War against mastitis: Current concepts on controlling bovine mastitis pathogens

Cristina Bogni1*, Liliana Odierno1, Claudia Raspani1, José Giraudo2, Alejandro Larriestra2, Elina Reínoso1, Mirta Lasagno1, Mirian Ferrari1, Edith Ducrós2, Cecilia Frigerio1, Susana Bettera1, Matías Pellegrino1, Ignacio Frola1, Silvana Dieser3 and Claudina Vissio2.

1Department of Microbiology and Immunology, Faculty of Physical-Chemical and Natural Sciences
2Department of Animal Pathology, Faculty of Agronomy and Veterinary Medicine. 3. Department of Chemical Technology, Faculty of Engineering. University of Río Cuarto. Ruta 36 Km 601, X5804ZAB Río Cuarto, Córdoba. Argentina.
*Corresponding author: E-mail: cbogni@exa.unrc.edu.ar

Mastitis, inflammation of the mammary gland, is one of the most costly and complex diseases of the dairy industry. The economic consequences of bovine mastitis are related to treatment, production losses, culling and changes in milk quality. These factors have a substantial impact on the farm business. The complexity is reflected in the numerous causative pathogens, the variety and magnitude of the physiological responses to these pathogens and the variation in efficacy of control measures for different causative organisms. Whether accompanied by clinical signs or not, an IMI (IMI) is associated with an increase in the somatic cell counts (SCC) in milk. The magnitude of this increase varies according to the bacteria involved in the IMI. The major pathogens (Streptococcus agalactiae, Staphylococcus aureus, Streptococcus spp., coliforms Escherichia coli and Klebsiella spp. and Mycoplasma spp.) are responsible, most of the time, for clinical mastitis. The minor pathogens, staphylococci other than Staphylococcus aureus (mostly Staphylococcus chromogenes, Staphylococcus hyicus, Staphylococcus epidermidis, and Staphylococcus xylosus) or Corynebacterium spp. are associated with a moderate infection and rarely with clinical signs. Based upon their primary reservoir and mode of transmission, mastitis agents are classified as contagious (Staphylococcus aureus and Streptococcus agalactiae), commonly transmitted among cows and environmental pathogens, streptococci other than Streptococcus agalactiae (mostly Streptococcus uberis) and coliform (e.g., Escherichia coli, Klebsiella spp.) which arise from the cows environment, entering into the udder between milkings, when teats are exposed to mud, manure, and dirty bedding materials. Mastitis is an evolving disease that must be discussed ecologically. As result of that, the causative agents, the milk production environment and the mammary gland as a reservoir, should be understood as a whole. These are the driving forces of the persistence and spread of the disease in the herd. The control of bovine mastitis is mainly based on prevention and the focus has to be on the reduction of new infection risk, the control of mammary gland state and the management of the clinical cases. All control tools are developed to deal with the disease and finally to manipulate the immunity and to reduce the carrier state. Even though some current management practices, such as proper milking hygiene and reduced exposure to environmental pathogens contribute to the decrease in the occurrence of the disease, the treatment for bovine mastitis relies heavily on the use of antibiotics. Antibiotics have been used routinely for the treatment of existing infections and also for preventing new ones mainly at the drying off. But, while dry cow antibiotic therapy has helped to reduce the incidence of mastitis, the emergency of antibiotic-resistant pathogens has provoked considerably damages. The development of preventives measures, like application of vaccines, immunomodulators, beneficial microorganisms (probiotics) or their metabolic products (bacteriocins, lactic acid, and hydrogen peroxide), are beginning to be highly considered and applied in many herds.

This chapter provides the opportunity to expand our knowledge of bovine mastitis and undoubtedly contributes to the development of novel approaches to mastitis diagnostics and control.

1. Global milk production

Since mid-1990th the world milk consumption has been growing in average 10-15 million tonnes per year, based on the population growth and the increasing income in many countries. The production of milk cow increased from 460 to 550 million tonnes in 2009. The consumption of milk is heterogeneous over the world and varies among the different countries. Even though, European Union and North America continue to be the largest milk consumers, since 1980 the demand for the dairy products has grown considerably, especially in China, India and several Asian countries. The governmental support of milk consumption like school milk programs and new dairy products favors this expansion. An increase of 25% on world milk demand between 2007 and 2020 is expected [1]. In this context, the production and quality of milk could be altered by mastitis, the inflammation of mammary gland. Milk somatic cell count (SCC) constitutes a useful tool to measure milk quality, health status of the mammary gland and the risk of milk changes composition. Increased SCC is associated with reductions in milk yield and it adversely affects cheese production, as a result of reduced of both, curd firmness and fat, and casein loss in whey. Several studies on the United States show that costs related to mastitis on dairy farms are approximately US $200 per cow/year, and this gives an annual lost of 2 billion dollar for dairy industry. Almost all authors agree that at least 70% of economic losses come from reduction on milk
production and discard of milk from sick animals. Other causes are the elimination of milk containing antibiotics used in treating sick animals, loss of genetic value by removing cows early and therefore more expensive replacement, veterinary fees, drug expenditures, payments of extra hours and the commercial value reduction of cows removed [2]. Thus, milk producers face the challenge of improving milk quality to reduce human health negative impact. There is no evidence that high SCC is directly associated with adverse effects on human health. However, high SCCs are associated with increased indirect risks, including poor farm hygiene, antibiotic residues and the presence of pathogenic organisms and toxins in milk [3].

2. Development of mastitis

The teat itself is the first line of defence against the penetration of bacteria into the udder. Normally, the sphincter muscle closes the teat canal tightly when the cow is not being milked. Infections begin when microorganisms penetrate the teat canal and multiply in milk-producing tissues. During mechanic milking, organisms present in the milk or at the teat end may be propelled into or through the teat duct into the cistern. After milking, the teat canal remains dilated for one to two hours. Organisms from the environment (manure, bedding, etc.) or those found on injured skin at the tip of the teat may easily invade an open canal. Bacteria may proceed into the udder by attaching and colonizing new tissue. Once bacteria are inside the udder, infection and inflammation of the damaged area begin. Bacteria initially affect tissues lining the large milk-collecting ducts and cisterns by the production of several virulence factors that cause swelling and death of milk producing cells. Milk-secreting cells damaged release substances that lead to increased permeability of vessels and attract leukocytes which can engulf and destroy bacteria. During this process, the leukocytes release substances that cause the recruitment of additional leukocytes from the blood into the milk. If bacteria are not entirely destroyed, they continue to multiply and begin to invade smaller ducts and alveolar areas. Sometimes the microorganisms are eliminated rapidly and the infection is cleared. In this case, the clogged ducts are opened and milk composition and production return to normal in several days. However, as the infection persists and ducts remain clogged, the entrapped milk makes the secretory cells revert to a resting (non-producing) state and the alveoli begin to shrink. Substances released by leukocytes lead to the complete destruction of alveolar structures, which are replaced by connective and scar tissues. The destruction of milk secretory tissue is, in effect, the cow’s third line of defence to bring the infection under control. Thus, as the disease progresses, the number of somatic cells in the milk becomes elevated and associated with a permanent reduction in milk yield [4].

3. Different ways of mastitis presentations

Whether accompanied by clinical signs or not, an intramammary infection (IMI) is associated with an increase in the SCC in milk which varies according to the bacteria involved in the IMI. Mastitis usually occurs primarily in response to intramammary bacterial infection, but also to mycoplasmal, fungal, or algal infections. Mechanical trauma, thermal trauma, and chemical injury, predispose the gland to IMI. Occurrence of mastitis depends on the interaction of host, microbial agent, and environmental factors. The severity of the inflammation can be classified into sub-clinical, clinical and chronic forms, and its degree depends on the nature of the causative pathogen and on the age, breed, immunological health and lactation state of the animal. Sub-clinical mastitis is difficult to detect due to the absence of any visible indications, and it has major cost implications. Clinical mastitis is a condition in which abnormalities of the udder and secretion are readily observable. Changes in the milk, such as flakes, clots, and a watery appearance are the most obvious abnormalities; heat, swelling and sensitivity of the udder are either slight or absent. Systemic symptoms may also be present and include fever, loss of appetite, reduced rumen function, weakness, and depression. Chronic mastitis is a form of udder infection that lasts long and results in persistent inflammation of the mammary gland [4].

4. Mastitis diagnostics

Monitoring udder health performance is impossible without reliable and affordable diagnostic methods. Therefore, there is a constant need to improve these methods, for accuracy, cost, or convenience. The most frequently used diagnostic methods are SCC and bacteriological culturing of milk. Currently, methods such as measurement of N-acetyl-β-D-glucosaminidase (NAGase), lactate dehydrogenase activity (LDH), electric conductivity (EC) on milk, are used less frequently. Mastitis diagnosis starts with visual observation of the udder and of the milk through the forestripping, which is also an important part of udder preparation [5]. If there is any detectable change in quarter and/or any observable abnormality in the milk, the quarter is defined as having clinical mastitis. It is important not to ignore clinical symptoms. An easy, cheap, and rapid “cow-side” mastitis test to estimate SCC on farm is the California Mastitis Test (CMT). This test could be used by farmers and veterinarians to diagnose and treat the inflammation in its early stages, thus having the potential to stop the propagation of the disease in the herd. An increase in temperature is one of the symptoms associated with mastitis. A thermal camera was used to diagnose experimentally-induced mastitis and could detect temperature changes of 1 to 1.58 °C. Infrared thermograph was also used to measure skin surface
temperatures in infected cows, and a strong correlation (R²=0.92) between skin surface temperature and SCCs was observed. However, the ambient temperature can affect this assay, and a rise in temperature might only occur in some cases of mastitis; therefore, temperature might only act as an indicator of infection. Estimation of the levels of inflammation-related enzymes might also be used for the detection of mastitis as these show good correlation with SCCs. Other enzymatic tests include the detection of an esterase secreted by somatic cells using an enzymatic assay on a dipstick*.

Bioluminescence- determination assays, based on estimation of the ATP concentrations in somatic cells or the recognition of somatic cell DNA by fluorescent staining, can also be used ‘on-site’ for the reliable determination of elevated SCC levels and thus the probable presence of mastitis [6].

The identification of pathogens causing mastitis is important for disease control and epidemiological studies. Bacteriological culturing can be carry out at herd, as well as cow and quarter level, each with its own specific goal. Conventional identification of the species is performed according to cultural, biochemical and serological properties. Commercial biochemical kits are available for identification but they have proved unreliable for the identification of veterinary pathogens. The use of molecular methods in pathogen detection has increased over the last years. For a number of mastitis pathogens, PCR-based techniques have been described [7]. Recently, multiplex PCR tests in which several pathogens can be tested at the same time have been developed [8]. Additionally, real-time PCR assays are being developed for detection and quantifying mastitis pathogens in milk. Advances in relevant proteomics techniques, such as two-dimensional gel electrophoresis and mass spectroscopy, have led to the identification of several new proteins involved in mastitis. The proteomics studies mentioned above resulted in information on the different protein expression pattern obtained from mastitis-infected milk and on the proteins expressed by invading pathogens. This information can be applied not only to the discovery of new therapeutic targets but also to the search for new diagnostic biomarkers. The successful application of these new biomarkers in a detection device still remains a challenge. Recent advances in microfluidics and so-called ‘biochips’ have the capacity to revolutionize diagnostics [9].

5. Mastitis associated pathogens

Over 135 different microorganisms (bacterial, algal or fungal) have been isolated from bovine IMI, but the majority of these are caused by staphylococci, streptococci, and gram-negative bacteria [10]. Microorganisms that cause mastitis are generally classified as either contagious or environmental based upon their primary reservoir and mode of transmission. *Staphylococcus aureus* and *Streptococcus agalactiae* are contagious pathogens and are commonly transmitted among cows by contact with infected milk. These pathogens are of particular importance because they cause mainly subclinical forms of IMI that are often difficult to detect. Primary environmental pathogens include different types of bacteria: species of streptococci other than *Strep. agalactiae* (*Streptococcus spp.*), coliform species (*Escherichia col*, *Klebsiella spp.*, *Enterobacter spp.*) and *Pseudomonas* spp. (Table 1). In regard to *Streptococcus dysgalactiae* and *Streptococcus uberis* classification, in some laboratories, the two species are grouped together as “environmental streptococci”. However, these species differ in many bacteriological and epidemiological characteristics. *Strep. uberis* has many characteristics of an environmental pathogen. For instance, the large variety of strains occurring in the environment makes it unlikely that a large proportion of cows in a herd would get infected with the same strain. On the other hand, *Strep. dysgalactiae*, largely conforms to the description of contagious pathogens. *Strep. dysgalactiae* is more contagious in nature than *Strep. uberis*, but is also found in the environment. The major pathogens (*Staph. aureus, Strep. agalactiae, Strep. dysgalactiae, Strep. uberis*) are responsible for clinical and subclinical mastitis, whereas coliforms and *Mycoplasma* spp. usually cause clinical mastitis. The minor pathogens are most often associated with a moderate infection rather than associated with clinical signs. These infections are mainly due to staphylococci other than *Staph. aureus* or *Corynebacterium bovis*.

5.1 *Staphylococcus aureus*

Recent studies of genetic homology (DNA sequences, DNA-rRNA hybridization, comparative sequencing of 16 rRNA) have shown that the genus *Staphylococcus*, together with the genera *Gemella, Macrococcus* and *Salinicoccus* is found within the family *Staphylococcaceae*. The *Staphylococcus* genus includes 42 species, divided by the coagulase test into coagulase-positive (CPS) and coagulase-negative (CNS) species. Some of them are part of the normal microbial flora of skin and mucous membranes of animals. *Staph. aureus* is the most prevalent mastitis causing agent in many parts of the world. However other CPS, including *Staphylococcus hyicus* and *Staphylococcus intermedius*, are also recognized as etiologic agents of bovine mastitis. In many studies and in routine diagnostics, Gram-positive cocci, catalase and coagulase-positive are merely confirmed as *Staph. aureus* [11]. Considering the similar morphological and biochemical characteristics (haemolysis, clumping factor, acetoin production, pyrrolidonyl arylamidase, acid production from maltose, sensitivity to aconitaine and polymyxin) among different CPS species isolated from bovine mastitis, phenotypic identification may be unreliable, and misidentification may happen [12]. The use of nucleic acid targets, with their high sensitivity and specificity, provides an alternative technique for the accurate identification and classification of *Staphylococcus* species. Apart from the 16S rRNA gene, the 16S-23S rRNA intergenic spacer region, and the heat shock protein 60 (hsp60) gene [13], other gene sequences have been used in genetic studies: the femA gene,
the sodA gene [14], the tuf gene, the rpoB gene [15], and the gap gene[16]. *Staph. aureus* produces a large number of potential virulence factors. Among them, several extracellular toxins (α, β, γ and δ hemolysins, enterotoxins), enzymes (staphylokinase, lipases, esterases, protease, nuclease), cell-wall associated proteins (protein A, collagen-binding protein, fibronectin-binding protein, elastin-binding protein), capsular polysaccharides and slime. In vitro studies have shown that *Staph. aureus* adheres to mammary epithelial cells and extracellular matrix components and invades into mammary epithelial. Adhesion is a prerequisite and crucial early step for mammary gland infection. Bacteria are found enclosed in membrane bound vacuoles in the cytoplasm of mammary epithelial cells. *Staph. aureus* escapes from the phagosome into the cytoplasm and induces apoptosis. The invasion into mammary epithelial cells may occur through an endocytic process. Thus, the recurrent subclinical infection may result from this intracellular existence of bacteria that are protected from host defenses and effects of antibiotics. In the last decade, several virulence genes (spa IgG-binding, spa X-region, nuc, clfA, coa, hla, hlb, fnbA, fnbB, cap, agrI, agrII, agr III, icaA, icaD) in *Staph. aureus* isolated from bovine have been described [17,18]. Virulence genes or gene elements may also have an association with resistance determinants (mecA, blaZ) and hypervirulence. In spite of the introduction of large-scale mastitis control programmes, *Staph. aureus* remains a major mastitis pathogen. It causes mastitis epidemics even in well-managed dairy herds and can persist for long periods in the mammary glands. The current control practices may fail to prevent the spread of particularly virulent strains. The control of *Staph. aureus* mastitis should be focused on specific strains causing infections in the herds.

### Table 1. Bovine mastitis associated microorganisms

<table>
<thead>
<tr>
<th>Contagious microorganism</th>
<th>Environmental microorganism</th>
<th>Others pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Escherichia coli*</td>
<td>Coagulase-negative staphylococci (CNS) **</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em></td>
<td>Klebsiella spp.*</td>
<td></td>
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<tr>
<td><em>Streptococcus dysgalactiae</em></td>
<td>Citrobacter spp.*</td>
<td></td>
</tr>
<tr>
<td><em>Mycoplasma</em> spp.*</td>
<td>Serratia spp. *</td>
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<tr>
<td><em>Corynebacterium</em> spp.*</td>
<td>Enterobacter spp.*</td>
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<tr>
<td></td>
<td>Proteus spp. *</td>
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<tr>
<td></td>
<td><em>Streptococcus</em> aiberis</td>
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<tr>
<td></td>
<td><em>Streptococcus</em> dssgalactiae</td>
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<td></td>
<td><em>Streptococcus</em> spp.*</td>
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<td></td>
<td><em>Enterococcus</em> faecalis</td>
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<tr>
<td></td>
<td><em>Enterococcus</em> faecium</td>
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<tr>
<td></td>
<td><em>Aerococcus</em> spp.*</td>
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<td></td>
<td><em>Pseudomonas</em> spp.*</td>
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<tr>
<td></td>
<td><em>Bacillus</em> spp.*</td>
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<tr>
<td></td>
<td><em>Arcanobacterium</em> pyogenes</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Nocardia</em> spp. **</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yeast spp.*</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Prototheca</em> spp.*</td>
<td></td>
</tr>
</tbody>
</table>

* Classified as major pathogen, **Classified as minor pathogen

5.2 *Streptococcus* spp.

*Streptococci* are a heterogeneous group of bacteria, consisting of as many as 48 species [19]. Identification of streptococcal species is currently based on observation of the cultural and morphological characteristics, determination of the biochemical pattern (production of enzymes and production of acid from various carbohydrate sources) and observation of the antigenic structure according to the classification of Rebecca Lancefield. PCR amplification of species-specific parts of the gene encoding the 16S rRNA, the 23S rRNA gene, the 16S-23S rDNA intergenic spacer region, tuf gene, cfb gene, as well as rRNA intergenic spacer length polymorphism analysis had been successfully used for the rapid and reliable identification of these species [20,21].
5.2.1 *Streptococcus agalactiae*

*Strep. agalactiae* is a beta-hemolytic Gram-positive bacteria corresponding to group B streptococci. In animals it is well known worldwide as a major contagious pathogen causing bovine subclinical mastitis. This bacterium can survive a very short time in the environment, but it can persist indefnitely within the mammary gland as an obligate pathogen of the udder [22]. *Strep. agalactiae* forms small 3 to 4 mm, grey-white colonies, which have a narrow zone of beta hemolysis on blood agar. *Strep. agalactiae* is identified in the veterinary laboratory by CAMP factor, hydrolysis of hippurate and lack of hydrolysis of esculin agar. This species is characterized as inulin-variable. Commercial antisera that recognize the group B antigen are used to identifc isolates. One of the most important virulence factors is the capsule polysaccharide of which nine antigenic variants have been identified [23]. Few studies have explored the presence of genetic determinants that encode potential virulence factors. The *bca* and *scpB* virulence-related genes were the most frequent among bovine isolates [24]. More recently, virulence genes *hylB* (hyaluronidase) and *lmh* (laminin-binding protein) were detected in bovine strains.

5.2.2 *Streptococcus dysgalactiae*

*Strep. dysgalactiae* is described as α-hemolytic or nonhemolytic (Lancefield group C) and associated only with IMI. Among the environmental streptococci, *Strep. dysgalactiae* is one of the most prevalent, which may infc mammary glands as favorable conditions arise [25]. This species is identifed as inulin and esculin-variable and by lack of CAMP factor production and hydrolysis of hippurate. Little is known about factors that contribute to the virulence of *Strep. dysgalactiae*. Several cell-associated and extracellular factors of this species have been identifed. This pathogen can interact with several plasma and extracellular host-derived proteins (among others immunoglobulin G, albumin, fibronectin, fbrinogen). These interactions are mediated by bacterial surface proteins. The virulence genes encoding hyaluronidase, fibrinolysin, streptolysin-S, glyceraldehyde-3-phosphate dehydrogenase, plasminogen-binding M-Like protein and the collagen-like protein were detected in the majority of bovine isolates. Additionally, group A streptococci bacteriophage-associated virulence genes encoding superantigens, DNase and/or streptodornase were detected in bovine isolates [26]. Furthermore, *Strep. dysgalactiae* adheres to and is internalized by bovine mammary epithelial cells *in vitro*. Involvement of host cell kinases is required for its internalization into bovine mammary epithelial cells; a process that apparently occurs by a receptor-mediated endocytosis mechanism. Finally, *Strep. dysgalactiae* survive within mammary epithelial cells for extended periods of time without losing viability or damaging the eukaryotic cell. Among the virulence factors, bovine S protein enhances streptococcal adherence to bovine epithelial cells. The binding of bovine complement S protein (vitronectin) to *Strep. dysgalactiae* isolates from mastitis cows and its role in adherence to bovine epithelial cells were observed. Vitronectin is a multifunctional protein that plays an important role in complement-dependent cell lysis, in the coagulation system, and in cellular adhesion.

5.2.3 *Streptococcus uberis*

*Strep. uberis* is an important environmental pathogen particularly because it is ubiquitous in the dairy environment. Identification of *Strep. uberis* is currently based on observation of the cultural and morphological characteristics, biochemical tests determination, and enzyme activity [27]. *Strep. uberis* is identified by hydrolysis of hippurate, esculin and inulin and characterized as CAMP factor-variable. On the other hand, several commercial microbial identification systems have also been used to differentiate *Strep. uberis* from other species of streptococci and enterococci isolated from bovine mastitis, and more recently, molecular tools such as PCR-based protocols have been proposed to provide an accurate identification of *Strep. uberis* isolates [28]. Among these, RFLP analysis of 16S rDNA described by [20,27] were proposed as a general method for bacterial identification. Virulence factors associated with pathogenesis are not well understood and constitute a major obstacle for the development of strategies to control this important mastitis pathogen. Several putative virulence associated genes have been described. Among them, virulence genes encoding hyaluronic acid capsule, plasminogen activator proteins such as PauA, PauB and streptokinase, lactoferrin binding proteins, SUAM, CAMP factor, a surface dehydrogenase protein GapC and Opp proteins. A recent study carried out by Reinoso et al., 2011 [28] reported the distribution of 10 virulence associated genes over various herds. The results showed that not all virulence genes were present in the strains but all detected genes were present in combination. Within this context, it will be of great interest to investigate new genes related to virulence in *Strep. uberis*.

5.3 Coagulase-negative *Staphylococcus*

Coagulase-negative *Staphylococcus* (CNS) has traditionally been considered to be a minor pathogen. However, during the last decade, they are being considered as emerging pathogens of bovine mastitis [29, 30]. CNS seems to be a particular problem especially in well managed and high producing farms, where udder infections caused by major mastitis pathogens have been successfully controlled. The highest prevalence of CNS infections is in heifers rather than in cows, and particularly around calving period.
CNS infections have been shown that can cause more severe and persistent processes due to mammary gland tissues damage. A wide variety of reservoirs of CNS have been identified. Species such as Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus simulans and Staphylococcus warneri belong to the normal bacterial flora of the teat skin, while Staphylococcus xylosus and Staphylococcus sciuri seem to come from the environment. Staphylococcus chromogenes may colonize the skin of the teat and other parts of animal’s body such as hair, vagina and teat canal. Since different species have different pathogenic effects, it is important to know which broad groups are present in a herd or area. Potential virulence factors of CNS have been investigated both by phenotypic and genotypic methods. CNS has shown to adhere to bovine mammary cells almost equally to Staph. aureus, although the invasive capacity of Staph. aureus is greater than that of CNS strains. In cell cultures, CNS isolates from mastitis showed cytotoxic activity, possibly caused by a metalloprotease. Biofilm associated proteins were found among bovine mastitis isolates, including Staph. epidermidis, Staph. chromogenes, Staphylococcus hyicus, and Staph. xylosus [31]. Among other virulence factors, exoproteins such as DNAsa, elastase, lecitinase, proteases, enterotoxins and shock toxic syndrome toxin (TSST) have been described.

5.4 Escherichia coli

Among coliforms bacteria, E. coli is the most frequently isolated from bovine milk in cows belonging to dairy farms with intensive systems of milk production. E. coli is a member of Enterobacteriaceae family. Its primary importance is its ability for lactose fermentation. Over 700 antigenic types or serotypes of E. coli have been recognised based on O, H, and K antigens. Two classes of coliforms have to be distinguished: strains that are harmless (non-pathogenic strains) and strains that cause a wide variety of typical clinical infections (pathogenic strains). Millions of non-pathogenic E. coli bacteria are living in the humans and animals normal intestinal microflora. E. coli is ubiquitous in the cow’s environment because is massively excreted with the faeces. E. coli causes infection and inflammation of the mammary gland in dairy cows mainly around parturition and during early lactation striking local and sometimes severe systemic clinical symptoms. Clinical signs vary from very severe, even fatal forms, or mild mastitis, where cows have only local signs in the udder. During mastitis, the host defense status is a factor determining the outcome of the disease. Particularly, during E. coli mastitis, the neutrophil is a key factor in the cows’ defense against IMI. However, virulence of the involved bacterial strain may also play a role. Most of the pathogenic E. coli strains posses several kinds of pathogenic mechanisms and virulence factors. A non-specific but potent factor that is important during the pathogenesis of E. coli is the endotoxin or lipopolysaccharide, which is responsible for most pathophysiological effects [31]. Major groups of E. coli virulence factors among bovine mastitis strains include, serum resistance associated factor, cytotoxic necrotizing factors (CNF1 and CNF2), aerobactin, capsule antigen K1, F17 fimbriae, TraT lipoprotein. A variety of virulence genes are present in E. coli mastitis strains. Recently, Wenz et al 2006 [32] reported that the eaeA gene ( intimin protein), CNF1 and CNF2 genes and cs31a gene (fimbrial antigen) were occassionally identified and were not associated with systemic disease severity. A high degree of genotypic variability is characteristic of E. coli strains causing clinical mastitis within and between different farms. Studies with pulse-field gel electrophoresis suggest that clinical bovine mastitis E. coli isolates may form a subset of the general environmental E. coli population. They are better able to multiply in the udder and to evade the host cellular innate immune response, and are genetically distinct from most environmental strains.

6. Preventive and control strategies against mastitis pathogens

The herd-level SCC is a result of many factors such as cow factors, management practices, and seasonal fluctuations. The pathogen distribution among the herd also influences the level of herd SCC. For instance, Staph. aureus-positive herds have higher bulk milk SCC than Staph. aureus-negative herds. To continuously monitor and interpret SCC on the herd level and to detect an increase or decrease in the trend over time would be ideal. Bonuses programs are applied in many countries based on a SCC threshold value varying from 150,000 to 250,000 cells/mL. The “Five Point Plan” proposed for the National Mastitis Council (2003) [4] summarizes several strategies for controlling herd mastitis, based upon adoption of preventive and control strategies including diagnosis, segregation of the animals and the use of improved hygiene and therapeutic protocols. Some of these practices to control mastitis in dairy herds are summarized in Table 2. Main objective of this plan is to reduce the degree and the durability of the infection and avoid new IMI. The “Five Point Plan” encloses:

6.1. Improvement of milking routine

One of the essential steps is the correct use of milking equipment and its periodic evaluation. This includes washing and disinfection of the milking machine and the production line before and after milking. Treatment of teat ends is based on cleaning and drying of teats before milking and disinfection pre and post milking (pre-dipping and teat-dipping).
6.2. Antibiotic treatment at the dry period
Intramammary dry cow therapy reduces the number of contagious infections during the dry period and environmental streptococcal infections during the early dry period. The dry cow preparations are formulated (vehicles, solvents, pH) to cause minimal tissue irritation, to avoid damaging the secretory tissue and to prevent fibrosis. An antibiotic which is active against Gram-positive organisms in low concentrations is chosen, and if combinations are used, antibiotics with bactericidal effects are preferable. Antibiotic combinations of cloxacillin, ampicillin, cephapirin, streptomycin, cephalaxin, penethamate, erythromycin, amoxicillin, penicillin, nafcillin are frequently used.

6.3. Treatment of clinical mastitis
Antibiotic treatment of subclinical mastitis during lactation is cost effective and increases the opportunities for drug residues in milk. According to the Food and Drug Administration/Center for Veterinary Medicine [34] the approved antibiotics for the treatment of bovine mastitis are: pirlimycin, methicillin, cloxacillin, amoxicillin, novobiocin, penicillin G, dihydrostreptomycin, cephapirin and erythromycin. The choice of the antimicrobial agents and the route of administration will be directed by the characteristics of the drug and regulatory issues. *Staph. aureus* is susceptible to a variety of antibiotics in vitro. However, farmers often complain that in vivo cure rates are disappointing. Several factors including the ability of bacterium to survive inside neutrophils, to form small-colony variants, to induce fibrosis and formation of micro abscesses, and to invade mammary epithelial cells are potential contributors to the poor response of chronic *Staph. aureus* infections to antimicrobial treatment. Many antimicrobial drugs used for mastitis treatment, including compounds that penetrate the mammary gland; are sulfonamides, penicillins with the exception of penethamate iodide, aminoglycosides, and early-generation cephalosporins.

6.4. Replacement of cows with chronic mastitis
Culling is used in mastitis control for the following reasons: 1) infected udders are sources of new infections in the herds and 2) susceptibility increases in those cows which have suffered mastitis. A cow which has had mastitis is usually more susceptible during subsequent lactations. A cow which has incurable mastitis at earlier lactations should be culled.

6.5. Vaccination plan
The prevention of bovine mastitis by using dry cow therapy has demonstrated that 80% of new infections caused by different pathogens during the dry period can be eliminated, but is not completely effective in *Staph. aureus* infected animals where less than 15% of infected cows respond to the antibiotic therapy. Traditional measures are able to reduce the incidence of *Staph. aureus* IMI. However, the control of the disease in many cases becomes difficult despite the application of these practices. Since the disease was first diagnosed, veterinarians and dairy herd’s producers claim for the development of an efficient vaccine against bovine mastitis. The main obstacles to achieve this objective are the large number of microorganisms (bacteria, viruses, fungi) involved in the production of the disease, an incomplete knowledge of the mammary gland’s immunity and the virulence factors of the main pathogens, the failure to maintain a high level of immunoglobulins in milk and the lack of proper vaccination schedule. Perhaps the greatest vaccine development progress has been achieved by the use of a vaccine based on mutant bacterin (*E. coli* J5 strain) currently available in several countries including U.S., France and Denmark, among others. This vaccine showed how to reduce the duration and severity of the symptoms of clinical mastitis after challenge with a virulent strain of *E. coli* [35].

Recently, many *Strep. uberis* virulence factor have been identified. Among them, vaccines made on the base of GapC, CAMP-factor, pauA, live *Strep. uberis* 0140J strain and bacterial surface extract of *Strep. uberis* were developed [36,37].

Several research works are conducted to avoid *Staph. aureus* bovine mastitis by using vaccines made on the base of wall cellular component, capsular exopolisacarides, bacterins, live attenuated bacteria and those DNA technology based [38]. Even though these vaccines increased the level of specific antibodies in blood, the levels reached in milk are very low and it is very difficult to prevent new infections and to induce both humoral and cellular immune response.
Table 2. Current practices to control mastitis in dairy herds and their effect on dairy cow health

<table>
<thead>
<tr>
<th>ATTRIBUTES</th>
<th>Use of germicidal teat dip</th>
<th>Antibiotic dry treatment</th>
<th>Clinical cows treatment</th>
<th>Proper hygiene practice in dairy herd</th>
<th>Chronic mastitis cows segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>% reduction of new infections</td>
<td>50-80</td>
<td>60-70</td>
<td>40-60</td>
<td>40</td>
<td>20-30</td>
</tr>
<tr>
<td>Frequency of use</td>
<td>50%</td>
<td>75%</td>
<td>70%</td>
<td>80%</td>
<td>No frequent</td>
</tr>
<tr>
<td>Practical difficulty</td>
<td>Easy</td>
<td>Easy</td>
<td>Easy</td>
<td>Easy</td>
<td>Easy</td>
</tr>
<tr>
<td>Acceptance by consumers</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Cost</td>
<td>Medium</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Incidence in the production</td>
<td>Medium</td>
<td>Medium</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Risk to operator</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Environment's risk</td>
<td>Pollutes water</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Risk to animal health</td>
<td>Eliminates normal flora</td>
<td>Selection of antibiotic resistance strains</td>
<td>Selection of antibiotic resistance strains</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Risk to milk products</td>
<td>Chemical residues</td>
<td>No</td>
<td>Antibiotic residues</td>
<td>No</td>
<td>Improves bulk tank milk quality</td>
</tr>
<tr>
<td>Risk to human health</td>
<td>No</td>
<td>No</td>
<td>Allergic reactions</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Prevention of new infections</td>
<td>For a short period</td>
<td>Until half-life of the antibiotic</td>
<td>No</td>
<td>Only at milking</td>
<td>Yes</td>
</tr>
</tbody>
</table>

7. Alternative approaches to control pathogen infection

Even though the above strategies contribute to decrease the occurrence of the disease, the treatment for bovine mastitis still relies heavily on the use of antibiotics, both for prophylaxis and therapy. But, while antibiotics have had a major impact on dairy cow health and consequently on milk production, their use is questioned because of traces of antibiotics in milk for human consumption. In order to reduce antibiotic residues in dairy products and in agreement with global pressure to limit their use in dairy cattle, research has been focussed on enhancing cows’ natural defence mechanisms through the development of innovative methods for the treatment and the prevention of bovine mastitis.

7.1. Probiotics: A Healthy Alternative

Probiotics are "live microorganisms, which administered in adequate amounts confer a health benefit on the host" according to World Health Organization and the Food and Agriculture Organization of the United Nations. Among lactic acid bacteria (LAB) the genera most often used as probiotics are Lactobacillus, Bifidobacterium, Lactococcus, Enterococcus, Streptococcus, Saccharomyces. They are all Gram-positive and the genera Lactobacillus and Bifidobacterium are the mainly selected because of their long history of safe use in dairy industry and their natural presence in the human intestine. Different criteria to define a strain as "potentially probiotic" include the following conditions: GRAS organism (Generally Recognized As Safe), viability during processing and storage, antagonistic effect against pathogens, tolerance to bile acid challenge and adherence to the intestinal epithelium of the host among others.

Recently the application of live bacteria as potential therapeutic against mastitis has gained interest. Probiotic bacteria can be used to control several inflammatory processes through antagonism and immunomodulation. Commensal bacteria, with a broad spectrum of antimicrobial activity, have previously been isolated from healthy bovine udders and suggested as potential anti-mastitis agents. Greene et al, 1991 [39] investigated the effects of treating bovine subclinical mastitis infections with intramammary infusions of Lactobacillus acidophilus and Lactobacillus casei (Lacto-bac, a commercial probiotic) and although an increase in SCC was observed, no increase in intramammary cure rate was detected. On the other hand lacticin 3147, a bacteriocin produced by Lactococcus lactis DPC 3147 exhibits a broad-antimicrobial spectrum against mastitis-causing pathogens in vitro. In combination with a bismuth-based teat seal,
provides protection against infection with *Strep. dysgalactiae* and *Staph. aureus* in dry period cows [40]. Recently, Klostermann et al, 2008 [41] demonstrated that a resuspended freeze-dried application of *Lc. lactis* is as effective as an antibiotic in curing clinical mastitis cases and Crispie et al, 2008 [42] showed that administration of the lactococcal culture into the mammary glands of uninfected animals elicits an immunomodulatory effect that substantially produces a recruitment of polymorphonuclear and lymphocytes to the infused quarters.

Other alternative strategies against mastitis pathogens are bacteriocins (polypeptide antibiotics) which usually only target closely related species. The advantage of bacteriocins over antibiotics in the treatments is that they could be targeted against specific pathogenic organisms. Bacteriocins identified for potential use as antimicrobials include lantibiotics, produced by LAB strains and, colicins and microcins, produced by gram-negative bacteria. Commercial products are currently available for the treatment of mastitis in dairy cattle and will be discussed in more detail. Table 3 summarises potential applications of some bacteriocins in veterinary fields.

<table>
<thead>
<tr>
<th>Bacteriocins</th>
<th>Producer/Commercial Product</th>
<th>Potencial use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin</td>
<td><em>Lactococcus lactis</em></td>
<td>Effective against a wide range of Gram positive bacteria, including mastitis pathogens. Used to treat mastitis in cattle</td>
</tr>
<tr>
<td>Nisin A</td>
<td>Origin Ambicin N®</td>
<td>Anti-bacterial activity against mastitis pathogens</td>
</tr>
<tr>
<td>Nisin A</td>
<td>Consept®</td>
<td>Germicidal activity against mastitis pathogens (<em>Staph. aureus, Strep. agalactiae, Strept. uberis, E. coli</em> and <em>Klebsiella pneumoniae</em>)</td>
</tr>
<tr>
<td>Nisin A</td>
<td>Mast out (Immucell, EEUU)</td>
<td>Intramammary infusion product containing Nisin for the treatment of subclinical mastitis in lactating dairy cows</td>
</tr>
<tr>
<td>Nisin A</td>
<td>Wipe Out (Immucell, EEUU)</td>
<td>Dairy Wipes made from a non-woven material that has been soaked through with a quick-acting and effective antimicrobial solution containing Nisin</td>
</tr>
<tr>
<td>Nisin Z</td>
<td>Nisin Z Silver-Elephant®</td>
<td>Germicidal activity against mastitis pathogens in clinical and subclinical mastitis</td>
</tr>
<tr>
<td>Lacticin 3147</td>
<td>DPC3147 isolated from a Irish kefir grain</td>
<td>Treat mastitis in cattle. Germicidal activity of bismuth and Lacticin 3147-based teat seal in dry cows challenged with <em>Staph. aureus</em></td>
</tr>
<tr>
<td>Lacticin 3147</td>
<td>DPC3251 isolated from a Irish kefir grain</td>
<td>Natural teat dip using a fermentate containing the live bacterium <em>Lc. lactis</em> DPC 3251</td>
</tr>
<tr>
<td>Lacticin NK34</td>
<td>Partially purified form of <em>lacticin NK34</em></td>
<td><em>In vivo</em> preventive and therapeutic effects on mouse infection model using mastitis pathogens</td>
</tr>
<tr>
<td>Mutacin B-Ny266</td>
<td><em>Streptococcus mutans</em></td>
<td>Bacterial infection caused by methicillin-resistant staphylococci</td>
</tr>
<tr>
<td>Lysostaphin</td>
<td>Recombinant lysostaphin</td>
<td>Therapeutic effect against <em>Staph. aureus</em> by intramammary application</td>
</tr>
</tbody>
</table>

Nisin was first identified attempted to find a practical application for the treatment of bovine mastitis. The interest for the use of nisin as a therapeutic agent was renewed in 1989, when Broadbent et al. showed its inhibitory effect to several gram-positive pathogens causing mastitis. The practical use of nisin was investigated by Sears et al, 1992 [43] in combination with lysostaphin by administration of intramammary infusions. In addition, promising results (cure rates of 66% for *Staph. aureus*, 95% for *Strep. agalactiae* and 100% for *Strep. uberis*) were obtained. Two nisins have been evaluated for prevention or treatment of bovine mastitis: the nisin A, origin Ambicin N® with germicidal activity against mastitis pathogens (*Staph. aureus, Strep. agalactiae, Strept. uberis, Klebsiella pneumoniae* and *E. coli*) and the nisin Z, origin nisin Z Silver-Elephant® with Germicidal activity against mastitis pathogens. Some of commercially available products containing nisin for bovine mastitis are Consept®, Mast out (Immucell, EEUU) and Wipe Out (Immucell, EEUU) all of them elaborated with nisin A. Recently, it has been demonstrated that lacticin 3147, a new bacteriocin produced by *Lactococcus lactis* ssp. *lactis* DPC3147 is also effective against a wide range of gram-positive bacteria, including many mastitis causing pathogens. Lacticin NK34 (partially purified form of lacticin NK34) has in vivo preventive and therapeutic effects on mouse infection model using mastitis pathogens.

Our research group focused on the prevention of bovine mastitis by promoting the colonization of mammary gland during dry cow period with indigenous probiotic strains. Based on the host specificity, one hundred and two LAB
strains from foremilk, stripping milk and teat canal scrapped were isolated. The production of antagonistic substances and bacterial-surface properties, such as hydrophobicity and self-aggregation were evaluated. Safety aspects as virulence traits and antibiotic resistance were also taken into account [44]. Finally, three strains: Lc. lactis subsp. lactis CRL1655, Lactobacillus perolens CRL1724 and Enterococcus hiriae CRL1835 were selected. Among lactobacilli probiotic properties, inhibition of mastitis pathogens by organic acid production and adhesion to teat canal cells were the most relevant. E. hiriae CRL1835 is a bacteriocinogenic strain and. Lc lactis subsp. lactis CRL1655 is a producer of nisin Z [44]. Furthers studies will be conducted to develop non antibiotic formulations for the treatment and prevention of bovine mastitis.

7.2. Quorum sensing

Quorum sensing (QS) is the regulation of gene expression in response to fluctuations in cell-population density. Bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. The detection of a minimal threshold stimulatory concentration of an autoinducer leads to an alteration in gene expression. In the natural environment, there are many different bacteria living together which use different signalling molecules. Today, several quorum sensing systems are intensively studied in different pathogenic bacteria [45]. Staph. aureus, an important mastitis pathogen, regulates its virulence factors in a coordinated manner, by the aid of well-characterized global regulatory elements, such as sar locus and two-component regulatory systems agr and sae locus. Staph. aureus initially infects by binding to epithelial cells using a series of extra-cellular binding proteins [46]. During the late initial stage of growth, self-generated signal molecules secreted by the bacteria serve as quorum sensing agents. When they reach certain concentration their interaction with specific receptors activates the transcriptional signalling molecules. Today, several quorum sensing systems are intensively studied in different pathogenic bacteria [45]. Staph. aureus, an important mastitis pathogen, regulates its virulence factors in a coordinated manner, by the aid of well-characterized global regulatory elements, such as sar locus and two-component regulatory systems agr and sae locus. Staph. aureus initially infects by binding to epithelial cells using a series of extra-cellular binding proteins [46]. During the late initial stage of growth, self-generated signal molecules secreted by the bacteria serve as quorum sensing agents. When they reach certain concentration their interaction with specific receptors activates the transcriptional

Recent studies in Streptococcus spp have shown the presence of QS genes such as luxS and comEA, EC, X (competence) responsible of group behaviour. Quorum sensing regulates diverse physiological processes including formation of surface-associated communities called biofilms, where bacteria are embedded in an extracellular polymeric matrix, and are protected against environmental stresses, antimicrobial treatment, and the host immune system. As Staphylococcus spp and Streptococcus spp. have ability to grow in infected tissues on biofilms developing an innate resistance to almost all therapeutic agents, the difficulties of treating recurrent infections might be related to this pathogen ability [47]. Understanding the virulence factors that influence the ability of major mastitis pathogens to colonize and maintain infections will allow finding effective and appropriate treatment protocols that could greatly decrease the impact of mastitis to the dairy industry. As QS is an important process involved in bacterial survival and infections, recent research has focused on the development of therapeutic agents which prevent or manage bacterial pathogenesis by inhibiting bacterial QS. Inhibition of quorum sensing offers an alternative to antibiotic mediated bactericidal or bacteriostatic approach and reduces the risk for resistance development.

7.3. Transgenic dairy cows

The ability to produce transgenic dairy cows opens the door to countless new strategies which aim at enhancing the efficiency of dairy production. The goals of these projects include increasing milk production efficiency, increasing milk protein content, and animal health through enhanced disease resistance. Current therapies for mastitis rely heavily on the use of β-lactam antibiotics such as penicillins and cephalosporins. A transgenic approach to enhance mastitis resistance would enable mammary epithelial cells to produce antibacterial enzymes that, in contrast to β-lactam antibiotics, would be degraded along with other milk proteins during the digestion process and would not represent a health risk to the consumer. Antibacterial proteins, such as lysostaphin, are not typically used as injection or oral therapeutics because of immune-mediated or digestive destruction of their activity. In contrast, the immune system of transgenic animals will not consider the transgenic protein as being foreign. The use of transgenesis to direct expression of a foreign protein into milk to prevent mastitis was first reported in a mouse model in 1987 by Gordon et al. Shortly thereafter, it was proposed that mammary production of lysozyme II or bacterial lysostaphin would be an effective means to enhance mastitis resistance. Both of these proteins have considerable antistaphylococcal activity. However, the initial applications of the technology were the generation of transgenic mice producing human lysozyme or human lactoferrin in milk. Clearly, there are potential risks in the production of foreign proteins in milk of dairy cows that could positively or negatively affect its antibacterial or functional characteristics. Transgenic technology has resulted in the production of mice and cows that secrete human lactoferrin into their milk. The transfer of a gene encoding human lactoferrin to the bovine mammary gland would, in theory, represent a good candidate approach. Results showed that lactoferrin transgenesis model did not provide protection against E. coli mastitis in dairy cows, but reduced the severity of the inflammatory reaction, which could be seen in the systemic signs and in the serum cortisol and haptoglobin concentrations. Thus, over expression of bovine lactoferrin does not seem to
be a candidate for enhancing mastitis resistance, although its gene regulatory region may be suitable to direct expression of new antibacterial proteins [48].

Another foreign protein, lysozyme, also altered the physical and functional properties of the milk. Unfortunately, human lysozyme has very limited potency against Staph. aureus and is ineffective against E. coli and Strep. uberis isolates obtained from mastitis milk. However, it seems unlikely that lysostaphin would be among the best candidate proteins for genetic engineering to enhance mastitis resistance in dairy cows if this approach were taken by the dairy industry in the future. Both, natural and bovine derived lysostaphin were equally effective in three different mouse infection models. Cows carrying a lysostaphin gene showed to be more resistant to Staph. aureus induced IMI and to CNS mastitis than normal cows. They eliminated Staph. chromogenes from the mammary gland faster and had a milder clinical and local inflammatory response [49].

Another transgenic approach to enhance cow disease resistance involves the production of pathogen-specific antibodies into milk. However, the increasing resistance of dairy cattle to infections by genetic engineering is a delicate issue with many ethical considerations. For animal welfare, one of the most important goals should be to prevent the most common diseases among production animals. Genetic engineering could be one means to control mastitis, but further studies are needed.

References