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REVIEW ARTICLE

Transgenic technology: the strategy for the control and prevention of bovine staphylococcal mastitis?

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Abstract Mastitis is the disease that most affects dairy cattle with losses above US$ 2 billion per year in the United States alone. It frequently presents bacterial origin, with *Staphylococcus aureus* (*S. aureus*) standing out as a pathogen challenging to eliminate because of the high resistance to antimicrobials. Antimicrobial therapy often demonstrates failure, with low cure rates, bacterial resistance and bacterial seclusion in the outbreaks of infection as well as leaving its residues in soil, water, and even animal products. Advances in research may provide benefits to animal welfare by increasing cow's resistance to mastitis by inducing mammary gland cells to secrete an antibacterial protein called lysostaphin, which is a potent staphylocolytic enzyme. Over the years, research groups have developed projects aimed at developing particular immunomodulators, as well as transgenic lysostaphin-secreting cows. The focus of this review is to compile studies on the use of lysostaphin and in the therapeutic and prophylactic control of staphylococcal mastitis using genetic engineering and biotechnology as an alternative tool. In the transgenic models of mice and cows, lysostaphin was able to prevent staphylococcal mastitis presenting little effect on the integrity of the mammary gland, animal physiology and milk produced. Further studies should be performed not only related to cases of prevention of staphylococcal mastitis, but also in the treatment and maintenance of the long-term action of lysostaphin.

Introduction

Dairy cattle represents a traditional productive structure, being considered a source of valuable protein resource and with significant economic importance (USDA, 2015). The activities related to the production, industrialization, and

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commercialization of milk and its derivatives, goes from the provision of food to the population until the generation of employment, income and at the consumer market, since the countryside as well as at the urban environment (Voges, Thaler Neto, & Kazama, 2015).

The introduction of new technologies, such as complete genome sequencing and genome manipulation in cattle, has opened a new era for industrial applications (Yum, Youn, Choi, & Jang, 2018). According to Vilela, De Resende, Leite, and Alves (2017), the productive sector must be improved to increase productivity and sustainability in the exploitation of milk. In this subject, the health aspects of the herd have been the subject of several discussions, to avoid changes in the elemental composition of the milk produced (Schwendel et al., 2015).

Bacteria of the genus *Staphylococcus* are among the major pathogens involved in cases of bovine mastitis, in which *Staphylococcus aureus* (*S. aureus*) is considered the principal agent responsible for clinical infections in dairy cows worldwide (Aarestrup, Seyfarth, Emborg, Pedersen, Hendriksen, & Bager et al., 2001). Although *S. aureus* can colonize the skin and mucosa of cows, the udder is characterized as the largest reservoir of this bacterium (Capurro, Aspán, Unnerstad, Waller, & Artursson, 2010). Intramammary infection caused by *S. aureus* can be widespread among cows of the same herd, and the use of genotyping has demonstrated that clonal groups can remain in the same herd over a long period (Mork et al., 2012; Piccinini et al., 2012).

As an alternative to the treatment and prophylaxis of staphylococcal mastitis, lysostaphin, which corresponds to a glycylglycine enzyme, found in the cell wall of staphylococci, can specifically cleave the peptidoglycan pentaglycine interconnecting bridges located in the cell wall of staphylococci (Browder, Zygmunt, Young, & Travormina, 1965). Lysostaphin is an antibacterial enzyme, peptidoglycan hydrolase that was first isolated and characterized from a culture of *Staphylococcus simulans* (*S. simulans*), formerly known as *Staphylococcus staphylolyticus* (Schindler & Schuhardt, 1964). Since *S. aureus* cell wall bridges have a high proportion of pentaglycine, lysostaphin becomes a highly efficacious agent in the lysis of actively growing and quiescent *S. aureus* cells with low specificity for other pathogens causing *S. aureus* mastitis (Kerr et al., 2001; Zygmunt & Tavormina, 1972).

Thus, this enzyme presents itself as an ideal candidate for antibacterial action, added to the fact that it does not degrade milk proteins, preserving its composition (Kerr et al., 2001). The potential of lysostaphin as a therapeutic and prophylactic control of staphylococcal mastitis was initially demonstrated in a mouse model (Bramley & Foster, 1990) and later in dairy cattle (Oldham & Daley, 1991).

Currently, biotechnology has been emerging as an instrument of new alternatives for the treatment and cure of several diseases (De Paula, Dos Santos, Pales, Castro, Lopes, & Santos, 2017). In this context, science has demonstrated the ability to manipulate the genome of animals, as well as the production of transgenics (Pinkert, 2004). Gordon, Lee, Vitale, Smith, Westphal, and Hennighusen (1987) reported the use of transgenic for the first time to direct the expression of a foreign protein in the milk of mice. The specific transcripts of lactation were already incorporated into the swine (Wall, Pursel, Shamay, Mcknight, Pittius, & Hennighausen, 1991), sheep (Wright, Carver, Cottom, Reeves, Scott, Simons, 1991), goats (Ebert et al., 1991) and cattle (Krimpenfort et al., 1991); mainly related to pharmaceutical interests with the objective of generating animal bioreactors (Kerr et al., 2001).

The purpose of this review was to report the scientific research related to the mechanism of action of lysostaphin and recombinant lysostaphin, as well as its biotechnological potential in the therapeutic and prophylactic control of staphylococcal mastitis, contributing to improve the quality of the daily activity and, consequently, the quality of the food produced.

Staphylococcal mastitis

Mastitis is defined as an inflammatory process of the mammary gland, as a response to glandular udder tissue, which occurs by physical aggression or the presence of infectious agents (Beloti, Nero, Moreira, Da Silva, Fagnani, & Reis, 2015). Despite technological and scientific advances, milk remains an excellent culture medium for the growth of almost all types of microorganisms (Kumar, Rahal, Dwivedi, & Gupta, 2010), such as bacteria, viruses, fungi and protozoa (Amin, Amouda, & Abdel-All, 2011). The body temperature of the animal also contributes to the growth of microorganisms (Sharma, Maiti, & Sharma, 2007).

The main route of entry of microorganisms in the udder is through the canal of the ceiling, which can reach internal portions of the mammary gland, establish and multiply, generating an inflammatory process (Harmon, 1994). The pathogens commonly involved include Gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas* spp. and *Klebsiella* spp.; and Gram-positive, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* (Hillerton & Berry, 2005).

S. aureus is a Gram-positive coccus, facultative anaerobic, immobile, hemolytic, non-spore-forming, catalase and coagulase-positive bacterium (Harmon, 1994), known as the main causative agent of bovine mastitis, including cases of clinical and subclinical mastitis. It generally exhibits antimicrobial resistance, due to its ability to form biofilms and the ability to take refuge within the host phagocytes and epithelial cells of the mammary gland, thus contributing to the persistence of the disease and/or the need for prolonged therapy (Kamaruzzaman, Chong, & Edmondson-Brown, 2017).

In the last decades, research has shown that *S. aureus* causing intramammary infections does not form a homogeneous group; and some strains are host-adapted and highly contagious, and others are sporadically isolated and tend to be the secondary cause of intramammary infections in a herd (Middleton & Fox, 2002; Ote, Taminiau, Duprez, Dizier, & Mainil, 2011). Usually, contamination occurs by cow-cow transmission, during manual or mechanical milking, and by interferences that come into contact with the ceiling (Fox, Gershman, Hancock, & Hutton, 1991; Saperstein, Hinckley, & Post, 1988).

Regarding antimicrobial therapy, the cure rate is usually less than 25% (Rainard, 2005), and the use of intramammary or parenteral antimicrobials during lactation and

non-lactation periods indicated variable results. Cure rates were reported ranging from 3 to 76%, using conventional antimicrobial treatment strategies in the lactation period (Barkema, Schukken, & Zadoks, 2006).

Often, cure rates after antimicrobial therapy are affected by advanced cow age, increased somatic cell count (SCC), evolution to chronicity of infection, elevation of bacterial counts and number of infected breast quarters (Barkema, Schukken, & Zadoks, 2006). Some authors point out the importance of the use of prolonged therapy to help improve cure rates (Barkema et al., 2006; Roy & Keefe, 2012).

This pathogen can transpose the immune defenses of the host and may evolve the disease into a chronic intramammary infection. *S. aureus* may become highly resistant to antibiotic therapy due to the development of antibiotic resistance, generation of microabscesses or staphylococcal invasion of phagocytes and mammary epithelial cells (Barkema et al., 2006).

According to Rainard (2005), because of the lack of effective vaccines and the lack of antibiotic therapy, the elimination of chronically infected cows is the most effective way to control the spread of the disease; and also contributes to the reduction of staphylococci in milk, since this represents a risk of occurrence of foodborne illnesses. Another factor is that the heritability of resistance to mastitis is low and for this reason, selective breeding is not considered as a tool to combat mastitis (Lund, Miglior, Dekkers, & Burnside, 1996).

Lysostaphin as an anti-staphylococcal therapeutic agent

Lysostaphin is a potent staphyllolytic enzyme (Rainard, 2005) containing a peptidase capable of cleaving specifically the polyglycine bridges unique to *S. aureus* cell walls (Dixon, Goodman, & Koenig, 1968). The activity of lysostaphin is measured by its ability to lyse *S. aureus* cells, being influenced by enzyme concentration, pH, temperature and ion concentration and salts (Schindler & Schuhardt, 1965).

S. aureus is encapsulated by a thick layer of peptidoglycan and lysostaphin acts to degrade the peptidoglycan layer, resulting in lysis and cell death (Kumar, 2008). According to Schindler and Schuhardt (1964), peptidoglycan confers strength and stiffness to the cell wall of Gram-positive microorganisms, allowing growth and division, maintaining cell shape and protecting against osmotic lysis in *S. aureus* (Fig. 1).

Biotechnological potential of recombinant lisostafine in staphylococcal mastitis

Recombinant lysostaphin (rLYS) is a zinc metalloenzyme that hydrolyzes glycylglycine bonds of pentaglycine cross-linked peptidoglycan of *S. aureus* cell wall (Recsei, Gruss, & Novick, 1987; Wadstrøm & Vesterberg, 1971). The first studies used rLYS in the treatment of *S. aureus* mastitis in rodent models and showed rates of reduction of mammary infection higher than 87%, suggesting *in vivo* staphyllolytic activity, without deleterious effects on the host and being an alternative to

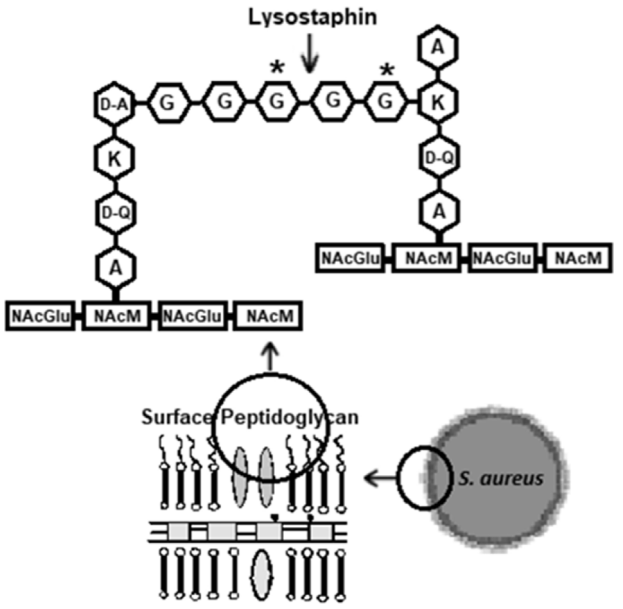

Fig. 1 Mechanism of action of lysostaphin on the cell wall of *Staphylococcus aureus*. Structure of peptidoglycan and primary hydrolysis site of lysostaphin on peptidoglycan. NAcGlu, N-acetylglucosamine; NAcM, N-acetylmuramic; A, L-alanine; D–Q, D-glutamine; K, L-lysine; D-A, D-alanine; G, L-glycine.

conventional antimicrobial agents (Bramley & Foster, 1990; Hoerning, Donovan, Pithua, Williams, & Middleton, 2016; Sears, Smith, Polak, & Blackburn, 1988). In addition to applications in transgenic cows, the study with lysostaphin is constantly advancing and may be useful in several biotechnological applications (Table 1).

Oldham and Daley (1991), described the efficacy of rLYS in lactating dairy cows with experimentally induced *S. aureus* intramammary infection (50–100 CFU strain ATCC 29740), where a single intramammary infusion of 100 mg rLYS in 60 ml of Phosphate-Saline Buffer Solution (PBS) eliminated 95% of *S. aureus* in the treated mammary quarters for a minimum of one milking (12 h). However, despite maintenance of the *in vitro* antimicrobial activity of rLYS for 72 h, *in vivo*, most of the infected mammary quarters fell within 72 h of treatment.

Oldham and Daley (1991), consider that the low cure rate of rLYS is because intracellular *S. aureus* has not been killed by rLYS and therefore contributing to the rapid relapse of the infection. Thus, rLYS was shown to be partially effective in the treatment of mastitis induced by *S. aureus* when infused directly into bovine udders.

The authors also pointed out that when compared to conventional intramammary antibiotic therapies (penicillin G, cefapirin sodium in PBS and cefapirin sodium in peanut oil), rLYS presented a 20% cure, being superior to penicillin G (0% curing) and equivalent to cefapirin sodium in PBS (29% cure). While cefapirin sodium in peanut oil was more effective at a cure rate of 57% (Oldham & Daley, 1991).

Hoerning et al. (2016) in order to evaluate the action of a fused rLYS on a protein transduction domain (rLYS-PDT), shown in preliminary *in vitro* studies to eliminate intracellular *S. aureus* (US Patent 8383102 B2; Donovan, 2013), chose a model of dry cow (out of the lactation period)

Table 1 Strategies for the use of lysostaphin and biotechnological applications.

Usage strategy	Applications	Reference
Pharmaceutical applications	Topical, subcutaneous and intraperitoneal treatment of *S. aureus* induced infections.	Harrison and Cropp (1967)
Incorporation in food	Control food poisoning by *S. aureus* directly	Metcalf and Deibel (1969)
Control of nasal colonization	In humans.	Quickel, Selden, Caldwell, Nora, and Schaffner (1971)
Presumptive tests	Differentiation with staphylococci and micrococci.	Langlois, Harmon, and Akers (1988)
Incorporation in food	Genetic engineering of lactic acid bacteria commonly used in food fermentations	Gaier, Vogel, and Hammes (1992); Cavadini, Hertel, and Hammes (1998)
Therapeutic agent	Anti staphylococcal.	Dajcs, Hume, Moreau, Caballero, Cannon, and O'callaghan (2000); Kerr et al. (2001)
Control of nasal colonization	In mice.	Kokai-Kun, Walsh, Chanturiya, and Mond (2003)
Immunogenic potential	Deimmunized lysostaphin using a computationally guided process that optimizes sets of mutations to delete immunogenic T cell epitopes without disrupting protein function.	Blazanovic et al. (2015)
Alternative chemotherapeutics	Use of fusion proteins of the Lysostaphin CWT domain considered as a promising source of alternative chemotherapeutic agents.	Schmelcher, Powell, Becker, Camp, and Donovan (2012); Osipovitch and Griswold (2015); Jagielska, Chojnacka, and Sabała (2016)
Incorporation of lysostaphin in disinfectant solutions	Increased bacteriolytic activity in commercial health-related disinfection products.	Wu, Kwon, Kim, Zha, Mora-Pale, Dordick (2018)

therapy in order to maximize enzyme concentrations and time of exposure of *S. aureus* to rLYS-PTD in the mammary gland. In this experiment, healthy cows were experimentally infected with *S. aureus* infusions (ATCC 29740). Cows were divided into two groups; group 1 received treatment (279 mg rLYS-PTD) and group 2 (control group). After 30 days, 14 cows were euthanized and submitted to macroscopic and histological examination. In conclusion, rLYS-PTD was not an effective treatment for dry cows for subclinical and chronic *S. aureus* mastitis in the dose and formulation tested, with a global cure rate of 0%, as well as ineffective in subsequent lactation.

Hoerning et al. (2016), such as Oldham and Daley (1991), used cows with intramammary infection induced by *S. aureus* (ATCC 29740). This challenge model was chosen to minimize the variation caused by different intramammary infections that could have occurred as a natural infection. However, in the dry cow, milk is not withdrawn 2–3 times a day, which means that removal of the residual drug does not occur as during lactation; thus, one can predict that drug concentrations are maintained because the drug is not removed. This assumes that most of the drug remains in breast tissue or milk (Hoerning et al., 2016).

An additional complication to determine the appropriate dose of rLYS-PTD is the fact that the cellular specificity of the attached PTD is unknown. If the rLYS-PTD construct is carrying rLYS in all cells of the mammary gland, not only those infected with *S. aureus*, it is assumed that the required dose of rLYS-PTD would become exponentially larger, thus providing another possible explanation for the lack of efficacy of treatment compared to rLYS. Although preliminary work confirms the in vitro efficacy against extracellular and intracellular *S. aureus*, it is difficult to know how the construction of rLYS-PTD works *in vivo* in the presence of milk components, immune system cells and mammary epithelium (Hoerning et al., 2016).

Expression of rLYS in transgenic cows

The creation of transgenic dairy cows aims to increase milk production, and quality and genetic engineering provide a faster course for this demand (Kolb, 2002). Several enzymes with antibacterial activity were the target of works to prevent bacterial infections. Lactoferrin, lysozyme, and lactoperoxidase are proteins present in bovine milk that have antibacterial activity but are insufficient to prevent mastitis (Reiter, 1978; Takahashi, Eisenhuth, Lee, & Schachtele, 1992).

Transgenic techniques may alter the ability of animals to produce differentiated kinds of milk, such as increased resistance to bacteria that cause mastitis. Transgenic mice bearing a beta-lactoglobulin-lysostaphin (βLG-Lys) fusion gene secrete lysostaphin at concentrations of 0.06–1.3 mg/ml of milk. Their resistance to mastitis induced by *S. aureus* has been shown to improve with increasing lysostaphin concentrations (Kerr et al., 2001), confirming the hypothesis that the transgenic production of lysostaphin by the lactating mammary gland confers substantial resistance to staphylococcal mastitis (Bramley & Foster, 1990; Williamson, Bramley, & Lax, 1994).

Kerr et al. (2001) further emphasize that when the native form of lysostaphin secreted by transfected eukaryotic cells, it becomes glycolyzed and inactive and that other substitution strategies may reveal an even more active lysostaphin variant. The production of lysostaphin by transgenic mice

has shown to have little effect on the integrity of the mammary gland, animal physiology, and milk produced. The transgenic animals showed similarity to non-transgenic animals, fertile and capable of normal lactation.

Histological examination detected normal mammary glands with milk protein profiles similar to non-transgenic animals. The milk was not processed to produce its derivatives, but the activity of lysostaphin is highly specific for staphylococcal bacteria and according to the author, should not have effects on the cultures used in the dairy industry. It is possible that bacterial strains develop resistance to a transgenic strategy and can reduced by the simultaneous production of two or more antibacterial proteins that have different bacteriostatic properties against the same pathogen species (Kerr et al., 2001).

According to Wall et al. (2005), transgenic cows producing only 3 mg of lysostaphin/ml of milk may protected against repeated intramammary challenges with *S. aureus*. The secretion of rLYS in milk is stable throughout lactation, and milk compositions and yields were similar between transgenic and non-transgenic herd groups (Van Hekken, Wall, Somkuti, Powell, Tunick, & Tomasula, 2009).

In this context, Wall et al. (2005), sought to increase the resistance of dairy cows to mastitis by inducing the mammary gland cells to secrete antibacterial lysostaphin. In this experiment, transgenic cows secreted lysostaphin in the milk at concentrations ranging from 0.9 to 14 mg/ml, the milk with the highest titer capable of inhibiting the growth of several species of *Staphylococcus*, including *S. chromogenes*, *S. hyicus*, *S. epidermidi!!s*, *S. simulans* and *S. xylosus*.

The intramammary infusions of *S. aureus* administered in three transgenic cows, and ten non-transgenic controls. Resistance evaluated in a forced infection experiment, where viable cells of this pathogen inoculated into the mammary glands. *In vitro* assays demonstrated the ability to kill *S. aureus*, with an udder infection rate of 71% in control cows and 14% in transgenic cows. It was also found that the severity and duration of infection were lower in transgenic cows (Wall et al., 2005).

Protection against *S. aureus* obtained with low expression levels of lysostaphin in milk (3 mg/ml). The results indicated that genetic engineering could provide a viable tool to improve disease resistance, providing animal welfare (Wall et al., 2005).

Wall et al. (2005), found that constitutive secretion of lysostaphin in the mammary gland at concentrations ranging from 0.9 to 14 mg/ml prevented the entry of staphylococci into the mammary gland after an experimental challenge. At higher concentrations, staphylococci could not be recovered in milk, most likely the bacteria were lysed almost instantaneously after challenge and before they could multiply sufficiently to induce a local inflammatory response, and possibly without the contribution of the innate immune system (Rainard, 2005).

Wall et al. (2005), cautiously note that this work represents an initial step to improve cow resistance to mastitis-causing bacteria and that, other pathogens that cause mastitis, such as *E. coli* and *Streptococcus uberis*, are not inhibited. Although encouraging, this study evaluated the ability of secreted rLYS to prevent intramammary infection by *S. aureus*, rather than treating an existing intramammary infection and despite the novelty character, the viability of rLYS secreting cows in commercial dairy products is currently limited in scope (Hoerning et al., 2016).

Van Hekken et al. (2009) determined the impact of industrial thermal processing on lysostaphin activity, with the objective of evaluating the possible technological changes in the processing of milk and its derivatives derived from transgenic cows. The distribution of lysostaphin in different stages of cheese production, the persistence of active lysostaphin in fresh serum and in cheese stored at 4 °C for up to 90 days, the effect of lysostaphin on cheese-producing properties, raw milk after heat treatment at 63 °C for 30 min (slow pasteurization), 72 °C for 15 s (rapid pasteurization), as well as 140 °C for 2 s (Ultra High Temperature - UHT).

For this study, three transgenic cows and three non-transgenic control cows (cows 5–8 weeks postpartum) used, of which semi-hard cheeses made. Samples were tested at each step of the processing to determine the amount (ELISA) and activity ability to inhibit the growth of lysostaphin *S. aureus* (Van Hekken et al., 2009).

The results of this study indicated that most of the lysostaphin was present in the aqueous portion of the milk and was not affected by pasteurization, although UHT treatment reduced enzyme concentration by 60%, as well as decreased activity and amount of lysostaphin during the manufacture of the cheese. Based on the amount of lysostaphin present at the start of cheese production, 10–15% of lysostaphin recovered in the serum, 21–55% in the cheese curd on the first day and 21–36% in the cheese stored at 4 °C for 90 days.

Van Hekken et al. (2009), demonstrated that lysostaphin persisted in typical dairy processing procedures, albeit at reduced levels, and remained viable in pasteurized milk, cheese, and whey. Lysostaphin did not prevent milk coagulation or inactivated the starter cultures used in the cheese manufacturing process, conferring potential value as a bioprotective agent against *S. aureus* in dairy foods.

Conclusions

Dairy cows produce large amounts of milk, presenting great potential for expression of recombinant proteins, allowing the development of the industrial and pharmaceutical sectors. The transgenic methodologies need more advances since the production of transgenic cows presents difficulties, such as long periods for detection of transgenes, expression of recombinant proteins and usually, only one calf generated at the end of each gestation.

Studies should be performed to evaluate the long-term efficacy of lysostaphin since it could reduce by the emergence of resistant strains, as well as the duration of transgenic cows resistant to staphylococcal mastitis. Additional research should be conducted to assess its impact on food safety and quality. Transgenic cows resistant to mastitis by *S. aureus* may allow considerable decreases in the use of antimicrobials and consequently contribute to the control of the dissemination of resistant bacteria.

Ethical and legal aspects should be considered and clarified by the scientific community that must present a return to consumers so that they can decide on the consumption of such products.

Future prospects

Genomic engineering technologies for the production of genetically modified cattle provide powerful advantages in the livestock industry. In the future, technological advances in this area should occur in harmony between man and animal for advances in genetic technologies, resistance, understanding of diseases and the production of recombinant proteins using animal bioreactors.

Conflicts of interest

The authors declare no conflicts of interest.

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