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Mestrado Integrado em Medicina Veterinária

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ESTE TRABALHO FOI REALIZADO COM A COLABORAÇÃO DE:

Microbial Diversity and Evolution (MDE)

SEGALAB, Laboratório de Sanidade Animal e Segurança Alimentar, S.A.

Centro de Investigação em Biodiversidade e Recursos Genéticos

Faculdade de ciências e Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Sozinhos, nunca chegaremos a lado nenhum.

Dedico por isso este trabalho aos meus pais. Por nunca me ter faltado nada e sempre terem acreditado em mim.

Sem vocês nada disto teria sido possível.

ABSTRACT

Streptococcus agalactiae and *Streptococcus uberis* are relevant mastitis pathogens, a prevalent and costly disease in dairy herds, which have a considerable impact on cow health, in milk quality and production. The aim of this study was to investigate the occurrence of some antimicrobials-related and virulence-related genes in *Str. uberis* and *Str. agalactiae* isolated from bovine milk and to assess the molecular epidemiology.

Several virulence genotypes were observed to be associated with mammary infections. These genotypes were found within the same herd and among different herds, showing that different virulence patterns were able to cause infection. The genetic variability was higher in *Str. uberis*, with the different virulence patterns being able to cause clinical mastitis and apparently none of the virulence genotypes seem to be dominant relative to the others. On the contrary, *Str. agalactiae* present less genetic variability.

The genes responsible for the adherence to epithelium were present in all tested strains, the *sua* gene in *Str. uberis* and the *fbsB* gene in *Str. agalactiae*. Markers derived from the fructose operon (FO1 and FO3) were specific to bovine isolates of *Str. agalactiae*. The nisin operon markers (NU1 and NU3) were able to discriminate strain-specific patterns of *Str. uberis*, but were not found in *Str. agalactiae*. The virulence-associated markers (V1, V3) were detected in all of *Str. uberis*. The *gapC* marker (V2) was able to discriminate virulence patterns of *Str. agalactiae*, and was also possible to detect the antimicrobials resistance gene *ermB* in this species. An apparent advantage in the frequency and severity of infection for strains containing the gene *gapC* was also suggested.

This work suggests reinforces the hypothesis from a previous study (Almeida *et al*., 2013) about the good consistency and stability of these markers to be used for identification of members of the *Streptococcus* genus, *Str. agalactiae* and *Str. uberi.* The obtained results also suggest the exchange of genetic material between *Str. agalactiae* and environmental bacteria. Finally it is expected that this work contributes for the implement of methodological ground to carry out epidemiological and molecular evaluations in causative agents of bovine mastitis.

Keywords: Antimicrobials Resistance; Bovine Mastitis; DNA Markers; Dot blot hybridization; *Streptococcus agalactiae*; *Streptococcus uberis*; Veterinary epidemiology; Virulence factors.

AGRADECIMENTOS

A realização deste relatório, do estágio curricular, bem como de todo o meu percurso académico só foram possíveis devido ao enorme apoio por parte de inúmeras pessoas que gostaria de destacar e endereçar os mais sinceros agradecimentos:

Em primeiro lugar queria agradecer ao meu orientador, Professor Doutor Niza Ribeiro, pela atenção e ajuda prestada durante o estágio.

Ao Professor Doutor Fernando Tavares pela forma como me recebeu, pela sua disponibilidade, pelo constante acompanhamento, dedicação e preocupação manifestada durante a realização do meu estágio e por todos os conhecimentos que me transmitiu.

À Drª. Helena Larisma Madeira, bem como a todos os veterinários da SEGALAB, pelo auxílio durante as visitas às explorações, pela simpatia e colaboração prestada para que este trabalho fosse possível. Um agradecimento especial também para a Engª. Paula Santos, pelo trabalho e paciência que teve em providenciar as estirpes necessárias para o meu estudo.

Um agradecimento para a pessoa que mais me marcou durante este estágio. Pela sua simpatia, pela forma como me recebeu, por toda a sua paciência e calma para ensinar e por toda a ajuda que me prestou. Contribuindo para o meu desenvolvimento, não só profissional, mas também pessoal. Por isso e por muito mais, quero deixar os meus sinceros agradecimentos ao meu companheiro de laboratório Pedro Albuquerque.

Ao Alexandre Almeida, pela sua orientação, paciência e disponibilidade que teve para me ensinar as técnicas e protocolos usados neste trabalho. À Maria João Fonseca e Ricardo Costa pela agradável companhia que me proporcionaram durante a minha estadia no laboratório, bem como a todo o grupo do MDE.

A todos os meus amigos da faculdade, vocês sabem quem são! Pela importância que tiveram ao longo deste meu percurso e que certamente vão continuar a ter. Em particular para o Bruno Lopes, Bruno Silva e Luís Carmo pela ajuda e companheirismo que demostraram nesta fase de estágio. Adriana Sequeira e Inês Xavier pela agradável companhia durante as longas horas de escrita da tese.

Aos meus colegas de casa, que me acompanharam de perto ao longo destes anos. Porque foram e continuarão a ser a minha segunda família: Fábio Pereira, Flávio Faustino e João Saraiva. E aos meus amigos de sempre: Ana Coelho, Cesar Gomes, Joana Felício e Vânia Teixeira.

A todos os professores que, de uma forma ou de outra, contribuíram para a minha formação. Deixando um especial agradecimento ao Professor Paulo Costa e à Professora Paula Proença pela disponibilidade demonstrada nas alturas em que mais precisei.

Por fim, quero agradecer a toda à minha família. Aos meus pais, avós, tios e primos. À minha tia Lúcia e tia Adélia por terem sido as minhas “segundas mães” em muitas ocasiões e aos meus irmãos Daniel, Edna e Patrícia.

A todos, muito obrigado.

LIST OF ABBREVIATIONS

Camp - Cyclic adenosine monophosphate

CAP - Catabolite gene activator protein

DNA Deoxyribonucleic acid

fbsA- Fibrinogen-binding protein A

fbsB - Fibrinogen-binding protein B

fbsB[C] - Fibrinogen-binding protein B C-terminal region

Fg – Fibrinogen

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase is a glycolytic

GBS - Group B Streptococcus

MLS - Macrolide–lincosamide–streptogramin

opps - Oligopepide permeases

PCR - Polymerase Chain Reaction

PMN - Polymorphonuclear neutrophils

rRNA - Ribosomal ribonucleic acid

SSC - Somatic cell count

Taq - DNA polymerase from *Thermus aquaticus*

VG - Virulence genotypes

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INTRODUTION

1. Mastitis

Despite the control strategies, bovine mastitis remains the most important economic disease affecting dairy herds throughout the world. This disease influences milk production, increases the treatment costs and decreases milk quality reducing its economic value.

Mastitis is an inflammation of the mammary gland that occurs as a response to injury. The inflammation process has the purpose of destroying or neutralizing the infectious agents allowing subsequent healing and return to normal function. Clinically It can be divided into clinical mastitis (symptomatic) and subclinical mastitis (asymptomatic) (Jones & Bailey, 2009). The inflammation can be caused by physical trauma and chemical irritants, but the most common causes of mastitis in dairy cows are infectious agents, usually bacteria but also Mycoplasma and Algae (Jones*& Bailey*, 2009; Ruth *et al*., 2011). Over 150 different species able to cause bovine mastitis have been identified, most commonly *Staphylococcus*, *Streptococcus* and Gram-negative bacteria including *Escherichia coli*. Other agents such as Mycoplasma, *Enterococcus*, pseudomonads and Algae are less frequently isolated (Watts, 1988; Ruth *et al*., 2011; Contreras & Rodriguez, 2011).

Commonly, pathogens are found either in the udder (contagious pathogens) or in the cow's surroundings (environmental pathogens). The reservoir for contagious pathogens is the udder of an infected cow. Pathogens are then spread from infected udders during milking process through contaminated teatcup liners or milkers' hands, leading to longer and prevalent infections in herds. Environmental pathogens are spread from soil, cow’s bed, manure but may also be transmitted during milking. The risk of infection of environmental pathogens is high if the cow lies down after milking, since the sphincter muscles in the teat canal remain dilated. Environmental infections are frequently associated with an unsanitary environment and are often responsible for the majority of the clinical cases (Jones & Bailey, 2009; McDonald, 1979). *Staphylococcus aureus* and *Streptococcus agalactiae* are the contagious pathogens most frequently detected in mastitis isolates. On the other hand the most prominent environmental agents are *Streptococcus uberis*, *Str. dysgalactiae*, coliforms such as *Escherichia coli* and *Klebsiella pneumonia* (Jones & Bailey, 2009; McDonald, 1979).

2. *Streptococcus*

As previously described, several streptococcal species are amongst the most frequently isolated udder pathogens. *Streptococci* are Gram-positive bacteria widespread in the environment and are also commensal organisms of the cow's udder, mucosa and skin. Some of the commensal *Streptococcus* are opportunistic bacteria causing infection if the balance between bacteria and host is broken. Others are considered primary pathogens of mastitis (Sandholm *et al.*, 1995; Cleary & Cheng, 2006). The most significant species are *Str. agalactiae* (contagious agent), *Str*. *uberis* (environmental agent) and to a lower extent *Str. bovis*, *Str. dysgalactiae* and other streptococci (Kuang *et al*., 2009; Jones & Bailey, 2009).

2.1. *Streptococcus agalactiae*

Str. agalactiae belong to Group B Streptococcus (GBS), pyogenic and haemolitic streptococci. In humans, *Str. agalactiae* is a commensal organism which is best known as a cause of postpartum infection and a common cause of meningitis in neonates. It also provokes urinary tract infections, pneumonia, osteomyelitis, meningitis and soft tissue infections (Devi *et al*., 2010; Woods & Levy, 2013). In ruminants, *Str. agalactiae* is a major cause of mastitis, causing mainly subclinical infections (Katholm & Rattenborg, 2010).

Str. agalactiae, such as others contagious pathogens, can be kept under control by preventive measures to avoid the entry of infected cows in herd, by preventing its spread during milking (e.g. milking the infected cows after the healthy cows) or by treatment and dry-cow therapy with antibiotics. In well-managed herds it is possible to completely eliminate *Str. agalactiae* (Jacobsson, 2003). Nevertheless, the epidemiological study of *Str. agalactiae* is particularly important since a single strain is able to infect multiple animals in a herd and the use of antibiotics in food production is now questioned (Jones *et al.*, 2003).

Infections caused by *Str. agalactiae* are usually asymptomatic and persistent, but efficiently eliminated with intramammary therapy (Keefe, 1997). However, new infections in the herd may lead to prevalent subclinical infections, with normal appearance of the milk and udder. Even though, milk production decreases and composition is altered (Jones & Bailey, 2009). Milk of healthy quarters generally contain below $200x10^3$ somatic cells/ml. During subclinical infections the influx of

inflammatory cells into milk, mostly polymorphonuclear neutrophil (PMN), increases, resulting in an elevation of somatic cell count (SCC) which is reflected in the elevation of SCC in bulk tank (Jones & Bailey, 2009).

Most of the strains are CAMP-positive (97%-99%) and approximately half of them are β-haemolytic (Devi *et al.*, 2010; Sandholm *et al*., 1995). The differences in pathogenicity between strains might be related to several putative virulence factors that will be described later.

2.2. *Streptococcus uberis*

It is possible to eradicate contagious pathogens in herds, but environmental pathogens are ubiquitous throughout the dairy environment and improving milking hygiene is not enough to prevent their spread. These traits makes *Streptococcus uberis* particularly problematic considering that it is an important cause of clinical and subclinical mastitis in both lactating and nonlactating cows (Reinoso, *et al.* 2011).

Str. uberis has been recently identified as a highly recombinant organism and some strains can establish infection more effectively than others (Richards *et al.*, 2011; Pryor *et al.*, 2009). The differences in pathogenicity between strains are related to several putative virulence factors. Among these, evasion of the host phagocytosis conferred by the hyaluronic acid capsule (Ward *et al*., 2001); ability to obtain essential nutrients to grow in the mammary gland environment by plasminogen activator proteins such as PauA (Rosey *et al*., 1999); adherence to and invasion of the host tissue mediated by SUAM (Almeida *et al.,* 2006); CAMP factor (Jiang *et al*., 1996); and a surface dehydrogenase protein GapC (Pancholi *et al*., 1993), have been described (Reinoso *et al*., 2011). Furthermore, recent studies have identified a nisin U operon with close similarity to *Str. agalactiae*, which has been suggested to provide a competitive advantage during mastitis infection (Richards *et al*., 2011). Most of the strains are CAMP-negative, but CAMP-positive strains also can be found (Sandholm *et al.*, 1995).

3. Udder defence

The udder has two defensive mechanisms: the primary defence mechanisms prevent the pathogenic agents from entering the mammary gland, and the secondary defence mechanisms consist in several immunological, chemical and cellular systems in milk. The teat canal serves as the udder first line of defence. It represents a physical barrier to the invasion of microorganisms, nevertheless the sphincter muscle in the teat canal remains dilated for approximately two hours after milking and the risk of ascending infection is high during this period. The use of a teat dipping after milking and preventing cows from lying down during this critical period are measures that reduce considerably the risk of ascending infections (Sandholm *et al.*, 1995; Jones & Bailey, 2009). When microorganisms invade the teat canal, the washing-out effect of milking and the epithelial desquamation are mechanisms that decrease bacterial colonization (Sandholm *et al.*, 1995). Moreover, the epithelial cells produce keratin, a fibrous protein with long chain fatty acids that present bacteriostatic properties (Jones & Bailey, 2009).

If the microorganisms pass through the first line of the udder defence, milk is not a good environment for bacterial growth due to the presence of antimicrobial factors in the udder secretion. The concentrations of these factors are under genetic control and depend on the udder health and lactation stage. Briefly, the most significant antibacterial factors of milk are Lactoferrin, Transferrin, Lysozyme, Lactoperoxidase, the complement system and the immunological defence mechanisms. (Sandholm *et al.*, 1995)

The iron binding proteins like Lactoferrin and Transferrin are a significant part of antibacterial defence. They are detected at relatively high concentrations in milk and during mastitis the concentration of these proteins in bovine mammary secretions greatly increases. They counteract the iron uptake by the bacteria and thus prevent bacterial growth. However, streptococci in general are less sensitive to the iron restriction because they have low iron requirement (Sandholm *et al.,* 1995; Fang & Oliver*,* 1999). Lysozyme is an antimicrobial enzyme which hydrolyses the β-glycosidic linkage between muramic acid and N-acetylglucosamine in the peptidoglycan structure of the bacterial wall, causing osmotic lysis of the bacteria. Nevertheless, the bacteriolytic effect in milk is weak due to his low concentration (Sandholm *et al.*, 1995). The Lactoperoxidase causes an oxidation of sensitive enzymes within the bacterial cell wall and it is effective against *Str. uberis* infections. Lactoperoxidase also prevents *Str. agalactiae* infections at some degree. However, the activity of Lactoperoxidase is limited by the concentration of peroxide. The oxygen pressure in milk is low, and

decreases more during inflammation, which inhibits the formation of peroxidase and limits the Lactoperoxidase activity during mastitis (Sandholm *et al.*, 1995). The complement system recognizes and destroys bacteria, particularly important against *E.coli* infections, and helps bacterial opsonisation by phagocytes (Sandholm *et al.*, 1995).

The immunological response is influenced by many factors, such as the immune and nutritional status of the cow, stage of lactation, age and the causative pathogen (Harmon, 1994). During mammary infection PMN leukocytes and phagocytes are attracted in large numbers into milk, thus increasing the somatic cell count (SCC) as well as damaging secretory cells. Somatic cells consist mainly of PMN and white blood cells. Substances released by PMN completely destroy the alveolar structure which is replaced by scar tissue. This process may conduct to the occurrence of small focus of infection walled of scar tissue that becomes difficult to reach with antibiotics (Jones *&* Bailey, 2009).

4. Factors in Bacteria Promoting Infection

The infection of mammary glands occurs when bacteria invade the teat canal and spread towards the upper milk tract. The severity of infection depends on the adaptation to the milk environment and on various virulence factors (Sandholm *et al.*, 1995). Despite the large range of bacteria present in the dairy environment, usually only one strain is isolated within the same mammary quarter (or at most two) and infections with multiple strains are rare (Pryor *et al.*, 2009).

To occur an infection may not be enough for bacteria to penetrate the udder defence as other bacterial species may also entry to mammary gland and compete with each other to become the dominant species. The selection of a dominant species is linked to several bacterial factors. Among these, the ability to adhere to and invade epithelial cells; to avoid the bovine immune system or to resist phagocytosis and the ability to obtain essential nutrients to growth in milk seems to play an important role. Within the same species direct competition between different strains can also occur through the production of small antimicrobial peptides and bacteriocins, which destroy bacteria of the same or closely related species (Pryor *et al.,* 2009). The characterization and study of these factors could give some information about the strains of *Str. agalactiae* and *Str. uberis* more adapted to mammary gland environment and be the dominant species in mastitis. The factors that were investigated in this work are described below.

4.1. Adherence and invasion

Bacteria causing chronic mastitis are able to adhere to the mamary gland tissue and resist to the milk flow. Furthermore, the virulence of bacteria is diminished when the adherence factors are not present (Sandholm *et al.*, 1995). Bacterial adherence to the host via its surface adhesins is considered important during early stages of infection in *Str. agalactiae* and *Str. uberis*, since it prevents the flushing effect during milking and reduces the effectiveness of phagocytic defences (phagocytes cannot reach the intracellular environment) (Jacobsson *et al.*, 2003; Prado *et al.*, 2010).

A subfamily of these adhesins known as Microbial Surface Component Recognizing Adhesive Matrix Molecules, binds specifically to extracellular matrix molecules like fibrinogen, fibronectin, laminin and collagen (Patti *et al.*, 1994). These molecules are located in the intercellular tissue bellow the epithelium. Consequently, epithelial lesions promote the adherence of the bacteria (Sandholm *et al.*, 1995).

Fibrinogen (Fg) is frequently a site of binding among the Gram-positive bacteria. In *Str. agalactiae* two subfamilies of proteins that bind to Fg have been studied: Fibrinogen-binding protein A (FbsA) and Fibrinogen-binding protein B (FbsB) (Devi *et al.*, 2010). The FbsA promotes binding of *Str. agalactiae* to the human Fg (Schubert *et al*., 2002). The FbsB has a conserved C-terminal region and N-terminal signal peptide. The region between the N-terminal peptide and C-terminal differs from one strain to other and this non-conserved region can bind Human Fg. However, it has been shown that the conserved C-terminal region (fbsB[C]) binds only to bovine Fg (Jacobsson *et al.*, 2003 and Gutekunst *et al*, 2004). Furthermore, milk has high calcium content and the presence of Ca^{2+} increases the FbsB[C] binding to bovine Fg (Devi *et al*, 2010). Some studies have demonstrated that FbsB also promotes the invasion of *S. agalactiae* into lung epithelial cells (Gutekunst *et al*, 2004). Nevertheless, its role in invasion of mammary epithelial cells in cows remains unknown and requires future studies.

The adherence of *Str. uberis* takes a different pathway. The *Streptococcus uberis* adhesion molecule (Suam), also called previously as lactoferrin-binding protein (Fang & Oliver, 1999), has high affinity to lactoferrin and is involved in the adherence and invasion of bovine mammary epithelial cells in *Str. uberis* (Almeida *et al.*, 2006). Lactoferrin is an iron binding protein, with low antibacterial effect against *Str. uberis* (streptococci have low requirement for iron), which is present at high concentrations in milk. Its main function is to counteract the iron uptake by the bacteria. However, in addition to the binding iron ability, Lactoferrin also binds to cellular membranes of

mammary epithelial cells, working as a bridging protein between *Str. uberis* and epithelial or phagocytes cells in the adhesion process (Fang & Oliver, 1999).

4.2. Capsule

Some pathogenic bacteria protect themselves against phagocytosis with intracellular growth, destruction of phagocytes and avoidance of the host cellular defences. In capsulated *S. aureus* and *E. coli* the antibodies and complement factors cannot reach the bacteria, impairing the recognition of the pathogen by phagocytes (Sandholm *et al.*, 1995). The mechanism of hyaluronic acid capsule to avoid phagocytosis is not totally clear for *Str. uberis*, and contrary to *S. aureus,* the amount of immunoglobulin bounds was not affected by the presence of capsule (Leight *et al*., 1994). Nonetheless, the strains that have an hyaluronic capsule around them appear to be resistant to phagocytosis by bovine neutrophils and establish infection more effectively (Ward *et al*., 2001; Leight, *et al.*, 1991). Moreover, these bacteria tolerate better the LP system, causing persistent infections (Sandholm *et al.*, 1995). However, recent studies reported that the hyaluronic acid capsule of *Str. uberis* seems to plays a minor role in the early stages of the infection with the resistance to phagocytosis being ascribed to an undefined component unconnected with the capsular phenotype (Ward *et al*, 2009).

A cluster of tree genes is involved in the production of the hyaluronic acid capsule: *hasA*, *hasB* and *hasC* (Ward *et al*., 2001). The gene *hasA* catalyzes the hyaluronic acid from N-acetylglucosamine and glucuronic acid (DeAngelis *et al*., 1993), *hasB* encondes UDP-glucose dehydrogenase (Dougherty *et al*., 1993) and *hasC* encodes UDP-glucose pyrophosphorylase (Ashbaugh *et al.,* 1998).

4.3. Fructose operon

Carbohydrates are an important source of carbon and energy for the growth of many bacteria. When grown in an environment with different carbon sources namely glucose, lactose and fructose, bacteria preferentially utilize only one class of carbohydrates, usually glucose. In the absence of the primary source of carbon, the cAMP concentration increases and forms a complex with CAP, the cAMP-CAP complex. This cAMP-CAP complex increases the expression of CAP-dependent operons, including some enzymes that can supply energy from lactose and fructose (Chu *et al.,* 1999).Recent studies have shown that the complete fructose operon is present in most of bovine *Str. agalactiae* and is absent in all human strains. This suggests a unique fructose utilization pathway for bovine strains of *Str. agalactiae,* and may facilitate survival in nutritionally limited environments, surviving longer in extrammary reservoirs

(Richards *et al.,* 2011). A four-gene operon is involved in fructose utilization: phosphotransferase system fructosespecific IIA component (*fruD*), fructose-specific IIBC PTS component (*fruC*), fructose-1-phosphate kinase (*fruP*) and transcriptional regulator (*fruR*) (Richards *et al*., 2011).

4.4. Obtaining essential nutrients to grow in milk

Extracellular proteins are commonly related to pathogenicity. These proteins facilitate the bacterial growth and protect them from the immune system. Some authors have associated the presence of one of these proteins, the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein as a virulence factor for *Str. uberis* and *Str. agalactiae* (Ling *et al*, 2004; Maeda *et al.*, 2004; Reinoso *et al.*, 2011). This enzyme is involved in bacterial energy production, breaking down glucose for energy and carbon molecules which is essential for bacterial growth in environments without neoglucogenic substrates (Madureira *et al.,* 2007; Kim *et al.*, 2005). Furthermore, besides the immunomodulatory properties, has been reported that GAPDH has the ability to bind to several host proteins and to resist against reactive oxygen produced by phagocytic cells. Moreover, it has imunomodulatory properties (Madureira *et al.,* 2007; Holzmuller *et al.*, 2006; Pancholi & Fischetti, 1992).

Str. uberis is a fastidious organism unable to grow in media where the amino acid composition consists only of those found in free or peptide form in milk. Therefore, to grow in milk this bacterium must be able to hydrolyse milk proteins (Kitt and Leigh 1997; Leigh *et al.*, 1993; Smith *et al.,* 2002). The hydrolysis of plasminogen to plasmin, through extracellular plasminogen activator (PauA), is an important route to facilitate the acquisition of amino acids and peptides essential for bacterial growth. However, for the acquisition and accumulation of amino acids internally an intact oligopetide transport system it is necessary. Without this system bacterial growth in milk may not be possible. These bacterial oligopepide permeases (opps) have been identified in several streptococci species, comprising a complex of five proteins: oppA, oppB, oppC, oppD and oppF. Among them, the oppF, which provides energy for transport of the peptide substrate, seems to play an important role during growth of *Str. uberis* in milk (Smith *et al.*, 2002; Reinoso *et al.,* 2011).

4.5. Nisin U operon

Many Gram-positive bacteria are capable of producing a broad range of molecules that may be inhibitory either to themselves or to other bacteria. Some of these bacterial toxins are categorized as bacteriocins, i.e. antimicrobial peptides that typically inhibit the growth of the same or closely related species (Jack *et al.*, 1995; Pryor *et al.,* 2009). Within the streptococcal mastitis, nisin is the most widely studied bacteriocin and a new variant of nisin was discovered in *Str. uberis*: the nisin U (Wirawan *et al.,* 2006).

Studies conducted in experimentally infected cows with multiple strains of *Str. uberis* showed that, when infused alone, all the strains were capable of causing mastitis. However, when infused together, a single nisin U producer strain usually predominated in intramammary infection. These results indicate that multiple strains can be reduced to only one strain during development of infection and suggest that the ability to produce nisin U may have provided these strains with a competitive advantage during infection (Pryor *et al*., 2009). Interestingly, only a few strains of *Str. uberis* seem to possess the ability to produce nisin (Wirawan *et al.*, 2006).

Lateral transference of genes mediated via integrative conjugative elements may occur between Streptococcus species (Davies *et al.*, 2009), and it is possible that the nisin U operon was laterally exchanged between *Str. uberis* and *Str. agalactiae* (Richards *et al.,* 2011). In theory, *Str. agalactiae* strains containing the nisin U operon might have a similar advantage during infection. Although, none of *Str. agalactiae* strains possessing the nisin operon studied by Richards *et al*. (2011), could produce nisin. Nonetheless, it is possible that some bovine *Str. agalactiae* may possess functional nisin operons providing them a similar competitive advantage during mastitis infection (Richards *et al.*, 2011). Also the nisin U operon is not present in *Str. agalactiae* human strains indicating that this specificity to bovine strains is correlated with a bovine environment (Richards *et al.*, 2011).

5. Resistance to antimicrobials

The widespread use of antimicrobials for prevention and treatment of dry-cow and clinical mastitis resulted in an overall increase in resistance of mastitis pathogens to these components (Erskine *et al.*, 2004). The choice of antimicrobial products for mastitis treatment depends on the availability and regulation of veterinary drugs and differs across countries. Many antimicrobials are authorized for mastitis treatment in cattle, namely penicillin, cefazolin, cefoperazone, pirlimycin, gentamicin, streptomycin and amoxicillin–clavulanic acid. For human treatment: rifaximin, erythromycin, vancomycin, chloramphenicol and tetracycline are commonly used (Rato *et al.*, 2012). Macrolides, mainly erythromycin, are also used to treat bovine mastitis in countries like France and the USA (Haenni *et al.,* 2010).

Several studies have reported a decrease in susceptibility of environmental streptococci to compounds from the macrolide–lincosamide–streptogramin (MLS) group (Tikofsky *et al.*, 2004), and the emergence and spread of resistance of group B streptococcus to macrolides, usually in association with resistance to tetracycline (Duarte *et al.*, 2004). To study this spread of MLS resistance in bovine *Str. uberis* and *Str. agalactiae* previous works explored the genes responsible for the resistance to erythromycin and pirlimycin, i.e. the most commonly used macrolide and lincosamide antibiotics, as well as for tetracycline (Loch *et al.*, 2005).

The most common macrolide resistance mechanism in Streptococci is ribosomal modification by a methylase, which is encoded by an *erm* gene (*ermA* and *ermB*), mostly by *ermB* (Weisblum *et al.*, 1985; Roberts *et al.*, 2002) The ermB methylase confers resistance to erythromycin and inducible or constitutive resistance to lincosamines and streptogramin B (Duarte *et al.*, 2004). Resistances to tetracycline are often found on the same motile unit as the erythromycin resistance genes and are associated to a variety of genes that encode either a protein which pumps tetracycline out of the cell or a ribosomal protein which protects the ribosomes from the action of tetracycline (Speer *et al.*, 1992). In streptococcal species, the tetracycline resistance genes *tetM*, *tetO*, *tetT*, *tetW*, *tetL*, *tetQ*, *tetK* and *tetS* have been found (Pires *et al.*, 2005; Aminov *et al.*, 2001; Ng *et al.*, 2001; Rato *et al.*, 2012). This work, for practical reasons and due to lack of time and resources, focused only in the *tetS* gene, because it was associated to phenotypes not related to *ermB* gene on data obtained by Rato *et al.* (2012).

The resistance to lincosamide antibiotics, such as pirlimycin, occurs due to an alteration of a specific adenine in the 23S rRNA, and also confers cross-resistance to macrolide, lincosamide, and streptogramin B type antibiotics (Lecercq *et al.*, 1991). This resistance is related with the *linB* gene, known to be carried by a plasmid of *Enterococcus faecium* (Bozdogan *et al.*, 1999), emphasizing the implication of horizontal gene transfer of antimicrobial resistance genes between bovine mastitis pathogens (Rato *et al.*, 2012).

6. Integration of molecular tools into veterinary epidemiology

Molecular epidemiology is defined as an interdisciplinary approach to the study of the distribution, patterns, causes, and effects of infectious or non-infectious disease in human and animal populations through the use of molecular biology methods. In this context molecular epidemiology encompass disciplines such as epidemiology, molecular biology and population genetics (Muellner *et al.,* 2011; Riley, 2004).

Nowadays, with the advancement of technology, it is increasingly faster and easier to generate molecular typing data and to efficiently analyse such data with new and better bioinformatics tools. Moreover, these technologies are becoming more widely available, offering new and powerful tools to increase our understanding of the epidemiology of important pathogens. A major advantage of molecular tools is the capacity to process a large number of strains or a large number of loci in same pathogen at the same time, yielding unambiguous data that can easily be stored and shared (Muellner *et al.,* 2011; Archie *et al.*, 2009). The integration of these data into epidemiological models and investigations could help understanding the key epidemiological factors between persistent pathogens and their evolution through time and space (Muellner *et al.,* 2011).

When an outbreak of mastitis occurs, the molecular study and characterization of differences between and within species, the presence of adaptive mechanisms to the mammary environment and knowing of the transmission mechanisms is determinant for the veterinary to develop efficient control strategies and implement measures to prevent future outbreaks. This is especially important due to the fact that bovine mastitis can either be caused by contagious or environmental pathogens and is essential understand the diversity and behavior of *Str. uberis* and *Str. agalactiae* in herds (Muellner *et al.,* 2011; Rato *et al*, 2008).

Another practical application of molecular tools into veterinary epidemiology is the study of antibiotic resistance genes. It is increasingly important to perform a fast and accurate detection of emerging antibiotic resistance among the bacterial pathogens. However, some of them are fastidious organisms requiring enriched media and modified growth conditions for reliable susceptibility testing and clinical laboratories may not be able to rely on a single susceptibility testing method to detect all of these emerging resistances. The use of molecular tools provides a new and perhaps more definitive approach for detection of antimicrobial resistances through the use of genetic probes or nucleic acid amplification techniques for direct detection of genes known to encode certain resistance mechanisms, with high sensitivity and specificity (Frye *et al.*, 2000).

7. Objectives

The epidemiological and evolutionary studies of *Streptococcus agalactiae* and *Streptococcus uberis* within the context of bovine mastitis presented in this work were carried at the Microbial Diversity and Evolution (MDE) group, CIBIO. Overall, this study aimed to:

i. Characterize important virulence factors and antimicrobials resistances described in the literature for *Str. agalactiae* and *Str. uberis* within the context of bovine mastitis.
ii. Obtain and store a consistent number of strains to perform epidemiological and evolutionary studies.
iii. Obtain gene-specific DNA markers and determinate their presence in numerous isolates of *Str. agalactia* and *Str. uberis* obtained from cows in herds near to Porto, Portugal.
iv. Validate the selected markers using PCR-based techniques and dot blot hybridisation assays.
v. Evaluate if there are significant differences among strains, and try to correlate these data with the clinical information available (CCS and clinical mastitis).
vi. Implement the methodological ground to carry out epidemiological and molecular evaluations in causative agents of bovine mastitis.

MATERIALS AND METHODS

1. Milk samples and bacterial isolates

Milk sampling was performed in all lactating cows of 11 dairy herds in an intensive system, including in the sampling individuals not showing signs of mastitis and cows with clinical mastitis. The four teats were cleaned with a paper towel immersed in an antiseptic solution and then, the teat end was disinfected with cotton dampened with alcohol (70%). The foremilk was discarded and 20 ml of milk were collected, about 5 ml from each quarter. The samples were analyzed by SEGALAB (Laboratório de Sanidade Animal e Segurança Alimentar, S.A.) with species identification performed using the VITEK 2 system (bioMérieux, Durham, NC). A total of 61 strains of *Streptococcus agalactiae* and 10 strains of *Streptococcus uberis* were isolated (Table I).

Table I: Streptococcus strains isolated for this study.

Strain	Species	Location	Herd	CCS ($x10^3$) *	Clinical mastitis (Yes/No) *	Source
SA35	*Streptococcus agalactiae*	Barcelos	A	56	Y	SEGALAB
SA36	*Streptococcus agalactiae*	Barcelos	A	662	N	SEGALAB
SA37	*Streptococcus agalactiae*	Barcelos	A	1716	N	SEGALAB
SA38	*Streptococcus agalactiae*	Barcelos	A	253	N	SEGALAB
SA39	*Streptococcus agalactiae*	Barcelos	A	7197	N	SEGALAB
SA40	*Streptococcus agalactiae*	Barcelos	A	31	N	SEGALAB
SA41	*Streptococcus agalactiae*	Barcelos	A	327	N	SEGALAB
SA42	*Streptococcus agalactiae*	Barcelos	A	1067	N	SEGALAB
SA43	*Streptococcus agalactiae*	Barcelos	A	91	N	SEGALAB
SA44	*Streptococcus agalactiae*	Barcelos	A	186	N	SEGALAB
SA45	*Streptococcus agalactiae*	Barcelos	A	1232	N	SEGALAB
SA46	*Streptococcus agalactiae*	Barcelos	A	157	N	SEGALAB
SA47	*Streptococcus agalactiae*	Barcelos	A	254	N	SEGALAB
SA48	*Streptococcus agalactiae*	Barcelos	A	141	N	SEGALAB
SA49	*Streptococcus agalactiae*	Barcelos	A	2795	Y	SEGALAB
SA50	*Streptococcus agalactiae*	Barcelos	A	1815	N	SEGALAB
SA51	*Streptococcus agalactiae*	Barcelos	A	719	Y	SEGALAB
SA52	*Streptococcus agalactiae*	Barcelos	A	52	N	SEGALAB
SA55	*Streptococcus agalactiae*	Barcelos	A	375	N	SEGALAB
SA56	*Streptococcus agalactiae*	Barcelos	A	328	N	SEGALAB
SA57	*Streptococcus agalactiae*	Barcelos	A	254	N	SEGALAB
SA58	*Streptococcus agalactiae*	Barcelos	A	273	N	SEGALAB
SA59	*Streptococcus agalactiae*	Barcelos	A	132	N	SEGALAB
SA60	*Streptococcus agalactiae*	Barcelos	A	1862	N	SEGALAB
SA62	*Streptococcus agalactiae*	Barcelos	A	17431	N	SEGALAB
SA65	*Streptococcus agalactiae*	Barcelos	A	1564	N	SEGALAB
SA66	*Streptococcus agalactiae*	Barcelos	A	1150	N	SEGALAB
SA67	*Streptococcus agalactiae*	Barcelos	A	15719	Y	SEGALAB
SA68	*Streptococcus agalactiae*	Barcelos	A	9153	Y	SEGALAB
SA69	*Streptococcus agalactiae*	Barcelos	A	2247	N	SEGALAB
SA74	*Streptococcus agalactiae*	Barcelos	A	979	N	SEGALAB
SA76	*Streptococcus agalactiae*	Barcelos	B	1784	N	SEGALAB
SA77	*Streptococcus agalactiae*	Barcelos	B	251	N	SEGALAB
SA78	*Streptococcus agalactiae*	Barcelos	B	1749	N	SEGALAB
SA79	*Streptococcus agalactiae*	Barcelos	B	927	N	SEGALAB
SA80	*Streptococcus agalactiae*	Barcelos	B	3713	N	SEGALAB
SA82	*Streptococcus agalactiae*	Póvoa Varzim	C	334	N	SEGALAB
SA83	*Streptococcus agalactiae*	Póvoa Varzim	C	712	N	SEGALAB

Table 1 (Continued)

Strain	Species	Location	Herd	CCS (x10^3) *	Clinical mastitis (Yes/No) *	Source
SA84	*Streptococcus agalactiae*	Póvoa Varzim	C	1937	N	SEGALAB
SA85	*Streptococcus agalactiae*	Póvoa Varzim	C	9215	N	SEGALAB
SA86	*Streptococcus agalactiae*	Póvoa Varzim	C	3735	N	SEGALAB
SA87	*Streptococcus agalactiae*	Póvoa Varzim	C	213	N	SEGALAB
SA88	*Streptococcus agalactiae*	Póvoa Varzim	C	1017	N	SEGALAB
SA89	*Streptococcus agalactiae*	Póvoa Varzim	C	909	N	SEGALAB
SA90	*Streptococcus agalactiae*	Barcelos	A	5882	N	SEGALAB
SA91	*Streptococcus agalactiae*	Penafiel	D	997	N	SEGALAB
SA92	*Streptococcus agalactiae*	Penafiel	D	919	N	SEGALAB
SA93	*Streptococcus agalactiae*	Barcelos	E	346	N	SEGALAB
SA94	*Streptococcus agalactiae*	Barcelos	E	328	N	SEGALAB
SA95	*Streptococcus agalactiae*	Póvoa Varzim	F	78	N	SEGALAB
SA96	*Streptococcus agalactiae*	Póvoa Varzim	F	4512	N	SEGALAB
SA97	*Streptococcus agalactiae*	Póvoa Varzim	F	107	N	SEGALAB
SA98	*Streptococcus agalactiae*	Póvoa Varzim	F	2463	N	SEGALAB
SA99	*Streptococcus agalactiae*	Póvoa Varzim	F	56	N	SEGALAB
SA100	*Streptococcus agalactiae*	Póvoa Varzim	G	381	N	SEGALAB
SA101	*Streptococcus agalactiae*	Póvoa Varzim	G	520	N	SEGALAB
SA102	*Streptococcus agalactiae*	Póvoa Varzim	G	670	N	SEGALAB
SA103	*Streptococcus agalactiae*	Póvoa Varzim	G	6475	N	SEGALAB
SU10	*Streptococcus uberis*	Barcelos	J	**	Y	SEGALAB
SU11	*Streptococcus uberis*	Amarante	H	**	Y	SEGALAB
SU13	*Streptococcus uberis*	Amarante	H	11744	Y	SEGALAB
SU14	*Streptococcus uberis*	Vila Verde	I	2134	N	SEGALAB
SU15	*Streptococcus uberis*	Barcelos	J	***	Y	SEGALAB
SU16	*Streptococcus uberis*	Barcelos	E	541	N	SEGALAB
SU17	*Streptococcus uberis*	Barcelos	E	16665	Y	SEGALAB
SU18	*Streptococcus uberis*	Amarante	H	311	N	SEGALAB
SU19	*Streptococcus uberis*	Póvoa Varzim	K	15650	Y	SEGALAB
SU20	*Streptococcus uberis*	Barcelos	J	25522	Y	SEGALAB
SA53*	*Streptococcus agalactiae*	Barcelos	A	74	N	SEGALAB
SA54*	*Streptococcus agalactiae*	Barcelos	A	2969	N	SEGALAB

(*) These strains were after confirmed as *Staphylococcus pasteuri* by sequencing of the 16S rRNA gene and withdrawn from the study.

Seventeen strains characterized in the work of Almeida *et al.,* (2013), were used for controls. (Table II).

Table II: Bacterial strains used for controls (Almeida *et al.*, 2013).

Strain	Species	Location	Source
SAA9	*Streptococcus agalactiae* LMG 15083	-	LMG
SA7	*Streptococcus agalactiae*	Vila do Conde	SEGALAB
SA8	*Streptococcus agalactiae*	Vila do Conde	SEGALAB
SA11	*Streptococcus agalactiae*	Trofa	SEGALAB
SUA12	*Streptococcus uberis* LMG 9465	-	LMG
SU2	*Streptococcus uberis*	Barcelos	SEGALAB
SU3	*Streptococcus uberis*	Barcelos	SEGALAB
SB A10	*Streptococcus bovis* LMG 8518	-	LMG
SS4	*Streptococcus dysgalactiae*	-	SEGALAB
SPA11	*Streptococcus parauberis* LMG 12174	-	LMG
SAUR1	*Staphylococcus aureus* LMG 8224	-	LMG
SHAA8	*Staphylococcus haemolyticus* LMG 13349	-	LMG
SPA1	*Staphylococcus pasteuri*	Vila do Conde	SEGALAB
E2	*Enterococcus faecium*	Barcelos	SEGALAB
EFA2	*Enterococcus faecalis* LMG 7937	-	LMG
LLA6	*Lactococcus lactis subsp. lactis* LMG 6890	-	LMG
VFA13	*Vagococcus fluvialis* LMG 12318	-	LMG

LMG - Belgian Co-ordinated collections of microorganisms, Gent, Belgium;

2. Culture conditions and DNA extraction

All strains were cultured in Brain Heart Infusion (BHI) (biolab®, Hungary) medium at 37°C. Bacterial DNA was extracted using the EaZy Nucleic Acid bacterial DNA purification KIT (Omega Bio-Tek, Norcross, GA), according to the manufacturer's instructions. Quantification of DNA samples was done using the Qubit 2.0 Fluorometer HS Assay (Invitrogen, Carlsbad, CA) and quality was assessed by electrophoresis in 1% agarose gels.

3. PCR amplification

The oligonucleotide primers for the detection of the *ermB*, *linB*, *tetS*, *sua* and *fbsB* genes (Table III) were designed using with Primer3 software (http://frodo.wi.mit.edu/) and synthesized by STABVida (Lisbon, Portugal). Amplicon specificity was assessed using BLAST (Basic Local Alignment Search Tool) (Altschul 1990) (BLAST, http://blast.ncbi.nlm.nih.gov/).

Table III: NCBI Reference Sequence or GeneBank accession number used to design primers.

Gene	Designed in	NCBI / GeneBank accession no.
ermB	*Streptococcus uberis* strain 330 *ermB* gene, partial cds	EF422362.1
tetS	*Streptococcus uberis* TetS (*tetS*) gene, partial cds	EF092839.1
linB	*Streptococcus uberis* isolate QMP Z3-369 lincosamide nucleotidyltransferase (*linB*) gene, partial cds	EF539833.1
sua	*Streptococcus uberis* 0140J chromosome, complete genome	NC_012004.1
fbsB	*Streptococcus agalactiae* NEM316, complete genome	NC_004368.1

The PCR master mix contained: 25ng of bacterial DNA, 0,2 µM of each primer, 1x DreamTaq buffer, containing 1.5 mM of $MgCl_2$ (Fermentas, Ontario, Canada), 1 U of DreamTaq DNA polymerase (Fermentas), 0.2 mM of each dNTPs (Fermentas). The reaction was performed with an initial denaturation of 95°C for 5 min, followed by 35 cycles at 95° for 30s, 55°C for 30s and 72°C for 45s with a final extension of 10 min at 72°C. PCR products were visualized on 1.5% agarose gels stained with GelRed (Biotum) at 80V. PCR amplicons were purified from agarose gels using the GFX PCR DNA and gel band purification kit (GE Healthcare, Buckinghamshire, United Kingdom) and the identity of these amplicons was confirmed by sequencing (STAB Vida, Portugal).

Table IV: Taxa-specific markers with PCR primer sequence used in this study.

Marker	Primer Sequence (5´-3')	T_a	Amplicon length	Specificity	References
F1	TTATGCTCGTCTTGCTCTTTACGG GCACACGTCCAAGTGATGTAGCTG	54.6°C	285bp	Streptococcaceae	Almeida *et al.*, 2013
A1	ATGTAGCTGCTGATTCTGTCATAA AATAGCTGGTGTAGATTTGACTGC	52.6°C	314bp	*Str. agalactiae*	Almeida *et al.*, 2013
SU	TCGTTTGTATACGCTTGATGCT CACGTCTCTATAAAAGGAATTCCC	50.6°C	229bp	*Str. uberis*	Almeida *et al.*, 2013

Table V: Functional markers with PCR primer sequence used in this study.

Marker	Target gene	Primer Sequence (5´-3')	T_a	Amplicon length	References
sua	*sua*	TCAGTTGTTGTGATTGCTGACGTC CAAACAAGTGGTTTCAGGTCCATT	56°C	600bp	This study
fbsB	*fbsB*	ACAAAGTTCAGTTGCGCAAACT CGCGATGAGATTGATTTACTCA	52.9°C	525bp	This study
ermB	*ermB*	AAAGCCATGCGTCTGACATC TGTGGTATGGCGGGTAAGTT	52.5°C	194bp	This study
linB	*linB*	CCTGATACGAAGGCTATGCTT GGTGACTTTGCAAATCCATAACT	50.0°C	403bp	This study
tetS	*tetS*	AGGACAAACTTTCTGACGACA CTGAATTGAGTTGTGTGGGTGA	49.1°C	306bp	This study
FO1	*fruR*	CGACATCAAAAAAACAACTAACAC TCCACCACGTTATTGAGAGTTT	50.6°C	331bp	Richards *et al.*, 2011
FO3	*fruD*	TCTCAATTTCTTCGATCTCATGTGC CAGGTCTTGTTGTCGAAAACGATTA	52.6°C	348bp	Richards *et al.*, 2011
NU1	*nsuR*	CCAAGGTTGCAGCGCATTT CCCCTTATTGTCTTGATGGGATT	51.5°C	331bp	Richards *et al.*, 2011
NU3	*nsuf*	AATCAAATCGTTGATGAAAATGACC AAACTTCTCCGTAATCCCAAACTTC	50.6°C	502bp	Richards *et al.*, 2011
V1	*hasC*	TGCTTGGTGACGATTTGATG GTCCAATGATAGCAAGGTACAC	58.0°C	300bp	Ward *et al.*, 2001
V2	*gapC*	GCTCCTGGTGGAGATGATGT GTCACCAGTGTAAGCGTGGA	55.0°C	189bp	Reinoso. et al. 2011
V3	*oppF*	GGCCTAACCAAAACGAAACA GGCTCTGGAATTGCTGAAAG	54.0°C	419bp	Smith *et al.*, 2002

4. Dot Blot screening

DNA probes were obtained from purified PCR amplicons (≈ 150 ng), using the using the DIG-High Prime labelling kit (Roche, Basel, Switzerland) according to manufacturer's instructions.

For Dot Blot hybridisation assays, 100ng of heat-denatured DNA from each bacteria was spotted into a nylon membrane using a Bio-Dot apparatus (Bio-Rad) (Table VI, Figure 1). Hybridisation was carried out overnight at 68°C with a probe concentration of 100ng/ml. Washing and detection were carried out according to the DIG system instructions (Roche). DIG-labelled nucleic acids were detected by chemiluminescence using a Molecular Imager Chemi-Doc system (Bio-Rad).

. **Table VI:** Layout of the membranes used in the dot blot hybridisation assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	C+	SA35	SA36	SA37	SA38	SA39	SA40	SA41	SA42	SA43	SA44	C+
B	C-	SA45	SA46	SA47	SA48	SA49	SA50	SA51	SA52	SA55	SA56	C-
C	SA57	SA58	SA59	SA60	SA62	SA65	SA66	SA67	SA68	SA69	SA74	SA76
D	SA77	SA78	SA79	SA80	SA90	SA93	SA94	SA81	SA82	SA83	SA84	SA85
E	SA86	SA87	SA88	SA89	SA95	SA96	SA97	SA98	SA99	SA100	SA101	SA102
F	SA103	SA91	SA92	-	SU10	SU15	SU16	SU17	SU20	SU19	SU11	SU13
G	C-	SU18	SU14	-	SAA9	SA7	SA8	SA11	SUA12	SU2	SU3	C-
H	C+	SBA10	SS4	SPA11	SAUR1	SHAA8	SPA1	E2	EFA2	LLA6	VFA13	C+

.NOTE: SA53 and SA54 were withdrawn in this final dot blot membrane

The Dot Blot hybridisation analysis was performed using an image-processing algorithm (MATLAB-based algorithm), that calculates the probability of each dot being a positive result, using as references the positive and negative controls present in each membrane. The exposure time of the Chemidoc system was adjusted so that all dots were below pixel saturation (Marcal *et al.*, 2009; Albuquerque *et al.*, 2011)

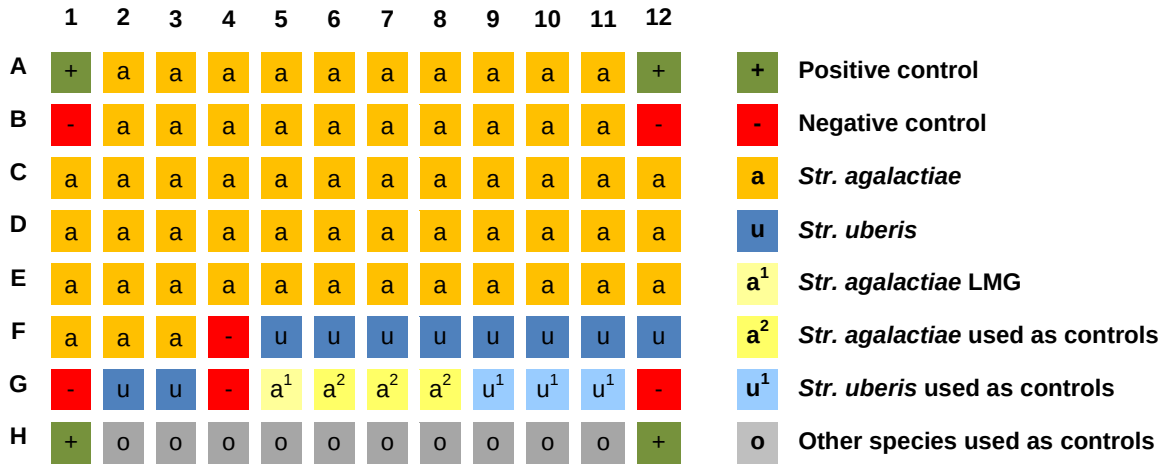

Figure 1: Layout of the membranes used in the dot blot hybridisation assays.

RESULTS

1. Frequency in herds

The implementation of milk quality protocols is usual in dairy herds, establishing control measures to reduce the number of clinical mastitis and to decrease the CCS in milk. In this context, veterinarians perform one or two visits per year in order to collect samples from all lactating cows. This measure allows to detect cows and teats infected with pathogenic agents causing clinical and sub-clinical infections.

The results obtained from 5 of these visits, performed by SEBALAB veterinarians, are represented in Table VII. In which the frequency of *Str. agalactiae* and *Str. uberis* in some of the studied herds can be observed.

Table VII: Number of *Str. agalactia* (SA) and *Str. uberis* (SU) found in consecutive visits in which all the lactating cows were tested.

Visit	Herd A				Herd E				Herd F				Herd H			
	Year	n	SA	SU	Year	n	SA	SU	Year	n	SA	SU	Year	n	SA	SU
1	2010	149	29	0	2010	83	35	2	2010	62	26	0	2010	98	0	8
2	2011	175	26	3	2011	79	14	4	2011	65	12	3	2010	66	0	4
3	2011	181	24	0	2013	91	9	4	2012	71	28	0	2011	106	0	11
4	2012	195	9	0	-	-	-	-	2013	53	14	0	2012	126	0	2
5	2012	173	46	0	-	-	-	-	-	-	-	-	-	-	-	-

NOTE: These data only comprises the lactating cows at the moment of visit.

2. Preliminary identification of isolates

A preliminary test was carried out using taxonomic markers specific for *Streptotoccus* (F1), *Str. agalactiae* (A1) and *Str. uberis* (SU) to confirm the identity of the 61 isolates of *Str. agalactiae* and 10 isolates of *Str. uberis* obtained from cows. The obtained results showed that hybridisation with these taxonomic markers was not observed in 2 strains: SA53 and SA54 (Fig.1-Appendix). These isolates, identified as *Staphylococcus pasteuri* by sequencing of the 16S rRNA gene, were withdrawn from the study.

3. Taxonomic markers

Regarding marker F1, dot blot hybridisation showed a specificity of 100% for all 79 species belonging to the *Streptococcus* genus, including *Str. agalactia*, *Str. uberis* and the control strains *Str. dysgalactiae, Str. bovis* and *Str. parauberis*. Marker A1 was specific for all 63 (100%) *Str. agalactiae* strains. Finally, marker SU presented positive hybridisation in all 13 *Str. uberis* and negative signal for all the other species, with the

exception of 1 *Str. agalaciae* strain, which showed a very small hybridisation (average probability of 0.26; Table I-Appendix) with this marker. The hybridisation results obtained for taxonomic markers are represented in Table VIII, and Figure 2-Appendix.

Table VIII: Results obtained from the dot blot assays for taxa-specific markers (F1, A1 and SU).

Species	n	Taxa-specific markers		
		F1	A1	SU
Str. agalactiae	59	59 (100%)	59 (100%)	1 (1.7%)*
Str. uberis	10	10 (100%)	-	10 (100%)
***Str. agalactiae* LMG[(A)]**	1	1 (100%)	1 (100%)	-
***Str. agalactiae* [(B)]**	3	1 (100%)	3 (100%)	-
***Str. uberis*[(C)]**	3	1 (100%)	-	3 (100%)
Str. bovis	1	1 (100%)	-	-
Str. dysgalactiae	1	1 (100%)	-	-
Str. parauberis	1	1 (100%)	-	-
S. aureus	1	-	-	-
S. haemolyticus	1	-	-	-
S. pasteuri	1	-	-	-
E. feacium	1	-	-	-
E. faecalis	1	-	-	-
L. lactis	1	-	-	-
Vagococcus fluvialis	1	-	-	-

(A) *Str. agalactiae* LMG from human origin; (B) *Str. agalactiae* used as positive controls; (C) *Str. uberis* used as positive controls; (*) It was observed a small hybridisation.

4. Virulence factors

4.1. Research and selection of virulence factors

Several putative virulence factors have been identified in *Str. agalactiae* and *Str. uberis*. A survey of the literature allowed to select virulence factors that may have an important role, or at least provide some competitive advantage, during infection of the mammary gland (Table IX and X). Since it was not possible to test all of these virulence factors in this study, only the regions related to adhesion and invasion, antiphagocytosis, ability to grown in milk and production of bacteriocin were selected for further analysis.

Regarding adhesion and invasion to epithelium, the fibrinogen binding protein (FbsB) for *Str. agalactiae* and the adhesion molecule (Suam) for *Str. uberis* were considered for this study. The hyaluronic acid capsule (V1) was tested due to its antiphagocytosis properties. Concerning the ability of *Str.agalactiae* to grow in milk and survive in environment, the fructose operon (FO1 and FO3), Glyceraldehyde-3-phosphate (V2) and oligopetide transport system (V3) were selected. Finally, for study of bacteriocin production the nisin U region (NU1 and NU3) was chosen.

Table IX: Virulence factors for *Str. agalactiae*, identified in the literature.

Str. agalactiae

Function and name	Gene	Source
Adhesion		
Fibrinogen binding protein	*fbsB*	Jacobsson *et al,* 2003; Gutekunst *et al,*
Laminin-binding protein	*lmb*	Chen *et al*, 2005
Fibrinnectin-binding protein	*pavA*	Chen *et al*, 2005
Invasion		
α-C protein	*bca*	
Fibrinogen binding protein	*fbsB*	Gutekunst *et al,* 2004
Antiphagocytosis		
β-C protein	*cba*	Chen *et al*, 2005
Capsule	*	Chen *et al*, 2005
Ability to grown in milk/environment		
Fructose operon	*fruC, fruD, fruP, fruR*	Richards *et al*, 2011
Lactose operon		Richards *et al*, 2011
Glyceraldehyde-3-phosphate	*gapC*	Reinoso *et al*, 2011
Oligopetide transport system	*opp*	Smith *et al*, 2002
Complement protease		
C5a peptidase	scpB	Chen *et al*, 2005
Toxin		
CAMP factor	*cfb*	Chen *et al*, 2005
Bacteriocins		
Nisin U	*nsu*	Richards *et al*, 2011; Wirawan *et al,* 2006

(*) Several gens were identified and are listed in Chen *et al.,* 2005.

Table X: Virulence factors for *Str. uberis*, identified in the literature.

Str. uberis

Function and name	Gene	Source
Adhesion and Invasion		
Str. uberis adhesion molecule	*sua*	Almeida *et al*, 2006
Lactoferrinbinding proteins	*lbp*	Moshynskyy *et al*, 2003
Antiphagocytosis		
Hyaluronic acid capsule	*hasA, hasB, hasC*	Ward *et al*, 2001
Ability to grown in milk/environment		
Plasminogen activator	*pauA/ pauB, skc*	Rosey *et al, 1999* ; Ward & Leigh, 2002; Johnsen et al., 1999
Fructose operon	*fruC, fruD, fruP, fruR*	Richards *et al*, 2011
Lactose operon		Richards *et al*, 2011
Glyceraldehyde-3-phosphate	*gapC*	Reinoso *et al*, 2011
Oligopetide transport system	*opp*	Smith *et al*, 2002
Toxin		
CAMP factor	*cfu*	Reinoso *et al*, 2011
Bacteriocin		
Nisin U	*nsu*	Wirawan *et al,* 2006
Uberolysin	*ublA*	Wirawan *et al,* 2007

4.2. Virulence markers

Results obtained with markers from the fructose operon (FO1 and FO3) revealed that these genes were present in all 62 (100%) *Str. agalactiae* from bovine origin. However, the control strains *S. agalactiae* LMG 15083 of Human origin presented a lower signal for this operon. The fructose operon was also found in *Str. dysgalactiae* and *Str. parauberis.* The *fbsB* gene was specific for all 63 (100%) *Str. agalactiae.* The nisin U operon markers (NU1 and NU3) were present in 5 (38%) strains of *Str. uberis* (3 in the

studied strains and 2 in control strains). The remaining 8 (62%) strains of *Str. uberis* presented a small hybridisation for NU3 and none for NU1. The nisin U operon was also found in 2 (3.2%) *Str. agalactiae* from control strains. The hyaluronic acid capsule (V1) and *Str. uberis* adhesion molecule (sua) were specific for all 13 (100%) *Str. uberis*. The *gapC* marker (V2) was present in all 13 (100%) *Str. uberis* tested. Regarding *Str agalactiae,* the V2 marker provided positive signals for 54 (85.7%) strains, 50 in the studied ones and 4 in the control strains. A lower signal for V2 was observed in 6 (9.5%) and no hybridisation for the remaining 3 (4.8%) strains of *Str agalactiae*. In control strains, *gapC* gene was present in the environmental streptococci *Str. parauberis*. Dot blots using the *oppF* gene marker (V3) were specific for all 13 (100%) *Str. uberis* strains tested, in addition to *Str. parauberis.*

The strains were aggregated in virulence genotypes (VG), according to the results of the dot blot assays for virulence markers, with the respective average CCS and number of clinical mastitis reported for each genotype. In this work, 10 different VG for the studied strains were observed: 3 VG for *Str. agalactiae* and 7 VG for *Str. uberis*. The most frequent virulence genotype in *Str agalactiae* was VG1, positive for: FO1, FO3, V2 and fbsB. VG1 was present in 50 (84.8%) strains, with an average CCS of 2043±3167 $x10^3$ cells/ml and 4 of these 50 strains (8%) were isolated from clinical mastitis. With a similar pattern to VG1 but with a lower hybridisation for the *gapC* gene, VG2 was observed in 6 of 59 strains (10.2%) with an average CCS of 3062±6233cells/ml and 1 (16.7%) strain isolated from clinical mastitis. Finally, VG3 differs from the other two by presenting negative hybridisation for the *gapC* gene. This genotype appears with less frequency, only present in 3 (5%) strains with an average CCS of 370±330cells/ml. None of the VG3 strains were isolated from clinical mastitis (Table XI).

Str. uberis showed a larger genetic variety, with 7 different virulence genotypes in only 10 strains. The hyaluronic acid capsule (V1), Glyceraldehyde-3-phosphate (V2), oligopetide transport system (V3) and the ability to adhere and invade epithelium conferred by gene *sua* are common to all genotypes. On the other hand, the fructose operon and nisin U operon vary between genotypes (Table XI).

In control strains 10 more different VG for all species were found. Particular relevance should be given to VG11, in which the nisin U operon in *Str. agalactiae* was found (Table XII).

Table XI: Number of strains observed for each virulence genotype pattern (VG) obtained from the dot blot assays for virulence and functional markers (FO1, FO3, NU1, NU3, V1, V2, V3, fbsB and sua) with the average CCS and number of stains isolated from clinical mastitis, in the studied strains. Green (+): Positive hybridisation, average probability above 0.75; Yellow (i): Intermediate hybridisation, average probability between 0.25 and 0.75; Red (-): Negative hybridisation, average probability below 0.25.

Virulence Genotype	Result for markers									*Str. agalactiae* (n= 59)	*Str. uberis* (n=10)	Average CCS (x10^3) for VG	Strains isolated from clinical mastitis
	FO1	FO3	NU1	NU3	V1	V2	V3	fbsB	Sua				
VG1	+	+	-	-	-	+	-	+	-	50(85%)	-	2043±3167	4(8%)
VG2	+	+	-	-	-	i	-	+	-	6(10%)	-	3062±6233	1(16.7%)
VG3	+	+	-	-	-	-	-	+	-	3(5%)	-	370±330	0(0%)
VG4	i	i	+	+	+	+	+	-	+	-	1(10%)	25522	1(100%)
VG5	i	i	-	i	+	+	+	-	+	-	2(20%)	16152±717	2(100%)
VG6	i	-	-	i	+	+	+	-	+	-	1(10%)	541	0(0%)
VG7	-	i	+	+	+	+	+	-	+	-	1(10%)	311	0(0%)
VG8	-	i	-	i	+	+	+	-	+	-	1(10%)	11744	1(100%)
VG9	-	-	+	+	+	+	+	-	+	-	1(10%)	*	1(100%)
VG10	-	-	-	i	+	+	+	-	+	-	3(30%)	2134 **	2(66.7%)

(*) The CCS was not performed due to insufficient amount of milk. (**) In 2 of 3 samples, milk was considered improper for consumption and the CCS was not performed.

Table XII: Results observed for each virulence genotype pattern (VG) obtained from the dot blot assays for functional and virulence markers (FO1, FO3, NU1, NU3, V1, V2, V3, fbsB and sua) in the control strains. Green (+): Positive hybridisation, average probability above 0.75; Yellow (i): Intermediate hybridisation, average probability between 0.25 and 0.75; Red (-): Negative hybridisation, average probability below 0.25.

Virulence genotypes	Result for markers									*Str. agalactiae LMG*[A]	*Str. Agalactiae*[B]	*Str. uberis*[C]	*Str. bovis*	*Str. dysgalactiae*	*Str. parauberis*	*S. aureus*	*S. haemolyticus*	*S. pasteuri*	*E. feacium*	*E. faecium*	*L. lactis*	*V. fluvialis*
	FO1	FO3	NU1	NU3	V1	V2	V3	fbsB	sua													
VG1	+	+	-	-	-	+	-	+	-	-	1	-	-	-	-	-	-	-	-	-	-	-
VG9	-	-	+	+	+	+	+	-	+	-	-	1	-	-	-	-	-	-	-	-	-	-
VG11	+	+	+	+	-	+	-	+	-	-	2	-	-	-	-	-	-	-	-	-	-	-
VG12	i	i	-	-	-	+	-	+	-	1	-	-	-	-	-	-	-	-	-	-	-	-
VG13	+	+	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
VG14	+	+	-	-	-	+	+	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
VG15	-	-	-	-	-	-	i	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
VG16	i	i	-	+	+	+	+	-	+	-	-	1	-	-	-	-	-	-	-	-	-	-
VG17	-	-	+	+	+	+	+	-	+	-	-	1	-	-	-	-	-	-	-	-	-	-
VG18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	1	1	1	1
									Number of strains:	1	3	3	1	1	1	1	1	1	1	1	1	1

(A) *Str. agalactiae* LMG from human origin; (B) *Str. agalactiae* used as positive controls; (C) *Str. uberis* used as positive controls

Regarding the relation between virulence genotypes and herds, it was observed that VG1 was present in 6 (86%) of the 7 herds infected with *Str. agalactiae* (herd E was the only exception). Whereas VG2 was present in 4 (57%) herds and VG3 in only 3 (42%) herds. Within the same herd VG1 strains, if present, were always in greater number than VG2 and VG3 strains. A great diversity of different virulence patterns was present in *Str. uberis* strains, with different VG found in the same herd. On the other hand, strains with identical VG were also found in different herds (table XIII).

Table XIII: Distribution of virulence genotypes from *Str. agalactiae* (VG1, VG2 and VG3) and *Str. uberis* (VG4 to VG10) in the studied herds (A to J).

Virulence genotype	Number of strains	Herd										
		A	B	C	D	E	F	G	H	I	J	K
		n=32	n=5	n=9	n=2	n=4	n=5	n=4	n=3	n=1	n=3	n=1
Str. *agalactiae*	**59**	**32**	**5**	**9**	**2**	**2**	**5**	**4**	**0**	**0**	**0**	**0**
VG1	**50**	28	4	8	2	-	5	3	-	-	-	-
VG2	**6**	3	1	-	-	1	-	1	-	-	-	-
VG3	**3**	1	-	1	-	1	-	-	-	-	-	-
Str. uberis	**10**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**3**	**1**	**2**	**1**
VG4	**1**	-	-	-	-	-	-	-	-	-	1	-
VG5	**2**	-	-	-	-	1	-	-	-	-	-	1
VG6	**1**	-	-	-	-	1	-	-	-	-	-	-
VG7	**1**	-	-	-	-	-	-	-	1	-	-	-
VG8	**1**	-	-	-	-	-	-	-	1	-	-	-
VG9	**1**	-	-	-	-	-	-	-	-	-	1	-
VG10	**3**	-	-	-	-	-	-	-	1	1	1	-

The complete data obtained for virulence markers, with the average probability for each marker in all strains, can be consulted in Table I-Appendix and Figure 3-Appendix.

5. Antimicrobials markers

The erythromycin resistance gene *ermB*, the tetracycline resistance gene *tetS* and the pirlimycin resistance gene *linB* were screened by PCR in some strains from each herd. Positive amplification was possible for the *ermB* gene and after amplicon identity confirmation by sequencing, it was possible to obtain a probe for dot blot analysis. However, it was not possible to obtain an amplicon with the intended size for the genes *linB* and *tetS* in none of the tested strains.

Considering the studied strains, the dot blot assays with the ermB marker (Figure 4-Appendix) revealed positive results for all 10(100%) *Str. uberis*. In *Str. agalactia* a positive hybridisation was observed in 5 (8.5%) strains and a lower signal in 2 (3.4%)

other. These 7 strains were isolated from herd C, where 7 out of 9 (77.8%) isolates presented the resistance gene for erythromicyne. All the remaining 52 *Str. agalactiae* strains showed negative signal with this marker. In the control strains, all *Str. uberis* and 2 (50%) *Str. agalactiae* presented a positive signal with no signal observed in the remaining strains (Table XIV).

Table XIV: Number of strains observed for each in the dot blot assays for antimicrobial markers (ermB) in the studied strains.

Species	n	ermB marker	
		Positive hybridisation	Intermediate hybridisation
Str. agalactiae	59	5 (8.5%)	2 (3.4%)
Str. uberis	10	10 (100%)	-
***Str. agalactiae* LMG[(A)]**	1	-	-
***Str. agalactiae* [(B)]**	3	2 (66.7%)	-
***Str. uberis*[(C)]**	3	2 (66.7%)	1 (33.3%)
Str. bovis	1	-	-
Str. dysgalactiae	1	-	-
Str. parauberis	1	-	-
S. aureus	1	-	-
S. haemolyticus	1	-	-
S. pasteuri	1	-	-
E. feacium	1	-	-
E. faecalis	1	-	-
L. lactis	1	-	-
Vagococcus fluvialis	1	-	-

(A) *Str. agalactiae* LMG from human origin; (B) *Str. agalactiae* strains used as positive controls; (C) *Str. uberis* strains used as positive controls;

DISCUSSION

1. Frequency in mastitis

Despite the several control measures implemented in herds for the prevention and treatment of mammary infections, the frequency of clinical and sub-clinical mastitis remains high. Among the etiological agents causing mastitis, *Str. agalactiae* and *Str. uberis* are important causes of infection in Portuguese herds. By analysing the frequency of isolation of these agents in some herds studied it can be observed that they are often isolated from lactating cows (Table VII). Therefore, it is important to conduct molecular epidemiological studies in order to understand if the new outbreaks of *Str. agalactia* and *Str. uberis* are related to some virulence factors, cow's genetics, failures in control measures or a combination of all these factors.

The data of prevalence rates in mastitis as well as the biological characteristics of *Str. uberis* and *Str. agalactia* isolated from dairy herds in the Portuguese northwest region is still scarce. In this work, the genotypic properties of several strains isolated from cows of dairy farms from the regions near Porto (Portugal) was investigated, in an attempt to define the pathogenic mechanisms and the presence of antimicrobials genes in these species. The identification of these putative virulence factors could provide relevant data about the evolution, the strategies for evasion of the host defences and determinants that allow pathogens to persist throughout time in herds. This work also intended to contribute for the development of non-antibiotic disease control approaches, of particular importance in dairy herds since the control of mastitis is mainly based on the use of antibiotics.

2. Taxonomic genetic markers as detection methods

The taxa-specific markers F1, SA1 and SU correctly identified all *Streptococcaceae*, *Str. agalactiae* and *Str. uberis*, respectively. Despite strain SA86 presenting a small hybridisation signal for marker SU, the calculated average probability was only 0.26 (Table I-Appendix), corresponding to the lower limit of what is considered an intermediate hybridisation Also one cannot exclude the possibility that this may be due to a technical error when processing the samples in the dot blot assay or due to an artefact in the analysis performed by the image-processing algorithm. The results of this work reinforce the hypothesis from a previous study (Almeida *et al*., 2013) concerning the good consistency and stability of these markers to be used for

identification of the *Streptococcus* genus, *Str. agalactiae* and *Str. uberis*, because they show high specificity and sensitivity (Table XV).

Table XV: Specificity and sensitivity of markers F1, A1 and SU for characterization of the *Streptococcus* genus, *Str. agalactiae* and *Str. uberis*.

	Markers					
	F1 (*Streptococcus*)		**A1** (*Str. agalactiae*)		**SU** (*Str. uberis*)	
	Positive strains n=79	**Negative strains** n=7	**Positive strains** n=63	**Negative strains** n=23	**Positive strains** n=13	**Negative strains** n=72
Positive identification	79(100%)	0	63(100%)	0	13 (100%)	1(1.4%)*
Negative Identification	0	7 (100%)	0	23(100%)	0	71(98.6%)

(*) It was observed a small hybridisation.

3. Virulence genotypes

Functional traits responsible for pathogenicity are usually located in unstable or dynamic genomic regions, which are less reliable for detection purposes. Nevertheless, they may provide additional information of the virulence potential of pathogenic species. In this study several virulence genotypes (VG) were identified, according to the results obtained for the presence of specific virulence-associated genes.

The results showed that all strains isolated from bovine mammary gland, have the genes responsible for adherence and hypothetical invasion of epithelial cells, the gene *sua* in *Str. uberis* and the gene *fbsB* in *Str. agalactiae*. These results differ from other studies, in which *sua* was not present in 20% of the *Str. uberis* strains isolated from udders of cows with mastitis (Reinoso et al, 2011), suggesting that strains with this gene have a greater probability of causing mastitis, but is not an obligatory factor to establish infection.

The genes *fruD* and *fruR,* involved in fructose utilization, were present in all *Str. agalactiae* strains obtained from bovine samples. The ability to use fructose it is also present in other environmental pathogens closely related to *Str. agalactiae* (including *Str. dysgalactiae* and *Str. parauberis).* Interestingly, no hybridisation was observed with these markers in none of the studied *Str uberis*, suggesting a different pathway for carbon sources in this species that has the capacity to hydrolyse a wide range of sugars (Ward *et al,* 2009).

It should be noted that the human strain SAA9 showed a significantly lower hybridisation for the fructose operon. Richards *et al.* (2011), found the complete fructose operon in 95% of the bovine samples, but they also did not detected the operon in any of the human strains. This data suggests a unique fructose utilization pathway for the bovine population of *Str. agalactiae*, which might facilitate survival in the extramammary environment. The fructose operon was present in all virulence genotypes found for *Str. agalactiae* strains tested in this study, supporting the hypothesis that, although it may not confer an advantage during infection, strains using the fructose pathway survives longer outside the mammary gland, obtaining an important adaptive advantage during spread.

Another factor included in this study was the nisin U operon that may provide a competitive advantage during infection (Pryor *et al*, 2009). The complete nisin U operon was found in 3 (30%) strains of *Str. uberis* isolated for this work, belonging to VG4, VG7 and VG8. Interestingly, all of them were isolated from clinical mastitis and presented very high CCS. This operon was not observed in the *Str. agalactiae* collected in this work. Nonetheless, it was detected in 2 *Str. agalactiae* from control strains, both from the same herd (Almeida *et al*, 2013). Despite epidemiological information of these 2 strains are not available, this data confirms the exchange of genetic material between separate mastitis pathogens species. That might have contributed to the continued adaptation of *Str. agalactiae* to the bovine environment through the incorporation of potential virulence factors or antibiotic resistance in the genome (Richards et al, 2011).

The only difference observed in the virulence genotypes of *Str agalactiae,* was the presence (or absence) of the gene *gapC*. Most of the isolated *Str. agalactiae* strains carry the gene *gapC*, which has been described as being associated with virulence (Ling et al., 2004; Maeda et al., 2004).This virulence is due to the ability to bind several host proteins and to resist against reactive oxygen produced by phagocyts (Holzmuller et al, 2006). Moreover, it is involved in bacterial energy production and it has imunomodulatory properties (Madureira *et al,* 2007). Comparing the average somatic cell and the number of clinical mastitis, it can be observed that there is a difference in the severity of infection between the strains with and without the gene. The average CCS in infections caused by VG1 strains (with *gapC gene)* and VG2 strains (with possibility to have complete or parcial *gapC* gene*)* is substantially higher than the observed in the VG3 strains (absence of *gapC* gene). Furthermore, 8% of VG1 and 16.7% of the VG2 were isolated from clinical mastitis, whereas none of the VG3 strains were isolated from symptomatic infections. Interestingly, the most severe infections

were caused by VG2 strains, which showed an intermediate hybridisation for *gapC* gene, with an average CCS of 3062±6233cells/ml and 16.7% from clinical mastitis. This might be due to the reduced number of strains of this genotype, which does not allow a very representative statistical data analysis. On the other hand, it is possible that some kind of mutation in the gene confers these strains a greater virulence potential. Nonetheless, these results support the hypothesis that its presence confers an advantage during infection and might increase its severity. VG3 strains found in this work showed less serious infections. Nevertheless, due to the reduced number of strains and based to the fact that clinical information is restricted to the time of sampling, not taking into account the evolution of the infection throughout time, we cannot set aside the possibility of VG3 strains also being able to cause more serious infections.

Regarding *Str. uberis*, some studies showed prevalence of the *gapC* gene in 80% of the isolates (Reinoso *et* al, 2011).In this work the gene was present in all studied strains, so it is not possible to compare the severity of infection in strains with and without the gene.

Taking into account this increase in the severity of infection, combined with the high prevalence of the gene in bovine population of *Str. agalactia* and *Str. uberis*, some authors have suggested that a GapC protein may be a good target for vaccine development (Perez-Casal *et al*, 2004).

Strains with hyaluronic acid capsule around them seem to be resistant to phagocytosis by bovine neutrophils and establish infection more effectively (Ward et al, 2001; Leight, et al, 1991). Nonetheless, some authors argue that hyaluronic acid capsule of *Str. uberis* plays only a minor role in the early stages of infection and is not required for the development of infection and clinical mastitis. (Field et al, 2003; Ward et al, 2009). The hyaloronic acid capsule production is dependent on the *has* operon (*hasA*, *hasB* and *hasC*). Despite some authors mention that the role of *hasC* is unclear to capsule biosynthesis, it is found with high frequency in capsular phenotypes (Reinoso *et al*, 2011). In this work the gene *hasC* is present in all VGs of *Str. uberis*, confirming the high prevalence of capsulated strains of *Str. uberis* causing mastitis.

Finally, the *oppF* gene, which plays an important role during growth in milk (Smith et al, 2002), was found in all *Str. uberis* strains. Comparatively, Reinoso *et al.* (2011), found the *oppF* gene only in 64.1% of the strains. Once again, due to the absence of strains without the gene, we cannot compare the severity patterns of strains with or without

thise gene. It is however possible to assume that, based on our study, the prevalence of the *oppF* gene in the dairy herds analysed is higher than in other locations.

In conclusion, several virulence genotypes were observed associated with mammary infections. These genotypes were found within the same herd and among different herds, showing that different virulence patterns were able to cause infection. The genetic variability is higher in *Str. uberis*, in which the different virulence patterns are able to cause clinical mastitis and apparently none of the virulence genotypes seems to be dominant relative to the others. It is important to consider that *Str. uberis* infections may also be dependent on cow's factors (Reinoso *et al*, 2011). On the contrary, *Str. agalactiae* presents less genetic variability, with an apparent advantage in the frequency and severity of infection for strains containing the gene *gapC*.

4. Antimicrobials resistance

In order to study the emergence and spread of MLS and tetracycline resistance in bovine *Str.agalactiae* and *Str. uberi* strains the following genes were tested: macrolide resistance gene *ermB*; lincomicine resistance gene *linB* and tetracycline resistance gene *tetS*. *linB* and *tetS* were not detected by PCR in any of the tested strains. Moreover, the *in vitro* disk diffusion method with tetracycline and pirlimycin, using the interpretative standards available for these organisms in the European Society of Clinical Microbiology and Infection Diseases (EUCAST, http://mic.eucast.org/) also showed that the strains tested were susceptible for these compounds.

In this study, erythromycin resistance mediated by the *ermB* gene was shown to be widespread in *Str. uberis,* with the 13 (100%) tested strains presenting the gene. This confirms the emergence and spread of the macrolide resistance in the environmental streptococci.

Importantly, the *ermB* gene was found in 7 (11.1%) *Str. agalactiae* strains. This suggests the exchange of genetic material between *Str. agalactiae* and environmental bacteria. *Str. agalactiae* infections are currently treated effectively with penicillin and other compounds, however, there are other mastitis-causing pathogens, or environment bacteria, that are less susceptible to antibiotics used against *Str. agalactiae*. This is a cause for concern since that lateral transfer of genes between *Str. agalactia* and other pathogens is frequent and plays a significant role in its evolution (Richards *et al.*, 2011). Furthermore, this may result in the acquisition of increased antibiotic resistances, penicillin for instance, which would greatly difficult the treatment of infections.

5. Future Perspectives

Data from this study should be interpreted with caution because this work has evaluated a small number of strains, within a restricted number of herds and to the timeframe of the study. Nevertheless, the results obtained in this work provide a methodological ground to carry out epidemiological and molecular evaluations of the causative agents of bovine mastitis, which may contribute to an efficient control, treatment and prevention of bovine mastitis. In the future, it will be important to:

1) Increase the number of tested isolates in order to obtain a more robust number of strains to allow comprehensive epidemiological and evolutionary studies.
    a. Collect samples from cows that were previously subjected to milk sampling. This will allow obtaining strains isolated from the same cow in different times, providing a unique and important set of data to study the bacterial evolution.
    b. Increase our culture collection with isolates originated from a wide range of herds, in order to assess biogeographical data.
2) Develop an inverted dot blot platform with the most discriminatory markers. That will permit to simultaneously evaluate all the selected probes with a single hybridisation assay.
3) Investigate more genes related to virulence factors and antimicrobials resistance.
4) Standardize the clinical information gathered from infected cows, regarding severity, chronicity and resistance to treatment, in order to better assess the epidemiological features of the farms.
5) Infer geographical and temporal dissemination patterns of streptococci causing bovine mastitis, and correlate these with bacterial genomic traits.

APPENDIX

1. References

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2. Figures, Graphs and Tables

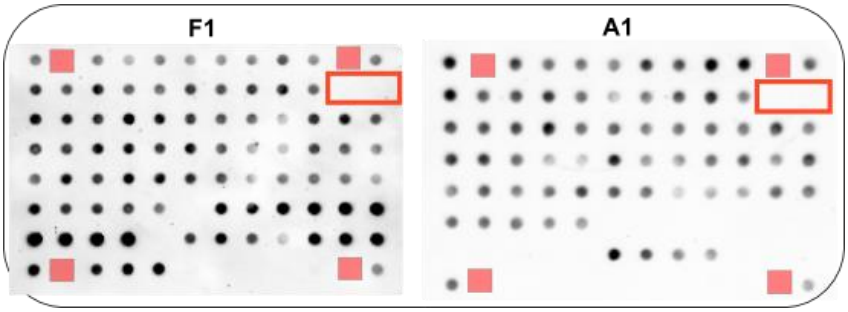

Figure 1: Preliminary Dot blot assays of taxa-specific markers for Streptococcaceae (F1), *Str. agalactiae* (A1) in isolates.

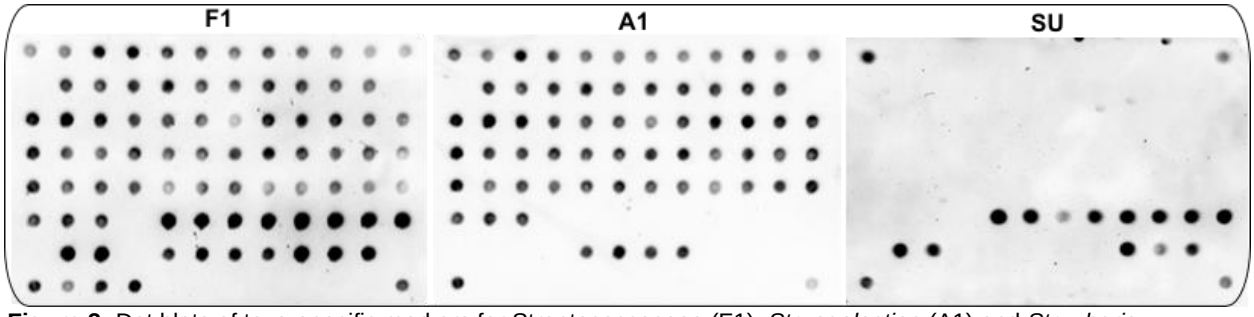

Figure 2: Dot blots of taxa-specific markers for Streptococcaceae (F1), *Str. agalactiae* (A1) and *Str. uberis*

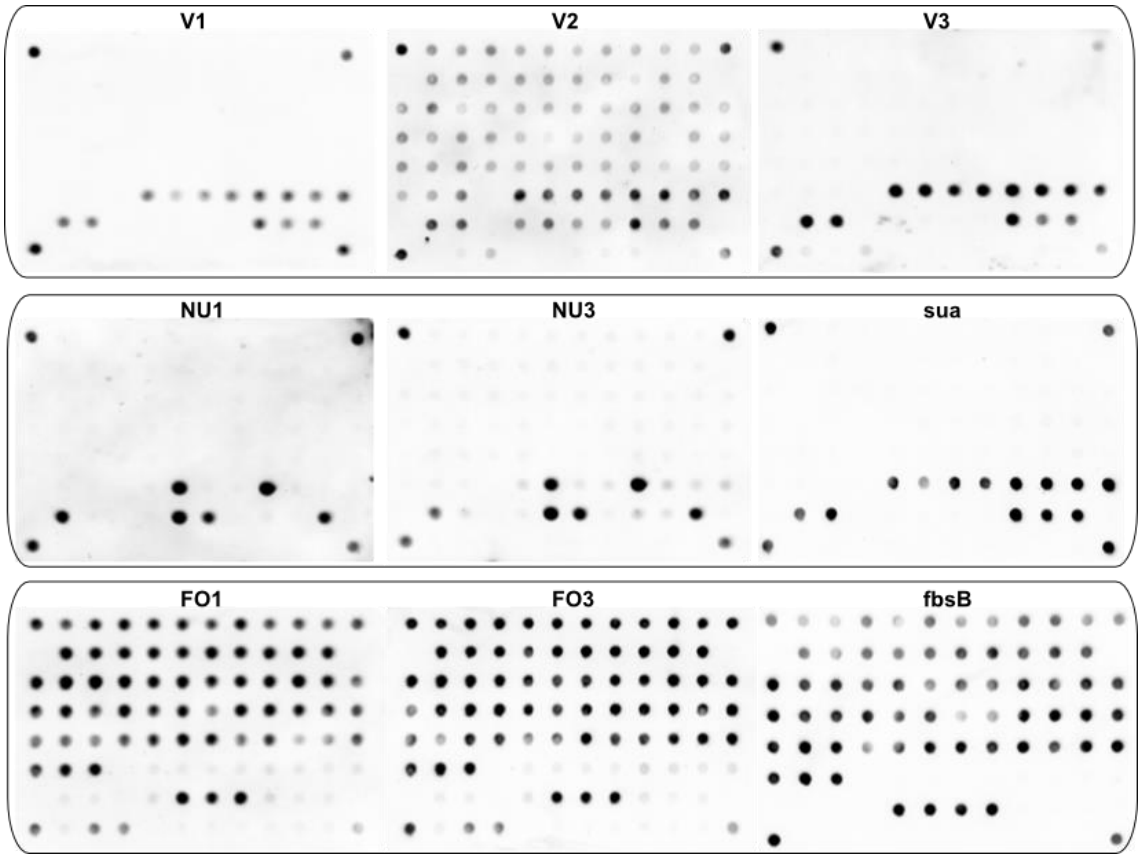

Figure 3: Dot blots of virulence and functional markers from the *hasC* (V1), *gapC* (V2), *oppF* (V3), nisin U operon (NU1 and NU3), *sua* (sua) fructose operon (FO1 and FO3) and *fbsB* (fbsB)

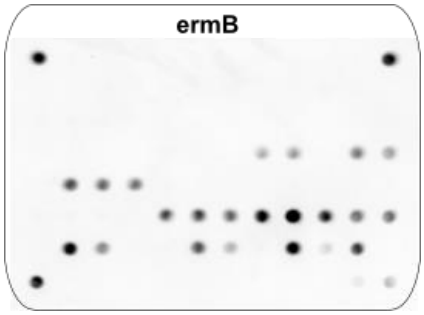

Figure 4: Dot blots of antimicrobials marker for *ermB* (V1).

Table I: Average probability values of the results obtained from the dot blot assays, with the respective herd and virulence genotype (VG). Values below 0.25 are indicated as red, values between 0.25 and 0.75 as yellow and values above 0.75 are shown as green.

Strain	Herd	VG	Calculated probability for markers												
			F1	A1	SU	FO1	FO3	NU1	NU3	V1	V2	V3	sua	fbsB	ermB
SA35	A	1	1	1	0,03	1	1	0	0,05	0	0,99	0,03	0,01	0,93	0,01
SA36	A	1	0,98	1	0,02	1	1	0	0,04	0,01	0,97	0,02	0,01	0,79	0,01
SA37	A	1	1	1	0,08	1	1	0	0,05	0,01	1	0,03	0,01	1	0,01
SA38	A	1	1	1	0,04	1	1	0	0,02	0	0,93	0,02	0,01	0,82	0,01
SA39	A	1	1	1	0,01	1	1	0	0,04	0	0,92	0,08	0,01	1	0,01
SA40	A	2	1	1	0,08	1	1	0	0,04	0	0,72	0,08	0,01	0,98	0,01
SA41	A	1	1	1	0,02	1	1	0	0,04	0	0,93	0,03	0,01	0,98	0
SA42	A	1	1	1	0	1	1	0	0,03	0	0,87	0,02	0,01	1	0
SA43	A	1	1	1	0	1	1	0,01	0,03	0	0,78	0,01	0,01	1	0
SA44	A	1	1	1	0	1	1	0,02	0,02	0	0,79	0,01	0	0,99	0
SA45	A	1	1	1	0,02	1	1	0,02	0,08	0	0,98	0,04	0,01	1	0
SA46	A	1	1	1	0,01	1	1	0,02	0,07	0	0,98	0,02	0,01	0,95	0,01
SA47	A	1	1	1	0,02	1	1	0,02	0,24	0,01	0,98	0,05	0,01	1	0,01
SA48	A	1	1	0,98	0,13	1	1	0,02	0,09	0	0,98	0,03	0,01	1	0,01
SA49	A	1	1	1	0,05	1	1	0,05	0,07	0,01	0,97	0	0,01	1	0,01
SA50	A	1	1	1	0,05	1	1	0,02	0,05	0,01	0,95	0,04	0,01	1	0,02
SA51	A	1	1	1	0,05	1	1	0,03	0,06	0	0,91	0,07	0	1	0
SA52	A	3	1	1	0,03	1	1	0,04	0,07	0	0,11	0,01	0,01	1	0,01
SA55	A	1	1	1	0,02	1	1	0,04	0,05	0	0,96	0,01	0,01	1	0,01
SA56	A	1	1	1	0,02	1	1	0,02	0,04	0	0,82	0,02	0,01	1	0,01
SA57	A	1	1	1	0,08	1	1	0,03	0,08	0,01	0,94	0,02	0,01	1	0,01
SA58	A	1	1	1	0,02	1	1	0,04	0,12	0,02	1	0,13	0	1	0,03
SA59	A	2	1	1	0,04	1	1	0,02	0,05	0,01	0,27	0,02	0,01	1	0,01
SA60	A	1	1	1	0,04	1	1	0,01	0,1	0	0,86	0,02	0,01	1	0
SA62	A	1	1	0,99	0,06	1	1	0,01	0,05	0	0,9	0,03	0,01	1	0,01
SA65	A	1	1	1	0,05	1	1	0,01	0,05	0	0,91	0,03	0,01	1	0
SA66	A	1	0,93	1	0	1	1	0,01	0,03	0	0,86	0,02	0,01	1	0
SA67	A	2	1	1	0	1	1	0,02	0,05	0	0,58	0,02	0,01	1	0
SA68	A	1	1	1	0,03	1	1	0,02	0,1	0	0,92	0,01	0,01	1	0
SA69	A	1	1	1	0,02	1	1	0,05	0,06	0	0,98	0,01	0,02	1	0
SA74	A	1	1	1	0,03	1	1	0,05	0,05	0	0,88	0,02	0,01	1	0
SA76	B	2	1	1	0,17	1	1	0,03	0,04	0	0,64	0,01	0,01	1	0,01
SA77	B	1	1	1	0,09	1	1	0,01	0,01	0	1	0,02	0,01	1	0,01
SA78	B	1	1	0,99	0,01	1	1	0,01	0,02	0	0,94	0,02	0	1	0
SA79	B	1	1	0,97	0,03	1	1	0,01	0,02	0	0,99	0,01	0	1	0
SA80	B	1	1	1	0,02	0,98	1	0,02	0,01	0	0,97	0,02	0,01	1	0,01
SA81	C	1	1	0,95	0	1	1	0,04	0,05	0	0,91	0	0	0,97	0,85
SA82	C	1	0,98	0,96	0	1	1	0,01	0,05	0	0,99	0,01	0,01	1	0,7
SA83	C	3	0,96	1	0,03	1	1	0,04	0,05	0	0	0,05	0,02	1	0,03
SA84	C	1	1	1	0,03	1	1	0,02	0,04	0	0,92	0,01	0,03	1	0,97
SA85	C	1	1	1	0,05	1	1	0,02	0,06	0	0,78	0	0,03	1	0,65

Table 1 (Continued)

Strain	Herd	VG	Calculated probability for markers												
			F1	A1	SU	FO1	FO3	NU1	NU3	V1	V2	V3	sua	fbsB	ermB
SA86	C	1	1	1	0,26	1	1	0,01	0,02	0,01	0,99	0,04	0,01	1	0,01
SA87	C	1	1	1	0,13	1	0,98	0,02	0,04	0	0,97	0,03	0,01	1	1
SA88	C	1	1	1	0,06	1	1	0,01	0,04	0	1	0,02	0,01	1	1
SA89	C	1	1	1	0,07	1	1	0,01	0,04	0	0,93	0,03	0	0,98	0,99
SA90	A	1	1	1	0,03	1	1	0	0,04	0	0,83	0,02	0	1	0,01
SA91	D	1	1	1	0,19	1	1	0,03	0,08	0,01	0,94	0,05	0	1	0,02
SA92	D	1	1	1	0,05	1	1	0,03	0,07	0	1	0,08	0,01	1	0,03
SA93	E	3	1	1	0,02	1	1	0	0,02	0	0,04	0,01	0	1	0
SA94	E	2	0,95	0,95	0	1	1	0,02	0,01	0	0,5	0,02	0,01	0,84	0
SA95	F	1	0,99	0,98	0,03	1	0,99	0,01	0,05	0	0,9	0,03	0,01	1	0,02
SA96	F	1	1	0,99	0	1	1	0,01	0,01	0	0,92	0,04	0	1	0
SA97	F	1	1	1	0	1	1	0	0,01	0	0,95	0,04	0,01	1	0,01
SA98	F	1	0,98	1	0	1	1	0,01	0,02	0	0,91	0	0,01	1	0
SA99	F	1	1	1	0	1	1	0,01	0,04	0	0,92	0,01	0,01	1	0,02
SA100	G	2	1	1	0,03	0,97	1	0,01	0,03	0	0,46	0,01	0,01	1	0,01
SA101	G	1	1	1	0,23	1	0,98	0,02	0,02	0	0,79	0,01	0,04	1	0,01
SA102	G	1	0,95	1	0,04	1	1	0,01	0,02	0	0,76	0	0,01	1	0
SA103	G	1	1	1	0,15	1	1	0,01	0,05	0	0,92	0,03	0,01	1	0,02
SU10	J	10	1	0	1	0,13	0,2	0,04	0,6	0,99	1	1	1	0,01	1
SU11	H	10	1	0	1	0,14	0,12	0,01	0,5	1	1	1	1	0,02	1
SU13	H	8	1	0	1	0,24	0,29	0,04	0,72	1	1	1	1	0,03	1
SU14	I	10	1	0	1	0,16	0,13	0,03	0,33	0,96	1	1	1	0,01	1
SU15	J	9	1	0,01	1	0,17	0,2	1	1	0,88	1	1	0,95	0,01	1
SU16	E	6	1	0,01	0,99	0,26	0,23	0,03	0,37	0,95	1	1	1	0,01	1
SU17	E	5	1	0,01	1	0,29	0,27	0,03	0,48	1	1	1	1	0,01	0,97
SU18	H	7	1	0,01	1	0,25	0,35	1	1	1	1	1	1	0,01	1
SU19	K	5	1	0,01	1	0,36	0,5	0,04	0,75	1	1	1	1	0,01	1
SU20	J	4	1	0,01	1	0,6	0,61	1	1	1	1	1	1	0,03	1