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Characterization of bacteria isolated from Portuguese traditional cheeses

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Abstract

Azeitão and Nisa cheeses are products with protected designation of origin (PDO) traditionally manufactured in Portugal with sheep's raw milk. The predominant group of bacteria in cheeses is lactic acid bacteria (LAB) which includes genera like *Lactococcus* spp. and *Enterococcus* spp., these are the microorganisms our work will be focusing on. The fact that these bacteria have important roles both in foods and the gut of animals (including humans) makes them conductors for both positive impact, such as probiotic features, and negative impact, such as transference of virulence factors or antibiotic resistances. Our aim with this work was to analyse the diversity of LAB in Azeitão and Nisa cheeses from several origins and years of production and assess the negative traits these bacteria could have and transfer such as antibiotic resistances, hemolysis and production of gelatinase.

Enumeration of previously mentioned bacteria was performed, and results were compared to other years of production. During the years studied no unit had consistently higher CFU counts. *Lactococcus* spp. was the group with highest bacteria counts in the majority of units during the three years studied and *Enterococcus* spp. was the one with the lowest CFU. For diversity analysis, RAPD-PCR were performed in order to create dendrograms for each cheesemaking unit and bacterial group. Just like for bacterial enumeration, no specific trends were observed in the diversity values for the units throughout the studied years. Concerning 2018 cheeses, the group with the highest diversity was *Enterococcus* spp. even though it was also the one with less CFU. This independence between CFU counts and diversity was noted all through our work in different years, units and groups of bacteria. Identification of 2017 enterococci representatives was performed using a multiplex PCR and showed a predominance of *E. faecium*, followed by *E. faecalis* and *E. durans* that were equally represented.

Pathogenic potential of representative isolates was assessed through the search of antibiotic resistances and virulence factors. Antibiotic susceptibility was studied through disc diffusion assays. Significant differences were observed in the number of resistances found in studied years for lactococci and enterococci. Between units there was also significant differences in the total number of resistances during 2016, 2017 and 2018 in enterococci. In LAB isolates resistances considered non intrinsic were found, to clindamycin and erythromycin. Furthermore, enterococci isolates resistant to teicoplanin, ciprofloxacin, tetracycline, chloramphenicol, erythromycin and vancomycin were observed. Isolates resistant to three or more antimicrobial agents were observed but these didn't comply with the characteristics necessary to be classified as multidrug-resistant (MDR) bacteria. Virulence factors were studied and hemolysis was detected in 11% of representative isolates from 2016, 6% from 2017 and 12% in isolates from 2018 cheeses. Furthermore, only two positive results for gelatinase were found in 2017 representative isolates.

In conclusion, our results showed that no pattern of bacterial enumeration or microbiome diversity is present throughout different years of production in artisanal cheeses such as the ones studied. Moreover, although alarming resistances were found in enterococci no multi-drug resistance isolates were observed. Further work should be performed to continue the characterization of the pathogenic potential of isolates present in these cheeses.

Keywords: traditional Portuguese cheese; protected designation of origin; lactic acid bacteria; microbiome diversity; antibiotic resistance

Resumo

A fermentação tradicional de queijos é realizada em muitos países do mundo, nomeadamente Portugal, sendo que consiste no aproveitamento dos microrganismos naturalmente presentes no leite cru. Estes microrganismos encontram-se no leite devido às diferentes etapas de colheita e manuseamento durante e após esse processo. Sendo que, alteram as moléculas presentes no leite através da fermentação dando novas propriedades ao produto final que será posteriormente curado. As condições destas fases de fermentação e maturação diferem entre tipos de queijos e zonas de produção, determinando as propriedades organoléticas do produto final.

Os queijos de Azeitão e Nisa fazem parte dos 10 queijos portugueses que possuem a categoria de denominação de origem protegida (DOP). As zonas de produção dos queijos de Azeitão são Palmela, Sesimbra e Setúbal e as de Nisa são Nisa, Crato, Castelo de Vide, Marvão, Portalegre, Monforte, Arronches e Alter do Chão. Ambos são produzidos com leite de ovelha cru. No caso de Nisa o leite provém de uma raça de ovelha chamada Merina Branca e no caso de Azeitão não se especifica uma raça. O coalho vegetal usado na produção de ambos queijos é obtido de *Cynara cardunculus* L. Devido às diferentes fases e condições de produção o queijo de Azeitão consiste numa pasta semi-mole e amanteigada, de cor branca ou ligeiramente amarelada com um sabor ácido e salgado. O queijo de Nisa consiste numa pasta semi-dura de cor branca amarelada com um sabor ligeiramente ácido e um cheiro intenso.

As bactérias ácido lácticas (BAL) são um grupo de vários géneros que partilham características em comum como serem gram-positivas, catálase negativas, não formam esporos, anaeróbicas facultativas e terem um nível G+C baixo. Para além disto, o seu nome provém da sua capacidade para fermentar açucares, transformando-os em ácido láctico através de homo- ou heterofermentação. Este grupo é o predominante em leite cru e, portanto, em queijos produzidos de forma artesanal e essas bactérias vão desempenhar diversos papéis ao longo da fermentação e maturação deste tipo de queijo. Os géneros de BAL que predominam em comidas fermentadas como os queijos são *Lactococcus, Streptococcus, Pediococcus, Leuconostoc, Lactobacillus* e *Enterococcus*. Tendo em conta que estas bactérias formam parte tanto da cadeia alimentar como da microbiota de animais e seres humanos podem servir como veículo de transmissão de genes ao existir uma transferência genética entre espécies ou géneros, como descrito na literatura. A problemática desta possível transferência ocorre quando esses genes conferem resistência a antibióticos ou fatores de virulência que anteriormente essas bactérias não possuíam. Essa transferência pode ocorrer para algumas bactérias que formam parte da nossa microbiota e são responsáveis por infeções oportunistas ou para bactérias patogénicas presentes no nosso corpo devido a uma infeção. Resistências adquiridas a antibióticos têm sido estudadas e observadas em BAL, em concreto em *Enterococcus* spp. devido ao seu papel patogénico oportunista. Foram descritas resistências a antibióticos de diferentes classes como ß-lactâmicos, cefalosporinas, aminoglicósidos, lincosamidas e estreptograminas. Em concreto, resistências à tetraciclina e eritromicina são das mais preocupantes devido à importância destes antibióticos e porque esta resistência tem sido atribuída ao uso indevido de antibióticos em comida de animais.

Este trabalho teve como objetivo continuar o estudo de queijos de Azeitão e Nisa DOP que começou numa dissertação anterior onde se caracterizaram estes queijos pela sua microbiota e propriedades físico-químicas. Seguido deste estudo foi também analisada a diversidade dos microbiomas destes produtos como parte de outra dissertação. Assim, o presente trabalho consistiu em comparar os resultados anteriores de diversidade e caracterização microbiológica assim como completar essa análise com a procura de resistências a antibióticos e fatores de virulência.

Durante o ano de 2018 foram recolhidos queijos de seis queijarias de Azeitão e de duas queijarias de Nisa. A partir destes foram feitas contagens de unidades formadoras de colónias (UFC) e isoladas as bactérias de diferentes grupos, nomeadamente, BAL isoladas com meio MRS (maioritariamente

Lactobacillus spp.), *Lactococcus* spp. com meio M17 e *Enterococcus* spp. isoladas com meio SBA. Depois foi extraído o ADN desses isolados para realizar a técnica de RAPD-PCR e análise de dendrogramas criados com os perfis de bandas obtidos. A partir estes dendrogramas foi analisada a semelhança entre os isolados dos distintos queijos e foram obtidos índices de diversidades dos diferentes grupos de bactérias e queijarias estudadas. Esta diversidade e as contagens de bactérias foram comparadas às obtidas noutras dissertações deste mesmo projeto nas quais se estudaram anos de produção anteriores. Partindo destes mesmos dendrogramas, foram escolhidos isolados representantes de cada queijaria e realizados testes de resistência a antibióticos assim como a presença de fatores de virulência.

Relativo aos diferentes anos de produção estudados não foi observada uma tendência clara de contagens de UFC quando comparados os anos ou queijarias, isto é, não houve queijos que tivessem consistentemente maior ou menor número de bactérias sendo que este número foi variável ao longo dos anos. Enquanto à diversidade, foram observadas poucas coincidências nas queijarias com maior ou menor índice de diversidade. No caso dos lactococos foi observado o menor índice correspondente aos anos 2018 e 2016 na mesma queijaria, A4. Nos enterococos foi também encontrada a menor diversidade na mesma queijaria, N9, nos queijos dos anos 2018 e 2016. Nas amostras de 2018 o grupo com maior diversidade foi o dos enterococos mas cabe destacar que os três grupos bacterianos tiveram índices muito parecidos. A identificação dos representantes deste grupo de bactérias dos queijos de 2017 foi realizada com uma multiplex PCR e a maioria de isolados foi identificado como *E. faecium*, seguido por *E. faecalis* y *E. durans* estando igualmente representados em número de isolados.

Foram encontradas diferenças significativas no número de resistências ao longo dos anos nos grupos *Lactococcus* spp e *Enterococcus* spp. No caso deste último género, ocorreram também diferenças significativas entre o número de isolados resistentes de diferentes queijarias durante os três anos estudados. As resistências a antibióticos encontradas no grupo de BAL foram à clindamicina e eritromicina, no caso dos lactococos as resistências que foram observadas neste trabalho são consideradas intrínsecas pelo que não haveria possibilidade de transferência dessas resistências para outras bactérias. As resistências extrínsecas encontradas nos enterococos foram à teicoplanina, ciprofloxacina, tetraciclina, cloranfenicol, eritromicina e vancomicina. Apesar de terem sido observados isolados resistentes a três antibióticos de três classes diferentes estes não cumpriam todos os requisitos de modo a serem considerados multirresistentes. No estudo de fatores de virulência foi detetada a capacidade de hemólise em 11% dos representantes de 2016, 6% nos de 2017 e 12% nos de 2018. No teste para identificar a produção de gelatinase só foram observados dois resultados positivos e esses pertenciam a isolados de queijos de 2017.

Concluindo, a diversidade do microbioma e as contagens dos queijos de Azeitão e Nisa estudados não seguiram nenhum padrão nos anos que foram comparados. O número de *Enterococcus* spp. resistentes diminuiu desde 2016 até ao último ano estudado neste trabalho, 2018, mas as resistências encontradas nos isolados de 2018 foram a antibióticos mais relevantes para a saúde pública como são a vancomicina, eritromicina e tetraciclina. No mesmo sentido, também não foram encontrados isolados multirresistentes e os fenótipos dos fatores de virulência estudados não foram observados em grande quantidade. Contudo, deve ser referido que o estudo destes queijos continuará e serão feitos mais testes tanto no âmbito da diversidade como do potencial patogénico dos isolados das bactérias ácido lácticas presentes.

Palavras-chave: queijo tradicional Português; denominação de origem protegida; bactérias ácido lácticas; diversidade de microbioma; resistência a antibióticos

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List of relevant abbreviations:

CFU – Colony-forming units
CLSI - Clinical Laboratory Standards Institute
DNA – Deoxyribonucleic acid
EUCAST - European Committee on Antimicrobial Susceptibility Testing
FDA – Food and drug administration
HE – Hektoen enteric
HGT – Horizontal gene transfer
LAB – Lactic acid bacteria
MDR – Multi-drug resistance
MRS – Man Rogosa and Sharpe
PCR – Polymerase chain reaction
PDO – Protected designation of origin
RAPD – Random amplification of polymorphic DNA
SBA – Slanetz and Bartley agar
TSA – Trypticase soy agar
TSI – Triple sugar ion
VRE – Vancomycin-resistant enterococci
XLD – Xylose lysine deoxycholate

1- Introduction

1.1. Cheese characteristics and manufacture

Fermented foods and beverages have been produced and consumed by humans since the first civilizations. In particular, cheese may be one of the most important fermented milk products and all around the world there are a lot of cheeses still made through a traditional process of fermentation and maturation. According to *Codex Alimentarius* (Codex standard 283, 1978) cheese is the ripened or unripened soft, semi-hard, hard, or extra-hard product that may be coated, and in which the whey protein/casein ratio doesn't exceed that of milk. It is obtained by coagulating wholly or partly the protein of milk, skimmed milk, partly skimmed milk, cream, whey cream or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents. Partially the whey resulting from the coagulation is drained, while respecting the principle that cheese-making results in a concentration of milk protein (in particular, the casein portion). Consequently, the protein content of the cheese will be distinctly higher than the protein level of the blend of the above milk materials from which the cheese was made.

Inside a healthy udder milk is sterile, but the nutrient composition of milk makes it a good growth medium for many microorganisms. Milk contamination happens from three sources: from within the udder, exterior of the udder and/or from the surface of milk handling and storage equipment (Quigley et al., 2013). The most represented group of bacteria in raw milk are lactic acid bacteria (LAB), which are bacteria that ferment lactose to lactate. Within this group the dominant genera in raw milk include *Lactococcus, Lactobacillus, Leuconostoc, Streptococcus* and *Enterococcus*. The different microbiota associated to the fermented raw milk to obtain cheese has a direct impact in its organoleptic properties, quality and time until spoilage (Devirgiliis, Zinno, & Perozzi, 2013; Quigley et al., 2013).

The traditional process of fermentation in cheese is essentially the exploitation of the microorganisms naturally present in raw milk, meaning that no starter microbial cultures are added. These microorganisms will break down complex molecules into simpler ones through fermentation, giving new properties to the final product that will be further enhanced with subsequent production stages, such as cheese maturation (Macori & Cotter, 2018). Differences between cheeses, Serra da Estrela and Irish cheeses, manufactured from pasteurized or raw milk have been studied because the replacement of natural microbiota for starter cultures eventually changes the sensory properties of such cheeses (Macedo, Tavares, & Malcata, 2004; Quigley et al., 2012).

Starter bacteria need a proteolytic system to hydrolyze the milk proteins to the amino acids and peptides required for bacterial growth. This proteolysis and the capacity to produce acid rapidly are important properties of these bacteria, since it will help reduce the propensity of spoilage and the development of flavor in cheese (Cogan et al., 1997).

Due to the fact that this is a traditional process of manufacture, differences between cheesemaking units are expected and are likely to be related to different methods, raw materials and handling which can affect the final concentrations or presence of certain groups of microorganisms. This will also affect the organoleptic properties of the cheeses and emphasize variation between cheeses from different units (Pintado et al., 2008). In a study with traditional raw milk Camembert cheeses (Henri-Dubernet, Desmasures, & Guéguen, 2008) variations were observed between units where lactobacilli species had a high diversity and its dynamics varied among those dairies contributing to a specific microbiota in each cheese. Differences in bacterial development was also observed in Nostrano di Primiero cheeses (Poznanski, Cavazza, Cappa, & Cocconcelli, 2004) that were manufactured with raw milk from different regions.

1.1.1. Azeitão and Nisa protected designation of origin (PDO) cheeses

In Portugal the artisanal production of regional cheeses is an important part of cultural heritage and 10 of those traditional cheeses have PDO (https://europa.eu/rapid/press-release_IP-96-153_en.htm. Consulted: August 28, 2019). In this work we will be focusing in two of those PDO cheeses: Azeitão and Nisa. Production of Azeitão cheese is restricted to counties of Palmela, Sesimbra and Setúbal whereas Nisa cheese is produced in Nisa, Crato, Castelo de Vide, Marvão, Portalegre, Monforte, Arronches and Alter do Chão.

PDO Azeitão and Nisa cheeses are obtained from sheep raw milk, in Nisa's case the milk comes from a concrete breed of sheep called Merina Branca while in Azeitão's cheese no breed is specified. Vegetable rennet used in both cheeses is obtained from *Cynara cardunculus* L.

Production method for Azeitão cheese begins by filtering the raw milk, after this the rennet and salt are added, and the milk is stored at 30ºC for 45 min. After coagulation is completed the serum excess is manually removed and a compressed bulk is obtained, which remains 20 days at 10ºC-12ºC under a relative humidity of 85% to 90%.

On the other hand, the production method for Nisa cheese consists in adding the vegetable rennet to the milk at 25ºC-28ºC during 60 min. After this some of the serum is removed and the salt is added. The maturation consists of two phases, the first one is up to 18 days long at 8ºC-10ºC with a relative humidity of 80%-90% while the second phase lasts up to 40 days at 10ºC-14ºC and a relative humidity of 85%-90%.

As a consequence of these different production methods Azeitão cheese is semi-soft and buttery with a characteristic spicy, acidic and salted flavor, while Nisa cheese is semi-hard, with a slightly acidic flavor and intense smell. Both cheeses have white or slightly yellow color (https://tradicional.dgadr.gov.pt. Consulted: June 15, 2019).

1.1.2. Fermentative bacteria

Lactic acid bacteria (LAB) constitute a group of multiple genera that share physiological features and owe their designation to the capacity to ferment sugar into lactic acid through homo- or heterofermentative metabolism. This group is characterized by being Gram-positive, catalase negative, non-spore forming, facultative anaerobic and having low G+C content. Their natural habitats are usually nutritionally rich environments, like plants and animal raw materials, fermented food products, animal skin and mucous membranes (Settanni & Moschetti, 2010). Concerning cheese production these bacteria can play different roles such as participation in the fermentation process or maturation of cheese. As mentioned before, proteolysis is very important in cheese production for final texture and flavor. Hence, LAB possess a complex proteolytic enzymatic system and plays an important role in degradation of casein and peptides producing free aminoacids that contribute directly to the basic taste of cheese and indirectly to the production of volatile aroma compounds (Herreros, Fresno, González Prieto, & Tornadijo, 2003). Apart from their role in texture, flavor and smell development these microorganisms have also a protective role in improving food safety and as probiotic bacteria that confer health benefits for humans (Settanni & Moschetti, 2010). The most relevant LAB in fermented foods belong to the genera *Lactococcus, Streptococcus, Pediococcus, Leuconostoc* and *Lactobacillus*. Likewise, several species from these groups are part of the gut microbiota of healthy humans (Devirgiliis et al., 2013).

In a review (Quigley et al., 2013), about the microbiota of raw milk it was documented that in sheep milk, which is very used throughout Europe for cheese production, LAB is the predominant group of bacteria. The genera that dominates cheeses produced with this milk are lactococci, lactobacilli and leuconostoc.

Pico cheese is a Portuguese PDO cheese (Domingos-Lopes, Stanton, Ross, Dapkevicius, & Silva, 2016), although manufactured with raw cow milk all its process is traditional and the predominant genera from LAB are the same as mentioned before. However, in this case *Enterococcus* genus was more dominant than in previously mentioned cheeses. Terrincho cheese is also a PDO cheese traditionally manufactured with raw sheep milk. In this type of cheese (Pintado et al., 2008) *Lactobacillus* spp. and *Lactococcus* spp. were the predominant genera and enterococci were also found at considerable high numbers. São Jorge is a traditional cheese from Azores island (Portugal) (Kongo, Ho, Malcata, & Wiedmann, 2007) produced with raw bovine milk in which lactobacilli and enterococci were identified as the dominant groups of bacteria found in all phases of production. Manchego cheese, for example, is produced in Spain and some of it is manufactured from raw sheep milk. It has been documented that *Lactococcus* spp.*,* *Lactobacillus* spp. and *Leuconostoc* spp. are predominant while *Enterococcus* spp. is also present but not as much as the aforementioned (Cabezas, Sanchez, Poveda, Seseña, & Palop, 2007; Nieto-arribas et al., 2011).

Impact of different LAB in cheese's microbial community has also been studied. For example, in a review about Serra da Estrela cheeses (Macedo et al., 2004) it was observed that the addition of *Lactococcus lactis* and/or *Lactobacillus plantarum* reduces the numbers of *Enterobacteriaceae* but changes the flavor of Serra cheeses reinforcing the idea that the presence and proportion of different groups of bacteria is important to the unique characteristics of each cheese.

Enterococcus spp. is the most controversial genus in LAB due to its concerns regarding food safety and antibiotic resistances and because of this it's also one of the most studied. High levels of these bacteria usually result from poor hygienic practices during manufacture, but it has been proven they play a major role in ripening and aroma development in many cheeses such as Manchego, Mozzarella, Kefalotyri, Serra da Estrela or Cebreiro (Franz, Holzapfel, & Stiles, 1999). Persistence of these bacteria during stressful stages like ripening can be attributed to their wide range of growth temperatures, high tolerance of heat, salt and acid (Cogan et al., 1997). Although some *Enterococcus* spp. are associated to human diseases they have also an important role in food safety due to their capacity to produce enterocins that inhibit other pathogenic bacteria (Moraes et al., 2012). Moreover, much like other LAB this genus has also probiotic characteristics beneficially affecting the host by improving the properties of its microbiota (Franz et al., 1999).

Fermentative bacteria present in raw milk, and subsequently in cheeses manufactured with that milk, have different roles during fermentation. Primary role of *Lactotoccus* spp. is the acidification of cheese through the production of L-lactate but they also contribute to proteolysis, conversion of amino acids into flavor compounds, citrate utilization and fat metabolism (Smit, Smit, & Engels, 2005). *Lactobacillus helveticus* is known to have a rapid autolysis resulting in the release of intracellular enzymes and reduction in bitterness which leads to increased and desirable flavor notes in cheese (Broadbent et al., 2011). This species is also characterized for being the most proteolytic of the LAB group and the release of free fatty acid after lipolysis introduces important flavor compounds to cheese (Hickey, Kilcawley, Beresford, & Wilkinson, 2007). In Italian and Swiss-type cheeses it was observed that a consortium between different subspecies of *L. delbrueckii* and other thermophilic LAB are likely involved in correct acidification and casein degradation during starter preparation and into the cheese curd (G. Giraffa et al., 2004). Furthermore, there are several other lactobacilli that increase in number during manufacture of dairy products and become dominant during the ripening of cheese (Henri-Dubernet et al., 2008). *Streptococcus thermophilus* is widely used as a starter culture in dairy products (Ott, Germond, & Chaintreau, 2000) being considered one of the most important bacteria in this industry. Its importance is due to the ability to rapidly decrease pH through lactate formation and the production of important metabolites. Other *Streptococcus* spp. have also been isolated from artisanal raw milk cheeses (De Vuyst & Tsakalidou, 2008; Lombardi et al., 2004) and studied their technologically relevance such as ability to acidify and produce peptidases while lacking antibiotic resistance and hemolytic activity. Several *Propionibacterium* spp. have been isolated from different cheeses or milk (Meile, Le Blay, & Thierry, 2008), this group has been proposed as probiotics since the only isolates with human clinical relevance belong to the "acnes group" and dairy propionibacteria have a long documented history of use in foods. *Leuconostoc* spp. are present in milk probably due to contamination during collection or storage and processing since they have the ability to survive on surfaces and tools for long periods of time and to resist hot and cold temperatures (Hemme & Foucaud-Scheunemann, 2004). These bacteria grow poorly in milk due to a lack of proteolytic activity and require amino acids or peptides to stimulates growth provided by other microorganisms (Hemme & Foucaud-Scheunemann, 2004; Vedamuthu, 1994). Even so, genome sequencing of a strain of *L. pseudomesenteroides* isolated from dairy products (Victoria, Valentin, & Renaulta, 2012) showed genes involved in carbohydrate fermentation, protein and amino acid metabolism and a key pathway in production of aromatic compounds. Concerning *Enterococcus* spp. we have already commented the positive influence these bacteria have in many cheeses, they comprise a major part of the fresh cheese curd microbiota and, in some cases, they are the predominant microorganisms in the fully ripened product (Giorgio Giraffa, 2003). Furthermore, studies in raw milk cheese (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006) have showed that this group is an important component of the natural cultures involved in fermentations and contribute to ripening, taste and flavor. This is attributed not only to their primary and secondary metabolisms, these bacteria can also produce several enzymes that interact with milk components and promote other important biochemical transformations (Giorgio Giraffa, 2003) and contribute to fermentation due to their proteolytic activity and contribution to the development of flavor compounds (Franz et al., 1999). Finally, even though they are not bacteria, fungi and yeasts also play a major role in dairy fermentations concretely in cheese, yeasts secrete enzymes that play a key role in texture and produce various aromas during ripening (Quigley et al., 2013).

1.1.2.1. Fingerprinting and identification of LAB

Polymerase chain reaction (PCR) is a method very used in molecular biology to obtain copies of a specific DNA segment. Using this method, we are able to identify isolates to a level of genus, species or sometimes even strain. Furthermore, one molecular method widely used in biology is random amplification of polymorphic DNA (RAPD) that is a type of PCR where the segments of amplified DNA are random. This method is also used to identification usually through fingerprinting where profiles from amplified segments are obtained and grouped to find similarities between isolates.

A study with Taleggio cheese (Feligini et al., 2012) used RAPD-PCR analysis to characterize LAB isolates at various stages of cheese production. RAPD was also used in a study that investigated the origin of *Lactobacillus plantarum* from different points in the manufacture of Roncal cheese (Oneca, Irigoyen, Ortigosa, & Torre, 2003) and results allowed to conclude that those bacteria didn't come from the milk. In other foods such as sausages (Cocolin et al., 2004), RAPD has also been used to characterize *L. sakei* isolates and study the different strains found in this food. A study with isolates from traditional French cheeses (Cibik, Lepage, & Tailliez, 2000) used RAPD to differentiate strains of *Leuconostoc* isolates and allowed investigators to observe that *L. mesenteroides* was the dominant species present. Representative isolates from Manchego cheese (Nieto-arribas et al., 2011) were chosen after analysis of RAPD-PCR profiles and then a species-specific PCR was used to identify the different *Enterococcus* species present.

Through a multiplex PCR several *Lactobacillus* species were identified in a study (Kwon, Yang, Yeon, Kang, & Kim, 2004) that aimed to create this type of one-step method to identify the major probiotic species of this genera. Other study (Plessas et al., 2017) developed a multiplex PCR based on a RAPD analysis to detect *L. paracasei* in food products. Multiplex PCR have also been developed for other bacteria such as *Leuconostoc* species, in this study (Lee, Park, & Kim, 2000) it was possible to identify several species both from pure cultures and mixed populations. In a study with Bryndza cheese (Jurkovič et al., 2006) PCR was used to identify the *Enterococcus* species present and know which species dominated the different kinds of cheeses analyzed.

Overall, studies previously mentioned demonstrate the applicability of PCR-based amplification methodologies to identify and characterize cheese-related bacteria.

1.2. Pathogenic potential in cheese microbiota

1.2.1. Antibiotic resistance

The food chain has been considered as the main route of transmission of antibiotic resistant bacteria between the animal and human population (Witte, 1997). Concretely, fermented foods that aren't submitted to heat before consumption provide a vehicle for antibiotic resistant bacteria with a direct link between the animal microbiota and the human gastrointestinal tract (Mathur & Singh, 2005).

Taking this into consideration, the main problem with foodborne bacteria is their possible role as reservoir of antibiotic resistance genes that can either present a problem if this bacteria act at some point as opportunistic pathogens or be transferred to commensal bacteria and from those to human/animal pathogens and thus impairing antibiotic treatment of common infections (Devirgiliis et al., 2013; Mathur & Singh, 2005). Studying the pathogenic potential and antibiotic susceptibility of food microbiota has become more and more relevant in recent years. However, in a review about published data of antibiotic resistance in lactobacilli and lactococci (Devirgiliis et al., 2013) it was stated that, when tested in conjugation experiments, the potential of horizontal transmission to pathogens or opportunistic pathogens was low in these genera.

Resistance to antibiotics can be intrinsic to a bacterial genus or species and lead to that microorganism capacity to survive in the presence of an antimicrobial agent due to inherent characteristics of its genome. This type of resistance is usually not relevant because it's not horizontally transferable to other potentially pathogenic bacteria and in its original non-pathogenic bacteria it poses no risk. On the other hand, resistance to antibiotics can be acquired and spread horizontally among different bacteria. This type of resistance can arise from genome mutations or through the acquisition of additional genes coding for a resistance mechanism.

Lactobacilli isolates from Pico cheese (Domingos-Lopes et al., 2016) were found to be resistant to cephalosporins and in other traditional dairy products (Guo et al., 2017) high resistance to ciprofloxacin, gentamicin and streptomycin were found whereas resistance to ampicillin, penicillin, chloramphenicol and tetracycline were low level. LAB isolates from a traditional Turkish white cheese (Erginkaya, Turhan, & Tath, 2018) showed resistances to erythromycin, chloramphenicol, gentamicin and ciprofloxacin. A study that evaluated several European probiotic products (Temmerman, Pot, Huys, & Swings, 2003) detected resistances to kanamycin, vancomycin, tetracycline, penicillin, erythromycin and chloramphenicol in LAB isolates. Vancomycin resistance has been found in several studies with lactobacilli isolates from dairy products (Domingos-Lopes et al., 2016; Erginkaya et al., 2018; Gad, Abdel-hamid, & Farag, 2014) giving credibility to the theory that this resistance is intrinsic in this genus. However, resistance results in lactococci, lactobacilli and leuconostoc must be carefully interpreted since there is little consistency among researchers for phenotypical assays and official cut off values (M. Álvarez-Cisneros & Ponce-Alquicira, 2019).

Susceptibility to antibiotics present in food-related microorganisms has recently become more and more relevant especially concerning enterococci. As mentioned before this genus is highly widespread because of their adaptability to different environments and they're especially important as part of the gastrointestinal tract of humans and animals. *Enterococcus* spp. are also the only genus of LAB known as opportunistic pathogens, being a major cause of healthcare associated infections (Russo et al., 2018). This controversial role is accentuated due to their part in humans and animal microbiota, being part of the food chain, having intrinsic and acquired resistance to different antibiotics and their possible involvement in food-borne illnesses due to the presence of virulence factors. Moreover, the use of antibiotics as growth promoters in food animals has been revealed as one of the most important factors in creating reservoirs of transferable antibiotic resistance in this group (Giorgio Giraffa, 2002).

In enterococci from artisanal Portuguese cheeses (Porto, Fujimoto, Borges, Maria, & Döering, 2016) several resistances have been described such as to erythromycin, vancomycin, teicoplanin and tetracycline. A study with isolates from Terrincho cheese (Pimentel et al., 2007) showed that only 8% had some form of resistance, specifically to tetracycline and ciprofloxacin, with an overall low resistance to the antibiotics analysed. Strains isolated from French raw milk cheeses (Bertrand, Mulin, Viel, Thouverez, & Talon, 2000) showed a high-level resistance to kanamycin and gentamicin. Isolates from Italian PDO cheeses have also been studied (Russo et al., 2018), specifically species *E. faecium, E. faecalis, E. durans* and *E. hirae.* In this study the highest incidence of resistance was observed against rifampicin and erythromycin followed by chloramphenicol and tetracycline while a low resistance to vancomycin was detected. Other Italian cheeses (G. Giraffa, Olivari, & Neviani, 2000) showed resistance to vancomycin in 25% of its isolates. Patterns of antibiotic resistance in enterococci from food of animal origin in Germany (Peters, Mac, & Wichmann-schauer, 2003), including cheeses, showed resistances to penicillin, tetracycline, quinupristin/dalfopristin, chloramphenicol and erythromycin. Artisanal Turkish white cheeses were also studied (Ispirli, Demirbas, & Dertli, 2017) and the only high-level resistance identified was from two *E. durans* isolates to vancomycin. However, enterococci antibiotic resistance profiles isolated from different foods of animal origin also from Turkey (Mus et al., 2017), including dairy products, described high resistance to tetracycline followed by quinupristin/dalfopristin, ciprofloxacin, penicillin, linezolid, ampicillin, streptomycin and gentamicin. In this study many *E. faecalis* isolates were resistant to one or more antibiotics and resistance to tetracycline was especially important among *E. faecium* isolates. Isolates from European raw milk cheeses (Teuber, Meile, & Schwarz, 1999) exhibited resistance to penicillin (18%), erythromycin (48%), gentamicin (80%), tetracycline (59%), rifampicin (7%), chloramphenicol (32%), fusidic acid (14%) and vancomycin (4%). In a study about enterococci in artisanal food (Delpech et al., 2012), including cow and goat cheeses, the most frequently detected resistance among *E. faecium* was tetracycline, high resistance to erythromycin and ciprofloxacin were also observed. There were also resistances to relevant classes of antibiotics such as beta-lactams, aminoglycosides and glycopeptides some of these being known as intrinsic resistances. Moreover, resistance to linezolid in some *E. faecium* strains was also observed.

Enterococcus spp. intrinsic resistances to several antibiotic classes have been described such as ß-lactams, cephalosporins, aminoglycosides, lincosamides and steptogramins (Hollenbeck & Rice, 2012). Taking all this into account, from the resistances described above the relevant ones considering them as acquired resistances would be erythromycin, linezolid, chloramphenicol, tetracycline, teicoplanin, vancomycin and ciprofloxacin. Resistances to tetracycline and erythromycin have also been observed in isolates from animal facilities and in food of animal origin, moreover, resistance to tetracycline has been attributed to the overexploitation of these antibiotics in veterinary practices (Chopra & Roberts, 2001).

1.2.2. Virulence factors

Bacteria can act as reservoir for virulence factors genes just like with antibiotic resistance genes as seen before. Concretely, within the group of LAB, *Enterococcus* spp. has been the genus in which most studies about virulence factors have focused due to their duality in food and human infections, as we've commented before. As mentioned before, horizontal gene transfer is a process in which an organism transfers genetic material to another organism that's not its offspring. This mechanism has been shown to represent a crucial factor in bacterial evolution and a major driver of adaptation in food systems (Andam, Carver, & Berthrong, 2015). Evidence of this has been provided in cheese-associated bacteria species (Bonham, Wolfe, & Dutton, 2017), many of the transferred regions are multi-gene islands and shared by numerous. HGT enhances the evolution of antibiotic resistances and virulence factors in bacteria communities due to its capacity to transfer these genes even over species and genus borders. Furthermore, transfer of virulence determinants from enterococci to other bacteria via natural conjugation has been demonstrated before (Eaton & Gasson, 2001).

Several species of enterococci have been studied for their role on human diseases although *Enterococcus faecalis* has been considered responsible for 65% to 80% of all enterococcal healthcare associated infections and *E. faecium* for the remainder (Jett, Huycke, & Gilmore, 1994; Malani, Kauffman, & Zervos, 2002; Murray, 1990). So, numerous studies have been done searching for virulence factors on enterococci, specifically in those isolates found in food, to have a better understanding of the possible danger of treating these bacteria as probiotic.

Gelatinase is an important virulence factor since it's a protease involved in the hydrolysis of gelatine, casein, collagen, haemoglobin and small proteins. Production of gelatinase is usually associated with clinical isolates and its expression is important for the infectious process. A study about enterococcal isolates from food (Soares-Santos, Salvador Barreto, & Semedo-Lemsaddek, 2015) revealed that 39% of those isolates were gelatinase producers and all isolates harboured the gene responsible for that protein. Gelatinase activity in *Enterococcus* spp. from Pico cheese (Domingos-Lopes et al., 2016) was positive in 64% of those isolates and in isolates from Bryndza cheese (Jurkovič et al., 2006) only 4% of those showed a positive phenotype for this assay.

Hemolysin is an extracellular protein that is a toxin active against both eukaryotic and prokaryotic cells and plays an important role in enterococcal virulence. This toxin is also referred to as cytolysin because of its wide target cell range and is one of the most studied virulence traits attributed to *Enterococcus* spp. (Franz et al., 2001; Semedo et al., 2003). This role in virulence has been documented before (Ike, Hashimoto, & Clewell, 1987) since clinical isolates have been reported as hemolytic in percentages much higher than isolates from uninfected sources. In a recent study (Porto et al., 2016) production of hemolysins has been observed in all *Enterococcus* spp. isolates from traditional cheeses although results varied according to blood origin used on growth medium. Other study with raw milk and cheeses (Moraes et al., 2012) obtained similar results where most of the isolates presented ß-hemolysis. Contrary results have also been found in Turkish white cheeses (Ispirli et al., 2017) where no isolate exhibited ß-hemolysis. It has also been reported that there is a higher incidence of this virulence trait in *E. faecalis* than for *E. faecium*, which correlates with this species causing more enterococcal infections (Franz et al., 2001).

Taking this into account, it seems the percentage of phenotypically positives for gelatinase and hemolysin in food isolates is variable.

1.3. Aims of the study

The purpose of this study was to continue the experimental work previously performed by two other master students (Batista, 2017; Ruivo, 2018). The first one focused on the physical, chemical and microbiological characterization of these cheeses collected during 2016. Meanwhile, the second thesis focused on microbiological characterization and microbiome diversity of 2016 and 2017 cheeses. Our work consisted on comparing the characterization of lactic acid bacteria and microbiome diversity from 2016, 2017 and 2018 cheeses. Furthermore, we extended our study to the search of pathogenic potential in Azeitão and Nisa cheeses microbiota from all the years of production previously mentioned.

Briefly, our aim was to address the diversity present in Azeitão and Nisa PDO-cheeses due to differences in manufacture (processing-units) and years of production. We also studied the antibiotic resistances present in the different groups of bacteria and virulence factors in enterococci throughout the studied years. We were able to identify new resistances found each year, how those resistances evolved, and which ones were the most worrying. The same approach was taken with virulence factors. Overall, a characterization of bacterial diversity and pathogenic potential of isolates from Azeitão and Nisa PDO-cheeses through different years of production was performed.

2- Material and methods

2.1. Conventional microbiological procedures

PDO-cheeses produced in Azeitão and Nisa were collected from different cheesemaking units, six from Azeitão and two from Nisa, and kept in sterile recipients at -20ºC until microbiological characterization. Preparation of samples was performed adding 225 ml of ISO Peptone Water (Scharlau) to 25 g of cheese in a *Stomacher* bag and then processed in a peristaltic blender (Stomacher Lab-Blender 400) for 90 sec. In order to have a representative sample of every analyzed cheese both the rind and the interior were part of the total 25 g used. The product of this first step is the mother solution (10^{-1}), subsequently used to prepare serial dilutions, which were inoculated in different growth media to quantify cheese microorganisms, as shown in Table 2.1. For further characterization, approximately 20% of *Enterococcus* spp., lactic acid bacteria (LAB) and *Lactococcus* spp. were isolated.

Table 2.1. Growth media, incubation details and characteristic colonies to consider for enumeration.

Microorganism	**Growth medium**	**Growth conditions**	**Inoculation**	**Characteristic colonies**	**ISO**
Enterococcus spp.	Slanetz and Bartley (SBA)	37ºC±2ºC/ 44h±4h	0.1mL/ Superficial	Red color	7899-2:2002
Fungi and yeast	Rose Bengal Chloramphenicol	23ºC±2ºC/ 72h	1 mL distributed in 5 dishes (0.2mL each)	Filamentous for fungi and pink for yeasts	3277: 1987
Lactic Acid Bacteria	Man Rogosa and Sharpe (MRS)	30ºC/72h (anaerobiose)	0.1mL/ Superficial	All	15214: 1998
Lactococcus spp.	M17	30ºC/72h (anaerobiose)	0.1mL/ Superficial	All	

In addition to enumeration and isolation mentioned above a search for pathogenic microorganisms such as *Listeria* spp. and *Salmonella* spp. was performed according to ISO standards *11290-2:1998/Amd.1:2004* and *6579:2002* respectively. The first step was submitting the mother solution to an enrichment at 37ºC for 24 h. Afterwards, the enriched mother solution was inoculated in Chromogenic Listeria Agar (ALOA) at 37ºC for 24 h to search for *Listeria* spp. Furthermore, the enriched mother solution was inoculated in two different broths in order to search for *Salmonella* spp: Rappaport-Vassiliadis soya peptone (RVS) and Mueller Kauffmann Tetrathionate Novobiocin (MKTTn). After an incubation period of 24 h at 37ºC for MKTTn and 42ºC for RVS, broths were inoculated in Xylose Lysine Deoxycholate (XLD) agar and Hektoen Enteric (HE) agar at 37ºC for 24 h. When suspected colonies appeared in XLD and HE agar they were inoculated in Triple Sugar Iron (TSI) agar and if this test was positive according to ISO standards those colonies were isolated in a general growth medium such as Trypticase Soy Agar (TSA). Finally, those suspicious colonies were identified with API 20E (Biomerieux) tests.

2.2. Molecular procedures

2.2.1. DNA extraction

Genetic material from purified isolates was extracted using the boiling method (Millar, Jiru, Moore, & Earle, 2000). The first step was to suspend a colony in 50 µl of Tris-EDTA buffer with 0.1% (v/v) Tween 20 (Merck) and incubate for 10 min at 100ºC. Immediately after this, samples were put in ice for 5 to 10 min in order to cause a thermic shock. The final step was to centrifuge the samples at 13000 rpm for 2 min in a Hermle® Z233 MK-2 centrifuge (Hermle, Germany) and store the supernatant at -20ºC or directly used in PCR reactions.

2.2.2. Genus and species identification

In order to confirm genus and identify the species of suspected *Enterococcus* spp. a multiplex polymerase chain reaction (PCR) was performed. A set of primers, Ent1 and Ent2, were used to confirm genus (Ke et al., 1999). As to species identification, three sets of primers were used in order to identify the three most common species found in traditional cheeses as shown in Table 2 (Arias et al., 2006; Jurkovič et al., 2006). The reaction mixture had a total volume of 20 µl, containing 4 µl of Buffer 5x for Taq II polymerase (NZYtech, Portugal), 0.2 µl of primer Ent1 and 2 at 50 pmol, 0.3 µl of each of the rest primers at 50 pmol, 0.8 µl of $MgCl_2$ at 50 mM, 0.3 µl of dNTPs at 10 mM, 1U of NZYTaq II DNA polymerase and 1 µL of DNA.

Table 2.2. PCR amplification details for enterococcal genus and species identification.

Target bacteria	**Primer**	**Sequence (5' to 3')**	**Product (bp)**
E. faecalis	ddlE1	ATCAAGTACAGTTAGTCTT	941
	ddlE2	ACGATTCAAAGCTAACTG	
E. faecium	ddlF1	GCAAGGCTTCTTAGAGA	550
	ddlF2	CATCGTGTAAGCTAACTTC	
E. durans	mur2edF	AACAGCTTACTTGACTGGACGC	177
	mur2edR	GTATTGGCGCTACTACCCGTATC	
Enterococcus spp.	ent1	TACTGACAAACCATTCATGATG	112
	ent2	AACTTCGTCACCAACGCGAAC	

Amplification was performed using a Doppio thermocycler (VWR, USA) and the following conditions: 95ºC for 5 min, followed by 35 cycles consisting of 95ºC for 1 min, annealing at 57ºC for 1 min, extension at 72ºC for 1 min, and a final step at 72ºC for 10 min. After this samples were stored at

4ºC until electrophoresis. Reference strains used as a positive control were *Enterococcus faecalis* MMH 594, *E. faecium* DSMZ 20477 and *E. durans* DSMZ 20633.

Electrophoresis was performed adding 2 µl of GelStar (stock solution 10X) fluorochrome to 8 µl of sample and applying it to 1.2% agarose gel in 0,5X TBE buffer. The conditions were 80 V for 50 min and the molecular weight marker used was NZYDNA Ladder VI also mixed with GelStar. After electrophoresis agarose gel was then imaged at ChemiDoc XRS+ with the software ImageLab.

2.2.3. Genomic diversity

2.2.3.1. RAPD-PCR

The reaction mixture had a total volume of 20 µl, containing 2 µl of Buffer 10x for Taq II polymerase Supreme (NZYtech, Portugal), 1 µl of M13 primer (5'-GAG GGT GGC GGT TCT-3') at 50 pmol (Cocolin et al., 2004), 1.25 µl of $MgCl_2$ at 50 mM, 0.5 µL of dNTPs at 10 mM, 1U of NZYTaq II DNA polymerase Supreme and 2 µL of DNA.

Amplification was performed using a Doppio thermocycler (VWR, USA) and the following conditions: 94ºC for 5 min, followed by 40 cycles consisting of 94ºC for 1 min, annealing at 40ºC for 2 min, extension at 72ºC for 2 min, and a final step at 72ºC for 10 min. Once amplification was finished products were stored at 4ºC until electrophoresis.

For electrophoresis 8 µl of product were mixed with 2 µl of GelStar (stock solution 10X) fluorochrome and applied to 1.2% agarose gel with 0,5X TBE buffer. The conditions for this run were 110V for 2h 15 min. Afterwards an image of the gel was taken at ChemiDoc XRS+ with the software ImageLab.

2.2.3.2. Data analysis

In order to analyse the different profiles obtained we used the software BioNumerics (version 6.6.5, Applied Maths, Belgium). All images were normalized, Pearson correlation coefficient was calculated and dendrograms were created through unweighted pair group method with arithmetic mean (UPGMA). These dendrograms were used to choose representative isolates for subsequent analysis, as described below.

Two diversity indexes were calculated for all three groups analysed Simpson (D) (Hunter PR & Gaston MA, 1988) and Shannon-Weaver (J') (Tramer, 1969). Simpson index (D) measures the probability of two randomly picked isolates belonging to the same genomic group or cluster, however the index used in this work is the complement (D'=1-D) that tells us the probability of randomly choosing two isolates that belong to two different clusters or groups.

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j - 1)$$

In this formula "N" is the total number of analysed isolates, "s" represents the total number of groups formed and "n_j" the total number of isolates in each group.

Shannon-Weaver (J') index allows us to assess the groups richness and the uniformity of abundance within the different formed groups. It represents the proportion between observed and maximum possible diversity.

$$H' = \frac{N \log_2 N - \sum_{j=1}^{s} n_j \log_2 n_j}{N} \qquad H' \max = \log_2 s \qquad J' = \frac{H'}{H'max}$$

In this formula, “N” represents the total number of analysed isolates, “s” the number of formed clusters, “n_j” number of isolates inside every cluster and “J’” the ratio of observed diversity over maximum possible diversity.

2.3. Antibiotic resistance

The disc diffusion technique was used to study antibiotic resistance in LAB, grown in MRS medium; *Lactococcus* spp., grown in M17 medium; and *Enterococcus* spp., grown in Brain Heart Infusion (BHI) medium. Briefly, each bacterial culture, collected from overnight grown plates, was suspended in 500 µl of Ringer (Oxoid, UK) until the suspension reached a concentration of 0.5 in the McFarland scale (approximately 10^8 CFU/ml). This bacterial suspension was distributed on a squared petri dish with a sterile swab. A disc from each antibiotic (Oxoid, UK) was placed sufficiently separated from each other so that resulting halos could be easily read. Petri dishes were incubated at 37ºC for 24 h for *Enterococcus* spp. and at 30ºC with anaerobic atmosphere for 48 h for LAB and *Lactococcus* spp. After incubation transparent halo diameters were measured and interpreted according to Clinical Laboratory Standards Institute (CLSI, 2016) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019).

All antibiotics used, their class, cell target and concentrations for the three bacterial groups studied are shown in Table 3.

Table 2.3. Antibiotics used and their concentration in µg for disc diffusion assays.

Antibiotic	**Concentration for *Enterococcus* spp.**	**Concentration for LAB**	**Concentration for *Lactococcus* spp.**	**Class**	**Target**
Gentamicin	120	10	10	Aminoglycosides	Protein synthesis inhibition
Kanamycin	-	30	30		
Streptomycin	300	10	10		
Clindamycin	-	2	2	Lincosamides	
Erythromycin	15	15	15	Macrolides	
Linezolid	30	-	-	Oxazolidinones	
Chloramphenicol	30	30	30	Chloramphenicol	
Quinupristin /Dalfopristin	15	-	-	Streptogramins	
Tetracycline	30	30	30	Tetracyclines	Cell wall synthesis inhibition
Teicoplanin	30	-	-	Glycopeptides	
Vancomycin	30	30	30		
Amoxicillin/ Clavulanic acid	30	-	-	β-lactams	
Ampicillin	10	10	10		
Ciprofloxacin	5	-	-	Quinolones	DNA synthesis inhibition
Levofloxacin	5	-	-		

Using the software JASP (Version 0.10.2) chi-square statistics were performed and p-values were calculated to know if differences between the number of resistances in different years and units were significant. The chi-square formula is:

$$\chi_c^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

Where “c” are degrees of freedom, “O” is the observed value and “E” is the expected value.

2.4. Assessment of virulence factors

Enterococcus spp. were submitted to two tests to assess virulence potential. One of these tests was a hemolysis assay in order to know if these isolates had hemolytic properties. Columbia Agar with 5% sheep blood plates (Frilabo, Portugal) were inoculated and then incubated at 37ºC for 48 h. Hemolytic activity was determined according to the agar color observed around the colonies: green color corresponded to α-hemolytic (partial hemolysis), white to γ-hemolytic (no hemolysis) and transparent to ß-hemolytic (total hemolysis).

The other test performed was for the evaluation of gelatinase production, which was performed in gelatin agar medium, prepared with 1.25 g of peptone (Scharlau, Spain), 0.75 g of yeast extract (Scharlau, Spain), 0.75 g of gelatin (Difco), 5 g of bacteriological agar (Scharlau, Spain) and 250 ml of distilled water. Isolates were incubated for 48 h at 37ºC, after incubation a saturated solution of ammonium sulfate was added covering the medium surface and after waiting 5 min results were observed. If transparent halos appeared surrounding the colonies it indicated gelatin digestion and diameters were measured.

3- Results and discussion

The study of Azeitão and Nisa PDO-cheeses started with the physico-chemical and microbiological characterization of 2016 samples from different cheesemaking units (Batista, 2017). Afterwards, this project was followed by the study and comparison of microbiome diversity of these cheeses from 2016 and 2017 produced in the same cheesemaking units (Ruivo, 2018). In the present work we compared the microbiological characterization and microbiome diversity of 2016, 2017 and 2018 cheeses from the same cheesemaking units. One unit (A6 from Azeitão) was added to this study in 2018 so data is only available from this year and can’t be compared to prior years.

We also extended the study to the evaluation of bacterial pathogenic potential studying antibiotic resistances and virulence factors such as hemolysis and gelatinase production for cheese enterococci.

3.1. Enumeration of bacteria

3.1.1. Lactic acid bacteria: comparison between production years and cheesemaking units

As part of the large group of lactic acid bacteria (LAB) there are several genera which include *Lactococcus* spp. and *Enterococcus* spp. and so although all studied bacteria belong to LAB we will be referring to three separated groups. LAB were isolated from MRS agar which allows lactobacilli growth although other lactic acid bacteria can also appear (Corry, Curtis, & Baird, 2003). Isolation of *Lactococcus* spp. was performed using a selective growth medium for this genus, M17 agar. For *Enterococcus* spp. the medium used was Slanetz and Bartley agar, a selective medium for these bacteria.

Isolation and enumeration of bacteria from 2018 samples was done in the context of the present work while for 2016 and 2017 microbial isolation and enumeration was performed in the context of previous thesis as mentioned before (Batista, 2017; Ruivo, 2018).

Concerning LAB we could observe, as shown in Figure 3.1., that in all units except N9 from Nisa samples from 2016 had a higher number of these bacteria while data from 2017 and 2018 years were very similar. Values of colony-forming units (CFU) observed in this work were very much alike to data previously published about LAB in Manchego cheeses (Cabezas et al., 2007) that have similar characteristics with PDO-cheeses analyzed in this work such as being manufactured with sheep milk, having PDO designation and being artisanal.

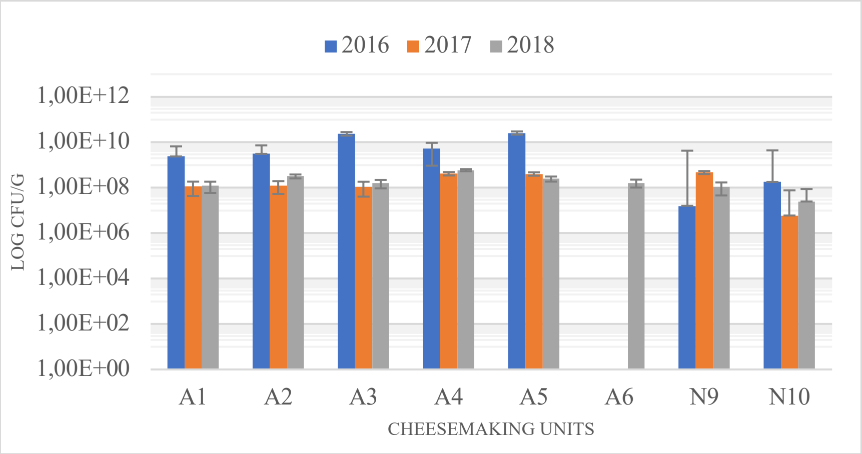

Figure 3.1. Enumeration of lactic acid bacteria.

A study that compared Terrincho Portuguese PDO-cheeses (Pintado et al., 2008), which is also made with raw sheep milk, from five dairy farms showed significant differences between farms for all microbial groups studied (lactobacilli, lactococci, enterococci, staphylococci, enterobacteria, pseudomonads, yeasts and molds). In a previous study (Quigley et al., 2012) that worked with 62 artisanal Irish cheeses from cow, goat or sheep milk it was also noted how *Lactobacillus* populations had a significant increase, even when produced in the same farm, due to differences in ripening temperatures. One hypothesis that could explain the differences that we observed between years and units could be that temperature and humidity conditions oscillated even between established limits. It has already been suggested for artisanal raw milk cheeses like Camembert (Henri-Dubernet et al., 2008) that there is strong variability in lactobacilli not only because of the composition of raw milk but also due to the practices of each cheesemaking unit.

Lactococcus spp., which were isolated with M17 growth medium, CFU counts are represented in Figure 3.2., presented similar values to LAB. This was expected since this genus is one of the predominant in lactic acid bacteria found in raw sheep milk and cheeses (Cabezas et al., 2007; Quigley et al., 2013). Furthermore, in a study with 4379 isolates from 35 different cheeses (Cogan et al., 1997) 38% of those isolates were identified as lactococci, making this group of bacteria the predominant. Higher CFU counts were observed in 2017 samples from Nisa units although this didn’t occur in 2016 and 2018.

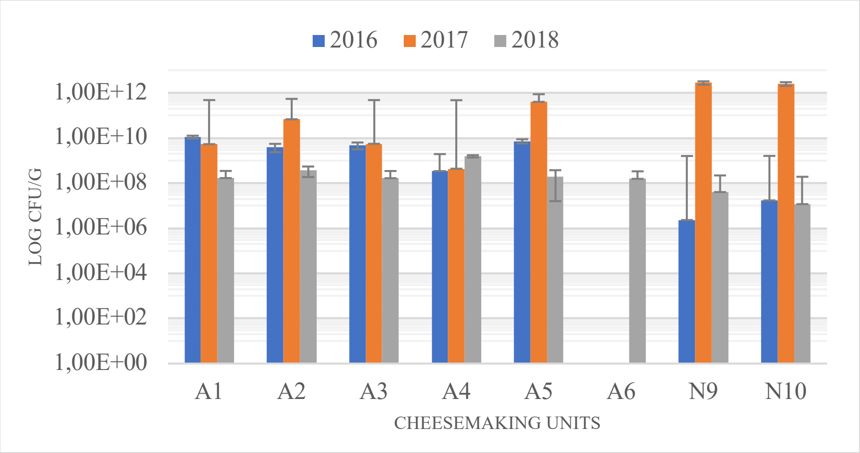

Figure 3.2. Enumeration of *Lactococcus* spp.

These differences could be due to variations on the maturation phases of 2017 Nisa cheeses as it has been observed in a study about flavor formation by lactic acid bacteria (Smit et al., 2005) that *Lactococcus* spp. are predominant in starter cultures for cheese-making. Most definitely the flavor of these cheeses would be different from other years since acidification is the primary role of this genus in cheese fermentation. In our case, the year that had higher CFU in most of cheesemaking units is 2017, unlike what we've seen above with LAB, where 2016 had higher values than the other two years. So, although we have similar CFU counts in LAB and *Lactococcus* spp. the years of production with highest counts do not match in both groups. An explanation for this could be the fact that although lactococci are part of LAB, MRS medium favors the growth of other bacteria such as *Lactobacillus* spp. and M17, the medium growth used to isolate *Lactococcus* spp., is selective for this genus. Moreover, in a study with Serra da Estrela cheese (Macedo et al., 2004) it was observed that lactobacilli survive better when their counts are similar to those of lactococci, as we see in cheeses analyzed in this work, since it seems that pH reduction caused by lactococci didn't affect negatively lactobacilli survival.

Regarding *Enterococcus* spp. CFU counts, represented in Figure 3.3., high variability was observed through the three years and the different units. In half of the units the higher CFU's were observed in 2016 samples, in one unit (N9 from Nisa) it was observed in 2017 samples and other one (A5 from Azeitão) in 2018.

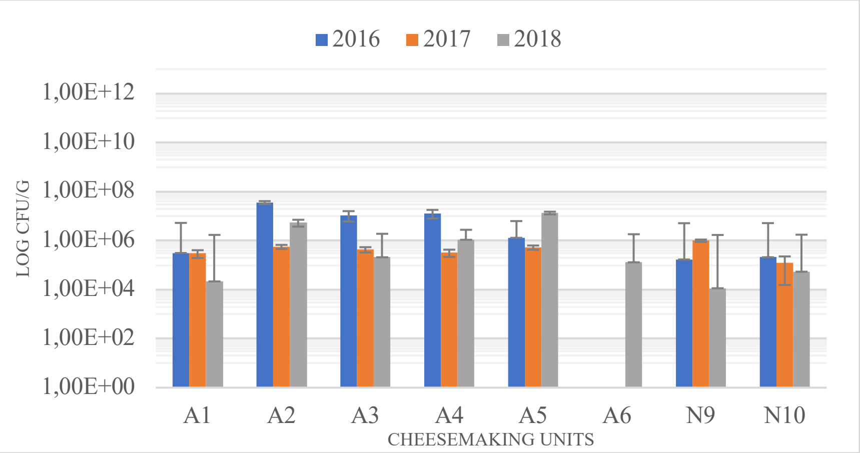

Figure 3.3. Enumeration of *Enterococcus* spp.

Overall, CFU counting for this group was much lower than the other groups and the numbers we observed in this work were consistent with previous studies that analyzed Terrincho, Manchego, Cebreiro, La Serena, White-brined, Kefalotyri, Teleme and bryndza cheeses where CFU/g varied between 10^4 to 10^7 (Franz et al., 1999; Jurkovič et al., 2006; Pintado et al., 2008).

Studies with Manchego, Armada, Cebreiro, Picante, Majoero, Feta, Telemem Mozzarella, Monte Veronese, Fontina, Caprino, Serra, Venaco and Comté cheeses (Giorgio Giraffa, 2003) show that this genus is predominant in the fully ripened product. However, we clearly saw that this was not the case for Azeitão and Nisa cheeses since enterococci had consistently lower counts than the other studied bacteria. In a study with Serra da Estrela cheeses (Macedo et al., 2004) it was observed that lactobacilli and lactococci caused a reduction in enterococci counts. This reduction was due to the importance of pH value for the survival of enterococci in cheeses, lactobacilli and lactococci in high counts cause a drop in pH that allows a natural control of enterococcal growth.

3.1.2. Genus and species identification

In the context of the previous thesis included in this project (Ruivo, 2018), *Enterococcus* spp. were quantified in SBA medium from 2017 cheese samples and 20% of the colonies obtained were further isolated. RAPD-PCR were performed with the DNA extracted from randomly picked isolates from each cheesemaking unit. Band profiles obtained were used to create dendrograms where clusters of similar profiles were observed. Isolates from the same cluster had similar genetic material and probably belong to the same species or strains. From those clusters isolates were selected as representatives from each cheesemaking unit and identified at species level.

In the context of this work, most of the 32 enterococcal representative isolates from 2017 were identified at genus and species level. Although for the 34 representatives from 2016 the same procedure was also performed results were inconclusive. This could be due to lack of sufficient genetic material for amplification so DNA extraction of those 2016 isolates and identification should be performed again to obtain better results.

A multiplex PCR with primers for *Enterococcus* spp. genus and three species of this genus that usually are the predominant in cheeses was performed. Those species were *E. faecalis, E. faecium* and *E. durans*. Since isolates were obtained using a selective growth medium, *Enterococcus* spp. genus primers were only used as confirmation.

All 32 isolates from 2017 were confirmed as *Enterococcus* spp., 14 were identified as *E. faecium*, 5 as *E. faecalis* and also 5 as *E. durans*. The remaining isolates had inconclusive results as to species identification, since there was amplification with genus primers but not with species primers. For identification of those isolates a PCR with specific primers for other *Enterococcus* spp. could be performed since they could belong to species that are less common in cheese's microbiota. It was expected that most of the isolates belonged to *E. faecium* or *E. faecalis* since these are the species more commonly described in cheeses produced with raw milk such as Pico, Serra da Estrela, Picante da Beira Baixa, Manchego, Cebreiro, Kefalotyri and White-brined (Domingos-Lopes et al., 2016; Franz et al., 1999; Nieto-arribas et al., 2011), although usually *E. faecalis* is the predominant species. As to *E. durans* this species has been found in several artisanal cheeses from around the world such as Pico, Ragusano, Pecorino Siciliano, Turkish white, Bryndza and Chinese yak milk cheese (Bao et al., 2012; Ispirli et al., 2017; Jurkovič et al., 2006; Russo et al., 2018) but usually it's not as predominant as the other two mentioned species, as we can also see in this work. It's worth mentioning that in cheeses from both Nisa cheesemaking units all isolates but one were identified as *E. faecium*. Since these cheeses are different in texture and flavor from Azeitão cheeses the predominance of this species could influence those variations in organoleptic characteristics, as previous studies (Foulquié Moreno et al., 2006; Franz et al., 1999) have showed that enterococcal species are involved in ripening, taste and flavor of raw milk cheeses. It’s also interesting that all isolates from A2 (Azeitão) were identified as *E. durans* even more so knowing that this species usually is not predominant in cheese microbiota. As discussed before this could also mean that this species plays an important role in specific characteristics of Azeitão cheeses produced in that specific unit.

Identification of representative isolates from enterococci from 2016 and 2018 should be done in following works. LAB and lactococci from all years studied (2016, 2017 and 2018) should also be identified for a better understanding of Azeitão and Nisa PDO-cheeses diversity and how it changes through the years and units.

3.1.3. Other microorganisms of interest

Yeast and molds play an important role in dairy fermentations such as cheese production since they secrete important enzymes during ripening (Quigley et al., 2013). Taking this into consideration, enumeration of these microorganisms was performed for 2018 cheeses in this work and for 2016 cheeses in the context of a previous thesis (Batista, 2017). In Figure 3.4. are represented the count comparison of both yeast and molds between cheesemaking units for 2018 samples. The number of yeasts was always superior to molds and similar between units while molds counts were more variable between the different units. Both yeasts and molds have a broad pH requirement for growth while moisture requirements differ since yeasts generally require a higher water activity (Tournas, Stack, Mislivec, Koch, & Bandler, 2001). Taking into consideration that water activity is similar in Azeitão and Nisa cheeses (Batista, 2017) no disparities were expected both between yeast and molds counts as well as between units or regions. However, in both Nisa cheeses there were no filamentous fungi or yeasts, this could be due to Nisa’s cheeses texture properties being different than Azeitão cheeses. Nisa’s matrix would make it more difficult to yeasts and molds to survive during later stages of ripening because these cheeses are harder than Azeitão cheeses. These results don’t necessarily imply that these microorganisms were not present at early stages of fermentation, since studies about ferment dairy foods (Macori & Cotter, 2018; Marco et al., 2017) have reported their presence in starter cultures both natural and commercial. Comparing these 2018 results to molds and yeasts counts obtained in 2016 cheeses (data not shown) values are similar in Azeitão units but when it comes to Nisa units in the previous year analyzed there were counts of these microorganisms and as we’ve seen before this didn’t happen in 2018 cheeses. Specifically, in unit N10 from 2016 a high count of molds and yeasts were observed which contradicts results obtained in the present work. This accentuates the discrepancies in enumeration results we observed in all microorganisms studied when comparing different years of production.

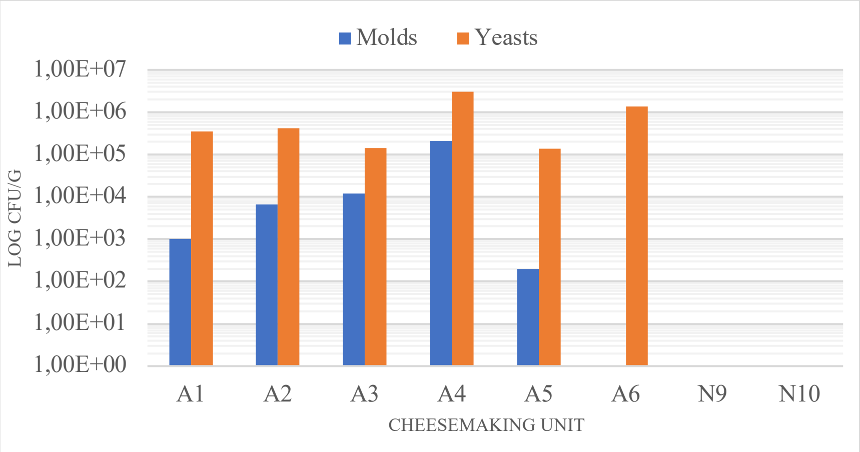

Figure 3.4. Enumeration of yeast and molds.

A screening for food safety related microorganisms such as *Listeria* spp. and *Salmonella* spp. was performed in 2018 cheeses, these two genera having pathogenic species important for human health. Cheeses from 2016 were also screened for these bacteria in one of the previous thesis in the context of this same project (Batista, 2017) and although suspected colonies of *Listeria* spp. were found in selective growth medium ALOA, when carried out confirmatory PCR tests results were negative. In the case of *Salmonella* spp. there were no suspected colonies.

Concerning 2018 cheeses, no suspected colonies of *Listeria* spp. were found. In two cheesemaking units from Azeitão suspected colonies of *Salmonella* spp. were obtained in XLD and HE agar. These colonies were considered positive due to a turn in the agar color and a black color in the center of the colony. Suspected colonies were then inoculated in a TSI slant and results were positive for glucose fermentation, negative for lactose and sucrose fermentation and production of hydrogen sulfide was observed. Since these results indicate a presumptive positive, a confirmatory test was performed after isolating the suspecting colonies in TSA. API 20E test kit was negative for *Salmonella* spp. for all suspicious colonies and identification was positive for *Proteus* spp.. This genus has species known to be human opportunistic pathogens, usually causing urinary infections, such as *P. mirabilis* (Schaffer & Pearson, 2015) although they've also been isolated from the human gastrointestinal tract in healthy patients and other animals (Drzewiecka, 2016). Besides, this genera has also been found in cheeses from United Kingdom and France (Deetae, Bonnarme, Spinnler, & Helinck, 2007; Yunita & Dodd, 2018) and has been proven their contribute to cheese flavor (Yu, Bai, Fan, Zheng, & Cai, 2019) so their role is yet controversial.

3.2. Cheese microbial diversity

3.2.1. Comparison between production years

Random amplification of polymorphic DNA (RAPD) is a type of PCR where the segments of amplified DNA are random. In the present work this method was applied to LAB, *Lactococcus* spp. and *Enterococcus* spp. isolates from 2018 cheeses. After isolation of 20% of CFU obtained of each group of bacteria for every cheesemaking unit, DNA extraction of those isolates was performed and used for PCR amplification. From the resulting band profiles obtained for each isolate, dendrograms were created with software BioNumerics (version 6.6.5, Applied Maths, Belgium) for each cheesemaking unit and bacterial group. Those dendrograms allowed the analysis of similarity between different isolates and definition of clusters. Also, from these dendrograms Simpson (D') and Shannon-Weaver (J') indexes were calculated to assess the diversity present in 2018 Azeitão and Nisa cheeses. The same approach was performed for 2016 and 2017 isolates by another master student and the results were part of her dissertation (Ruivo, 2018). In the present work we will be comparing those diversity results (2016 and 2017 samples) with the ones obtained for 2018 cheeses. Since we'll be comparing results through the three years the same cut-off value of similarity that Ruivo chose for 2016 and 2017 isolates was selected, that cut-off was 60%.

3.2.1.1. Lactic acid bacteria

The cheesemaking unit with higher diversity in 2018 was A4 (D'= 0.9; J'= 0.909) with 11 clusters formed (Figure 3.5) of which 5 were single-member clusters. That high Simpson diversity index value means that when picking two isolates randomly we have a 90% chance of those isolates belonging to different clusters. This result didn't match the ones obtained in the years before since for 2016 the highest diversity was found in N9 (D'= 0.218; J'= 0.392) and for 2017 in N10 (D'= 0.793; J'= 0.8). It's interesting that in that N9 sample of 2016 the diversity index was very low and that same unit has the lowest CFU counts of LAB of all three years together with N10 from 2017 but this cheese, as we've seen, had a much higher diversity. So, not necessarily a low CFU count means low diversity.

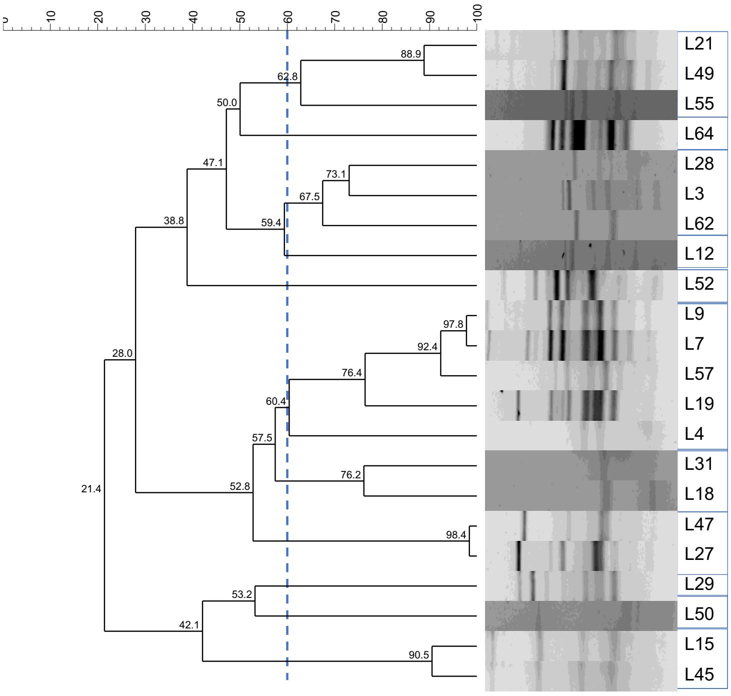

Figure 3.5. Dendrogram of LAB isolates from A4 unit (2018) with 60% similarity cut-off and formed clusters indicated. "L" letter and following number identify each isolate.

As to the unit with the lowest diversity found in 2018 that was A5 (D'= 0.41; J'= 0.653) with only 3 clusters formed (Figure 3.6). For 2016 the unit with the lowest diversity was A2 with null values of both indexes and for 2017 samples the lowest was A1 (D'= 0.696; J'= 0,738). Once again, diversity values of 2017 and 2018 were very different than 2016 values and the low diversity indexes didn't match low CFU counts compared to the rest of cheeses.

Concerning this group of bacteria, 2016 samples had much less diversity than posterior years although for Azeitão units this year of production had consistently higher CFU counts. This contrast between low diversity and high CFU counts was observed in other groups of bacteria in the present work. Logically, the diverse species or strains in these cheeses are independent of the number of bacteria present since there could be a high number of the same species or vice versa. As proven in a previous study with Serra da Estrela cheeses (Macedo et al., 2004) low diversity would most certainly affect organoleptic properties of produced cheeses. Different values of diversity through the years and between cheesemaking units have also been observed in previous studies that used RAPD-PCR (Henri-Dubernet et al., 2008) in lactobacilli from raw milk cheeses like French Camembert cheese. This could be explained due to the fact that raw milk cheeses, such as Azeitão and Nisa, have a variable microbiome since starter cultures consist of microorganisms naturally present in milk and diversity is not controlled.

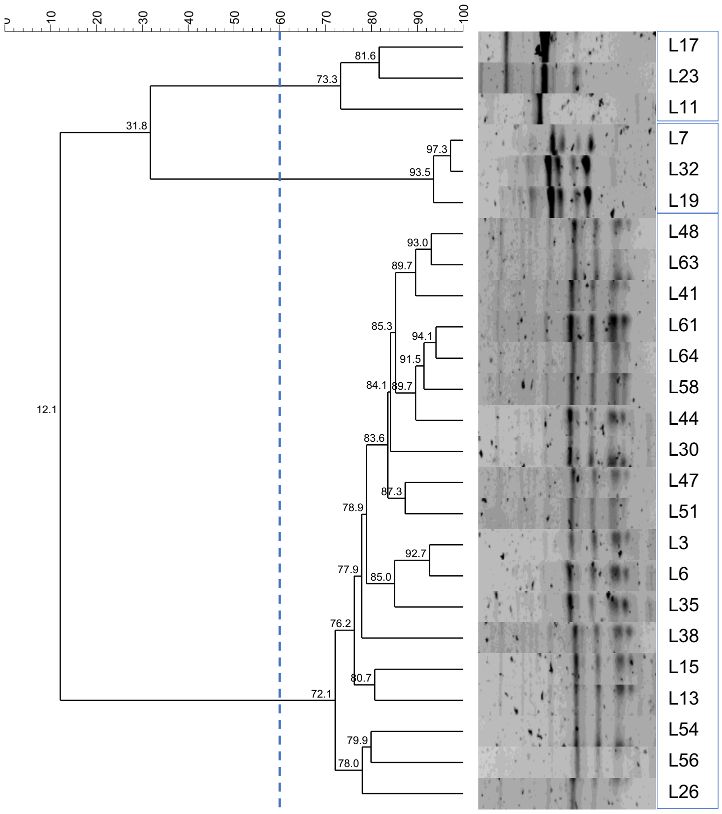

Figure 3.6. Dendrogram of LAB isolates from A5 unit (2018) with 60% similarity cut-off and formed clusters indicated. "L" letter and following number identify each isolate.

3.2.1.2. *Lactococcus* spp.

As one of the predominant groups of lactic acid bacteria present in artisanal cheeses like Terrincho or Manchego (Cabezas et al., 2007; Pintado et al., 2008; Smit et al., 2005) the diversity of this genera was also studied.

Cheesemaking unit N10 was the one with the highest diversity in 2018 samples (D'= 0.937; J'= 0.944) with a total of 13 different clusters (Figure 3.7) of which 6 were single-member clusters. This cheese was also the one with lowest CFU counts compared to the other 2018 cheeses although the same didn't apply to 2016 and 2017 cheeses. In 2016 samples the unit with the highest diversity was A3 (D'= 0.704; J'= 0.801) and in 2017 it was A1 (D'= 0.541; J'= 0.436). In this group we see that when comparing the highest diversity values 2017 samples are not so similar to 2018 samples, in fact the diversity index observed in 2017 is low compared to the ones obtained in 2016 and 2018.

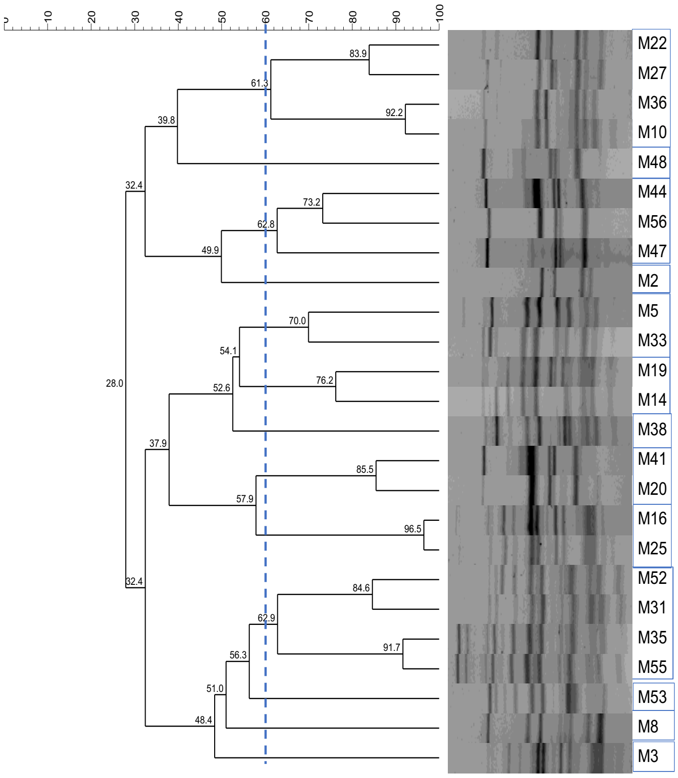

Figure 3.7. Dendrogram of *Lactococcus* spp. isolates from N10 unit (2018) with 60% similarity cut-off and formed clusters indicated. "M" letter and following number identify each isolate.

The unit with the lowest diversity in 2018 was A4 (D'= 0.753; J'= 0.886) where only 5 clusters were formed (Figure 3.8). In this case, the lowest diversity in 2016 samples was also found in cheesemaking unit A4 (D'= 0.237; J'= 0.241) although the diversity found in 2018 sample was still much higher. On the other hand, the lowest diversity found in 2017 was in N10 unit with a null diversity value. The same unit had the lowest diversity in 2016 and 2018 samples but the unit that had the lowest diversity in 2017 is the unit that had the highest in 2018. So, it seems that in this particular group of bacteria 2018 results had more consistency with 2016. Although N10 unit from 2017 was the one with lowest diversity it was also one of the two with higher CFU counts. So, what we see is that even though CFU counts were high that didn't mean the diversity would necessarily be high too since those high counts could be from the same microorganism. In a study with Serra da Estrela cheeses (Macedo et al., 2004) it was observed that lactococci at high viable levels inhibit their own growth due to the drastic pH reduction. This could mean that a highest competition between species takes place when counts of lactococci are high and that could be why the diversity is low or null.

Hence, in this genus we saw more resemblance between 2018 and 2016, although diversity values were much higher for 2018 samples, there were similarities as to which units had lowest diversity. Likewise, we could be observing the results of a competition between several species or strains of this group that could explain the contrast between low diversity and high CFU counts in some cheesemaking units.

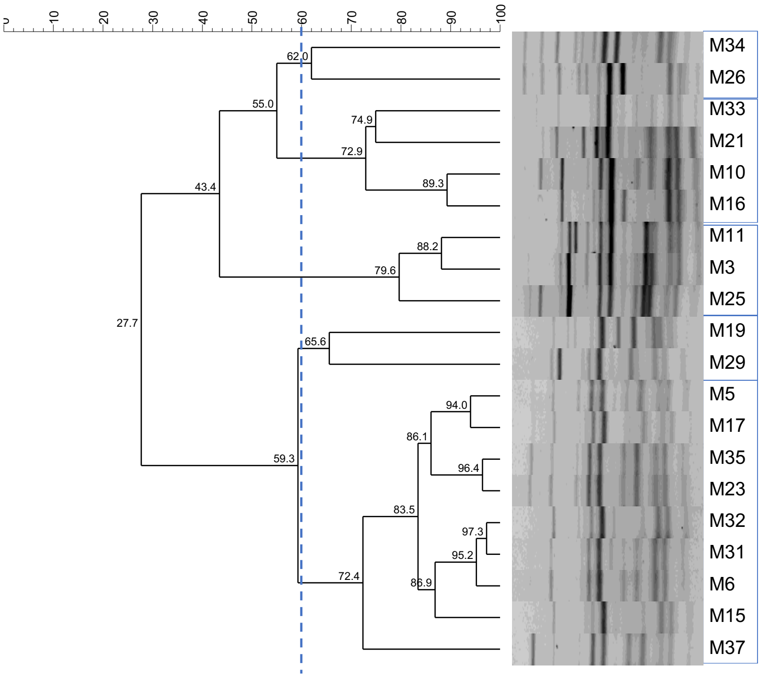

Figure 3.8. Dendrogram of *Lactococcus* spp. isolates from A4 unit (2018) with 60% similarity cut-off and formed clusters indicated. "M" letter and following number identify each isolate.

3.2.1.3. *Enterococcus* spp.

This genus is also part of the lactic acid bacteria group and so its diversity was also studied in samples from the three years previously mentioned. In 2018 the cheesemaking unit with the highest diversity was N10 (D'= 0.956; J'=964) which had 12 clusters (Figure 3.9) of which 8 were single-member clusters. Highest diversity in 2016 samples was found in A2 unit (D'= 0.54; J'= 0.999) and in 2017 the unit with the highest value was A4 (D'= 0.78; J'= 0.854). In this group we observed, as before in other groups, that samples from 2018 had more diversity than previous years. In essence, while in the most diverse unit from 2016 if we picked two random isolates we had 54% of chance they would belong to different groups, in the most diverse unit from 2018 that value would be 95%. We also observed that unit N10 from 2018 is one of the cheeses with the lowest CFU counts from all three years and yet the diversity it shows is very high. This proves, as observed before in other groups studied, that CFU counts are not directly related with the diversity in the sample.

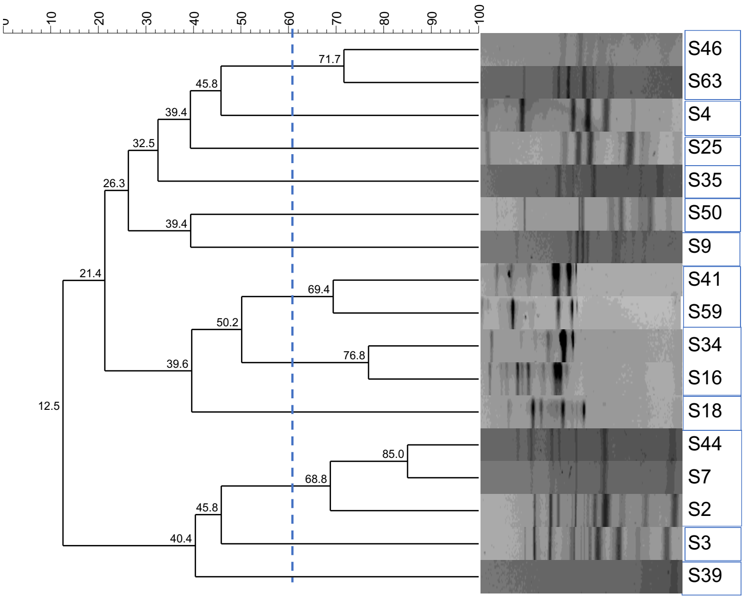

Figure 3.9. Dendrogram of *Enterococcus* spp. isolates from N10 unit (2018) with 60% similarity cut-off and formed clusters indicated. "S" letter and following number identify each isolate.

The lowest diversity in 2018 was found in unit N9 (D'= 0.713; J'= 0.806) with the formation of 5 different clusters of isolates (Figure 3.10) of which 2 were single-member clusters. As to 2016 samples the unit with the lowest diversity was also N9 with null diversity values and in 2017 it was N10 (D'= 0.46; J'= 0.714). Again, values for 2016 are a lot different and much lower than for the two other years although lowest diversity was found in the same cheesemaking unit in 2018 and 2016 samples. This also happened with *Lactococcus* spp. diversity. Another interesting fact is that in this group we find that N10 has the highest diversity in 2018 and the lowest in 2017, just like we've seen before when analyzing the results for LAB.

Overall, the diversity of the different studied units compared throughout three years had no apparent correlation and varied a lot although in the case of this group of bacteria we observed that in all studied years the lowest diversity values were found in Nisa units. This could mean that Nisa cheeses had a lowest diversity of enterococci due to their distinctive properties like texture and more acid flavor. However, we couldn't affirm this since in 2018 cheeses N10 unit (from Nisa) had the highest diversity value from that year. These fluctuations are not surprising since no starter cultures are added in the manufacturing of these artisanal cheeses and so there is really no control over the microorganisms that constitute the final cheese microbiota. In a study with Serra da Estrela cheeses (Macedo et al., 2004) it was observed that lactobacilli and lactococci caused a reduction in enterococci counts as we've discussed before but diversity values are not so different from the other two groups analyzed. Therefore, that reduction could have happened for CFU counts but not for enterococcal diversity found in studied cheeses. As seen in studies with Manchego cheeses (Nieto-arribas et al., 2011) this group usually shows high diversity in cheeses and that is what we also see in Azeitão and Nisa cheeses from 2017 and 2018.

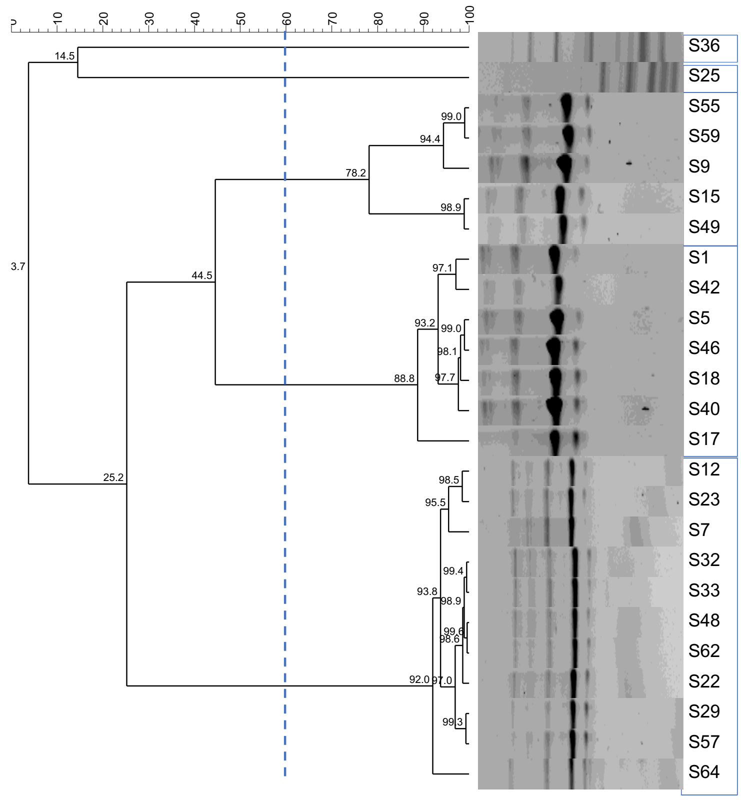

Figure 3.10. Dendrogram of *Enterococcus* spp. isolates from N9 unit (2018) with 60% similarity cut-off and formed clusters indicated. "S" letter and following number identify each isolate.

3.2.2. Comparison between production units and groups of bacteria

All band profiles from 2018 samples were compared in a dendrogram for each group of bacteria (LAB, *Lactococcus* spp. and *Enterococcus* spp.). This way we were able to study if there were similarities between isolates from different units and if cluster formation was origin dependent. The cut-off chosen was 60% of similarity in order to be consistent with results previously analyzed in this work.

The group with higher diversity was *Enterococcus* spp. with D'= 0.955 and J'= 0.876. The number of clusters formed was 46 (Appendix A) and although many of these clusters grouped isolates from the same cheesemaking unit, others had isolates from different units with high similarity values. This means that while some strains would be characteristic of a unit, others are found across several units. As to diversity between units, half of them (A2, A3, A6 and N10) had a diversity index (D') higher than 0.9. As observed there was no relation between the two different regions and their diversity value, in other words, we didn't see units from a region (Azeitão or Nisa) having less or more diversity than the other. Furthermore, linking these results with CFU counts obtained for this group, we already saw that enterococci was the group with less counts from all three studied. So, even though this group of bacteria was the less predominant in Azeitão and Nisa cheeses it was also the most diverse. Studies in European raw milk cheeses (Foulquié Moreno et al., 2006; Franz et al., 1999; Giorgio Giraffa, 2003) have shown the importance of enterococci in the fermentation and ripening of cheeses and so what we see in our work is that even more than a high count of these bacteria what really seems to make a difference is the diversity that we find in cheeses. On the other hand, this diversity could also be important due to their pathogenic potential and capacity to act as a reservoir for antibiotic resistances and virulence factors as we will be studying later in this work.

Lactococcus spp. group had the following higher diversity value, D'= 0.953 and J'= 0.894, with the formation of 37 clusters (Appendix B). We could easily observe that the number of clusters compared to the other group already analyzed was much lower. Moreover, in this group we saw that the majority of the clusters were made up of isolates from the same cheesemaking unit. This could mean that there were species or strains characteristic of their origin. When it comes to each unit diversity with the exception of A4, the unit with the lowest diversity as mentioned before, all units had a Simpson index superior to 0.8 which means almost every unit had a considerable high diversity when it comes to lactococci. In a study with two Greek cheeses (Pavlidou, Bozoudi, Hatzikamari, Tzanetakis, & Litopoulou-Tzanetaki, 2011), Graviera Kritis and Feta, it was proved that each cheese possesses a characteristic community of *Lactococcus* spp., even if it comes from the same cheesemaking unit, and that each community has influence on the final properties of those cheeses. In our work it was noted that many clusters grouped isolates from a unit, meaning that, just like in the study mentioned before, each cheese seemed to have its own characteristic microbial community.

Lowest diversity was found in the LAB group (D'= 0.937; J'= 0.856) with the formation of 33 clusters (Appendix C). Considering each unit and their diversity indexes it was noted that except for three units the rest had a Simpson index higher than 0.8. This high diversity was expected since in many studies that investigated lactobacilli (Broadbent et al., 2011; G. Giraffa et al., 2004; Hickey et al., 2007; Oneca et al., 2003) different species and subspecies of *Lactobacillus* spp. were identified and their different roles in cheese manufacture assessed. Furthermore, the growth medium used to isolate this group (MRS) allows the growth of other genera besides *Lactobacillus* spp., although in lower numbers, as discussed before. Since many of the big clusters formed have isolates from at least two cheesemaking units that could be explained due to the fact that isolates with similar profiles could be from the same genera, species or strain as part of LAB (Devirgiliis et al., 2013).

In all groups we observed that many clusters, especially the larger ones, had isolates from different units, sometimes even from three or more units. This means that many isolates from similar or identical species were found across different units and it would be interesting to identify these isolates at species or strain level to know how they relate to each unit and cheese.

3.3. Evaluation of pathogenicity potential

3.3.1. Antibiotic resistance

Three groups, LAB, *Lactococcus* spp. and *Enterococcus* spp.., were studied regarding their antibiotic resistances. Representative isolates were chosen using dendrograms made with RAPD-PCR band profiles. Similar isolates were grouped, and from each cluster representatives were chosen since similarities probably mean those isolates belong to the same species or strain. In the case of LAB, from 2016 a total of 35 representatives were studied and from 2017 there were 22 representatives. Lactococci group had 22 representatives in 2016 samples and 23 in 2017 samples. Enterococci number of representatives in 2016 samples was 34, in 2017 samples 32 and in 2018 we chose 58 representatives. All representatives of 2016 and 2017 samples were chosen in the context of Ruivo's dissertation (Ruivo, 2018).

LAB and lactococci antibiotic resistances were only analyzed for 2016 and 2017 samples but will be performed for 2018 samples in future works. In the case of *Enterococcus* spp. antibiotic resistances were screened in 2016, 2017 and 2018 representatives. For all three groups chi-square tests were performed to analyze if the number of resistances were significantly different between units and years of cheese production. Breakpoints used for resistance and susceptibility in LAB and lactococci were established in a previous master thesis about lactic acid bacteria and their probiotic potential (Touret, 2016) since there are no official breakpoints for lactobacilli and lactococci.

3.3.1.1. Lactic acid bacteria

For this group of bacteria, nine different antibiotics were tested from seven different classes: aminoglycosides, lincosamides, macrolides, chloramphenicol, tetracycline, glycopeptides and ß-lactams. With the chosen antibiotics three different targets were studied such as protein synthesis inhibition, cell wall synthesis inhibition and DNA synthesis inhibition (for details see Table 2.3. on page 11). Besides, these antibiotics have been previously tested for LAB in studies about antibiotic resistances in dairy products (Devirgiliis et al., 2013; Domingos-Lopes et al., 2016; Erginkaya et al., 2018; Gad et al., 2014; Guo et al., 2017; M. Álvarez-Cisneros & Ponce-Alquicira, 2019; Temmerman et al., 2003).

Regarding resistance results from representative isolates from both 2016 and 2017 (Figure 3.11.) we could see that resistance to gentamicin, kanamycin, streptomycin, tetracycline and vancomycin appears in both years although with differences as to the percentages observed. The highest frequencies observed in 2016 isolates are resistances to streptomycin and tetracycline while in 2017 isolates are to kanamycin, streptomycin and vancomycin. All these resistances to the previously mentioned antibiotics have been described in recent studies and some authors even consider most of these resistances intrinsic to LAB like *Lactobacillus* spp. (Domingos-Lopes et al., 2016; Erginkaya et al., 2018).

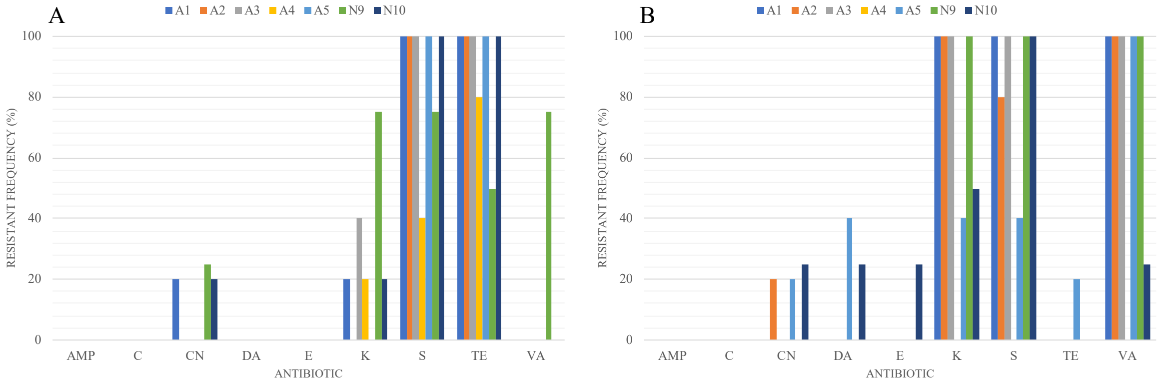

Figure 3.11. Lactic Acid Bacteria (LAB) antibiotic resistance frequencies in 2016 (A) and 2017 (B) for representative isolates. AMP: Ampicillin; C: Chloramphenicol; CN: Gentamicin; DA: Clindamycin; E: Erythromycin; K: Kanamycin; S: Streptomycin; TE: Tetracycline; VA: Vancomycin

On the other hand, resistance to clindamycin and erythromycin are only found in samples from 2017 and even though isolates resistant to clindamycin have been found in a work previously referenced (Erginkaya et al., 2018), susceptibility to these two antibiotics have been described in other works (Gad et al., 2014; Guo et al., 2017). None of these isolates had multiresistance since, according to a group of international experts (Magiorakos et al., 2012), multi-drug resistance was defined as acquired non-susceptibility to three or more antibiotics from different classes and with different targets. Even so, resistance to erythromycin is especially worrying because it’s one of the most prescribed antibiotic and since these bacteria can act as reservoir and transfer resistance genes to other bacteria (Devirgiliis et al., 2013; Mathur & Singh, 2005) this could become a problem.

These two resistances found in isolates from 2017 samples should be further investigated to see if these could possibly be new common resistances found in LAB isolates from food.

There were no significant differences in the number of resistant isolates between year ($X^2 = 2.950$; p = 0.086) or cheesemaking unit ($X^2 = 4.550$; p = 0.603) as we can see in Table 3.1. A higher percentage (31%) of resistant isolates were found in 2017 and the cheesemaking unit with higher percentage (32%) of resistant isolates through both years was N9.

Table 3.1. Chi-square test results of resistant isolates obtained through 2016 and 2017 LAB isolates analyzed and between cheesemaking units.

Year	**Resistant n (%)**	**p-value**
2016	74 (24)	0.086
2017	62 (31)	
Cheesemaking unit		
A1	24 (30)	0.603
A2	23 (28)	
A3	15 (28)	
A4	7 (16)	
A5	25 (25)	
N10	22 (27)	
N9	20 (32)	

3.3.1.2. *Lactococcus* spp.

In this group of bacteria, we chose the same antibiotics studied for LAB because the same studies (Devirgiliis et al., 2013; Domingos-Lopes et al., 2016; Erginkaya et al., 2018; Gad et al., 2014; M. Álvarez-Cisneros & Ponce-Alquicira, 2019; Temmerman et al., 2003) that studied other LAB genera such as lactobacilli also studied lactococci. Therefore, antibiotics studied are usually the same for these genera, lactobacilli and lactococci, due to proximity and both being considered safe for foods.

In isolates from 2016, even though we had much lower resistances than in 2017 isolates we still observed resistance to all antibiotics studied except gentamicin. Furthermore, almost every isolate showed resistance to more than two antibiotics although isolates can only be considered multi-drug resistant if resistance to three or more antimicrobials from different classes is observed, as explained before (Magiorakos et al., 2012). Likewise, intrinsic resistances shouldn't be taken into account to determine if an isolate is multi-drug resistant (MDR).

In Figure 3.12. and Table 3.2. we can clearly observe that there were much more resistances in 2017 samples both in frequency and presence of resistant isolates in cheesemaking units. Logically, since more resistances were observed in 2017 samples more isolates with resistance to more than one antibiotic were observed too. In these samples, resistances were detected to all antibiotics except for gentamicin and most of the isolates were resistant to more than five antibiotics.

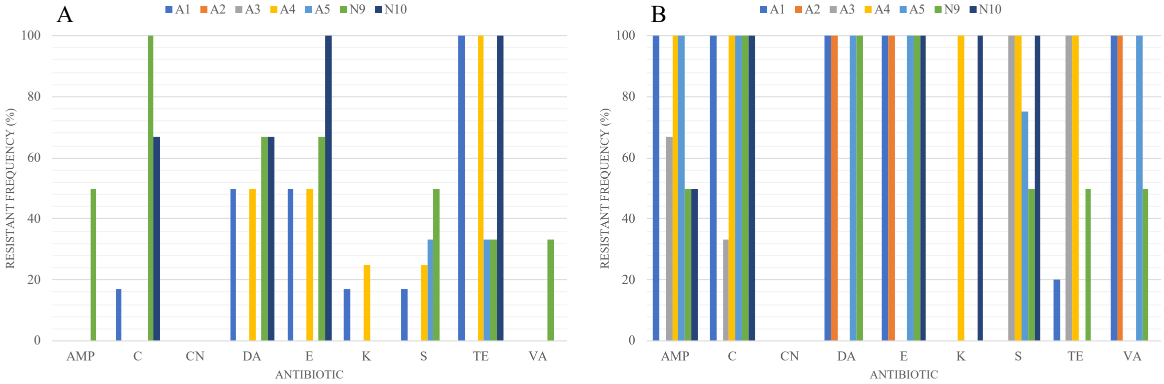

Figure 3.12. *Lactococcus* spp. antibiotic resistance frequencies in 2016 (A) and 2017 (B) for representative isolates. AMP: Ampicillin; C: Chloramphenicol; CN: Gentamicin; DA: Clindamycin; E: Erythromycin; K: Kanamycin; S: Streptomycin; TE: Tetracycline; VA: Vancomycin

On one hand there have been some resistances described in *Lactococcus* spp., such as to vancomycin, streptomycin or tetracycline in isolates from dairy products (Gad et al., 2014). On the other hand, several studies and general scientific opinion as to this genus safety have showed that *Lactococcus* spp. are sensitive to most antibiotics tested against them (Devirgiliis et al., 2013; Domingos-Lopes et al., 2016; Mathur & Singh, 2005).

The fact that we have a significant rise in resistant isolates from one year to another could be a concern because these microorganisms are gaining resistances and could present a problem to human health. This concern is due to the fact that even though in conjugation experiments with both lactobacilli and lactococci (Devirgiliis et al., 2013) the potential of horizontal transmission to other bacteria was low it still happens. So, in this particular genus it’s not intrinsically concerning the fact that we observe these resistances, it would be a problem if the genes responsible for these resistances are transmitted to potentially pathogenic bacteria. Although HGT has only been described for tetracycline in lactococci species isolated from dairy products (Devirgiliis et al., 2013).

Considering that there isn’t much consensus as to which resistances are intrinsic in this genus it becomes challenging to assign the MDR category to these isolates. Nevertheless, there were isolates in every unit from 2016 and 2017 resistant to at least three antibiotics from different classes but only with two different targets. Taking this into account, and the previously mentioned definition, these isolates can’t be considered MDR.

It’s important to point out that isolates from A2 and A3 units from 2016 weren’t analyzed for resistances because it was impossible to regrow those isolates. This same problem happened to some isolates from 2017 and that’s why for this genus less isolates were studied.

As shown in Table 3.2., resistant number of representative isolates studied from 2016 and 2017 were significantly different from each other ($X^2 = 21.022$; $p < 0.001$) but there were no significant differences in the number of resistant isolates between cheesemaking units ($X^2 = 6.023$; $p = 0.421$). Representative isolates from 2017 show a higher percentage of resistances (50%) and as to cheesemaking units the one with the highest percentage (49%) throughout both years is N9.

Table 3.2. Chi-square test results of resistant isolates obtained through 2016 and 2017 *Lactococcus* spp. isolates analyzed and between cheesemaking units.

Year	**Resistant n (%)**	**p-value**
2016	58 (28)	< .001*
2017	102 (50)	
Cheesemaking unit		
A1	41 (41)	0.421
A2	12 (36)	
A3	9 (25)	
A4	15 (33)	
A5	24 (39)	
N10	38 (42)	
N9	21 (49)	

*Significant value $p<0.05$

3.3.1.3. *Enterococcus* spp.

In the case of enterococci, resistance to thirteen antibiotics was studied, belonging to nine different classes: aminoglycosides, macrolides, oxazolidinones, chloramphenicol, streptogramins, tetracyclines, glycopepetides, ß-lactams and quinolones. With the chosen antibiotics three different targets were studied such as protein synthesis inhibition, cell wall synthesis inhibition and DNA synthesis inhibition (for details see Table 2.3. on page 11). Moreover, resistance to these antibiotics have been studied for different foods and cheeses from Portugal, Germany, Italy, Turkey and other parts of Europe (Bertrand et al., 2000; Delpech et al., 2012; G. Giraffa et al., 2000; Ispirli et al., 2017; Mus et al., 2017; Peters et al., 2003; Pimentel et al., 2007; Porto et al., 2016; Russo et al., 2018; Teuber et al., 1999).

In this work two classifications were used with different breakpoint values for antibiotic susceptibility, EUCAST ("The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0. 2019.," n.d.) and CLSI ("Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; nineteenth informational supplement," n.d.). These organizations have different breakpoints for some antibiotics, for example, EUCAST breakpoint for vancomycin resistance is below than 12 mm while CLSI breakpoint for the same antibiotic is below than 14 mm. This originates discrepancies causing isolates to be resistant according to one classification but susceptible according to the other. In the case of EUCAST some of the antibiotics we studied don't have breakpoints established such as gentamicin, streptomycin, chloramphenicol, tetracycline, amoxicillin and erythromycin. While EUCAST is more used in some parts of Europe, CLSI is the preferred system for the United States of America and other regions outside Europe. Taking into consideration that CLSI has breakpoint values for all antibiotics used in this work it would make more sense to use this one although in this work we compared results obtained with both classifications to be able to know if resistances found are present using both classifications.

In Figure 3.13.A we can see that resistance to quinupristin/dalfopristin was the most prevalent in samples from 2016, most of isolates being resistant to this antibiotic. However, resistance to this antibiotic is usually high in enterococci isolates and considered part of intrinsic resistances. Besides, as we will discuss after, this resistance diminishes in the following years and this could mean that even though these bacteria probably have the gene responsible for this resistance, phenotypically it could progressively be irrelevant.

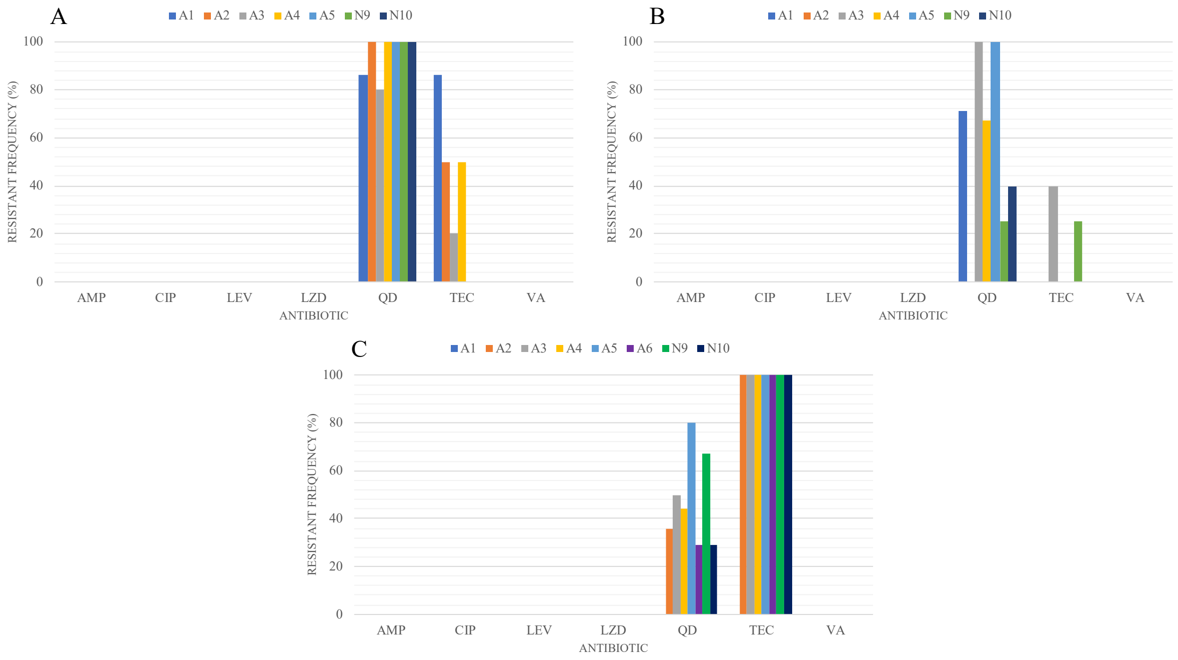

Figure 3.13. *Enterococcus* spp. antibiotic resistance frequencies in 2016 (A), 2017 (B) and 2018 (C) cheeses isolates, according to EUCAST breakpoints. AMP: Ampicillin; CIP: Ciprofloxacin; LEV: Levofloxacin; LZD: Linezolid; QD: Quinupristin/Dalfopristin; TEC: Teicoplanin; VA: Vancomycin

In all three years resistances to quinupristin/dalfopristin and teicoplanin were observed, considering that in 2017 (Figure 3.13.B) there were fewer resistant isolates in general, as discussed before. Even though we observed resistances to the same antibiotics as in 2016, for teicoplanin for example only two units had resistant isolates. Even so, one of the isolates showed resistance to both antibiotics. As discussed before, resistance to quinupristin/dalfopristin is considered intrinsic but teicoplanin resistance is considered acquired. The fact that we had less resistances in 2017 could be due to not showing that phenotype but the genes responsible for those resistances being in those isolates. This would explain the resistant isolates increase in 2018, which in the case of teicoplanin was even higher than in 2016.

All isolates analyzed from 2018 (Figure 3.13.C) showed resistance to teicoplanin and there were also isolates resistant to quinupristin/dalfopristin in all units from that year. Furthermore, even though it seems teicoplanin resistance has risen from 2016 until now, resistance to quinupristin/dalfopristin has diminished. Teicoplanin and vancomycin belong to the same class, glycopeptides, but according to EUCAST breakpoints no isolate was found to be resistant to vancomycin. There were only resistant isolates to vancomycin when taking into account CLSI breakpoints. The opposite happens with teicoplanin since with CLSI values no isolate was found to be resistant. This shows how variable results can be, even for antibiotics from the same class.

Resistance to quinupristin/dalfopristin has been found in *Enterococcus* spp. isolates from clinical sources (Wang et al., 2016) and in foods from animal origin (Mus et al., 2017; Peters et al., 2003). In this last study a small percentage of resistant isolates to linezolid was also found, but not in our case. Resistant isolates to teicoplanin have also been found before in artisanal foods of animal origin (Delpech et al., 2012). On the other hand, a recent study with *Enterococcus* spp. from human and red meat sources

(Golob et al., 2019) found no isolates resistant to this antibiotic. Resistance to teicoplanin is alarming because this antibiotic is commonly used to fight multiple antibiotic resistant strains or in presence of allergies to other antibiotics in clinical therapy (Giorgio Giraffa, 2002). Even though these isolates aren't clinical it's worrying that resistances to antibiotics that are considered as last therapeutic options, such as teicoplanin or quinupristin/dalfopristin, were found. However, as mentioned before resistance to quinupristin/dalfopristin is considered intrinsic in many studies, this means that only teicoplanin, as extrinsic resistance, would be a concern as to horizontal transfer of antibiotic resistance genes.

Starting off comparing these results with the ones obtained with CLSI breakpoint values (Figure 3.14.) we can see that there weren't any resistant isolates to teicoplanin, so resistance to this antibiotic is only observed if we take into account EUCAST values. As to resistance to quinupristin/dalfopristin according to CLSI we observe that the frequency of resistant isolates grows through the years until 2018 when half of the cheesemaking units have at least 20% of isolates resistant to this antibiotic. This way, resistance to this antibiotic is observed with both classifications (EUCAST and CLSI) although with clear differences, being that with CLSI breakpoint less resistant isolates are observed.

In samples from 2016 (Figure 3.14.A), almost every isolate showed resistance to tetracycline and although this wasn't the case for the rest of the years it is an important resistance since it's considered acquired. Ciprofloxacin resistant isolates were only found in one cheesemaking unit from 2016 and this resistance has been previously described in isolates from artisanal food (Delpech et al., 2012) and animal origin foods (Mus et al., 2017). The isolate resistant to that antibiotic showed resistance to tetracycline too. Both resistances are considered acquired and of importance in enterococci although both have been described in several past studies as mentioned before.

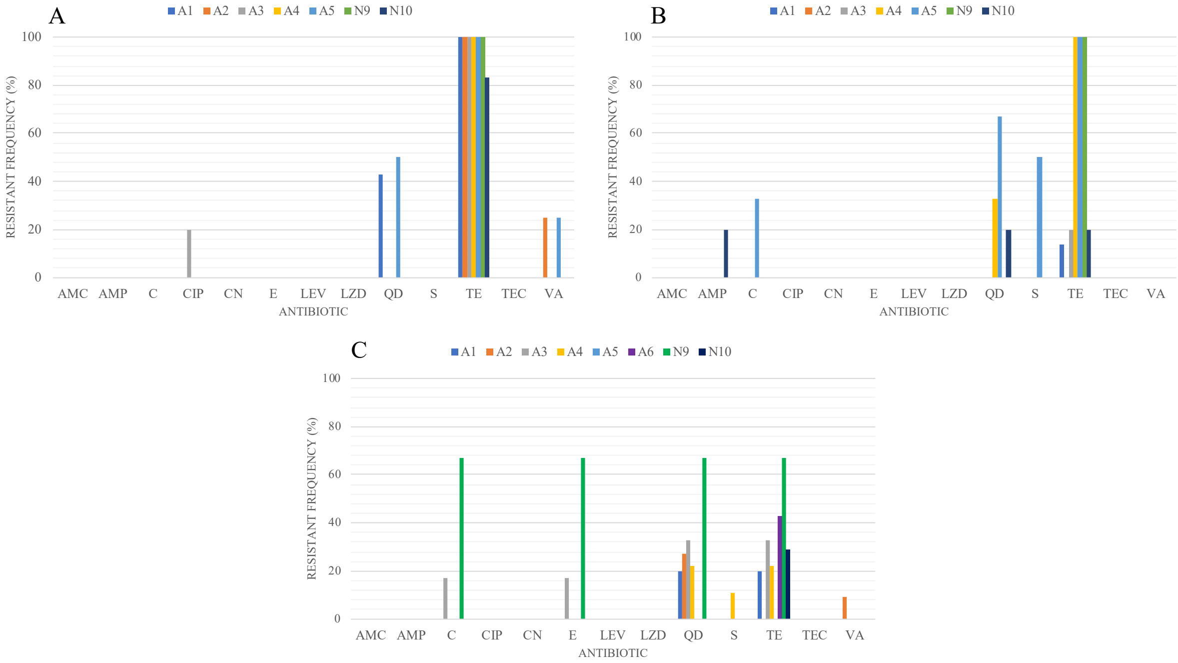

Figure 3.14. *Enterococcus* spp. resistance frequencies in 2016 (A), 2017 (B) and 2018 (C) cheese isolates, according to CLSI breakpoints. AMP: Ampicillin; C: Chloramphenicol; CIP: Ciprofloxacin; CN: Gentamicin; E: Erythromycin; LEV: Levofloxacin; LZD: Linezolid; QD: Quinupristin/Dalfopristin; S: Streptomycin; TE: Tetracycline; TEC: Teicoplanin; VA: Vancomycin

In 2017 samples (Figure 3.14.B) new resistances were observed such as ampicillin, chloramphenicol and streptomycin and in the case of these two last resistances they were found in the same isolate. We have to take into consideration that both low level streptomycin and ampicillin are considered intrinsic resistances. So, in this year the new relevant data was the resistance to chloramphenicol that has been found in isolates from humans (Golob et al., 2019), isolates from food of animal origin (Peters et al., 2003) and in isolates from cheeses (Giorgio Giraffa, 2002) and is considered an acquired resistance. This is important too because in 2018 samples two other units had isolates resistant to this antibiotic. Resistance to streptomycin is also important because in this study the dose tested was very high so it would be considered acquired resistance.

Resistance to erythromycin was only observed in isolates from 2018 samples (Figure 3.14.C) and in two cheesemaking units so it could be the rise of a new resistance in the analyzed cheeses. This resistance has been previously described in food and milk isolates (Delpech et al., 2012; Giorgio Giraffa, 2002; Różańska, Piłat, Kubajka, & Weiner, 2019) and it has also been observed in isolates from animal farms (Chopra & Roberts, 2001). Therefore, its resistance has been associated with overexploitation of these antibiotics in veterinary practices. Tetracycline resistance was also observed in 2018 samples and in the same study mentioned before it was also linked to the use of these antibiotics in animal environments. Moreover, all isolates resistant to erythromycin were also resistant to tetracycline and chloramphenicol. All three resistances are considered acquired and could have been horizontally transferred to these isolates or potentially be transferred to other bacteria and cause a problem with the treatment of clinical infections.

Resistance to vancomycin was found in isolates from two cheesemaking units from 2016 and 2018 samples. Resistance to this antibiotic has been previously described in isolates from artisanal cheeses (Porto et al., 2016) and foods with animal origin (Delpech et al., 2012; Mus et al., 2017). This antimicrobial agent is a “last resort drug” against clinical infections and it could potentially be horizontally transferred since it’s considered an acquired resistance in enterococci. Even though in 2018 samples we saw a lower percentage of resistance to this antibiotic, these results were still relevant due to its importance in clinical infections because there has been an increase in infections caused by vancomycin-resistant enterococci (VRE) (Vehreschild, Haverkamp, Biehl, Lemmen, & Fätkenheuer, 2019). Teicoplanin, that belong to the same class as vancomycin, is also considered a “last resort drug” but in this case no isolate resistant to this antibiotic was found when taking into consideration CLSI breakpoints.

Intrinsic antibiotic resistances to low-level aminoglycosides such as gentamicin or streptomycin and ß-lactams such as amoxicillin/clavulanic acid or ampicillin have been widely described (Hollenbeck & Rice, 2012) and were observed in this work. Regarding these intrinsic resistances only one isolate was resistant to ampicillin and other isolate to streptomycin from 2017 samples. From 2018 isolates only one was resistant to streptomycin and no other intrinsic resistance was observed. As mentioned before, resistant isolates to streptomycin in our work were relevant due to the high-level dose applied in our tests. Therefore, resistance to this antibiotic in our work would be considered acquired.

Isolates resistant to tetracycline were found in 2016, 2017 and 2018 samples although it seems this resistance has a downward trend. Resistance to this antibiotic has been described in isolates from cheeses and other foods with animal origin (Giorgio Giraffa, 2002; Mus et al., 2017). This is an antibiotic that is becoming less and less popular precisely due to increased resistance (Grossman, 2016) and because since it is an acquired resistance it has the potential to be horizontally transferred to other bacteria and it’s probably what has occurred precipitating the less clinical application of this drug.

Multidrug-resistant (MDR) bacteria has been defined by scientists that belong to several international organisms such as CLSI, EUCAST and the United States Food and Drug Administration (FDA) as acquired non-susceptibility to at least one agent in three or more antimicrobial categories or classes with different targets (Magiorakos et al., 2012). According to this definition and EUCAST breakpoints we had no MDR isolates in any studied cheeses. Taking into account CLSI breakpoints one isolate from 2017 and A5 unit showed resistance to three antibiotics from different classes but two of them had the same target. From 2018 cheeses two isolates from two different cheesemaking units (A3 and N9) were found to be resistant to three different antibiotics from three different categories but, again, two of those had the same cell target. So, in conclusion, no enterococci isolate was found to be MDR in our work.

For 2017 isolates that were identified at species level we could see if there was some connection between species and certain resistances. Resistance to quinupristin/dalfopristin, tetracycline and teicoplanin was observed both in *E. faecium* and *E. faecalis* isolates from different units. Intermediate values (neither resistant nor susceptible) for vancomycin appeared in three isolates identified as *E. faecalis* from three different units (data not shown). Moreover, intermediate values for erythromycin and ciprofloxacin were found in several isolates identified as *E. faecium* from different cheesemaking units. The only isolate that presented resistance to ampicillin was identified as *E. faecium* and showed resistance to quinupristin/dalfopristin and tetracycline as well as intermediate values for ciprofloxacin and erythromycin. The isolate resistant to four antibiotics (streptomycin, chloramphenicol, quinupristin/dalfopristin and tetracycline) according to CLSI breakpoints was identified as *E. faecalis*. In a recent study enterococci isolates from two Italian cheeses were identified and it was observed that *E. faecalis* is the most resistant species while the most variability was found in *E. faecium* isolates (Russo et al., 2018). This could be tested for our cheeses if in the future we identify the rest of isolates and compare the resistances from all studied years but for now we confirm that data from previous studies.

Throughout the three studied years there were significant differences as to number of resistant isolates as shown in Table 3.3., according to EUCAST ($X^2 = 14.323$; $p < 0.001$) and CLSI ($X^2 = 18.500$; $p < 0.001$). It's important to keep in mind that CLSI has breakpoints for all antibiotics studied but EUCAST doesn't so CLSI results could be more representative. According to EUCAST values practically the same percentage of resistances were found in 2016 and 2018 isolates but there was a significant drop in 2017. As to CLSI parameters, similar values appeared in 2017 and 2018 while in 2016 there were more resistant isolates.

As to significant differences on resistant isolates between cheesemaking units through the three years studied these were only found when taking into account CLSI values ($X^2 = 15.104$; $p = 0.035$). Regarding these results the two units with higher percentage of resistant isolates were A5 and N9. Furthermore, the unit with less resistant isolates is A6 but since this unit was added to this study in 2018 there were no other resistance results to compare with. It could be interesting to see if this low percentage of resistances remains throughout future years of production.

Table 3.3. Chi-square test results of resistant isolates obtained through 2016, 2017 and 2018 in *Enterococcus* spp. isolates analyzed and between cheesemaking units.

Year	**Resistant n (%) EUCAST**	**p-value EUCAST**	**Resistant n (%) CLSI**	**p-value CLSI**
2016	43 (10)	< .001*	45 (10)	< .001*
2017	18 (4)		20 (5)	
2018	82 (11)		32 (4)	
Cheesemaking unit				
A1	30 (10)	0.952	17 (5)	0.035*
A2	21 (9)		9 (4)	
A3	18 (9)		13 (6)	
A4	21 (10)		14 (6)	
A5	16 (10)		15 (10)	
A6	9 (10)		3 (3)	
N10	17 (7)		10 (4)	
N9	11 (8)		16 (11)	

*Significant value p<0.05

In conclusion, it seems that through the years until 2018 there has been less percentage of resistant isolates per antibiotic, but new resistances have arisen and some to very important antibiotics used nowadays as treatment to clinical infections. We also observed in 2018 samples resistances linked with the overexploitation and bad use of antibiotics that were not present in past years. This supports the growing concern of the scientific community as to the use of antibiotics in animal environments.

3.3.2. Virulence factors

Enterococci has been the group within LAB in which most studies about virulence factors have focused because they are important not only in food but also in clinical infections (Franz et al., 2001; Ike et al., 1987; Jett et al., 1994; Malani et al., 2002; Murray, 1990). Gelatinase production and hemolysis were the factors chosen to work with due to their importance in *Enterococcus* spp. and the existence of easy methods to study their phenotype. Hemolysis was studied for representatives of 2016, 2017 and 2018 samples whereas gelatinase test was only performed for 2016 and 2017 representatives.

Isolates that produced ß-hemolysis were the only ones considered hemolysis positive. As shown in Figure 3.15. the percentage of isolates hemolysis positive was practically the same for 2016 (11.8%) and 2018 (12.1%) whereas for 2017 the percentage was nearly half of that (6.5%).

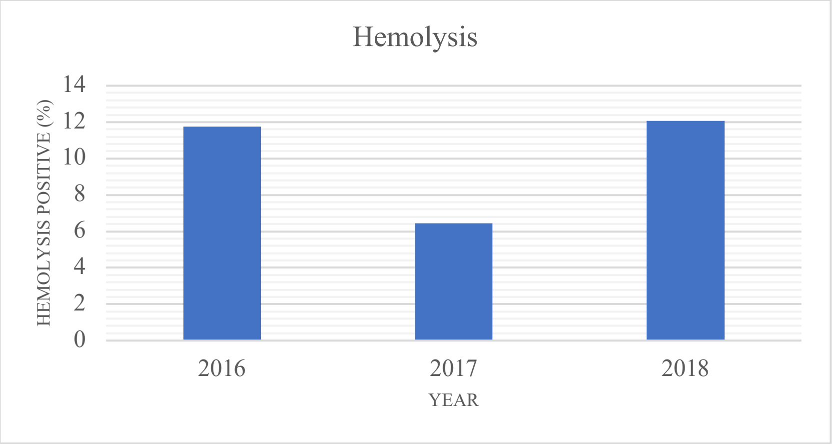

Figure 3.15. Percentage of ß-hemolytic in representative *Enterococcus* spp. from 2016 (34 isolates), 2017 (31 isolates) and 2018 (58 isolates).

As shown before in this work species identification for most 2017 representative isolates was performed. Linking that identification to these results, one of the positives obtained was identified as *Enterococcus durans* and 7 isolates that were α-hemolytic were identified as *E. faecium*.

A previous study that performed this same assay with isolates from cheese produced with sheep milk (Semedo et al., 2003) found that 6% of the food isolates were ß-hemolytic, the same percentage that we obtained in 2017 isolates. Further investigation of virulent factors with molecular methods such as PCR could give us the answer to why there are differences in percentage between years since in previous studies (Ispirli et al., 2017; Porto et al., 2016) about this virulent factor in enterococcal isolates there are contradictory results such as in one study no hemolysis was detected and in the other all isolates studied showed hemolysis. The most likely explanation to this is that even though isolates can have the gene responsible for hemolysins production that doesn't mean phenotypically we would see hemolysis.

Gelatinase assay showed only two positive results in 2017 isolates and no positives in 2016 isolates. These results were not expected since in previous studies a high percentage of isolates from food samples were gelatinase positive (Moraes et al., 2012; Soares-Santos et al., 2015). However, it's important to have in mind that studied isolates might not be gelatinase positive phenotypically but still have the gene that encodes for this virulent factor, *gelE*, and this can be attributed to a chromosomal deletion of part of loci *fsr* (Semedo-Lemsaddek & Mato, 2011).

It would be useful to perform a search for virulent genes with molecular methods in all the representative isolates because in many of the previously cited works even if phenotypically isolates don't show the virulent factor, they still have the responsible gene. Relevant virulence genes that could be studied would be the ones responsible for aggregation protein (*agg*), gelatinase (*gelE*) , cytolisin/hemolysin (*cyl*), cell wall protein involved in immune evasion (*esp*), adhesins (*efaAfs/efaAfm*) and sex pheromons (*cpd, cob, ccf, cad*) (Eaton & Gasson, 2001).

4- Conclusions

Enumeration of LAB, lactococci and enterococci in this work was consistent with previous studies that analyzed CFU counts of those bacteria in traditional cheeses. Differences were observed through the studied years that can be explained by the fact that these cheeses were made with raw milk and the particular practices of each cheesemaking unit. Similar counts were observed for LAB isolates (isolated with MRS) and lactococci while enterococci has consistently lower counts than the other studied bacteria in our cheeses probably due to the variation on pH values caused by the other bacteria.

As to enterococci identification the majority of identified isolates were *E. faecium*, followed by *E. faecalis* and *E. durans*. We also observed that some species seemed to be characteristic of certain units in 2017 samples, such as *E. durans* in A2 (Azeitão) and *E. faecium* in both Nisa units. Yeasts and molds were analyzed for 2018 cheeses, more counts of yeasts were observed and there was much more variability in molds counts. Finally, Nisa cheeses didn't report any molds or yeasts probably due to the characteristics of those cheeses since they had a harder matrix. Furthermore, no pathogenic bacteria such as *Listeria* spp. or *Salmonella* spp. were found in any of 2018 cheeses.

One of the aims of this work was to compare the diversity observed through different years until now in PDO cheeses from different cheesemaking units from Azeitão and Nisa. Concerning this, there was no observable trend between the three years studied as to the units with highest and lowest diversity index. Although two exceptions were noted, in the group *Lactococcus* spp. the same unit had the lowest diversity in 2016 and 2018 and the same happened for *Enterococcus* spp.. We can conclude that LAB diversity through the years and different units is variable and follows no specific trend. This was expected since there is no control over the microbiota of these artisanal cheeses or the relative proportion between microorganisms. Even though the method of manufacture was the same, hygienic conditions through all the process change between units and times of production. Diversity of the different groups of bacteria and cheesemaking units was not directly related to CFU counts, meaning that, in this work we observed high diversity in cheeses with high CFU counts and low CFU counts and the same happened with low diversity. There was no connection between a high or low CFU counts and the diversity of that sample. In 2018 samples the group of bacteria with highest diversity was *Enterococcus* spp.. This is relevant due to their duality when it comes to their importance for organoleptic characteristics in food environments and pathogenic potential in clinical infections. With the analysis of dendrograms of each group of bacteria from 2018 we also conclude that many isolates from different units had similar RAPD profiles which could mean that some species or strains were transversally found in several units and cheeses.

Significant differences in the number of resistances between studied years were found in *Enterococcus* spp. and *Lactococcus* spp.. In the case of the first group variations between resistances found in different cheesemaking units were also significant. In LAB isolates two relevant resistances were observed, clindamycin and erythromycin, this last one being the most worrying if horizontal transfer of those genes occur due to being one of the most prescribed antibiotics. Lactococci data from both years studied showed isolates with several resistances but since those resistances have been described as intrinsic in some previous studies, we can't be sure if this would be a concern as to gene transfer. In enterococci isolates important resistances were found such as to teicoplanin, ciprofloxacin, tetracycline, chloramphenicol, erythromycin and vancomycin. We consider these important due to their category as acquired resistances that could potentially be transferred to other bacteria. An isolate resistant to ciprofloxacin and tetracycline was observed in 2016 sample from A3 and from 2017 cheese produced in A5 a *E. faecalis* isolate was found to be resistant to streptomycin, chloramphenicol, quinupristin/dalfopristin and tetracycline. In 2018 samples resistances linked with the overexploitation and bad use of antibiotics were observed. However, no multidrug-resistant isolate was found in any of the studied cheeses.

As to virulence factors studied in enterococci no high values of hemolysis or gelatinase production were observed in this work taking into account results from previous studies where most or all isolates showed positive phenotypes for this virulence factors.

In conclusion, Azeitão and Nisa cheeses diversity and CFU counts didn't follow any concrete pattern throughout the studied years. Differences between years were found in the number of lactococci and enterococci resistant isolates. Furthermore, percentage of enterococci resistant isolates diminished since 2016 to 2018 but antibiotic resistances observed this last year were more relevant to human health such as to vancomycin, erythromycin and tetracycline. There were no MDR isolates found in this work and percentage of positive phenotypes for studied virulence factors was low. More studies about Azeitão and Nisa cheeses will be performed to complete the results shown in this work and to observe how diversity and pathogenic potential continues to evolve in bacteria from these cheeses.

5- References

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Appendix A – Dendrogram of *Enterococcus* spp. isolates

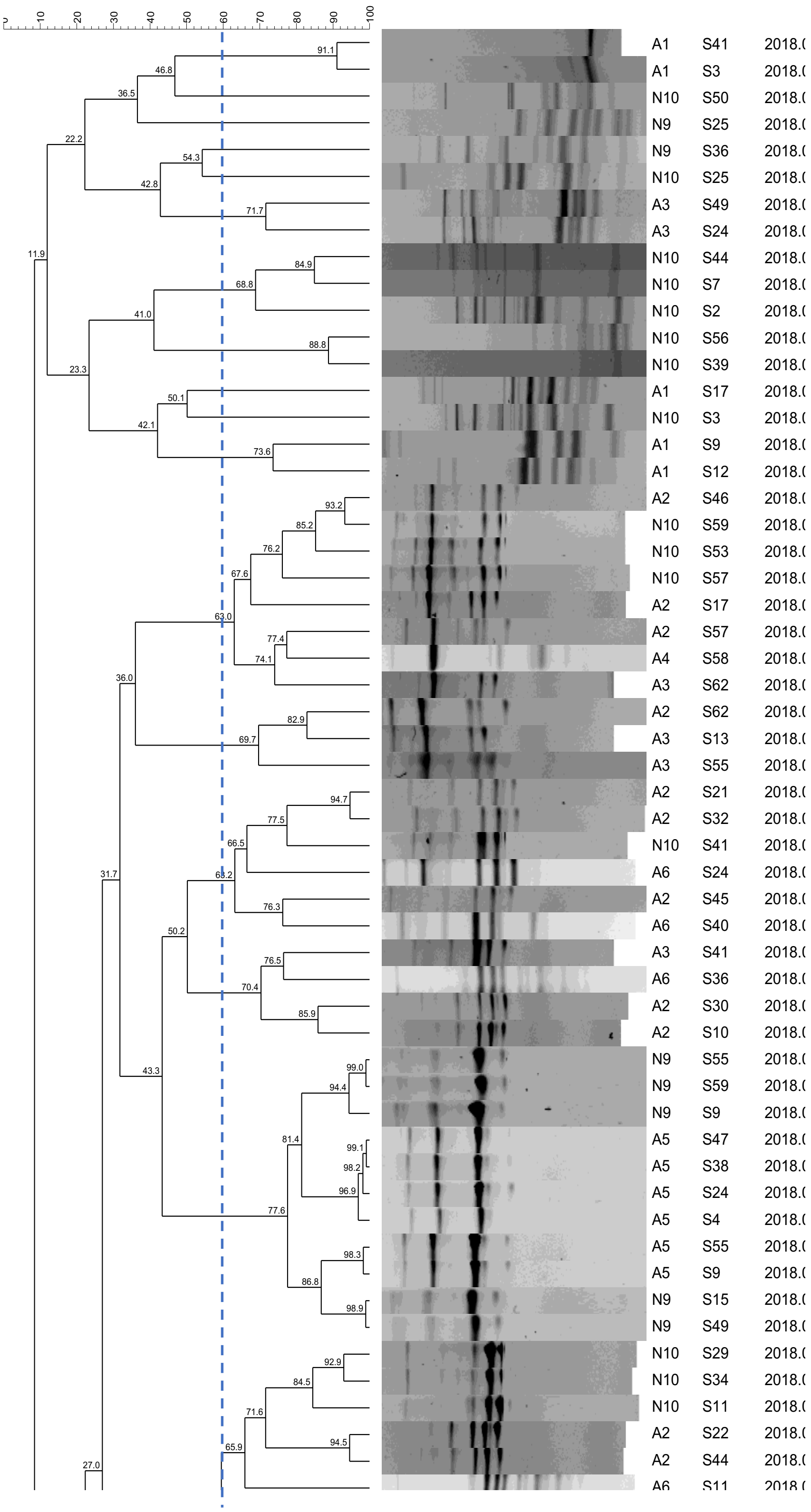

Figure 1.1. First part of dendrogram with Enterococcus spp. isolates and 60% cut-off value indicated.

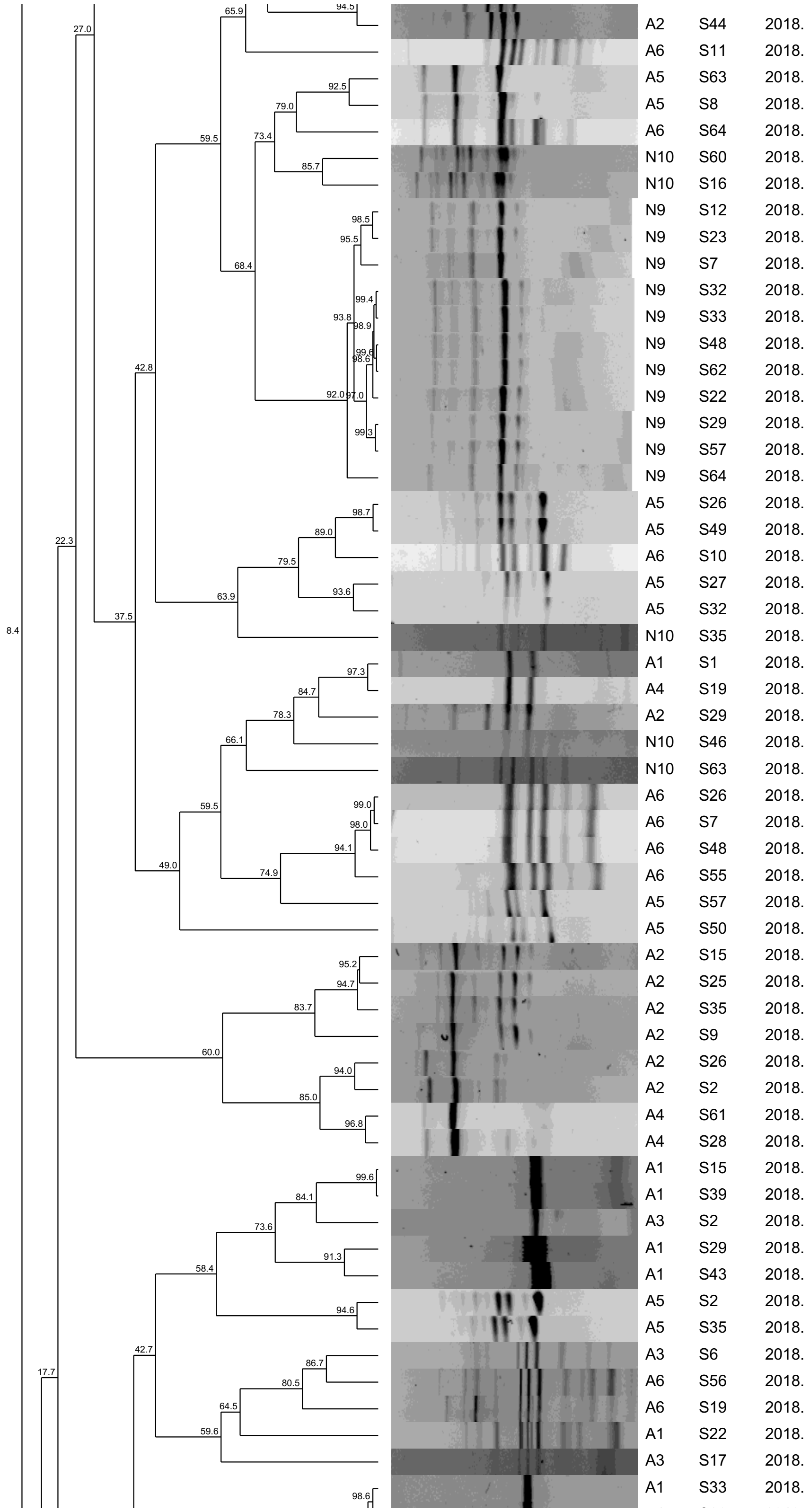

Figure 1.2. Second part of dendrogram with *Enterococcus* spp. isolates and 60% cut-off value indicated.

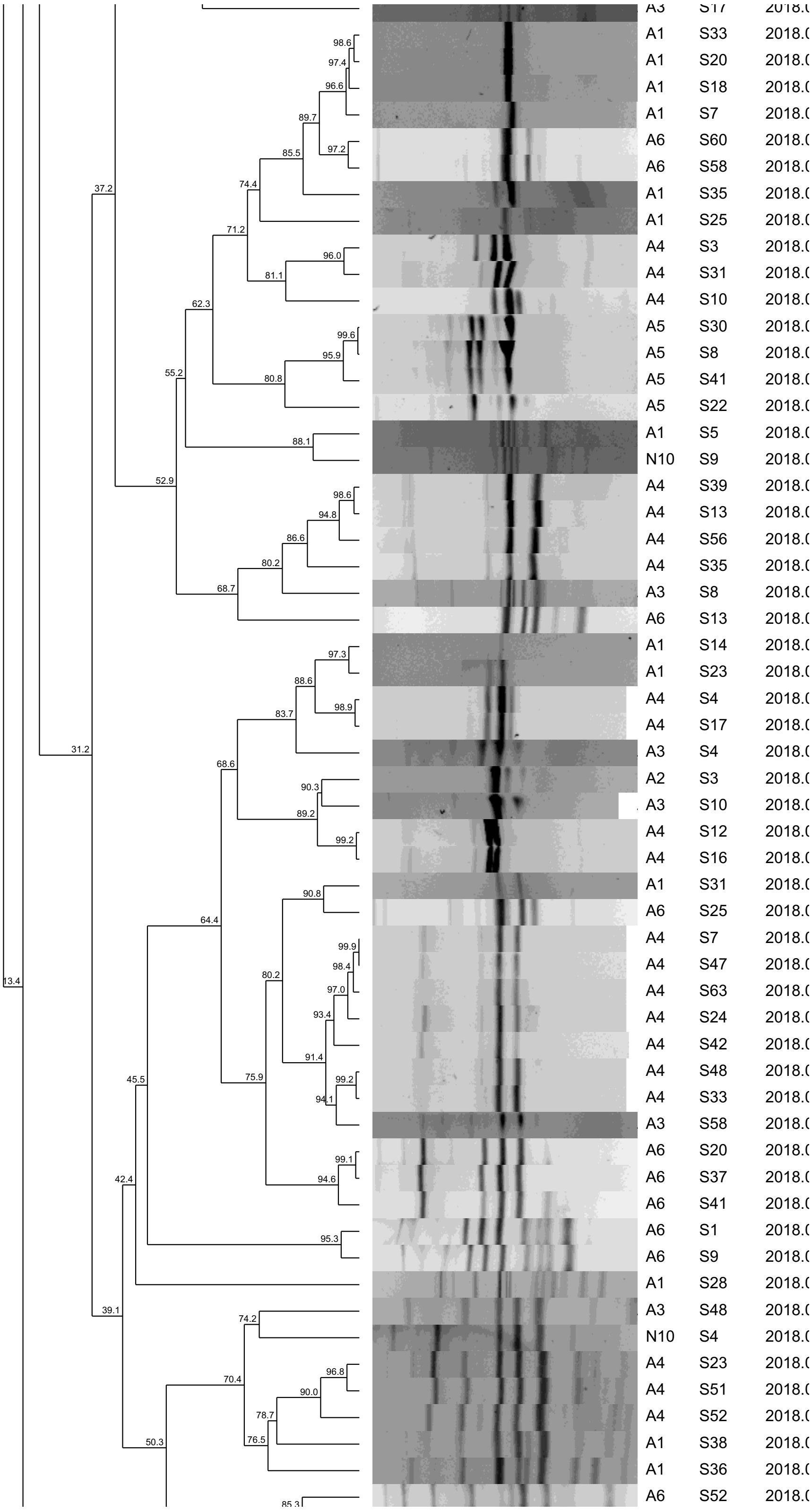

Figure 1.3. Thrid part of dendrogram with *Enterococcus* spp. isolates and 60% cut-off value indicated.

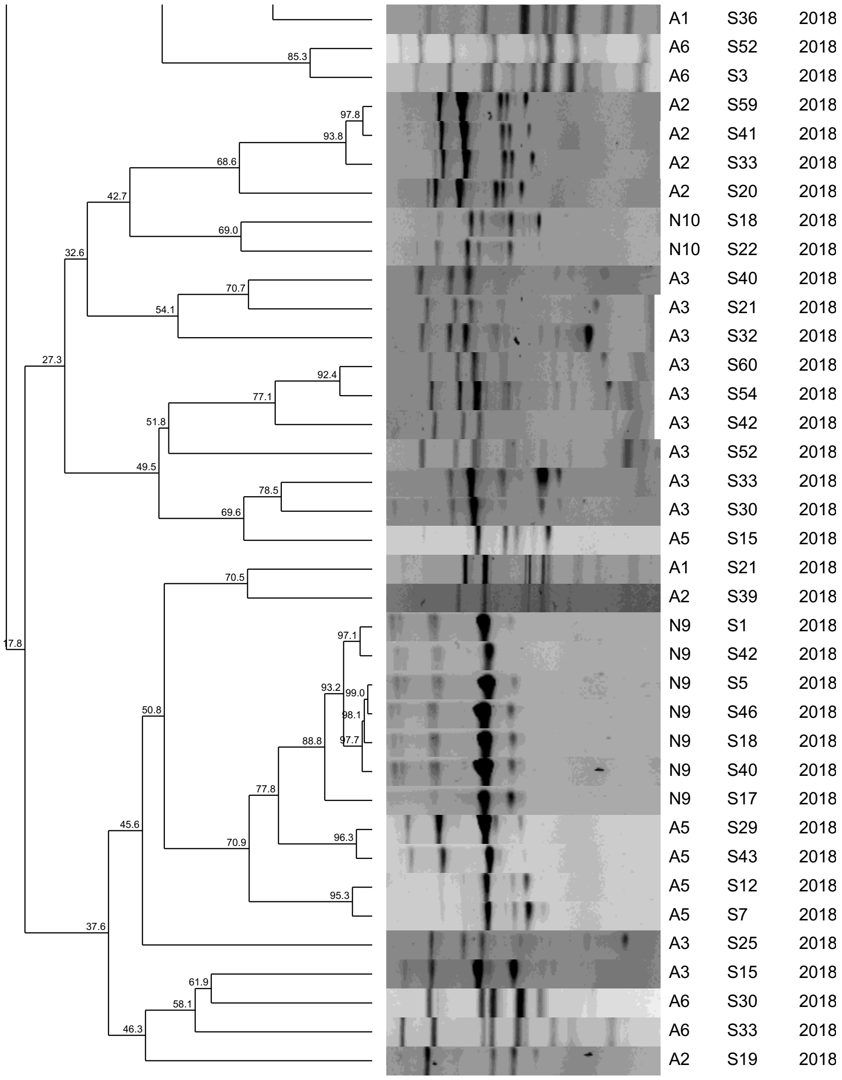

Figure 1.4. Fourth part of dendrogram with *Enterococcus* spp. isolates and 60% cut-off value indicated.

Appendix B – Dendrogram of *Lactococcus* spp. isolates

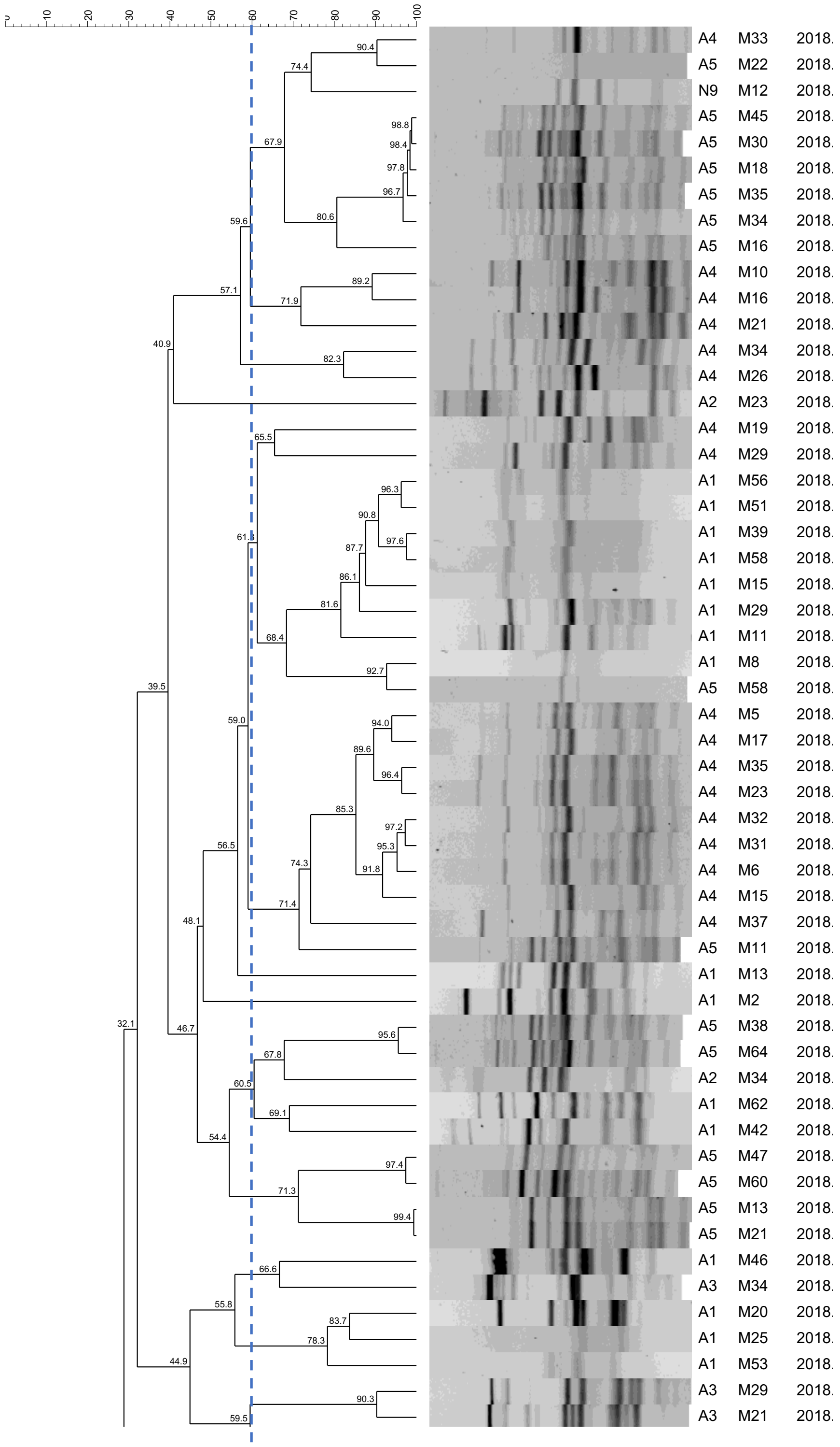

Figure 2.1. First part of dendrogram with *Lactococcus* spp. isolates and 60% cut-off value indicated.

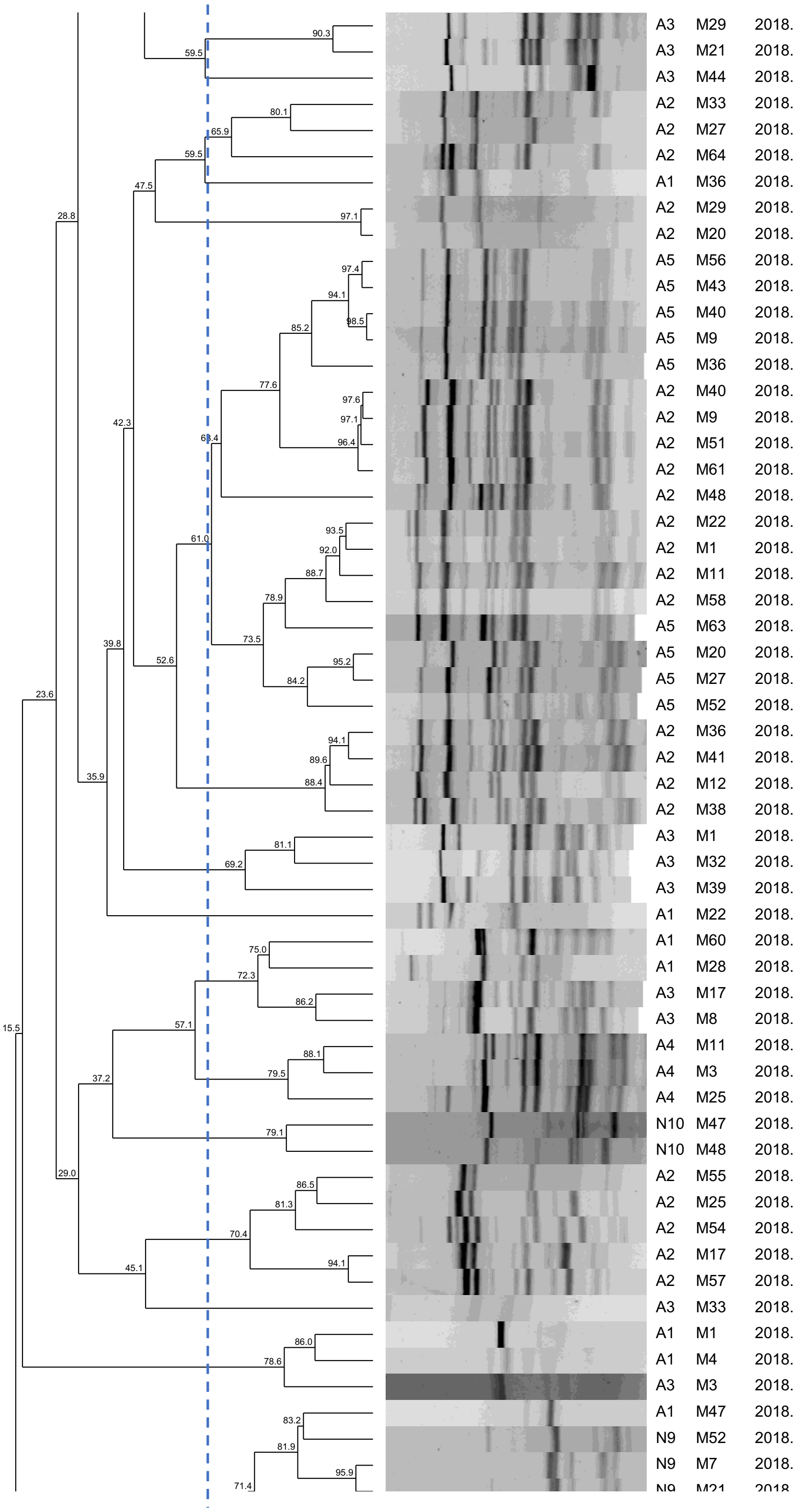

Figure 2.2. Second part of dendrogram with *Lactococcus* spp. isolates and 60% cut-off value indicated.

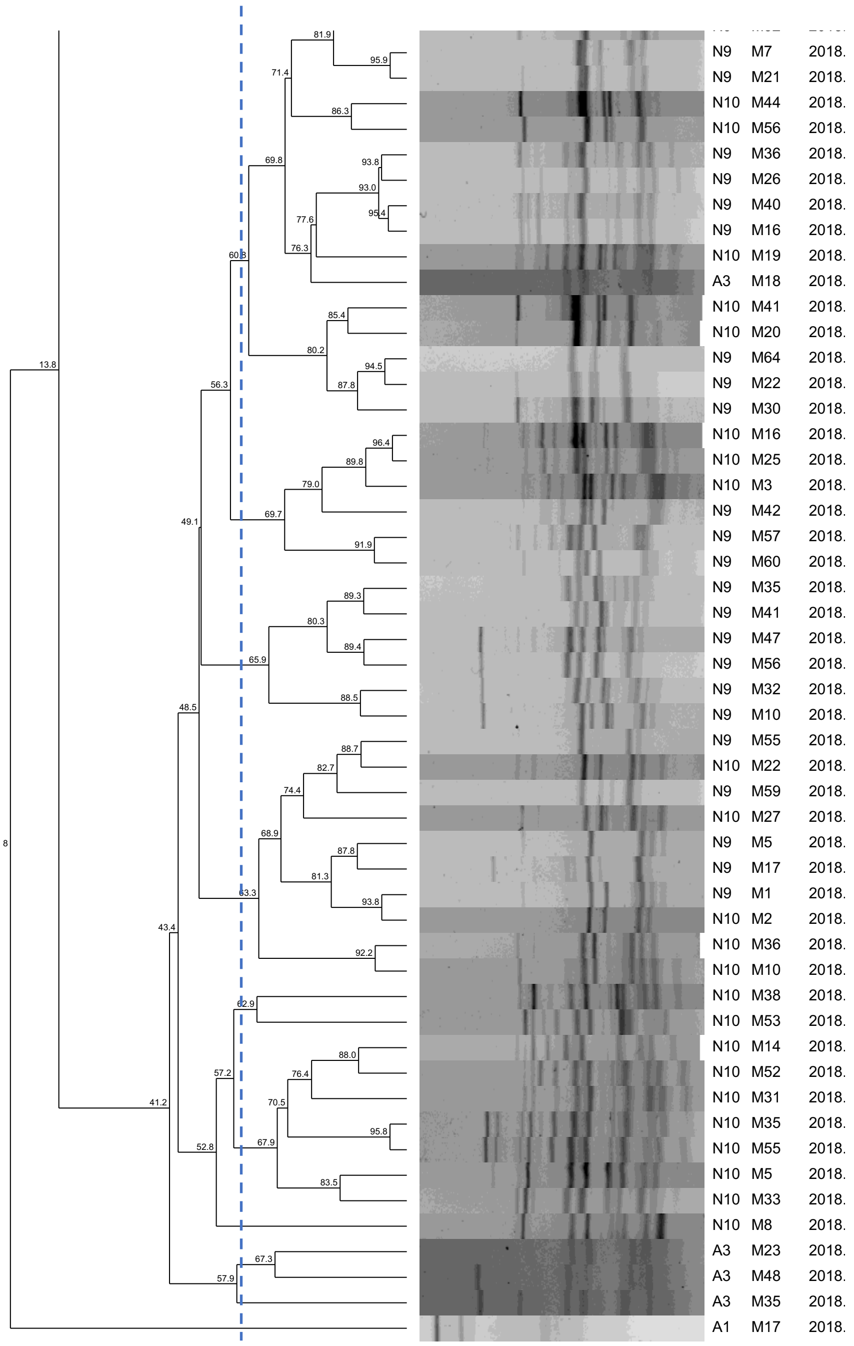

Figure 2.3. Third part of dendrogram with *Lactococcus* spp. isolates and 60% cut-off value indicated.

Appendix C – Dendrogram of LAB isolates

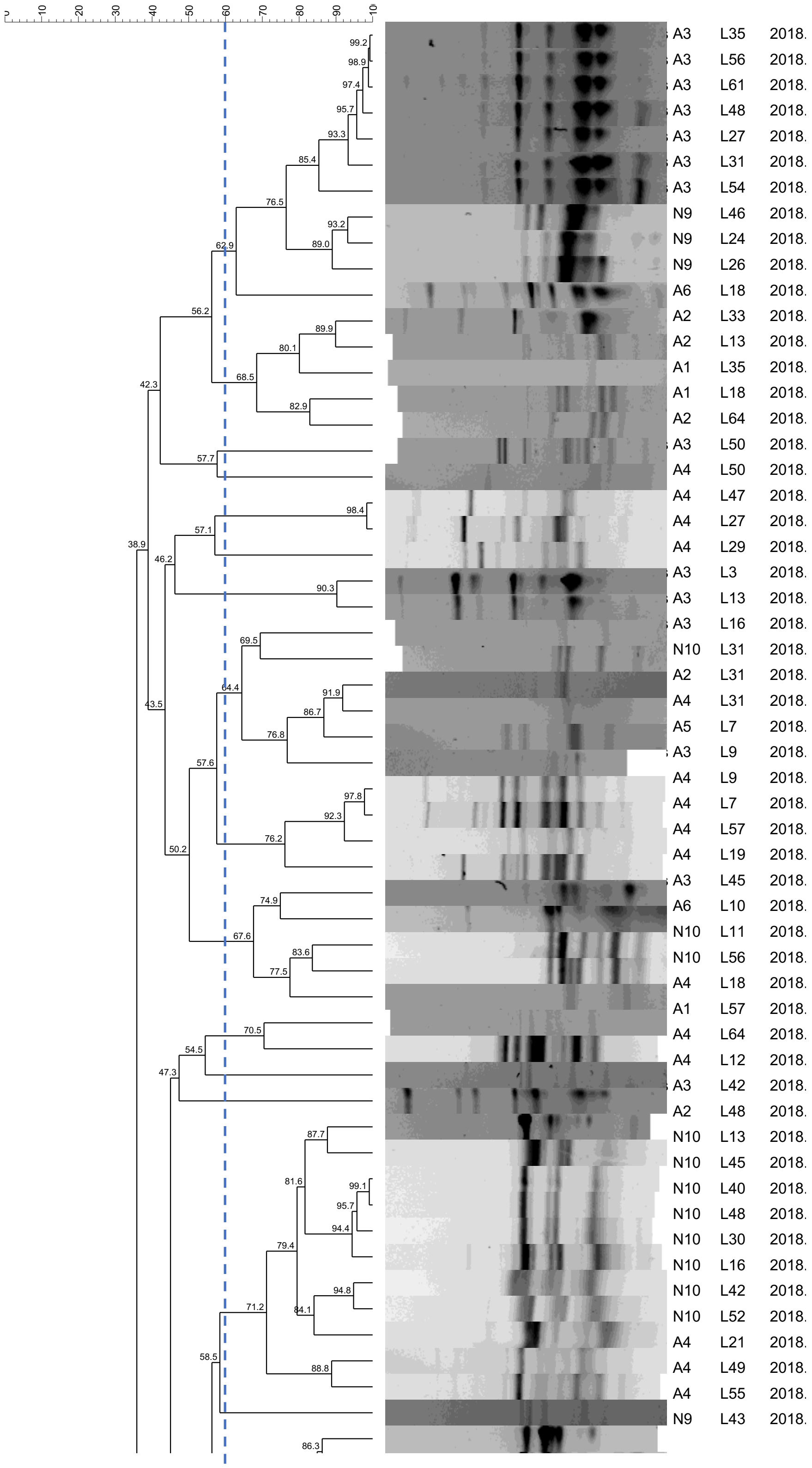

Figure 3.1. First part of dendrogram with LAB isolates and 60% cut-off value indicated.

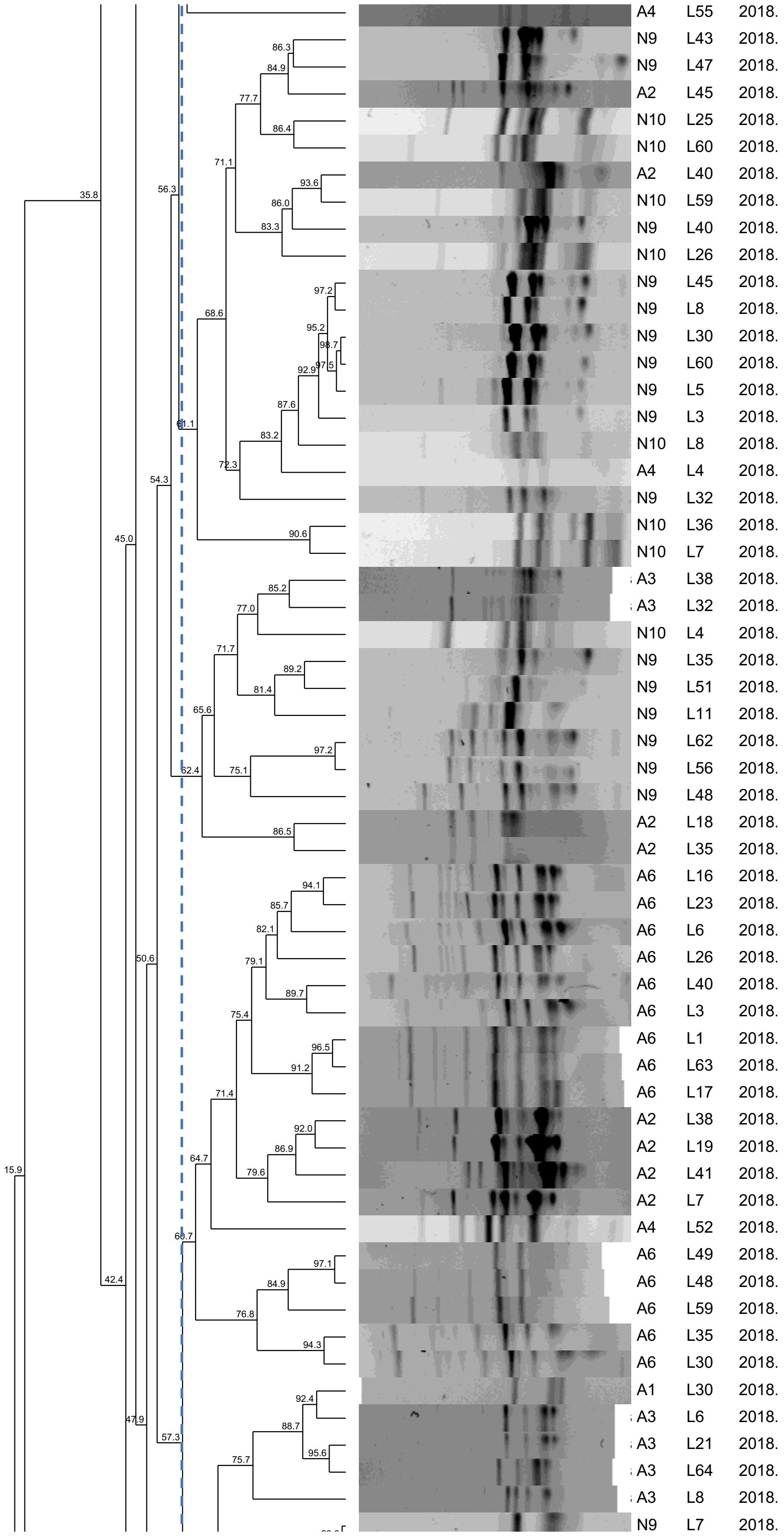

Figure 3.2. Second part of dendrogram with LAB isolates and 60% cut-off value indicated.

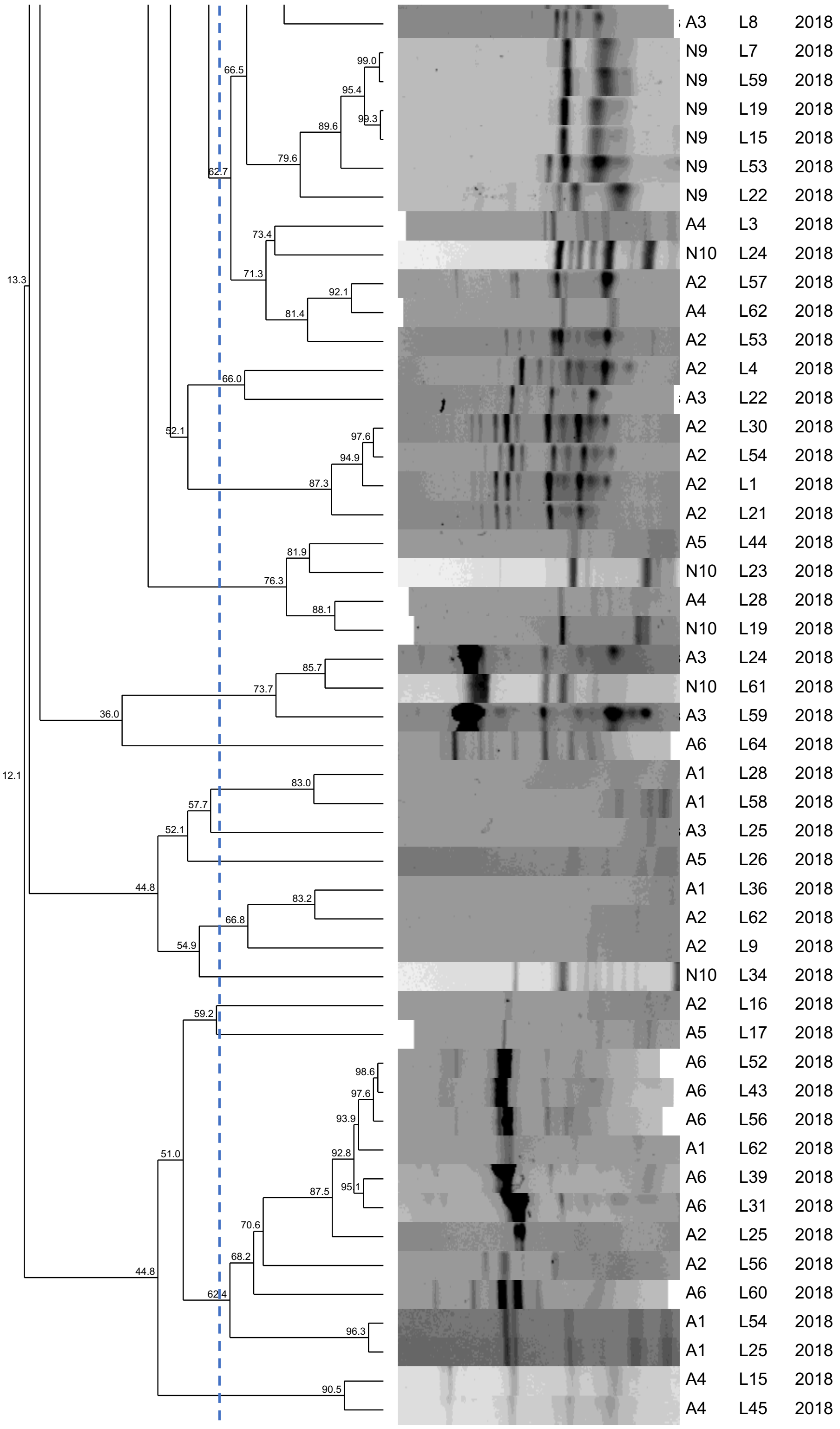

Figure 3.3. Thrid part of dendrogram with LAB isolates and 60% cut-off value indicated.