THE MILK WITHHOLDING TIME OF SALICYLIC ACID FOR TREATMENT OF DIGITAL DERMATITIS IN DAIRY CATTLE

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Digital dermatitis is a top cause of lameness in dairy cattle that results in ulcerative lesions on the feet. Topical salicylic acid has been shown to provide similar efficacy to the antibiotic drugs used previously, but there is no milk withholding time established in the United States. The objective of this study was to provide data in order to establish this withholding period. A secondary objective was to evaluate outcomes among treatments. Treatment groups were topical applications of the following drugs: salicylic acid paste, salicylic acid powder, and tetracycline. The lesions were scored at day 0, day 7, and day 28 post-treatment. Milk samples were collected the day before treatment, 4 hours, 8 hours, 24 hours, 36 hours, and 48 hours post-treatment. Results indicated that most cows did not show detectable levels of salicylic acid after 24 hours.

Key Words: digital dermatitis, dairy cow, salicylic acid, milk withdrawal

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-Kelsey Wirt

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

DD.....Digital dermatitis

SA .....Salicylic acid

SPA .....Salicylic acid paste

SPO .....Salicylic acid powder

TPO.....Tetracycline powder

ng.....nanogram

ml .....milliliter

h.....hour

d.....day

ELDU.....Extra Label Drug Use

VFD.....Veterinary Feed Directive

VCPR.....Veterinary Client Patient Relationship

g.....gram

LLOQ.....Lower Limit of Quantification

LOD .....Limit of Detection

CHAPTER 1. INTRODUCTION

The objective of this thesis document is to evaluate the different components of the research that was completed to provide data towards a milk withholding time for the use of salicylic acid for digital dermatitis in dairy cows. This document is composed of a literature review, a manuscript identifying milk withholding times and clinical efficacy of tetracycline and salicylic acid, and a conclusion chapter.

The literature review highlights the current concepts of digital dermatitis including the disease process, common treatment options, comparisons of the different treatments, economic impact of the disease, and the overall purpose of the need for alternative treatments.

The main objective of the manuscript is to establish data for a milk withholding period for salicylic acid in milk. A secondary objective is to compare the efficacy of tetracycline versus salicylic acid in treating digital dermatitis in dairy cattle.

The conclusion chapter depicts the overall experience of working towards a Master's degree. This will include what I've learned, challenges along the way, and how this all played a role in final product.

CHAPTER 2. LITERATURE REVIEW

Lameness is defined as any abnormality in gait or stance and it remains one of the top welfare issues in the dairy cattle industry (Cramer et al., 2008). This condition is most commonly due to contagious or non-contagious sources resulting in pain upon locomotion in the legs and feet of dairy cows (Murray et al., 1996). As a result of this condition, many cows are unable to perform as well as they would when healthy. They spend more time lying down, and consequently have a decreased consumption of feed since they are not spending as much time at the feed bunks. This can have a negative effect on their milk production, as well as their reproductive capabilities (Evans et al., 2016). Quality milk production and reproduction two key objectives when having a successful dairy operation, so this is an important area of interest to improve on.

Lameness can be caused by a number of conditions including infectious and non-infectious sources. Of the infectious sources, digital dermatitis (DD) has the highest prevalence (Cramer et al., 2008; USDA, 2009; Solano et al., 2016). The most common non-infectious sources include sole ulcers, sole hemorrhage, and white line disease (DeFrain et al., 2013). DD is an ulcerative or hyperkeratotic lesion that affects the plantar aspect of the interdigital cleft space of the foot (Cramer et al., 2008). An ulcerative lesion has a red, strawberry-like appearance that is equal to or larger than 2 cm, and is painful to the touch (Capion et al., 2018). These red lesions are considered to be the active and "infective" stage where they can spread the disease to other animals. The hyperkeratotic lesions are considered to be the "healing" stages of this disease and exhibit a large scab covering the previously affected area. The typical population of animals affected by this disease are lactating cows, being more susceptible during their first lactation (DeFrain et al., 2013). Lesions are most commonly found on the hind limbs

and can result in a great amount of pain to the animal which can lead to decreased milk production, reproductive capabilities, and overall welfare (Evans et al., 2016). The causative agent has been thought to be a multifactorial group of *Treponome* spp. spirochete bacteria (Dopfer et al., 1997; Capion et al., 2018). Many studies that have collected histopathology samples have found these species of bacteria present in active lesions. It is thought that the bacteria can encyst deep into the layers of the epidermis and form a protective layer against antibodies or other drugs, making it a nearly impossible disease to fully eradicate (Hartshorn et al., 2013). These bacteria can also live in areas of feces or debris making dairy barns a high risk of keeping the disease around. It is also thought that the more painful the cow's feet are, the more time they will spend off of them by laying down. By doing this, they potentially expose their hind limbs to feces in the alleyways more frequently. Many studies have found that keeping a clean and dry housing area is best to help manage the spread of DD.

This disease has been found to show a natural progression between various stages. These stages have been defined and categorized by multiple researchers (Dopfer et al., 1997; Berry et al., 2012), most commonly including the distinction of no lesion being present (M0) followed by the remaining stages: early stage lesion being less than 2 cm in size (M1), infective and clinical stage being red in appearance and equal to or greater than 2 cm in size (M2), healing stage being characterized by a scab or hyperkeratosis around the lesion (M3-M4), and chronically infective stage being a hyper keratinized scab with a small focal active lesion within it (M4.1).

Figure 2.1. Photos of the lesion scores: (A) M1, (B) M2, (C) M3, (D) M4, (E) M4.1; Letter A is from Zinicola et al., 2015 while letters B-E are photos from the project described in Chapter 3.

Research has shown that the natural progression of a new lesion to develop from M0 to ≥ M2 takes an estimated 133-146d (Krull et al., 2016; Relun et al., 2013). Certain studies have evaluated the length of time it takes for the lesion to transition between each stage. Based on their results, it is thought that these lesions can transition between stages within a week time frame (Biemans et al., 2018). Biemans et al. established that the M4 stage occurs for the longest period of time before transitioning to another stage. The two stages that are considered infective are M2 and M4.1, however there could be potential that the M4 stage plays the largest role in reinfection due to the fact that lesions stay in this stage the longest (Biemans et al., 2018). Biemans et. al (2018) found a transmission rate for every one cow infected with DD, there were two cows that developed DD lesions. It is thought that a lesion becomes chronic once it reaches the M4 stage. The likelihood of it returning to M0 (healthy skin, no lesion) is low once the lesion is seen at this stage due to the difficulty of treatment penetrating through the hyper keratinized skin layers. This aspect will be looked at further in Chapter 3. Once DD is established on a farm, it is very hard if not impossible to eradicate it completely. One of the best ways to manage this disease is frequent and early detection of these lesions in order to treat them.

The economic impact of lameness on farms is of concern. According to Cha et al. (2010), the average cost of a mild lameness-causing lesion ranged from $53 to $232 per cow per

year while severe lesions could cost producers $402 to $622 for each lesion. The economic losses associated with DD are estimated to cost the farmer between $95 to $133 per lesion in lost production, treatments, and discarded milk (Cha et al., 2010). DD affects over 70% of herds and approximately 20% of cows found at the time of hoof trimming in the United States and Canada (Cramer et al., 2018). Within herd prevalence has been estimated at 20 to 25% for herds living in free stall barns. (Cramer et al., 2008). When comparing the number of animals affected to the amount of money lost, it is easy to see that this disease can greatly alter a producer's bottom line. One of the objectives of this study is to explore treatment options that are most cost effective for the producers to use when treating DD.

There has been a large amount of research done regarding the best way to treat DD. The most common forms of treatment/management of DD are antibiotic drugs in the form of a footbath or topical treatment (Plummer and Krull, 2017). Footbaths have been a good option for producers to control the spread of the disease, as they can be implemented with minimal labor requirements. However, footbaths are not very effective as an overall treatment strategy. The most common additives used for footbaths have been antibiotic drugs, copper sulfate, formalin, and other inorganic compounds (Laven and Hunt, 2002). A negative aspect of footbaths is that the solution needs to be disposed of, and the materials being used are not ideal to be discarded into the environment.

Another form of treatment is using topical application of various substances to the lesion. This has been considered the "gold standard" due to the fact that the hoof trimmer (or other observer) can identify the lesion and what stage it is currently in. The most commonly used topical treatments have been tetracycline, oxytetracycline, lincomycin, and salicylic acid (SA) in the form of a powder or paste applied topically with or without a bandage (Berry et al., 2012;

Cutler et al., 2013; Shultz and Capion, 2013). Previous research has shown that tetracycline has healing rates that range from 68-87% at a dose of 2-5g per lesion (Plummer and Krull, 2017). Cure rates for other substances such as lincomycin and oxytetracycline have been 73% and 68% one month after treatment, respectively (Berry et al., 2010). In 2013, Schultz and Capion found that the topical treatment of salicylic acid had healing rates of 13.6% at 34 days post-treatment compared to chlortetracycline which had healing rates of 5.1% at the same time point. In contrast, other studies found healing rates using oxytetracycline between 70-90% (Read and Walker, 1998; Manske et al., 2002). These discrepancies likely exist depending on what each study defined as "healing". Some studies define healing as a transition away from the M2 stage to an M3, M4, or M4.1 while others may define healing only as a transition to M0.

Although tetracyclines have been effective in treating DD, there are also concerns about its use, including frequent recurrence of lesions, drug residues in milk, and the development of antimicrobial drug resistance. A study done by Krull et al. in 2016 evaluated 43 cows for a minimum of 50 days after therapy with topical oxytetracycline. The data showed that only 9% of lesions returned to normal skin. Over the following year, half of these animals still did not have complete skin healing and required retreatment. Another study done by Cramer et al. in 2019 tested the amount of residue of tetracycline in the milk at varying concentrations for treatment. They showed that 22% of cows from all treatment groups showed amounts of tetracycline in the milk post-treatment for DD, which is why there is a need to dump the milk after treatment (Cramer et al., 2019). In addition, there is a need for an alternative treatment due to concerns about the potential development of antibiotic drug resistance due to the use of antibiotic drugs in livestock. The use of topical tetracycline to treat DD is considered to be extra label drug use, otherwise known as "off-label" in the United States (Cramer and Johnson, 2019).

Veterinarians are the only individuals that have the authority to utilize drugs in an off-label manner, if they have deemed no other approved drugs appropriate for treatment. This requires that producers follow exact instructions for dose, frequency, and duration. If the instructions are not followed appropriately, it could potentially contribute to antimicrobial resistance or residues in food products. With this concern on the rise, the Veterinary Feed Directive (VFD) was established and implemented in 2015 in order to reduce the role of animal agriculture's potential contribution to antibiotic resistance by prohibiting producers from buying and using antibiotics without the knowledge or consent from a veterinarian. The VFD requires that the farm have a current Veterinarian Client Patient Relationship (VCPR) in order to utilize these therapeutics on their operation. This requires more frequent visits from the veterinarian, which ultimately costs more time and money. In addition to the VFD, the demand for "antibiotic-free" products from consumers is at an all-time high. In light of this, salicylic acid has been at the forefront of options to replace antibiotics when treating DD due to its comparable treatment effects to traditionally used antibiotic drugs.

A promising alternative to tetracycline drug for the treatment of DD is salicylic acid. Salicylic acid (SA) is a derivative of aspirin and is an ingredient commonly found in skincare products to aid with the treatment of acne in humans (Madan and Levitt, 2013). SA has been used for many years in Denmark as a non-antibiotic option for treatment of DD (Capion et al., 2018). It is a useful option due to its keratolytic, bacteriostatic, fungicidal, and photoprotective properties (Madan and Levitt, 2013). In addition, it is thought to have anti-inflammatory characteristics, which may aid in ridding the bacteria in the deeper layers of the epidermis as well as promoting epidermal repair (Weber et al., 2019). It is also thought to be a cheaper option of treatment when compared to drugs such as tetracycline (Laven and Logue, 2006). The toxic

dose of SA in humans is when blood concentrations are greater than 35 mg/dL. (Madan and Levitt, 2013). This can result in side effects of nausea, reduced brain function, respiratory distress, and metabolic abnormalities (Madan and Levitt, 2013). In addition, some people are allergic to salicylates. It is important for us to keep food safety in mind when we are using substances that could end up in the products that humans are consuming. In order for us to ensure these products are safe, there needs to be research done in order to establish the necessary milk withdrawal time for the topical use of SA in the treatment of DD in dairy cattle. In Germany, there is a commercial product of SA in a paste formulation mixed with methylsalicylate that requires a 1d withdrawal period for meat and milk (Weber et al., 2019). However, there has been no data published to support any milk withdrawal time specific to the therapeutic dose in the United States. The main objective of Chapter 3 is to provide data in order to aid in the establishment of a milk withdrawal time for the use of topical SA at the therapeutic dose.

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CHAPTER 3. TOPICAL SALICYLIC ACID TREATMENT OF DIGITAL DERMATITIS IN DAIRY COWS: DRUG RESIDUES IN MILK AND CLINICAL EFFICACY[1]

Interpretive Summary (IS)

Digital dermatitis is one of the leading causes of lameness in dairy cattle. The therapeutic practices for these lesions have included the use of antibiotic drugs along with regular footbaths to diminish the prevalence. There is a rising concern of resistance to antibiotic drugs, so it is ideal that a non-antibiotic alternative treatment option is established. Salicylic acid has been shown to provide similar treatment efficacy to the antibiotic drug tetracycline, but there is no milk withdrawal time established in the United States to use this product safely. This study was done to provide data in order to establish this withdrawal period.

Abstract

Digital dermatitis is one of the most prevalent causes of lameness in dairy cattle that causes painful and ulcerative lesions on the feet. The therapeutic practices for these lesions have included the use of topical antibiotic drugs along with regular footbath routines to diminish the prevalence on farms. Topical salicylic acid has been shown to provide similar treatment efficacy to the antibiotic drugs used previously, but there is no milk withholding time established in the

[1] A manuscript prepared for submission to the *Journal of Dairy Science*. The material in this chapter was co-authored by K.M. Wirt, J.M. Young, J.D. Peterson, G. Cramer and S.A. Wagner. K.M. Wirt, J.M. Young are affiliated with the Department of Animal Sciences at North Dakota State University. J.D. Peterson is affiliated with the Veterinary Diagnostics Laboratory at Iowa State University College of Veterinary Medicine. G. Cramer is affiliated with the College of Veterinary Medicine at the University of Minnesota. S.A. Wagner is affiliated with Texas Tech University School of Veterinary Medicine. K. M. Wirt was responsibile for collecting milk samples, animal handling, data management and collection, and preparation and revision of this chapter. J.M Young was responsible for data management, statistical analysis, and proofreading. J.D. Peterson was responsible for analyzing the milk samples for drug residue and methodologies for drug residue analysis. G. Cramer was responsible for study design, data collection, and statistical input. S.A. Wagner was responsible for study design, data collections, study management, and revisions to the text.

United States to use this product safely. The objective of this study was to provide data in order to establish this withholding period. A secondary objective was to evaluate outcomes among treatments. The three treatment groups were topical applications of the following drugs: salicylic acid paste, salicylic acid powder, and tetracycline with 18 cows per treatment. The lesions were scored (M-stage scoring system) at day 0, day 7, and day 28 post-treatment. Milk samples were collected the day before treatment, 4 hours, 8 hours, 24 hours, 36 hours, and 48 hours post-treatment. Results indicated that most cows did not show detectable levels of salicylic acid after 24 hours, however there were three cows that showed detectable levels through 36 hours post treatment. Results showed that the treatments did not differ significantly from one another in regards to their treatment effect on the M2 lesions (P=0.5443).
Key Words: digital dermatitis, dairy cow, salicylic acid, milk withdrawal

Introduction

Digital dermatitis (DD) is a contagious disease that causes painful and ulcerative lesions on the feet that commonly lead to lameness. It remains as one of the top issues in welfare and overall productivity of the dairy industry. Amongst the infectious sources of lameness, DD has the highest prevalence on dairy farms across the globe (Cramer et al., 2008; USDA, 2009). It has been estimated that for every lesion, there is a financial loss of approximately US$133 to the producer (Cha et al., 2010). The probable causative agent of DD is thought to be the *Trepenoma spp.* of spirochete bacteria, but researchers have not been able to consistently induce DD using pure cultures (Evans et al., 2008). This demonstrates that it is a multifaceted disease process, and there are likely other bacterial agents involved.

In North America, DD has commonly been treated with antibiotic drugs. Tetracyclines have been a treatment of choice by a one-time topical application of 2 to 5 g per affected lesion

in the form of a powder or paste, with or without a bandage (Cutler et al., 2013). The use of tetracyclines to treat DD is considered to be extra-label drug use (ELDU) in the United States. This type of use requires a valid veterinarian-client-patient relationship and a written prescription. Currently, there is a 24-h recommended milk withdrawal interval for the topical use of tetracycline by the United States Food Animal Residue Avoidance Databank (FARAD), provided there is no oral ingestion (FARAD, 2020). In addition, the Veterinary Feed Directive (VFD) makes the powdered dosage forms of tetracyclines unavailable without a prescription from a veterinarian, as there are concerns about antibiotic drug resistance. With these types of restrictions, an alternative to antibiotic drugs is desirable for the treatment of DD. Salicylic acid (SA) is a good contender for a non-antibiotic form of treatment for DD due to its keratolytic and anti-inflammatory effects (Weber et al., 2019). It is typically applied directly onto the lesion in the form of a powder or paste with a bandage. There is no recommended milk withholding interval for the topical use of SA in the United States, along with no publications providing data on residues of this drug in the milk. It has been found that a thermographic camera can be a useful tool in identifying inflammation in the hooves (Gianesella et al., 2018). Thermographic images were used in an exploratory fashion in order to understand potential for thermography to become a validated tool to assist in the evaluation of DD. Our hypothesis was that we would note higher temperatures with M2 lesions versus the other stages since these lesions are more inflamed. The primary objective of our study was to determine the amount of SA in milk after treatment at various time points. This data will aid in developing a recommendation for a withholding interval after the topical use of SA in treating DD in dairy cattle. Secondary objectives were (1) to evaluate the efficacy of SA as a treatment for DD and (2) to compare the efficacy of SA to tetracycline when treating DD.

Materials and Methods

All procedures involving animals were approved by the North Dakota State University Institutional Animal Care and Use Committee, protocol #A19088

Animals

This study was performed at the North Dakota State University Dairy Research and Teaching Unit in Fargo, ND. All animals were housed indoors in free stall housing. All cows had ad libitum access to water and a ration formulated to meet the nutritional demands of lactating cows, which was delivered once daily at 6:00 am and pushed up throughout the day. Cows were milked twice daily in a tandem milking parlor at 4:00 am and 3:00 pm. Cows were screened for enrollment based upon observation for active DD lesions in the milking parlor by study personnel and/or observations by management on the farm. Cows that were flagged for screening were examined by a veterinarian in a standing hydraulic chute; those with active lesions (M2) were enrolled in the study. Cows were enrolled in the months August, October, and March in 3 cohorts of 33, 12, and 9 cows. Cows that had illnesses other than lameness (e.g. mastitis, metabolic disease) or a history of medical treatment that had not completed the drug withholding period(s) + 7 days were excluded from enrollment.

Treatment Groups

Cows were blocked by day of enrollment then randomly assigned to one of 3 treatment groups:

- 2g Tetracycline hydrochloride powder mixed with 6 mL glycol, applied topically and left unbandaged (TPO)
- 5g of SA powder applied topically and covered with a coflex bandage (SPO)

- 6 mL SA paste (Hoof Gel with Salicylic Acid 38% JorVet) applied topically then covered with a coflex bandage. (SPA)

Randomization was achieved by using the Randomizr software (version 0.20.0 package in R (version 3.60). Because DD is painful condition, there was no untreated control group. If there was more than one lesion on a cow/hoof, the largest and most active stage was used for the study. Milk was not marketed for 24 hours after treatment in all groups.

Study Design

The sample size for this study was based on the US regulatory agency standards for establishing withdrawal times for veterinary drugs, FDA guidance for industry #207, which uses the Veterinary International Conference on Harmonization guideline 48. This guideline recommends 20 cows per treatment group for the determination of residues and was the goal for the study. The study procedures were as follows:

Day -1: Baseline milk sample was collected from potential candidates

Day 0: The affected foot was cleaned, and a digital photo & thermography image were obtained while restrained in a hydraulic chute. A red leg band was placed on the affected limb in order to denote milk withholding. The following were recorded: affected limb, stage of lesion, time of digital photo, number of thermography image, treatment type, and treatment time. Milk samples were collected at approximately 4 and 8 h post treatment.

Day 1: Milk samples were collected at approximately 24 h and 36 h post treatment. Red leg bands were taken off after the 24 h post-treatment milking and replaced with green leg bands so that treated cows could be easily identified by study personnel.

Day 2: Milk samples were collected at 48 h post treatment. Bandages were removed after the 48 h milk sample was collected.

Days 7 and 28: Each cow enrolled was restrained via a hydraulic suspending trimming chute and had their lesion scored by a veterinarian. The lesion was photographed via digital and thermographic camera. The green leg bands were taken off after the 28 d evaluation. Cohort 3 was excluded from 28 d collections due to travel restrictions related to the SARS-COV-2 pandemic.

Digital and Thermographic Photography

A digital photo of the lesion was taken via an IPad Air 2 (Apple, 2014) on day 0 and on the 7 and 28 day rechecks. A thermographic image using a FLIR E8 Wifi camera (FLIR Systems OÜ, Estonia) was taken of the lesion on the day prior to treatment, 7 day recheck, and 28 day recheck. Each thermographic image had the lesion outlined with the location indicating the interdigital cleft location utilizing the FLIR software (Sp1) (Figure 3.1). The software then established a minimum, maximum, and average temperature of the foot surface within the outlined lesion.

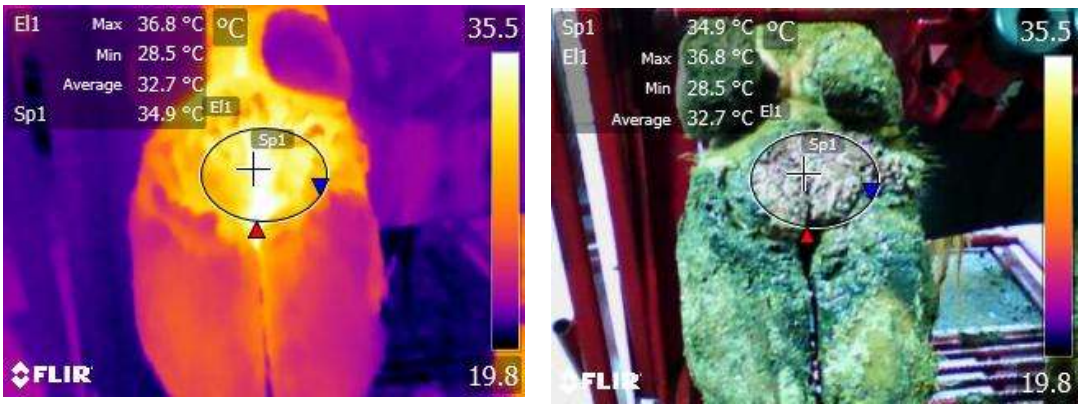

Figure 3.1. An example of one of the thermographic and digital images generated by the thermographic camera.

Milk Collection and Drug Assays

Milk samples taken at milking times were collected in the parlor via hand-stripping immediately after milking was completed. At the 8-hour sampling time, which was not a milking

time, animals were simply hand-stripped for the sample. Each sample collected ranged from 5-10 mLs in volume per the laboratory requirements. All milk samples were stored in a -80 degrees F freezer shortly after collection until they were shipped overnight on dry ice to the laboratory.

Tetracycline

Bovine milk concentrations of tetracycline were determined using UHPLC mass spectrometry. Oxytetracycline was used as the internal standard. A Q Exactive Focus orbitrap was coupled to a Dionex Ultimate 3000 (Thermo Scientific, San Jose, CA, USA). The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in methanol. The mobile phase began at 7.5% B with a linear gradient to 95% B from 0.5-4 min. The gradient was maintained at 95% B for 1 min followed by re-equilibration to 7.5% B. The flow rate was maintained at 0.5 mL/min. An Accucore C18 column was used (100 mm x 2.1 mm, 2.6 μm particles) from Thermo (Thermo Scientific, San Jose, CA, USA) with column temperature set to 35 °C. The injection volume was 5 µL. The following ions were used for identification and quantification: Tetracycline (m/z 445.161) 154.05 and 410.13 and Oxytetracycline (m/z 461.155) 381.06 and 426.12. The retention times were 2.21 and 2.25 for tetracycline and oxytetracycline respectively.

Calibration curves were calculated using Quan Browser portion of the Xcalibur software and a linear fit. Calibration curves were 5-300 ng/mL. The correlation coefficient (r^2) exceeded that of 0.99. The calibrators used were within a tolerance of ±15% of the nominal value except for the lower limit of quantification, which was < 20%. The QCs were within a tolerance of ±15% of the nominal value. The limit of detection (LOD) was 3 ng/mL and the limit of quantitation (LOQ) which was based on the calibration curve was 5 ng/mL.

Salicylic Acid

Salicylic Acid concentration was determined using ultra high-pressure liquid chromatography (UHPLC) with fluorescence detection. The UHPLC used was a Thermo Vanquish Flex system consisting of a Binary Pump, Autosampler, Column compartment, Variable wavelength UV detector, and Variable wavelength Florescence detector. 0.2mL of Milk was aliquoted for extraction of calibrators, quality controls, and unknown samples. Calibrators were spiked into blank matrix at seven concentrations ranging from 20 to 5000 ng/mL. Three quality controls were spiked into blank matrix at 150, 1500, and 3500 ng/mL. 20µL of 12% Formic Acid was added to each extraction tube, followed by 0.78mL of Acetonitrile. Tubes were placed on a multitube vortex mixer for 10 minutes followed by centrifugation for 5 minutes at 4°C. 0.5 mL of the upper layer was transferred and concentrated to dryness at 25°C. Samples were reconstituted in 0.1% Formic Acid in Water**.**

The mobile phases consisted of: A) 3.5mM phosphate solution with 0.1% formic acid in Water and B) HPLC grade Acetonitrile. Separation was accomplished using an Accucore aQ, 100 x 2.1, 2.6µm particle size column (Thermo Scientific, San Jose, Ca, USA) maintained at 45°C. The autosampler was maintained at a temperature of 6°C and the injection volume was set to 5µL. The separation was performed at a flow rate of 0.3mL/min at a starting solvent composition of 25% B increasing linearly to 35 % B over 3.5 minutes. The solvent composition was then increased to 95% B over 0.5 minutes and held at 95% B for two minutes before equilibrating to 25% B. Salicylic Acid was detected at an Excitation wavelength of 295nm and an Emission wavelength of 410nm and a retention time of 1.92 (± 0.014) min. Thermo Chromeleon software was used to process quantitative results. All calibrations consisting of seven points between 20ng/mL and 5000ng/mL and a blank resulted in linear

curves with $r^2 \geq 0.997$. The lower limit of quantification (LLOQ) was 20ng/mL (the concentration of the lowest calibrant). All QC samples were calculated within 10% of their nominal value.

Statistical Analyses

The drug residue data was analyzed using PROC MIXED in SAS (version 9.4; SAS Institute Inc., Cary, NC). For the cure rate data, we counted animals within each category then used PROC FREQ in SAS to determine if the distribution of animals within category differed between treatments. The lesion score data was analyzed using PROC GLIMMIX in SAS. Fixed effects included were treatment, day, cohort, and the interactions of treatment with day and treatment with cohort. The two-way interaction of day with cohort and the three-way interaction of treatment, day, and cohort were tested and removed from the model because $P > 0.1$. DIM was included as a covariate. A repeated measures was included using the random statement in GLIMMIX with cow as the subject. Different covariance structures were tested and the best fit based on AICc was chosen. The temperature data was analyzed using PROC CORR and PROC REG in SAS. We ran a simple linear regression including all four temperature measurements to predict lesion score and did a simple linear regression by treatment. Statistical significance was defined as $P \leq 0.05$ for all measures.

Results

Fifty-four cows (18 per treatment group) completed all study procedures as described other than cohort 3 for 28 day observations. Our treatment group totals (18) fell below the recommended FDA guideline for 20 cows per treatment group. Twenty-eight day lesions data were not collected for one cow in cohort 2 due to being culled for reasons unrelated to the treatment. The average DIM for each cohort was as follows: 190 days = cohort 1; 198 days = cohort 2; 240 days = cohort 3. The mean lactation number for each cohort was 2.

Drug Residue

There were 36 cows that did not show any drug residue amounts at any time point (SA Paste = 8, SA Powder = 8, Tetracycline = 8). The other 18 cows showed amounts above the LLOQ for each drug (Figure 3.2). Of those 18 cows with detectible drugs in their milk, 12 cows showed detectable amounts of drug ranging from 20.4 ng/ml to 87.4 ng/ml for SA and 6 ng/ml to 26 ng/ml for tetracycline starting between 4-8 hours post-treatment and then no detectable amounts by 24 hours (SA Paste = 5, SA Powder = 3, Tetracycline = 5). Two cows had levels above the LLOQ on the baseline sample (SA Paste =1, 39.8 ng/ml; cohort 2, SA Powder = 1, 23.5 ng/ml; cohort 1). Two cows did not have a detectable amount until 36 hours (SA Powder = 2). One cow had 87.4 ng/ml at 8 hours, a non-detectable amount at 24 hours, and then a level of 22.1 ng/ml at 36 hours.

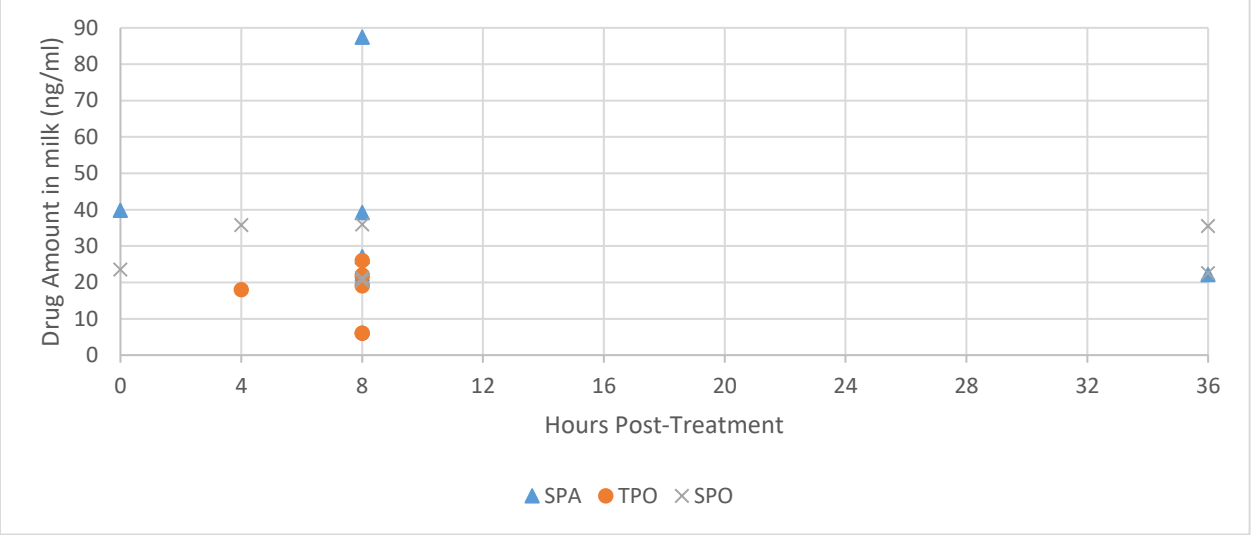

Figure 3.2. Drug amounts found above the LOD (3 ng/ml) for Tetracycline and LLOQ (20 ng/ml) for Salicylic Acid in milk samples at each time point post-treatment amongst all cohorts.

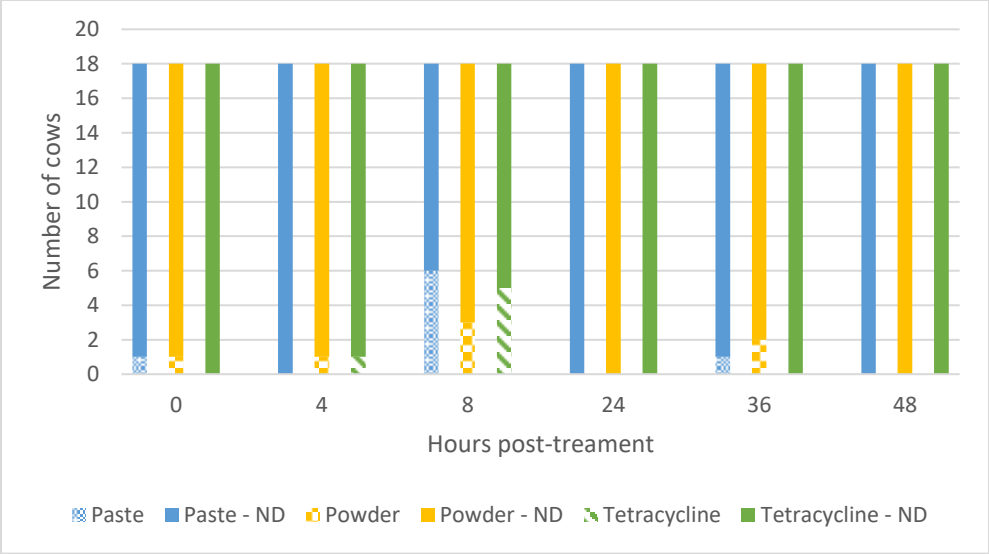

Figure 3.3. The number of cows that showed positive (detectable) results of drug and the cows that had no detectable drug (ND) at all of the various time points for each treatment.

Clinical treatment outcomes

All lesions were scored as an M2 on day 0 in order to be enrolled in the study. A transition from M2 to stages M3, M4, or M4.1 was considered "improvement". There were no lesions that resolved completely to M0. Among all cohorts, there were 20 cows that remained an M2 lesion on day 7: 7 from SA paste, 7 from SA powder, and 6 from Tetracycline. There were 8 cows that transitioned from M2 to M3 by day 7: 3 from SA Paste, 3 from SA powder, and 2 from Tetracycline. There were 26 cows that transitioned from M2 to M4 by day 7: 8 from SA paste, 8 from SA powder, and 10 from Tetracycline. In cohorts 1 & 2 where there was a day 28 collection, there were four groupings noted:

(A) cows that improved between d0 to d7 and remained improved on d28;

- SA paste: 5, SA powder: 4, tetracycline: 7

(B) improved between d7 to d28;

- SA paste: 2, SA powder: 0, tetracycline: 0

(C) improved between d0 to d7, but then regressed back to an M2 between d7 to d28; and

- SA paste: 4, SA powder: 5, tetracycline: 4

(D) those that showed no improvement between the time periods.

- SA paste: 4, SA powder: 6, tetracycline: 3

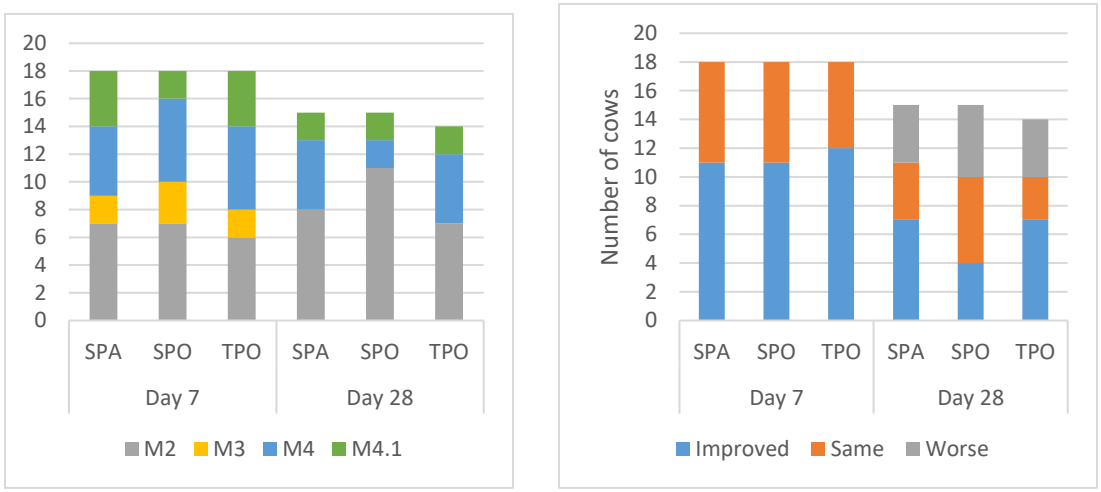

Figure 3.4. All cows enrolled were staged an M2 on day 0. The first image is showing the number of each lesion at day 7 and day 28 for each treatment. The second image is categorizing the number of cows that had lesions that improved, stayed the same, or worsened by the previous evaluation (day 7 from day 0 and day 28 from day 7). Any stage other than an M2 was considered "improved".

Lesion Score

Results showed that the treatments did not differ from one another in regards to treatment effect on M2 lesions (P=0.54). There was a treatment x day effect on mean lesion scores showing improvement on day 7 across all treatments analyzed together (P<0.0001).

Temperature

Thermographic data were not affected by treatment. The simple linear regression did not show that thermographic images could be used to predict lesion score (R^2 = 0.20). We then looked within treatments which showed the same results: SA Paste (R^2 = 0.50), SA Powder and tetracycline (R^2 = 0.15). More acute lesions (M2) had higher overall temperatures compared to the other lesion scores. The correlations for each factor evaluated by the camera relating to

lesion score were as follows: Sp1 = -0.27350, Min = -0.21961, Max = -0.35128, and AVG = -0.36980. These negative numbers indicate the lower the lesion score, the higher the temperature as they are an inverse relationship.

Discussion

Our primary objective was to establish parameters to suggest a milk withholding time for the topical treatment of SA for DD. The authors could find no published data of this kind. If deemed a suitable treatment, this would broaden the non-antibiotic treatment options for producers, hoof trimmers, and veterinarians. Drug residue in products for human consumption continues to grow as a public health concern, especially with the antibiotic class of drugs, due to the worry of resistance (Silbergeld et al., 2008). The risk of antimicrobial drug resistance development is an important reason to try and find an alternative treatment that is not an antimicrobial drug. In addition, there are further potential risks to fetuses of pregnant women. According to the FDA, oral dosage of tetracycline falls under category D which states: "There is evidence of adverse fetal risks in humans based on investigational or marketing data. Potential benefits may warrant use of this medication despite risks" (Lee et al., 2013). SA falls under category C for pregnant women which states: "Animal studies have shown an adverse effect on the fetus, and there is a lack of well-controlled studies in humans. Potential benefits may warrant use of this medication despite risks" (Lee et al., 2013). Topical over-the counter facewash products have been used in pregnant women for many years without any adverse event or effect (Lee et al., 2013). Both SA and tetracycline are considered to be safe while breastfeeding (Lee et al., 2013). It is important to note that higher dosages of any drug, including tetracycline and SA, will pose a greater risk to the fetus or infant as these higher dosages would likely stay in the human milk longer compared to low dosages. There is not a specific time period established for

peak levels of salicylates to be found in human milk after treatment of SA or aspirin. There is no drug approved by the FDA for the treatment of DD. The current recommended milk withholding period for tetracycline after topical use for the treatment of DD is 24 hours (FARAD, 2020). Our data showed that the amounts of tetracycline equaled or fell below the LOD (6 ng/ml) after 24 hours in all cows only showing amounts at the 8 hr milking. This agrees with previous research. In 2019, Cramer et al. found that cows treated with 5g tetracyline paste, should follow a withholding time of 24 hours.

Our results also indicated the majority of cows (67%) never had quantifiable levels of SA in their milk at any time after treatment, and that most of those that did had levels below the LLOQ of SA by 24 hours post treatment. However, there were three cows that still showed SA in their milk for up to 36 hours. Of those cows, one had a level of 87.4 ng/ml at 8 hours post treatment, an amount below the LLOQ at 24 hours post treatment, and an amount of 22.1 ng/ml at 36 hours post treatment. It is probable that that there may have been SA below the LLOQ (20 ng/ml) at 24 hours, since the 36 hour amount was in the low twenties as well. There were two cows that had detectable levels of SA (23.5 ng/ml and 39.8 ng/ml) during the baseline milk sample collections. After discussion with the farm, there was no risk of treatment with SA prior to our sampling. The chemist at the lab that analyzed the samples did mention the chance of a little naturally occurring amount of SA in the milk. In humans, small amounts of SA are found to be naturally occurring in their serum with no salicylates being taken prior to the blood sample being taken (Battezzati et al., 2006). SA is a phenolic compound found naturally in plants that plays a central role in disease resistance to pathogen infection (Battezzati et al., 2006). Those with a vegetarian diet were found to have a higher level of salicylates in their serum compared to non-vegetarians (Blacklock et al., 2001). This may lead as an explanation as to why dairy cows

may have some levels of naturally occurring SA due to being an herbivorous species. In addition, the numbers that were reported were not extremely higher than the LLOQ. It is also possible that samples were mis-labelled during research procedures or mis-coded at the analytical laboratory.

Our findings indicate that the milk withholding time for SA are comparable to the withholding times for tetracycline, though to ensure food safety, a withholding time of 36 hours should be observed.

All tested treatments had similar effects on clinical outcomes. SA has been shown to be an effective tool in treating DD when compared to tetracycline (Capion et al., 2018; Schultz & Capion, 2013), and it has been used for many years in Denmark as a non-antibiotic option for treatment of DD (Capion et al., 2018). It is thought to have anti-inflammatory characteristics, which may aid in ridding the bacteria in the deeper layers of the epidermis as well as promoting epidermal repair (Weber et al., 2019). It may also be a cheaper option of treatment when compared to drugs such as tetracycline (Laven and Logue, 2006). All treatment groups showed improvement by day 7 with no treatment differences in lesion score on day 7. Our results are similar to those of previous studies comparing SA and tetracyclines. In a study done by Jacobs et al. in 2018, they compared the effects of three different treatments: tetracycline, HealMax (AgroChem Inc., Saratoga Springs, NY), and HoofSol (Diamond Hoof Care Ltd., Intracare BV, Veghel, the Netherlands). One of the ingredients in HealMax is methyl salicylate, which is a methyl ester of SA. They found that HealMax was just as effective as tetracycline at clinically curing active lesions with 1 treatment. In 2013, Schultz and Capion found that topical treatment with SA proved to be 1.75-fold better than chlortetracycline in terms of clinical improvements, and 2.5 times greater at reducing lesion size by day 14 and 34. Our results

support this finding as cows that were treated with SA powder and tetracycline regressed similarly between days 7 and 28 resulting in similar lesion scores on day 28 while SA paste prevented worsening of lesions between day 7 to day 28. SA paste was the only treatment that did not show lesion regression over time back to the more infective stage of M2.

It is important to note that we did not observe healing for the overall disease, as a large portion of the lesions regressed back to an M2 lesion by day 28 amongst all treatments and none of the lesions ever transitioned to a healthy M0. Multiple studies have evaluated the transition periods between lesion stages. Krull et al. (2016) found that the average time for a lesion to develop was 133 days in 2016 whereas others have found that transitions happen weekly/biweekly (Nielson et al., 2011, Holzhauer et al., 2008). Our results seem to agree with the shorter transition period, as we had lesions transition between stages from 0 to 7 days and from 7 to 28 days. Iit has proven to be difficult to isolate the issue that results in the reoccurrence of this disease due to its multifactorial process of *Treponome spp*. spirochete bacteria that can encyst deep into the epidermal layers (Dopfer et al., 1997; Capion et al., 2018). Our results agree with the difficulty of healing this disease and showed that the sole treatments used were not enough to fully cure the lesion by day 28. This could be due to the fact that the treatments were not able to penetrate the deeper epidermal layers of the lesions.

We chose to evaluate the thermographic images because it has been shown that there is a significant increase in temperature on lame feet caused by DD lesion or claw horn lesions compared to healthy feet, and that the heel area is the most accurate in detecting this temperature difference (Harris-Bridge et al., 2018). A study done by Oikonomou et al. in 2014 found that a decreased digital cushion thickness was associated with increased sole temperature. Our primary goal for using this tool was to see if the temperature was associated with lesion score, and we did

not find any particular connection. However, there was a weak correlation that indicated the M2 lesions were associated with higher temperatures. One thing to note was that our study did not have any M0 or M1 lesions noted. This created a high variation due to the range of temperatures compared to the lesion scores. Future studies would want to be sure to have all stages of the disease included. This finding and the others discussed from previous research indicate that this could be a helpful tool in identifying lameness events. However, our sample size was much smaller than that study, so there is potential there could have been associations that weren't observed.

Previous studies have noted 47.8% of lameness events occurring before 180 DIM, indicating the need to focus on the dry period to mid-lactation (DeFrain et al., 2013). By contrast, our results indicated that DIM did not have a specific effect on lameness as all cohorts enrolled had an average DIM higher than 180 days with lesion scores (M2). We feel that we still saw these lameness events after 180 DIM due to the disease being extremely persistent and difficult to eradicate.

Conclusion

In conclusion, we were able to identify that the withholding time for topical use of SA would likely be 36 hours.

Acknowledgements

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CHAPTER 4: CONCLUSION

Although I had worked under Dr. Wagner as a research technician, I had no idea about how much work it takes to complete a research project from start to finish. This process allowed me to be much more involved in the process of understanding funding the project, the choices that you have to make along the way to ensure scientific integrity, and then finally putting together a clear message to share the answers that you found. I was surprised about the amount of times that changes had to be made to the original design of the project. With each change I learned how to overcome those challenges by being creative and flexible.

In regards to the topic, I learned that digital dermatitis is a very complicated disease process that affects so many dairy cows and producers across the globe. I did not realize how many different factors can play a role in the outcome of this disease. This led to one of my biggest challenges which was how to determine what part to focus on. As I researched this topic, I realized that there are so many variances in how people categorize, treat, and prevent digital dermatitis. This made it very difficult to make any direct comparisons to our specific project. Luckily, I had two lameness experts that helped guide me through deciphering these different aspects and now I feel that I have my own scientific opinion on these different factors that I learned about.

Most importantly, I learned a lot about myself. With each new challenge, I was forced out of my comfort zone and was required to adapt and grow as a person and a professional. This experience was extremely humbling and it really required me to lean on the experts that were on my team. Their knowledge and expertise really molded how I approach science and research, and I know I still have a lot to learn about these areas.

All of these aspects have taught me that working towards this Master's degree is a lot of learning and minimal perfection. There is always room for revisions, and those that have

differing ideas will ultimately make you a better scientist. It can open your eyes to something that you may not have thought of, or it will make you more confident in your current position. The biggest lesson that I learned, and that I aim to take with me in the future, is that I want to be involved in research that helps solve real problems for animals and producers with the highest level of scientific integrity.