Alternative approach to mastitis management – How to prevent and control mastitis without antibiotics?

Abordagem alternativa para o manejo da mastite – Como prevenir e controlar a mastite sem antibióticos?

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Abstract

Mastitis affects a high proportion of dairy cows throughout the world and is one of the greater problems faced by the dairy industry today. The disease is still a major cause of economic loss on a dairy farm. Mastitis poses not only negative consequences for the dairy farmer but also for the dairy industry as a number of issues threaten the reputation of milk as a healthy product from healthy animals. The use of antimicrobials is one of those concerns and threats. Antimicrobial usage on dairy farms is most often related to udder health as most medicines are used in prevention and control of mastitis. Antimicrobials remain vital for treatment of bacterial infections in dairy cattle, but in light of the upcoming debate instigated by the potential link between the use of antimicrobial products in animal husbandry and the development of antimicrobial resistance in both animal and human pathogens, there is an urgent need for innovation and alternatives to antibiotic therapy for mastitis treatment and control. Alternative approaches include vaccination, probiotics or beneficial microorganisms and inhibitory substances, immunomodulation, bacteriophages, homeopathy, and plant-derived inhibitory substances, yet only when scientifically-proven evidence is available indicating these alternatives are effective.

Keywords: Alternative. Mastitis. Treatment. Vaccination.

Resumo

A mastite ocorre em uma alta proporção de vacas leiteiras em todo o mundo e é um dos grandes problemas que atinge a indústria leiteira. A doença ainda é uma das maiores causas de perdas econômicas das granjas leiteiras. A mastite não tem apenas consequências negativas para os fazendeiros, mas atinge também a indústria leiteira, particularmente pela expectativa de que o leite deve ser um produto saudável produzido por animais saudáveis. O uso de antimicrobianos é uma grande preocupação. O emprego de antimicrobianos nas granjas leiteiras está frequentemente relacionado com a saúde do úbere uma vez que a maioria dos medicamentos são usados na prevenção e controle da mastite. Os antimicrobianos ainda são vitais para o tratamento das infecções bacterianas do gado leiteiro, mas na atualidade há um grande questionamento para a ligação potencial entre o uso de produtos antimicrobianos no manejo animal e o desenvolvimento de resistência antimicrobiana tanto de patógenos de animais como de humanos, há portanto a necessidade urgente da introdução de alternativas para o tratamento e controle da mastite com antibióticos. As possibilidades incluem vacinação, probióticos ou microorganismos benéficos e substâncias inibidoras, imunomodulação, bacteriófagos, homeopatia, fitoterápicos inibidores. Contudo todos esses procedimentos necessitam ser cientificamente provadas para demonstrar que são alternativas realmente efetivos.

Palavras-chave: Alternativas. Mastite. Tratamento. Vacinação.

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Introduction

Mastitis affects a high proportion of dairy cows throughout the world and is one of the greater problems faced by the dairy industry today (BRADLEY, 2002). The disease is still a major cause of economic loss on any dairy farm. The main financial losses are due to the expense of antibiotic treatment, along with the associated costs of decreased milk production and decreased fertility or, in cases where antibiotic treatment is ineffective or unwanted, culling or death (HALASA et al., 2007). Additionally, farmers supplying milk with a high bulk milk somatic cell count may be losing out on bonus payments and may also incur penalties. Treating infected cows also increases labor (e.g. time and effort) for the farmer and causes stress, consequences that should not be underestimated as both are perceived as the two most annoying aspects of mastitis by farmers (JANSEN et al., 2009).

Mastitis has consequences beyond the dairy farm as well as a number of issues related to the disease that threaten the positive reputation of milk as a healthy, high-quality and nutritious product produced by healthy and contented animals. First, udder health accounts for the largest proportion of antimicrobial drug use on a dairy farm (STEVENS et al., 2016). Second, herds struggling with udder health problems have a higher risk of delivering milk containing antibiotic residues because of their increased (and often injudicious) antibiotic usage (RUEGG; TABONE, 2000). Third, cows suffering from even mild cases of clinical mastitis are in pain, which obviously has its implications for animal welfare. In fact, because

of worries about potential antimicrobial residues in milk, emerging antimicrobial resistance, issues with milk quality and animal welfare, mastitis has become a valid concern to consumers and society, a development everyone involved in the dairy industry should be aware of.

Ongoing political debate and public concerns about the emergence of antimicrobial resistance and drug residues in milk emphasize the need for innovation and alternatives to antibiotic therapy. The focus of this manuscript is to describe and discuss mastitis vaccines and alternatives that can potentially be used in the prevention and control of bovine mastitis with a focus on the potential relevance of medium-chain fatty acids (MCFA).

Vaccines

What is vaccination?

The immune response of the mammary gland consists of an innate and adaptive immune response arm. The adaptive immune response arm specifically responds to a specific antigen. The innate immune system recognizes conserved microbial molecules via their receptors (RAINARD; RIOLLET, 2006). Besides the macrophages, neutrophils, dendritic cells, and natural killer cells, it also includes physical barriers such as the teat sphincter, and chemical barriers such as the keratin of the teat canal and lactoferrin (SCHUKKEN et al., 2011). Interestingly, milk in healthy cows has a resident population of immune cells. The population is usually dominated by macrophages but also contains neutrophils and lymphocytes (SORDILLO, 2005). Lymphocytes are divided in two groups: T- and B-lymphocytes. The T-lymphocytes are further categorized into $\alpha\beta$ T-lymphocytes, including CD4$^+$ T-helper lymphocytes and CD8$^+$ T-cytotoxic lymphocytes, and $\gamma\delta$ T-lymphocytes. In the lactating mammary gland, $\alpha\beta$ T-lymphocytes dominate and primarily express the CD8$^+$ phenotype (SHAFER-WEAVER; SORDILLO, 1996). Those activated cytotoxic CD8$^+$ T-lymphocytes kill are

assumed to kill host cells infected with a pathogen, as detected by antigens expressed on the surface of the infected cells. On the contrary, helper CD4^{+} T-lymphocytes have a more indirect but equally important effect on the invaded mastitis-causing pathogens. A CD4^{+} T-helper lymphocyte that matures usually develops into one of four types of T-helper cells. Stimulation of the latter mature T-helper cells results in the expression of a large variety of cytokines that can direct the immune response towards a B-cell-mediated (Th2 response), a pro-inflammatory cytotoxic T-cell-mediated response (Th1 response) or a neutrophil-mediated response (Th17 response) or in the expression of cytokines that counter-regulate the response (Treg response). It is well-known that when bacteria invade the mammary gland and cause an intramammary infection, large numbers of leukocytes migrate into the udder, resulting in the establishment of a host response against the pathogen. Although the increasing body of research that focuses on the immunological response of the mammary gland, the specific leukocyte populations mediating this immune response are currently not yet well defined.

Perhaps the most important consequence of an adaptive immune response is the establishment of a state of immunological memory. Immunological memory is the ability of the immune system to respond more rapidly and effectively to pathogens that have been previously encountered, which happens in case of vaccination (JANEWAY et al., 2001). A distinguishing feature of immunological memory is the irreversible genetic change of B-lymphocytes from IgM producers to producers of other antibody isotypes, including IgG1 and IgG2 (BURTON et al., 2001). In several species including the bovine, an immune response with a higher production of IgG2 antibody has been recognized as part of a Th1 pro-inflammatory response, while a response with a higher production of IgG1 is part of a Th2 or anti-inflammatory response. Because IgG2 is an important opsonizing antibody aiding in neutrophil phagocytosis of bacteria, and IgG2 has the ability to readily fix complement, it has been suggested that an IgG2 Th1-type response might be beneficial against bovine mastitis (THOMPSON-CRISPIE et al., 2013).

Each vaccine contains a killed or weakened form of the specific organism (e.g. *Staphylococcus aureus*, *Escherichia coli*, …) that causes a disease such as mastitis. Even though the organism in the vaccine has been altered so that it will not cause sickness, the part of the organism that stimulates the immune system to respond to ("antigen") is still present. Mastitis vaccines against *E. coli* primarily contain the inactive J5 *E. coli* strain, resulting in the formation of antibodies against the uniform component lipopolysaccharide (LPS) in the outer membrane of all Gram-negative bacteria causing the severe symptoms associated with hyperacute *E. coli* mastitis cases. Mastitis vaccines against *S. aureus* consist of either bacterins (= killed or avirulent/weakened *S. aureus* strains) or exopolysaccharides (= sugar residues secreted by bacteria in the surrounding environment). One of those exopolysaccharides is poly-N-acetylglucosamine (PNAG), a surface polymer produced by a variety of bacterial species, including *S. aureus* and *Staphylococcus epidermidis,* and facilitating bacterial cell-to-cell contact in biofilms (PÉREZ et al., 2009).

What is vaccine efficacy?

Efficacy of a vaccine refers to the reduction in disease measured in a carefully monitored, randomized controlled clinical trial conducted in a homogeneous population according to a defined protocol. In essence, the vaccine efficacy is determined by four parameters. The first parameter is the impact of vaccinations on the rate of new infections. This represents the classic vaccine effect, whereby the vaccine reduces the susceptibility of non-infected individuals such that no or fewer new infections take place. The second parameter is the impact of vaccination on the infectiousness of an infected individual. The vaccine may reduce the amount of shedding of infected but vaccinated individuals compared to nonvaccinated infectious individuals. For

example, considering *S. aureus* is a mammary pathogen that may be transmitted cow-to-cow, a reduction in the infectiousness of a vaccinated individual would be valuable. The third parameter is the impact of vaccination on the cure of existing infections. Vaccinations may result in a shorter duration of infection. The duration is essentially the inverse of cure, so a higher cure will result in a shorter duration. The fourth and final parameter of vaccine efficacy is the reduction in progression of infection from subclinical to clinical mastitis. As clinical mastitis results in milk discard, treatment and animal sickness, a reduction in progression of infection would be of value to the dairy industry.

Vaccines against mastitis

Commercial mastitis vaccines are currently available for immunization against mastitis caused by *S. aureus* and *E. coli*. In the U.S., there are two *S. aureus* bacterins available. The vaccines are marketed as Somato-Staph® and Lysigin® and are labeled as somatic antigen containing phage types I, II, III, IV and miscellaneous groups of *S. aureus*. There are also three coliform mastitis vaccines available. Two are identical and marketed as J-5 Bacterin® (Hygieia Biological Laboratories, Woodland, California, USA), ENVIRACOR J-5 (Zoetis, Parsippany, New Jersey, US). A separate bacterin-toxoid (J-Vac®, Merial Inc., Duluth, Georgia, USA) is also available. The 4th Gram-negative mastitis vaccine contains re-17 mutant *Salmonella typhimurium* bacterin toxoid. On the European market, there is only one labeled vaccine against mastitis available (Startvac®, Hipra S.A., Girona, Spain). The vaccine contains inactivated *E. coli* (J5), inactivated *S. aureus* (CP8) SP 140 strain expressing Slime Associated Antigenic Complex (SAAC) and adjuvant. The vaccine has a label claim for reducing the incidence of subclinical mastitis and incidence and severity of clinical signs of clinical mastitis caused by coliform, *S. aureus*, and non-*aureus* staphylococci (NAS).

At this time, there are no commercial vaccines available that have a proven efficacy against streptococcal mastitis. Still, the increased frequency of mastitis caused by environmental streptococci such as *Streptococcus uberis* has resulted in a number of unsuccessful attempts to produce vaccines against this pathogen. The wide strain variety together with the strain-specific protection in particular slows the development of vaccines specifically oriented against mastitis-causing streptococci (DENIS et al., 2009).

Vaccine efficacy

Vaccine efficacy against S. aureus

A number of studies have been published on the efficacy of vaccination against *S. aureus* mastitis. In one of the first field trials including 30 heifers, a 3-fold decreased risk of *S. aureus* infections was observed in heifers vaccinated with an experimental vaccine formulation based on inactivated *S. aureus* cells and exopolysaccharides at 5w and 1w before calving. Still, no effects on the somatic cell count were found (GIRAUDO et al., 1997). Nickerson et al. (1999) vaccinated heifers with the commercially available Lysigin® (Boehringer Ingelheim Vetmedica Inc., St. Joseph, Missouri, USA) at six months of age followed by a booster dose two weeks later and subsequent vaccinations every six months until calving. Vaccinated heifers had a 45% reduction in both new *S. aureus* intramammary infections during pregnancy and new *S. aureus* intramammary infections at calving relative to controls. Middleton et al. (2006) compared the efficacy of the same commercially available vaccine with two experimental formulations and nonvaccinated controls in primiparous heifers as well. Heifers were vaccinated twice, 28 days apart in late gestation with either a 3-isolate experimental bacterin (Group I; n = 11), a 5-isolate experimental bacterin (Group II; n = 11), or the commercially available Lysigin® (Boehringer Ingelheim Vetmedica Inc., St. Joseph, Missouri, USA) (Group III; n = 14). Group IV consisted of 11 nonvaccinated control animals. All

groups (vaccinated animals and nonvaccinated ones) were challenged with a heterologous strain of *S. aureus* by intramammary infusion on days 6-8 of lactation in a single infection-free mammary quarter. All animals became infected with *S. aureus* after challenge. In contrast to the results obtained by Nickerson et al. (1999), no differences in *S. aureus* clearance rates were observed between groups. Animals vaccinated with Lysigin® (Boehringer Ingelheim Vetmedica Inc., St. Joseph, Missouri, USA) had a lower mean duration of clinical mastitis and showed less severe symptoms than nonvaccinated control animals. There was no evidence that any of the vaccinated groups had a lower somatic cell count than nonvaccinated control animals, and no evidence that vaccinates had a greater milk yield than controls post-challenge. Still, significantly higher concentrations of milk antibodies against *S. aureus* were observed in the Lysigin® (Boehringer Ingelheim Vetmedica Inc., St. Joseph, Missouri, USA) vaccinated animals than in the nonvaccinated control animals (LUBY et al., 2007). Similar results were obtained in studies that used avirulent (PELLIGRINO et al., 2008) or inactivated *S. aureus* (TENHAGEN et al., 2001) vaccine formulations or vaccines including insoluble bacterial fragments of two field *S. aureus* field strains and secreted antigens of a third field strain (LEITNER et al., 2013). In the latter study, however, the milk somatic cell count was almost 50% lower in vaccinated animals compared to nonvaccinated animals. Also, the average daily milk yield was 0.5 kg/day higher in vaccinated animals than in nonvaccinated control cows.

In a recently published study (SCHUKKEN et al., 2014), the efficacy of the novel commercially available vaccine Startvac® (Hipra S.A., Girona, Spain) was evaluated on two commercial dairy farms. In total, 1,156 lactations from 809 cows were enrolled. During the first phase of the trial, all cows that were due to calve were vaccinated until approximately 50% of the cows in the milking herd was vaccinated. At that point, when 50% vaccination coverage was reached, cows that were due to calve were randomly assigned to be vaccinated or left as negative controls. Vaccination of cows was done according to label, with a total of three vaccine doses, with the first injection at 45 days before the expected parturition date, the second injection 35 days thereafter (corresponding to 10 days before the expected parturition date), and the third injection 62 days after the second injection (equivalent to 52 days post-parturition). The vaccine efficacy for the rate of new infections was relatively low and depending on the parity. For heifers, the vaccine efficacy for transmission was 25%, indicating that the new infection rate was 25% lower in vaccinated heifers than in nonvaccinated heifers. However, the vaccine efficacy for transmission was still positive but nonsignificant for animals in second lactation (+ 16%) and even negative (- 30%) for animals in third or higher lactation. The latter suggests an even higher transmission and thus higher new infection rate in vaccinated animals than in nonvaccinated animals. The vaccine efficacy for cure was moderate with a value of 41%, meaning that the cure rate of *S. aureus* infections in vaccinated animals was 41% higher than in nonvaccinated animals. The latter resulted in a shorter duration of *S. aureus* infections. Still, a significant difference in vaccine efficacy for cure was present between the two herds. Combining the transmission parameter and cure rate parameter into the overall basic reproduction ratio, R_0, resulted in a value of 0.89 for vaccinated animals and a value of 1.72 for control cows. For NAS, the R_0 value for vaccinated animals was 0.91 and 1.40 for control animals. For both NAS and *S. aureus*, vaccination resulted in moving the basic reproduction ratio from above to below the threshold of one. The overall vaccine efficacy was estimated at 45%.

In an experimental clinical trial including eight clinically healthy heifers and cows, four animals were vaccinated with Startvac® (Hipra S.A., Girona, Spain) at 45 days and 10 days before calving (PIEPERS et al., 2017). At 15 days after calving, two contralateral quarters of each of the eight animals were challenged with 2 x 10⁹ cfu/ml of the formaldehyde-killed *S. aureus*

strain. The other two quarters were infused with a phosphate buffered solution and served as control quarters. Blood samples were collected at 45 days before calving, 10 days before calving and 15 days after calving just before inoculation. Quarter milk samples were collected at two hours before inoculation and four hours, 12 hours, 24 hours and 48 hours after inoculation. All animals remained clinically healthy during the trial period. Twelve hours post-challenge, all quarters inoculated with the killed *S. aureus* strain were PCR-positive for *S. aureus*. Still, quarters from vaccinated animals had odds up to 20 times higher to be free of *S. aureus* two days post-challenge than nonvaccinated animals. In the nonvaccinated group, the average daily milk yield tended to decrease from 34.2 kg/day at 15 days after calving to 30.5 kg per day at 16 days after calving. In the vaccinated group, no significant difference in the average daily milk yield was observed over time. On the contrary, the average daily milk yield even increased from 33.7 kg per day at 15 days after calving to 36.5 kg per day at 16 days after calving. Interestingly, in the nonvaccinated animals, *S. aureus*-challenged quarters had a significantly higher somatic cell count than the control quarters whereas in the vaccinated group, the quarter milk somatic cell count was not significantly different from the quarter milk somatic cell count in the control quarters. The milk neutrophil viability was significantly higher in the *S. aureus*-challenged quarters than in the control quarters though was not influenced by the vaccination status of the animal. The blood concentration of both SAAC- and J5-specific antibodies increased between 45 days before the expected calving date and 15 days after calving in the vaccinated animals only. Consequently, vaccinated animals had significantly higher SAAC- and J5-specific antibody blood concentrations at 15 days after calving (pre-challenge) than the nonvaccinated animals. The milk concentration of SAAC-specific antibodies was significantly higher in vaccinated animals than in nonvaccinated animals, independently from the inoculation status of the quarters and the time of

sampling. The latter findings support the results of Prenafeta et al. (2010). The higher anti-SAAC blood concentrations suggest a more pronounced humoral specific immune response which might explain the shorter duration of the *S. aureus* infections as was found in the study of Schukken et al. (2014). Also, the higher anti-SAAC concentrations in milk might potentially trigger the opsonization of the inoculated *S. aureus* bacteria and partly explain why vaccinated animals suffered from a less severe inflammatory reaction than the nonvaccinated animals. In this regard, Camussone et al. (2014) immunized 17 pregnant heifers with one of two vaccine formulations composed of either *S. aureus* whole or lysed cells formulated with Immune Stimulation Complex Matrix (ISCOM matrix). Both immunogens induced a strong humoral immune response in blood and milk characterized by a substantial increase in antibody concentration. Neutrophil phagocytosis was much more pronounced in the vaccinated animals than in the nonvaccinated ones, suggesting an increased opsonization of the *S. aureus* bacteria in case of increased antibody concentrations. The milk concentration of J5-specific antibodies observed in vaccinated animals was not significantly different at 15 or at 17 days after calving. Vaccination with Startvac® (Hipra S.A., Girona, Spain) evoked a shift primarily toward the IgG1 antibody subtype (Th2-response). The IgG1 antibodies are well-known for their strong opsonizing capacity facilitating the recognition of the bacteria by the immune cells as well as their phagocytosis and killing by those immune cells. Regarding the response against *E. coli*, a weak though significant increase in J5-specific IgG2 concentrations (Th1-response) was observed.

Vaccine efficacy against E. coli

Despite the development of several vaccines against *E. coli* mastitis in the 1980s, based on the J5 *E. coli* mutant, such vaccines to date, although demonstrating an ability to reduce the severity of clinical signs and duration of infection, have failed to

demonstrate a reduction in the rate of new intramammary infections (HOGAN et al., 1992; WILSON et al., 2007a). Investigation of the use of J5 coliform vaccines has also demonstrated a positive effect on production in a way that vaccinated cows have been shown to recover milk yield after a clinical case more quickly than unvaccinated cows (WILSON et al., 2007b, 2008, 2009). The latter research group also provided evidence that increased production of both J5-specific IgG1 and IgG2 antibodies are relevant mechanisms of J5 vaccine protection, including the production of a higher proportion of IgG2 in vaccinated animals than in non-vaccinates which points towards a Th1-response. A significant constraint on the use of *E. coli* mastitis vaccines has been the relatively onerous vaccination regimens necessary to achieve the desired level of efficacy. These often necessitate vaccination both before and after calving. This has led to the development of more practical, farmer-friendly approaches to vaccination when J5 core antigen vaccines have been deployed in the field, such as a rolling schedule of vaccination of all cows in the herd on a quarterly basis. In a recent study, the efficacy of a multivalent mastitis vaccine in the control of bovine mastitis under UK field conditions using both the label regimen and a schedule of quarterly vaccination (BRADLEY et al., 2015). 3,130 cows were recruited between September 2010 and January 2012 to develop this study, from seven farms in the southwestern United Kingdom, and were randomly allocated, within farm, to one of three groups. The first group received the vaccine (Startvac®, Hipra S.A., Girona, Spain) following the label regimen, the second group was vaccinated every 90 d following an initial vaccination course, and the third group was left unvaccinated to act as controls. Vaccine efficacy was assessed in the first 120 d of lactation. No strict criteria were applied pertaining to bulk milk somatic cell count or clinical mastitis incidence. All cows and heifers approaching their first calving were eligible for recruitment to the study, contingent on being in good health, having four functional quarters, teats free of significant teat lesions, and an estimated calving date to allow vaccination at predicted times before calving. Data were available for analysis from 1,696 lactations in 1,549 cows. In total, 779 cases of clinical mastitis occurred in the three study groups, and no significant differences were observed in the incidence or prevalence of clinical or subclinical mastitis between any of the three groups. Mastitis vaccination following the label regimen was associated with a significant reduction in the severity of clinical cases. Cows in this group were at significantly decreased odds of developing moderate to severe clinical mastitis (i.e. more than just visual changes of the milk). When the analysis was extended with the effect of vaccination on culling to encompass the first 305 d of lactation, this revealed a significant difference in the total number of cows culled between the treatment groups, with 26.2, 18.3, and 24.2% of cows being culled in the unvaccinated, label, and rolling groups, respectively. Significantly fewer cows were culled from the label group than from either the unvaccinated or the rolling groups. Analysis of milk production data demonstrated that, on average, cows on the label regimen produced a higher volume of milk (231 L) and more milk solids (12.36 kg) than unvaccinated cows in the first 120 d of lactation.

Other alternatives

Bacteriophages

Bacteriophage therapy can be an alternative to antibiotics in the fight against intramammary infections. Bacteriophages are viruses able to infect and kill bacteria (CHIBANI-CHENNOUFI et al., 2004). Phages were demonstrated to yield potential as new antimicrobial agents for veterinary applications. One of the well-known bacteriophages is *Phage K*, an anti-staphylococcus phage, with lytic and antimicrobial action. *Phage K* has been used as prophylactic measure against intramammary infections caused by *S. aureus*. One of the main disadvantages of the latter phage is, however, that it is inhibited by milk and udder

secretions (O'FLAHERTY et al., 2005). Other virulent phages with an anti-staphylococcal activity are *MSA6* (KWIATEK et al., 2012) and *CHAP_k* (FENTON et al., 2013). *CHAP_k* showed effectiveness against biofilms either by preventing biofilm formation or by disrupting established biofilms of staphylococcal strains associated with bovine mastitis.

Nanoparticles

Nanoparticles can be used as delivery vehicles for antimicrobial agents. Several researchers demonstrated that nanoparticles have potential as an alternative for the treatment of bovine mastitis. As an example, it has been demonstrated that silver nanoparticles inhibited *S. aureus* bacteria isolated from milk samples of cows with subclinical mastitis (DEHKORDI et al., 2011). The synergistic effect of silver nanoparticles and antibiotics has been evaluated as well. A combination of the antimicrobial erythromycin with silver nanoparticles was found to be successful against *S. aureus* in *in vitro* work of Kazemi et al. (2014). Other nanoparticles showing potential as alternative treatment for *S. aureus* intramammary infections are the tilmicosin-solid lipid nanoparticles (WANG et al., 2012) as well the amoxicillin nanoparticles that were demonstrated to be biologically active against *S. aureus*, *E. coli* and *Streptococcus agalactiae* (XUEFENG et al., 2009). Cardozo et al. (2014) revealed that nitric oxide-nanoparticles were able to inhibit *S. aureus*, and in general could be used in the treatment and prevention of bovine mastitis.

Cytokines

The emergence of antibiotic resistance has prompted the investigation of the immunotherapeutic use of recombinant cytokines in the treatment of bovine mastitis. Cytokines are small proteins that have an important role in cell signaling. Some cytokines (such as IL-2, IFN-γ, and TNF-α) are responsible for stimulating innate and acquired immunity in the mammary gland. Unfortunately, the enhanced immunity was not adequate to successfully treat bovine mastitis. When combined with antibiotics, however, an additive effect was found, suggesting a synergistic effect and potential applications of cytokines as adjuvant in mastitis therapy (ALLUWAIMI, 2004). A more recent cytokine-derived molecule that has been investigated and is now commercially available is pegbovigrastim. Pegbovigrastim is the pegylated form of the cytokine bovine granulocyte colony stimulation factor. The cytokine bovine granulocyte colony stimulating factors is a cytokine that regulates the growth and differentiation of the precursor cell in the bone marrow committed to forming neutrophils. PEGylation increases the half-life of the protein messenger molecule from hours to days. In a very recent study of Canning et al. (2017), pegbovigrastim-treated animals exhibited a 35% decrease in the incidence of clinical mastitis relative to controls during the first 30 days of lactation (Figure 1).

Homeopathy

Homeopathy was developed by the German physician Samuel Hahneman (1810) and is commonly referred to as a "complementary or alternative medicine". Producers see advantages in the use of homeopathic remedies to avoid milk withdrawal periods (BOLDYREVA, 2003), milk residues (ENBERGS, 1998), or development of antibiotic resistance (SMITH, 2002). Still, scientific evidence for the effectiveness of homeopathy is lacking. Recent studies in veterinary medicine focused on the difference of the cure risk between therapy and control groups. While some studies found no effects of homeopathic therapy for mastitis (HEKTOEN, 2004; WERNER et al., 2010), other studies showed negative results in the homeopathic group (GARBE et al., 2000; VARSHNEY; NARESH, 2005), and still others showed positive results (DAY, 1986; MERCK et al., 1989). In a meta-analysis of Mathie and Clausen (2015), no significant differences were found between homeopathic and conventional therapies in veterinary medicine. Interestingly, many of the studies included

in the meta-analysis lacked a proper scientific approach. Only 28% of the studies using homeopathy in veterinary medicine were randomized and placebo-controlled (CLAUSEN et al., 2013). In a very recently executed triple-blind, randomized controlled trial, the specific treatment effects of homeopathic mastitis in dairy cows was investigated. However, no significant differences were observed in time to recovery, somatic cell count, risk of clinical cure within 14 days after disease occurrence, mastitis recurrence risk, or culling risk. The results indicated no additional effect of homeopathic treatment compared with placebo (EBERT et al., 2017). The findings are in accordance with those obtained in a meta-analysis study conducted by Dufour and co-workers who concluded that homeopathic treatments are not efficient for management of clinical mastitis (DUFOUR et al., 2017).
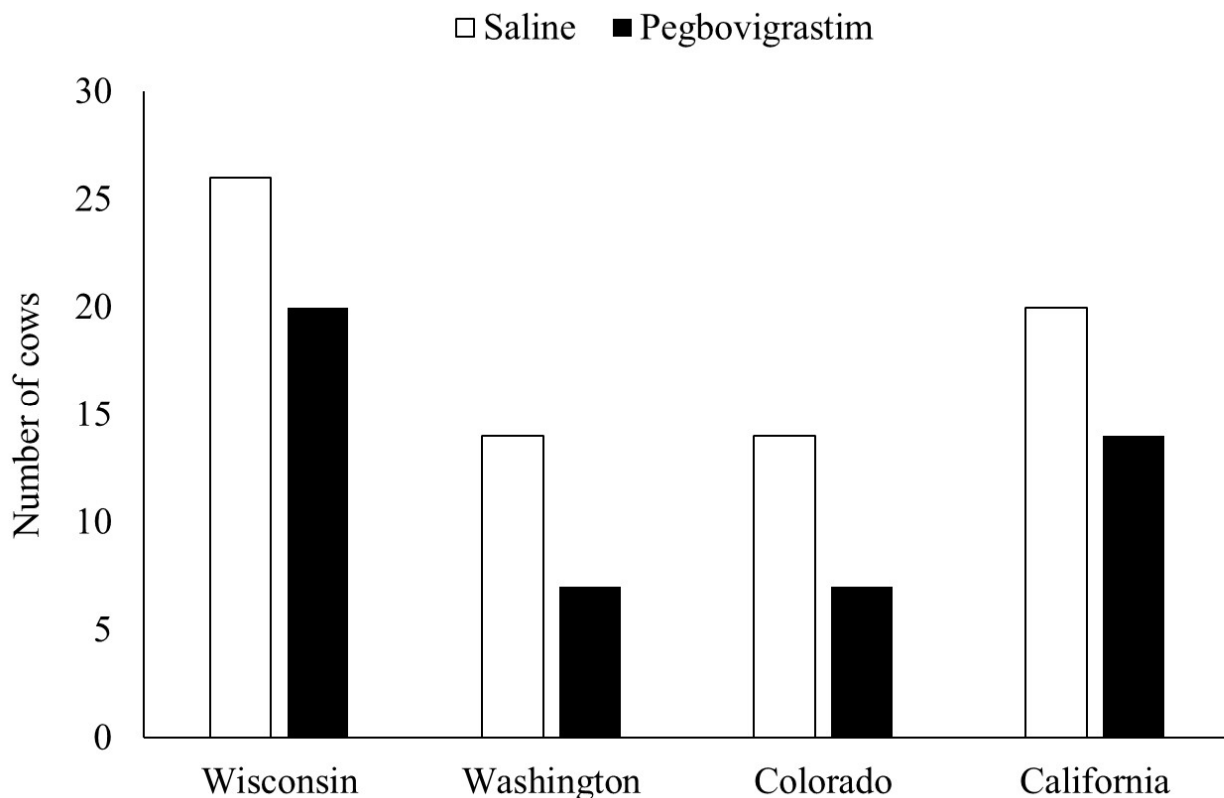

Figure 1 – Effect of pegbovigrastim on number of periparturient cows and heifers suffering from clinical mastitis at four United States dairies
Source: adapted from Canning et al. (2017)

Bacteria and bacteria-derived inhibitory substances

Several researchers suggest natural compounds produced by bacteria and presenting antimicrobial action as potential alternatives for antibiotics in the treatment of bovine mastitis. In 2003, field observations of De Vliegher et al. (2003) suggested that teat apex colonization by *Staphylococcus chromogenes* prepartum in dairy heifers protects the udder quarters against elevated somatic cell count. Interestingly, two out of 10 *S. chromogenes* isolates, both originating from two different teats from the same heifer, consistently inhibited growth of all *S. aureus*, *Streptococcus dysgalactiae*, and *S. uberis* strains, but none of the *E. coli* strains *in vitro* (DE VLIEGHER et al., 2004). *Staphylococcus chromogenes* belongs to the heterogenous group of NAS and is one of the five most prevalent NAS causing intramammary infections in bovine (VANDERHAEGHEN et al., 2015). Piccart et al. (2016) recently found that lactic acid bacteria

recovered from honeybees inhibited the *in vitro* growth of all major mastitis pathogens. Even the mastitis pathogens that were displaying antimicrobial resistance to one or more antimicrobial compounds were inhibited to some extend by the honey and lactic acid bacteria. Also, Bouchard et al. (2013) found that live *Lactobacillus casei* were able to prevent the internalization of *S. aureus* into mammary epithelial cells. Nisin (SEARS et al., 1992), Lacticin 3147 (TWOMEY et al., 2000) (Figure 2) and lysostaphin (HENG et al., 2007) are bacteriocins that have shown to be effective against *S. aureus*.
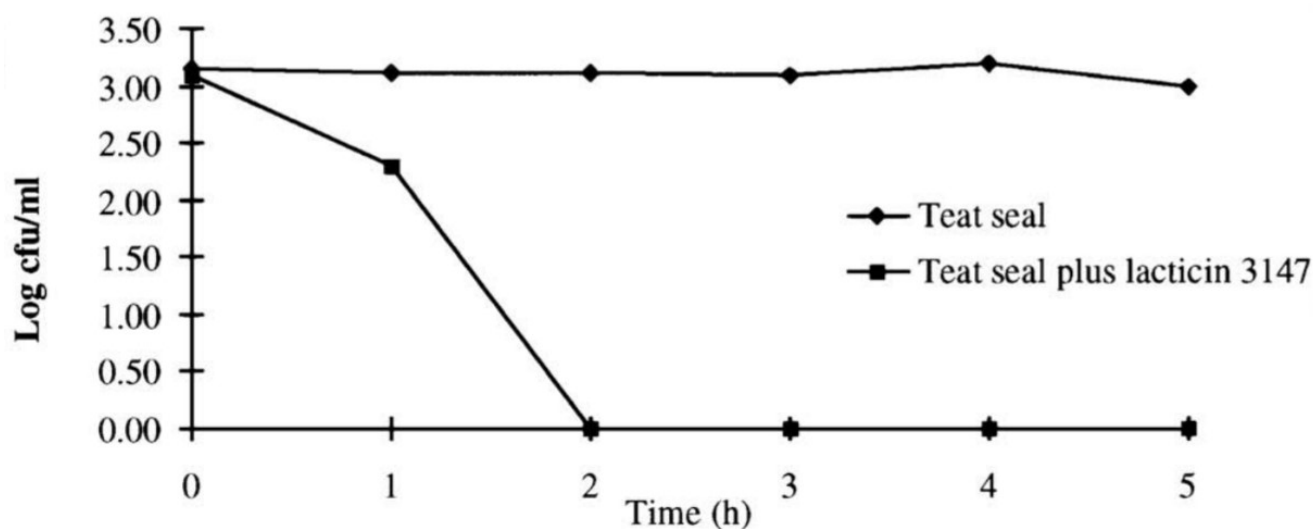

Figure 2 – The inhibitory effect of teat seal plus lacticin 3147 against *Staphylococcus aureus* DPC5246 resuspended in buffer at pH 7.0
Source: based on Twomey et al. (2000)

Animal-derived inhibitory substances

The use of immunomodulators, naturally produced by mammals, as potential non-antibiotic antimicrobial agents for the treatment and prevention of bovine mastitis has also received attention. Lactoferrin is probably the most well-known natural immunomodulator. Lactoferrin is a glycoprotein found in several body secretions such as saliva, tears, bronchial mucus, and milk. This molecule exhibits an antibacterial effect against some major mastitis-causing pathogens namely *E. coli*, *S. aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*, and also NAS (HAFEZ et al., 2013) (Figure 3). Another example is recombinant bovine soluble CD14. Recombinant bovine soluble CD14 reduced the severity of experimental *E. coli* mastitis in mice (LEE et al., 2003). There is increasing evidence that the soluble form of CD14 competes with membrane-bound CD14 for lipopolysaccharide and plays a pivotal role in regulating bacterial infection and septic shock caused by Gram-negative bacteria (LEE et al., 2003).
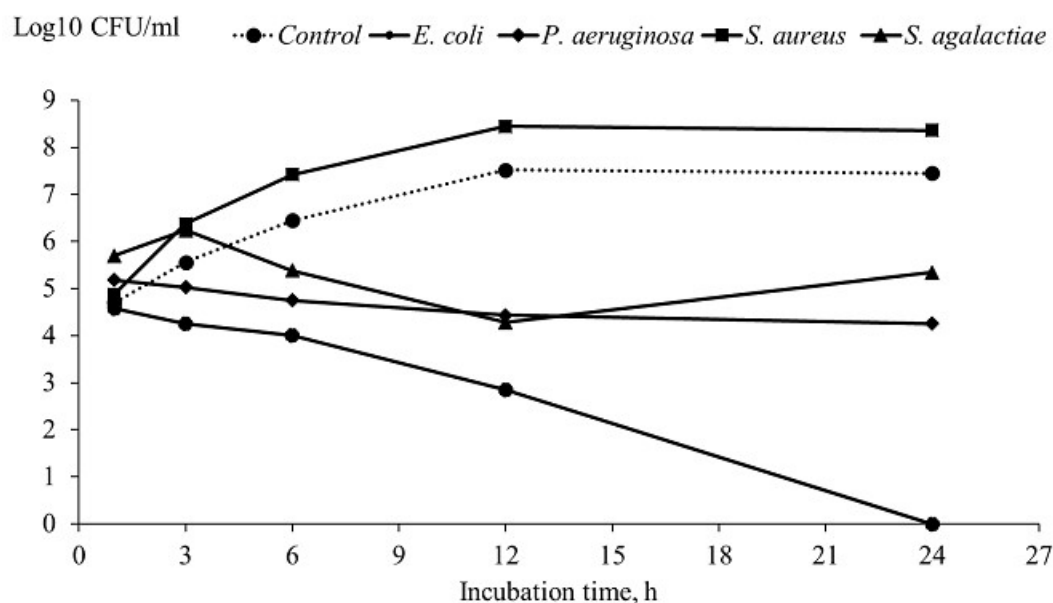
Figure 3 – Antimicrobial effect of bovine lactoferrin on *E. coli*, *S. aureus*, *Streptococcus agalactiae* and *Pseudomonas aeruginosa* counts after 1, 3, 6, 12, and 24 h of incubation

Plant-derived inhibitory substances and medium-chain fatty acids

Plants are promising sources of new biologically active agents with antimicrobial action. One of the major advantages of plant-derived drugs is that they do not induce resistance after prolonged exposure (DOMADIA et al., 2007). Some examples of plant-derived natural compounds are diterpenes (FONSECA et al., 2013), *trans*-cinnamaldehyde, eugenol, carvacrol, and thymol (BASKARAN et al., 2009). All show antibacterial activity against the major mastitis pathogens including *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, *S. aureus*, and *E. coli*.

MCFA are another form of plant-derived antimicrobials. MCFA are derived from medium-chain triglycerides. Natural sources rich in medium-chain fatty acids are coconut oil, palm kernel oil and camphor tree drupe. Coconut oil is composed of approximately 66% of medium-chain triglycerides. Horse milk is another rich source of medium-chain triglycerides. On the contrary, the milk fats of humans, dogs and guinea pigs are largely made up of long-chain fatty acids (BRECKENRIDGE; KUKSIS, 1967). The milk fats of cows, sheep and goats are primarily rich in short-chain fatty acids. MCFA and the corresponding number of carbons found in medium-chain triglycerides are caproic acid (C6), caprylic acid (C8), capric acid (C10) and lauric acid (C12). Caprylic, capric and lauric acid have been extracted as well, although in relatively small amounts, from the teat keratin lining the teat canal (TREECE et al., 1966). Besides the microbicidal activity, in humans there is increasing evidence that MCFA have immunomodulating activities.

A substantial body of literature exists on the antimicrobial properties of free medium- and long-chain fatty acids and their monoglycerides that exert *antimicrobial activity* towards an array of pathogenic micro-organisms. Still, information on their efficacy in killing mastitis-causing bacteria is limited. Probably the first study that provided some information published on the microbicidal activity of long-chain fatty acids which are naturally present in the teat canal keratin towards several common mastitis pathogens was that of Hogan et al. (1987). A more recent study specifically investigated the efficacy of the MCFA caprylic acid (C8), and its monoglyceride, monocaprylin, to inactivate a number of mastitis

pathogens, including *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, *S. aureus*, and *E. coli* (NAIR et al., 2005). Populations of surviving bacteria were determined at 0 min, 1 min, 6 h, 12 h, and 24 h of incubation at a temperature of 39°C. Both caprylic acid and monocaprylin reduced growth of all five pathogens > 5.0 log cfu/mL after 6 h of incubation (Figure 4). Interestingly, the microbicidal activity of fatty acids decreases with increasing chain length, with MCFA exhibiting stronger activity than long-chain fatty acids (WANG; JOHNSON, 1992; ISAACS et al., 1995). The decreasing effectiveness with longer chain length is most probably related to increased hydrophobicity and decreased solubility. Inhibitory fatty acids must be sufficiently water soluble to reach an effective concentration in the aqueous solution and yet be sufficiently hydrophobic to interact with hydrophobic proteins or lipids on the bacterial cell surface (WANG; JOHNSON, 1992). The latter reasoning might hold true for the different MCFA mutually as well (NAIR et al., 2005).
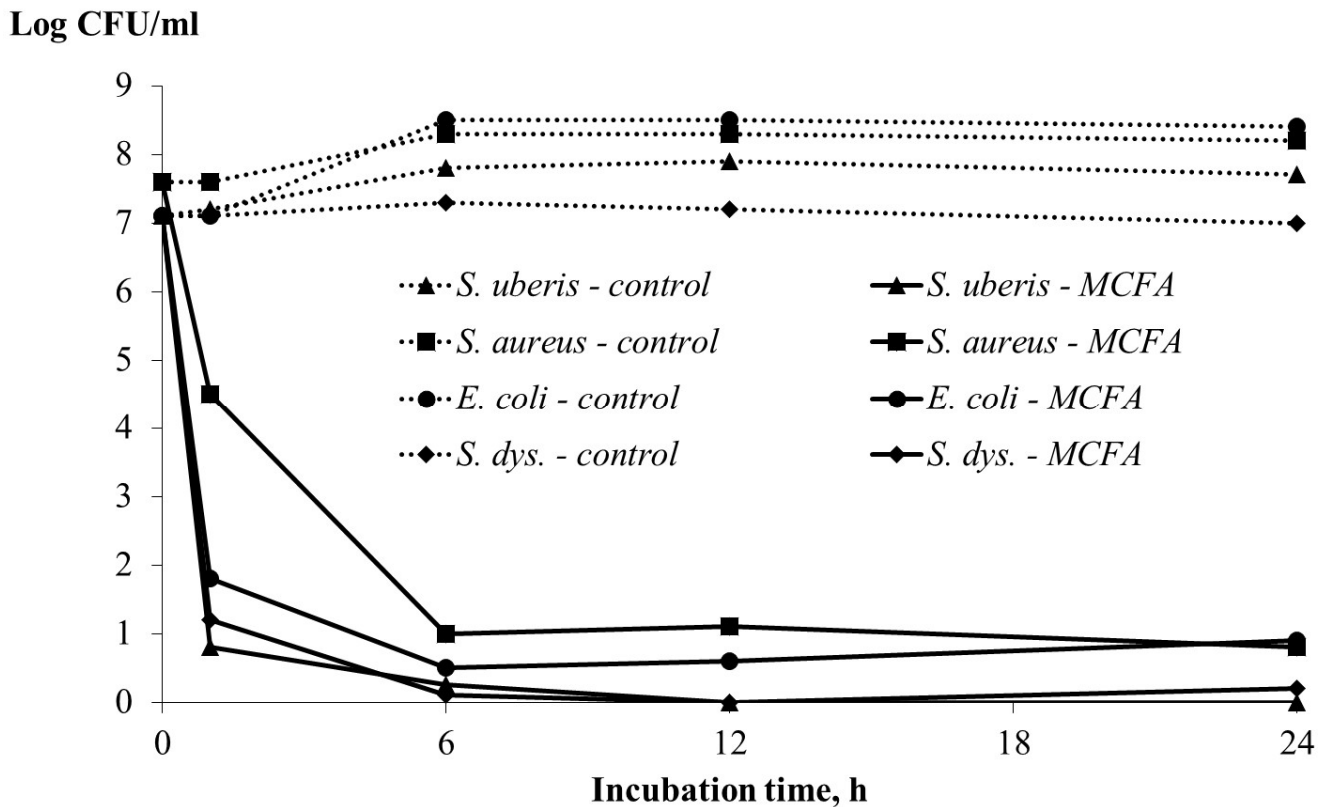

Figure 4 – Effect of 50 mM of the medium-chain fatty acid (MCFA) caprylic acid on *Streptococcus uberis*, *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus dysgalactiae* in milk. Surviving bacterial populations (expressed in log cfu/mL) in milk samples stored at 39°C for 24 h were enumerated at specified time points on tryptic soy agar plates containing 0.6% yeast extract

Source: adapted from Nair et al. (2005)

The exact mechanism behind the bactericidal activity of free fatty acids on bacteria has not yet been unraveled. Cellular membranes have long been regarded as a primary target for antimicrobial fatty acids. Monoglycerides have been demonstrated to penetrate into the bacteria as non-ionic surfactants and to incorporate themselves into the bacterial plasma membrane, thereby altering membrane permeability and affecting transport of nutrients. Electron microscopic studies with 43 bacterial cells exposed to monoglycerides have shown plasma membrane disintegration leaving the peptidoglycan cell wall intact (BERGSSON et al., 1998). Other studies indicated that micromolar concentrations of fatty acids can affect the activity of enzymes in the cell membrane (WARTH, 1989; VIEGAS; SA-CORREIA, 1991). MCFA, in particular, have also been hypothesized to diffuse into bacterial cells in their undissociated form and dissociate within the protoplasm, thereby leading to intracellular acidification (SUN et al., 2002). A lower intracellular pH can lead to inactivation of intracellular enzymes (VIEGAS; SA-CORREIA, 1991), and inhibition of amino acid transport (FREESE et al., 1973). At sublethal concentrations, monoglycerides such as monolaurin can interfere with bacterial signal transduction, and inhibit the expression of virulence factors and antibiotic resistance genes (PROJAN et al., 1994; RUZIN; NOVICK, 1998).

The sensitivity to fatty acids seems to vary among bacterial species and possibly also among strains within a species. In general, Gram-negative bacteria like *E. coli* are less sensitive to the antimicrobial effect of fatty acids and monoglycerides than Gram-positive bacteria (KABARA, 1978; MONK et al., 1996). Also, *S. aureus* seems to be more tolerant than mastitis-causing streptococci such as *S. agalactiae*, *S. dysgalactiae*, and *S. uberis* (NAIR et al., 2005). In the latter study, caprylic acid at 100 Mm and

monocaprylin at 50 mM levels resulted in a rapid decline (4 to 6 log cfu/ml) in the populations of streptococci after 1 min of treatment. At the same time point, the effect of monocaprylin on *E. coli* and *S. aureus* was clearly less pronounced (3 to 4 log cfu/ml). The comparatively lesser instantaneous antibacterial effect of MCFA on Gram-negative bacteria can possibly be attributed to the well-known differences in cell wall structure between Gram-positive and Gram-negative bacteria. With respect to *S. aureus*, it is well established that some strains are able to produce slime once they start growing (SUTRA et al., 1990). It is very plausible that the layer of exopolysaccharide or pseudocapsule slime surrounding them could interfere with the lipids coming in direct contact with the bacterial cell membranes and exerting the antimicrobial activity.

The inhibitory activity of fatty acids and monoglycerides probably also depends on the composition of the medium in which they are supposed to act. Wang and Johnson (1992) found that although lauric acid and its monoglyceride monolaurin were bactericidal against *Listeria monocytogenesis* in brain-heart infusion broth, none of the two were still inhibitory in milk. Certain nutrients in relatively high concentrations such as lipophilic proteins (e.g. albumin), fat globules, starch, or others could interact with fatty acids and thereby decrease their bioavailability (KABARA, 1978). Glassman (1949) reported that proteins reduce the bactericidal activity of surface-active agents like monoglycerides due to the formation of lipid-protein complexes. On the contrary, reduced antimicrobial activity of MCFA when evaluated in milk could not be observed by Nair et al. (2005).

The discrepancy in the findings between those two studies might again be due to differences in chain length between the MCFA that were tested. Caprylic acid, an 8-carbon MCFA, is less

hydrophobic and potentially more soluble than lauric acid, a 12-carbon MCFA, and could therefore be more effective in killing bacteria under less optimal conditions, i.e. milk, than MCFA with a longer chain (NAIR et al., 2005).

Although a plethora of information is available on the *immunomodulating effects* of MCFA in humans, little information is available on these effects in the bovine. Piepers and De Vliegher (2013) explored the effect of commercially available orally supplemented MCFA (Aromabiotic®, Vitamex n.v., Drongen, Belgium) to heifers and multiparous cows starting at 6 to 8 weeks prior to calving on the blood and milk neutrophil apoptosis at 1 to 3 days after calving in a double-blinded clinical trial. Neutrophils play an important role in the first line immune defense of the mammary gland (RAINARD et al., 2006). Apoptosis of bovine neutrophils implies an impaired phagocytotic and oxidative burst activity (MEHRZAD et al., 2004). A reduction in neutrophil apoptosis has therefore been proposed to be beneficial for the elimination of the bacteria in the early stages of intramammary infections as increased numbers of more viable neutrophils enhance the defense against the invading bacteria (DIBBERT et al., 1999). In this trial, twelve lactating cows and 10 end-term heifers were randomly assigned to either an untreated control (n = 11) or MCFA supplemented (treated) group (n = 11). Oral supplementation with MCFA started at dry-off for multiparous cows and 7 weeks prior to expected calving date for the end-term heifers. Interestingly, oral supplementation of MCFA to heifers and multiparous cows suppressed the natural drop in the systemic as well as the local innate immunity shortly after calving as evident from the better neutrophil quality in both blood and milk (Figure 5). In the control animals, blood neutrophil apoptosis significantly increased between start of supplementation and the first days after calving, whereas no substantial change in blood neutrophil apoptosis could be observed in the treated animals. Also, the proportion of apoptotic milk neutrophils in early lactation was lower in the treated group compared to the control group. The mechanism behind these potential immunomodulating effects is unknown and even more intriguing than the questions related to the microbicidal activity of MCFA. In humans, MCFA such as lauric acid seems to have moderate to strong antioxidant properties, delaying or even preventing cell/tissue damage due to oxidative stress (BELLINATIE-PIRES et al., 1993; HENRY et al., 2002; VERSLEIJEN et al., 2005). Oxidative stress is the result of an imbalance between increased production of reactive oxygen species (ROS) causing cell and tissue damage, and reduced availability of antioxidants usually protecting cells from the damaging effects of ROS. Besides their anti-oxidative activities, caprylic and lauric acid also exhibit anti-inflammatory properties via the inhibition of cyclo-oxygenase I (COX-I) and II (COX-II) (HENRY et al., 2002).

When looking at the potential applications of MCFA in practice, treatment of mastitis should be distinguished from prevention of mastitis:

Treatment of mastitis. The effectiveness of MCFA in inhibiting the most common mastitis pathogens under *in vitro* circumstances looks promising in view of the development of novel strategies in the control of mastitis. Similar to what has already been done with bacteriocins (RYAN et al., 1999), MCFA could be incorporated in intramammary infusions whether or not combined with internal teat sealants used at dry-off. Still, there is much more research required to ascertain the *in vivo* effectiveness as well as their potential negative effects on the udder tissue. In addition, MCFA might affect the organoleptic and functional properties of milk which would

obviously make the use of MCFA as microbicidal less desirable (AL-SHABIBI et al., 1964). Oral supplementation of MCFA will most likely not affect the organoleptic properties of milk, but will, as a drawback, probably neither result in an increase of the antibacterial properties of milk. Grummer and Socha (1989) evaluated the effects of medium-chain triglycerides supplementation on milk fatty acid composition. Although changes in milk fatty acid composition were observed, they were minor and mostly unexplained. In contrast to long-chain fatty acids, which are absorbed via the lymphatic system as chylomicrons, MCFA are absorbed via the hepatic portal vein. As a consequence, the efficiency of transfer of absorbed MCFA to milk is low.

Prevention of mastitis. Oral supplementation of MCFA could offer an opportunity to stimulate cellular immunity and support cows in meeting their every-day challenges, particularly in the critical period around calving. Negative energy balance and increased milk production demands in high yielding dairy cows during transition often result in an accumulation of ROS that exceeds the antioxidant capabilities of the cow. The increased oxidative stress during transition has been proposed a number of times as a major underlying factor of the immunosuppression that cows suffer from around parturition (BERNABUCCI et al., 2005; CASTILLO et al., 2005; SORDILLO; AITKEN, 2009). As antioxidants, MCFA could possibly help in minimizing the detrimental effects of the excessive accumulation of ROS and thus keeping oxidative stress and negative effects on the cellular immune functions under control. Because of their anti-inflammatory properties, MCFA could also be beneficial in channeling the sometimes overzealous inflammatory response to intramammary infections that occurs during transition, thereby limiting udder tissue damage and milk production losses (BURVENICH et al., 2007). However, further research is needed to value the relevance of both the anti-oxidative and anti-inflammatory effects of MCFA in terms of potential reduction of antibiotic usage on dairy farms in relation to udder health.

Conclusions

Currently, the administration of antimicrobials is still the most common method to treat bovine mastitis. Still, this kind of strategy has some important disadvantages including the potential development of antimicrobial resistance in both animal and human pathogens, and the risk for antimicrobial residues in milk. Therefore, a wide range of alternatives to antimicrobials have been investigated by several groups of researchers in order to find an effective and equivalent approach for the treatment of bovine mastitis. Bacteriophages, vaccines, nanoparticles, cytokines, and natural compounds of plants, animals and bacteria are some examples of potentially valid substitutes for antimicrobials. For most of those alternatives, *in vitro* studies showed encouraging results but more research, primarily conducted *in vivo*, is still lacking though critical and required. Currently, vaccination in the periparturient period against mastitis caused by *E. coli* and *S. aureus*, injection of pegbovigrastim in periparturient period and oral supplementation of MCFA across dry period are the only alternatives that have yet been shown to yield decent prevention, though not curative efficacy *in vivo*.
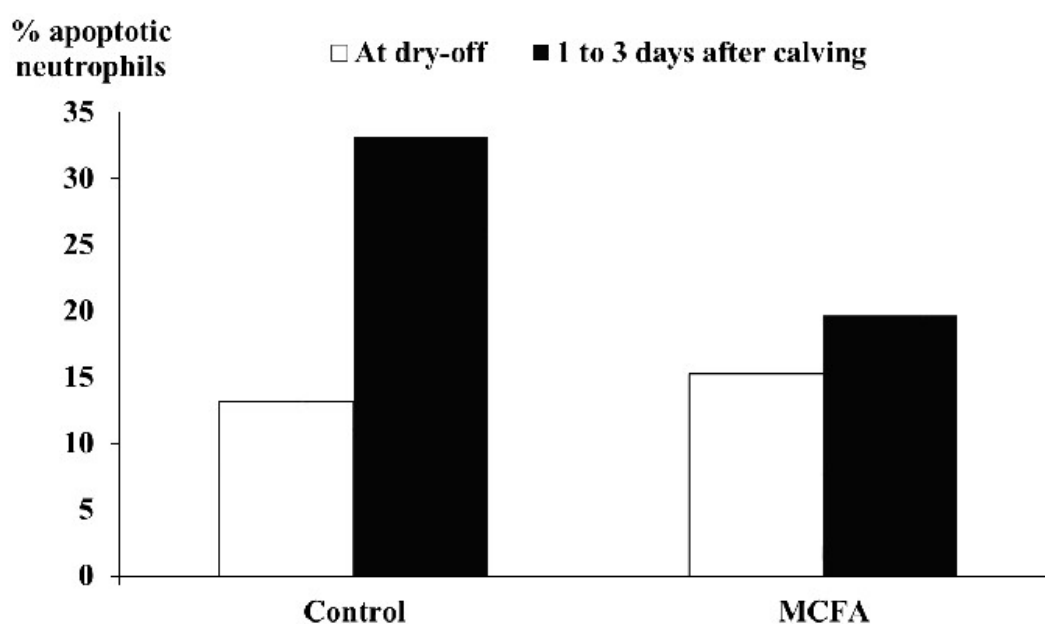
Figure 5 – Percentage apoptotic blood polymorphonuclear neutrophilic leukocytes in both control and medium-chain fatty acid (MCFA) supplemented animals between the start of supplementation [6-8 weeks before expected calving date (= at dry-off)] and early lactation (1-3 days in milk)

Source: adapted from Piepers and De Vliegher (2013)

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