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Genetics of Animal Health and Disease in Livestock

Edited by
Bianca Castiglioni

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Bianca Castiglioni
National Research Council of Italy
Italy

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About the Editor

Bianca Castiglioni is a senior research scientist at the National Research Council (CNR) of Italy, Institute of agricultural biology and biotechnology. She has expertise in livestock genomics, transcriptomics, epigenetics and metagenomics. She has participated as scientific coordinator and WP or Task leader in several European and national research projects. She published over 100 peer-reviewed papers (WoS Researcher ID: G-9856-2013). Her activities span from the study of genes and mechanisms that control the livestock production traits to the study of animal microbiome, with special focus on animal health, and the traceability and food safety of animal products.

Preface to "Genetics of Animal Health and Disease in Livestock"

Livestock diseases adversely affect animal production throughout the world. Although there are some examples of genetic resistance to disease in livestock, disentangling the genetic effects is a compelling task. Indeed, in most cases, animals are not resistant to a disease, but they vary in their susceptibility to the disease agents. Therefore, it is still difficult to demonstrate the potential of the genetic approach and to be able to identify genetic variation that accounts for disease resistance and/or tolerance.

An additional issue is that resistance is measurable only in the presence of the disease-causing pathogen. Moreover, for most livestock, the genes and products of the innate and adaptive immune system are not fully known or functionally annotated. Many immune-related genes exist as multiple copies within an individual animal, and their number, sequence, and regulation are difficult to characterize. The lack of methods to follow specific genes or to functionally measure outputs at a cellular or animal level reduces our ability to fill the knowledge gaps. Many diseases are complex, and their causative pathogens are unknown. Furthermore, the influence of a healthy microbiome on pathogen virulence is only now beginning to be understood.

Nevertheless, the role of genetics in improving animal health will become increasingly important as the focus on tackling antimicrobial drug resistance increases. This research will result in greatly reduced direct and indirect costs associated with animal disease, maintenance of a secure, and safe food supply, improved animal welfare, production efficiency, and resilience to environmental changes, and reductions in antimicrobial use and improved vaccines or other measures that can mitigate or prevent existing, new, and re-emerging infectious pathogens.

Bianca Castiglioni
Editor

The Role of Innate Immune Response and Microbiome in Resilience of Dairy Cattle to Disease: The Mastitis Model

Valerio Bronzo [1,†]**, Vincenzo Lopreiato** [2,†]**, Federica Riva** [1]**, Massimo Amadori** [3,*]**, Giulio Curone** [1]**, Maria Filippa Addis** [1]**, Paola Cremonesi** [4]**, Paolo Moroni** [1,5]**, Erminio Trevisi** [2] **and Bianca Castiglioni** [4]

[1] Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, 26900 Lodi, Italy; valerio.bronzo@unimi.it (V.B.); federica.riva@unimi.it (F.R.); giulio.curone@unimi.it (G.C.); filippa.addis@unimi.it (M.F.A.); pm389@cornell.edu (P.M.)

[2] Dipartimento di Scienze animali, Alimentazione e Nutrizione, Facoltà di Agraria, Scienze Alimentari e Ambientali, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy; vincenzo.lopreiato@unicatt.it (V.L.); erminio.trevisi@unicatt.it (E.T.)

[3] Rete Nazionale di Immunologia Veterinaria, 25125 Brescia, Italy

[4] Institute of Biology and Biotechnology in Agriculture, National Research Council (CNR), 26900 Lodi, Italy; paola.cremonesi@ibba.cnr.it (P.C.); bianca.castiglioni@ibba.cnr.it (B.C.)

[5] Quality Milk Production Services, Animal Health Diagnostic Center, Cornell University, 240 Farrier Road, Ithaca, NY 14850, USA

[*] Correspondence: m_amadori@fastwebnet.it; Tel.: +39-347-462-4837

[†] These authors contributed equally to this work.

Simple Summary: A major concern for the development of livestock activities is represented by the gradual reduction of antibiotic usage in farm animals, which may disturb the fragile balance between animal health and production. Therefore, it is necessary to maintain the immunocompetence of farm animals within the structure of this new trend toward reduced drug usage. High-yielding dairy cattle often experience more disease prevalence associated with short life expectancy and reduced environmental fitness. These signs of immunosuppression can be linked to metabolic changes observed around calving, which confirms the crucial link between immunity and milk production levels. The immunocompetence of these animals should be re-appraised and new disease control strategies should be based on creating a more efficient immune system. This review summarizes the dairy cow's metabolic response to stress and what role the innate immune system and microbiome play. The review also discusses how new approaches to animal health based on specific intervention at dry-off and in the first weeks after calving are needed as the relevant stressors are pivotal to disease occurrence.

Abstract: Animal health is affected by many factors such as metabolic stress, the immune system, and epidemiological features that interconnect. The immune system has evolved along with the phylogenetic evolution as a highly refined sensing and response system, poised to react against diverse infectious and non-infectious stressors for better survival and adaptation. It is now known that high genetic merit for milk yield is correlated with a defective control of the inflammatory response, underlying the occurrence of several production diseases. This is evident in the mastitis model where high-yielding dairy cows show high disease prevalence of the mammary gland with reduced effectiveness of the innate immune system and poor control over the inflammatory response to microbial agents. There is growing evidence of epigenetic effects on innate immunity genes underlying the response to common microbial agents. The aforementioned agents, along with other non-infectious stressors, can give rise to abnormal activation of the innate immune system, underlying serious disease conditions, and affecting milk yield. Furthermore, the microbiome also plays a role in

shaping immune functions and disease resistance as a whole. Accordingly, proper modulation of the microbiome can be pivotal to successful disease control strategies. These strategies can benefit from a fundamental re-appraisal of native cattle breeds as models of disease resistance based on successful coping of both infectious and non-infectious stressors.

Keywords: dairy cattle diseases; innate immune system; metabolic stress; microbiome

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1. Introduction

In the last decade, ensuring animal health and welfare with the progressive reduction of drug usage has become a key issue for farmers as well as consumers worldwide. Dairy cattle diseases cause morbidity, mortality, and often decreased profitability for farmers, but antibiotics are now used more responsibly for treatment and control of these diseases [1,2]. Due to the known difficulties in developing novel antibiotic classes, the prudent use of the same products must be targeted. Public concerns have been raised regarding animal disease control, how animals for human consumption are treated with drugs, and the environment in which these animals are raised.

Alternative methods for preventing animal diseases are needed. One idea is through the modulation of the immune system. It has been documented that it is rare for every animal exposed to the same infection to develop symptoms that are clinical; furthermore, different breeds exhibit different traits related to disease [3–6]. It is difficult to explain why some animals in the same group develop varying degrees of the same illness. Genetics, the immune system, management, age, and other factors influence the health of an animal [7,8]. More variables play a role in animal health, making it difficult to pinpoint any single factor (Figure 1).
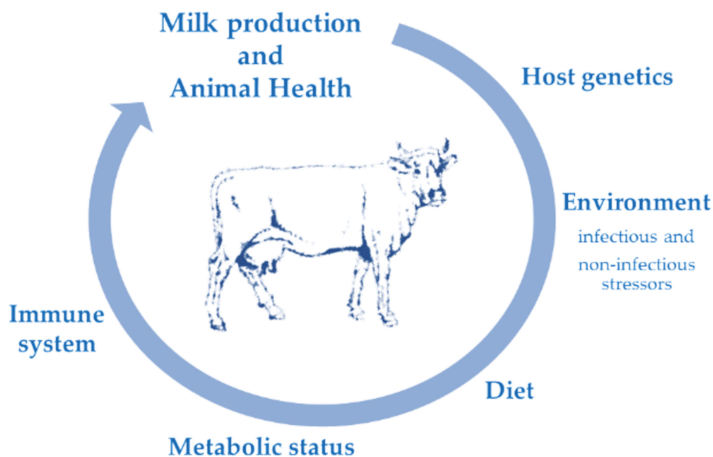
Figure 1. Milk production and animal health are influenced by and correlated to many factors such as genetics, environment stressors, diet, metabolic *status,* and the immunological system that all interact.

During the periparturient period, dairy cows undergo a number of metabolic-, endocrine-, physiologic-, and immune-related changes, rendering cows more susceptible to disease and less efficient. Health problems occurring before and after calving lead to severe negative effects on the productive efficiency of lactating cows. Reductions in the cow's production and increased mortality rates are associated with periparturient health disorders. The costs of antimicrobial drugs, vaccines, labor, and preventive measures must be taken into consideration. During this period, immune system efficiency together with good liver functionality as well as the capability of cows to minimize the

gap between nutrient intake (increasing dry matter intake)and nutrient output (milk production) determines the disease resistance capability of the animal [9]. The most important roles of the immune system are to prevent microbial diffusion and to reduce or eliminate infections.

2. Immunocompetence of High Genetic Merit Dairy Cattle and Disease Control Strategies

The immune system has developed along with the phylogenetic evolution as a refined sensing and response system, aimed at neutralizing all the possible noxa affecting or potentially affecting the host's homeostasis [10]. The system has evolved from the recognition of conserved patterns of microbial pathogens to having great potential for recognizing fine specificities of microbial agents. Adaptive immunity rose with phylogenesis approximately 500 million years ago in jawed fish and proceeded to mammals as a result of selective pressures derived from the increased complexity of organs and apparata [11]. As a result, mammals avail themselves of innate immune mechanisms to deal with a plethora of infectious and non-infectious stressors. Adaptive mechanisms (antibodies and antigen-specific T lymphocytes) are used whenever the primary non-adaptive mechanisms fail to control a challenge to homeostasis [12].

Ruminants are no exception to this general rule. Domestication of ruminants began some 10,000 years ago [13] and has since played a vital part in the economic and social advancement of mankind. It can be argued that domestication was an advantage to ruminants in terms of easier access to feeding resources and protection against climatic challenges [14]. The relationship with mankind became complex with the advent of intensive farming and genetic selection for higher production levels. This relationship gave rise to a substantial worsening of animal welfare, and the historical relationship between domestication and welfare has become a bell-shaped dose-response curve [14]. We must find credible solutions to the major problem of ethics and the sustainability of farming activities. These solutions must take into consideration the diverging needs of environmental constraints and high production levels brought about by the increasing world population and its growing demand for animal products.

In this conceptual framework, a major concern for the development of farming activities is represented by the gradual reduction of antibiotic usage in farm animals, which may disturb the fragile balance between animal health and production. It is necessary to stimulate the immunocompetence of farm animals within the structure of this new trend toward reduced drug usage. High-yielding dairy cattle often experience high disease prevalence associated with short life expectancy [15]. Most importantly, they show distinct signs of reduced environmental fitness, shown as coping poorly with both infectious and non-infectious stressors, as observed, e.g., in the hot summer season of 2003 [16]. The immunocompetence of these animals should be re-evaluated and new disease control strategies should be based on increasing the efficiency of the immune system.

2.1. The Concept of Immunocompetence

Immunocompetence is the ability of the body to produce a normal immune response following exposure to an antigen. This process involves complex genetic traits [17]. To produce an effective immune response, different cells and genes are necessary along with the ability of innate and adaptive immunities to coordinate. Danger describes the force that dictates the reaction profile of the immune system [18] for both infectious and non-infectious stressors [10]. Microbial infections entail some overlapping signals triggered by both PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns). Within this operational framework, the innate immune system begins to destroy the stressors affecting the host's homeostasis. Innate immunity must not cause substantial tissue damage as a result of a disproportionate inflammatory response. If pathogens persist after the innate response, adaptive immunity is induced to control the ongoing infection. The B and T cell receptor activity directed against specific antigens is the main component of immunocompetence. Innate immunity also plays an important part in the recruitment and orientation of receptor responses. These are used sparingly by the host as the response of secondary antibodies and immunological

memory benefits represent high energetic cost [19]. Immunocompetence depends on factors such as a diet with adequate protein, energy, and multiple micronutrients. Immunocompetence presents sexual dimorphism where females present a general increased immunoreactivity compared to males [20]. Sexual dimorphism is due to genetic differences (several immune genes are in the X chromosome) and hormonal selective pressure. To achieve good reproductive fitness, females are selected to have a long life span due to a stronger immune system, whereas males need to maximize sexual mating early in life without investing in the immune system [21]. The immunocompetence of an individual undergoes some changes during the lifespan. Calves can adequately react to environmental pathogens through the transfer of colostrum immunoglobulins. Subsequent immunodeficiency or immune-compromised status in calfhood can occur following infections, drug treatments, and prolonged environmental stressful conditions. It is possible to enhance the immune response with three different general approaches: vaccination, passive immunization, and immunomodulation.

Evidence of reduced immunocompetence of high-yielding dairy cattle derives from epidemiological data and experimental studies [22–24]. As for the epidemiological data, the dramatic improvement of milk quality in terms of somatic cell counts was paired with an impressive increase in the milk yield of Holstein cows [25,26]. The impact of these performances on animal welfare and health has been considerable. In this respect, as the genetic ability to produce milk increases, more cows develop production diseases; the associations between increased milk production and increased risk of production diseases, as well as reduced fertility, are clearly documented, but less is known about the biological mechanisms behind these relationships [22]. Cows alive in the North-Eastern part of the USA at 48 months of age decreased from 80% in 1957 to 13% in 2002; on the same farms and in the same time period, the mean calving interval increased from 13 to 15.5 months [22]. As for experimental studies, high-yielding dairy cattle showed distinct signs of immunosuppression, which can be linked to the dramatic metabolic changes observed around calving [23,24]. Metabolic stresses associated with lactation influence the composition of peripheral blood mononuclear cell populations, as opposed to cows submitted to mastectomy [27], which confirms the crucial link between immunity and milk production levels.

2.2. Metabolic Stress and the Innate Immune System

Innate and adaptive immune mechanisms are complementary and synergistic. This operational framework has been jeopardized by genetic selection for high milk yield, which led to reduced serum concentrations of lysozyme compared with the other cattle breeds [28]. Lysozyme plays a fundamental antimicrobial role and is part of important regulatory circuits of the inflammatory response [29]. Metabolic priority for offspring survival demands the maintenance of milk yield to the detriment of other functions [30] as the fetus and placenta have the same priority during pregnancy as the brain and Coagulase-negative Staphylococci (CNS) [12]. The high levels of milk yield exceed the potential of dry matter intake and the subsequent negative energy balance gives rise to metabolic stress, shown as a disequilibrium in the homeostasis of a living organism as a result of anomalous utilization of nutrients [31]. The unsatisfactory profiles of the immune response in high-yielding dairy cattle can be either primary (i.e., associated with the genetic selection for high milk/fat/protein yields), or secondary to metabolic stress (Figure 2). Two main signaling pathways monitor nutrient availability, control metabolic stress responses, and exert a central role in modulating innate immunity: the mTOR-(mammalian target of rapamycin) and eIF2α (eukaryotic initiation factor-2α)-dependent signal transduction cascades [31]. The most important regulators of mTOR and eIF2α are cellular energy status (ATP/AMP ratio), amino acid availability, oxygen tension, and oxidative stress. A direct link between metabolism and innate immunity is the binding of free fatty acids to Toll-like Receptors (TLR) 4, which is implicated in the development of inflammation in states of hyperlipidemia; the subsequent cascade of signaling events is very similar to that observed after exposure to microbial stressors [32].
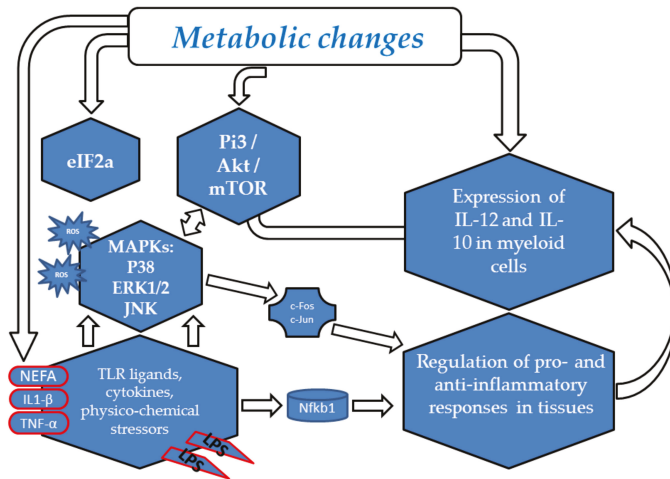
Figure 2. Metabolic stress is perceived by the innate immune system. The Pi3/Akt/mTOR- and eIF2α-dependent signal transduction cascades are the main signaling pathways monitoring nutrient availability and controlling metabolic stress responses. Toll-like receptors (TLRs) are expressed on innate immune cells, such as neutrophils, macrophages, and dendritic cells, and respond to the membrane components of Gram-positive or Gram-negative bacteria and to saturated free fatty acids mainly included in the NEFA (Non-esterified Fatty Acid) that are released by adipose tissue during status of negative energy balance. TLRs provoke rapid activation of innate immunity by inducing production of proinflammatory cytokines and upregulation of costimulatory molecules by both MAPK activation, which in turn activates c-Fos and c-Jun, and NF-kappa B activation through a MyD88-independent pathway. In addition, interleukin-1β (IL1-β) and tumor necrosis factor alpha (TNF-α), as critical cytokines, can induce a wide range of intracellular signal pathways as well as inflammation and immunity as nearly all cells express the respective receptors.

Metabolic stress in the framework of Negative Energy Balance (NEB) should be seen as a crucial element underlying the occurrence of diverse disease conditions of dairy cows. In this respect, the roles of the p38a MAPK/mTOR and Pi3/Akt/mTOR signaling pathways are pivotal to regulating the balance between pro and anti-inflammatory cytokines in tissues in response to environmental stress [33]. This confirms the central role of the innate immune system in response to environmental stressors. Additionally, the potent regulatory actions of the immune system might be conveniently exploited in the future toward vaccines for metabolic diseases, like type 2 diabetes mellitus [34], which is reminiscent of insulin resistance in dairy cows.

2.3. The Influence of the Microbiome on the Immune System of Dairy Cattle

The microbiome contributes to the architecture and function of tissues, influences the host energy metabolism, and plays an important role in the balance between health and disease. The microbiota and its metabolites are crucial in the maintenance of host homeostasis [35]. At the beginning of the host's life, the composition of the microbiota evolves into a healthy and viable community that strengthens itself and the host. Early development disturbances such as antibiotics, infections, or poor feed may lead to greater disease susceptibility [36].

Next-generation sequencing has enabled several groups to investigate microbiome influences on disease development. Bovine microbial communities have been described across many anatomical sites [37–40], including the mammary gland [41–43] and the uterus [44–47]. The composition of the bovine microbiome can affect the health [48–50] and performance of animals [51]. In ruminants such as dairy cows, the intestinal community of calves changes rapidly after birth and constantly during the

first 12 weeks of life. Bacteroides–Prevotella and Clostridium coccoides–Eubacterium rectale species dominate the calf microbiota in this period [52]. After weaning, the microbiota must also compensate for the change in diet. This is a critical period when several events may affect the microbiota and health of the animal. During weaning Bacteroidetes decreased (remaining the dominant phyla) while Proteobacteria and Firmicutes increased [38]. These changes in microbial community composition, in part, are due to host physiological changes but are also likely due to the introduction of solid feeds because diet is a large driver of microbial community composition and modulation [38]. Weaning in dairy calves elicited an immune response in the lower gastrointestinal tract, but adding solid feed in addition to milk replacer resulted in changes to the immune response as well as gut bacteria [53]. Increased solid feed resulted in an increased total amount of bacteria present in the gastrointestinal tract. Weaning age and method of weaning can also change rumen or gastrointestinal tract microbiome establishment and community structure [54]. Weaning strategies can influence the ability of a calf to adapt to the dietary shift and can influence the severity of production losses. The effects of various feeding strategies and age as determining factors in the extent of microbial shifts in the rumen and feces during weaning still need to be studied.

Studies indicate that bovine gut microbiome can change immune responses [55]. These changes may be through direct and indirect mechanisms, such as through bacterial secretion of antimicrobial compounds or through influencing the expression of genes underlying host mucosal immune responses [56]. While much research on commensal microbes modulating host immune responses exists, studies investigating pathogen and pathobiont impact on host health are few [55,57]. The process of antimicrobial resistance is a relevant public health issue and, although antimicrobial use in human medicine arguably contributes to antimicrobial resistance much more than corresponding use in the livestock sector, it is important that farms proactively apply principles of prudent and judicious use of antimicrobials [58]. Farm animals and humans alike are threatened by this development. To promote early life wellness, pre- and probiotics are used to establish and restore microbiota health. Several studies demonstrated that probiotics and prebiotics achieved a positive balance in the gastrointestinal microbiota of cattle [59]. The functional interactions between gut microbes and relationships between microbes and host cells have yet to be fully investigated. Other pre-existent factors may play a crucial role, including host genetics, environmental conditions, and the resident, established microbiota [36].

3. Immunocompetence in Bovine Mastitis

Immunocompetence in the mammary gland is the outcome of a complex, coordinated network of anatomical, humoral, and cellular factors that are both specific and non-specific [60]. Immunocompetence can vary during lactation, showing depression in the peripartum period due to the hormonal and metabolic stress of calving and milk production [61]. The teat duct epithelium produces keratin that physically traps bacteria and blocks their migration to the mammary cistern. Keratin also has antimicrobial activity due to some bacteriostatic fatty acids (lauric, myristic, palmitoleic, and linoleic), as well as fibrous proteins that bind and damage the microorganism cell wall [62]. The duct epithelium can also modulate the expression of Pathogen Recognition Receptors (PRRs), such as Toll-like Receptors (TLRs), that after binding to PAMPs lead to the expression of inflammatory genes, and the release of cytokines (IL-1beta, TNF-alpha, IL-8) and acute phase proteins (haptoglobin, HP) but also antimicrobial peptides (pentraxin 3 PTX3, lipopolysaccharide-binding protein LBP) [6,63,64]. The mammary gland displays both innate and adaptive immune mechanisms that collaborate to defend the tissue against microbial invasions. The innate immune system is able to recognize the pathogens through TLRs and trigger an inflammatory response to kill them by phagocytosis (facilitated by the expression of surface receptors for Ig and complement proteins), or expression of antimicrobial molecules (lactoferrin, transferrin, lysozyme, defensins, cathelicidin, myeloperoxidases, complement system). Neutrophils respond with the up-regulation of adhesion molecules (L-selectin and beta2-integrin) to reach the damaged tissue [65]. Macrophages can perform phagocytosis, similar to

neutrophils, but also act as antigen-presenting cells by exposing to lymphocytes the microbial antigens associated with Major Histocompatibility Complex MHC class II [66]. In the mammary gland of dairy cattle, the prevalence of lymphocyte populations (Th1, Th2, Th17, Treg, T cytotoxic, gamma-delta T cells, B cells) varies during lactation and the role of lymphocytes and natural killer cells is not fully understood under both health and disease conditions [67–69]. The main lymphocyte subset found in the bovine mammary gland is gamma-delta T cells [70]. During the peripartum period, lymphocytes assume a regulatory or suppressor phenotype, whereas at mid-lactation they shift to a cytotoxic phenotype and produce Interferon IFN-gamma [71]. In general, lymphocytes of the mammary gland are less responsive compared to the circulating ones; this could be partly due to a lower efficiency in antigen presentation in this area [61,67]. B cells do not change in number during the lactation stages [65]. B cells of the mammary gland serve as antigen-presenting cells but can also differentiate to plasma cells that produce antibodies (Ig) of four main isotypes: immunoglobulin G (IgG)1, IgG2, IgA, and IgM [72]. The concentrations of Ig in milk vary greatly during lactation. The activation of Th cells induces the expression of different cytokine repertoires that in turn influences the activity of T cytotoxic, B cells, macrophages, neutrophils, and Natural Killer NK cells [72]. The contribution to the immune response of mammary epithelial cells (MEC) is particularly important. MEC express PRRs; once they recognize microbial components, they can activate an innate immune response by expressing pro-inflammatory mediators (IL-1beta, TNF-alpha, IL-6, IL-8, acute phase proteins) and antimicrobial molecules (defensins, cathelicidin, calprotectin) [73]. Depending on the cytokine pattern in the inflamed mammary microenvironment, different responses can be started: Th1 (favoring cell-mediated responses), Th2 (favoring humoral responses), or Th17 (favoring activation and functions of granulocytes). This polarization drives the expression of specific molecules with different biological effects [74].

Decreased immunocompetence in the mammary gland, for instance in the peripartum period, predisposes cows to develop mastitis. In order to ameliorate the immune reactivity of the mammary gland, different strategies have been investigated. Diets can be fortified with minerals and micronutrients. These potentiate the activity of immune cells (such as Se and Vit E) and act as antioxidants (Vit A, Zn, Cu) to protect against the toxic effects of Reactive Oxygen Species (ROS) [75,76]. Another strategy consists in the administration of recombinant cytokines; for example, Granulocyte-Colony Stimulating Factor (G-CSF) results efficacious in the recruitment and differentiation from bone marrow reserves of a high number of polymorphonuclear leukocyte (PMN), as well as in the enhancement of their action at calving, which underlies a major reduction of mastitis prevalence [77]. Finally, vaccines were developed to prevent the insurgence of new infections and reduce the tissue damage induced by pathogens. The controversial results of different types of vaccines have cast doubts on this strategy [78]. It can be argued that merely using vaccines shows poor efficacy, which suggests their association with sanitary measures such as milking hygiene, teat dipping, confinement, and culling of chronically infected cows [79].

3.1. Epigenetics and Trained Immunity: Implications for the Control of Mastitis

Recently, evidence demonstrated that the innate immune system has the capability to develop "memory", once attributed only to adaptive immunity. Innate immune memory is known as "trained immunity". Studies show that the innate immune system can modify its response after the first encounter with both infectious and non-infectious stressors [80]. This is reminiscent of the cross-protection observed following different pathogen infections, described previously [81]. The peculiarities of trained immunity consist of the involvement of specific cell types (monocytes, macrophages, NK cells, innate lymphoid cells, ILC) and in epigenetic mechanisms that induce long-lasting adaptation. As a result, these cells remain highly responsive versus non-specific insults after the first recognition of a stressor [80]. In trained immunity, innate immune cells undergo epigenetic re-arrangement, leading to gene- or locus-specific changes in their chromatin profiles after a previous stimulation [80]. The major epigenetic mechanism active in trained immunity is histone modification with chromatin

reconfiguration but other processes such as DNA methylation, modulation of microRNA, and long noncoding RNA expression seem to play a role [82]. This provides a transcriptional profile that modifies signaling and metabolism of innate immune cells [80]. Evidence of trained immunity is also described in dairy cows. Mammary epithelial cells stimulated with either Lipopolysaccharides (LPS) or Pam2CSK4 (two TLR ligands) develop endotoxin tolerance by epigenetic mechanisms; this response protects mammary tissues by enhancing the expression of beta-defensins and membrane protectors (Serum Amyloid A3 SAA3, Transglutaminase 3 TGM3) and down-regulating the expression of proinflammatory cytokines (TNF-alpha, IL-1beta) at a subsequent challenge [83]. This suggests that priming epithelial mammary cells with PAMPs induces a protective status by dampening exaggerated inflammation and enhancing bactericidal activity in a subsequent infection.

Trained immunity needs to be dissected to better understand its relevant subtended mechanisms. The identification of epigenetic changes increasing immunocompetence should be conducive to a promising mastitis control strategy. Furthermore, trained immunity in the mammary gland could be helpful in the design of efficacious vaccines combining both memory of adaptive immunity and "trained" innate responses.

3.2. The Milk Microbiome and the Mammary Gland Health

The bioactive molecules in milk play an integral role in training the immune system of recently born animals. The intestinal equilibrium of newborns is maintained by a synergistic relationship between antimicrobial peptides, lactoferrins, lysozymes immunoglobulins, and oligosaccharides [84,85]. The nutrient-rich ecosystem of milk allows growth of a wide range of microorganisms [41,86]. These microorganisms may contribute to the development of neonate gut microbiota, interact with the immune system, and regulate inflammatory responses and infection susceptibility [87].

Inflammation of the mammary gland, mastitis, is a response to intramammary infection, metabolic disorders, and trauma. Intramammary infection often occurs from the passage of pathogens beyond the teat canal [62], activating immune responses. Several factors can trigger mammary gland defenses against pathogens [88]. Commensal microbiota residing in the udder [41] may govern mastitis susceptibility. Bacteriocins produced by certain non-aureus Staphylococci (NAS) and *Corynebacterium* species colonizing the teat apices and teat canals may inhibit growth of major mastitis pathogens [89]. Within complex ecosystems, ecosystem diversity can increase resiliency against an influx of external species by supporting favorable interactivity [90]. The complexity of microbe to microbe communications concerning the functional properties of the mammary ecosystem are difficult to understand. It is essential to identify those bacterial species in the milk microbiota that contribute to mammary homeostasis and mastitis pathogen susceptibility [91]. While exploring milk microbiota diversity in relation to udder health, studies have shown a connection between dysbiotic microbiota and mastitis incidence [48,92–94]. In clinical *versus* non-clinical milk samples, clinical sample microbiota had reduced richness and evenness [48,93]. Despite these studies, much remains unknown concerning the ability of commensal microbiota to maintain mammary gland balance and modulate mastitis susceptibility. Derakhshani et al. [91] provided new insight into bacterial community composition and structure, which inhabit the mammary gland. This study shows the possible relationship of bacterial taxa with the inflammatory status of the udder. The identification of that possible hub species and candidate foundation taxa were associated with the inflammatory status of the mammary gland and/or future incidences of clinical mastitis.

In conclusion, more research is necessary to understand the interactions between the microbial world and its hosts. The dissection of these relationships may result in new ways to repair microbial community structure in animals that are affected by organism imbalance.

4. Metabolic Response of Dairy Cows to Challenges: Insights into the Transition Period

It has been well established that high yielding dairy cows undergo several challenges throughout the whole gestation-lactation cycle with the most challenging time frame being in the transition period

with the onset of lactation. Complex adaptation processes take place to enable the maintenance of the animals' energy and nutrient homeostasis but many cows fail to successfully cope with the genetically imposed burden of meeting the requirements for the metabolically prioritized mammary gland in early lactation [28,95,96]. Metabolic stress produces a series of effects on productive and reproductive performance, on the immune system, and overall, on the well-being of the dairy cows [97]. Production diseases that imply a metabolic response are not necessarily related to performance level. Concerning the early lactation, Bertoni et al. [98] were able to develop a novel and clear interpretation of the relationship between milk production levels and health status. The authors demonstrated that high-yielding dairy cows with the highest milk production in the first month of lactation were characterized by better liver functionality and a less pronounced inflammatory status. Considerable biological variation in metabolic adaptation exists among individual animals, particularly in the period between late gestation and early lactation, which is accompanied by distinct levels of metabolic stress [99]. In this context, the successful adaptation to the lactational challenges relies on the metabolic robustness and the activation of the immune machinery [9], with performance levels that should not become another disturbing element of this adaptation. The period from late pregnancy over the course of calving is accompanied by a significant reduction in feed intake, causing the entrance into mild/severe NEB of dairy cows. The latter is the result of the sudden increase of milk secretion in early lactation, which is not compensated by a sufficient increase in feed intake, resulting in the metabolism and immunity (mainly the innate) being out of balance until the level of feed intake is able to cover the energy output with milk production.

Looking at later lactational stages, metabolic adaptation responses to environmental (heat stress, facilities, overcrowding, etc.) and immunometabolic stressors (acidosis, mastitis, etc.) have less detrimental effect on animal health because of the favorable energy balance and the unimpaired immune system. In this section, we presented an overview of the metabolic adaptations to different lactational stages, focusing on the transition period and on the effect of the negative energy balance (NEB) at mid and late lactation in comparison with the early lactation, all from a metabolic standpoint.

4.1. A Multifaceted Challenge Called Transition Period

Drackley [95] argued that the biology underlying the transition to lactation was the "final frontier" in our understanding of the dairy cow. Since that time, a number of relevant in-depth studies uncovered most of the "obscured field" of the transition period with researchers demonstrating that immune cells are directly involved in a surprising array of metabolic functions including the maintenance of gastrointestinal function, control of adipose tissue lipolysis, which in turn determine liver functionality, and regulation of insulin sensitivity in multiple tissues [100–103]. It was also postulated and highlighted that metabolic changes related to energy and calcium supply in support of lactation occurring concurrently impair the innate immune response [9]. The NEB during the transition period explains this reduced immune function, which is also associated with increased concentrations of some blood metabolites as a result of tissue mobilization [96,104]. PMN and lymphocyte functions decrease gradually, starting about 2 weeks before calving, with the lowest efficiencies between the time of calving and 2 days after [104,105]. According to Kehrli et al. [104], the impaired neutrophil function during the periparturient period can be attributed to many of the hormonal and metabolic changes that prepare the mammary gland for lactation. Around this critical period, metabolism shifts from the demands of pregnancy to those of lactation, increasing demands for energy and protein. Together, these metabolic and immunologic challenges during the peripartal period are important factors that limit the ability of most cows to achieve optimal performance and immune-metabolic status [95,106]. Several "exploratory" studies on the immune function during the peripartum period led researchers to investigate potential interventions that might mitigate the immune dysfunction occurring immediately before and after parturition. The focus has been on stimulation of the circulating numbers and possibly the function of neutrophils using the recombinant bovine granulocyte colony-stimulating factor (rbG-CSF) as reported previously [58,107–110]. Treatment with rbG-CSF, starting from approximately

a week before parturition with one injection (15 mg of rbG-CSF) and the second within 24 h after parturition, was able to increase neutrophils, basophils, eosinophils, and monocytes count [108–111]. From a molecular point of view, mRNA abundance of most genes involved in the cell adhesion, migration, recognition, antimicrobial activity, and inflammation cascade was increased. This suggested a complete activation of the immune machinery against the critical period post-partum, at least as a first response of leukocytes to transcriptional regulation [110]. Recently, Lopreiato et al. [102], for the first time, have highlighted the effect of rbG-CSF in maintaining stable cytokine levels during the first month after parturition, reflecting greater regulation of neutrophil recruitment, trafficking, and maturation during the inflammatory response, providing evidence of the immunomodulatory action of rbG-CSF around parturition, when dairy cows are highly immune hypo-reactive. A novel outcome reported by Lopreiato et al. [102] was that increasing the release of pro-inflammatory cytokines, interleukin-6 (IL-6) and interleukin-1β (IL-1β), after parturition upon rbG-CSF treatment did not result in increased systemic inflammation, as shown by haptoglobin and ceruloplasmin plasma levels. This latter finding points out that other mechanisms and/or molecules are likely to drive the inflammation after parturition. Plasma concentrations of IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) have been shown to be 1.5- to five-fold higher prepartum compared to the early lactation period [112]. Further studies should be undertaken to uncover the unknown mechanisms behind this controversial aspect of inflammation within the transition period.

Besides a systemic inflammation, pro-inflammatory cytokines also act on peripheral cells inducing insulin resistance [113,114]. Under these conditions, circulating glucose is prioritized to the non-insulin-dependent glucose transporters that are expressed on immune cells and mammary glands only [115]. The massive glucose requirements of an activated immune system during systemic inflammation could further reduce the energy available for the other tissues, as the mammary gland does not markedly reduce the glucose uptake, aggravating the NEB occurring in early lactation [115]. When NEB occurs, mobilization of body fat and proteins is induced and free fatty acids (FFA) and amino acids are used as gluconeogenic sources by the liver [116]. A severe NEB occurring in the transition period could induce an FFA overload in the liver, inducing the release of beta-hydroxybutyrate (BHB) into the blood following ineffective oxidation of FFA and impaired pivotal functions [95,103,117]. This systemic inflammation is also known to induce the acute-phase response in liver, implying reduced constitutive protein expression (e.g., albumin, lipoproteins, paraoxonase, and retinol-binding protein), counterbalanced by augmented production of positive acute-phase proteins (APP) such as haptoglobin, ceruloplasmin, serum amyloid A, and C-reactive protein [118]. Oxidative stress also occurs during this period and is driven by the imbalance between the production of reactive oxygen metabolites (ROM), reactive nitrogen species (RNS), and the neutralizing capacity of antioxidant mechanisms in tissues and blood, caused by the increased immune response and the metabolic intensification to support lactogenesis [9,88]. When oxidative stress overwhelms cellular antioxidant capacity, ROM induces an inflammatory response. The increase in oxidative stress and inflammation during this period is also negatively associated with a reduction in liver functionality, and measurement of APP can provide a useful tool to assess liver function as well as inflammation [118]. Liver function is often impaired in transition dairy cows. In this context, it is relevant to point out the scenario occurring in the rumen during the transition period. Few studies have investigated the molecular adaptations of ruminal epithelium during the peripartum period [119–123]. These studies revealed the existence of interactions among genes of the immune system and those involved in the preparation for the onset of lactation [119,121,122], as well as the presence of growth factors that seem to be regulated after parturition [120]. The connections among ruminal fermentation, microbiota, the ensuing ruminal epithelium adaptations, and the consequence on systemic responses (e.g., immunometabolism) of the cow remain unclear. Whether microbial metabolism could affect epithelial gene expression via metabolites remains uncertain. The interaction of rumen content and epithelium with the systemic immune response opens a new scenario in the management of forestomaches. The role of saliva and its composition in terms of immune cells and immunogenic molecules should be further investigated

as a potential factor of the reduced immunocompetence in dairy cows, mainly in the transition period [64,124]. Secondly, the role of diet appears crucial not only for an accurate formulation (e.g., fermentability of carbohydrates, protein degradability) but also for nutrient imbalance and/or for microbiota composition, which might alter epithelium functions (e.g., increasing its permeability). Several attempts to improve energy intake and thus avoiding detrimental effects especially after parturition have been proposed. Controlling energy intake during the dry period to near calculated requirements leads to transition success, with fewer diseases and disorders than cows fed high-energy diets [125–127], but also greater DMI around parturition [128]. Prolonged over-consumption of energy during the dry period can decrease post-calving DMI, resulting in negative responses of metabolism with higher NEFA and BHB in blood and greater triacylglycerol in the liver after calving [126]. The diet must be formulated to limit energy intake and meet the requirements for protein, minerals, and vitamins. To date, there little knowledge about diet formulation for the immediate postpartum period in order to optimize transition success and consequently reproduction efficiency. Proper dietary formulation in both dry and close-up periods would maintain or enable rumen adaptation to higher grain diets after calving, which in turn reflects a greater level of energy intake and energy utilization.

Further perspectives are created by the opportunity to control rumen microbiomes by the host animal (genotype) as a result of genetic selection [129].

4.2. The Association between Rumen Microbiome, Cattle Production, and Health Traits

The rumen contains trillions of bacteria, protozoa, and methanogenic archaea as major components. A symbiotic relationship exists between a ruminant host and the microbiota where bacteria are provided shelter and nutrients and the host benefits from essential nutrients released by bacterial fermentation activities. Along with beneficial and essential nutrients, bacterial activity may be connected to the release of harmful compounds including bacterial endotoxins.

Recent studies using an omics-based structure have suggested that differences in rumen microbiota are associated with cattle production and health traits, such as feed efficiency [130,131], methane (CH_4) yield [132], milk composition [133], and ruminal acidosis [134]. Manipulating rumen microbiota may improve cattle productivity and health and reduce CH_4 emissions. Transfaunation of ruminal contents is regularly used to enhance rumen function and milk production [135]. Although studies show that ciliated protozoa responsible for plant material digestion may be successfully transferred, there is more resistance within the bacterial community perhaps due to host-specific properties [136,137], suggesting the importance of the host's genetics influence on rumen microbiota. More studies are needed to provide convincing information about associations between host and rumen microbiota.

5. Native Cattle Breeds, an Interesting "Case Study"

An interesting model for the study of the susceptibility to production diseases could be represented by native cattle breeds. These breeds have been part of livestock history until the 21st century when they were abandoned in favor of more productive cosmopolitan breeds (Holstein, Brown Swiss and Jersey) [138–141]. Some native breeds survived, thanks mainly to the efforts of many small traditional farmers residing mainly in rural marginal areas. The intense genetic selection received by the cosmopolitan breeds in order to improve the productive characteristics led them to develop peculiar physiological features, which have likely impaired some immune defense mechanisms, increasing the incidence of metabolic and infectious diseases, and worsening both fertility and longevity [76,142–144]. These phenomena have been studied in Holstein Friesian (HF) cows, the most widespread and highly selected dairy cattle breed. The intense genetic selection of HF for milk production has been associated with relevant physiological dysfunctions, e.g., reduction of the immune competence, severe NEB, inflammatory-like status, oxidative stress, and hypocalcemia [9]. The metabolic pressure caused by the high, energetic requests of the mammary gland combined with the stress resulting from the pregnancy-calving period in the context of severe NEB can lead to serious disruptions of physiological homeostatic balance [103,104]. All these physiological perturbations seem to be less

intense in the "lower productive" native breeds. The scientific community is comparing breeds with different selective pressure in order to improve the comprehension of regulatory mechanisms of cattle physiology. The literature is not very extensive and has some limitations (e.g., number of animals, different environments, diet, and management), but important physiological information can be deduced. Mendoca et al. [145] compared HF and Montbéliarde-sired crossbred cows in the peripartum; no differences were found in terms of metabolic and inflammatory (haptoglobin concentration) responses, milk yield and incidence of typical peripartum diseases (retained fetal membranes, metritis, and subclinical endometritis). Despite that, the HF showed more pyrexia events in early lactation (50.0 vs. 31.4%) and a higher incidence of purulent vaginal discharge (44.2 vs. 26.5%) than crossbred cows. Curone et al. [6] compared HF with Rendena cows, an Italian breed native of the Rendena Valley in Northeastern Italy (Trentino), and observed that HF in the postpartum showed a more severe systemic inflammatory response in terms of haptoglobin, total proteins, globulins, and bilirubin, a more severe fat mobilization associated with lower body muscle mass and lower amino acid mobilization. In this study and in that of Cremonesi et al. [146], detailed insights into the milk microbial population of HF and Rendena along the transition period were also provided. The results highlighted the existence of differences in terms of general microbial diversity, taxonomy, and predicted functional profiles. Those differences might also have an impact on their mammary gland health concerning disease and pathogen resistance. These differences seem related to inflammo-metabolic changes occurring around calving, which suggest a possible relationship among these responses and the mechanisms of resistance in the mammary gland.

The local breed Simmental, when compared with HF cows during the transition period, presented a different metabolic adaptation, in terms of different energy, inflammatory, and oxidative pattern responses. Simmental showed a lower value of BHB and higher mobilization of muscle protein (creatinine) [101]. Simmental cows seemed more sensitive to induction of the immune system after calving, with a greater transcript abundance of proinflammatory cytokines and receptor genes, cell migration- and adhesion-related genes [102,110]. Begley et al. [147] showed that, when infected with Candida albicans, Norwegian Red cows have a greater primary antibody-mediated immune response, producing greater concentrations of immunoglobulin G (IgG) compared to HF cows. One of the largest comparative studies was performed by Bieber et al. [148]. They compared the production, fertility, longevity, and health-associated traits of local native and modern breeds of dairy cattle in 4 different European nations: Austria, Switzerland, Poland, and Sweden. They compared Original Braunvieh and Grey Cattle with Braunvieh (Brown Swiss blood >60%) in Switzerland; Grey Cattle with Braunvieh (Brown Swiss blood >50%) in Austria; Polish Black and White, Polish Red and White, and Polish Red with Polish Holstein Friesian in Poland; and Swedish Red with Swedish Holstein in Sweden. Average milk yields were substantially lower for local compared with commercial breeds in all countries. Local breeds showed a longer productive lifetime and a shorter calving interval with a lower insemination index than commercial breeds. Another approach to re-appraise the native breeds is the use of crossbreed cattle. Several studies showed how dairy producers may improve the longevity, robustness, and fertility of cows and the profitability of dairying by crossing pure HF cows with bulls of different native breeds [149–151]. The lower production level of local breeds is partly compensated by advantages in fertility, health status, and longevity. It is important to remember that the breeding goals should balance productivity with functional traits [152], and the choice of appropriate dairy breeds can be regarded as a key factor for successful health management in dairy farming.

6. Conclusions

The immune system has evolved along with the phylogenetic evolution as a highly refined sensing and response system poised to react against diverse infectious and non-infectious stressors for better survival and adaptation. This operational framework is jeopardized when high-yielding dairy cattle are poorly managed. Metabolic priority for offspring survival is affected by the levels of milk yield, exceeding the potential of dry matter intake. Secondly, the subsequent negative energy balance gives

rise to metabolic stress, e.g., a disequilibrium in the homeostasis of a living organism as a result of anomalous utilization of nutrients. It can be argued that high genetic merit for milk yield is correlated with a defective control of the inflammatory response underlying the occurrence of several production diseases. This is evident in the mastitis model where high-yielding dairy cows show high disease prevalence in the framework of reduced effectiveness of the innate immune response.

Effective monitoring tools, immunomodulators, and nutraceuticals should be combined with proper farm management and feeding regimes. Specific intervention protocols should be implemented in the first weeks after calving and at dry-off because the relevant stressors are pivotal to disease occurrence and early culling of high-yielding dairy cattle.

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Review

Overview of Research Development on the Role of NF-κB Signaling in Mastitis

Muhammad Zahoor Khan [1], Adnan Khan [2], Jianxin Xiao [1], Jiaying Ma [1], Yulin Ma [1], Tianyu Chen [1], Dafu Shao [3] and Zhijun Cao [1,*]

[1]   State Key Laboratory of Animal Nutrition, Beijing Engineering Technology Research Center of Raw Milk Quality and Safety Control, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China; zahoorkhattak91@163.com (M.Z.K.); dairyxiao@gmail.com (J.X.); majiaying@cau.edu.cn (J.M.); ma18810318038@163.com (Y.M.); 18355593440@163.com (T.C.)

[2]   Key Laboratory of Animal Genetics, Breeding, and Reproduction, Ministry of Agriculture & National Engineering Laboratory for Animal Breeding, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China; dr.adnan93@cau.edu.cn

[3]   Institute of Agricultural Information of CAAS, Beijing 100081, China; shaodafu@caas.cn

*   Correspondence: caozhijun@cau.edu.cn; Tel.: +86-10-62-733-746

Simple Summary: NF-κB signaling has been widely studied for its role in inflammatory and immunity-related diseases. Mastitis is considered one of the inflammatory and immunity associated diseases which are a serious threat to the global dairy industry. Having such a critical role in immunity and inflammation, NF-κB signaling is currently under target for therapeutic purposes in mastitis control research. The virulent factor, lipopolysaccharides (LPS), of bacteria after attachment with relevant Toll-like receptors (TLRs) on mammary epithelial cells starts its pathogenesis by using NF-κB signaling to cause mastitis. Several studies have proved that the blocking of NF-κB signaling could be a useful strategy for mastitis control.

Abstract: Mastitis is the inflammation of the mammary gland. *Escherichia coli* and *Staphylococcus aureus* are the most common bacteria responsible for mastitis. When mammary epithelial cells are infected by microorganisms, this activates an inflammatory response. The bacterial infection is recognized by innate pattern recognition receptors (PRRs) in the mammary epithelial cells, with the help of Toll-like receptors (TLRs). Upon activation by lipopolysaccharides, a virulent agent of bacteria, the TLRs further trigger nuclear factor-κB (NF-κB) signaling to accelerate its pathogenesis. The NF-κB has an essential role in many biological processes, such as cell survival, immune response, inflammation and development. Therefore, the NF-κB signaling triggered by the TLRs then regulates the transcriptional expression of specific inflammatory mediators to initiate inflammation of the mammary epithelial cells. Thus, any aberrant regulation of NF-κB signaling may lead to many inflammatory diseases, including mastitis. Hence, the inhibiting of NF-κB signaling has potential therapeutic applications in mastitis control strategies. In this review, we highlighted the regulation and function of NF-κB signaling in mastitis. Furthermore, the role of NF-κB signaling for therapeutic purposes in mastitis control has been explored in the current review.

Keywords: mastitis; bovine mammary epithelial cells; inflammatory cytokines; NF-κB signaling; PRRs; TLRs

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1. Introduction

Mastitis is the inflammation of the mammary gland, which is associated with pathological changes in udder tissue and decreases in the quantity and quality of milk [1,2]. Based on its duration and

symptoms, mastitis might be acute or chronic [3,4]. Udder swelling, reduced milk yield, clots and increase somatic cell counts in milk are the most common clinical signs of mastitis [5]. All these factors are associated with pathogenic invasion, which is followed by the involvement of neutrophils under a specific stimulus. The inflammatory conditions may lead to chronic inflammation if not properly controlled and treated [6,7]. Different types of etiological invading bacterial pathogens are involved in bovine mastitis, of which *Coliforms, Escherichia coli, Streptococci* and *Staphylococcus aureus* are the most common bacteria [8–11]. Gram-negative bacteria, such as *E. coli*, can often cause clinical mastitis, and Gram-positive bacteria, such as *S. aureus*, are involved in subclinical mastitis infection [12–14].

Previous reports have documented that mammary epithelial cells work as the first line of defense of the mammary gland by generating multiple inflammatory cytokines against bacteria invading the epithelial cells [15,16]. Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) expressed by many cell types, including mammary epithelial and immune cells [17]. In addition, it has been reported that innate immune systems recognize pathogens through TLRs [18–20].

The TLRs are distributed on the host cell surface that regulates the initial sensation of infection [21,22]. Every pathogen uses specific receptors on host cells—for example, *S. aureus* uses TLR2 and TLR6 [23], while *E. coli* utilizes TLR2 and TLR4—to transmit their signals inside the cell [21]. This specificity to TLRs depends on the virulent factor of pathogens. The cell wall of *S. aureus* is composed of lipoteichoic acid and peptidoglycan [24], while Gram-negative bacteria, such as *E. coli*, have lipopolysaccharides (LPS) in their cell wall [25]. The binding of pathogenic virulent factors to TLRs leads to the activation of several signaling components, including nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) [26], which is considered one of the key players associated with inflammatory action. Besides, NF-κB signaling has been widely studied for its role in regulation of immunity and inflammation. Keeping in view the versatile functions of NF-κB signaling, the current review has specifically concentrated on summarizing possible research development on the role of NF-κB signaling activation and regulation of immunity and inflammation in bovine mastitis.

2. Materials and Methods

All studies which have discussed the role of NF-κB signaling in mammary gland infection, mainly bovine mastitis, were screened through authentic sources, such as PubMed, ScienceDirect, Web of Science, SpringerLink, Scopus and Google Scholar. The major keywords used for the search of literature were milk production, mastitis, NF-κB signaling, TLRs, MYD88, PPRs, cytokines, *E. coli-* and *S. aureus*-mastitis. The related data published in the English language in well-reputed peer-reviewed journals have been included for discussion in the current review. Furthermore, we excluded all content available in the form of conference abstracts, books, book chapters and unpublished findings.

3. General Regulatory Pattern of NF-κB Signaling

NF-κB is a common term used for inducible dimeric transcription factors. It is composed of a Rel family DNA binding protein which distinguishes common sequence motifs. Mammals express 5 Rel (NF-κB) proteins which are composed of two classes including Rel A (p65), c-Rel and Rel-B proteins which do not need proteolytic processing as the class is composed of NF-κB1 and NF-κB2 genes, encoded for p105 and p100, respectively, which do not require proteolytic processing to synthesize mature p50 and p52 NF-κB proteins [27]. The NF-κB protein was first found in murine B-lymphocytes, but currently, it has been identified in many cell types, including mammary epithelial cells [28]. Different external stimuli, such as tumor necrosis factor Alpha (TNF-α) [29], interleukin 1-beta (IL-1β) [30], LPS and reactive oxygen species (ROS) [31] after attachment with TLRs, activate NF-κB [32]. NF-κB signaling has an essential role in the regulation of immunity and inflammation [33], cell apoptosis, cell survival and proliferation (Figure 1) [34,35].
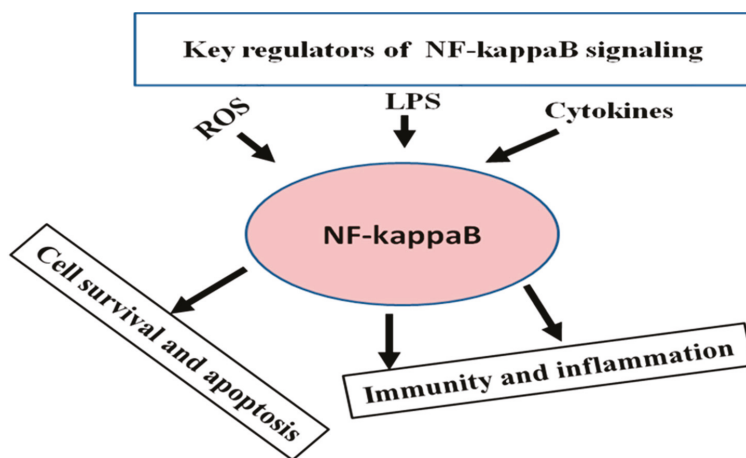
Figure 1. The key inducers of the NF-κB pathway and regulation of immunity, inflammation, cell survival and apoptosis by NF-κB signaling.

In addition, NF-κB signaling plays a vital role in the regulation of inflammatory cytokines, adhesion molecules, chemokines and growth factors involved in mammary gland inflammation [36]. Adhesion molecules are important proteins of tight junctions [37], which are closely related to the link between cell membranes and are required for normal lactation in mammals [38]. Song et al. has shown that LPS disrupt the permeability of the blood–milk barrier by activating the NF-κB signaling pathway. The pro-inflammatory cytokines regulated by the NF-κB signaling pathway promote the process of inflammation and interrupt the integrity of tight junction structures in the mammary epithelial cells [39]. The disruption in the blood–milk barrier has been reported during mastitis, which might be due to damage of the tight junctions responsible for normal lactation [40]. The disruption of tight junctions also may lead to loss of milk which is one of the common signs of mastitis in dairy cattle. Having such a critical role in inflammation and immunity, the NF-κB pathway has been widely targeted in mastitis research [41–46].

4. Role of NF-κB Signaling in Normal Physiology of Mammary Gland Development

A regulated pattern of activation of NF-κB during the various stages of the development of mammary glands has been demonstrated [46]. NF-κB activation rises during pregnancy and decreases during lactation, followed by elevation during the mammary gland involution, again [47,48]. This change in pattern suggests that NF-κB plays a significant role during pregnancy and involution. Mammary gland involution is associated with apoptosis of the secretory alveolar epithelium [49], and NF-κB has been explored to mediate the anti-apoptotic proteins [50]. These findings revealed the role of NF-κB in promoting the survival of epithelial cells [51]. It has been demonstrated that NF-κB activates the two essential lactogenic hormones, namely prolactin and oxytocin [52,53]. In addition to playing a role in the developmental process of normal mammary glands, NF-κB activation was found to be associated with mammary gland infections.

5. Role of NF-κB Signaling in Mastitis

The murine model and bovine reports have shown the link of NF-κB regulation with mastitis [43]. Most of the studies investigated the role of NF-κB in mastitis as a regulator of inflammatory cytokines [54,55]. Considerable losses of milk have been observed during mastitis and mammary gland involution which showed the link of both with the up-regulation of NF-κB during a time of milk loss and mammary gland remodeling.

5.1. Mechanism of NF-κB Signaling Activation by Bacteria during Mastitis

LPS, a bacterial virulence factor, interacts with TLRs which are residing on surface mammary epithelial cells [56]. Upon activation, the TLRs further engage myeloid differentiation factor 88 (MyD88) [57] and c-Jun N-terminal kinase (JNK) [58], which triggers NF-κB [32] and mitogen-activated protein kinase (MAPK) signaling. The translocation of NF-κB and MAPK signaling further regulates the production of target inflammatory genes [59–62]. The mechanism of NF-κB signaling activated by *S. aureus* and *E. coli* during mastitis is shown in Figure 2.
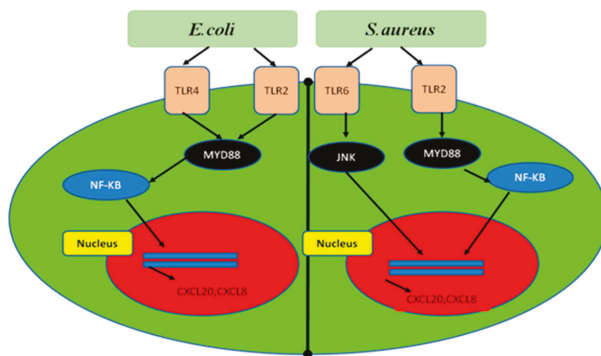
Figure 2. The interactive mechanism of *E. coli* and *S. aureus* with TLR2, TLR4 and TLR6, and the regulation of NF-κB signaling to activate the inflammatory genes.

5.2. Mechanism of NF-κB Signaling Activation by Inflammatory Cytokines

Besides *S. aureus* and *E. coli,* various inflammatory cytokines activate NF-κB signaling regulation in mammary epithelial cells. The NF-κB and MAPK pathways activate pro-inflammatory cytokines interleukin 6 (IL-6), IL-1β and TNF-α [63]. Nuclear factor-κB is a nuclear transcription factor that exists in an inactive form in the cytoplasm and is bound to its inhibitor IκB [64,65]. Once activated, the NF-κB unit p65 separates from IκB and translocates from the cytoplasm to the nucleus, where it regulates inflammatory gene expression [66]. The pathogenic message usually causes the liberation of NF-κB from IκB [65]. The regulation of the inflammation through NF-κB by pro-inflammatory cytokines is shown in Figure 3. The promoter of the inflammatory genes contains binding sites for NF-κB, and thus mostly depends on NF-κB for its regulation [67]. It has been reported that active NF-κB complexes cannot be detected in healthy cow milk cells, while the NF-κB elevated level was noticed in the milk cells of cows with acute mastitis. In addition, the activity of NF-κB in milk cells varies from low to high in chronic mastitis [67]. Stimulation of LPS causes mammary epithelial cells to produce cytokines TNF-α, IL-6 and IL-1β [68]. The increased levels of TNF-α, IL-6 and IL-1β have been observed in LPS-infused mammary glands [69]. Furthermore, Blum et al. reported the high level of cytokines (TNF-α, IL-6 and IL-17), somatic cell count (SCC), and up-regulation of TLR4 expression in leukocytes of the milk of an *E. coli*-induced mastitic cow [70]. In the mammary glands, inflammation is associated with an increased level of neutrophil chemo-attractants and the cytokines IL-1ß, IL-6, IL8 and TNF-α [71,72]. The expression level reported for IL8 and TNF-α in *E. coli* induced-mastitis in bovine mammary epithelial cells (BMECs) was much higher than for *S. aureus,* which is due to the weak Lipoteichoic acid (LTA) induction of TNF-α, or inactivation of NF-κB signaling [73]. Boulanger et al. observed that NF-κB was highly associated with the level of the expression of interleukin-8 and granulocyte/macrophage colony-stimulating factors, two NF-κB-dependent cytokines critically linked to the regulation and continuation of neutrophilic inflammation. Altogether, these findings suggested the crucial role of NF-κB in the pathogenesis of mastitis.
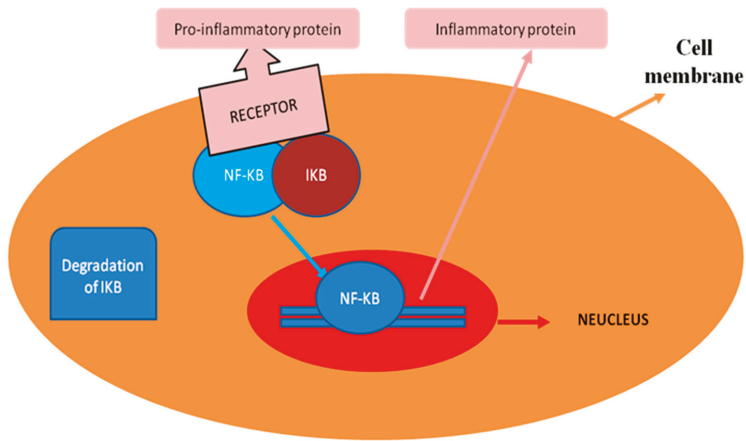
Figure 3. The regulation of the inflammation process by pro-inflammatory cytokines through NF-κB signaling; the cytokines, after attachment with receptors, cause the degradation of IKB from NF-κB. Upon activation, NF-κB directly binds to the promoters of target genes on DNA in the nucleus and regulates the specific inflammatory proteins.

5.3. Bovine Myeloid Differentiation Primary Response 88 (MYD88), NFKBIA and TRAPPC9 Role as a Regulator of Lipopolysaccharide (LPS)-Induced NF-κB Signaling Pathways

MYD88 is the main adopter molecule for TLR2, 4, 5, 7, 8 and 9 signaling [74]. The TLRs, when activated by mastitis-induced bacteria, pass the signal to MYD88, which is considered the critical immune regulator adapter molecule against various pathogens [75,76]. MYD88 acts as the key regulator of NF-κB by causing the degradation of IKB. Wang and his co-authors compared the expression level of MYD88 in healthy and mastitic cows. It was observed that MYD88 expression, which works as a bridge between TLRs and NF-κB, was elevated in mastitic cows compared to healthy ones [69,77,78]. It was noticed in a study that inhibition of MYD88, along with its inhibitor, Pepinh-MYD, significantly reduced the level of NF-κB [63].

Another essential protein is the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA), which encodes IκB and is responsible for the negative activation of NF-κB transcription factors. It has been shown in a report that LPS cause the degradation of IκBα; they facilitate the translocation of NF-κB in the nucleus, which in response accelerates the re-synthesis of IκBα [79]. Fang et al. noticed the up-regulation of NFKBIA in *S. aureus*-induced mastitis [80]. The trafficking protein particle complex 9 (TRAPPC9), also called NIK-and-IKK2-binding protein (NIBP), is a key regulator of NF-κB signaling [72,81,82]. An in-vitro study revealed NIBP low expression results in the down-regulation of TNF-α-induced NF-κB [83]. Wang et al. noticed through a genome-wide association study (GWAS) that the mutation in TRAPPC9 is associated with milk SCS [84]. The high expression level of the TRAPPC9 gene was reported in mammary epithelial cells infected with *S. aureus*. Furthermore, it was revealed that the TRAPPC9 gene might be considered a potential marker against mastitis [85]. The above-published studies showed that MYD88, NFKBIA and TRAPPC9 might work as a bridge between cell surface receptors and NF-κB. Thus, any change in these genes may disturb NF-κB signaling, which facilitates the pathogenesis of mastitis.

5.4. NF-κB Regulates the Immunity and Inflammatory Linked Genes during Mastitis

When NF-κB signaling is activated by external stimuli, such as bacteria or cytokines, it starts to regulate the production of inflammatory chemokines (IL-8, CXCL1, CXCL10, etc.), cytokines (IL-6, TNF-α, IFN-gamma and IL-1β), adhesion molecules (ICAM-1 and MMPs), growth factors (CSF) and apoptotic associated genes [76,86,87]. For the site of infection, many proteins are required; these

proteins are: adhesion factors, such as ICAM-1 and VCAM-1, which facilitate neutrophil margination, diapedesis and transepithelial migration; chemokines, such as interleukin (IL)-8, which are responsible for chemotactic of neutrophils; IL-1β and TNF-α, which regulate neutrophils [67]. When bacteria enter the teat, the mammary epithelial cells secrete chemokines (CXCL8 and CXCL20) and cytokines (TNF-α and IL-1β). Production of cytokines and chemokines in the milk of the mastitic mammary gland is considered the key player of inflammation [72,88]. The TNF-α and fatty acid synthetase (FAS) mRNA expression was significantly up-regulated in LPS-challenged quarters [89]. A study reported the up-regulation of CXCL8 and TNF-α in *E. coli* induced mastitis in mammary epithelial cells [88]. In addition, the high expression of CXCL10, CCL2, CCL5 *and* CCL20 was noticed in bovine mammary epithelial cells in *E. coli* induced mastitis, which is essential for the recruitment of leucocytes [90]. The expression levels of IL-6, complement factor 3 (C3), NFKBIA and MMP9 were also elevated during mammary gland infection [90]. It has been reported that monocytes, natural killer cells and activated lymphocytes are majorly regulated by the chemokines CXCL10 and CCL5 [91]. Apart from the above functions, CXCL10 directs the recruitment and activation of neutrophils towards LPS-infection spots in mice and humans [83,92,93]. In addition, CXCL10 was reported as a highly expressed gene in response to *E. coli* infection in mammary glands [94]. The levels of CXCL10 and ICAM1 were noticed to be significantly elevated in the *S. aureus*-mastitic mammary glands of cows [95]. Similarly, the high expression of CCl5 has also reported in *E. coli*-induced mastitis in BMECs [96]. The expression levels of CXCL8, IL6 and CSF3 were higher in *S. aureus* challenged BMECs [80]. Additionally, many other immunity and inflammatory associated genes, such as SAA3, CCL5, C3 and CSF3, were also documented in mastitis-infected mammary glands [69]. Furthermore, the high expressions of CXCL10, IL6, CXCL8, IFN-gamma and IL-1β induced by LPS in BMECs are able to regulate inflammation [97]. It has been demonstrated in previous reports that inflammatory cytokines and chemokines create protection against foreign invading pathogens in bovine mammary glands, by increasing the movement of leucocytes from the blood into the mammary tissue [98]. Similarly, a study reported the protecting role of IL-1β by recruiting neutrophils into the mammary gland [99].

5.5. Research Progress on Target of NF-κB Signaling as a Therapeutic in Mastitis Control

It is well known that TLRs, upon recognition of external stimuli, activate NF-κB regulation to produce inflammatory linked genes to eliminate the cause of infection in mammary epithelial cells. TLR4, a pro-inflammatory cytokine, and LPS, a component of the cell wall of bacteria, are common inducers of NF-κB signaling. The LPS-induced inflammation in mammary epithelial cells causes the up-regulation of TLR4 [100,101]. Recently, NF-κB signaling is being widely targeted as a therapeutic choice against mastitis resistance. A study proved, experimentally, that selenium restricts *S. aureus*-induced mastitis through inhibition of the MAPK and NF-κB pathways and TLR2 [102]. Cytokines, an important group of inflammatory mediators, play a major role in the process of inflammation [103]. Stimulation by LPS causes mammary epithelial cells to produce the cytokines TNF-α, IL-6 and IL-1β [60]. Increased levels of TNF-α, IL-6 and IL-1β have been observed in LPS-infused mammary glands. Similarly, Akhter et al. [104] noticed the up-regulation of pro-inflammatory cytokines in *S. aureus*-induced mammary epithelial cells. Further, they proved that the expression levels of genes associated with TLR2/TLR4-mediated NF-κB/MAPKs pathways were higher in *S. aureus*-infected mammary epithelial cells. The excessive expression of pro-inflammatory IL1β may lead to pathological conditions [105]. Dai et al. noticed that methionine and arginine attenuated the proinflammatory action by preventing the regulation of NF-κB. Furthermore, methionine and arginine down-regulated the levels of TLR4 and IL1β in LPS-induced mastitis, which caused the excessive regulation of inflammatory changes, and thus damaged the cells [106]. Taken together, it has been concluded here that methionine and arginine, being blockers of NF-κB, can be considered as prophylactic agents of mastitis.

Exogenous hydrogen sulfide has the ability to suppress inflammatory cytokine production, ROS [107–109], and promotes anti-inflammatory proteins [110]. The high level of ROS is associated with the imbalance between cellular redox states and oxidative stress, which has a significant role

in the promotion of inflammation [111]. It was noticed that LPS alone diminished cell viability and caused inflammatory changes in mammary epithelial cells. However, it was found that the hydrogen sulfide (H2S) combined with LPS restored the viability of the cells [112]. Sun et al. revealed that H2S, after entry into the cells, first blocked the TLR4 and ROS, and thereby no signal was given for NF-κB to produce a high level of inflammatory proteins in mammary epithelial cells [113]. In addition, the mRNA expression of TNF-α, IL-1β, IL-8 and IL-6 was also very low in H2S-treated mammary epithelial cells.

Morin has anti-inflammatory properties [114] and inhibits the release of the inflammatory cytokines IL-6 and IL-8 and tumor necrosis factor (TNF) from mast cells [115]. It was experimentally proved that morin is associated with inhibition of TNF-α, IL-6 and IL-1β in LPS-induced bovine mammary epithelial cells (bMECs). To suppress the level of cytokines, morin down-regulates the levels of MAPK and NF-κB pathways in LPS-induced mammary epithelial cells [78]. NLRP3 inflammasome is the key regulator of IL-1β, while a recent study noticed that morin significantly down-regulated the level of IL-1β [116] in LPS-induced bovine mammary epithelial cells by suppression of NF-κB and nucleotide-binding domain, leucine-rich repeat-containing family, pyrin domain containing 3 (NLRP3) inflammasome [117]. Furthermore, it has been noticed that morin also maintained the integrity of the tight junction from the action of the inflammatory cytokines regulated by NF-κB [63]. Likewise, polydatin has anti-inflammatory efficiency and can be used to control *S. aureus*-induced mastitis. The most in-depth mechanism showed that polydatin decreased the expression of TLR2 and MyD88, which further suppressed the level of NF-κB in mammary epithelial cells of *S. aureus*- induced mastitis [60].

Tea tree oil (TTO) is an essential oil which has antibacterial and anti-inflammatory properties and promotes the movement of polymorphonuclear leukocytes towards the infection. TTO also inhibits NFKBIA and TNF-α [118]. In addition, TTO act as an inhibitor of the NF-κB pathway, which is essential for the regulation of immunity and inflammatory responses in mammary glands. Nucleotide-binding oligomerization domain (NOD) is a type of PRR that plays an important role in the regulation of innate immunity [119]. Recently, it was documented that by blocking NOD1/NF-κB signaling, LPS stimulation reduced neutrophil migration and phagocytic killing ability. Further, it was proved that the activation of NOD1/NF-κB in vitro restricted the action of LPS by promoting the functional capacity of neutrophil [60]. Chlorogenic acid has anti-inflammatory and antibacterial effects [120,121]. A study reported that chlorogenic acid inhibits cytokine production in LPS-stimulated RAW264.7 cells by suppressing the phosphorylation of NF-κB [122]. Similarly, chlorogenic acid was noticed to reduce the level of cytokines followed by inhibition of TLR4 and phosphorylation of NF-κB in LPS-induced mastitic mammary epithelial cells [123]. Thymol was found to be very effective in mastitis treatment. The mechanism for the association of thymol was tested in BMECs. The western blot result showed that thymol treatment significantly inhibited the production of IL-6 and TNF-α, followed by suppression of the NF-κB pathway [124]. In Table 1, we summarized those studies which targeted the NF-κB signaling to control mastitis.

Table 1. Chemicals and their anti-inflammatory effect in mastitis by suppression NF-κB signaling.

Authors	Agent	Function	Targets
Sun et al. [113]	H2S	Antibacterial	Block TLR4, ROS, NF-κB
Garcia et al. [125]	Citrus oils		Down-regulate TLR2, NFKBIA, IL8, TNF-α
Wang et al. [78]	Morin	Anti-inflammatory	Inhibit IL-6, TNF-α, IL-1β, suppress NF-κB phosphorylation
Li et al. [126]	8-Methoxypsoralen	Anti-inflammatory	Inhibit IL-6, TNF-α, IL-8, IL-1β, suppress NF-κB phosphorylation
Chen et al. [36]	Nuciferine	Anti-inflammatory	Inhibit TLR4, TNF-α, IL-1β, suppress NF-κB phosphorylation
Yang et al. [127]	Oxymatrine	Anti-inflammatory	Suppress NF-κB phosphorylation
Ershun et al. [128]	Cepharanthine	Anti-inflammatory	Inhibit IL6, TNF-α, IL-1β, suppress NF-κB phosphorylation
Su et al. [129]	Rutin		Decrease level of IL-1β, IL-6, and TNF-α, suppress NF-κB phosphorylation
Liu et al. [112]	Sodium houttuyfonate	Antinflammatory	Inhibit NF-κB phosphorylation
Li et al. [130]	Emodin ameliorates	Anti-inflammatory, antibacterial	Decrease level of IL-1β, IL-6, and TNF-α, suppress NF-κB phosphorylation
Hu et al. [42]	Cynatratoside-C from Cynanchum atratum	Anti-inflammatory	Suppress TLR4, inhibit NF-κB phosphorylation
He et al. [131]	Docosahexaenoic acid	Anti-inflammatory	Decrease level of IL-1β, IL-6, and TNF-α, suppress NF-κB phosphorylation
He et al. [132]	Baicalein	Anti-inflammatory	Suppress TLR4, inhibit NF-κB phosphorylation

From the above discussion, it has been cleared that NF-κB signaling plays a role of backbone in the pathogenesis of mastitis by promoting cytokine production. Thus, by targeting NF-κB, mastitis can be effectively controlled [124].

6. Conclusions

Overall, the current review, based on published studies, revealed that activation of NF-κB resulted in decreased of milk and apoptotic signaling, which could be minimized through selective modulation of NF-κB signals. Furthermore, the review suggested that NF-κB is a vital regulator of milk loss during mammary gland involution and infection, and recognized the NF-κB signaling pathway as a possible target for preventing mastitis-induced milk loss in dairy cattle. In addition, based on published literature, we concluded that TLR4, IL-1β, IL-6, TNF-α and MYD88 are key players in NF-κB signaling and also have an essential role in mastitis development. From the literature studies, it was revealed that *S. aureus* and *E. coli,* after attachment with TLRs, used NF-κB pathway for pathogenesis. Thus, the utilization of NF-κB as a therapeutic target in mastitis control showed successful outcomes. In addition, TLR4, IL-1β, IL-6, TNF-α, MYD88 and NF-κB might be a useful addition as markers in mastitis control strategies.

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Role of the JAK-STAT Pathway in Bovine Mastitis and Milk Production

Muhammad Zahoor Khan [1], Adnan Khan [2], Jianxin Xiao [1], Yulin Ma [1], Jiaying Ma [1], Jian Gao [3] and Zhijun Cao [1,*]

[1] State Key Laboratory of Animal Nutrition, Beijing Engineering Technology Research Center of Raw Milk Quality and Safety Control, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China; zahoorkhattak91@163.com (M.Z.K.); dairyxiao@gmail.com (J.X.); ma18810318038@163.com (Y.M.); majiaying@cau.edu.cn (J.M.)

[2] Key Laboratory of Animal Genetics, Breeding, and Reproduction, Ministry of Agriculture & National Engineering Laboratory for Animal Breeding, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China; dr.adnan93@cau.edu.cn

[3] Department of Clinical Veterinary Medicine, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China; gaojian2016@cau.edu.cn

* Correspondence: caozhijun@cau.edu.cn; Tel.: +86-10-62733746

Simple Summary: The cytokine-activated Janus kinase (JAK)—signal transducer and activator of transcription (STAT) pathway has an important role in the regulation of immunity and inflammation. In addition, the signaling of this pathway has been reported to be associated with mammary gland development and milk production. Because of such important functions, the JAK-STAT pathway has been widely targeted in both human and animal diseases as a therapeutic agent. Recently, the *JAK2*, *STATs*, and inhibitors of the JAK-STAT pathway, especially cytokine signaling suppressors (SOCSs), have been reported to be associated with milk production and mastitis-resistance phenotypic traits in dairy cattle. Thus, in the current review, we attempt to overview the development of the JAK-STAT pathway role in bovine mastitis and milk production.

Abstract: The cytokine-activated Janus kinase (JAK)—signal transducer and activator of transcription (STAT) pathway is a sequence of communications between proteins in a cell, and it is associated with various processes such as cell division, apoptosis, mammary gland development, lactation, anti-inflammation, and immunity. The pathway is involved in transferring information from receptors on the cell surface to the cell nucleus, resulting in the regulation of genes through transcription. The Janus kinase 2 (*JAK2*), signal transducer and activator of transcription A and B (STAT5 A & B), STAT1, and cytokine signaling suppressor 3 (*SOCS3*) are the key members of the JAK-STAT pathway. Interestingly, prolactin (Prl) also uses the JAK-STAT pathway to regulate milk production traits in dairy cattle. The activation of *JAK2* and *STATs* genes has a critical role in milk production and mastitis resistance. The upregulation of *SOCS3* in bovine mammary epithelial cells inhibits the activation of *JAK2* and *STATs* genes, which promotes mastitis development and reduces the lactational performance of dairy cattle. In the current review, we highlight the recent development in the knowledge of JAK-STAT, which will enhance our ability to devise therapeutic strategies for bovine mastitis control. Furthermore, the review also explores the role of the JAK-STAT pathway in the regulation of milk production in dairy cattle.

Keywords: bovine mastitis; JAK-STAT pathway; *JAK2*; *STATs*; *SOCS3*; immunity; milk production

1. Introduction

Bovine mastitis is a seriously infectious and contagious disease, which is a massive threat to the dairy industry throughout the globe [1]. Mastitis is the inflammation of the mammary gland, which is characterized by physical, chemical, and microbiological alterations in milk, following pathological changes in udder tissue [2]. Bovine mastitis is described as acute or chronic based on inflammation, redness, and localized heat at the infected area, with more severe symptoms, such as fever, leading to septicemia, and the formation of abscesses [3,4]. There are two types of mastitis: clinical and subclinical mastitis. In most cases, infection with Gram-negative bacteria such as *Escherichia coli* (*E. coli*) can often cause clinical mastitis, and Gram-positive bacteria such as *Staphylococcus aureus* (*S. aureus*) are involved in subclinical mastitis infection [5,6].

Bovine mastitis is considered one of the costly diseases of dairy cattle because of milk losses, treatment costs, and rare death [7,8]. In China, the annual losses of 15–45 billion Chinese Yuan (CNY) have been documented [7], while in the US and India, the dairy industry has experienced losses of 2 billion and 526 million dollars, respectively [9]. In Europe, collectively, the cost due to mastitis has reached 1.55 billion euros per year [10]. This increased frequency was linked to public concerns for animal welfare and has made mastitis the key disease of the dairy sector [11]. In addition, bovine mastitis has a major zoonotic risk, correlated with the shedding of bacteria and their toxins into milk [12].

Mammary epithelial cells are the first line of defense of the mammary gland to invading bacteria. They not only act as physical barriers but also are capable of producing inflammatory mediators. While interacting with invading bacteria, mammary epithelial cells generate multiple inflammatory cytokines [13,14]. Several genes and pathways have been reported to be associated with the regulation of bovine mastitis [15]. It is well known that the innate immune system recognizes the presence of pathogens ligands through a membrane receptor family known as Toll-like receptors (TLRs) [16]. TLRs are pattern recognition receptors (PRRs) on the host cell surface that recognize bacterial-pathogen-associated molecular patterns [17]. Upon activation, TLRs further mediate different important signaling, such as that of the JAK-STAT pathway.

Any disruption in the JAK-STAT pathway may lead to various diseases, including bovine mastitis that compromises the immune system of the host. Furthermore, it has also been documented that *STAT5A* works as a mediator for extracellular prolactin receptors. At the same time, *JAK2* plays a role as a bridge between *STAT5A* and prolactin receptor (PrlR), which is essential for milk production and mammary gland development. Keeping in view the vital role of JAK-STAT signaling in immunity, inflammation, and milk production, the current review paper is designed with aims to summarize the role of the JAK-STAT pathway in bovine mastitis and milk production.

2. General Mechanism of the JAK-STAT Pathway Regulation

There are three main components of the JAK-STAT pathway: receptors, Janus kinases (JAKs), signal transducers, and activators of transcription proteins (*STATs*) [18]. The mammalian JAK family consists of *JAK1*, *JAK2*, *JAK3*, and tyrosine kinase 2 (*TYK2*), which are linked to the cytoplasmic domains of diverse cytokine receptors [19]. Among the seven members of *STATs* (*STAT1*-4, 5a, 5b, and 6) in mammalian cells, *STAT5A* and *STAT5B* show high sequence identity and lie closest in a head-to-head pattern next to *STAT3* [19,20]. The members of the STAT family are involved in cell growth, differentiation, cell survival and apoptosis, and mammary gland development. The cytokines, after attachment with receptors on the cell surface, activate JAKs. The two JAKs come close through receptor oligomerization. Furthermore, these JAKs phosphorylate the receptor complex's intracellular tyrosines, generating the docking sites for STATs. Consequently, the activated *STATs* form hetero- or homodimers, where the Src-homology 2 (SH2) domain of each STAT binds the phosphorylated tyrosine of the opposite STAT, and the dimers then translocate to the cell nucleus to induce transcription of the target genes. JAK-STAT has been revealed to operate downstream of several peptide hormones and cytokines that are necessary for the development of the postnatal and secretory

function of the mammary gland [21]. The phosphorylated *STAT5A* and *STAT5B* form homodimers and heterodimers in mammary epithelial cells in order to regulate the process of differentiation, survival, and proliferation through the modification in cellular gene expression [22]. The rapamycin target phosphatidylinositol 3-kinase-protein kinase B/mammalian signaling pathway (PI3K-Akt/mTOR) mediates many cellular processes such as cell proliferation, growth, survival, and metastasis [23], and it is necessary for the development of the mammary gland [24]. A conditional knockout of Akt1 prevents the extensive survival of mammary epithelial cells, which express hyperactive *STAT5*, indicating that the PI3K-Akt/mTOR pathway is a crucial downstream signaling effector of JAK-STAT signaling [25]. To find out the interconnection between different genes and their biological functions in the JAK-STAT pathway, we exploited an online software database for annotation, visualization, and integrated discovery (DAVID; https://david.ncifcrf.gov/) [26], which are summarized in Figure 1.
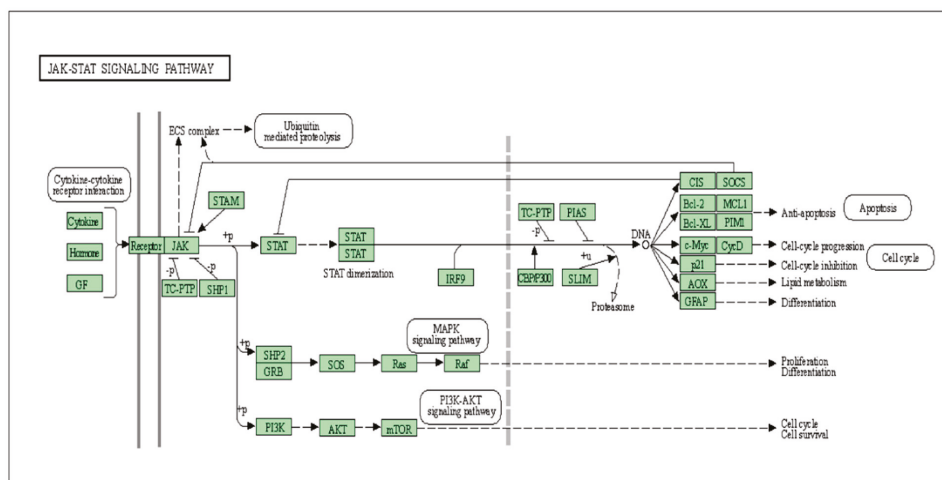
Figure 1. The regulation of the cytokine-activated Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway by cytokines, hormones, and growth factors; engagement of the JAK-STAT pathway in the process of differentiation, survival, and proliferation through the modification in cellular gene expression.

STAT5, being the main gene of the JAK-STAT inflammatory signaling pathway, has an essential role in prolactin-induced mammary gland factor and is assumed to be associated with mammary gland development in transgenic mice [27]. Consequently, upon activation, JAK regulates the cellular mechanisms such as cell migration, apoptosis, cell proliferation, and differentiation, which are essential for hematopoietic responses, immune development, mammary gland development, and the lactation process [28]. Cytokines play a vital role in the regulation of the JAK-STAT pathway, which further facilitates immunity and inflammation. Consequently, the JAK-STAT pathway has been widely studied for its critical role in immunity and inflammation [29,30], and evidence indicates that persistent activation of this pathway might lead to many immune- and inflammatory-related diseases [31,32]. Performing a critical role in immunity, cell proliferation, cell differentiation, and inflammation, the JAK-STAT pathway has been widely targeted for therapeutic purposes in several inflammatory diseases [33].

3. The JAK-STAT Pathway Role in Milk Production in Dairy Cattle

The JAK-STAT pathway regulates lactation [34], while PI3K/Akt within the JAK-STAT pathway shows overexpression in lactating cows [35]. Gene deletion analysis in mice has documented an important role of the JAK-STAT signaling pathway in the lactation and development of the mammary

gland [36,37]. In the mammary gland, the JAK-STAT pathway, along with *SOCS* signaling, plays a critical role in controlling cytokine signals and has shown an association with mammary gland development and milk production [38]. Moreover, studies have documented the essential role of the JAK-STAT pathway in blood cell differentiation and casein gene regulation during milk production [39,40]. It has been shown that some JAK-STAT-associated proteins are regulated by PrlR, which may establish a balance between growth hormone and milk protein yield [41]. It has been illustrated that by using the JAK-STAT pathway, the lactogenic hormones, through their receptors on cell membranes, regulate milk proteins [42]. Prolactin also uses JAK-STAT signaling and regulates the processes of lactation and reproduction in mammals [43]. It has been documented that a higher concentration of Prl in blood circulation is associated with an increased level of milk production in dairy cattle [44]. During hypothyroidism, a severe decrease in milk production has been documented. Furthermore, it has been found that hypothyroidism decreases the level of prolactin, resulting in lower expression of the JAK-STAT pathway, which is responsible for lower milk production in hypothyroidized rats [45].

3.1. Role of JAKs in Milk Production in Dairy Cattle

JAK2 is the tyrosine kinase responsible for phosphorylation of both PrlR and Stat5, based on tissue culture cell studies. According to one report, in the absence of the *JAK2* gene, mammary epithelium proliferation and differentiation were reduced by 95% around parturition [46]. The endocrine factor prolactin attaches to the PrlRs and causes their dimerization. JAK protein kinases are linked to these receptors and these JAK proteins alter a receptor into a tyrosine kinase receptor. The regulated receptors may specifically phosphorylate inactive *STATs*, which result in dimerization. These dimers are further translocated into the nucleus. The *STATs* attach to the upstream promoter elements of the casein gene and cause their transcription. Growth hormones (GHs) control the growth and development of the mammary gland and regulate milk production and milk protein levels in cattle [41,47]. *STAT5* passes on messages from cytokines and growth factors outside the cell to the nucleus of the mammary gland epithelial cells and thereby mediates the transcription of the gene during pregnancy, lactation, and weaning [48].

It has been consistently reported that the polymorphisms T-C39652459 and T-C39645396, at intron 15 and exon 20, respectively, in the *JAK2* gene, are significantly associated with milk lactose production in dairy cows [49]. Furthermore, the variant *JAK2/RsaI* is involved in the regulation of milk and milk protein and can be considered a milk production marker in dairy cattle [50]. The variants 39630048C/T and 39631175T/C in the *JAK2* gene significantly influence milk fat and milk proteins, respectively, in Chinese Holsteins [51]. PrlR uses *STAT5A* and *JAK2* as mediators to activate the proteins associated with milk production traits [52].

3.2. Role of STATs in Milk Production in Dairy Cattle

STATs are activated by specific ligands, i.e., *STAT5A* is regulated by Prl, while STAT5B regulation is mediated through growth hormones (GHs) [53]. STAT5 is an important intracellular mediator of prolactin signaling and can activate transcription of milk proteins in response to Prl. *STAT5* has been suggested to be candidate marker genes for milk protein yield and composition in dairy cattle [54]. During pregnancy, *STAT5A* and PrlR play essential roles in mammary epithelium proliferation and differentiation [55,56]. Consequently, it has been found that PrlR has a positive impact on lactation performance in cows, possibly due to its involvement in steroid synthesis and cholesterol regulation [57]. During pregnancy and lactation, STAT5A and STAT5B are the essential proteins required for the synthesis of luminal progenitor cells from mammary stem cells and the differentiation of milk-producing alveolar cells [58]. *STAT5A* and *STAT5B* have been linked with the development of the mammary gland during pregnancy [59]. It was previously found that *STATs* promote the mammary gland cells' survival by mediating the promoters of genes essential for milk proteins [34,60]. *STATs* facilitate various peptide hormones and cytokines in targeted cells such as Prl and GH and are linked to milk production. Whey acidic protein (WAP) is expressed in the mammary gland and is associated with

the improvement of milk protein. *STAT5* has been considered an important transcription factor that is responsible for the regulations of Prl at 5' flanking regions of *WAP* [61]. It has been observed that the downregulation of Prl in hypothyroidized rats causes the inhibition of the transcriptional activity of *STAT5*. Consequently, any abnormality in the thyroid gland severely affects milk production efficiency in rats because of the low level of Prl [45]. In addition, GH also regulates the STAT1 gene and its expression has been reported during mammary gland development [62,63]. Furthermore, a study has reported the combination effect of STAT1 with other JAK-STAT signaling members on milk production traits [38]. Keeping in view the important role of *STATs* as a mediator of prolactin signaling, the polymorphisms in these genes were further studied for its role in milk production.

The mutations in the *STAT5A* gene have been reported for their effect on milk yield [64]. Consistently, the *STAT5A/AvaI* polymorphism at position C-T 6853/exon7 was documented to be associated with milk production and could be used as a significant marker for milk improvement [65]. In addition, the *STAT5A/MslI* locus has been found to be correlated with milk yield, milk fat, and protein [65–67]. The polymorphic site A14217G and 17266indelCCT in *STAT5A* have shown significant associations with milk protein percentage and milk yield, respectively [68]. Consequently, Schennink et al. documented that single nucleotide polymorphism (SNP) 9501G>A in *STAT5A* significantly influenced milk fat composition [69]. Khatib et al. noticed that variant 12195T/C in *STAT5A* was significantly linked to a decrease in milk fat and protein percentage in dairy cattle [70]. The variant 31562 T>C in *STAT5B* was reported to be associated with milk yield and milk protein [71]. The association of *CD4* and *STAT5B* with milk traits might be due to their role in the regulation of prolactin-induced mammary gland factor [72]. Moreover, the variant in the STAT 1 gene has been documented to be linked with milk fat, milk protein, and milk yield in dairy cattle [73]. Consequently, the polymorphism STAT1/BspHI has been reported to be associated with milk production traits in Jersey cows [74]. Similarly, Deng et al. reported that SNPs in STAT1 have a significant association with milk production traits and could be a useful addition to the marker-assisted selection for milk production [75].

The above findings reveal that the JAK-STAT pathway plays a central role in the regulation of milk production traits.

4. The JAK-STAT Signaling Role in Bovine Mastitis

As mastitis is an immunity- and inflammatory-related disease, scientists have widely targeted the JAK-STAT pathway in bovine mastitis control research. Besides having a critical role in mammary gland development, any abnormal regulation may disturb the normal function of the JAK-STAT pathway, resulting in impairment of mammary gland development and exposure to mammary infections. Buitenhuis et al. found the altered expression of the JAK-STAT pathway in the mammary gland tissue of cows challenged with *E. coli* [76]. It is well known that the JAK-STAT pathway is regulated by IFN, LPS, or growth factors. In its turn, JAK-STAT signaling mediates proinflammatory cytokines. Tiezzi et al. documented the JAK-STAT pathway as a key pathway that regulates clinical mastitis [77]. Recently, it has been reported that cirsimarin (an extract of *Cirsium japonicum* var. ussuriense) treatment suppressed the expression of inflammatory cytokines by downregulating the phosphorylation of the JAK-STAT pathway in the mammary gland. Thus, this substance can be targeted as a therapeutic agent in many inflammatory diseases, including bovine mastitis [78]. It has been shown that 8-methoxypsoralen treatment protects bovine mammary epithelial cells against lipopolysaccharide-induced inflammatory injury by inhibition of the JAK/STAT and NF-κB pathways [79]. JAK-STAT suppression by xanthotoxin resulted in the downregulation of IL-6, nitric oxide (NO), and tumor necrosis factor (TNF-α) induced by LPS in bovine mammary epithelial cells [80]. This mechanism is essential for regulating udder reactions to infection as it controls the chronic accumulation of neutrophils in the bovine mammary gland [81], whereas JAK also serves as a signaling agent for hormones and interleukin receptors [82] and *JAK2* is considered one of the top-rated genes of bovine mastitis tolerance [83].

4.1. Association of JAK2 Activity with Bovine Mastitis

Any dysfunctions of the JAK-STAT pathway may expose cattle to mastitis because of abnormal activation of the proliferation and apoptosis of cells. From this point of view, it can be expected that mutations in genes involved in the JAK-STAT pathway might be a target in bovine mastitis research. The inflammatory- and immunity-associated diseases are polygenic traits [71], and polymorphisms in immunity-linked genes can regulate the immune responses of the host to pathogens [84]. Two major approaches are dominantly targeted by animal scientists to control mastitis. The first approach is to look for major genes associated with mastitis resistance, while the second one is to target the polymorphisms within genes and their links with mastitis resistance traits.

Many types of mutations in the JAK-STAT pathway have been identified; most of them are related to *JAK2*.

As demonstrated in Table 1, the polymorphism 39630048C/T in *JAK2* is associated with interleukin-17 (IL-17) [85], IL-6, and interferon-gamma (IFN-γ) expression [51]. Furthermore, the SNPs (39652267A/G, 39631175T/C) in the *JAK2* gene have been documented for their significant links with milk somatic cell counts (SCCs), IL-6, and IFN-γ [51,85]. Mutation 39631044G/A in the *JAK2* gene was noticed to be significantly associated with milk somatic cell scores (SCSs) in Chinese Holsteins [85]. Moreover, the polymorphism 39645396C/T in the *JAK2* gene was linked to milk SCCs, IL-6, and IFN-γ [86], while SNP-39631044G/A in *JAK2* was associated with milk SCSs [85]. SCCs and SCSs are widely targeted as early mastitis indicators [7]. Increased SCCs in early lactation can signify the presence of intramammary infection, and, in many countries, the indirect selection against mastitis using milk SCCs is practiced [87]. However, in the early phase of infectivity, the neutrophil and inflammatory cytokine levels increase quicker than milk SCCs [88]. That is why, nowadays, more interest is paid to the increase in cells and cytokine levels in milk and blood, respectively, rather than just the overall SCC, which may provide an early status of udder health [89]. A study showed that inflammatory cytokines (IL-6, IL-17, and IFN-γ, TNF-α) could be used as subclinical mastitis indicators, in addition to SCSs and SCCs [51,86,90]. In addition, it is predicted that the 39645396C/T SNP changes lysine to asparagine [86]. The expression of IL-6 was higher in plasma cell mastitis (PCM), which indicated that the IL-6/STAT3 pathway could play a key role in the pathogenesis of PCM [22,91]. The IL-17 family consists of cytokines that participate in acute and chronic inflammation and provoke the host's defense against microbial organisms [92]. T-helper 17 cells are thought to be a significant source of IL-17A; furthermore, IL-17, producing innate immune cells, activate the fast release of IL-17A [93] in response to pathogens or tissue injury [94].

Table 1. Association of SNPs in *JAK2* with bovine mastitis resistance phenotypic traits.

Gene	Mutation	Reference	Position	Phenotypic Traits	Authors
JAK2	C-T/EXON16	rs210148032	Chr8:39652267	SCC	[51,85]
JAK2	C-T/EXON20	rs110298451	Chr8:39645396	IL-6, IFN-γ, SCC	[51,86]
JAK2	C-T/3′ flanking region	rs135128681	Chr8:39630048	IL-6, IFN-γ, SCC	[51,85]
JAK2	T-C/3′ flanking region	Novel	Chr8:39631175	IL-6, SCC	[51,85]
JAK2	G-A/3′ flanking region	Novel	Chr8:39631044	SCS	[85]
JAK2	5′ upstream	rs379754157	Chr8:39750638	SCC	[49]

IL-17 has been shown to be significantly upregulated in goat milk infected with *E. coli* or *S. aureus* [95]. *IL-17A* production was documented during *S. uberis* mastitis [96], and slightly increased expression was also noticed in the somatic cells of cows infected with *S. aureus* [97]. Furthermore, an in-vitro study illustrated that IL-17A reinforces the ability of mammary epithelial cells (MECs) to resist the consequences produced by *S. aureus* [98]. It has been reported that *IL-17A* and *IL-17F* play a critical role in regulating host–pathogen interactions during the development of mastitis [99]. The SNPs in *IL-17F* and *IL-17A* have been shown to be associated with milk SCCs [90]. Moreover, IL-17 also activates IL-6 with IFN-γ and tumor necrosis factor-alpha (TNF-α) [100]. Usman et al. revealed that

IL-6 is the best indicator of mastitis and can be a target in mastitis control strategies [85]. Altogether, the above-published studies show that IL-17, IL-6, IL-4, IFN-γ, SCS, and SCC are the key indicators of mastitis. The interactions of polymorphisms in *JAK2* with bovine mastitis resistance phenotypic traits (IL-17, IL-6, IL-4, IFN-γ, SCS, and SCC) show that *JAK2* might be considered a useful marker in bovine mastitis resistance strategies.

4.2. Role of STATs in Bovine Mastitis

A variety of cytokines and growth factors activate *STATs*, which are a family of latent transcription factors. During the process of inflammation, *STAT5B* regulates CD4+ T-cells differentiation [101]. STAT1 raised the expression of *SOCS3* and SOCS1 in *S. aureus*-infected mammary epithelial cells [102]. Furthermore, it was reported that upon treatment with JAK inhibitors, the plasma cells in PCM decreased considerably due to the suppression of IL6/STAT/JAK signaling, resulting in the reversion of pathogenesis [91]. Accordingly, it was found that the inflammatory cytokines regulate the JAK-STAT pathway in the mammary gland; in response, the phosphorylation of STAT takes place. The phosphorylated STAT translocates into the nucleus and mediates the production of proinflammatory genes that facilitate mastitis's pathogenesis [78]. It is known that the inflammatory cells are recruited towards the site of infection, in which T-cells, particularly CD4+ cells, are predominantly observed in bovine mastitis [103]. Rivas et al. revealed that *S. aureus*-infected dairy cows showed a remarkable elevation in the level of CD4+ T-cells at the early stage of infection in the mammary gland [104]. Eder's team recently proved that the CD4+T-cell level was higher in dry cows compared to lactating cows. These findings show that a decrease in the level of CD4+ T-cells in lactating dairy might be one of the reasons for susceptibility to infection during this stage [105]. Usman et al. reported a significant association of variant T104010752C in the *CD4* gene with milk SCCs [90]. In the previous study, it was noticed that polymorphisms in *CD4* and *STAT5B* genes are significantly linked with mastitis-resistance phenotypic traits [83]. Furthermore, the polymorphism in *CD4* at locus g.13598C>T showed a significant association with SCS, which is the crucial indicator of mastitis.

The combination geneotype analysis of CD4 g.13598C>T and STAT5b g.31562 T>C is associated with milk SCSs in Chinese Holsteins. Furthermore, it was reported that cows with combination genotypes of CCTT show the highest estimated breeding value (EBV) for SCSs [71]. Another study documented that the silencing of the *CD4* gene through DNA methylation influences the progress of CD4+ T-cells in inflammatory conditions [106]. These findings demonstrate that CD4 protein and CD4+ T-cells play essential roles in host defense during the development of mastitis.

As demonstrated in Table 2, the polymorphism in *STAT5A* (43046497A/C) is associated with IL-6 and also changes the amino acid isoleucine to valine [85]. Similarly, mutation at point 43673888A>G in the *STAT5B* gene was significantly linked to mastitis-resistance phenotypic traits (IL-4 and SCC) [86]. Bochniarz et al. reported the elevated level of IL-6 and decreased level of IL-4 in the milk and serum of cows infected with *S. aureus* [107]. In addition, the polymorphism STAT5A-AvaI was associated with milk SCCs and electrical conductivity (EC) in the milk of mastitic cows [108]. EC in milk is one of the essential indicators of bovine mastitis because of its association with Na and Cl levels, which increase during mastitis. Cai et al. also reported a STAT5A gene through genomewide association studies (GWAS) as a potential candidate marker for bovine mastitis resistance [109]. Based on the above-published findings, we concluded that *STAT5A* and *STAT5B* might be target mastitis-resistance markers in dairy cattle.

Table 2. Association of SNPs in *STAT5A* and *STAT5B* with bovine mastitis resistance phenotypic traits.

Gene	Mutation	Reference	Position	Phenotypic Traits	Authors
STAT5A	A-C/Intron 9	rs109358395	Chr19:43046497	IL-6	[85]
STAT5B	A-G/Intron 4	rs41915686	Chr19:43673888	IL-4, SCC	[86]
STAT5b	T-C/EXON 8		Chr19:31562	SCS	[71]

5. Inhibitors of the JAK-STAT Pathway: Role in Mastitis and Milk Production

The protein inhibitors of activated STAT (PIAS) [110], protein tyrosine phosphatases (PTPs) [111], and cytokine signaling suppressors (SOCSs) [112] are three major classes used by cells to control the JAK-STAT pathway [113]. PIAS proteins are considered important transcriptional coregulators of JAK-STAT signaling because of their significant contribution to the control of gene expression [114]. PIAS proteins restrict the regulation of the JAK-STAT pathway in three ways: (1) by adding a small ubiquitin-like modifier (SUMO) group to STAT and blocking its phosphorylation, (2) by preventing the binding of STAT to DNA [115], and (3) by recruiting histone deacetylase to remove acetyl changes to histones by lowering gene expression [116]. Similarly, PIAS3, a member of the PIAS family, has been identified to inhibit STAT3 signaling after regulation by the cytokine IL-6 [117]. Moreover, PIAS1 could inhibit NF-κB and JAK-STAT activity regulated by cytokine TNF and the LPS endotoxin [110]. PIAS has a major role in cell proliferation [118], cell apoptosis, and the immune response [115]. Protein tyrosine phosphatases (PTPs) are a group of enzymes that remove the phosphate group from the JAK-STAT pathway and prevent the action of signaling [119]. The *STATs* are deactivated by PTPS in both the nucleus and cytoplasm. Src homology phosphatase 2 (SHP-2) is one of the members of PTPs that inactivate STAT5 in the cytoplasm. Similarly, SHP1 prevents the phosphorylation of the JAK-STAT pathway and blocks its further action [120,121]. The general role of JAK-STAT inhibitors has been summarized by recently published reviews in more detail [31,122]. Although the two groups of PTPs and PIAS have essential roles in the regulation of the JAK-STAT pathway, their tasks have not been evaluated in milk production or bovine mastitis to date. Therefore, we have only focused on cytokine signaling suppressors (SOCSs) in our current review.

Some SOCS proteins are triggered by cytokines and pathogenic mediators and, thus, function in a classical negative-feedback loop to impede the transduction of cytokine signals. Consequently, they represent an effective mechanism for the negative regulation of the cytokine-mediated JAK-STAT pathway [123]. The DNA binding of STAT protein regulates the mRNA expression of SOCSs [124]. *SOCS3* can inhibit JAK tyrosine kinase activity directly via its kinase-inhibitory region (KIR), which has been proposed to serve as a pseudosubstrate and is essential for cytokine signal suppression [125]. Undeniably, both a KIR and a KIR-mimetic peptide, classified as the tyrosine kinase inhibitor peptide (TKIP), have been described to inhibit *JAK2*-regulated transcription factor STAT1 phosphorylation [126,127]. The SH2 domain of SOCS can also directly bind to the receptors and prevent the signal from passing to JAK-STAT signaling [128]. Moreover, Kimura et al. revealed that LPS could activate *JAK2* and *STAT5*, which participate in the induction of IL-6, while SOCS1 inhibits this process selectively [129].

The suppression of IL-6 and IFN-γ usually occurs around parturition, which depresses immunity and exposes dairy cattle to mastitis [130]. Normal levels of IL-6 and IFN-γ are necessary for the maintenance of bovine immunity. Moreover, *SOCS3* has been reported to be one of the key inhibitors of IL-6 and IFN-gamma. This evidence shows that *SOCS3* might have a potential role in mastitis development in dairy cattle [131]. Moreover, Fang et al. found that *SOCS3* was significantly upregulated after the mammary gland had been infected with *S. aureus*. The authors further supposed that *SOCS3* could negatively regulate the JAK-STAT pathway, which might be one of the reasons for its critical role in mastitis development [132]. Huang et al. also reported that *SOCS3* is a negative regulator of the JAK-STAT pathway. Furthermore, it was demonstrated that overexpression and inhibition of *SOCS3* brought visible changes in milk protein, which might be due to the action of *SOCS3* on the JAK-STAT pathway [133]. The Huang team further suggested that a low level of *SOCS3* is essential for the regulation of milk synthesis. Similarly, a study reported that *SOCS3* inhibits the induction of Prl and activation of STAT5 [134]. Zahoor et al. found that merTK reduces the inflammatory changes induced by *S. aureus* through *STATs*/SOCS3 signaling [102]. Furthermore, it has been revealed that impaired SOCS1/3 has a crucial role in the susceptibility of mammary epithelial cells to *S. aureus* infections. Additionally, a study reported a polymorphism in SOCS2, which was significantly associated with susceptibility to inflammation of the mammary gland [135]. *SOCS3* also has an inhibitory role in STAT5

regulation, which is one of the strong reasons for their influence on lactational performance in dairy cattle. Further study is highly recommended to find out the specific variants in *SOCS3* that interact with *STAT5* and *JAK2* during mastitis development and milk production in dairy cattle.

6. Conclusions

Altogether, it can be concluded that a delicate equilibrium must be achieved for the effective activation of the JAK/STAT pathway, when the immune system is needed for action against infection, and proper restoration when the infection is diminished. Thus, the JAK-STAT pathway can be considered as a therapeutic option in mastitis control and enhancement of milk production strategies. Furthermore, it is suggested that the interactive mechanism of *SOCS3*, *STATs*, and *JAK2*, *STAT5A*, and *STAT5B* during milk production and mastitis development should be considered in future rodent-knockout research models. It is highly recommended that further polymorphisms in STAT1 and *SOCS3* and their associations with milk production and mastitis resistance traits be found out. Finally, PTPs and PIAS are critical inhibitors of the JAK-STAT pathway, so research on the evaluation of their role in bovine mastitis would be an interesting development.

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Review

Improvement of Disease Resistance in Livestock: Application of Immunogenomics and CRISPR/Cas9 Technology

Md. Aminul Islam [1,2,3], Sharmin Aqter Rony [4], Mohammad Bozlur Rahman [5], Mehmet Ulas Cinar [6,7], Julio Villena [2,8], Muhammad Jasim Uddin [1,9,*] and Haruki Kitazawa [2,3,*]

[1] Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh; aminul.vmed@bau.edu.bd

[2] Food and Feed Immunology Group, Graduate School of Agricultural University Science, Tohoku University, Sendai 980-8572, Japan; jcvillena@cerela.org.ar

[3] Livestock Immunology Unit, International Research and Education Centre for Food and Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai 980-8572, Japan

[4] Department of Parasitology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh; s.a.rony@bau.edu.bd

[5] Department of Livestock Services, Krishi Khamar Sarak, Farmgate, Dhaka 1215, Bangladesh; mbozlur@gmail.com

[6] Department of Animal Science, Faculty of Agriculture, Erciyes University, 38039 Kayseri, Turkey; mucinar@erciyes.edu.tr

[7] Department of Veterinary Microbiology & Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA 99164, USA

[8] Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli, (CERELA), Tucuman 4000, Argentina

[9] School of Veterinary Science, Gatton Campus, The University of Queensland, Brisbane 4072, Australia

*   Correspondence: m.uddin2@uq.edu.au (M.J.U.); haruki.kitazawa.c7@tohoku.ac.jp (H.K.); Tel.: +61-07-3870-0830 (M.J.U.); +81-22-757-4372 (H.K.)

Simple Summary: Disease resistance is the ability of animals to inhibit the growth of invading pathogens within the body, which is influenced by the interaction of the host immune system, host genetics, and the pathogens. Resistant animals can be produced by molecular breeding by introducing the genomic marker responsible for disease resistance or immunocompetence. Immunogenomics is an information science that enables the genome-scale investigation of host immune response to pathogenic infection thereby identification of the genomic marker for disease resistance. Once the genomic marker is determined, it could be implemented in producing disease resistance animals by applying the advanced reproductive biotechnology like genome editing. The technical ease and decreasing cost over time might enhance the application of genome editing techniques for producing disease resistance livestock.

Abstract: Disease occurrence adversely affects livestock production and animal welfare, and have an impact on both human health and public perception of food–animals production. Combined efforts from farmers, animal scientists, and veterinarians have been continuing to explore the effective disease control approaches for the production of safe animal-originated food. Implementing the immunogenomics, along with genome editing technology, has been considering as the key approach for safe food–animal production through the improvement of the host genetic resistance. Next-generation sequencing, as a cutting-edge technique, enables the production of high throughput transcriptomic and genomic profiles resulted from host-pathogen interactions. Immunogenomics combine the transcriptomic and genomic data that links to host resistance to disease, and predict the potential candidate genes and their genomic locations. Genome editing, which involves insertion, deletion, or modification of one or more genes in the DNA sequence, is advancing rapidly and

may be poised to become a commercial reality faster than it has thought. The clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) [CRISPR/Cas9] system has recently emerged as a powerful tool for genome editing in agricultural food production including livestock disease management. CRISPR/Cas9 mediated insertion of *NRAMP1* gene for producing tuberculosis resistant cattle, and deletion of *CD163* gene for producing porcine reproductive and respiratory syndrome (PRRS) resistant pigs are two groundbreaking applications of genome editing in livestock. In this review, we have highlighted the technological advances of livestock immunogenomics and the principles and scopes of application of CRISPR/Cas9-mediated targeted genome editing in animal breeding for disease resistance.

1. Introduction

Foods from the livestock are a vital source of high-quality protein. Efficient production of animal-originated food is one of the major issues for global food safety, a long desire for a healthy population. The occurrence of infectious diseases in livestock affects not only the farm production, economics, and animal health-welfare; but it also increases the risk of zoonoses. Therefore, the outbreak of infectious diseases in food animals is a major threat to food safety and public health. The emergence of antimicrobial drug resistance along with the unavailability of effective vaccines and spontaneous genetic mutation of infectious pathogens are the major perils for breaking down the disease control strategies [1]. On the other hand, there is a growing consumer demand to produce organic animal food by securing animal welfare standards. Raising disease-free healthy livestock is the key to produce safe meat and milk. Good husbandry practices including optimum housing, ration, use of probiotics, biosecurity, and vaccination should be taken into account for the maintenance of health and production of farmed animals [2]. However, the improvement of host genetic resistance to disease may potentially contribute to the profitability through improved animal welfare and reduced antibiotic usage in livestock production [3].

Disease resistance is the host's ability to restrict or inhibit the establishment of infections and/or pathological processes of infectious pathogens [4]. Interactions among the host immune system and invading pathogens, and host genome determine the fate of infection and disease pathogenesis (Figure 1). Innate immunity is the frontline host defense against invading pathogens, and genes associated with the induction of innate immune responses are considered as the potential candidates for disease resistance [5]. Innate immune responses provide immediate and non-specific defense against a wider range of pathogens; therefore, innate immune response traits are the first choice to be incorporated in the disease-resistant breeding plans. Notably, the heritability of the innate immune response is medium to moderate [6]. The contribution of host genetic factors to disease occurrence is one of the fundamental issues in understanding disease pathogenesis and host resistance. The variation in the genetic resistance to disease is due largely to the variability in the host's immune response to infection. Thus, information on immunology and genomics together would better characterize the disease phenotype [4]. The application of genomic technologies to understand the immunology is termed as immunogenomics. Immunogenomics include integrated analysis of immunologic and genomic data on host response to infectious pathogens, and thereby contribute to identifying potential candidate genes for disease resistance in livestock [7]. The single nucleotide polymorphisms (SNPs) within the candidate gene associated with host immunocompetence to infection can subsequently be considered as a DNA marker for disease resistance.

Genome editing is a bio-engineering technology that involves insertion, deletion, or modification of a specific section of DNA sequence in the genome. The genome-editing technology encompasses

nuclease enzyme for cutting DNA sequence, in addition to a targeting mechanism that guides the enzyme to a particular site on the genome [8]. The clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) [CRISPR/Cas9] system is one of the latest genome-editing tools that has been widely used over the last couple of years (reviewed by Pellagatti et al. [9]). Genome editing in livestock has become a commercial reality due to the emergence of CRISPR/Cas9 technology. Advance use of CRISPR/Cas9 could facilitate the improvement of disease resistance in livestock through (a) enhancing the frequency of favorable trait-associated alleles, (b) introgression of favorable alleles from other breeds/species, and (c) by generating de novo favorable alleles [10]. However, the main challenge is the identification of genome-editing targets for a disease-resistant trait, which will require a combination of high-quality annotated livestock genomes, well-powered genome-wide association studies, and robust knowledge of molecular genetics of pathogen-host immune system interactions. In this review, we have systematically explained how the gift of immunogenomics could be applied to grasp the disease resistance candidate genes and use of the biological scissor, CRISPR/Cas9 technology, for insertion or deletion of desired genes in the host genome (Figure 1). The prospects, regulations, and social acceptance of genome editing technology concerning improving the livestock health and production are also discussed.
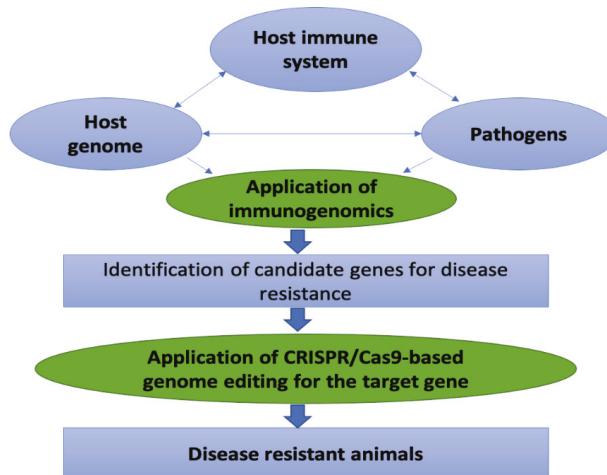
Figure 1. Schematic diagram showing the applications of immunogenomics and genome editing to produce disease-resistant animals. Severity and pathogenesis of disease depend on the interaction of the host immune system and the invading pathogens, where host genetic has potential influence. Immunogenomics employ the integrated bioinformatics tools to explore the influence of host genetic on the interaction between the host immune system and invading pathogens and subsequently identify the candidate gene (s) for disease resistance. The CRISPR/Cas9 mediated genome editing technology could subsequently be employed for targeted modification of the host genome to produce disease-resistant animals.

2. Disease Resistance: The Phenotype

Host-pathogen interactions result in either death or survival of the affected animal. Survivors either remain healthy and unaffected by the pathogens or experience a course of a disease that recover afterward. The immune system plays a crucial role in maintaining a balance between health and disease pathogenesis. In general, disease resistance demonstrates the host's ability to limit or prevent the replication of invading pathogens [11,12], and the relationship between the host and pathogens is better understood from an ecological point of view [13]. This concept includes several ways through which a host becomes comparatively more robust. For example, to display less possibility of infection, to have

a reduced proliferation rate once infected, to possess a reduced rate of shedding or transmission [4]. It is not easy to measure the disease resistance trait, which is a major challenge for the investigator. It is possible that phenotype of disease resistance is relative rather than absolute and altered resistance impacts on the population as a whole because few attributes favor the individual host in certain cases, but other attributes (such as lower rate of transmission and infection) favor other population members. There is another associated phenotype called disease resilience, the capacity of the host to suppress the establishment or development of infection. Disease resilience is a physiological state in which an infected animal is capable of sustaining an acceptable degree of efficiency despite the burden of the pathogen [14]. The prospects and possibilities of using disease resilience in animal breeding programs are reviewed by Berghof et al. [15]. In addition, tolerance is another closely related phenotype that indicates the ability of the host to maintain the body homeostasis in the presence of replicating pathogens, with limited pathological consequences [12]. In a mixed population, the presence of asymptomatic and disease tolerant individuals may increase the genetic resistance of individual hosts, but there is a risk of increasing the prevalence of disease on the farm. Therefore, the disease resistance phenotype could consider to be incorporated into the host genetic improvement strategy.

Absolute quantification of the disease resistance phenotype under field condition is very difficult due to logistic and economic constraints, which is one of the major rate-limiting steps in animal breeding for disease resistance. Therefore, targeting immunocompetence trait is the indirect approach for improving disease resistance in livestock, and thereby producing safe milk and meat in the age of antibiotic resistance. Immunocompetent animals possess higher metabolic function and resilience to a wider range of infectious diseases, thereby improving the production performance in terms of quality and quantity. The host resistance to infectious diseases in livestock could be enhanced by incorporating resistance genes in the host genetics. The most economically important diseases in different livestock species could be considered first in the breeding goals for more sustainable production of disease-resistant animals [3]. Some infectious diseases of economic importance are mastitis, foot and mouth disease (FMD), tuberculosis, John's disease, and tick-borne diseases in cattle, Peste des petits ruminant (PPR) in goats, porcine reproductive and respiratory syndrome (PRRS), African swine fever in pigs, and gastrointestinal (GI) parasite infection in cattle and sheep [16].

Breeds of different animal species have a great influence on the innate resistance to infectious disease. Native breeds usually exert a higher degree of resistance to pathogens than the high-yielding exotic breed of the same species, which is believed to be due to the baseline immune competence, mainly innate immunity which could be transmitted down the progeny via genetic information and colostrum. Indigenous breeds of cattle (*Bos indicus*) were found to have a higher resistance to tick infestation and tick-borne diseases compared to high-yielding crossbred cattle as evidenced by higher hemolytic complement activity [17]. Native breed's resilience to local pathogens could be gained through evolution and adaptability when raised in the extensive farming system [18]. On contrary, intensive farming may weaken hosts' innate immunity. The relative difference in immunocompetence to infectious disease was reported among porcine breeds [19,20]. The genetic components associated with disease resistance should incorporate into the breeding program. Advances in tools and techniques for studying immunology and genetics of livestock and data analyses by immunogenomics enabled animal scientists to implement the molecular breeding technique including marker-assisted selection, genomic selection, and targeted genome editing for sustainable livestock production.

3. Advances of Immunogenomics

Immunogenomics is an information science that deals with big data from immunology and genomics. Immunogenomics integrates the molecular interactions among the host genome, immune system, and the invading pathogens. Understanding the function of the mammalian immune system has been broadening since the intersection of immunology and high-throughput sequencing technologies. Immunogenomics combines the transcriptome, DNA variants, polymorphisms/SNPs, and quantitative trait loci (QTL) mapping data resulted from host-pathogen interactions through a series of integrated

bioinformatics [7]. Thus, immunogenomics have been considered as an efficient omics tool for identifying disease resistance genes or DNA markers [7]. Transcriptome profile provides an overview of expressed genes associated with particular phenotypes and is used as guidelines for subsequent analyses by proteomics, metabolomics, epigenomics, and other omics approaches. Since transcriptome can explore the relationship between genotype and phenotype of an organism, it has been considered as the most informative assay to start with for the functional genomics. Microarray and next-generation sequencing (NGS) are the cutting-edge technologies for transcriptomics or immunogenomics in many areas of the life sciences [21]. In parallel to the revolutionary progress of NGS technology, some advanced tools for cell biology studies are equally impactful. For example, flow cytometry and mass spectrometry/cytometry can provide a better picture of the phenotypic diversity among immune cell subsets [22]. Besides, the development of the induced pluripotent stem cell (iPSC) model from peripheral T cells has opened up another exciting avenue of immunology research [23].

3.1. Sequencing Technology

As a cutting-edge tool for immunogenomics, the NGS platform has rapidly evolved over the past 15 years, and exponentially increased amounts of sequence data generated per instrument run at ever-decreasing costs [24]. NGS-based RNA sequencing (RNA-Seq) can quantify all sorts of RNA species including messenger RNA (mRNA), microRNA, small interfering RNA, and long non-coding RNA, which enables the researcher to discover novel RNA forms and variants. The NGS methodology has been extended through the development of single-cell RNA sequencing (scRNA-seq) and in-situ RNA sequencing [24–26]. A single cell is the smallest structural and functional unit of a living organism, as such a particular cell represents a specific unit of molecular coding across the DNA, RNA, and protein expression [27]. Therefore, the omics-based investigations are highly expected to be carried out at the single-cell level for more precise results. The scRNA-seq has tremendous progress in the last couple of years owing to overcoming the difficulties of isolation of a single cell population [28]. The technological progress and application of the single-cell sequencing platform about cancer research have been well-summarized in Huang et al. [28]. The scRNA-seq has recently advanced along with the development of whole-genome sequencing such as scRNA methylation sequencing and single-cell assay for transpose-accessible chromatin sequencing (Single-cell ATAC-seq) [28]. Recently, the direct RNA sequencing using high throughput nanopore sequencing technology has emerged as the latest state-of-the-art RNA-Seq technique [29]. However, the single-molecule, long-read sequencing-based NGS technology is coming soon which may replace the ongoing platforms [30].

Currently, Illumina and Thermo Fisher's (Ion Torrent) standard and commonly used NGS platforms are based on short-read sequencing technologies [31]. To build sequencing ready libraries with an average length of 300 bp (ranging from 200–700 bp), short-read RNA-seq requires either fragmentation followed by reverse transcription or full-length cDNA synthesis followed by fragmentation. Since most mammalian RNA transcripts are 1–2 kb in length [32], getting complete RNA sequencing information relies on agreement with the annotated whole transcriptomic sequence or de novo transcriptome assembly approaches. In addition, genes have more than a transcriptional isoform that confronts the performance of the NGS in accurate gene expression quantification. The mRNA molecules transcribed from the same locus are referred to as transcriptional isoforms because mRNA can be produced from different transcriptional start sites, terminated at different polyadenylation sites, or as a result of alternative splicing [33]. Due to the limitations of short-read sequencing, it is difficult to assemble all expressed isoform for each gene and quantify the expression of all the isoforms with currently available bioinformatics tools [34,35]. To address these limitations, two commercial companies have recently launched the single-molecule, long-read sequencing technology-based NGS platform: Pacific Bioinformatics (PacBio), and Oxford Nanopore Technologies (ONT). With these technologies, the read length achieved (~15 kb for PacBio and >30 kb for ONT) exceeds the length of most mammalian transcripts. Combined with the benefit of full-length cDNA synthesis [36]. Especially SMARer (Switching Mechanism at RNA Termini) technology, long-read technologies are commercially available

from Clontech (Mountain view, CA, USA), makes full-length mRNA sequencing possible with more precise transcriptomic studies. While a very powerful approach to unraveling the full spectrum of gene expression profiles is represented by long-read technologies, the relatively high costs of these technologies have prevented the broader spectrum of use. Oikinomopoulos et al. [30] reviewed the recent scientific and methodological developments in transcriptome profiling using the newly introduced single-molecule, long-read sequencing technology.

3.2. Bioinformatics Tools

While in the NGS platform, sequencing has become relatively straightforward with overcoming the technological limitations, the processing of upstream samples and downstream data analyses are still labor-intensive. To make a meaningful biological analysis, big data obtained from microarray and RNA-seq experiments requires high-power statistics and rigorous bioinformatics. Although most of the RNA-Seq data analysis algorithms can be run either in a Unix environment or inside the R/Bioconductor environment [37] from a command-line interface, some web-based menu-driven interfaces (e.g., Galaxy (www.usegalaxy.org), Geneious (www.geneious.com)) also support NGS data analysis. For the identification of DNA variants/SNPs associated with features like disease resistance, NGS sequence reading has been an area of rapid development [38]. Data from DNA variants (SNPs) or genome-wide association studies can be incorporated to explore the association between genomic architecture and the traits of interest once the potential candidate genes have become available from transcriptome analysis. The Animal QTL database (QTLdb) has been developed to bridge genotypes and phenotypes by repositing all publicly accessible QTLs and SNP/gene association data on animal species [39]. Approximately 191,422 QTLs have been curated to date, including 142,261 for cattle, 30,580 for pigs, 12,246 for chicken, 3305 for sheep and 2446 for horses (https://www.animalgenome.org/cgi-bin/QTLdb/index, Release 41, 26 April 2020). In order to analyze, annotate and visualize such genomic data for complex phenotype, such as immune repertoires, including disease resistance, bioinformatics software tools are essential components [40].

The keystone of immunogenomics is data integration. Accordingly, the scientific community can benefit from data sharing strategies that facilitate the integration of datasets among research groups. However, reliable methods for data integration are needed and require a broad range of expertise such as in mathematical and statistical models, computational methods, visualization strategies, and deep understanding of complex phenotypes. The commonly used tools and databases for immunogenomics of animals are summarized in Table 1.

Table 1. Bioinformatic tools or database commonly used for transcriptomics or immunogenomics studies aiming to identify the key regulatory genes in animals.

Bioinformatics Tools/Databases	Potential Implications	References
'Bowtie', 'msa'	Sequence-read alignment,	https://cran.r-project.org/
R/Bioconductor, limma, DESeq2	Differential gene expression analyses	https://cran.r-project.org/
GSEA-Gene Set Enrichment Analysis	Gene set enrichment analysis	[41] https://www.gsea-msigdb.org/gsea/index.jsp
DAVID	Gene ontology and pathway analysis	[42] https://david.ncifcrf.gov/
KEEG-Kyoto Encyclopedia of Genes and Genomes	Gene ontology and pathway analysis	https://www.genome.jp/kegg/

Table 1. *Cont.*

Bioinformatics Tools/Databases	Potential Implications	References
InnateDB	Database for gene ontology, pathway analysis and prediction interactome	[43] https://www.innatedb.com/
REACTOME	Database for gene ontology and pathway analysis	[44] https://reactome.org/
QTLdb	Database of quantitative trait loci of animals	[39] https://www.animalgenome.org/ cgi-bin/QTLdb/index
BovineMine	Annotation and functions of gene	[45] http://128.206.116.13: 8080/bovinemine/begin.do
bioDBnet-Biological database network	Interconnected access to many types of biological databases, conversion of gene or protein identifies	[46] https: //biodbnet-abcc.ncifcrf.gov/
STRING	Functional protein association network analysis and visualization	[47] https://string-db.org/
NetworkAnalyst	Co-regulatory gene or protein network analysis and visualization	[48] https://www.networkanalyst.ca/
WGCNA	Weighted gene co-expression network analysis	[49] https://cran.r-project.org/
Cytoscape	Creation and visualization gene network	[50] https://cytoscape.org/

While in silico genomics tools and databases for the human genome have been developed well, those for the genomes of livestock are still growing. Thus, an ID conversion tool, such as bioDBnet [46], is required to convert gene ID to human orthologous identifiers to perform the downstream functional analysis using human database. BovineMine, for instance, is a useful database and web portal for data mining for the annotation of bovine genes and genomes [45]. Standardize laboratory workflows, raw data formats, experimental designs, and methods of biostatistical analyses are, however, required. The technological shortcomings of immunogenomics are being resolved and are likely to be put in the life sciences among the largest 'big data' enterprises [51]. The Functional Annotation of ANimal Genomes (FAANG www.faang.org) was developed in this regard to support the standard protocol for core research, data research and meta-data analysis of standards for swine immunogenomics studies [52]. In addition, the 1000 Bull Genome Project has provided the bovine research community with a huge volume of data on bovine variants that will be useful for GWAS and the identification of causal mutations (http://1000bullgenomes.com). These initiatives pave the way for a systemic incorporation of the findings of bovine immunogenomics studies and for making them available online.

4. Applications of Immunogenomics in Livestock Disease Management

Sustainable management of livestock diseases requires breeding techniques that protect the environment, animal welfare, and public health, as well as providing adequate financial rewards for farmers. Several efforts are in progress to develop a disease-resistant stock through molecular breeding. By dissecting the genetic makeup, a typical first stage of molecular breeding by marker-assisted selection, genomic selection, or genome editing is to determine the extent in genetic variation on the individual trait. Simple understanding of host immunology and genetics better characterize the disease-resistant phenotype. The advent of immunogenomics provides more accurate identification of candidate biomarkers for disease resistance, which makes it easier to enhance disease resistance by genome editing techniques.

In a real-life situation, the direct estimation of disease resistance phenotype is very difficult because a precise animal identification and disease regression method and infection challenge routines are required [53]. The disease resistance phenotype is typically quantified in the direct disease model by calculating the magnitude of the infection, such as burden of the inside-host pathogen (e.g., viral load), and these are technically difficult to incorporate in the intensive commercial production system. In addition, the direct measurement techniques target the host's resistance to a particular infection. However, when introduced by molecular breeding, it can result in increased susceptibility to other infections [54]. Appropriate indirect methods are therefore highly desirable for estimating disease resistance. One method may be to estimate the phenotype of disease resistance by defining the genomic marker associated with the immunocompetence of the host produced from vaccination that is inheritable and linked to improved health and production performance [55,56]. Vaccination does not cause disease, but it allows memory T-lymphocytes, B-lymphocytes, and antibodies to be formed by the host immune system, enabling hosts to defend the subsequent infection [57]. The most suitable candidates for disease resistance traits are possibly genes regulating the first few hours of the host's response during innate immunity to infection or vaccination [5,58]. Due to the rapid onset and wider range of defense, innate immune traits have the potentials to be used in the selection of livestock for disease resistance [53], and the innate immune traits display significant genetic variation among the breeds of livestock species [6,59]. Thus, as a possible indirect indicator of disease resistance, enhancing innate immunocompetence is of great importance.

To identify the potential biomarkers for immunocompetence as indirect measures of disease resistance in livestock, the study population could be divided into two contrast phenotypes correlated to disease resistance (Figure 2). The contrast phenotypes may be achieved by either taking animals with the extreme immune response phenotype following vaccination (extremely high antibody titre vs. extremely low antibody titre) or vaccinated vs. control animals. In order to obtain adequate statistical power from the experiment, the use of an optimum number of animals as biological replicates for each group should be considered. Schurch et al. [60] proposed that for the detection of substantially differentially expressed gene between two contrast phenotypes using RNA sequencing technology, at least six biological replicates should be used. The single population of target primary cells should be separated once the experimental group is fixed in order to prevent cell-type-specific gene expression. Then, a pure single-cell population should be subjected to the extraction of total RNA and genomic DNA separately. To scan the full spectrum of gene expression between the contrast phenotypes, the quality-assured total RNA samples can be subjected to holistic transcriptome profiling by RNA-seq. The RNA samples could be used for profiling for proteomics and metabolomics. On the other hand, SNP sequencing and genotyping, quantitative trait loci (QTL) mapping, and genome-wide association study (GWAS) may also be subjected to DNA samples, and epigenomics analysis targeting the phenotype of disease resistance. Rigorous integrated bioinformatics framework on all sets of omics data together helps us to identify the molecular biomarkers for intended immunocompetence trait. Those could be suggested as the targets for CRISPR/Cas9 mediated genome editing technology after functional validation of the identified biomarkers in the independent population.

Applications of immunogenomics in human disease particularly in cancer have much progress such as the in-silico prediction of human leukocyte antigen (HLA) gene has been accelerated through a combination of high-throughput sequencing technology and specialized computational approaches [61]. Immunogenomics have been employed to explore the porcine resistance to Gram-negative bacterial infection in porcine [7] and gastrointestinal nematode infection in ruminant [62]. Knowledge of immunogenomics has been applied in the identification of several immune response genes in livestock based on their association with resistance or susceptibility in several diseases and their proven function in disease pathogenesis [16]. Several GWAS have been performed aiming to identify the QTLs or SNP profiles associated with resistance or susceptibility to bovine mastitis [63], and milk somatic cell count as an indicator of mastitis [64,65]. Many immune response genes are associated with mastitis resistance including cytokines IL-4, IL-8, IL-13, and IL-17 [66,67]. Nevertheless, further fine-tuning of

the genomics tools and in silico omics software recourses in near future would enable the identification of disease resistance candidate biomarkers in a more precise way.
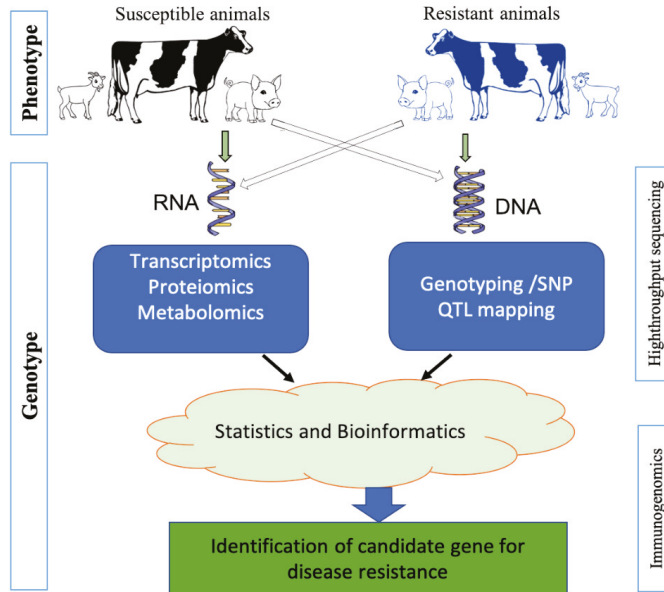

Figure 2. A working pipeline of (in vivo, in vitro, and in silico) immunogenomics for identification of disease resistance candidate gene/marker as prospective targets for genome editing. Isolation of single-cell population of a target from both phenotypic groups followed by RNA and DNA extraction separately. The RNA samples could be employed for proteomics and metabolomics profiling. On the other hand, DNA samples could also be subjected to single nucleotide polymorphisms (NSP) sequencing and genotyping, quantitative trait loci (QTL) mapping, and genome-wide association study, and epigenomics study targeting the disease resistance phenotype. Rigorous integrated bioinformatics application on all sets of omics data together enables us to identify the molecular biomarker for the target immunocompetence trait. After functional validation of the identified biomarkers in the independent population, those could be recommended as the targets for CRISPR/Cas9 mediated genome editing technology.

5. Advances of Genome Editing Technology

The premier seed for the possibility of a desired improvement of the mammalian genome has sown by Palmiter and Brinster in 1988 [68]. The active insertion into the mouse embryos by pronuclear microinjection of a foreign DNA fragment, a growth hormone gene called metallothionein-I, resulted in the rapid growth of the animals as targeted [68]. This technique of genome engineering based on cells was restricted to injecting plasmids or gene fragments into the embryos pronucleus. Subsequent advances in genome engineering have been achieved by the introduction of transposons or retroviral vectors [69], followed by the development of homing endonuclease (HEs), natural meganuclease capable of introducing double-strand breaks (DSBs) at 14–40 bp target sites [70]. Nevertheless, in vivo application of HE-based genome modification has been limited due to their off-target cutting propensity. In general, modern genome editing relies on DNA insertion, deletion, or substitution in the genome of a living organism using programmable nuclease-based editors (Figure 3). Zinc finger nuclease (ZFNs) [71,72] and transcription activator-like effector nuclease (TALENS) [73] were the most widely used genome editors until recently. Zinc fingers (ZFs) are among the most well-characterized DNA-binding protein domains found in the nature that have enhanced the programmed modification

repertoire of enable any target genome to be accurately cut and repair [72]. A second group of naturally occurring proteins containing a DNA-binding domain and formed by proteobacteria of the genus Xanthomonas is transcription activator-like effector (TALE) [73]. A pair of ZFNs must bind regions flanking the target locus for genome editing to form a FokI dimer, which is required to induce DSBs (Figure 3A) [74]. Similarly, TALENs are also modular proteins that contain two domains: a customizable DNA-binding domain (TALE) and the FokI nuclease domain. The TALE-binding DNA sequence is cut by dimerization and thus produces DSBs in a similar manner to ZFNs (Figure 3B) [75]. However, both ZFNs and TALENs are limited by targeting multiple sites in the same genome. The comprehensive interaction of ZFNs with protein-DNA and the highly repetitive nature of TALENS warrant the advent of new instruments.
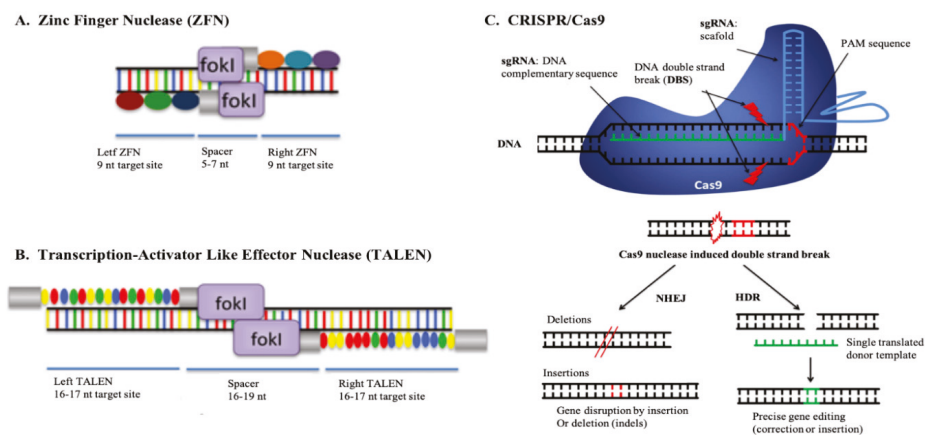

Figure 3. Nuclease-based genome editors. (**A**). Zinc Finger Nuclease (**B**). Transcription-Activator Like Effector Nuclease (TALEN). (**C**). Schematic diagram showing genome editing using CRISPR/Cas9 system. The Cas9 induces DNA double-strand break (DSB) which are repaired either by imperfect nonhomologous end-joining (NHEJ) to generate insertion or deletion (indels) or if a repair is provided, by homology-directed repair (HDR) (Adapted from Moore et al. [71], and Pellagatti et al. [9].

The clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system, which was built from an inherent antiviral mechanism found in bacteria [76], is the latest addition to the genome toolbox. In comparison to a classical genetic modification that involves moving genes from species to another, the CRISPR/Cas9 system relies on the use of molecular 'scissors' to introduce changes in existing DNA sequences [75,77]. The CRISPR/Cas9 system uses a single guided RNA (sgRNA) to help the Cas9 nuclease to classify the particular genomic sequence, unlike the ZFNs and TALENs, which use proteins to recognize specific sequences in the genome (Figure 3C). In the presence of sgRNA complementary sequence and the Porto-spacer adjacent motif (PAM) sequence [9], the Cas9 protein binds to the sgRNA scaffold and generates a DSB. The DSB activates the machinery for the repair of endogenous cellular DNA that catalyzes non-homologous end joining (NHEJ) and homology direct repair (HDR) (Figure 3C). The NEHJ pathway is preferably used by most cell types, which is possibly an error-prone mechanism that generally results in minor insertions or deletions at the site of repair. NEHJ also produces a mutation and induces encoded protein fragmentation or functional gene knockout by producing DNA break in the coding sequence of a gene. On the other hand, the HDR pathway can be activated through flooding the target cell with a DNA repair template, which allows the implementation of specific sequence changes proximal to the cut site ranging from single base changes to the insertion of transgenes. In order to make minor change, a synthetic single-stranded oligodeoxynucleotide is used, whereas larger modification requires the plasmid/dsDNA template. Finally, two simultaneous breaks are produced and transcriptional profiles are altered either by

removing regulatory elements or by deleting exons. Protein domains are subsequently deleted, leaving the remaining reading frame intact [78]. One of the CRISPR/Cas9 system's key advantage is that the Cas9 nuclease is not covalently fused to a DSB, so it is possible to use same protein to attack multiple target sites by combining it with a different sgRNA package. Besides, most of the necessary reagents can be made in the molecular biology laboratory, while Cas9 nuclease and sgRNA molecules can be purchased commercially. The CRISPR/Cas9 system has made the genome-editing technique a practical reality in recent years due to its methodological simplicity, performance, precision, flexibility, and a greater degree of accuracy.

A variety of techniques are available to produce the genetically modified animals depending on the species and cell type used to deliver the Cas9 genome-editor (reviewed by Harwood et al. [79]). The technique of sperm mediated gene transfer (SMGT) is used to deliver the genome-edited reagents into a zygote. Manipulation of spermatogonial stem cells (SSCs) or primordial germ cells (PGCs) is often used for the development of genome-edited organisms. For the development of genome-manipulated animals, the pronuclear and intracytoplasmic injection of genome editors into zygotes accompanied by either direct transfer to surrogates or in vitro maturation and embryo transfer could be applied. In addition, somatic cell nuclear transfer (SCNT) is another approach that enables precise edits to be selected in somatic cells before nuclear transfer to surrogates or in vitro maturation. The injection of edited embryonic or iPSC into blastocyst may also be used for the development of chimeric animals examined by Harwood et al. [79]. Like the genome-editor tool use, the techniques follow is also important to achieve the intended efficiency and precision in generating genome-edited animal species. The introduction of genome editing in the research animal model for the treatment of human disease has also made tremendous progress, in addition to the development of CRISPR/Cas9 techniques in animal disease management. Recent reviews have summarized the methodological advances of CRISPR/Cas-9 mediated genome editing in large animals (pigs, monkeys, dogs, rabbits, mice, rats) with a focus on the creation of model animals for studying human diseases [80,81].

6. Applications of Genome Editing in Livestock Disease Management

The CRISPR/Cas9-based genome editing has been applied in livestock for several specific (but not limited to) purposes: (a) for inactivation or alteration of expression of targeted genes in the model animals to confirm their functions (b) for producing research animals for studying human disease pathogenesis in controlled setup and evaluate potential therapies, (c) and producing genetically modified animals for industrial, pharmaceutical, and biotechnological implications [82]. Nevertheless, the success of the gene-editing system in controlling animal diseases would be affected by several factors. For instance, the proportion of gene-edited animals and how they are distributed within and across the population. According to epidemiological theory, a certain proportion of gene-edited animals required to achieve herd immunity, and to prevent disease transmission within the population [83]. Disease-specific epidemiological modeling could provide information on the required number of genome-edited animals for preventing certain disease/infectious agents. Such modeling should consider the influence of population structure, demographic characteristics, diverse environmental factors, and management strategies that affect the disease transmission dynamics to estimate the size of the population for genome editing. The latest advance in genome editing with programmable nucleases, such as CRISPR/Cas9, has opened up new avenues for animal breeding targeted with disease resistance. However, the efficiency of genome editing varies due to the variation in reproductive physiology among different livestock species. Genome editing in cattle accompanied by major challenges due to high market value, a small number of offspring, and longer gestation period (9 months gestation, 12–18 months to reach puberty). While pigs, sheep, and goats have several advantages as they are smaller, cheaper, and produce more offspring at a time, shorter gestation lengths, and shorter ages of puberty. Over time, genome editing has been successfully applied in livestock species to improve different traits like growth, production performance, and resistance to certain diseases.

Myostatin (*MSTN*) gene is known to be associated with growth and skeletal muscle development. *MSTN* was targeted in the earlier attempts of genetic manipulation in farm animals because the disruption of this single gene has significant effects on meat production, an economically important trait. To date, genome editing has been successfully applied to knockout the *MSTN* from the genomes of cattle, pigs, sheep, and goats (reviewed by Petersen [8]). In cattle, the introduction of antibiotic lysostaphin by SCNT resulted in secretion of milk protein that has bactericidal activity against mastitis-causing bacteria *Staphylococcus aureus* [84]. The gene encoded with bovine whey protein ß-lactoglobulin (BLG), which is a major milk protein and a dominant allergen, was successfully knocked out in the bovine genome using ZFNs technique [85]. Another genome editing study has described the production of swine with mutation RELA (p56) gene using ZFNs, which confer the tolerance of African swine fever [86]. Production of a hornless strain of dairy cattle (Holstein Frisian) by inserting the pooled gene (P) of beef cattle (Angus) through the HDR pathway of gene editing has also been reported [87]. With the evolution of CRISPR/Cas9-based genome editing and a deeper understating of how to gear up its potential, it is now possible to introduce extremely precise changes to the genome with better accuracy and efficiency than any previous attempts. The CRISPR/Cas9 mediated insertion of *NRAMP1* gene for producing tuberculosis resistant cattle and deletion of *CD163* gene for producing porcine reproductive and respiratory syndrome (PRRS) resistant pigs are two groundbreaking application of genome editing technique in livestock. Hereinbelow, we summarized some prominent examples of implication of CRISPR/Cas9-based genome editing to produce disease-resistant livestock (Table 2).

Table 2. Reported application of genome editing techniques for disease resistance livestock breeding.

Species	Disease	Targets of Genome Modification	Reference
Goat	Mastitis	Lysozyme (human)	[88]
Cattle	Mastitis	Lysostaphin (*Staphylococcus simulans*)	[84]
	Enzootic pneumonia	Cluster of differentiation 18 (CD18)	[89]
	Tuberculosis	The natural resistance to infection with intracellular pathogens 1 (NRAMP1) gene	[90]
Pigs	African swine fever	RELA	[86]
	PRRS	Histone deacetylase HDAC6	[91]
		Cluster of differentiation 163 (CD163)	[92,93]

6.1. Porcine Reproductive and Respiratory Syndrome (PRRS) in Pigs

PRRS is considered the most economically important infectious disease of the swine industry worldwide affecting the production, reproduction, health, and welfare of pigs. The global transcriptome profiling of peripheral blood mononuclear cells revealed that pigs can induce innate and subsequent adaptive immune response to PRRS virus infection or vaccination [92,93]. The host transcriptomic response to PRRSV has been found to have substantial genetic variation [19,20]. The cluster of differentiation 163 (CD163) is a cell surface receptor gene, which is a member of scavenger receptor cysteine-rich superfamily (SRCR) having one intracellular domain and nine extracellular SRCR domains [94]. The CD163 facilitates the PRRS virus to enter into the pulmonary alveolar macrophage through endocytosis, where the virus replicates and induces disease pathogenesis [94,95]. Whitworth et al. [96] reported for the first time that CRISPR/Cas9 mediated CD163-knockout pigs were fully protected against the clinical outcome of PRRS virus infection with a single isolate [96]. A subsequent experiment demonstrated that the SRCR5 domain is crucial for establishing viral infection [97]. The implication of reproductive biotechnology for the production of genome-modified pigs might therefore significantly reduce the PRRS-associated economic losses in the pork industry.

6.2. African Swine Fever Resistance in Pig

Another economically relevant infectious disease caused by the African swine fever virus (ASFV) in pigs is African swine fever (ASF). Many areas of sub-Saharan Africa are endemic to the ASF. It has been detected recently in Eastern Europe, from where it is rapidly spreading to both Western Europe and China. Native wild swine breeds including the Warthog, have been reported to be resilient to ASFV infection, whereas domestic pigs experience cytokine storm-related lethal hemorrhagic fever. Significant variation in the expression of the RELA gene between resilient and susceptible pigs is associated with host response to ASFV infection [98]. The RELA is a part of the Nuclear Factor kappa Beta (NFkB) transcription factor, considered to play an important role in stress management and immune defense. By using the ZFN-based genome editing technique [99], the sequence of RELA gene in domestic pigs could be translated to that of Warthog pigs. However, the phenotypic data supporting the genetic resistance to ASFV infection yet to be reported.

6.3. Tuberculosis Resistance in Cattle

Bovine tuberculosis (bTB) is a chronic bacterial disease that is crippling and caused by *Mycobacterium bovis* in cattle. With a wide host range, *M. bovis* infection create considerable hardship for livestock producers with estimates of more than 50 million cattle infected worldwide [100]. This zoonotic infection can be transmitted to humans mainly through the ingestion of milk products that are not pasteurized, resulting in a 10–15% prevalence of human TB [99]. Compulsory testing accompanied by the slaughter of test-positive animals, which accounts for as huge economic loss, is an important means of bTB regulation. The bTB is as a crucial target for genome editing for producing tuberculosis resistant cattle, due to its economic and zoonotic importance, endemic existence, and failure of conventional control strategies. As a strong candidate gene for tuberculosis resistance, natural resistance to infection with intracellular pathogens 1 (*NRAMP1*) gene has been reported by several studies. In a recent study, Cas9 nickase (nCas9) was used by scientist to insert the *NRAMP1* into the genome of the bovine fetal fibroblast [90]. These engineered fibroblast cells were then used as donor cells in somatic cell nuclear transfer, where the *NRAMP1*-containing donor cell nucleus was inserted into the cow's ovum. Before being transferred to recipient cows following a physiologically natural estrous cycle, ova were then nurtured in the laboratory up to embryos. The inserted *NRAMP1* gene was correctly expressed and provided cattle by showing a higher degree of resistance to *M. bovis* infection [90]. It has also been reported that resistance against *M. bovis* infection could be achieved in cattle through TALEN-mediated insertion of mouse SP110 gene into an intergenic region of the bovine genome [101].

6.4. Enzootic Pneumonia Resistance in Cattle

Pasteurellosis in cattle also called shipping fever or enzootic pneumonia is a respiratory disease complex mostly found in recently weaned, single-sucked beef calves after housing or transport to a new house. Following infection, *P. hameolytica* secretes a leukotoxin that is cytotoxic and it binds to the uncleaved signal peptide of the CD18 protein on the surface of leukocytes. However, the mature CD18 lacks the signal peptide in the leukocytes of other species (e.g., mouse and human) that do not suffer from this disease. ZFNs have been used to introduce a single amino acid change in the bovine CD18 protein and leukocytes from the resultant cattle were able to inhibit the *P. hameolytica* leukotoxin-induced cytotoxicity [102].

7. Ethics, Regulations, and Social Acceptance of Genome-Edited Livestock Products

Manipulation of genomes in farm animals is becoming a lucrative and materialistic alternative. However, problems such as regulatory legislation, market pay off, and performance acceptability of users are still unresolved. The societal attitude towards genome-edited animal products worldwide will depend on whether the handling was carried out with due regard to the ethical value of target customer community and the issue of animal welfare [103]. The resulting foods after genome-edited

livestock should appear on the commercial market ready to be distributed to consumers immediately, with the required legislative approval of a country. However, the contested factors linked to genetically modified (GM) as well as cloned animals suggest that food derived from genome-edited animals is socially rejected or unable to accept it. Psychological studies have shown that several factors such as the consumer's perceived risks of the consumer, the recognized benefits and/or the confidence in regulatory legislation, can cause the acceptability of GM animals by researchers [104]. The public's skeptical view of GMOs (GM organisms) is partly related to the degree of skepticism of both researchers and state policymakers [105]. Off-target mutagenesis seems to be a big problem due to the widespread use of the CRISPR/Cas9 tool. In relation to the breeding of disease resistant animals, the problem is of more serious concern. The debate on closed animals, stimulated by so many tests, prompted the study of off-target mutation. Exploration of off-target mutations tends to be crucial from the point of view of animal health and ethics to the broader implications of genome-editing techniques in animal breeding [103]. Therefore, in addition to scientific ethics, ethical concerns of animal health and welfare to minimize the imminence of off-target mutations are required to enhance public understanding. This could advocate the social acceptance of genome-edited livestock products gradually.

8. Potentials and Prospects of CRISPR/Cas9 Technology in Livestock Production

The CRISPR/Cas9 exhibits the potentials for substantial improvement over the gene-editing technologies in the ease of application, speed, efficiency, and cost involved. The genome-editing technique has been extensively used for elucidating the gene function in disease pathogenesis and host immune responses. Indeed, the CRISPR/Cas9-based correction of gene mutation in a mouse model of human disease and the primary adult stem cells derived from patients suffering monogenic hereditary defects are being considered the cornerstones for future gene therapy technique [9]. The CRISPR/Cas9-mediated knockout libraries could also potentially be applied to target regions of interest in the noncoding regulatory segment of the genome, such as promoter and enhancer. Moreover, the application of CRISPR/Cas9 in genome-wide studies will facilitate the holistic screening of disease resistance markers. The CRISPR/Cas9 technique would also enable a much wider range of modifications, for instance, gene knockout, base-pair substitution, targeted insertion/deletion of larger genomic regions, and modulation of gene expression [8]. Despite many challenges, remarkable advancements in the field of gene therapy and CRISPR/Cas9-based genome editing technique have been observed in recent years, which paves the way for the development of sustainable disease control strategies for humans, crops, fish, and livestock. Though unlikely in crops, where the whole population can rapidly be replaced, the application of genome editing in the livestock population is a more complex and time-consuming process. Moreover, increasing the efficiency of the CRISPR/Cas9-based repairing process, particularly to increase the rate of gene correction and reduce resultant off-target effects and the development of more effective delivery methods would require for its wider application.

9. Conclusions

Raising immunocompetent healthy livestock is crucial for the sustainable production of safe food. Understanding the genetics behind the host immunocompetence to infectious pathogens is the key to improve the level of disease resistance through molecular breeding approaches. Exploring the variation of host innate immunocompetence to economically important infectious diseases among indigenous, exotic, and cross-bred animals of the same species can be of important starting point toward estimating the genetic components of disease resistance phenotype. The cutting-edge techniques for immunological and molecular genetics study may create a direct linkage between disease-resistant phenotype and the host genotype. Continuous advancement of open-source in silico omics tools will identify the potential genomic marker, the target for genome editing for disease resistance in livestock. The application of modern reproductive biotechnology, such as CRISPR/Cas9 mediated genome editing, is a breakthrough tool for improving disease resistance in livestock due to its high

precision. Minimizing the risk of off-target mutations would restore the animal welfare standard and increase consumer acceptance of food products derived from genome-edited livestock.

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Identification of SNPs Associated with Somatic Cell Score in Candidate Genes in Italian Holstein Friesian Bulls

Riccardo Moretti [1,†], Dominga Soglia [1,†], Stefania Chessa [1,*], Stefano Sartore [1], Raffaella Finocchiaro [2], Roberto Rasero [1] and Paola Sacchi [1]

[1] Department of Veterinary Science, University of Turin, 10095 Turin, Italy; riccardo.moretti@unito.it (R.M.); dominga.soglia@unito.it (D.S.); stefano.sartore@unito.it (S.S.); roberto.rasero@unito.it (R.R.); paola.sacchi@unito.it (P.S.)

[2] Associazione Nazionale Allevatori Razza Frisona e Jersey Italiana—ANAFIJ, 26100 Cremona, Italy; raffaellafinocchiaro@anafi.it

* Correspondence: stefania.chessa@unito.it; Tel.: +39-011-6709-255

† Co-first author: these authors contributed equally to this work.

Simple Summary: Mastitis is a worldwide diffused disease usually treated with an excessive use of antibiotics. Therefore, antimicrobial resistance is an important issue to be addressed by scientists. One of the possible solutions to decrease the use of drugs is genetic selection of resistant animals, that is, individuals that can be more resistant to mastitis. In our survey we analyzed Single Nucleotide Polymorphisms (SNPs) in genes known to be involved in both infection resistance and immune system activity. We found a group of SNPs that can be associated to mastitis related phenotypes (namely SCS) and that can be used for selecting resistant animals. An efficient selection is able to improve both animal welfare and quality and safety of animal products

Abstract: Mastitis is an infectious disease affecting the mammary gland, leading to inflammatory reactions and to heavy economic losses due to milk production decrease. One possible way to tackle the antimicrobial resistance issue stemming from antimicrobial therapy is to select animals with a genetic resistance to this disease. Therefore, aim of this study was to analyze the genetic variability of the SNPs found in candidate genes related to mastitis resistance in Holstein Friesian bulls. Target regions were amplified, sequenced by Next-Generation Sequencing technology on the Illumina® MiSeq, and then analyzed to find correlation with mastitis related phenotypes in 95 Italian Holstein bulls chosen with the aid of a selective genotyping approach. On a total of 557 detected mutations, 61 showed different genotype distribution in the tails of the deregressed EBVs for SCS and 15 were identified as significantly associated with the phenotype using two different approaches. The significant SNPs were identified in intergenic or intronic regions of six genes, known to be key components in the immune system (namely *CXCR1*, *DCK*, *NOD2*, *MBL2*, *MBL1* and *M-SAA3*.2). These SNPs could be considered as candidates for a future genetic selection for mastitis resistance, although further studies are required to assess their presence in other dairy cattle breeds and their possible negative correlation with other traits.

Keywords: Holstein Friesian cattle; mastitis resistance; candidate genes; SNP selection; next-generation sequencing

1. Introduction

Mastitis is an infectious disease that affects the mammary gland of cattle, leading to an inflammatory reaction and therefore to negative economic consequences due to a marked decrease in milk production [1]. This infection is usually caused by microorganisms penetrating the mammary gland via teat canal [2]: pathogens can be transmitted either between cows (e.g., *Staphylococcus aureus*) or picked up from the environment (e.g., *Escherichia coli*) [3]. Bovine mastitis is considered as one of the costliest diseases affecting dairy cattle

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worldwide, with antimicrobial therapy representing the major impact on sustained costs [4]. Furthermore, given the high mastitis frequency worldwide, the subsequent antibiotic use in dairy cows is under constant control due to its association with antimicrobial resistance increase [5].

One possible way to tackle the antimicrobial resistance issue is to select animal with a higher genetic resistance to this disease [6]. Starting from the 50's, along with its genetic relationship with additional infection-related phenotypes, the possible inheritance of genetic resistance to mastitis was studied through the years [7,8]. However, the first traits to be selected, like mammary gland characteristics and somatic cell count (SCC), revealed to have low to moderate heritability [9]. Therefore, new genetic approaches to find markers able to allow a faster and more accurate selection are requested, with two potential available candidates: genome scanning and single nucleotide polymorphisms (SNPs) in candidate genes [6]. A holistic approach such as genome scanning and/or a Genome Wide Association Study (GWAS) requires a large effect and/or a large number of animals to detect loci associated with traits of interest, while complex diseases are determined by many loci or genes with a small or almost negligible effect [10]. Therefore, the SNP approach in candidate genes involved in organism recognition, leukocyte recruitment, pathogen elimination and resolution seem to be more direct and reliable.

At first, we selected nine genes that are all involved in the immune response to mastitis infection according to literature. Pentraxin 3 (*PTX3*) gene maps on *Bos taurus* autosome 1 (BTA1) and its encoded 45 kDalton glycosylated protein is expressed by mononuclear phagocytes, dendritic and endothelial cells in response to primary inflammatory signals, due to complement activation and pathogen recognition [11]. Chemokine (C-X-C motif) receptors 1 and 2 (*CXCR1* and *CXCR2*, respectively) are paralogous genes coding for major proinflammatory cytokine receptors [12]. They both map on BTA2 and are separated by a 23 Kb fragment. *CXCR1* and *CXCR2* have been considered as prospective genetic markers for mastitis resistance in dairy cows [13,14]. Deoxycytidine kinase (*DCK*) maps on BTA6 and has a functional role in drug resistance and sensitivity [15]. Toll like receptor 4 (*TLR4*) maps on BTA8 and is associated with the early innate immune response. Specifically, *TLR4* is recognized as the key transmembrane receptor for the detection of gram-negative bacteria [16]. Nucleotide binding oligomerization domain containing 2 (*NOD2*) maps on BTA18 and is a key component in the innate immune system, inducing the activation of proinflammatory signaling pathways [17]. Mannose binding lectin 1 and 2 (*MBL1* and *MBL2*, respectively) map on BTA28 and BTA26, respectively. MBLs are collagenous lectins involved in the innate immune response to various microbial pathogens and are potential candidate gene to mastitis resistance [18]. Lastly, mammary Serum amyloid A3.2 (*M-SAA3.2*) maps on BTA29 and belongs to a superfamily of apolipoproteins expressed in bovine mammary gland as a response to pathogens associated to mastitis [19]. Another candidate gene found in a genomic region close to *DCK*, namely the immunoglobulin J (joining) chain gene (*JCHAIN*), was then included in the re-sequencing to find new possible candidate SNPs: this gene plays an important role in the assembly of polymeric immunoglobulins (dimeric IgA and pentameric IgM) and in their selective transport across epithelial cell layers [20].

The aim of this research was to analyze the genetic variability of these genes in Italian Holstein bulls and to study the effects of their SNPs in relation to resistance to mastitis [21].

2. Materials and Methods

2.1. Animal Data

The deregressed Estimated Breeding Values for Somatic Cell Score (dEBVs) were obtained by the National Breeding Association (ANAFIJ) for a total of 15,562 Holstein bulls. The dEBVs were calculated as for the genomic national evaluation with the algorithm developed by ANAFIJ. We chose to analyze individuals within the two extreme tails of the dEBVs distribution (± 1 SD) of bulls born between 2002 and 2012. Given the 11 years range of bulls' birth year, dEBVs were expressed as deviation from the mean dEBVs updated in

April 2014. Only bulls with a reliability index > 90 and with availability of semen were retained, resulting in the two following subsets: 37 bulls with low dEBVs (group L, <95) and 58 bulls with high dEBVs (group H, >105). Group L dEBVs ranged from 85 to 94 (92.0 ± 2.3, mean ± SD), while group H was composed by bulls with dEBVs values ranging from 106 to 112 (107.9 ± 1.8, mean ± SD). Reliability of the two groups was (mean ± SD) 92.4 ± 0.2 and 92.6 ± 0.2 (L and H, respectively). The mean number of daughters for each bull was 310 ± 74 and 300 ± 57 (L and H, respectively). Semen doses of the selected bulls were obtained from specialized laboratories for semen cryo-conservation.

2.2. Genes Data and Re-Sequencing

The DNA of the 95 bulls was re-sequenced in order to detect all the SNPs present in the sequences of the investigated genes. Information were obtained from NCBI database [22], "Gene" section (available from https://www.ncbi.nlm.nih.gov/gene/).

The assembly of the UMD Bovine Genome 3.1.1 was taken as a reference for all the genes (Table 1). The *PTX3* gene mapped on the strand AC_000158.1, corresponding to the sequence of BTA1 in position 1: 111,027,803–111,033,868. The *CXCR1* and *CXCR2* genes both mapped on the strand AC_000159.1, corresponding to the sequence of BTA2 in position 106,936,887–106,938,583 and 106,900,465–106,915,876, respectively. *JCHAIN* was located upstream *DCK* in position 87,759,435–87,768,832 on BTA 6, while *DCK* gene mapped on the strand AC_000163.1 in position 88,049,498–88,077,488. The *TLR4* gene mapped on the strand AC_000165.1, corresponding to the sequence of BTA8 in position 108,828,899–108,839,913. The *NOD2* gene mapped on the strand AC_000175.1, corresponding to the sequence of BTA18 in position 19,177,563–19,212,607. The *MBL1* and *MBL2* genes mapped on the strands AC_000185.1 (BTA28) and AC_000183.1 (BTA26), respectively, in position 35,840,848–35,846,070 and 6,343,615–6,348,912, respectively. Lastly, *M-SAA3.2* gene mapped on the large strand AC_000186.1, corresponding to the sequence of BTA29 in position 26,755,567–26,759,547.

Table 1. Position of the selected genes on UMD bovine genome 3.1.1, target region selected for resequencing, genes' upstream and downstream regions sequenced, and gene sequencing coverage (COV).

GENE (CHR)	Gene Position		ReSeq Region		Upstream	Downstream	COV
PTX3 (BTA1) [1]	111,027,803	111,033,868	111,026,949	111,032,707	854	−1161	64%
CXCR1 (BTA2)	106,936,878	106,938,583	106,935,752	106,942,024	1126	3441	88%
CXCR2 (BTA2)	106,900,475	106,915,876	106,899,301	106,917,188	1174	1312	73%
JCHAIN (BTA6)	87,759,435	87,768,832	87,758,532	87,770,133	903	1301	85%
DCK (BTA6)	88,049,498	88,077,488	88,043,812	88,077,721	5686	233	78%
TLR4 (BTA8)	108,828,899	108,839,913	108,818,057	108,841,671	10,842	1758	81%
NOD2 (BTA18)	19,177,563	19,212,607	19,166,798	19,213,798	10,765	1191	83%
MBL2 (BTA26)	6,343,615	6,348,912	6,332,528	6,349,772	11,087	860	64%
MBL1 (BTA28) [1]	35,840,848	35,846,070	35,839,722	35,856,132	10,062	1126	88%
M-SAA3.2 (BTA29)	26,755,567	26,759,547	26,749,896	26,760,832	5671	1285	82%

[1] reverse oriented gene.

Genomic DNA was extracted from semen by using NucleoSpins Tissue kit (Macherey-Nagel, Düren, Germany). The primers were designed using the Design Studio web application by Illumina® to sequence the entire genes and about 10,000 bp of the upstream regions to search for polymorphisms that could be responsible of the gene expression and be related with resistance to mastitis also in the 5′ UTR regulatory regions. The maximum length of the amplicons for each gene was 450 bp, trying to maximize the coverage of the target region. The primers generated by the software were included in a TruSeq® kit custom amplicon. All the obtained amplicons were sequenced by Next-Generation Sequencing technology on the Illumina® MiSeq platform at the IGA technology Services (Udine, Italy), which also performed the output data processing the variant and genotype call and generated a Variant Call File (VCF) for each gene. Polymorphisms were filtered on the base of locus GQX (genotype quality assuming position, <10,000), GQ (genotype

quality, <30.00), R8 (indel repeat length, >8), and MQ (mapping quality, <0.00). Other considered parameters were indel, site conflicts, and read depth. The genotype table was set up in R environment inferring all the identified allelic variants from the VCF files. Allelic frequencies were calculated and mutations with Minor Allele Frequency (MAF) < 0.05 were removed from the dataset.

2.3. SNP Analysis

An investigation in the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov) was carried out on the SNPs with MAF $\geq$ 0.05 to verify if they were already present in available databases, to define their gene position and, if detected in exon regions, to evaluate their effect on protein translation. The Wilcoxon-Mann-Whitney (WMW) non-parametric test was used to check if there was a difference between the two groups in terms of genotype distribution for all the loci. Subsequently, the generalized heteroscedastic effects regression model (HEM) was used to estimate the contribution of each SNP to the differentiation of the individuals into the two tails of the distribution of the dEBVs [23]. All the SNPs were also simultaneously tested with a multiple gene approach (MG), where association analysis under an additive model was performed considering the phenotype as binary trait (high or low dEBVs) and using the GRAMMAR approach as implemented within the GenABEL package v.1.8 [24] for R [25]. Since the analyzed SNPs were not actually scattered all over the genome, but in 10 selected genes (considering the introduction of *JCHAIN*), polymorphisms with a correlation higher than 0.80 with any others were excluded. Also, the SNPs not in Hardy-Weinberg Equilibrium (HWE) were excluded. Moreover, four individuals having identity by state (IBS) > 0.95, as revealed by the genomic relationship matrix calculated using the entire dataset, were not included in the following analysis. Then, a polygenic analysis was conducted using a genomic kinship matrix based on SNP genotypes to account for relationship between individuals. Residuals from the polygenic analysis were then used as dependent, quantitative variables in single marker, linear regression analyses to test the significance of marker effects.

In order to understand the possible role of the significantly associated SNPs a first check was performed on NCBI database to see if the SNPs fell within regions from which an RNA transcription through RNAseq analysis was obtained so far. Then, short interspersed nuclear elements (SINE), long terminal repeat elements (LTR), and RNA repeats were analyzed using UCSC Genome Browser [26]. Finally, RNA Central was used to search for potential similarity with non-coding RNA [27], while miRbase was used to search for micro-RNA (miRNA) [28].

3. Results

The final re-sequenced regions are listed in Table 1. Only for *TLR4*, *NOD2*, *MBL2*, and *MBL1* we obtained sequences for about 10,000 bp upstream the selected genes, whereas for *M-SAA3.2* and *DCK* we obtained reads for about 5600 bp upstream. In the remaining genes, namely *PTX3*, *CXCR1*, *CXCR2*, and *JCHAIN*, we could re-sequence about 1000 bp of the upstream region. For *PTX3* gene we could not obtain the last 1000 bp of the gene. This occurred mainly because some regions were highly repeated while for other regions the software failed in finding specific primers. For the same reasons we could not obtain the 100% coverage, but only a mean gene coverage of 78.6% of the selected regions, with *PTX3* and *MBL2* sequenced with 64% and *CXCR1* and *MBL1* with 88% of coverage respectively. Despite gene selection, other genes are included in the sequenced regions of the bovine genome assembly: *PTX3* gene is included in a region within the ventricular zone expressed PH domain homolog 1 gene (*VEPH1*) and the collectin surfactant protein A (SP-A) gene (*SFPTA1*) is located downstream *MBL1*. Moreover, one significant SNP in *M-SAA3.2* was mapped in an intergenic region that seems to be closer to *SAA4* than to *M-SAA3.2*, about 14,000 bp far from the re-sequencing chosen region. Considering the high similarity of the SAA superfamily, further work is needed to verify if there were mapping errors or we obtained re-sequencing of non-specific products. Therefore, we considered the discovered

SNPs as belonging to the selected genes and specified in the annotation column of Table 2 their position referring to other genes.

Table 2. SNPs resulted significantly different in frequency in the two tail of the deregressed EBVs for SCS as calculated with Wilcoxon-Mann-Whitney (WMW) test. The SNP resulted significantly associated with the phenotype with the Heteroscedastic Effects regression Mode (HEM) and the multiple gene approach (MG) are also reported together with the SNP effect.

Gene	Chr	Position	rs	Allele	Annotation	WMW p [5]	HEM	MG p [5]
PTX3 [1]	1	111,028,365	rs378618073	G/T	3′ UTR	***	ns	ns
		111,028,516	rs208223246	C/T	exon 3 (E$_{347}$K)	***	ns	ns
		111,028,532	rs43263271	A/C	exon 3 (D$_{341}$E)	*	ns	ns
		111,030,195	rs207576885	A/T	intron 2	***	ns	ns
		111,030,376	rs210764862	G/A	intron 2	***	ns	ns
		111,030,399	rs381383694	A/C	intron 2	***	ns	ns
		111,030,410	NA [2]	A/G	intron 2	***	ns	ns
		111,030,413	rs208776659	C/G	intron 2	***	ns	ns
		111,030,423	NA	T/C	intron 2	***	ns	ns
		111,030,988	rs381920578	C/T	intron 2	**	ns	ns
		111,031,525	rs207709330	T/A	intron 2	*	ns	ns
		111,031,892	rs43263268	A/C	intron 2	***	ns	ns
CXCR1	2	106,939,924	rs109694601	G/A	intron 1	**	ns	−0.159 (A) *
JCHAIN	6	87,762,375	rs110597692	C/G	intron 2	*	ns	ns
		87,762,415	rs110854643	G/A	intron 2	*	ns	ns
		87,764,301	rs382005122	C/T	intron 2	*	ns	ns
DCK	6	88,043,981	rs1115177107	G/A	intergenic	*	ns	ns
		88,044,420	NA	G/T	intergenic	*	0.432 (T) *	0.125 (T) *
		88,048,414	rs137327740	C/G	−1.084 5′ UTR	*	ns	ns
		88,054,256	rs43472176	T/C	intron 1	***	ns	−0.205 (C) **
		88,054,483	rs43472177	C/T	intron 1	***	ns	ns
		88,055,035	rs43472180	T/C	intron 1	***	−0.451 (C) *	ns
		88,059,177	rs379452380	-/T	intron 2	*	ns	ns
		88,069,402	rs452449360	T/C	intron 4	*	−0.418 (C) *	ns
		88,069,428	NA	T/A	intron 4	**	−0.699 (T) *	−0.186 (T) **
TLR4	8	108,822,089	rs43578057	T/C	intergenic	*	ns	ns
		108,822,381	rs43578059	A/C	intergenic	*	ns	ns
		108,822,406	rs43578060	C/T	intergenic	*	ns	ns
		108,822,446	rs43578061	G/A	intergenic	*	ns	ns
		108,822,466	rs43578062	T/A	intergenic	*	ns	ns
		108,822,579	rs43578063	T/A	intergenic	*	ns	ns
NOD2	18	19,168,779	rs111017375	A/C	intergenic	*	ns	ns
		19,168,902	rs109352180	T/C	intergenic	**	ns	0.162 (C) *
		19,186,138	rs209462767	G/A	splice exon 4	*	ns	ns
		19,205,258	rs209159307	G/A	intron 11	*	ns	−0.195 (A) *
		19,210,095	rs110918103	T/A	intron 12	**	0.303 (A) *	0.159 (A) **
		19,210,136	rs210362219	G/A	intron 12	**	0.303 (A) *	ns
MBL2	26	6,332,839	rs110884426	C/T	intergenic	ns	−0.421 (T) *	ns
		6,332,909	rs208727559	T/A	intergenic	*	ns	ns
		6,332,959	rs209975765	C/T	intergenic	*	ns	ns
		6,334,846	rs520561418	C/T	intergenic	*	ns	ns
		6,335,302	rs442274187	G/T	intergenic	*	ns	ns
		6,341,013	rs380597712	A/C	intergenic	*	ns	ns
		6,341,147	rs465968175	T/C	intergenic	*	ns	ns
		6,342,415	rs459506838	TTAA/-	−1200 5′ UTR	*	ns	ns
		6,342,906	rs482417200	T/A	−709 5′ UTR	*	ns	ns
		6,343,309	rs798205710	C/T	−306 5′ UTR	*	ns	ns
		6,343,517	rs384805952	T/C	−98 5′ UTR	*	ns	ns
		6,343,820	rs438573157	G/T	intron 1	*	ns	ns
		6,344,627	rs436853860	A/G	intron 1	*	ns	ns

Table 2. *Cont.*

Gene	Chr	Position	rs	Allele	Annotation	WMW *p* [5]	HEM	MG *p* [5]
		6,344,678	rs455369386	C/T	intron1	*	ns	ns
		6,344,920	rs209940244	G/A	exon 2 ($P_{42}P$)	*	ns	ns
		6,344,929	rs210820536	T/C	exon 2 ($N_{45}N$)	*	ns	ns
		6,345,350	rs438686412	A/G	intron 2	*	ns	ns
		6,345,401	rs463533307	C/T	exon 3 ($P_{73}P$)	*	ns	ns
		6,345,502	rs475632625	C/G	intron 3	*	ns	ns
		6,349,735	rs136687134	A/C	+823 3′ UTR	ns	0.242 (C) *	ns
MBL1 [1]	28	35,842,351	rs208247354	G/A	intron 3	*	ns	ns
		35,844,679	rs208491630	G/T	intron 1	*	ns	ns
		35,852,741	rs211629255	C/T	intron 2 *SFPTA1* [3]	**	0.323 (T) *	ns
M-SAA3.2	29	26,741,245	rs42175273	A/T	intergenic *SAA4* [4]	ns	ns	−0.126 (T) *
		26,755,302	rs136687125	T/C	−265 5′ UTR	*	ns	ns
		26,755,410	rs137746604	G/A	−157 5′ UTR	*	ns	ns
		26,755,477	rs210417381	T/C	−90 5′ UTR	*	ns	−0.181 (C) *

[1] reverse oriented gene; [2] NA = not available; [3] the SNP falls in intron 2 of the collectin surfactant protein A gene *SFPTA1*; [4] the SNP falls in an intergenic region mapped near *SAA4* gene; [5] *p*-values: * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, ns = not significant.

In the sequenced region the total number of called variants was 25,200, but after quality filtering 2585 SNPs were retained. Out of these 2585 SNPs only 557 had a MAF > 0.05 and therefore were used to analyze their associations with the selected phenotype: 20 SNPs within *PTX3* (BTA1-Supplementary Table S1), three of which located in intron 2 and not found on NCBI SNPs databases (1: 111,030,410, 1: 111,030,420, and 1: 111,030,423); 91 SNPs in *CXCR1* and *CXCR2* (BTA2-Supplementary Table S2), three not found on NCBI SNPs databases (2: 106,913,554, 2: 106,913,717, and 2: 106,913,727) and supposed to lead to missense ($V_{125}A$), synonymous ($L_{179}L$), and missense ($V_{183}I$) mutations, respectively; 76 SNPs within *JCHAIN* and *DCK* (BTA6-Supplementary Table S3), nine not found on NCBI SNPs databases (6: 88,044,038, 6: 88,044,298, 6: 88,044,381, 6: 88,044,420, 6: 88,044,746, 6: 88,064,952, 6: 88,069,416, 6: 88,069,428, and 6: 88,069,437), the first five located in intergenic region and the last four in intron regions; 81 SNPs within *TLR4* (BTA8-Supplementary Table S4); 43 SNPs in *NOD2* (BTA18-Supplementary Table S5); 54 SNPs in *MBL2* (BTA26-Supplementary Table S6); 64 in *MBL1* (BTA28-Supplementary Table S7); 128 SNPs in *M-SAA3.2* (BTA29-Supplementary Table S8). After pruning for correlation and HWE, 182 of these 557 polymorphisms were tested for associations with the MG approach.

WMW test revealed 61 SNPs with a significant different genotype distribution in the two tails of the dEBVs: 12 SNPs out of 20 in *PTX3*, 1 SNP out of the 91 on BTA2 in *CXCR1*, 12 SNPs (three in intron 2 of *JCHAIN*, three in intergenic regions of *DCK*, and six in *DCK* introns) out of 76 on BTA6, six SNPs out of 81 in *TLR4*, 18 SNPs out of 54 in *MBL2*, 3 SNPs out of 64 in *MBL1*, four SNPs out of 128 in M-SSA3.2. Both the HEM and the MG analysis showed nine SNPs significantly associated with the phenotype each. Since two SNPs obtained by HEM and one SNP obtained by MG approach had no significant differences in genotype distribution by WMW, the total number of SNPs included in Table 2 is 64. The SNPs position refers to the UMD Bovine Genome 3.1.1 assembly and, where available, the SNPs rs are reported together with the description of the type of variant, the level of significance of the three tests and the effect of the SNP for HEM and MG approaches. The HEM approach revealed significant SNPs in *DCK* (4), *NOD2* (2), *MBL2* (2), *MBL1* (1), whereas the MG approach revealed significant SNPs in *CXCR1* (1), *DCK* (3), *NOD2* (3), and *M-SAA3.2* (2). None of the SNPs in *PTX3*, *CXCR2*, *JCHAIN* and *TLR4* resulted significantly associated with the SCS levels in the analyzed population. Nevertheless, one SNP in *PTX3* mapped within 3′ UTR and others two corresponded to the non-synonymous mutations responsible for the aminoacidic exchanges $D_{341}E$ and $E_{347}K$, therefore they could lead to changes in the expressed protein and be matter of interest although they were not significantly associated with the SCS levels in the analyzed population.

3.1. CXCR1

The only SNP showing a different genotype distribution in L and H groups by WNW test, rs109694601, also resulted significantly associated with SCS levels by MG approach. This SNP, located within intron region in *CXCR1*, is mapped 24 pb upstream a Mammalian-wide Interspersed Repeats (MIR) element of the MIR3 subfamily. MIRs represent an ancient family of tRNA-derived Short INterspersed Elements (SINEs) found in all mammalian genomes, whose core region may serve some general function, although they have ceased to amplify by retro-transposition. Despite their massive presence in mammalian genomes, their contribution to the transcriptome is still largely unexplored even in humans, and in particular, elements controlling their transcription have never been systematically studied [29], thus we cannot even hypothesize a role of this SNP yet.

3.2. DCK

None of the SNPs located in *JCHAIN* showed significant associations with the considered phenotype, whereas a total of five SNPs in *DCK* were found significantly associated with dEBVs levels with HEM (four SNPs) and MG (three SNPs) approach. It has to be noted that despite the pruning of the high correlated SNPs and the removal of four individuals because of high IBS by MG approach, which could lead to slightly different results with respect to HEM, the two SNPs not found on NCBI databases (6: 88,044,420 and 6: 88,069,428) resulted significant with both HEM and MG approach (Table 2). The intergenic region comprising the SNP at position 88,044,420 and SNP rs1115177107 includes a transcribed RNA, as shown by NCBI database, and could therefore have some not yet described regulatory effects. In *DCK* intron 1, where two significant SNPs (rs43472176 and rs43472180) were described, two ncRNA-like sequences were also found. Moreover, in-between rs43472177 and rs43472180 we found a sequence showing high homology with BTA-mir-2452, a miRNA found expressed upon induced viral infection [30], indicating that it should have a role in the immune response.

3.3. NOD2

Four out of the six SNPs with significant genotype distribution in H and L groups, were also found significantly associated with dEBVs levels using MG (rs109352180, rs209159307, and rs110918103) and HEM (rs110918103, and rs210362219) approaches. As for *DCK* also for *NOD2* there was at least one SNP in common between the two approaches (rs110918103). The intergenic region comprising rs109352180 shows the presence of transcribed RNA and could therefore have regulatory effects. Moreover, rs109352180 flanks a region partially aligning with three human miRNAs and four bovine lncRNAs, three of which are precursors of miR-185, a miRNA that could influence the expression of different genes [31,32]. The region is also rich in SINEs, whereas rs210362219 is in a sequence aligning with 3 bovine lncRNA and one human miRNA (68% identity).

3.4. MBL2

None of the 18 SNPs that could be of interest on the basis of WMW test resulted significantly associated with SCS levels by MG approach, neither the three mapped within exon regions (rs209940244, rs210820536, rs463533307), all synonymous mutations, nor the three in proximity of 5′ UTR (Table 2). With HEM approach two SNPs resulted significantly associated with the phenotype: rs110884426, mapped in the intergenic region and rs136687134, located about 1000 bp downstream the 3′ UTR. Both SNPs are found in a transcribed RNA, as shown by NCBI database. Moreover, rs136687134 is included in a region of Long INterspersed Elements (LINEs), transposable elements that take a large proportion of eukaryotic genomes, once regarded as nonfunctional sequences, and now considered to play pivotal roles in gene regulation [33,34].

3.5. MBL1

None of the 3 SNPs that could be of interest on the basis of WMW test resulted significantly associated with SCS levels by MG approach, whereas rs211629255, the SNP that falls into intron 2 of *SFPTA1*, resulted significant by the HEM approach (Table 2).

3.6. M-SAA3.2

The WMW test showed that genotypes distribution in the two sample groups was significantly different for three SNPs, namely rs136687125, rs137746604, and rs210417381. Of the three SNPs, only rs210417381, with also rs42175273, was found to be significantly associated with the phenotype in the MG approach, (Table 2). It has to be considered that the three SNPs located near the 5′ UTR of *M-SAA3.2* have a mean correlation of about 0.77 and were therefore all included in the MG analysis, but due to the high correlation among them it is difficult to define which SNPs has actually an effect or if they are all involved in the response to mastitis resistance. The 3 SNPs are included in an RNA transcribed region containing 4 ncRNAs and also a long terminal repeated (LTR) element that should belong to endogenous retrovirus KERVK family. There are evidences that LTR sequences derived from distantly related endogenous retroviruses (ERVs) act as regulatory sequences for many host genes in a wide range of cell types throughout mammalian evolution [35,36]. Re-activation of ERVs is often associated with inflammatory diseases, thus the region could actually be related with mastitis resistance/susceptibility.

4. Discussion

Starting from the knowledge of the correlation between SCC and mastitis [37], the aim of this study was to analyze the genetic variability of candidate genes and to investigate the association of the identified SNPs with SCS EBVs. As reported by Koeck et al. [38], SCS EBVs are a valuable alternative predictor for mastitis resistance. Candidate genes selected for this study were already known in literature to be related to immune response to mastitis infections [11–19]. To maximize the effects of the different SNPs, bulls to be sequenced were selected between the low and high tails of distribution for SCS. We chose to use the selective genotyping approach: only individuals from the high and low extremes of the trait distribution are selected for genotyping, reducing genotyping work and costs maintaining nearly equivalent efficiency to complete genotyping [39,40].

An overall total of 64 SNPs showed significantly different genotype distributions in the H and L groups or were identified as significantly associated with dEBVs for SCS. Among these SNPs, 15 resulted significantly associated with HM or MG approach and 3 of them with both approaches. The main difference between the two approaches used in this study lies in MG that takes into account the genomic relationship matrix and the correlation between SNPs. Indeed, when correlation is considered, several SNPs associations could actually belong to correlated SNPs that were excluded from the analysis rather than to the reported SNP. Moreover, with the MG approach, given the general small allele substitution effect when considering simultaneously SNPs in different genes, and using a correction for the genomic relationship, a low number of significant SNPs is expected. The remaining SNPs should be, therefore, the ones where the association is stronger. Of the 15 SNPs identified by both approaches, the most significant were located inside intronic regions within *DCK* (rs43472176 and one at position 6: 88,069,428 not previously available on NCBI SNPs databases) and *NOD2* (rs110918103). The newly discovered SNP at position 6: 88,069,428, together with rs110918103, are two of the three SNPs resulted significantly associated with both HM and MG approach. No significantly associated SNPs were found in exon regions, while eight SNPs were located in the intergenic regions and could possibly be related to distal regulatory element. Six genes (*CXCR1*, *DCK*, *NOD2*, *MBL2*, *MBL1* and *M-SAA3.2*) included SNPs that were identified as significantly associated by HEM and/or MG and that are known to be key components in the immune system. As reported by Siebert et al. [41], several studies identified different SNPs in gene associated with mastitis and, furthermore, with SCS. Similarly, *DCK* gene was suggested as candidate gene

associated with mastitis [42]. On the other hand, the direct association between mastitis and *MBL1*, *MBL2*, *NOD2* and *M-SAA3.2* genes has not been fully explored so far. Nevertheless Wang et al. [43], using a GWAS approach found a significant SNP in *SAA2* gene, indicating an important role of the superfamily of these apolipoproteins. These results suggest that, just like *CXCR1* and *DCK* genes, also *MBL1*, *MBL2*, *NOD2* and *M-SAA3.2* genes could be eligible as candidate genes for genetic selection of mastitis resistant cows. Many studies demonstrated that SNPs associated with diseases are mainly located in non-coding regions, making it difficult to link them to specific biological pathways. A recent study, in which a genotyping-by-sequencing approach was used to find novel SNPs associated with milk traits, showed that the majority of identified SNPs were located within intergenic regions (69%), followed by intronic regions (25%), with only 3.46% of SNPs being coding variants [44]. Moreover, different studies found that conserved non-coding regions in introns and near genes show large allelic frequency shifts, similar in magnitude to missense variations, suggesting that they are critical for gene function regulation and evolution in many species [45,46]. Most of the significant SNPs detected in our investigation are located inside or in proximity to complete or partial lncRNA-like or miRNA-like sequences, or to repeated regions containing SINEs or LINEs, and it is now established that both intronic and LINE/SINEs repeats could lead to transcriptional regulation of the affected genes [47]. Thus, although the role of some of these elements, especially in the bovine species, has still to be verified, and further studies are needed to better understand the role of these SNPs, the results obtained here are a starting point. Contrary to the findings of Welderufael et al. [48], no SNPs were identified as significantly associated with dEBVs level within *PTX3* gene in our population. Similarly, no significant SNPs were detected within candidate genes *TLR4* [49].

5. Conclusions

In this study, next generation sequencing technology was used to discover new SNPs in candidate genes related to mastitis resistance and to identify those associated with dEBVs for SCS. We analyzed the genetic variability of several SNPs found in candidate genes and identified 15 that are associated with the phenotype. Two of them maps within *DCK* gene and were not previously available on NCBI database, whereas other SNPs strengthen the role of *CXCR1*, *NOD2*, *MBL1*, *MBL2*, and *M-SAA3.2* as candidate genes. These results suggest that the possibility to use SNPs as markers for genetic selection of mastitis resistant cattle is plausible.

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Heritability of Teat Condition in Italian Holstein Friesian and Its Relationship with Milk Production and Somatic Cell Score

Francesco Tiezzi [1], Antonio Marco Maisano [2], Stefania Chessa [3], Mario Luini [2,4] and Stefano Biffani [4,*]

[1]   Department of Animal Science, North Carolina State University, Raleigh, NC 27695, USA; f_tiezzi@ncsu.edu
[2]   Istituto Zooprofilattico Sperimentale Lombardia Emilia Romagna "Bruno Ubertini"—I.Z.S.L.E.R. Territorial Section of Lodi and Brescia Sector Diagnostic, Animal Health and Welfare, 26900 Lodi, Italy; antoniomarco.maisano@izsler.it (A.M.M.); mariovittorio.luini@ibba.cnr.it (M.L.)
[3]   Dipartimento di Scienze Veterinarie, Università degli Studi di Torino, 10095 Grugliasco, Italy; stefania.chessa@unito.it
[4]   Consiglio Nazionale delle Ricerche (CNR), Istituto di biologia e biotecnologia agraria (IBBA), Via Edoardo Bassini, 20133 Milano, Italy
*   Correspondence: biffani@ibba.cnr.it

Simple Summary: Mammary infections in dairy cattle are still a major problem which impair animal health and jeopardize breeders' efforts to attain sustainable production. The first natural protection against pathogens' access to udder tissue is the teat canal. Teat canal morphology can be influenced by several environmental factors but the present study confirmed the existence of a genetic component. Moreover it was observed that the teat canal morphology is related to milk production and somatic cell count, the latter being an indirect indicator of mammary infections. Considering that the current selection objectives implemented in dairy cattle worldwide have shifted toward a more balanced breeding goal, with a larger emphasis on health traits, a further genetic deterioration in teat condition is not expected.

Abstract: In spite of the impressive advancements observed on both management and genetic factors, udder health still represents one of most demanding objectives to be attained in the dairy cattle industry. Udder morphology and especially teat condition might represent the first physical barrier to pathogens' access. The objectives of this study were to investigate the genetic component of teat condition and to elucidate its relationship with both milk yield and somatic cell scores in dairy cattle. Moreover, the effect of selection for both milk yield and somatic cell scores on teat condition was also investigated. A multivariate analysis was conducted on 10,776 teat score records and 30,160 production records from 2469 Italian Holstein cows. Three teat scoring traits were defined and included in the analysis. Heritability estimates for the teat score traits were moderate to low, ranging from 0.084 to 0.238. When teat score was based on a four-classes ordinal scoring, its genetic correlation with milk yields and somatic cell score were 0.862 and 0.439, respectively. The scale used to classify teat-end score has an impact on the magnitude of the estimates. Genetic correlations suggest that selection for milk yield could deteriorate teat health, unless more emphasis is given to somatic cell scores. Considering that both at national and international level, the current selection objectives are giving more emphasis to health traits, a further genetic deterioration in teat condition is not expected.

Keywords: dairy cattle; teat-end hyperkeratosis; udder health; somatic cell; genetic correlation; selection response

1. Introduction

Udder health still represents one of the most demanding objectives to be attained in the dairy cattle sector. In spite of the impressive advancements observed on both management and genetic factors, the diseases of the mammary gland in response to an infection, i.e., a clinical (CM) or a subclinical mastitis (SCM), is one of the most common and expensive diseases faced [1–6]. In 2015 the estimated overall cost per case of clinical mastitis in the first 30 days of lactation was $444 [7]. Moreover, 71% of the costs of a case of clinical mastitis were indirect costs. Among the several factors which can mitigate or increase the occurrence of intramammary infections, udder and teat morphology play a substantial role [8–11]. Indeed, the most relevant pathogens affecting dairy cattle, e.g., *Staphylococcus aureus (S. aureus)* [12] gain access to udder tissues primarily via the teat canal. Teat-end hyperkeratosis (THK), i.e., the hyperplasia of the teat orifice's keratin layer [13], is considered one of the most common teat condition changes observed in dairy cattle [14]. THK is commonly evaluated using an ordinal scale ranging from 1 (no THK) to 4 (severe THK) [15]. Results presented in a recent review show that a condition of severe THK is an important risk factor for both CM and SCM, while a mild THK can mitigate possible SCM [11]. Alterations in teat condition across lactation and parities are expected as a consequence of machine milking [14] but the existence of a genetic component has also been investigated [16,17]. In a first study enrolling 1740 US Holstein cows from nine herds, Chrystal and colleagues [16] found that heritability estimates of teat end shape for first, second, and all lactations combined were 0.53, 0.44, and 0.56, respectively. Teat diameter heritability estimates were 0.23, 0.27, and 0.35, respectively across the three lactations. Teat-end shape and diameter repeatability estimates were 0.75 and 0.36, respectively. In a subsequent study with 1259 cows from a single herd, Chrystal et al [17] found heritability estimates of teat-end shape for first, second, and third and later lactations of 0.34, 0.21, and 0.13, respectively. Repeatability ranged from 0.34 to 0.46.

An additional and widely used phenotypic indicator of udder health is somatic cell count (SCC). After being transformed to somatic cell score (SCS) using a logarithmic transformation [18], it is used as a predictor in the selection for mastitis resistance [3,19]. Some studies [20–27] have investigated the relationship between THK and SCC and most of them reported positive associations, especially when THK is scored as severe. However THK scoring can be confounded with other factors, e.g., parity, and its relationship with SCC might not be clearly identified. Chrystal and colleagues investigated the relationship between teat end shape and SCS in two subsequent studies [16,17]. They used SCS as a dependent variable in a mixed model and included teat end shape as a covariate. In both cases, the effect of teat end shape was not significant. Among the possible explanations the authors hypothesized some genetic factors that would control SCS and prevail over the effect of teat end shape.

The main objectives of this work were to further investigate the genetic component of THK and to elucidate its relationship with both milk yield (MY) and SCS. Moreover, the genetic change in THK when selecting for both MY and SCS was also investigated.

2. Materials and Methods

2.1. Dataset

Animal welfare and use committee approval was not needed for this study as datasets were obtained from pre-existing databases based on routine animal recording procedures. Data used in the present study were collected between September 2011 and August 2012 and between March 2016 and January 2017 in 48 Italian Holstein dairy farms located in the Lombardy region (Northern Italy, 45.4791° N, 9.8452° E). The average herd size was 106 milking cows, ranging from 38 to 285 cows. The original data set included 3087 cows [28].

Teat condition score (TCS) was evaluated visually for each cow during milk sampling and assigned a score using the methodology proposed by Neijenhuis et al. [15]: an absent callosity was evaluated as TCS = 1, a smooth callous ring around the orifice was evaluated as TCS = 2; a rough and very rough callous rings were evaluated as TCS = 3 and 4, respectively. Scores were applied on each single teat,

generating four records for each cow scored, each one was assigned to the respective teat position (FL: front left; FR: front right; RL: rear left; RR: rear right). In addition to scoring values, date of scoring and hygiene scoring were obtained. Hygiene of udder, flanks and legs was scored based on a 4-point scale system [29], from very clean (score 1) to very dirty skin (score 4). Both TCS and hygiene score were collected by a group composed of four technicians who were previously trained to harmonize the scoring procedure.

Production records for daily milk yield (MY) and SCC per ml were obtained by the Regional Breeders' Association (ARAL, Crema, Italy). The variable SCC was log-transformed into SCS by using the formula $SCS = log_2(SCC/100,000) + 3$ [18]. Together with production records, calving dates, herd number and pedigree information were obtained. Using calving date, it was possible to extract the parity order and days in milk at teat scoring and production recording.

Teat scoring was organized in a dataset where the four quarters appeared as repeated information of the same phenotype. Each herd was visited and scored only once although the scoring was conducted over two consecutive days in two of the herds. Teat position information was assigned to each record, so that each cow showed four records with the respective position indicator. Only cows showing all four records were included in the analysis.

Production records from the same cows were retained and were organized as repeated measures on the same individuals. For each cow, only production records from the same lactation number of the scoring were retained. The median number of records per cow was 10, each record reported MY and SCS for that cow in that test-day.

The final dataset was built stacking the teat score and production datasets. Records that showed teat scoring did not show production records, and vice versa.

The final dataset included 10,776 scoring records and 30,160 production records, for a total of 40,936 records available for analyses. All records came from 2649 cows, daughters of 869 sires and 2408 dams, while 275 cows appeared also as dams. All animals were raised in a total of 48 herds.

The choice of building a dataset where test scores and production records were not aligned was dictated by practical modeling decisions. The alignment of the teat score records with the closest production record could have been done. However, the repeated nature of the teat score record would have forced the production record to be repeated four times, which was not appropriate. At the same time, repeated records for both teat scores and production records allowed us to disentangle the additive genetic and permanent environmental effects. By not aligning records, the residual covariance between the teat scores and the production records could not be estimated. Nevertheless, such covariance should be forced to zero, because the phenotypes came from different data capture and recording systems. The cow permanent environmental effect allowed us to estimate a non-genetic covariance between the two sets of traits.

2.2. Statistical Analysis

Three teat scoring traits were defined and included in the analysis. A four-classes ordinal scoring (1, 2, 3, 4) was maintained for the trait hereinafter called TS. In addition, two binary traits were created. Classes 2, 3 and 4 were combined into a single class creating the trait hereinafter called TS_a; classes 1 and 2 vs. 3 and 4 were combined to create trait TS_b. Relative frequencies for all trait classes are reported in Table 1.

A tri-variate threshold-linear model was used for analyzing traits combination such as TS-MY-SCS, TS_a-MY-SCS, TS_b-MY-SCS. Model specifications were:

$$y(\lambda) = \mathbf{X}b + \mathbf{Z}_h h + \mathbf{Z}_p p + \mathbf{Z}_s s + e$$

where y is the phenotype vector for MY and SCS, λ is the underlying liability for TS, TS_a or TS_b, $\mathbf{X}$ and b are the incidence matrix and vector of solutions for fixed effects, $\mathbf{Z}_h$ and h are the incidence matrix and vector of solutions for the herd environmental random effect, $\mathbf{Z}_p$ and p are the incidence matrix

and vector of solutions for the cow permanent environmental random effect, $\mathbf{Z}_s$ and s are the incidence matrix and vector of solutions for the sire additive genetic random effect, e is the vector of residuals. Fixed effects common to all traits were parity by stage of lactation class (36 classes), defined by pasting the parity class (first, second and later lactations) with the stage of lactation month (last class including records until the 15th month of lactation). Additional fixed effects for TS, TS_a and TS_b were hygiene (4 classes) and teat position (4 classes). The vector of solutions for random effects were assumed to be normally and independently distributed. Specifically for the herd random effect:

$$\begin{bmatrix} h_1 \\ h_2 \\ h_3 \end{bmatrix} \sim N(0,\ \mathbf{I} \otimes \mathbf{H})$$

where $\mathbf{I}$ is an identity matrix and $\mathbf{H}$ is the following variance-covariance matrix:

$$\begin{bmatrix} \sigma^2_{h11} & \sigma_{h12} & \sigma_{h13} \\ \sigma_{h12} & \sigma^2_{h22} & \sigma_{h23} \\ \sigma_{h13} & \sigma_{h23} & \sigma^2_{h33} \end{bmatrix}$$

Similarly, for the cow permanent environmental effect:

$$\begin{bmatrix} p_1 \\ p_2 \\ p_3 \end{bmatrix} \sim N(0,\ \mathbf{I} \otimes \mathbf{P})$$

where $\mathbf{I}$ is an identity matrix and $\mathbf{P}$ is the following variance-covariance matrix:

$$\begin{bmatrix} \sigma^2_{p11} & \sigma_{p12} & \sigma_{p13} \\ \sigma_{p12} & \sigma^2_{p22} & \sigma_{p23} \\ \sigma_{p13} & \sigma_{p23} & \sigma^2_{p33} \end{bmatrix}$$

For the sire additive genetic effect:

$$\begin{bmatrix} s_1 \\ s_2 \\ s_3 \end{bmatrix} \sim N(0,\ \mathbf{A} \otimes \mathbf{S})$$

where $\mathbf{A}$ is the numerator relationship matrix constructed on the pedigree and $\mathbf{S}$ is the following variance-covariance matrix:

$$\begin{bmatrix} \sigma^2_{s11} & \sigma_{s12} & \sigma_{s13} \\ \sigma_{s12} & \sigma^2_{s22} & \sigma_{s23} \\ \sigma_{s13} & \sigma_{s23} & \sigma^2_{s33} \end{bmatrix}$$

and for the residual effect:

$$\begin{bmatrix} e_1 \\ e_2 \\ e_3 \end{bmatrix} \sim N(0,\ \mathbf{I} \otimes \mathbf{R})$$

where $\mathbf{I}$ is an identity matrix and $\mathbf{R}$ is the following variance-covariance matrix:

$$\begin{bmatrix} 1 & 0 & 0 \\ 0 & \sigma^2_{e22} & \sigma_{e23} \\ 0 & \sigma_{e23} & \sigma^2_{e33} \end{bmatrix}$$

Therefore, residual variance for any teat scoring trait was fixed to 1 for identifiability in the threshold liability model and the covariance between the teat scoring and production traits was fixed to 0, due to lack of records showing both traits.

Variance components estimates were obtained using software THRGIBBS1F90 version 3.1 [30]. Solutions for TS_a and TS_b were then obtained using the package 'MCMCglmm' implemented in R version 3.6.1 [31] by fixing the variance components to the estimated values. This package was chosen for easier manipulation of solutions in the R environment. In both cases, a total of 300,000 iterations were run, removing the first 50,000 as burn-in and thinning every 50 iterations. Convergence was assessed by visual inspection of trace plots and Geweke's test conducted using the 'coda' package in R [32].

Table 1. Frequency of observations for each teat score in the dataset ($n = 10,776$).

Score	Number of Records	Relative Frequency		
		TS [1]	TS_a [1]	TS_b [1]
1	7078	65.7	65.7	90.6
2	2687	24.9		
3	838	7.78	34.3	9.4
4	173	1.61		

[1] TS = teat scoring trait based on a four-classes ordinal scoring (1 = absent callosity, 2 = smooth callous ring, 3 = rough callous ring, 4 = very rough callous ring). TS_a: binary teat scoring trait where classes 2, 3 and 4 were combined into a single class. TS_b: binary teat scoring trait where classes 1 and 2 vs. 3 and 4 were combined.

2.3. Genetic Parameters

Heritability was calculated as:

$$h^2 = \frac{4\sigma_s^2}{\sigma_s^2 + \sigma_p^2 + \sigma_h^2 + \sigma_e^2}$$

Intra-herd heritability was calculated as:

$$h_{IH}^2 = \frac{4\sigma_s^2}{\sigma_s^2 + \sigma_p^2 + \sigma_e^2}$$

Cow repeatability was calculated not considering the genetic effect as a source of cow repeatability, therefore:

$$r^2 = \frac{\sigma_p^2}{\sigma_s^2 + \sigma_p^2 + \sigma_h^2 + \sigma_e^2}$$

Herd repeatability was calculated as:

$$he^2 = \frac{\sigma_h^2}{\sigma_s^2 + \sigma_p^2 + \sigma_h^2 + \sigma_e^2}$$

In all formulas, σ_e^2 was '1' for the teat score traits. Genetics, cow and herd correlations were calculated by dividing the covariance by the product of the standard deviations. Parameters were calculated at every iteration and posterior mean and 95% empirical confidence intervals were used as estimates of their standard error.

Least square means for all effects were calculated on the liability scale at every iteration and transformed to the phenotypic (probability) scale. Posterior mean and 95% empirical confidence intervals were used as estimates and their error. Plots were created using package 'ggplot2' implemented in R [33].

2.4. Genetic Change in Teat Score Traits

Expected selection response on the teat score traits was calculated for economic indices that combined MY and SCS with different values of relative emphasis. One economic index for each of the three teat score traits was built, including three traits (the teat score traits, MY and SCS). Relative emphasis was applied on MY and SCS, starting with 100% emphasis on MY and 0% emphasis on SCS and ending with 0% emphasis on MY and 100% on SCS, with step increases of 1% unit. While MY was given positive emphasis, SCS was given negative emphasis. Following [34–36] the genetic response on the teat score trait was calculated as:

$$r = \frac{w'g_{\circ 1}}{\sqrt{w'Gw}}$$

where *r* is the genetic response for the teat score trait (TS, TS$_a$ or TS$_b$); w is the vector of index weight; **G** is the variance-covariance between the teat score trait, MY and SCS; g$_{\circ 1}$ is the first column of **G**. The genetic response was then expressed in genetic standard deviation units for the respective teat score trait.

3. Results

3.1. Data

Descriptive statistics for the teat score traits are in Table 1. Mean values and SD for daily milk production (MY) and daily linear score (SCS) were 29.73 ± 9.05 for and 3.97 ± 1.99 (not reported in table). The observed phenotypic trend for MY and SCS by parity and month of lactation are in Figures 1 and 2, respectively.
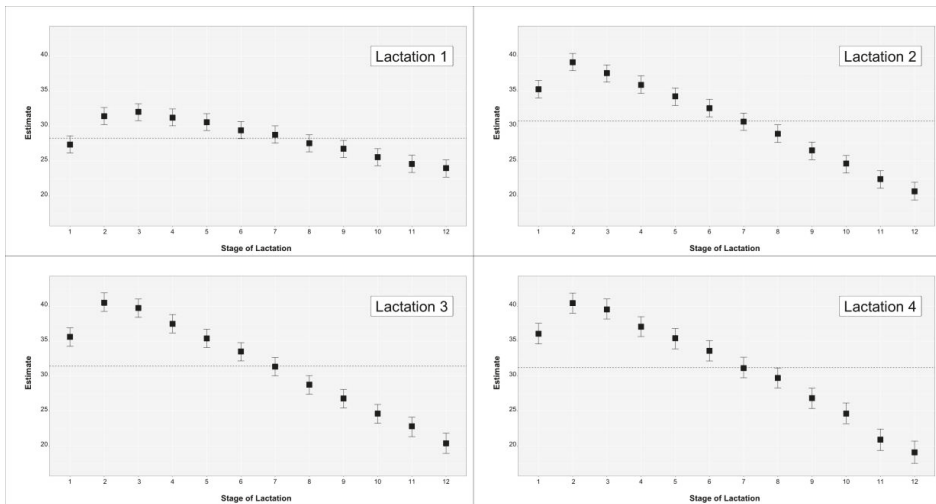

Figure 1. Milk yield across parity and stage of lactation for cows with a teat-score evaluation.
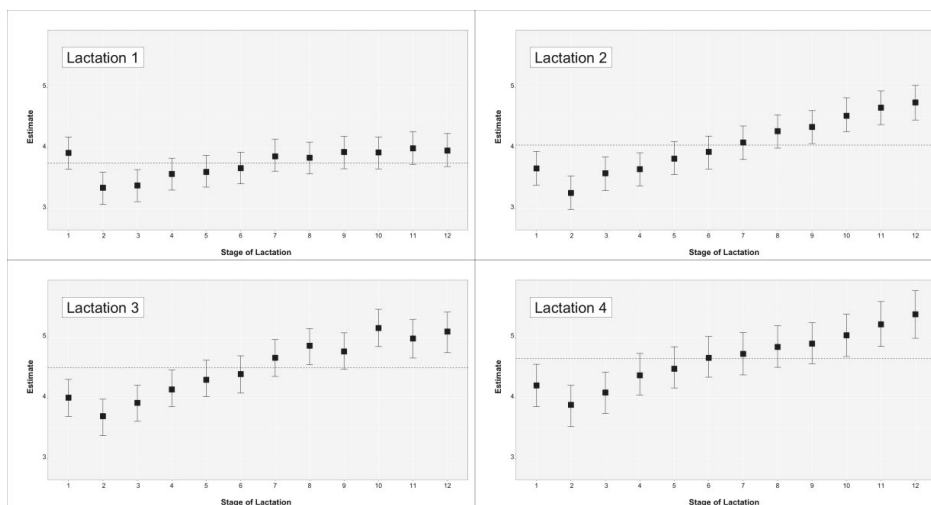
Figure 2. Somatic cell score across parity and stage of lactation for cows with a teat-score evaluation.

3.2. Heritability and Environmental Effects Estimates

Estimates of variance components, heritability, intra-herd heritability, cow repeatability and herd repeatability for all traits are reported in Table 2. Estimates for MY and SCS came from the model where TS was the correlated trait, though posterior distribution estimates for production traits in the other models largely overlapped the values shown.

Table 2. Estimates (posterior mean and 95% highest probability density intervals) of variance components and genetic parameter estimates for the traits considered in this study.

Parameter	Trait				
	TS	TS$_a$ [1]	TS$_b$ [1]	MY [1]	SCS [1]
Sire genetic variance	0.438 (0.233; 0.702)	0.128 (0.072; 0.188)	0.036 (0.010; 0.066)	0.958 (0.450; 1.608)	0.148 (0.084; 0.214)
Cow permanent environmental variance	3.998 (3.465; 4.532)	1.449 (1.324; 1.577)	0.430 (0.371; 0.488)	15.12 (14.05; 16.15)	1.488 (1.398; 1.591)
Herd-test day variance	1.648 (0.902; 2.548)	0.506 (0.288; 0.768)	0.160 (0.078; 0.249)	15.04 (7.85; 23.06)	0.624 (0.292; 0.925)
Residual variance	1.281 (1.217; 1.343)	1.073 (1.051; 1.095)	1.085 (1.060; 1.105)	28.01 (27.57; 28.51)	1.788 (1.758; 1.817)
Heritability (h^2)	0.238 (0.116; 0.364)	0.162 (0.090; 0.235)	0.084 (0.025; 0.155)	0.065 (0.030; 0.109)	0.146 (0.080; 0.210)
Intra-Herd heritability (h^2-IH)	0.289 (0.153; 0.45)	0.248 (0.061; 0.434)	0.250 (0.135; 0.363)	0.126 (0.025; 0.217)	0.234 (0.114; 0.343)
Cow repeatability	0.544 (0.469; 0.603)	0.460 (0.417; 0.503)	0.251 (0.222; 0.282)	0.257 (0.220; 0.290)	0.368 (0.333; 0.401)
Herd-test-day repeatability	0.221 (0.141; 0.313)	0.159 (0.096; 0.224)	0.093 (0.051; 0.141)	0.251 (0.164; 0.353)	0.153 (0.093; 0.224)

[1] TS = teat scoring trait based on a four-classes ordinal scoring (1 = absent callosity, 2 = smooth callous ring, 3 = rough callous ring, 4 = very rough callous ring). TS$_a$: binary teat scoring trait where classes 2, 3 and 4 were combined into a single class. TS$_b$: binary teat scoring trait where classes 1 and 2 vs. 3 and 4 were combined. MY = daily milk yield. SCS = daily somatic cell score.

Heritability estimates for the teat score traits were moderate to low, with TS showing the largest value (0.238) followed by TS_a with 0.162 and TS_b with 0.084. A similar but weaker trend was found for the intra-herd heritability, TS showed an estimate of 0.289, TS_a showed 0.248 and TS_b showed 0.250. The weaker trend was due to the herd effect also decreasing from 0.221 for TS to 0.159 for TS_a to 0.093 for TS_b. Cow repeatability (free of genetic variance) was 0.554 for TS, 0.46 for TS_a and 0.251 for TS_b.

Heritability and intra-herd heritability estimates were larger for SCS than MY (0.146 vs. 0.065 and 0.234 vs. 0.126, respectively). Also cow repeatability was stronger for SCS than MY (0.368 vs. 0.257) while herd effect was weaker for SCS than MY (0.153 vs. 0.251, respectively).

3.3. Genetic and Environmental Correlations

Correlations between teat scoring and production traits for all random effects are reported in Table 3. Correlations were considered significant when the 95% empirical confidence intervals did not include the '0' value.

Table 3. Estimates (posterior mean and 95% highest probability density intervals) of genetic correlations between teat score and production traits.

Item		MY [1]			SCS [1]	
	TS [1]	TS_a [1]	TS_b [1]	TS [1]	TS_a [1]	TS_b [1]
Genetic correlation	0.86 (0.71; 0.98)	0.89 (0.61; 0.99)	0.54 (−0.11; 0.96)	0.44 (0.10; 0.80)	0.36 (−0.02; 0.70)	0.38 (−0.09; 0.90)
Cow permanent environmental correlation	0.01 (−0.04; 0.06)	0.02 (−0.03; 0.07)	−0.003 (−0.08; 0.06)	0.14 (0.09; 0.19)	0.12 (0.07; 0.18)	0.15 (0.08; 0.22)
Herd-test day Correlation	0.30 (−0.06; 0.64)	0.30 (−0.03; 0.62)	0.46 (0.13; 0.72)	−0.25 (−0.57; 0.10)	−0.25 (−0.58; 0.08)	−0.47 (−0.76; −0.17)

[1] TS = teat scoring trait based on a four-classes ordinal scoring (1 = absent callosity, 2 = smooth callous ring, 3 rough callous ring, 4 = very rough callous ring). TS_a: binary teat scoring trait where classes 2, 3 and 4 were combined into a single class. TS_b: binary teat scoring trait where classes 1 and 2 vs. 3 and 4 were combined. MY = daily milk yield. SCS = daily somatic cell score.

Genetic correlations for TS and TS_a with MY were positive and significant, showing values of 0.862 and 0.893, respectively. The correlation between MY and TS_b was not significant. Only the genetic correlation between TS and SCS was significant showing a value of 0.439.

None of the cow permanent environmental correlations of teat scoring traits with MY were significant. All correlations with SCS were significant and positive but weak, showing values of 0.141, 0.125 and 0.152 for TS, TS_a and TS_b.

For the herd environmental correlations, the only significant estimate was found between TS_b and MY with a value of 0.463. However, for all estimates the tendency was for a positive relationship between teat scoring traits and MY and a negative relationship with SCS.

3.4. Influence of Fixed Effects on Teat Scores

Least square mean estimates for the hygiene score are reported in Table 4 while estimates for the teat position effect are reported in Table 5. Estimates are expressed on the phenotypic scale (probability of a TS greater than 1 for TS_a or TS greater than 2 for TS_b).

The hygiene effect did not appear to be relevant for any traits, since the posterior distributions of the estimates overlapped.

A relationship was observed among teat positions and TS_a. Indeed front quarters showed a higher probability of a TS greater than 1 or TS greater than 2. The same pattern was found for TS_b, with probabilities for front left and front right quarters at 0.095 and probabilities for rear left and rear right quarters at 0.094. While the front-back contrast could not be declared significant at the chosen α threshold of 0.05, the trend appeared evident.

Table 4. Least square mean estimates (posterior mean and 95% highest probability density intervals) for the influence of Hygiene score on TS_a and TS_b traits. Estimates are expressed on the phenotypic scale (probability of a TS greater than 1 for TS_a or TS greater than 2 for TS_b).

Trait	Hygiene Score [2]			
	1	2	3	4
TS_a [1]	0.326 (0.285; 0.375)	0.364 (0.314; 0.423)	0.382 (0.309; 0.451)	0.317 (0.248; 0.397)
TS_b [1]	0.093 (0.092; 0.094)	0.094 (0.094; 0.094)	0.094 (0.094; 0.096)	0.095 (0.094; 0.097)

[1] TS_a: binary teat scoring trait where TS classes 2, 3 and 4 were combined into a single class. TS_b: binary teat scoring trait where TS classes 1 and 2 vs. 3 and 4 were combined. [2] Hygiene of udder, flanks and legs was scored based on a 4-point scale system, from very clean (score 1) to very dirty skin (score 4).

Table 5. Least square mean estimates (posterior mean and 95% highest probability density intervals) for the influence of udder quarter on on TS_a and TS_b traits. Estimates are expressed on the phenotypic scale (probability of a TS greater than 1 for TS_a or TS greater than 2 for TS_b).

Trait	Udder Quarters			
	Front Left	Front Right	Rear Left	Rear Right
TS_a [1]	0.393 (0.327; 0.462)	0.388 (0.323; 0.455)	0.302 (0.265; 0.34)	0.312 (0.271; 0.356)
TS_b [1]	0.095 (0.093; 0.097)	0.095 (0.093; 0.097)	0.094 (0.092; 0.096)	0.094 (0.092; 0.096)

[1] TS_a: binary teat scoring trait where TS classes 2, 3 and 4 were combined into a single class. TS_b: binary teat scoring trait where TS classes 1 and 2 vs. 3 and 4 were combined.

Least square mean estimates expressed on the phenotypic scale (i.e., probability) for each parity by month of lactation effect are in Figure 3. Results for TS_a are in the upper part while results for TS_b in the lower part of the figure.
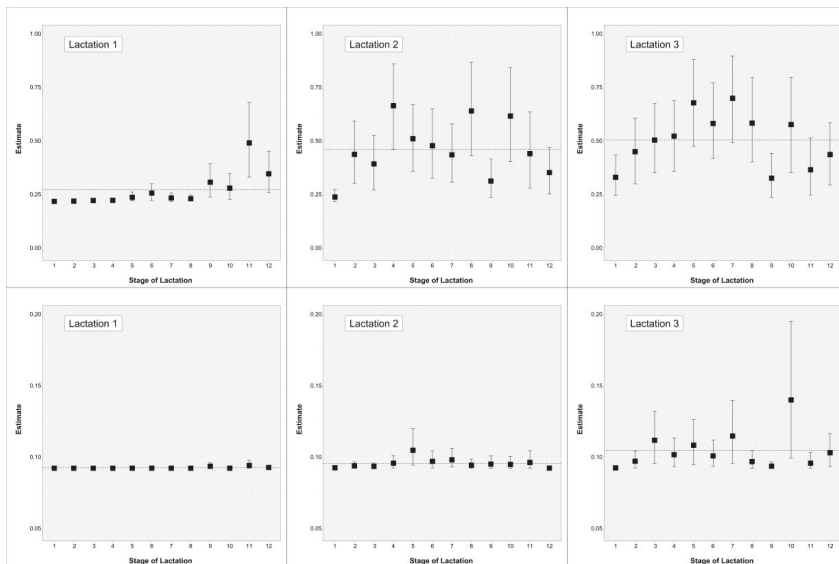

Figure 3. Least square mean estimates expressed on the phenotypic scale (posterior mean and 95% highest probability density intervals) for the lactation by stage of lactation effect on TS_a (upper figures) and TS_b (lower figures) traits. The dashed horizontal line represents the estimate for that lactation across stages.

Estimates for TS_a in lactation 1 showed an increase in the higher scores towards the end of lactation with estimates going from below 0.25 to 0.30. Values similar to the end of lactation 1 were maintained in lactation 2 and subsequently, suggesting that the deterioration of teats is carried over (it is worth considering that this is a cross-sectional study, and not a longitudinal study). Estimates within lactations 2 and subsequently did not show as clear a pattern, though a small increase was shown until the peak of lactation. Lactation estimates across stages for TS_a increased from 0.28 to 0.47 to 0.51 in lactation 1, 2 and 3, respectively. Similarly, lactation estimates across stages for TS_b increased from 0.094 to 0.097 to 0.106 for lactation 1, 2 and subsequently. Conversely, estimates within-lactation for TS_b did not show relevant differences.

3.5. Genetic Response in Teat Score Traits

The genetic response in teat score traits (TS, TS_a and TS_b) after selecting for both MY and SCS applying different relative emphasis is reported in Figure 4. The x-axis gives, from left to right, the relative emphasis as shifting from MY to SCS, the y-axis gives the genetic response in genetic standard deviation units for the respective trait given the overall relative emphasis. The values on the left side of the graph show the response when low emphasis is given to MY, which is positively (unfavorably) correlated with the three traits, and high emphasis is given to SCS, which is still positively correlated but it was given negative emphasis.
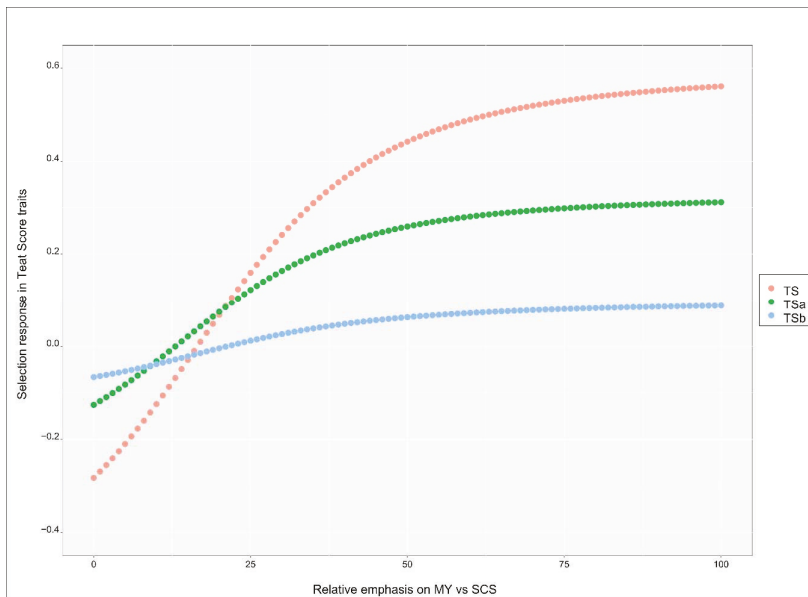
Figure 4. Expected genetic progress in Teat Score traits (y-axis) when shifting relative economic emphasis (x-axis) on milk yield (MY) vs. somatic cell score (SCS). Negative values mean an improvement in teat score (less teat-end hyperkeratosis).

When the emphasis on MY was below 12 (and subsequently the emphasis on SCS was over −88) genetic response for all three teat score traits was negative, i.e., teat score improved. The response for TS became positive at the value of MY emphasis of 17 (i.e., −83 for SCS), became positive for TS_a at the MY emphasis value of 14 (i.e., −86 for SCS) and at the value of 22 for TS_b (i.e., −78 for SCS). All the response curves reached a plateau around the value of 40 for emphasis on MY. At large values of MY emphasis, genetic response became strong and positive (unfavorable) for TS (0.56 genetic standard deviation units), moderately positive for TS_a (0.32 genetic standard deviation units) and almost null

for TS_b (0.09 units). The genetic response was therefore mostly unfavorable for all traits unless most of the emphasis was given to SCS (at least 90%). While genetic correlations with other productive and reproductive traits are missing, we could infer that strong index emphasis should be given to udder health if the deterioration of teat score at the genetic level needs to be avoided. The response was stronger, in either direction, for TS than TS_a, probably due to the larger heritability since the genetic correlations with MY and SCS were about the same for both traits. The response was small to null for TS_b.

4. Discussion

The results of the present study confirm that THK has a genetic component. Indeed, heritability ranged from 0.084 for TS_b (binary trait) to 0.238 for TS (scored using a 1 to 4 scale). These results agree well with the estimates previously reported by Chrystal et al. [17] but are lower than those presented by Lojda et al. [37], Seykora and McDaniel [38], and Chrystal et al. [16]. An interesting aspect regards the scale used to score THK which might influence heritability estimates. Pantoja et al [11], in their systematic review of the association between THK and mastitis, support the idea that THK should be scored using a reduced number of categories. This approach will guarantee an adequate number of records per THK score, hence a better statistical power. However reducing the number of THK scores will partly jeopardize its actual variation and, as [17] suggested, possibly lead to lower heritability estimates. In the present study, the relative frequency of a TS = 4 (i.e., very rough callous ring) was below 2%, lower than that reported by Pantoja et al [11]. However, the 95% highest probability density intervals of the posterior mean heritability estimate for TS trait ranged from 0.116 to 0.364, suggesting that a 4-point scale is still a viable solution. A similar pattern was observed for cow repeatability, with larger estimates when THK was scored on a 4-point scale. This result is quite interesting because it suggests that reducing the scale and treating the THK as a binary trait could be a less accurate measure if recorded only once per cow.

Parity and DIM strongly affect THK [11] and the results found in the present study, even if modulated by the scoring scale, confirm this finding.

The relationship between THK and other breeding goals (e.g., milk yields and somatic cell score) has been mainly investigated considering the former as the dependent variable (Y) and the latter as possible factors affecting Y. The relationship, especially with SCS, was not always clear and significant [11,16,17]. In the present study a different approach has been applied. Indeed, previous studies, not only in dairy cattle [3] but also in other dairy species [39], have suggested a non-zero genetic correlation between udder traits and milk and/or SCS. This aspect is not negligible and should be taken into consideration when implementing a selection index. If the genetic correlation is statistically different from zero there could have been a correlated favorable or unfavorable response on THK. This is particularly true if we consider that selection on milk yield has been the primary objective in dairy cattle for many years [40]. The genetic correlation between THK and milk yield was large and positive, i.e., unfavorable, confirming previous findings [41] in dairy cattle. However, a recent and large study by Tribout [42] in dairy cattle did not confirm the existence of a single quantitative trait locus (QTL) with pleiotropic effect on both milk production and udder morphology suggesting instead the presence of neighboring QTLs that show linkage disequilibrium, eventually leading to a non-null genetic correlation. When using THK scored on 1–4 scale, the genetic correlation was positive and significant (95% empirical confidence intervals not including the '0' value). A positive genetic correlation means that an increase in SCS causes an increase in THK hence selection for lower **SCS** should have a positive impact on teat-end condition. When the relative emphasis on milk yield is below 15%, the response in teat score traits is favorable. Nowadays the focus of selection has moved away from being purely production oriented toward a more balanced breeding goal, even if milk yield has still a relative emphasis not less than 40–50% [40].

In this study we also estimated cow-level correlations as well as herd-test-day correlations. While the former expresses trait associations that are driven by any cow-related factor other than

additive genetics, the latter expresses an association at the herd-level, which is likely due to management alone. In management, we include any strategic choice made by the farmer as well as the equipment used for milking, which is known to affect the insurgence of THK.

Cow correlations were not significant when MY was the correlated trait, but were positive, though weak, when SCS was the correlated trait. This suggests that, beyond genetics, there is a common factor that drives SCS to increase and THK to appear more frequently. At the herd level, correlations were not significant when TS and TS_a were the correlated traits. TS_b was positively correlated with MY and negatively correlated with SCS. This suggest that herd management (and equipment) that leads to higher MY also leads to higher THK. Also, the herd management that leads to lower SCS also leads to higher THK (moving from classes 1 and 2 to classes 3 and 4, specifically).

The different direction of the correlation between TS_b and SCS depending on the additive genetic, cow permanent or herd level suggests that there are counter-acting factors. While cows themselves could show a positive association for which higher SCS means higher THK, the herd management (and equipment) shows the opposite direction, i.e., herds with lower SCS have higher THK and vice versa. The milking practice, for example, could be a factor determining this association.

5. Conclusions

In the current study, multivariate analysis conducted on 10,776 scoring records and 30,160 production records from 2469 Italian Holstein cows enabled the estimation of the genetic parameters for teat-end score and its relationship with milk yields and SCS. Teat-end score has a genetic background and is genetically related to both production and SCS. The scale used to classify teat-end score has an impact on the magnitude of the estimates which however were always statistically different from zero. Teat-end score was also genetically related to SCS, reinforcing the idea that udder morphology is still a fundamental piece in the control of mammary infection. Finally, an unfavorable genetic correlation of teat score with milk yield was observed. However, considering that the current selection objectives implemented in dairy cattle worldwide have shifted toward a more balanced breeding goal a further genetic deterioration in teat-score is not expected. The importance of the negative effects of some environmental aspects, such as milking routine, should not be forgotten.

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Article

RNA Sequencing (RNA-Seq) Based Transcriptome Analysis in Immune Response of Holstein Cattle to Killed Vaccine against Bovine Viral Diarrhea Virus Type I

Bryan Irvine Lopez [1,†], Kier Gumangan Santiago [2,3,†], Donghui Lee [2], Seungmin Ha [4] and Kangseok Seo [2,*]

[1] Division of Animal Genomics and Bioinformatics, National Institute of Animal Science, Rural Development Administration, Wanju 55365, Korea; irvinelopez@korea.kr

[2] Department of Animal Science and Technology, Sunchon National University, Suncheon 57922, Korea; santiagokier2015@gmail.com (K.G.S.); a3832737@naver.com (D.L.)

[3] Department of Animal Science, College of Agriculture, Central Luzon State University, Science City of Muńoz 3120, Philippines

[4] Dairy Science Division, National Institute of Animal Science, Rural Development Administration, Cheonan 31000, Korea; justusha@korea.kr

* Correspondence: sks@sunchon.ac.kr

† These authors contributed equally to this work.

Simple Summary: Due to the undeniable detrimental impact of bovine viral diarrhea virus (BVDV) on cattle worldwide, various preventive approaches are carried out to control the spread of this disease. Among the established preventive approaches, vaccination remains the most widely used cost-effective method of control. Hence, a deeper study into the host immune response to vaccines will further refine the efficacy of these vaccines; the identification of differentially expressed genes (DEGs) related to immune response might bring a long-lasting solution. Thus far, studies showing the genes related to the immune response of cattle to vaccines are still limited. Therefore, this study identified DEGs in animals with high and low sample to positive (S/P) ratio based on the BVDV antibody level, using RNA sequencing (RNA-seq) transcriptome analysis, and functional enrichment analysis in gene ontology (GO) annotations and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Results revealed that several upregulated and downregulated genes were significantly annotated to antigen processing and presentation (MHC class I), immune response, and interferon-gamma production, indicating the immune response of the animals related to possible shaping of their adaptive immunity against the BVDV type I. Moreover, significant enrichment to various KEGG pathways related to the development of adaptive immunity was observed.

Abstract: Immune response of 107 vaccinated Holstein cattle was initially obtained prior to the ELISA test. Five cattle with high and low bovine viral diarrhea virus (BVDV) type I antibody were identified as the final experimental animals. Blood samples from these animals were then utilized to determine significant differentially expressed genes (DEGs) using the RNA-seq transcriptome analysis and enrichment analysis. Our analysis identified 261 DEGs in cattle identified as experimental animals. Functional enrichment analysis in gene ontology (GO) annotations and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways revealed the DEGs potentially induced by the inactivated BVDV type I vaccine, and might be responsible for the host immune responses. Our findings suggested that inactivated vaccine induced upregulation of genes involved in different GO annotations, including antigen processing and presentation of peptide antigen (via MHC class I), immune response, and positive regulation of interferon-gamma production. The observed downregulation of other genes involved in immune response might be due to inhibition of toll-like receptors (TLRs) by the

upregulation of the Bcl-3 gene. Meanwhile, the result of KEGG pathways revealed that the majority of DEGs were upregulated and enriched to different pathways, including cytokine-cytokine receptor interaction, platelet activation, extracellular matrix (ECM) receptor interaction, hematopoietic cell lineage, and ATP-binding cassette (ABC) transporters. These significant pathways supported our initial findings and are known to play a vital role in shaping adaptive immunity against BVDV type 1. In addition, type 1 diabetes mellitus pathways tended to be significantly enriched. Thus, further studies are needed to investigate the prevalence of type 1 diabetes mellitus in cattle vaccinated with inactivated and live BVDV vaccine.

1. Introduction

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of domestic and wild ruminants affecting multiple organ systems, incurring most economic losses due to respiratory diseases, low reproductive performance (due to reduced conception rates), early embryonic deaths, abortion, congenital weak calves, and high costs of control programs [1–4]. BVDV belongs to the genus *Pestivirus* within the family *Flaviviridae*, with two species, namely BVDV1 and BVDV2; both consist of strains, belonging to biotypes non-cytopathogenic or cytopathogenic, based on cell-cultured characteristics [5]. The non-cytopathogenic strain has the ability to cross the maternal placenta, infecting the growing fetus at early gestation (before 150 gestation days) due to the undeveloped immune system and failure of recognizing the virus as foreign [6]. This fetal infection affects fetal development [7], and results in persistently infected born calves, shedding lifelong reservoir of BVDV in the herd, while cytopathic BVDV plays a vital role by superinfecting persistently infected cattle, leading to mucosal disease [5]. In addition, recent studies reported that the immunosuppressive ability of BVDV heightens other viral disease potentiators, particularly the bovine respiratory disease [8–10]. The differences in genotypes and biotypes of BVDV, a wide range of susceptible hosts, the ability to induce persistent infection, and intervene with both innate and adaptive immunity, makes the prevention and control program difficult [4].

To date, modified live viral and inactivated viral vaccines are widely used to prevent the consequences of BVDV infection [11]. However, vaccine efficacy varies, depending on the animal's nutritional status [12], maternal antibody from the dam colostrum [13], and the presence of persistently infected cattle in the herd. Thus far, numerous studies were conducted to improve vaccine efficacy against BVDV, yet still remains prevalent among the cattle herd worldwide.

Numerous studies focusing on the transcriptomic analysis of animals infected with various diseases were carried out. In the study of Li et al. [4], various differentially expressed genes (DEGs) related to goat immune response, including inflammation, defense response, cell locomotion, and cytokine/chemokine-mediated signaling were revealed by transcriptome analysis with samples from BVDV2 artificially infected goat peripheral blood mononuclear cells (PBMCs). Similar methodology was done in the study of Singh et al. [14], where various significant DEGs related to the immune system processes of goat and sheep against bluetongue virus serotype 16 (BTV-16) were revealed, such as NFκB, MAPK, Ras, NOD, RIG, TNF, TLR, JAK-STAT, and VEGF signaling pathways. Meanwhile, comparative transcriptomic analyses between infected and non-infected animals were conducted by Barreto et al. [15], where infected bovine were observed to have massive changes in the expression profiles of keratinocyte, immune system, cell proliferation, and apoptosis genes.

All of these studies used transcriptome analysis and next-generation sequencing (NGS) approach particularly the RNA sequencing (RNA-Seq). RNA sequencing is a developed method that uses deep sequencing technology for transcriptome profiling [16]; aside from providing a comprehensive picture of the transcriptome, it also reveals the activity and mechanism of the molecular structure and explores the biological function of a gene [17]. Furthermore, future works using this technology are considered,

such as genetic linkage mapping, quantitative trait analysis, disease-resistant strains, effective vaccines, and therapies development [18].

Although genotyping and RNA sequencing still remain costly, such studies may provide novel insights and solid foundation in improving herd performance through the robustness of animals against viral diseases. However, studies pertaining to the immune responses of the animal to the vaccine were not fully explored. Thus, the objective of this study is to identify differentially expressed genes (DEGs) related to the functional immune response of the host vaccinated with the inactivated BVDV type I vaccine, and to provide insight on how vaccines improve the immunity of animals against diseases.

2. Materials and Methods

2.1. Experimental Animals and Vaccination

A total of 107 vaccinated Holstein Cattle (*Bos taurus*) was used in the study. Multivalent killed vaccine Bar Vac Elite 4-HS (Boehringer Ingelheim Vetmedica, Inc., St Joseph, MO, USA) containing antigen of infectious bovine rhinotracheitis virus, bovine viral diarrhea virus (BVDV, type I), bovine respiratory syncytial virus (BRSV), Myxovirus parainfluenza type 3 (PI3) and *Haemophilus somnus* bacterin were given intramuscularly, as prescribed by the manufacturer.

Blood samples were collected from the jugular vein at 7, 28, and 168 d post-vaccination. Subsequently, blood was allowed to coagulate for 1–2 h at 4 °C and centrifugate for 20 min at room temperature with the relative centrifugal force of $1800 \times g$. Serum was collected and aliquoted into 1.5 mL tubes and stored below −60 °C until ELISA test.

2.2. Serological Antibody Detection

Competitive ELISA, using VDPro BVDV AB ELISA (Median Diagnostics Inc., Chuncheon, Republic of Korea) was used to assay the antibody responses of each animal against the vaccine. Assaying was performed as per manufacturer protocols. Concisely, the BVDV gp63 antigen was allowed to absorb in the polystyrene plate and bind with antibodies in serum samples. It was competed for corresponding hydrogen peroxide conjugated monoclonal antibodies. The chromogenic change after the addition of 3, 3′, 5, 5′-Tetramethylbenzidine substrate was measured at 450 nm optical density using BioTek ELISA reader and Gen5 2.07 software; results with lower color development signify a higher level of antibody. The optical density (OD) value was measured using a microplate reader set at 405 nm and concentration was valued with the corresponding standard references. The obtained OD value of BVDV type I antibodies were evaluated for the comparative value, related to the positive control value, to get antibodies level in the sample to positive (S/P) ratio form by applying the equation as below.

$$\frac{S}{P} = \frac{\text{Sample O.D. value } - \text{ Negative Control O.D. value}}{\text{Positive control O.D. value } - \text{ Negative Control O.D. value}} \tag{1}$$

The obtained BVDV type I S/P ratio, and other immune-related parameters, such as TNF-alpha, IFN-gamma, IL-17A, IL-1b, IL-4, IL-2, and IL-6 were used as the basis for selecting experimental animals. Among the 107 experimental animals, only ten (10) animals were selected and grouped into two groups, namely the low and high BVDV type I groups; each group had five (5) animals (Figure 1).

2.3. RNA Isolation, Library Preparation, and RNA Sequencing (RNA-seq)

Total RNA was stabilized and isolated from the blood samples (collected after vaccination) of the selected animal groups using Tempus Blood RNA Tube (Applied Biosystems, Seoul, Korea), according to the manufacturer's instructions. RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA) was used to purify and concentrate the previously isolated RNA. RNA quality based on RNA integrity number (RIN) was determined using Agilent Technologies 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) with an acceptable RIN value of ≥7. These RNA samples were used to generate RNA-Seq transcriptome libraries, using the TruSeq Stranded Total RNA LT sample preparation kit (Globin) of Illumina (San Diego, CA,

USA). To ensure the quality of prepared libraries, the size of the PCR enriched fragments were verified by checking the template size distribution, by running on Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip, and were quantified using qPCR according to the Illumina qPCR quantification protocol guide. After a series of quality control and quantification, prepared paired-end libraries for animals with high (test) and low (control) BVDV type I antibody were then sequenced with the Illumina NovaSeq 6000 platform, performed by TNT Research Corporation Limited (Anyang, South Korea).
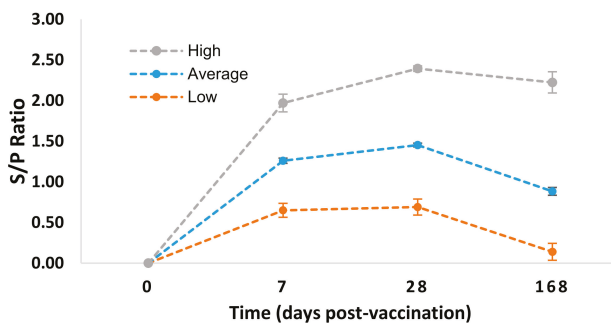

Figure 1. Sample to positive (S/P) ratio of cattle groups identified as high ($n = 5$), low ($n = 5$) and average ($n = 107$) bovine viral diarrhea virus (BVDV) type I antibody level at different time points. The error bars indicate standard error.

2.4. Reads Trimming, Mapping, and Assembly of Sequenced RNA Reads

Quality control (QC) of raw paired-end reads were done by trimming reads using Trimmomatic 0.38 (http://www.usadellab.org/cms/?page=trimmomatic). QC included the removal of adapter sequence, contaminant DNA, and low-quality reads with lengths below 36 bp. Clean reads (cDNA) were indexed to reference (*Bos taurus*) cattle genome GCF_002263795.1 ARS-UCD1.2 and were mapped against the reference genome using HISAT2 version 2.1.0 (Bowtie2 aligner) (https://ccb.jhu.edu/software/hisat2/index.shtml). Reference-based aligned read assembly of transcripts was performed using the StringTie 1.3.4d (https://ccb.jhu.edu/software/stringtie/). This allowed the identification of transcript or genes with annotation information in the assembled genome, while genes without annotated information were defined as new transcripts [19]. On the other hand, mapping of each sample without the –e option of StringTie allowed the prediction of novel transcript and novel alternative splicing transcript. The gffcompare program from GFF utilities was used to compare existing annotations and distinguish novel transcript types.

2.5. Differential Expression Genes (DEGs) Analysis and Clustering

The identification of DEGs between case and control samples was based on the expression level on each transcript, which was calculated using the fragments per kilobase of exon per million mapped reads (FPKM) method. The DESeq2 package, equipped with fold change and negative binomial (nbinom) Wald test, was used for differential expression analyses. DEGs were identified based on the following parameters: the logarithmic fold change was greater than or equal to 2 ($|fc|>=2$) and nbinom Wald test raw $p < 0.05$. In addition, hierarchical clustering of significant genes was done to determine the similarity level of each sample.

2.6. Functional Annotation and Enrichment Analysis

DEGs were based on several functional annotation databases, specifically gene ontology (GO) (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://kegg.jp). Enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery 6.8

(DAVID) tool (http://david.abcc.ncifcrf.gov/) equipped with the modified Fisher's exact test. DEGs with a *p*-value of less than 0.05 were significantly considered enriched in GO terms and KEGG pathways.

3. Results

3.1. Experimental Animals

Table 1 shows the two groups of experimental animals identified in this study, namely, low and high group, based on the level of BVDV type I antibody and level of immune responses, including TNF-alpha, IFN-gamma, IL-17A, IL-1b, IL-4, IL-2, and IL-6. For the earlier group, there were five identified animals namely (ID number), 13064, 13083, 13090, 14010, and 14017, while a similar number of animals belong to the latter group, namely, 14107, 15060, 15071, 15083, and 15094.

3.2. Transcriptome Sequencing Data

An average of 9.0G bp (Table 1) raw data for each sample was obtained from paired-end transcriptome sequencing from the Illumina NovaSeq 6000 platform. Prior to further analysis, raw data were subject to quality control using Trimmomatic version 0.38. The trimmed results show that the total read bases, GC (%), and Q30 (%) of each sample have values ranging from 7.0 G to 11.0 G, 44.86% to 45.99%, and 94.65% to 95.31%, respectively, as shown in Table 1. Trimmed data were mapped against the reference genome (GCF_002263795.1 ARS-UCD1.2) using the HISAT2 program. Obtained mapped reads were then assembled using StringTie-e option version 1.3.4d, which resulted in a total of 100,685 transcripts and 39,127 genes successfully mapped against the reference genome. Thereafter, the removal of low-quality transcripts and genes was done, leaving only 10,000 transcripts and 35,000 genes for differentiation analysis. Furthermore, a total of 1452 novel transcripts, 13,060 novel splicing variants, and 4199 novel genes were identified using the StringTie software.

Table 1. Summary of the mapping information for each sample.

Sample ID	Total Raw Reads	Total Clean Reads	GC (%)	Q30 (%)	No. of Processed Reads	No. of Mapped Reads	No. of Unmapped Reads
13064	1.13E + 10	1.12E + 08	45.52	95.28	1.11E + 08	1.08E + 08	2.32E + 06
13083	9.59E + 09	9.50E + 07	45.73	95.27	9.40E + 07	9.12E + 07	2.74E + 06
13090	9.77E + 09	9.67E + 07	45.22	94.65	9.55E + 07	9.38E + 07	1.70E + 06
14010	8.52E + 09	8.44E + 07	45.00	95.31	8.36E + 07	8.17E + 07	1.82E + 06
14017	9.38E + 09	9.29E + 07	45.99	95.30	9.19E + 07	8.93E + 07	2.55E + 06
14107 *	9.67E + 09	9.58E + 07	45.03	95.22	9.47E + 07	9.27E + 07	2.02E + 06
15060 *	8.31E + 09	8.22E + 07	45.54	95.08	8.13E + 07	7.96E + 07	1.62E + 06
15071 *	9.01E + 09	8.92E + 07	45.87	95.23	8.83E + 07	8.18E + 07	6.37E + 06
15083 *	8.37E + 09	8.29E + 07	44.48	95.19	8.20E + 07	8.02E + 07	1.77E + 06
15094 *	7.42E + 09	7.35E + 07	44.86	94.98	7.26E + 07	7.07E + 07	1.92E + 06

* Animals belongs to high immune responses and BVDV type I antibody group.

3.3. Differentially Expressed Genes

The resulting good quality genes from StringTie software were filtered by excluding genes with at least one zero count, leaving only 45% (16,315 genes) for DEG analysis. Prior to DEG analysis, expression levels between genes of each sample were first normalized using the Relative Log Expression (RLE) normalization method, based on raw read counts (Figure 2).

The analysis of the differently expressed genes (DEGs) were done by comparing the normalized values using the DESeq2 package, equipped with log fold change and nbinom Wald test. A total of 261 genes were considered differentially expressed based on the threshold level (fold change (log2) ≥ 2 and *p*-value < 0.05) (Figure 3). Results of comparison analysis between animals with high (test) and low (control) BVDV type I antibody groups revealed that 143 genes were classified as up-regulated while the remaining 118 genes were down-regulated (Figure 3).
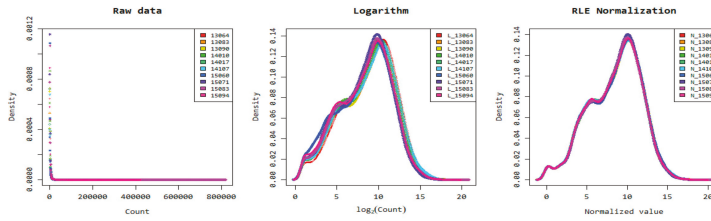
Figure 2. Density plot of normalized data using Relative Log Expression (RLE) normalization method based on read count and log2.
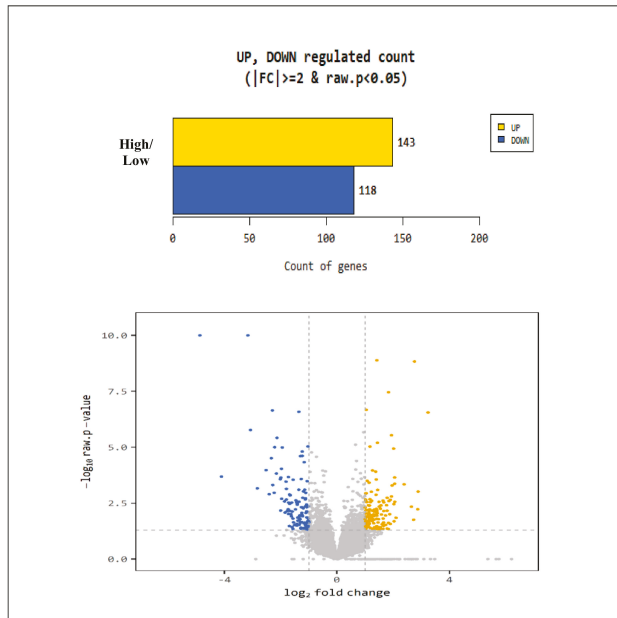

Figure 3. Number of up- and down-regulated genes after comparison of normalized values using the DESeq2 package.

3.4. Functional Enrichment Analysis of Identified DEGs

RNA-seq transcriptome analysis successfully identify a total of 261 DEGs. Several functional annotation databases, such as gene ontology (GO) and KEGG pathway using the Database for Annotation, Visualization and Integrated Discovery 6.8 (DAVID) tool, were used to determine the biological function of these identified DEGs. DAVID gene enrichment analysis revealed 28 significant GO terms throughout the differentiation analysis ($p < 0.05$). However, there were only three GO major categories, namely biological process (GOTERM_BP), cellular component (GOTERM_CC), and molecular function (GOTERM_MF) where the significant DEGs were grouped based on their functionality (Figure 4). In this study, 38 significant DEGs were distributed to top 10 GOTERM_BP, namely antigen processing and presentation of peptide antigen (via MHC class I), immune response, positive regulation of gene expression, negative regulation of gene expression, negative regulation of cell growth, negative regulation of oxidoreductase activity, positive regulation of interferon-gamma production, collagen biosynthetic process, and caveola assembly. In GOTERM_MF, 21 DEGs were distributed into the following top 5 GO terms; calcium ion binding, calcium-dependent cysteine-type endopeptidase activity, SH3 domain binding, superoxide-generating Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase activator activity, and protein complex scaffold.

Meanwhile, 78 DEGs were distributed to the top 7 GOTERM_CC as follows; integral component of membrane, class I protein complex, extracellular region, proteinaceous extracellular matrix, membrane raft, axon and anchored component of the external side of the plasma membrane.

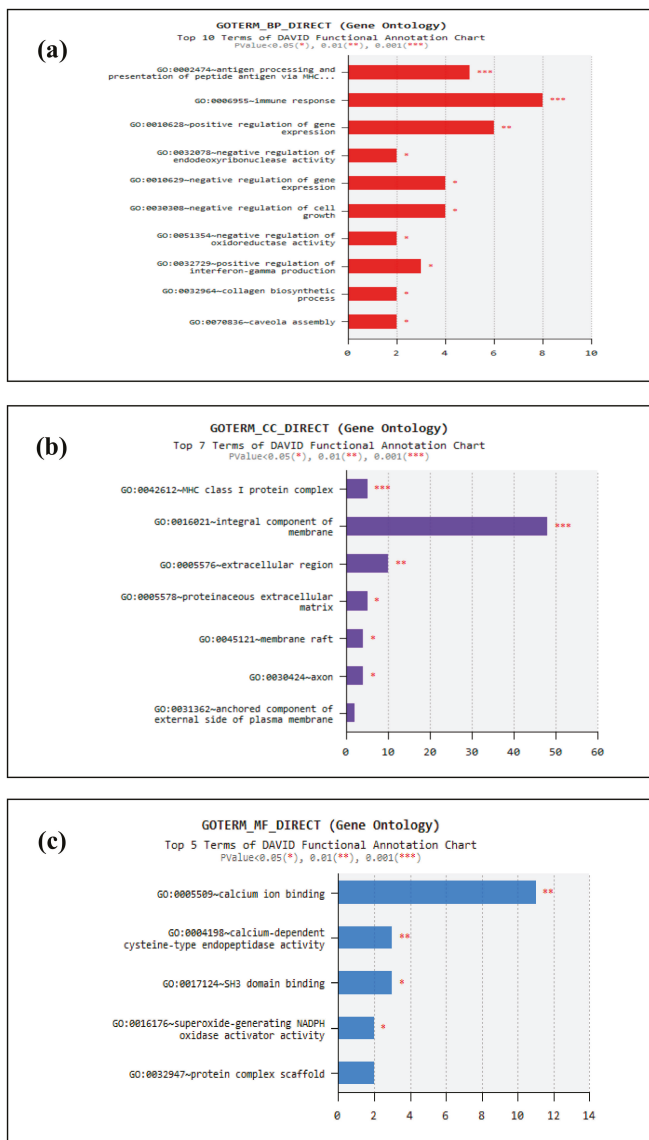**Figure 4.** Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in vaccinated cattle, selected based on BVDV type I antibody level (**a**) GOTERM_Biological Process, (**b**) GOTERM_Cellular Component, and (**c**) GOTERM_Molecular Function. GO terms are located on the y-axis and terms with (*) (**) (***) means significant enrichment with a *p*-value of <0.05, 0.01, and 0.001, respectively.

3.5. KEGG Pathway Enrichment Analysis

To allow deeper understanding of the biological function of significant DEGs, KEGG pathway enrichment analysis was done using DAVID 6.8 tool. Initially, KEGG pathways analysis successfully annotated DEGs into 10 pathways which later reduced into 5 significantly enriched pathways ($P<0.05$) namely; cytokine-cytokine interaction, platelet activation, ECM-receptor interaction, hematopoietic cell lineage, and ABC transporters (Table 2). Cytokine-cytokine interaction pathway involved 2 upregulated (IL18, IL1RAP) and 4 down-regulated DEGs (CCR8, CCL3, IL20RA, TGFB2); hematopoietic cell lineage pathway linked 4 upregulated DEGs (GP5, GP1BA, CD24, GP9); platelet activation pathway with 5 up-regulated DEGs (GP5, P2RX1, MAPK12, GP1BA, GP9); ECM-receptor interaction pathway with 3 up-regulated (GP5, GP1BA, GP9) and 1 down-regulated (ITGB4) DEGs and ABC transporters pathway with 1 upregulated (ABCB11) and 2 downregulated DEGs (LOC100296627, CFTR). Results obtained from GO and KEGG analysis indicated that various DEGs were involved in host immune response to inactivated BVDV Type I vaccine.

Table 2. List of significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with immune response.

	Pathways	ID	DEGs No.	*p*-Value	Up-Regulated Genes	Down-Regulated Genes
1	Platelet activation	bta04611	5	1.34E-02	GP5, P2RX1, GP1BA, GP9	MAPK12
2	Cytokine-cytokine receptor interaction	bta04060	6	2.07E-02	IL18, IL1RAP	CCR8, CCL3, IL20RA,TGFB2
3	ECM-receptor interaction	bta04512	4	2.52E-02	GP5, GP1BA, GP9	ITGB4
4	Hematopoietic cell lineage	bta04640	4	2.91E-02	GP5, GP1BA, CD24, GP9	-
5	ABC transporters	bta02010	3	3.73E-02	ABCB11	LOC100296627, CFTR
6	Type I diabetes mellitus	bta04940	2	5.48E-02	BOLA, PTPRN2	-

ECM—Extra Cellular Matrix, ABC—ATP Binding Cassette

4. Discussion

BVDV infection is undeniably detrimental to bovine raisers by reducing milk yield, and is associated with low reproductive performance and growth retardation, allowing the occurrence of other disease potentiators, premature culling, and a high rate of mortality to young stock [20]. Houe et al. [20] and Carman et al. [21] estimated that national herd could experience economic loss ranging between $10 million and $40 million per million calvings, and $40,000–$100,000 (USD) per herd, respectively. Thus, to prevent such negative effects of BVDV, the development of cost-effective controls, including vaccines and eradication schemes were considered [22]. However, despite effective control programs, BVDV infection remains rampant in most cattle herd worldwide. Evidence reveals that variability of the BVDV strains, cross placental ability of the virus leading to persistent infections, wide spectrum of susceptible hosts, and the ability to interfere both innate and adaptive immunity makes prevention and control, such as vaccination, less effective [4,23].

Recently, similar studies that used next-generation sequencing technology (NGS) purported various DEGs related to animal immune response against viral diseases, providing a deeper understanding of immune responses. Since the development of microarray-based analysis and completion of the Human Genome Project, more advanced sequencing technology has come about, such as RNA-Seq based transcriptome analysis [24]. Compared to DNA microarray-based technology, RNA–Seq provide greater dynamic range by directly revealing sequence identity crucial for annotation quantification of unknown genes and novel transcript isoforms [25,26]. In studies by Li et al., Singh et al., and Barreto et al. [4,14,15], RNA-Seq based transcriptome analyses were used to successfully identify

both up- and down-regulated genes related to the host immune response during BVDV, bluetongue virus of sheep and goats, and bovine papillomatosis infection, respectively.

In this study, cattle vaccinated with inactivated multivalent vaccine (BVDV type I, BRSV, Myxovirus parainfluenza type 3 (PI3), *Haemophilus somnus* bacterin) were evaluated days after the last vaccination, to identify and understand changes in gene expressions related to the immune response brought by the vaccine. Specifically, this study examines the only animal with a high and low BVDV type I antibody level; thus, DEGs identified in the transcriptome analysis were highly attributed to the immune response of the animal to inactivated BVDV type I vaccine.

Vaccination is considered an effective tool in preventing and controlling infectious diseases involving the cooperative action of innate and adaptive immunity [27]. Innate immunity plays a key role in triggering adaptive immune response by involving hematopoietic cells, such as macrophages, mast cells, neutrophils, eosinophils, dendritic cells, natural killer cells, and non-hematopoietic cells, such as skin and epithelial linings of the gastrointestinal, genitourinary, and respiratory tract [28]. Meanwhile, adaptive immunity plays its vital role in the immune system as it involves a tightly regulated interaction between antigen -presenting cells and T and B lymphocytes that facilitate pathogen-specific immunologic effector pathways, immunologic memory, and regulation of host immune homeostasis [29].

In this study, functional enrichment analysis revealed upregulated DEGs related to both innate and adaptive immune responses, such as BoLA, IL18, and BCL3. Among identified immune-related genes, the bovine lymphocyte antigen (BoLA) caught the attention of the researcher, as it was directly involved in antigen presentation. The BoLA gene located on chromosomes BTA 23 [30], and generally known as the MHC of cattle, was reported to play an integral role in immune responsiveness and susceptibility to the diseases of the host animal [31]. MHC is a cell surface glycoprotein molecule, having the binding ability to foreign peptides, such as viral proteins, and provides context for the recognition of T-lymphocytes responsible for cell-mediated immunity [32,33]. In studies conducted by Gutierrez et al. [34] and Weigel et al. [35], it was discovered that MHC genes are strongly associated with disease resistance and susceptibility to a wide range of diseases; thus, it can be a natural strategy in controlling infectious diseases, by incorporating it to the selection index and in genetic manipulation techniques.

The IL18 and Bcl-3 gene was also identified, upregulated in this study; IL18 gene play an important role in the T-cell-helper type 1 (Th1) and are involved in the regulation of innate and adaptive immune response by inducing IFN-gamma in natural killer cells (NKC) and T helper (Th1) lymphocytes [36,37]. Primary precursors of IL-18 are expressed in epithelial cells of the body, while the primary sources are macrophages and dendritic cells [38]. In a study conducted on laboratory mice, IL-18 was considered an effective adjuvant by enhancing immunogenicity through its relevant activities, such as activator of NK cells, a strong stimulator of Th1 responses, and other immunoactive cytokines in Th1 cells, monocytes, and NK Cells [38].

Whereas, the BCL3 is a proto-oncogene member of the IkB family, and also reportedly plays an important role in immune responses. In a study by Schwarz et al., it was purported that the Bcl-3 protein interacts specifically with the NFkB subunits (p50 and p52). It was also reported that mice lacking the Bcl-3 gene exhibit normal development and immunoglobin levels, but the humoral immune response was severely affected, and fail to produce antigen-specific antibodies [39]. Further, in the study of Fredericksen et al. [40], it was observed that the BVDV-1 infected Madin-Darby bovine kidney cell line induces immune marker production, such as BCL3, IL-1, IL-8, IL-15, IL-18, Mx-1, IRF-1, and IRF-7 through the NF-kB signaling pathway. Furthermore, Carmody et al. [41] reported that Bcl-3 limits the strength of toll-like receptors (TLRs) that are responsible for triggering inflammatory cytokines production and development of both adaptive and innate immunity through p50 subunit ubiquitination stabilization. Thus, this limitation of TLR responses might be responsible for the downregulation of other DEGs related to inflammatory responses.

Enrichment analysis through KEGG pathways of DEGs was done to understand signal transduction pathways activated and repressed by inactivated antigen (vaccine). Results of KEGG pathway enrichment analysis revealed five (5) significantly enriched pathways, such as platelet activation, cytokine-cytokine receptor interaction, ECM receptor interaction, hematopoietic cell lineage, and ABC transporters. Among identified significant pathways, cytokine-cytokine receptor interaction involved the most number of DEGs. The cytokine-cytokine (c-c) receptor interaction plays a vital role in health during immunological and inflammatory responses to diseases through the synergistic convergence of signaling pathways and divergence of the cytokine signal, which activates another cytokine system [42]. In this study, six (6) significant DEGs under c-c receptor interaction pathways were identified, namely; CCR8, CCL3, IL20RA, TGFB2, IL18, and IL1RAP with only the last two (2) DEGs identified as upregulated (IL18, IL1RAP). Downregulation of other DEGs belonging to c-c receptor interaction and linked to TLR may be attributed to previously reported upregulation of Bcl-3, which limits the duration of TLR responses that control deleterious inflammatory diseases. However, further studies are warranted to fully support this claim.

Another significantly enriched pathway observed in this study is the extracellular matrix (ECM) receptor interaction pathway, which includes four (4) upregulated DEGs, namely, glycoprotein (GpV), GpIba, GpIX, and ITGB4. Briefly, ECM is a non-cellular component found in all tissue and organs, providing cellular constituents its physical framework, and it also plays a vital role in tissue morphogenesis, differentiation, and homeostasis by initiating crucial biochemical and biomechanical signals [43]. Additionally, ECM conveys specific signals to cells resulting in the modulation of basic functions that are important for the early steps of inflammation, particularly the migration of immune cells during tissue inflammation and immune cell differentiation [44]. These functions of ECM are believed to be mediated primarily by integrins under the family of cell surface receptors [45]. In support, Kroll et al. [46] and Englund et al. [47] reported that platelet membranes, such as GpIb and GpIX, when bound to the von Willebrand factor (vWF), would help transmit signals to the platelet that leads to platelet activation and adhesion. Whereas, the platelet glycoprotein (GP) Ib-IX-V complex is responsible for platelet rolling and adhesion to the site of injury [48]. As such, the literature suggests that the upregulation of integrin subunit beta 4 (ITGB4), GpV, GpIba, and GpIX in this study might be involved in the immune-related functions of ECM.

Fascinatingly, enrichment of ECM receptor pathways supported the succeeding enriched pathways, such as the hematopoietic cell lineage and platelet activation pathways. In a study by Klein [49], it was reported that the ECM matrix molecules (collagen, proteoglycans, and glycoproteins) are part of the bone marrow microenvironment that plays a very significant role in promoting hematopoietic cell proliferation and differentiation. Thus, the upregulation of all DEGs under the hematopoietic cell lineage pathway (GP5, GP1BA, CD24, GP9) supports the observed upregulation of some DEGs under the ECM pathway.

On the other hand, the platelet activation pathway, which are believed to be related to ECM glycoprotein, was reported to be triggered during viral antigen-antibody complexes, from which virus-induced platelet activation can modulate platelet count that help shape immune response through their released products that suppressed infection [50]. Under this pathway, there were four identified upregulated DEGs, namely, GP5, P2RX1, GP1BA, and GP9, with only MAPK12 as down-regulated.

Another important enriched pathway is the ATP-binding cassette (ABC) transporter, which purportedly plays a crucial role in adaptive immunity by its ability to shuttle degrade proteasomal products into the endoplasmic reticulum (ER), which then loaded to MHC class I before antigen presentation on the cell surface [51,52]. In the study of Hinz and Tampé [53], it was also reported that transporters associated with antigen processing (TAP) could be challenged with a number of viral factors, which prevent antigen translocation and loading MHC class I in virally infected cells. Thus, this literature suggests that the upregulation of ABCB11 (ABC transporter pathways) previously observed in this study was associated with the development of adaptive immunity against BVDV Type I.

Another interesting pathway that tended to be significantly enriched was the type 1 diabetes mellitus pathway. This information catches the attention of researchers due to a previous report that cattle infected with the BVD-mucosal disease virus can induce insulin-dependent diabetes mellitus [54].

5. Conclusions

The results of this study showed significantly identified DEGs under different immune-related gene ontologies and signaling pathways in response to BVDV type 1 antigen. These observed findings will surely provide assistance by enlightening end-users and other researchers on the changes happening in the animal immune system brought by vaccination. In addition, the potential inclusion of DEGs to animal improvement programs, such as breeding, selection, and genetic manipulation techniques will surely help improve the efficacy of the vaccine. Furthermore, the DEGs, annotation, and pathways identified in this study can be utilized for future studies concerning the immune response of cattle to vaccines.

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Genetics of Arthrogryposis and Macroglossia in Piemontese Cattle Breed

Liliana Di Stasio [1,*], Andrea Albera [2], Alfredo Pauciullo [1], Alberto Cesarani [3], Nicolò P. P. Macciotta [3] and Giustino Gaspa [1]

[1] Department of Agricultural, Forest and Food Sciences, University of Torino, Largo Baccini 2, 10095 Grugliasco (TO), Italy; alfredo.pauciullo@unito.it (A.P.); giustino.gaspa@unito.it (G.G.)

[2] Associazione Nazionale Allevatori Bovini di Razza Piemontese, strada provinciale Trinita' 31/A, 12061 Carrù (CN), Italy; andrea.albera@anaborapi.it

[3] Department of Agriculture, University of Sassari, Via De Nicola 9, 07100 Sassari, Italy; acesarani@uniss.it (A.C.); macciott@uniss.it (N.P.P.M.)

* Correspondence: liliana.distasio@unito.it

Simple Summary: The study was carried out in order to investigate the genetic background of arthrogryposis and macroglossia in the Piemontese cattle breed, for which limited information is available so far. The genotyping of affected and healthy animals with a high-density chip and the subsequent genome-wide association study did not evidence a single strong association with the two pathologies. Therefore, for arthrogryposis, the results do not support the existence of a single-gene model, as reported for other breeds. Rather, 23 significant markers on different chromosomes were found, associated to arthrogryposis, to macroglossia, or to both pathologies, suggesting a more complex genetic mechanism underlying both diseases in the Piemontese breed. The significant single nucleotide polymorphisms (SNPs) allowed the identification of some genes (*NTN3*, *KCNH1*, *KCNH2*, and *KANK3*) for which a possible role in the pathologies can be hypothesized. The real involvement of these genes needs to be further investigated and validated.

Abstract: Arthrogryposis and macroglossia are congenital pathologies known in several cattle breeds, including Piemontese. As variations in single genes were identified as responsible for arthrogryposis in some breeds, we decided: (i) to test the hypothesis of a similar genetic determinism for arthrogryposis in the Piemontese breed by genotyping affected and healthy animals with a high-density chip and applying genome-wide association study (GWAS), F_{ST} and canonical discriminant analysis (CDA) procedures, and (ii) to investigate with the same approach the genetic background of macroglossia, for which no genetic studies exist so far. The study included 125 animals (63 healthy, 30 with arthrogryposis, and 32 with macroglossia). Differently from what reported for other breeds, the analysis did not evidence a single strong association with the two pathologies. Rather, 23 significant markers on different chromosomes were found (7 associated to arthrogryposis, 11 to macroglossia, and 5 to both pathologies), suggesting a multifactorial genetic mechanism underlying both diseases in the Piemontese breed. In the 100-kb interval surrounding the significant SNPs, 20 and 26 genes were identified for arthrogryposis and macroglossia, respectively, with 12 genes in common to both diseases. For some genes (*NTN3*, *KCNH1*, *KCNH2*, and *KANK3*), a possible role in the pathologies can be hypothesized, being involved in processes related to muscular or nervous tissue development. The real involvement of these genes needs to be further investigated and validated.

Keywords: Piemontese breed; arthrogryposis; macroglossia; genetic model

1. Introduction

Arthrogryposis and macroglossia have long been known as congenital abnormalities observed in several cattle breeds [1,2]. Arthrogryposis is characterized by joints contractures with different degrees of severity, which can affect one to four legs, with various associated clinical signs, the most frequent being cleft palate [3]. More than one etiologic event, such as plant toxicosis [4], prenatal viral infections, and a possible hereditary component, have been reported as responsible for the disease occurrence [5]. Less information is available for macroglossia, which consists in the swelling of the tongue that may interfere with the calf's ability to nurse. The defect is thought to have a genetic basis, but no scientific evidence is available so far.

For both defects, double muscling is considered as a predisposing factor. Already back in the 1963, Lauvergne et al. [6] listed rickets-like troubles and macroglossia among the clinical signs displayed by the hypertrophied animals. The observation that the manipulation of the myostatin gene and, more specifically, the downregulation of its expression resulted in a series of adverse effects, including leg problems and macroglossia, which seems to confirm the negative influence of double muscling [7]. Moreover, macroglossia is one of the primary features of the human Wiedemann-Beckwith syndrome (OMIM 130650), which is clinically similar to muscular hypertrophy in cattle [8].

Both arthrogryposis and macroglossia have been reported for decades in the hypertrophied Piemontese cattle breed. Since the end of 1980s, the National Association of the Piemontese cattle Breeders (ANABORAPI) started to select against these two pathologies by culling Artificial Insemination (AI) bulls with a high percentage of affected progeny. A decrease from 2.74% to 0.34% and from 2.36% to 0.28% in the occurrence of arthrogryposis and macroglossia, respectively, were obtained in the period 1990–2017 (Supplemental Figure S1) as a consequence of this selection strategy (ANABORAPI).

These data seem to support the hypothesis of a genetic background for the defects, but the few investigations in the Piemontese breed did not give conclusive results. Huston et al. [3] suggested that, in the Piemontese, arthrogryposis could be determined by an incompletely penetrant recessive allele, with higher penetrance in males, which seems to be consistent with the ANABORAPI data (Supplemental Figure S1). However, a genome-wide association study carried out on Piemontese calves affected by arthrogryposis and macroglossia genotyped with a medium density (50 K) single nucleotide polymorphism (SNP) BeadChip did not detect clear signals of association for both pathologies [9]. On the contrary, recent studies detected variations in single genes as responsible for arthrogryposis in Angus [10], Swiss Holstein [11], Belgian Blue [12], and Red Danish [13] cattle breeds.

Therefore, the aims of this study were: (i) to test the hypothesis of a similar monogenic determinism for arthrogryposis in the Piemontese cattle breed by genotyping affected and healthy animals with a high-density chip never used in previous studies and applying genome-wide association study (GWAS), F_{ST} and canonical discriminant analysis (CDA) procedures, and (ii) to investigate with the same approach the genetic background of macroglossia, for which no information is available so far.

2. Materials and Methods

2.1. Ethics Statement

No animals were used in the present study. The biological samples belonged to collections available from the ANABORAPI institutional activity. For this reason, the Animal Care and Use Committee approval was not necessary.

2.2. Animals, Genotyping, and Data Editing

Animals affected by arthrogryposis or macroglossia were found and sampled by the veterinarians of the ANABORAPI during the routine inspections in the farms registered in the Herd Book of the Piemontese breed. The phenotypic expression of arthrogryposis in the Piemontese breed is very variable, ranging from moderate contracture of the legs to more severe expressions that can be only surgically corrected. As this variability could represent a confounding factor, only animals with

extreme expressions of the defect (Supplemental Figure S2a) were considered. Moreover, as in about 3% of the affected animals the two pathologies coexist, only animals affected by a single pathology, arthrogryposis or macroglossia (Supplemental Figure S2), were included in the study, in order to avoid a further confounding effect.

Blood samples were collected from a total of 98 Piemontese male veals: 17 affected by arthrogryposis (Ar), 18 affected by macroglossia (Ma), and 63 healthy (He). The Ar, Ma, and He subjects were distributed in 17, 15, and 59 herds, respectively. All these animals were genotyped with the customized GeneSeek® Genomic Profiler™ Bovine 150 K (Lincoln, NE, USA). As it was difficult to collect a larger number of affected animals for their low incidence due the selection policy, we decided to include 31 additional subjects (16 affected by arthrogryposis and 15 by macroglossia) previously genotyped with the Illumina® BovineSNP50v2 (San Diego, CA, USA). Both 150 K and 50 K markers were mapped on the ARS-UCD1.2 and subjected to quality checks (QC). The QC was performed using PLINK v.1.923 [14] independently for each dataset using the following filters: SNPs with missing rate > 0.02, minor allele frequency (MAF) < 0.05 or deviating from Hardy-Weinberg equilibrium (*p*-values < 10^{-6}) were discarded; moreover, only autosomal SNPs with known genomic positions were considered for further analysis. On the subjects' side, both SNP missingness per individual (>0.02) and individual heterozygosity deviations caused an animal to be removed from the datasets. After data editing, 94 and 31 veals were left in 150 K and 50 K, respectively. Then, the default settings of Beagle software [15] were applied for imputing the 50-K genotypes at 150 K on the whole dataset, obtaining a group of 125 animals (63 He, 30 Ar, and 32 Ma). This dataset was then split into two subsets of 93 (He + Ar) and 95 (He + Ma) individuals each that underwent a new round of QC with the aforementioned settings. A total of 100,791 and 100,907 SNPs were analyzed for the Ar and Ma subsets, respectively.

2.3. Statistical Analysis

A genome-wide association study (GWAS) was conducted using two separate case-control designs for the two syndromes following the approaches of [16,17]. The individuals involved in this study were weakly or not related. The genomic relationship matrix off-diagonal elements were, on average, −0.03, −0.03, and −0.02 for Ar, Ma, and He animals, respectively. The negative relatedness values signify that the individuals have genotypes less similar in expectation than the average. The affected and healthy animals were respectively assigned to cases or controls prior to test the allelic associations (–assoc flag in PLINK) performed by a X^2 test. Genomic-control adjusted *p*-values were plotted against the genomic position for the two analyzed diseases. To tackle the multiple testing issue that arises when thousands of hypotheses are simultaneously tested, we decided to consider as significant the associations with false discovery rate (FDR) [18] below 0.05 using the –adjust flag in PLINK [14]. On the same two datasets, F_{ST} analysis was conducted using the Weir and Cockerham [19] estimator implemented in PLINK, and those SNPs exceeding the 99.9th percentile threshold were retained. Then, the F_{ST} outliers were merged with SNPs resulting from the GWAS, obtaining two sets of SNPs associated to Ar and Ma, respectively. In order to corroborate the GWAS results, the canonical discriminant analysis (CDA) was run on the top significant SNPs associated to both diseases, jointly considering all the animals and using the CANDISC procedures of SAS software (Sas Institute, Cary, NC, USA). The correlation structure between the top ranked SNPs was used to derive new variables, namely canonical discriminant variables (CAN), able to maximize the separation between predefined groups [20]. If S is the n × p matrix of the p SNP genotypes (coded as 0, 1, or 2 for the "aa", "Aa", or "AA" genotypes, respectively) measured in n animals belonging to k groups (three in our cases: Ar, Ma, and He), the ith CAN may be calculated as

$$CAN_i = w_{i1}s_1 + w_{i2}s_2 + \cdots + w_{ip}s_p \tag{1}$$

where s_i are the centered SNP genotypes and w_{ip} are the raw canonical coefficients for the p analyzed SNP (i.e., the weights of each SNP in the discriminant function). The vectors of coefficients w were obtained with a procedure that involves the eigen-decomposition of a linear transformation of between- and within-group SNP (co)variance matrices [20].

2.4. Candidate Gene Detection

Annotated genes were retrieved in the region of 100-kilo base pairs (kb) (50-kb down- and upstream, respectively) surrounding each SNP highlighted by the combined use of GWAS and F_{ST}. The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and UCSC Genome Browser Gateway (http://genome.ucsc.edu/) databases were used.

3. Results

3.1. Genome-wide Association Study and F_{ST}

The results of the genome-wide case-control study are shown in Figure 1, which reports the −log10 of genomic-control adjusted *p*-values for the two diseases; in green are highlighted the SNPs that exceeded the chosen threshold. The factor λ was 1.11 and 1.17 for Ar and Ma, respectively, indicating a slight inflation of the statistical test. Quantile-quantile (QQ) plots of the ordered *p*-values for the Ar and Ma case-control GWAS (Supplemental Figure S3) showed that few SNPs strongly deviate from the diagonal identity lines for both diseases.
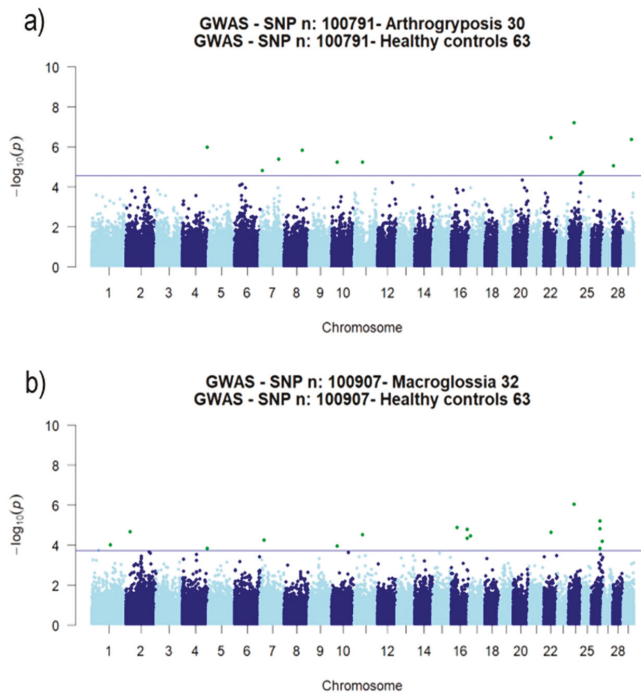
Figure 1. Manhattan plots. (**a**) Healthy vs. arthrogryposis and (**b**) healthy vs. macroglossia. GWAS: genome-wide association study and SNP: single nucleotide polymorphism.

The F_{ST} analysis enabled to highlight 102 and 101 outlier SNPs for Ar and Ma, respectively, (Supplemental Tables S1 and S2) that overcame the 99.9th percentile threshold (Ar vs. He: 0.1401 and Ma vs. He: 0.1425) (Supplemental Figure S4). The combined GWAS and F_{ST} approaches were able to identify 23 SNPs that were considered in a further analysis. In particular, seven SNPs were exclusively associated with arthrogryposis (on *Bos taurus* chromosomes, BTAs, 7, 8, 24, 25, 28, and 29) and 11 exclusively with macroglossia (on BTAs 1, 2, 7, 16, 17, and 26), whereas five markers (on BTAs 4, 11, 22, and 24) were in common to both pathologies (Table 1). Consistently, those SNPs presented MAF

values markedly deviating in the two groups of animals, as also highlighted by the F_{ST} values (from 0.217 to 0.336 and from 0.200 to 0.319 for Ar and Ma, respectively) compared with healthy samples (Supplemental Table S3).

Table 1. Relevant markers found by the genome-wide association study (GWAS) and F_{ST} analysis and raw canonical coefficients (CC) associated to significant single nucleotide polymorphisms (SNPs). Within-class average scores for the two discriminant functions (Can1 and Can2). CC: canonical coefficients.

SNP	GWAS		Raw CC	
	BTA [1]	Disease [2]	Can1	Can2
ARS-BFGL-NGS-13673	1	Ma	0.63	0.52
ARS-USDA-AGIL-chr2-17084934-000418	2	Ma	0.27	0.59
ARS-USDA-AGIL-chr4-114395607-000108	4	Ar, Ma	−0.19	0.05
ARS-USDA-AGIL-chr7-12174899-000752	7	Ar	−0.18	−0.14
Hapmap44668-BTA-119022	7	Ar	0.62	−0.26
ARS-USDA-AGIL-chr7-18201332-000761	7	Ma	0.66	0.88
ARS-USDA-AGIL-chr8-84099468-000783	8	Ar	−0.15	−0.72
ARS-USDA-AGIL-chr10-25594159-000176	10	Ar, Ma	−0.37	0.15
ARS-USDA-AGIL-chr11-36809347-000009	11	Ar, Ma	−0.16	0.22
ARS-BFGL-NGS-15423	16	Ma	0.02	0.02
BovineHD1600007856	16	Ma	0.37	0.25
BovineHD4100012725	16	Ma	0.72	−0.11
ARS-USDA-AGIL-chr17-6999864-000318	17	Ma	−0.09	−0.17
ARS-USDA-AGIL-chr22-32285822-000477	22	Ar, Ma	0.19	−0.08
BovineHD2400015279	24	Ar	−0.57	0.56
ARS-USDA-AGIL-chr24-25995108-000530	24	Ar, Ma	−0.17	−0.47
ARS-USDA-AGIL-chr25-1930875-000536	25	Ar	−0.12	0.45
BovineHD4100017966	26	Ma	−0.02	−0.78
BovineHD2600011259	26	Ma	0.31	0.42
BovineHD2600011282	26	Ma	0.42	0.5
BovineHD2600014129	26	Ma	0.59	0.85
BovineHD2800000629	28	Ar	0.56	−1.17
ARS-USDA-AGIL-chr29-39842168-000583	29	Ar	−0.40	−0.65
Within-class average				
Arthrogryposis		Ar	1.23	−1.55
Macroglossia		Ma	2.03	1.13
Healthy control		He	−1.62	0.16

[1] *Bos taurus* chromosome; [2] Ar: arthrogryposis and Ma: macroglossia.

3.2. Canonical Discriminant Analysis

The analysis of the squared Mahalanobis distances computed using the top significant SNPs indicated that affected (Ar or Ma) and healthy controls statistically differ ($p < 0.001$) as well as Ar and Ma did within affected animals (Supplemental Table S4).

The results of the CDA carried out using the significant markers identified by the GWAS and F_{ST} are presented in Figure 2 and Table 1. The two canonical functions explained 75% and 25% of the variance, respectively. When the individual samples were plotted in the new coordinate system defined by the first two discriminant functions, a clear, albeit imperfect, separation between the affected (regardless of the pathology) and the healthy subjects was highlighted (Figure 2). The animals with positive CAN1 scores are mostly the affected animals. In addition, CAN2 allowed to separate the subjects affected by arthrogryposis (with negative scores on CAN2) from those affected by macroglossia (with positive scores), as confirmed by the within-class (Ar, Ma, or He) average scores for CAN1 and CAN2 (Table 1).
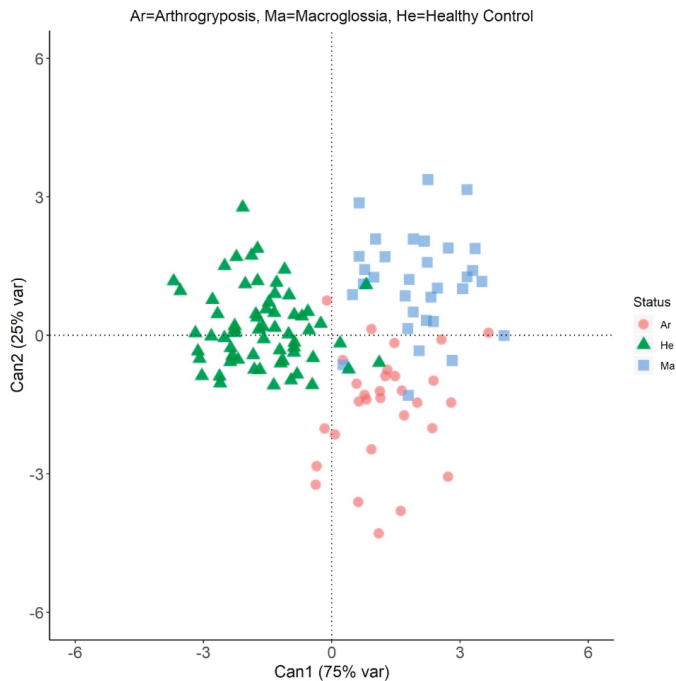
Figure 2. Plot of the individual scores on the two canonical variables (Can1 and Can2) depicted in different colors and symbols according to their health status.

The raw canonical coefficients for the identified SNPs had mainly large positive weights for Ma (ranging from −0.09 to 0.72) and negative weights for Ar, even with some exceptions (from −0.57 to 0.62). A rather elusive pattern was ascertained looking at the most deviating canonical weight; conversely, the selected SNPs jointly were able to discriminate between groups of animals. This seems also confirmed looking at the canonical correlation between the SNP genotype and CAN variables (Supplemental Figure S5).

3.3. Candidate Gene Detection

The lists of genes that mapped in the interval of 100 kb surrounding the significant SNPs and that may be putatively associated with the diseases are reported in Tables 2 and 3. As for arthrogryposis, a total of 20 genes were identified in the considered interval, and eight of them included a significant marker, while, for macroglossia, 26 genes were mapped in the highlighted regions, with 10 of them including a marker. Of the identified genes, 12 were in common to both investigated diseases (Tables 2 and 3).

Table 2. Genes near the SNPs associated to arthrogryposis. The genes including a marker are in bold.

BTA	SNP Name	Position	Symbol	Gene Name	Location
4	ARS-USDA-AGIL-chr4-114395607-000108	113,596,650	KCNH2	Potassium voltage-gated channel, sub-family H, member 2	113,526,185..113,562,025
			NOS3	Nitric oxide synthase 3	113,577,075..113,595,527
			ATG9B	**Autophagy-related 9B**	113,594,821..113,605,878
			ABCB8	ATP binding cassette subfamily B member 8	113,605,775..113,623,178
			ASIC3	acid sensing ion channel subunit 3	113,624,088..113,629,085
7	ARS-USDA-AGIL-chr7-121174899-000752	11,085,449	**ZNF333**	**zinc finger protein 333**	11,071,151..11,102,144
			ADGRE3	adhesion G protein-coupled receptor E3	11,116,555..11,181,632
	Hapmap44668-BTA-119022	85,227,970	//	no genes in the considered interval	
8	ARS-USDA-AGIL-chr8-84099468-000783	82,677,581	ERCC6L2	ERCC excision repair 6 like 2	82,557,942..82,712,671
10	ARS-USDA-AGIL-chr10-25594159-000176	25,539,231	**OR4E2**	**olfactory receptor, family 4, subfamily E, member 2**	25,539,229..25,540,170
			OR10G2	olfactory receptor, family 10, subfamily G, member 2	25,587,007..25,587,963
11	ARS-USDA-AGIL-chr11-36809347-000009	36,957,396	ACYP2	acylphosphatase 2	36,831,155..37,010,456
			TSPYL6	**TSPY like 6**	36,954,224..36,957,858
22	ARS-USDA-AGIL-chr22-32285822-000477	32,169,050	**FRMD4B**	**FERM domain containing 4B**	32,022,767..32,382,939
24	ARS-USDA-AGIL-chr24-25995108-000530	25,684,356	DSG2	desmoglein 2	25,602,973..25,653,973
			DSG3	**desmoglein 3**	25,666,298..25,699,081
			DSG4	desmoglein 4	25,725,711..25,755,364
	BovineHD2400015279	53,288,962	//	no genes in the considered interval	
25	ARS-USDA-AGIL-chr25-1930875-000536	1,929,340	CCNF	cyclin F	1,947,654..1,965,422
			TEDC2	tubulin epsilon and delta complex 2	1,966,625..1,970,947
			NTN3	netrin 3	1,975,804..1,978,415
28	BovineHD2800000629	2,661,658	//	no genes in the considered interval	
29	ARS-USDA-AGIL-chr29-398421168-000583	39,215,494	PAG9	pregnancy-associated glycoprotein 9	39,237,412..39,246,707
1	ARS-BFGL-NGS-13673	86,784,250	//	no genes in the considered interval	
2	ARS-USDA-AGIL-chr2-17084934-000418	17,077,000	**ZNF385B**	**zinc finger protein 385B**	16,957,646..17,442,243
			TRNAC-ACA	transfer RNA cysteine	17,082,352..17,082,423

Table 3. Genes near the SNPs associated to macroglossia. The genes including a marker are in bold.

BTA	SNP Name	Position	Gene Symbol	Gene Name	Location
4	ARS-USDA-AGIL-chr4-114395607-000108	113,596,650	KCNH2	potassium voltage-gated channel, sub-family H, member 2	113,526,185..113,562,025
			NOS3	nitric oxide synthase 3	113,577,075..113,595,527
			ATG9B	**autophagy related 9B**	113,594,821..113,605,878
			ABCB8	ATP binding cassette subfamily B member 8	113,605,775..113,623,178
			ASIC3	acid-sensing ion channel subunit 3	113,624,088..113,629,085
7	ARS-USDA-AGIL-chr7-18201332-000761	16,970,401	CERS4	ceramide synthase 4	16,897,965..16,933,292
			CD320	CD320 molecule	16,952,355..16,957,071
			NDUFA7	NADH:ubiquinone oxidoreductase subunit A7	16,960,703..16,967,936
			RPS28	ribosomal protein S28	16,968,061..16,969,193
			KANK3	**KN motif and ankyrin repeat domains 3**	16,969,360..16,980,814
			ANGPTL4	angiopoietin like 4	17,005,585..17,012,655
10	ARS-USDA-AGIL-chr10-25594159-000176	25,539,231	**OR4E2**	**olfactory receptor, family 4, subfamily E, member 2**	25,539,229..25,540,170
			OR10G2	olfactory receptor, family 10, subfamily G, member 2	25,587,007..25,587,963
11	ARS-USDA-AGIL-chr11-36809347-000009	36,957,396	**ACYP2**	**acylphosphatase 2**	36,831,155..37,010,456
			TSPYL6	**TSPY like 6**	36,954,224..36,957,858
16	BovineHD1600007856	27,494,192	NVL	nuclear valosin-containing protein-like	27,322,130..27,502,627
			CNIH4	cornichon family AMPA receptor auxiliary protein 4	27,529,034..27,543,276
	ARS-BFGL-NGS-15423 BovineHD4100012725	72,258,249 72,266,300	**KCNH1**	**potassium voltage-gated channel subfamily H member 1**	72,205,829..72,642,416
17	ARS-USDA-AGIL-chr17-6999864-000318	7,013,884	**LRBA**	**lipopolysaccharide responsive beige-like anchor protein**	6,799,299..7,556,716
22	ARS-USDA-AGIL-chr22-32285822-000477	32,169,050	**FRMD4B**	**FERM domain containing 4B**	32,022,767..32,382,939
24	ARS-USDA-AGIL-chr24-25995108-000530	25,684,356	DSG2	desmoglein 2	25,602,973..25,653,973
			DSG3	**desmoglein 3**	25,666,298..25,699,081
			DSG4	desmoglein 4	25,725,711..25,755,364
26	BovineHD4100017966 BovineHD2600011259 BovineHD2600011282	40,441,709 40,460,199 40,517,972	**PLPP4**	**phospholipid phosphatase 4**	40,509,729..40,656,785
	BovineHD2600014129	48,680,201	//	no genes in the considered interval	

4. Discussion

The present study provides new data on the genetics of arthrogryposis and the first insight into the analysis of macroglossia in the Piemontese breed. An important systematic bias in GWAS often reported in the literature is caused by population stratification due to ethnic/breed admixture and/or close relationships among individuals of case-control studies [21,22]. In our case, a limited inflation of the statistical tests was observed; thus, a genomic-control approach was adopted, since the individuals included in the design belonged to the same breed and were weakly or not related [21].

Interestingly, the combined use of case-control GWAS, F_{ST}, and CDA highlighted several markers potentially associated with the investigated syndromes. The use of multiple approaches is generally advised in genome-wide analysis [17,23,24]. The use of CDA was recently proposed as an effective tool for improving the discovery rate either alone or in a combination with GWAS, especially when the sample size is reduced [25].

As for arthrogryposis, the results depict a situation different from what was observed in the other investigated breeds [10–13], where variations in single genes were identified as responsible for the disease. In fact, our data did not evidence a single strong association with the pathology, while they highlighted a number of significant markers located on different chromosomes, suggesting a polygenic mechanism underlying the disease. The joint role of these markers is supported by their ability to separate the three groups of animals according to their health status.

None of the markers for arthrogryposis identified in the Piemontese breed are located within or near the genes reported as causing the disease in the other breeds. In this respect, it is important to underline that also the causal variations found in those breeds were of different types and in different genes: a large deletion encompassing three genes (BTA 16) in Angus [10], a missense mutation in the *MYBPC1* gene (BTA 5) in Swiss Holstein [11], a splicing variant in the *PIGH* gene (BTA 10) in Belgian Blue [12], and a small deletion in the *CHRNB1* gene (BTA 19) in Red Danish [13]. This implies that the genetic determinism of arthrogryposis is not the same in the affected breeds. On the other hand, it must be considered that a large variability in the phenotypic expression of what is called "arthrogryposis" was observed in the breeds studied so far, from lethal consequences, as in Belgian Blue or Angus breeds, to less severe problems, as in the Piemontese. Additionally, at least six types of arthrogryposis with different clinical signs and grades of severity were reviewed by Huston et al. [3] in cattle. Such heterogeneity makes it difficult to clearly define the trait that could explain the differences observed at the genetic level. In all cases, however, the findings of the different studies are compatible with the autosomal recessive mode of inheritance suggested since the earliest studies. The incidences of the two pathologies in the Piemontese breed in the last decades also showed a trend compatible with the case of selection against the recessive phenotype, and this led us to hypothesize the existence of a monogenic determinism similar to what was observed in the other cattle breeds. However, the present data do not support this hypothesis, suggesting that a more complex mechanism is responsible for the disease in the Piemontese breed.

For macroglossia, no previous genetic data exist. The results of the current study highlight a situation comparable to that obtained for arthrogryposis, so that a multifactorial mechanism can be hypothesized also for macroglossia.

The identified SNPs were located within or close to 33 genes, of which 9 and 13 were exclusive for arthrogryposis and macroglossia, respectively, and 11 common to the two pathologies. This is worthy of note, considering that, in the Piemontese breed, both pathologies are sometimes observed in the same animal. Thus, the findings of this study might suggest that the putative candidate genes common to both diseases could be involved in basic physiological processes common to both defects.

In the case of arthrogryposis, seven of the relevant SNPs mapped in coding genes, whereas, for macroglossia, 11 SNPs were located within coding genes. In some cases, it is unclear from the gene annotations their possible involvement in the pathologies. Instead, for other genes, a possible role can be hypothesized, as their products are part of processes related to muscular or nervous

tissue developments whose defects are included among the common causes of the pathologies here considered [26].

Among these genes, Netrin3 (*NTN3*) encodes a member (NTN3) of a family of extracellular proteins that act as chemotropic guidance cues for migrating cells and axons during neural development [27]. In mice, it was demonstrated that NTN3 is expressed in muscle cells, and therefore, it may play a role in guiding peripheral axons to their corrected muscle targets [28].

Additionally, *KCNH1* (potassium voltage-gated channel subfamily H member 1) and *KCNH2* (potassium voltage-gated channel subfamily H member 2) genes code for proteins that belong to a complex protein superfamily widely distributed during embryonic development and involved in a wide variety of cell functions. In mice, the two genes are co-expressed in the skeletal muscle during embryogenesis, including the cranial, thoracic, and limb regions [29]. In man, KCNH1 was shown to be involved in myoblast fusion, a complex process that includes withdrawal from the cell cycle, cell-cell interactions, adhesion, alignment, and a final membrane fusion to form the multinucleated skeletal muscle fiber [30].

A possible role can be also suggested for the *KANK3* (KN motif and ankyrin repeat domains 3) gene strongly expressed in different body compartments, including the skeletal muscle, and involved in the control of cytoskeleton formation by negatively regulating actin polymerization [31].

5. Conclusions

The overall findings indicate that the genetic determinism of arthrogryposis and macroglossia in the Piemontese breed is more complex than previously believed. In fact, the results do not support the existence of a single-gene model, while suggesting a multifactorial genetic mechanism underlying the investigated pathologies. Several markers significantly associated with both diseases were found, and genes possibly affecting the traits were identified. The real involvement of these genes needs to be further investigated and validated.

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Insight into the Possible Formation Mechanism of the Intersex Phenotype of Lanzhou Fat-Tailed Sheep Using Whole-Genome Resequencing

Jie Li [1,†], Han Xu [1,2,†], Xinfeng Liu [1], Hongwei Xu [3,4,*], Yong Cai [5,6] and Xianyong Lan [1,*]

[1]   Animal Genome and Gene Function Laboratory, College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China; lijie95@nwafu.edu.cn (J.L.); xuhan23@mail2.sysu.edu.cn (H.X.); liuxinfen227@nwafu.edu.cn (X.L.)
[2]   School of Medicine, Sun Yat-sen University, Guangzhou 510275, China
[3]   College of Life Science and Engineering, Northwest Minzu University, Lanzhou 730030, China
[4]   Gansu Tech Innovation Center of Animal Cell, Biomedical Research Center, Northwest Minzu University, Lanzhou 730030, China
[5]   Science Experimental Center, Northwest Minzu University, Lanzhou 730030, China; caiyong@xbmu.edu.cn
[6]   Key Laboratory of Biotechnology and Bioengineering of State Ethnic Affairs Commission, Biomedical Research Center, Northwest Minzu University, Lanzhou 730030, China
*   Correspondence: xuhongwei@xbmu.edu.cn (H.X.); lanxianyong79@nwsuaf.edu.cn (X.L.)
†   These authors equally contributed to this work.

Simple Summary: Individuals with hermaphroditism are a serious hazard to animal husbandry and production due to their abnormal fertility. The molecular mechanism of sheep intersex formation was unclear. This study was the first to locate the homologous sequence of the goat polled intersex syndrome (PIS) region in sheep and found that the intersex traits of Lanzhou fat-tailed sheep were not caused by the lack of this region. By detecting the selective sweep regions, vital genes associated with androgen biosynthesis and the follicle stimulating hormone response entry were found, including *steroid 5 alpha-reductase 2* (*SRD5A2*), and *pro-apoptotic WT1 regulator (PAWR)*. Additionally, the copy number variations of the four regions on chr9, chr1, chr4, and chr16 may affect the expression of the gonadal development genes, *zinc finger protein*, *FOG family member 2* (*ZFPM2*), *LIM homeobox 8* (*LHX8*), *inner mitochondrial membrane peptidase subunit 2* (*IMMP2L*) and *slit guidance ligand 3* (*SLIT3*), respectively, and further affect the formation of intersex traits.

Abstract: Intersex, also known as hermaphroditism, is a serious hazard to animal husbandry and production. The mechanism of ovine intersex formation is not clear. Therefore, genome-wide resequencing on the only two intersex and two normal Lanzhou fat-tailed (LFT) sheep, an excellent but endangered Chinese indigenous sheep breed, was performed. Herein, the deletion of homologous sequences of the goat polled intersex syndrome (PIS) region (8787 bp, 247747059–247755846) on chromosome 1 of the LFT sheep was not the cause of the ovine intersex trait. By detecting the selective sweep regions, we found that the genes related to androgen biosynthesis and follicle stimulating hormone response items, such as *steroid 5 alpha-reductase 2* (*SRD5A2*), *steroid 5 alpha-reductase 3* (*SRD5A3*), and *pro-apoptotic WT1 regulator (PAWR)*, may be involved in the formation of intersex traits. Furthermore, the copy number variations of the four regions, chr9: 71660801–71662800, chr1: 50776001–50778000, chr4: 58119201–58121600, and chr16: 778801–780800, may affect the expression of the *zinc finger protein*, *FOG family member 2* (*ZFPM2*), *LIM homeobox 8* (*LHX8*), *inner mitochondrial membrane peptidase subunit 2* (*IMMP2L*) and *slit guidance ligand 3* (*SLIT3*) genes, respectively, which contribute to the appearance of intersex traits. These results may supply a theoretical basis for the timely detection and elimination of intersex individuals in sheep, which could accelerate the healthy development of animal husbandry.

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1. Introduction

Intersexuality, also known as hermaphroditism, refers to the phenomenon that a dioecious animal is characterized by female-to-male sex reversal or abnormal gonad development. Intersex individuals are unable to reproduce, which poses certain obstacles to the protection and breeding of endangered species, and causes production loss to animal husbandry. In vertebrates, the mechanisms of sex determination are mainly divided into two types, genetic sex determination and environmental sex determination [1]. Intersex mostly occurs in the goat population, with high occurrence frequencies (about 3%–10%) [2], while related reports on horses, donkeys, pigs, and sheep are few. In goats, it is also named polled intersex syndrome (PIS) for the phenomenon of intersex individuals often found in hornless goat populations [2]. In the previous study, a 11.7 kb deletion fragment containing a repeat sequence (AF404302) was cloned by PCR, and the complete absence of this fragment resulted in goat polled syndrome [3,4]. A recent study about long-read whole-genome sequencing of a PIS-affected goat and a horned control goat revealed the presence of a more complex structural variant consisting of a 10,159 bp deletion and an inversely inserted 480 kb duplicated segment containing two genes, *potassium inwardly rectifying channel subfamily J member 15* (*KCNJ15*) and *ETS transcription factor ERG* (*ERG*) [5]. The deletion of the PIS region was identified affecting the development of germ cell support cells, and it can also affect the expression of genes, including *Forkhead box L2* (*FOXL2*), *PIS-regulated transcript 1* (*PISRT1*), and *promoter FOXL2 inverse complementary* (*PFOXic*) [6], indicating that the lack of the PIS region is closely related to goat intersex traits.

Compared to goats, reports of intersex sheep are quite rare. Domestic sheep and domestic goats, diverging about 4 to 5 million years ago and evolving into two different branches, are relatively close in genetic distance, and they have many similar genetic targets during domestication [7]. Therefore, it was suspected that the cause of sexual traits in sheep may be similar to that of goat sex. The location of homologous sequences of goat PIS regions should be detected in sheep.

Genetic variations or regulatory regions may affect an individual's phenotypic traits by affecting the transcription or translation of key genes [8]. In bovines, a 110 kb deletion in the *MER1 repeat containing imprinted transcript 1* (*MIMT1*) gene was a prominent cause of bovine abortion and stillbirth [9]. Bovine osteosclerosis may be associated with a deletion of approximately 2.8 kb in exon 2 and part of exon 3 of the *solute carrier family 4 member 2* (*SLC4A2*) gene encoding an anion exchanger [10]. An increased copy number of the *prolactin receptor* (*PRLR*) and *sperm flagellar 2* (*SPEF2*) genes in the K locus on the Z chromosome in chickens were closely related to the slow feathering trait of the chicken [11,12]. Additionally, the copy number variation of the *sperm flagellar 2* (*ASIP*) gene in sheep was closely related to the coat color [13]. It was speculated that some mutations may lead to the abnormal expression of certain genes that affect sex formation, leading to the generation of intersex traits.

As an excellent sheep variety of meat and wool, Lanzhou fat-tailed (LFT) sheep are famous for their large and fat tail and a number of excellent characteristics, such as the crude feed tolerance, higher disease resistance, and higher resilience than other domestic sheep. In recent years, only two surviving intersexual sheep were found in the LFT sheep population. In this study, the genome-wide resequencing of two intersex LFT sheep and two normal LFT sheep was performed, and combined with the resequencing data of four normal Tan sheep to find potential genes or regions related with the formation of ovine intersex traits, and thus provide an important basis for the timely detection and elimination of intersex individuals in sheep conservation and expansion.

2. Materials and Methods

2.1. Ethics Statement

All implemented experiments were approved by the Institutional Animal Care and Use Committee and were in strict accordance with good animal practices as defined by the Northwest A&F University (protocol number NWAFAC1008).

2.2. Animal and Sequencing

In this study, the ear tissues of four sheep, including two surviving intersex (LZ1 and LZ2) and six normal (LZ3 to LZ8) Lanzhou fat-tail sheep from Lanzhou City were collected and stored in 70% alcohol at −80 °C. Genomic DNA was extracted from sheep ear tissues using the phenol-chloroform method according to a previously reported protocol [14]. The DNA samples were quantified using a Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA). Four DNA libraries, two intersex (LZ1 and LZ2) and two normal LFT sheep (LZ3 and LZ4), with insert sizes of approximately 350 bp, were constructed following the manufacturer's instructions, and 150 bp paired-end reads were generated using the Illumina HiSeq X10 platform.

2.3. Sequence Quality Checking and Mapping

Considering the relatively close genetic relationship between LFT sheep and Tan sheep, the previous four resequencing data of Tan sheep were also used as a normal control group for further analysis [15,16]. Before alignment, the FastQC software was used (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to detect the joint information, the length information, and the quality information for each base on each read of all the raw data. Based on the results of the above quality control, the adapter and low quality raw paired reads were filtered using Trimmomatic (v0.36) [17]. The high-quality reads were mapped to the sheep version 4.0 reference genome (GCF_000298735.2_Oar_v4.0_genomic.fna) using the 'mem' algorithm of the Burrows–Wheeler Alignment Tool (BWA) software [18]. SAMtools (http://samtools.sourceforge.net/) was used to remove replicate sequences [19].

2.4. Calling and Validation of SNPs and CNVs

The calling of single nucleotide polymorphisms (SNPs) was performed with Genome Analysis Toolkit (GATK, version 2.4–9) UnifiedGenotyper [20], and was annotated by ANNOVAR [21]. The called SNPs with reads >4 and quality ≥20 were used for further analysis. CNVcaller software (https://github.com/JiangYuLab/CNVcaller) was used for the detection of whole-genome copy number variation (CNV) [22]. The specific steps were in accordance with a reported study [23], including segmenting the reference genome into a window of a specified size (800 bp) with a step size of 400 bp. Herein, Vst was used to measure the difference in the size of each copy number variation between different groups [24,25]. The mean log2 ratio across all probes falling within a specific CNV region was calculated. The variance of the means was for the entire set (Vt). The average variance within populations was then calculated (Vs) by taking the mean between populations (i.e., the V intersex sheep and Vnormal sheep). Vst values were finally calculated using the standard formula Vst = (Vt − Vs)/Vt.

2.5. Regional Localization and Depth Statistics for Ovine Homologous Sequences of PIS

The sequence of the PIS region was extracted from the goat reference genome (GCF_001704415.1_ASM170441v1_genomic.fna) by using SAMtools and then aligned to the sheep reference genome using BLAT v. 36 × 1 software (BLAST-Like Alignment Tool) to obtain the ovine homologous regions [26].

After that, we used the SAMtools-depth to count the reads depth for each locus in the candidate region. Furthermore, we corrected it based on the total depth of sequencing to obtain the reads depth in the homologous regions of the eight samples, including two intersex LFT sheep, two LFT sheep,

and four reported Tan sheep [15,16]. If the reads depth was essentially 0, the candidate region was completely missing on the ovine genome.

2.6. PCR Amplification of PIS Candidate Region Sequences in Sheep

In order to further verify whether the PIS candidate region of the intersex individual was really missing, primers, namely PIS-1, PIS-2, PIS-3, and PIS-4, for the homologous sequence in the ovine PIS region were designed to reference the second generation genome of the sheep chr1: 247747059–247755846 sequence (Supplemental Table S1). We performed assays of amplification on two intersex and six normal LFT sheep according to previous reaction volume and amplification procedure [27]. The products were separated on 2.5% agarose gels.

2.7. Sweep Analysis of SNPs in Selected Regions

In order to identify the selection signatures in the genomes of sheep, two sequenced pools based on genetic differentiation (Fst) of each 150 KB genome window were separately performed. The specific formula of Fst was consistent with the previous studies [28,29].

After Z-transformation of Fst, the candidate selection windows were used by selecting the top 1% in Fst score intersections [30]. Finally, the geneview module of python and R were utilized for data visualization.

2.8. Gene Annotation and Functional Enrichment Analysis of Selected Signal Regions

Gene functional enrichment analysis was performed primarily on the KOBAS 3.0 website (http://kobas.cbi.pku.edu.cn/index.php) [31]. Herein, considering that the sheep database in KOBAS was not complete, a Perl script was used to compare the longest protein sequences of each gene in sheep and human by invoking Blastp, and the gene extracted in the previous step (2.4 and 2.7) was converted into a gene homologous to "human", and then human was selected as a species for annotation.

3. Results

3.1. Sequencing, Filtering, and Mapping

Whole-genome sequencing of two normal Tan sheep, as well as two intersex sheep and four normal LFT sheep, was performed on an Illumina HiSeq X10 platform using genomic DNA and 198.5 Gb of high quality paired-end reads were generated. After getting the raw data, we utilized FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) for quality testing. According to the test results, the clean reads were obtained using trimmomatic (http://www.usadellab.org/cms/index.php/page=trimmomatic) to filter the low-quality reads (Supplemental Table S2). The clean reads of eight sheep were mapped to the sheep reference genome (GCF_000298735.2_Oar_v4.0_genomic.fna) using BWA-MEM [17,31], with an average mapping rate above 94% (Supplemental Table S3).

3.2. Single Nucleotide Polymorphisms (SNPs) Calling and Annotation

A total of 29,732,629 SNPs were obtained among eight sheep, among which, the most distributed SNPs were on chromosome 1 (n = 3,186,739), and the least distributed, lowest number of SNPs were on chromosome 24 (n = 459,707) (Supplemental Table S4). According to the results of the annotation, the proportions of transition (ts) mutations (A/G 10333679 and T/C 10308562) and transversion (tv) mutations (A/T 2050141, A/C 2457813, G/T 2450228, and G/C 2132206) were 69.4% and 30.6%, respectively, which met the 3 to 1 ratio (Figure 1).
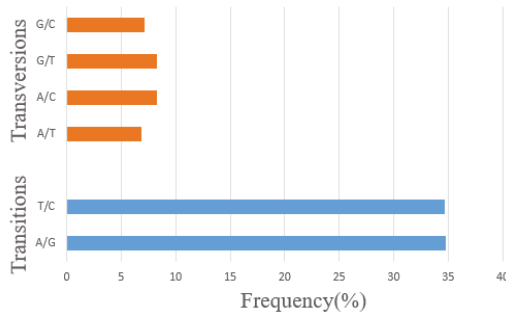
Figure 1. The statistics of mutation type of single nucleotide polymorphisms (SNPs). Note: The proportion of translation (ts) mutations and transversion (tv) mutations meet the 3 to 1 ratio.

3.3. The Localization Results of Homologous Sequences of Goat PIS Region in Sheep

According to the specific information of the goat PIS region (AF404302.1: 27015–38775) in the NCBI, the obtained sequence of this 11.76 kb segment [2] was aligned to the sheep genome using BLAT, and then the score was calculated using the pslScore.pl script provided by the BLAT software to evaluate the results of the comparison.

The BLAT results show that there were 79 alignment regions, and the alignment region with the highest score was located in the region of the ovine chromosome1 (start to end position: 247747059–250146105, 2399.046 kb) (Supplemental Table S5). Then, the detailed analysis of the 79 region revealed that the first 58 of them were a closely connected 8787 bp region (chr1: 247747059–247755846), corresponding to a segment of the 11.7 kb region of the goat (AF404302.1:30003–38775) with 100% coverage (Figure 2a). Further localization revealed that this region was located approximately 340 kb upstream of the *FOXL2* gene (chr1: 248088730–248095868) in the sheep genome, which was consistent with the position of the goat *FOXL2* gene. Therefore, the 8787 bp sequence (chr1: 247747059–247755846) was served as a candidate region for ovine intersex traits.

(**a**)

Figure 2. *Cont.*

(b)

(c)

Figure 2. Study on the homologous sequences of the polled intersex syndrome (PIS) regions in sheep and goats. (**a**) The comparison results between the sheep chromosome 1: 247747059–250146105 and goat PIS area (the color line represents the matching relationship: goat 8773 bp matched sheep 8787 bp, and goat 2987 kb matched sheep 1813.4 kb). (**b**) The statistics of the read depth in the sheep 8787 bp candidate area. (**c**) Electrophoresis results of the four pairs of primer amplification products, indicating that the four regions of intersex sheep were not missing.

3.4. Statistics of Reads Depth and PCR Amplification of Sheep 8787 bp Candidate Sequences

In the two intersex sheep and normal individuals, the read depth was between 5 and 20, and there was no significant difference between them (Figure 2b).

According to the results of agarose gel electrophoresis, in two intersex LFT sheep (LZ1 and LZ2) and six normal LFT sheep (LZ3, LZ4, LZ5, LZ6, LZ7, and LZ8), four pairs of primers (PIS-1, PIS-2, PIS-3, and PIS-4) were able to amplify the target band in the candidate region, and the product lengths were 118, 376, 380, and 454 bp (Figure 2c), respectively, indicating that the candidate region of intersex sheep was not missing. It is further explained that the intersex traits of LFT sheep was not caused by the lack of the region.

3.5. Genome-Wide Selection Sweeping Analysis in Intersex and Normal Populations

ZFst score were calculated for the only two intersex LFT sheep and six normal sheep populations (including two LFT sheep and four Tan sheep). The top 1% percent of the windows with the highest *ZFst* score were defined as candidate selective sweep regions. The results show that most chromosomes contained windows with a higher differentiation coefficient, and chromosomes 2, 6, 7, 10, and 11 were strongly selected (Figure 3a). The region with the largest *ZFst* value was located on the X chromosome (chrX: 99675001–99825000, *ZFst* = 10.69), and the second one was located on chromosome 3 (chr3: 105000001–105150000, *ZFst* = 10.38) (Figure 3a; Supplemental Table S6).
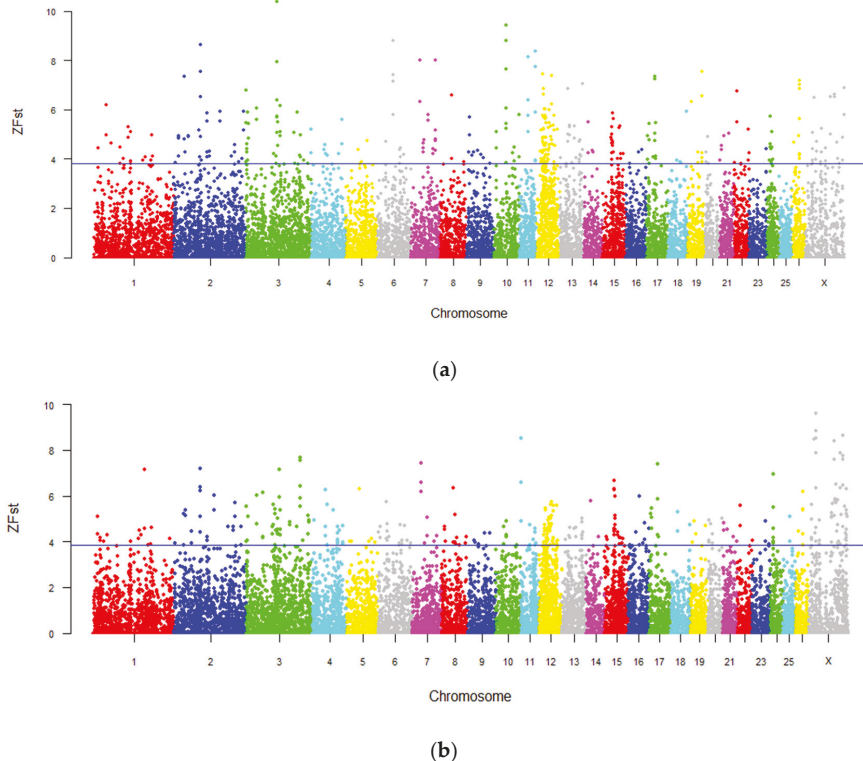

(**a**)

(**b**)

Figure 3. The detection of genome-wide selection signals in intersex and normal populations. (**a**) The genome-wide distribution of *ZFst* between intersex sheep and normal sheep (including normal Lanzhou large-tailed sheep and Tan sheep). (**b**) The genome-wide distribution of *ZFst* between intersex and normal individuals of Lanzhou large-tailed sheep. Note: The blue line represents the top 1% of the ZFst value.

A total of 466 genes were obtained by gene annotation (Supplemental Table S7), and then those genes were functionally enriched by KOBAS3.0. A total of 1757 significant entries were found ($p < 0.05$), and 1038 significant entries remained after the false discovery rate (FDR)-correction (corrected p-Value < 0.05), mainly including items, such as muscle development, fat development, and immunity. Furthermore, the pathways were screened and two more significant entries related to female gonadal development (GO: 0008585) and the development of primary female sexual characteristics (GO: 0046545) were found ($p < 0.05$) (Supplemental Table S7). Nevertheless, neither entry was significant after correction.

3.6. Genome-Wide Selection Sweeping Analysis in Intersex LFT Sheep and Normal LFT Sheep

To exclude the effects of SNP locus frequencies between different breeds, the study then compared two intersex individuals and two normal LFT sheep. Consistent with the previous section, the region with the largest *ZFst* value was located on the X chromosome (Figure 3b). By contrast, the second region was also in the X chromosome, rather than chromosome 3 (Figure 3b). Moreover, a total of 451 genes were obtained by annotation (Supplemental Table S8). After functional enrichment analysis, 1680 significant entries were found ($p < 0.05$) and 969 significant terms remained after false discovery rate (FDR)-correction (corrected p-value < 0.05) (Supplemental Table S9). Screening of the pathway revealed five significant entries related to the synthesis and response of the sex hormone ($p < 0.05$). After correction (corrected p-value < 0.05), the genes, such as *SRD5A2*, *SRD5A3*, and *PAWR*, were significantly rich in the androgen biosynthesis process and their responses to follicle stimulating hormones (Supplemental Table S9). Additionally, the genes involved in androgen receptor signaling pathways, androgen metabolism, and the regulation of intracellular estrogen receptor signaling pathways, and gonadotropin response processes, such as *UFM1 specific ligase 1* (*UFL1*), *GTP-binding nuclear protein Ran-like* (*LOC105609617*), *mediator complex subunit 14* (*MED14*), *DEAD-box helicase 5* (*DDX5*), etc. (Supplemental Table S9).

3.7. Detection of Genome-Wide Copy Number Variation (CNV) in Intersex and Normal Populations

As copy number variation regions (CNVRs) can be separated by gaps or poorly assembled regions, the adjacent initial calls were merged if their reads depth were highly correlated. The default parameters were as follows: the distance between the two initial calls was less than 20% of their combined length, and the Pearson's correlation index of the two CNVRs was significant at the $p = 0.01$ level [21]. After calculation and combination through the CNVcaller software, 87,729 CNVRs were obtained, of which 1817 CNVRs were located on the scaffold sequence (not assembled into chromosomes) and 11,170 CNVRs were located on the X chromosome (Supplemental Table S10).

As the number of X chromosomes is different in females and males, the copy number variation on the scaffold and X chromosomes was not considered in the results. Therefore, 74,302 CNVRs located on autosomes were used for the subsequent analysis. As shown in Supplemental Table S10, the smallest proportion of CNVRs was chromosome 26 (5.8%), and the largest was chromosome 11 (9.92%). In addition, the number of CNVRs distributed on chromosome 1 ($n = 8267$) was the highest with a total length of 19,584,800 bp, while the smallest was on chromosome 26 ($n = 1116$) with 2,556,800 bp. The longest CNVR was 380,800 bp and located on chromosome 13 (Supplemental Table S10).

The copy number of the normal and intersex populations was screened with variation length >2000 bp and Vst value >0.25, and a total of 238 candidate regions were obtained. Gene annotation of the above 238 candidate CNVRs revealed that 140 were located in the intergenic region, and the remaining 98 overlapped with the genes (Supplemental Table S11). Functional enrichment analysis was performed on the annotated genes, revealing a total of 1838 significant entries ($p < 0.05$) and 1039 significant entries after FDR-correction (corrected p-value < 0.05) (Supplemental Table S12).

Through further GO enrichment analysis of CNVRs, four GO items related to female gonad development were found. Based on functional enrichment analysis, the enriched genes were mainly *ZFPM2, LHX8* (located at 43,507 nt downstream of its corresponding copy number region), IMMP2L (located at 400835 nt upstream of its corresponding copy number region), and *SLIT3* (Supplemental Table S12). The above four genes corresponded to four CNVRs, respectively. Furthermore, in the intersex individuals, the copy number region associated with the *LHX8*, *IMMP2L*, and *ZFPM2* genes were gained, while the copy number region associated with the *SLIT3* gene was lost (Table 1).

Table 1. The information of the four candidates copy number variation region and the genotype of eight samples.

CNV Regions			Related Genes	Vst-Values	Sample Genotype							
Chromosome	Start Position	End Position			LZ1	LZ2	LZ3	LZ4	Tan1	Tan2	Tan3	Tan4
1	50776001	50778000	*LHX8*	0.5713	AB	AB	AA	AA	AA	AA	AA	AA
4	58119201	58121600	*IMMP2L*	0.7097	AB	AB	AA	AA	AA	AA	AA	AA
9	71660801	71662800	*ZFPM2*	0.8611	AB	AB	AA	AA	AA	AA	AA	AA
16	778801	780800	*SLIT3*	0.4357	Ad	Ad	AA	AA	AA	AA	AA	AA

Note: AA means two copies; AB means three copies; Ad means just one copy. *LHX8, LIM homeobox 8* gene; *IMMP2L, inner mitochondrial membrane peptidase subunit 2* gene; *ZFPM2, zinc finger protein FOG family member 2* gene; *SLIT3, slit guidance ligand 3* gene. The *V*st-values measure the difference in the size of each copy number variation between different groups.

4. Discussion

It is well-known that the mutation of the goat PIS region contributes to the absence of horns and sex-reversal [4]. Herein, through the reads depth and the PCR amplification experiments, the PIS homologous segments of two intersex sheep and normal sheep were not in a missing state, which indicated that the intersex trait of LFT sheep may not be caused by the absence of this region. The intersexuality of goats was always accompanied by hornlessness, manifested as non-interval syndrome, and the probability of the intersex appearance in the hornless goat population was 3% to 10% [32].

Genetic variations or regulatory regions may affect an individual's phenotypic traits by affecting the transcription or transition of key genes. We speculated that some mutations may lead to the abnormal expression of certain genes that affect sex formation, leading to the appearance of intersex. Furthermore, a previous study reported that the replication of the *SRY-box transcription factor 3* (*SOX3*) gene may also cause human developmental disorders [33]. An increased copy number of the *SRY-box transcription factor 9* (*SOX9*) gene may trigger the probability of intersex individuals [34], so it is suspected that the intersexuality of the sheep may be due to other reasons, such as genetic variations.

Functional enrichment analysis revealed significant entries for the androgen biosynthesis processes and follicle stimulating hormone responses, including *SRD5A2*, *SRD5A3*, and *PAWR* genes. Among them, mutations of the *SRD5A2*, which were closely related to testicular decline, could affect the formation of the urethra and external genitalia, leading to hypoplasia of the male reproductive organs [35]. Therefore, polymorphisms of the *SRD5A2* gene may be a key point leading to intersexuality in sheep.

Functional enrichment of the sequencing data for intersex sheep and normal sheep populations (including Tan sheep) did not find significant entries related to gonadal development or sex hormone metabolisms. A previous study reported that testicular tissue dysplasia was a key cause of human gender developmental disorders [35]. Additionally, the excessive synthesis of estrogen in vivo also affected the formation and development of female reproductive organs. Therefore, it was speculated that various mutations of genes related to the synthesis and secretion of androgen led to the fact that the testis and its accessory reproductive organs could not be maintained.

As hermaphroditism is not conducive to animal reproduction, it is speculated that intersexual individuals are selected to be eliminated in evolution, so the occurrence in the current group is low. A major limitation of this study is that there were only two intersexual individuals sequenced, which may result in a certain number of false positives in the sequencing results. If the limitation of the sample size is eliminated, the study may obtain more mutation regions or copy number variation regions that are more reliable than in this paper, and may even lock in the major genes that lead to intersex traits. It is undeniable that this study provides a direction and reference basis for further in-depth exploration of the molecular mechanism of sheep intersex traits.

5. Conclusions

This study was the first to locate the homologous sequence of the goat PIS region in sheep and found that the intersex traits of LFT sheep were not caused by the lack of this region. Through detecting the selective sweep regions, the vital genes associated with androgen biosynthesis and the follicle stimulating hormone response entry were found, including *SRD5A2*, *SRD5A3*, and *PAWR*. Additionally, the copy number variations of the four regions on chr9, chr1, chr4, and chr16 may affect the expression of the gonadal development genes, *ZFPM2*, *LHX8*, *IMMP2L*, and *SLIT3*, respectively, which contribute to the appearance of intersex traits.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/6/944/s1, Table S1: PCR amplification primer information for candidate region on sheep chromosome 1; Table S2: The statistics of resequencing samples from 4 Lanzhou large-tailed sheep; Table S3: The statistics of resequencing samples from 8 sheep; Table S4: Chromosome distribution of SNPs in sheep; Table S5: Goat PIS regional positioning on sheep genome (the top 10 score); Table S6: List of genes in the selected overlapping regions by top 1% highest Z(Fst) between intersex sheep and normal sheep (including normal Lanzhou large-tailed sheep and Tan sheep); Table S7: GO term of the candidate genes associated with the intersex; Table S8: List of genes in the selected overlapping regions by top 1% highest Z(Fst) between intersex and normal individuals of Lanzhou large-tailed sheep; Table S9: GO term of the candidate genes associated with the intersex; Table S10: Autosomal distribution of CNVRs in sheep; Table S11: Gene annotation of the candidate CNVRs; Table S12: Functional enrichment analysis of annotated genes.

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Influence of Age and Immunostimulation on the Level of Toll-Like Receptor Gene (*TLR3, 4,* and *7*) Expression in Foals

Anna Migdał [1,*], Łukasz Migdał [1], Maria Oczkowicz [2], Adam Okólski [3] and Anna Chełmońska-Soyta [4,5]

[1] Department of Genetics, Animal Breeding and Ethology, Faculty of Animal Sciences, University of Agriculture in Krakow, al. 29 Listopada 46, 31-425 Kraków, Poland; lukasz.migdal@urk.edu.pl

[2] Department of Animal Molecular Biology, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice, Poland; maria.oczkowicz@izoo.krakow.pl

[3] Institute of Veterinary Science, University Centre of Veterinary Medicine UJ-UR, University of Agriculture in Krakow, al. Mickiewicza 24/28, 30-059 Kraków, Poland; adam.okolski@urk.edu.pl

[4] Laboratory of Reproductive Immunology, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Weigla 12 Street, 53-114 Wroclaw, Poland; anna.chelmonska-soyta@hirszfeld.pl

[5] Department of Immunology, Pathophysiology and Veterinary Preventive Medicine, Division of Immunology and Veterinary Preventive Medicine, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Norwida 31 Street, 50-375 Wroclaw, Poland

* Correspondence: anna.migdal@urk.edu.pl; Tel.: +48-(12)-662-53408

Simple Summary: Detailed knowledge of the molecular mechanisms of immunoglobulin synthesis appears necessary for a better understanding of foal immunity maturity and its influencing factors. At the same time, it encourages studies regarding the influence of the signaling cascade's proteins on the primary immunological response, which provides an opportunity to develop extremely precise methods of regulating acquired immunity. The results revealed that the expression of the*TLR3* and *TLR4* genes, as well as the levels of immunoglobulins and interleukins, can be modulated by stimulation with the pharmacological agent, and that the expression of the *TLR3* and *TLR4*genes in peripheral blood cells is dependent on age.

Abstract: The aim of this study was to investigate the molecular mechanisms leading to the identification of pathogens by congenital immune receptors in foals up to 60 days of age. The study was conducted on 16 foal Polish Pony Horses (Polish Konik) divided into two study groups: control (*n* = 9) and experimental (*n* = 7). Foals from the experimental group received an intramuscular duplicate injection of 5 mL of Biotropina (Biowet) at 35 and 40 days of age. The RNA isolated from venous blood was used to evaluate the expression of the*TLR3*, *TLR4*, and *TLR7* genes using RT-PCR. The results of the experiment demonstrated a statistically significant increase in the level of *TLR3* gene expression and a decrease in the level of*TLR4* gene expression with foal aging. The level of *TLR7* gene expression did not show age dependence. Immunostimulation with Biotropina had a significant impact on the level of the genes' expression for Toll-like receptors. It increased the level of *TLR4* expression and decreased *TLR3* expression. Thus, it was concluded that the expression of the*TLR3* and *TLR4*genes in peripheral blood cells is dependent on age. This experiment demonstrated a strong negative correlation between *TLR3* and *TLR4* gene expression.

Keywords: *TLR3*; *TLR4*; *TLR7*; foals; immunostimulation; gene expression

1. Introduction

Immune response differs in newborn and adult horses. Despite involving similar components, the regulation of immunity and the response to antigens vary. Foals are born with small, non-protective amounts of endogenous serum immunoglobulins (i.e., IgM and IgG) [1]. Immunoglobulin transport is limited due to the structure of the horse placenta (placenta spuria). That is why the suckling of colostrum is essential in the first hours of a foal's life. Immunological outcomes in newborn foals differ as compared to adults and are distinguished by modified cytokine profiles, as well as reduced antibody and T-cell responses [2]. Moreover, foals have a very low level of immunoglobulins in their blood plasma. An innate immune system composed of pre-existing or rapidly induced defenses is critical for newborn foals, while an antigen-specific response requires exposure to pathogens and time for development after birth [3]. The effectiveness of the immunological reaction is controlled by a complexity of direct and indirect mechanisms involving interactions among various cells and cytokine-induced actions. Many different immune cells are involved in maintaining a balanced immune response [4]. Receptors called pathogen recognition receptors (PRRs) represent very important elements found in immune cells. Thanks to their conservative structure, these receptors can recognize signals associated with a variety of pathogens. Pathogen-associated molecular patterns (PAMPs), PRR ligands, are molecules specific to viruses, bacteria, and other microorganisms with evolutionarily conserved structures [5]. Toll-like receptors (TLRs) are the most closely investigated PRRs and are one of the most essential components of immune responses [6,7]. Toll-like receptors play a key role in activating and stimulating innate as well as acquired immunity [8]. Identification of the threat and activation of TLRs triggers an immune response, leading to the elimination of this threat from the organism, which involves two basic reactions, namely, inflammatory, and antiviral. Cells with activated receptors release large amounts of proinflammatory cytokines, chemokines, and defensins, and these released factors initiate the migration and aggregation of immune cells (e.g., leukocytes, macrophages, mast cells, and dendritic cells) at the site of the pathogen invasion [7]. Activated TLRs present on the surface of macrophages lead to increased synthesis of the proinflammatory cytokines IL-1, -6, -8, -12, and TNF-α. In addition, complexes of ligands and TLR4 receptors increase the phagocytic activity of macrophages and stimulate the production of reactive oxygen species (ROIs) and the synthesis of nitric oxide (NO). TLR-activated macrophages enhance the expression of major histocompatibility complexes I and II (MHCI and MHCII), CD80, CD86, and co-stimulators that make immune cells more efficient in displaying T-cell antigens that induce specific immune responses [9,10].

Approximately 20% of foals die before the end of the second month, which brings significant economic and breeding losses [11,12]. This justifies undertaking research into new and novel techniques for stimulation of the immune system. Gaining insight into the behavior of TLRs seems to be necessary for expanding our understanding of the mechanism responsible for the development of foal resistance/immunity, and the identification of determinants for further building it up. In addition, it is also an important element contributing to finding innovative solutions in the fight against infections and new ways to improve the prevention and treatment of infections in animals. The paradox of neonatal vaccination is the need of immediate protection during early days, the perceived limitations of the immune system of neonate foals, and the theory of maternal antibody interference [13]. Studies have shown that the immune system of neonatal foals is also naive and immature relative to juvenile and adult horses [14–17]. Several studies have suggested that basal TLR expression in full-term neonatal blood monocytes is similar to that of adults [18,19]. The TLR-mediated production of cytokines by neonatal monocytes, however, is very different in newborns compared to that of adults [19]. Thus far, little is known about the development of the horse immune system during pre- and postnatal periods, which negatively affects the ability to devise strategies for maintaining and improving foal health. Based on its biological properties, as well as the influence of Toll-like receptors on the immune response traits of farm animals and humans, we hypothesized that gene expression for Toll-like receptors TLR-3, TLR-4, and TLR-7 in foals is dependent on factors such as age and immunostimulation. The aim of this work was to investigate the molecular mechanisms leading to the identification of pathogens by

congenital immune receptors in foals up to 60 days of age, including the verification of the hypothesis concerning age-related expression of the *TLR3*, *TLR4*, and *TLR7* genes.

2. Materials and Methods

This experiment was granted permission from the Local Ethics Committee in Kraków (no 37, 30 May 2016).

2.1. Animals and Feeding

Studies were carried out on 16 foals representing Polish Pony horses (Polish Konik). This primitive horse breed is genetically and phenotypically closely related to its wild ancestor, the Tarpan Horse (Eurasian wild horse) [20].

All foals with mares were kept in the same stable in individual boxes (size 2.15 × 3.50 m) on permanent straw bedding at the Experimental Station of the University of Agriculture in Krakow. All animals were clinically healthy throughout the experimental period. Mares of 5–17 years of age and 270–340 kg live body weight were not vaccinated during pregnancy. Foal birth weight was 27–35 kg, and weight loss on the first day of life was <1.5%. The horses had all been used by university students in the teaching program. No horses were used for equestrian purposes. Inclusion criteria consisted of foals born from healthy mares with no placentitis, a normal gestational period, an uneventful birth, and normal physical and neurological examination findings. The foals had to successfully stand and nurse within 2 h of birth and remain clinically healthy during the study period.

Mares were fed ad libitum with hay (*Lolium* 40% and *Trifolium L.* 20%) with the addition of oats in the amount of 1.5 kg/mare/day [21]. Foals were fed only with colostrum and mother's milk ad libitum, without additional supplementation. Water was offered from automatic water drinkers (flow ~ 10 L/min).

2.2. Experimental Design

Two weeks before delivery, birth alarms (Abfohlsystem, Jan Wolters, Steinfeld, Germany) were placed in the labia, and mares were moved to box stalls inside a stable lit with natural light (Figure S1). During the experiment, foals were kept with their mothers in individual boxes, and when leaving the stalls with their mothers for the pasture, they were randomly assigned into the following groups:

- The control group (Group C) (*n* = 9)—foals without any pharmacological and feed additives that may influence immune system;
- The experimental group (Group E) (*n* = 7)—foals that were administered an immunostimulating agent.

For the immunostimulation, a commercially available immunostimulator was used in the present study, namely, Biotropine (Biowet Drwalew S.A., Drwalew, Poland), which consists of a mixture of inactivated Gram-positive bacteria, e.g., *Staphylococcus aureus* (74 mg/mL), *Streptococcus zooepidermicus* (24.6 mg/mL), *Streptococcus equi* (24.6 mg/mL), *Streptococcus equisimilis* (24.6 mg/mL), *Streptococcus agalactiae* (24.6 mg/mL), *Streptococcus dysgalactiae* (24.6 mg/mL), *Erysipelothrix insidiosa* (49 mg/mL), and Gram-negative bacteria, e.g., *Escherichia coli* (123 mg/mL) and *Pasteurella multocida* (123 mg/mL) as well as pork spleen extract (10 mg/mL). On days 35 and 40 after birth, the foals from the experimental group received an intramuscular (*m. pectoralis descendens*) injection of 5 mL of Biotropine.

2.3. Blood Sampling and Blood Analysis

Blood samples were collected from foals by jugular venipuncture. Blood samples were obtained from foals up until 60 days of age according to the following scheme: After birth before the first suckling and then on the 1st, 3rd, 5th, 10th, 20th, 30th, 40th, 50th, and 60th days of age. Three milliliters of blood were collected into TEMPUS tubes (Applied Biosystems, Foster City, CA, USA) with RNA stabilizing factor. Samples were stored at −20 °C until further processing. Isolation of RNA was carried out using

TEMPUS SPIN (Ambion, Waltham, MA, USA) according to the manufacturer's protocol (Supplementary File 1). One microgram of RNA was transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol.

A "No-RT" (non-reverse transcriptase) control was used for selected RNA samples to analyzed contamination in samples.

Gene expression analyses (Table S2 presents the reaction efficiency of each gene) were performed on an Illumina Eco system (Illumina, San Diego, CA, USA, Country)using TaqMan®MGB (Applied Biosystems, Foster City, CA, USA) probes (Table 1). Every sample was analyzed in triplicate in a final volume of 10 µL (Table S1). Amplification was performed according to the following protocol: polymerase activation at 95 °C (2 min) and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The *SDHA* and *HPRT* genes were used as housekeeping genes (Table 1).

Table 1. Probes used for amplification of Toll-like receptor (TLR) genes and housekeeping genes.

Gen.	Full Name of the Gene	Access Number GenBank	TaqMan Gene Expression Assay ID	Dye
TLR3	Toll-like receptor 3	NC_009170.2	Ec03467747_m1	FAM
TLR4	Toll-like receptor 4	NC_009168.2	Ec03468993_m1	FAM
TLR7	Toll-like receptor 7	NC_009175.2	Ec03467530_m1	VIC
SDHA	Succinate dehydrogenase complex subunit A	XM_001490889	Ec03470479_m1	VIC
HPRT	Hypoxanthinephosphoribosyl transferase	AY372182.1	Ec03470217_m1	VIC

In addition, an analysis of the blood morphotic parameters was performed (Supplementary File 1).

2.4. Statistical Analysis

Data are presented as means ± standard error. The data were analyzed using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). The Shapiro–Wilk test was considered the best test to check the normality of the distribution of random variables. Because the data did not have a normal distribution, the Kruskal–Wallis test was used with immunostimulation and age as the effects. The degree of association between the parameters was examined using a non-parametric Spearman's rank correlation coefficient. Values ranging from 0.0 to 0.5, from 0.5 to 1.0, from −0.5 to 0.0, and from −1.0 to −0.5 indicate weak positive, strong positive, weak negative, and strong negative correlations, respectively.

3. Results

3.1. Influence of Age on the Expression of TLR3, 4, and 7 mRNA

The lowest expression of *TLR3* was observed during delivery (6.20 ± 0.89) (Figure 1—data presented from control group). After delivery, the level of *TLR3* mRNA increased. In the period between delivery and 60 days of age, the level of *TLR3* expression increased by 94.34%. We found a highly statistically significant difference between the age and expression of *TLR3* mRNA ($p > 0.01$), as presented in Table 2. From the 5th day of age, we found statistical differences between samples, with the highest expression level on the 60th day after delivery.

The highest level of *TLR4* mRNA expression was observed during the delivery (18.3 ± 2.6) of newborn foals. From the day of the delivery to 20th day of age, we observed a steady significant decrease of *TLR4* mRNA expression (Figure 1) in the blood of the examined foals. During the subsequent days of observation, the expression of the mRNA of this receptor remained at a similar level. The lowest expression value was observed at the 60th day of age (5.82 ± 0.96) (Table 2). Between the delivery day and the 60th day of age, the expression of mRNA for *TLR4* decreased by 76.89%. Statistical analysis showed highly statistically significant differences between the age of foals and the expression of *TLR4* mRNA ($p < 0.01$).
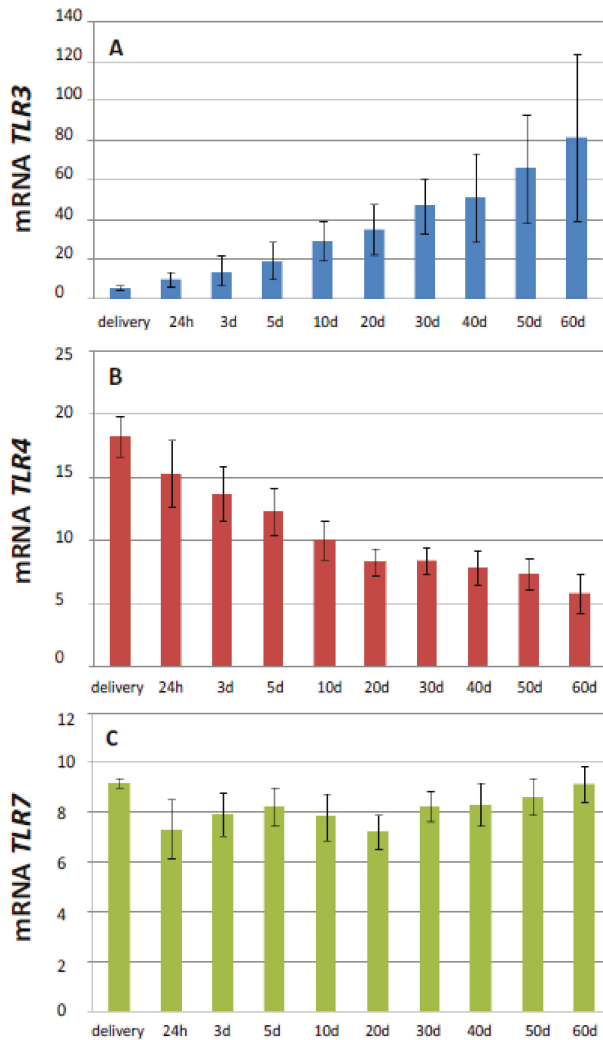
Figure 1. Trends in the change of TLR3 (**A**), TLR4 (**B**), and TLR7 (**C**) expression during foals' growth. Delivery, sample collected at delivery; 24 h, sample collected 24 h after delivery; 3 days, sample collected every 3rd day after delivery; 5 days, sample collected every 5 days after delivery; 10 days, sample collected every 10 days after delivery; 20 days, sample collected every 20 days after delivery; 30 days, sample collected 30 days after delivery; 40 days, sample collected every 40 days after delivery; 50 days, sample collected every 50 days after delivery; 60 days, sample collected 60 days after delivery. The means are reported with their standard errors.

The expression of the *TLR7* gene remained statistically unchanged throughout the experiment (Figure 1). The highest values were observed during the day of delivery (9.20 ± 1.20), while the lowest was observed 20 days after delivery (7.20 ± 0.61). There was no statistically significant correlation between the age and the expression of *TLR7* mRNA ($p > 0.2366$) (Table 2).

Table 2. Expression of *TLR3*, *TLR4*, and *TLR7* mRNA over the foals' subsequent days of age from the control group (mean ± standard error (SE)).

Age	TLR3	TLR4	TLR7
Delivery [1]	6.2 [A] ± 0.9 [2]	18.3 [A] ± 2.6	9.2 ± 1.2
24 h	9.8 [B] ± 1.7	15.3 [B] ± 2.2	7.3 ± 0.9
3 days	14.1 [A,C] ± 2.1	13.7 [C] ± 1.8	7.9 ± 0.8
5 days	19.1 [A,B,D] ± 2.2	12.3 [A,D] ± 1.5	8.2 ± 0.9
10 days	29.3 [A,B,C,E] ± 2.8	10.1 [A] ± 1.1	7.8 ± 0.7
20 days	35.1 [A,B,C,F] ± 3.1	8.3 [A,B] ± 1.1	7.2 ± 0.6
30 days	48.4 [A,B,C,D,G] ± 4.9	8.4 [A,B] ± 14	8.3 ± 0.9
40 days	53.6 [ABCDEF] ± 5.8	7.8 [A,B,C] ± 1.2	8.3 ± 0.7
50 days	80.4 [ABCDEFG] ± 8.7	7.3 [A,B,C] ± 1.5	8.6 ± 0.7
60 days	87.9 [A,B,C,D,E,F,G] ± 8.0	5.8 [A,B,C,D] ± 0.9	9.2 ± 07

[1] Delivery, sample collected at delivery; 24 h, sample collected 24 h after delivery; 3 days, sample collected 3 days after delivery; 5 days, sample collected 5 days after delivery; 10 days, sample collected 10 days after delivery; 20 days, sample collected 20 days after delivery; 30 days, sample collected 30 days after delivery; 40 days, sample collected 40 days after delivery; 50 days, sample collected 50 days after delivery; 60 days, sample collected 60 days after delivery. [2] Means are reported with their standard errors. Means with same letter in column show highly statistically significant differences ($p < 0.01$).

3.2. Influence of Stimulation with Biotropina on the Expression of TLR3, 4, and 7 mRNA

Analysis of changes in the expression of *TLR3* mRNA after the injection of the immunostimulant (Group E) showed a decrease by 41.65% (Table 3), while in the control group (Group C), a dynamic increase in the expression was observed until the last day of observation (116.22 ± 13.93). Highly statistically significant differences were found between immunostimulation and the expression of *TLR3* mRNA.

Table 3. Influence of stimulation with Biotropina on the expression of mRNA for selected Toll-like receptors (*TLR3*, *TRL4*, *TLR7*) (mean ± SE).

Age	TLR3		TLR4		TLR7	
	Group C	Group E	Group C	Group E	Group C	Group E
Delivery [1]	8.3 ** ± 1.8 [2]	4.1 ** ± 0.55	19.9 * ± 4.0	13.7 * ± 0.5	12.9 * ± 2.9	7.6 * ± 0.9
24 h	14.7 ** ± 3.8	6.5 ** ± 1.24	17.5 * ± 3.3	11.5 * ± 0.6	10.2 ± 2.1	7.0 ± 0.8
3 days	20.0 ** ± 4.9	8.7 ** ± 2.24	16.7 ** ± 2.4	9.9 ** ± 1.1	9.6 ± 1.7	8.3 ± 1.1
5 days	28.0 ± 2.6	15.1 * ± 1.70	14.5 * ± 1.9	9.7 * ± 1.1	11.4 * ± 2.2	7.4 * ± 0.9
10 days	32.7 ± 3.3	30.1 ± 5.43	10.9 ± 0.7	7.1 ± 1.3	9.7 ± 1.4	7.4 ± 0.6
20 days	36.7 ± 4.6	36.4 ± 4.93	8.0 ± 1.2	7.2 ± 1.5	8.7 ± 1.5	7.0 ± 0.7
30 days	62.2 * ± 9.6	39.6 * ± 7.43	8.2 ± 2.3	7.1 ± 1.9	10.0 ± 2.0	7.1 ± 0.9
40 days	78.2 ** ± 6.8	23.1 ** ± 8.82	6.5 * ± 1.3	10.0 * ± 2.4	9.7 ± 1.6	7.9 ± 0.8
50 days	107.0 ** ± 15.3	26.0 ** ± 9.71	5.5 ± 1.9	6.8 ± 1.9	11.2 * ± 1.2	6.9 * ± 0.9
60 days	116.2 ** ± 13.9	44.6 ** ± 10.20	4.2 ± 1.4	6.5 ± 1.7	11.5 ± 1.2	7.6 ± 0.7

[1] Delivery, sample collected at delivery; 24 h, sample collected 24 h after delivery; 3 days, sample collected 3 days after delivery; 5 days, sample collected 5 days after delivery; 10 days, sample collected 10 days after delivery; 20 days, sample collected 20 days after delivery; 30 days, sample collected 30 days after delivery; 40 days, sample collected 40 days after delivery; 50 days, sample collected 50 days after delivery; 60 days, sample collected 60 days after delivery. [2] Means are reported with their standard errors; Group C, control group; Group E, experimental Biotropina-stimulated group (injection at the 35th and 40th days after delivery). * Means in row/line for receptor show significant differences ($p < 0.05$); ** Means in row/line for receptor show highly statistically significant differences ($p < 0.01$).

The level of *TLR4* mRNA expression at 30 days of age was similar in both groups (Table 3). After Biotropina injection, *TLR4* mRNA expression increased by 41.33% (Group E), while the expression of *TLR4* mRNA in foals from Group C decreased by 20.22%. On the following days after immunostimulation, *TLR4* mRNA expression in the foals from Group E was higher, but we did

not find statistically significant differences between groups. Statistical analysis showed statistically significant differences in *TLR4* mRNA expression after immunostimulation.

The initial expression of *TLR7* mRNA before first suckling was higher in Group C. In Group E, the highest level of expression was observed during delivery at 7.57 ± 0.88 (Table 3). In Group C, the expression was higher during the experiment compared to Group E, and we found highly statistically significant differences between groups ($p < 0.001$). No statistically significant differences were found between the expression of *TLR7* mRNA and immunostimulation.

The Spearman's rank correlation test showed a strong negative correlation between the *TLR3* and *TLR4* genes, and lack of correlation between *TLR3* and *TLR7* as well as *TLR4* and *TLR7* (Table 4).

Table 4. Correlations (*p*-value) of the expression of *TLR3*, *TLR4*, and *TLR7* mRNA over the subsequent days of age of the foals from the control group.

Gene	Age	TLR4	TLR7
TLR3	<1 h	−0.160 (0.6273)	0.62857 (0.1631)
	24 h	−0.191 (0.4199)	0.462 (0.1400)
	3 days	−0.100 (0.6726)	0.492 (0.1276)
	5 days	−0.095 (0.6912)	0.328 (0.1582)
	10 days	**−0.350 (0.0299)**	0.567 (0.1917)
	20 days	**−0.582 (0.0071)**	0.423 (0.1634)
	30 days	**−0.04361 (0.0085)**	0.368 (0.1098)
	40 days	**−0.56992 (0.0087)**	**0.472 (0.0355)**
	50 days	**−0.46466 (0.0039)**	**0.76541 (0.0251)**
	60 days	**−0.35338 (0.0026)**	**0.82105 (0.0341)**
TLR4	<1 h		−0.340 (0.1376)
	24 h		−0.385 (0.0936)
	3 days		−0.472 (0.1355)
	5 days		−0.341 (0.1408)
	10 days		−0.191 (0.4199)
	20 days		−0.319 (0.1707)
	30 days		−0.271 (0.2468)
	40 days		−0.53083 (0.1600)
	50 days		**−0.67519 (0.0111)**
	60 days		**−0.360 (0.0116)**

<1 h, sample collected at delivery; 24 h, sample collected 24 h after delivery; 3 days, sample collected 3 days after delivery; 5 days, sample collected 5 days after delivery; 10 days, sample collected 10 days after delivery; 20 days, sample collected 20 days after delivery; 30 days, sample collected 30 days after delivery; 40 days, sample collected 40 days after delivery; 50 days, sample collected 50 days after delivery; 60 days, sample collected 60 days after delivery. Correlations (*p*-value) bolded show significant differences ($p < 0.05$) while underlined show highly significant differences ($p < 0.01$).

3.3. Influence of Age and Stimulation with Biotropina on the Level of Blood Morphotic Elements

Statistical analysis showed (Table 5):

- A significant influence of age on the hematocrit level;
- A highly significant influence of age on the hemoglobin level;
- A significant influence of age on the level of erythrocytes;
- A highly significant influence of age and immunostimulation on the level of leukocytes;
- A highly significant influence of age and immunostimulation on the level of lymphocytes;
- A highly significant influence of immunostimulation on the number of monocytes;
- A significant influence of age and immunostimulation on the number of neutrophils.
- A highly significant influence of immunostimulation, significant influence of age on the number of basophils;
- A highly significant influence of immunostimulation and age on the number of basophils.

Table 5. Level of blood morphotic elements in foals (mean ± SE).

Parameters	Age	<1 h [1]	24 h	3 Days	5 Days	10 Days	20 Days	30 Days	40 Days	50 Days	60 Days
Hematocrit (PCV) %	C	50.00 [2] ± 1.2	43.33 ± 1.2	39.83 ± 1.5	41.11 ± 1.1	41.56 ± 1.9	34.44 ± 1.6	37.33 ± 1.1	36.33 ± 1.3	37.72 ± 1.2	37.56 ± 1.7
	E	50.50 ± 1.2	44.50 ± 1.8	43.67 ± 1.7	39.58 ± 1.5	41.17 ± 0.9	38.20 ± 1.1	39.75 ± 2.9	36.50 ± 1.7	37.25 ± 1.3	37.75 ± 0.9
Hemoglobin (g/dL)	C	15.46 ± 0.7	14.55 ± 0.7	13.26 ± 0.5	13.75 ± 0.7	13.63 ± 0.7	14.57 * ± 1.2	15.11 * ± 0.9	14.85 * ± 1.3	13.30 ± 0.6	13.98 * ± 1.1
	E	13.94 ± 0.9	13.04 ± 1.7	13.50 ± 0.8	12.71 ± 0.6	12.39 ± 0.2	13.03 ± 0.9	11.80 * ± 0.3	11.70 * ± 0.4	12.39 ± 0.5	10.08 * ± 0.3
RBC count (10^6/µL)	C	10.93 ± 0.9	10.62 ± 0.7	9.99 ± 0.6	10.55 ± 0.6	9.53 ± 0.4	10.38 ± 1.3	11.24 ± 1.5	9.98 ± 0.8	10.43 ± 0.8	9.86 ± 0.8
	E	11.50 ± 0.5	10.01 ± 0.3	9.17 ± 0.4	10.24 ± 0.9	9.33 ± 0.8	9.04 ± 0.7	9.59 ± 1.0	9.69 ± 0.7	10.29 ± 0.3	10.22 ± 0.3
WBC count (10^3/µL)	C	7.35 ± 0.8	7.71 ± 0.7	10.35 ± 1.2	9.86 ± 1.0	11.03 ± 0.8	11.30 ± 0.8	12.60 ± 1.0	14.31 ** ± 0.8	14.62 * ± 0.7	13.07 ** ± 0.7
	E	6.25 ± 0.6	8.17 ± 0.5	9.15 ± 0.8	10.64 ± 1.4	12.40 ± 1.1	12.48 ± 0.9	10.91 * ± 1.6	21.75 ** ± 0.5	18.98 * ± 0.4	14.89 ** ± 0.2
Eosinophils (/µL)	C	105 ** ± 2.2	116 ** ± 2.4	72 ** ± 1.5	296 ** ± 6.2	110 ** ± 2.3	226 ** ± 4.7	315 ** ± 6.6	286 ** ± 6.0	292 ** ± 6.1	327 ** ± 6.9
	E	0 ** ± 0.0	16 ** ± 0.3	22 ** ± 0.4	31 ** ± 0.6	37 ** ± 0.7	42 ** ± 0.8	55 ** ± 1.0	206 ** ± 3.7	105 ** ± 1.9	133 ** ± 2.4
Basophils (/µL)	C	44 ± 0.9	39 ± 0.8	31 ± 0.6	99 ± 2.1	55 ± 1.2	113 * ± 2.4	94 * ± 1.9	72 * ± 1.5	146 ** ± 3.1	196 ** ± 4.1
	E	18 ± 0.3	29 ± 0.5	38 ± 0.7	39 ± 0.7	52 ± 0.9	64 * ± 1.1	75 * ± 1.3	308 ** ± 5.5	205 ** ± 3.7	76 ** ± 1.4
Neutrophils (/µL)	C	4471 * ± 59.5	4488 * ± 59.3	7041 ± 93.4	6903 ± 91.6	5898 * ± 78.3	6104 ± 81.0	7150 * ± 94.9	8583 ± 113.9	8186 ± 108.6	6403 * ± 84.9
	E	5650 ± 50.8	6942 ± 62.5	7374 ± 66.4	7645 ± 68.8	8804 * ± 79.2	7773 ± 69.9	5705 ** ± 51.3	9499 * ± 85.5	9109 ± 81.9	7523 ± 67.7
Lymphocytes (/µL)	C	2554 ** ± 35.7	2852 * ± 39.9	3003 * ± 42.0	2465 ± 34.5	4686 ** ± 65.6	4635 * ± 64.9	4945 ** ± 69.2	5150 ** ± 72.1	5701 ** ± 79.8	5880 ** ± 82.3
	E	470 ** ± 4.2	1090 * ± 9.8	1609 ** ± 14.5	2802 ± 25.2	3110 ** ± 28.0	4280 * ± 38.5	4850 ** ± 43.6	10953 ** ± 98.6	9355 ** ± 84.2	7054 ** ± 63.5
Myelocytes (/µL)	C	162 ± 2.3	231 ** ± 3.2	207 * ± 2.9	99 ± 1.4	276 * ± 3.7	226 * ± 3.2	94 * ± 1.3	215 ** ± 3.0	292 * ± 4.1	261 * ± 3.7
	E	112 ± 1.0	93 ** ± 0.8	107 * ± 0.9	123 ± 1.1	397 * ± 3.6	321 * ± 2.9	225 * ± 2.0	784 ** ± 7.1	206 * ± 1.8	104 * ± 0.9

[1] <1, sample collected at delivery; 24 h, sample collected 24 h after delivery; 3 days, sample collected 3 days after delivery; 5 days, sample collected 5 days after delivery; 10 days, sample collected 10 days after delivery; 20 days, sample collected 20 days after delivery; 30 days, sample collected 30 days after delivery; 40 days, sample collected 40 days after delivery; 50 days, sample collected 50 days after delivery; 60 days, sample collected 60 days after delivery. [2] Means are reported with their standard errors. Group C, control group; Group E, experimental Biotropina-stimulated group (injection on the 35th and 40th days after delivery). * Means in row/line for receptor show significant differences ($p < 0.05$); ** means in row/line for receptor show highly statistically significant differences ($p < 0.01$).

4. Discussion

Infectious diseases are common in foals between the first and fifth months of age. Analysis of the concentrations of immune system components during this period in healthy and infected foals may help understand the basics of the maturation of the immune system and also better understand infection mechanisms [22]. To the best of our knowledge, there are very limited reports where weekly collections and the expression of immune-related genes have been performed. Therefore, our results may be interesting for better understanding the changes during the first weeks of a foal's life and the changes it undergoes during this time. Most studies report data from the first 24 h of a foal's life, from the first 42 days, and very often from adult horses. Moreover, most data include thoroughbreds, while we performed our analysis on a primitive horse breed that is known for their adaptation to harsh conditions. While this study was performed on a primitive domestic breed, the results from this study may differ from potential results if performed on selectively breed domestic horses. Flaminio et al. [22] reported that healthy lymphocytes of healthy foals were the lowest at birth and that values increased until the sixth month of age. In our study, we obtained similar results; however, in Polish Pony, lymphocyte counts were higher (Table 5).

4.1. Changes in TLR4 Gene Expression

Because of the increased vulnerability of foals to some pathogens (e.g., *Rhodococcus equi*), it seems reasonable to analyze changes in the expression of the genes that are responsible for the recognition of the conserved constituents of pathogens [23]. In the present study, a highly significant influence of age and stimulation with Biotropina was observed for *TLR4*. Data available in the literature indicate that the influence of age on *TLR4* expression in horses is contradictory. Vendrig et al. [24] found no differences between the expression of *TLR4* in the blood mononuclear cells of foals at 12 h of life or in adult horses. Stimulation with lipopolysaccharides (LPS) resulted in higher *TLR4* mRNA expression in adult horses, while no response to LPS stimulation was found in foals in an in vivo study [24]. In contrast, Tessier et al. [25], having compared *TLR4* mRNA expression in umbilical cord blood and peripheral blood from adult horses, demonstrated higher*TLR4* mRNA expression in umbilical cord blood in response to LPS administration. The influence of age on the expression of *TLR4*was observed by Hansen et al. [26] in horses aged between 5 and 27 years. Higher *TLR4* mRNA levels were found in younger horses, but the decrease in mRNA levels with age were not statistically significant. *TLR4* mRNA levels were higher in blood mononuclear cells compared to the same cells from pulmonary vascular secretions. Osorio et al. [27] and Strong et al. [28] evaluated the expression of the *TLR4* gene in the first weeks of a calf's life, and their results were similar to the one produced in our study. The highest level of*TLR4* gene expression was observed after birth, and a statistically significant decrease in expression was reported during the following days. A similar trend was identified in our study. Yerkovich et al. [29] and Levy et al. [30] showed that the expression of the *TLR4* gene was significantly higher in peripheral blood in premature and full-term infants than in adults, both before and after LPS stimulation [31]. This trend, indicating a decreasing expression of *TLR4* with age, was also found in humans and mice [32,33]. On the other hand, some results illustrate a higher expression of *TLR4* in newborns compared to adults [34] or decreasing expression of *TLR4* after stimulation [35]. The differences in *TLR4* expression revealed in these studies and in our experiment may be caused by different concentrations of LPS in the stimulation.

4.2. Changes in TLR3 Gene Expression

We found that *TLR3* mRNA levels increased with the age of the foals. Our results are in agreement with other reports about horses and mostly human newborns [25,36–38]. Interestingly, there are reports proving epigenetics control *TLR3* expression mechanisms [36]. As Porras et al. [36] reported in their results from healthy donors, it can be presumed that a low level of *TLR3* in newborns is a developmentally desirable trend. In a mouse model, Zhang et al. [37] reported higher abortion rates

linked with higher *TLR3* levels. It was also mentioned that *TLR3* expression was age-dependent, which can be confirmed by our results. As *TLR3* binds double-stranded RNAs (dsRNAs), its decreased level may increase susceptibility to viral infection in young foals. In our study, the lowest level was recorded before the first suckling, which is in agreement with other reports in premature infants [38] and newborns [39,40]. The data presented above, and the results obtained in our study of the *TLR3* gene, may explain the higher incidence of equine herpesvirus-1 (EHV-1) and equine herpesvirus-4 (EHV-4), responsible for massive respiratory tract infections in foals and young horses. A severe course and high mortality due to contracting equine viral arteritis (EVA) in young horses may also be a result of the decreased expression of *TLR3*. Hussey et al. [41] suggested that *TLR3* plays an essential role in recognizing EHV-1 infections. In our study, a decrease in *TLR3* gene expression was observed after stimulation with Biotropina. We analyzed the level of expression in foals up to 60 days of age, but the literature indicates that the immune system of horses develops most intensively until about 90 days of life [42]. Foals have all of the components of an immune system characteristic of adult horses—but many mechanisms of the immune response have yet to mature. The results indicate that activation of horse monocytes by ligands for the *TLR2* and *TLR4* genes increases their expression, but not that of *TLR3*. Additionally, *TLR3* gene expression decreases with the increase of *TLR4* gene expression after the stimulation of monocytes [43].

4.3. Changes in TLR7 Gene Expression

TLR7 is responsible for recognizing guanidine-rich, single-stranded viral RNA (ssRNA) and is an important mediator of the peripheral immune response. Asquith at al. [41] and Slavica at al. [39] observed no effect of age on*TLR7* gene expression in newborns, similar to Talmadge et al. [22] in horses. Belnoue et al. [44] reported significantly higher levels of *TLR7* gene expression in two-week-old foals compared to adult horses. Harrington et al. [9] found neither an age-dependent pattern in the expression of the *TLR7/8* genes, nor did they detect the effect of imidazoquinol R848 stimulation on its expression, despite increasing the levels of IL-6 and IL-8. Our results also did not confirm any relationship between a foal's age and expression of *TLR7*. Until now, little was known about the signaling mechanisms of Toll-like receptors in foals. Identifying the receptors and describing ligands that react with them can provide new insights into immunological responses and can also point to new pathways in the field of therapy and prevention of diseases, particularly infectious ones.

5. Conclusions

In summary, on the basis of the results obtained, it was concluded that the expression of the *TLR3* and *TLR4* genes in peripheral blood cells is dependent on age. The expression of the *TLR3* and *TLR4* genes, as well as the levels of immunoglobulins and interleukins, can be modulated by stimulation with the pharmacological agent Biotropina. This experiment demonstrated a strong negative correlation between *TLR3* and *TLR4* gene expression. Detailed knowledge of the molecular mechanisms of immunoglobulin synthesis appears necessary for a better understanding of foal immunity maturity and its influencing factors. At the same time, this experiment encourages studies regarding the influence of the signaling cascade's proteins on the primary immunological response, providing an opportunity to develop extremely precise methods of regulating acquired immunity. There is still little information about the maturity of a horse's immune system in the pre- and postnatal period, which negatively affects the planning of health protection strategies for foals.

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Genome-Wide DNA Methylation Changes of Perirenal Adipose Tissue in Rabbits Fed a High-Fat Diet

Jiahao Shao [1,†], Xue Bai [1,†], Ting Pan [2], Yanhong Li [1], Xianbo Jia [1], Jie Wang [1] and Songjia Lai [1,*]

[1] College of Animal Science and Technology, Sichuan Agricultural University, Chengdu 611130, China; shaojh1997@163.com (J.S.); baixue333work@163.com (X.B.); lyh81236718@163.com (Y.L.); jaxb369@sicau.edu.cn (X.J.); wjie68@163.com (J.W.)

[2] College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China; panting555666@163.com

[*] Correspondence: laisj5794@163.com

[†] These authors contributed equally to this work.

Simple Summary: Obesity is spreading rapidly in most countries and regions, becoming a considerable public health concern because it is associated with type II diabetes mellitus, fatty liver disease, hypertension, and even certain cancers. The biological effects of caloric restriction are closely related to epigenetic mechanisms, including DNA methylation. Here, rabbits were used as a model to study the effect of a high-fat diet on the DNA methylation profile of perirenal adipose tissue. The results indicate that 2906 genes associated with differentially methylated regions were obtained and were involved in the PI3K-AKT signaling pathway (KO04151), linoleic acid metabolism (KO00591), DNA replication (KO03030), and MAPK signaling pathway (KO04010). In conclusion, high-fat diet may cause changes in the DNA methylation profile of adipose tissue and lead to obesity.

Abstract: DNA methylation is an epigenetic mechanism that plays an important role in gene regulation without an altered DNA sequence. Previous studies have demonstrated that diet affects obesity by partially mediating DNA methylation. Our study investigated the genome-wide DNA methylation of perirenal adipose tissue in rabbits to identify the epigenetic changes of high-fat diet-mediated obesity. Two libraries were constructed pooling DNA of rabbits fed a standard normal diet (SND) and DNA of rabbits fed a high-fat diet (HFD). Differentially methylated regions (DMRs) were identified using the option of the sliding window method, and online software DAVID Bioinformatics Resources 6.7 was used to perform Gene Ontology (GO) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis of DMRs-associated genes. A total of 12,230 DMRs were obtained, of which 2305 (1207 up-regulated, 1098 down-regulated) and 601 (368 up-regulated, 233 down-regulated) of identified DMRs were observed in the gene body and promoter regions, respectively. GO analysis revealed that the DMRs-associated genes were involved in developmental process (GO:0032502), cell differentiation (GO:0030154), and lipid binding (GO:0008289), and KEGG pathway enrichment analysis revealed the DMRs-associated genes were enriched in linoleic acid metabolism (KO00591), DNA replication (KO03030), and MAPK signaling pathway (KO04010). Our study further elucidates the possible functions of DMRs-associated genes in rabbit adipogenesis, contributing to the understanding of HFD-mediated obesity.

Keywords: DNA methylation; high-fat diet; rabbits

1. Introduction

From the last 5 decades, the incidence of obesity has sharply increased, becoming one of the most considerable threats to human health because it is associated with the risk of type II diabetes mellitus, fatty liver disease, hypertension, and even certain cancers [1]. Obesity is a multifactorial pathological process, and genetic, environmental, and behavioral factors influence the development of obesity [2]. Nowadays, an imbalance between energy intake and expenditure is a major contributor to fat deposition in individuals predisposed to obesity [3]. Fat deposition is characterized by an increase in the number and size of adipocytes, and its process is closely related to physiological homeostasis, far beyond simple fat storage [4]. HFD has been shown to induce obesity in animal models and humans, and further induce a variety of obesity-related clinical diseases, such as osteoporosis, inflammation, and even neurodegeneration [5–7]. Perirenal fat, as part of abdominal visceral fat, is often used to elucidate the molecular and pathophysiological mechanisms of metabolic disorders associated with obesity or adipose development, because it is closely related to kidney injury, metabolism of triacylglycerol, and other metabolic regulation [8]. For example, detailed studies have shown that the perirenal fat thickness in obese patients could be a valuable marker to define the risk of developing hypertension and kidney dysfunction [9,10]. The expression profile of perirenal fat microRNA was changed during different growth stages of rabbits, and the differential microRNA expression was enriched for the MAPK signaling pathway, Wnt signaling pathway, aldosterone synthesis, and secretion pathways [11].

First proposed by Waddington in 1942, epigenetics refers to heritable changes in gene expression without an altered DNA sequence [12]. Epigenetics is caused by the interaction of environmental factors and intracellular genetic material, such as dietary factors, microRNA, and genomic imprinting, etc. Noteworthily, the biological effects of caloric intake are closely related to epigenetic mechanisms, including chromatin remodeling and DNA methylation [13]. DNA methylation of leptin and adiponectin promoters in obese children is associated with BMI, dyslipidemia, and insulin resistance [14]. These observations support the hypothesis that epigenetic modifications might underpin the development of obesity and related metabolic disorders. Hypermethylation of the pro-opiomelanocortin and serotonin transporter genes has been positively associated with childhood or adult obesity [15]. HFD changes the methylation status of *Casp1* and *Ndufb9* genes in obese mice, which are related to liver lipid metabolism and liver steatosis [16]. In addition, the leptin promoter was hypermethylated and *Ppar-α* promoter was hypomethylated in oocytes of mice fed with HFD, and the same changes were also observed in the liver of female offspring [17]. However, few studies have reported the changes in perirenal adipose tissue methylation profile in HFD-induced obese rabbits.

To further understand the epigenetic mechanisms influencing fat metabolism in obese rabbits, we investigated the role of DNA methylation in perirenal adipose tissue by sequencing and analyzing DNA methylation libraries from rabbits fed a standard normal diet (SND) and a high-fat diet (HFD).

2. Materials and Methods

2.1. Animals

A total of 24 female Tianfu black rabbits from a strain breed at the Sichuan Agricultural University in China were randomly divided into two groups and fed either a standard normal diet (SND) or a high-fat diet (HFD; 10% lard was added to the standard normal diet) for four weeks. The composition and nutrient content of the standard normal diet (SND) and the high-fat diet (HFD) were described in our previous report [18]. At the beginning of the trial, all rabbits were 35 days of age and housed individually in a clean iron cage (600 × 600 × 500 mm) and kept in an environmentally controlled room. Rabbits were free to access water and fed twice a day. At the end of the trial, rabbits were screened for obesity using the body mass index (BMI; BMI = bodyweight (kg)/height^2 (m)), and three rabbits from each group meeting the experimental requirements were selected for sampling. All experimental protocols were performed under the direction of the Institutional Animal Care and

Use Committee from the College of Animal Science and Technology, Sichuan Agricultural University, China (DKY-B2019202015, 5 December 2019).

2.2. DNA Extraction

Perirenal adipose tissue samples were collected immediately after rabbits were euthanized (shock and bleed treatment). Tissue blocks were placed in 4 mL EP tubes and stored in a −80 °C freezer. Total DNA from perirenal adipose tissue was extracted using a commercial TIANamp Genomic DNA extraction kit (Tiangen, Beijing, China), following the manufacturer's instructions. Subsequently, the purity and concentration of DNA were assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Carlsbad, CA, USA), and only DNA meeting quality criteria (thresholds: $A_{260}/A_{280} \approx 1.8$; concentration $\geq$ 200 ng/μL) was used for the trial.

2.3. DNA Methylation Library Construction and Sequencing

To identify genome-wide DNA methylation changes in perirenal adipose tissue induced by HFD, two libraries were constructed by pooling the DNA samples from three SND rabbits and three HFD rabbits. Briefly, DNA was fragmented by sonication to 100 to 500 bp fragments. The fragments were end-repaired using T4 DNA polymerase and Klenow enzyme and adaptors were ligated after generating 3'dA overhangs. Bisulfite treatment was conducted using the ZYMO EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA), following the manufacturer's protocol. After desalting, fragments of sizes ranging from 220 to 320 bp were isolated using a 15% PAGE gel and amplified by adaptor-mediated PCR. Lastly, the libraries were sequenced using the Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA) by Chengdu Life Baseline Technology Corporation, China.

2.4. Processing and Comparison of Sequencing Data

By removing adapter sequences and low-quality reads containing more than 50% low-quality bases (quality score < 5), clean reads were retained. Clean reads were aligned to the rabbit reference genome (GCF_000003625.3) with software BSMAP 2.90 (http://code.google.com/p/bsmap). Two forward strands, i.e., BSW (++) and BSC (−+) were used as references. The accuracy of DNA methylation detection depends on the conversion efficiency of cytosine, and the incomplete transformation of cytosine in sequences may lead to false-positive results. Here, lambda phage DNA was used as a control group to calculate the bisulfite conversion rate.

2.5. Methylation Site Detection

The methylation C sites were determined using the method described in a previous study [19]. Briefly, a binomial distribution test was performed for methylated reads number and non-methylated reads number at C sites. C sites were identified as the methylation C sites when the number of reads was greater than or equal to the binomial distribution expected value and the total effective coverage was greater than or equal to four.

2.6. Methylation Level Analysis

The average genome-wide methylation level reflects the overall characteristics of the methylation pattern of the genome. DNA methylation occurs in three sequence contexts: CG, CHG, and CHH (H = A, C, or T). The average methylation levels of CG, CHG, and CHH were calculated based on the percentage of methylated cytosine in the entire genome, chromosome, and genomic functional elements. For each type of sequence (CG, CHG, and CHH), the average methylation level was calculated according to the following formula: the average methylation level = methylated reads / (methylated reads + non-methylated reads) × 100%. To assess the association between sequence characteristics and methylation bias, we calculated the methylation percentage of nine bases upstream and downstream of the methylation site.

2.7. Searching for Differentially Methylated Regions (DMRs)

DMRs were identified using the option of a sliding window method. Briefly, the sliding windows, which were used for further analysis, had to meet the following criteria: (a) the depth in each cytosine should be more than four in each sample, and each C site should cover at least four methylation reads; (b) the number of selected cytosine should be larger than five; (c) after calculating mean methylation level of each sample, the fold change of mean methylation level between the two samples should be larger than two. After repeating extension steps, the merged regions with $p < 0.05$ were defined as DMRs.

2.8. Functional Enrichment Analysis of Differentially Methylated Genes

To explore the role of epigenetic variation in biological processes and pathways, online software DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) was used to perform Gene Ontology (GO) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis of DMRs-associated genes. GO analysis can be used to identify the performance of the gene product and contains three types of information: cellular component, molecular function, and biological processes. KEGG is the main public database that integrates the genome, chemistry, and system function information, particularly the set of genes associated with the systemic functions of cells, organisms, and ecosystems. Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Quality Assessment of Sequencing Data

After raw reads were processed, a total of 1,221,455,488 clean reads were obtained from methylation sequencing libraries (Table S1). The clean reads were mapped to the rabbit reference genome, and the mapping rate was 84.910% in the SND group and 84.730% in the HFD group, respectively. The bisulfite conversion rate was 99.550% for SND, and 99.520% for HFD. In addition, the effective coverage rate of C base in each chromosome ranged from 89.892% to 97.577% and ranged from 91.822% to 97.804% in different functional genomic elements (Tables S2 and S3).

3.2. Methylation Level Analysis

Genome-wide methylation level analysis showed that the methylation level of C, CHG, and CHH in the HFD group was higher than in the SND group but the CG methylation level in the HFD group was lower than in the SND group (Table S4). Results of the methylation level C, CG, CHG, and CHH on different chromosomes are shown in Table S5. The greatest differences in C, CG, CHG, and CHH between the two groups were found on chromosome 20, chromosome X, chromosome 1, and chromosome 11, reaching 0.569%, 2.736%, 0.056%, and 0.047%, respectively. In addition, we classified the various functional genomic elements into promoter, CDS, intron, mRNA, downstream, CpGIsland, ncRNA, and transposons. Compared with the SND group, the methylation level of C, CHG, and CHH in each functional genomic element was increased in the HFD group (Table S6). However, based on comparison with the SND group, promoter, intron, mRNA, downstream, and ncRNA methylation levels were decreased in CG, and only CDS, CpGIsland, and transposons methylation levels were increased in CG.

3.3. Genome-Wide Characteristics of Methylated C Bases

The percentage of methylated C bases in CG were highest, reaching 94.795% (SND) and 94.843% (HFD) but rarely cytosine methylation was found in CHH and CHG. In addition, we calculated the methylation percentage of nine bases (methylated C at the fourth base) upstream and downstream of the methylated site. As shown in Figure 1, CG, CAG, and CAC were the most likely sites to be methylated in both SND and HFD groups.
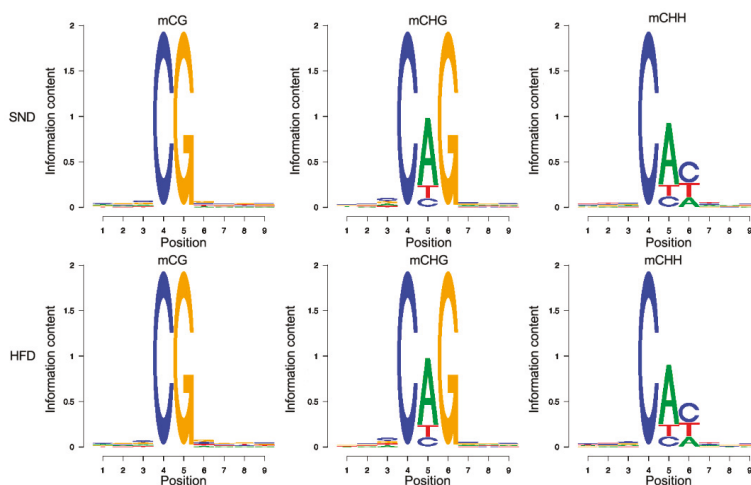
Figure 1. Genome-wide characteristics of methylated C bases. Sequence characteristics of bases near mCG, mCHG, and mCHH in the SND and HFD group.

3.4. Analysis of Differentially Methylated Regions (DMRs)

A total of 12,230 DMRs were identified in the genome of the HFD group compared to the SND group. Chromosome 21 was the chromosome with the least amount of DMRs and chromosome 13 was the chromosome with the most amount of DMRs (Figure 2a,b). The total length of DMRs in each chromosome is shown in Table S7. In addition, the DMRs were mapped to the gene body and promoter regions, and 2305 (1207 up-regulated, 1098 down-regulated) and 601 (368 up-regulated, 233 down-regulated) methylated genes were obtained, respectively. Some genes involved in adipocyte growth and development have also been identified, including *ACE2*, *AGTR1*, *IGF1R*, and *ACSL4*.

3.5. GO and KEGG Enrichment Analysis

To better study the biological functions of the DMRs-associated genes, we used online software DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) to carry out gene ontology (GO) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis. GO analysis of the overlapping DMRs-associated genes in the gene body regions found a total of 6310 enriched GO terms (4796 biological processes (BP), 579 cellular components (CC), and 935 molecular functions (MF)), of which 12.570% were significantly enriched ($p < 0.05$) (Table S8). The main GO terms involved in overlapping DMRs-associated genes in the gene body regions included the developmental process (GO:0032502), cell differentiation (GO:0030154), and lipid binding (GO:0008289). The top 10 significantly enriched terms in the BP, CC, and MF categories are shown in Figure 3a. The KEGG pathway analysis showed that overlapping DMRs-associated genes in the gene body regions were enriched in 314 pathways including the PI3K-AKT signaling pathway (KO04151), linoleic acid metabolism (KO00591), and pathways for DNA replication (KO03030). Thirty-nine of these pathways (12.420%) were significantly enriched ($p < 0.05$, Table S9). In addition, a scatter analysis was carried out for the 20 most significant pathways to intuitively show the significance of these pathways (Figure 3b).
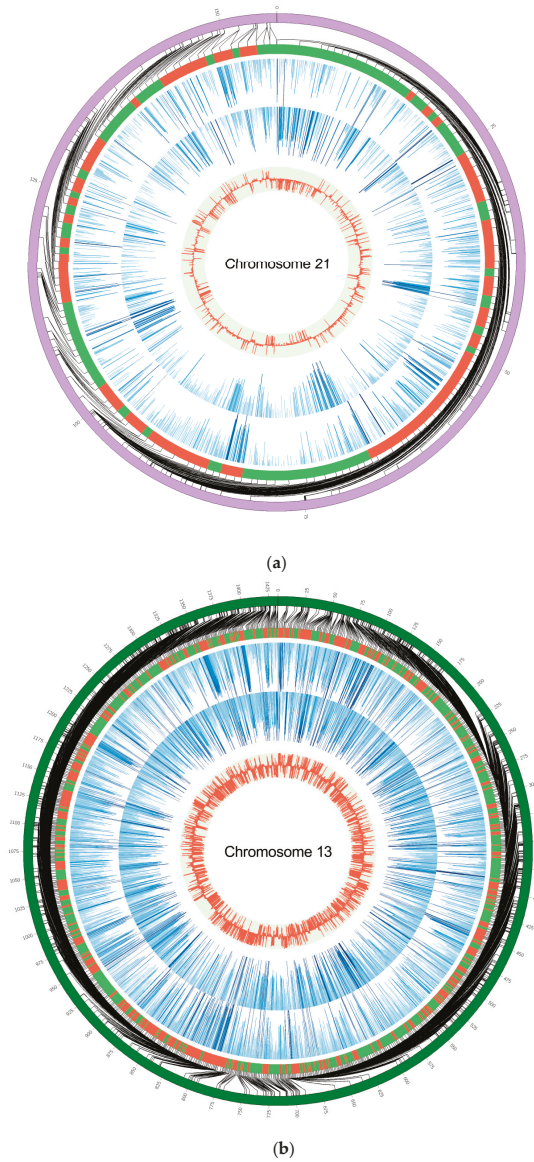
(**a**)

(**b**)

Figure 2. Analysis of differentially methylated regions (DMRs). Two chromosomes with the least (**a**) and the most (**b**) amount of DMRs. The outer ring represents the position of the genomic chromosome; the second circle is the DMRs region: the red area represents the higher methylation level of HFD compared to the SND group and the green area represents the lower methylation level of HFD compared to the SND group; the third circle represents the methylation rate of each site of sample HFD; the fourth circle represents the methylation rate of each site of sample SND; the fifth circle represents the difference of methylation rate.

Figure 3. GO and KEGG enrichment analysis. (**a**) GO analysis of the overlapping DMRs-associated genes in the gene body regions and promoter regions. (**b**) KEGG pathway analysis of the overlapping DMRs-associated genes in the gene body regions and promoter regions. Rich factor = (DMRs-associated genes annotation in term/genes annotation in term)/(DMRs-associated genes with KEGG annotation / all genes with KEGG annotation).

GO analysis of the overlapping DMRs-associated genes in the promoter regions showed enrichment of 2223 biological processes (BP), 311 cellular components (CC), and 405 molecular functions (MF), of which 173 BP (7.780%), 20 CC (6.430%), and 37 MF (9.140%) were significantly enriched (Table S10).

The significantly enriched GO terms mainly include positive regulation of lipid biosynthetic process (GO:0046889), regulation of cholesterol metabolic process (GO:0090181), and regulation of lipid biosynthetic process (GO:0046890). The top 10 significantly enriched GO terms in the BP, CC, and MF categories of GO analysis are shown in Figure 3a. KEGG pathway analysis found 266 enriched pathways including the MAPK signaling pathway (KO04010) (Table S11). The top 20 significantly enriched pathways are presented in Figure 3b.

4. Discussion

DNA methylation represents an important epigenetic marker because it is associated with chromosomal structural changes, embryonic development, expression of imprinted genes, and causing corresponding diseases, including X chromosome inactivation and DNA unwinding [20–22]. Nowadays, obesity prevention and treatment strategies have been unsuccessful, and DNA methylation is one of the epigenetic modifications associated with obesity [23]. The rabbit is an ideal material to study obesity due to its lipid metabolism and obesity-related clinical manifestations similar to those of humans [24,25]. Thus far, some DNA methylation related studies have been investigated in rabbit models but studies of the changes in perirenal adipose tissue methylation profile in HFD-induced obese rabbits have not been carried out. In this study, DNA methylation patterns were investigated in rabbit models to understand obesity-related DNA methylation changes.

Here, the mapping rates were 84.910% and 84.730% in the SND group and HDF group, respectively. The bisulfite conversion rates were 99.550% (SND) and 99.520% (HFD) in the two groups, which was consistent with previous research, indicating that the libraries were high quality and reliable [26,27]. Methylation is a dynamic process in cells, which can be regulated by methylation and demethylation. The average methylation level of the whole genome reflects the overall characteristics of the genome methylation profile. The results of the genome-wide methylation level analysis in this study were similar to those in mice [28]. The methylation level of CG was higher than the methylation level in C, CHG, and CHH. However, this result is different from that of plant Arabidopsis Thaliana. The plant genome has extensive methylation at the CHG site [29]. CG methylation was maintained by Dnmt1. CHH methylation and some CHG methylation is usually maintained by the activity of the conserved Dnmt3. The high level of CHG methylation seen in Arabidopsis thaliana is maintained by plant-specific methyltransferase [30]. In addition, research on chickens suggests that promoter DNA methylation generally affects chromatin structure and is a signal to inhibit gene transcription, and promoter regions are lowly methylated [31]. Our study also found that promoter regions showed a lower methylation level than other regions. However, a study in mice fed with HFD showed that promoter regions are hypermethylated [32]. Therefore, we hypothesized that differences in methylation level may be species-specific.

The results of genome-wide characteristics of methylated C bases showed that the proportion of mCG was the highest, while the cytosine methylation was low in CHH and CHG. Some studies have shown that no enzyme can maintain mCHG during DNA replication in animals, so the sites of CHG type in animal cells generally show a very low level of methylation. CHH can only rely on the methylation mechanism, so CHH methylation is easily lost in the process of DNA replication and is generally in the state of hypomethylation [33]. The results of this study showed that the characteristics of methylation in the rabbit genome were similar to those in other animals.

DMRs refer to the regions of DNA molecules with different methylation status in two samples. The identification of DMRs is the first step towards the study of DMRs-associated genes [34]. In our study, a total of 2906 DMRs were identified, and 2305 (1207 up-regulated, 1098 down-regulated) and 601 (368 up-regulated, 233 down-regulated) methylated genes were associated with differentially methylated regions. Many genes are related to adipocyte growth and development. For example, as the members of the renin-angiotensin system (RAS), *ACE2* and *AGTR1* were reported to participate in the development and progression of obesity [35,36]. *PPARγ* and *aP2* are important transcription factors in the development and function of the adipose tissue and marker of lipogenesis [37]. Previous studies

showed that inhibition of *IGF1R* decreased the expression of PPARγ, thereby inhibiting lipogenesis [38]. Moreover, *ACSL4* plays a role in the regulation of lipid metabolism. *ACSL4* was expressed throughout the entire differentiation process in pig preadipocytes and showed a similar expression trend with lipogenesis-associated genes *PPARγ* and *aP2* [39].

Gene ontology (GO) analysis is a reliable bioinformatics tool for understanding the characteristics of genes and gene products. The significantly enriched terms in the BP, CC, and MF categories indicated the possible roles of the DMRs-associated genes in regulating obesity. The significantly enriched GO terms showed correlation with adipocyte lipid metabolism and metabolisms, such as lipid binding (GO:0008289), positive regulation of lipid biosynthetic process (GO:0046889), regulation of cholesterol metabolic process (GO:0090181), developmental process (GO:0032502), and cell differentiation (GO:0030154). Some terms were related to adipocyte development, including cytoskeletal protein binding (GO:0008092), tubulin binding (GO:0015631), calcium ion binding (GO:0005509). Cytoskeletal remodeling and cell–cell interaction are a necessary step in the transformation of preadipocytes into mature adipocytes, and adipocyte development is dependent on α-tubulin [40,41]. Calcium is a complex mediator in adipogenesis because it regulates numerous cellular processes [42]. Furthermore, other GO items related to hormones and enzymes were also significantly enriched, such as regulation of glucocorticoid secretion (GO:2000849), N-acetyltransferase activity (GO:0008080), and phosphoric diester hydrolase activity (GO:0008081). Increasing evidence suggests that excess glucocorticoids leads to increased fat mass and obesity through the accumulation of adipocytes [43]. Acetyltransferase is a regulator of adipogenesis and lipid metabolism, and its regulatory mechanism is mainly transcription and post-translation modifications [44]. Phosphoric diester hydrolase is a regulator of systemic glucose and insulin homeostasis [45]. Interference of phosphoric diester hydrolase expression in 3T3-L1 adipocytes caused a dramatic decrease in adipocyte differentiation key gene (*PPARγ*, *aP2*) and lipid accumulation [46].

Adipogenesis is a complex process involving an elaborate network of transcription factors and signaling pathways. Results of KEGG analysis showed that DMRs-associated genes were mainly involved in the PI3K-AKT signaling pathway (ko04151), linoleic acid metabolism (KO00591), DNA replication (KO03030), and MAPK signaling pathway (KO04010). The PI3K-AKT signaling pathway is a key regulator in cell proliferation, differentiation, and apoptosis [47]. Activation of the PI3K-AKT signaling pathway promotes the expression of marker genes involved in adipogenesis and glucose uptake [48]. In our study, 70 DMRs-associated genes were enriched in the PI3K-AKT signaling pathway, thereby revealing that these DMRs-associated genes may be essential for adipogenesis. Linoleic acid metabolism (KO00591) is also associated with adipogenesis. Linoleic acid can be converted to the metabolically active arachidonic acid, which has roles in inducing inflammation and adipogenesis. Excessive intake of linoleic acid results in increasing magnitudes of adiposity, inflammatory cytokines, and insulin resistance [49]. In addition, it is becoming clear that DNA replication (KO03030) and the MAPK signaling pathway (KO04010) play an important role in adipocyte growth and development [50,51]. Thus, the results of our study indicate that these DMRs-associated genes might be an important regulator in adipogenesis. However, due to the limitation of experimental conditions, such as pooled samples, only one library per group, sequencing methods, etc., functional verification of these DMRs-associated genes will be important to consider in the future.

5. Conclusions

In conclusion, our study indicates that a high-fat diet may affect genes associated with adipogenesis by altering DNA methylation patterns. We identified 2906 methylated genes, of which, *ACE2*, *AGTR1*, *IGF1R*, and *ACSL4* may have a key role in adipogenesis. These genes may be involved in the regulation of adipogenesis through the PI3K-AKT signaling pathway (KO04151), linoleic acid metabolism (KO00591), DNA replication (KO03030), and MAPK signaling pathway (KO04010).

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Embryonic Thermal Manipulation Affects the Antioxidant Response to Post-Hatch Thermal Exposure in Broiler Chickens

Khaled M. M. Saleh [1], Amneh H. Tarkhan [1] and Mohammad Borhan Al-Zghoul [2],*

[1] Department of Applied Biological Sciences, Faculty of Science and Arts, Jordan University of Science and Technology, P.O. Box 3030, Irbid 22110, Jordan; khaledmousa93@gmail.com (K.M.M.S.); amneht92@gmail.com (A.H.T.)

[2] Department of Basic Medical Veterinary Sciences, Faculty of Veterinary Medicine, Jordan University of Science and Technology, P.O. Box 3030, Irbid 22110, Jordan

* Correspondence: alzghoul@just.edu.jo; Tel.: +962-79034-0114

Simple Summary: The broiler chicken is one of the most important livestock species in the world, as it occupies a major role in the modern human diet. Due to uneven artificial selection pressures, the broiler has increased in size over the past few decades at the expense of its ability to withstand oxidative damage, the latter of which is often a byproduct of thermal stress. In order to attenuate the effects of heat stress, thermal manipulation (TM), which involves changes in incubation temperature at certain points of embryonic development, is increasingly being presented as a way in which to improve broiler thermotolerance. Therefore, the objective of this study was to investigate how TM might affect broiler response to post-hatch thermal stress in the context of the genes that help combat oxidative damage, namely the catalase, NADPH oxidase 4 (*NOX4*), and superoxide dismutase 2 (*SOD2*) genes. Expression of all three aforementioned genes differed significantly between TM and control chickens after exposure to cold and heat stress. Conclusively, TM may act as a viable mode of preventative treatment for broilers at risk of thermally induced oxidative stress.

Abstract: Thermal stress is a major source of oxidative damage in the broiler chicken (*Gallus gallus domesticus*) due to the latter's impaired metabolic function. While heat stress has been extensively studied in broilers, the effects of cold stress on broiler physiologic and oxidative function are still relatively unknown. The present study aimed to understand how thermal manipulation (TM) might affect a broiler's oxidative response to post-hatch thermal stress in terms of the mRNA expression of the catalase, NADPH oxidase 4 (*NOX4*), and superoxide dismutase 2 (*SOD2*) genes. During embryonic days 10 to 18, TM was carried out by raising the temperature to 39 °C at 65% relative humidity for 18 h/day. To induce heat stress, room temperature was raised from 21 to 35 °C during post-hatch days (PD) 28 to 35, while cold stress was induced during PD 32 to 37 by lowering the room temperature from 21 to 16 °C. At the end of the thermal stress periods, a number of chickens were euthanized to extract hepatic and splenic tissue from the heat-stressed group and cardiac, hepatic, muscular, and splenic tissue from the cold-stressed group. Catalase, *NOX4*, and *SOD2* expression in the heart, liver, and spleen were decreased in TM chickens compared to controls after both cold and heat stress. In contrast, the expression levels of these genes in the breast muscles of the TM group were increased or not affected. Moreover, TM chicks possessed an increased body weight (BW) and decreased cloacal temperature (T^C) compared to controls on PD 37. In addition, TM led to increased BW and lower T^C after both cold and heat stress. Conclusively, our findings suggest that TM has a significant effect on the oxidative function of thermally stressed broilers.

Keywords: broiler; thermal manipulation; antioxidant; heat stress; cold stress

1. Introduction

The term broiler refers to any member of the red junglefowl subspecies, *Gallus gallus domesticus*, that has been reared for the purposes of meat production and consumption [1]. Constituting the largest standing avian population, the broiler has become a vital component of modern human nutrition as a result of the rapid industrialization of the poultry production process [2]. Since the mid-twentieth century, broilers have undergone intensive breeding in order to enhance their growth rates, meat yield, and feed conversion ratios [3]. However, these artificial selection pressures have been largely consumer-driven, focusing solely on improving certain commercially attractive parameters at the expense of immune, metabolic, and skeletal function [4]. As a result, the modern broiler has become increasingly susceptible to the effects of thermal and, in turn, oxidative stresses [5].

Due to impaired metabolic function and expensive energetics, broilers are especially vulnerable to heat stress, which occurs when the broiler is unable to adequately dissipate body heat to the environment [6]. Rising global temperatures have consolidated the threats of heat stress to the development and wellbeing of broiler chickens, and such increases in temperature are exacerbated during the hotter seasons [7]. In addition, broiler heat stress can be caused by certain stages of the poultry production process, especially during their transport from rearing to processing facilities [8]. Broilers subject to heat stress will have a lower body weight due to decreased feed intake, and their innate immune function will be impaired as a result of decreased immune organ weight [9]. In fact, it has been illustrated that heat stress results in oxidative stress in broilers, resulting in a number of adverse metabolic changes [10].

Heat stress is a major cause of oxidative stress in broilers, and oxidative damage deteriorates the appearance, flavor, and nutritional value of broiler meat [11]. Oxidative stress can be defined as the imbalance that occurs when the amount of reactive oxygen species (ROS) in an animal cell exceeds the latter's antioxidant capacity [12]. To prevent oxidative stress, several genes are involved in the maintenance of cellular homeostasis, including NADPH oxidase 4 (*NOX4*), superoxide dismutase (*SOD2*), and catalase [13,14]. Primarily expressed in renal and vascular cells, the *NOX4* gene is constitutively active and codes for an oxygen-sensing enzyme that can also play a role in antimicrobial defense [15–17]. If overexpressed, *NOX4* leads to oxidative stress due to its production of superoxide (O_2^-) radicals and hydrogen peroxide (H_2O_2) molecules [18,19]. To prevent NOX4-associated oxidative stress from occurring, SOD2 and catalase act to dismutate O_2^- and break down H_2O_2, respectively [20].

Unlike heat stress, cold stress in broilers has not been the subject of much research in the context of its relation to oxidative stress. Nonetheless, cold stress has been found to induce oxidative stress and modulate immune function in broilers while also increasing their susceptibility to necrotic enteritis and ascites development [21–25]. Moreover, cold stress was found to affect the thigh muscle of broilers more severely than the breast muscle, and it significantly reduced the feed intake and body weights of broilers but increased their feed conversion ratios [26,27]. As outdoor rearing systems gain more popularity, preventing cold stress will become increasingly costly to the poultry industry, and such costs often fluctuate depending on fuel prices, season, and existing heating systems [28].

To mitigate the damage caused by heat and cold stress, thermal manipulation (TM), which involves embryonic exposure to high or low temperatures, has been found to improve thermotolerance and enhance physiological parameters of broilers [13,29–33]. However, further research needs to be carried out in order to understand the effects of heat- and cold-induced oxidative stress in thermally manipulated broilers. Therefore, the main purpose of the present study was to investigate the effects of both cold and heat stress on the antioxidant defense mechanisms of thermally manipulated broiler chickens.

2. Materials and Methods

Ethical approval for all experimental procedures was obtained from the Animal Care and Use Committee at Jordan University of Science and Technology (approval # 16/3/3/418).

2.1. Egg Procurement and Incubation

Fertile Cobb eggs (n = 600) were obtained from local distributors based in Madaba, Jordan. Before incubation, eggs were thoroughly examined, and eggs were excluded if they displayed abnormality or damage (n = 69). The remaining eggs (n = 531) were then randomly divided into two groups, control (n = 266) and thermal manipulation (TM) (n = 265), and incubated in semi-commercial incubators (Masalles S.L., Barcelona, Spain). In the control group, eggs were incubated under standard conditions (37.8 °C and 56% relative humidity (RH)) throughout embryogenesis. In contrast, TM eggs were only incubated under standard conditions from embryonic days (ED) 1 to 9 and 19 to 21, as TM was applied from ED 10 to 18 by incubating the eggs at 39 °C and 65% RH for 18 h/day. On ED 7, candling was performed on each egg in order to exclude infertile and/or nonviable eggs.

2.2. Hatchery Management

On hatch day, the hatchability, which is the percentage of fertile eggs that hatch, was calculated according to the following equation: hatchability = (number of hatched chicks/total number of incubated eggs) × 100. Chicks were left in the incubator to dry for the first 24 h of their post-hatch life, after which they were transported to a special area designated for the field experiments. On post-hatch days (PD) 1 and 37, the cloacal temperatures (T^C) and body weights (BW) were recorded, and the number of chicks that died within the whole field experimental period was noted. Dead chickens were histopathologically examined, but no significant obvious findings were reported. Before exposure to thermal stress, chicks were randomly distributed into their coops in groups of ten. In the first week, the temperature of the enclosures was kept at 33 ± 1 °C and was steadily reduced to 24 °C by the end of the third week. The RH during the rearing period was maintained within a range of 45%–52%. Water and appropriate feed were supplied to the chicks ad libitum during the whole field experiment period. On PD 8 and 20, chicks were vaccinated against Newcastle disease, and, on PD 15, the chicks were vaccinated against infectious bursal disease. The overall experimental design is illustrated in Figure 1.
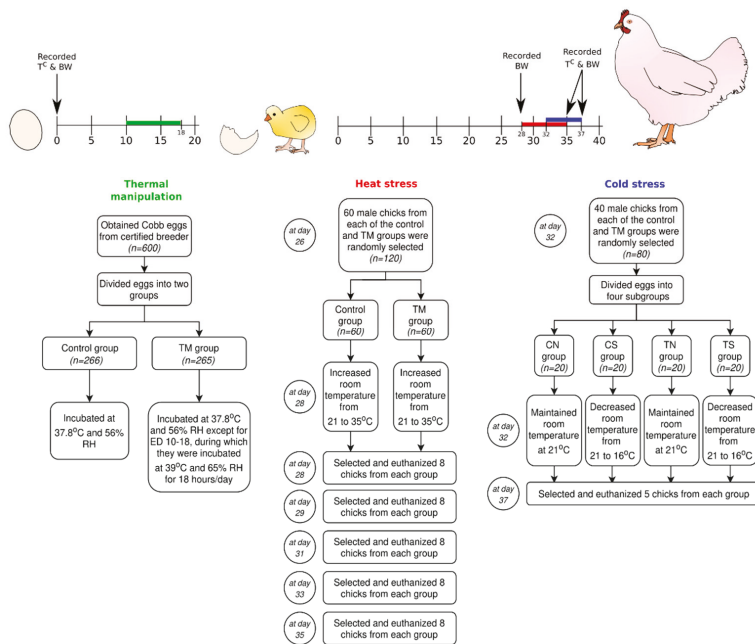

Figure 1. Experimental design showing the three main phases: thermal manipulation, heat stress, and cold stress.

2.3. Experiment 1: Post-Hatch Heat Exposure

On PD 26, male chicks (n = 60) were randomly selected from each of the control and TM groups to be transported to the experimental room for the induction of heat stress. On PD 28, heat stress was induced by raising the temperature of the experimental room to 35 °C and 45%–52% RH until PD 35. During this period, male chicks (n = 60) from each of the control and TM groups were subject to normal conditions (not shown in Figure 1). The experimental and rearing rooms were located on the same floor in order to mitigate transport stress. After 0, 1, 3, 5, and 7 days of heat exposure, chicks (n = 8) were randomly chosen from the control and TM groups. The T^C and BW of the chicks were recorded, after which they were euthanized in order to collect hepatic and splenic organs. Samples were snap-frozen on-site using liquid nitrogen, transferred to the laboratory, and stored at −80 °C.

2.4. Experiment 2: Post-Hatch Cold Exposure

On PD 32, chicks (n = 40) from each of the two incubation groups (control group and TM group) were randomly chosen and subdivided into four subgroups: control exposed to cold stress (CS), TM exposed to cold stress (TS), control exposed to normal conditions (CN) and TM exposed to normal conditions (TN). Cold stress was achieved by lowering the room temperature to 16 °C and 45%–52% RH from PD 32 to 37. At PD 37, BW and T^C were recorded for chicks (n = 5) from each subgroup, and a number of chicks (n = 5) were humanely euthanized in order to collect the liver, spleen, heart, and breast muscle organs. Samples were snap-frozen on-site using liquid nitrogen, transferred to the laboratory, and stored at −80 °C.

2.5. cDNA Synthesis

The Direct-Zol™ RNA MiniPrep (Zymo Research, Irvine, CA, USA) was utilized alongside TRI Reagent® (Zymo Research, Irvine, CA, USA) in order to isolate total RNA from all the collected samples. The Biotek PowerWave XS2 Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) was employed to determine the quantity and quality of the samples, after which 2 μg of total RNA from each sample were inputted into the Superscript III cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) to synthesize cDNA.

2.6. Primer Design and Relative mRNA Quantitation Analysis by Real-Time RT-PCR

The primer sequences that were used for real-time RT-PCR analysis are listed in Table 1. Primers were taken from previous reports [34] and were designed using the PrimerQuest tool on the Integrated DNA Technologies website (Coralville, IA, USA) (https://eu.idtdna.com/pages) and the Nucleotide database on the NCBI (Bethesda, MA, USA) website (https://www.ncbi.nlm.nih.gov/nucleotide/). The QuantiFast SYBR® Green PCR Kit (Qiagen, Hilden, Germany) was utilized on a Rotor-Gene Q MDx 5 plex instrument (Qiagen, USA) according to the manufacturer's protocol. For the internal control, fold changes in gene expression were normalized against the 28S ribosomal RNA. Single target amplification specificity was ensured by the melting curve, and relative quantitation was calculated automatically by the software on the Rotor-Gene Q MDx 5 plex instrument.

Table 1. Primer sequences used for real-time RT-PCR analysis.

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
NOX4	CCAGACCAACTTAGAGGAACAC	TCTGGGAAAGGCTCAGTAGTA
SOD2	CTGACCTGCCTTACGACTATG	CGCCTCTTTGTATTTCTCCTCT
Catalase	GAAGCAGAGAGGTTCCCATTTA	CATACGCCATCTGTTCTACCTC
28S rRNA	CCTGAATCCCGAGGTTAACTATT	GAGGTGCGGCTTATCATCTATC

2.7. Statistical Analysis

IBM SPSS Statistics v23.0 (IBM, USA) was used for all statistical analyses performed in the current study. The chi-squared test was used to analyze hatchability and mortality rates. T^C, BW, and the fold changes in mRNA levels of the catalase, *NOX4*, and *SOD2* genes are portrayed as means ± SD. An independent t-test compared between the control and TM groups with respect to several parameters at each time interval (PD 1, 3, 5, 7, 9, 11, 13, 15, 19, 22, 25, 28, 30, 33 and 35). Within the treatment group itself, two-way ANOVA was also used to compare between different parameters at specific time intervals after thermal stress. Statistical significance for parametric differences was set at 0.05.

3. Results

3.1. Effect of Thermal Manipulation (TM) on Hatchability and Physiological Parameters of Broiler Chicks

No significant effect was observed in either the mortality (control = 1.7; TM = 1.7) or the hatchability (control = 85.71; TM = 83.02) rates between the control and TM groups. However, TM led to significantly lower cloacal temperatures (T^C) on PD 1 and 37 and to higher body weights (BW) on PD 37. However, no significant change was observed in hatchling BW (Table 2).

Table 2. Effects of embryonic thermal manipulation (TM) on post-hatch body weight (BW) and cloacal temperature (T^C) of broiler chickens.

	Post-Hatch Day	Control	TM
T^C (°C)	1	39.63 ± 0.24 [a]	39.48 ± 0.23 [b]
	37	39.05 ± 0.24 [a]	38.38 ± 0.22 [b]
BW (g)	1	44.2 ± 3.8 [a]	42.7 ± 2.8 [a]
	37	2302.8 ± 79.7 [a]	2440 ± 82.5 [b]

[a,b] within the same row, means ± SD with non-identical superscripts are significantly different.

3.2. Effect of Post-Hatch Heat Stress on Physiological Parameters of Thermally Manipulated Broilers

Table 3 illustrates the effects of heat stress for 7 days (PD 28 to 35) on T^C, BW, and BW gain in the controls and TM broiler chickens. TM significantly decreased the mortality rate during post-hatch heat exposure (control = 12%; TM = 8%). Moreover, heat stress significantly increased the T^C in both groups, but the T^C of controls was significantly higher compared to TM chicks. On day 0 (PD 28) of heat stress, the BW of the TM group was significantly higher than that in controls. Similarly, the BW of controls was significantly lower compared to TM chicks on day 7 (PD 35) of heat stress, but the subgroups exposed to heat stress possessed significantly lower BW and BW gain compared to those exposed to normal conditions.

Table 3. Effects of post-hatch heat stress for 7 days (post-hatch day (PD) 28 to 35) on cloacal temperature (T^C), body weight (BW), and BW gain in broiler chickens subjected to embryonic thermal manipulation and controls.

	Normal Conditions		Heat Stress	
	(21 °C; RH 45%–52%)		(35 °C; RH 45%–52%)	
	Control	TM	Control	TM
T^C (°C)	39.65 ± 0.28 [a]	39.08 ± 0.21 [b]	41.35 ± 0.24 [c]	40.15 ± 0.26 [d]
BW (g)				
Day 0 (PD 28)	1373.3 ± 51.3 [a]	1675.7 ± 83.8 [b]	1456.7 ± 82.8 [a]	1704 ± 74.4 [b]
Day 7 (PD 35)	1847.9 ± 108.1 [a]	2108.8 ± 95.8 [b]	1645 ± 40.6 [c]	1930 ± 50.2 [d]
BW gain (g)	474.6 ± 70.9 [a]	433 ± 39.1 [a]	188.3 ± 47.3 [b]	226 ± 34.7 [b]

[a–d] within the same row, means ± SD with non-identical superscripts are significantly different.

3.3. Effect of Post-Hatch Heat Stress on Antioxidant Enzyme mRNA Levels in Thermally Manipulated Broilers

Figure 2 represents the effects of heat stress on the hepatic and splenic mRNA levels of certain antioxidant enzymes in broiler chicks subjected to embryonic TM. Tables S1 and S2 includes the mRNA levels of the same antioxidant genes for broiler chicks (TM and controls) not exposed to heat stress during the same timeframe.
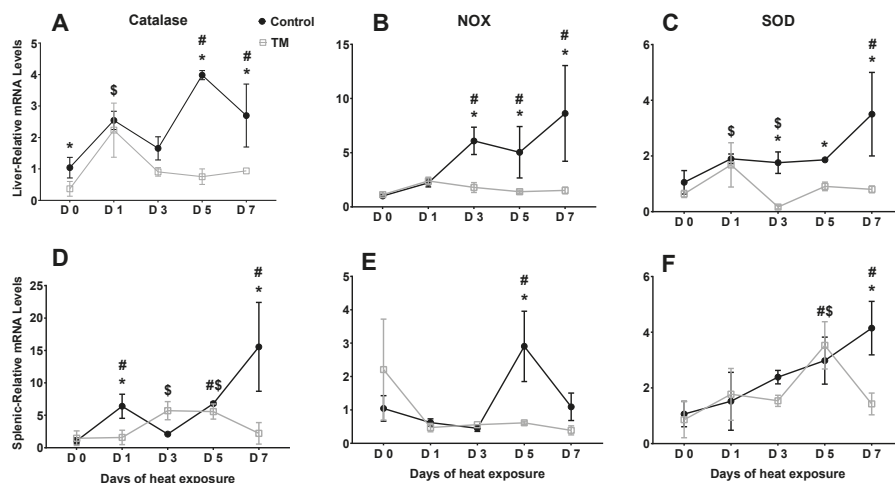

Figure 2. Effect of post-hatch heat stress for 7 days (PD 28 to 35) on the mRNA levels of catalase, *NOX4*, and *SOD2* in the liver (**A–C**) and spleen (**D–F**) of TM broiler chicks (n = 5). * within the same day, means ± SD of TM and control chicks are significantly different. # within the control group, means ± SD of non-identical days differ significantly. $ within the TM group, means ± SD of non-identical days differ significantly.

Catalase. On day 0 (PD 28) of heat stress, TM led to significantly lower catalase mRNA levels in the liver. In the control group, the hepatic mRNA levels of catalase were significantly higher after 5 (PD 33) and 7 (PD 35) days of heat stress compared to day 0, while, in the TM group, the level was significantly higher only after 1 day (PD 29) of heat exposure. The hepatic catalase mRNA level was significantly lower in TM chicks compared to controls after 5 (PD 33) and 7 (PD 35) days of heat stress.

The splenic mRNA level of catalase was not significantly different between TM and control chicks on day 0 (PD 28) of heat stress. However, the level was significantly lower in TM chicks compared to controls after 1 (PD 29) and 7 (PD 35) days of heat stress. Within the control group, the splenic mRNA level of catalase was significantly higher after 1 (PD 29), 5 (PD 33), and 7 (PD 35) days of heat exposure compared to day 0 (PD 28), whereas, in the TM group, the splenic mRNA level of catalase was significantly higher after 3 (PD 31) and 5 (PD 33) days (vs. day 0 (PD 28)).

NOX4. In the liver, the mRNA level of *NOX4* was not significantly different between the TM and control groups on day 0 (PD 28) of heat stress. In contrast, the *NOX4* mRNA level was significantly lower in TM chicks compared to controls after 3 (PD 31), 5 (PD 33), and 7 (PD 35) days of heat stress. Within the control group, the mRNA level was significantly increased after 3 (PD 31), 5 (PD 33), and 7 (PD 35) days of heat stress (vs. day 0 (PD 28)), but, in the TM group, the level did not significantly change during heat stress compared to day 0 (PD 28).

Similarly, the splenic mRNA level of *NOX4* was not significantly different between TM and control chicks on day 0 (PD 28) of heat stress. However, the level was significantly lower in the TM group compared to controls after 5 days (PD 33) of heat stress. Within the control group, the mRNA level of *NOX4* was significantly increased after 5 days (PD 33) of heat exposure in comparison with

day 0 (PD 28), while in the TM group, the level did not significantly change during heat exposure in comparison with day 0 (PD 28).

SOD2. The liver mRNA level of *SOD2* was not significantly different between the TM and control groups on day 0 (PD 28) of heat stress. However, *SOD2* levels were significantly lower in TM chicks compared to controls after 3 (PD 31), 5 (PD 33), and 7 (PD 35) days of heat stress. Within the control group, the hepatic mRNA level of *SOD2* was significantly higher after 7 days (PD 35) of heat stress compared to day 0 (PD 28), while, in the TM group, the level significantly increased only after 1 day (PD 29) of heat exposure (vs. day 0 (PD 28)).

The splenic mRNA level of *SOD2* did not significantly differ between the TM and control groups on day 0 (PD 28) of heat stress. Contrastingly, the splenic level was significantly lower in TM chicks compared to controls after 7 days (PD 35) of heat exposure. Within the control group, the splenic mRNA level of *SOD2* was significantly higher after 7 days (PD 35) of heat stress compared to day 0 (PD 28), whereas, in the TM group, the level was significantly higher only after 5 days (PD 33) of heat exposure (vs. day 0 (PD 35)).

3.4. Effect of Post-Hatch Cold Stress on Physiological Parameters of Thermally Manipulated Broilers

Table 4 represents the effects of cold stress for 5 days (PD 32 to 37) on T^C, BW, and BW gain in thermally manipulated broiler chickens and controls. Application of TM significantly decreased the mortality rate during post-hatch exposure to cold stress (control = 5%; TM = 0). In contrast, cold stress did not significantly affect T^C, but, in both the TC and TN subgroups, controls exhibited significantly higher T^C compared to TM chicks. On day 0 (PD 32) of cold exposure, there was no significant change was observed in BW between the control and TM groups. After 5 days (PD 37) of cold stress, the BW of controls was significantly lower in comparison with control chicks exposed to cold stress. Furthermore, cold stress significantly decreased the BW gain in both the control and TM chicks, although the weight gain was significantly lower in controls compared to TM chicks.

Table 4. Effect of post-hatch cold stress for 5 days (post-hatch day (PD) 32 to 37) on cloacal temperature (T^C), body weight (BW), and body weight gain in broiler chickens subjected to embryonic thermal manipulation and controls.

	Normal Conditions		Cold Stress	
	(21 °C; RH 45%–52%)		(16 °C; RH 45%–52%)	
	Control (CN)	TM (TN)	Control (CS)	TM (TS)
T^C (°C)	39.18 ± 0.35 [a]	38.5 ± 0.34 [b]	39.3 ± 0.2 [a]	38.93 ± 0.32 [ab]
BW (g)				
Day 0 (PD 32)	1717.9 ± 137.5 [a]	1834 ± 112.8 [a]	1720 ± 147.5 [a]	1845.5 ± 119.9 [a]
Day 5 (PD 37)	2244.3 ± 134.4 [a]	2281 ± 101.9 [a]	1993.1 ± 131.3 [b]	2179 ± 134.8 [a]
BW gain (g)	526.4 ± 41.1 [a]	447 ± 42.2 [b]	273.1 ± 23.9 [c]	333.5 ± 53.2 [d]

[a–d] within the same row, means ± SD with non-identical superscripts are significantly different.

3.5. Effect of Post-Hatch Cold stress on mRNA Levels of Antioxidant Enzymes in Thermally Manipulated Broilers

Figure 3 represents the effects of cold stress on the mRNA levels of antioxidant enzymes in the liver, spleen heart and breast muscle of broiler chickens subjected to embryonic thermal manipulation.
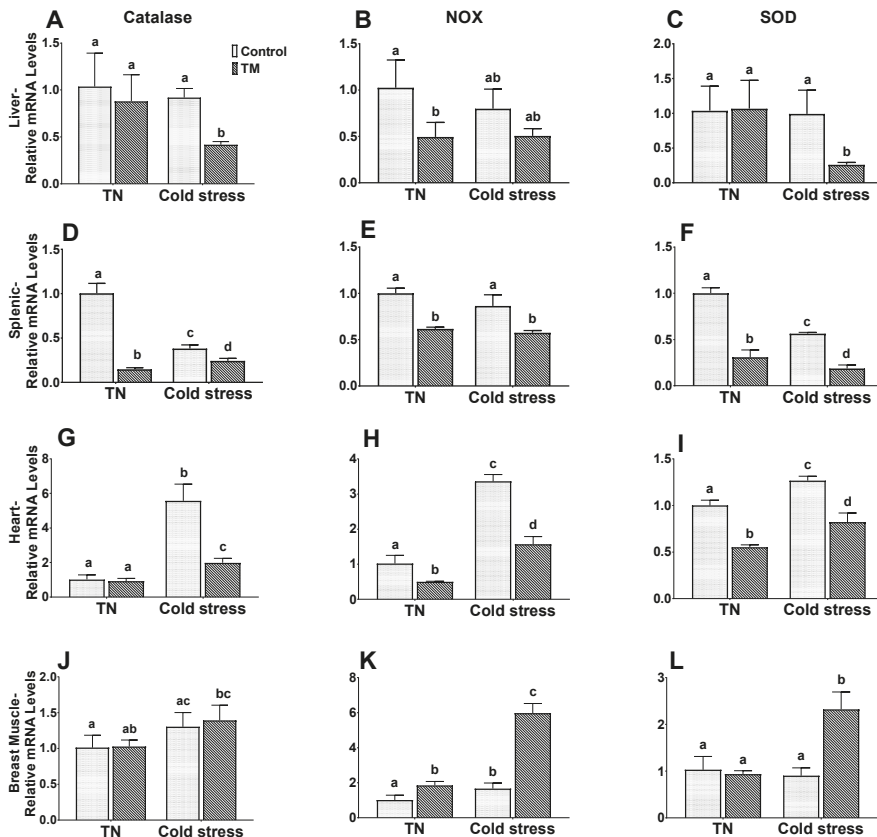
Figure 3. Effect of post-hatch cold stress for 5 days (PD 32 to 37) on the mRNA levels of catalase, *NOX4*, and *SOD2* in the liver (**A**–**C**), spleen (**D**–**F**), heart (**G**–**I**), and breast muscle (**J**–**L**) in TM broiler chickens (n = 5). [a–d] means ± SD with non-identical superscripts are significantly different.

Catalase. TM did not significantly change the cardiac, hepatic, and muscular mRNA levels of catalase in chicks kept under normal environmental temperatures. However, TM significantly decreased the catalase mRNA level in the spleen. Regarding those chicks exposed to cold stress, the TM group possessed a significantly lower mRNA level of catalase in the liver, spleen, and heart compared to controls.

NOX4. In the chicks of the TN subgroup, TM chicks possessed significantly lower splenic, hepatic, and cardiac mRNA levels of *NOX4* compared to controls. Despite this, muscular NOX4 mRNA levels were significantly higher in the TM chicks. Similar results were observed in the chicks exposed to cold stress.

SOD2. No significant changes were seen in the hepatic and muscular *SOD2* mRNA levels between the TM and control groups exposed to normal conditions. However, the splenic and cardiac levels of *SOD2* mRNA were significantly higher in controls compared to TM chicks. After cold exposure, the cardiac, hepatic, and splenic mRNA levels of *SOD2* were significantly higher in controls compared to TM chicks, but the level in breast muscle was significantly higher in the TM group.

4. Discussion

Oxidative damage is caused by excess reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which are a necessary product of aerobic metabolism [35]. Heat stress is a major cause of oxidative damage in poultry, and it is associated with a modulation in the expression of antioxidant genes, including catalase, *NOX4*, and *SOD2* [10,13]. Thermal manipulation (TM) has often been suggested as a viable method of improving the acquisition of thermotolerance in heat-stressed broilers [31,36–38]. However, the effects of TM and subsequent heat challenge on broiler antioxidant capacity has not been extensively explored. Similarly, a dearth of information exists with regard to the effects of cold stress on broiler gene expression, especially within the context antioxidant gene expression. The objective of the present study was two-fold: it aimed to ascertain the effects of embryonic TM on broilers under conditions of post-hatch heat stress as well as cold stress.

During heat stress, the behavior of broilers is altered as they attempt to decrease their body temperature (T^C), resulting in myriad negative effects on performance [39]. In the current study, TM was found to result in significantly lower T^C on post-hatch days (PD) 1 and 37 and higher body weights (BW) on PD 37 compared to controls. Correspondingly, it has often been reported that TM treatments significantly increased broiler BW [37,40,41] and improved their abilities to regulate their T^C in periods of heat challenge [31,32,42]. Lower T^C during heat stress also improved feed conversion ratios in TM broilers, and it has been suggested that the lower T^C in TM broilers is due to slower metabolic rates as a result of the TM treatment [43].

With regard to post-hatch heat stress, our findings show that TM chickens had significantly decreased mortality rates and BT as well as increased BW compared to controls. Heat stress has been extensively reported to affect broiler physiological parameters [8,44,45]. On a similar note, cold-stressed TM chickens had significantly lower mortality rates than cold-stressed controls. Previously, TM has been found to reduce the mortality rates of broilers during heat challenge [46,47]. Contrastingly, one study reported that heat-stressed TM broilers experienced higher mortality rates than their control counterparts [48]. These differences in findings may be attributed to the fact that there is no one single type of TM treatment, and different studies employ different periods and conditions of TM.

The catalase enzyme is found in the majority of aerobic organisms as well as in some obligate anaerobes [49]. Catalase is responsible for the breakdown of hydrogen peroxide (H_2O_2) into oxygen and water, thereby preventing oxidative damage from occurring in a cell [50]. In the present study, catalase expression was significantly modulated in heat- and cold-stressed TM and control chickens. In fact, heat stress resulted in decreased hepatic and splenic catalase expression in TM chickens compared to controls, while cold stress led to significantly lower cardiac, hepatic, and splenic catalase expression levels in the TM group. Compared to controls, heat-stressed TM chickens were previously reported to exhibit decreased catalase mRNA levels [13]. Moreover, broilers were found to exhibit higher levels of catalase activity during acute heat stress, but this antioxidant capacity decreased with age [51]. In female broilers, cardiac catalase activity was reduced after cold stimulation [52].

To maintain homeostasis, the NOX4 enzyme is heavily involved in the oxygen-sensing process, the latter of which causes it to generate significant amounts of ROS [17]. Additionally, *NOX4* over-expression is often associated with oxidative stress in a number of different organs [18,53,54]. In avian muscle cells, ROS production during heat stress and subsequent oxidative damage has been tentatively attributed to *NOX4* up-regulation [55]. In the present study, hepatic and splenic *NOX4* expression levels were significantly lower in heat-stressed TM chickens compared to controls. Similarly, after cold stress, TM chickens exhibited decreased cardiac, hepatic, and splenic but increased muscular *NOX4* expression than that in controls. Hepatic *NOX4* mRNA expression was previously reported to be lower in TM chickens exposed to heat stress compared to controls [13]. In cultured avian cells, heat stress was found to upregulated *NOX4* mRNA expression [55].

The SOD2 enzyme functions to transform the superoxide (O_2^-) radical into hydrogen peroxide and water, and it plays an important cytoprotective role against oxidative stress [56]. Our findings indicate that both heat and cold exposure led to generally decreased *SOD2* expression in several

organs. Compared to controls, heat-stressed TM chickens displayed lower hepatic and splenic *SOD2* expression levels, while cold-stressed TM chickens showed decreased cardiac, hepatic, and splenic *SOD2* expression. Like *NOX4*, however, muscular *SOD2* expression levels were higher in cold-stressed TM chickens compared to their control counterparts. A previous study found that hepatic *SOD2* expression and enzymatic activity were decreased in TM chickens exposed to heat stress [13]. In contrast, another study found that *SOD* mRNA levels in two broiler strains (Cobb and Hubbard) were unaffected by heat stress [57]. Additionally, *SOD2* levels remained unchanged in avian cell cultures exposed to heat stress [55]. The present findings may suggest that lower levels of oxidative *NOX4* expression may lead to lower expression of the anti-oxidative catalase and *SOD2* genes.

Interestingly, mRNA expression levels of the catalase, *NOX4*, and *SOD2* genes in the breast muscle differed from those in the heart, liver, and spleen in cold-stressed TM chickens. Such inter-organ variation in gene expression is to be expected, as expression varies to a larger degree between organs of a single species than between different species [58]. However, in broilers, the breast muscle in particular has been subject to rapid changes in size and conformation over the past few decades due to the artificial selection pressures applied by the commercial poultry industry [59]. This has resulted in a number of abnormalities and myopathies of the breast muscle that is estimated to affect up to 90% of broilers worldwide [60–62]. In fact, broiler breast muscle cells were suggested to constantly undergo hypoxic stress, as the transcriptional profiles of non-stressed broiler breast muscle and heat-stressed layer breast muscle were similar [63].

A number of strengths can be found in the current study. All samples were taken from male Cobb chicks in order to reduce inter-strain and inter-sex genetic variation. Moreover, any non-experimental stress was minimized by ensuring that the rearing and experimental rooms were in close proximity to one another. However, there are some limitations of the present study. Firstly, the effect of TM on the developmental parameters of broiler embryos was not investigated, requiring future research. Secondly, the oxidation levels of lipids, proteins, and DNA in different tissues must still be measured in order to ascertain the final balance of catalase, *NOX4*, and *SOD2* expression. Lastly, the exact impact of TM on embryonic mortality was not considered, which mandates future lines of research in this context.

5. Conclusions

Our findings indicate that TM at 39 °C and 65% RH for 18 h/day from days 10 to 18 of embryonic development might result in positive long-lasting effects on broiler antioxidant capacity. Future research should focus on the effects of TM and subsequent thermal challenge on various types of broiler muscle, as the expression dynamics of the breast muscle was found to differ from those of other organs.

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