Monitor and modulate immune responses in bovine mastitis to improve antibiotic stewardship and efficiency in milk production

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抗菌性物質の適正使用と乳生産効率の改善に向けた
牛乳房炎における免疫応答の監視と調節

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Abbreviations

Abbreviation	Explanation
A/G ratio	Albumin/Globulin ratio or Albumin-to-Globulin ratio
ABH group	Antibiotic-treated, Healthy group
ABI group	Antibiotic-treated, Infected group
ABIS group	Antibiotic-treated, Infection-Suspected group
BDCT	Blanket Dry Cow Therapy
CI	Confidence Interval
CMT	California Mastitis Test
CNS	Coagulase-Negative *Staphylococci*
DHI	Dairy Herd Improvement (program)
DIM	Days In Milk
DSCC	Differential Somatic Cell Count
EMM	Estimated Marginal Means
GAM	Generalized Additive Model
GNR	Gram-Negative Rods
H group	Antibiotic-untreated, Healthy group
Ig(s)	Immunoglobulin(s)
IMI	Intramammary Infection
MAC	Macrophage proportions (in somatic cells); equivalent to 100-DSCC
NAGase	N-acetyl-β-D-glucosaminidase
NS (NG)	No significant growth
OR	Odds Ratio
OS	Streptococci other than *Streptococcus uberis* and *Streptococcus dysgalactiae*
PMN(s)	Polymorphonuclear leukocyte(s)
SA	*Staphylococcus aureus*
SCC	Somatic Cell Count
SCS	Somatic Cell Score; equivalent to $\log_2(SCC/100{,}000) +3$
SD	*Streptococcus dysgalactiae*
SDCT	Selective Dry Cow Therapy
Str.	Streptococci and Streptococcus-like bacteria
SU	*Streptococcus uberis*
T_c	Cytotoxic T cells
T_h	Helper T cells
WBC	White Blood Cell

1. Research Background

Mastitis is one of the commonest diseases in dairy cows, impacting animal health and milk production significantly. A majority of antibiotics given to dairy cows are thus for controlling mastitis. However, huge antibiotic use in animal production has raised public concerns over the emergence of antimicrobial resistance. On the other hand, current antibiotic therapies for mastitis control are fairly inefficient. This thesis aims to improve antibiotic stewardship in mastitis control by monitoring or modulating the immune responses of cows.

1.1. Mastitis and its Impact

Mastitis refers to the inflammation of mammary glands. The inflammation response was usually triggered by invading microorganisms into the glands. Common mastitis pathogens include gram-positive bacteria such as *Staphylococcus aureus* (**SA**), *Streptococcus* spp., *Enterococcus* spp., *Corynebacterium* spp., and coagulase-negative staphylococci (**CNS**), gram-negative bacteria such as *Escherichia coli, Klebsiella* spp., *Enterobacter* spp., and *Serratia marcescens,* as well as *Mycoplasma* spp., such as *Mycoplasma bovis* (NMC, 2017). Occasionally, yeast such as *Candida* spp. and algae such as *Prototheca* spp. also cause intramammary infections (**IMI**) and the consequent mastitis. These pathogens come from either the udder of infected cows (i.e., SA, *Streptococcus agalactiae*, *Corynebacterium* spp., and *Mycoplasma* spp.) or the environment (i.e., most of the other pathogens), leading to a broad classification of pathogens based on the transmission route, namely *contagious* or *environmental pathogens*. As a consequence of the diverse transmission route of pathogens, no single measure can completely prevent the occurrence of mastitis. Generally, mastitis occurred more frequently after parturition (Sordillo and Streicher, 2002; Waller, 2002), which is related to IMI occurring during the dry period (Green et al., 2002) and impaired immunity of cows during the periparturient period (Burton and Erskine, 2003), highlighting the importance of dry period management in mastitis control.

The differences in pathogenicity between causative pathogens lead to varied clinical presentations in mastitis. Clinical signs in mastitis can range from systemic (hyperthermia, anorexia, and depression) to local (edematous udders), or presented only in milk (flakes or clots in milk), resulting in a simple classification based on the severity of the disease (Wenz et al., 2001a; Wenz et al., 2006). Besides *clinical mastitis*, mastitis can be either asymptomatic, namely *subclinical mastitis*, which we could not identify through clinical signs, but only through cow's immune responses, for instance, by somatic cell count released in milk. Gram-negative coliforms, such as *E. coli,* were more likely to cause acute, severe clinical mastitis, but

Corynebacterium spp. and CNS instead mostly caused subclinical or mild mastitis (Oliveira et al., 2013). Studies have shown that mastitis caused by SA and *Streptococcus uberis* (**SU**) is hard to cure and likely to recur. On some occasions, the intention to treat mastitis caused by SA has thus been discouraged (Zadoks et al., 2001a, b; Barkema et al., 2006).

Distinct characteristics between pathogens consequently impact milk production loss caused by mastitis (Gröhn et al., 2004; Schukken et al., 2009b; Hertl et al., 2014; Heikkilä et al., 2018) and the survival of animals (Bar et al., 2008; Hertl et al., 2011; Cha et al., 2013). Based on the pathogenicity, mastitis pathogens can be roughly categorized into *minor* and *major pathogens*. Milk yield losses caused by major pathogens, including *E. coli,* SA, and *Streptococcus* spp*.,* are often greater than the losses caused by minor pathogens, such as *Corynebacterium* spp. and CNS (Gröhn et al., 2004); however, due to the high prevalence of minor pathogens, their impacts should not be underestimated (Heikkilä et al., 2018). Milk production loss caused by mastitis also depends on the cow's status, namely its parity and lactation stage. Mastitis occurring at the early and end of lactation caused more milk losses than that occurring in mid-lactation, and high-parity cows lose more due to their great milk yield (Dürr et al., 2008; Hagnestam-Nielsen et al., 2009; Gonçalves et al., 2018).

According to Hogeveen et al. (2019), 20~40% of dairy cows encountered one or more cases of clinical mastitis in their life, and whenever, around 10~30% of lactating cows are suffering from subclinical mastitis (indicated by somatic cell count). For one case of clinical mastitis, it costs $US325.76 for primiparous cows or $US426.50 for multiparous cows (Liang et al., 2017), although the estimation ranges from $US3.80 to $US360 between studies (Halasa et al., 2007; Bar et al., 2008; Cha et al., 2011). Mastitis costs farmers through the impairment of milk production and quality, drug and veterinarian expenditures, milk discarded due to antibiotics, labor costs, culling of cows, and so on (Halasa et al., 2007). Among these, the major cost results from reductions in milk production (an average of 58% of total losses), followed by the culling of cows (an average of 26% of total losses). On average, mastitis causes a $US131 loss per cow per year as estimated (Hogeveen et al., 2019). Generally, the high frequency and significant impact of mastitis cause it one major concern in dairy farming, in either the aspect of economics or animal welfare (Ruegg, 2017b).

1.2. Mastitis Control and Recent Focuses on Antibiotic Stewardship

In response to the great magnitude of mastitis impact, practitioners have devoted significant efforts to controlling the disease. In the 1960s, National Mastitis Council proposed the "Five-Point Plan" targeting the control of contagious mastitis (i.e., mastitis caused by *Streptococcus agalactiae* and SA), whose points include: (i) disinfect teats after milking; (ii)

administrate antibiotics to *every* quarter at dry-off, namely blanket dry cow therapy (**BDCT**); (iii) record and treat clinical cases; (iv) cull chronically infected cows; (v) maintain milking machine regularly (Ruegg, 2017b). Besides hygiene practices, antibiotics play an important role in mastitis control, either in dry (i.e., point ii) or lactating periods (i.e., point iii). Consequently, antibiotics used for controlling mastitis account for a majority of antibiotics use on dairy farms (Ruegg, 2017b). The Five-Point Plan indeed succeeded, as we can see the prevalence of contagious mastitis pathogens, especially *Streptococcus agalactiae,* has decreased in recent years (Makovec and Ruegg, 2003); however, the large number of antibiotics used in dairy farming have raised public attention, related to the concerns over the emergence of antimicrobial resistance (Rajala-Schultz, 2004; McDougall et al., 2021). In response to the concerns, researchers considered replacing BDCT with selective dry therapy (**SDCT**), which allocates antibiotics *selectively* to cows with higher risks of mastitis rather than all cows at dry-off (McCubbin et al., 2022). Researchers have also introduced protocols to treat clinical mastitis *selectively* based on on-farm culture results (Lago et al., 2011a, b; Ruegg, 2018) and developed vaccines protecting cows against gram-negative IMI (Hogan et al., 1992a, b). Most of these studies successfully demonstrated that antibiotic use can be reduced without sacrificing the udder health of animals. Nonetheless, unsatisfactory implementation rates and results of these practices have been observed in certain studies, as reviewed by Erskine (2012), Lago and Godden (2018), and McCubbin et al. (2022). Therefore, we require additional research to develop alternative measures for mastitis control, ensuring a balance between antibiotic stewardship and animal health.

1.3. Immune Responses and Immunological Indicators in Mastitis

Understanding immune responses toward IMI is the key to mastitis management, either for detecting, preventing, treating, or estimating the impact/prognosis of the disease. Different mastitis pathogens cause distinct immune responses (De Haas et al., 2002; Bannerman, 2009; Schukken et al., 2011). Varied immune responses toward IMI lead to different clinical presentations in mastitis. Due to the great variety of causative pathogens, immune responses in mastitis are difficult to clarify, and the host-dependent response toward the same pathogen further increases the challenge (Wenz et al., 2001a; Burvenich et al., 2003); fortunately, with significant research efforts in this area, nowadays we can get a glance into immune response patterns in mastitis via the following indices.

1.3.1. Somatic cell count and related tests

Somatic cell count

Somatic cell count (**SCC**) is an index incorporated regularly in dairy herd improvement (**DHI**) testing. Due to the accessibility and preciseness of this indicator, it has been the most important tool for detecting IMI for decades (MacLeod et al., 1953; Dohoo and Leslie, 1991; Pyorala, 2003; Adkins and Middleton, 2018). The somatic cells in milk consisted mainly of immune cells, namely polymorphonuclear leukocytes (**PMN**s), macrophages, and lymphocytes, in the addition to a small proportion of epithelial cells. In healthy cows, macrophages are the predominant cells, playing an important role in sensing invading pathogens and recruiting PMNs (Paape et al., 2002; Oviedo-Boyso et al., 2007; Elazar et al., 2010). As a result, udders with IMI are usually characterized by increases in SCC and PMN proportions. Due to the strong association between SCC and IMI, an elevation of SCC has been used to define subclinical mastitis. Studies showed that a cut-off of 200,000 cells/mL has the highest accuracy for detecting IMI (Dohoo and Leslie, 1991; Schepers et al., 1997), thus this value is commonly used to define subclinical mastitis in the literature (Harmon, 1994; Schukken et al., 2003; Rhoda and Pantoja, 2012; Adkins and Middleton, 2018). Nonetheless, besides the IMI status, SCC can be affected by cow's factors such as parity or stage of lactation (Harmon, 1994; Laevens et al., 1997; Schepers et al., 1997), as cows with higher parity or at the beginning or end of lactations would have a higher SCC. The necessity for varying the threshold for individual cows has thus been questioned (Bradley and Green, 2005).

The effect of IMI on SCC is pathogen-specific. Major pathogens usually cause a more pronounced increase in SCC than minor pathogens. Consequently, the sensitivity of the test for detecting major pathogen infections is higher than for detecting minor pathogen infections (Sargeant et al., 2001). De Haas et al. (2002) demonstrated that the lactation curves for SCC are affected by the occurrence of clinical mastitis, of which the effect is pathogen-specific. Before the outbreak of mastitis caused by *E. coli* and non-specific mastitis (i.e., culture-negative mastitis), SCC levels were within the normal range. Contrastingly, before the outbreak of mastitis caused by SA and streptococcal mastitis, continuous higher SCC levels were observed, suggesting that subclinical mastitis caused by these pathogens had been presented before the identification of clinical cases.

The SCC test implementation also enables us to quantify the negative impacts of subclinical mastitis easily. Numerous studies attempted to clarify the association between SCC levels, milk losses, and the related milk compositional changes (Jones et al., 1984; Hamann, 2002; Dürr et al., 2008; Forsbäck et al., 2009; Hagnestam-Nielsen et al., 2009; Hand et al., 2012;

Malek Dos Reis et al., 2013; Cinar et al., 2015; Bobbo et al., 2016; Gonçalves et al., 2018; Hadrich et al., 2018; Franzoi et al., 2020; Bonestroo et al., 2022). The negative association between subclinical mastitis and milk production is a result of the damage of inflammation and infection to the mammary glands, causing impaired milk synthesis and leakages of milk into the blood. Moreover, an elevation of SCC negatively impacts the reproductive performance of dairy cows (Lavon et al., 2011; Hudson et al., 2012; Albaaj et al., 2017; Rearte et al., 2022), probably resulting from the detrimental effects of inflammation on ovaries (Gilbert et al., 1990; Lavon et al., 2010). The impaired milk production and reproductive performance consequently result in shortened longevity of cows with elevated SCC (Caraviello et al., 2005; Sewalem et al., 2006).

Differential somatic cell count

As briefed in the last section (*Somatic cell count*), somatic cells in milk include PMNs, macrophages, and lymphocytes. Each type of cell plays a different role in immune responses toward IMI (Sordillo et al., 1997). When the udder is infected, the macrophages detect the infection and initiate inflammatory responses, recruiting PMNs (mainly neutrophils) to eliminate the invaders. Due to this, several studies suggest that changes in the subpopulation (composition) of somatic cells would be an alternative sign of mastitis, which may appear earlier than changes in SCC (Rivas et al., 2001; Schwarz et al., 2011; Pilla et al., 2013). Ideally, after eradicating the infection, the inflammation will be suppressed, and macrophages will take the responsibility for removing apoptotic neutrophils and repairing injured udder tissues caused by mastitis damage (Sladek and Rysanek, 2001; Sladek et al., 2005; Bratton and Henson, 2011). However, if PMNs fail to eradicate the infection, the inflammation will progress to chronic stages, thus we can observe both high levels of SCC and macrophage proportions in this stage of mastitis (Leitner et al., 2000; Rivas et al., 2001).

Despite the potential values provided by differentiating somatic cells, procedures for that had been laborious and costly, necessarily counting cells with either a microscope or a flow cytometer following immunostaining. In 2017, Damm et al. (2017) introduced a new technique and the corresponding trait, namely differential somatic cell count (**DSCC**). The technique can be incorporated into regular DHI testing, opening up opportunities for analyzing somatic cell composition on a large scale. DSCC (%) represents the combined proportion of PMNs and lymphocytes in somatic cells and thus 100 – DSCC approximates macrophage proportions (%). Since lymphocytes generally maintain a low proportion during mastitis, we can consider a change in DSCC as a consequent change in PMNs (Damm et al., 2017). Studies showed that DSCC level varies depending on the mastitis pathogen (Schwarz et al., 2019; Kirkeby et al.,

2020; Schwarz et al., 2020b; Pegolo et al., 2022) and mastitis stages (Kirkeby et al., 2021). Specifically, DSCC increases more markedly in the early stage of major pathogen infections and decreases in either the healing or chronic stage of mastitis.

As such, together with SCC, DSCC has been used for estimating mastitis impact and predicting the udder health status of cows. Schwarz et al. (2020a) investigated the association of these two indicators by categorizing cows into the following 4 udder health groups: healthy = SCC ≤ 200,000 cells/mL and DSCC ≤ 65%; suspicious mastitis = SCC ≤ 200,000 cells/mL and DSCC > 65%; mastitis = SCC > 200,000 cells/mL and DSCC > 65%; and chronic mastitis = SCC > 200,000 cells/mL and DSCC ≤ 65%. They showed that cows with high SCC and low DSCC (chronic mastitis) were with the least favorable milk yield and quality (Schwarz et al., 2020a) and were most likely to leave the herd (Schwarz et al., 2021). Lastly, Halasa and Kirkeby (2020) provide an overview of previous research on DSCC.

California mastitis test

The California mastitis test (**CMT**) was developed in the 1950s (Schalm, 1957). After its development, the test rapidly becomes the most popular cow-side test (i.e., performed immediately after collecting samples) for diagnosing mastitis, because it is easy and fast to be performed and has fair-to-good preciseness for detecting IMI (Barnum and Newbould, 1961; Wesen et al., 1968; Dingwell et al., 2003). To perform the CMT, we simply mix milk and test reagents, and after seconds we can tell whether the quarter is healthy by the CMT reaction. The CMT reaction is closely related to SCC. After mixing the CMT reagent with milk, it lyses somatic cell membranes, precipitating cell DNA, and resulting in elevated viscosity and gel formation. We can score this reaction to estimate SCC. Since the CMT is easy to perform, it has been applied widely for guiding SDCT (Bhutto et al., 2012; Swinkels et al., 2021; McDougall et al., 2022) and monitoring mastitis prognosis (Lam et al., 2009). Nonetheless, the estimation of SCC based on the CMT is imprecise (Ruegg and Reinemann, 2002), thus the accuracy of the CMT for detecting IMI is unsatisfactory compared to tests using proper thresholds of SCC (Sargeant et al., 2001; Middleton et al., 2004).

Lysosomal enzymes

PMNs eliminate mastitis pathogens by either phagocytosis or cell lysis. Both processes are accompanied by the release of lysosomal enzymes, such as N-acetyl-β-D-glucosaminidase (**NAGase**) and β-glucuronidase. Several studies found that NAGase can be used to detect IMI with an accuracy comparable with SCC (Kitchen, 1981; Mattila and Sandholm, 1985; Miller and Paape, 1988; Berning and Shook, 1992; Pyörälä and Pyörälä, 1997; Hovinen et al., 2016). Moreover, studies suggested that NAGase activity increases with the amount of bacterial DNA

in milk and the severity of mastitis, also differing between the causative pathogen of mastitis (Pyörälä et al., 2011; Kalmus et al., 2013). With the recent progress in biosensors (Viguier et al., 2009), lysosomal enzymes such as NAGase are potential biomarkers for detecting mastitis.

1.3.2. Systemic immunological indicators

Serum proteins

Protein is a major component in the serum. Serum proteins can be classified into either albumins or globulins, each has its physiological functions. Albumin is the majority of serum proteins, constituting 35% to 50% of the total (Eckersall, 2008), essential for regulating blood osmotic pressures and transporting nutrients. Globulins, comprising α, β, and γ globulins, instead play an important role in immune responses. Proteins involved in innate immunity (acute response proteins; mostly α globulins) and adaptive immunity (immuno-globulin [**Ig**]; mostly γ globulins) all belong to the globulin family (see also section 1.3.2. Cytokines). Thus the concentration of globulin and its ratio relative to the albumin are affected by immune status, making the Albumin/Globulin (**A/G**) ratio an important tool for disease identification and prognosis evaluation in animals (Eckersall, 2008). Bobbo et al. (2017) demonstrated that the A/G ratio is related to udder health. They observed a lower serum A/G ratio in cows with high SCC, attributable to both an increase in globulin concentrations and a decrease in albumin concentrations. Cattaneo et al. (2021) further found that the A/G ratio is associated with dairy cows' performance. Cows with a high A/G ratio before dry-off generally have greater milk yield and better reproductive performance in the subsequent lactation.

Leukogram

Changes in leukogram (i.e., white blood cell [**WBC**] count, neutrophil count, lymphocyte count, monocyte count, and so on) in mastitis differ depending on both the causative pathogen and disease severity. Bannerman et al. (2004a, b, c; 2005) conducted a series of experiments to investigate pathogen-specific innate immune responses in mastitis. Generally, gram-negative IMI (i.e., *E. coli, Klebsiella pneumoniae, Serratia marcescens*, and *Pseudomonas aeruginosa*) induced neutropenia, but this was absent in gram-positive IMI (i.e., SA and SU). On the other hand, Wenz et al. (2001a) investigated the effect of disease severity on the leukogram in coliform mastitis, and they found that cows with moderate or severe symptoms were with lower numbers of WBC and neutrophils compared to cows with mild symptoms. Neutropenia occurs in cows due to endotoxemia, which causes neutrophils to rapidly redistribute from the circulation to the site of inflammation and also the consequent bone marrow depletion (Sharkey and Heinrich, 2020). This explains why neutropenia is commonly observed in severe coliform mastitis. Since the leukogram changes depending on

the mastitis pathogen and severity, it has been proposed as a tool for differentiating causative pathogens (Smith et al., 2001) and evaluating the prognosis of mastitis (Braun et al., 2021).

1.3.3. Cytokines

Cytokines are a group of low-molecular-weight, short-lived glycoproteins, almost produced by and working on every type of cell. Cytokines mediate immune responses, such as mastitis, interactively with other cytokines. Each stage of mastitis, including initiation and resolution, is carefully regulated by these glycoproteins. Mastitis may be out of control due to the imbalance of cytokines, leading to either inactivated or overactivated immune responses, which consequently damage cows' health and milk production. As we will see below, cytokine response patterns in mastitis highly correspond to changes in the above-mentioned immunological indicators, and a thorough understanding of cytokines toward IMI provides potential preventions and cures of mastitis. To describe the roles of cytokines in mastitis, I summarize reviews reported by Burton and Erskine (2003), Bannerman (2009), Schukken et al. (2011), and Sordillo (2018) as follows.

Immune responses can be classified into innate and adaptive immunity in a broader sense, that the former is more immediate and unspecific, and the latter is instead specific and antigen-dependent. Despite the convenience of this simplified classification, it is important to remember that they act cooperatively to defend our bodies. Although cytokines can be broadly categorized into *pro-inflammatory* and *anti-inflammatory cytokines*, some cytokines have both pro- and anti-inflammatory properties making them *pleiotropic cytokines*. Figure 1 illustrates how cytokines regulate immune responses in mastitis.

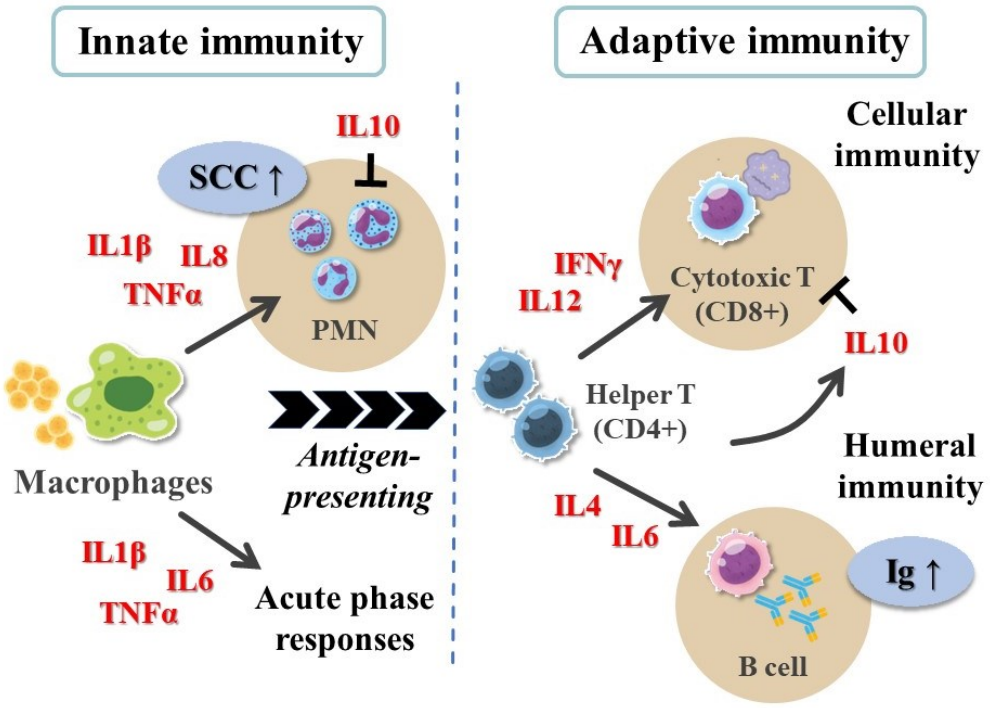

Figure 1. Regulation of immune responses in mastitis through cytokines.

Innate immunity is the front line of the fight against mastitis pathogens. Upon IMI, Toll-like receptor 2 and Toll-like receptor 4 on immune cells respectively recognize lipoteichoic acid from gram-positive bacteria and lipopolysaccharide from gram-negative bacteria. The activated Toll-like receptors pass the signal through NF-κB pathways and eventually promote the production of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α. In the case of mastitis, these cytokines are mainly produced by mammary epithelial cells and macrophages/monocytes resident in the alveolar space of the udder. IL-8 is a chemokine, which causes neutrophil migration to the udder and the consequent increase in SCC. IL-1β, TNF-α, and IL-6 are responsible for stimulating *acute-phase responses* (see Ceciliani et al. (2012) and Eckersall (2008) for reviews of acute-phase responses in animals) and inflammation responses such as hyperthermia and increases in vascular permeability, also notorious for causing septic shock in coliform mastitis. The effects of pro-inflammatory cytokines IL-1β and TNF-α overlap with each other, and they act synergistically to enhance inflammatory responses and neutrophil recruitment by inducing IL8. IL-6 is instead a pleiotropic cytokine with anti-inflammatory properties, as it inhibits the expression of IL-1β and TNF-α. IL-6 also modulates adaptive immunity, as it induces T-cell activation, B-cell differentiation, and the consequent antibody (i.e., Ig) production.

Adaptive immunity on the other hand constructs a more specific line of defense through the recognition of antigens. If cows repeatedly encounter a particular pathogen, this specific action will be more rapid, pronounced, and effective through immunological memory. This is the basis for vaccination. Adaptive immunity can be mediated by either immune cells or Igs (i.e., *cellular immunity* and *humoral immunity*), both of which are initiated and regulated by CD4+ helper T cells (**T_h**). By recognizing antigens presented by antigen-presenting cells (B cells, macrophages, dendritic cells, and so on), type 1 T_h activates CD8+ cytotoxic T cells (**T_c**) and a variety of phagocytes (e.g., neutrophils, macrophages) via IFN-γ and IL-12. IFN-γ and IL-12 are the cytokines linking innate and adaptive immunity, as they promote both phagocytosis and the activation of T_h and T_c. IL-12 also promotes the differentiation of T cells into type 1 T_h to produce IFN-γ, which further enhances cellular immunity.

Contrastingly, type 2 T_h promotes the proliferation and differentiation of B cells via IL-4, IL-6, and IL-10, enabling them to produce Igs, namely IgG1, IgG2, IgM, and IgA. These Igs not only provide newly-born calves immunity but also get involved in the fight against IMI. As a consequence, the concentration of each Ig fluctuates depending on lactation stages and the infection status of the udder. IL-4 is essential for Igs production (especially IgG and IgA) by stimulating B cells. IL-10 is also known as an anti-inflammatory cytokine responsible for the resolution of inflammation. It limits the inflammatory response and the recruitment of neutrophils by inhibiting the production of pro-inflammatory cytokines (e.g. IL-1β, TNF-α). IL-4 and IL-10 also alter the balance between type 1 and type 2 T_h by inhibiting IFN-γ and IL-12 production. As such, a balance exists between cellular and humoral immunity, and immune responses are dominated by either of each depending on the progress of the disease or the animal's status. For instance, humoral immunity dominates in periparturient cows, leading to high IgG concentration in colostrum, reduced neutrophil recruitment, and the consequent higher risk of IMI.

Cytokines induced by IMI are pathogen-specific, in terms of concentration, release timing as well as duration (Bannerman, 2009; Schukken et al., 2011). Significant differences have been found between cytokine responses toward gram-positive and gram-negative bacteria due to their distinct structures of cell walls (i.e., lipoteichoic acid and lipopolysaccharide). Among these, the differences between responses toward *E. coli* and SA have been most investigated. As reported by Bannerman et al. (2004c), *E. coli* induced a more acute and pronounced elevation of all analyzed pro-inflammatory cytokines, namely TNF-α, IL-1β, IL-6, IL-8, IL-12, and IFN-γ, contributing to severe systemic symptoms, such as hyperthermia and neutropenia. In contrast, SA provoked a delayed, mild and short elevation of cytokines. Further, increases in IL-8 and TNF-α were absent in SA infections, which explained why increases in

SCC were delayed in SA infections. In cows infected with SA, systemic signs, such as hyperthermia or reductions in milk yields, were observed in a relatively insignificant manner. The difference between cytokines responses toward *E. coli* and SA probably leads to the distinct systemic signs and SCC patterns observed in these infections (see also section *Somatic Cell Count*). Table 1 summarizes the functions of cytokines and immune cells in mastitis.

Table 1. Functions of cytokines and immune cells in mastitis

Cytokine/ Cell	Innate or Adaptive	Pro- or Anti-inflammatory	Cellular or Humoral	Functions
TNF-α	Innate	Pro	Cellular	Stimulate acute phase response; Sickness; Septic shock; Promote IL-8
IL-1β	Innate	Pro	Cellular	Stimulate acute phase response; Sickness; Septic shock; Promote IL-8
IL-4	Adaptive	Anti	Humoral	Stimulate B cells and Igs production; Inhibit IFN-γ production
IL-6	Both	Pleiotropic	Humoral	Stimulate acute phase response; Sickness; Septic shock; Promote B cell differentiation
IL-8	Innate	Pro	Cellular	Neutrophil recruitment
IL-10	Both	Anti	Humoral	Impair antigen presentation; Inhibit IL-1β, TNF-α, IFN-γ & IL-12
IL-12	Both	Pro	Cellular	Promote phagocytosis; Activate type 1 T_h; Suppress type 2 T_h
IFN-γ	Both	Pro	Cellular	Promote phagocytosis; Stimulate T_c; Suppress type 2 T_h
Neutrophils	Innate	-	Cellular	Phagocytosis
Macrophages	Both	-	Cellular	Produce TNF-α, IL-1β, IL-6; Phagocytosis; Present antigen; Repair damaged tissues
Type 1 T_h	Adaptive	-	Cellular	Activate T_c; Promote phagocytosis
Type 2 T_h	Adaptive	-	Humoral	Stimulate B cells and Igs production
T_c	Adaptive	-	Cellular	Lyse infected endogenous cells
B cell	Adaptive	-	Humoral	Present antigen; Produce Igs

Abbreviations:
TNF = Tissue necrosis factor; IL = Interleukin; T_h = Helper T cells; T_c = Cytotoxic T cells; Igs = Immunoglobulins.

1.4. Summary of Research Background

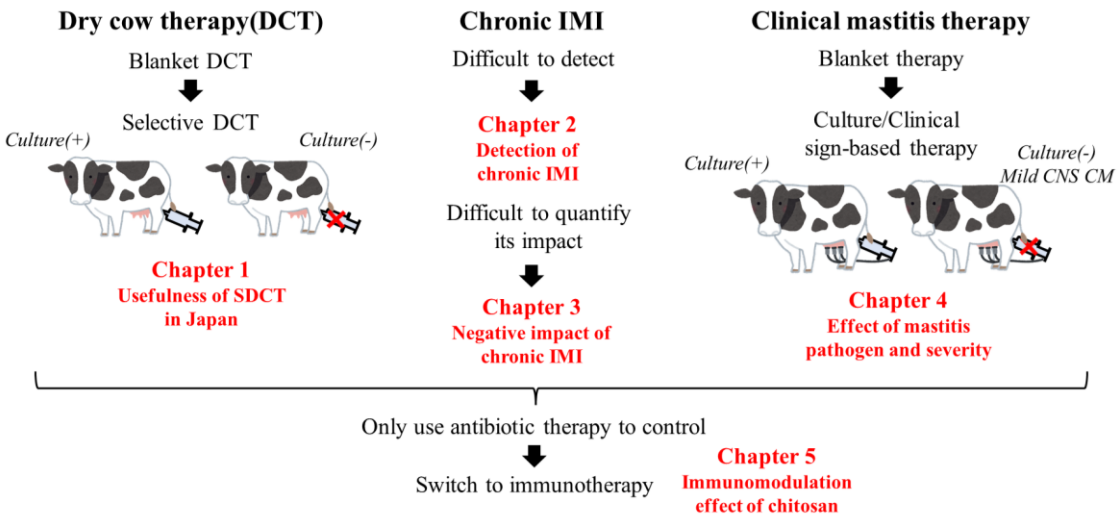

Figure 2. Research background: how can we improve antibiotic stewardship and efficiency in milk production? IMI: intramammary infection; CM: clinical mastitis.

Figure 2 shows research background of this thesis. To reduce economic losses caused by mastitis and to improve the udder health of dairy cows, antibiotic treatment has been applied to lactating and dry cows. This successfully controlled mastitis; however, the high demand for antibiotics was accompanied by concerns over the emergence of antimicrobial resistance. For these reasons, the selective use of antibiotics, such as SDCT, has been recommended, although its usage and effects were not well investigated in Japan (Chapter 1). To effectively allocate antibiotics, it is necessary to identify the cows most benefiting from the therapy. This can be facilitated by using tests such as the CMT (Chapter 2); however, the sensitivity of this test would consequently affect the outcome of SDCT. To identify cows benefiting most from antibiotic treatment, it is also helpful to quantify milk production loss caused by mastitis, and this can be conducted conveniently using immune indicators included in monthly DHI testing, such as SCC and DSCC (Chapter 3). Furthermore, immune responses in mastitis largely depend on the causative pathogen and disease severity (Chapter 4), which consequently affect treatment decisions and the outcome of the disease. Eventually, a therapy alternative to antibiotics, such as immunotherapy, is necessary for mastitis control to improve antibiotic stewardship. It is thus worth investigating the effects of *chitosan*, a biomaterial with immunomodulatory properties, on mastitis (Chapter 5).

The thesis aims to investigate immune responses in mastitis to quantify mastitis impacts and to improve the identification, prevention, and treatment of the disease. The final goal is to improve antibiotic stewardship without sacrificing animal health and economic benefits.

2. Chapter 1. Effect and Limitation of Selective Dry Cow Therapy

This chapter contained the work included in the paper:

Huang, C. H., I. Fujiwara, and N. Kusaba. 2022. *Effect of selective dry cow therapy on dry period intramammary infection dynamics and their association with management factors in Japan.* Anim. Sci. J. 93. https://doi.org/10.1111/asj.13718.

2.1. Introduction

As proposed in the 1960s, the "Five-Point Plan" encouraged farmers to administrate antibiotics to every quarter of dry cows, namely BDCT. BDCT aims to control IMI during the dry period, the main cause of postpartum clinical mastitis (Green et al., 2002), by both treating existing infections and preventing new infections. It indeed succeeded because bulk SCC (Berry et al., 2006) and the prevalence of major mastitis pathogens (Makovec and Ruegg, 2003) have decreased considerably over time. However, due to the general improvement of udder health and the concerns over the emergence of antimicrobial resistance (Rajala-Schultz, 2004; McDougall et al., 2021), the preventive use of antibiotics in dairy farming has been discouraged or even prohibited in certain countries, including The Netherlands and Nordic countries (Vanhoudt et al., 2018; Vilar et al., 2018). SDCT has thus been investigated as a substitute for BDCT in western countries (McCubbin et al., 2022).

SDCT allocates antibiotics selectively to cows (i.e., cow-level) or quarters (i.e., quarter-level) with higher risks of mastitis, identified with either bacterial culture (Cameron et al., 2014; Kabera et al., 2020), SCC records in monthly milk testing (Bradley et al., 2010; Vasquez et al., 2018), or the CMT (Swinkels et al., 2021; McDougall et al., 2022). In most trials, SDCT achieved a considerable reduction in antibiotic use without sacrificing animal health, as long as healthy quarters were inserted with a teat sealant in lieu of antibiotics to prevent new infections. Unfortunately, although SDCT is promising for reducing antibiotic use and the corresponding costs (Scherpenzeel et al., 2018), currently still not a common practice in Japan. The hesitation to apply SDCT is mostly due to concerns over its negative impacts on udder health and milk production (Scherpenzeel et al., 2016). The unsatisfactory effects of SDCT may result from poor hygiene practices (Green et al., 2007), or the missingness of teat sealants implementation (Winder et al., 2019). Thus, in this study, we aimed to investigate the effect of SDCT on antibiotic use and dry period IMI, and its limitations, which can be attributed to

differences in management factors, such as hygiene conditions and the efficacy of teat sealants, between Japan and other countries.

2.2. Materials and Methods

We conducted a cow-level SDCT trial, in which we used milk culture, the CMT, and clinical mastitis records to identify cows with higher mastitis risks and selectively allocated antibiotics to all quarters of these cows. The trial enrolled cows that entered the dry period from February to November 2020. To elucidate the influence of management factors on SDCT, we evaluated herd hygiene and the duration of teat sealant adherence right after the dry-off of each cow. All procedures followed the Guidelines for the Care and Use of Agricultural Animals of Obihiro University (Permission number: #20-55).

2.2.1. Study herd

The study herd comprised around 70 lactating Holstein cows kept at the Field Center of Animal Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine in Hokkaido, Japan (42° 52′ 34″ 106 N, 143° 10′ 23″ E). These cows were raised in a free-stall barn, fed a total mixed ration, milked twice daily in a parlor, and produced average milk of 11,000 kg annually. All lactating cows entered a DHI program with monthly milking tests, clinical mastitis as well as culling events were regularly recorded, and bulk SCC had been no more than 150,000 cells/mL during the study period.

2.2.2. Experimental procedures

We allocated treatment and grouped quarters of enrolled cows for investigation following procedures described in Figure 3. We identified cows with IMI by milk culture, the CMT, and mastitis records. More specifically, we collected quarter milk samples and performed the CMT for every quarter of enrolled cows 2 d before dry-off. Subsequently, we recognized the cow as high-risk based on the following criteria: (i) at least one quarter showed positive culture result; (ii) at least one quarter showed positive CMT reaction; (iii) at least one quarter with records of clinical mastitis caused by SA or caused by streptococci at >200 DIM in the current lactation; all other cows were recognized as low risk. We performed the CMT using a commercial reagent (P.L tester; Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan), following a method described by Schalm (1957). At dry-off, we required technicians to wear disposable gloves and allocate antibiotic infusions (250 mg of cefazolin, Cefamezin DC; Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan) into the quarters of the high-risk cows after cleaning the teat ends with 70% isopropyl alcohol-soaked swabs; however, no antibiotics were allocated to

the low-risk cows. Instead, technicians applied an external teat sealant (Teatoner®; Tokuyama Corporation, Shunan, Japan) to the teat ends of all enrolled cows following the label directions.

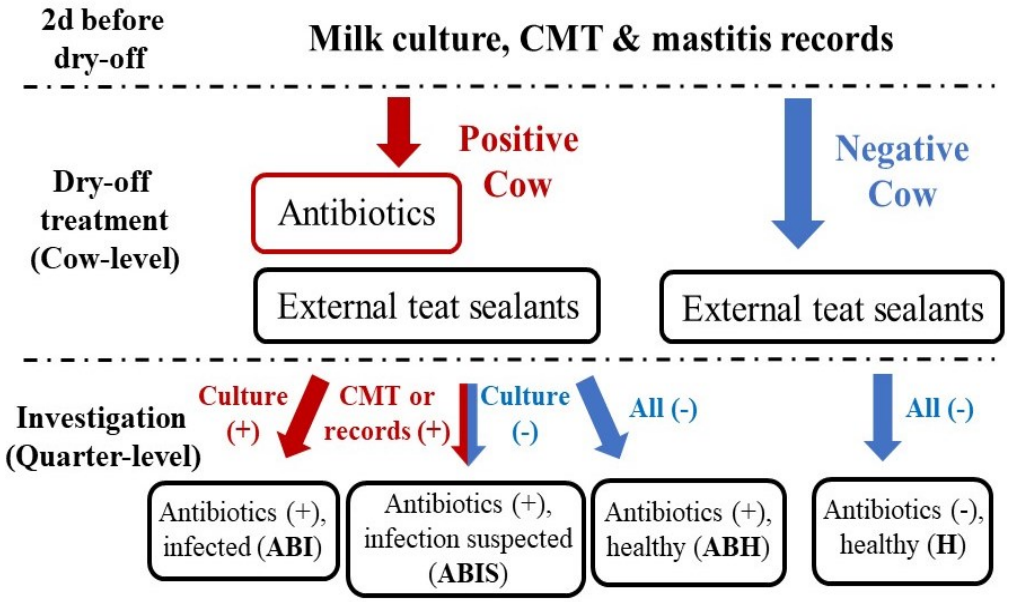

Figure 3. Procedures for treatment allocation and investigation in our SDCT trial. Culture (+) = culture-positive in the bacterial culture of milk; CMT (+) = positive CMT reaction; Records (+) = with records of clinical mastitis caused by SA or caused by streptococci at >200 DIM in the current lactation.

To assess the effect of SDCT on dry-period IMI, we classified the quarters of enrolled cows as follows: (i) Antibiotic-treated, infected (**ABI)** group: quarters with IMI at dry-off, which received antibiotics; (ii) Antibiotic-treated, infection-suspected (**ABIS**) group: quarters without IMI at dry-off but showed positive reaction in the CMT or were with at least one record of clinical mastitis caused by SA or caused by streptococci at >200 DIM in the current lactation. These quarters received antibiotics; (iii) Antibiotic-treated, healthy (**ABH**) group: quarters with neither IMI nor the suspicion of IMI at dry-off but received antibiotics since it belonged to the high-risk cows; (iv) Antibiotic-untreated, healthy (**H**) group: quarters without IMI and did not receive antibiotics at dry-off. Afterward, to investigate how antibiotics and management factors affected dry period IMI, we evaluated herd hygiene and the duration of external teat sealant adherence after dry-off and sampled mammary secretions 2 wk before parturition.

2.2.3. Sampling

We collected samples aseptically from quarters of enrolled cows 2 d before dry-off (dry-off), and 2wk before the due date (prepartum). For dry-off samples, after milking, we thoroughly cleaned teat ends with 70% isopropyl alcohol-soaked swabs and collected samples

into sample tubes. After sampling, post-dipping was applied. For prepartum samples, after cleaning cow teats by pre-dip containing 0.1% iodine and a sterilized towel, we forestripped, scrubbed teat ends with alcohol-soaked, and collected mammary secretions into sample tubes. After sampling, we applied external teat sealants to all teats after post-dipping to protect quarters from IMI.

2.2.4. Bacterial culture of milk

Immediately after sample collection, we performed bacterial culture following the National Mastitis Council guidelines (NMC, 2017) with some modifications. Briefly, we smeared 50 μL (instead of 10 μL used routinely) of milk onto a 5% sheep blood agar (TSA II®; BD Japan) and incubated it at 37°C. As summarized in Figure 3, we categorized bacteria into either staphylococci, streptococci, gram-negative rods (GNR), or gram-positive rods based on their morphology, catalase reaction, and Gram staining. We differentiated SA from CNS based on hemolysis patterns and a tube coagulase test. We further cultured streptococci on a chromogenic agar (CHROMagar™ Orientation, BD Japan) (Garcia et al., 2021), a sorbitol-containing andrade peptone broth, and an SF broth (Streptococcus uberis diagnosis kit; Kanto Chemical, Japan) (Kusaba, 2014) to make a presumptive diagnosis. Despite that, due to the lack of a final diagnosis, we categorized these isolates into streptococci and streptococcus-like pathogens (Str.). For gram-positive rods, we differentiated Trueperella pyogenes from Corynebacterium spp. based on catalase tests and hemolysis patterns (Hogan et al., 1999). If we observed two or fewer colonies on the plate (≤40 CFU/ml), the sample was deemed no significant growth (NS). Last, we considered samples as contaminated when 3 or more distinct colonies were presented on a single plate.

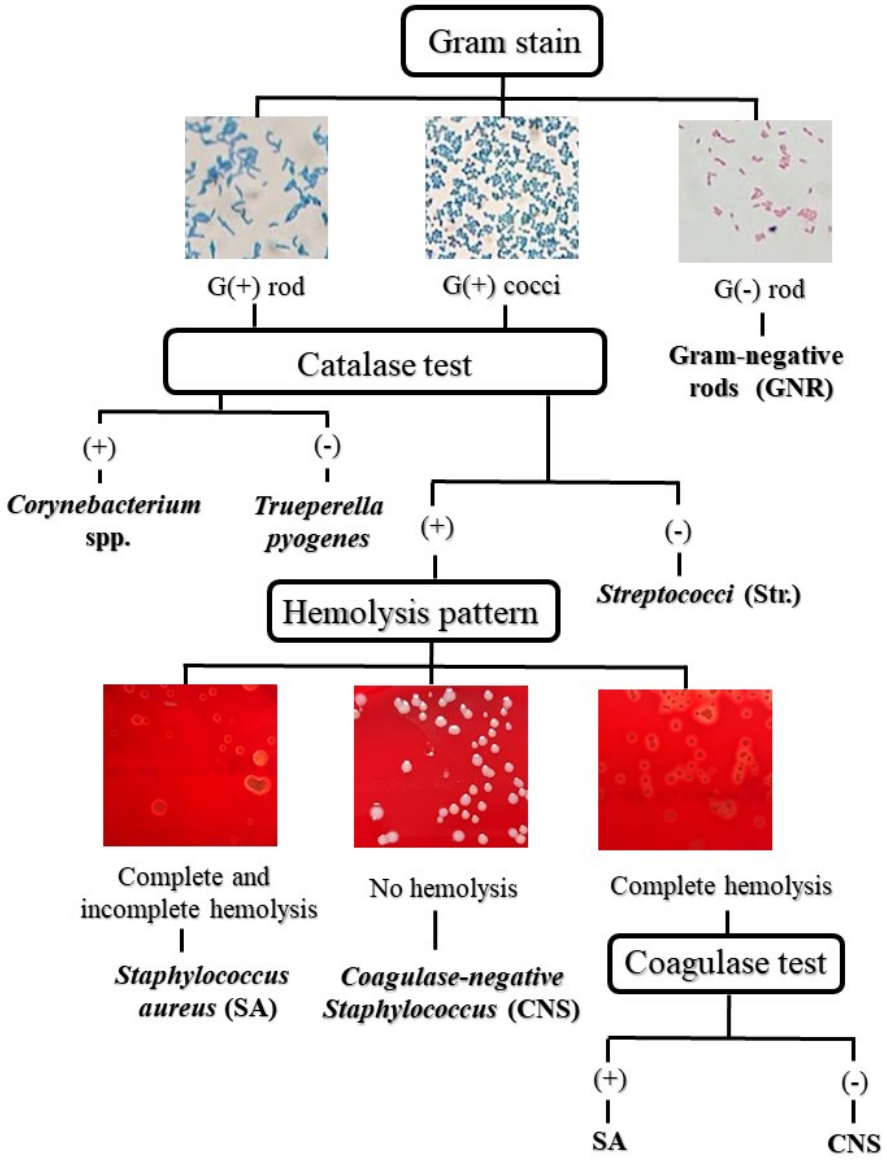

Figure 4. A flowchart showing the bacteriologic diagnosis procedures.

2.2.5. Evaluating herd hygiene and the duration of external teat sealant adherence

We evaluated the hygiene conditions of dry cows by scoring the udders and hindlegs cleanliness of dry cows using a scoring regime described in Schreiner and Ruegg (2003). More specifically, we scored the lateral aspects and the posterior aspects of udders separately, and we scored the upper aspect and the lower aspect of hindlegs separately as well (Figure 5A). We averaged scores from the herd and used them as the herd hygiene scores for each dry-off period. Nevertheless, we excluded hygiene scores from recently dried (i.e., less than 2 days) cows since these scores could not represent the hygiene condition of the dry cow herd. This approach was inspired by studies showing that the herd hygiene score is related to herd SCC (Dohmen et al., 2010) and clinical mastitis incidence (Verbeke et al., 2014).

We recorded whether the external teat sealant still adhered to the teat end for the first 2 wk after dry-off for each dry cow. We supposed that the external teat sealant lost its functions when it could no longer cover the teat orifice (Figure 5B).

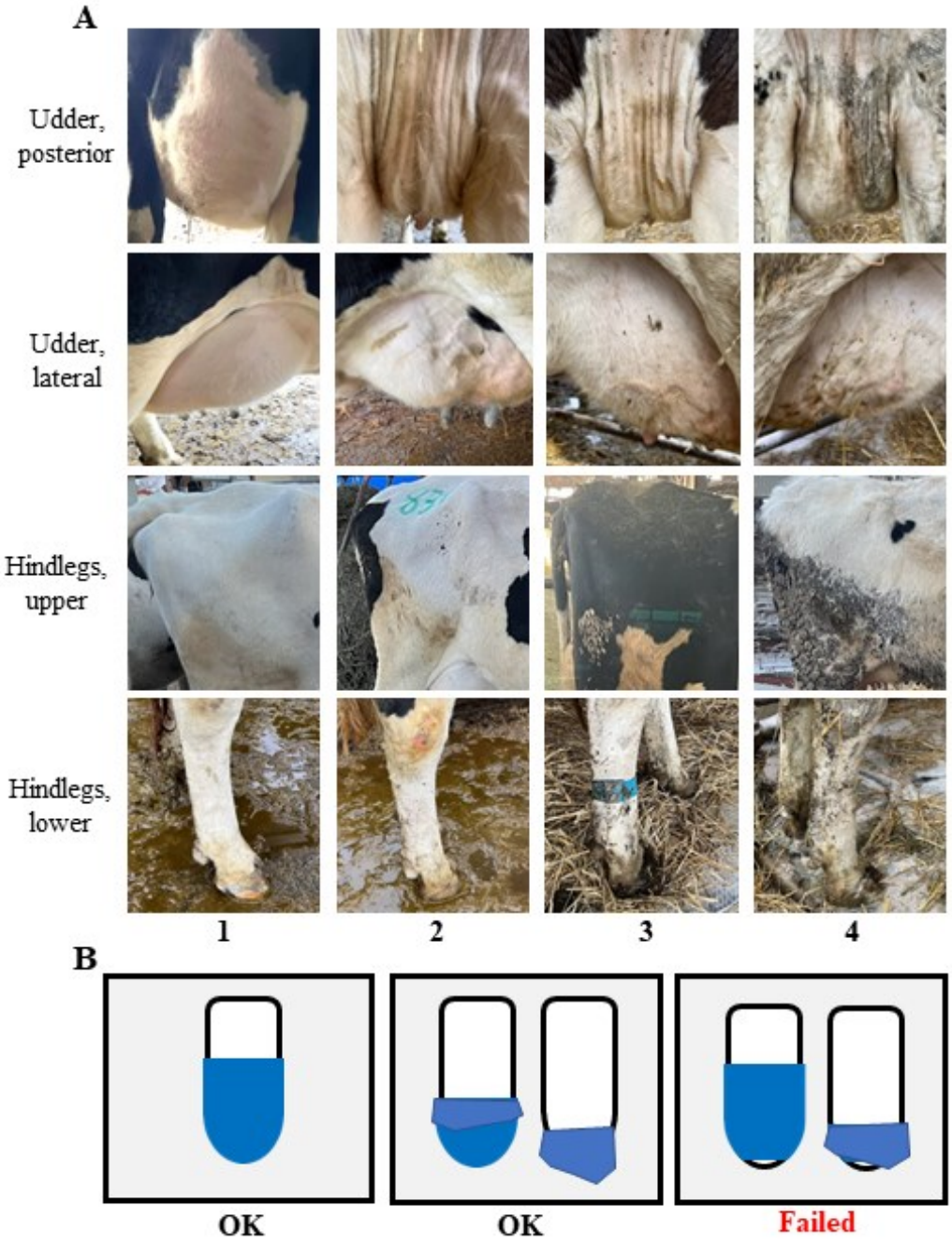

Figure 5. The scoring regime used to evaluate hygiene condition (A) and external teat sealant adherence (B).

2.2.6. Statistical analysis

We considered each quarter as an independent observation in the analysis. We used Fisher's exact test to compare proportions from two categories (e.g., infection rates from two groups). We applied Mann–Whitney U test and Welch's t-test to compare herd hygiene score and duration of external teat sealant adherence between two groups of quarters, respectively. We performed all analyses with the statistical software R version 4.0.5 (R Core Team, 2021) and determined significance when $p < .05$.

2.3. Results

2.3.1. Antibiotic use and the dynamic of dry period IMI in an SDCT trial

The trial enrolled 44 cows (n=166 quarters), of which 17 cows (38.6%) were with IMI at dry-off. An additional 13 cows (29.5%) showed positive CMT reaction at dry-off or were with clinical mastitis records, consequently recognized as high-risk cows. In total, we allocated antibiotics to 30 cows (68.2%).

Of the 166 enrolled quarters, 27 (16.3%) were with IMI at dry-off, of which 7 were with SA (25.9% of quarters with IMI), 17 were with CNS (63.0%), 4 were with Str. (14.8%), and 2 were with *Corynebacterium* spp. (7.4%). Since 3 quarters were infected with 2 pathogens, the pathogen prevalence does not sum to one. The infected quarters belonged to the **ABI** group. An additional 31 quarters (18.7%) showed positive CMT reaction at dry-off or were with clinical mastitis records, thus being categorized into the **ABIS** group. Another 52 quarters (31.3%) of high-risk cows without IMI but receiving antibiotics were allocated to the **ABH** group. The other 56 quarters (33.7%) of low-risk cows instead received no antibiotics and belonged to the **H** group. As a result, the current SDCT trial achieved a 33.7% of reduction in antibiotic use compared to a BDCT approach.

At 2wk before the due date (prepartum), we found IMI in 49 quarters (29.6%) of 166 enrolled quarters. Also, we identified two pathogens in 10 quarters. Table 2 shows the distribution of IMI before calving, in which Str. was the most prevalent pathogen (14.5%), and CNS (13.3%) was the second. Among quarters treated with antibiotics (i.e., ABI, ABIS, and ABH), the high-risk quarters (i.e., ABI and ABIS) were with significantly higher IMI rates compared to the low-risk quarters (i.e., ABH) ($p = 0.023$, tested by Fisher's exact test). Among low-risk quarters (i.e., ABH and H), antibiotic treatment did not significantly affect the prevalence of prepartum IMI ($p = 0.27$, tested by Fisher's exact test).

Table 2. Distribution of intramammary infections before calving [n (%)].

	Prepartum IMI				
Pathogen [a]	ABI [b]	ABIS	ABH	H	Total
NS	16 (59.3)	19 (61.3)	42 (80.8)	40 (71.4)	117 (70.4)
SA	1 (3.7)	-	1 (1.9)	1 (1.8)	3 (1.8)
CNS	4 (14.8)	2 (6.5)	5 (9.6)	11 (17.8)	22 (13.3)
Str.	5 (18.5)	9 (29.0)	4 (7.7)	6 (10.7)	24 (14.5)
GNR	3 (11.1)	4 (12.9)	1 (1.9)	2 (3.6)	10 (6.0)
N	27	31	52	56	166
Prevalence	40.8 %	38.7 %	19.2 %	28.6 %	29.6 %

[a] NS = No significant growth; SA = S*taphylococcus aureus*; CNS = Coagulase-negative staphylococci; Str. = Streptococci and streptococcus-like bacteria; GNR = gram-negative rods.
[b] ABI = antibiotic-treated, infected; ABIS = antibiotic-treated, infection-suspected; ABH = antibiotic-treated, healthy; H = antibiotic-untreated, healthy.

2.3.2. Association between CMT reaction, mastitis records, and dry period IMI

Of the 166 quarters undergoing the CMT at dry-off, 16 (9.6%) showed a positive reaction. Of the quarters with a positive CMT reaction, we found IMI in 10 quarters (62.5%) before calving, which was significantly higher than quarters with a negative CMT reaction (39/150 = 27.9%; p = 0.007, tested by Fisher's exact test). In addition, 33 quarters were with at least one clinical mastitis record (19.9%). Of quarters with mastitis records, we found IMI in 13 quarters (39.4%) before calving, which was not significantly different from that had no mastitis records (36/133 = 27.1%; p = 0.201, tested by Fisher's exact test).

2.3.3. Impacts of herd hygiene and external teat sealant adherence on dry period IMI

In the analysis of herd hygiene scores, we excluded 2 cows and their 8 quarters because these cows did not enter the herd of dry cows immediately after dry-off. We compared the herd hygiene scores in ABH and H groups by their IMI status before calving. Figure 6 shows that the median herd hygiene scores were not significantly different regardless of the prepartum IMI status of quarters in the ABH group; however, in the H group, the median herd hygiene scores were generally higher in quarters with prepartum IMI than in those without prepartum IMI.

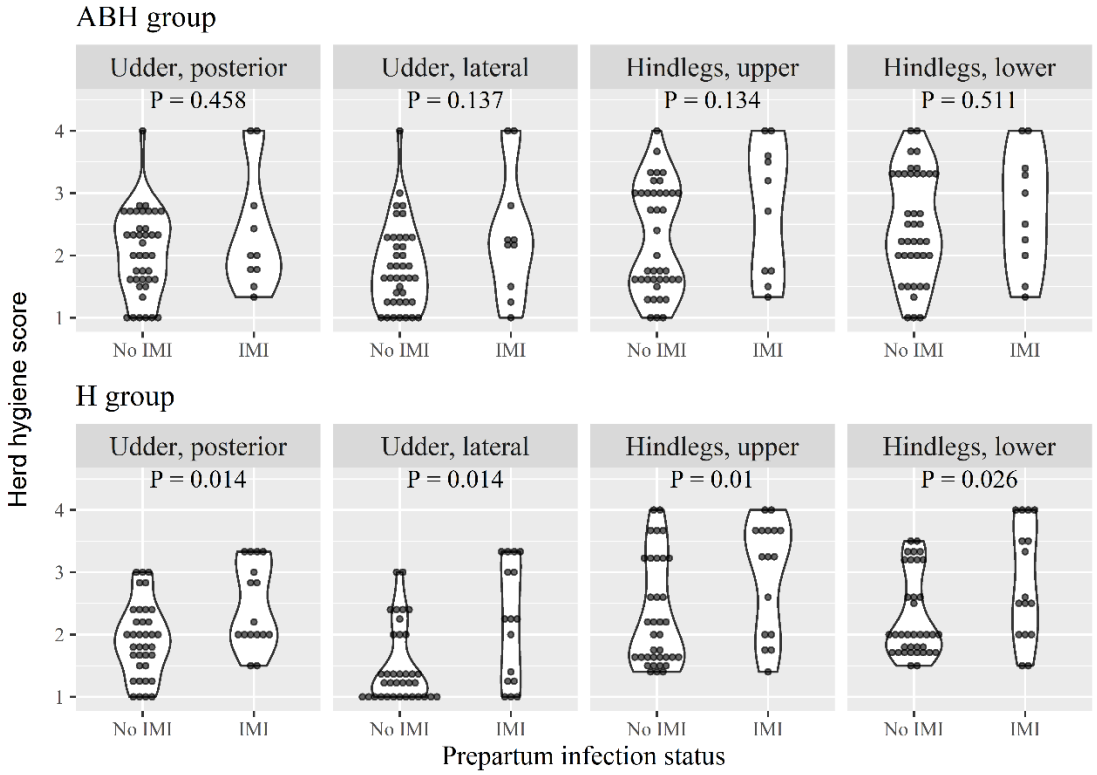

Figure 6. Violin plots showing herd hygiene scores at day-off for quarters in the antibiotic-treated, healthy (ABH; top) and antibiotic-untreated, healthy (H; bottom) group. Quarters are categorized by their intramammary infection (IMI) status before calving. A dot represents one quarter. P-values were given by Mann–Whitney U tests.

When analyzing the duration of external teat sealant adherence, we excluded one cow and its 2 quarters because we could not apply the external teat sealant to the cow at dry-off due to her resistance. We compared the duration of external teat sealant adherence in ABH and H groups by their IMI status before calving. For the ABH group, the mean durations of external teat sealant adherence were not significantly different between quarters with or without prepartum IMI (IMI vs. No IMI = 5.5 vs. 6.0; p = 0.67, tested by Welch's t-test); nonetheless, for the H group, the mean duration of external teat sealant adherence was shorter in quarters with prepartum IMI than in those without IMI (IMI vs. No IMI = 4.3 d vs. 5.5 d; p = 0.01, tested by Welch's t-test).

2.4. Discussions

In the current SDCT trial, antibiotic use was reduced by 33.7% compared with a BDCT approach. We conducted the SDCT at the cow level, meaning that we allocated antibiotics to all quarters of the high-risk cows. SDCT can be either performed at the quarter level, in which antibiotics were only allocated to quarters with high IMI risks. Apparently, a quarter-level SDCT potentially achieves an additional reduction in antibiotic use compared to a cow-level

approach, since uninfected quarters of high-risk cows can still avoid antibiotic treatments. Nonetheless, a quarter-level approach requires to be facilitated by either bacterial cultures of milk (Rowe et al., 2020) or the CMT (Swinkels et al., 2021), which may be impracticable in large commercial farms. Moreover, a quarter-level SDCT may fail when the concurrent use of internal teat sealants is absent (McCubbin et al., 2022) or when contagious pathogens are presented (Rowe et al., 2020), possibly because pathogens may spread between quarters.

We noticed a significantly higher prepartum IMI prevalence in quarters presumed with higher risks (i.e., ABI and ABIS) than in those presumed with lower risks (ABH), despite that all these quarters received antibiotics at dry-off. Furthermore, a positive CMT reaction at dry-off also indicated a higher IMI risk in the dry period even when no pathogens were isolated at dry-off. Studies showed that the bacterial examination of milk does not always identify major mastitis pathogens, such as SA (Sears et al., 1990) and streptococci (Dohoo et al., 2011), even though the quarter is truly infected. For these reasons, we further utilized the CMT (Bhutto et al., 2012; Swinkels et al., 2021), and the records of clinical mastitis caused by SA and streptococci (Zadoks et al., 2001; Vasquez et al., 2018; McParland et al., 2019), to reduce the false-negative rate, ensuring that cows received antibiotics when IMI existed. Alternatively, the selection of high-risk cows can be facilitated by either SCC (Bradley et al., 2010; Vasquez et al., 2018; Rowe et al., 2020) or DSCC records (Schwarz et al., 2019). We choose the CMT and mastitis records because these approaches do not require monthly DHI milking tests. The on-farm use of the CMT makes it applicable for most dairy farms in Japan currently, although using SCC and DSCC records to conduct SDCT is likely to be the mainstay in the recent future with the popularity of DHI milk tests.

In the current study, we observed a numerically higher prevalence of prepartum IMI in the H group (28.6%) than that in the ABH group (19.2%). Although the difference was statistically insignificant due to the small sample size in the current study, the result shows that quarters without antibiotics' protection may be more likely to be infected in the dry period. We thus conducted further investigations on herd hygiene conditions of dry cows and the duration of external teat sealant adherence to explain the unfavorable results from our SDCT trial, in which we found that quarters with prepartum IMI were characterized by worse herd hygiene conditions and shorter durations of external teat sealant adherence. As reviewed by McCubbin et al. (2022), herd characteristics, such as hygiene practice at dry-off (Green et al., 2007) or the prevalence of major pathogen IMI (Cameron et al., 2014), play an important role in the success of an SDCT. Our results support their opinions, and we recommend improving the hygiene conditions of dry herds and overall mastitis management before implementing SDCT to limit new IMI risks (Bradley et al., 2018).

The success of an SDCT trial also depends on whether teat sealants, especially internal teat sealants, were applied concurrently, as evidenced by several meta-analyses (Winder et al., 2019; Kabera et al., 2021). This becomes more clear by comparing early trials of SDCT conducted without teat sealants (Browning et al., 1990; Berry and Hillerton, 2002; McDougall, 2010; Scherpenzeel et al., 2014) with recent trials implemented with internal teat sealants (Bradley et al., 2010; Cameron et al., 2014; Patel et al., 2017; Kabera et al., 2020; Rowe et al., 2020), as unsatisfactory results have only been observed in trials conducted without teat sealants. Teat sealants serve as a physical barrier between the udder and the environment, essential for preventing new IMI (Huxley et al., 2002). Our results support this viewpoint since the low durability of external teat sealant adherence contributed to the new IMI introduced in the dry period. Due to the law, internal teat sealants are not permitted in Japan and thus external teat sealants must be applied instead. However, there are several disadvantages of external teat sealants as compared with internal teat sealants, namely that they are difficult to apply, likely to be ineffective without proper application, and could not last as long as internal ones, thus reapplication is necessary (Crispie et al., 2004; Lim et al., 2007). Taken together, an SDCT must be conducted concurrently with teat sealants. To promote SDCT implementation in Japan, a revision of the law to allow the use of internal teat sealants is probably necessary.

2.5. Conclusions

The SDCT trial achieved a reduction in antibiotic use by around 33%. Previous studies showed that SDCT improved antibiotic stewardship without negative impacts on either udder health or milk production. However, we observed relatively unfavorable impacts of our SDCT trial on the udder health of dry cows. Our results suggest that the success of an SDCT may be limited by the hygiene conditions of dry cows, the efficacy of teat sealants, or the sensitivity of methods in selecting cows to receive antibiotics. We thus encourage improving hygiene practices and permitting internal teat sealants before implementing SDCT in Japan. Further, in the next chapter, we will discuss the effect of DSCC on the accuracy of the CMT for detecting IMI, which provides novel insights into using the CMT for making decisions on dry cow therapy or other antibiotic treatment of mastitis.

3. Chapter 2. Differential Somatic Cell Count Affects the California Mastitis Test

This chapter contained the work included in the paper:

Huang, C. H. and N. Kusaba. 2022. Association between differential somatic cell count and California Mastitis Test results in Holstein cattle. JDS Communications 3:441 - 445. https://doi.org/10.3168/jdsc.2022-0249.

3.1. Introduction

As discussed in Chapter 1, a successful SDCT program relies on tests accurately identifying cows or quarters with IMI. Likewise, in the other aspects of mastitis control, for example, the treatment of clinical mastitis, a reliable and convenient test for detecting IMI, such as the CMT, can facilitate the allocation of antibiotics, which is at the core of antibiotic stewardship in mastitis control. As a reminder of the section *California mastitis test*, the CMT is a fast and cheap cow-side test (Schalm, 1957) widely used for screening IMI (Sargeant et al., 2001), guiding SDCT (Bhutto et al., 2012), and monitoring therapeutic response in clinical mastitis (Lam et al., 2009). We can estimate the SCC level in milk by scoring the CMT reaction, a reaction caused by the precipitation of cell DNA and protein (Nageswararao and Derbyshire, 1969). Due to the close association between SCC and IMI, treatment decisions have been made using the CMT. Nonetheless, the CMT reaction is an imprecise estimation of SCC because a huge variation in SCC values has been observed in milk samples with a similar CMT reaction (Ruegg and Reinemann, 2002). Consequently, the CMT has lower accuracy for detecting IMI compared with tests using proper SCC thresholds (Sargeant et al., 2001), likely leading to improper allocation of antibiotics in clinical practice.

Because the CMT works through the precipitation of DNA and proteins, and structural differences exist between cell types, the proportions of each cell type in milk may influence the CMT reaction. The somatic cells in milk comprise PMN, macrophages, and lymphocytes. Each type of cell plays a different role in mastitis, and consequently, their proportions vary between stages of mastitis (refer to section *Differential somatic cell count*). As a result, when samples are with similar SCC values but from different stages of mastitis, the CMT reaction may differ. Herein, we aimed to investigate how differential cell counts influence the CMT result by using DSCC (the combined proportion of PMN and lymphocytes) and macrophage proportions (MAC[%]; equals 100-DSCC). We hypothesized that both total and differential cell counts

influence the CMT. The conclusion is potentially useful for understanding the variation in the CMT results between stages of mastitis.

3.2. Materials and Methods

To understand how varied total and differential cell counts between stages of mastitis affect the CMT result, we performed the CMT on 0, 3, 5, 7, 14, and 21 d after identifying mastitis, and simultaneously collected milk samples from the affected quarter to determine SCC, DSCC, MAC, as well as the IMI status. We conducted the study from June 2021 to February 2022. All experimental procedures followed the Guidelines for the Care and Use of Agricultural Animals of Obihiro University (Permission number: #21-156).

3.2.1. Study herd and enrolled cows

The study herd used in the current study was consistent with that described in section 2.2.1. Study herd. The study enrolled cows confirmed with clinical or subclinical mastitis but excluded cows with mastitis caused by the same pathogen as the previous occurrence (i.e., recurrent mastitis). Farm staff identified clinical mastitis during milking through clinical signs, such as clots in milk or swollen udder, and study personnel confirmed the case within 6 h. Subclinical mastitis was identified by the monthly DHI test (>200,000 cells/mL), and the affected quarter was further confirmed through the CMT. Cows with clinical mastitis caused by major pathogens (i.e., SA, Str., and GNR) were treated with antibiotics once a day following afternoon milking for 5 consecutive days (i.e., from d 1 to 5), but the other cows (i.e., cows with subclinical mastitis or cows with clinical mastitis caused by minor pathogens or non-specific pathogens) remained untreated.

3.2.2. Sampling and the determination of SCC, DSCC, and IMI status

When confirming mastitis (d 0), we collected the quarter milk sample and performed the CMT after cleaning the udder and forestripping. We collected another 30 ~ 50 mL sample, which was preserved at 4°C until analysis. Within 4 d of sample collection, we sent the sample to the laboratory of Tokachi Federation of Agricultural Cooperatives to determine SCC (cells/mL), DSCC (%), and MAC (%) using the Fossomatic 7 DC (Foss Analytical A/S). We collected another 3 to 5 mL of sample aseptically to determine IMI status through bacterial culture, which was performed immediately after sample collection, following the method described in section 2.2.4. Bacterial culture of milk. On 3, 5, 7, 14, and 21 d after identifying mastitis, the same procedure (i.e., the CMT, bacterial culture, and the determination of SCC and DSCC) was conducted immediately before afternoon milking.

3.2.3. Procedures for the CMT

The CMT was performed with a commercial reagent (8.5 g/L sodium dodecylbenzene sulfonate containing bromothymol blue; P.L. tester; Nippon Zenyaku Kogyo Co. Ltd.), following a standard method (Schalm, 1957). Milk was injected into a paddle and adjusted to 3 mL. An equal volume of CMT reagent was added and mixed well. The reaction was interpreted within 15 s and scored from 0 to 3, where 0 was no thickening, 0.5 (trace reaction) was slight thickening, 1 was thickening without gel formation, 2 was immediate thickening with slight gel formation, and 3 was distinct gel formation. All CMTs were performed by myself (the author), and I was blinded to SCC and DSCC values when conducting the CMT.

3.2.4. Statistical analysis

All statistical analyses were performed using R software (v4.0.5; R Core Team, 2021). We excluded mastitis events from the analysis where follow-up was interrupted due to culling (n = 1), dry-off (n = 2), or receiving systemic medications for lameness (n = 2). SCC was unmeasurable in milk full of clots (n = 4), and DSCC measurements were unreliable in samples with ≤50,000 cells/mL (n = 37), thus we excluded such records as well.

Before model fitting, we transformed SCC to somatic cell score (**SCS**), which equals $\log_2(\text{SCC}/100{,}000) + 3$, to fit a normal distribution. Visual inspection of the quantile-quantile plots for DSCC and MAC values indicated left- and right-skewed distributions, respectively. Because MAC equals 100-DSCC, MAC was log-transformed and used for analysis (Osborne, 2002). To determine the association between the SCS, MAC, and CMT score (an ordinal categorical outcome), we analyzed data in a *cumulative logit mixed model* using the "clmm" function within the "ordinal" package (Christensen, 2019). The model was of the form

$$\textbf{\textit{Equation 1}}\textit{: Logit } [P(Y_i \leq j)] = \theta_j - \beta_1 SCS_i - \beta_2 \log(MAC_i) - day_k - \gamma_m - \varepsilon_i ,$$

where Y_i is the response variable (CMT score) for the i^{th} observation, taking on a value of either 0, 0.5, 1, 2, or 3, and j denotes the 5 possible values of Y_i. The parameter θ_j provides a separate intercept for each category j; SCS_i, MAC_i, and day_k are fixed effects of SCS, MAC, and sampling day (6 levels: d 0, 3, 5, 7, 14, 21), respectively, for the i^{th} observation on day k. β_1 and β_2 are regression coefficients for SCS_i and MAC_i respectively; γ_m is the random effect allowing random intercept for each mastitis event to account for the correlation of effects within the mastitis event, and ε_i is the error term. The significance of each term in the model was tested by likelihood ratio tests. The random effect of each cow and 2-way interactions of the fixed effects were also tested but later dropped from the model due to the lack of significance. The effects were deemed significant when $P < .05$.

3.3. Results and Discussions

3.3.1. Descriptive statistics

During the study period, we followed 58 mastitis events occurring in 41 cows and obtained 348 quarter-level records. After excluding data where SCC or DSCC values were inaccessible, the data set included 307 records. Table 3 provides descriptive statistics of SCS and DSCC (%) by CMT scores. Despite a positive association between SCS and CMT scores, SCS varied greatly in each score, showing that the scores may not depend solely on SCS. All missing values in category CMT score 0 were due to the unavailability of DSCC values and with SCS ≤ 2, leading to an overestimation of the mean of SCS. However, we do not believe that these missing values would affect the estimation of the model because samples with SCS ≤ 2 are likely to show negative reactions in the CMT regardless of DSCC. Figure 7 shows changes in CMT score according to SCS and MAC (100-DSCC). From the figure, we noticed that the CMT score increased as macrophage proportions increased.

Table 3. The average somatic cell score and differential somatic cell count (DSCC) within each california mastitis test (CMT) score (n = 307; *SD = standard deviation).

			Somatic cell score				DSCC	
CMT score	n	NA	Mean	SD*	Minimum	Maximum	Mean, %	SD
0	102	36	4.48	1.45	2.16	8.77	69.74	13.3
0.5	58	1	5.97	1.56	2.64	10.00	71.58	10.0
1	78	0	7.07	1.51	4.00	11.09	72.25	14.5
2	45	1	8.85	1.57	5.00	11.67	71.28	17.1
3	24	3	11.25	0.92	9.56	12.37	69.59	7.3

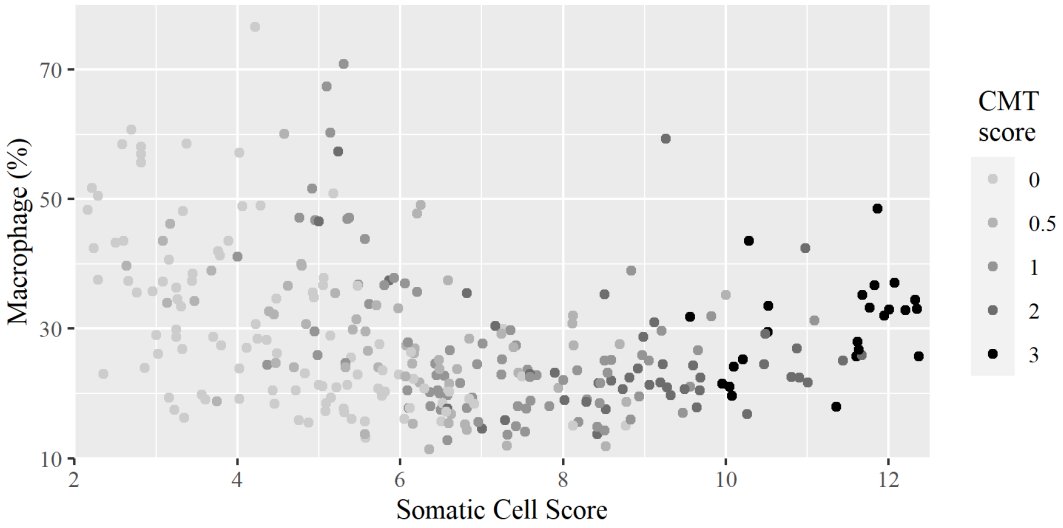

Figure 7. Variations in california mastitis test (CMT) score as a function of somatic cell score and macrophage proportions.

3.3.2. Model summary

Table 4 shows the model summary on analyzing the association between SCS, the logarithm of MAC, and the CMT score. Both SCS and MAC significantly influenced CMT results (P = 2.71E-44 and P = 5.08E-4 in ANOVA, respectively). Specifically, CMT scores were positively associated with the logarithm of MAC (odds ratio [**OR**]: 4.35, 95% confidence interval [**CI**]: 1.91–9.91). This phenomenon can be explained by the different cellular structures between macrophages and PMN. Nagahata et al. (1987) reported that the activity of lysosomal enzymes (i.e., NAGase and β-glucuronidase) is higher in milk macrophages than in milk PMN, and the activity of these enzymes was strongly related to the CMT reaction. The difference in the activity of lysosomal enzymes between macrophages and PMN could be a potential mechanism causing their distinct behavior in the CMT.

Table 4. Summary of the model on analyzing the association between somatic cell score (SCS), macrophage proportions (MAC), and california mastitis test (CMT) scores

Parameters	Coefficients (SE[b])	Odds ratio (95% CI[c])	ANOVA		
			LR Chisq[d]	Df [e]	P-value
SCS	1.30 (0.12)	3.66 (2.89, 4.64)	194.90	1	2.71E-44
log (MAC)[a]	1.47 (0.42)	4.35 (1.91, 9.91)	12.09	1	5.08E-4
Days after identifying mastitis (Reference: d 0)			11.10	5	0.049
D 3	-1.58 (0.51)	0.21 (0.08, 0.56)			
D 5	-1.12 (0.53)	0.33 (0.12, 0.92)			
D 7	-1.48 (0.56)	0.23 (0.08, 0.69)			
D 14	-1.39 (0.58)	0.25 (0.08, 0.78)			
D 21	-1.11 (0.58)	0.33 (0.11, 1.02)			
Intercepts of cut-points					
0|0.5	10.31 (1.95)				
0.5|1	12.11 (1.99)				
1|2	14.99 (2.07)				
2|3	18.62 (2.23)				

[a] Logarithm of macrophage proportions; [b] Standard error; [c] Confidence interval;
[d] Likelihood ratio χ^2 value; [e] Degree of freedom.

Compared with samples collected on the day mastitis was identified (i.e., d 0), samples collected on other days tended to have a lower CMT score (OR ranging from 0.21 to 0.33, and except for D 21, in which the 95% CI of OR did not include 1). This can be attributed to a different sampling timing (d 0 samples were collected after milking) or expectation from the investigator (expecting that quarters with mastitis would have high CMT scores). Furthermore, the random effect of mastitis events was highly significant (P = 1.49E-4), suggesting that variance in CMT results exists between mastitis events.

We also provide intercepts of cut points (θ_j) for the fitted model. These values may be inessential for interpreting results but can be used for prediction. In a cumulative logit model, the threshold parameter (θ_j) is the intercept for the j^{th} cumulative logit: logit($P(Y_i \leq j)$). Given a sample with known values of SCS, MAC, and day, we can predict which CMT score it would show. For example, for a sample collected on day 5, with an SCS value of 4 and a MAC value of 20, the possibility of it showing a CMT score ≤ 0 would thus be anti-logit(10.31 - 1.3×4 - 1.47×log(20) - (-1.12)) = anti-logit(1.826) = 0.862 (see *Equation 1* & coefficients in Table 4), and the possibility that it shows CMT score ≤ 0.5 would be anti-logit(12.11 - 1.3×4 -

1.47×log(20) - (-1.12)) = anti-logit(1.826) = 0.974, thus the possibility it shows CMT = 0.5 would be 0.974 - 0.862 = 0.112. In this manner, we can predict the possibility that the CMT shows a score of 1 ~ 3 for any samples with known values of SCS and MAC, as visualized in Figure 8. Remember that the SCS is a logarithmic conversion of SCC, thus the same CMT reaction may represent hugely varied SCC values depending on the macrophage proportions.

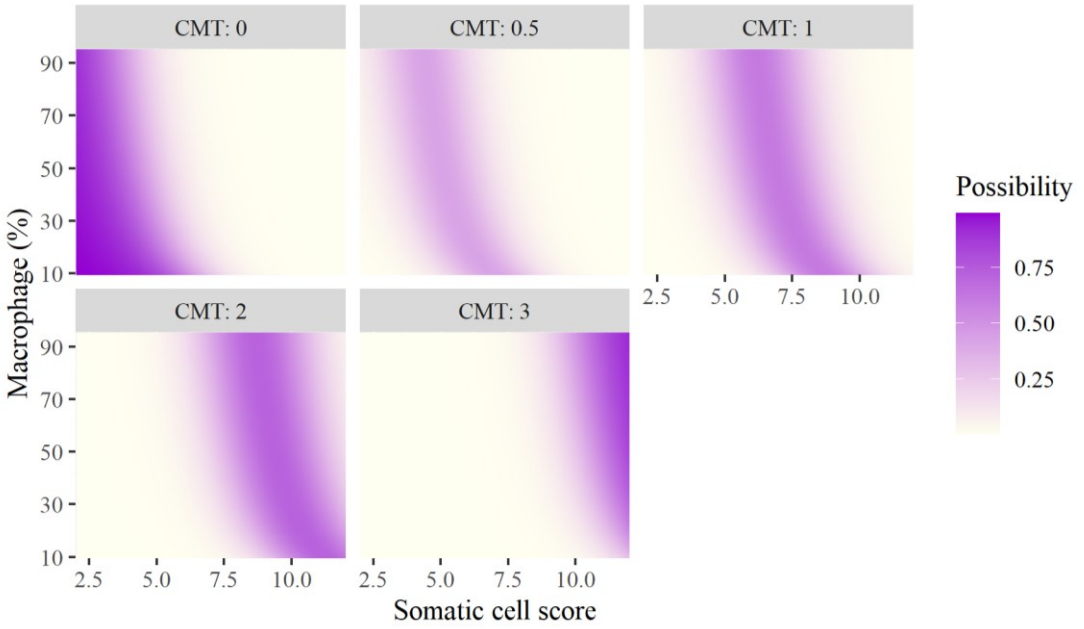

Figure 8. Predictions of the california mastitis test (CMT) score according to somatic cell score and macrophage proportions for samples collected at d 5 after mastitis occurred.

3.3.3. Concerns over the current use of the CMT for mastitis control

Our results raise some concerns over the current use of the CMT for mastitis control. To explain this, we selected 4 cases (i.e., 912B, 922B, 753D, and 781C) to illustrate, with the progress of mastitis, how changes in SCS and macrophage proportions may affect CMT results (Figure 9). In cases 912B and 922B (upper 2 panels), SCS decreased gradually with a corresponding increase in MAC, indicating a healing process of mastitis (see section *Differential somatic cell count*). This was supported by the negative bacterial culture results on d 7, 14, and 21 in both cases. However, in case 922B, the CMT result would lead us to the opposite conclusion. Specifically, the CMT showed positive reactions on d 14 and 21, but SCS had been decreasing gradually since d 7 in this case. This conflicting finding is thought to arise from the increasing macrophage proportions during the healing process of mastitis, leading to high CMT scores at relatively low levels of SCC. Because the CMT result conflicted with

bacterial culture results and SCC, the treatment period would have been extended unnecessarily if the treatment decision were based solely on CMT.

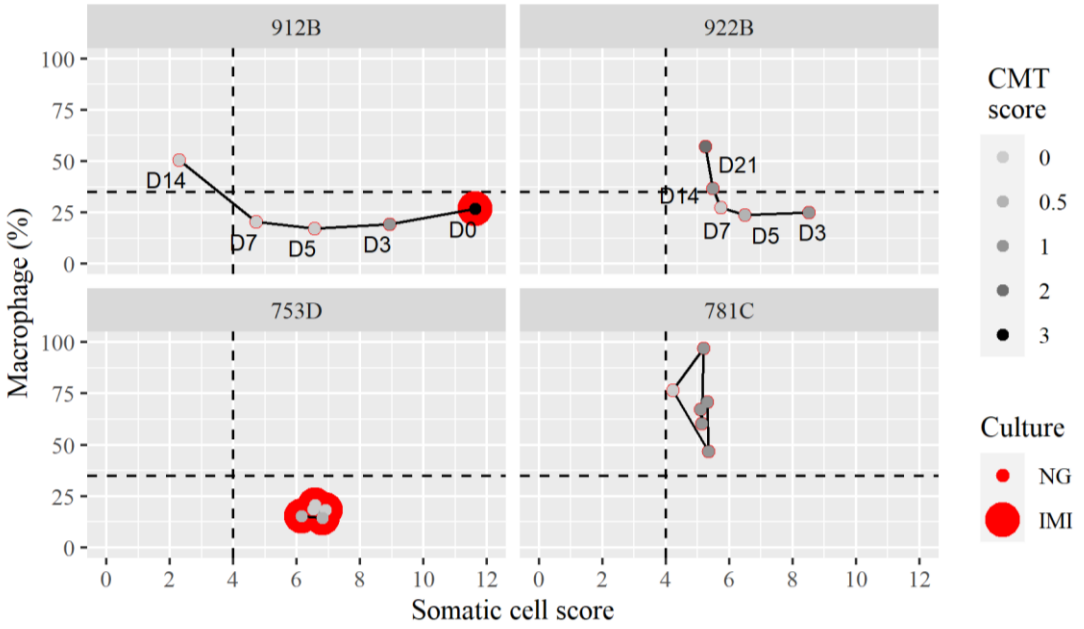

Figure 9. Examples showing how changes in somatic cell score and macrophage proportions affect california mastitis test (CMT) results. The 2 reference lines correspond to where SCS equals 4 and macrophage proportions equal 35% (i.e., DSCC equals 65%). In the upper 2 panels, points are labeled with days (D) after identifying mastitis. Milk culture results (i.e., no significant growth [NG] or IMI detected [IMI]) were labeled by red circles.

The disagreement between CMT results and SCC level existed in subclinical mastitis as well. Cases 753D and 781C (lower 2 panels in Figure 9) were cases of subclinical mastitis. An IMI of *Streptococcus* spp. had been detected persistently in 753D, whereas no IMI had been detected in 781C (i.e., nonspecific subclinical mastitis) during the study period. Although case 753D had continually higher SCS than case 781C, the latter showed stronger CMT reactions. This discrepancy probably resulted from the distinct DSCC between the 2 cases. Studies have reported DSCC values to be higher in cows with major pathogen IMI compared with cows without IMI or with minor pathogen IMI (see section *Differential somatic cell count*), consistent with the current observation. The findings on case 781C explain why positive CMT reactions were occasionally observed in some quarters from cows with low SCC (< 200,000 cells/mL), but antibiotic treatment (dry cow therapy) scarcely improve the health of these quarters (Swinkels et al., 2021). Notably, using the CMT to guide an SDCT program may lead to higher antibiotic use compared with an SCC-guided SDCT (McDougall et al., 2022).

The 2 dotted reference lines in Figure 9 were drawn based on cut-offs suggested by Schwarz et al. (2020a), in which they showed that cows with high SCS and low DSCC (chronic

mastitis; such as Case 781C in our example) are the least productive and the most likely to leave the herd (see also section *Differential somatic cell count*), and we further clarified this relationship with another approach in Chapter 3. Accordingly, identifying cows and quarters with chronic mastitis potentially benefits herd management, and the CMT can be a screening tool when quarter-level information is inaccessible.

3.3.4. Limitations of this study

Since we used the CMT to identify quarters with subclinical mastitis, selection bias may thus be introduced. Another potential source of bias is the expectation of the CMT reaction based on mastitis progression, which we addressed by considering sampling days from identifying mastitis. Potential confounders can be milk components correlated with DSCC (e.g., lactose; Bobbo et al. (2020); Pegolo et al. (2021)); however, milk components other than nucleated cells are less likely to affect the CMT reaction, in which polymerized DNA is required (Nageswararao and Derbyshire, 1969). Notably, our findings require further validation by performing the CMT with other reagents, because the choice of reagent is known to affect the sensitivity of the CMT (Leach et al., 2008). We selected several cases to illustrate how changes in macrophage proportions can affect the field use of the CMT, but the generalizability of these findings warrants further research.

3.4. Conclusions

Results from this study demonstrate a positive association between MAC and the CMT result. In the recovery of mastitis, MAC tended to increase as SCC decreased, possibly leading to a false-positive CMT reaction and, consequently, an unnecessary extension of treatment. Furthermore, in some cases of subclinical mastitis, the high MAC leads to positive CMT results; however, antibiotics may be unhelpful in such cases due to the absence of IMI. Thus, we advise not making treatment decisions based on the CMT alone. Instead, bacterial culture and SCC or DSCC tests may be helpful aids. From another perspective, we noted that the CMT is especially sensitive to quarters with chronic mastitis. Therefore, the CMT can be used to screen cows with chronic mastitis. We believe results from the present study can be applied to improve antibiotic stewardship in both lactation and dry cow therapy.

4. Chapter 3. Estimate Mastitis Impact using Total and Differential Somatic Cell Count

The manuscript regarding the work in this chapter has been prepared for publication:

Huang, C. H., K. Furukawa, and N. Kusaba. *Estimating the nonlinear interaction between somatic cell score and differential somatic cell count on milk production by parity using generalized additive models.* Manuscript.

4.1. Introduction

Due to the significant impact of mastitis on economics, estimating the negative effects of mastitis on milk yield and quality is one of the important subjects in dairy herd management (Halasa et al., 2007; Huijps et al., 2008; Le Maréchal et al., 2011; Hogeveen et al., 2019). The impact of subclinical mastitis has been evaluated mostly based on SCC since the information is highly accessible in herds with regular DHI milk testing. For decades, a threshold value of SCC, 200,000 cells/ml has been commonly used to indicate subclinical mastitis and is considered detrimental to milk yield and quality (Dohoo and Leslie, 1991). However, the applicability of this threshold value has been questioned (Bradley and Green, 2005), as a sound threshold value varies depending on the cows' situation (e.g., parity or lactation stage), and several studies observed significant milk losses even when SCC was well below this threshold (Dürr et al., 2008; Halasa et al., 2009; Gonçalves et al., 2018; Bonestroo et al., 2022). Moreover, when SCC increases, milk losses increase (Gonçalves et al., 2018; Bonestroo et al., 2022). To clarify the negative impacts of mastitis, we believe that dichotomizing SCC values based on a predefined threshold should be avoided, and this practice should be extended to other mastitis indicators as well.

As briefed in the section Differential somatic cell count, Schwarz et al. (2020a) and Bobbo et al. (2020) and other scientists (Pegolo et al., 2021; Mariani et al., 2022) demonstrated that cows with high SCC and low DSCC tend to produce less milk, possibly an effect of chronic mastitis. Despite their important contributions to the literature, the analyses were, unfortunately, conducted either by dichotomizing SCC and DSCC values based on predefined thresholds or by categorizing these values based on their percentiles. An appropriate threshold value of DSCC currently has yet to be supported by sufficient evidence. Due to the close relationship between SCC and DSCC, the threshold probably should be adjusted depending on SCC. Specifically, the meaning of a DSCC of 50% may differ when SCC is 50,000 cells/ml or 500,000 cells/ml, therefore the interaction between SCC and DSCC should be considered.

Increasing the number of categories for SCC and DSCC values and including their interaction seemingly solved some problems, as has been done by Mariani et al. (2022). However, modern statisticians discouraged categorizing continuous variables as well because it causes potential bias and loss of statistical power (Frank, 2015).

Recently, Bonestroo et al. (2022) have demonstrated that generalized additive models (GAM) are suitable for describing the nonlinear relationship between SCC and milk yield. This approach essentially eliminates the necessity of predefining a threshold or categorizing any continuous variables. This is helpful because continuous variables are common in observational studies. For example, in the present study, continuous variables range from interests of the investigation, such as SCC and DSCC, to confounding factors, such as DIM and sampling date. In GAM, the effects of these confounding factors are well quantified and controlled, and consequently, the effects of research interests can be carefully clarified.

To our knowledge, the effects of SCC, DSCC, and their interaction on milk production have yet to be investigated by using GAM. Additionally, previous studies have seldom quantified lactation curves and seasonal effects on milk yield and milk components, namely fat, lactose, and protein percentages, with mastitis effects into consideration. Consequently, this information is absent on dairy farms in Japan. Hence, in this study, we aim to analyze test-day records with GAM rather than pre-defined thresholds to investigate: (i) the nonlinear effects of SCC, DSCC, and their interaction; (ii) lactation curves and seasonal effects, on milk yield and milk components by different parity of cows.

4.2. Materials and Methods

4.2.1. Data

Data was provided by the Tokachi Federation of Agricultural Cooperatives, the largest DHI association in Tokachi Subprefecture, Hokkaido, Japan. The original dataset consisted of 460,580 test-day records collected from 96,075 lactating cows in 278 herds between January 2021 and March 2022. Most herds kept solely Holstein cattle. On average, there were 347 (ranging from 7 to 2,065) cows' records available for each herd, and 4.79 test-day records available for each cow. The test-day records include information on milk yield (kg/d), milk components (fat, protein, and lactose percentages), SCC (cells/ml), and DSCC (%), in addition to cow information such as lactation number and DIM. Each cow and herd were assigned a unique identifier, allowing us to estimate the random effect of cows and herds resulting from unobservable genetic and management differences.

4.2.2. Sample collection and analysis

Composite milk samples were collected cow by cow, where the sample timing (during morning or evening milking) was alternated for each herd from month to month. After collection, samples were preserved using bronopol and analyzed mostly within 4 days. SCC, DSCC, and milk composition were determined using CombiFoss 7 DC (FOSS Analytical A/S) in the testing center of the Tokachi Federation of Agricultural Cooperatives.

4.2.3. Data cleaning and editing

There were two purposes for data cleaning: first, to obtain reliable and informative data; second, to reduce the computational burden for model fitting. For this, we excluded one record with missing values, records outside the range of 6 to 305 DIM (378,583 records left in the cohort), and records where SCC was outside the range of 50,000 to 1,500,000 cells/mL (166,924 records left in the cohort). Although Schwarz et al. (2020a) considered including measurements outside this range, the DSCC determination was most reliable for samples within the range chosen (Damm et al., 2017). Last, we excluded cows with less than 5 observations, and herds with less than 50 cows recorded in the dataset, resulting in 50,296 test-day records of 7,484 cows (average of 6.72 records/cow) in 71 herds. This step aimed to reduce the computational burden resulting from random effect estimation in GAM, which increased with the number of groups squared (Pedersen et al., 2019).

Several steps were taken to aid statistical analysis. Yields of each component, namely protein (kg/d), fat (kg/d), and lactose (kg/d) yields, were calculated by multiplying their percentages by milk yield, and SCC was transformed to SCS. Further, we aggregated records based on lactation numbers, namely first, second, and third or more lactation (labeled as 1, 2, and 3+, respectively). We expected greater milk losses in cows with high parity due to their greater milk yield (Gonçalves et al., 2018; Bonestroo et al., 2022), and expected varied lactation curves in cows with different parity (Schutz et al., 1990), thus we estimated milk yield and components separately for each parity group as described in the following section. In a preliminary analysis, we found that subdividing the last group (i.e., third or more lactation) contributed little to the model fitting.

4.2.4. Statistical analysis

All analyses were performed in R 4.0.5 (R Core Team, 2021). We fit GAM using the "bam" function within the "mgcv" package (Wood, 2017). This function enabled us to save substantial computational time and memory when fitting GAM to large datasets (Wood et al., 2015).

To describe the statistical analysis in the present study, a brief on the role of GAM is required. GAM relaxed the linearity assumption in the generalized linear model by replacing parametric coefficients (β_j) with *smooth functions* (f_j) of the independent variables. These smooth functions were not restricted to any parametric forms and thus were very flexible in describing the nonlinear dependence between the response and predictors. Also, the smoothness of these functions is controlled properly through penalization via a *smoothness parameter*. In practice, this parameter can be selected automatically typically by the restricted maximum likelihood, which largely prevents overfitting, ensuring that these smooth functions approximate the true relations between the predictors (i.e., SCS, DSCC, DIM, and day of the year) and the response (i.e., milk yield or components) (Wood, 2017; Pedersen et al., 2019). *Random effects* in GAM are also estimated via smooth functions, in the sense that the coefficients of each group (i.e., individual cow and herd) are shrunk towards zero through the smoothness parameter, and the degree of shrinkage is inversely proportional to the random effect variance (Wood, 2017; Pedersen et al., 2019). *Interaction effects* can be estimated using tensor products in GAM (Wood, 2006). This approach allows each predictor to have its own smoothness penalty and smooth functions, making it very applicable as one of our purposes is to estimate the interaction between SCS and DSCC, which are on completely different scales. Moreover, mgcv allows us to construct *group-specific* smooth functions using the "by" argument (Wood, 2017; Pedersen et al., 2019). This is useful since we expected that changes in milk yield due to SCS/DSCC, DIM, or seasons differ *by* the parity group.

Based on the above, we constructed the following models:

Equation 2:

$$y_i = \beta_0 + \beta_{parity} + f_1(SCS_i) \times \text{Parity} + f_2(\text{D}SCC_i) \times \text{Parity} + f_3(\text{SCS}_i, \text{D}SCC_i) \times \text{Parity} + f_4(DIM_i) \times \text{Parity} + f_5(DoY_i) \times \text{Parity} + \gamma_{herd} + \gamma_{cow} + \varepsilon_i ,$$

where y is the response variable, and i represents the ith observation. y_i is either milk yield, milk component percentages, or milk component yields of the ith observation. We assumed that these responses would have a scaled t distribution (i.e., heavy-tailed). β_0 is a general intercept, and β_{parity} are parity group-specific intercepts. f_1, f_2, f_3 and f_4 are smooth functions for SCS, DSCC, the interaction between SCS and DSCC, and DIM, respectively. These smooth functions were constructed with *cubic regression splines*.f_5 is the smooth function for the day of the year, which was constructed with *cyclic cubic regression splines*, forcing the beginning and end of the year to be joined to estimate the cyclic seasonal effect. $f_1 \sim f_5$ are all group-specific smoothers, meaning that each parity group (i.e., 1, 2, and 3+) was fitted separately and consequently has its own smooth functions for each predictor. γ_{herd} and γ_{cow} are random

effects of herd and cow to account for variation between herds and cows, giving each herd and cow their own intercepts, and ε_i is the residual for the ith observation. Smooth functions usually consist of more than one parameter and are better interpreted by visualization. To visualize the interaction between SCS and DSCC, we plotted predictions by excluding DIM and seasonal effects as well as the random effects of herd and cow. To visualize the DIM and seasonal effects, we plotted predictions at a value of SCS equivalent to 2 and DSCC equivalent to 65%, which is close to the average in the original dataset, while excluding other effects.

All models were checked with the "gam.check" function in mgcv for the distribution and homoscedasticity of residuals, as well as potential outliers. Outliers were detected occasionally. However, due to the large sample size, keeping these outliers was not considered to alter the inference of results, and we believe discussing the causes of these outliers is beneficial to this study. We also assessed autocorrelation using autocorrelation plots. Slight autocorrelation was detected (|rho| < .1) and solved using the "itsadug" package (Van Rij et al., 2022) with the approach described in Bonestroo et al. (2022).

4.3. Results and Discussions

Previous studies assumed that SCC elevates in mastitis, DSCC elevates in early infections, and macrophages are predominant in healthy or chronically infected udders. They thus divided cows into 4 groups based on one threshold of SCC along with one threshold of DSCC (see section Differential somatic cell count). However, there were no clearly defined thresholds for distinguishing between infected/uninfected cows or acute/chronic infections. Herein, we aimed to investigate the nonlinear interaction between SCS (a logarithm transformation of SCC) and DSCC and its effect on milk yield and milk composition (i.e., milk component percentages) without predefined thresholds. The effects of SCS and DSCC on milk component yields were also analyzed, and we supplemented the results as figures in the appendix (Figure S1~S3). Additionally, lactation curves and seasonal variations in milk production have not been investigated with the effects of mastitis in consideration, thus we will discuss these phenomena as well.

4.3.1. The distribution of observations and descriptive statistics

After data cleaning, the distribution of the observations is shown as a function of each independent variable (i.e., SCS/DSCC, DIM, and sampling date) in

Figure 10. Since the investigation had been conducted from January 2021 to March 2022, there were more observations in the first three months of the year. Interestingly, as parity

increased, observations with high SCS and low DSCC increased. This corresponds to the findings of Schwarz et al. (2020a) and may result from the higher risk of chronic mastitis in cows with higher parity.

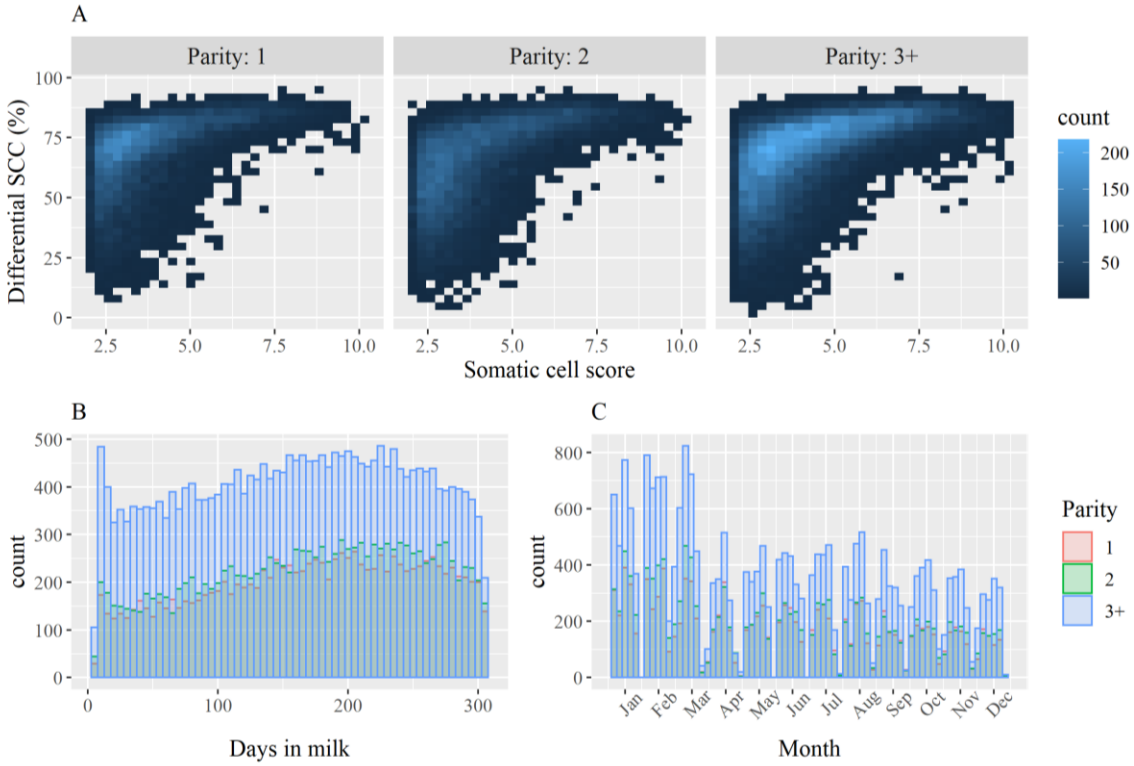

Figure 10. The distribution of observations used in the analysis of the impacts of somatic cell score (SCS), differential somatic cell count (DSCC), days in milk (DIM), and season on milk production. Counts of observations were shown as a function of SCS/DSCC (A), DIM (B), and sampling date (C) by parity of cows.

Descriptive statistics for SCS, DSCC, milk yield, and milk composition are given by each parity group (Table 5), and as a function of DIM (Figure 11) and month of the year (Figure 12). The averaged SCS (i.e., 3.8 ~ 4.2, depending on the parity) was high compared to previous studies (Franzoi et al., 2020; Pegolo et al., 2021) as an effect of excluding observations with SCC lower than 50,000 cells/ml. Before data cleaning the average SCS was indeed around 1.9 ~ 2.6 depending on the parity, showing that mastitis management was optimal in investigated dairy herds. SCS increased as parity increased. Surprisingly, DSCC did not follow the same trend, despite the high association between these two traits found in other studies (Damm et al., 2017; Pegolo et al., 2021). Figure 11 shows that SCS maintained the same level during the entire lactation, but DSCC was lower in both the early and late lactation. Both of them suggest that parity and lactation stage are factors that affect the relationship between SCS and DSCC.

Notably, the low DSCC values observed in early lactation may be due to dysfunctions in neutrophil recruitment during the periparturient period (Nagahata et al., 1988; Shuster et al., 1996; Burton and Erskine, 2003), while the low DSCC values in late lactation may be due to higher risk of chronic mastitis (Olde Riekerink et al., 2007) during the time.

In addition, milk and milk component yields increased as parity increased, but lactose percentages were higher in primiparous cows (Table 5), corresponding to the findings of Costa et al. (2018). Lactation curves (Figure 11) and seasonal variations (Figure 12) for milk yield and milk composition followed other researchers' observations (Friggens et al., 2007; Salfer et al., 2020).

Table 5. Descriptive statistics of somatic cell score (SCS), differential somatic cell count (DSCC), milk yield, and milk component percentages and yields by different parity.

	1			2			3+		
Traits	Mean ± SD [a]	P5	P95	Mean ± SD	P5	P95	Mean ± SD	P5	P95
Cows	2300			2954			4141[b]		
N	12088			13495			24666 [c]		
SCS	3.8 ± 1.4	2.24	6.75	3.9 ± 1.5	2.26	6.97	4.2 ± 1.6	2.29	7.51
DSCC; %	68.7±14.3	39.5	85.7	64.6±16.3	33.9	85.5	67.9±15.2	37.6	86.1
Milk yield; kg/d	28.7 ± 7	16.6	39.8	32.4 ± 9.1	17.2	47.1	33.9 ± 9.8	17.4	49.8
Fat; %	4.1 ± 0.7	3.18	5.34	4.1 ± 0.7	3.13	5.29	4.1 ± 0.7	3.07	5.29
Fat yield; kg/d	1.2 ± 0.3	0.73	1.59	1.3 ± 0.4	0.72	1.9	1.4 ± 0.4	0.72	2.01
Protein; %	3.5 ± 0.4	2.92	4.05	3.5 ± 0.4	2.9	4.06	3.4 ± 0.4	2.79	3.99
Protein yield; kg/d	1 ± 0.2	0.61	1.31	1.1 ± 0.3	0.63	1.52	1.1 ± 0.3	0.61	1.57
Lactose; %	4.5 ± 0.2	4.19	4.76	4.4 ± 0.2	4.06	4.67	4.3 ± 0.2	3.94	4.64
Lactose yield; kg/d	1.3 ± 0.3	0.73	1.81	1.4 ± 0.4	0.73	2.11	1.5 ± 0.4	0.72	2.21

[a] Standard deviation.
[b] Parity 3: 2486 cows; 4: 1526 cows; 5: 755 cows; 6: 358 cows; 7+: 235 cows.
[c] Parity 3: N=11456; 4: N=6996; 5: N=3417; 6: N=1663; 7+: N=1134.

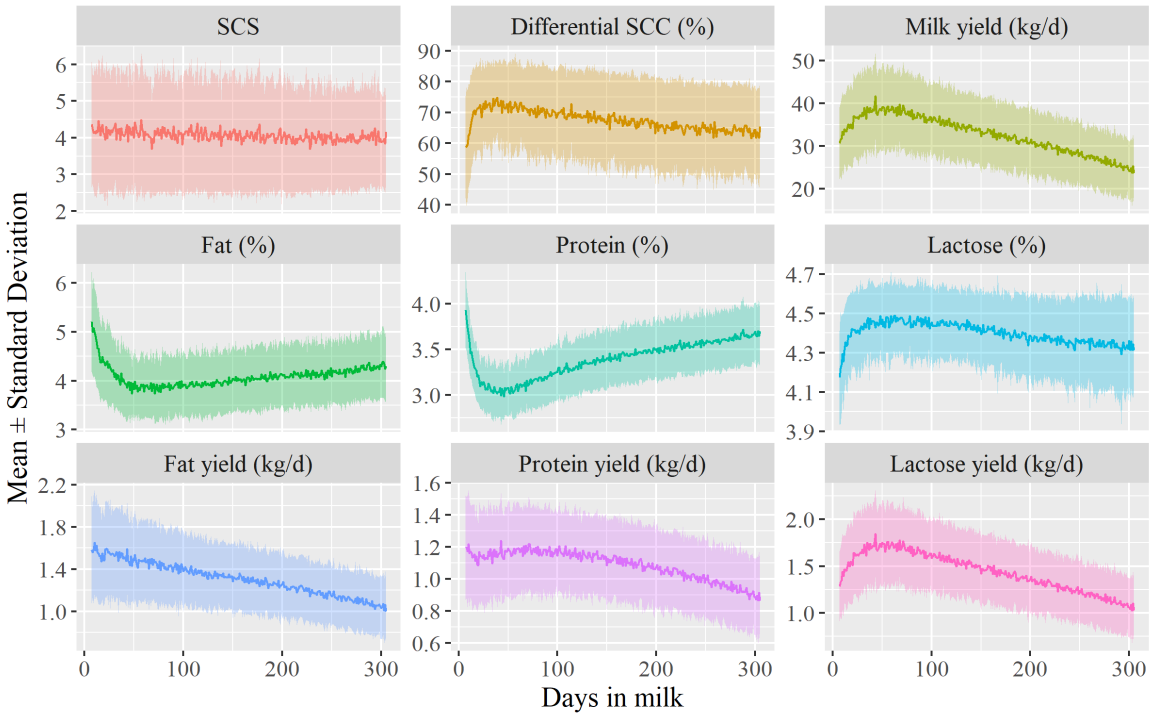

Figure 11. Descriptive statistics of somatic cell score (SCS), differential somatic cell count (DSCC), milk yield, and milk component percentages and yields by days in milk.

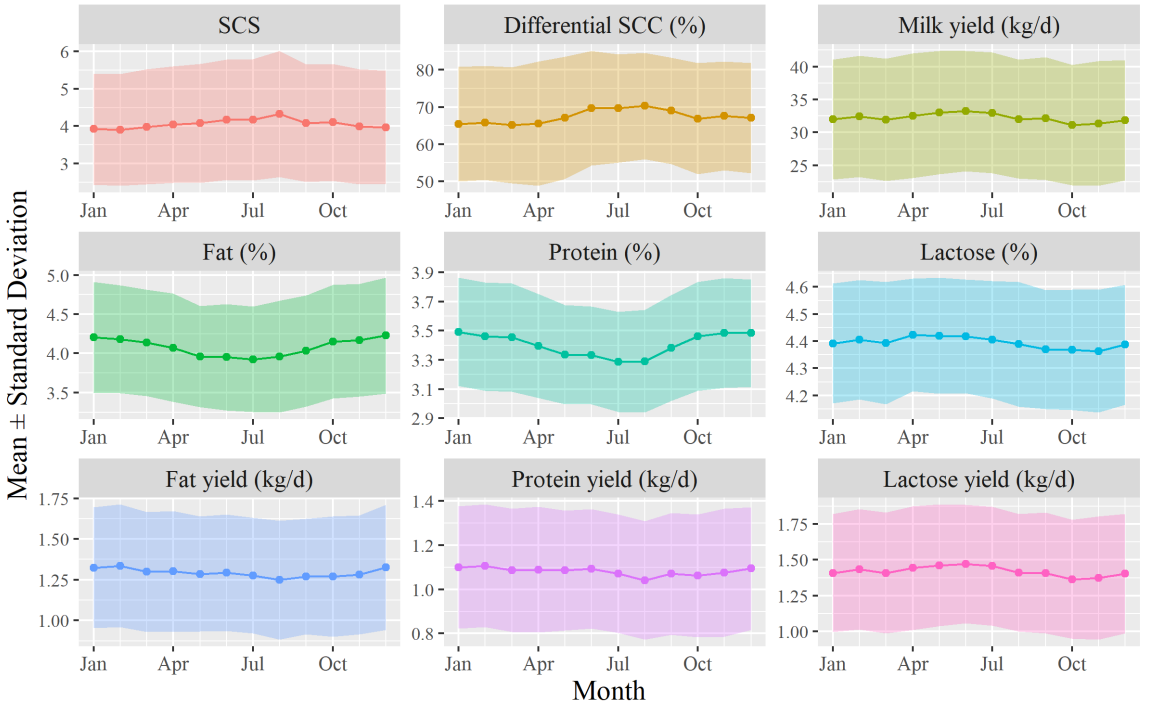

Figure 12. Descriptive statistics of somatic cell score (SCS), differential somatic cell count (DSCC), milk yield, and milk component percentages and yields by sampling month.

4.3.2. Summary of models

Table S2 summarizes fitted GAM. As expected, both SCS and DSCC significantly affected milk yield and composition in all parity groups (all p-values were very close to zero), parallel to previous studies (Bobbo et al., 2020; Schwarz et al., 2020a; Pegolo et al., 2021), indicating that DSCC can provide information inaccessible solely through SCS. Furthermore, effective degrees of freedom provided in the table revealed a degree of nonlinearity for the relationship between the predictor and the response, where an effective degree of freedom of 1 indicated a linear relationship, and a higher effective degree of freedom indicated a more complex, nonlinear relationship. The relationship between either SCS or DSCC and analyzed traits was mostly nonlinear. The former supports the findings of Bonestroo et al. (2022), while the latter is a novel finding. The interaction between SCS and DSCC was also significant in all parity groups. This finding corresponds to Mariani et al. (2022), highlighting the necessity of investigating this interaction.

Regarding confounding factors, the effects of DIM and day of the year (i.e., seasonal effect) were also highly significant and nonlinear, which we will address in the following sections. Random effects of cow and herd were significant in all models. The estimated standard deviations of these random terms are given in Table S2. Results show that variations between cows were greater than variations between herds, especially for lactose percentage, suggesting that lactose concentration mainly depends on genetic differences between animals rather than management differences between herds. In contrast, traits such as milk yield may still depend on herd-level management factors, such as milking system or frequency. Finally, adjusted R2 of fitted models ranged from 0.50~0.76 depending on the analyzed trait, indicating an overall fair to good goodness-of-fit.

4.3.3. Factors affecting milk yield

Effects of SCS and DSCC on milk yield

Figure 13A shows predicted milk yield as a function of SCS and DSCC. Milk yield was negatively related to SCS but positively related to DSCC. Interestingly, even when SCS was as high as 8 (3,200,000 SCC/ml), cows with high DSCC had productivity similar to healthy cows (30~40 kg/d). One explanation is that these cows may be in the early stages of mastitis, and their milk yields had yet to be affected. In addition, milk yields increased as DSCC increased even when SCS was low, despite the small difference. Considering these findings, a high DSCC value may indicate an active immune system and well-functioning neutrophil recruitment, likely beneficial to milk production (Burton and Erskine, 2003). This conflicts with the findings

of Schwarz et al. (2020a), where they reported that cows with high DSCC and low SCC were slightly less productive than cows with low DSCC and low SCC. In the study, cows with SCC < 50,000 cells/mL and cows with low DSCC/SCC (i.e., DSCC < 65%; SCC = 50,000 ~ 200,000 cells/mL) were all included in the group "healthy". This may cause significant bias due to the heterogeneity in the group. The detrimental effect of SCC on milk yield was observed when SCC was as low as 7,400 cells/mL (Dürr et al., 2008), thus cows with low DSCC/SCC were probably not as healthy as cows with SCC < 50,000 cells/mL. On the other hand, milk losses in cows with high SCS and low DSCC were critical, especially in cows with high parity (reduced from 40 kg/d to 20 kg/d). Previous studies demonstrated the detrimental effects of high SCS and low DSCC, which can be attributed to the impact of chronic mastitis (Bobbo et al., 2020; Schwarz et al., 2020a). However, for the first time, we demonstrated that this effect is more critical in cows with high parity, probably due to their great milk yields. Thus, we believe that avoiding chronic mastitis in these cows is the key to efficient milk production.

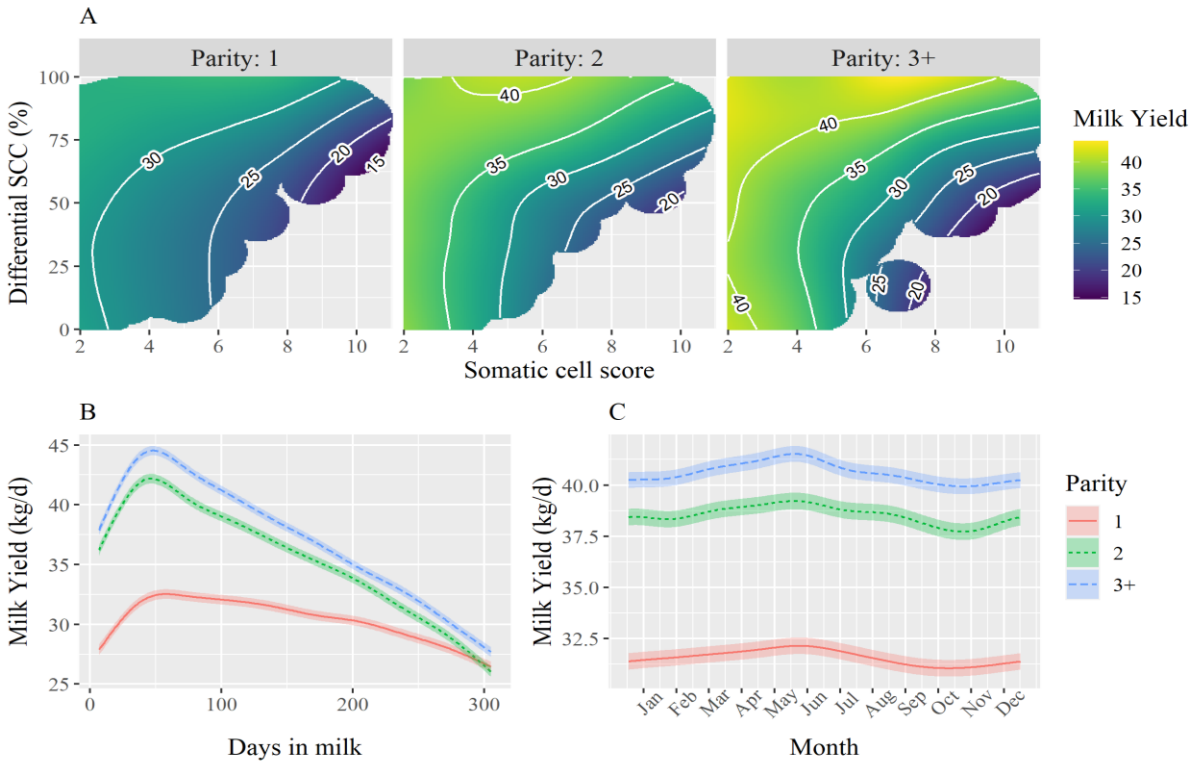

Figure 13. Predictions (± standard error) of milk yield as a function of SCS/Differential somatic cell count (DSCC; A), DIM (B), and day of the year (C) by parity of cows.

Lactation curves and seasonal variations in milk yield

Estimated lactation curves (Figure 13B) are similar to Wood's lactation curves (Wood, 1967). The peak of milk yield occurred around 48 DIM for multiparous cows but relatively later for primiparous cows (58 DIM), consistent with a recent study (Masía et al., 2020). Peak milk yields were 32.5, 42.2, and 44.5 kg/d for cows in first, second, and third and more lactations, respectively. As previous studies suggested, peak milk yield increased as parity increased, but persistency was higher in primiparous cows (Schutz et al., 1990). Even without discretizing DIM (Schutz et al., 1990) or assuming a particular form of relationship between DIM and milk yield (Wood, 1967), GAM estimated lactation curves reasonably well.

Figure 13C shows seasonal variations in milk yield. Seasonal variations in milk yield were similar in cows of all parity, with a peak in the middle of June and a nadir at the end of October. The peak occurred considerably later than that observed in US dairy herds (peaked in April; Salfer et al., 2020), but the amplitude of variations (around 0.6 ~ 0.9 kg/d, depending on parity) was close to that observed in Pennsylvania and Minnesota (around 1.2 kg/d), possibly due to the similar latitude between Tokachi and these two regions. Seasonal variations in milk yield were often attributed to heat stress; however, the nadir did not occur in mid-summer, suggesting that other factors, such as changes in day length (Salfer et al., 2020), may play a role in these variations.

4.3.4. Factors affecting milk lactose percentage

Effects of SCS and DSCC on milk lactose

Figure 14A shows the effects of SCS and DSCC on lactose percentage, which is similar to that on milk yield; except that when SCS was low, the effect of DSCC was almost negligible, which agreed with Bobbo et al. (2020). The effect of mastitis on lactose percentage has been well documented, as reviewed by Costa et al. (2019). Decreased lactose contents in mastitic milk can be attributed to two reasons: (i) impaired lactose synthesis of mammary epithelial cells; (ii) lactose leakages due to increased blood-milk barrier permeability, both resulting from the damage of inflammation and infection to the mammary glands. Lactose leakages directly relate to milk losses, because lactose is the main osmotic regulator between blood and milk (Costa et al., 2019). Pronounced lactose reduction in cows with high SCS and low DSCC may be an effect of chronic mastitis, as previous studies suggested (Bobbo et al., 2020; Pegolo et al., 2022). Alternatively, pathogen-specific effects may play a role. Some pathogens, such as *Prototheca* spp., can cause severe damage to the blood-milk barrier (see more detailed discussions in 5.4.4 The dynamics of DSCC in clinical mastitis); consequently, a large number of macrophages are

necessary for the repair of damaged mammary tissues (Wawron et al., 2013). This explains why low DSCC values (high macrophage proportions) and severe lactose reductions were observed during *Prototheca* spp. infections (Pegolo et al., 2022). In summary, the strong association between lactose and SCS/DSCC observed in our study supports the argument that lactose percentage is a potential alternative to monitoring udder health (Costa et al., 2019).

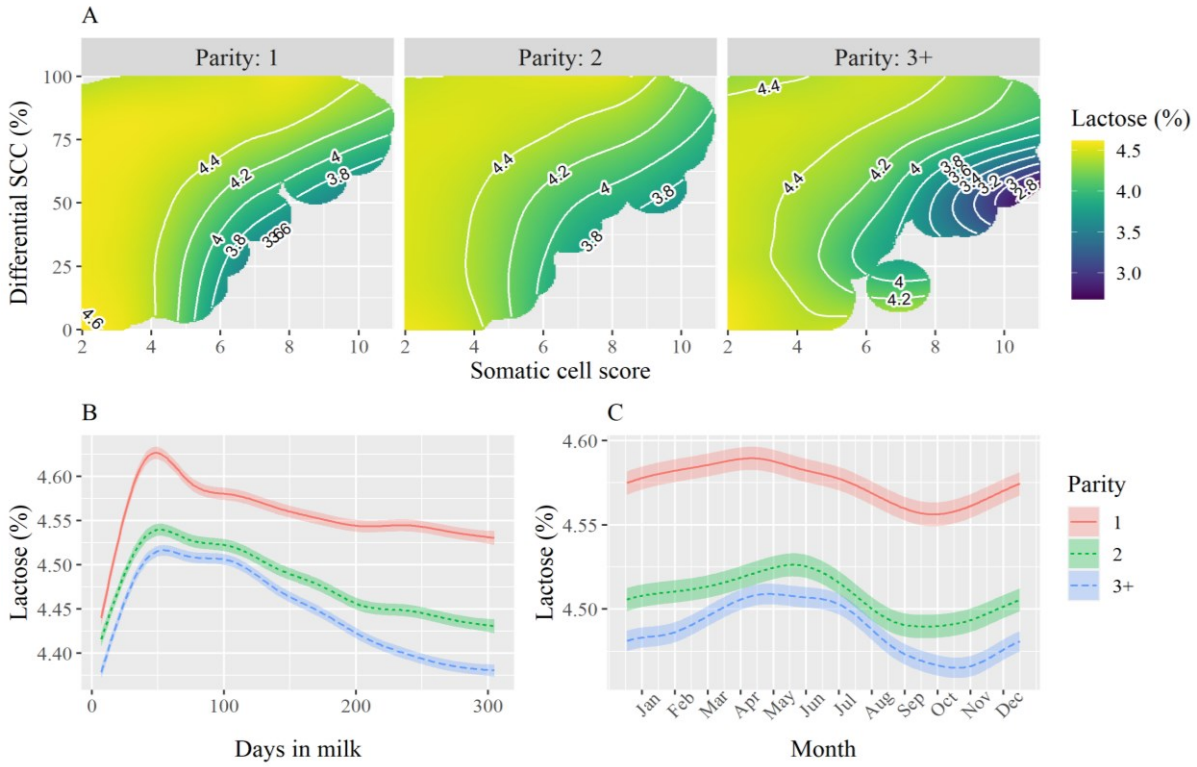

Figure 14. Predictions (± standard error) of lactose (%) as functions of SCS/Differential somatic cell count (DSCC; A), DIM (B), and day of the year (C) by parity of cows.

Lactation curves and seasonal variations in milk lactose

Lactation curves for lactose percentage (Figure 14B) were similar to that for milk, except that peak and persistence for lactose percentage were the highest in primiparous cows and decreased as parity increased, in agreement with Costa et al. (2018). Since we have considered the effects of mastitis on milk production using SCS and DSCC in our analysis, the difference here was unlikely due to the higher mastitis risk in multiparous cows. Instead, other physiological mechanisms, such as changes in mammary epithelium integrity and permeability with increasing parity, may be involved (Costa et al., 2019). Seasonal variations in lactose percentage were detected (Figure 14C), following a similar trend as in milk yield, with a peak in spring and a nadir in autumn.

4.3.5. Factors affecting milk fat and protein percentages

Effects of SCS and DSCC on milk fat and protein

Figure 15A and Figure 16A show the effects of SCS and DSCC on milk fat and protein percentages, respectively. The effects were similar to each other but opposed to that of milk yield and lactose percentage. As observed by Bobbo et al. (2020), milk from cows with high DSCC and low SCS was with the lowest fat (3.4~3.6%, depending on parity) and protein percentages (2.9~3%, depending on parity). There are two explanations for this phenomenon: (i) the dilution effect due to the high milk yield from these cows, as shown in Figure 13A; (ii) the proteolytic (Le Roux et al., 2003) and lipolytic (Gargouri et al., 2008) effects of PMN. To clarify, we evaluated the association between SCS, DSCC, and yields of fat and protein (Figure S2A & Figure S3A). Results were ambiguous as patterns differed depending on the parity of cows, suggesting that both mechanisms may be involved. Interestingly, milk with the highest fat (4.6~4.8%, depending on parity) and protein percentages (3.5~3.9%, depending on parity) was produced by cows with high SCS and low DSCC (Figure 15A & Figure 16A). This can be attributed to the concentration effect due to reduced milk yield in these cows. According to Figure S2A & Figure S3A, protein and fat yields from these cows were indeed the lowest.

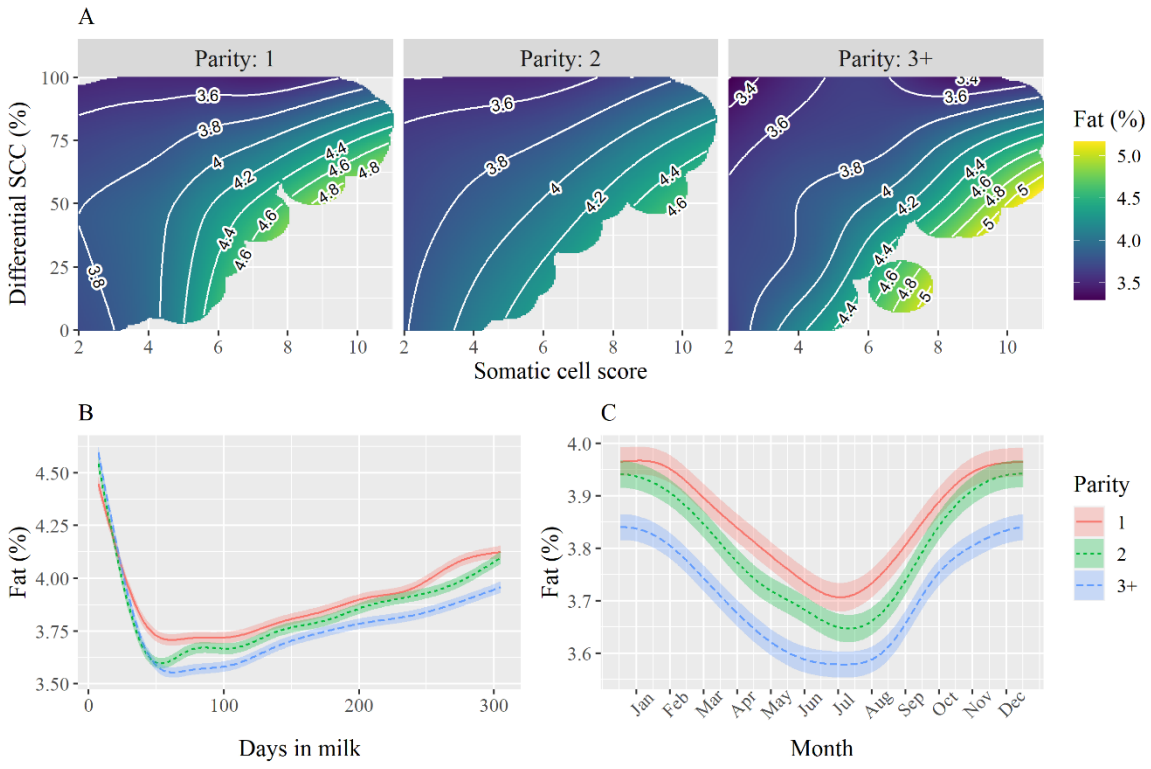

Figure 15. Predictions (± standard error) of fat (%) as functions of SCS/Differential somatic cell count (DSCC; A), DIM (B), and day of the year (C) by parity of cows.

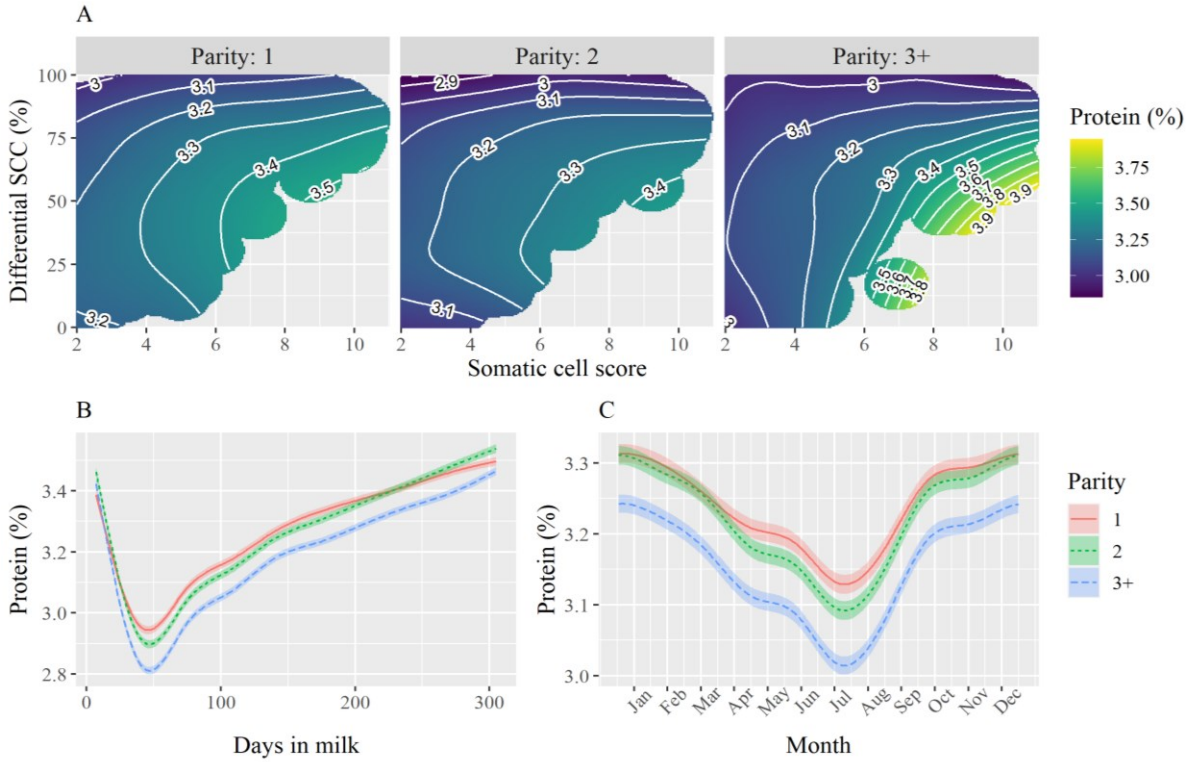

Figure 16. Predictions (± standard error) of protein (%) as functions of SCS/Differential somatic cell count (DSCC; A), DIM (B), and day of the year (C) by parity of cows.

Lactation curves and seasonal variations in milk fat and protein

Figure 15B & Figure 16B show that lactation curves for protein and fat percentages were opposed to that for milk yield and lactose percentage. Protein and fat percentages declined after calving until a nadir of around 50 DIM and increased steadily until the end of lactation. Fat percentage (Figure 15B; -0.7 ~ -1.0% from 7 DIM until the nadir, depending on parity) declined more rapidly than protein percentage (Figure 16B; -0.4 ~ -0.6% from 7 DIM until the nadir, depending on parity) before the nadir, explaining why a higher fat-to-protein ratio was observed in early lactations (Friggens et al., 2007). Both percentages decreased as parity increased, but the difference between primipara and multipara was minor compared to that for lactose percentages and milk yields. Figure 15C & Figure 16C show that seasonal variations in protein (amplitude: around 0.10%) and fat (amplitude: around 0.15%) percentages were much greater than that in lactose percentage (amplitude: around 0.02%). Also, in agreement with previous studies (Heck et al., 2009; Salfer et al., 2020), seasonal variations in protein and fat percentages exhibited a different pattern compared with milk yield and lactose percentage, with a peak at the beginning of January and a nadir near the end of July. Salfer et al. (2020) reported that annual changes in fat and protein percentages were mainly affected by changes in day length, thus gradual changes in these components throughout the year were expected. This is close to

our observations on fat percentage; however, for protein percentage, we observed a sharp decline at the beginning of June. The additional decrease in protein percentage possibly resulted from heat stress (Wheelock et al., 2010) in this period. Figure 17 shows temperature trends in Tokachi across the study period.

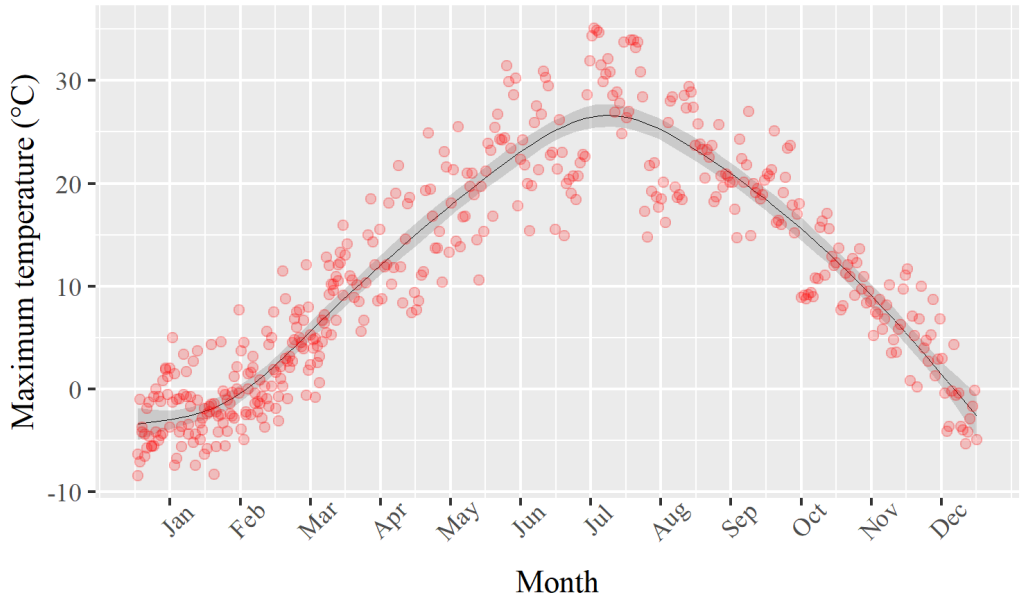

Figure 17. Changes in daily maximum temperature in Shikaoi town, a representative of Tokachi Subprefecture, across the study period. A smooth trend (predictions ± SE) constructed by a generalized additive model is also shown. Data was collected from Japan Meteorological Agency (https://www.data.jma.go.jp/gmd/risk/obsdl/)

4.3.6. Limitations and future perspectives

Several limitations exist in the present study. We encountered difficulty in the scarcity of test instruments available, as only a single instrument among three in the test center could measure DSCC. As a result, relatively sparse test-day records per cow were obtained during the study period (i.e., an average of 4.79 records per cow for 15 months). We addressed this issue in the process of data cleaning, where we deliberately selected cows with more than 5 observations in the dataset to reduce the computational burden of model fitting. However, this introduced potential selection bias and reduced the generalizability of results, as enrolled cows were those with a relatively high SCC level (i.e., >50,000 cells/mL) across the study period.

As shown in Figure 18, some outliers, especially underestimated values, were identified during model fitting. Besides measurement error, potential causes of these underestimated values are clinical mastitis, and other production diseases, such as retained placenta, left-displaced abomasum, lameness, etc., whose negative impacts on milk production are well

documented (Liang et al., 2017). Except for clinical mastitis, these diseases are unlikely to be identified through DHI milk testing thus causing bias. However, if milk yields substantially deviate from model-based predictions, we can reasonably suspect that underlying reasons exist. As such, we may apply models produced here to detect diseases affecting milk production.

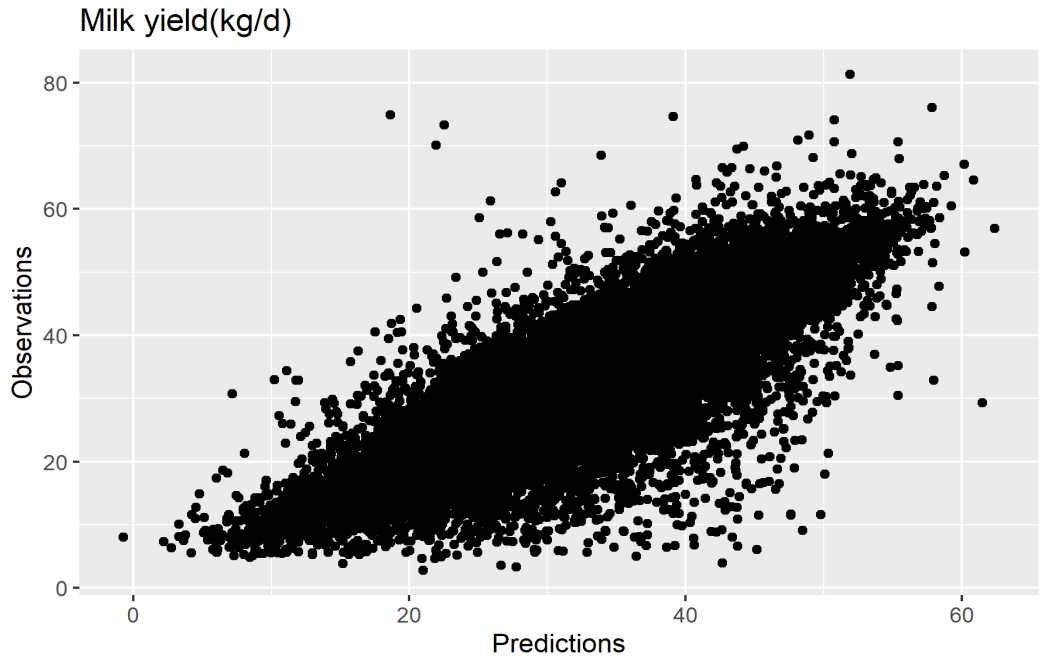

Figure 18. Predictions versus observations of milk yield in a generalized additive model.

Another source of bias is the interaction between SCS/DSCC and DIM because there is evidence that milk losses in the same level of SCS differ by lactation stages (Gonçalves et al., 2018). Fitting a model that includes this interaction is theoretically possible using GAM in mgcv; however, we could not afford the huge computational burden. Interestingly, Gonçalves et al. (2018) found that milk losses at the same level of SCS were great at the beginning and end of lactation compared with mid-lactation. Our data, on the other hand, showed that DSCC was lower at the beginning and end of lactation while SCS remained stable (Figure 2). Since milk yield was negatively impacted by low DSCC (Figure 4A), the findings of Gonçalves et al. (2018) and ours corroborated.

Although we considered several effects and their interactions in our models, GAM is far more flexible. For instance, we can fit a model allowing each cow/herd to have its own smooth functions which are shrunk towards global smooth functions (Pedersen et al., 2019), close to the random slope model in mixed-effect models. This setting is reasonable as we expect the SCS/DSCC, DIM, or seasonal effects to differ by cow due to genetic differences, and the

seasonal effect to differ by herd due to management differences, but such effects are random compared to the parity effect. We believe this approach may be helpful in future studies in the field of genetics and herd management.

In this study, we focused on how SCS and DSCC affect cows' productivity. Future studies could aim to investigate factors causing changes in SCS and DSCC, as well as the outcomes of cows with different levels of SCS and DSCC. Previous studies have postulated that cows with high SCS and low DSCC are caused by chronic mastitis; however, this has not been verified. In fact, along with our study (see Chapter 4), other studies suggest that pathogen-specific effects may be involved (Kirkeby et al., 2021; Pegolo et al., 2022). Schwarz et al. (2021) used the combination of SCS and DSCC to predict the future survival and udder health status of cows, but with an approach based on predefined thresholds. We believe that both questions can be answered with a more flexible approach.

4.4. Conclusions

We investigated the effects of SCS and DSCC on milk yield and composition using GAM. Results showed that DSCC can provide additional information besides that offered by SCS. Notably, the nonlinear interaction between SCS and DSCC and its effect on milk yield and composition were elucidated by GAM, where the conventional approach by which cows were categorized based on predefined thresholds produced unclear results. Our results indicated that the negative effect of high SCS and low DSCC on milk yield was most obvious in cows with high parity, where their productivity was reduced by half (from 40kg/d to 20kg/d). Milk composition was also significantly impacted by the effect of high SCS and low DSCC, either due to the direct effect of chronic mastitis (i.e., on lactose percentage) or due to the concentration effect resulting from reduced milk yield (i.e., on protein and fat percentages). By measuring DSCC and SCC, we demonstrated that immune responses toward IMI are highly associated with impaired milk production and quality, which can be linked to chronic mastitis. To achieve efficient milk production, we should prevent cows from entering a state of high SCS and low DSCC.

5. Chapter 4. Severity and Pathogen Dependent Immune Responses in Mastitis

The manuscript regarding the work in this chapter has been prepared for publication:

Huang, C. H., M. Kayano, and N. Kusaba. *Pathogen and severity-dependent immune responses in bovine mastitis: highlight the dynamics of differential somatic cell count.* Manuscript.

5.1. Introduction

In Chapters 2 and 3, we mentioned that the level of immune indicators, such as DSCC and SCC, varies dynamically after mastitis occurred, as we observed that SCC and DSCC decreased in the healing and chronic stages of mastitis. Indeed, as described extensively in section 1.3. Immune Responses and Immunological Indicators in Mastitis, immune responses in mastitis depend also on the causative pathogen and disease severity. The most obvious difference is between immune responses toward gram-negative and gram-positive bacteria. Infections of gram-positive bacteria are often accompanied by the release of a great amount of pro-inflammatory cytokines, resulting in peracute mastitis with severe symptoms. Contrastingly, gram-positive bacteria can avoid being detected by innate immunity to some degree. In such infections, increases in pro-inflammatory cytokines are obscure or even absent, thus chronic subclinical infections have frequently been observed in gram-positive IMI (De Haas et al., 2002; Schukken et al., 2011). Besides the pathogen-specific immune responses, the same pathogen can cause distinct immune responses as well, likely due to host factors (Burvenich et al., 2003). Higher concentrations of pro-inflammatory cytokines and acute-phase proteins were detected in the blood of cows with severe mastitis (Ohtsuka et al., 2001; Wenz et al., 2010).

Such difference in immune responses leads to varied outcomes of the disease. Each mastitis pathogen affects milk production to a different degree (Heikkilä et al., 2018), and the inflammation impairs milk production (Hand et al., 2012; Gonçalves et al., 2018; Hadrich et al., 2018) and reproductive performance (Lavon et al., 2011; Hudson et al., 2012; Fuenzalida et al., 2015) proportional to its severity. Consequently, treatment outcome depends on both the severity and the causative pathogen of mastitis (Oliveira et al., 2013). Antibiotics used for controlling mastitis account for a majority of antibiotic use on dairy farms, but outcomes were often not as expected (Ruegg, 2017b). To achieve an effective therapy and avoid unnecessary economic losses and pain in animals, we hence need to make treatment decisions based on pathogen susceptibility and disease prognosis, of which the evaluation can be made by

monitoring immune responses. Cytokines are the direct mediator affecting immune responses, while they are difficult to be measured. Nonetheless, variations of cytokine also reflect in the dynamics of other immunological indicators, including SCC, DSCC, serum proteins, and the leukogram. Compared to cytokines, we can easily quantify the level of these indicators, making them applicable in clinical. Understanding the dynamics of these indicators in mastitis thus helps us clarify pathogenesis, evaluate prognosis, and make clinical decisions, consequently improving antibiotic stewardship and animal welfare in dairy farming.

In this study, we aim to investigate the dynamics of immune indicators, namely WBC, A/G ratio, SCC, and DSCC, in naturally occurring clinical mastitis. The effects of causative pathogens and severity were taken into concern. We will highlight the dynamics of DSCC because it has yet to be studied after mastitis occurred.

5.2. Materials and Methods

To evaluate immune responses in mastitis with different severity and causative pathogen, after identifying clinical mastitis, we evaluated disease severity based on clinical signs and diagnosed the causative pathogen by milk culture. Also, we collected blood samples and milk samples from the affected quarter immediately (d 0) as well as 3, 5, 7, 14, and 21 days after the identification to evaluate IMI status and the level of WBC, A/G ratio, SCC as well as DSCC. We conducted the study from June 2021 to February 2022. All experimental procedures followed the Guidelines for the Care and Use of Agricultural Animals of Obihiro University (Permission number: #21-156).

5.2.1. Study herd and the enrollment of cows

The study herd used in the current study was consistent with that described in section 2.2.1. Study herd. The study enrolled cows confirmed with clinical mastitis but excluded cows concurrently receiving antibiotics or anti-inflammatory medications for other diseases (e.g., lameness) or with mastitis caused by the same pathogen as the previous occurrence (i.e., recurrent mastitis). Cows showing clinical signs of mastitis, including abnormal milk and/or swollen udder, were first identified by farm staff and later confirmed by study personnel within 6 hr. To confirm the mastitis, we performed a physical examination and the CMT, collecting milk samples aseptically from the affected quarter for the bacteria culture test. We treated mastitis based on the milk culture result (Lago and Godden, 2018). Namely, mastitis caused by major pathogens (i.e., *Streptococcus* spp., SA, GNR) had been treated with antibiotics once a day for 5 consecutive days (i.e., d 1-5 after mastitis occurrence) or otherwise left untreated.

5.2.2. Blood sampling and analysis

Peripheral blood samples were collected from the coccygeal veins of cows. A subset of the sample was anticoagulated with EDTA for measuring WBC, which was performed immediately after sample collection with an automated hematology analyzer (Celltac-α®; Nihon Kohden Co., Tokyo, Japan). Another subset of the sample was collected in plain serum tubes and allowed to clot for 10 min at 37°C, followed by centrifugation at 1600g, 4°C for 15 min for serum isolation. Isolated serum was stored at -30 °C until analysis. The analysis was performed with an automatic photometer analyzer (TBA-120FR®, Toshiba Co., Japan) for total protein and albumin. We calculated globulin concentration by subtracting the concentration of albumin from serum total protein, and subsequently determined the A/G ratio.

5.2.3. Milk sampling, culture, and analysis

We collected milk samples and determined SCC and DSCC based on the method described in section 3.2.2. Sampling and the determination of SCC, DSCC, and IMI status. After sample collection, the bacterial culture of milk was performed based on the method described in section 2.2.4. Bacterial culture of milk. Except that for isolates presumed to be *Streptococcus uberis* (**SU**) and *Streptococcus dysgalactiae* (**SD**), we made the confirmation using API 20 Strep® (Biomerieux inc., Japan), and the other streptococci-like isolates were categorized into other streptococci and related genera (**OS**). Also, mastitis arising from gram-positive rods has not been identified in this study. As such, mastitis caused by GNR, SA, CNS, SU, and OS, as well as non-specific mastitis (no significant growth detected; NG) were identified during the study period.

5.2.4. Mastitis severity evaluation

We evaluated the severity of clinical mastitis based on a scoring system as described in Pinzón-Sánchez and Ruegg (2011), where clinical mastitis was defined as mild when only the milk was abnormal (e.g., containing clots), as moderate when the affected quarter was abnormal (e.g., swollen) but without systemic signs of disease, or as severe when cow showed systemic signs such as hyperthermia (>39.5°C), dehydration, anorexia or depression.

5.2.5. Statistical analyses

All analyses were performed with the statistical software R version 4.0.5 (R Core Team, 2021). Data was first explored and transformed with the package “tidyverse” (Hadley et al., 2019). For modeling purposes, SCC was transformed to SCS to fit a normal distribution, and days in milk (**DIM**) were scaled in a range from 0 to 1 through min-max normalization, which

was transformed back to the original scale after modeling for interpretation purposes. The statistical analyses aimed to clarify how mastitis pathogens and severity affected immune responses over time.

Statistical analyses of SCS, WBC, and A/G ratio

We fitted data in linear mixed models using the 'lmer' function within the 'lme4' package (Douglas et al., 2015). Models were of the form:

$$\textbf{Equation 3}: Y_{ijklm} = \beta_0 + \beta_1 Path_k + \beta_2 Sev_m + \beta_3 Time_l + \beta_4 Path_k \times Time_l + \beta_5 Sev_m \times Time_l + \beta_6 DIM_i + \beta_7 DIM_i^2 + \beta_8 Parity_{ij} + \gamma_j + \varepsilon_{ijklm},$$

where $\boldsymbol{Y_{ijklm}}$ is the response variable, SCS, WBC, and A/G ratio, for the ith observation in the jth mastitis event. $\boldsymbol{Path_k}$, $\boldsymbol{Sev_m}$, and $\boldsymbol{Time_l}$ are fixed effects of mastitis pathogen (7 levels: NG, CNS, SA, SU, SD, OS, and GNR), severity (3 levels: mild, moderate, and severe), and days after mastitis (6 levels: d 0, 3, 5, 7, 14 and 21), respectively. Interactions between $\boldsymbol{Path_k}$, $\boldsymbol{Sev_m}$, and $\boldsymbol{Time_l}$ were included to evaluate the effect of pathogen and severity on each time point. $\boldsymbol{DIM_i}$ and $\boldsymbol{DIM_i^2}$ are the linear and quadratic terms of days in milk, $\boldsymbol{Parity_{ij}}$ is the term of parity (a numerical variable), and $\boldsymbol{\gamma_j}$ is a random effect term allowing the intercept to vary for each mastitis case. The random effect of each cow was also tested but later excluded due to insignificance in all tested traits, which has been done to reduce model complexity.

Statistical analyses of DSCC

As previous studies suggested (Damm et al., 2017; Pegolo et al., 2021), DSCC values are strongly associated with SCS. Consequently, analyzing DSCC alone probably concludes similarly to what we can draw from analyzing SCS (Schwarz et al., 2020b). Since DSCC represents the proportion of PMNs and lymphocytes in SCC, the deviation (residual) from this relationship may have a specific meaning, such as an indication of the mastitis stage. To test this hypothesis, we included linear and quadratic terms of SCS in the model:

$$\textbf{Equation 4}: DSCC_{ijklm} = \beta_0 + \beta_1 SCS_{ijklm} + \beta_2 SCS_{ijklm}^2 + \beta_3 Path_k + \beta_4 Sev_m + \beta_5 Time_l + \beta_6 Path_k \times Time_l + \beta_7 Sev_m \times Time_l + \beta_8 DI_i + \beta_9 DIM_i^2 + \beta_{10} Parity_{ij} + \gamma_j + \varepsilon_{ijklm},$$

where $\boldsymbol{DSCC_{ijklm}}$ is the observed DSCC, $\boldsymbol{SCS_{ijklm}}$ and $\boldsymbol{SCS_{ijklm}^2}$ are linear and quadratic terms of SCS. The other terms remain as they were in Equation 3.

We tested the significance of each term ($P < .05$) in these models by type III ANOVA using the package "lmerTest" (Alexandra et al., 2017), in which Kenward-Roger's approximation was used to calculate denominator degrees of freedom. The effect of numerical variables was visualized using the package "visreg" (Breheny and Burchett, 2017). To explore the effect of categorical variables, estimated marginal means (**EMMs**) were computed,

compared, and plotted using the "emmeans" package (Lenth, 2022). When conducting multiple comparisons, we adjusted p values with an appropriate method (i.e., for pairwise comparisons, Tukey's method was used; for many-to-one comparisons, Dunnett's method was used). In the following text, the number following "±" represents the SE of the estimate if not specified otherwise.

5.3. Results

5.3.1. Details of clinical mastitis cases

We followed 49 cases of clinical mastitis occurring in 38 cows and obtained 294 records during the investigation. The number of cases within each pathogen or severity group is detailed in Table 6. The most isolated pathogen was SU (n=15; 30.6%), followed by OS (n=8; 16.3%), and CNS (n=7; 14.3%). The number of cases with mild, moderate, and severe symptoms was 32 (65.3%), 9 (18.4%), and 8 (16.3%), respectively. Except for mastitis caused by GNR, cases mostly presented mild clinical signs, accounting for 50% (SD) to 85.7% (CNS) of cases within each pathogen group. Table 6 also summarizes the parity and the DIM at the occurrence of each pathogen-specific CM. CM caused by SA and SD tended to occur in earlier lactation but no distinct differences in the parity existed between each group.

Table 6. The number of cases within each pathogen and severity group, and summarized parity and days in milk of cows when cases were identified.

	Severity				**Days in milk**	**Parity**
	Mild	Moderate	Severe	Total		
Pathogens*	Number of cases (%)				Mean ± SD	
CNS	6 (85.7)	1 (14.3)	0	7 (14.3)	87.6 ± 66	2.6 ± 1
GNR	0	1 (50)	1 (50)	2 (4.1)	154 ± 4.2	3.5 ± 2.1
NG	4 (57.1)	3 (42.9)	0	7 (14.3)	170.4 ± 98.8	3.1 ± 1.3
OS	5 (62.5)	1 (12.5)	2 (25)	8 (16.3)	187.6 ± 104.1	3.2 ± 1.5
SA	4 (66.7)	1 (16.7)	1 (16.7)	6 (12.2)	39 ± 48.5	2.2 ± 1
SD	2 (50)	0	2 (50)	4 (8.2)	43.8 ± 74.6	2.2 ± 0.5
SU	11 (73.3)	2 (13.3)	2 (13.3)	15 (30.6)	110.7 ± 70.6	2.4 ± 0.9
Total (%)	32 (65.3)	9 (18.4)	8 (16.3)	49 (100)	116 ± 89.7	2.7 ± 1.1

* NG = no significant growth; CNS = couagulase-negative staphylococci; GNR = Gram negative rods; OS = other streptococci; SA = *Staphylococcus aureus*; SD = *Streptococcus dysgalactiae*; SU = *Streptococcus uberis*.

5.3.2. Factors affecting the dynamics of SCS, WBC, and the A/G ratio

Factors affecting somatic cell score (SCS)

SCS in four samples could not be measured due to abundant clots in the samples. Results from ANOVA (Table 7) show that time affected SCS most significantly. EMMs of SCS at d 0, 3, 5, 7, 14, and 21 were 10.34 ± 0.50, 7.67 ± 0.43, 6.54 ± 0.43, 5.74 ± 0.42, 4.48 ± 0.42, and 4.8 ± 0.42, respectively. Mastitis pathogen and severity affected SCS; however, although SCS varied on average depending on the pathogen or the severity of the disease (in pairwise comparisons, NG - SU = -2.3 ± 0.68, P = 0.023; Mild - Severe = -1.5 ± 0.63, P = 0.05), we observed similar downward trends in all groups (Figure 19 & Table S1).

Table 7. ANOVA for the effect of time, mastitis pathogen, and severity on somatic cell score, white blood cell count, and serum albumin/globulin ratio (A/G ratio).

		Somatic cell score			**White blood cell count**			**A/G ratio**		
Terms	NuDF[a]	DeDF[b]	F[c]	P value	DeDF	F	P value	DeDF	F	P value
Time	5	198.9	36	3.60E-26	201.4	8.6	2.00E-07	203.5	4	1.80E-03
Pathogen	6	37.6	2.4	0.047	37.1	2.3	0.06	37.3	2.6	0.032
Severity	2	37.6	3.4	0.046	37.2	0.8	0.45	37.3	3.7	0.034
Parity	1	37.1	0.2	0.7	37.2	10.2	2.90E-03	37.4	14.6	4.80E-04
DIM[d]	1	42.3	1.8	0.18	40.9	2.9	0.1	47.3	1.7	0.19
DIM^2	1	42.8	0.3	0.62	41.5	5.1	0.029	49.1	3	0.09
Time x Pathogen	30	196.7	0.9	0.61	200.2	1.7	0.015	200.4	0.9	0.66
Time x Severity	10	196.5	1.2	0.31	199.9	1.9	0.05	199.8	2.6	5.00E-03

[a] NuDF = Numerator degrees of freedom; [b] DeDF = Adjusted denominator degrees of freedom.
[c] F = F value; [d] DIM = Days in milk.

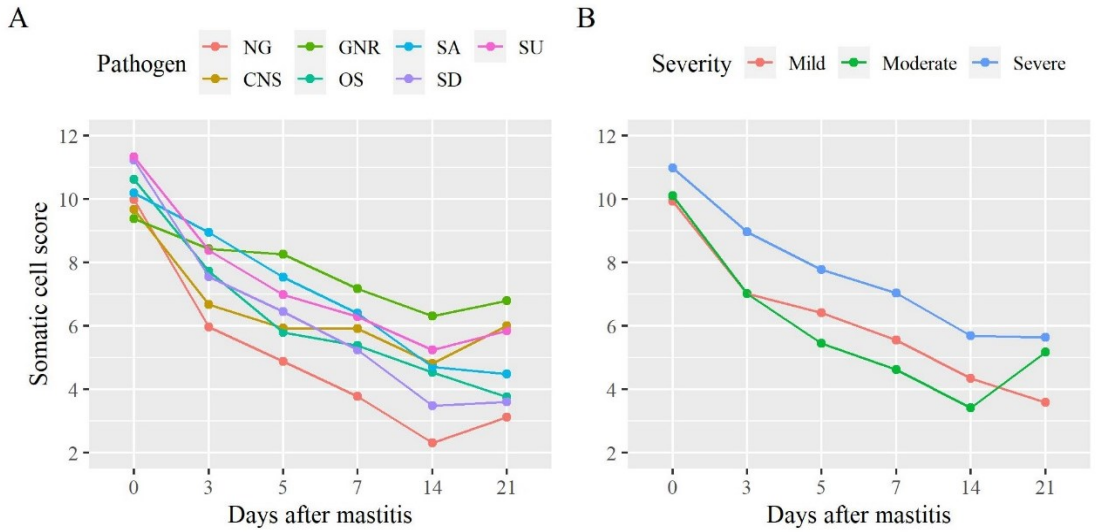

Figure 19. Estimated marginal means of somatic cell score over time in each pathogen (A) and severity (B) group. NG = no significant growth; CNS = coagulase-negative staphylococci; GNR = gram-negative rods; OS = other streptococci; SA = *Staphylococcus aureus*; SD = *Streptococcus dysgalactiae*; SU = *Streptococcus uberis*.

Factors affecting white blood cell count (WBC)

Time, parity, DIM, the interaction between time and pathogen as well as the interaction between time and severity significantly affected the WBC level (Table 7). Results show that WBC decreased as parity increased (Figure 20A), and a non-linear relationship existed between DIM and WBC, namely that the WBC level was lower at the lactation peak (Figure 20B). Both the pathogen and the severity of mastitis affected the dynamics of WBC. On the day GNR mastitis occurred, WBC was lower than normal (Figure 21A & Table S1), but it increased dramatically at d 3 and decreased gradually in the following days. In streptococcal mastitis (i.e., OS, SD & SU), WBC started to rise at d 5 and reach the peak at d 7. On the contrary, in the other mastitis (i.e., NG, CNS & SA), WBC remained at the same level as on d 0 (Figure 21A). We confirmed this finding by computing polynomial contrasts for each pathogen as a function of time. Results show that WBC in GNR, OS, SD, and SU mastitis were with significant linear, quadratic, or cubic trends, while no significant trend of WBC was observed in NG, CNS, or SA mastitis (Table 8).

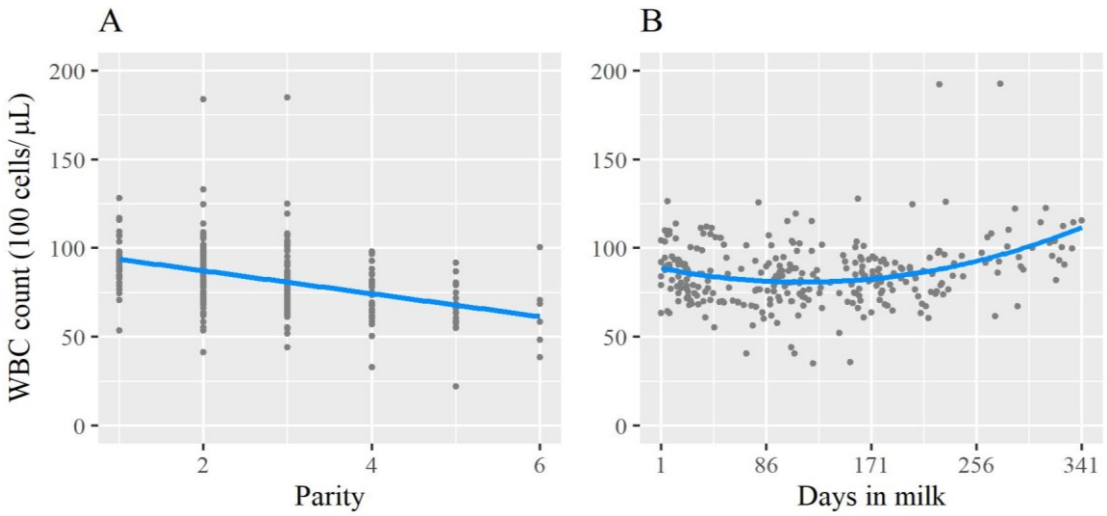

Figure 20. The effect of parity (A) and days in milk (B) on white blood cell (WBC) count.

Moreover, the same analysis was conducted to compare the dynamic of WBC in each severity group, and there was a substantial difference between the contrasts for each group (Table 8). To verify this difference, we further compared them between groups. Results show a statistical difference in the quadratic trend between the severe group and the other groups (Mild - Moderate = -57.4 ± 73.1, P = .43; Mild - Severe = 192.5 ± 79.1, P = .016; Moderate - Severe = 249.9 ± 96.6, P = .01), showing that the WBC level changed more aggressively in cows with severe mastitis (Figure 21B).

Table 8. Trends of white blood cell count in each pathogen and severity group. Estimated coefficients (±SE) for polynomial contrasts are shown.

	Polynomial terms in trends of white blood cell count					
	Linear		Quadratic		Cubic	
Group	Estimate ± SE	P value	Estimate ± SE	P value	Estimate ± SE	P value
Pathogens*						
NG	73.6 ± 67.6	0.28	-106 ± 73.9	0.15	-78.9 ± 108.1	0.47
CNS	-14.7 ± 69.8	0.83	-63.5 ± 76.3	0.41	-24.2 ± 111.6	0.83
GNR	254.8 ± 119.5	0.034	-543.4 ± 130.9	4.9E-05	521.2 ± 191.6	7.1E-03
OS	20.9 ± 61.5	0.73	-183.7 ± 67.1	6.7E-03	-98.4 ± 98.2	0.32
SA	-36.3 ± 71	0.61	-102.2 ± 77.3	0.19	-19.3 ± 113.1	0.86
SD	196 ± 87	0.025	-161.8 ± 94.9	0.09	-44.6 ± 139	0.75
SU	193.7 ± 48.4	8.7E-05	-151.9 ± 52.8	4.5E-03	-270.1 ± 77.3	5.8E-04
Severity						
Mild	95.2 ± 36.9	0.011	-142.5 ± 40.2	4.8E-04	29.2 ± 58.8	0.62
Moderate	-28.4 ± 59.1	0.63	-85.1 ± 64.6	0.19	-28.8 ± 94.5	0.76
Severe	228.1 ± 62.2	3.1E-04	-335 ± 67.9	1.7E-06	-6.6 ± 99.4	0.95

*NG = no significant growth; CNS = coagulase-negative staphylococci; GNR = gram-negative rods; OS = other streptococci; SA = *Staphylococcus aureus*; SD = *Streptococcus dysgalactiae*; SU = *Streptococcus uberis*.

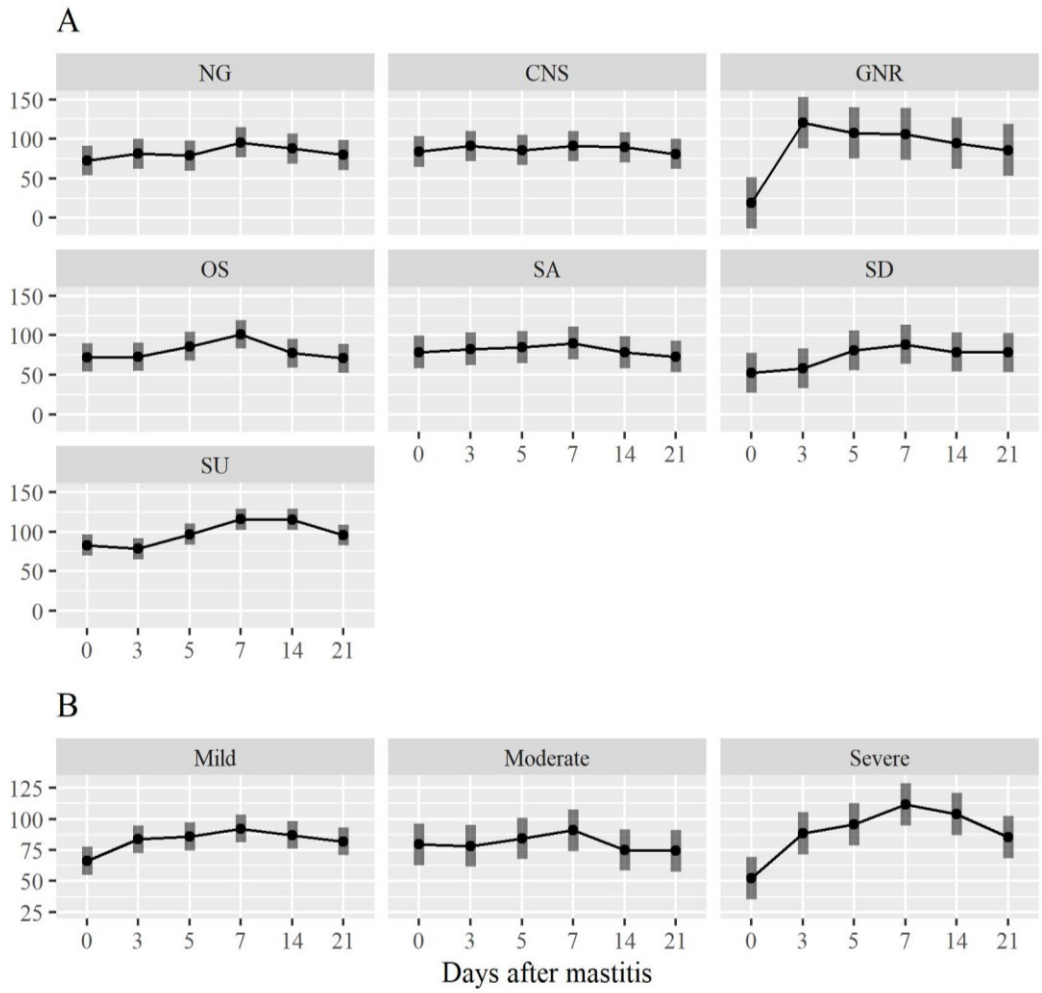

Figure 21. Estimated marginal means ± 95% confidence intervals of white blood cell count over time in each pathogen (A) or severity (B) group. Corresponding polynomial contrasts for each group are reported in Table 8. Abbreviations: NG = no significant growth; CNS = coagulase-negative staphylococci; GNR = gram-negative rods; OS = other streptococci; SA = *Staphylococcus aureus*; SD = *Streptococcus dysgalactiae*; SU = *Streptococcus uberis*.

Factors affecting the Albumin/Globulin (A/G) ratio

Time, parity, the severity and pathogen of mastitis, and the interaction between time and the severity significantly affected the A/G ratio (Table 7). A strong negative relationship existed between parity and A/G ratio (-0.053 ± 0.014; P = 5.2E-04). Although the A/G ratio varied on average depending on the causative pathogen, their dynamics were similar, namely that the A/G ratio dropped slightly from d 3 in most pathogen groups except for SD mastitis (Table S1). In contrast, mastitis severity had a more obvious effect on the dynamics of the A/G ratio. The A/G ratio in severe mastitis dropped significantly from d 3 till the end of sampling (d 21), whereas remained at the same level in both mild and moderate mastitis during the investigation (Figure 22 & Table S1).

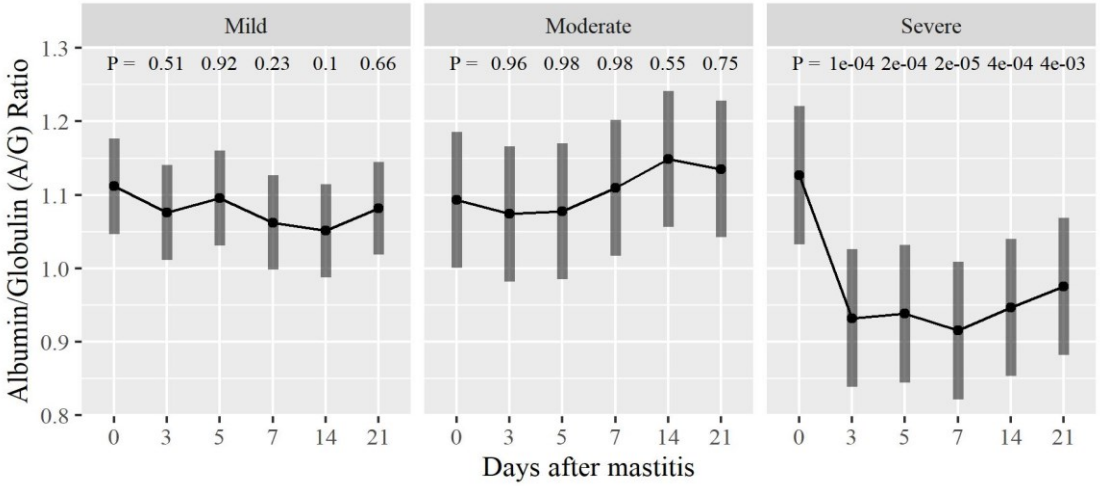

Figure 22. Estimated marginal means (EMMs) ± 95% confidence intervals of serum albumin/globulin (A/G) ratio over time in each severity group. In each group, EMMs on d3 ~ d21 were compared with EMM on d 0, and P-values adjusted by Dunnett's method are shown.

5.3.3. Factors affecting the dynamics of DSCC

We excluded 34 records where DSCC measurements were unreliable (in samples with SCC ≤ 50,000 cells/mL) or unmeasurable (in samples with abundant clots), resulting in 260 observations. We found a strong non-linear relationship between DSCC and SCS (Table 9 & Figure 23A). Also, parity significantly affected DSCC. Figure 23B shows that as parity increased, DSCC values tended to be higher. Interestingly, the dynamic of DSCC remarkably differed in mastitis caused by SD. This was confirmed by comparing polynomial contrasts in NG mastitis (as a reference) with those in the other types of mastitis, in which a statistical difference was observed in the quadratic trend between NG mastitis and SD mastitis (-212.3 ± 55.6, P = 1.9E- 04). To visualize the effect, we plotted model-based predictions of DSCC as a function of SCS by pathogen group (Figure 24), which shows that, for SD mastitis, DSCC decreased while SCS remained high.

Table 9. ANOVA for the effect of time, mastitis pathogen, and severity on differential somatic cell count.

	Differential somatic cell count (DSCC)			
Terms	NuDF [a]	DeDF [b]	F value	P value
SCS [c]	1	191.5	81.0	2.3E-16
SCS^2	1	186.6	55.7	3.1E-12
Time	5	173.5	2.2	0.06
Pathogen	6	39.4	1.1	0.36
Severity	2	39.7	2.1	0.14
DIM [d]	1	40.0	3.4	0.07
DIM^2	1	38.7	1.7	0.2
Parity	1	37.6	4.6	0.038
Time x Pathogen	30	168.4	1.6	0.029
Time x Severity	10	168.1	0.6	0.82

[a] NuDF = Numerator degrees of freedom; [b] DeDF = Adjusted denominator degrees of freedom; [c] SCS = Somatic cell score; [d] DIM = Days in milk.

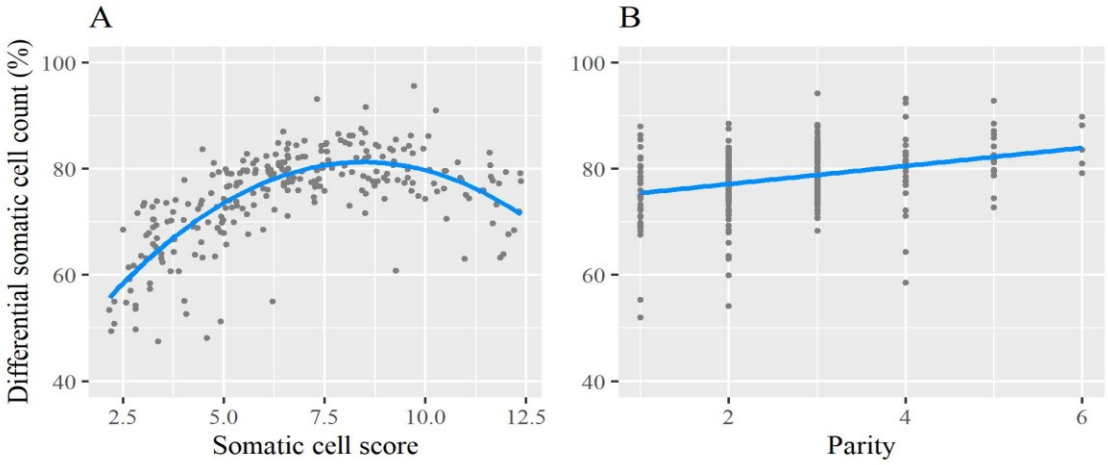

Figure 23. The relationship between somatic cell score and differential somatic cell count (DSCC; A), and the effect of parity on DSCC (B).

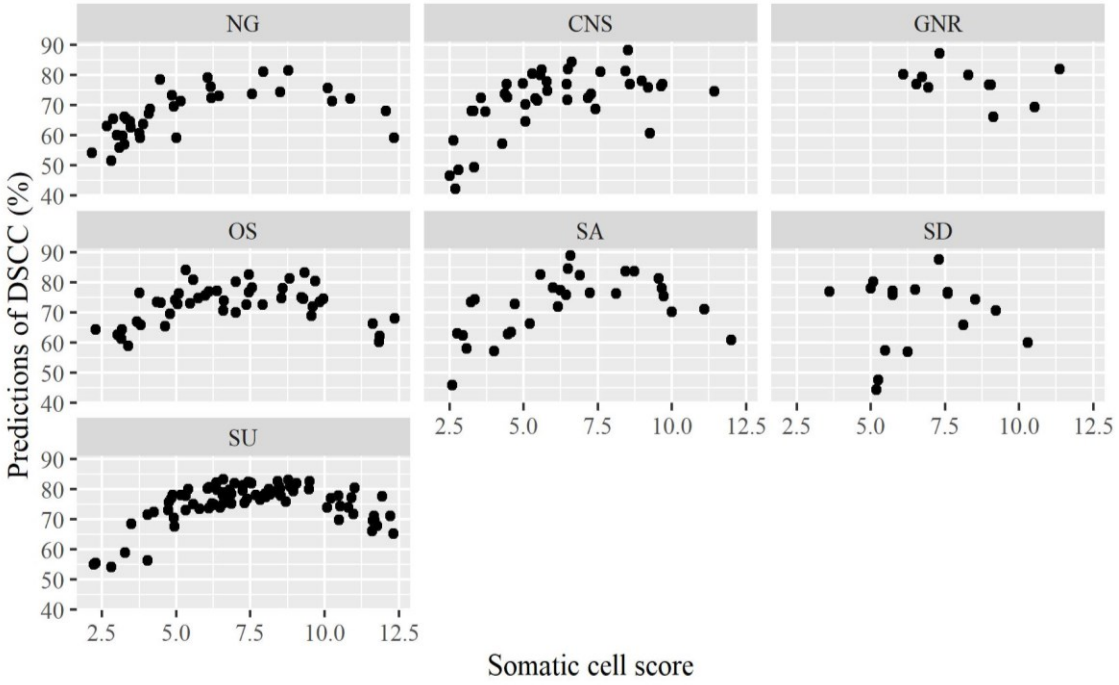

Figure 24. Predictions of differential somatic cell count (DSCC) as a function of somatic cell score in mastitis caused by different pathogens.

5.4. Discussions

5.4.1. Details of clinical mastitis cases

Investigated clinical mastitis was mainly caused by gram-positive pathogens, dissimilar from studies conducted in either China (Gao et al., 2017), Belgium (Verbeke et al., 2014), or the US (Oliveira et al., 2013). In those studies, gram-negative pathogens were more commonly isolated. The high prevalence of SU can result from the fact that straw was used as bedding in our study herd (Unnerstad et al., 2009). Similar to previous studies, symptoms of clinical mastitis were mostly mild (Oliveira et al., 2013; Verbeke et al., 2014). However, in our study mastitis caused by gram-positive was more likely to be evaluated as a severe case. Because clinical signs were evaluated by a trained investigator in this study, the sensitivity for detecting systemic clinical signs may be higher as compared to other studies, where clinical signs were mostly recorded by producers. In addition, mastitis caused by SD and SA was prone to occur in early lactation, corresponding to the previous report (De Haas et al., 2002).

5.4.2. Pathogen-specific immune responses

At first glance, the similar patterns of SCC in mastitis caused by major and minor pathogens (i.e., CNS) might be unexpected (Figure 19), due to the well-known differences between them in virulence (Schukken et al., 2009a) and the ability to elevate SCC (Djabri et

al., 2002). However, we only collected cases with clinical signs, and in such a case, minor pathogens caused a comparable elevation of SCC (De Haas et al., 2002) and milk yield loss (Heikkilä et al., 2018) as major pathogens. Although the level of SCC slightly varied depending on the pathogen (Figure 19), the difference was trivial, hence challenging to be applied to differentiate the causative pathogen of clinical mastitis. On the other hand, we observed a more specific effect of pathogens on the dynamic of WBC. Mastitis caused by GNR triggered the most immediate and dramatic immune responses, and streptococcal infections induced delayed, less intensive, but significant responses (Figure 21). In contrast, immune responses toward SA and CNS infections were the most obscure. These findings correspond to changes in cytokines in milk (Bannerman, 2009) as well as Toll-like receptor gene expressions in mammary epithelial cells during IMI (Petzl et al., 2008), likely related to the impairment of NF-κB activation in staphylococcal infections (Yang et al., 2008). The leukogram (i.e., WBC, neutrophil count, monocyte count, etc.) has been proposed as a tool for differentiating gram-positive and gram-negative mastitis, with an overall accuracy of 91% (Smith et al., 2001). In addition to that, we demonstrate the potential use of WBC for differentiating streptococcal and staphylococcal mastitis.

5.4.3. Using the A/G ratio to evaluate mastitis severity

Our results show that mastitis severity affects the dynamics of the A/G ratio, as the A/G ratio dropped sharply only in severe mastitis (Figure 22). Bobbo et al. (2017) reported an inverse relationship between the A/G ratio and SCS. They concluded that decreased A/G ratio can be attributed to an increase in globulin production due to inflammation and an albumin loss due to the damaged blood-milk barrier. Since higher concentrations of cytokine were detected in cows with severe mastitis (Wenz et al., 2010), an increase in globulin production was expected. Also, the damage to the blood-milk barrier was supposedly serious in severe mastitis, thus our observations were probably a result of both. Our results show that mastitis severity was revealed more by the A/G ratio (Figure 22) than by SCS (Figure 19B), suggesting that compared to SCS, the A/G ratio provides more information regarding patients' general health status.

The severity of mastitis affected its outcomes, including the number of treatments (Oliveira et al., 2013), the culling rate (Wenz et al., 2001a), and the breeding performance (Fuenzalida et al., 2015) of cows, thus evaluating mastitis severity is essential for herd management. In this respect, the A/G ratio can be used to determine the disease's severity afterward, since the decrease in A/G ratios remains detectable for at least 3 wk (Figure 22). Combining the findings of a recent study (Cattaneo et al., 2021), our results show how severe

clinical mastitis impairs dairy cows' performance. Cattaneo et al. (2021) demonstrated that cows with a low A/G ratio before dry-off perform worse compared to their pen mates in the next lactation, both in terms of milk yield and fertility. Because a pronounced decrease in the A/G ratio was observed in severe mastitis (Figure 22), we can thus presume that severe clinical mastitis occurring in the late lactation would deteriorate cows' milk yield and fertility in the subsequent lactation. This stress the importance of mastitis severity evaluation in dairy herd management, and the evaluation can be facilitated by measuring the A/G ratio.

5.4.4. The dynamics of DSCC in clinical mastitis

To our knowledge, this is the first study to describe the dynamics of DSCC in clinical mastitis. Surprisingly, we identified a non-linear relationship between SCS and DSCC (Figure 23A). Since PMNs are the first cells to arrive at the site of inflammation, we expected the proportions of PMNs and DSCC (PMN + lymphocytes) would increase as SCS increased. Although most studies found a linear association between SCS and DSCC (Damm et al., 2017; Schwarz et al., 2019; Pegolo et al., 2021), an indistinct non-linear pattern between them can be recognized in the figures presented by Kirkeby et al. (2020) (Figure 2 in that study). Because we collected samples immediately after clinical mastitis occurred, some of our samples contained extremely high numbers of somatic cells, and consequently, this non-linear relationship was revealed. As a possible explanation of this phenomenon, mammary epithelial cells shedding into milk due to mastitis damage, and these cells are counted as SCC but not included in DSCC (Damm et al., 2017). In one study, the proportion of epithelial cells in SCC could be up to 44% (Leitner et al., 2000). Both sufficiently explain why DSCC decreased when SCS was extremely high. However, the cell differentiation assay used in that study differs from that used in this study, thus the non-linear relationship discovered here requires further validation.

We applied an alternative strategy to analyze the dynamics of DSCC. As mentioned in the section *Statistical analyses of DSCC*, this was to avoid a similar result as that from the analysis of SCS, due to the high association between these two traits. With this strategy, we found that the relationship between DSCC and SCS differs in the healing process of SD mastitis. In such cases, DSCC decreased while SCS remained high (Figure 24). In the healing process of mastitis, macrophages are required to remove apoptotic neutrophils (Sladek and Rysanek, 2001) and to repair damaged tissue (Tizard, 2017). SD can release hyaluronidase and fibrinolysin, and invade mammary epithelial cells (Calvinho et al., 1998), explaining how SD can cause stronger damage to the mammary gland, and as a result, the healing process required more macrophages. Pegolo et al. (2022) described a similar pathogen-specific effect on DSCC.

They showed that *Prototheca spp.* caused lower DSCC values compared to *Streptococcus agalactiae.* Interestingly, another study showed that *Prototheca spp.* caused irreversible damage to the mammary gland, inducing chronic granulomatous inflammation (infiltration of macrophages) following the failure of healing (Wawron et al., 2013). Lastly, the "low DSCC and high SCS" situation described here is associated with economic loss and poor prognosis of mastitis. Studies showed that cows with high SCS and low DSCC produced the least milk and were most likely to be culled (Schwarz et al., 2020a; Schwarz et al., 2021), attributable to the irreversible mammary tissue damage.

We were unable to find a significant effect of the severity of mastitis on the dynamics of DSCC, but this effect could not be neglected. In our investigation, most severe mastitis cases had yet to enter the healing process at the end of follow-up, as shown by their high SCS values on d 21 in Figure 19B. The sampling scheme applied here was insufficient to describe the dynamics of DSCC in severe mastitis, in particular regarding their healing process, thus an extended follow-up is required in the future.

5.5. Conclusions

In this study, we investigated the dynamics of several immunological indicators after clinical mastitis occurred. We found that differences in the severity and causative pathogen of mastitis lead to variations in these indicators. We observed significant differences in the dynamics of WBC between clinical mastitis caused by coliforms, streptococci, and staphylococci, while the A/G ratio dropped dramatically only in animals with severe mastitis. Furthermore, this study provides a novel insight that, when analyzed together with SCC, DSCC can provide additional information regarding the udder health status. In future works on DSCC, we thus better consider the information provided by SCC in the analysis. Overall, these findings indicate that immune responses in mastitis varied depending on both the severity and causative pathogen, supporting the importance of severity evaluation and pathogen identification in mastitis control. The results also help us elucidate the pathogenesis of reduced productivity due to mastitis. Now we understand cows with high SCS and low DSCC (Chapter 3) and cows with low A/G ratios tend to perform worse, thus we require measures to rescue cows from these unfavorable statuses. In the next chapter, we will discuss the effect of a biomaterial with immunomodulatory properties, namely chitosan, on preventing dry period IMI and recurrent clinical mastitis. Eventually, we possibly can prevent or remedy cows in such status (Chapter 5).

6. Chapter 5. Immunomodulatory Effects of Oral-administrated Chitosan on Mastitis

6.1. Introduction

For several decades, mastitis has been controlled mainly by antibiotics; however, the treatment outcomes can be unsatisfactory (Ruegg, 2017b), especially when mastitis is in a chronic stage (Barkema et al., 2006; Bradley and Green, 2009) or when it recurrently occurred (Pinzón-Sánchez and Ruegg, 2011). Such findings are unsurprising as both chronic infections and repeated episodes of clinical mastitis are related to ineffective antibiotic treatment, and consequently, repeating the identical treatment likely leads to another failure. Antibiotic treatment often fails due to antimicrobial resistance in microbes, an inherent property of microbes allowing them to survive in nature, which is further strengthened when the microbe was under selective pressure from antimicrobial agents. In light of the emerging antimicrobial resistance, the concept of antibiotic stewardship was constructed, aiming to prevent antibiotic abuse and elevate the efficacy of antibiotic treatment. As drugs used for mastitis control contributed to the majority of antibiotic use in dairy farming, antibiotic stewardship for mastitis is of significant concern.

To prevent antimicrobial resistance caused by antibiotic overuse, an alternative therapy is required to help animals fight against pathogens. In this aspect, immunotherapy attracts particular attention. Immunotherapy cures the disease either by enhancing or suppressing the immune response of the host. An immunostimulant stimulates the immune system to enhance host resistance against invaders. On the other hand, agents with anti-inflammatory properties suppress the immune system to avoid the harmful effects of prolonged and excessive inflammatory responses. Although the saying goes “you cannot sell the cow and drink the milk”, some biomaterials, such as chitosan, indeed have both properties, enabling them to facilitate the therapy by up or down-regulating the immune system.

Chitosan is a polysaccharide polymer obtained by the deacetylation of chitin, which is the second most copious biomass following cellulose. Chitosan is commonly isolated from crustacean shells. The ease of obtaining crustaceans' shells as a by-product of seafood processing drives abundant research efforts to clarify the properties and the usefulness of chitosan. The activities of chitosan include antimicrobial, antioxidant, anti-inflammatory, anticancer, immuno-stimulatory, wound healing, hypocholesterolemic, and prebiotic (Liaqat and Eltem, 2018). Among these, the immunomodulatory properties of chitosan (Moran et al.,

2018) are of particular interest because our aim was to develop immunotherapy against mastitis. As an immunostimulant, chitosan was reported to promote the release of TNF-α (Otterlei et al., 1994), IL-1β, and the consequent lysosomal destabilization (Bueter et al., 2014). The induction of these cytokines by chitosan may be Toll-like receptor 2 and Toll-like receptor 4-dependent (Bueter et al., 2013; Zhang et al., 2014) due to the structure resembling lipopolysaccharides (Otterlei et al., 1994). Studies also demonstrated that chitosan promotes cellular immunity by inducing IL-12 (Mori et al., 2012), IFN- γ and type 1 interferon (Carroll et al., 2016), possibly through the IRF-3 rather than the typical NF-κB pathway. Compared to alum, a classical adjuvant used in vaccines, chitosan induced a more robust cellular immunity as a vaccine adjuvant, showing its potential as an alternative to alum (Mori et al., 2012). This is further evidenced by the findings of Neimert-Andersson et al. (2011). They found that when *Haemophilus influenzae* type b vaccine was administrated with chitosan adjuvant, levels of IFN- γ , IL-4, IL-6 IL-17, and IL-10 cytokines, as well as serum IgG1 and IgG2 were elevated. The anti-inflammatory activities of chitosan have also been well documented in plentiful studies. To name a few, Ma et al. (2011) found that chitosan inhibited gene expression of IL-6 and TNF-α induced by lipopolysaccharides, possibly through blocking mitogen-activated protein kinase and PI3K/Akt signaling pathways and the downstream NF-κB nuclear translocation (Li et al., 2014). Chung et al. (2012) demonstrated that chitosan protected the mice from the lung inflammation induced by ovalbumin, through the inhibition of pro-inflammatory cytokines IL-4, IL-5, IL-13, and TNF-α.

Despite the attractive properties of chitosan, studies focusing on the effects of chitosan on ruminant immune functions are rather scarce, consequently limiting its use in the dairy industry. Still, some promising results support its potential as an alternative to antibiotics for mastitis control. Using a mice model, Moon et al. (2007) showed that the peritoneal inoculation of chitosan elevated levels of pro-inflammatory cytokines IL-6 and IFN- γ . In the same study, oral administration of chitosan protected mice from *Staphylococcus aureus* isolated from bovine mastitis, thus the authors concluded that chitosan can be applied for mastitis control due to its immunostimulatory properties. Experimented in bovine, Liu et al. (2007) supplemented 0.1 ~ 0.3% chitosan in the feed of dry cows for 60 days before calving, resulting in higher concentrations of serum immunoglobulin and glucose in the dry period, which can protect cows from IMI occurring in this period. Recently, an inconsistent finding was reported in lactating cows. Zheng et al. (2021) supplemented 500 ~ 2000 mg/kg in the feed of lactating cows, which instead leads to a decrease in the expression of IL-1 and NF-κB genes. They concluded that the anti-inflammatory effects of chitosan supplementation may be beneficial for milk production.

Until now, the immunomodulatory effects of chitosan have yet to be demonstrated beneficial for mastitis control in dairy cows. To avoid antibiotic abuse, we discussed the effect of selective dry cow therapy in Chapter 1; however, as we have observed, the outcome of an SDCT can be affected by the hygiene practices of farms and other management factors, thus a therapy alternative to antibiotic treatment is required. Furthermore, treating clinical mastitis with antibiotics is insufficiently effective, and the consequent recurrence of mastitis leads to even further antibiotic overuses, economic losses (Bar et al., 2007; Schukken et al., 2009b; Hertl et al., 2014), as well as shortened life spans of animals (Bar et al., 2008; Hertl et al., 2011; Cha et al., 2013). To overcome this, immunotherapy modulating immune systems may help eliminate IMI and prevent recurrent mastitis. In this study, we aimed to investigate: (1) whether chitosan can reduce IMI occurring in the dry period; (2) whether chitosan can improve the efficacy of antibiotic treatment and prevent the recurrence of clinical mastitis; (3) whether chitosan affects the immune status of lactating and/or dry cows.

6.2. Materials and Methods

We conducted two experiments, namely experiment A and experiment B, to evaluate the effects of chitosan on preventing dry period IMI and recurrent clinical mastitis. We performed both experiments following the Guidelines for the Care and Use of Agricultural Animals of Obihiro University using the study herd described in section 2.2.1 study herd. A low molecular weight (i.e., 50-200 kDa) chitosan provided by Hokkaido Soda Co., Ltd. (Tomakomai, Hokkaido, Japan) was used in this study.

6.2.1. Experiment A. Effects of chitosan on preventing dry period IMI

Experimental design

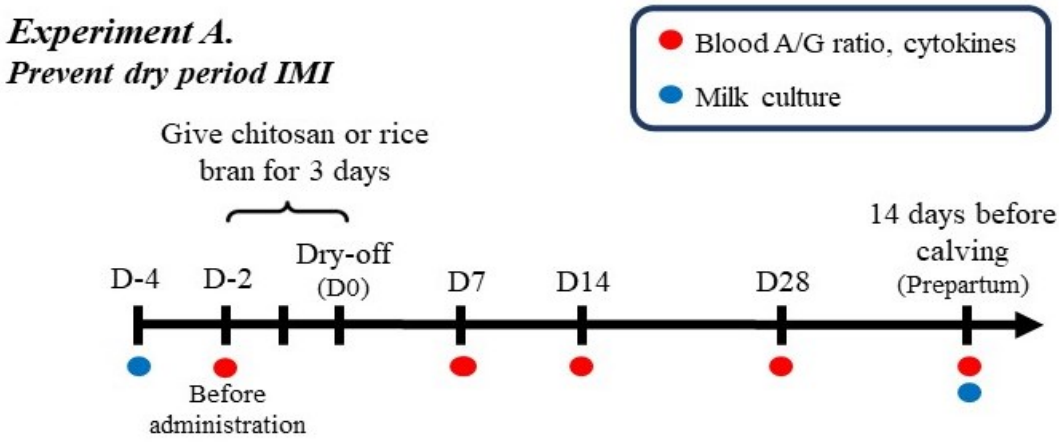

Figure 25. Experiment design of experiment A, which aimed to investigate the effects of orally administrated chitosan on preventing dry period intramammary infections (IMI). Abbreviations: A/G ratio = albumin/globulin ratio.

Figure 25 briefs experiment design of experiment A. In experiment A, we examined cows that entered the dry period between June 2021 to June 2022. Four days before dry-off, we collected milk samples aseptically from all quarters of cows and cultured them separately. Only cows without any IMI in all quarters were enrolled and allocated alternately to either the chitosan group or the control group. Two days before dry-off, we collected blood samples from the enrolled cows to evaluate levels of immune markers (i.e., serum A/G ratio and cytokines) before treatment administration (labeled as before administration). After blood collection, we orally administrated 10 g low molecular weight chitosan and 30 g rice bran to cows in the chitosan group, and 30 g rice bran to cows in the control group. We gave the chitosan and/or rice bran once a day, from 2 days before dry-off until dry-off for 3 days in total. To evaluate levels of the immune markers after treatment administration, we collected blood samples on 7, 14, and 28 days after dry-off (d 7, d 14, and d 28), and 14 days before the due date (labeled as prepartum). Last, we collected milk samples on the last sampling date to evaluate IMI status before calving.

Our purpose was to evaluate the effect of chitosan on preventing dry period IMI, but other management factors, such as the efficacy of external teat sealant and herd hygiene, would affect the dynamic of dry period IMI as well (Chapter 1), whose effects need to be considered. Therefore, we recorded herd hygiene scores and the duration of external teat sealant adherence as described in 2.2.5. Evaluating herd hygiene and the duration of external teat sealant

adherence. Because we found high correlations between each herd hygiene score, only herd hygiene scores from the posterior aspect of the udder were used in the analysis.

Sample collection and analysis

In experiment A, milk samples were collected based on the procedures previously described in section 2.2.3. Sampling. After collection, milk culture was performed immediately using the technique described in section 2.2.4. Bacterial culture of milk. On the other hand, blood samples were collected from the coccygeal veins of cows into plain serum tubes. Collected samples were allowed to clot for serum isolation, and the isolated serum was stored at -30 °C until the analysis. Methods for isolating serum and determining the A/G ratio level were detailed in section 4.2.2. Collection and analysis of blood samples. Moreover, cytokines (IL-6, IL-12, IFN-γ, IL-4, and IL-10) were quantified using commercial sandwich ELISA kits (BT LAB, Shanghai, China) following the manufacturer's instructions. Each sample was examined twice in an assay to calculate the average and %CV. Measurements with a %CV higher than 20% were considered invalid and discarded.

Statistical analysis

Statistical analyses were performed using R software (v4.0.5; R Core Team, 2021). First, we evaluated the effect of chitosan on preventing dry period IMI with the consideration of the effects of management factors, namely the duration of teat sealant adherence and herd hygiene scores. We fitted data in a generalized linear mixed model using the 'glmer' function within the 'lme4' package (Douglas et al., 2015). In this analysis, the outcome is whether the quarter was infected in the dry period (i.e., with IMI before calving), which is a binary outcome (i.e., infected or not). Therefore, we chose a Bernoulli distribution and a logit link function to fit the model, which was of the form:

Equation 5: $$logit(Inf_{ijkl}) = \beta_0 + \beta_1 Group_k + \beta_2 Hygiene_l + \beta_3 Sealant_i + \gamma_j + \varepsilon_{ijkl}$$,

where Inf_{ijkl} is the IMI status before calving (2 levels: infected or not infected), for the ith quarter in the jth cow. $Group_k$, $Hygiene_l$, and $Sealant_i$ are fixed effects of the experiment group (2 levels: chitosan group or control group), hygiene scores (continuous variable), and the duration of teat sealant adherence (continuous variable). γ_j is the random effect of the cow allowing the intercept to vary for each cow, and ε_{ijkl} is the residuals.

Another aim was to investigate the effects of chitosan on the immune status of dry cows, which we evaluated through the levels of serum proteins and cytokines. Different from infection status, levels of serum proteins and cytokines are continuous values. We thus fitted data in a

linear mixed model, using the 'lmer' function within the 'lme4' package (Douglas et al., 2015). Models were of the form:

Equation 6: $Y_{ijk} = \beta_0 + \beta_1 Group_k + \beta_2 Time_i + \beta_3 Group_k \times Time_i + \gamma_j + \varepsilon_{ijk}$,

where Y_{ijk} is the response variable, serum protein (i.e., globulin, albumin, total protein, and the A/G ratio) and cytokine concentrations (i.e., IL-6, IL-12, IFN-γ, IL-4, and IL-10), for the ith observation in the jth cow. $Group_k$ and $Time_i$ are fixed effects of the experiment group (2 levels: chitosan group or control group), and time points (5 levels: before administration, d 7, d 14, d 28 after dry-off, and prepartum), respectively. Interactions between $Group_k$ and $Time_i$ was included to evaluate the effect of chitosan on each time point. γ_j is a random effect term allowing the intercept to vary for each cow. EMMs of serum protein and cytokine concentrations were computed and compared between groups at each time point using the "emmeans" package (Lenth, 2022).

6.2.2. Experiment B. Effects of chitosan on preventing recurrent clinical mastitis

Experimental design

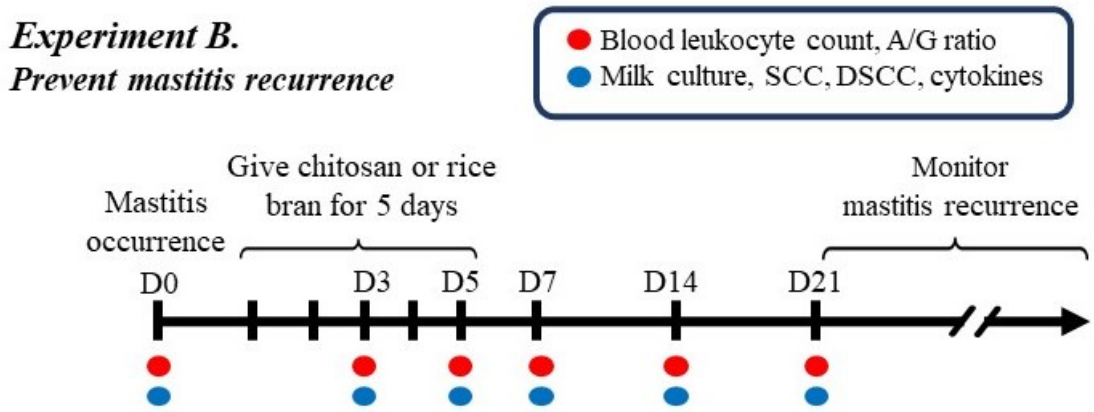

Figure 26. Experiment design of experiment B, which aimed to investigate the effects of orally administrated chitosan on preventing recurrent mastitis. Abbreviations: A/G ratio = albumin/globulin ratio; SCC = somatic cell count; DSCC = differential SCC.

Figure 26 briefs the experiment design of experiment B. In experiment B, we enrolled cows identified with clinical mastitis from June 2021 to February 2022; however, we excluded cows concurrently receiving medications for other concurrent diseases (e.g., lameness) and cows with recurrent mastitis. The identification and treatment of these cows followed the protocol described in section 4.2.1. Study herd and the enrollment of cows. Briefly, mastitis caused by major pathogens had been treated with antibiotics once daily for 5 consecutive days

but other mastitis cases (i.e., non-specific mastitis and mastitis caused by minor pathogens) were left untreated. After confirming mastitis, we evaluated disease severity as previously defined in section 4.2.4 Evaluate mastitis severity. Concurrently, we collected blood and quarter milk samples to evaluate the immune and IMI status. Later, we allocated cows with mastitis caused by the same pathogen alternately to either the chitosan group or the control group. From the next day, we orally administrated either 10 g low molecular weight chitosan (50-200 kDa) and 30 g rice bran to cows in the chitosan group or 30 g rice bran to cows in the control group once daily, continuing for 5 days. We collected milk samples from the affected quarter and blood samples from the cow on d 3, 5, 7, 14, and 21 after mastitis occurred to evaluate IMI status and the levels of WBC, serum proteins, SCC, and DSCC. After that, we continually monitored whether these mastitis cases recurred.

Sample collection and analysis

In experiment B, milk samples were collected for determining SCC, DSCC, and IMI status following the procedures described in section 4.2.3. Milk sampling, culture, and analysis. Based on the culture results, we categorized mastitis pathogens into the following groups: GNR, SA, CNS, SU, OS, SD, and NG. Additionally, a subset of milk samples was collected for determining cytokine levels in whey. Whey cytokines (TNFα, IL-1β, IL-6, IL-12, IFN-γ, IL-4, and IL-10) were quantified using commercial sandwich ELISA kits (BT LAB, Shanghai, China) following the manufacturer's instruction. Each sample was examined twice in an assay to calculate the average and %CV. Measurements with a %CV higher than 20% were considered invalid.

Concurrent with the collection of milk samples, blood samples were collected from the coccygeal veins of cows. Samples anticoagulated with EDTA were used for measuring WBC level, and samples collected in plain serum tubes were allowed to clot for serum isolation. Obtained serum was stored at -30 °C until the analysis. Details on determining the level of A/G ratio and WBC can be found in section 4.2.2. Collection and analysis of blood samples.

Statistical analysis

In experiment B, we aimed to investigate the effect of chitosan on preventing recurrent mastitis. As reviewed by Jamali et al. (2018), clinical mastitis cases in which a bacteriological cure was absent were much more likely to recur. Therefore, firstly, we investigated the effect of chitosan on the D21 bacteriological cure rate using a generalized linear model, where a Bernoulli distribution and a logit link function were chosen to fit the model, using the "glm" function within basic R (R Core Team, 2021). The model was of the form:

Equation 7: $$logit(Cure_{ijk}) = \beta_0 + \beta_1 Group_j + \beta_2 Pathogen_k + \varepsilon_{ijk},$$

where $Cure_{ijk}$ is the result of the bacteriological test on D21, in which NG was defined as cured, otherwise considered as uncured (2 levels: cured or not). $Group_j$ and $Pathogen_k$ are the effects of the experiment group (2 levels: chitosan or control group) and the causative pathogen (2 levels: minor or major pathogen), respectively. ε_{ijk} is the residuals. The effect of pathogens was considered since different treatment protocols were applied depending on the causative pathogen (only quarters infected with major pathogens received antibiotics).

Secondly, we investigated the effect of chitosan on time from mastitis occurrence until mastitis recurrence using survival analysis, namely Cox proportional hazards regression models. The Cox regression model enables us to assess the effects of several factors simultaneously on survival times, which can be fitted using the 'coxph' function within the 'survival' package (Therneau and Grambsch, 2000). As in Equation 7, we include both effects of the experiment group and the causative pathogen in the model. The model was of the form:

Equation 8: $$h(t) = h_0(t) \times \exp\left(\beta_1 Group_j + \beta_2 Pathogen_k\right),$$

where **t** is the time from mastitis occurrence until mastitis recurrence. Mastitis was deemed recurrent when the same pathogen was isolated from the same quarter as the previous occurrence. However, when the different pathogen was isolated from the same quarter, the recurrence was rejected and the case was deemed censoring. Similarly, when the affected quarter was dried or blinded, the case was deemed censoring. As in Equation 7, $Group_j$ and $Pathogen_k$ are the effects of the experiment group and the causative pathogen. Additionally, **h(t)** is the hazard function, and h_0 is the baseline hazard, which refers to the hazard when the effects of all risk factors equal zero.

Finally, we investigated the effects of chitosan on cows' immune status after mastitis occurred, which we evaluated through changes in serum protein, WBC, SCS, DSCC, and whey cytokine levels. We fitted data in a linear mixed model using the 'lmer' function within the 'lme4' package (Douglas et al., 2015). Models were of the form:

Equation 9:

$$\begin{aligned} Y_{ijklmn} = \beta_0 &+ \beta_1 Pathogen_k + \beta_2 Severity_l + \beta_3 Group_m + \beta_4 Time_n \\ &+ \beta_5 Pathogen_k \times Time_n + \beta_6 Severity_l \times Time_n \\ &+ \beta_7 Group_m \times Time_n + \beta_8 DIM_i + \beta_9 DIM_i^2 + \beta_{10} Parity_{ij} + \gamma_j \\ &+ \varepsilon_{ijklmn}, \end{aligned}$$

where $\boldsymbol{Y_{ijklmn}}$ is the response variable, SCS, DSCC, WBC, A/G ratio, or cytokine concentrations, for the i[th] observation in the j[th] mastitis event. $\boldsymbol{Pathogen_k}$, $\boldsymbol{Severity_l}$, $\boldsymbol{Group_m}$, and $\boldsymbol{Time_n}$ are fixed effects of mastitis pathogen (7 levels: NG, CNS, SA, SU, SD, OS, and GNR), severity (3 levels: mild, moderate, and severe), experiment group (2 levels: chitosan group or control group), and days after mastitis (6 levels: d 0, 3, 5, 7, 14 and 21), respectively. Interactions between $\boldsymbol{Pathogen_k}$, $\boldsymbol{Severity_l}$, $\boldsymbol{Group_m}$, and $\boldsymbol{Time_n}$ were included to evaluate the effect of pathogen, severity, and experiment group on each time point. $\boldsymbol{DIM_i}$ and $\boldsymbol{DIM_i^2}$ are the linear and quadratic terms of days in milk, $\boldsymbol{Parity_{ij}}$ is the term of parity, and $\boldsymbol{\gamma_j}$ is a random effect term allowing the intercept to vary for each mastitis case.

The significance of each term ($P < .05$) in Equation 9 was tested by type III ANOVA using the package "lmerTest" (Alexandra et al., 2017). Kenward-Roger's approximation was used to calculate denominator degrees of freedom. EMMs were computed and compared between the two experiment groups at each time point. Additionally, the effects of parity, DIM, mastitis pathogen, and disease severity were presented in Chapter 4.

6.3. Results

6.3.1. Experiment A. Effects of chitosan on preventing dry period IMI

We enrolled 21 cows without IMI before dry-off, of which, we allocated 10 cows to the control group and the other 11 cows to the chitosan group. Two quarters in the control group and two quarters in the chitosan group were blind and excluded from the analysis.

IMI rate before calving

Two weeks before the due date, 9 cows (9/10; 90%) in the control group and 5 cows (5/11; 45.5%) in the chitosan group were with IMI. Among their quarters, 12 of 38 quarters (31.6%) in the control group and 9 of 42 quarters (21.4%) in the chitosan group were with IMI. Table 10 provides the distribution of pathogens in each group. Noted that some quarters were infected with two pathogens thus the percentages do not sum to one. Table 11 shows the summary of fitted models, which indicates that chitosan has no effects (Estimate = -0.38, p = 0.48) on the dynamics of dry period IMI. Additionally, the duration of teat sealant adherence and herd hygiene respectively had no (Estimate = 0.04, p = 0.59) and marginal effects (Estimate = 0.69, p = 0.09) on the occurrence of dry period IMI.

Table 10. Distribution of intramammary infections before calving in quarters of the control and the chitosan group [number of quarters (%)].

	Pathogens of intramammary infections before calving *							
Group	NG	CNS	GNR	OS	SA	SD	SU	Total
Control	26 (68.4%)	3 (7.9%)	1 (2.6%)	0 (0%)	1 (2.6%)	5 (13.2%)	3 (7.9%)	38
Chitosan	33 (78.6%)	3 (7.1%)	0 (0%)	1 (2.4%)	3 (7.1%)	2 (4.8%)	3 (7.1%)	42

* NG = no significant growth; CNS = couagulase-negative staphylococci; GNR = Gram negative rods; OS = other streptococci; SA = *Staphylococcus aureus*; SD = *Streptococcus dysgalactiae*; SU = *Streptococcus uberis*.

Table 11. Effects of chitosan, the duration of teat sealant adherence, and herd hygiene on the occurrence of dry period intramammary infections.

	Estimate	Standard error	z value	P value
Intercept	-2.81	1.40	-2.00	0.05
Group (*Reference = control*)	-0.38	0.53	-0.71	0.48
Teat sealant	0.04	0.07	0.53	0.59
Herd hygiene score	0.69	0.40	1.69	0.09

Serum protein concentrations

Due to insufficient lengths of the dry period, we could not collect D14 blood samples from one cow in the control group, and D28 blood samples from 4 cows in the control group and 4 cows in the chitosan group. Results from ANOVA (Table 12) show that time affected serum protein levels most significantly, but chitosan had no apparent effects. Figure 27 shows EMMs of serum protein levels in the control and the chitosan group at each time point. We noted that albumin concentrations descended over time, and globulin concentrations increased over the dry period but decreased sharply before calving. Consequently, the A/G ratio decreased over the dry period but increased before calving. On the other hand, chitosan had no effects on the dynamics of serum proteins. We compared serum protein levels between the experimental groups at each time point but could not identify any significant differences.

Table 12. ANOVA for the effects of time, chitosan (Group), and their interaction on levels of total protein, albumin, globulin, and serum albumin/globulin ratio (A/G ratio).

		Total protein			Albumin		
	NuDF [a]	DeDF [b]	F value	P value	DeDF	F value	P value
Time	4	67.41	7.68	3.67E-05	67.77	12.34	1.36E-07
Group	1	19.18	0.10	0.76	19.30	0.01	0.91
Time X Group	4	67.41	0.54	0.71	67.77	1.27	0.29
		Globulin			A/G ratio		
	NuDF	DeDF	F value	P value	DeDF	F value	P value
Time	4	67.28	8.58	1.17E-05	67.35	9.70	2.96E-06
Group	1	19.13	0.08	0.78	19.16	0.00	0.97
Time X Group	4	67.28	0.43	0.79	67.35	0.45	0.77

[a] NuDF = Numerator degrees of freedom; [b] DeDF = Adjusted denominator degrees of freedom.

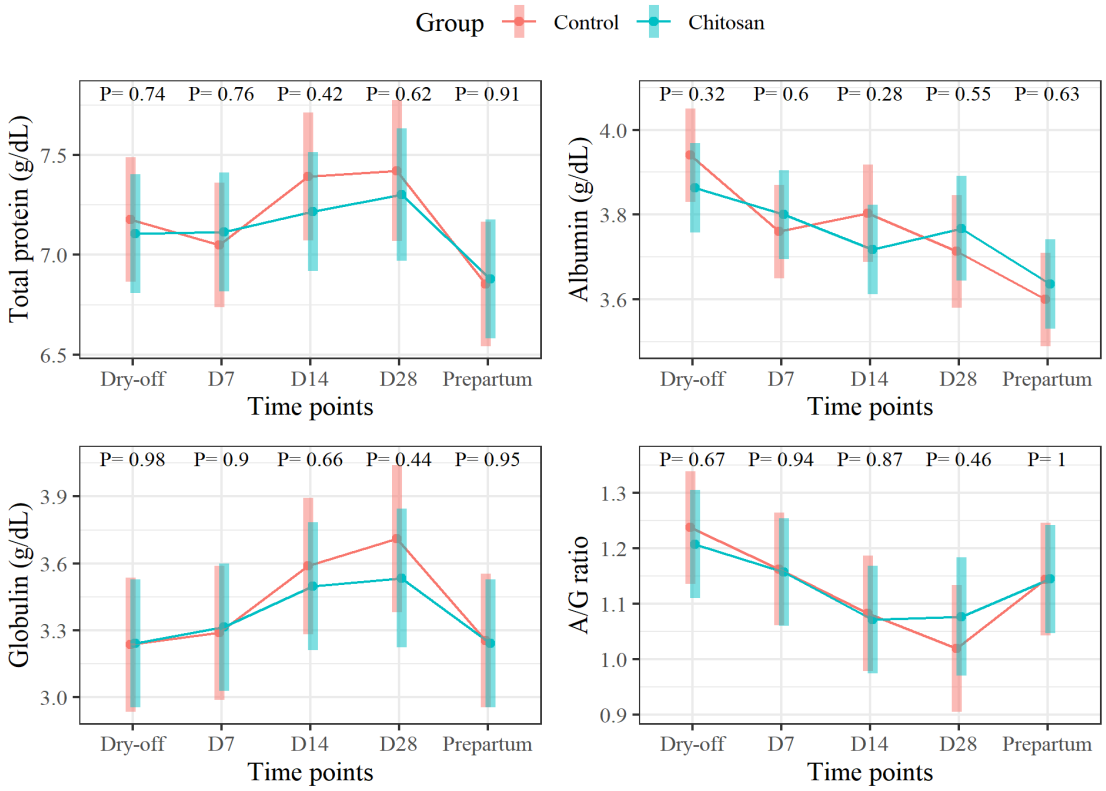

Figure 27. Estimated marginal means (EMMs) ± 95% confidence intervals of total protein, albumin, globulin, and serum albumin/globulin ratio (A/G ratio) levels in the control and the chitosan group over the dry period. EMMs were compared between groups at each time point, and P-values are shown.

Serum cytokine concentrations

Only a limited number of samples can be measured within a cytokine kit, thus we excluded blood samples collected on D28 after dry-off. Results from ANOVA (Table 13) show that time affected IL-6 concentrations but not on the other measured cytokines. Also, administrating chitosan before dry-off did not significantly affect the dynamics of cytokines. Figure 28 shows EMMs of cytokine concentrations in the control and the chitosan group at each sampling time point. Most of the cytokines exhibited a descending trend over time, although only in IL-6 this relationship was demonstrated statistically. On the other hand, the oral administration of chitosan had no obvious effects on cytokine concentrations. We compared cytokine concentrations between groups at each time point, but the difference between the control group and the chitosan group was insignificant in all comparisons.

Table 13. ANOVA for the effects of time, chitosan (Group), and their interaction on serum cytokine concentrations during the dry period.

		IL-6			IL-12			IFN-γ		
	NuDF[a]	DeDF[b]	F value	P value	DeDF	F value	P value	DeDF	F value	P value
Time	3	52.06	5.89	1.5E-03	55.07	2.21	0.10	55.05	1.98	0.13
Group	1	19.00	1.16	0.29	19.01	0.53	0.48	19.00	0.51	0.49
Time X Group	3	52.06	0.59	0.62	55.07	1.23	0.31	55.05	1.49	0.23
		IL-4			IL-10					
	NuDF	DeDF	F value	P value	DeDF	F value	P value			
Time	3	55.05	1.71	0.18	56.02	0.92	0.44			
Group	1	19.00	0.53	0.47	19.00	2.27	0.15			
Time X Group	3	55.05	0.25	0.86	56.02	0.55	0.65			

[a] NuDF = Numerator degrees of freedom; [b] DeDF = Adjusted denominator degrees of freedom.

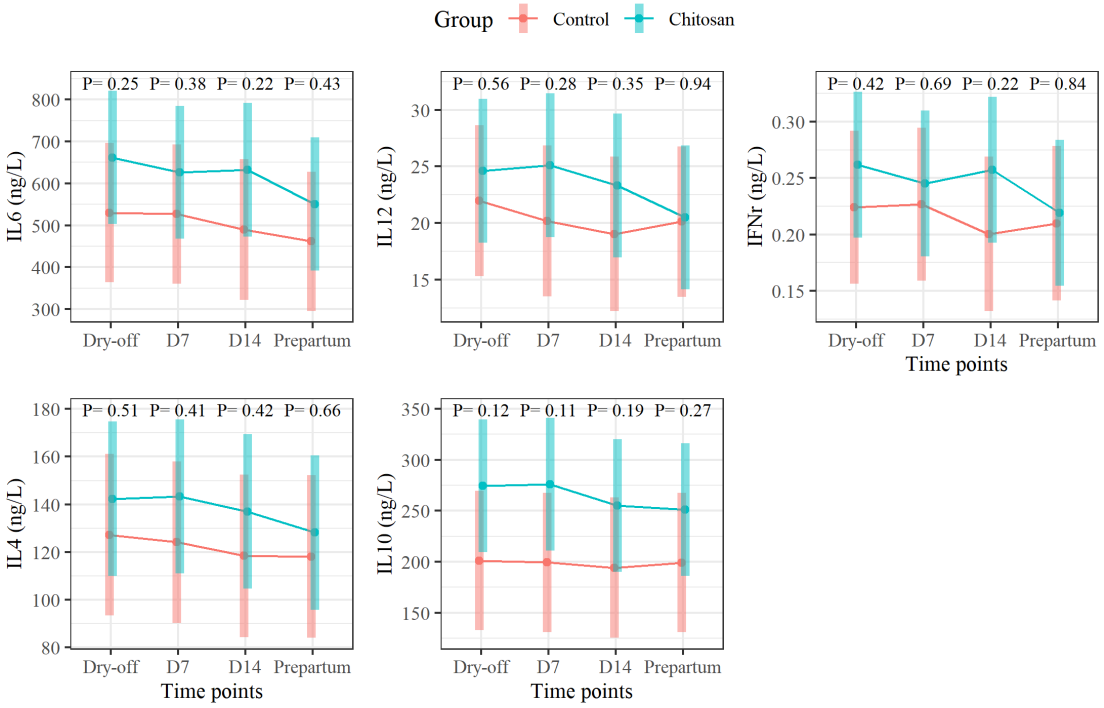

Figure 28. Estimated marginal means (EMMs) ± 95% confidence intervals of cytokine levels in the control and the chitosan group over the dry period. EMMs were compared between groups at each time point, and P-values are shown.

6.3.2. Experiment B. Effects of chitosan on preventing recurrent clinical mastitis

Allocation of cows into the control and the chitosan group

As discussed in Chapter 4, immune responses in mastitis depend on the causative pathogen and disease severity, consequently affecting mastitis outcomes. To evaluate the effectiveness of chitosan on the recurrence of mastitis, we need to take these effects into account. Therefore, we allocated cows with clinical mastitis caused by the same pathogen and with a similar severity alternately into the control and the chitosan group. Table 14 shows the characterization of mastitis cases occurring in cows allocated in each group. Generally, cows with clinical mastitis caused by the same pathogen were equally distributed in two groups, except for cows with OS mastitis. Diagnosing streptococci at the species level typically takes 48 hours, but we are required to administrate treatment on the next day after mastitis occurred. As such, when we administrated chitosan, we could only speculate the species of streptococci, which results in the unequal distribution of OS mastitis in the two groups.

Table 14. Characterization of mastitis cases in the control and chitosan group.

Pathogen[a]	Group	Severity			Total[b]
		Mild	Moderate	Severe	
CNS	Control	4	0	0	4
	Chitosan	2	1	0	3
GNR	Control	0	1	0	1
	Chitosan	0	0	1	1
NG	Control	1	2	0	3
	Chitosan	3	1	0	4
OS	Control	3	1	2	6
	Chitosan	2	0	0	2
SA	Control	2	1	0	3
	Chitosan	2	0	1	3
SD	Control	1	0	1	2
	Chitosan	1	0	1	2
SU	Control	6	1	1	8
	Chitosan	5	1	1	7

[a] NG = no significant growth; CNS = couagulase-negative staphylococci; GNR = gram-negative rods; OS = other streptococci; SA = *Staphylococcus aureus;* SD = *Streptococcus dysgalactiae*; SU = *Streptococcus uberis.*
[b] 27 cases in the control group; 22 cases in the chitosan group

Effects of chitosan and pathogen on D 21 bacteriological cure rate

On the analysis of the bacteriological cure rate, we excluded cases from that we could not isolate bacteria on D 0 (i.e., cases of NG). In the control group, 75% (15/20) cases of major pathogen-caused mastitis and 75% (3/4) cases of minor pathogen-caused mastitis were cured on d 21 after mastitis occurrence. Contrastingly, in the chitosan group, 80% (12/15) cases of major pathogen-caused mastitis and 0% (0/3) cases of minor pathogen-caused mastitis were cured. Table 15 shows the model summary on the effects of orally administrated chitosan and the causative pathogen on D 21 bacteriological cure rate. The results show that mastitis caused by major pathogens tended to have a higher D 21 bacteriological cure rate (OR = 4.58; 95% CI = 0.83 - 25.16; P = 0.08), which may result from the use of antibiotics in these cases. On the other hand, the administration of chitosan had no obvious effects on D 21 bacteriological cure rate (OR = 0.64; 95% CI = 0.16 - 2.63; P = 0.54).

Table 15. Effects of chitosan and the mastitis pathogen on D21 bacteriological cure rate.

	Estimate ± Standard error	Odds ratio (95% confidence interval)	P value
Intercept	-0.1 ± 0.82	0.9 (0.18 - 4.53)	0.90
Group (*Reference = control*)	-0.44 ± 0.72	0.64 (0.16 - 2.63)	0.54
Pathogen (*Reference = minor*)	1.52 ± 0.87	4.58 (0.83 - 25.16)	0.08

Effects of chitosan and pathogen on mastitis recurrence

To evaluate the effectiveness of chitosan in preventing mastitis recurrence, we conducted a cox proportional hazards survival analysis on the time between mastitis occurrence and mastitis recurrence. The results were visualized in Figure 29. Mastitis caused by major pathogens tended to be more unlikely to recur (OR = 0.33; 95% CI = 0.09 - 1.18; P = 0.09), likely an effect of antibiotic treatment; however, administrating chitosan had no obvious effects on preventing the recurrence (OR = 0.91; 95% CI = 0.26 – 3.21; P = 0.88).

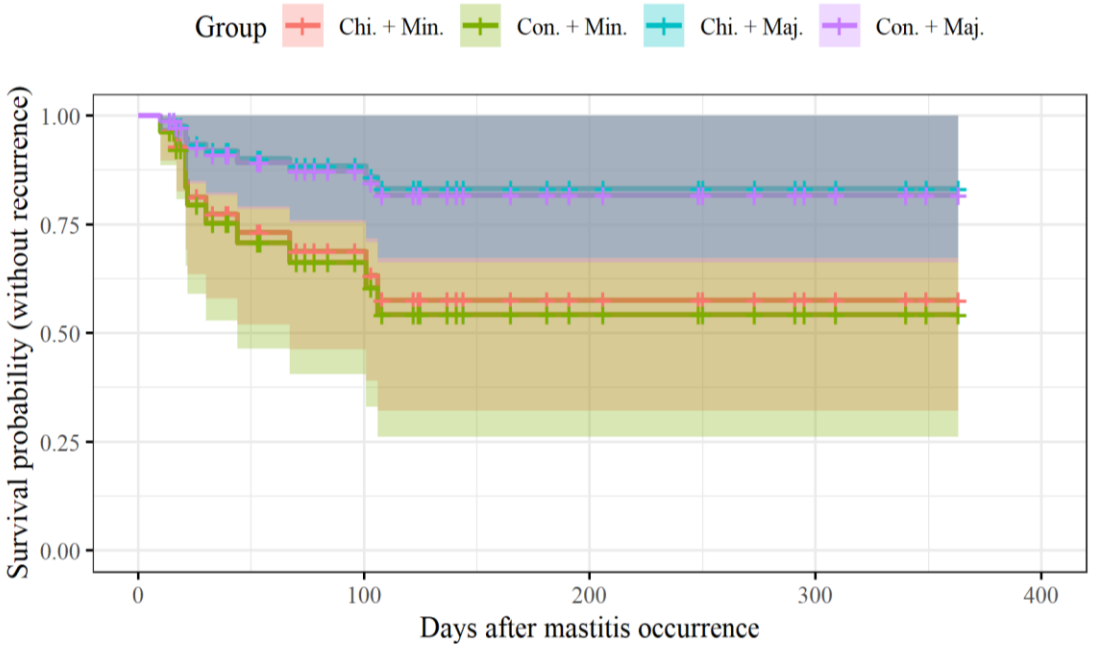

Figure 29. A survival plot showing the effects of chitosan and causative pathogen on the survival probability (without recurrent mastitis). Abbreviations: Chi. = chitosan; Con. = control; Min. = minor pathogens; Maj. = major pathogens. The colorful shadows show 95% confidence intervals, and the plus marks (+) represent censoring observations.

Effect of chitosan on dynamics of SCS, DSCC, WBC, and the A/G ratio

We could not measure SCS and DSCC for four samples since abundant clots were presented in these samples, and we could not measure DSCC for another 30 samples since these samples were with lower than 50,000 cells/mL and the measurements were unreliable. Table 16 shows that time after mastitis occurrence had a significant effect on the level of SCS, DSCC, WBC, and the A/G ratio, but administrating chitosan did not affect the level of these indicators. Specifically, both the effects of the experiment group and the interaction between time and the experiment group were insignificant on all the evaluated traits. Figure 30 shows the level of these immune indicators in the control and the chitosan group at each time point. The administration of chitosan had no apparent effects on the dynamics of these indicators. We

compared their levels between groups at each time point. The only significant difference was found at D0 for the DSCC level. However, the measurement was performed before administrating chitosan, making the difference impossibly due to the effect of chitosan.

Table 16. ANOVA for the effects of the experiment group (chitosan) and time after mastitis occurrence on the level of somatic cell score (SCS), differential somatic cell count (DSCC), white blood cell count (WBC), and the albumin/globulin ratio (A/G ratio).

	SCS		DSCC		WBC		A/G ratio	
	F value	P value	F value	P value	F value	P value	F value	P value
Time	35.12	1.8E-25	6.24	2.5E-05	9.15	7.6E-08	4.37	8.7E-04
Group	1.2	0.28	1.8	0.19	0.28	0.6	0.69	0.41
Time X Group	0.48	0.79	1.3	0.27	1.65	0.15	1.54	0.18

* Note: effects of days in milk, parity, mastitis pathogen, and severity were not shown.

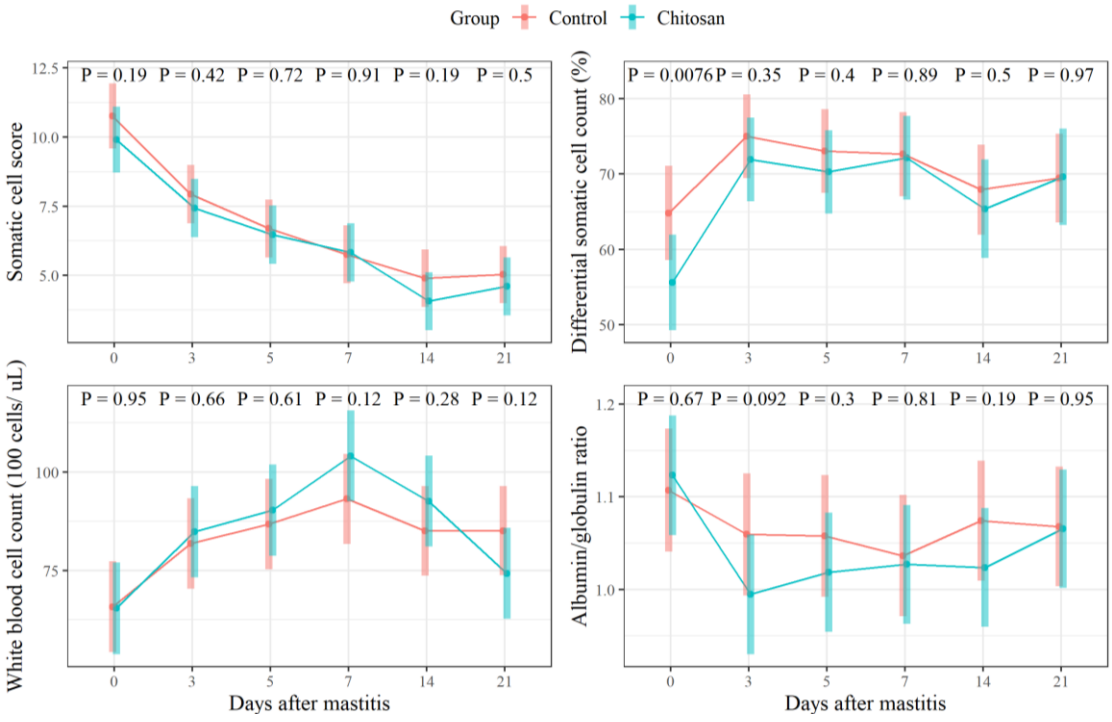

Figure 30. Estimated marginal means (EMMs) ± 95% confidence intervals of somatic cell score, differential somatic cell count, white blood cell count, and the albumin/ globulin ratio in the control and the chitosan group over time after mastitis occurrence. EMMs were compared between groups at each time point, and P-values are shown.

Whey cytokine concentrations

Due to the limited number of cytokine measurements, we selected specific cases (Table 17) to measure cytokine concentrations in whey on D0, D3, and D7 after mastitis occurred. We selected these cases due to the similarity of severity and causative pathogen between them. Table 18 shows that whey cytokine concentrations were affected by neither time, the administration of chitosan nor their interaction. As shown in Figure 31, we compared whey cytokine concentrations between experiment groups at each time point; however, we could not identify any apparent differences. Although a marginally significant difference was identified at D0 for the IL-12 level, the measurement was performed before administrating chitosan, thus the difference cannot be attributed to the effect of chitosan.

Table 17. Characterization of mastitis cases selected for whey cytokine measurements.

		Severity			
Pathogen[a]	**Group**	Mild	Moderate	Severe	**Total[b]**
CNS	Control	2	0	0	2
	Chitosan	2	0	0	2
SA	Control	2	1	0	3
	Chitosan	2	0	1	3
SD	Control	1	0	1	2
	Chitosan	1	0	1	2
SU	Control	5	1	1	7
	Chitosan	5	1	1	7

[a] CNS = coagulase-negative staphylococci;
SA = *Staphylococcus aureus;*
SD = *Streptococcus dysgalactiae;*
SU = *Streptococcus uberis.*
[b] 14 cases in the control group;
14 cases in the chitosan group

Table 18. ANOVA for the effects of chitosan (Group) and time after mastitis occurrence on whey cytokine concentrations.

	TNFα		IL-1β		IL-6		IL-12	
	F value	P value	F value	P value	F value	P value	F value	P value
Time	0.24	0.79	0.70	0.50	0.78	0.46	0.23	0.79
Group	0.16	0.69	0.24	0.63	0.44	0.52	0.94	0.34
Time X Group	0.67	0.51	0.05	0.95	1.01	0.37	1.71	0.19

	IFN-γ		IL-4		IL-10	
	F value	P value	F value	P value	F value	P value
Time	0.53	0.59	0.77	0.47	0.11	0.89
Group	0.52	0.47	0.31	0.58	0.17	0.68
Time X Group	0.26	0.77	0.07	0.93	0.82	0.45

* Note: effects of days in milk, parity, mastitis pathogen, and severity were not shown.

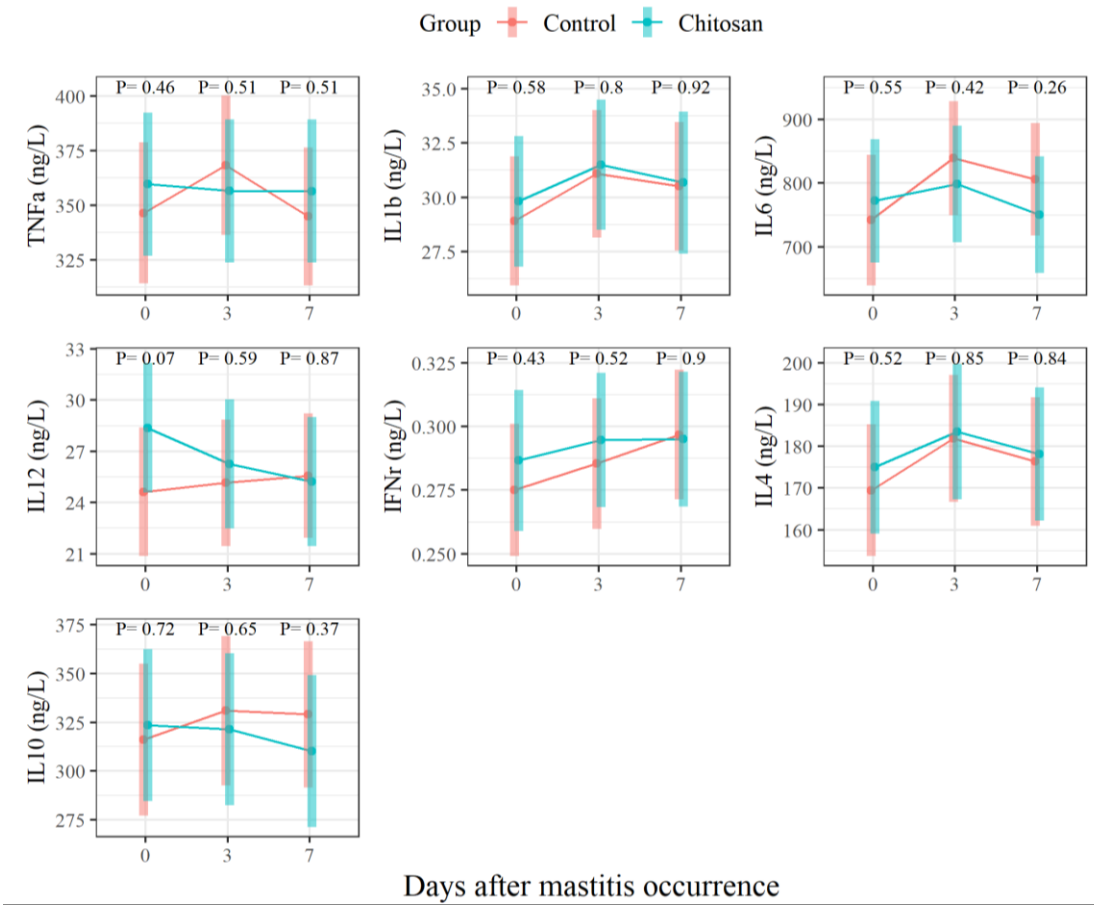

Figure 31. Estimated marginal means (EMMs) ± 95% confidence intervals of cytokine concentrations in the control and the chitosan group over time after mastitis occurrence. EMMs were compared between groups at each time point, and P-values are shown.

6.4. Discussions

In the two experiments of the present study, we could not identify any effects of chitosan on the immune status of either dry or lactating cows. As a consequence, neither prevention nor treatment of mastitis could benefit from the oral administration of chitosan, at least in the current study. In the following, we will discuss possible reasons that can lead to the ineffectiveness of chitosan in modulating cows' immune systems. Also, we will introduce some alternative administration routes to oral for giving chitosan, which may be beneficial for future studies to develop novel strategies to apply chitosan for mastitis control.

6.4.1. Possible reasons causing the ineffectiveness of chitosan

Administration dose and duration

In the present study, the duration of chitosan administration (i.e., 3 d in experiment A and 5 d in experiment B) was relatively short and the administration dose (i.e., 10 g/d) was

relatively low as compared with other studies. In a study aiming to evaluate the effect of chitosan on milk production and immune functions of dairy cows (Zheng et al., 2021), 500 ~ 2000 mg/kg chitosan was supplemented for 56 d in the feed, and results showed that the effect of chitosan increased with supplementation dosage. Similarly, in a trial investigating chitosan's effect on nutrition and immune functions of dry cows (Liu et al., 2007), chitosan supplementation accounted for 0.1 ~ 0.3% of dry matter intake and lasted for 60 d. Generally, in trials that aimed to investigate the chitosan effect on ruminants, oral administration of chitosan mostly lasted for more than 3 weeks (Harahap et al., 2022). Insufficient duration and dose of supplementation possibly caused the ineffectiveness of chitosan in this trial.

The molecular weight of chitosan

The effect of chitosan depends largely on its molecular weight. Numerous studies demonstrated that the biological activities of chitosan are molecular weight-dependent (No, 2002; Park et al., 2004; Lee et al., 2009; Wu et al., 2015; Zheng et al., 2016). Chitosan with lower molecular weight and degree of polymerization would have a higher solubility, and chitosan with molecular weights > 30 kDa is poorly soluble in neutral aqueous solutions, possibly disabling its biological activities (Kim and Rajapakse, 2005). However, the rumen environment is slightly acidic to acidic. It ranges from pH 6.2 to 7.0 in cows without ruminal acidosis and is less than 5.6 in cows with subacute ruminal acidosis (Oetzel, 2017). Thus, further investigation is required to clarify the bioavailability of the currently used low molecular weight chitosan (50-200 kDa) in dairy cows. However, in a previous study, supplementing chitosan with 50 kDa molecular weight successfully elevated dry cow's Igs concentrations (Liu et al., 2007), suggesting that the molecular weight of chitosan may not be the main reason causing the ineffectiveness observed here.

6.4.2. Other possible applications of chitosan for mastitis control

Antimicrobial activities of chitosan: supplemented in intramammary infusions and teat dips

Besides the immunomodulatory effect, the antimicrobial effects of chitosan have been supported by tremendous evidence (Rabea et al., 2003; Raafat and Sahl, 2009; Abd El-Hack et al., 2020). Although the specific mechanism still requires elucidation, scientists proposed that chitosan may destroy bacteria by altering the permeability of cell walls, causing leakages of intracellular contents, or by inhibiting the synthesis of RNA, DNA, and proteins (Rabea et al., 2003). As such, chitosan has been proposed as an alternative antimicrobial agent used for treating bovine mastitis. Moon et al. (2007) conducted an *in vitro* experiment, finding that low molecular weight chitosan successfully destroyed cell walls of SA isolated from bovine mastitis

and inhibited their growth within 10 min of treatment. Asli et al. (2017) further demonstrated that 2.6 kDa chitosan inhibited biofilm formation in SA, and no adverse effects occurred when it was infused into the mammary glands of cows. In that study, they also showed that chitosan had a synergistic effect with antibiotics using an *in vivo* experiment conducted in mice. This synergistic effect of chitosan and antibiotics was later validated in chronic CNS mastitis *in vitro* by Breser et al. (2018). Additionally, they found that chitosan can stimulate mammary epithelial cells to release proinflammatory cytokine, IL-6. Due to these findings, Zhang et al. (2021) evaluated the effect of a teat dip containing chitosan, both *in vitro* and *in vivo*, where they confirmed that supplementing 1% 50 kDa chitosan in teat dip increased its antimicrobial efficacy and the modified teat dip successfully reduced the prevalence of subclinical mastitis. However, until now, we still require a large-scale *in vivo* investigation on the effects of chitosan or chitosan-supplemented antibiotics on treating mastitis, which may be a very attractive option to improve antibiotic efficacy and antibiotic stewardship in the future.

Immunostimulatory activities of chitosan: supplemented in internal teat sealants or vaccines

Instead of administrating chitosan orally, chitosan can be given parenterally through either mucosal (including mammary glands), subcutaneous, or muscular tissues (Moran et al., 2018). Giving chitosan through mucosal tissues stimulates local immune responses, thus a stronger and more immediate immune response can be expected. To be given through mucosa, we have to prepare chitosan as hydrogels (Bhattarai et al., 2010), allowing them to be easily infused into mammary glands. Lanctôt et al. (2017) developed a chitosan hydrogel and administrated it into cow's mammary glands at dry-off. They hypothesized that the intramammary infusion of chitosan can stimulate local immune responses, speeding up the involution of mammary glands, thus reducing IMI risks during the dry period. Results showed that SCC, lactate dehydrogenase, and other inflammation markers were elevated in quarters infused with chitosan hydrogels. Although a direct relationship between the treatment and dry period IMI risk was not confirmed in that study, chitosan successfully induced a strong local inflammatory response in mammary glands. This result indirectly supported the viewpoint that chitosan is a potential candidate for vaccine adjuvants because it strongly induced cellular immunity as compared to alum (Moran et al., 2018). For several decades, researchers dedicated themselves to developing mastitis vaccines against major pathogens; however, the outcomes varied and were mostly unsatisfactory as reviewed by Rainard et al. (2021). In the review, the authors also pointed out that humoral immunity in the mammary gland would be inefficient since in a healthy mammary gland, the blood-milk barrier inhibits the transudation of antibodies from blood to milk, and the large volume of milk dilutes the antibody titers. Consequently, the authors suggested that adjuvants promoting cellular immunity and intramammary

immunization (Herry et al., 2017) are potentially helpful. In this context, an intramammary vaccine with chitosan-based adjuvants is worth developing.

6.5. Conclusions

We evaluated the effect of orally supplemented chitosan on preventing dry period IMI and recurrent clinical mastitis; however, the oral administration of chitosan failed to modulate cow's immune status, and we have not observed any beneficial effects of the administration on mastitis control. The ineffectiveness of chitosan can be related to several factors, including the dosage and the duration of administration, the molecular weight of chitosan, and the route of administration. In contrast to the unfavorable results obtained here, previous studies have shown that, due to its antimicrobial activities, chitosan can either prevent or treat bovine mastitis when supplemented in teat dips or intramammary antibiotic infusions. Further, chitosan strongly induced local cellular immunity in mammary glands, making it a potential candidate for mastitis vaccine adjuvants. Overall, the usefulness of chitosan in mastitis control still needs comprehensive investigation.

7. General Discussions

This thesis aimed to improve mastitis control strategies, to achieve sound antibiotic stewardship without sacrificing animal health and economic benefits. To summarize our findings, in Chapter 1, we found that an SDCT approach reduced antibiotic use by around 33%, but this reduction was accompanied by an increase in the IMI rate in the dry period. The increased IMI risk was related to poor herd hygiene and lower duration of external teat sealant adherence. In Chapters 2 to 4, we aimed to optimize methods used for selecting cows who can benefit most from antibiotic therapy, using a novel somatic cell trait, DSCC, and other immune markers. In Chapter 2, we found a positive association between MAC (equaling 100-DSCC) and the CMT result. MAC tends to increase in the healing stage and the chronic stage of mastitis, and consequently, the CMT result tends to be positive in these stages, making the CMT an unreliable guide if used for monitoring clinical treatment outcomes, but likely able to be used for detecting chronic mastitis. In Chapter 3, we elucidated the detrimental effect of high SCS and low DSCC on milk production with a proper statistical model. The effect was most pronounced in cows with high parity, where the milk yield was reduced by half (from 40 kg/d to 20 kg/d), with a corresponding change in milk composition. To achieve efficient milk production, we should prevent or recover high parity cows from a state of high SCS and low DSCC (chronic mastitis). In Chapter 4, we investigated the dynamics of several immune markers after clinical mastitis occurred, aiming to understand the effect of mastitis severity and causative pathogen. Changes in WBC differed between mastitis arising from coliforms, streptococci, and staphylococci. The A/G ratio dropped dramatically only in severe mastitis. We thus believe that these markers can facilitate pathogen identification and severity evaluation in mastitis, as well as the decision-making of mastitis treatment. Moreover, in the healing process of SD mastitis, DSCC decreased but SCS remained high, which suggests that, due to the severe damage from this pathogen, more macrophages are required to heal the mammary gland. This finding helps us clarify the pathogenesis of impaired productivity in cows with high SCS and low DSCC. Antibiotic treatment is ineffective in treating chronic and recurrent mastitis. We thus investigated whether the immunomodulatory effect of chitosan can be applied to prevent IMI and recurrent mastitis in Chapter 5. However, the oral administration of chitosan failed to modulate the cow's immune system, and the results also indicated that we cannot control mastitis with this approach. In the following section, we will discuss the current approaches for prudent antibiotic use in mastitis control, which is highly related to our findings.

The most common reason for administering antibiotics to dairy cows is to control mastitis (Ruegg, 2017b), and in most studies, antibiotics used for treating clinical mastitis are equivalent

to or more than those used for dry cow therapy (Pol and Ruegg, 2007; Saini et al., 2012; Stevens et al., 2016). However, antibiotic therapies are beneficial only for one-third of non-severe clinical mastitis cases (Ruegg, 2018), thus improving the efficiency of antibiotic treatment for clinical mastitis is essential for antibiotic stewardship. Ruegg (2018) has presented practicable guidelines to determine proper antibiotic therapies for clinical mastitis. All these guidelines follow a very simple principle, namely that "antibiotic treatment should be administrated only if the administration will result in a better clinical outcome than what can be expected without the administration". Nonetheless, this principle would be violated if mastitis was treated with antibiotics under several situations:

(1) **Causative pathogen was NG**: due to the absence of an active bacterial infection, antibiotic treatment cannot improve clinical outcomes (Fuenzalida and Ruegg, 2019b; c).

(2) **Causative pathogen was GNR**, including *E. coli* and *Klebsiella spp.*: mastitis caused by these bacteria is more likely to cure spontaneously, and thus the intramammary treatment is usually not encouraged for non-severe cases, although a lower spontaneous cure rate of *Klebsiella spp.* as compared to *E. coli* has been reported (Pyörälä and Pyörälä, 1998; Lago et al., 2011a; Fuenzalida and Ruegg, 2019a).

(3) **Causative pathogen was those resistant to any available antibiotics**, such as yeast, *Prototheca spp.*, and *Mycoplasma spp.*: these cases are not expected to benefit from any approved antibiotics due to the unsuitable mechanisms of action of drugs (Ruegg, 2018).

(4) **Extend antibiotic therapy for CNS infections**: CNS IMI usually locates on mucosal surfaces of mammary ducts but not invades mammary glands, and consequently, a 2-d of treatment can effectively eradicate the infection (Oliver et al., 2004b; Pyorala and Taponen, 2009). Extending treatment for CNS IMI has seldom improved clinical outcomes, instead increasing treatment costs and the amount of milk discarded (Pinzon-Sanchez et al., 2011). On the other hand, extending antibiotic treatment for mastitis caused by bacteria invading mammary glands, such as streptococci and SA, improves the cure rate considerably (Oliver et al., 2004a; Oliver et al., 2004b; Barkema et al., 2006; Lago and Godden, 2018).

(5) **Extend antibiotic therapy solely based on clinical signs (e.g., the appearance of milk) or indirect immune indicators (e.g., the CMT, SCC)**: The inflammatory response does not necessarily represent an active infection. Based on clinical observations by the author of the guidelines (Ruegg, 2018), milk can remain abnormal for 7 days after non-severe coliform mastitis. Treating mastitis solely based on clinical outcomes or immune responses instead of the presence of an active IMI certainly leads to ineffective therapies.

(6) **Treat non-severe mastitis systemically**: Systemic antibiotic treatment is beneficial for cows with severe mastitis (i.e., with systemic signs) because septicemia has been commonly observed in these cases (Wenz et al., 2001b; Erskine et al., 2002). However, when the infection is constrained in the udder (i.e., in non-severe cases), the benefit of systemic treatment is obscure because it is difficult to achieve therapeutic concentrations in the udder (Wenz et al., 2005; McDougall et al., 2007). The only exception may be SA mastitis. Some studies showed that systemically-administered penicillin G can efficiently eradicate SA IMI in young cows (Pyörälä and Pyörälä, 1998; Taponen et al., 2003; Pyörälä, 2009).

(7) **Treat animals without reviewing the medical history**: The success of antibiotic therapy depends largely on cow factors, namely immunity. Cows with high parity, chronically elevated SCC or recurrent mastitis records, and periparturient cows are expected to respond poorly to the IMI, consequently resulting in a lower cure rate (Pyörälä and Pyörälä, 1998; McDougall et al., 2007; Bradley and Green, 2009; Pinzón-Sánchez and Ruegg, 2011). Therefore, the therapeutic decision should be made considering these factors. For instance, the author recommended treating GNR infection occurring in periparturient cows because in these cows a spontaneous cure is not very expectable. Also, treating SA infections occurring in young cows results in better clinical outcomes than treating those occurring in cows with high parity or with chronic infections (Barkema et al., 2006).

(8) **Treat subclinical mastitis during lactation:** Due to the necessity of discarding milk during the treatment, treating subclinical mastitis during lactation is generally not recommended (Pyörälä, 2009). It is only justifiable when a highly contagious pathogen, such as SA or *Streptococcus agalactiae*, is prevalent in the herd. However, cows with subclinical mastitis produce less, low-quality milk, and may respond poorly to antibiotic therapies as the infection progresses to the chronic stage. Therefore, the benefit of treating subclinical mastitis during lactation may still require a comprehensive investigation (Ruegg, 2017a).

The prudent use of antibiotics for mastitis treatment is based on a thorough understanding of the causative pathogen (points 1 ~ 4). The pathogen identification can be easily facilitated by the technique of on-farm culture (Lago and Godden, 2018), as we have implemented (2.2.4. Bacterial culture of milk). Due to the abundance of gram-positive infections in our trial, antibiotic use can only be reduced by roughly 16.3% (i.e., 8/49 cases were non-severe NG/GNR mastitis; Table 6) if the treatment had been allocated following guidelines described by Ruegg (2018). However, we adopted a slightly different approach, where we treated non-severe GNR mastitis but omitted the treatment of non-severe CNS mastitis. As a result, antibiotic use was reduced by 28.6% (i.e., 14/49 cases were non-severe NG/CNS mastitis; Table 6) but

accompanied by a possibly higher recurrence rate (Figure 29). Recently, Lago et al. (2016) conducted a trial with similar approaches, in which only environmental streptococci clinical mastitis cases were treated with antibiotics. The result shows that, with this approach, a reduction of antibiotic use has been achieved without any negative effects on the rate of recurrent mastitis and culling. Although the result seemingly conflicts with ours, the benefit of omitting treatment of CNS mastitis is promising and requires more investigations. Extended or secondary antibiotic treatment can be justified with milk culture results, which is more proper than making decisions solely based on clinical impressions or indirect indicators including the CMT (point 5). We have intensively discussed the mechanism possibly causing conflicts between the CMT result and the IMI status/disease prognosis in Chapter 2. The results support the use of on-farm culture.

Severity evaluation and medical history review are both required for making proper treatment decisions. Concurrent with the identification of mastitis, the severity should be evaluated (4.2.4. Mastitis severity evaluation), and the treatment should be planned correspondingly (point 6). Parenteral antibiotics, anti-inflammatory, and fluid therapies should be given immediately to cows with systemic symptoms (Ruegg, 2017a). In Chapter 3, we demonstrated that high-parity cows with chronic infections are with most impaired productivity (Figure 13). In Chapter 4, we showed that severe mastitis led to decreases in the A/G ratio, possibly an effect of bacteremia. As a result, milk production was impaired in cows with decreased A/G ratios. It is interesting to know whether these therapies (parenteral antibiotics and other symptomatic therapies) alleviate the decrease in the A/G ratio and the impairment of productivity. Medical history review is important because cows with high parity and cows with chronic infections respond poorly to antibiotic therapies (point 7). Alternative to medical history review, stages of infection can be recognized by DSCC values. Considering the ineffectiveness of antibiotic therapy, alternative decisions, such as early dry-off or culling, should be considered. Future studies can focus on the effect of antibiotics on different stages of IMI (point 8), where the chronicity of mastitis can be judged objectively by DSCC values.

The prudent use of antibiotics in dry cow therapy is another important aspect of antibiotic stewardship in mastitis control, which we have discussed in Chapter 1. To summarize, we found that the success of SDCT depended on herd characteristics such as herd hygiene conditions, and the efficacy of teat sealant. We thus advise farmers to review herd characteristics before implementing SDCT, and on the other hand, we encourage the government to permit the use of internal teat sealants in Japan.

8. Conclusions

This thesis aimed to improve antibiotic stewardship in mastitis control by monitoring and modulating immunity. The research purpose of each study seems diverse, but their results indeed all contribute to the same goal, namely reducing antibiotic use in mastitis control without sacrificing animal health and economic benefits. We listed the major findings of this thesis and proposed some suggestions correspondingly:

(1) Consider herd characteristics, such as hygiene conditions, before implementing SDCT.

(2) Whenever possible, use (internal) teat sealants concurrently with SDCT implementation.

(3) Avoid making clinical decisions solely based on the CMT result.

(4) Consider using the CMT to rapidly screen chronically infected quarters.

(5) Dynamics of WBC and DSCC vary depending on the causative pathogen of mastitis.

(6) The A/G ratio dropped dramatically in cows with severe mastitis, which is possibly related to the impairment of milk production.

(7) WBC, DSCC, and the A/G ratio can facilitate pathogen identification and severity evaluation in mastitis, and the consequent treatment planning and prognosis evaluation.

(8) GAM successfully clarified the nonlinear interaction between SCS and DSCC.

(9) The negative effect of high SCS and low DSCC (chronic mastitis) on productivity was most pronounced in high-parity cows.

(10) Antibiotic therapy is ineffective for chronic infections in high-parity cows, thus early dry-off and culling should also be considered.

(11) Orally administrated chitosan was not beneficial for mastitis control, but other routes of administration (e.g., supplemented in teat dips or intramammary infusions) may be effective.

(12) Chapter 7. General Discussions presents other evidence-based approaches to the prudent use of antibiotics for mastitis control.

We believe that antibiotic stewardship in mastitis control can be further improved with continuing research efforts. A society that ensures both animal and human health is promising.

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Abstract

The major reason for giving dairy cows antibiotics is to prevent or treat mastitis, but among these therapies, some are probably unnecessary or inefficient. This thesis aimed to improve antibiotic stewardship in mastitis control. In Chapter 1, we investigated the usefulness of selective dry cow therapy (**SDCT**). SDCT reduced antibiotic use by 33%, but poor herd hygiene and shortened duration of external teat sealant adherence led to increased risks of dry-period intramammary infections (**IMI**). In Chapter 2, we investigated the dynamics of somatic cell score (**SCS**) and macrophage proportion (**MAC**; equivalent to 100 – differential somatic cell count [**DSCC**]) during mastitis and their effects on the California Mastitis Test (**CMT**). Results showed positive associations between SCS, MAC, and CMT scores. The high MAC during healing and chronic stages of mastitis may cause samples with relatively low SCS to show positive CMT reactions. In Chapter 3, we used generalized additive models (**GAM**) to estimate the nonlinear interaction between SCS and DSCC and its effect on milk production. Milk loss and composition change were severe in cows with high SCS and low DSCC. Especially for high-parity cows, milk yields were reduced by half. In Chapter 4, we investigated how the severity and causative pathogen of mastitis influence immune responses. We found a sharp drop in the albumin/globulin ratio after severe mastitis occurred, and that blood leukocyte count changed drastically in coliform or severe mastitis, slightly increased in streptococcal mastitis but remained stable in staphylococcal mastitis. There was a pathogen-dependent, non-linear relationship between DSCC and SCS. When cows recovering from *Streptococcus dysgalatiae* mastitis, DSCC decreased while SCS remained high, indicating a healing process with a large number of macrophages involved. In Chapter 5, we conducted two experiments to evaluate the immunomodulatory effects of chitosan on preventing dry period IMI and recurrent clinical mastitis. The oral administration of chitosan failed to modulate cows' immune systems or to benefit mastitis management. To summarize, to implement SDCT, sound herd hygiene and the concurrent use of teat sealants are required to prevent new IMI. Treatment of clinical mastitis should not be extended solely based on a positive CMT reaction. Instead, a milk culture test is necessary to determine the cure for mastitis. Since the immune response in mastitis differs depending on the causative pathogen and severity, treatment should be planned correspondingly. The damage of chronic IMI, characterized by high SCS and low DSCC, to milk yield is most significant in high-parity cows. Antibiotics may be ineffective in this case, early drying and culling should thus be considered. We believe antibiotic use in mastitis control can benefit from our findings but further research efforts are required.

要約

乳房炎は、酪農産業に最大の経済的損失をもたらす疾病の一つであり、従前から、抗菌性物質がその被害の低減、すなわち、乳房炎の治療や予防に用いられてきた。しかし、近年、生産性の向上と共に抗菌性物質の慎重使用が求められている。本研究は、乳房炎における免疫応答の監視と調節を通し、抗菌性物質の使用削減および生産性向上を目的として行われた。第 1 章では、日本における選択的乾乳期治療(SDCT)の有用性を検討した。SDCT を用いた場合、盲目的に行われている乾乳期治療と比べ、抗菌性物質の使用が約 33%減少した。SDCT の成功の要因として、新たな乾乳期乳腺感染を防ぐため、乾乳期の環境衛生管理と内部乳頭シーラントの注入の必要性が示された。また、治療の必要性を正確に判断するための検査法の精度の検討も必要と考えられた。そこで、第 2 章では、乳汁体細胞スコア(SCS)とマクロファージの割合が CMT 変法の結果に及ぼす影響を調査した。SCS とマクロファージの割合はどちらも CMT スコアと正の相関があった。乳房炎の回復期と慢性期において、マクロファージの割合が高くなるため、SCS が低くても CMT 変法の陽性反応を引き起こす可能性が明らかとなった。この結果から、単独に CMT 変法を用いて乳房炎の回復状況を判断するのは危険であり、また、慢性乳房炎の摘発には CMT 変法が有用であることが示された。慢性乳房炎が乳量への被害を引き起こす可能性が従前から指摘されてきたが、詳細に検証されていなかった。第 3 章では、体細胞数および種別体細胞数（DSCC；多形核好中球とリンパ球の割合；%）と乳量の関係を検討し、乳房炎の各ステージにおける乳量の変化を調査した。SCS と DSCC が両方とも高い（初期乳房炎）牛では乳量の減少はほとんど見られなかったが、SCS が高く DSCC が低い（慢性乳房炎）牛では乳量の損失が深刻であった。乳房炎のステージによって、乳牛の生産性が大きく変化し、乳房炎における免疫応答は生産性に深刻な影響を及ぼすことが明らかとなった。第 4 章では、乳房炎の重症度と原因菌が牛の免疫応答に与える影響を調査した。大腸菌群や重度の乳房炎では、白血球数の急激な上昇が見られたが、ブドウ球菌による乳房炎では変化が認められなかった。重度の乳房炎ではA/G比が急激に低下し、この低下は乳生産の低下にも関連していると考えられた。第3章と第4章により、効率的な乳生産を達成するには、重症度と慢性乳房炎をコントロールする必要がある。しかし、乳房炎が慢性期に推移すると、抗菌性物質の効果が低下すると言われ、抗菌性物質の適正使用を達成するには、免疫療法など抗菌性物質に代わる治療法が必要であると考えられる。そこで、第 5 章では、乾乳期乳腺感染と乳房炎再発予防に対するキトサンの免疫調節効果を評価した。しかし、キ

トサンによる乾乳期乳腺感染と乳房炎の発生抑制は認められず、免疫機能の調節効果も認められなかった。

以上の結果から、乳房炎の病態により、免疫応答が異なることが判明し、ＣＭＴ変法、SCS、DSCC および A/G 比などの動態から、生産性に問題のある慢性や重度の乳房炎の個体を特定できる可能性が示唆された。このことは、乳房炎対策における治療や淘汰方針の確立に有効である。また、適切な治療や淘汰などの牛群管理は抗菌性物質の使用削減および生産性の向上につながると考えられた。

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Appendix

Table S1. Estimated marginal means (± standard error) of somatic cell score, white blood cell count, and albumin/globulin ratio as a function of time in each pathogen or severity group.

	Somatic cell score						White blood cell count						Albumin/Globulin ratio					
Time point	D 0	D 3	D 5	D 7	D 14	D 21	D 0	D 3	D 5	D 7	D 14	D 21	D 0	D 3	D 5	D 7	D 14	D 21
Pathogens*																		
NG	10 ± 0.9	6 ± 0.9	4.9 ± 0.9	3.8 ± 0.9	2.3 ± 0.9	3.1 ± 0.9	72.4 ± 9.6	81.2 ± 9.6	78.7 ± 9.6	95.7 ± 9.6	87.9 ± 9.6	79.7 ± 9.7	1.17 ± 0.05	1.07 ± 0.05	1.08 ± 0.05	1.1 ± 0.05	1.12 ± 0.05	1.11 ± 0.05
CNS	9.7 ± 0.9	6.7 ± 0.9	5.9 ± 0.9	5.9 ± 0.9	4.8 ± 0.9	6 ± 0.9	83.9 ± 9.7	90.9 ± 9.7	85.8 ± 9.7	91.1 ± 9.7	89.2 ± 9.7	80.9 ± 9.6	1.02 ± 0.05	0.96 ± 0.05	0.93 ± 0.05	0.94 ± 0.05	1 ± 0.05	0.99 ± 0.05
GNR	9.4 ± 1.5	8.4 ± 1.5	8.3 ± 1.5	7.2 ± 1.5	6.3 ± 1.5	6.8 ± 1.5	19.1 ± 16.5	120.4 ± 16.5	107.3 ± 16.5	106.1 ± 16.5	94.4 ± 16.5	85.9 ± 16.6	1.21 ± 0.09	1.09 ± 0.09	1.1 ± 0.09	1.04 ± 0.09	0.99 ± 0.09	1.02 ± 0.09
OS	10.6 ± 0.8	7.7 ± 0.8	5.8 ± 0.8	5.4 ± 0.8	4.5 ± 0.8	3.8 ± 0.9	72.2 ± 9	72.8 ± 9.1	86.1 ± 9.1	101.1 ± 9.1	77.2 ± 9.2	70.7 ± 9.4	1.2 ± 0.05	1.13 ± 0.05	1.15 ± 0.05	1.15 ± 0.05	1.09 ± 0.05	1.13 ± 0.05
SA	10.2 ± 1.1	8.9 ± 1	7.5 ± 1	6.4 ± 0.9	4.7 ± 0.9	4.5 ± 0.9	78.7 ± 10.5	82.5 ± 10.5	84.8 ± 10.4	90.2 ± 10.4	78.2 ± 10.2	73 ± 10.1	1.09 ± 0.06	0.99 ± 0.06	0.99 ± 0.06	0.97 ± 0.06	1 ± 0.06	1.05 ± 0.06
SD	11.2 ± 1.5	7.6 ± 1.2	6.4 ± 1.2	5.2 ± 1.2	3.5 ± 1.1	3.6 ± 1.1	52.6 ± 12.8	58.2 ± 12.7	81.1 ± 12.7	88.4 ± 12.6	78.6 ± 12.5	78.1 ± 12.4	1.05 ± 0.07	1 ± 0.07	1.02 ± 0.07	1.05 ± 0.07	1.13 ± 0.07	1.17 ± 0.07
SU	11.3 ± 0.6	8.4 ± 0.6	7 ± 0.6	6.3 ± 0.6	5.2 ± 0.6	5.8 ± 0.6	82.8 ± 6.7	78.4 ± 6.7	96.8 ± 6.7	115.6 ± 6.7	115.3 ± 6.7	95.5 ± 6.7	1.05 ± 0.04	0.95 ± 0.04	0.97 ± 0.04	0.96 ± 0.04	1 ± 0.04	0.98 ± 0.04
Severity																		
Mild	9.9 ± 0.5	7 ± 0.5	6.4 ± 0.5	5.6 ± 0.5	4.3 ± 0.5	3.6 ± 0.5	66.2 ± 5.7	83.6 ± 5.7	85.9 ± 5.7	92.2 ± 5.7	87 ± 5.6	81.9 ± 5.6	1.11 ± 0.03	1.08 ± 0.03	1.1 ± 0.03	1.06 ± 0.03	1.05 ± 0.03	1.08 ± 0.03
Moderate	10.1 ± 0.8	7 ± 0.8	5.4 ± 0.8	4.6 ± 0.8	3.4 ± 0.8	5.2 ± 0.8	79.4 ± 8.4	78.2 ± 8.4	84.3 ± 8.4	90.8 ± 8.4	75.1 ± 8.4	74.3 ± 8.4	1.09 ± 0.05	1.07 ± 0.05	1.08 ± 0.05	1.11 ± 0.05	1.15 ± 0.05	1.14 ± 0.05
Severe	11 ± 1	9 ± 0.8	7.8 ± 0.8	7 ± 0.8	5.7 ± 0.8	5.6 ± 0.8	52.3 ± 8.7	88.6 ± 8.7	95.8 ± 8.6	111.9 ± 8.6	104 ± 8.6	85.4 ± 8.6	1.13 ± 0.05	0.93 ± 0.05	0.94 ± 0.05	0.91 ± 0.05	0.95 ± 0.05	0.98 ± 0.05

* NG = no significant growth; CNS = coagulase-negative staphylococci; GNR = gram-negative rods; OS = other streptococci; SA = *Staphylococcus aureus*; SD = *Streptococcus dysgalactiae*; SU = *Streptococcus uberis*.

Table S2. Summary of generalized additive models for analyzing the impacts of somatic cell score (SCS), differential somatic cell count (DSCC), days in milk (DIM), and season by parity of cows on milk production.

	Milk yield		Lactose		Fat		Protein	
Terms [a]	EDF (Ref.df) [b]	F value [c]	EDF (Ref.df)	F value	EDF (Ref.df)	F value	EDF (Ref.df)	F value
f(SCS, parity 1)	5.16 (6.09)	27.1	6.78 (7.64)	98.9	4.45 (5.35)	25.1	1 (1)	97.2
f(SCS, parity 2)	3.43 (4.23)	74.7	6.27 (7.21)	96.4	3.83 (4.7)	39.4	1 (1.01)	134.4
f(SCS, parity 3+)	4.53 (5.58)	107.3	5.8 (6.83)	140	4.48 (5.48)	69.1	6.29 (7.29)	45.9
f(DSCC, parity 1)	4.48 (5.55)	43.3	7.22 (8.03)	44.1	4.15 (5.14)	23.8	5.56 (6.61)	32
f(DSCC, parity 2)	5.83 (6.93)	40.7	6.44 (7.48)	47.5	3.01 (3.83)	40.9	7.17 (8.1)	39.6
f(DSCC, parity 3+)	6.1 (7.18)	139.5	8.36 (8.74)	323.5	1 (1.01)	244.8	4.47 (5.59)	136.2
f(SCS×DSCC, parity 1)	2.89 (3.24)	13.7	7.28 (8.52)	74.6	2.8 (3.12)	12.8	5.61 (7.07)	8.2
f(SCS×DSCC, parity 2)	6.45 (7.85)	29.8	7.71 (8.98)	58.3	1 (1)	27.4	4.86 (6.14)	8.7
f(SCS×DSCC, parity 3+)	9.53 (10.81)	42	12.89 (13.53)	165.5	9.97 (11.41)	15	9.26 (10.59)	15.9
f(DIM, parity 1)	8.5 (8.93)	213.6	8.88 (9)	118.2	8.76 (8.98)	235.7	8.97 (9)	1222
f(DIM, parity 2)	8.86 (8.99)	1389.4	8.72 (8.98)	171.7	8.9 (9)	306.3	8.98 (9)	1662.2
f(DIM, parity 3+)	8.93 (9)	3404.7	8.85 (8.99)	602.9	8.93 (9)	764.8	8.99 (9)	3643.2
f(DoY, parity 1)	4.61 (8)	49.9	5 (8)	80.1	6.09 (8)	450.2	7.6 (8)	1256
f(DoY, parity 2)	6.6 (8)	89.1	5.63 (8)	108.5	6.41 (8)	747.8	7.75 (8)	2358.5
f(DoY, parity 3+)	6.81 (8)	246.8	6.6 (8)	334.4	6.82 (8)	1237.8	7.86 (8)	4928.7
γ(Cow)	6872.39 (7483)	23.2	6921.04 (7483)	18.1	6832.85 (7483)	16	7030.43 (7483)	27.1
SD	5.34		0.13		0.43		0.24	
γ(Herd)	67.2 (71)	4733.1	59.88 (70)	1433.1	64.19 (70)	2281.1	64.84 (70)	5232.5
SD	2.85		0.03		0.16		0.09	
Adjusted R^2	0.70		0.65		0.50		0.76	

[a] f(SCS), f(DSCC), f(SCS×DSCC), f(DIM), and f(DoY) are smooth functions for SCS, DSCC (differential SCC), the interaction of SCS and DSCC, DIM and DoY (day of the year), respectively; γ(Cow) and γ(Herd) are random effects for cow and herd, and rows named 'SD' provide their estimated standard deviations.
[b] EDF = effective degrees of freedom; Ref.df = reference degree of freedom.
[c] Significance of smooth functions and random effects was determined as described in Wood (2013a) and Wood (2013b), respectively; all p-values < 1×10^{-6}.

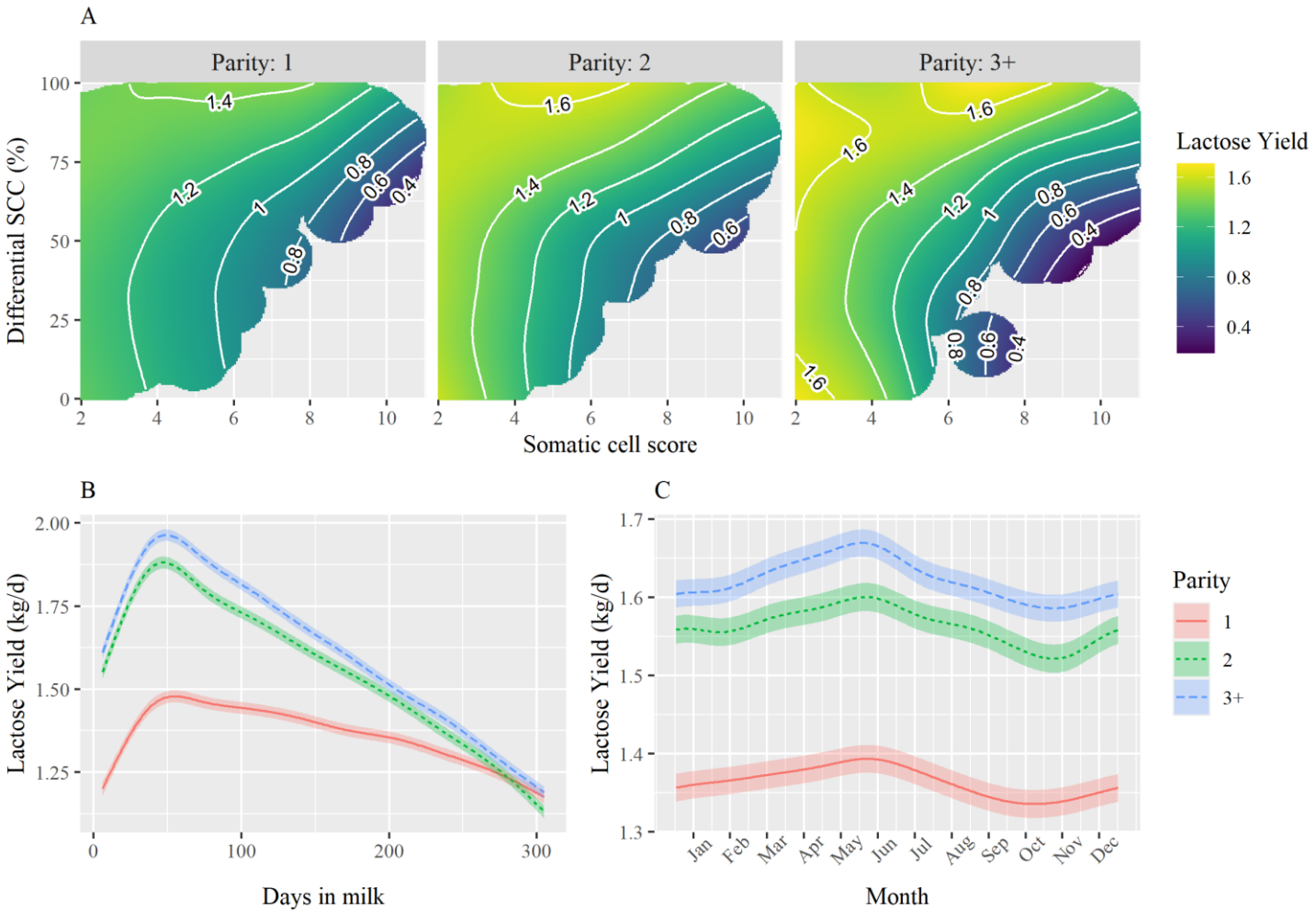

Figure S1. Predictions (± standard error) of lactose yield (kg/d) as functions of SCS/Differential somatic cell count (DSCC; A), DIM (B), and day of the year (C) by parity of cows.

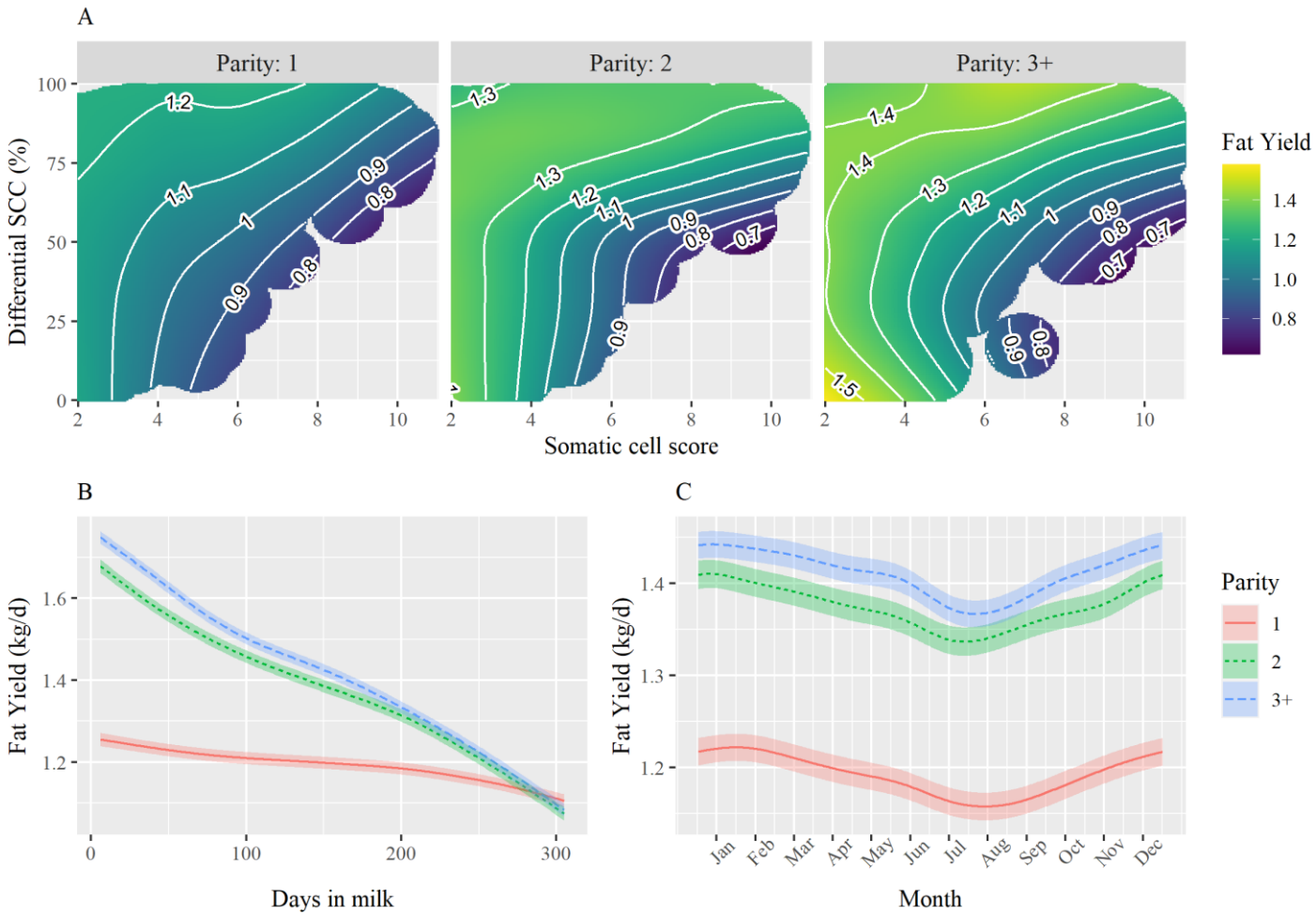

Figure S2. Predictions (± standard error) of fat yield (kg/d) as functions of SCS/Differential somatic cell count (DSCC; A), DIM (B), and day of the year (C) by parity of cows.

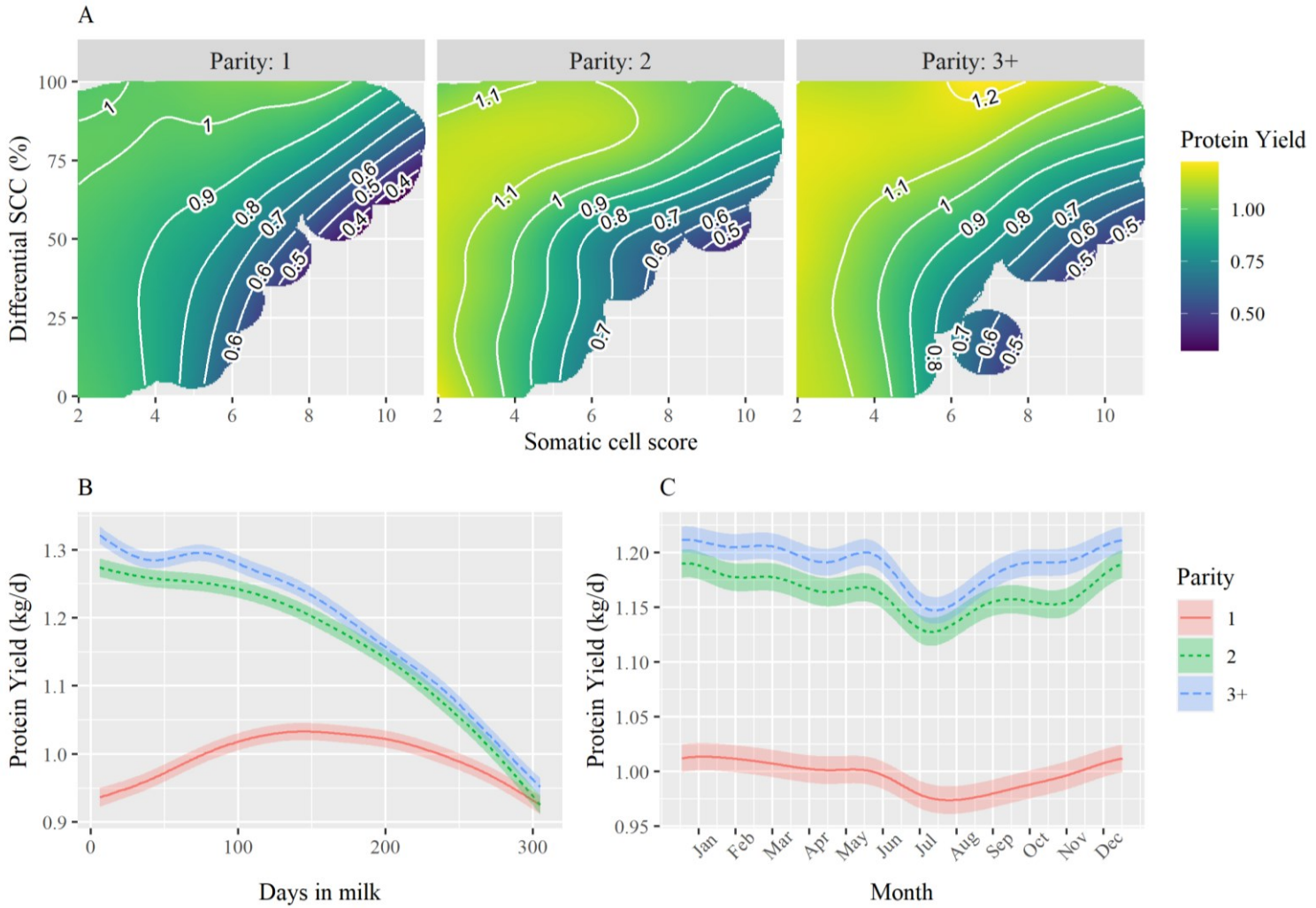

Figure S3. Predictions (± standard error) of protein yield (kg/d) as functions of SCS/Differential somatic cell count (DSCC; A), DIM (B), and day of the year (C) by parity of cows.