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Gene Network and Pathway Analysis of Transcriptional Signatures Characterizing Sole Ulcer and Digital Dermatitis in Dairy Cows

Roshin Anie Mohan
South Dakota State University

GENE NETWORK AND PATHWAY ANALYSIS OF TRANSCRIPTIONAL SIGNATURES CHARACTERIZING SOLE ULCER AND DIGITAL DERMATITIS IN DAIRY COWS

BY

ROSHIN ANIE MOHAN

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DISSERTATION ACCEPTANCE PAGE

Roshin Anie Mohan

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Johan Osorio
Advisor Date

Vikram Mistry
Department Head Date

Nicole Lounsbery, PhD
Director, Graduate School Date

This dissertation is dedicated to my family.

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LIST OF ABBREVIATIONS

BCS	Body condition score
BP	Biological processes
CBT	Coronary band
CC	Cellular Component
CK	Cytokeratin
DD	Digital dermatitis
DE	Differentially expressed
DEG	Differentially expressed genes
EGF	Epidermal Growth Factors
FC	Fold change
FDR	False discovery rate
FLIR	Forward-looking infrared
FRAP	Ferric reducing ability of plasma
GGT	Gamma glutamyl transferase
GMCSF	Granulocyte macrophage colony stimulating factor
GO	Gene Ontology
GOT	Glutamic-oxaloacetic transaminase
HC	Healthy cows
Hp	Haptoglobin
ICS	Intracellular Cementing substance
IFNG-γ	Interferon gamma
IL	Interleukin

KRT	Keratin
KRTPA	Keratin associated protein
LPS	Lipopolysaccharide
MF	Molecular Function
MMP	Matrix metalloproteases
MPO	Myeloperoxidase
negAPP	Negative acute phase protein
NF-kβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen associated molecular patterns
PCA	Principal component analysis
PCR	Polymerase chain reaction
PON	Paraoxonase
posAPP	Positive acute phase protein
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
ROM	Reactive Oxygen Metabolites
rRNA	Ribosomal Ribonucleic acid
RT-qPCR	Real-time quantitative polymerase chain reaction
SAA	Serum amyloid A
SD	Standard deviation
SE	Standard error
SU	Sole ulcer
TB	Total bilirubin

TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TPM	Transcripts per kilobase Million
VFA	Volatile fatty acids
WLD	White line disease

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ABSTRACT

GENE NETWORK AND PATHWAY ANALYSIS OF TRANSCRIPTIONAL SIGNATURES CHARACTERIZING SOLE ULCER AND DIGITAL DERMATITIS IN DAIRY COWS

ROSHIN ANIE MOHAN

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This study aimed to evaluate the transcriptomic profile of both corium tissue from lactating dairy cows experiencing sole ulcers and skin lesions from lactating dairy cows diagnosed with digital dermatitis (DD). Hoof biopsies were performed in multiparous Holstein dairy cows selected based on their condition as clinically healthy or diagnosed with sole ulcers (n=7/group). Similarly, skin biopsies were taken from the center of active (M2/M4.1) DD lesions or non-active (M0/M4/M1) from multiparous Holstein dairy cows (n = 7/group) to assess the impact of DD on the skin transcriptome via RNA-seq analysis. All RNA samples were sequenced using Illumina, NovaSeq S4 at the University of Minnesota Genomics Center. Principal component analysis (PCA) and analysis of variance for each gene was calculated using the DESeq2 package in R to determine the differentially expressed (DE) genes, fold change (FC), and *P*-value based on the comparison of sole ulcer (SU) over healthy cows (HC) and digital dermatitis (DD) over apparently healthy (HDD). The DE genes with at least ± 2-fold change were retained for an enrichment pathway analysis using gene ontology (GO) terms and the enrichGO function in the Clusterprofiler package in R. The FDR cut-off value was set to < 0.01. The PCA analysis clearly discriminated the hoof transcriptomes between HC and SU cows. The number of DE genes in SU and HC was 10,274 ± 22 and 10,289 ± 36

(mean±SD), respectively. Several genes coding for keratins (e.g., *KRT34* and *KRT85*) were downregulated (FC < -15) in SU cows, while the highest upregulated (FC = 10.9) gene in SU was *IL6*. The GO analysis showed significant downregulation of GO terms in SU cows compared to HC in the Cellular Component category, including intermediate and keratin filaments and intermediate filament cytoskeleton. Upregulated genes in SU cows resulted in a significant enrichment of GO terms in the Biological Process category, including extracellular matrix organization and vasculature development, and GO terms in the Cellular Component category such as extracellular matrix and cell surface. The GO terms enrichment across biological process, cellular components, and molecular function categories were highly involved in the keratinization process, inflammation, and transcriptional regulation. These processes are crucial for the development of sole ulcers causing lameness in dairy cows. Similarly, in the case of digital dermatitis also, the PCA analysis clearly discriminated the skin transcriptome between DD cows and NA. Genes coding for several keratins-associated proteins, including *KRT34*, *KRTAP21-1*, and *KRTAP4-9,* were downregulated in DD cows. The GO terms associated with downregulated genes in the Cellular Component category were mainly correlated with collagen-containing extracellular matrix and keratin filament, while GO terms in the Biological Process category were related to humoral immune response and biomineral tissue development. Upregulated genes were highly enriched in the Molecular Function GO terms of cell-adhesion and skin development as well as intermediate filament cytoskeleton in the Cellular Component category. Overall, this skin transcriptome evaluation comparing NA and DD cows identified key biological pathways in which there was a dysregulation in the skin extracellular matrix, encompassing the

keratinization process, collagen binding, and immune response caused by DD lesions. This transcriptomic dataset captures the molecular adaptations in the development of sole ulcer and bovine DD. In the future, this information could be further utilized to generate nutritional and management strategies to reduce this infectious disease.

INTRODUCTION

Lameness is a clinical symptom recognized by a change in locomotion with associated lesions in the pelvic limb (Langova et al., 2020). Lameness is considered one of the top three of the costliest diseases in the dairy industry, besides fertility problems and mastitis (Ranjbar et al., 2016). Lameness is also a major concern in dairy cattle in terms of animal welfare (Huxley, 2012), and it can lead to economic losses due to reduced productive and reproductive performance (Nocek, 1997). Lameness is an animal wellbeing concern as many dairy producers are unaware of the proper management and treatment.

The two most common causes of lameness include sole ulcers and digital dermatitis (Green et al., 2002; Bruijnis et al., 2010). The sole ulcer is a noninfectious condition predisposed by metabolic conditions and physical wear. In contrast, digital dermatitis is an infectious lesion (Bruijnis et al., 2010). Both of these conditions cause animal welfare concerns (Gomez and Cook, 2010). Metabolic disorders like ruminal acidosis will eventually lead to excessive production of lactic acid, histamines, and release endotoxins, which deteriorates the hoof health (Langova et al., 2020). The sole ulcer will cause the animals to take a cow-hocked position to compensate for the pressure on the hoof (Greenough, 1985). The cows infected with digital dermatitis also exhibit lameness depending on the severity of infection (Read and Walker, 1998).

There are several predisposing factors for claw lesions, including ruminal acidosis, coriosis/laminitis, activation of metalloproteases, and changes in hormone levels (relaxin) and estrogen in the peripartum period (Lischer et al., 2002; Shearer et al., 2015). Cows around parturition are highly susceptible to sole ulcers because of hormone level changes (Nocek, 1997; Räber et al., 2004; Knott et al., 2007).

Only a few studies are currently available on the transcriptomic alterations due to sole ulcer and DD. Alterations in the blood biomarkers in response to sole ulcer and DD conditions are also scarce. An inflammatory pathway involved in the pathogenesis of DD has not been elucidated (Vermeersch and Opsomer, 2019). Transcriptomic data revealed via RNA-seq analysis can provide deeper insights into the changes in expression patterns in disease conditions like sole ulcer and DD. In addition, RNA-seq data can provide novel molecular targets for nutritional interventions to reduce sole ulcer incidence and prevalence in dairy herds. Transcriptomic data on DD can demonstrate how the permeability and keratinization in paracellular epithelial cells in skin are being affected. Similarly, in the case of sole ulcer, these data can provide details about the progression of the disease. Enrichment pathway analyses will help understand these findings better and identify molecular targets for nutritional or management interventions.

Therefore, this study's overall goal was to evaluate the physiological alterations in cows with sole ulcer and digital dermatitis via transcriptomics related to inflammation, keratinization, and hoof conformation in correlation with the blood biomarkers analysis.

CHAPTER 1. LITERATURE REVIEW

Overview of lameness in dairy cows

Lameness is a clinical symptom recognized by a change in locomotion with associated lesions in the pelvic limb (Langova et al., 2020). It has been observed that more than 90% of the lesions occur in the distal part of the hind limb (Sadiq et al., 2017; Griffiths et al., 2018; Daros et al., 2019). Lameness is considered one of the top three of the costliest diseases in the dairy industry, besides fertility problems and mastitis (Ranjbar et al., 2016). Lameness is also a major concern in dairy cattle in animal welfare (Huxley, 2012). The prevalence of lameness is reported to be between 25-55% in dairy farms, and it can lead to overall productive and reproductive losses in dairy cattle. The most commonly employed method to detect lameness in cows is by observing dairy cows' walking gait(Flower and Weary, 2006). There are different systems to score lameness (Sprecher et al., 1997). In these scoring systems, the raking of animals is done from score 1 to score 5, based on the presence or absence of some behaviors indicative of lameness (Sprecher et al., 1997). Some examples of lameness behaviors include arched back, poor tracking up with short strides, asymmetric gait, inability to bear weight on one or more limbs(Flower and Weary, 2006). According to Denis-Robichaud et al. (2020), a cow is lame if at least two of the lameness behaviors were observed in one-minute observation. Flower and collaborators established a method to assess the severity of such condition utilizing a locomotion score based on a scale (1 = normal, symmetrical gait and flat back to 5 = lame, asymmetric gait, extremely arched back) (Flower and Weary, 2006).

Lameness leads to economic losses due to reduced productive and reproductive performance (Nocek, 1997). Lameness is an animal wellbeing concern as many dairy

producers are unaware of the proper management and treatment. Cow behavior has been correlated with lameness, as reported by Gomez and Cook (2010) that the lame cows spent more time lying down and standing up. The time spent eating will also be reduced, and 27% of the costs of production diseases are due to the problems with lameness (Burger, 2017).

The two most common causes of lameness include sole ulcers and digital dermatitis (DD)(Green et al., 2002; Bruijnis et al., 2010). The sole ulcer is a noninfectious condition predisposed to metabolic conditions and physical wear. In contrast, DD is an infectious lesion (Bruijnis et al., 2010). Both of these conditions cause animal welfare concerns (Gomez and Cook, 2010). Metabolic disorders like ruminal acidosis will eventually lead to excessive production of lactic acid, histamines, and release endotoxins, which deteriorates the hoof health (Langova et al., 2020). The sole ulcer will cause the animals to take a cow-hocked position to compensate for the pressure on the hoof (Greenough, 1985). The cows infected with DD also exhibit lameness depending on the severity of infection (Read and Walker, 1998).

As discussed previously, lameness is a major welfare concern in dairy cattle, and it is considered one of the costliest diseases affecting dairy cattle. It leads to major productive and reproductive losses. Hence the two major causes of lameness, sole ulcer and DD, are reviewed here.

Hoof development and hoof health in dairy cattle

Keratin is fibrous or amorphous proteinaceous material produced by epidermal cells (Steinert and Idler, 1975; Tomlinson et al., 2004). The hoof of dairy cattle is made of keratin (Steinert and Idler, 1975). The skin, hair, hoof, and horn are also formed by

keratinization and programmed cell death (cornification) (Fraser and Macrae, 1980). The distal part of the limb is protected by the hoof, formed by the epithelial layer's keratinization and modification of the dermis (Langova et al., 2020). The epidermal cells responsible for the synthesis of keratin are called keratinocytes (Dale et al., 1985). The hoof is composed of a wall and sole, and the junction between them is called white line (Kempson and Logue, 1993). The dermis or corium is the supportive living layer of the epidermis, and it forms collagen and elastin. The epidermis' four distinct layers are stratum basal, stratum spinosum, stratum granulosum, and stratum corneum. The cell division in the basal layer generates horny tissue by keratinization and cornification (Tomlinson et al., 2004). Stratum spinosum is several layers thick, and the continuous replacement of cell contents by a rapid rate of keratin synthesis (Kempson and Logue, 1993). The synthesis of the intercellular cementing substance (ICS), a lipid-rich extracellular substance, helps the keratinocytes be glued together, and it is the final step of keratinization (Kempson and Logue, 1993). Therefore, lipids and especially long-chain fatty acids (> 24 C) are crucial for bovine claw integrity. Because of the role of long-chain fatty acids in the bovine claw, biotin is a vital B vitamin for the carboxylation reactions for fatty acid elongation (Langova et al., 2020).

Keratins are mainly classified as α and β keratins (Spearman, 1966). The secondary structures like α helices and β sheets are the internal supportive structures of keratins and are used to classify them (Lodish et al., 1995). The α keratins are mainly found in mammals in the wool, hair, hooves, nails, horns, and the outer layer of the skin (i.e., stratum corneum) (Chen et al., 2012). The β keratins are mainly found in avian and reptilian tissues like feathers, claws, and beaks of birds and scales and claws of reptiles

(Chen et al., 2012). Based on the biochemical properties, the keratins are separated to type I (acidic) and type II (basic to neutral) keratins (Schweizer et al., 2006).

Keratin proteins are expressed systematically as the differentiation proceeds. Bovine keratins include the cytokeratins (CK) CK1, CK6, CK7, CK10, CK14, and CK16 (Kvedar et al., 1986). The CK 4, CK5/6, CK10, and CK14 are lightly expressed in bovine claw tissue (Hendry et al., 2001). The CK5/6 and CK14 were present in the basal epidermis of healthy tissue and CK10 in supra basal layers (Hendry et al., 2001). The keratinization process takes place in epidermal keratinocytes by bundling of keratin filaments to form regular macromolecular arrays, subsequently by the enucleation of cells (Budras et al., 1989; Kempson and Logue, 1993). The rate of synthesis of keratins is altered in claws affected by a diseased or condition. For example, CK16, which was not usually found in healthy tissue, was found in cows affected with a sole ulcer (Hendry et al., 2001). In addition, Hendry et al. (2001) observed a change in the expressed cytokeratin profile in cows with sole ulcerated tissues.

Dietary nutrients such as minerals, vitamins, amino acids (AA), and fatty acids play a substantial role in the structural integrity of the hoof (Mulling et al., 1999; Van Marle-Köster et al., 2019; Langova et al., 2020). These components are involved in the keratinization process, ensuring healthy horn growth and structural binding of the keratin proteins (Tomlinson et al., 2004; Van Marle-Köster et al., 2019; Langova et al., 2020). The nutrients that are essential for the process of keratinization include AA (e.g., cysteine and methionine), fatty acids (e.g., linoleic and arachidonic acid), macro minerals (e.g., Ca), trace minerals (e.g., zinc), and vitamins (e.g., biotin)(Mulling et al., 1999). Imbalances in the availability of trace minerals such as (e.g., Zn, Cu, Se, and Mn),

vitamins (e.g., A, D, and Biotin), and the other nutrients can lead to fragile horn formation (Langova et al., 2020).

It has been observed that dietary lipid may affect lameness due to an association between the fat layer of the heel and nutritional status (Lean et al., 2013; Alvergnas et al., 2019). The digital cushion in cows will be more vulnerable if cows are fed a low-fat diet and a lower concentration of antioxidants like vitamin E (Lean et al., 2013). Potential regulators of epidermal keratinization, include hormones (e.g., hydrocortisone, prolactin, and thyroid), growth factors [e.g., epidermal Growth Factors (EGF)], transforming growth factors, and micronutrients (e.g., vitamin D3 and Retinoic acid) (Ekfalck et al., 1990).

Minerals play an important role in determining the strength of the hooves due to their involvement in biochemical pathways associated with keratin synthesis (Tomlinson et al., 2004). Trace minerals are important for keratinization because they are essential co-factors for many enzymes, including the enzymes involved in keratin production. Among the trace minerals, Zn plays a crucial role in hoof formation by performing catalytic and structural, and regulatory functions in keratinization (Zhao et al., 2014; Langova et al., 2020). The Cu is also an essential trace mineral for activation of the Thiol oxidase enzyme, which helps in the formation of chemical bonds between keratin fibers (Zhao et al., 2014). Also, Cu regulates the maturation of keratins (Noori et al., 2013). Manganese has an indirect role in the keratinization process (Zhao et al., 2014). Selenium helps in the protection of the sole from oxidative damage (Lean et al., 2013).

Macrominerals such as Ca has a significant role in the keratinization process, especially in the final stage of mature horn cell formation (Zhao et al., 2014). Insufficient

Ca during the maturation process of a keratinocyte, can predispose to the formation of a dyskeratotic horn (Langova et al., 2020). Other macro minerals such as P and Mg also play an integral part in the building of healthy hoof and bone (Langova et al., 2020).

Vitamins such as A, D, and E play a critical role in structure and quality of hoof (Burger, 2017). Biotin or vitamin B7 also plays a significant role in keratinization (Singh et al., 2019). Biotin is also called ad vitamin H, and it acts as a cofactor in gluconeogenesis, fatty acid synthesis, and protein synthesis (Randhawa et al., 2008). Biotin also plays a role in the production of lipids of ICS, enabling the growth of resistant horns (Lean et al., 2013). The corium is the dermal layer located just below the epidermis, and it is the real living layer of skin (Tomlinson et al., 2004). According to Mulling and Lischer (2002), changes in the dermal vascular system during acute laminitis will lead to disruption in cell differentiation, leading to inferior horn production.

It is evident from the above studies that hoof is formed from an amorphous proteinaceous material produced by the epidermal cells, and the proper supply of nutrients is needed for the appropriate development of keratins. Any disruption in the supply of nutrients to the extremities will eventually lead to the development of inferior hoof conformation and eventually associated with lameness.

Nutritional factors leading to laminitis in dairy cattle

Nutritional factors will predispose laminitis in dairy cows, especially during the transition period. Feeding of highly fermentable carbohydrate diet is a typical example (Vermunt and Greenough, 1996). Rumen acidosis usually occurs following the feeding of cows with a highly fermentable carbohydrate diet (Nocek, 1997; Burger, 2017). This will result in an increased rate of production of volatile fatty acids in the rumen, which will

decrease the rumen's pH (Nocek, 1997). Normal ruminal fermentation yields 65% acetate, 20% propionate, and 15% butyrate (Church, 1988). Readily fermentable carbohydrate sources will increase the propionate proportion by around 40%, with a drop in acetate. The decreased pH in the rumen will increase the population of *Streptococcus bovis* (Church, 1988). This, in turn, will produce more lactate which will result in a further drop in rumen pH. This will damage the ruminal epithelial wall resulting in the escape of the bacterial endotoxins and bacteria from the rumen to the blood, causing sepsis and causing vascular constriction in extremities leading to the development of laminitis (Nocek, 1997). Lipopolysaccharide (LPS) released from the cell walls of gram-negative bacteria stimulates histamine release causing vasoconstriction and insufficient blood supply to hoof dermis, causing the inadequate supply of oxygen (Blowey and Chesterton, 2012). Further, it will result in systemic acidosis resulting in the reduced oxygen-carrying capacity of the blood. This will reduce the supply of oxygen to the extremities, including limbs, contributing to laminitis in dairy cows (Nocek, 1997).

In addition to the above, other nutritional factors such as low fiber diets will eventually reduce the ruminal pH. With the increased consumption of carbohydrates, fiber consumption, chewing time, and saliva production decrease, resulting in a subsequent reduction in the pH of the rumen (Burger, 2017; Langova et al., 2020). A decrease in insulin sensitivity in early lactation could compromise keratin production due to reduced glucose and AA uptake (Hendry et al., 1999). The partial hydrolysis of inulin-type fructan, one of the fructans found in pasture grasses, yields a carbohydrate called oligofructose (Pavis et al., 2001). The overload of oligofructose also leads to laminitis in dairy cows (Danscher et al., 2009).

High levels of rumen degradable protein sources in the ration can increase lameness risk (Burger, 2017). The reasons suggested are allergic histaminic reactions to certain types of proteins or a link between high protein supplementation and protein degradation (Nocek, 1997) . However, dietary protein-induced lameness in dairy cattle is still unclear (Nocek, 1997) . The excess protein level in the cow's ration can also be problematic due to faster horn growth (Gelasakis et al., 2019).

The supply of readily fermentable carbohydrates will lead to a decrease in pH of the rumen leading to an increase in the population of *Streptococcus bovis.* This will eventually lead to a further drop in ruminal pH leading to ruminitis and escape of bacterial endotoxins leading to laminitis.

Sole ulcer in Dairy Cattle

An ulcer is a full-thickness defect in the epithelium exposing the underlying corium (Shearer et al., 2015). The very first description of sole ulcer dates to 1920 by Rusterholtz. Later, the scientific designation was given to this condition as *Pododermatites circumscripta*. Depending on the area in which ulcers appear, different terms have been coined to explain the region of the lesion, such as toe ulcer (zone 5), sole ulcer in the typical place (zone 4), and heel ulcers (zone 6). There are different stages in the development of sole ulcer-like *Pododermatitis hemorrhagica* (first stage), which is identified by a hemorrhage in the corium without a break in the horn. Then, in the second stage, there will be a perforation in the corium (*Pododermatitis perforata*). The final stage with trauma and granular tissue formation is termed *Pododermatitis circumscripta* (Shearer et al., 2015).

Sole ulcers are most commonly found on the hind lateral claw, frequently bilateral (Amstel and Shearer, 2006). The typical location of sole ulcers is at the junction of the heel and sole in the hind feet (Manske et al., 2002). The ulcer is not evident in some cases, but there will be hemorrhage beneath the sole, or the sole can be yellow and soft (Manske et al., 2002).

There are several predisposing factors for claw lesions, including rumen acidosis, coriosis/laminitis, activation of metalloproteases, and changes in hormone levels such as relaxin and estrogen in the peripartum period (Lischer et al., 2002; Shearer et al., 2015). Cows around the time of parturition are highly susceptible to sole ulcers because of hormone levels changes (Räber et al., 2004). Sole ulcer results in reduced reproductive and milk yield performance while increasing culling rate in dairy farms (Pavlenko et al., 2011).

The prevalence of sole ulcers was directly associated with the thickness of the digital cushion, which is positively associated with the body condition score of the animals (Bicalho et al., 2009; Iqbal et al., 2016). Holstein dairy cows are the most susceptible animals (Enevoldsen et al., 1991). The sole ulcer occurrence in a given lactation increases the risk of developing a sole ulcer in the subsequent lactation (Enevoldsen et al., 1991). Sole ulcer pathogenesis is based on the injury to the corium in the third phalanx resulting in a failure to provide suspensory aid and cushion to the claw.

The weakening of this tissue may be of biochemical origin, e.g., lactogenic hormones like prolactin and hydrocortisone, which have been observed to decrease the synthesis of proteins in bovine claw explants (Hendry et al., 1997).

The digital cushion composition is important in the pathogenesis of sole ulcers (Lischer et al., 2002). The digital cushion in heifers has loose connective tissue with a small amount of fat consisting of saturated fatty acids. In contrast, older cows' digital cushion has more fat consisting mainly of monosaturated fatty acids (Lischer et al., 2002). In cows with sole ulcers, both corium and digital cushion are thinner with less fat (Lischer et al., 2002; Amstel and Shearer, 2006). This might be the reason for the greater incidence of sole ulcers in heifers on the concrete floor.

The inflammatory-based injury to the dermal-epidermal interface occurs in three phases. In the first phase, circulatory changes occur, leading to tissue hypoxia, edema, and activation of matrix metalloproteinases resulting in collagen degradation (Lischer et al., 2002). Phase two is characterized by the sinking of the 3rd phalanx and dermis compression (Amstel and Shearer, 2006). The subsequent changes are caused by nutrient supply interference resulting in other abnormalities in cell proliferation and differentiation. In the third phase, hemorrhages occur, which leads to full-thickness horn defects.

Sole hemorrhage is the early clinical sign of sole ulcer, although it becomes visible several weeks after the initial injury (Bergsten and Herlin, 1996; Amstel and Shearer, 2006). The hemorrhagic lesions are most commonly found in the junction of the heel and sole. The animals affected with sole ulcers exhibit a typical posture with the hind foot placed well behind or with a ‘cow hocked’ stance in order to place the weight on the medial claws. Animals with a severe sole ulcer will have serious locomotion problems and will be reluctant to move, resulting in low feed intake and weight loss (Greenough, 1985).

The keratin synthesis is disturbed as the epidermal cells overlying the ulcer become dyskeratotic. Similarly, the structure and synthesis of ICS will also be disturbed (Amstel and Shearer, 2006). The most common method employed to treat sole ulcer includes the elevation of the sound claw by a claw block, which helps to relieve the weight from the affected claw (Amstel and Shearer, 2006). The main strategies to prevent sole ulcers include nutrition and management. The hormonal changes occurring during the peripartal period also predispose dairy cows to sole ulcers (Amstel and Shearer, 2006).

Digital dermatitis in dairy cows

Digital dermatitis is considered one of the most common skin conditions leading to significant economic loss worldwide (Bruijnis et al., 2010; Cha et al., 2010). The average cost of DD per case averages $132.96, and the subclinical DD cases cost around $269 (Cha et al., 2010).

The usual dermatitis site is between the heel bulbs in the plantar aspects of the foot (Read and Walker, 1998; Holzhauer et al., 2006; Pavlenko et al., 2011). Digital dermatitis lesions appear more on hindlegs than forelegs (Read and Walker, 1998). The most important clinical sign is lifting the foot and walking on the tip of the toe (Blowey and Sharp, 1988).

It has been observed that the incidence of DD is more common in cows housed in dry lots than the cows with access to pasture (Wells et al., 1999). Textured floors also show decreased DD incidence compared to smooth floors (Wells et al., 1999). Because of the pathogenic etiology of DD, introducing new cows to the herd may cause an increase in the incidence of DD if such cows are carriers of pathogens causing DD (Wells et al.,

1999). Similarly, improper cleaning of hoof trimming instruments can be a vector for spreading pathogens causing DD (Wells et al., 1999). Primiparous cows are more prone to DD, which might be due to the suboptimal immune response due to high energy expenditure towards lactation (Read and Walker, 1998).

The spread of DD between animals is mainly attributed to bacterial burden on useful equipment and stall (Wells et al., 1999; Somers et al., 2005; Holzhauer et al., 2006). Individual variability has also been observed among cows in susceptibility to DD (El-Shafaey et al., 2017). The degree of susceptibility varies with skin characteristics, hoof conformation, and immune system properties (Scholey et al., 2013).

Pringle et al. (2009) and Döpfer et al. (1997) proposed a clinical-M stage scale to classify different stages of DD, and it was slightly altered by Berry et al. (2012), going from M0 to M4.1. Döpfer et al. (1997) classified the clinical of DD in 5 different stages M0-M4, mainly based on visual appearance of the lesions. M0 represents normal skin without any DD lesions. M1 is characterized by an early small pink area of 4.2 cm diameter. M2 represents an acute classical ulcerative lesion of > 2 cm diameter. M3 represents the healing stage, covered by the scab, and M4 is the late chronic stage which shows dyskeratosis or a proliferative growth or both.

Etiology

DD has a complex etiology (Choi et al., 1997). Digital dermatitis is contagious and is caused mainly by *Treponema* sp. (Pringle et al., 2009). The causative agent of DD is thought to be *Treponema denticola*, *T. phagedenis*, or *T. medium* (Willis et al., 1999). It was further confirmed that these agents cause DD by 16s rRNA sequencing (Evans et al., 2008). Later on, several other *Treponema* sp. were isolated from active lesions of DD

(Klitgaard et al., 2013) and some variants are difficult to treat *(Klitgaard et al., 2013).* The severity of the infection will increase as the number of species formed at the infection site increases (Klitgaard et al., 2013).

Various species of bacteria like *Fusobacterium necrophorum, Dichelobacter nodosus, Bacteroides* sp., *Campylobacter* sp., *Mycoplasma* sp., *Borrelia* sp. and *Porphyromonas* spp. have been found in DD lesions (Blowey et al., 1994; Döpfer et al., 1997). These might be involved in the pathogenesis or a shift in *Treponema* sp's phylotypes (Zinicola et al., 2015).

Several bacteria species such as *T. denticola*, *T. phagedenis*, *T. medium*, and *T. putidum* are predominant in the active lesions, while *Porphyromonas* spp., *Alkaliphilus crotontoxidans, Soehngenia saccharolytica*, and *Telmatospirillum siberiense* are found in advanced DD lesions from M3-M4 (Zinicola et al., 2015). The *Treponema* sp. found at different depths of the skin also varies. While *T. phagedenis*-like and *T. denticola*-like are situated deep in the epidermis, *T. vincentii* can be detected more superficially (Klitgaard et al., 2008). Temperature may play a role in the *Treponema* sp. profile in a given DD lesion. For instance, the optimal temperature for *T. phagedenis* growth was about 40˚C. The temperature of the animal's hoof is around 22˚C, which increases to around 6˚C during DD (Stokes et al., 2012; Wilson-Welder et al., 2013).

Immunity

The typical lipopolysaccharide found in gram-negative bacteria seems to be absent in *Treponema* sp. while some have a functionally similar molecule lipo-oligosaccharide (LOS), which implicates weaker stimulation of immunity (Refaai et al., 2013).

Innate immunity displays an upregulated continuous response in DD, whereas the adaptive immune response shows a delayed onset (Refaai et al., 2013). The epidermal hyperplasia, along with the massive surge of interleukin-8 (IL-8), occurs throughout the clinical stages of DD, which could be the reason for the continuous activation of the innate immune system (Refaai et al., 2013). There will be an increase in keratinocyte-derived IL-8 in M2 and M3 lesions of DD (Refaai et al., 2013; Watts et al., 2018). IL-8 is an early response pro-inflammatory chemokine which stimulates neutrophils (Evans et al., 2014). The antibody levels also vary widely within and between affected animals (Gomez et al., 2004; Moe et al., 2010). This could be due to the dynamic poly bacterial burden during DD development (Krull et al., 2014).

Biomarkers associated with lameness

The acute-phase proteins are common biomarkers of inflammation because their blood concentration changes in response to inflammation (Schneider, 2015). There are two types of acute-phase proteins, positive acute-phase proteins (posAPP), which increase in response to inflammation, and negative (negAPP), decreasing during inflammation (Bagga et al., 2016). Serum Amyloid A (SAA) is an important posAPP related to inflammation (Bagga et al., 2016). SAA1 and SAA2 are mainly synthesized in the liver, and SAA3 is the main extrahepatic isoform (Uhlar and Whitehead, 1999). Bagga et al. (2016) observed a significant increase (4.6 times) of SAA in lame crossbred dairy cattle compared with healthy cows. Haptoglobin is a posAPP, and in cattle, serum haptoglobin concentration increases in the case of acute, subacute, and chronic inflammation (Alsemgeest et al., 1994). O'Driscoll et al. (2015) found that lame cows tended to have a higher haptoglobin level than healthy cows.

Matrix Metalloproteases are involved in extracellular protein degradation (Almeida et al., 2007). The basal membrane disruption could be partially ascribed to extracellular proteolysis from matrix metalloproteases (MMPs). Connective tissue degradation in the claw dermis shows the involvement of metalloproteases (Hendry et al., 2001). MMPs such as MMP2 and MMP9 are known to degrade extracellular collagen (Nagase and Woessner, 1999; Hendry et al., 2001). However, O'Driscoll et al. (2015) did not find any difference in expression of MMP9 between the lame cows and healthy cows. MMP13, also called Collagenase-3, also plays a pivotal role in collagen degradation (Almeida et al., 2007). Almeida et al. (2007) found an increased expression of *MMP13* in cows with impaired locomotion. O'Driscoll et al. (2015) also found an increased expression of *MMP13* in cows diagnosed with a sole ulcer. Collagenase-1 or Matrix Metalloprotease-1 (*MMP1*) is found to increase in all forms of cutaneous wounds (Sudbeck et al., 1997).

The cytokines IL-1 and IL1-β are known to increase the expression of growth factors, which drive the proliferation of keratinocytes in the epidermal cell layers. The changes in the expression of cutaneous cytokines such as IL-1 and IL-6 and tumor necrosis factor (TNFα), in turn, will result in changes in the expression of keratinocyte growth factor (GF) (Chedid et al., 1994). Granulocyte macrophage colony-stimulating factor (GMCSF) and other cytokines will lead to a disturbance in keratin proliferation and keratinization (Chedid et al., 1994).

High concentrate diets have been observed to increase *IL1B, IL6, TNFA,* and *MMP2* mRNA expression in lamellar tissues (Langova et al., 2020). High-grain diets have been reported to lead to changes in the composition of bacteria leading to LPS in

blood, activating the inflammatory response in the lamellar tissue and eventually lamellar damage (Bergsten et al., 2003; Burger, 2017; Zhang et al., 2018). O'Driscoll et al. (2015) did not find any difference in expression of *IL2*, *IL4*, *IFNG*, or *TNFA* between lame and healthy cows.

In a study by Refaai et al. (2013) in cows with DD, an upregulation of the proinflammatory cytokines *IL1B* and *IL6* were observed, reaching a peak at the M2 stage, without significant differences between the other stages of the disease. A 328-fold upregulation of *IL8* was observed during M2 stage and 190-fold upregulation during the M3 stage (Refaai et al., 2013). The expression level of *IL10*, *IL17*, and TNFA was not affected. In the same study, Refaai et al. (2013) reported downregulation of *IL4*, *IL5*, *IL13*, and chemokine receptor-3 (*CCR3*) in cows with DD compared with healthy cows.

The acute-phase proteins, SAA and haptoglobin, seem to increase in lame cows. Similarly, certain metalloproteases such as MMP13, which plays a pivotal role in the degradation of collagen, increases in cows with impaired locomotion. A high-grain diet in dairy cows was associated with an increase in proinflammatory cytokines like *IL1B* and *IL6*. Similarly, DD lesions upregulated *IL1B* and *IL6* in the M2 stage, while upregulated *IL8* was observed in M2 and M3 stage.

Molecular factors associated with sole ulcer and digital dermatitis

Molecular factors associated with sole ulcer

The cytokeratins, CK4, CK5/6, CK10, and CK14, are the most abundant in healthy bovine tissue, as observed by Hendry et al. (2001), while CK16 was present in diseased claws but not in healthy. It was reported by (Osorio et al., 2012) that the damage

caused to the corium during sole ulcer and subclinical laminitis affects the expression of cytokeratins while triggering oxidative stress and inflammation (Hendry et al., 2001).

An increased protein synthesis (including keratin) and cellular proliferation were observed in ulcerated cows (Hendry et al., 2001; Osorio et al., 2016), also observed a downregulation of keratin 5 (*KRT5*) between healthy and ulcerated bovine tissues. However, there was a significant presence of KRT5 protein in basal and supra basal layers of necrotic tissue instead of basal layers only, as usually observed in the healthy tissue (Hendry et al., 2001). This observation indicates that tissue damage results in abnormal distribution of cytokeratins throughout the corium (Osorio et al., 2016)

It has also been found that the genes related to inflammation and oxidative stress like *STAT3* (Signal transducer and activator of transcription 3), *MYD88* (Myeloid differentiation primary response 88), *SOD2* (Superoxide Dismutase 2), and *TLR4* (Toll-like receptor 4) were more abundant in the corium tissue of lame cows(Osorio et al., 2016).

Molecular factors associated with digital dermatitis

Toll-like receptors (TLRs) bind with specific pathogen associated molecular patterns (PAMPs) on the pathogens' surface. TLR4 binds to LPS on the surface of gram-negative bacteria, whereas TLR2 recognizes acetylated lipoprotein (Brightbill, 1999). *Treponema* sp., the causative agent of DD, generally lacks LPS (Seshadri et al., 2004). However, they express lipoproteins that are likely to bind to TLR2/1 heterodimer. This, in turn, will initiate the cascade leading to *NFKB1* transcription (Medzhitov and Horng, 2009).

In the case of cows with DD, the expression of keratins generally decreases, while the expression of some keratins increases, such as keratin 6A in M2 lesions. Similarly, the expression of monocyte chemoattractant factor also shows an increased expression in the DD M2 lesions (Vermeersch and Opsomer, 2019). The expression of Filaggrin 2, which acts as an epidermal barrier, also decreases in the DD M2 lesions (Vermeersch and Opsomer, 2019). An increase in expression of α2- macroglobulin-like 1 was found in the skin tissues from the DD lesions (Vermeersch and Opsomer, 2019). The related α2-macroglobulin gene has also been shown to be crucial for the proliferation of other treponema species, e.g., *Treponema denticola* in human periodontitis (Socransky and Hubersak, 1967).

In short, there is a wide variation in expression of keratin type between the healthy and lame cows. A significant change in gene expression in relation to inflammation and oxidative stress was also noticed in lame cows. In the cows with digital dermatitis, there was a significant dysregulation of keratin synthesis with a decrease in most of the keratins except keratin 6A.

RATIONALE AND OBJECTIVES

The overall goal of the project was to have a better understanding of the physiological and transcriptomic alterations in dairy cows with the two most common conditions related to lameness, sole ulcer and digital dermatitis. The average annual incidence of lameness is reported to be 25% (Flower and Weary, 2006). The high level of lameness in dairy herds leads to significant economic losses for dairy producers and is an important animal welfare issue. Sole ulcers and white line disease, when combined, are either the first or second most common cause of lameness in dairy cattle (Shearer et al.,

2015). During early lactation, cows undergo a common state of negative energy balance coupled with an abrupt switch to a lactation diet typically high in carbohydrates, and this scenario can compromise hoof health and induce lameness over time. Thus, nutrition plays a major role in reducing lameness incidence and prevalence in confined transition dairy cattle.

Bovine DD is a painful condition of the foot and can lead to lameness in dairy cattle. This condition can reduce productive and reproductive performance in dairy cattle leading to major economic losses. Digital dermatitis has multiple etiology, and it usually appears between the heel bulbs of hind feet (Blowey and Sharp, 1988).

Although there are a few studies on the alterations in the gene expression patterns in cows with these two conditions, the availability of transcriptomic data is limited. Furthermore, the alterations in the blood biomarkers during these two conditions are also scarce. The inflammatory pathway involved in the pathogenesis of DD has not been elucidated (Vermeersch and Opsomer, 2019). The detailed analysis of the transcriptomic data with various bioinformatics tools gives a clear picture of the regulatory pathways involved in the development of sole ulcer or DD. Transcriptomic data revealed via RNA seq analysis can provide deeper insights into the changes in expression patterns during disease conditions such as sole ulcer and DD. Further analysis of this RNA-seq data can provide novel molecular targets for nutritional interventions to reduce sole ulcer incidence and prevalence in dairy herds. The DD data can demonstrate how the skin paracellular epithelial permeability and keratinization are being affected. Similarly, in the case of sole ulcer transcriptomics can provide specific details about the progression of the

disease. Enrichment pathway analyses will help better understand these findings and further identify molecular targets for nutritional or management interventions.

Therefore, the objectives of this study were to i) evaluate the physiological alterations in cows with sole ulcer via transcriptomics related to inflammation, keratinization, and hoof conformation in correlation with the blood biomarkers ii) evaluate the physiological alterations in cows with digital dermatitis via transcriptomics of the skin tissue from the lesion in correlation with blood biomarker analysis.

CHAPTER 2

TRANSCRIPTIONAL ALTERATIONS DUE TO SOLE ULCER REVEALED VIA RNA-SEQ ANALYSIS OF CORIUM TISSUE IN LACTATING DAIRY COWS

ABSTRACT

This study aimed to evaluate the transcriptomic profile of the corium tissue of lactating dairy cows experiencing sole ulcers disease. Hoof biopsies were performed in multiparous Holstein dairy cows selected based on their condition as clinically healthy or diagnosed with sole ulcers (n=7/group). All RNA samples were sequenced using Illumina, NovaSeq S4 at the University of Minnesota Genomics Center. Principal component analysis (PCA) and analysis of variance for each gene was calculated using the DESeq2 package in R to determine the differentially expressed (DE) genes, fold change (FC), and *P*-value based on the comparison of sole ulcer (SU) over healthy cows (HC). The DE genes with at least ± 2-fold change were retained for an enrichment pathway analysis using gene ontology (GO) terms and the enrichGO function in the Clusterprofiler package in R. The FDR cut-off value was set to < 0.01. The PCA analysis clearly discriminated the hoof transcriptomes between HC and SU cows. The number of DE genes in SU and HC was 10,274 ± 22 and 10,289 ± 36 (mean±SD), respectively. Several genes coding for keratins (e.g., *KRT34* and *KRT85*) were downregulated (FC < -15) in SU cows, while the highest upregulated (FC = 10.9) gene in SU was *IL6*. The GO analysis showed significant downregulation of GO terms in SU cows compared to HC in the Cellular Component category, including intermediate and keratin filaments and intermediate filament cytoskeleton. Upregulated genes in SU cows resulted in a significant enrichment of GO terms in the Biological Process category, including

extracellular matrix organization and vasculature development, as well as GO terms in the Cellular Component category such as extracellular matrix and cell surface. The GO terms enrichment across biological process, cellular components, and molecular function categories were highly involved in the keratinization process, inflammation, and transcriptional regulation. These processes are crucial for the development of sole ulcers causing lameness in dairy cows. This study provides an in-depth molecular and functional foundation for future nutritional interventions to reduce sole ulcer incidence in dairy herds.

INTRODUCTION

Lameness is a very common illness of confined dairy cattle, particularly after parturition when the weight of the udder places additional physical constraints on rear-leg locomotion. The average annual incidence of lameness is reported to be 25% (Flower and Weary, 2006). The high level of lameness in dairy herds leads to significant economic losses for dairy producers and is an important animal welfare issue (Huxley, 2012). Sole ulcers and white line disease, when combined, are either the first or second most common cause of lameness in dairy cattle (Shearer et al., 2015). During early lactation, cows undergo a common state of negative energy balance coupled with an abrupt switch to a lactation diet typically high in carbohydrates, and this scenario can compromise hoof health and induce lameness over time (Nocek, 1997). Thus, nutrition plays a major role in reducing lameness incidence and prevalence in confined transition dairy cattle.

The distal part of the limb is protected by the hoof, formed by keratinization of the epithelial cell layer and modification of the dermis (Langova et al., 2020). Many nutritional factors will predispose lameness in dairy cattle, especially during the transition period. For

instance, rumen acidosis following the intake of highly fermentable carbohydrates can lead to increased production of volatile fatty acids (VFA) and can decrease the rumen pH (Nocek, 1997). It will increase the population of *Streptococcus bovis,* leading to more lactic acid production. In turn, this will further drop the rumen pH *(Church, 1988)*. This will eventually lead to rumenitis, damage of the ruminal wall, and escape of bacterial endotoxins to blood, leading to laminitis (Nocek, 1997). Partial hydrolysis of inulin-type fructan found in the pasture grass yields a carbohydrate oligofructose leading to laminitis in dairy cattle (Danscher et al., 2009).

Prevalence of sole ulcers is directly associated with the thickness of the digital cushion, which is positively associated with the body condition score (Bicalho et al., 2009; Iqbal et al., 2016). Holstein dairy cows are more susceptible to sole ulcers compared to other breeds (Enevoldsen et al., 1991). The keratin synthesis will be disturbed during the progression of sole ulcer as the epidermal cells become dyskeratotic (Amstel and Shearer, 2006). The typical location of the sole ulcer lesion is between the junction of the heel and sole. The tissue damage will result in abnormal distribution of cytokeratins throughout the corium (Osorio et al., 2016).

There will also be changes associated with the blood biomarkers, including haptoglobin and serum amyloid A (SAA) (Uhlar and Whitehead, 1999; Bagga et al., 2016). Similarly, the increase in the concentration of matrix metalloproteases, which play an important role in the degradation of extracellular collagen, has also been observed (O'Driscoll et al., 2015). There will also be changes associated with proinflammatory cytokines such as IL-1β and IL-6 (Chedid et al., 1994).

Although a few studies on the gene expression profiling of the cows with sole ulcers are available, the transcriptomic data from the corium tissue sample of the cows with sole ulcers and healthy cows are limited. Recently, a method was validated to obtain the corium tissue biopsy from hoof to obtain biological information through gene expression profiling (Osorio et al., 2016). Therefore, the objectives of this study were to i) evaluate the physiological alterations in cows with sole ulcer via transcriptomics related to inflammation, keratinization, and hoof conformation in correlation with the blood biomarkers

MATERIALS AND METHODS

Animal selection

Holstein dairy cows from a commercial dairy herd in South Dakota (Elkton, SD, USA) were used in the experiment. All animal protocols were approved by the South Dakota State University Institutional Animal Care and Use Committee and cows were enrolled with owner consent. Locomotion score was recorded while the cows came out of the milking parlor by two trained personnel prior to the biopsy procedure. Selected cows were restrained in a standing hoof-trimming chute. The claws of each cow were assessed by the foot trimmer and by a trained professional.

Twelve Holstein dairy cows were allocated depending on their condition as clinically healthy or healthy cows (HC; n = 6) and cows diagnosed with sole ulcer (SU; n = 6). Lameness assessment was conducted to establish the severity of such condition utilizing a locomotion score based on a scale (1 = normal, symmetrical gait and flat back to 5 = lame, asymmetric gait, extremely arched back) described by Flower and Weary (2006). Cows were diagnosed with sole ulcers by a professional hoof trimmer. After cows have

been identified as healthy or lame (i.e., sole ulcers), hoof biopsies were performed according to procedures described by Osorio et al. (2016).

Infrared Thermography

The thermal images of the ulcerated and healthy sole tissue of the cows were taken before biopsy using a high-resolution FLIR camera mode C2 (FLIR Systems Inc., Wilsonville, OR). The settings of emissivity and reflectance were set according to the reference, and the images were taken when the animals were secured in the chute. All the images were analyzed by FLIR Tools 5.6 software (FLIR System Inc.). Using the FLIR Tools software, a geometric figure was drawn in each image in designated areas of interest for temperature analysis. With this feature, the minimum, maximum, and average temperatures were calculated for each image. For accurate analysis of the thermal images, the emissivity value was set to 0.98, the reflective temperature was set to 20°C, and the relative humidity to 75%. All images were obtained from 1 m distance from the object and exclusively captured by one individual.

Hoof biopsy

Hoof hardness was assessed using a durometer (Shore A 0-100HA). Biopsies were performed in a hydraulic squeeze chute by immobilizing the limbs. All surgical instruments were autoclaved and kept sterile until use. The hair of the pastern area of the animals was clipped. The dorsal and abaxial walls of the claw and the pastern area were cleaned with a povidone-iodine scrub allowing at least 3 minutes of contact time. A tourniquet was applied to the leg in the midway between the fetlock and tarsus/carpus to reduce bleeding during biopsy collection. Regional anesthesia of the foot was accomplished by injecting approximately 5 mL of 2% lidocaine in the area of each of the following nerves: a) if front

limb: axial and abaxial dorsal nerve of digit and axial and abaxial palmar digital nerve of the digit, b) if hind limb: axial and abaxial dorsal proper nerve of digit and axial and abaxial plantar proper nerve of the digit. Adequacy of the regional anesthesia was assessed by two or three needle pricks on the skin in the region of the pastern. If the digital nerve blocks were not effective, the following regional nerve blocks were done with approximately 5 ml of 2% lidocaine injected in each location: a) if front limb: superficial branch of the radial n., dorsal branch of the ulnar n., palmar branch of the ulnar n., and median n.; b) if hind limb: superficial peroneal n, medial plantar n, and lateral plantar n. Biopsies were not performed if more proximal nerve blocks did not produce anesthesia of the digit.

As described in Osorio et al. (2016), a sterile 12 mm drill bit was be used to drill a hole through the sole of the hoof in claw zone 4, if a healthy cow, or near the necrotized area adjacent to the sole ulcer. There was an ease of passage of the drill through the hoof as the drill approached the laminar corium. The hole was drilled until just prior to penetrating the deepest layer of the hoof wall (i.e., just prior to reaching the laminar corium). A 6 mm biopsy punch (KRUUSE Group, Denmark) was inserted through the hole and used to cut through the remaining hoof wall and the underlying corium. The biopsy instrument was rotated 2-3 times to cut through the tissues completely. A forceps and #15 scalpel blade were used to finish severing the laminar corium and to remove the biopsy specimen. Sterile, dry gauze was placed in the hole and held in place with tape and changed as needed based on accumulation of blood or soiling removed after three days. A wood block was affixed to the sole of the opposite claw (claw with no biopsy) to relieve weight bearing on the biopsy site in the sole.

The cows were returned to the standard operating procedures of the dairy farm after the procedure. Post-surgical care included flunixin meglumine treatment immediately following the procedure for pain relief. The animals were monitored daily for 7 days for signs of lameness and treated with further flunixin meglumine if needed. The inspection of wounds was done when the bandage was changed on day 3 and 7 post-biopsy. The wound was cleaned with dilute antiseptic solution (chlorhexidine diluted 1:40 in sterile saline) in case of any abnormal discharge (something other than blood).

Analysis of blood biomarkers

Blood samples (20 mL) were collected before biopsies by puncture of the coccygeal or jugular vein with 18G needles for blood biomarker analysis. The blood biomarkers analyzed were albumin, total bilirubin (TB), cholesterol, non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), creatinine, urea, glucose, ceruloplasmin, gamma-glutamyl transferase (GGT), glutamic-oxaloacetic transaminase (GOT), haptoglobin (Hp), Reactive Oxygen Metabolites (ROM) *Myeloperoxidase* (*MPO*), Paraoxonase (PON), and ferric reducing ability of plasma (FRAP) (Osorio et al., 2014).

RNA Extraction and RNA Sequencing

The tissue samples were stored in liquid nitrogen immediately after the biopsy until further analysis. Total RNA isolation was performed from 50 mg of hoof tissue (Osorio et al., 2012). RNA quality was measured using TapeStation technology with an average RIN of 8.0 ± 1.02 for all the samples. The RNA samples were submitted to Next-Generation Sequencing (Illumina, NovaSeq S4) at the University of Minnesota Genomics Center. Processed reads were aligned to the bovine genome using Kallisto. Differential

transcript analysis was performed using the DESeq2 package in R, and a false discovery rate < 0.05 correction was applied.

Enrichment Pathway Analysis

The differentially expressed (DE) genes with at least ± 2-fold change were utilized to perform an enrichment pathway analysis using gene ontology (GO) terms. The GO terms are classified according to their category: BP = biological processes, CC = cellular component, and MF = molecular function. Gene enrichment analysis classified the differentially expressed genes under these three GO terms and categories. The biological processes category represents the biological objective, molecular function represents the biochemical activity of the gene and cellular component reveals the place in the cell where the gene products are active.

Statistical Analysis

Data for the infrared imaging and blood biomarkers were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC), with the group classification as the fixed effect and animals as the random effect. Statistical significance was declared at $P \leq 0.05$ and tendencies at $P < 0.10$. Locomotion scores were calculated using descriptive statistical methods.

Analysis of differential gene expression raw transcript counts was normalized and analyzed using the Bioconductor package edgeR (Robinson et al., 2010). The DESeq package in R was used to analyze transcripts between SU and HC cows, and a false discovery rate < 0.05 correction was applied.

RESULTS

Clinical parameters associated with sole ulcer

The first parameter observed to identify the animals in lameness was the locomotion score. The HC cows had a locomotion score of 1, compared to the SU cows with a locomotion score of 2.5 ±0.5 (mean ±SD). The hoof hardness was measured by Durometer (Shore A 0-100HA), and it was 79.8±10.6 for the HC cows and 69.9±9.9 (mean ±SD) for SU cows. The infrared analysis images showed that the average temperature of SU cows was $30.47 \pm 1^{\circ}C$, (mean ±SD), which was greater ($P = 0.01$) than the $26.76 \pm 1.18^{\circ}C$ in HC cows.

Blood Biomarkers

The results of blood biomarkers are presented in Table 1. Among the blood biomarkers associated with liver function, serum GOT showed an increase ($P = 0.04$) in SU cows compared to HC cows. Haptoglobin showed a trend ($P = 0.07$) for greater concentration in SU cows in comparison to HC. Similar to GOT, the serum concentration of ceruloplasmin was greater ($P = 0.05$) in SU cows than in HC. Other liver function biomarkers such as GGT ($P = 0.43$) and albumin ($P = 0.75$) were no affected by sole ulcer effects.

The biomarkers of muscle mass and nitrogen metabolism, including creatinine ($P=0.73$) and urea ($P = 0.87$), were not affected by sole ulcer effects. The concentration of biomarkers for energy balance, including NEFA ($P = 0.04$) and BHB ($P < 0.01$) was greater in SU cows than in HC. The serum levels of glucose ($P < 0.27$) and cholesterol ($P = 0.34$) were similar between groups. The serum levels of FRAP ($P = 0.03$) and TB ($P < 0.01$) showed a greater concentration in SU cows in comparison to HC. ROM showed a

decrease ($P = 0.04$) in HC cows in comparison to SU. However, MPO ($P = 0.85$) and PON ($P = 0.87$) were similar between groups.

RNA-seq Analysis

The concentration of RNA and RNA quality measured as RIN factor in the hoof samples was appropriate to run an RNA-seq analysis (Table 2). The RNA sequence was run on a NovaSeq S4 using 50 PE (paired-end reads) for an estimated yield of ~30M reads/sample. The actual reads/sample were close to the targeted 30M (Table 3; number of paired-end reads), with the exception of sample Sole 1, which generate 16.3M reads. However, this still represents a significant yield of reads for a typical RNA-seq data. Final reads were aligned to the bovine genome using the genome-guided alignment software, Kallisto. The final % of mapped reads were 84.4% ranging from 80.6 to 89.6%. The aligned counts or TPM (Transcripts per kilobase Million) for each gene from each sample were used for subsequent analysis.

The number of differentially expressed (DE) genes in sole ulcer and healthy cows were 10,274 ± 22 and 10,289 ± 36, respectively (Figure 2). The analysis of variance for each gene was calculated using the DESeq2 package after performing the variance stabilizing transformation. This analysis allows us to determine DE genes, fold change, and P-value based on the comparison of sole ulcers over healthy cows (fold change = SU/HC). This returned an overall 4,269 and 3,799 upregulated and downregulated genes (Figure 3). The mean of normalized counts for each gene with its respective log fold change is presented in Figure 3, and DE genes at FDR < 0.05 are colored blue. The volcano plot (Figure 5) presents those genes found statistically significant (FDR < 0.05) with at least ± 2-fold-change in purple. Notice that while there is a greater number of

upregulated genes that are primarily contained within 10-fold-change, a greater number of downregulated genes with < -10-fold-change can be observed in Figure 5. The principal components analysis in Figure 6 clearly differentiates those healthy cows from sole ulcer cows. The top 50 downregulated and upregulated genes in Table 4 are graphically represented in the heatmap in Figure 7. These data show that the majority of downregulated genes are keratin and keratin-associated proteins, while the upregulated genes are with genes related to inflammation (e.g., *IL6*, *IL19*, *SAA*) and extracellular matrix (e.g., *MMP1* and *MMP13*).

Enrichment Pathway Analysis

The DE genes with at least ± 2-fold change (984 genes) were utilized to perform an enrichment pathway analysis using gene ontology (GO) terms. The number of DE genes with > 2-fold change was 645, while the number of DE genes with < -2-fold change was 339. Figure 8 shows all the GO terms that were statistically enriched based on the 984 DE genes with at least ± 2-fold change. The GO terms are classified according to their category: BP = biological processes, CC = cellular component, and MF = molecular function. In the same figure, the bars are colored from green to red to denote that such GO terms are either inhibited (or decreasing) or activated (or increasing), based on their z-score enrichment. The -log (adj p-value) in Figure 8 indicates the level of statistical significance [e.g., adj-pvalue = 0.05 will correspond to -log (adj p-value) = 1.3] for each GO term enrichment. The same GO terms in Figure 8 are presented in Figure 9, but as a bubble graph where the y-axis is the -log (adj p-value) and the x-axis is the z-score, while the area of the displayed circles is proportional to the number of genes assigned to the term and the color corresponds to the category.

A circular visualization is presented in Figure 8 for the same GO terms enriched with the same 984 DE genes. The outer circle shows a scatter plot for each term with the colored logFC assigned to each gene, where red circles display upregulated and blue ones downregulated genes. Then, the z-score is represented by the colored bars in the circle, which denote if the GO term is inhibited or activated and the level of the z-score. Figures 8 and 9 are similar to Figure 7; however, these graphs were built using only the downregulated (339) and upregulated (645) genes, respectively.

The pathway analysis of DEG reveals that the genes related to extracellular matrix were the most impacted, followed by collagen-containing extracellular matrix. The most impacted pathways under the biological process subontology were related to blood vessel development, vasculature development, and cell adhesion.

DISCUSSION

Clinical parameters associated with sole ulcer

Locomotion Score. Out of the 12 cows, the six healthy cows had an average locomotion score of 1, while the SU cows had a locomotion score of 2.5 ± 0.5. This was in accordance with the findings of Norring et al. (2008) and Danscher et al. (2009), who reported an increase in locomotion score associated with lameness. It was also observed by Orman and Endres (2016) that the locomotion score of cows with sole ulcer (3.0 ± 0.8) was greater than the locomotion score of healthy cows (2.2 ± 0.7).

Hoof Hardness. Hoof harness was showing a little higher value overall compared to the previous studies. The healthy animals had hoof hardness of 79.8 ± 6.1, compared to the sole ulcerated cows with a hoof hardness of 69.9 ± 4.4. The hoof hardness score was in the range of 40 ± 0.6 to 43.9 ± 0.6 in the study by O'Driscoll et al. (2008). However, it

was measured by a shore D (harder elastomer measurements with a pointed end needle) scale in contrast to our study with a shore A scale (softer elastomer measurements with a blunt end needle).

***Infrared Thermography*.** Infrared thermography allows the non-invasive measurement of the surface temperatures of the objects. It effectively detects the differences in temperatures due to underlying problems (Alsaaod et al., 2015). In our study, we observed a greater temperature in SU cows than HC. Orman and Endres (2016) used infrared thermography for the detection of foot lesions in the rear feet of 139 lactating dairy cows. Foot lesions identified in that study included white line disease (WLD), sole ulcer (SU), and digital dermatitis (DD). Temperatures at the coronary band (CBT) and the skin (ST) were recorded. Cows were scored for locomotion on a scale of 1–5 (1 = normal and 5 = severely lame). CBT was higher for all types of foot lesion (34.1 ± 2.3, 33.8 ± 1.6 and 33.1 ± 1.6°C for WLD, SU and DD, respectively) than for healthy (32.6 ± 1.9°C) feet. The greater CBT was associated with inflammation related to the hoof disease. In our study, we also observed an overall increase in temperature in the sole of SU cows in comparison to HC, which can be associated with an inflammatory condition.

Analysis of Blood biomarkers

The inflammatory response is characterized by an increase in the production of positive acute-phase proteins (posAPP) such as Hp and SAA and a concomitant decrease in the production of negative APP (negAPP) such as albumin (Bertoni et al., 2008). Acute-phase proteins are blood proteins which show a change in concentration in response to various inflammatory and non-inflammatory condition (Schneider, 2015).

The hepatocytes release APPs after activation by proinflammatory cytokines (IL1, IL6, and TNFα) secreted by monocytes in response to increased bacterial toxins/tissue injury (Bagga et al., 2016).

Bovine SAA and Hp levels have been observed to be elevated more in acute inflammation than in chronic inflammation. Hp has also been recognized as a valuable marker of disease in ruminants (Murata et al., 2004). In our study, Hp showed a trend to be greater in SU cows in comparison to HC. It was observed by Bagga et al. (2016) that in crossbreed cows, Holstein Friesian-Sahiwal, the serum Hp concentration was 20 times greater in lame cows (21.7 ± 3.3 mg/dL) as compared to non-lame cows (1.2 ± 0.1 mg/dL). In the same study, the authors observed Hp levels were approximately 30 times greater in 12 animals out of 30 lame cows, and in the rest of the animals, the Hp levels were above 1.75 and below 25 mg/dL. Similarly, the increase in Hp was elevated in lame cows in studies by Jawor et al. (2008). O'Driscoll et al. (2015) also observed that lame cows had higher haptoglobin levels than healthy cows.

Bertoni et al. (2008) reported that TB, GOT, GGT, albumin, and PON are reliable biomarkers of liver function around calving. The clearance of bilirubin depends on liver function, and high levels of GOT and GGT indicate liver cell damage (Bertoni et al., 2008). In our study, among the blood biomarkers associated with liver function, serum GOT showed an increase in SU cows compared to HC. In our study, the serum levels of TB showed an increase in SU cows compared to HC. This coupled with the greater GOT in SU cows indicates that SU cows had a degree of liver damage enough to affect the liver clearance of TB. This is partially confirmed by Lischer et al. (2001), who reported that cows with prolonged recovery from sole ulcers had higher serum levels of TB.

The level of blood urea and creatinine are used as biomarkers of muscle mass and nitrogen metabolism (Osorio et al., 2014). In our study, these biomarkers were similar between groups. O'Driscoll et al. (2015) observed lower urea concentration in lame cows than sound cows (4.35 ± 0.27 versus 5.17 ± 0.26 μmol/L).

Similarly, the concentration of NEFA in the blood is used as a biomarker for lipid metabolism (Osorio et al., 2014). In the present study, the biomarkers for lipid metabolism, including NEFA and BHB, showed an increase in SU cows than HC. The other biomarkers found to increase in lame cows are glucose and creatinine kinase associated with tissue breakdown and the rise in response to tissue injury (Weissman, 1990). The serum levels of glucose and cholesterol were similar between groups. O'Driscoll et al. (2015) observed that lame cows had higher glucose levels than healthy cows.

In our study, the serum levels of FRAP showed an increase in SU cows than HC. Transition dairy cows supplemented with amino acid complexed trace minerals had a trend for greater FRAP than cows supplemented with inorganic trace minerals (Batistel et al., 2016). In the same study, it was observed that the supplementation of organic trace minerals improves the hoof health in transition dairy cows (Osorio et al., 2016).

RNA-seq analysis

The principal components analysis shown in Figure 2.6 is based on differentially expressed genes and depicts an evident clustering of SU cows against HC cows. The heat map analysis of the differentially expressed genes between the healthy and sole ulcer cows also reveals that expression is completely different in the sole ulcer group and healthy group.

Expression of genes related to inflammation. The actual reads/sample were close to the targeted 30M, except sample Sole 1, which generates 16.3M reads. The number of differentially expressed (DE) genes in SU and HC cows was 10,274 ± 22 and 10,289 ± 36, respectively. Further analysis of DE returned an overall 4,269 and 3,799 upregulated and downregulated genes. In our study, the transcripts found to be upregulated in sole ulcerated cows were *IL6*, *IL19*, *SAA*, *MMP1*, and *MMP13*. The *IL6* was found to be the most upregulated gene. The *IL6* is a pro-inflammatory cytokine that increases the release of acute-phase proteins (Murata et al., 2004). O'Driscoll et al. (2015) observed that cows with low locomotion scores tended to have lower expression of genes such as *IL1A*, *IL1B*, *IL8*, and *IL10* than lame cows. O'Driscoll et al. (2015) also observed no difference in the expression of *IL2*, *IL4*, and *IFNG* between the lame cows and healthy cows. The expression of *SAA3* was also found to be significantly upregulated in our study. SAA is an important acute phase protein related to inflammation (Heegaard et al., 2000). SAA1 and SAA2 are mainly synthesized in the liver and SAA3 is the main extrahepatic isoform (Uhlar and Whitehead, 1999) Bagga et al. (2016) also observed a significant increase (4.6 times) in SAA in lame cows than healthy cows.

Expression of genes related to keratinization. Keratins are mainly classified as α and β keratins (Spearman, 1966). The secondary protein structures such as α helices and β sheets are the internal supportive structures of keratins (Lodish et al., 1995). Based on the biochemical properties, the keratins are separated to type I (acidic) and type II (basic to neutral) keratins (Schweizer et al., 2006).There are a set of keratin proteins that are expressed systematically as the differentiation proceeds. Bovine keratins include cytokeratins (CK) CK1, CK6, CK7, CK10, CK14, and CK16 (Kvedar et al., 1986). The

CK4, CK5/6, CK10, and CK14 are highly expressed in bovine claw tissue (Hendry et al., 2001). The CK5/6 and CK14 were present in the basal epidermis of healthy tissue and CK10 in supra basal layers (Hendry et al., 2001). The keratinization process occurs in epidermal keratinocytes by bundling keratin filaments to form regular macromolecular arrays, subsequently by the enucleation of cells (Budras et al., 1989; Kempson and Logue, 1993). The rate of synthesis of keratin is altered in diseased bovine claws. For example, CK16, which was not usually found in healthy tissue, was found in sole diseased cows (Hendry et al., 2001). Hendry et al. (2001) also observed that there is cytokeratin imbalance in ulcerated tissues.

In our study, among the downregulated genes, the most downregulated one was keratin 35 (*KRT35)*, followed by keratin 85 *KRT85*, keratin-associated protein 11-1(*KRTAP11-1*), keratin 33A(*KRT33A*), keratin-associated protein 3-1(*KRTAP3-1*), and keratin-associated protein 3-3 (*KRTAP3-3*). This finding is in accordance with Belge et al. (2012) that foot diseases of cattle result in inhibition of normal keratin synthesis coupled with increased vascular reactivity. Dyskeratotic changes were seen in the stratum basale and stratum spinosum were observed in the cows with uncomplicated sole ulcer lesions (Belge et al., 2012).

Expression of genes related to extracellular matrix. It was reported by Dufour (2015) that the activation of pro-inflammatory cytokines would upregulate the activity of matrix metalloproteases that have collagenase, which will impair the integrity of the suspensory apparatus of the hoof capsule. Matrix Metalloproteases are involved in extracellular protein degradation. Collagenase-3 or *MMP13* has a pivotal role in collagen degradation (Almeida et al., 2007). The increase in expression of *MMP13* in our study is

consistent with the findings of Almeida et al. (2007), who found an increased expression of *MMP13* in cows with impaired locomotion. This finding was also in accordance with the findings of O'Driscoll et al. (2015), who found an increase in expression of *MMP13* in cows with sole ulcer. Collagenase-1 or Matrix Metalloprotease1(*MMP1*) is found to increase in all forms of cutaneous wounds (Sudbeck et al., 1997). In our study also, *MMP1* was found to be significantly increased in SU cows than HC. Hyaline degeneration and mineralization, especially around the ulcer sites, were noticed in collagen bundles in cows with uncomplicated sole ulcers (Belge et al., 2012).

Enrichment Pathway analysis.

The GO analysis reveals that the 984 DEG were ascribed to at least one of the three categories (e.g., BP, MF, and CC). The GO terms plotted with log (adjusted-P) value against the Z-score revealed 32 GO terms under the BP subontology. Six GO terms were classified under the category of CC, and nine GO terms were under MF. Figure 2.9 reveals that the GO term with the highest impact was 'Extracellular matrix' (GO:0031012), which corresponds to the CC category. The most impacted ones under the BP category were 'Blood vessel development' (GO:0001568), which was followed by 'Vasculature development' (GO:0001944) and 'Cardiovascular system development' (GO:0072358).

Figure 2.11 for the 339 downregulated genes based on adjusted p-value and Z-score denoted that within the CC category, the 'Intermediate filament' (GO:0005882) was the most impacted one, followed by 'Intermediate filament cytoskeleton' (GO:0045111) and 'Keratin filament' (GO:0045095). In contrast, in the MF category,

‘Multicellular organismal water homeostasis’ (GO:0050891) and ‘Water homeostasis’ (GO:0030104) were the only impacted GO terms in this category.

In Figure 2.12, for the 645 upregulated genes, the most impacted GO term was again ‘Extracellular matrix’ (GO:0031012), which belonged to the CC category, followed by ‘Blood vessel development’ (GO:0001568) term belonging to the BP category.

The circular visualization in Figure 2.10 confirms the greater amount of upregulated genes (denoted as red dots) ascribed to the impacted GO term such as ‘Blood vessel development’ (GO:0001568) followed by ‘Vasculature development’ (GO:0001994) and ‘Cardiovascular system development’ (GO:0072358).

Taken together, the GO analysis of DEG reveals that the genes related to the 'Extracellular matrix’ term were the most impacted, followed by ‘Collagen-containing extracellular matrix’. The most impacted pathways under the BP category were related to ‘Blood vessel development’, ‘Vasculature development’, ‘Cardiovascular system development’. The analysis of the most impacted GO terms reveals that there will be an overall vasculature need during the development of sole ulcer, while having a considerable reduction in structural cellular programs due to the consistent decrease in keratin and cytoskeleton formation in the corium. Eventually, the proper development and differentiation of keratinocytes will be affected, resulting in disruption of the integrity of claw dermis.

CONCLUSIONS

Our preliminary data further confirmed that there was an increase in the systemic inflammatory response associated with increased lipid metabolism and decreased liver

function compared to healthy cows. It was also confirmed that sole ulcerations could cause transcriptomics alterations leading to inflammatory-like conditions while altering keratinization of the hoof. There was a dysregulation of keratin synthesis in SU cows which was associated with extracellular matrix degradation of collagen.

Table 2.1. Blood biomarkers analysis showing concentrations of metabolism, liver health, and oxidative status in cows with sole ulcer (SU) and healthy cows (HC).

	Group			
Parameters	HC	SU	SEM[1]	*P*-value
Metabolism				
Cholesterol, mmol/L	4.14	4.73	0.42	0.34
Glucose, mmol/L	5.16	5.49	0.21	0.27
BHB, mmol/L	0.41	0.55	0.05	0.04
NEFA, mmol/L	0.18	0.50	0.07	< 0.01
Nitrogen Metabolism				
Creatinine, µmol/L	86.40	84.39	4.22	0.73
Urea, mmol/L	5.49	5.40	0.38	0.87
Liver Function				
Albumin, g/L	34.64	33.70	2.17	0.75
Total bilirubin, µmol/L	1.49	3.33	0.38	< 0.01
Ceruloplasmin, µmol/L	2.63	3.03	0.13	0.05
GGT, U/L	26.5	22.38	2.68	0.43
GOT, U/L	121.39	162.35	13.33	0.04
Haptoglobin, g/L	0.32	0.71	0.14	0.07
Oxidative status				
FRAP, µmol/L	138.00	164.11	7.83	0.03
MPO, U/L	488.84	482.70	24.13	0.85
ROM, mg of H_2O_2/100mL	19.33	22.15	0.91	0.04

[1]Largest standard error of the mean.

[2]BHB = beta-hydroxybutyrate, NEFA = non-esterified fatty acids, GGT = gamma-glutamyl transferase, GOT = glutamic-oxaloacetic transaminase, FRAP = ferric reducing ability of plasma, MPO = myeloperoxidase, ROM = reactive oxygen metabolites.

Table 2.2. Summary of RNA concentration and quality measured via Nanodrop and TapeStation, respectively.

Sample	Group	RNA concentration (ng/uL)	RNA integrity number (RIN)
Sole 1	Sole ulcer	373	8.5
Sole 9	Sole ulcer	356	9.0
Sole 14	Sole ulcer	179	8.3
Sole 17	Sole ulcer	70.5	8.5
Sole 23	Sole ulcer	370	7.5
Sole 28	Sole ulcer	381	9.1
Sole 13	Healthy	148	8.9
Sole 15	Healthy	266	6.0
Sole 18	Healthy	1,015	7.6
Sole 19	Healthy	612	7.6
Sole 25	Healthy	140	8.2
Sole 26	Healthy	197	8.0

Table 2.3. Summary of read counts, alignment of reads to the Bos taurus genome using Kallisto.

Sample	Group	Number of paired-end reads	Remaining reads after quality filtering	Unmapped reads	Uniquely Mapped reads	Uniquely mapped (%)
Sole 1	Sole ulcer	16,373,342	15,961,956	1,467,634	13,601,029	85.2
Sole 9	Sole ulcer	32,831,911	32,152,612	2,343,338	28,811,212	89.6
Sole 14	Sole ulcer	30,105,046	29,452,498	2,841,167	24,840,511	84.3
Sole 17	Sole ulcer	29,308,797	28,595,801	2,572,260	25,000,687	87.4
Sole 23	Sole ulcer	29,648,542	29,006,101	2,364,082	25,551,830	88.1
Sole 28	Sole ulcer	27,629,296	27,028,186	2,043,276	22,491,132	83.2
Sole 13	Healthy	31,451,741	30,770,854	2,094,720	25,057,395	81.4
Sole 15	Healthy	38,053,141	37,262,428	2,541,140	30,046,172	80.6
Sole 18	Healthy	28,161,391	27,612,290	1,687,520	22,898,187	82.9
Sole 19	Healthy	36,448,776	35,769,824	2,489,453	29,869,879	83.5
Sole 25	Healthy	28,669,534	28,117,678	2,114,408	23,419,870	83.3
Sole 26	Healthy	30,823,788	30,321,891	1,683,162	25,130,557	82.9

Table 2.4. List of selected top 50 downregulated and upregulated significantly differentially expressed genes from sole ulcer and healthy corium samples.

Gene symbol	Gene name	Log2 FC	lfcSE	pvalue	padj
IL6	interleukin 6	10.9	1.30	4.12E-17	3.68E-15
MMP13	matrix metallopeptidase 13	9.9	1.52	6.41E-11	2.56E-09
SAA3	serum amyloid A 3	8.2	0.70	6.35E-32	1.53E-29
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	7.2	1.44	5.04E-07	7.68E-06
PTX3	pentraxin 3	7.2	1.46	7.55E-07	1.08E-05
THBS2	thrombospondin 2	6.9	1.29	8.90E-08	1.60E-06
M-SAA3.2	mammary serum amyloid A3.2	6.9	1.19	7.12E-09	1.73E-07
PRSS35	serine protease 35	6.8	1.46	2.89E-06	3.49E-05
IL19	interleukin 19	6.8	1.75	1.13E-04	7.52E-04
SFRP4	secreted frizzled related protein 4	6.7	1.08	4.21E-10	1.43E-08
THBS2	thrombospondin 2	6.6	1.37	1.71E-06	2.22E-05
ACTR2	actin related protein 2	6.6	1.41	3.06E-06	3.66E-05
FGF7	fibroblast growth factor 7	6.6	1.15	8.81E-09	2.11E-07
FAP	fibroblast activation protein alpha	6.6	1.21	4.29E-08	8.46E-07
PTGS2	prostaglandin-endoperoxide synthase 2	6.5	1.19	6.26E-08	1.17E-06
TMEM190	transmembrane protein 190	6.5	1.74	1.97E-04	1.20E-03
FBLN1	fibulin 1	6.5	1.01	1.73E-10	6.37E-09
ALDH1A3	aldehyde dehydrogenase 1 family member A3	6.5	1.06	8.29E-10	2.58E-08
VCAN	versican	6.5	1.19	4.22E-08	8.35E-07
KRTAP1-1	keratin associated protein 1-1	-12.9	1.16	4.64E-29	9.72E-27
KRT81	keratin 81	-13.0	1.09	1.05E-32	2.61E-30
KRTAP27-1	keratin associated protein 27-1	-13.3	1.01	4.63E-40	1.47E-37
KRTAP13-1	keratin associated protein 13-1	-13.4	1.00	1.00E-40	3.32E-38
MT4	metallothionein 4	-14.2	0.91	1.81E-55	1.80E-52
KRT31	keratin 31	-14.5	1.05	2.70E-43	9.77E-41
KRT81	keratin 81	-14.6	1.02	1.42E-46	6.63E-44
KRT86	keratin 86	-15.1	1.01	2.56E-50	1.46E-47
KRT84	keratin 84	-15.1	0.85	8.70E-71	6.92E-67
KRTAP3-1	keratin associated protein 3-1	-15.2	0.98	6.11E-54	5.40E-51
KRTAP3-3	keratin associated protein 3-3	-15.2	1.01	3.04E-51	2.01E-48
KRT33A	keratin 33A	-15.3	1.01	1.48E-51	1.07E-48
KRTAP11-1	keratin associated protein 11-1	-15.6	0.96	1.99E-59	3.97E-56
KRT85	keratin 85	-15.9	1.03	2.28E-53	1.81E-50
KRT34	keratin 34	-16.1	1.00	3.23E-58	4.28E-55

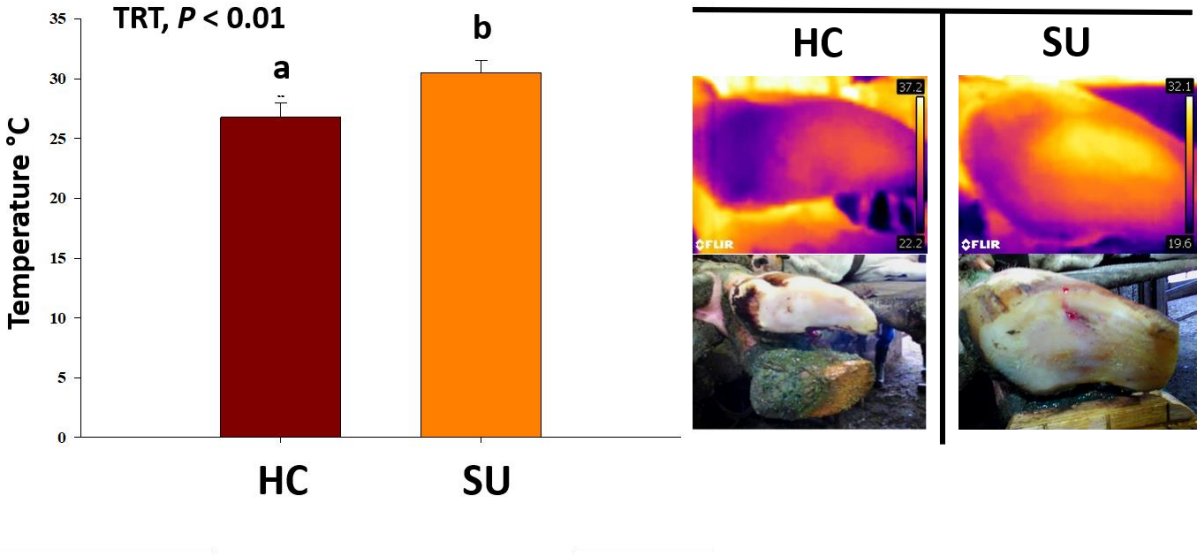


Figure 2.1. Results of infrared thermography in healthy cows (HC) and sole ulcer (SU) cows (n=7/group). The average temperatures of the cows with sole ulcer were 30.5 ± 1˚C, which was significantly different from the healthy cows with an average temperature of 26.8 ± 1.2.

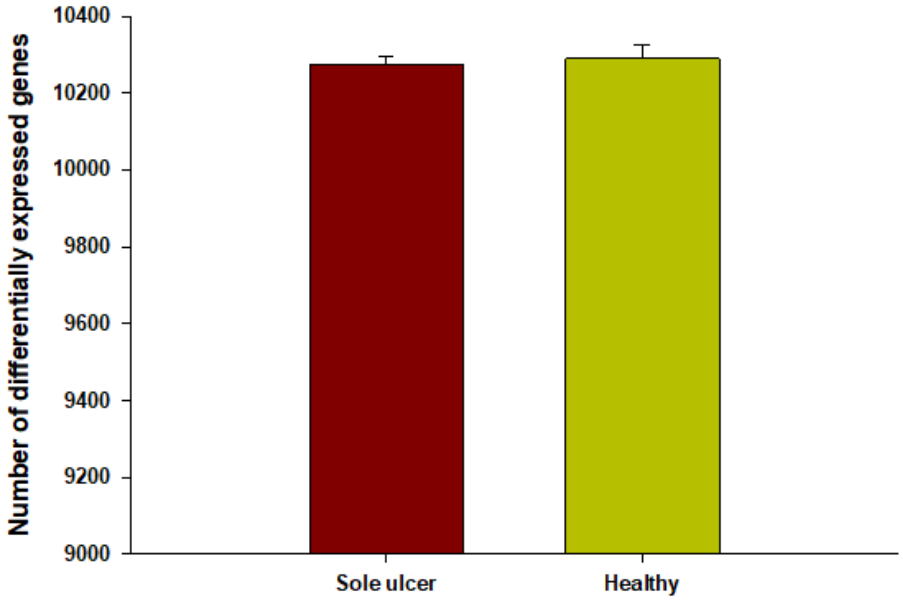


Figure 2.2. Number of differentially expressed genes across all samples. The number of differentially expressed (DE) genes in sole ulcer and healthy cows were 10,274 ± 22 and 10,289 ± 36, respectively.

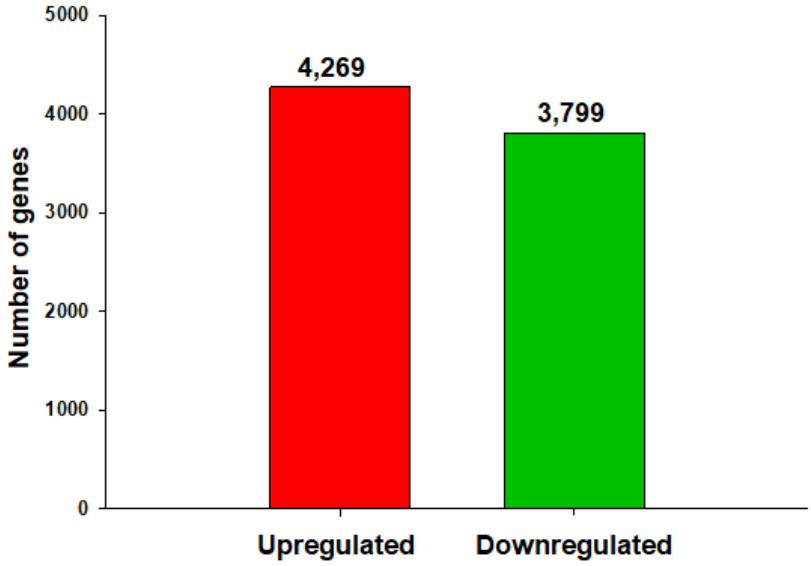


Figure 2.3. Number of upregulated and downregulated genes. Among the differentially expressed (DE) genes in sole ulcer and healthy cows, 4,269 genes were upregulated, and 3,799 genes were downregulated.

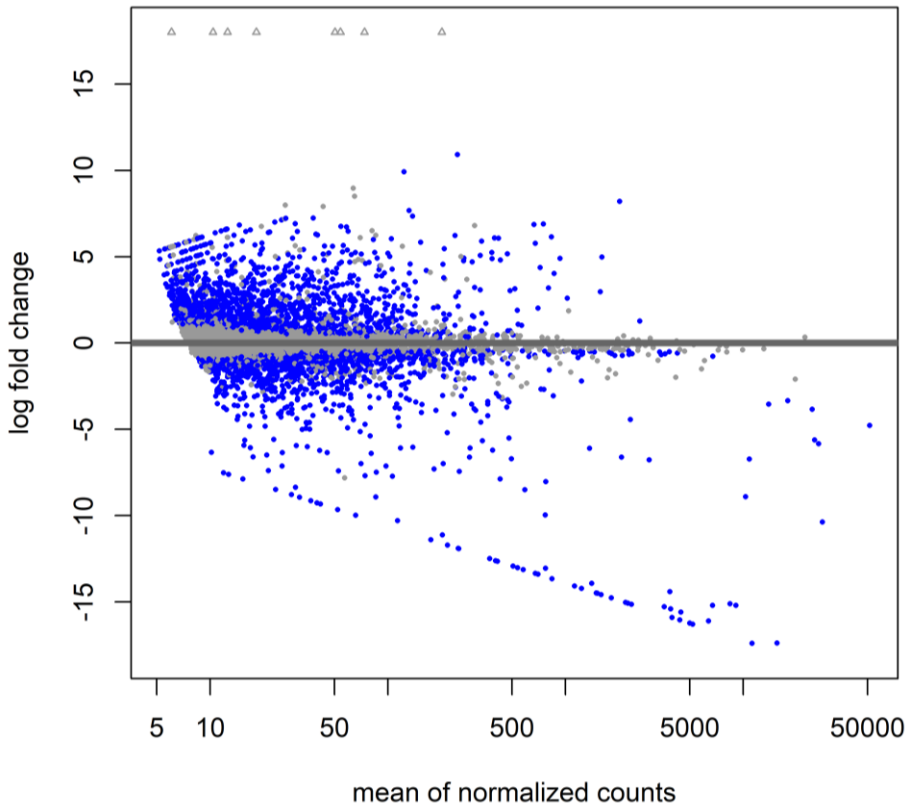


Figure 2.4. MA-plot for fold change of normalized gene counts. MA plots demonstrate the expression of genes identified as differentially expressed (DE) in the transcriptome from the corium tissue of healthy cows versus the cows with sole ulcer.

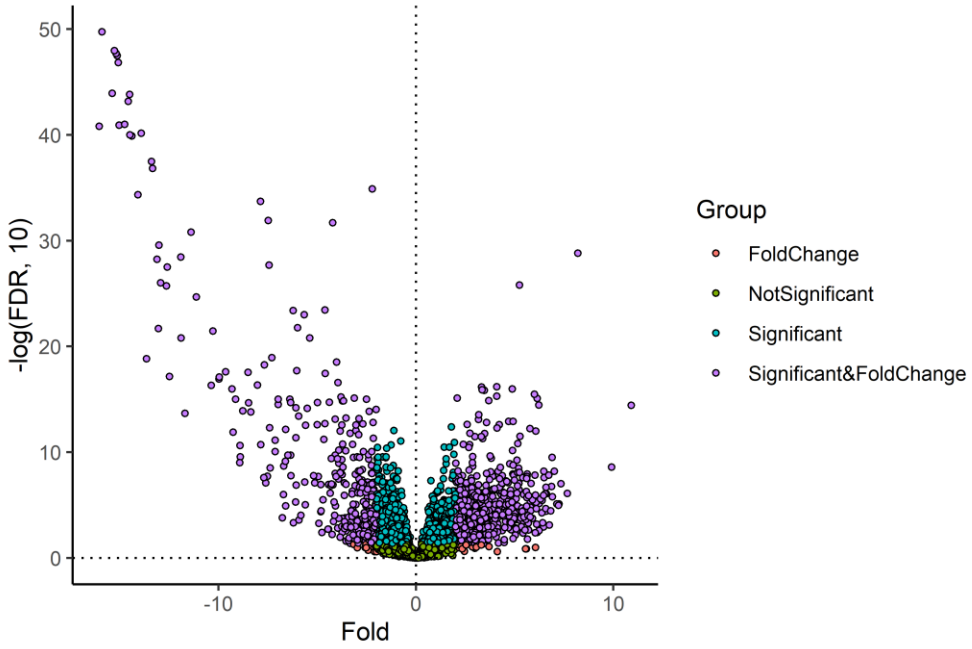


Figure 2.5. Volcano plot of differentially expressed genes. The genes which are having a statistically significant fold change is represented by purple color dots. The genes with a fold change are denoted by orange dots and the genes which are significantly different is denoted by the cyan dots.

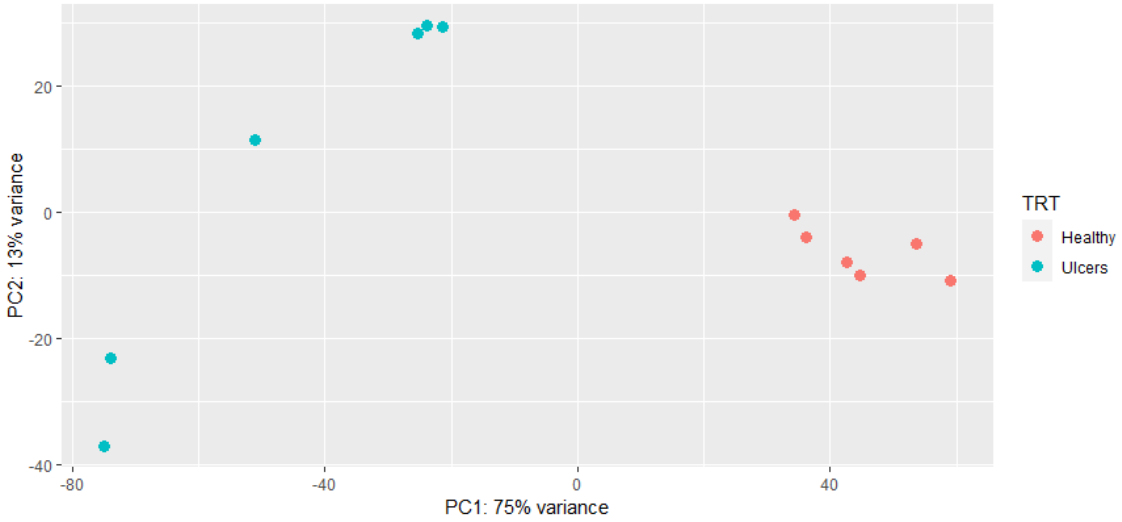


Figure 2.6. Principal components analysis. The gene expression profile of the animals with sole ulcer shows a different special groping compared to the healthy ones. Each point corresponds to one of the 12 samples. The samples from the healthy cows are represented by the orange dots and the ones from cows with sole ulcer is represented by cyan dots.

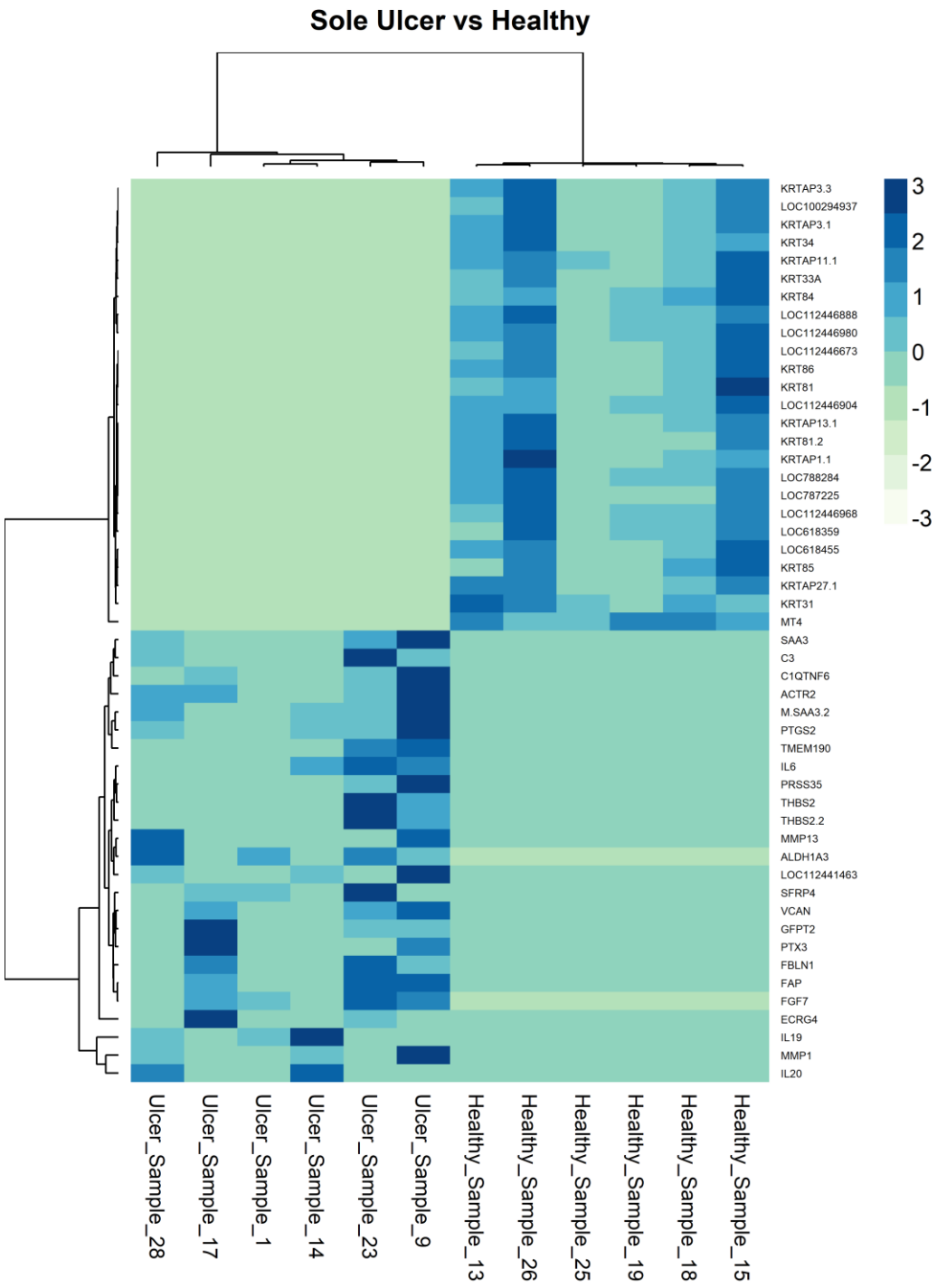


Figure 2.7. Heatmap of top 25 upregulated and downregulated genes representing differential expression of genes across the samples. Each horizontal bar represents the genes and the vertical bars, the samples.

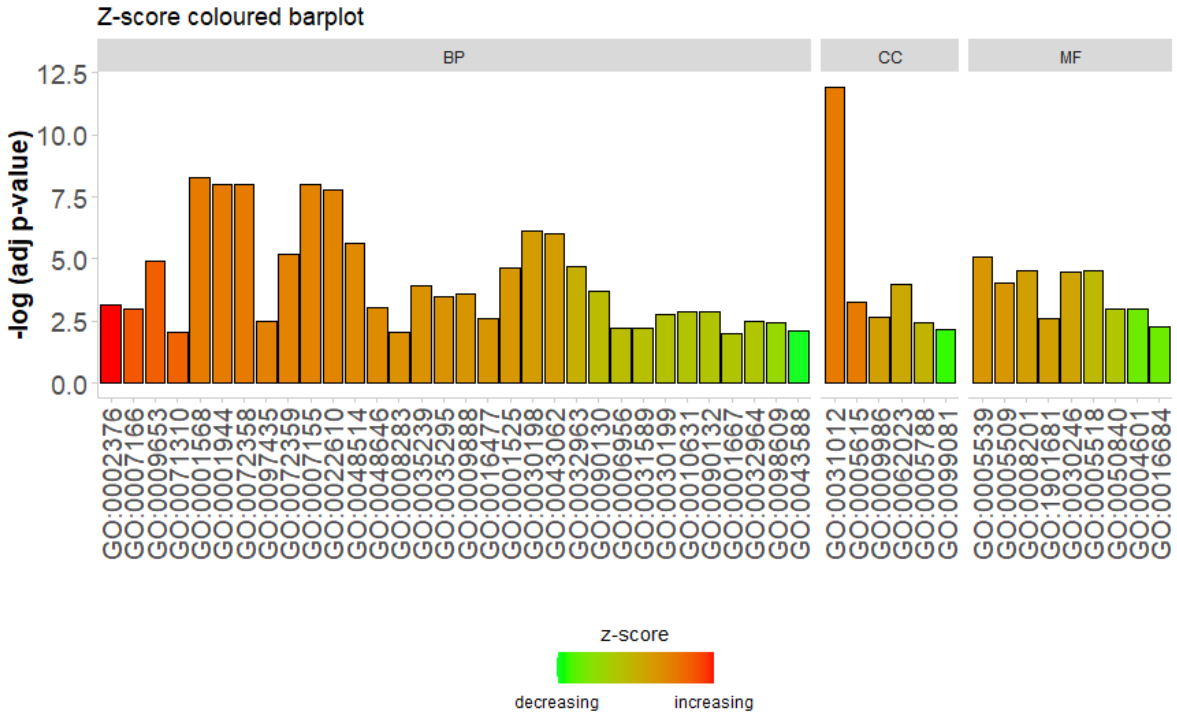
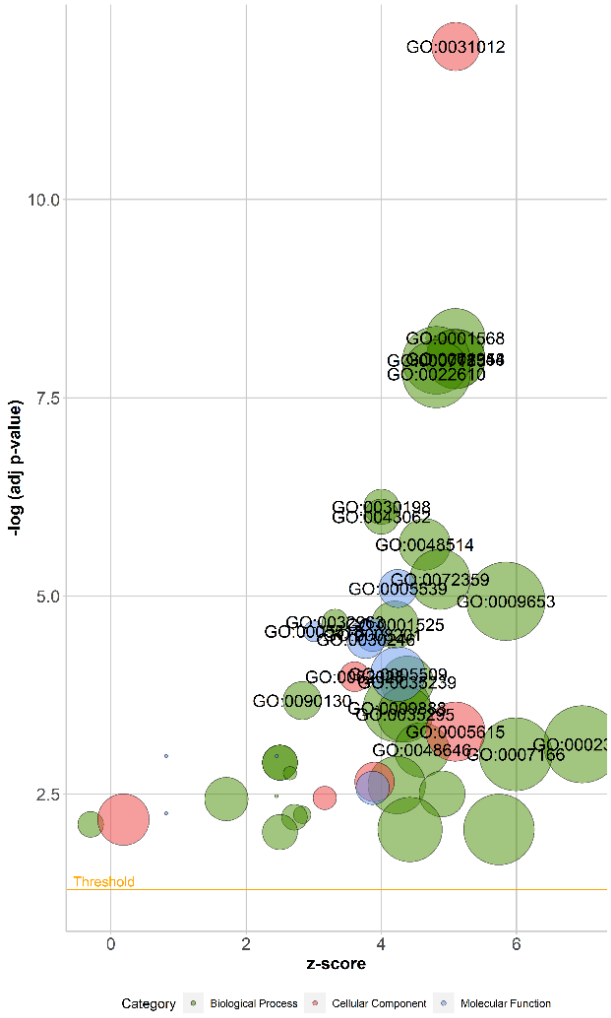
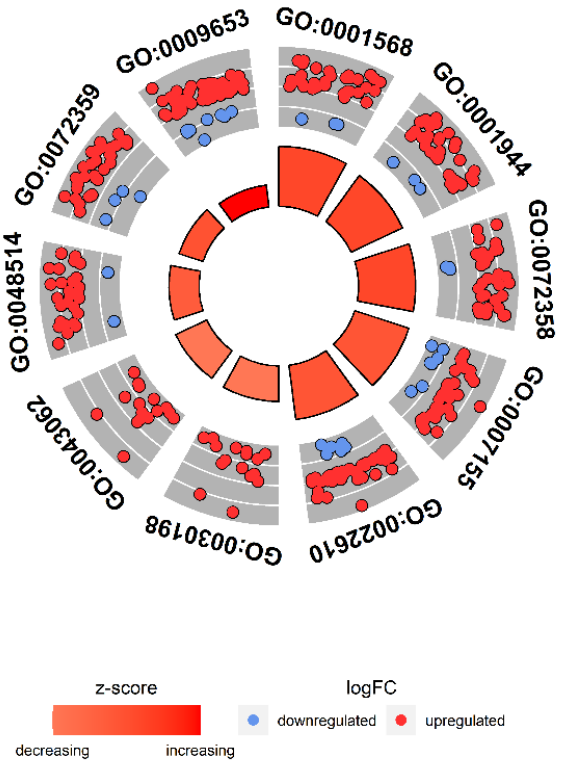


Figure 2.8. Gene Ontology analysis of 984 DEG – Gene enrichment analysis by GO term and categories: Biological process (BP), Cellular component (CC), Molecular function (MF). Bar chart showing the top GO terms for each category, ranked by fold enrichment following analysis of differentially expressed genes.



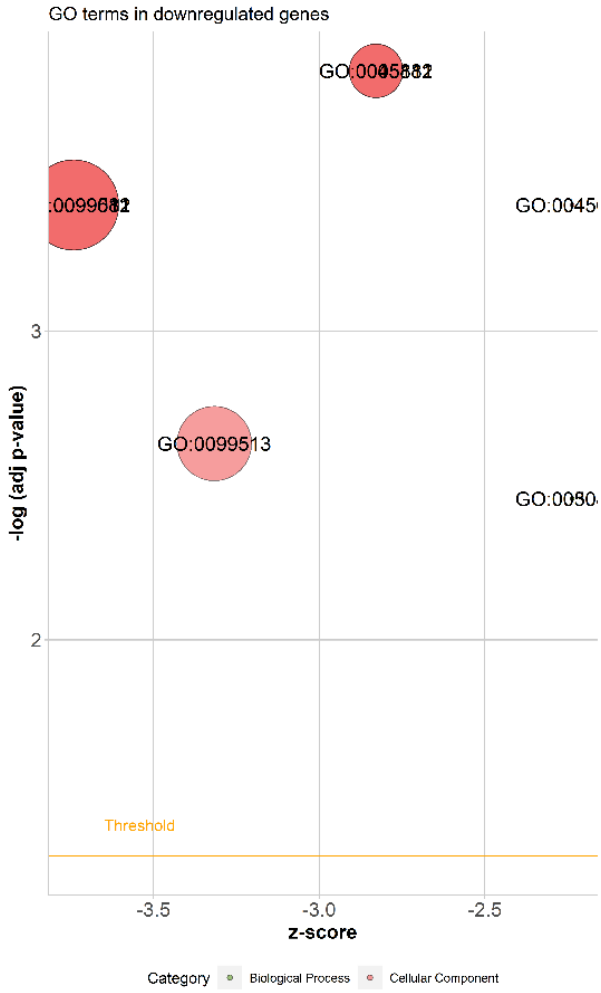
ID	Description
GO:0001568	blood vessel development
GO:0001944	vasculature development
GO:0072358	cardiovascular system development
GO:0007155	cell adhesion
GO:0022610	biological adhesion
GO:0030198	extracellular matrix organization
GO:0043062	extracellular structure organization
GO:0048514	blood vessel morphogenesis
GO:0072359	circulatory system development
GO:0009653	anatomical structure morphogenesis
GO:0032963	collagen metabolic process
GO:0001525	angiogenesis
GO:0035239	tube morphogenesis
GO:0090130	tissue migration
GO:0009888	tissue development
GO:0035295	tube development
GO:0002376	immune system process
GO:0048646	anatomical structure formation involved in morphogenesis
GO:0007166	cell surface receptor signaling pathway
GO:0031012	extracellular matrix
GO:0062023	collagen-containing extracellular matrix
GO:0005615	extracellular space
GO:0005539	glycosaminoglycan binding
GO:0005518	collagen binding
GO:0008201	heparin binding
GO:0030246	carbohydrate binding
GO:0005509	calcium ion binding

Figure 2.9. Bubble plot showing significantly enriched Gene Ontology (GO) terms of 984 DEG based on adj P-value and Z-score. The y-axis represents the negative logarithm of the adjusted p value [false discovery rate (FDR)] for the GO terms, and the x-axis displays the z-score.



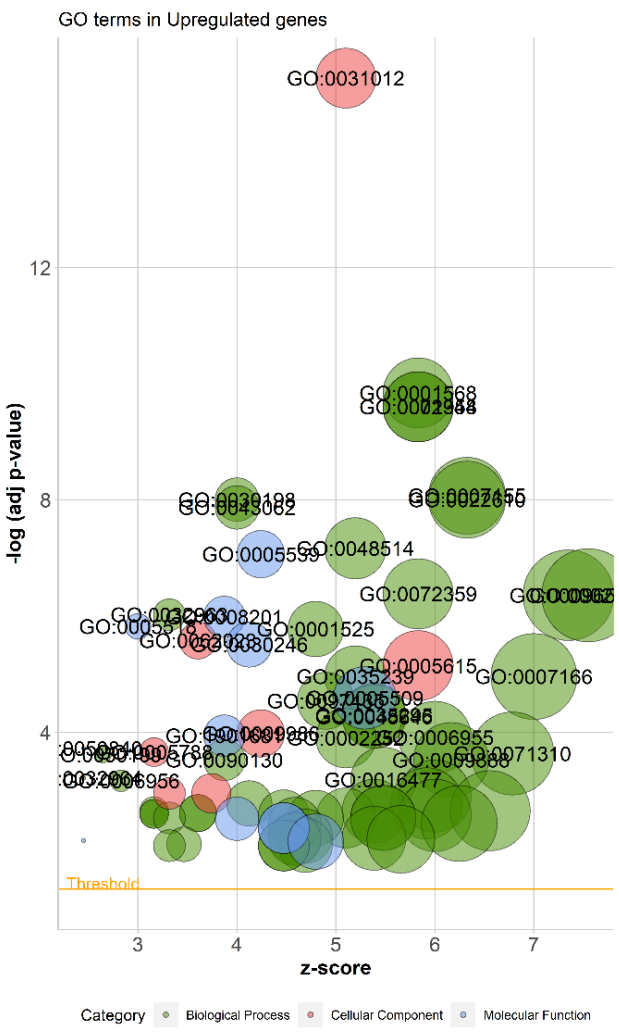
ID	Description
GO:0001568	blood vessel development
GO:0001944	vasculature development
GO:0072358	cardiovascular system development
GO:0007155	cell adhesion
GO:0022610	biological adhesion
GO:0030198	extracellular matrix organization
GO:0043062	extracellular structure organization
GO:0048514	blood vessel morphogenesis
GO:0072359	circulatory system development
GO:0009653	anatomical structure morphogenesis

Figure 2.10. The circular visualization of the results of gene annotation enrichment analysis in the Biological Process category from Gene Onthology. The outer circle shows a scatter plot for each term of the logFC of the assigned genes. Red circles display upregulation, and blue ones display downregulation. The inner circle is the representation of Z-score. The size and the color of the bar correspond to the value of the Z-score.



ID	Description
GO:0050891	multicellular organismal water homeostasis
GO:0030104	water homeostasis
GO:0005882	intermediate filament
GO:0045111	intermediate filament cytoskeleton
GO:0045095	keratin filament
GO:0099512	supramolecular fiber
GO:0099081	supramolecular polymer
GO:0099513	polymeric cytoskeletal fiber

Figure 2.11. Bubble plot showing significantly enriched Gene Ontology (GO) terms for the 339 downregulated genes based on adj P-value and Z-score. The y-axis represents the negative logarithm of the adjusted p value [false discovery rate (FDR)] for the GO terms, and the x-axis displays the z-score.



GO:0001944	vasculature development
GO:0072358	cardiovascular system development
GO:0007155	cell adhesion
GO:0022610	biological adhesion
GO:0030198	extracellular matrix organization
GO:0043062	extracellular structure organization
GO:0048514	blood vessel morphogenesis
GO:0072359	circulatory system development
GO:0009653	anatomical structure morphogenesis
GO:0002376	immune system process
GO:0032963	collagen metabolic process
GO:0001525	angiogenesis
GO:0035239	tube morphogenesis
GO:0007166	cell surface receptor signaling pathway
GO:0097435	supramolecular fiber organization
GO:0035295	tube development
GO:0048646	anatomical structure formation involved in morphogenesis
GO:0006955	immune response
GO:0002252	immune effector process
GO:0071310	cellular response to organic substance
GO:0030199	collagen fibril organization
GO:0009888	tissue development
GO:0090130	tissue migration
GO:0032964	collagen biosynthetic process
GO:0016477	cell migration
GO:0006956	complement activation
GO:0031012	extracellular matrix
GO:0062023	collagen-containing extracellular matrix
GO:0005615	extracellular space
GO:0009986	cell surface
GO:0005788	endoplasmic reticulum lumen
GO:0005539	glycosaminoglycan binding
GO:0008201	heparin binding
GO:0005518	collagen binding
GO:0030246	carbohydrate binding
GO:0005509	calcium ion binding

Figure 2.12. Bubble plot showing significantly enriched Gene Ontology (GO) terms for the 645 upregulated genes based on adj P-value and Z-score. The y-axis represents the negative logarithm of the adjusted p value [false discovery rate (FDR)] for the GO terms, and the x-axis displays the z-score.

CHAPTER 3

SKIN TRANSCRIPTOME IMPACTED BY DIGITAL DERMATITIS IN LACTATING DAIRY COWS

ABSTRACT

Bovine digital dermatitis (DD) is a complex multibacterial disease that is a major cause of lameness in cattle. In this study, skin tissue biopsies from the center of active (M2/M4.1) DD lesions or non-active (M0/M4/M1) skin of multiparous Holstein dairy cows (n = 7/group) were performed to assess the impact of DD on the skin transcriptome via RNA-seq analysis. The skin biopsies were performed using a sterile biopsy punch, and the RNA samples were sequenced using Illumina, NovaSeq S4 at the University of Minnesota Genomics Center. Principal component analysis (PCA) and the differentially expressed (DE) genes based on the comparison of DD over non-active cows (NA) were calculated using the DESeq2 package in R. To investigate further the biological function of the DE genes (FDR < 0.05) with at least ± 2-fold change were retained for an enrichment pathway analysis using gene ontology (GO) terms and the enrichGO function in the Clusterprofiler package in R. The FDR cut-off value was set to < 0.01. The PCA analysis clearly discriminated the skin transcriptome between DD cows and NA. Genes coding for several keratins-associated proteins, including *KRT34*, *KRTAP21-1*, and *KRTAP4-9* were downregulated in DD cows. The GO terms associated with downregulated genes in the Cellular Component category were mainly correlated with collagen-containing extracellular matrix and keratin filament, while GO terms in the Biological Process category were related to humoral immune response and biomineral tissue development. Upregulated genes were highly enriched in the Molecular Function GO terms of cell-

adhesion and skin development as well as intermediate filament cytoskeleton in the Cellular Component category. Overall, this skin transcriptome evaluation comparing NA and DD cows identified key biological pathways in which there was a dysregulation in the skin extracellular matrix, encompassing the keratinization process, collagen binding, and immune response caused by DD lesions. This transcriptomic dataset captures the molecular adaptations in the development of bovine DD, which can be further utilized to target suitable management and nutritional strategies to reduce this infectious disease.

INTRODUCTION

Bovine digital dermatitis (DD) is a painful condition of the foot and can lead to lameness in dairy cattle. This condition leads to reduced productive and reproductive performance in dairy cattle leading to major economic losses. It has multiple etiology, and it usually appears between the heel bulbs of hind feet (Blowey and Sharp,2008). DD is considered as one of the most common skin conditions leading to significant economic loss worldwide (Bruijnis et al., 2010; Cha et al., 2010). The average cost of DD per case averages $132.96 and the subclinical DD cases cost around $269 (Cha et al., 2010).

Improper cleaning of hoof trimming instruments can also lead to DD (Wells et al., 1999). Primiparous cows are more prone to DD, which might be due to the suboptimal immune response due to high energy expenditure towards lactation (Read and Walker, 1998). The degree of susceptibility varies with skin characteristics, hoof conformation, and immune system properties (Scholey et al., 2013). DD has a complex etiology (Choi et al., 1997). Digital dermatitis is contagious and is caused mainly by *Treponema* species (Pringle et al., 2009). The causative agent of DD is thought to be *Treponema denticola, T. phagedenis, or T. medium* (Willis et al., 1999).

In the case of cows with DD, the expression of keratins generally decreases, while the expression of a few keratins such as keratin 6A increases in M2 lesions of DD. Although there are a few studies on gene expression profiling during DD in dairy cows, the available transcriptomic data in this area is limited. Furthermore, the alterations in the blood biomarkers in the cows with digital dermatitis are also scarce. The inflammatory pathway involved in the pathogenesis of DD has not been elucidated (Vermeersch and Opsomer, 2019). The detailed analysis of the transcriptomic data with various bioinformatics tools gives a clear picture of the regulatory pathways involved in digital dermatitis development. Therefore, the objective of the study was to evaluate the physiological alterations in cows with digital dermatitis via transcriptomics of the skin tissue from the lesion in correlation with blood biomarker analysis.

MATERIALS AND METHODS

Experimental Design

Selection of Animals and Locomotion score

Twelve lactating Holstein dairy cows from a commercial dairy herd in South Dakota (Elkton, SD) were used in the experiment. All animal protocols were approved by the South Dakota State University Institutional Animal Care and Use Committee and cows were enrolled with owner consent. Locomotion score was recorded while the cows came out of the milking parlor by two trained personnel prior to the biopsy procedure. Selected cows were restrained in a standing hoof-trimming chute. The interdigital area between the heel bulbs in the plantar aspects of the foot was first inspected by the foot trimmer and then by a trained professional veterinarian prior to the biopsy of the skin. The classification of the lesions to different grades of digital dermatitis was also carried

out by the veterinarian. The lesions were classified as M0, if the area was apparently healthy. The digital dermatitis lesions varied from M2 to M4.1 stage in the selected animals.

The classification by Döpfer et al. (1997) was followed to classify the clinical of DD in 5 different stages M0-M4, mainly based on the visual appearance of the lesions. M0 represents normal skin without any DD lesions. M1 is characterized by an early small pink area of 4.2 cm diameter. M2 represents an acute classical ulcerative lesion of > 2 cm diameter. M3 represents the healing stage, covered by the scab, and M4 is the late chronic stage which shows dyskeratosis or a proliferative growth or both. Chronic stage with small active painful M1 focus is classified as M4.1(Berry et al., 2012)

Twelve Holstein dairy cows were allocated depending on their condition as clinically healthy or non-active DD (NA; n = 6) and cows diagnosed with an active/painful digital dermatitis (DD; n = 6). Lameness assessment was conducted to establish the severity of such condition utilizing a locomotion score based on a scale (1 = normal, symmetrical gait and flat back to 5 = lame, asymmetric gait, extremely arched back) described by(Flower and Weary, 2006). After cows have been identified as NA or DD, skin biopsies were performed according to procedures described by Marcatili et al. (2016).

Infrared Thermography

The thermal images of the ulcerated and healthy sole tissue of the cows were taken before biopsy by using a high-resolution FLIR camera model C2 (FLIR Systems Inc., Wilsonville, OR). The settings of emissivity and reflectance were set according to the reference, and the images were taken when the animals were secured in the chute. All the images were analyzed by FLIR Tools 5.6 software (FLIR System Inc.). Using the FLIR

Tools software, a geometric figure was drawn in each image in designed areas for the temperature analysis. With this feature, the minimum, maximum, and average temperatures were calculated for each image. For accurate analysis of the thermal images, the emissivity value was set to 0.98, the reflective temperature was set to 20°C, and the relative humidity to 75%. All images were obtained from 1 m distance from the object and exclusively captured by one individual.

Skin biopsy

This procedure was done as described by Marcatili et al. (2016) with some modifications. Cows were restrained in a hydraulic chute, and each limb was immobilized. All surgical instruments were autoclaved and kept sterile until used. The hair of the pastern area was clipped. Before sampling, the surface area was cleaned with water and disinfected with a povidone-iodine solution allowing for at least 3 minutes of contact time. Biopsies were taken from the center of the lesion with a 6 mm sterile biopsy punch and were immediately transferred into a cryovial and placed in liquid nitrogen. Sterile, dry gauze was placed in the hole and was held in place with tape. The gauze was changed as needed based on accumulation of blood or soiling and was removed after three days.

The cows were returned to the standard operating procedures of the dairy farm after the procedure. Post-surgical care included flunixin meglumine (Banamine) treatment immediately following the procedure for pain relief. The animals were monitored daily for 7 days for signs of lameness and treated with further flunixin meglumine if needed. The inspection of wounds was done when the bandage was changed on day 3 and 7 post-biopsy. The wound was cleaned with dilute antiseptic solution (chlorhexidine diluted 1:40 in sterile saline) in case of any abnormal discharge (something other than blood).

Analysis of blood biomarkers

Blood samples (20 mL) were collected before biopsies by puncture of the coccygeal or jugular vein with 18G needles for blood biomarker analysis. The blood biomarkers analyzed were albumin, total bilirubin, cholesterol, non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), creatinine, urea, glucose, ceruloplasmin, gamma-glutamyl transferase (GGT), glutamic-oxaloacetic transaminase (GOT), haptoglobin (Hp), Myeloperoxidase *(*MPO*),* Paraoxonase (PON), Interleukin 6 (IL-6) and ferric reducing ability of plasma (FRAP) (Osorio et al., 2014).

RNA Extraction and RNA Sequencing

The tissue samples were stored in liquid nitrogen immediately after the biopsy until further analysis. Total RNA isolation was performed from 50 mg of skin tissue (Osorio et al,2012). RNA quality was measured using TapeStation technology with an average RIN of 8.0±1.02 for all the samples. The RNA samples were submitted to Next-Generation Sequencing (Illumina, NovaSeq S4) at the University of Minnesota Genomics Center. Processed reads were aligned to the bovine genome using Kallisto.

Enrichment Pathway analysis

The DE genes with at least ± 2-fold change were utilized to perform an enrichment pathway analysis using gene ontology (GO) terms. The GO terms are classified according to their category: BP = biological processes, CC = cellular component, and MF = molecular function. Additionally, panther enrichment analysis was used with the aim of providing an alternative enrichment analysis. The -log (adj p-value) indicated the level of statistical significance [e.g., adj-pvalue = 0.05 will correspond to -log (adj p-value) = 1.3] for each GO term enrichment.

Statistical Analysis

Data for the infrared imaging and blood biomarkers were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC), with the group classification as the fixed effect and animals as the random effect. Statistical significance was declared at $P \leq 0.05$ and tendencies at $P < 0.10$. Locomotion scores were calculated using descriptive statistical methods.

Analysis of differential gene expression raw transcript counts was normalized and analyzed using the Bioconductor package edgeR (Robinson et al., 2010). The DESeq package in R was used to analyze transcripts between DD and NA skin, and a false discovery rate < 0.05 correction was applied.

RESULTS

Clinical Parameters associated with digital dermatitis

Lameness was assessed based on locomotion score (scale 1 to 5), and it was 1 and 2.3 ± 0.8 (mean ± SD) for NA and DD, respectively. The infrared analysis images showed that the average temperature of DD cows was 27.9 ± 9.4˚C, which was similar (P=0.91) to NA cows with 26.8 ± 5.0˚C. The active lesions of the digital dermatitis were classified from stage M2 to stage M4.1, while the healthy cows had non-active lesions of DD.

Analysis of Blood Biomarkers

The results of blood biomarker analysis are presented in Table 1. Among the blood biomarkers of liver function, serum albumin was lower (P = 0.03) in DD cows than NA. A trend (P = 0.07) for greater Hp in DD cows than NA was observed (Table1). Other biomarkers of liver function, such as GGT (P = 0.59), GOT(P = 0.41), TB (P = 0.54), and ceruloplasmin (P = 0.96) were not affected by DD effects.

Among the nitrogen metabolism biomarkers, creatinine tended ($P = 0.06$) to be lower in DD cows than NA, while urea concentration was not affected ($P = 0.60$) by DD lesions. The biomarkers for energy metabolism, including NEFA ($P = 0.94$), BHB ($P = 0.47$), cholesterol ($P = 0.25$), and glucose ($P = 0.60$) were not affected by DD lesion. Similarly, the serum levels of FRAP ($P = 0.39$) and TB ($P = 0.54$) were not affected by DD lesion.

RNA-seq Analysis

The concentration of RNA and RNA quality measured as RIN factor in the skin samples was appropriate to run an RNA-seq analysis (Table 3.2). The RNA sequence was run on a NovaSeq S4 using 50 PE (paired-end reads) for an estimated yield of ~30M reads/sample. The actual reads/sample were close to the targeted 30M (Table 3.3; number of paired-end reads). The sample Skin 3 had the lowest reads/sample of 18M, which still represents a yield of reads for typical RNA-seq data. Final reads were aligned to the bovine genome using the genome-guided alignment software, Kallisto. The final % of mapped reads was 86.1% ranging from 83.7 to 88.2%. The aligned counts or TPM (Transcripts per kilobase Million) for each gene from each sample were used for subsequent analysis.

The number of differentially expressed (DE) genes in DD and NA cows were 10,284 ± 18 and 10,268 ± 4, respectively (Figure 3.1). The analysis of variance for each gene was calculated using the DESeq2 package after performing the variance stabilizing transformation. This analysis allows us to determine DE genes, fold change, and P-value based on the comparison of DD over non-active cows (fold change = DD/NA). This returned an overall 4,769 and 4,463 upregulated and downregulated genes (Figure 3.2).

The mean of normalized counts for each gene with its respective log fold change is presented in Figure 3.3, and DE genes at FDR < 0.05 are colored blue. The volcano plot (Figure 3.4) presents those genes found statistically significant (FDR < 0.05) with at least ± 2-fold-change in purple. Notice that while there is a fairly similar number of upregulated and downregulated genes, there is a cluster of six genes highly downregulated in DD cows, and this is evident in Figure 3.4. The principal components analysis in Figure 3.5 differentiates those NA cows from DD cows, and this separation is more noticeable in terms of the PCA1 except for one cow. The top 50 downregulated and upregulated genes in Table 3.4 are graphically represented in the heatmap in Figure 3.6. These data show a transcriptional shift among keratins; while some keratins are upregulated (e.g., *KRT10*), others are downregulated (e.g., *KRT34*, *KRTAP27*-1) under DD conditions. Additionally, these data suggest that DD induces programmed cell death and cornification of the affected skin.

Enrichment pathway analysis

The DE genes with at least ± 2-fold change (1,268 genes) were utilized to perform an enrichment pathway analysis using gene ontology (GO) terms (Figure 3.7). The number of DE genes with > 2-fold change was 749 (i.e., upregulated), while the number of DE genes with < -2-fold change was 519 (i.e., downregulated). Figure 3.7 shows all the GO terms that were statistically enriched based on the 1,268 DE genes with at least ± 2-fold change. The GO terms are classified according to their category: BP = biological processes, CC = cellular component, and MF = molecular function. In the same figure, the bars are colored from green to red to denote that such GO terms are either inhibited (or decreasing) or activated (or increasing), based on their z-score enrichment. The -

log(adj p-value) in Figure 3.7 indicates the level of statistical significance [e.g., adj-pvalue = 0.05 will correspond to -log(adj p-value) = 1.3] for each GO term enrichment. The same GO terms in Figure 3.7 are presented in Figure 3.8, but as a bubble graph where the y-axis is the -log(adj p-value) and the x-axis is the z-score, while the area of the displayed circles is proportional to the number of genes assigned to the term and the color corresponds to the category.

A circular visualization is presented in Figure 3.9 for the same GO terms enriched with the same 1,268 DE genes. The outer circle shows a scatter plot for each term with the colored logFC assigned to each gene, where red circles display upregulated and the blue ones downregulated genes. Then, the Z-score is represented by the colored bars in the circle, which denote if the GO term is inhibited or activated and the level of the z-score.

Figure 3.10 is a circular visualization that displays the relationship between highly impacted GO terms and genes upregulated or downregulated. For instance, *KRT10* was an upregulated gene (in red) that is associated with the GO terms of 'Tissue development' (GO:0009888), 'Intermediate filament' (GO:0005882), and 'Intermediate filament cytoskeleton' (GO:0045111). Figures 3.11 and 3.12 are similar to Figure 3.8; however, these graphs were built using only the downregulated (519) or upregulated (749) genes, respectively.

In short, the Gene Ontology analysis of 1,268 differentially expressed genes, based on the adjusted p-value and Z score, revealed that the most important pathways belonging to the CC category were 'Intermediate filament' and 'Intermediate filament cytoskeleton'. In the BP category, the most impacted ones were 'Tissue development',

'Cell-cell adhesion', and 'Cell adhesion'. The only impacted GO term in MF category was 'Calcium ion binding' (GO:0005509). In the upregulated pathways, the most impacted part of the CC category was the 'Apical part of the cell' (GO:0045177) and the 'Apical plasma membrane' (GO:0016324), while the most important GO term in the BP category was 'Cell to cell adhesion' and 'Cell adhesion'.

DISCUSSION

Clinical parameters associated with digital dermatitis

Stage of digital dermatitis of selected cows. Döpfer et al. (1997) classified DD in 5 different stages M0-M4, mainly based on the visual appearance of the lesions. M0 represents normal skin without any DD lesions, M1 is characterized by an early small pink area of 42cm diameter .M2 represents acute classical ulcerative lesion of >2 cm diameter. M3 represents the healing stage, covered by the scab and M4 is the late chronic stage which shows dyskeratosis or a proliferative growth or both. In our study, the healthy animals were in the M0 stage, while the ones with digital dermatitis were in M2 to M4.1 grades. The chronic stage with small active painful M1 focus is classified as M4.1 (Berry et al., 2012).

Locomotion Score. Lameness was assessed based on locomotion score (scale 1 to 5), and it was 1 and 2.3 ± 0.8 (mean ± SD) for healthy cows and cows with digital dermatitis, respectively. This was in agreement with the findings of Danscher et al. (2009), who reported an increase in locomotion score associated with lameness. Danscher et al. (2009) conducted a study on clinical orthopedic implications of oligofructose overload. In this study, the lame cows had a locomotion score ≥ 2, which was in accordance with our findings. Similarly, in the study by Krull et al. (2016), it was

reported that cows with DD had an increase in locomotion score associated with the progression of different clinical stages of DD.

Infrared Thermography. Infrared thermography allows the non-invasive measurement of the surface temperatures of the objects. It effectively detects the differences in temperatures due to underlying problems (Alsaaod et al., 2015). The infrared imaging analysis showed that the average temperature of DD cows was 27.9 ± 9.4˚C, and NA cows showed an average temperature of 26.8 ± 5.0˚C. In a study by Orman and Endres (2016) infrared thermography was used for the detection of foot lesions in dairy cattle on the rear feet of 139 lactating dairy cows. Foot lesions identified in the study included white line disease (WLD), sole ulcer (SU), and digital dermatitis (DD). Temperatures at the coronary band (CBT) and the skin (ST) were recorded. Cows were scored for locomotion on a scale of 1–5 (1 = normal and 5 = severely lame). CBT was higher for all types of foot lesion (34.1 ± 2.3, 33.8 ± 1.6, and 33.1 ± 1.6°C for WLD, SU, and DD, respectively) than for healthy (32.6 ± 1.9°C) feet. In this study, the coronary band's temperature was higher in cows with digital dermatitis, which could be due to inflammation of the affected region. In our study, the affected area did not show any increase in temperature in animals with digital dermatitis compared to the healthy ones.

Blood biomarkers in cows with digital dermatitis

The inflammatory response is characterized by an increase in the production of positive acute-phase proteins (posAPP) such as Hp and SAA and a concomitant decrease in the production of negative APP (negAPP) such as albumin (Bertoni et al., 2008). Bertoni et al. (2008) reported that TB, GOT, GGT, albumin, and PON are reliable biomarkers of liver function in transition dairy cows. The rate of clearance of TB

(Bertoni et al., 2008) is dependent on liver function, and greater GOT and GGT are related to liver cell damage. In our study, serum albumin was lower in DD cows than NA. In contrast, Hp was greater in DD cows than NA. These combined effects indicate that DD cows were under a significant amount of stress, given that their albumin and Hp levels are comparable with low liver function cows in Bertoni et al. (2008).

RNA-seq Analysis

The principal components analysis of the DE genes revealed that the gene expression profile of the animals with DD showed a distinct clustering compared to NA cows. The heat map analysis of the differentially expressed genes between NA and DD cows also confirms the previous observation in the PCA data.

The actual reads/sample were close to the targeted 30M. The number of DE genes in DD and healthy cows was 10,284 ± 18 and 10,268 ± 4.24, respectively. Further analysis of the differentially expressed genes returned an overall 4,769 and 4,463 upregulated and downregulated genes, respectively.

The top five upregulated genes with the greatest fold change were peptide inhibitor 3 (*PI3*), tracheal antimicrobial peptide (*TAP*), S100 calcium binding protein A9 (*S100A9*), lysozyme (*LYZ1*), and *KRT10* (Keratin 10). The top five downregulated genes with a significant fold change include mucin-like1(*MUCL1*), major allergen BDA20 (*BDA20*), secretoglobin family ID member 2 (*SCGBID*), keratin 34 *(KRT34),* and trichohyalin (*TCHH*).

The *PI3* gene encodes an elastase-specific inhibitor that functions as an antimicrobial peptide against gram-positive and gram-negative bacteria and fungal

pathogens (Correnti et al., 2018). Laminar tissue of horses with chronic laminitis also showed an upregulation of antimicrobial peptide-like *PI3* mRNA expression (Steelman et al., 2013; Carmona et al., 2018). The *PI3* and defensins are defined as the major keratinocyte antimicrobial proteins which defend against microbes by disrupting bacterial cell membrane integrity. The *PI3* also acts as a serine protease inhibitor that protects against excessive tissue damage by neutrophil degranulation (Steelman et al., 2013). They are produced in response to inflammation resulting from infection/trauma. These are chemoattractants for several types of immune cells (Wilkinson et al., 2009; Wiesner and Vilcinskas, 2010). The tracheal antimicrobial peptide or *TAP* is an antimicrobial peptide from the tracheal mucosa (Diamond et al., 1991). The damage-related genes like superoxide dismutase 2 (*SOD2*), *S100A9*, and matrix metalloproteinase 13 (*MMP13*) are essential components of epithelial cells that respond to infection or trauma (Steelman et al., 2013). The S100 proteins are involved in the regulation of several cellular processes such as cell cycle progression and differentiation (Xia et al., 2018). Steelman et al. (2013) reported that *S100A9* was dramatically overexpressed in the laminar tissue of horses with chronic laminitis. Usually, *S100A8* and *S100A9* are co-regulated in laminar keratinocytes as a part of proinflammatory calprotectin complex (Faleiros et al., 2011). Lysozyme is an anti-bacterial agent (Ragland and Criss, 2017).The enzyme lysozyme can damage bacterial cell walls by attacking the peptidoglycan layer and is also a biomarker of immune response (Sotirov et al., 2005; Miglio et al., 2018). *KRT10* gene encodes a member of type I (acidic) cytokeratin family (Jorcano et al., 1984). The *KRT10* is a keratinocyte differentiation maker (Zhao et al., 2017). In general, there was an

upregulation of the expression of antimicrobial peptides for defending skin tissue from bacterial invasion along with keratins like *KRT10* in DD cows.

MUCL1 is a protein-coding gene involved in diseases of glycosylation (Sabry and Moussa, 2019). *BDA20* is a major dander allergen in bovine (Rautianinen et al., 1997; Wang et al., 2007), which has been reported to be upregulated in the skin following infestations with tick larvae. The *KRT34* is a type I hair keratin, and it is an acidic protein that heterodimerizes with type II keratins to form hair and nails (Saitou and Gokcumen, 2019). Scholey et al. (2013) also observed the downregulation of *KRT34* in skin transcriptome profiling of cows with digital dermatitis. The protein encoded by the *TCHH* gene forms crosslinked complexes with itself and keratin intermediate filaments to provide mechanical strength to the hair follicle inner root sheath (Schmutz et al., 1998). Nowicka and Nawrot (2019) have also reported that a cornified envelope is formed during keratin development, which protects the epidermal barrier against lytic enzymes. Trichohyalin acts as a part of this envelop. Most of the downregulated genes reveal a downregulation of most of the keratins, causing dysregulation of keratin synthesis in the samples from the active DD lesions.

The top upregulated and downregulated genes were different in our study than those reported by Scholey et al. (2013). In this study, gene expression in skin biopsies from five bovine digital dermatitis lesions and five healthy bovine feet was compared using RNA-Seq technology. This kind of differential transcriptomic expression might be because in the acute bovine DD lesions with multiple bacterial etiology, the expression of many genes will be altered. Moreover, Scholey et al. (2013) collected the samples from DD lesions equivalent to an M2 grade. In our study, the samples varied from M2 to M4.1

grades. Scholey et al. (2013) observed an upregulation of many MMP-related genes such as *MMP1*, *MMP3*, *MMP4*, and *MMP13* and downregulation of *MMP7*. MMPs are implicated in many disease processes where connective tissue turnover is dysregulated (Kushlinskii et al., 2012). An increased mRNA expression of *KRT6A* and *IL1B* was also observed in bovine DD lesions. Except for *KRT6A*, the expression of most of the other keratins and keratin-associated genes were downregulated (Scholey et al., 2013). In our study, although the most upregulated genes related to keratinization was *KRT10*, most of the other keratins were downregulated, which is generally in agreement with Scholey et al. (2013). This was also in agreement with the review by Vermeersch and Opsomer (2019) on the cows with DD that the expression of keratins generally decreased, whereas the expression of *KRT6A* was increased in M2 lesions of digital dermatitis. The expression of monocyte chemoattractant factor also showed an increased expression in the M2 lesions of digital dermatitis. (Vermeersch and Opsomer, 2019).

Enrichment pathway analysis

The Gene Ontology analysis revealed activation of GO terms mostly in the BP category with fewer terms activated CC and MF categories. Among the 1,268 DEG, the GO enrichment analysis revealed 67 GO terms belonging to the BP category, 14 GO terms in the CC category, and only one in the MF. In Figure 3.10, it is evident that the most impacted GO term was ‘Intermediate filament’ (GO:0005582), followed by ‘Intermediate filament cytoskeleton’ (GO:0045111) and ‘Keratin filament’ (GO:0045095), all under the CC category. Among the 519 downregulated DEG (Figure 3.11), the most impacted one was ‘Intermediate filament’ (GO:0005882) followed by ‘Intermediate filament cytoskeleton’ (GO:00045111) and ‘Keratin filament’

(GO:0045095) in the CC category. Among the 749 upregulated DEG, the most impacted GO term was ‘Apical part of cell’ (GO:0045177) and ‘Apical plasma membrane’ (GO:0016324) belonging to the CC category.

The circular visualization in Figure 3.9 of the GO enrichment plotted against logFC, and Z-score denotes that maximum Z-score with more upregulated genes were under GO term of ‘Tissue development’ (GO:0009888), ‘Cell-cell adhesion’ (GO:0098609), and ‘Cell adhesion’ (GO:0007155). In short, the GO analysis reveals an overall downregulation of most of the keratins coupled with dysregulation of the intermediate filaments. These disruptive changes during the progression of the lesion reduce the integrity of the skin in the affected region resulting in further complications.

CONCLUSION

The skin transcriptome profile of DD cows in comparison with the healthy cows reveals that DD conditions induce a transcriptional shift among keratins. Some keratins were upregulated (e.g., *KRT10*), while others were downregulated (e.g., *KRT34*, *KRTAP27*-1). Additionally, our data suggest that DD induces programmed cell death and cornification of the affected skin. There was also an upregulation of the antimicrobial peptides in the cows with active painful digital dermatitis lesions compared to healthy ones. This was coupled with a concomitant decrease in albumin and an increase in Hp in DD cows, indicating a systemic inflammatory response. Our data suggest that digital dermatitis can alter skin paracellular epithelial permeability and keratinization.

Table 3.1. Details of blood biomarkers analysis showing concentrations of metabolism, liver health, and oxidative status in cows with Digital dermatitis (DD) and non-active digital dermatitis cows (NA).

	Group			
Parameters	NA	DD	SEM[1]	*P*-value
Energy metabolism				
Cholesterol, mmol/L	4.91	5.63	0.43	0.25
Glucose, mmol/L	5.14	4.84	0.11	0.60
BHB, mmol/L	0.42	0.42	0.05	0.94
NEFA, mmol/L	0.14	0.20	0.05	0.47
Nitrogen metabolism				
Creatinine, µmol/L	86.58	76.60	3.69	0.06
Urea, mmol/L	5.99	5.64	0.46	0.60
Liver Function				
Albumin, g/L	36.99	33.45	1.08	0.03
Total bilirubin, µmol/L	1.10	1.29	0.23	0.54
Ceruloplasmin, µmol/L	2.70	2.69	0.16	0.96
GGT, U/L	29.25	26.44	2.58	0.59
GOT, U/L	133.62	147.44	11.58	0.41
Haptoglobin, g/L	0.18	0.80	0.22	0.07
Oxidative status				
FRAP, µmol/L	144.80	154.70	7.88	0.39
MPO, U/L	437.44	450.90	18.18	0.60
ROM, Mg of H_2O_2/100mL	20.91	17.67	7.18	0.74

[1]Largest standard error of the mean.

[2]BHB = beta-hydroxybutyrate, NEFA = non-esterified fatty acids, GGT = gamma-glutamyl transferase, GOT = glutamic-oxaloacetic transaminase, FRAP = ferric reducing ability of plasma, MPO = myeloperoxidase, ROM = reactive oxygen metabolites.

Table 3.2. Summary of RNA concentration and quality measured via Nanodrop and TapeStation, respectively.

Sample	Group	RNA concentration (ng/uL)	RNA integrity number (RIN)
Skin 3	Digital dermatitis	477	9.4
Skin 11	Digital dermatitis	777	9.2
Skin 12	Digital dermatitis	486	9.4
Skin 21	Digital dermatitis	188	8.4
Skin 22	Digital dermatitis	688	8.2
Skin 29	Digital dermatitis	438	6.8
Skin 2	Non-active	41.3	7.7
Skin 6	Non-active	50.4	7.7
Skin 7	Non-active	38.9	7.0
Skin 8	Non-active	60.8	6.5
Skin 10	Non-active	35.4	6.9
Skin 20	Non-active	78.0	6.0

Table 3.3. Summary of read counts, alignment of reads to the Bos taurus genome using Kallisto.

Sample	Group	Number of paired-end reads	Remaining reads after quality filtering	Unmapped reads	Uniquely Mapped reads	Uniquely mapped (%)
Skin 3	Digital dermatitis	21,928,686	21,532,605	1,531,967	18,410,692	85.5
Skin 11	Digital dermatitis	25,624,137	25,029,243	1,460,078	21,228,030	84.8
Skin 12	Digital dermatitis	27,762,792	27,198,335	2,296,964	23,352,251	85.9
Skin 21	Digital dermatitis	29,425,224	28,720,202	2,109,770	24,149,056	84.1
Skin 22	Digital dermatitis	26,662,904	26,108,703	2,206,987	21,848,113	83.7
Skin 29	Digital dermatitis	28,698,590	28,005,055	3,208,725	23,496,531	83.9
Skin 2	Healthy	39,066,887	38,237,548	3,024,786	33,687,555	88.1
Skin 6	Healthy	32,931,055	32,178,270	2,711,767	28,392,606	88.2
Skin 7	Healthy	26,030,042	25,510,975	2,459,664	22,253,946	87.2
Skin 8	Healthy	34,534,211	33,806,461	2,832,739	29,650,722	87.7
Skin 10	Healthy	33,758,161	33,151,224	3,298,737	28,777,478	86.8
Skin 20	Healthy	33,682,666	32,913,812	2,948,921	28,675,564	87.1

Table 3.4. List of selected top 50 downregulated and upregulated significantly differentially expressed genes from digital dermatitis and healthy skin samples.

Gene symbol	Gene name	Log2FC	lfcSE	pvalue	Padj
PI3	peptidase inhibitor 3, skin-derived (SKALP)	13.5	1.29	1.94E-25	1.98E-22
TAP	tracheal antimicrobial peptide	12.7	2.08	1.03E-09	2.14E-08
S100A9	S100 calcium binding protein A9	12.2	0.90	6.41E-42	5.88E-38
LYZ1	lysozyme 1	11.8	2.00	3.82E-09	6.73E-08
KRT10	keratin 10	11.8	1.32	4.10E-19	1.05E-16
A2ML1	alpha-2-macroglobulin like 1	11.4	1.28	6.49E-19	1.61E-16
SERPINB4	serpin family B member 4	11.1	1.08	9.66E-25	8.87E-22
LAP	lingual antimicrobial peptide	10.8	1.48	2.65E-13	1.59E-11
CASP14	caspase 14	10.6	1.47	5.96E-13	3.17E-11
APOBEC3Z1	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A	10.4	1.38	4.19E-14	2.98E-12
CNFN	cornifelin	10.3	1.41	3.01E-13	1.76E-11
C28H10orf99	chromosome 28 C10orf99 homolog	10.2	1.04	7.38E-23	4.52E-20
RHCG	Rh family C glycoprotein	10.0	2.03	8.92E-07	7.78E-06
S100A12	S100 calcium binding protein A12	9.7	1.00	2.63E-22	1.15E-19
IL36A	interleukin 36 alpha	9.4	1.47	1.88E-10	4.87E-09
S100G	S100 calcium binding protein G	-7.4	1.19	5.90E-10	1.33E-08
THRSP	thyroid hormone responsive	-7.6	1.97	1.14E-04	5.11E-04
KRTAP27-1	keratin associated protein 27-1	-7.7	2.60	3.25E-03	9.02E-03
CHRDL1	chordin like 1	-8.0	0.95	3.76E-17	6.28E-15
AKR1C3	aldo-keto reductase family 1, member C3	-8.3	3.00	5.98E-03	1.51E-02
CCL26	C-C motif chemokine ligand 26	-8.3	0.68	1.01E-33	2.33E-30

GLYATL2	glycine-N-acyltransferase like 2	-8.6	3.00	4.22E-03	1.13E-02
OGN	osteoglycin	-8.6	1.03	7.95E-17	1.22E-14
SCGB2A2	secretoglobin, family 2A, member 2	-9.6	2.39	6.66E-05	3.19E-04
TCHH	trichohyalin	-9.9	2.59	1.39E-04	6.06E-04
KRT34	keratin 34	-22.2	3.00	1.53E-13	9.60E-12
SCGB1D	secretoglobin, family 1D, member 2	-24.9	3.00	1.21E-16	1.66E-14
BDA20	major allergen BDA20	-27.9	2.75	3.86E-24	2.95E-21
MUCL1	mucin-like 1	-28.7	2.80	1.11E-24	9.25E-22

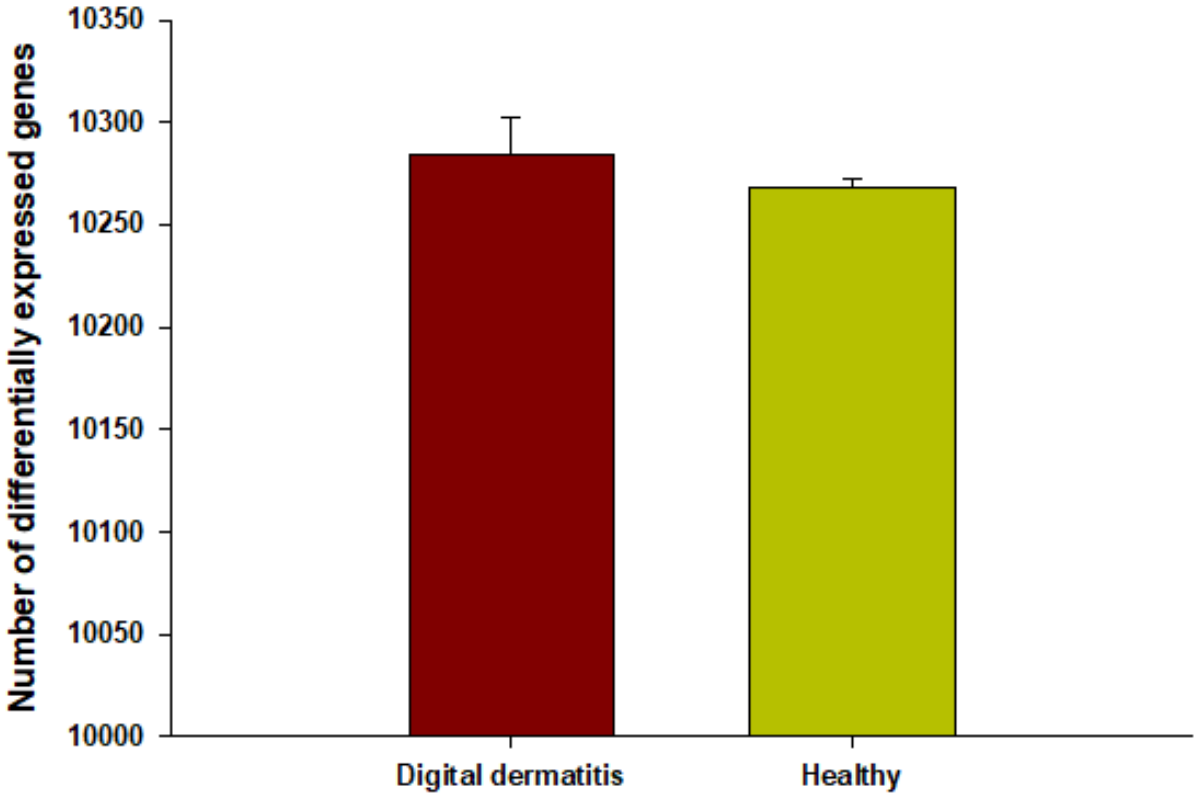


Figure 3.1. Number of differentially expressed genes. The number of differentially expressed (DE) genes in DD and healthy cows were 10,284 ± 18 and 10,268 ± 4, respectively.

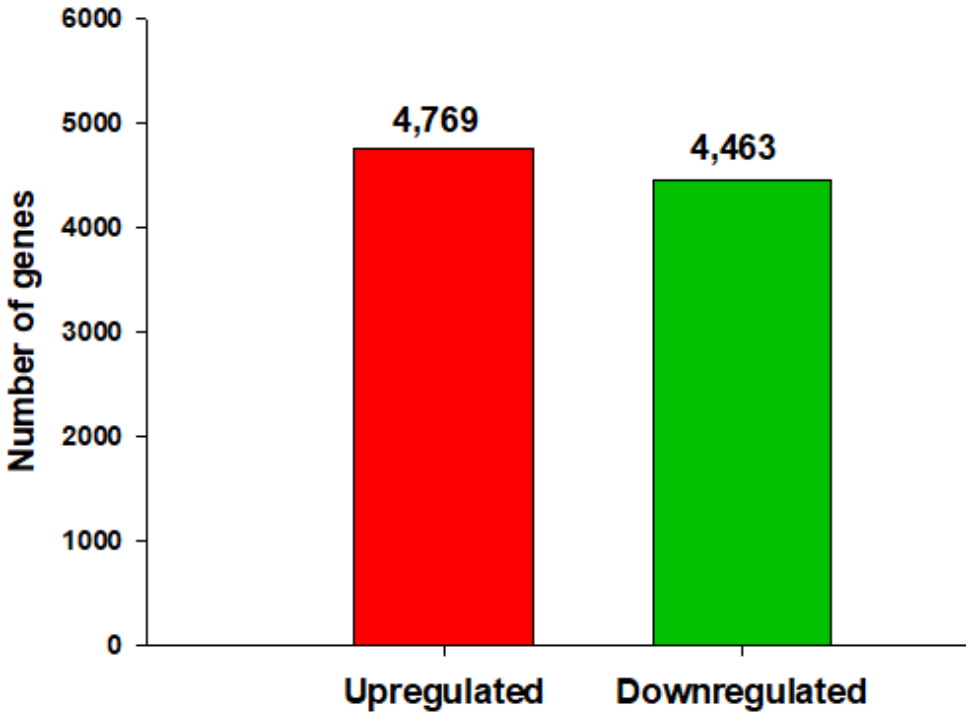


Figure 3.2. Number of upregulated and downregulated genes. Among the differentially expressed (DE) genes in sole ulcer and healthy cows, 4,769 genes were upregulated, and 4,463 genes were downregulated.

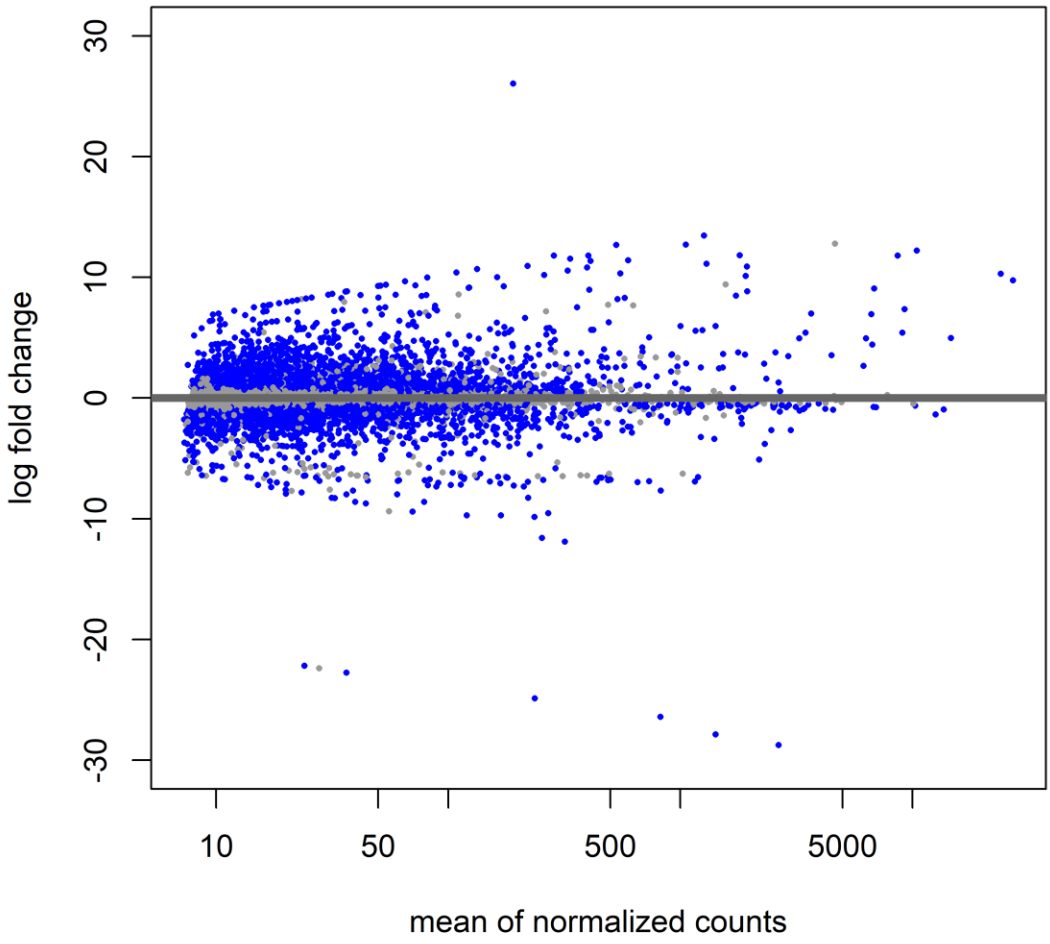


Figure 3.3. Fold change of normalized gene counts. MA plots demonstrate the expression of genes identified as differentially expressed (DE) in the transcriptome from the corium tissue of healthy cows versus the cows with sole ulcer. The points above the zero represents the upregulated genes and the points below downregulated ones. The points with a significant fold change are blue in color and the ones without significant fold change gray.

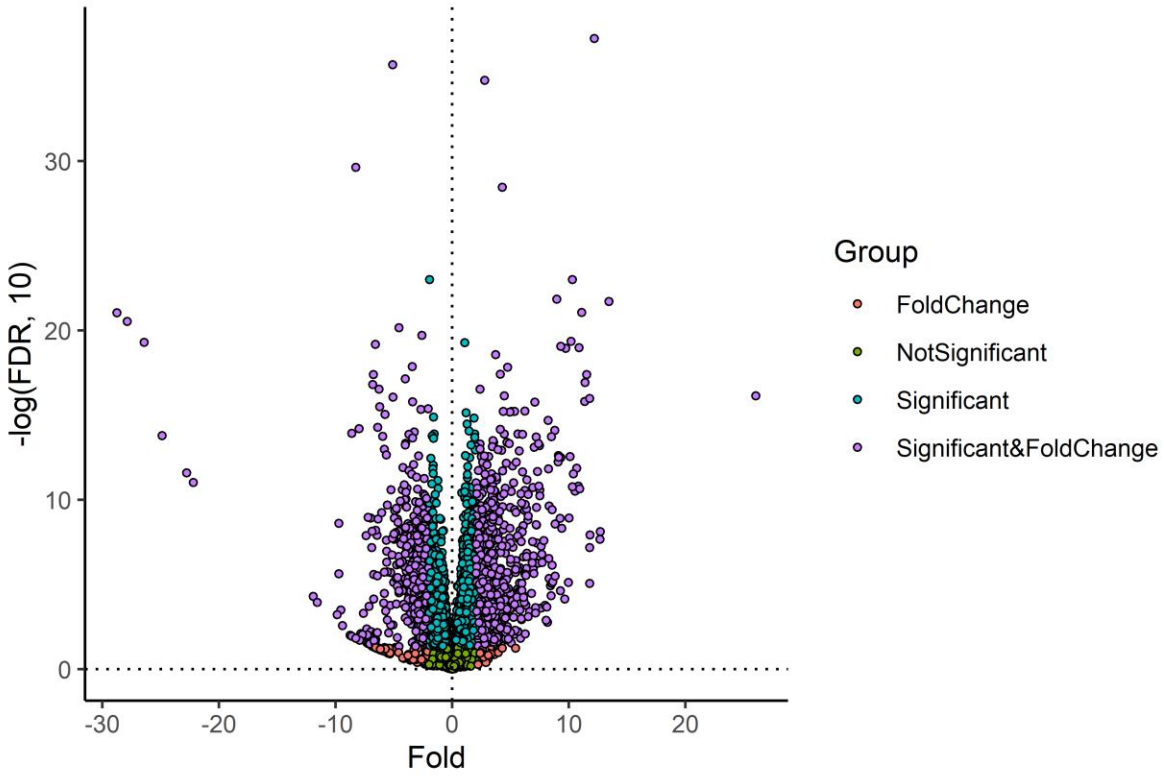


Figure 3.4. Volcano plot of differentially expressed genes. The genes which are having a statistically significant fold change is represented by purple color dots. The genes with a fold change are denoted by orange dots and the genes which are significantly different is denoted by the cyan dots.

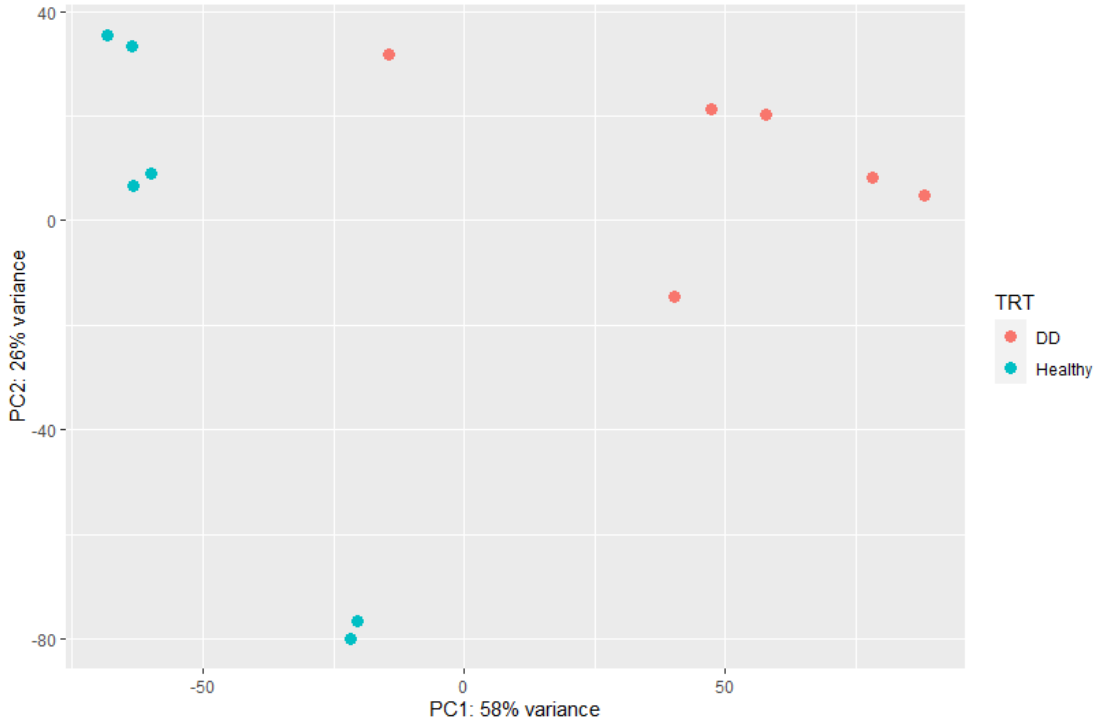


Figure 3.5. Principal components analysis. The gene expression profile of the animals with sole ulcer shows a different special groping compared to the healthy ones. Each point corresponds to one of the 12 samples. The samples from the healthy cows are represented by the orange dots and the ones from cows with sole ulcer is represented by cyan dots.

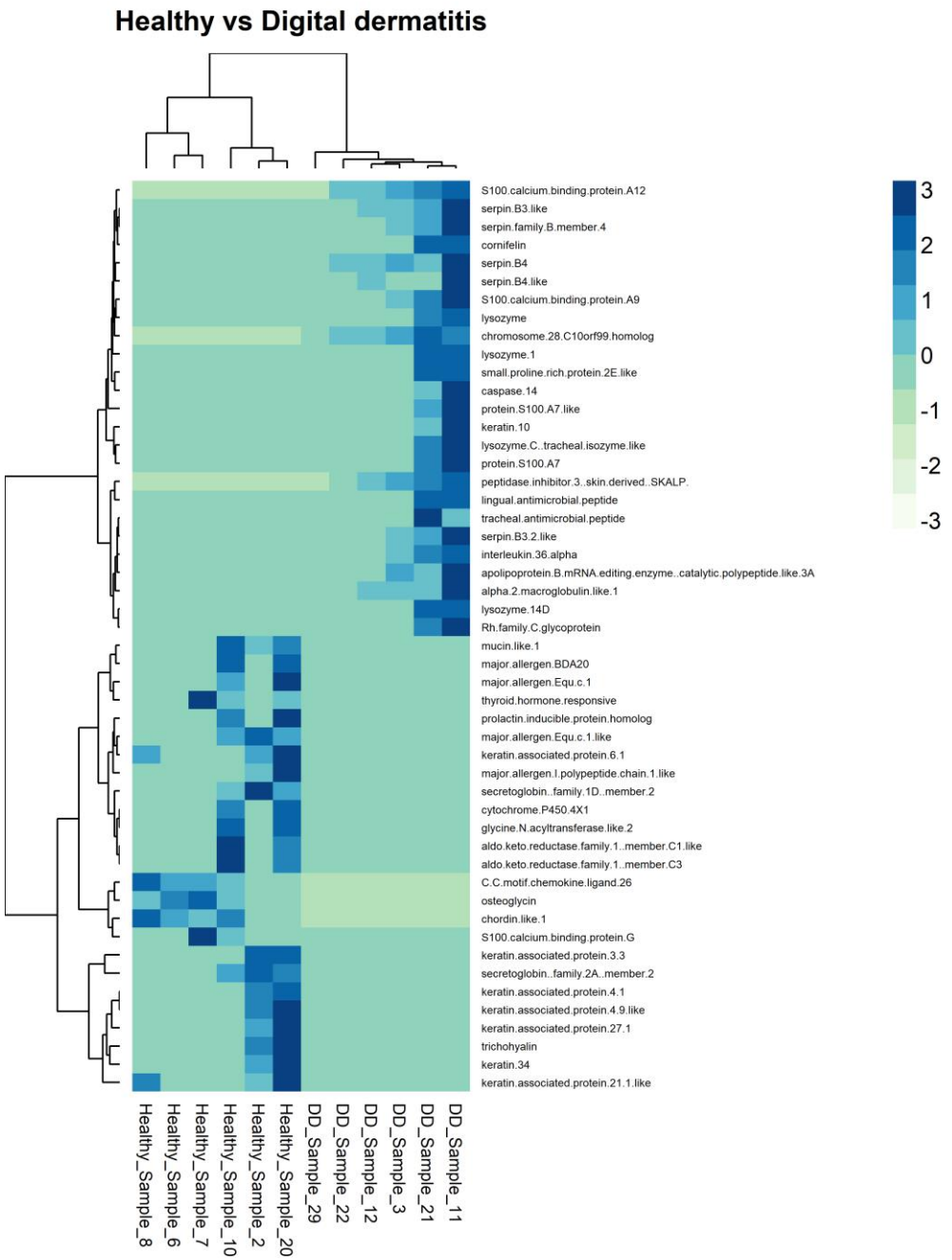


Figure 3.6. Heatmap of top 25 upregulated and downregulated genes representing differential expression of genes across the samples. Each horizontal bar represents the genes and the vertical bars, the samples.

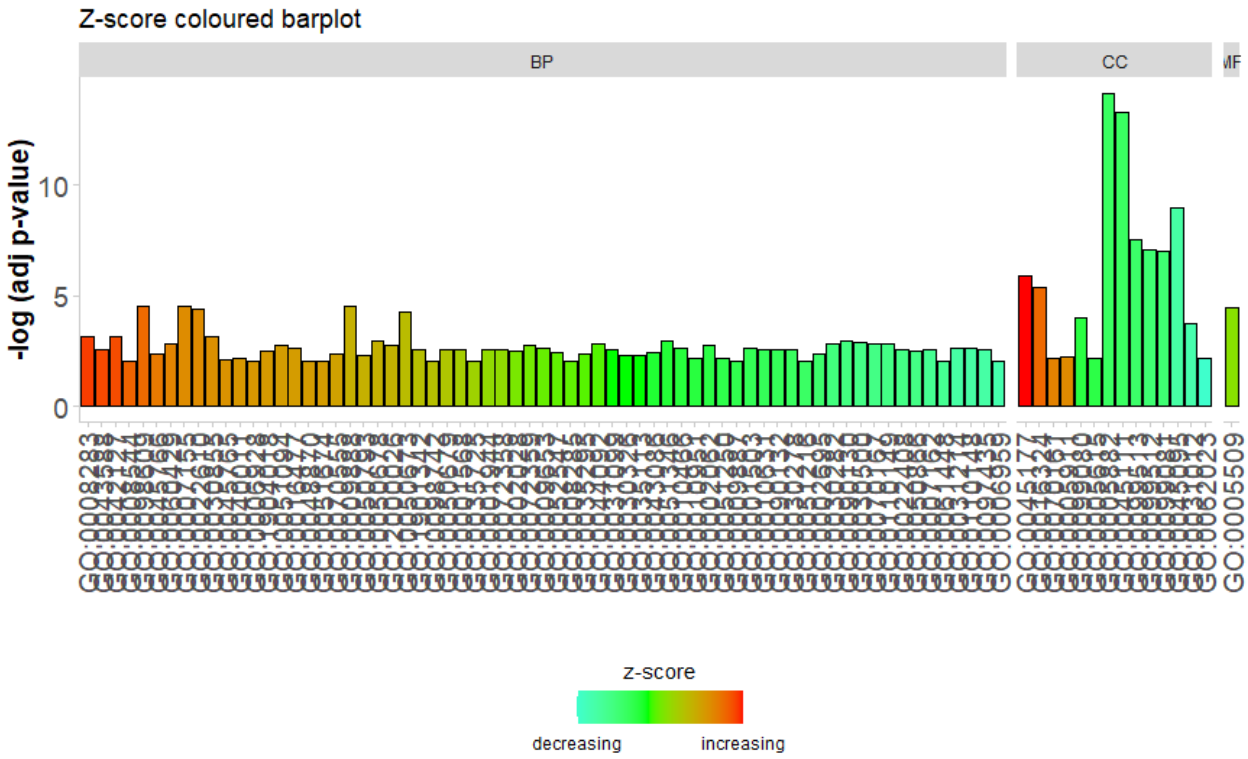
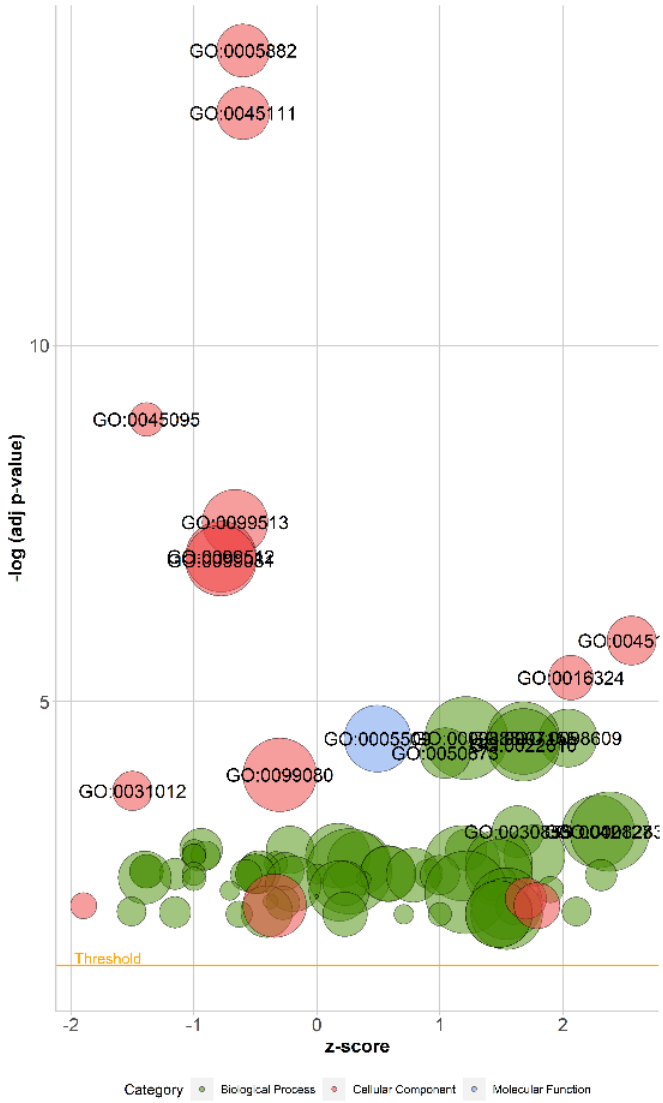
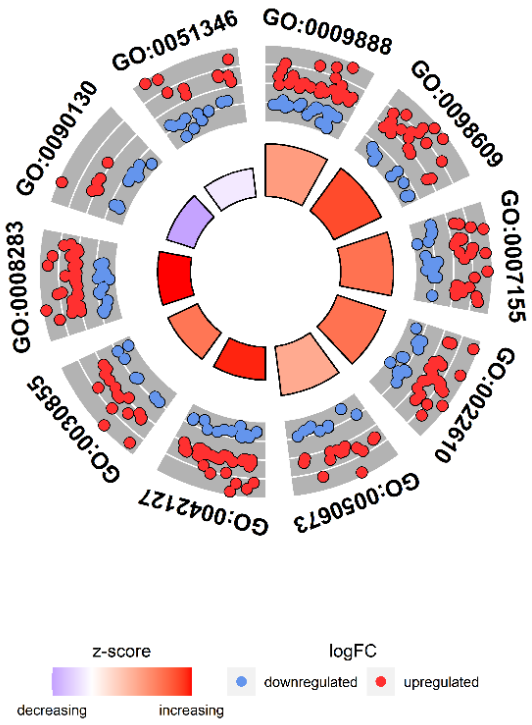


Figure 3.7. Gene Ontology analysis of 1,268 DEG – Gene enrichment analysis by GO term and categories: Biological process (BP), Cellular component (CC), Molecular function (MF). Bar chart showing the top GO terms for each category, ranked by fold enrichment following analysis of differentially expressed genes.



ID	Description
GO:0009888	tissue development
GO:0098609	cell-cell adhesion
GO:0007155	cell adhesion
GO:0022610	biological adhesion
GO:0050673	epithelial cell proliferation
GO:0042127	regulation of cell population proliferation
GO:0030855	epithelial cell differentiation
GO:0008283	cell population proliferation
GO:0005882	intermediate filament
GO:0045111	intermediate filament cytoskeleton
GO:0045095	keratin filament
GO:0099513	polymeric cytoskeletal fiber
GO:0099512	supramolecular fiber
GO:0099081	supramolecular polymer
GO:0045177	apical part of cell
GO:0016324	apical plasma membrane
GO:0099080	supramolecular complex
GO:0031012	extracellular matrix
GO:0005509	calcium ion binding

Figure 3.8. Bubble plot showing significantly enriched Gene Ontology (GO) terms of 1,268 DEG based on adj P-value and Z-score. . The y-axis represents the negative logarithm of the adjusted p value [false discovery rate (FDR)] for the GO terms, and the x-axis displays the z-score.



ID	Description
GO:0009888	tissue development
GO:0098609	cell-cell adhesion
GO:0007155	cell adhesion
GO:0022610	biological adhesion
GO:0050673	epithelial cell proliferation
GO:0042127	regulation of cell population proliferation
GO:0030855	epithelial cell differentiation
GO:0008283	cell population proliferation
GO:0090130	tissue migration
GO:0051346	negative regulation of hydrolase activity

Figure 3.9. The circular visualization of the results of gene annotation enrichment analysis. The outer circle shows a scatter plot for each term of the logFC of the assigned genes. Red circles display upregulation, and blue one's display downregulation. The inner circle is the representation of Z-score. The size and the color of the bar correspond to the value of the Z-score.

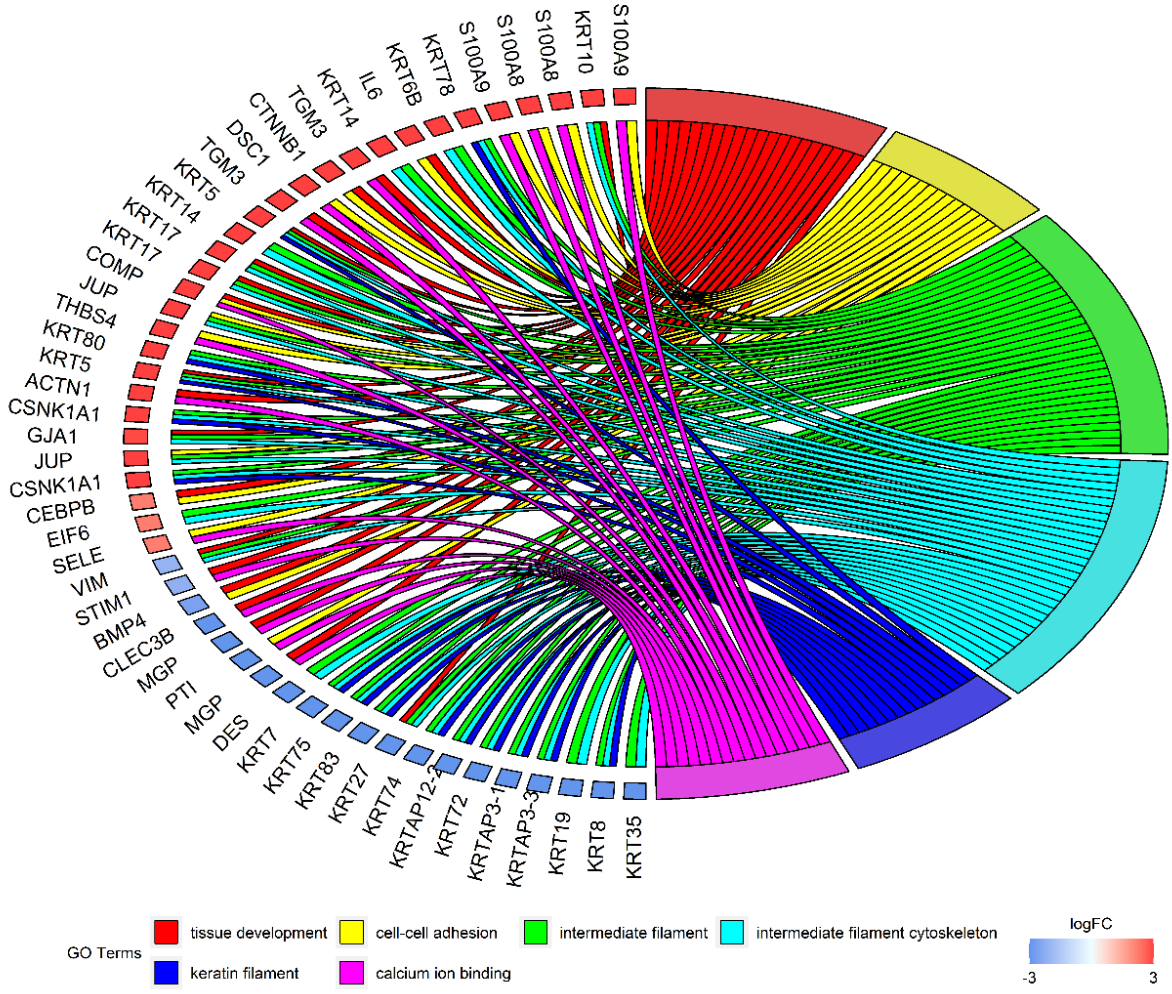
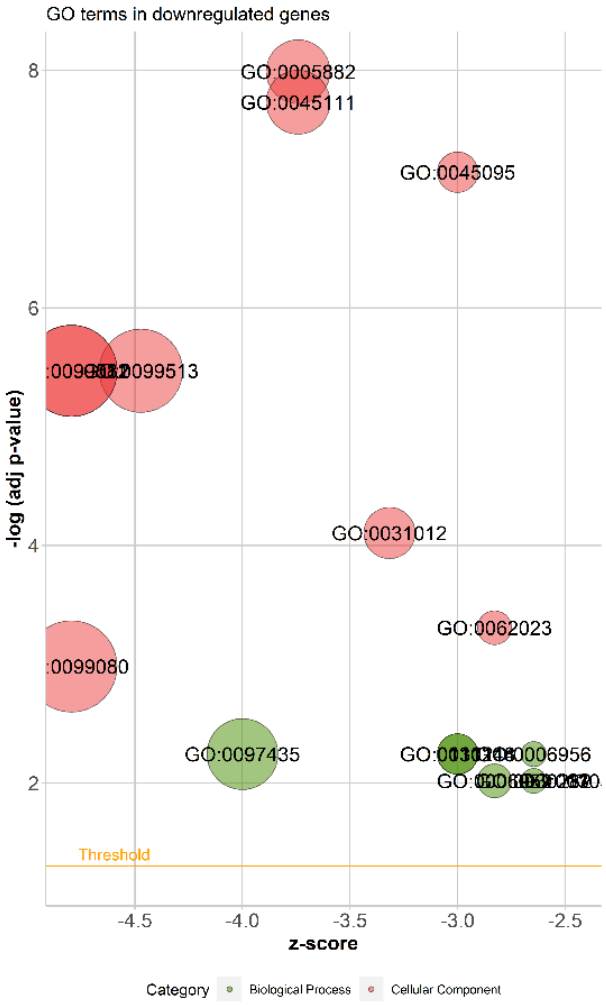
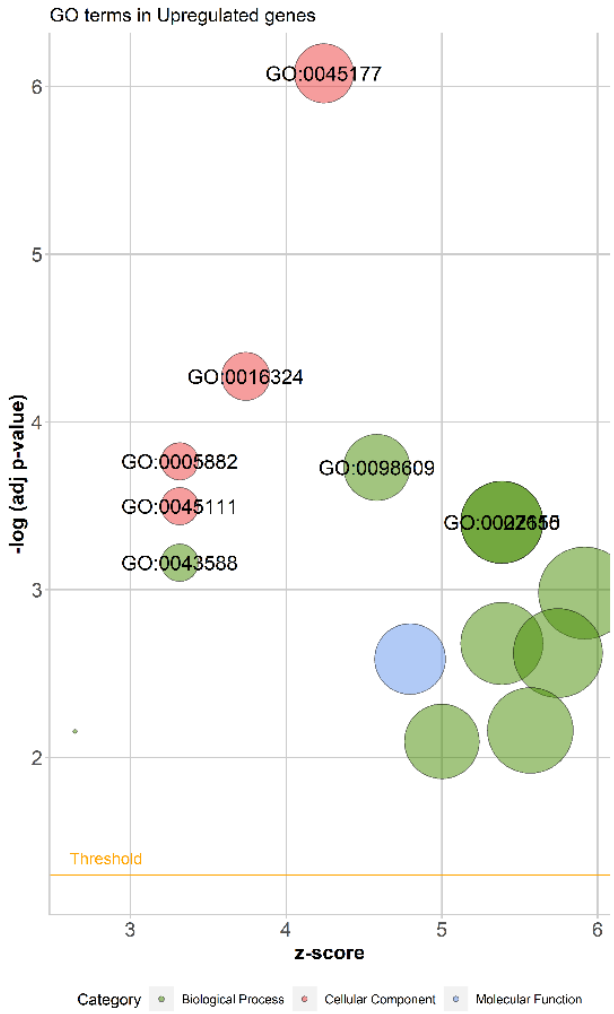


Figure 3.10. GO Chord plot of top overrepresented GO terms belonging to the Biological Process subontology. The genes are linked to their assigned terms via colored ribbons. Genes are ordered according to the observed log-fold change (logFC), which is displayed in descending intensity of red squares displayed next to the selected genes.



ID	Description
GO:0006958	complement activation
GO:0097435	supramolecular fiber organization
GO:0031214	biomineral tissue development
GO:0110148	biomineralization
GO:0030500	regulation of bone mineralization
GO:0070167	regulation of biomineral tissue development
GO:0110149	regulation of biomineralization
GO:0030282	bone mineralization
GO:0006959	humoral immune response
GO:0005882	intermediate filament
GO:0045111	intermediate filament cytoskeleton
GO:0045095	keratin filament
GO:0099512	supramolecular fiber
GO:0099513	polymeric cytoskeletal fiber
GO:0099081	supramolecular polymer
GO:0031012	extracellular matrix
GO:0062023	collagen-containing extracellular matrix
GO:0099080	supramolecular complex

Figure 3.11. Bubble plot showing significantly enriched Gene Ontology (GO) terms for the 519 downregulated genes based on adj P-value and Z-score. The y-axis represents the negative logarithm of the adjusted p value [false discovery rate (FDR)] for the GO terms, and the x-axis displays the z-score.



ID	Description
GO:0098609	cell-cell adhesion
GO:0007155	cell adhesion
GO:0022610	biological adhesion
GO:0043588	skin development
GO:0045177	apical part of cell
GO:0016324	apical plasma membrane
GO:0005882	intermediate filament
GO:0045111	intermediate filament cytoskeleton

Figure 3.12. Bubble plot showing significantly enriched Gene Ontology (GO) terms for the 749 upregulated genes based on adj P-value and Z-score. The y-axis represents the negative logarithm of the adjusted p value [false discovery rate (FDR)] for the GO terms, and the x-axis displays the z-score.

OVERALL SUMMARY AND CONCLUSION

Our data indicate that sole ulcerations can cause transcriptomics alterations leading to inflammatory-like conditions while altering keratinization of the hoof. There was a dysregulation of keratin synthesis in cows with sole ulcer associated with extracellular degradation of collagen. The transcriptomic data from the cows with digital dermatitis also showed a transcriptional shift among keratins. Some keratins were upregulated (e.g., *KRT10*) while others downregulated (e.g., *KRT34*, *KRTAP27*-1) under digital dermatitis conditions. Additionally, these data suggest that digital dermatitis induces programmed cell death and cornification of the affected skin. There was also an upregulation of the antimicrobial peptides in the cows with active painful digital dermatitis lesions compared to healthy ones. This was coupled with a decrease in the negative acute-phase protein, albumin, and a trend for an increase in the positive acute-phase protein haptoglobin, indicating a systemic inflammatory response. Our data suggest that digital dermatitis can alter skin paracellular epithelial permeability and keratinization. In short, the transcriptomic profiling from sole ulcer and digital dermatitis shows that there was a dysregulation of keratin synthesis coupled with extracellular degradation of collagen.

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