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Immune mechanisms, resistance genes, and their roles in the prevention of mastitis in dairy cows

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Abstract. Mastitis is one of the most important diseases of the mammary gland. The increased incidence of this disease in cows is due to the breeding of dairy cattle for higher yields, which is accompanied by an increased susceptibility to mastitis. Therefore, the difficulty involved with preventing this disease has increased. An integral part of current research is the elimination of mastitis in order to reduce the consumption of antibiotic drugs, thereby reducing the resistance of microorganisms and decreasing companies' economic losses due to mastitis (i.e. decreased milk yield, increased drug costs, and reduced milk supply). Susceptibility to mastitis is based on dairy cows' immunity, health, nutrition, and welfare. Thus, it is important to understand the immune processes in the body in order to increase the resistance of animals. Recently, various studies have focused on the selection of mastitis resistance genes. An important point is also the prevention of mastitis. This publication aims to describe the physiology of the mammary gland along with its immune mechanisms and to approximate their connection with potential mastitis resistance genes. This work describes various options for mastitis elimination and focuses on genetic selection and a closer specification of resistance genes to mastitis. Among the most promising resistance genes for mastitis, we consider *CD14*, *CXCR1*, lactoferrin, and lactoglobulin.

1 Introduction

A new direction in breeding practice should be the resilience of cows; this should focus on reproduction and morphology, especially udders and hooves, allowing for an increased production age and improving the welfare of the animals. Cow resistance is the ability to maintain normal production, fertility, and health, even under changing environmental conditions, such as sudden temperature fluctuations, changes in feed composition and quantity, or exposure and infectious agents (Poppe et al., 2020).

Direct annual economic losses per cow in the event of clinical mastitis range from EUR 115.4 to EUR 193. These costs include the increased need for veterinary treatment and medicines, breeder's time, discarded milk, and increased cow culling (Krupova et al., 2019).

In a dairy herd, it is possible to find healthy cows with high production and cows with one or more infected udder quarters during every lactation. This variability suggests that the incidence and prevalence of mastitis depends on differences in bovine mammary gland susceptibility to intramammary infection (Bannerman et al., 2008). Many factors affect mammary infection, including parity, nutrition, stage of lactation, milk production, breed, and genes (Zadoks et al., 2001; Ogorevc et al., 2009; Zhang et al., 2016; Hamel et al., 2021).

The severity of the inflammation of the mammary gland is influenced by invasive microorganisms and the subsequent immune response (Pighetti and Elliott, 2011; Gogoi-Tiwari et al., 2017). Some cows show greater or lesser susceptibility and sensitivity to the same infectious pathogen, and the incidence of mastitis also varies depending on the stage of lactation (initial and stable lactation period) (Burvenich et al., 2000). The speed, strength, and duration of the immune reaction and the susceptibility to disease are critically linked to the animal's genetic background (Pighetti and Elliott, 2011; Bobbo et al., 2019). The results of endocrine and

genetic studies can lead to a better understanding of the susceptibility to mastitis (Weigel and Shook, 2018; Miles and Huson, 2021). However, the task of effectively integrating information from molecular and quantitative genetics into existing breeding programmes remains (García-Ruiz et al., 2016). Genetic selection of mastitis-resistant cows could become an important alternative for the prophylaxis of mastitis in the future (Burvenich et al., 2000; Weigel and Shook, 2018).

2 Natural defence mechanisms of the mammary gland against infection

The first line of defence of the mammary gland against infection includes the udder and teat morphology, milking speed, innate immunity, and the mechanical and antimicrobial teat canal barrier, through which bacteria must penetrate to cause intramammary infection (Burvenich et al., 2000; Rainard and Riolett, 2006; Günther and Seyfert, 2018). The first defensive cells have various basic functions to maintain tissue integrity in health and disease (Günther and Seyfert, 2018). Teat sphincters isolate the interior of the mammary gland from the external environment and prevent the passage of external contaminants and microorganisms. Damage to this structure is accompanied by an increased incidence of mastitis (Blowey and Edmondson, 2010). The teat canal is lined with folds of keratinized skin epidermis covered by a thin layer of lipid-esterified and non-esterified fatty acids (myristic, palmitoleic, and linoleic acids). Keratin and the lipid-lined teat canal have antibacterial properties, which (along with the teat plug) provide another physical barrier, preventing bacterial migration into the mammary cistern (Sordillo and Streicher, 2002; Blowey and Edmondson, 2010; Senthilkumar et al., 2020). Certain keratin-associated cationic proteins may bind to pathogenic mastitis microorganisms, thereby increasing their sensitivity to osmolarity modification (Paulrud, 2005). For this reason, the teat canal is considered to be an important barrier against intramammary infection.

The inner surface of the mammary gland is covered by epithelial cells, which play an essential role in early interactions between a pathogen and the host (Derakhsani et al., 2018b). Their task is to create a final barrier between the external environment and the inside of the body (Korhonen et al., 2000). Epithelial cells express toll-like receptors (TLRs) that are responsible for the initial identification of invasive pathogens (Rainard and Riolett, 2006; Deb et al., 2013). The TLR family of genes contains members 1–10 and allows individual cells to recognize bacterial, viral, and dangerous signals (Khan et al., 2019).

Within the mammary gland, mastitis-causing microorganisms are recognized primarily by toll-like receptors (TLRs) such as TLR2 and TLR4, which are expressed by epithelial cells (Rainard and Riolett, 2006; Ezz et al., 2019). The activation of TLRs leads to a cascade of reactions: the release of the transcription factor, nuclear factor-κB, which is subsequently translocated to the nucleus and regulates the expression of many pro-inflammatory signalling molecules necessary to initiate a mammary immune response. These reaction molecules include cytokine tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-12 (IL-12); chemokines IL-8 and RANTES (regulated upon activation, normal T cells expressed and secreted); oxygen radicals; tissue factors; and anti-inflammatory factors IL-10 and transforming growth factor (TGF) (Boudjellab et al., 2000; Bannerman et al., 2004b). Furthermore, epithelial cells secrete two important components of innate immunity, complement factor C3 and lactoferrin (Hagiwara et al., 2003; Griesbeck-Zilch et al., 2009).

Monocytes and phagocytic cells produce cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8). L- Lipopolysaccharides (LPSs) are the main component of the cell wall of Gram-negative bacteria, which are released during bacterial cell division or death. LPSs induce the release of cytokines from mononuclear phagocytes. The overexpression of such cytokines can be implicated in mediating acute septic shock (Boudjellab et al., 2000; Ohtsuka et al., 2001). IL-8 is a chemoattractant of cytokines that activates polymorphonuclear neutrophils (Wu et al., 2019a). The most important effectors in the bovine mammary immune system are somatic cells, including epithelial cells from the gland and leukocytes from the blood (Li et al., 2014). Leukocytes migrate from the bloodstream in varying numbers, depending on the corresponding pathogen (Sordillo and Streicher, 2002; Oviedo-Boyso et al., 2007).

Macrophages naturally occur in the mammary gland and, along with neutrophils, are mainly activated during its inflammation (Langrová et al., 2005; Bassel and Caswell, 2018). Macrophages predominate in healthy mammary glands and signal the invasion of pathogenic microorganisms (Paape et al., 2002; Li et al., 2014). During the invasion of pathogens into the mammary gland, they are detected mainly through monocytes/macrophages, although also through mammary gland epithelial cells, which release chemoattractants, controlling the migration of polymorphonuclear neutrophil leukocytes (neutrophils) to the inflammatory region (Paape et al., 2002; Langrová et al., 2005; Li et al., 2014; Bassel and Caswell, 2018). As already mentioned, the level of neutrophils in the blood of cows in the puerperal period is highly heritable and is associated with susceptibility to clinical mastitis (Burvenich et al., 2000; König and May, 2019).

Macrophages are mainly activated by bacterial lipopolysaccharides (LPSs) and interferon-γ (IFN-γ) (Toman et al., 2009; Elazar et al., 2010). Bacterial LPSs (mainly Gram-negative bacteria) induce an inflammatory response (Bannerman et al., 2003; He et al., 2017) initiated by LPS/TLR4 signalling (Li et al., 2015) by alveolar macrophages (Persson et al., 1996). This signalling induces

an increased release of inflammatory cytokines to prompt polymorphonuclear neutrophil (PMN) migration into the mammary gland (Persson et al., 1996; Zhao and Lacasse, 2008; Bassel and Caswell, 2018). In the mammary gland, the total number of somatic cells and the basal PMN level in milk increases by 5 %–25 % to approximately 90 % (Leitner et al., 2000).

Amino acids, especially methionine (Met) and arginine (Arg), are important for functional immunity and recovery during infectious diseases. However, stress or inflammation reduces their intake (Wu et al., 2016). Studies have reported more effective pro-inflammatory responses elicited by the LPS challenge, with increased Arg supply (Wu et al., 2016; Zhao et al., 2018).

The second line of defence consists of a network of memory cells and immunoglobulins reacting with the first line of defence. An important factor in the rapid and adequate response of the organism to infection is the regulation of the immune response. Rapid elimination of pathogenic microorganisms in the mammary gland is essential, whereby damage to the mammary gland by bacterial toxins and oxidative products released by PMNs is minimized. The number of circulating PMNs that affects the susceptibility of dairy cows to mastitis is highly hereditary and is closely related to their susceptibility to clinical mastitis. In particular, the period around birth is critical, during which time the number of circulating PMNs in the blood is reduced (Paape et al., 2002; Vangroenweghe, 2005).

Macrophages are the key cells of innate immunity, and their main role is regulation. They are multifunctional cells involved in the management of hematopoiesis, homeostasis, wound healing, the destruction of microorganisms and the regulation of inflammation (through the synthesis of acute tissue proteins), the removal of dead cells and tissue regeneration, cytotoxic reactions, antigen presentation to T lymphocytes, response regulation of T lymphocytes, and the regulation of tolerance (Toman et al., 2009; Sharifi et al., 2019).

Although their bactericidal competence appears to be limited, they actively phagocytize, indicating that they can recognize bacteria. Opsonin receptors (the IgG1 Fc receptor and the IgG2 Fc receptor) have been confirmed on milk macrophages. Macrophages are stimulated by *Escherichia coli* lipopolysaccharides (LPSs) and respond by secreting interleukins (IL-1). Macrophages in cattle express a gene that produces the cluster of differentiation 14 (CD14) protein. It is deposited on the surface of the membrane as membrane-bound CD14 (mCD14) or excreted into the environment. "Resident" neutrophils could also be involved in the signalling of bacterial elements, although this signalling has not been demonstrated. Both cell types can release chemotactic and inflammatory mediators that seek out and recognize bacteria (Paape et al., 2002; Rainard and Riolett, 2006; Gilbert et al., 2013).

Monocytes and neutrophils penetrate from the capillaries into the tissues by diapedesis and adhere to the endothelium (Reece, 2011; Rudziak et al., 2019). In tissues, monocytes are then able to differentiate into macrophages. After phagocytosis of apoptotic neutrophils, anti-inflammatory cytokines (TGF-β and IL-10) are produced by macrophages. The process of gene transcription of anti-inflammatory cytokines is inhibited, and the process of resolving inflammation begins (Toman et al., 2009).

Cytokines are low-molecular-weight proteins or glycoproteins (Su et al., 2016). They arise from the activity of cells of various tissues and organs, although primarily from cells of the immune system. Cytokines serve to communicate between cells and form the so-called "intercellular information network". In certain microenvironments, there is a greater number of cytokines that can interact (e.g. potentiate, inhibit, and induce synthesis of other cytokines). The biological activity of cytokines is the result of mutual current local concentration, target cell type, 19 regulatory factors, genetic predisposition, current individual health, age, nutrition, sex, and drugs, among others. Cytokines are classified into three classes according to their effects on interferon-α (INF-α; antiviral and antiproliferative effect), interferon-γ (INF-γ; immunostimulatory effect), and interleukins (IL-2; T-cell stimulation). IFN-γ is the major cytokine of the immune response. It is involved in the activation of cytotoxic T cells, natural killer (NK) cells, and macrophages (Mladosievicova et al., 2015).

Both bacterial host factors and the immune response contribute to tissue epithelial damage. With the increased migration of immune cells into the mammary gland and the disruption of the blood–milk barrier, there is greater damage to the mammary gland epithelium. The degradation of the extracellular matrix can lead to the death of epithelial cells. In addition, polymorphonuclear neutrophils and macrophages can damage mammary tissue by releasing reactive intermediates of oxygen and proteolytic enzymes. In vitro and in vivo studies have suggested that the use of antioxidants and other preservatives in mastitis control programmes helps alleviate secretory cell damage, thereby reducing subsequent milk loss (Zhao and Lacasse, 2008).

3 Prevention of mastitis

It is important to focus on preventing the development of mastitis, both clinical and subclinical. Among the options for prevention and the procedure of minimizing inflammation of the mammary gland, we include (1) the minimization of bacteria in the environment by maintaining environmental hygiene, especially during the pre-and postpartum periods, which are the periods of the highest risk of intramammary infection with coliform bacteria (Herrera, 2009); and (2) improving nutrition, housing, and the environment, and, thus, minimizing the stress, the negative-energy balance, and the physiological imbalance on dairy cattle (Pyorala, 2002; Ing-

vartsen and Moyes, 2013; Esposito et al., 2014; Gombart et al., 2020).

One means of preventing mastitis is vaccination, as it strengthens the immune system of animals and reduces the incidence of mastitis; moreover, if disease occurs, vaccination can prevent a severe course (Herrera, 2009; Rainard et al., 2018). Vaccination is the controlled exposure of a host defence system to a pathogen or toxin in an attenuated form to teach the immune system to recognize specific antigens for that pathogen. This speeds up the immune system response if a particular antigen reappears, preventing the pathological course of the infection (Erskine, 2012). However, vaccine development has been difficult because the immune response to a natural infection does not effectively protect against subsequent infection. In recent years, reports have been published describing prototype vaccines against *Streptococcus uberis*, *Streptococcus agalactiae*, *Escherichia coli*, and *Staphylococcus aureus*. These vaccines are based on either the use of a bacterial extract that contains antigens from the most common bacterial serotypes or that contains only cell fragments (antigens) that may or may not be conjugated to carriers to increase their immunological efficacy (Talbot and Lacasse, 2005; Rainard et al., 2018). Vaccination does not prevent intramammary infection. The immunization performed does not reduce the prevalence of Gram-negative bacterial infection, but it does reduce the incidence of clinical mastitis and the severity of the disease. As for the difference between vaccinated and non-vaccinated cows, 66.7 % of coliform clinical intramammary infections were reported in unvaccinated cows, whereas they were reported in only 20 % of vaccinated cows (Hogan and Smith, 2003).

There are also available commercial monovalent (against one pathogen) and polyvalent (against more than one pathogen) vaccines produced against *E. coli* and coliform bacteria, for example ENVIRACOR J-5, ENDOVAC-Dairy coliform vaccine, J-VAC, and STARTVAC (Guccione et al., 2017). Another type of vaccine is the so-called autogenous vaccine, which is prepared from native strains isolated from cows in the herd suffering from mastitis and then applied to the herd. These vaccines are mainly produced against the *S. aureus* and *S. uberis* pathogens. These vaccines are not commercial; however, commercial autogenous mastitis vaccines are also available, e.g. BESTVAC and IDT (Ismail, 2017).

The next type of vaccine against mastitis is the so-called pegbovigrastim (commercially Imrestor) (Ruiz et al., 2017). This vaccine contains granulocyte colony-stimulating factor (G-CSF). G-CSF is a cytokine responsible for neutrophil maturation and the stimulation of neutrophil release from bone marrow stores (Mayadas et al., 2014). This vaccine is used in the transition period, during which time the number of neutrophils in the blood is reduced. The recommended application is 7 d before the expected delivery and on the day of delivery. Studies have shown that there is an increase in the number of neutrophils, which remain elevated even after calving (Ruiz et al., 2017).

4 Genetic selection

It is generally known that there is a positive genetic correlation between milk production and the incidence of mastitis (as well as somatic cell scores – SCSs), estimated to range from 0.24 to 0.55 (mean of 0.43). In the literature, the genetic correlation between milk production and clinical mastitis ranges from slightly negative to 0.66, with the average of seven studies being 0.30 (Heringstad et al., 2000; Vallimont et al., 2009; Chegini et al., 2018).

If the influence of mastitis is ignored in the breeding programme, the emphasis on milk production will negatively affect mastitis resistance. Studies have shown that ignoring the effects of mastitis in the steaming plan results in a genetic increase in mastitis cases of 0.02 per cow per year, assuming a genetic correlation between mastitis and milk yield of 0.30. Therefore, the inclusion of resistance in breeding programmes is needed in order to suppress undesirable correlations resulting from selection based only on milk production. The choice to increase mastitis resistance contributes to reducing production costs and maximizing overall economic profit (Heringstad et al., 2000). There is considerable genetic variation for clinical mastitis. Health records relating to udder disease may be used to genetically evaluate cow health (Vallimont et al., 2009). Mastitis is the most common reason for the use of antibiotics in lactating dairy cows. Genetic improvement in mastitis resistance or vaccination against mastitis can reduce the need for treatment (and, consequently, the use of antibiotics) as well as reducing the risk of bacterial resistance to antibiotics. The high consumption of antibiotics is a global problem (Heringstad et al., 2000; Tiwari et al., 2013; Cheng and Han, 2020).

The mapping of genes and variants involved in innate immune responses is essential in order to understand this inflammatory disease and to identify the potential genetic markers of mastitis resistance. The benefit of the subsequent generation of dairy cows would be to obtain favourable alleles promoting greater resistance to infection and a reduction in antibiotic use (Alain et al., 2009; Mahmood et al., 2017).

Mastitis resistance is a complex system involving a variety of pathways, under the influence of a large number of candidate genes (Karthikeyan et al., 2016). Low heritability (Pighetti and Elliott, 2011), high environmental impact, and differences in farm management make it difficult to identify the linkage between genetic variants and mastitis resistance. These causes have led researchers to attempt the identification of genes and genetic variability related to mastitis resistance in dairy cattle in individual studies (Sahana et al., 2014; Tiezzil et al., 2015).

Researchers have focused on identifying mastitis-related genes through activities such as pathogen recognition, leukocyte recruitment, migration, elimination, and pathogen differentiation. For mastitis resistance, it is difficult to identify candidate genes that determine traits due to the polygenic nature of the disease. Complex properties are mainly con-

trolled by minor genes (many low-effect genes), compared with a limited number of major genes (Hayes et al., 2010; Karthikeyan et al., 2016).

A database of candidate bovine genes and genetic markers for milk production and mastitis has been compiled to provide an integrated research tool, containing a variety of research and information supporting a genomic approach to lactation study, udder development, and health. The database includes 943 genes and genetic markers involved in the development and function of the mammary gland (Ogorevc et al., 2009).

5 Lactoferrin

Lactoferrin (*LTF*) is one of the minor genes that may affect the health of the mammary gland. Dairy cows with clinical and subclinical mastitis show an average higher concentration of lactoferrin in milk than healthy cows (Musayeva et al., 2018). These data correlate with the SCS (Hagiwara et al., 2003). Researchers state that there is a possible relationship between *LTF* and dairy mastitis (Soyeurt et al., 2012; Mohammadnezhad et al., 2021).

Research also suggests that the *LTF AA* genotype may be associated with increased resistance to intramammary infections in Awassi sheep (Alekish et al., 2019). Sharma et al. (2015) report that the *LTF* gene polymorphism showed a significant association with the somatic cell count (SCC). Dairy cows with genotypes *GG* and *GC* showed a higher SCC than cows with genotype *CC*. Further research on dairy cows has shown that the *CC* genotype is found with a frequency of 0.33; dairy cows with subclinical mastitis showed both the *GG* (0.17) and *GC* (0.50) genotype, and cows with clinical mastitis showed the *GC* genotype.

Lactoferrin is a multifunctional iron-binding glycoprotein. It is synthesized in the epithelium of the mammary gland and has bactericidal and bacteriostatic effects (Huang et al., 2012; Shimazaki and Kawai, 2017). It is contained in body secretions – milk and fluids of the intestinal tract – where it represents an important part of the host's first line of defence (Harmon and Newbould, 1980). Lactoferrin is contained in secondary granules and large granules of neutrophils. It is released in the infected mammary gland, where it makes up about 5 % of milk during acute inflammation (Harmon and Newbould, 1980; Shimazaki and Kawai, 2017), and ensures the migration, maturation, and function of immune cells (Shimazaki and Kawai, 2017).

6 Keratin 5

Keratin 5 (*KRT5*) is involved in the formation of the teat plug. Teat sphincters isolate the inside of the mammary gland from the outside environment and prevent the passage of external contaminants and microorganisms. Damage to this structure is accompanied by an increased incidence of mastitis (Blowey and Edmondson, 2010). The teat canal is lined with folds of keratinized skin epidermis, covered with a thin layer of lipids of esterified and non-esterified fatty acids (myristic, palmitoleic, and linoleic acids). Keratin and the lipid-lined teat canal have antibacterial properties, which provide (along with the plug) another physical barrier, preventing bacterial migration into the mammary cistern (Sordillo and Streicher, 2002; Blowey and Edmondson, 2010). In addition, certain keratin-associated cationic proteins may bind to pathogenic microorganisms of mastitis, increasing their sensitivity to osmolarity modifications (Paulrud, 2005).

Clinical mastitis arising during the dry period was shown to be most common (97 %) in quarters with open teat canals (Williamson et al., 1995). During lactation, dilatation of the teat canal sphincter after milking may compromise the animal's first line of immune defence, thereby increasing their susceptibility to udder invasion and colonization by a wide range of microorganisms from various sources (Jones and Bailey, 2009). For this reason, the teat canal and keratin plug are considered important barriers against intramammary infection (Freu et al., 2020).

There are many microorganisms, which are a potential source of mammary gland infection, on the skin surface of the teat and keratin from the teat canal (Nickerson, 1991). The results in the literature support the plausibility of the theory that the reduction in SCSs is due to increased keratin synthesis and improved immune defence (Jones, 1995). Calcium is a factor that increases differentiation and promotes the expression of differentiation-specific keratin genes (Paulrud, 2005; Bikle et al., 2012).

The mammary gland becomes infected by environmental pathogens, especially during the dry period (Bradley and Green, 2004). The susceptibility of the udder during this period is attributed to the failure of the formation of the keratin plug in the teat canal in the early stage of the dry period or its loss in the late stage of the dry period (Williamson et al., 1995; Bradley and Green, 2004; Paulrud, 2005). The resulting infections during the dry period are especially pronounced after calf birth. It is reported that 50 % of environmental mastitis diagnosed within 100 d of calving occurred during the dry period (Bradley and Green, 2000, 2004).

At the moment of teat canal disruption/crossing by invasive microorganisms, especially bacteria, access to the milk is gained despite a wide range of innate and acquired immune responses (Sordillo and Streicher, 2002). The intramammary environment provides the bacteria with a suitable environment, as evidenced by the ability of most mastitis pathogens to proliferate rapidly despite the inflammatory responses of the immune system (Rainard, 2017; Derakhshani et al., 2018a).

7 Interferon-γ receptor 2 (IFNGR2)

Interferon, induced during mastitis, is secreted by helper CD4+ and CD8+ lymphocytes, and promotes cell-mediated immunity through the increased phagocytic and antigenic capacity of macrophages (Bannerman, 2009). Interferon-γ receptor 2 is an important cytokine that mediates the inflammatory response during infection (Bannerman, 2009; Pant et al., 2011; Mann et al., 2019).

Elevated IFNG levels are associated with the onset of intramammary infection (Bannerman et al., 2004a, b; Pant et al., 2011) Increased IFNG is accompanied by increased production of nitric oxide monocytes. Oxidizing molecules are crucial in the defence against bacteria, although they are concurrently harmful to host tissues (Burvenich et al., 2003).

8 Lactoglobulin (LGB)

The β-lactoglobulin (*BLG*) gene is a protein commonly present in milk at normal pH (Singh and Gallier, 2017). BLG is a whey protein and member of the lipocalin family present in cow's milk that easily adheres to hydrophobic molecules. The *BLG* gene binds iron through siderophores; thus, it is fundamentally involved in the immune response in the fight against pathogens in milk (Alim et al., 2015). Studies have reported that bovine prolactin (PRL) and β-lactoglobulin are associated with milk quality and the SCC in dairy breeds (Luhar et al., 2006; Sing et al., 2015). The increased SCC results in a decline in the amount of *BLG* gene in milk (Litwińczuk et al., 2011). *BLG* was highly expressed in healthy udders; it is one of the major whey proteins of ruminants: in combination with α-LG, it makes up approximately 14 % of the protein in milk (Coulon et al., 1998; Kamiński et al., 2007).

BLG antagonizes the *LTF* gene. However, together they have antibacterial effects. As a result, their immune mechanisms help protect the mammary gland against bacterial infection (Chaneton et al., 2011; Singh et al., 2014). The *BLG* gene has an inhibitory effect on mastitis agents such as *Streptococcus* spp. and *Staphylococcus aureus*; thus, it reduces the rate of spread of infections, thereby contributing to improving milk quality (Chaneton et al., 2011; Ateya et al., 2016).

Singh et al. (2015) observed that genotypes *AB* and *BB* *BLG* had a significant ($p < 0.05$) effect on overall milk yield and maximum yield compared with genotype *AA*. Thus, genotypes *AB* and *BB* may be favourable to better milk production characteristics. *BLG* polymorphism has previously been associated with the SCC in dairy cows (Luhar et al., 2006). Kriventsov et al. (1975) reported a lower milk microbiota in *BLG B*-expressing animals than in *BLG A*-expressing animals; in contrast, Luhar et al. (2006) found that the *B* allele is associated with mastitis, and Singh et al. (2015) agreed that the SCC of *AA* genotype cows is significantly ($p < 0.05$) lower than in *AB*- and *BB*-genotype cows. Therefore, the *AA* group of animals may be favourable in terms of the selection of animals resistant to mastitis.

9 Casein (CSN)

The most expressed genes in the healthy mammary gland are β-casein (*CSN2*), κ-casein (*CSN3*), α S1 casein (*CSN1S1*), and α S2-casein (*CSN1S2*). β-casein and whey are the two main protein groups in cows' milk (Kamiński et al., 2007). Although *CSN1S1* and *CSN2* are not the most expressed, they are still highly expressed in the mastitis mammary gland (Wentato et al., 2019). These expression values were found to be lower compared with a healthy mammary gland (Coulon et al., 1998; Kamiński et al., 2007). In the mastitis mammary gland, *CSN3* genes were not in the highly expressed group. A possible consequence of the lower expression of these genes is that the protein content of mastitis cows' milk would be reduced, which could have economic consequences (Asselstine et al., 2019). Similar results have been reported by Heringstad et al. (2005), who found an antagonistic genetic relationship between clinical mastitis and protein yield. The percentage of protein and fat correlates positively (0.67); this means that if the protein content of milk decreases, the percentage of fat also decreases, which has a greater impact on the profitability of cows' milk (Carlén et al., 2004).

10 *CD14*

The *CD14* gene product is an important part of host innate immunity. It provides sensitivity to lipopolysaccharides to individual cell types, such as neutrophils, monocytes, and macrophages (Wang et al., 2006; Ibeagha-Awemu et al., 2008; Wu et al., 2019b). The *CD14* gene product mediates host defences against Gram-negative bacterial infections, provides immunity against viral infections, and determines the effect of changes in protein expression on the surface of monocytes and neutrophils in healthy dairy cows. It occurs on the cell membrane of monocytes and, to a lesser extent, on the membrane of neutrophils. *CD14* ensures the sensitivity of cells to lipopolysaccharide (LPS), including epithelial cells and endothelial cells (Wang et al., 2002; Ibeagha-Awemu et al., 2008; Wu et al., 2019b). LPS, when bound to host membrane proteins such as CD14, causes the release of proinflammatory cytokines that recruit neutrophils as an early innate immune response. Excessive levels of proinflammatory cytokines cause tissue damage and compromise the mammary gland function (Wall et al., 2009; Ranoa et al., 2013; Wu et al., 2019b).

There are two forms of the gene product, the membrane-bound form of mCD14 and the soluble form of sCD14 (Ibeagha-Awemu et al., 2008; Hirsch et al., 2021). sCD14 is thought to be derived from monocytes by direct exocytosis and from proteolytic cleavage of mCD14 on the cell surface. Researchers believe that soluble and membrane-bound forms

of CD14 compete for LPS binding. The result is increased concentrations of sCD14 modulating humoral and cellular responses that otherwise elicit when LPS and associated proteins bind to membrane-bound CD14 and interact with TLR4 (Wall et al., 2009; Wu et al., 2019b). It has been shown that sCD14 and LPS-binding protein (LBP) can bind LPS and transport it to high-density serum lipoproteins, thereby reducing (detoxifying) serum LPS levels. The protective nature of sCD14 has been demonstrated in challenge studies in cows fed exogenous sCD14. Based on the demonstrated ability of sCD14 to attenuate innate immune responses to LPS exposure, it is hypothesized that increasing the concentration of sCD14 in the mammary glands would increase the sensitivity of the immune control and, thus, modulate the immune response that acts during mastitis. The concentration of sCD14 can be increased by introducing a transgene encoding sCD14 expressed in mammary epithelium during lactation. Increasing innate immunity against the microorganism by increasing the recognition of the host immune response of the nucleus of the conserved molecule to the pathogen (e.g. LPS) would be less likely to develop antibiotic resistance in microorganisms (Lee et al., 2003; Wall et al., 2009). Recombinant bovine sCD14 can increase the sensitivity of mammary epithelial cells to low LPS concentrations in vivo and in vitro, suggesting that sCD14 plays an important role in initiating host responses to Gram-negative bacterial infections (Wang et al., 2002; Ibeagha-Awemu et al., 2008; Védrine et al., 2018).

The main component of the outer membrane of Gram-negative bacteria, such as *E. coli*, is LPS (also called endotoxins) (Whitfield and Trent; 2014; Sperandeo et al., 2017), which occupies 75 % of the bacterial surface, with the remaining part being made up of proteins (Nikaido, 1987). The innate immune system is easily prepared to detect LPS and responds to Gram-negative bacterial infections (Wall et al., 2009; Faraj et al., 2017). LBP and CD14, among other helper molecules, facilitate LPS activation of TLR4 and adequate regulation of proinflammatory cytokine expression, including tumour necrosis factor-α (TNF-α) and interleukins (IL-1, IL-6, and IL-8) (Wall et al., 2009; Rossol et al., 2011; Akhtar et al., 2020). These cytokines, in turn, contribute to the recruitment and activation of neutrophils, which are among the first leukocytes to prevent the host from being attacked by bacteria. Excessive local release of proinflammatory cytokines, including TNF-α, in response to LPS, can cause local tissue damage, while excessive systemic release can lead to toxic shock, which can be fatal in severe cases (Wall et al., 2009; Akhtar et al., 2020).

During the peripartum period, individual changes in cow response were noted for Gram-negative and Gram-positive bacterial infections. Therefore, there are various variations in *CD14* gene sequences that may play an important role in the presentation of CD14 molecules and, thus, sensitivity to LPS (Wang et al., 2002; Ibeagha-Awemu et al., 2008; Akhtar et al., 2020).

SCCs in mammary glands injected with recombinant sCD14 were increased earlier compared with cows injected with saline (in the same regions). This was attributed to the binding of LPS produced by *E. coli* to sCD14 and the binding of the LPS/rbosCD14 complex to epithelial cells. This binding initiated the recruitment of somatic cells to the affected regions and the elimination of *E. coli*. sCD14 quarters remained normal compared with the two quarters without its application. It follows that intramammary injection of sCD14 prevented *E. coli* infection (Wang et al., 2006; Védrine et al., 2018).

11 *CXCR1*

The innate immune response plays an important role during bacterial infections. Inherent immune efficacy relies on the expression of many genes and is associated, inter alia, with neutrophil activity (Pawlik et al., 2015; Alhussien and Dang, 2020). The interleukin 8 (IL-8) α receptor, encoded by the *CXCR1* gene, is present on the neutrophil surface and binds pro-inflammatory IL-8 with high affinity (Teijeira et al., 2020). Hence, the gene for bovine *CXCR1* offers the potential for use as a marker of bovine mastitis. Previous studies on *CXCR1* polymorphism have yielded conflicting results (Pawlik et al., 2015; Alhussien and Dang, 2020).

Pro-inflammatory cytokines can induce an antimicrobial response to epithelial cells and ensure the migration of neutrophils and dendritic cells to the site of infection (Kolls et al., 2008; Huang, 2021). As noted above, IL-8 plays an important role in the migration of neutrophils and other inflammatory cells from the blood to the mammary gland (Swain et al., 2014). In addition, other pathways directly involved in the immune response (i.e. IL-6 signalling and IL-10 signalling) are represented overall and in the late phase of the response (Lewandowska-Sabat et al., 2012).

Increased expression of IL-10 and IL-6 has been reported at an earlier stage of the response to mammary gland infection. IL-6 regulates cells to control and terminate the inflammatory response during late-stage mastitis infection (Lewandowska-Sabat et al., 2012).

Rambeaud and Pighetti (2005) consider that the incidence of the *CC* genotype of the *CXCR1* gene is accompanied by impaired neutrophil migration in vitro, which explains the physiological mechanism of genetic differences in susceptibility to mastitis. In the herd studied, the highest SCC was more associated with the *CXCR1 C* allele than with the *G* allele, although the differences between genotypes were insignificant.

A study by Pawlik et al. (2015) reported that this polymorphism did not affect sensitivity to mastitis, with respect to SCS or *S. aureus*. The frequency of the *C* allele in the population was relatively low (i.e. 28.73 %). In other studies, the frequency of the *CXCR1 C* allele ranged between 36 % and 67 % for Holstein cattle. Only two cows carried the *CXCR1*

CC genotype; this could mean that the *C* allele of the *CXCR1* gene was excluded from the herd studied.

One should bear in mind that a low daily SCC test is not necessarily associated with a reduced incidence of mastitis. As mentioned earlier, the *CC* variant of the *CXCR1* polymorphism is associated with impaired neutrophil migration, and this could mean that a lower SCC day in cows of this genotype is associated with chronic mastitis due to low neutrophil counts and/or impairment in somatic cells. Not all previous studies have linked the *C* allele of *CXCR1* to susceptibility to mastitis (Pawlik et al., 2015). Galvão et al. (2011) found that cows with the *CXCR1 CC* or *CG* genotype have a lower incidence of clinical mastitis compared with the *GG* genotype, which contradicts the results of Youngerman et al. (2004), who did not find a significant association between clinical mastitis, SCC, or SCCs and the *CXCR1* genotype. However, Youngerman et al. (2004) did find an association between higher SCS and the heterozygous *GC* genotype compared with the *GG* genotype in the Holstein cow population. Interestingly, Holstein cows with a high SCS have been linked with reduced daily fat production in a farm study (Youngerman et al., 2004).

12 Others genes

A total of 117 candidate single-nucleotide polymorphisms (SNPs) and 27 quantitative trait loci (QTLs) associated with mastitis resistance within a population of phenotypically well-characterized dairy cattle were identified. The three QTLs most suggestive of genome-wide significance are located on BTA26 and overlap the *SORCS3* gene and a previously identified QTL for teat length (Kurz et al., 2019).

SNPs UA-IFASA-8493 and ARS-BFGL-NGS-5022, found on BTA14 and BTA15, are found within stathmin-like 2 (*STMN2*) and the nuclear mitotic apparatus genes for protein 1 (*NUMA1*). They are considered to be new polymorphisms related to SCSs (Iung et al., 2015). Stathmin is involved in cell-cycle regulation (Rubin and Atweh, 2004).

ARS-BFGL-NGS-117704 is located on BTA6, and is an SNP associated with the SCS (Meredith et al., 2012; Sahana et al., 2013). This region of the chromosome corresponds to the interleukin 8 (*IL8*) gene, which is involved in the immune response, especially in the early stages, against bacterial invasion (Hedrick et al., 1999).

These SNPs were located in quantitative trait locus (QTL) mapping studies on BTA5, BTA6, BTA8, BTA11, BTA18, and BTA23 were frequently associated with the SCC (Lund et al., 2008; Hu et al., 2019).

13 Conclusions

Dairy immunity is currently one of the most important points of research in the context of global pressure to reduce the consumption of antibiotics. It occurs through a variety of processes and signals. Therefore, it is currently a scientific challenge to reveal the most important genes and gene combinations in order to ensure high resistance among animals.

Data availability. The data are available from the corresponding author upon request.

Author contributions. MZ and ZH conceptualized the study, prepared the original draft of the paper, and acquired funding. PD and IV were responsible for carrying out validation. MZ undertook the formal analysis and investigation and was responsible for acquiring resources. LL, IN, PD, and IV reviewed and edited the paper. ZH supervised the study. All authors read and agreed upon the published version of the paper.

Competing interests. The contact author has declared that none of the authors has any competing interests.

Ethical statement. This experiment was conducted in compliance with the Czech National Council Act No. 246/1992 Coll. to protect animals from cruelty and with the amended Act No. 162/1993 Coll. This work was approved by the Commission to protect Animals against Cruelty of Mendel University in Brno and the Ministry of Agriculture of the Czech Republic (statement no. 35537/2020-MZE-18134, serial no. MZe 2165).

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