (Dias, 1998; Roseiro, Wilbey et al., 2003). The curd continues to be worked until it becomes sufficiently dry (Roseiro, Wilbey et al., 2003).

Then it is removed from the molds and crumbled between the fingertips, in an operation called “repiso”. This operation transforms the curd into a homogeneous paste, which can then be placed in the final molds. Some cheesemakers take advantage of this operation to make or finish salting (Canada, 2001; Dias, 1998). In Serpa PDO cheese manufacture, a total of approximately 1500 g of salt are added to 100 L of milk (Alvarenga, 2008).

Once in the new molds, the cheeses are then turned over several times, to drain the remaining whey (Alvarenga, 2008; Roseiro, Wilbey et al., 2003). This operation has the dual function of reducing the air spaces inside the mass, and shape the cheese (Dias, 1998). In the semi-industrial scale of Serpa cheese production, the molds are regularly placed in a hydraulic press to facilitate whey drainage (Roseiro, Wilbey et al., 2003), subjected to 3 kg/m^2 pressure for 4 to 5 hours (Dias, 1998).

I.2.5.4. Ripening

The conditions under which Serpa cheese is ripened or matured are very variable between dairies, depending on time of year, size of cheese, cheesemaker rating and market demand (Dias, 1998).

The production rules document (Vieira, 1994) contains some conditions to be met at this stage (Table 3), particularly a minimum cure time of 30 days and, in the case of herds where the health status of brucellosis is unknown, the minimum cure time should be 45 days. This latter period is not in line with the current Regulation (EC) Nº 1662/2006 (EC, 2006b), which states that under these conditions raw sheep milk may only be used in the

manufacture of cheeses with a minimum ripening period of 2 months (60 days), provided that authorized by the competent authority.

Generally, after whey draining, the cheese will go through two maturation rooms. The first with lower temperature and higher humidity, in order to control the onset of microbial growth, and to prevent the cheese surface from drying out quickly. The second, with higher temperature and lower humidity, in order to provide rind consolidation and favour the development of other microbiological species (Dias, 1998; Roseiro, Wilbey et al., 2003). After curing, the cheese is stored in a chamber at a temperature of 0 to 5 ° C, a relative humidity of 77 to 83% and a ventilation of 3 m/s, where it remains until dispatch (Bettencourt et al., 2008).

In the first maturation room of a dairy without control means, average temperature values of 13° C and relative humidity of 90% were recorded (Roseiro, Wilbey et al., 2003). The same authors state that in dairy factories with controlled curing conditions, namely temperature, humidity and air velocity, average temperature values are usually lower and mention 10°C and 88% humidity, in the case studied. At this stage, between the first and second week of curing, a brace is placed (Dias, 1998). Serpa cheese is a semi-soft cheese with lateral bulging, the strapping being placed to preserve its physical characteristics and prevent deformation until consumption (Bettencourt et al., 2008). The length of stay in the first maturation room varies widely and depends on environmental factors, milk type and cheesemaker sensitivity, but is usually between two and three weeks (Dias, 1998; Roseiro, Wilbey et al., 2003).

The cheeses are then transferred to the second ripening room, where it stay until they are finished. Under controlled conditions, the average temperature recorded was 15 ° C and the relative humidity 82%. However, under uncontrolled conditions, temperature values between 9 and 17 ° C and relative humidity between 67 and 100% were recorded for a minimum maturation period of 30 days (Roseiro, Wilbey et al., 2003). Generally, curing takes between three and four weeks in the second chamber, making up a total curing time of five to six weeks (Canada, 2001; Dias, 1998).

Throughout the ripening period, the cheese will be turned and washed with a frequency that depends on the appearance of the crust, which should be kept smooth and clean

(Bettencourt et al., 2008; Dias, 1998). The washing is done with running drinking water, with the aid of a clean brush used exclusively for this purpose (Bettencourt et al., 2008).

I.3. Raw milk cheese microbiology

I.3.1. Raw milk microbiota

Due to its composition, milk is an excellent medium for the development, reproduction, conservation and transmission of many microbial groups. It is usually considered that milk is sterile while in the upper udder of a healthy lactating female, given the absence of contamination sources under these conditions (Montel et al., 2014; Quigley et al., 2013). However, recent research on cow milk (Young, Hine, Wallace, Callaghan, & Bibiloni, 2015) reveals that, even at this level, milk is not as sterile as previously thought and already contains a wide variety of microorganisms. These include *Ruminococcus*, *Bifidobacterium* and *Peptostreptococcaceae*, which probably enter the milk via the enteromammary route.

Before reaching the cheese manufacturing site, milk continues to enrich its own microbiota, which may result from additional contamination or simply from the development of existing flora (Montel et al., 2014; Quigley et al., 2013). Contamination comes from animals, humans, equipment and the environment (Calasso et al., 2016; Montel et al., 2014; Skeie, Haland, Thorsen, Narvhus, & Porcellato, 2019). In turn, milk is a direct and indirect source of microorganisms in cheese and enriches the entire cheese production environment with its microbiota. The biodiversity of cheese is thus linked to the complexity of microbiota in raw milk (Kousta, Mataragas, Skandamis, & Drosinos, 2010).

Raw milk microflora is defined as a dynamic community highly subject to variation. This variability seems to be quite profound between producers, whereas for the same producer there seems to be variability between seasons (Chen, Lewis, & Grandison, 2014; Montel et al., 2014; Nalepa, Olszewska, & Markiewicz, 2018). Skeie et al. (2019) obtained results confirming variability both within and between farms. The author notes that despite the near constant level of bacteria identified in milk (cow`s) from each individual farm, the dominant microbiota differed significantly between the samples. While populations of *Pseudomonas* and *Lactococcus* had similar compositions, *Bacillus* and, especially,

Streptococcus populations changed between samples from the same farm, and from different farms and geographical areas. Of course, hygiene and conservation practices also interfere with this variability (Kousta et al., 2010).

Total culturable counts currently range from 3 to 4 log cfu/ml in raw cow's milk (D'Amico, Druart, & Donnelly, 2010; EFSA, 2015; Montel et al., 2014). Raw milk from sheep and goats generally has a higher bacterial count than milk from cows, ranging from 4 to 5 log cfu/ml (Verraes et al., 2014). Microbiology studies of sheep milk used to make Serpa cheese lead to total count values between 3 and 6 log cfu/ml (Canada, 2001; Roseiro, Wilbey, et al., 2003). In general, regardless of the type of milk, the bacterial counts are far higher than fungal counts (Montel et al., 2014). Table 8 presents the counts of the main microbial groups commonly investigated in milk.

Table 8 - Microbial counts and types of microorganisms in cow, goat and sheep milk (Source: Montel et al. (2014) and references therein).

Microbial Groups	**Cow Milk**	**Goat Milk**	**Sheep Milk**
	(log cfu/ml)		
***Staphylococcus spp.* and coryneform bacteria**	2-3	3	2-4
Lactococcus spp.	1-2	2-3	4
Lactobacillus spp.	1-2	2	3-4
Streptococcus spp.	1-4	_	_
Leuconostoc spp.	1-2	2-3	4-5
Enterococcus spp.	1-2	1-3	3-5
Propionic bacteria	1-2	nd	nd
Enterobacteriaceae	1	5-6	2-4
Pseudomonas spp.	2-3	1-2	2-4
Yeasts	1-2	1-2	2-5
Moulds	<1	<1	_
Aerobic spores	<1	<1	_
Coliform bacteria	<1	2-3	_

nd - Not done

In qualitative terms, Montel et al. (2014) mentions more than 100 genera and 400 species that have been identified among the raw milk microbial flora. These are essentially Gram-negative bacteria (> 90 species), Gram positive catalase bacteria (> 90 species), LAB (> 60 species), yeast (> 70 species) and even mold (> 40 species). The latest molecular

techniques through high-throughput sequencing (HTS) have identified many species other than the normal ones (Masoud et al., 2012; Quigley et al., 2012), pointing to thousands of different taxa detected in bovine raw milk samples, as revised by Skeie et al. (2019). A single milk sample can contain as many as 36 dominant microbial species (Montel et al., 2014).

Refrigeration practices of raw milk before cheese manufacture can, however, completely alter the microbial balance, especially when it is not processed directly at the place of production. Under these conditions, psychotropic bacteria naturally present in milk can proliferate, reaching levels higher than 5 log cfu/ml (Montel et al., 2014; Skeie et al., 2019). These are mainly Gram-negative bacteria of the genus *Pseudomonas spp.*, but also *Acinetobacter spp.* or *Enterobacteriaceae* such as *Hafnia alvei*, all identified as causing milk changes due to their lipolytic and proteolytic activity. These counts may increase by more than 3 log cfu/ml after storage of milk for 3 days at 8º C or 7 days at 4º C, altering the balance of the natural milk flora (Montel et al., 2014). The presence of these microorganisms in milk at concentrations of about 6 log cfu/ml decreases curd yield and quality (Ladenbach & Marshal, 2009).

The microbiological quality of raw milk provides information on both the sanitary conditions of the product and its hygienic quality. At European level (EC, 2006a), on microbiological criteria for foodstuffs, concerning the use of raw milk of species other than cow in the manufacture of products made from raw milk by a process that does not include heat treatment, establishes as a criterion the plate count at 30 ° C, a count ≤ 5.70 log cfu/ml (Table 4).

I.3.2. Microbiology of raw milk cheese

One of the main characteristics of cheeses produced from raw milk is that they harbor a complex microbial community. Over the past 15 years, microbial diversity studies of several varieties of cheeses that combine both genotypic and phenotypic approaches have partly described the complexity of such communities (Irlinger, Layec, Hélinck, & Dugat-Bony, 2015). It has been shown that each cheese has a specific, dense microbiota made up of a few to several dozen species. Importantly, the microorganisms detected originated from the milk itself and/or the manufacturing environment (Calasso et al., 2016; Jin et al., 2018; Kousta et al., 2010; Wouters, Ayad, Hugenholtz, & Smit, 2002).

Species/strains present can survive, develop and even become dominant throughout the cheese production process. This will depend on each one's metabolic potential under the different environmental conditions to which they will be subjected (Calasso et al., 2016; Montel et al., 2014; Pereira et al., 2010). The microbial dynamics will be conditioned mainly by nutrient availability, temperature, acidity, salt concentration, relative humidity and gas concentration in the environment (Beresford et al., 2001; Caplice & Fitzgerald, 1999; Wouters et al., 2002). These conditions provide intense and constant modifications of the microbiota present throughout the process and even between the inside and the surface of the cheese (Montel et al., 2014).

I.3.2.1. Cheese core

I.3.2.1.1. Firmicutes

Dominant species differ according to ripening time and between varieties of cheese. However, LAB are usually the dominant microbial group, reaching levels of about 8-9 log cfu/g on the first day of production and remaining dominant until the end of cure, despite changes in species balance throughout maturation (Calasso et al., 2016; Montel et al., 2014; Luca Settanni & Moschetti, 2010). At least 21 species spanning 7 different genera have already been identified in this product (Beresford et al., 2001; Montel et al., 2014). Dominant genera are *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Enterecoccus*. Among these, the most frequent species are *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, L*actobacillus casei*, *Enterococus faecalis* and *Enterococus faecium* (Montel et al., 2014). Different research works on microbiological characterization of raw milk cheese refer to the presence of this type of microorganisms, despite the specificity of each cheese (Feutry, Oneca, Berthier, & Torre, 2012; Fuka, Engel, Skelin, Redzepovic, & Schloter, 2010; Ordiales, Benito, et al., 2013; Picon, Garde, Ávila, & Nuñez, 2016; Riquelme et al., 2015; Tavaria & Malcata, 1998).

The LAB involved in cheese production are divided into dominant ones, also known as starters lactic acid bacteria (SLAB), and secondary, also known as adjuvant or non starter (NSLAB) cultures. SLAB dominant LAB have as their main function the production of acid during fermentation, with the consequent decrease of pH, essential for curd formation and inhibition of undesirable microorganisms, but also contributing to the maturation process (Afzal et al., 2017; Beresford et al., 2001; Montel et al., 2014; Pogacic

et al., 2015). The SLAB group mostly includes *Lactococcus lactis* and *Leuconostoc spp.* among mesophilic species and *Streptococcus thermophilus*, *Lactobacillus delbrueckii* and *Lactobacillus helveticus* among thermophilic species (Fox, McSweeney, Cogan, & Guinee, 2004).

NSLAB is a more heterogeneous group that participates in the maturation process mainly due to their proteolytic action and also in the development of specific aromas using amino acids (Afzal et al., 2017; Beresford et al., 2001; Montel et al., 2014; Pogacic et al., 2015). They are mostly mesophiles of the genera *Lactobacillus*, but also *Pediococcus*, *Enterococcus* and *Leuconostoc*, with long milk generation times and little acidification (Marco Gobbetti, De Angelis, Di Cagno, Mancini, & Fox, 2015; M. Gobbetti et al., 2002). SLAB are high in number at the beginning of ripening and decrease regularly by two or more log cycles during ageing. On the contrary, NSLAB are present at low concentrations after pressing which, however, may increase of about four or five orders of magnitude within a few months (Fox et al., 2004). NSLAB are traditionally selected as a means of determining the organoleptic characteristics of the final cheese (Beresford et al., 2001; Fox et al., 2004), but also on the basis of their health benefits (enhancement of intestinal probiosis, production of bioactive peptides, generation of gammaaminobutyric acid (GABA) and inactivation of antigenotoxins (Settanni & Moschetti, 2010).

To a lesser extent, the presence of bacteria of the genus *Staphylococcus* is observed in the core. Normal is the presence of at least four species and counts of 5 log ufc/g. Recent studies show that this concentration may increase if there is a high contamination in the milk and / or skin of the handlers, reaching the final product at 6-7 log ufc/g. The presence and increased concentration of this microorganism throughout manufacture is due to the fact that it easily attaches to the curd matrix and its optimum growth in milk (Rola, Czubkowska, Korpysa-Dzirba, & Osek, 2016). Results of a study on a Portuguese raw sheep milk cheese with 30 days of ripening showed concentrations of this group of 5,8 and of 6,0 log ufc/g, respectively on the surface and in the cheese core (Soares, Marques, Tavaria, Malcata, & Pintado, 2009; Soares et al., 2011). In this work, the identified species were *S. saprophyticus*, *S. aureus*, *S. epidermidis*, *S. chromogenes*, *S. simulans*, *S. lentus*, *S. sciuri*, *S. equorum*, *S. haemolyticus* and *S. caprae*. The predominant ones were *S. equorum* (32,7%) and *S. saprophyticus* (25,2%). Some coagulase positive staphylococci appear to be of technological importance, notably, *S. xylosus*, *S. carnosus*,

and S. *equorum;* however, some species in this group may pose a medical risk at various levels, including food poisoning (Soares et al., 2011). In quantitative terms follow the bacteria of the group *Clostridiales.*

I.3.2.1.2. Proteobacteria

Montel et al. (2014) and references therein, refers to the group of Proteobacteria as the second most important in number, reaching counts as high as 8 log ufc/g. This group includes genera belonging to the *Enterobacteria*, such as *Enterobacter, Klebsiella, Citrobacter* and *Hafnia alvei*, but also the genera *Pseudomonas, Stenotrophomonas* and *Psychrobacter*. Enterobacterial counts, in particular, can reach 6-7 log cfu/g in the first days of ripening, but then begin to evolve more or less slowly, depending on the type of cheese (Coton et al., 2012; Tabla et al., 2016). The same evolution was observed for the presence of *E. coli* but with lower initial concentrations (1-2 log ufc/g) and a more abrupt reduction in its concentration; it may disappear within 30-60 days of maturation (Peng, Schafroth, Jakob, Stephan, & Hummerjohann, 2013; Tabla et al., 2016). These authors state that, over the course of ripening, this group tends to become more homogeneous, being dominated by *H. alvei* in the case of raw milk sheep cheeses.

Although *Enterobacteria* are recognized as the microbiota of this type of product, their presence generates some controversy for both sanitary and technological reasons. On the one hand, given their intense proteolytic and lipolytic activity, they are related to specific characteristics of artisanal cheeses, namely in their aromatic composition (increasing concentration of aldehydes, ketones and sulfur compounds). However, this activity is not always beneficial for cheese. Additionally, this group includes potential pathogens and their presence is of concern. They are related to faecal contamination and, therefore, the level of this contamination is used as an indicator of hygiene. Finally, gas production by many of these bacteria may be related to early cheese defects, namely eye overflow and destruction of structure by bursting and cracking (Coton et al., 2012; Montel et al., 2014; Tabla et al., 2016).

I.3.2.1.3. Other bacteria

The group *Acrinobacteria* has also been detected at concentrations of about 4 lof ufc/g, belonging to at least 4 genera, the most common being *Corynebacterium, Arthrobacter* and *Brevibacterium.* Other prokaryotes considered as smaller populations but sometimes identified in this product were *Chryseobacterium* and *Prevotella* (Montel et al., 2014).

I.3.2.1.4. Fungi

Fungi, namely yeast and mould, although growing slower than LAB, are also important, especially in the ripening phase. Due to the lipolytic and proteolytic activity, fermentation of residual lactose and assimilation of citric and lactic acids contribute to the development of cheese aroma and rheological properties (Andrade, Melo, Genisheva, Schwan, & Duarte, 2017; Delamare, Andrade, Mandelli, Almeida, & Echeverrigaray, 2012.; Padilla, Belloch, López-Díez, Flores, & Manzanares, 2014; Pereira-Dias, Potes, Marinho, Malfeito-Ferreira, & Loureiro, 2000). For the same reasons, they can also contribute to cheese deterioration (Pereira-Dias et al., 2000). It was also reported that they are able to inhibit growth of the pathogens or have a probiotic potential. Isolates with antimicrobial activity against food-borne pathogens may be helpful in ensuring the hygienic quality of dairy products, if used as adjunct cultures. This is very important, especially on the surface of the cheese where they act as a barrier against pathogens and spoilage microorganisms, most often through the production of killer toxins or “mycocins”.

The occurrence of yeast in cheeses is usually associated with conditions of low pH, low moisture content, high salt concentration and low temperatures. These are mainly on the surface and appear in cheese via a variety of sources such as milk, curd, equipment, brine and others (Padilla et al., 2014). *Debaryomyces hansenii* is the dominant yeast species in many cheeses but *Kluyveromyces lactis* and *Kluyveromyces marxianus* are also found. Other species present in cheese are *Yarrowia lipolytica, Geotrichum candidum* and *Saccharomyces cerevisiae* (Cardoso et al., 2015; Padilla et al., 2014).

In the cheese core there is the development of the yeast population in the order of 2-6 log cfu/g. Data available in different publications on yeast counts (log ufc/g) during cheese ripening period or in the final product and identification of the isolated yeasts in raw ewes milk cheeses with vegetal renet from the Iberian Peninsula are reported in Table 9.

Table 9 - Yeast counts (log ufc/g) during cheese ripening period or in the final product and identification of the isolated yeasts in raw ewes milk cheeses whit vegetal renet from the Iberian Peninsula.

CHEESE	Yeast counts (log ufc/g)						Yeasts Identification	Identification Methodology	Bibliographic Source
		Days of Ripening							
		0	2	30	30-45	60			
Torta del Casar PDO	–		3,85-4,38	3,53-4,48	–	2,92-3,86	*Candida zeylanoides*; *Candida parapsilosis*; *Rhodotorula mucilaginosa*; *Yarrowia lipolytica*	16S rRNA gene sequencing analysis and rRNA gene internal transcribed spacer (ITS) regions Sequences were compared with the EMBL and GenBank database using the BLAST algorithm	(Ordiales, Benito, et al., 2013)
La Serena PDO	_		_	_	_	3,24-4,69	_	_	(Canada, 2001)
Los Pedroches	_		3.5	1,77	_	0,87	_	_	(Vioque et al., 2000)
Serra da Estrela PDO	0,47-7 (Decreased by 15% from day 1 to the end of the maturation period)						*Cryptococcus humicola*; *Cryptococcus curvatus*; *Sporidiolobus johnsonii*; *Tremella foliaceae*; *Torulaspora delbrueckii*; *Rhodotorula glutinis*; *Rhodotorula hylophila*; *Rhodotorula minuta*; *Rhodotorula ingeniosa*; *Pichia membranaefaciens*; *Kluveromyces lactis*; *Debaryomyces hansenii*; *Trichosporon aquatile*; *Leucosporidium scotii*; *Sporolobomyces roseus*; *Candida rugosa*; *Candida zeylanoides*; *Candida etchellsii*	API ID 32C (BioMérieux)	(Dahl, Tavaria, & Malcata, 2000; Reis & Malcata, 2011; F. K. Tavaria & Malcata, 1998; Freni K. Tavaria & Malcata, 2000)
Évora PDO	2.7- 6.4 (With the higher counts observed after a ripening period of 30 days)						*Candida curvata*; *Candida famata*; *Debaryomyces hansenii*; *Candida humicola*; *Candida intermedia*; *Candida parapsilosis*; *Candida zeylanoides*; *Rhodotorula minuta*; *Rhodotorula glutinis*; *Rhodotorula rubra*; *Pichia carsonii*; *Pichia etchelsii*; *Trichosporon cutaneum*	API ID 32C (BioMérieux)	(Pereira-Dias et al., 2000)
Azeitão PDO	_		_	_	_	6,00	_	_	(Freitas & Malcata, 2000)
Serpa artesanal PDO	_	5,64	_	5,1	3,28-4,33*	3,00	_		(Canada, 2001; Roseiro, Wilbey, et al., 2003)
Serpa Semi-industrial PDO	_	4,04	_	3,36	2,60*	2,46	_	_	(Canada, 2001; Roseiro, Wilbey, et al., 2003)

I.3.2.2. Cheese rind

Unlike the core, the cheese surface is a very different and more open ecosystem where a greater diversity of genera and species can be observed, whether eukaryotic or prokaryotic. The characteristics of the cheese rind help define the type of cheese and largely determine its taste. They range from simple to complex assemblages harbouring *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, yeasts and moulds (Irlinger et al., 2015). The latter, as well as aerobic bacteria like *Corynebacteriaceae* and *Micrococcaceae*, seem to develop more easily here than inside cheese.

At the beginning of the cheese-making process, LAB are dominant in the cheese rind. However, in the early days (2-7 days) of ripening, yeast and/or mould begin colonization of the cheese surface. The yeast count is on the order of 6 to 8 log cfu/cm^2 and remains approximately constant until the end of ripening.

Yeasts and moulds metabolize lactic acid and produce ammonia, which increases the pH from 4,8-5,2 to 6,0-8,2 (Montel et al., 2014). This change facilitates the development of acid-sensitive but salt-tolerant bacteria (Irlinger et al., 2015; Montel et al., 2014). The final bacterial cell count is 1–2 log units higher than the yeast cell count (Montel et al., 2014).

It can be recognized that yeasts are in greater amount in the surface of the cheese, where they can reach a maximum in the first few days of ripening. These numbers can reach 10^6–10^9 cfu/g or 10^6–10^8cfu/cm^2. Thereafter, the population remains at a nearly constant level of about 10^7cfu/g or decrease slightly during ripening. According to Irlinger et al. (2015) and references therein, and having in mind the results obtained from 33 investigations into the cheese rind, 104 bacterial genera can be distinguished, ranging from 3 to 30 depending on the cheese variety (1 *Acidobacteria*, 28 *Actinobacteria*, 5 *Bacteroidetes*, 24 *Firmicutes* and 46 *Proteobacteria*) and 39 fungal genera, ranging from 1 to 11 (21 moulds and 18 yeasts), on the surface of cheese.

Among the yeast's genera identified, *Debaryomyces*, *Yarrowia*, *Candida* and *Geotrichum* were the most frequent, followed by *Kluyveromyces* and *Pichia*. Montel et al. (2014) and references therein, refers to species *D. hansenii*, G. *candidum*, *C. catenulata*, *K. lactis* and

Y. lipolytica. The filamentous fungi *Penicillium* was the most frequent, followed by *Scopulariopsis* and *Fusarium* (Irlinger et al., 2015 and references therein).

Between the *Firmicutes*, *Staphylococcus* and the LAB of the genus *Lactococcus*, *Enterococcus*, *Lactobacillus*, *Streptococcus* and *Vagococcus* and halophilic LAB such as *Marinilactibacillus* and *Facklamia*, were the most frequent (Irlinger et al., 2015; Montel et al., 2014). Considering the *Actinobacteria*, *Brevibacterium*, *Corynebacterium* and *Arthrobacter* were the most frequent genera, followed by *Brachybacterium*, *Microbacterium*, *Agrococcus* and *Micrococcus*. The genera *Psychrobacter*, *Halomonas*, *Pseudoalteromonas* and *Vibrio* (which are all halotolerant *Proteobacteria*) are also major cheese rind microorganisms (Irlinger et al., 2015 and references therein).

I.3.3. Raw milk cheeses microbiological safety

Cheeses are characterized by a set of intrinsic factors that contribute to their preservation and make them less perishable than milk, including a low pH (less than 5.3), salt content from 1,5 to 5,0% which reduces water activity (a_w) and low redox potential. Low temperature, as an extrinsic factor during maturation and conservation, also contributes to this stability (ICMSF, 2006). In addition, the large amount and diversity of microorganisms naturally present in cheese control pathogen proliferation. LAB, the main endogenous flora of this type of cheese, produce antimicrobial compounds such as bacteriocins, organic acids and hydrogen peroxide, which also contributes to the microbiological safety of cheese (ICMSF, 2006; Yoon, Lee, & Choi, 2016).

However, cheeses made from raw milk are among the products that give rise to some discussion and concern about their safety, being classified by some authors as risky foods. Indeed, cases and outbreaks of food poisoning have been linked to their consumption (Brooks et al., 2012; Verraes et al., 2015; Yoon et al., 2016).

Food safety hazards (physical, chemical and microbiological) can enter the dairy supply chain at various points, via animal feed, through the dairy farm environment, or during further processing. In raw milk cheese contamination, the raw material constitutes a potential source of microbiological hazards. While many of these microorganisms reach the milk from the surrounding environment, equipment and/or personnel, zoonotic pathogens can also be introduced into milk from unhealthy animals (Asselt, der Fels-

Klerx, Marvin, Bokhorst-van de Veen, & Groot, 2017; Guerreiro, Velez, Alvarenga, Matos, & Duarte, 2013; Kousta et al., 2010; Quigley et al., 2013). Figure 5 shows the main contamination routes and hazards associated with various dairy products, including raw milk cheese regardless of the producing species.

Data available in literature concerning microbiological hazards associated with milk and cheese specifically from goats and sheep are quite scarce (Asselt et al., 2017; Verraes et al., 2014). Some sources indicate that this milk can contain the human pathogens *Salmonella* spp., *Campylobacter* spp., Shiga-toxin-producing *Escherichia coli* (STEC), L*isteria monocytogenes*, *Staphylococcus aureus*, *Streptococcus* spp., *Coxiella burnetii*, *Helicobacter pylori*, *Brucella* spp., *Toxoplasma gondii*, *Mycobacterium* spp., or tick-borne encephalitis virus (TBEV) (Fotou et al., 2011; Verraes et al., 2014). This list largely corresponds to the most relevant hazards for raw milk in general, presented in Figure 5. Verraes et al. (2014) concludes that the main microbiological hazards that appear to be associated with raw sheep and goat milk are STEC and *Campylobacter* spp., followed by *Brucella* spp. and TBEV, for being associated with outbreaks. However all outbreaks identified by the author were due to the consumption of raw goat milk. According to a recent EFSA scientific opinion report, *Salmonella spp.* was also a main hazard for goat and sheep milk, and *Campylobacter spp.* the leading cause of outbreaks in the period concerned (EFSA, 2015). These contaminants may manifest themselves in the cheese produced.

Indeed, raw milk cheese may be associated with the transmission of diseases such as brucellosis, tuberculosis, botulism and infections caused by *E. coli*, *Salmonella* spp., *St. aureus* or *Campylobacter*, being the biggest fears those related to the possible transmission of *L. monocytogenes* (Figure 5) (Verraes et al., 2015; West, 2008; Yoon et al., 2016). Unlike hard cheeses, soft and semi-soft cheeses with high moisture content allow different pathogens to grow (Asselt et al., 2017). Additionally, *L. monocytogenes* can grow even during refrigerated storage. For this reason, children, the elderly, people with a weakened immune system, and especially pregnant women are advised to avoid consuming this type of product (FDA, 2018; West, 2008).

EFSA/ECDC (2018) report, on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017, stresses that for *Campylobacter* in milk and cheeses the overall occurrence was lower than 2%. The only positive cheese samples (3/522) were from retail

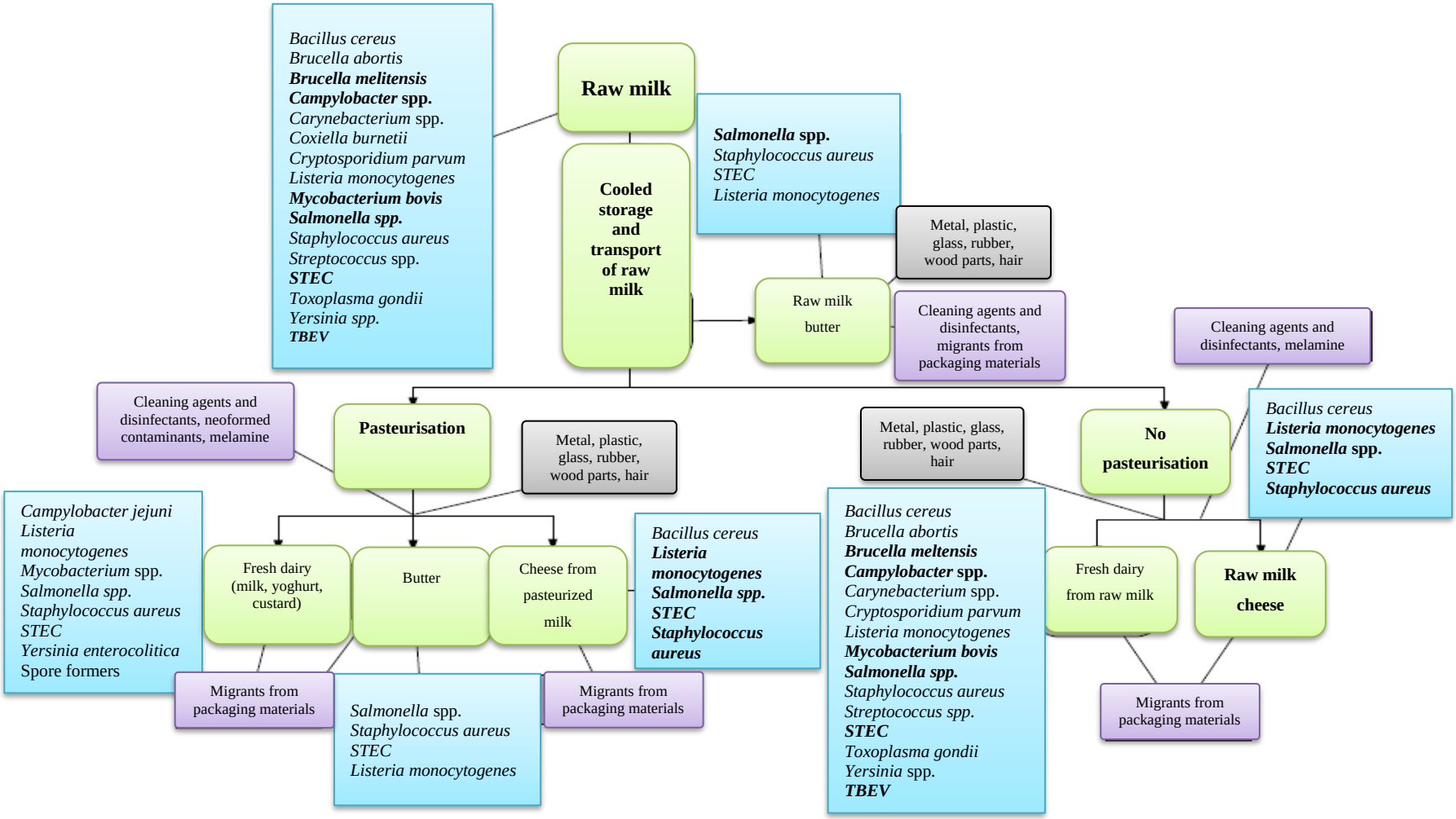

Figure 5 - Overview of microbiological (blue), chemical (purple), and physical (green) hazards (most important in bold) reported in scientific literature for dairy products processed at the dairy farm. Data available at European Food Safety Authority (EFSA), *Listeria monocytogenes* Raiid Alert System for Food and Feed (RASFF) for data notifications of food safety hazards in milk and milk products within the EU during 2009 to 2014, and the Dutch monitoring program on chemical hazards (2009 to 2013) as well as expert information (Adapted from Asselt et al., 2017).

level sheeps cheese reported by a single member state (MS). Regarding STEC, the highest proportion of positive units was reported in treated milk samples (4,0%) followed by cheeses (1,3%). None of the samples of dairy products were positive for STEC O157.

In the same report, and considering the data of occurrence in ready to eat (RTE) food samples from all sampling stages (processing, retail, border inspections and unspecified) in 2017, *L. monocytogenes* occurrence in soft and semi-soft cheeses and hard cheeses was respectively of 0,9% and 0,6%.

Among retail samples, positive results for sheep cheese are reported by MS Portugal (EFSA/ECDC, 2018). Regarding the occurrence of *L. monocytogenes* in different types of cheese, the number of positive units was higher in soft and semi-soft cheeses made from raw or low-heat-treated (LHT) milk (Figure 6).

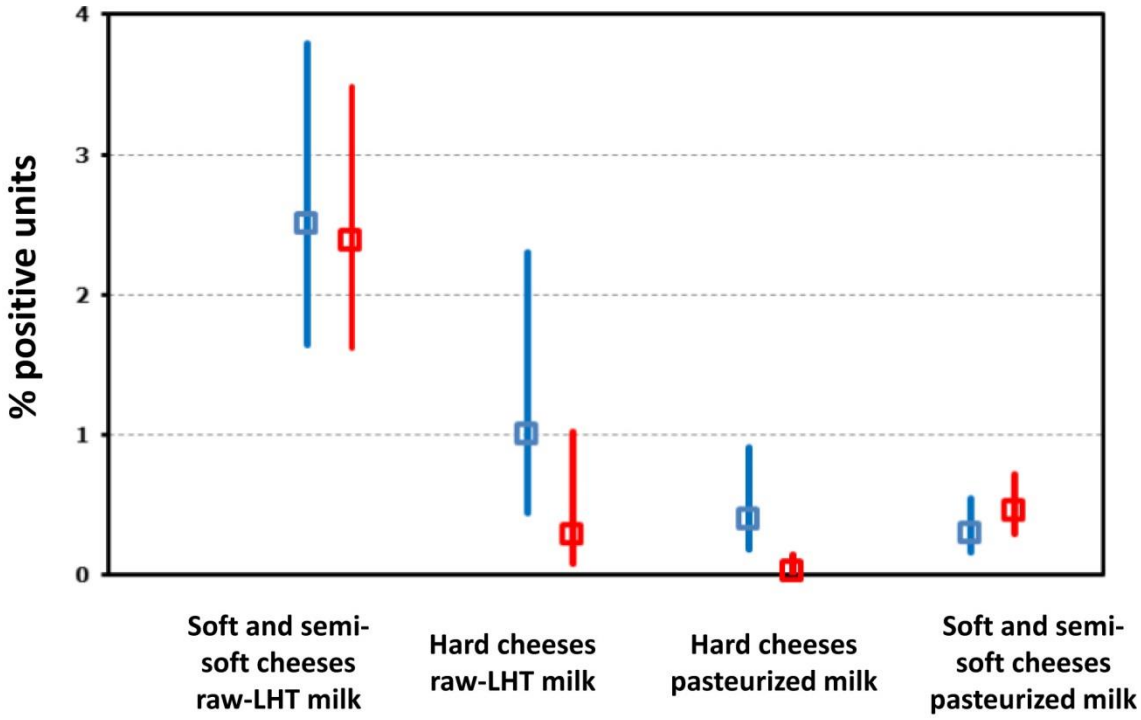

Figure 6 - Proportion of *L. monocytogenes* positive sampling units in cheeses in 2017 (red) and in 2016 (blue) across all sampling stages (overall), retail and processing plant levels (Source: EFSA/ECDC, 2018).

Comparing cheeses made from raw or LHT milk or pasteurised milk, the positive case results were higher for raw or LHT milk cheeses, whether soft and semi-soft or hard cheeses (Figure 6). These results are generally consistent with the 2016 and previous data (Asselt et al., 2017; EFSA/ECDC, 2018). The positive cases in hard cheese with raw milk

were found in cow cheese, both in 2016 and 2017. In the same period, the positive cases in soft and semi-soft cheese with raw milk were mostly observed in sheep cheese.

With regard to food-borne outbreaks (FBO) by food vehicle in 2017 (EFSA/ECDC, 2018) (Figure 7), the set 'Milk and milk products' was associated with a large variety of causative agents. However, *Campylobacter* was the causative agent implicated in most of the strong-evidence outbreaks of this food group mainly associated with the consumption of milk, such as STEC (Figure 7), namely raw unpasteurised and unheated milk, as in recent years. The causative agent associated with cheese consumption was mainly *Salmonella*, but also *L. monocytogenes* and STEC.

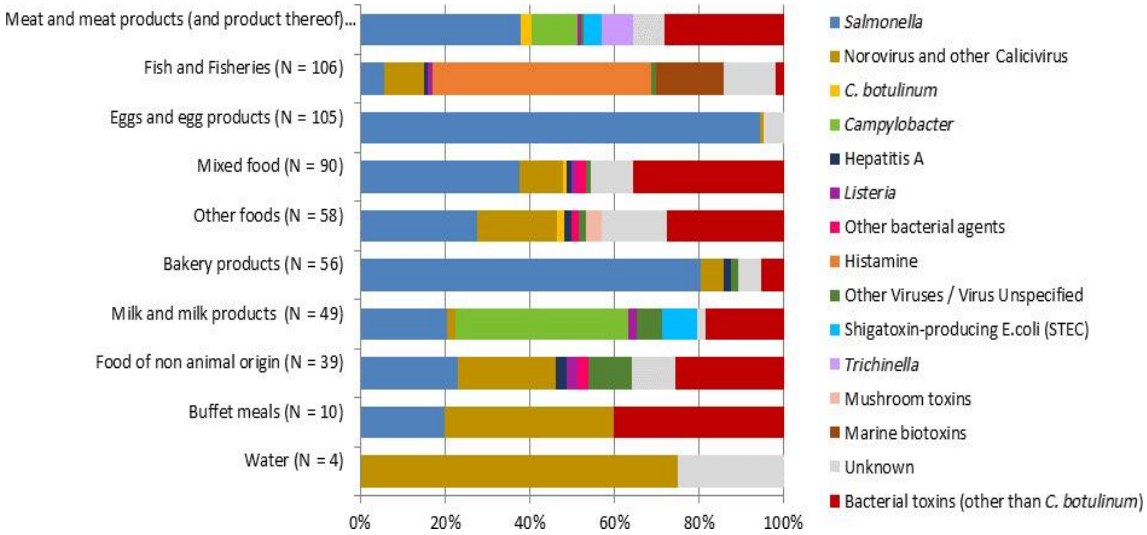

Figure 7 - Frequency distribution of causative agents associated with strong evidence food borne and waterborne outbreaks, by food vehicle. (Milk and milk products include cheese, dairy products other than cheeses and milk) (Source: EFSA/ECDC, 2018).

The food vehicle pair "Milk and milk products" is among the 2017 top ten of pairs causing the highest number, cases, hospitalizations and deaths following strong-evidence FBO. In most of these situations in 2017, there was an increase in the number of cases compared to the annual average observed in the period between 2010 and 2016, which may be over 50%. Regarding the number of strong-evidence FBO, this pair was responsible for 9,9% of total cases (Cheeses-6,4%; Milk-3,2%; Other- 0,3%). It was the food vehicle associated with the highest number of cases (487) of food poisoning by bacterial toxins other than *Clostridium botulinum* (toxins produced by *Bacillus*, *Clostridium* other than *Clostridium botulinum* and *Staphylococcus* and other unspecified bacterial toxins). It was also among the vehicles most associated with hospitalizations (38), in this case due to the transmission

of *Salmonella*. Finally, it was also associated with strong-evidence FBO which resulted in deaths (1) due to STEC (EFSA/ECDC, 2018).

The data collected in recent years thus seem to point to unsatisfactory results more associated with raw milk cheeses. Yoon et al. (2016), comparing this type of cheese with cheese from pasteurized milk, points out advantages and disadvantages of each concerning microbiological safety. They point out that the presence and antimicrobial activity of the natural flora of raw cheese, especially LAB, seems to reduce the microbiological risk compared to cheeses made with pasteurized milk; however, this antagonistic activity is not consistent as the contaminating microflora varies according to the origin of the cheese. Additionally, not all potential cheese contaminant pathogens are inhibited and some remain at detectable levels. This effect also depends on the type of pathogen present and its initial concentration. On the other hand, pasteurizing milk destroys the harmful but also the beneficial flora. In addition, sporulated pathogens such as *Clostridium spp.* can resist pasteurization and multiply actively, as there is no competitive flora. Also, contamination can happen after heat treatment. The same author concludes that the microbiological risk associated with raw milk cheese can always be reduced by constantly monitoring hygiene in the milk and cheese production environment and storage places and, if necessary, with a maturation of at least 60 days.

The United States law on soft ripened cheeses (FDA, 2019), maintains the minimum maturation period of 60 days for that kind of cheese made with unpasteurized milk and maturation at temperatures not below 35 deg. F (Boor, Wiedmann, Murphy, & Alcaine, 2017; FDA, 2016). This procedure has been adopted by other countries, namely Canada (Boor et al., 2017). European rules on food hygiene only require two months of ripening if raw milk for cheese production comes from goats or sheep from herds that do not meet the requirements for brucellosis and tuberculosis control (EC, 2004a).

Some authors argue that 60 days of maturation may not guarantee the absence of pathogens, namely *L. monocytogenes* (D'Amico, Druart, & Donnelly, 2008; D'Amico et al., 2010). However, studies conducted by some researchers and the FDA between 2014 and 2016 (Brooks et al., 2012; FDA, 2016) conclude that the risk after this time is minimal. This agency points out that, to ensure consumer safety, it is sufficient to identify and monitor more rigorously producers with indicators of poor hygiene and safety, testing both raw milk and finished product (FDA, 2015, 2016).

Interestingly, the study on the evaluation of the risk of listeriosis associated with the consumption of soft raw milk cheeses carried out by Health Canada and the FDA (FDA, 2015), despite recognizing the greater risk of listeriosis associated with the consumption of this type of cheese, even at 60 days of age, also reveals a slight decrease in this risk associated with the consumption of the same cheeses with less than 60 days of ripening. They justify that a shorter maturation period does not allow the growth of *L. monocytogenes* to the point of being harmful. This analysis supports the decision in some countries to remove 60-day aging periods for certain cheeses (Boor et al., 2017).

Effectively controlling the microbiological quality of cheese made from raw milk provides information on both the sanitary conditions of the product and its hygienic quality. At European level, Regulation (EC) Nº 2073/2005 (EC, 2005a) on microbiological criteria for foodstuffs defines the microbiological safety and hygiene criteria to be met by this product. The microbiological criteria referred to are presented in Table 5. In order to ensure consumer safety, as already defined for Serpa cheese, it imposed the implementation of the HACCP preventive system (Regulation (EC) Nº 852/2004) (EC, 2004a) and the monitoring of hygiene procedures, from herd feeding and health to the final stages of production and distribution and marketing (Regulation (EC) Nº 853/2004) (EC, 2004b), bearing in mind that these are "food products with traditional characteristics" (Regulation Nº 2074/2005) (EC, 2005b).

I.3.4. High throughput sequencing in the study of dairy microbial ecology

As stated above, one of the main features of cheeses made from raw milk is that they harbor a complex microbial community, which can be highly variable in the composition and abundance of its constituent species, being considered as a microbiologically dynamic matrix. Thus, molecular approaches involve DNA, and occasionally RNA, based molecular methods, with high throughput sequencing (HTS) emerging technologies, now offer an unprecedented opportunity to profile dominant as well as subdominant cheese microbial populations on a large scale (Calasso et al., 2016; De Filippis, Parente, & Ercolini, 2017; Eric Dugat-Bony et al., 2016; Jonnala, McSweeney, Sheehan, & Cotter, 2018; Kamimura, De Filippis, Sant'Ana, & Ercolini, 2019). Additionally, metagenomics conducted with shotgun libraries can aid in the exploration of both taxonomic composition and metabolic activities and interactions within the cheese microbial community (Ercolini, 2017).

Initial surveys of microbial community diversity, including in dairy products, applied cultivation-based methods. These techniques begin with plating samples on suitable agar and picking isolated colonies for subsequent identification using morphological, biochemical and later, molecular characterization, followed by the characterization of the technological properties of the isolates (Londono-Zapata, Durango-Zuleta, Sepulveda-Valencia, & Herrera, 2017; Lusk et al., 2012). This approach, besides being very laborious, is often biased and of limited value due to the inability to cultivate most (approximately 99%) naturally occurring species (Beresford et al., 2001; Bokulich & Mills, 2012; Cocolin & Ercolini, 2015; Degnan & Ochman, 2012; Giraffa & Neviani, 2001; Jünemann et al., 2017; Lusk et al., 2012; Quigley et al., 2011). In addition, the stressful conditions of some food systems, particularly resulting from fermentations, may induce a viable but uncultivable state in microorganisms, preventing culture-based detection (Lusk et al., 2012).

These techniques have been replaced by a second approach focused on culture-independent methods, in which the total DNA from a sample is extracted and analysed to characterize the microbial community (richness and relative abundance) using molecular techniques (Londono-Zapata et al., 2017). However, cultivation and isolation of microorganisms is strictly necessary when, in addition to microbiological characterization, it is intended to develop for example starter cultures, as well as studies on food spoilage. In these cases, the identified microbial isolates are essential for assessing individual contribution, potential interactions and sensitivity to food processing conditions (Cocolin & Ercolini, 2015). Additionally, many publications highlight the benefits of using a polyphasic approach, ie the parallel use of culture-dependent and culture-independent strategies in characterizing microbial populations, claiming their complementarity (Quigley et al., 2011). This dual strategy has been used in several studies on the microbiological characterization of cheeses (Aldrete-Tapia, Escobar-Ramirez, Tamplin, & Hernandez-Iturriaga, 2014; Delcenserie et al., 2014; Ercolini, De Filippis, La Storia, & Iacono, 2012; Lusk et al., 2012).

Molecular tools, based on nucleic acids sequence detection, came to be used to characterize microbial communities due to their higher speed and accuracy. Indeed, the advent of the era of molecular biology, in the 1950s, and the subsequent emergence of new technologies, like Sanger sequencing in the 1970s and polymerase chain reaction

(PCR) in the 1980s, had a positive impact on all areas of biology (Bokulich & Mills, 2012; Liu et al., 2012; O'Flaherty & Klaenhammer, 2011). Subsequently, numerous culture-independent methods were developed using as their basis PCR amplification, cloning and Sanger sequencing of universally conserved molecules (first generation sequencing), usually the 16S ribosomal RNA gene (16S rRNA) in the case of bacteria, and internal transcribed spacer (ITS) genes in the case of fungi (De Filippis et al., 2017; Degnan & Ochman, 2012; Kergourlay, Taminiau, Daube, & Verges, 2015; O'Flaherty & Klaenhammer, 2011). In general, culture-independent methods were faster, more sensitive and less susceptible to bias than culture-dependent methods (Quigley et al., 2011), allowing the identification of microbial groups not detected by conventional methods.

Among the culture-independent methods for microbial communities characterization, we can now distinguish between first generation, second or next-generation sequencing (NGS) or “short-read” technologies, and newer third generation technologies (TGS) or "long-read" sequencing (Bokulich & Mills, 2012; van Dijk, Jaszczyszyn, Naquin, & Thermes, 2018). Table 10 provides a brief characterization of the three generations of sequencing technologies most commonly used in the context of independent culture methods for the characterization of microbial profiles.

First generation profiling methods, developed at the beginning of 1990s, include for example denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (TRFLP), restriction fragment length polymorphism (RFLP) and several other automated PCR-based techniques (Bokulich & Mills, 2012; Degnan & Ochman, 2012; Kergourlay et al., 2015; Liu et al., 2012; O'Flaherty & Klaenhammer, 2011; Quigley et al., 2011). PCR-DGGE has received a great deal of attention since it was first adopted for studying bacterial communities (Bokulich & Mills, 2012), being in widespread use in the microbiological characterization of cheese (Table 10). In this technique, a fingerprint analysis is performed, in which the microbiota is visualized as band patterns (Cocolin & Ercolini, 2015). The resolution of these techniques is limited, as only intense and well separated bands can be sequenced in the profiles. So, the information obtained about the microbial consortia and their individual members is also limited (Cocolin, Alessandria, Dolci, Gorra, & Rantsiou, 2013; Jünemann et al., 2017).

Further evolution was stimulated by the advent of high-throughput sequencing (HTS) and information technologies in the mid 2000s, which gave rise to NGS (Table 10), that, combined with powerful bioinformatic approaches, became ubiquitous in microbial ecology studies (Bokulich & Mills, 2012; De Filippis et al., 2017; Jagadeesan et al., 2019; Kergourlay et al., 2015; Liu et al., 2017), including those of cheese (Table 10). HTS entails higher sensitivity allowing in-depth community measurement at relatively low cost compared with traditional first-generation culture-independent methods, uncovering also low abundant microorganisms (De Filippis et al., 2017; Kergourlay et al., 2015). A study in which this technology was applied (Masoud et al., 2012) has revealed a diverse subdominant population which went undetected by DGGE analysis.

Second generation sequencing or NGS collectively describes new technologies that achieve massively parallel sequencing of heterogeneous DNA fragments (Bokulich & Mills, 2012). Unlike Sanger sequencing, NGS technologies do not require the cloning of template DNA into vectors and the electrophoretic separation of sequencing products, but rather the DNA template is fragmented, amplified by PCR, and subsequently sequenced, being the microbial consortia described as nucleic acid sequences (Cocolin & Ercolini, 2015; O'Flaherty & Klaenhammer, 2011).

In food microbiology, NGS technology is used for profiling microbial communities but also for identifying isolates (e.g. a bacterial colony, a virus or any other organism), by determining the entire sequence of the genome which is commonly referred to as "whole genome sequencing" (WGS) (Jagadeesan et al., 2019). In order to investigate microbial communities, two NGS approaches can now be used: the most common is rRNA amplicon-based HTS sequencing (metabarcoding or metagenetics), which involves the amplification and sequencing of specific marker gene families, but also the metagenomic microbiome profiling, the random shotgun sequencing of the entire genomic content of the communities (Cocolin & Ercolini, 2015; Jagadeesan et al., 2019).

Vermote et al. (2018) used these two approaches in parallel to obtain a holistic view of cheese brines microbial diversity, since they are complex habitats due to composition and salinity, which implies a specific and diverse microbial composition, as well as the possible occurrence of non-cultivable living microorganisms.

Table 10 - Characterization of the three generations of sequencing technologies most commonly used in the context of the characterization of microbial profiles (Sources: Bokulich & Mills, 2012; Ercolini, 2017; O'Flaherty & Klaenhammer, 2011; Scholz, Lo, & Chain, 2012).

SEQUENCING TECHNOLOGY CLASSIFICATION		Tool	Sequencing technology bases	Maximum target size (bp or kb)	Sensitivity (cells/ml or reads/run)	Output /run (Gb)	Error rate	Starting DNA	Run time (h)	Advantages	Disadvantages	Dairy applications (Bibliographic source)
Taditional Sanger capillary electrophoresis sequencing	**1rd generation**	**DGGE**	Based on the separation of PCR amplicons of the same size but different sequences.Fragments are separated in a denaturing gradient gel based on their differential denaturation (melting) profile	100-300bp	10^2-10^3cells/ml	0,001	–	–	2	Single-nucleotide fragment separation	Technically difficult; non-quantitative	(Alegria, Szczesny, Mayo, Bardowski, & Kowalczyk, 2012; Alessandria et al., 2010; Bassi, Puglisi, & Cocconcelli, 2015; Bonetta, Carraro, Rantsiou, & Cocolin, 2008; Carpino et al., 2017; Casalta, Sorba, Aigle, & Ogier, 2009; Dolci et al., 2013; El-Baradei, Delacroix-Buchet, & Ogier, 2007; Ercolini, Frisso, Mauriello, Salvatore, & Coppola, 2008; Ercolini, Mauriello, Blaiotta, Moschetti, & Coppola, 2004; Ercolini, Moschetti, Blaiotta, & Coppola, 2001; Fontana, Cappa, Rebecchi, & Cocconcelli, 2010; Gala et al., 2008; Giannino, Marzotto, Dellaglio, & Feligini, 2009; Gkatzionis, Yunita, Linforth, Dickinson, & Dodd, 2014; Masoud et al., 2012; Nikolic et al., 2008; Pangallo et al., 2014; Randazzo, Vaughan, & Caggia, 2006; Ryssel et al., 2015; Van Hoorde, Heyndrickx, Vandamme, & Huys, 2010; Koenraad Van Hoorde, Verstraete, Vandamme, & Huys, 2008)
		TRFLP	Based on the digestion of amplified ribosomal DNA using one or more restriction enzymes.	1000bp	10^2cells/ml		–	–		High-throughput, pseudoquantitative	Lower taxonomic resolution;	(Arteau, Labrie, & Roy, 2010; Mirna Mrkonjic Fuka et al., 2013; Gala et al., 2008; Gkatzionis et al., 2014; Rademaker, Hoolwerf, Wagendorp, & te Giffel, 2006; Rademaker, Peinhopf, Rijnen, Bockelmann, & Noordman, 2005)
High-Throughput Sequencing (HTS)	**2rd generation or Next Generation Sequencing (NGS) or "short read" sequencing**	**454 Life Sciences platforms**										
		FLX Titanium	Sequencing by synthesis (Pyrosequencing - detection of pyrophosphates release during DNA synthesis)	300-400bp	10^6reads/run	> 0,6	Low	1 µg for shotgun library and 5 µg for pair-end	24	Longer fragment length, theoretically higher taxonomic resolution	Low coverage, expensive	(Aldrete-Tapia et al., 2014; Delcenserie et al., 2014; Ercolini et al., 2012; Fuka et al., 2013; Lusk et al., 2012; Masoud et al., 2012; Quigley et al., 2012; Riquelme et al., 2015)
		FLX+		500-600bp	10^6reads/run							(Calasso et al., 2016; De Pasquale, Calasso, et al., 2014; De Pasquale, Di Cagno, Buchin, De Angelis, & Gobbetti, 2014, 2016; Guidone et al., 2016; Guzzon et al., 2017; Lo, Xue, Weeks, Turner, & Bansal, 2016; Ryssel et al., 2015)
		Illumina platforms										
		GAIIx	Polymerase-based sequencing-by synthesis	Short reads 1 × 36 – 2 × 300bp	10^8reads/run	0.3–1000	Low	<1 µg for single or pair-end	2–29	Superior sequence coverage, lowest sequencing cost	Shorter fragment length, theoretically lower taxonomic resolution	–
		HiSeq2000			10^9reads/run							(Almeida et al., 2014; Escobar-Zepeda, Sanchez-Flores, & Quirasco Baruch, 2016)
		MiSeq			10^7reads/run							(Bassi et al., 2015; Ceugniez et al., 2017a, 2017b; Dalmasso et al., 2016; Eric Dugat-Bony et al., 2016; Duru et al., 2018; Marino et al., 2017; Parente et al., 2016; Vermote, Verce, De Vuyst, & Weckx, 2018)
		NextSeq			10^8reads/run							(Haastrup et al., 2018)
		Ion Torrent platforms	Sequencing by synthesis	Short reads 200-400bp		0.6–15	Low	–	2–4	Low cost and fast run	High rate of sequencing errors	(Berthoud et al., 2017; Murugesan et al., 2018; Ribani et al., 2018; Vermote et al., 2018)
	3rd generation (TGS) or "long-read" sequencing	**PacBio platforms**	Single molecule real time (SMRT) sequencing	Long reads Up to 60kb	10^8reads/run	0.5–10	High	~1.5 µg (ideally 2-3 µg)	0.5–4	Real long reads; Extremely high accuracy (CCS> 99.999 % /20 passes); Direct detection of epigenetic modifications; No problem with repeats, low/high % G	High instrument cost; low number of sequence read per run; highest error rates compared with other NGS chemistrie	(Jin et al., 2018; Li et al., 2017)
		Oxford Nanopore Technologies (ONT)	Single molecule sequencing	Long reads Up to 100kb	10^6reads/run	0.1–20	High		0,02-48	Promise to permit on-site, long-read, real-time sequencing	Laborious sample preparation requirements and high error rates	–

The main purpose of metagenomics (DNA-seq or RNA-seq) is to obtain, at the same time, information about the microbe composition and the gene content without any PCR bias (Cocolin & Ercolini, 2015; Ferrocino & Cocolin, 2017). Metagenomics can be combined with metaproteomics (detection and categorization of proteins), metabolomics (metabolite concentration) and meta-transcriptomics (measurement of mRNA expression), with great potential for the survey of food production and the evaluation of food safety, authenticity and quality (Jagadeesan et al., 2019). The application of these technologies in the food area has been designated as "foodomics" (Cifuentes, 2009). These approaches have already been used in some cheese investigations (Almeida et al., 2014; De Filippis et al., 2017; Dugat-Bony et al., 2015; Lessard, Viel, Boyle, St-Gelais, & Labrie, 2014; Monnet et al., 2016; Wolfe, Button, Santarelli, & Dutton, 2014). Figure 8 shows a summary of potential NGS use by the food industry. Although these techniques potentially provide a much higher amount of information, their cost is still substantially higher compared with the amplicon-based approaches (De Filippis et al., 2017; Ferrocino & Cocolin, 2017). Additionally, specific bioinformatics and biostatistics skills for the data analysis may be lacking (Ferrocino & Cocolin, 2017).

Amplicon-based HTS sequencing, which targets genes of taxonomic relevance, has become the most widely used approach in food microbial ecology. Dairy is by far the most explored environment and a broad variety of cheeses were studied through these approaches (De Filippis et al., 2017; Ercolini, 2017). In this technique, DNA/RNA extracted directly from samples undergoes targeted PCR amplification of phylogenetic marker genes using universal PCR primers which target known marker genes, commonly the 16S rRNA gene for Archaea and Bacteria and the internal transcribed spacer (ITS) of the ribosomal gene cluster sequences for fungal species. Amplicons obtained are physically partitioned and sequenced, and sequences are compared to reference databases to identify the operational taxonomic units (OTUs) through well established bioinformatics pipelines (Bokulich & Mills, 2012; Cocolin & Ercolini, 2015; Ercolini et al., 2012; Parente et al., 2016). Although PCR-dependent, metabarcoding is considered quantitative as the number of reads for each operational taxonomic unit (OTU) is proportional to the abundance of that OTU in the sample and the higher sensitivity also allows the identification of sub-populations previously difficult to detect (De Filippis et al., 2017).

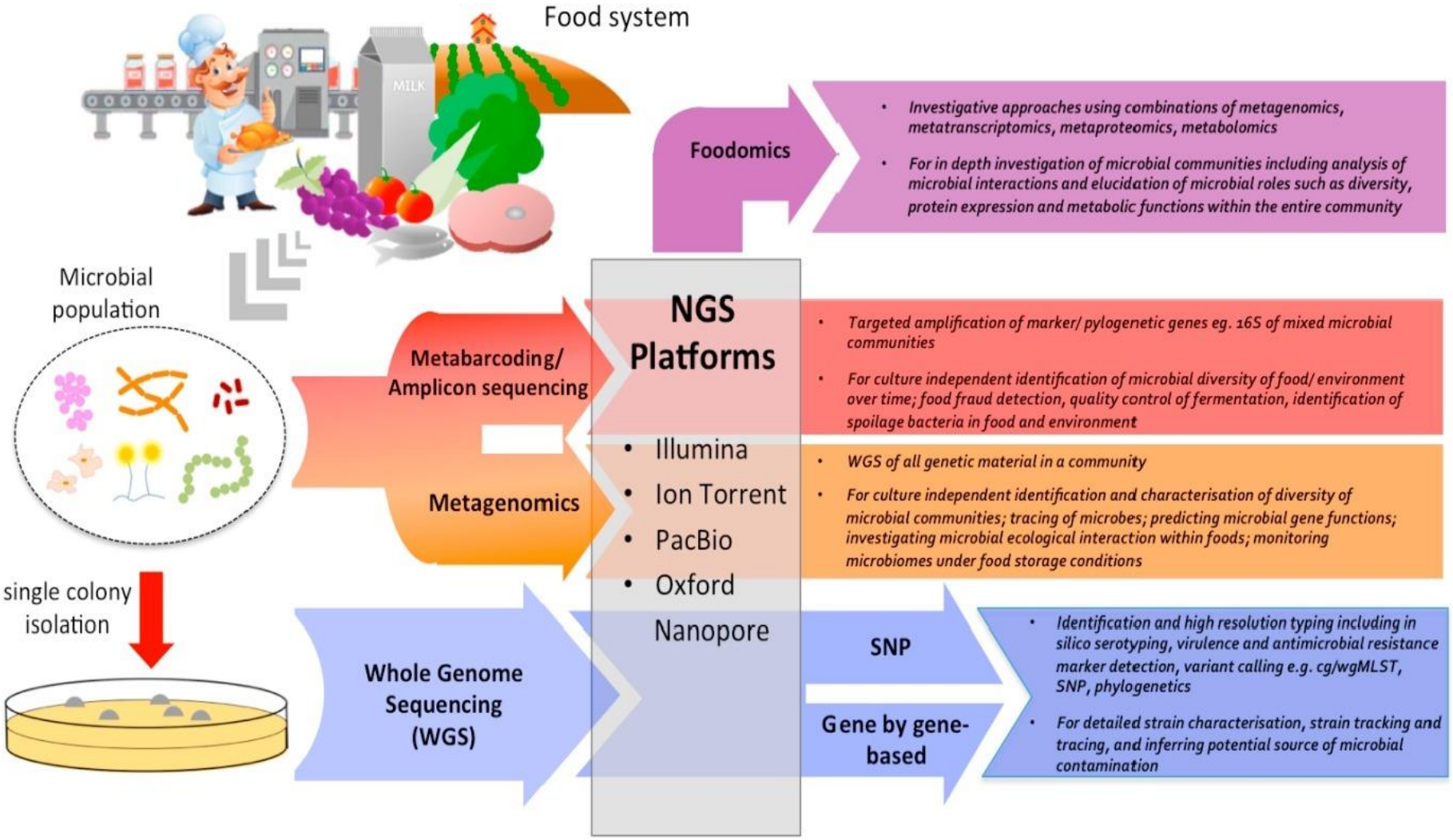

Figure 8 - Summary of potential NGS use by the food industry (Source: Jagadeesan et al., 2019).

In practice, after the sequencing step, the raw data obtained are preprocessed through a quality checking (QC) procedure. The purpose of QC is to improve the analysis accuracy by removing low quality and erroneous reads as well as amplification artifacts, which is essential to prevent an overestimation of the community species diversity in the next steps. After the generation of error-free (and de-replicated) reads, the actual analysis is initiated by encompassing, in essence, three major blocks: clustering into operational taxonomic units (OTUs), taxonomic classification and statistical evaluation (Jünemann et al., 2017).

The 16S rRNA gene is used as an universal marker, since it is ubiquitous in prokaryotes being considered a 'bacterial barcode' (Jünemann et al., 2017). With a lot of information (1500 bp long), it consists of highly conserved as well as hypervariable regions (HVRs) V1 to V9 facilitating both the amplification by universal primers and a high specificity to distinguish between organisms (Janda & Abbott, 2007). Finally, it relies upon an impressive archive of reference sequences (Jagadeesan et al., 2019). In cheese, V1-V3 HVR is the target that has been the most extensively used (Parente et al., 2016).

However, because of the shorter read lengths of NGS, only a single HVR or a combination of neighbouring HVRs of the rRNA gene is amplified by PCR and sequenced, which limits the taxonomic resolution (Jünemann et al., 2017). It should also be noted that the use of different targeted HVRs of the same 16S rRNA gene eventually results in different classification results (Mizrahi-Man, Davenport, & Gilad, 2013; Youssef et al., 2009). So, there is a need for increasing taxonomic resolution, as most HTS studies limit taxonomic affiliation to the genus level due to technological (e.g., sequence length) and/or data processing issues (e.g., sequence database availability) (Dugat-Bony et al., 2016; Meola et al., 2019).

This limitation in taxonomic annotation may affect dairy microbiome studies. Dairy environments are often characterized by only a few abundant genera belonging to LAB. Thus, species or even strain level taxonomic annotation is crucial to better characterize microbial diversity in these products. Under these conditions it is also of paramount importance to select the correct HVRs to maximize species-level resolution, as short fragment strategies, on single HVRs or HVR pairs, often fail to reliably assign the correct taxonomy at the species level (Meola et al., 2019). An alternative application to this issue

is the selection of genes with high polymorphism within species, potentially allowing discrimination between different strains within a species (Ercolini et al., 2012).

Meola (2019) proposes, as an alternative to available universal 16S databases (Silva, The Ribosomal Database Project - RDP, and Greengenes) (BalvoĆiūtė & Huson, 2017), a manually curated database for 16S OTUs classification of NGS short reads, restricted to the biodiversity expected in dairy products. This alternative would greatly improve the accuracy and reproducibility of phylogenetic classification at the level of all taxonomic categories, offsetting the limitations of short reading sequences. Additionally metagenomics is one method that overcomes that limitation by sequencing all the nucleic acids directly from a sample and is, therefore, not restricted to the 16S rRNA gene (O'Flaherty & Klaenhammer, 2011).

At the level of fungal discrimination, the choice of ITS region (fungi) is essentially due to the availability of databases (De Filippis et al., 2017). However, the uneven ITS length among species may promote preferential amplification of shorter fragments during the PCR step and therefore lead to an incorrect estimation of OTU abundance (Bokulich & Mills, 2012; De Filippis et al., 2017; Ercolini et al., 2012). Therefore, the use of different targets would be advisable, such as the 26S or the 18S rRNA genes (De Filippis et al., 2017).

As regards equipment, there are two main NGS platforms currently used for microbial community profiling, the 454 Life Sciences pyrosequencing and Illumina (formerly Solexa) sequencing platforms (Bokulich & Mills, 2012; O'Flaherty & Klaenhammer, 2011) (Table 10). Both use sequencing of both ends of the DNA fragment, termed pair-end sequencing: 454 Life Sciences pyrosequencing technology by synthesis via pyrophosphate detection and Illumina by synthesis chemistry with reversible terminator nucleotides, each labeled with a different fluorescent dye (Bokulich & Mills, 2012; O'Flaherty & Klaenhammer, 2011). These systems deliver a functionally identical product, raw DNA sequence, but with varying error rates, sequence lengths, yields, and costs (Bokulich & Mills, 2012). The superior per-base cost efficiency and high sequencing accuracy of Illumina sequencing has prompted its growing application in the study of microbial ecology over pyrosequencing (Bokulich & Mills, 2012; Degnan & Ochman, 2012; Jünemann et al., 2017; van Dijk et al., 2018), and food microbiology is no exception, including cheese (Table 10).

So, NGS technology is developing at a rapid pace from being solely a research tool to becoming routinely applied in food microbiology, including diagnostics, outbreak investigations, antimicrobial resistance, forensics and food authenticity (Jagadeesan et al., 2019; Kergourlay et al., 2015). The application of this technology to cheese, in addition to the study of cheese microflora, allows for further specific information, namely on the authenticity of a product (Aldrete-Tapia et al., 2014; Delcenserie et al., 2014), understanding of the manufacturing process (Bokulich & Mills, 2012; Ercolini et al., 2012; Fuka et al., 2013), the impact of their alteration (Aldrete-Tapia et al., 2014; Fuka et al., 2013) and identification of spatial and temporal variations during cheese processing (O'Sullivan et al., 2015).

Although NGS technologies are extremely advantageous, they also have some disadvantages, as already mentioned throughout the text. These limits can be summarized in the relatively short reads, the depth of sequencing effort, and bias on DNA extraction and amplification (Jünemann et al., 2017; Kergourlay et al., 2015). One major limitation is precisely the length of sequencing fragments. As genomes often contain numerous repeated sequences that are longer than the NGS reads, this may lead to misassemblies and gaps (Goodwin, McPherson, & McCombie, 2016). Also, larger nucleotide structural variations are more challenging to detect and characterize. The fact that NGS methods rely on PCR also causes difficulties with regions of extreme GC%, as these are inefficiently amplified by PCR. Some of these limits can be overcomed by introducing some innovations in the methodology as mentioned above, or by using metagenomics, since this is not restricted to the 16S rRNA gene and does not need to use PCR (O'Flaherty & Klaenhammer, 2011).

Another alternative are third generation sequencing (TGS)/long read sequencing techniques (Table 10). TGS technologies provide a way to study genomes, transcriptomes and metagenomes at an unprecedented resolution, in addition to the absence of PCR amplification and the production of long reads (van Dijk et al., 2018). The distinct features of TGS are single molecule sequencing (SMS) and real time sequencing (RT) (as opposed to NGS, where sequencing is paused after each base incorporation) (Schadt, Turner, & Kasarskis, 2010). Two types of sequencers are currently available, PacBio (single-molecule real-time - SMRT Sequencing) and Oxford Nanopore Technologies - ONT (Nanopore Sequencing) (Table 10), but they are not yet routinely used for amplicon

metabarcoding studies (Meola et al., 2019; van Dijk et al., 2018) (Table 10). A weakness of TGS sequencing is the high error rate (van Dijk et al., 2018). However, in a recent work (Singer et al., 2016), the results of microbiota profiling of samples taken from a lake using the PacBio SMRT sequencing and Illumina platform show that PacBio SMRT sequencing resulted in less ambiguous classification, while allowing a more encompassing identification of species diversity. Thus, they conclude that the PacBio SMRT platform could be used to describe the microbial communities more accurately, with higher phylogenetic resolution (Singer et al., 2016). .

I.4. Functional foods concept

I.4.1. Concept of functional food and general aspects

The concept of functional food (FF) resulted from the well-known synergy between health and diet and was based on the expansion of knowledge about physiologically active food components (Serafini, Stanzione, & Foddai, 2012). Foods are no longer intended to simply satisfy hunger and to provide the necessary nutrients, but also and especially to prevent nutrition-related diseases and to improve physical and mental well-being (Bigliardi & Galati, 2013). Disease prevention by increased consumption of functional foods may substantially reduce medical costs, which makes functional foods also interesting from a socio-economic perspective (Moors, 2012).

This concept has emerged in the eighties of the last century in Japan, to describe processed foods containing ingredients that enhance specific body functions, designated as Foods for Specified Health Use (FOSHU). They did this in a national effort to reduce the escalating cost of healthcare (Abdel-Salam, 2010; Serafini et al., 2012). In the nineties, the European Union (EU) founded the Functional Food Science in Europe (FUFOSE) and produced a consensus report with a working definition of functional food (Diplock et al., 1999; EC/DGRI, 2010). This document has been widely used as a basis for discussion and further evolution of the thinking on the topic. At the beginning of this century, the Food and Agriculture Organization of the United Nations (FAO) also published a report on functional foods which concluded on the need to clearly define functional food, since there is no universal consensus (Subirade, 2007).

In Europe there is still no official definition accepted by all states, but the EU Project FUFOSE working definition is generally well accepted: “A food can be regarded as ‘functional’ if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. FF must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in a varied diet on a regular basis: they are not pills, capsules or any form of dietary supplement, but part of a normal food pattern” (Abdel-Salam, 2010; Diplock et al., 1999; EC/DGRI, 2010; Eussen et al., 2011; Daniel Granato, Nunes, & Barba, 2017; Roberfroid, 2002; Serafini et al., 2012).

There are a number of other working definitions for FF which are essentially in line with the previous one (Bigliardi & Galati, 2013; Clydesdale, 2004; Crowe & Francis, 2013; Hasler & Brown, 2009; Kapsak, Rahavi, Childs, & White, 2011). All agree that FF must be part of the usual diet, in a food form and contain bioactive or functional ingredients that provide a health benefit.

In this context, we can consider that all foods are functional at some physiological level. Although there are many ways in which FF can be categorized, we can divide these into three categories based on how the bioactives are found in, or incorporated into. Thus FF may be unmodified whole or basic foods, the simplest or natural form of functional foods, or modified, such as processed foods altered with added bioactives (the bioactive does not exist naturally in this food and is added during processing), or foods enhanced (fortified or enriched) to have more of a bioactive (Abdel-Salam, 2010; Bigliardi & Galati, 2013; Clydesdale, 2004; Crowe & Francis, 2013; Hasler & Brown, 2009; Kapsak et al., 2011; Martins, Pinho, & Ferreira, 2004; Subirade, 2007).

The most important challenge is to ensure that functional or bioactive ingredients will survive or remain active and bio-available after the processing and storage (Day, Seymour, Pitts, Konczak, & Lundin, 2009; Subirade, 2007; Tripathi & Giri, 2014). The main substances that give the characteristic of functionality to the food are probiotics (microbial cultures), prebiotics, antioxidants, dietary fiber, fatty acids and phytosterols, but also vitamins, minerals, proteins, peptides, amino acids and carbohydrates not included in those categories (Abdel-Salam, 2010; Martins et al., 2004; Serafini et al., 2012). Table 11 presents the characterization of the main functional ingredients, some

Table 11 - Main classes of functional or bioactive food ingredients, their characterization, the health benefits normally attributed to it and examples of each type of ingredients and the respective health claim approved under applicable European legislation (EC, 2012a) as it is considered scientifically proven (Source: Abdel-Salam, 2010; EC/DGRI, 2010; Gibson et al., 2017; Martins et al., 2004; Serafini et al., 2012).

Functional or Bioactive Ingredients	Definition / Characterization; Possible beneficial health functions; Functional nutrient, substance, food or food category/Authorized health claim [Commission Regulation (EU) Nº 432/2012]
Probiotics	**Definition -** Live microorganisms which when administered in adequate amounts can alter intestinal microbiota and confer a health benefit to the host. The most common include strains of *Lactobacillus* and *Bifidobacterium* (note that health benefits are strain-specific). **Possible beneficial health functions:** Improved immune function; treatment of gastrointestinal disorders; reduced risk of colon cancer, urinary tract infections and hypertension; cholesterol lowering properties; prevention and treatment of allergies. **Functional nutrient, substance, food or food category with authorized health claim:** Live yoghurt or fermented milk cultures.
Prebiotics	**Definition -** A substrate that is selectively utilized by host microorganisms conferring a health benefit. The majority are **disaccharide** (e.g. lactulose), short polysaccharides (e.g. inulin, dextrin), oligosaccharides **(fructo-oligosaccharides-FOS** and galacto-oligosaccharides-GOS) or polysaccharides (**e.g. polydextrose).** **Possible beneficial health functions:** improved of intestinal functions; regulation and modulation of immune; increase in bone calcium content and bone mineral density; reduced risk of obesity, type 2 diabetes, metabolic syndrome, coronary heart disease, colon cancer; reduced risk and/or improved management of intestinal inflammation and inflammatory bowel conditions. **Functional nutrient, substance, food or food category with authorized health claim:** Native chicory inulin.
Antioxidants	**Definition -** Compounds that inhibit or delay oxidation of a substrate and therefore minimize the production of reactive oxygen species (ROS) which can cause damage to DNA, protein and lipids. Diet is the most important source of antioxidants providing vitamins C and E, carotenoids, copper and selenium, flavonoids and many other phytochemicals. **Possible beneficial health functions:** Control of aging; control of many chronic diseases often associated with increased production of ROS and increased oxidative stress. **Functional nutrient, substance, food or food category with authorized health claim:** Vitamins C e E; Copper; Selenium; Olive oil polyphenols; Riboflavin (Vitamin B2); Zinc.
Dietary Fibre	**Definition -** Polysaccharides that are resistant to hydrolysis by digestive enzymes (e.g. cellulose, pectin, gums, beta-glucans, inulin, oligosaccharides, fructans, lignin). **Possible beneficial health functions:** improvements in bowel function; improvements of fermentability of colonic microbioata; reduced risk of gastrointestinal disorders; reduced risk of cardiovascular disease; reduced blood pressure; improved lipid levels; reduced inflammation; improved glycemic control and reduced risk of type 2 diabetes; reduced risk of certain types of cancer; help with weight loss as it can increase satiety and reduce absorption time. **Functional nutrient, substance, food or food category with authorized health claim:** Arabinoxylan (hemicelulose); beta-glucans from oats and barley; hydroxypropyl methylcellulose (hpmc); pectins; alpha-cyclodextrin; Barley grain fibre; oat grain fibre; wheat bran fibre; sugar beet fibre; Beta-glucans; chitosan; glucomannan (konjac mannan); guar gum; hydroxypropyl methylcellulose (hpmc); pectins; Glucomannan (konjac mannan); Rye fibre; native chicory inulin; Wheat bran fibre.
Fatty Acids	**Definition -** Monounsaturated (MUFA) and/or polyunsaturated fatty acids (PUFA); Omega-3 fatty acids: long-chain PUFA that are incorporated into cell membranes (e.g. A-linolenic acid (ALA), eicosapentanoic acid (EPA), docosahexanoic acid (DHA). **Possible beneficial health functions:** improvements of cardiovascular health; reduce risk of certain cancers; management of rheumatoid arthritis. Incorporated into cell membranes affect: membrane fluidity; enzyme activity; cell signaling; gene expression; eicosanoid production (regulation of inflammation, platelet aggregation and vasodilation/constriction). **Functional nutrient, substance, food or food category with authorized health claim:** ALA; DHA; EPA plus DHA; Monounsaturated and/or polyunsaturated fatty acid, oleic acid; DHA plus EPA.
Phytosterols	**Definition -** Phytochemicals structurally similar to cholesterol but are not readily absorbed. Are found naturally in plants (fruits, vegetables, nuts, seeds, grains, legumes). **Possible beneficial health functions:** compete and interfere with dietary and endogenous cholesterol absorption and reduce circulating LDL and total-cholesterol, thereby: reducing cardiovascular disease risk; reducng risk of cancer (lung, stomach, colon, breast and prostate); antioxidant; anti-inflammatory; anti-atherogenic properties. **Functional nutrient, substance, food or food category with authorized health claim:** Plant sterols and plant stanols.

examples of possible beneficial health functions assigned to each, regardless of whether they are scientifically documented in humans, and examples of each type of ingredients and the respective health claim approved under applicable European legislation (EC, 2012a), as it is considered scientifically proven. It is important to add the concept of synbiotic functional product, in which the prebiotic selectively supports the growth of probiotic component (Kolida & Gibson, 2011). These are defined as mixtures of probiotics and prebiotics that beneficially affect host health by improving the implantation, growth and survival of specific living health promoting microorganisms in the gastrointestinal system (Gibson et al., 2017; Kolida & Gibson, 2011) .

FF products can be found in virtually all segments of the food and drink market, but many of the functional food products developed are in the dairy, confectionary, soft drink and baby food markets (Liang, Sarabadani, & Berenjian, 2016). According to a consensus document (Diplock et al., 1999), yoghurt and other fermented milks can be considered the first functional foods.

The most recognized FF products on the markets today are probiotics and prebiotics (Liang et al., 2016). Interestingly, these are the categories of functional ingredients that present the fewest approved health claims in EU under applicable European legislation (EC, 2012a) (Table 11).

Innovations introduced in the food industry in recent years mainly refer to the introduction of novel foods, among which functional foods play an outstanding role, as contribution to a healthy life, but also from the increasing cost of healthcare that is necessary to overcome, the steady increase in life expectancy and the consequent desire of older people for greater quality of life (Betoret, Betoret, Vidal, & Fito, 2011; Bigliardi & Galati, 2013). Kapzak et al. (2011) adds the pervasive media attention to food innovation and medical discovery and the easy access to information through new, highly-targeted and portable media.

Researchers also agree in that functional food represents one of the most interesting areas of research and innovation in the food industry (Annunziata & Vecchio, 2011; Betoret et al., 2011; Bigliardi & Galati, 2013; Daniel Granato et al., 2017; Kaur & Singh, 2017; Santeramo et al., 2018)

I.4.2. European regulatory systems governing of functional foods

In the EU, no legislation is in place on functional foods, so there is no legal power of this definition but, in order to prevent consumers being misled by unclear information on food products and to harmonize health claims made in commercial communications in each member state, the EU has formulated regulation on nutrition and health claims (HCR), including reduction of disease risk claims, or any statement about a connection between food and health [Regulation (EC) Nº 1924/2006] (EC, 2006c), applicable from 1 July 2007 (Baňares, 2016; Martinez & Siani, 2017; Vicentini, Liberatore, & Mastrocola, 2016; Wong, Lai, & Chan, 2015). This regulation is consistent with the broader legal framework outlined by the general principles and requirements of food law [Regulation (EC) Nº 178/2002] (EC, 2002) and the general provisions relating to the labelling, presentation and advertising of foodstuffs [Directive 2000/13/EC; Regulation (EU) Nº 1169/ 2011] (EC, 2000, 2011). The latter is formally named Regulation on "food information to consumers" (FIC) (Martinez & Siani, 2017).

According to this regulation and other bibliographic sources (CAC, 2004; Subirade, 2007), health claims consist of front-of-package information, which link the product with specific health-related functions. So, health claims play an important role in purchase decisions (Santeramo et al., 2018). Under the HCR, medicinal claims on food (i.e. claims attributing to any food the property of preventing, treating or curing a human disease) are forbidden, whereas function health claims, reduction of disease risk claims (i.e. any health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease) and claims referring to children's development, are allowed. In addition, food information provided on a voluntary basis shall not be ambiguous or confusing, shall not mislead the consumer, and shall be based on relevant scientific data (EC, S/D; Martinez & Siani, 2017; Subirade, 2007).

Building a relevant scientific basis for functional food claims relies on the ability to demonstrate the bio-efficacy of functional food components in vivo, and not only by performing in vitro tests. These two conditions also determine toxicological effects and therapeutic dosage and assess the physicochemical, chemical, sensory and shelf life properties of a developed food before stating it is functional (Martinez & Siani, 2017;

Wong, Lai, et al., 2015). It is a complex and costly task, but it is essential to the acceptance of functional foods (Granato et al., 2017; Subirade, 2007).

As no legal definition for functional foods has been adopted, as we have said before, one must consider legal functional foods to comprise foods with approved health claims, thus, based on relevant scientific data (Martinez & Siani, 2017; Van Loveren, Sanz, & Salminen, 2012). The Article 13º of the Regulation (EC) Nº 1924/2006 (EC, 2006c), regarding the use of health claims, establishes that such claims can only be authorised for use in the EU after a scientific assessment by the Panel on Nutrition, Novel Foods and Food Allergens (NDA), a specific body of the European Food Safety Authority (EFSA). The EFSA assesses the health claim dossiers and advises the European Commission, which makes the final decision (Moors, 2012).

The same Article provides for the existence of a list of permitted health claims made about foods, other than those referring to the reduction of disease risk and to children's development and health, established by nutrient, substance, food or food category, which has already been published in Regulation (EU) Nº 432/2012 (EC, 2012a), now with amendments M1 to M13. To prepare this document, since HCR came into force in 2007, EFSA has evaluated more than 4000 health claims, from a list of 44000 provided by the different EU countries to the Commission, and has approved until now around 220 (EC, 2012a, S/D). These health claims have become a means to communicate to consumers the health benefits of foods that contain specific formulations, conveying relevant information that would otherwise remain unknown, and they also act as key factors for the development of the functional food market (Martinez & Siani, 2017; Vicentini et al., 2016).

Additionally, the production or introduction of novel functional foods in the EU was controlled by Regulation (EU) Nº 1169/2011 (EC, 2011), now amended by Regulation (EU) 2015/2283 (EC, 2015), specifically on novel foods. This establishes rules for the placing of novel foods on the EU market and is aimed at the proper functioning of the internal market, while ensuring a high level of protection of human health and consumer interests. In accordance with these regulations, novel foods are foods or food ingredients not yet significantly used for human consumption in the European Union before 15 May 1997, which have to undergo a risk assessment and get authorisation before it is they are placed on the market.

Given the demands and difficulties for the approval of health claims, EFSA recently published General Scientific Guidance for stakeholders on health claim applications (EFSA/NDA, 2016a) that summarises ten years of experience in this field. It goes some steps beyond the previous guidance and spells out the scientific reading of a legal text. Also updated was the guidance on the scientific requirements for health claims related to gut and immune function, which clarifies applicants in preparing applications for the authorisation of health claims related to the immune system, the gastrointestinal tract and defence against pathogenic microorganisms (EFSA/NDA, 2016b).

Following these revisions, the scientific and technical guidance for the preparation and presentation of an application for authorisation of a health claim was also updated taking into account the new scientific and technical developments in this field (EFSA/NDA, 2017). This guidance outlines the information and scientific data which must be included in the application, the hierarchy of different types of data and study designs, and the key issues which should be addressed in the application to substantiate the health claim.

I.4.3. Concept of probiotic, probiotic food, dairy probiotics and general aspects

The first references to the use of microorganisms in the resolution of health problems arose between the late eighteenth and early nineteenth centuries. It consisted in the suppression and displacement of harmful bacteria in the intestine by orally administered beneficial ones that improved microbial balance, health and longevity (Vrese & Schrezenmeir, 2008). Elie Metchnikoff (Nobel Prize in 1908) claimed that the intake of yoghurt containing lactobacilli results in a reduction of toxin-producing bacteria in the gut and is associated with increased longevity of the host (Metchnikoff, 1908). The first industrially produced yoghurt was developed in accordance with the ideas of Metchnikoff, to help children suffering from diarrhea, and was sold in pharmacies (Gasbarrini, Bonvicini, & Gramenzi, 2016; Vrese & Schrezenmeir, 2008).

The word probiotic, meaning “for life”, was first used by Kollath in 1953 to designate “active substances that are essential for a healthy development of life”. In 1989, Fuller presented the first accepted definition as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Gasbarrini et al., 2016; Vrese & Schrezenmeir, 2008). But probiotics act in organs other than the intestine, so a broader definition arises in 2002 by an expert panel of the Food

and Agriculture Organization of the United Nations (FAO) and the World Health Organisation (WHO), which defines probiotic as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2006; Hill et al., 2014; Salvetti & O'Toole, 2017). This definition is inclusive of a broad range of microbes and applications, whilst capturing the essence of probiotics (microbial, viable and beneficial to health). Thus, it has been widely adopted and has proven valuable to researchers, regulators and consumers (Ashwell, 2004; Hill et al., 2014; Roberfroid, 2002). By October 2013 the definition was reinforced as relevant and sufficiently accommodating for current and anticipated applications by an expert panel of the International Scientific Association for Probiotics and Prebiotics (ISAPP) (Hill et al., 2014). EFSA uses the FAO/WHO definition when referring to probiotics (Baňares, 2016; Hill et al., 2014). Regardless of some differences between various existing definitions, almost all argue that probiotic microorganisms must be alive and the health effects scientifically proven.

The ISAPP expert panel recommends that undefined faecal microbiota transplants and live cultures traditionally associated with fermented foods and for which there is no evidence of a health benefit should stay outside the probiotic framework. In the case of the live microbes in such foods, the panel suggests that they are only described as “live and active cultures”, since it is not always possible to clearly distinguish the contribution to health of live microbes from that of the food matrix, and potentially beneficial microbes might often represent a diverse community that is not well-defined in terms of strain composition and stability (Hill et al., 2014). However, this panel recognizes the beneficial relationship between some foods, especially fermented dairy products and the reduced risk of certain diseases. It seems reasonable to argue that if a food contains large numbers of live cells belonging to a species for which health benefits have been demonstrated, these foods should be considered to have similar health benefits (Marco et al., 2017; Sanders, Merenstein, Merrifield, & Hutkins, 2018).

It is also important to distinguish between probiotic or probiotic microorganisms and probiotic foods, which are often used as synonyms. Foods are only one of the potential delivery vehicles of probiotic microorganisms to a host (Salvetti & O'Toole, 2017; Vandenplas, Huys, & Daube, 2015; Vrese & Schrezenmeir, 2008). Probiotic foods can be defined as those containing living probiotic microorganisms in an adequate matrix and

in sufficient concentration, so that after their ingestion the postulated effect is obtained and goes beyond that of usual nutrient suppliers (Vrese & Schrezenmeir, 2008). Milk is one of the matrices being used as a probiotics vehicle, so it is appropriate to use the designation dairy probiotics when describing the majority of probiotics that can actively ferment milk or survive in milk or fermented milk products in high numbers (Zoumpopoulou, Pot, Tsakalidou, & Papadimitriou, 2017).

However, foods with probiotics also includes juices, sour milk, oat-based products, ice-creams, nutrition bars, infant formulas, relishes and condiments, sweeteners, waters, pizza crust, and other products such as gum and lozenges (Begum et al., 2017; Salvetti & O'Toole, 2017). Mayonnaise, meat-based products, cheese, and cheese-based dips are the most recently developed foods having probiotics (Begum et al., 2017). Probiotics available today comprise a much broader range of products that can be delivered to a host also through dietary supplements, or as active components of a registered medication (Salvetti & O'Toole, 2017; Vandenplas et al., 2015; Vrese & Schrezenmeir, 2008).

The mínimum requirements for probiotic status include the assessment of strain identity (genus, species and strain level), in vitro tests to screen potential probiotics (e.g. resistance to gastric acidity, bile acid, and digestive enzymes), safety assessment (requirement for proof that a probiotic strain is safe and without contamination in its delivery form) and in vivo studies for substantiation of the health benefits in the target host (Hill et al., 2014; Van Loveren et al., 2012; Vandenplas et al., 2015).

The benefits of probiotics have mainly been related to the health of the gastrointestinal tract and also the urogenital system (Hill et al., 2014; Van Loveren et al., 2012; Vandenplas et al., 2015; Zoumpopoulou et al., 2017). These benefits are associated with regulation of the intestinal and vaginal microbiota composition by reducing the numbers or colonization of pathogens through competition for nutrients and binding sites and by the in situ production of antimicrobials (Van Loveren et al., 2012; Zoumpopoulou et al., 2017). Other body sites, such as the mouth and the skin, are also considered targets for probiotic applications. Probiotics may play an important role in oral medicine and dentistry (Vandenplas et al., 2015; Vrese & Schrezenmeir, 2008).

Specific probiotics may also be associated with other health benefits such as immune and allergy response, modulation of lactose intolerance, reduction of serum cholesterol and

constipation, diarrhea prevention and symptom alleviation and mineral absorption (Hill et al., 2014; Van Loveren et al., 2012; Zoumpopoulou et al., 2017). Some of them produce vitamins that may become available for the host. Whatever the benefit, the effect may be therapeutic or prophylactic (Vandenplas et al., 2015; Zoumpopoulou et al., 2017). The goal can be to fight the cause of the disease or metabolic alterations, or to lessen the symptoms associated with the occurrence or progression of a disease or metabolic alteration (Vandenplas et al., 2015).

Zoumpopoulou (2017) emphasizes that probiotics could have applications beyond those already mentioned. The huge research on microbiota composition in health and disease, have suggested new potential applications of probiotics in the fields of metabolic syndromes (obesity, diabetes, cardiovascular disease, etc.), psychotropic activity through the gut-brain axis, and anti-mutagenic or anti-cancerous activities. Another interesting application being developed concerns the use of genetically modified probiotics to deliver therapeutic molecules to the host.

I.4.4. The use of health claims in probiotic foods – legal issues

The increase in demand, as well as the lack of a well-established regulatory status of functional foods, including probiotic products, has led to the misuse of the term "probiotic", which has been used for some foods without any pre-market approval and in the absence of an approved health claim (Sanders, 2015; Van Loveren et al., 2012). In this context, also the number of proclaimed probiotic microorganisms in the absence of human tests found in the scientific literature increased rapidly (Zoumpopoulou et al., 2017). Because of this, the scientific criteria to judge health claims for probiotics or the use of the term "probiotic" as a health claim, despite differing from country to country, are generally becoming more and more strict (Reid, 2015).

In Europe, the regulations applicable to probiotics are those that apply to functional foods, and were already presented earlier in this paper (EC, 2006c, 2011, 2012a). According to the guidelines on the implementation of such regulation on health claims (EC, 2007), anything that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health is a health claim. In this sense the term "probiotic" was considered as a health claim itself, since, when used on a food label, this implies that the product contains a substance that may be beneficial for health. Terms like

‘live’ or ‘active’ used to describe microorganisms present in a food, imply a probiotic function and therefore are also considered to be health claims (Baňares, 2016; EC, 2007; Foligné, Daniel, & Pot, 2013).

The essential base criteria for the establishment of human health claims on probiotic strains go against what was defined for probiotic status: (a) characterization of the strain or each of the strains in a probiotic mix or combination (identification, safety, resistance), (b) identification of the health relationship to benefit the general population or a defined part of it, and (c) demonstration of health effects in a normal healthy target population (FAO/WHO, 2006; Van Loveren et al., 2012).

Considering the European regulations on functional foods mentioned above, 150 applications for health claims on probiotics have been submitted for evaluation to EFSA and no application has received a positive opinion (Baňares, 2016; IPA, 2017; Miquel et al., 2015). Therefore, no claims on probiotics are listed on the EU register as authorised for use. In Europe, not a single probiotic product, food or food supplement can mention the health benefits of the strains it includes (Foligné et al., 2013; Glanville, King, Guarner, Hill, & Sanders, 2015).

Although some health claims have not been approved due to the failure to characterize the strain(s) involved, other justifications have been presented in relation to the other criteria. The main reasons given by the EFSA panels for rejecting the proposed claims are laid out in Table 12.

The probiotic claims that have been fully evaluated and rejected are listed as non-authorised on the EU Register on nutrition and health claims (EC, 2018) (Appendix 2 presents some unauthorized probiotic claims in the context of dairy foods). The only exception being the authorization concerning the claim “Improve lactose digestion of the product in individuals who have difficulty digesting lactose”, attributed specifically to the live yoghurt cultures and applicable in yoghurts and other fermented milks (Appendix 3) (EC, 2018; Smug, Salminen, Sanders, & Ebner, 2014).

Table 12 - Main reasons given by the EFSA panels for rejecting the proposed health claims on probiotics (Source: EC, 2018).

• Lack of characterization of the strain or, when a combination of bacterial strains has been used, only part of them have been sufficiently characterized.
• Absence of evidence of health claims by human studies.
• Relationship between claim and health considered too general.
• Considered by EFSA not to be beneficial. e.g. EFSA has not accepted that merely increasing the proportion of lactobacilli or bifidobacteria in the gut should be considered a beneficial health effect.
• Considered by EFSA as pertaining to treatment of pathological situations rather than maintaining normal physiological conditions or reducing disease risk factors.
• Claims oriented to subjects beyond the scope of the claims regulation.
• Studies provided to substantiate claims with flaws in their design. e.g.: intervention studies have not always been sufficiently randomized, measures for blinding subjects and/or observers have not always been adequate, and often statistical analysis has been inadequate.
• Flaws in the actual measurements have been encountered. e.g. the immunologic basis for a runny nose or a rash to corroborate the allergic nature of these measures has been absent, and the infectious nature of diarrhea has not been addressed.

Nutrition guidelines given by governments or government-related expert organizations of EU member states usually include yoghurt as part of a healthy diet. Interestingly, none of those countries mentioned yoghurt as an alternative for people with lactose intolerance, despite this claim approved (Ebner, Smug, Kneifel, Salminen, & Sanders, 2014; Smug et al., 2014).

These restrictions contrast with the increasing knowledge and medical interest in probiotics. In fact, despite the large number of studies on the effects of probiotics on health, most have been considered as exploratory by EFSA. Mechanisms of probiotic action appear to remain insufficiently designed to fulfill the criteria for substantiation of a health claim under the current EU claims regulation (Salvetti & O'Toole, 2017; Van Loveren et al., 2012). This has worried the scientific community, which believes that some allegations supported by solid scientific evidence were also rejected. It also discourages producers to invest in further research that could substantiate the health benefits of the strains. Additionally, the food industry has been greatly affected by the stagnating sales of these products in Europe, while experiencing an increase in other parts of the world (Bañares, 2016; Foligné et al., 2013; Thomas, 2016).

For Bañares (2016), the result of the application of EU regulations is that the food labels provide little or no information about the purchased product. So we went from a situation, before 2006, where the messages were quite aggressive and almost therapeutic, to nearly empty labels, without any health claims. Far from providing enough information, the labels can offer just an extraneous name (strain designation) and some numbers (millions of units) followed by the acronym CFU (colony forming units), that consumers are unable to interpret (Sanders et al., 2018).

Recently, some Member States (Belgium, Czech Republic, Denmark, Italy, Slovenia and the UK) forwarded a letter to the Commission in which they requested the use of the term "probiotic" as well as some guidance on the conditions of use. These countries propose, within the current legal situation, to change the FIC so that probiotic products can be included as additional labeling data, or that the expression "contains probiotics" becomes a nutrition, rather than a health claim (Baňares, 2016).

However, some countries, despite being EU members, publish official nutrition guidelines or recommendations on the use of prebiotics and probiotics or fermented milks (Smug et al., 2014). These include, for example, Italy and the United Kingdom (Hill et al., 2014; IPA, 2015; Ministry of Health, 2018; NHSUK, 2018).

Outside the EU the regulations governing probiotic claims vary by geographical region and countries being, in some of these, of much simpler application than in the EU. Figure 9 presents some aspects that characterize the regulation on probiotics in different countries where the use of favourable claims relating to probiotics and their labelling is authorized. Canada has one of the most progressive and well-developed regulatory schemes on probiotics (Hill et al., 2014; IPA, 2017). But health claims in the field of functional foods were first defined in Japan and came into force with the law on FOSHU. Following the Japanese system, several countries have decided to regulate health messages or other information of the health benefits of foods or food components (Thomas, 2016). In general, probiotics are sold as foods or supplement-type products and, as in the EU, no mention of disease or illness is allowed. Normally, claims tend to be general and products are aimed at the generally healthy population (WGO, 2017).

It is interesting to underline that also some associations and scientific societies defend the use of the "probiotic" denomination under certain circumstances, without being

considered as a “health claim” (Baňares, 2016; Hill et al., 2014). This is the case of ISAPP, the International Spanish Society of Probiotics and Prebiotics (SEPyP), the International Probiotic Association (IPA) and IPA Europe.

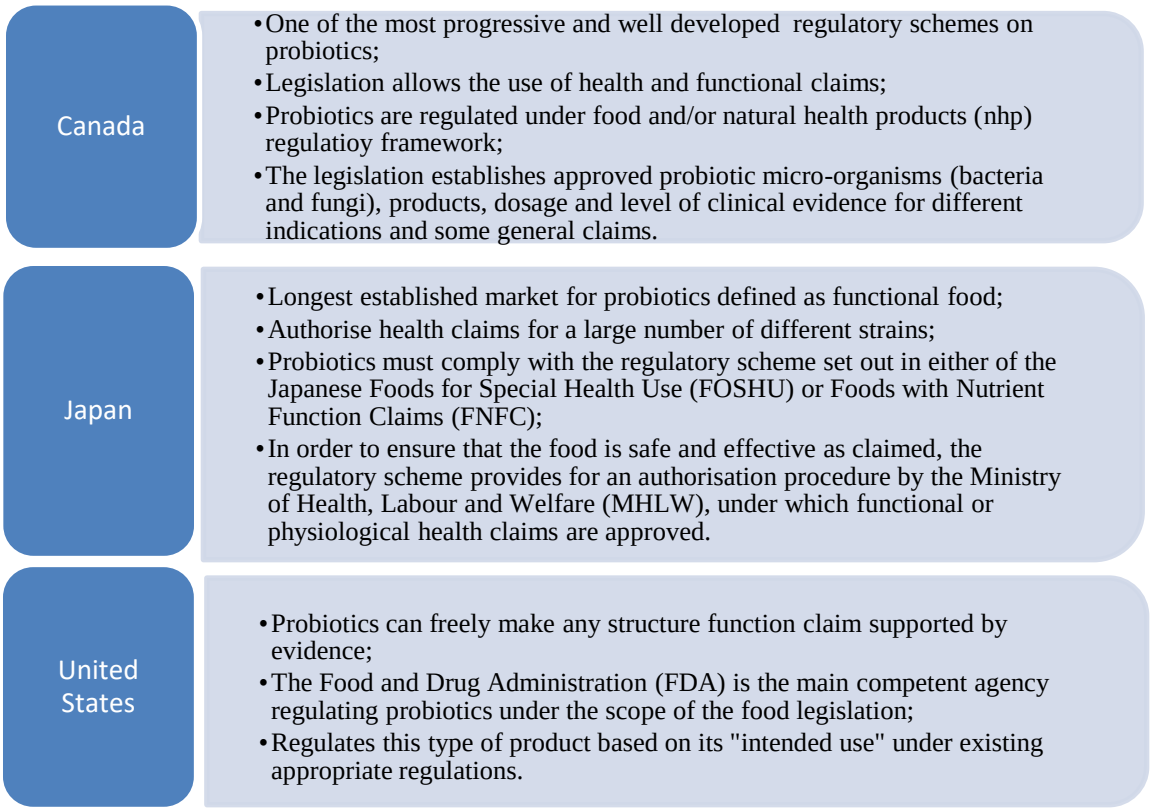

Figure 9 - Regulation on probiotics in different countries outside the European Union (Sources: Hill et al., 2014; IPA, 2017; WGO, 2017).

Also several clinical organizations have assessed probiotics and probiotic foods for their evidence-based health effects and have published guidelines for probiotic use. These are the European Society for Primary Care Gastroenterology, World Gastroenterology Organisation, National Institute for Health and Care, the European Society of Paediatric Gastroenterology, Hepatology and Nutrition, the European Society for Paediatric Infectious Diseases and the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (Ebner et al., 2014; Guarner et al., 2012; Hungin et al., 2013). These institutions have in common the interest in generating high quality scientific information for the probiotic and prebiotic fields and providing guidance for collaborative and multidisciplinary research and/or in the development of a clear legal framework.

There is a consensus among those responsible for the health and expert panels from those countries and institutions that the core benefit of probiotics is supporting a healthy digestive tract and that this effect can be ascribed to probiotics as a general class (Hill et al., 2014). Thus, for food and food supplements with probiotics for which nonstrain-specific claims might be made, the use of the word probiotics can be allowed according to some conditions. The conditions normally required are being traditionally used in humans, considered safe for human consumption, viable at the time of ingestion and in the gut, containing strains which are characterised using specific methods and containing at least 10^9 live cells of at least one strain of bacteria per daily serving of the product to guarantee an effect. The amount of live cells may be different as long as demonstrated by scientific studies (Health Canada, 2015; Hill et al., 2014; IPA, 2015; Ministry of Health, 2018; NHSUK, 2018). However claims on strain-specific benefits can only be made if the mechanistic basis has been demonstrated (Baňares, 2016; Hill et al., 2014). These aspects require previous scientific consensus, and for such a purpose some accurate guidelines or scientific open databases should be implemented in order to establish the strains considered as probiotic, similar to what already happens in some countries (Baňares, 2016).

The minimum probiotic count recommended by the United States Food and Drug Administration (US FDA) in probiotic food products is 10^6 CFU/ml (Begum et al., 2017; FAO/WHO, 2006). Depending on the quantity ingested and considering the effect of storage on the viability of probiotics, a daily dosage of 10^8 to 10^9 probiotic microorganisms is crucial to attain probiotic action in human beings. It has also been stated that probiotic products should be consumed daily, with an approximate amount of 100 g/day to deliver about 10^9 viable cells to the intestine (Begum et al., 2017).

A simpler and more straightforward EU regulatory system would stimulate probiotic research and innovation, ensure effective communication to consumers and healthcare providers and strengthen their confidence in probiotic and health claims through coherent recommendations and product labels. Finally, it would improve the industry with high quality and profitable products (Salvetti & O'Toole, 2017). It would also help to harmonize the use of these claims among EU member states, as some countries even prevent the use of the term 'live' on packaging, while others allow 'probiotic' as a general statement (Thomas, 2016).

I.4.5. Mechanisms underlying probiotic effects on health

Based on the body of available research, we can consider two common general benefits often associated with probiotics: supporting a healthy digestive tract and a healthy immune system (Hill et al., 2014; Nagpal et al., 2012; WGO, 2017). In fact, the main function of probiotics, but also prebiotics, is to interact with microorganisms that colonize humans, especially at the level of the gastrointestinal tract (GIT). In doing so, they improve or restore microbial homeostasis, ensuring proper balance between pathogens and beneficial microorganisms and contributing to host health (Nagpal et al., 2012; Vandenplas et al., 2015; WGO, 2017). On the other hand, the intestinal microbiota is also a vital part of the body's defence system.

The GIT is described as the body's largest immune organ, since it represents the host's greatest area of mucosal contact with the environment and contains as many as 80% of all antibody-producing cells (Binns, 2013; Hug, Mohajeri, & La Fata, 2018). The single layer of specialized epithelial cells present in the gut, designated as intestinal barrier, form a highly complex structured network that is the major intestinal defense system against pathogens. The epithelium of gut uses different defense mechanisms against the patogens such as microbiota, immune response (innate or acquired), mucus layer secretion, as well as integrity and turnover of the epithelial cell. In order to maintain the intestinal barrier function, adjacent epithelial cells of the gut form tight junctions with each other. These junctions act as a barrier that is impermeable to particulate things and liquid materials. Other cells of epithelium e.g. Goblet and Paneth cells also support barrier function, thereby contributing as a part of the innate immune system. Collectively, all these barriers decrease the load of pathogens at the interface between epithelium and lumen (Binns, 2013; Bron et al., 2017; Purchiaroni et al., 2013).

The indigenous microbial communities or microbiota are host specific, location specific, very complex in composition and normally play a beneficial role in the host with which they establish a symbiotic relationship. The microbial composition of these communities is established early in life and is determined by the host genotype, initial colonization at birth via vertical transmission, age, dietary habits, therapies and by the health status, being reasonably stable over time in healthy adults, despite considerable inter-individual variation (Aguirre et al., 2016; Binns, 2013; Bäckhed et al., 2012; D'Argenio & Salvatore,

2015; Doré & Blottière, 2015; Nagpal et al., 2012; WGO, 2017). Most of the human adult microbiota lives in the gut (D'Argenio & Salvatore, 2015).

The GIT in an adult contains about 40 trillion of microbial cells, comensal and transient, located mainly in the colon (10^{12} cells per gram of luminal contents - 1–2 kg of body weight) and comprising hundreds of species (> 1000) (Binns, 2013; D'Argenio & Salvatore, 2015; WGO, 2017). Bacteria predominate, including less than 1% of archaea, and can only be overcome by viruses/phages. *Firmicutes* and *Bacteroidetes* predominate and those that remain are *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Fusobacteria* and Cyanobacteria. Fungi and protists are also present but with a negligible contribution (Bäckhed et al., 2012; WGO, 2017). Potentially commensal beneficial genera include *Bifidobacterium*, *Eubacterium* and *Lactobacillus*; these are characterized by fermenting carbohydrates and do not produce toxins. However, all individuals also harbor microbes that have opportunistic or pathogenic potential (e.g. *Clostridium difficile*) (Binns, 2013). Human gut bacteria quickly respond to changes in diet and these changes can potentially have an impact on the health of the host (Aguirre et al., 2016). In Table 13 we present additional information about the human GIT microbiota.

So, there is little certain knowledge about what constitutes the normal microbial composition (eubiosis) of the gut due to the difficulty of this study and the great inter-individual variation. Recent research suggests that the normal microbiota is not simply a collection of micro-organisms, but reflects an inter-relationship between different groups that may work together. But in certain diseases, dysbiosis can be observed (Table 13), that is, deviations in composition or function from the usual microbiota. Dysbiosis has been connected, among others to obesity, metabolic syndrome, psychiatric disease, oral and skin health disturbances and cancer (Table 13) (Zoumpopoulou et al., 2017), although it is difficult to predict whether the change in the microbiota causes, or partly causes, the disease state or whether the change in microbes is a result of the disease itself (Binns, 2013).

Table 13 - Human GIT conditions affecting microbial growth, quantitative and qualitative GIT microbiota, description in eubiosis and diseases associated with dysbiodis (Sources: Binns, 2013; Bäckhed et al., 2012; D'Argenio & Salvatore, 2015; Zoumpopoulou et al., 2017).

GIT component		GIT conditions affecting microbial growth	Nº of microrganisms (cfu/g of content) and main inhabitants in eubiosis	Most represented Phyla and their relative abundance (%)	Dysbiosis Associated Diseases or Conditions
Stomach		Low pH – Acid secretions suppress most ingested microbes. Rapid transit impedes stable colonization of the lumen.	~10^3 Lactobacilli, enterococci, *Helicobacter* and bacilli	• Firmicutes (38.8), • Bacteroidetes (27.8), • Actinobacteria (8.2), • Proteobacteria (2.1), • Other: Fusobacteria, Verrucomicrobia, Cyanobacteria.	• Obesity • Metabolic syndrome • Nonalcoholic steatohepatitis • Inflammatory bowel diseases (IBD) (Crohn's disease, ulcerative colitis, pouchitis) • Irritable bowel syndrome (IBS), • Functional bowel disorders • Atherosclerosis • Type I diabetes • Type II diabetes • Autism • Schizophrenia • Depression • Anxiety • Allergy • Asthma • Celiac disease • HIV infection • Colon cancer • GIT infections • Antibiotic-associated diarrhea (AAD) • Necrotising enterocolitis • Rheumatoid arthritis
Small intestine	**Duodenum**	Low pH – acid, bile and pancreatic secretions suppress most ingested microbes.	~10^2 - 10^4 Lactobacilli and enterococci		
	Jejunum	Rapid transit impedes stable colonization of the lumen.	~10^4		
	Ileum		~10^6 - 10^7		
Large intestine (colon)		_	~10^{11} - 10^{12} • Great diversity of microbes • Anaerobes		

On the other hand, recent research stresses that GIT commensal bacteria such as *Roseburia*, *Akkermansia*, *Bifidobacterium* and *Faecalibacterium prausnitzii* appear to be associated more commonly with health (WGO, 2017). Other examples are the presence of bacteria of the genus *Barnesiella* in the colon and the resistance to invasion by vancomycin-resistant enterococci (Caballero et al., 2015) and the antagonistic effect of secondary bile salts produced by *Clostridium scindens* on the proliferation of *Clostridium difficile* (Buffie et al., 2015). Doré & Blottière (2015), states that low bacterial diversity consistently appears as a risk factor associated with detrimental effects on health, and is characteristic for numerous chronic diseases. So, the modulation of the gut microbiota to

gain a healthy status is the challenge facing metagenomic research in coming years (D'Argenio & Salvatore, 2015).

When we consume probiotics, we are ingesting live microorganisms in a range of dosages, spanning from 10^8 to 10^{12} cells/day, depending on the product (Binns, 2013). So, consuming probiotics on a daily basis could be equivalent to introducing new, albeit transient microbes, into the indigenous intestinal microbiota as a relatively large fraction of those microbes survives passage through the human digestive tract. However, one must take into account that these ingested microbes are likely to be affected by the host's diet and not all are able to replicate and persist in the gut and promote long-lasting effects on the resident colonic microbiota. Normally they disappear a few days after cessation of their intake (Binns, 2013; Tachon, Lee, & Marco, 2014)

At this level comensal and transient microorganisms proliferate by fermenting available substrates from the diet or endogenous secretions and contribute to host nutrition. The presence and development of these microorganisms affect the intestinal ecosystem in a beneficial way, where five basic mechanisms for biological probiotic functionality can be considered (Table 14). These are microbiological, nutritional, physiological, immunological and a fifth functional effect related to the ability to decrease detrimental compounds in the gut (Binns, 2013; Bron et al., 2017; Granato, Branco, Cruz, Faria, & Shah, 2010; Markowiak & Śliżewska, 2017; Vandenplas et al., 2015). In the Table 14 we can see the mechanisms associated with each and some examples.

The effects may be local in the gut lumen, where probiotic bacteria can interfere with the growth or survival of pathogenic micro-organisms. At a more internal level, probiotics can improve the mucosal barrier function and mucosal immune system, or the effects can be systemic with repercussions on the systemic immune system, as well as on other cell and organ systems such as the liver and the brain (Rijkers et al., 2010).

Table 14 - Biological functionality of probiotics and mechanisms of action (Binns, 2013; Bron et al., 2017; D. Granato et al., 2010; Landete et al., 2016; Markowiak & Śliżewska, 2017; Vandenplas et al., 2015; WGO, 2017).

Biological effect of probiotics	Mechanisms of action of probiotics in the GIT	
Microbiological functionality **General Effect:** To improve or restore microbial homeostasis with exclusión of opportunistic and potential pathogens and selection of commensal organisms.	**Competitive exclusión**	• The large number of microbes ingested has the potential for a greater impact in the upper GIT where lower densities of micro-organisms are found, but also in the colon through competition for nutrients, physical sites (e.g. mucus adhesion) or receptors, thus reducing the ability opportunistic and potential pathogens to colonise the intestine, adhering or translocating.
	Antagonistic exclusión	• Low pH - Mainly bifidobacteria, lactobacilli and streptococci in the colon ferment carbohydrates and dietary fibre that escape digestion in the upper GIT, with production of organic acids (e.g. lactate) and short chain fatty acids - SCFA (e.g acetate, butyrate, propionate); • Production of bacteriocins, which are small antimicrobial peptides; • Production of reactive oxygen species, such as hydrogen peroxide, that are highly reactive; • Alteration of gene expression - Some probiotics have demonstrated this ability in vitro, thereby reducing pathogen virulence.
Nutritional functionality	**Production of vitamins**	• Vitamin K, vitamin B12, pyridoxine, biotin, folate, nicotinic acid and thiamin, with increasing nutrient bioavailability.
	Increased lactose tolerance	• Lactase-positive strains increase lactose tolerance and relieve discomfort caused by the same.
	Production of health-promoting compounds	• Production of health-promoting conjugated linoleic acids (e.g. SCFA, CLA and other fatty acids, bioactve peptides, GABA); • Conversion of phytoestrogen precursors to bioactive metabolites.
Physiological functionality	• Enhance GI transit with regulation of the intestinal flow and gut motility; • Digestion of food with easy digestion and increasing nutrient bioavailability (e.g. SCFA are used as a source of energy by the host); • Reduction of bloating or gas production; • Enhancement of ion absorption by intestinal epithelial cells with e.g. prevention of osteoporosis (SCFA are absorbed, enhancing the uptake of water and salts); • Decrease of bile salt toxicity by bile salt hydrolases and reduction of other catabolic products eliminated by kidney and liver; • Decrease of serum cholesterol levels by bile salt hydrolase positive probiotics with possible prevention of arteriosclerosis; • Improvement of intestinal barrier function. The disruption of this function may increase the risk of certain intestinal disorders or diseases. Some mechanisms: o Production of butyrate (SCFA) that is the major source of energy of the epithelial cells lining the colon can have an impact on growth and differentiation of these cells. Butyrate can also stimulate the production of epithelial mucin (high molecular weight glycoproteins) by mucus-producing Goblet cells, that helps protect underlying epithelial cells from mechanical and chemical damage, potential pathogen translocation, and may improve the release of pathogens from the GIT; o Certain probiotics may enhance the ability of specialised Paneth cells in the small intestine to produce the antibacterial peptides known as defensins; o In vitro studies suggest that certain probiotics may increased the expression of genes encoding tight junction proteins (e.g. occludins and claudins) enhancing the capacity of tight junctions (small intercellular space between epithelial cells) to control access by foreign molecules and particles.	

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Table 14 – Continuation - Biological functionality of probiotics and mechanisms of action (Binns, 2013; Bron et al., 2017; D. Granato et al., 2010; Landete et al., 2016; Markowiak & Śliżewska, 2017; Vandenplas et al., 2015; WGO, 2017).

Biological effect of probiotics	Mechanisms of action of probiotics in the GIT	
Lowering health detrimental components in GIT	**Sorption of the compound to microbial biomass**	• Mycotoxins (e.g. aflatoxin B1); • Xenobiotics with toxic properties as unwanted residues from environmental contamination of the food chain; • Hazardous compounds from the food production process (e.g. PAH production during grilling of meat).
	Direct detoxification of the hazardous compound	• Direct breakdown of toxic substances (e.g. breakdown of fumonisin a carcinogenic mycotoxin); • Scavenge superoxide radicals.
	Indirect by probiotic modulation of GIT microenvironment	• Production of organic acids by probiotic microorganisms was reported to negatively affect the production of Shiga-toxin 2 from enterohemorrhagic *E. coli* O157:H7; • Saccharolytic fermentation concomitantly reduces the potentially adverse effects of protein fermentation and other processes, which originate nitrogen and sulphur containing compounds (ammonia, N-nitroso- and azo-compounds, sulphides).
Immunological functionality **General Effect**: Interaction with the GIT immune cells and lymphoid tissue to modulate the immune and inflammatory responses of the host and protect from pathogens (bacteria, viruses, fungi), other foreign materials (antigens) and also from tumour cells arising in the host.		• **Action on the innate immune response**: various probiotics can modulate the activity of phagocytic cells (neutrophils, macrophages and dendritic cells) and natural killer (NK) cells (non-T non-B lymphocytes); • **Action on the acquired immune response**: The interaction between microbial cells and host cells is mediated by the interaction with specific receptors such as Toll-like receptors (TLR) that are associated with cells lining the mammalian GIT. The activation of these receptors initiates a cascade of concerted immune signals leading to different responses: ○ Maturation of T cells (Th1 versus Th2) and T-regulatory cells, which allows an appropriate response to potential pathogens and food antigens (an inappropriate T cell response is thought to be one of the features of allergic conditions). The production of butyrate (SCFA) has been reported to support regulatory T-cell functions in the gut; ○ B cell differentiation and production of protective antibodies, such as IgA production locally and systemically; • **Changes in pro/antiinflammatory cytokine profiles**: specific probiotic strains or prebiotics have been found to stimulate an increase in the anti-inflammatory cytokines, such as IL-10 and TGF-β, and a decrease in the expression of pro-inflammatory cytokines, such as TNF-α and IFN-γ. It is proposed that these changes in cytokine balance could be a mechanism by which prebiotics and probiotics may be able to mitigate chronic intestinal inflammation.

These mechanisms can lead to competitive and antagonistic exclusion of opportunistic and potential pathogens, an improved intestinal environment, intestinal barrier reinforcement (by decreasing permeability due to the stimulation of tight junction functionality, and by proliferation of cells or inhibition of epithelial cells apoptosis), down-regulation of inflammation, and up-regulation of the immune response to antigenic challenges since probiotics can activate both the innate and adaptive immune systems (Table 14) (Lebeer et al., 2018; WGO, 2017).

In any case, these phenomena are thought to mediate most beneficial effects, among which the most widely recognized is a reduction in the incidence and severity of infectious diarrea with different causes, including antibiotic-associated diarrea (AAD) and *Clostridium difficile* associated diarrea (WGO, 2017; Zoumpopoulou et al., 2017).

Evidence is gradually developing for the potential for probiotics to impact other conditions of the GIT, such as inflammatory bowel disease (IBD) like ulcerative colitis and Crohn's disease, irritable bowel syndrome (IBS) and colon cáncer (Bron et al., 2017; Principi, Cozzali, Farinelli, Brusaferro, & Esposito, 2018; Zoumpopoulou et al., 2017).

The influence on the immune system may enhance resistance to infections, particularly those of the GIT or respiratory tract, but also help to mitigate allergies including to food, particularly in infants and young children (Majamaa & Isolauri, 1997; Zoumpopoulou et al., 2017). An expanding area of interest for both prebiotics and probiotics is the investigation of their potential for an anti-inflammatory role in places other than the gut, such as cardiovascular disease, obesity and metabolic syndrome (associated with the risk of developing cardiovascular disease, obesity and type 2 diabetes), also psychotropic activity through the gut-brain axis, and anti-mutagenic or anti-cancerous activities (Gallego & Salminen, 2016; Wang & Kasper, 2014; Zoumpopoulou et al., 2017). There is more and more evidence for the influence of dysbiosis on mental health and disease, and it has been connected to psychiatric conditions like autism, schizophrenia and stress-related disorders like depression and anxiety (Fond et al., 2015).

However, these mechanisms are not common to all probiotics. Supporting a healthy digestive tract is widely considered a common role among a large number of different probiotic strains normally studied, while a healthy immune system is also widely acknowledged, but more strain-specific (Hill et al., 2014; Nagpal et al., 2012; WGO,

2017). In general, it is considered that other benefits are promising but the evidence is not yet sufficient to consider these effects shared by all probiotics (Hill et al., 2014; Kechagia et al., 2013; WGO, 2017; Zoumpopoulou et al., 2017) and more research is needed to clarify the mechanisms of this action (Zoumpopoulou et al., 2017).

Based on this principle, in Figure 10 we present a possible classification of the mechanisms underlying probiotic effects as Widespread, Frequent and Rare, as they can be observed, respectively, in several studied species, or only in some specific species or strains. It is also considered that although multiple mechanisms are often represented in a single strain, no individual probiotic would be expected to have all the effects listed in Figure 10 (Hill et al., 2014).

I.4.6. Resistance and safety aspects in the selection of probiotic microorganisms

Taking into consideration the minimum requirements for probiotic status or for the use of health claims by specific microbial strains, the assessment of probiotic strains usually begins by evaluating of their tolerance to the hostile environment of the human gastrointestinal tract (GIT) and their ability to colonize the host, as well as safety issues. Thereafter, this assessment continues on beneficial health aspects such as antimicrobial activity, immunomodulatory capacity and activity leading to the release of biologically active compounds (Papadimitriou et al., 2015; Zoumpopoulou et al., 2018).

Initial screening for probiotics essentially relies on *in vitro* testing. It is usually the preferred choice because of the simplicity and low cost, as well as for the ability to screen multiple strains simultaneously. The use of these tests has revealed reproducibility problems, making it difficult to rely solely on the outcomes of *in vitro* tests for the selection of probiotic strains. Additional use of *in vivo* models may be necessary and appropriate, but in most cases they can not be used due to the increased cost and for ethical reasons (Ouwehand & Salminen, 2003; Papadimitriou et al., 2015).

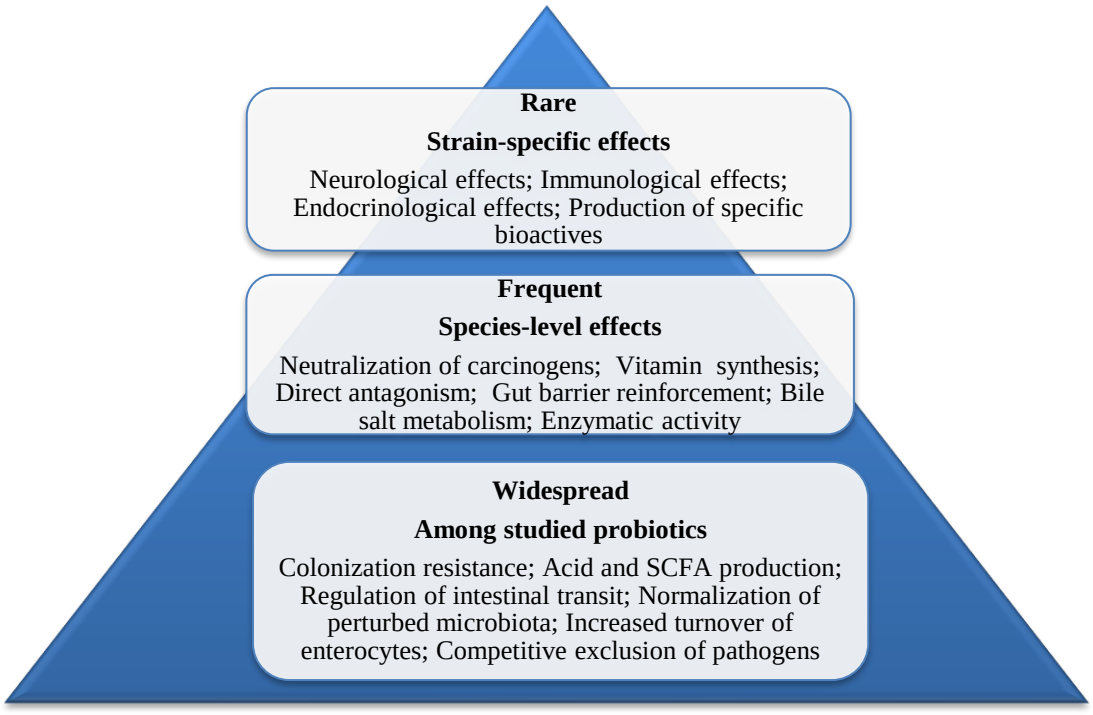

Figure 10 - Classification of the mechanisms underlying probiotic effects as Widespread, Frequent and Rare as they can be observed, respectively, in several studied probiotic species, or only in some specific species or strains (Source: Hill et al., 2014).

Several authors point out to the tendency to replace traditional screening methods with omics-based technologies (as genomics, proteomics or metabolomics) or, at least, to use them as a complementary tool for screening with current methods (Papadimitriou et al., 2015; Vinderola, Gueimonde, Gomez-Gallego, Delfederico, & Salminen, 2017).

At this point, we will address the more general aspects of selecting probiotics related to tolerance of strains to the conditions of the gastrointestinal tract, essential for their physiological effects and the safety of strains and also essential for their use without compromising consumer safety. More specific aspects of the characterization of strains related to the synthesis of bioactive compounds that may have a beneficial effect on health will be addressed in the following point (cheese as a probiotic food), as their production also depends on the matrix involved.

In practice the selection of probiotic microorganisms requires a systematic approach using a step-by-step strategy consisting of a sequence of tests in order to select the strains that have the highest number of positive properties as probiotics and, concomitantly, without negative characteristics (Melo Pereira, de Oliveira Coelho, Magalhães Júnior, Thomaz-Soccol, & Soccol, 2018).

Figure 11 presents a complete possible screening approach to use in order to characterize probiotic potentials strains (PPS) according to WHO/FAO (2006) and additional screening for beneficial health effects and industrial requirements, and the possibility of using omics-based technologies in the identification and characterization of strains, that is, indirectly through the collective characterization and quantification of biological molecule sets that translate its structure, function and dynamics.

I.4.6.1. Tolerance to the conditions of the stomach and small intestine

Firstly, potential probiotic strains (PPS) should be evaluated for the characteristics that allow them to cope with the stress conditions that the human body creates (Figura 11). One of the proposed golden rules for a correct use of probiotics is that only microbial strains able to resist gastrointestinal conditions should be considered (Grigoryan, Bazukyan, & Trchounian, 2018; Klopper, Deane, & Dicks, 2018; Marco Toscano, De Grandi, Pastorelli, Vecchi, & Drago, 2017).

After ingestion, the first obstacle that the PPS must overcome is oral cavity enzymes (amylase and lysozyme), although they only contact for a short time (Melo Pereira et al., 2018). Gram-positive bacteria are generally sensitive to lysozyme, but some LAB are more resistant than other gram-positive bacteria (Angmo, Kumari, Savitri, & Bhalla, 2016; Soares et al., 2019; Solieri, Bianchi, Mottolese, Lemmetti, & Giudici, 2014). At this level microorganisms may also be affected by the slight thermal shock caused by the internal body temperature (Melo Pereira et al., 2018). The next line of defense against microorganisms entering the gastrointestinal tract is stomach with a pH between 1.5 and 3.0 and the presence of pepsin, although the presence of enzymes has a negligible effect on microorganisms compared to pH (Martinsen, Bergh, & Waldum, 2005; Sumeri, Adamberg, Uusna, Sarand, & Paalme, 2012). Therefore, the survival of strains will depend essentially on their ability to tolerate low pH.

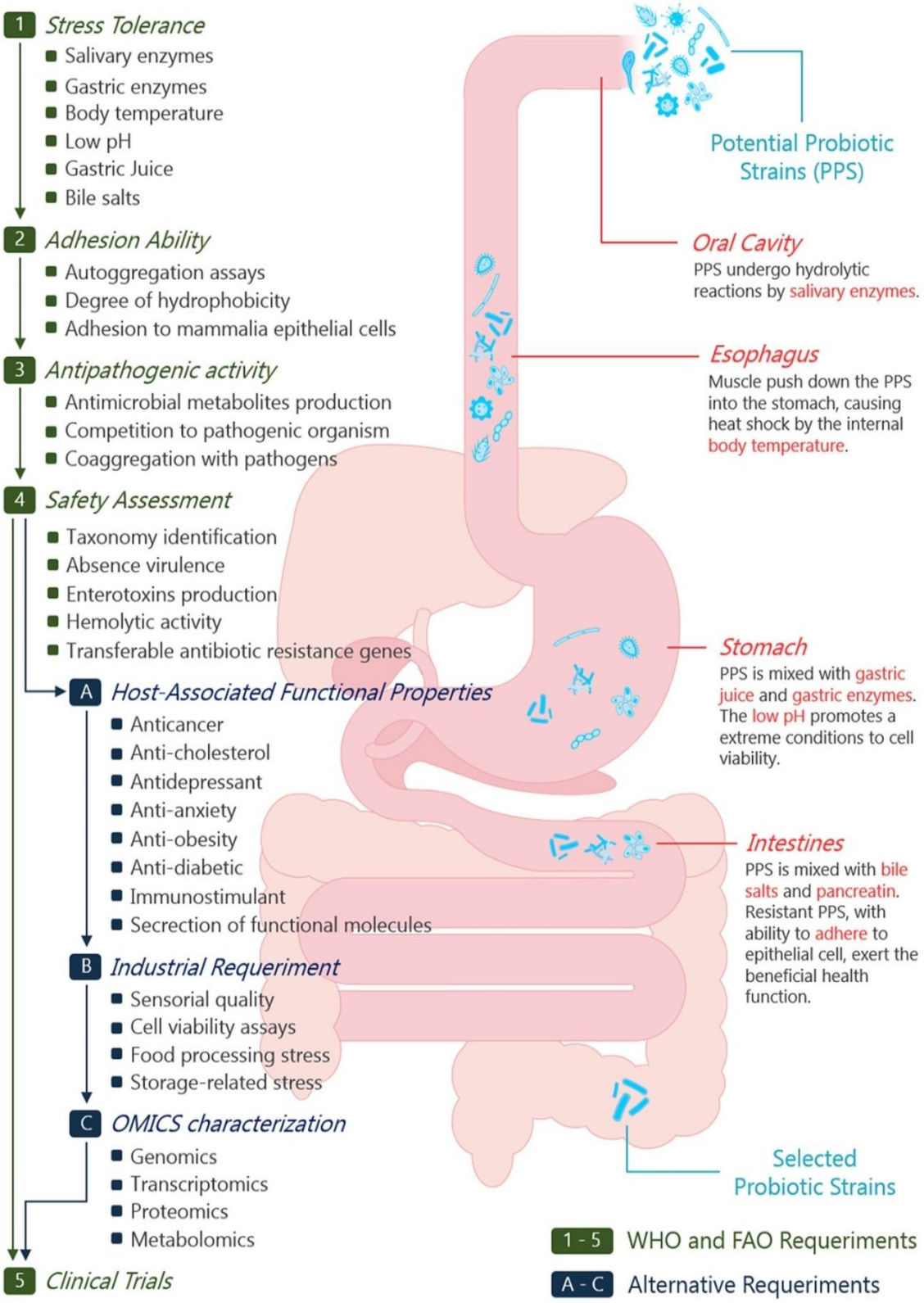

Figure 11 - Screening approaches used for characterization of potential probiotic strains (PPS) according to WHO/FAO (2006) and alternative screening for beneficial health effects and industrial requirements and the possibility of using OMICS technologies in identification and characterization of strains (Source: Melo Pereira et al., 2018).

It is known that the composition of the carrier matrices can influence the viability of probiotics. The pH in the stomach is lower (1.5), when the bolus is insufficient and higher when the bolus is large (5.5) (Peterson, Mackowiak, Barnett, Marlingcason, & Haley, 1989). Ingestion of foods with high fat content, high pH and or high buffering capacity (like milk) may reduce stomach acidity, contributing to the survival of probiotic microorganisms. Moreover, liquid foods are digested faster than solid foods, consequently reducing the contact time of probiotics with stressors (Klu & Chen, 2015).

Some references point to lactobacilli as more resistant to low pH and adapted to milk and other food substrates, and bifidobacteria as more sensitive, showing low or no survival rates at pH 2 and pH 3 (Van Loveren et al., 2012). However, in a recent study Toscano et al. (2015) evaluated the probiotic characteristics of three *Bifidobacterium* strains, reporting the strong ability of these strains to resist acidic conditions, as shown by a high rate of viability after one hour of incubation in a gastric solution. Resistant bacteria have several protective mechanisms, which allow them to have an adaptive response to low pH exposure, such as the ability to exclude protons from inside by increasing H+-ATPase activity. The same mechanism is involved in the acid tolerance of *Lactococcus lactis* (Toscano et al., 2017 and references therein).

The next obstacle for bacteria that survive stomach conditions are bile secretions (bile salts and pancreatin) in the duodenum (Figure 11). Bile secretions have a detergent function emulsifying and solubilizing lipids, playing a fundamental role in the digestion of fat. This property of bile salts gives them a potent antimicrobial activity due to the dissolution of cell membranes composed of lipids and fatty acids (Klopper et al., 2018; Melo Pereira et al., 2018). However, some microorganisms can reduce the effects of bile salt emulsification by means of bile salt hydrolase enzymes (BSHs), reducing their activity or by alternative mechanisms to counteract bile damage (Nagpal et al., 2012; Solieri et al., 2014; Marco Toscano et al., 2017; Toscano, De Vecchi, Gabrieli, Zuccotti, & Drago, 2015). Furthermore, epithelial cells of the small intestine, especially in the proximal region, are covered by a thin, and unevenly distributed, layer of mucus (Juge, 2012) and do not provide an ideal support for the adhesion of microorganisms. Thus, bacteria in the duodenum are mostly transient (Holzapfel, Haberer, Snel, Schillinger, & Huis in't Veld, 1998).

The low tolerance of strains to these inhibitory conditions generally excludes a considerable number of PPS. In summary, resistance to pH between 2 and 5 and tolerance to bile salt concentrations between 0,15 and 0,5% has been recommended for probiotics, which is in the range of the physiological concentrations met in the gastrointestinal tract (Papadimitriou et al., 2015). These resistance properties can be tested by cultivating the relevant strain of interest at a different pH with the presence of enzymes, such as pepsin, lysozyme and amylase, phenol, NaCl, Oxgall, porcine gastric juice, pancreatic, and taurodeoxycholic acid. Resistance to these compounds is measured by the colony count or absorbance at different time intervals (Melo Pereira et al., 2018). According to Vinderola et al. (2017), these in vitro static experiments might be more inhibitory than a real upper digestion process since real conditions are difficult to mimic. The author proposes that a standardized protocol should be developed to study the gastrointestinal resistance of PPS.

Since the PPS overcomes the previous barriers, it has the possibility of developing in the less rigorous environment of the distal portion of the small intestine (ileum) and in the large intestine (colon). The large intestinal tract is lined with a thick mucosal layer of varying densities and hosts approximately 70% of the gut microbiota (Ouwehand & Salminen, 2003). Thus, the strain must compete with the resident microbial community (10^4 to 10^{11} cells/ml) for available substrates (Gorbach & Goldin, 1992; Marco Toscano et al., 2017; Vandenplas et al., 2015). The ability to adhere, colonize the gut and survive over time in the intestinal environment is thus a fundamental feature for probiotic microorganisms, which allows them to exert their beneficial activities

I.4.6.2. Adherence to the intestinal mucosa and persistence in the intestinal tract

In PPS selection, the ability to adhere to epithelial cells and intestinal mucus are key features to be considered as these mechanisms are species and strain-dependent (Grigoryan et al., 2018; Melo Pereira et al., 2018; Toscano et al., 2017). As we said adherence of probiotics to the intestinal epithelium may contribute to their persistence on the mucosal surface and therefore facilitates their action at this level (Figure 11). However, colonization of the gut by orally administered probiotics appears to be only temporary. Nevertheless, transient colonization was observed (Vinderola et al., 2017).

Microbial adhesion to epithelial cells is a complex contact process involving two membranes (i.e., microbial and human cells) that depend on the physiochemical composition of the strain cell surface (Melo Pereira et al., 2018). More specifically, this accession to epithelial cells is related to both the autoaggregation capacity and non-specific hydrophobic properties of the cell surface (Collado, Meriluoto, & Salminen, 2008). Microbial cell autoaggregation ability ensures that the probiotic reaches a high cell density in the gut contributing to the adhesion mechanism to different kinds of host cells, while cell surface hydrophobicity allows for an improved interaction between microbe and human epithelial cells (Dlamini, Langa, Aiyegoro, & Okoh, 2019; Melo Pereira et al., 2018).

Autoaggregation is considered a desirable characteristic for preliminary PPS screening (Dlamini et al., 2019; Klopper et al., 2018); it's also simple and efficient (Papadimitriou et al., 2015). It can be achieved by measuring absorbance of bacterial suspensions in phosphate-buffered saline (PBS) that are left standing, in certain time intervals (Collado et al., 2008). Regarding the hydrophobicity study, controversial results have been obtained, showing that this feature may be questionable. In general, assessing the adhesive capacity of probiotic strains based on surface hydrophobicity is quite outdated (Papadimitriou et al., 2015; Vinderola et al., 2017).

Intestinal epithelial cell lines are often presumed to better represent conditions in the tissues of the gastrointestinal tract, so direct adhesion capacity to mammalian epithelial cells, such as Caco-2, HT-29, fetal I-407, is another way to assess the property of microbial adhesion (Melo Pereira et al., 2018; Papadimitriou et al., 2015). Another model to study in vitro adhesion is based on the use of immobilized intestinal mucus (Ouwehand & Salminen, 2003; Vinderola et al., 2017). The probiotic *Lactobacillus rahmnosus* GG strain is commonly used as a control to evaluate probiotic adhesion to intestinal cells since it is able to colonize the human intestine for more than one week (Alander et al., 1999). In general, *in vitro* testing of the adhesion potential is considered experimentally difficult (Papadimitriou et al., 2015).

The human adeno carcinoma cell line Caco-2, which expresses several markers characteristic of normal small villi cells (colonocytes and enterocytes), has been extensively used to study bacterial adhesion mechanisms for pathogens and probiotic strains (Imran et al., 2019; Ouwehand & Salminen, 2003; Vlková, Rada, Šmehilová, &

Killer, 2008), including cheese strains (Holzapfel et al., 1998; Imran et al., 2019; Losio et al., 2015; Solieri et al., 2014). Some studies have revealed a high correlation between in vitro assays with Caco-2 cells and in vivo assays (Jacobsen et al., 1999). However, tumorigenic cell lines have an altered metabolism and different surface sugar composition than normal cells, being of controversial use (Papadimitriou et al., 2015; Vinderola et al., 2017).

Additionally, reproducibility issues have been observed among laboratories depending on the bacterial strain, cell line or mucus employed. Another drawback concerns the very simple in vitro test model (prokaryotic-eukaryotic coculture) relative to the complex bowel system in vivo. For example, fierce competition for adhesion sites between different microbes in vivo cannot be reproduced (Papadimitriou et al., 2015; Vinderola et al., 2017). Nevertheless, strains that have been shown to adhere to human cells with high efficiency in vitro generally behave similarly in vivo (Papadimitriou et al., 2015). Vinderola et al. (2017) proposes, as a potential improvement, the use of epithelial cell lines from normal epithelial tissues and primary cultures from small intestine explants, and the use of intestinal tissue segments.

I.4.6.3. Safety Aspects - Origin and identification of strain

Assessing the safety of a PPS is essential, as there is always a risk of infection when introducing live microorganisms to the diet. This is of particular importance in the case of new strains which do not have a long history of safe use, and of probiotics belonging to species for which general assumption of safety cannot be made (EFSA/BIOHAZ, 2017; Lahtinen, Boyle, Margolles, Frias, & Gueimonde, 2009). Furthermore, strain safety will remain a concern in relation to infants, elderly people, patients in hospitalized condition, and those with immunodeficiency due to a genetic or acquired disease (Castro, Tornadijo, Fresno, & Sandoval, 2015; Lahtinen et al., 2009; Sanders et al., 2010; Sotoudegan, Daniali, Hassani, Nikfar, & Abdollahi, 2019). Reported cases of bacteremia and fungemia associated with probiotic use, involve these high risk populations (Lahtinen et al., 2009; Sotoudegan et al., 2019).

In the EU, a priori safety is generally accepted for microorganisms that have been awarded the qualified presumption of safety (QPS) approach established by EFSA (EFSA/SC, 2005, 2007; Salvetti & O'Toole, 2017; Van Loveren et al., 2012). This list

forms the basis of organisms at the species level which are considered safe for foods and feeds and is updated annually (EFSA/BIOHAZ, 2019; Kumar et al., 2015). Some probiotic strains are also classified as generally regarded as safe (GRAS) and listed in the U.S. Food and Drug Administration GRAS Inventory (FDA, 2020; Thomas, 2016).

Assessment of the safety of a probiotic begins with the correct identification of the strain (EFSA/SC, 2007; Melo Pereira et al., 2018; Toscano et al., 2017). Coupled with knowledge about the source of the microorganism, identification guarantees prior knowledge of bacteria concerning the pathogenicity potential (Melo Pereira et al., 2018).

The conventional source of probiotics for human use, recommended by FAO/WHO (2006), is the human gastrointestinal tract (GIT). Thus most of the probiotics available on the market were isolated from healthy humans to increase compatibility with and survival in the GIT notably by better adherence (e.g. *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Shirota, *Lactobacillus acidophilus* LA-1). However, several commercially explored, well-studied probiotic strains are not native human colonizers (e.g. *Bifidobacterium animalis* subsp. *Lactis*, *Saccharomycescerevisiae* var. *boulardii*) (Dorota & Danuta, 2018).

A range of unconventional sources of PPS is currently being used to isolate novel probiotic strains including such as the gastrointestinal tract of an animal, human breast milk, food (fermented and unfermented), air, or soil (Dorota & Danuta, 2018). Among foods, fermented and unfermented dairy products such as sheep and camel milk, cheese, traditional dairy products, camel milk and kefir grains, constitute one of the main resources (Melo Pereira et al., 2018). Research reports point out that *Lactobacillus* strains isolated from cheese and fermented vegetables show adhesion properties better or comparable to strains from human faeces (Boricha, Shekh, Pithva, Ambalam, & Vyas, 2019; Monteagudo-Mera et al., 2012).

For the selection of probiotic strains to be used in a given matrix, it is important to have a compromise between strain survival in the matrix and maintaining the desired health benefits. In addition, if the matrix is a food, such as cheese, the probiotic strain to be used should not alter its characteristics compromising its acceptance by the consumer (Plessas et al., 2017). Thus screening for PPS between the native bacteria of the matrix concerned, or similar, is a viable and possible way to achieve this compromise.

The identification of individual strains is performed as early as possible in the screening process. Each microorganism contained in probiotic products must be identified at species and strain level by internationally accepted molecular methods, according to the International Code of Nomenclature (nomenclature for bacteria is kept at the International Committee on Systematics of Prokaryotes (http://icsp.org/) and the International Code of Nomenclature of fungi is kept by the International Commission on the Taxonomy of Fungi – ICTF (www.fungaltaxonomy.org) (EFSA/BIOHAZ, 2017; Marco Toscano et al., 2017). EFSA/NDA (2016a) propose (Annex B) techniques for the identification and molecular characterisation of microorganisms, taking into consideration the current state-of-art. Moreover, it is widely recognized that the comparison of the results obtained by using different molecular methodologies (polyphasic approach) is the best way to establish strain identity (Lahtinen et al., 2009).

As a general rule, it is also recommended that strains are deposited in an internationally recognised culture collection with access number for control purposes. It can be in a biodepository such as the ATCC (American Type Culture Collection), or the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), so that microbiologists and the industry can secure a backup of their own cell cultures with their inherent physiological characteristics (FAO/WHO, 2006; Simone, 2019).

In the case of a combination of two or more microorganisms, it is considered that if one of the microorganisms used in the combination is not sufficiently characterised, the combination proposed is also not sufficiently characterised (EFSA/NDA, 2016a). This document also proposes (Annex B) techniques for the identification and molecular characterisation of microorganisms, taking into consideration the current state-of-art.

I.4.6.4. Transmission of antibiotic resistance genes and safety

In 2007, the EFSA introduced antimicrobial resistance as a safety concern associated with probiotic consumption (EFSA/FEEDAP, 2008). Consequently, in 2012, the QPS program also included this criterion (EFSA/FEEDAP, 2012). This document recommends that commercial microbial strains used as food supplements should not harbour any transferable antibiotic resistance and, consequently, the determination of Minimum Inhibitory Concentrations (MICs) of the main antimicrobial agents must be performed for each strain.

Antibiotic resistance is supposed to be a positive feature in a probiotic because by resisting the antibiotic, it would be able to restore intestinal eubiosis. However, if antibiotic resistant genes and determinants can be transferred from the probiotic to pathogenic, a serious risk for human health may arise, as pathogens can become resistant to antimicrobial agents, nullifying antibiotic therapies and further contributing to the spread of antibiotic resistance (Marco Toscano et al., 2017). Indeed, there is evidence to support the horizontal transfer of resistant genes among beneficial commensal bacteria including LAB and from these to pathogenic bacteria in the gut of the host (Gueimonde, Sanchez, de los Reyes-Gavilan, & Margolles, 2013; Jose, Bunt, & Hussain, 2015; Wong, Saint Ngu, Dan, Ooi, & Lim, 2015). Additionally, the use of massive antibiotic therapies in parallel with the use of probiotics can turn the latter into a dangerous reservoir of antibiotic resistance determinants (Mathur & Singh, 2005).

Only the ability of probiotics to conduct horizontal gene transfer by mobile elements (eg, plasmids, conjugative transposons, integrons, and bacteriophages) allows the development of antibiotic resistant strains. When resistance is the result of a chromosomal mutation (intrinsic) probiotic bacteria do not constitute a safety concern in itself, because this information can only be passed on to the next generation of mutant bacteria (Jose et al., 2015; Mathur & Singh, 2005). Thus, a crucial aspect in studying antibiotic resistance in probiotic bacteria is to separate intrinsic resistance from acquired resistance.

Given the importance of the subject, several studies have evaluated probiotics or PPS, either in commercial preparations or in food including dairy products, regarding the presence of genes associated with antibiotic resistance (Ammor, Florez, & Mayo, 2007; Guo et al., 2017; Li et al., 2019; Sharma et al., 2017; Sharma, Tomar, Goswami, Sangwan, & Singh, 2014; Sharma, Tomar, Sangwan, Goswami, & Singh, 2016; Wong, Saint Ngu, et al., 2015; Xu et al., 2012; Yang & Yu, 2019; Zheng et al., 2017).

Susceptibility to antibiotics can be measured by several phenotypic methods, including agar disk diffusion and agar overlay diffusion, E-test, agar dilution or broth dilution. Diffusion tests use antibiotic discs with inhibitory concentrations in agar plates, while E-test and dilution methods are based on the minimum inhibitory concentration (MIC) assay, which determines the minimum necessary concentration of an antimicrobial to inhibit microorganism growth (Guo et al., 2017; Mathur & Singh, 2005). The introduction

of the LAB susceptibility medium (LSM) facilitated the performance of these tests on LAB (Klare et al., 2005).

Phenotypic screening can have low reproducibility among laboratories (Nawaz et al., 2011), thus, PCR became the gold standard to detect the presence of resistance genes in probiotic bacteria because of its high sensitivity and convenience. In the PCR assay, DNA isolated from resistant strains are amplified with primers specific for the respective antibiotic resistance gene, and expected amplicons are analyzed by gel electrophoresis. The PCR products can also be sequenced for confirmation of the amplified sequences (Guo et al., 2017; Yang & Yu, 2019).

More recently, whole-genome sequencing analysis has emerged as a cost-effective approach to test the antibiotic resistance of probiotic bacteria, allowing for the differentiation between intrinsic and acquired resistance (Melo Pereira et al., 2018). Another way to make this distinction is analyzing gene transfer by filter mating experiments, where the probiotic with a detected antibiotic resistance gene is cultured with recipient cells (not possessing the gene); the transfer ratio to the recipient cell can be then analyzed by phenotypic and molecular methods (Gevers, Huys, & Swings, 2003; Guo et al., 2017; Nawaz et al., 2011).

I.4.7. Functional probiotics market trends

Although the supply of novel functional foods in the market is varied and abundant, probiotics still remain the most popular (Liang et al., 2016; Salvetti & O'Toole, 2017). Rising incidences of digestive and gastrointestinal disorders in a growing population, increasing consumer awareness regarding holistic health and understanding the importance of the microbiota in maintaining health are just some of the factors responsible for the growth of the probiotics market worldwide (IPA, 2017; Salvetti & O'Toole, 2017).

The global probiotic market is growing and there are significant opportunities for further growth (IPA, 2017; Reid, 2015; Thomas, 2016). It has been estimated that probiotic foods comprise about 60–70% of the total functional food market (Begum et al., 2017). The market for probiotic ingredients is projected to reach USD 46.55 billion by 2020, with Europe and the Asian-Pacific region estimated to be the largest and the fastest-growing markets, respectively (Salvetti & O'Toole, 2017). Given that probiotics are naturally

found in some foods, it is important to emphasize that market studies on global consumer trends survey, revealed that products that are naturally functional are the most sought after attributes among consumers purchasing functional food and beverage products, with 44% of participants choosing it (Euromonitor International, 2015). Also the market of probiotic dairy foods is increasing annually (Granato et al., 2010; Homayouni, Alizadeh, Alikhah, & Zijah, 2012; Reid, 2015). Thomas (2016) underlines that 79% of new dairy products launched were probiotic foods and beverages.

However, European probiotic market growth has fallen short of expectations, probably due to the complexity of the applicable regulations. The EU was the top global market for probiotic yoghurt and supplement sales until 2009, but current data show that probiotic yoghurts and fermented milk have lost more than € 1 billion in projected sales during the period 2009-2017 (Euromonitor data) in this region, despite a growing economy since 2013. The loss for probiotic yoghurt and fermented milk sales have been almost 19,3% in value. Meanwhile, in the same period, double-digit growth in probiotic sales has been recorded elsewhere in the world (+138,5% in North America, +49,1% in Latin America, +74,9% in the Asia-Pacific region) (Begum et al., 2017; Pecere, 2018; Thomas, 2016).

Considering the expanding market, it becomes important to establish the profile of the usual consumer of FF. This may be important for food manufacturers as they need to ensure that the development of these products, although founded in science, meet the expectations of the consumers. Santeramo et al. (2018) stresses that the development of new functional foods is a risky activity, because it is often driven by technical feasibility and not by the potential acceptance among consumers. Consumer attitudes are a key consideration in whether a functional food will be accepted and consumed by various sub-groups (Duncan, Dunn, Stratton, & Vella, 2014; Kaur & Singh, 2017).

Clearly, high income and high education are associated with positive consumer behavior in relation to FF (Kaur & Singh, 2017; Santeramo et al., 2018). With regard to gender and age, most of the results are not conclusive. However, recently Bimbo et al. (2017), by focusing on functional dairy products, concluded that females and older consumers are more likely to be willing to try and to include FF in their diet. The price is another important and limiting factor for their acquisition, mainly by lower socioeconomic consumer groups and young adults (Annunziata & Vecchio, 2011; Samoggia, 2016). Relatively high price premiums can be regarded as one reason for the limited market

success of several FF products introduced in recent years in Europe (Annunziata & Vecchio, 2011), and price/value criteria are the basis for purchasing decisions (Samoggia, 2016).

Annunziata y Vecchio (2011), in their study on the FF market in Europe, conclude that healthiness is the main factor affecting consumer attitude towards FF, so marketing strategies should focus on reinforcing FF properties and trying to communicate them clearly and less scientifically, in order to avoid confusion with other generic health foods, such as light or diet products. They underline the importance of consumer trust in health claims and suggest the introduction of a specific logo on the label and education and communication campaigns conveyed by public authorities, which could better contribute to the promotion and distinction of these products in the market. In order to target different consumer segments, namely young adults, companies can develop tailor-made pricing policies.

I.5. Cheese potencial as functional food

I.5.1. Dairy as functional food

Many existing and traditional dairy foods, like milk, yoghurt and cheese, can be considered functional in that they have long been considered nutrient dense and health-promoting foods that impart health benefits (Homayouni et al., 2012; Playne, Bennett, & Smithers, 2003). Because of this, milk has an outstanding position in the development of functional foods, being the basis of many of these products (Homayouni et al., 2012). Indeed, a variety of functional dairy foods (FDF) beyond the traditional have been conquering the market worldwide. Examples include probiotic, prebiotic, and symbiotic products, omega-3, low-cholesterol and low lactose or lactose free products and products that can control or manage hypertension and immune functions, among others.

In the worldwide functional food (FF) market, the FDF are key products with the highest sales and sales increase worldwide. Among nutrition-modified and functional products, FDF account for nearly 43% of the total sales, being one of the most investigated markets (Bimbo et al., 2017; Granato et al., 2017; Rodrigues, Rocha-Santos, Freitas, Duarte, & Gomes, 2012; Samoggia, 2016). This acceptance is due to its high nutritional value plus organoleptic characteristics that are highly appreciated by consumers (Shiby & Mishra,

2013). Additionally, dairy products are considered as one of the most credible product carriers to host functional ingredients (Bimbo et al., 2017; Granato et al., 2010; Homayouni et al., 2012; Reid, 2015). Also, they are available all year round (Reid, 2015).

Shiby & Mishra (2013) stresses that the FDF market worldwide is almost entirely made up of fermented dairy products. These foods mainly include yoghurts, fermented dairy beverages and cheeses (Granato et al., 2010; Hess, Jonnalagadda, & Slavin, 2016), being yoghurt and by-products considered top food trends (Sloan, 2016). These findings appear to be in line with the trend revealed in a recent study, that consumers are more receptive to traditional foods and products made via traditional procedures instead of products made with technologically advanced production methods or "atypical raw materials" (Settanni & Moschetti, 2014). The author refers to the example of skyr's recent popularity, because it is a traditional fermented dairy product. Evidence of health benefits associated with the presence of specific components or bacteria in dairy products is progressively gaining established scientific credibility (Bhat & Bhat, 2011; Granato et al., 2010; Hill et al., 2014; Marco et al., 2017; Zoumpopoulou et al., 2017). Because of the health benefits and the fact that fermented dairy products have been consumed all over the world for millennia, these are usually included in dietary recommendations (Chilton, Burton, & Reid, 2015; Ebner et al., 2014; Hess et al., 2016).

Indeed the functional role of fermented dairy products, including cheese, result either from direct action of microrganisms present (probiotic effect) or from effects related to the presence of biologically active components of milk (Appendix 4) or produced by the action of probiotic microorganisms on milk compounds, mainly during fermentative processes (biogenic effect) (Bhat & Bhat, 2011). In fact, in any fermented food, nutritional and functional properties are improved by the fermentation process. The presence, proliferation and biochemical action of naturally occurring or added beneficial microorganisms involves breaking down complex constituents into simpler ones, many of which may be bioactive. Final products also have greater stability (shelf-life), sensory attributes and safety (Macori & Cotter, 2018).

For all of the above reasons fermented dairy products have traditionally been used as delivery vehicles for probiotics naturally present or added, and are also one of the most extensively explored sources of proficient probiotics (Dorota & Danuta, 2018; George Kerry et al., 2018; Gupta & Bajaj, 2018; Macori & Cotter, 2018). Different types of

cheese have been much investigated in these two aspects, despite some health concerns normally associated with their consumption, namely high fat content, lactose intolerance and allergies to milk proteins, as well as the possible accumulation of toxic compounds (Gupta & Bajaj, 2018).

Biologically active components in this type of products, i.e. with a positive impact on body functions or conditions and which may ultimately influence health, are essentially functional proteins, bioactive peptides, cytokines, essential fatty acids, organic acids, oligosacarides and polysaccharides, minerals (mainly calcium) and vitamins (folate, riboflavin, B12) (Bhat & Bhat, 2011; Marco et al., 2017; Sultan, Huma, Butt, Aleem, & Abbas, 2018). Dairy bioactive compounds can be classified, among others, as immunomodulant, antimicrobial, antihypertensive, anti-oxidative, anti-mutagenic, opioid, anti-thrombotic, anti-obesity and mineral-binding agents, depending upon biological functions (Mohanty, Mohapatra, Misra, & Sahu, 2016; Sultan et al., 2018). Appendix 4 presents a table with the bibliographical research carried out on main biologically active components of milk and/or fermented dairy products and their posible beneficial effects on health, as suggested in different bibliographic sources.

Available scientific information suggests that its consumption contributes to meeting nutrient recommendations, contribute to the gastrointestinal and bone health and may protect against some transmissible diseases and the most prevalent chronic health non-communicable diseases like hypertension, coronary heart disease (CHD), obesity, osteoporosis, cancer, diabetes and metabolic syndrome (Bhat & Bhat, 2011; Bordoni et al., 2017; Da Silva & Rudkowska, 2016; Goede, Soedamah-Muthu, Pan, Gijsbers, & Geleijnse, 2016; Hess et al., 2016; Marco et al., 2017; Mohanty et al., 2016; Radha & Megha, 2016; Sultan et al., 2018; Thorning et al., 2016).

Despite the vast extent of research that has been conducted on dairy products and their connections to human health, including their associations with chronic disease prevention, the EFSA has yet to approve most directly applicable health claims on dairy products (including fermented dairy foods in general but also yoghurt, cheese, natural or added probiotics and other functional ingredients). The scientific opinion of the panel of experts on the substantiation of the health claims submitted has been negative on the basis of different justifications such as the lack of food/food constituents characterization, insufficient existing or provided scientific evidence or claimed effect not considered as a

beneficial physiological effect. In all these situations, those experts consider that a cause and effect relationship cannot be established between the foods/food constituents which are the subject of the opinion and the claimed effects. Appendix 2 (A2) lists the dairy nutrients, substances or foods for which the authorisation request to use the health claim, submitted to EFSA under Article 13º of the Regulation (EC) Nº 1924/2006 (EC, 2006c), was not granted. It also presents the EFSA bibliographic reference, which justifies the (not-) granted status.

Some dairy products meet the criteria required by EFSA, taking into account their nutritional content, and may make use of certain authorized claims (Appendix 3). However, these health claims do not cover all possible health benefits attributed to the consumption of dairy foods, especially with regard to probiotics. Therefore, further scientific research on nutrition on dairy products and derived components may be of great advantage in order to promote their use for the benefit of public health.

However, recognizing the beneficial properties of dairy products, dietary guidelines from many countries recommend its consumption (Bordoni et al., 2017; Da Silva & Rudkowska, 2016; Hess et al., 2016). The current recommendations in the United States, Canada, Australia, United Kingdom and also Portugal and Spain are that adults should consume at least the minimum recommended of 2–3 or 4 portions/day to exert its beneficial effects, within a well-balanced diet and a healthy lifestyle (Da Silva & Rudkowska, 2016; Gregório, Santos, Ferreira, & Graça, 2012; Hess et al., 2016; SENC, 2016; USDHHS/USDA, 2015). In most cases, competent authorities recommend giving priority to low-fat dairy products without added sugars. Some authors add that higher dairy product consumption (4–5 servings per day) may have additional beneficial effects on oxidative outcomes, but more welldesigned clinical studies are needed to ascertain these effects (Da Silva & Rudkowska, 2016).

I.5.2. Cheese as a matrix for probiotics

The delivery of high numbers of microorganisms to the GIT is supported by the matrix of some fermented foods which promote the long-term survival of organisms during distribution and storage (Marco et al., 2017). The health-modulating potential of some strains also might be enhanced by the delivery matrix. For example, the colitis levels in mice fed *L. casei* BL23 incubated in milk were very low compared to that when using the

same probiotic inoculated in nonnutritive buffer (Lee, Yin, Griffey, & Marco, 2015). Fermented foods also have a particular potential as a practical vehicle in which to provide probiotic strains to people in lowincome countries (Mpofu et al., 2014).

According to Granato et al. (2010), dairy products are the main vehicle for probiotic supplementation and a good vehicle to transfer probiotics to the human intestinal tract. Among these products, various types of cheese have characteristics that are favorable for the survivability of probiotics, that may even be part of its own native flora (Blaiotta et al., 2017; De Prisco & Mauriello, 2016; Granato et al., 2010; Vrese & Schrezenmeir, 2008). The reasons why cheese represents a suitable vehicle for the supply of probiotics in the human gut are both its matrix, that provides favorable conditions and high protection to living cells, and its compliance with worldwide long-term diet (De Prisco & Mauriello, 2016; Thomas, 2016).

This can be challenging in terms of the dairy industry because cheeses naturally enriched or supplemented with probiotic bacteria have added value when compared to traditional ones and represent a current trend especially when using sheep milk as a raw material (Balthazar et al., 2017; Granato et al., 2010). Castro et al (2015) and Thomas (2016) suggest that some steps forward seem to be the development of new varieties, the incorporation of new probiotic and well characterized strains, or the manufacture of symbiotic cheeses.

The use of cheese for this purpose may pose some challenges related to the viability of probiotics in this matrix. The factors that most affect the survival of probiotics in cheese could be categorized in three main groups including formulation factors, process factors, and packaging (Figure 12) (De Prisco & Mauriello, 2016; Karimi, Mortazavian, & Da Cruz, 2011). Below we will address the most important.

Indeed, viability is a key parameter for the efficacy of probiotic products. So, probiotic cells must remain viable in cheese above a standard threshold level between 10^6 and 10^7cfu/g until the time of consumption, in order to ensure a daily intake of at least 10^8–10^9 viable cells, essential to provide a therapeutic effect (FAO/WHO, 2006; HC, 2009; Melo Pereira et al., 2018; Settanni & Moschetti, 2010).

In addition, this amount of probiotics should not adversely alter the sensory attributes of cheese (Albenzio et al., 2010; Grattepanche, Miescher-Schwenninger, Meile, & Lacroix, 2008; Karimi et al., 2011). Results of some current studies prove these conditions in cheese even at the level of industrial production (Blaiotta et al., 2017; Martins et al., 2018; Speranza et al., 2018).

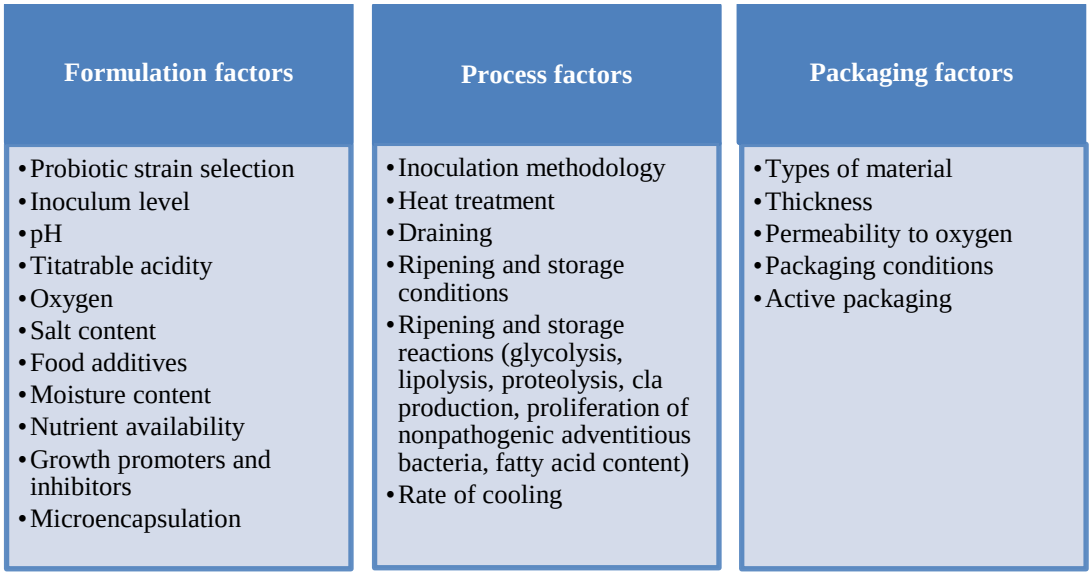

Figure 12 - Main factors that may affect the viability of probiotic bacteria in cheese (Adapted from Karimi et al., 2011 and de Prisco & Mauriello, 2016).

Intrinsic factors favorable to the development of probiotics in this matrix in relation to milk or yogurt and other fermented milks (pH 3.7- 4.5), are higher pH (4.8-5.6 or more), lower titratable acidity, higher buffering capacity, greater fat content, higher nutrient availability, lower oxygen content and a denser matrix (Castro et al., 2015; De Prisco & Mauriello, 2016; Gomes da Cruz, Alonso Buriti, Batista de Souza, Fonseca Faria, & Isay Saad, 2009; Madureira et al., 2008; Thomas, 2016). Under these conditions the probiotic can survives throughout the shelf life of the product and reaches the consumer in the appropriate high quantities (Blaiotta et al., 2017; Homayouni et al., 2012; Martins et al., 2018; Speranza et al., 2018).

Bifidobacteria and lactobacilli are the most commonly used probiotics in functional dairy foods and are generally classified as strictly anaerobic or microaerophilic but their resistance to oxygen is strain and species-dependent. They usually show poor resistance under prolonged acidic conditions, with lactobacilli (optimum pH 5,5-6) being less affected than bifidobacteria (optimum pH 6-7) (Gomes & Malcata, 1999; Grattepanche

et al., 2008). These characteristics explain the higher survival of probiotic cultures in cheese compared with other fermented dairy products. In fact, the cheese core can be considered an anaerobic environment with very low redox potential (Eh) of about –250 mV. Furthermore, the pH of hard and semi-hard cheeses, generally nearly 5.0 after production, remained relatively constant throughout the ripening period or increased slightly (Caldeo & McSweeney, 2012; Grattepanche et al., 2008). In some soft cheeses, the pH increases rapidly during ripening and can reach values around 7,0 in core and rind (Grattepanche et al., 2008). In this way, cheese is also a promising carrier for enlarging the range of probiotics added to functional foods, mainly bifidobacteria that are usually more sensitive. Additionally, predominant fatty acids in dairy such as butyric acid, palmitic acid and stearic acid promote the growth of some probiotic bacteria (Gómez-Cortés, Juárez, & de la Fuente, 2018; Plessas et al., 2017).

Cheese also provides an additional protection to probiotic bacteria against the severe gastrointestinal conditions, especially throughout the gastric transit, due to the dense matrix, relatively high total solids and fat content, as well as to the creation of a buffer against the high acidic environment (Bergamini, Hynes, Quiberoni, Suarez, & Zalazar, 2005; Granato et al., 2010; Karimi et al., 2011; Ross, Fitzgerald, Collins, & Stanton, 2002). This protection is superior with the use of sheep milk, due to the high protein and fat levels. Balthazar et al. (2017) considers that the benefits of sheep cheese as the matrix remain unexplored by the dairy industry. For this author, sheep's milk, besides being an excellent source of nutrients, allows a high yield in cheese production and a dense matrix due to its high protein and fat content. For the same reasons and also due to the low oxygen permeability, it can provide very effective protection for probiotic bacteria upon passage through the gastrointestinal tract and during commercial storage of the product. Appendix 5 presents a table with the results of the literature search on the use of sheep's cheese as a matrix for different probiotics. The type of cheese and the strain (s) tested are given, as well as a summary of the results obtained as regards the viability of the strains concerned in this matrix and the consequences on the characteristics of the cheese.

One factor that can negatively affect the development of probiotics in cheese may be salt content (Margarita, Fernando, Milton, & Obeimar, 2016). High salt conditions in cheese appeared to reduce the viability and enzymatic activity due to osmotic stress effects on the bacterial cell membrane (Thomas, 2016). The viability of probiotic bacteria decreased

drastically in cheeses with a salt concentration of over 4% (Karimi, Sohrabvandi, & Mortazavian, 2012). Possible solutions include microencapsulation and careful strain selection (Castro et al., 2015; Karimi et al., 2011). Another drawback related to probiotic viability during storage is the cold stress, when temperatures reduce the membrane fluidity and the enzymatic activity and increase of sensitiveness toward sodium chloride (Corcoran, Stanton, Fitzgerald, & Ross, 2008).

Additionally the microbial interaction among autochthonous, starter, protective or beneficial cultures have to be deeply investigated. LAB, for example, produce a range of compounds including bacteriocins, organic acids, antimicrobials and hydrogen peroxide that can inhibit not only pathogenic or spoilage bacteria but also that belonging to the same genus, including probiotics (De Prisco & Mauriello, 2016). Desfosses-Foucault et al. (2012), when studying the interaction between probiotic strains and starters of the genus *Lactococcus*, emphasizes the inhibition of the growth of the latter by the probiotic *Lb. rhamnosus*.

Although cheese is likely to be one of the best carriers for probiotics, the addition of high numbers of viable and metabolically active probiotic cells can affect product quality, especially organoleptic properties (Castro et al., 2015; Cuffia et al., 2017; Dantas et al., 2016; Grattepanche et al., 2008). However the gross chemical composition of cheese (i.e. salt, protein, fat and moisture) and pH are generally not influenced by added probiotic bacteria (Burns et al., 2012; Grattepanche et al., 2008; Martins et al., 2018; Mushtaq, Gani, Masoodi, & Ahmad, 2016; Pino et al., 2017; Speranza et al., 2018).

Some studies on this subject (Albenzio, Santillo, Caroprese, Braghieri, et al., 2013; Albenzio et al., 2010; Cuffia et al., 2017; Felicio et al., 2016; Grattepanche et al., 2008) mention a higher moisture level, increases in free amino acid and high acetic acid content. The first is explained by a rapid acidification during cheese manufacture, leading to low body/texture scores in sensory analysis, the last is due to heterofermentation carried out mainly by bifidobacteria. Acetic acid contributes to the typical flavor of different cheeses, but excessive concentrations can also result in off-flavors (Zabaleta, Albisu, & Barron, 2017). Probiotic cultures in cheese do not generally affect primary proteolysis but may interfere in secondary proteolysis with the release of peptides and free amino acids that directly contribute to cheese flavor (such as sweet, bitter or malty) and can be precursors

for the synthesis of other flavors or volatile aroma, resulting in off flavors (Grattepanche et al., 2008).

The viability of probiotic strains through processing can be improved by cell immobilisation on natural carriers or microencapsulation (De Prisco & Mauriello, 2016; Ningtyas, Bhandari, Bansal, & Prakash, 2019). In the bibliographic research carried out by Terpou et al. (2017) various natural supports such as apple or pear pieces, *Pistacia terebinthus* resin and whey protein, were successfully used as carriers for probiotic cell immobilisation in dairy products production. Owing to their biocompatibility, using milk proteins for this purpose is an attractive method (Ehsannia & Sanjabi, 2016; Sarao & Arora, 2017). In a recent study mentioned before (Terpou et al., 2017), the orange berries of sea buckthorn, which is considered a "superfood", were successfully used as immobilisation carriers of the probiotic strain *Lactobacillus casei* ATCC 393. The cheeses obtained had enriched aroma with terpenes and carbonyl compounds and higher probiotic cell population.

Apart from the protection of the cells, microencapsulation can further facilitate the handling of probiotic cultures as well as the masking of taste and aroma given by the production of different metabolic compounds (e.g. acetic acid) during fermentation in foods where they are not required (De Prisco & Mauriello, 2016; Sarao & Arora, 2017). Food-grade biopolymers (i.e. alginate, chitosan, pectin, starch, carrageenan and milk proteins) are the most investigated and used matrices for cell encapsulation by working as real physical barriers or by exercising a buffered action (De Prisco & Mauriello, 2016). Among the technologies applied for cell encapsulation, emulsion, spray drying and extrusion are the most studied and applied on both a laboratory and an industrial scale. However, new technologies, such as complex coacervation and vibrational extrusion technology, are emerging (De Prisco & Mauriello, 2016).

Furthermore, the use of prebiotics (e.g. inulin, polydextrose, wheat dextrin, fructooligosaccharides-FOS, galactooligosaccharides-GOS, lactulose) to improve probiotic viability during microcapsules production and storage is gaining great attention. Their application is regarded as highly useful, especially when relatively invasive techniques for cell viability such as spray or freeze-drying are applied for microencapsulation purposes (De Prisco & Mauriello, 2016; Speranza et al., 2018). Applied directly to cheese, they can also be useful (Rodrigues, Rocha-Santos, Gomes,

Goodfellow, & Freitas, 2012). In the case of petit-suisse cheese, the presence of the prebiotics inulin and oligofructose seem to promote growth rates of bifidobacteria and lactobacilli, besides increased lactate and Short Chain Fatty Acids (SCFA) production (Cardarelli, Buriti, Castro, & Saad, 2008).

As already reported, cheese is a promising food matrix for probiotics, however, only a few probiotic cheeses have been successfully developed for the market compared with yoghurts or fermented milks. So, strain selection and possible process adjustments should be carefully evaluated to maximize probiotic cell viability during cheese manufacture and storage, as well as to limit possible changes in organoleptic properties (Castro et al., 2015; Thomas, 2016).

I.5.3. Cheese as a source of probiotics

Some of the most familiar fermented foods contain viable cells in notable quantities ranging between 10^6 and 10^9 cells/g or ml, which potentially increase the numbers of microbes in the diet by up to 10000-fold (Adouard et al., 2016). Taking into account that some cheeses contain, on average, 10^8 to 10^9 microorganisms/g (Montel et al., 2014), that a relatively large fraction of those microbes survives passage through the human digestive tract and that europeans eat between 25 and 30 kg of cheese per capita per annum, the annual intake of viable cells provided by cheese can be estimated at 10^{13} and 10^{14} per capita per annum (Adouard et al., 2016). According to Marco et al. (2017), consumption of cheese and other fermented foods can provide exposure to micro-organisms that could, at least, counteract the hygienic, sanitized Western diet and lifestyle.

Many of the species found in fermented foods may belong to or be identical to commensal species relevant to promoting health (Binns, 2013; Bron et al., 2017; George Kerry et al., 2018; Marco et al., 2017). Strains of species with recognized probiotic activity by Health Canada (HC, 2019), like *Lactobacillus acidophilus*, *Lb. plantarum*, *Lb. paracasei*, *Lb. casei*, *Lb. rhamnosus*, Lb. *johnsonii*, *Lb. reuteri*, are usually in high concentration in fermented foods, including several cheese varieties, essentially as part of the group of the NSLAB some of which have been characterized as probiotics (Marco et al., 2017; Papadopoulou, Argyri, Varzakis, Tassou, & Chorianopoulos, 2018; Summer et al., 2017).

Indeed there are many studies that prove the probiotic potential of microorganisms from cheese, like *Lb. rhamnosus* (Caggia, De Angelis, Pitino, Pino, & Randazzo, 2015; Ricciardi et al., 2015; Succi et al., 2005; Summer et al., 2017), *Lb. plantarum* (Ribeiro, Stanton, Yang, Ross, & Silva, 2018; Zoumpopoulou et al., 2018), *Lb. fermentum* (Ricciardi et al., 2015; Tulumoğlu, Kaya, & Şimşek, 2014), *Lb. paracasei* (Caggia et al., 2015; Carafa et al., 2015; Ricciardi et al., 2015; Zorica et al., 2010), *Lb. brevis* (Carafa et al., 2015), *Enterococcus* and *Pediococcus* (Ricciardi et al., 2015), *Kluyveromyces marxianus and Kl. lactis* (Fadda, Mossa, Deplano, Pisano, & Cosentino, 2017).

Adouard et al. (2016) show that some cheese microorganisms survive when subjected to in vitro digestive stress in a dynamic simulator of the gastrointestinal tract. The yeasts *Geotrichum candidum*, *Kl.lactis* and *Debaryomyces hansenii*, and bacteria *Hafnia alvei*, *Corynebacterium casei*, *Staphylococcus equorum*, *Brevibacterium aurantiacum* and *Arthrobacter arilaitensis* were resistant, while *Lactococcus lactis* displayed poorer survival rates in gastric and duodenal compartments. A specific strain of *Propionibacterium freudenreichii*, used both as a cheese starter and as a probiotic in food supplements, with an immunomodulatory profile, continues to demonstrate these properties even when provided within a cheese (Plé et al., 2015). Promising immunomodulatory properties were also uncovered in selected strains of the dairy starter *Lb. delbrueckii* that can modulate innate immune responses (Rocha et al., 2014; Rocha et al., 2012).

Effectively one of the traditional trends in cheese research, especially on raw milk cheese, has been to screen among autochthonous starter and nonstarter strains to determine whether they could have potential health benefits. Bacteria selected from this source will be more likely be tolerant to the technological process and to this hostile environment. This fraction is particularly attractive as a bioreservoir for potential probiotic strains suitable to survive GIT condition (Castro et al., 2015). The isolation and characterization of strains from traditional fermented dairy foods from different culinary cultures and geographical regions may reveal autochthonous strains with interesting functional traits (Zoumpopoulou et al., 2017). Many regional cheeses have been used to isolate microorganisms with health promoting properties (Caggia et al., 2015; Ribeiro, Domingos-Lopes, Stanton, Ross, & Silva, 2018; Zoumpopoulou et al., 2017).

I.5.4. Most common cheese probiotics

Most current probiotics are LAB of the genus *Lactobacillus* and the genus *Bifidobacterium*, which are among the first identified probiotic microorganisms (Gomes & Malcata, 1999; Vrese & Schrezenmeir, 2008). They are members of the human and animal microbiome and are thought to be implicated in the maintenance of the host's health (Tripathi & Giri, 2014; Ventura, Canchaya, Fitzgerald, Gupta, & van Sinderen, 2007; Vrese & Schrezenmeir, 2008; Zoumpopoulou et al., 2017). These Gram positive lactic acid-producing bacteria are also part of the microflora of many foods and LAB are also traditionally used in the production of various fermented foods including dairy products (Salvetti & O'Toole, 2017; Zoumpopoulou et al., 2017).

The association between these two bacterial groups and humans through food or as part of the commensal microbiota has caused many of them to be classified in the qualified presumption of safety (QPS) european list (EFSA/BIOHAZ, 2019), or as generally regarded as safe (GRAS) (FDA, 2020; Frestedt, 2018; Tripathi & Giri, 2014). Additionally they also have the advantage of being only weakly proteolytic and lipolytic, not causing the accumulation of organoleptically unpleasant products (Narvhus & Axelsson, 2003).

These probiotics are thus the most common and used in dairy matrices, including cheese (Rodrigues, Rocha-Santos, Gomes, et al., 2012). Other microorganisms that may play an important role in cheese as probiotics, include bacterial strains of the genera *Enterococcus*, although controversial, and *Propionibacterium*. Other microbial genera used include LAB of the genera *Lactococcus*, *Streptococcus* and *Leuconostoc*, and yeasts of the genera *Debaryomyces* and *Kluyveromyces*. Table 15 lists the most relevant species/subspecies used or to be used as probiotics in cheese.

Table 15 - The most relevant bacterial and yeast species/subspecies, with potential for use as probiotics in cheese (Source: Castro et al., 2015; Fadda et al., 2017; García-Tejedor, Sánchez-Rivera, Recio, Salom, & Manzanares, 2015; Karimi et al., 2011).

Lactobacillus	***Bifidobacterium***	**Others (∗)**
Lb. acidophilus	*B. animalis*	*Enterococcus faecalis*
Lb. casei	*B. animalis ssp. lactis*	*E. faecium*
Lb. casei ssp.pseudoplantarum	*B. infantis*	*Lactococcus lactis*
Lb. casei ssp. rhamnosus	*B. breve*	*Leuconostoc paramesenteroides*
Lb. delbrueckii ssp. bulgaricus	*B. lactis*	*Propionibacterium freudenreichii ssp. shermanii*
Lb. delbrueckii ssp. Lactis	*B. longum*	*Streptococcus thermophilus*
Lb. gasseri	*B. bifidum*	*Debaryomyces hansenii*
Lb. paracasei		*Kluyveromyces lactis*
Lb. plantarum		*Kl. marxianus*
Lb. rhamnosus		
Lb. salivarius		
Lb. brevis		
Lb. mucosae		

I.5.4.1. *Bifidobacterium* genus

The use of strains of *Bifidobacterium* in the dairy industry has become popular by the end of the 1970s as a result of the increased knowledge about their taxonomy and ecology, reduced acidication during post-processing storage and their relatively high yield of L(+) lactic acid compared with D(-) lactic acid, which is more easily metabolized by humans (Gomes & Malcata, 1999; McCartney, 2003).

Bifidobacteria belong to the phylum Actinobacteria, that is characterized by the high guanine plus cytosine (G+C) content (54 - 67 mol%) of their DNA (Gomes & Malcata, 1999), which differentiates them from LAB. Currently, the genus *Bifidobacterium* encompasses 70 species (NCBI, 2018; Ventura, Turroni, & van Sinderen, 2015; Zhou & Li, 2015). They are strictly anaerobic, catalase-negative, nonmotile and nonsporulating rods with varying appearance (Gomes & Malcata, 1999; Ventura et al., 2015; Vrese & Schrezenmeir, 2008; Zhou & Li, 2015). In fact its shapes include short, curved rods, club-shaped rods and bifurcated Y and V-shaped rods. The optimum growth pH is between 6-7, with no growth below 4.5-5.0, or above 8.0-8.5. The optimal temperature is around 37-41ºC, with a maximum of 43-45ºC (Gomes & Malcata, 1999; Zhou & Li, 2015).

They are heterofermentative saccharolytic organisms that produce acetic and lactic acids usually without generation of CO_2 (Gomes & Malcata, 1999; Vrese & Schrezenmeir, 2008). All bifidobacteria from human origin are also able to utilize galactose, lactose and, usually, fructose as carbon sources. Some species ferment complex carbohydrates such as D-galactosamine, D-glucosamine, amylose and amylopectin (Crociani, Alessandrini, Mucci, & Biavati, 1994). A recent study (Ose et al., 2018) concluded that bifidobacteria readily metabolized shorter fructooligosaccharide (FOS) (prebiotic), with end-products linked with host health.

This bacterial genus constitutes a major part of the normal intestinal microflora in humans throughout life, whose presence may modulate the microbiota of the human intestinal tract, mainly in the colon, where they may exert beneficial health effects (Gonzalez-Sanchez, Azaola, Gutierrez-Lopez, & Hernandez-Sanchez, 2010), which mainly include the eradication of pathogenic microorganisms and immune modulation. They appear in the stools a few days after birth and increase in number thereafter. The number of bifidobacteria in the colon of adults is 10^{10}–10^{11}cfu/g, but this number decreases with age (Vrese & Schrezenmeir, 2008). However, they are distributed by other habitats as the oral cavity, insect and animal intestines, sewage and food (Ventura et al., 2015; Vrese & Schrezenmeir, 2008). Bifidobacteria are known to produce complex B vitamins, slowly absorbed in the human body, and vitamin K. Also, the nutritional properties of fermented milks containing bifidobacteria indicate lower residual lactose and higher levels of free amino acids than nonfermented milks (McCartney, 2003).

In general, the use of bifidobacteria in food is technologically limited due to its nutritional requirements, sensitivity to oxygen, refrigeration temperatures and low pH values (4.2–4.6), which makes it difficult to maintain the concentration or to keep the count in the range of 10^6 –10^7 cfu⁄ml (Gonzalez-Sanchez et al., 2010), Its use as probiotics has been limited to fermented milks. They have also been used in some types of cheese, although maintaining bifidobacteria in this type of product is mainly conditioned by appropriate temperature (4°C) and a moderate salt concentration (Aranceta, Bixquert, & Burnat, 2002).

I.5.4.2. *Lactobacillus* genus

LAB are the other most used group of probiotics. This group includes catalase-negative cocci, coccobacilli, or rods, in single cells or coupled tetrads and short or long chains (Narvhus & Axelsson, 2003; Settanni & Moschetti, 2010). They present limited biosynthetic abilities and complex nutritional requirements, and therefore depend on the presence of specific nutrients and carbohydrates, which they use through homo or heterolactic fermentation. Following the first, they produce copious amounts of lactic acid. Through the second fermentation pathway, they produce acetic acid, ethanol, and carbon dioxide in addition to lactic acid (Narvhus & Axelsson, 2003).

Lactobacillus is the largest genus within the group of LAB. This genus is found in a variety of habitats where rich carbohydrate-containing substrates are available, such as human and animal mucosal membranes, on plants or materials of plant origin, fermenting or spoiling food, sewage and fermented milk products, including cheese (Tripathi & Giri, 2014; Vrese & Schrezenmeir, 2008). In cheese, depending on the species, it can act as SLAB or NSLAB, and is the most used genus as probiotic.

The genus *Lactobacillus* belong to the Phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*. To date (November 2018), it contains 229 species (NCBI, 2018). The various species can be grouped in obligately homofermentative (e.g. *Lb. acidophilus, Lb. delbrueckii, Lb. helveticus, Lb. salivarius*), facultatively heterofermentative (e.g. *Lb. casei, Lb. curvatus, Lb. plantarum, Lb. sakei*) and obligately heterofermentative (e.g. *Lb. brevis, Lb. buchneri, Lb. fermentum, Lb. reuteri*) (Ibrahim, 2016).

Lactobacilli are more resistant to low pH than Bifidobacteria and are better adapted to milk and other dairy. A large number of probiotic *Lactobacillus* species are therefore technologically suitable for dairy applications compared to *Bifidobacteria* (Tripathi & Giri, 2014). Some strains, either mesophilic or thermophilic, such as *Lb. acidophilus, Lb. casei, Lb. rhamnosus, Lb. delbrueckii, Lb. helveticus* and *Lb. plantarum,* are technologically better suited for use as probiotics in cheese and are the most commonly used for this purpose (Ibrahim, 2016; Tripathi & Giri, 2014).

I.5.4.4. *Enterococcus* genus

The enterococci comprise the third-largest genus of LAB after the genera *Lactobacillus* and *Streptococcus*. Until the 1980s they were described as streptococci, but these were reclassified as *Streptococcus*, *Lactococcus* and *Enterococcus* on the basis of phylogenetic evidence strengthened by 16S rRNA DNA sequencing and/ or DNA–DNA hybridization studies (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006; Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2011). In this context, the faecal streptococci that can be found in the gastrointestinal tract of humans and animals, but also in various other habitats, were grouped into the new genus *Enterococcus* (phylum *Firmicutes*, clase *Bacilli*, orden *Lactobacillales*, familia *Enterococcaceae*) (Franz et al., 2011).

Zhong et al. (2017) concluded that humans and mammals may be the original hosts of *Enterococcus*, and then species from humans and mammals made a host-shift to plants, birds, food and other environments. Currently, 61 species of *Enterococcus* are validly described (NCBI, 2018). Enterococci are Gram-positive, non-sporeforming, catalase-negative, oxidase-negative, facultative anaerobic cocci that occur singly, in pairs, or in chains (Moreno, 2006).

A considerable number of strains belonging to different species of *Enterococcus* are highly competitive due to their resistance to a wide pH, salts and temperature range (Hasna, Walid, Khaled, & Riadh, 2018). The optimum temperature for this genus is about 35ºC, although most species will grow at temperatures ranging from 10 to 45ºC and survive heating at 60ºC for 30 min. They can also grow in the presence of 6,5% NaCl and at an extended range of pH, up to pH 9.6 (Foulquié Moreno et al., 2006).

Another competitive factor in this genus is its ability to produce bacteriocins recognized for their wide-range effectiveness on pathogenic and spoilage bacteria. Enterococcal bacteriocins are of great interest as natural antimicrobial agents in the food industry, and as a potential drug candidate for replacing antibiotics in order to treat multiple drug-resistant pathogens (Brandão et al., 2010; Hasna et al., 2018). The production or induction of the production of butyrate, a short-chain fatty acid (SCFA), by some strains of *E. durans* is also very important given the significant anti-inflammatory effects of this substance, which can be useful as prophylactic treatment to avoid inflammatory bowel disease (Avram-Hananel, Stock, Parlesak, Bode, & Schwartz, 2010; Carasi et al., 2017).

The antihypertensive sequence LHLPLP, identified as one of the major peptides responsible for ACE inhibitory and antihypertensive effects, is produced by *Enterococcus faecalis* in fermented milk (García-Tejedor et al., 2015).

Some strains of this genus are already commercially available as food and feed additives and supplements in the form of pharmaceutical preparations, either for animals or for humans, for the treatment of diseases such as irritable bowel syndrome, diarrhoea or antibiotic associated diarrhoea, or for health improvements such as lowering cholesterol levels or immune regulation (Hanchi, 2018; Franz et al., 2011). These bacteria are thus ingested in high numbers to exert these effects at the level of the gastrointestinal system, through the interaction with the gut microbial populations and with the gastrointestinal immune system (Franz et al., 2011).

Enterococci are important in the dairy industry and are often associated with the microflora of cheeses made from raw milk from goats, sheep or cows in Southern Europe acting as NSLAB (Hanchi, 2018). They are important in the maturation of different cheese varieties, probably due to their proteolytic or lipolytic activity, their ability to ferment citrate to produce diacetyl and other volatile compounds that contribute to the flavor or to provide a characteristic flavor and taste (Foulquié Moreno et al., 2006). For instance, *E. faecium, E. durans* and *E. faecalis* are commonly found in cheese (Amaral, Silva, Casarotti, Nascimento, & Penna, 2017; Hasna et al., 2018; Ispirli, Demirbaş & Dertli, 2017).

However Enterococcal probiotics are not usually used as starter cultures or co-cultures in foods because their role as probiotics is still controversial. This is due to their increased association with nosocomial infections and because they harbor multiple antibiotic-resistant genes, which are transmissible by conjugation to pathogenic microorganisms (Franz et al., 2011; Hasna et al., 2018; Puchter et al., 2018). In fact, several putative virulence factors have been described in enterococci, such as aggregation substance proteins, gelatinase, cytolysin, enterococcal surface proteins, hyaluronidase, accessory colonization factors and endocarditis antigens (Daria Van, Melissa, & Michael, 2013; Foulquié Moreno et al., 2006; Strateva, Atanasova, Savov, Petrova, & Mitov, 2016). So, from the current regulatory point of view, this genus is not part of the QPS or GRAS lists (EFSA/BIOHAZ, 2019; FDA, 2020; Hasna et al., 2018).

It should be noted that recent advances in molecular biology and the recommended methods for the safety evaluation of *Enterococcus* strains made the distinction between comensal and clinical clades possible (Hasna et al., 2018). In the work presented by Ghattargi et al. (2018), it is concluded that comparative genome analyses can be applied to find potential probiotic candidates. This study particularly identified genes that are responsible for imparting probiotic, non-pathogenic and pathogenic features to the strains of *E. faecium*. It identified a strain that is a potential probiotic candidate due to its high genomic stability, absence of known virulence factors or antibiotic resistance genes and close genomic relatedness with marketed probiotics. Nevertheless, the development of highly adapted methods and legislations are still required (Hasna et al., 2018).

I.5.4.5 *Propionibacterium* genus

Dairy propionibacteria are extensively used as cheese starters, for the manufacture of Swiss-type cheeses where they are responsible for the characteristic flavor and eye formation, but there is a growing interest in the strain-dependent probiotic potential of propionibacteria (Campaniello, Bevilacqua, Sinigaglia, & Altieri, 2015; Darilmaz & Beyatli, 2012a, 2012b; Poonam, Pophaly, Tomar, De, & Singh, 2012; Rabah, do Carmo, & Jan, 2017; Zárate & Pérez Chaia, 2012). They are Gram-positive, anaerobic, aerotolerant, catalase-positive, non-motile bacteria, belonging to the *Propionibacteriales* order (NCBI, 2018; Piwowarek, Lipińska, Hać-Szymańczuk, Kieliszek, & Ścibisz, 2018; Poonam et al., 2012; Rabah et al., 2017). *Propionibacterium freudenreichii* and *P. acidipropionici* hold QPS and GRAS status (EFSA/BIOHAZ, 2019; FDA, 2020; Piwowarek et al., 2018; Rabah et al., 2017).

Propionibacteria are small rod-shaped bacteria. In the presence of oxygen, they demonstrate pleomorphism, in which club-shaped cells are observed; they can also take the form of letters V and Y. In anaerobiosis, they can take the form of spherical shapes (cocci). The optimal pH revolves around 7.0 (range 4.5–8.0). Most *Propionibacterium spp*. are mesophiles with an optimum temperature for growth of about 30 °C. It is relatively resistant to high temperatures, being able to survive up to 20s at 70 °C (certain strains withstand temperatures of up to 76 °C for 10s (Piwowarek et al., 2018).

Propionibacteria use several peculiar metabolic pathways, the main one being the central carbon metabolic pathway through which they use lactate (produced by LAB) during

growth and produce propionate, acetate and carbon dioxide (Poonam et al., 2012). But they are known not only for their ability to produce propionic acid with antimicrobial properties particularly against fungi but also for adequately producing a variety of bacteriocins (propionicins; jenseniins; thoeniicina), with a wider antimicrobial spectrum covering other Gram-positive bacteria including LAB, Gram-negative bacteria, yeasts and filamentous fungi (Campaniello et al., 2015; Darilmaz & Beyatli, 2012a, 2012b; Fillipe et al., 2018; Poonam et al., 2012).

Other health effects of propionibacteria have been related to their ability to produce vitamins (e.g. B12; folate; riboflavin), bifidogenic compounds (e.g. 1,4-dihydroxy-2-naphthoic acid (DHNA), 2-amino-3-carboxy-1,4-napthoquinone (ACNQ) which promotes the growth of bifidobacteria in the colon region, the improvement of the intestinal microbiota composition and their metabolic activities (the prebiotics GOS β(1 → 6), β(1 → 3) and β(1 → 4) linked trisaccharides and OsLu containing mainly β(1 → 6) linked trisaccharides), as well as their immunomodulatory, antimutagenic and anticarcinogenic properties (Piwowarek et al., 2018; Poonam et al., 2012; Rabah et al., 2017; Sabater et al., 2019; Zárate & Pérez Chaia, 2012). The last two properties are due to propionic and acetic acids (SCFA) derived from lactate fermentation (Plé et al., 2015; Rabah et al., 2017). These two acids also impart background flavor to cheese (Poonam et al., 2012).

P. freudenreichii, traditionally used as a cheese-ripening starter, is currently considered as an emerging probiotic that has recently revealed promising immunomodulatory properties, suggesting a powerful role in the context of inflammatory bowel diseases (Fillipe et al., 2018; Rabah et al., 2017). In Rabah et al. (2018) assumes that this effect may be matrix-dependent and related to surface proteins that will be protected by the cheese matrix. These considerations open perspectives on the favorable use of cheese with this probiotic, in the treatment of those diseases.

Propionibacteria can endure harsh technological and physiological conditions. They have been reported to survive low pH as well as high bile concentrations during GIT transit in several in vitro and in vivo studies (Campaniello et al., 2015; Darilmaz & Beyatli, 2012a). Technological stresses such as reconstitution in milk, fermentation of a wide range of carbohydrate substrates, microencapsulation, spray-drying, freeze-drying, and storage at low temperatures do not seem to affect this bacterial group (Darilmaz & Beyatli, 2012a,

2012b). In Zárate & Pérez Chaia (2012), it has been demonstrated that the β-galactosidase of *P. acidipropionici* resisted to the manufacture of a Swiss type cheese and to adverse gastrointestinal conditions. It can be considered the hypothesis of the use of using these strains in the manufacture of a probiotic product aimed at lactose intolerant individuals. So, probiotic cheeses for the delivery of beneficial propionibacteria may represent an alternative to other conventional fermented dairy products, such as yoghurt and fermented milks.

The combined probiotic properties of lactic acid and propionic acid bacteria were employed by Plé and colleagues who developed a pressed cheese containing *P. freudenreichii* and *Lb. delbrueckii* strains previously selected for their anti-inflammatory properties (Plé et al., 2016).

I.5.4.6. Yeasts

Yeasts make up a large and heterogeneous group of eukaryotic microorganisms that are widespread in natural environments, including the GIT of humans, plants, airborne particles, and food products. The high content of proteins, vitamin B, traces minerals, and various immune-stimulant compounds (proteases, β-glucans, and mannan oligosaccharides) has increased the interest in the use of yeasts as a probiotic (Arévalo-Villena, Fernandez-Pacheco, Castillo, Bevilacqua, & Briones Pérez, 2018; Fadda et al., 2017; Gil-Rodríguez, Carrascosa, & Requena, 2015). Yeasts also have the advantages of nonsusceptibility to antibiotics and good tolerance for industrial processing conditions (i.e., lyophilization and high temperatures) (Abdel-Salam, 2010; Joshi & Thorat, 2011; Morgunov, Kamzolova, & Lunina, 2013). *Saccharomyces boulardii* has a QPS status (EFSA/BIOHAZ, 2019) and is the most used and studied probiotic (Hudson et al., 2016). However, several other potential probiotic yeasts (*Kluyveromyces*, *Debaryomyces*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces* and *Candida*) are constantly being identified (Melo Pereira et al., 2018).

For example, *D. hansenii* is one of the predominant yeast species in all types of cheese, where it plays an important role in manufacturing. The results obtained by García-Tejedor et al. (2015), point to the feasibility of classifying some strains from milk as GRAS microorganisms that have strain-specific caseinolytic systems for the production of HLPLPL and HLPLP antihypertensive sequences. Also, *Kluyveromyces* strains isolated

from artisanal cheese have shown interesting functional traits and the absence of undesirable properties; they can be considered as potential probiotics (Fadda et al., 2017).

I.5.5. Cheese as matrix for the synthesis of bioactive compounds

During cheese fermentation and ripening processes, the enzymatic activity of the raw material and microorganisms involved can change the nutritive and bioactive properties of this food matrix in a manner that has beneficial consequences for human health. In Figure 13 is outlines the role of microorganisms in the synthesis of some bioactive compounds from milk constituents.

Microorganisms, especially LAB and yeast act as precursors of organic acids, peptides, fatty acids, γ-aminobutyric acid (GABA), exopolysaccharides and vitamins that have beneficial effects on health. Different mechanisms and factors are involved during the release of bioactive compounds, including enzymes, pH conditions, ripening time, and temperature (Santiago-López et al., 2018).

I.5.5.1. Organic acids (lactate)

Lactose is one of the most important components of milk. In fermented dairy products LAB ferment this oligosaccharide into lactic acid in amounts often reaching 1%. In cheese production, the remaining lactose is further separated into the whey fraction. Thus, most cheeses are typically well-tolerated by lactose-intolerant individuals due to insufficient activity of β-galactosidase (Marco et al., 2017). The acidification also changes the physical properties of casein which coagulates and promotes digestibility, improves the utilization of calcium and other minerals and inhibits the growth of potentially harmful bacteria (Bhat & Bhat, 2011; Marco et al., 2017).

A recent study has shown that in concentrations similar to those found in large bowel lumen, lactates and some SCFAs (acetate, propionate and butyrate) can reduce pro-inflammatory cytokine secretion of Tool Like Receptors (TLR)-activated and of sentinel cell types like bone-marrow-derived macrophages and dendritic cells, in a dose-dependent manner (Hug et al., 2018; Iraporda et al., 2015; Marco et al., 2017).

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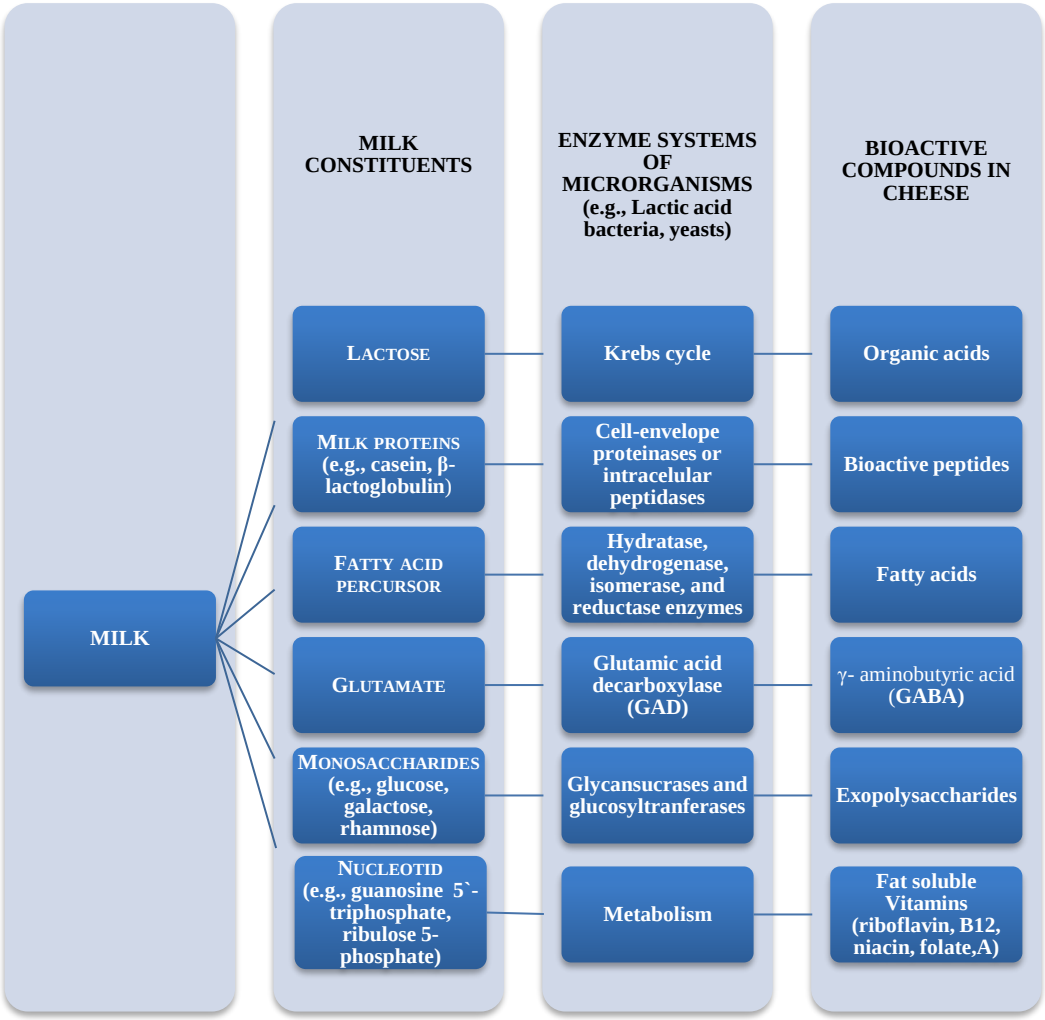

Figure 13 - Role of microorganisms during the cheese production process as precursors of bioactive compounds beneficial to health from milk components. Microorganisms, especially LAB and yeast act as precursors of bioactive compounds such as organic acids, peptides, fatty acids, γ-aminobutyric acid (GABA), exopolysaccharides and vitamins (Adapted from Santiago-López et al., 2018).

This anti-inflammatory action makes SCFAs particularly associated with a reduced risk of some diseases, including the irritable bowel syndrome, inflammatory bowel disease (IBD), cardiovascular diseas, and cancer (Iraporda et al., 2015). Lactobacilli-derived physiological lactate concentration also alters redox status by reducing the reactive oxygen species burden in intestinal enterocytes (Kahlert et al., 2016; Marco et al., 2017).

However in this process, LAB can produce L (+) lactic acid and/or D (-) lactic acid (Garvie, 1980). The type of isomer produced will depend on the presence of the specific enzyme NAD-dependent lactate dehydrogenase (nLDH) and its activity (D-nHDL or L-nHDL). The conversion of L-lactic acid to D-lactic acid is catalysed by the enzyme racemase; however few microorganisms synthesize this enzyme (Garvie, 1980). The lactic D (-) isomer is not hydrolyzed by the LDH enzyme in humans so large amounts of this isomer can cause consumer acidosis. For this reason its consumption is not recommended for infants and young children. The WHO recommends not exceding daily amounts of 100 mg / kg of body weight (Ewaschuk, Naylor, & Zello, 2005; Uribarri, Oh, & Carroll, 1998; WHO, 1974). Therefore, for the use in food processing, those bacteria that preferentially produce the lactic L (+) isomer should be selected (Garvie, 1980).

I.5.5.2 Bioactive peptides

Peptides and peptide fractions having bioactive properties, hidden and inactive in the primary protein structure, can easily be released and activated by both milk and starter LAB proteolytic activity during cheese processing. This may occur during fermentation, cheese ripening or during digestion in the gastrointestinal tract (Albenzio & Santillo, 2013; Egger & Ménard, 2017; Sultan et al., 2018; Summer et al., 2017). Active peptides are natural components of cheeses like Feta, Swiss, 'Festivo', Edam, Emmental, Parmigiano Reggiano, Comte and Cheddar (Summer et al., 2017). In a functional Scamorza ovine cheese ripened for 15 days, the proteolytic ability of probiotics was verified with the formation of bioactive sequences in the cheese matrix, which could be supplied with cheese consumption (Albenzio et al., 2015). *L. helveticus* is one of the main species responsible for the formation of these bioactive compounds (Sadat-Mekmene, Genay, Atlan, Lortal, & Gagnaire, 2011).

These peptides are derived from milk proteins (αs1-casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin, immunoglobulins, lactoferrin, phosphoglycoproteins, transferrin and serum albumin) and consist of 2–30 amino acid residues. Each bioactive peptide may exert one or more different beneficial physiological functions: opioid peptides are opioid receptor ligands which can modulate absorption processes in the intestinal tract; immunomodulating casein peptides stimulate the activities of the cells of the immune system; caseinophosphopeptides may function as carriers for different minerals, especially calcium, angiotensin-converting-enzyme (ACE) inhibitors peptides

are blood pressure regulators and exert an antihypertensive effect in patients with blood pressure problems (Marco et al., 2017; Sadat-Mekmene et al., 2011; Sultan et al., 2018; Summer et al., 2017).

The latter are of particular interest in fermented dairy products (Hess et al., 2016). They are generated by the activity of specific LAB enzymes that cut proteins like β-casein and κ-casein. Several ACE-inhibitory peptides (VPP, IPP, LHLPLP and HLPLP) were found in Parmigiano Reggiano cheese samples, in their relative intestinal digestate (Summer et al., 2017) and in the plasma of subjects who had ingested 100 g of this cheese per day for one week (Caira et al., 2016). Several *Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. casei* strains isolated from Bulgarian white brined cheese showed strong ACE-inhibitory activity (Dimitrov, Chorbadjiyska, Gotova, Pashova, & Ilieva, 2015). In *D. hansenii*, one of the predominant yeast species in cheeses, it was detected caseinolytic systems for the production of the antihypertensive sequences HLPLPL and HLPLP were detected (García-Tejedor et al., 2015). Moreover, the antihypertensive activity of these products remained constant after gastrointestinal digestion with pepsin and corolase PP (Sultan et al., 2018).

However, there is still a high level of uncertainty on the role of these peptides in the in vivo biological effect (Egger & Ménard, 2017; Sultan et al., 2018). Thus, EFSA had a negative scientific opinion on the substantiation of health claims related to dairy bioactive peptides IPP, VPP and C12 on the maintenance of normal blood pressure and elastic properties of the arteries (EFSA/NDA, 2009d, 2010b, 2011b, 2012; Li-Chan, 2015) and αS1-casein tryptic hydrolysate and alleviation of psychological stress (EFSA/NDA, 2011g) (Appendix 4). The main reason for negative opinion lies in the poor characterization of the bioactive peptides, the lack of dose-response relationships, the low bioavailability of the peptides and their low-potency.

I.5.5.3. Fatty acids

Some authors underline the functionality of dairy products by taking into account their fat content. They consider it the most complex fat in the human diet, consisting of more than 400 distinct fatty acids (FA), many of these being supplied in our diet only by dairy products (Bhat & Bhat, 2011; Summer et al., 2017). In particular, it has been found that some cheeses can be an important source of FA, which appears to be particularly

beneficial to human health. In fact, during the ripening of cheese, a partial lipolysis of fats which can be attributed to the lipases associated with lactic acid microbiota makes a certain amount of fatty acids available in a free form, facilitating their absorption and action (Fernández-García, Carbonell, Calzada, & Nuñez, 2006; Summer et al., 2017). Among these, conjugated linoleic acid (CLA), SCFA like butyric acid (C4:0), trans-palmitoleic acid (tC16:1) and possibly also the branched chain fatty acid phytanic acid (C20:0) (Bhat & Bhat, 2011; Gómez-Cortés et al., 2018; Marco et al., 2017; Summer et al., 2017).

CLA in particular is a FA with atheroprotective, anti-cancer, anti-obesity and anti-inflammatory properties that can be enriched in fermented products by LAB from linoleic acid through the action of linoleate isomerase (Castro et al., 2015; Marco et al., 2017; Ribeiro, Stanton, et al., 2018; Summer et al., 2017). CLA is an acronym comprising a group of linoleic acid (cis-9 cis-12 18:2) isomers with conjugated double bonds that differ in position and geometry (cis or trans) (Gómez-Cortés et al., 2018). The main isomer (79 to 94 %) is rumenic acid (RA) (cis-9, trans-11 CLA - c9, t11) (Gómez-Cortés et al., 2018; Hess et al., 2016; Summer et al., 2017), abundant in milk and cheese fat (Gómez-Cortés et al., 2018).

In cheeses with *Lb. paracasei* and Lb. *acidophilus,* the CLA content increased during the storage period due to the lipolysis of free linoleic acid by these bacteria (Albenzio, Santillo, Caroprese, Ruggieri, et al., 2013; Bergamini et al., 2005). In *Lb. plantarum* isolated from a traditional Azorean cheese the ability to convert free linoleic acid into CLA was identified (Ribeiro, Stanton, et al., 2018). Carafa et al. (2019) found that the presence of a strain of *Lb. rhamnosus* in cow cheese stimulated the increase of CLA. In a research it has been shown that the use of prebiotics (fructooligosaccharides - FOS or a mixture of FOS and inulin) in combination with probiotic strains (Rodrigues, Rocha-Santos, Gomes, et al., 2012) may increases the production of SCFA and functional CLA compounds, so with a lower atherogenicity index.

However, the recommended intake of CLA as well as other fatty acids is still under scrutiny (Gómez-Cortés et al., 2018). According to the same authors, CLA as several of the above mentioned FA, are included only in the human diet from ruminant fats, so the incorporation of whole milk products in our menu should be encouraged. Future research needs to establish how much dairy is needed to have positive health effects.

EFSA scientific opinión on the substantiation of submitted health claims related to CLA isomers c9, t11 and t10, c12, on weight management, fat metabolism enhancement, support of lean body mass, insulin sensitivity, antioxidativity and immune health (EFSA/NDA, 2010e) and reduction in body fat mass (EFSA/NDA, 2015a), is negative. The main reason for this is that a cause and effect relationship has not been established between the consumption of an equimolar mixture of the CLA isomers c9, t11 and t10, c12, and a beneficial physiological effect, although it is considered that the subject of the health claims, is sufficiently characterised.

Recent literature also suggests that the FAs present in dairy products can have a potential role in preventing diabetes and some scientific studies have shown that the incidence of diabetes is inversely associated with dairy products with high fat content (Díaz-López et al., 2016; Gómez-Cortés et al., 2018; Mozaffarian et al., 2010; Talaei, Pan, Yuan, & Koh, 2018). Díaz-Lópes et al. (2016) argues that a healthy dietary pattern that incorporates a high consumption of dairy products, and particularly yoghurt, may be protective against type 2 diabetes in older adults at high cardiovascular risk.

I.5.4.4. Gamma-aminobutyric acid (GABA)

GABA is a non-proteinogenic amino acid synthesized from glutamate, considered a potent bioactive compound. It has been widely studied because of its well-characterized physiological functions and positive effects on many metabolic disorders (Diana, Quilez, & Rafecas, 2014; Pessione & Cirrincione, 2016). Its main action is as antihypertensive, but it is also associated with immunomodulatory, antidiuretic, antidepressant and relaxing effects. Additionally, some studies have shown that GABA can induce the secretion of insulin and therefore help prevent or manage diabetes (Diana et al., 2014; Dinan & Cryan, 2012; Foster & Neufeld, 2013; Lebeer et al., 2018; Wang & Kasper, 2014).

LAB play a very important role in the proteolysis that takes place during cheese ripening since they contain proteinases and peptidases that can lead to the production of free amino acids (Fox, Guinee, Cogan, & McSweeney, 2016). Thus a high level of amino acid L-glutamate may be theoretically liberated since native caseins contain a high proportion of this amino acid. The accumulation of L-glutamate, low pH, and anaerobiosis can promote GABA production by LAB via glutamic acid decarboxylase (GAD), depending on the type of LAB present (Santiago-López et al., 2018). So this transformation can result in

the accumulation of GABA and CO_2 and the consumption of a proton, increasing the alkalinity of cytosol and the environment.

Thus, the screening of LAB for the ability to produce GABA is becoming increasingly important in cheese application (Luca Settanni & Moschetti, 2010). *Lb. casei*, *Lb. brevis*, *Lb. plantarum*, *Lb. rhamnosus*, *Lb. otakiensis*, and *Lb. paracasei* are reported to produce GABA in cheese (Carafa et al., 2015; Lyte, 2011; Ribeiro, Domingos-Lopes, et al., 2018). Lactobacilli are the best GABA-producers; however, Lactococci, Streptococci, and Bifidobacteria can synthesize GABA as well (Carafa et al., 2015; Lyte, 2011; Zoumpopoulou et al., 2017).

In a recent study (Renes, Ladero, Tornadijo, & Fresno, 2019), GABA production was monitored in pasteurized sheep's milk cheese made with four different autochthonous co-cultures of LAB strains. High average concentrations of GABA (1296.75 mg/kg of cheese) were found at 240 days of ripening. They conclude that the co-culture containing four autochthonous LAB strains (*Lactococcus lactis* subsp. *lactis*; *Lc. lactis* subsp. *cremoris*; *Lb. plantarum*; *Lb. casei* subp. *casei*) can bring this benefit and additionally provide reduction of the ripening time. Recent studies on Spanish and Italian artisanal cheese including raw milk ewe's cheese, found GABA concentrations above 300 mg/kg (Diana et al., 2014; Rosanna et al., 2019). In strains from Pico cheese *Lb. plantarum* displayed the highest GABA-producing capacity, followed by *Lb. Otakiensis* and *Lb. paracasei* (>300 mg/Kg) which also displayed appropriate technological properties for the manufacture of cheese with health-promoting properties. None of the strains from the genera *Lactococcus*, *Leuconostoc* and *Enterococcus* evaluated were GABA producers (Ribeiro, Domingos-Lopes, et al., 2018).

I.5.6. Cheese as a source of harmful compounds and mitigating factors

The presence of some chemical compounds in cheese, whether due to its composition or the metabolic action of microorganisms, may jeopardize its functionality.

I.5.6.1. Saturated fatty acids (SFA)

Although the functionality of dairy products is partly associated with their fat content, one of the main reasons why milk and dairy products consumption is associated with disease is the high proportion of saturated fatty acids (SFA), that may imply atherogenic

blood profile conditions and thereby an increased risk of coronary heart disease (CHD). Recent works suggest that dairy may display features that can counteract the negative effect of SFA on CHD (Marco et al., 2017; Radha & Megha, 2016; Soerensen, Thorning, Astrup, Kristensen, & Lorenzen, 2014).

For example, only three (lauric, myristic and palmitic) of the different SFA in milk have the ability to raise blood cholesterol; on the other hand, at least one-third of the FA present are unsaturated, so with a cholesterol lowering tendency. Furthermore, dairy products contain calcium, linoleic acid, CLA, antioxidants and probiotic bacteria, all these components with at least a protective if not hypocholesterolemic effect (Bhat & Bhat, 2011; Marco et al., 2017; Radha & Megha, 2016; Soerensen et al., 2014). Radha & Megha (2016) adds the presence of β–lactorphin, a peptide derived from β-lactoglobulin, which has angiotensin converting enzym (ACE) inibitory activity that improves vascular relaxation and, therefore, decreases hypertension.

The common association between a diet rich in SFA and the risk of CHD is also contradicted by the so-called French paradox, which describes the observation of low CHD mortality in France, despite high intake of SFA (Lallès, 2016; Petyaev & Bashmakov, 2012; Zheng et al., 2015). The first hypothesis to explain this was the high consumption of red wine in France, with the consequent ingestion of the cardio-protective biomolecules polyphenols and especially resveratrole (Biagi & Bertelli, 2015; Renaud & Delorgeril, 1992). However, Petyaev & Bashmakov (2012) consider that this paradox seems to be a multifactorial phenomenon for which the consumption of dairy products, especially cheese, can contribute. They claim that this dairy product and derivatives are essential in diets characterized by low cardiovascular mortality, such as the typical French and Mediterranean ones. Regular cheese consumption unifies all regional dietary cultures in France, which is the second highest cheese consumption country in the world (>26 kg/capita/year) (Lallès, 2016; Petyaev & Bashmakov, 2012; Zheng et al., 2015). The anti-inflammatory activity and the ACE inibitory activity by the dairy bioactive compounds are pointed out as the pharmacological basis for this phenomenon (Marco et al., 2017; Petyaev & Bashmakov, 2012; Radha & Megha, 2016; Soerensen et al., 2014).

Lallès (2016) hypothesised that this action of dairy products may be due to the stimulating effect of many of its constituents (e.g. casein, calcium, lactose, fat, vitamins A and D, methyl-donors: folate and vitamin B12, SCFA: butyrate and propionate) on the intestinal

alkaline phosphatase (AP), an enzyme with a potent anti-inflammatory action. AP may also be present in raw milk and dairy products or produced by fungi from moulded cheeses. At the intestinal level, this enzyme would dephosphorylate and thus detoxify the pro-inflammatory microbial components, such as lipopolysaccharides (LPS). In this way, they would be unable to trigger inflammation and thus to generate insulin resistance, glucose intolerance, type 2 diabetes, metabolic syndrome and obesity, known risk factors for CHD.

Results obtained by Zheng et al. (2015) in a metabolomics study suggest that cheese consumption is associated with an increased level of SCFA in the gut, as well as an increased extent of lipid excretion which leads to beneficial effects on cholesterol metabolism. SCFA can be formed in colon from lactate or from dietary carbohydrates that are not previously digested, possibly induced by beneficial gut microbiota. As already mentioned, these fatty acids have anti-inflammatory properties with beneficial effects on health, including cardiovascular health (Iraporda et al., 2015). Overall, this study suggests that cheese could be an important piece in the French paradox puzzle.

I.5.6.2. Biogenic amines (BA)

During cheese fermentation, the proteolytic activity of starter microorganisms is important for the provision of energy and a source for carbon, essential amino acids, and nitrogen in order to ensure their active growth and to accelerate milk acidification and gelation. The consequent use by microorganisms of the amino acids released in this process as an energy source by their decarboxylation also appears to be associated with microbial survival under acidic stress conditions, and in the regulation of osmotic and oxidative stresses. In the continuation of the manufacturing process during the maturation stage, adjunct cultures (NSLAB) proteolytic activity is essential for the development of sensory attributes and structural characteristics of cheese (Benkerroum, 2016; Spano et al., 2010; Zuljan et al., 2016). However, from a food safety standpoint this may also lead to the formation of toxic BA. Given the dual importance of this process (beneficial implications on cheese quality but possible health damage), it is difficult to control.

BA are biologically active non-volatile nitrogenous organic bases of low molecular weight with aliphatic, aromatic or heterocyclic structure, that include monoamines, diamines and polyamines. They are formed mainly by the decarboxylation of free amino

acids (FAA) through the action of decarboxylase enzymes in all living organisms (EFSA/BIOHAZ, 2011). Endogenous amines or natural BA synthesized physiologically as a result of normal cellular metabolic activities play vital roles in the bioregulation of cell growth and gene expression, protein synthesis, membrane division and stabilization, tissue repair, and modulation of intracellular signaling pathways and ion channels (Benkerroum, 2016). In animals and humans, they are involved in synaptic transmission, blood pressure control, allergic response and cellular growth control (EFSA/BIOHAZ, 2011).

Nonetheless, ingestion of BA of exogenous origin in large amounts may be hazardous to animal and human's health through the toxic effect of these compounds (EFSA/BIOHAZ, 2011; Santos, 1996; Spano et al., 2010). In this condition's BA can enter the systemic circulation and cause the release of adrenaline and noradrenaline, provoking gastric acid secretion, increased cardiac output, headaches or migraines, tachycardia, increased blood sugar levels and increased or decreased blood pressure (EFSA/BIOHAZ, 2011; Santos, 1996; Spano et al., 2010). Toxicological effects can have varying degrees of severity, ranging from a limited headache to organ failure and death (Benkerroum, 2016).

The main source of exogenous amines is dietary, through the uptake of foods with high levels of biogenic amines like fish, fish products and fermented foodstuffs (meat, dairy, vegetables, beers and wines) (EFSA/BIOHAZ, 2011). The most important BA found in food are heterocyclic and aromatic mono or diamines (histamine, tyramine, phenylethylamine) and aliphatic polyamines like diamines (putrescine and cadaverine), which are products of the decarboxylation of histidine, tyrosine, phenylalanine, ornithine and lysine, respectively. Aliphatic polyamines with more than two amino groups (agmatine, spermidine, and spermine) may also be present (Erdag, Merhan, & Yıldız, 2018; Giorgio, Di Trana, & Claps, 2018; Victor Ladero, Martín, Fernández, & Alvarez, 2010; Ruiz-Capillas & Herrero, 2019; Zuljan et al., 2016). Microorganisms that contain the enzyme decarboxylases are responsible for the formation of biogenic amines in foods (Benkerroum, 2016; Spano et al., 2010; Zuljan et al., 2016).

The main factors influencing the BAs production in foods are the availability of precursors (i.e. amino acids), the presence of microrganisms synthetising amino acid decarboxylases, and favourable conditions for their growth and decarboxylating activity (Benkerroum, 2016; EFSA/BIOHAZ, 2011). These are usually optimal in fermented

foods including cheese, especially the ripened varieties from raw milk, which therefore, may contain hazardous BA levels (Combarros-Fuertes et al., 2016; Victor Ladero et al., 2010; Linares et al., 2012; Zuljan et al., 2016). In cheese not only precursor amino acids and decarboxylating microorganisms are available, but there are also favorable conditions such as the presence of cofactors (pyridoxal phosphate), adequate water activity (0.90 to 0.99), pH (5.0 to 6.5) and temperature during fermentation (25 to 44°C), maturation (10 to 20°C) or storage (temperature-abuse conditions, refrigeration) (Benkerroum, 2016; Linares et al., 2012).

The most common BA mainly present in cheese are tyramine, histamine, putrescine and cadaverin (Ladero et al., 2010; Linares et al., 2012; Mayer & Fiechter, 2018; Renes et al., 2019). Buňková et al. (2013) mentions to ewe`s milk cheese as the one with the highest amounts of BA (tyramine, putrescine, cadaverine). Indeed cheese is one of the most prevalent foods associated with amine poisoning mainly due to the high level of tyramine (relevant vasoactive amine), that may result in a dangerous intoxication characterized by an increase in blood pressure known as the "cheese reaction" with a particular focus on consumers who are using monoamine oxidases (BA detoxification enzyme) inhibitory compounds as a medicine (Ladero et al., 2010; Santos, 1996).

Numerous microorganisms, contaminants of different origins, normal microbiota, but also advantageous, intentionally added as starter or adjunct starter cultures in cheese production, including LAB, yeasts, and molds have been reported to produce BA (Linares et al., 2012; Spano et al., 2010). Although dairy contaminants such as *Enterobacteriaceae*, psychrophilic and psychrotrophic bacteria (such as *Pseudomonas spp*. and *Proteus spp*.) are known to be major BA-producing microorganisms, dairy strains of lactobacilli, enterococci, streptococci, leuconostoc and yeasts have been associated with high levels of BAs in cheese and other dairy products, namely tyramine (Linares et al., 2012; Spano et al., 2010). Yeast species that contribute to the fermentation and/or maturation of many cheese varieties and fermented also produce BAs (Benkerroum, 2016).

Efforts are being made to control BAs in food products but there is still no legal limit for biogenic amines in dairy products (Renes et al., 2019). Specific EU legislation only covers histamine in fishery products (EC, 2005a) and no criteria have been established for other BA or other food products. In the U.S. the FDA has set histamine limits at 50

mg/kg applicable to all food products (Ruiz-Capillas & Herrero, 2019). Suggested limits for cheese in the bibliography are of 100–417 mg/kg for histamine (considering a daily consumption of 60 g) (Rauscher-Gabernig, Grossgut, Bauer, & Paulsen, 2009), for tyramine of 100–800 mg/kg (ten Brink, Damink, Joosten, & Huis in 't Veld, 1990) or until 1000 mg/kg (considering a female user, 60 kg body mass, 95 percentile), and for total biogenic amines a maximum of 900 mg/kg (Valsamaki, Michaelidou, & Polychroniadou, 2000).

Controlling BAs in cheese is important for both food safety and the economy,, as high values can make business transactions difficult. The main BA control strategies are focused on prevention of BA formation in foods at all relevant points in the food chain (EFSA/BIOHAZ, 2011). Therefore, the risk of incorporating biogenic amineproducing strains should be avoided by the hygienic quality requirement of the raw material and throughout the process and using well-characterised inoculants (EFSA/BIOHAZ, 2011; Linares et al., 2012). Samková, Dadáková, & Pelikánová (2013) found that simply washing the cheese outside resulted in a reduced BA content.

In this direction novel strategies are under investigation that including the use of LAB adjunt cultures which are able to degrade biogenic amines or the use of phages to reduce the number of specific undesirable microorganisms present in the cheese matrix. In this context Tittarelli, Perpetuini, Di Gianvito, & Tofalo (2019) isolated two strains of LAB (*Lb. casei* and *E. casseliflavus*) from raw milk cheese that show a quite high % of degradation of BAs, and did not show decarboxylase activity. Renes et al. (2019) proposes the use of a co-culture formed by autochthonous LAB as starters and as adjunct culture as a good approach to the development of functional sheep milk cheeses with reduced biogenic amine content.

Polyamines (PA) such as putrescine, agmatine, spermidine and spermine, although BA, are regarded as a different group, given the specific biosynthesis and the roles they play in eukaryotic cells (Kalač, 2014). These compounds are essential when at physiological concentrations in the stabilization of DNA, RNA, membranes and some proteins being essential for cellular growth, differentiation, proliferation and regeneration. In this context, polyamines participate in numerous favourable physiological processes namely in the reduction of some age-associated cardiovascular diseases (Handa, Fatima, & Mattoo, 2018; Kalač, 2014; Pegg, 2016). These conditions indicate the possibility of

using PA-based therapies (Pegg, 2016), even though they also seem to be implicated in harmful processes for human health (Handa et al., 2018; Kalač, 2014; Pegg, 2016).

Indeed, the excess of some PA seems to cause acute reductions in blood pressure, respiratory symptoms and nephrotoxicity (Pegg, 2016). Deregulation of PA homeostasis is associated with a number of pathological conditions such as neurological disorders (Alzheimer and Parkinson), inflammation, cerebral stroke, kidney failure and cancer (Handa et al., 2018; Kalač, 2014; Pegg, 2016). The latter is the most debated and involves carcinogenesis, tumour invasion and metastasis, with cellular PA concentrations clearly increased in different types of cancer (e.g., colorectal and breast cancer) (Handa et al., 2018; Pegg, 2016).

Thus, the bodily pool of polyamines must be strictly controlled. This is maintained by endogenous or de novo biosynthesis within the cells, and with exogenous sources, i.e., PA-producing intestinal microorganism and dietary intake. The body appears to rely on a continuous supply of polyamines from food (Kalač, 2014; Santos, 1996). Diet may be a useful source of these substances (Handa et al., 2018; Kalač, 2014).

Cheeses are regarded as one of the sources of PA. In cheese, the content of PAs is usually higher than in the original milk (Kalač, 2014; Linares et al., 2012; Linares, Martín, Ladero, Alvarez, & Fernández, 2011; Summer et al., 2017). Extremely high levels of putrescine and spermine are present in matured cheese (1 year), however it is much lower in other types of cheese, namely soft cheese with 4-8 weeks of ripening (Handa et al., 2018; Kalač, 2014). The same is true for the use of raw versus pasteurized milk (Benkerroum, 2016). The lowest levels of PA are found in sheep's milk (Giorgio et al., 2018). As for the other BA, the level of PA in cheese seems to increase mainly due to improper processing and storage conditions, associated whit a high level of contamination by PA-producing spoilage microorganisms such as *Pseudomonas spp*., *Aeromonas spp.*, *Bacillus spp.* and clostridia, or by the naturally occurring polyamines in milk. Yeasts and molds are another potential source for these polyamines in dairy products. However, LAB appear not to contribute significantly to the accumulation of these polyamines in dairy (Benkerroum, 2016).

A diet that includes cheese is thus a good source of PA under circumstances of rapid growth and cell proliferation (as in newborns or during the recovery of injured tissues) as

it provides more polyamines than endogenous biosynthesis (Handa et al., 2018). Furthermore, dietary polyamines may become important with ageing, as cell proliferation and ornithine decarboxylase (the first rate-limiting enzyme in PA biosynthesis) activity, slows with age (Giorgio et al., 2018; Minois, Carmona-Gutierrez, & Madeo, 2011). However, foods with high PA contents, which include ripened cheeses, are contraindicated for patients with tumours (Kalač, 2014).

A recent investigation (del Rio et al., 2018) noted that toxic concentrations in food would only be found very exceptionally, whereby the intake of PA-rich food does not seem to be harmful, at least for healthy people. However, in view of the risks presented, a maximum putrescine tolerable levels of 180 mg/kg was proposed (Austria) for cheese. Average putrescine daily intake from fermented foods was established to be 6.8 and 8.8 mg per female and male adults, respectively (Kalač, 2014).

II – OBJETIVES / OBJETIVOS

II.1 Justification - The Serpa cheese problem

Serpa cheese is a ripened traditional cheese produced in the Alentejo region, a province in southern Portugal, in a geographical area of production established in the Regulatory Decree Nº 39/87 (DR, 1987) and referred to as a Protected Designation of Origin (PDO) product (ECC, 1992a). Due to its specificity Serpa cheese is unique among traditional Portuguese cheeses and is renowned and appreciated (Alvarenga, 2008; Alvarenga, Canada, & I. Sousa, 2011; Amaral, 1996; Canada, 2001; Reis & Malcata, 2011), as can be seen in the increased demand in recent years (DGADR, 2016a, 2017, 2018). It's one of the most appreciated Portuguese raw ewes' milk semi-soft cheeses, with high acceptance by consumers and, so, economically important. Serpa cheese production benefits the rural economy, boosts farmers` income and maintains the population in less favored or remote areas.

Even though Serpa cheese's designation brings many advantages like a significant economic potential, the research has been dedicated to, in particular, its physical and chemical aspects (Alvarenga, 2008; Alvarenga et al., 2011; Alvarenga et al., 2008; Amaral, 1996; Canada, 2001; Roseiro, Wilbey et al., 2003), sensory (Canada, 2001) and technological characteristics (Alvarenga, 2008; Alvarenga et al., 2011; Alvarenga et al., 2008; Amaral, 1996; Canada, 2001; Roseiro, Wilbey et al., 2003). As with other traditional portuguese cheeses the study of its microbial diversity, including the study of specific flora along the maturation and benefits that can be drawn from this knowledge is limited (Dias, 1998; Roseiro, Wilbey et al., 2003). Indeed, scientific knowledge gained from research of Portuguese traditional cheeses trails behind other countries (Reis & Malcata, 2011).

It is a creamy, fat and semi-soft cheese, originated from a slow curd syneresis, after coagulation with a vegetable rennet infusion (*Cynara cardunculus* L.) and fermented by natural microbial populations. Its rind is thin, soft, uniform and slightly rough, with a soft straw-yellow color. Cheese bulk has buttery closed texture, color yellowish-white or straw yellow with humidity of 61-69% and fat in the dry residue of 45-60% (DR, 1987, 1994; Vieira, 1994). Thirty days is the minimum time of ripening set in Regulatory Decree Nº 39/87 (DR, 1987). Serpa differs from other PDO Portuguese cheeses due, among other factors, the milking times and distance between milking and cheesemaking

areas, which lead to different microflora and consequently to variable organoleptic characteristics (Reis & Malcata, 2011; Roseiro, Wilbey et al., 2003).

Cheeses manufactured from raw milk without starter culture and following traditional manufacturing procedures, like Serpa, may possess a very miscellaneous and rich microflora, including bacteria, yeasts and molds, forming a complex microbiota ecosystem. They arise from the milk and from the environment which contaminate the milk or cheese curd during manufacture and ripening (Beresford et al., 2001; Calasso et al., 2016; Kousta et al., 2010; Settanni et al., 2013). This biodiversity can be a fundamental factor for the maintenance of the typical features of traditional cheeses (Montel et al., 2014; Yoon et al., 2016). Several works have shown that the indigenous microflora of raw milk and other adventitious microorganisms originating from the environment are believed to be responsible for most of the physicochemical and aromatic transformations that take place during cheese making, especially LAB including various genera like Lactobacillus, Lactococcus, Streptococcus, Enterococcus and Leuconostoc (Aponte, Fusco, Andolfi, & Coppola, 2008; Fuka, Engel, Skelin, Redzepovic, & Schloter, 2010; Gala et al., 2008; Nikolic et al., 2008; Ordiales, Benito, et al., 2013; Poznanski, Cavazza, Cappa, & Cocconcelli, 2004; Serhan et al., 2009). This is also true for artisanal Portuguese cheeses (Pereira et al., 2010; Reis & Malcata, 2011; Roseiro, Wilbey et al., 2003).

Thus, the microbiological characterization is essential to understanding the kind and evolution of microbial groups involved in the production and maturation stages of this cheese, not only the lactic flora, but also other kinds of bacteria, yeasts and molds, prevalent or otherwise in the process, but whose presence and performance is crucial to attain the unique characteristics of this cheese (Alegría et al., 2016; Atanassova et al., 2016; Carafa et al., 2019; Maifreni et al., 2013; Padilla et al., 2014; Pogačić et al., 2016).

However, the biodiversity that characterizes this type of cheese also determines a great heterogeneity of the final product (Zabaleta et al., 2017; Zabaleta et al., 2016; Zabaleta, Gourrat, Barron, Albisu, & Guichard, 2016). Furthermore, the consumption of products made with raw milk, raises some suspicions from the point of view of food security, which hinders the acceptance of these products in some markets (Almeida et al., 2007; Carrascosa et al., 2016; Hymery et al., 2014; Ombarak et al., 2016; Verraes et al., 2015; Yoon et al., 2016).

Serpa cheese microbiota are also probably originated from raw milk and contamination from the environment by adventitious microorganisms. Methods of cheese making are artisanal and involve a lot of manual processing, that makes the microflora dependent on hygiene of different cheesemakers and subjected to much variability and consequently little product uniformity and security (Almeida et al., 2007; Roseiro, Barbosa, et al., 2003). To overcome this, some authors suggest the pasteurization of milk and the use of starter cultures to increase the uniformity and safety (Serhan et al., 2009). However, heat treatments reduce the number of strains, particularly the adventitious microflora which is considered as a special feature of PDO cheeses (Aponte et al., 2008). Recent studies argued or the use of a specific starter culture composed by specific and representative strains isolated from native flora, which ensures sensorial properties similar to those of the artisanal cheese, retaining the authenticity and improving safety (Aponte et al., 2008; Fuka et al., 2010; West, 2008). Even the autochthonous strains can be used to impart certain properties to the cheese so as not to harm the health or they may be used by risk groups (Tidona et al., 2016). Additionally, several investigations indicate that probiotic species are LAB commonly found in dairy products (Gonzalez et al., 2007).

Therefore, knowledge on specific microbial population of Serpa cheese and it characterization with respect to technological and probiotic properties, has to be acquired to better improve and manipulate its production and safety.

On all previous works on Serpa cheese's flora and in most other artisanal Portuguese cheeses were used only cultural methods (Amaral, 1996; Canada, 2001; Dias, 1998; Pereira-Dias et al., 2000; Reis & Malcata, 2011; Roseiro, Wilbey, et al., 2003). Thus, for better establishing the specific flora involved in this type of product and also for results comparison, an important innovation can be used: the association between culture-based microbiological analyses and sequencing technology. A combined approach is the best current strategy to study microbial ecology. No molecular method is a stand-alone tool for describing microbial communities (Bokulich & Mills, 2012). This is the first molecular ecological investigation into the microbial community of Serpa cheese using sequencing technology.

Considering what has been said before, the purpose of the present work is to explore the natural microbiological ecosystem of Serpa cheese with culture-dependent and

independent methods in order to isolate specific autochthonous strains which can be used in the improvement and innovation of its production.

That is why the general objective of this thesis includes the characterization of the microbiota of PDO Serpa cheese after thirty days of ripening, the minimum time set in Regulatory Decree Nº 39/87 (DR, 1987) and a selection of native strains with probiotic aptitude.

II.2. Partial objetives/objetivos

To achieve this general objective, the following partial objectives have been proposed:

1. Characterizing and studying the bacterial community including foodborne pathogens present in Serpa cheese with PDO label in comparison with similar non-PDO registered cheeses of the area by culture dependent and independent methods, using for strain identification 16S rRNA Gene Sequencing and High-Throughput Sequencing (HTS) Analysis.

1. Caracterización e identificación de la flora microbiana, beneficiosa y patogena presente en el queso Serpa con DOP, en comparación con quesos similares no registrados la DOP, utilizando técnologías tradicionales de cultivo e identificación mediante técnicas biología molecular con secuenciación de genes 16S rARN, así como tecnicas más novedosas de secuenciación masiva (HTS).

2. Characterizing and studying the yeast community present in Serpa cheese with PDO label in comparison with similar non-PDO registered cheeses of the area, by culture dependent and independent methods, using for strain identification PCR-RFLP analysis and sequencing of the 26S rRNA by HTS.

2. Caracterización e identificación de la población de levaduras presentes en el queso Serpa con DOP en comparación con quesos similares sin DOP, usando métodos tradicionales de cultivo e identificación mediante el análisis de las cepas aisladas por PCR-RFLP, así como mediante tecnologías de secuenciación masiva (HTS) con el 26S rARN.

3. Evaluation of the probiotic properties of native BAL strains isolated from artisanal Serpa cheese, as well as its ability to adapt to different prebiotic substances, for its possible application during the manufacturing process of a probiotic functional cheese.

3. Evaluación de las propiedades probióticas de cepas de BAL autóctonas aisladas del queso Serpa artesanal, asi como su capacidad de adaptacion a diferentes sustancias prebioticas, para su posible aplicación durante el proceso de fabricación de un queso funcional probiotico.

III - MATERIAL AND METHODS

III.1. Biological material

Serpa cheeses were manufactured after raw ewes' milk coagulation with a *Cynara cardunculus* L. flowers infusion as described by (Alvarenga, J. Canada, & I. Sousa, 2011). The sample under analysis consisted of several units of this cheese collected in five distinct dairy industries located in the geographical area of production. Three industries, identified as A, C and G, belonged to the PDO "Serpa cheese", while non-PDO registered industries were designated as V and B. The sample units were taken at the end of the ripening process (30 days). Two different batches by season, winter and spring, were analysed for the PDO industries, whereas only samples produced in winter were considered for the non-PDO industries.

The analyzed ripened cheeses are cylindrical in shape with around 800 g each, 15 cm diameter and 5 cm height and samples were taken from the core. Each assay was performed in three different cheeses by batch, making each determination in triplicate.

III.2. Physicochemical analysis

In the physicochemical characterization of the sample, pH, moisture content and water activity (a_w) were determined by officially established techniques. The moisture content of the samples was determined by dehydration at 102 $\pm$ 2ºC to a constant weight according to the official method established in ISO 5534:2004E (ISO, 2004a). The water activity (a_w) was determined using a GBZ FA-St/1 apparatus (Scientific Instruments, Romans sur Is`ere, France). The pH was measured using a Crison mod. 2002 pH meter (Crison Instruments, Barcelona, Spain).

III.3. Microbiological analysis

III.3.1. Bacterial counts

To the count of total and specific microorganisms, 10g of the core of each cheese was placed aseptically into a sterile plastic bag with 90 mL of sterile diluent and homogenized for 120s in a sample homogenizer. The cheese homogenates were serially diluted and aliquots from each dilution were inoculated into appropriate culture medium in a Petri dish or test tube and incubated at the appropriate temperature/time and atmosphere, as

established by international standards or appropriate bibliography. For the enumeration of sulfite-reducing *Clostridium*, the diluted cheese samples were heated at 80ºC for 10 min in water bath to kill vegetative forms. After incubation, all colonies present or only characteristic were quantified and the results were calculated in cfu/g. Table 16 shows the culture media and incubation conditions (temperature/time and atmosphere), used to quantify the different bacteria or bacterial groups.

Table 16 - Culture media and incubation conditions (temperature / time and atmosphere), used in the quantification of different bacteria or bacterial groups within the scope of this work.

Bacteria/ Bacterial Group	Culture Medium	Incubation Temperature/ Time	Incubation Atmosphere
Mesophilic aerobic bacteria	Plate Count Agar (PCA; Oxoid, Hampshire, UK)	30ºC/48 h	Normal
Mesophilic LAB	de Man, Rogosa and Sharpe agar (MRS; Oxoid) acidified (pH 5.6)	30ºC/48 h	10% CO_2
Lactococci	M17 agar (Oxoid)	30ºC/48 h	10% CO_2
Enterococci	Slanetz and Bartley agar (SB; Oxoid)	37ºC/48 h	Normal
***Leuconostoc* spp.**	Mayeux, Sandine, and Elliker agar (MSE; Biokar Diagnostic, Beauvais, France)	21ºC/72 h	10% CO_2
Enterobacteria	Violet Red Bile Glucose agar (VRBG; Oxoid)	30ºC/24 h	Normal
Escherichia coli	Bile X-Glucuronide agar (TBX; Serco, Mexico)	44ºC/24 h	Normal
Sulfite-reducing clostridia	Sulfite– Polymyxin–Sulfadiazine agar (SPS, Oxoid),	44ºC/72 h	Anaerobic
Staphylococci	Baird-Parker agar (BP; Oxoid) supplemented with potassium tellurite and egg yolk emulsion	37ºC/48h	Normal

III.3.2. Fungus counts

For fungus counts, the decimal dilutions of each sample were inoculated on a selective media (Rose Bengal Chloramphenicol agar - RBC; Oxoid, Hampshire, UK) in Petri dishes. The enumeration of characteristic yeast colonies was performed after incubation at 25ºC/72 h, all according to the current international regulations. The results were calculated in cfu/g.

III.3.3. Pathogen detection and confirmation

For *Salmonella* spp detection, 25g of each sample were homogenized in 225 mL of buffered peptone water and incubated at 30ºC for 24 hours, for subsequent detection

according to ISO 6785:2001 (ISO, 2001). The detection of *Listeria monocytogenes* was also carried out on 25g of each sample, according to the standard ISO 11290–1:2004 (ISO, 2004b). Counting and isolation of *S. aureus* and *E. coli* were performed according to the general technique described above using selective culture media (Table 16). Presumptive *Listeria spp*. or *Salmonella spp*. isolates and *S. aureus* characteristic colonies from BP agar tested for catalase and coagulase activity (Staphylex, Oxoid), were confirmed by sequencing the rRNA 16S gene as as described below. In the case of *E. coli*, presumptive colonies were confirmed in brilliant green lactose bile broth (Oxoid) (35ºC/24 to 48hr) before identification by 16S rRNA sequencing. Enterohemorrhagic *E. coli* serotype O157:H7 was then detected using a multiplex PCR for verotoxins (*E. coli* CECT4267- positive control) (Fratamico, Bagi, & Pepe, 2000).

III.3.4. Bacteria identification by DNA sequencing analysis

From each plated culture medium with isolated colonies at the highest dilutions, 10 colonies were randomly selected and isolated in a non-selective culture medium (nutrient agar - Oxoid) and further investigated for their identification at the species level. For such, the genomic DNA of the pure isolates previously grown in nutrient broth (Oxoid) was obtained and the 16S rRNA gene sequences analyzed as described by (Benito et al., 2008). The sequences were compared with the EMBL/GenBank database using the BLAST algorithm. The isolates identifications were confirmed based on the highest identity score (highest sequence homology).

III.3.5. Fungi identification by PCR-RFLP analysis of ITS region and sequencing analysis of the 26S rRNA region

For fungal identification, ten colonies of each RBC (Oxoid) agar plate containing the highest dilutions were randomly selected and isolated in the same culture medium for further identification at the species level. The genomic DNA of the 471 yeast isolates from the core cheese samples was obtained from a pure culture of each isolate in yeast extract peptone dextrose broth (YPD, Pronadisa).

From the DNA of pure isolates, fragment profiles of yeast isolates were grouped by PCR-RFLP (restriction fragment length polymorphism) of the ITS1-5.8S rRNA-ITS2 region,

using the restriction enzymes TaqI, Sau3AI, DdeI and HaeIII (Thermo Fisher Scientific, Waltham, MA, USA), as described by Gallardo et al. (2014). The resulting fragments were separated on 1.5% agarose gels and PCR fragment sizes were estimated by comparison with the GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific), using GeneTools image analysis software (SynGene, Cambridge, UK. The fragment profiles obtained were grouped into operational taxonomic units (OTUs) (P1 to P17).

Then, five representative isolates of each OTU were identified to the species level, by sequencing of the 26S LSU rRNA D1/D2 domains (O'Donnell, 1993), according to the PCR conditions described by Gallardo et al. (2014). The PCR products were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), sequenced by the Facility of Applied ioscience 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).

III.3.6. Identification of bacterial community by HTS of the 16S rRNA gene

The DNA from cheeses was extracted using a PowerFood microbial DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA). For this purpose the cheese (5 g) was previously homogenized in a suitable buffer (2% trisodium citrate buffer - VWR, Dublin, Ireland), enzymatically lysed with lysozyme (1 mg/mL), mutanolysin (50 U/mL) and proteinase K (800 μg/mL) followed by an incubation at 55ºC for 1 hour (Quigley et al., 2011).

The genomic DNA was amplified for the hypervariable V3V4 region with specific primers and further re-amplified in a limited-cycle PCR reaction to add a sequencing adaptor and dual-indexed barcodes. Second PCR reactions added the indexes and sequencing adaptors to both ends of the amplified target region. Negative controls were included for all amplification reactions. The amplicons were quantified by fluorimetry with PicoGreen dsDNA quantitation kit (Invitrogen, Life Technologies, Carlsbad, CA, USA), pooled at equimolar concentrations and pair-end sequenced with MiSeq R_ V3

chemistry, according to the manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

Sequenced reads were demultiplexed automatically by the Illumina® MiSeq® sequencer using the CASAVA package (Illumina, San Diego, CA, USA) and quality-filtered with PRINSEQ software (Schmieder & Edwards, 2011) using the following parameters: 1) bases with average quality lower than Q25 in a window of 5 bases were trimmed, and 2) reads with less than 150 bases were discarded. The forward and reverse reads were merged by overlapping paired-end reads using the AdapterRemoval v2.1.5 t (Schubert, Lindgreen, & Orlando, 2016) software with default parameters.

The QIIME package v1.8.0 (Caporaso et al., 2010) was used for OTU generation, taxonomic identification, sample diversity and richness indices calculation. Sample identifications were assigned to the merged reads and converted to FASTA format (split libraries fastq.py, QIIME). Chimeric merged reads were detected and removed using UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011) against the Greengenes v13.8 database (DeSantis et al., 2006) (script identify chimeric seqs.py, QIIME).

Operational taxonomic units (OTUs) were selected at 97% similarity threshold using the open reference strategy. First, merged reads were pre-filtered by removing sequences with a similarity lower than 60% against the Greengenes v13.8 database (DeSantis et al., 2006). The remaining merged reads were then clustered at 97% similarity against the same databases. Merged reads that did not cluster in the previous step were *de novo* clustered into OTUs at 97% similarity. A representative sequence of each OUT was then selected for taxonomy assignment (pick rep set.py, assign taxonomy.py; QIIME).

III.3.7. Identification of yeast community by HTS of the 16S rRNA gene

DNA extraction from cheese for yeast identification was similar to that used for bacterial identification by this technology (section III.3.5.), but in this case the the genomic DNA was amplified for the hypervariable ITS region with specific primers (Tedersoo et al., 2014).

Further reamplification and quantification, pooling in equimolar concentrations and pair-end sequencing of amplicons was also similar to that used for bacteria (section III.3.5.) and carried out at Genoinseq (Cantanhede, Portugal). The next steps were also the similar but in the final stage the chimeric merged reads were detected and removed, using UCHIME (Edgar et al., 2011) against UNITE/QIIME ITS v12.11 database (Abarenkov et al., 2010) (script identify_chimeric_ seqs.py, QIIME). The OTUs selection at 97% similarity steps until a representative sequence of each select OTU could be obtained for taxonomy assignment, were performed taking into account the referred database (pick_rep_set.py, assign_taxonomy.py; QIIME).

III.4. Selection of autochthonous lactic acid bacteria strains for symbiotic cheese production

III.4.1. Latic acid bacteria isolates for study

From the lactic acid bacteria (LAB) isolated from Serpa cheese, 116 were selected based on genre (*Lactobacillus*, *Lactococcus*, *Leuconostoc and Enterococus*) and origin (industry A, C, G, B, V) to be studied for their probiotic potential. Table 17 shows the selected isolates of Serpa cheese LAB to study the probiotic characteristics and selected strains following the application of the gastrointestinal tract resistance and safety tests.

Each LAB to be studied as potential probiotic strains (PPS) were previously cultured in MRS (Scharlab, Barcelona, Spain) under anaerobic conditions. After growth they were harvested and washed twice with phosphate buffered saline (PBS; Thermo Fisher Scientific) at pH 7,2. Cell concentration was adjusted to about 10^8 cfu/mL. In all tests three biological replicates of the cultures were used and the assays for each PPS were performed in duplicate.

III.4.2. Tolerance to the gastrointestinal tract conditions

III.4.2.1. Tolerance to low pH

Acidity resistance was evaluated in PBS with pH adjusted to 2,5, 2,75, 3,0 or 5,0, supplemented with pepsin (3,5 g/L, w/v), with PBS at pH 5 as control (Han, Kong, Chen,

Sun, & Zhang, 2017; Ruiz-Moyano, Martin, Jose Benito, Perez Nevado, & de Guia Cordoba, 2008). Strains were considered resistant as long as viable counts were not less than 2 log cfu/ml relative to the control, after 2 hours of contact.

III.4.2.2. Bile salt tolerance

The capacity of each selected strains to grow in the presence of bile salt (Sigma Chemical Co. St. Louis, MO, USA) were evaluated in MRS agar supplemented with those salts in the range of 0,3-2% (w/v) for 72 h, incubated at 37ºC under 10% CO_2.

III.4.2.3. Survival on complete gastrointestinal transit

To know the capacity of the acid and bile salt-tolerant strains to overcome the barriers in the GIT, they were subjected to the multiple stresses at once by the method of (Bao et al., 2010). Strains are subjected, at 37ºC under 10% CO_2, sequentially to the following conditions: simulated gastric juice (3,5 g/L of pepsin and 0,2% NaCl) at pH 2,75 for 2 h, and simulated intestinal juice (1 g/L of trypsin, 5 g/L bile salt, 2 g/L of pancreatin (Sigma), 11 g/L of sodium bicarbonate and 2 g/L of NaCl) at pH 8 for 6 h. The viable bacteria were counted on MRS media after 0, 2, 4 and 6 h. Strains with viable counts lower than 2 log cfu/mL respective to the control were considered not resistant.

III.4.3. Capacity to colonise the intestine

III.4.3.1. Aggregation capacity

The autoaggregation capacity has been determined using the autoaggregation assay described by Xu, Jeong, Lee, & Ahn (2009) with modifications. The LAB suspension (2 mL) was vortexed for 10 s and absorbance (Shimadzu UV 1800 spectrophotometer at 600 nm) was measured immediately (0 h). Then the suspension was incubated at 30ºC for 2 h and the absorbance of the supernatant was monitored at different time intervals (1 h, 2 h). The autoaggregation was calculated (Autoaggregation (%) = $(1 - A_t / A_0) \times 100$; A_t - absorbance at a determined interval, 1 h or 2 h; A_0 - absorbance at the beginning of the assay).

III.4.3.2. Cell surface hydrophobicity

This assay was carried out by the method described by Lee & Puong (2002) with slight modifications. LAB strain suspensions were adjusted in PBS to obtain an optical density (OD) at 600 nm of 1±0,05. This suspension were mixed with 99% n-hexadecane (Sigma) at high speed (1 min.) and left undisturbed (1 h/37ºC) to allow the phase separation. The lower aqueous phase was carefully removed with a sterile Pasteur pipette and final absorbance (A1) was recorded (Shimadzu UV 1800 at 600 nm) spectrophotometer. The decreased absorbance in the aqueous phase was taken as a measure of cell surface hydrophobicity (H%), (H(%) = [(A0 - A1)/ A0] x 100; A0, A1 - absorbance values before and after extraction with n-hexadecane).

III.4.4. Safety assays

III.4.4.1. Antibiotic susceptibility

The selected acid-tolerant strains were tested for antibiotic susceptibility by the disc diffusion method according to guidelines of National Committee for Clinical Laboratory Standards (NCCLS, 2012). For such antibiotic discs were applied on Petri dish cultures and incubated under anaerobic conditions at 37ºC/24 h. A range of twelve clinically important antibiotics (Oxoid, Basingstoke, England) were used: ampicillin (10 μg), gentamicin (10 μg), kanamycin (30 μg), trimethoprim (5 μg), erythromycin (15 μg), tetracycline (30 μg), clindamycin (2 μg), chloramphenicol (30 μg), penicillin G (10 units), polymixin B (300 units), nalidixic acid (30 μg) and vancomycin (30 μg). The diameters of the inhibition zones were measured and the results interpreted according to the criteria proposed by Charteris, Kelly, Morelli, & Collins (1998).

III.4.4.2. Biogenic amine production

The capacity to produce biogenic amines (BA) of selected strains was determined by the improved medium described by Bover-Cid & Holzapfel (1999). According to this, in a first phase, decarboxylase activity is induced by subcultivating the strains, under anaerobic conditions at 37ºC/4 days, in MRS broth containing 0,1% of each precursor amino acid (tyrosine, histidine, lysine, tyrosine, arginine, glutamine tryptophan, phenylalanine and ornithine; Sigma) and 0,005% of pyridoxal-5-phosphate. After induction of decarboxylase activity, the strains were grown in the improved medium

(without agar; 0,25% of each precursor amino acid) described by Bover-Cid & Holzapfel (1999) under anaerobic conditions at 37ºC/4 days. The filtrate obtained from this previously centrifuged culture will be used to quantify and determine the type of BA produced (tyramine, tryptamine, histamine, putrescine, phenylethylamine, spermine, spermidine and cadaverine) by high-performance liquid chromatography (HPLC)–electrospray ionisation (ESI)-mass spectrometry, according to the method described by Fernández et al. (2016). BA in samples was distinguished by their mass spectrum and retention time.

III.4.5. Growth on prebiotic

Was evaluated the *in-vitro* growth of the pre-selected LAB on three commercial prebiotics, lactulose (Sigma), short-chain fructo-oligosaccharide (FOS, Orafti® P95 with a degree of polymerisation (DP) 2–8, Beneo-Orafti, Belgium) and long-chain inulin (Orafti® GR with DP 2-60 and average ≥10, Beneo-Orafti). To assess growth under these conditions a suspension of each strain was inoculated in semi-solid culture medium (MRS) devoid of glucose, and supplemented with 2% (w/v) of each sterile-filtered prebiotic as the sole carbohydrate source. The positive control for growth consisted of the same media supplemented with 2% (w/v) of glucose or lactose, whereas the negative control was a carbohydrate-free semi-solid MRS. Growth was evaluated in a automated turbidimeter BioscreeC Microbiology for 96 h at 37ºC by reading the OD at 600 nm at regular intervals without shaking. The ability of each strain to grow in the presence of different prebiotics was evaluated by comparing the OD 600nm of each carbohydrate with the value obtained on glucose.

III.4.6. Short-chain fatty acid production

The assessment of the production capacity of short-chain fatty acid (SCFA) by selected LAB strains began with the growth in modified MRS broth (devoid of glucose and sodium acetate; with 2% (w/v) of the carbohydrate source - glucose, lactose, lactulose, FOS or inulin), in anaerobiosis at 37ºC, until stationary phase. The concentration of lactic acid (D-lactic and L-lactic), was evaluated in the filtrate obtained from the previously centrifuged culture supernatant, by the enzymatic method K-DLATE (Megazyme, Bray, Ireland).

The amount of the remaining SCFA was determined in a gas chromatograph (model 4890 Series II; Hewlett-Packard, Palo Alto, CA, USA) equipped with a split/splitless injector and a flame ionisation detector. SCFA were separated on a Carbowax™ fused silica capillary column (30 m × 0,25 mm id; 0,25 μm film thickness; Ohio Valley). The initial oven temperature was held at 80ºC for 2 min and was increased at a rate of 20ºC/min to 200ºC and retained for 12 min. Injector and detector temperatures were 250ºC. The carrier gas was nitrogen at 1,8 mL/min. The individual SCFA were identified by comparison of their retention times with those of reference standard mixtures (Sigma Chemical Co., St. Louis, MO, USA). SCFA concentrations were calculated by using the peak area ratio of the analyte to the internal standard (2-ethyl butyric acid), based on the methodology described by (Brighenti, 1997).

IV – RESULTS

IV.1. Bacterial Communities in Serpa Cheese by Culture Dependent Techniques, 16S rRNA Gene Sequencing and High-throughput Sequencing Analysis

Bacterial Communities in Serpa Cheese by Culture-Dependent Techniques, 16S rRNA Gene Sequencing and High-throughput Sequencing Analysis

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Abstract: Serpa cheese is one of the traditional regional Portuguese cheeses having the Protected Denomination of Origin (PDO) designation. This study investigated the bacterial community in the traditional Portuguese Serpa cheese. The microorganisms identified at the end of ripening (30 days) mainly were lactic acid bacteria (LAB). *Lactobacillus paracasei/Lactobacillus casei* was the main species in cheese from PDO registered industries, whereas in non-PDO registered industries *Lactobacillus brevis* was highlighted, among other LAB. *Enterobacteriaceae* species were detected at 20% to 40% of the total isolates. The results obtained by high-throughput sequencing analysis confirmed that LAB was the main microbial group, with *Lactococcus* genus contributing to approximately 40% to 60% of the population, followed by *Leuconostoc* and *Lactobacillus*. The *Enterobacteriaceae* family was also important. The differences between bacterial communities from PDO and non-PDO registered industries suggest that the lack of regulation of the cheese-making practices may influence unfavorably. The new knowledge about bacterial diversity in Serpa cheese could be useful to set up new ripening conditions, which favor the development of desirable microorganisms.

Keywords: cheese, microbial population, traditional cheese

Practical Application: The control of the manufacturing process of traditional cheeses can be improved through the knowledge of the bacterial diversity that develops. Thus, the growth of desirable microorganisms can be promoted to homogenize the final product.

Introduction

Serpa is an artisanal ripened Portuguese cheese granted the Protected Designation of Origin (PDO) label (Council Regulation EEC 2081/92), with six industries making cheese under this designation, although there are also other producers in the area without following the PDO regulation. It is produced within the Alentejo province (south of Portugal) from raw ewes' milk using aqueous infusion of the dried flowers from *Cynara cardunculus* L. plant as coagulant and without the addition of a starter culture. The absence of thermal process and starter microorganisms means that its quality and characteristics depend mainly on the endogenous microbiota, which arises primarily from the raw milk, vegetable coagulant and the cheese dairy environment (Aquilanti et al., 2011; Bokulich & Mills, 2013; Ordiales et al., 2013b; Pereira, Graça, Ogando, Gomes, & Malcata, 2010a).

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Most of the microbial community present in raw milk are lactic acid bacteria (LAB) (*Lactococcus* spp., *Lactobacillus* spp., and *Enterococcus* spp.), but microorganisms, such as *Enterobacteriaceae*, coliforms, *Staphylococcus aureus*, *Pseudomonas* spp., or even *Listeria monocytogenes* may also be present, and this raises the potential of public health risks (Pereira et al., 2010a). Hence, controlling the microbial communities during cheese production is the main factor to ensure food safety and the sensorial properties of the final product (Coton et al., 2012). During cheese ripening, the microbiota is mainly dominated by various LAB species, and to a lesser extent by cocci Gram-positive catalase-positive, diverse Gram-negative bacteria (*Citrobacter* spp., *Enterobacter* spp., *Pseudomonas* spp., *Serratia* spp. and *Hafnia* spp.), yeasts and molds (Abriouel, Martín-Platero, Maqueda, Valdivia, & Martínez-Bueno, 2008; Ordiales et al., 2013a; Dos Santos, Benito, Córdoba, Alvarenga, & Ruiz-Moyano, 2017). The contribution of LAB to the cheese final organoleptic characteristics is a consequence of their ability to ferment lactose and their proteolytic activity (Menéndez, Centeno, Godínez,& Rodríguez–Otero, 2000).

The properties of the resulting cheese can be expected to vary between producers due to small differences in the cheese making technology and slight variations in the chemical and microbial composition of the milk associated with the conditions of milk production, such as hygiene, geographical area, animal breed, season, and the microbial population of the cheese making environment (Alessandria et al., 2016; Bokulich & Mills, 2013; Guiné, Tenreiro, Correia, Correia, & Barracosa, 2016; Pereira

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et al., 2010b). A thorough microbial survey of similar cheeses regarding their origin and production technology, as PDO and non-PDO cheeses, could be very important for the dairy industry, as the PDO regulation enables knowledge of the production technology and milk production conditions to guarantee the quality of the product.

Accurate identification of microorganisms requires a culture-dependent approach combined with mainly DNA-based molecular techniques (Bokulich & Mills, 2013; Ordiales et al., 2013a). Sequencing the 16S rRNA gene in bacteria is a suitable tool for describing the microbial diversity of food process. Identifying live microorganisms along the cheese making process may contribute to establish strategies to control the microbial population and their influence on the final cheese characteristics (Ordiales et al., 2013b). However, the advent of culture independent technique such as high-throughput sequencing (HTS) technology has revolutionized the study of microbial ecosystems, including food fermentations. High-throughput sequencing enables comprehensive microbial surveys with detection sensitivities and throughputs several orders of magnitude greater than earlier molecular techniques via massive parallel sequencing of short amplicons of universally conserved DNA fragments, typically the 16S rRNA gene in bacteria (Bokulich & Mills, 2013). Thus, HTS tools have superior sequence coverage (live and death cells during the whole process) and lower sequencing cost, but the shorter fragment length results in lower taxonomic resolution (Bokulich & Mills, 2012).

Although Serpa cheese is considered one of the most appreciated Portuguese cheeses for its sensorial characteristic, little is known about the microbial diversity of this traditional cheese. Therefore, the aim of the present work was to study the bacterial community and foodborne pathogens presence in Serpa cheese with PDO label in comparison with similar non-PDO registered cheeses of the area by culture dependent and independent methods.

Materials and Methods

Serpa cheese samples

Samples were taken at the end of the ripening process (30 days) from 5 different dairy industries located in the geographical area of production. Three industries, identified as A, C, and G belonged to PDO "Serpa cheese," while the non-PDO registered industries were designated as V and B. Serpa cheeses were manufactured after raw ewes' milk coagulation with a *Cynara cardunculus* L. flowers infusion as described Alvarenga, Canada, and Sousa (2011). Ripened cheeses have a cylindrical shape with around 800 g each, 15 cm diameter and 5 cm height.

The ripening process was performed in 2 stages. In PDO registered industries, the cheeses were ripened in rooms with temperature and relative humidity controlled. The first stage was at temperature around 8 to 9 °C and relative humidity between 92% and 97% during 8 to 10 days, followed by second period until the end of ripening at temperature around 10 to 13 °C and relative humidity between 85% and 90%. In non-PDO registered industries, the cheese-making process is more artisanal, and the production is limited to winter season. In the initial stage, the ripening was performed in a room with temperature around 12 to 13 °C and relative humidity of 90% during 8 to 10 days, followed by a second period until the end of the ripening in a room at ambient conditions. Consequently, the temperature and relative humidity varied depending on the weather conditions, with values ranging from 8 to 18 °C and relative humidity between 65% and 95%.

Two different batches by season, winter and spring, were analyzed for the PDO industries, whereas only winter was used in the non-PDO industries. Each physicochemical and microbiological assay was performed in three different cheeses by batch ($n = 48$), making each determination in triplicate.

Physicochemical analysis

The moisture content of the samples was determined by dehydration at 102 ± 2 °C to a constant weight according to the official method of the International Organization for Standardization protocols ISO 5534:2004E. The water activity (a_w) was determined using a GBZ FA-St/1 apparatus (Scientific Instruments, Romans sur Isère, France). The pH was measured using a Crison mod. 2002 pH meter (Crison Instruments, Barcelona, Spain).

Culture dependent analysis: microbial counts and pathogens detection

For the isolates and microbial counts, each cheese sample (10 g) was placed aseptically into a sterile plastic bag with 90 mL 1% peptone water (Pronadisa, Alcobendas, Madrid, Spain), and homogenized for 120 s in a Stomacher instrument (Lab-Blender 400 Seward Lab., London, England). The cheese homogenates were serially diluted and aliquots from each dilution were inoculated onto agar plates. Mesophilic aerobic bacteria were counted on plate count agar (PCA; Oxoid, Hampshire, UK) after incubation at 30 °C for 48 hr. Mesophilic lactobacilli and lactococci were enumerated on de Man, Rogosa and Sharpe (MRS; Oxoid) agar acidified to pH 5.6 with acetic acid (10%) and M17 agar (Oxoid), respectively, under 10% CO_2 atmosphere at 30 °C after 48 hr. Enterococci, typical pink or dark red colonies with a narrow whitish border, were counted on Slanetz and Bartley (SB; Oxoid) agar at 35 °C for 48 hr. The transparent and gelatinous colonies of *Leuconostoc* spp. were enumerated on Mayeux, Sandine, and Elliker (MSE) agar medium (Biokar Diagnostic, Beauvais, France) at 21 °C after 72 hr. The growth of black staphylococci colonies on Baird-Parker agar (BP; Oxoid) supplemented with potassium tellurite and egg yolk emulsion was assessed after incubation at 37 °C for 48 hr. Enterobacteria (Gram-negative and cytochrome oxidase negative), identified as rose-coloured colonies surrounded by a halo of purple precipitate, were counted on violet red bile glucose agar (VRBG; Oxoid), after incubation at 30 °C for 24 hr. *Escherichia coli* were selectively grown on chromogenic tryptone bile X-glucuronide (TBX) agar (Serco, Mexico) and the typical blue-green colonies, indicating the presence of ß-D-glucuronidase activity, were enumerated after incubation at 44 °C for 24 hr. For the enumeration of sulfite-reducing *Clostridium* spp., 10 mL aliquots of 1:10 diluted cheese samples were heated at 80 °C for 10 min in water bath to kill vegetative forms. Then, 0.1 and 1 mL aliquots of the suspensions were mixed with 9.9 and 9 mL of sulfite–polymyxin–sulfadiazine agar (SPS, Oxoid), respectively, while a 5 mL aliquot was mixed with 5 mL of SPS agar 2X. After solidification of the media, another 2 to 3 mL of SPS agar was overlaid and the test tubes were incubated under anaerobic conditions at 44 °C for 72 hr. Colonies with appearances similar to black cotton wool and approximately 2 to 3 mm in size were counted as suspected sulfite-reducing *Clostridia*.

Finally, for *Listeria* spp. and *Salmonella* spp. analysis, 25 g of each cheese sample were placed aseptically into sterile plastic bags and homogenized in 225 mL of buffered peptone water and incubated at 30 °C for 24 hr. Then, *Listeria* spp. and *Salmonella* spp. were detected by ISO 11290–2:1998+A1:2004 (1998) and ISO 6785:2001 (2001), respectively.

Microbial identification of isolates by DNA sequencing analysis

Various colonies isolated from the cheeses were identified by rRNA 16S gene sequencing analysis. From each plated medium with colonies in the highest dilutions, 10 colonies were isolated at random on nutrient agar (Oxoid) and finally grown in 5 mL of nutrient broth (Oxoid). The genomic DNA of the pure isolates was obtained and the 16S rRNA gene sequences analyzed as described by Benito et al. (2008a, b). The sequences were compared with the EMBL/GenBank database using the BLAST algorithm. The isolates were confirmed based on the highest identity score (highest sequence homology).

For pathogens identification, presumptive *Listeria* spp. or *Salmonella* spp. isolates and *S. aureus* colonies from BP agar with a black appearance and surrounded by a clear zone and tested for catalase and coagulase activity (Staphylex, Oxoid) were confirmed by sequencing the rRNA 16S gene as above. Finally, presumptive *E. coli* from TBX plates were transferred to tubes of brilliant green lactose bile broth (Oxoid) and incubated at 35 °C for 24 to 48 hr. The confirmed *E. coli* colonies were transferred to eosin-methylene-blue lactose sucrose agar (Oxoid) before identification by 16S rRNA sequencing. Enterohemorrhagic *E. coli* serotype O157:H7 was then detected using a multiplex PCR for verotoxins as described by Fratamico, Bagi, and Pepe (2000). The primers used in the multiplex PCR and the sizes of the expected PCR products were as follows: Shiga 1 toxin (210 bp) stx1F (5′-TGTAACTGGAAAGGTGGAGTATACA-3′) and stx1R (5′-GCTATTCTGAGTCAACGAAAATAAC-3′); and Shiga 2 toxin (484 bp) stx2F (5′-GTTTTTCTTCGGTATCCTATTCC-3′) and stx2R (5′-GATGCATCTCTGGTCATTGTATTAC-3′). Primers for 16S rRNA gene amplification were used as the reaction control and the verotoxin-producing *E. coli* CECT4267 as the positive control.

Identification of bacterial community by HTS of the 16S rRNA gene

Cheese (5 g) was homogenized in 45 mL of a 2% trisodium citrate buffer (VWR, Dublin, Ireland). A 2 mL aliquot of the homogenate was enzymatically lysed with lysozyme (1 mg/mL), mutanolysin (50 U/mL), and proteinase K (800 μg/mL), followed by incubation at 55 °C for 1 hr, as previously described by Quigley, O'Sullivan, Beresford, Ross, and Fitzgerald (2011). After, the DNA was extracted using a PowerFood microbial DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA).

The genomic DNA was amplified for the hypervariable V3V4 region with specific primers and further re-amplified in a limited-cycle PCR reaction to add a sequencing adaptor and dual-indexed barcodes. The initial PCR reactions were performed for each sample using 2X KAPA HiFi HotStart ready mix, 0.2 μM of each PCR primer: forward primer Bakt_341F 5′-CCTACGGGNGGCWGCAG-3′' and reverse primer Bakt_805R 5′-GACTACHVGGGTATCTAATCC-3' (Herlemann et al., 2011; Klindworth et al., 2013) and 12.5 ng of template DNA. The PCR conditions involved denaturation at 95 °C for 3 min, followed by 35 cycles of 98 °C for 20 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min. Negative controls were included for all amplification reactions. Second PCR reactions added the indexes and sequencing adaptors to both ends of the amplified target region by the use of 2X KAPA HiFi HotStart ready mix, 5 μL of each index (i7 and i5) (Nextera XT Index Kit, Illumina, San Diego, CA) and 5 μL of the first PCR product, in a total volume of 50 μL. The PCR conditions involved a 95 °C denaturation for 3 min, followed by 8 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min. The amplicons were quantified by fluorimetry with PicoGreen dsDNA quantitation kit (Invitrogen, Life Technologies, Carlsbad, CA, USA), pooled at equimolar concentrations and pair-end sequenced with MiSeq® V3 chemistry, according to the manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

Sequenced reads were demultiplexed automatically by the Illumina® MiSeq® sequencer using the CASAVA package (Illumina, San Diego, CA, USA) and quality-filtered with PRINSEQ software (Schmieder & Edwards, 2011) using the following parameters: 1) bases with average quality lower than Q25 in a window of 5 bases were trimmed, and 2) reads with less than 150 bases were discarded. The forward and reverse reads were merged by overlapping paired-end reads using the AdapterRemoval v2.1.5 (Schubert, Lindgreen, & Orlando, 2016) software with default parameters. The QIIME package v1.8.0 (Caporaso et al., 2010) was used for OTU generation, taxonomic identification, sample diversity and richness indices calculation. Sample identifications were assigned to the merged reads and converted to FASTA format (split_libraries_fastq.py, QIIME). Chimeric merged reads were detected and removed using UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011) against the Greengenes v13.8 database (DeSantis et al., 2006) (script identify_chimeric_seqs.py, QIIME). Operational taxonomic units (OTUs) were selected at 97% similarity threshold using the open reference strategy. First, merged reads were pre-filtered by removing sequences with a similarity lower than 60% against the Greengenes v13.8 database (DeSantis et al., 2006). The remaining merged reads were then clustered at 97% similarity against the same databases. Merged reads that did not cluster in the previous step were *de novo* clustered into OTUs at 97% similarity. A representative sequence of each OTU was then selected for taxonomy assignment (pick_rep_set.py, assign_taxonomy.py; QIIME).

Statistical analyses

SPSS for Windows 21.0 (SPSS Inc Chicago, IL, USA) was used. The physicochemical and microbiological data were analyzed by a one-way analysis of variance (ANOVA). The means were separated by Tukey's honestly significant difference test.

Results and Discussion

Physicochemical data

The physicochemical characteristics of the cheeses (Table 1) revealed some differences among samples from different industries and seasons. The cheese samples had a pH range between 4.95−5.49, with the highest values for industry V and A in spring season. These pH data are in concurrence with those reported for soft cheeses (Alvarenga, Silva, Garcia, & Sousa, 2008; Ordiales et al., 2013a; Roseiro, Wilbey, & Barbosa, 2003). At 30 days of ripening, the moisture content of the cheeses ranged from 45.02 to 48.76% ($p < 0.05$) for PDO industries and 39.10-51.90% for non-PDO registered industries, while the a_w values were around 0.90 to 0.98. Significant differences in moisture and a_w between samples from industry V (non-PDO registered) and the rest of the industries were observed. Overall the results obtained in PDO registered industries agreed with the findings presented by Alvarenga et al. (2008) in Serpa Portuguese cheese.

Table 1–Mean values of pH, moisture and a_w values in cheese samples.

Serpa Cheese			Physicochemical parameters		
Season	PDO registered	Industries	pH Mean ± SD*	Moisture (%) Mean ± SD	a_w Mean ± SD
Winter	Yes	A	5.08 ± 0.09^{a}	48.76 ± 0.62^{a}	0.96 ± 0.02^{a}
		C	5.03 ± 0.03^{a}	47.71 ± 1.53^{ab}	0.98 ± 0.01^{a}
		G	4,95 ± 0.01^{a}	47.21 ± 0.96^{ab}	0.97 ± 0.03^{a}
	No	V	5.49 ± 0.04^{c}	39.10 ± 1.34^{c}	0.90 ± 0.03^{b}
		B	5.02 ± 0.09^{a}	51.90 ± 0.99^{d}	0.96 ± 0.01^{a}
Spring	Yes	A	5.48 ± 0.04^{c}	47.25 ± 1.37^{ab}	0.98 ± 0.01^{a}
		C	4.99 ± 0.16^{a}	45.02 ± 3.69^{a}	0.98 ± 0.01^{a}
		G	5.36 ± 0.10^{b}	46.6 0 ± 1.17^{ab}	0.98 ± 0.01^{a}
P values			***0.000***	***0.038***	***0.046***

^{a,b,c}For a given determination (column), values with different superscript numbers are significantly different ($P < 0.05$).

Table 2–Mean values of the microbiological counts (log cfu/g) in Serpa cheese from different PDO and non-PDO registered industries.

Season	Winter					Spring		
PDO registered	Yes			No		Yes		
Industries	A	C	G	V	B	A	C	G
Mesophilic aerobic bacteria (PCA*)	8.52	8.65	8.61	8.20	8.53	8.51	8.59	8.92
Lactobacilli (MRS)	8.03^{b}	8.53^{bc}	9.54^{d}	7.81^{b}	7.33^{a}	8.19^{bc}	8.32^{bc}	8.63^{b}
Lactococci (M17)	8.23^{ab}	8.44^{ab}	8.56^{bc}	8.46^{ab}	8.02^{a}	8.04^{a}	8.57^{bc}	9.01^{d}
Leuconostoc (MSE)	2.24^{b}	2.83^{b}	7.96^{c}	n.d.**^{a}	7.21^{c}	7.01^{c}	7.82^{c}	7.20^{c}
Enterococci (SB)	6.54^{ab}	7.15^{c}	6.32^{a}	7.65^{d}	6.66^{ab}	6.83^{bc}	7.04^{c}	6.51^{ab}
Staphylococci (BP)	n.d.^{a}	n.d.^{a}	n.d.^{a}	3.04^{b}	n.d.^{a}	6.01^{b}	4.78^{b}	2.18^{a}
***Staphylococcus aureus* (BP)**	n.d.^{a}	n.d.^{a}	n.d.^{a}	3.04^{b}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}
Enterobacteria (VRBG)	7.28^{b}	5.20^{b}	6.33^{b}	5.97^{b}	5.30^{b}	2.55^{a}	6.47^{b}	6.89^{b}
***Escherichia coli* (TBX)**	3.93^{d}	2.20^{b}	2.44^{bc}	3.88^{d}	3.12^{abc}	0.86^{a}	3.55^{cd}	3.14^{abc}
Sulfite-reducing Clostridia (SPS)	n.d.^{a}	n.d.^{a}	1.88^{b}	n.d.^{a}	n.d.^{a}	0.88^{b}	n.d.^{a}	0.83^{b}

^{a,b,c,d}For a given determination, values (row) with different superscript letters are significantly different ($P < 0.05$).
*PCA: Plate count agar; MRS: de Man, Rogosa and Sharpe Agar; M17: M17 agar; MSE: Mayeux, Sandine and Elliker agar; SB: Slanetz and Bartley agar; BP: Baird-Parker agar; VRBG: Violet Red Bile Glucose agar; TBX: chromogenic tryptone bile X-glucuronide agar; SPS: sulfite–polymyxin–sulfadiazine agar.
**n.d. not detected.

Microbial counts and identifications. The microbiological counts revealed some significant differences among cheeses from different industries and seasons (Table 2). Total aerobic bacteria counts ranged from 8.20 to 8.92 log cfu/g at the end of ripening process. Overall, the microbiota of raw milk cheese without the addition of starter is commonly dominated by a limited number of LAB genera, which have important roles in the organoleptic properties of artisanal cheeses (Feutry, Oneca, Berthier, & Torre, 2012; Ordiales et al., 2013a, b). The presumptive lactobacilli ranged from 7.33 to 9.54 log cfu/g, with the lower counts among the samples for non-PDO industries, V and B (7.33 and 7.81 log cfu/g respectively). The counts of lactococci were similar to lactobacilli, whereas the levels of *Leuconostoc* spp. were lower in all samples. This last LAB group showed the most variation between the industries. The enterococci population was also detected at a considerable level, with counts ranging from 6.32 to 7.65 log cfu/g. The presence of high numbers of enterococci is typical of artisan raw milk cheese, which, due to their ubiquitous presence, can easily contaminate the raw milk during its collection or at various stages of cheese making, due to poor manufacturing practices (Ordiales et al., 2013a, b; Ortigosa et al., 2008). However, they are considered a common member of cheese microbiota, with important influence in the ripening process (Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006).

For secondary microbiota, staphylococci were detected at a significantly lower level in winter cheeses compared to the spring samples. The staphylococci population is common in artisanal cheese with high variability in the counts (Galan, Cabezas, & Fernández-Salguero, 2012; Ordiales et al., 2013a).

Finally, the enterobacteria counts were between 5.20 and 7.28 log cfu/g with no significant differences between the industries, except for industry A in the spring, which scored statistically lower counts at 2.55 log cfu/g. A high level of enterobacteria is usually considered an indication of poor hygienic practices. However, the aforementioned values are in agreement with the range found by other workers for similar soft cheeses at around 30 days of ripening (Ordiales et al., 2013a, b; Tavaria & Malcata, 1998). Importantly, the presence of enterobacteria in high number at the end of the ripening may also be involved in the development of the sensory properties of the final cheese. Conversely, some species of this microbial group are considered spoilage organisms or even may be human pathogens, such as *E. coli* or *Salmonella* spp. Therefore, the level of *E. coli* was also investigated. Although much lower counts were detected, *E. coli* followed a similar tendency to the enterobacteria counts between the industries, with industry A in spring displaying the lowest counts.

The isolates from the PCA, MRS, M17, MSE, and SB plates were further investigated for their identification at the species level

Table 3–Identification of the isolates from PCA, MRS, M17, MSE, and SB plates and percent of distribution of each species by industry.

Identification	Industry							
	Winter					Spring		
	PDO			Non-PDO		PDO		
Sequencing of 16S rRNA gene Species-Accession no. (% identity)	A	C	G	V	B	A	C	G
	% of isolates on PCA*							
Lb. paracasei – CP013921.1/ *Lb. casei* – /KT897918.1 (100%)	26.6	71.4	61.8	30.1		47.2	35.4	32.5
Lb. plantarum – KT946604.1 (100%)	26.6		12.4		16.7			32.5
Lb. brevis – KP793171.1 (100%)				15.1	50.2		6.4	
L. mesenteroides – KP742818.1 (99%)						5.9	30.1	8.1
E. faecalis – KU321632.1 (100%)	17.8	5.1			8.3	6	5.9	
E. faecium – CP014449.1 (100%)						17.8		
E. hirae – LC122277.1 (100%)				15.1				
Hafnia alvei – KT767875.1 (100%)	29.2	23.6	25.9	21.7	13.5	23.1	23.1	26.8
Escherichia coli – CP015076.1 (99%)				18.1	11.3			
	% of isolates on MRS							
Lb. paracasei – CP013921.1/ *Lb. casei* – /KT897918.1 (100%)	37.5	100	71.4	40		61.3	50	40
Lb. plantarum – KT946604.1 (100%)	37.5		14.3		22.2			40
Lb. pentosus – KU945826.1 (100%)			14.3					
Lb. curvatus – KT763326.1 (100%)								10
Lb. brevis – KP793171.1 (100%)				20	66.7		8.3	
L. mesenteroides – KP742818.1 (99%)						7.7	41.7	10
E. faecalis – KU321632.1 (100%)	12.5				11.1	7.7		
E. faecium – CP014449.1 (100%)	12.5			20		23.2		
E. hirae – LC122277.1 (99%)				20				
	% of isolates on M17							
Lb. paracasei – CP013921.1 /*Lb. casei* – /KT897918.1 (100%)						50	14.3	
Lb. plantarum – LC125266.1 (100%)							14.3	
Lb. brevis – KT757228.1 (100%)	25							
L. mesenteroides – KP742818.1 (99%)			100			25	57.1	100
Lc. lactis – KT757263.1 (100%)							14.3	
E. faecalis – KU321632.1 (100%)	75	50		20	50	25		
E. faecium – CP014449.1 (100%)				40				
E. hirae – LC122277.1 (100%)		50		40	50			
	% of isolates on MSE							
L. mesenteroides- KP742818.1 (99%)	100	100	100	100	100	100	100	100
	% of isolates on SB							
E. faecalis – KU321632.1 (100%)	87.5	71.4			100	87.5	100	57.2
E. faecium – CP014449.1 (100%)		14.3						42.8
E. hirae – LC122277.1 (100%)	12.5	14.3	16.7	75				

*PCA: Plate count agar; MRS: de Man, Rogosa and Sharpe Agar; M17: M17 agar; MSE: Mayeux, Sandine and Elliker agar; SB: Slanetz and Bartley agar.

Table 4–Identification of the isolates from BP, VRBG, and TBX plates and percent of distribution of each species by industry.

Identification	Industry							
	Winter					Spring		
	PDO			Non-PDO		PDO		
Sequencing of 16S rRNA gene Species-Accession no. (% identity)	A	C	G	V	B	A	C	G
	% of isolates on BP*							
S. epidermidis – KT989845.1 (100%)						25	100	
S. caprae – KT387321.1 (100%)						12.5		
S. hominis – LN774575.1 (100%)						12.5		
S. warneri – KC139448.1 (100%)						37.5		33.3
S. simulans – CP014016.1 (100%)						12.5		
S. cohnii – KX023361.1 (100%)								66.7
S. aureus – CP015173.1 (100%)				100				
	% of isolates on VRBG							
H. alvei – KT767875.1 (100%)	100	100	100	30	60	100	100	100
E. coli – CP015076.1 (99%)				60	40			
K. oxytoca – KU761531.1 (100%)				10				
	% of isolates on TBX							
E. coli – CP015076.1 (100%)	100	100	100	100	100	100	100	100

*BP: Baird-Parker agar; VRBG: Violet Red Bile Glucose agar; TBX: chromogenic tryptone bile X-glucuronide agar.

(Table 3). Counts on the PCA medium provided a guide to the prevalence of live bacteria in the final cheese. The microorganisms identified in the PCA medium mainly corresponded to LAB and to a lesser extent, enterobacteria. *Lactobacillus paracasei/Lb. casei* were the main species in cheese samples from PDO industries (A, C and G) in both seasons. *Lb. casei* and *Lb. paracasei* are closely related species and, therefore, difficult to distinguish, with 99.4% similarity (Öztürk & Meterelliyöz, 2015). In cheeses from non-PDO registered industries, *Lb. brevis* and *Lb. paracasei/casei* were the dominant species in industries B and V, respectively. Other LAB identified were *Lb. plantarum, Leuconostoc mesenteroides,* only identified in spring season, and species belonging to the genus *Enterococcus*. *E. faecium* appeared in cheeses from industries A and V, while *E. faecalis* has a higher prevalence in A and B. These genera and species are among the most common LAB found in raw milk cheese (Feutry et al., 2012; Fuka et al., 2013; Ordiales et al., 2013a; Picon, Garde, Ávila, & Nuñez, 2016). Finally, some species from the *Enterobacteriaceae* family were detected, which contributed to around 20 to 40% of the total isolates identified. The species, *Hafnia alvei*, was identified at around 20 to 30% in all the industries. This species has demonstrated its ability to survive during the ripening process of soft cheese and other types of cheese with a long maturation process (Abriouel et al., 2008; Coton et al., 2012; Ordiales et al., 2013a; Tabla et al., 2016). Moreover, it is known to positively contribute to the aromatic properties of cheeses (Irlinger et al., 2012). The most disturbing was the presence of *E. coli* in the final product at appreciable level, particularly those obtained from industries V and B (both non-PDO registered industries), which may be due to poor milking hygiene or poor hygienic processing as consequence of different manufacturing practices suggested by PDO "Cheese Serpa".

These results differ slightly from the results obtained with HTS (Figure 1). During whole cheese processing, the predominant bacteria comprised *Lactococcus*, *Lactobacillus*, and *Leuconostoc* genera. Most of the identifications matched with those obtained in PCA, except for *Lactococcus*. Studies to assess the persistence and viability of this microorganism throughout manufacturing and ripening of cheeses have been done (Ruggirello, Cocolin, & Dolci, 2016; Ruggirello, Dolci, & Cocolin, 2014). The authors observed that at the end of ripening, lactococci were detected at a minimal amount and this discrepancy was explained with the knowledge that during the ripening process, lactococci enter in a stressed physiological state (viable not culturable, VNC), which might cause their inability to grow on synthetic medium despite their viability in the cheese matrix.

The identification of microorganisms isolated from the specific media MRS, M17, MSE and SB for various LAB genera is shown in Table 3. In MRS, corresponding mainly to *Lactobacillus* spp., *Lb. paracasei/Lb. casei* was the most common species in this medium, followed by sporadic identification of *Lb. platarum*, *Lb. brevis*, *Lb. pentosus* and *Lb curvatus*. In particular, *Lb. brevis* was present at a relatively high percentage in samples from V and B (both non-PDO registered industries). *L. mesenteroides*, *E. faecalis*, *E. faecium* and *E. hirae* were also identified in MRS agar but not in all samples. *L. mesenteroides* was also detected in PCA agar but was also only associated with spring season samples.

In M17, only *Lactococcus lactis* was detected in samples from industry C. This is in discordance with the HTS results (Figure 1B), where *Lactococcus* was the most abundant genera in the cheeses. The viability of *Lactococcus* at the end of ripening is minimal because they succumb to a stressed physiological state during the ripening process (Ruggirello et al., 2014, 2016). On the other hand, it has been reported that M17 medium it is not such a selectivity for lactococci species growth (Ruggirello et al., 2014).

All the isolates identified in MSE and SB medium corresponded to *Leuconostoc* spp. and *Enterococcus* spp., respectively, showing both media were highly selective for enumerating these genera of LAB (Table 3).

Leuconostoc spp. isolates were found in all industries and were identified as *L. mesenteroides* (Table 3). This species is usually found as a subdominant LAB species with raw milk cheese (Aquilanti et al., 2011; Fuka et al., 2013; Picon et al., 2016) and, alongside *Leucnostoc lactis,* is the most relevant species of this genus in the dairy industry (Hemme & Foucaud-Scheunemann, 2004). Although these species grow poorly in milk, they are more competitive in the late stage of the cheese ripening process. In particular, it is known that these bacteria contribute to the peculiar aroma of traditional cheeses, mainly due to their ability to metabolize citrate and lactate (McSweeney & Sousa, 2000). Additionally, they may influence the texture of cheese by the synthesis of dextrans. These valuable technological properties are the reason some researchers have considered them as starters for cheese manufacture (Alegría, Delgado, Flórez, & Mayo, 2013; Nieto-Arribas, Seseña, Poveda, Palop, & Cabezas, 2010) and may positively influence the organoleptic properties in Serpa cheese from spring season.

The enterococci population was dominated by *E. faecalis*, followed by *E. faecium* and *E. hirae*, which was only identified in winter samples and was the predominant species in industry V (Table 3). *E. faecalis* and *E. faecium* are common enterococci species found in raw milk cheese, whereas *E. hirae* is more sporadically present at an important level (Feutry et al., 2012; Ordiales et al., 2013a). Among the LAB genera, enterococci raise some controversy because some members, particularly *E. faecalis*, have been linked to various virulence factors, antibiotic resistance and gene transfer mechanisms related to human pathogenesis (Moreno et al., 2006). Moreover, they also present important tyrosine decarboxylase activity, responsible for tyramine accumulation in cheese (Picon et al., 2016). However, in many artisanal cheeses, their presence has been correlated with a positive contribution to the ripening process, influencing the development of the typical organoleptic properties, and inhibiting foodborne pathogens by producing the bacteriocins, enterocins (Giraffa, 2003; Ordiales et al., 2013b).

Table 4 shows the species identified in the BP, VRBGA and TBX plates. *S. aureus* is a foodborne pathogen and was the unique species identified in the samples originating from industry V, which is a non-PDO registered. *Staphylococcus epidermidis*, *S. warneri* and *S. cohnii* were mainly identified in cheeses prepared during spring. *Staphylococcus* spp. has been detected in most cheese varieties as a major component of the secondary flora that remains throughout ripening, probably due to their resistance to salt and dehydration (Little et al., 2008; Ordiales et al., 2013a).

The predominance of the *Enterobacteriaceae* family was also observed by HTS analysis during the ripening process (Figure 1A). *Enterobacteriaceae* have frequently been found during cheese manufacture (Coton et al., 2012; Ordiales et al., 2013a). The enterobacteria identified were mainly dominated by *H. alvei*, except for industries V and B (both non-PDO registered). For these two industries *E. coli* was also identified at a high level (Table 4), some strains of this species are considerate important foodborne pathogens. *H. alvei* has often been described as the dominant Gram-negative bacteria in cheeses (Abriouel et al., 2008; Coton et al., 2012; Ordiales et al., 2013a). These bacteria have been shown to display high proteolytic activities, which

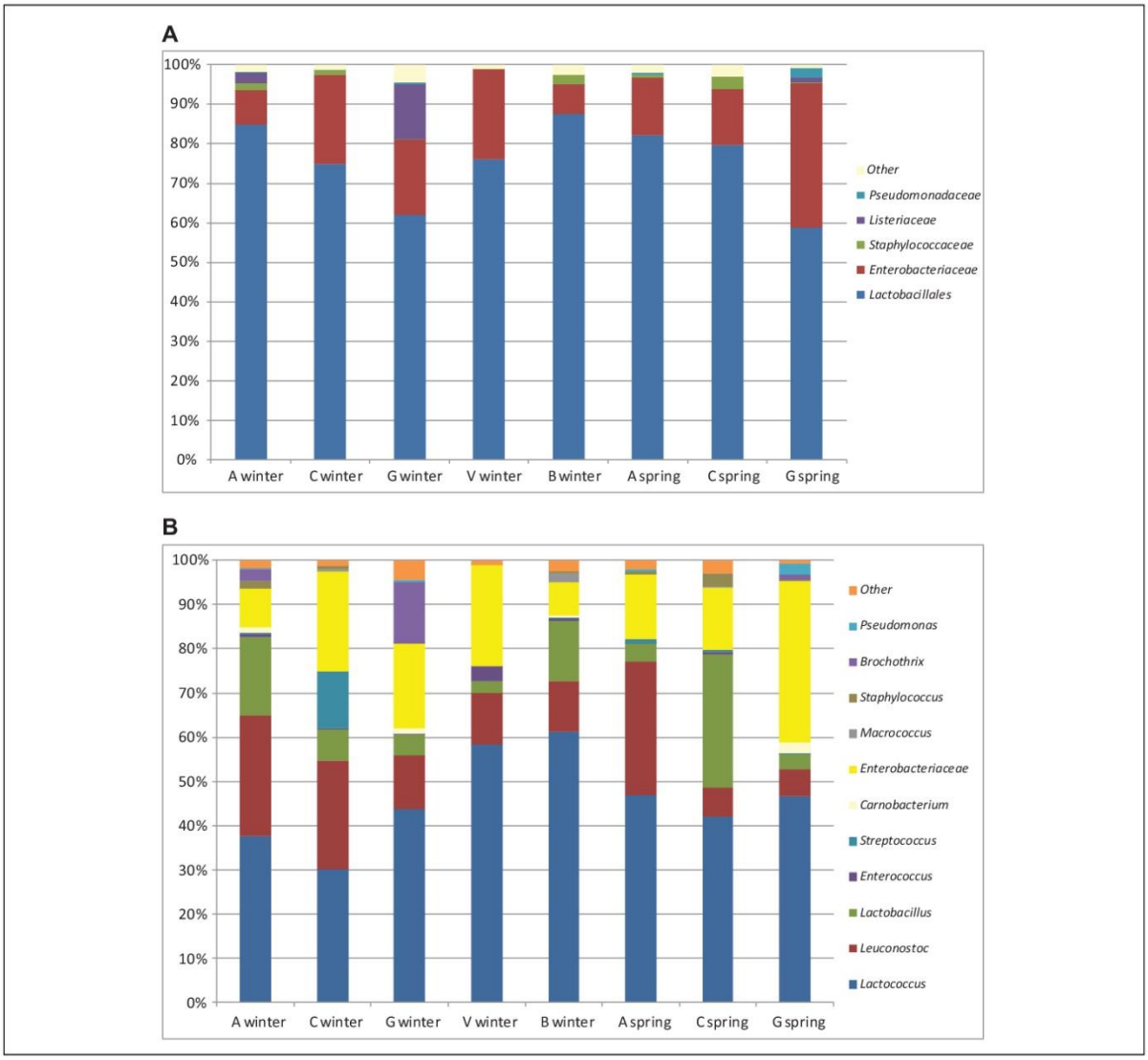

Figure 1–Mean relative abundance of bacteria in cheeses from different industries (capital letters) analyzed in two different seasons (winter and spring) using high-throughput sequencing of the 16S rRNA gene. (A) at the family level; (B) at the genus level.

could affect the sensory quality of cheese, such as creamy cheeses (Ordiales et al., 2013b).

Pathogen detection

The most relevant foodborne pathogens in raw milk cheeses, *Listeria monocytogenes*, *Salmonella* spp. and enterohemorrhagic *E. coli* were not detected in any of the cheese samples after 30 days of ripening. The absence of these pathogens in the cheese indicates the safety quality of the final product. The bacterial populations at the end of the ripening time depend on the adaptation of the microorganisms to the stress conditions found during the maturation process, such as salt concentration, pH decrease and competitive interaction with the microbiota present (Donnelly, 2004).

E. coli was found in all cheese samples at 30 days of ripening (Table 4) and all the isolates from the TBX medium were identified as *E. coli* by sequencing. This could have been due to the use of contaminated milk. *E. coli* is also used as an indicator of direct or indirect fecal contamination of foods, suggesting possible presence of enteric pathogens. In cheese, the presence of *E. coli* may indicate poor hygiene conditions (Kornaki & Johnson, 2001). Little et al. (2008) found initial levels of *E. coli* ranging from 1.1 $\times$ 10^5 to 4.6 $\times$ 10^6 cfu/g in raw milk cheese, and Ordiales et al. (2013a) found *E. coli* at 30 days of ripening in soft cheese "Torta del Casar," but these were not detected at the end of ripening process (60 days). So, these reports highlight the importance of the ripening period in raw milk cheese to ensure the safety of the final product, particularly considering the ability of some pathogens, such as *E. coli* O157:H7 and *Listeria monocytogenes* to tolerate periods of similar maturity to Serpa cheese (Almeida et al., 2007; Montet et al., 2009). Although no relevant foodborne pathogens were detected at 30 days, the *E. coli* level found in all samples at the end of the ripening (Table 2), highlighted its identification among

the dominants species on PCA agar on non-PDO industries (Table 3), suggests that a longer period, close to 60 days of ripening, would be recommended.

S. aureus was only found in cheeses from industry V (non-PDO registered) (Table 3). In raw milk cheese, *S. aureus* contamination can be caused by raw milk produced by animals suffering from mastitis, contaminated milk tank, and poor hygiene practices or the cheese handlers who are *S. aureus* carriers (Guerreiro, Velez, Alvarenga, Matos, & Duarte, 2013; Rola, Czubkowska, Korpysa-Dzirba, & Osek, 2016). *S. aureus* has been reported in cheese made from raw milk with a higher incidence than other foodborne pathogens (Little et al., 2008; Ordiales et al., 2013a). However, although *S. aureus* can produce an enterotoxin that causes illness, high numbers of the organism are necessary to produce the toxin in sufficient quantities to be a threat to public health (Le Loir, Baron, & Gautier, 2003). *S. aureus* can grow during cheese processing, but once a_w and pH are decreased, its growth is generally inhibited (Stewart et al., 2002).

Sulfite-reducing clostridia are widespread in the environment and can contaminate milk and cheese. They were detected in some cheese samples from industries G and A, but at low levels (Table 2). Clostridia include pathogenic representatives, such as *Clostridium perfringens, C. botulinum, C. difficile*, and *C. tetani*, as well as the spoilage species, *C. tyrobutyricum*, which is the main cause of the late-blowing defect in cheese, responsible for relevant financial losses in the dairy industry (Brändle, Domig, & Kneifel, 2016).

Conclusions

The data reported in this study showed higher differences among the identified microorganisms isolated from cheeses obtained from PDO and non-PDO industries. Hence, the production regulation gives guarantees of standardization to the product. The microorganisms identified at the end of ripening were LAB and to a lesser extent, enterobacteria, with *Lb. paracasei/Lb. casei* being the main species in cheese from PDO industries, while in non-PDO industries *Lb.brevis* was also identified at high level. However, by culture independent methodology (HTS), the predominating genera were mainly *Lactococcus*, followed by *Lactobacillus*, and *Leuconostoc* genus. Finally, although high level of enterobacteria were found, the main foodborne pathogens, *L. monocytogenes*, *Salmonella* spp. and enterohemorrhagic *E. coli* were not detected in cheese samples at the end of maturation, which guarantee the microbiological safety of the final product. Moreover, the study of technological and functional properties of autochthonous strains of lactic acid bacteria could be also an important tool, starter cultures to control the ripening process and ensure the final quality and safety of the cheese.

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Authors' Contributions

Maria Teresa P. Gonçalves carried out experiments, interpreted the results and drafted the manuscript. María José Benito designed the study, interpreted the results and wrote the manuscript. María de Guía Córdoba collaborated in designing experiments and drafted the manuscript. Conceição Egas developed high-throughput sequencing analysis and drafted the manuscript. Almudena V. Merchán carried out experiments and participated in data analysis. Ana I. Galván carried out experiments and participated in data analysis. Santiago Ruiz-Moyano participated in designing the study and experiments, interpreted results and drafted the manuscript.

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IV.2. Yeast community in traditional Portuguese Serpa cheese by culture dependent and independent DNA approaches

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Yeast community in traditional Portuguese Serpa cheese by culture-dependent and -independent DNA approaches

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ABSTRACT

This study investigated the yeast community present in the traditional Portuguese cheese, Serpa, by culture-dependent and -independent methods. Sixteen batches of Serpa cheeses from various regional industries registered with the Protected Designation of Origin (PDO) *versus* non-PDO registered, during spring and winter, were used. Irrespective of the producer, the yeast counts were around 5 log CFU/g in winter and, overall, were lower in spring. The yeast species identified at the end of ripening (30 days), using PCR-RFLP analysis and sequencing of the 26S rRNA, mainly corresponded to *Debaryomyces hansenii* and *Kluyveromyces marxianus*, with *Candida* spp. and *Pichia* spp. present to a lesser extent. The culture-independent results, obtained using high-throughput sequencing analysis, confirmed the prevalence of *Debaryomyces* spp. and *Kluyveromyces* spp. but, also, that *Galactomyces* spp. was relevant for three of the five producers, which indicates its importance during the early stages of the cheese ripening process, considering it was not found among the dominant viable yeast species. In addition, differences between the identified yeast isolated from cheeses obtained from PDO and non-PDO registered industries, showed that the lack of regulation of the cheese-making practice, may unfavourably influence the final yeast microbiota. The new knowledge provided by this study of the yeast diversity in Serpa cheese, could be used to modify the cheese ripening conditions, to favour desirable yeast species. Additionally, the prevalent yeast isolates identified, *Debaryomyces hansenii* and *Kluyveromyces* spp., may have an important role during cheese ripening and in the final sensorial characteristics. Thus, the study of their technological and functional properties could be relevant, in the development of an autochthonous starter culture, to ensure final quality and safety of the cheese.

1. Introduction

Serpa is an artisanal ripened Portuguese cheese granted the Protected Designation of Origin (PDO) label (Council Regulation (EEC) No 2081/92, 2017), with six industries making cheese under this designation, although there are also many producers in the area that make it without following the PDO regulation. It is produced within the Alentejo province (south of Portugal) from raw ewes' milk, using an aqueous infusion of the dried flowers from *Cynara cardunculus* L. as rennet and without the addition of a starter culture. Cheese ripening is a complex fermentation process involving a wide range of biochemical reactions. Industrial-scale cheese production usually applies a thermal treatment to standardise the microbial diversity, followed by starter culture inoculation to ensure the safety and reduce the variability in the final product but affecting the original sensorial characteristics (Montel et al., 2014). In contrast, there is no standardisation for the thermal process and starter microorganisms application in traditional raw milk cheese, which, therefore, possess a complex microbial community that arises primarily from the raw milk, vegetable rennet and the cheese dairy environment (Aquilanti et al., 2011; Bokulich and Mills, 2013; Ordiales et al., 2013a, 2103b; Pereira et al., 2010; Roseiro et al., 2003), leading to desirable sensorial properties highly appreciated by consumers. The development of the initial microbial population during the cheese-making process and ripening period, strongly contribute to the quality and safety of the cheese, through their metabolic activities (Montel et al., 2014). Most of the microbial community present in raw

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milk are lactic acid bacteria (*Lactococcus* spp., *Lactobacillus* spp. and *Enterococcus* spp.) and their importance in cheese ripening is well recognised (Beresford et al., 2001). Additionally, the yeast population is also important in raw milk (Delavenne et al., 2011; Quigley et al., 2013) and is associated with the secondary microbiota of diverse types of cheeses, where they play an important role during the ripening.

Artisanal cheeses possess a large assortment of yeast species, mainly belonging to the genera *Debaryomyces*, *Geotrichum* (= *Galactomyces*), *Kluyveromyces*, *Candida*, *Pichia* and *Yarrowia* (Banjara et al., 2015; Binetti et al., 2013; Ceugniez et al., 2015; Padilla et al., 2014), although the prevalence of certain yeast species can be influenced by the type of cheese (Dugat-Bony et al., 2016). Yeasts contribute to the cheese ripening by lactate consumption, alkaline metabolite formation, lactose fermentation, lipolysis, proteolysis, production of aromatic compounds and by their positive or negative interactions with other members of the microbial cheese community, which are important for the typical characteristics of cheese (Jakobsen and Narvhus, 1996; Rossi et al., 1997). Conversely, yeasts can also cause cheese spoilage, by generating undesirable flavours, texture losses, excessive gas formation, acidity increase and brown surface discolourations (Carreira et al., 1998; Jakobsen and Narvhus, 1996; Liu and Tsao, 2009; Wyder et al., 1999).

Differences in artisanal cheese can be expected among producers, due to small differences in the cheese-making technology and slight variations in the chemical and microbial composition of the milk associated with the conditions of milk production, such as hygiene, geographical area, animal breed, season and the microbial population of the cheese-making environment (Alessandria et al., 2016; Bokulich and Mills, 2013; Guiné et al., 2016). Thus, considering the relevance of the microbial community, particularly, the fungi, on the organoleptic properties, as well as in the safety of cheese, the various genera and species present must be identified and quantified, to determine their influence on maturation, alteration or deterioration of cheese. A thorough microbial survey of similar cheeses, regarding their origin and production technology, as PDO and non-PDO cheeses, could be highly valuable for the dairy industry, as the PDO accreditation enables knowledge of the production technology and milk production conditions, to guarantee the quality of the product.

For several decades, the diverse composition of cheese fungi has been investigated by the application of conventional culture-dependent approaches combined with molecular tools based on the polymerase chain reaction (PCR) of the short non-coding ribosomal ITS regions (ITS I and ITS II), which are extremely variable spacers in both sequence and length that provide an excellent tool to differentiate between and within species, and the D1/D2 domain of the large subunit ribosomal RNA (LSU rRNA) (Álvarez-Martín et al., 2007; Blackwood et al., 2003; Tofalo et al., 2014). However, the development of culture-independent molecular methods has changed the approach to study microbial communities during food fermentations processes. In particular, high-throughput sequencing (HTS) technology, has revolutionised the study of microbial ecosystems (De Filippis et al., 2017). HTS enables comprehensive microbial surveys, with detection sensitivities and throughputs several orders of magnitude greater than earlier molecular techniques (Bokulich and Mills, 2013). Among HTS possibilities, PCR amplification and sequencing of universally conserved DNA fragments, typically the ITS gene in fungi, is the most common approach exploited in food microbiology ecology studies (De Filippis et al., 2017). The advantages of this HTS approach, are its superior sequence coverage (live and dead cells throughout the process) and lower sequencing cost, however, it is restricted by primer amplification bias and by short read length, which results in a lower taxonomic resolution (Bokulich and Mills, 2012). Culture-dependent and -independent molecular biology techniques are complementary rather than contradictory. Thus, an in-depth study of the fungal diversity involved in the cheese maturation process by combining these approaches, may contribute to better understand the metabolic activities of this microbial community and their possible interactions with other members, to establish strategies to control the microbial population.

Most of the studies of microbial ecology using HTS technologies in cheese have addressed bacterial communities, whereas, scarce have investigated the fungal ecology, despite the relevance of yeasts during cheese ripening (Bokulich and Mills, 2013; Ceugniez et al., 2017; De Filippis et al., 2017; Stellato et al., 2015; Wolfe et al., 2014). Although Serpa cheese is highly valued for its sensorial characteristics, little is known about its yeast diversity. In this context, the present study aimed to compare the yeast community in Serpa cheese with a PDO label with similar non-PDO registered cheeses of the area, by culture-dependent and -independent methods.

2. Materials and methods

2.1. Serpa cheese samples

Samples were taken from the core of ripened cheeses (30-days-old) produced by five different dairy industries located in the geographical area of production. Three industries, identified as A, C and G, belonged to PDO "Queijo Serpa", while the non-PDO registered industries were designated V and B. Two different batches and seasons, winter and spring, were analysed for the PDO industries, whereas only samples produced in winter were considered for the non-PDO industries (Table 1). Each assay was performed in three different cheeses by batch (n = 48), making each determination in triplicate.

2.2. Physicochemical analysis

The moisture content of the samples was determined by dehydration at 104 °C to a constant weight, according to the official method of

Table 1
Mean values of pH, moisture, water activity (a_w) and yeast counts (log CFU/g) in the core of cheese samples.

Serpa cheese			Physicochemical parameters			Yeast count
Season	PDO registered	Industries	pH Mean ± SD*	Moisture (%) Mean ± SD	a_w Mean ± SD	(log CFU/g) Mean ± SD
Winter	Yes	A	5.08 ± 0.09^{a}	48.76 ± 0.62^{a}	0.96 ± 0.02^{a}	5.66 ± 0.11^{d}
		C	5.03 ± 0.03^{a}	47.71 ± 1.53^{ab}	0.98 ± 0.01^{a}	5.81 ± 0.45^{d}
		G	4,95 ± 0.01^{a}	47.21 ± 0.96^{a,b}	0.97 ± 0.03^{a}	4.62 ± 0.20^{c}
	No	V	5.49 ± 0.04^{c}	39.10 ± 1.34^{c}	0.90 ± 0.03^{b}	4.44 ± 0.79^{b,c}
		B	5.02 ± 0.09^{a}	51.90 ± 0.99^{d}	0.96 ± 0.01^{a}	4.20 ± 0.19^{b}
Spring	Yes	A	5.48 ± 0.04^{c}	47.25 ± 1.37^{ab}	0.98 ± 0.01^{a}	2.55 ± 0.63^{a}
		C	4.99 ± 0.16^{a}	45.02 ± 3.69^{a}	0.98 ± 0.01^{a}	3.93 ± 0.27^{b}
		G	5.36 ± 0.10^{b}	46.60 ± 1.17^{ab}	0.98 ± 0.01^{a}	5,80 ± 0.10^{d}
P values			*0.000*	*0.038*	*0.046*	*0.000*

^{a,b,c}For a given determination (column), values with a different superscript number are significantly different ($P < 0.05$).
*SD: standard deviation.

the International Organisation for Standardisation (ISO Norm R-1442., 1979). The water activity (a_w) was determined using a GBZ FA-St/1 apparatus (Scientific Instruments, Romans-sur-Isère, France). The pH was measured using a Crison mod. 2002 pH meter (Crison Instruments, Barcelona, Spain).

2.3. Yeast counts and identification

For yeast counts, 10 g of each cheese sample was aseptically placed into a sterile plastic pouch with 90 mL of 1% peptone water (Pronadisa, Alcobendas, Madrid, Spain), followed by homogenisation for 120 s in a stomacher (Lab Blender 400, Seward Medical, London, UK). The cheese homogenates were serially diluted and aliquots from each dilution were inoculated on rose bengal chloramphenicol agar plates (RBC; Oxoid, Hampshire, UK). The enumeration was performed after incubation at 25 °C for 72 h.

2.3.1. PCR-RFLP analysis of the ribosomal internal transcribed spacers (ITS)

Ten colonies from each agar plate containing the highest dilutions were isolated at random, on RBC agar (Oxoid) and finally 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, until they were sufficiently grown to allow species identification.

Genomic DNA of the pure isolates was obtained by centrifugation (10,000 *g* for 5 min at 4 °C) of 1 mL of 24 h culture in YPD broth. The supernatant was discarded. The cell pellet was ground with a motor-driven pestle in 0.6 mL of hot (65 °C) extraction buffer (50 mM Tris-HCl, pH 8; 50 mM EDTA; 3% SDS; 1% β-mercaptoethanol) at 65 °C. Then, 50 μL proteinase K (10 μg/mL) was added, followed by incubation at 65 °C for 1 h, cooling on ice and, finally, extraction with phenol–chloroform–isoamyl alcohol (25:24:1). This suspension was centrifuged at 3000 *g* for 5 min. The upper phase, containing DNA, was washed with phenol–chloroform–isoamyl alcohol (25:24:1), transferred to a 1.5-mL centrifuge tube and precipitated by addition of 3 M sodium acetate to a final concentration of 10% (*w/v*) and two volumes of cold ethanol. Then, the tubes were mixed gently by inversion and overnight incubate at − 20 °C. After centrifugation, the pellet was washed with ethanol (70% w/v), dried under vacuum at 37 °C, and suspended in 50 μL of sterile water. Finally, the DNA was incubated with 20 μL of RNAse A (10 μg/mL) at 37 °C for 30 min.

Afterwards, yeast isolates were identified by PCR-RFLP of the ITS1-5.8S rRNA-ITS2 region, using the restriction enzymes *Taq*I, *Sau*3AI, *Dde*I and *Hae*III (Thermo Fisher Scientific, Waltham, MA, USA), as previously described (Gallardo et al., 2014). The resulting fragments were separated on 1.5% agarose gels and PCR fragment sizes were estimated by comparison with the GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific), using GeneTools image analysis software (SynGene, Cambridge, UK). Fig. 1. show an example of PCR-RFLP profiles obtained after digestion with enzymes *Taq*I and *Sau*3 AI. The fragment profiles obtained were grouped into operational taxonomic units (OTUs).

2.3.2. Sequencing analysis of the 26S rRNA region

Five representative isolates of each OTU were identified to the species level, by sequencing the D1/D2 domain of the 26S LSU rRNA, using the primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell, 1993), according to the PCR conditions described by Gallardo et al. (2014). The PCR products were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), 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).

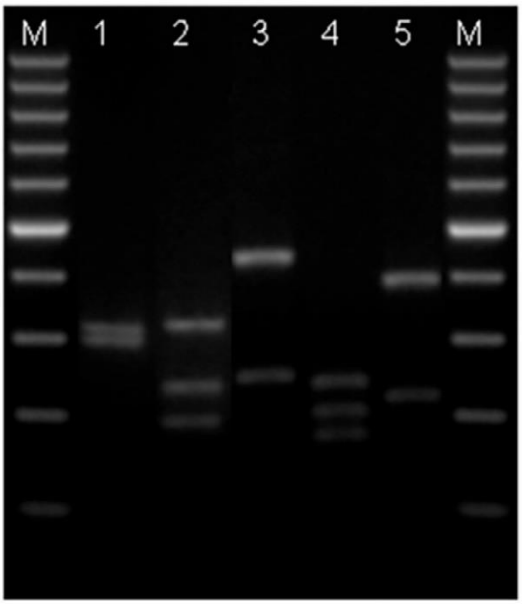

Fig. 1. Restriction profiles of ITS1-5.8S rRNA-ITS2 region with enzyme *Taq*I of *D. hansenii* profile T1 (lane 1) and *K. lactis/marxianus* profile T3 (lane2) and with enzyme *Sau* 3AI of *K. lactis/marxianus* profile S2 (lane 3), *Cryptococcus oeirensis* profile S10 (lane 4) and *D. hansenii* profile S1 (lane 5). Lanes M correspond to the GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific) from 100 bp to 1000 bp.

2.4. Identification of fungal community by HTS of the ITS rRNA gene

Cheese (5 g) was homogenised in 45 mL of 2% trisodium citrate buffer (VWR, Dublin, Ireland). A 5-mL aliquot of the homogenate was centrifuged at 10000 *g* for 5 min and the supernatant discarded. The cell pellet was suspended in lysis buffer and disrupted with 400 − 600 μm silica grinding beads in 1600 MiniG® (SPEX SamplePrep, Metuchen, NJ) at 1500 rpm for 2 min, followed by enzymatic lysis with lysozyme (1 mg/mL), mutanolysin (50 U/mL) and proteinase K (800 μg/mL) at 55 °C for 1 h, as previously described (Quigley et al., 2011). Next, the DNA was extracted using a PowerFood Microbial DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA).

The genomic DNA was amplified for the hypervariable ITS region with specific primers and further reamplified in a limited-cycle PCR reaction, to add a sequencing adapter and dual-indexed barcodes. The initial PCR reactions were performed for each sample using 2X KAPA HiFi HotStart ReadyMix, 0.2 μM of a pool of forward primers: ITS3NGS1_F 5′-CATCGATGAAGAACGCAG-3′, ITS3NGS2_F 5′-CAACGATGAAGAACGCAG-3′, ITS3NGS3_F 5′-CACCGATGAAGAACGCAG-3′, ITS3NGS4_F 5′-CATCGATGAAGAACGTAG-3′, ITS3NGS5_F 5′-CATCGATGAAGAACGTGG-3′, and ITS3NGS10_F 5′-CATCGATGAAGAACGCTG-3′ and reverse primer ITS3NGS001_R 5′-TCCTSCGCTTATTGATATGC-3′ (Tedersoo et al., 2014) and 12.5 ng of template DNA. in a total volume of 25 μL. The PCR conditions involved denaturation at 95 °C for 3 min, followed by 30 cycles at 98 °C for 20 s, 65 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min. Negative controls were included for all amplification reactions. Second PCR reactions added the indices and sequencing adapters to both ends of the amplified target region, by the use of 2X KAPA HiFi HotStart ReadyMix, 5 μL of each index (i7 and i5) (Nextera XT Index kit, Illumina, San Diego, CA) and 5 μL of the first PCR product, in a total volume of 50 μL. The PCR conditions involved a 95 °C denaturation for 3 min, followed by 8 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 5 min. The amplicons were quantified by fluorimetry with

Table 2
Restriction band profile of ITS1-5.8S rRNA-ITS2 with the various restriction enzymes.

Dde I[a]		*Hae* III[a]		*Sau* 3AI[a]		*Taq* I[a]	
Profile	PCR RFLP bands (bp)	Profile	PCR RFLP bands (bp)	Profile	PCR RFLP bands (bp)	Profile	PCR RFLP bands (bp)
D1	462 + 216	H1	421 + 176 + 81	S1	397 + 221	T1	319 + 300
D2	587 + 171	H2	646 + 112	S2	448 + 250	T2	300 + 211 + 108
D3	301 + 199 + 155	H3	628 + 113	S3	390 + 215	T3	329 + 235 + 192
D4	453	H4	453	S4	246 + 107	T4	312 + 294
D5	557	H5	400 + 143	S5	325 + 172	T5	267 + 186
D6	514	H6	390 + 124	S6	350 + 104	T6	251 + 191
D7	288 + 183	H7	366 + 70	S7	301 + 101	T7	209 + 183
D8	521 + 160	H8	422 + 91	S8	272 + 101	T8	188 + 177
D9	405	H9	540 + 86	S9	363 + 125	T9	228 + 144
D10	539 + 65	H10	405	S10	239 + 206 + 175	T10	367 + 255
D11	336 + 130	H11	381 + 144 + 79	S11	144 + 112 + 82 + 67	T11	237 + 168
D12	493	H12	358 + 90 + 72	S12	229 + 163 + 152	T12	303 + 301
		H13	493	S13	280 + 129	T13	208 + 115 + 81
				S14	298 + 119	T14	295 + 198

[a] By columns, letters with a different number indicate different sizes of PCR RFLP bands, or different restriction profiles.

PicoGreen dsDNA Quantitation kit (Invitrogen, Life Technologies, Carlsbad, California, USA), pooled at equimolar concentrations and pair-end sequenced with MiSeq® V3 chemistry, according to the manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

Sequenced reads were demultiplexed automatically by the Illumina® MiSeq® sequencer, using the CASAVA package (Illumina, San Diego, CA, USA) and quality-filtered with PRINSEQ software (Schmieder and Edwards, 2011), using the following parameters: 1) bases with average quality lower than Q25 in a window of 5 bases were trimmed, and 2) reads with < 150 bases were discarded. The forward and reverse reads were merged by overlapping paired-end reads, using the AdapterRemoval v2.1.5 software (Schubert et al., 2016) with default parameters. The QIIME package v1.8.0 (Caporaso et al., 2010) was used for OTU generation, taxonomic identification, sample diversity and richness indices calculation. Sample identifications were assigned to the merged reads and converted to FASTA format (split_libraries_fastq.py, QIIME). Chimeric merged reads were detected and removed, using UCHIME (Edgar et al., 2011) against UNITE/QIIME ITS v12.11 database (Abarenkov et al., 2010) (script identify_chimeric_seqs.py, QIIME). The OTUs were selected at 97% similarity threshold, using the open reference strategy. First, merged reads were pre-filtered by removing sequences with a similarity lower than 60%, against the UNITE/QIIME ITS v12.11 database (Abarenkov et al., 2010). The remaining merged reads were then clustered at 97% similarity against the same databases listed above. Merged reads that did not cluster in the previous step, were *de novo* clustered into OTUs at 97% similarity. A representative sequence of each OTU was then selected for taxonomy assignment (pick_rep_set.py, assign_taxonomy.py; QIIME).

2.5. Statistical analyses

SPSS for Windows 21.0 (SPSS Inc. Chicago, IL, USA) was used. The physicochemical data were analysed by a one-way analysis of variance (ANOVA), and a three-way ANOVA was used for the microbiological data. The means were separated by Tukey's honestly significant difference test.

3. Results and discussion

3.1. Physicochemical changes and yeast counts

The cheese samples had a pH range between 4.95 – 5.49. These pH data concur with those reported for other soft body cheeses (Alvarenga et al., 2008; Ordiales et al., 2013a). At 30 days, the moisture content of the cheeses ranged from 45.02 – 48.76%, while the a_w values were around 0.90 – 0.98. Among the samples, those from industry V, non-PDO registered, showed the highest pH value and a significantly lower a_w and moisture ($P < 0.05$) (Table 1). Overall, these results agree with the findings presented by Roseiro et al. (2003) in Serpa Portuguese cheese made using a semi-industrial process.

The mean RBC yeast counts ranged between 4.24 – 5.66 log CFU/g in the winter, with non-PDO producers having the lowest mean values. In spring, the counts for producers A and C were significantly lower than in the winter season, whereas samples from producer G showed a contrary tendency (Table 1). The observed counts in the core of the cheese at the end of the process (30 days) were slightly higher than that reported by other authors in similar types of cheeses at around 30 days of ripening (Ordiales et al., 2013a; Tavaria and Malcata, 1998). Moreover, the differences in the yeast counts in the final cheese between producers and seasons are expected, due to several factors, such as the initial population of the raw materials, the hygiene practices during the cheese-making process and the industry environment, which have a strong influence on the final cheese fungal populations (Bokulich and Mills, 2013; Ordiales et al., 2013a, 2013b; Quigley et al., 2013).

3.2. Culture-dependent identifications by PCR-RFLP analysis of ITS region

The isolates from the RBC plates were further investigated for their identification at the species level (Table 2). The 471 yeast isolates from the core cheese samples were grouped by PCR-RFLP analysis of the ITS1-5.8S rRNA-ITS2 and identified by subsequent sequencing of the 26S LSU rRNA D1/D2 domains. This provided a guide to the prevalence of live yeast in the final cheeses from different industries and seasons.

Based on the molecular identification of the ITS region by RFLP, the endonucleases *Dde*I, *Hae*III, *Sau*3AI and *Taq*I showed different results in the restriction analyses (Table 2). Twelve different restriction profiles were obtained with *Dde*I (D1 – D12), while thirteen were observed with *Hae*III (H1 – H13) and fourteen with *Sau*3AI (S1 – S14) and *Taq*I (T1 – T14). The high numbers of restriction profiles obtained and the different sizes of the PCR products from the ITS region, means a wide diversity of yeast species exist in Serpa cheese (Tables 2 and 3). The combination of restriction analyses using these four enzymes, allowed separation of 471 isolates into seventeen different OTUs. The sequencing of the 26S LSU rRNA and BLAST sequence comparison of representatives isolates from each OTU obtained by PCR-RFLP, allowed their identification at the species level (Table 3). The PCR-RFLP profiles P1, P2, P3 and P4, contained the majority of the isolates, approximately 71.1%, which belonged to *Debaryomyces hansenii* (OTUs 1 and 2), *Kluyveromyces lactis* and *Kluyveromyces marxianus*, respectively. The remaining 28.9% of the total isolates comprised twelve different species

Table 3
Identification of the yeast isolated from Serpa cheese samples by a culture-dependent method.

			Restriction analysis of ITS1-5.8S rRNA-ITS2				Identification		
PCR-RFLP profiles (OTUs)	% of isolates	PCR ITS size (bp)	*DdeI*[a]	*HaeIII*[a]	*Sau*3AI[a]	*TaqI*[a]	26S rRNA sequencing	Accession numbers	% identification
P1	30.5	678	D1	H1	S1	T1	*Debaryomyces hansenii*	KY107562.1	100%
P2	5.3	678	D1	H1	S1	T2	*Debaryomyces hansenii*	KY107562.1	100%
P3	18.7	756	D2	H2	S2	T3	*Kluyveromyces lactis*	KY108048.1	100%
P4	16.6	756	D2	H3	S2	T3	*Kluyveromyces marxianus*	KY108098.1	100%
P5	9.1	665	D3	H1	S3	T4	*Candida zeylanoides*	KY106915.1	100%
P6	1.9	453	D4	H4	S4	T5	*Candida pararugosa*	HE660073.1	100%
P7	1.9	557	D5	H5	S5	T6	*Candida parapsilosis*	KY106676.1	100%
P8	1.3	514	D6	H6	S6	T7	*Candida cabralensis*	NG_055163.1	99%
P9	4.4	470	D7	H7	S7	T8	*Pichia fermentans*	KY108815.1	100%
P10	1.3	470	D7	H7	S8	T8	*Pichia fermentans*	KY108815.1	100%
P11	1.3	551	D5	H8	S9	T9	*Pichia kudriavzevii*	KY108855.1	100%
P12	3.2	681	D8	H9	S10	T10	*Cryptococcus oeirensis*	AF181519.1	100%
P14	1.9	405	D9	H10	S11	T11	*Yarrowia lipolytica*	KY110199.1	100%
P15	0.6	604	D10	H11	S12	T12	*Cyberlindnera jadinii*	KC844835.1	100%
P16	1.3	520	D11	H12	S13	T13	*Moniliella suaveolens*	LC004102.1	100%
P17	0.6	493	D12	H13	S14	T14	*Magnusiomyces capitatus*	NG_055400.1	100%

[a] By columns, letters with a different number indicate different sizes of PCR bands, or different restriction profiles.

from seven different genera. Of these, *Candida zeylanoides* and *Pichia fermentans* represented around 9.1 and 5.7% of the total, whereas the remaining species were minor contributors (Table 3). Overall, the majority of the species found are very common in different cheese variety (Álvarez-Martín et al., 2007; Atanassova et al., 2016; Banjara et al., 2015; Gardini et al., 2006; Padilla et al., 2014; Pereira-Dias et al., 2000; Tofalo et al., 2014).

For *D. hansenii*, two different profiles for the restriction enzyme *Taq*I were obtained, generating two OTUs (P1 and P2). Gallardo et al. (2014) also acquired different profiles using the *Taq*I enzyme, for isolates from *D. hansenii*. Likewise, two different OTUs were ascribed to *P. fermentans* species (P9 and P10) due to different restriction profiles obtained by *Sau*3AI (Table 3). Thus, the restriction analysis of the ITS-5.8S rRNA, combining the enzymes *Taq*I and *Sau*3AI, allows an accurate and simple way to identify common yeast species from cheese.

Regarding the distribution of yeast species according to industry and season, Table 4 shows the percentage of isolates from each species detected. The microbiota of yeast in cheese samples from PDO registered industries, were dominated by *D. hansenii* and *Kluyveromyces* spp., with different percentages depending on the season. In industry A, *D. hansenii* was the only species present in spring samples, while, in winter, although *D. hansenii* was dominant, other yeast species commonly associated with cheese including *Cryptococcus oeirensis*, *P. fermentans*, *Pichia kudriavzevii* and *Candida pararugosa*, were also found at noticeable levels. The appearance of the ubiquitous species *Magnusiomyces capitatus* (teleomorph form of *Saprochaete capitata*), previously named *Geotrichum capitatum*) in samples from industry A, is concerning, given this organism has been associated with human pathology in immunocompromised patients (Brunetti et al., 2016). However, in the remaining two PDO registered industries, the yeast community was composed mainly of species from the genera *Kluyveromyces*. In particular, *K. marxianus* was mainly found in winter samples and *K. lactis* in spring. Moreover, *P. fermentans* in cheese from industry G in winter and *C. zeylanoides* in cheese produced by industry C in spring, were also present at important levels, with minor percentages of the other isolated yeast species.

On the contrary, the yeast population in non-PDO industries (V and B) was different to PDO industries. In industry V, *C. zeylanoides* was the major species, with *Candida parapsilosis* and *Moniliella suaveolens* present to a lesser extent, whereas in industry B, although around 60% of the isolates belonged to *D. hansenii* and *K. marxianus*, remarkable levels of three other species were also present. Two species, *Candida cabralensis* and *M. suaveolens*, were only associated with non-PDO industries. The presence of these species in cheese have been reported sporadically, with *C. cabralensis* found in traditional Cabrales cheese (Flórez et al., 2010), and *M. suaveolens* in artisanal Fiore Sardo cheese (Fadda

Table 4
The percentages of each yeast species identified in Serpa cheese samples according to the producer and season.

Season		Winter					Spring		
Industries		PDO			Non PDO		PDO		
		A	C	G	V	B	A	C	G
Identified species	*Debaryomyces hansenii*	25.0				58.4	100.0		6.0
	Kluyveromyces marxianus		71.5	32.0		8.3		28.5	
	Kluyveromyces lactis	5.0	24.0	26.0				28.5	88.0
	Pichia fermentans	15.0		33.0					
	Pichia kudriavzevii	15.0							
	Candida pararugosa	15.0							
	Candida cabralensis					16.7			
	Candida zeylanoides				63.0			35.0	6.0
	Candida parapsilosis			9.0	25.0				
	Yarrowia lipolytica					8.3		8.0	
	Cryptococcus oeirensis	20.0							
	Magnusiomyces capitatus	5.0							
	Moniliella suaveolens				12.0	8.3			
	Cyberlindnera jadinii		4.5						

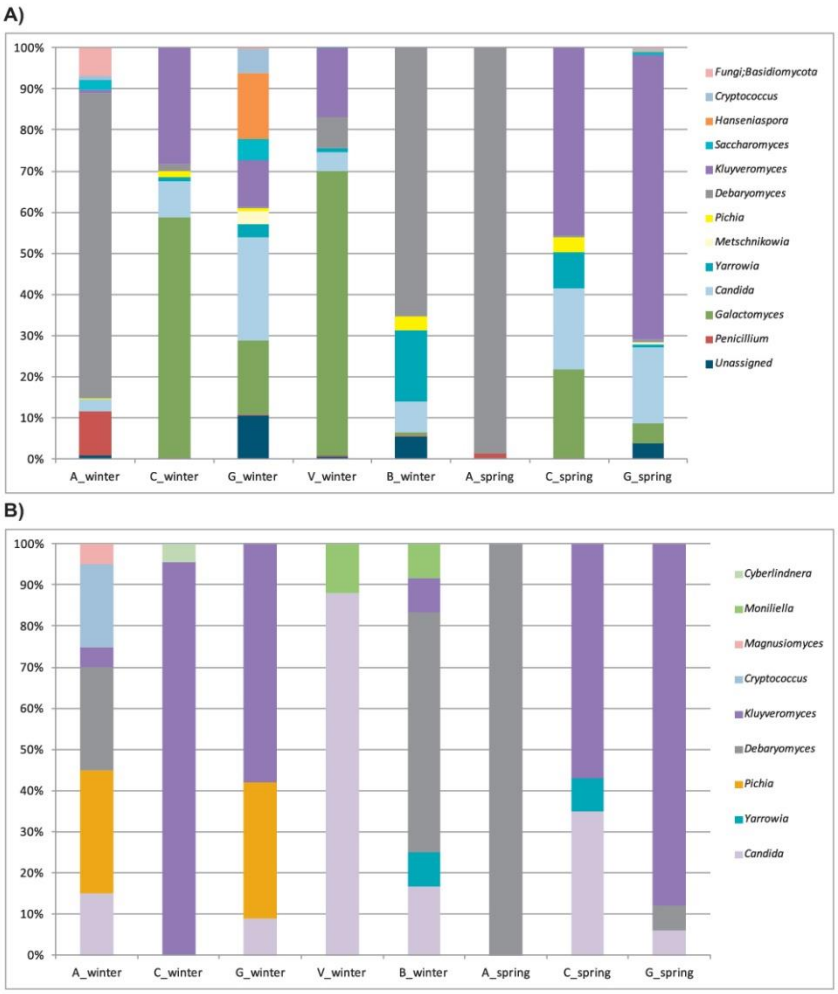

Fig. 2. Mean relative abundance of yeast in cheeses from different industries (capital letters) analysed in two different seasons (winter and spring) using high-throughput sequencing of the ITS rRNA gene at the genus level (A), and culture dependent methods (B).

et al., 2004) and Mexican artisanal Cotija cheese (Chombo-Morales et al., 2016). Furthermore, although yeasts are rarely associated with foodborne infections, *C. parapsilosis* is among the most common yeast pathogens and responsible for various mycoses (Jacques and Casaregola, 2008). This species was isolated in winter, at a low level in cheese obtained from industry G (PDO registered) but it was found at a higher level in cheese produced by industry V (non-PDO registered). However, *C. parapsilosis* has been detected in various types of cheese and infection for the arising from the consumption of food contaminated with this species, has not yet been documented (Banjara et al., 2015; Padilla et al., 2014; Pereira-Dias et al., 2000).

3.3. Culture-independent identification by HTS of the ITS rRNA gene

The HTS identifications at the genera level, of the yeast community in the cheeses obtained from the various industries and different seasons, are presented in Fig. 2A. In agreement with the culture-dependent tools, a wide diversity of yeasts was found. The sequenced isolates were clustered into 11 main OTUs. Low percentages of sequences were associated with the phyla Basidiomycota and this was mainly found in cheese from industry A in winter. Most of the sequences were assigned to phyla Ascomycota and it is remarkable that between 4 and 10% of the sequences (industry G in both seasons and industry B) were not matched to any yeast genera of the UNITE/QIIME database. These species may be entrenched in the environment of these industries and it could be of interest to perform isolates from these buildings to obtain information about these unknown yeasts.

Regarding the yeast OTUs identified, most of the yeast species detected by the culture-dependent approach belonged to the genera found by HTS (Table 4; Fig. 2). Among the new OTUs, *Galactomyces* spp. (= *Geotrichum* spp.) was found at an important level in cheeses from industries C, G and V in winter and from industry C in spring. Among the species belonging to the genera *Galactomyces*, *G. candidus* (anamorph *Geotrichum candidum*) is a ubiquitous yeast species commonly found in cheese and with important technological application in the cheese-making process (Boutrou and Guéguen, 2005; Ceugniez et al., 2017). However, the salt sensitivity of this species is well known and, in general, its growth is limited at levels above 1% (Wyder, 1998). HTS of target genes from genomic DNA, cover live and dead microorganisms. Hence, *G. candidus* was probably dominant at the beginning but was out-competed by other yeast species, such as those detected by the culture-dependent method, due to the decrease in moisture and consequent increase in the salt concentration with the progression of cheese ripening. In addition, another three genera detected by HTS, *Metschnikowia*, *Saccharomyces* and *Hanseniaspora*, were also not found among the dominant isolates.

Among the industries, although slight differences in the abundance of each genus, except *Galactomyces*, were found, overall, a high correlation between both approaches used in this work to study the Serpa cheese yeast community, was obtained (Table 4 and Fig. 2). Regarding the PDO registered industries, *Debaryomyces* spp. was dominant in cheese from industry A and *Kluyveromyces* spp. from industry C, in both seasons. However, in cheese from industry G, although *Kluyveromyces* spp. was also the most important yeast genera in spring, in winter, although HTS showed an important level of this organism, its abundance was lower than *Candida* spp. that was isolated from culture media at a lower level. A similar tendency was observed for the non-PDO industries, where *Debaryomyces* spp. was dominant in cheese from industry B in agreement with the culture-dependent method, however, in industry V, *Candida* spp. was not the major yeast and it was out-competed by other yeast genera.

The results of this study demonstrate that the yeast community of Serpa cheese is composed of a wide diversity of species, similar to the results reported in the literature in other artisanal cheese (Atanassova et al., 2016; Padilla et al., 2014; Pereira-Dias et al., 2000; Tofalo et al., 2014). The most common species detected, belonged to *Debaryomyces* and *Kluyveromyces* genera and *Galactomyces* may also be important during the initial stage of ripening. These species are among the most frequent yeasts found in cheese. In addition, they are recognised as safe and contribute positively to the ripening and sensorial characteristics (Fleet, 2011). Interestingly, in general, the same yeast genera were prevalent in PDO registered industries in both seasons, which, despite the expected differences in the yeast community of the raw materials, indicates that the cheese-making environment may be an important source of yeast with relevant functions during the ripening period. These results confirmed the evidence reported by other authors, who studied the influence of the industry environment (Bokulich and Mills, 2013; Gori et al., 2013; Stellato et al., 2015).

4. Conclusion

The data reported in this study showed the complex community of yeasts in artisanal Serpa cheese. The differences between the identified yeast isolated from cheeses obtained from PDO and non-PDO registered industries, reveal that the lack of regulation of the cheese-making practice may unfavourably influence the final yeast microbiota. The combination of culture-dependent and -independent techniques, demonstrated that the prevalent yeast belonged to *Debaryomyces* and *Kluyveromyces* genera. In addition, the high levels of *Galactomyces* detected by HTS, which may be not viable at the end of the process, indicate its importance during the early stages of cheese ripening. The new knowledge of the yeast diversity in Serpa cheese, could be used to modify the cheese ripening conditions, to favour desirable yeast species. Additionally, the prevalent yeast isolates identified, *D. hansenii* and *Kluyveromyces* spp., may have an important role during cheese ripening and in the final sensorial characteristics. Thus, the study of their technological and functional properties could be relevant to develop an autochthonous starter culture, to ensure the final quality and safety of the cheese.

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IV.3. Suitability of autochthonous lactic acid bacteria strains and prebiotics for the development of a symbiotic artisanal soft cheese

Suitability of autochthonous lactic acid bacteria strains and prebiotics for the development of a symbiotic artisanal soft cheese

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Abstract

Today there is a growing interest in functional foods for their positive impact in human health. Here, we evaluated the probiotic properties of autochthonous lactic acid bacteria from artisanal Serpa cheese considering their tolerance to gastrointestinal transit, capacity to colonise the intestine, safety properties and prebiotic metabolism. Twenty strains presented better tolerance to stress conditions found in the gastrointestinal tract, highlighting *Lactobacillus. brevis* C1Lb21 for its adequate autoaggregating ability and significant higher hydrophobicity. Unfortunately, eight strains were discarded for their safety characteristics. The fermentation of prebiotics showed that lactulose supported better growth of lactobacilli and induced the production of short-chain fatty acids (SCFA). During lactulose fermentation, *Lb. pentosus* G4Lb7 produced statistically higher amounts of SCFA and *Lb. plantarum* G1Lb5 lactic acid. Thus, considering the probiotic characteristics studied *Lb. brevis* C1Lb21, *Lb. plantarum* G1Lb5 and *Lb. pentosus* G4Lb7 are promising probiotic candidates in combination with lactulose for developing a symbiotic Serpa cheese.

Keywords: lactic acid bacteria, probiotic, prebiotic, traditional cheese, functional food.

1. Introduction

Nowadays, there is a growing interest in nutrition interventions to improve health, and food is not only intended to provide the necessary nutrients, but also to have a beneficial effect on consumers' health (Linares et al., 2017). This has led to an increased interest and demand for functional food in the markets mainly in developed countries such as the USA, Europe and Japan. Among them, probiotics have been preferred by consumers due to the numerous studies that have demonstrated their beneficial effects (Ouwehand, 2015). Probiotics are defined by an expert panel of The International Scientific Association for Probiotics and Prebiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Lactic Acid Bacteria (LAB) are the most common microorganisms applied as probiotics because they are a desirable member of the gastrointestinal tract (GIT) microbiota and have the status of GRAS "Generally Recognised As Safe" (Linares et al., 2017). Although several LAB species have shown probiotic characteristics, these are not generally linked with a particular one, and it is well known that they are strain-dependent (Vasiljevic & Shah, 2008).

It is widely accepted that probiotics for human application should be of human or food origin. Microorganisms, mainly LAB, have been used in fermentation processes to ensure food safety and to improve sensory attributes since the development of human civilisations (Hutkins, 2008). Fermented food may contain important levels of viable microbes that can mostly survive passage through the human GIT where they may exert influence on human health (Derrien & van Hylckama Vlieg, 2015). The food matrix of some fermented products is an excellent environment for the survival of microorganisms, acting as a vehicle for beneficial microbes through GIT transit. As a consequence, fermented artisanal foods are gaining popularity and have even been proposed as part of dietary recommendations (Ebner, Kneifel, Salminen, & Sanders, 2014). Among them, fermented dairy products have been by far the most investigated as a source of probiotics and how to carry them efficiently through GIT (Giraffa, 2012). In particular, the most traditional products are potential sources of novel probiotics.

Serpa is a Protected Designation of Origin (PDO; Council Regulation EEC 2081/92) soft-cheese produced within the Alentejo province, located in the south of Portugal. It is made from raw ewe's milk using vegetable coagulant obtained from the infusion of dried flowers from *Cynara cardunculus* L. and without the addition of starter cultures. Its quality and specific characteristic arises mainly from the endogenous microbiota coming from the raw materials employed, the cheese dairy environment and the traditional

technology used in the cheese-making process. In our previous research, the microbial community of this artisanal cheese have been described by culture-dependent and -independent approaches (Gonçalves et al., 2017, 2018). LAB were clearly the main microbial group with viable counts around 10^8 cfu/g at the end of the ripening process. LAB are involved in the ripening process of the cheese and contribute to the final organoleptic properties (O'Sullivan & Cotter, 2017). Additionally, the LAB strains present in Serpa cheese may possess probiotic potential.

The primary criteria for selecting strains as suitable probiotics are survival to restrictive conditions of the GIT, ability to adhere to the intestinal mucosa and colonise the colon, at least temporarily, and capacity to exert potential health benefits on the host. Moreover, although LAB have the status of GRAS, for probiotic application their safety should be evaluated and verified (FAO/WHO, 2014). It should be highlighted that the probiotics also have to be adapted to the fermentation process and storage to be ingested at levels above 10^6-10^7 cfu/g in order to positively influence consumer health (Tripathi & Giri, 2014). Cheese is an excellent food matrix to transport probiotic due to its specific physical and chemical characteristics such as pH between 5-6, high fat content and nutrient availability and low oxygen content. These conditions favour their survival through gastrointestinal transit (Karimi, Mortazavian, & Da Cruz, 2011) and hence, the application of probiotics has been optimised in different types of cheeses (Minervini et al., 2012; Albenzio et al., 2013). Therefore, the aim of this study was to evaluate probiotic properties, widely used in their screening and selection, of autochthonous LAB isolated at the end of the ripening process of artisanal Serpa cheese, for potential use during its manufacture process.

2. Material and methods

2.1 Bacterial isolates

A total of 116 LAB belonging to the *Lactobacillus, Lactococcus, Leuconostoc* and *Enterococus* genus isolated from Serpa cheese (Gonçalves et al., 2017) were selected in based to species identification and industry origin to study their probiotic characteristics (Table 1). LAB were routinely grown in Mann Rosa Sharpe (MRS; Scharlab, Barcelona, Spain) broth at 37 ºC under 10% CO_2 for 24 h. All strains were subcultured twice prior to the experiments, harvested at 21,500 *g* 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 10^8 cfu/mL. For all strains, three biological replicates of the cultures were performed and the assays for each strain were conducted in duplicate.

2.2 Probiotic assays *in-vitro*

2.2.1 Tolerance to low pH

The resistance to acid pH of each isolate was measured in PBS supplemented with 3.5 g/L (w/v) of pepsin and the pH adjusted to 2.5, 2.75, 3 or 5 following the method described by Ruiz-Moyano, Martín, Benito, Nevado, & Córdoba (2008). PBS at pH 5 was used as control. Strains with viable counts lower than 2 log cfu/mL respective to the control were considered not resistant.

2.2.2 Bile salt growth

The capacity of each strain to grow in the presence of bile salt (Sigma Chemical Co. St Louis, MO, USA) was evaluated by inoculating 5 µL of cell suspension to MRS agar supplemented with bile salt concentrations of 0%, 0.3%, 0.6%, 1% and 2% (w/v). The inoculated plate was incubated at 37 ºC under 10% CO_2 for 72 h and the presence of growth was observed every 24 h.

2.2.3 Transit tolerance to simulated gastric juice

The strains resistant to low pH and bile salts were used to determine the survival on complete gastrointestinal transit by the method of Bao et al. (2010). Briefly, strains were inoculated in simulated gastric juice (3.5 g/L of pepsin and 0.2% NaCl) at pH 2.75 for 2 h, followed by 6 h in simulated intestinal juice (1 g/L of trypsin, 5 g/L bile salt, 2 g/L of pancreatin; (Sigma), 11 g/L of sodium bicarbonate and 2 g/L of NaCl) at pH8. The plates were incubated at 37 ºC under 10% CO_2 and the viable bacteria were counted on MRS media after 0, 2, 4 and 6 h. Strains with viable counts lower than 2 log cfu/mL respective to the control were considered not resistant.

2.2.4 Aggregation activity

The specific cell–cell interactions were determined using the autoaggregation assay described by Xu, Jeong, Lee and Ahn (2009) with modifications. The LAB suspension (2 mL) was vortexed for 10 s and incubated at 30 °C for 2 h. Absorbance was measured immediately and incubated at 30 °C for 2 h. The absorbance of

the supernatant was monitored using Shimadzu UV 1800 spectrophotometer at 600 nm at different time intervals (0h, 1 h and 2 h). The autoaggregation was calculated using the following equation:

$$\text{Autoaggregation (\%)} = (1 - A_t / A_0) \times 100$$

where A_t represents absorbance at a determined interval,1 h or 2 h and A_0 represents absorbance at the beginning of the assay (0 h).

2.2.5 Cell surface hydrophobicity

This assay was carried out by the method described by Lee and Puong (2002) with slight modifications. LAB strain suspensions were adjusted in PBS to obtain an optical density (OD) at 600 nm of 1±0.05. Five millilitres of cell suspension in PBS were mixed with 1 mL of 99% n-hexadecane (Sigma) by vortexing at high speed for 1 min. Then, the test tubes were left undisturbed for 1 h at 37 °C to allow the phase separation. The lower aqueous phase was carefully removed with a sterile Pasteur pipette and final absorbance (A1) was recorded at 600 nm using a Shimadzu UV 1800 spectrophotometer. The decreased absorbance in the aqueous phase was taken as a measure of cell surface hydrophobicity (H%), calculated using the following equation:

$$\text{Hydrophobicity (\%)} = [(A0 - A1)/ A0] \times 100$$

where A0 and A1 were absorbance values before and after extraction with n-hexadecane, respectively.

2.3 Safety assays

2.3.1 Antibiotic susceptibility

The selected acid-tolerant strains were tested for antibiotic susceptibility by the disc diffusion method according to guidelines of National Committee for Clinical Laboratory Standards (NCCLS, 2012). A range of twelve clinically important antibiotics (Oxoid, Basingstoke, England) were used: ampicillin (10 μg), gentamicin (10 μg), kanamycin (30 μg), trimethoprim (5 μg), erythromycin (15 μg), tetracycline (30 μg), clindamycin (2 μg), chloramphenicol (30 μg), penicillin G (10 units), polymixin B (300 units), nalidixic acid (30 μg) and vancomycin (30 μg). One hundred microlitres of overnight culture (OD = 0.5) was spread onto MRS agar (Scharlab). The antibiotic discs were applied to the culture plates using an antibiotic disc

dispenser (Oxoid) and incubated at 37 ºC under 10% CO_2 for 24 h. The diameters of the inhibition zones were measured and the results interpreted according to the criteria proposed by Charteris, Kelly, Morelli, and Collins (1998).

2.3.2 Biogenic amine production

The capacity to produce biogenic amines (BA) of selected strains was determined by the improved medium described by Bover-Cid and Holzapfel (1999). Previously, decarboxylase activity was induced by subcultivating the strains five times in MRS broth containing 0.1% of each precursor amino acid (tyrosine, histidine, lysine, tyrosine, arginine, glutamine tryptophan, phenylalanine and ornithine; Sigma) and 0.005% of pyridoxal-5-phosphate. Five microlitres of each strain was inoculated in triplicate on the decarboxylase medium plates with and without amino acids (as a control) before incubating for 4 days at 37 °C under 10% CO_2.

The amount and type of BA produced (tyramine, tryptamine, histamine, putrescine, phenylethylamine, spermine, spermidine and cadaverine; Sigma) was also measured by HLPC-ESI-MS - according to the method described by Fernandez et al. (2016) on an Agilent series 1100 apparatus (Agilent Technologies, Palo Alto, CA, USA). LAB, after induction of decarboxylase activity, were grown in the improved medium described by Bover-Cid and Holzapfel (1999) without agar and containing 0.25% of each precursor amino acid for 4 days at 37 ºC under 10% CO_2. Two millilitres of improved broth medium was centrifuged at 8000 *g* for 5 min and filtered through 0.22µm filters (Thermo Fisher Scientific, Waltham, MA, USA). BA in samples was distinguished using an Agilent series 6100 Series Single Quad LC/MS (Agilent Technologies) equipped with a multimode source in electrospray ionisation mode by their mass spectrum and retention time.

2.4 Growth on prebiotic

The LAB selected on the basis of their probiotic and safety properties were tested for growth in the presence of three commercial prebiotics; lactulose (Sigma), short-chain fructo-oligosaccharide (FOS, Orafti® P95 with a degree of polymerisation (DP) 2–8, Beneo-Orafti, Belgium) and long-chain inulin (Orafti® GR with DP 2-60 and average ≥10, Beneo-Orafti). Two µL of each LAB strain suspension was inoculated in 200 µL of semi-solid MRS medium containing 0.125% agar (w/v), devoid of glucose, and supplemented with

2% (w/v) of each sterile-filtered prebiotic as the sole carbohydrate source. The positive control for growth consisted of semi-solid MRS supplemented with 2% (w/v) of glucose or lactose, whereas the negative control was a carbohydrate-free semi-solid MRS. The automated turbidimeter BioscreeC Microbiology reader set up at 37 ºC was used to monitor the growth for 96 h by reading the OD at 600 nm at regular intervals without shaking. The ability of each strain to grow in the presence of different prebiotics was evaluated by comparing the OD600nm of each carbohydrate with the value obtained on glucose.

2.5 Short-chain fatty acid production

To determine the capacity to produce short-chain fatty acid (SCFA), selected LAB strains were grown on modified MRS (mMRS) broth at 37 ºC under 10% CO_2 until stationary phase. The mMRS was formulated as commercial MRS devoid of glucose and sodium acetate and supplemented with 2% (w/v) of the carbohydrate source (glucose, lactose, lactulose, FOS or inulin). The supernatants of the cultures were obtained by centrifugation of the media at 8000 *g* for 5 min before filtering through 0.22 µm filters (Thermo Fisher Scientific). The concentration of lactic acid in the supernatant, D-lactic and L-lactic acid, was quantified by the enzymatic method K-DLATE (Megazyme, Bray, Ireland) according to the manufacturer's instructions. To measure the amount of the remaining SCFA, 800 µL of supernatant was mixed with 100 µL of internal standard solution (2-ethyl butyric acid (Sigma) at 5 mM prepared in 12% of formic acid) and 100 µL of meta-phosphoric acid solution (16% w/v). SFCA were extracted with 500 µL of diethyl ether by vortexing for 1 min and centrifugation at 17000 *g* for 5 min at 4 ºC. One microlitre of the upper phase was injected into a gas chromatograph (model 4890 Series II; Hewlett-Packard, Palo Alto, CA, USA) equipped with a split/splitless injector and a flame ionisation detector. SCFA were separated on a Carbowax™ fused silica capillary column (30 m × 0.25 mm id; 0.25 µm film thickness; Ohio Valley). The initial oven temperature was held at 80 °C for 2 min and was increased at a rate of 20 °C/min to 200 °C and retained for 12 min. Injector and detector temperatures were 250 °C. The carrier gas was nitrogen at 1.8 mL/min. The individual SCFA were identified by comparison of their retention times with those of reference standard mixtures (Sigma Chemical Co., St Louis, MO, USA). SCFA concentrations were calculated by using the peak area ratio of the analyte to the internal standard (2-ethyl butyric acid), based on the methodology described by Brighenti (1997).

2.6 Statistical analysis

Autoaggregating and hydrophobicity data were analysed by a one-way analysis of variance (ANOVA) and SCFA production by two-way ANOVA using the program SPSS for Windows 21.0 (SPSS Inc Chicago, IL, USA). The means were separated by Tukey's honestly significant difference (HSD) test ($p \leq 0.05$).

3. Results and discussion

3.1 Tolerance to the gastrointestinal tract conditions

Microorganisms to be considered as candidates for probiotic use must be first adapted to the restrictive conditions of the GIT and subsequently colonise the intestinal tract. The low pH of the stomach can vary from 1.5 during fasting, to values around 4 after a meal: acting as an effective barrier against the entry of microorganisms (Simonian, Vo, Doma, Fisher, & Parkman, 2005). In order to evaluate the acid tolerance of the LAB *in-vitro* they were exposed to a pH between 2.5 and 3 during 2 h. These conditions are commonly used in the literature to study the survival of bacteria to stomach environment and have demonstrated to be an adequate criterion to select acid-tolerant strains intended for probiotic application (Han, Kong, Chen, Sun, & Zhang, 2017; Ruiz-Moyano et al., 2008). In the current study, all LAB isolates tolerate pH 3, however, when the pH dropped to 2.5, acid stress was critical, and none survived at the levels required. At an intermediate pH, 2.75, 20 of the 116 strains exhibited satisfactory levels of viability after 2 h of exposure (Table 2). Several authors have reported that viability of LAB is dramatically affected at low pH, especially below pH 2.5 (Reale et al., 2015; Zoumpopoulou et al., 2018). The acid tolerance of LAB is strain specific and mediated via a number of mechanisms, in particular, F_0F_1-ATPase is an important element in the response and tolerance to low pH in *Lactobacillus* spp. (Corcoran, Stanton, Fitzgerald, & Ross, 2005). In addition to the intrinsic characteristics of strain, the survival of a potential probiotic to the acid environment of the stomach is also strongly influenced by the composition of the food that it contains. In particular, foods with high levels of fat, high nutrient availability and determined physical conditions, like the ones given in cheese, provide additional protection of the microorganism against the restrictive conditions of the GIT transit (Karimi et al., 2011). In this study, a pH value of 2.75 was found to be highly discriminating and although the pH of the stomach during a meal is normally above this value, in order to guarantee the viability of the potential probiotic strains, the most acid-tolerant LAB were selected to study their behaviour under the conditions of the small intestine.

After passage through the stomach, the potential probiotics are exposed to the toxicity of bile salts. The amount of bile salts varies with the digestion time and the physiological concentration is usually between 0.15-0.5% (Papadimitriou et al, 2015). The selected strains were evaluated to grow in the presence of bile salts in the range of 0.3-2% for 72 h. They were able to grow well in the presence of bile salt concentrations up to 2 % after 24 h, except the two *Lb. casei/paracasei* strains, V1Lb8 and A2Lb1, which showed a clear sensitivity to bile salts at a concentration above 1% (Table 2). Concentrations around 1.5% may occur at the beginning of digestion (Davenport, 1977). Bile salts have antimicrobial potential and decrease the viability of the microorganisms by their effect on the cell membrane and DNA (Merritt and Donaldson, 2009). These results are consistent with those previously recovered from other probiotic *in-vitro* testing where most of the strains were more resistant to bile salts than low pH (Han et al., 2017; Reale et al., 2015; Ruiz-Moyano et al., 2008; Zoumpopoulou et al., 2018).

Previous works display different strategies to assess the *in-vitro* capacity of a potential probiotic to reach the distal part of the intestinal tract where they positively influence the host's health. In general, the main consideration is tolerance to low pH and bile salts, individually or as combined stress factors, but the digestive enzymes are not always included in the test. In this work, to know the capacity of the acid and bile salt-tolerant strains to overcome the barriers in the GIT, they were subjected to the multiple stresses at once. Most of the selected strains showed good survival to complete GIT transit (Table 2). However, two of the twenty strains, *Lb. casei/paracasei* strains V1Lb8 and A2Lb1, decreased their viability dramatically when they were exposed to simulated gastric juice for 90 min follow by 120 min in simulated intestinal juice. This emphasises the effect of bile salt on bacterial viability after the exposition to artificial gastric juice. With respect to the remaining strains, the majority maintain viability during the whole assay, although *Lb. brevis* V1Lb10 and *Lb. plantarum* B1Lb2 presented a moderate reduction afterwards of 6 h under simulated intestinal juice. These data agree with those previously obtained after single stress exposure and confirm that gastric juice is the main hurdle in the GIT transit.

3.2 Capacity to colonise the intestine

Adhesion to intestinal tissue is one of the main features for a probiotic candidate: since it is involved in host colonisation and allows the probiotic to exert its beneficial effects (Papadimitriou et al., 2015). Cell surface characteristics, autoaggregation and hydrophobicity have been associated with epithelial adhesion (Collado, Meriluoto, & Salminen, 2008) and they are considered a reliable *in-vitro* system to initially assess the potential adhesion ability of GIT transit tolerant LAB.

The percentage of autoaggregation after 1 and 2 h of incubation are presented in Figure 1. In general, the values varied between 1.05 and 8.13 % at 1 h and 2.7-17.14 % at 2 h. All strains increased the autoaggregation percentages with the incubation time that is congruent with other authors (García-Cayuela et al., 2014; Han et al., 2017). Among strains, significant differences were observed ($p \leq 0.05$). At 2 h seven of the twenty strains, *Lb. brevis* C1Lb21 and B2Lb5, *Lb. crustorum* A3Lb18, *E. faecium* V2Lb3, A1Lb9 and G1Et4 and *E. durans* G1Et3, exhibited better autoaggregation with percentages higher than 12%, corresponding to a noteworthy capacity based upon previous studies in LAB (García-Cayuela et al., 2014; Han et al., 2017). Besides, García-Cayuela et al. (2014) reported that autoaggregation ability of lactobacilli correlated highly with co-aggregation with pathogens. So, the LAB strains with higher autoaggregation capacity may also contribute to prevent the colonisation of the human gut by foodborne pathogens.

With respect to hydrophobicity capacity, the values were highly variable ranging from 5.42% to 76.5% (Figure 2). The highest hydrophobicity, 76.5%, was recorded by *Lb. brevis* C1Lb21 followed by six strains, *E. faecium* A1Lb9 and G1Et4, *Lb. crustorum* V1Lb9, *Lb. brevis* B1Lb3 and B2Lb5 and *Lb. plantarum* G4Lb1 that possessed moderate capacity with percentages ranging between 25-50%. As depicted in Figure 2, thirteen strains showed low values (<25%). The results obtained in both cell surface properties did not always show the same trend for all the assayed strains. This conflict between autoaggregation and hydrophobicity has been previously reported (García-Cayuela et al., 2014), suggesting that autoaggregation capacity is strain-dependent, results in complex physical and chemical interactions as well as being influenced by environmental conditions (Collado et al., 2008; García-Cayuela et al., 2014; Goh & Klaenhammer, 2010;). Moreover, in the literature, bacterial adhesion to host epithelial cells has been widely linked to cell surface properties: however, although they play a key role, adhesion is a very complex mechanism influenced by multiple factors and they are not always correlated (García-Cayuela et al., 2014;

Lee et al., 2016). On this basis, although cell surface properties provide important information, LAB which lack suitable data may also have high adhesion capacity and need to be further investigated.

3.3 Safety aspects

3.3.1. Antibiotic susceptibility

Nowadays, antibiotic resistance is considered a serious concern in medicine. According to the European Food Safety Authority (EFSA), intrinsic resistance in bacteria present a minimal risk for horizontal spread whereas acquired resistance is considered a high risk (EFSA, 2012). The antibiotic susceptibility of selected LAB strains against twelve common antibiotics is shown in Table 3. All lactobacilli strains were found susceptible or moderately susceptible to penicillin G, chloramphenicol, erythromycin, tetracycline, ampicillin, gentamycin and clindamycin, but resistant to vancomycin and nalidixic acid. In addition, eleven lactobacilli strains were resistant to kanamycin, all *Lb. brevis* and *Lb. casei/paracasei* strains to polymyxin B and three strains, *Lb. brevis* B1Lb2, *Lb. casei/paracasei* V1lb8 and A2Lb1, to trimethoprim. In general, although there may be differences between species and strains, most of *Lactobacillus* spp. are considered to be naturally resistant to vancomycin, nalidixic acid, kanamycin, polymyxin B and trimethoprim. So, the patterns of antibiotic susceptibility obtained are in accordance with the results obtained in other works for these lactobacilli species and these strains can be used in food systems (Abriouel et al., 2015; Sharma, Tomar, Goswami, Sangwan, & Singh, 2014). Regarding the enterococci strains, these were detected as susceptible or moderately susceptible to penicillin G, chloramphenicol, erythromycin and ampicillin, but resistant to kanamycin, polymyxin B and nalidixic acid. However, they presented a variable pattern of antibiotic resistance against vancomycin, tetracycline, trimethoprim, gentamycin and clindamycin. The resistance profiles of enterococci from food or clinical isolates vary widely, containing many acquired traits (Mathur & Singh, 2005). Enterococci are naturally resistant to cephalosporins, low level aminoglycosides (kanamycin and gentamycin), polymixins, lincomycin, clindamycin and often quinolones (nalidixic acid) (Mathur & Singh, 2005; Sharma et al., 2014). Major concern is the detection of acquired resistance to trimethoprim in three strains, G3Et3, A1Lb9 and V2Lb3, and especially to relevant clinical antibiotic such as tetracycline in two strains, A1Lb9 and G3Et3, and vancomycin in G1Et4. Consequently, strains with acquired resistance to the clinically relevant antibiotics may potentially contribute to their pathogenesis and must not be used as adjunct to food.

3.3.2 Biogenic amine production

The occurrence of BA in food can have toxicological effects and lead to different types of intoxication. The ability of the LAB strains to produce BA *in-vitro* is presented in Table 3. Eight out of the twenty strains, three *Lactobacillus* spp., *Lb. brevis* B1Lb3 and B2Lb5, *Lb. crustorum* V1Lb9 and the five *Enterococcus* spp. strains were positive on the decarboxylase medium. The confirmatory analysis by HPLC-MS showed that all positive strains tested produced high levels of tyramine and scarce amounts of tryptamine, whereas the negative strains did not produce detectable amounts of BA. Most of the enterococci isolated from cheese and other origins are tyramine producers and these characteristics could be considered species-level traits for the main species found in fermented food: *E. faecium*, *E. faecalis* and *E. durans* (Ladero et al., 2012). Likewise, a high number of *Lb. brevis* strains isolated from cheese are tyramine producers (Ladero et al., 2015). Although the BA production of positive strains *in-vivo* may differ from those obtained *in-vitro,* due to cheese being a complex food matrix with an extensive number of physico-chemical factors that have a strong influence in BA formation (Gardini, Özogul, Suzzi, Tabanelli, & Özogul, 2016), the use of BA producer strains as potential probiotics may be problematic and, therefore, they were discarded.

3.4 Prebiotic growth and production of SCFA

Prebiotics are defined as "non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health" (Gibson & Roberfroid, 1995). Thus, growth in prebiotics is considered a positive feature for probiotic bacteria. The capacity of the twelve pre-selected LAB to grow *in-vitro* on three commercial prebiotics and positive substrate controls (glucose and lactose) is presented in Table 4. Growth behaviour on different carbon sources and maximum OD values obtained were arbitrarily classified in four growth levels.

In general, all LAB strains reached maximum OD 600nm values in lactulose comparable to the positive controls, although *Lb. brevis* C1Lb21 and V1Lb10 presented slower growth rate and longer lag phase. Of the two fructooligosaccharides types examined, the growth was less pronounced and more variable between strains than lactulose. Interestingly, *Lb. casei/paracasei* V1Lb8 was able to grow on FOS and inulin at the same level reached by the positive controls, whilst intermediate growth was observed in the remaining strains except for *Lb. brevis* strains *Lb. plantarum* B2Lb1 and *Lb. crustorum* A3Lb18, which showed low

ability to use these prebiotics (Table 4; Figure. S1). Metabolism of prebiotics is species- and strain-specific depending on the oligosaccharide structure (Goh & Klaenhammer, 2015). The microorganisms that use prebiotic have to degrade them into monosaccharides and subsequently metabolise them. Therefore, the presence of transporters and glycoside hydrolases are key factors in their utilisation. In this study, lactulose was the most effective prebiotic for supporting bacterial growth followed by inulin and FOS. Contrary to the expected, growth of LAB on inulin was, in general, greater than in FOS, despite having a higher DP. We suppose that this result may be influenced for the higher purity of FOS (Orafti® P95). The capacity to ferment lactulose is widespread between lactobacilli species and β-galactosidase activity has been related with its hydrolysis and metabolisation (Mao et al., 2014; Sharma & Kanwar, 2018). However, FOS utilisation is generally more specific. Among *Lactobacillus* species, genetic mechanisms for FOS utilisation have been well described in *Lb. acidophilus* and *Lb. plantarum*, where the hydrolysis is conducted by a cytoplasmic β-fructofuranosidase (Barrangou, Altermann, Hutkins, Cano, & Klaenhammer , 2003; Saulnier, Molenaar, de Vos, M., Gibson, & Kolida, 2007), and in *Lb. paracasei* and *Lb. pentosus* by an extracellular glycosidase (Goh et al., 2006; Paludan-Muller, Gram, & Rattray, 2002). This supports the growth phenotype found in our work for strains of these species grown in FOS and inulin. Our results suggest that lactulose is a suitable prebiotic to support lactobacilli growth. In addition, this prebiotic has demonstrated to be selective to avoid pathogenic bacterial growth (Sharma & Kanwar, 2017), to improve the response of probiotics to acid and bile stresses (Adebola, Corcoran, & Morgan, 2014), and positive effect in the treatment of intestinal disorders (Gibson et al., 2004; Vilela et al., 2008).

The end-products of carbohydrate metabolism by lactobacilli are organic acids, ethanol and gases. Among these compounds, SCFA display distinct positive physiological effects on the host. Butyric acid has been the most widely studied for its anti-carcinogenic and anti-inflammatory effects, although acetic and propionic acid also exhibit health-promoting effects (Russell, Hoyles, Flint, & Dumas, 2013). In fact, some of the beneficial effects of probiotics are associated with their production in the gut. Table 5 shows the mean values of lactic acid and SCFA production by carbon source. Overall, lactic acid was the principle metabolite detected followed by small amounts of acetic, butyric, isobutyric, propionic and isovaleric acids. The limited capacity of most of the LAB strains to ferment FOS and inulin was clearly revealed by the lower SCFA concentration in mMRS broth. Interestingly, although the amount of lactic acid was statistically lower in lactulose compared to glucose and lactose, this prebiotic induced a noteworthy level

of it, significantly increased the production of SCFA detected and decreased the ratio of the isomer D-lactic that may cause health problems.

Regarding the strain capacity, Figure 3 shows the mean values and statistical differences of the organic acids produced by the pre-selected LAB strains in lactulose and carbohydrate source references, lactose and glucose. Significant differences ($p \leq 0.05$) were found between strains in the amount of SCFA produced in lactulose. For all tested strains, acetic acid was the second major metabolite with approximately 10-fold less concentration than lactic acid, except for *Lb. pentosus* G4Lb7 that produced similar amount of both metabolites. For the rest of compounds detected, the concentration in mMRS broth media was approximately similar with values around 0.10 mM. However, outstandingly similar trends to that found in acetic acid were detected for the tested strains. Besides acetic acid, *Lb. pentosus* G4Lb7 also produced significantly higher ($p \leq 0.05$) amount of butyric, isobutyric, propionic and isovaleric acids, whilst similar patterns were found among the other strains. Among them, *Lb. casei/paracasei* A2Lb1 stood out. This was due to propionic acid level was comparable to *Lb. pentosus* G4Lb7 and the average amount of the rest of the SCFA were higher; however, this strain was especially sensitive to GIT transit (Table 2).

With respect to lactic acid production, in lactose and glucose *Lb. plantarum* G2Lb9 produced significantly higher amounts ($p \leq 0.05$) with values around 175 mM. This was followed by a second group of strains composed of *Lb. plantarum* G1Lb5, C1Lc12 and G4Lb7, *Lb. casei/paracasei* V1Lb8 and *Lb. pentosus* G4Lb7, with values ranging from 130 to 155 mM. Lactic acid production from lactose fermentation by cheese microbiota has technological applications during cheese ripening to drop the pH and provide an acidic environment. This ensures food safety and favours the coagulation process (McSweeney, 2004). On the other hand, in lactulose, *Lb. platarum* G1Lb5 produced significantly higher amounts of lactic acid ($p \leq 0.05$), reaching levels of 153 mM without statistical difference in comparison to glucose and lactose. The rest of the strains, with the exception of the two *Lb. casei/paracasei*, exhibited an evident decrease in the production of this metabolite. Lactulose is not consumed in the upper part of the intestine and can stimulate the growth of probiotics in the colon and lactic acid production. Although in general LAB produced low amounts of the most interesting SCFA for human health, it must be considered that a potential probiotic will be a member of the intestinal microbiota where it may establish possible cross-feeding interactions with lactate-consuming butyrate-producing colon bacteria (Moens, Verce, & De Vuyst, 2017; Weir et al., 2013). Thus, to select a potential probiotic aside from SCFA production capacity in assay

conditions is also relevant as their ability to produce lactic acid from non-digestible oligosaccharides may contribute to increase the daily SCFA amount in the colon environment by the activity of the intestinal microbiota.

In conclusion, considering the *in-vitro* tests performed in this study for screening the potential probiotic characteristics, *Lb. brevis* C1Lb21, *Lb. plantarum* G1Lb5 and *Lb. pentosus* G4Lb7 are promising candidates for further validation *in-vitro* and *in-vivo* investigations, in order to be used in a new functional Serpa cheese. Furthermore, lactulose was found to be an appropriate prebiotic to support LAB growth and induce SCFA production suggesting its application in combination with autochthonous probiotic to development a symbiotic commercial cheese. However, their influence in the ripening process and final cheese characteristics should be previously verified.

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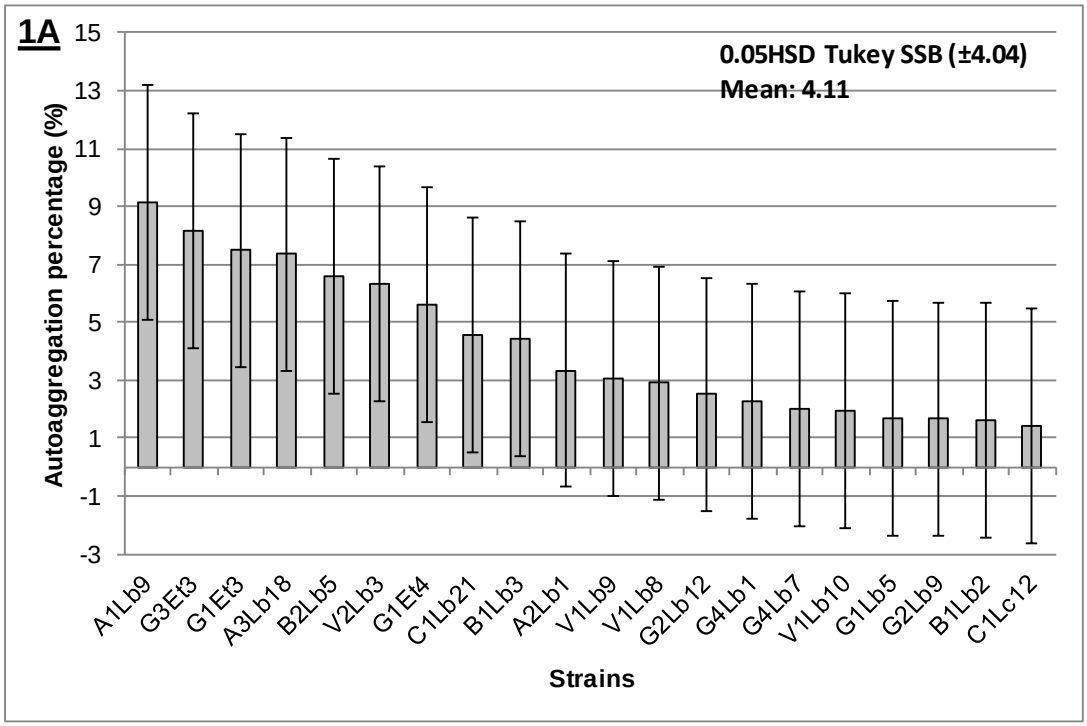
FIGURES



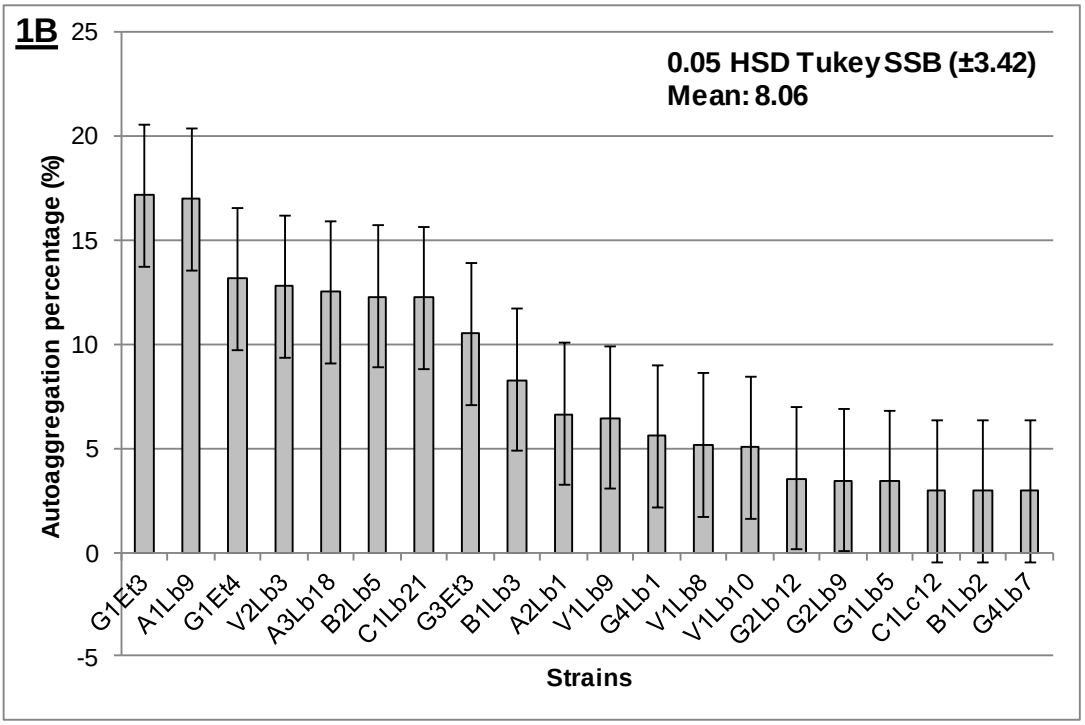


Figure1. Autoaggregation percentages of LAB strains after 1 h (1A) and 2 h (2B). SSB: statistical significance bar using the Tukey's HSD test ($p \leq 0.05$).

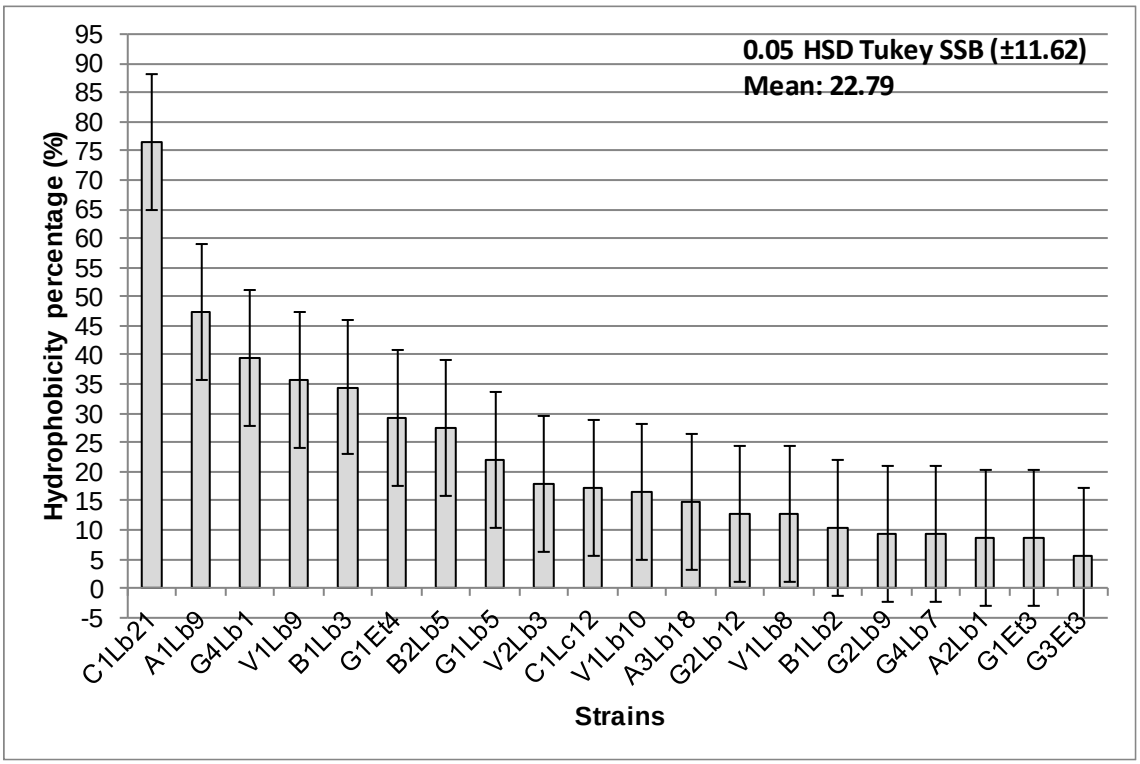


Figure 2. Hydrophobicity percentages of LAB strains against hexadecane. SSB: statistical significance bar using the Tukey's HSD test ($p \leq 0.05$).

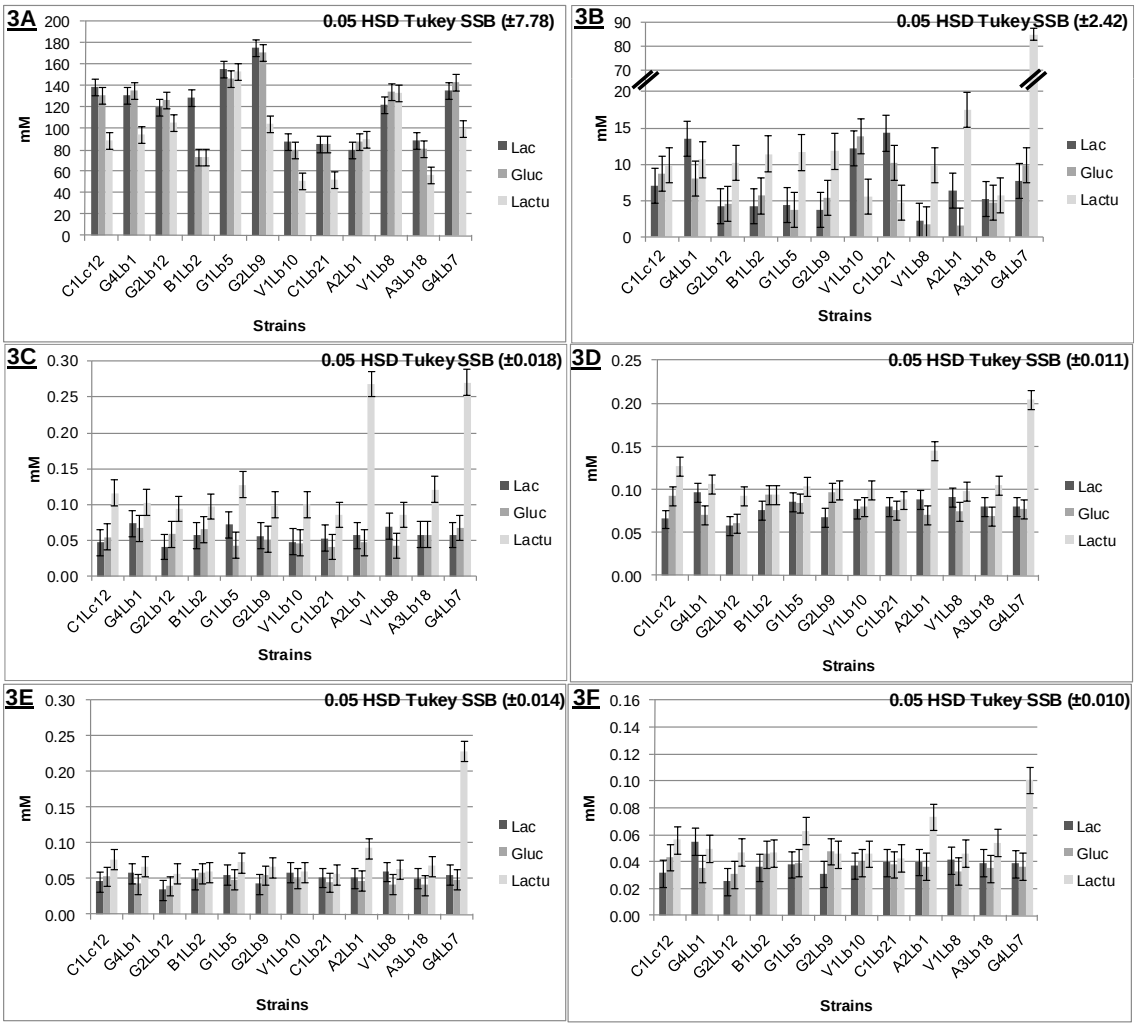


Figure 3. Quantification of lactic acid and SCFA by selected LAB in glucose (Gluc), lactose (Lac) and lactulose (Lactu). (3A) Lactic acid, (3B) acetic acid, (3C) propionic acid, (3D) butyric acid, (3E) isobutyric acid, (3E), and (3F) isovaleric acid. SSB: statistical significance bar using the Tukey's HSD test ($p \leq 0.05$).

V - GENERAL DISCUSSION

V.1. Sample physicochemical characterization

In the physicochemical characterization of the sample the pH, moisture content and water activity (a_w) the values obtained revealed some differences among samples from different industries and seasons.

The cheese samples presented pH values between 4.95-5.49, with the highest values for industry V and A in spring season. These pH data concur with those reported for soft cheeses from raw sheep's milk (Ordiales, Benito, et al., 2013), including Serpa cheese (Alvarenga et al., 2008; Roseiro, Wilbey, et al., 2003). The moisture content of the cheeses ranged from 45,02 to 48.76% ($p < 0,05$) for PDO industries and 39,10- 51,90% for non-PDO registered industries, while the a_w values were around 0,90 to 0,98. Significant differences in moisture and a_w between samples from industry V (non-PDO registered) and the other industries were observed. Overall the results obtained in PDO registered industries agreed with the findings presented by Alvarenga et al. (2008) and Roseiro, Wilbey, et al. (2003), in Portuguese Serpa cheese.

V.2. Serpa cheese microbiota quantitative characterization

V.2.1. Bacterial counts

Microbiological counts have shown some significant differences among cheeses from different industries and seasons. Total aerobic bacteria counts ranged from 8,20 to 8,92 log cfu/g at the end of ripening process. Overall, the microbiota of raw milk cheese without the addition of a starter is commonly dominated by lactic acid bacteria (LAB) genera, which play important roles in the organoleptic properties of artisanal cheeses (Feutry et al., 2012; Ordiales, Benito et al., 2013; Ordiales, Martín et al., 2013). The presumptive lactobacilli ranged from 7,33 to 9,54 log cfu/g, with the lower counts among the samples for non-PDO industries, V and B (7,33 and 7,81 log cfu/g respectively). Lactococci counts were similar to lactobacilli, whereas the levels of *Leuconostoc* spp. were lower in all samples. This last LAB group showed the biggest variation between the industries. The enterococci population was also detected at a considerable level, with counts ranging from 6,32 to 7,65 log cfu/g. The presence of high numbers of enterococci is typical of artisan raw milk cheese, which, due to their ubiquitous presence, can easily contaminate the raw milk during its collection or at various stages of cheese making, due

to poor manufacturing practices (Ordiales, Benito et al., 2013; Ordiales, Martín et al., 2013; Ortigosa et al., 2008). However, they are considered a common member of cheese microbiota, with a significant influence in the ripening process (Foulquié Moreno et al., 2006). For secondary microbiota, staphylococci were detected at a significantly lower level in winter cheeses compared to the spring samples. The staphylococci population is common in artisanal cheese with high variability in the counts (Galán, Cabezas, & Fernández-Salguero, 2012; Ordiales, Benito et al., 2013; Soares et al., 2011). Finally, the enterobacteria counts were between 5,20 and 7,28 log cfu/g with no significant differences between the industries, except for industry A in the spring, which scored statistically lower counts at 2,55 log cfu/g. A high level of enterobacteria is usually considered an indication of poor hygienic practices. However, the aforementioned values agree with the range found by other workers for similar soft cheeses at around 30 days of ripening (Ordiales, Benito et al., 2013; Tavaria & Malcata, 1998). Importantly, the presence of enterobacteria in high number at the end of the ripening may also be involved in the development of the sensory properties of the final cheese (Irlinger et al., 2012). Conversely, some species of this microbial group are considered spoilage organisms (Tabla et al., 2016) or may even be human pathogens, such as *E. coli* or *Salmonella* spp.. Therefore, the level of *E. coli* was also investigated. Although much lower counts were detected (0,86-3,93 log cfu/g), *E. coli* followed a similar tendency to the enterobacteria counts between the industries, with industry A in spring displaying the lowest counts (0,86 log cfu/g).

V.2.2. Yeast counts

The mean yeast counts ranged from 4,24 to 5,66 log cfu/g in winter, with non-PDO producers presenting the lowest mean values. In spring, producers A and C counts were significantly lower than in winter, while producer G samples showed a contrary trend. The observed counts in the core of the cheese were slightly higher than that reported by other authors in similar types of cheeses at around 30 days of ripening (Ordiales, Benito et al., 2013; Tavaria & Malcata, 1998). Moreover, differences in the yeast counts in the final cheese between producers and seasons are expected, due to several factors, such as the initial population of the raw materials, hygiene practices during the cheese-making process and the industry environment, which have a strong influence on the final cheese

fungal populations (Bokulish & Mills, 2013; Ordiales, Benito et al., 2013; Ordiales, Martín, et al., 2013; Quigley et al., 2013).

V.3. Serpa cheese microbiota qualitative characterization

V.3.1. Bacterial microbiota

The identification of microorganisms isolated from the PCA medium following the total mesophilic aerobic count, allows to evaluate the prevalence of live bacteria in the final Serpa cheese. From these results we can see the prevalence of LAB and, to a lesser extent, of enterobacteria.

Lactobacillus paracasei/Lb. casei were the main species in cheese samples from PDO industries (A, C and G) in both seasons. *Lb. casei* and *Lb. paracasei* are closely related species and, therefore, difficult to distinguish, with 99,4% similarity (Oztürk & Meterelliyöz, 2015). In cheeses from non-PDO registered industries, *Lb. brevis* and *Lb. paracasei/casei* were the dominant species in industries B and V, respectively. Other LAB identified were *Lb. plantarum, Leuconostoc mesenteroides,* only identified in the spring season, and species belonging to the genus *Enterococcus*. *E. faecium* appeared in cheeses from industries A and V, while *E. faecalis* has a higher prevalence in A and B. These genera and species are among the most common LAB found in raw milk cheese (Feutry et al., 2012; Fuka et al., 2013; Ordiales, Benito et al., 2013; Picon et al., 2016).

Among the total mesophilic microorganisms, some species from the *Enterobacteriaceae* family were also detected, which contributed to around 20 to 40% of the total isolates identified. The species, *Hafnia alvei*, was identified at around 20 to 30% in all the industries. This species has demonstrated its ability to survive during the ripening process of soft cheese and other types of cheese with a long maturation process (Abriouel, Martín-Platero, Maqueda, Valdivia, & Martínez-Bueno, 2008; Coton et al., 2012; Ordiales, Benito, et al., 2013; Tabla et al., 2016). Moreover, it is known to positively contribute to the aromatic properties of cheeses (Irlinger et al., 2012). The most disturbing finding was the presence of *E. coli* in the final product at an appreciable level, particularly those obtained from industries V and B (both non-PDO registered industries), which may be due to poor milking hygiene or poor hygienic processing as a consequence of different manufacturing practices.

The identification of microorganisms isolated from selective culture media is also important as it can provide better discrimination against the species present. So, on the de Man, Rogosa and Sharpe (pH 5,6) (MRS; Oxoid) agar, the identified species corresponding mainly to *Lactobacillus* spp., *Lb.paracasei/Lb.casei* was the most common species in this medium, followed by sporadic identification of *Lb. plntarum, Lb. brevis, Lb.pentosus, Lb. curvatus, L. mesenteroides, E. faecalis, E. faecium and E. hirae*. Notably, *Lb. brevis* was present at a relatively high percentage in both non-PDO registered industries samples (V and B). The results obtained with the identification of isolates from M17 agar (Oxoid), were similar to those obtained from MRS, except for the presence of *Lactococcus lactis* among isolates from industry C.

All the isolates identified in the (MSE) agar medium (Biokar Diagnostic, Beauvais, France) corresponded to *Leuconostoc mesenteroides*, which confirms this medium as selective for this genus. This species is generally found as a subdominant LAB species in raw milk cheese (Aquilanti et al., 2011; Fuka et al., 2013; Picon et al., 2016) where it is most competitive in the final stage of the cheese ripening process. Some researchers suggest using this species as a starter for cheese manufacture (Alegría, Delgado, Flórez, & Mayo, 2013; Nieto-Arribas, Seseña, Poveda, Palop, & Cabezas, 2010), as they may contribute to the peculiar aroma of cheese, mainly due to their ability to metabolize citrate and lactate (McSweeney & Sousa, 2000) and may influence cheese texture by dextran synthesis.

The Slanetz and Bartley medium (SB; Oxoid) also proved to be highly selective for *Enterococcus* spp. The enterococcal population was dominated by *E. faecalis*, followed by *E. faecium* and *E. hirae*. The latter, unlike the previous ones, are sporadic in cheese y (Feutry et al., 2012; Ordiales, Benito et al., 2013). In this work, it was identified only in winter isolates, but predominated in the V industry. In artisanal cheeses, the presence of this genus has been correlated with the development of typical organoleptic properties and inhibiting foodborne pathogens by the production of bacteriocins and enterocines a (Brandão et al., 2010; Giraffa, 2003; Hasna et al., 2018; Ordiales, Martín et al., 2013). They have also been associated with the production of anti-inflammatory (eg, butyrate and short-chain fatty acid) substances (Avram-Hananel et al., 2010; Carasi et al., 2017) and antihypertensive substances, in the latter case by *E. faecalis* himself in fermented milk (García-Tejedor et al., 2015). However, the presence and use of some of these

microorganisms in cheese, particularly *E. faecalis*, raises some controversy due to the association with virulence factor production, antibiotic resistance and gene transfer mechanisms related to human pathogenesis (Foulquié Moreno et al., 2006; Franz et al., 2011; Hasna et al., 2018; Puchter et al., 2018). In addition, they also have important tyrosine decarboxylase activity, which is responsible for tyramine accumulation in cheese (Picon et al., 2016).

The enterobacteria identified in PDO registered industries were mainly dominated by *H. alvei*. That is often described as the dominant Gram-negative bacteria in cheeses (Abriouel et al., 2008; Coton et al., 2012; Ordiales, Benito, et al., 2013). These bacteria have been shown to display high proteolytic activity, which could affect the sensory quality of cheese, such as creamy cheeses (Ordiales, Martín et al., 2013). In both non-PDO registered industries *E. coli* was also identified at a high level. Some strains of this species are considered important foodborne pathogens.

Staphylococcus spp. has been detected in most cheese varieties as a major component of the secondary flora that remains throughout ripening, probably due to its resistance to salt and dehydration (Little et al., 2008; Ordiales, Benito et al., 2013). In the isolates from the Baird-Parker agar (BP; Oxoid) supplemented with potassium tellurite and egg yolk emulsion, *Staphylococcus epidermidis*, *S. warneri* and *S. cohnii* were mainly identified in cheeses prepared during spring. *S. aureus* is a foodborne pathogen and was the only species identified in the samples originating from V industry (non-PDO registered).

Through the high-throughput sequencing (HTS) technology used it was only possible to identify the bacteria present at the gender level. This condition constitutes a limitation to its use as already noted in the presented bibliographic review and is usually due to technological (e.g., sequence length) and/or data processing issues (e.g., sequence database availability) (Dugat-Bony et al., 2016; Meola et al., 2019). This limitation in taxonomic annotation may affect dairy microbiome studies.

Results obtained by HTS differ slightly from results obtained by culture-dependent assessment. Through the processing of the whole DNA, the predominant bacterial genera identified were *Lactococcus*, *Lactobacillus* and *Leuconostoc and Enterobacteriaceae*. This information matches the identifications obtained in PCA, except for the presence of *Lactococcus*. However, this seems to be justified by the fact that *Lactococcus* succumb

to a stressed physiological state during the ripening process (Ruggirello, Cocolin, & Dolci, 2016) which might cause their inability to grow in a synthetic medium despite their viability in the cheese matrix (Ruggirello et al., 2016; Ruggirello, Dolci, & Cocolin, 2014).

The greater sensitivity of this type of technique to cultural methods, and even to earlier HTS technologies, allows for a more detailed community assessment also identifying low abundance microorganisms (De Filippis et al., 2017; Kergourlay et al., 2015). In fact, in addition to the genera already mentioned, it is also identified in small proportions of *Pseudomonas*, *Brochothrix*, *Macrococcus*, *Carnobacterium*, *Streptococcus* and *Enterococcus* were also identified.

V.3.2. Fungal microbiota

With the obtained results it was possible to appreciate the prevalence of live yeast in the final cheeses from different industries and seasons. Based on the molecular identification of the ITS region by RFLP, the combination of restriction analyses using the four enzymes mentioned (TaqI, Sau3AI, DdeI and HaeIII), allowed for the separation of the 471 isolates into seventeen different OTUs. The sequencing of the 26S LSU rRNA and BLAST sequence comparison of representatives isolates from each OTU obtained by PCR-RFLP, allowed their identification at the species level. Thus, approximately 71,1%, of the isolates belonged to *Debaryomyces hansenii* (P1 and 2), *Kluyveromyces lactis* and *Kluyveromyces marxianus*, respectively. The remaining 28,9% comprised twelve different species from seven different genera. Of these, *Candida zeylanoides* and *Pichia fermentans* represented around 9,1 and 5,7% of the total. The remaining species were minor contributors (*Candida pararugosa*; *Candida parapsilosis*; *Candida cabralensis*; *Pichia kudriavzevii*; *Cryptococcus oeirensis*; *Yarrowia lipolytica*; *Cyberlindnera jadinii*; *Moniliella suaveolens*; *Magnusiomyces capitatus*). Overall, most species found are very common in different cheese varieties (Alvarez-Martín, Flórez, López-Díaz, & Mayo, 2007; Atanassova et al., 2016; Banjara, Suhr, & Hallen-Adams, 2015; Gardini et al., 2006; Padilla et al., 2014; Pereira-Dias et al., 2000; Tofalo et al., 2014).

As in this work, also Gallardo et al. (2014) acquired different profiles using the TaqI enzyme, for isolates from *D. hansenii* (P1 and P2). Likewise, two different OTUs were ascribed to *P. fermentans* species (P9 and P10) due to different restriction profiles

obtained by Sau3AI. Thus, the restriction analysis of the ITS-5.8S rRNA, combining the enzymes TaqI and Sau3AI, allows an accurate and simple way to identify common yeast species from cheese.

Regarding the distribution of yeast species according to industry and season, we could see that the yeast microbiota in cheese samples from PDO registered industries, were dominated by *Kluyveromyces spp and D. hansenii*, with different percentages depending on the season. However, *D. hansenii* is only dominant in industry A, especially in spring, while in the remaining two PDO registered industries (C, G), the yeast community was composed mainly of species from the genera *Kluyveromyces*. In particular, *K. marxianus* was mainly found in winter samples and *K. lactis* in spring ones. Moreover, *P. fermentans* in cheese from industry G in winter and *C. zeylanoides* in cheese produced by industry C in spring, were also present at important levels, with minor percentages of the other isolated yeast species.

The appearance of the ubiquitous species *Magnusiomyces capitatus* (teleomorph form of *Saprochaete capitata*), previously named *Geotrichum capitatum*) in samples from industry A raises concerns, since this organism has been associated with human pathologies in immunocompromised patients (Brunetti et al., 2016). Furthermore, *C. parapsilosis* is among the most common yeast pathogens and responsible for various mycoses (Jacques & Casaregola, 2008). This species was isolated in winter, at a low level in cheese obtained from industry G (PDO registered) but it was found at a higher level in cheese produced by industry V (non-PDO registered). However, this yeast has been detected in various types of cheese and infection arising from the consumption of food contaminated with this species, has not yet been documented (Banjara et al., 2015; Padilla et al., 2014; Pereira-Dias et al., 2000).

Regarding to the yeast population in non-PDO industries (V and B), also around 60% of the isolates belonged to *D. hansenii* and *K. marxianus* in industry B with noteworthy levels of three other species. But in industry V, *C. zeylanoides* was the major species, with *C. parapsilosis* and *Moniliella suaveolens* present to a lesser extent. Two species, *C. cabralensis* and *M. suaveolens*, were only associated with non-PDO industries. The presence of these species in cheese have been reported sporadically, with *C. cabralensis* found in traditional Cabrales cheese (Flórez, Belloch, Alvarez-Martín, Querol, & Mayo, 2009), and *M. suaveolens* in artisanal Fiore Sardo cheese (Fadda et al., 2004) and Mexican

artisanal Cotija cheese (Chombo-Morales, Kirchmayr, Gschaedler, Lugo-Cervantes, & Villanueva-Rodríguez, 2016).

As with culture-dependent tools, a large diversity of yeast communities of Serpa cheese has been identified (11 main OTUs). A high correlation between both approaches used in this work was obtained. The identified species are among the most common found in cheese and the results are in accordance with results reported in the literature for other artisanal cheese (Atanassova et al., 2016; Padilla et al., 2014; Pereira-Dias et al., 2000; Tofalo et al., 2014). The most common species detected, belonged to the *Debaryomyces* and *Kluyveromyces* genera. *Galactomyces* may also be important during the initial stage of ripening. These species are recognised as safe and contribute positively to the ripening and sensorial characteristics of cheese (Fleet, 2011). Interestingly, in general, the same yeast genera were prevalent in PDO registered industries in both seasons, which, despite the expected differences in the yeast community of the raw materials, indicates that the cheese-making environment may be an important source of yeasts with relevant functions during the ripening period (Bokulish & Mills, 2013; Gori, Ryssel, Arneborg, & Jespersen, 2013; Stellato, De Filippis, La Storia, & Ercolini, 2015).

Most of the sequences were assigned to phyla *Ascomycota*, low percentages were associated with the phyla *Basidiomycota* (mainly industry A/winter) and it is remarkable that between 4 and 10% of the sequences (industry G in both seasons and industry B) were not matched to any yeast genera of the UNITE/QIIME database. Among the OTUs only identified by this technique, *Galactomyces spp*. (=*Geotrichum spp*.) was found at an important level in cheeses from industries C, G and V in winter and from industry C in spring. Among the species belonging to the genera *Galactomyces*, *G. candidus* (anamorph *Geotrichum candidum*) is an ubiquitous yeast species commonly found in cheese and with important technological applications in the cheese-making process (Boutrou & Guéguen, 2005; Ceugniez et al., 2017a). However, the salt sensitivity of this species is well known and, in general, its growth is limited at levels above 1% (Wyder, 1998). HTS of target genes from genomic DNA, cover live and dead microorganisms. Hence, *G. candidus* was probably dominant at the beginning but was out-competed by other yeast species, such as those detected by the culture-dependent method, due to the decrease in moisture and consequent increase in the salt concentration with the progression of cheese ripening. In

addition, another three genera detected by HTS, *Metschnikowia*, *Saccharomyces* and *Hanseniaspora*, were also not found among the dominant isolates.

Regarding the PDO registered industries, *Debaryomyces spp*. was dominant in cheese from industry A and *Kluyveromyces spp*. from industry C, in both seasons. In industry G, *Kluyveromyces spp.* was also the most important yeast genera in spring, but in winter its abundance was lower than *Candida spp*. that was isolated from culture media at a lower level. A similar tendency was observed for the non-PDO industries, where *Debaryomyces spp*. was dominant in cheese from industry B in agreement with the culture-dependent method, however, in industry V, *Candida spp*. was not the major yeast and it was out-competed by other yeast genera, mainly *Galactomyces spp*., possibly for the aforementioned reasons.

V.4. Safety assessment results

Regarding the results obtained, it should be emphasized that the most relevant foodborne pathogens in raw milk cheeses, *Listeria monocytogenes*, *Salmonella spp*. and enterohemorrhagic *E. coli* were not detected in the amount of cheese sample under analysis. The absence of these pathogens in the cheese indicates the safety quality of the final product with 30 days of ripening. The bacterial populations at the end of the ripening time depend on the adaptation of the microorganisms to the stress conditions found during the maturation process, such as salt concentration, pH decrease and competitive interaction with the microbiota present (Fox et al., 2004).

Although no relevant foodborne pathogens were detected, *E. coli* was identified by sequencing in all samples, thus being present in an amount between 0,86 and 3,93 log CFU/g. In cheese, the presence of *E. coli* may indicate poor hygiene conditions (Kornacki, Gurtler, & Stawick, 2013). Little et al. (2008) found initial levels of *E. coli* ranging from $1{,}1 \times 10^5$ to $4{,}6 \times 10^6$ cfu/g in raw milk cheese, and Ordiales, Benito et al. (2013) found *E. coli* at 30 days of ripening in soft "Torta del Casar" cheese, but these were not detected at the end of ripening process (60 days). So, these reports highlight the importance of the ripening period in raw milk cheese to ensure the safety of the final product, particularly considering the ability of some pathogens, such as *E. coli* O157:H7 and *L. monocytogenes* to tolerate periods of similar maturity to Serpa cheese (30 days minimum) (Almeida et al., 2007). These results suggest that a longer maturation period should be considered.

S. aureus was only found in cheeses from industry V (non-PDO registered) in a mean concentration of 3,04 log CFU/g, which still meets the limits set by the European Union for this type of product (EC, 2005a). In raw milk cheese, *S. aureus* contamination can be caused by raw milk produced by animals suffering from mastitis, contaminated milk tank, and poor hygiene practices or by cheese handlers who are *S. aureus* carriers (Guerreiro et al., 2013; Rola et al., 2016). *S. aureus* can grow during cheese processing, but once a_w and pH decrease, its growth is generally inhibited (Stewart et al., 2002). However, this microorganism has been reported in cheeses made from raw milk with a higher incidence than other foodborne pathogens (Little et al., 2008; Ordiales, Benito et al., 2013). Although *S. aureus* can produce an enterotoxin that causes illness, high numbers of the organism are necessary to produce the toxin in sufficient quantities to be a threat to public health (Le Loir, Baron, & Gautier, 2003).

Sulfite-reducing clostridia were detected in some cheese samples from industries G and A, but at low levels. They are widespread in the environment and can contaminate milk and cheese. Gender *Clostridium* include pathogenic representatives, such as *Clostridium perfringens, C. botulinum, C. difficile*, and *C. tetani*, as well as the spoilage species, *C. tyrobutyricum*, which is the main cause of the late-blowing defect in cheese, responsible for relevant financial losses in the dairy industry (Brändle, Domig, & Kneifel, 2016).

V.5. Selection of autochthonous lactic acid bacteria strains for symbiotic cheese production

V.5.1. Lactic acid bacteria autochthonous isolates for study

From all the lactic acid bacteria (LAB) isolated from Serpa cheese, 116 were selected based on species (*Lactobacillus*, *Lactococcus*, *Leuconostoc and Enterococus*) and origin (industry A, C, G, B, V) to be studied for their probiotic potential. Table 17 shows the selected isolates of Serpa cheese LAB to study the probiotic characteristics and selected strains following the application of the gastrointestinal tract resistance and safety tests.

Table 17 - Selected isolates of Serpa cheese LAB to study and strains selected following the application of the gastrointestinal tract (GIT) resistance and safety tests.

Serpa cheese LAB isolates		Strains selected in each test performed			
Species	**Nº isolates**	**Code of acid-tolerant strains**	**Code of bile salt and complete GIT transit - tolerant strains**	**Code of strains selected based on antibiotic susceptibility**	**Code of strains selected based on biogenic amine production**
Lactobacillus casei/paracasei	34	V1LB8, A2Lb1	_	_	_
Lactobacillus plantarum	16	G4Lb1, G2Lb12, B1Lb12, G1Lb5, G2Lb9	G4Lb1, G2Lb12, B1Lb12, G1Lb5, G2Lb9	G4Lb1, G2Lb12, B1Lb12, G1Lb5, G2Lb9	G4Lb1, G2Lb12, B1Lb12, G1Lb5, G2Lb9
Lactobacillus brevis	12	V1Lb10, B1Lb3, C1Lb21, B2Lb5	V1Lb10, B1Lb3, C1Lb21, B2Lb5	V1Lb10, B1Lb3, C1Lb21, B2Lb5	V1Lb10, C1Lb21
Lactobacillus curvatus	4	_	_	_	_
Lactobacillus crustorum	4	A3Lb18, V1Lb9	A3Lb18, V1Lb9	A3Lb18, V1Lb9	A3Lb18, V1Lb9
Lactobacillus coryniformi	2	_	_	_	_
Lactobacillus pentosus	2	G4Lb7	G4Lb7	G4Lb7	G4Lb7
Leuconostoc mesenteroides	12	_	_	_	_
Lactococcus lactis	2	_	_	_	_
Enterococcus faecium	14	V2Lb3, A1Lb9, G1Et4	V2Lb3, A1Lb9, G1Et4	_	_
Enterococcus hirae	12	G3Et3	G3Et3	_	_
Enterococcus durans	2	G1Et3	G1Et3	G1Et3	_
Nº Selected Strains: Total	116	19	17	13	10

V.5.2. Tolerance to the gastrointestinal tract conditions

V.5.2.1. Tolerance to low pH

One of the proposed golden rules for a correct use of probiotics is that only microbial strains able to resist gastrointestinal conditions should be considered (Toscano et al., 2017). So, in oeder to be considered as candidates for probiotic use, microorganisms must first be adapted to the restrictive conditions of the GIT and subsequently colonise the intestinal tract.

The low pH of the stomach can vary from 1,5 during fasting, to values around 4 after a meal: acting as an effective barrier against the entry of microorganisms (Martinsen et al., 2005; Peterson et al., 1989; Simonian, Vo, Doma, Fisher, & Parkman, 2005; Sumeri et

al., 2012). The pH in the stomach is lower (1,5), when the bolus is insufficient, and higher when the bolus is large (5,5) (Peterson et al., 1989). So a resistance to pH between 2 and 5 has been recommended for probiotics, which is in the range of the physiological concentrations met in the gastrointestinal tract (Papadimitriou et al., 2015).

In the current study, all LAB isolates tolerate pH 3, however, when the pH dropped to 2,5, acid stress was critical, and none survived at the levels required. At an intermediate pH level of 2,75, 19 of the 116 strains exhibited satisfactory levels of viability after 2 h of exposure (Table 16). Most resistant strains are of the genus *Lactobacillu*s spp., especially *Lb. plantarum* (5), followed by *Lb. brevis* (4), *Enterococcus faecium* (3), *Lb. casei/paracasei* (2), *Lb. crustorum* (2), *Lb. pentosus* (1), *E. hirae* (1) and *E. durans* (1). None of the *Leuconostoc mesenteroides* strains resisted these conditions. In fact, some references point to lactobacilli as more resistant to low pH and adapted to milk and other food substrates (Tham, Peh, Bhat, & Liong, 2012; Van Loveren et al., 2012).

Several authors have reported that viability of LAB is dramatically affected at low pH, especially below pH 2,5 (Zoumpopoulou et al., 2018). However, according to Vinderola (2017), the in vitro static experiments might be more inhibitory than a real upper digestion process since real conditions are difficult to mimic. In this research work the pH value of 2,75 was found to be highly discriminating and although the pH of the stomach during a meal is normally above this value, in order to guarantee the viability of the potential probiotic strains, the most acid-tolerant LAB were selected to study their behaviour under the conditions of the small intestine.

The acid tolerance of LAB is strain specific and mediated via a number of mechanisms, in particular, F_0F_1-ATPase is a particularly important element in the response and tolerance to low pH in *Lactobacillus* spp. (Corcoran, Stanton, Fitzgerald, & Ross, 2005; Toscano et al., 2017). In addition to the intrinsic characteristics of the strain, the survival of a potential probiotic to the acid environment of the stomach is also strongly influenced by the composition of the food that it contains. In particular, foods with high levels of fat, high nutrient availability and some physical conditions (Klu & Chen, 2015) like the ones in cheese, provide additional protection of the microorganism against the restrictive conditions of the GIT transit (Karimi et al., 2011).

V.5.2.2. Bile salt tolerance

The next obstacle for bacteria that survive stomach conditions are bile secretions in the duodenum. Bile salts have potent antimicrobial activity due to their ability to dissolve lipids and fatty acids from cell membranes (Klopper et al., 2018; Melo Pereira et al., 2018). The amount of bile salts varies with digestion time and the physiological concentration is usually between 0,15-0,5%, but concentrations around 1,5% may occur at the beginning of digestion. Resistance to that amount of bile salts has been recommended for probiotics (Papadimitriou et al., 2015).

Except for the two *Lb. casei/paracasei* strains (V1Lb8, A2Lb1), which showed a clear sensitivity to bile salts at a concentration above 1%, all other strains were able to grow well in the presence of bile salt concentrations up to 2% after 24 h (Table 17). These results are consistent with those previously recovered from other probiotic *in-vitro* testing where most of the strains were more resistant to bile salts than low pH (Han et al., 2017; Ruiz-Moyano et al., 2008; Zoumpopoulou et al., 2018).

V.5.2.3. Survival on complete gastrointestinal transit

Most of the selected strains showed good survival to complete GIT transit (Table 16). However, two of the nineteen strains, *Lb. casei/paracasei* strains (V1Lb8, A2Lb1), dramatically decreased their viability when exposed to simulated gastric juice for 90 min followed by 120 min in simulated intestinal juice. This emphasises the effect of bile salt on bacterial viability after the exposition to artificial gastric juice. With respect to the remaining strains, the majority maintained viability during the whole assay, although, *Lb. brevis* V1Lb10 and *Lb. plantarum* B1Lb2 presented a moderate reduction after 6 under simulated intestinal juice. These data agree with those previously obtained after single stress exposure and confirm that gastric juice is the main hurdle in the GIT transit. There are 17 strains left to proceed with this assessment (Table 16).

V.5.3. Capacity to colonise the intestine

V.5.3.1. Aggregation ability

Adhesion to intestinal tissue is one of the main features for a probiotic candidate: since it is involved in host colonisation and allows the probiotic to exert its beneficial effects

(Papadimitriou et al., 2015). The accession to epithelial cells is related to both the autoaggregation ability and non-specic hydrophobic properties of the cell surface (Collado et al., 2008). Microbial cell autoaggregation ability ensures that the probiotic reaches a high cell density in the gut contributing to the adhesion mechanism to different kind of host cells, while cell surface hydrophobicity allows for an improved interaction between microbe and human epithelial cells (Dlamini et al., 2019; Melo Pereira et al., 2018). These tests are considered a reliable *in-vitro* system to initially assess the potential adhesion ability of GIT transit tolerant LAB (Collado et al., 2008; Dlamini et al., 2019; Klopper et al., 2018). They are also simple and efficient (Papadimitriou et al., 2015).

In general, the percentage of autoaggregation varied between 1,05 and 8,13 % at 1 h and 2,7-17,14 % at 2 h. All strains increased the autoaggregation percentages with the incubation time, an observation that is congruent with other authors (García-Cayuela et al., 2014; Han et al., 2017). Among strains, significant differences were observed ($p \leq 0.05$). At 2 h seven of the twenty strains, *Lb. brevis* C1Lb21 and B2Lb5, *Lb. crustorum* A3Lb18, *E. faecium* V2Lb3, A1Lb9 and G1Et4 and *E. durans* G1Et3, showed better autoaggregation with percentages higher than 12%, corresponding to a noteworthy abiliy based upon previous studies in LAB (García-Cayuela et al., 2014; Han et al., 2017). Besides, García-Cayuela et al. (2014) reported that the autoaggregation ability of lactobacilli correlated highly with co-aggregation with pathogens. So, the LAB strains with higher autoaggregation ability may also contribute to prevent the colonisation of the human gut by foodborne pathogens.

V.5.3.2. Cell surface hydrophobicity

Hydrophobicity was were highly variable ranging from 5,42% to 76,5%. The highest hydrophobicity, 76,5%, was recorded in *Lb. brevis* C1Lb21 followed by six strains, *E. faecium* A1Lb9 and G1Et4, *Lb. crustorum* V1Lb9, *Lb. brevis* B1Lb3 and B2Lb5 and *Lb. plantarum* G4Lb1, that possessed moderate abilities with percentages ranging between 25-50%. Thirteen strains showed low values (<25%).

The results obtained in both cell surface properties did not always show the same trend for all the assayed strains. This conflict between autoaggregation and hydrophobicity has been previously reported (García-Cayuela et al., 2014), suggesting that autoaggregation ability is strain-dependent, results in complex physical and chemical interactions and is

influenced by environmental conditions (Collado et al., 2008; García-Cayuela et al., 2014; Goh & Klaenhammer, 2010). In the literature, bacterial adhesion to host epithelial cells has been widely linked to cell surface properties: however, although it plays a key role, adhesion is a very complex mechanism influenced by multiple factors and they are not always correlated (García-Cayuela et al., 2014; Lee & Puong, 2002). On this basis, although cell surface properties provide important information, LAB which lack suitable data may also have high adhesion abilities and need to be further investigated. In general, assessing the adhesive cability of probiotic strains based on surface hydrophobicity is quite outdated (Papadimitriou et al., 2015; Vinderola et al., 2017).

V.5.4. Safety assays

V.5.4.1. Antibiotic susceptibility

In 2007, the EFSA introduced antimicrobial resistance as a safety concern associated with probiotic consumption (EFSA/SC, 2007). Consequently, in 2012, the QPS program included also this criterion (EFSA/FEEDAP, 2012). This document recommends that commercial microbial strains used as food supplements should not harbour any transferable antibiotic resistance.

In general, although there may be differences between species and strains, most *Lactobacillus* spp. are considered to be naturally resistant to vancomycin, quinolones (nalidixic acid), kanamycin, polymyxin B and trimethoprim. The Enterococci are naturally resistant to cephalosporins, low level aminoglycosides (kanamycin and gentamycin), polymixins, lincomycin, clindamycin and often quinolones (nalidixic acid) (Mathur & Singh, 2005; Sharma et al., 2014). But the resistance profiles of enterococci from food or clinical isolates vary widely, containing many acquired traits (Mathur & Singh, 2005).

The antibiotic susceptibility of the selected LAB strains against twelve common antibiotics is shown in Table 18. All lactobacilli strains were found to be susceptible or moderately susceptible to penicillin G, chloramphenicol, erythromycin, tetracycline, ampicillin, gentamycin and clindamycin, but resistant to vancomycin and nalidixic acid. In addition, eleven lactobacilli strains were resistant to kanamycin, all *Lb. brevis* and *Lb. casei/paracasei* strains to polymyxin B and three strains, *Lb. brevis* B1Lb2, *Lb.*

casei/paracasei V1lb8 and A2Lb1, to trimethoprim. So, the patterns of antibiotic susceptibility obtained are in accordance with the results obtained in other works for these lactobacilli species and these strains can be used in food systems (Abriouel et al., 2008; Sharma et al., 2014).

Regarding the enterococci strains, these were detected as susceptible or moderately susceptible to penicillin G, chloramphenicol, erythromycin and ampicillin, but resistant to kanamycin, polymyxin B and nalidixic acid. However, they presented a variable pattern of antibiotic resistance against vancomycin, tetracycline, trimethoprim, gentamycin and clindamycin.

A major concern is the detection of acquired resistance to trimethoprim in three strains, G3Et3, A1Lb9 and V2Lb3, and especially to relevant clinical antibiotics such as tetracycline in two strains, A1Lb9 and G3Et3, and vancomycin in G1Et4. Consequently, these enterococci strains with acquired resistance to the clinically relevant antibiotics may potentially contribute to their pathogenesis and must not be used as adjunct to food (Table 18).

Indeed enterococci are not usually used as starter cultures or co-cultures in foods because they harbor multiple antibiotic-resistant genes, which are transmissible by conjugation to pathogenic microorganisms (Franz et al., 2011; Hasna et al., 2018; Puchter et al., 2018). So, from the current regulatory point of view, this genre is not part of the QPS or GRAS lists (Hasna et al., 2018).

Table 18 - Antibiotic susceptibility and biogenic amines (BA) production of acid-tolerant LAB strains.

LAB Strains	*Lb. plantarum*							*Lb. brevis*			*Lb. casei/paracasei*			*Lb. crustorum*	*Lb. pentosus*	*E. faecium*			*E. durans*	*E. hirae*
	C1Lc12	G4Lb1	G2Lb12	B1Lb2	G1Lb5	G2Lb9	V1Lb10	B1Lb3	C1Lb21	B2Lb5	V1Lb8	A2Lb1	A3Lb18	V1Lb9	G4Lb7	V2Lb3	A1Lb9	G1Et4	G1Et3	G3Et3
Antibiotic	**Antibiotic susceptibility**																			
Kanamycin	R*	R	R	MS	R	R	R	R	R	MS	R	R	R	MS	MS	R	R	R	R	R
Penicillin G	S	S	S	S	MS	MS	MS	S	MS	MS	MS	S	S	S	MS	S	S	S	S	S
Polymyxin B	R	R	R	R	R	R	S	S	S	S	R	R	S	S	MS	R	R	R	R	R
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Erythromycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Vancomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	S	S
Tetracycline	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R
Trimethoprim	S	S	S	R	S	S	S	S	S	S	R	R	S	S	S	R	R	S	S	R
Ampicillin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	S	R
Clindamycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	MS
Nalidixic acid	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	Biogenic amines production (mg/L)																			
Decarboxylase medium	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	+	+	+	+	+
Biogenic amines																				
Cadaverine	n.d.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Putrescine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Spermine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Spermidine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Histamine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenylethylamine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tryptamine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**2.53**	n.d.	**3.58**	n.d.	n.d.	n.d.	**1.58**	n.d.	**12.86**	**12.67**	**9.46**	**7.88**	**14.46**
Tyramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**523.61**	n.d.	**2523.06**	n.d.	n.d.	n.d.	**2593.25**	n.d.	**2150.42**	**3529.65**	**3851.75**	**2907.17**	**4167.67**

*R: resistant; MS: moderately susceptible; S: susceptible; ** n.d.: not detected.

It should be noted that recent advances in molecular biology and the recommended methods for the safety evaluation of *Enterococcus* strains allowed for the distinction between comensal and clinical clades (Hasna et al., 2018). In the work presented by Ghattargi et al. (2018), they identified a strain that is a potential probiotic candidate due to the high genomic stability, absence of known virulence factors and antibiotic resistance genes and close genomic relatedness with marketed probiotics. Nevertheless, the development of highly adapted methods and legislations are still required (Hasna et al., 2018).

V.5.4.2. Biogenic amine production

The proteolytic activity of microorganisms is important for their survival and for the development of sensory attributes and structural characteristics of cheese (Benkerroum, 2016; Spano et al., 2010; Zuljan et al., 2016). However, from a food safety standpoint this may lead to the formation of biogenic amines (BA), which can lead to different types of intoxication.

The ability of the LAB strains to produce BA *in-vitro* is presented in Table 17. Eight out of the twenty strains, three *Lactobacillus* spp., *Lb. brevis* B1Lb3 and B2Lb5, *Lb. crustorum* V1Lb9 and the five *Enterococcus* spp. strains were positive on the decarboxylase medium. The confirmatory analysis by HPLC-MS showed that all positive strains tested produced high levels of tyramine and small amounts of tryptamine, whereas the negative strains did not produce detectable amounts of BA.

Most of the enterococci isolated from cheese and other origins are tyramine producers and these characteristics could be considered species-level traits for the main species found in fermented food: *E. faecium*, *E. faecalis* and *E. durans* (Linares et al., 2012). Likewise, a high number of *Lb. brevis* strains isolated from cheese are tyramine producers (Ladero, Fernandez, Cuesta, & Alvarez, 2010). Although the BA production of positive strains *in-vivo* may differ from those obtained *in-vitro*, because cheese is a complex food matrix with an extensive number of physico-chemical factors that have a strong influence in BA formation (Fausto Gardini, Özogul, Suzzi, Tabanelli, & Özogul, 2016), the use of BA producer strains as potential probiotics may be problematic and, therefore, they were discarded (Table 16).

V.5.5. Growth on prebiotic

Prebiotics are defined as "non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health" (Roberfroid, 2002). Growth in prebiotics is considered a positive feature for probiotic bacteria. The metabolism of prebiotics is species- and strain-specific depending on the oligosaccharide structure (Goh & Klaenhammer, 2015). The microorganisms that use prebiotic have to degrade them into monosaccharides and subsequently metabolise them. Therefore, the presence of transporters and glycoside hydrolases are key factors in their use.

In general, all LAB strains reached maximum OD 600nm values in lactulose comparable to the positive controls, although *Lb. brevis* C1Lb21 and V1Lb10 presented slower growth rates and longer lag phase. Of the two fructooligosaccharides types examined, the growth was less pronounced and more variable between strains than with lactulose. Interestingly, *Lb. casei/paracasei* V1Lb8 was able to grow on FOS and inulin at the same level reached by the positive controls, whilst intermediate growth was observed in the remaining strains except for *Lb. brevis* strains *Lb. plantarum* B2Lb1 and *Lb. crustorum* A3Lb18, which showed low ability to use these prebiotics.

In this study, lactulose was the most effective prebiotic for supporting bacterial growth followed by inulin and FOS. Contrary to what was expected, growth of LAB on inulin was, in general, greater than in FOS, despite having a higher DP. We suppose that this result may be influenced by the higher purity of FOS (Orafti® P95).

The ability to ferment lactulose is widespread between lactobacilli species and β-galactosidase activity has been linked with its hydrolysis and metabolisation (Mao et al., 2014). However, FOS utilisation is generally more specific. Among *Lactobacillus* species, genetic mechanisms for FOS utilisation have been well described in *Lb. acidophilus* and *Lb. plantarum*, where the hydrolysis is conducted by a cytoplasmic β-fructofuranosidase (Barrangou, Altermann, Hutkins, Cano, & Klaenhammer, 2003; Saulnier, Molenaar, De Vos, Gibson, & Kolida, 2007), and in *Lb. paracasei* and *Lb. pentosus* by an extracellular glycosidase (Goh & Klaenhammer, 2015; Paludan-Müller, Gram, & Rattray, 2002). This supports the growth phenotype found in our work for strains of these species grown in FOS and inulin. Our results suggest that lactulose is a suitable

prebiotic to support lactobacilli growth. In addition, this prebiotic has demonstrated to be selective to avoid pathogenic bacterial growth (Sharma et al., 2017), to improve the response of probiotics to acid and bile stresses (Adebola, Corcoran, & Morgan, 2014), and to have a positive effect on the treatment of intestinal disorders (Gibson, Probert, Loo, Rastall, & Roberfroid, 2005).

V.5.6. Short-chain fatty acid production

The end-products of carbohydrate metabolism by lactobacilli are organic acids, ethanol and gases. Among these compounds, SCFA display distinct positive physiological effects on the host. Butyric acid has been the most widely studied for its anti-carcinogenic and anti-inflammatory effects, although acetic and propionic acids also show health-promoting effects (Russell, Hoyles, Flint, & Dumas, 2013). Indeed, some of the beneficial effects of probiotics are associated with the production of these compounds in the gut or in fermented foods, so we evaluated the production capacity of these acids by the selected strains.

Overall, lactic acid was the main metabolite detected followed by small amounts of acetic, butyric, isobutyric, propionic and isovaleric acids. The limited ability of most of the LAB strains to ferment FOS and inulin was clearly revealed by the lower SCFA concentration in mMRS broth. Interestingly, although the amount of lactic acid was statistically lower in lactulose compared to glucose and lactose, this prebiotic induced a noteworthy level of it, significantly increased the production of SCFA detected and decreased the ratio of the isomer D-lactic that may cause health problems.

Regarding the strain capacity, significant differences ($p \leq 0.05$) were found between strains in the amount of SCFA produced in lactulose. For all tested strains, acetic acid was the second major metabolite with approximately 10x lower concentration than lactic acid, except for *Lb. pentosus* G4Lb7 that produced similar amounts of both metabolites. For the others compounds detected, the concentration in mMRS broth media was approximately similar to values around 0,10 mM. However, outstandingly similar trends to that found in acetic acid were detected for the tested strains. Besides acetic acid, *Lb. pentosus* G4Lb7 also produced significantly higher ($p \leq 0.05$) amounts of butyric, isobutyric, propionic and isovaleric acids, whilst similar patterns were found among the other strains. Among them, *Lb. casei/paracasei* A2Lb1 stood out. This was due to the

fact that the propionic acid level was comparable to *Lb. pentosus* G4Lb7 and the average amounts of the rest of the SCFA were higher; however, this strain was especially sensitive to GIT transit.

With respect to lactic acid production, in lactose and glucose *Lb. plantarum* G2Lb9 produced significantly higher amounts ($p \leq 0,.05$) with values around 175 mM. This was followed by a second group of strains composed of *Lb. plantarum* G1Lb5, C1Lc12 and G4Lb7, *Lb. casei/paracasei* V1Lb8 and *Lb. pentosus* G4Lb7, with values ranging from 130 to 155 mM. Lactic acid production from lactose fermentation by cheese microbiota has technological applications during cheese ripening to drop the pH and provide an acidic environment. This ensures food safety and favours the coagulation process (McSweeney, 2004). On the other hand, in lactulose, *Lb. plantarum* G1Lb5 produced significantly higher amounts of lactic acid ($p \leq 0,05$), reaching levels of 153 mM without any statistical difference in comparison to glucose and lactose. The other strains, with the exception of the two *Lb. casei/paracasei*, showed a clear decrease in the production of this metabolite. Lactulose is not consumed in the upper part of the intestine and can stimulate the growth of probiotics in the colon and lactic acid production.

Although in general LAB produced low amounts of the most interesting SCFA for human health, it must be considered that a potential probiotic will be a member of the intestinal microbiota where it may establish possible cross-feeding interactions with lactate-consuming butyrate-producing colon bacteria (Moens, Verce, & De Vuyst, 2017). Thus, selecting a potential probiotic aside from SCFA production capacity in assay conditions is also relevant as their ability to produce lactic acid from non-digestible oligosaccharides may contribute to increase the daily SCFA amount in the colon environment by the activity of the intestinal microbiota.

VI – CONCLUSIONS / CONCLUSIONES

1. In the physicochemical characterization of the sample used, pH values ranged from 4,95 to 5,49 and a_w values from 0.90 to 0.98. Cheese moisture content ranged from 45,02 to 48,76% for the PDO industries and 39,10-51,90% for non-PDO industries. Significant differences in moisture and/or a_w were observed among non-PDO industries and between these and other industries. Overall, the results obtained in the PDO-industries agreed with the findings of other studies on sheep cheese and even Serpa cheese. Considering the physicochemical characterization, the sample used appeared to be representative and adequate given the objectives of this work.

   Los resultados de caracterización fisicoquímica de las muestras analizadas mostraron valores de pH que oscilaron entre 4,95 a 5,49 y de a_w de 0,90 a 0,98. El contenido de humedad en las muestras de queso varió de 45,02 a 48,76% para las industrias acogidas a las DOP y 39,10-51,90% para las industrias sin DOP. Se observaron diferencias significativas en la humedad y a_w entre las industrias no DOP y entre estas y las otras industrias. En general, los resultados obtenidos en las industrias acogidas a DOP coincidieron con los hallazgos de otros estudios sobre queso de oveja e incluso queso Serpa. Es por ello, que las muestras utilizadas resultaron ser representativas y adecuadas para los objetivos planteados en esta tesis.

2. Average bacterial counts at the end of the ripening process (30 days) reveal total mesophilic bacteria ranging from 8,20 to 8,92 log cfu/g, and the predominance of lactic acid bacteria (LAB) with some significant differences among cheeses from different industries and seasons. In this context, presumptive lactobacilli ranged from 7,33 to 9,54 log cfu/g, with the lowest counts among the samples from non-PDO industries (7,33 to 7,81 log cfu/g), followed by enterococci (6,32 to 7,65 log cfu/g) and *Leuconostoc* spp (not detected to 7,96 log cfu/g). This last gender presented the largest variation among the industries. As for secondary microbiota, enterobacteria have significant representation (5,20 to 7,28 log cfu/g) without major differences, whereas staphylococci were detected at a significantly lower level in winter cheeses (not detected to 3.04 log cfu/g), compared to the spring samples (2,18 to 6,01 log cfu/g). *Escherichia coli* level is lower (0,86-3,93 log cfu/g), but its presence was confirmed by sequencing in all samples.

Los recuentos bacterianos al final del proceso de maduración revelan niveles de bacterias aerobias mesófitas totales que oscilaron entre 8,20 a 8,92 log ufc/g, siendo la flora predominante las bacterias ácido lácticas (BAL) con algunas diferencias significativas entre los quesos de diferentes industrias y estaciones. En este contexto, los niveles de lactobacilos oscilaron entre 7,33 y 9,54 log ufc/g, siendo los recuentos más bajos los obtenidos en las muestras de quesos sin DOP (7,33 a 7,81 log ufc/g), seguidos de los niveles de enterococos (6,32 a 7,65 log ufc/g) y *Leuconostoc* spp (no detectado a 7,96 log ufc/g). Este último género presentó la mayor variación entre las industrias. En cuanto a la flora secundaria, las enterobacterias mostraron una representación mayoritaria (5,20 a 7,28 log ufc/g) sin diferencias significativas, mientras que los estafilococos se detectaron a un nivel significativamente más bajo en los quesos de invierno (no se detectaron a 3,04 log ufc/g), en comparación a las muestras de primavera (2,18 a 6,01. log ufc / g). El nivel de *E. coli* fue inferior (0,86-3,93 log ufc/g), pero su presencia se confirmó mediante secuenciación en todas las muestras.

3. Regarding the fungal count at the end of the process (30 days), yeasts dominated, since the sample analyzed was from the cheese core. Irrespective of the producer, the mean yeast counts were around 5,0 log cfu/g. The yeast counts ranged between 4,24−5,81 log cfu/g in the winter, with non-PDO producers having the lowest mean values (4,20 to 4,44 log cfu/g) and between 2,55−5,80 log cfu/g in the spring. There seems to be a trend towards lower values in the spring, but this trend is contradictory among producers.

Con respecto al recuento de la población fúngica, las levaduras predominaron en todas las muestras analizadas, dado que estas provenían del núcleo del queso. Independientemente del productor, los recuentos medios de levadura fueron de 5,0 log ufc/g. Los niveles de levadura oscilaron entre 4,24−5,81 log ufc/g en invierno, en productores no acogidos a DOP y con valores medios más bajos (4,20 a 4,44 log ufc/g) en los acogidos a DOP y entre 2,55−5,80 log ufc/g en la primavera. Esto indica un evolución hacia recuentos más bajos en la primavera, aunque no se observó una tendencia clara entre los diferentes productores.

4. The most relevant foodborne pathogens in raw milk cheeses, *Listeria monocytogenes*, *Salmonella spp*. and enterohemorrhagic *E. coli* were not detected in the sample under analysis. The absence of these pathogens in the cheese indicates the safety quality of the final product with 30 days of ripening. *S. aureus* was only found in cheeses from a non-PDO registered industry in a mean concentration of 3,04 log cfu/g, which still meets the limits set by the EU regulation for this type of product. Sulfite-reducing clostridia were detected in some cheese samples but at low levels.

En ningunas de las muestras analizadas no se detectaron patógenos como *Listeria monocytogenes*, *Salmonella* spp. y *E. coli* enterohemorrágico. La ausencia de estos patógenos en el queso indica la calidad higiénico sanitaria del producto final con 30 días de maduración. *S. aureus* solo se encontró en quesos de un productor no acogido por la DOP, con niveles medios de 3,04 log ufc/g, que aún cumple con los límites establecidos por la regulación de la UE para este tipo de producto. Así mismo, tambien se detectaron clostridios sulfito reductores en algunas muestras de queso pero a niveles bajos.

5. The identification of bacteria present at the end of ripening (30 days) by 16S RNA following culture-dependent methods confirms LABs as the dominant group. Identifications of isolates from both general and selective culture media reveal *Lactobacillus paracasei/casei* as the main species in cheese from PDO industries, whereas in non-PDO industries *Lactobacillus brevis* stood out among the remaining. Other LAB identified were *Lb. plantarum*, *Lb. brevis, Lb.pentosus, Lb. curvatus*, *Leuconostoc mesenteroides (*only in spring), and species belonging to the genus *Enterococcus, E. faecalis, E. faecium and E. hirae.* Among the secondary microorganisms *Hafnia alvei* was the main species among *Enterobacteriaceae* identified at around 20 to 30% in all the industries. The genus *Staphylococcus*, isolated only in spring, was discriminated as *S.epidermidis*, *S. warneri* and *S. cohnii*.

La identificación de las cepas aisladas del final del proceso de maduración (30 días) por el rARN 16S siguiendo métodos dependientes del cultivo, confirma que las BAL fueron el grupo dominante. Las identificaciones de los aislamientos de medios de cultivo tanto generales como selectivos revelan que *Lactobacillus paracasei /*

casei fue la especie principal en las muestras de queso de industrias acogidas a DOP, mientras que en las industrias no DOP, *Lactobacillus brevis* fue el mayoritario. Otras BAL identificadas fueron *Lb. plantarum, Lb. brevis, Lb.pentosus, Lb. curvatus, Leuconostoc mesenteroides* (solo en primavera) y especies pertenecientes al género *Enterococcus, E. faecalis, E. faecium* y *E. hirae*. Entre los microorganismos secundarios, *Hafnia alvei* fue la especie principal identificada entre las *Enterobacteriaceae* con alrededor del 20 al 30% en todas las industrias. El género *Staphylococcus,* aislado solo en primavera, fue discriminado como *S.epidermidis, S. warneri* y *S. cohnii*.

6. The results obtained by high-throughput sequencing (HTS) analysis confirmed that LAB was the main bacterial group represented by the *Lactobacillales* order which includes approximately 60 to 85% of the microbial cheese population, followed by *Enterobacteriales* (10% - 36%), but up to 5% of unidentified sequences can be observed. However, this technology reveals the genus Lactococcus as 40% to 60% of the population, followed by genera *Leuconostoc* (5%-30%) and *Lactobacillus* (2%-30%). This technology allows for a more detailed community assessment also identifying the presence of less abundant (0-24%) genera as *Streptococcus* and *Brochothrix* and trace genres (0-<5%), like *Pseudomonas*, *Brochothrix*, *Macrococcus*, and *Carnobacterium*.

Los resultados obtenidos mediante el análisis de secuenciación masiva de alto rendimiento (HTS) confirmaron a las BAL como el principal grupo bacteriano representado por *Lactobacillus* con aproximadamente del 60 al 85% de la población microbiana del queso, seguido de las enterobacterias (10% - 36%). Tambien se obtuvo un 5% de secuencias no identificadas. Sin embargo, esta tecnología ha perimitido revelar que el género *Lactococcus* contituye del 40% al 60% de la población, seguido de los géneros *Leuconostoc* (5% -30%) y *Lactobacillus* (2% - 30%). Esta tecnología permite una evaluación comunitaria más detallada que también identifica la presencia de géneros menos abundantes (0-24%) como *Streptococcus* y *Brochothrix* y trazas de géneros (0- <5%), como *Pseudomonas, Brochothrix, Macrococcus* y *Carnobacterium.*

7. Regarding the yeast in the final cheese, the molecular identification of the ITS region by the RFLP showed a great diversity. Approximately 71%, of the isolates belonged to *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus*. The remaining 29% comprised eleven different species from seven different genera. Of these, *Candida zeylanoides* and *Pichia fermentans* amounted to around 9,1 and 5,7% of the total. The other species are minor contributors and include *Candida pararugosa*; *Candida parapsilosis*; *Candida cabralensis*; *Pichia kudriavzevii*; *Cryptococcus oeirensis*; *Yarrowia lipolytica*; *Cyberlindnera jadinii*; *Moniliella suaveolens*; *Magnusiomyces capitatus*.

Con respecto a población de levaduras presentes en el queso final, la identificación molecular de la región ITS mediante PCR-RFLP mostró una gran diversidad. Aproximadamente el 71% de los aislamientos pertenecían a *Debaryomyces hansenii*, *Kluyveromyces lactis* y *Kluyveromyces marxianus*. El 29% restante comprendía once especies diferentes de siete géneros distintos, donde *Candida zeylanoides* y *Pichia fermentans* representaron alrededor del 9,1 y el 5,7% del total, respectivamente. Las otras especies menos representativas fueron *Candida pararugosa, Candida parapsilosis, Candida cabralensis, Pichia kudriavzevii, Cryptococcus oeirensis, Yarrowia lipolytica, Cyberlindnera jadinii, Moniliella suaveolens, Magnusiomyces capitatus*.

8. In the distribution of yeast species according to industry and season, *Kluyveromyces spp* or *D. hansenii* prevail, with different percentages depending on producer and season, except in one of the non-PDO industries where the only dominant genus is *Candida spp.*. *K. marxianus* and *P. fermentans* were mainly found in winter samples and *K. lactis* and *C. zeylanoides* in spring. Two species, *C. cabralensis* and *M. suaveolens*, were only associated with non-PDO industries.

En relación a la distribución de las especies de levaduras según la industria y la estación, se observó una prevalecia de *Kluyveromyces* spp o *D. hansenii*, con diferentes porcentajes dependiendo del productor y la estación, excepto en una de las industrias no acogidas a la DOP donde el único género dominante fue *Candida* spp. Las especies *K. marxianus* y *P. fermentans* se encontraron principalmente en muestras de invierno y *K. lactis* y *C. zeylanoides* en primavera. Dos especies, *C. cabralensis* y *M. suaveolens*, solo se asociaron con industrias sin DOP.

9. The results obtained by high-throughput sequencing (HTS), reveals a greater diversity of yeasts, in spite of the high correlation between both approaches used. Thus, most of the sequences (90%-100%) were assigned to phyla *Ascomycota*, a minority (0-8%) to *Basidiomycota*, but up to 10% of unidentified sequences can be observed. The genera *Debaryomyces* and *Kluyveromyces* were present and identified as the most common, the genus *Galactomyces spp*. (=*Geotrichum spp*.) was also identified at an important level in both PDO and non-PDO cheese, especially in winter. Three other non-dominant genera detected by HTS were, *Metschnikowia, Saccharomyces* and *Hanseniaspora*.

Los resultados obtenidos por secuenciación de alto rendimiento (HTS), han revelado una mayor diversidad de levaduras, a pesar de la elevada correlación entre ambas tecnologías utilizadas. La mayoría de las secuencias (90% -100%) se asignaron al filo ascomicetos, una minoría (0-8%) a basidiomicetos y hasta el 10% de secuencias no identificadas. Los géneros *Debaryomyces* y *Kluyveromyces* estuvieron presentes e identificados como los más comunes, mientras que el género *Galactomyces* spp. (= *Geotrichum* spp.) también fué identificado en un nivel importante, tanto en queso con DOP como sin DOP, especialmente en quesos de invierno. Otros tres géneros no dominantes detectados por secuenciacion masiva HTS fueron, *Metschnikowia, Saccharomyces* y *Hanseniaspora.*

10. In the study for screening the potential probiotic characteristics, it was possible to select three potential probiotic strains (PPS) from a total of 115. Considering the in-vitro tests performed, *Lb. brevis* C1Lb21, *Lb. plantarum* G1Lb5 and *Lb. pentosus* G4Lb7 are promising probiotic candidates for further validation in-vitro and in-vivo investigations, in order to be used in a new functional cheese. Furthermore, lactulose was found to be an appropriate prebiotic to support LAB growth and induce SCFA production suggesting its application in combination with autochthonous probiotic to develop a symbiotic commercial cheese.

Tras los ensayos in vitro realizados para evaluar las características probióticas del total de 115 aislados autóctonos, fue posible seleccionar tres como potenciales probióticos (PPS). *Lb. brevis* C1Lb21, *Lb. plantarum* G1Lb5 *y Lb. pentosus* G4Lb7 son candidatos prometedores como cepas probióticas para ser utilizadas

en la elaboración de un nuevo queso funcional tras una validación adicional en investigaciones in vitro e in vivo. Además, se descubrió la lactulosa como el prebiótico más apropiado para apoyar el crecimiento de estas BAL e inducir la producción de ácidos grasos de cadena corta SCFA, lo que sugiere su aplicación en combinación con las cepas probióticas autóctonos seleccionadas para desarrollar un queso comercial funcional simbiótico

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APPENDICES

APPENDIX 1

Appendix 1 - European and national legislation applicable to the production of Serpa cheese.

Applicable European Legislation	Regulation (EU) Nº 1151/2012 of the European Parliament and of the Council of 21 November 2012, on quality schemes for agricultural products and foodstuffs.
	Commission Implementing Regulation (EU) Nº 668/2014 of 13 June 2014 laying down rules for the application of Regulation (EU) Nº 1151/2012 of the European Parliament and of the Council on quality schemes for agricultural products and foodstuffs.
	Commission Communication — Guidelines on the labelling of foodstuffs using protected designations of origin (PDOs) or protected geographical indications (PGIs) as ingredients (2010/C 341/03).
	Regulation (EC) Nº 882/2004 of the European Parliament and of the Council of 29 April 2004, on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Amended by: Commission Regulation (EC) Nº 776/2006 of 23 May 2006 amending Annex VII to Regulation (EC) Nº 882/2004 of the European Parliament and of the Council as regards Community reference laboratories.
	2007/363/EC: Commission Decision of 21 May 2007, on guidelines to assist Member States in preparing the single integrated multi-annual national control plan provided for in Regulation (EC) Nº 882/2004 of the European Parliament and of the Council (notified under document number C(2007) 2099).
	Regulation (EC) Nº 852/2004 of the European Parliament and of the Council of 29 April 2004, on the hygiene of foodstuffs.
	Regulation (EC) Nº 853/2004 of the European Parliament and of the Council of 29 April 2004, laying down specific hygiene rules for food of animal origin. Amended by: Commission Regulation (EC) Nº2074 of 5 December 2005; Commission Regulation (EC) Nº 2076/2005 of 5 December 2005; Comission Regulation (EC) Nº 1662/2006 of 6 November 2006; Council Regulation (EC) Nº 1791/2006; Commission Regulation (EC) Nº 1243/2007 of 24 October 2007; Commission Regulation (EC) Nº 1020/2008 of 17 October 2008; Regulation (EC) Nº 219/2009 of the European Parliament and of the Council of 11 March 2009; Commission Regulation (EC) Nº 1161/2009 of 30 November 2009; Commission Regulation (EU) Nº 558/2010 of 24 June 2010; Corrected by: OJ L 226, 25.6.2004, p. 22 (853/04).
	Commission Regulation (EC) Nº 2074/2005 of 5 December 2005, laying down implementing measures for certain products under Regulation (EC) Nº 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) Nº 854/2004 of the European Parliament and of the Council and Regulation (EC) Nº 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) Nº 852/2004 of the European Parliament and of the Council and amending Regulations (EC) Nº 853/2004 and (EC) Nº 854/2004. It provides, in its Article 7, a derogation from Regulation (EC) Nº 852/2004 as regards foods with traditional characteristics, defining what foods with traditional characteristics are and laying down the requirements that may be granted to establishments manufacturing foods with such characteristics.
	Commission Regulation (EC) Nº 2073/2005 of 15 November 2005, on microbiological criteria for foodstuffs. Amended by: COMMISSION REGULATION (EC) Nº 1441/2007 of 5 December 2007; Commission Regulation (EU) Nº 365/2010 of 28 April 2010; Commission Regulation (EU) Nº 1086/2011 of 27 October 2011; Commission Regulation (EU) Nº 209/2013 of 11 March 2013; Commission Regulation (EU) Nº 1019/2013 of 23 October 2013; Commission Regulation (EU) Nº 217/2014 of 7 March 2014; Commission Regulation (EU) Nº 2015/2285 of 8 December 2015; Commission Regulation (EU) Nº 2017/1495 of 23 August 2017; Commission Regulation (EU) Nº 2019/229 of 7 February 2019; Corrected by: Corrigendum, OJ L 278, 10.10.2006, p. 32 (2073/2005); Corrigendum, OJ L 283, 14.10.2006, p. 62 (2073/2005); Corrigendum, OJ L 068, 13.3.2015, p. 90 (1086/2011); Corrigendum, OJ L 195, 20.7.2016, p. 82 (1441/2007); Corrigendum, OJ L 195, 20.7.2016, p. 83 (1019/2013).
Applicable National Legislation	Legislative order nº 9/2015 - Repeals Normative Order Nº38/2008 of 4 July and establishes procedures for the recognition of foods with traditional characteristics and traditional production methods, for granting adaptations to hygiene requirements applicable to food production, and for granting the derogations provided for in Article 7 of Commission Regulation (EC) Nº 2074/2005 of 5 December.
	Interpretative Note Nº 1/2015 of Legislative order Nº 9/2015.
	Legislative order nº 11/2018 - Determines that under Regulation (EC) Nº 882/2004 of the European Parliament and of the Council of 29 April 2004, the Directorate-General for Agriculture and Rural Development (DGADR) is the competent authority responsible for carrying out official controls.

APPENDIX 2

Appendix 2 - Dairy nutrients, substances or foods for which the authorization request to use the health claim referred, submitted to EFSA under Regulation (EC) Nº 1924/2006 , was not accepted (EC, 2018).

Dairy Nutrient, substance or food	Claim Required	Status	EFSA Reference
Artigo 13 (1) - Health claims other than those referring to the reduction of disease risk			
(Cow's) Milk And dairy products for which milk is the main ingredient and no sugar has been added – e.g. yoghurt, cheese.	• (Cow's) Milk products help: ○Support the normal and healthy development of teeth. ○Contributes to dental health.	Non-authorised	(EFSA/NDA, 2011h)
Calcium in dairy products	• Calcium naturally present in dairy products: ○Is important for weight management ○Has been shown to stimulate lipolysis. ○Aids weight loss. ○Modulates fat metabolism. ○Helps promote fat loss.	Non-authorised	(EFSA/NDA, 2010c)
Carbohydrates in dairy products	• Carbohydrates in dairy products: ○Have very low glycemic index (GI). ○Give a very low and slow blood glucose response.	Non-authorised	(EFSA/NDA, 2010d)
Dairy* (low fat dairy) *Dairy refers to cow's milk, yoghurt and cheese	• Dairy in an energy restricted diet or as part of a weight loss diet: ○Weight loss. ○Weight maintenance.	Non-authorised	(EFSA/NDA, 2011h)
Fermented dairy products (food not covered by specific food legislation - was food on Irish market before 1st July 2007)	Exact wording of claim as it appears on product: • Dairy in an energy restricted diet or as part of a weight loss diet: ○Weight loss. ○Weight maintenance.	Non-authorised	(EFSA/NDA, 2011h)
Fermented dairy products (food not covered by specific food legislation - was food on Irish market before 1st July 2007)	• Exact wording of claim as it appears on product: ○Enhances natural resistance. ○Strengthen your immunity.	Non-authorised	(EFSA/NDA, 2011f)
Fermented dairy products (food not covered by specific food legislation - Was food on Irish market before 1st July 2007)	• Probiotic: ○Enhances levels of beneficial microflora ○Balances intestinal microflora ○Beneficially affects the intestinal flora ○Supports a balanced beneficial gi microflora.	Non-authorised	(EFSA/NDA, 2009c)
Fermented milk with *Lactobacillus paracasei* lpc 37	• Microflora of the gastrointestinal tract ○Natural defence /immune system.	Non-authorised	(EFSA/NDA, 2010h)
Fermented whey	• For stomach health. ○Maintains a healthy gut bacteria population and aids the metabolism. ○The (L+) lactic acid is a natural prebiotic and makes the metabolism more effective.	Non-authorised	(EFSA/NDA, 2010f)
Peptides (milk products fermented with *L. helveticus* lactic acid bacteria)	○Help to control blood pressure.	Non-authorised	(EFSA/NDA, 2009d, 2009e, 2012)
Dairy products containing the combination of three probiotic ingredients*: Lactobacillus casei* F19, *Bifidobacterium lactis* Bb12, *Lactobacillus acidophilus* La5	○Balances the gut flora. ○Supports a healthy gut flora. ○Support gastrointestinal conditions during antibiotic treatment.	Non-authorised	(EFSA/NDA, 2009e)
Fat free dairy products with reduced sugars or without added sugars	○Help control body weight as part of a balanced diet. ○Other examples of wordings In English: ○Help maintain body weight. ○Help keep your figure. ○Nutritional partner of your figure.	Non-authorised	(EFSA/NDA, 2011h)

Cheese (three probiotic cheese products marketed by The Irish Dairy Board which contain *Lb. paracasei* (NFBC 338) (food not covered by specific food legislation - was food on Irish market before 1st July 2007)	Exact wording of claim as it appears on product: **Claims:** o Enjoyed regularly, it will help maintain a healthy digestive system. For best results and full benefits of probiotic culture, consume cold. o Helps maintain a healthy digestive lifestyle and a balanced diet. To guarantee the benefits, this cheese should be eaten uncooked. • **Examples of any alternative wording that may be used in relation to claim:** • Probiotic Culture *Lb. paracasei* (NFBC 338): o Help maintain a healthy digestive system. o Helps ensure a healthy digestive lifestyle. o Helps maintain a balanced digestive system.	Non-authorised	(EFSA/NDA, 2010j)
Raw or processed food products of animal origin, plus bread and panification products, among these (cow's, goat's and ewe's milk and related products:(milk, cream, butter, cheese, yoghurt, fresh dairy products). (products which fit into a diet naturally rich in omega 3 marketed by The Tradilin network)	Products which fit into a diet naturally rich in omega 3 which: o Participates to improve/decrease carbohydrate metabolic disorders. o Participates in the control of parameters of the metabolic syndrome. o Participates/contributes/helps to regulate carbohydrate metabolism. o Participates/contributes/helps to regulate carbohydrate metabolism. o Helps to better to regulate carbohydrate metabolism/ control the parameters on the metabolic syndrome.	Non-authorised	(EFSA/NDA, 2011h)
Bifidobacterium (BB12) fortified cultured milk (Hodzeko-Amasi)	Enhances: o Natural immune function o Helps maintain blood cholesterol o Reduces symptoms of inflammatory bowel conditions.	Non-authorised	(EFSA/NDA, 2011c)
Calcium	o Diet which includes several daily servings of low-fat milk products (about 1200 mg of calcium/day) helps to control blood pressure.	Non-authorised	(EFSA/NDA, 2009a)
Milk fat globule membrane/Milk phospholipids	Helps to: o Support/strengthen the natural defence o Support/strengthen the body's defences o Functioning of the digestive system.	Non-authorised	(EFSA/NDA, 2010k)
Artigo 13 (5) – Health claims referring to newly developed scientific data and/or which include a request for the protection of proprietary data.			
Dairy product (milk beverage) enriched with milk peptide and magnesium	o Helps to moderate signs of anxiety in mildly stress-sensitive adults.	Non-authorised	(EFSA/NDA, 2008c)
Fat-free yoghurts and fermented milks with live yoghurt cultures	• With added vitamin D, and with no added sugars in the context of an energy-restricted diet help to: o Maintain lean body mass (muscle and bone) o Reduce body and visceral fat.	Non-authorised	(EFSA/NDA, 2015b)
Low-fat fermented milk with a combination of fructo-oligosaccharides (FOS) and live *Lactobacillus rhamnosus* GG (ATCC53103), *Streptooccus thermophilus* (Z57) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB2).	o Helps to reduce recurrence of lip cold sores caused by Herpes simplex virus infection in healthy susceptible individuals.	Non-authorised	(EFSA/NDA, 2016c)

Live *Lactobacillus casei* strain Shirota as present in a fermented milk product	○Daily consumption helps maintain the upper respiratory tract defences by helping to support immune functions	Non-authorised	(EFSA/NDA, 2010a)
Milk product, rich in fibre and protein	○Reduces the feeling of hunger	Non-authorised	(EFSA/NDA, 2008e)
Artigo 14 (1) (a) – Health claims referring to reduction of a risk factor in the development of a disease.			
Lactobacillus helveticus fermented Evolus® low-fat milk products	○Reduces arterial stiffness.	Non-authorised	(EFSA/NDA, 2008d)
Artigo 14 (1) (b) - Health claims referring to child development and health.			
Dairy products (milk and cheese)	○Promote dental health in children.	Non-authorised	(EFSA/NDA, 2008a)
Dairy products (milk, cheese and yoghurt)	○Three portions of dairy food every day, as part of a balanced diet, may help promote a healthy body weight during childhood and adolescence.	Non-authorised	(EFSA/NDA, 2008b)

APPENDIX 3

Appendix 3 - Health claims authorized for nutrient, substance or food, under Article 13º of the Regulation (EC) Nº 1924/2006 (EC, 2006c), which can be applied to dairy products. Source: (EC, 2012a, 2018; Hess et al., 2016).

Nutrient, substance or food	Claim	Status	EFSA Reference
Foods with a low or reduced content of saturated fatty acids The claim may be used only for food which is low in saturated fatty acids as listed in the Annex to (EC, 2006c)	Reducing consumption of saturated fat contributes to the maintenance of normal blood cholesterol levels.	**Authorised** *Applicable to dairy products: ○Milk (fat-free); ○Yoghurt (fat-free); ○Cottage cheese (fat-free).	(EFSA/NDA, 2011d)
Foods with a low or reduced content of sodium The claim may be used only for food which is low in sodium/salt as listed in the Annex to (EC, 2006c)	Reducing consumption of sodium contributes to the maintenance of normal blood pressure	**Authorised** *Applicable to dairy products: ○Milk (2%, 1%, fat-free); ○Yoghurt (low-fat, fat-free).	(EFSA/NDA, 2011e)
Live yoghurt cultures In order to bear the claim, yoghurt or fermented milk should contain at least 10^8 ufc/g of live starter microorganisms (*Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*)	Improve lactose digestion of the product in individuals who have difficulty digesting lactose.	**Authorised** *Applicable to dairy products: ○Yoghurt; ○Fermented milk products.	(EFSA/NDA, 2010g)
Phosphorus The claim may be used only for food which is at least a source of phosphorus as as listed in the Annex to (EC, 2006c)	Contributes to: • Normal energy-yielding metabolism • Normal function of cell • The maintenance of normal bones • The maintenance of normal teeth • The maintenance of muscle mass.	**Authorised** *Applicable to dairy products: ○Yoghurt (low-fat, fat-free) ○Cottage cheese (2%, 1%, fat-free).	(EFSA/NDA, 2009f)
Protein The claim may be used only for food which is at least a source of protein in the Annex to (EC, 2006c)	Contributes to the maintenance of normal bones.	**Authorised** *Applicable to dairy products: ○Milk (2%, 1%, fat-free) ○Yoghurt (low-fat, fat-free) ○Cottage cheese (2%, 1%, fat-free).	(EFSA/NDA, 2010i, 2011a)
Vitamin D The claim may be used only for food which is at least a source of vitamin D as listed in the Annex to (EC, 2006c)	Contributes to: • Normal absorption/utilization of calcium and phosphorus • Normal blood calcium levels • Maintenance of normal bones • Maintenance of normal muscle function • Maintenance of normal teeth • Normal function of the immune system • Process of cell division.	**Authorised** *Applicable to dairy products: ○Milk (2%, 1%, fat-free) ○Vitamin D-fortified milk.	(EFSA/NDA, 2009b, 2009g, 2010l, 2011a)

* all calculations for applicable dairy products meeting EFSA health claim standards were completed using the USDA Natl. Nutrient Database for Standard Reference. The following specific products were used:milk, fluid 2% with added vitamin A and vitamin D; milk, fluid 1% with added vitamin A and vitamin D; milk, nonfat, fluid, with added vitamin A and vitamin D (fat-free or skim); yogurt, plain, low-fat, 12 g protein per 8 ounce; yogurt, plain, skim milk, 13 grams protein per 8 ounce; cheese, cottage, low-fat, 2% milk fat; cheese, cottage, low-fat, 1% milk fat; cheese, cottage, non fat, uncreamed, dry, large or small curd. Vitamin D amounts used in the above calculations were the values for D2 + D3 (listed in µg) and for vitamin D the values used were the RAE values (also listed in µg).

APPENDIX 4

Appendix 4 - Main biologically active components of milk and dairy products and some of their potential beneficial effects on health fects.

Biologically active components in milk and/or fermented dairy products	Biological activity or health benefit	Bibliography
Calcium	• Bone mass density; • Blood pressure regulation; • Decreased risk of central adiposity.	(Hess et al., 2016)
Enzymes	• Digestion; • Antioxidante; • Antimicrobial activity.	(Bhat & Bhat, 2011; Da Silva & Rudkowska, 2016)
β-lactoglobulin	• Emulgator; • Immunomodulatory; • Facilitates calcium, vit. A, vit. D, FA reabsorption, because it binds to these compounds.	(Bhat & Bhat, 2011)
Bioactive peptides of β-lactoglobulin **(β-lactorphin; lactokinins; met-his-lle-arg-leu; lle-lle-ala-glu-lys; β- lg f(15-20), f(25-40); f(78-83); f(92-100); β-lactotensin; dykky(98-102)**	• Antihipertensive; • Antithrombotic; • Opioid; • Cognitive health benefits; • Antimicrobial; • Immunomodulatory; • Hypocholesterolemic; • Radical-scavenging activity; • Anxiolitic; • Anti-inflammatory; • Better oral tolerance (because low IgE specific for the protein)	(Bhat & Bhat, 2011; Hernández-Ledesma, Recio, & Amigo, 2008; Hess et al., 2016; Sultan et al., 2018)
α-lactalbumin	• Anticancer activity; • Probable anti ulcerative properties; • Low allergy inducing potencial	(Bhat & Bhat, 2011)
Bioactive peptides of α-lactalbumin **(tripepetid gly-leu-phe; α-lacthorphin; lactokinins; met-his-lle-arg-leu; lle-lle-ala-glu-lys)**	• Important antimicrobial activity; • Opioid; • Hypotensive; • Antioxidant.	
Lactoferrin **(glycoprotein)**	• Regulation of iron homeostasis; • Defense against a broad range of infections; • Anti-inflammatory activity; • Cancer proteccion; • Antiviral activity (hepatitis, papillomavirus, herpes simplex); • Preventive effects on chemically induced colon carcinogenese and transplanted carcinoma cells metastasis in the mouse.	
Bioactive peptide of lactoferrin – lactoferricin	• Immunomodulatory; • Antiviral activity (hepatitis, papillomavirus, herpes simplex).	
Bioactive peptide of caseins – Phosphopeptides	• Originates organophosphact salts which act as carriers of cations in the intestine; • Anticariogenic effect (recalcification, inhibiting adhesion and growth of plaque forming bacteria).	(Bhat & Bhat, 2011; Da Silva & Rudkowska, 2016; García-Tejedor et al., 2015; Hess et al., 2016; Summer et al., 2017; Wu et al., 2016)
Tripeptides – **IPP (Ile-Pro-Pro) and VPP (Val-Pro-Pro); LHLPLP; HLPLP; C12 peptide**	• Opioid; • Cognitive health benefits; • Immunomodulatory; • Anti-hypertensive (due to inhibition of ace); • Anti-inflammatory; • Insulin-mimetic; • So, possible protection against metabolic disease.	

Immunoglobulins **(IgG1, IgG2, IgM, IgA)**	• General protection agains pathogens; • Activation of complement; • Stimulation of phagocytosis; • Preventing adhesion of microbes; • Neutralization of viruses and toxins; • Increases levels of glutathione a key cell antioxidant and activity against different microbial infeccions.	
Cytokines, chemokines (that operate in network)	• Contributes to: o orchestration, development and functions of the immune system.	(Bhat & Bhat, 2011)
Lactulose **(originates from lactose during heat processing of milk oligosaccharides i.e. short-chain non-digestible carbohydrates that act as prebiotics)**	• Prebiotic effect; • Immunostimulatory; • Anti-inflammatory; • Antiviral; • Immunological function.	
Polysaccharides **Some polysaccharides in dairy also act as prebiotics and are fermented by the intestinal microbiota to short chain fatty acids (SCFA)**	• Antioxidant; • preventing adhesion of microbes; • Immunostimulatory; • Hypocholesterolemic.	
Lactic acid **(originates from lactose throughout the fermentation process. The low pH and consequent destabilization of casein alters the physical properties of milk and explains some beneficial effects on health)**	• Tolerateded by lactose intolerant people; • Promotes digestibility; • Improves utilization of calcium and other minerals; • Inhibits the growth of potencially harmful microrganisms.	(Marco et al., 2017)
Dairy Fat	• Milk fat is always a delivery medium for fat soluble nutrients (e.g.vitamins).	(Bhat & Bhat, 2011; Marco et al., 2017)
Milk fat globule membrane	• Cognitive health benefits; • Delayed onset of Alzheimer's disease.	(Bhat & Bhat, 2011; Gómez-Cortés et al., 2018; Hess et al., 2016; Park et al., 1997; Summer et al., 2017),
Conjugated linoleic acid (CLA), sphingomyelin, butyric acid (only in small ruminant milk), ether lipids, β-caroten, vitamins A and D	• Anticarcinogenic potencial; • Preventing diabetes; • CLA plus: o Preventing atherosclerosis; o Modulating immune system; o Fat mass reductions; • Sphingolipids and metabolits plus: o Antimicrobial; o Immunomodulatory; o Inhibition of cholesterol adsorption.	
Caprylic acid (C8:0) **Capric acid (C10:0)**	• Antiviral activities; • Vasodilation inducer (capric acid).	
Lauric acid (C12:0)	• Antiviral and antibacterial activities • Anticariogenic and antiplaque effect • Increases the levels of HDL-C	
Palmitoleic acid (C16:1)	• Preventing diabetes.	
Phytanic acid (C20:0)		

APPENDIX 5

Appendix 5 - Results of the bibliographic search performed regarding the use of sheep cheese as a matrix for different probiotics. The type of cheese and the strain (s) tested are given, as well as a summary of the results obtained as regards the viability of the strains concerned in this matrix and the consequences on the characteristics of the cheese.

Cheese	Probiotic strain	Conclusion	Reference
Pecorino cheese	*Lb. acidophilus* (LA-5), *B. lactis* (BB-12), and *B. longum* (BB-46)	*Lb. acidophilus* and *Bifidobacterium* mix displayed counts of 8 log cfu/g and 9 log cfu/g, respectively, in cheese during ripening; The cheese with the bifidobacteria mix displayed a greater degradation of casein (CN) matrix, resulting in the highest values of non-CN, N and water soluble N and the highest amount αs-CN degradation products in cheese at 60 d of ripening. The cheese with L*b. acidophilus* displayed intermediate levels of LAB and of nitrogen fractions; There were no differences in odor and taste scores for sampled added with probiotics.	(Santillo & Albenzio, 2008)
Pecorino cheese	*B. longum* 46, *B. lactis* BB-12, and *L. acidophilus* La-5	Lower pH in curd and cheese containing probiotics during ripening; Lower hardness in probiotic cheeses, mainly related to the primary proteolysis, a breakdown of both α- and β-CN fractions; More intense lipolysis was detected in cheese containing probiotics in terms of both free fatty acids and CLA content; the latter was found at the highest levels in cheese containing *Lb. acidophilus*. Cheese acceptability was expressed for color, smell, taste, and texture perceived during cheese consumption; all these attributes scored values over 7 on a 9-point scale and no differences were reported among cheeses.	(Albenzio et al., 2010)
Pecorino cheese	*Lb. acidophilus* (LA-5), *B. lactis* (BB-12), and *B. longum* (BB-46)	Ovine cheese containing probiotic strains presented a more intense proteolysis and a greater level of short chain free fatty acids and CLAdue to the metabolic activity of the adjunct microflora; The sensorial profile of ovine cheese showed lower humidity and gumminess in cheeses containing probiotics as a consequence of differences in the maturing process; furthermore, probiotic cheeses scored higher ratings for salty and pungent attributes.	(Santillo & Albenzio, 2015)
Pecorino Siciliano cheese	*Lb. rhamnosus* H25, *Lb.paracasei* N24	Both strains exhibited heat resistance and survival throughout cheese production and ripening; The presence of these strains positively influenced the physical-chemical, microbiological and sensorial characteristics of the final product; The molecular typing of lactobacilli obtained from faeces of healthy volunteers that consumed the cheese revealed a high survival of the strains, evidencing their persistence during passage through the GIT.	(Pino et al., 2017)
Scamorza cheese	*B. longum* 46, *B. lactis* BB-12, and *L. acidophilus* La-5	*B. longum* and *B. lactis* strains sustained greater proteolysis in cheese, leading to more complex soluble peptide and free amino acid profiles; *Lb. acidophilus* strain ruled lipolysis and was able to significantly increase vaccenic and oleic acids and CLA content in cheese; *Lb. acidophilus* and the mix of the *B. longum* and *B. lactis* mean values were 7.55 ± 0.07 and 9.09 ± 0.04 log cfu/g, respectively. In addition, probiotic cheeses had lower pH values.	(Albenzio, Santillo, Caroprese, Ruggieri, et al., 2013)
Scamorza cheese	*B. longum* 46, *B. lactis* BB-12, and L*b. acidophilus* La-5	Probiotic cheese showed higher color and structural uniformities, lower friability and adhesively, and was more creamy and grainy evaluated by descriptive sensory analysis; Bifidobacteria determined the greatest differences compared with the control product, whereas the incorporation of *Lb. acidophilus* LA-5 produced intermediate characteristics.	(Albenzio, Santillo, Caroprese, Braghieri, et al., 2013)
Canestrato Pugliese hard cheese	*B. bifidum* Bb02 and/or *B. longum* Bb46	*B. bifidum* cells survived in cheeses at concentrations up to 6 log cfu/g for at least 56 d of ripening, while *B. longum* survived for 35 d; No significant differences were observed in the main chemical composition, and only a slightly higher concentration of acetic acid was found in cheeses with bifidobacteria added; Cheeses with bifidobacteria added contained significantly higher α- and β-galactosidase activities; No differences were found neither for the	(Corbo, Albenzio, De Angelis, Sevi, & Gobbetti, 2001)

		free amino acid and free fatty acid contents nor in the sensory acceptance.	
Semi-hard ewe's cheese	*B. animalis* subsp. *lactis* BB-12 and *Lb. acidophilus* LA-5.	The number of live probiotic bacteria remained at about 10^6-10^7 cfu/g during 45 days of ripening; *Lb. acidophilus* LA-5 improved the taste of cheeses; Addition of probiotic cultures did not significantly influence the chemical properties and microbiological quality of produced cheeses.	(Blazic et al., 2011)
Argentinean cheese	*L. acidophilus* (LA-5), *B. lactis* (BB-12)	*Lb. acidophilus* La-5 and *B. animalis spp. lactis* BB-12 strains reached satisfactory levels (> 10^6 cfu/g).The added probiotic cultures did not modify the different parameters evaluated: gross composition, nitrogen fractions, lipolysis, fatty acids profiles of cheese fat including CLA and volatile profile of cheeses.	(Perotti et al., 2014)
Feta cheese	*Lb. casei* ATCC 393	Data demonstrated that both free and immobilized *Lb. casei* survived during the maturation process and were detected at the essential concentration for providing health benefits.	(Dimitrellou, Kandylis, Sidira, Koutinas, & Kourkoutas, 2014)
Feta cheese	*Lb. plantarum* T571	Results showed that the probiotic strain survived during storage at 4º and 12ºC and was detected in adequate amounts until the end of storage; promoted the reduction of *L. monocytogenes* in a shorter time period compared to the control samples; new products inoculated were of high quality and with sensory characteristics similar to the typical characteristics of Feta cheese.	(Papadopoulou et al., 2018)
Lighvan cheese	*B. lactis* subsp. animalis	*B. lactis* subsp. *animalis* cells survived in cheese at concentrations up to 6.84 log10 CFU/g for at least 60 days of ripening. *B. lactis* subsp. *animalis* affected proteolysis and lipolysis, characteristics of the traditional Lighvan cheese but it did not have any effect on sensory properties of probiotic Lighvan cheese. Besides meeting precise consumer demand, the production of functional or probiotic cheeses may be useful for differentiating and increasing the market popularity of various Iranian cheeses such as traditional Lighvan, which still have a strict regional tradition. If eaten daily, probiotic Lighvan cheese can be considered as a probiotic vector or as an additional variety supporting other probiotic foods that are eaten daily but we can conclude that in cheeses ripened in brine, a significant part of ripening products is transferred into brine and their effects on sensory properties of the final product are limited.	(Lavasani, 2018)