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ACUTE PHASE RESPONSE AND MILK YIELD IN DAIRY COWS DURING TOPICAL TREATMENT OF DIGITAL DERMATITIS

AKUUTSE FAASI VASTUS JA PIIMATOODANG PIIMAVEISTEL DIGITAALDERMATIIDI LOKAALRAVI AJAL

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Digital dermatitis (DD) is a worldwide spread contagious disease of cattle causing painful lesions on the interdigital skin of the animals' feet. Aetiology of the disease is not fully known yet, but according to current knowledge DD is a polytreponemal disease. DD is treated with topical antibiotic or non-antibiotic substances. Decreased milk yield has been observed together with DD. Lameness related to DD is considered to be one factor resulting in the loss of production and impaired welfare. Acute phase response (APR) is organisms' way to fight against trauma or pathogens via acute phase proteins (APPs). APPs are activated by cytokines, the protein hormones that have multiple crucial roles in maintaining homeostasis. There has been some evidence of systemic APR in relation with DD. The aim of the thesis was to evaluate the effects of topical DD treatment to the APR during a two-week study period. Also, the effect of DD to milk yield was evaluated. During the study period, fibrinogen was significantly elevated in cows with DD in comparison to the healthy control group. Haptoglobin and serum amyloid-A were not significantly elevated. Milk yield was observed for a 28-day period, but there wasn't a significant difference between study and control groups. These results indicate that topical DD treatment causes local inflammation which activates APR via fibrinogen.

Keywords: digital dermatitis, fibrinogen, milk yield

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Digitaaldermatiit (DD) on kogu maailmas levinud veiste nakkushaigus, mis põhjustab valulikke kahjustusi loomade sõranahal. Haiguse etioloogia ei ole veel täielikult teada, kuid praeguste teadmiste kohaselt on DD polütreponemiline haigus. DD-d ravitakse lokaalsete antibiootiliste või mitteantibiootiliste ainetega. Koos DD-ga on täheldatud piimatoodangu vähenemist. DD-ga seotud lonkamist peetakse üheks teguriks, mis põhjustab toodangu vähenemist ja heaolu halvenemist. Akuutse faasi vastus (*acute phase response* - APR) on organismi viis võidelda traumade või patogeenide vastu akuutse faasi valkude (*acute phase proteins* - APP) abil. APP-d aktiveerivad tsütokiinid, valguhormoonid, millel on homöostaasi säilitamisel mitu olulist rolli. On leitud tõendeid süsteemse APR-i kohta seoses DD-ga. Lõputöö eesmärk oli hinnata lokaalse DD-ravi mõju APR-le kahenädalase uuringuperioodi jooksul. Samuti hinnati DD mõju piimatoodangule. Uuringuperioodi jooksul oli DD-ga lehmadel võrreldes terve kontrollrühmaga märkimisväärselt suurenenud fibrinogeeni tase veres. Haptoglobiin ja seerumi amüloid A ei olnud märkimisväärselt tõusnud. Piimakogust jälgiti 28 päeva jooksul, kuid uuringu- ja kontrollrühma vahel ei olnud märkimisväärset erinevust. Need tulemused näitavad, et lokaalne DD ravi põhjustab lokaalset põletikku, mis aktiveerib APR-i fibrinogeeni kaudu.

Märksõnad: digitaaldermatiit, fibrinogeen, piimatoodang

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LIST OF ABBREVIATIONS

AMS	automated milking system
APP	acute phase proteins
APR	acute phase response
AUC	area under the curve
BCS	body condition score
CHDL	claw horn disruption lesions
CODD	contagious ovine digital dermatitis
CRP	c-reactive protein
DAMP	damage associated molecular pattern
DD	digital dermatitis
DIM	days in milk
ECM	energy corrected milk
Fb	fibrinogen
Hb	haemoglobin
Hp	haptoglobin
IL-1	interleukin 1
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IL-8	interleukin 8
IL-17	interleukin 17
INF-γ	interferon gamma
IP	interdigital phlegmon
LBP	lipopolysaccharide binding protein
NK	natural killer
NSAID	non-steroidal anti-inflammatory drug
PAMP	pathogen associated molecular pattern
PMR	partial mixed ratio
PRR	pattern recognition receptor
SAA	serum amyloid A
SU	sole ulcer
TNF-α	tumor necrosis factor alfa
UCD	udder cleft dermatitis
WLD	white line disease

INTRODUCTION

Bovine digital dermatitis (DD) is an infectious disease in dairy cattle and causes discomfort and lameness. Characteristic for DD are painful lesions in the interdigital commissure in hind legs (Evans *et al.*, 2016). DD has spread throughout the world and causes economical losses via decreased milk yield and fertility problems (Evans *et al.*, 2016). Despite being a local infection, some authors have described elevated cytokine and acute phase protein (APP) levels in the serum of affected animals, which indicates the presence of acute phase response (APR) (Ilievska *et al.*, 2019; Pirkkalainen *et al.*, 2024). Since DD has spread across the globe and its aetiology is not yet fully understood, it is important that the disease mechanism and how the body reacts to the disease is being studied further.

Purpose of this study was to examine the effects of topical DD treatment to the acute phase response during a two-week study period. Also, the effect of DD to milk yield was evaluated.

1. LITERATURE REVIEW

1.1.Hoof disorders

Lameness is considered one of the main causes of economic losses and early culling in dairy farms (Puerto *et al.,* 2021). Most common causes for dairy cattle lameness are hoof disorders which can be divided into infectious and non-infectious by their origin (Smith *et al*., 2010). Infectious hoof diseases are of bacterial origin and this kind of diseases are DD and interdigital phlegmon (IP). Non-infectious diseases include claw horn disruption lesions (CHDL), like sole ulcer (SU) and white line disease (WLD) (Murray *et al.*, 1996; Hoblet and Weiss, 2001; Amory *et al.*, 2008; Smith *et al.*, 2010).

1.2. Digital dermatitis

1.2.1. Pathogenesis of digital dermatitis

DD causes skin lesions, that are usually found in the interdigital commissure in the hind legs and that can be very painful (Evans *et al*., 2016). It was first described in 1974 in Italy by Cheli and Mortellaro (Cheli and Mortellaro, 1974). Pathogenesis of the disease is not fully understood. Some publications describe DD as a polytreponemal infection because of the presence of many different treponemes (Zinicola *et al*., 2015). Most frequently isolated treponemes from DD lesions include *Treponema medium*/*T. vincentii*-like, *T. phagedenis*-like and *T. putidum*/*T. denticola*-like (currently called as *T. pedis*) (Zinicola *et al*., 2015; Evans *et al*., 2016). Since these bacteria resemble treponemes found in humans the suffix "-like" is added (Evans *et al*., 2016). Treponemes are anaerobic gram-negative spirochetes (Newbrook *et al*., 2021). Researchers have been able to isolate these same bacteria also from the oral cavity, rumen and faeces of the cows which indicates that the gastrointestinal tract of the animals might serve as a reservoir for the bacteria causing DD (Zinicola *et al*., 2015; Evans *et al*., 2016). *Treponema phagedenis*-like bacteria are proven to be one of the factors which causes the poor healing of the lesion via immunosuppression (Zinicola *et al*., 2015; Evans *et al*., 2016). Treponemes have been associated to other hoof diseases with impaired wound healing (Staton *et al*., 2020). Unsuccessful skin inoculation of only *Treponema* spp. bacteria suggests that there are other factors contributing to the disease (Zinicola *et al*., 2015). Krull *et al*. (2014) described in their study the pathogenesis to be mainly bacterial based since the lesions are successfully

treated with antibiotics, inefficacy of monovalent vaccines and the inability to find fungal or viral isolates from the lesions.

Similar diseases in other species with the involvement of treponemes are contagious ovine digital dermatitis (CODD) in sheep and equine hoof canker in horses, but also syphilis in humans (Sullivan *et al*., 2015; Palmer and O'Connell, 2015; Marčeková *et al*., 2021). Treponemes have also been found in conditions like udder cleft dermatitis (UCD), ischemic teat necrosis and hock lesions (Sobhy *et al*., 2020). In addition to Treponemes, other bacteria like *Bacteroides* spp., *Borrelia burgdorferi*, *Campylobacter* spp., *Fusobacterium* spp. and *Guggenheimella bovis* are found from the DD lesions (Palmer and O'Connell, 2015; Evans *et al*., 2016).

1.2.2. Risk factors

Since the aetiology of DD is not fully comprehended, the risk factors are still partly to be solved. Palmer and O'Connell (2005) have summed up possible risk factors in their review article: early postpartum period, poor slurry removal, moisture in the barn, Holstein-Friesian breed or its crossbreeds, first or second lactation, increased herd size and conformation of the hooves. Since the transmission of the disease is thought to be skin-to-skin or from slurry to skin, increased contact time of slurry with skin increases the risk to develop DD (Palmer and O'Connell, 2015).

1.2.3. Scoring system

M-stage scoring system – the most common scoring system in use - has six stages and was created by Döpfer *et al*. (1997) and modified by Berry *et al*. (2012). The M comes from Mortellaros first initial (the veterinarian who discovered DD), and the disease is sometimes referred to as Mortellaro disease in the literature. The scoring is made based on the macroscopic changes on the skin. Lesions can be divided into active and chronic so that M1, M2 and M4.1 are active and M3 and M4 are chronic (Biemans *et al*., 2018). Active lesions, also described as ulcerative lesions, have characteristic strawberry-like appearance (Losinger, 2006; Evans *et al*., 2016). Active lesion that is under 2cm in diameter is described as M1 lesion and lesions over 2cm in diameter are described as M2; M4.1 is chronic lesion that has small active area on top of the chronic lesion; M3 is the healing phase, and its characteristic is non-painful brown rubbery scab; M4 is the chronic stage of DD lesion, where the appearance can vary (Döpfer *et al*., 1997 and modified by Berry *et al*., 2012). The colour of a chronic can range from tan to black and it might have hair-like hyperkeratotic papillomatous projections, or mass-like

elevation and the consistency of the lesion is usually rubbery and shaped irregularly (Döpfer *et al*., 1997). These chronic lesions are not painful (Berry *et al*., 2012). Because of these hair-like projections, some articles describe DD as hairy footwarts - this name is often used especially in North and South Americas and Australasia (Evans *et al*., 2016).

1.2.4. Treatment

The common treatment for DD lesions is topical. The treatment contains either antimicrobial products, like broad-spectrum tetracyclines, or products without antimicrobials, like copper and zinc chelates and salicylic acid (Schultz and Capion, 2013; Vanhoudt *et al*., 2022). The treatment might be combined with bandage on the hoof. Results of either treatment might not lead to complete recovery from the disease but turns the painful active lesions to non-painful chronic lesions (Vanhoudt *et al*., 2022). DD is also controlled with hoof bathing, and various substances are used in hoof baths.

1.3. Acute phase response

APR is a systemic reaction, and it is an organism's way to defend itself from pathogens and traumas and is activated when the local defence system is not able to fight against local inflammation (Gabay and Kushner, 1999; Ceciliani *et al*., 2012). APR activates multiple actions throughout the body including behavioural, physiological, nutritional and biochemical factors. Such actions are fever, leucocytosis, thrombocytosis, anorexia, decreased gluconeogenesis and cachexia (Gabay and Kushner, 1999; Ceciliani *et al*., 2012). The cascade of actions is initiated either by pathogen associated molecular patterns (PAMP) or damage associated molecular patterns (DAMP). In case of PAMP, parts of pathogen or its toxins and in the case of DAMP parts of destroyed cells are recognized by body's innate immune system via pattern recognition receptors (PRRs) (Ceciliani *et al*., 2012). Innate immune system consists of two stages: the body surfaces (skin and mucous membranes) with their physiologic properties like different pH or presence of enzymes act as a barrier for any invaders, and nonspecific immune defence which includes cells like monocytes, neutrophiles and macrophages. Innate immunity doesn't recognize antigens but works via PRRs and through those receptors it can help with activation of the adaptive immune system (Li and Wu, 2021). PRRs are not only present on the cell membranes but also inside the cells (Li and Wu, 2021).

APR is described in many conditions, like clinical mastitis, respiratory infections in calves and postpartum metritis in dairy cows (Suojala *et al*., 2008; Chan *et al*., 2010; Prohl *et al*., 2015)

1.3.1. Acute phase proteins

APPs can be categorized in two different ways: type 1 APPs are proteins that are produced after activation by interleukin 1 (IL-1) and type 2 APPs are proteins that are released after activation by interleukin 6 (IL-6) (Petersen *et al*., 2004). The other way to categorize APPs is how they act during the APR: either their concentration is increasing, which makes them positive APPs, or their concentration is decreasing, which makes them negative APPs (Gabay and Kushner, 1999; Petersen *et al*., 2004). These positive APPs are among others serum amyloid A (SAA), C-reactive protein (CRP), fibrinogen (Fb), alpha 1-acid glycoprotein and caeruloplasmin (Murata et al., 2004).

Type 1 APPs includes SAA and CRP. These are so called rapid reacting APPs, which means their concentration increases rapidly after inflammation occurs, but they also normalise fast when the inflammation has dissolved (Petersen *et al*., 2004). CRP is not a significant APP for cattle since its level doesn't change during inflammatory processes (Petersen *et al*., 2004). SAA is currently one of the main APPs used in inflammatory diagnostics in cattle (Constable *et al*., 2016). Fb and haptoglobin (Hp) are type 2 APPs - the level in the serum doesn't rise as fast as type 1 APPs, but it stays elevated longer (Petersen *et al*., 2004).

There are more positive APPs than there are negative APPs. Albumin is the most common negative APP but also transferrin is included in this group. The positive APPs are produced in hepatocytes in the liver, and they are released when pro-inflammatory cytokines are activated (Murata et al., 2004).

Different APPs have different tasks during inflammation, but their general role is to assists in elimination of foreign objects or organisms, repair tissues and degrade dead tissue to regain homeostasis in the body (Kushner, 1982). Hp is not normally present in serum of bovines but in case of inflammation or trauma its concentration in serum can increase over ten times (Petersen *et al*., 2004). Its main task is to avoid the loss of iron when haemolysis is occurring by binding haemoglobin (Hb), but also immunomodulation, inhibition of phagocytosis, bacterial activity, and chemotaxis of granulocytes (Petersen *et al*., 2004). Fb is crucial in maintaining the homeostasis of the body and repairing occurred damage in the tissues. (Murata *et al*., 2004). For SAA, the specific task is yet to be discovered but there are suggestions that it

inhibits fever, immune response, and oxidative effects of neutrophilic granulocytes (Petersen *et al*., 2004).

APPs have significant role in the diagnostics in veterinary medicine and they are the easily measurable characteristic for implementing the severity of inflammation (Constable *et al*., 2016). Commonly measured APPs from bovines are SAA, Hp and Fb. Occasionally also other APPs, like albumin, are measured (Constable *et al*., 2016).

1.3.2. Cytokines

When PRRs on the outer membrane of circulating monocytes recognizes either PAMPs or DAMPs, they start to secrete pulses of pro-inflammatory cytokines to the bloodstream (Petersen *et al*., 2004). These pro-inflammatory cytokines are protein hormones and different cytokines have different tasks. Pro-inflammatory cytokines include interleukin 1 beta (IL-1β), IL-6, interferon gamma (INF-γ) and tumor necrosis factor alfa (TNF-α) (Petersen *et al*., 2004; Ilievska *et al*., 2019). TNF-α enhances the IL-1 production, activates the T- and B- lymphocytes and natural killer (NK) cells, provokes endothelial damage locally and suppress the synthesis of proteins which leads to destruction of cells which leads to cachexia (severe condition in which the body mass is lost due to chronic aggressive inflammatory reaction) (Gabay and Kushner, 1999; Petersen *et al*., 2004). IL-1 cytokines have various tasks in various locations. In bone marrow, they regulate the production of B-lymphocytes, in the liver they initiate the release of type 1 APPs, and they stimulate the release of IL-6 from many different cells (Petersen *et al*., 2004). IL-6 inhibits the production of IL-1 and TNF-α, induces fever, causes pathologic pain, stimulates the differentiation of T lymphocytes and stem cells, and induce type 2 APP production (Petersen *et al*., 2004).

1.4. Acute phase response and hoof disorders

Inflammatory response is widely studied in other pathologies of bovines but there are relatively few publications about hoof disorders. Studies including hoof diseases like SU, WLD and IP have indicated the presence of APR in association with clinical presentation of these hoof diseases (Tóthová *et al*., 2011; Kontturi *et al*., 2020).

Bagga *et al*. (2016) examined in their study the APR in lame dairy cows. The causes of the lameness in this study were SU, white line fissure and sole haemorrhage. They discovered significant increase in serum APPs (SAA, Hp, Fb and CRP) in the lame cows compared to the

non-lame control group. Ilievska *et al*. (2019) showed similar results in their study as cows with SU had significantly elevated SAA and Hp levels compared to control group. Pirkkalainen *et al*. (2022) discovered also increased SAA values in their study on cows with SU but not significantly higher Hp values.

Kontturi *et al*. (2020) studied APR in cows with naturally occurring IP and they discovered that the Hp and SAA levels increased, and albumin decreased significantly in affected animals. They also discovered association between the severity of the disease and level of elevation of the positive APPs. Smith *et al*. (2010) also described significant APR in cows with IP.

1.4.1. Acute phase response and DD

Studies of APPs and hoof disease often summarize the hoof diseases and there are not many results related to specific hoof disease. In this kind of studies, it is described that cows with DD show increased values of APPs in their blood (Tóthová *et al*., 2011). Smith *et al*. (2010) studied the Hp levels in cows with DD, but the problem was the small population of animals infected with DD. Of the four cows having DD lesions, one cow had elevated Hp levels which decreased three days after the treatment. More recent study has been able to identify increased SAA values in cows with DD lesions compared to healthy control group, but the results haven't been significant (Pirkkalainen *et al*., 2022). Ilievska *et al*. (2019) were able to identify significantly elevated Hp and SAA values in group of cows (n=15) with DD suggesting that DD causes strong acute phase response. Possible reason for such controversial results might be due to different stage of the disease (Pirkkalainen *et al*., 2022). In a recent study, the authors detected elevated cytokine levels in cows with DD, but not elevated APP levels (Pirkkalainen *et al*., 2024).

Recruiting of neutrophiles and other immune cells to epidermis and dermis is most likely initiated by pro-inflammatory cytokines like IL-6 and interleukin 8 (IL-8) (Newbrook *et al*., 2021). In case of treponemes, the immune response is suggested to be activated also by interleukin 17 (IL-17) (Wang *et al*., 2014). IL-17 is known in different diseases of humans, like psoriasis, and it is the targeted mechanism in the treatment of this diseases (Krueger *et al*., 2012). Newbrook *et al*. (2021) described in their study how the different phylogroups of treponemes act in DD lesions. They found out that *T. phagdenis* and *T. pedis* might be the factors that inhibit the acute phase response. In the study they also discovered that treponemes target the actin cytoskeleton, which is important component in repairing tissues and maintaining

the structures of the cells. Treponemes can also cause loss of focal adhesions vie gene upregulation which results in loss in cell-to-cell adhesions and thus, the bacteria are able to go and persist deep within the skin (Newbrook *et al*., 2021).

1.5. Hoof disorders and milk yield

Green *et al*. (2002) studied the effect of lameness to milk yield in dairy cows. The most common reasons for lameness in their study was SU, WLD, IP or DD. Similar study was performed by Hernandez et. al (2002) and the same hoof disorders were also the main reason for lameness in their study.

High yielding cattle are more prone to develop SU and WLD, than those with average or belove average milk production (Amory *et al*., 2008). SU and WLD are one of most common hoof disorders encountered in dairy cattle (Murray *et al*., 1996; Amory *et al*., 2008). Amory *et al*. (2008) described in their study the average estimated loss of energy corrected milk (ECM) during one lactation to be 570 kg in cows affected with SU and 370 kg in cows affected with WLD. Hernandez *et al*. (2002) described approximately 200 kg decrease in production within cows affected by sole or white line lesions compared to healthy control group.

Cows with IP had significantly decreased milk yield (approximately 855 kg less) compared to healthy control group in a study performed by Hernandez *et al*. (2002). In their study only 9 % of the study population of 167 cows showing signs of lameness had IP but of these IP cows, 60 % was culled during the lactation because of the disease (Hernandez *et al*., 2002). This shows the severity of the condition.

In case of SU and WLD, the cows tend to produce more milk before the diagnosis but fail to return back to high production after diagnosis and treatment (Amory *et al*., 2008). The gradual decrease of production in SU cows happens already 3 months prior to the diagnosis, but in cows with WLD the drop of production starts more closer to the date of diagnosis (Amory *et al*., 2008).

1.5.1. Digital dermatitis and milk yield

Murray *et al*. (1996) stated in their study which included 13,828 cows of which 8991 individuals showed the clinical signs of lameness that DD was the most common of the skin lesions to cause lameness within this study group. The study was performed between the years of 1989

and 1991 when DD was just recently discovered in the UK. Cows with DD showed behavioural changes, like standing while ruminating, not lying as much as the control group, drinking more water, showing vigilance and decrease in milk yield. Behavioural changes were especially present at 2-3 weeks after the treatment for DD (Pavlenko *et al*., 2011). Salem *et al*. (2023) recently published a study in Egypt, and they found out that 6,4 % of the cows with DD were clinically lame. The prevalence of lameness of cows diagnosed with DD in a Dutch study was 39 % (Frankena *et al*., 2009). This variation between studies can be partly explained by the difference in housing and management of the cows (Plummer and Krull, 2017). Lameness is solemnly cause for decreased milk yield in dairy cows (Rajala-Schultz *et al*., 1999)

Amory *et al*. (2008) described in their study the association of DD to the ECM produced. In the study, a significant decrease was discovered in produced ECM in cows with DD compared to the control group in both, two to three weeks after hoof trimming and five to six weeks after hoof trimming. In their study they also discovered that commonly cows that get DD aren't the highest yielding cows, but rather below the average of the herd in production 5 months before the DD diagnose. After successful treatment for DD, the infected cow's milk yield rose just a little above the average of the herd five months after the diagnosis (Amory *et al*., 2008). In French study they discovered 0,57 kg/day decrease in milk yield in cows with DD (Relun *et al*., 2013).

2. AIM OF THE THESIS

The aim of this randomized clinical trial was to evaluate the effects of topical DD treatment to the acute phase response in dairy cows during a two-week study period. Also, the effect of DD to milk yield was evaluated.

3. MATERIALS AND METHODS

3.1. Study farm

The study was performed in a commercial Estonian dairy farm. The farm consisted of two buildings which were connected to each other via corridor. In one building there were two separate sections for cows in milk, one with milking parlour and other with automated milking system (AMS). Each section had approximately 60 cubicles covered with rubber mattresses and sawdust was used as bedding material. The floors were covered with rubber mats and automated scraper was used to remove manure from the aisles. Cows in the AMS section had partial mixed ratio (PMR) ad libitum and parlour side had regulated feeding system of the same PMR. Both groups had additional concentrate feeders. In the other building, the dry cows and youngstock were housed.

3.2. Study population

The study population was chosen during the regular hoof trimmings, which were performed every 4-5 months. The data was collected during three different hoof trimmings that took place in February 2022, November 2022 and April 2023. Date of the hoof trimming was the day 0 (d0) of the study. Every cow was visually evaluated before they entered the trimming chute for lameness in the scale of 1-3, in which 1 was non-lame and 3 was strong lameness. Also body condition score (BCS) was evaluated in the scale of 1-5. Cows were also visually inspected for any signs of other conditions. In the trimming chute, all the hooves were inspected for DD lesions. In a case lesion was found, it was scored according to the M-scoring system and examined for lesion pain in scale 1-3 in which one was no pain and three was strong pain. After evaluation, the cows were divided into two different treatment groups so that every other cow was treated with DD-Delete White Line paste (salicylic acid; DD-Delete; CowCare, Valmiera, Latvia) and the others with Intra Hoof-fit gel (chelated copper and zinc; Intra Hoof-fit; Intracare, Veghel, The Netherlands). After the administration of treatment, gauze was placed on top of the lesion and the hoof was bandaged with elastic wrap and on top of that self-adhesive wrap. From every cow included into the study group, EDTA and serum blood samples were drawn from the milk vein with vacuum method. Control group was created by choosing the individuals with no hoof lesions or any signs of other conditions, and EDTA and serum blood samples were drawn. In total the study group consisted of 41 DD cows and control group consisted of 28 cows.

The cows belonging to the control or study groups were visually examined in a trimming chute again at days 3 (d3), 7 (d7), 10 (d10) and 14 (d14). Additionally, EDTA and serum blood were taken; BCS, lameness score and lesion pain score were evaluated. DD lesions were examined, scored blindly by the study veterinarian via smartphone and the animal was retreated if the lesion was still in active form. The data was collected after each hoof trimming to Excel-file as shown in the Table 1. Daily milk yield, days in milk (DIM), parity and date of birth were gathered from the farms data base, Vissuke (Estonian Production Animal Recording System). All this data was then managed together in a separate Excel-file.

Blood samples were stored in the fridge in the farm and transported to the Estonian University of Life Sciences. The serum and plasma were separated by centrifugation (2500 rpm) and stored in the freezer at -20 ºC. When all the samples were collected, they were analysed for Fb, Hp and SAA.

Table 1. Data recording sheet of dairy cows with digital dermatitis lesions during the study period.

Evaluation day	Animal ID	Evaluation date	Feet	Diagnosis	Treatment	Pain score	Lameness score	BCS
d0	3578	28/11/2022	2	5	2	2	-	2.5
d3		01/12/2022		5	2	2	2	-
d7		05/12/2022		1	2	2	2	3
d10		08/12/2022		3	-	1	2	-
d14		12/12/2022		5	-	1	2	3
d0	8661	28/11/2022	1	5	2	2	1	3
d3		01/12/2022		5	2	1	1	-
d7		05/12/2022		5	2	1	1	3.25
d10		08/12/2022		3	-	1	1	-
d14		12/12/2022		5	-	1	1	3
d0	8676	28/11/2022	1	5	1	2	-	3.5
d3		01/12/2022		5	1	1	1	-
d7		05/12/2022		1	1	1	1	3.5
d10		08/12/2022		1	1	1	1	-
d14		12/12/2022		3	-	1	1	3.5

Code explanation: Feet: 1 – Left hind foot, 2 – Right hind foot
Diagnosis: 0 – Healthy, 1 – DDM1, 2 – DDM2, 3 – DDM3, 4 – DDM4, 5 – DDM4.1/M4.2
Treatment: 1 – DD-Delete, 2 – Intra Hoof-fit
Pain score: 1 – No pain, 2 – Moderate pain, 3 – Strong pain
Locomotion score: 1 – No lameness, 2 – Moderate lameness, 3 – Strong lameness
BCS – Body condition score: Scale from 1-5

3.3. Laboratory methods

For SAA analysis from the serum sample, a commercial enzyme linked immunosorbent assay (ELISA) kit (Phase Serum Amyloid A Assay (SAA) – Multispecies, Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland) was used according to the manufacturer's instructions. Detection limit of the assay was 0.3 mg/l.

Serum Hp analyses were performed according to the Hb-binding assay suggested by Makimura & Suzuki (1982) with a modification when tetramethylbenzine (0.06 mg/ml) was used for a substrate and microtitration plates were used (Alsemgeest *et al*., 1994). For the standard curves pooled lyophilized aliquots of bovine acute phase serum with known Hp concentration were used. Detection limit of the assay was 60 mg/l.

From EDTA blood plasma Fb concentrations were analysed using the heat precipitation method (Millar *et al*., 1971). Fb concentrations were expressed as g/l.

3.4. Statistical analysis

For the raw data management and descriptive statistics Microsoft Excel (Microsoft, Redmond, Washington, USA) was used.

To analyse DD treatment effect to the cow's inflammatory response the area under the curve (AUC) was calculated using a trapezoid method for APPs (Fb, Hp and SAA) concentrations during study period (from treatment day to 14 days). AUC values of APPS were used as response variable in tree linear regression models were cow group (healthy controls and DD treatment with DD-Delete or Intra Hoof-fit groups) was included as categorical 3-level explanatory variable. For eliminating possible effect of different initial APPs concentrations at the beginning of the study cows APPs concentration at treatment was included as continuous variable to every model. For controlling possible confounding effect initially days in milk was included as continuous and cow lactation number as 2-level (first lactation cows and two or more lactations cows) categorical explanatory variables in all models. A stepwise backward elimination technique was used for final models. As APPs AUC variables were not normally distributed logarithmic transformation on Fb AUC and SAA AUC was used. For Hp AUC reciprocal square root transformation was used.

To analyse possible effect of DD to the cow's milk production linear regression model was used. In this model cows average day milk production over the study period (day milk production from the treatment day until 28 days after the treatment) was used as response variable and 2-level cow group variable (healthy controls and DD cows) were used as categorical explanatory variable. For controlling possible confounding effect days in milk was included as continuous and cow lactation number as 2-level (first lactation cows and two or more lactations cows) categorical explanatory variables were included, and a stepwise backward elimination technique was used for final model.

The results were considered statistically significant when the p-value was ≤ 0.05. Statistical analyses were conducted by Stata® IC14.2 (StataCorp, Texas, USA) management.

3.5. Ethics

This study has been approved by the Agriculture and Food Board of Republic of Estonia (1.2-17/39).

4. RESULTS

4.1. Acute phase response

The study group consisted of 69 cows in total, 28 control cows and 41 cows with DD. The DD group was divided into two different treatment groups according to the treatment that was used. 21 cows were in treated with DD-Delete and 20 cows with Intra Hoof-fit. Fibrinogen was elevated at d0 on both treatment groups when compared to control group (Table 2).

Table 2. Linear regression analysis for evaluating the differences of serum fibrinogen area under the curve (log(g/l)) during the study period (from hoof trimming (day 0) to 14 days) in control and study groups.

Variables	**n**	**Coefficient**	**SE**	**p-value**	**Wald test p-value**
Fibrinogen at day 0 (g/l)	69	0.104	0.009	<0.001	
Treatment group:					<0.001
Control	28	0			
DD-Delete	21	0.096	0.029	0.002	
Intra Hoof-fit	20	0.119	0.031	<0.001	
Intercept		3.591	0.050	<0.001	

SE – standard error

AUC method was used to evaluate the presence of Fb, Hp and SAA in the blood in the study cows in comparison to the control cows during the study period. Figure 1 illustrates the results of this analysis. In both treatment groups, the Fb was elevated throughout the study period compared to the control group. In comparison between the treatment groups, Intra Hoof-fit showed bigger elevation in Fb levels than DD-Delete group. In SAA and Hp levels there weren't significant (p-values >0.238) differences between the study and control groups.

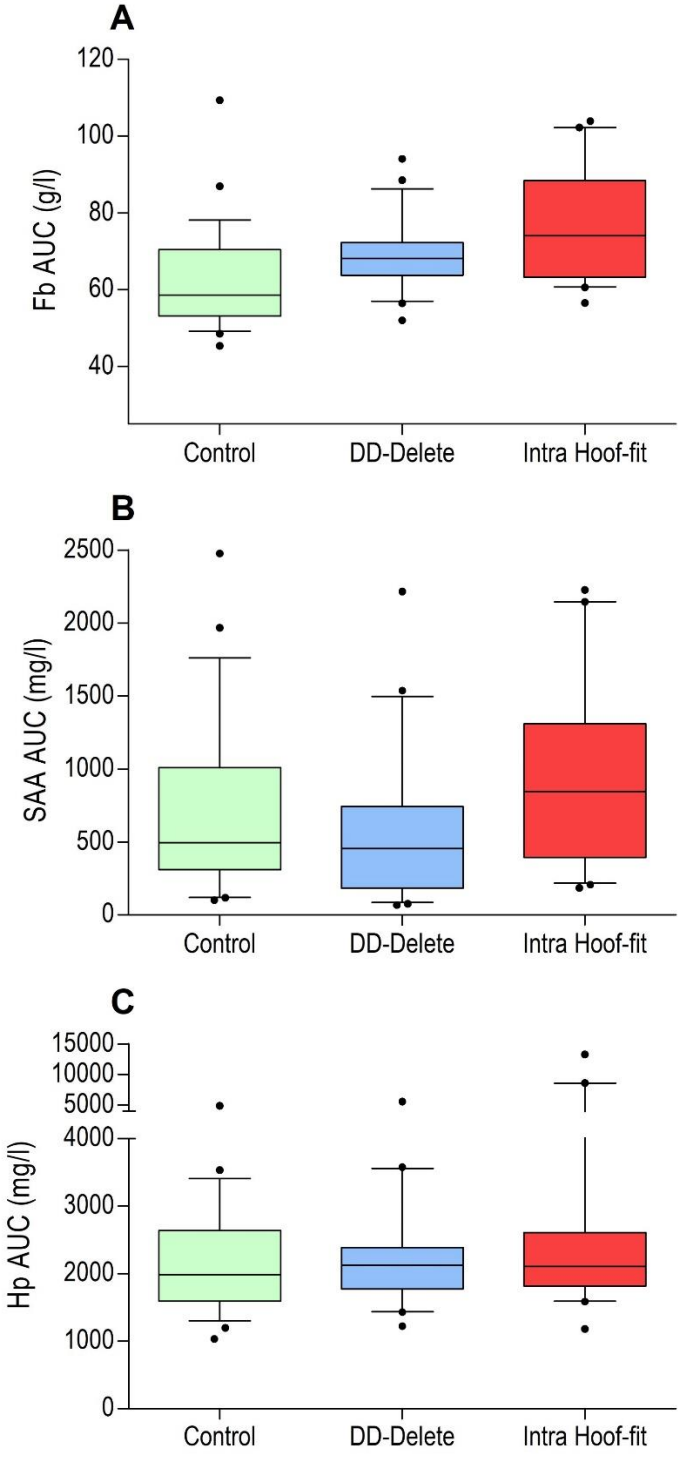

Figure 1. Area under the curve (AUC) during study period (from treatment to 14 days) of fibrinogen (Fb; A), serum amyloid A (SAA; B) and haptoglobin (Hp; C) concentrations by study groups: healthy control cows (n = 28), digital dermatitis cows DD-Delete (n = 21) and Intra Hoof-fit (n = 20) treatment groups. Boxes represents median and interquartile range, and the whiskers indicate 10-90% of the samples.

4.2.Milk yield

For clarity, the two treatment groups were united for one DD group for milk yield comparison. The study group for milk yield consisted of 37 cows in total, 20 with DD and 17 control cows. Regression model was used to evaluate the influence on milk yield between certain factors as shown in Table 3. DIM and cows in their second or later lactations were shown to be significant factors in relation to milk yield (p-values <0.001 and 0.011). Having DD was not affecting the milk yield significantly (p-value 0.731).

Table 3. Linear regression analysis to evaluate the effect of lactation number and digital dermatitis on the milk yield of dairy cows.

Variables	n	Coefficient	SE	p-value
Days in milk[1]	37			<0.001
Lactation category:				
1	18	0		
>1	19	5.38	2.01	0.011
Digital dermatitis:				
No	17	0		
Yes	20	-0.72	2.08	0.731
Intercept		37.38	2.79	<0.001

SE – standard error

Table 4. Average milk yield in kilograms between 0-28 days after the hoof trimming in control and study groups.

Variable	Control (n = 17) mean (±SD)	Digital dermatitis (n = 20) mean (±SD)
Milk 0	29.5 (10.84)	30.6 (9.99)
Milk 3	30.1 (10.84)	33.1 (9.33)
Milk 7	31.9 (11.15)	32.5 (8.38)
Milk 10	25.3 (9.02)	30.5 (7.51)
Milk 14	28.4 (10.33)	31.9 (7.35)
Milk 21	29.6 (9.52)	29.3 (8.44)
Milk 28	27.7 (10.62)	27.5 (7.67)
Mean milk (0-28)	28.9 (10.03)	30.8 (7.02)

Milk 0 – milk yield (kg) on the day of hoof trimming, Milk 3 – milk yield (kg) three days after hoof trimming, Milk 7- milk yield (kg) seven days after hoof trimming, Milk 10- milk yield (kg) ten days after hoof trimming, Milk 14- milk yield (kg) 14 days after hoof trimming, Milk 21- milk yield (kg) 21 days after hoof trimming, Milk 28- milk yield (kg) 28 days after hoof trimming, Mean milk – average milk yield during the study period (0-28 days). Study groups were merged for this analysis.
SD – standard deviation

Results were also analysed day-by-day basis on days 0, 3, 7, 10, 14, 21 and 28. Average milk yields of each group on each day were measured and the mean milk yield for the study period was calculated for both groups (Table 4). During this period the control group had average milk yield of 28.9 kg and the DD group had 30.8 kg.

5. DISCUSSION

We discovered that serum fibrinogen levels were significantly elevated during the topical treatment of DD lesions in both treatment groups. Similar statistical analysis was performed to Hp and SAA, but there wasn't significant difference compared to the control group. This supports the theory that both treatments activate the APR in the body and participate in the healing process of DD lesion. Schultz and Capion (2013) described in their study that salicylic acid is effective as treatment for DD and Klawitter *et al.* (2019) described that Intra Hoof-fit is also an effective treatment. These effective nonantibiotic treatments are important in decreasing the usage of antibiotics to prevent the antimicrobial resistance, and thus, it is crucial to understand why these treatments works for DD. Our findings suggests that both of these treatments cause some form of local irritation in the area of the lesion and thus activate the APR and help the lesion to heal. Fibrinogen might also have a crucial role in the healing process of the lesions, since one of its derivates, fibrin, is important factor in the wound healing process (Clark, 2001). This might be one of the reasons why fibrinogen was elevated, and other measured APPs not.

Salicylic acid and its derivates have been used for centuries as non-steroidal anti-inflammatory drugs (NSAIDs) and as topical agents in various of conditions in humans and animals (Thomas, 2006). Takiya *et al.* (2019) used oral administration of sodium salicylate – sodium salt of salicylic acid – in cows postpartum and they discovered enhanced APR via elevated Hp in the blood, and elevated TNF-α and lipopolysaccharide binding protein (LBP) levels in the adipose tissue of the treatment group when compared to nontreated control group. Their findings support our assumption that salicylic acid may activate the APR. However, it must be noted that the administration route is different in our study.

Copper (and zinc) sulphate footbaths have been used to prevent and control DD, and the results have been better than with formalin-based footbaths (Holzhauer *et al.*, 2012). The issue with copper and zinc sulphate is their detrimental effects on environment (Holzhauer *et al*., 2012). In their other study, Holzhauer *et al.* (2011) stated, that despite copper and zinc are harmful to the environment, the use of small amounts directly on the lesions as treatment, didn't pose a risk for the environment. In Estonia Intra Hoof-fit gel is registered as a topical drug and has no withdrawal time. Zinc has been used in different forms to aid in wound healing for a long time

(Schwartz *et al.*, 2005). Zinc has been described to aid in wound healing, to have anti-inflammatory properties and even participate in cytokine regulation locally (Schwartz *et al*., 2005). There are relatively few studies about copper in relation to wound healing and APR. Since in Intra Hoof-fit the copper is in chelated form, it needs to go through demetallation to form copper ion, which is the active form (Kirsipuu *et al.*, 2020). In human serum this takes approximately 30 minutes (Kirsipuu *et al*., 2020). The exact mechanism of how copper assists in wound healing is not completely understood, but copper takes part in cytokine modulation, has antimicrobial properties, and has a role in angiogenesis (Kornblatt *et al.*, 2016). Further research to understand the mechanism of action for Intra Hoof-fit is important.

For the comparison of the milk yield the two treatment groups were united to one DD group. In our study there wasn't significant difference in the milk yield between the control and study groups. There wasn't any significant difference between the study and control group even when we did the comparison on daily basis. This might be due to the small population size. However, there was a difference between milk yield of older cows when compared to first calvers, indicating the data was accurate. Also DIM showed to be significant factor affecting the milk yield. To get reliable results regarding milk yield in DD cows, the study population needs to be significantly larger.

Despite in our study the production didn't drop in DD cows, the discomfort and pain that DD causes is a significant welfare issue in dairy cattle worldwide. Since the disease and its pathophysiology and healing process are not fully understood yet, it is important that DD is studied even further. Especially, the participation of APR in the local disease is crucial to investigate even more. For future studies, it would be interesting to measure the APPs more frequently and already from day one after the treatment, since this kind of studies where APPs were measured as early as day three after the treatment haven't been done before. Pirkkalainen *et al*. (2022) did not find elevated APP levels in DD cows, but they took samples on days seven and ten after hoof trimming. The measurement of cytokines already on early days after the treatment would be interesting, since Pirkkalainen *et al.* (2024) had discovered the elevated cytokine levels in DD cows. In addition to these above mentioned, also the histological samples of the lesions would be interesting to study since it is suspected that some treponemes can alter the wound healing process (Zuerner *et al.*, 2007). In our study the lesions were macroscopically healed so we can make assumption that both of the treatments had some effects on these treponemes, either directly causing alterations to the bacteria or via activating the local

inflammation and APR, or both. By examining the histology, it might give more information about the bacteria in the lesion and how the tissues respond to treatment. By understanding the disease better, it might be possible to try to prevent and cure it more efficiently and enhance the wellbeing of the animals.

In this randomized clinical study, the admitted cows were randomly divided into two treatment groups. It can be seen as a strength as well as the fact that there was control group, and that the scoring of the lesions was blinded. Also, this study had a practical approach – treatment of DD – and thus, gives important and useful information to the practitioners working in the field. Weakness in our study was small study population and due to technical issues, some of the milk data was not available. To compare the treatment effect, it would be ideal to have a positive control group – cows with active DD lesions, that are not treated. However, it would be unethical to leave painful lesions untreated.

CONCLUSIONS

Cows with DD had a higher serum fibrinogen level compared to healthy control group. This indicates that topical treatment activates APR and healing process of DD lesions. No difference was found between milk yield of DD cows and healthy control cows.

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APPENDICES

APPENDIX 1. Non-exclusive licence for depositing the final thesis and opening it for the public and the supervisor's (supervisors') confirmation for allowing the thesis for the defence

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In Tartu, 09.05.2024

The core supervisor's approval for the final thesis to be allowed for defence

This is to confirm that the final thesis is allowed for defence.

Hertta Pirkkalainen 09.05.2024
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Supervisor's name and signature Date

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Supervisor's name and signature Date