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Distribution of Treponema species and antimicrobial resistance genes in digital dermatitis lesions

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Distribution of *Treponema* species and antimicrobial resistance genes in digital dermatitis lesions

by

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Abstract

Digital dermatitis lesions are ulcerative or proliferative masses between the heel bulbs. The most significant clinical outcome of digital dermatitis (DD) is lameness, leading to animal welfare concerns and economic loss for dairy producers worldwide due to premature culling, milk loss, and decreased fertility. While there is insufficient evidence to determine the etiological agent(s) responsible for DD, it is widely accepted that DD is a polymicrobial disease significantly associated with anaerobic bacteria, *Treponema*. Difficulties in obtaining pure cultures and the nearly exclusive presence of *Treponema* in diseases as members of a polytreponemal or polymicrobial communities has led to insufficient species identification within lesions and incomparable prevalence estimates based on phylotype. Our primary research objectives were to develop a diagnostic tool to identify *Treponema spp.* within DD lesions to associate presence and abundance with DD lesion grades. Further, we examined the distribution of antimicrobial resistance genes (AMGs) within sequenced isolates of DD-associated *Treponema* and designed targeted PCR for *Treponema* AMGs ($AMGs^{Trep}$) within lesions.

We provide significant evidence that the absolute quantities of *Treponema* and $AMGs^{Trep}$ are lower in chronic and early, active DD lesions suggesting *Treponema spp.* within these lesions may be more susceptible to antimicrobials (AMs) compared to species in advanced, active lesions. We have identified potential interactions among *Treponema spp.* that may facilitate DD progression and enhance pathogenicity and may affect AM susceptibility and lesion chronicity. With the novel diagnostic tool developed here, future research should elucidate interactions among *Treponema spp.* and *in vitro* susceptibility profiles and the efficacy of treating early and chronic lesions compared to advanced, active lesions on infection resolution.

Preface

This thesis consists of two manuscripts prepared for submission. The manuscripts included are:

Chapter 2

Beninger, C., Naqvi, S.A., Naushad, S., Orsel, K., Luby, C., Derakhshani, H., Khafipour, E., and De Buck, J. 2018. Associations between digital dermatitis lesion grades and the quantities of four *Treponema* species. Submitted.

Chapter 3

Beninger, C., Naqvi, S.A., Naushad, S., Wit, J., Luby, C., and De Buck, J. 2018. Distribution of antimicrobial resistance genes in digital dermatitis lesions and *Treponema* isolates, In Progress

The following manuscripts that I have co-authored while a master's student at the University of Calgary in the Faculty of Veterinary medicine were not included in this thesis:

Watts, K., Fodor, C., Beninger, C., Lahiri, P., Fernandez, R., De Buck. J., Knight, C., Orsel, K., Barkema, H., and Cobo, E., 2018, A differential innate immune response in active and chronic stages of bovine infectious digital dermatitis, *Frontiers in Microbiology*, Under review.

Jacobs, C., Beninger, C., Hazlewood, G., Orsel, K., and Barkema, H., 2018, Effect of footbath protocols for the prevention and treatment of digital dermatitis in dairy cattle: a systematic review and network meta-analysis, In progress.

Statement of Work

- Organizing farm visits and contacting producers with Elbert Koster, collecting biopsy samples on farm, all sample processing, including DNA extractions, *Treponema* culture, and sample preservation for chapters 2 and 3.
- *Treponema* culture and isolation from co-infecting bacterial contaminants.
- Utilized species-specific genes identified by Dr. Naushad to design, test, optimize, and validate novel PCR and qPCR in chapter 2.
- Clones were created by CB to generate standard curve for qPCR to relate cq to copy number. Spiking experiments and analysis done by CB.
- Microbiome analysis samples were prepared by CB and run by Drs. Khafipour and Derakhshani at the University of Manitoba whom also preliminarily analyzed data and assigned ASVs based on 2 algorithms they selected. Further analysis and comparison to qPCR data was done by CB.
- Statistical analyses were designed by CB and Ali Naqvi and run in R by Ali Naqvi.
- *Treponema* were cultured and gDNA extracted for Illumina MiSeq Next Generation Sequencing for chapter 3 performed by CB.
- Sequencing was performed with the assistance of Dr. Fernandez. Designed AMG^{Trep} PCR based upon genes found through targeted gene searches in NCBI and a literature review determining relevance to DD treatment and control and *Treponema spp*.
- AMGs were identified by Dr. Naushad from 7 genomes provided by Dr. Döpfer. Ali Naqvi aligned MiSeq data with the curated AMG^{Trep} created.
- All PCR reactions were tested, optimized and representative amplicons sequenced by CB with the assistance of Sandi Nishikawa.

- Nanopore sequencing (data not included) done by CB with the guidance of Dr. Wit. Statistical analyses were designed by CB and Ali Naqvi and run in R by Ali Naqvi.
- All manuscripts and chapters included in this thesis were written by CB, edited primarily by JDB, followed by co-authored manuscripts edits by those indicated.
- Work contributed to co-authored paper with Jacobs et al., consisted of reviewing abstracts and full texts for meta-analysis and edits.
- Work contributed to co-authored paper with Watts et al., consisted of developing protocol and generating *Treponema* polyclonal antibodies in rabbits including culture, inoculum, and titre testing, for IHC; images done by Dr. Fernandez. *Treponema* culture, identification, sample collection and processing. Designed biopsy sampling, transport, and isolation protocols. Corresponding materials and methods written by CB and manuscript edits.

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I would like to thank Elbert Koster, for teaching me everything I could ever want to know (and sometimes more) about hooves and hoof-trimming. Thank you for teaching me how to handle cows safely and only occassionally mentioning I was a little scared of them our first day on the farm. You have given me immense confidence in my skills as a future veterinarian. Thank you for making me laugh, listening to my half-hearted struggles, and always offering support in a sincere, yet sarcastic tone. Without your guidance and assistance, this research would not have been possible. Thank you to Alberta dairy producers for participating in our research and continued support of UCVM research. I would especially like to thank their cows for putting up with us on the farm, I truly hope this research will help improve DD and welfare.

I would like to thank my incredible friends and family. Thank you for always supporting me; whether I want to be a veterinarian, a physician, a researcher, or give up and move to the

beach to teach surfing lessons, you are always on my side and ready to offer beach suggestions and location rationale. Thank you to Ali Naqvi, for having the unfortunate task of being the voice of reason, dragging me back to reality against my will, and informing me the piña colada song is about communication in relationships for absolutely no reason when I tell you I plan to play it in my beachfront surf shop while enjoying a piña colada. Thank you for only looking mildly horrified when, after 16 hour farm and lab days, I'm overtired and begin to laugh (probably at my own joke, that isn't funny) which leads to crying, followed by simultaneous, indistinguishable laugh-crying for reasons we will never begin to know or understand. Thank you for being the bad guy and convincing me to stick out the hard times and follow my dreams, I love you for it and would never have made it here without you… no matter what I may have said at the time. Thank you for studying with me for the MMIs and convincing me to re-apply for vet school. Thank you for being my shoulder to laugh-cry on and never letting me give up on myself. You are truly incredible and I consider myself lucky to have you. To my fellow grad students, especially my officemates, thank you for offering advice, support, listening to my rants, and showing me laugh-crying is completely reasonable. I am incredibly grateful to have had you by my side through this experience.

There are far too many people I am fortunate enough to have in my life to metion here, but know your love and support in all my endeavours is indispensadable.

To my Dad, Ronald Beninger, who has always been my biggest advocate and always believed I could accomplish anything I set my mind to, even when I did not.

Table of Contents

List of tables and figures

List of abbreviations

aad(3")	Streptomycin 3"-adenyltransferase
AM	Antimicrobials
AMG	Antimicrobial Resistance Genes
AMG^{Trep}	*Treponema spp.* AMGs
AMR	Antimicrobial Resistance
ANT	Aminoglycoside nucleotidyltransferases
ASV	Amplicon Sequence Variants
ATCC	American Type Culture Collection
ATM	Anaerobic Transport Media
bp	Base pair
CTAB	Cetrimonium bromide
DD	Digital Dermatitis
De	*T. denticola*
DIC	Differential Interference Contrast
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FDH	Formaldehyde Dehydrogenase
flaB2	Flagellin B2
HGT	Horizontal Gene Transfer
HMA	Heavy Metal ATPase
MBC	Minimum Bactericidal Concentrations

Me	*T. medium*
MFS	Major Facilitator Superfamily
MGE	Mobile Genetic Elements
MLS_B	Macrolide, lincosamide, streptogramin B
MβL	Metallo-β-lactamase hydrolase
NCBI	National Center for Biotechnology Information
nim(5')	5'-nitroimidazole
nlpE	Copper resistance protein
OD	Optical Density
OTEB	Oral Treponeme Enrichment Broth
OTU	Operational Taxonomic Unit
PBP	Penicillin Binding Protein
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
Pe	*T. pedis*
Ph	*T. phagedenis*
qPCR	quantitative Polymerase Chain Reaction
R	Correlation coefficient
R_0	Reproduction rate
RNase	Ribonuclease
RPPs	Ribosomal Protection Proteins
rRNA	Ribosomal RNA
TetO/M/W	Tetracycline ribosomal protection proteins

TetR	Tetracycline efflux pump
WGS	Whole Genome Sequencing
ZnuA	Zinc resistance ABC transporter

Chapter 1. General Introduction to digital dermatitis and antimicrobial resistance genes

Chapter 1: General Introduction

Digital Dermatitis

Digital dermatitis was first identified in Italy in1974 and has since been reported as a highly prevalent disease globally (Cheli and Mortellaro, 1974; Toholj et al., 2012). Digital dermatitis (DD) is a cause of infectious lameness in dairy cattle worldwide and has spread from dairy cattle to susceptible ungulates including sheep, beef cattle, and some wild ungulates (Evans et al., 2016). Digital dermatitis is characteristically a moist, ulcerative lesion associated with a raw-red granular appearance and pain upon manipulation but can present as a chronic, proliferative lesion with papilliform hair-like projections (Berry et al., 2012; Orsel et al., 2017). The most significant manifestation of clinical DD is lameness which is the second most common health problem in intensively managed dairy cattle (Krull et al., 2014; Orsel et al., 2017). Lameness associated with DD lesions compromises animal welfare and leads to direct and indirect economic losses. Economic loses associated with DD include animal weight loss, decreased milk production and fertility, treatment expenses, and premature culling; lameness is the leading cause of culling in the dairy industry worldwide (Orsel et al., 2017; Toholj et al., 2012). It has been more than 40 years since the disease was first described, and despite efforts to prevent the disease with improved hygiene practices, regular footbaths and bedding modifications, effective treatments that eliminate the disease have not been identified (Dopfer et al., 1997; Evans et al., 2009a).

Lesion Scoring

Lesions from DD are found primarily on the hind legs and located on the plantar surface between the heel bulbs (Berry et al., 2012; Dopfer et al., 1997). There have been multiple lesion scoring systems developed to describe stages of DD including the Iowa DD scoring system, a ‘simplified’ M-stage scoring system in which M3 and M4 are treated as a single lesion grade,

binary active and inactive lesion classifications, and the original and most common, the M-stage scoring system (Dopfer et al., 1997; Krull et al., 2014; Relun et al., 2011; Zinicola et al., 2015b). There are five M lesion stages of DD including active, ulcerative stages and chronic, proliferative stages which progress non-linearly. M0 refers to normal skin without evidence of a pre-existing DD lesion (Krull, 2015). M1 is a small, ulcerative lesion less than 2cm while an M2 is the characteristic 'strawberry' lesion stage with an ulcerative lesion larger than 2cm (Dopfer et al., 1997; Krull, 2015). M1 and M2 are active, painful lesion stages prone to bleeding (Berry et al., 2012). Histologically, there is invasion of stratum spinosum by spirochetes, epidermal hyperplasia, and reactive inflammation in active DD stages (Wilson-Welder et al., 2015a). M3 is the healing stage, which typically occurs after antibiotic treatment, characterized by the transformation of the ulcerative surface to acanthotic or a firm-rubbery, brown eschar with no pain upon manipulation (Berry et al., 2012). M4 lesions are raised by brown, rubbery, irregular, proliferative hyperkeratotic growths with papilliform to hair-like projections (Berry et al., 2012). M4.1 are chronic stage M4 lesions with an active focus (Berry et al., 2012). Chronic, non-active DD lesions are considered less painful and less prone to bleeding (Berry et al., 2012). Lesions can alternate between chronic and active stages in the absence of treatment. Current treatment regimens have variable outcomes, and many treated lesions reoccur as clinical ulcerative stages, consistent with non-linear disease progression.

Etiology

Currently, it is believed the complex nature of DD is related to the changes in microbial community composition between establishment, early, active and chronic lesion stages (Krull et al., 2014; Wilson-Welder et al., 2015a). Although the etiological agents of DD have not been definitively identified, numerous genomic approaches have determined viral and fungal

pathogens are unlikely causative agents (Krull et al., 2014). Additionally, lesions tend to improve following treatment with antibiotics suggesting a bacterial etiology that is largely supported in the scientific community (Krull et al., 2014; Zinicola et al., 2015a). Visual, biochemical and molecular techniques have consistently identified multiple *Treponema spp.* in DD lesions (Plummer and Krull, 2017). Previous studies have associated the morphological changes associated with DD lesion progression with significant changes in the microbial population (Krull et al., 2014; Zinicola et al., 2015b). While it is likely that *Treponema* play a significant role in DD disease etiology and progression, DD is increasingly recognized as a polymicrobial disease with consistent shifts in community structure between lesions stages (Krull et al., 2014). *Treponema* are relatively absent from DD-free feet and become increasingly abundant in active, ulcerative and chronic, proliferative DD lesions which are dominated by *Spirochaetaceae* representing up to 90% of the bacterial community (Krull et al., 2014).

The inability to satisfy Koch's postulates for DD is likely due to the polymicrobial nature of the disease and the inability to culture a large proportion of species identified in natural infections (Orsel et al., 2017; Wilson-Welder et al., 2015). Further, *Treponema spp.* exist in DD lesions within polytreponemal communities in which up to 17 distinct phylotypes have been postulated (Rasmussen et al., 2012). When *Treponema* are successfully isolated from co-infecting members of the bacterial community, they are rarely isolated as a single species and tend to be cultured as a sub-population of *Treponema spp.* found in DD lesions. The difficulty to isolate single species of *Treponema* makes identifying the contribution of each species to virulence, tissue pathological changes, and community interactions nearly impossible to model *in vitro* and *in vivo*. There is increasing evidence in non-DD diseases associated with *Treponema* infections that supports the hypothesis that there are synergies and dependencies between

Treponema spp. and potentially non-treponemal members in DD polymicrobial communities. Similarities between periodontal disease and DD, such as complex polymicrobial communities and a high proportion of fastidious spirochetes that degrade host tissues, suggests interactions observed between oral pathogenic *Treponema* may be applicable to DD (Wilson-Welder et al., 2015). These studies have shown oral pathogens *Porphyromonas gingivalis* and *T. denticola* are metabolically dependent *in vitro*, exhibit synergistic virulence in animal models of periodontitis and improved growth in co-culture (Kesavalu et al., 1997; Tan et al., 2014). To improve current research efforts examining DD etiology, pathology, and treatment and control, a consistent and reliable method of identifying *Treponema spp.* present in DD lesions is required.

Research of DD is hindered by the inability to culture and isolate most DD-associated *Treponema spp.* in pure culture which leads to a deficit in genomic data required for species identification, phylogeny, and identification of virulence factors and antimicrobial resistance genes. The absence of complete, annotated *Treponema* genomes is likely responsible for *Treponema* isolate phylotyping rather than species identification. These factors further contribute to many DD-associated spirochetes remaining unknown and unidentified. *Treponema* phylotypes are based on culture independent methods such as 16S rRNA and/or *flaB2* sequencing and 16S-23S intergenic spacer region sequence analysis which distinguishes phylotypes based on size (Döpfer et al., 2012; Evans et al., 2008; Scandinavica et al., 2008; Stamm et al., 2002). Phylotypes are defined as clusters of *Treponema* in which the 16S rRNA sequence differs by approximately 2% from known species and species within a cluster must share $\geq$ 99% sequence similarity with other members of their cluster (Wilson-Welder et al., 2015a). Phylotypes are then named based upon their sequence similarity to human *Treponema* isolates, primarily periodontal disease isolates. While the number of *Treponema* phylotypes associated with DD is inconsistent

and variable depending on geographical location and method of grouping, *Treponema medium*/*T. vincentii*-like, *T. phagedenis*-like, *Treponema pedis*-like, and *Treponema putidum*/*Treponema denticola*-like appear to be among the most prevalent species in DD (Döpfer et al., 2012; Klitgaard et al., 2013; Rasmussen et al., 2012; Scandinavica et al., 2008; Stamm et al., 2002). The inconsistency and variability in species typing alone suggests understanding the nature of DD is greatly hindered by the inability to identify and characterize strongly correlated species. Whole genome sequencing (WGS) allows species characterization based on standards established by the International Committee on Systematics of Prokaryotes which form the basis of microbial discrimination, nomenclature, and phylogenetic relationships (Struelens et al., 2001). Complete genomes of infecting species would greatly enhance research efforts by providing consistent, unambiguous nomenclature which allows meaningful comparisons between studies. Methods of identifying both the *Treponema spp.* present in DD lesions and their relative proportions in relation to the grade of the lesion would enhance the current understanding of DD and the likelihood of effective control methods. Therefore, elucidating interactions between infecting species of *Treponema* and virulence may explain the non-linear disease progression and variable treatment outcomes

Transmission

Digital dermatitis may be transmitted between animals and herds through environmental reservoirs, host reservoirs, and fomites (Orsel et al., 2017). *Treponema*-associated with DD have been shown to undergo a morphological change from a spiral to an encysted form which increases their environmental survival time in addition to functioning as a host immune system evasion mechanism (Biemans et al., 2017; Döpfer et al., 2012). *Treponema* encyst as spherical bodies when host and environmental conditions are unfavourable for propagation and release

motile, spiral bacteria when conditions improve (Al-Qudah et al., 1983). It is currently uncertain whether the large, spherical bodies formed by *Treponema spp.* are restricted to a single species or multiple interacting species. Examining the nature of DD-*Treponema* spherical bodies may elucidate infection mechanisms and modes of transmission.

Treponema spp. can be transmitted between cattle without direct contact. The environmental survival time for *Treponema* is an important risk factor for DD. Beimans et al., (2017) recently modelled the reproduction rate (R_0) for DD lesions and found cows may be infected through infected feet or environmental reservoirs. Multiple experimental models of DD infection transmission have shown DD can be induced in naïve sheep and cattle by mixing and intermingling (Krull et al., 2016; Orsel et al., 2017). Previous studies have found *Treponema* in bovine gingiva and rectum tissues as well as rumen fluid, fecal samples, and slurry leading to a hypothesis that the gut may act a reservoir for *Treponema* (Evans et al., 2012). While the survival time of *Treponema spp.* in the environment is unknown, *Treponema* have been cultured from fomites such as hoof-trimming equipment before disinfection (Sullivan et al., 2014). Therefore, if hoof-trimming equipment is not adequately disinfected, the equipment may act as a transmission vector within and between herds. As DD is an infectious disease, introducing new cattle with DD to the herd may act as a transmission vector between farms (Refaai et al., 2013). Given the difficultly in eradicating DD from DD-infected herds, determining mechanisms of transmission and environmental survival time of DD-associated *Treponema spp.* could improve current control programs.

Risk Factors

Many questions remain regarding DD disease etiology and transmission which challenges defining risk factors to infection; however, many environmental, bacterial and host factors are

thought to play an important role in disease development. An important risk factor, surprisingly, is the grade of DD lesions present on the farm (Biemans et al., 2017). The only M-stage lesions with an $R_0>0$, indicating for every infection on the farm additional cattle will be infected, were M4 lesions; this finding is significant because treatment regimes and footbaths tend to improve the painful clinical symptoms of active DD lesions but do not reduce the prevalence of M4 lesions (Biemans et al., 2017). The infectivity of M4 lesions suggests they are an especially important lesion grade to treat and control but have not been emphasized for treatment and control as they don't contribute to lameness as significantly as the more painful, active lesions.

Individual level risk factors for DD include parity, lactation, breed and genetics (Refaai et al., 2013). Primiparous cows have an increased risk for DD lesions compared to multiparous cows, potentially related to inadequate acquired immunity against DD and undergoing drastic changes in environment, nutrition and metabolism around the time of calving with the latter two contributing to an increased risk in lactating cows (Refaai et al., 2013; Somers et al., 2005). Finally, breed and genetics have been associated with differential risk for DD due to differences in claw formation and undefined genetic factors influencing susceptibility (Refaai et al., 2013).

Herd level risk factors are primarily related to management practices such as hygiene, hoof-trimming, housing type, and purchasing heifers (Refaai et al., 2013). Poor hygiene and moist conditions weaken the hooves, increasing disease susceptibility by creating excess fluid maceration of the epidermis and allowing pathogen establishment (Refaai et al., 2013). Therefore, flooring type also plays an important role in DD prevalence. Cows on pasture are least likely to develop DD, followed by cattle on slatted, scrapped floors, and cows on solid concrete floors are associated with the highest DD prevalence (Refaai et al., 2013; Somers et al., 2005). Nutrition and aspects of cow comfort such as adequate bedding space and stocking

density are also associated with a reduced DD incidence attributed to decreased time spent standing with wet feet (Refaai et al., 2013; Somers et al., 2005). Therefore, changes in management and housing characteristics can greatly decrease the incidence of DD in infected herds.

Antibiotic Resistance

Antibiotic resistance originated in the environment where organisms evolved under a constant barrage of diverse cytotoxic and inhibitory chemicals. Before the first clinical use of antibiotics, resistant organisms were isolated from the environment suggesting that resistance genes evolved initially in antibiotic producing organisms to play a protective role or roles outside of antibiotic resistance (Vogwill and Maclean, 2015). It is clear that environmental bacteria are more often intrinsically drug resistant than the commensal and pathogenic organisms of importance to human and animal health (Wright, 2011). However, the widespread use of antibiotics in the past 70 years has provided selection pressures to mobilize highly efficient resistance genes to pathogenic bacteria (Wright, 2011). It is increasingly recognized that multi-drug resistant commensal bacteria in the gastrointestinal tract of humans and animals act as resistance reservoirs to pathogenic bacteria (Hawkey and Jones, 2009).

The use and overuse of antibiotics has contributed to the increase and wide spread distribution of antibiotic resistance genes by exerting a strong selection pressure on human and animal pathogens. However, the evolution of antibiotic resistance carries a fitness cost, expressed in terms of reduced competitive ability, reduced growth rate, or virulence to bacteria harbouring resistance genes (Vogwill and Maclean, 2015). The cost of harbouring resistance genes on bacteria fitness plays a key role in the evolutionary dynamics of resistance by generating selection against bacteria carrying resistance genes through a fitness disadvantage.

Therefore, the frequency and rate of dissemination of antibiotic resistance in bacterial populations is directly related to the potency, concentration and volume of antibiotic used and inversely related to the cost resistance poses on the fitness of bacteria (Debabov, 2013). However, bacterial and mobile genetic element (MGE) coevolution has facilitated various compensatory and adaptive mechanisms that have lessened the competitive disadvantage of resistant strains (Andersson and Hughes, 2010). Plasmid coevolution and compensatory mutations challenge the notion that resistance gene prevalence will decrease in the absence of antibiotics because this notion relies on the assumption that resistant strains have a fitness disadvantage over susceptible wildtype strains (Schrag et al., 1997).

Antibiotic use in Agriculture and Digital Dermatitis

Maintenance of healthy animals requires prevention of infection by pathogenic and opportunistic organisms (Merck Veterinary Manual, 2013). In addition to decreasing morbidity and mortality from clinical and subclinical infections, sub-therapeutic administration of antibiotics is used to increase growth rate and improve feed utilization with minimal additional labour input while preventing infections during high stress periods such as transport (Gersema and Helling, 1986). Lesions of the sole, including digital dermatitis, are typically controlled at the herd-level using regular footbaths. Footbaths typically contain copper sulfate, zinc sulfate, formaldehyde, or salicylic acid, with copper being the industry standard (Jacobs et al., 2017). Studies have shown utilizing footbaths along with good hygiene and management practices decreases the incidence of DD but is not 100% effective at preventing new infections in herds with a history of DD (Cutler et al., 2013; Laven and Logue, 2006; Speijers et al., 2013). Clinical cases of DD, typically large active, ulcerative lesions (M2 lesions and M4.1 lesions) can be treated topically with antibiotics. Topical antibiotics, oxytetracycline or lincomycin-

spectinomycin typically, are the most common and effective treatment for DD; unfortunately, neither have a high cure rate over time (Gomez et al., 2012; Krull et al., 2014; Wilson-Welder et al., 2015a). Systemic antibiotics can be used to treat DD, such as amoxicillin, erythromycin, and cefquinome, but this is infrequent due to mixed reports of cure rate, increased milk withdrawal times, cost, and concern of antibiotic resistance (Laven and Proven, 2000; Speijers et al., 2012). In large herds with many DD affected cattle, the most cost-effective mechanism to treat DD is antibiotic footbaths, typically containing tetracycline (or a derivative to increase efficacy) (Speijers et al., 2012). While antibiotic footbaths can be effective to treat DD while reducing time and labour costs, they likely enhance AMR on the farm compared to individual treatment and only provide short-lived symptomatic relief with many cattle remaining acutely or chronically infected (Bell, et al., 2017).

Resistance Co-occurrence

There is growing concern that environmental contamination with heavy metals, antibiotics and chemicals is leading to proliferation of metal and antibiotic resistance co-selection. Previous studies have identified a correlation between the amount and type of metal contamination and specific patterns of antibiotic resistance through co-resistance, where resistance determinants are present on the same genetic element, and cross-resistance, where the same resistance determinant confers resistance to both antibiotics and metals (Baker-Austin et al., 2006). This suggests use of heavy metal sulfate footbath solutions in combination with group or individual antibiotic treatment for DD could lead to increases in multi-drug and -chemical resistant bacteria. While AMR itself is not a virulence factor, both are necessary for bacteria to survive in competitive, adverse environments (Beceiro et al., 2013). As virulence mechanisms are necessary to overcome host defenses, harbouring AMR is key for the development of

infections in competitive environments under the pressure of antimicrobials (Beceiro et al., 2013). Furthermore, virulence factors and AMR genes are both transmitted primarily through horizontal gene transfer (HGT) on MGEs indicating they have both co-evolved with bacteria; the similarity in selection pressures and co-evolutionary history suggests similar compensatory mechanisms and mutations that cause AMR and virulence to be indirectly correlated.

Resistance Mechanisms

Bacteria may be either intrinsically resistant to an antibiotic or antimicrobial (AM) chemical, resisting activity of an AM through inherent structural or functional characteristics, or they may acquire AMR through genetic mutation or HGT of MGEs that confer resistance. These resistance mechanisms can be categorized according to the biochemical route which confers resistance: 1) modifications of the antibiotic, 2) modifications of the antimicrobial target sites, and 3) prevention of antibiotic from reaching target site (Munita et al., 2016). An effective way for bacteria to evade antibiotic pressures is to produce enzymes that add chemical moieties, such as acetylation, adenylation and phosphorylation, that inactivate the antibiotic or remove chemical moieties by breaking bonds between key chemical groups, preventing the antibiotic from interacting with its target (Munita et al., 2016). Similarly, bacteria may interfere with antibiotic action by modifying the target site to decrease binding affinity, protecting the target site by direct competition or inducing target site conformational changes, or dislodging the antibiotic from its target site (Donhofer et al., 2012). Finally, bacteria may decrease intracellular concentrations of an AM by decreasing permeability, preventing influx, and removing the AM through efflux pumps which may be substrate-specific or have broad substrate specificity found in multidrug resistant bacteria (Munita et al., 2016).

Antibiotic modification

Enzymatic modification of the antibiotic is the main resistance mechanism to β-lactams, such as penicillin. β-lactams are broad spectrum antibiotics that inhibit transpeptidase enzymes, referred to as penicillin binding proteins (PBPs), that are essential for the synthesis of peptidoglycan in the cell wall of both gram negative and positive bacteria (Palzkill, 2014). The inhibition of peptidoglycan synthesis results in the death of growing bacteria through a cellular stress response that leads to bacterial lysis (Fair and Tor, 2014). Metallo-β-lactamases (MβL) fold metallo-hydrolases are a sub-class in a diverse group of β-lactamase enzymes differentiated by mono or di-zinc ion dependent hydrolysis of the amide bond of the β-lactam ring (Palzkill, 2014). MβL fold metallo-hydrolases have broad substrate binding capacity, catalysing the hydrolysis of most β-lactams into biologically inert β-amino acids (Page and Badarau, 2008).

Spectinomycin binds to the bacterial 30S ribosomal subunit, interfering with the initiation of protein synthesis and elongation, resulting in a bacteriostatic effect and ultimately bactericidal activity. Cross-resistance to aminoglycoside antibiotics such as streptomycin and spectinomycin is conferred through modification enzymes, aminoglycoside nucleotidyltransferases (ANTs), that transfer an adenyl group to the antibiotic, inactivating the antibiotic (Prabhu et al., 2017). ANTs are classified based on the site of hydroxyl adenylation from the substrate ATP. Streptomycin 3″-adenylyltransferase confers cross resistance to spectinomycin and streptomycin (Lovering and Reeves, 2011). Lincomycin and spectinomycin are combined to treat many polymicrobial infections in veterinary medicine including digital dermatitis and respiratory diseases. Lincomycin is a small spectrum bacteriostatic antibiotic active against gram-positive bacteria and spectinomycin shows bactericidal activity against gram-negative bacteria and

Mycoplasma; the combination is used for broad spectrum antimicrobial activity with high blood and tissue concentrations.

Nitroimidazole drugs have selective toxicity towards anaerobic bacteria and protozoa due to the requirement of an anaerobic 1-electron reduction of the nitro group controlled by the target cell (Leiros et al., 2004). Nitroimidazoles are administered as prodrugs and their bactericidal activity requires the reduction of the nitro group; after entering susceptible cells by diffusion, the reduced, free radical antibiotic causes DNA strand breaks, helix destabilization and unwinding (Jenks, 2010; Leiros et al., 2004). Reduction is mediated by ferredoxin in the redox electron transport chain resulting in a short lived radical anion (Leiros et al., 2004). Resistance to nitroimidazole drugs is conferred through the presence of a gene encoding a 5-nitroimidazole reductase that prevent the formation of free radicals responsible for bactericidal activity. 5'-nitroimidazole reductases convert the necessary nitro group on the antibiotic into a non-bactericidal amine derivative ($R\text{-}NH_2$)(Leiros et al., 2004). The presence of 5-nitroimidazole on bacterial chromosomes or plasmids pose a significant risk to potential treatments of anaerobic bacteria associated with digital dermatitis.

Formaldehyde cellular toxicity results from its reactivity as an electrophile to free thiol and amine groups on protein and DNA resulting in the formation of irreversible formaldehyde adducts and DNA-DNA, DNA-protein and protein-protein crosslinks (Chen et al., 2016). Formaldehyde resistance is primarily mediated by glutathione and NAD-dependent formaldehyde dehydrogenases that detoxify formaldehyde to formate (Chen et al., 2016; Kümmerle et al., 1996). While formaldehyde is not utilized as frequently as copper or zinc sulfate solutions due to animal and environmental toxicity, it is still a common active ingredient in footbaths used to control DD.

Antibiotic Target Modification

Bacteria may resist the effects of antibiotics by modifying the antibiotic target site permanently through mutation or temporarily through transient target site conformational changes. Tetracyclines are broad spectrum antibiotics that bind to the elongating ribosomes during translation and inhibit the delivery of elongation factor Tu (Ef-Tu) carrying GTP and aminoacylated-tRNA to the A-site (Donhofer et al., 2012). The commonly used chlortetracycline and oxytetracycline for treatment of DD are natural compounds produced by *Streptomyces* species and are classified as first generation tetracyclines (Chopra and Roberts, 2001). The tetracycline resistance superfamily consists of ribosomal protection proteins (RPPs) such as TetO and TetM (Donhofer et al., 2012). TetO has 45% structural similarity to Ef-G and has been shown to interact with helix 34 adjacent to the tetracycline binding site which induces a conformational change to tetracycline binding site leading to tetracycline dissociation from the ribosome and lasting conformational changes preventing tetracycline rebinding (Donhofer et al., 2012). TetM and TetW have been shown to interact directly with the ribosomal tetracycline binding site leading to direct tetracycline dislodge and release and competitive inhibition (Alekshun and Levy, 2007; Donhofer et al., 2012).

Antibiotic Efflux Pumps

Bacterial multidrug efflux pumps are classified into five families: MFS (major facilitator superfamily), ABC (ATP-binding-cassette), resistance nodulation cell division (RND), small multidrug resistance, and multidrug and toxic compound extrusion based on sequence similarity (Nishino et al., 2010). Of the tetracycline-specific resistance mechanisms, RPPs (ribosomal protection proteins) and efflux pumps are the most common (Donhofer et al., 2012). TetR is a transcriptional regulator that controls the expression of a tetracycline efflux pump responsible for

multidrug resistance in addition to regulating osmotic stress and virulence and pathogenicity (Deng et al., 2013).The diverse C-terminal region of the TetR family of regulators allows different regulators to accommodate specific sets of inducing ligands (Deng et al., 2013). TetR binds to the promoter of efflux pump genes resulting in a conformational change of the protein which relieves transcriptional repression (Deng et al., 2013). Control of the TetR repressors is mainly controlled by ligands; in the absence of tetracycline, TetR binds to the promoter and represses transcription of the of the efflux pump genes (Deng et al., 2013). In the presence of tetracycline, tetracycline enters the cell and binds to TetR, leading to a conformational change that abolishes protein binding and relieves transcriptional repression (Deng et al., 2013). The multidrug resistance efflux pump then removes toxic substances, such as tetracycline, from the cell.

Acquisition of resistance to hydrophobic compounds such as macrolides are mediated by two classes of efflux pumps, ABC transporter superfamily and MFS (Leclercq, 2002). The MFS efflux proteins encoded are secondary transporters driven by the proton motive force and confer inducible resistance to 14- and 15- membered- ring macrolides and type B streptogramins (Leclercq, 2002; Reynolds et al., 2003). MLS_B MFS efflux pumps provide functional resistance to macrolides, lincosamides (lincomycin, clindamycin, and pirlimycin), and B type streptogramins, which are all chemically distinct but have a similar mode of action (Spížek and Řezanka, 2017). MLS_B antibiotics bind to the 50S ribosomal subunit resulting in the inhibition of protein synthesis and share at least one binding site (CLSI, 2013). Lincosamides are used to treat infections in which synergistic effects of mixed anaerobic bacteria are anticipated (Spížek and Řezanka, 2017).

Copper is an essential ion for most living organisms but has cytotoxic effects outside of the limited tolerable range (Hernández-Montes et al., 2012). Copper has been shown i*n vitro* to activate oxygen and hydrogen peroxide, performing Fenton reactions, leading to biological damage, such as lipid peroxidation and protein damage, from hydroxyl radicals and high ion oxidation states (Dupont et al., 2011; Winterbourn, 1995). The gene *nlpE* encodes an outer membrane lipoprotein involved in copper resistance and surface adhesion. The protein encoded by *nlpE* is part of the Cpx stress response system and has been shown to induce expression of multidrug resistance efflux pumps, increasing the overall resistance of bacteria to copper and antibiotics (Nishino et al., 2010). Heavy metal P-type ATPases are conserved protein domains involved in the transport and detoxification of heavy metals such as copper, cadmium, zinc, and cobalt (Marchler-Bauer et al., 2017). Like copper, zinc is an essential micronutrient for most living organisms. ZnuABC transporter is an essential periplasmic ABC transporter for zinc uptake, homeostasis and resistance to innate host defence mechanisms in bacteria, and increases host pathogenicity (Cerasi et al., 2013; Ilari et al., 2011). ZnuABC, a high affinity zinc transporter, functions to protect intracellular targets from metal poisoning (Ilari et al., 2011). Copper sulfate, followed by zinc sulfate, is the industry standard for herd-level control of DD; however use may lead to copper resistance in addition to multidrug resistance activation (Jacobs et al., 2017; Solano et al., 2015).

Significance of Research

This dissertation represents significant studies aimed to examine the contribution of *Treponema spp.* to DD lesion grades and the efficacy of current DD treatment and control regimes through the distribution of AMGs within DD lesions. To our knowledge, this is the first

study examining the contribution of *Treponema* species present and their absolute quantities to different disease manifestations described using the M-lesion scoring system. It is also the first study examining the distribution of *Treponema* harbouring AMGs within DD lesions. We have determined *Treponema* in DD lesions may be identified reliably and consistently at the species level using species-specific genes, providing a method to improve current nomenclature of DD-associated *Treponema*.

Objectives

1. Design a diagnostic multiplex species-specific qPCR and PCR to identify species of *Treponema* in DD lesions
2. Determine *Treponema* species presence and proportions in DD lesions and examine relative contribution to disease states
3. Sequence *Treponema* genomes to validate species-specific genes and examine the distribution of antimicrobial resistance genes in DD lesion

Chapter 2: Associations between digital dermatitis lesion grades and the quantities of four *Treponema* species

Associations between digital dermatitis lesion grades and the quantities of four *Treponema* species

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Running Title: *Treponema* quantification and DD lesions

Abstract

Digital dermatitis (DD) presents as painful, ulcerative or proliferative lesions that lead to lameness affecting economic efficiency and animal welfare. Although DD etiological agent(s) have not been established, it is widely accepted that DD is a polymicrobial disease significantly associated with species of *Treponema* and the non-linear disease progression may be attributed to interactions among infecting bacteria.

We postulated the morphological changes associated with DD lesion grades are related to interactions among infecting species of *Treponema*. We developed a novel species-specific qPCR that can identify the absolute abundance of the four of the most common species of *Treponema* in DD*, T. phagedenis, T. medium, T. pedis* and *T. denticola,* in a single reaction. We found both the abundance of species and the number of different *Treponema* species present is higher in active, ulcerative lesions than in healing lesions, chronic lesions, and DD-free skin. We have also found positive correlations among *T. phagedenis, T. medium* and *T. pedis* indicating they are significantly more likely to be found together than apart and their absolute quantities tend to increase together, a relationship which is not present with *T. denticola*. Our findings suggest that pathogenicity may be more closely associated with *Treponema* abundance, particularly of *T. phagedenis, T. medium and T. pedis,* and interactions among them, independent of *T. denticola*. Our results provide a novel, consistent method to identify species of *Treponema* within DD lesions and associate *Treponema* abundance and the number of species present with morphological changes related to host pathogenicity.

Introduction

Globally, digital dermatitis (DD) is a leading cause of infectious lameness in dairy cattle (Wilson-Welder et al., 2015). Digital dermatitis is mainly found on the plantar aspect of a cow's foot, presenting as an ulcerative or proliferative lesion along the coronary band, affecting the area between the heel bulbs (Wilson-Welder et al., 2015). A review paper on lameness prevalence studies throughout the world between 1993 and 2014 found between 25 and 55% of dairy cattle are clinically lame on average, 15-22% of cows have one or more DD lesions, and up to 94% of herds have DD (Cook, 2016; Solano et al., 2017a; Underwood et al., 2015). The infectious, ulcerative or proliferative lesions caused by DD contribute to animal welfare concerns and significant economic losses due to premature culling, milk loss, decreased fertility and treatment costs (Evans et al., 2009a; Solano et al., 2017a). The complex polymicrobial nature of the disease and non-linear lesion progression have contributed to the inability to determine the etiological agent(s) for DD despite significant economic incentives (Evans et al., 2009b; Plummer and Krull, 2017). Species of *Treponema,* fastidious, anaerobic bacteria, are consistently isolated from DD lesions in polytreponemal communities (Evans et al., 2008; Nielsen et al., 2016; Zinicola et al., 2015). While there is considerable evidence implicating *Treponema spp.* as causative agents in DD, Koch's postulates have yet to be satisfied (Gomez et al., 2012; Krull et al., 2016; Nielsen et al., 2016; Wilson-Welder et al., 2015). It has been more than 40 years since DD was first described, and despite efforts to prevent the disease with improved hygiene practices, regular footbaths and bedding modifications, effective treatments that eliminate the disease from herds have not been identified (Dopfer et al., 1997; Evans et al., 2009a). Furthermore, treatments for DD with 100% cure rate have not been identified and treated lesions

are prone to relapsing into active disease states or remaining chronic (Angell et al., 2015; Döpfer et al., 2012; Murray and Carter, 2015).

Methods to simultaneously identify multiple species of *Treponema* within DD lesions are currently limited to 16S rRNA gene sequencing-based microbiome approaches which cannot reliably resolve species composition due to partial 16S gene coverage, limited complete genomes available and many unidentified *Treponema* species. *Treponema* phylotypes are based on culture independent methods such as 16S rRNA and/or *flaB*2 sequencing and 16S-23S intergenic spacer region sequence analysis which distinguishes phylotypes based on size (Döpfer et al., 2012; Evans et al., 2008; Pringle et al., 2008; Stamm et al., 2002). Phylotypes are defined as clusters of *Treponema* in which the 16S rRNA sequence differs by approximately 2% from a known treponeme species and species within a cluster must share $\geq$ 99% sequence similarity with other members of their cluster (Wilson-Welder et al., 2015). Phylotypes are mostly named based upon their sequence similarity to human *Treponema* isolates, primarily periodontal disease isolates (Nielsen et al., 2016). Because researchers use different sequences for clustering, isolates are clustered differently, impacting prevalence data between studies (Döpfer et al., 2012; Evans et al., 2008; Pringle et al., 2008; Stamm et al., 2002).

Previous studies have associated *Treponema* abundance with lesion severity and temporal progression, demonstrating that the composition of species present changes considerably as lesions progress (Krull et al., 2014). The microbial community present within DD lesions has been shown to correlate with morphological changes of lesions; however, while this relationship is significantly correlated with the 5 grade Iowa DD scoring system, which primarily differs from M-grade scoring by having a temporal aspect to lesion progression and adding 2 early grade lesions, it has not been examined using the M-scoring system (Krull et al., 2014).

Therefore, current evidence suggests *Treponema spp.* and bacterial community composition may be related to morphological changes associated with grades of DD.

There is increasing evidence implicating multiple species of *Treponema* in DD etiology and pathogenicity; however, there is a knowledge deficit regarding potential interactions among species present (Orsel et al., 2017). Based on current evidence, we postulate a relationship between the *Treponema spp.* present and DD lesion grades and/or progression that may be facilitated by synergistic interactions among species. The aims of this study were to examine the correlations among *Treponema spp.* within and between lesion grades. For this purpose, a novel species-specific qPCR was developed to identify species present, their abundance, and relative proportions in DD lesions. Identifying correlations between species of *Treponema* present in DD lesions may provide essential targets for vaccines and treatment development by indicating species synergies required for enhanced virulence or pathogenicity.

Materials and Methods

Farm and animal details

Farms were selected based on previous DD prevalence information and geographical location, facilitated by two certified Alberta hoof trimmers. Samples were collected from 10 Alberta farms and one abattoir between Calgary and Ponoka throughout a 1.5-year period. Additionally, 10 samples were collected from a Saskatchewan dairy herd that has been a closed farm for over 50 years with a reported zero DD prevalence (Rayner Dairy Research and Teaching Facility, University of Saskatchewan). All animal use was approved under protocol #AC16-0070, by the University of Calgary Veterinary Services Animal Care Committee (VSACC) under the guidance of the Canadian Council on Animal Care (CCAC) prior to the onset of the study.

DD Scoring

Digital dermatitis lesions were identified on farm in the trimming chute according to the M grade scoring system and limited to lesions located between the heel bulbs (Berry et al., 2012; Dopfer et al., 1997). Briefly, M1 and M2 lesions were ulcerative masses along the coronary band, distinguished by a lesion smaller or larger than 2cm, respectively (Berry et al., 2012). Due to the difficulty in characterizing M3 lesions without treatment history, we classified M3 lesions as active lesions approximately 5 days after antibiotic treatment. M4 lesions were raised, hyperkeratotic, and proliferative, with papilliform projections and M4.1 lesions were M4 lesions with an active ulcerative area.

Biopsy

Identified lesions were scrubbed twice using a chlorohexidine scrub followed by a 70% ethanol wipe while animals were in a no-tilt trimming chute to remove caked on debris and clean the area. Lidocaine Neat (2-3mL) (Lidocaine HCl 2%, DIN 00712884, Zoetis Canada Inc., Kirkland, QC, Canada) was administered subcutaneously with a 20g needle. Small, 4mm biopsy punches with a maximum coring depth of 7mm (Standard Disposable Biopsy Punch, Miltex, Integra Life Sciences Corporation, York, PA, USA) were taken from various DD lesion grades from dairy cows on 10 Alberta farms and DD-free feet from the abattoir and 1 closed herd in Saskatchewan (N=142). For lesions with an ulcerative focus, M1, M2, and M4.1, the biopsies were collected from the active area. “M3 biopsies” were collected from M2 lesions approximately 5 days after antibiotic treatment with soluble tetracycline250 powder (250mg/g) (DIN 0052777, Vetoquinol, Lavaltrie, QC, Canada). Samples between the heel bulbs from non-infected hooves (N=21) were collected from culled Holstein cows at the abattoir less than 2 hours post-mortem. Two samples from DD-infected feet at the abattoir were taken to ensure

Treponema could be cultured from tissue samples collected post-mortem, if present. Biopsies were immediately placed into semi-solid anaerobic transport media (ATM) (Anaerobe Systems, Morgan Hill, CA, USA) with the interior portion inserted first to avoid contaminating the biopsies with microorganisms from the outer skin and transported at room temperature. The transport medium, ATM, contains sodium thioglycolate and cysteine reducing agents to sustain viability of anaerobic microbes during transport with minimal multiplication. Biopsy samples from within Alberta were transported to the lab and processed within 8h of sampling and biopsies from Saskatchewan were processed within 24h of sampling.

Culture and Isolation

In the anaerobic cabinet (Bactron3000, Sheldon Manufacturing, Inc., Cornelius, OR, USA) (25% CO_2, 5% H_2, balance nitrogen), on a sterile petri dish with sterile tissue forceps and a no.10 scalpel blade, the outermost portion of the epidermis was removed from the biopsies and discarded. The remaining interior portion was sectioned longitudinally into 4 fragments of approximately equal size and macerated. Biopsy fragments for DNA extraction and storage were inoculated into Tris-EDTA buffer (TE) (pH=8.0) and OTEB + 20% glycerol, and stored at -20 ºC and -80 ºC, respectively. Biopsy fragments for culture were inoculated into oral treponeme enrichment broth (OTEB) (Anaerobe Systems, Morgan Hill, CA, USA) with 5% enrofloxacin ($\geq$ 98% HPLC, powder, Sigma-Aldrich, Burlington, MA, USA) dissolved in DMSO, 10% rifampicin ($\geq$ 97% HPLC, powder, Sigma-Aldrich, Burlington, MA, USA) dissolved in DMSO, and 10% equal parts bovine and rabbit serum (Gibco, Life Technologies, New Zealand and USA, respectively) (OTEBSER) and MTGE media supplemented with 5% enrofloxacin in DMSO and 10% rifampicin by spread plating (MTGEER) (Anaerobe Systems, Morgan Hill, CA, USA). Biopsy fragments were inserted into MTGE medium using a no.10 scalpel blade and sterile

forceps to ensure the fragments were laterally and distally surrounded by agar. MTGEER plates were wrapped in parafilm and incubated for 7 days at 37°C. Motile bacteria (those that migrate away from the biopsy) forming white-translucent, small, and irregular colony morphologies on the surface of, or embedded within, the agar were selected from each plate and subcultured into 1mL of OTEBSER in 1.5 mL Eppendorf tubes and incubated for up to 10 days at 37ºC in the anaerobic cabinet. Subculture purity, absence of contaminating non-treponeme bacteria, was assessed using dark field or differential interference contrast (DIC) microscopy based on *Treponema* morphological characteristics (thin corkscrew-shaped bacteria between 0.1-0.4 μm in width and 4-15 μm in length, and rotational movement about the longitudinal axis) (Radolf, 1996). Subcultures were screened for *T. phagedenis, T. medium, T. pedis* and *T. denticola* with an in-house species-specific multiplex PCR outlined below. Cultures containing other bacteria in addition to *Treponema* were spread plated onto MTGEER, incubated for 7 days at 37ºC in the anaerobic cabinet, and subcultured into OTEBSER as above until contaminant free cultures were obtained.

Macerated biopsy fragments in 200 uL of TE were weighed twice and averaged prior to freezing and DNA extraction. DNA from macerated biopsy fragments in TE was extracted using DNeasy Blood and Tissue Extraction Kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). DNA was eluted into nuclease-free ultra pure water and stored at -20ºC.

qPCR design and optimization

A 4-plex *Treponema* species-specific qPCR was designed to quantify four treponeme species and their absolute quantities in bovine tissue samples. Genomic DNA extracted from 7 DD *Treponema spp.,* which were identified as *T. phagedenis, T. pedis,* and *T. medium,* from the

University of Wisconsin, provided by Dr. Döpfer, were sequenced, assembled and annotated as described previously (Naushad et al., 2016). For species-specific gene identification, an in-house database containing newly sequenced *Treponema* genomes and representative genome sequences from all known bacterial species was constructed from NCBI, EZBioCloud, PATRIC, and JGI-IMG (Markowitz et al., 2012; Pruitt et al., 2005; Wattam et al., 2014; Yoon et al., 2017). Potential species-specific genes were identified according to Naushad et al., 2014. Briefly, BLASTn searches were conducted on all Open Reading Frames (ORFs) from *T. pedis, T. phagedenis, T. medium* and *T. denticola* against the in-house database. The ORFs which were detected in a single *Treponema* species and were not found in any other representative bacterial species were considered potential species-specific genes. The specificity of potential species-specific genes was validated after blast searching against the NCBI nr database. The ORFs detected in all known strains of each species were considered unique genes. Unique genes selected for each *Treponema* species were CP004120 for *T. pedis*, WP_002698807.1 for *T. phagedenis*, WP_016523385.1 for *T. medium*, and EGC77593.1 for *T. denticola.* Based on BLAST results and available complete reference genomes for the four *Treponema* species, we assume there is a single species-specific gene copy found in each species. Primer and probes against species-specific genes were designed with idtDNA PrimerQuest software (Integrated DNA Technologies, 2017). Primer sequences were designed with an equal melting temperature (Tm) of approximately 60°C and probes had a Tm of approximately 70°C or 10°C above the primer Tm. The length of the qPCR products was set between 90 and 150bp. Runs of consecutive nucleotides and GC clamps were avoided, and GC content was below 60% in the primers and probes (TaqMan Probe Design, Premier Biosoft). Primer and probe sequences that met the above criteria were analyzed for hairpins, self-dimers, and hetero-dimers using

PriDimerCheking 0.1.0 software (Shen et al., 2010) to avoid secondary structure between primers and probes. Only sequences without hairpins at the Tm of the sequence and ΔG values, which indicate energetic favourability of a given structural conformation, more positive than -9kcal/mol were selected.

Conventional PCR using the genes identified above were designed to verify species composition to validate the qPCR and culture composition (Table 1). Final forward and reverse primer concentrations were 0.3μM in 23 uL of TopTaq mastermix (12.5 uL/reaction), nuclease free water, (6.5 uL/reactions) and coral load (2.5 uL/reaction) (TopTaq™ Master Mix Kit, Qiagen, Hilden, Germany) and 2 uL of template DNA. Reaction conditions were 95°C for 5m, (94°C 30s, 57°C for 30s 72°C 40s) x35, 72°C 5m.

To multiplex the qPCR, the likelihood of all combinations of primer and probe sequences to form secondary structure were calculated using PriDimerCheking 0.1.0 software (Table 1) (Shen et al., 2010). Sequences with the lowest alignment score and the most positive ΔG value were selected. Each species was designated a distinct fluorophore with minimal overlap in the absorption and emission spectra based on the Spectral Overlay Tool (Biosearch Technologies). All qPCR reactions were performed in TaqMan® Fast Advanced Master Mix (Applied Biosystems®, ThermoFisher, CA, USA). The annealing temperature of the qPCR reaction was optimized using a temperature gradient 3°C above and below the mean annealing temperature of all primer and probe sets (CFX96 Touch™ System, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The optimal annealing temperature was the highest temperature at which the lowest quantitation cycle (cq) value is found for all reactions (59-60.4°C); if the optimal annealing temperature was not the same in all reactions, the temperature was averaged without weighting (59.6°C). The primer and probe concentrations were optimized by varying the forward and

reverse primer and probe concentrations (50, 100, 200, 400, 600, and 800 nM); the optimal concentration was the lowest concentration for both primers that achieved the lowest cq value and >98% assay efficiency (1 μM). Assay efficiency was calculated using $E = 10^{(-1/slope)}$, where slope is the slope of the standard curve of each assay (Figure 1) (CFX Manager™ Software, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reactions were optimized as single reactions and then as multiplex reactions. Single and multiplex reactions were run in parallel and conditions were accepted when cq values differed by less than 0.5 between single and multiplex reactions and efficiency remained $\geq$ 95%. Final qPCR conditions for multiplex reactions was: 50°C 2m, 95°C 20s, (95°C 10s, 59.6°C 50s) x 39, 72°C 5m.

qPCR absolute quantification

To relate the cq value to gene copy number, clones containing the species-specific genes of interest were generated. Species- specific PCR products were purified using Qiagen QIAquick PCR Purification Kit and TOPO cloned into commercially available NEB 5-α F' I^q chemically competent *Escherichia coli* cells according to the manufacturer's recommendations (TOPO® TA Cloning® Kit, Invitrogen, Carlsbad, CA, USA). Transformants were identified following blue-white screening by direct colony PCR using the primer sets in Table 1.

Absolute quantification of qPCR products was determined using a standard curve of known concentrations of the above-mentioned clones and the corresponding cq values. The amount of plasmid DNA following purification was determined using a Qubit Fluorometer (Qubit® dsDNA HS Assay Kits, Life Technologies, Carlsbad, CA, USA). To ensure the calculated copy number is representative of the number of species in a biopsy sample and quantify sensitivity, we performed spiking experiments. To spike biopsy samples, known numbers of *Treponema* cells, roughly estimated using a Neubauer counting chamber with DIC

and 40x magnification, were serially diluted 10^{0}-10^{-7} and spiked into lesion-free biopsy samples (N=10) that did not contain the species of interest according to our species-specific PCR and qPCR. *Treponema* cultures of various species composition were grown for 7 days in OTEB in the anaerobic cabinet as described above. Cultures were pelleted by centrifugation at 4500× *g* at 8°C for 20 min, the supernatant removed, and the pellet resuspended in 200 uL of PBS. The biopsy samples were macerated as described above to mimic biopsy processing procedures and spiked with serially diluted *Treponema* samples or an equal volume of PBS as a negative control. Each culture serial dilution was divided into two samples; one was spiked into qPCR-negative bovine tissue while the other was directly extracted without bovine tissue. DNA from cultures, spiked and negative control tissue was extracted using Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The results from the spiked tissue samples were compared to an equal amount of the culture used to inoculate the tissue sample. The efficiency of the multiplex reactions was determined by converting the resulting cq values to copy numbers to determine if there was significant DNA loss at any point in the experimental procedure and accurately quantify the number of *Treponema* species per gram of biopsy tissue.

Microbiome Analysis

Microbiome analysis of the 16S rRNA V1-V2 hypervariable region was performed using Illumina MiSeq at the University of Manitoba with DNA extracted from 16 biopsy samples and *T. denticola, T. medium,* and *T. phagedenis* ATCC culture DNA provided by Dr. Luby of the University of Saskatchewan (ATCC #33520, 700293, 27087, respectively). UNOISE2 algorithm was selected to cluster sequences based on 100% sequence similarity to create amplicon sequence variants (ASVs) (Edgar, 2016) whereas UPARSE created OTUs *de novo* based on 97% sequence similarity (Edgar, 2013). Representative ASV reads were then aligned to the

GreenGenes (release May 2013) (DeSantis et al., 2006) database to assign taxonomies at the genus level. ASVs identified as *Treponema* and *Spirochaeates* were blasted using the NCBI database and EzBioCloud to assign species level classifications (>97% identity) and ASVs between 93-97% identities were grouped into "other".

qPCR validation

Microbiome analysis data was used to validate the qPCR by testing samples determined to contain *T. denticola*, *T. phagedenis*, *T. pedis* and/or *T. medium* and samples without the above species to ensure agreement. In the case that multiple ASVs corresponded to a single *Treponema* species, any hits for that species were included as a positive result. Positive and negative predictive values were calculated using microbiome analysis data as the standard for comparison (Plaire et al., 2017). Specificity of the qPCR products were verified by a single band at the respective base pair length using 1.4% agarose gel electrophoresis. Species-specific gene primers were used to amplify gene fragments from 3 *Treponema* cultures of biopsies originating from geographically distinct farm locations and Sanger sequenced to ensure desired gene product was amplified. Global pairwise alignments with free end gaps were generated between all available online and in-house copies of species-specific genes and sequenced fragments in Geneious (10.1.3, Biomatters Ltd., 2017) based on dynamic programming (Smith and Waterman, 1981). We assumed high similarity between sequences and used 93% similarity cost matrix (5.0/-9.02), gap open penalty of 12 and gap extension penalty of 3.

Statistical analysis

Statistical analyses were conducted in R v.3.4.2 (R Core Team 2017) and a P-value < 0.05 was considered statistically significant. As multiple samples were collected from each herd, the degree of clustering at the herd-level was first determined using a multivariate linear

regression with herd included as a predictor. Based on the limited magnitude of variance explained by herd as a predictor for each species (approximately 5%), herd effects were not included in subsequent models. Additionally, stratification by herd resulted in a significant loss of power since within-herd sample sizes were much smaller than the total sample size, making it impossible to investigate the main variable of interest – lesion grade. Prior to statistical analysis, qPCR copy numbers were standardized by unit of weight using the mean sample weight and the corresponding DNA yield in ng (18.15 mg/ng).

Pairwise associations between species presence were assessed using bivariate logistic regression in the statistical package "zeligverese" (Gandrud, 2017) for each pair of species. The outcome for each model was the presence or absence of both species from each pair, with lesion grade as a predictor. The bivariate logistic regression allowed us to determine the probability of finding a given pair of species together relative to the probability of isolating them separately. Pairwise inter-species correlations of the copy number per mg of tissue were calculated using Pearson's correlation coefficient.

Prior to analysis a natural logarithm transformation was used to normalize the data as observations were strongly skewed to the right. A multinomial regression using the "nnet" package (Ripley and Venables, 2016) was used to assess differences between lesion grades in the combinations of species that were found. Lesion grade was used as the outcome for this regression, with each distinct combination of species isolated being used as a categorical predictor. This model allowed for pairwise comparisons between combinations of species to determine the most common combinations found within each lesion grade and overall. To compare differences between qPCR copy-numbers per mg of species between lesion grades, a multivariate normal regression using the "lm" function in base R. A multivariate normal

regression allowed for the unbiased assessment of statistical differences by accounting for the inter-species correlation.

Results

In total, 142 biopsies were collected from 132 cows; most cows sampled twice were from the slaughterhouse with 6 cows on farms sampled on separate occasions or from lesions on different feet (Table 2).

Microbiome analysis data

In total, 56 distinct ASVs from 16 biopsy samples belonged to the genus *Treponema* when UPARSE (31 OTUs) and UNOISE2 (55 ASVs) data were combined. When assigned species identification, *T. phagedenis, T. denticola, T. medium, T. pedis,* and AM980447_s, which is most closely related to *T. denticola* with 94% identity, were the most common species of *Treponema* representing 37.3, 25.9, 7.6, 7.3 and 6.4% of total *Treponema* ASVs, respectively (Figure 1). Only 6 *Treponema* ASVs could be identified at the species level (>97% identity), *T. refringens* and *T. putidum* in addition to the above four species. Ultimately, *T. refringens* and *T. putidum* made up 0.61% and 0.09% of the *Treponema* species populations, respectively (Figure 1). All other *Treponema* species (>90% sequence similarity) could not be identified (Figure 1) and are reported as the closest hit or accession number on EzBioCloud. Sequences that could not be clustered into an accession number (less than 97% similarity to an accession number) were grouped together as ‘other’, which consisted of 5% of the *Treponema* species populations (Figure 1). *Treponema* identified made up just over 21% of the *Treponema* populations in biopsy samples.

qPCR validation

The sequenced gene fragments for *T. phagedenis* (785bp) had 99.6% pairwise identity among Alberta isolates (N=4) and 95.3% to the 3 species-specific gene sequences from which the primers were designed. The sequenced gene fragments for *T. medium* (515bp) had 99.8% pairwise identity among Alberta isolates (N=3) and 94.5% identical to 2 species-specific gene sequences from which the primers were designed. The sequenced gene fragments for *T. pedis* (295bp) had 93.7% pairwise identity to each other (N=4) and 97.5% pairwise identity to 5 species-specific gene sequences from which the primers were designed. The sequenced gene fragments for *T. denticola* (458bp) had 90.3% pairwise identity among Alberta isolates (N=3) and 94.7% pairwise identity to 5 species-specific gene sequences from which the primers were designed.

Microbiome analysis data were analyzed to verify the specificity of the 4-plex qPCR reaction (Figure 1). Regardless of the algorithm used to identify species, UPARSE or UNOISE2, the qPCR specificity was equal. The qPCR for *T. phagedenis, T. medium, T. pedis* and *T. denticola* agreed with microbiome analysis results 100%, 100%, 93.4%, and 87.5% of the time, respectively, based on 16 samples. In the case of *T. pedis*, there was a single false negative in the qPCR, where the sample had 0.015% of its total ASVs determined to be *T. pedis* by microbiome analysis. However, there were 2 and 3 distinct ASVs in UPARSE and UNOISE2, respectively and the ASV with 100% identity to *T. pedis* was 0 in both algorithms. There was a false positive and a false negative for *T. denticola*. The false negative comprised of 15 hits (0.06% of sample ASVs) and the false positive was just above the threshold (cq=40) of detection (cq=39.72). Sensitivity for *T. phagedenis, T. medium, T. pedis* and *T. denticola* were 100%, 100%, 93.0% and 93.3%, respectively. Specificity for *T. phagedenis, T. medium, T. pedis* were 100% whereas

T. denticola was 67% due to a single false positive. Our spiking experiment indicated the average DNA extraction and qPCR detection efficiency were between 96-99.5% for all species (Table 3).

Number of *Treponema* species cultured or detected in DD lesions

There were *Treponema* cultured or detected by qPCR from 100% of active (M1 lesions, M2 lesions, and M4.1 lesions) and chronic DD lesions (M4 lesions). Unless otherwise specified, direct detection refers to *Treponema* detected by qPCR directly from biopsy tissue before culture. A small proportion (15.4%) of healing/M3 lesions did not contain any of the 4 *Treponema* species or two species of *Treponema* (15.4%) and most (69.2%) contained 1 species of *Treponema* (Figure 3). The majority of M1 lesions and M4 lesions contained 2 species of *Treponema* (37.5% and 46.7%, respectively), followed by 3 species (31.3% and 33%, respectively), 4 species (25% and 13.3%, respectively), and a very small proportion containing 1 species (6.25% and 6.67%, respectively) (Figure 3). Conversely, the majority of M2 lesions and M4.1 lesions contained 3 (30% and 32.4%, respectively) or 4 species of *Treponema* (47.5% 16.4%, respectively). Similarly, 10% of M2 lesions and 10.8% of M4.1 lesions contain only 2 species while only 5% of M2 lesions and 18.9% of M4.1 lesions contain only 1 species of *Treponema* (Figure 3). The majority of M0 samples did not contain any of the 4 species of *Treponema* listed above (74.2%) (Table 4); M0 biopsies that contained species of *Treponema* typically contained only 1 and never exceeded 2 of the tested species (Figure 3). *Treponema* could not be isolated from M0 samples despite being initially detected visually within the sample tissue by dark field microscopy and qPCR. However, *Treponema* were detected and cultured from all DD-infected biopsies (N=2) collected from the abattoir indicating *Treponema* could be cultured from tissue collected post-mortem.

Culture and qPCR detection results indicated that, irrespective of lesion grade, *T. phagedenis* was the most commonly identified and isolated species of *Treponema*, followed by *T. medium, T. pedis* and *T. denticola* (Table 4). Multiple species of *Treponema* were detected in M3 samples by qPCR before culture; however, the only viable species detected in M3 lesions 5 days post-treatment with tetracycline was *T. denticola* which was detected in 100% of M3 cultures (Table 4). Culture and PCR results from isolated *Treponema* showed at least two cultures containing one or more unidentified species by positive microscopic detection and negative PCR results for *T. denticola, T. medium, T. pedis* and *T. phagedenis.* The two unknown samples were sequenced with two universal primer sets, resulting in sequence identity between 79 and 94% to known species of *Treponema*; most closely related to *T. medium*.

The presence or absence of each of the four species of *Treponema* was analyzed by lesion grade. Within lesion grades, *T. phagedenis* was consistently the most commonly identified species except for M3 lesions in which *T. denticola* was the most commonly identified species. Within M1 lesions and M2 lesions, *T. medium* was the second most commonly identified species of the four *Treponema* species, present in 87.5% of samples. Within M4 lesions, *T. pedis* is the second most common species (73.3%) followed by *T. medium* (66.7%) (Table 4). Overall, *T. denticola* was the least common species identified (36.2%) (Table 4).

Direct detection of *Treponema* in biopsies

Despite *T. phagedenis* being the most common of the four *Treponema* species to be found in M0 samples, it was significantly (3.3-3.6 times) more likely that *T. phagedenis* would be present in M1 lesions, M2 lesions, and M4.1 lesions than M0 samples (P= 0.004, 0.0001, and 0.0001, respectively) (Table 6). Interestingly there were no significant differences between *T. phagedenis* presence or absence in M4 lesions, M3 lesions and M0 samples (P>0.99). It was 2-

3.7 times more likely to find *T. pedis* in M1 lesions, M2 lesions, M4 lesions, and M4.1 lesions than M0 samples (p = 0.03, 0.001, 0.04, and 0.08). It was significantly more likely to find *T. pedis* (2.4 times) in M2 lesions than M3 lesions (P=0.048) and there were no significant differences between M0 samples and M3 lesions for *T. pedis* presence or absence (Table 5). Surprisingly, there were no significant differences in the presence or absence of *T. medium* in any lesion grades (P>0.6). M2 lesions, M3 lesions, M4 lesions and M4.1 lesions were significantly more likely to contain *T. denticola* than M0 samples (P=0.005, 0.001, 0.037, and 0.018, respectively). Further, *T. denticola* was significantly more likely to be found in M3 lesions and M2 lesions than M1 lesions (P=0.015 and 0.056) and there were no significant differences between the presence of *T. denticola* between M0 samples and M1 lesions (P>0.99) (Table 5).

Treponema species composition: direct detection in biopsies

A multinomial analysis of the presence or absence of a species irrespective of lesion grade indicated significant relationships between *T. phagedenis, T. pedis,* and *T. medium* that were independent of *T. denticola*. *Treponema phagedenis* was 26.2 times more likely to be found with *T. medium* than without (P=0.003) and *T. medium* was 7.5 times more likely to be found with *T. pedis* than without (P<0.001). Interestingly, *T. phagedenis* is only 2.5 times more likely to be found with *T. pedis* than without; however, the latter result was only suggestive of a relationship and did not reach statistical significance (P=0.10). Odds ratios for *T. denticola* in the presence of *T. pedis, T. medium* and *T. phagedenis*, were 1.75, 0.99 and 0.84, respectively, but failed to reach significance (P=0.27, 0.99, and 0.78, respectively).

The composition of *Treponema* species was analyzed between DD lesion grades to examine potential interactions among species. Overall, the most common species compositions

were *T. phagedenis, T medium,* and *T. pedis* (Ph/Me/Pe) and *T. phagedenis, T medium, T. pedis,* and *T. denticola* (Ph/Me/Pe/De) (21.1% and 20.4%, respectively) (Figure 4). Within lesions, all four species of *Treponema* were the most common species composition within M2 lesions (47.5%). However, in M1 lesions *T. medium, T. phagedenis,* and *T. pedis* (Ph/Me/Pe) was the most common species composition (31.3% of M1 lesions), followed by all four species (Ph/Me/Pe/D) and *T. medium* and *T. phagedenis* together which were equally likely at 25% of M1 lesions each (Figure 4). Similarly, Ph/Me/Pe and *T. phagedenis* and *T. pedis* were equally likely species composition in M4 lesions comprising 20% (to a total of 40%) of M4 biopsies collected. Ph/Me/Pe was the most common species compositions in M4.1 lesions comprising 37.8% of biopsies followed by *T. phagedenis* alone and all 4 species (Ph/Me/Pe/De) comprising 18.92% and 16.2% of samples, respectively. In M3 lesions, *T. denticola* alone was the most common species composition (69.2%) followed by no species and *T. denticola* and *T. pedis* together which were equally likely at 15.4% (Figure 4). Most M0 samples did not contain any species of the 4 most common species of *Treponema* (74.2%) and those that did contained a single species of *T. phagedenis, T. medium, T. pedis* or *T. denticola* (9.7%, 3.23%, 6.45% and 6.45%, respectively) (Figure 4). Finally, if *T. denticola'*s presence or absence is ignored, *T. medium, T. phagedenis,* and *T. pedis* are found together in 54.06-72.5% of active lesions compared to 0% of M0 samples and M3 lesions and 33.3% of M4 lesions (Figure 4).

Treponema **abundance and species composition**

Absolute quantification of *Treponema* in biopsies by qPCR after weight standardization indicated the total number of *Treponema* increased in active lesions compared to chronic and healing DD lesions, regardless of species (Figure 5). Pairwise comparisons between the total number of *Treponema* between lesion grades indicated significant differences between active

lesions, DD-free skin, and healing lesions. M1 lesions, M2 lesions, and M4.1 lesions had significantly more *Treponema* than M0 samples (P<0.001, <0.001, and =0.01, respectively) by 4.2, 3.8 and 2.6 times, respectively (Figure 5). M1 lesions and M2 contained significantly more *Treponema* than M3 lesions (P=0.01 and 0.009, respectively) by 3.4 and 3.0 times, respectively (Figure 5).

Counts of *T. phagedenis* per mg of tissue were significantly higher than *T. denticola, T. medium,* and *T. pedis* (P<0.001). Counts of *T. denticola* were significantly (1.63 times) lower than *T. medium* and *T. pedis* regardless of lesion grade (P<0.001). There were no significant differences between *T. medium* and *T. pedis* counts irrespective of lesion grade (P>0.99). *Treponema* counts between species indicated *T. phagedenis* and *T. medium* were the most correlated (R^2=0.71), followed by *T. medium* and *T. pedis* (R^2=0.49) and *T. phagedenis* and *T. pedis* (R^2=0.49). The amount of *T. denticola* was weakly correlated with *T. medium, T. phagedenis* and *T. pedis* (R^2=0.40, 0.32, and 0.30, respectively) indicating counts of *T. medium, T. pedis* and *T. phagedenis* have little effect on *T. denticola* counts and vice-versa.

Species composition: direct absolute quantification of *Treponema* species in biopsies

A multivariate analysis compared the number of an individual *Treponema* species according to lesion grade. Quantities (*Treponema*/mg of tissue) of *T. phagedenis* and *T. pedis* are highest in M2 lesions, M1 lesions and M4.1 lesions and lowest in M0 samples, M3 lesions, and M4 lesions (Figure 4). Quantities of *T. phagedenis* per mg of tissue were significantly higher in M1 lesions, M2 lesions, M4 lesions, and M4.1 lesions compared to M0 samples (P<0.001) but were not significantly different in M3 lesions (P=0.97) compared to M0 samples (Figure 5) (Table 6). Quantities of *T. pedis* were significantly higher in M1 lesions and M2 lesions compared to M0 samples (P=0.018 and 0.052, respectively); however, *T. pedis*/mg was not

significantly higher in M41.s compared to M0 samples (P=0.12) (Table 6). M1 lesions contained significantly more *T. pedis* than M3 lesions (P=0.05) (Table 6). Similarly, *T. medium* was present in significantly higher quantities per mg of tissue in M1 lesions and M2 lesions compared to M0 samples (P=0.02 and 0.003, respectively) (Table 6). However, there was no significant difference between *T. medium* counts in M4.1 lesions, M4 lesions, and M3 lesions and M0 samples (P=0.32, 0.47, and 0.67, respectively). Finally, *T. denticola* abundance per mg of biopsy tissue were not significantly different between lesion grades (Table 6).

Discussion

To our knowledge, this is the first study examining the distribution and absolute quantities of *Treponema* species among DD lesion grades. From the results presented here, we suggest that the four most common species of *Treponema* found in digital dermatitis, *T. phagedenis, T. pedis, T. medium* and *T. denticola*, can be identified at the species level using species-specific genes. Our qPCR results and validation demonstrate *Treponema* species can be identified in DD-lesion tissue at the species level in a single reaction, which may improve current prevalence estimates as a consistent, reliable way to identify species as opposed to phylotyping. We have found the number of *Treponema* species and their absolute quantities are higher in active lesions than in healing, chronic or DD-free skin suggesting *Treponema* abundance may influence host pathogenicity. Further, we have found strong relationships between the presence and absolute quantities of *T. phagedenis, T.pedis* and *T. medium* that are independent of T. *denticola*, suggesting there may be interactions between *Treponema* species found in DD.

We performed microbiome analysis on biopsies to validate our qPCR and look for additional species of *Treponema.* Our microbiome analysis results are consistent previous studies indicating *T. phagedenis, T. pedis, T. medium,* and *T. denticola* are the most prominent species

within lesions and are the most consistently isolated (Evans et al., 2008; Klitgaard et al., 2013). The species-specific qPCR confirms that the four species of *Treponema* are consistently identified in DD lesions. The variation in *Treponema* abundance may have important implications to DD lesion scoring as well as host pathology. We found *T. phagedenis* is present in all lesions grades and typically the most readily isolated species, consistent with previous literature (Krull et al., 2014; Trott et al., 2003). Our results show *T. denticola* was identified the least frequently and with the lowest average copy numbers, but in some studies it is one of the most frequent with *T. phagedenis* (Yano et al., 2010). Further, *T. pedis* was the third most common species identified and very close to *T. medium*; previous studies have found it is either identified in very low amounts (Klitgaard et al., 2013) or high (Evans et al., 2009a). The second most common species was *T. medium*; previous studies have found *T. medium*-like to be among the most prevalent species and seeing as it is typically clustered with *T. vincentii,* which we found in very low amounts, these results are comparable (Evans et al., 2009a; Klitgaard et al., 2013). We did not find *T. brennaborense* or *T. socranski,* or any closely related ASVs, in our microbiome analysis and did not investigate them further. However, many studies have found *T. brennaborense* in DD lesions in relatively low amounts and a geographical component or presence as a fecal contaminant has been suggested to account for this discrepancy (Evans et al., 2011; Klitgaard et al., 2008; Rasmussen et al., 2012; Wilson-Welder et al., 2015). These findings help resolve interactions between species that were previously unnoticed because *T. pedis* and/or *T. putidum* and *T. denticola* were clustered into a single phylotype (*T. denticola/T. putidum/T. pedis*-like) (Evans et al., 2008; Klitgaard et al., 2008; Rasmussen et al., 2012).

Our species-specific qPCR also demonstrated that the presence and absolute quantities of *Treponema* species are correlated with one another irrespective of lesion grade. The likelihood of

finding *T. pedis, T. medium* and *T. phagedenis* together is much higher than finding them apart. The presence or absence of the above-mentioned species have considerable predictive power on the presence or absence of the others. Additionally, there is a positive correlation between the quantities of the above-mentioned species with one another in that if one increases the other two tend to increase as well. The presence or absence of one species is a better predictor of the presence or absence of another species than their absolute values suggesting interactions between species may not be strictly density dependent. However, there is weak to no correlations between the quantities and presence or absence of *T. denticola* with *T. phagedenis, T. pedis,* and *T. medium*.

The designations of active, chronic, healing and DD-free heel bulb groupings are related to the total number of *Treponema* cells (abundance). We found the total number of *Treponema* cells were highest in M1 lesions, M2 lesions, and M4.1 lesions and lowest in M0 samples, M3 lesions, and M4 lesions. Previous studies have suggested that due to the high variation of phylotypes identified within lesions between studies, *Treponema* abundance may have a greater effect on pathology than species diversity (Rasmussen et al., 2012). Krull et al., 2014 found *Treponema* diversity increased following treatment with tetracycline and *T. denticola* was more abundant relative to active lesions grades. While we did not notice a significant increase in *T. denticola* in M3 samples, they were consistently among the only *Treponema* still viable by culture following lesion treatment with tetracycline. This difference may be due to a decreased time allotted between treatment and sampling. There was no difference between *Treponema* abundance in M0 samples, M3 lesions and M4 lesions; if healing lesions have approximately equal *Treponema* abundances to chronic lesions this may provide an insight into the chronic, cyclical nature of DD lesions.

Both the total number of *Treponema* cells and the number of *Treponema* species increases in active lesions compared to chronic, healing or DD-free skin. We found the number of *Treponema* species is most similar between M0 samples and M3 lesions, M4 lesions and M1 lesions, and M4.1 lesions and M2 lesions where larger, active lesions have higher numbers of *Treponema*, chronic and small active lesions have intermediate, and healing and DD-free have the fewest. Because most active lesions contain *T. phagedenis, T. pedis,* and *T. medium* and our results suggest these species are not significantly correlated with *T. denticola,* it is possible the pathogenicity of *Treponema* associated with DD is related to interactions between these three species regardless of *T. denticola*. The number of *T. denticola* cells isolated does not differ significantly between lesions grades, including M0. Furthermore, most biopsies do not contain *T. denticola,* ranging from 40-94% over all lesion grades with the exception of M3 lesions, supporting a negative association between macroscopic signs of pathogenicity and the presence of *T. denticola*. Interestingly, the most significant increase in *Treponema* abundance between lesions grades at the species level were *T. pedis* and *T. phagedenis* from healthy to active lesions. Further, the macroscopic changes associated with transitioning from active to a healing lesions are accompanied by a decrease in *T. pedis* abundance. While *T. phagedenis* is present throughout grades of DD, *T. pedis* and *T. phagedenis* counts are significantly higher and correlated to one another in active, but not in chronic lesions, suggesting pathogenicity associated with active lesions may be related to interactions between these two species.

If we ignore potential interactions among species of *Treponema* isolated from DD lesions, there are significant differences in species presence or absence between lesion grades when species are treated independently. *T. phagedenis* and *T. pedis* are significantly more likely to be found in active lesions than healthy, healing, and chronic DD grades, supporting a

relationship between host ulcerative pathology and both the presence and amount of *T. pedis* and *T. phagedenis*. The likelihood of *T. denticola* presence does not increase in any lesion in comparison with M0 except for M3 lesions, providing further indication *T. denticola* may not be related to the characteristic pathology associated with active DD lesions. Taken together, the absence of an association among *T. denticola* and the other three *Treponema* species, insignificant changes in *T. denticola* abundance between lesions grades, and *T. denticola* abundance increasing while the other 3 species of *Treponema* decrease following antibiotic treatment, may also suggest negative associations between *T. denticola* and *T. phagedenis*, *T. pedis* and *T. medium*.

Increasing *Treponema* diversity from healing to chronic to active DD lesions is supported by deep-sequencing findings in Krull et al., 2014. However, when lesions are grouped based on the single most common species composition M1 lesions, M4 lesions, and M4.1 lesions have the same most common species composition (Ph/Me/Pe), whereas all four species are most likely to be found together in M2 lesions. Conversely, the distribution of species compositions is most similar between M1 lesions and M4 lesions where all species and *T. medium* and *T. phagedenis* alone are equally likely as the second most common composition and the most common species is shared. Similarly, M3 lesions and M0 samples have distinct species compositions which is likely because of antibiotic treatment selecting against some, but not all, species. Further, lesions following antibiotic treatment are more similar in species profile to M0 samples than active or chronic lesions but not significantly different in terms of *Treponema* cell count, suggesting treatment decreases *Treponema* diversity but not *Treponema* cell count. However, culture results suggest *T. denticola* and to a lesser extent *T. phagedenis*, remain viable after treatment with tetracycline unlike *T. pedis* and *T. medium. In vitro* minimum bactericidal concentrations

(MBCs) of *T. denticola*-like DD isolates is between 3 and 6 mg/L and is not a recommended antibiotic to treat periodontal infections with *T. denticola* (Evans et al., 2009b; Hardham and Rosey, 2000). Conversely, MBCs for *T. phagedenis*-like and *T. medium*/ *T. vincentii*-like groups are 1.5-6mg/L and 0.75mg/L, respectively, supporting our culture viability results following tetracycline treatment and suggesting *T. denticola* may be more resistant to treatment with oxytetracycline than *T. medium, T. pedis* and *T. phagedenis* (Evans et al., 2009b). Taken together, our findings and previous literature suggest *T. denticola* has a smaller impact on host ulcerative pathology than *T. phagedenis, T. pedis* and *T. medium* but may resist antibiotic treatment, facilitating chronic infection states that allow subsequent lesion recolonization of susceptible species with greater influences on host pathogenicity.

The results presented here suggest there is a relationship between the macroscopic morphological changes between DD lesion grades and species composition, individual species abundances, and total *Treponema* abundance. While these results represent a subset of the *Treponema* species present in DD lesions, and it is possible the nature of these interactions is much more intricate and complex, the four species analyzed here are consistently the most prevalent and abundant in DD globally. We have demonstrated species of *Treponema* in DD can be identified based on species-specific genes and believe future research will be greatly enhanced by determining species of *Treponema* precisely. Many researchers are currently sequencing *Treponema* genomes which will allow currently unidentified species to be identified, potentially using species-specific genes, and contribute to future research analyzing correlations between species and morphological shifts of DD lesions. Our results support a significant role of *Treponema* in macroscopic pathology of DD lesions; however, because many bacteria are

consistently found in addition to *Treponema*, we believe future research would benefit from analyzing additional genera of bacteria.

Author Contributions

CB and JDB designed protocols and hypotheses. KO demonstrated sampling techniques on farm. CB conducted animal sampling under protocol created by KO with certified hoof-trimmers for Alberta farms and Abattoir and CL conducted sampling in Saskatchewan. CB cultured and isolated *Treponema* from biopsies and designed and species-specific qPCR and PCR with genes identified by SN. EK and HD performed microbiome analysis and algorithm for qPCR validation. CB and SAN designed statistical analysis and figures generated by SAN. Manuscript written by CB and JDB, and edited and reviewed by KO, SAN, SN, CL, EK, and HD.

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List of Figures

Table 2.1. Primer and probe sequences for *Treponema* species-specific fourplex qPCR and conventional PCR.

Species	Forward Primer (sense)	Probe (sense)	Reverse Primer (antisense)	Size (bp)
***T. denticola*[1]**	GGAAACTTAGGAATTCGATATGTAG	AGCATACAGCGATTATAACAAAGCCCTCGA	CCTTCTTTAGTTTCTTTGTGAGG	113
***T. medium*[1]**	AAAGCGCTACGAATCCTAAG	TGCACCCTTGTTTACTACTGCACAGCC	ATCATTACCCGTCCACAAAG	119
***T. phagedenis*[1]**	CCCGCAGGAAGGTATAATC	AATCCGCCTACGACTGCGATACCA	CACAGCTGTTGTGGTATTAAG	90
***T. pedis*[1]**	ACACCGATTGTACTGAATGA	ACTACACGTGGAGTACCGAATGCT	CCACGAGCTTTCTACAGATT	118
***T. denticola*[2]**	AGGAATGGCCTTTGAACCCGCA		CCGATGAACCCGTATCTTCACCGA	458
***T. medium*[2]**	GGAACAGGCAGCCGCATTGGAT		CCGCCCATGTGAGGCTTGTGAT	515
***T. phagedenis*[2]**	TCCGCCTACGACTGCGATACCA		CGGAACTGTCACAACTGGCGGA	785
***T. pedis*[2]**	TGGATGTTACGGAAGAGACACCGA		TGCCCCACTCTTACAAGTTCATCCCA	295

[1]Primer and probe sequences for species of *Treponema* for qPCR.
[2]Conventional PCR primer sequences for *Treponema* species.

Table 2.2. Representation of all biopsies collected and cultured from 10 Alberta farms, 1 Saskatchewan farm, and an Alberta slaughterhouse, by lesion grade.

Farm	No. cows		Lesion Grade					
			M0	M1	M2	M3	M4	M4.1
1	4	0	0	3	0	0	1	
2	20	0	2	6	0	6	10	
3	7	0	1	1	0	1	4	
4	12	0	6	5	0	0	1	
5	22	0	0	11	13	0	0	
6	6	0	1	2	0	0	3	
7	15	0	4	7	0	1	4	
8	3	0	0	0	0	2	1	
9	8	0	0	0	0	1	7	
10	26	0	1	5	0	3	6	
11[S]	10	10	0	0	0	0	0	
Abattoir	9	21	1	0	0	1	0	
Total	142	31	16	40	13	15	37	

[S] Indicates Saskatchewan farm.

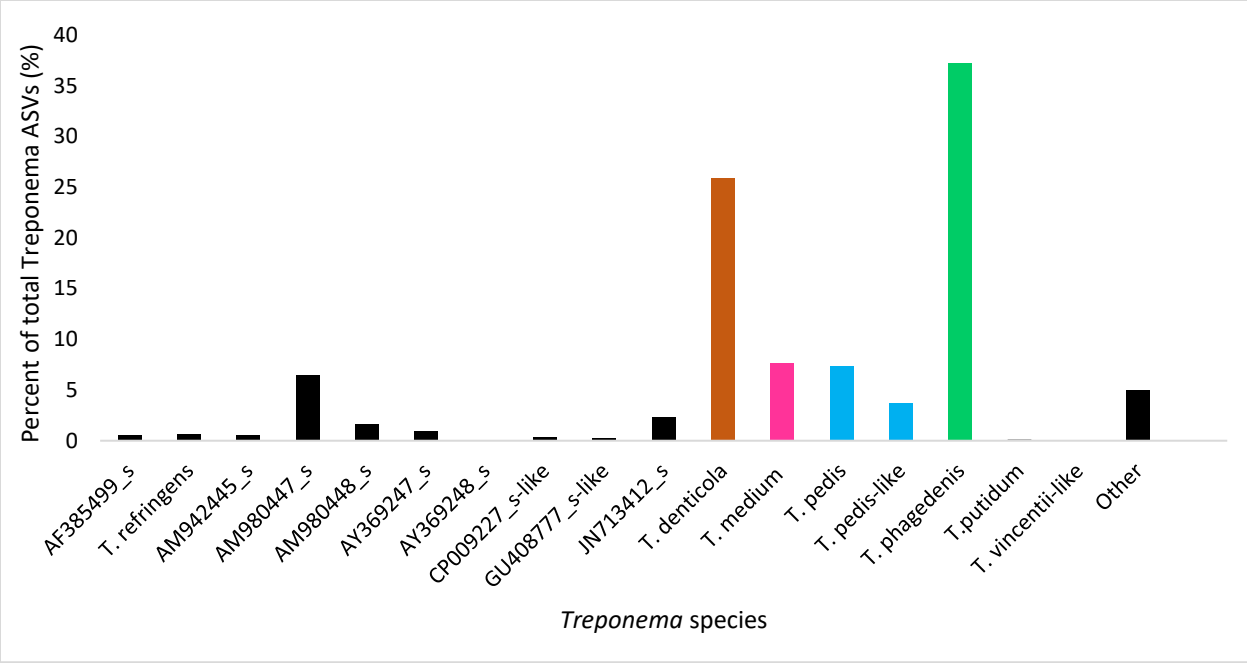

Figure 2.1. Microbiome analysis indicating ASVs designated to the genus *Treponema* from DNA directly extracted from DD-infected biopsies (N=16).

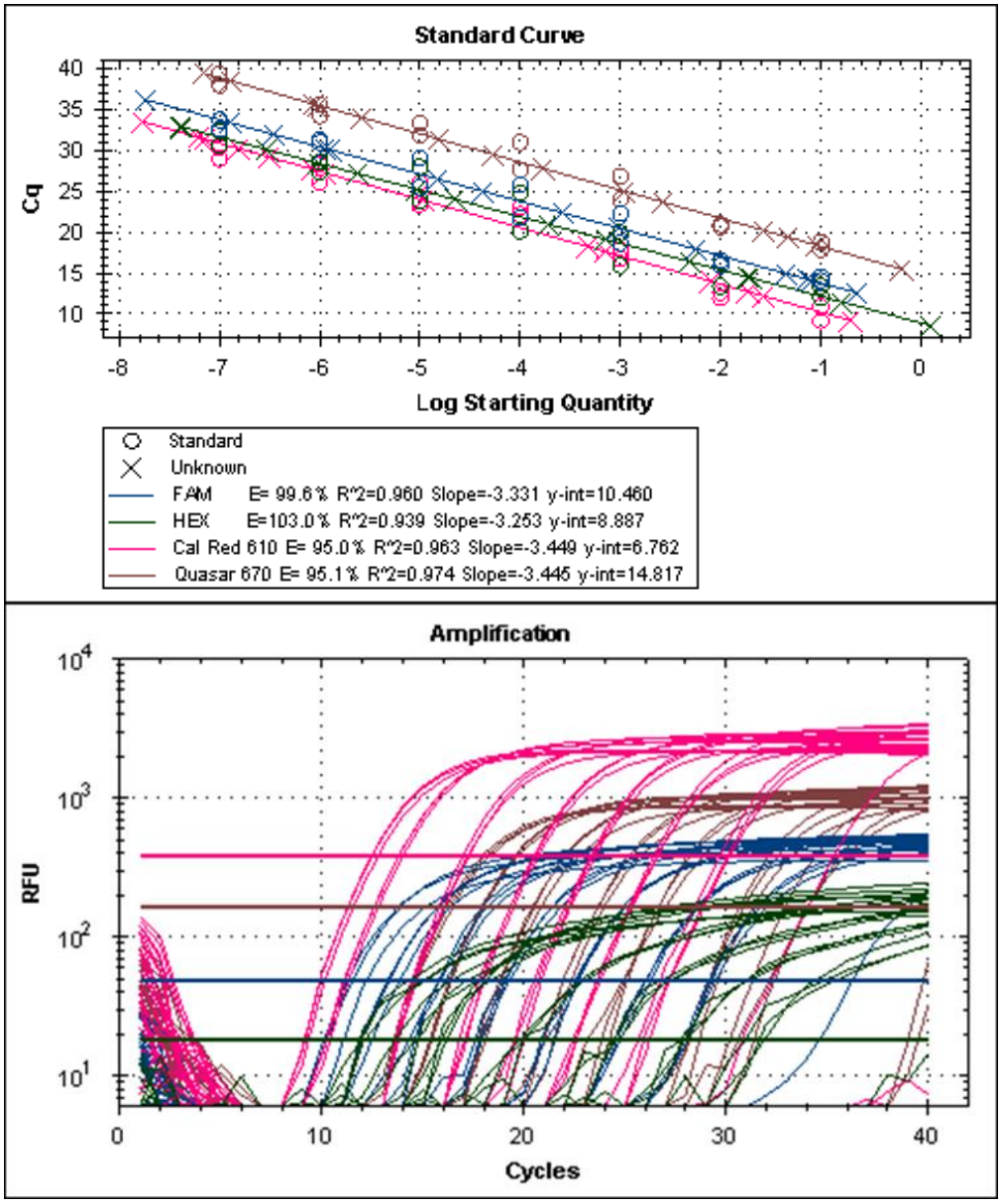

Figure 2.2. Standard curve for species-specific qPCR of *T. phagedenis* (HEX), *T. medium* (Cal Red 610), *T. pedis* (FAM) and *T. denticola* (Quasar 670).

Table 2.3. Spiking experiment results indicating the efficiency of *Treponema* DNA extraction and detection, with and without bovine tissue, using species-specific qPCR.

Species	1 Mean count[1]		1/10 Mean count		1/100 Mean count		Average Efficiency (%)
	Culture	Tissue (count/mg)	Culture	Tissue (count/mg)	Culture	Tissue (count/mg)	
T. phagedenis	$2.32x10^8$	$4.22x10^7$	$2.78 x10^8$	$3.32 x10^7$	$2.26 x10^7$	$1.04 x10^7$	99.5
T. medium	$8.72x10^7$	$9.76 x10^5$	$1.40 x10^8$	$6.80 x10^6$	$1.22 x10^7$	$9.45 x10^5$	98.8
T. pedis	$1.41x10^9$	$7.93 x10^7$	$6.85 x10^5$	$7.80 x10^4$	$9.31 x10^4$	$5.25 x10^3$	96.0
T. denticola	$2.58x10^6$	$1.24 x10^5$	$4.14 x10^5$	$8.36 x10^4$	$1.23 x10^5$	$1.59 x10^4$	98.2

[1] Cq values were converted into gene copy numbers and the mean count between samples without tissue was compared to the mean count with tissue samples of ten-fold serially diluted *Treponema* cultures.

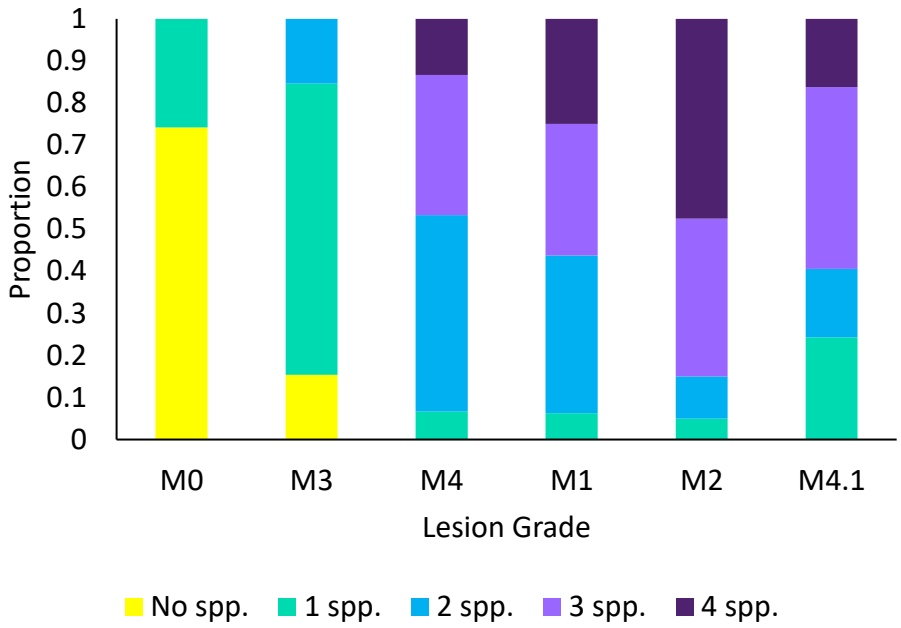

Figure 2.3. The proportion of DD lesions or lesion-free biopsies containing 1, 2, 3 or 4 species of *Treponema*. Lesion-free, healing and chronic lesions (M0, M3, and M4, respectively) depicted on the left and active lesions on the right (M2 and M4.1 lesions).

Table 2.4. Identification of four *Treponema* species (N=142) according to lesion grade by culture and species-specific PCR.

Lesion grade	No. Samples with species present (%)					
	T. phagedenis	*T. medium*	*T.pedis*	*T. denticola*	No spp.	Total
M0	3 (9.7)	1 (3.2)	2 (6.5)	2 (6.5)	23 (74.2)	31 (100)
M1	15 (93.8)	14 (87.5)	11 (68.8)	4 (25)	0 (0)	16 (100)
M2	40 (100)	35 (87.5)	32 (80)	24 (60)	0 (0)	40 (100)
M3	0 (0)	0 (0)	2 (15.4)	13 (100)	2 (15.4)	13 (100)
M4	13 (86.7)	10 (66.7)	11 (73.3)	4 (26.7)	0 (0)	15 (100)
M4.1	35 (94.6)	26 (70.3)	24 (64.9)	10 (27)	0 (0)	37 (100)
Total	106 (69.7)	86 (56.6)	82 (53.9)	55 (36.2)	25 (16.4)	152 (100)

Table 2.5. Difference between lesion grades in log odds of finding *Treponema* species across lesion grades assuming no relationships or dependence among species.

Lesion Comparison[1]	Species			
	T. denticola	*T. medium*	*T. pedis*	*T. phagedenis*
M0 - M1	-0.77	-19.72	-2.55*	-3.28**
M0 - M2	-3.86***	-19.80	-2.82***	-3.26***
M0 - M3	-4.96***	0.00	-0.37	16.49
M0 - M4	-3.95	-20.26	-3.69*	-20.65
M0 - M4.1	-3.52**	-18.94	-1.99ǂ	-3.64***
M1 - M2	-3.09ǂ	-0.08	-0.27	0.03
M1 - M3	-4.19**	19.72	2.17	19.77
M1 - M4	-3.18	-0.54	-1.14	-17.36
M1 - M4.1	-2.75	0.78	0.56	-0.35
M2 - M3	-1.10	19.80	2.44*	19.74
M2 - M4	-0.09	-0.46	-0.87	-17.39
M2 - M4.1	0.34	0.86	0.82	-0.38
M3 - M4	1.01	-20.26	-3.31	-37.13
M3 - M4.1	1.44	-18.94	-1.62	-20.12
M4 - M4.1	0.43	1.32	1.70	17.01

[1] Negative value indicates lesion grade on the left is exp(log odds) times less likely to contain the given species; positive value indicates lesion grade on the left is exp(log odds) times more likely to contain the given species. **P*<0.05, ***P*<0.01, ****P*<0.001, ǂ 0.051 < *P* < 0.90.

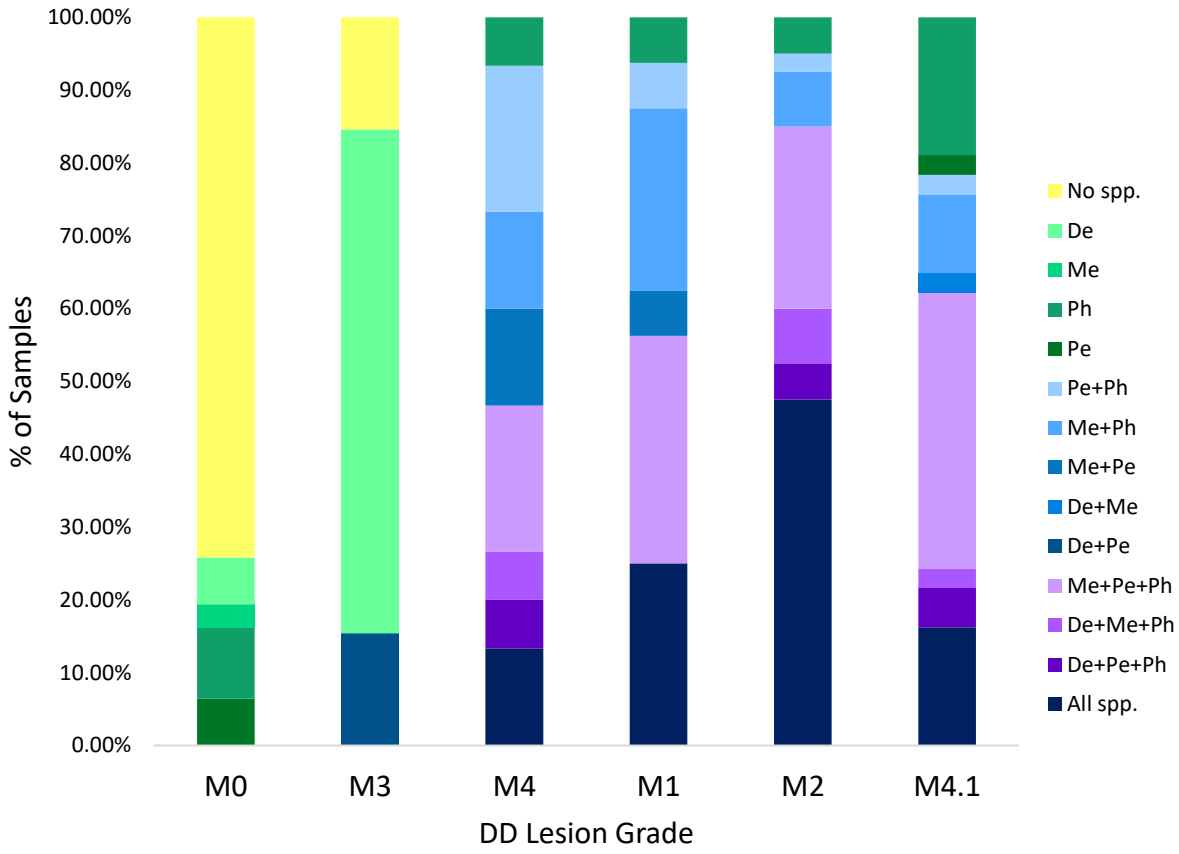

Figure 2.4. Species combinations within digital dermatitis lesions according to lesion grade. Each colour represents a mutually exclusive species composition (singlet, pair, triplet or quadruplet) within a lesion. Lesion-free, healing and chronic lesions depicted on the left and active lesions on the right. De = *T. denticola*; Me = *T. medium;* Pe = *T. pedis*; Ph = *T. phagedenis.*

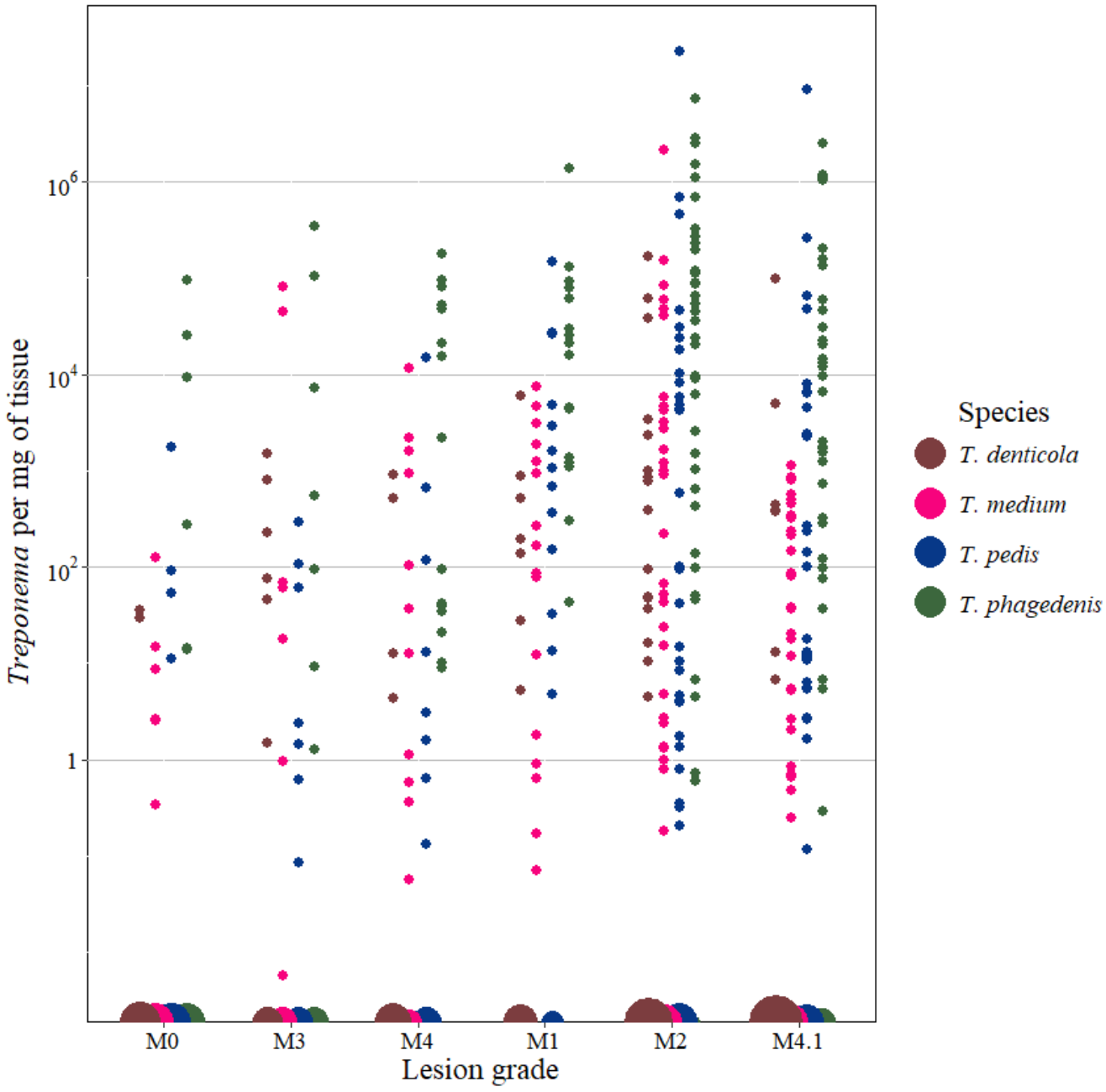

Figure 2.5. Species-specific bacterial cell numbers standardized by biopsy tissue weight (mg) of four *Treponema* species according to DD lesion grades.

Table 2.6. Difference between the natural logarithm of abundance (copies/mg of tissue) of *Treponema* species across lesion grades[1].

Lesion Comparison	Species				
	T. denticola	*T. medium*	*T. pedis*	*T. phagedenis*	Total
M0 - M1	-2.07	-3.70**	-4.44***	-6.30***	-4.20***
M0 - M2	1.97	-3.72***	4.21ǂ	-6.75***	-3.82***
M0 - M3	-1.91	1.67	-3.20	-4.77	-0.84
M0 - M4	1.22	-2.02	3.76	5.13**	-2.07
M0 - M4.1	1.80	-2.17	-2.90	5.58***	-2.64*
M1 - M2	1.33	1.83	2.97	-4.69	0.39
M1 - M3	1.06	1.70	2.52*	-3.60***	3.36**
M1 - M4	1.16	1.53	-2.68ǂ	-3.52	2.14
M1 - M4.1	-0.84	-1.86	-2.23	1.53	1.57
M2 - M3	-0.74	1.86	1.53	1.98***	2.98***
M2 - M4	-0.74	1.56	1.24	2.07	1.75
M2 - M4.1	-0.64	-0.30	-0.68	1.62	1.18
M3 - M4	-0.16	-0.16	0.29	-1.17	-1.23
M3 - M4.1	-0.10	0.14	-0.45	0.45ǂ	-1.79
M4 - M4.1	-0.10	0.02	-0.23	-0.08	-0.57

[1] Negative values indicate the mean log abundance was that amount less the lesion grade on the right; positive values indicate the mean log abundance was that amount greater than the lesion grade on the right. *P<0.05, **P<0.01, ***P<0.001, ǂ 0.051 < P < 0.9

Chapter 3: Distribution of antimicrobial resistance genes in digital dermatitis lesions and *Treponema* isolates

Distribution of antimicrobial resistance genes in digital dermatitis lesions and *Treponema* isolates

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Keywords: Digital dermatitis, *Treponema*, Antimicrobial resistance genes, treatment, lesion grade

Abstract

Digital dermatitis (DD) was first described in dairy cattle 1974, and despite considerable research efforts, effective methods for treatment and control of the disease that lead to lasting lesion resolution have not been identified. Inability to eradicate the disease from infected herds and to identify effective treatment options may be attributed in part to the remaining uncertainty regarding disease etiology. Treatment and control for DD is heavily reliant on broad-spectrum antimicrobials to which there is inherently more resistance among environmental bacteria and pathogens. Due to the nature of DD and the fastidious bacteria, *Treponema,* which have been strongly associated with DD lesions, conventional methods examining bacterial susceptibility to antimicrobials is difficult and for many species, impossible. The majority of *Treponema spp.* within DD lesions cannot be cultured, characterized, or identified; these species and their interactions with known species of *Treponema* may affect susceptibility *in vivo*. Further, the *Treponema spp.* that can be grown *in vitro* are rarely separated from co-infecting *Treponema spp.* which impairs identifying susceptibility profiles of species. Therefore, we have combined *Treponema* isolate sequencing and PCR to identify antimicrobial resistance genes ($AMGs^{Trep}$) in *Treponema spp.* in DD lesions. We selected resistance genes relevant to current treatment and control antimicrobials (AMs) and previously described AMs to which *Treponema spp.* had *in vitro* susceptibility. We have found significant correlations between the lesion grade and the number of *Treponema spp.* present to the number of resistance genes identified. Significantly, we have found evidence of heavy metal and antibiotic resistance gene co-occurrence within lesions and *Treponema* isolates. Future studies should examine the genes identified here and their relationships to *in vitro* susceptibility and treatment outcomes in field efficacy trials.

Introduction

Digital dermatitis (DD) is one of the greatest challenges to the dairy industry worldwide. Additionally, DD is increasingly recognized as affecting beef cattle and other production ungulates (Orsel et al., 2017; Sullivan et al., 2015). As a significant cause of infectious lameness, DD is responsible for considerable economic loss and animal welfare concerns due to the painful, ulcerative lesions associated with infection. The consequences of DD in dairy cattle include lameness, reduced longevity and fertility, decreased cow comfort, hoof conformational changes, and losses in milk production (Gomez et al., 2015; Menno Holzhauer et al., 2008; Jacobs et al., 2017). While DD is prevalent globally, treatment and control mechanisms that effectively remove the disease without relapse have not been identified (Laven and Logue, 2006). Most treatment and control regimes for DD over the last 40 years have consisted of antibiotics and heavy-metal (copper and zinc) sulfate solution footbaths but few have resulted in long term infection resolution in most animals. The lack of effective, long-term solutions to the increasingly large problem of DD may be due in part to the inability to identify a definitive etiological agent. While most evidence currently supports the anaerobic spirochetes of the genus *Treponema* as being primarily involved in DD infections, others have suggested a complex, polymicrobial etiology with multiple interacting bacteria that reflect macroscopic morphological changes to lesions (Krull et al., 2014). Both theories of disease etiology involve multiple species, whether of the genus *Treponema* or a broader range of bacteria, which likely have variable susceptibilities to antimicrobials.

Currently, herd-level footbathing is the most common prevention strategy using zinc sulfate or copper sulfate footbaths with the latter being the industry standard (Jacobs et al., 2017; Solano et al., 2015). Due to the increasing concern regarding environmental and health impacts

of harsh chemicals and antibiotics used in footbaths, there have been considerable increases in the variety of footbath solutions available but few have the same efficacy as the above standard. Treatment of clinical DD typically involves topical application of broad spectrum antibiotics to the affected area. Globally, tetracycline and lincomycin-spectinomycin appear to be the most commonly used antibiotics to treat DD despite increasing evidence that they are ineffective at clearing the infection, suggested by lesion relapses in the weeks following treatment (Cutler et al., 2013; Laven and Logue, 2006; Speijers et al., 2013; Toholj et al., 2012). Furthermore, *in vitro* susceptibility of *Treponema* frequently isolated from DD are variable and some strains are more resistant than others to common treatment antibiotics (Evans et al., 2009b). The inability to clear DD infections has fueled *in vitro* susceptibilities testing of *Treponema* and field trials of antibiotics and commercial products with variable results. However, antibiotics demonstrating the highest *in vitro* susceptibility against *Treponema* have been those with considerable milk withdrawal times, making them unrealistic for use in production dairy cattle (Evans et al., 2012).

Based on previous literature and anecdotal observations, we hypothesize species of *Treponema* consistently found in digital dermatitis have variable susceptibility to antibiotic treatment and footbath chemicals, contributing to lesion relapse and infection recurrence (Angell et al., 2015; Laven and Logue, 2006; Solano et al., 2017b, 2015). We have identified ten antimicrobial resistance genes, which mediate resistance through various protein-based mechanisms such as efflux pumps and target modifiers, of significant relevance to the dairy cattle industry. Through literature reviews, field experience, and informal communications with stakeholders, we have identified tetracycline, lincomycin and spectinomycin as the most common antibiotics used to treat clinical DD (Holzhauer et al., 2008; Kulow et al., 2015; Logue et al., 2012; Solano et al., 2017b; Wilson-Welder et al., 2015b). Similarly, we have selected

heavy metal resistance genes, particularly zinc and copper sulfates which are the most common active ingredients in footbath solutions (Jacobs et al., 2017; Kulow et al., 2015; Logue et al., 2012; Smith et al., 2014; Speijers et al., 2012; Teixeira et al., 2010; Thomsen, 2015). Our objective was to analyze the prevalence of microorganisms harbouring AMR genes (AMGs) in lesion-free and DD-infected biopsy samples with newly designed PCRs based on AMGs found in DD-isolated *Treponema* genomes through in-house MiSeq whole genome sequencing (WGS). We postulated AMGs will vary between species of *Treponema* present in DD samples and analyzed the number of AMGs present with four of the most common species of *Treponema* found in DD lesions, *T. phagedenis, T. medium, T. pedis* and *T. denticola.* We further analyzed the correlations and combinations among AMGs present in *Treponema* isolate sequences. Our results may improve treatment efficacy and decrease inappropriate use of antimicrobials by identifying the most and least prevalent AMGs harboured in microorganisms found in DD lesions for future efficacy trials.

Materials and Methods

Treponema Culture

Biopsy samples (N=146) comprising all M-stage lesions, with at least 13 samples per lesion grade, were collected and processed from 10 Alberta farms according to Beninger et al., 2018. Biopsy samples were sliced longitudinally and stored in Tris-EDTA (TE) (pH=8.0) (BioUltra, Sigma-Aldrich, Burlington, MA, USA) at -20°C for PCR, immediately cultured as outlined briefly below, and stored in 20% glycerol and OTEB at -80°C for future culture. Biopsy samples were transported in anaerobic transport media (ATM) and were handled exclusively in the anaerobic cabinet (Bactron3000, Sheldon Manufacturing, Inc., Cornelius, OR, USA) (25% CO2, 5% H2, balance nitrogen).

Biopsy samples stored in TE were directly extracted with DNeasy Blood and Tissue Extraction Kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany) and eluted in 200 uL nuclease free water. Following extraction, biopsy DNA was stored at -20°C prior to AMG PCR and species-specific qPCR (Beninger et al., 2018).

Treponema spp. were isolated, cultured and identified according to Beninger et al., 2018 with a novel species-specific qPCR. Biopsy fragments for culture were inoculated into oral treponeme enrichment broth (OTEB) (Anaerobe Systems, Morgan Hill, CA, USA) with 5% enrofloxacin (> 98% HPLC, powder, Sigma-Aldrich, Burlington, MA, USA) dissolved in DMSO, 10% rifampicin (> 97% HPLC, powder, Sigma-Aldrich, Burlington, MA, USA) dissolved in DMSO, and 10% equal parts bovine and rabbit serum (Gibco, Life Technologies, New Zealand and USA, respectively) (OTEBSER). To improve isolation of *Treponema* from the polymicrobial community found in DD lesions, biopsies were inserted into MTGE agar supplemented with 5% enrofloxacin in DMSO and 10% rifampicin by spread plating (Anaerobe Systems, Morgan Hill, CA, USA). Cultures were monitored visually using darkfield microscopy for the presence and growth of bacteria with characteristic *Treponema* morphology including corkscrew shape and rotational movement about the longitudinal axis (Beninger et al., 2018; Radolf, 1996). Cultures containing bacteria other than *Treponema* were sub-cultured as outlined in detail in Beninger et al., 2018 until cultures containing only *Treponema* were obtained.

Cultures were selected for WGS based on culture purity, known species composition of each culture, and two uncharacterized *Treponema* isolates of interest which were visually positive using darkfield microscopy as mentioned above, qPCR negative, and unidentifiable using 16S universal primers (Beninger et al., 2018). Further, equivalent samples, those that contained similar species profiles, were sub-selected to ensure samples chosen for WGS

originated from the most farms possible and results would be representative and generalizable. *Treponema* were cultured as above from cultures in OTEB + 20% glycerol stored at -80°C for DNA extractions approximately 7 days to an OD_{550}>0.25 prior to extraction.

DNA extraction

Two *Treponema* cultures of the same isolate were transferred into a single 15 mL tube and spun for 15 minutes at 3000 *xg* and all supernatant was removed. DNA extraction was performed according to a modified protocol from Wright et al., 2017. Buffer P1 and all solutions used were made immediately before use as follows: 500mM Tris-HCl (pH=8) (Trizma® hydrochloride,≥ 99% powder, Sigma-Aldrich, Burlington, MA, USA), 10mM EDTA (BioUltra, ≥ 99% powder, Sigma-Aldrich, Burlington, MA, USA), (pH=8), and 100µg/mL ribonuclease A (PureLink™ RNase A, 20 mg/mL, Invitrogen, Life Technologies, CA, USA). The bacterial pellet was resuspended in 467 µL of Buffer P1 and transferred to a sterile 1.5 mL Eppendorf tube (Eppendorf AG., Hamburg, Germany). Lyophilized lysozyme from chicken egg white (≥ 90% protein powder, ≥ 40,000units/mg, Sigma-Aldrich, Burlington, MA, USA), was suspended in sterilized PBS (100 mg/mL) and stored at -20°C before use; 8 uL of lysozyme was added to the resuspended pellet, gently mixed by inversion, and incubated at 37°C for 1h. Following incubation, 3uL of proteinase K (20 mg/mL, Invitrogen, Life Technologies, CA, USA) and 30uL of freshly prepared 10% SDS in Tris-EDTA (BioXtra, ≥ 99% powder, Sigma-Aldrich, Burlington, MA, USA), (pH=8) were added, gently mixed, and incubated for 1h at 56°C. Following incubation, 525 uL of phenol: chloroform: isoamyl (25:24:1 v/v) (pH=8.0, saturated with 10mM Tris, 1mM EDTA, Sigma-Aldrich, Burlington, MA, USA), was added and mixed for ten minutes by gentle inversion (Millipore Sigma, Merck kGaA, Darmstadt, Germany). Once samples were thoroughly mixed they were centrifuged for 15 minutes at 12,000 x*g*. The aqueous

layer was removed and transferred to a sterile 1.5 mL Eppendorf tube with a 1000 uL wide-orifice pipette and phenol: chloroform: isoamyl was added at approximately 1:1 ratio and mixed and centrifuged as described in the previous step. This step was repeated until the white protein layer was incomplete or no longer visible, typically twice. An equal volume of chloroform: isoamyl alcohol (24:1) (Sigma-Aldrich, Burlington, MA, USA) was added to the aqueous layer and mixed by gentle inversion for 5 minutes and centrifuged for 5min at 12,000 x*g*. The aqueous layer was transferred into a sterile 1.5mL tube and 2.5 volume of 100% ice cold ethanol was added to each sample and left at -20°C overnight. Overnight samples were centrifuged for 20 minutes at 12,000x*g* and the liquid was removed, taking care not to disturb the DNA pellet. The DNA was spun again for 30s at 12,000x*g* to remove residual ethanol and washed with ice-cold 70% ethanol, incubated at RT for 2mins, and centrifuged for 15 minutes at 12,000x*g*. The ethanol was completely removed and left to air dry in the biosafety cabinet at room temperature. Once the DNA was completely dry, the pellet was resuspended in 100 uL of freshly prepared 50mM Tris-HCl (pH=8). DNA was resuspended for 8 – 48 hours at 4°C before measuring DNA quality and concentration.

If there was a gelatinous substance that remained after the ethanol was removed from the pelleted DNA and the DNA pellet could be distinguished with the naked eye the NaCl (BioXtra, ≥99.5% (AT), Sigma-Aldrich, Burlington, MA, USA) concentration of the resuspended DNA was adjusted to 0.7M and 0.1 volume of 10% CTAB (BioXtra, ≥99%, Sigma-Aldrich, Burlington, MA, USA) in 0.7M NaCl was added. CTAB/NaCl solution: 4.1g NaCl in 80mL nuclease free water and 10g of CTAB was slowly added while constantly stirring and heated to 65°C. The final volume was adjusted to 100mL with nuclease free water as above and cooled to

RT prior to use (Wilson, 1997). The protocol above was then repeated beginning with the first phenol: chloroform: isoamyl addition.

DNA Quality and Quantity for Sequencing

DNA quality was accessed using a Nanophotometer NP80 (Implen, Los Angeles, CA, USA). Samples required an 260/280 absorption of 1.80 and a 260/230 absorption of 2.0-2.20. Samples meeting the above criteria were measured for DNA quantity using a Qubit® 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) for dsDNA. Samples with a sufficiently high DNA concentration were then run on a 0.8% agarose gel for 20h at 30V and samples with a single band, indicating the sample was not sheared, were accepted. DNA samples were stored at 4°C for less than 2 weeks prior to sequencing and the above quality and quantity assurance was repeated less than 24 hours before sequencing.

MiSeq

Thirty-eight *Treponema* isolates, originating from biopsies collected on 6 Alberta farms and a abattoir, were selected for sequencing using MiSeq according to the criteria mentioned above. Where possible, samples from the same biopsy with distinct species compositions were selected to allow assembly of individual species genomes.

Genomic *Treponema* DNA was tagged, amplified, and cleaned according to Illumina Nextera XT protocol (Document #6566 v01, Illumina Inc., CA, USA) within 3 days. Library size and concentration was verified using D5000 Agilent Tape Station Protocol on Agilent 2200 TapeStation instrument and software (Agilent Technologies, CA, USA). Samples with fragment sizes below 200bp on average or incompletely fragmented, resulting in multiple peak averages, were removed. Samples were normalized using the Bead-Based Normalization Method

(Document # 15039740 v3, MiSeq System Denature and Dilute Libraries Guide 2017) using MiSeq Reagent Kit v6 with 24uL Library Pool and 576uL of chilled HT1.

Sequencing, and all sequencing steps including cluster generation, paired-end sequencing (2 × 250bp), and primary data analysis for quality control, were performed using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). MiSeq sequences were trimmed of adapter sequence tags and poorly sequenced regions (quality score <20) using the CutAdapt database (Martin, 2011) and Trim Galore! 0.4.0 (Naushad et al., 2016). Trimmed sequences with quality scores above 20 were then *de novo* assembled into contigs and error-corrected using SPAdesversion3.6.0 (Nurk et al., 2013). Genomes were annotated with prokaryotic annotation software Prokka, which utilizes multiple protein databases such as BlastP, RefSeq, and Markov (Seemann, 2014).

AMG selection

First, 7 samples of genomic DNA from *Treponema* isolated from DD lesions, provided by Dr. Dopfer at the University of Wisconsin, were sequenced and annotated as above. Once annotated, coding DNA sequences (CDS) were analyzed using an in-house pipeline and database containing all known bacterial antimicrobial resistance genes from 4 databases: (1) ARG-ANNOT v3 (Antibiotic Resistance Gene-ANNOTation) (Gupta et al., 2014); (2) MegaRES v1.0.1 (Lakin et al., 2017); (3) Comprehensive Antibiotic Resistance Database v1.1.6 (CARD) (Jia et al., 2017); and (4) ResFinder from the Center for Genomic Epidemiology (Zankari et al., 2012) according to Nobrega et al., 2018 (Figure 3.1). Each query sequence was blasted against the 7 WGS mentioned above using an offline blast server containing the databases mentioned above with 30% similarity and 60% query coverage thresholds (Nobrega et al., 2018). The

sequences returned were blasted through Geneious 10.2.2 (Biomatters Ltd., 2017) and genes with above 90% pairwise identity were selected (Figure 3.1).

AMGs for PCR primer design were selected based on relevance to current treatment and control regimes for DD. AMGs selected all conferred protein-based resistance such that genes would not need to be sequenced to analyze resistance profile and gene presence alone was a resistance determinant. Further, AMGs with relevance to DD treatment and control that were not identified using the method described above were found on the NCBI protein database through targeted searches based on a literature review of relevant resistance determinants. Selected genes that were >10% different in pairwise alignments between species of *Treponema* were included in the database to ensure all target genes would be identified with the above criterion. Of 38 isolates sequenced with MiSeq, 33 passed quality testing ($Q \geq 20$) and were assembled using SHOVILL GPLv3 with SPAdes assembly default parameters (Seemann, 2017). The DIAMOND algorithm was used to align MiSeq reads against our AMG protein reference database generated as mentioned above using the high sensitivity mode where the e-value is less than $1x10^{-5}$ (Figure 3.1) (Buchfink et al., 2015).

Genes found in MiSeq data with over 90% sequence similarity to known resistance genes were aligned in Geneious 10.2.2 (Biomatters Ltd., 2017). Global pairwise alignments of all >90% complete genes were assumed to be highly similar and aligned using a 93% similarity cost matrix (5.0/-9.02), gap open penalty of 12 and gap extension penalty of 3. Consensus sequences of global pairwise alignments were used to design primers using idtDNA PrimerQuest software with equal melting temperatures (Tm) of 62°C (Figure 3.1) (Integrated DNA Technologies, 2017). Primers were then aligned with consensus sequence to ensure they bound to regions

without single nucleotide polymorphisms and blasted (blastx) in the NCBI database to ensure primers were specific to desired gene sequence in *Treponema*.

AMG PCR design

AMG primers were initially tested on AMG positive DNA isolates from MiSeq followed by specificity testing on DNA extracted directly from biopsy samples. Annealing temperature optimization was performed by running positive biopsy samples on a temperature gradient from the calculated annealing temperature to 10°C above the calculated annealing temperature according to Breslauer et al., 1986. The highest annealing temperature that yielded a positive result was selected for all subsequent reactions (Table 3.2). PCR products were run on 1.2% agarose gels at 120V for 35 minutes and a positive result was a single band at the appropriate fragment size (Table 3.2). Amplicons from each primer set were purified, quantified using a nanodrop as outlined above, adjusted to 50ng in nuclease free water with 100pmol of forward primer, and sanger sequenced the same day. Amplicon sequences were aligned and blasted in Geneious as above to ensure amplicon was a fragment of the desired gene sequence.

Statistical analyses

Statistical analyses were conducted in R v.3.4.2 (R Core Team 2017) and a P-value < 0.05 was considered statistically significant. Regression analyses were conducted using the "lme4" package (Bates et al., 2015) in R.

Pairwise correlations between genes were computed using the Pearson correation coefficient. Mixed-effects logistic regression was used to determine the association between the odds of finding a gene and the lesion grade. The outcome in the model was predicted odds of finding the given gene with lesion grade as a predictor, and herd random effects to account for unmeasured within-herd correlations. The association between presence of species and

identification of a given gene was assessed using mixed-effects logistic regression, with the predicted odds of finding the gene as the outcome again, and the presence of each species included as predictors additively. No between-species interaction effects on odds of finding a species were assessed. The correlation between the number of *Treponema* species and the number of resistance genes identified was computed using the Spearman rank correlation coefficient. The correlation between AMGs present within *Treponema* isolates was computed using Pearson's correation coefficient.

Results

AMGs identified in *Treponema* WGS

Two distinct genes conferring resistance to β-lactams, FprA family A-type flavoprotein and metallo-β-lactam hydrolase, were identified in *Treponema* genomes in the NCBI database and a representative selection of genes with >10% pairwise differences were included in DIAMOND alignment against MiSeq sequences. Metallo-β-lactam hydrolase was selected as it confers resistance to nearly all β-lactam antibiotics and was identified in 79% of *Treponema* isolates and aligned with 100% pairwise identity among MiSeq MβL sequences (N=26). Similarly, *znuA* and *zntA* which confer resistance to zinc were identified in 9.1 and 12.1% of MiSeq sequences, respectively (N=3 and 4, respectively), however, PCR for *zntA* failed and thus *znuA* was selected. Resistance to 15- and 16- membered macrolide rings conferred by *MacB* and MFS were found in 82% and 27% of MiSeq sequences, respectively (N=27 and 9, respectively). MFS was selected for PCR design as it confers resistance to macrolides in addition to lincosamides and streptogramins which are more relevant to DD treatment. MFS sequences aligned with 98.3% pairwise identity among MiSeq isolates (N=9) and 95.7-100% identity with *Treponema* MFS in the NCBI protein database.

Formaldehyde dehydrogenase was identified in 88% of *MiSeq sequences* sequenced using MiSeq and aligned with 96.4% pairwise identity among isolates (N=29). 5'-nitroimidazole was identified in only 12.1% of *Treponema* isolates and aligned with 100% pairwise identity among isolates and 82% identity to NCBI 5'-nitroimidazole sequences (N=4). Heavy metal ATPase involved in heavy metal tolerance and resistance was found in 85% of MiSeq sequences and aligned with 100% pairwise identity among isolates (N=28). Copper resistance protein *nlpE* was identified in 82% of MiSeq isolates and aligned with 99.5% pairwise identity among MiSeq *nlpE* sequences (N=27). Streptomycin and streptogramin resistance conferred by *aad*(3") was identified in 73% of MiSeq isolates and aligned with 97.5% pairwise identity among isolates (N=24). Tetracycline resistance conferred by TetR was identified in only 12.1% of MiSeq isolates and were 99.5% identical pairwise among isolates (N=4). Tetracycline resistance conferred by TetO/M/W superfamily of ribosomal protection proteins was identified in 15.2% of MiSeq isolates and aligned with 99.9% pairwise identity among isolates (N=5). TetR and TetO/M/W were not identified together in any MiSeq isolates and in total 27.3% of isolates contained a tetracycline resistance determinant (N=9).

Ten *Treponema* AMGs were selected for PCR on all biopsy samples. The respective AMG functions and relevance to DD treatment and control are given in Table 3.1. DD-infected biopsies were collected from 10 Alberta farms (N=126), DD-free biopsies from a closed herd in Saskatchewan with self-reported DD free status (N=10), and DD-free (N=9) and DD-infected (N=1) from an Alberta abattoir to a total of 146 samples.

AMG amplicon sequencing

The pairwise identities reported here are the highest sequence homologies found on NCBI for the amplicons sequenced. Two PCR positive amplicons, from biopsy samples

originating from geographically distinct farms, were sequenced for each primer set. Heavy metal translocating P-type ATPase (HMA489) PCR product had 98% pairwise identity to WP_082048175 from *T. phagedenis.* Metallo-β-Lactamase Fold Protein (MBL532) had 98.4% pairwise identity to WP_002699780 from *T. phagedenis*. Major Facilitator Superfamily transporter (MLSB381) had 98.4% pairwise identity to WP_024753562 and WP_002695472 from *T. phagedenis* and WP_046177016 from *Streptococcus dysgalactiae*. 5'-nitroimidazole sequenced fragment (N247) had 98.7% sequence identity to WP_006187569 in *T. vincentii* and 91% to WP_044016233 from an uncultured species of *Treponema*. TetR/AcrR family of transcriptional regulators gene sequence fragment had 99 and 92.9% pairwise identity to WP_016522767 and WP_016518855 in *T. medium* and *T. vincentii*, respectively. TetM/TetW/TetO/TetS family tetracycline resistance ribosomal protection proteins (Tet791) had 100% pairwise identity to WP_078934043 from *T. porcium* and 89.7% sequence identity with MiSeq data query sequence. Formaldehyde dehydrogenase (FDH333) resistance gene fragment had 100% sequence similarity to WP_024752284 from *T. phagedenis*. Zinc ABC transporter substrate-binding protein fragment (*ZnuA*294) had 87.3% sequence pairwise identity to WP_016523877 from *T. medium* and 94.4% sequence similarity to *ZnuA* fragment from MiSeq data. Streptomycin 3"-adenylyltransferase (Strep109) had 96% pairwise identity to WP_024753548 from *T. phagedenis*. Copper resistance protein *NlpE* (*NlpE*26) fragment had 92.1% pairwise identity to WP_044634613 from *T. phagedenis*.

Proportion of lesions with AMGs by treatment type

Treatment antibiotics

Advanced, active lesions had the highest proportion of samples with microorganisms harbouring AMGs of interest. Tetracycline ribosomal protection proteins (TetO/M/W) were

present in a higher proportion of M1 lesions, M2 lesions and M4 lesions at 93, 91 and 100%, respectively, than TetR MDR efflux pumps which were present in 73, 89, and 80% of samples, respectively. TetO/M/W and TetR were present equally in 77% of M3 lesions and 84 and 78% of M4.1 lesions, respectively (Table 3.3). Tetracycline resistance genes were present in 10 and 50% of M0s conferred by TetR and TetO/M/W, respectively (Table 3.3). Overall, TetO/M/W superfamily was present in a larger proportion of samples than TetR. If resistant determinants for tetracycline are combined where a sample containing either or both genes are considered genotypically resistant, 89% of all samples harbour bacteria resistant to tetracycline and 94.4% of all DD-lesions carry at least one tetracycline resistance determinant.

Streptomycin had the second highest proportions of samples harbouring resistant bacteria over all lesion grades. Advanced, active lesions had the highest proportion of samples harbouring streptomycin AMG; 97 and 96% of M4.1 lesions and M2 lesions harboured streptomycin 3"-adenyltransferase, respectively (Table 3.3). 93% of M4 lesions and 85% of M3 lesions harboured bacteria with streptomycin resistance compared to only 73% of M1 lesions and 45% of M0s. Resistance genes to macrolides, lincosamides, and streptogramin$_B$ (MLS$_B$) and TetO/M/W were the AMGs found in the highest proportion of M0s, with 50% of samples harbouring bacteria with these genes. MLS$_B$ antibiotic resistance was present in the lowest proportions of samples over all lesion grades. Active lesions, M1 lesions, M2 lesions, and M4.1 lesions had the highest proportions of samples with MLS$_B$ resistance with 80, 83, and 81% of samples harbouring MFS, respectively. Healing lesions had a lower proportion of samples with MFS than M0s at 31% and 50%, respectively, and 67% of M4 lesions harboured MFS (Table 3.3).

Footbath active ingredient resistance

Formaldehyde dehydrogenase was the most common footbath resistance gene in all lesion grades with 79% of samples harbouring the enzyme. Chronic and advanced, active lesions were most likely to carry resistance to formaldehyde with 100%, 95% and 93% of M2 lesions, M4.1 lesions and M4 lesions harbouring resistance, respectively, followed by early, active lesions, M1 lesions, with 87% harbouring bacteria with FDH. M0s and M3 lesions had the lowest proportion of samples with bacteria harbouring FDH resistance, at 35 and 62% on average.

Copper resistance protein, *nlpE*, was the second most common footbath resistance gene present in lesion grades on average at 65% (Table 3.3). M2 lesions had a substantially higher proportion of samples with bacteria harbouring *nlpE* than all other lesion grades at 98%. M1 lesions, M3 lesions, and M4 lesions had a similar number of samples harboring bacteria with *nlpE* at 73, 69 and 65% of samples, respectively. Finally, 45% of M0s harboured bacteria with *nlpE*. Heavy metal exporter (HMA) and zinc resistance protein (*ZnuA*) were in the lowest proportion of samples of average at 51 and 58%, respectively. HMA and *ZnuA* were present in 83% of M2 lesions and 93% and 67% of M1 lesions, respectively. HMA and *ZnuA* were present in 62% and 78% of M4.1 lesions, respectively, followed by approximately equal proportions in M4 lesions, at 47% and 53% of samples, respectively (Table 3.3). The proportion of M3 samples containing *ZnuA* is substantially higher than those with HMA, at 62 and 15%, respectively. Finally, *ZnuA* and HMA in M0s were present in the lowest proportions of all genes examined at 5% of M0 samples containing *Treponema* harbouring resistance to footbath chemicals (AMG^{Trep}) (Table 3.3).

Susceptible *in vitro*

Penicillin and nitroimidazole were identified as antibiotics with high *Treponema in vitro* susceptibility in previous studies and anaerobe specific antibiotics, respectively, that were suggested as alternatives to treat DD lesions. β-lactamase (MβL) was present in 78% of samples on average across lesion grades. The lesion grades with the highest proportion of samples containing *Treponema* that harbour penicillin binding proteins were active lesions, M1 lesions, M2 lesions, and M4.1 lesions at 87, 96, and 89% of samples, respectively (Table 3.3). M3 and M4 samples had approximately equal proportions of samples harbouring bacteria with MβL at 77 and 80% of samples, respectively. Finally, 40% of M0s harboured bacteria carrying MβL. 5'-nitroimidazole was the least common resistance gene across lesions grades of all genes of interest present in only 50% of samples on average. M1, M2, and M4.1 samples had the highest proportion of 5'-nitroimidazole resistance gene at 67, 72, and 76% on average (Table 3.3). M3 lesions and M4 lesions had approximately equal proportions of samples harbouring 5'-nitroimidazole at 38 and 40% respectively. Along with HMA and *ZnuA*, only 5% of M0s harboured bacteria with 5'-nitroimidazole (Table 3.3).

Odds of harbouring AMG^{Trep} by lesion grade

The odds of *Treponema* harbouring resistance genes of interest were examined by lesion grade; all lesion grades were compared to DD-free M0s. Except for MLS_B in M3 lesions and *nlpE* in M4 lesions (P=0.28 and 0.77, respectively), all lesion grades had positive odds of harbouring the specified resistance gene compared to M0s (P<0.05) (Table 3.4). The odds of identifying all resistance genes examined were negative in M0s, indicating you are less likely to find a given resistance gene in M0s compared to DD-infected samples (Table 3.4). The lesion grades with the highest odds of harbouring AMG^{Trep} were M4 lesions and M2 lesions, 59.1 and

56.24 times more likely than M0s, respectively (Table 3.4). M1 lesions and M4.1 lesions had the second highest odds of identifying resistance genes, 30.5 and 34.3 more likely than M0s, respectively (Table 3.4). Finally, M3 lesions had the lowest positive odds of identifying resistance genes at 15.4 times more likely than M0s (Table 3.4). All lesions grades, including tetracycline treated M3 lesions, were significantly more likely to contain tetracycline resistance determinant TetR than M0s (Table 3.4). Similarly, all lesion grades were significantly more likely to harbour streptomycin 3"-adenyltransferase compared to M0s, except for M1 which was suggestive but not significant. All active lesion stages were significantly more likely to harbour TetO/M/W superfamily resistance genes to tetracycline, however healing (M3) and chronic lesions (M4) were not. All lesion grades except for M3 lesions were significantly more likely to harbour resistance to 5'-nitroimidazole compared to M0s (Table 3.4). Similarly, all lesion grades except M3 lesions harbour heavy metal resistance significantly more than M0s (Table 3.4). Only advanced, active lesions (M4.1 and M2 lesions) harboured significantly more major facilitator superfamily resistance genes than M0s. All lesions harboured significantly more resistance to β-lactams (MBL) than M0s (Table 3.4). Finally, all lesions except M3 lesions were significantly more likely to carry zinc export proteins (*ZnuA*) than M0s.

Antibiotic resistance combination correlations in DD lesions

We examined the correlations between resistance genes in DD lesions. The highest correlation coefficient between all genes was for 5'nitroimidazole and *ZnuA* (R=0.59). Following 5'nitroimidazole, *ZnuA* was most correlated with TetR and FDH (R=0.43 and 0.42, respectively), MβL (R=0.40), and HMA (R=0.39); the lowest correlation coefficient was with MFS (R=0.10). TetR resistance gene was most strongly correlated with 5'-nitroimidazole (R=0.44), *ZnuA* (R=0.43), and FDH (R=0.40). Correlations (R) with TetR ranged from 0.44 to 0.18. TetO/M/W

was most correlated with TetR (R=0.38), FDH (R=0.32), and MβL (R=0.28); the lowest coefficient was with streptomycin (R=0.19). Formaldehyde dehydrogenase was most strongly correlated with MBL (R=0.44), *ZnuA* (R=0.42), and TetR (R=0.40) as above. Correlation coefficients (R) for FDH ranged from 0.44 to 0.18. Streptomycin 3"-adenyltransferase was most strongly correlated with FDH (R=0.30), MBL (R=0.27), and *ZnuA* (R=0.26). Correlation coefficients (R) for streptomycin 3"-adenyltransferase ranged from 0.30 to 0.08 (MFS). Copper resistance protein was most correlated with heavy metal resistance protein (HMA) R=0.32, TetR (R=0.28), and TetO/M/W (R=0.25). Following *ZnuA*, HMA was most correlated with TetR (R=0.34), 5'nitroimidazole (R=0.33), and *nlpE* (R=0.32). Following FDH and *ZnuA*, respectively, MβL was most correlated with 5'-nitroimidazole (R=0.39). MFS was the least correlated with all the resistance genes of interest, with correlations (R) ranging from 0.08 to 0.29. Ignoring the effects of lesion grade, 20% of all samples collected contained bacteria harbouring all the AMGs of interest, followed by 5% of samples containing all genes except HMA, and 4% containing all AMGs except MFS.

Advanced, active lesions were the most likely to harbour bacteria that collectively contained all resistance genes of interest. 39% of M2 lesions and 19% of M4.1 lesions contained all 10 genes of interest. All genes except for HMA and *nlpE* (9 genes total) were found in 11% of M4.1 lesions, respectively. Finally, all genes except MFS and HMA were identified in 7% of M2 lesions and 8% of M4.1 lesions respectively. Other AMG^{Trep} combinations were approximately equally likely and not associated with lesions grades.

AMG correlations within sequenced *Treponema* isolates

Significant correlations between the presence of resistance determinants within sequenced *Treponema* isolates were identified (Figure 3.2). Forty-one percent of *Treponema*

isolates contained FDH, HMA, MβL and nlpE, 31% of which contained the above mentioned and *aad*(3") Figure 3.2). The most strongly correlated genes in *Treponema* isolates were TetR and *nim*(5') (R=1). TetR was found in only a single isolate without *znuA* present (R=0.85). Similarly, *nim(*5') and *znuA*, were also found together in 75% of isolates where only a single sample contained *nim(*5') without *znuA* and all samples with *znuA* contained *nim*(5') (R=0.85). Further,100% of isolates with *znuA* contained *nim*(5') and TetR. Similarly, *nlpE* was found in 100% of *Treponema* isolates containing *aad*(3") (N=24) (R=0.77) and 100% of *Treponema* isolates harbouring FDH (N=29) (R=0.54). MβL and *nlpE* were strongly correlated in sequenced *Treponema* isolates (R= 0.72). *nlpE* was strongly correlated with HMA (R=0.68); however, both genes were negatively correlated with *znuA* (R= -0.12 and -0.16, respectively). HMA was strongly correlated with FDH (R=0.62) and *aad*(3"). Surprisingly, FDH was negatively correlated with MFS and TetO/M/W (R=-0.19 and -0.10, respectively). HMA and TetO/M/W were the most negatively correlated genes in *Treponema* isolates indicating the presence of one gene is more likely when the other is absent (R=-0.29). MFS was among the least correlated with the other genes of interest in *Treponema* isolates. However, TetO/M/W correlation coefficients were between -0.29 and 0.12 suggesting very weak or negative correlations.

Odds of identifying AMGs according to *Treponema* species present

The number of resistance genes of interest identified increases with the number of *Treponema* species identified according to a species-specific qPCR targeting *T. phagedenis, T. medium, T. pedis* and *T. denticola* (R=0.63) (Figure 3.3). The presence of certain species of *Treponema* significantly increases the odds of identifying resistance genes which vary between species (Figure 3.4). The odds of identifying MFS, FDH, TetO/M/W, and *ZnuA* are significantly higher when *T. phagedenis* is present than when none of the identifiable *Treponema* species are

present (P<0.05). The odds of identifying 5'-nitroimidazole and TetR were higher when *T. phagedenis* is present, suggestive of a relationship, but did not reach statistical significance (P<0.10) (Figure 3.4). The odds of identifying HMA, TetR, and *nim*(5') are significantly higher when *T. medium* is present than when it is absent (Figure 3.4). Further, the odds of identifying *ZnuA* was suggestively higher when *T. medium* was present but did not reach statistical significance (Figure 3.4). There were no significant differences in the odds of identifying resistance genes when *T. pedis* was present, however, the odds of identifying *nim*(5') was suggestive of a relationship (P=0.06). The odds of identifying MFS were significantly higher in lesions with *T. denticola* compared to lesions without *T. denticola*. The odds of identifying *aad*(3") and HMA were suggestively higher in lesions without the 4 species of *Treponema* of interest but was not statistically significant (P<0.08) (Figure 3.4).

AMG^{Trep} in MiSeq sequences compared to biopsies

As expected, resistant determinants present in biopsies are not present in all *Treponema* isolates from that biopsy. Conversely, TetO/M/W was not identified by PCR in one biopsy but was identified in all *Treponema* isolates sequenced from that biopsy (N=3). Similarly, MFS was detected in 3 *Treponema* isolates cultured from biopsies where it was not identified in by AMG^{Trep} PCR. HMA was found in 4 isolates from 3 biopsies where it was not identified by AMG^{Trep} PCR. Finally, *nlpE* was found in 6 isolates, 2 of which were from the same biopsy, (N_{biopsy}=5) in which it was not identified by AMG^{Trep} PCR.

Cultures containing one or more species of *Treponema* were sequenced using MiSeq and the annotated genome was mined for *Treponema* AMGs and compared to AMG^{Trep} PCR on the biopsy from which they were isolated. Agreement between presence of HMA, FDH, MβL and *aad*(3") biopsies and corresponding cultures was high, varying between only 7 and 27%,

respectively. While many biopsies harboured *Treponema* containing both tetracycline resistance determinants, MiSeq sequenced *Treponema* isolates contained only a single gene within an isolate and never both. Tetracycline resistance determinant prevalence in biopsies was high compared to only 31% in cultures. One of the most significant differences between *Treponema* isolates and AMG^{Trep} PCR results was for 5'-nitroimadazole where 68% of biopsies contained the gene but only 14% of isolates from these biopsies harboured 5'-nitroimidazole. Similarly, 63% of biopsies with sequenced cultures contained MFS but only 28% of MiSeq cultures contained MFS. Most sequenced cultures contained FDH, *nlpE*, MβL and HMA at 90, 83, 83, and 86%, respectively. Similarly, 72% of sequenced cultures contained *aad*(3") and 100% of their corresponding biopsies contained the gene.

Discussion

We have identified significant relationships between the number of *Treponema* species present and the odds to identifying resistance genes of interest relevant to DD treatment and control. Further, the presence of certain species of interest significantly increased the likelihood of identifying specific resistance genes, suggesting there may be differences in the resistance genes species of *Treponema* harbour and susceptibility profiles. We have found significant differences between bacterial AMG presence and the grade of DD lesions which further suggests variation in the AMGs harboured by *Treponema.* Finally, based on MiSeq results, subsets of cultured *Treponema* from DD lesions appear to harbour only a subset of $AMGs^{Trep}$ identified in DD lesions, suggesting variable antimicrobial susceptibility between *Treponema* species and other DD-associated bacteria. Significantly, our results suggest co-occurrence of heavy metal and antibiotic resistance determinants within isolates and DD lesions. Our results may suggest

potential interactions between overall susceptibility of the bacterial community to antimicrobials and lesion chronicity and cyclicity.

Comparing AMGs present within *Treponema* isolates and AMGs^{Trep} present in DD lesions allowed us to examine the broader resistance gene profiles contained within the polytreponemal community of DD lesions. Due to the fastidious nature of treponemes prohibiting isolation of pure cultures, analyzing WGS of individual species and their corresponding resistance genes has not been possible. However, designing and utilizing *Treponema* AMG specific primers to identify antimicrobial resistance determinants relevant to DD treatment and control allowed the identification of AMG^{Trep} within DD lesions rather than those present in *Treponema* isolates alone. Our results suggest that if we relied on MiSeq sequences alone we likely would have underestimated the prevalence of resistance determinants in our samples. Further, previous evidence has suggested the microbial community shifts significantly between lesion grades and active/chronic lesion designations and a method examining AMG^{Trep} within lesions allowed comparisons between lesion grades and AMGs that may be present in transient, unculturable species. Analyzing shifts in the proportion of samples containing AMGs may lead to increased treatment and control efficacy based on the nature of the lesions and identify optimal grade for intervention (Beninger et al., 2018; Evans et al., 2012; Krull et al., 2014). However, PCR-based analyses on uncultured *Treponema* can only provide insight into genotypic prevalence of resistance determinants among lesion grades and does not necessarily reveal functional and phenotypic resistance. Similarly, due to the inability to resolve mixtures of *Treponema* species from WGS data, we can relate AMG presence with species of *Treponema* present, identified utilizing a species-specific qPCR, but cannot definitively determine species contributing AMGs.

Discrepancies between prevalence of resistance determinants among MiSeq sequences from *Treponema* isolates of a given biopsy and those identified by AMG^{Trep} PCR from within the same biopsy were identified. Given MFS and *nim*(5') were present in substantially more biopsies than cultures, it is likely these genes are not commonly found in all 4 species of *Treponema* examined. There are many phylotypes of DD-associated *Treponema* that have not been successfully cultured and identified (Krull et al., 2014; Moreira et al., 2018; Wilson-Welder et al., 2015); because our data suggests *Treponema* have variable resistance profiles, the above-mentioned genes may be found in other *Treponema* or DD- associated bacteria. There are many bacteria present within DD lesions and in the case of MFS, sequencing results suggest the gene fragment is identical in *T. phagedenis* and *Streptococcus dysgalactiae*. Therefore, some of the MFS genes identified in lesions may not belong to treponeme species. Conversely, sequenced gene fragments conferring resistance to 5'-nitroimidazole were only identified in *Treponema* when blasted on NCBI with pairwise identity to *T. medium, T. vincentii, T. denticola, T. putidum,* and an uncultured treponeme (WP_044016233). While it was expected that the number of resistance determinants identified would be higher in the biopsy directly than in the subset of *Treponema* isolates cultured and sequenced, a few genes were consistently identified in MiSeq sequences that were not identified by AMG^{Trep} PCR on biopsies. The difference in identification of AMGs between methodologies is likely attributable to significantly more bacterial DNA, including AMGs, used in sequencing compared to the total amount of DNA in each PCR reaction. Furthermore, *Treponema* DNA is present in significantly lower proportions in directly extracted biopsy samples where bovine DNA makes up the majority of DNA present, which may account for decreased detection. Finally, *T. phagedenis* was the most readily cultured and isolated species of *Treponema,* present in the highest proportions in most lesions (Beninger et al.,

2018) which biases sequencing by synthesis-based methodologies due to unequal DNA contribution (Elbrecht et al., 2017). Indeed, *nim*(5') was present in significantly more samples containing *T. medium* than those without and MFS was found in significantly more samples containing *T. denticola* than those without; both species were present in the lowest proportions within lesions (Beninger et al., 2018). Therefore, our sequencing data likely underestimates prevalence of resistance determinants that are present in *Treponema* other than *T. phagedenis* and may not detect genes present in relatively low proportions. This may also contribute to considerably stronger AMG correlations within isolates than in biopsies. The largest discrepancy between methodologies was the identification of *nlpE* copper resistance protein suggesting our estimate of *nlpE* prevalence within lesions is likely an underestimate. Similarly, MFS and HMA were identified in 16% and 21% of *Treponema* cultures (N= 3 and 4, respectively) where they were not detected in biopsies suggesting heavy metal resistance genes and major facilitator superfamily proteins are more prevalent within biopsies than represented in our results, particularly in *Treponema* within lesions.

Sequencing results suggest that the primer sets designed are capable of recognizing AMGs from multiple species of *Treponema* but did not amplify resistance genes from other species of bacteria in the limited subset of samples sequenced with the exception of MFS as indicated above. We found that the number of *Treponema* species present significantly increased the odds of identifying resistance genes and the number of resistance genes identified, suggesting the resistance genes identified here can be attributed, at least in part, to various species of *Treponema*. Furthermore, previous studies have found species of *Treponema* increase in chronic and advanced, active lesions which supports an increase in the likelihood of identifying AMGs associated with *Treponema* in those lesions. DD is widely accepted as a polymicrobial disease

and the notion that infections are caused by *Treponema* in addition to other bacteria, particularly anaerobes, is increasingly examined. Therefore, even if resistance genes identified here are not specific to *Treponema,* they represent AMGs present within the dermis of DD-infected cattle and thus have significant implications for treatment and control.

The prevalence of resistance genes according to lesion grade may have significant impacts on viable treatment options for DD assuming genotypic data is predictive of phenotypic effect. There are significant differences between the odds of identifying resistance genes dependent on lesion grade, where advanced, active lesions are most likely to harbour most, if not all, of the resistance genes of interest. This suggests early, active lesions (M1) and chronic lesions (M4) may be the most likely to improve from antibiotic treatment compared to their advanced, active counterparts. Furthermore, because bacterial diversity tends to decrease in chronic lesions where spirochetes represent up to 90% of bacteria present, chronic and early lesions are less likely to harbour bacteria with resistance determinants to all treatments than advanced, active lesions (Krull et al., 2014). Macrolide, lincosamide, and streptogramin$_B$ resistance, conferred by MFS genes, and *nlpE,* and *aad*(3") were not significantly different in M1 lesions and M0s supporting the potential for increased bacterial susceptibility with early lesion treatment. However, antibiotic treatment was only applied for advanced, active lesions and many chronic lesions still harboured resistance genes to tetracycline and Lincomycin-spectinomycin.

For the farms sampled, excluding the abattoir, we found the treatment and control combinations used were tetracycline and copper sulfate, tetracycline and zinc sulfate, Lincomycin-spectinomycin and formaldehyde, tetracycline and formaldehyde, formaldehyde-copper sulfate mixed and tetracycline, and copper sulfate alone (DD-free farm); ordered most

common to least common. Our data suggests widespread resistance to tetracycline in all lesion grades which may be due to consistent treatment with oxytetracycline on most farms (Angell et al., 2015; Cutler et al., 2013; Laven and Logue, 2006; Solano et al., 2017b). The highest proportion of DD-lesions carry at least one tetracycline resistance gene when compared to other resistance genes and 77% of M3 lesions, which were collected 5 days post treatment with tetracycline, harboured genotypically tetracycline resistant bacteria. The second antibiotic used to treat advanced, active DD was linospectinomycin which also had widespread resistance determinants in all stages of clinical DD. Our findings may provide an explanation for previous literature demonstrating antibiotic treatment, particularly with tetracycline and Lincomycin-spectinomycin, was only temporarily effective to relieve active lesions signs and resulted in relapsing clinical DD lesions (Cutler et al., 2013; Laven and Proven, 2000; Speijers et al., 2012). Further, tetracycline and Lincomycin-spectinomycin are the most commonly used treatments and the presence of significantly more genes that may confer resistance to the above-mentioned antibiotics within M4 lesions may suggest a connection to lesion chronicity. Furthermore, the presence of HMA and *znuA* in M4 lesions supports previous literature suggesting footbaths improve signs of active lesions but do not improve M4 lesions. Previous studies have suggested penicillin and derivatives or macrolides to treat DD based on *in vitro* susceptibility testing of *Treponema*. We examined resistance to nitroimidazoles, which include metronidazole, tinidazole, and ornidazole, because they are an antibiotic specific to anaerobic bacteria and used to treat many polymicrobial infections with anaerobes (Jenks, 2010). Due to their narrow-spectrum of activity, resistance to nitroimidazoles is rare and nitroimidazoles may have less prevalent resistance genes harboured within pathogenic and environmental bacteria because aerobic bacteria are intrinsically resistant due to the inability to attain a sufficiently low

intracellular redox environment required to reduce the nitro group. Therefore, pressures that accelerate resistant determinant acquisition and transfer are lower than in antibiotics with broad-spectrum activity (Fair and Tor, 2014). Unfortunately, our results suggest widespread resistance to all the above-mentioned antibiotics in DD lesions compared to healthy tissues. However, our sequencing results suggest resistance genes to nitrimadazoles and macrolides conferred by MFS were significantly lower in *Treponema* cultures compared to lesions suggesting if *Treponema* are the major contributors to lesion severity and chronicity, the above-mentioned antibiotics may be considered for further efficacy testing.

Interestingly, there was no significant difference between the abundance of footbath resistance genes between M0s and M3 lesions. This may suggest decreasing bacterial abundance with an antibiotic followed by regular footbaths or topical administration of solutions or gels with heavy metals as active ingredients may be an effective regime to treat and control DD assuming AMGs reflect susceptibility. De-escalating antibiotic activity spectrum and cycling antimicrobials has been shown to be an effective method to decrease antibiotic resistance and improve therapeutic outcomes (Fair and Tor, 2014; Karam et al., 2016). Despite copper sulfate being the most commonly used footbath chemical, copper resistance protein was only significantly increased in M2 lesions but not M1 lesions, M3 lesions, M4 lesions or M4.1 lesions suggesting regular footbaths with copper sulfate may not be effective to significantly decrease symptom severity, likely associated with bacterial abundance, in M2 lesions but may be effective for other lesion grades. However, previous literature suggests symptomatic relief of active lesions following copper sulfate footbaths and symptom severity has been associated with *Treponema* and bacterial abundance and diversity which is not consistent with the above statement (Beninger et al., 2018; Krull et al., 2014). Although cure rate has not been recorded as

significantly different between active chemical ingredients in treatment and control regimes, copper sulfate was significantly more effective at preventing new active lesions than formalin in footbaths (Holzhauer et al., 2012). However, studies examining the efficacy of treatment and control chemicals must be regarded stringently as cure rate may be defined as transitioning from an active lesion state to any other lesion grade and rarely true abolition of the infection (Holzhauer et al., 2011; Jonesa et al., 2017; Speijers et al., 2012). Despite only a single farm utilizing zinc sulfate in footbaths, which were done infrequently, zinc resistance was widespread throughout lesions grades but found in relatively low proportions within sequenced *Treponema* isolates. Interestingly, despite use on two farms, alone or in combination with copper sulfate, resistance to formaldehyde was not significantly increased in M2 lesions or M3 lesions. However, M4.1 lesions had significantly higher odds of harbouring bacteria with FDH and the log odds for M2 lesions was substantially higher but insignificant. Further, formaldehyde damages the health of humans, animals, and the environment and should be avoided when possible (Orsel et al., 2017; Speijers et al., 2010). Finally, the effect of footbaths are often indiscriminate or interchanged between DD treatment and DD control and are comparably difficult due to variation in active chemicals and footbath type (static, spray, etc.) effecting outcomes and must be examined further to improve therapeutic outcomes.

Our results present considerable evidence supporting heavy metal resistance genes and treatment antibiotic resistance gene co-occurence. While within lesions the strongest correlations hold some relevance to treatment and control AM combinations, the correlations are stronger and more relevant within DD-associated *Treponema* isolates. The strongest correlations of all AMGs examined were between TetR and *znuA, nlpE* and *aad*(3”), and *nim*(5’) with TetR and *znuA,* and MβL and *nlpE*. Previous studies have demonstrated that metal exposure and the resistance

proliferating from that exposure leads to co-selection of unrelated resistance genes, likely do to presence of the genes on the same plasmid or transposon. The physical linkage results in the selection for genes on the same element in the absence of selective pressure for those elements (Baker-Austin et al., 2006). Co-selection for broad spectrum β-lactamases and heavy metals are among the most common (Baker-Austin et al., 2006; Romero et al., 2017). In the case of *Treponema* isolated from DD-infected cattle, there appears to be directed co-occurrence related to common treatment and control regimes reflecting known selective pressures. The presence of AMGs alone do not necessarily provide functional resistance; however, studies examining co-selection among biocides, disinfectants and antimicrobials found significant correlations between metal and antibiotic functional resistance (Pal et al., 2015; Romero et al., 2017). It is presently unknown where the AMGs examined in this study are found within DD *Treponema* genomes; however, naturally occurring plasmids have been found in periodontal associated *Treponema* and the same plasmids were found in multiple species of *Treponema* which suggests DD-associated *Treponema* may harbour mechanisms for HGT (Chan et al., 1996; Reedy et al., 1994). Furthermore, the polymicrobial nature of DD makes genetic exchange probable between species of *Treponema*, co-infecting species, and environmental bacteria. Genetic exchange between *T. denticola* and *Streptococcus ghondii* has been found which may provide an explanation for identical MFS genes in *T. phagedenis* and *S. dysgalectiae* (Wang et al., 2002). While the location of the resistance determinants examined are not known, because there is variability between isolates containing the same species, it is likely they are mostly acquired via HGT. Despite strong correlations within *Treponema* isolate AMGs, it is possible that the genes are not physically linked and co-selected but rather acquired for their distinct selective advantages against treatment antibiotics and control chemicals.

The research presented here represents a novel effort to identify *Treponema* AMGs found in DD lesions and attempts to identify differential genotypic susceptibility in species of *Treponema*. Our research suggests, assuming *Treponema* and AMG abundance are predictive of treatment success, that treating early and chronic lesions may be more effective than treating advanced, active lesions which are presently emphasized for treatment, presumably due to their association with severe clinical signs. Our results further suggest significant co-occurrence of heavy metal and antibiotic resistance genes present within lesions and particularly evident in sequenced *Treponema* isolates. While the implications of this research to DD treatment and control are uncertain, we believe the genes selected here should be examined further based on their abundance, relevance to DD treatment and control, and the effect of potential AMG co-occurrence on treatment efficacy.

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Figure 3.1. Workflow to identify AMGs found in *Treponema* relevant to DD treatment and control.

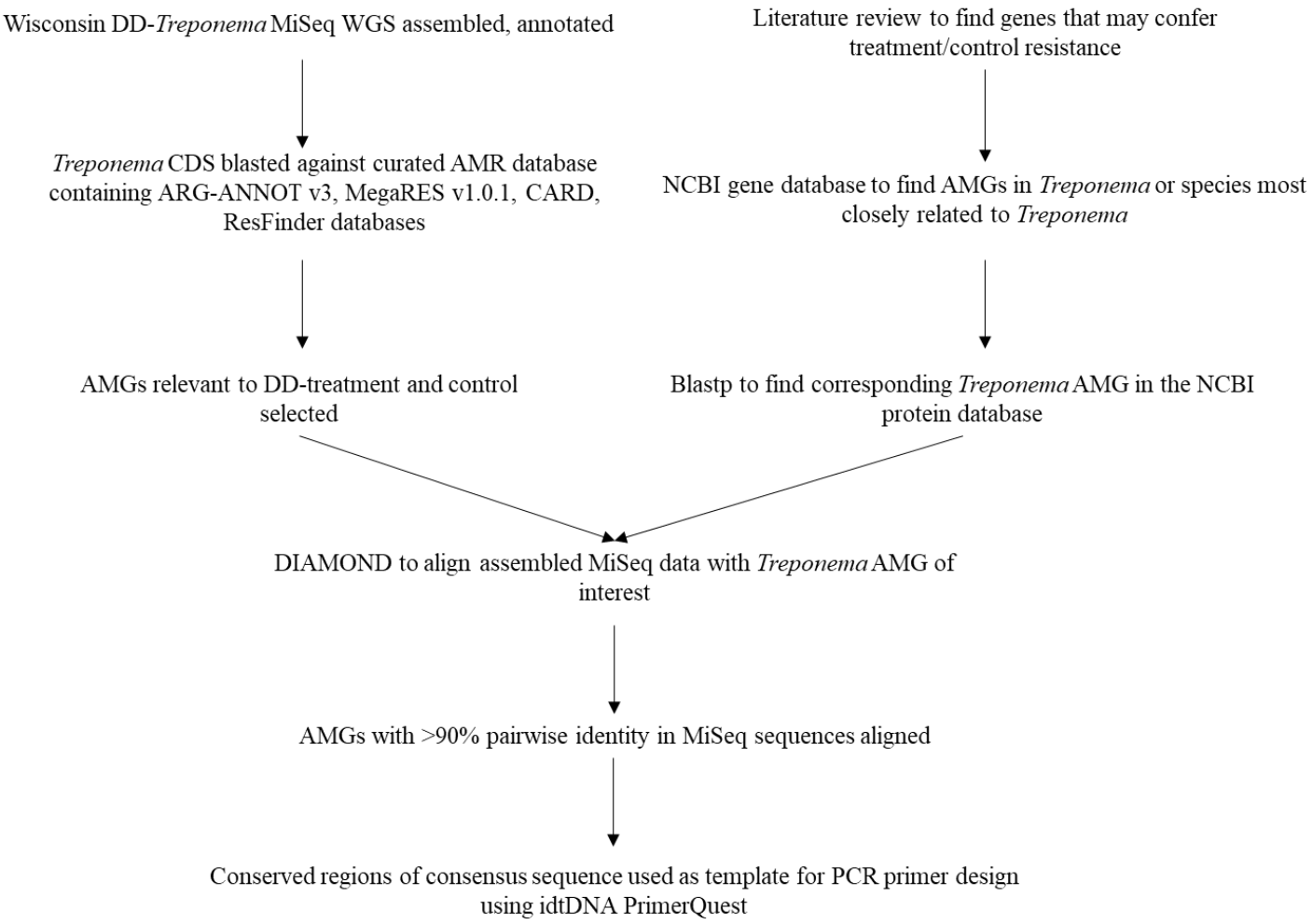


Table 3. 1. AMR genes selected for primer design for DD-infected biopsies.

AMR gene	Accession Number	Mechanism of Resistance	Relevance to DD treatment	Citation
Major Facilitator Superfamily (MFS)	AGT44704.1	Antibiotic Efflux Pump MLS_B (Macrolide, Lincosamide, Streptogramin)	Confers resistance to spectinomycin and lincomycin	(Leclercq, 2002)
Streptomycin 3''-adenylyltransferase (*aad*(3”))	WP_024753548.1	Adenylation enzyme that modifies streptomycin and spectinomycin	Confers cross-resistance to spectinomycin and lincomycin	(Sandvang, 1999)
***nlpE* copper resistance protein**	WP_044634613.1	Copper and multidrug efflux pump	Copper sulfate footbath resistance	(Nishino et al., 2010)
TetM/TetO/TetW Superfamily	WP_078934043.1	Ribosomal protein protection	Tetracycline resistance	(Arenz et al., 2015)
TetR	EFW36799.1	TetR-mediated multidrug efflux pumps	Tetracycline resistance	(Thekkiniath et al., 2016)
5-nitroimidazole (*nim*(5’))	AGT45133.1	Reductases that convert the nitro group on the antibiotic into a non-bactericidal amine	Anaerobe-specific antimicrobial	(Leiros et al., 2004; Pringle et al., 2011)
***znuA* zinc resistance protein**	AGT45229.1	ZnuABC zinc transporter	Zinc sulfate footbath and host-defence resistance	(Ilari et al., 2011)
Metallo-β-Lactamase Fold Protein (MβL)	WP_002688379.1	Catalyze hydrolysis of almost all β-lactam antibiotics	DD treatment and *Treponema* have shown *in vitro* susceptibility	(Evans et al., 2009b; Pettinati et al., 2016; Read and Walker, 1998)
heavy metal translocating P-type ATPase (HMA)	WP_024753385.1	Heavy metal export	Footbath resistance	(Jacobs et al., 2017)
Formaldehyde Dehydrogenase (FDH)	WP_104792647.1	Metabolizes formaldehyde into formate or assimilates into carbon metabolism pathways	Footbath resistance	(Chen et al., 2016; Jacobs et al., 2017; Wilson-Welder et al., 2015b)

Table 3. 2. PCR reactions conditions for all AMR primer sets.

AMR Gene	Primer name	Forward (5'-3')	Reverse (5'-3')	Annealing Temperature (°C)
MFS	MLSB 381	CTGAGTCCGCCTTTATCTATCG	CCTTGCGAATCGTCCCTTAT	53
Streptomycin 3"-adenylyltransferase (*aad*(3"))	Strep 109	CTCACGCATAGCATCCTCTATT	TGCACCTATCAAGTCGGTATTT	60
nlpE copper resistance protein	nlpE 26	CCATTATTTGTATGGCAGTCTTACTTG	TTCTCCGCTGTAAATTCCCATT	60
TetM/TetO/TetW Superfamily	Tet 791	GGCGTGTCTATCTACGGTTATAC	AATGACGGAGGGTTCCTTTAC	63
TetR	TetR 352	GTGAATCGAATCTGCCGTATCA	TCCACCTTTATATCGGATTTGGAA	62
5-nitroimidazole (nim(5'))	N247	CTGCCATCTGTTCGGTTATTTC	GACGGTACGTTGACTCTCTATG	62
znuA zinc resistance protein	ZnuA 294	GCTTATTGCTGATGGTGCTTTC	CTGCAAATGCGGAGACCTATAA	55
Metallo-β-Lactamase Fold Protein (MβL)	MBL 532	TCCGTGTCCGGGATATACTT	GGAGCATGAGATGCTGGTAAT	58
heavy metal translocating P-type ATPase (HMA)	HMA 489	TACTTTGTTCCCGCCGTAATC	GAGGGAAGGCGCATCAATTA	61
Formaldehyde Dehydrogenase (FDH)	FDH 333	TAGAAGTCGGCTCTCTCGTAAA	GCCCGATACCGATAACCAATAC	64

Table 3. 3. Proportion of samples harbouring resistance genes by lesion grade and relevance to DD treatment

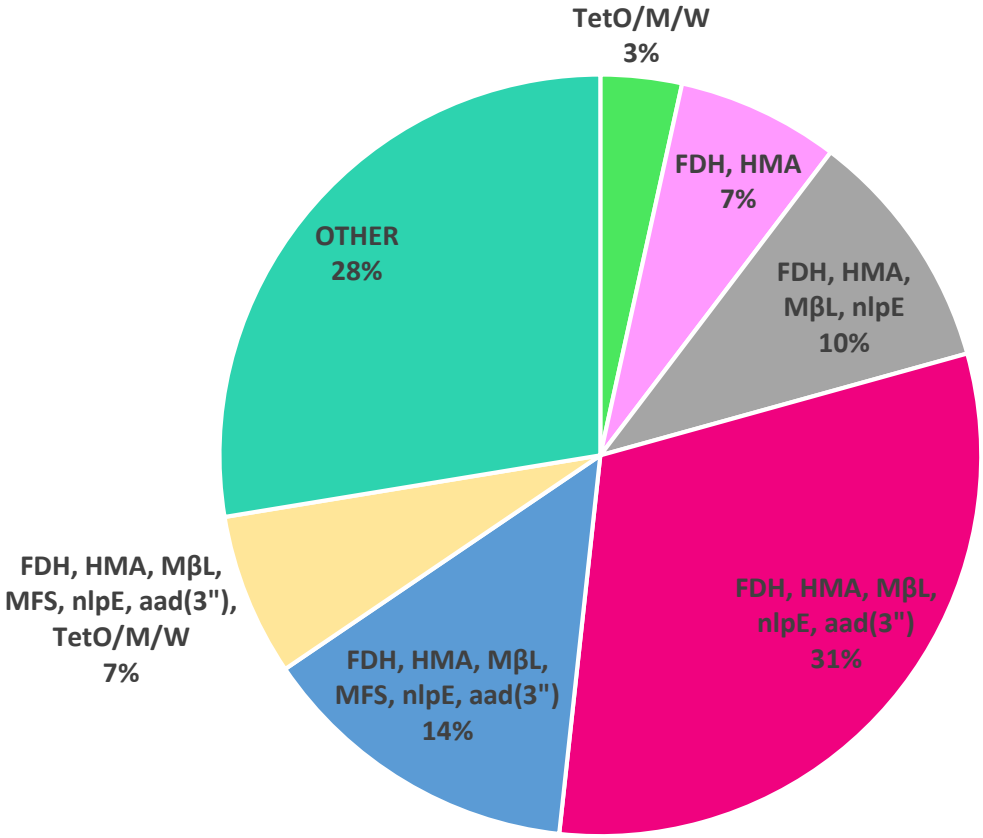
	Resistance Genes									
	Treatment Resistance				Footbath Resistance				Susceptible *In vitro*	
Grade	***aad*(3")**	**TetO/M/W**	**TetR**	**MFS**	**HMA**	***znuA***	**FDH**	***nlpE***	**MβL**	***nim*(5')**
M0	0.45	0.50	0.10	0.50	0.05	0.05	0.35	0.45	0.40	0.05
M1	0.73	0.93	0.73	0.80	0.93	0.67	0.87	0.73	0.87	0.67
M2	0.96	0.91	0.89	0.83	0.83	0.83	1.00	0.98	0.96	0.72
M3	0.85	0.77	0.77	0.31	0.15	0.62	0.62	0.69	0.77	0.38
M4	0.93	1.00	0.80	0.67	0.47	0.53	0.93	0.40	0.80	0.40
M4.1	0.97	0.84	0.78	0.81	0.62	0.78	0.95	0.65	0.89	0.76

Table 3. 4. Difference in log odds[1] of finding resistance gene determinant in a given stage of DD

		Lesion Grade					
	Resistance Gene	**M0[1]**	**M1**	**M2**	**M3**	**M4**	**M4.1**
Treatment Antibiotics	***aad*(3")**	-0.20	1.21ǂ	3.29***	1.91*	2.84*	3.78***
	TetO/M/W	0.00	2.64*	2.35***	1.20	34.36	1.64***
	TetR	-2.51*	3.68***	4.89***	3.59**	4.51***	4.19***
	MFS	0.00	1.39	1.56***	-0.81	0.69	1.46*
Footbath Chemicals	**FDH**	-0.69	2.61*	19.64	1.18	3.57***	3.85***
	HMA	-3.09*	5.76***	4.79***	1.44	3.21*	3.95***
	nlpE	-0.20	1.21ǂ	4.01***	1.01	-0.20	0.81
	znuA	-3.11*	3.69*	4.85**	2.43	3.88*	5.14***
Susceptible *in vitro*	**MβL**	-0.41	2.28***	3.50***	1.61*	1.79*	2.52***
	***nim*(5')**	-2.94*	3.65*	3.80***	0.81	3.20*	4.77***

[1] M0s are reference values to which all other lesion grades are compared. Negative value indicates lesion grade is exp(log odds) times less likely to harbour the resistance gene; positive value indicates lesion grade is exp(log odds) times more likely to harbour the resistance genes. *P<0.05, **P<0.01, ***P<0.001, ǂ 0.051 < P < 0.90.

Figure 3.2. AMG combinations found within *Treponema* isolates from DD.



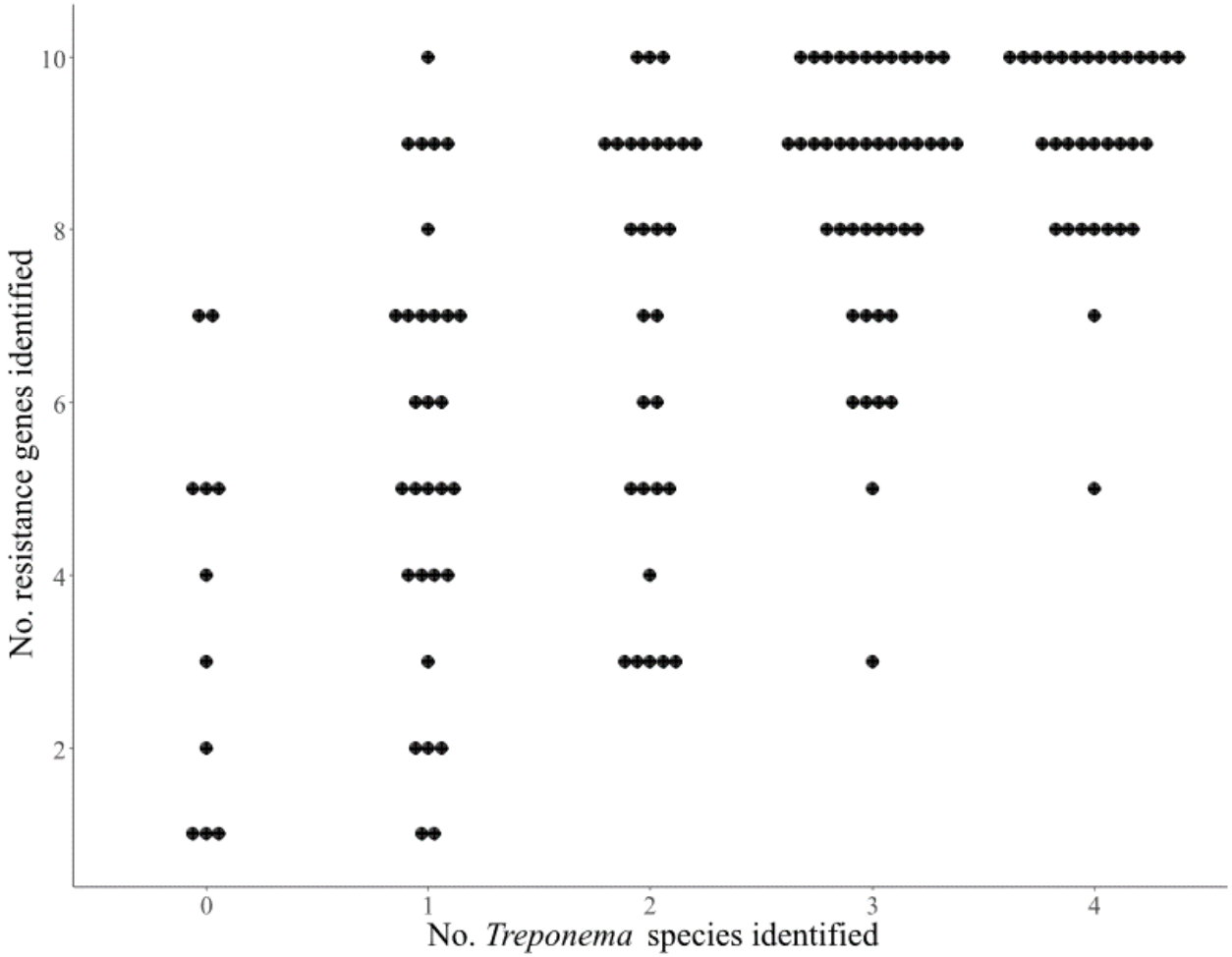


Figure 3.3. Number of resistance genes of interest identified according to the number of *Treponema* species identified within biopsies of DD lesions. Each dot represents a distinct biopsy.

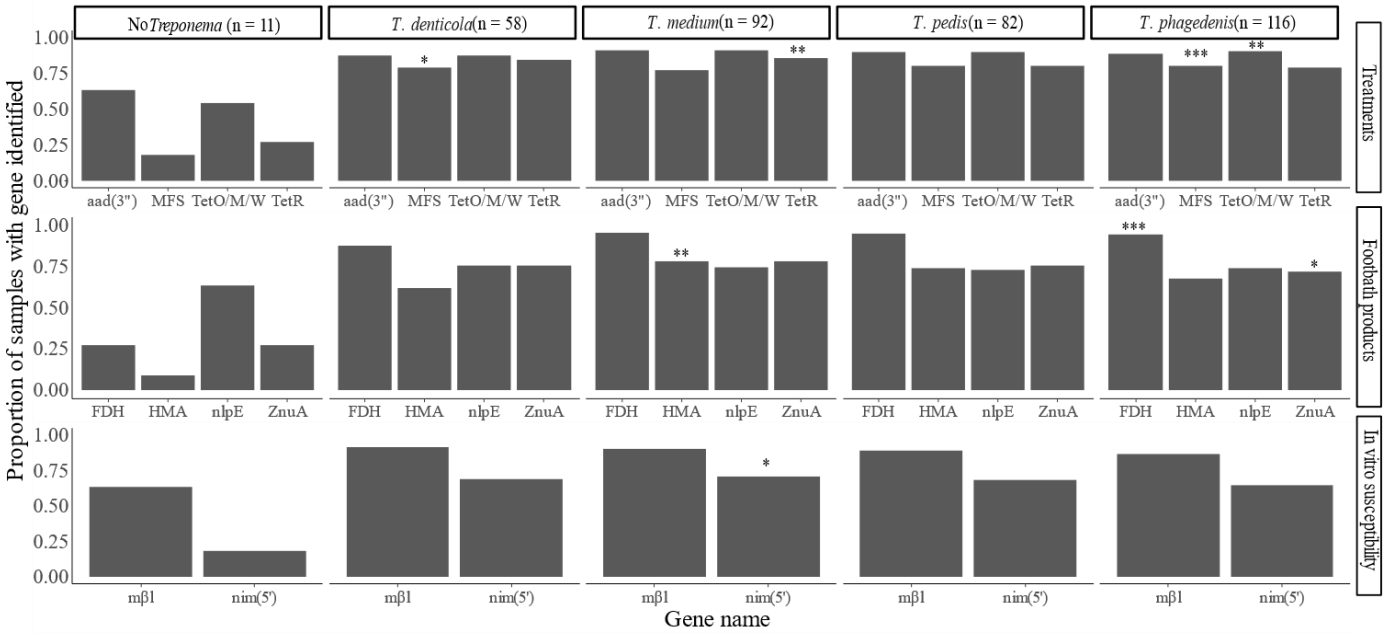


Figure 3.4. Proportion of samples containing resistance gene of interest and four species of *Treponema* according to relevance to treatment and control. Stars* indicate statistical difference in proportion with the gene in samples with the given species and proportion with the gene in samples with no *Treponema* isolated. *P<0.05, **P<0.01, ***P<0.001.

Chapter 4: Summarizing Discussion

Summarizing Discussion

The research presented in this thesis makes significant contributions to the study of digital dermatitis and may contribute to improving treatment and control regimes, while also defining areas of future research. We have found both the number of *Treponema* species present and their absolute abundance are related to painful advanced, active lesions which suggests *Treponema* abundance and species composition may have significant implications in lesion development and pathogenicity. We have further demonstrated that the number of *Treponema* species present, which is directly related to lesion grade, significantly increases the odds of identifying resistance determinants. Due to the predictive nature of increasing *Treponema* species and abundance corresponding to increases in the diversity and number of resistance genes present, we postulate that species of *Treponema* may harbour different resistance genes and have variable susceptibilities to antimicrobials. Differential resistance profiles among DD-associated *Treponema* implies learning the impact and contribution of each *Treponema* species to pathogenicity and chronicity may significantly improve treatment and control. Furthermore, we have identified significant correlations between the most common species of *Treponema* found within DD-lesions where both the presence of a *Treponema* species and the abundance of a species can predict the presence and relative abundance of another species. These findings further emphasize the importance of understanding the roles of *Treponema* species to lesions development, chronicity, and pathogenicity to target treatment appropriately. Through next generation sequencing and single-molecule sequencing technology, we were unable to distinguish and close hybrid assemblies of whole genome sequences from mixed cultures of *Treponema* species. The inability to close WGS of characterized and uncharacterized DD-associated *Treponema* identifies an important and active area of current research with the

potential to resolve interactions between species, identify important virulence factors and species AM susceptibility profiles, and targets for vaccine and treatment development.

Treponema species variation and abundance

The results presented in chapter 2 indicate significant correlations among 3 of the 4 species of *Treponema* most commonly identified in DD lesions. These results suggest that there are likely interactions among the bacterial community present in DD lesions that facilitate infection establishment, progression and chronicity. While we speculate the nature of these interactions are related to nutritional dependencies or complementary virulence factors, there is insufficient research in DD-associated *Treponema* synergies (Wilson-Welder et al., 2015b; Yano et al., 2010). However, the above interactions have been found in *Treponema spp.* present in DD lesions that were isolated from other *Treponema*-associated diseases such as periodontitis (Tan et al., 2014; Zhu et al., 2013). RNAseq and WGS are necessary to further explore and confirm the nature of these interactions among co-infecting species found in DD. We have identified significant correlations between the presence of *T. phagedenis, T. medium* and *T. pedis* and their predictive power on the presence of one another. While the abundance of the above-mentioned species are predictive of the abundance of the other species, this relationship was not as strong as mere presence or absence alone, suggesting interactions among particular species of *Treponema* may not be strictly density dependent. We have identified the presence and abundance of *T. phagedenis* and *T. pedis* as the most significantly correlated with lesion pathology such as large painful ulcers and bleeding upon touch associated with advanced active lesions, M2 lesions and M4.1 lesions. Although *T. phagedenis* is present throughout grades of DD lesions, *T. pedis* and *T. phagedenis* counts are significantly higher and correlated to one another in active, but not in chronic lesions, suggesting pathogenicity associated with active lesions may be related to

interactions between these two species. Further, *T. pedis* in particular has been implicated in many ulcerative diseases including porcine skin ulcers, bovine ischaemic teat necrosis, horse cankers, and perioral and genital ulcers in rabbits (Clegg et al., 2016; Wilson-Welder et al., 2015). However, these 4 species of *Treponema*, while globally present in DD, represent only a subset of the larger polymicrobial community and therefore interactions within the community affecting disease progression may be more intricate and complex than suggested here. It may be suggested that due to previously reported interactions among *Treponema* and other co-infecting bacteria that effect virulence and pathogenicity in other diseases, synergies related to DD pathology may involve other species of bacteria that are consistently present. While future research is needed to determine the contribution of individual species found in DD to lesion pathology, these species of *Treponema* may represent promising targets for future research and potentially treatment and control.

Treponema are found in variable abundance throughout grades of DD-lesions, however, their absolute abundance and the number of species present is significantly higher in advanced, active lesions than in chronic, healing or DD-free skin. However, the absolute abundance of *T. denticola* is not significantly different between any lesion grades and DD-free skin suggesting *T. denticola* abundance and/or presence may not be related to lesion pathogenicity. Furthermore, preliminary co-culture experiments between DD isolated *T. phagedenis, T. medium* and *T. pedis,* and combinations thereof, suggested the addition of variable ratios of an ATCC strain of *T. denticola* resulted in cultures encysting in OTEB broth and no detectable growth on solid media (data not shown). Taken together, these results may suggest a negative association between the growth of *T. phagedenis, T. pedis,* and *T. medium* and the presence of *T. denticola*. This further the supports the notion that examining the nature of interactions between species of *Treponema*

in DD-lesions may reveal key determinants of virulence and pathogenicity that can ultimately be exploited for treatment and control. However, while some of the 3 correlated species listed above have been shown to contribute to enhanced pathogenicity, *T. denticola* has been consistently related to periodontal pathogenicity and tissue degradation. With that said, it must also be mentioned *T. denticola* pathogenicity is typically reported in the presence of other bacteria that have not been identified in DD lesions and this may account for an insignificant correlation to pathogenicity.

Treponema and AMGs

As expected, we found pathogenicity was related to the abundance and number of *Treponema* species. It may then follow that with increased species diversity and absolute abundance, significant increases in the AMGs present are also expected.

The number of samples taken from each farm varied based upon DD lesion prevalence and the distribution of lesion grades. Therefore, farms with lower DD prevalence unavoidably are less represented and therefore we had insufficient power to determine if on-farm treatment was related to DD treatment and control antimicrobials. Further, the antimicrobials used on farm may have differed within, or immediately prior to, the sampling period and there is insufficient literature to suggest AMG acquisition periods based on changing selective pressures. However, as indicated in chapter 3, advanced, active DD lesions tended to have significantly higher proportions of AMGS than their healing and DD-free counterparts on all farms. We also found our DD-free closed herd had the lowest proportion of samples containing the AMGs of interest despite *Treponema* detected visually within biopsies (data not shown).

There is little to no evidence that treating advanced, active lesions is the best way to treat and control DD and to our knowledge the effect of treating different lesion grades has not been

explored. Treating active stages of DD is likely an attempt to decrease disease severity and pain that leads to lameness. Lameness is a major concern in the dairy industry due to decreased production related to increased lying time and shorter feeding time and feed intake and impaired fertility (Palmer and O'Connell, 2015). Further, studies examining the efficacy of treatment antimicrobials are confounded by including chronic lesions as 'cured' in their cure rate calculations. Our results in chapter 2 suggest *Treponema* abundance and species diversity remains high in M4 lesions and treating them as cured until active lesion recurrence may be a significant factor contributing to the cyclical nature of DD. Recently, a study on DD transmission rates between lesion grades found the M4 lesions were the only lesion grade that contributed to increased incidence of DD (Biemans et al., 2017). Given that M4 lesions contribute to higher incidence of new DD infections and antimicrobial treatment of M2 lesions typically ultimately results in a transient M4 lesion, current evidence suggests targeting bacteria present in M4 lesions and treating M4 lesions may decrease the incidence of new infections and may increase the likelihood of infection clearance without active lesion recurrence. We believe treatment and control of DD may benefit from targeted *in vitro* susceptibility testing of *Treponema* and field trials examining the effect of early and chronic lesion treatment compared to treatment of advanced, active lesions.

Treponema species identification

Among the contributions of this thesis to the study of digital dermatitis, the identification of species-specific genes to identify species may be the most significant. Due to the inability to culture and isolate the majority of *Treponema* species found in DD, there is a very limited number of identifiable species and most remain unknown. We have designed a qPCR that identifies 4 of the most common species of *Treponema* identified in DD-lesions. Based on in-

house and available genomic data, we believe these genes are present in a single copy. However, as highlighted throughout, the limited genomic data for a small subset of all species present may not be representative of DD associated *Treponema*. Further, due to the high number of *Treponema* that have not been cultured or characterized, these genes may be present in unknown *Treponema* species and affect interpretation. However, if it is determined these genes are present in other subsets of *Treponema*, these genes may still represent a consistent mechanism of identifying *Treponema* and provide insight into phylogeny.

Previous research on DD-associated *Treponema spp.* has relied on inconsistent phylotyping to cluster species of *Treponema* due to the difficulty of culture and isolation leading to few WGS and strain types available. Regrettably, this has led to 16S rRNA gene-based phylotyping which clusters *Treponema* differently based on the gene(s) or method used to assign a phylotype. While this itself hinders research by making results incomparable, it leads to highly variable results that impair progress in the field. By utilizing species-specific genes to identify *Treponema*, research examining the relationships among species of *Treponema* may be able to identify key nutrients and factors preventing isolation of pure cultures. Further, examining relationships between species present and AMGs and susceptibility, we may identify differential susceptibility assisting in the isolation of pure cultures. Isolating and sequencing pure cultures of *Treponema* is essential for progress in DD research including identification of novel species, virulence factors and their roles and contribution to DD.

Significance and implications

For producers

The tools we identified, optimized and validated in this thesis may lead to the identification of bacteria, *Treponema spp.*, that enhance DD progression and identify co-

occurrence of footbath and antibiotic resistance within DD lesion *Treponema spp.* We have found significantly fewer resistance determinants in samples which harbour fewer *Treponema spp.,* which are early and chronic disease states (M1 lesions and M4 lesions), as well as healing lesions (M3 lesions). Taken together this suggests early and chronic DD lesion treatment, which is atypical, may have greater likelihood of lesion resolution compared to advanced, active lesions which frequently reoccur. Further, we identified resistance gene combinations in DD lesions to heavy metals and formaldehyde (DD control chemicals in footbaths) and antibiotics used to topically treat active or 'strawberry' lesions. Resistance co-occurrence might suggest an explanation for chronic, reoccurring, and relapsing lesions due to the infection not resolving completely after treatment. While the presence of resistance genes alone does not indicate functional resistance to antibiotics and footbath chemicals, the number of widespread resistance genes within DD-lesions and species of *Treponema* is cause for concern. These results may imply consultation with the herd veterinarian to decrease unnecessary use of antimicrobials and improving hygiene may help decrease resistance presence on farms and enhance likelihood of successful interventions with antimicrobials when required

For the research community

Above we have highlighted significant correlations between the number of resistance genes present in lesions and the number of *Treponema* species present. While this may contribute new knowledge towards treatment and control efforts, *in vitro* and *in vivo* susceptibility testing of individual species could greatly enhance our understanding of the polytreponemal community, their relation to disease status and chronicity, and identification of viable treatment options. We have identified *T. phagedenis, T. medium* and *T. pedis* as

significantly correlated and their presence and absolute abundance is related to lesion stage. Conversely, *T. denticola* is not significantly correlated with the above-listed species nor is its presence or absolute abundance related to lesion stage. While this presents great, but contradictory, advancements in beginning to elucidate interactions among species and their relationship to pathogenicity, further studies are needed to determine their direct implications before examining their efficacy as vaccine and treatment targets.

The complex nature of DD lesion progression, etiology, transmission, and lesion recurrence suggest a polymicrobial etiology with variable AM susceptibilities. The research presented here suggests considerable differences between species of *Treponema* with regards to the AMGs they harbour. This may further treatment and control by allowing lesion grades and *Treponema* species typically found within them to be targeted specifically and reduce the use of unnecessary and ineffective antibiotic use should these relationships be reflective of species susceptibility.

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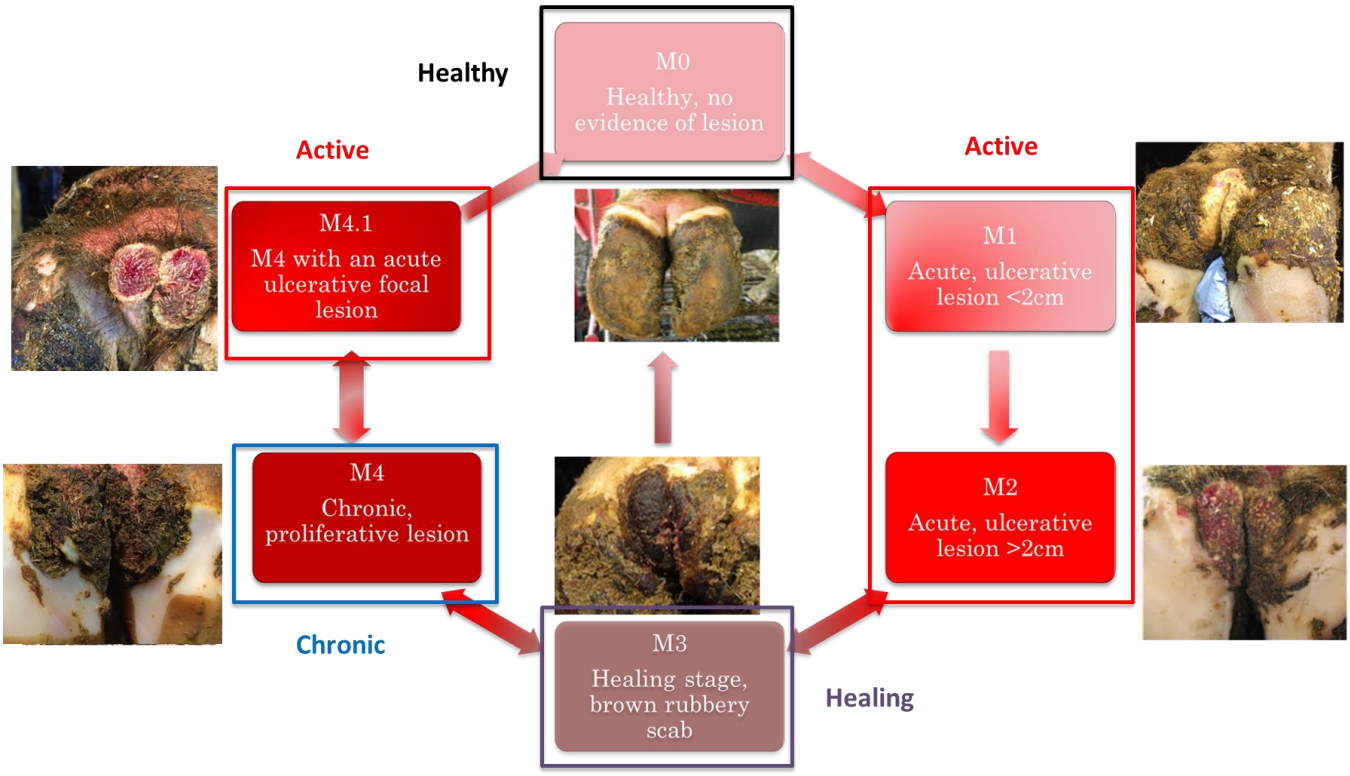
Appendices

Appendix 1. Non-linear disease progression of digital dermatitis. Active lesion stages have an ulcerative focus and chronic lesion stages are proliferative with hair-like projections.



Appendix 2. Differential interference contrast (DIC) 100x magnification of *Treponema spp.* isolated from digital dermatitis lesions.