UNIVERSITY OF LIVERPOOL

A Multifaceted Approach to Informing the Control of Digital Dermatitis in Dairy Herds

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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September 2022

Author's Declaration

Apart from the help and advice as acknowledged, all research described within this thesis has been completed solely by the author.

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September 2022

This research was carried out in the Department of Infection Biology and Microbiomes within the Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool

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For Edward.

It's such a privilege to watch you grow and learn.

You remind me every day of the importance of small things.

Amy Victoria Gillespie

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Bovine digital dermatitis (BDD) is a painful infectious foot disease of cattle, affecting a large proportion of dairy herds worldwide, compromising welfare and causing significant economic losses. Specific *Treponema* phylogroups are important in BDD aetiopathogenesis, however gaps in knowledge regarding transmission routes and pathogenesis limit our ability to control this endemic disease.

Hoof knives invariably become contaminated with treponemes during foot-trimming, although subsequent BDD transmission would depend on treponeme survival time on hoof knife blades, which here was found to be at least two hours. Disinfection during *in vitro* studies identified 2% Virkon®, 2% sodium hypochlorite and 1:100 FAM30® as effective at eliminating culturable treponemes from hoof knife blades with a 20 second contact time. These findings informed development of a disinfection protocol for use during foot-trimming, and its efficacy was confirmed using samples taken during foot-trimming of 133 BDD cases. The field data confirmed the relevance of hoof knife disinfection in preventing BDD transmission as culturable treponemes could be recovered in 1/22 (4.5%) cases where no contact was made between the hoof knife blade and the BDD lesion and 47/111 (42%) of cases where contact was made.

A questionnaire disseminated amongst foot-trimmers, farmers and veterinary surgeons showed more than half of respondents did not wash their hands or hoof knives during foot-trimming (79/143, (55%), and 80/143, (56%) respectively), and only a small proportion (26/143, (18%) and 30/143, (21%) respectively) did so after treatment of BDD- infected cattle. A follow-up questionnaire assessing the impact of recent research and knowledge exchange regarding foot-trimming hygiene showed that 36/80 respondents (45%) had enhanced their hygiene practices.

Previous work has used 16S rRNA gene sequencing of bovine foot skin swab samples from healthy foot skin to identify genera associated with future development of BDD lesions. Results from set analysis of this data indicated farm management practices influence the microbiome. Network analysis of the foot skin microbiome showed evidence of dysbiosis in swabs from healthy foot skin in cattle that later developed BDD lesions. Shotgun metagenomics identified higher abundance of genes that could be associated with collagen degradation in samples from cows that subsequently developed BDD lesions, suggesting early functional changes in the microbiome.

Formation of treponeme biofilms may contribute to BDD lesion chronicity and resistance to treatment. A microtiter plate model was used to study attachment ability of three BDD-associated treponeme strains (T19, T320A and T3552B) incubated under anaerobic conditions, and exposed to oxidative stress, using crystal violet staining. Evidence of biofilm formation, using crystal violet

staining, was found for T320A and dual species mixtures under anaerobic conditions, and for a dual species mixture of T19+T3552B incubated under microaerobic conditions. Transcriptome analyses corroborated evidence of biofilm phenotype characteristics in T320A.

Transcriptome analyses are also used to evaluate BDD treponeme gene expression changes triggered by oxidative stress. Increased expression of virulence factors relating to resistance to oxidative stress, chemotaxis and motility in BDD-associated treponemes compared to fewer changes observed in the human non-pathogenic *Treponema phagedenis* subsp. Reiter, indicated adaptations to resist ROS/ RNS when challenged by host immune cells. Ability to survive in oxygen would also be an important property allowing proliferation on bovine foot-skin during initiation of infection. Gene upregulation conferring resistance to oxidative stress was more marked in multispecies samples. Cooperation between species may enhance survival, potentially explaining the polytreponemal nature of BDD.

Finally, four conventional antimicrobials were identified as efficacious using *in vitro* sensitivity testing. Moreover, susceptibility data implicated six naturally derived compounds and one novel antibacterial metal may be suitable for use as topical preventative or treatment biocides in footbaths.

Overall, the data collected significantly contributes to our understanding of the role of foot-trimming in transmission of BDD. Detection of functional changes in the bovine foot skin microbiome prior to lesion appearance lends support to the importance of enhancing early preventative measures. A multifaceted approach dissected aspects of BDD treponeme pathobiology, revealing new information regarding their ability to form biofilms, thrive in microaerobic conditions, and to cooperate. Identification of novel antibacterial agents has potential to aid future treatment and control of this disease.

Acknowledgements

I think it's an understatement to say that a lot has happened in the last five years. Thank you to Nicholas Evans and Stuart Carter, who have provided exactly the right kind of support I needed to get this body of work completed, even against the backdrop of Covid, and becoming a new mum. I can honestly say this is the best job I have ever had, and I hope we can continue to collaborate in the future.

Thank you to Derek Armstrong and Jenny Gibbons from AHDB, and all the farmers, foot trimmers and veterinary surgeons who participated in aspects of this research. Also thank you to Roger Blowey, whose boundless enthusiasm is a true inspiration. I'm a firm believer that research should always be undertaken in the context of what could benefit our industry. I hope I can continue to contribute to advancing our understanding of bovine digital dermatitis, but more importantly to applying this knowledge to improving disease control on farms.

Thank you to Georgios Oikonomou and Veysel Bay for involving me in the microbiome work, and to Luca Lenzi and Sam Haldenby for the network and shotgun metagenomic analyses. Thank you to Ecaterina Vamos and Charlotte Nelson for carrying out the RNA sequencing, and to Yongxiang Fang for the transcriptomics analysis. Also thank you to Mark Senior and Jane Hodgkinson for your pragmatic approach to IPAP meetings.

To all my fellow postgrads and Leahurst colleagues- Covid taught me the importance of the people we surround ourselves with every day. I will miss our coffee breaks, quotes board and general office banter. A special mention to Helen Williams, Hayley Crosby-Durrani and Rachel Ridgway- your "vet mum" brand of friendship and support is invaluable.

And finally, thank you Alex, for putting up with me in general, but especially in the last six months whilst I've been glued to my laptop at every opportunity writing this. I simply wouldn't survive without you.

List of abbreviations

AHLs *N*-acyl-homoserine lactone compounds
AI-2 Autoinducer 2
AMP Antimicrobial peptide
BDD Bovine digital dermatitis
BlastKOALA Basic Local Alignment Search Tool (Kegg Orthology and Links Annotation)
bp Base pair
CGR Centre for Genomic Research
cm Centimetre
CODD Contagious ovine digital dermatitis
CO_2 Carbon dioxide
ddH_2O double distilled water
DEG Differentially expressed gene
DGE Differential gene expression analysis
dsDNA double stranded DNA
DNA Deoxyribonucleic acid
ECM Extracellular matrix
EPS Extracellular polymeric substances
EDTA Ethylenediaminetetraacetic acid
ELISA Enzyme-linked immunosorbent assay
FCS Foetal calf serum
FDR False discovery rate
Fg Bovine fibrinogen
g Centrifugal force
gDNA Genomic deoxyribonucleic acid
H2 Hydrogen
HS High sensitivity
kb Kilobase
KEGG Kyoto Encyclopaedia of Genes and Genomes
LDA Linear Discriminant Analysis
LefSe Linear discriminant analysis effect size
Log_2FC logfold change
LPS Lipopolysaccharide
mA Milliamps
MBC Minimum bactericidal concentration
MIC Minimum inhibitory concentration
mg Milligram
Mg^{2+} Magnesium ions
mm Millimetre
mM Millimolar
mRNA Messenger ribonucleic acid
M scoring system Mortellaro scoring system
ng Nano grams
N_2 Nitrogen
OD Optical density
OTEB Oral treponeme enrichment broth
PBS Phosphate buffered saline
PCA Principle component analysis
PCR Polymerase chain reaction
pM Picomoles
PNA Peptide nucleic acid

p value Probability value
QS Quorum sensing
QSI Quorum sensing inhibitor
q value FDR-adjusted *p* value
R^2 Linear correlation coefficient
RIN RNA integrity number
RNA Ribonucleic acid
RNase Ribonuclease
RNA-Seq Next-generation RNA sequencing
RS Rabbit serum
r2 Coefficient of determination
SOD Superoxide dismutase
T_m Melting temperature
UK United Kingdom
USA United States of America
UV Ultraviolet
V Volts
v/v Volume/volume
w/v Weight/volume
β Beta
µg Microgram
µl Microlitre
µm Micrometre
µM Micromolar

Chapter 1 Introduction

Bovine digital dermatitis (BDD) is an infectious foot disease of cattle, first reported by Cheli and Mortellaro in 1974 in Italy (Cheli & Mortellaro, 1974). It was first described in the USA in 1980 (Rebhun et al., 1980), in the Netherlands by Peterse in 1986 (Peterse, 1986), and in the UK in 1987 (Blowey, 1987). Thereafter, it rapidly became the most common lameness-causing skin lesion in cattle in the UK (Borgmann et al., 1996). The term "Digital Dermatitis" incorporates diseases referred to initially as "Interdigital Papillomatosis" and "Papillomatous Digital Dermatitis," since it was agreed that both in terms of gross pathology and histopathology, they are the same, with appearance varying depending on the stage of the disease (Read & Walker, 1998).

Lameness in dairy cattle is the top-ranked disease for economic impact and the top health and welfare challenge facing farmers and veterinary surgeons in the UK (Brigstocke, 2020). The most recent (2009) UK estimate of the economic cost of a case of BDD was made at £75.57 (Willshire & Bell, 2009). A recent meta-analysis estimated incidence rate in Britain at 53.6 cases per 100 cow years (Afonso et al., 2020). The overall impact when compared to the differing lesions that cause lameness in dairy cattle varies due to farm-specific factors, incidence rate, and duration of disease; however, BDD has been considered to have the greatest impact in terms of economics and animal welfare (Bruijnis et al., 2012).

1.1 Clinical presentation

BDD lesions are typically found at the skin-horn junctions of the bulbs of the heels and take the form of a wet exudative dermatitis (Blowey & Sharp, 1988). They are circular in appearance, usually 1-4cm in diameter (Rebhun et al., 1980), and most commonly affect a single hindlimb, but lesions can be found in any foot or multiple feet simultaneously (Read & Walker, 1998). Lesions are confined to the digits and are not found above the level of the dew claws. They affect the palmar/ plantar aspect of the foot and border the interdigital space (Read & Walker, 1998). Lesions were originally classified according to a four-stage scoring system (Döpfer et al., 1997) and later modified to include a fifth stage (Berry et al., 2012) (Table 1.1). Chronic lesions (M4) transition back to the "classical" infective M2 lesions (where "M" refers to Mortellaro), perpetuating the disease in a herd.

Table 1.1: Scoring system for digital dermatitis lesions

Table and Figures adapted from Döpfer et al., 1997.

Stage	Description
M1	Early stage of digital dermatitis with a circumscribed granulomatous area, 0.5 to 4 cm in diameter, which lies at the epithelial surface (Figure 1A) or up to 2 mm underneath it (Figure 1B)
M2	Classical ulceration of digital dermatitis, which is an area close to the coronary band affecting skin or horn, up to 7 cm in diameter, with granulomatous tissue when the lesion lies more than 2 mm underneath the epithelial level (Figure 1C)
M3	Classical ulceration of digital dermatitis in the process of healing covered by a scab (Figure 1D)
M4	Chronic stage lesion. Alteration of the skin close to the coronary band which can be observed in the endemic situation where there is a history or suspicion of digital dermatitis. The cutaneous lesions are hyperkeratotic and can present themselves with a proliferative aspect (Figure 1E)
M4.1	A chronic stage lesion with active M1 stage focus, which may progress to M2 (Berry et al., 2012)

Figure 1 Photographic appearance of BDD lesion stages

Figure 1A: M1 Lesion at the epithelial surface

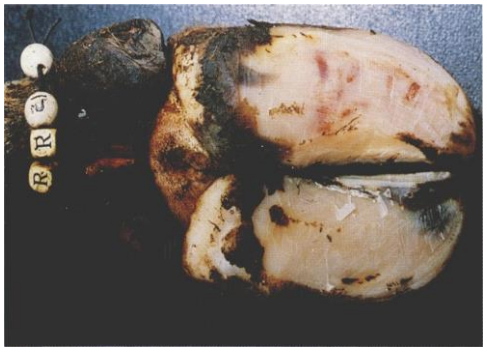


Figure 1B: M1 Lesion beneath the epithelial surface

Figure 1C: M2 Lesion

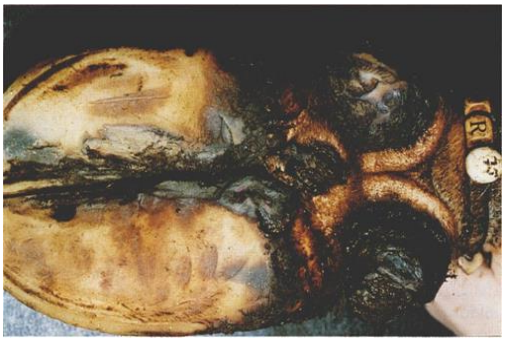


Figure 1D: M3 Lesion

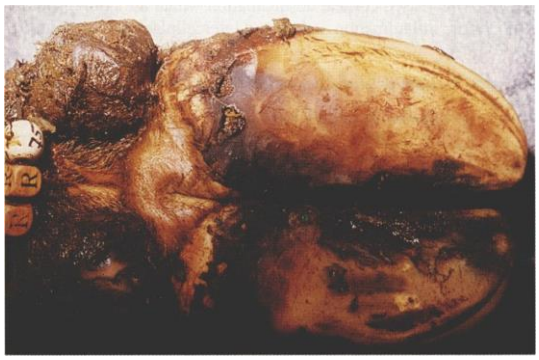


Figure 1E: M4 Lesion

1.2 Aetiology

The hyperkeratotic appearance of chronic lesions led to initial suspicion that BDD was caused by papillomavirus, but this viral aetiology could not be confirmed (Rebhun et al., 1980). Bacteriological investigation of aetiology cultured *Bacteroides* species from swabs of lesions but the significance of these was unclear. Spirochaetes were identified in smears from biopsy specimens, but their significance was also unknown (Blowey & Sharp, 1988). Later histological examination of similar lesion biopsies showed the invasive nature of these organisms, suggesting a possible role in pathogenesis, but further evidence was needed (Read et al., 1992). Canadian work identified a predominance of gram-negative spirochaete–like organisms in lesions, but further characterisation was needed (Sauvageau et al., 1994) and culturing these fastidious organisms proved problematic (Borgmann et al., 1996; Logue et al., 2005). Two distinct groups of spirochaetes were identified in lesions based on phenotypic, morphologic and antigenic characteristics; and were recognised as belonging to the genera *Treponema* (Walker et al., 1995). A humoral response to these two distinct spirochaete groups could be detected by Enzyme Linked Immunosorbent Assay (ELISA) in cows with visible disease (Walker et al., 1997), adding weight to their importance in the pathogenesis. In addition, the response of this disease to antimicrobial treatment suggested a bacterial aetiology (Read & Walker, 1998).

It is currently considered that there are specific *Treponema* phylogroups that are important in causing BDD (Evans et al., 2016). The taxonomy of the spirochaetes, including treponemes, has generally lagged behind that of other groups of bacteria because many cannot be sustained in culture. Despite this, hundreds of pathogenic and non-pathogenic species of treponeme have been identified, and advances in genomic technology have allowed improvement in our understanding and classification of these organisms.

The identification of specific BDD-associated spirochaetes was only initially possible through sequence analysis of the 16S rRNA gene. This technique identified five different BDD lesion-associated

Treponema phylotypes, some of which were closely related to human oral treponemes (Choi et al., 1997) (Table 1.2). A similar study found two phylotypes closely related to the human oral treponeme *T. denticola* (Collighan & Woodward, 1997).

Table 1.2 BDD phylotypes identified by Choi et al in 1997 and their relationship to human treponemes

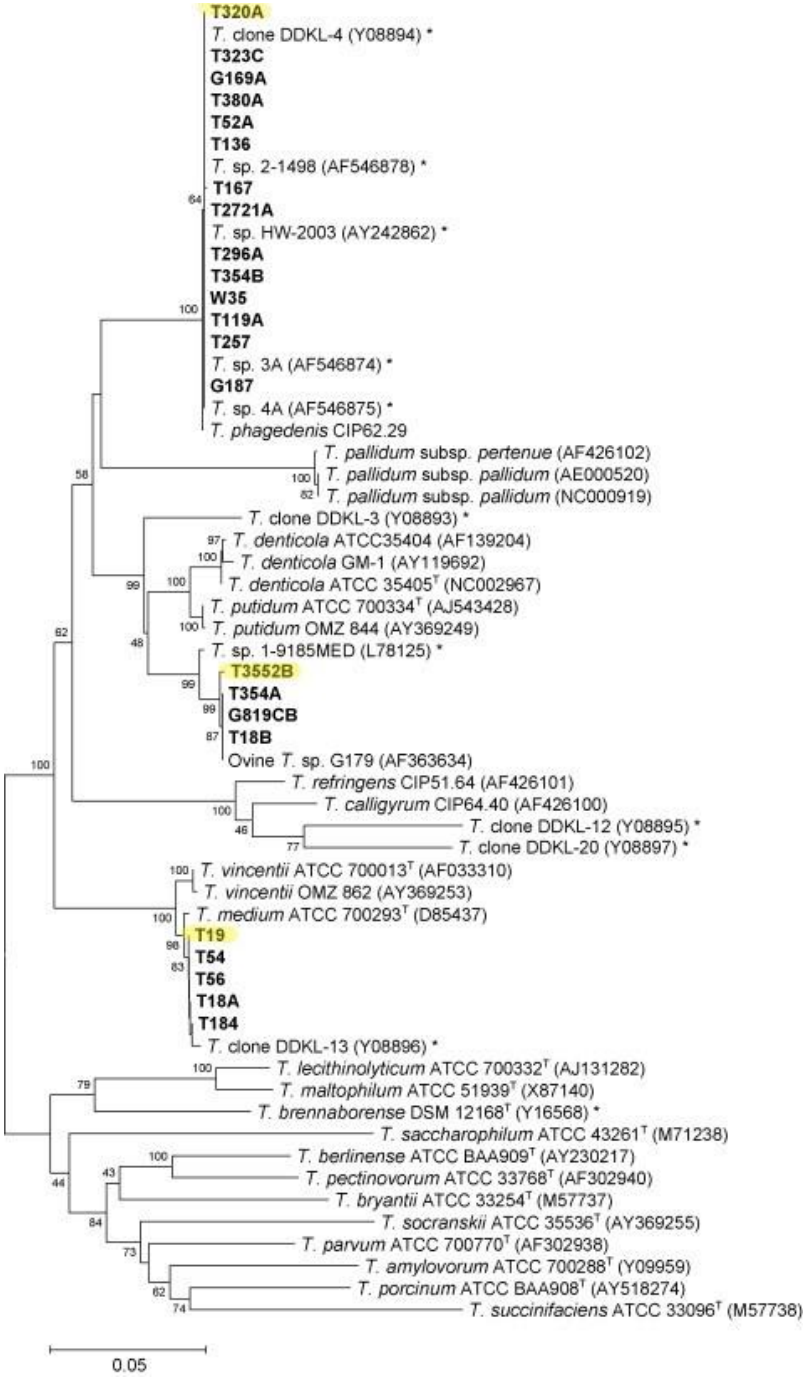
BDD Phylotype	Treponeme closely related to
DDKL-4	*T. phagedenis* (non-pathogenic in people, originally isolated from the urogenital tract).
DDKL-3	*T. denticola* (human oral treponeme)
DDKL-13	*T. vincentii* (human oral treponeme)
DDKL-12	No known cultivable close relatives but clustered close to group IV oral treponemes
DDKL-20	No known cultivable close relatives but clustered close to group IV oral treponemes

In the UK, further studies using 16SrRNA gene sequencing reported BDD spirochaetes to be genetically related to *T. vincentii, T. phagedenis* and *T. denticola* (Collighan & Woodward, 1997; Demirkan et al., 1999a; Demirkan et al., 1998). More detailed genotypic and phenotypic characterisation carried out on UK isolates demonstrated three distinct taxonomic groups, which were designated as *T. medium/ vincentii-like, T. phagedenis* -like, and *T. putidum/ denticola*- like (Evans et al., 2008). Perhaps significantly, these groups usually occur together in BDD lesions. More recently the latter phylogroup was re-classified as *T. pedis* (Evans et al., 2009a). Figure 2 shows a phylogenetic tree highlighting the three BDD strains from the *Treponema* genus that are used to represent each phylogroup and studied throughout this thesis. Interestingly, it has been suggested that symbiosis may exist between the three phylotypic groups; this synergism may be essential for survival and may even enhance pathogenicity (Evans et al., 2009b; Klitgaard et al., 2008; Nordhoff et al., 2008).

The designation of BDD treponemes into three distinct phylogroups, separate from human treponemes, has been confirmed using multilocus sequence typing, which made it possible to drop the "like" descriptions, simply leaving T. *medium*, T. *phagedenis* and T. *pedis* (Clegg et al., 2016). Further taxonomic scrutiny of the first and second groups was still needed to distinguish them from their similar human treponemes; bovine T. *phagedenis* has recently been distinguished from human counterparts by 16SrRNA sequencing (Kuhnert et al., 2020). Most recently, whole genome sequencing comparing representative bovine and human T. *medium* strains and comparing bovine T. *phagedenis* to human T. *phagedenis* biotype Reiter has been completed (Staton et al., 2021a).

Figure 2 Phylogenetic tree based on 16S rRNA gene sequence comparison over ~1420 aligned bases showing relationship between the three strains studied throughout this thesis (highlighted), and related 16S rRNA gene sequences. (Adapted from (Evans et al., 2008)).

Bootstrap confidence levels are displayed as percentages of nodes and only values above 40% are shown. Accession numbers are shown next to each strain/16S rRNA gene fragment clone in parentheses. *Previously reported 16S rRNA gene sequences from bovine digital dermatitis lesions.



1.3 Diagnosis

A reliable and affordable diagnostic test for BDD is not available and so the gold standard for diagnosis remains lifting feet for examination in a foot-trimming crush, which is time-consuming and therefore largely impractical from a labour perspective. Mobility scoring is often used to identify lame cows, but for digital dermatitis, only 39% of cattle with severe lesions are detected as lame (Frankena et al., 2009). Frequently, farms rely on identifying lesions during foot-trimming, but this means treatment will be delayed and often lesions will have become chronic before they are identified. Alternative methods for assessing within-herd prevalence and for identifying acute lesions for treatment are therefore required and this needs to be an active process.

M-scoring in the parlour after washing cows' feet and using a head lamp and mirror to aid inspections was sufficient for classifying presence/ absence of lesions with 90-92% sensitivity and 80-88% specificity (Relun et al., 2011; Solano et al., 2017), but this was reduced when classifying lesion stages, particularly for M1 and M4.1. Missing front foot lesions (approximately 10%) and those on the dorsal surface of the foot (approximately 2%) accounts for most of the decreased sensitivity compared to the gold standard (Solano et al., 2017). Scoring of youngstock and milking cows walking freely in their housing, or milking cows restrained in headlocks is less sensitive than scoring in the parlour (approximately 50-60% of those identified in a foot-trimming crush); however may still be suitable for assessing trends of BDD prevalence in a herd, for example following changes to disease control plans (Jacobs et al., 2017; Solano et al., 2017).

The use of *Treponema* serology for diagnosing BDD in individual animals is limited because the disease is endemic and therefore the presence of *Treponema* antibodies does not correlate with presence/ absence of lesions. A recent study showed increased levels of IgG1 are correlated with presence of lesions, but indirect ELISA still misclassified a substantial number of clinically affected animals as negative (81/204, 39.7%) as ELISA readings were so variable (Afonso et al., 2021). It may be more appropriate to apply these methods to estimate herd-level prevalence for disease monitoring purposes. It has been suggested that indirect ELISA using bulk tank milk samples is able to distinguish low ($\leq$ 10%), medium (10-40%) and high (>40%) prevalence herds with good sensitivity (84-97%) and specificity (86-100%) (Aubineau et al., 2021). If further validated, bulk tank sampling has potential to rival time-consuming BDD prevalence estimates made by other methods.

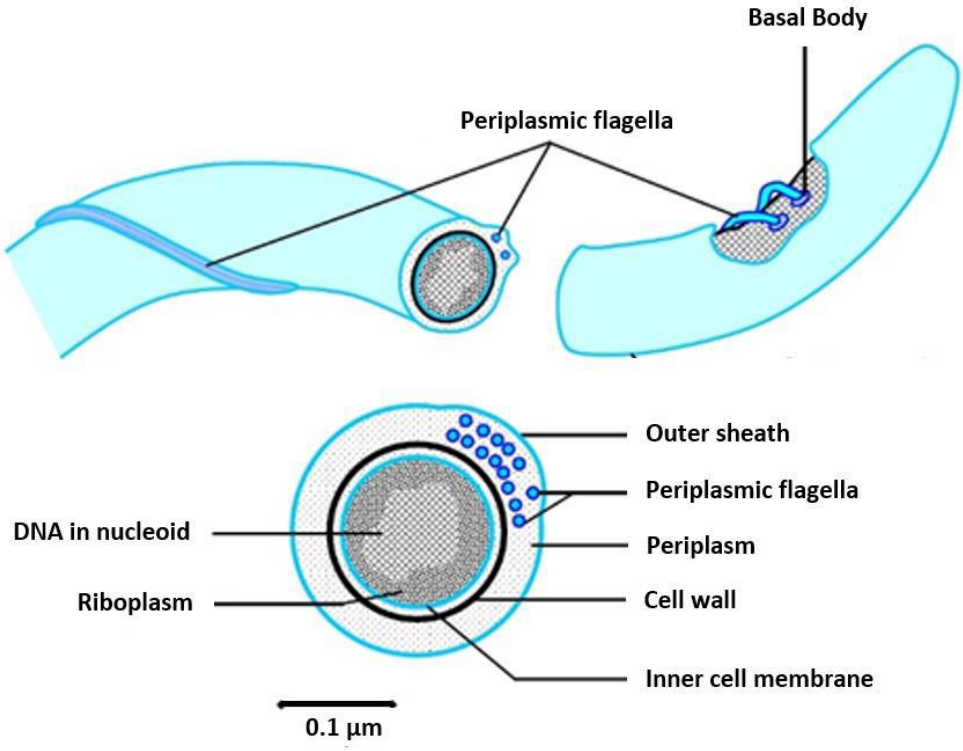
Progress has also been made using higher-tech solutions for detection of BDD which have potential to improve animal welfare. Computer vision methods have been developed using a database of images of the interdigital space from cows affected by BDD to train a BDD detection model to identify and classify lesions. It was able to correctly identify 70.8% of lesions according to internal validation, and

88.2% according to external validation. M2 lesions were less accurately classified than M4, likely due to fewer images available for model training (Cernek et al., 2020). There is also interest in the use of thermal imaging for detection of digital dermatitis. It has been shown that interdigital skin temperature pre-calving has a sensitivity of 77% and specificity of 66% for detecting active BDD lesions (Anagnostopoulos et al., 2021).

1.4 Morphology of the treponemes

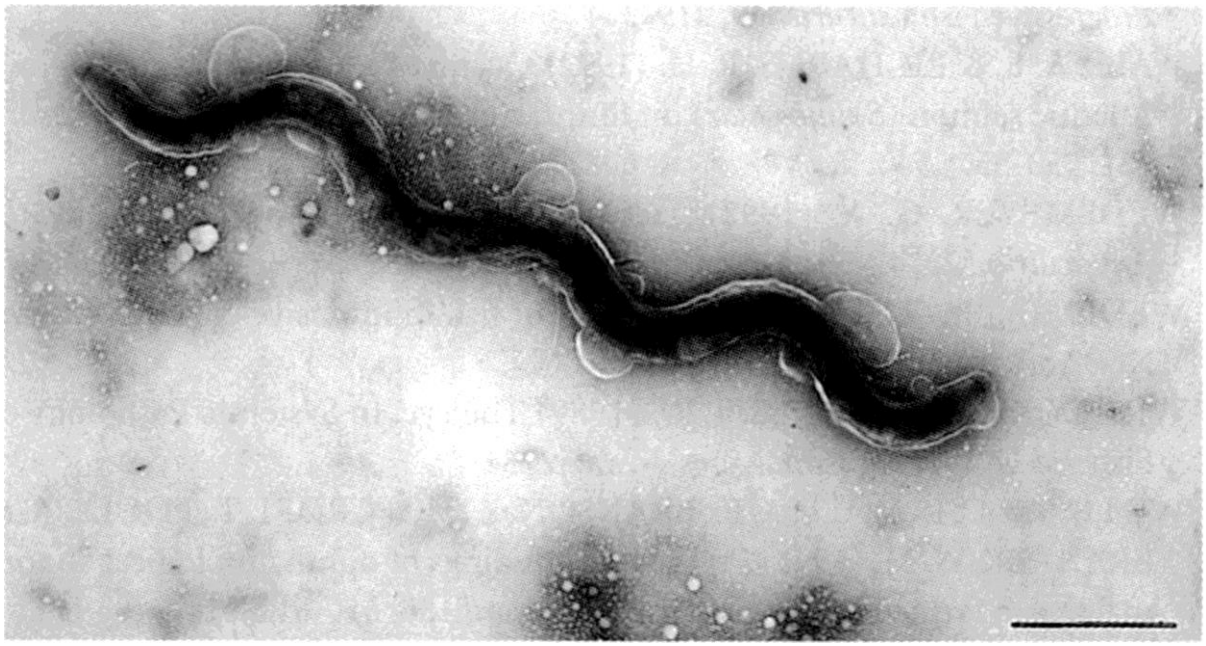
Treponemes belong to the bacterial phylum of Spirochaetes, which are characterised by their unique endoflagellae: flagellae that are located between the cell wall and outer sheath and commonly referred to as periplasmic flagellae or filaments. They are mainly anaerobic, although some are considered microaerophilic. Their general structure is most like Gram-negative bacteria: cytoplasm surrounded by a cytoplasmic membrane, and the presence of a thin cell wall surrounded by an outer sheath. However, the outer sheath exhibits several structural differences from Gram- negative bacteria, such as the presence of lipoteichoic acid, and many species lack lipopolysaccharide (LPS) (Norris et al., 2010). The spiral morphology conferred by the endoflagellae is illustrated in Figure 3.

Figure 3 Treponeme morphology. A schematic diagram illustrating typical morphological features of the *Treponema* species (Modified from Cronodon, (Cronodon, 2017)).



The morphology of BDD-associated treponemes has been observed using transmission electron microscopy. Those belonging to the *T. phagedenis*- like phylogroup are described as 10-15µm in length and 0.35-0.40µm wide with 7-9 periplasmic flagella at each end of the cell (Trott et al., 2003), although a later paper which examined treponemes with 100% sequence identity match in the 16S rRNA gene recorded their size at 6-8µm long and 0.2-0.3µm wide. It is possible these differences are due to variations in growth conditions. Spirochaetes belonging to the T. *pedis* phylogroup are recorded as 5-16µm long and 0.2-0.3µm wide (Evans et al., 2009a). A spirochaete isolate from a biopsy taken from a typical BDD lesion, with the appearance of an M4 lesion, is depicted in Figure 4.

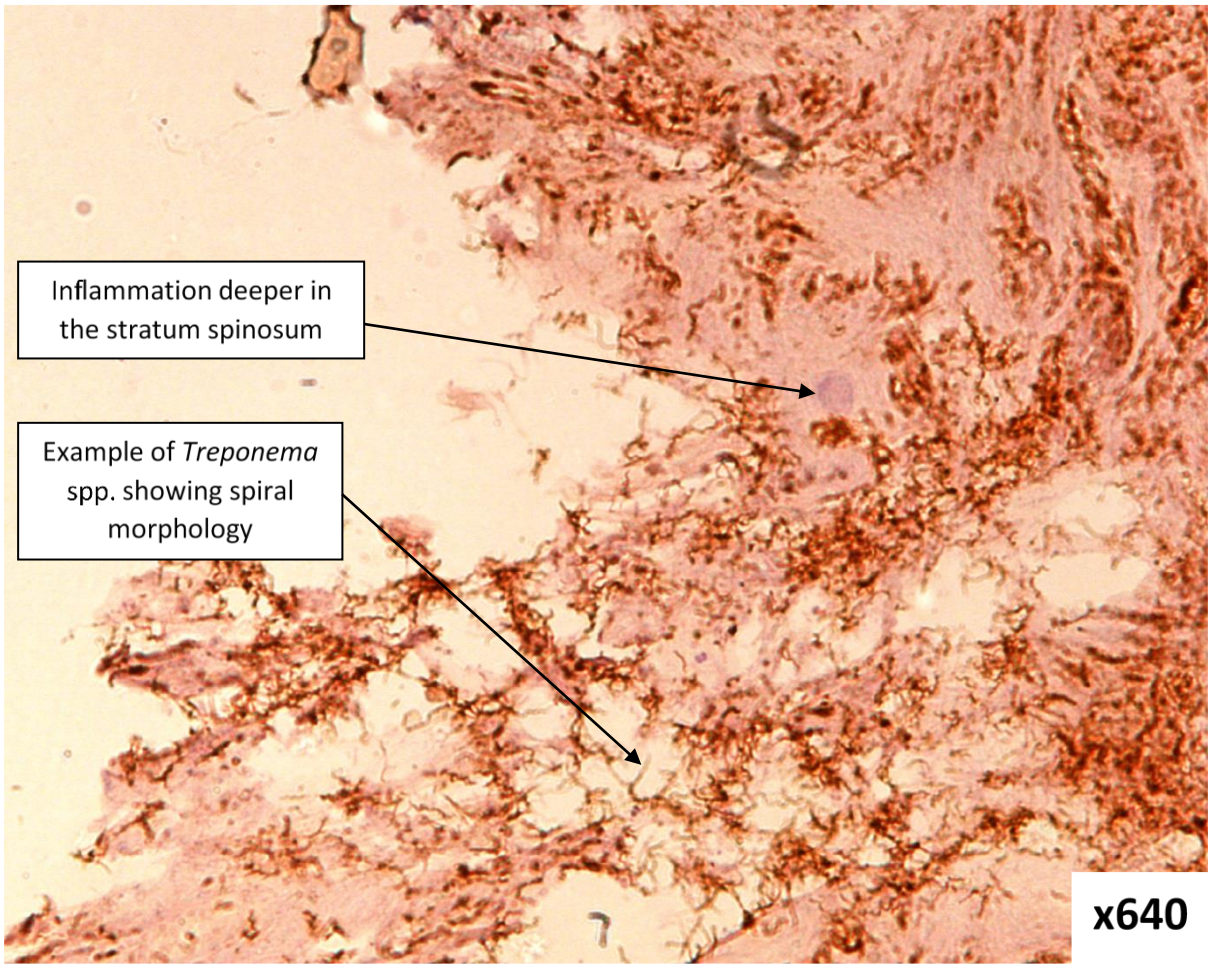
Figure 4 Electron micrograph of a digital dermatitis spirochaete isolated in the UK bar =1µm From (Demirkan, et al., 1999a).



1.5 Pathogenesis

Histopathology using biopsies from BDD lesions has demonstrated that treponemes are found deep in the epidermis, suggesting virulence as tissue invasion is considered a major virulence trait (Blowey et al., 1994; Moter et al., 1998) (Figure 5). Whilst less is known about pleomorphic forms of treponemes, an encysted form has been reported for BDD-associated strains which may have a role in persistence deep in the bovine foot skin (Döpfer et al., 2012a).

Figure 5 Histopathology of an active DD lesion, showing erosion of the stratum corneum (completely irregular surface), and deeper infection in the stratum spinosum prompting an inflammatory response (presence of polymorphonuclear leukocytes). Treponemes are abundant and visible in the eroded areas, showing their spiral morphology highlighted in brown due to immunohistochemical detection of *Treponema* spp. antigens. (Reproduced from Gillespie & Evans, 2019).



The importance of treponemes in BDD is supported by successful development of infection models, thus fulfilling another of Koch's postulates (Gomez et al., 2012; Krull et al., 2016; Read & Walker, 1996). Current evidence shows that the use of tissue homogenate prepared from lesion material is more effective in inducing BDD-like lesions than inoculation with pure *Treponema spp.* cultures alone, suggesting that the presence of a mixed culture is required for lesions to develop. Skin needs to be macerated to allow infection to become established. The reason for this is unknown, but it has been shown that a human oral *Treponema* species bind more favourably to actively dividing epithelial cells, implying that an actively repairing epithelium may be more susceptible to pathogen adhesion (Edwards et al., 2003a). It has also been shown that *T. denticola* adheres to the extracellular matrix proteins fibronectin, laminin and fibrinogen, which are found at the site of tissue damage (Edwards et al., 2003a).

Researchers are increasingly using next generation sequencing technologies to study disease pathogenesis. RNA sequencing is used to sequence mRNA transcripts which allows analysis of gene expression. The transcriptome is highly dynamic and responsive to changes in external conditions and therefore, reveals how functional elements of the genome are used in tissue changes such as development and disease (Raghavachari & Garcia-Reyero, 2018). Transcriptomics has been used to study the effects of BDD-associated treponemes on the local host immune response. The transcriptome of bovine macrophages exposed to bovine *T. phagedenis* showed dysregulation of genes related to innate immunity and wound repair which would enable bacteria to resist clearance and promote lesion formation (Zuerner et al., 2007). RNA sequencing of samples from BDD lesions showed evidence of decreased turnover of keratin, suggesting alterations to normal skin cell replenishment and compromise to the epidermal barrier, as well as evidence of downregulation of local immune and inflammatory responses (Scholey et al., 2013). Investigation using qPCR of genes in bovine keratinocytes and fibroblasts grown in tissue culture and exposed to BDD-associated treponemes showed that some common genes encoding inflammatory mediators such as RANTES/ CCL5 are upregulated in fibroblasts (Evans et al., 2014), again suggesting suppression of local immunity as a means of pathogenesis for this disease. RNA sequencing has identified fewer pro-inflammatory responses in bovine fibroblasts exposed to pathogenic BDD treponemes compared to a commensal treponeme, suggesting BDD treponemes avoid triggering (or suppress) substantial host inflammatory responses to enable tissue invasion and persistence (Newbrook et al., 2021).

1.6 Emerging perspectives on pathogenesis

1.6.1 The role of the microbiome

Whilst much of this thesis focuses on the BDD-associated treponemes, a wide variety of bacteria besides treponemes have been identified in BDD lesions. These include *Fusobacterium* spp*., Bacteroides* spp., *Dichelobacter nodosus, Guggenheimella bovis, Campylobacter* spp. and *Peptococcus* spp. (Blowey & Sharp, 1988; Döpfer et al., 1997; Rasmussen et al., 2012; Schlafer et al., 2008); and more recently *Porphyromonas levii, Mycoplasma* spp. *and Prevotella* spp. (Berry et al., 2010). This has led to the consideration of BDD as a polymicrobial disease; however, it is challenging to distinguish true pathogens from commensal bacteria and from contaminating bacteria originating from the environment. The development of next generation sequencing and other genomic technologies has enabled us to begin to make these distinctions by studying the microbiome of BDD lesions.

The term “microbiome” refers to the entire collection of genomes from all the microbes found in a particular ecological niche. The term is applied to a range of different anatomical locations in living

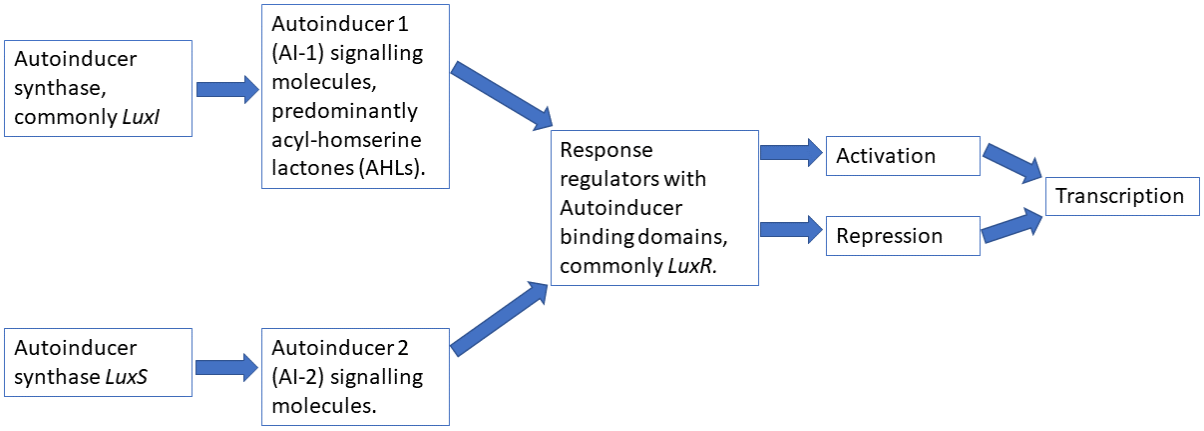
organisms, but also to many environmental locations from the depths of the Mariana trench to the heights of the International Space Station (Checinska et al., 2015; Liu et al., 2019).

Recent 16S rRNA gene and shotgun metagenomic sequencing of the bovine foot skin microbiome has shown differences between healthy skin and lesions, and how the bacterial composition in lesions changes as lesion morphology progresses (Caddey, et al., 2021; Ettema & Santos, 2004; Hesseling et al., 2019; Krull et al., 2014; Santos et al., 2012; Zinicola et al., 2015a; Zinicola et al., 2015b). Notably, *Treponema* species were found in low abundance in early lesions and became dominant in latter stage lesions (Krull et al. 2014; Zinicola et al., 2015a). This is analogous to the more widely studied human oral disease; which show progression of gingivitis to periodontal disease and the formation of periodontal pockets, which become more anaerobic and favour growth of *T. denticola* alongside *P. gingivalis* and *Tannerella forsythus* (Edwards et al., 2003a). Understanding the bovine foot skin microbiome would give insights into the importance of different bacterial species and the interactions between them in BDD pathogenesis, both in terms of initiation and perpetuation of disease.

1.6.2 The role of quorum sensing (QS)

Bacteria can communicate with each other to coordinate their behaviour in order to adapt to changes in their environment to improve survival despite changes in physiological conditions and nutrient availability, and to defend against presence of toxins. These communication processes, known as quorum sensing, occur in both in single and multispecies communities, in both planktonic and biofilm phenotypes. Signalling molecules are produced in a cell-density dependent manner. The most common intraspecies signalling molecules in Gram-negative bacteria are the acyl homoserine lactones (AHLs) and bacteria may have more than one AHL QS system. A group of molecules referred to as Autoinducer-2 (AI-2) are produced by a large cohort of Gram-positive and Gram-negative bacteria and can mediate both intra- and interspecies communication (Zhang et al., 2020). Figure 6 shows a simplistic schematic of the QS process and the most common proteins involved for Gram negative bacteria. *LuxI* and *luxR* homologs are usually tightly linked; however, there are examples of *luxR* homologs without paired *luxI* homologs, meaning that transcription factors in some bacteria can respond to AHLs produced by other bacteria (Schaefer et al., 2013).

Figure 6 Schematic of the most common quorum sensing pathways identified in Gram negative bacteria.



In oral biofilms, where *T. denticola* is considered instrumental to biofilm formation, evidence of AHLs or AI-2 production has not been reported. It is still possible that *T. denticola* participates in quorum sensing using receptors, without producing signalling molecules. There are examples of other bacteria where *luxR* response regulators can be found but are not coupled to *luxI* or *luxS* autoinducer synthase genes (Niazy, 2021; Schaefer et al., 2013). Quorum sensing pathways have not been studied or characterised in BDD associated treponemes.

The *luxS* system has been identified in *P. gingivalis* which is thought to produce AI-2 which leads to formation of oral biofilms (Niazy, 2021). It has been proposed that synergy between early colonising commensals and late pathogenic colonisers in the development of periodontitis is mediated by AI-2 from *F. nucleatum* (Jang et al., 2013). Since BDD is considered a polymicrobial disease in which *Fusobacterium* spp. and *Porphyromonas* spp. feature, it seems likely that communication between different species has a role to play in pathogenesis.

1.6.3 The role of bacterial biofilms

Pathogenic bacteria form biofilms as a survival strategy as they are protected in this phenotype from changes in environmental conditions, antibacterial chemicals, and components of the host immune system (Percival et al., 2012). It is thought that biofilms are part of most chronic infections (Vestby et al., 2020), therefore the ability to persist in this protected state could be relevant to BDD lesions which frequently become chronic.

Bacterial biofilms are surface-associated, adherent to a substratum or to each other. Direct examination reveals bacteria in clusters, encased in a matrix of extracellular polymeric substances, such as proteins (eg fibrin), polysaccharides (eg alginate), and extracellular DNA (Vestby et al., 2020). The infection is localised, and the infection is resistant to antimicrobials despite proven sensitivity *in*

vitro (Hall-Stoodley et al., 2004). There is evidence of altered phenotype with respect to growth rate and gene transcription. This distinguishes biofilms from "nonbiofilm" surface attached bacteria, such as those growing on agar plates, which behave like planktonic cells and do not exhibit the inherent resistance characteristics of true biofilms (Donlan & Costerton, 2002).

It is clear from epidemiological evidence that biofilms play an important role in infectious diseases; however, the detailed processes by which this happens are poorly understood. It is suggested that detachment of bacterial cells or cell aggregates from biofilms may allow dissemination to new sites of infection. Gram-negative bacteria within biofilms produce endotoxins which cause inflammation, initiating or aggravating tissue damage. There is evidence that organisms in biofilm are resistant to the antibody activity and phagocytosis. There is also evidence that the rate of genetic exchange between microorganisms in biofilm by conjugation and plasmid transfer can be accelerated, thus making this phenotype a hotbed for generation of antimicrobial resistant organisms (Donlan & Costerton, 2002).

Whilst biofilm-forming capability of BDD-associated treponemes has not been studied, it is well known that human oral treponemes behave synergistically as part of the "red complex" of bacteria comprising *Tannerella forsythus, Porphyromonas gingivalis* and *Treponema denticola*, and are instrumental to the pathogenesis of human periodontal disease (Ong et al., 2017; Socransky et al., 1998). It is thought that biofilm-grown bacteria produce quorum sensing signalling molecules which enable them to behave as a multicellular entity (Percival et al., 2012). The development of a model for the biofilm morphological form of BDD-associated treponemes and the potential role of biofilm and quorum sensing in BDD pathogenesis is a focus for this thesis.

1.6.4 Resistance to oxidative stress

Pathogenic bacteria face the metabolic challenge of exposure to reactive oxygen species (ROS) produced by host cells. The ability to resist the effects of ROS is intrinsic to pathogenicity (Gherardini et al., 2006). The BDD lesion transcriptome showed upregulation of bacterial genes involved in protection against oxidative stress was one of the major factors defining the disease (Marcatili et al., 2016). Study of BDD treponeme genomes, in comparison to non-pathogenic equivalent species from the same phylogroups, supports resistance to oxidative stress as a possible pathogenic mechanism (Staton et al., 2021a). It has been shown that *T. phagedenis* strain T320A and *T. pedis* strain T3552B can survive in faecal microcosms incubated aerobically for up to a week, and T320A can also survive for prolonged periods in common bedding materials, including up to a week in sand (Bell, 2017). The ability to survive exposure to oxygen would certainly confer an advantage on BDD treponemes when

colonising the bovine foot skin (Staton et al., 2021a), and survival in bedding or faeces would be key to maintaining an infection reservoir to perpetuate disease in a herd.

1.7 Current perspectives on treatment and control of bovine digital dermatitis

In the early years of disease characterisation, it was noted that most cases recovered without treatment within 7-10 days. Initial treatment suggestions were to clean the lesion and apply topical oxytetracycline and gentian violet spray, which resulted in resolution in 2-3 days (Blowey & Sharp, 1988). However, soon after footbathing with oxytetracycline at 2-4g/litre became the method of choice for many farmers, (Blowey, 1990) and later a higher dose of 6g/litre was suggested for severely affected herds. It was suggested that chronic proliferative lesions responded poorly to treatment compared to the early ulcerative lesions (Blowey et al., 1992).

In the intervening years, there has been a wide variety of prevention and treatment measures applied to BDD, sometimes based on reasonable trial work, and sometimes based on anecdotal evidence, but none have shown real potential for disease eradication as recurrence is common. The treatment options are limited by medicines licensing laws, practical considerations, and the increasing awareness of the need to reduce antimicrobial use (O'Neill, 2015). Current industry advice is based on the use of licensed topical oxytetracycline or thiamphenicol spray (Holzhauer et al., 2017) for acute cases, and the use of disinfectant footbaths as a collective treatment (on a group basis) to reduce shedding of treponemes into the environment from infected cows. It is known that early treatment is important for prevention of transition to the chronic M4 stage, which is responsible for most transmission and perpetuating this endemic disease (Biemans et al., 2018), whilst effective footbathing slows the transition from M4 back to M2 (Döpfer et al., 2012b).

There have been a wide variety of treatment trials carried out using antibiotic and non-antibiotic agents; applied on individual and group bases. Number of treatments applied, and follow-up periods also differ amongst trials, and they often lack a control group. This incongruous approach means that results are difficult to compare and overall, the existing body of evidence for effective treatment remains weak. A recent systematic review of randomised controlled trials concerning collective treatment found only thirteen valid papers, describing 18 prevention and 24 treatment protocols. There was wide variation in disinfectants used, methods of application and frequency of application. The study concluded that only 5% copper sulphate used at least four times weekly was superior to no footbath or water only for treatment of BDD, and there was no published evidence that any agent/ regime was superior to water or nothing for prevention of BDD (Jacobs et al., 2019). Footbaths have the added problem that contamination with organic material may inactivate the active ingredient, and

there is concern about several products regarding environmental contamination and human exposure (Ariza et al., 2019a). For example, it has been recognised that dairy manures contain high levels of zinc and copper, and this has been attributed to the use of heavy metals in footbaths for control of infectious foot diseases (McBride & Spiers, 2007). When these manures are spread on agricultural land, accumulation in soil leads to contamination of surface groundwater (Schipper et al., 2008). Due to these environmental concerns, copper and zinc sulphate are not approved biocides for veterinary hygiene purposes under EU legislation (Biocidal Products Directive 98/8/EC and Biocidal Products Regulation (Regulation (EU) No 528/2012) (Bell & Vanhoudt, 2020). Formalin is approved (37% formaldehyde), however a strict long term exposure limit of 0.3ppm is in place across the EU due to its carcinogenic properties (Bell & Vanhoudt, 2020).

1.7.1 Infection reservoirs

It is an important aspect of disease control that the sources of disease- infection reservoirs- are identified. It is widely considered that in BDD the most important sources of infection are the BDD lesions themselves, particularly M4 lesions. Although there is little difference in transmission rate depending on lesion class, as 70% of the infectious time is spent at M4, this class contributes 88.5% to R_0 (Biemans et al., 2018). Buying in replacement heifers or rearing heifers alongside those from other farms are significant risk factors, showing the importance of practising good external biosecurity (Oliveira et al., 2017; Rodríguez-Lainz et al., 1996; Rodriguez-Lainz et al., 1999; Yang et al., 2018). Inspecting purchased animals for BDD lesions offers little reassurance, since BDD may be subclinical. *Treponema spp.* have been identified in tissue samples from apparently healthy skin using fluorescent in-situ hybridisation (Rasmussen et al., 2012), and using PCR and immunohistochemistry (Bell, 2017).

Increases in BDD cases during housing and under conditions of poor hygiene suggest faeces as a source of treponemes. However, attempts at isolating treponemes or identifying them by PCR assays from environmental slurry samples and individual fresh faecal samples were unsuccessful (Evans et al., 2012a). Initial investigation of 44 sites in the bovine gastrointestinal tract as an infection reservoir found *T. phagedenis*- like BDD spirochaetes in one BDD-affected cow in oral gingival tissue, the rumen dorsal sac and the reticular pillar. The recto-anal junction from a healthy cow was positive for *T. medium*- like BDD spirochaetes. Further testing of oral gingival samples found 1/8 positive for *T. pedis,* and further testing of recto-anal junction samples found BDD spirochaetes in 4/21 samples (Evans et al., 2012a). This suggests possible carriage of pathogenic treponemes, but the lack of detection in faeces casts doubt on the importance of the gastrointestinal tract as an infection reservoir. Metagenomic studies of slurry, however, did identify small numbers of *Treponema* spp. in DD-infected herds and their absence in healthy herds, suggesting that slurry may be a vehicle for spread, but not

the primary infection reservoir (Klitgaard et al., 2017). Certainly, identification of the gastrointestinal tract as an infection reservoir gives a biological basis for many of the well-established links between BDD and poor hygiene (Evans et al., 2016).

Use of a primary hoof trimmer who trims cows' hooves at other farms, and lack of washing of hoof trimming equipment between cows being trimmed, has been associated with increased incidence (>5%) of DD in herds (Wells et al., 1999). A study of pasture-based herds in New Zealand supported these findings, and the authors concluded that farms with BDD should ensure that hoof trimming equipment is disinfected effectively between cattle (Yang et al., 2018). BDD treponeme DNA was detected on 17/17 hoof knives following foot trimming of clinical DD cases, and 7/8 gloves worn by a foot trimmer to trim feet of DD positive cows (Blowey et al., 2013). In addition, an isolate belonging to the *T. phagedenis*-like phylogroup was cultivated from a knife after trimming a DD positive cow (Sullivan et al., 2014) and they can be isolated in culture for up to three days from gloves after they were contaminated during handling of sheep feet affected by the analogous disease contagious ovine DD (Angell et al., 2017). Further investigation of foot trimming as a risk for spread of DD found BDD treponemes on 9/10 gloves worn by a foot trimmer to trim feet of BDD positive cows. BDD treponemes were also found on 1/24 hoof grinder discs, 1/19 hoof grinder handles and 2/20 hoof clipper blades (Bell, 2017). Overall, this data suggests hoof knives and gloves to be major control points for preventing transmission. This survival of viable BDD treponemes, which are typically describes as obligate anaerobes, under aerobic conditions could be very important in disease transmission.

1.7.2 The role of genetics

Traditional approaches to BDD prevention concentrate on controlling infection reservoirs and environmental hygiene; however, it is becoming clear that host genetics has an important role to play in this disease. Heritability of a phenotypic trait is the proportion that is attributable to genetics. Previous studies have estimated heritability of BDD at 0.07 – 0.16 with variation depending on the model type used (Heringstad et al., 2018). Including more detail about lesion types and lesion recurrence rather than classifying cows only as "affected" or "unaffected," increased this estimate to an upper limit of 0.52 (Schöpke et al., 2015). The serological immune response to infection also varies, and some heifers respond with skin proliferation which leads to chronic disease likely to perpetuate infection levels in a herd (Gomez et al., 2014). Four genomic regions have been identified as associated with BDD, harbouring genes involving inflammatory and fibroblastic processes (Sánchez-Molano et al., 2019). It therefore seems the host response to disease, as well as host susceptibility, has a genetic component.

1.8 Novel approaches to treatment

Antimicrobial therapy is the most applied method for treating bacterial infections in cattle, however we are entering an age of developing antimicrobial resistance, where even resistance to new antibiotics introduced to the market develops within ten years. This necessitates advancement of novel approaches to treatment (Kalia et al., 2019), especially in livestock where antibiotic use is becoming increasingly restricted in order to preserve them for human use.

1.8.1 Peptide Nucleic Acids

There are some examples of natural gene control mechanisms in bacteria known as antisense inhibition, or RNA- silencing. These antisense oligonucleotides, defined as being between 8 and 50 base pairs long, bind to RNA via Watson-Crick base pairing, thus modulating RNA function. Natural antisense RNAs typically form 12-30bp with sense mRNA, because binding is often complicated by mismatches and gaps in the structure. Oligonucleotide antisense agents have shown some efficacy against bacterial targets; however, this activity has been improved using synthetic Peptide Nucleic Acids (PNAs), which are oligomers attached to a pseudo-peptide backbone. For these synthetic molecules, size is important because delivery of the molecule is an issue- this may be overcome by conjugating the PNA to a peptide that allows the molecule to cross bacterial cell membranes (Good et al., 2001). These can be designed to exert antimicrobial effects by binding to and silencing RNA, thus inhibiting the translation of target genes important for bacterial survival. The simplest mechanism is to prevent translation by blocking the start codon of sense mRNA using antisense sequences and causing the mRNA to degrade (Good et al., 2001).

1.8.2 Antimicrobial peptides

Antimicrobial peptides (AMPs) are naturally occurring short chain amino acids produced by all living organisms and are part of the host innate immune system. Their small size allows them to penetrate bacterial membranes, causing intracellular leakage. They have potential as adjunctive therapies alongside conventional antimicrobials (Saeed et al., 2022) as they act as a first line of defence against invading pathogens, can modulate the host immune response, and promote wound healing. For example, bovine AMPs cathelicidins have shown promise as an adjunctive therapy for bovine mastitis (Tomasinsig et al., 2010). The whey protein lactoferrin, which is found in milk, yields lactoferrin-derived peptides which have shown synergy with several conventional antimicrobials against the bacteria *Staphylococcus spp.* and *Escherichia coli,* the fungi *Candida spp.,* and the pathogenic amoeba *Entamoeba histolytica* (Bruni et al., 2016).

1.8.3 Biofilm and quorum sensing inhibitors

Bacteria living in biofilms are more resistant to conventional antimicrobials. This can be due to the physical protection afforded by extracellular polymeric substances; matrix components such as alginate and eDNA are known to chelate antibiotics. Biofilm inhibitors aim to break down EPS, improving susceptibility of microorganisms to conventional antimicrobials. Biofilms also have physiological tolerance to antibiotics, firstly because their metabolic activity is low in the inner part of the biofilm, and secondly because many antibiotics do not work well under the hypoxic conditions of the inner layers. This can be solved by addition of stimulating anaerobic growth in these layers, or by physical dispersion of the biofilm. Since most pathogenic bacteria have a propensity to form biofilms, treatment of biofilm infections presents a considerable clinical problem (Bjarnsholt, Ciofu, et al., 2013).

Quorum sensing inhibitors (QSIs) disrupt microbial communication, decreasing or even completely inhibiting the production of virulence factors, including ability to form biofilms. Mechanisms to block QS in Gram negative bacteria focus on disruption to AHLs, such as inhibiting their synthesis, degrading them using lactonases or acylases, modifying their activity using reductases or oxidases, or competing with signal molecules for binding to receptor sites (Kalia et al., 2019; Paluch et al., 2020). QSIs can be of microbial, plant or animal origin, or synthetic. Monoclonal antibodies have also been used successfully to inactivate AHL production, thus inhibiting production of pyocyanin toxin produced by *Pseudomonas aeruginosa* in a mouse model. There have been a large number of preliminary studies testing the potential of QSIs *in vitro* and using *in vivo* mouse models. Field trials in the human health sector have been successful for prevention of biofilms on contact lenses, and for improving lung function in cystic fibrosis patients, however efforts need to be directed to large scale trials for evaluation QSI efficacy in treatment of infectious diseases (Kalia et al., 2019).

1.8.4 Probiotics

It was recognised early in microbiome research that germ-free organisms (such as germ-free mice) were more susceptible to disease, showing that commensal microorganisms are protective against disease. Probiotics are beneficial micro-organisms that are used in the context of restoring a healthy microbiome in order to combat pathogens; either by directly killing them, outcompeting them, or enhancing the immune response against them (Lukic et al., 2017). The World Health Organisation definition of a probiotic is “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host.” *In vitro* and *in vivo* studies have examined *Lactobacilli, Bifidobacteria* and *Staphylococcus epidermidis* as candidates for probiotics that promote wound

healing. Gut probiotics and commensals may modulate skin wound healing via effects on systemic immunity (Lukic et al., 2017).

1.9 Aims and Objectives

1. Improve understanding of the role of foot-trimming in transmission of BDD.
    a. Evaluate survival time of BDD treponemes on hoof knives
    b. Develop an effective hoof knife disinfection protocol and trial it under field conditions

2. Measure the changes in foot trimming hygiene practices that have occurred in industry as a result of this research.

3. Advance understanding of early BDD aetiology.
    a. Use co-occurrence analysis to describe the characteristics of bacterial ecological networks associated with susceptibility development of BDD lesions.
    b. Use shotgun metagenomics to elucidate functional pathways via which protective and detrimental bacteria may be acting.

4. Advance understanding of BDD treponeme pathogenesis.
    a. Develop a microtiter plate model for use in studies of single and multi-species BDD treponeme biofilms.
    b. Use transcriptomics to determine candidate genes responsible for formation of biofilms.
    c. Use transcriptomics to identify candidate genes involved in bacterial communication and synergy between species, both in planktonic and biofilm-form.
    d. Use transcriptomics to study the potential role of resistance to oxidative stress in BDD treponeme pathogenesis.

5. Evaluate conventional and novel antimicrobial treatments for BDD *in vitro.*
    a. Design and test novel PNA-peptides against BDD-associated treponemes for minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs).
    b. Collect data on *in vitro* sensitivity of BDD treponemes to previously untested conventional antimicrobials.
    c. Collect data on *in vitro* sensitivity of BDD treponemes to naturally occurring antibacterial compounds and antibacterial metals.

Chapter 2 General materials and methods

Table 2.1 Reagents

Solution	Preparation
1X phosphate buffered saline (PBS)	Prepared using commercially available Dulbecco's Phosphate Buffered Saline, (Oxoid, Basingstoke, UK). Each tablet diluted with 100 ml ddH_2O to make a working concentration of 1X PBS and autoclaved before use.
Agarose 1.0% (w/v)	1g of agarose powder (Biorad, Hemel Hempstead, UK) was added to 100ml 1X TAE buffer.
Chelex-100 resin 5% (w/v)	5g of Chelex-100 resin (BioRad, Hemel Hempstead, UK) was dissolved in 10ml of ddH_2O.
Enrofloxacin	50 mg of Enrofloxacin powder (Sigma-Aldrich, Dorset, UK) added to 5 ml of 1M potassium hydroxide (KOH) (Sigma-Aldrich, Dorset, UK) and balanced with equal 1M HCl (Sigma-Aldrich, Dorset, UK). Resulting 5mg/ml solution was filtered using a 200nm syringe filter (Minsart, Sartorius, Goettingen, Germany) and stored at 4°C in 1 ml aliquots.
Foetal calf serum (FCS) 10% (v/v)	10% (v/v) FCS (Gibco, Paisley, UK) was heated inactivated at 56°C for 30 minutes in a water bath, frozen at -20°C in 20ml aliquots, and defrosted and filtered using a 450nm filter (Minsart, Sartorius, Goettingen Germany) prior to use.
Glycerol	Glycerol (BDH, Dorset, UK) was used in 1ml aliquots for storage of treponemes at -80°C in 2ml screw top tubes. Glycerol was autoclaved prior to use.
Rifampicin	50mg of Rifampicin powder (Sigma-Aldrich, Dorset, UK) was dissolved in 10ml of 1M methanol (Sigma-Aldrich, Dorset, UK). The resulting 5mg/ml stock solution was filtered using a 200nm syringe filter (Minsart, Sartorius, Goettingen Germany) and stored at -20°C in 1 ml aliquots.
TAE (1X) electrophoresis buffer	100ml of TAE (40X) (molecular grade) (Promega, UK) was added to 3900ml of ddH_2O to give a working solution of 1X TAE.
Rabbit serum (RS) 10% (v/v)	RS (GE Healthcare Life Sciences, Buckinghamshire, UK) was heat inactivated in a 56°C water bath for 30 minutes, frozen at -20°C in 20 ml aliquots, and defrosted and filtered using a 450nm filter (Minsart, Sartorius, Goettingen, Germany) prior to use.

Ethidium bromide (1%)	Commercially available Ethidium bromide 1% (w/v) solution (Fisher Bioreagents, Fisher Scientific, UK).
Oral Treponeme Enrichment Broth (OTEB)	Commercially available liquid growth medium supplied in 7ml glass tubes (Anaerobe Systems, CA, USA).
Crystal violet	1% (w/v) crystal violet aqueous solution (Sigma-Aldrich, Dorset, UK) was diluted using distilled water to either 0.1% or 0.05% and stored at room temperature in 20ml aliquots.
Acetic acid	99.9% acetic acid (Sigma-Aldrich, Dorset, UK) was diluted using distilled water to 30% and stored in 20ml aliquots at room temperature.

2.1 Preparation of standard treponeme cultures in liquid media

All BDD treponemes used in this project originated from freezer stocks (-80^{o}C) of cultures stored in 10% glycerol, which were originally recovered in pure culture from field cases of BDD in the UK (Evans et al., 2008). The human strain *Treponema phagedenis* subsp. Reiter originated from the human genito-urinary tract (WHO, 1967) and was a kind gift from Professors H. Kuramitsu / C. Cameron, University of Victoria, Canada. Periodically throughout the project relevant cultures were aliquoted into autoclaved 10% glycerol to replenish stocks.

Treponemes were grown in tubes containing liquid media consisting of commercially available oral treponeme enrichment broth (OTEB, Anaerobe Systems, USA) supplemented with 10% foetal calf serum (FCS) or 10% rabbit serum (RS) in an anaerobic cabinet (Don Whitley Scientific, UK) (85% N_2, 10% H_2 and 5% CO_2, 36^{o}C). The OTEB was supplemented with 700µL of RS for growth of *Treponema medium/ vincentii*-like strains (group 1), and 700µL of FCS for growth of *Treponema phagedenis*-like (group 2) or *Treponema pedis* (group 3) strains. Serum was heat-inactivated for half an hour and filtered using 0.45µm syringe filters before use. For the purposes of the following projects, pure cultures of strains T19, T320A and T3552B will be used to represent groups 1, 2 and 3 respectively.

Plugged disposable 150mm sterile glass Pasteur pipettes (Volac^{TM}, Fisher Scientific, UK) were used to inoculate the appropriate medium with the strain of interest. For initial inoculation from freezer stocks, 15 drops (30µl per drop) were added to the appropriate medium. Treponemes typically take 4-9 days to grow to late exponential phase depending on the strain. To facilitate weekly inoculation, nine drops of pure culture was used for T19, three drops for T320A and five drops for T3552B. Following inoculation, tube caps were untwisted by half a turn to allow some gaseous transfer. Cultures could be used for experiments after a minimum of two passages.

Cultures were visualised weekly by phase contrast microscopy using a Diaplan Vario Orthomat-2 microscope (Leitz, Wetzlar, Germany) to assess the presence of treponemes, which are deemed viable if they were motile. The treponemes grow at the bottom of the tubes containing the liquid medium, necessitating mixing before withdrawal for this analysis. This was done using the sterile glass Pasteur pipettes to withdraw and expel approximately 1ml of the mixture three times to disperse the bacteria more equally. Cultures could be used for experiments if growth was considered good: at least three for T19 or four for other strains as described in Table 2.2.

Table 2.2 Description of treponeme growth and motility scores as determined by phase contrast microscopy (Bell, 2017)

Description		
Score	**Growth**	**Motility**
0	No treponemes	No movement, fixed to bottom of slide
1	Only a few treponemes visible (~1-10)	Brownian motion
2	>10 treponemes visible	<50% of treponemes motile
3	~50% of slide covered with treponemes	50% of treponemes motile
4	~75% of slide covered with treponemes	75% of treponemes motile
5	Dense growth of treponemes with almost 100% of slide covered	100% of treponemes motile

2.2 Spectrophotometry

Before use in all experiments, ODs were measured using a spectrophotometer (Multiskan®EX, Thermo Fisher Scientific, Massachusetts, USA) to calculate and subsequently adjust the number of bacterial cells present for inoculation. Table 2.3 shows the standard ODs at 540nm used and the corresponding bacterial cell numbers as previously determined using a Petroff-Hausser counting chamber (Evans et al., 2009c). Where necessary cultures were diluted to the standard ODs using liquid culture medium.

Table 2.3 Standard ODs for three strains of treponeme and the corresponding numbers of organisms per ml determined using a Petroff-Hauser counting chamber (Evans et al., 2009c)**.**

Phylogroup	**Optical density**	**Organisms/ ml**
T. medium (T19)	0.25	8.75×10^7
T. phagedenis (T320A, Reiter)	0.43	1.14×10^8
T. pedis (T3552B)	0.37	2.69×10^8

2.3 Preparation of field samples in liquid media

For collection of field samples, 2ml screw top tubes were filled with 1.5ml of liquid medium inside an anaerobic cabinet and the lids tightened. Each field sample had two corresponding tubes- one containing 10% OTEB FCS and one containing 10% OTEB RS. During field sampling, the tips of cotton swabs were removed using scissors, allowing them to drop immediately into liquid media. Lids were tightened and samples were transported at ambient temperature and transferred to an anaerobic cabinet. The antibiotics rifampicin and enrofloxacin were added to reach a final concentration of 5µg/ml and 1µg/ml respectively, in order to supress growth of contaminants, and lids were loosened half a turn to allow gaseous transfer.

2.4 DNA Extraction

For extraction of genomic DNA from laboratory cultures, 1.5ml of culture was placed in a sterile lock tube and centrifuged at 13,500g for two minutes (Prism microcentrifuge, Labnet International, Edison, USA). The supernatant was discarded, and the pellet re-suspended in 700µL of culture, and 250µL of 10% Chelex resin was added (Bio-Rad, Hemel Hempstead, UK). For extraction of genomic DNA from field cultures at six weeks post-inoculation, 750µL of culture was added to 250µL of 10% Chelex resin. In all cases suspensions were boiled for fifteen minutes, then centrifuged at 13,000g for ten minutes (Chua et al., 2005). Resulting supernatants were stored at -20^{o}C until needed for downstream testing.

Genomic DNA was extracted from cotton swabs using a DNeasy® Blood & Tissue Kit (Qiagen, UK) according to manufacturer's instructions and samples were stored at -20^{o}C for testing by nested PCR. Briefly, working inside a laminar flow cabinet, each swab tip was removed with a sterile scalpel blade in a petri dish and a proportion weighing less than 25mg was placed in a 1.5ml autoclaved microcentrifuge tube. Tubes were placed in a rocking incubator at 37^{o}C overnight after addition of 180µl Buffer ATL and 20µL proteinase K. The next day samples were vortexed and 200µl of Buffer AL and 200µl of molecular grade ethanol added (Sigma-Aldrich, Dorset, UK) before vortexing again. Supernatants were pipetted into DNeasy spin columns in 2ml collection tubes and centrifuged at 8000g for 1 minute. Spin columns were placed in new 2ml collection tubes and 500µl of Buffer AW1 was added. Samples were centrifuged at 8,000g for 1.5 minutes and columns placed in new collection tubes. 500µl Buffer AW2 was added, and samples were centrifuged at 20,000g for 3.5 minutes. The flow-through in the collection tubes was discarded and the samples centrifuged again at 20,000g for one minute to ensure all ethanol contamination had been removed from the spin column membranes. DNA was then eluted into 1.5ml microcentrifuge tubes using 100µl Buffer AE applied to the membranes and incubated at room temperature for 1 minute prior to loading into the centrifuge.

2.5 Polymerase Chain Reaction (PCR)

There are five PCR assays that are relevant to this thesis: universal amplification of the 16S rRNA gene which is used as the initial step in nested PCR protocols, the *Treponema* spp. genus specific assay which indicates the presence of treponemes but not necessarily those known to be pathogenic in BDD, and amplification reactions specific for 16S rRNA of each of the three groups/species of considered pathogenic BDD treponemes. Reaction mixes included 1µl of template DNA with 4.0µl Firepol® Taq (Solis Biodyne, Estonia), 13.8µl molecular grade double-distilled water (ddH_2O) and 0.6µl of both forward and reverse primers (Eurofins Genomics, UK) to make a final volume of 20µl. The nested PCR step for *Treponema* spp. used a different reaction mix: 4.0µl Firepol® Taq (Solis Biodyne, Estonia), 14.36µl ddH_2O and 0.32µl of both forward and reverse primers. Primers and reaction conditions are shown in tables 2.4 and 2.5 respectively. All reactions were carried out in triplicate and were validated using ddH_2O as a negative control, and positive controls relevant to the reaction. Specifically, these were genomic DNA of *T. denticola* for the *Treponema* spp. reaction, and T19, T320A and T3552B cultures for group one, two and three reactions respectively.

Table 2.4 Primers for standard PCRs used in this thesis

Reaction	Forward Primer	Reverse Primer	Predicted band size (bp)	Source
Universal 16S rRNA gene	(5'-AGAGTTTGATCCTGG-3')	(5'-TACCTTGTTACGACTT-3')	1,526	(Rurangirwa et al., 1999)
***Treponema* spp.**	(5'-AARCATGCAAGTCGARCG GCAAG-3')	(5'-TCCATTGCGGAATATTCTTA-3')	335	(Moore et al., 2005)
T. medium/T. vincentii-like	(5'-GAATGCTCA TCTGATGACGGTAATCGA CG-3')	(5'-CCGGCCTTAT CTAAGACCTTCTACTAG-3')	475	(Evans, et al., 2009b)
***T. phagedenis*-like**	(5'-GAAATACTCAAGCTTAAC TTGAGAATT GC-3')	(5'-CTACGCTACCATATCTCTA TAATATTGC-3')	400	(Evans et al., 2009b)
T. pedis	(5'-GGAGATGAG GGAATGCGTCTTCGATG-3')	(5'-CAAGAGTCGTATTGCTACG CTGATATATC -3')	475	(Evans et al., 2009b)

Table 2.5 Reaction conditions for five PCRs used throughout this thesis

	Universal 16S rRNA		***Treponema* spp.** (Moore et al., 2005)		***T. medium*/*T. vincentii*-like** (Evans et al., 2009b)		***T. phagedenis*- like** (Evans et al., 2009b)		***T. pedis*** (Evans et al., 2009b)	
	Temp(°C)	**Time**	**Temp(°C)**	**Time**	**Temp(°C)**	**Time**	**Temp(°C)**	**Time**	**Temp(°C)**	**Time**
Initial denaturation	95	5			95	5mins	95	5mins	95	5mins
Denaturation	94	1	95	15s	95	1min	95	1min	95	1min
Annealing	55	3	53	30s	68	2min	64	1min	68	30s
Extension	72	3	72	30s	72	2mins	72	2mins	72	2mins
	25 cycles		34 cycles		40 cycles		40 cycles		40 cycles	
	72	7	72	5mins	72	10mins	72	10mins	72	10mins

2.6 Agarose Gel Electrophoresis

PCR products were visualised after electrophoresis in 1% Agarose (Biorad, Hemel Hempstead, UK). Gels were supplemented with 0.5mg/ml ethidium bromide during preparation and placed within their gel cassettes into TAE (1X) electrophoresis solution in electrophoresis tanks (Geneflow Ltd, Staffordshire, UK). Electric current was supplied by Biorad Powerpac 300 (Biorad, Hemel Hempstead, UK) at 120V, 400mA for 45 minutes. Following electrophoresis, gels were transferred to a UV Illuminator, and images were visualised and captured using the Syngene Ingenius 3 gel documentation instrument and GeneSys software (Syngene, Cambridge, UK). 100bp and 1kb DNA ladders were used to compare and visualise the size of DNA samples (Promega, Wisconsin, USA).

2.7 Preparation of microtiter plates for development of a static biofilm model for *Treponema* spp.

All experiments used 96-well flat-bottomed polystyrene microtiter plates (Thermo Fisher Scientific, Loughborough, UK).

2.7.1 Microtiter plate coatings

A range of coatings were used to encourage biofilm formation. Four serum coatings were trialled: 100% FCS, 10% FCS, 100% RS and 10% RS. Serum was diluted with sterile PBS where appropriate. Five bovine fibrinogen concentrations were trialled. Bovine fibrinogen was prepared by weighing 1mg/ml Fibrinogen from Bovine Plasma (Sigma-Aldrich, Dorset, UK) into PBS pre-heated to 37^{o}C. The mixture was rocked slowly in an incubator at 37^{o}C for one hour to dissolve the fibrinogen. The final concentration was checked by using a NanoDrop™ ND-2000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK), according to the manufacturer's instructions. The spectrophotometer was blanked against 1µl RNase-free water and fibrinogen concentration was subsequently assessed from a single 1µl aliquot. Bovine fibrinogen was further diluted using sterile PBS to reach the following working concentrations: 250µg/ml, 50 µg/ml, 10 µg/ml, 5 µg/ml and 1 µg/ml. All experiments included columns coated using PBS only and uncoated columns to act as controls.

Coatings were added to plates at 50µl per well and incubated for one hour at 37^{o}C. Plates were stored at 4^{o}C overnight in humid containers (clear sealed polythene bags containing 4ml of distilled water) and washed three times before use using 100µl PBS per well each time.

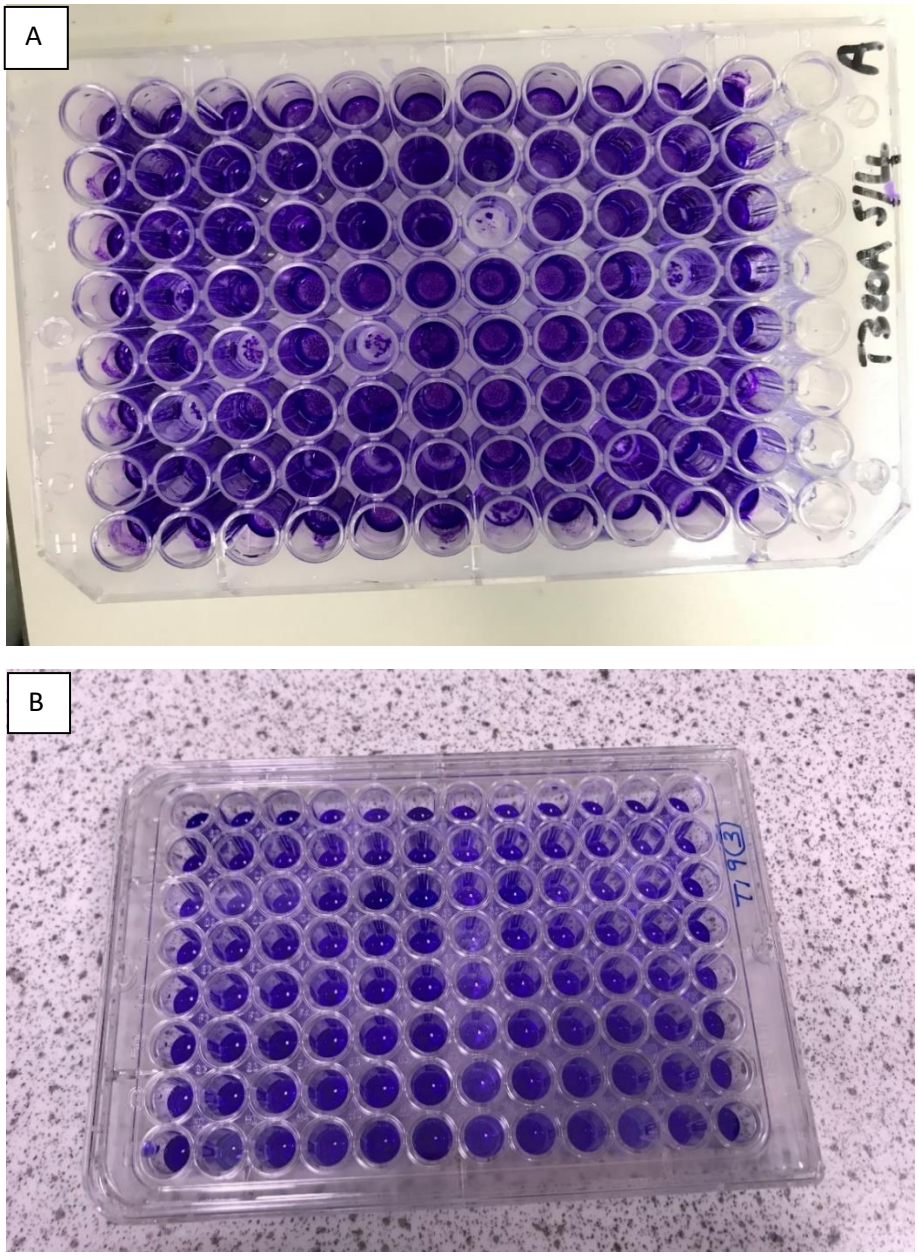
2.7.2 Loading of microtiter plates

All OTEB, serum, petri dishes and microplates were placed in an anaerobic cabinet one hour prior to use. Cultures were used at 7 days from their previous passage, unless otherwise required by the experiment. ODs of cultures were measured using the spectrophotometer with liquid medium as controls (2.2). Wells were filled using a multichannel pipette to dispense 150µl of liquid growth medium into each well. The medium used was OTEB + FCS prepared as described in 2.1, except for plates where only T19 was to be inoculated, in which case OTEB + RS was used. Control rows received a further 50µl of medium, whilst test rows received 50µl of the relevant treponeme culture. Microplates were removed from the anaerobic cabinet after inoculation and OD_{540nm} measured using the spectrophotometer (Multiskan EX, Thermo Fisher Scientific, USA). Once returned to the anaerobic cabinet, microplates were incubated inside polythene bags containing 5ml ddH_2O to maintain humidity and prevent evaporation from wells for two or seven days according to the experimental protocol.

2.7.3 Crystal violet assay

At the end of the incubation period, 165µl of liquid was aspirated from all wells and plates left to dry for one hour. 35µl of methanol was added per well and left to fix material for half an hour. Crystal violet stain was prepared by adding 2ml of liquid 1% crystal violet (Sigma-Aldrich, Dorset, UK) to 18ml of distilled water. Plates were then removed from the anaerobic cabinet and 150µl per well of the resulting 0.1% crystal violet stain added. For later experiments, a 0.05% crystal violet stain was used. After 20 minutes, all wells were washed three times using PBS from a wash bottle (Figure 1A). Plates were left to air dry for a few hours before adding 150µl of 30% acetic acid (Fisher scientific, UK) to solubilise the crystal violet. After 15 minutes, 125µl of the resulting coloured solution was transferred to new microtiter plates after mixing using a multichannel pipette to ensure the colour was homogenous (Figure 1B). ODs were read at 540nm on a spectrophotometer (Multiskan EX, Thermo Fisher Scientific, USA) (O'Toole, 2011).

Figure 1 A) Microtiter plate following staining with 0.1% crystal violet and washing three times with PBS from a wash bottle. B) Microtiter plate following solubilisation of crystal violet using 30% acetic acid



2.8 Ethical Approval

Ethical approval was sought for the hoof knife disinfection field trial (Chapter 3) Ref: VREC 662.

Ethical approval was also sought for both foot-trimming hygiene questionnaires (Chapter 4) Ref: VREC 786 and 786a.

Both applications were approved by the University of Liverpool veterinary research ethics committee.

Chapter 3 Survival of bovine digital dermatitis treponemes on hoof knives and their disinfection

3.1 Introduction

Herd-level risk factors associated with the presence of BDD include larger herd size (>100 cows), no access to pasture, calving at a site separate to the main farm, foot-trimmer working on other farms, and lack of washing of hoof trimming equipment (with water) between cows (Wells et al., 1999). There is evidence that poor cow comfort may increase BDD cases, but the reasons and mechanisms for this have not yet been identified (Somers et al., 2005). Support for housing, nutrition and environmental factors playing a part is provided by the fact that dry cows have fewer cases of BDD (Somers et al., 2005).

A substantial collective of literature has resulted in industry recommendations to concentrate on improving environmental hygiene to control BDD. The existing paradigm is that infection is spread predominantly from M2 and M4 lesions via the environment; however, treponemes have not been isolated in culture from environmental sources or detected by PCR (Evans et al., 2012a). Metagenomic studies of slurry, however, did identify small numbers of *Treponema* spp. in BDD infected herds and their absence in healthy herds, suggesting that slurry may be a vehicle for spread, but not the primary infection reservoir (Klitgaard et al., 2017). Hence, questions remain about the importance of slurry and the environment in transmission. Current industry advice regarding better slurry management may be beneficial due to reducing the susceptibility of skin to invasion by BDD, and effective footbathing protocols have generally proved useful in reducing case numbers; however, there are additional infection reservoirs such as hoof knives which may be key to BDD eradication initiatives.

An epidemiological study published in 1999 associated the use of a primary hoof trimmer who trims cows' hooves at other farms, and lack of washing of hoof trimming equipment between cows being trimmed, with increased incidence (>5%) of BDD in herds (Wells et al., 1999). A 2018 study of pasture-based herds in New Zealand supported these findings, and the authors concluded that farms with BDD should ensure that hoof trimming equipment is disinfected effectively between cattle (Yang et al., 2018). An analysis of external biosecurity factors affecting BDD prevalence on Danish dairy farms also found that use of external foot-trimmers raised the odds for increased BDD prevalence (OR 1.20), however this was compared to foot-trimming carried out by both a trained operator from the farm and a professional foot-trimmer. Use of a trained farm person alone was more detrimental (OR 1.40)

and may reflect the positive effects of a good hoof care regime with follow-up of BDD cases where professional trimmers are employed (Oliveira et al., 2017).

Microbiological studies have detected BDD treponeme DNA on 17/17 hoof knives following foot trimming of clinical BDD cases, and 7/8 gloves worn by a foot trimmer to trim feet of BDD positive cows (Blowey et al., 2013; Sullivan et al., 2014). In addition, an isolate belonging to the *T. phagedenis*-like spirochaetes was cultivated from a knife after trimming a BDD positive cow (Sullivan et al., 2014) and they can be isolated in culture for up to three days from gloves contaminated during handling of sheep feet affected by the analogous disease contagious ovine DD (CODD) (Angell et al., 2017). BDD-associated *Treponema* have also been identified on hoof-trimming equipment using 16S rRNA gene sequencing (Rock et al., 2015). The results from these studies may explain the epidemiological findings that associate poorer foot-trimming biosecurity with increased BDD prevalence. With dairy farm growth and amalgamation across the last fifty years, use of external foot-trimmers operating high-throughput systems where large numbers of cows are trimmed in succession has increased. This has raised concerns that hoof knives and gloves may be acting as fomites for BDD transmission as they are quickly transited from foot-to-foot. It is not known, however, whether *Treponema* detected by PCR or 16S rRNA sequencing corresponds to the presence of viable infectious organisms (Sullivan et al., 2014), and if they are viable, whether they remain at sufficient numbers to constitute an infectious dose. Survival time of BDD treponemes on hoof knife blades in aerobic conditions is also relevant to their ability to transmit between cows' feet via this route.

Disinfection of hoof knives between animals is not always carried out by farmers, foot-trimmers and veterinary surgeons, and there is currently no validated practical disinfection regime. Efficacy of disinfectants against BDD-associated treponemes under field conditions is unknown and may be complicated by the presence of manure contamination. EU Regulation 528/2012 specifies biocides must be effective against *Enterococcus hirae, Proteus vulgaris, Pseudomonas aeruginosa* and *Staphylococcus aureus* at contamination levels of 20g/L organic matter (Ariza et al., 2019a). Laboratory work testing disinfectants against a *Treponema phagedenis*-like spirochaete to determine minimum inhibitory concentrations and minimum bactericidal concentrations showed a range of disinfectants to be effective even in the presence of 20% manure (Hartshorn et al., 2013). It could therefore be expected that common disinfectants available in the farming industry would achieve effective concentrations for eliminating treponemes in a practical on-farm setting.

The work presented here uses prepared treponeme cultures inoculated onto hoof knife blades in two experiments. The first experiment tested survival times of treponemes on hoof knife blades under aerobic conditions. The second experiment was a disinfection study which tested a range of common

disinfectants at working concentrations for removing viable treponemes from hoof knife blades. Disinfectants deemed successful *in vitro* are tested under field conditions during foot-trimming of cows affected by BDD.

3.2 Methods

3.2.1 *In vitro* experiments

3.2.1.1 Treponeme culture preparation and inoculation onto hoof knives

These studies used two strains of BDD-associated treponeme bacteria: T320A, belonging to the *Treponema phagedenis* group, and T3552B belonging to the *Treponema pedis* group. Both were previously identified as associated with digital dermatitis lesions (Evans et al., 2008). Organisms were retrieved from our culture collection (originally isolated from active BDD cases) and inoculated into tubes containing liquid medium (Oral Treponeme Enrichment Broth, OTEB, Anaerobe Systems USA) with 10% heat-inactivated foetal calf serum (FCS, Thermo Fisher Scientific, USA). Cultures were grown, maintained and prepared for use according to standard practices (Chapter 2.1 and 2.2). 2.5ml of the prepared cultures for each replicate were transferred to sterile Eppendorf tubes using plugged disposable 150mm sterile glass Pasteur pipettes (Volac™, Fisher Scientific, UK). These were centrifuged for 5 minutes at 5000*g* and the supernatant discarded so that 0.5ml of liquid culture remained. Glass pipettes were used to re-suspend the pellet containing the bacteria, and 0.5ml cultures were applied to one side of each hoof knife blade, measured using 2ml serological pipettes (VWR, UK) (Illustrated in Figure 1).

Figure 1 Hoof knife with prepared treponeme culture applied to blade

3.2.1.2 Treponeme survival on hoof knife blades

Cotton swabs (Copan Italia, Italy) were used to take suspension samples two minutes after cultures were placed on hoof knife blades. These were placed into liquid culture medium (OTEB with FCS) to serve as positive controls. After a series of specified waiting times, two further swabs were taken, the first for inoculation into liquid culture medium and the second for direct detection of treponemes using nested PCR assays. Waiting times were 10 minutes, 1 hour, 2 hours, 4 hours and 18 hours. These times were chosen to represent the risk of transmitting treponemes to the next cow during a foot trimming visit, between farms visited on the same day, or between farms visited on consecutive days. Three replicates were carried out for each waiting time for each of the two treponeme strains. Tubes containing swabs in liquid medium were transferred to an anaerobic cabinet as soon as possible and swabs for direct PCR were stored at -20^{o}C.

3.2.1.3 Disinfection of hoof knife blades

Positive control swabs of blades were taken after two minutes treponeme contact time and placed in liquid culture medium as for the survival study. Hoof knife blades were then disinfected for 20 seconds by full immersion dipping in one of the following disinfectants: 2% (w/v) Virkon® (Dupont, Wilmington, USA), 2% (v/v) sodium hypochlorite, 2%(v/v) glutaraldehyde, 5% (w/v) copper sulphate, or 1%(v/v) FAM30® (Evans Vanodine, Preston, UK) (Illustrated in Figure 2). Since all these chemicals are diluted in water, the experiments were also carried out using water only for comparison. These disinfectants represent those commonly used on dairy farms. The short contact time was chosen to test efficacy under a condition that would minimise disruption caused by disinfection use during foot-trimming visits. As in the survival study, two swabs were taken post-disinfection: one for bacterial culture and one for nested PCR assays. Tubes containing swabs in liquid medium were transferred to an anaerobic cabinet as soon as possible and swabs for direct PCR were stored at -20^{o}C. For each different disinfectant and strain, treponemes were inoculated on to at least 15 different knives across a minimum of three different days.

Figure 2 Hoof knife in copper sulphate disinfectant

3.2.1.4 Phase contrast microscopy

For both studies, all cultures were examined weekly for six weeks using phase contrast microscopy to determine presence or absence of treponeme growth as described in Chapter 2.1. Cultures were considered positive for treponeme growth if at least ten motile treponemes were visible per field of view. Replicates that did not meet these criteria in the positive control culture by week six were discarded.

3.2.1.5 DNA extraction

Genomic DNA was extracted from cotton swabs using a DNeasy® minikit (Qiagen, UK) according to manufacturer's instructions and samples were stored at -20°C for testing by nested PCR, as described in Chapter 2.5. Extraction of genomic DNA from cultures was carried out as described in Chapter 2.4. Briefly, 1.5ml of culture was placed in a sterile lock tube and centrifuged at 13,500*g* for two minutes. The supernatant was discarded, and the pellet re-suspended in 700µL of culture, and 250µL of 10% Chelex resin was added (Bio-Rad, Hemel Hempstead, UK). The suspension was boiled for fifteen minutes, then centrifuged at 13,500*g* for ten minutes (Chua et al., 2005). Resulting supernatants were stored at -20°C until testing by nested PCR.

3.2.1.6 PCR assays

PCR assays specific for each respective culturable BDD treponeme phylogroup were carried out as previously described (Evans et al., 2009b) (Chapter 2.5). Briefly, reaction mixtures used Firepol® Taq (Solis Biodyne, Estonia) according to manufacturer's instructions. Reaction conditions were as previously described, with an initial step using universal 16SrRNA gene bacterial primers, followed by a phylogroup-specific nested PCR step, resulting in products which are 300-500bp elements of the 16S rRNA gene. PCR products were visualised after electrophoresis through 1% Agarose (Biorad, Hemel Hempstead, UK) and stained with 0.5mg/ml ethidium bromide (Chapter 2.6). All reactions were carried out in triplicate and were validated using positive and negative controls as previously described (Evans et al., 2009b) (Chapter 2.6).

3.2.2 Field Study

3.2.2.1 Sample collection for field study

The study included lactating dairy cattle during routine foot-trimming in three Holstein-Friesian commercial dairy farms sampled on various dates from July 2018 to October 2019. Farm 1 was milking approximately 200 cows (Cheshire, UK), Farm 2 approximately 280 milking cows (Cheshire, UK), and Farm 3 approximately 260 cows (Gloucestershire, UK). All cows were housed in cubicles and all herds were largely closed (rarely buying in animals). The case definition was any foot showing visible BDD lesions of any pathological classification (M1, M2, M3, M4 or M4.1) (Berry et al., 2012; Döpfer et al., 1997) or non-healing claw-horn disruption lesions (NH) that have been previously associated with BDD treponemes (Scholey et al., 2012; Sykora et al., 2015). Swab samples were taken during foot-trimming if a foot fitted the case definition. The hoof knives used belonged to the foot-trimmers who were participating in the study (Aesculap VC316V or VC300/ VC305, Neogen, USA). All foot-trimming was carried out according to the foot-trimmer's normal protocol. Initial studies were made where the knife blades either did or did not come into contact with BDD lesions. However, swabs of blades which did not make lesion contact had a very low culture rate of pathogenic treponemes meaning that efficacy of disinfectants could not be assessed. Consequently, the approach was revised so that in subsequent studies only cases where hoof knife contact had been made with a BDD lesion for treatment purposes were included.

At the beginning of each sample collection session and after each foot during foot-trimming, hoof knives were cleaned using washing-up detergent in water, then immersed in 70% ethanol for a minimum of ten minutes and air-dried prior to use. Plain cotton swabs (Copan, Italy) were passed back and forth three times over the whole length of the front and back of hoof knife blades to serve as pre-

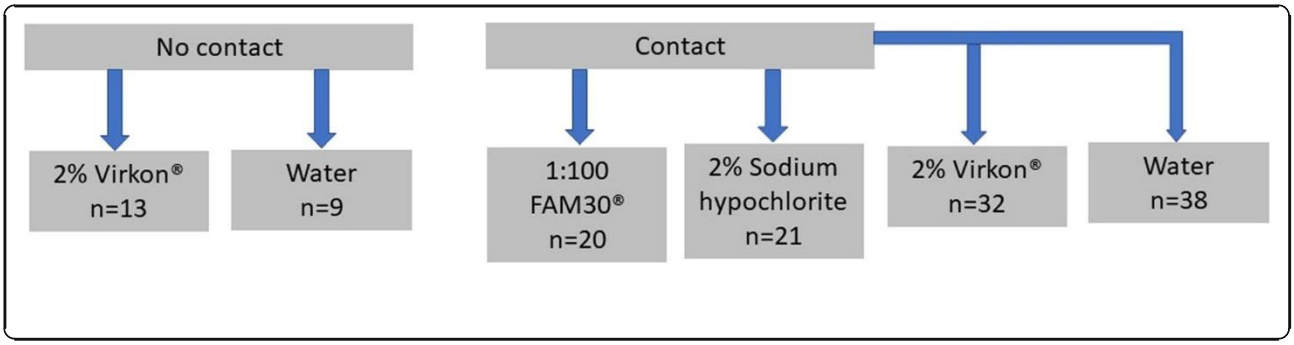
trimming control samples. Three swabs were taken per blade: one for inoculation into Oral Treponeme Enrichment Broth (OTEB, Anaerobe systems, USA) containing 10% heat-inactivated Foetal Calf Serum (FCS, Thermo Fisher Scientfic, USA), one for inoculation into OTEB containing 10% heat- inactivated Rabbit Serum (RS, Firstlink, UK) and one for direct testing by nested PCR without prior culture. Swab samples from the knives were taken again once foot-trimming of each foot was completed. Knives were rinsed briefly in water (three seconds) to remove gross contamination before immersing the blades in one of three disinfectants (2% Virkon®, 2% sodium hypochlorite, 1:100 FAM30®) or water (as a comparison) for 20 seconds. They were shaken dry, and three swab samples taken for a third time. Figure 3 illustrates a typical hoof knife after foot-trimming, after cleaning, and during disinfection.

Figure 3 Hoof knife before and after cleaning, and during disinfection

The number of blades disinfected with each agent, according to whether contact was made with the BDD lesion is shown in Figure 4. The culture protocol was designed to favour growth of the three cultivable BDD treponeme phylogroups so that the effect of disinfection could be evaluated. Liquid medium containing FCS favours the growth of *T. phagedenis*- like and *T. pedis* strains, whilst liquid medium containing RS favours growth of *T. medium/ vincentii*- like strains.

Figure 4 Experimental design of disinfection study.

Showing the number of samples cleaned using each agent according to whether or not contact was made between the hoof knife blade and the BDD lesion.



3.2.2.2 Field sample processing

On farm knife swabs for culture were immediately inoculated into their designated medium (OTEB) in 2ml screw top tubes (Chapter 2.3) and transported at ambient temperature. On return to the laboratory, they were placed in an anaerobic cabinet (Don Whitley Scientific, UK) (85% N_2, 10% H_2 and 5% CO_2, 36°C) and rifampicin and enrofloxacin added to a final concentration of 5µg/ml and 1µg/ml respectively to supress growth of contaminants. After six weeks in culture, bacterial genomic DNA (gDNA) was extracted from cultures by using a Chelex resin (Biorad, USA) protocol according to manufacturer's instructions (Chapter 2.4). Resulting gDNA-containing supernatant was frozen at -20°C.

Swabs for direct nested PCR analyses were placed on ice for transport and gDNA subsequently extracted using a DNeasy® minikit (Qiagen, UK) according to manufacturer's instructions and stored at -20°C (Chapter 2.4).

3.2.2.3 PCR assays for field samples

Detection of *Treponema* was undertaken via two methods: nested PCR of sample swabs taken directly from the hoof knife blades, and nested PCR of DNA extracted from cultures given six weeks to grow. The former method is very sensitive for detecting *Treponema* DNA and the latter provides a measure of treponemal viability determined by an ability to grow in liquid culture medium.

All gDNA samples were subjected to nested PCR assays to a) detect the *Treponema* genus, and to b) specifically detect each of the three cultivable BDD treponeme phylogroups (Chapter 2.5). PCR products were visualised after electrophoresis through 1% Agarose (Biorad, Hemel Hempstead, UK) and stained with 0.5mg/ml ethidium bromide (Chapter 2.6). All reactions were carried out in triplicate

and were validated using positive and negative controls as previously described (Evans et al., 2009b) (Chapter 2.5). Samples were considered positive if they tested positive to at least one of the cultivable phylogroup specific PCR assays. Samples which tested positive only to the *Treponema* genus PCR assay were deemed to contain non BDD associated treponemes.

3.2.2.4 Statistical analyses

Univariable logistic regression was performed using STATA v14 (Statacorp, USA) to test whether contact between the hoof knife blade and the lesion, or the lesion stage, explained the outcome of post-trimming cultures. Each disinfectant used was tested as an explanatory variable for the outcome of detection of BDD-associated phylogroup and/ or Treponema genus DNA post-disinfection.

3.3 Results

3.3.1 *In vitro* experiments

3.3.1.1 Treponeme Survival on hoof knife blades

It was consistently possible to culture both strains of treponeme from hoof knives for up to two hours post inoculation (PI) (2/3 replicates were positive using T320A and 3/3 replicates were positive using T3552B). Treponeme growth was visible by phase contrast microscopy after one week, and in all cases nested PCR carried out on genomic DNA extracted from these cultures confirmed microscopy findings. After four hours PI, treponemes could not be detected in culture, either by weekly phase contrast microscopy or by PCR testing of cultures after six weeks. All samples remained positive by direct PCR testing of swabs for the full 18 hours PI for both strains of treponeme (Table 3.1).

Table 3.1 Results showing BDD treponemes can be cultured from hoof knife blades at 2 hours PI, and detected by PCR for at least 18 hours PI

Treponeme strain (phylogroup)	**T320A** (*Treponema phagedenis*)			**T3552B** (*Treponema pedis)*		
Survival time PI	**PCR positive swabs**	**Phase contrast microscopy positive cultures**	**PCR positive cultures**	**PCR positive swabs**	**Phase contrast microscopy positive cultures**	**PCR positive cultures**
10 minutes	3/3	3/3	3/3	3/3	3/3	3/3
1 hour	3/3	3/3	3/3	3/3	3/3	3/3
2 hours	3/3	2/3	2/3	3/3	3/3	3/3
4 hours	3/3	0/3	0/3	3/3	0/3	0/3
18 hours	3/3	0/3	0/3	3/3	0/3	0/3

3.3.1.2 Disinfection of hoof knife blades

Three disinfectants completely prevented visible treponeme growth under laboratory conditions, as determined by phase contrast microscopy: 1% FAM30®, 2% Virkon® and 2% sodium hypochlorite. When using PCR of cultures after six weeks as an outcome, 1% FAM30® eliminated all detectable DNA, whilst there was detectable DNA in 1/13 T320A cultures post-disinfection with 2% Virkon®, and 2/15 T320A cultures post-disinfection with 2% sodium hypochlorite, suggesting some limited growth. Water alone was the least effective, leading to visible treponeme growth in 16/28 cases, and positive PCRs from cultures in 19/28 cases.

Contrastingly, 2% Virkon® and 2% sodium hypochlorite yielded the best DNA degradation results as determined by direct PCR of swabs in terms of removing/destroying all bacterial DNA in 18/26 and 20/31 of cases respectively, whilst 1% FAM30® did not destroy bacterial DNA by this measure (Table 3.2). Water resulted in positive results for treponemal DNA presence by direct PCR in 27/28 cases (Table 3.2).

Table 2: Efficacy of disinfectants (20 seconds exposure time) against BDD treponemes on hoof knife blades

Treponeme strain (phylogroup)	**T320A** (*Treponema phagedenis*)			**T3552B** (*Treponema pedis*)		
Disinfectant	**Post-disinfection**					
	PCR positive swabs	**Phase contrast microscopy positive cultures**	**PCR positive cultures**	**PCR positive swabs**	**Phase contrast microscopy positive cultures**	**PCR positive cultures**
Water	10/12	6/12	6/12	16/16	10/16	13/16
5% Copper sulphate	13/16	1/16	4/16	11/17	1/17	2/17
1:100 FAM30	11/11	0/11	0/11	12/12	0/12	0/12
2% Glutaraldehyde	15/15	2/15	6/15	11/11	0/11	0/11
2% Sodium hypochlorite	10/15	0/15	2/15	1/16	0/16	0/16
2% Virkon	6/13	0/13	1/13	2/13	0/13	0/13

3.3.2 Field Study Results

A total of 133 BDD cases with the following pathological lesion stages were used to collect samples: one M1, 11 M2, 10 M3, 101 M4 and 10 M4.1. One of three disinfectants was used on trimming blades for 86 cases, whilst water was used in the remaining 47 cases. For 22 cases, no blade contact was made with the BDD lesion during foot-trimming, whilst for the remaining 111 cases blade contact was made with the lesion which was done to remove crusting from lesions prior to the application of topical treatment (Terramycin Aerosol Spray, Zoetis, UK). Seven non-healing lesion (NH) cases were also used to collect samples: three cases of white line disease, two cases of toe necrosis, and two toe

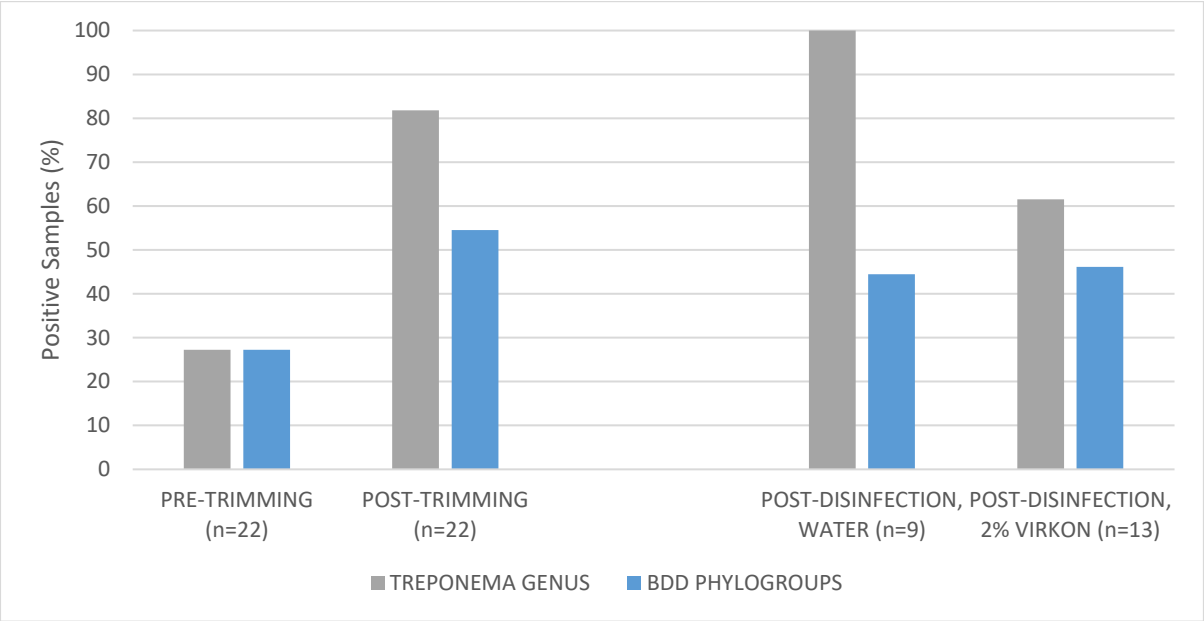
ulcers. In all seven NH cases appropriate trimming and treatment of lesions necessitated contact between the hoof knife blade and the lesion.

Where contact was not made with BDD lesions, 18/22 (81.8%) of post-trimming swabs taken directly from the hoof knife blades tested positive (using nested PCR) for the *Treponema* genus, and 12/22 (54.5%) tested DNA positive for at least one of the three pathogenic phylogroups after trimming (Figure 5). After disinfection 17/22 (77.3%) remained DNA positive for the *Treponema* genus and 10/22 (45.5%) remained DNA positive for at least one of the three pathogenic phylogroups. All knives cleaned with water- 9/9 (100%), and 8/13 (61.5%) of those disinfected with 2% Virkon® remained DNA positive for the *Treponema* genus. The number of knives testing positive for BDD phylogroup DNA post-disinfection with water increased from 2/9 (22.2%) to 4/9 (44.4%), whilst disinfection using 2% Virkon® reduced the number of positive samples from 10/13 (76.9%) to 6/13 (46.2%) (Figure 6). (2% sodium hypochlorite and 1:100 FAM30® were not tested in cases where no lesion contact had been made). Some samples taken pre-trimming (6/22 (27.3%)) tested DNA positive for *Treponema* genus and BDD phylogroups, suggesting some hoof knife blades were contaminated prior to trimming, including some that had not been previously used for hoof trimming that day.

For post swab cultures (6 weeks), where no blade contact was made with BDD lesions, *Treponema* genus and pathogenic treponeme DNA were detected in only 1/22 (4.5%) cases after trimming, indicating very low incidence of living organisms when sampled. Disinfection using 2% Virkon® removed culturable organisms in this case. No samples taken pre-trimming were found to contain culturable treponemes.

Figure 5: Direct PCR results showing disinfectant efficacy (no contact made between hoof knives and BDD lesions)

Treponema DNA Positive Samples (%) identified by direct PCR showing efficacy of 2% Virkon® (compared to water only) for disinfection of hoof knife blades for removing *Treponema* genus DNA and DNA from three BDD-associated treponeme phylogroups. No contact was made between the hoof knife blades and the BDD lesions (n=22).



Where blade contact was made with BDD lesions (Figure 6), all swabs (111/111) taken after trimming were DNA positive (using nested PCR) for the *Treponema* genus and at least one of the three pathogenic phylogroups. After disinfection, 69/111 (62.2%) remained PCR positive for the *Treponema* genus and 38/111 (34.2%) for at least one of the pathogenic phylogroups. Knives cleaned with water or disinfected with 1:100 FAM30® frequently remained contaminated with *Treponema* genus DNA (38/38 (100%) and 16/20 (75%) of samples respectively). Water and FAM30® also performed less well for removing BDD phylogroup DNA; with 15/38 (39.5%) and 9/20 (45.0%) of samples remaining positive (Figure 6). Univariable logistic regression showed that when knives were disinfected using 2% sodium hypochlorite or 2% Virkon® (compared to water) they had decreased odds of testing PCR positive for BDD-associated phylogroup and/ or *Treponema* genus DNA (Table 3.3).

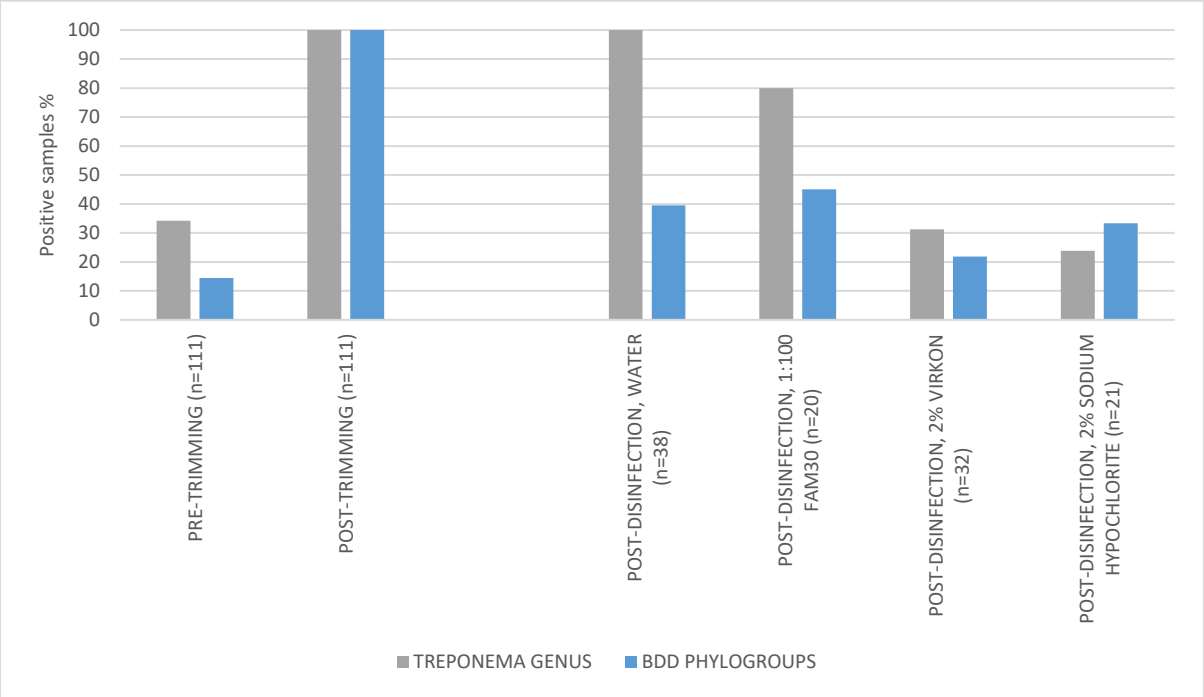
Table 3.3 Results of univariable logistic regression showing decreased odds of detection of Treponema genus and/ or BDD-associated phylogroup DNA where 2% sodium hypochlorite or 2% Virkon® were used to disinfect hoof knives compared to water

Disinfectant	Odds Ratio	95% Confidence Interval	P-value	Standard Error
1:100 FAM30®	−2.09	− 4.42- 0.24	0.078	1.19
2% sodium hypochlorite	−4.31	−6.48- -2.15	< 0.001	1.11
2% Virkon®	−4.23	−6.30- -2.16	< 0.001	1.06
Baseline (water)	3.83	1.85–5.81		1.01

For swabbing of blades where lesion contact was made, as found for the samples where no contact was made between the hoof knives and the BDD lesions, some samples taken pre-trimming tested positive for the *Treponema* genus (38/111 (34.2%)) and BDD phylogroups (16/111 (14.4%)), indicating some hoof knife blades had become contaminated prior to trimming (Figure 6). Positive pre-trimming samples came both from knives that were being used for the first time that day, and from knives that had already been used for foot-trimming during the session. Table 3.4 shows that these treponemes were not however, viable.

Figure 6: Direct PCR results showing disinfectant efficacy (contact made between hoof knives and BDD lesions)

Treponema DNA Positive Samples (%) identified by direct PCR, showing efficacy of three disinfectants (compared to water only) for disinfection of hoof knife blades for removing *Treponema* genus DNA and DNA from three BDD-associated treponeme phylogroups. Contact was made between the hoof knife blades and the BDD lesions (n=111).



Where contact was made with BDD lesions (n=111), the *Treponema* genus was detected by nested PCR of six-week cultures in 64/111 (57.7%) of cases and BDD-associated treponemes were detected in 47/111 (42.3%) of cases after trimming. Univariable logistic regression showed that making contact between the hoof knife blade and the lesion was statistically more likely to result in a positive culture (Odds Ratio 3.39, 95% confidence interval 1.35–5.43, $P = 0.001$). All three disinfectants (and water only) were effective at removing culturable organisms (Table 3.4).

Table 3.4: Culture PCR results showing disinfectant efficacy where contact was made between hoof knives and BDD lesions

The effect of disinfectants on viable treponemes on hoof trimming knives (determined by PCR of cultures), before use, post-trimming, and post-disinfection. In all samples knife-BDD lesion contact occurred during trimming.

Disinfectant	**PRE-TRIMMING**		**POST-TRIMMING**		**POST-DISINFECTION**	
	Treponema genus	**BDD phylogroups**	**Treponema genus**	**BDD phylogroups**	**Treponema genus**	**BDD phylogroups**
2% Virkon® n=32	0/32	0/32	18/32 (56.3%)	13/32 (40.6%)	0/32	0/32
2% sodium hypochlorite n=21	0/21	0/21	10/21 (47.6%)	10/21 (47.6%)	0/21	0/21
1:100 FAM30® n=20	0/20	0/20	12/20 (60.0%)	10/20 (50.0%)	0/20	0/20
Water n=38	0/38	0/38	24/38 (63.2%)	14/38 (36.8%)	0/38	0/38
Total (n=111)	0/111	0/111	64/111 (54.2%)	47/111 (39.8%)	0/111	0/111

For non-healing lesions, six knives used were disinfected using 2% Virkon. Direct PCR results showed 1/6 was positive for *Treponema* genus and BDD phylogroups pre-trimming, and 6/6 were positive for *Treponema* genus and BDD phylogroups post-trimming. Post-disinfection, 4/6 remained positive for *Treponema* genus, and 1/6 remained positive for BDD phylogroups. The blade used to trim one NH case was cleaned using water only. This blade was negative for *Treponema* genus and BDD phylogroups pre-trimming, positive for both post-trimming, and remained positive for *Treponema* genus after cleaning. No positive samples were identified from NH cases using culture PCR.

In an analysis of treponemes on blades used to trim different BDD lesion stages, there is a clear presence at all M grades (Table 3.5). All different BDD lesion stages showed detection of BDD treponemes in post-trimming cultures except for a single M1 case in which there was no contact between the hoof knife blade and the lesion. Some lesion stages yielded higher proportions of positive cultures where contact was made between the hoof knife blade and the lesion: 80%, 75% and 66.7% from M2, 3 and 4.1 lesions respectively compared to 36.2% for chronic M4 cases.

A full comprehensive list of cases showing both direct PCR and culture PCR results is available in Table 1 in Appendix A.

Table 3.5: Detection of BDD treponeme phylogroups according to lesion stage

The effect of BDD lesion stage on detection of BDD treponeme phylogroups in post-trimming and post-disinfection hoof knife samples (disinfected using water only, 2% Virkon®, 2% sodium hypochlorite or 1:100 FAM30®) as measured by both direct and culture PCR.

Lesion Type	Contact with lesion (Yes/No)	Post-trimming Direct PCR	Post-disinfection Direct PCR	Post-trimming Culture PCR	Post-disinfection Culture PCR
M1(n=1)	No	1/1(100%)	1/1(100%)	0	0
M2(n=6)	No	3/6(50.0%)	2/6(33.3%)	0	0
M2(n=5)	Yes	5/5(100%)	2/5(40.0%)	4/5(80.0%)	0
M3(n=6)	No	4/6(66.7%)	3/6(50.0%)	1/6(16.7%)	0
M3(n=4)	Yes	4/4(100%)	3/4(75.0%)	3/4(75.0%)	0
M4(n=7)	No	3/7(42.9%)	3/7(42.9%)	0	0
M4(n=94)	Yes	94/94(100%)	29/94(40.4%)	34/94(36.2%)	0
M4.1(n=1)	No	1/1(100%)	1/1(100%)	0	0
M4.1(n=9)	Yes	9/9(100%)	4/9(44.4%)	6/9(66.7%)	0
All lesions (n=22)	No	12/22(54.5%)	10/22(45.4%)	1/22(4.5%)	0
All lesions (n=111)	Yes	111/111(100%)	38/111(34.2%)	47/111(39.8%)	0

3.4 Discussion

The *in vitro* work shows that viable BDD treponemes can survive on hoof knife blades under aerobic conditions in the laboratory for two hours. The survival of bacteria, previously presumed to be obligate anaerobes, for at least two hours on trimming blades in aerobic conditions is probably key to their apparent ability to be transmitted between animals during foot trimming (Evans et al., 2009b; Wells et al., 1999) and will also have relevance for other means of BDD transmission between animals and farms. Recent study of the complete genome of treponeme species representative of the three BDD-associated phylogroups showed a greater number of oxidative stress genes when compared to their non-pathogenic equivalents (Staton et al., 2021a), suggesting that aerotolerance may be a key survival and pathogenic mechanism.

The *in vitro* work also demonstrates that three common disinfectants prevent visible growth under anaerobic conditions in the laboratory: 1:100 FAM30®, 2% Virkon® and 2% sodium hypochlorite. In addition, 1:100 FAM30® also eliminated all detectable DNA from cultures, suggesting this may be the preferred option for foot trimming tool disinfection. Interestingly FAM30® was also found to prevent treponeme growth by the same measure when used to wash gloves worn to handle feet of sheep

infected with CODD (Angell et al., 2017). However, 1:100 FAM30® did not eliminate DNA detectable by direct PCR and other disinfectants were more successful by this measure.

Interpretation of the direct PCR results from swabs are challenging for two reasons. Firstly, the modes of action of disinfectants are incompletely described so it is not clear whether all disinfectants would be expected to destroy DNA entirely. Glutaraldehyde, for example, has fixative properties and could therefore be expected to preserve DNA. The primary mode of action for aldehydes is to form cross-linkages with surface-exposed proteins and peptides, thus intracellular components may be preserved at least for a short time such as the 20 seconds tested in the present study (McDonnell, 2017). Conversely, halogens such as the iodine contained in FAM30® could be expected to have a dramatic effect on DNA structures, although the exact mode of action is unknown and likely to vary significantly depending on the formulation of the product (McDonnell, 2017). The second challenge in terms of interpretation is that the presence of detectable DNA does not necessarily correspond to the presence of viable bacteria that would be capable of growth in their normal biological context.

The role of foot-trimming in transmission of CODD which is associated with the same treponemes (Sullivan et al., 2015a) should also be considered in design of foot disease control measures in sheep. Current best practice advises against routine foot trimming of sheep, as trimming more than twice per year has been associated with presence of CODD on farms. In addition, causing sheep to bleed during trimming has been associated with increased period prevalence of CODD. It has been proposed that damage to the soft tissues of sheep feet leave them susceptible to this disease and that transmission may occur due to contaminated equipment (Dickins et al., 2016).

The finding that viable BDD treponemes can be grown from hoof knife blades for up to two hours confirms that contamination of knives during foot trimming is a potential risk for transmission of BDD, both between cows in the same herd, and between herds.

BDD infection models have shown that existing tissue damage and direct contact with fresh lesional material containing a viable polytreponemal bacterial load is needed to cause lesion development (Gomez et al., 2012; Krull et al., 2016; Read & Walker, 1996). Foot-trimming would appear to fulfil the criteria for the infection models as viable treponemes are transferred between the feet of cows in the herd in quick succession (assuming that effective disinfection is not practised), and these feet frequently demonstrate some evidence of damage either from slurry exposure or general mechanical damage. It is therefore important for the industry to apply a feasible and effective disinfection protocol for use during foot-trimming. The laboratory results suggested that the use of 1:100 FAM30®, 2% Virkon® or 2% sodium hypochlorite with 20 seconds contact time should be suitable for this purpose.

We carried out the field study to test these recommendations, since presence of manure contamination may alter the performance of disinfectants (Hartshorn et al., 2013). The field study has confirmed previous work that the BDD associated treponemes present in BDD lesions are readily transferrable to hoof trimming blades (Sullivan et al., 2014). It also shows they can be viable and therefore transmissible. Importantly, it has also shown that even brief disinfection of the blades is very efficient in eliminating viable treponemes from blades and presumably blocking at least this route of BDD transmission. This is a very practical outcome worthy of consideration for best practice as it can be achieved with minimal effort or investment. A disinfection protocol for industry use was developed alongside the dairy levy board AHDB Dairy and is available in Appendix B.

In over 90% of samples, direct nested PCR testing detected contamination of hoof knives with the *Treponema* genus DNA post-trimming and at least one of the three culturable BDD-associated treponeme phylogroups. This is consistent with findings from previous field work (Sullivan et al., 2014) and the present study achieved similar overall disinfection efficacy for the BDD phylogroups investigated as determined by the presence of BDD treponeme DNA within swab samples. However, detection of bacterial DNA direct from swabs does not measure the viability of organisms and therefore does not indicate whether they might be capable of transmission. For this reason, we introduced the use of bacterial culture of blade swabs, which showed that in 48/133 (36.1%) cases, post-trimming hoof knives were contaminated with BDD phylogroup treponemes that were viable. This is surprisingly high considering that treponemes are notoriously fastidious (Evans et al., 2008) and therefore their survival during sample collection and transport under aerobic conditions (especially when they are anaerobic organisms) would be expected to be low (Angell et al., 2017). In addition, field samples inevitably contain many contaminating bacteria that could be expected to out-compete treponemes in culture. For these reasons, those samples where DNA was detected but cultures were negative should not automatically be disregarded as not containing viable treponemes.

It is evident that contact with the BDD lesions during foot-trimming increases the frequency of detectable DNA contamination on hoof knife blades, and frequency of culturable BDD treponemes. Contamination rates may also vary according to BDD prevalence on farm, and management measures taken to reduce environmental contamination that are likely to reduce foot contamination. For example, recently footbathed feet may have fewer viable treponemes, which would result in a reduction in new BDD cases observed (Holzhauer et al., 2012).

In the field, water only was equally as effective at removing viable treponemes from hoof knives as any of the three disinfectants tested. Water was not successful during laboratory testing as the majority of knives remained contaminated with viable treponemes that were also visible using phase

contrast microscopy. The *in vitro* study also found that 1:100 FAM 30® eliminated all *Treponema phagedenis* or *Treponema pedis* from hoof knife blades that could be detected by culture PCR. FAM 30® was therefore considered superior to 2% sodium hypochlorite of 2% Virkon®, which did not eliminate the *Treponema phagedenis* phylogroup according to this measure in 2/15 and 1/13 experiments respectively. This difference in performance was not translated into our field data, however the finding that FAM 30® did not perform as well as the other disinfectants for removing DNA detectable by direct PCR did recur. *In vitro* experiments were carried out using pure treponeme cultures and are likely to represent a greater bacterial challenge which could explain these differences in the findings. Nevertheless, certainly water should still be considered inferior to the three disinfectants for hoof knife disinfection given the laboratory collected data.

Positive cultures came from cases with M2, M3, M4 and M4.1 lesions. It has been shown that treponeme numbers are higher in active ulcerative lesions (as previously determined using qPCR to quantify bacterial DNA, rather than using culture) (Beninger et al., 2018). Our results indicate that higher percentages of M2, M3 and M4.1 lesions lead to positive cultures post-trimming when compared to the chronic M4 stage; however, this effect could be due to smaller sample sizes in the other lesion categories. This distribution of BDD lesion stages in the field is consistent with a recent study that classified 63.4% of heel bulb lesions as M4 (Solano et al., 2017), and a recent study of BDD transmission dynamics which found that about 70% of infected time was spent as M4 (Biemans et al., 2018).

Interestingly we did not get any positive cultures from non-healing lesions, which may indicate that our culture techniques are less suitable for recovering treponemes found in these lesions. Previous attempts to culture treponemes from non-healing lesions also failed and it was suggested that this may be due to the more pronounced polymicrobial character of these diseases, or more contamination with slurry due to the differences in anatomical location of the lesions (Evans et al., 2011).

Pre-trimming contamination of hoof knives was an unexpected finding. It is possible that washing between cows was inadequate, and treponeme DNA was robust enough to remain intact after cleaning and immersion in 70% ethanol for ten minutes. This would not, on its own, be important for disease transmission (as no viable organisms were detected) but could explain our pre-trimming data sets. It is also possible that contamination was caused by aerosolisation of bacteria in the vicinity of the foot-trimming crush, contaminating knives as they were air dried for use. Although studies regarding bio-aerosols on dairy farms are limited, it has been shown that *Mycobacterium avium* subsp. *paratuberculosis* can be detected in settled dust particles inside dairy housing three weeks after introduction of infected cattle (Eisenberg et al., 2010). Furthermore, spirochaetes have been identified

in aerosols on a dairy farm, representing 1% of the total 16S rRNA gene sequences identified in aerosol samples (Ravva et al., 2011). Given this contamination did not result in viable treponeme cultures, we consider aerosols have limited ability to transmit *Treponema* spp.

3.5 Conclusion

Previous epidemiological studies identified use of an external foot-trimmer and lack of washing of hoof trimming equipment as risk factors for increased herd BDD prevalence (Wells et al., 1999; Yang et al., 2018). The findings that treponemes can survive on hoof knife blades for two hours and subsequently be recovered in culture, and the finding of viable treponemes post-trimming in field samples, even where no contact has been made between hoof knife and BDD lesion, provides evidence that reduced biosecurity during hoof trimming is a risk factor for BDD transmission. Collectively, the evidence provides a compelling argument for improving hygiene during foot-trimming. The use of disinfectants on trimming tools will not only assist in controlling transmission of treponemes between animals and farms but will also have the effect of reducing transmission of other bacteria which are known to contribute to digital dermatitis and other important foot lesions (Bay et al., 2018).

The disinfectants used here (2% Virkon®, 2% sodium hypochlorite, 1:100 FAM30®) have been shown to be effective against BDD treponemes on hoof knives both in the laboratory and on farm during foot-trimming of dairy cows with a short contact time of 20 seconds for removing viable treponemes. The disinfection protocol used in this study should therefore be considered reliable for adoption as standard industry practice.

Chapter 4 Current foot-trimming hygiene practices and research impact on knowledge and practice of biosecurity during cattle foot-trimming

Industry Group Abbreviations used in this chapter

AHDB Agricultural and Horticultural Development Board
BCVA British Cattle Veterinary Association
CHCSB Cattle Hoof Care Standards Board
HTA Hoof Trimmers Association
NACFT National Association of Cattle Foot Trimmers
NFU National Farmers Union
WOHT World of Hoof Trimming

4.1 Introduction

Increasing dairy herd sizes and decreased staff: cow ratios mean that farmers and their veterinary surgeons are no longer able to provide adequate hoof trimming care. Demand has grown for dedicated foot trimmers to carry out routine prophylactic foot trimming, which is recommended in the dairy herd to maintain correct claw shape and reduce claw horn lesions (Hernandez et al., 2007; Manske et al., 2002a, 2002b), and to treat lameness cases. There is also industry pressure to proactively identify lame cows using regular mobility scoring as it has been shown that early treatment of lesions improves prognosis as well as being essential for good animal welfare (Leach et al., 2012). There is no legal requirement for those operating as foot trimmers to be formally qualified, however the industry is working to improve professional standards via formal training.

Vocational training for cattle foot-trimming was developed in the Netherlands by the Utrecht University Veterinary School and the Dairy Training Centre in the 1980s and teaches the Dutch Five Step trimming method which was first published in English in 1985 (Touissant Raven, 1985). The Dutch Foot-trimming Diploma has become an internationally recognised qualification for certifying foot-trimmers and maintaining professional standards through continued auditing. In the UK since 2019, the Royal Agricultural University has offered a level 4 qualification in dairy cattle foot trimming, which requires attendance at training, submission of a case portfolio, theory and practical examinations, and ongoing audits for qualified trimmers. For both qualifications foot-trimmers are required to demonstrate knowledge and practice of good biosecurity between farms to prevent spread of infectious diseases, however prior to this current project there were no specific evidence- based recommendations relating to maintenance of good biosecurity during foot-trimming on a single farm. In addition, farm staff may be trained informally in-house to carry out foot-trimming within their own herd, and veterinary surgeons who also treat lameness cases receive training via veterinary schools.

Since internal biosecurity has historically not been consistently addressed in training and industry recommendations are not well established, it is unknown what current foot-trimming hygiene practices are being applied by individuals in the industry.

Previous research has identified hoof knives and gloves worn during the routine husbandry procedure of cattle foot-trimming as key infection reservoirs for BDD-associated treponemes (Blowey et al., 2013; Sullivan et al., 2014) which was concerning because of the potential risk for transmitting BDD on and between farms. After informing the foot-trimming community of this issue, funding was secured to develop the evidence base to advise the industry how to solve the problem. The current project was able to develop and test a disinfection protocol for use during foot- trimming using data from *in vitro* disinfection experiments and field studies reported in Chapters 3 (Gillespie et al., 2019, 2020) (a collaboration between AHDB Dairy and the University of Liverpool). To maximize impact from this research, a series of knowledge transfer activities were carried out to ensure that end users of the research would be able to incorporate the study findings into practice.

The value of research-based knowledge has long been recognized in the livestock industry, however, translating this knowledge into strategies that encourage behavioural change often proves challenging (Garforth et al., 2004). There are several popular health psychology models which endeavour to explain how individuals make decisions and translate these into action, such as the Theory of Reasoned Action, which considers the decision-makers beliefs and perception of action outcome, as well as recognizing the importance of the influence of the actions and behaviours of others (Garforth et al., 2004). In light of poorly implemented internal biosecurity practices on farms, there has been some exploration of the role of cattle farmers and dairy veterinarians specifically in on-farm biosecurity (Brennan & Christley, 2013; Sayers et al., 2014). However, there is no information in the literature regarding attitudes and beliefs of foot-trimmers regarding biosecurity. This should be considered especially important in controlling the spread of BDD, as those who carry out foot-trimming include a subset of farmers and veterinarians together with foot-trimming professionals who will frequently bring themselves and their equipment (known key infection reservoirs) into contact with large numbers of cattle feet.

In this study, we set out to measure the impact of our research (and associated knowledge transfer) upon enhancing both understanding and practice of all professionals who trim cows' feet to prevent the transmission of BDD between cattle during foot-trimming, and to identify future avenues of relevant research.

To this end, this chapter describes the three stages in this study: 1) an initial foot-trimming hygiene questionnaire and knowledge exchange, 2) dissemination of questionnaire results and further knowledge exchange, and 3) a follow-up questionnaire.

4.2 Methods

4.2.1 Questionnaire design

The study population were cattle foot health experts comprising dairy farmers, veterinary surgeons, and commercial cattle foot-trimmers. The foot-trimming hygiene questionnaire was undertaken between April and October 2019, and the follow-up questionnaire between January and May 2020. Questionnaires were designed for paper formats and adapted to electronic formats using SurveyMonkey software (SurveyMonkey Inc., San Mateo, California USA). Both questionnaires were checked for question validity by research and foot-trimming colleagues. Electronic versions were checked for usability and technical functionality. Participants were provided with information sheets (either printed where applicable, or electronically) explaining the purpose of the study and ensuring they were aware that participation was voluntary. Both studies were approved by the University of Liverpool Veterinary Ethics Committee (Ref: VREC 786 and 786a).

The foot-trimming hygiene questionnaire collected data about how many farms a participant trimmed at, the number of cows trimmed per week, and how many herds they worked with had BDD. Participants were asked whether they cleaned their hands during foot-trimming, and if so, how often and by what means. These questions were repeated concerning cleaning of hoof knives. Respondents were asked to give an email address if they were prepared to be contacted in the future with a follow-up questionnaire.

The questions regarding number of farms visited, cows trimmed and herds with BDD were repeated in the follow-up questionnaire. Participants were also asked which country they worked in as the questionnaire was promoted to industry groups outside the UK.

To assess the impact of the knowledge exchange activities, participants were asked if they had seen the hygiene protocol published by the University of Liverpool and AHDB Dairy, or other articles in the farming or veterinary press concerning hygiene during foot trimming. Participants were asked to respond on a Likert scale to statements regarding their awareness of the potential to spread digital dermatitis during foot trimming and awareness of appropriate hygiene and disinfection to prevent this.

Where respondents reported a change in their hygiene management, information was collected about which changes had been made, and whether they or their clients had perceived a reduction in BDD on

farm as a result. Responses were collected on a Likert scale regarding six barriers to improving foot trimming hygiene, which were selected based on dialogue between researchers and stakeholders throughout knowledge exchange events. Finally, participants were asked open questions to prompt suggested improvements to the foot-trimming hygiene protocol and give ideas for future research in this area. Three prizes of Amazon vouchers were offered as an incentive to participate.

4.2.2 Questionnaire advertising and distribution

The foot-trimming hygiene questionnaire was administered using in-person and online methods. In-person questionnaires were distributed at relevant industry events: NACFT conference 2019, CHCSB conference 2019, TotalDairy 2019, European Bovine Congress 2019 and BCVA Congress 2019. Online questionnaires were distributed via social media, specifically using posts to the NACFT, BCVA, Veterinary Voices UK and WOHT Facebook groups, and shared via Twitter. The questionnaire was uploaded to the BCVA website, and emailed to the NACFT, HTA and Dutch Foot Trimmers mailing lists. Links were also posted on The Farming Forum and University of Liverpool Bovine Digital Dermatitis internet sites.

The follow-up questionnaire was administered using only online methods, since all events were cancelled due to the Covid-19 pandemic. Advertising and distribution of questionnaires online was carried out as for the foot-trimming hygiene survey. It was also promoted via the "Healthy Feet Webinar" organised by BCVA and AHDB Dairy. We were able to directly contact via email 87 respondents from the foot-trimming hygiene survey who had given their consent and email addresses for this purpose. The paper format initial questionnaire, and the intended paper format follow-up questionnaire are available in Appendix B.

4.2.3 Knowledge exchange

Prior to opening of the first questionnaire, development of a foot-trimming hygiene protocol was undertaken in consultation with the UK dairy industry levy board, AHDB Dairy, based on our previous research findings (Blowey et al., 2013; Gillespie et al., 2019; Sullivan et al., 2014) (Available at https://ahdb.org.uk/reducing-spread-of-DD). The protocol was advertised and disseminated at the industry events attended by the authors to administer the questionnaire. Online methods targeted both nationally and internationally were also used for promotion (April- October 2019). In the second stage of the study, results from the foot-trimming hygiene questionnaire were disseminated to industry stakeholders and participants via a series of knowledge exchange articles in the farming, foot-trimming and veterinary presses, as well as using online delivery (January to May 2020). Details of the overall sampling and knowledge exchange strategies are show in Table 4.1.

Table 4.1: Sampling and knowledge exchange strategy and engagement with relevant industry stakeholders (foot trimmers, dairy farmers and veterinary surgeons) at the three stages of the study.

Study phase	*Initial questionnaire and knowledge exchange including advertising of foot-trimming hygiene protocol* April- October 2019	*Dissemination of initial questionnaire results and further knowledge exchange and advertising of foot-trimming hygiene protocol* January-May 2020	*Follow-up questionnaire (online only)* April-June 2020
Target group	Online	Online Video	(Online only)
Foot trimmers	NACFT Facebook Group, WOHT Facebook Group Emails to NACFT, HTA and Dutch foot trimmers	NACFT Facebook Group, WOHT Facebook Group, Emails to NACFT, HTA, Dutch foot trimmers and email address list collected from first questionnaire	NACFT Facebook Group, WOHT Facebook Group, Emails to NACFT, HTA and Dutch foot trimmers and email address list collected from first questionnaire
Farmers	Post on The Farming Forum	Post on The Farming Forum	Post on The Farming Forum
Veterinary Surgeons	BCVA Facebook Group and BCVA website, Veterinary Voices UK Facebook Group	BCVA Facebook Group and BCVA website, Veterinary Voices UK Facebook Group	BCVA Facebook Group, Veterinary Voices UK Facebook Group, promoted via the Healthy Feet Programme Webinar organised by BCVA.
All	Twitter, promoted by AHDB Dairy University of Liverpool Bovine Digital Dermatitis webpage	Twitter, promoted by AHDB Dairy Previously collected email addresses. University of Liverpool Bovine Digital Dermatitis webpage	Twitter, promoted by AHDB Dairy Previously collected email addresses. University of Liverpool Bovine Digital Dermatitis webpage
	Offline	Offline	
Foot-trimmers	Presentation and questionnaire at NACFT conference 2019, questionnaire at CHCSB CPD day 2019.	Articles for NACFT magazine and HTA magazine	
Farmers	Attendance at TotalDairy conference Article for Farmer's Weekly	(Engagement via NFU unsuccessful)	
Veterinary surgeons	Presentations at European Bovine	Article for Livestock CPD magazine	

	Congress 2019 and BCVA Congress 2019	
All		Poster Presentation AHDB conference 2020

4.2.4 Statistical analysis

For both questionnaires, data were initially inputted manually and imported from SurveyMonkey into Microsoft Excel (Microsoft, Washington, USA) and checked for inconsistencies. Descriptive statistics were calculated using Microsoft Excel.

For the initial questionnaire, participants were categorized according to whether their reported overall hygiene measures were likely to be adequate for preventing significant contamination hands and hoof knives with BDD-associated pathogens, which could consequently be passed to the next cow in a foot-trimming working session. Hygiene measures were considered adequate if undertaken at least after trimming of infected cows, and the hygiene measure included using soap or disinfectant. Changing gloves was also considered an adequate hygiene measure for hand washing.

For the follow-up questionnaire, data was imported in STATA v14 (Statacorp, USA) and univariable logistic regression was used to assess the association between the country the respondent was working in and three outcomes: whether they had seen the AHDB Dairy hygiene protocol, or other relevant articles, and whether they had changed practice. Univariable logistic regression was also used to assess the association between having seen the hygiene protocol and/ or other articles and whether hygiene management had changed. The association between number of cows trimmed each week and the outcome of a change in practice was also assessed using univariable logistic regression. Graphs highlighting respondents who were classified as practicing adequate hygiene were produced using RStudio version 1.2.5019-6 (RStudio, Boston, MA, USA).

4.3 Results

4.3.1 Initial questionnaire: Assessing current industry practice for hygiene during foot-trimming

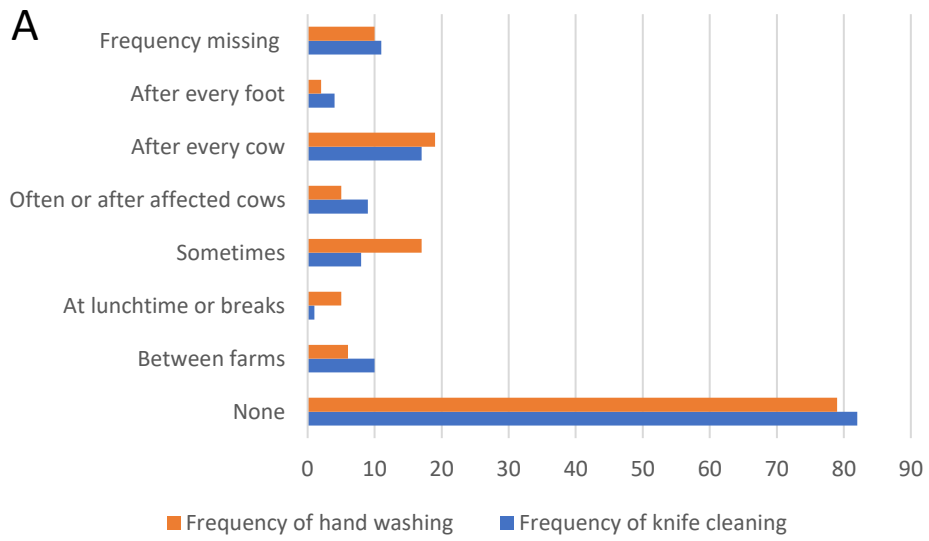
The first questionnaire had a total of 143 respondents: 84 online and 59 via paper questionnaire. Respondents reported working on 4951 farms (median 20, IQR 5-42, range 1-500). Respondents considered that 3635/ 4951 of these farms experienced cases of digital dermatitis (73.4%). Collectively, they trimmed an estimated 22,490 cows each week (median 123, IQR 10-250, range 1-2000). Respondents frequently reported that they did not wash their hands or hoof knives during foot-

trimming (79/143, 55.2% and 82/142, 57.7% respectively). For those that did report washing hands and/ or hoof-knives, the frequency of these practices is shown in Figure 1A. Participants reported using ten different hygiene methods for hand washing and twelve for cleaning hoof knives (Figure 1B).

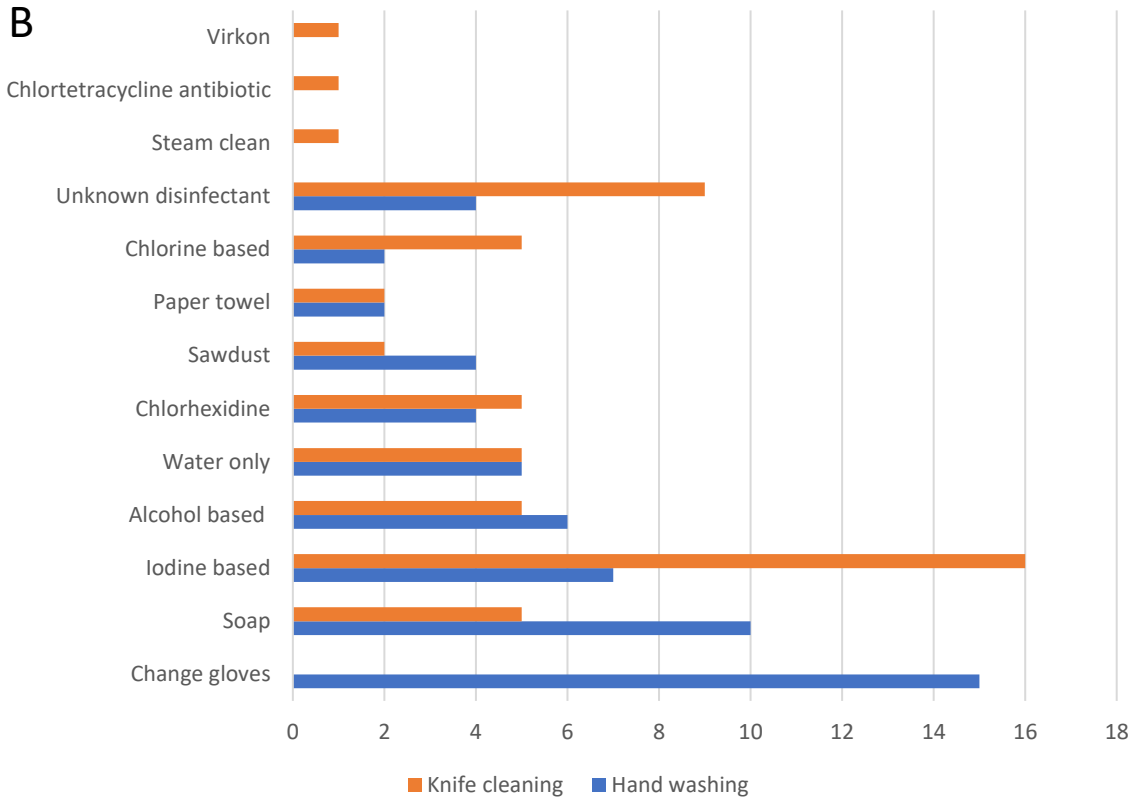
When we considered both their hand washing and knife cleaning procedures, thirteen participants were classified as practicing adequate hygiene (13/143, 9.1%). Collectively these thirteen accounted for trimming only 594/22,490 (2.6%) of the estimated number of cows trimmed by respondents each week and just four of these respondents were trimming more than 15 cows per week (Figure 1C), suggesting practical difficulties in implementation may be a barrier to widespread adoption of the hygiene protocol.

Figure 1: **Results from the initial questionnaire. 1A: Frequency of hand washing (n=143) and hoof-knife cleaning (n=142) amongst professionals trimming cows' feet, displayed in decreasing order of frequency.**

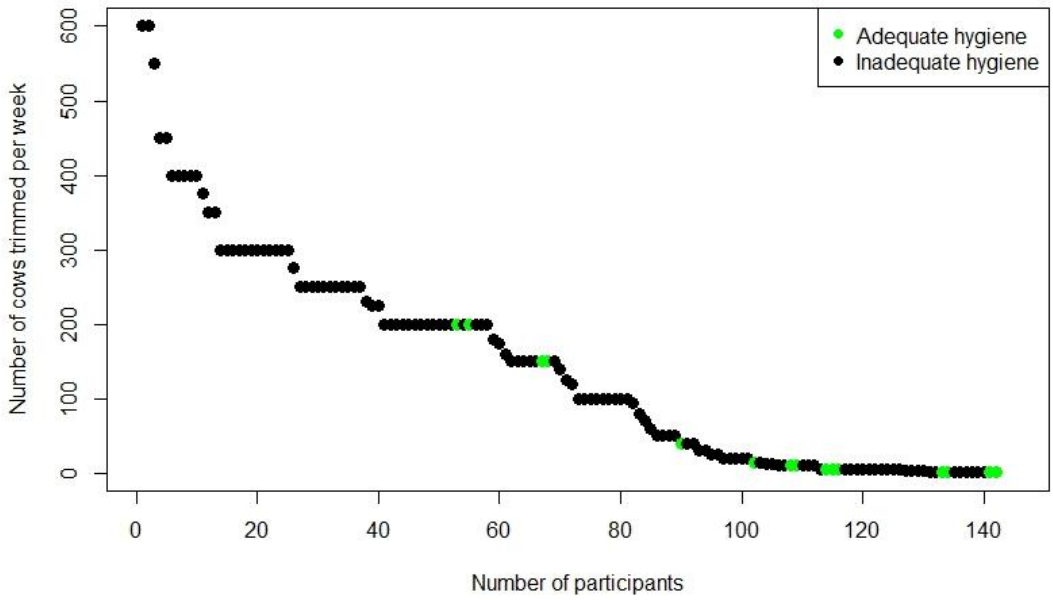
Some participants reported that they do wash their hands or hoof knives during foot-trimming but did not answer how frequently. 1B: Hygiene methods used by professionals for hoof-knife cleaning and hand washing during foot-trimming. 1C: Highlighting those participants who were classified as practicing hygiene adequate for preventing transmission of BDD during foot-trimming, plotted against the number of cows they were trimming each week.



B


Virkon
Chlortetracycline antibiotic
Steam clean
Unknown disinfectant
Chlorine based
Paper towel
Sawdust
Chlorhexidine
Water only
Alcohol based
Iodine based
Soap
Change gloves
0
2
4
6
8
10
12
14
16
18
Knife cleaning
Hand washing

C


Adequate hygiene
Inadequate hygiene
Number of cows trimmed per week
0
100
200
300
400
500
600
0
20
40
60
80
100
120
140
Number of participants

4.3.2 Follow-up questionnaire: Assessing the impact of research on foot-trimming hygiene practices

Eighty farmers, veterinary surgeons and foot trimmers responded to the follow-up survey and reported working on a total of 3,800 farms (median 28, IQR 10-50, range 1-600), collectively trimming approximately 12,660 cows per week (median 150, IQR 9-250, range 1-1,200). Respondents considered that 3458/3800 (91%) herds they visited experienced bovine digital dermatitis lesions. Participants were working in the UK (45/80, 56.0%), Europe (20/80, 25.0%), the USA and Canada (14/80, 17.5%), and New Zealand (1/80, 1.3%). Thirty respondents had seen both the foot-trimming hygiene protocol produced by the University of Liverpool and AHDB Dairy, and other articles regarding foot-trimming hygiene in the farming or veterinary press during the previous year. Twelve more (15%) had seen only the foot-trimming hygiene protocol, and thirteen reported seeing other articles only. Thirty-five participants (43.8%) agreed or strongly agreed that their awareness of the potential to spread BDD during foot-trimming had increased during the last year, whilst thirty-six (45.0%) agreed or strongly agreed that their awareness of appropriate hygiene and disinfection to prevent spread of BDD during foot-trimming had increased. Thirty-six respondents reported making changes to their hygiene management during foot-trimming in the previous year (36/80, 45.0%), totalling 64 improvements to hygiene between them (Figure 2A); affecting 1,383 farms (1,383/3,800, 36.4%) and 5,130 cows trimmed each week (5,130/12,660, 40.5%). Two respondents (2/36, 5.6%) reported that they had observed a reduction in BDD cases they attributed to the changed practice.

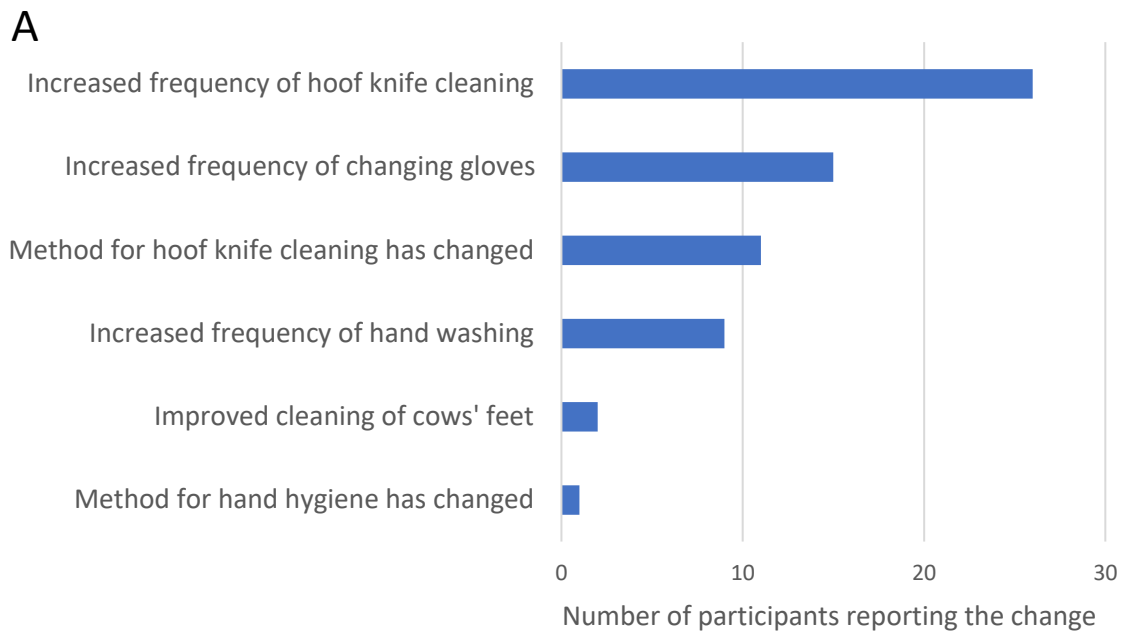
Univariate logistic regression showed that respondents were more likely to have seen the AHDB Dairy hygiene protocol if they were working in the UK (P<0.001), however, there was no association between country of origin and seeing other articles in the farming or veterinary press (P=0.65). Univariate logistic regression showed no association between the country of origin and changes in practice (P=0.32), however, having seen both the AHDB Dairy hygiene protocol *and* other articles increased the odds of changing the working practice (OR 1.49, 95%CI 0.34-2.64, P=0.011), suggesting that University of Liverpool research and knowledge exchange activities were effective, and had international impact, and multiple sources of information were needed. No association was found between the number of cows trimmed each week and changes made in practice (P=0.39) (Figure 2B).

Respondents were most likely to agree or strongly agree that difficulty in accessing water and cleaning facilities on farms was a barrier to improving foot-trimming hygiene, followed closely by those agreeing or strongly agreeing that they did not have time to disinfect each knife (Figure 2C).

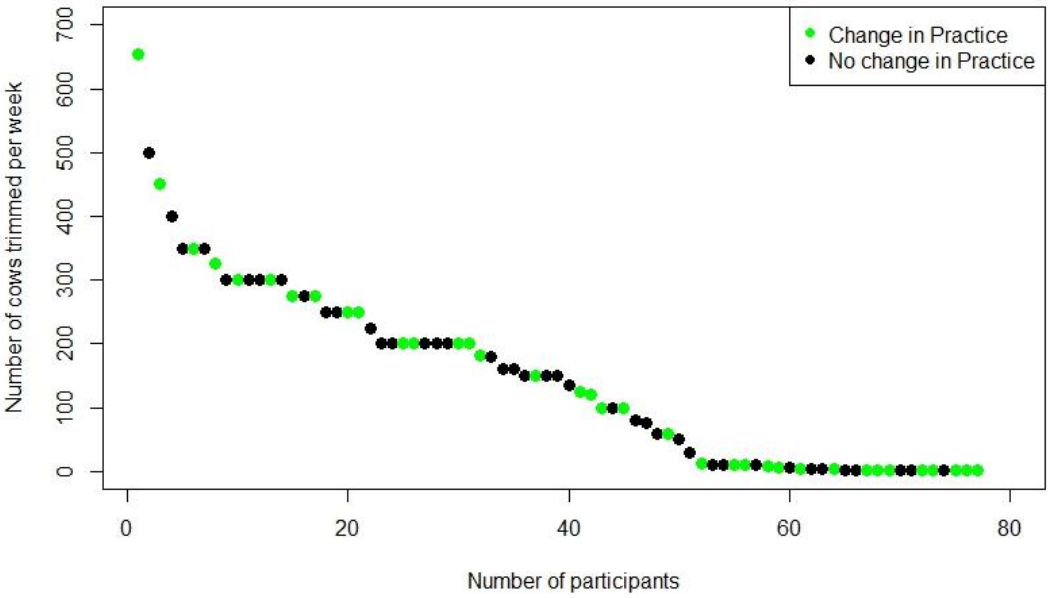
Thirty respondents suggested areas for future research. Seven (7/30, 23.3%) thought a trial of foot-trimming hygiene measures to assess the impact on BDD case numbers would be beneficial. Five (5/30,

16.7%) suggestions were concerned with further detailed aspects of the risk of BDD transmission during foot-trimming. Other popular themes were research on how to better engage farmers to help them improve management of BDD (4/30, 13.3%), and research on the effect of improving whole-herd foot hygiene on BDD case numbers (3/30, 10.0%).

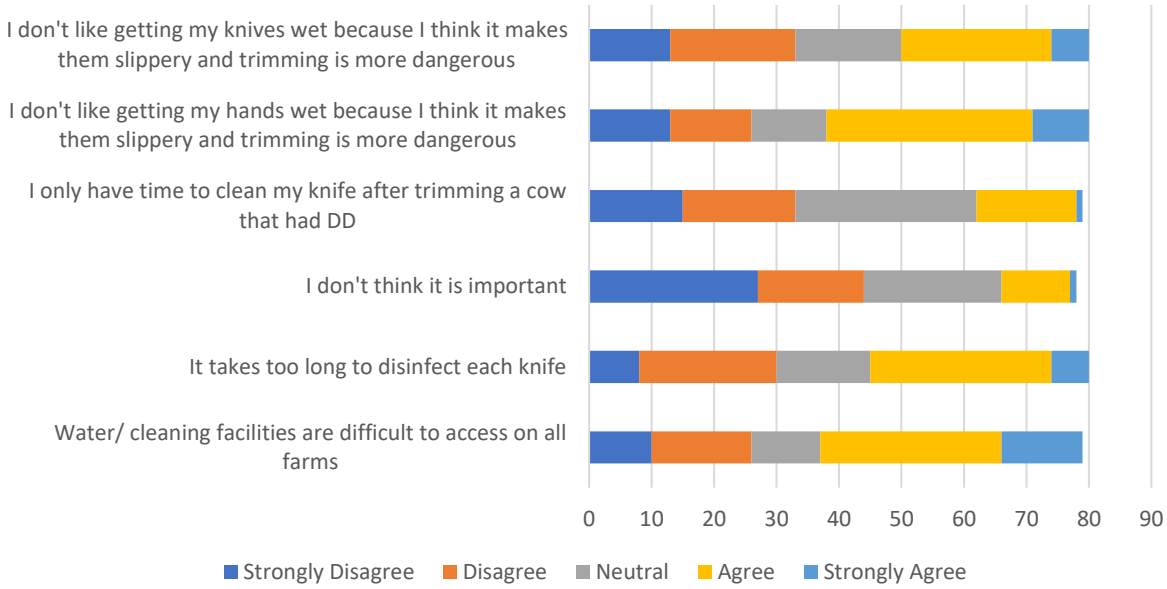
Figure 2: Results from the follow-up questionnaire. 2A. Number of industry professionals reporting different changes made to their foot-trimming hygiene practices in the last year. 2B: Highlighting those participants who reported changing their management of hygiene during foot-trimming, plotted against the number of cows they were trimming each week. 2C: Responses collected on a Likert scale to six statements describing barriers to improving foot-trimming hygiene which had been commonly identified via conversation with industry stakeholders.



B


Change in Practice
No change in Practice
Number of cows trimmed per week
0
100
200
300
400
500
600
700
0
20
40
60
80
Number of participants

C


I don't like getting my knives wet because I think it makes them slippery and trimming is more dangerous
I don't like getting my hands wet because I think it makes them slippery and trimming is more dangerous
I only have time to clean my knife after trimming a cow that had DD
I don't think it is important
It takes too long to disinfect each knife
Water/ cleaning facilities are difficult to access on all farms
0
10
20
30
40
50
60
70
80
90
Strongly Disagree
Disagree
Neutral
Agree
Strongly Agree

4.4 Discussion

The study purpose was to gauge current foot trimming hygiene practices and assess if recent research and knowledge exchange may have led to practical change. Questions were designed to measure awareness of the research, to determine extent of changes made to foot-trimming hygiene practices and to ascertain end user requirements for future research programmes.

The study has highlighted different attitudes and behaviours amongst professionals trimming cows' feet, with more than half of respondents not initially considering hand washing or hoof-knife cleaning in their workflow. Those who did reported a wide variety of different hygiene methods. Study participants came from a variety of career backgrounds with vocational training in cattle foot-trimming having varying degrees of formality, which may partly explain the spectrum of practices reported.

Those classified as practising adequate hygiene in the initial questionnaire were often trimming fewer than 15 cows per week, suggesting practical difficulties in implementation may be a barrier to widespread adoption of the protocol. Indeed, in the follow-up questionnaire, poor access to water and cleaning facilities on farms and lack of time to disinfect knives were most often identified as obstacles, supporting the concept that practical difficulties are considered a substantial barrier.

The follow-up questionnaire showed increased awareness of foot-trimming hygiene within the study year, which we attribute to our knowledge exchange work. Furthermore, we have shown that following both the AHDB Dairy hygiene protocol and other articles in the farming and veterinary press was associated with increased odds of changing practice, suggesting that the research and knowledge exchange activities were worthwhile and effective.

The main theme in suggestions for future research was to continue investigating transmission routes including ratifying the importance of fomites in BDD transmission (12/30, 40% of suggestions), indicating there is continued uncertainty in the industry surrounding the value of adopting good foot-trimming hygiene. Lack of proof of efficacy has previously been cited by farmers and veterinary surgeons as a perceived barrier to adoption of good biosecurity practices for prevention of spread of other diseases (Gunn et al., 2008). Although the request for future research ideas was presented as an open question, the subject of the questionnaire may have influenced this outcome.

The limitations of this study, including sampling methods, should be acknowledged. There is sample bias in the study population resulting from a non-random sampling strategy and convenience sampling, and therefore whilst we have targeted the major groups trimming cattle feet, the results should not be overly generalised. In particular, the online-only approach for advertising the follow-up

questionnaire may have selected for those who are more accustomed to use of social media and the internet. The second questionnaire may also have received biased responses, both in terms of those who responded and in terms of the impact reported on hygiene practices, because industry professionals who are already engaged with our research may have been more likely to participate (volunteer bias) (Eysenbach, 2004). Since both questionnaires were anonymous, however, we are not able to assess how many respondents answered both questionnaires.

In conclusion and notwithstanding these limitations, we identified that a comprehensive knowledge exchange programme of recent research has helped to rapidly increase knowledge and awareness of improving hygiene management during foot-trimming. The present survey results indicate a substantial uptake of suggestions contained in the foot-trimming hygiene protocol by the dairy industry, with the impact of preventing one possible route of transmission of BDD. There is acknowledgement of the continued difficulties with practical implementation of improved hygiene, and an expression of a continued requirement from industry stakeholders for BDD research. Areas of interest remain focussed on transmission routes and control of this important disease with associated knowledge exchange needed to maximize impact and help farmers to improve their herd management.

Chapter 5 Set, network and shotgun metagenomic analyses of the bovine foot skin microbiome; associations with development of Bovine Digital Dermatitis and differences between farms.

5.1 Preface

All samples used to produce the dataset for these analyses were collected and prepared for 16S rRNA gene sequencing by Dr. Veysel Bay as part of his degree of Doctor in Philosophy (Bay, 2018). The 16S rRNA gene sequencing was carried out by staff at the Centre for Genomic Research (CGR) at the University of Liverpool. Statistical analyses of the 16S rRNA sequencing data that is referred to throughout this chapter was carried out by Dr. Veysel Bay, and Dr. Luca Lenzi from CGR, and Dr. Erika Ganda from Penn State University Pennsylvania, USA. Modelling of the data for network analysis was carried out by Dr. Luca Lenzi, and data analysis for shotgun metagenomics was carried out by Dr. Sam Haldenby from CGR.

Further details of the 16S rRNA analysis can be found in the Supporting Publication, which is available as a preprint and is currently under peer review:

The bovine foot skin microbiota is associated with host genotype and the development of infectious digital dermatitis lesions
Bay V., Gillespie A., Ganda, E.K., Evans N., Carter S., Lenzi L., Lucaci, A., Haldenby S., Barden, M., Griffiths B., Sanchez-Molano E., Bicalho R., Banos G., Darby A., Oikonomou G.

The Bovine Foot Skin Microbiota is Associated with Host Genotype and the Development of Infectious Digital Dermatitis Lesions | Research Square

5.2 Introduction

A wide variety of bacteria besides treponemes have been identified in BDD lesions, often in combination. These include *Fusobacterium* spp*., Bacteroides* spp., *Dichelobacter nodosus, Guggenheimella bovis, Campylobacter* spp. and *Peptococcus* spp. (Blowey & Sharp, 1988; Döpfer et al., 1997; Rasmussen et al., 2012; Schlafer et al., 2008); and more recently *Porphyromonas levii, Mycoplasma* spp. *and Prevotella* spp. (Berry et al., 2010). This has led to the description of BDD as a polymicrobial disease, however little is known about the relationships and interactions between different bacterial genera or species, and how this might contribute to pathogenesis. It has been suggested that *Dichelobacter nodosus* is involved in the early stages of BDD lesions, perhaps by degrading superficial layers of the epidermis, making is accessible to colonisation by various phylotypes of *Treponema* (Rasmussen et al., 2012). The development of next generation sequencing and other genomic technologies has enabled us to begin to examine BDD pathogenesis considering the whole bacterial population, by studying the microbiome of BDD lesions.

The term “microbiota” refers to the community of microorganisms found in a particular habitat (for example on the bovine foot skin). The term “microbiome” refers to both the microbiota and their “theatre of activity” which includes for example their metabolites and signalling molecules, and the surrounding environmental conditions. In other words, the “microbiome” is comprised of more than simply the identity of the microorganisms found there (Berg et al., 2020). The microbiome is continuously exposed to disturbances and environmental factors which may lead to changes known as dysbiosis (Ferrer et al., 2017). Dysbiosis is defined as any alteration in the taxonomic composition of resident commensal microbial communities relative to those found in healthy individuals. There are three types: loss of beneficial microbes, overgrowth of harmful microbes, and loss of diversity in the microbial population; and it is likely that aspects of all three are required to promote disease (Petersen & Round, 2014).

There is a growing body of information published on the normal composition of the bovine foot skin microbiome. Skin biopsies of normal skin from dairy cows and fattening beef cattle, used as controls to compare to the microbiome of BDD lesions, showed phyla mainly belonged to *Firmicutes, Actinobacteria, Proteobacteria* and *Bacteroidetes* (Caddey et al., 2021; Nielsen et al., 2016). Amongst the four dominant phyla in dairy cows, there were four families that reached a prevalence of more than 5%: *Cornynebacteriaceae, Ruminococcaceae, Carnobacteriaceae* and *Moraxellaceae* (Nielsen et al., 2016). The sequences from BDD lesions reported in the same study were dominated by the phyla *Spirochaetes, Tenericutes, Fusobacteria and Bacteroidetes.* There were four families exceeding 5% abundance in lesions: *Spirochaetaceae, Mycoplasmataceae, Fusobacteriaceae* and *Porphyromonadaceae.* The four most abundant genera in lesions were *Treponema, Mycoplasma, Fusobacterium* and *Porphyromonas.* A different study found that the families *Moraxellaceae, Corynebacteriaceae, Lachnospiraceae* and *Ruminococcaceae* were overrepresented in healthy feet compared to BDD lesions in dairy cows (Krull et al., 2014). Another study compared the microbiome of 51 healthy foot skin biopsy samples with 89 biopsies from BDD lesions in dairy cows (58 active lesions and 31 inactive lesions). There were eight major phyla identified: *Firmicutes, Spirochaetes, Bacteroidetes, Actinobacteria, Proteobacteria, Tenericutes, Cyanobacteria* and *Fusobacteria.* In healthy skin *Firmicutes* and *Actinobacteria* were dominant, which was corroborated in results from shotgun metagenomic sequencing of 16 of the healthy skin samples (Zinicola et al., 2015a).

In beef cattle, BDD lesions were associated with increased relative abundance of the phyla *Spirochaetes* and *Tenericutes* and the families *Spirochaetaceae, Porhyromonadaceae, Mycoplasmataceae* family XI and *Fusobacteriaceae* compared to healthy skin. At genera level *Treponema, Porphyromonas, Mycoplasma* and *Fusobacterium* relative abundances were significantly higher in most lesion stages compared to healthy skin (Caddey et al., 2021). Active BDD lesions in dairy

cattle had reduced relative abundance of the phylum *Firmicutes* and increased relative abundance of *Spirochaetes* compared to inactive BDD lesions and healthy skin. Both active and inactive BDD lesions had reduced relative abundance of *Actinobacteria* compared to healthy skin (Zinicola et al., 2015a).

It would therefore appear that (in order of relative abundance) *Firmicutes, Actinobacteria, Proteobacteria* and *Bacteroidetes* are the core phyla of the healthy bovine foot skin microbiome and the families *Corynebacteriaceae, Ruminococcaceae,* and *Moraxellaceae* are candidates for core taxa at family level. At genus level *Treponema, Porphyromonas, Mycoplasma* and *Fusobacterium* are more abundant in most BDD lesion stages in fattening beef cattle (Caddey et al., 2021) and dairy cattle after slaughter (Nielsen et al., 2016). A recent meta-analysis of BDD microbiota reported that this applied consistently across studies, and these were considered the best genera for differentiating diseased from healthy skin (Caddey & de Buck, 2021). However, we should remain wary that technical variation in sample type, and experimental methods from DNA extraction to sequencing itself causes variation amongst datasets. The analyses of different datasets may not be comparable, as there are differences between the reference databases used and databases are constantly updated.

The human skin microbiome has been more extensively studied and papers commonly report the same four phyla as the core phyla for bovine foot skin (Egert & Simmering, 2016), although *Actinobacteria* appear to dominate, driven by the genera *Corynebacteria* and *Propionibacteria,* and are followed by *Firmicutes, Proteobacteria* and *Bacteroidetes* (Grice et al., 2009). In terms of skin lesions there are no studies of the microbiome of yaws lesions- the treponemal skin disease most directly comparable with BDD. However, microorganisms in Diabetic Foot Ulcers (DFUs) have been well studied. The bacterial populations differ from shallow to deeper ulcers, with deeper ulcers associated with more anaerobic bacteria and less abundance of *Staphylococcus,* and DFUs of longer duration had higher relative abundance of anaerobic bacteria and Gram negative *Proteobacteria* (Gardner et al., 2013). To draw a parallel with BDD lesions, it has been shown that the microbiome differs depending on lesion stage (Krull et al. 2014; Zinicola et al., 2015b).

It is also known that the microbial community in human skin differs depending on the physiology of the skin site, so a distinction is made between moist, dry, and sebaceous sites (Byrd et al., 2018). Applied to cows' feet, the physiology such as the pH of the skin could be affected by presence of slurry, and the common management practice of footbathing, increasing the influence that housing and management conditions could have on the bovine foot skin microbiome.

Since housing and the inevitable increase in exposure to slurry is associated with higher BDD incidence (Blowey & Sharp, 1988; Rodríguez-Lainz et al., 1996; Rodriguez-Lainz et al., 1998; Wells et al., 1999), it is possible that the presence of faeces could cause dysbiosis of the foot skin microbiome and make

cows more prone to development of BDD lesions. It is also possible that many of the bacteria inhabiting the foot skin originate from faeces, particularly in housed dairy cattle. The phyla *Firmicutes* and *Bacteroidetes* have the highest relative abundance in the bovine faecal microbiome (Hagey et al., 2019; Li et al., 2020; Uchiyama et al., 2020). Dominant families from the *Firmicutes* phylum include *Peptostreptococcaceae, Ruminococcaceae, Clostridiaceae* and *Lachnospiraceae.* Dominant families from the *Bacteroidetes* phylum include *Coriobacteriaceae, Rikenellaceae, Prevotellaceae, Paraprevotellaceae, Porphyromonadaceae* and *Bacteroidaceae* (Hagey et al., 2019). The bacterial genera *Prevotella*, *Ruminococcus, Lachnospiraceae* UCG-008, and *Eubacterium coprostanoligenes* group are present in more than 90% of all bovine faecal samples, whilst other genera such as *Alistipes, Bacteroides, Clostridium, Faecalibacterium* and *Escherichia/ Shigella* are also strongly faeces-associated (Holman & Gzyl, 2019).

Information about the microbiome of healthy foot skin comparisons with BDD lesions has been collected using gene sequencing, however sequencing does not differentiate between live colonising bacteria and dead transient bacteria. The role of bacteria in the microbiome and the overall structure and function of the community can be inferred by undertaking network analysis. This identifies and visualises significant co-occurrence patterns such as co-operative and competitive interactions between bacteria (Faust & Raes, 2012). An understanding of such microbe-microbe interactions is a prerequisite for understanding microbiome function and designing ways to manipulate the skin microbiota for preventing or treating skin disease using prebiotic or probiotic strategies (Egert & Simmering, 2016).

Whole metagenome sequencing offers two main advantages over marker gene sequencing: increased sequencing depth facilitating taxonomic resolution to species or even strain level; and the ability to assign functional capacity at gene level (Knight et al., 2018). There have been two studies of bovine foot skin that use shotgun metagenomics to examine the microbiome of different BDD lesion stages. Both identified taxonomic differences between lesions and healthy skin, particularly highlighting the increased abundance of *Treponema* spp. as lesion morphology progresses (Krull et al. 2014; Zinicola et al., 2015a). This is analogous to the more widely studied human oral treponematoses; which show progression from gingivitis to periodontal disease and the formation of periodontal pockets, which favour growth of *Treponema denticola* alongside *Porphyromonas gingivalis* and *Bacteroides forsythus* (now *Tannerella forsythus)* as the pockets become anaerobic (Edwards et al., 2003a). An increase in virulence factors relating to motility/ chemotaxis and iron acquisition were noted; and an increase in abundance of zinc and copper resistance genes in samples from BDD lesions compared to healthy skin (Zinicola et al., 2015a).

It has been shown using analysis of 16S rRNA gene sequencing data from cows at three commercial dairy farms that some genera of bacteria found in the bovine foot-skin microbiome are associated with future development of BDD lesions, whilst other genera appear protective (Bay et al., 2021). This chapter uses a multidisciplinary approach to provide further biological insights into the same bovine foot-skin microbiome data. First it reports further information on the composition of the microbiome by using set analysis to compare the most abundant taxa from feet that were healthy and those that were affected by BDD at the time of sampling. Since the previous work also showed that β-diversity of the foot skin microbiome differed depending on farm of origin, set analysis is also used to examine these differences. Network analysis is carried out to find differences in correlations amongst bacteria in the microbiota depending on whether the foot went on to develop a BDD lesion or not. Then shotgun metagenomics is used to understand in further detail possible roles for protective and detrimental bacteria in the bovine foot skin microbiome and the functional pathways via which they may be acting.

5.3 Methods

5.3.1 Sample collection

The 16S rRNA gene sequencing data was generated using swab samples from the heel bulbs of the back-left feet of 259 cows from three commercial dairy farms, 3-4 weeks prior to expected calving (Bay et al., 2021). Longitudinal inspection of the feet of enrolled cows was carried out at one, four- and 8-10-weeks post-calving. Although samples were collected only from the back-left foot (for reasons associated with project logistics), all four feet were inspected and lesions recorded of any of the five clinical BDD stages according to the established M-scoring system (Berry et al., 2012). This resulted in classification of the population into four foot-health groups according to the presence/ absence of BDD lesions on any foot as follows: HtHt: The cows which remained healthy during the study, HtIn: The cows which were healthy at sampling, then developed BDD, InIn: The cows which had BDD at all checkpoints, InHt: The cows which had BDD at the initial check point, but then recovered. In this study, the sequencing data was analysed by clustering sequences into operational taxonomic units (OTUs) based on 97% sequence identity. Inferences about the microbiome from different foot-health groups were made by comparing the relative abundance and diversity of OTUs (Bay et al., 2021).

5.3.2 Set analysis

The unfiltered table listing operational taxonomic units (OTUs) was generated by QIIME as previously described (Bay, 2018). The metadata was used to compile a list of OTUs present in each of the 12 sample groups at cow level (HtHt, HtIn, InHt and InIn for each of the three farms). An OTU was defined as present in a group if its overall relative abundance across all samples in the group was greater than 0.01%. Once lists of OTUs were compiled for the sample groups, they were uploaded to Intervene (asntech.shinyapps.io/intervene) (Khan & Mathelier, 2017) and intersections between sets of OTUs were visualized using UpSet (Lex et al., 2014). An UpSet plot was generated, which shows intersections in OTUs between farms, between disease classes, and considering both farm of origin and disease class. OTUs found to be unique to farm three were examined for taxa responsible for differences in β-diversity previously detected in farm three samples compared to farms one and two (Bay et al., 2021). Data was filtered in Microsoft Excel to show the top 20 genera found in samples taken from macroscopically healthy feet (HtHt and HtIn groups), and the top 20 genera found in samples taken from BDD-infected feet (InIn and InHt groups). This was used to identify whether more OTUs from the top 20 genera were associated with healthy or infected samples as a proxy for detrimental or protective genera.

5.3.3 Network analysis

16S rRNA gene sequencing techniques produce compositional data, meaning that the abundance counts are normalized to the total number of counts in the sample, and so microbial counts are not independent and conventional statistical procedures such as correlation (for detecting OTU-OTU relationships) can produce spurious results. 16S rRNA gene sequencing techniques produce sparse datasets because they measure hundreds of OTUs on a relatively small number of samples, leading to datasets where inference of OTU-OTU association networks is severely under-powered. Therefore, to identify the ecological interactions among the microbial taxa in the samples (Faust & Raes, 2012), co-occurrence analysis for the previously identified OTUs was performed using the SParseInversE Covariance Estimation for Ecological ASsociation Inference tool, a model that uses a sparse inverse covariance selection strategy to reconstruct the network (Kurtz et al., 2015), which was implemented in R (version 3.6.3) using the SpiecEasi package. OTUs below 0.005% of the total frequency were excluded (Bokulich et al., 2013), reducing the number of OTUs from 75,643 to 3,039. Then, starting with the OTU abundance table, data is transformed using a centred log-ratio transformation, so that the data is converted to a scale to which it is possible to apply conventional statistical procedures. Two OTUs (nodes) are linked if doing so provides additional information about the other, given the state of all other nodes in the network. It avoids detection of correlated OTUs that are indirectly

connected, for example via another OTU. This is considered the most accurate and informative way to construct an undirected network and is one of the few tools designed to work with compositional and sparse data sets (Layeghifard et al., 2017). The interaction graphs are estimated from the transformed data. This is done using the Stability Approach to Regularisation Selection (StARS) scheme to identify the relevant co-occurrences. This method repeatedly uses random subsamples of 80% of the data to estimate an interaction graph. The stability of edges is then calculated, and they are ranked according to their confidence to find a network in which the subsampled graphs have the least variable edges (Liu et al., 2010). The graphs produced are undirected, so although they describe interactions between nodes as positive or negative, they do not describe which partner is exerting the influence, or the magnitude of that influence. Co-occurrences with magnitude of correlation of less than 0.15 were excluded.

Network visualisation was carried out using Cytoscape Version 3.6.1 (Shannon et al., 2003). Comparisons were made between HtHt and HtIn groups to identify mutualistic or competitive interactions that differ between the two disease groups at phylum level, and therefore may influence development of BDD lesions. Nodes were filtered to include those from the six phyla contributing >1% of OTUs and network statistics were computed using NetworkAnalyzer and compared.

Genus level analysis was restricted to the following genera: *Acholeplasma* spp., *Anaerococcus* spp., *Fastidiosipila* spp., *Peptoclostridium* spp., *Prevotella* spp., *Porphyromonas* spp. and *Succiniclasticum* spp. according to those found by response screening to be more prevalent in HtIn groups compared to HtHt groups in the 16S rRNA gene microbial analysis (Bay et al., 2021). *Macrococcus* spp. and *Brachybacterium* spp. were included as they were found to be less prevalent in HtIn samples. *Treponema* spp. were also investigated as they were absent from the top 20 most prevalent genera in HtHt samples, but present in the top 20 for HtIn samples, and they are widely considered to be pathogenic in BDD.

5.3.4 Shotgun metagenomic analysis

Shotgun metagenomic analysis was undertaken for five samples from each of the HtHt and HtIn groups to compare cows that developed BDD with those that didn't with higher taxonomic resolution, and to investigate differences in functional profiles. To ensure the two groups were comparable for this small sample size, cows were selected from one farm. To maximise the chances of achieving sufficient sequencing depth, cows were selected at random from those with >5ng/µl DNA content in samples after initial DNA extraction was carried out for 16S rRNA sequencing, as measured using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Fair Lawn, NJ, USA). Microbial DNA was extracted from a second set of swabs that had been collected parallel to those used in the 16S rRNA gene analysis.

The DNA extraction method was the same, using the PureLink™ Microbiome DNA Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Agarose gel electrophoresis was carried out using SYBR green as the nucleic acid stain (Thermo Fisher Scientific Fair Lawn, NJ, USA) to ensure presence of clear DNA bands.

gDNA samples were submitted to the Centre for Genomic Research for library preparation using the Nextera XT kit (Illumina). gDNA input was quantified using Qubit™ to ensure 1ng of each sample was submitted for tagmentation. Whole samples were used in a limited amplification step (12 cycles) which incorporates the indexes at the priming step. Libraries were cleaned using 0.6x AMPure beads. After elution, the quantity of the library was checked by Qubit™ assay and the quality checked on a DNA high sensitivity bioanalyser chip. The final libraries were pooled in equimolar ratio and the quantity and quality of the final pool assessed by Qubit™ and Bioanalyzer, and subsequently by qPCR using the Illumina Library Quantification Kit from KAPA (KK4854) on a Roche Light Cycler LC480II according to manufacturer's instructions. The final loading concentration of 300pM was reached by adding 35µl of exclusion amplification enzyme mix. The libraries were sequenced on an Illumina HiSeq 4000 platform using sequencing by synthesis (SBS) technology to generate 2 x 150bp paired-end reads.

Data files were demultiplexed and converted to FASTQ format using Casava v.1.8.2 (Illumina). FASTQ files were trimmed using option _O3 Cutadapt version 1.2.1 (Marcel Martin, 2011) to exclude those matching Illumina adaptor sequences by $\geq$ 3bp at the 3' end. Reads were further trimmed to remove low quality bases, using Sickle version 1.200 with a minimum window quality score of 20. After trimming, reads shorter than 20bp were removed, and single reads were excluded as length distributions showed they were of poor quality. Host reads were removed following alignment against the host Bos Taurus genome ARS-UCD1.2 (GCA_002263795.2) using Bowtie2 v2.2.6 (Langmead & Salzberg, 2012): read pairs where one or both reads aligned were removed. The remaining reads in pairs were merged using PEAR v0.9.11 (Zhang et al., 2014) to form a single long read based on overlapping homology. Those that could not be merged in this way were concatenated with an intervening N-base. The resulting sequences underwent taxonomic assignment using Kraken v0.10.6 (Wood & Salzberg, 2014) and results were filtered using a confidence threshold of 0.1. Results were analysed using Linear discriminant analysis effect size (LefSe) (Segata et al., 2011) to determine the taxa most likely to explain differences between the two classes HtHt and HtIn. The HUMAnN2 search strategy (Franzosa et al., 2018) was used to functionally annotate read data and abstracts to show biological pathway abundance and completeness. HUMAnN2 uses the MetaPhlAn2 computational package (Truong et al., 2015) to screen DNA reads to identify known microbial species. It then constructs a pangenome database specific to each sample and maps reads against the sample's pangenome database (which is functionally annotated). Finally, reads that do not align to their

pangenomes are submitted to a protein database (UniRef) for translated searching (Franzosa et al., 2018). The gene families identified are further analysed using the MetaCyc database to reconstruct and quantify complete metabolic pathways (Caspi et al., 2015).

Samples were renormalized for sequence depth and terms converted using GO slim (EMBL-EBI, Cambridgeshire, UK) to generate heatmaps representing biological processes, molecular functions, and cellular components.

5.4 Results

After 16S rRNA gene sequencing, samples were rarefied to 135,000 samples per sequence leading to exclusion of 17 samples; consequently, 242 samples remained in the final dataset with 235 classified into four groups according to foot health status. HtHt cows never had digital dermatitis lesions, HtIn were healthy pre-calving, but subsequently developed BDD, InIn had BDD pre-calving and did not recover at any sampling point, and InHt had BDD pre-calving but recovered at a subsequent sampling point. This resulted in 12 sample groups for analysis (four disease categories from each of three farms). Disease classifications were made at cow-level considering the health status of all four feet. Table 5.1 shows this classification of the cows by foot health status and by farm of origin.

Table 5.1 Classification of cows into groups according to their BDD status and farm of origin

Farm	**HtHt* n=112**	**HtIn n=48**	**InIn n=58**	**InHt n=17**	**Unknown n=7**
1 n=83	40	16	14	12	1
2 n=51	13	18	14	1	5
3 n=108	59	14	30	4	1

*HtHt: The cows which remained healthy during the study, HtIn: The cows which were healthy at sampling, then developed BDD, InIn: The cows which had DD in all checkpoints, InHt: The cows which had BDD at initial check point then recovered, Unknown: The cows which could not be followed adequately, died or were sold during the study.

5.4.1 Set analysis

After selecting OTUs that were present at a minimum of 0.01% prevalence in all samples, the data for set analysis contained 2,234 OTUs in 12 categories according to BDD health status and farm (Figure 1). OTUs were classified into 338 taxonomic groups. The largest number of OTUs (n=378) were shared amongst all 12 sample categories. The second largest intersection (n= 234 OTUs) was between all disease groups on farm three. Table 5.2 shows the ten most common genera identified as unique to

farm three. These are predominantly obligate anaerobes which are strongly faeces- associated (Hagey et al., 2019; Li et al., 2020; Uchiyama et al., 2020).

Figure 1 UpSet plot generated by Intersection analysis showing the distribution of 2,234 OTUs amongst 12 categories.

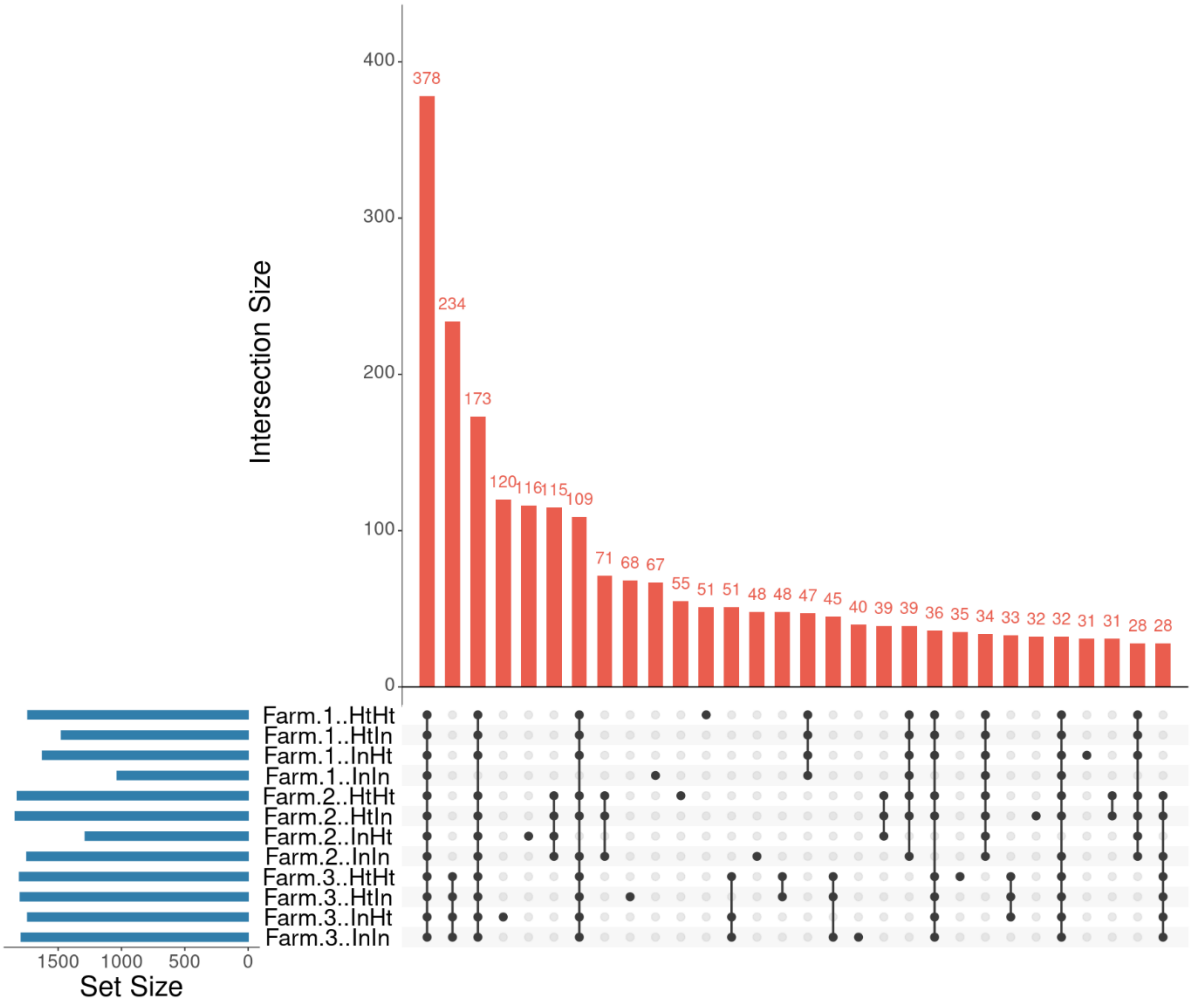


Table 5.2 Number of OTUs in the top ten genera identified as unique to farm three samples using Set Analysis.

* Lachnospiraceae family not identified to genus level

Genera	Number of OTUs
Ruminococcaceae UCG-010	42
uncultured bacterium	19
Rikenellaceae RC9 gut group	18
Lachnospiraceae*	18
Ruminococcaceae UCG-013	15
Ruminococcaceae UCG-005	12
Christensenellaceae R-7 group	12
Ruminococcus 2	7
[Eubacterium] coprostanoligenes group	6
Alistipes	6

A comparison was made between swabs taken from macroscopically healthy feet (HtHt and HtIn groups) and those taken when the foot was infected (InIn and InHt groups). Examining the top 20 genera from each group identified 29 different genera. Most of the 29 genera identified had more OTUs in common between healthy and diseased samples than differed (Table 5.3). Eleven genera showed more differences than similarities in OTUs when comparing healthy and diseased samples, indicating different bacterial species between the groups which may indicate pathogenic or protective roles. These included five potential pathogenic genera more abundant in infected samples, *Treponema* spp., *Acholeplasma* spp., *Fastidiosipila* spp., *Helcococcus* spp. and *Proteiniclasticum* spp., and one group of OTUs that could only be identified at phylum level (Bacteroidetes). *Flavobacterium* spp. had more OTUs identified in healthy samples, potentially indicating a protective effect of some species from this genus.

Table 5.3 Comparison of top twenty most prevalent genera identified as unique to healthy or BDD-infected feet using Set analysis of 16S rRNA gene sequencing data from skin swab samples (n= the number of samples in each group).

Key:

	Members of the top 20 most prevalent genera in both groups (HtHt, HtIn, InIn and InHt cows)
	Members of the top 20 most prevalent genera in the healthy groups only (HtHt and HtIn cows)
	Members of the top 20 most prevalent genera in the infected groups only (InIn and InHt cows)
	Genera showing more differences than similarities between healthy and infected groups

* Lachnospiraceae and Ruminococcaceae families OTUs not identified to genus level

^ Bacteroidetes phylum OTUs not identifiable at lower taxonomic levels

Genera	No. of OTUs from healthy feet (n=160)	No. of OTUs from infected feet (n=75)	No. of OTUs shared between healthy and infected feet
Ruminococcaceae UCG-010	32	16	132
uncultured bacterium	16	20	61
*Lachnospiraceae	16	14	62
Rikenellaceae RC9 gut group	14	26	80
Christensenellaceae R-7 group	11	9	49
[Eubacterium] coprostanoligenes group	10	5	49
Ruminococcaceae UCG-005	9	7	101
uncultured	5	15	37
Ruminococcaceae UCG-014	4	11	14
Corynebacterium 1	3	5	24
Lachnospiraceae NK3A20 group	3	5	20
*Ruminococcaceae	10	3	26
Pseudomonas	9	3	27
Flavobacterium	7	2	8
Ruminococcaceae UCG-013	5	3	39
Alistipes	5	2	35
dgA-11 gut group	5	1	9
Porphyromonas	3	5	13
Akkermansia	3	1	11
Ruminiclostridium 5	3	3	4
Treponema 2	0	15	12
Fastidiosipila	0	14	13
Helcococcus	0	10	1
Ruminococcus 2	2	6	17
Acholeplasma	1	6	4
Psychrobacter	2	5	6
^Bacteroidetes	1	5	5
Bacteroides	2	4	48
Proteiniclasticum	0	4	2

5.4.2 Network analysis

A comparison of network analysis statistics is shown in Table 5.4. Networks using OTUs defined to phylum level for the six phyla contributing more than 1% of OTUs for the HtHt and HtIn groups at cow level are shown in Figure 2.

Table 5.4. Simple Parameters from Network Analyses for HtHt and HtIn groups, carried out using the six phyla that accounted for more than 1% of total OTUs.

Group	HtHt n=112	HtIn n=48	Definition
Parameter			
Number of nodes	2339	2802	Nodes represent OTUs
Number of edges	2311	3564	Edges represent correlations (positive or negative) between Nodes (OTUs)
Isolated nodes	18	18	Nodes (OTUs) that are not correlated with any others in the network
Connected components	244	66	Lower = stronger connectivity
Network diameter	51	28	Largest distance between two nodes
Network centralisation	0.003	0.002	How central the most central node is compared to how central all the other nodes are.
Network heterogeneity	0.561	0.503	Reflects the tendency of the network to contain hub nodes: 1 would mean uniformity
Characteristic path length	17.228	9.821	Shorter = stronger connectivity
Average number of neighbours	1.976	2.559	More = stronger connectivity
Clustering coefficient	0.024	0.008	Nodes with <2 neighbours are assumed to have a clustering coefficient of 0
Network density	0.001	0.001	Solely isolated nodes would score 0, cliques would score 1

Both groups have low network density and network centralisation with no hub nodes, identifying these as random networks (Layeghifard et al., 2017). Network heterogeneity is slightly lower for the HtIn group as there is a higher average number of neighbours. The HtIn group has fewer connected components, shorter characteristic path length, more edges, and smaller network diameter despite a larger number of nodes, showing stronger connectivity and shorter expected distances between nodes. The more highly connected nature of the bacterial network in samples from the HtIn feet is apparent from the network images shown in Figure 2. When the ten genera that were known to differ in relative abundance between groups were selected and examined alongside their adjacent nodes, it was noted that more negative interactions (denoted by red edges) existed in HtIn groups: 641/ 3564 (18.0%) compared to the HtHt samples 160/ 2311 (6.9%). There was no clear pattern to the identity

of adjacent nodes, but it was apparent that the negative interactions originated from the eight genera (*Acholeplasma* spp., *Anaerococcus* spp., *Fastidiosipila* spp., *Peptoclostridium* spp., *Prevotella* spp., *Porphyromonas* spp., *Succiniclasticum* and *Treponema* spp) that were more abundant in the HtIn samples compared to the HtHt samples (Figure 3).

Figure 2 Bacterial networks for the six phyla contributing >1% of total OTUs as measured by 16S rRNA gene sequencing in (A) HtHt and (B) HtIn samples

(HtHt: The cows which remained healthy during the study. HtIn: The cows which were healthy at initial sampling, then developed BDD). Green edges represent positive correlations between nodes; red edges represent negative correlation.

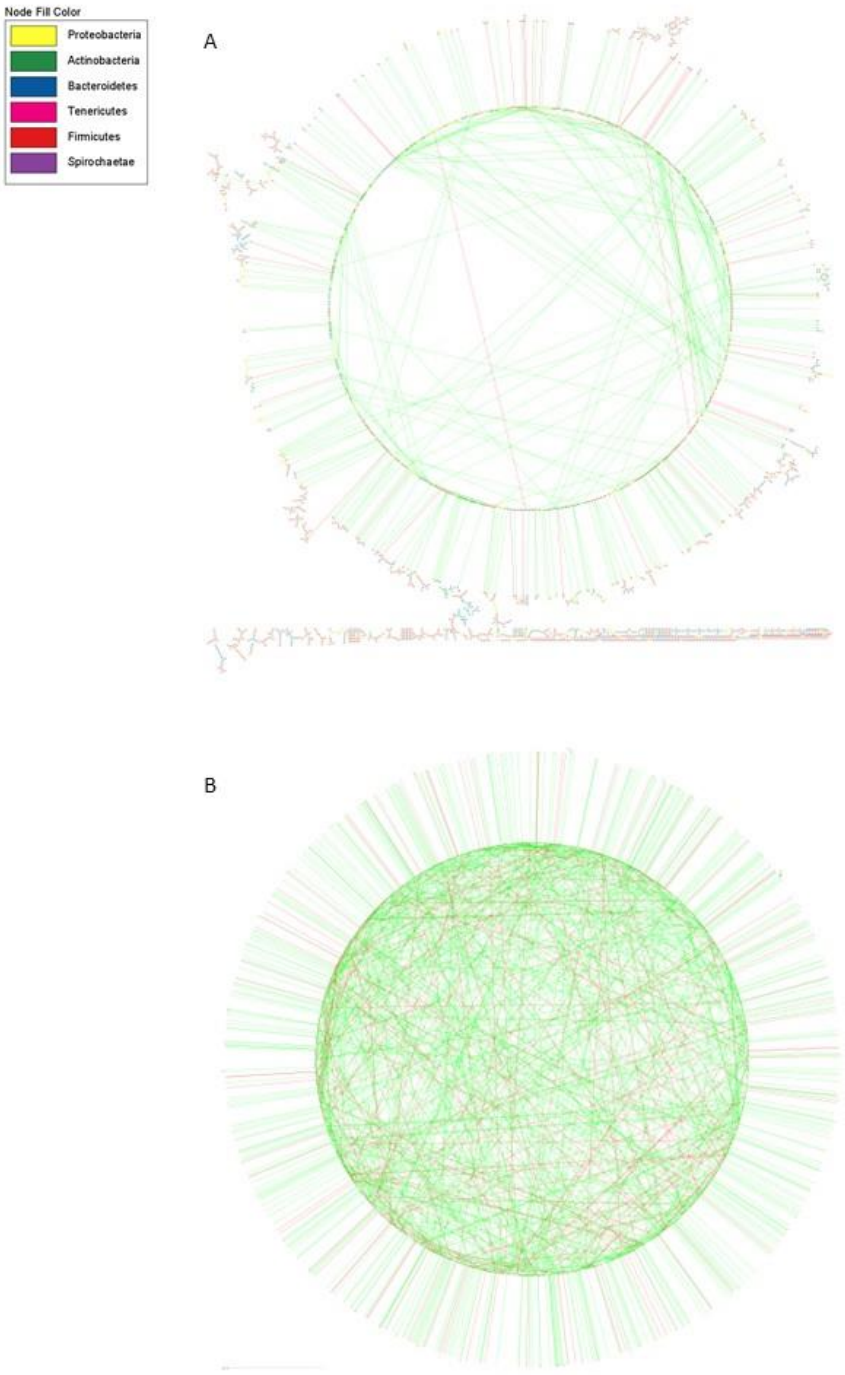
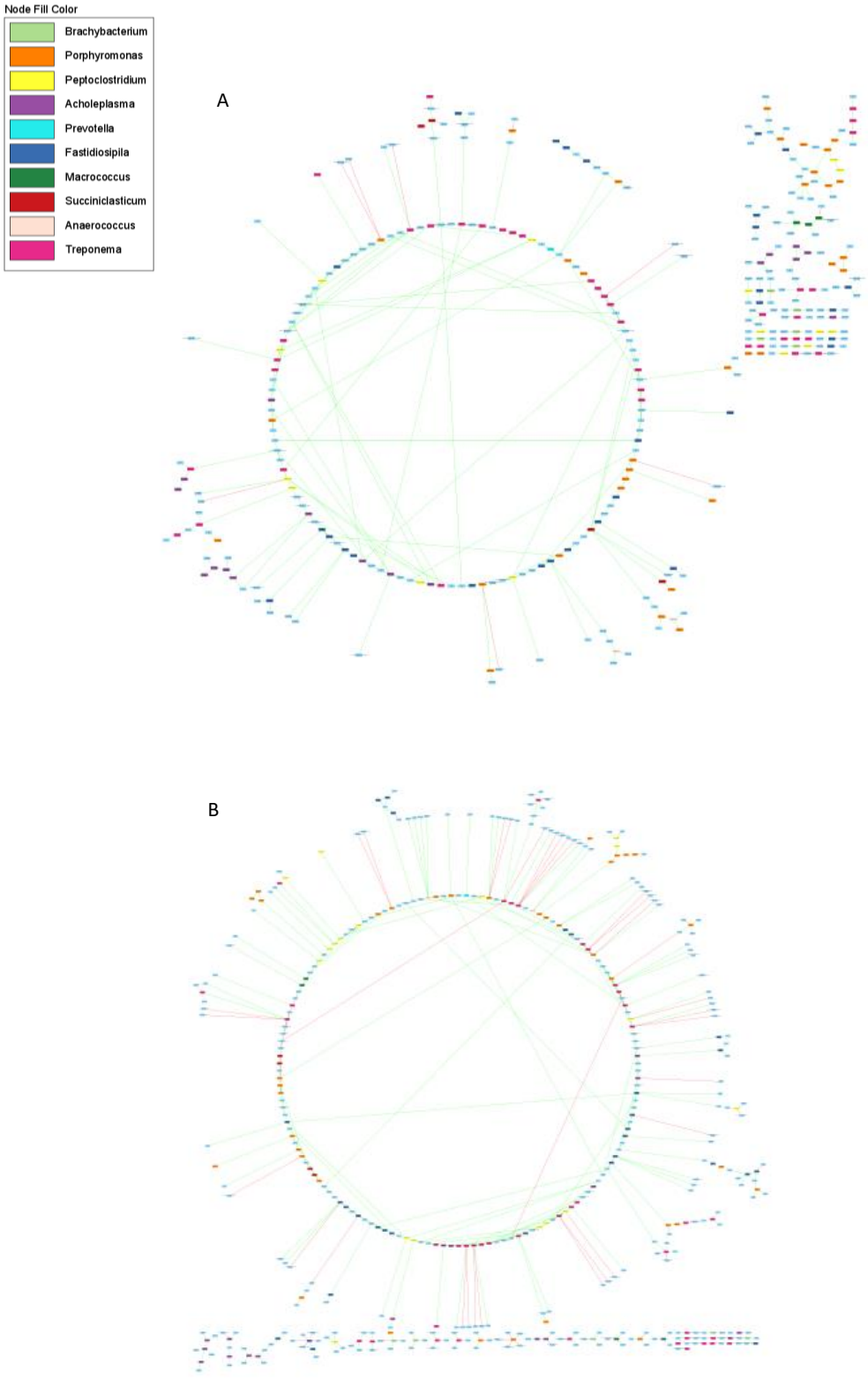


Figure 3 Bacterial networks for the ten genera found to differ in relative abundance when measured by 16S rRNA gene sequencing in (A) HtHt and (B) HtIn samples

(HtHt: Cows which remained healthy during the study. HtIn: Cows were healthy at initial sampling, then developed BDD). Green edges represent positive correlations between nodes; red edges represent negative correlations.

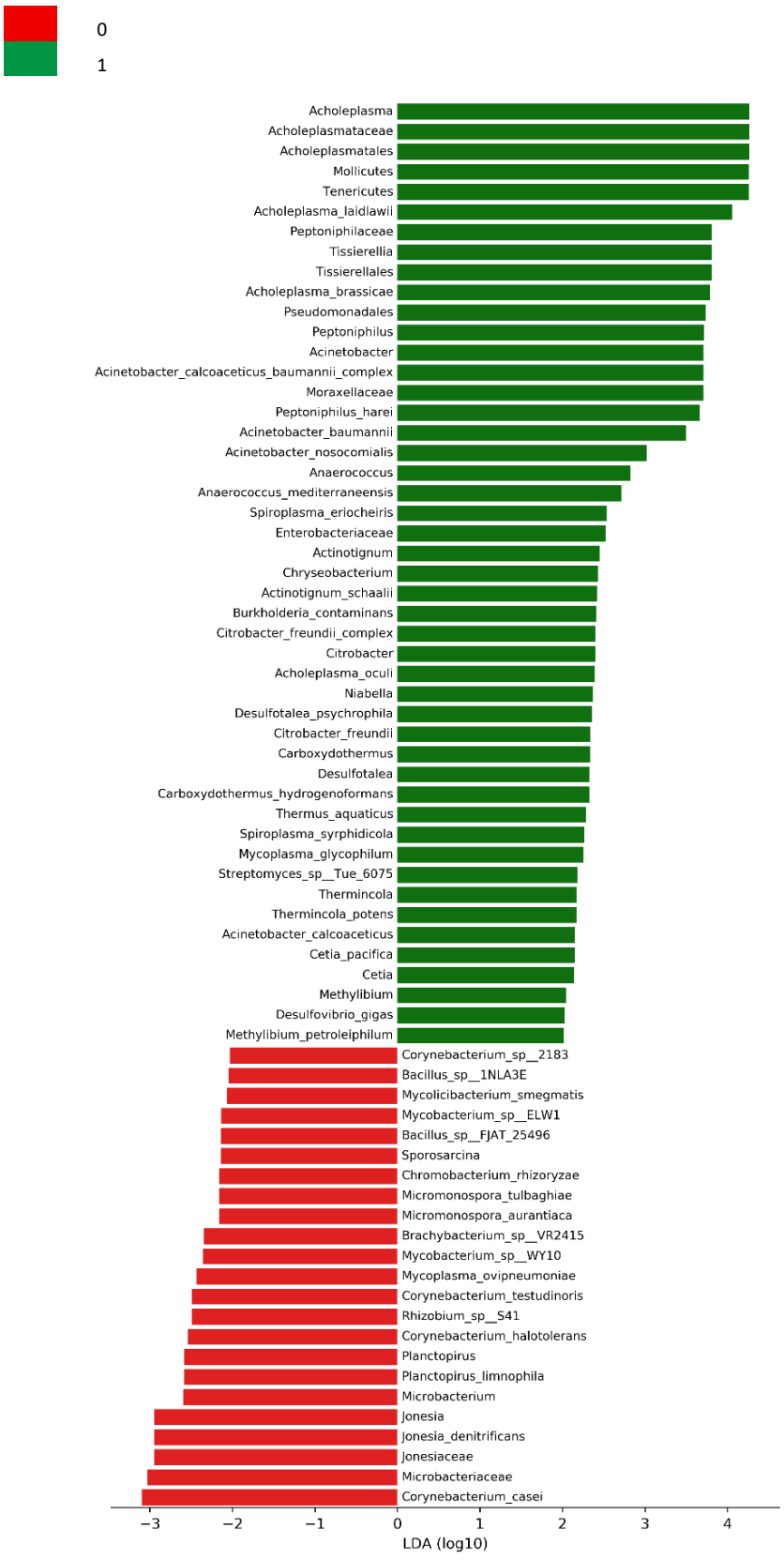


5.4.3 Shotgun metagenomics

DNA concentrations and sample quality for the ten samples selected (five from HtHt cows and five from HtIn cows) are shown in Table 1 in Appendix C. Samples yielded numbers of read pairs in the range 21,491,970 - 40,468,932. The percentage of host reads removed was low, in the range 0.09 - 8.73%. Taxonomic assignment using Kraken, which showed a precision of 98.25% and a sensitivity of 75.13% in test sets, identified only 4.03-6.42% of the remaining reads.

LefSe analysis revealed some differences in the taxa between the HtHt and HtIn group. Twenty-three taxa were significantly associated with the HtHt group using LDA score of >2. Forty-seven taxa were significantly associated with the HtIn group (Figure 4). *Brachybacterium* spp. was identified as significantly associated with HtHt samples, and several taxa from the *Acholeplasmataceae* family were significantly associated with HtIn samples. These findings may correlate with the 16S rRNA sequencing analysis findings of increased relative abundance of *Brachybacterium* spp. in HtHt samples, and the genera *Acholeplasma* spp. and *Porphyromonas* spp. at cow level in HtIn samples (Bay et al., 2021).

Figure 4 LefSe analysis showing taxa that were most likely to explain differences between the microbial communities in the HtHt (0) group and HtIn (1) group.

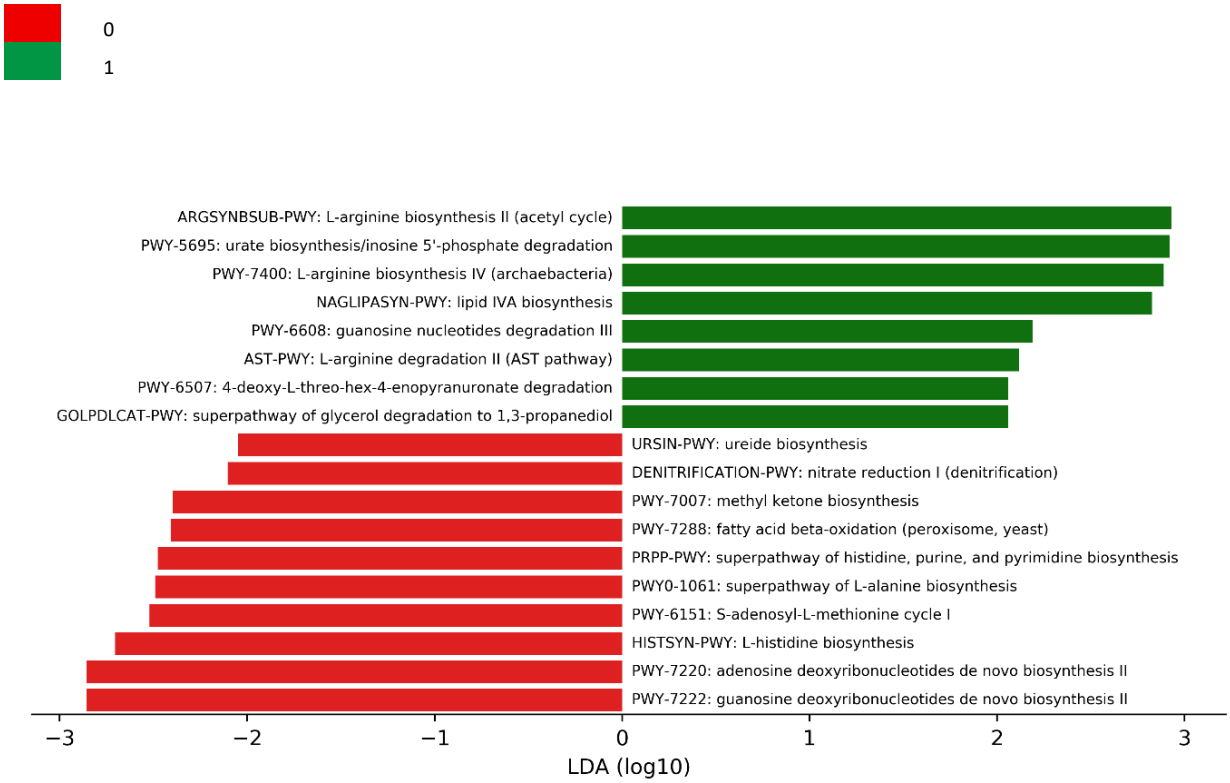


Ten functional pathways were identified as significantly more abundant in the HtHt group (Figure 5). All were metabolic pathways for synthesis or degradation of amino acids or fatty acids, or pathways involved in nucleotide synthesis. One of these pathways is found only in yeast. The presence of a yeast-associated pathway is plausible as cows' feet could be contaminated with feed and bedding material, and taxonomic classification identified members of the order Rhizobiales.

Eight functional pathways were identified as significantly more abundant in the HtIn group (Figure 5), of which three were associated with degradation of nucleotides and one indicated production of 4-deoxy-L-*threo*-hex-4enopyranuronate, which is a uronic acid resulting from the degradation of many polymers. These include plant polymers such as pectin and gellan, but also important components of connective tissue such as heparin, heparin sulfate, hyaluronan and chondroitin sulfate (Maruyama et al., 2015). Further explanation of the functional pathways identified can be found in Table 2 in Appendix C.

Despite detection of some differences in individual functional pathways, no overall differences in abundance for gene families in the GO slim categories of biological processes, molecular functions or cellular components were detected (Appendix C, Figure 1).

Figure 5 Biologically relevant differences between HtHt (0) and HtIn (1) samples in functional pathways identified using HUMAnN2.



5.5 Discussion

It is known that subclinical BDD exists, as histologically identifiable skin changes occur before lesion appearance (Rasmussen et al., 2012). This work shows that the microbiome is also changing before lesion appearance, both structurally and functionally. Understanding mechanisms of early pathogenesis is key to development of preventative strategies.

5.5.1 Set Analysis

Intervene is a useful tool for visualising and comparing data which falls into multiple categories (Khan & Mathelier, 2017). The resulting plot (Figure 1) highlights the taxonomic differences found in samples from farm three, which may be due to environmental conditions in which the dry cows were housed. This unknown effect of the farm environment has been previously reported as a limitation in microbiome studies of the bovine foot skin (Ariza et al., 2019b). In our case the main differences in farm management between farm three and farms one and two that could explain the observed differences in foot skin microbiota profiles were the following: sampled animals in farm three were housed in deep sand bedded cubicles and were walking through a 2% formalin footbath three times a week; sampled animals in farms one and two were housed in deep straw bedded yards and were not walking through a footbath when they were not milked. Given that the majority of OTUs unique to farm three were strongly faeces-associated, these differences could also be attributed to significant differences in dry cow diet, however these were not documented as part of this study. It is also possible that the findings reflect the fact that a greater proportion of farm three samples belonged to the HtHt foot-health group than on the other two farms (Table 5.1).

Set analysis also identified five genera with more OTUs unique to infected samples than shared with healthy samples, suggesting pathogenicity. The Acholeplasma genus is part of the class of wall-less bacteria Mollicutes, alongside the genus Mycoplasma (Holt et al., 2000b). In addition to the genus being present in BDD lesions (Zinicola et al., 2015b), the Acholeplasma type strain *A. laidlawii* has been associated with bovine infertility and has been isolated from cases of infectious bovine keratoconjunctivitis (Baptista, 1979; Doig, 1981). The Helcococcus genus includes *Helcococcus ovis*, a catalase negative gram-positive aerobic coccus, which has been associated with bovine uterine disease and endocarditis, and ovine mastitis (Songer & Post, 2005). Comparatively little is known about the genera Fastidiosipila and Proteiniclasticum as they are relatively recently described. Proteiniclasticum is a genus of gram-negative anaerobes belonging to the Clostridiaceae family. The only recognised species currently is *P. ruminis,* a proteolytic species isolated from a yak rumen (K. Zhang et al., 2010). Fastidiosipila is a genus of gram-positive cocci related to the *Clostridium*

subphylum, for which the type strain is *F. sanguinis,* which has been isolated from human blood (Falsen et al., 2005).

Treponema spp., *Acholeplasma* spp. and *Helcococcus* spp. which have been previously identified as associated with BDD lesions (Caddey et al., 2021; Choi et al., 1997; Demirkan et al., 1998, 1999; Evans et al., 2008, 2009; Zinicola et al., 2015b) and future development of BDD lesions (Bay et al., 2021). *Fastidiosipila* spp. have been previously reported as significantly more abundant in bovine sole ulcers, toe necrosis and white line disease foot lesions; and *Helcococcus* spp. as more abundant in bovine interdigital phlegmon (footrot) samples (Bay et al., 2018).

Flavobacterium spp., found widely distributed in soil and water (Holt et al., 2000a), was associated with healthy samples (HtHt and HtIn). This genus was the second most highly associated with HtHt samples using Songbird analysis of the original 16S rRNA data analysis and appeared to be driving the differences in β-diversity between farm three and the other two farms (Bay et al., 2021).

5.5.2 Network analysis

Network analysis is used as a technique for visualising ecological interactions between microbes which collectively are more important that the presence of the constituent individual species (Layeghifard et al., 2017). This technique has been carried out for eight BDD-associated bacterial species found at high abundance in BDD lesions in beef cattle and reported possible functional differences within lesions between *Fusobacterium* spp. (Caddey et al., 2021). In our data genera identified as detrimental or protective for future development of BDD are interacting differently in the HtHt samples compared to HtIn samples. The increase in negative interactions in the HtIn group may indicate a more competitive environment in the foot skin microbiome of these cows. This could be attributable to overgrowth of the eight genera identified as more abundant in HtIn samples (Faust et al., 2012). Whilst we were unable to identify specific interactions or bacterial complexes that may contribute to BDD pathogenesis, it is possible an increase in competitive interactions could correspond to dysbiosis and could identify these eight genera as pathogens that contribute to early BDD lesion development.

5.5.3 Shotgun metagenomics

Shotgun metagenomic analysis showed differences in taxa present in HtHt compared to HtIn samples. LEfSe analysis reveals biologically relevant differences between microbial communities rather than only statistical differences in features, in this project differences in relative abundance between clades and functional pathways. The scores assigned can be interpreted as the degree of consistent difference identified in the two classes that explain the greatest differences between the communities (Segata et al., 2011). HtHt samples had increased relative abundance of many Gram-positive bacteria

from the phylum *Actinobacteria* which would be expected to be part of the healthy foot skin flora (Nielsen et al., 2016; Zinicola et al., 2015a), for example *Corynebacteriaceae,* which were previously found to be over-represented in healthy skin samples (Krull et al., 2014). HtIn samples showed biologically relevant increases in cell wall-less bacteria from the *Tenericutes* phyla, which have previously been found to be more abundant in BDD lesions (Nielsen et al., 2016). Taxonomic assignment of reads was low and therefore the significance of these findings is uncertain, however increased relative abundance of several taxa from the Acholeplasmataceae family in the HtIn group may represent early overgrowth of species such as *Porphyromonas* spp. and *Acholeplasma* spp. which have been previously associated with BDD lesions (Zinicola et al., 2015b).

Functional differences in the microbiome, for example increases in genes for flagellar motility and zinc and copper resistance, have been previously reported in biopsies taken from BDD lesions compared to healthy skin (Zinicola et al., 2015a). Our data may suggest an increase in pathways relating to degradation of connective tissues, but the same pathways could indicate increased degradation of plant polymers in HtIn samples, which could be due to increased contamination with bacteria from faeces or bedding materials rather than the foot skin. It is unknown whether bacteria from faeces which would normally degrade plant polymers are also capable of degrading mammalian connective tissues. Significant differences in the abundance of gene families responsible for upregulation of these pathways were not detected. Either functional differences in the skin microbiome do not materialise before development of morphological lesions or are undetectable from our data, perhaps due to small sample sizes and a large percentage of unassigned sequences.

Overall, greater resolution to look at strain level may be required to uncover the true differences in the foot skin microbiome between those cows that develop BDD and those that do not. This is because gene content and expression differences between strains will determine the functional differences between health and disease. Functional genomics studies are needed, which will require the development of more detail in the reference genomes. Functional pathways identified are limited by the contents of the MetaCyc database. Interpretation also needs to be cautious as pathways have not been experimentally elucidated (Caspi et al., 2015).

5.6 Conclusion

Set analysis added to the evidence that *Acholeplasma* spp., *Fastidiosipila* spp., *Helcococcus* spp., and *Proteiniclasticum* spp. can be considered pathogenic in BDD lesions, and their role alongside the *Treponema* spp., which are widely considered to be causative, needs further investigation. It suggested *Flavobacterium* spp. as associated with healthy feet, which alongside the original 16S rRNA analysis indicates a protective effect. Set analysis also indicated that the influence of the faecal microbiome on the foot-skin microbiome could have important implications for BDD pathogenesis and requires further investigation. Network analysis showed evidence of dysbiosis occurring in the bovine foot-skin microbiome in advance of appearance of lesions; development of BDD preventative measures need to take account of the fact that the microbiome is changing in advance of lesion appearance. Shotgun metagenomics identified higher abundance of genes that could be associated with collagen degradation in samples from cows that subsequently developed BDD lesions, suggesting early functional changes in the microbiome.

Chapter 6 Development of an *in vitro* biofilm model for studying aspects of BDD treponeme pathogenesis.

6.1. Introduction

This chapter describes the development of an *in vitro* microtiter plate static biofilm model for study of bovine digital dermatitis (BDD) treponeme phylogroups in this morphological form. It also uses the model to compare growth and biofilm formation in dual and triple species combinations to single species in microtiter plate cultures.

Several criteria have been used to characterise biofilm-induced pathogenesis (Hall-Stoodley et al., 2004; Sapi et al., 2019). Pathogenic bacteria are typically considered surface-associated or adherent to a substratum. Direct examination reveals bacteria in clusters forming a three-dimensional structure. They are encased in a matrix of extracellular polymeric substances (EPS), which may be composed of host or bacterial constituents, and form a protective 'skin' of hydrophobic molecules such that bacterial cells in biofilms are able to withstand various environmental stressors. An example of such resilience is that they are commonly resistant to antimicrobials (Hall-Stoodley et al., 2004; Sapi et al., 2019).

The lesions of BDD exhibit many characteristics which suggest formation of biofilms is contributing to their pathogenesis. Lesions commonly fail to cure (Berry et al., 2012) despite proven sensitivity of BDD-associated treponemes to several antimicrobials *in vitro* (Evans et al., 2009b; Evans et al., 2012b). Cure rates after topical or parenteral antibiotic treatment are 60-70% in the short term (Berry et al., 2010; Holzhauer et al., 2011; Read & Walker, 1998), reducing to around 50% in the longer term (Berry et al., 2012; Read & Walker, 1998), which is low compared to cure rate achieved for the human skin treponematosis yaws using oral or intramuscular treatment (Mitjà et al., 2012). When data has been collected longitudinally it is unclear whether lesions are a recurrence of old lesions, or new lesions. Lesions frequently become chronic; a study of the contribution of different disease stages (M-stages (Berry et al., 2012)) showed that about 70% of the time lesions are at the chronic M4 stage (Biemans et al., 2018). It is also considered that the aetiology of the disease is polytreponemal as microbiological studies of lesions consistently identify multiple treponeme phylotypes (Evans et al., 2009b).

BDD treponemes have not yet been examined for ability to form biofilms, however oral bacteria which include treponemes are known to take this morphological form and have become the paradigm of multispecies biofilms (Kolenbrander et al., 2010). There are many parallels between human periodontitis and bovine digital dermatitis as both are diseases of collagenous tissues; and aspects of their pathology, immunology and bacterial aetiology are similar (Edwards et al., 2003b). Given these

similarities we hypothesise that treponeme behaviour in digital dermatitis lesions may approximate treponeme behaviour in oral biofilm models.

The Gram-negative spirochaete *Borrelia burgdorferi,* which is the aetiological agent of Lyme disease, has also been shown to form biofilms *in vitro.* The model was established in 48-well tissue culture plates, both in uncoated wells and in the presence of various matrices (Sapi et al., 2012). The presence of *B. burgdorferi* biofilms has also been demonstrated *in vivo* in skin lesions known as borrelial lymphocytomas; a cutaneous complication of Lyme disease. Presence of biofilm in borrelial lymphocytomas was demonstrated using immunohistochemistry and fluorescent in-situ hybridisation on skin biopsies, identifying mucopolysaccharides including alginate which are well characterized biofilm surface markers in other pathogenic bacteria (Sapi et al., 2016).

Like BDD, Lyme disease has a high recurrence rate after discontinuing antibiotic treatment (Sapi et al., 2016). Borrelia can assume different morphological forms and has a spiral motile form, a persister form known as round bodies, and they can form biofilms or biofilm-like aggregates (Sapi et al., 2011). Whilst less is known about pleomorphic forms of treponemes, an encysted form has been reported for BDD-associated strains and may have a similar role in persistence deep in the bovine foot (Döpfer et al., 2012a). It is possible that BDD lesions contain aggregates of treponemes which exhibit a monospecies biofilm phenotype as part of a multispecies infection, similar to bacterial organisation in other chronic wounds (Bjarnsholt, Alhede, et al., 2013).

Less than 5% of the biofilm literature is devoted to methodology (Magana et al., 2018). However, a simple microtiter plate model can be used with a short incubation time of 1-2 hours to investigate whether an organism will attach to a surface. Longer incubation times of around 20 hours will show if biofilm formation occurs (O'Toole, 2011). This method has the advantage of being high throughput and is useful for testing the effects of different environmental factors on the ability of bacteria to form biofilms. Microtiter plates can also be coated with biologically relevant substances so that attachment to different surfaces can be evaluated. This method is not suitable for studies of biofilm structure due to difficulties with imaging biofilms (Peterson et al., 2011).

Crystal violet is used to stain adherent cells, and the absorbance of the stain is measured to indicate quantity of bacteria that have formed a biofilm (Peterson et al., 2011). Crystal violet staining is the most used method for measuring biofilm mass and works by using basic dye to stain negatively charged molecules. It is favoured in screening experiments because it is rapid, simple and inexpensive (Magana et al., 2018), however there are many steps in the method that can lead to variability and therefore low reproducibility (Pantanella et al., 2013). Because the dye binds to both live and dead

cells as well as matrix, this method is not suitable for evaluating killing of biofilm cells such as an antimicrobial resistance assays (Peeters et al., 2008).

Although biofilms may form on abiotic surfaces such as polystyrene, biotic surface coatings can play a role in biofilm formation (Magana et al., 2018). *Treponema* spp. adhere to a range of host proteins. The interactions of *Treponema denticola and Treponema pallidum* with fibronectin have been most fully studied; and it is known that *T. denticola* cells also adhere to laminin and fibrinogen with similar affinities as to fibronectin, and to collagen types I and IV (Edwards et al., 2003a). Adhesion studies have shown that animal BDD strains bind to these substrates in smaller numbers, however they adhere to fibrinogen at equivalent or greater levels than *T. denticola* (Edwards et al., 2003b). Outer membrane proteins from BDD-associated treponemes have been shown to bind specifically to bovine fibrinogen (Staton et al., 2020).

In vitro models of biofilms including treponemes have been developed in the human dental research field to mimic plaque. Development of a biofilm model even for *T. denticola,* which is one of the more easily cultured species of the genus, has proved difficult. It has been achieved in a static system using 96-well flat-bottomed polystyrene plates with or without a coating of heat- inactivated rabbit serum, allowing five days for growth. Biofilm formation was quantified using crystal violet staining (Bian et al., 2013). It has also been achieved in a continuous culture system containing glass rods coated in fibrinogen allowing fourteen days for growth (Mitchell et al., 2010). This second example was designed to allow transcriptomic analyses of a mature biofilm. This latter disease model was considered important as it is known that *T. denticola* is more prevalent in more advanced cases of periodontitis (Socransky et al., 1998). Since it is known that BDD-associated treponemes increase in number to predominate in chronic BDD lesions (Krull et al., 2014), we draw the parallel that a more mature biofilm may also be more relevant to study of BDD.

The objective here was to develop a static *in vitro* biofilm model for BDD-associated treponemes, both as single-species and mixed-species. This model could be used in future work for transcriptomics studies to gather information regarding biofilm-associated pathogenesis. Understanding the pathogenesis of BDD-associated treponemes could inform approaches to treatment and prevention of BDD. This chapter describes the optimisation of this model design and the challenges encountered.

6.2 Methods

6.2.1 Coating optimisation experiments

Microtiter plates were coated and washed, and relevant control media and one of three species representing the three major BDD treponeme phylogroups (*T. medium* T19, *T. phagedenis* T320A or *T. pedis* T3552B) inoculated according to a standard method (Chapter 2.7.1 and 2.7.2). Serum and fibrinogen coatings were trialled as these had been previously shown to facilitate adherence of *T.denticola* in biofilm models (Bian et al., 2013; Mitchell et al., 2010). Plates were incubated for seven days in an anaerobic cabinet (Don Whitley Scientific, UK) (85% N_2, 10% H_2 and 5% CO_2, 36^{o}C). This incubation time was chosen to allow growth of bacteria to stationary phase, and to allow the opportunity for mature biofilm formation. Microtiter plate layouts for coating optimisation experiments are shown in templates in Appendix D Figure 1. Coatings used were neat foetal calf serum (FCS), 10% FCS, neat rabbit serum (RS), 10% RS and five different concentrations of bovine fibrinogen (Fg): 250, 50, 10, 5 and 1µg/ml Fg. 1x Phosphate buffered saline (PBS) was used as a control coating since sera and fibrinogen were diluted using 1x PBS, and uncoated wells were used as negative controls.

Bacterial growth was determined by subtracting the average optical density at 540nm (OD_{540nm}) of the three control wells from the average OD of the five technical replicates in each column at day 0. Microplates were removed from the anaerobic cabinet after inoculation and ODs were determined using a spectrophotometer as described in Chapter 2.7.2. The same calculations were applied to the ODs measured at day 7, and the difference between the two determined. The average of two experimental replicates was calculated for each species and standard deviations were calculated using the STDEV.P function in Microsoft Excel (Microsoft Corporation, USA).

Biofilm growth was determined using the crystal violet method described in Chapter 2.7.3.

6.2.2 Timing optimisation experiments

There is no precedent in the literature for defining how long a treponeme biofilm takes to progress to stationary phase. We therefore tested alternative incubation times of two, four, six, eight and ten days. These experiments were carried out using the T320A and T3552B species of treponeme. One serum and one fibrinogen coating were selected according to those that favoured growth in 6.2.1. Two experimental replicates were carried out for each time point. Microtiter plate layouts are shown in Appendix D, Figure 2.

6.2.3 Investigation of high background levels of crystal violet staining of liquid growth medium

Given difficulties with control wells retaining high levels of crystal violet stain, making interpretation of presence of biofilm difficult, wells were visualised using a light microscope (Olympus, Tokyo) with 200x magnification. Images were captured using a Toupcam™ Camera (UCMOS series) with a microscope adaptor (ToupTek Photonics, Zhejiang, China) to aid interpretation of the phenotypic data.

It was also suspected during timing optimisation experiments that crystal violet staining of control wells was increased after longer incubation times. This was investigated using control material (OTEB+FCS) inoculated (200µl per well) into microtiter plates that were coated as shown in Figure 1 in Appendix D. Two experimental replicates were carried out at two, six-, and ten-days' incubation.

6.2.4 Investigation of biofilm formation by dual and triple species mixtures under optimised conditions

Information from model optimisation experiments informed conditions for investigating biofilm formation using mixed species cultures. Combinations investigated were: T19+T320A, T19+T3552B, T320A+T3552B and T19+T320A+T3552B. Microtiter plates were prepared with coatings corresponding to those which produced the best growth for each species individually (10% FCS and 50µg/ml Fg for T19 and T320A; 10% RS and 1µg/ml Fg for T3552B) and inoculated as shown in Figures 3 and 4 in Appendix D, including single species wells as positive controls for comparison, and wells containing only liquid medium as negative controls. Treponeme cultures were adjusted to double or treble standard ODs (Chapter 2, Table 2.3), and the volume inoculated was reduced to 25µl or 17µl, depending on whether a dual or triple-species mix was being investigated. Cultures were placed in 2ml tubes using glass pipettes and centrifuged for five minutes at 2,350g. Supernatant was removed by pipetting and bacteria resuspended to reach the required OD. Inoculated microtiter plates were incubated for two days. The concentration of crystal violet stain used was decreased from 1 to 0.5% in these experiments to try to reduce background staining.

6.2.5 Effect of oxygen exposure on biofilm growth

It has been suggested that biofilm formation may be enhanced by adverse environmental conditions as they are known to protect cells from a harsh environment (Philips et al., 2017). To investigate whether biofilm formation could be enhanced by oxygen exposure, four different incubation conditions were trialled using T320A. "Group A" conditions refers to incubation of microplates under what could be considered the most favourable conditions for planktonic growth: inside an anaerobic cabinet with no removal for OD measurement on day 0. "Group B" conditions refer to inoculation of microplates with T320A bacteria that had been incubated at 37°C under aerobic conditions (still in

liquid culture tubes) for 24 hours. “Group C” conditions used T320A bacteria grown and inoculated using standard anaerobic procedures, but microplates were incubated at 37°C under aerobic conditions (0.3%CO_2, 37°C, Sanyo CO_2 incubator MCO-175, Sanyo, Osaka, Japan) for 24 hours before being returned to the anaerobic cabinet. “Group D” conditions also used T320A bacteria grown and inoculated using standard anaerobic procedures, but microtiter plates were incubated at 37°C under microaerobic conditions for 48 hours (5%CO_2, 5%O_2, 37°C, Sanyo O_2/CO_2 incubator MCO-18M, Sanyo, Osaka, Japan). ODs were measured on day 0 for all microplates except “Group A.” All four conditions were trialled with a two-day incubation time. Six technical replicates and two experimental replicates were included for each experiment. Investigation of microaerobic conditions (Group D) was extended to dual (T19+T320A, T19+T3552B, and T320A+T3552B) and triple species (T19+T320A+T3552B) mixtures to compare any differences to T320A alone. Plate coatings and microplate layouts used are shown in Figures 2, 3 and 4 in Appendix D, according to the species inoculated.

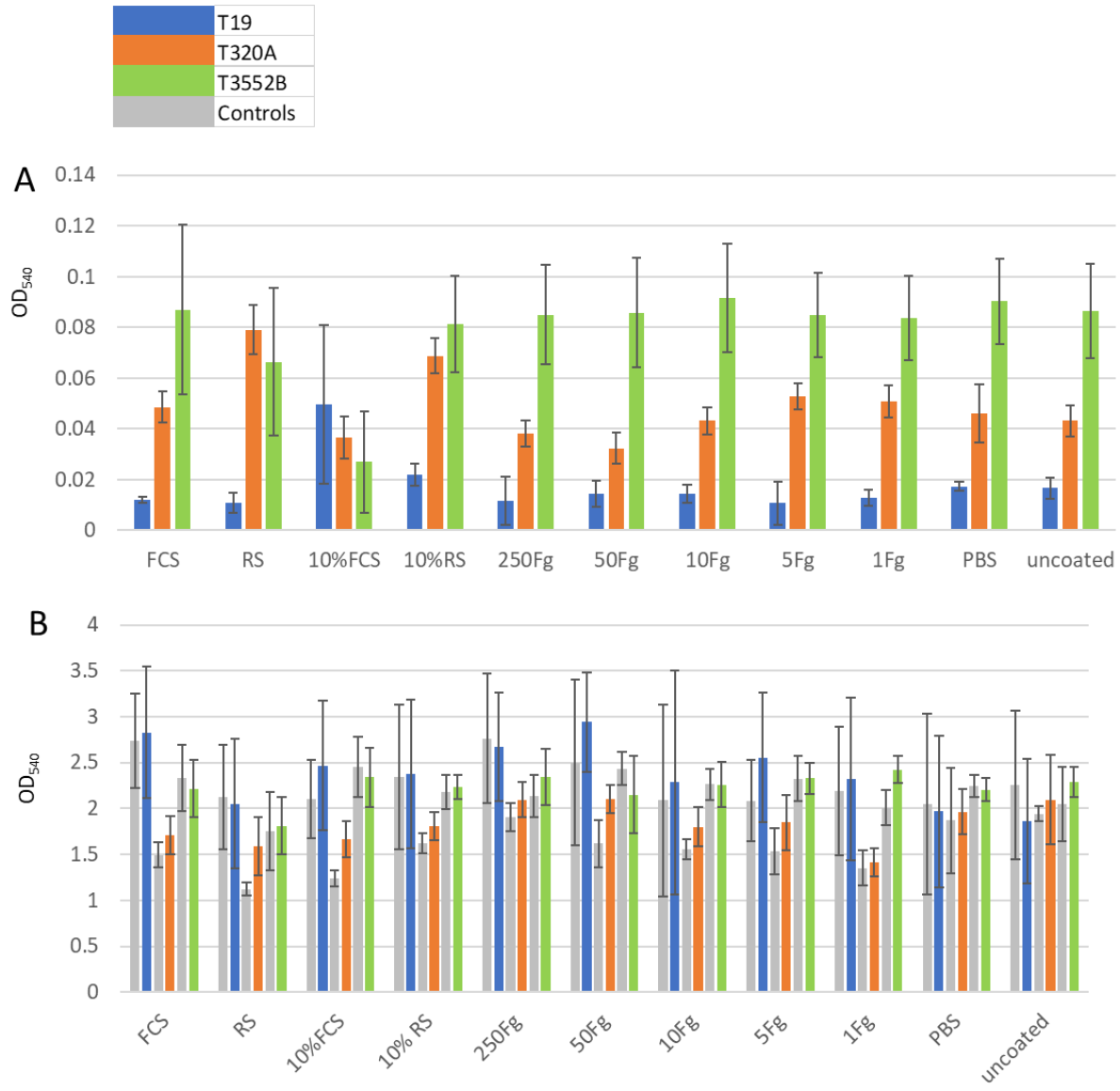
6.3 Results

6.3.1 Coating optimisation experiments

Growth was greatest for T3552B, although it is also the most variable according to the standard deviations. Coating wells with 10% FCS favoured T19 growth, RS favoured T320A growth, and 10µg/ml Fg favoured T3552B growth (Figure 1A).

Based on highest crystal violet OD and most clearly differentiated from the relevant control ODs, the most favourable serum coating for T320A was 10% FCS and the most favourable bovine fibrinogen coating was 50µg/ml Fg. This is considered a weak biofilm former as the staining in experimental wells is less than double that in control wells (Stepanovic et al., 2007). It can be considered that neither T19 nor T3552B clearly formed biofilms on any serum or fibrinogen-coated surfaces as standard deviations overlapped for all coatings, however 10% FCS and 50µg/ml Fg were considered most favourable for T19, and 10%RS and 1µg/ml Fg for T3552B (Figure 1B). Conclusions were not made in terms of comparing species because variation in control well staining makes different experiments incomparable.

Figure 1A) Average growth and 1B) Average crystal violet staining of three BDD treponeme species on microtiter plates determined by ODs read at 540nm after 7 days incubation with nine different plate coatings. The error bars represent standard deviation calculated from two experimental replicates.

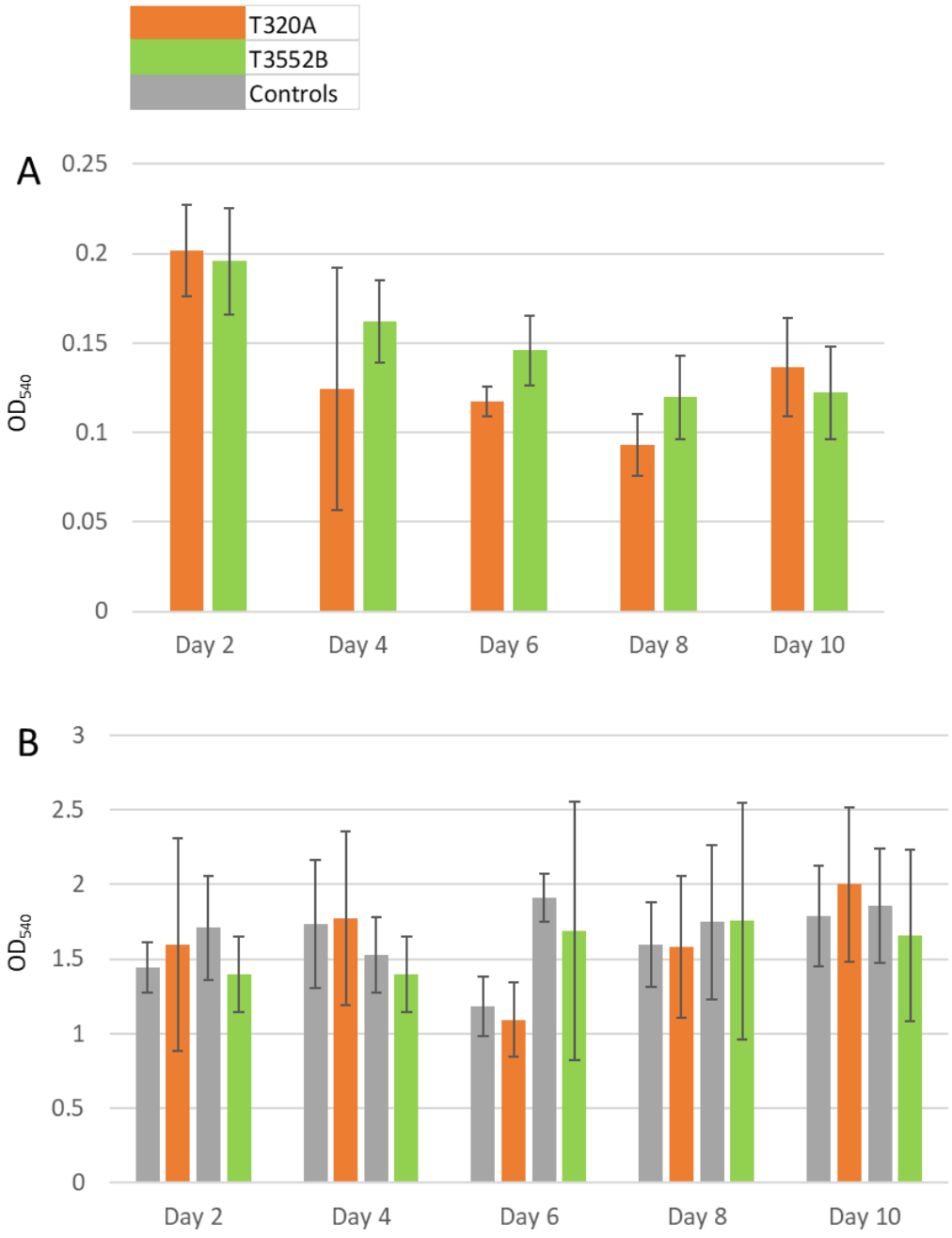


6.3.2 Timing optimisation experiments

Biofilm formation is described as having an initial attachment phase, which progresses to mature biofilm or "stationary phase" analogous to what would occur in planktonic culture. Since a strong biofilm was not observed during initial coating optimisation experiments, T320A was used to investigate the growth curve for biofilms and optimise length of incubation. Times tested were 2, 4, 6, 8 and 10 days. Despite no evidence of biofilm formation occurring during coating optimisation experiments for T3552B, testing was also pursued for this species to investigate whether biofilm would form at shorter or longer incubation times.

For T320A, growth was greatest on day 2 and most variable on day 4 as shown by larger standard deviations, and starts to increase again on day 10. Growth for T3552B was also optimised at day two and declined as the time interval increased (Figure 2A). There were no differences between plate coatings; bar charts showing growth by plate coatings are available in Figure 5, Appendix D. Results from crystal violet staining did not indicate biofilm formation for any time point for either T320A or T3552B (Figure 2B) and highlighted the extent of experimental variation in this method.

Figure 2A) Average growth and 2B) Average crystal violet staining for T320A and T3552B incubated for five different times under anaerobic conditions. The error bars represent standard deviation calculated from two experimental replicates.



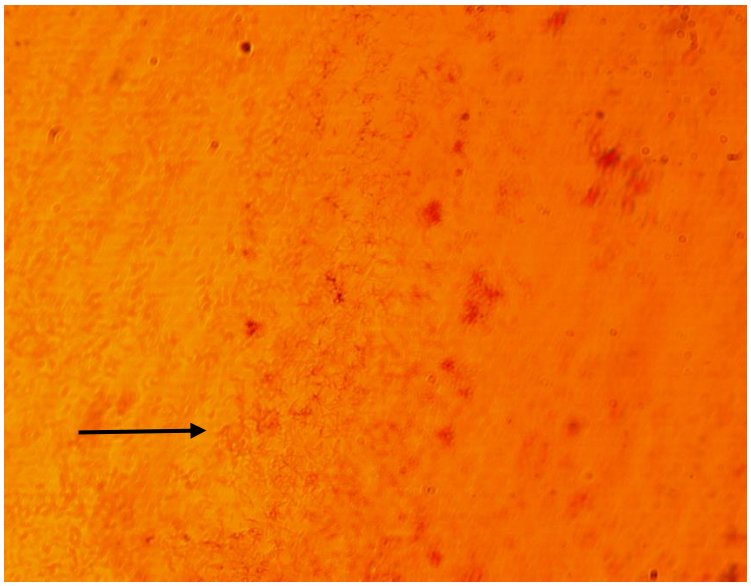
6.3.3 Investigation of high background levels of crystal violet staining of liquid growth medium

Microscopic examination of microtiter plate control wells after crystal violet staining showed granules of stain remaining in the wells (Figure 3A). Bacteria remaining in wells can be seen after crystal violet staining in Figure 3B. Background staining of media remaining in control wells complicates the interpretation of ODs used to determine presence or absence of biofilm.

Figure 3 Crystal violet granules staining microtiter plate wells in the absence (A) and presence (B) of BDD treponemes highlighted by the arrow, 200x magnification.

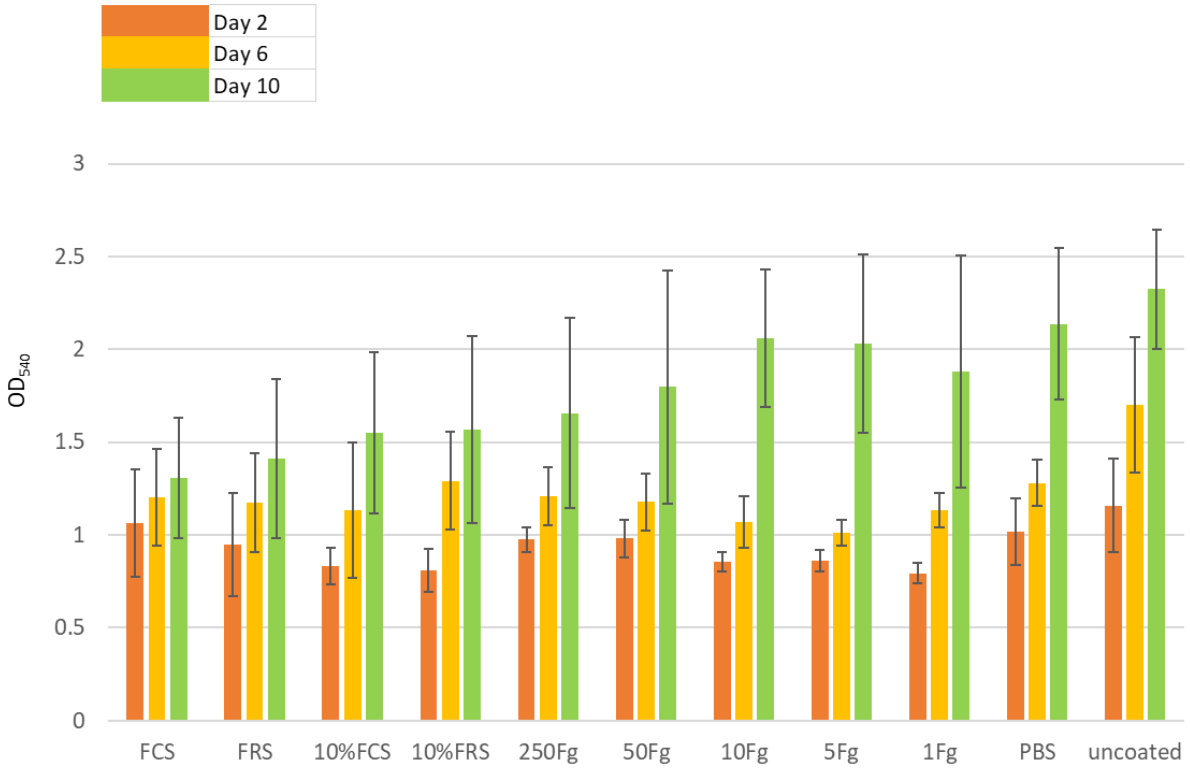
A

B



Further investigation of high levels of control staining found that the liquid medium (OTEB + FCS) stained darker and with more variability for all coatings if it had been incubated for a longer time (Figure 4). Further experiments were limited to two days incubation time to avoid the effects of increased staining in older control wells.

Figure 4. Optical densities due to crystal violet staining of liquid growth medium after two, six- and ten-days' incubation.



6.3.4 Investigation of biofilm formation by dual and triple species mixtures under optimised conditions

Dual and triple species cultures showed more bacterial growth (higher OD_{540nm}) in microplates at day two than single-species constituents, except for T19+T320A (Figure 5). Results from crystal violet quantification of microtiter plates (Figure 6) suggested presence of biofilm for T19+T320A with PBS coating, and T320A+T3552B with 50µg/ml Fg coating or no coating. This is not directly correlated with increased growth, as mixed species wells in T19+T320A microtiter plates did not show increased OD compared to T320A wells.

Figure 5 Average growth for dual and triple species mixes of BDD treponemes compared to their single species constituents after 2 days incubation under anaerobic conditions. The error bars represent standard deviation calculated from two experimental replicates.

A) T19+T320A, B) T19+T3552B, C) T320A+T3552B, D) T19+T320A+T3552B

For some experimental replicates, T19 did not grow, leading to negative optical density readings compared to control wells

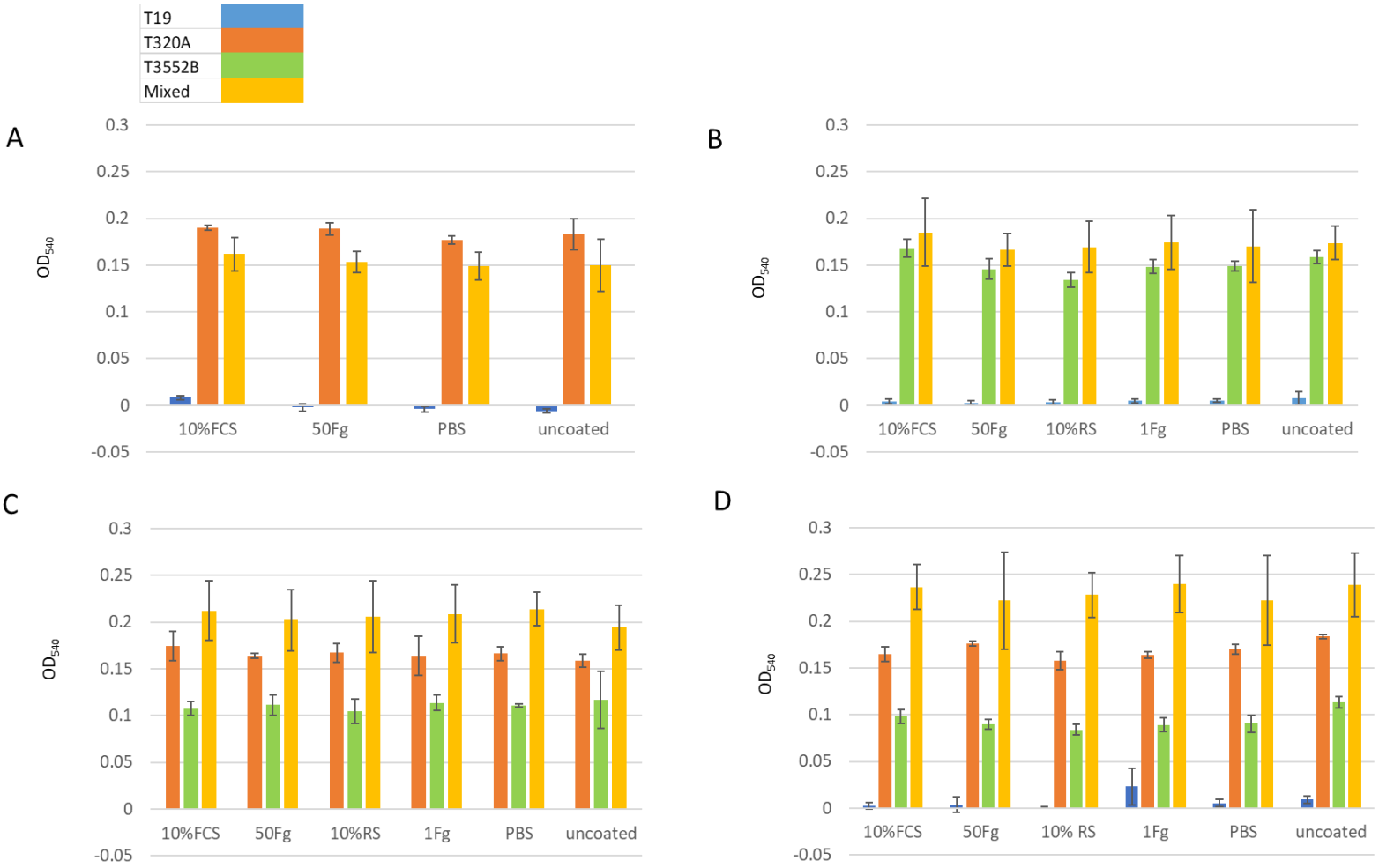
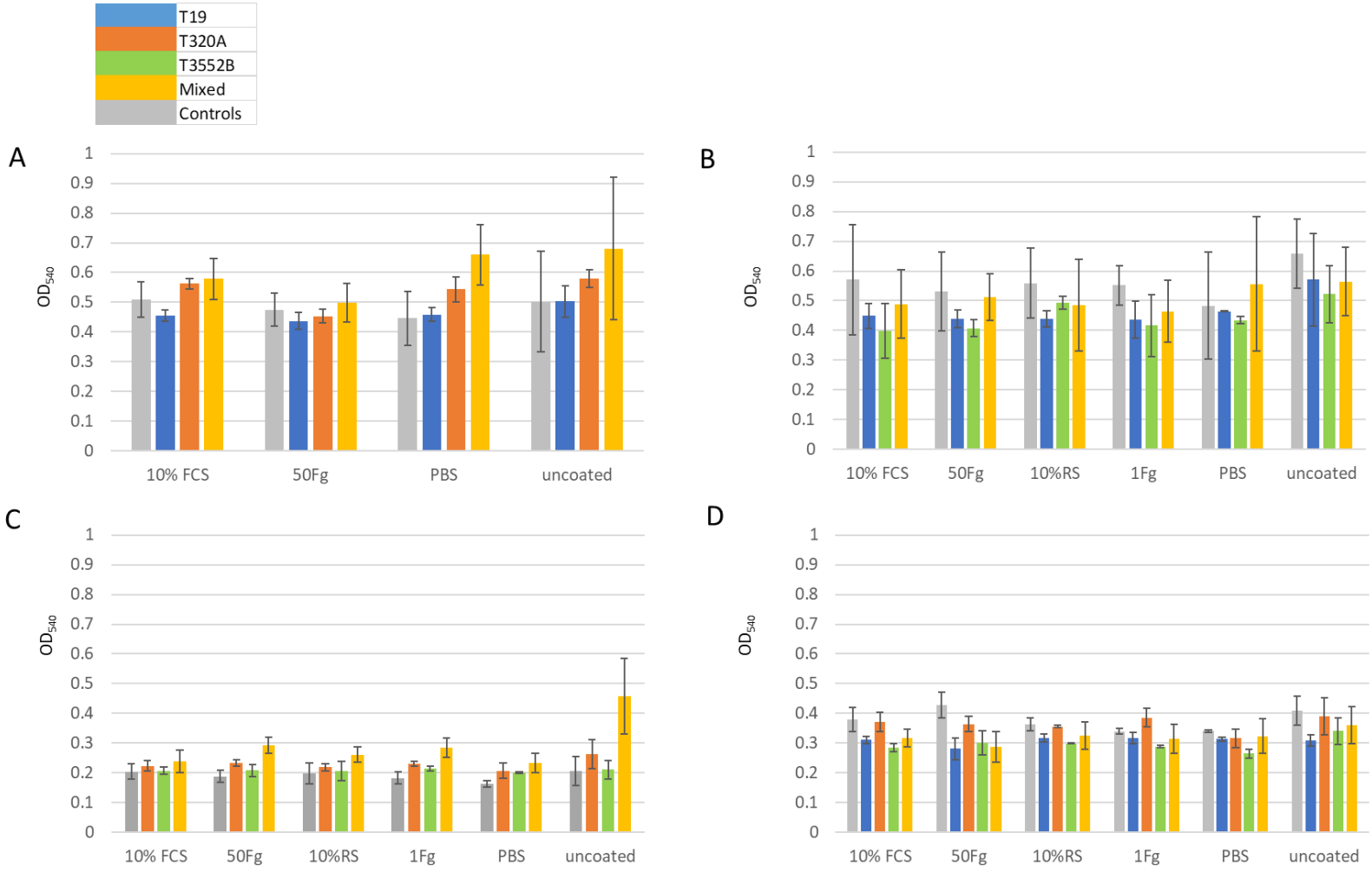


Figure 6 Average crystal violet staining for dual and triple species mixes of BDD treponemes compared to their single species constituents after 2 days incubation under anaerobic conditions. The error bars represent standard deviation calculated from two experimental replicates.

A) T19+T320A, B) T19+T3552B, C) T320A+T3552B, D) T19+T320A+T3552B

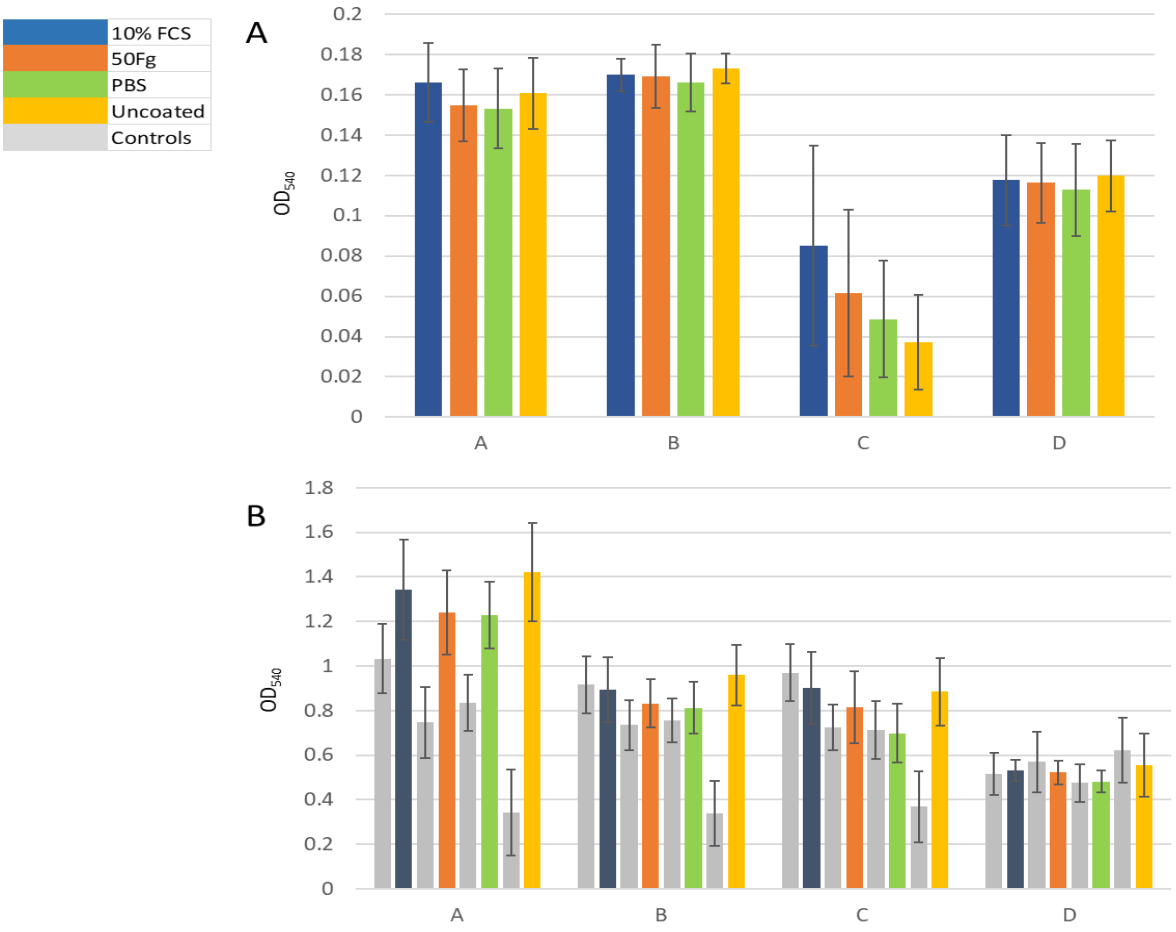


6.3.5 Effect of oxygen exposure on biofilm growth

Bacterial growth for T320A was most reduced under Group C conditions, with two days' exposure to microaerobic conditions (Group D) having a small detrimental effect (Figure 7A). Whilst Groups A and B conditions lead to similar growth, Group A conditions lead to more crystal violet staining, suggesting biofilm formation is occurring in T320A microtiter plate cultures after 48 hours incubation using plate coatings of 50Fg, PBS or no coating. This was not observed at day 2 in timing optimisation experiments, however planktonic inoculating cultures and microtiter plates were not removed from the anaerobic cabinet on day 0 for Group A, indicating this may be important for facilitating biofilm formation (Figure 7B).

Figure 7A) Average growth and B) average crystal violet staining of T320A after two days incubation using four different plate coatings under four different environmental conditions. The error bars represent standard deviation calculated from two experimental replicates.

Environmental conditions: Group A) microtiter plates never removed from anaerobic cabinet, Group B) planktonic cultures incubated under aerobic conditions for 24 hours prior to inoculating microtiter plates, Group C) microtiter plates incubated under aerobic conditions for 24 hours after inoculation before returning to the anaerobic cabinet for the second 24 hours, Group D) microtiter plates incubated under microaerobic conditions for 48 hours.



For dual and triple species mixes, exposure to microaerobic conditions attenuated growth, with no suggestion of increased growth except for a small effect in T320A+T3552B (Figure 8). T320A growth was not affected by incubation under microaerobic conditions in this experiment, yielding similar absorbance values to those seen under anaerobic conditions (Figure 5). T3552B growth was reduced under microaerobic conditions compared to anaerobic conditions (Figure 5). T19 growth was low under both incubation conditions, however, appears slightly improved by microaerobic conditions. Crystal violet staining suggested biofilm formation under microaerobic conditions for T19+T3552B for all plate coatings except 50µg/ml Fg. Staining of single species wells for comparison suggested this effect may be attributable to T19 (Figure 9).

Figure 8: Average growth of dual and triple species combination of BDD treponemes compared to their single species constituents incubated under microaerobic conditions for two days. The error bars represent standard deviation calculated from two experimental replicates.

A) T19+T320A, B) T19+T3552B, C) T320A+T3552B, D) T19+T320A+T3552B

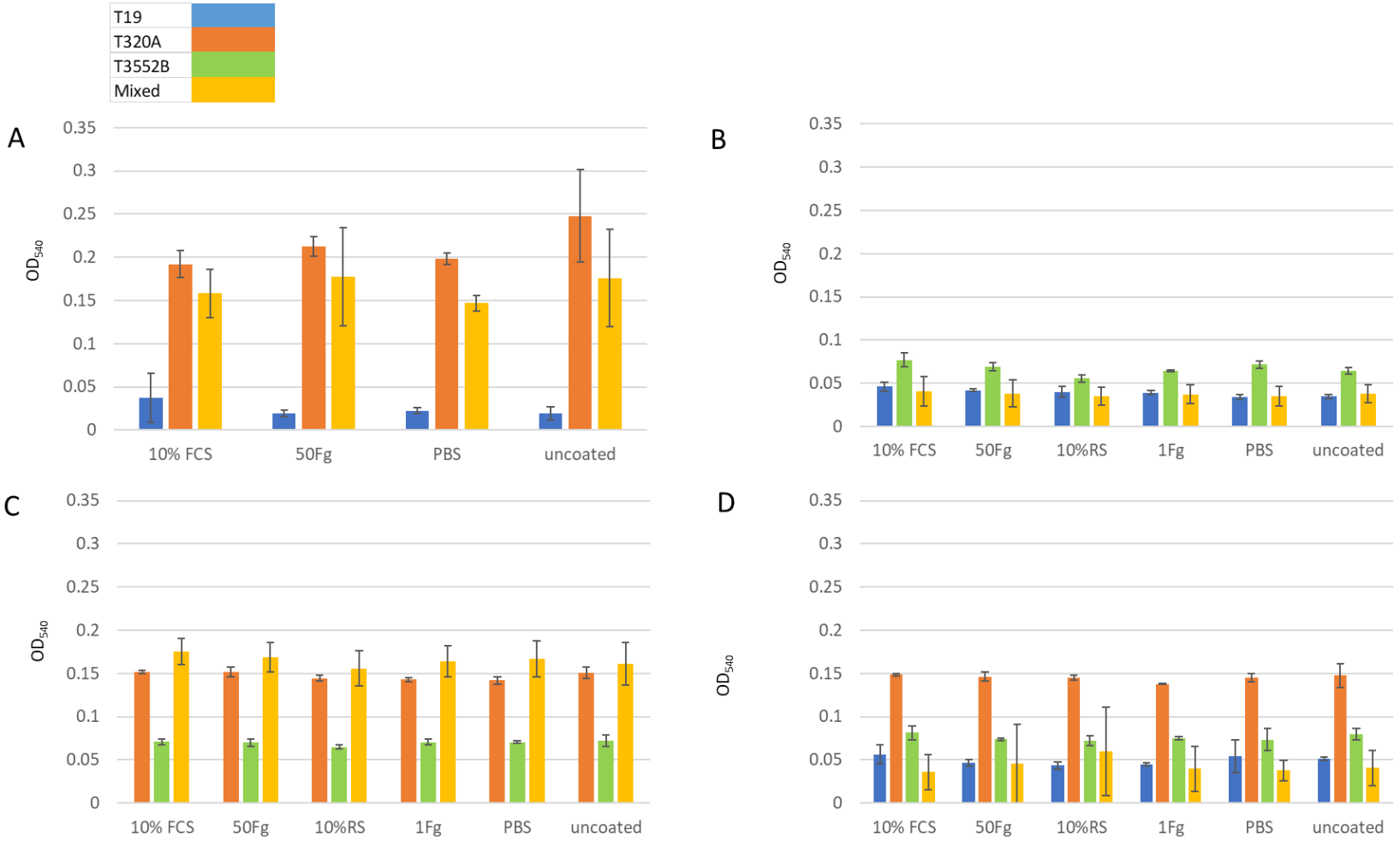
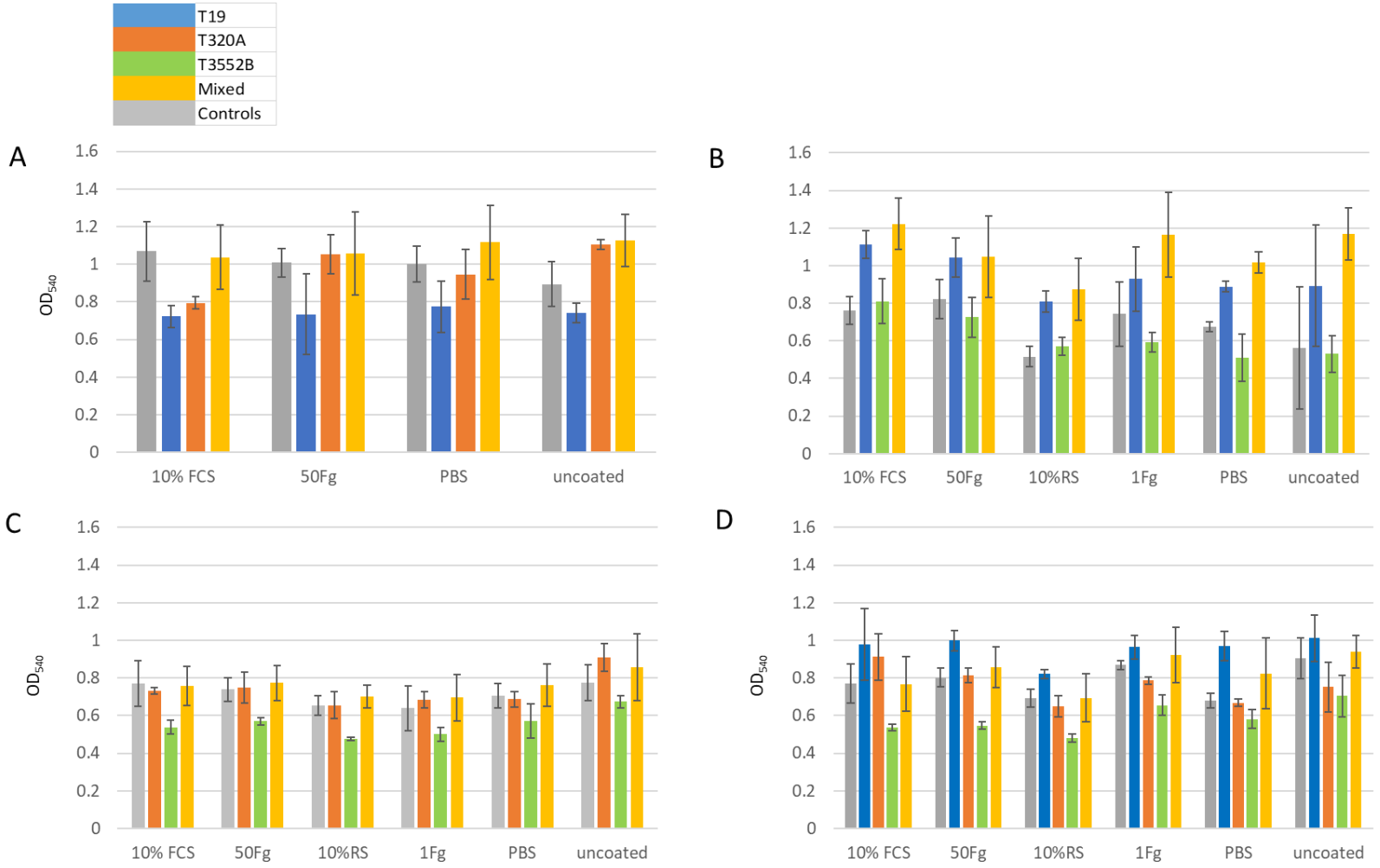


Figure 9: Average crystal violet staining of dual and triple species combination of BDD treponemes compared to their single species constituentsmicr incubated under microaerobic conditions for two days. The error bars represent standard deviation calculated from two experimental replicates.

A) T19+T320A, B) T19+T3552B, C) T320A+T3552B, D) T19+T320A+T3552B



6.4 Discussion

This chapter shows potential biofilm formation by T320A under anaerobic conditions, and in dual mixtures with the other BDD treponemes T19 or T3552B. It also shows potential biofilm formation for a dual species mix of T19+T3552B incubated under microaerobic conditions. Variability in absorbance used to measure treponeme growth and crystal violet staining of adherent cells limits interpretation of the data, and further work is needed to confirm presence of BDD treponeme biofilm phenotypes in this *in vitro* model for surface adherence.

6.4.1 Growth

The initial plate coating optimisation experiments showed that T3552B had grown to a higher OD by day seven than T320A, whereas in planktonic culture T320A typically grows to a higher OD by day seven (Table 2.3, Chapter 2). Timing optimisation experiments showed growth was highest for both T320A and T3552B after 2 days. It is possible that cultures reach stationary phase in microtiter plates more quickly than in macro-culture, especially since the concentration of bacteria in microtiter plate wells is much higher and we can expect the nutrient supply to become exhausted much faster (Evans et al., 2009c; Evans et al., 2008). It is also possible that increased OD may reflect changes other than increased bacterial growth, such as changes to plate coatings and the composition of the growth medium which may affect light absorbance. It is possible that clumping of bacteria or condensing of DNA in advance of cell death may increase absorbance readings (Schleheck et al., 2009). Two days was used as the optimum incubation time for subsequent experiments to mitigate for these complexities, however this did not favour T19 growth, which was poor in this short timeframe, making it difficult to assess presence of biofilm for this species.

We expected growth to be enhanced in dual and triple species mixes compared to the constituent single species wells because more bacteria were inoculated initially, which was the case, except for T19+T320A. This could be attributed to poor T19 growth due to short incubation time, however there was an additive effect observed for T19+T3552B, therefore growth characteristics between species was not uniform. The magnitude of increased growth for all combinations was smaller than expected given the double or triple quantities of inoculating bacteria; it is possible that growth was limited by nutrient availability.

Exposure of T320A microtiter plates to aerobic conditions for 24 hours markedly reduced growth; microaerobic conditions for 48 hours caused some attenuation; however, this was not consistent in later experiments where T320A growth after microaerobic incubation was comparable to previous data after anaerobic incubation. This experiment highlighted that T320A growth is more resistant to oxidative stress than the other species. This could be expected due to a larger number of oxidative

stress genes in the genome compared to T19 and T3552B (Staton et al., 2021a). Exposure of T320A to aerobic conditions for 24 hours prior to inoculation into microtiter plates did not affect growth, showing either those bacteria in liquid culture tubes were able to defend against oxidative stress more effectively, or that cultures were able to recover and grow within 24 hours after exposure to oxidative stress. Exposure of dual and triple species mixes to microaerobic conditions for 48 hours reduced growth compared to anaerobic conditions, except for T320A+T3552B, again suggesting T320A may have a role in resistance to oxidative stress.

6.4.2 Crystal violet staining

Initial plate coating optimisation experiments yielded high levels of crystal violet staining, including in the control wells which did not contain any bacteria. Different incubation times were trialled instead to see if crystal violet staining was reduced at different time points. Because there was marked staining of control wells, we examined the effect of incubation time on media alone and concluded that longer incubation times increased crystal violet staining and consistently resulted in increased staining of control wells. For this reason, subsequent experiments used only two-day incubation times and crystal violet concentration was reduced to 0.05%, which is half the recommended concentration (O'Toole, 2011). Nevertheless, high background staining and variability of control wells continued to feature in experiments. The crystal violet method works by using basic dye to stain negatively charged molecules, which may include bacteria and a range of polysaccharides (Pantanella et al., 2013). It is possible that crystal violet staining of constituents of the growth medium adhered to the microtiter plates, or extracellular proteins, interfered with biofilm measurements. Other limitations of the crystal violet method include variation in results where biofilms are loosely adherent, as they are easily dislodged by washing with PBS, and low reproducibility due to variation in experimental conditions (Pantanella et al., 2013).

Crystal violet staining provided some evidence that T320A is capable of biofilm formation, and in combination with T19 or T3552B. Incubation under microaerobic conditions increased crystal violet staining which suggested biofilm formation for the dual species mix T19+T3552B, however the biofilm phenotype was not detected in other dual species wells, or in the triple species wells. The relationship between oxidative stress and biofilm formation is unclear; for some bacteria genes upregulated in response to oxidative stress also enhance biofilm formation, whilst (Gambino & Cappitelli, 2016)rs (Gambino & Cappitelli, 2016). If biofilm formation is a response to adverse environmental conditions, this would suggest T19 is least able to cope with oxidative stress and forms biofilm as a means of protection; whilst T320A assumes the biofilm phenotype only under anaerobic conditions, although its growth is unaffected by incubation under microaerobic conditions. The links between oxidative

stress and biofilm formation in treponemes could be important for BDD pathogenesis and require further study.

6.4.3 Further work

Measurement of the biofilm phenotype presented here relies on a single biochemical method, where it is recommended to combine different experimental approaches (Pantanella et al., 2013). One route for development of the BDD treponeme biofilm model is to trial further conditions that could be enhance biofilm formation. There are many factors influencing bacterial adhesion: environmental conditions, the chemistry of the attachment material surface, roughness of the material surface, and electrostatic and hydrophobic interactions between the bacteria and the surface (Filipović et al., 2020). We targeted environmental conditions (eg. time of exposure, bacterial concentration, oxidative stress) to accelerate adhesion. We also altered the surface chemistry by using adsorbed bovine fibrinogen to promote adhesion. Further strategies could target surface roughness or pH, for example, to find out if these promote attachment.

It is possible that bacteria had attached and already detached from microtiter plate surfaces, as attachment in a study of human oral treponeme *T. denticola* showed adherence was greatest at only one hour after inoculation, compared to two or three hours, due to degradation of the fibronectin substrate used in the model (Vesey & Kuramitsu, 2004). Our objective was to produce established biofilms rather than to promote only initial attachment. Future work would need to promote adhesion. Continuous culture systems could be used in the future as they are considered useful for the study of more mature biofilms as they tend to lead to formation of biofilms with greater biomass promoted by high shear forces (Donlan & Costerton, 2002; Mitchell et al., 2010).

Imaging using confocal laser scanning microscopy is commonly applied to confirm presence of viable bacteria using live/ dead staining, and presence of extracellular polymeric substances using biofilm matrix staining (Philips et al., 2017). This provides robust data on structure and viability which cannot be assessed using crystal violet staining (Thurnheer et al., 2004). This is pertinent for BDD biofilm studies because they exist in chronic wounds, where reduced vascularity and compromised host defences allow persistence of bacterial aggregates. It is possible that monospecies aggregates confer a biofilm phenotype within the context of this multispecies infection (Bjarnsholt, Alhede, et al., 2013). Imaging could be used to describe the architecture of bacterial species persisting in BDD lesions, giving insights into pathogenesis. Given the polybacterial nature of BDD lesions, polybacterial biofilm models may also be useful to study potential roles of other bacterial species in BDD lesion formation. Previous research showed that *Porphyromonas gingivalis* was synergistic with the human oral treponeme *T.*

denticola in forming biofilms, resulting in more biofilm mass and tighter adherence to the substratum (Kuramitsu et al., 2005).

Chapter 7 Global transcriptome analysis of BDD treponemes during oxidative stress and biofilm growth

7.1 Introduction

Transcriptomics is the study of the transcriptome- the RNA transcribed from genomic DNA, which reflects the current state of gene expression within a target population. Studying the transcriptome reveals the functional elements of the genome and is essential for understanding cell biology and pathogenesis of disease. For BDD-associated treponemes, pathogenesis has been inferred by describing the serological immune response (Demirkan et al., 1999b; Walker et al., 1997) and by development of infection models for inducing skin lesions in cattle (Gomez et al., 2012; Krull et al., 2016; Read & Walker, 1996). Research to date has looked at the differential gene expression in the host during infection to describe host-pathogen interactions (Evans et al., 2014; Newbrook et al., 2021; Scholey et al., 2012); however, there has been no examination of the transcriptome of BDD-associated treponemes. Genome-wide studies using microarray or RNA sequencing (RNAseq) using next-generation sequencing technology, have become the main tools used for transcriptomics (Raghavachari & Garcia-Reyero, 2018). The advantages of RNA-seq over microarray includes the ability to identify unannotated transcripts, even those from organisms that do not have existing genomic sequences. Gene expression levels can be more accurately quantified, and the method requires relatively low amounts of RNA (Wang et al., 2009).

An improved understanding of the pathogenesis and pathobiology of BDD-associated treponeme phylotypes is key to improving disease control. Here, we analyse the transcriptome of BDD-associated treponemes grown under different conditions to study three concepts relevant to their pathogenesis: response to oxidative stress, biofilm formation, and quorum sensing.

7.1.1 Oxidative stress

Oxidative stress is caused by an imbalance of reactive oxygen species (ROS) compared with the ability of a biological system to detoxify them. Their strong redox potential can damage biomolecules and compromise cell homeostasis when cell envelope integrity is impaired, eventually leading to cell death (Chautrand et al., 2022). Bacteria possess regulatory systems such as periredoxin, glutaredoxin and thioredoxin which are needed to survive environmental oxidative stress. A range of transcriptional regulators such as OxyR and PerR sense and transduce these survival responses. Antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) also play a significant role in the management of environmental oxidative stress (Sudharsan et al., 2023).

An ability to continue growth when exposed to oxygen would be an important property allowing BDD-associated treponemes to proliferate on the bovine foot skin during initiation of infection; a potential mechanism to explain their role in this polymicrobial disease. A similar property has been described for the obligate anaerobe *Bacteroides fragilis,* enabling proliferation in host tissues in advance of abscess formation (Baughn & Malamy, 2004). An ability of BDD-associated treponemes to utilise oxygen could also create anoxic conditions enabling infection with other obligate anaerobes commonly identified in BDD lesions such as *Porphyromonas* spp*., Fusobacterium* spp*., Bacteroides* spp*., and Prevotella* spp. (Krull et al., 2016). An understanding of genetic control of the response to oxidative stress could help identify virulence factors.

Capacity to cope with oxidative stress posed by ROS produced by host immune cells is central to bacterial ability to colonise a host and cause disease (Gherardini et al., 2006). ROS have antimicrobial actions as a result of their role in the phagocyte oxidase complex. For example, Superoxide $O_2^{-\cdot}$ Is produced by the NADPH phagocyte oxidase complex in both polymorphonuclear and mononuclear phagocytes (Fang, 2004). Analysis of the complete genomes of the three species of BDD treponemes used in the present study has revealed key differences in oxidative stress genes compared to non-pathogenic equivalent species (Staton et al., 2021a), further supporting their relevance to pathogenesis.

7.1.2 Biofilm

Bacteria prefer to live in close proximity and favour adhering to a surface rather than remaining in planktonic form in liquid, a phenomenon first observed in aquatic bacteria during the 1940s (Heukelekian & Heller, 1940; Zobell, 1943). The ability to form biofilms is considered an evolutionary advantage which allows bacteria to survive in diverse environmental conditions. (Hall-Stoodley et al., 2004). In this state, they produce extracellular polymeric substances (EPS) which protect them from environmental changes and confer tolerance to the immune system and to antimicrobials. They constitute both fast-growing and slow growing metabolically inactive populations. This heterogeneous environment in bacterial biofilms leads to localised selective pressures and high mutation rates which accelerate the development of antimicrobial resistance (Ciofu et al., 2022).

As introduced in Chapter 6, BDD lesions exhibit many characteristics that suggest the manifestation of a biofilm phenotype. Based on our results, *T. phagedenis* T320A appears capable of biofilm formation by attachment to a fibrinogen-coated surface. We therefore use the fibrinogen-coated microtiter plate model to look for gene dysregulation which could provide evidence of attachment to fibrinogen and other biofilm formation and growth characteristics. An understanding of the genetic

control of biofilm formation in BDD-associated treponemes would facilitate novel approaches to BDD treatment targeted at preventing treponemes from establishing these protected communities.

7.1.3 Quorum sensing

Bacteria communicate in a cell-density dependent manner in several ways using small, diffusible organic molecules called autoinducers. These mechanisms, collectively known as quorum sensing (QS), influence bacterial phenotype by regulating virulence genes and biofilm formation. QS increases the overall fitness of a bacterial community by promoting phenotypic heterogeneity, allowing adaptation to specific conditions in varying environments (Striednig & Hilbi, 2022). The most well-known examples of signalling molecules in Gram negative bacteria are *N*-acyl-homoserine lactone compounds (AHLs, also known as AI-1) which give species-specific signals, and autoinducer-2 (AI-2) which is considered to give cross-species signals. Signalling molecules are recognised by sensors found in the plasma membrane known as sensor histidine kinases, or by transcription factors found in the cytosol (Striednig & Hilbi, 2022).

Treponema denticola is considered essential to the initiation of human periodontal disease alongside the other Gram-negative anaerobes *Porphyromonas gingivalis* and *Tannerella forsythia* as part of the bacterial consortium known as the "red complex" (Socransky et al., 1998). Transcriptional responses of *T. denticola* to other associated bacteria found in subgingival plaque have been examined and showed that the presence of the most closely associated pathogens suppressed expression of major antigens, thus suggesting immune evasion is enhanced by the presence of other pathogens (Sarkar et al., 2014). However, typical AHL and AI-2 signalling molecules have not been identified in *T. denticola* (Niazy, 2021). Here we examine differences between a dual and a triple species mix of BDD treponemes compared to single species cultures and hypothesise that there is synergy between species that promotes immune evasion, or which might enable pathogenesis. Identification of quorum sensing pathways (signalling molecules or receptors) would enable design of quorum sensing inhibitors (QSIs) which provide treatment alternatives to conventional antimicrobials.

7.1.4 Applications in disease control

Antimicrobial resistance to a variety of conventional antimicrobials has been reported in dairy cattle and reducing their use in agriculture was one of the overarching principles of the Review on Antimicrobial Resistance (O'Neill, 2015). Most conventional antimicrobials are derived from microbes or fungi; therefore, it follows that naturally occurring resistance mechanisms will exist and continue to spread in bacterial populations where there is selection pressure in their favour. Specifically, BDD treponemes appear to have intrinsic resistance to colistin and moreover appear to be able to develop

resistance to spectinomycin (Evans et al., 2009c; Evans et al., 2012b). *T. pallidum* subsp. *pertenue,* the causative bacteria of the human analogous skin disease yaws, rapidly develops azithromycin resistance when this macrolide is used in eradication campaigns (Mitjà et al., 2018). This has increased the impetus to find alternative approaches to developing antibacterial agents against treponemes.

Quorum sensing is an area of focus for research into the possibility of a new generation of antimicrobials. This is concentrated on inhibition of biofilm formation using QSIs. Strategies that have shown promise include disrupting AI-2 generation, mimicking signalling molecules, inhibiting efflux transporters involved in QS pathways, and removing AI-2 processing enzymes. The group of QSIs known as furanones, derived from the marine alga *Delsia pulchra,* have been most extensively investigated as potential next generation antimicrobials (Lyons et al., 2020). For example, the synthetic furanone C-30 inhibits induction of virulence factors in *Pseudomonas aeruginosa,* thereby attenuating the organism and facilitating immune cell-mediated killing of bacteria (Bjarnsholt et al., 2013). Generally combining QSI use with antibiotics improves sensitivity of bacteria and therefore provides a good strategy for enhancing the antimicrobial effects and preventing bacterial resistance (Cheng et al., 2014a). For treatment of biofilms, combination therapy using antibiotics with biofilm inhibitors such as enzymes that can degrade the EPS matrix or efflux pump inhibitors has shown some success. However, biofilm inhibitors are not bactericidal, therefore biofilm production restarts once treatment is discontinued (Cheng et al., 2014a).

Another emerging strategy for antibiotic alternatives is to develop compounds designed to inhibit bacterial virulence factors. This may cause pathogens to lose their virulence and enable eradication by the host immune system (Bjarnsholt et al., 2013). There has been some success in trials to inhibit type three secretion systems in *Yersinia pseudotuberculosis* and *Salmonella spp.* Prevention of adhesion using pilicides to inhibit bacterial pili formation, and inhibiting toxin and pilus in *Vibrio cholerae* have been shown to be effective. For BDD, this may be a useful prophylactic approach to preventing biofilms from developing. In addition, improved knowledge of surface proteins, which are important virulence determinants in spirochaetes, is a valuable strategy for vaccine development and therefore disease prevention (Pulzova & Bhide, 2014).

This chapter examines global differential mRNA expression in *T. phagedenis* strain T320A during attachment to fibrinogen in microtiter plates as a model for biofilm formation during adhesion to bovine foot skin. It also compares the transcriptome from one species of each of the three cultivable phylogroups of BDD pathogenic treponemes under anaerobic and microaerobic conditions; to identify genes which may be responsible for resistance to oxidative stress. For comparison, the human nonpathogenic *T. phagedenis* Reiter is also studied, since investigation of its genome suggests the

presence of fewer genes likely to allow adaptation to microaerobic conditions compared to the bovine species, which may therefore explain absence of pathogenicity (Staton et al., 2021a). Since BDD is considered a polytreponemal disease, in which multiple bovine *Treponema* species are identified simultaneously (Evans et al., 2009b; Klitgaard et al., 2008; Nordhoff et al., 2008), we also study biofilm formation and response to oxidative stress in RNA samples from mixed treponeme species samples. The biofilm phenotype is simulated with a *T. phagedenis* T320A/ *T. pedis* T3552B dual species mix; whilst the response to oxidative stress in the same mix, and in a *T. phagedenis* T320A/ *T. pedis* T3552B/ *T. medium* T19 triple species mix is also studied.

7.2 Materials and methods

The BDD spirochaetes *T. medium* strain T19, *T. phagedenis* strain T320A and *T. pedis* strain T3552B, which are considered representative of the three major phylogroups of cultivable BDD-associated treponemes (Evans, et al., 2009b) were grown to late exponential phase under anaerobic conditions as described in Chapter 2.1. The human commensal strain *T. phagedenis* Reiter was also grown for 7 days to late exponential phase as described previously (Evans et al., 2009c).

7.2.1 Determining optimum incubation conditions and incubation time

Experiments have shown that RNA biosynthesis in *T. pallidum* is fastest in the first three hours and largely finished by 12 hours post-induction, suggesting the fastest rate of metabolism in the short term (Cox & Radolf, 2006). A study of the *T. denticola* transcriptome used short incubation times to capture transcriptional changes during early interactions (Sarkar et al., 2014). To determine the suitability of a six-hour incubation time for RNA sample collection from the *Treponema* species in this project, phenotypic data was collected. Pairs of 96-well microtiter plates were inoculated as described in Chapter 2.7.2 with three strains of *Treponema*: T320A, T3552B and Reiter grown under standard conditions (Chapter 2.1) with two columns (16 wells) for each strain. Microtiter plates were incubated in an anaerobic cabinet (Don Whitley Scientific, UK) (85% N_2, 10% H_2 and 5% CO_2, 36^{o}C) for 48 hours to correspond to growth of bacteria to mid-end logarithmic phase. ODs were read at 540nm on a spectrophotometer (Multiskan®EX, Thermo Fisher Scientific, Massachusetts, USA). Plates were incubated under microaerobic (5%CO_2, 5%O_2, 37^{o}C, Sanyo O_2/CO_2 incubator MCO-18M, Sanyo, Osaka, Japan) or aerobic conditions (0.3%CO_2, 37^{o}C, Sanyo CO_2 incubator MCO-175, Sanyo, Osaka, Japan) for six hours and ODs were re-measured at 540nm. Three experimental replicates were carried out on different days.

The results informed a second experiment to collect phenotypic data using Cell Titer-Blue® (CTB, Promega, Wisconsin, USA) to assess cell viability (which is based on measurement of a colour change

which occurs as living cells convert blue resazurin to fluorescent pink resorufin) as previously used for *T. denticola* (McHardy et al., 2010). Three 96-well microtiter plates were inoculated with three strains of *Treponema*: T320A, T3552B and Reiter grown under standard conditions with four columns (32 wells) for each strain. Microtiter plates were incubated in an anaerobic cabinet for 48 hours to correspond to growth of bacteria to mid-end logarithmic phase. A control plate remained in the anaerobic cabinet whilst one was incubated under aerobic conditions and the third under microaerobic conditions. Incubation times tested were three, six and 24 hours, and one week. At each time point, 50µl of culture was removed from each of 6 wells and pooled in an Eppendorf tube for each species and incubation condition, resulting in nine samples. 30µl CTB was added, tubes inverted and incubated at room temperature for ten minutes whilst colour change developed. Microtiter plates were returned to their respective incubators. Samples were centrifuged (Prism microcentrifuge, Labnet International, Edison, USA) at 13,500*g* for five minutes and 100µl from each transferred to a microtiter plate and read using a spectrophotometer at 620nm. Three experimental replicates were carried out with initial inoculations on different days.

7.2.2 Preparation of material for RNA extraction

Samples collected for RNA extraction included single, dual and triple species samples as Planktonic (P) or Biofilm (B) phenotypes. Planktonic samples incubated for 48 hours under anaerobic conditions were used as controls for comparing the treatment effects of exposure to microaerobic conditions (M) or growth as biofilm phenotypes (B) (Table 7.1). Material was prepared and RNA extracted from three experimental replicates for each sample type.

Table 7.1 Samples collected for RNA extraction

Species	Anaerobic conditions	Microaerobic conditions
Single species T19	Planktonic, T19 P	Planktonic, T19 M
Single species T320A	Planktonic and Biofilm, T320A P and T320A B	Planktonic, T320A M
Single species T3552B	Planktonic, T3552B P	Planktonic, T3552B M
Single species Reiter	Planktonic, RP	Planktonic, RM
Dual species T320A + T3552B	Planktonic and Biofilm, T320A+T3552B P and T320A+T3552B B	Planktonic T320A+T3552B M
Triple species T19+T320A+T3552B	Planktonic, T19+T320A+T3552B P	Planktonic T19+T320A+T3552B M

96-well polystyrene microtiter plates were coated with 50µg/ml bovine fibrinogen as described in Chapter 2.7.1.

Microtiter plates were inoculated in groups of four, leaving all outermost wells containing 200µl of growth medium and the 60 inner wells containing 150ul of growth medium and 50µl of the relevant bacterial culture. For multispecies samples, bacteria were inoculated at double (for T320A +T3552B) or treble (for T19 + T320A +T3552B) their standard ODs (Chapter 2, table 2.2) and the volume of each species reduced accordingly to maintain the 50µl volume. This resulted in 240 wells being available to comprise each RNA sample collected. Microtiter plates intended for use as anaerobic samples were incubated for 48 hours in an anaerobic cabinet. Those intended as microaerobic samples were incubated anaerobically for 42 hours before transferring to an incubator set to microaerobic conditions for six hours prior to RNA extraction. This timeframe has previously been used for capturing transcriptional changes in the early stages of interaction between *T. denticola* and other species of oral bacteria (Sarkar et al., 2014) and results from phenotypic experiments (7.2.1) supported this as a suitable incubation time. All microtiter plates were stored in sealed polythene bags containing 3ml of distilled water to minimise evaporation from wells.

Bacteria for RNA samples were collected from one microtiter plate at a time to minimise the risk of changes to gene expression occurring. For planktonic samples, 50µl of supernatant was removed from each well using a pipette and added to 1.5ml Eppendorf tubes. For biofilm samples, 130µl of supernatant was carefully removed from all wells using a multichannel pipette and discarded. Cells adhered to the bottom of wells were disrupted by pipetting up and down three times using a multichannel pipette, then 50µl of material was removed from each well individually and added to 1.5ml Eppendorf tubes. Tubes were centrifuged at 13,500*g* for five minutes to pellet the bacterial cells. The supernatant was poured off and the tube blotted on absorbent paper. 1ml 1X Phosphate Buffered Saline was added to each pellet, tubes were vortexed and centrifuged for a second time at 13,500*g* for five minutes. The supernatant was discarded again, and each tube blotted on absorbent paper. This procedure resulted in eight pellets for each sample, which were later pooled as part of the RNA extraction procedure. Pellets were stored at 4^{o}C for a maximum of one hour until extraction.

7.2.3 RNA extraction protocol

RNA extraction was carried out using chloroform extraction and ethanol precipitation as per previously published protocol with some modifications introduced during pilot studies to optimise quantity and quality of RNA (Clements et al., 2006). 240µl Trizol™ reagent (ThermoFisher Scientific, Massachusetts, USA) was added to each pellet and pipetted up and down several times to homogenise and ensure lysis. All pellets were incubated at room temperature for five minutes to permit complete

dissociation of the nucleoproteins complex before adding 48µl of chloroform and shaking by hand for 15 seconds. All samples were incubated for 3 minutes then centrifuged at 4°C for 15 minutes at 12,000g (Sigma laboratory centrifuge 2K15, Philip Harris Scientific). 80µl of the aqueous phase from each of eight tubes was pooled to complete each sample and 320ul of isopropanol added and samples frozen at -80°C overnight.

Samples were centrifuged at 12,000*g* for 10 minutes at 4°C, the supernatant discarded, and the tube blotted on absorbent paper. Pellets were washed with 1ml of 100% ethanol (Sigma-Aldrich, Missouri, USA), vortexed to dislodge the pellet, and centrifuged at 7,500*g* for 5 minutes at 4°C. The supernatant was discarded in the same fashion and pellets washed with 1ml of 75% ethanol. Samples were vortexed and centrifuged again at 7,500*g* for 5 minutes at 4°C. The supernatant was discarded again, this time carefully removing any remaining supernatant using a pipette. Samples were air dried in a fume hood for ten minutes and then at 65°C in a heat block for 2-3 minutes. Pellets were re-suspended in 50µl RNase free water (Thermo Fisher Scientific, Massachusetts, USA) warmed for 2-3 minutes at 65°C in a heat block, and vortexed for 20 seconds to ensure pellets were fully resuspended.

Total RNA contents were quantified (µg) using Nanodrop spectrophotometer readings (Thermo Fisher Scientific, Massachusetts, USA) to calculate the mixture required for gDNA digestion. 1µl of RNasin Plus (Promega, Southampton, UK) was added to each sample and the Thermo Scientific DNase I, RNase-free kit (Thermo Fisher Scientific, Massachusetts, USA) used according to manufacturer's instructions, except doubling the DNase content used to 2µl of enzyme per µg of RNA. Samples were warmed at 37°C for 40 minutes using a heat block prior to addition of EDTA stop buffer, and heating for 10 minutes at 65°C.

To improve RNA quality, samples were purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) as follows. Sample volumes were adjusted to 100µl by addition of calculated quantities of RNase-free water. 350µl Buffer RLT was added, and samples were vortexed for 20 seconds. 250µl 100% ethanol was added and mixed well by pipetting before 700µl of each sample was transferred to an RNeasy mini spin column (supplied within collection tubes). Columns were centrifuged at 8000*g* for 30 seconds (Prism microcentrifuge, Labnet International, Edison, USA) and transferred to new collection tubes. 500µl Buffer RPE was added, samples were centrifuged again at 8000*g* for 30 seconds and columns placed in new collection tubes. A second wash was carried out with 500µl Buffer RPE, this time centrifuging at 12,000*g* for 2 minutes and columns were once more placed in new collection tubes. Samples were centrifuged again for a further minute at 12,000*g* to ensure the membranes were fully dry. Columns were transferred to 1.5ml collection tubes (Eppendorf) and air dried in a fume hood

for 5 minutes. Finally, RNA was eluted by addition of 40µl of RNase free water to the columns, which were centrifuged at 8000*g* for 1 minute.

Final RNA quantities, and 280/ 260 and 260/ 230 ratios were measured using a Nanodrop spectrophotometer. Additionally, RNA was also quantified using the Qubit™ fluorometer high sensitivity RNA assay kit (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA).

7.2.4 RNA sequencing

Quality control, rRNA depletion, sample purification, and Illumina® sequencing, were performed by Dr Ecaterina Vamos and Charlotte Nelson at the University of Liverpool Centre for Genomic Research (CGR). Bioinformatic analysis was provided by Dr Yongxiang Feng at CGR.

Cytoplasmic and mitochondrial rRNA was removed from samples using 300ng of total RNA as input material to the NEBNext® rRNA depletion (bacteria) protocol according to manufacturer's instructions. RNA libraries were generated using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (New England Biolabs, Massachusetts, USA). Following 11 amplification cycles, libraries were purified using Ampure XP beads (Beckman Coulter Life Sciences, Indianapolis, USA) and labelled with unique double barcode sequences for sample identification. The Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific, Fair Lawn, NJ, 39 USA) and Fragment Analyser data (Agilent, Agilent Technologies 40 Inc., Santa Clara, CA, USA) was used to assess quantity and quality of final libraries, and pool them in equimolar amounts. Quantity and quality were also assessed by qPCR using the Illumina® KAPA Library Quantification Kit (KK854, Kapa Biosystems, Wilmington, USA) on a Roche LightCycler® (LC4811, Roche Diagnostics Ltd, Burgess Hill, UK) according to manufacturer's instructions. Briefly, a 10µl PCR reaction (performed in triplicate for each pooled library) was prepared on ice with 8µl SYBR Green I Master Mix and 2µl diluted pooled DNA (1:1000 to 1:100,000 depending on the initial concentration determined by the Qubit® dsDNA HS Assay Kit). PCR thermal cycling conditions consisted of initial denaturation at 95°C for 5 minutes, 35 cycles at 95°C for 30 seconds (denaturation) and 60°C for 45 seconds (annealing and extension), melt curve analysis to 95°C (continuous) and cooling at 37°C.

Following calculation of the molarity using qPCR data, template DNA was diluted to 300pM and denatured for 8 minutes at room temperature using freshly diluted 0.2N sodium hydroxide (NaOH) and the reaction subsequently terminated by addition of 400mM TrisCl pH8. To improve sequencing quality control 1% PhiX was added. The libraries were sequenced on the Illumina® NovaSeq 6000 platform (Illumina®, San Diego, USA) following the standard workflow over one lane of an S1 flow cell, generating 2 x 150 bp paired end reads.

7.2.5 Bioinformatic Analysis

Basecalling and de-multiplexing of indexed reads was performed by CASAVA version 1.8.2 (Illumina) to produce the sequence data for the samples, in FASTQ format. Raw FASTQ files were trimmed to remove Illumina adapter sequences using Cutadapt version 1.2.1 (Marcel Martin, 2011). The option "-O 3" was set, so the 3' end of reads which matched the adapter sequence over at least 3bp was trimmed away. Reads were further trimmed to remove low quality bases, using Sickle version 1.200 with a minimum window quality score of 20. After trimming, reads shorter than 20bp were removed. Read pairs were subsequently analysed if both reads from the pair passed this filter.

Reads were aligned to reference sequences appropriate for each of the four *Treponema* strains included in the project and annotated using GTF files. TopHat 2.1.0 was used as the alignment tool with option "-g1" which instructs for best hits, or randomly select a best hit where there are multiple best hits. Further KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway annotation information was obtained using the eggNOG-mapper tool (Huerta-Cepas et al., 2019). Reads aligning to the reference genome sequences were counted according to the gene features that they mapped to, as defined in the GTF files, using HTSeq-count version 0.6.1p1 (Anders et al., 2015).

7.2.6 Differential Gene Expression Analysis

Differential gene expression (DGE) analysis was performed on the gene count table in the R enviro (Anders & Huber, 2010) package (Anders & Huber, 2010). Planktonic anaerobic samples (Table 7.1) were considered the baseline for assessing within-group variation attributable to technical and biological variation, compared to between-group variation attributable to different growth conditions. Correlation analysis, Principal Component Analysis (PCA) and pairwise scatter plots were used to assess and visualise within sample group variation. Samples were excluded from further analysis if the within-group variation was greater than between-group variation to improve statistical power in DGE detection. An additional assessment was used to contrast the read count contributions from different strains among samples and sample groups. The function hclust was used in R (Rstudio v2022.02.1+461, Boston, USA) to generate heatmaps depicting clustering of samples by growth condition for each of the four single treponeme species, as well as for dual and triple species samples.

For each gene count data set, the DGE analysis employed a Generalised Linear Model (GLM) which takes each group mean as a model coefficient. The required contrasts were then performed based on model fit results using the contrast fit technique. Normalisation factors were calculated to correct for differences in library size among samples using the default method Trimmed Mean M values (TMM) in edgeR. The gene-wise dispersions were estimated and adjusted considering a fitted dispersion and

abundance relationship from the data. The GLM was parametrized using the count data and then used to obtain the log_2FC values for each required comparison. The estimated log_2 Fold Changes (log_2FC) were tested using the LR (likelihood ratio) test. P-values associated with log_2FC were adjusted for multiple testing using the False Discovery Rate (FDR) approach (Benjamini & Hochberg, 1995). Significantly differentially expressed genes (DEGs) were defined as those with FDR-adjusted P-value < 0.05.

All differentially expressed genes where $-1>log_2FC>1$ and FDR<0.05 were matched to proteins using the annotated genomes in R (Rstudio v2022.02.1+461, Boston, USA). The accession numbers for the genome sequences used are GenBank: CP027017, CP027018, CP045670 and CP031394. Protein lists containing the amino acid sequences were converted from .txt to FASTA format using Batch Entrez (NCBI, Maryland, USA) and then functionally annotated at species level using the KEGG (Kyoto Encyclopaedia of Genes and Genomes) Orthology database via the BlastKOALA search tool (Kanehisa et al., 2016). Database results were examined individually to identify trends then reviewed alongside the BlastKOALA data annotation summaries, and pathways identified by the KEGG Mapper Reconstruction Results and BRITE Hierarchies (Kanehisa et al., 2022).

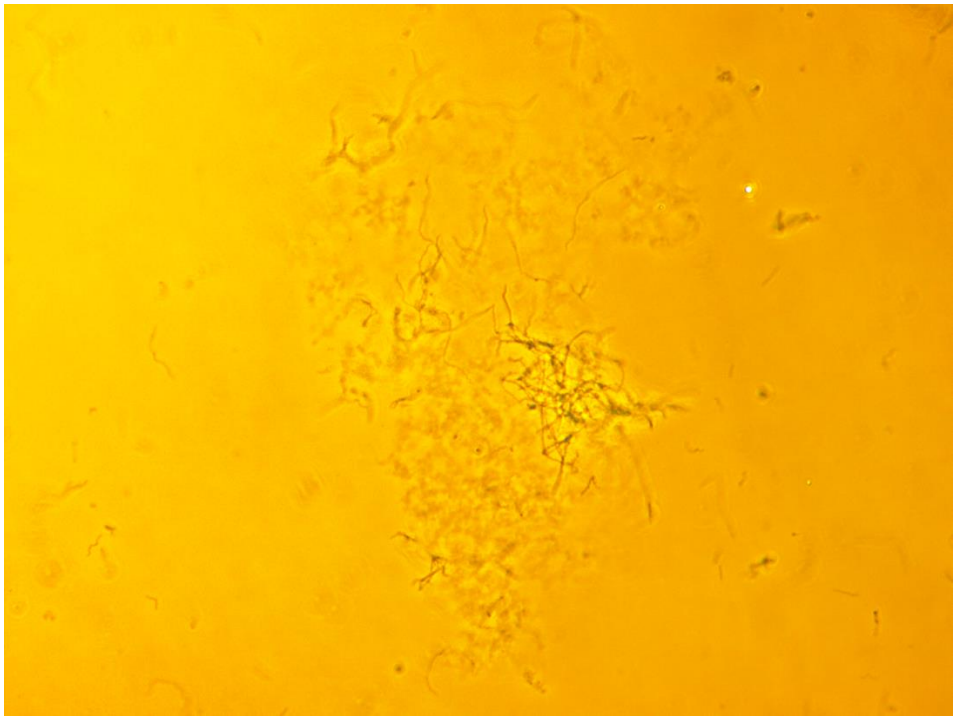
Mauve Multiple Genome Alignment (version 10, The Darling lab, University of Technology, Sydney) was used to align the four treponeme species and examine orthologs of genes of interest to aid in comparison of differences in DEGs between species (Darling et al., 2004).

7.3 Results

7.3.1 Determining optimum incubation conditions and incubation time

Here, exposure to oxygen increased the relative OD of treponemal cultures, when OD would be expected to decrease as bacterial cells die. Median and interquartile range for each species were: T320A 112.78% (107.4-124.57), T3552B 132.04% (118.07-128.89), and Reiter 110.18% (110.55-110.63). Phase contrast microscopy images suggested this turbidity increase may be due to clustering of treponemes, which was marked in T3552B, fitting with the finding that the relative OD increased the most for this species (Figure 1). Relative OD was therefore not an appropriate proxy for cell viability under oxidative stress so the Cell Titer-Blue® Cell Viability Assay was trialled instead.

Figure 1 Clustered appearance of T3552B using phase contrast microscopy after six hours' exposure to aerobic conditions.



7.3.2 Cell Titer Blue Data Analysis

Data from three experimental replicates was formatted in Microsoft Excel and imported into STATA(v14) (Statacorp, USA). Optical Density (620nm) data measured during the CTB experiments followed normal distribution; therefore, univariable regression was used to assess the effect of species, incubation condition and incubation time on OD. Variables where $P<0.1$ (incubation condition and time) were offered to a final multivariable linear regression model, which showed no significant differences in OD at 6 hours compared to 3 hours, but statistically significant increases in OD for longer incubation times. Both microaerobic and anaerobic conditions increased OD at all time points ($P<0.05$, Table 7.2).

Lower ODs were observed to correspond to more marked colour change from blue towards pink, so lower OD is used as a proxy for increased metabolic activity. Incubation times of 24 hours or one week decreased metabolic activity. Microaerobic incubation conditions decreased metabolic activity, and aerobic incubation decreased it further.

Table 7.2: Linear regression model with OD_{620nm} of Cell Titer Blue as the outcome variable, showing significant differences attributed to incubation time and incubation conditions.

		Coefficient Absorbance (OD_{620})	Standard Error	t	P value	95% confidence interval
Incubation time Baseline = 3 hours	6 hours	0.005	0.014	0.37	0.714	-0.023-0.033
	24 hours	0.054	0.014	3.76	<0.001	0.025-0.082
	1 week	0.040	0.014	2.76	0.007	0.011-0.070
Incubation conditions Baseline = anaerobic	microaerobic	0.038	0.012	3.03	0.003	0.013-0.062
	aerobic	0.072	0.012	5.80	<0.001	0.047-0.096
Baseline (3 hours, anaerobic)		0.228	0.012	18.4	<0.001	0.203-0.253

OD_{620nm} data was summarised for each species using mean and standard deviation. Considering anaerobic, microaerobic and aerobic data for the three species, variation in OD_{620nm} was lowest at six hours after initial readings were taken (Table 7.3). T3552B was most metabolically active under all conditions; Reiter remained more metabolically active under microaerobic and aerobic conditions compared to T320A. When species was forced into the linear regression model, it showed that T3552B was more metabolically active (lower OD_{620nm}) than T320A (P=0.017) whereas there was no significant difference when comparing Reiter to T320A (P=0.473).

Considering the phenotypic data, RNA samples were collected after six hours' exposure of *Treponema* to microaerobic conditions. This was deemed optimum for allowing detectable changes in transcription without markedly decreasing cell viability.

Table 7.3: Means and standard deviations for OD_{620nm} measured at four time points during Cell Titer Blue experiments.

Timepoint	Mean OD_{620nm}	Standard Deviation
3 hours	0.264	0.056
6 hours	0.270	0.042
24 hours	0.318	0.071
1 week	0.304	0.067

7.3.3 Quality Control results

A summary of the quality and quantity of final libraries is shown in Table 7.4. Sample quality and quantity for individual samples is available in Appendix E, Table 1. RNA quantity varied from 30.4-1019ng/µl, and RIN numbers from 1.1-9.5. It was not possible to improve RNA quality for T19 samples, therefore we had to allow much lower values for these sample types (range 1.1-3.3).

Table 7.4 Summary of median RNA quantity and quality for each sample type

Sample contents	Median RNA quantity (ng/µl)	Median RIN
T19 P	34.4	1.8
T19 M	56.5	1.2
T320A P	73.6	6.6
T320A M	62.6	5.9
T320A B	212.5	6.1
T3552B P	356	7.6
T3552B M	310	7.2
Reiter P	161.8	5.9
Reiter M	134.4	6.2
T320A+T3552B P	236	6.4
T320A+T3552B M	372	7.2
T320A+T3552B B	260	6.8
T19+T320A+T3552B P	598	7.2
T19+T320A+T3552B M	462	7.2

7.3.4 Analysis of Sequenced Reads

Number of raw reads ranged from 11,671,276 - 117,520,464 resulting in 5,829,511 - 58,609,384 trimmed read pairs. Full data for sequenced reads, trimmed read pairs and mapped read pairs are recorded in Appendix E, Table 1.

Anaerobic planktonic samples were used as controls for comparison of their microaerobic and/ or biofilm equivalents. For dual and triple-species mixes, data was analysed using each species as the baseline due to differences in the contribution of each species to samples. DGE analysis was therefore performed after splitting dual and triple species samples further according to each species. Heatmaps depicting hierarchical cluster analysis of samples for single, dual and triple species samples are available in Appendix E, Figure 1. One experimental replicate from each of the following sample types were excluded following within and between-group analyses: T19 M, T320A P, T320A B, T320A + T3552B P and T320A +T3552B B.

7.3.5 Differential Gene Expression Analysis

Differential Gene Expression Analysis is reported after applying the criteria $-1>\log_2FC>1$ and FDR<0.05. Table 7.5 shows that the dual and triple species cultures upregulated notably more genes (286 and 294 respectively) than the single-species samples (78, 38, 21 and 28 for T19, T320, T3552B and Reiter respectively). Dual and triple species samples also downregulated more genes (72 and 67) compared to single species samples (14, 11, 0 and 1). More differentially expressed genes were detected for the pathogenic BDD T320A strain (58) compared to its non-pathogenic human equivalent Reiter (29) under microaerobic conditions. When compared to standard anaerobic planktonic samples, samples incubated under microaerobic conditions yielded higher numbers of DEGs than biofilm phenotype samples. Biofilm phenotype samples showed more downregulated (40) than upregulated genes (10). The 20 most upregulated and 20 most downregulated genes for each of the six sample types (according to $\log_2FC$) are illustrated in bar charts in Figure 2 in Appendix E.

Table 7.5 Number of Differentially Expressed Genes in Sample Comparisons ($-1>\log_2FC>1$, FDR<0.05)

For each sample type, comparisons were made to the anaerobic planktonic samples for the same species or mix of species. For mixed species samples, component species were considered separately since they did not uniformly contribute to the contents of each sample.

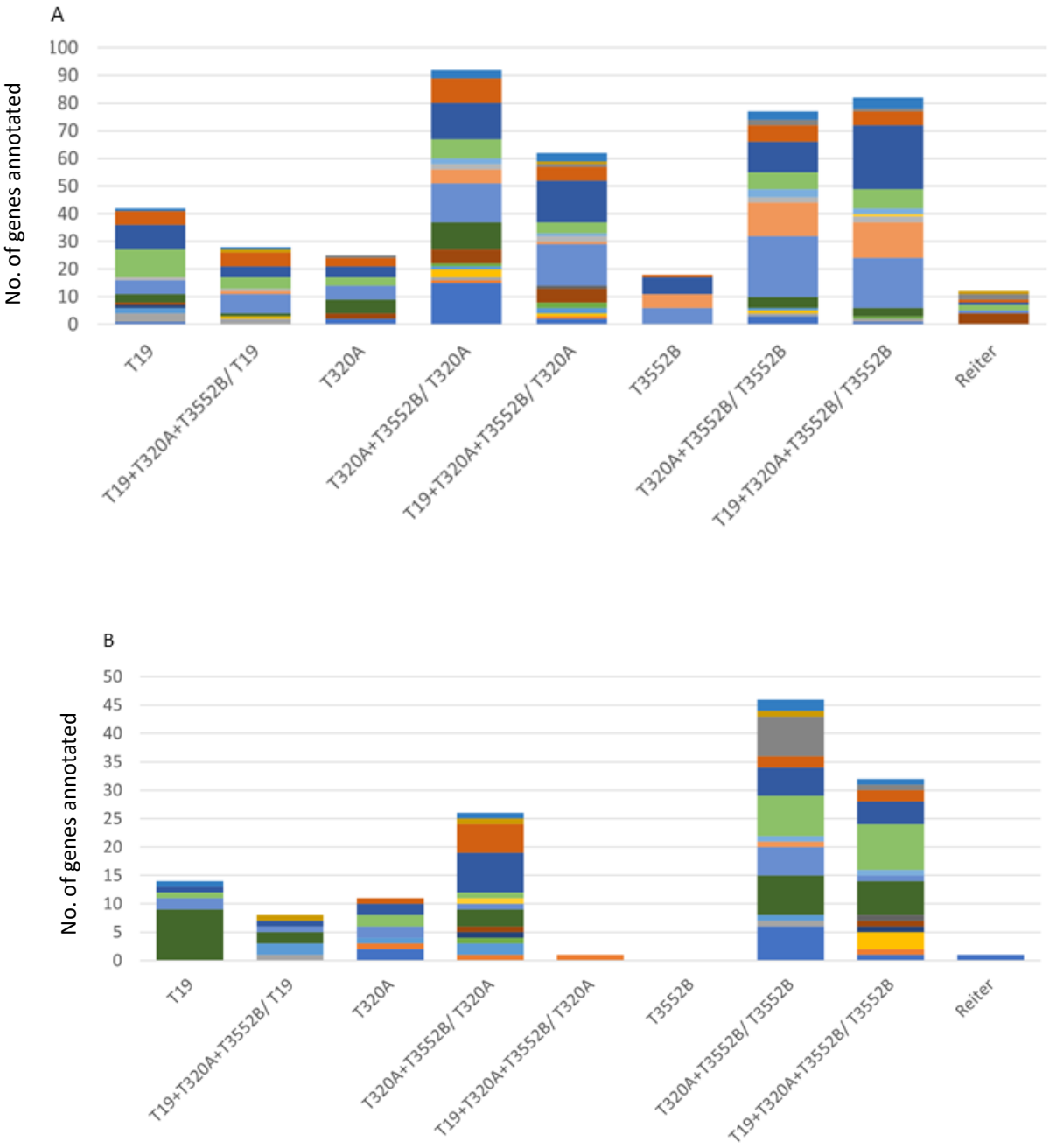
Sample	Total Upregulated genes	Upregulated genes annotated using BlastKOALA	Total Down-regulated genes	Down-regulated genes annotated using BlastKOALA
T19 M	78	42	30	14
T320A M	38	25	20	11
T3552B M	21	18	3	0
Reiter M	28	12	1	1
T320A+T3552B/T320A M	163	92	130	46
T320A+T3552B/T3552B M	123	77	87	26
T19+T320A+T3552B/T19 M	45	28	11	8
T19+T320A+T3552B/T320A M	92	62	3	1
T19+T320A+T3552B/T3552B M	157	82	50	32
TOTAL FOR MICROAEROBIC SAMPLES	745	438	335	139
T320A B	10	7	20	11
T320A+T3552B/T320A B	0	0	12	7
T320A+T3552B/T3552B B	0	0	8	1
TOTAL FOR BIOFILM PHENOTYPE	10	7	40	19

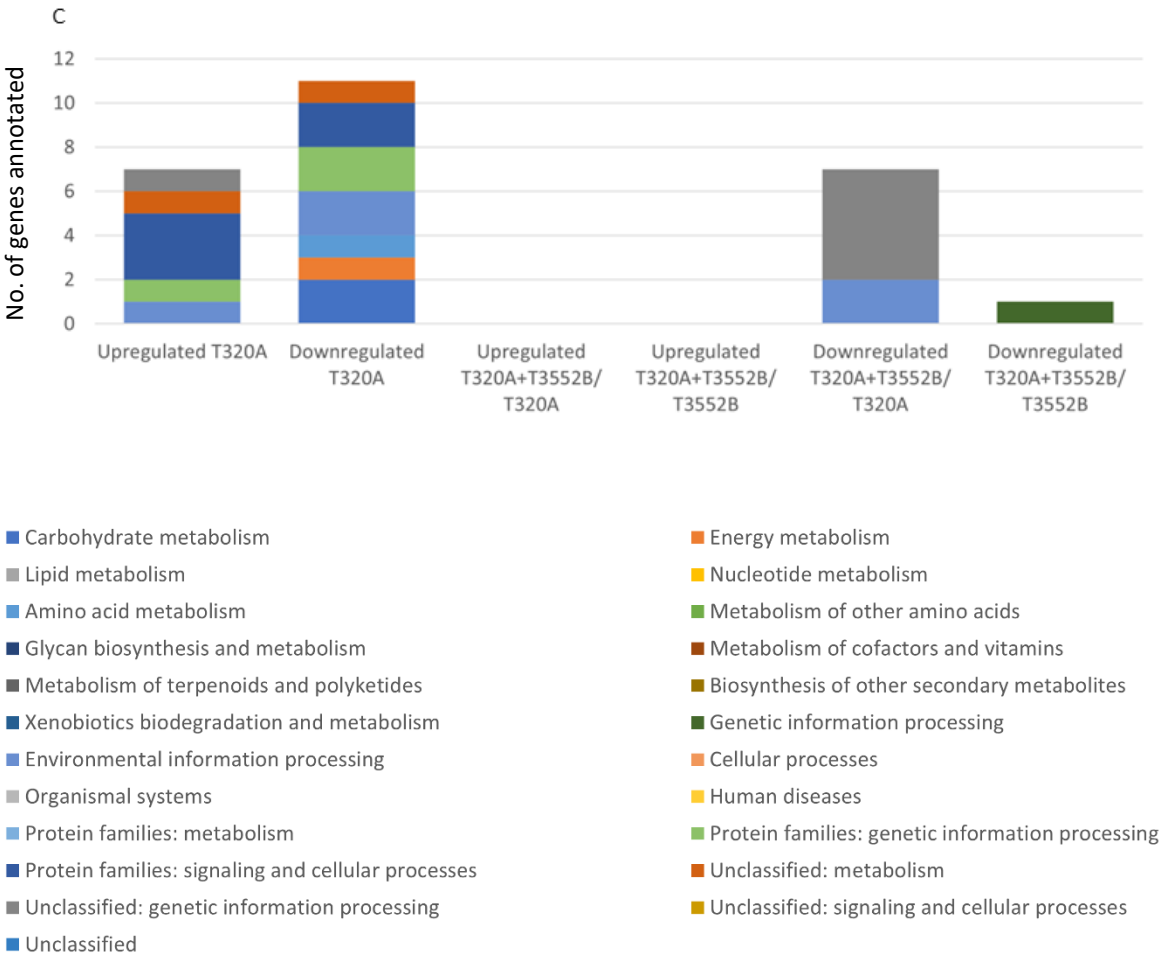
7.3.5.1 Functional annotation of genes using BlastKOALA

Following matching of DEGs to the proteins they encode using the annotated genomes, BlastKOALA classified 438/745 (58.8%) predicted upregulated proteins and 139/465 (29.9%) predicted downregulated proteins. Overall, under microaerobic conditions, proteins in the categories: Environmental information processing (93/438), Protein families: signalling and cellular processes (86/438), and Cellular processes (43/438) were most commonly upregulated. There is likely to be significant overlap in the most common two categories as membrane transporters and proteins associated with their processes could be classified in both categories. Genes in the categories: Genetic information processing (27/139), Protein families: signalling and cellular processes (20/139, and Protein families: genetic information processing (19/139) were most commonly downregulated. No environmental information processing or amino acid metabolism predicted proteins were annotated for the non-pathogenic *T. phagedenis* Reiter. As shown in Table 7.5, the number of upregulated genes was smaller for the biofilm phenotype samples compared to downregulated genes. Genes for carbohydrate, energy and amino acid metabolism, and those for genetic information processing were downregulated. Bar graphs in Figure 2 illustrate the functional annotation of DEGs using BlastKOALA.

Figure 2 Bar Graphs showing classification of proteins into KEGG categories functionally annotated using BlastKOALA.

A) Classification of differentially expressed genes upregulated under microaerobic conditions compared to equivalent anaerobic samples
B) Classification of differentially expressed genes downregulated under microaerobic conditions compared to equivalent anaerobic samples
C) Classification of genes upregulated or downregulated in the biofilm phenotype compared to equivalent planktonic samples
(For dual and triple-species samples, baseline strains are denoted by the denominators)

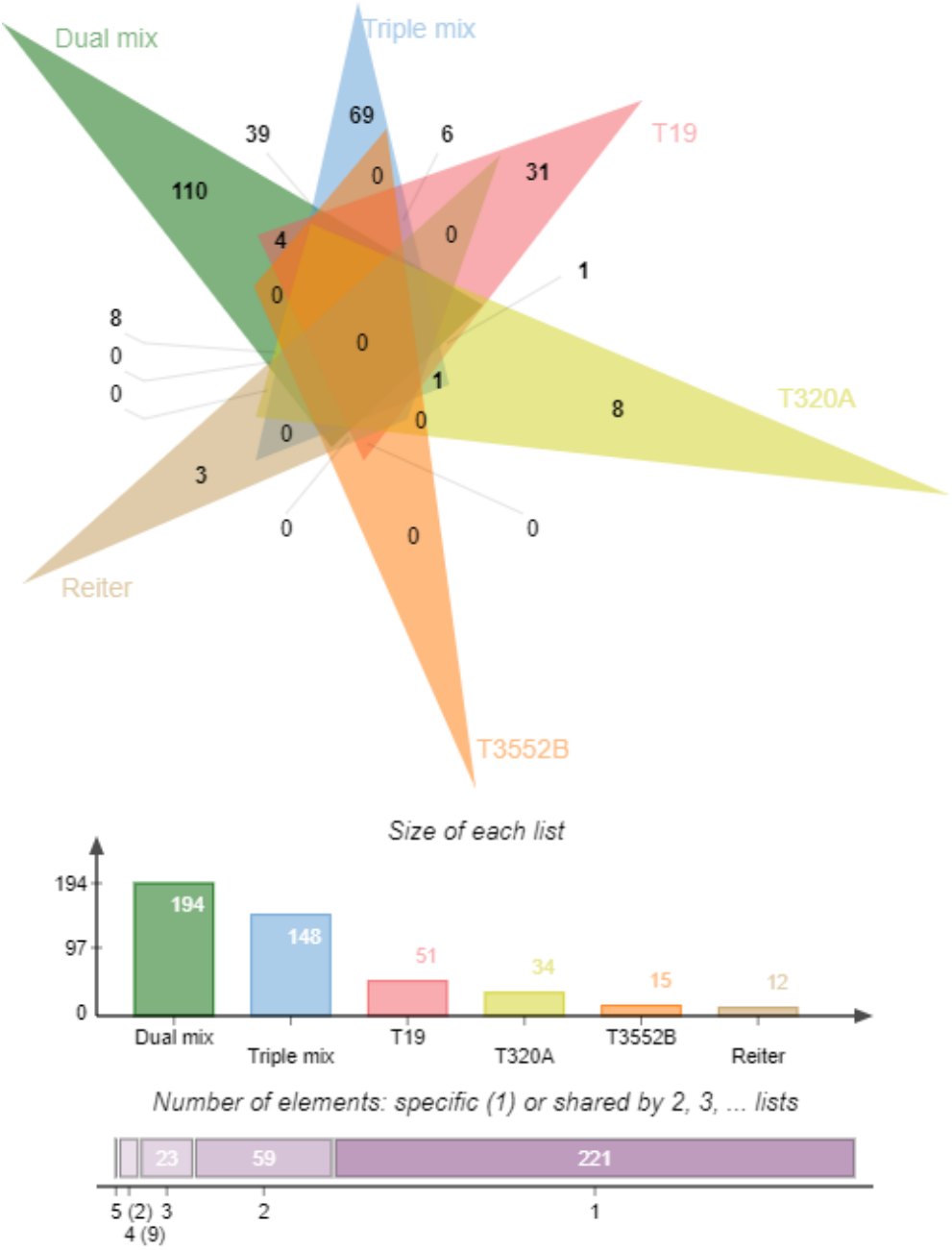



C
No. of genes annotated
12
10
8
6
4
2
0
Upregulated T320A
Downregulated T320A
Upregulated T320A+T3552B/ T320A
Upregulated T320A+T3552B/ T3552B
Downregulated T320A+T3552B/ T320A
Downregulated T320A+T3552B/ T3552B
Carbohydrate metabolism
Energy metabolism
Lipid metabolism
Nucleotide metabolism
Amino acid metabolism
Metabolism of other amino acids
Glycan biosynthesis and metabolism
Metabolism of cofactors and vitamins
Metabolism of terpenoids and polyketides
Biosynthesis of other secondary metabolites
Xenobiotics biodegradation and metabolism
Genetic information processing
Environmental information processing
Cellular processes
Organismal systems
Human diseases
Protein families: metabolism
Protein families: genetic information processing
Protein families: signaling and cellular processes
Unclassified: metabolism
Unclassified: genetic information processing
Unclassified: signaling and cellular processes
Unclassified

7.3.5.2 Response to Oxidative stress

KEGG Orthology pathways identified using BlastKOALA showed minimal overlap between functional pathways identified in the six sample types exposed to microaerobic conditions (Figure 4). We were not able to detect any "core" predicted pathways affected by oxidative stress shared by all sample types from our data. There were no predicted pathways shared by all three BDD single species sample types.

Figure 4: Venn diagram showing differences in dysregulated KEGG orthology functional pathways identified by BlastKOALA for the six sample types exposed to microaerobic conditions.



To further investigate differences in the response to oxidative stress, dysregulated genes were examined to identify presence/ absence of orthologs amongst the four treponeme species. Species differences were identified for 38 genes. One was exclusive to T19, two were exclusive to T320A, and ten were exclusive to T3552B, including Superoxide Dismutase. Their presence/ absence and log_2FC where they were dysregulated under microaerobic conditions are presented in Table 7.7.

The use of BRITE hierarchies in BlastKOALA identified four key themes associated with bacterial survival: upregulation of transport proteins, especially those needed for iron acquisition and nickel transport, upregulation of genes for proteins protecting against oxidative stress, upregulation of molecular chaperones and folding catalysts, and downregulation of DNA repair and recombination proteins. There is some evidence of increased expression of genes responsible for bacterial virulence and motility. Details from this categorisation are available in Table 2 in Appendix E. Key genes of interest are listed in Table 7.7 showing whether they were upregulated or downregulated under microaerobic conditions and values for log_2FC. Tables 3 and 4 in Appendix E give additional information regarding functions of upregulated and downregulated genes respectively.

Table 7.7: Genes dysregulated when treponemes are exposed to oxidative stress, showing log_2 fold change for each species in single, dual or triple-species samples.

"ND" Gene present in the genome but not dysregulated.
"no ortholog" denotes genes where no ortholog is present in the genome for that species.

Gene	Gene locus tag	T19 single	T320A single	T3552B single	Reiter single	T320A dual	T3552B dual	T19 triple	T320A triple	T3552B triple
BACTERIAL SURVIVAL										
ROS scavenging										
Flavodoxin I fldA/nifF/isiB	T19: C5N99_00510							+1.777474		
	T19: C5N99_05280							+2.721183		
	T320A: C5O78_02690					+4.036524			+3.563171	
	T320A: C5O78_08060								+3.345655	
	T3552B: DYQ05_02690						+1.900296			+2.690211
	Reiter: DWQ65_11830				+1.466326					
rnfG; NAD+ oxido-reductase	T19: ND									
	T320A: C5O78_03800					+1.272339				

	T3552B DYQ05_05610						+1.140114			
	Reiter: ND									
rnfD; NAD+ oxido-reductase subunit D	T19: C5N99_04805	+1.498755								
	T320A: ND									
	T3552B: ND									
	Reiter: ND									
Dps, starvation inducible DNA binding protein	T19: C5N99_03050	+1.629183						+1.428541		
	T320A: C5O78_04955								+1.582672	
	T3552B: DYQ05_04440									+3.395691
	Reiter: ND									
Dfx; Superoxide Reductase	T19: no ortholog									
	T320A: C5O78_13450		+2.315364						+1.355905	
	T3552B: DYQ05_00300						+1.58783			+2.336406
	Reiter: ND									
SOD (Cu-Zn); Superoxide dismutase	T19: no ortholog									
	T320A: no ortholog									

	T3552B: DYQ05_02635						+1.94399			+3.342183
	Reiter: no ortholog									
Rubredoxin	T19: ND									
	T320A: ND									
	T3552B: DYQ05_08940						-1.05786			
	Reiter: ND									
Protein Repair										
trxA/ trxB	T19: ND									
	T320A: C5O78_04865 C5O78_13070					+1.466286 +1.162459			+2.024007 +3.60359	
	T3552B: DYQ05_02775						+2.550049			+3.52321
	Reiter: ND									
HSP90A/ molecular chaperone HtpG	T19: ND									
	T320A: ND									
	T3552B: DYQ05_02575						+1.157034			+1.514518
	Reiter: ND									

dnaK/HSPA9 molecular chaperone	T19: ND									
	T320A: ND									
	T3552B: DYQ05_09730						+1.550867			
	Reiter: ND									
dnaJ molecular chaperone	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_10620						+1.24379			
	Reiter: no ortholog									
GrpE molecular chaperone	T19: C5N99_00995							+1.485186		
	T320A: C5O78_01920		+1.564380							
	T3552B: DYQ05_09735									+2.58899
	Reiter: ND									
groES	T19: ND									
	T320A: C5O78_12975		+1.853675							
	T3552B: DYQ05_00835									+2.108161
	Reiter: DWQ65_02890				+1.044985					
groEL/ HspD1	T19: ND									
	T320A: ND									

	T3552B: DYQ05_11195						+1.453863			+1.573513
	Reiter: ND									
Hsp20	T19: C5N99_05270							+1.683940		
	T320A: ND									
	T3552B: DYQ05_06930									+2.148058
	Reiter: ND									
msrB	T19: C5N99_07955							+1.587649		
	T320A: ND									
	T3552B: DYQ05_10800						+1.013745			
	Reiter: ND									
Hsp33	T19: ND									
	T320A: C5O78_01465								+1.480962	
	T3552B: ND									
	Reiter: ND									
Lipid repair										
ahpC/ Peroxi-redoxin	T19: C5N99_05075 C5N99_09195							+2.797458 +1.439925		

	T320A: C5O78_04840		+2.19308			+2.467545			+4.565728	
	T3552B: DYQ05_12520						+1.834569			+2.461882
	Reiter: ND									
DNA repair										
uvrABC	T19: ND									
	T320A: C5O78_06720 C5O78_11155					-1.187037 -1.402478				
	T3552B: ND									
	Reiter: ND									
ligA/ ligB	T19: ND									
	T320A: C5O78_05200					-1.187204				
	T19: ND									
	Reiter: ND									
Metal utilisation										
troB/mntB/znuC	T19: ND									
	T320A: C5O78_01815					+1.696205			+2.735185	
	T3552B: DYQ05_13315			+1.7409			+2.411111			+3.194513
	Reiter: ND									

troC/mntC/znuB	T19: ND									
	T320A: C5O78_01810					+2.32204			+3.18374	
	T3552B: DYQ05_13310			+2.056604			+2.659599			+2.542677
	Reiter: ND									
copA	T19: ND									
	T320A: C5O78_02275					+1.451611			+2.279195	
	T3552B: DYQ05_01975						+2.652126			
	Reiter: DWQ65_06250				+1.325757					
tonB	T19: ND									
	T320A: C5O78_13275		+2.130253			+1.971890			+2.081269	
	T3552B: no ortholog									
	Reiter: ND									
yclQ/ceuA	T19: no ortholog									
	T320A: C5O78_03445								+1.895491	
	T3552B: no ortholog									
	Reiter: ND									
yclN/ceuB	T19: no ortholog									

	T320A: C5O78_03450					+1.985480				
	T3552B: no ortholog									
	Reiter: ND									
irtA/ybtP	T19: C5N99_05495 C5N99_10065 C5N99_11755							+3.078599 +2.872999 +1.836555		
	T320A: C5O78_04915 C5O78_11550 C5O78_11595		+2.642106			+2.573844 +2.678753 +1.526385			+3.288488 +2.059774 +1.711581	
	T3552B: DYQ05_02285 DYQ05_04135			+2.710265			+4.364786 +2.308671			+3.073966 +1.6162
	Reiter: ND									
irtB/ybtQ	T19: C5N99_10070							+2.423960		
	T320A: C5O78_04910 C5O78_11555 C5O78_11590 C5O78_11595		+2.429926			+1.466286 +1.925490 +1.680049 +1.526385			+3.715573 +1.576306 +1.439741 +1.711581	
	T3552B: DYQ05_02265			+3.306199			+3.16427			
	Reiter: ND									
TC.FEV.OM2, cirA, cfrA; hmuR	T19: no ortholog									
	T320A: C5O78_09625		+3.767698			+4.237698			+3.838127	
	T3552B: ND									

	Reiter: ND									
ABC.PE.S	T19: no ortholog									
	T320A: C5O78_04875 C5O78_09935					+1.882645			+1.393513	
	T3552B: DYQ05_04555 DYQ05_10355		+2.168674				+1.851716			+3.096781
	Reiter: ND									
feoB	T19: no ortholog									
	T320A: C5O78_12600					+3.113990			+4.026626	
	T3552B: DYQ05_04555						+1.851716			
	Reiter: ND									
feoA	T19: no ortholog									
	T320A: C5O78_12590								+1.403458	
	T3552B: DYQ05_01795 DYQ05_01800						+1.114627			+1.620824
	Reiter:ND									
ABC.PE.P	T19: no ortholog									
	T320A: no ortholog									

	T3552B: DYQ05_00625 DYQ05_04560 DYQ05_10360			+1.904182 +2.443647		+2.105255 +2.546352 +3.593182				+3.992826 +1.907922 +4.745541
	Reiter: no ortholog									
ABC.FEV.P	T19: C5N99_04425	+1.729207								
	T320A: ND									
	T3552B: DYQ05_04165 DYQ05_06980						+2.55712			+1.823182 +2.035446
	Reiter: ND									
ABC.PE.P1	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_04565 DYQ05_10365			+2.09602			+2.213153			+2.123598 +3.443935
	Reiter: no ortholog									
ddpD	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_04570			+1.677515			+2.230003			+2.988603
	Reiter: no ortholog									
ddpF	T19: no ortholog									
	T320A: no ortholog									

	T3552B: DYQ05_04575						+1.858231			+1.689589
	Reiter: no ortholog									
ABC.FEV.A	T19: no ortholog									
	T320A: ND									
	T3552B: DYQ05_04160 DYQ05_06975			+1.808938			+2.509387			+3.626577 +3.341471
	Reiter: DWQ65_10415				+1.044840					
ABC.FEV.S	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_06985									+1.194983
	Reiter: no ortholog									
sufC	T19: C5N99_06030									+1.058298
	T320A: ND									
	T3552B: DYQ05_07410						+2.267503			+2.631984
	Reiter: ND									
sufB	T19: ND									
	T320A: ND									
	T3552B: DYQ05_07415						+2.126714			+2.361507
	Reiter: ND									

htsA	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_07880						+1.480628			+1.01584
	Reiter: no ortholog									
htsB	T19: ND									
	T320A: ND									
	T3552B: DYQ05_07875						+1.875863			+1.859951
	Reiter: ND									
znuA	T19: ND									
	T320A: C5O78_05920								+1.308046	
	T3552B: DYQ05_07960						+2.693038			
	Reiter: ND									
troA/mntA/znuA	T19: ND									
	T320A: C5O78_01820								+1.643371	
	T3552B: DYQ05_13320						+2.330616			+3.376127
	Reiter: ND									
nikA/ cntA	T19: C5N99_11670	+2.131836								

	T320A: no ortholog									
	T3552B: DYQ05_12335			+1.238124			+2.695095			+1.777822
	Reiter: no ortholog									
nikB/ cntB	T19: ND									
	T320A: no ortholog									
	T3552B: DYQ05_12330						+2.592948			+1.484269
	Reiter: no ortholog									
nikC/ cntC	T19: ND									
	T320A: no ortholog									
	T3552B: DYQ05_12325						+2.881445			+1.186794
	Reiter: no ortholog									
nikD/ cntD	T19: ND									
	T320A: no ortholog									
	T3552B: DYQ05_12320						+2.419226			
	Reiter: no ortholog									
nikE/cntF	T19: ND									
	T320A: no ortholog									

	T3552B: DYQ05_12315						+1.595648			
	Reiter: no ortholog									
cbiK	T19: no ortholog									
	T320A: C5O78_12610 C5O78_12615		+3.379769 +4.064311			+3.617999 +3.251610			+4.215718 +4.35108	
	T3552B: DYQ05_01820									+1.031422
	Reiter: ND									
sitA	T19: C5N99_10620							+3.794933		
	T320A: no ortholog									
	T3552B: no ortholog									
	Reiter: no ortholog									
sitB	T19: C5N99_10625							+3.865719		
	T320A: no ortholog									
	T3552B: no ortholog									
	Reiter: no ortholog									
sitC	T19: C5N99_10630							+3.910731		
	T320A: no ortholog									
	T3552B: no ortholog									

	Reiter: no ortholog									
FTR/ FTH1/ efeU	T19: C5N99_12135							+1.418996		
	T320A: C5O78_10310								+1.399046	
	T3552B: ND									
	Reiter: no ortholog									
P19/ ftrA	T19: ND									
	T320A: C5O78_10305								+1.490926	
	T3552B: no ortholog									
	Reiter: ND									
rcnA	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_03845									+1.069143
	Reiter: no ortholog									
ftnA (ferritin)	T19: ND									
	T320A: ND									
	T3552B: DYQ05_03935									+1.121207
	Reiter: ND									
afu/ fbpA	T19: ND									

	T320A: C5O78_04555		-1.767618			-1.538699				
	T3552B: ND									
	Reiter: ND									
ABC.FEV.A	T19: no ortholog									
	T320A: C5O78_09470					-1.669160				
	T3552B: no ortholog									
	Reiter: no ortholog									
ABC.FEV.S	T19: no ortholog									
	T320A: C5O78_09475					-1.725156				
	T3552B: no ortholog									
	Reiter: no ortholog									
ABC.FEV.P	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_00220									-1.00064
	Reiter: no ortholog									
sitB	T19: C5N99_10625	-2.100129								

	T320A: no ortholog									
	T3552B: no ortholog									
	Reiter: no ortholog									
TRANSCRIPTIONAL REGULATOR										
perR, Fur family transcriptional regulator	T19: C5N99_08040							+2.048473		
	T320A: C5O78_05940								+1.990431	
	T3552B: no ortholog									
	Reiter: ND									
VIRULENCE ASSOCIATED GENES										
cheY	T19: no ortholog									
	T320A: C5O78_05810					+1.322324				
	T3552B: ND									
	Reiter: ND									
Mcp	T19: C5N99_00730	-1.482997								
	T320A: C5O78_06860 C5O78_01365 C5O78_09260 C5O78_09285					+1.167960 -1.063188 -1.228017 -2.729438				
	T3552B: DYQ05_03855 DYQ05_04445						+1.198159 +1.370634			

	DYQ05_06765 DYQ05_10570						+2.174452			+1.731695
	Reiter: ND									
Wbpl and wlbD	T19: ND									
	T320A: C5O78_00355					+1.089619				
	T3552B: ND									
	Reiter: no ortholog									
yajC	T19: ND									
	T320A: C5O78_09980		+1.767245			+1.128968				
	T3552B: ND									
	Reiter: ND									
tlyC	T19: C5N99_05095	+1.48914								
	T320A: ND									
	T3552B: ND									
	Reiter: ND									
RelB/ dinJ	T19: C5N99_05410	+1.489140								
	T320A: ND									
	T3552B: ND									
	Reiter: ND									

RelE/ stbE	T19: ND									
	T320A: no ortholog									
	T3552B: DYQ05_01570 DYQ05_01575									+1.036922 +1.066451
	Reiter: no ortholog									
Outer membrane protein	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_07395						+1.088262			
	Reiter: no ortholog									
Exfoliative toxin eta A/B	T19: ND									
	T320A: ND									
	T3552B: DYQ05_04455						-1.38437			
	Reiter: ND									
yoeB toxin	T19: ND									
	T320A: ND									
	T3552B: DYQ05_08895						-1.69896			
	Reiter: ND									
PilZ	T19: C5N99_06360 C5N99_07085	-1.802437 -1.794708								

	T320A: ND									
	T3552B: ND									
	Reiter: ND									
BapA	T19: ND									
	T320A: C5O78_07915					-2.330350				
	T3552B: ND									
	Reiter: ND									
cheW	T19: ND									
	T320A: ND									
	T3552B: DYQ05_09885						-1.01861			
	Reiter: ND									
yidC	T19: C5N99_01905	-1.236264								
	T320A: ND									
	T3552B: ND									
	Reiter: ND									
Motility										
flgK flagellar hook protein	T19: ND									
	T320A: C5O78_05545					+1.153616				

	T3552B: ND									
	Reiter: ND									
flgABCD flagellar basal-body rod proteins	T19: ND									
	T320A: C5O78_02075 C5O78_02080 C5O78_13690					+1.169123 +1.037461 +1.424788				
	T3552B: ND									
	Reiter: ND									
flgM negative regulator of flagellin synthesis	T19: ND									
	T320A: C5O78_03315					+1.671467				
	T3552B: ND									
	Reiter: ND									
FliL flagellar protein	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_08000						+1.260282			
	Reiter: no ortholog									
gldF	T19: C5N99_04760	+3.084417								
	T320A: no ortholog									
	T3552B: no ortholog									

	Reiter: no ortholog									
gldA	T19: no ortholog									
	T320A: C5O78_07275					-1.093312				
	T3552B: no ortholog									
	Reiter: ND									

7.3.5.3 Biofilm Phenotype

Only T320A genes were identified as differentially expressed in the biofilm phenotype samples. The Venn diagram in Figure 3, produced in jvenn open access software (Bardou et al., 2014), illustrates there was no overlap between functional pathways dysregulated in the single and dual species biofilm samples. BlastKOALA identified that genes for carbohydrate, energy and amino acid metabolism, and those for genetic information processing were downregulated. Further details of genes of interest including log_2FC values and descriptions of gene function are shown in Table 7.6. As no dysregulated genes were detected in the biofilm phenotype for T3552B, genomes were searched for orthologous genes in MAUVE to establish if this was due to differences in genomes. No orthologs were found in T3552B for the upregulated membrane transporter TC.FEV.OM3, the downregulated Transposase IS-5 family, or the downregulated flagellar motor protein flbB (Table 7.6).

Figure 3: Venn diagram illustrating number of functionally annotated pathways dysregulated in single and dual species biofilm samples.

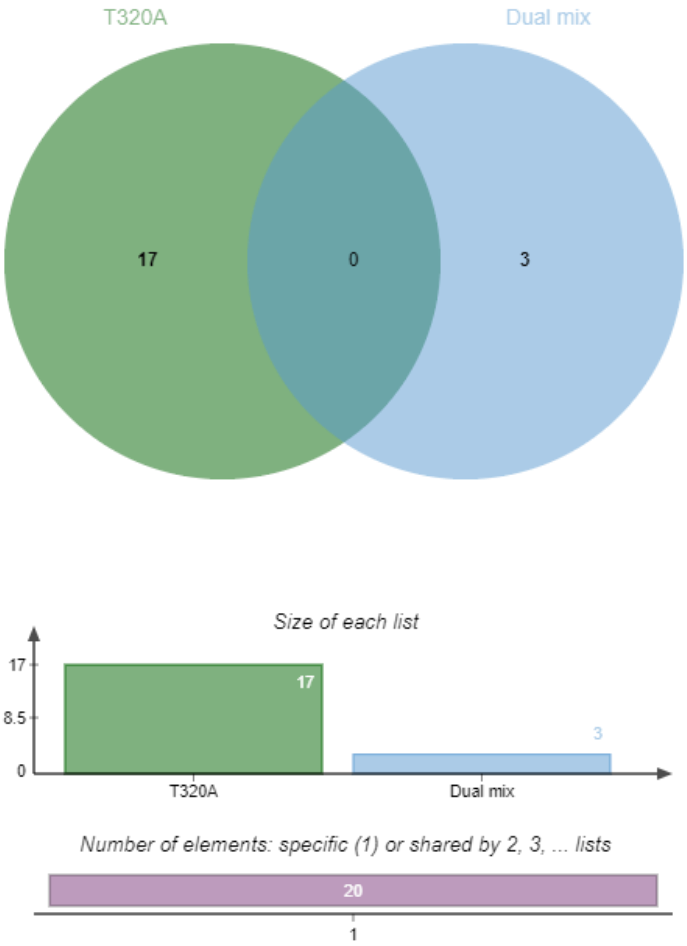


Table 7.6 Biofilm phenotype dysregulated genes showing locus tags, log_2 fold change and description of gene functions, indicating where orthologs were absent from genomes.

Gene	Gene locus tag	T320A single	T320A dual	T3552B dual	Gene description
BACTERIAL SURVIVAL					
ROS Scavenging					
Dfx: Superoxide reductase	T320A: C5O78_13450	+2.315364			(Superoxide reductase) Catalyses the reduction of superoxide to hydrogen peroxide (via NADP and rubredoxin), which is then reduced to water by peroxidases. This is in contrast to the mechanism SOD uses by which superoxide is reduced to oxygen (Jenney et al., 1999).
	T3552B: ND				
Protein repair					
grpE molecular chaperone	T320A: C5O78_01920	+2.117460			GrpE is a molecular chaperone helping to regulate the DnaK reaction cycle (Kim et al., 2013).
	T3552B: ND				
Transporters					
TC.FEV.OM3	T320A: C5O78_09625	+3.772664			Outer membrane receptor for the iron-siderophore complex ferrienterochelin, and colicins.
	T3552B: no ortholog				
irtA	T320A: C5O78_11550	+2.642106			ABC transporter for iron acquisition and assimilation described in *Mycobacterium tuberculosis* (Ryndak et al., 2010).
	T3552B: ND				

bioY	T320A: C5O78_05035	-1.133515			Proteins with transmembrane domains which play a role in biotin uptake (Guillén-Navarro et al., 2005).
	T3552B: ND				
REGULATOR					
Crp/ Fnr	T320A: C5078_13835	-2.792278			Global transcriptional regulator, which typically function in response to environmental change to optimise metabolism and enhance survival (A. Zhou et al., 2012).
	T3552B: ND				
VIRULENCE ASSOCIATED GENES					
Mcp	T320A: C5O78_09260 C5O78_09285		-1.562066 -2.644217		Transmembrane chemoreceptors which continually monitor the environment (Lux & Shi, 2006).
	T3552B: ND				
Transposase IS-5 family	T320A: C5O78_04810 C5O78_06855 C5O78_08215 C5O78_09555 C5O78_10880		-1.867186 -2.224450 -2.571656 -2.841716 -2.412370		Mobile genetic elements: specific functions of this family are unknown (Cordaux, 2008).
	T3552B: no ortholog				
Motility					
flbB	T320A: C5O78_05470	-1.080665			Flagellar motor protein important for normal orientation of periplasmic flagella (Moon et al., 2016).
	T3552B: no ortholog				

ND= Not detected (but ortholog is present in the genome)

7.3.5.4 Quorum sensing

DGE analysis clearly showed increased dysregulation of genes in dual and triple species samples exposed to microaerobic conditions compared to single species samples. Examination of BRITE Hierarchies in BlastKOALA (Appendix E, Table 2) identified more evidence of transport protein upregulation in dual and triple species samples. Proteins protective for oxidative stress expected to be found in exosomes were identified only in dual species samples. Upregulation of methyl-accepting chemotaxis proteins, cheY chemotaxis proteins and flagellar proteins associated with signalling and cellular processes were identified only in dual species samples. KEGG pathway reconstruction was used to identify annotated quorum sensing pathways. Full results for KEGG pathway reconstruction for genes upregulated and downregulated under oxidative stress can be found in Appendix E, Tables 5 and 6 respectively. QS pathways were detected in BlastKOALA for T320A, T3552B and Reiter single-species microaerophilic samples, as well as dual and triple-species microaerophilic samples. Eight genes were upregulated in QS pathways; four only in T3552B (ABC.PE.P, ABC.PE.P1, ddpD, ddpF), three in both T320A and T3552B (ABC.PE.S, yajC, fadD), and one in both T320A and Reiter (ribD) (Table 7.8). One gene, yidC, was found to be downregulated in T19 in triple species samples. No QS pathways were identified as dysregulated in biofilm phenotype samples using KEGG pathway reconstruction.

The common QS autoinducer (signalling molecule) synthase genes *lux I* and *lux S* were not identified in any of the four treponeme genomes using MAUVE. The response regulator capable of binding autoinducers, *Lux R,* was identified in the T19 genome.

Table 7.8 Dysregulated genes associated with quorum sensing pathways showing log_2 fold change for each species in single, dual or triple-species samples.

ND: Not Detected

Gene	Gene locus tag	T19 single	T320A single	T3552B single	Reiter single	T320A dual	T3552B dual	T19 triple	T320A triple	T3552B triple
ABC.PE.S	T19: no ortholog									
	T320A: C5O78_04875 C5O78_09935					+1.88265			+1.39351	
	T3552B: DYQ05_04555 DYQ05_10355			+2.16867			+3.09678			+1.80285 +2.76146
	Reiter: ND									
ABC.PE.P	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_00625 DYQ05_04560 DYQ05_10360			+1.90418 +2.44365			+2.10526 +2.54635 +3.59318			+3.99283 +1.90792 +4.74554

	Reiter: no ortholog									
ABC.PE.P1	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_04565 DYQ05_10365			+2.09602			+2.21315			+2.12360 +3.44394
	Reiter: no ortholog									
ddpD	T19: no ortholog									
	T320A: no ortholog									
	T3552B: DYQ05_04570			+1.67752			+2.23000			+2.98860
	Reiter: no ortholog									
ddpF	T19: no ortholog									
	T320A: no ortholog									

	T3552B: DYQ05_04575						+1.85823			+1.68959
	Reiter: no ortholog									
ribD	T19: no ortholog									
	T320A: C5O78_13240					+1.85602			+3.91413	
	T3552B: no ortholog									
	Reiter: DWQ65_11830				+1.46633					
yajC	T320A: C5O78_09980		+1.76725			+1.12897				
	T320A: ND									
	T3552B: ND									
	Reiter: ND									
fadD	T19: ND									
	T320A: C5O78_08325					+1.02775				
	T3552B: ND									

	Reiter: ND									
yidC	T19: C5N99_01905							-1.23626		
	T320A: ND									
	T3552B: ND									
	Reiter: ND									

7.4 Discussion

7.4.1 Microaerobic conditions

Increased turbidity as measured using ODs in the short term on exposure to oxygen may indicate an increase in metabolic activity in advance of cell death. Alternatively, bacteria can grow as planktonic cellular aggregations in response to stressful environmental conditions, which also fits with our observation of clumping of bacteria after exposure to oxygen as part of cell viability assays (Schleheck et al., 2009).

The phenotypes from the CTB experiments suggest that Reiter is superior to T320A at coping with exposure to oxygen. This appears to be at odds with the presence of more oxidative stress genes in T320A which was expected to confer better oxygen resistance (Staton et al., 2021a). However, the presence of oxidative stress genes could be interpreted as a sign of pathogenicity via better ability to defend against ROS/ RNS when challenged by host immune cells. In addition, the lesser oxidative stress response exhibited by Reiter under microaerobic conditions may indicate innate resistance to ROS, which has been recognised in the spirochaete *Borrelia burgdorferi* (Gherardini et al., 2006). Another possibility is that additional oxidative stress genes in T320A not only defend against the deleterious effects of oxygen but are able to actively use metabolic pathways for ATP synthesis under aerobic conditions. An ability to continue growth when exposed to oxygen would be an important property allowing pathogenic treponemes to proliferate on the bovine foot skin during initiation of infection. A similar property has been described for the anaerobe *Bacteroides fragilis,* enabling proliferation in host tissues in advance of abscess formation (Baughn & Malamy, 2004).

It is possible that T320A exhibits phenotypic heterogeneity, meaning there is a range of reversible cell-to-cell variation in the clonal population, conferring individual traits. This could explain why its survival under microaerobic conditions (assessed using Cell Titer Blue to measure metabolic activity) is reduced compared to Reiter despite presence of more oxidative stress genes in the genome and yet there is a clearer response to microaerobic conditions observed in the transcriptome compared to Reiter. It is known that BDD treponemes exhibit morphological changes during growth, existing as the classic spiral form, but also as encysted forms and as spiral structures with spherical bodies at the tip which are thought to represent an intermediate form (Döpfer et al., 2012a). It is possible that encysted forms behave as persister cells, analogous to the persister form known as round bodies in the spirochete *Borrelia burgdorferi* (Sapi et al., 2011).

Overall, many of the genes upregulated under microaerobic conditions are protecting cells from oxidative stress. We interpret that increased expression of oxidative stress genes in dual and triple-species samples could also correspond to better resistance to ROS/ RNS when challenged by host

immune cells. This may go some way towards explaining the well-established phenotype that BDD is a polytreponemal disease (Evans 2009b; Klitgaard et al., 2008; Nordhoff et al., 2008).

The upregulation of metal transporters has occurred because the oxidative stress response and metal metabolism are interrelated (Gherardini et al., 2006). Redox reactions which involve electron transfers between molecules are central to many biological processes, for example cell signalling and protein folding. Many of these redox reactions use vitamins and metals as cofactors, with riboflavin and iron being the most abundant and important cofactors, each responsible for approximately 17% of reactions (Sepúlveda Cisternas et al., 2018). Iron homeostasis is particularly important to bacterial survival and is often incorporated into proteins as a biocatalyst or electron carrier, making iron essential for gene expression. Growing cells therefore require iron, and our data shows upregulation of iron transporters TonB and feoAB, as well as ATP binding cassette metal transporters TroABC and irtAB, indicating active uptake. Too much iron in cells is toxic in the presence of oxygen, therefore protective mechanisms are also upregulated. In our data, this is exemplified by upregulation of the Fur transcriptional repressor which will protect against oxidative stress caused by increased ferric iron. It also shows upregulation of ferritin which is used for iron storage to mitigate against its toxic effects (Andrews et al., 2003). Upregulation of transporters TonB, TC.FEV.OM2 and htsAB could also be interpreted as upregulation of virulence factors, since heme iron acquisition contributes to pathogenesis of systemic *Staphylococcus* infections (Mason & Skaar, 2009) and heme is also used as a source of essential iron by pathogenic Group A *Streptococci* (Lu et al., 2012).

Molecular chaperones are responsible for diverse aspects of protein quality control. Newly translated proteins use them in the process of folding to their correct three-dimensional structure, avoiding misfolding which leads to protein aggregation, especially under conditions of cellular stress. They are also needed for protein unfolding, disaggregation, and for targeting misfolded proteins for proteolytic degradation (Kim et al., 2013). Under microaerobic conditions, we hypothesise that anaerobic treponemes are increasing molecular chaperone production- evidenced by an increase in Hsp90, DNaK and its co-factor DnaJ, Hsp20 and Hsp33, the nucleotide exchange factor GrpE, and the chaperonins GroEL and GroES- as a survival mechanism.

There is also upregulation of some virulence genes which may suggest exposure to oxygen is promoting pathogenicity: cheY, Mcp, wbpl/ wlbD, yajc, tlyC, Rel B and Rel E. Upregulation of the RelBE toxin:antitoxin system is likely to result in a reduction of translation at a global level (Christensen et al., 2001), increasing persistence of treponemes in these unfavourable conditions. Upregulation of flagellar genes suggests increased cell motility, which may be a component of increased pathogenicity by enhancing invasion. T19 was also the only species to contain pilZ, a receptor for the common

bacterial messenger cyclic-di-GMP (Galperin & Chou, 2020). A *T. denticola* mutant where a PilZ-like protein gene was deleted showed abnormal swimming behaviours, repression of biofilm formation and reduced ability to induce skin abscesses and host humoral immune response in an *in vivo* mouse model (Bian et al., 2013). Downregulation of pilZ in T19 under microaerobic conditions may have the same effect, especially since upregulation of flagellar proteins seen in the other BDD species are not seen in T19. There is little known about the specific pathogenic mechanisms of BDD-associated treponemes although it is known that some phylogroups are more invasive than others (Moter et al., 1998; Stamm & Trott, 2006).

7.4.2 Biofilm phenotype

The biofilm phenotype downregulated carbohydrate, amino acid and energy metabolism, and genes encoding proteins involved in genetic information processing. The flagellar motor protein flbB was also downregulated which may provide evidence of decreased motility associated with biofilm formation. Overall, there is some evidence that changes normally associated with biofilm formation are occurring ie. slower growth and absence of motility (Sauer & Camper, 2001). Conversely downregulation of the transposase IS5 family suggests reduced horizontal gene transfer, when the opposite is normally associated with biofilm formation (Madsen et al., 2012); however, the IS5 family has not been characterised and therefore this result is difficult to interpret. Interestingly, only T320A genes were dysregulated in the dual species samples, indicating no biofilm characteristics occurring in T3552B. This reflects the findings in Chapter 6 that T320A appeared to form biofilms whilst T3552B did not as a single species.

Sparsity of DEGs identified in biofilm samples may be due to lack of metabolic activity in biofilms which may have reached stationary phase within 48 hours. We did not detect evidence of adhesion in the biofilm phenotype; however, a previous study showed greatest biofilm formation using *T. denticola* attached to fibronectin after just 1 hour (compared to 2 or 3 hours) (Vesey & Kuramitsu, 2004). Upregulation of genes responsible for attachment may have been detectable much more quickly, earlier in the exponential phase. It is also possible that BDD-associated treponemes in this model are surface-associated without being surface-adherent; therefore, genes promoting adhesion would not be upregulated. Biofilms found in chronic wounds are characterised by immature biofilms that are non-surface-attached, and mature biofilms that become embedded in host materials, which would fit with the clinical presentation of BDD (Bjarnsholt et al., 2013).

We did not observe an upregulation of genes associated with EPS production (Sun & Zhang, 2021). A biosynthetic pathway for a rare mannuronic acid sugar has been identified in BDD treponemes as important for pathogenesis due to absence in comparative commensal treponemes (Staton et al.,

2021a). The extracellular polymeric substance alginate secreted by microorganisms in biofilms, is composed of mannuronic acid and guluronic acid. Production of mannuronic acid by pathogenic treponemes suggests they may be capable of alginate production, although genes for mannuronic acid synthesis were not shown to be upregulated in the transcriptomics data from biofilm samples. It is possible that the energy-dependent generation of EPS which protect biofilms does not occur in pure cultures as they confer no selective advantage in this environment (Costerton et al., 1978). The ability to form biofilms is considered a virulence factor, and it is possible that patterns of gene expression in laboratory strains of *Treponema* become altered after serial passage, therefore not reflecting the true phenotypes that occur in disease (Duangurai et al., 2020). Comparison of the transcriptome from samples using *Treponema* from clinical isolates after low and high numbers of passages would be needed to evaluate these effects.

7.4.3 Quorum sensing

Dual and triple species samples showed more changes to gene expression, which could be interpreted as evidence of cooperation between species to enhance community survival. This could be an example of bacterial collective behaviour which is coordinated using quorum sensing. More genes associated with quorum sensing pathways were identified as upregulated using BlastKOALA in dual and triple species samples, and methyl accepting chemotaxis proteins were upregulated in the dual species samples. These are chemoreceptors which are part of two-component regulatory systems which trigger gene expression and could indicate increased cell-cell communication (Lux & Shi, 2006).

Quorum sensing pathways for *Treponema* have not been described, and autoinducer molecule production has not been widely studied for this genus. We identified *LuxR* in the T19 genome in the absence of *Lux I* or *LuxS.* There are examples of other pathogenic bacteria, such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, where *LuxR* response regulators can be found which are not coupled to *LuxI* or *LuxS* autoinducer synthase genes (Schaefer et al., 2013).

7.5 Conclusion

This study has demonstrated that BDD treponemes survive exposure to microaerobic and aerobic conditions for at least six hours, using a range of bacterial survival mechanisms to protect against ROS and repair damaged proteins. *T. phagedenis* T320A and *T. pedis* T3552B showed an increase in virulence- associated gene transcription under microaerobic conditions, including increased motility gene expression, which may indicate increased pathogenicity under adverse environmental conditions and innate ability to resist cell mediated immunity. It has also demonstrated evidence of biofilm phenotype characteristics in *T. phagedenis* T320A which may explain its ubiquitous nature in BDD lesions compared to other treponeme species. We also identify upregulation of more genes associated with quorum sensing pathways in dual and triple species samples, which alongside an increased response to oxidative stress suggests cooperation between species to enhance survival.

Chapter 8 *In vitro* susceptibility of bovine digital dermatitis treponemes to conventional and novel antimicrobial agents.

8.1 Introduction

The conventional antimicrobials penicillin and macrolides have been reported as the most efficacious agents against BDD treponemes (Evans 2009c; Evans et al., 2012b). Whilst there has only been a single report of antibiotic (spectinomycin) resistance in BDD treponemes (Evans et al., 2009c), and none thus far for β-lactam or macrolide antibiotics, many clinically associated bacteria develop an arsenal of antibiotic resistance mechanisms. Resistance mechanisms against penicillin typically include production of β-lactamases and alteration of the drug targets (penicillin-binding proteins) in bacterial cell walls (Reygaert, 2018). Moreover, resistance to the macrolide antibiotic azithromycin has been reported in *Treponema pallidum* subsp. *pertenue,* the cause of the human treponemal skin disease yaws and this threatens the ongoing eradication campaign for this disease (Mitjà et al., 2018). Resistance to macrolides has also been reported for *T. pallidum* subsp. *pallidum,* the cause of human syphilis (Venter et al., 2021). These studies highlight the need to explore alternative treatments for treponemes. In the context of BDD, which is endemic in most UK dairy herds, there are added considerations of the application of milk withdrawal periods when antimicrobial products are used, meaning milk from a treated animal cannot be sold whilst it contains pharmacologically active substances exceeding the Maximum Residue Limits (MRLs) as defined in EU regulation (EC) No 470/2009. Since there is little evidence that systemic use of antibiotics for BDD treatment is superior to topical applications (Laven & Logue, 2006), topical treatments are usually recommended as direct treatment costs are lower and there are no resulting medicine residues in milk. However, there is interest in alternatives to conventional antimicrobial treatments because it is possible to contaminate cows' teats during topical application of sprays, necessitating application of a withdrawal period (Cramer et al., 2009).

8.1.1 Conventional antimicrobials

In a previous trial, BDD treponemes did not appear to be particularly susceptible to third-generation cephalosporins (Evans et al., 2012b). Here, we trial two further examples from this group of antimicrobials: ceftriazone and cefixime. We also test the antimicrobials nitazoxanide and metronidazole, from the nitroimidazole class. Metronidazole is a well-established anti-protozoal treatment with good activity against anaerobes (Gupta et al., 2022). There have been no reports of resistance to nitazoxanide, which could indicate potential longevity for its use (Reed et al., 2018). Nitazoxanide has been tested against 241 human strains of anaerobic bacteria *in vitro* and its activity

was comparable to metronidazole (Dubreuil et al., 1996), however, it has not been tested against bovine strains of anaerobic bacteria, or bovine treponeme strains.

We also test polymixin B, which is a lipopeptide antibiotic in the same class as colistin, which has a narrow spectrum of activity against Gram-negative bacteria. For each, the target is the outer membrane as it destabilises lipopolysaccharide (LPS); however, the exact mechanisms of action remain unclear (Poirel et al., 2017). Previous testing *in vitro* of colistin against bovine and ovine treponemes showed colistin was not effective, with the Minimum Inhibitory Concentration (MIC) >384mg/L (Evans et al., 2012b), perhaps reflecting the unconventional structure of the treponeme outer sheath and the fact that many treponeme species are considered to lack typical LPS (Norris et al., 2010). There is concern regarding emerging resistance to colistin because it has historically been used orally as a growth promoter in food-producing animals; however, the use of polymyxin B topically as a BDD treatment may be justifiable as topical applications do not expose the gut microbiome to antimicrobials and reach a higher therapeutic concentration at the site of application (Constable et al., 2017).

We also test bacitracin, a polypeptide antibiotic also referred to as an antimicrobial peptide alongside polymyxin B and colistin. It is usually used for treatment of Gram-positive infections as the primary target is the cell wall (Kapoor et al., 2017), but is also effective against many Gram-negative bacteria including spirochetes (Cheng et al., 2014b).

The final conventional antimicrobial tested here is linezolid, a synthetic antibiotic from the oxazolidinone class which inhibits bacterial protein synthesis by blocking translation initiation. It is predominantly active against Gram-positive bacteria, with limited activity against Gram-negative bacteria (Pletz et al., 2010); however, it has been shown to have good *in vitro* and *in vivo* activity against *T. pallidum* (Haynes et al., 2021).

8.1.2 Naturally derived antibiotic agents

This study also examines naturally occurring compounds which have been previously identified as antibacterial to investigate their efficacy against BDD treponemes. The main component of honeybee (*Apis mellifera)* venom, melittin, has been shown to severely disrupt the surface envelope and halt motility in the spirochaete *Borrelia burgdorferi* (Lubke & Garon, 1997). Moreover, both melittin and whole bee venom have been shown to significantly reduce the viable cell count during the logarithmic phase of growth for *B. burgdorferi* as well as reducing stationary phase persister cells, and the attached biofilm phenotype (Socarras et al., 2017).

Furthermore, we investigate the polyphenolic compound tannic acid derived from Chinese gall nuts, and compounds found in four essential oils: cinnamaldehyde, menthol, eugenol and carvacrol. Tannins are naturally occurring compounds in several plant species which confer resistance to pathogens (Scalbert, 1991). They act by depriving the target organism of iron and interacting with vital proteins such as enzymes. They have shown antimicrobial activity against the Gram-negative bacteria *Campylobacter jejuni* (Anderson et al., 2012) and Salmonella Typhimurium (Costabile et al., 2011). Eugenol and carvacrol are other naturally occurring compounds with antioxidant properties found in essential oils. Eugenol originates from clove buds, and carvacrol from plants such as oregano and thyme (Silva et al., 2021). Menthol is a monoterpene compound found for example in peppermint plants. It is thought that it can disrupt lipids in microorganism plasma membranes leading to changes in permeability and leakage of intracellular materials (Trombetta et al., 2005). Cinnamaldehyde is found in cinnamon, which is derived from the dry bark and twig of *Cinnamomum* spp. (Davis & Yokoyama, 2011). Cinnamaldehyde has been identified as a quorum-sensing inhibitor (QSI), reducing biofilm formation in *Pseudomonas aeruginosa* and affecting AI-2 mediated quorum sensing in *Vibrio* spp. (Nazzaro et al., 2013). Eugenol has also been identified as a QSI capable of inhibiting the *las* and *pqs* quorum sensing systems (L. Zhou et al., 2013). Of 21 essential oils screened against *Salmonella enterica* and *Listeria monocytogenes* strains, oregano, cinnamon and clove were the most effective, hence their selection for this study (Mazzarrino et al., 2015). Eugenol, carvacrol and cinnamaldehyde were also identified as effective against porcine strains of another clinically relevant spirochete *Brachyspira hyodysenteriae* (Maele et al., 2016).

8.1.3 Antibacterial metals

Heavy metals, particularly copper and zinc sulphate, are commonly used as footbathing disinfectants for prevention of BDD lesions in dairy herds (Cook et al., 2012; Solano et al., 2015). Recent genomic sequencing of BDD-associated treponemes identified phosphate transporters and a phosphate utilisation operon in *T. phagedenis* isolates (Staton et al., 2021a). It has been suggested that phosphate transport systems confer heavy metal resistance by transporting metal-phosphate complexes out of the cell (Navarro et al., 2013). In addition, genes for resistance to copper and zinc were expressed in BDD lesion biopsies at greater normalized abundance compared to healthy skin (Zinicola et al., 2015a). Previous *in vitro* susceptibility data showed that *T. phagedenis* was less susceptible to zinc sulphate compared to copper sulphate (Hartshorn et al., 2013). This chapter collects *in vitro* susceptibility data for zinc, nickel, and copper sulphate for *T. phagedenis* and *T. pedis,* to extend the evidence for differences in efficacy between heavy metals which may be linked to genetic tolerance mechanisms and have implications for field application.

We also collect *in vitro* susceptibility data for the antibacterial metal gallium. Gallium (III) inhibits microbial growth by disrupting ferric iron-dependent pathways which are crucial for bacterial metabolism. It is effective in iron limited conditions against a range of pathogens known to cause multidrug-resistant infection in people, including strains of the Gram-negative bacteria *Klebsiella pneumoniae* and *Enterobacter cloacae* (Hijazi et al., 2018). Gallium maltolate was successful as a topical application in preventing development of lesions caused by *T. pallidum* subsp *pertenue* in a rabbit model of yaws (Giacani et al., 2019).

8.1.4 Peptide nucleic acids (PNAs)

Given the emergence of resistance mechanisms to conventional antimicrobials, alternative routes to halting pathogenic bacteria growth are needed. Antisense inhibition of RNA is a natural mechanism by which bacteria control gene expression and therefore has been targeted as a means of inhibiting cell growth (Good et al., 2001). PNAs are antisense molecules designed to match the translation initiation regions (TIRs) of essential genes, thus inhibiting translation initiation and possibly also promoting mRNA degradation (Goltermann & Nielsen, 2020). The PNAs used here were designed against the TIRs of the two housekeeping genes *acpP* (role in fatty acid biosynthesis) and *ftsZ* (role in cellular division) which have been identified as efficacious targets for preventing bacterial growth in *Escherichia coli* (Goh et al., 2009). These are also known to be highly specific and effective for the Gram-negative bacteria *Salmonella spp.* and *Klebsiella spp.* but have not been previously tested on spirochetes (Monde et al., 2014).

This chapter reports results from broth microdilution *in vitro* susceptibility experiments against BDD treponemes using previously untrialled conventional antimicrobials (synthetic and largely derived from microbes/ fungi). It also reports susceptibility results using two novel therapeutic agents PNA-peptides. In addition, we investigate treponeme susceptibility to compounds derived from bee venom and essential oils, and for four antimicrobial metals, two of which (copper and zinc sulphate) are widely used for BDD prevention in footbaths.

8.2 Materials and Methods

8.2.1 Bacterial strains and growth conditions

Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) were determined for each therapeutic candidate (TC) against *T. phagedenis* strain T320A and *T. pedis* strain T3552B, representing two of the BDD treponeme phylogroups. Cultures were prepared in liquid media and diluted for use to standard optical densities (ODs) as described in Chapter 2.1-2.2.

8.2.2 Broth microdilution method

Therapeutic candidates (TCs) were tested using a previously described microdilution method (Angell et al., 2015; Evans et al., 2009c; Evans et al., 2012b) Under anaerobic conditions, 96-well microplates were inoculated with 150µl of Oral Treponeme Enrichment Broth containing 10% Foetal calf serum (OTEB+FCS) using a multichannel pipette. All plates contained a column supplemented with 50µl of OTEB+FCS per well added as a negative control, and a column with 50µl bacteria (only) supplemented in each well added as a positive control. For copper, zinc and nickel sulphate, an additional column of control wells was inoculated to allow for comparison of ODs affected by the colour of the solutions. 150µl of each TC was added in row A. A multichannel pipette was used to mix well contents three times before moving 150µl to the next row. This serial dilution continued down the microplate and the final 150µl of well contents discarded. Prepared cultures (8.2.1) were added at 50µl to each test well.

Microtiter plates were incubated in an anaerobic cabinet (Don Whitley Scientific, UK) (85% N_2, 10% H_2 and 5% CO_2, 36^{o}C) for four days to determine MICs for TCs against T320A, and three days for TCs against T3552B as previously described (Evans et al., 2009c; Evans et al., 2012b). Microplates were incubated inside polythene bags containing 5ml double distilled water (ddH_2O) to maintain humidity and prevent evaporation from wells. Plates were read using a spectrophotometer at 540nm to visualise the inhibition of bacterial growth. MICs are identified at wells where ODs show growth inhibition compared to positive control wells and are reported as the median of three experimental replicates.

Minimum Bactericidal Concentrations were determined by transferring 20µl from each well to a second microtiter plate freshly inoculated with 180µl of OTEB+FCS and observing for growth in wells after a further four days. Wells identified as containing MBCs by visual turbidity were inspected using phase contrast microscopy to confirm absence of treponemes as described in Chapter 2.1. MBCs are reported as the median of three experimental replicates.

Laboratory-supplied versions of naturally occurring products were obtained from Sigma Aldrich (St Louis, Missouri, USA). Testing ranges and methods for dilution of stock solutions for TCs were determined using existing literature (including reference tables contained in the Clinical and Laboratory Standards Institute (CLSI) guidelines where appropriate) and manufacturer's instructions as described in Table 8.1. Testing ranges were adjusted, if necessary, after a pilot experimental replicate. For study validation penicillin, oxytetracycline and erythromycin were included as controls to enable comparison to results from a previous treponeme microdilution MIC study (Evans et al., 2009c).

Table 8.1 Stock solutions and testing ranges for therapeutic candidates tested against treponeme species *T. phagedenis* strain T320A and *T. pedis* strain T3552B.

Therapeutic candidate	**Stock solution**	**Testing range**	**References**
Control antimicrobials			
Penicillin	100mg/ml in ddH_2O	0.00183-0.234µg/ml	(Angell et al., 2015; Evans et al ,2009c)
Oxytetracycline	5mg/ml in ddH_2O	0.0938-12µg/ml	(Angell et al., 2015; Evans et al., 2009c)
Erythromycin	1mg/ml in ddH_2O	0.0146-1.88µg/ml	(Angell et al., 2015; Evans et al., 2009c)
Conventional antimicrobials			
Cefixime	1mg/ml in PBS	0.235-3µg/ml	(Lewis et al., 2022)
Ceftriaxone	1mg/ml in ddH_2O	0.235-3µg/ml	(Lewis et al., 2022)
Bacitracin	50mg/ml in ddH_2O	0.733-93.8µg/ml	(Charlebois et al., 2012)
Polymixin B	5mg/ml in ddH_2O	7.313-937.5µg/ml	(Simar et al., 2017; Zavascki et al., 2007)

Metronidazole	1mg/ml in ethanol	0.0586-7.5µg/ml	(Lewis et al., 2022; Love et al., 2017)
Nitazoxanide	1mg/ml in ddH_2O	1.831-234.4µg/ml	(Dubreuil et al., 1996)
Linezolid	0.5mg/ml in ethanol	0.0469-6µg/ml	(Haynes et al., 2021)
Naturally derived antibiotic agents			
Apis mellifera (bee) venom	20mg/ml in PBS	11.7-1500µg/ml	(Socarras et al., 2017)
Tannic acid	50mg/ml in ddH_2O	7.31-937.5µg/ml	(Kolodziej et al., 1999)
Eugenol	400mg/ml in 70% ethanol	117-15000µg/ml	(Shapiro et al., 1994; Silva et al., 2021)
Menthol	100mg/ml in 70% ethanol	7.31-937.5µg/ml	(Trombetta et al., 2005)
Carvacrol	1M in ddH_2O	1.56mM-200mM	(Maele et al., 2016)
Cinnamaldehyde	100mM in ddH_2O	0.0781-10mM	(Maele et al., 2016)
Antibacterial metals			
Copper sulphate	1M in ddH_2O	0.00391-0.5mM	(Sütterlin et al., 2018)
Nickel sulphate	1M in ddH_2O	0.586-75mM	(Sütterlin et al., 2018)

Zinc sulphate	1M in ddH_2O	0.586-75mM	(Sütterlin et al., 2018)
Gallium (III) nitrate hydrate	100mM in ddH_2O	0.0781-10mM	(Hijazi et al., 2018)

8.2.3 Peptide PNA Design

The nucleotide sequences were designed complementary to the relevant mRNA regions between the Shine Dalgarno sequences and the start codons of the two target genes (Campion et al., 2021). Optimum PNA size is 10-12 nucleobases, which is a compromise between increased RNA target affinity and uptake by target cells (Goltermann et al., 2019). They are tested alongside mismatched controls which have the same nucleobase content. Two nonadjacent bases are switched to lower T_m and avoid target binding. Use of mismatched controls rules out the possibility of bacterial killing by general toxicity rather than by specific antisense activity (Goltermann & Nielsen, 2020). Genome sequences for *T. phagedenis* strain T320A and *T. pedis* strain T3552B used for PNA design were obtained from NCBI, accession numbers GCA_017161245.1 and GCA_017161325.1 respectively.

The PNA oligomers were conjugated to cationic carriers known as bacteria penetrating peptides (BPPs) to allow transport across the bacterial envelope (Good et al., 2001). Bacterial LPS is a major barrier against cell permeability (Good et al., 2000); however, more recently this has been questioned as the inner membrane SbmA protein was shown to be a necessary transporter for some BPP-PNA conjugates (Ghosal et al., 2013). Therefore, the BPP used in this project H-(R-Ahx-R)$_4$-Ahx-βAla (where AHx is 6-aminohexanoic acid and βAla is β-alanine) has an alternative uptake mechanism that allows the PNA to cross the inner cell membrane independent of the inner membrane SbmA protein (Ghosal et al., 2013).

PNAs were synthesised using established solid-phase synthesis methods (Christensen et al., 1995) and gifted by the Center for Peptide-Based Antibiotics, University of Copenhagen, under the guidance of Professor Peter Nielsen. The nucleotide sequences and carriers are detailed in Table 8.2.

Table 8.2: Carriers and PNA sequences used to target the essential *acpP* and *ftsZ* genes in *T. phagedenis* and *T. pedis.* Deliberately mismatched nucleotides are shown in red.

Carrier	bp sequence 5' to 3'	Target Gene	Explanation
H-(R-eg1-R)$_4$-eg1 [NH_2 at the 3'end]	TCC ATT GTT AT	*acpP*	Match to *T. phagedenis*
	TCC TTT GAT AT		Mismatch to *T. phagedenis*
	TCC ATA AAA TA		Match to *T. pedis*
	TCA ATA ACA TA		Mismatch to *T.pedis*
	TCC ATA CAT TT	*ftsZ*	Match to *T. phagedenis*
	TCA ATA CCT TT		Mismatch to *T. phagedenis*
	GTC ATT GAA TC		Match to *T. pedis*
	GTA ATT GCA TC		Mismatch to *T.pedis*

PNAs were diluted to 1mM stock solutions in ddH_2O. Before use, the concentration of an aliquot of stock solution was determined using the Nanodrop spectrophotometer and further ddH_2O added accordingly to ensure the final concentration of PNA on the microtiter plate would be 20µM. The ranges tested were therefore 0.156µM-20µM. The upper limit of the test range was chosen to exceed that in previous literature by one dilution (Monde et al., 2014).

8.3 Results

MICs for control antimicrobials used for study validation were comparable to those reported in previous microdilution method studies (Angell et al., 2015; Evans et al., 2009c). Specific comparison of T320A and T3552B results from a previous study using linear regression showed high correlation between results from the two studies, indicating the efficacy and reproducibility of this microdilution method. R^2 values were 0.992 for penicillin, 0.997 for oxytetracycline, and 0.739 for erythromycin. The current study found MICs to be one dilution higher for activity of erythromycin against T320A and T3552B (Evans et al., 2009c).

MICs and MBCs were reached for all conventional antimicrobials except for nitazoxanide, identifying existing molecules that could potentially be useful for BDD treatment. The MBC recorded for bacitracin against T320A (18.8µg/ml) was above the 1-10µg/ ml range typically considered to denote susceptibility (Simões et al., 2009). MICs for nitazoxanide (>234.4µg/ml) and Polymixin B (468.8µg/ml)

were distinctly higher than this susceptible range indicating resistance. Cefixime, metronidazole, ceftriaxone and linezolid exhibited MICs and MBCs suggesting susceptibility; ceftriaxone and linezolid exhibited MICs and MBCs below 1µg/ml, indicating they are very effective at inhibiting the BDD treponemes.

Although MICs and MBCs were reached for all naturally derived antibiotic agents tested, they required high concentrations of solution to inhibit the BDD treponemes, suggesting that it may not be achievable *in vivo* to reach therapeutic tissue concentrations using these substances. This was also true for the antibacterial metals, except for copper sulphate, which is already an established biocide used for foot disinfection on dairy farms. MICs and MBCs for *T. phagedenis* were 75 times higher for zinc and nickel sulphate compared to copper sulphate; and for *T. pedis* the values were 150 times higher. Although there are no standard values regarding metal resistance in treponemes and reported values vary between bacterial genera (Argudín et al., 2019), it is possible this indicates resistance, or at least tolerance, to zinc and nickel. Gallium (III) nitrate hydrate was not as efficacious as copper sulphate but *in vitro* susceptibility values (0.469-1.88mM) suggest this could be a useful treatment for BDD. PNA Peptides directed against the *acpP* or *ftsZ* housekeeping genes did not show efficacy in the range tested. *T. pedis* was equally or more susceptible to all TCs compared to *T. phagedenis* except for ceftriaxone, bee venom and tannic acid.

Table 8.3 Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations for a range of conventional antimicrobials, naturally derived antibiotic agents, antibacterial metals, and peptide PNAs against *T. phagedenis* strain T320A and *T. pedis* strain T3552B.

ND not done

Therapeutic candidate	**MIC**		**MBC**	
Species	**T320A**	**T3552B**	**T320A**	**T3552B**
Control antimicrobials				
Penicillin	0.0146µg/ml	0.0293µg/ml	ND	ND
Oxytetracycline	0.375µg/ml	0.75µg/ml	ND	ND
Erythromycin	0.0586µg/ml	0.0293µg/ml	ND	ND
Conventional antimicrobials				
Cefixime	0.75µg/ml	0.375µg/ml	1.5µg/ml	0.75µg/ml
Ceftriaxone	0.0938µg/ml	0.188µg/ml	0.188µg/ml	0.188µg/ml
Bacitracin	2.34µg/ml	2.34µg/ml	18.8µg/ml	4.69µg/ml
Polymixin B	468.8µg/ml	468.8µg/ml	937.5µg/ml	468.8µg/ml
Metronidazole	0.235µg/ml	0.235µg/ml	0.938µg/ml	0.235µg/ml

Nitazoxanide	>234.4µg/ml	>234.4µg/ml	>234.4µg/ml	>234.4µg/ml
Linezolid	0.188µg/ml	0.0938µg/ml	0.375µg/ml	0.188µg/ml
Naturally derived antibiotic agents				
Apis mellifera (bee) venom	93.8µg/ml	375µg/ml	187.5µg/ml	750µg/ml
Tannic acid	117µg/ml	468.8µg/ml	468.8µg/ml	937.5µg/ml
Eugenol	468.8µg/ml	468.8µg/ml	937.5µg/ml	937.5µg/ml
Menthol	468.8µg/ml	2343.8µg/ml	937.5µg/ml	468.8µg/ml
Carvacrol	351.8µg/ml	176.3µg/ml	704.3µg/ml	351.8µg/ml.
Cinnamaldehyde	123.8µg/ml	123.8µg/ml	495µg/ml	247.5µg/ml
Antibacterial metals				
Copper sulphate	0.0938mM	0.0117mM	0.188mM	0.00235mM
Nickel sulphate	7.031mM	1.76mM	14.06mM	3.51mM
Zinc sulphate	7.031mM	1.76mM	14.06mM	3.51mM
Gallium (III) nitrate hydrate	0.469mM	0.469mM	1.88mM	0.938mM
Peptide PNAs				
acpP matched to T320A	>15µM		>15µM	
ftsZ matched to T320A	>15µM		>15µM	
acpP matched to T3552B		>15µM		>15µM
ftsZ matched to T3552B		>15µM		>15µM

8.4 Discussion

This study identifies four conventional antimicrobials and five phytochemicals that could be useful as novel therapeutic agents for BDD. It also shows potential resistance of BDD treponemes to zinc and nickel sulphate. This first trial of PNA-peptides targeted at the TIRs of two housekeeping genes was unsuccessful.

Interestingly, ceftriaxone and cefixime were effective against BDD treponemes in this trial, when comparable third generation cephalosporins found in licensed cattle products, previously showed poorer efficacy (MICs for ceftiofur 3-6mg/L and cefalexin 12-48mg/L) (Evans 2012b). Use of this class of antibiotics is restricted in livestock and permissible only as a last resort after sensitivity testing in much of Europe (Responsible use of medicines in agriculture alliance, 2020), nevertheless for refractory BDD cases ceftriaxone or cefixime offer a more targeted option than other third generation cephalosporins. Polymixin B yielded high MIC values, in common with colistin, a lipopeptide antibiotic from the same class to which BDD treponemes have been previously considered intrinsically resistant (Evans et al., 2012b). Here, the BDD treponemes were unexpectedly resistant to nitazoxanide, since it is targeted at the PFOR pathway which is an important component of many metabolic pathways for anaerobic bacteria and has previously shown efficacy against a wide range of strict anaerobes (Dubreuil et al., 1996). The nitazoxanide derivative amixicile, which also inhibits PFOR, was effective against human oral treponemes, however it was ineffective against *Borrelia burgdorferi* which lacks the PFOR pathway (Reed et al., 2018). Since our transcriptomics data show changes in T320A and T3552B metabolism in response to oxidative stress, it is possible that the PFOR pathway is not utilised under anaerobic conditions used in these broth microdilution assays. The mechanisms for BDD treponeme resistance to nitazoxanide found here warrant further investigation.

Although they are not recognised by the medical community as therapeutic agents, many phytochemicals such as those tested in this study have antibacterial potential. They commonly have weak or narrow spectra of activity which precludes interest in clinical applications. Antibacterial properties are commonly reported for phytochemicals when MICs are in the range 100-1000µg/ ml whereas typical antibiotics derived from bacteria or fungi display MICs in the range 0.01-10µg/ml (Simões et al., 2009). The human oral treponemes *T. denticola* and *T.vincentii* are more susceptible to eugenol than other obligate anaerobes, *in vitro,* showing MICs of 800µg/ml, which is comparable to MICs reported here for phytochemicals (Shapiro et al., 1994). They were considered susceptible to eugenol because this concentration of active ingredient could be reached in the context of oral hygiene products. Indeed, if topical application of phytochemicals is considered for BDD treatment, then all five of those tested here, may be suitable for use. Footbathing for BDD prevention and

treatment is common practice on dairy farms, and disinfectants are used in footbaths with concentrations of active ingredients in the range 2-5% (Cook et al., 2012; Jacobs et al., 2019; Solano et al., 2015). MICs and MBCs for thymox (with the active ingredient thymol) have been evaluated in this context and deemed effective as MICs and MBCs after 10 minutes contact time were <0.01%, distinctly below the 1% manufacturer-recommended working concentration (Kulow et al., 2015). If the same criteria are applied, then bee venom may also be considered as a potentially suitable footbathing agent.

Susceptibility *in vitro* has been previously reported for copper sulphate against *Treponema phagedenis* at <0.01% (Hartshorn et al., 2013). Our findings show similar values indicating high sensitivity of *T. phagedenis* to copper sulphate, which we also found for *T. pedis*. MICs and MBCs for *T. phagedenis* were 75 times higher for zinc and nickel sulphate compared to copper sulphate; and for *T. pedis* the values were 150 times higher. Zinc sulphate footbaths have been used in sheep as treatment for infectious foot diseases, since copper sulphate cannot be used due to copper toxicity problems. Ovine footrot cases respond well to this, whereas cases of the BDD analogous disease contagious ovine digital dermatitis (CODD) respond poorly (Bernhard et al., 2021; Tegtmeyer et al., 2020). Involvement of treponeme species (many common to both CODD and BDD (Duncan et al., 2014; Sayers et al., 2009)) distinguishes CODD from footrot, therefore these bacteria are considered phenotypically resistant to zinc. In fact, treatment with zinc sulphate can be used to differentiate these two similar clinical presentations; since *Dichelobacter nodosus,* one of the primary causative agents of ovine footrot, is susceptible to zinc sulphate in the range 8-16µg/ml (Gradin et al., 1983). As a better alternative than zinc or nickel sulphate to copper sulphate, our results suggest that gallium (III) nitrate hydrate may be suitable as a new antibacterial metal formulation in footbaths for both cattle and sheep.

The PNA-peptides trialled here did not inhibit treponeme growth. One of the major limitations of this technology is the ability of these synthetic molecules to penetrate the cell membrane as these oligonucleotides are too large for passive cellular uptake. It is possible that the unusual structure of the treponeme outer sheath prevented entry to cells (Norris et al., 2010). The OTEB medium used to grow treponemes here contains cations, particularly Mg^{2+}, which may reduce cell permeability (Eriksson et al., 2002).

8.5 Conclusion

This chapter shows efficacy against two strains of BDD treponeme for conventional antimicrobials ceftriaxone, cefixime, linezolid and metronidazole. It also suggests six naturally derived products and the antibacterial metal gallium (III) nitrate hydrate as possible alternatives for footbath treatments of infectious foot diseases in ruminants, particularly sheep where use of copper sulphate causes toxicity. Continued surveillance of new therapeutic candidates *in vitro* and trialling of identified products *in vivo* should enable for the continued development of treatments for this important severe infectious disease.

Chapter 9 Discussion and further work.

In the most recent industry-wide survey, cattle lameness was considered the top syndrome impacting cattle production and welfare in the UK with bovine digital dermatitis (BDD) considered the most important disease (Miller, 2021). BDD is endemic in more than 90% of UK dairy farms and the most recent review estimated an incidence rate of 53.6 cases per 100 cow years (Afonso et al., 2020). Pain caused by BDD is manifested as lameness, which was detected during mobility scoring in 26.3% of mildly affected and 39.5% of severely affected cattle (Frankena et al., 2009). BDD is also associated with reduced milk yield and reproductive performance (de Jesús Argáez-Rodríguez et al., 1997; Losinger, 2006). BDD is endemic worldwide, and in the US and the EU the annual economic loss from BDD could exceed US$1 billion (Zinicola et al., 2015b). Improving control of this painful infectious disease is crucial from both welfare and economic perspectives.

Bacteria of the genus *Treponema* are considered the main pathogen associated with BDD and BDD is described both as a polytreponemal disease, which features multiple treponeme phylotypes, and as a complex polymicrobial disease (Marcatili et al., 2016; Rasmussen et al., 2012). The aetiopathogenesis, genomic architecture, host immune response, and transmission routes of the disease have not been fully elucidated (Evans et al., 2016; Orsel et al., 2018). Current disease control measures rely on labour intensive methods for diagnosis of acute lesions and treatment with topical antimicrobials. Treatment is often unsuccessful in the long term, with high lesion recurrence rates and many becoming chronic, remaining as a source of infection within the herd (Berry et al., 2010, 2012; Holzhauer et al., 2011; Jacobs et al., 2018). Prevention is centred on prophylactic footbathing strategies, despite lack of evidence that they are effective (Jacobs et al., 2019). They commonly rely on use of copper sulphate, which poses an environmental hazard, or formalin which is a carcinogen (Laven & Logue, 2006; Speijers et al., 2010). There is also evidence of resistance to conventional antimicrobials in BDD treponemes (Evans et al., 2009c). Therefore, there is an urgent need to develop novel evidence-based preventive and control strategies to improve animal health and welfare and safeguard human health and the environment.

This thesis contributes to the development of novel DD control strategies by considering several aspects of foot infections in cattle. The first studies were an investigation of hoof knives as a vector for disease and identifying an effective disinfection protocol for mitigating the risk of transmitting treponemes during foot-trimming. The thesis also presents set, network and shotgun metagenomic analyses of the bovine foot-skin microbiome, providing compelling evidence that the disease process begins before morphological appearance of lesions. Furthermore, new information regarding BDD pathogenesis with respect to biofilm formation and quorum sensing opens new avenues for control

strategies. Finally, *in vitro* investigation of novel antimicrobial agents highlights new possibilities for future treatment and prevention.

9.1 The role of foot-trimming in BDD transmission

The development of *in vivo* models for reliable induction of BDD have demonstrated that foot application of macerated BDD lesion material is the most consistent approach for initiating disease (Krull et al., 2016). When successive foot-trimming is carried out on cattle in herds where BDD is endemic, it is highly likely that similar material could be unwittingly transferred between feet. As demonstrated by field trials reported in Chapter 3, this is particularly high risk when contact has been made with BDD lesions for trimming/ treatment purposes.

The survey reported in Chapter 4 demonstrated relatively poor consideration of hygiene protocols amongst professionals responsible for cow foot health. Whilst we were able to attribute some improvements in the sector to the research work and subsequent programme of knowledge exchange reported in this thesis, respondents to the follow-up questionnaire expressed a wish for future research to continue to investigate the risk of BDD transmission during foot-trimming, which is perhaps a reflection of reluctance to accept responsibility for a role in BDD transmission. Generally, people are less willing to change their behaviour if they deny the negative effects of their behaviour and do not perceive it as a threat. Table 9.1 rationalises the probable mechanisms for BDD transmission and makes the argument that foot-trimming is the only event during which it is known that viable treponemes contact the feet of other cows in the herd (assuming that effective disinfection is not practised). Lame cows presented for foot trimming are more likely to exhibit existing tissue damage, that could make them more susceptible to acquiring the pathogens needed to cause BDD lesions, fulfilling the Krull infection model criteria. “Proving” causation without doubt would require a large scale randomised controlled trial to measure the effect of adopting good hygiene during foot-trimming by assessing incidence rate longitudinally in many dairy herds. Given that many environmental risk factors linked to BDD could not be controlled in such studies and the high costs associated, it is unlikely that such a comprehensive study would be feasible. The microbiological evidence of transmission risk presented here and elsewhere, should provide enough scientific rationale to influence foot-trimmers’ behaviour and should be incentive enough to at least adopt minimum hygiene standards. It is widely acknowledged, however, that decisions are not based on knowledge alone, but on socio-psychological factors, and external influences from advisors and peers (Ritter et al., 2017).

Table 9.1. Identified sources of infection on farm, fulfilment of infection model criteria and associated risk factors and microbiological evidence. Reproduced from Gillespie & Evans (2019).

Source of Infection	PERCEIVED LIKELIHOOD OF DIRECT CONTACT	VIABLE BACTERIAL LOAD	Associated risk factor Evidence	Tissue damage/ Infection reservoir evidence
Foot-trimming tools	HIGH	YES	Not washing hoof trimming tools in between animals=increased DD risk (Wells et al., 1999). Use of hoof trimmer who works on multiple farms= increased risk of BDD (Wells et al., 1999; Yang et al., 2018).	Tools will make direct contact with damaged feet. BDD is highly contagious The BDD treponemes are identified frequently on hoof trimming equipment and can be cultured and can survive for up to two hours (Gillespie et al., 2019; Sullivan et al., 2014).
Foot trimming hands/gloves	HIGH	YES	Use of hoof trimmer who works on multiple farms= increased risk of BDD (Wells et al., 1999; Yang et al., 2018).	Hands/gloves will make direct contact with damaged feet. BDD is highly contagious. The BDD treponemes are identified frequently on hands/gloves and can be cultured (Angell et al., 2017; Blowey et al., 2013).
Farm slurry	HIGH	UNKNOWN	Increased environment slurry= increased DD risk (Rodriguez-Lainz et al., 1996). Increased hygiene = reduced BDD risk (Hultgren & Bergsten, 2001).	Exposure of skin to moisture (such as moist slurry environment) needed for BDD transmission (Gomez et al., 2012; Krull et al., 2016; Read & Walker, 1996). BDD treponemes in oral and rectal cavities suggests faecal shedding (Evans et al., 2012b), although cultivable treponemes not isolated from slurry.
Oral or rectal cavity	LOW	YES	Increased hygiene = reduced BDD risk.	BDD treponemes in oral and rectal cavities (Evans et al., 2012b) and can be cultured from ruminant rectal tissue (Sullivan et al., 2015b).
Lesions	LOW	YES	Mathematical model shows M4 to be the most significant lesion stage for transmission (Biemans et al., 2018).	BDD treponemes culturable from lesions (Evans et al., 2008).

Foot trimming respondents also expressed opinions that future research should concentrate on how to better engage farmers to improve their management of BDD. This could reflect an element of "shifting the blame" onto farmers, as foot-trimmers feel there is little incentive to practice good biosecurity during foot trimming when cows immediately return to slurry-contaminated housing, which is a prominent risk factor for high prevalence of BDD (Somers et al., 2005; Wells et al., 1999). It is true that even the generic advice used in the industry for BDD control is rarely applied as effectively as possible on individual farms. This could be because despite high prevalence, BDD control is not prioritised by most producers, as it is not considered as vital as other problems (such as mastitis or infertility) to herd productivity. It is possible that veterinary input on this subject is also less than in other areas, such as calf health, reproduction and mastitis, where progress is much easier and quicker to measure. For multifactorial chronic conditions such as BDD that require a substantial amount of effort to improve, difficulties with assessing the effect of adopted management changes may reinforce the perception that they are ineffective (Jansen & Lam, 2012).

A recent study attempted to engage farmers in the Netherlands in a BDD management programme using a risk assessment and a one-page summary of farm-specific recommendations. This was insufficient to initiate the behaviour change needed to decrease BDD prevalence and the study concluded this may have been improved if there had been follow-up to advice from the farms' own veterinarians (Vanhoudt et al., 2021). In the context of the UK, the industry-led Healthy Feet Programme encourages farmers to engage in all aspects of lameness control, including BDD control. However, this programme is voluntary and as such may not be prioritised by farmers due to more pressing disease control initiatives. For example, 95% of dairy farmers are engaged in the national management plan for control of Johne's disease, thanks to its incorporation in the UK national farm assurance program (Red Tractor) (Orpin et al., 2021), and all are obliged to undertake compulsory testing for Bovine Tuberculosis in line with national legislation. As an adaptation of the conceptual framework provided by the widely acclaimed Health Belief Model (Janz & Becker, 1984), consideration of four major factors is required in encouraging adoption of disease control measures by farmers: enhance problem awareness and sense of responsibility, enhance belief in the effectiveness of proposed strategies, enhance ability to implement recommended changes, and enhance the perceived benefits of disease prevention and control (Ritter et al., 2017).

9.2 Further investigation of biofilm formation in BDD pathogenesis

This thesis investigates the role of biofilm formation as a pathogenic mechanism in BDD associated treponemes. It presents evidence that BDD treponemes are capable of biofilm formation; however, the simplest form of a static *in vitro* biofilm model was used, and biofilms were quantified using the crystal violet assay, interpretation of which was hindered by staining of growth medium. Analysis of the transcriptome from this model provided some evidence of biofilm characteristics; however, some key features were not detected.

Investigation of the structure and function of biofilms in biopsies from different BDD lesion stages could be productive for directing the development of *in vitro* models. Techniques could include detection of alginate expression using anti-alginate antibodies and detection of extracellular DNA by staining with DDAO (7-hydroxy-9H-(1, 3-dichloro-9, 9 dimethylacridin-2-one)) DNA binding fluorescent dye. These techniques have been used to show biofilm characteristics in aggregates of the spirochaete *Borrelia burgdorferi* grown in tissue culture plates (Sapi et al., 2012); however, they would need adapting for use in tissue sections.

The biofilm model presented here approximates a surface-attached biofilm; however, for BDD, it may be more relevant to pursue development of a chronic wound model to investigate the structure and function of tissue-associated biofilm. There are several existing biofilm wound models which might be adapted for BDD study, including a dynamic 3 dimensional model using a colony- drip flow reactor in which a three-species biofilm model was successfully grown, which included the obligate anaerobe *Clostridium difficile* (Brackman & Coenye, 2016; Woods et al., 2012).

The four genera tentatively described as core bacterial species in BDD lesions are *Treponema spp., Porphyromonas* spp*, Fusobacterium* spp. and *Mycoplasma* spp., (Caddey & de Buck, 2021; Nielsen et al., 2016), and the first three have been detected and visualised in lesions using fluorescent in situ hybridisation (Nielsen et al., 2016). *Fusobacterium* spp. was not associated with foot skin swabs from cows that later developed BDD lesions (Bay et al., 2021), whilst *Mycoplasma* spp. and *Porphyromonas* spp. were. Although we cannot distinguish between primary pathogenic and opportunistic bacteria, current evidence suggests *Porphyromonas* spp. and *Mycoplasma* spp. as primary candidates for involvement in initiation of disease. *Porphyromonas levii* has been reported in BDD lesions, both from bacteriological cultures and detection in acute and chronic lesions using shotgun metagenomic analysis (Berry et al., 2010; Zinicola et al., 2015b). Addition of this obligate anaerobe to biofilm models may promote biofilm formation *in vitro,* improving the value of the model for investigating BDD pathogenesis and potential treatments. This was shown to be the case when *Porphyromonas gingivalis* was added to a model with *T. denticola*, resulting in more biofilm mass and tighter

adherence to the substratum (Kuramitsu et al., 2005). Confocal microscopy demonstrated attachment of *P.gingivalis* to the substratum first, followed by aggregation with *T. denticola* (Yamada et al., 2005). Whilst *Treponema* are considered the primary pathogens in BDD, lesions are polymicrobial, and co-infection with *Porphyromonas* may promote formation of biofilm and facilitate progression to chronic lesions. In addition, the same species associated with BDD lesions, *P. levii,* has been shown to form biofilms with *Fusobacterium necrophorum* and impair the oxidative response of bovine neutrophils *in vitro* compared to planktonic equivalent cultures (Lockhart et al., 2017).

Multispecies biofilms involving *Mycoplasma* spp. have not been widely studied; however, it is known that persistence of *Mycoplasma* is a hallmark of several prevalent livestock diseases. In cattle, these are severe respiratory diseases caused by *Mycoplasma bovis* (enzootic pneumonia) and *Mycoplasma mycoides* subspecies *mycoides* (contagious bovine pleuropnemonia). The ability to form biofilms is a known mechanism for persistence in the porcine pathogen *M. hyopneumoniae,* but this has not been studied for bovine disease (Hoelzle et al., 2020). Furthermore, the ability of the BDD-relevant pathogen *Mycoplasma fermentans* to form biofilms has been recently demonstrated (Awadh et al., 2021). Consequently, given that *Mycoplasma* are common in farm environments (Justice-Allen et al., 2010; Stärk et al., 1998), it is distinctly possible that they may be important in maintaining BDD lesions by forming biofilms with existing infecting bacteria and essentially keeping the wound open.

9.3 The future of BDD control

Network analysis of the bovine foot skin microbiome detected dysbiosis already occurring prior to visible lesion development, and shotgun metagenomic analysis showed the presence of functional changes. The fact that there was no correlation in bacterial networks between presence of *Treponema* spp. and other bacterial genera associated with disease prior to visible appearance of lesions suggests there is an opportunity for intervention to halt the progress of disease, before cooperative bacterial communities become established. This highlights that BDD control is best targeted to animals unaffected by visible lesions. A Canadian study identified BDD in youngstock on 11 of 28 farms and recorded 11% prevalence in heifers over 12 months old (Jacobs et al., 2017). A Dutch study showed BDD prevalence in heifers less than one year old was just 0.4% but increased to 12.9% between one year old and first calving. It is therefore crucial that preventative footbathing is extended to heifers during the rearing period.

This thesis reports evidence of biofilm formation and quorum sensing capabilities in BDD treponemes. Searching of BDD treponeme genomes in MAUVE did not reveal the presence of the *LuxS* gene responsible for production of the signalling molecule autoinducer-2 (AI-2). To establish whether BDD treponemes produce AI-2, the signalling molecule most commonly produced by Gram-negative

bacteria, *Vibrio harveyi* BB170 could be used as a reporter strain as it exhibits bioluminescence in response to AI-2 (Jang et al., 2013).

A unique feature of quorum sensing is that it triggers upregulation of virulence factors once bacterial cells reach a threshold density (Buch et al., 2021). Early intervention to inhibit quorum sensing molecules before they reach this critical threshold may offer an alternative preventative approach for BDD; for example, by inclusion of QSIs in preventative footbaths. Phytochemicals and the antibacterial metal gallium nitrate identified in this thesis as potential topical therapeutic agents offer possibilities in this field. Furthermore, in human oral bacterial communities, accumulation of AI-2 is thought to be instrumental to progression of biofilm formation and eventual encouragement of pathogen growth (Kolenbrander et al., 2010). Thus, biofilm formation and quorum sensing are intrinsically linked.

Inhibiting bacterial communication systems are considered a promising target for control of human oral disease in which biofilms are key to pathogenesis. For example, D-galactose has been shown to reduce biofilm formation in the "red complex" of bacteria composed of *F. nucleatum, P. gingivalis* and *T. denticola*, and QSIs furanone and D-ribose decreased bone destruction and total bacterial numbers, reducing periodontitis progression in a mouse model of *P. gingivalis* (Muras et al., 2022). Biofilm formation is another common mechanism shared by periodontal diseases and chronic wounds (Mancl et al., 2013). Consequently, it is likely that joint studies of periodontal disease and BDD will reveal key information on the pathogenesis of both diseases, especially the bacteriological aspects.

Breeding for resistance to BDD presents another future possibility for enhancing disease control. Genetic models have estimated heritability of BDD to an upper limit of 0.52 (Schöpke et al., 2015) and genomic regions associated with BDD house genes for inflammatory and fibroblastic processes which may affect the host response to infection (Sánchez-Molano et al., 2019). Two further genomic regions containing genes involved in immunological processes have also been identified as associated with disease susceptibility and probability of chronic progression (Oelschlaegel et al., 2022). Recently, a genome wide association study and regional heritability mapping associated regions of the bovine genome with relative abundance of the members of the foot skin microbiota *Peptoclostridium* spp. and *Treponema* spp., which are associated with future development of BDD lesions (Bay et al., 2021). This indicates that host genetic control of the foot skin microbiota may also have a role in BDD susceptibility.

9.4 Novel treatments

An understanding of biofilm formation and quorum sensing mechanisms in BDD treponemes opens new possibilities for enhanced treatments using biofilm and quorum sensing inhibitors. Two of the phytochemicals tested in this thesis have been described as biofilm inhibitors; tannic acid for preventing *Staphylococcus aureus* from colonising surfaces, and eugenol for inhibiting biofilm formation by *Klebsiella pneumoniae* (Roy et al., 2018). Two antimicrobial peptides (AMPs) engineered from jelleine, a family of AMPs from the "royal jelly" secretion of *Apis mellifera,* have also shown anti-biofilm activity, which is interesting in the context of *Apis mellifera* venom anti-treponeme properties shown in this thesis (Lim et al., 2013). A range of anti-biofilm molecules have been tested *in vitro*, with enzymes for dispersion of EPS, various AMPs and quorum sensing inhibitors showing promise for prevention and dispersion of biofilms. Development of a multispecies biofilm model for BDD would enable *in vitro* sensitivity testing to yield results more relevant to disease in the field.

This thesis also identified the conventional antimicrobials ceftriaxone, cefixime, linezolid and metronidazole as potential candidates for BDD treatment. Use of ceftriaxone or cefixime for BDD treatment could be recommended above ceftiofur or cefalexin as the MICs are much lower, reducing antimicrobial use, and reducing the risk of exposure to subtherapeutic concentrations that may drive antimicrobial resistance (Holman & Chénier, 2015). BDD treponemes were unexpectedly resistant to nitazoxanide. It is possible that genes encoding the PFOR pathway targeted by nitazoxanide were not expressed during experiments. Transcriptomics data showed that exposure to oxidative stress markedly changes BDD treponeme metabolism; therefore, repeating MIC and MBC testing after exposure to oxidative stress, which mimics exposure to ROS produced by neutrophils, could give a more accurate estimation of expected treatment efficacy in the field.

Emerging understanding of the bovine foot skin microbiome creates possibilities for developing probiotics for BDD treatment. Promoting growth of bacteria that appear protective against lesion development and reducing the presence of pathogens through competition with non-pathogenic species could help to maintain a healthy foot-skin microbiome. Preserving beneficial microbiota is recognised as essential for preserving health, giving impetus to pursuit of next-generation precision antimicrobials which can specifically target pathogens, rather than killing non-target organisms (de la Fuente-Nunez et al., 2017). The PNA peptides trialled in this thesis are an example of a nucleic acid-based precision antimicrobial. Another prominent example is the use of manipulated versions of the widespread CRISPR-*Cas* bacterial immune mechanism. These have shown efficacy when directed against antimicrobial resistance genes and delivered via bacteriophages or phagemids. This

technology could be adapted to target virulence factors such as toxin-encoding genes (de la Fuente-Nunez et al., 2017).

In human medicine, the use of lasers and ultrasonic therapy are being explored for treatment of chronic wounds, both with the underlying hypothesis that they are capable of disrupting biofilms (Mancl et al., 2013). These alternative treatment approaches could become relevant to BDD treatment, particularly if biofilms are shown to be key to BDD pathogenesis.

9.5 Vaccine development

Improved understanding of disease pathogenesis would also direct vaccine development. Targeting identification of immunogenic outer membrane proteins is a rational approach because spirochaete OMPs are frequently implicated in adherence to host extra-cellular matrix proteins and preventing this adherence would halt disease initiation. Recently four outer membrane proteins have been identified as immunogenic *in vivo* using calves (Staton et al., 2020). Analysis of BDD associated treponeme genomes identified two OMPs as possible vaccine candidates as the recombinant proteins bound to host ECM proteins, and an ELISA-based serological assay showed an IgG2 response to both in cows with BDD (Staton et al., 2021a). Metatranscriptomics of BDD lesions also detected upregulation of bacterial virulence genes involved in chemotaxis, flagellar synthesis and protection against oxidative stress (Marcatili et al., 2016), in common with the treponeme-specific data presented in this thesis. It is possible that reducing treponeme motility, or ability to survive oxidative stress, may attenuate virulence and prevent disease. For example, NADH oxidase mutants of the pathogenic spirochaete *Brachyspira hyodysenteriae* showed attenuated virulence in a porcine infection model, demonstrating that oxidative stress genes are important for virulence (Stanton et al., 1999).

To date there have been no successful trials of BDD vaccines. A prototype bacterin vaccine was manufactured and trialled in two dairy herds. Although there was a detectable serological response, there was no significant clinical response (Ertze et al., 2006). Two herd-specific vaccines, either containing *Treponema* spp. antigen or not, were trialled in a dairy herd in Germany and yielded no significant clinical effect (Metzner et al., 2001). A vaccine containing *Dichelobacter nodosus* designed for prevention of footrot in sheep (Footvax, MSD Animal Health) showed efficacy of 32% against contagious ovine digital dermatitis (CODD), the infectious foot disease of sheep analogous to BDD. The fact that some efficacy was noted even though the vaccine does not contain *Treponema* or *Fusobacterium* antigens, both of which have a role in CODD pathogenesis (Staton et al., 2021b), shows that inclusion of antigens from other bacterial species are also likely to be relevant to BDD vaccines. Limitations to vaccine development include high variability between individual cows in lesion

microbiome composition and gene expression, and host antibody responses (Marcatili et al., 2016). Variability in the microbiome is composed both of differences in the number and identity of *Treponema* spp. present, and differences in the other bacterial species present.

9.6 Conclusion

The information presented in this thesis emphasises the importance of implementing good hygiene practices during foot-trimming. Continued knowledge exchange in this area is essential for changing many of the socio-psychological factors which limit acceptance of the recommended hygiene protocol. Studying BDD pathogenesis at the initiation of disease has emphasised the importance of applying control measures to heifers during the rearing period before the appearance of BDD lesions. Further understanding regarding the polytreponemal and polymicrobial nature of BDD is key to determining mechanisms for biofilm formation and quorum sensing. Description of these pathogenic processes would also be key to advancing research into new control strategies, including innovation concerning novel treatments and vaccine targets.

References

Afonso, J. S., Bruce, M., Keating, P., Raboisson, D., Clough, H., Oikonomou, G., & Rushton, J. (2020). Profiling Detection and Classification of Lameness Methods in British Dairy Cattle Research: A Systematic Review and Meta-Analysis. *Frontiers in Veterinary Science*, *7*, 542.

Afonso, J. S., Oikonomou, G., Carter, S., Clough, H. E., Griffiths, B. E., & Rushton, J. (2021). Diagnosis of Bovine Digital Dermatitis: Exploring the Usefulness of Indirect ELISA. *Frontiers in Veterinary Science*, *8*, 1250.

Almiron, M., Link, A. J., Furlong, D., & Kolter, R. (1992). A novel DNA-binding protein with regulatory and protective roles in starved Escherichia coli. *Genes & Development*, *6*(12b), 2646–2654.

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biology*, *11*(10), 1–12.

Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics*, *31*(2), 166–169.

Anderson, R. C., Vodovnik, M., Min, B. R., Pinchak, W. E., Krueger, N. A., Harvey, R. B., & Nisbet, D. J. (2012). Bactericidal effect of hydrolysable and condensed tannin extracts on Campylobacter jejuni in vitro. *Folia Microbiologica*, *57*(4), 253–258.

Andrews, S. C., Robinson, A. K., & Rodríguez-Quiñones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiology Reviews*, *27*(2–3), 215–237.

Angel. Ertze, R., Read, D., Hird, D. W., & Berry, S. L. (2006). Field evaluation of prophylactic and therapeutic effects of a vaccine against (Papillomatous) digital dermatitis in dairy cattle on two California dairies. *Bovine Pract.*, *40*, 76–82.

Angell, J. W., Clegg, S. R., Grove-White, D. H., Blowey, R. W., Carter, S. D., Duncan, J. S., & Evans, N. J. (2017). Survival of contagious ovine digital dermatitis (CODD)-associated treponemes on disposable gloves after handling CODD-affected feet. *The Veterinary Record*, *181*(4), 89.

Angell, J. W., Clegg, S. R., Sullivan, L. E., Duncan, J. S., Grove-White, D. H., Carter, S. D., & Evans, N. J. (2015). In vitro susceptibility of contagious ovine digital dermatitis associated Treponema spp. isolates to antimicrobial agents in the UK. *Veterinary Dermatology*, *26*(6).

Argudín, M. A., Hoefer, A., & Butaye, P. (2019). Heavy metal resistance in bacteria from animals. *Research in Veterinary Science*, *122*, 132–147.

Ariza, J. M., Bareille, N., Oberle, K., & Guatteo, R. (2019). Current recommendations for footbath solutions renewal rates in dairy cattle: the need for adaptation? *Animal : An International Journal of Animal Bioscience*, *13*(6), 1319–1325.

Ariza, J. M., Döpfer, D., Anklam, K., Labrut, S., Oberle, K., Bareille, N., Relun, A., & Guatteo, R. (2019). Do footbath disinfectants modify the dynamics of the skin microbiota in dairy cattle affected by digital dermatitis? *BioRxiv*, *accepté*.

Arnér, E. S. J., & Holmgren, A. (2000). Physiological functions of thioredoxin and thioredoxin reductase. *European Journal of Biochemistry*, *267*(20), 6102–6109.

Aubineau, T., Relun, A., Gentin, B., & Guatteo, R. (2021). Short communication: Informative value of an ELISA applied to bulk tank milk to assess within-herd prevalence of digital dermatitis in dairy herds. *Journal of Dairy Science*, *104*(1), 963–968

Awadh, A. A., Kelly, A. F., Forster-Wilkins, G., Wertheim, D., Giddens, R., Gould, S. W., & Fielder, M. D. (2021). Visualisation and biovolume quantification in the characterisation of biofilm formation in Mycoplasma fermentans. *Scientific Reports 2021 11:1*, *11*(1), 1–9.

Baptista, P. J. H. P. (1979). INFECTIOUS BOVINE KERATOCONJUNCTIVITIS A REVIEW. *Brazilian Veterinary Journal*, *135*, 225.

Bardou, P., Mariette, J., Escudié, F., Djemiel, C., & Klopp, C. (2014). Jvenn: An interactive Venn diagram viewer. *BMC Bioinformatics*, *15*(1), 1–7.

Baughn, A. D., & Malamy, M. H. (2004). The strict anaerobe Bacteroides fragilis grows in and benefits from nanomolar concentrations of oxygen. *Nature 2004 427:6973*, *427*(6973), 441–444.

Bay, V. (2018). *A study on the role of the foot skin microbiome and the host's genetics in the development of lameness causing foot lesions in dairy cattle*. [PhD thesis]. University of Liverpool.

Bay, V., Gillespie, A., Ganda, E. K., Evans, N., Carter, S., Lenzi, L., Lucaci, A., Haldenby, S., Barden, M., Griffiths, B. E., Sánchez-Molano, E., Bicalho, R., Banos, G., Darby, A., & Oikonomou, G. (2021). The Bovine Foot Skin Microbiota is Associated with Host Genotype and the Development of Infectious Digital Dermatitis Lesions. In *Research Square*. https://doi.org/10.21203/rs.3.rs-650860/v1

Bay, V., Griffiths, B., Carter, S., Evans, N. J., Lenzi, L., Bicalho, R. C., & Oikonomou, G. (2018). 16S rRNA amplicon sequencing reveals a polymicrobial nature of complicated claw horn disruption lesions and interdigital phlegmon in dairy cattle. *Scientific Reports*, *8*(1).

Bell, J. (2017). *Identifying Infection Reservoirs of Digital Dermatitis in Dairy Cattle.* [PhD thesis]. University of Liverpool.

Bell, N., & Vanhoudt, A. (2020). Treating and controlling digital dermatitis in dairy cattle. *In Practice*, 554–567.

Beninger, C., Naqvi, S. A., Naushad, S., Orsel, K., Luby, C., Derakhshani, H., Khafipour, E., & De Buck, J. (2018). Associations between digital dermatitis lesion grades in dairy cattle and the quantities of four Treponema species. *Veterinary Research*, *49*(1), 111.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, *57*(1), 289–300.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M. C. C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G. H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J. A., Maguin, E., Mauchline, T., McClure, R., … Schloter, M. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome*, *8*(1), 1–22.

Bernhard, M., Frosth, S., & König, U. (2021). First report on outbreaks of contagious ovine digital dermatitis in Sweden. *Acta Veterinaria Scandinavica*, *63*(1), 1–9.

Berry, S. L., Read, D. H., Famula, T. R., Mongini, A., & Döpfer, D. (2012). Long-term observations on the dynamics of bovine digital dermatitis lesions on a California dairy after topical treatment with lincomycin HCl. *Veterinary Journal*, *193*(3), 654–658.

Berry, S. L., Read, D. H., Walker, R. L., & Famula, T. R. (2010). Clinical, histologic, and bacteriologic findings in dairy cows with digital dermatitis (footwarts) one month after topical treatment with lincomycin hydrochloride or oxytetracycline hydrochloride. *Journal of the American Veterinary Medical Association*, *237*(5), 555–560.

Bian, J., Liu, X., Cheng, Y.-Q., & Li, C. (2013). Inactivation of cyclic Di-GMP binding protein TDE0214 affects the motility, biofilm formation, and virulence of Treponema denticola. *Journal of Bacteriology*, *195*(17), 3897–3905.

Biegel, E., & Müller, V. (2010). Bacterial Na+-translocating ferredoxin: NAD+ oxidoreductase. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(42), 18138–18142.

Biemans, F., Bijma, P., Boots, N. M., & de Jong, M. C. M. (2018a). Digital Dermatitis in dairy cattle: The contribution of different disease classes to transmission. *Epidemics*, *23*.

Biemans, F., Bijma, P., Boots, N. M., & de Jong, M. C. M. (2018b). Digital Dermatitis in dairy cattle: The contribution of different disease classes to transmission [Elsevier B.V.]. In *Epidemics* (Vol. 23).

Bjarnsholt, T., Alhede, M., Alhede, M., Eickhardt-Sørensen, S. R., Moser, C., Kühl, M., Jensen, P. Ø., & Høiby, N. (2013). The in vivo biofilm. *Trends in Microbiology*, *21*(9), 466–474.

Bjarnsholt, T., Ciofu, O., Molin, S., Givskov, M., & Høiby, N. (2013). Applying insights from biofilm biology to drug development — can a new approach be developed? *Nature Reviews Drug Discovery 2013 12:10*, *12*(10), 791–808.

Blowey, R., Done, S., & Cooley, W. (1994). Observations on the pathogenesis of digital dermatitis in cattle. *Veterinary Record*, *135*(5), 115–117.

Blowey, R., Sullivan, L., Evans, N., & Carter, S. D. (2013). Detection of Bovine Digital Dermatitis on Hoof Knives and Hands and their Removal by Disinfection. In J. (University of B. Whay, B., Hockenhull (Ed.), *27th International Symposium and 9th International Conference on Lameness in Ruminants.* (p. 181).

Blowey, R. W. (1987). Digital dermatitis in dairy cattle. *The Veterinary Record*, *121*(13), 311.

Blowey, R. W. (1990). Digital Dermatitis Control. *Veterinary Record*, 120.

Blowey, R. W., & Sharp, M. W. (1988). Digital dermatitis in dairy cattle. *The Veterinary Record*, *122*(21), 505–508.

Blowey, R. W., Sharp, M. W., & Done, S. H. (1992). Digital dermatitis. *Veterinary Record*, *131*(2), 39 LP – 39.

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A., & Caporaso, J. G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods*, *10*(1), 57–59.

Borgmann, I. E., Bailey, J., & Clark, E. G. (1996). Spirochete-associated bovine digital dermatitis *Canadian Veterinary Journal,* 37(1), 35-37

Boyer, E., Bergevin, I., Malo, D., Gros, P., & Cellier, M. F. M. (2002). Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium. *Infection and Immunity*, *70*(11), 6032–6042.

Brackman, G., & Coenye, T. (2016). In vitro and in vivo biofilm wound models and their application. *Advances in Experimental Medicine and Biology*, *897*, 15–32.

Brennan, M. L., & Christley, R. M. (2013). Cattle producers' perceptions of biosecurity. *BMC Veterinary Research*, *9*, 71.

Brigstocke, T. (2020). *GB Cattle Health and Welfare Group Fifth Report, Top Cattle Health and Welfare Issues*.

Bruijnis, M. R. N., Beerda, B., Hogeveen, H., & Stassen, E. N. (2012). Foot disorders in dairy cattle: Impact on cow and dairy farmer. *Animal Welfare*, *21*(SUPPL. 1), 33–40.

Bruni, N., Capucchio, M. T., Biasibetti, E., Pessione, E., Cirrincione, S., Giraudo, L., Corona, A., & Dosio, F. (2016). Antimicrobial Activity of Lactoferrin-Related Peptides and Applications in Human and Veterinary Medicine. *Molecules 2016,* 21(6), 752.

Buch, P. J., Chai, Y., & Goluch, E. D. (2021). Bacterial chatter in chronic wound infections. *Wound Repair and Regeneration*, *29*(1), 106–116.

Byrd, A. L., Belkaid, Y., & Segre, J. A. (2018). The human skin microbiome. *Nature Reviews Microbiology,* 16, 143-155.

Caddey, B., & de Buck, J. (2021). Meta-Analysis of Bovine Digital Dermatitis Microbiota Reveals Distinct Microbial Community Structures Associated With Lesions. *Frontiers in Cellular and Infection Microbiology*, *0*, 597.

Caddey, B., Orsel, K., Naushad, S., Derakhshani, H., & Buck, J. de. (2021). Identification and Quantification of Bovine Digital Dermatitis-Associated Microbiota across Lesion Stages in Feedlot Beef Cattle. *MSystems*, *6*(4).

Caddey, B., Orsel, K., Naushad, S., Derakhshani, H., & De Buck, J. (2021). Identification and Quantification of Bovine Digital Dermatitis-Associated Microbiota across Lesion Stages in Feedlot Beef Cattle. *MSystems*.

Campbell, R. E., Mosimann, S. C., Tanner, M. E., & Strynadka, N. C. J. (2000). The structure of UDP-N-acetylglucosamine 2-epimerase reveals homology to phosphoglycosyl transferases. *Biochemistry*, *39*(49), 14993–15001.

Campion, C., Charbon, G., Thomsen, T. T., Nielsen, P. E., & Løbner-Olesen, A. (2021). Antisense inhibition of the Escherichia coli NrdAB aerobic ribonucleotide reductase is bactericidal due to induction of DNA strand breaks. *Journal of Antimicrobial Chemotherapy*, *76*(11), 2802–2814.

Cao, J., Woodhall, M. R., Alvarez, J., Cartron, M. L., & Andrews, S. C. (2007). EfeUOB (YcdNOB) is a tripartite, acid-induced and CpxAR-regulated, low-pH Fe2+ transporter that is cryptic in Escherichia coli K-12 but functional in E. coli O157:H7. *Molecular Microbiology*, *65*(4), 857–875.

Caspi, R., Billington, R., Ferrer, L., Foerster, H., Fulcher, C. A., Keseler, I. M., Kothari, A., Krummenacker, M., Latendresse, M., Mueller, L. A., Ong, Q., Paley, S., Subhraveti, P., Weaver, D. S., & Karp, P. D. (2015). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Research*, *44*(2), 471–480.

Cernek, P., Bollig, N., Anklam, K., & Döpfer, D. (2020). Hot topic: Detecting digital dermatitis with computer vision. *Journal of Dairy Science*, *103*(10), 9110–9115.

Chan, A. C. K., Doukov, T. I., Scofield, M., Tom-Yew, S. A. L., Ramin, A. B., MacKichan, J. K., Gaynor, E. C., & Murphy, M. E. P. (2010). Structure and Function of P19, a High-Affinity Iron Transporter of the Human Pathogen Campylobacter jejuni. *Journal of Molecular Biology*, *401*(4), 590–604.

Charlebois, A., Jalbert, L. A., Harel, J., Masson, L., & Archambault, M. (2012). Characterization of Genes Encoding for Acquired Bacitracin Resistance in Clostridium perfringens. *PLOS ONE*, *7*(9), e44449.

Charon, N. W., Chunhao, L., & Goldstein, S. F. (2006). The Beguiling Motity of the Genus Treponema. In J. D. Radolf & S. A. Lukehart (Eds.), *Pathogenic Treponema, Molecular and Cellular Biology* (pp. 127–146). Caister Academic Press.

Chautrand, T., Souak, D., Chevalier, S., & Duclairoir-Poc, C. (2022). Gram-Negative Bacterial Envelope Homeostasis under Oxidative and Nitrosative Stress. *Microorganisms*, *10*(5).

Checinska, A., Probst, A. J., Vaishampayan, P., White, J. R., Kumar, D., Stepanov, V. G., Fox, G. E., Nilsson, H. R., Pierson, D. L., Perry, J., & Venkateswaran, K. (2015). Microbiomes of the dust particles collected from the International Space Station and Spacecraft Assembly Facilities. *Microbiome*, *3*, 50.

Cheli, R., & Mortellaro, C. (1974). La dermatite digitale del bovino. In *Proceedings of the 8th International Conference on Diseases of Cattle* (pp. 208–213).

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L., & Yuan, Z. (2014a). Antibiotic alternatives: The substitution of antibiotics in animal husbandry? *Frontiers in Microbiology*, *5*(MAY), 217.

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L., & Yuan, Z. (2014b). Antibiotic alternatives: The substitution of antibiotics in animal husbandry? *Frontiers in Microbiology*, *5*(MAY), 217.

Choi, B. K., Nattermann, H., Grund, S., Haider, W., & Gobel, U. B. (1997). Spirochetes from digital dermatitis lesions in cattle are closely related to treponemes associated with human periodontitis. *International Journal of Systematic Bacteriology*, *47*(1), 175–181.

Christensen, L., Fitzpatrick, R., Gildea, B., Petersen, K. H., Hansen, H. F., Koch, T., Egholm, M., Buchardt, O., Nielsen, P. E., Coull, J., & Berg, R. H. (1995). Solid-Phase synthesis of peptide nucleic acids. *Journal of Peptide Science*, *1*(3), 175–183.

Christensen, S. K., Mikkelsen, M., Pedersen, K., & Gerdes, K. (2001). RelE, a global inhibitor of translation, is activated during nutritional stress. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(25), 14328–14333.

Chua, P. K. B., Corkill, J. E., Hooi, P. S., Cheng, S. C., Winstanley, C., & Hart, C. A. (2005). Isolation of Waddlia malaysiensis , A Novel Intracellular Bacterium, from Fruit Bat (Eonycteris spelaea). *Emerging Infectious Diseases*, *11*(2), 271–277.

Ciofu, O., Moser, C., amp, P., Jensen, strup, & Hamp, N. (2022). Tolerance and resistance of microbial biofilms. *Nature Reviews Microbiology*.

Clegg, S. R., Carter, S. D., Birtles, R. J., Brown, J. M., Anthony Hart, C., & Evans, N. J. (2016). Multilocus sequence typing of pathogenic treponemes isolated from cloven-hoofed animals and comparison to treponemes isolated from humans. *Applied and Environmental Microbiology*, *82*(15), 4523–4536.

Clements, D. N., Vaughan-Thomas, A., Peansukmanee, S., Carter, S. D., Innes, J. F., Ollier, W. E. R., & Clegg, P. D. (2006). Assessment of the use of RNA quality metrics for the screening of articular cartilage specimens from clinically normal dogs and dogs with osteoarthritis. *American Journal of Veterinary Research*, *67*(8), 1438–1444.

Collighan, R. J., & Woodward, M. J. (1997). Spirochaetes and other bacterial species associated with bovine digital dermatitis. *FEMS Microbiol Lett*, *156*(1), 37–41.

Constable, P., Hinchcliff, K., Done, S., & Grunberg, W. (Eds.). (2017). Practial Antimicrobial Therapeutics. In *Veterinary Medicine* (11th ed., pp. 153–174). Elsevier.

Cook, N. B., Rieman, J., Gomez, A., & Burgi, K. (2012). Observations on the design and use of footbaths for the control of infectious hoof disease in dairy cattle. *Veterinary Journal (London, England : 1997)*, *193*(3), 669–673.

Cordaux, R. (2008). ISWpi1 from Wolbachia pipientis defines a novel group of insertion sequences within the IS5 family. *Gene*, *409*(1–2), 20–27.

Costabile, A., Sanghi, S., Martin-Pelaez, S., Mueller-Harvey, I., Gibson, G. R., Tastall, R. A., & Klinder, A. (2011). Inhibition of Salmonella Typhimurium by tannins in vitro. *Journal of Food, Agriculture and Environment*, *9*(1), 119–124.

Costerton, J. W., Geesey, G. G., & Cheng, K.-J. (1978). *How Bacteria Stick*. *238*(1), 86–95.

Coulter, E. D., & Kurtz, J. (2001). A Role for Rubredoxin in Oxidative Stress Protection in Desulfovibrio vulgaris: Catalytic Electron Transfer to Rubrerythrin and Two-Iron Superoxide Reductase. *Archives of Biochemistry and Biophysics*, *394*(1), 76–86.

Cox, D. L., & Radolf, J. D. (2006). Metabolism of the Treponema. In J. D. Radolf & S. A. Lukehart (Eds.), *Pathogenic Treponema Molecular and Cellular Biology* (pp. 61–100). Caister Academic Press.

Cramer, G., Lissemore, K. D., Guard, C. L., Leslie, K. E., & Kelton, D. F. (2009). Herd-level risk factors for seven different foot lesions in Ontario Holstein cattle housed in tie stalls or free stalls. *Journal of Dairy Science*, *92*(4), 1404–1411.

Cronodon. (2017). *Spirochaetes*. http://cronodon.com/BioTech/Bacteria_motility3.html

Cullen, P. A., Haake, D. A., & Adler, B. (2004). Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiology Reviews*, *28*(3), 291–318.

Dang, W., Hu, Y. hua, & Sun, L. (2011). HtpG is involved in the pathogenesis of Edwardsiella tarda. *Veterinary Microbiology*, *152*(3–4), 394–400.

Darling, A. C. E., Mau, B., Blattner, F. R., & Perna, N. T. (2004). Mauve: Multiple Alignment of Conserved Genomic Sequence with Rearrangements. *Genome Research*, *14*(7), 1394.

Davis, P. A., & Yokoyama, W. (2011). Cinnamon intake lowers fasting blood glucose: Meta-analysis. *Journal of Medicinal Food*, *14*(9), 884–889.

de Jesús Argáez-Rodríguez, F., Hird, D. W., Hernández De Anda, J., Read, D. H., & Rodríguez-Lainz, A. (1997). Papillomatous digital dermatitis on a commercial dairy farm in Mexicali, Mexico: Incidence and effect on reproduction and milk production. *Preventive Veterinary Medicine*, *32*(3–4), 275–286.

de la Fuente-Nunez, C., Torres, M. D., Mojica, F. J., & Lu, T. K. (2017). Next-generation precision antimicrobials: towards personalized treatment of infectious diseases. *Current Opinion in Microbiology*, *37*, 95–102.

Demirkan, I., Carter, S. D., Hart, C. A., & Woodward, M. J. (1999). Isolation and cultivation of a spirochaete from bovine digital dermatitis. *Veterinary Record*, *145*(17), 497–498.

Demirkan, I., Carter, S. D., Murray, R. D., Blowey, R. W., & Woodward, M. J. (1998). The frequent detection of a treponeme in bovine digital dermatitis by immunocytochemistry and polymerase chain reaction. *Veterinary Microbiology*, *60*, 285–292.

Demirkan, I., Walker, R. L., Murray, R. D., Blowey, R. W., & Carter, S. D. (1999). Serological evidence of spirochaetal infections associated with digital dermatitis in dairy cattle. *Public Health*, *157*, 69–77.

Dickins, A., Clark, C. C. A., Kaler, J., Ferguson, E., O'Kane, H., & Green, L. E. (2016). Factors associated with the presence and prevalence of contagious ovine digital dermatitis: A 2013 study of 1136 random English sheep flocks. *Preventive Veterinary Medicine*, *130*, 86–93.

Doig, P. A. (1981). Bovine Genital Mycoplasmosis. *The Canadian Veterinary Journal*, *22*(11), 339–343.

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, *15*(2), 167–193.

Döpfer, D., Anklam, K., Mikheil, D., & Ladell, P. (2012a). Growth curves and morphology of three Treponema subtypes isolated from digital dermatitis in cattle. *Veterinary Journal*, *193*(3), 685–693.

Döpfer, D., Anklam, K., Mikheil, D., & Ladell, P. (2012b). Growth curves and morphology of three Treponema subtypes isolated from digital dermatitis in cattle. *The Veterinary Journal*, *193*(3), 685–693.

Döpfer, D., Holzhauer, M., & Boven, M. van. (2012). The dynamics of digital dermatitis in populations of dairy cattle: Model-based estimates of transition rates and implications for control. *Veterinary Journal*, *193*(3), 648–653.

Döpfer, D., Koopmans, A., Meijer, F. A., Szakall, I., Schukken, Y. H., Klee, W., Bosma, R. B., Cornelisse, J. L., van Asten, A. J., & ter Huurne, A. A. (1997). Histological and bacteriological evaluation of digital dermatitis in cattle, with special reference to spirochaetes and Campylobacter faecalis. *The Veterinary Record*, *140*(24), 620–623.

Duangurai, T., Reamtong, O., Rungruengkitkun, A., Srinon, V., Boonyuen, U., Limmathurotsakul, D., Chantratita, N., & Pumirat, P. (2020). In vitro passage alters virulence, immune activation and proteomic profiles of Burkholderia pseudomallei. *Scientific Reports 2020 10:1*, *10*(1), 1–16.

Dubbs, J. M., & Mongkolsuk, S. (2007). Peroxiredoxins in bacterial antioxidant defense. *Sub-Cellular Biochemistry*, *44*, 143–193.

Dubreuil, L., Houcke, I., Mouton, Y., & Rossignol, J.-F. O. (1996). In Vitro Evaluation of Activities of Nitazoxanide and Tizoxanide against Anaerobes and Aerobic Organisms. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY*, *40*(10), 2266–2270.

Duncan, J. S., Angell, J. W., Carter, S. D., Evans, N. J., Sullivan, L. E., & Grove-White, D. H. (2014). Contagious ovine digital dermatitis: An emerging disease. *The Veterinary Journal*, *201*(3), 265–268.

Edwards, A. M., Dymock, D., & Jenkinson, H. F. (2003a). From tooth to hoof: Treponemes in tissue-destructive diseases. *Journal of Applied Microbiology*, *94*(5), 767–780.

Edwards, A. M., Dymock, D., & Jenkinson, H. F. (2003b). From tooth to hoof: Treponemes in tissue-destructive diseases. *Journal of Applied Microbiology*, *94*(5), 767–780.

Edwards, A. M., Dymock, D., & Jenkinson, H. F. (2003c). From tooth to hoof: treponemes in tissue-destructive diseases. *Journal of Applied Microbiology*, *94*(5), 767–780.

Edwards, A. M., Dymock, D., Woodward, M. J., & Jenkinson, H. F. (2003). Genetic relatedness and phenotypic characteristics of Treponema associated with human periodontal tissues and ruminant foot disease. In *Microbiology* (Vol. 149, Issue 5, pp. 1083–1093).

Egert, M., & Simmering, R. (2016). *The Microbiota of the Human Skin* (pp. 61–81). Springer, Cham.

Eisenberg, S. W. F., Nielen, M., Santema, W., Houwers, D. J., Heederik, D., & Koets, A. P. (2010). Detection of spatial and temporal spread of Mycobacterium avium subsp. paratuberculosis in the environment of a cattle farm through bio-aerosols. *Veterinary Microbiology*, *143*(2–4), 284–292.

Eitinger, T., & Mandrand-Berthelot, M. A. (2000). Nickel transport systems in microorganisms. *Archives of Microbiology*, *173*(1), 1–9. https://doi.org/10.1007/S002030050001

Eriksson, M., Nielsen, P. E., & Good, L. (2002). Cell Permeabilization and Uptake of Antisense Peptide-Peptide Nucleic Acid (PNA) into Escherichia coli *. *Journal of Biological Chemistry*, *277*(9), 7144–7147.

Ettema, J. F., & Santos, J. E. P. (2004). Impact of Age at Calving on Lactation, Reproduction, Health, and Income in First-Parity Holsteins on Commercial Farms. *Journal of Dairy Science*, *87*(8), 2730–2742.

Evans, N. J., Blowey, R. W., Timofte, D., Isherwood, D. R., Brown, J. M., Murray, R., Paton, R. J., & Carter, S. D. (2011). Association between bovine digital dermatitis treponemes and a range of 'non-healing' bovine hoof disorders. *Veterinary Record*, *168*(8), 214–214.

Evans, N. J., Brown, J. M., Demirkan, I., Birtles, R., Hart, C. A., & Carter, S. D. (2009a). In vitro susceptibility of bovine digital dermatitis associated spirochaetes to antimicrobial agents. *Veterinary Microbiology*, *136*(1–2), 115–120.

Evans, N. J., Brown, J. M., Demirkan, I., Birtles, R., Hart, C. A., & Carter, S. D. (2009b). In vitro susceptibility of bovine digital dermatitis associated spirochaetes to antimicrobial agents. *Veterinary Microbiology*, *136*(1–2), 115–120.

Evans, N. J., Brown, J. M., Demirkan, I., Birtles, R., Hart, C. A., & Carter, S. D. (2009c). In vitro susceptibility of bovine digital dermatitis associated spirochaetes to antimicrobial agents. *Veterinary Microbiology*, *136*(1–2), 115–120.

Evans, N. J., Brown, J. M., Demirkan, I., Birtles, R., Hart, C. A., & Carter, S. D. (2009d). In vitro susceptibility of bovine digital dermatitis associated spirochaetes to antimicrobial agents. *Veterinary Microbiology*, *136*(1–2), 115–120.

Evans, N. J., Brown, J. M., Demirkan, I., Murray, R. D., Birtles, R. J., Hart, C. A., & Carter, S. D. (2009). Treponema pedis sp. nov., a spirochaete isolated from bovine digital dermatitis lesions. *International Journal of Systematic and Evolutionary Microbiology*, *59*(Pt 5), 987–991.

Evans, N. J., Brown, J. M., Demirkan, I., Murray, R. D., Vink, W. D., Blowey, R. W., Hart, C. A., & Carter, S. D. (2008). Three unique groups of spirochetes isolated from digital dermatitis lesions in UK cattle. *Veterinary Microbiology*, *130*(1–2), 141–150.

Evans, N. J., Brown, J. M., Demirkan, I., Singh, P., Getty, B., Timofte, D., Vink, W. D., Murray, R. D., Blowey, R. W., Birtles, R. J., Hart, C. A., & Carter, S. D. (2009). Association of unique, isolated treponemes with bovine digital dermatitis lesions. *Journal of Clinical Microbiology*, *47*(3), 689–696.

Evans, N. J., Brown, J. M., Hartley, C., Smith, R. F., & Carter, S. D. (2012a). Antimicrobial susceptibility testing of bovine digital dermatitis treponemes identifies macrolides for in vivo efficacy testing. *Veterinary Microbiology*, *160*(3–4), 496–500. https://doi.org/10.1016/j.vetmic.2012.06.001

Evans, N. J., Brown, J. M., Hartley, C., Smith, R. F., & Carter, S. D. (2012b). Antimicrobial susceptibility testing of bovine digital dermatitis treponemes identifies macrolides for in vivo efficacy testing. *Veterinary Microbiology*, *160*(3–4), 496–500.

Evans, N. J., Brown, J. M., Scholey, R., Murray, R. D., Birtles, R. J., Hart, C. A., & Carter, S. D. (2014a). Differential inflammatory responses of bovine foot skin fibroblasts and keratinocytes to digital dermatitis treponemes. *Veterinary Immunology and Immunopathology*, *161*(1–2), 12–20.

Evans, N. J., Brown, J. M., Scholey, R., Murray, R. D., Birtles, R. J., Hart, C. A., & Carter, S. D. (2014b). Differential inflammatory responses of bovine foot skin fibroblasts and keratinocytes to digital dermatitis treponemes. *Veterinary Immunology and Immunopathology*, *161*(1–2), 12–20.

Evans, N. J., Murray, R. D., & Carter, S. D. (2016). Bovine digital dermatitis: Current concepts from laboratory to farm. *Veterinary Journal*, *211*, 3–13.

Evans, N. J., Timofte, D., Isherwood, D. R., Brown, J. M., Williams, J. M., Sherlock, K., Lehane, M. J., Murray, R. D., Birtles, R. J., Hart, C. A., & Carter, S. D. (2012). Host and environmental reservoirs of infection for bovine digital dermatitis treponemes. *Veterinary Microbiology*, *156*(1–2), 102–109.

Eysenbach, G. (2004). Improving the quality of web surveys: The Checklist for Reporting Results of Internet E-Surveys (CHERRIES). In *Journal of Medical Internet Research* (Vol. 6, Issue 3, p. e34). Journal of Medical Internet Research.

Falsen, E., Collins, M. D., Welinder-Olsson, C., Song, Y., Finegold, S. M., & Lawson, P. A. (2005). Fastidiosipila sanguinis gen. nov., sp. nov., a new Gram-positive, coccus-shaped organism from human blood. *International Journal of Systematic and Evolutionary Microbiology*, *55*(2), 853–858.

Fang, F. C. (2004). Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nature Reviews Microbiology,* 2(10), 820-32.

Faust, K., & Raes, J. (2012). Microbial interactions: From networks to models. In *Nature Reviews Microbiology* (Vol. 10, Issue 8, pp. 538–550).

Faust, K., Sathirapongsasuti, J. F., Izard, J., Segata, N., Gevers, D., Raes, J., & Huttenhower, C. (2012). Microbial co-occurrence relationships in the Human Microbiome. *PLoS Computational Biology*, *8*(7).

Ferrer, M., Méndez-García, C., Rojo, D., Barbas, C., & Moya, A. (2017). Antibiotic use and microbiome function. *Biochemical Pharmacology*, *134*, 114–126.

Filipović, U., Dahmane, R. G., Ghannouchi, S., Zore, A., & Bohinc, K. (2020). Bacterial adhesion on orthopedic implants. *Advances in Colloid and Interface Science*, *283*, 102228.

Flower, A. M. (2007). The SecY translocation complex: convergence of genetics and structure. *Trends in Microbiology*, *15*(5), 203–210.

Frankena, K., Somers, J. G. C. J., Schouten, W. G. P., van Stek, J. v., Metz, J. H. M., Stassen, E. N., & Graat, E. A. M. (2009). The effect of digital lesions and floor type on locomotion score in Dutch dairy cows. *Preventive Veterinary Medicine*, *88*(2), 150–157.

Franzosa, E. A., McIver, L. J., Rahnavard, G., Thompson, L. R., Schirmer, M., Weingart, G., Schwarzberg Lipson, K., Knight, R., Caporaso, J. G., Segata, N., & Huttenhower, C. (2018). Species-level functional profiling of metagenomes and metatranscriptomes. *Nature Methods*, *15*, 962–968.

Fraser, T., & Brown, P. D. (2017). Temperature and oxidative stress as triggers for virulence gene expression in pathogenic Leptospira spp. *Frontiers in Microbiology*, *8*(MAY), 783.

Galperin, M. Y., & Chou, S. H. (2020). Structural conservation and diversity of PILZ-related domains. *Journal of Bacteriology*, *202*(4).

Gambino, M., & Cappitelli, F. (2016). Mini-review: Biofilm responses to oxidative stress. *Https://Doi-Org.Liverpool.Idm.Oclc.Org/10.1080/08927014.2015.1134515*, *32*(2), 167–178.

Gardner, S. E., Hillis, S. L., Heilmann, K., Segre, J. A., & Grice, E. A. (2013). The neuropathic diabetic foot ulcer microbiome is associated with clinical factors. *Diabetes*, *62*(3), 923–930. https://doi.org/10.2337/db12-0771

Garforth, C., Rehman, T., McKemey, K., Tranter, R., Cooke, R., Yates, C., Park, J., & Dorward, P. (2004). Improving the Design of Knowledge Transfer Strategies by Understanding Farmer Attitudes and Behaviour. *Journal of Farm Management*, *12*(1), 17–32.

Genest, O., Wickner, S., & Doyle, S. M. (2019). Hsp90 and Hsp70 chaperones: Collaborators in protein remodeling. *Journal of Biological Chemistry*, *294*(6), 2109–2120.

Gherardini, F. C., Boylan, J. A., & Brett, P. J. (2006). Metal Utilization and Oxidative Stress. In J. D. Radolf & S. A. Lukehart (Eds.), *Pathogenic Treponema Molecular and Cellular Biology* (pp. 101–126). Caister Academic Press.

Ghosal, A., Vitali, A., Stach, J. E. M., & Nielsen, P. E. (2013). Role of SbmA in the uptake of peptide nucleic acid (PNA)-peptide conjugates in E. coli. *ACS Chemical Biology*, *8*(2), 360–367.

Giacani, L., Bernstein, L. R., Haynes, A. M., Godornes, B. C., Ciccarese, G., Drago, F., Parodi, A., Valdevit, S., Anselmi, L., Tomasini, C. F., & Baca, A. M. (2019). Topical treatment with gallium maltolate reduces Treponema pallidum subsp. pertenue burden in primary experimental lesions in a rabbit model of yaws. *PLoS Neglected Tropical Diseases*, *13*(1).

Gillespie, A., Carter, S. D., Blowey, R. W., & Evans, N. (2019). Survival of bovine digital dermatitis treponemes on hoof knife blades and the effects of various disinfectants. *Veterinary Record*, *186*, 67.

Gillespie, A., & Evans, N. (2019). Infection reservoirs and transmission of digital dermatitis in the dairy herd. *Livestock*, *25*(3).

Gillespie, A. v., Carter, S. D., Blowey, R. W., Staton, G. J., & Evans, N. J. (2020). Removal of bovine digital dermatitis-associated treponemes from hoof knives after foot-trimming: a disinfection field study. *BMC Veterinary Research*, *16*(1), 330.

Goh, S., Boberek, J. M., Nakashima, N., Stach, J., & Good, L. (2009). Concurrent growth rate and transcript analyses reveal essential gene stringency in Escherichia coli. *PloS One*, *4*(6), e6061.

Goltermann, L., & Nielsen, P. E. (2020). PNA Antisense Targeting in Bacteria: Determination of Antibacterial Activity (MIC) of PNA-Peptide Conjugates. *Methods in Molecular Biology*, *2105*, 231–239.

Goltermann, L., Yavari, N., Zhang, M., Ghosal, A., & Nielsen, P. E. (2019). PNA length restriction of antibacterial activity of peptide-PNA conjugates in Escherichia coli through effects of the inner membrane. *Frontiers in Microbiology*, *10*(MAY), 1032.

Gomez, A., Anklam, K. S., Cook, N. B., Rieman, J., Dunbar, K. A., Cooley, K. E., Socha, M. T., & Döpfer, D. (2014). Immune response against Treponema spp. and ELISA detection of digital dermatitis. *Journal of Dairy Science*, *97*(8), 4864–4875.

Gomez, A., Cook, N. B., Bernardoni, N. D., Rieman, J., Dusick, A. F., Hartshorn, R., Socha, M. T., Read, D. H., & Döpfer, D. (2012). An experimental infection model to induce digital dermatitis infection in cattle. *Journal of Dairy Science*, *95*(4), 1821–1830.

Good, L., Kumar Awasthi, S., Dryselius, R., Larsson, O., & Nielsen, P. E. (2001). *Bactericidal antisense effects of peptide-PNA conjugates*. http://biotech.nature.com

Good, L., Sandberg, R., Larsson, O., Nielsen, P. E., & Wahlestedt, C. (2000). Antisense PNA effects in Escherichia coli are limited by the outer-membrane LPS layer. *Microbiology*, *146*(10), 2665–2670.

Gradin, J. L., Schmitz, M. S., & Schmitz, J. A. (1983). Susceptibility of Bacteroides nodosus to various antimicrobial agents. *Journal of the American Veterinary Medical Association*, *183*(4), 434–437.

Grice, E. A., Kong, H. H., Conlan, S., Deming, C. B., Davis, J., Young, A. C., Bouffard, G. G., Blakesley, R. W., Murray, P. R., Green, E. D., Turner, M. L., & Segre, J. A. (2009). Topographical and temporal diversity of the human skin microbiome. *Science*, *324*(5931), 1190–1192.

Grudniak, A. M., Markowska, K., & Wolska, K. I. (2015). Interactions of Escherichia coli molecular chaperone HtpG with DnaA replication initiator DNA. *Cell Stress and Chaperones*, *20*(6), 951–957.

Guillén-Navarro, K., Araíza, G., García-De Los Santos, A., Mora, Y., & Dunn, M. F. (2005). The Rhizobium etli bioMNY operon is involved in biotin transport. *FEMS Microbiology Letters*, *250*(2), 209–219.

Gunn, G. J., Heffernan, C., Hall, M., McLeod, A., & Hovi, M. (2008). Measuring and comparing constraints to improved biosecurity amongst GB farmers, veterinarians and the auxiliary industries. *Preventive Veterinary Medicine*, *84*(3–4), 310–323.

Gupta, R., Sharma, S., Singh, R., Vishwakarma, R. A., Mignani, S., & Singh, P. P. (2022). Functionalized Nitroimidazole Scaffold Construction and Their Pharmaceutical Applications: A 1950–2021 Comprehensive Overview. *Pharmaceuticals 2022, Vol. 15, Page 561*, *15*(5), 561.

Hagey, J. v, Bhatnagar, S., Heguy, J. M., Karle, B. M., Price, P. L., Meyer, D., & Maga, E. A. (2019). Fecal Microbial Communities in a Large Representative Cohort of California Dairy Cows. *Frontiers in Microbiology*, *10*, 1093.

Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004a). Bacterial biofilms: From the natural environment to infectious diseases. In *Nature Reviews Microbiology* (Vol. 2, Issue 2, pp. 95–108).

Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004b). Bacterial biofilms: From the natural environment to infectious diseases. In *Nature Reviews Microbiology* (Vol. 2, Issue 2, pp. 95–108).

Hartshorn, R. E., Thomas, E. C., Anklam, K., Lopez-Benavides, M. G., Buchalova, M., Hemling, T. C., & Döpfer, D. (2013). Short communication: Minimum bactericidal concentration of disinfectants evaluated for bovine digital dermatitis-associated Treponema phagedenis-like spirochetes. *Journal of Dairy Science*, *96*(5), 3034–3038.

Haynes, A. M., Giacani, L., Mayans, M. V., Ubals, M., Nieto, C., Pérez-Mañá, C., Quintó, L., Romeis, E., & Mitjà, O. (2021). Efficacy of linezolid on Treponema pallidum, the syphilis agent: A preclinical study. *EBioMedicine*, *65*

Heringstad, B., Egger-Danner, C., Charfeddine, N., Pryce, J. E., Stock, K. F., Kofler, J., Sogstad, A. M., Holzhauer, M., Fiedler, A., Müller, K., Nielsen, P., Thomas, G., Gengler, N., de Jong, G., Ødegård, C., Malchiodi, F., Miglior, F., Alsaaod, M., & Cole, J. B. (2018). Invited review: Genetics and claw health: Opportunities to enhance claw health by genetic selection. *Journal of Dairy Science*, *101*(6), 4801–4821.

Hernandez, J. A., Garbarino, E. J., Shearer, J. K., Risco, C. A., & Thatcher, W. W. (2007). Evaluation of the efficacy of prophylactic hoof health examination and trimming during midlactation in reducing the incidence of lameness during late lactation in dairy cows. *Journal of the American Veterinary Medical Association*, *230*(1), 89–93.

Hesseling, J., Legione, A. R., Stevenson, M. A., McCowan, C. I., Pyman, M. F., Finochio, C., Nguyen, D., Roic, C. L., Thiris, O. L., Zhang, A. J., van Schaik, G., & Coombe, J. E. (2019). Bovine digital dermatitis in Victoria, Australia. *Australian Veterinary Journal*.

Heukelekian, H., & Heller, A. (1940). Relation between Food Concentration and Surface for Bacterial Growth. *Journal of Bacteriology*, *40*(4), 547–558.

Hijazi, S., Visaggio, D., Pirolo, M., Frangipani, E., Bernstein, L., & Visca, P. (2018). Antimicrobial activity of gallium compounds on ESKAPE pathogens. *Frontiers in Cellular and Infection Microbiology*, *8*(SEP), 316.

Hoelzle, K., Ade, J., & Hoelzle, L. E. (2020). Persistence in Livestock Mycoplasmas—a Key Role in Infection and Pathogenesis. *Current Clinical Microbiology Reports*, *7*(3), 81–89.

Holman, D. B., & Chénier, M. R. (2015). Antimicrobial use in swine production and its effect on the swine gut microbiota and antimicrobial resistance. *Canadian Journal of Microbiology* 61(11), 785-798

Holman, D. B., & Gzyl, K. E. (2019). A meta-analysis of the bovine gastrointestinal tract microbiota. *FEMS Microbiology Ecology*, *95*(6).

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., & Williams, S. T. (2000a). Gram-negative aerobic/ microaerophilic rods and cocci. In W. R. Hensyl (Ed.), *Bergey's Manual of Determinative Bacteriology* (Ninth Edition, p. 83). Lippincott, Williams and Wilkins.

Holt, J. G., Krieg, N. R., Sneath, P. H., Staley, J. T., & Williams, S. T. (2000b). The Mycoplasmas (or Mollicutes): cell wall-less bacteria. In W. R. Hensyl (Ed.), *Bergey's Manual of Determinative Bacteriology* (Ninth Edition, pp. 705–706). Lippincott Williams and Wilkins.

Holzhauer, M., Bartels, C. J., Bergsten, C., van Riet, M. M. J., Frankena, K., & Lam, T. J. G. M. (2012). The effect of an acidified, ionized copper sulphate solution on digital dermatitis in dairy cows. *Veterinary Journal (London, England : 1997)*, *193*(3), 659–663.

Holzhauer, M., Bartels, C. J., Van Barneveld, M., Vulders, C., & Lam, T. (2011). Curative effect of topical treatment of digital dermatitis with a gel containing activated copper and zinc chelate. *Vet Rec.*

Holzhauer, M., Bartels, C. J., van Barneveld, M., Vulders, C., & Lam, T. (2011). Curative effect of topical treatment of digital dermatitis with a gel containing activated copper and zinc chelate. *Vet Rec.*

Holzhauer, M., Ploegmakers-van Deventer, R., Smits, D., & Swart, W. (2017). Comparing the curative efficacy of topical treatment with thiamphenicol and oxytetracycline on digital dermatitis lesions in dairy cattle. *The Veterinary Record*, *180*(20), 500. https://doi.org/10.1136/vr.103758

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S. K., Cook, H., Mende, D. R., Letunic, I., Rattei, T., Jensen, L. J., von Mering, C., & Bork, P. (2019). eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Research*, *47*(D1), D309–D314.

Hultgren, J., & Bergsten, C. (2001). Effects of a rubber-slatted flooring system on cleanliness and foot health in tied dairy cows. *Preventive Veterinary Medicine*, *52*(1), 75–89.

Iwig, J. S., Rowe, J. L., & Chivers, P. T. (2006). Nickel homeostasis in Escherichia coli – the rcnR-rcnA efflux pathway and its linkage to NikR function. *Molecular Microbiology*, *62*(1), 252–262.

Jacobs, C., Beninger, C., Hazlewood, G. S., Orsel, K., & Barkema, H. W. (2019). Effect of footbath protocols for prevention and treatment of digital dermatitis in dairy cattle: A systematic review and network meta-analysis. *Preventive Veterinary Medicine*, *164*, 56–71.

Jacobs, C., Orsel, K., & Barkema, H. W. (2017). Prevalence of digital dermatitis in young stock in Alberta, Canada, using pen walks. *Journal of Dairy Science*, *100*(11), 9234–9244.

Jacobs, C., Orsel, K., Mason, S., & Barkema, H. W. (2018). Comparison of effects of routine topical treatments in the milking parlor on digital dermatitis lesions. *Journal of Dairy Science*, *101*(6),

Jang, Y. J., Choi, Y. J., Lee, S. H., Jun, H. K., & Choi, B. K. (2013). Autoinducer 2 of Fusobacterium nucleatum as a target molecule to inhibit biofilm formation of periodontopathogens. *Archives of Oral Biology*, *58*(1), 17–27.

Jansen, J., & Lam, T. J. G. M. (2012). The Role of Communication in Improving Udder Health. *Veterinary Clinics of North America: Food Animal Practice*, *28*(2), 363–379.

Janz, N. K., & Becker, M. H. (1984). *The Health Belief Model: A Decade Later*.

Jenney, F. E., Verhagen, M. F. J. M., Cui, X., & Adams, M. W. W. (1999). Anaerobic microbes: Oxygen detoxification without superoxide dismutase. *Science*, *286*(5438), 306–309.

Justice-Allen, A., Trujillo, J., Corbett, R., Harding, R., Goodell, G., & Wilson, D. (2010). Survival and replication of Mycoplasma species in recycled bedding sand and association with mastitis on dairy farms in Utah. *Journal of Dairy Science*, *93*(1), 192–202.

Kalia, V. C., Patel, S. K. S., Kang, Y. C., & Lee, J. K. (2019). Quorum sensing inhibitors as antipathogens: biotechnological applications. *Biotechnology Advances*, *37*(1), 68–90.

Kanehisa, M., Sato, Y., & Kawashima, M. (2022). KEGG mapping tools for uncovering hidden features in biological data. *Protein Science*, *31*(1), 47–53.

Kanehisa, M., Sato, Y., & Morishima, K. (2016). BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *Journal of Molecular Biology*, *428*(4), 726–731.

Kapatral, V., Olson, J. W., Pepe, J. C., Miller, V. L., & Minnich, S. A. (1996). Temperature-dependent regulation of Yersinia enterocolitica Class III flagellar genes. *Molecular Microbiology*, *19*(5), 1061–1071.

Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of Anaesthesiology, Clinical Pharmacology*, *33*(3), 300.

Kędzierska, B., Lian, L. Y., & Hayes, F. (2007). Toxin–antitoxin regulation: bimodal interaction of YefM–YoeB with paired DNA palindromes exerts transcriptional autorepression. *Nucleic Acids Research*, *35*(1), 325–339.

Khan, A., & Mathelier, A. (2017). Intervene: A tool for intersection and visualization of multiple gene or genomic region sets. *BMC Bioinformatics*, *18*(1).

Kim, Y. E., Hipp, M. S., Bracher, A., Hayer-Hartl, M., & Ulrich Hartl, F. (2013). Molecular Chaperone Functions in Protein Folding and Proteostasis. *Annual Review of Biochemistry, 82*, 323–355.

Klitgaard, K., Boye, M., Capion, N., & Jensen, T. K. (2008). Evidence of multiple Treponema phylotypes involved in bovine digital dermatitis as shown by 16S rRNA gene analysis and fluorescence in situ hybridization. *Journal of Clinical Microbiology*, *46*(9), 3012–3020.

Klitgaard, K., Strube, M. L., Isbrand, A., Jensen, T. K., & Nielsen, M. W. (2017). Microbiota Analysis of an Environmental Slurry and Its Potential Role as a Reservoir of Bovine Digital Dermatitis Pathogens. *Applied and Environmental Microbiology*, *83*(11).

Knight, R., Vrbanac, A., Taylor, B. C., Aksenov, A., Callewaert, C., Debelius, J., Gonzalez, A., Kosciolek, T., McCall, L. I., McDonald, D., Melnik, A. v., Morton, J. T., Navas, J., Quinn, R. A., Sanders, J. G., Swafford, A. D., Thompson, L. R., Tripathi, A., Xu, Z. Z., … Dorrestein, P. C. (2018). Best practices for analysing microbiomes. In *Nature Reviews Microbiology* (Vol. 16, Issue 7, pp. 410–422). Nature Publishing Group.

Kolenbrander, P. E., Palmer, R. J., Periasamy, S., & Jakubovics, N. S. (2010a). Oral multispecies biofilm development and the key role of cell-cell distance. In *Nature Reviews Microbiology* (Vol. 8, Issue 7, pp. 471–480).

Kolenbrander, P. E., Palmer, R. J., Periasamy, S., & Jakubovics, N. S. (2010b). Oral multispecies biofilm development and the key role of cell-cell distance. In *Nature Reviews Microbiology* (Vol. 8, Issue 7, pp. 471–480).

Kolodziej, H., Kayser, O., Latté, K. P., Berlin-Brandenburg, L., & Ferreira, D. (1999). *Article in Planta Medica*, 65(5) 444-446

Krewing, M., Stepanek, J. J., Cremers, C., Lackmann, J. W., Schubert, B., Müller, A., Awakowicz, P., Leichert, L. I. O., Jakob, U., & Bandow, J. E. (2019). The molecular chaperone Hsp33 is activated by atmospheric-pressure plasma protecting proteins from aggregation. *Journal of the Royal Society Interface*, *16*(155).

Krull, A. C., Cooper, V. L., Coatney, J. W., Shearer, J. K., Gorden, P. J., & Plummer, P. J. (2016a). A Highly Effective Protocol for the Rapid and Consistent Induction of Digital Dermatitis in Holstein Calves. *PloS One*, *11*(4), e0154481.

Krull, A. C., Cooper, V. L., Coatney, J. W., Shearer, J. K., Gorden, P. J., & Plummer, P. J. (2016b). A Highly Effective Protocol for the Rapid and Consistent Induction of Digital Dermatitis in Holstein Calves. *PloS One*, *11*(4), e0154481.

Krull, A. C., Shearer, J. K., Gorden, P. J., Cooper, V. L., Phillips, G. J., & Plummer, P. J. (2014). Deep Sequencing Analysis Reveals the Temporal Microbiota Changes Associated with the Development of Bovine Digital Dermatitis. *Infection and Immunity*, *82*(8), 3359–3373.

Krull, A. C., Shearer, J. K., Gorden, P. J., Cooper, V. L., Phillips, G. J., & Plummera, P. J. (2014). Deep sequencing analysis reveals temporal microbiota changes associated with development of bovine digital dermatitis. *Infection and Immunity*, *82*(8), 3359–3373. https://doi.org/10.1128/IAI.02077-14

Kuhnert, P., Brodard, I., Alsaaod, M., Steiner, A., Stoffel, M. H., & Jores, J. (2020). Treponema phagedenis (Ex noguchi 1912) brumpt 1922 sp. nov., nom. rev., isolated from bovine digital dermatitis. *International Journal of Systematic and Evolutionary Microbiology*, *70*(3), 2115–2123.

Kulow, M., Zibaee, F., Allard, M., & Döpfer, D. (2015). Short communication: Determination of the ability of Thymox to kill or inhibit various species of microorganisms associated with infectious causes of bovine lameness in vitro. *Journal of Dairy Science*, *98*(11), 7899–7905.

Kuramitsu, H. K., Chen, W., & Ikegami, A. (2005). Biofilm Formation by the Periodontopathic Bacteria Treponema denticola and Porphyromonas gingivalis. *Journal of Periodontology*, *76*(11-s), 2047–2051.

Kurtz, Z. D., Müller, C. L., Miraldi, E. R., Littman, D. R., Blaser, M. J., & Bonneau, R. A. (2015). *Sparse and Compositionally Robust Inference of Microbial Ecological Networks*.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359.

Latasa, C., Roux, A., Toledo-Arana, A., Ghigo, J. M., Gamazo, C., Penadés, J. R., & Lasa, I. (2005). BapA, a large secreted protein required for biofilm formation and host colonization of Salmonella enterica serovar Enteritidis. *Molecular Microbiology*, *58*(5), 1322–1339.

Laven, R. A., & Logue, D. N. (2006). Treatment strategies for digital dermatitis for the UK. *Veterinary Journal, 171*(1), 79–88.

Layeghifard, M., Hwang, D. M., & Guttman, D. S. (2017). Disentangling Interactions in the Microbiome: A Network Perspective. In *Trends in Microbiology*. 25(3), 217-228.

Layer, G., Aparna Gaddam, S., Ayala-Castro, C. N., Choudens, S. O. de, Lascoux, D., Fontecave, M., & Outten, F. W. (2007). SufE Transfers Sulfur from SufS to SufB for Iron-Sulfur Cluster Assembly. *Journal of Biological Chemistry*, *282*(18), 13342–13350.

Leach, K. A., Tisdall, D. A., Bell, N. J., Main, D. C. J., & Green, L. E. (2012). The effects of early treatment for hindlimb lameness in dairy cows on four commercial UK farms. *The Veterinary Journal*, *193*(3), 626–632.

Lessard, I. A. D., & Walsh, C. T. (1999). VanX, a bacterial D-alanyl-D-alanine dipeptidase: Resistance, immunity, or survival function? *Proceedings of the National Academy of Sciences of the United States of America*, *96*(20), 11028–11032.

Lewis, J. S., Weinstein, M. P., Bobenchik, A. M., Campeau, S., Cullen, S. K., Gold, H., Humphries, R. M., Kirn, T. J., Limbago, B., Mathers, A. J., Mazzuli, T., Richter, S. S., Satlin, M., Schuetz, A. N., Sharp, S., & Simner, P. J. (2022). *CLSI M100-ED32:2022 Performance Standards for Antimicrobial Susceptibility Testing*. CLSI.

Lex, A., Gehlenborg, N., Strobelt, H., Vuillemot, R., & Pfister, H. (2014). UpSet: Visualization of intersecting sets. *IEEE Transactions on Visualization and Computer Graphics*, *20*(12), 1983–1992.

Li, H., Li, R., Chen, H., Gao, J., Wang, Y., Zhang, Y., & Qi, Z. (2020). Effect of different seasons (spring vs summer) on the microbiota diversity in the feces of dairy cows. *International Journal of Biometeorology*, *64*(3), 345–354. https://doi.org/10.1007/s00484-019-01812-z

Li, X., Pal, U., Ramamoorthi, N., Liu, X., Desrosiers, D. C., Eggers, C. H., Anderson, J. F., Radolf, J. D., & Fikrig, E. (2007). The Lyme disease agent Borrelia burgdorferi requires BB0690, a Dps homologue, to persist within ticks. *Molecular Microbiology*, *63*(3), 694–710.

Lim, K., Rong, R., Chua, Y., Saravanan, R., Basu, A., Mishra, B., Tambyah, P. A., Ho, B., Su, S., & Leong, J. (2013). Immobilization Studies of an Engineered Arginine−Tryptophan-Rich Peptide on a Silicone Surface with Antimicrobial and Antibiofilm Activity. *ACS Applied Materials and Interfaces*, 5(13), 6412-6422.

Liu, H., Roeder, K., & Wasserman, L. (2010). Stability Approach to Regularization Selection (StARS) for High Dimensional Graphical Models. *Advances in Neural Information Processing Systems,* 24(2).

Liu, J., Zheng, Y., Lin, H., Wang, X., Li, M., Liu, Y., Yu, M., Zhao, M., Pedentchouk, N., Lea-Smith, D. J., Todd, J. D., Magill, C. R., Zhang, W. J., Zhou, S., Song, D., Zhong, H., Xin, Y., Yu, M., Tian, J., & Zhang, X. H. (2019). Proliferation of hydrocarbon-degrading microbes at the bottom of the Mariana Trench. *Microbiome*, *7*(1), 47.

Lockhart, J. S., Buret, A. G., Ceri, H., Storey, D. G., Anderson, S. J., & Morck, D. W. (2017). Mixed species biofilms of Fusobacterium necrophorum and Porphyromonas levii impair the oxidative response of bovine neutrophils in vitro. *Anaerobe*, *47*, 157–164.

Logue, D. N., Offer, J. E., Laven, R. A., & Ellis, W. A. (2005). Digital dermatitis – The aetiological soup. *The Veterinary Journal*, *170*(1), 12–13.

Losinger, W. C. (2006). Economic impacts of reduced milk production associated with papillomatous digital dermatitis in dairy cows in the USA. *Journal of Dairy Research*, *73*(2), 244–256.

Love, D., Fajt, V. R., Hairgrove, T., Jones, M., & Thompson, J. A. (2017). Metronidazole for the treatment of Tritrichomonas foetus in bulls. *BMC Veterinary Research*, *13*(1), 1–6. https://doi.org/10.1186/S12917-017-0999-2/TABLES/1

Lu, C., Xie, G., Liu, M., Zhu, H., & Lei, B. (2012). Direct Heme Transfer Reactions in the Group A Streptococcus Heme Acquisition Pathway. *PLOS ONE*, *7*(5), e37556.

Lubke, L. L., & Garon, C. F. (1997). The Antimicrobial Agent Melittin Exhibits Powerful In Vitro Inhibitory Effects on the Lyme Disease Spirochete. *Clinical Infectious Diseases*, *25*(Supplement_1), S48–S51.

Lukic, J., Chen, V., Strahinic, I., Begovic, J., Lev-Tov, H., Davis, S. C., Tomic-Canic, M., & Pastar, I. (2017). Probiotics or pro-healers: the role of beneficial bacteria in tissue repair. *Wound Repair and Regeneration*, *25*(6), 912–922.

Lux, R., & Shi, W. (2006). Chemotaxis Signalling Systems in Spirochetes: Their Role in Directed Movement and Pathogenesis. In *Pathogenic Treponema, Molecular and Cellular Biology* (pp. 147–170). Caister Academic Press.

Lyon, W. R., Gibson, C. M., & Caparon, M. G. (1998). A role for Trigger Factor and an Rgg-like regulator in the transcription, secretion and processing of the cysteine proteinase of Streptococcus pyogenes. *The EMBO Journal*, *17*(21), 6263–6275.

Lyons, T., Gahan, C. G. M., & O'Sullivan, T. P. (2020). Structure-activity relationships of furanones, dihydropyrrolones and thiophenones as potential quorum sensing inhibitors. *Future Medicinal Chemistry*, *12*(21), 1925–1943.

Madsen, J. S., Burmølle, M., Hansen, L. H., & Sørensen, S. J. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology & Medical Microbiology*, *65*(2), 183–195.

Maele, L. vande, Heyndrickx, M., Maes, D., de Pauw, N., Mahu, M., Verlinden, M., Haesebrouck, F., Martel, A., Pasmans, F., & Boyen, F. (2016). In vitro susceptibility of Brachyspira hyodysenteriae to organic acids and essential oil components. *J. Vet. Med. Sci*, *78*(2), 325–328.

Magana, M., Sereti, C., Ioannidis, A., Mitchell, C. A., Ball, A. R., Magiorkinis, E., Chatzipanagiotou, S., Hamblin, M. R., Hadjifrangiskou, M., & Tegos, G. P. (2018). Options and limitations in clinical investigation of bacterial biofilms. In *Clinical Microbiology Reviews* (Vol. 31, Issue 3). American Society for Microbiology.

Mancl, K. A., Kirsner, R. S., & Ajdic, D. (2013). Wound biofilms: Lessons learned from oral biofilms. *Wound Repair and Regeneration*, *21*(3), 352–362.

Manske, T., Hultgren, J., & Bergsten, C. (2002a). The effect of claw trimming on the hoof health of Swedish dairy cattle. *Preventive Veterinary Medicine*, *54*(2), 113–129.

Manske, T., Hultgren, J., & Bergsten, C. (2002b). Prevalence and interrelationships of hoof lesions and lameness in Swedish dairy cows. *Preventive Veterinary Medicine*, *54*(3), 247–263.

Marcatili, P., Nielsen, M. W., Sicheritz-Pontén, T., Jensen, T. K., Schafer-Nielsen, C., Boye, M., Nielsen, M., & Klitgaard, K. (2016). A novel approach to probe host-pathogen interactions of bovine digital dermatitis, a model of a complex polymicrobial infection. *BMC Genomics*, *17*(1).

Marcel Martin. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.17:*, 5–7.

Maruyama, Y., Oiki, S., Takase, R., Mikami, B., Murata, K., & Hashimoto, W. (2015). Metabolic fate of unsaturated glucuronic/iduronic acids from glycosaminoglycans: Molecular identification and structure determination of streptococcal isomerase and dehydrogenase. *Journal of Biological Chemistry*, *290*(10), 6281–6292.

Mason, W. J., & Skaar, E. P. (2009). Assessing the Contribution of Heme-Iron Acquisition to Staphylococcus aureus Pneumonia Using Computed Tomography. *PLOS ONE*, *4*(8), e6668.

Mazzarrino, G., Paparella, A., Chaves-López, C., Faberi, A., Sergi, M., Sigismondi, C., Compagnone, D., & Serio, A. (2015). Salmonella enterica and Listeria monocytogenes inactivation dynamics after treatment with selected essential oils. *Food Control*, *50*, 794–803.

McBride, M. B., & Spiers, G. (2007). Trace element content of selected fertilizers and dairy manures as determined by ICP–MS. *Communications in Soil Science and Plant Analysis,* 32(1-2), 139-156.

McBride, M. J., Braun, T. F., & Brust, J. L. (2003). Flavobacterium johnsoniae GldH Is a Lipoprotein that Is Required for Gliding Motility and Chitin Utilization. *Journal of Bacteriology*, *185*(22), 6648–6657.

McDonnell, G. E. (2017). *Antisepsis, Disinfection, and Sterilization: Types, Action, and Resistance, Second Edition*. American Society of Microbiology.

McHardy, I., Keegan, C., Sim, J.-H., Shi, W., Lux, R., Lee Ho, P., & Butantan, I. (2010). *Transcriptional Profiles of Treponema denticola in Response to Environmental Conditions*.

Metzner, M., Schutz, W., Pijl, R., & Klee, W. (2001). Investigations on the efficacy of herd-specific vaccines against digital dermatitis of cattle. *Tierarzliche Praxis Ausgabe G, Grosstierenutztiere*, *29*(6), 345–350.

Miller, N. (2021). Cattle and sheep health and welfare priorities- a ‘grassroots’ survey across the four nations of the UK. *Ruminant Health and Welfare Group.*

Mitchell, H. L., Dashper, S. G., Catmull, D. v, Paolini, R. A., Cleal, S. M., Slakeski, N., Tan, K. H., & Reynolds, E. C. (2010). Treponema denticola biofilm-induced expression of a bacteriophage, toxin-antitoxin systems and transposases. *Microbiology (Reading, England)*, *156*(Pt 3), 774–788.

Mitjà, O., Godornes, C., Houinei, W., Kapa, A., Paru, R., Abel, H., González-Beiras, C., Bieb, S. v., Wangi, J., Barry, A. E., Sanz, S., Bassat, Q., & Lukehart, S. A. (2018). Re-emergence of yaws after single mass azithromycin treatment followed by targeted treatment: a longitudinal study. *The Lancet*, *391*(10130), 1599–1607.

Mitjà, O., Hays, R., Ipai, A., Penias, M., Paru, R., Fagaho, D., De Lazzari, E., & Bassat, Q. (2012). Single-dose azithromycin versus benzathine benzylpenicillin for treatment of yaws in children in Papua New Guinea: An open-label, non-inferiority, randomised trial. *The Lancet*, *379*(9813), 342–347.

Monde, M., Chessher, A., Goh, S., Good, L., & Stach, J. E. Me. (2014). Species-Selective Killing of Bacteria by Antimicrobial Peptide-PNAs. *PloS One*, *9*(2), e89082.

Moon, K. H., Zhao, X., Manne, A., Wang, J., Yu, Z., Liu, J., & Motaleb, M. A. (2016). Spirochetes flagellar collar protein FlbB has astounding effects in orientation of periplasmic flagella, bacterial shape, motility, and assembly of motors in Borrelia burgdorferi. *Molecular Microbiology*, *102*(2), 336–348.

Moore, L. J., Woodward, M. J., & Grogono-Thomas, R. (2005). The occurrence of treponemes in contagious ovine digital dermatitis and the characterisation of associated Dichelobacter nodosus. *Veterinary Microbiology*, *111*(3–4), 199–209.

Motaleb, M. A., Pitzer, J. E., Sultan, S. Z., & Liu, J. (2011). A novel gene inactivation system reveals altered periplasmic flagellar orientation in a Borrelia burgdorferi flil mutant. *Journal of Bacteriology*, *193*(13), 3324–3331.

Moter, A., Leist, G., Rudolph, R., Schrank, K., Choi, B. K., Wagner, M., & Göbel, U. B. (1998). Fluorescence in situ hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions. *Microbiology*, *144*(9), 2459–2467.

Muras, A., Mallo, N., Otero-Casal, P., Pose-Rodríguez, J. M., & Otero, A. (2022). Quorum sensing systems as a new target to prevent biofilm-related oral diseases. *Oral Diseases*, *28*(2), 307–313.

Navarro, C. A., von Bernath, D., & Jerez, C. A. (2013). Heavy Metal Resistance Strategies of Acidophilic Bacteria and Their Acquisition: Importance for Biomining and Bioremediation. *Biological Research*, *46*(4), 363–371.

Nazzaro, F., Fratianni, F., & Coppola, R. (2013). Quorum Sensing and Phytochemicals. *International Journal of Molecular Sciences, 14*(6), 12607–12619.

Newbrook, K., Carter, S. D., Crosby-Durrani, H., & Evans, N. J. (2021). Challenge of Bovine Foot Skin Fibroblasts With Digital Dermatitis Treponemes Identifies Distinct Pathogenic Mechanisms. *Frontiers in Cellular and Infection Microbiology*, *10*, 824.

Niazy, A. A. (2021). LuxS quorum sensing system and biofilm formation of oral microflora: A short review article. *The Saudi Dental Journal*, *33*(3), 116–123.

Nielsen, M. W., Strube, M. L., Isbrand, A., Al-Medrasi, W. D. H. M., Boye, M., Jensen, T. K., & Klitgaard, K. (2016a). Potential bacterial core species associated with digital dermatitis in cattle

herds identified by molecular profiling of interdigital skin samples. *Veterinary Microbiology*, *186*.

Nielsen, M. W., Strube, M. L., Isbrand, A., Al-Medrasi, W. D. H. M., Boye, M., Jensen, T. K., & Klitgaard, K. (2016b). Potential bacterial core species associated with digital dermatitis in cattle herds identified by molecular profiling of interdigital skin samples. *Veterinary Microbiology*, *186*, 139–149.

Nielsen, M. W., Strube, M. L., Isbrand, A., Al-Medrasi, W. D. H. M., Boye, M., Jensen, T. K., & Klitgaard, K. (2016c). Potential bacterial core species associated with digital dermatitis in cattle herds identified by molecular profiling of interdigital skin samples. *Veterinary Microbiology*, *186*, 139–149.

Noinaj, N., Guillier, M., Barnard, T. J., & Buchanan, S. K. (2010). TonB-dependent transporters: regulation, structure, and function. *Annual Review of Microbiology*, *64*, 43.

Nordhoff, M., Moter, A., Schrank, K., & Wieler, L. H. (2008). High prevalence of treponemes in bovine digital dermatitis-A molecular epidemiology. *Veterinary Microbiology*, *131*(3–4), 293–300.

Norris, S. J., Paster, B. J., & Smibert, R. M. (2010). *Bergey's Manual of Systematic Bacteriology* (N. R. Krieg, J. T. Staley, D. R. Brown, B. P. Hedlund, B. J. Paster, N. L. Ward, W. Ludwig, & W. B. Whitman, Eds.; Second). Springer.

Obuchowski, I., Piróg, A., Stolarska, M., Tomiczek, B., & Liberek, K. (2019). Duplicate divergence of two bacterial small heat shock proteins reduces the demand for Hsp70 in refolding of substrates. *PLOS Genetics*, *15*(10), e1008479.

Oelschlaegel, D., Wensch-Dorendorf, M., Kopke, G., Jungnickel, R., Waurich, B., Rosner, F., Döpfer, D., Brenig, B., & Swalve, H. H. (2022). Functional Variants Associated With CMPK2 and in ASB16 Influence Bovine Digital Dermatitis. *Frontiers in Genetics*, *0*, 1286.

Oliveira, V. H. S., Sørensen, J. T., & Thomsen, P. T. (2017). Associations between biosecurity practices and bovine digital dermatitis in Danish dairy herds. *Journal of Dairy Science*, *100*(10), 8398–8408.

O'Neill, J. (2015). *Antimicrobials in agriculture and the environment: reducing unnecessary use and waste.* Review on Antimicrobial Resistance chaired by Jim O'Neill.

Ong, H. S., Oettinger-Barak, O., Dashper, S. G., Darby, I. B., Tan, K. H., & Reynolds, E. C. (2017). Effect of azithromycin on a red complex polymicrobial biofilm. *Journal of Oral Microbiology*, *9*(1), 1339579.

Orino, K., Lehman, L., Tsuji, Y., Ayaki, H., Torti, S. v, & Torti, F. M. (2001). Ferritin and the response to oxidative stress. *Biochem. J*, *357*, 241–247.

Orpin, P., Sibley, D., & Taylor, N. (2021). *An update on the UK National Johne's Management Plan*. An-update-on-the-UK-NJMP-IDF-1-Final.pdf (johnes.org)

Orsel, K., Plummer, P., Shearer, J., de Buck, J., Carter, S. D., Guatteo, R., & Barkema, H. W. (2018). Missing pieces of the puzzle to effectively control digital dermatitis. *Transboundary and Emerging Diseases*, *65*(May 2017), 186–198.

O'Toole, G. A. (2011). Microtiter Dish Biofilm Formation Assay. *Journal of Visualized Experiments*.

Paluch, E., Rewak-Soroczyńska, J., Jędrusik, I., Mazurkiewicz, E., & Jermakow, K. (2020). Prevention of biofilm formation by quorum quenching. *Applied Microbiology and Biotechnology*, *104*(5), 1871–1881.

Pantanella, F., Valenti, P., Natalizi, T., Passeri, D., & Berlutti, F. (2013). Quantitative analysis of microbial biofilm colonizing abiotic surfaces Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use. *Annales Di Igiene*, 31–42.

Peeters, E., Nelis, H. J., & Coenye, T. (2008). Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *Journal of Microbiological Methods*, *72*(2), 157–165.

Percival, S. L., Hill, K. E., Williams, D. W., Hooper, S. J., Thomas, D. W., & Costerton, J. W. (2012). A review of the scientific evidence for biofilms in wounds. *Wound Repair and Regeneration*, *20*(5), 647–657.

Peterse, D. J. (1986). No. *Proceedings of the 14th International Meeting on Diseases of Cattle*.

Petersen, C., & Round, J. L. (2014). Defining dysbiosis and its influence on host immunity and disease. In *Cellular Microbiology* (Vol. 16, Issue 7, pp. 1024–1033). Blackwell Publishing Ltd. https://doi.org/10.1111/cmi.12308

Peterson, S. B., Yasuhiko, I., Borlee, B. R., Murakami, K., Harrison, J. J., Colvin, K. M., & Parsek, M. R. (2011). *Biofilm Infections* (T. Bjarnsholt, C. Moser, P. Ostrup Jensen, & N. Holby, Eds.; First). Springer, New York.

Philips, J., Rabaey, K., Lovley, D. R., & Vargas, M. (2017). Biofilm formation by clostridium ljungdahlii is induced by sodium chloride stress: Experimental evaluation and transcriptome analysis. *PLoS ONE*, *12*(1).

Pletz, M. W., Burkhardt, O., & Welte, T. (2010). Nosocomial methicillin-resistant Staphylococcus aureus (MRSA) pneumonia: Linezolid or vancomycin? - Comparison of pharmacology and clinical efficacy. *European Journal of Medical Research*, *15*(12), 507–513.

Poirel, L., Jayol, A., & Nordmanna, P. (2017). Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. *Clinical Microbiology Reviews*, *30*(2), 557.

Pulzova, L., & Bhide, M. (2014). Outer Surface Proteins of Borrelia: Peerless Immune Evasion Tools. *Current Protein & Peptide Science*, *15*(1), 75–88.

Raghavachari, N., & Garcia-Reyero, N. (2018). Overview of Gene Expression Analysis:Transcriptomics. In N. Raghavachari & N. Garcia-Reyero (Eds.), *Overview of Gene Expression Analysis:Transcriptomics* (pp. 1–6). Springer Science and Business Media, LLC.

Rasmussen, M., Capion, N., Klitgaard, K., Rogdo, T., Fjeldaas, T., Boye, M., & Jensen, T. K. (2012). Bovine digital dermatitis: Possible pathogenic consortium consisting of Dichelobacter nodosus and multiple Treponema species. *Veterinary Microbiology*, *160*(1–2), 151–161.

Ravva, S. V., Sarreal, C. Z., & Mandrell, R. E. (2011). Bacterial Communities in Aerosols and Manure Samples from Two Different Dairies in Central and Sonoma Valleys of California. *PLoS ONE*, *6*(2), e17281.

Read, D. H., & Walker, R. L. (1998). Papillomatous digital dermatitis (footwarts) in California dairy cattle: Clinical and gross pathologic findings. *Journal of Veterinary Diagnostic Investigation*, *10*(1), 67–76.

Read, D. H., Walker, R. L., Castro, A. E., Sundbergh, J. P., & Thurmond, M. C. (1992). An invasive spirochaete associated with interdigital papillomatosis of dairy cattle. In *Veterinary Record* (pp. 130: 59-60).

Read, D., & Walker, R. (1996). Experimental transmission of papillomatous digital dermatitis (footwarts) in cattle. *Veterinary Pathology*, *33*, 607.

Rebhun, W. C., Payne, R. M., King, J. M., Wolfe, M., & Begg, S. N. (1980). Interdigital papillomatosis in dairy cattle. *Journal of the American Veterinary Medical Association*, *177*(5), 437–440.

Reed, L. A., O'bier, N. S., Oliver, L. D., Hoffman, P. S., & Marconi, R. T. (2018). Antimicrobial activity of amixicile against Treponema denticola and other oral spirochetes associated with periodontal disease. *Journal of Periodontology*, *89*(12), 1467–1474.

Relun, A., Guatteo, R., Roussel, P., & Bareille, N. (2011). A simple method to score digital dermatitis in dairy cows in the milking parlor. *Journal of Dairy Science*, *94*(11), 5424–5434.

Responsible use of medicines in agriculture alliance, RUMA. (2020, January 31). *HP-CIAs- 'last resort' antibiotics*.

Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology*, *4*(3), 482.

Ritter, C., Jansen, J., Roche, S., Kelton, D. F., Adams, C. L., Orsel, K., Erskine, R. J., Benedictus, G., Lam, T. J. G. M., & Barkema, H. W. (2017). Invited review: Determinants of farmers' adoption of management-based strategies for infectious disease prevention and control. *Journal of Dairy Science*, *100*(5), 3329–3347.

Rock, C., Krull, A., P, G., Shearer, J., & Plummer, P. (2015). *Proceedings of the International Symposium and Conference on Lameness in Ruminants - Valdivia, Chile - 2015*.

Rodionov, D. A., Hebbeln, P., Gelfand, M. S., & Eitinger, T. (2006). Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: Evidence for a novel group of ATP-binding cassette transporters. *Journal of Bacteriology*, *188*(1), 317–327.

Rodriguez-Lainz, A., Hird, D. W., Carpenter, T. E., & Read, D. H. (1996). Case-control study of papillomatous digital dermatitis in southern California dairy farms. *Preventive Veterinary Medicine*, *28*(96), 117–131.

Rodríguez-Lainz, A., Hird, D. W., Carpenter, T. E., & Read, D. H. (1996). Case-control study of papillomatous digital dermatitis in southern California dairy farms. *Preventive Veterinary Medicine*, *28*(2), 117–131.

Rodriguez-Lainz, A., Melendez-Retamal, P., Hird, D. W., Read, D. H., & Walker, R. L. (1999). Farm- and host-level risk factors for papillomatous digital dermatitis in Chilean dairy cattle. *Preventive Veterinary Medicine*, *42*(2), 87–97.

Roy, R., Tiwari, M., Donelli, G., & Tiwari, V. (2018). Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. *Virulence,* 9(1), 522-554.

Rurangirwa, F. R., Dilbeck, P. M., Crawford, T. B., McGuire, T. C., & McElwain, T. F. (1999). Analysis of the 16S rRNA gene of microorganism WSU 86-1044 from an aborted bovine foetus reveals that it is a member of the order Chlamydiales: Proposal of Waddliaceae fam. nov., Waddlia chondrophila gen. nov., sp. nov. *International Journal of Systematic Bacteriology*, *49*(2), 577–581.

Ryndak, M. B., Wang, S., Smith, I., & Rodriguez, G. M. (2010). The Mycobacterium tuberculosis high-affinity iron importer, IrtA, contains an FAD-binding domain. *Journal of Bacteriology*, *192*(3), 861–869.

Sabri, M., Caza, M., Proulx, J., Lymberopoulos, M. H., Brée, A., Moulin-Schouleur, M., Curtiss, R., & Dozois, C. M. (2008). Contribution of the SitABCD, MntH, and FeoB metal transporters to the virulence of avian pathogenic Escherichia coli O78 strain χ7122. *Infection and Immunity*, *76*(2), 601–611.

Saeed, S. I., Kamaruzzaman, N. F., Mergani, A., & Aklilu, E. (2022). Antimicrobial Peptides: Bringing Solution to the Rising Threats of Antimicrobial Resistance in Livestock. *Frontiers in Veterinary Science, 1*, 851052.

Sánchez-Molano, E., Bay, V., Smith, R. F., Oikonomou, G., & Banos, G. (2019). Quantitative Trait Loci Mapping for Lameness Associated Phenotypes in Holstein-Friesian Dairy Cattle. *Frontiers in Genetics*, *10*, 926.

Santos, T. M. A., Pereira, R. V., Caixeta, L. S., Guard, C. L., & Bicalho, R. C. (2012). Microbial diversity in bovine Papillomatous digital dermatitis in Holstein dairy cows from upstate New York. *FEMS Microbiology Ecology*, 79(2), 518-529.

Sapi, E., Balasubramanian, K., Poruri, A., Maghsoudlou, J. S., Socarras, K. M., Timmaraju, A. V., Filush, K. R., Gupta, K., Shaikh, S., Theophilus, P. A. S., Luecke, D. F., MacDonald, A., & Zelger, B. (2016). Evidence of in vivo existence of Borrelia biofilm in borrelial lymphocytomas . *European Journal of Microbiology and Immunology*, *6*(1), 9–24.

Sapi, E., Bastian, S. L., Mpoy, C. M., Scott, S., Rattelle, A., Pabbati, N., Poruri, A., Burugu, D., Theophilus, P. A. S., Pham, T. V, Datar, A., Dhaliwal, N. K., MacDonald, A., Rossi, M. J., Sinha, S. K., & Luecke, D. F. (2012a). Characterization of biofilm formation by Borrelia burgdorferi in vitro. *PloS One*, *7*(10), e48277.

Sapi, E., Bastian, S. L., Mpoy, C. M., Scott, S., Rattelle, A., Pabbati, N., Poruri, A., Burugu, D., Theophilus, P. A. S., Pham, T. v, Datar, A., Dhaliwal, N. K., MacDonald, A., Rossi, M. J., Sinha, S. K., & Luecke, D. F. (2012b). Characterization of biofilm formation by Borrelia burgdorferi in vitro. *PloS One*, *7*(10), e48277.

Sapi, E., Gupta, K., Wawrzeniak, K., Gaur, G., Torres, J., Filush, K., Melillo, A., & Zelger, B. (2019). Borrelia and Chlamydia can form mixed biofilms in infected human skin tissues . *European Journal of Microbiology and Immunology*, *9*(2), 46–55.

Sapi, E., Kaur, N., Anyanwu, S., Luecke, D. F., Datar, A., Patel, S., Rossi, M., & Stricker, R. B. (2011a). Evaluation of in-vitro antibiotic susceptibility of different morphological forms of Borrelia burgdorferi. *Infection and Drug Resistance*, *4*(1), 97–113. https://doi.org/10.2147/IDR.S19201

Sapi, E., Kaur, N., Anyanwu, S., Luecke, D. F., Datar, A., Patel, S., Rossi, M., & Stricker, R. B. (2011b). Evaluation of in-vitro antibiotic susceptibility of different morphological forms of Borrelia burgdorferi. *Infection and Drug Resistance*, *4*(1), 97–113.

Sarkar, J., Mchardy, I. H., Simanian, E. J., Shi, W., & Lux, R. (2014). Transcriptional Responses of Treponema denticola to Other Oral Bacterial Species. *PLoS ONE*, *9*(2).

Sarkar, J., McHardy, I. H., Simanian, E. J., Shi, W., & Lux, R. (2014). Transcriptional responses of Treponema denticola to other oral bacterial species. *PLoS ONE*, *9*(2).

Sauer, K., & Camper, A. K. (2001). Characterization of phenotypic changes in Pseudomonas putida in response to surface-associated growth. *Journal of Bacteriology*, *183*(22), 6579–6589.

Sauvageau, R., Higgins, R., Jacques, M., & Bouchard, E. (1994). Bovine digital dermatitis. *The Canadian Veterinary Journal*, *35*(8), 522.

Sayers, G., Marques, P. X., Evans, N. J., O'grady, L., Doherty, M. L., Carter, S. D., & Nally, J. E. (2009). Identification of spirochetes associated with contagious ovine digital dermatitis. *Journal of Clinical Microbiology*, *47*(4), 1199–1201.

Sayers, R. G., Good, M., & Sayers, G. P. (2014). A survey of biosecurity-related practices, opinions and communications across dairy farm veterinarians and advisors. *Veterinary Journal*, *200*(2), 261–269.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry*, *30*(12), 3875–3883.

Schaefer, A. L., Lappala, C. R., Morlen, R. P., Pelletier, D. A., Lu, T. Y. S., Lankford, P. K., Harwood, C. S., & Greenberg, E. P. (2013). LuxR- and LuxI-Type Quorum-Sensing Circuits Are Prevalent in Members of the Populus deltoides Microbiome. *Applied and Environmental Microbiology*, *79*(18), 5745.

Schipper, P. N. M., Bonten, L. T. C., Plette, A. C. C., & Moolenaar, S. W. (2008). Measures to diminish leaching of heavy metals to surface waters from agricultural soils. *Desalination*, *226*(1–3), 89–96.

Schlafer, S., Nordhoff, M., Wyss, C., Strub, S., Hübner, J., Gescher, D. M., Petrich, A., Göbel, U. B., & Moter, A. (2008). Involvement of Guggenheimella bovis in digital dermatitis lesions of dairy cows. *Veterinary Microbiology*, *128*(1–2), 118–125. https://doi.org/10.1016/j.vetmic.2007.09.024

Schleheck, D., Barraud, N., Klebensberger, J., Webb, J. S., McDougald, D., Rice, S. A., & Kjelleberg, S. (2009). Pseudomonas aeruginosa PAO1 Preferentially Grows as Aggregates in Liquid Batch Cultures and Disperses upon Starvation. *PLoS ONE*, *4*(5), e5513.

Scholey, R. A., Blowey, R. W., Murray, R. D., Smith, R. F., Cameron, J., Massey, J. P., Ollier, W. E., & Carter, S. D. (2012). Investigating host genetic factors in bovine digital dermatitis. *Veterinary Record*, *171*(24), 624–624.

Scholey, R. A., Evans, N. J., Blowey, R. W., Massey, J. P., Murray, R. D., Smith, R. F., Ollier, W. E., & Carter, S. D. (2013). Identifying host pathogenic pathways in bovine digital dermatitis by RNA-Seq analysis. *Veterinary Journal*, *197*(3), 699–706.

Schöpke, K., Gomez, A., Dunbar, K. A., Swalve, H. H., & Döpfer, D. (2015). Investigating the genetic background of bovine digital dermatitis using improved definitions of clinical status. *Journal of Dairy Science*, *98*(11), 8164–8174.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011a). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011b). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60.

Sepúlveda Cisternas, I., Salazar, J. C., & García-Angulo, V. A. (2018). Overview on the Bacterial Iron-Riboflavin Metabolic Axis. *Frontiers in Microbiology*, *0*, 1478.

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N., Schwikowski, B., & Ideker, T. (2003). Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Research*, *13*(11), 2498–2504.

Shapiro, S., Meier, A., & Guggenheim, B. (1994). The antimicrobial activity of essential oils and essential oil components towards oral bacteria. *Oral Microbiology and Immunology*, *9*(4), 202–208.

Silva, C. G., Yudice, E. D. C., Campini, P. A. L., & Rosa, D. S. (2021). The performance evaluation of Eugenol and Linalool microencapsulated by PLA on their activities against pathogenic bacteria. *Materials Today Chemistry*, *21*, 100493.

Simar, S., Sibley, D., Ashcraft, D., & Pankey, G. (2017). Colistin and Polymyxin B Minimal Inhibitory Concentrations Determined by Etest Found Unreliable for Gram-Negative Bacilli. *The Ochsner Journal*, *17*(3), 239.

Simões, M., Bennett, R. N., & Rosa, E. A. S. (2009). Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. *Natural Product Reports*, *26*(6), 746–757.

Singh, V. K., Singh, K., & Baum, K. (2018). The Role of Methionine Sulfoxide Reductases in Oxidative Stress Tolerance and Virulence of Staphylococcus aureus and Other Bacteria. *Antioxidants*, *7*(10), 128.

Socarras, K. M., Theophilus, P. A. S., Torres, J. P., Gupta, K., & Sapi, E. (2017). Antimicrobial Activity of Bee Venom and Melittin against Borrelia burgdorferi. *Antibiotics 2017, Vol. 6, Page 31*, *6*(4), 31.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., & Kent, R. L. (1998). Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology*, *25*(2), 134–144. https://doi.org/10.1111/J.1600-051X.1998.TB02419.X/FORMAT/PDF

Solano, L., Barkema, H. W., Jacobs, C., & Orsel, K. (2017). Validation of the M-stage scoring system for digital dermatitis on dairy cows in the milking parlor. *Journal of Dairy Science*, *100*(2), 1592–1603.

Solano, L., Barkema, H. W., Pajor, E. A., Mason, S., LeBlanc, S. J., Zaffino Heyerhoff, J. C., Nash, C. G. R., Haley, D. B., Vasseur, E., Pellerin, D., Rushen, J., de Passillé, A. M., & Orsel, K. (2015). Prevalence of lameness and associated risk factors in Canadian Holstein-Friesian cows housed in freestall barns. *Journal of Dairy Science*, *98*(10), 6978–6991.

Somers, J. G. C. J., Frankena, K., Noordhuizen-Stassen, E. N., & Metz, J. H. M. (2005). Risk factors for digital dermatitis in dairy cows kept in cubicle houses in The Netherlands. *Preventive Veterinary Medicine*, *71*(1–2), 11–21.

Songer, J. G., & Post, K. W. (2005). The Genera Streptococcus and Enterococcus. In *Veterinary Microbiology, Bacterial and Fungal Agents of Animal Disease* (pp. 51–52). Elsevier.

Speijers, M. H. M., Baird, L. G., Finney, G. A., McBride, J., Kilpatrick, D. J., Logue, D. N., & O'Connell, N. E. (2010). Effectiveness of different footbath solutions in the treatment of digital dermatitis in dairy cows. *Journal of Dairy Science*, *93*(12), 5782–5791.

Stamm, L. v., & Trott, D. J. (2006). Treponema and Bovine Skin Disease: Papillomatous Digital Dermatitis and Ulcerative Mammary Dermatitis. In J. D. Radolf & S. A. Lukehart (Eds.), *Pathogenic Treponema Molecular and Cellular Biolodgy* (pp. 403–420). Caister Academic Press.

Stanton, T. B., Rosey, E. L., Kennedy, M. J., Jensen, N. S., & Bosworth, B. T. (1999). Isolation, oxygen sensitivity, and virulence of NADH oxidase mutants of the anaerobic spirochete Brachyspira (Serpulina) hyodysenteriae, etiologic agent of swine dysentery. *Applied and Environmental Microbiology*, *65*(11), 5028–5034.

Stärk, K. D. C., Nicolet, J., & Frey, J. (1998). Detection of Mycoplasma hyopneumoniae by air sampling with a nested PCR assay. *Applied and Environmental Microbiology*, *64*(2), 543–548.

Staton, G. J., Angell, J. W., Grove-White, D., Clegg, S. R., Carter, S. D., Evans, N. J., & Duncan, J. S. (2021). Contagious Ovine Digital Dermatitis: A Novel Bacterial Etiology and Lesion Pathogenesis. *Frontiers in Veterinary Science*, *8*, 1013.

Staton, G. J., Carter, S. D., Ainsworth, S., Mullin, J., Smith, R. F., & Evans, N. J. (2020). Putative β-barrel outer membrane proteins of the bovine digital dermatitis-associated treponemes: Identification, functional characterization, and immunogenicity. *Infection and Immunity*, *88*(5).

Staton, G. J., Clegg, S. R., Ainsworth, S., Armstrong, S., Carter, S. D., Radford, A. D., Darby, A., Wastling, J., Hall, N., & Evans, N. J. (2021). Dissecting the molecular diversity and commonality of bovine and human treponemes identifies key survival and adhesion mechanisms. *PLOS Pathogens*, *17*(3), e1009464. https://doi.org/10.1371/JOURNAL.PPAT.1009464

Stepanovic, S., Vukovic, D., Hola, V., Bonaventura, G. di, Djukic, S., Ćirkovic, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS*, *115*(8), 891–899.

Striednig, B., & Hilbi, H. (2022). Bacterial quorum sensing and phenotypic heterogeneity: how the collective shapes the individual. *Trends in Microbiology*, *30*(4), 379–389.

Sudharsan, M., Rajendra Prasad, · N, & Saravanan, R. (2023). Bacterial redox response factors in the management of environmental oxidative stress. *World Journal of Microbiology and Biotechnology*, *39*(3), 11.

Sullivan, L. E., Blowey, R. W., Carter, S. D., Duncan, J. S., Grove-White, D. H., Page, P., Iveson, T., Angell, J. W., & Evans, N. J. (2014a). Presence of digital dermatitis treponemes on cattle and sheep hoof trimming equipment. *The Veterinary Record*, *175*(8), 201.

Sullivan, L. E., Blowey, R. W., Carter, S. D., Duncan, J. S., Grove-White, D. H., Page, P., Iveson, T., Angell, J. W., & Evans, N. J. (2014b). Presence of digital dermatitis treponemes on cattle and sheep hoof trimming equipment. *Veterinary Record*, *175*(8), 201.

Sullivan, L. E., Carter, S. D., Duncan, J. S., Grove-White, D. H., Angell, J. W., & Evans, N. J. (2015). The gastrointestinal tract as a potential infection reservoir of digital dermatitis-associated treponemes in beef cattle and sheep. *Applied and Environmental Microbiology*, *81*(21), 7460–7469.

Sullivan, L. E., Evans, N. J., Blowey, R. W., Grove-White, D. H., Clegg, S. R., Duncan, J. S., & Carter, S. D. (2015). A molecular epidemiology of treponemes in beef cattle digital dermatitis lesions and comparative analyses with sheep contagious ovine digital dermatitis and dairy cattle digital dermatitis lesions. *Veterinary Microbiology*, *178*(1–2).

Sun, X., & Zhang, J. (2021). Bacterial exopolysaccharides: Chemical structures, gene clusters and genetic engineering. *International Journal of Biological Macromolecules*, *173*, 481–490.

Sütterlin, S., Téllez-Castillo, C. J., Anselem, L., Yin, H., Bray, J. E., & Maiden, M. C. J. (2018). Heavy Metal Susceptibility of Escherichia coli Isolated from Urine Samples from Sweden, Germany, and Spain. *Antimicrobial Agents and Chemotherapy*, *62*(5).

Sykora, S., Kofler, J., Glonegger-Reichert, J., Dietrich, J., Auersperg, G., & Brandt, S. (2015). Treponema DNA in bovine 'non-healing' versus common sole ulcers and white line disease. *Veterinary Journal*, *205*(3), 417–420.

Takei, Y., Iizuka, R., Ueno, T., & Funatsu, T. (2012). Single-molecule Observation of Protein Folding in Symmetric GroEL-(GroES)2 Complexes. *Journal of Biological Chemistry*, *287*(49), 41118–41125.

Tegtmeyer, P. C., Staton, G. J., Evans, N. J., Rohde, J., Punsmann, T. M., & Ganter, M. (2020). First cases of contagious ovine digital dermatitis in Germany. *Acta Veterinaria Scandinavica*, *62*(1), 46. https://doi.org/10.1186/s13028-020-00544-0

Theis, K., Skorvaga, M., MacHius, M., Nakagawa, N., van Houten, B., & Kisker, C. (2000). The nucleotide excision repair protein UvrB, a helicase-like enzyme with a catch. *Mutation Research - DNA Repair*, *460*(3–4), 277–300.

Thurnheer, T., Gmür, R., & Guggenheim, B. (2004). Multiplex FISH analysis of a six-species bacterial biofilm. *Journal of Microbiological Methods*, *56*(1), 37–47.

Tomasinsig, L., de Conti, G., Skerlavaj, B., Piccinini, R., Mazzilli, M., D'Este, F., Tossi, A., & Zanetti, M. (2010). Broad-spectrum activity against bacterial mastitis pathogens and activation of mammary epithelial cells support a protective role of neutrophil cathelicidins in bovine mastitis. *Infection and Immunity*, *78*(4), 1781–1788.

Touissant Raven, E. , H. R. T. , P. D. J. (1985). *Cattle Footcare and Claw Trimming*. Farming Press.

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G., & Bisignano, G. (2005). Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial Agents and Chemotherapy*, *49*(6), 2474–2478.

Trott, D. J., Moeller, M. R., Zuerner, R. L., Goff, J. P., Waters, W. R., Alt, D. P., Walker, R. L., & Wannemuehler, M. J. (2003). Characterization of Treponema phagedenis-like spirochetes isolated from papillomatous digital dermatitis lesions in dairy cattle. *Journal of Clinical Microbiology*, *41*(6), 2522–2529.

Truong, D. T., Franzosa, E. A., Tickle, T. L., Scholz, M., Weingart, G., Pasolli, E., Tett, A., Huttenhower, C., & Segata, N. (2015). MetaPhlAn2 for enhanced metagenomic taxonomic profiling. In *Nature Methods* (Vol. 12, Issue 10, pp. 902–903). Nature Publishing Group.

Uchiyama, J., Murakami, H., Sato, R., Mizukami, K., Suzuki, T., Shima, A., Ishihara, G., Sogawa, K., & Sakaguchi, M. (2020). Examination of the fecal microbiota in dairy cows infected with bovine leukemia virus. *Veterinary Microbiology*, *240*, 108547.

Vanhoudt, A., Orsel, K., Nielen, M., & van Werven, T. (2021). An observational study on the management of digital dermatitis through a repeated risk assessment on 19 Dutch dairy herds. *Journal of Dairy Science*, *104*(1), 947–956.

Venter, J. M. E., Müller, E. E., Mahlangu, M. P., & Kularatne, R. S. (2021). Treponema pallidum Macrolide Resistance and Molecular Epidemiology in Southern Africa, 2008 to 2018. *Journal of Clinical Microbiology*, *59*(10), e0238520.

Vesey, P. M., & Kuramitsu, H. K. (2004). Genetic analysis of Treponema denticola ATCC 35405 biofilm formation. *Microbiology (Reading, England)*, *150*(Pt 7), 2401–2407.

Vestby, L. K., Grønseth, T., Simm, R., & Nesse, L. L. (2020). Bacterial Biofilm and its Role in the Pathogenesis of Disease. *Antibiotics 2020, 9*(2), 59.

Walker, R. L., Read, D. H., Loretz, K. J., Hird, D. W., & Berry, S. L. (1997). Humoral response of dairy cattle to spirochetes isolated from papillomatous digital dermatitis lesions. *American Journal of Veterinary Research*, *58*(7), 744—748.

Walker, R. L., Read, D. H., Loretz, K. J., & Nordhausen, R. W. (1995). Spirochetes isolated from dairy cattle with papillomatous digital dermatitis and interdigital dermatitis. *Veterinary Microbiology*, *47*(3–4), 343–355.

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics 2008 10:1*, *10*(1), 57–63.

Wells, S. J., Garber, L. P., & Wagner, B. A. (1999). Papillomatous digital dermatitis and associated risk factors in US dairy herds. *Prev. Vet. Med.*, *38*, 11–24.

WHO. (1967). Reiter Treponeme: A review of the literature: Introduction. *Bulletin of the World Health Organization*, *36* (Suppl 2), 5.

Willshire, J. A., & Bell, N. J. (2009). An economic review of cattle lameness. *Cattle Practice*, *17*, 136–141.

Wood, D. E., & Salzberg, S. L. (2014). Kraken: Ultrafast metagenomic sequence classification using exact alignments. *Genome Biology*, *15*(3), R46.

Woods, J., Boegli, L., Kirker, K. R., Agostinho, A. M., Durch, A. M., deLancey Pulcini, E., Stewart, P. S., & James, G. A. (2012). Development and application of a polymicrobial, in vitro, wound biofilm model. *Journal of Applied Microbiology*, *112*(5), 998–1006.

Yamada, M., Ikegami, A., & Kuramitsu, H. K. (2005). Synergistic biofilm formation by Treponema denticola and Porphyromonas gingivalis. *FEMS Microbiology Letters*, *250*(2), 271–277.

Yang, D. A., Laven, R. A., Heuer, C., Vink, W. D., & Chesterton, R. N. (2018). Farm level risk factors for bovine digital dermatitis in Taranaki, New Zealand: An analysis using a Bayesian hurdle model. *The Veterinary Journal*, *234*, 91–95. https://doi.org/10.1016/j.tvjl.2018.02.012

Zagorski, N., & Wilson, D. B. (2004). Characterization and comparison of metal accumulation in two Escherichia coli strains expressing either CopA or MntA, heavy metal-transporting bacterial P-type adenosine triphosphatases. *Applied Biochemistry and Biotechnology 2004 117:1*, *117*(1), 33–48.

Zavascki, A. P., Goldani, L. Z., Li, J., & Nation, R. L. (2007). Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *Journal of Antimicrobial Chemotherapy*, *60*(6), 1206–1215.

Zawadzka, A. M., Kim, Y., Maltseva, N., Nichiporuk, R., Fan, Y., Joachimiak, A., & Raymond, K. N. (2009). Characterization of a Bacillus subtilis transporter for petrobactin, an anthrax stealth siderophore. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(51), 21854–21859.

Zhang, J., Kobert, K., Flouri, T., & Stamatakis, A. (2014). PEAR: A fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics*, *30*(5), 614–620.

Zhang, K., Song, L., & Dong, X. (2010). Proteiniclasticum ruminis gen. nov., sp. nov., a strictly anaerobic proteolytic bacterium isolated from yak rumen. *International Journal of Systematic and Evolutionary Microbiology*, *60*(9), 2221–2225.

Zhang, L., Li, S., Liu, X., Wang, Z., Jiang, M., Wang, R., Xie, L., Liu, Q., Xie, X., Shang, D., Li, M., Wei, Z., Wang, Y., Fan, C., Luo, Z. Q., & Shen, X. (2020). Sensing of autoinducer-2 by functionally distinct receptors in prokaryotes. *Nature Communications 2020 11:1*, *11*(1), 1–13.

Zhou, A., Chen, Y. I., Zane, G. M., He, Z., Hemme, C. L., Joachimiak, M. P., Baumohl, J. K., He, Q., Fields, M. W., Arkin, A. P., Wall, J. D., Hazen, T. C., & Zhou, J. (2012). Functional Characterization of Crp/Fnr-Type Global Transcriptional Regulators in Desulfovibrio vulgaris Hildenborough. *Applied and Environmental Microbiology*, *78*(4), 1168. https://doi.org/10.1128/AEM.05666-11

Zhou, L., Zheng, H., Tang, Y., Yu, W., & Gong, Q. (2013). Eugenol inhibits quorum sensing at sub-inhibitory concentrations. *Biotechnology Letters*, *35*(4), 631–637.

Zinicola, M., Higgins, H., Lima, S., Machado, V., Guard, C., & Bicalho, R. (2015a). Shotgun Metagenomic Sequencing Reveals Functional Genes and Microbiome Associated with Bovine Digital Dermatitis. *PloS One*, *10*(7), e0133674

Zinicola, M., Higgins, H., Lima, S., Machado, V., Guard, C., & Bicalho, R. (2015b). Shotgun Metagenomic Sequencing Reveals Functional Genes and Microbiome Associated with Bovine Digital Dermatitis. *PloS One*, *10*(7), e0133674.

Zinicola, M., Lima, F., Lima, S., Machado, V., Gomez, M., Döpfer, D., Guard, C., & Bicalho, R. (2015a). Altered microbiomes in bovine digital dermatitis lesions, and the gut as a pathogen reservoir. *PloS One*, *10*(3), e0120504.

Zinicola, M., Lima, F., Lima, S., Machado, V., Gomez, M., Döpfer, D., Guard, C., & Bicalho, R. (2015b). Altered microbiomes in bovine digital dermatitis lesions, and the gut as a pathogen reservoir. *PloS One*, *10*(3), e0120504.

Zobell, C. E. (1943). The Effect of Solid Surfaces upon Bacterial Activity. *Journal of Bacteriology*, *46*(1), 39–56.

Zuerner, R. L., Heidari, M., Elliott, M. K., Alt, D. P., & Neill, J. D. (2007). Papillomatous digital dermatitis spirochetes suppress the bovine macrophage innate immune response. *Veterinary Microbiology*, *125*(3–4), 256–264.

Appendix A Chapter 3 Survival of bovine digital dermatitis treponemes on hoof knives and their disinfection

Table 1: Detection of the Treponema genus and three BDD treponeme phylogroups on 140 hoof knives during foot-trimming, using direct PCR of swabs and PCR of gDNA extracted from samples cultured for six weeks under anaerobic conditions.

SAMPLE ID	FARM ID	DISINFECTANT	LESION CLASSIFI-CATION	CONTACT WITH LESION, YES OR NO	DIRECT PCR SWABS												CULTURE RESULTS											
					PRE-TRIM				POST-TRIM				POST-DISINFECTION				PRE-TRIM				POST-TRIM				POST-DISINFECTION			
					T	1	2	3	WT	1	2	3	T	1	2	3	T	1	2	3	T	1	2	3	T	1	2	3
1	1	water	1	N	-	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1	water	4.1	N	-	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	water	3	N	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
4	1	water	2	N	+	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
5	1	water	2	N	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	1	2% Virkon	2	N	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	1	2% Virkon	4	N	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
8	1	2% Virkon	2	N	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
9	1	2% Virkon	3	N	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
10	1	water	3	N	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	1	water	2	N	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	1	water	4	N	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	1	water	4	N	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	1	2% Virkon	4	N	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	1	2% Virkon	3	N	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	1	2% Virkon	4	N	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	1	2% Virkon	3	N	+	-	+	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

18	1	2% Virkon	4	N	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
19	1	2% Virkon	3	N	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
20	2	2% Virkon	WLD	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	2	2% Virkon	TN	Y	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	2	2% Virkon	TU	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	2	2% Virkon	TU	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	2	2% Virkon	2	N	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	2	2% Virkon	2	N	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	2	2% Virkon	WLD	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	2	2% Virkon	WLD	Y	+	-	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
28	2	2% Virkon	4	N	+	-	+	+	+	-	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
29	3	2% sodium hypochlorite	4	Y	+	-	+	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
30	3	2% sodium hypochlorite	4	Y	+	-	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
31	3	2% sodium hypochlorite	4	Y	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
33	3	2% sodium hypochlorite	4	Y	+	-	+	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
34	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
36	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

40	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41	3	water	4	Y	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	3	water	4	Y	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
43	3	water	TN	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	3	2% Virkon	4	Y	+	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-	-	-	-
45	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	3	2% Virkon	4	Y	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
47	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
49	3	2% sodium hypochlorite	4	Y	-	-	+	-	+	+	+	+	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-
50	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-
51	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
52	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
53	3	2% sodium hypochlorite	2	Y	+	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
54	3	2% sodium hypochlorite	4	Y	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
55	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	+	+	+	+	-	-	-	-
56	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-
57	3	2% sodium hypochlorite	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-
58	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
59	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
60	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
61	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
63	3	2% Virkon	4	Y	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
64	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-

65	3	water	4	Y	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
66	3	water	4	Y	-	+	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
67	3	FAM	4	Y	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
68	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
69	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
70	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
71	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
72	3	FAM	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
73	3	FAM	4.1	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
74	3	FAM	4.1	Y	-	-	-	-	+	+	+	+	+	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-
75	3	FAM	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
76	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
77	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
78	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-
79	3	FAM	4.1	Y	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-
80	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	+	-	+	+	-	-	-	-
81	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-
82	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
83	3	FAM	4	Y	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
84	3	FAM	4	Y	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
85	3	FAM	2	Y	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	-	-
86	3	FAM	4	Y	-	-	-	-	+	+	+	+	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-
87	3	water	4.1	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
88	3	water	4	Y	+	-	-	-	+	+	+	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
89	3	water	4	Y	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-
90	3	water	4	Y	+	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	+	-	+	+	-	-	-	-
91	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-
92	3	water	4	Y	+	-	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
93	3	water	4	Y	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
94	3	water	4	Y	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-

95	3	water	2	Y	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-
96	3	water	3	Y	-	-	-	-	+	+	+	+	+	-	+	-	-	-	-	-	+	+	+	+	-	-	-	-
97	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	+	-	+	-	-	-	-	-
98	3	water	4	Y	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
99	3	water	4	Y	+	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
100	3	water	4	Y	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
101	3	water	4	Y	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
102	3	2% Virkon	4	Y	+	-	-	-	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-
103	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
104	3	2% Virkon	4.1	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
105	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
106	3	2% Virkon	3	Y	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
107	3	2% Virkon	2	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
108	3	2% Virkon	2	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
109	3	2% Virkon	4	Y	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
110	3	2% Virkon	4	Y	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
111	3	2% Virkon	4	Y	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
112	3	2% Virkon	4	Y	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
113	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
114	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
115	3	2% Virkon	4	Y	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
116	3	2% Virkon	3	Y	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
117	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
118	3	water	3	Y	+	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-
119	3	water	4	Y	+	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
120	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
121	3	water	4	Y	+	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-
122	3	water	4.1	Y	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	+	-	+	+	-	-	-	-
123	3	water	4.1	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
124	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-

125	3	water	4	Y	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
126	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
127	3	water	4	Y	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
128	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
129	3	water	4	Y	+	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
130	3	water	4	Y	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
131	3	water	4	Y	-	-	-	-	+	+	+	+	+	-	+	-	-	-	-	-	+	+	+	+	-	-	-	-
132	3	water	4.1	Y	-	-	-	-	+	+	+	+	+	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-
133	3	2% Virkon	4	Y	+	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-
134	3	2% Virkon	4	Y	+	-	-	-	+	+	+	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
135	3	2% Virkon	2	Y	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
136	3	2% Virkon	3	Y	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
137	3	2% Virkon	4	Y	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
138	3	2% Virkon	4	Y	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-
139	3	2% Virkon	4	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
140	3	2% Virkon	4.1	Y	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

WLD= White Line Disease

TN= Toe Necrosis

TU= Toe Ulcer

Appendix B Chapter 4 Current foot-trimming hygiene practices and research impact on knowledge and practice of biosecurity during cattle foot-trimming

Initial Questionnaire

Foot trimming Hygiene Questionnaire

1. How many farms do you foot trim on?.....
2. How many cows do you trim in a typical week?.....
3. How many herds that you trim have digital dermatitis?.....
   Don't know/ Prefer not to say
4. How many do not have digital dermatitis?.....
   Don't know/ Prefer not to say
5. Do you clean your hands during foot trimming? **YES** **NO**

   If YES, how often?.....

   If YES, what do you use and what is your method?

   .....

   .....

   .....

   .....

6. Do you clean you hoof knives during foot trimming? **YES** **NO**

   If YES, how often?.....

   If YES, what do you use and what is your method?

   .....

   .....

   .....

   .....

It would help us enormously if you would be prepared to help further with this study. If you are happy to be contacted in the future with a follow-up questionnaire, please provide your email address: …….

Many thanks for completing the questionnaire!

Follow-up Questionnaire

Foot trimming Hygiene Questionnaire

1. Which country do you work in?

2. How many farms do you foot trim on?

3. How many cows do you trim in a typical week?

4. How many herds that you trim have digital dermatitis (DD)?

5. How many do not have digital dermatitis?

6. Have you seen the protocol published by the University of Liverpool and AHDB Dairy for best hygiene practices during foot trimming? (Please circle)

   YES/NO

7. Have you seen any articles in the farming or veterinary press about best hygiene practice during foot trimming? (Please circle)

   YES/NO

8. Please circle your response to each statement:

   My awareness of the following has increased across the last year:

   a) The potential to spread DD during foot trimming

   Strongly disagree/ disagree/ neutral/ agree/ strongly agree

   b) Appropriate hygiene and disinfection to prevent spread of DD during foot trimming

   Strongly disagree/ disagree/ neutral/ agree/ strongly agree

   9.a) Please circle your response to the following:

   My management of hygiene during foot trimming has changed in the last year

   Strongly disagree/ disagree/ neutral/ agree/ strongly agree

b) If your management of hygiene during foot trimming has changed, please tick all changes that apply. If not, please go to question 10.

□Increased frequency of hand washing

□ Increased frequency of changing gloves

□Increased frequency of hoof knife cleaning

□Method for hand hygiene has changed

(Please specify ……..)

□Method for hoof knife cleaning has changed

(Please specify……..……)

□Other

(Please specify .....)

c) If you have changed practice, do you consider that you/your client(s) have observed a reduction in DD on farm as a result?

YES NO DON'T KNOW

10. What are the barriers to improving foot-trimming hygiene?
Please tick your response to each statement.

	Strongly disagree	Disagree	Neutral	Agree	Strongly Agree
Water/ cleaning facilities are difficult to access on all farms					
It takes too long to disinfect each knife					
I don't think it is important					
I only have time to clean my knife after trimming a cow that had DD					
I don't like getting my **hands** wet because I think it makes them slippery and trimming is more dangerous					
I don't like getting my **knives** wet because I think it makes					

them slippery and trimming is more dangerous					

11. If you wish, please suggest improvements to the foot-trimming hygiene protocol.

12. What future research do you believe would be beneficial in this area?

If you wish to be entered into our prize draw, please provide your email address. We will only use this information to contact you if you win a prize.*

……..

Many thanks for completing the questionnaire!

*Winners will be contacted within one month of the questionnaire end date. If you do not respond within 14 days your prize will be void and another winner will be chosen.

Reducing the spread of digital dermatitis by disinfection of hoof-trimming equipment

Hygiene standard operating procedure for foot trimming:

The following protocol has been shown to eliminate the viable bacteria from foot-trimming knives and user gloves, thereby minimising the spread of digital dermatitis (DD) via this route.

As some (~12.5%) healthy feet may have subclinical DD, this protocol should be used at all times during foot trimming, i.e. when trimming feet with any signs of DD (including healed), any other foot/claw horn presentations and also healthy feet.

This procedure should be used, together with additional control strategies, such as routine footbathing and effective slurry management on farm to help prevent the spread of DD.

Before you start

One option is to wear arm-length gloves with wrist-length gloves over the top. This makes it easier to change wrist-length gloves when they are damaged. Wrist protectors can still be worn over the arm-length gloves, as only the hand gloves would be changed, as needed.

Equipment

- **Arm-length disposable gloves**
- **Wrist-length disposable gloves**
- **A bucket of soapy water**
- **Two pairs of clean hoof knives**
- **Container for disinfectant**
- **Disinfectant: 2% Virkon®, 2% sodium hypochlorite, or 1% FAM30® are suitable for this purpose**

Protocol

1) Prior to use, ensure all knives are free from faecal material and visible dirt. Fill the container with suitable disinfectant and then submerge the blades for at least 20 seconds.

2) After trimming each foot, clean your hands and knives by swilling in the bucket of soapy water to remove visible dirt.

3) Dry knives with paper towel. This will also help to remove visible dirt.

4) Return the knife blades to the disinfectant for at least 20 seconds before next use.

5) To minimise disruption to work flow, use alternate pairs of knives for each foot, leaving the first pair in disinfectant.

UNIVERSITY OF LIVERPOOL

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Appendix C (Chapter 5)

Table 1: DNA concentrations of samples used for shotgun metagenomics, as measured using the Qubit Bioanalyser and Nanodrop; and measures of sample quality from Nanodrop.

Sample ID	Qubit (ng/µl)	Nanodrop 260/230	Nanodrop 260/280	Nanodrop (ng/µL)
28 (HtIn)	4.22	0.49	2.17	4.5
29 (HtIn)	6.32	0.74	1.71	10.6
31 (HtIn)	15.2	0.96	1.76	18.4
32 (HtIn)	28.6	1.27	1.85	38.3
100 (HtIn)	1.69	1.16	1.87	24.5
102 (HtHt)	6.8	0.90	1.90	14.8
105 (HtHt)	6.17	0.81	1.42	10.8
116 (HtHt)	5.5	0.67	2.09	8.0
191 (HtHt)	12.0	0.93	2.27	12.1
193 (HtHt)	3.38	0.75	1.84	7.1

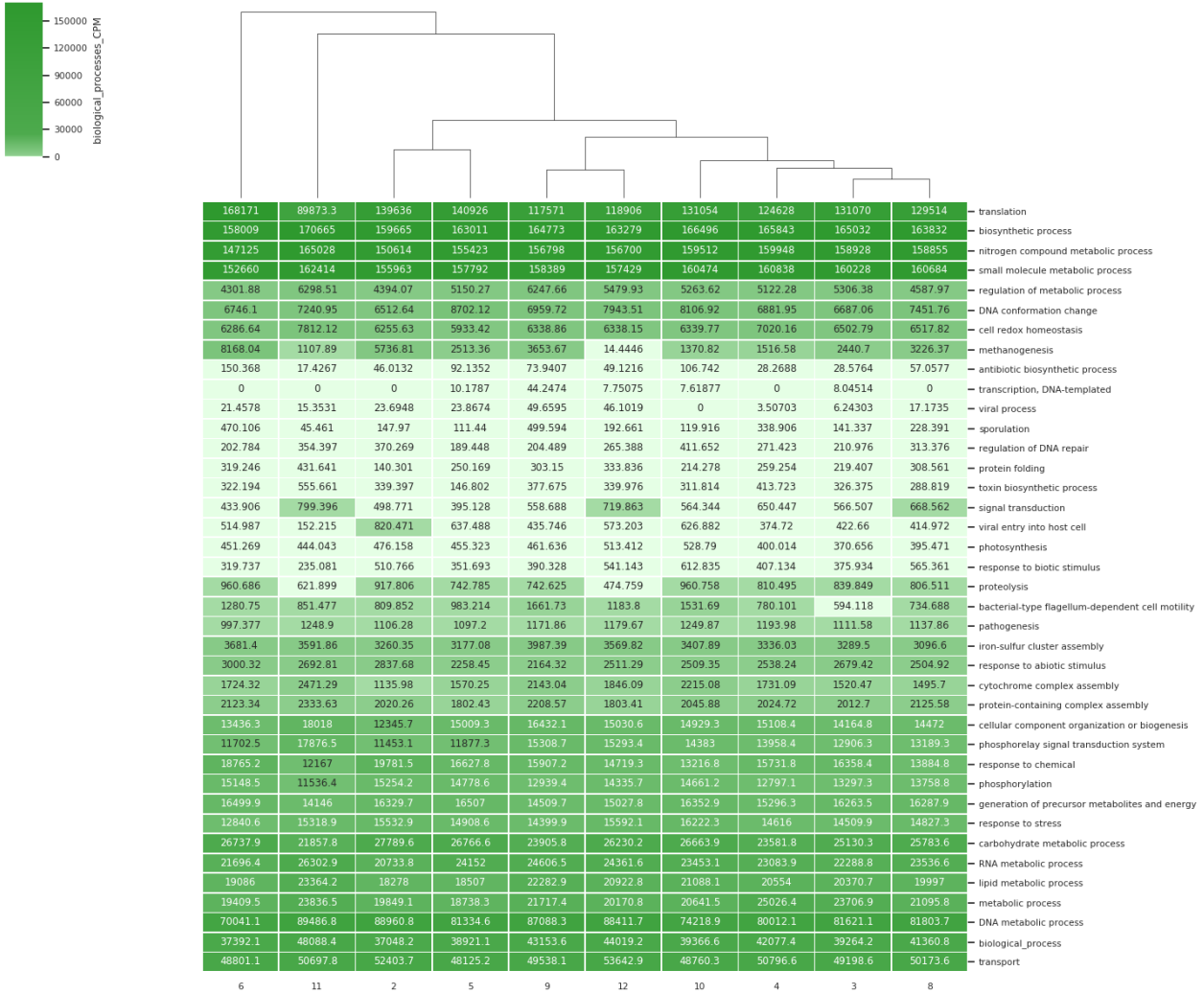
Table 2: Functional Pathways found to differ between HtHt and HtIn groups using the HUMAnN2 search strategy carried out on metagenomic sequences

Pathways	Explanation	Upregulated in HtHt or HtIn?
Arginine biosynthesis via L-ornithine	Amino acid synthesis	HtIn
Urate biosynthesis inosine 5 phosphate degradation	Purine degradation	HtIn
L- arginine biosynthesis IV archaebacteria	Amino acid synthesis	HtIn
Lipid IV A biosynthesis	Bacterial cell wall synthesis	HtIn
Guanosine nucleotides degradation III	Purine degradation	HtIn
L arginine degradation AST pathway	Amino acid degradation	HtIn
4 deoxy L threo hex 4 enopyranuronate degradation	Degradation of large complex polymers such as plant pectins or connective tissue components for example hyaluronan and chondroitin sulfates.	HtIn
Superpathway of glycerol degradation to 1,3 propanediol	Glycerol degradation	HtIn
Ureide biosynthesis	Amine/ polyamine synthesis	HtHt
Nitrate reduction I denitrification	Denitrification- a respiratory process in conditions of oxygen depletion	HtHt
Methylketone biosynthesis	Product of metabolism	HtHt
Fatty acid beta oxidation peroxisome yeast	Fatty acid degradation in yeast	HtHt
Superpathway of histidine, purine and pyrimidine biosynthesis	Bacterial metabolic pathway	HtHt
Superpathway of L alanine biosynthesis	Bacterial amino acid synthesis	HtHt

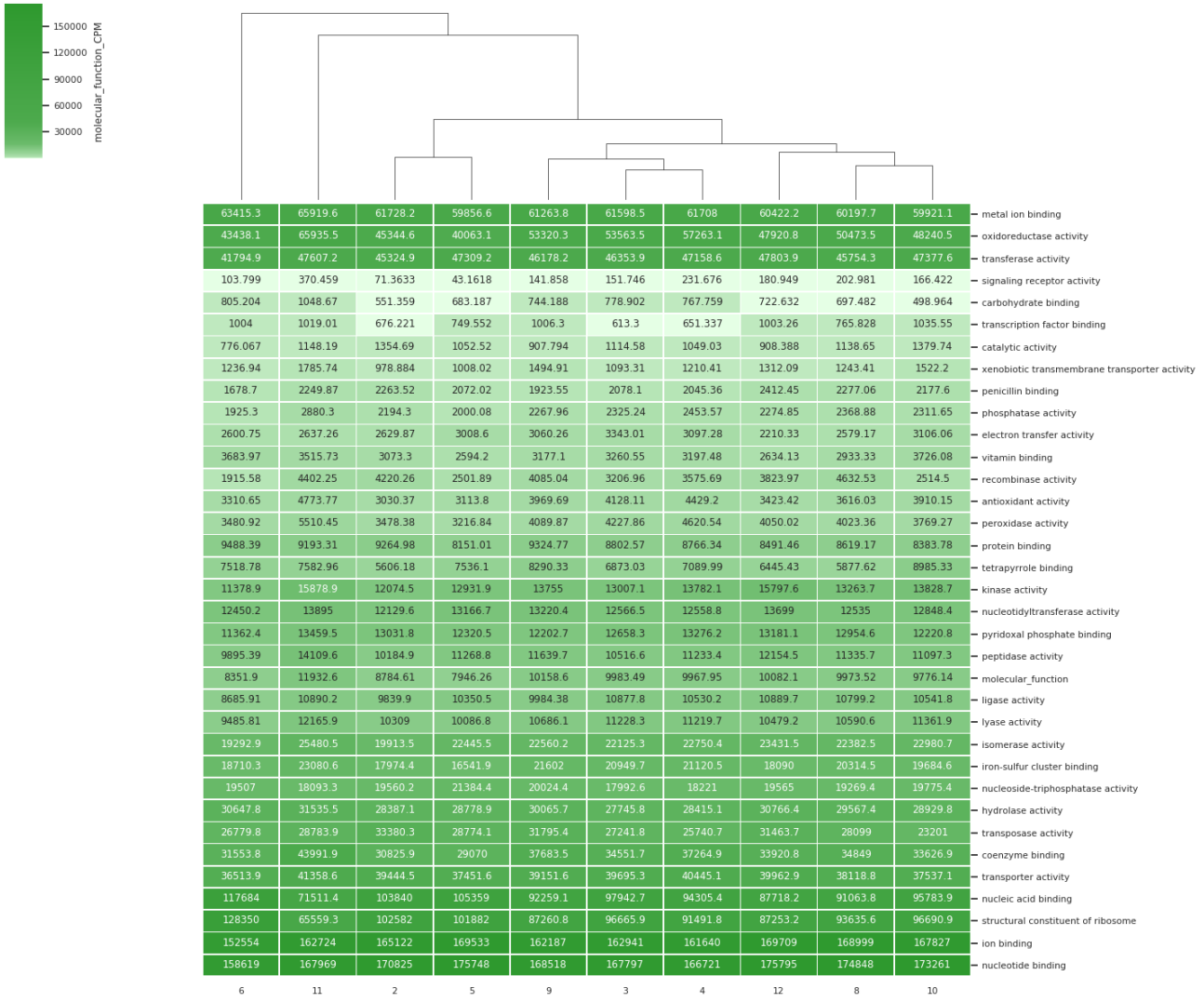
S- adenosyl L methionine cycle I	Amino acid metabolism	HtHt
L-Histidine biosynthesis	Amino acid synthesis	HtHt
Guanosine deoxyribonucleotides de novo biosynthesis II	Nucleotide synthesis	HtHt
Adenosine deoxyribonucleotides de novo biosynthesis II	Nucleotide synthesis	HtHt

Figure 1 Heatmaps comparing frequency of detection of genes responsible for A. Biological processes B. Molecular functions C. Cellular components
Columns 2-6 correspond to samples from HtHt cows, whilst columns 8-12 correspond to samples from HtIn cows.

A



B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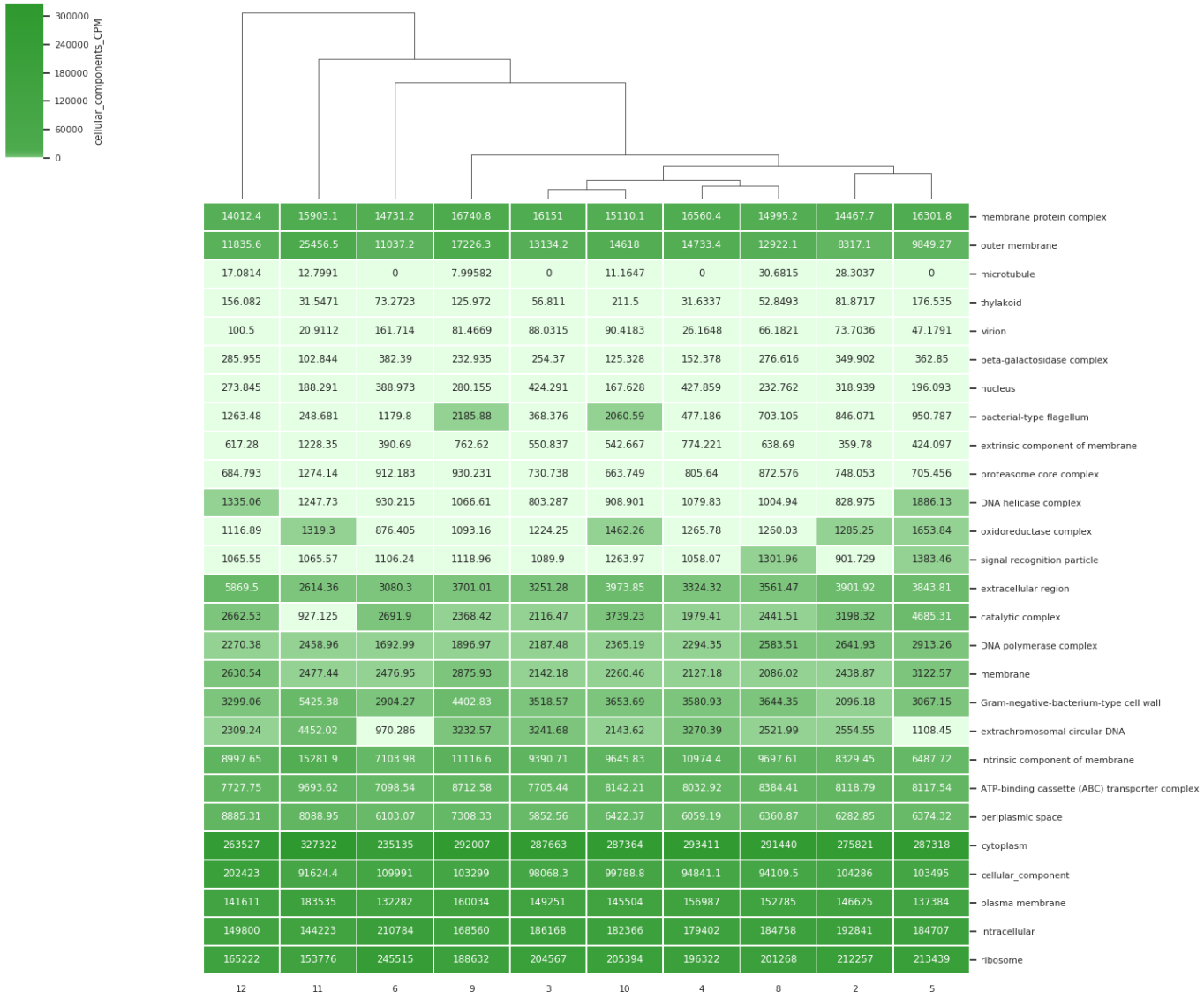
150000
120000
90000
60000
30000
63415.3 65919.6 61728.2 59856.6 61263.8 61598.5 61708 60422.2 60197.7 59921.1 metal ion binding
43438.1 65935.5 45344.6 40063.1 53320.3 53563.5 57263.1 47920.8 50473.5 48240.5 oxidoreductase activity
41794.9 47607.2 45324.9 47309.2 46178.2 46353.9 47158.6 47803.9 45754.3 47377.6 transferase activity
103.799 370.459 71.3633 43.1618 141.858 151.746 231.676 180.949 202.981 166.422 signaling receptor activity
805.204 1048.67 551.359 683.187 744.188 778.902 767.759 722.632 697.482 498.964 carbohydrate binding
1004 1019.01 676.221 749.552 1006.3 613.3 651.337 1003.26 765.828 1035.55 transcription factor binding
776.067 1148.19 1354.69 1052.52 907.794 1114.58 1049.03 908.388 1138.65 1379.74 catalytic activity
1236.94 1785.74 978.884 1008.02 1494.91 1093.31 1210.41 1312.09 1243.41 1522.2 xenobiotic transmembrane transporter activity
1678.7 2249.87 2263.52 2072.02 1923.55 2078.1 2045.36 2412.45 2277.06 2177.6 penicillin binding
1925.3 2880.3 2194.3 2000.08 2267.96 2325.24 2453.57 2274.85 2368.88 2311.65 phosphatase activity
2600.75 2637.26 2629.87 3008.6 3060.26 3343.01 3097.28 2210.33 2579.17 3106.06 electron transfer activity
3683.97 3515.73 3073.3 2594.2 3177.1 3260.55 3197.48 2634.13 2933.33 3726.08 vitamin binding
1915.58 4402.25 4220.26 2501.89 4085.04 3206.96 3575.69 3823.97 4632.53 2514.5 recombinase activity
3310.65 4773.77 3030.37 3113.8 3969.69 4128.11 4429.2 3423.42 3616.03 3910.15 antioxidant activity
3480.92 5510.45 3478.38 3216.84 4089.87 4227.86 4620.54 4050.02 4023.36 3769.27 peroxidase activity
9488.39 9193.31 9264.98 8151.01 9324.77 8802.57 8766.34 8491.46 8619.17 8383.78 protein binding
7518.78 7582.96 5606.18 7536.1 8290.33 6873.03 7089.99 6445.43 5877.62 8985.33 tetrapyrrole binding
11378.9 15878.9 12074.5 12931.9 13755 13007.1 13782.1 15797.6 13263.7 13828.7 kinase activity
12450.2 13895 12129.6 13166.7 13220.4 12566.5 12558.8 13699 12535 12848.4 nucleotidyltransferase activity
11362.4 13459.5 13031.8 12320.5 12202.7 12658.3 13276.2 13181.1 12954.6 12220.8 pyridoxal phosphate binding
9895.39 14109.6 10184.9 11268.8 11639.7 10516.6 11233.4 12154.5 11335.7 11097.3 peptidase activity
8351.9 11932.6 8784.61 7946.26 10158.6 9983.49 9967.95 10082.1 9973.52 9776.14 molecular_function
8685.91 10890.2 9839.9 10350.5 9984.38 10877.8 10530.2 10889.7 10799.2 10541.8 ligase activity
9485.81 12165.9 10309 10086.8 10686.1 11228.3 11219.7 10479.2 10590.6 11361.9 lyase activity
19292.9 25480.5 19913.5 22445.5 22560.2 22125.3 22750.4 23431.5 22382.5 22980.7 isomerase activity
18710.3 23080.6 17974.4 16541.9 21602 20949.7 21120.5 18090 20314.5 19684.6 iron-sulfur cluster binding
19507 18093.3 19560.2 21384.4 20024.4 17992.6 18221 19565 19269.4 19775.4 nucleoside-triphosphatase activity
30647.8 31535.5 28387.1 28778.9 30065.7 27745.8 28415.1 30766.4 29567.4 28929.8 hydrolase activity
26779.8 28783.9 33380.3 28774.1 31795.4 27241.8 25740.7 31463.7 28099 23201 transposase activity
31553.8 43991.9 30825.9 29070 37683.5 34551.7 37264.9 33920.8 34849 33626.9 coenzyme binding
36513.9 41358.6 39444.5 37451.6 39151.6 39695.3 40445.1 39962.9 38118.8 37537.1 transporter activity
117684 71511.4 103840 105359 92259.1 97942.7 94305.4 87718.2 91063.8 95783.9 nucleic acid binding
128350 65559.3 102582 101882 87260.8 96665.9 91491.8 87253.2 93635.6 96690.9 structural constituent of ribosome
152554 162724 165122 169533 162187 162941 161640 169709 168999 167827 ion binding
158619 167969 170825 175748 168518 167797 166721 175795 174848 173261 nucleotide binding
6 11 2 5 9 3 4 12 8 10

C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300000
240000
180000
120000
60000
0
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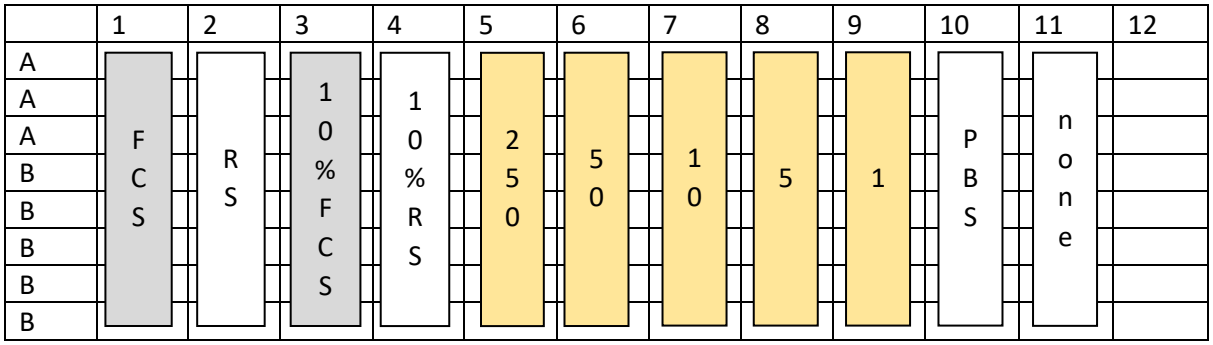
outer membrane
microtubule
thylakoid
virion
beta-galactosidase complex
nucleus
bacterial-type flagellum
extrinsic component of membrane
proteasome core complex
DNA helicase complex
oxidoreductase complex
signal recognition particle
extracellular region
catalytic complex
DNA polymerase complex
membrane
Gram-negative-bacterium-type cell wall
extrachromosomal circular DNA
intrinsic component of membrane
ATP-binding cassette (ABC) transporter complex
periplasmic space
cytoplasm
cellular_component
plasma membrane
intracellular
ribosome
12
11
6
9
3
10
4
8
2
5

Appendix D: Chapter 6 Development of an *in vitro* Biofilm Model for Studying aspects of BDD treponeme pathogenesis

Figure 1. Layout of microtiter plates used to determine the optimum coating for growth and biofilm formation of three species of BDD treponeme.



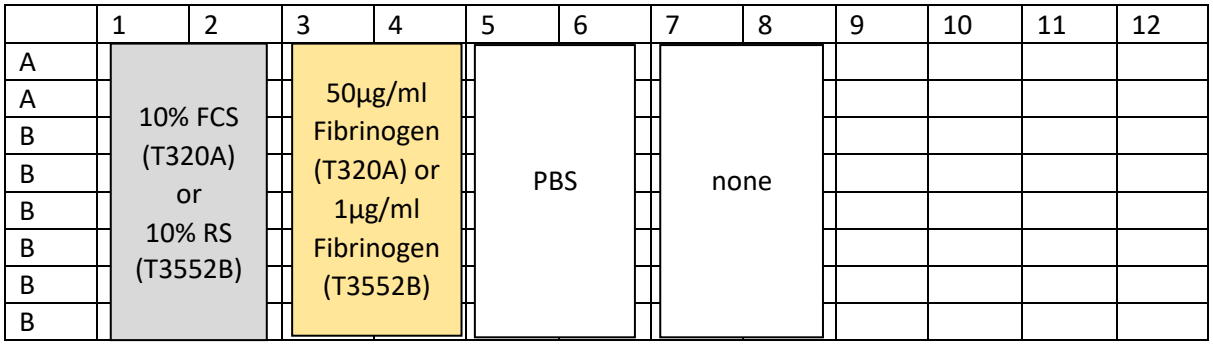
Key:
Rows

A. Control wells containing 200µl of liquid growth medium Oral Treponeme enrichment broth with 10% Foetal calf serum (OTEB+FCS) for T320A and T3552B, and Oral Treponeme enrichment broth with 10% Rabbit serum (OTEB+RS) for T19.
B. Wells containing 150µl of liquid growth medium and 50µl of bacteria.

Columns

1. Foetal Calf Serum
2. Rabbit serum
3. 10% Foetal calf serum (diluted using PBS)
4. 10% Rabbit serum (diluted using PBS)
5. 250µg/ml Bovine Fibrinogen (diluted using PBS)
6. 50µg/ml Bovine Fibrinogen (diluted using PBS)
7. 10µg/ml Bovine Fibrinogen (diluted using PBS)
8. 5µg/ml Bovine Fibrinogen (diluted using PBS)
9. 1µg/ml Bovine Fibrinogen (diluted using PBS)
10. Control coating with 1x phosphate buffered saline (PBS)
11. Control uncoated wells

Figure 2 Layout of microtiter plates used to determine the optimum timing for growth and biofilm formation of T320A and T3552B.



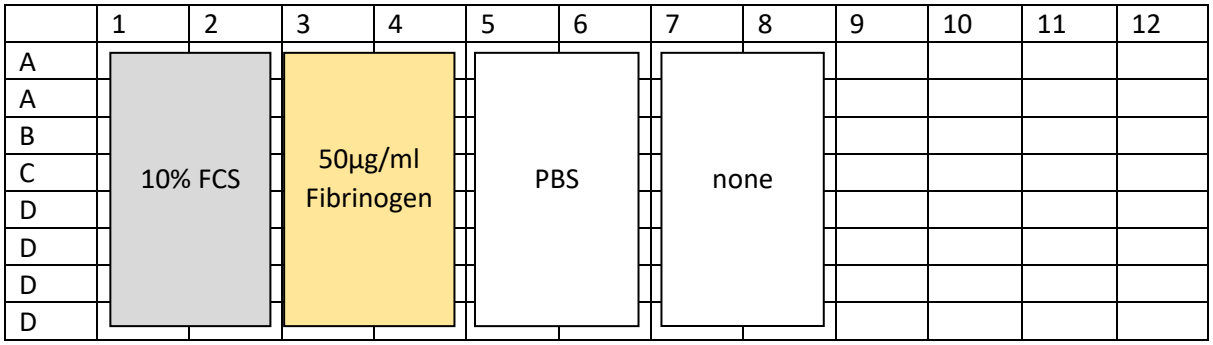
Key:
Rows

A. Control wells containing 200µl of liquid growth medium OTEB+FCS
B. Wells containing 150µl of OTEB+FCS and 50µl of T320A or T3552B.

Columns

1-2. 10% Foetal calf serum or 10% Rabbit serum (both diluted using PBS)
3-4. 50µg/ml or 1µg/ml Bovine Fibrinogen (both diluted using PBS)
5-6. Control coating with PBS
7-8. Control uncoated wells

Figure 3 Microtiter plate layout for T19+T320A experiments



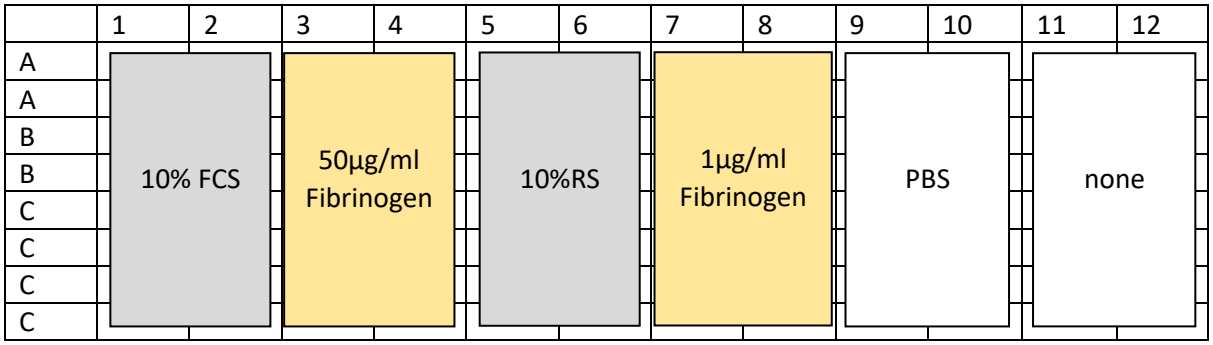
Key:
Rows

A. Control wells containing 200µl of liquid growth medium OTEB+FCS
B. Wells containing 150µl of OTEB+RS and 50µl of T19.
C. Wells containing 150µl of OTEB+FCS and 50µl of T320A.
D. Wells containing 150µl of OTEB+FCS and 50µl of T19+T320A.

Columns/ Coatings

1-2. 10% Foetal calf serum (diluted using PBS)
3-4. 50µg/ml Bovine Fibrinogen (diluted using PBS)
5-6. Control coating with PBS
7-8. Control uncoated wells

Figure 4. Microtiter plate layout for T19+T3552B, T320A+T3552B and T19+T320A+T3552B* experiments



Key:

Rows

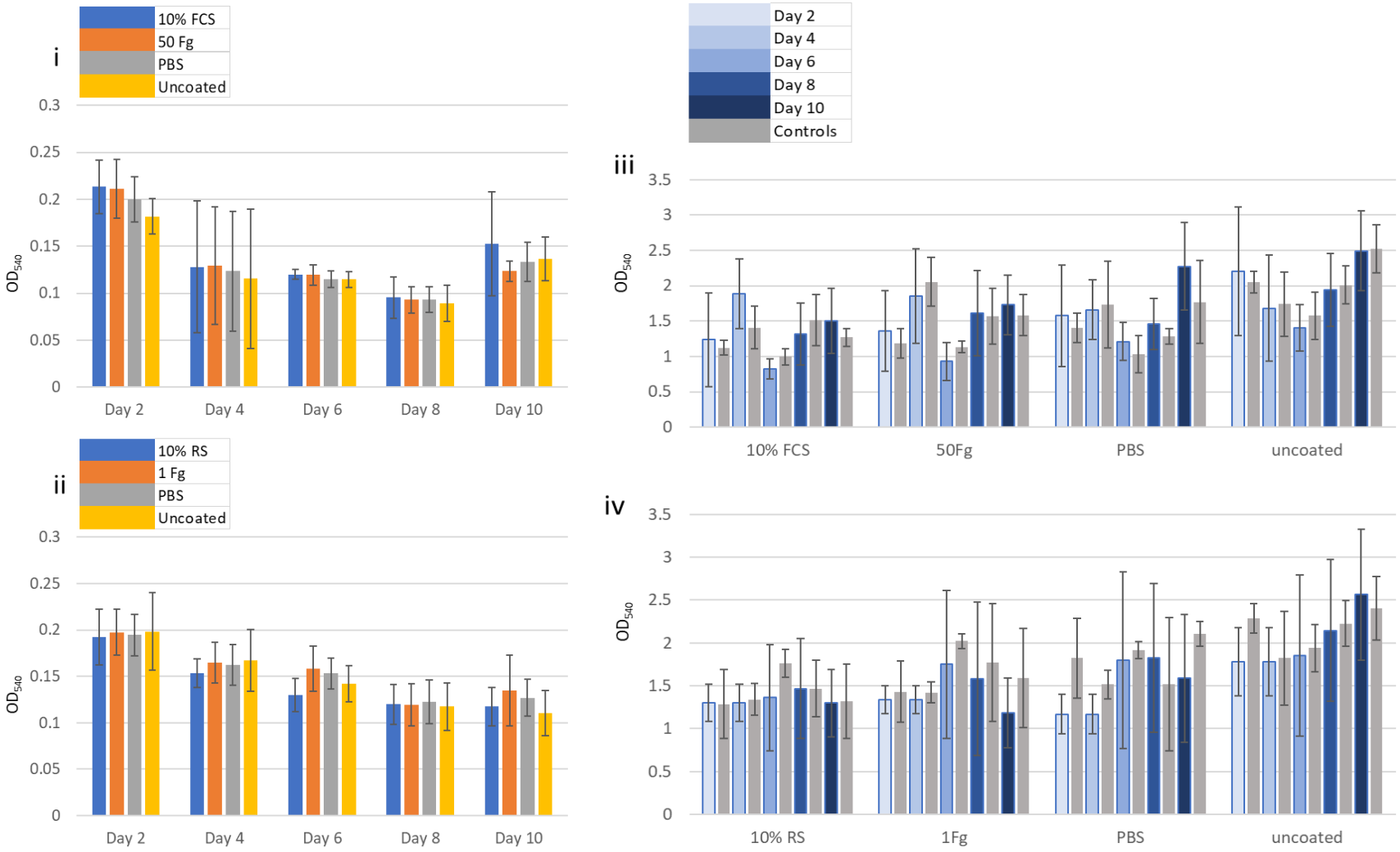
A. Control wells containing 200µl of liquid growth medium OTEB+FCS
B. Wells containing 150µl of OTEB+FCS and 50µl of single species of bacteria.
C. Wells containing 150µl of OTEB+FCS and 50µl of dual or triple species cultures.

*Triple-species plates contained only one row of control medium, and three rows of single-species.

Columns/ Coatings

1-2.10% Foetal calf serum (diluted using PBS)
3-4.50µg/ml Bovine Fibrinogen (diluted using PBS)
5-6.10% Rabbit serum
7-8.1µg/ml Bovine Fibrinogen (diluted using PBS)
9-10. Control coating with PBS
11-12. Control uncoated wells

Figure 5: OD_{540} representing growth for (i) T320A and (ii) T3552B after 2, 4, 6, 8 and 10 days incubation, and OD_{540} showing crystal violet staining of microtiter plate wells for (iii) T320A and (iv) T3552B after 2, 4, 6, 8 and 10 days incubation.



Appendix E Chapter 7 Global transcriptome analysis of BDD treponemes during biofilm growth and oxidative stress

Table 1: Quality control data for RNA samples, sequenced reads, trimmed read pairs and mapped read pairs.

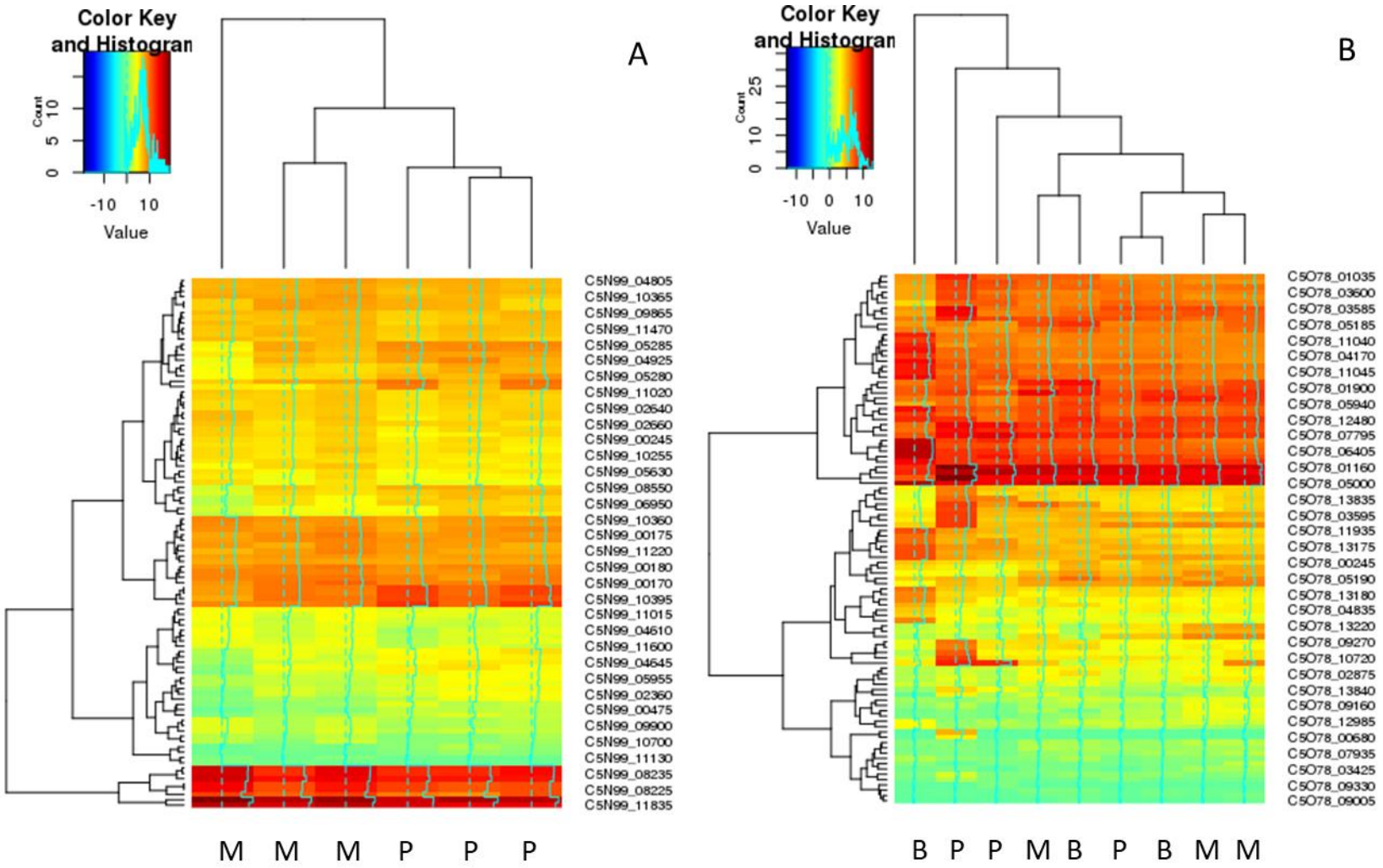
Sample contents	Qubit quantity (ng/ul)	Nanodrop 260/280	Nanodrop 260/230	RIN number	Raw read number	Trimmed read number (% raw)	Trimmed Read Pairs Number	Mapped Read Pairs (% total)
T19 P	34.4	2.07	2.13	3.3	85 632 990	85 484 194 (99.83)	42 702 792	36 049 757 (87)
T19 P	35.8	2.21	2.18	1.8	47 567 454	47 494 704 (99.85)	23 718 683	19 719 264 (85.61)
T19 P	7.42	2.85	0.76	1.2	15 069 434	15 021 142 (99.68)	7 496 299	5 828 722 (80.41)
T19 M	84.2	2.11	1.34	2.3	16 198 292	16 161 908 (99.78)	8 067 854	4 712 064 (59.99)
T19 M	77.2	2.15	1.32	1.1	22 361 080	22 305 151 (99.75)	11 132 683	3 064 333 (28.19)
T19 M	134.4	2.2	0.63	1.2	39 322 066	39 260 416 (99.84)	19 607 448	16 592 348 (87.16)
T320A P	250	2.2	2.16	6.6	68 670 360	68 560 574 (99.84)	34 245 704	31 068 650 (92.72)
T320A P	32.4	2.26	1.8	7.8	47 541 064	47 474 815 (99.86)	23 711 602	10 072 196 (43.47)
T320A P	73.6	2.96	1.67	5.4	45 092 760	45 024 738 (99.85)	22 486 659	19 919 933 (90.67)
T320A M	30.4	1.94	1.69	4.4	59 793 434	59 702 687 (99.85)	29 818 097	26 815 489 (91.95)
T320A M	51.6	2.19	2.19	3.0	83 037 700	82 881 502 (99.81)	41 407 662	37 971 855 (93.79)

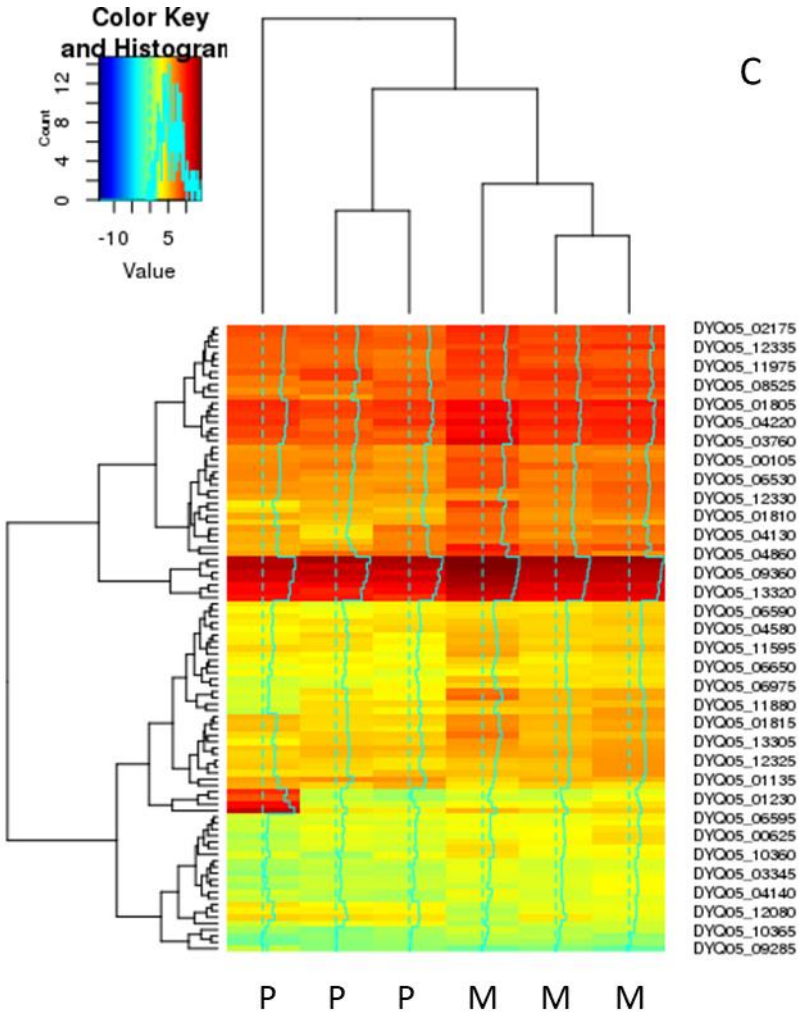
T320A M	85.6	2.19	2.11	6.3	67 395 136	67 249 850 (99.78)	33 600 665	30 840 270 (93.67)
T320A B	252	2.2	2.32	5.6	70 441 572	70 321 165 (99.83)	35 130 155	32 051 023 (93.25)
T320A B	514	2.17	2.33	7.3	55 934 532	55 846 388 (99.84)	27 891 802	25 371 333 (93.07)
T320A B	175	2.17	1.51	5.7	38 971 948	38 891 683 (99.79)	19 427 246	18 273 658 (95.54)
T3552B P	256	2.16	2.01	7.1	72 540 832	72 446 322 (99.87)	36 190 436	32 415 564 (91.77)
T3552B P	356	2.18	2.88	9.4	43 227 624	43 169 670 (99.87)	21 565 979	18 555 791 (88.12)
T3552B P	446	2.22	1.7	7.6	27 972 158	27 936 058 (99.87)	13 954 080	12 177 090 (89.46)
T3552B M	464	2.2	2.49	7.3	48 611 056	48 547 486 (99.87)	24 252 126	21 131 781 (89.39)
T3552B M	264	2.21	2.42	6.7	57 018 744	56 938 201 (99.86)	28 444 574	25 204 030 (90.85)
T3552B M	42.8	2.15	2.09	2.1	52 575 646	52 491 660 (99.84)	26 220 074	23 058 746 (90.11)
Reiter P	280	2.2	2.3	6.4	60 981 316	60 899 042 (99.87)	30 422 817	25 445 154 (83.64)
Reiter P	62.4	2.16	1.54	5.9	84 037 502	84 191 954 (99.86)	42 061 889	35 051 574 (83.33)
Reiter P	161.8	2.2	0.87	3.2	46 059 908	45 996 328 (99.86)	22 974 527	19 364 812 (84.29)
Reiter M	226	2.21	2.43	6.5	60 627 944	60 540 053 (99.86)	30 236 218	25 144 938 (83.16)
Reiter M	53.6	2.2	2.04	5.9	64 162 390	64 066 557 (99.85)	32 007 705	27 054 070 (84.52)
Reiter M	107	2.21	2.23	6.6	117 520 464	117 331058 (99.84)	58 609 384	49 063 654 (83.71)

T320A+ T3552B P	99.4	2.19	2.38	3.5	46 925 760	46 857 265 (99.85)	23 401 941	20 642 595 (90.19)
T320A+ T3552B P	236	2.18	2.03	6.4	61 295 844	61 204 744 (99.85)	30 574 220	27 348 338 (91.66)
T320A+ T3552B P	1019	2.09	2.2	9.5	41 643 894	41 592 143 (99.88)	20 775 681	18 459 097 (91.08)
T320A+ T3552B M	664	2.21	2.33	7.2	49 853 550	49 783 341 (99.86)	24 864 977	22 273 400 (91.78)
T320A+ T3552B M	434	2.18	2.44	8.1	35 071 082	35 028 838 (99.88)	17 500 245	15 840 916 (92.59)
T320A+ T3552B M	310	2.16	1.92	7.1	46 267 788	46 207 545 (99.87)	23 401 941	20 916 269 (92.74)
T320A+ T3552B B	672	2.21	2.37	7.2	44 876 168	44 820 413 (99.88)	22 391 231	20 210 928 (92.36)
T320A+ T3552B B	284	2.12	2.29	7.5	38 959 158	38 910 274 (99.87)	19 437 187	17 425 448 (91.9)
T320A+ T3552B B	162.4	2.03	1.12	6.4	66 659 526	66 562 694 (99.85)	33 243 929	29 827 556 (91.75)
T19+T320A +T3552B P	700	2.21	1.49	7.4	11 688 032	11 671 276 (99.86)	5 829 511	5 206 576 (91.46)
T19+T320A +T3552B P	462	2.2	1.83	7.2	27 601 546	27 562 786 (99.86)	13 767 969	11 845 550 (88.08)
T19+T320A +T3552B P	598	2.21	2.49	6.0	28 998 956	28 957 638 (99.86)	14 463 323	7 873 255 (55.69)
T19+T320A +T3552B M	462	2.2	2.22	6.9	19 355 056	19 328 686 (99.86)	9 655 179	8 654 800 (91.89)
T19+T320A +T3552B M	370	2.21	1.94	7.5	23 088 416	23 059 061 (99.87)	11 518 108	9 993 742 (88.96)
T19+T320A +T3552B M	408	2.2	2.08	7.1	16 909 736	16 885 446 (99.86)	8 434 172	7 425 695 (90.17)

Figure 1: Hierarchical cluster analysis for samples using counts per million (CPM) to show normalized expression for the top ranked dysregulated genes

A T19 single species, B T320A single species, C T3552B single species, D Reiter single species, E Dual species, F Triple species

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-10 5
Value
C
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DYQ05_01805
DYQ05_04220
DYQ05_03760
DYQ05_00105
DYQ05_06530
DYQ05_12330
DYQ05_01810
DYQ05_04130
DYQ05_04860
DYQ05_09360
DYQ05_13320
DYQ05_06590
DYQ05_04580
DYQ05_11595
DYQ05_06650
DYQ05_06975
DYQ05_11880
DYQ05_01815
DYQ05_13305
DYQ05_12325
DYQ05_01135
DYQ05_01230
DYQ05_06595
DYQ05_00625
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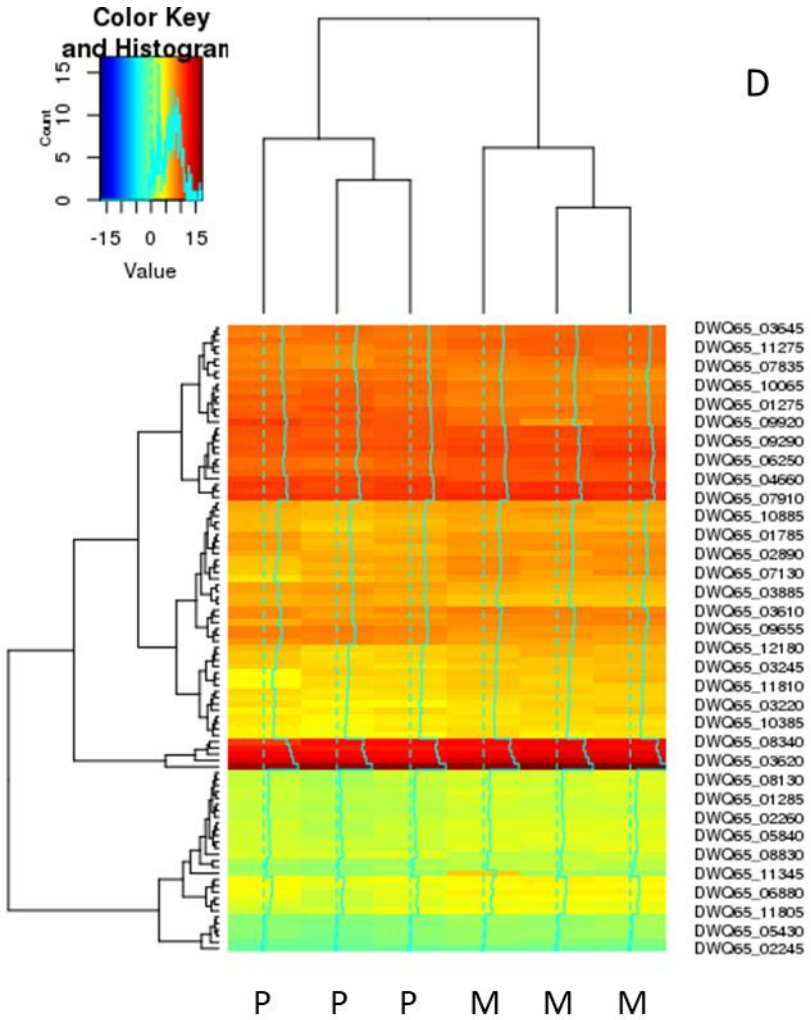
P P P M M M


Color Key and Histogram
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-15 0 15
Value
D
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DWQ65_11275
DWQ65_07835
DWQ65_10065
DWQ65_01275
DWQ65_09920
DWQ65_09290
DWQ65_06250
DWQ65_04660
DWQ65_07910
DWQ65_10885
DWQ65_01785
DWQ65_02890
DWQ65_07130
DWQ65_03885
DWQ65_03610
DWQ65_09655
DWQ65_12180
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DWQ65_11810
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DWQ65_08340
DWQ65_03620
DWQ65_08130
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DWQ65_02260
DWQ65_05840
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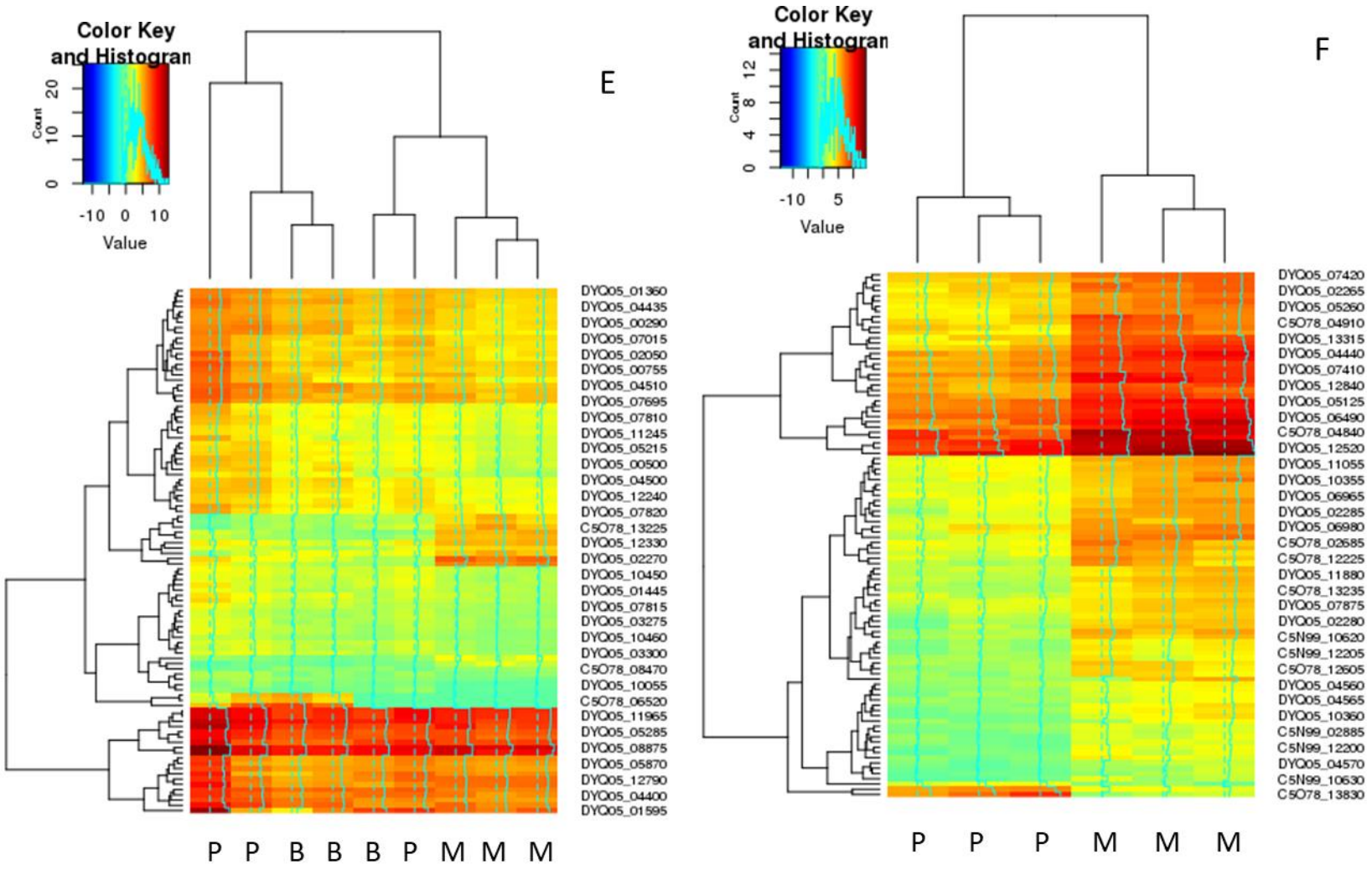
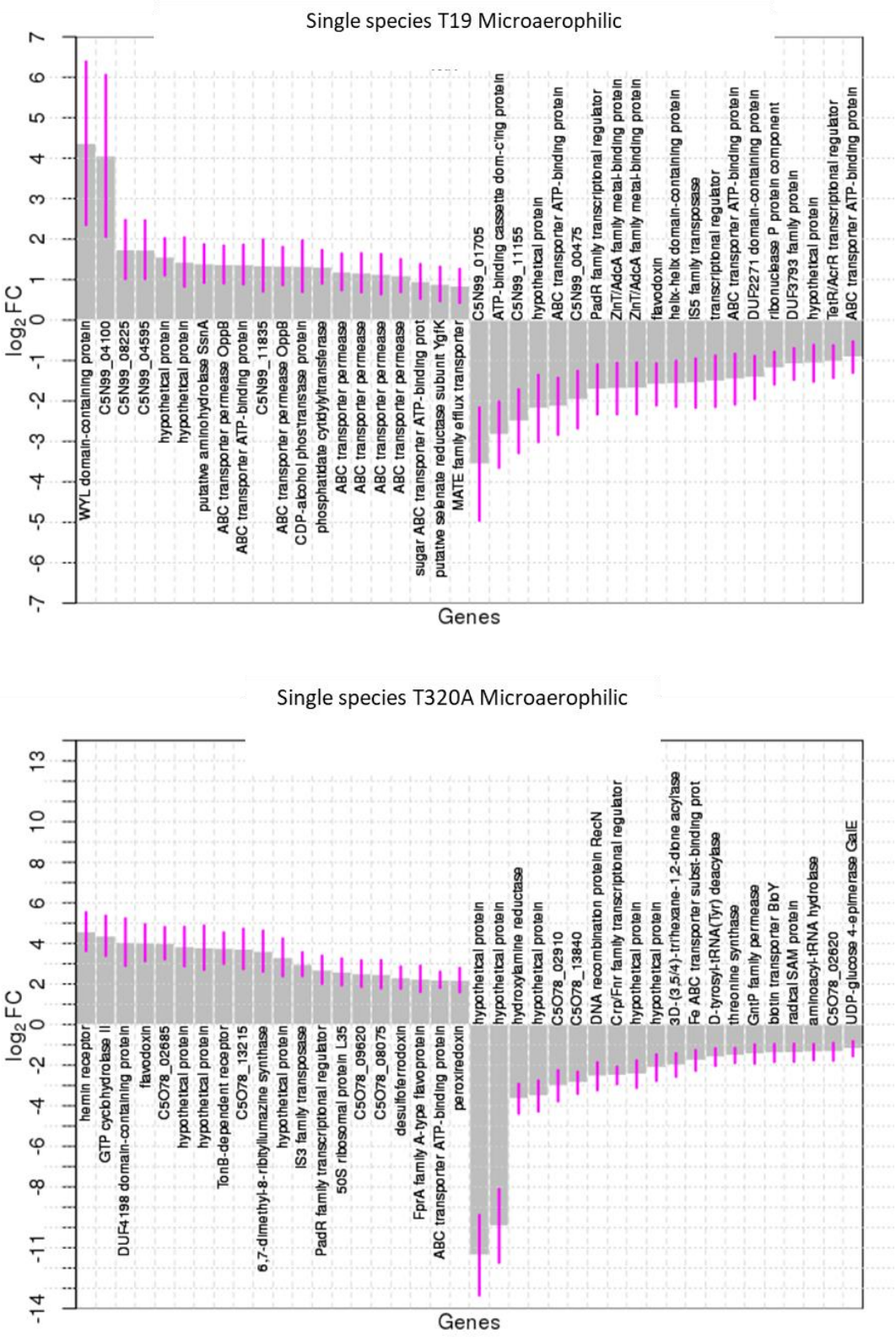
DWQ65_11805
DWQ65_05430
DWQ65_02245
P P P M M M



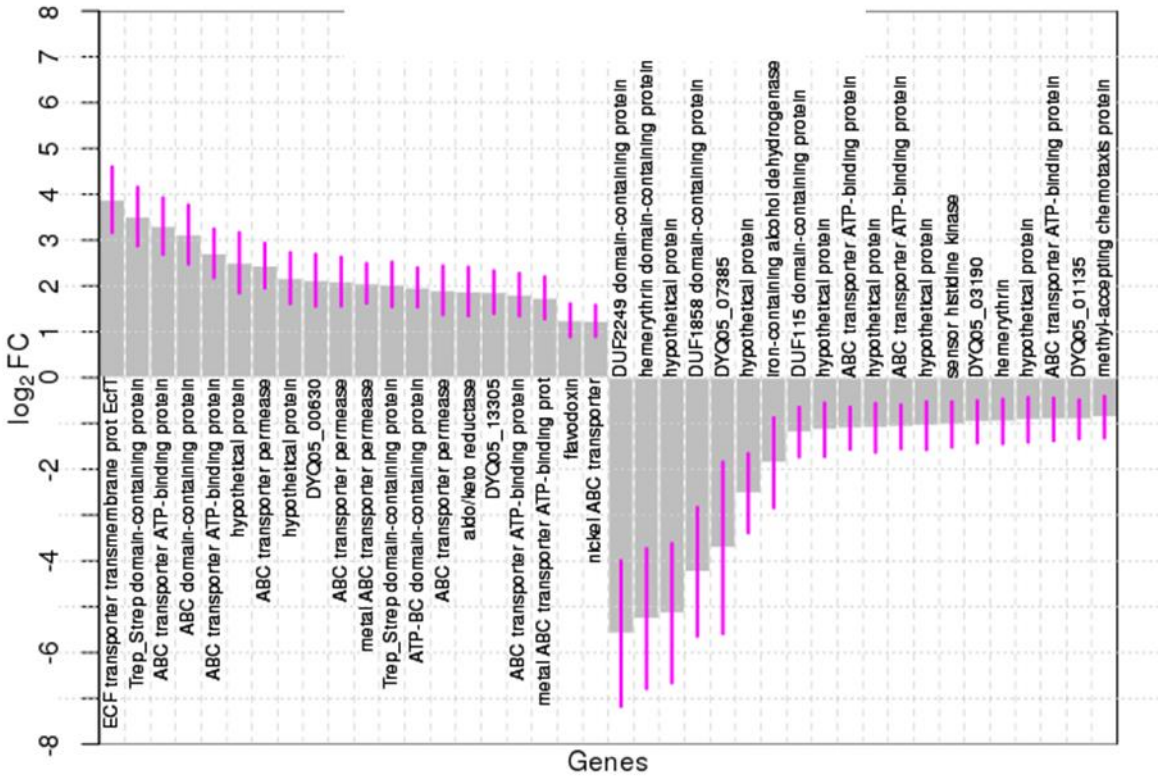
P= Planktonic sample, M= Microaerobic sample, B= Biofilm sample

Figure 2: Twenty most upregulated and twenty most downregulated genes (Logfold Change, log_2FC) for each sample type comparison

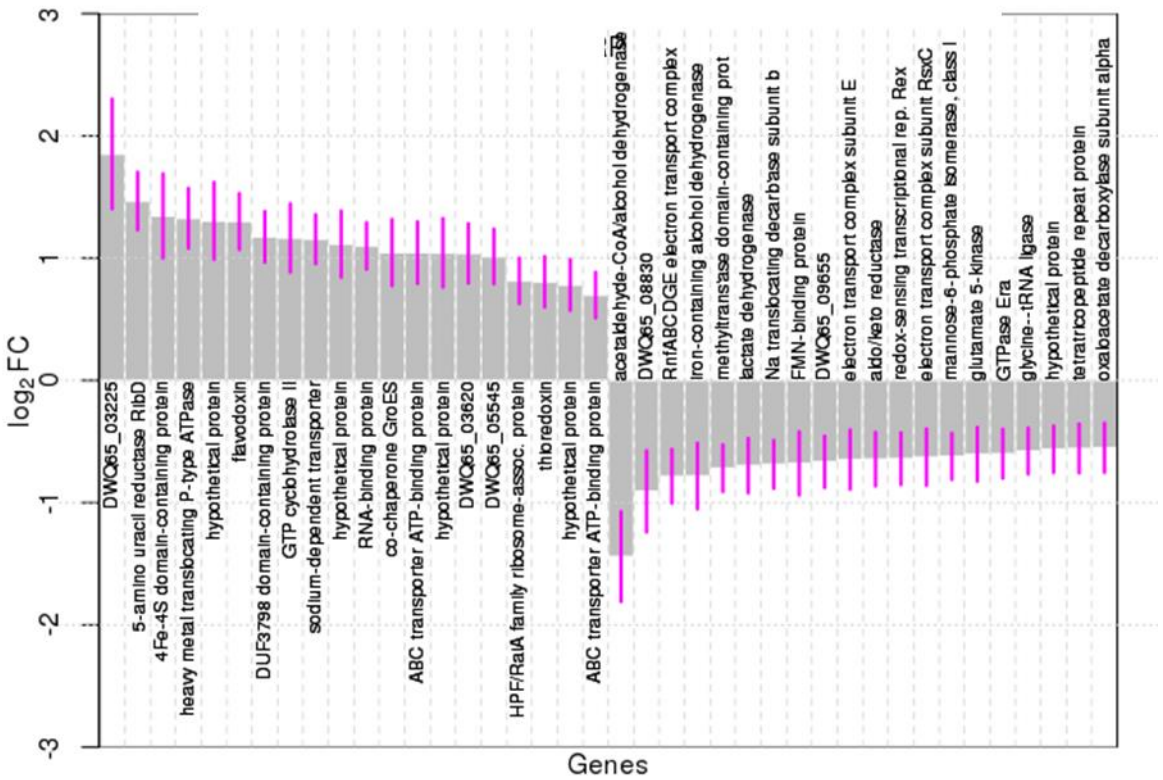
Purple bars represent standard errors for log_2FC
Genes not annotated in the database are labelled using locus tags

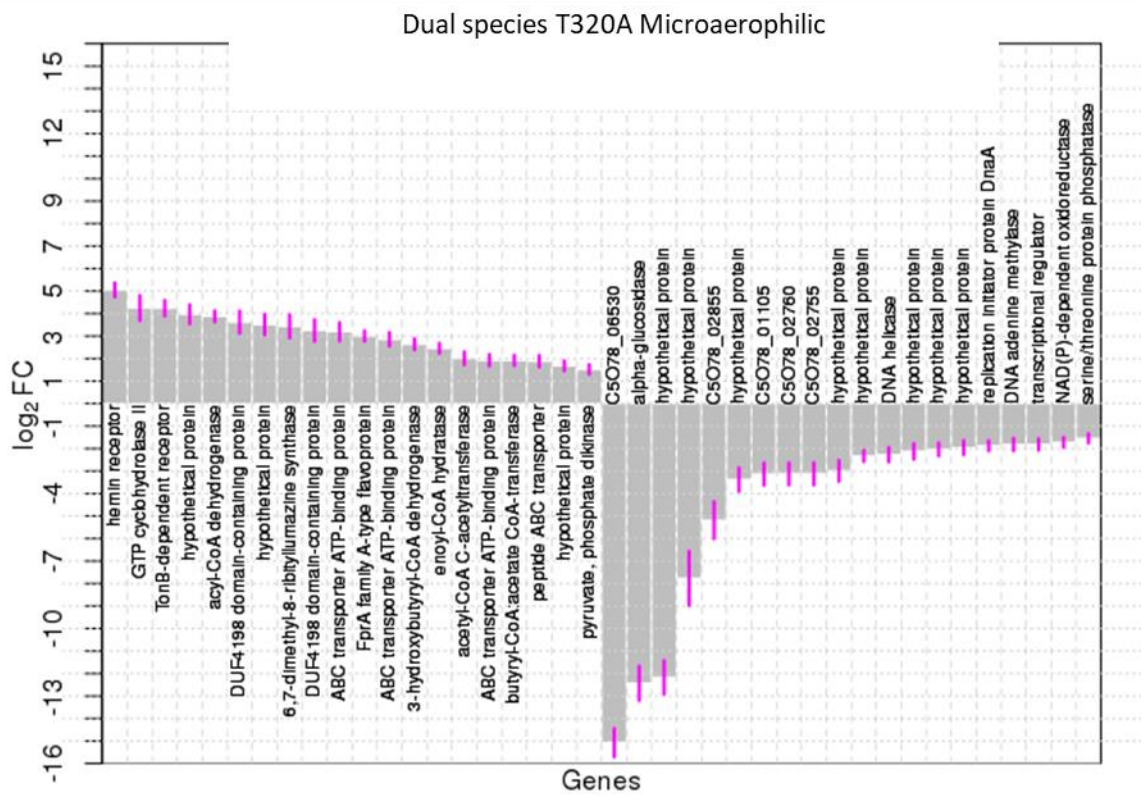


Single species T3552B Microaerophilic



Single species Reiter Microaerophilic




Dual species T320A Microaerophilic
log2 FC
15
12
9
7
5
3
1
-1
-4
-7
-10
-13
-16
hemin receptor
GTP cyclohydrolase II
TonB-dependent receptor
hypothetical protein
acyl-CoA dehydrogenase
DUF4198 domain-containing protein
hypothetical protein
6,7-dimethyl-8-ribityllumazine synthase
DUF4198 domain-containing protein
ABC transporter ATP-binding protein
FprA family A-type flavoprotein
ABC transporter ATP-binding protein
3-hydroxybutyryl-CoA dehydrogenase
enoyl-CoA hydratase
acetyl-CoA C-acetyltransferase
ABC transporter ATP-binding protein
butyryl-CoA:acetate CoA-transferase
peptide ABC transporter
hypothetical protein
pyruvate, phosphate dikinase
C5O78_06530
alpha-glucosidase
hypothetical protein
hypothetical protein
C5O78_02855
hypothetical protein
C5O78_01105
C5O78_02760
C5O78_02755
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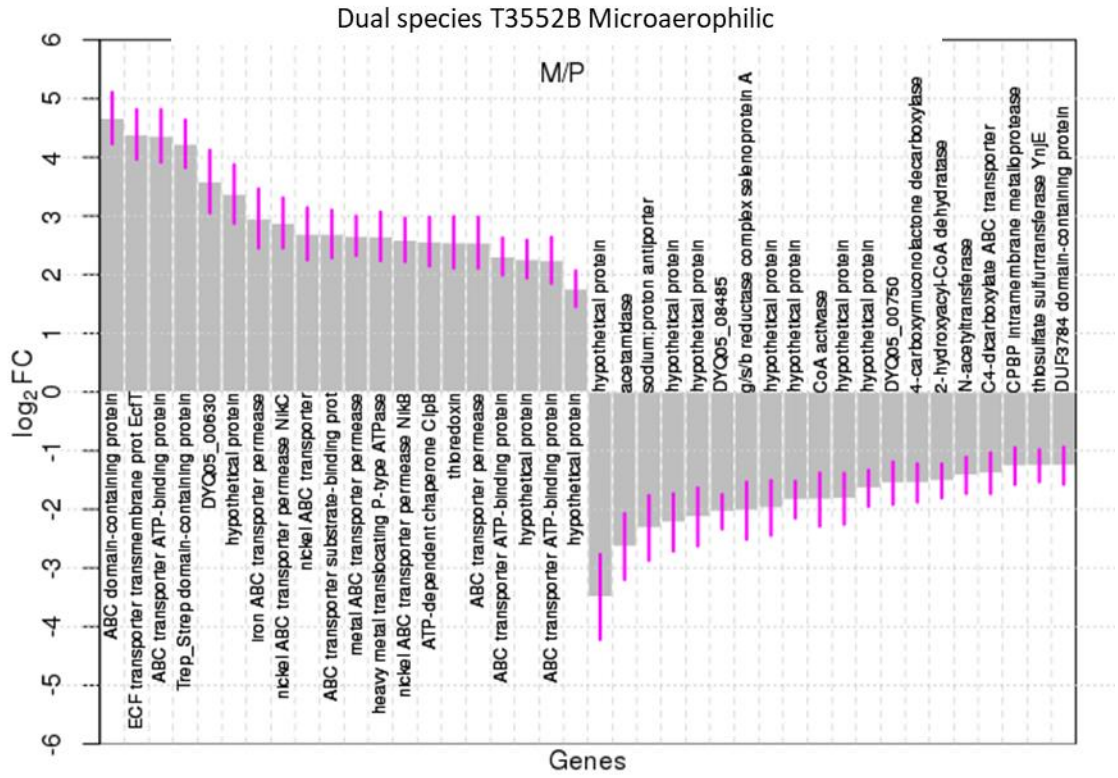
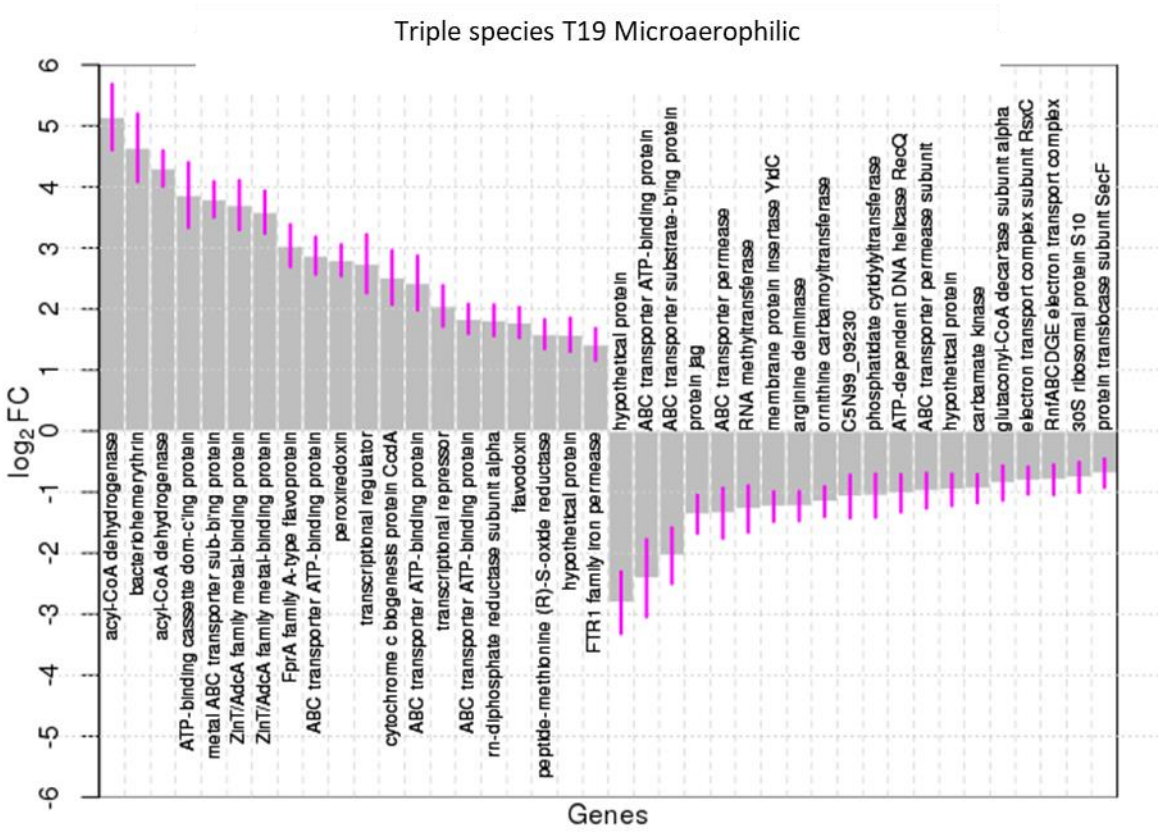
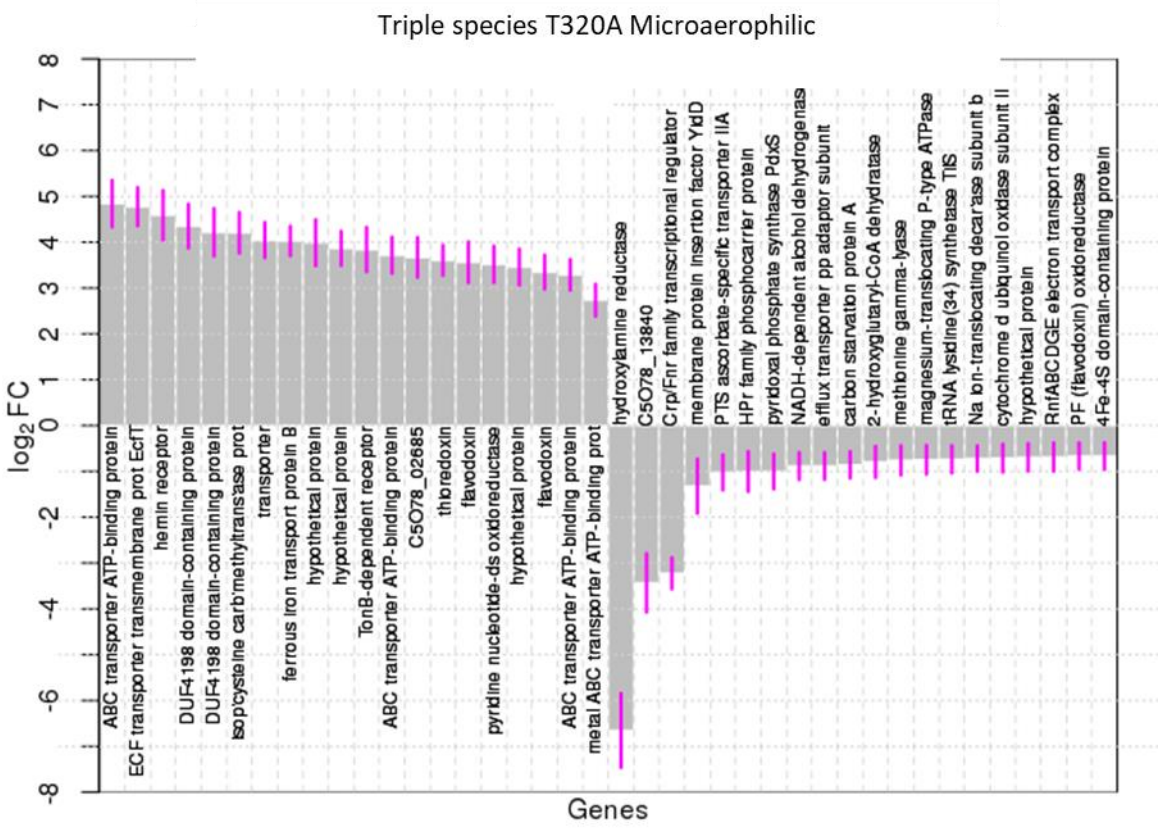
hypothetical protein
DNA helicase
hypothetical protein
hypothetical protein
hypothetical protein
replication initiator protein DnaA
DNA adenine methylase
transcriptional regulator
NAD(P)-dependent oxidoreductase
serine/threonine protein phosphatase
Genes


Dual species T3552B Microaerophilic
M/P
log2 FC
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
-6
ABC domain-containing protein
ECF transporter transmembrane prot EcfT
ABC transporter ATP-binding protein
Trep_Strep domain-containing protein
DYQ05_00630
hypothetical protein
Iron ABC transporter permease
nickel ABC transporter permease NikC
nickel ABC transporter
ABC transporter substrate-binding prot
metal ABC transporter permease
heavy metal translocating P-type ATPase
nickel ABC transporter permease NikB
ATP-dependent chaperone ClpB
thioredoxin
ABC transporter permease
ABC transporter ATP-binding protein
hypothetical protein
ABC transporter ATP-binding protein
hypothetical protein
hypothetical protein
acetamidase
sodium:proton antiporter
hypothetical protein
hypothetical protein
DYQ05_08485
g/s/b reductase complex selenoprotein A
hypothetical protein
hypothetical protein
CoA activase
hypothetical protein
hypothetical protein
DYQ05_00750
4-carboxymuconolactone decarboxylase
2-hydroxyacyl-CoA dehydratase
N-acetyltransferase
C4-dicarboxylate ABC transporter
CPBP intramembrane metalloprotease
thiosulfate sulfurtransferase YnjE
DUF3784 domain-containing protein
Genes


Triple species T19 Microaerophilic
log2 FC
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
-6
acyl-CoA dehydrogenase
bacterbhemerythrin
acyl-CoA dehydrogenase
ATP-binding cassette dom-c'ing protein
metal ABC transporter sub-b'ing protein
ZnT/AdcA family metal-binding protein
ZnT/AdcA family metal-binding protein
FprA family A-type flavoprotein
ABC transporter ATP-binding protein
peroxiredoxin
transcriptional regulator
cytochrome c biogenesis protein CcdA
ABC transporter ATP-binding protein
transcriptional repressor
ABC transporter ATP-binding protein
rn-diphosphate reductase subunit alpha
flavodoxin
peptide-methionine (R)-S-oxde reductase
hypothetical protein
FTR1 family iron permease
hypothetical protein
ABC transporter ATP-binding protein
ABC transporter substrate-b'ing protein
protein tag
ABC transporter permease
RNA methyltransferase
membrane protein insertase YidC
arginine deiminase
ornithine carbamoyltransferase
C5N99_09230
phosphatidate cytidylyltransferase
ATP-dependent DNA helicase RecQ
ABC transporter permease subunit
hypothetical protein
carbamate kinase
glutaconyl-CoA decar'ase subunit alpha
electron transport complex subunit RsxC
RnfABCDGE electron transport complex
30S ribosomal protein S10
protein translocase subunit SecF
Genes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ECF transporter transmembrane prot EcfT
hemin receptor
DUF4198 domain-containing protein
DUF4198 domain-containing protein
isopcysteine carb'methyltransase prot
transporter
ferrous iron transport protein B
hypothetical protein
hypothetical protein
TonB-dependent receptor
ABC transporter ATP-binding protein
C5O78_02685
thioredoxin
flavodoxin
pyridine nucleotide-ds oxdoreductase
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flavodoxin
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metal ABC transporter ATP-binding prot
hydroxylamine reductase
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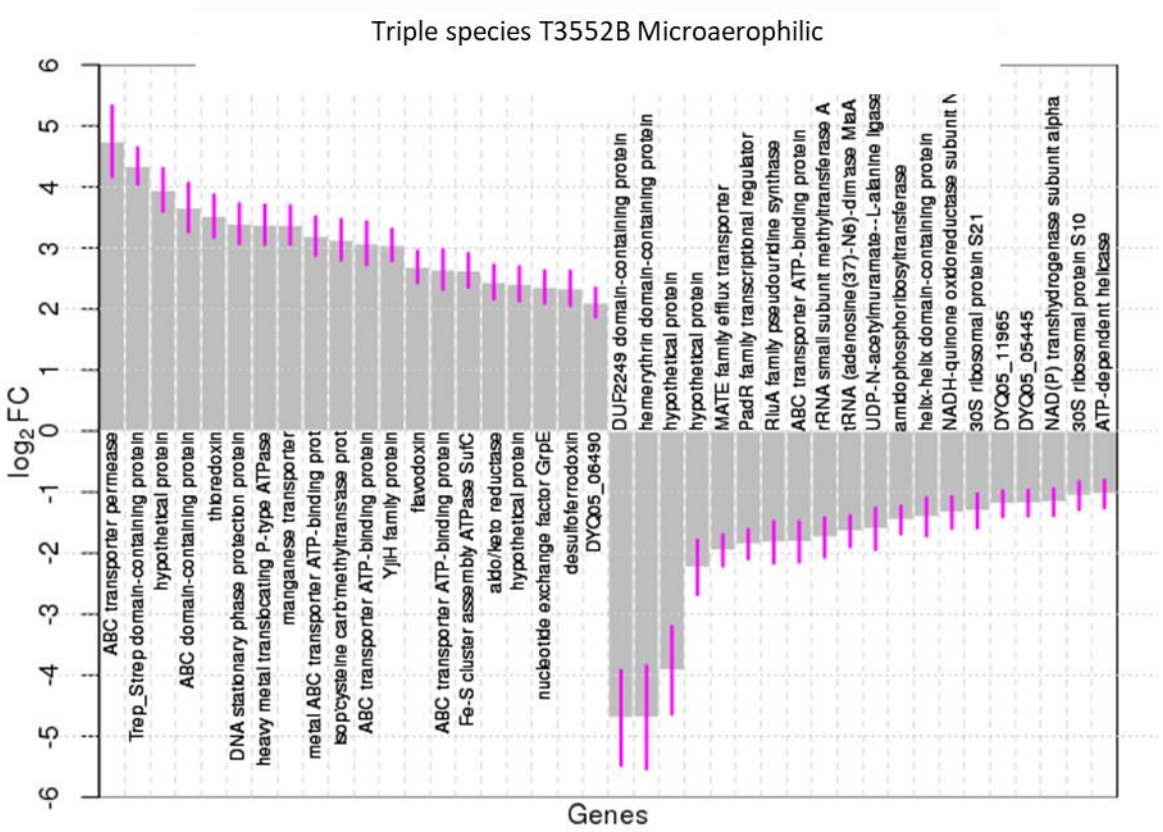
Crp/Fnr family transcriptional regulator
membrane protein insertion factor YidD
PTS ascorbate-specific transporter IIA
HPr family phosphocarrier protein
pyridoxal phosphate synthase PdxS
NADH-dependent alcohol dehydrogenas
efflux transporter pp adaptor subunit
carbon starvation protein A
2-hydroxyglutaryl-CoA dehydratase
methionine gamma-lyase
magnesium-transbcating P-type ATPase
tRNA lysidine(34) synthetase TilS
Na ion-transbcating decar'ase subunit b
cytochrome d ubiquinol oxdase subunit II
hypothetical protein
RnfABCDGE electron transport complex
PF (flavodoxin) oxdoreductase
4Fe-4S domain-containing protein
Genes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Trep_Strep domain-containing protein
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ABC domain-containing protein
thioredoxin
DNA stationary phase protection protein
heavy metal translocating P-type ATPase
manganese transporter
metal ABC transporter ATP-binding prot
isopcysteine carbmethyltransase prot
ABC transporter ATP-binding protein
YjiH family protein
flavodoxin
ABC transporter ATP-binding protein
Fe-S cluster assembly ATPase SufC
aldo/keto reductase
hypothetical protein
nucleotide exchange factor GrpE
desulfoferrodoxin
DYQ05_06490
DUF2249 domain-containing protein
hemerythrin domain-containing protein
hypothetical protein
hypothetical protein
MATE family efflux transporter
PadR family transcriptional regulator
RluA family pseudouridine synthase
ABC transporter ATP-binding protein
rRNA small subunit methyltransferase A
tRNA (adenosine(37)-N6)-dimase MtaA
UDP-N-acetylmuramate--L-alanine ligase
amidophosphoribosyltransferase
helix-helix domain-containing protein
NADH-quinone oxidoreductase subunit N
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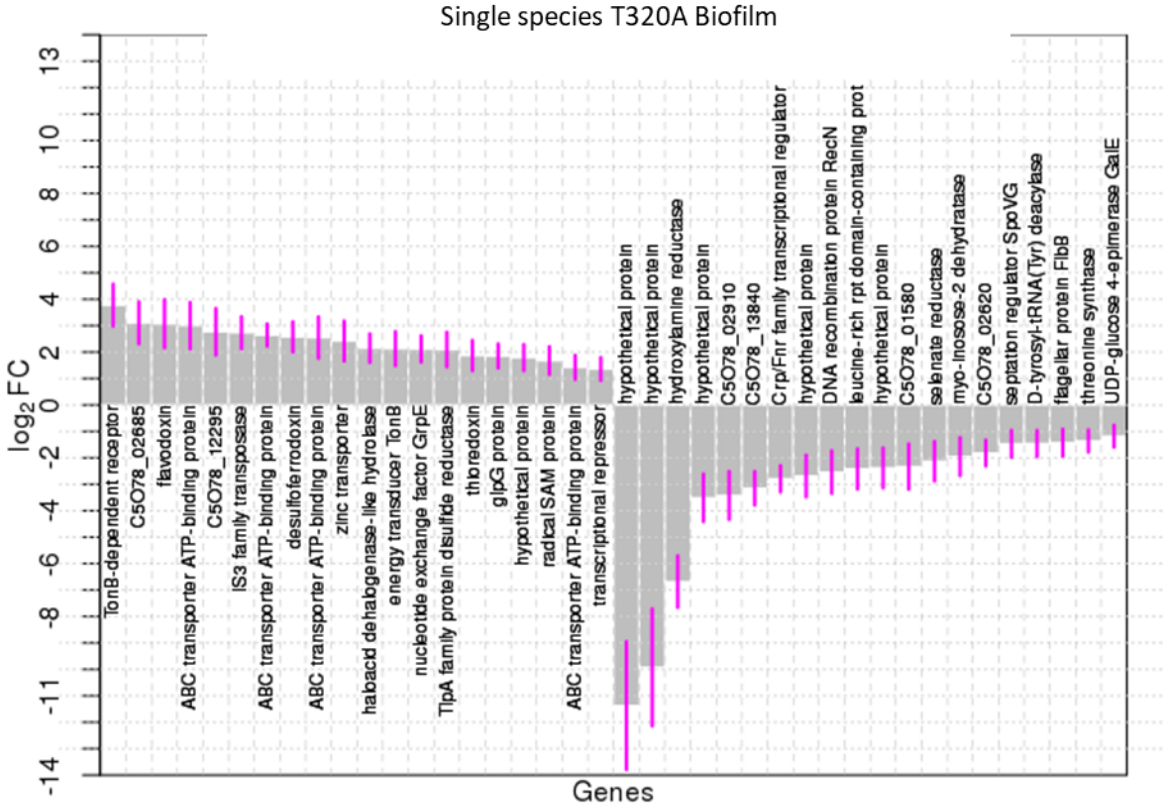
DYQ05_11965
DYQ05_05445
NAD(P) transhydrogenase subunit alpha
30S ribosomal protein S10
ATP-dependent helicase
Genes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flavodoxin
ABC transporter ATP-binding protein
C5O78_12295
IS3 family transposase
ABC transporter ATP-binding protein
desulfoferrodoxin
ABC transporter ATP-binding protein
zinc transporter
haloacid dehalogenase-like hydrolase
energy transducer TonB
nucleotide exchange factor GrpE
TlpA family protein disulfide reductase
thioredoxin
glpG protein
hypothetical protein
radical SAM protein
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transcriptional repressor
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DNA recombination protein RecN
leucine-rich rpt domain-containing prot
hypothetical protein
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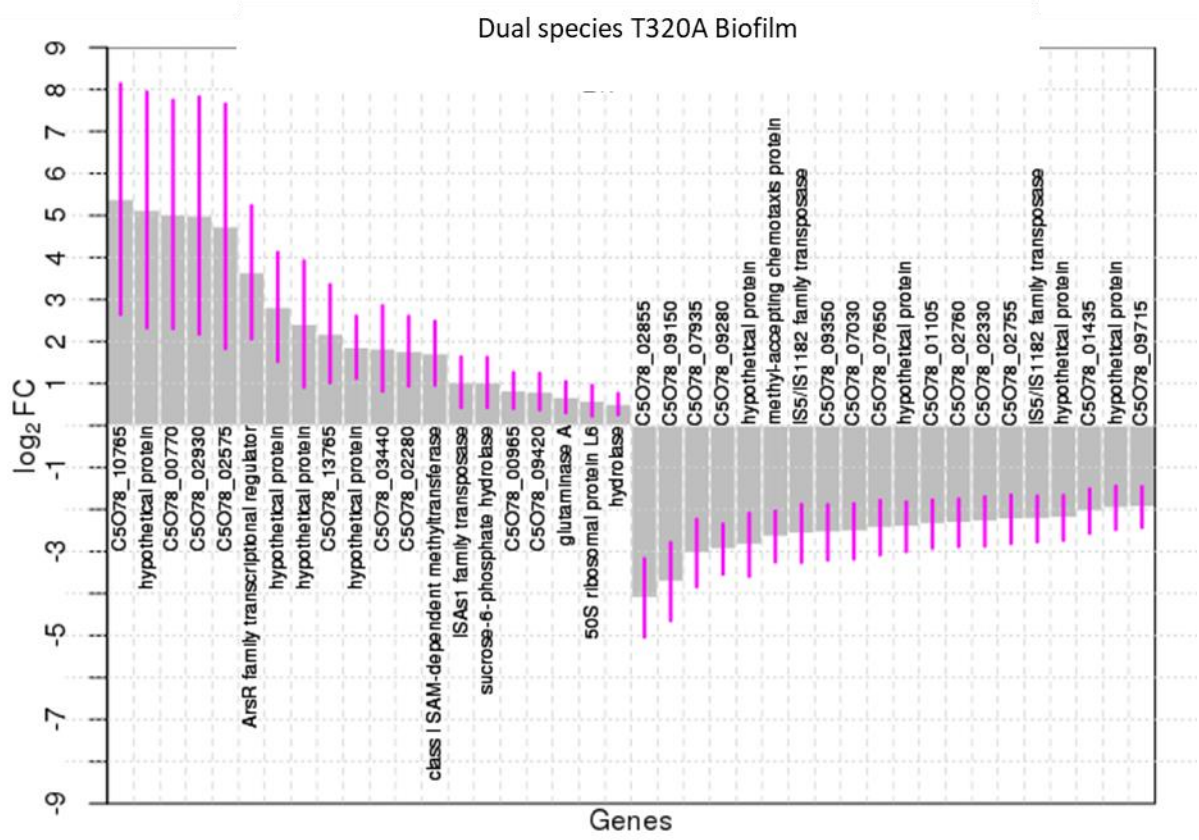
selenate reductase
myo-inosose-2 dehydratase
C5O78_02620
septation regulator SpoVG
D-tyrosyl-tRNA(Tyr) deacylase
flagellar protein FlbB
threonine synthase
UDP-glucose 4-epimerase GalE
Genes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C5O78_02930
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ArsR family transcriptional regulator
hypothetical protein
hypothetical protein
C5O78_13765
hypothetical protein
C5O78_03440
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class I SAM-dependent methyltransferase
ISAs1 family transposase
sucrose-6-phosphate hydrolase
C5O78_00965
C5O78_09420
glutaminase A
50S ribosomal protein L6
hydrolase
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hypothetical protein
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IS5/IS1182 family transposase
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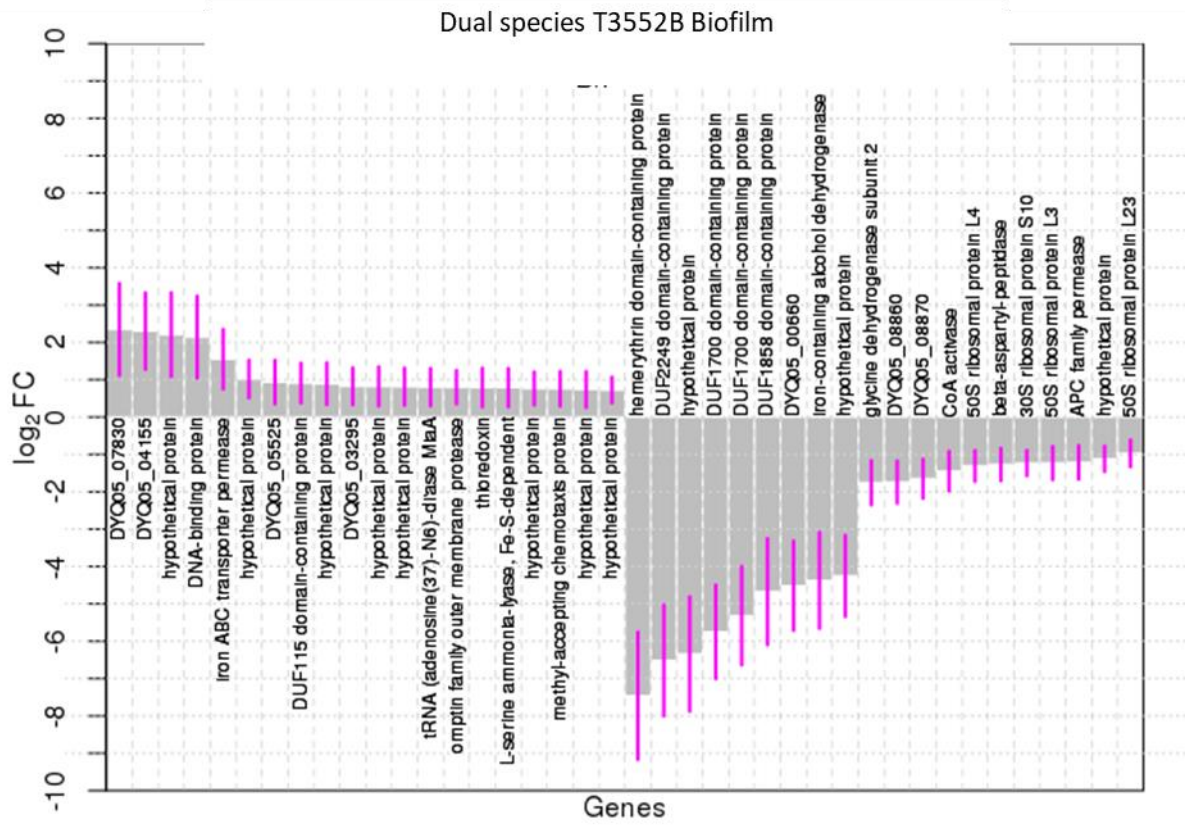
C5O78_09715
Genes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DYQ05_04155
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DNA-binding protein
iron ABC transporter permease
hypothetical protein
DYQ05_05525
DUF115 domain-containing protein
hypothetical protein
DYQ05_03295
hypothetical protein
hypothetical protein
tRNA (adenosine(37)-N6)-dtase MtaA
ompdn family outer membrane protease
thioredoxin
L-serine ammonia-lyase, Fe-S-dependent
hypothetical protein
methyl-accepting chemotaxis protein
hypothetical protein
hypothetical protein
hemerythrin domain-containing protein
DUF2249 domain-containing protein
hypothetical protein
DUF1700 domain-containing protein
DUF1700 domain-containing protein
DUF1858 domain-containing protein
DYQ05_00660
iron-containing alcohol dehydrogenase
hypothetical protein
glycine dehydrogenase subunit 2
DYQ05_08860
DYQ05_08870
CoA activase
50S ribosomal protein L4
beta-aspartyl-peptidase
30S ribosomal protein S10
50S ribosomal protein L3
APC family permease
hypothetical protein
50S ribosomal protein L23
Genes

Table 2 Protein categories identified from differentially expressed genes (-1>log$_2$FC>1, FDR<0.05) under microaerobic conditions using BRITE hierarchies in Blast KOALA.

Upregulated categories have a white background, downregulated categories are shaded light grey.

Category	Subcategory	Sample type	No of KO identifiers in each sample set	No of Proteins	Additional information/ interpretation
Signalling and cellular processes	Transporters	T19 M	12	14	Various transport systems for sugars, iron/nickel, magnesium/ cobalt, LPS, thiamine.
		T320A M	6	8	Including iron and nickel transporters
		T3552B M	14	17	ABC transporters
		Reiter M	1	1	Iron complex transporter
		T320A+T3552B M	40	61	Dominated by proteins with roles in iron acquisition and nickel transport systems
		T320A+T3552B M	7	10	Includes the four methyl-accepting chemotaxis protein receptors, the cheY chemotaxis protein and flagellar proteins
		T19+T320A+T3552B M	45	84	Dominated by proteins with roles in iron acquisition and nickel transporters. Also includes a formate transporter protein; formate functions to combat oxidative stress.
		T320A M	3	3	ABC transporters for iron and biotin
		T19+T320A+T3552B M	5	6	ABC transporters and a phosphotransferase system

	Bacterial motility proteins	T320A M	1	1	Flagellin synthesis
	Exosome	T320A+T3552B M	34	39	Enzymes needed for metabolism, molecular chaperones and the enzyme peroxiredoxin; protection against oxidative stress.
	Ribosome	T320A+T3552B M	8	8	Proteins for large and small ribosomal subunits
Genetic information processing	Chaperones and folding catalysts	T320A+T3552B M	9	11	Mostly heat shock proteins
		T19+T320A+T3552B M	10	15	Mostly heat shock proteins but includes the protein folding catalyst thioredoxin I which indicates a response to oxidative stress; potentially acting to refold polypeptides misfolded due to cellular stress.
		T19 M	6	8	Large and small ribosomal subunits
	Transcription factors	T320A+T3552B M	5	5	Transcriptional regulators
	Unclassified	T19 M	14	15	Proteins needed for ribosome and tRNA biogenesis, protein folding, DNA replication, repair and recombination.
		T320A M	7	7	Transcriptional regulator, Large ribosomal subunit, Heat shock proteins
		Reiter M	3	2	Transcriptional regulator, Heat shock proteins
		T19+T320A+T3552B M	20	21	Various categories indicating downregulation of transcription and translation.

	DNA repair and recombination proteins	T320A+T3552B M	7	7	Correlates with identified KEGG pathways for DNA replication and repair
Metabolism	Enzymes	T19 M	17	18	Including enzymes needed in sugar, vitamin and amino acid metabolism, and DNA helicase for DNA replication
		T320A M	6	6	Oxidoreductases, transferases, hydrolases, lyases
		Reiter M	5	5	Oxidoreductases, transferases, hydrolases, lyases
		T320A+T3552B M	56	59	Oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases and translocases. Highlighting superoxide dismutase, protecting against oxidative stress.
		T19+T320A+T3552B M	34	39	Oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases and translocases. Various functions in metabolism and transport. Also includes peroxiredoxins (antioxidant enzymes) and superoxide dismutase.
		T320A M	7	7	Oxidoreductases, hydrolases, lyases, isomerases
		Reiter M	1	1	Acetaldehyde dehydrogenase, and oxidoreductase
		T320A+T3552B M	31	32	Oxidoreductases, transferases, hydrolases, lyases,

					isomerases, ligases, translocases
		T19+T320A+T3552BM	25	26	Oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, translocases. Reflects the downregulation of amino acid metabolism, arginine biosynthesis and purine and pyrimidine metabolism.

Table 3 Description of genes upregulated under oxidative stress conditions

Gene	**Explanation and references**
Flavodoxin I fldA/nifF/isiB	Transfer electrons in metabolic reactions. They do require riboflavin to function and are like ferredoxin (Sepúlveda Cisternas et al., 2018).
rnfG; H+/Na+-translocating ferredoxin:NAD+ oxidoreductase subunit G rnfG; H+/Na+-translocating ferredoxin:NAD+ oxidoreductase subunit G	An electron transport pathway containing ferredoxin, enabling anaerobic respiration (Biegel & Müller, 2010).
Dps, starvation inducible DNA binding protein	Protects DNA during stationary phase making it DNase resistant (Almiron et al., 1992), and confers resistance to hydrogen peroxide which can cause oxidative damage to DNA. Also needed for *Borrelia burgdorferi* persistence in ticks (Li et al., 2007) and was identified as a putative virulence factor in BDD treponemes (Staton, Clegg, et al., 2021)
Dfx; superoxide reductase	Catalyses the reduction of superoxide to hydrogen peroxide (via NADP and rubredoxin), which is then reduced to water by peroxidases. This is in contrast to the mechanism SOD uses by which superoxide is reduced to oxygen(Jenney et al., 1999).
SOD(Cu-Zn)	Protects anaerobes from the toxic effects of oxygen (Jenney et al., 1999)
trxA/ trxB	This is the thioredoxin system, which is ubiquitous in all living organisms and is a defence against oxidative stress and has roles in growth and apoptosis (Arnér & Holmgren, 2000). It has similar functions to the glutathione peroxidase system, thus raising the question of why T19 and T3552B have GSH, but T320A and Reiter don't according to MAUVE. trxA/B is present in T19 and Reiter, but not upregulated, so how does this affect them?!

	Linked to protein repair as thioredoxin is the hydrogen donor for methionine sulfoxide reductases (msrAB). Many peroxiredoxins (which catalyse reduction of H_2O_2) require reduction by thioredoxin (Arnér & Holmgren, 2000).
HSP90A/ molecular chaperone HtpG	Optimum folding of newly synthesised cellular proteins under stress conditions (Grudniak et al., 2015), including the effects of reactive oxygen species (Dang et al., 2011). May also have a role in virulence (Genest et al., 2019).
DnaK molecular chaperone, DnaJ molecular chaperone and GrpE nucleotide exchange factor	All essential to Hsp90 function for remodelling and reactivating proteins (Genest et al., 2019). DnaJ and GrpE regulate the DnaK reaction cycle(Kim et al., 2013). DNAK is important at entry to stationary phase.
groES groEL/ HspD1	GroES and GroEL are also referred to as chaperonins. They protect proteins from the aggregation-promoting cytosol during folding and operate post-translationally (Kim et al., 2013). GroES is a co-factor for GroEL (Takei et al., 2012). GroEL is known to be fairly resistant to oxygen as it remains fully functional, even when all 23 of its surface methionine residues are oxidised. GroEL has been identified as a putative virulence factor in BDD treponemes (Staton, Clegg, et al., 2021).
Hsp 20 Hsp33	Small heat shock proteins play an important role in maintaining protein homeostasis (proteostasis) within cells. They protect substrates from further aggregation until they can be refolded by chaperones from the larger Hsp families(Obuchowski et al., 2019). Hsp 33 is activated when oxidative stress and unfolding conditions coincide (Krewing et al., 2019)
MsrAB; peptide methionine sulfoxide reductase	Methionine is particularly prone to oxidation compared to other amino acids. Msr enzymes salvage proteins by catalysing the reduction of oxidised methionine residues (Singh et al., 2018).
ahpC or Peroxiredoxin	Multiple protective effects during oxidative stress including protection of DNA from oxidative damage and molecular chaperone activity. Possibly also a virulence factor. AhpC may also be induced by other general stresses including glucose starvation and entry into stationary phase although to a lesser degree (Dubbs & Mongkolsuk, 2007).
TroA/mntA/znuA TroB/mntB/znuC TroC/mntC/znuB	Contained on the tro operon, these form metal transporters analogous to ABC transporters and are responsible for acquiring essential metals from host tissues (Gherardini et al., 2006)
CopA	Copper membrane transporter (Zagorski & Wilson, 2004)
TonB	Transports hemin-binding proteins that have bound heme into cells (Cullen et al., 2004)
yclQ/ceuA yclN/ceuB	Part of an ABC transporter system reported in *Bacillus subtilis* for petrobactin uptake; a siderophore which functions to acquire iron (Zawadzka et al., 2009)
irtA/ybtP irtB/ybtQ	ABC transporter for iron acquisition and assimilation described in *Mycobacterium tuberculosis* (Ryndak et al., 2010).
TC.FEV.OM2, cirA, cfrA; hmuR	TonB-dependent transport system, an outer membrane iron transporter. These can also be used as a means of entry to cells by phage and colicins (Noinaj et al., 2010).
feoA feoB	ABC ferrous iron transporters, shown to have a role in virulence of *Salmonella enterica* serovar Typhimurium (Boyer et al., 2002).

ABC.PE.P ABC.PE.P1 ABC.PE.S	Components of ABC peptide transport systems
ABC.FEV.P ABC.FEV.S ABC.FEV.A	Components of ABC iron transport systems
ddpD ddpF	The ddpABCDF operon is thought to be turned on at the start of stationary phase enabling use of the dipeptide D-Ala-D-Ala, which could then be oxidized as an energy source for cell survival under starvation conditions (Lessard & Walsh, 1999). Also has a role in transcription of cysteine protease(Lyon et al., 1998).
sufC sufB	Components of the Suf pathway required for biogenesis of iron-sulphur (Fe-S) clusters which are essential for many cell functions. Fe-S are susceptible to disruption by iron starvation or oxidative stress. The Suf pathway is a specialised system for Fe-S cluster biosynthesis under stress (Layer et al., 2007).
htsA htsB	Heme transport system protein permease described in Group A *Streptococcus* and in *Staphylococcus aureus* (Lu et al., 2012; Mason & Skaar, 2009).
znuA	
nikA/ cntA nikB/ cntB nikC/ cntC nikD/ cntD nikE/cntF	Components of an ABC transporter family providing Ni^{2+} for the anaerobic biosynthesis of hydrogenases. Nickel-containing enzymes are involved in at least five metabolic processes including detoxification of superoxide anion radicals (Eitinger & Mandrand-Berthelot, 2000).
cbiK	Transport of nickel and cobalt ions needed as cofactors for enzymes in a variety of metabolic processes (Rodionov et al., 2006).
sitA sitB sitC (Salmonella iron transporter)	ABC divalent metal ion transporter responsible for manganese and iron uptake. May also contribute to virulence and have a role in resistance to oxidative stress, as demonstrated in *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (APEC) 078 (Boyer et al., 2002; Sabri et al., 2008).
FTR/ FTH1/ efeU	High-affinity iron transporter (Cao et al., 2007).
P19/ ftrA	High-affinity iron transporter (Chan et al., 2010)
rcnA	Nickel and cobalt efflux protein, also required in low nickel growth conditions for nickel import via NikABCDE (Iwig et al., 2006).
ftnA (ferritin)	Intracellular iron storage, which may be a mechanism for reducing accumulation of ROS under oxidative stress conditions (Orino et al., 2001).
perR, Fur family transcriptional regulator (repressor in original data)	Metal-responsive transcriptional repressor (Ferric uptake regulator). Peroxide exposure prevents PerR from binding to DNA, thus allowing transcription of ahpC and other genes involved in defense against oxidative stress (Dubbs & Mongkolsuk, 2007).
cheY	Chemotaxis protein, found in motile and non-motile bacteria, therefore may comprise a more general means of regulating cellular processes that are not limited to motility (Lux & Shi, 2006).
Mcp	Methyl-accepting chemotaxis proteins. These are generally transmembrane proteins that monitor the environment, functioning as chemoreceptors, and ultimately effecting changes in motility which are mediated via cheY(Lux & Shi, 2006).
Wbpl/ wlbD	Involved in synthesis of the bacterial capsule precursor UDP-N-acetyl-D-mannosamine (Campbell et al., 2000).

yajC	A membrane protein which is part of the SecY translocation complex which has secretory function (Flower, 2007).
tlyC	Virulence gene involved in haemolysis (Fraser & Brown, 2017).
RelE/ stbE RelB/ dinJ	RelE/RelB is a toxin-antitoxin system. Overexpression of RelE is triggered by amino acid starvation. The effect is to inhibit translation and provoke stimulation of gene expression to increase amino acid biosynthesis. RelB counteracts the toxic effect of RelE. It is an autorepressor of RelE transcription (S. K. Christensen et al., 2001).
FlgK FlgB and FlgC FlgD	Parts of the motility apparatus: Hook-filament interface Rod Hook formation (Charon et al., 2006)
FlgM negative regulator of flagellin synthesis	Negative regulator of flagellin synthesis. Unknown for Treponema whether this is a virulence factor. $FlgM^-$ mutants are not virulent in *Salmonella typhimurium but* are fully virulent for *Yersinia enterocolitica*(Kapatral et al., 1996).
FliL flagellar protein	Precise function remains unknown and likely varies depending on bacterial species, however it is suggested that it coordinates or regulates the orientation of periplasmic flagellae in *Borrelia burgdorferi*(Motaleb et al., 2011).
GldF	Described in *Flavobacterium johnsoniae* as responsible for gliding motility; thought to encode components of an ABC transporter required for motility (McBride et al., 2003).

Table 4 Description of genes downregulated under oxidative stress conditions

Gene	
uvrABC	DNA repair by nucleotide excision repair; removing damaged DNA through excision of an oligonucleotide that contains the lesion (Theis et al., 2000).
ligA/ ligB	DNA ligases needed for DNA replication and repair
rubredoxin	Electron donor for reduction of superoxide to hydrogen pe(Coulter & Kurtz, 2001) stress (Coulter & Kurtz, 2001).
afu/ fbpA	Component of and iron transport system
ABC.FEV.A ABC.FEV.S ABC.FEV.P	Components of ABC iron transport systems
sitB	ABC divalent metal ion transporter responsible for manganese and iron uptake. May also contribute to virulence and have a role in resistance to oxidative stress, as demonstrated in *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (APEC) 078(Boyer et al., 2002; Sabri et al., 2008). T19 ONLY
Exfoliative toxin eta A/B	Identified in *Staphylococcus aureus,* these toxins are proteases which cause exfoliative skin disease by unknown mechanisms which result in epidermal detachment at the stratum granulosum
yoeB toxin	yoeB toxin is involved in induction of programmed cell death. It is an autorepressor, meaning that the presence of the protein itself will repress the expression of that protein (Kędzierska et al., 2007).
pilZ	PilZ domain-containing proteins are receptors for c-di-GMP, a ubiquitous bacterial messenger that regulates many processes including motility, biofilm formation, DNA repair, cell development and virulence(Galperin & Chou, 2020). T19 ONLY
BapA	Large protein associated with the cell surface and needed for recruitment into a biofilm matrix, and for host cell invasion (Latasa et al., 2005).

cheW	Coupling factor connecting MCPs with the central histidine kinase CheA in chemotaxis signal transduction pathways (Lux & Shi, 2006).
MCP	Transmembrane chemoreceptors which continually monitor the environment (Lux & Shi, 2006).
yidC	Part of the Sec operon for protein translocation
gldA	Described in *Flavobacterium johnsoniae* as responsible for gliding motility; thought to encode components of an ABC transporter required for motility (McBride et al., 2003).

Table 5 Pathways upregulated in microaerobic samples as identified by BlastKOALA using KEGG Pathway Reconstruction

Pathway	**Sample type**	**No of KO identifiers in each sample set**	**No of Proteins**	**Additional information/ interpretation**
Metabolism>Global and overview maps>01100 Metabolic Pathways	T19 M T320A M T320A+T3552B M T19+T320A+T3552B M	7 4 28 15	7 4 28 15	Amino acid, sugar and fatty acid metabolism Amino acid, sugar and riboflavin metabolism Predominantly carbohydrate metabolism Predominantly carbohydrate metabolism
Metabolism>Global and overview maps>01110 Biosynthesis of secondary metabolites	T19 M T320A+T3552B M T19+T320A+T3552B M	3 14 11	3 14 11	Includes peptidoglycan biosynthesis Predominantly carbohydrate and riboflavin metabolism Including fatty acids, amino acids and riboflavin
Genetic Information Processing>03030, 03420, 03420 Replication and Repair	T19 M	3	3	Replicative DNA helicase, nucleotide excision repair (during transcription), mismatch repair exodeoxyribonuclease
Environmental Information Processing>Membrane transport>02010 ABC transporters	T19 M T320A M T3552B M T320A+T3552B M T19+T320A+T3552B M	4 3 6 15 16	5 4 6 27 35	Including nickel, LPS and thiamine transport Iron and nickel transporters Manganese/ zinc/ iron, nickel and iron transporters

				Iron, nickel, zinc, manganese and riboflavin transporters. Iron, nickel, zinc, manganese and riboflavin transporters.
Cellular Processes>Cellular community- prokaryotes	T320A M T3552B M T320A+T3552B M T19+T320A+T3552B M	2 4 8 6	2 5 12 11	02024 Quorum sensing preprotein translocase subunit yajC 02025, 02026 negative regulator of flagellin synthesis FlgM found in *Escherichia coli* and *Pseudomonas aeruginosa* 02024 Quorum sensing Peptide/ nickel transport system substrate-binding protein 02024 Quorum sensing Mostly peptide/ nickel transport systems 02024 Quorum sensing Mostly peptide/ nickel transport systems
Genetic Information>Translation>03010 Ribosome	T320A M T320A+T3552B M	4 8	4 8	Large ribosomal subunit Large and small ribosomal subunits
Metabolism>Metabolism of cofactors and vitamins>Riboflavin metabolism	Reiter M	4	4	Riboflavin metabolism
Metabolism>Global and overview maps>01240 Biosynthesis of cofactors	T320A+T3552B M T19+T320A+T3552B M	8 5	8 5	Linked to riboflavin synthesis and flavodoxins Riboflavin metabolism
Metabolism>Global and overview maps>01120	T320A+T3552B M	11	11	Predominantly carbohydrate metabolism
Environmental Information Processing>Signal transduction>02020 Two-component system	T320A+T3552B M	5	8	Including four methyl-accepting chemotaxis protein receptors

Cellular Processes>Cellular community-prokaryotes>02026 Biofilm formation	T320A+T3552B M	1	1	Starch synthase previously identified in *Escherichia coli*
Cellular Processes>Cell motility>02030 Bacterial chemotaxis	T320A+T3552B M	2	5	The four methyl-accepting chemotaxis protein receptors, and the cheY chemotaxis protein
Cellular Processes>Cell motility>02040 Flagellar assembly	T320A+T3552B M	5	5	Flagellar proteins

Table 6 Pathways downregulated in microaerobic samples as identified by BlastKOALA using KEGG Pathway Reconstruction

Pathway	**Sample type**	**No of KO identifiers in each sample set**	**No of Proteins**	**Additional information/ interpretation**
Genetic Information Processing>Translation>03010 Ribosome	T19 M T19+T320A+T3552B	6 3	8 3	Large and small ribosomal subunits Small ribosomal subunits
Genetic Information Processing>Replication and Repair>03030, 03430, 03440	T19 M T320A+T3552B M	3 10	2 10	DNA polymerases Enzymes needed for DNA replication and repair
Metabolism>Global and overview maps>01100 Metabolic pathways	T320A M Reiter M T320A+T3552B M T19+T320A+T3552B	4 1 12 12	4 1 13 12	Sugar, amino acid and nitrogen metabolism Acetaldehyde dehydrogenase Includes ribokinase Mainly amino acid and nitrogen metabolism, arginine biosynthesis; also purine and pyrimidine metabolism
Environmental Information Processing>Membrane transport>ABC transporters	T320A M	2	2	Iron and biotin transport systems
Genetic Information Processing>Folding, sorting and degradation>RNA degradation	T19+T320A+T3552B	2	2	RNA and DNA helicase

Table 7: Full list of Differentially Expressed Genes -1≥ log_2FC ≥1, false discovery rate <0.05 for microaerobic samples

Sample Type	gene_id	location	logCPM	logFC.G1M/G1P	PV.G1M/G1P	FDR.G1M/G1P
T19	C5N99_11130	CP027017.1(+)	-2.70836	6.30836	0.000155	0.008704
	C5N99_08225	CP027017.1(+)	12.96808	4.069845	8.78E-09	7.79E-06
	C5N99_04595	CP027017.1(+)	12.96882	4.061012	9.90E-09	7.79E-06
	C5N99_04605	CP027017.1(+)	14.58729	3.747035	1.58E-07	7.49E-05
	C5N99_08235	CP027017.1(+)	14.3661	3.597505	3.69E-07	0.000145
	C5N99_00040	CP027017.1(+)	14.42568	3.36251	9.98E-07	0.000337
	C5N99_01590	CP027017.1(-)	17.65592	3.286222	2.53E-06	0.000665
	C5N99_04760	CP027017.1(+)	4.850189	3.084417	9.42E-10	2.23E-06
	C5N99_10700	CP027017.1(+)	1.181747	3.06471	4.86E-06	0.000971
	C5N99_09900	CP027017.1(+)	2.251553	2.941918	3.83E-06	0.000905
	C5N99_01190	CP027017.1(+)	2.384363	2.923324	6.53E-06	0.000971
	C5N99_05520	CP027017.1(+)	0.327712	2.898151	0.000195	0.00979
	C5N99_01010	CP027017.1(-)	1.131287	2.797226	0.00017	0.008921
	C5N99_03810	CP027017.1(+)	0.201033	2.748596	0.000166	0.008921
	C5N99_05045	CP027017.1(+)	11.07616	2.714312	0.000272	0.013314
	C5N99_11835	CP027017.1(-)	15.75758	2.678512	8.13E-06	0.00113
	C5N99_03290	CP027017.1(+)	1.688982	2.614369	0.000141	0.008334
	C5N99_01195	CP027017.1(-)	3.263502	2.584853	9.53E-05	0.006626
	C5N99_04610	CP027017.1(+)	3.537722	2.489547	0.000504	0.018047
	C5N99_07295	CP027017.1(-)	6.399282	2.401461	6.57E-06	0.000971
	C5N99_08240	CP027017.1(+)	3.578825	2.349702	0.000925	0.028026
	C5N99_03855	CP027017.1(-)	3.597103	2.346025	1.38E-05	0.001717
	C5N99_03045	CP027017.1(+)	2.688411	2.308204	4.35E-05	0.003955
	C5N99_06590	CP027017.1(+)	6.39899	2.301514	1.31E-05	0.001717
	C5N99_05050	CP027017.1(+)	5.791433	2.238113	4.67E-05	0.004086
	C5N99_10365	CP027017.1(+)	7.520575	2.211353	4.23E-08	2.50E-05
	C5N99_01285	CP027017.1(-)	3.11706	2.192812	0.000184	0.009457
	C5N99_04460	CP027017.1(+)	1.359379	2.18662	0.001309	0.034406
	C5N99_11320	CP027017.1(+)	3.462937	2.169658	0.000578	0.01925
	C5N99_11670	CP027017.1(+)	1.644638	2.131836	0.000332	0.014786
	C5N99_11600	CP027017.1(-)	3.213145	2.113877	0.000364	0.015474
	C5N99_10200	CP027017.1(+)	5.093804	2.082987	2.25E-06	0.000665
	C5N99_11015	CP027017.1(+)	3.864721	2.054699	5.93E-05	0.005005
	C5N99_02660	CP027017.1(-)	6.290323	1.99195	2.98E-05	0.003057
	C5N99_11270	CP027017.1(-)	8.298055	1.985326	2.12E-05	0.002309
	C5N99_12385	CP027017.1(-)	6.639768	1.969253	5.21E-06	0.000971
	C5N99_03730	CP027017.1(+)	5.836261	1.935725	5.97E-06	0.000971
	C5N99_10360	CP027017.1(+)	8.469114	1.918923	0.000121	0.007931
	C5N99_04825	CP027017.1(-)	6.824392	1.892264	2.09E-05	0.002309
	C5N99_03345	CP027017.1(-)	8.318062	1.879848	6.20E-06	0.000971
	C5N99_06055	CP027017.1(+)	8.28743	1.834993	0.000381	0.015775
	C5N99_05630	CP027017.1(-)	5.360484	1.829947	0.000138	0.008334

	C5N99_10255	CP027017.1(+)	5.572945	1.821667	4.24E-05	0.003955
	C5N99_00320	CP027017.1(-)	10.03915	1.804353	0.002358	0.046426
	C5N99_00315	CP027017.1(-)	5.691477	1.756731	0.001151	0.032755
	C5N99_04425	CP027017.1(-)	5.882211	1.729207	0.00045	0.017444
	C5N99_11045	CP027017.1(-)	7.423888	1.709872	2.15E-05	0.002309
	C5N99_00245	CP027017.1(-)	5.705582	1.68226	0.00013	0.008092
	C5N99_00795	CP027017.1(-)	8.576232	1.647913	0.000596	0.019554
	C5N99_03850	CP027017.1(-)	4.259188	1.634788	0.001465	0.035372
	C5N99_03050	CP027017.1(-)	9.04223	1.629183	0.001998	0.041058
	C5N99_11220	CP027017.1(-)	8.12315	1.609709	8.91E-05	0.006402
	C5N99_03430	CP027017.1(-)	5.587165	1.605116	8.12E-05	0.00619
	C5N99_01580	CP027017.1(+)	3.808004	1.602867	0.002117	0.042497
	C5N99_01265	CP027017.1(-)	8.877898	1.598243	0.000785	0.024395
	C5N99_11890	CP027017.1(+)	5.584075	1.595352	0.000298	0.013818
	C5N99_11020	CP027017.1(+)	6.39956	1.56368	0.000328	0.014786
	C5N99_04400	CP027017.1(+)	8.351512	1.55699	0.000126	0.008024
	C5N99_08825	CP027017.1(+)	5.338411	1.555806	0.000559	0.019146
	C5N99_01295	CP027017.1(-)	6.326885	1.536078	0.000419	0.016482
	C5N99_10235	CP027017.1(-)	5.14139	1.513954	0.001108	0.032018
	C5N99_08810	CP027017.1(-)	3.841902	1.508802	0.00157	0.036366
	C5N99_02520	CP027017.1(-)	5.350098	1.501393	0.000147	0.008453
	C5N99_04805	CP027017.1(-)	7.568254	1.498755	7.78E-05	0.006125
	C5N99_05095	CP027017.1(+)	6.311638	1.48914	0.000463	0.017576
	C5N99_09865	CP027017.1(-)	6.936376	1.489005	0.001437	0.035372
	C5N99_11620	CP027017.1(+)	4.315244	1.483529	0.001701	0.03791
	C5N99_11535	CP027017.1(+)	4.038017	1.482163	0.002402	0.046902
	C5N99_03425	CP027017.1(-)	5.733881	1.447095	0.000541	0.018812
	C5N99_03335	CP027017.1(-)	8.039178	1.446836	0.00028	0.013314
	C5N99_02535	CP027017.1(-)	8.784227	1.431438	0.00154	0.036029
	C5N99_04615	CP027017.1(+)	7.551747	1.399238	0.001204	0.033169
	C5N99_08815	CP027017.1(-)	5.548012	1.393168	0.001482	0.035372
	C5N99_12350	CP027017.1(-)	5.410705	1.392974	0.001377	0.034671
	C5N99_12280	CP027017.1(-)	7.335302	1.38698	0.000469	0.017576
	C5N99_11000	CP027017.1(+)	6.156573	1.386316	0.000498	0.018047
	C5N99_02640	CP027017.1(-)	6.071214	1.381655	0.000668	0.021357
	C5N99_00170	CP027017.1(-)	9.481035	1.378925	0.001013	0.029908
	C5N99_00180	CP027017.1(-)	9.008975	1.377164	0.000528	0.018618
	C5N99_05400	CP027017.1(-)	6.50711	1.352008	0.001602	0.036749
	C5N99_00175	CP027017.1(-)	8.579862	1.346505	0.000282	0.013314
	C5N99_10915	CP027017.1(-)	9.347362	1.333718	0.001471	0.035372
	C5N99_11005	CP027017.1(+)	6.723793	1.322012	0.002458	0.047617
	C5N99_02485	CP027017.1(-)	8.333081	1.307562	0.002309	0.045841
	C5N99_11415	CP027017.1(-)	8.596908	1.292231	0.001773	0.038802
	C5N99_10795	CP027017.1(+)	5.640947	1.267654	0.001954	0.041058
	C5N99_02255	CP027017.1(-)	8.161117	1.267115	0.00131	0.034406
	C5N99_11470	CP027017.1(-)	6.875101	1.246895	0.001683	0.03791

	C5N99_03325	CP027017.1(+)	8.489407	1.184872	0.001969	0.041058
	C5N99_02525	CP027017.1(-)	7.392182	1.1703	0.001891	0.040631
	C5N99_10865	CP027017.1(+)	7.895292	-1.32538	0.001833	0.039733
	C5N99_00145	CP027017.1(+)	9.248893	-1.40419	0.001212	0.033169
	C5N99_00335	CP027017.1(-)	8.237971	-1.41657	0.001379	0.034671
	C5N99_04035	CP027017.1(+)	7.118984	-1.41985	0.001916	0.040782
	C5N99_00065	CP027017.1(+)	7.842931	-1.43753	0.000669	0.021357
	C5N99_12560	CP027017.1(-)	5.649004	-1.43802	0.001353	0.034671
	C5N99_00730	CP027017.1(+)	6.463329	-1.483	0.000416	0.016482
	C5N99_00485	CP027017.1(-)	5.788268	-1.5176	0.001231	0.033169
	C5N99_05285	CP027017.1(-)	8.257704	-1.56403	0.000348	0.015212
	C5N99_05995	CP027017.1(+)	7.959651	-1.56608	0.002122	0.042497
	C5N99_07730	CP027017.1(+)	7.167756	-1.67578	0.001221	0.033169
	C5N99_04180	CP027017.1(-)	7.936393	-1.68239	0.001359	0.034671
	C5N99_10775	CP027017.1(+)	6.11603	-1.68708	0.000571	0.01925
	C5N99_08550	CP027017.1(+)	6.709269	-1.69989	0.001726	0.038125
	C5N99_04365	CP027017.1(+)	6.148276	-1.73751	0.001235	0.033169
	C5N99_07085	CP027017.1(+)	6.596739	-1.79471	0.001529	0.036029
	C5N99_06360	CP027017.1(+)	6.581139	-1.80244	0.000941	0.028135
	C5N99_10060	CP027017.1(+)	8.163116	-1.80677	3.36E-05	0.003307
	C5N99_06630	CP027017.1(+)	5.397484	-1.82415	0.001691	0.03791
	C5N99_07670	CP027017.1(-)	5.673789	-1.91806	0.000765	0.024087
	C5N99_04355	CP027017.1(+)	6.164786	-1.95426	0.000891	0.027338
	C5N99_06950	CP027017.1(-)	5.656354	-2.00482	7.53E-05	0.006125
	C5N99_01920	CP027017.1(-)	8.282525	-2.02986	8.94E-05	0.006402
	C5N99_08310	CP027017.1(-)	5.837887	-2.03177	0.000107	0.007207
	C5N99_10625	CP027017.1(+)	8.357554	-2.10013	0.002072	0.042209
	C5N99_09705	CP027017.1(-)	4.239633	-2.11982	0.000164	0.008921
	C5N99_07350	CP027017.1(+)	5.428234	-2.13847	0.001985	0.041058
	C5N99_07285	CP027017.1(-)	8.17584	-2.21104	0.000367	0.015474
	C5N99_06560	CP027017.1(-)	8.153984	-2.23177	0.000387	0.015779
	C5N99_02360	CP027017.1(+)	3.251793	-2.41296	0.000476	0.017585
	C5N99_11155	CP027017.1(-)	2.961944	-2.8383	0.001454	0.035372
	C5N99_00475	CP027017.1(+)	1.376116	-3.17764	0.001111	0.032018
T320A	C5O78_06875	CP027018.1(+)	8.225031	-1.18856	0.000589	0.034232
	C5O78_02620	CP027018.1(-)	5.571521	-1.34154	0.001007	0.04967
	C5O78_02210	CP027018.1(+)	6.734868	-1.35715	0.000294	0.023386
	C5O78_07795	CP027018.1(+)	9.097238	-1.37214	0.001017	0.04967
	C5O78_11355	CP027018.1(+)	7.875408	-1.391	0.000816	0.042992
	C5O78_05035	CP027018.1(-)	9.824475	-1.40002	0.000671	0.037348
	C5O78_05470	CP027018.1(+)	7.569524	-1.45257	0.000995	0.04967
	C5O78_05595	CP027018.1(+)	8.689335	-1.52628	1.41E-05	0.00261
	C5O78_02205	CP027018.1(+)	7.056986	-1.60586	9.11E-05	0.011527
	C5O78_04555	CP027018.1(+)	5.851584	-1.76762	0.00022	0.021079
	C5O78_05425	CP027018.1(-)	1.903833	-1.99394	0.000275	0.022525
	C5O78_09270	CP027018.1(+)	4.522802	-2.12149	0.000561	0.034232

	C5O78_09455	CP027018.1(+)	5.782383	-2.44313	0.000149	0.016619
	C5O78_13835	CP027018.1(+)	5.316246	-2.50464	1.90E-09	5.28E-06
	C5O78_03575	CP027018.1(+)	5.911625	-2.54811	0.000103	0.012483
	C5O78_13840	CP027018.1(+)	2.115985	-2.86651	5.49E-08	3.05E-05
	C5O78_02910	CP027018.1(+)	4.893757	-3.01484	4.13E-05	0.006393
	C5O78_10720	CP027018.1(+)	5.596296	-3.52002	1.90E-06	0.00048
	C5O78_13830	CP027018.1(-)	7.064889	-3.65619	2.74E-07	9.53E-05
	C5O78_00680	CP027018.1(-)	0.652906	-9.92429	2.20E-08	1.53E-05
	C5O78_00675	CP027018.1(-)	2.105419	-11.3686	4.55E-09	6.34E-06
	C5O78_12630	CP027018.1(+)	9.281366	4.583619	7.74E-07	0.000215
	C5O78_13225	CP027018.1(-)	4.819031	4.374751	4.52E-06	0.000968
	C5O78_12615	CP027018.1(+)	3.989042	4.064311	0.000245	0.021079
	C5O78_02690	CP027018.1(-)	10.27799	4.036524	4.26E-06	0.000968
	C5O78_02685	CP027018.1(-)	6.290112	4.012682	2.17E-07	8.63E-05
	C5O78_09630	CP027018.1(+)	5.404598	3.844573	3.79E-05	0.006393
	C5O78_12620	CP027018.1(+)	1.946982	3.791656	0.000238	0.021079
	C5O78_09625	CP027018.1(+)	7.841868	3.767698	5.39E-07	0.000167
	C5O78_13215	CP027018.1(-)	1.313934	3.730861	8.34E-05	0.011051
	C5O78_13220	CP027018.1(-)	4.020675	3.618574	0.000159	0.016668
	C5O78_13315	CP027018.1(-)	1.054263	3.502951	0.00025	0.021079
	C5O78_12610	CP027018.1(+)	3.344851	3.379769	0.000819	0.042992
	C5O78_12635	CP027018.1(+)	5.259313	3.312204	0.000176	0.017469
	C5O78_11485	CP027018.1(-)	4.996308	2.974661	1.80E-07	8.33E-05
	C5O78_12220	CP027018.1(-)	5.333372	2.77551	0.000454	0.032312
	C5O78_02680	CP027018.1(-)	4.378627	2.702262	4.82E-05	0.007063
	C5O78_13580	CP027018.1(+)	6.100668	2.595339	3.96E-05	0.006393
	C5O78_09620	CP027018.1(+)	11.64434	2.512697	5.92E-05	0.008235
	C5O78_08075	CP027018.1(+)	0.324322	2.482797	0.000162	0.016668
	C5O78_04910	CP027018.1(-)	7.421478	2.429926	0.000583	0.034232
	C5O78_13450	CP027018.1(+)	9.803287	2.315364	1.05E-05	0.00209
	C5O78_07420	CP027018.1(+)	8.957323	2.251165	0.00023	0.021079
	C5O78_11550	CP027018.1(+)	7.849523	2.209479	1.32E-08	1.23E-05
	C5O78_04840	CP027018.1(+)	12.46507	2.19308	0.000114	0.013274
	C5O78_04860	CP027018.1(-)	6.047274	2.189354	0.000818	0.042992
	C5O78_12625	CP027018.1(+)	7.081681	2.070525	0.000321	0.024134
	C5O78_08795	CP027018.1(-)	5.023597	2.015786	0.000623	0.035365
	C5O78_13345	CP027018.1(-)	7.151019	2.003414	0.000535	0.034232
	C5O78_13275	CP027018.1(+)	5.609535	1.994584	0.000578	0.034232
	C5O78_07425	CP027018.1(+)	7.804872	1.951373	0.00059	0.034232
	C5O78_02635	CP027018.1(+)	2.182294	1.864074	0.000476	0.032312
	C5O78_12975	CP027018.1(+)	7.983768	1.853675	0.000526	0.034232
	C5O78_09980	CP027018.1(-)	6.470988	1.767245	0.000844	0.043482
	C5O78_03315	CP027018.1(+)	6.115402	1.671467	0.000317	0.024134
	C5O78_08070	CP027018.1(+)	5.636686	1.64087	0.000469	0.032312
	C5O78_01920	CP027018.1(-)	10.28449	1.56438	0.000468	0.032312
	C5O78_00165	CP027018.1(-)	6.396308	1.559079	0.001241	0.054819

	C5O78_05190	CP027018.1(+)	5.793488	1.087036	0.012461	0.176008
	C5O78_02525	CP027018.1(+)	6.083135	1.085595	0.007235	0.136042
T3552B	DYQ05_01235	CP045670.1(+)	7.754097	-5.14085	0.000365	0.036734
	DYQ05_01240	CP045670.1(+)	6.72827	-5.25911	0.000287	0.032833
	DYQ05_01245	CP045670.1(+)	9.639376	-5.58574	0.000219	0.027619
	DYQ05_00630	CP045670.1(-)	4.703494	2.124944	8.78E-05	0.013811
	DYQ05_02265	CP045670.1(+)	8.267521	3.306199	4.54E-08	3.81E-05
	DYQ05_02270	CP045670.1(+)	7.305886	3.51537	2.13E-08	3.81E-05
	DYQ05_02275	CP045670.1(+)	5.766077	3.881372	3.27E-08	3.81E-05
	DYQ05_02280	CP045670.1(+)	5.517714	3.121733	6.37E-07	0.000267
	DYQ05_02285	CP045670.1(+)	6.654484	2.710265	1.95E-07	0.000123
	DYQ05_03345	CP045670.1(-)	1.937257	2.033755	1.52E-05	0.004252
	DYQ05_03760	CP045670.1(-)	9.443101	2.506467	7.23E-05	0.012142
	DYQ05_04555	CP045670.1(+)	6.136584	2.168674	5.14E-05	0.009946
	DYQ05_04560	CP045670.1(+)	3.896891	2.443647	3.04E-07	0.000153
	DYQ05_04565	CP045670.1(+)	3.007261	2.09602	4.15E-05	0.00949
	DYQ05_13310	CP045670.1(-)	7.420463	2.056604	1.05E-06	0.000377
	DYQ05_03350	CP045670.1(-)	3.3502	1.968033	2.08E-06	0.000655
	DYQ05_00625	CP045670.1(-)	2.982164	1.904182	0.00017	0.023779
	DYQ05_06530	CP045670.1(-)	7.152693	1.88024	0.000199	0.026339
	DYQ05_13305	CP045670.1(-)	5.313978	1.864707	3.19E-05	0.008043
	DYQ05_06975	CP045670.1(-)	3.67611	1.808938	5.06E-05	0.009946
	DYQ05_13315	CP045670.1(-)	9.108713	1.7409	7.23E-05	0.012142
	DYQ05_01810	CP045670.1(+)	6.356049	1.715901	0.000311	0.034056
	DYQ05_04570	CP045670.1(+)	1.903524	1.677515	0.000442	0.042845
	DYQ05_11880	CP045670.1(+)	4.775041	1.636949	0.000359	0.036734
	DYQ05_02690	CP045670.1(-)	7.892197	1.249499	0.000271	0.032465
	DYQ05_12335	CP045670.1(-)	8.518864	1.238124	0.000152	0.022524
Reiter	DWQ65_09920	CP031394.1(-)	9.674894	-1.44063	4.54E-05	0.007845
	DWQ65_11345	CP031394.1(-)	3.29628	3.943654	0.000119	0.012519
	DWQ65_03225	CP031394.1(+)	-0.07084	1.851334	1.95E-05	0.003784
	DWQ65_07155	CP031394.1(-)	-0.20669	1.470963	0.000434	0.029646
	DWQ65_11830	CP031394.1(-)	7.26188	1.466326	3.01E-10	7.82E-07
	DWQ65_07130	CP031394.1(+)	7.422724	1.344873	4.97E-05	0.007855
	DWQ65_06880	CP031394.1(+)	4.00237	1.331379	0.000125	0.012519
	DWQ65_06250	CP031394.1(-)	9.801345	1.325757	3.47E-08	1.50E-05
	DWQ65_03835	CP031394.1(-)	1.163128	1.319541	0.000337	0.025682
	DWQ65_06415	CP031394.1(-)	5.606203	1.304363	1.85E-05	0.003784
	DWQ65_05540	CP031394.1(+)	10.33506	1.299514	7.85E-09	4.07E-06
	DWQ65_01995	CP031394.1(+)	1.484716	1.241907	0.000108	0.012178
	DWQ65_00105	CP031394.1(+)	2.432219	1.21818	0.000368	0.027273
	DWQ65_11825	CP031394.1(-)	6.747783	1.207818	0.000124	0.012519
	DWQ65_11275	CP031394.1(-)	9.055785	1.173721	7.69E-09	4.07E-06
	DWQ65_11815	CP031394.1(-)	7.425143	1.164952	1.63E-05	0.003784
	DWQ65_10160	CP031394.1(-)	9.71849	1.154876	4.97E-09	4.07E-06
	DWQ65_10255	CP031394.1(-)	2.786017	1.130934	0.000662	0.035796

	DWQ65_03230	CP031394.1(+)	7.402288	1.114572	2.04E-05	0.003784
	DWQ65_02560	CP031394.1(-)	8.838733	1.099312	4.34E-09	4.07E-06
	DWQ65_00065	CP031394.1(-)	2.560439	1.082672	0.000524	0.031599
	DWQ65_11810	CP031394.1(-)	5.474363	1.076772	0.000462	0.030733
	DWQ65_05840	CP031394.1(-)	2.637592	1.070871	0.000488	0.030879
	DWQ65_06750	CP031394.1(+)	2.7553	1.059676	0.000564	0.033228
	DWQ65_02890	CP031394.1(+)	7.281973	1.044985	6.05E-05	0.008724
	DWQ65_10415	CP031394.1(-)	6.043325	1.04484	1.89E-05	0.003784
	DWQ65_06315	CP031394.1(+)	4.61183	1.042703	0.000104	0.012178
	DWQ65_00205	CP031394.1(-)	2.497941	1.040964	0.000608	0.033937
	DWQ65_03620	CP031394.1(-)	14.19484	1.038561	9.69E-06	0.002793
	DWQ65_03220	CP031394.1(+)	5.142132	1.024148	0.000832	0.043143
	DWQ65_05545	CP031394.1(+)	6.607388	1.01295	3.09E-06	0.001143
T320A +T3552B/ T320A	C5O78_06530	CP027018.1(+)	7.496419	-15.0759	1.99E-127	5.50E-124
	C5O78_06535	CP027018.1(-)	4.903965	-12.4323	1.09E-59	1.01E-56
	C5O78_06520	CP027018.1(-)	4.617039	-12.1708	3.56E-60	4.91E-57
	C5O78_06515	CP027018.1(+)	0.419695	-7.76829	5.53E-11	4.02E-09
	C5O78_00675	CP027018.1(-)	-1.52777	-5.8827	0.001113	0.007442
	C5O78_01585	CP027018.1(-)	-2.47952	-5.4449	0.000936	0.006413
	C5O78_02855	CP027018.1(+)	3.83235	-5.18272	1.52E-10	9.10E-09
	C5O78_10875	CP027018.1(+)	0.278979	-4.05176	1.98E-07	4.71E-06
	C5O78_11615	CP027018.1(-)	2.563203	-3.61601	8.74E-07	1.75E-05
	C5O78_07935	CP027018.1(+)	2.924541	-3.61195	1.41E-07	3.41E-06
	C5O78_09150	CP027018.1(-)	2.713622	-3.49732	5.14E-06	7.93E-05
	C5O78_14320	CP027018.1(+)	3.221306	-3.454	5.61E-07	1.16E-05
	C5O78_10880	CP027018.1(-)	4.078728	-3.37557	5.02E-11	3.74E-09
	C5O78_09610	CP027018.1(-)	3.962099	-3.37033	1.42E-09	6.58E-08
	C5O78_01870	CP027018.1(-)	4.816446	-3.34606	1.20E-08	4.34E-07
	C5O78_07780	CP027018.1(+)	5.319853	-3.33535	8.72E-09	3.30E-07
	C5O78_07030	CP027018.1(-)	5.227799	-3.27349	5.13E-09	2.18E-07
	C5O78_08215	CP027018.1(-)	5.219724	-3.25838	2.47E-08	8.00E-07
	C5O78_10890	CP027018.1(+)	4.385226	-3.23248	8.11E-09	3.11E-07
	C5O78_07345	CP027018.1(-)	5.040402	-3.22237	3.52E-08	1.03E-06
	C5O78_04520	CP027018.1(+)	4.982697	-3.20238	2.83E-08	8.67E-07
	C5O78_07650	CP027018.1(-)	4.785508	-3.1757	8.99E-09	3.35E-07
	C5O78_09145	CP027018.1(+)	2.923879	-3.16303	2.51E-05	0.00032
	C5O78_10865	CP027018.1(+)	2.259668	-3.16224	3.27E-06	5.50E-05
	C5O78_10555	CP027018.1(-)	1.498144	-3.15657	5.15E-08	1.44E-06
	C5O78_10840	CP027018.1(+)	5.009616	-3.15451	1.46E-08	5.22E-07
	C5O78_09350	CP027018.1(+)	5.252131	-3.15096	1.67E-08	5.69E-07
	C5O78_01105	CP027018.1(-)	8.453093	-3.13615	1.45E-10	8.92E-09
	C5O78_02760	CP027018.1(-)	6.17443	-3.12399	1.22E-10	7.65E-09
	C5O78_02755	CP027018.1(-)	7.885359	-3.12192	2.13E-10	1.20E-08
	C5O78_09280	CP027018.1(+)	8.70822	-3.07061	9.71E-10	4.70E-08
	C5O78_09555	CP027018.1(-)	3.182691	-3.04375	1.58E-06	2.85E-05
	C5O78_06500	CP027018.1(-)	4.770631	-3.0256	2.52E-08	8.00E-07

	C5O78_09655	CP027018.1(-)	6.687863	-2.99209	6.61E-11	4.62E-09
	C5O78_02330	CP027018.1(+)	4.858257	-2.97359	3.53E-09	1.55E-07
	C5O78_09285	CP027018.1(+)	9.87945	-2.72944	7.45E-08	1.96E-06
	C5O78_09805	CP027018.1(-)	2.605108	-2.71813	0.000306	0.002638
	C5O78_06855	CP027018.1(-)	5.966845	-2.69949	5.54E-09	2.32E-07
	C5O78_09455	CP027018.1(+)	5.787355	-2.68049	6.49E-09	2.56E-07
	C5O78_09700	CP027018.1(-)	7.133877	-2.57209	6.33E-10	3.23E-08
	C5O78_09495	CP027018.1(-)	3.031463	-2.56179	5.92E-06	8.83E-05
	C5O78_01435	CP027018.1(-)	6.624434	-2.48537	2.80E-08	8.67E-07
	C5O78_04810	CP027018.1(+)	5.242853	-2.4691	1.34E-07	3.28E-06
	C5O78_01100	CP027018.1(-)	6.621363	-2.46331	1.62E-08	5.59E-07
	C5O78_07915	CP027018.1(+)	9.240637	-2.33035	3.68E-21	8.47E-19
	C5O78_10560	CP027018.1(-)	5.379472	-2.27227	1.01E-12	1.12E-10
	C5O78_08635	CP027018.1(+)	0.993124	-2.25861	0.010324	0.043191
	C5O78_09300	CP027018.1(+)	8.520291	-2.12753	4.42E-10	2.34E-08
	C5O78_04695	CP027018.1(-)	0.891441	-2.09071	7.35E-06	0.000107
	C5O78_10225	CP027018.1(-)	10.38591	-2.03789	1.07E-12	1.14E-10
	C5O78_01035	CP027018.1(-)	7.621537	-1.96125	2.12E-10	1.20E-08
	C5O78_08535	CP027018.1(-)	8.668737	-1.92579	3.26E-07	7.26E-06
	C5O78_11625	CP027018.1(+)	1.436883	-1.908	1.19E-05	0.000164
	C5O78_05135	CP027018.1(-)	8.6989	-1.87418	3.40E-17	7.22E-15
	C5O78_05220	CP027018.1(-)	1.807939	-1.87265	0.001646	0.010076
	C5O78_09305	CP027018.1(+)	8.064864	-1.85351	9.18E-07	1.82E-05
	C5O78_05230	CP027018.1(-)	5.873489	-1.82154	1.84E-11	1.54E-09
	C5O78_10415	CP027018.1(-)	5.957425	-1.81674	2.75E-13	3.16E-11
	C5O78_05225	CP027018.1(-)	3.27127	-1.80685	3.50E-06	5.81E-05
	C5O78_09775	CP027018.1(+)	3.119663	-1.79868	6.20E-06	9.21E-05
	C5O78_09480	CP027018.1(+)	6.130798	-1.75855	5.68E-09	2.34E-07
	C5O78_05895	CP027018.1(+)	2.617861	-1.73786	8.40E-05	0.000895
	C5O78_01295	CP027018.1(+)	5.21084	-1.72816	5.86E-09	2.38E-07
	C5O78_09475	CP027018.1(+)	7.226642	-1.72516	1.24E-07	3.09E-06
	C5O78_00385	CP027018.1(-)	8.787073	-1.72263	2.26E-15	3.90E-13
	C5O78_08555	CP027018.1(-)	4.548003	-1.71731	3.49E-05	0.000423
	C5O78_05880	CP027018.1(-)	4.801944	-1.70603	1.03E-07	2.64E-06
	C5O78_03740	CP027018.1(-)	13.07965	-1.70197	5.27E-08	1.44E-06
	C5O78_09270	CP027018.1(+)	4.304251	-1.68336	5.28E-07	1.11E-05
	C5O78_01580	CP027018.1(-)	-0.01128	-1.68092	0.008595	0.037373
	C5O78_09470	CP027018.1(+)	6.078503	-1.66916	2.08E-06	3.68E-05
	C5O78_11255	CP027018.1(+)	2.997374	-1.65193	4.81E-06	7.54E-05
	C5O78_14015	CP027018.1(-)	3.754865	-1.61068	5.02E-06	7.78E-05
	C5O78_08530	CP027018.1(-)	7.151503	-1.57866	9.60E-05	0.001008
	C5O78_04500	CP027018.1(+)	1.226479	-1.57101	0.000815	0.005798
	C5O78_07185	CP027018.1(-)	8.57737	-1.54776	4.69E-14	6.48E-12
	C5O78_10565	CP027018.1(-)	0.259288	-1.5452	0.009585	0.040653
	C5O78_09265	CP027018.1(+)	4.186483	-1.54335	1.13E-06	2.21E-05
	C5O78_04555	CP027018.1(+)	5.722819	-1.5387	4.05E-06	6.46E-05

	C5O78_00810	CP027018.1(+)	3.95041	-1.51833	4.19E-07	9.12E-06
	C5O78_10230	CP027018.1(-)	8.079677	-1.50236	2.52E-08	8.00E-07
	C5O78_10420	CP027018.1(-)	5.772043	-1.47619	2.43E-09	1.10E-07
	C5O78_10595	CP027018.1(-)	7.576723	-1.47143	2.97E-09	1.32E-07
	C5O78_09205	CP027018.1(+)	8.194476	-1.45714	7.50E-10	3.70E-08
	C5O78_10850	CP027018.1(+)	7.62245	-1.44647	4.02E-08	1.17E-06
	C5O78_08560	CP027018.1(-)	2.767343	-1.44402	0.00085	0.005987
	C5O78_06035	CP027018.1(+)	5.883193	-1.4376	4.81E-07	1.03E-05
	C5O78_09245	CP027018.1(+)	5.657603	-1.42001	3.91E-06	6.33E-05
	C5O78_08500	CP027018.1(-)	1.933101	-1.41918	0.001732	0.010469
	C5O78_09715	CP027018.1(-)	9.484753	-1.40672	0.000534	0.004161
	C5O78_01015	CP027018.1(-)	7.892181	-1.40554	1.58E-08	5.53E-07
	C5O78_11155	CP027018.1(-)	8.87493	-1.40248	5.94E-09	2.38E-07
	C5O78_07910	CP027018.1(+)	6.065028	-1.39994	2.99E-08	9.07E-07
	C5O78_01995	CP027018.1(+)	9.128771	-1.38813	1.21E-07	3.05E-06
	C5O78_08540	CP027018.1(-)	5.293013	-1.382	0.00014	0.001348
	C5O78_09705	CP027018.1(-)	9.560276	-1.36881	0.000113	0.00115
	C5O78_02325	CP027018.1(+)	3.918422	-1.36092	4.30E-05	0.000507
	C5O78_05240	CP027018.1(-)	1.806774	-1.32218	0.009237	0.03948
	C5O78_11250	CP027018.1(-)	2.329068	-1.31317	0.000571	0.00437
	C5O78_08565	CP027018.1(-)	6.919336	-1.30819	5.84E-05	0.000648
	C5O78_11855	CP027018.1(+)	5.274718	-1.30375	5.68E-06	8.52E-05
	C5O78_01865	CP027018.1(-)	6.809041	-1.29939	3.82E-07	8.36E-06
	C5O78_01010	CP027018.1(+)	2.390756	-1.29524	0.00129	0.008296
	C5O78_08525	CP027018.1(-)	6.333519	-1.28976	0.000568	0.00436
	C5O78_03840	CP027018.1(+)	7.924902	-1.28361	3.15E-08	9.47E-07
	C5O78_00375	CP027018.1(-)	7.056171	-1.25906	5.21E-06	7.95E-05
	C5O78_11320	CP027018.1(-)	1.716741	-1.2469	0.001726	0.010469
	C5O78_11360	CP027018.1(+)	7.047402	-1.24423	1.30E-06	2.43E-05
	C5O78_06030	CP027018.1(+)	6.384578	-1.23317	1.27E-06	2.41E-05
	C5O78_01030	CP027018.1(+)	2.578835	-1.22907	0.004249	0.021485
	C5O78_09210	CP027018.1(+)	5.795483	-1.22817	1.89E-06	3.37E-05
	C5O78_09260	CP027018.1(+)	11.65433	-1.22802	0.002014	0.011831
	C5O78_08455	CP027018.1(-)	3.793946	-1.22717	0.005934	0.027817
	C5O78_11245	CP027018.1(-)	5.174488	-1.22409	0.000106	0.001083
	C5O78_05470	CP027018.1(+)	7.316428	-1.22057	0.000158	0.001501
	C5O78_01890	CP027018.1(+)	8.884841	-1.2177	3.48E-08	1.03E-06
	C5O78_13950	CP027018.1(-)	5.288164	-1.2129	1.55E-05	0.000211
	C5O78_05120	CP027018.1(-)	1.653736	-1.21128	0.008348	0.036471
	C5O78_11355	CP027018.1(+)	7.997441	-1.20976	2.77E-05	0.000351
	C5O78_09710	CP027018.1(-)	11.27402	-1.20117	0.001288	0.008296
	C5O78_05145	CP027018.1(-)	4.475714	-1.20075	4.72E-05	0.000545
	C5O78_05750	CP027018.1(+)	8.143504	-1.19819	4.88E-08	1.37E-06
	C5O78_06010	CP027018.1(+)	6.063261	-1.19793	5.28E-06	8.01E-05
	C5O78_01625	CP027018.1(+)	7.338742	-1.19442	9.27E-06	0.000131
	C5O78_11695	CP027018.1(-)	8.737443	-1.19068	5.91E-07	1.22E-05

	C5O78_05200	CP027018.1(+)	9.17505	-1.1872	2.35E-08	7.71E-07
	C5O78_06720	CP027018.1(-)	10.04474	-1.18704	3.37E-05	0.000412
	C5O78_00365	CP027018.1(-)	7.302633	-1.1749	3.25E-05	0.0004
	C5O78_05820	CP027018.1(+)	2.861951	-1.17298	0.001184	0.007803
	C5O78_09550	CP027018.1(+)	3.674042	-1.17237	0.00145	0.009174
	C5O78_03535	CP027018.1(-)	5.27848	-1.16764	5.36E-05	0.000602
	C5O78_02620	CP027018.1(-)	5.562015	-1.16621	4.59E-05	0.000533
	C5O78_06915	CP027018.1(-)	3.27978	-1.16608	0.000203	0.00186
	C5O78_03275	CP027018.1(+)	6.176037	-1.14765	1.25E-06	2.39E-05
	C5O78_14230	CP027018.1(+)	6.629624	-1.12243	2.48E-06	4.26E-05
	C5O78_14255	CP027018.1(-)	6.662714	-1.11226	1.59E-05	0.000214
	C5O78_07190	CP027018.1(-)	7.03653	-1.1101	2.04E-05	0.000265
	C5O78_06965	CP027018.1(+)	5.733004	-1.10613	0.001886	0.011256
	C5O78_09130	CP027018.1(-)	10.82267	-1.09932	1.43E-06	2.61E-05
	C5O78_04775	CP027018.1(+)	9.229142	-1.09481	5.41E-05	0.000605
	C5O78_00505	CP027018.1(+)	5.811254	-1.09453	3.67E-05	0.000442
	C5O78_07275	CP027018.1(+)	8.529796	-1.09331	2.99E-05	0.000372
	C5O78_02285	CP027018.1(+)	8.15817	-1.08228	2.59E-07	6.07E-06
	C5O78_07670	CP027018.1(+)	4.396123	-1.07849	0.00034	0.002862
	C5O78_00370	CP027018.1(-)	9.651621	-1.07484	4.42E-06	7.01E-05
	C5O78_08580	CP027018.1(+)	8.844639	-1.06702	6.09E-08	1.65E-06
	C5O78_00875	CP027018.1(+)	7.199061	-1.06454	1.76E-05	0.000234
	C5O78_09440	CP027018.1(+)	3.000622	-1.06402	0.009505	0.040499
	C5O78_01365	CP027018.1(+)	8.17344	-1.06319	1.64E-07	3.94E-06
	C5O78_02655	CP027018.1(-)	5.234615	-1.06088	0.000921	0.006339
	C5O78_12550	CP027018.1(+)	5.659512	-1.05762	0.000378	0.003106
	C5O78_05235	CP027018.1(-)	2.678413	-1.05068	0.007365	0.033126
	C5O78_05050	CP027018.1(-)	8.953447	-1.03562	1.71E-06	3.06E-05
	C5O78_14300	CP027018.1(-)	4.253118	-1.03092	0.00175	0.010548
	C5O78_02220	CP027018.1(+)	7.356094	-1.02963	0.000201	0.001853
	C5O78_05055	CP027018.1(-)	7.232305	-1.02886	3.28E-05	0.000403
	C5O78_05440	CP027018.1(+)	6.027318	-1.02235	0.001058	0.007155
	C5O78_11640	CP027018.1(+)	6.825227	-1.02042	0.00012	0.001194
	C5O78_00300	CP027018.1(+)	7.520869	-1.01367	0.00013	0.001279
	C5O78_00210	CP027018.1(-)	4.767126	-1.00885	0.00091	0.006312
	C5O78_07495	CP027018.1(+)	6.239663	-1.00754	0.000126	0.001249
	C5O78_00380	CP027018.1(-)	9.223542	-1.005	7.36E-06	0.000107
	C5O78_07260	CP027018.1(-)	9.094934	-1.00352	6.32E-06	9.33E-05
T320A+T3552B/T3552B	DYQ05_00045	CP045670.1(-)	7.425305	-1.01822	0.000631	0.010187
	DYQ05_09885	CP045670.1(+)	8.079476	-1.01861	0.000339	0.006231
	DYQ05_06155	CP045670.1(+)	10.02092	-1.02077	0.000901	0.0132
	DYQ05_09550	CP045670.1(-)	8.874209	-1.03038	0.001353	0.017571
	DYQ05_00510	CP045670.1(+)	6.242286	-1.03826	0.001269	0.016649
	DYQ05_04495	CP045670.1(+)	5.945099	-1.03921	0.000654	0.010359
	DYQ05_08240	CP045670.1(+)	5.859811	-1.04341	0.004382	0.0429
	DYQ05_03560	CP045670.1(-)	4.941384	-1.05092	0.002968	0.031413

	DYQ05_05135	CP045670.1(+)	5.199409	-1.051	0.002698	0.029551
	DYQ05_08940	CP045670.1(-)	7.143661	-1.05786	0.00087	0.012873
	DYQ05_08520	CP045670.1(+)	6.677595	-1.06659	0.000322	0.00597
	DYQ05_04460	CP045670.1(+)	7.036566	-1.06843	0.000191	0.003937
	DYQ05_09820	CP045670.1(-)	6.370466	-1.0764	0.000641	0.010223
	DYQ05_11730	CP045670.1(+)	3.677099	-1.0825	0.00503	0.047817
	DYQ05_04725	CP045670.1(+)	7.510991	-1.08908	0.001035	0.01465
	DYQ05_05130	CP045670.1(-)	4.583404	-1.0942	0.004506	0.043656
	DYQ05_04475	CP045670.1(+)	7.341989	-1.0952	0.000162	0.003423
	DYQ05_06895	CP045670.1(-)	4.40671	-1.0961	0.002935	0.031413
	DYQ05_07220	CP045670.1(+)	7.656489	-1.09947	0.000316	0.005896
	DYQ05_09060	CP045670.1(-)	7.955719	-1.11009	0.000115	0.002606
	DYQ05_04435	CP045670.1(-)	6.161543	-1.13769	0.000933	0.013501
	DYQ05_11625	CP045670.1(+)	4.629086	-1.1408	0.004394	0.0429
	DYQ05_08380	CP045670.1(-)	7.742591	-1.14651	8.44E-05	0.002105
	DYQ05_08700	CP045670.1(-)	5.000407	-1.14895	0.003395	0.034908
	DYQ05_05080	CP045670.1(+)	7.945724	-1.14908	0.001651	0.020999
	DYQ05_08500	CP045670.1(+)	7.022207	-1.16016	0.000448	0.007631
	DYQ05_04050	CP045670.1(+)	8.761701	-1.16237	0.002542	0.028591
	DYQ05_09215	CP045670.1(-)	7.07308	-1.16977	0.000171	0.003582
	DYQ05_07520	CP045670.1(+)	4.545084	-1.18278	0.00294	0.031413
	DYQ05_03000	CP045670.1(+)	6.020155	-1.19644	0.002006	0.024176
	DYQ05_06465	CP045670.1(-)	2.867055	-1.21447	0.002502	0.028258
	DYQ05_03300	CP045670.1(+)	2.640854	-1.22079	0.005177	0.04903
	DYQ05_02330	CP045670.1(-)	7.048455	-1.23961	8.44E-05	0.002105
	DYQ05_11620	CP045670.1(+)	3.838425	-1.24925	0.001123	0.015627
	DYQ05_04420	CP045670.1(+)	6.87499	-1.25557	3.90E-05	0.001197
	DYQ05_07815	CP045670.1(+)	2.687127	-1.25693	0.002963	0.031413
	DYQ05_08490	CP045670.1(+)	7.834564	-1.25835	2.20E-06	0.000109
	DYQ05_12595	CP045670.1(-)	7.682298	-1.26297	0.000204	0.004148
	DYQ05_11945	CP045670.1(-)	8.352558	-1.26391	2.74E-05	0.000886
	DYQ05_05215	CP045670.1(+)	4.282067	-1.27417	0.00166	0.02101
	DYQ05_00290	CP045670.1(+)	6.257119	-1.2866	9.22E-05	0.002256
	DYQ05_04405	CP045670.1(+)	5.732566	-1.28662	0.002637	0.029374
	DYQ05_09615	CP045670.1(+)	4.382061	-1.30827	0.001679	0.021049
	DYQ05_04470	CP045670.1(+)	4.669721	-1.33752	0.000192	0.003937
	DYQ05_00520	CP045670.1(+)	3.439139	-1.34668	0.001255	0.01662
	DYQ05_04500	CP045670.1(+)	5.032963	-1.35115	0.000404	0.007162
	DYQ05_04450	CP045670.1(+)	10.30966	-1.35333	0.003106	0.032073
	DYQ05_02040	CP045670.1(-)	10.48412	-1.36186	7.76E-05	0.002014
	DYQ05_08245	CP045670.1(+)	3.871672	-1.36886	0.001255	0.01662
	DYQ05_09655	CP045670.1(-)	4.40506	-1.37592	0.001119	0.015627
	DYQ05_09955	CP045670.1(-)	4.552935	-1.37628	0.000598	0.009725
	DYQ05_04455	CP045670.1(+)	8.521763	-1.38437	3.59E-05	0.001129
	DYQ05_00755	CP045670.1(-)	6.065122	-1.40354	0.000101	0.002347
	DYQ05_03305	CP045670.1(-)	6.542286	-1.42149	2.33E-06	0.000113

	DYQ05_07510	CP045670.1(+)	4.540836	-1.42693	0.000557	0.009294
	DYQ05_08270	CP045670.1(+)	5.455957	-1.4414	0.000284	0.005378
	DYQ05_12340	CP045670.1(+)	4.148723	-1.44329	0.000819	0.012432
	DYQ05_04505	CP045670.1(+)	4.936364	-1.44856	9.87E-05	0.002324
	DYQ05_04335	CP045670.1(+)	4.735794	-1.45222	0.000676	0.010649
	DYQ05_00745	CP045670.1(-)	3.345873	-1.47404	0.00042	0.007298
	DYQ05_10390	CP045670.1(-)	4.826927	-1.49875	0.000261	0.004976
	DYQ05_04510	CP045670.1(+)	6.897483	-1.51525	8.35E-08	7.01E-06
	DYQ05_08495	CP045670.1(+)	6.426731	-1.55004	1.12E-06	7.02E-05
	DYQ05_00750	CP045670.1(-)	6.501948	-1.55332	8.89E-06	0.000367
	DYQ05_11560	CP045670.1(+)	2.786033	-1.60595	0.000206	0.004148
	DYQ05_12610	CP045670.1(-)	5.773428	-1.62483	0.002203	0.026049
	DYQ05_04830	CP045670.1(-)	6.487718	-1.6403	4.84E-08	4.52E-06
	DYQ05_00480	CP045670.1(+)	1.538931	-1.64059	0.002869	0.031148
	DYQ05_06215	CP045670.1(+)	4.056871	-1.68002	0.00095	0.013678
	DYQ05_04825	CP045670.1(-)	3.881494	-1.68546	0.000445	0.007621
	DYQ05_08895	CP045670.1(+)	4.442569	-1.69896	8.26E-05	0.002102
	DYQ05_00500	CP045670.1(+)	4.494582	-1.70099	6.42E-05	0.001757
	DYQ05_10050	CP045670.1(+)	1.761406	-1.77552	0.000639	0.010223
	DYQ05_04715	CP045670.1(+)	10.2072	-1.79138	9.50E-05	0.002279
	DYQ05_04845	CP045670.1(-)	4.60741	-1.81943	1.47E-05	0.000528
	DYQ05_02050	CP045670.1(-)	6.075926	-1.83347	2.61E-05	0.000852
	DYQ05_04465	CP045670.1(+)	8.491854	-1.83689	2.48E-09	3.47E-07
	DYQ05_11630	CP045670.1(+)	3.806923	-1.97647	1.21E-05	0.000468
	DYQ05_05865	CP045670.1(+)	17.11204	-2.02283	1.76E-05	0.000598
	DYQ05_08485	CP045670.1(+)	6.288409	-2.04191	1.78E-12	4.97E-10
	DYQ05_03280	CP045670.1(-)	2.825335	-2.05072	0.000527	0.008849
	DYQ05_02045	CP045670.1(-)	4.140865	-2.12993	8.03E-06	0.000337
	DYQ05_04850	CP045670.1(-)	2.114239	-2.20951	0.001376	0.01777
	DYQ05_12085	CP045670.1(-)	4.625416	-2.22229	2.75E-06	0.000128
	DYQ05_04865	CP045670.1(-)	10.87654	-2.31764	1.44E-05	0.000524
	DYQ05_04860	CP045670.1(-)	9.037391	-2.63488	1.38E-06	7.90E-05
	DYQ05_01595	CP045670.1(-)	9.71087	-2.99547	0.002343	0.026831
	DYQ05_01235	CP045670.1(+)	5.0969	-3.28585	0.002458	0.027928
	DYQ05_10045	CP045670.1(+)	-0.61686	-3.37541	0.004638	0.04476
	DYQ05_09000	CP045670.1(-)	7.799577	-3.49362	7.12E-07	4.85E-05
	DYQ05_10055	CP045670.1(+)	-0.56909	-4.11869	0.004208	0.04156
	DYQ05_01240	CP045670.1(+)	4.110732	-4.17294	0.000382	0.006879
	DYQ05_01245	CP045670.1(+)	7.048171	-4.35221	7.25E-05	0.001903
T320A+T3552B/T320A	C5O78_10300	CP027018.1(-)	6.235349	1.006484	4.05E-05	0.000482
	C5O78_10515	CP027018.1(+)	4.263464	1.007517	0.002585	0.014301
	C5O78_13350	CP027018.1(-)	8.525272	1.01482	8.70E-06	0.000123
	C5O78_04745	CP027018.1(+)	6.858947	1.01504	8.63E-05	0.000917
	C5O78_04260	CP027018.1(+)	8.304004	1.020477	1.21E-06	2.35E-05
	C5O78_03885	CP027018.1(-)	5.632825	1.02653	0.000209	0.001888
	C5O78_08325	CP027018.1(+)	9.386056	1.02775	2.22E-06	3.90E-05

	C5O78_08115	CP027018.1(+)	10.78645	1.028406	3.46E-06	5.79E-05
	C5O78_09940	CP027018.1(-)	7.800479	1.031333	1.68E-05	0.000225
	C5O78_10295	CP027018.1(-)	5.526295	1.033926	0.000259	0.002297
	C5O78_11100	CP027018.1(-)	3.887904	1.035975	0.007829	0.034642
	C5O78_10500	CP027018.1(+)	8.932838	1.037286	1.23E-06	2.38E-05
	C5O78_02080	CP027018.1(+)	7.161492	1.037461	0.000101	0.001045
	C5O78_07180	CP027018.1(+)	8.486925	1.038078	1.44E-06	2.61E-05
	C5O78_04640	CP027018.1(+)	11.35427	1.043437	0.000708	0.005239
	C5O78_13590	CP027018.1(+)	7.331727	1.044431	0.000303	0.002625
	C5O78_06085	CP027018.1(-)	4.332118	1.044433	0.001212	0.007909
	C5O78_13865	CP027018.1(+)	2.161631	1.050369	0.011375	0.046529
	C5O78_10445	CP027018.1(+)	4.816011	1.052607	0.002166	0.012562
	C5O78_10135	CP027018.1(-)	7.594036	1.055631	0.000159	0.00151
	C5O78_10740	CP027018.1(+)	4.466095	1.057681	0.003249	0.017187
	C5O78_13600	CP027018.1(+)	7.121886	1.061228	1.57E-05	0.000213
	C5O78_04240	CP027018.1(+)	8.24057	1.068908	1.10E-05	0.000153
	C5O78_07475	CP027018.1(-)	4.715131	1.072018	0.00168	0.010247
	C5O78_04005	CP027018.1(+)	9.992557	1.07331	7.65E-06	0.00011
	C5O78_12145	CP027018.1(+)	2.466035	1.075342	0.011107	0.045799
	C5O78_12080	CP027018.1(-)	5.495982	1.076124	0.000467	0.003738
	C5O78_11065	CP027018.1(-)	3.300527	1.082485	0.002617	0.014424
	C5O78_02065	CP027018.1(+)	8.935743	1.08469	2.65E-08	8.30E-07
	C5O78_13450	CP027018.1(+)	9.086761	1.086135	0.003397	0.017932
	C5O78_10150	CP027018.1(-)	6.243402	1.087654	0.001681	0.010247
	C5O78_11760	CP027018.1(+)	6.237963	1.08841	2.93E-05	0.000366
	C5O78_00355	CP027018.1(-)	9.092691	1.089619	9.89E-08	2.55E-06
	C5O78_09420	CP027018.1(-)	4.374222	1.100456	0.001939	0.011463
	C5O78_01650	CP027018.1(+)	4.569843	1.102707	0.002364	0.013458
	C5O78_11310	CP027018.1(-)	5.685513	1.102781	6.77E-05	0.000741
	C5O78_04210	CP027018.1(+)	5.346063	1.117274	0.000189	0.00176
	C5O78_02035	CP027018.1(+)	6.918349	1.117402	6.97E-05	0.000758
	C5O78_05970	CP027018.1(+)	11.55576	1.123779	3.78E-06	6.17E-05
	C5O78_09980	CP027018.1(-)	6.004875	1.128968	0.000812	0.005796
	C5O78_00180	CP027018.1(+)	7.66815	1.131698	0.000125	0.00124
	C5O78_12285	CP027018.1(-)	2.961935	1.135071	0.002891	0.015577
	C5O78_05545	CP027018.1(+)	9.523651	1.153616	1.24E-05	0.000171
	C5O78_13070	CP027018.1(-)	8.507669	1.162459	0.000493	0.003902
	C5O78_09645	CP027018.1(+)	2.525825	1.165568	0.006454	0.029651
	C5O78_06860	CP027018.1(+)	6.9159	1.16796	0.00026	0.002299
	C5O78_02075	CP027018.1(+)	8.292458	1.169123	4.02E-06	6.46E-05
	C5O78_04040	CP027018.1(+)	3.833537	1.170968	0.000593	0.004461
	C5O78_11330	CP027018.1(-)	5.458603	1.17407	0.000207	0.001886
	C5O78_04245	CP027018.1(+)	8.174008	1.180779	7.39E-08	1.96E-06
	C5O78_09965	CP027018.1(+)	3.112662	1.188907	0.005355	0.025896
	C5O78_13695	CP027018.1(-)	8.816298	1.190581	9.57E-09	3.52E-07
	C5O78_04225	CP027018.1(+)	8.969917	1.205968	4.34E-09	1.87E-07

	C5O78_10290	CP027018.1(-)	7.040026	1.213821	3.72E-06	6.11E-05
	C5O78_05010	CP027018.1(-)	5.591641	1.21467	2.07E-05	0.000268
	C5O78_01425	CP027018.1(+)	2.854018	1.219996	0.001003	0.006806
	C5O78_00175	CP027018.1(-)	6.122726	1.227059	0.000343	0.002875
	C5O78_13330	CP027018.1(-)	3.877467	1.229403	0.000736	0.005359
	C5O78_13310	CP027018.1(-)	7.154813	1.233741	1.36E-06	2.50E-05
	C5O78_02885	CP027018.1(+)	11.04493	1.240565	4.94E-07	1.05E-05
	C5O78_10175	CP027018.1(-)	6.687208	1.242443	4.23E-05	0.000502
	C5O78_01430	CP027018.1(+)	3.503333	1.253853	0.000274	0.002404
	C5O78_02520	CP027018.1(+)	5.760047	1.255597	1.77E-05	0.000234
	C5O78_00015	CP027018.1(-)	5.742876	1.257519	0.008113	0.035613
	C5O78_04795	CP027018.1(+)	7.284638	1.261522	1.30E-06	2.43E-05
	C5O78_03800	CP027018.1(+)	7.542904	1.272339	2.48E-06	4.26E-05
	C5O78_10105	CP027018.1(-)	4.678892	1.282673	0.000114	0.001155
	C5O78_08155	CP027018.1(+)	12.31934	1.283365	9.51E-08	2.48E-06
	C5O78_10980	CP027018.1(+)	6.517766	1.284315	4.53E-07	9.78E-06
	C5O78_12675	CP027018.1(+)	7.589791	1.290931	2.24E-06	3.92E-05
	C5O78_04345	CP027018.1(-)	4.809829	1.302573	9.29E-05	0.000983
	C5O78_02060	CP027018.1(+)	5.930237	1.303072	4.67E-06	7.37E-05
	C5O78_07695	CP027018.1(-)	6.198478	1.303178	7.79E-06	0.000111
	C5O78_08100	CP027018.1(+)	9.89378	1.306202	2.46E-07	5.80E-06
	C5O78_02845	CP027018.1(-)	2.745719	1.313976	0.003959	0.020433
	C5O78_01255	CP027018.1(+)	10.54632	1.319209	4.96E-06	7.73E-05
	C5O78_13465	CP027018.1(+)	3.370464	1.320068	0.000537	0.004174
	C5O78_05810	CP027018.1(-)	4.943363	1.322324	2.93E-05	0.000366
	C5O78_02195	CP027018.1(+)	2.630871	1.352387	0.001292	0.008296
	C5O78_02265	CP027018.1(-)	7.976512	1.363698	3.92E-06	6.33E-05
	C5O78_04350	CP027018.1(-)	5.24843	1.366728	2.85E-05	0.00036
	C5O78_04255	CP027018.1(+)	8.889784	1.391833	1.98E-11	1.56E-09
	C5O78_13555	CP027018.1(+)	8.590177	1.412481	1.85E-11	1.54E-09
	C5O78_13690	CP027018.1(-)	6.559634	1.424788	3.69E-07	8.14E-06
	C5O78_13200	CP027018.1(-)	2.600303	1.440598	0.000774	0.005577
	C5O78_13955	CP027018.1(-)	5.980616	1.440651	1.82E-08	6.13E-07
	C5O78_11305	CP027018.1(+)	9.430208	1.450151	1.43E-09	6.58E-08
	C5O78_02275	CP027018.1(-)	9.182084	1.451611	5.21E-08	1.44E-06
	C5O78_09575	CP027018.1(-)	7.326983	1.452493	2.24E-08	7.44E-07
	C5O78_00190	CP027018.1(+)	8.242383	1.465027	1.57E-11	1.40E-09
	C5O78_04865	CP027018.1(-)	9.835584	1.466286	1.10E-07	2.79E-06
	C5O78_06165	CP027018.1(-)	1.117803	1.475088	0.006147	0.028623
	C5O78_10315	CP027018.1(-)	1.02224	1.478655	0.005879	0.027664
	C5O78_07535	CP027018.1(+)	1.80104	1.494276	0.001955	0.011534
	C5O78_08165	CP027018.1(+)	9.982238	1.517081	2.45E-12	2.42E-10
	C5O78_10575	CP027018.1(-)	6.207644	1.517907	8.01E-05	0.000861
	C5O78_11595	CP027018.1(-)	6.514542	1.526385	0.002303	0.013138
	C5O78_07925	CP027018.1(+)	8.166067	1.529763	9.83E-07	1.94E-05
	C5O78_03405	CP027018.1(+)	7.87322	1.529828	3.24E-11	2.48E-09

	C5O78_01260	CP027018.1(+)	9.097549	1.533006	2.56E-10	1.41E-08
	C5O78_04250	CP027018.1(+)	9.638868	1.550684	9.90E-11	6.51E-09
	C5O78_12095	CP027018.1(-)	8.764903	1.575766	6.54E-07	1.34E-05
	C5O78_00185	CP027018.1(+)	8.651723	1.580235	1.89E-11	1.54E-09
	C5O78_07450	CP027018.1(+)	3.548938	1.582766	1.43E-05	0.000196
	C5O78_13345	CP027018.1(-)	6.740601	1.590868	4.78E-08	1.36E-06
	C5O78_06090	CP027018.1(-)	1.93236	1.615899	0.001274	0.00826
	C5O78_08160	CP027018.1(+)	7.682429	1.633606	9.57E-11	6.45E-09
	C5O78_00705	CP027018.1(-)	1.858331	1.657678	0.000329	0.002797
	C5O78_10320	CP027018.1(-)	4.406797	1.668469	0.000133	0.001296
	C5O78_05865	CP027018.1(-)	1.454723	1.67585	0.005734	0.02738
	C5O78_10435	CP027018.1(+)	10.03231	1.677791	2.89E-14	4.20E-12
	C5O78_11590	CP027018.1(-)	5.691524	1.680049	3.26E-07	7.26E-06
	C5O78_01815	CP027018.1(-)	7.063287	1.696205	6.78E-08	1.82E-06
	C5O78_13580	CP027018.1(+)	5.417119	1.705399	0.000116	0.001164
	C5O78_07700	CP027018.1(-)	7.569801	1.762413	5.81E-10	3.03E-08
	C5O78_03445	CP027018.1(+)	5.236742	1.845312	0.004345	0.021853
	C5O78_13240	CP027018.1(-)	4.47386	1.85602	7.38E-06	0.000107
	C5O78_09935	CP027018.1(-)	6.671869	1.882645	1.86E-13	2.34E-11
	C5O78_12230	CP027018.1(-)	5.451248	1.882941	0.006074	0.028424
	C5O78_10005	CP027018.1(-)	5.365388	1.886008	7.25E-09	2.82E-07
	C5O78_09140	CP027018.1(-)	6.290055	1.902461	0.000116	0.001166
	C5O78_07435	CP027018.1(+)	8.176162	1.916603	1.53E-16	3.01E-14
	C5O78_11555	CP027018.1(+)	6.428745	1.92549	6.87E-14	9.03E-12
	C5O78_13275	CP027018.1(+)	5.320159	1.97189	4.10E-08	1.18E-06
	C5O78_04355	CP027018.1(-)	3.333014	1.972625	3.00E-07	6.83E-06
	C5O78_03450	CP027018.1(+)	3.560437	1.98548	5.21E-06	7.95E-05
	C5O78_07410	CP027018.1(+)	9.166936	2.013724	1.79E-12	1.83E-10
	C5O78_04860	CP027018.1(-)	5.779875	2.018623	6.69E-11	4.62E-09
	C5O78_00905	CP027018.1(+)	0.772949	2.051639	0.002888	0.015577
	C5O78_11880	CP027018.1(-)	0.878151	2.130171	0.000973	0.00663
	C5O78_02680	CP027018.1(-)	3.766769	2.355009	8.69E-07	1.75E-05
	C5O78_12605	CP027018.1(+)	3.380587	2.356777	1.31E-07	3.23E-06
	C5O78_01810	CP027018.1(-)	4.10803	2.432204	2.03E-10	1.19E-08
	C5O78_07430	CP027018.1(+)	8.177623	2.458603	6.97E-28	2.41E-25
	C5O78_04840	CP027018.1(+)	11.95594	2.467545	3.14E-06	5.31E-05
	C5O78_12150	CP027018.1(+)	0.843083	2.560567	5.14E-05	0.000586
	C5O78_04915	CP027018.1(-)	8.022384	2.573844	0.00252	0.013997
	C5O78_07425	CP027018.1(+)	8.016069	2.63942	1.96E-26	6.01E-24
	C5O78_11550	CP027018.1(+)	7.167356	2.678753	5.40E-07	1.13E-05
	C5O78_02685	CP027018.1(-)	5.78795	2.687332	1.57E-08	5.53E-07
	C5O78_08060	CP027018.1(+)	8.322479	2.694761	2.08E-05	0.000268
	C5O78_04855	CP027018.1(-)	1.787491	2.777613	2.63E-07	6.10E-06
	C5O78_02690	CP027018.1(-)	9.784533	2.828355	1.14E-09	5.44E-08
	C5O78_04910	CP027018.1(-)	7.158669	2.857589	1.02E-21	2.82E-19
	C5O78_12225	CP027018.1(-)	5.159718	2.934826	1.10E-10	7.05E-09

	C5O78_07420	CP027018.1(+)	9.204063	3.00238	2.81E-39	1.29E-36
	C5O78_09255	CP027018.1(+)	-0.56864	3.059871	0.005773	0.027392
	C5O78_12600	CP027018.1(+)	6.378534	3.113991	3.06E-07	6.92E-06
	C5O78_13235	CP027018.1(-)	3.669226	3.175994	3.10E-10	1.68E-08
	C5O78_12220	CP027018.1(-)	4.748761	3.196301	1.42E-15	2.62E-13
	C5O78_12615	CP027018.1(+)	4.006062	3.25161	6.63E-12	6.31E-10
	C5O78_12625	CP027018.1(+)	6.899551	3.251978	9.80E-05	0.001021
	C5O78_13215	CP027018.1(-)	1.029199	3.359883	5.62E-06	8.48E-05
	C5O78_13220	CP027018.1(-)	3.917185	3.433192	1.57E-11	1.40E-09
	C5O78_12635	CP027018.1(+)	5.062997	3.512777	7.08E-15	1.15E-12
	C5O78_12610	CP027018.1(+)	3.192494	3.617999	2.46E-13	2.95E-11
	C5O78_12155	CP027018.1(+)	-0.23476	3.682621	0.000435	0.003492
	C5O78_12620	CP027018.1(+)	2.037452	3.708283	6.75E-10	3.39E-08
	C5O78_07415	CP027018.1(+)	9.923361	3.877469	3.22E-55	1.78E-52
	C5O78_09630	CP027018.1(+)	5.366207	3.964681	1.33E-21	3.33E-19
	C5O78_09620	CP027018.1(+)	11.17233	4.168607	6.73E-06	9.88E-05
	C5O78_09625	CP027018.1(+)	7.618243	4.237698	2.13E-34	8.41E-32
	C5O78_13225	CP027018.1(-)	4.349291	4.2488	1.05E-14	1.61E-12
	C5O78_12630	CP027018.1(+)	9.454047	5.052759	1.57E-58	1.09E-55
	C5O78_06210	CP027018.1(-)	-1.65679	5.678374	0.008558	0.03727
	C5O78_02745	CP027018.1(-)	-1.35714	6.237166	0.002929	0.015701
T320A +T3552B/ T3552B	DYQ05_02280	CP045670.1(+)	5.175709	4.669281	9.37E-27	2.36E-23
	DYQ05_02275	CP045670.1(+)	5.472704	4.390736	2.43E-25	2.04E-22
	DYQ05_02285	CP045670.1(+)	6.526626	4.364786	1.33E-22	8.37E-20
	DYQ05_02270	CP045670.1(+)	7.298938	4.229664	1.49E-25	1.88E-22
	DYQ05_04140	CP045670.1(+)	1.526129	3.85096	1.65E-06	9.05E-05
	DYQ05_10360	CP045670.1(+)	1.874294	3.593182	3.34E-07	2.47E-05
	DYQ05_00630	CP045670.1(-)	2.947958	3.586301	1.12E-11	2.34E-09
	DYQ05_03760	CP045670.1(-)	8.219158	3.374828	7.83E-12	1.79E-09
	DYQ05_02265	CP045670.1(+)	8.149512	3.16427	5.72E-05	0.001619
	DYQ05_10355	CP045670.1(+)	4.977945	3.096781	0.001185	0.01622
	DYQ05_08530	CP045670.1(-)	0.957998	3.031946	0.002902	0.031378
	DYQ05_04165	CP045670.1(-)	2.995231	2.955712	2.26E-09	3.35E-07
	DYQ05_12325	CP045670.1(-)	4.56851	2.881445	7.15E-12	1.79E-09
	DYQ05_04155	CP045670.1(+)	3.445725	2.712934	0.002316	0.026638
	DYQ05_12335	CP045670.1(-)	7.494048	2.695095	6.95E-10	1.09E-07
	DYQ05_07960	CP045670.1(-)	10.16534	2.693038	1.83E-11	3.54E-09
	DYQ05_13310	CP045670.1(-)	6.688916	2.659599	1.18E-15	5.94E-13
	DYQ05_01975	CP045670.1(-)	9.567967	2.652126	8.00E-11	1.44E-08
	DYQ05_12330	CP045670.1(-)	5.605624	2.592948	1.68E-12	4.97E-10
	DYQ05_05125	CP045670.1(-)	10.59072	2.56203	3.76E-10	6.31E-08
	DYQ05_02490	CP045670.1(-)	3.433716	2.555068	0.000157	0.003372
	DYQ05_02775	CP045670.1(+)	8.973582	2.550049	3.21E-09	4.04E-07
	DYQ05_04560	CP045670.1(+)	3.361436	2.546352	2.71E-09	3.59E-07
	DYQ05_08535	CP045670.1(-)	1.766745	2.516084	9.20E-06	0.000374
	DYQ05_06525	CP045670.1(-)	2.937734	2.514839	7.59E-06	0.000324

	DYQ05_06975	CP045670.1(-)	3.057353	2.509387	1.75E-06	9.36E-05
	DYQ05_12320	CP045670.1(-)	4.440244	2.419226	1.74E-08	1.91E-06
	DYQ05_13315	CP045670.1(-)	8.393711	2.411111	2.30E-08	2.32E-06
	DYQ05_13410	CP045670.1(-)	4.050023	2.410006	0.000116	0.002606
	DYQ05_03355	CP045670.1(-)	1.442858	2.360607	0.000745	0.01144
	DYQ05_13040	CP045670.1(-)	3.648397	2.340717	1.18E-06	7.23E-05
	DYQ05_13320	CP045670.1(-)	10.43461	2.330616	3.54E-07	2.54E-05
	DYQ05_04135	CP045670.1(+)	8.154305	2.308671	1.79E-13	7.51E-11
	DYQ05_07955	CP045670.1(-)	5.815583	2.281238	3.08E-08	2.98E-06
	DYQ05_08525	CP045670.1(-)	7.346251	2.269882	8.87E-08	7.21E-06
	DYQ05_07410	CP045670.1(+)	8.958039	2.267503	1.25E-06	7.31E-05
	DYQ05_02780	CP045670.1(+)	6.802726	2.264954	1.20E-12	4.30E-10
	DYQ05_06970	CP045670.1(-)	4.995054	2.243392	6.23E-09	7.13E-07
	DYQ05_04570	CP045670.1(+)	1.604573	2.230003	3.99E-05	0.001209
	DYQ05_04565	CP045670.1(+)	2.4662	2.213153	1.59E-06	8.92E-05
	DYQ05_05260	CP045670.1(-)	7.038332	2.175532	2.99E-07	2.28E-05
	DYQ05_10570	CP045670.1(+)	7.99821	2.174452	7.91E-08	6.87E-06
	DYQ05_07415	CP045670.1(+)	10.13537	2.126714	5.42E-08	4.88E-06
	DYQ05_05640	CP045670.1(-)	2.017241	2.106148	0.000103	0.002374
	DYQ05_00625	CP045670.1(-)	1.803702	2.105255	8.92E-05	0.002204
	DYQ05_04440	CP045670.1(+)	9.491144	2.026855	2.13E-06	0.000107
	DYQ05_05265	CP045670.1(-)	5.463555	2.025019	1.14E-05	0.000452
	DYQ05_00105	CP045670.1(+)	6.684408	2.014085	1.25E-06	7.31E-05
	DYQ05_12835	CP045670.1(-)	3.904116	1.998112	4.22E-05	0.001236
	DYQ05_12840	CP045670.1(-)	7.764655	1.996467	1.27E-05	0.000481
	DYQ05_06530	CP045670.1(-)	5.88228	1.981425	9.03E-07	5.98E-05
	DYQ05_02635	CP045670.1(+)	10.87397	1.94399	1.82E-07	1.43E-05
	DYQ05_04130	CP045670.1(+)	5.696402	1.941368	0.001243	0.01662
	DYQ05_04145	CP045670.1(+)	4.751484	1.939713	4.29E-06	0.000193
	DYQ05_11880	CP045670.1(+)	4.695242	1.906729	1.04E-06	6.69E-05
	DYQ05_02690	CP045670.1(-)	7.168435	1.900296	1.15E-05	0.000452
	DYQ05_07875	CP045670.1(-)	4.709205	1.875863	1.49E-05	0.00053
	DYQ05_02175	CP045670.1(+)	8.206423	1.859744	1.82E-08	1.91E-06
	DYQ05_03340	CP045670.1(-)	2.152419	1.858513	4.22E-05	0.001236
	DYQ05_04575	CP045670.1(+)	1.87693	1.858231	0.000705	0.010958
	DYQ05_01805	CP045670.1(+)	9.206402	1.856925	3.07E-06	0.000141
	DYQ05_08005	CP045670.1(-)	7.981049	1.854235	7.51E-06	0.000324
	DYQ05_04555	CP045670.1(+)	5.535498	1.851716	2.95E-05	0.000941
	DYQ05_12520	CP045670.1(+)	12.59213	1.834569	4.79E-06	0.000212
	DYQ05_11230	CP045670.1(-)	8.556903	1.75967	3.66E-09	4.39E-07
	DYQ05_13305	CP045670.1(-)	4.166426	1.752572	1.62E-05	0.000559
	DYQ05_10580	CP045670.1(+)	10.25434	1.713578	1.61E-05	0.000559
	DYQ05_01830	CP045670.1(+)	9.800374	1.69302	1.83E-06	9.61E-05
	DYQ05_03350	CP045670.1(-)	2.674632	1.663047	0.000233	0.004587
	DYQ05_07420	CP045670.1(+)	7.741737	1.653455	2.21E-05	0.000731
	DYQ05_03670	CP045670.1(+)	8.206497	1.629427	4.01E-07	2.81E-05

	DYQ05_09735	CP045670.1(-)	10.10283	1.619923	6.72E-05	0.001783
	DYQ05_01810	CP045670.1(+)	6.01148	1.605405	1.28E-05	0.000481
	DYQ05_12315	CP045670.1(-)	3.883389	1.595648	0.000288	0.005414
	DYQ05_12465	CP045670.1(-)	9.112784	1.590241	1.97E-05	0.000662
	DYQ05_00300	CP045670.1(+)	12.41636	1.58783	6.25E-05	0.001731
	DYQ05_07360	CP045670.1(-)	5.897471	1.553018	6.25E-05	0.001731
	DYQ05_09730	CP045670.1(-)	10.90806	1.550867	0.000153	0.003347
	DYQ05_10780	CP045670.1(+)	5.140477	1.527863	0.002635	0.029374
	DYQ05_01885	CP045670.1(-)	6.742035	1.520965	0.000218	0.004366
	DYQ05_01835	CP045670.1(+)	6.32604	1.512804	2.38E-06	0.000113
	DYQ05_00770	CP045670.1(-)	3.003496	1.50802	0.004095	0.040768
	DYQ05_09565	CP045670.1(+)	10.83247	1.489703	6.64E-05	0.00178
	DYQ05_07880	CP045670.1(-)	6.589018	1.480628	0.000352	0.006425
	DYQ05_00305	CP045670.1(+)	8.071549	1.476371	1.36E-05	0.000503
	DYQ05_01815	CP045670.1(+)	4.956974	1.469321	0.000444	0.007621
	DYQ05_11195	CP045670.1(+)	11.42498	1.453863	9.44E-05	0.002279
	DYQ05_04585	CP045670.1(+)	3.855729	1.445747	0.000226	0.00448
	DYQ05_04540	CP045670.1(-)	5.427115	1.441767	5.13E-05	0.001485
	DYQ05_02185	CP045670.1(+)	3.438246	1.434435	0.000682	0.010677
	DYQ05_01565	CP045670.1(+)	9.075045	1.401706	4.03E-05	0.001209
	DYQ05_01335	CP045670.1(-)	7.679073	1.401541	8.02E-05	0.00206
	DYQ05_04220	CP045670.1(+)	9.164421	1.393104	6.52E-05	0.001765
	DYQ05_01310	CP045670.1(+)	5.055202	1.378088	0.003019	0.031819
	DYQ05_04445	CP045670.1(+)	7.827573	1.370634	2.03E-06	0.000104
	DYQ05_01340	CP045670.1(-)	3.633087	1.364642	0.004861	0.04656
	DYQ05_10585	CP045670.1(+)	9.228672	1.355589	0.000136	0.002998
	DYQ05_01055	CP045670.1(-)	3.599718	1.334123	0.000749	0.01144
	DYQ05_05795	CP045670.1(-)	5.137392	1.320731	0.002679	0.029469
	DYQ05_06520	CP045670.1(-)	4.486806	1.31903	0.000873	0.012873
	DYQ05_01760	CP045670.1(-)	6.359684	1.31164	0.00016	0.003423
	DYQ05_11055	CP045670.1(+)	5.705836	1.304086	0.001244	0.01662
	DYQ05_10595	CP045670.1(+)	7.775518	1.302204	0.000129	0.002869
	DYQ05_05730	CP045670.1(+)	5.337062	1.281461	0.000582	0.009519
	DYQ05_00150	CP045670.1(+)	5.998988	1.271055	0.00058	0.009519
	DYQ05_08000	CP045670.1(-)	5.386108	1.260282	0.001279	0.016694
	DYQ05_02830	CP045670.1(+)	5.939759	1.256898	5.40E-05	0.001545
	DYQ05_10650	CP045670.1(+)	9.531529	1.255103	0.000851	0.012842
	DYQ05_10620	CP045670.1(-)	6.649788	1.24379	0.000156	0.003372
	DYQ05_01330	CP045670.1(-)	8.566449	1.240313	0.00024	0.00465
	DYQ05_13470	CP045670.1(-)	5.881192	1.207649	0.000515	0.008712
	DYQ05_01825	CP045670.1(+)	6.633673	1.200288	0.000238	0.004646
	DYQ05_03855	CP045670.1(+)	8.376591	1.198159	3.67E-05	0.001141
	DYQ05_05685	CP045670.1(-)	4.995759	1.19416	0.00097	0.013884
	DYQ05_11270	CP045670.1(-)	6.298013	1.178421	0.000358	0.006496
	DYQ05_10625	CP045670.1(-)	8.183414	1.168617	9.67E-05	0.002297
	DYQ05_05055	CP045670.1(+)	6.186608	1.161645	0.000746	0.01144

	DYQ05_02575	CP045670.1(+)	9.216435	1.157034	0.000932	0.013501
	DYQ05_13385	CP045670.1(-)	6.991153	1.152047	0.000391	0.006984
	DYQ05_08075	CP045670.1(-)	6.983707	1.151344	0.000417	0.007288
	DYQ05_04925	CP045670.1(+)	5.95709	1.144163	0.001698	0.02117
	DYQ05_05610	CP045670.1(+)	6.903572	1.140114	0.000105	0.002401
	DYQ05_01795	CP045670.1(+)	6.508476	1.114627	0.001042	0.014666
	DYQ05_00310	CP045670.1(+)	7.980833	1.114442	0.00102	0.014513
	DYQ05_07395	CP045670.1(+)	7.201149	1.088262	0.000874	0.012873
	DYQ05_09175	CP045670.1(-)	8.911972	1.081112	0.000189	0.003937
	DYQ05_01990	CP045670.1(-)	5.658724	1.076827	0.002004	0.024176
	DYQ05_01630	CP045670.1(+)	9.588239	1.059304	0.001529	0.019557
	DYQ05_05985	CP045670.1(-)	8.558696	1.048822	0.000858	0.012864
	DYQ05_02835	CP045670.1(+)	9.425555	1.043525	0.00126	0.01662
	DYQ05_12045	CP045670.1(-)	7.999406	1.01935	0.001159	0.015952
	DYQ05_10800	CP045670.1(+)	8.584388	1.013745	0.004451	0.043294
	DYQ05_12500	CP045670.1(+)	8.083505	1.001994	0.003674	0.03732
T19 + T320A + T3552B/ T19	C5N99_00100	CP027017.1(-)	9.125204	-1.01995	0.000455	0.017936
	C5N99_10365	CP027017.1(+)	7.53497	-1.05941	0.001435	0.044176
	C5N99_09230	CP027017.1(-)	7.204849	-1.07241	0.001205	0.038665
	C5N99_05885	CP027017.1(+)	10.71269	-1.15576	1.21E-06	9.69E-05
	C5N99_05880	CP027017.1(+)	12.4755	-1.23035	1.70E-07	1.66E-05
	C5N99_01905	CP027017.1(-)	9.876538	-1.23626	2.07E-07	1.93E-05
	C5N99_08940	CP027017.1(-)	6.974963	-1.27477	0.000406	0.017227
	C5N99_09460	CP027017.1(+)	6.829802	-1.3454	0.00055	0.019926
	C5N99_01900	CP027017.1(-)	8.316263	-1.36374	6.53E-06	0.000445
	C5N99_09095	CP027017.1(+)	8.476681	-2.04215	4.03E-06	0.000302
	C5N99_11895	CP027017.1(+)	5.946908	-2.41089	7.16E-05	0.004023
	C5N99_11890	CP027017.1(+)	7.018963	-2.81152	1.37E-08	1.62E-06
T19 + T320A + T3552B/ T320A	C5O78_13835	CP027018.1(+)	6.442589	-3.22311	3.01E-21	9.11E-19
	C5O78_13840	CP027018.1(+)	2.748123	-3.43192	4.16E-08	2.56E-06
	C5O78_13830	CP027018.1(-)	8.272586	-6.65305	1.06E-16	1.69E-14
T19 + T320A + T3552B/ T3552B	DYQ05_00220	CP045670.1(+)	3.633695	-1.00064	0.002098	0.016794
	DYQ05_10615	CP045670.1(+)	7.111628	-1.0035	0.000111	0.001475
	DYQ05_11840	CP045670.1(+)	3.443757	-1.01493	0.002718	0.020375
	DYQ05_08875	CP045670.1(+)	11.71855	-1.02142	9.98E-05	0.001359
	DYQ05_05865	CP045670.1(+)	16.79752	-1.02629	8.55E-06	0.00016
	DYQ05_02700	CP045670.1(+)	9.155039	-1.03061	1.26E-05	0.00023
	DYQ05_10315	CP045670.1(+)	9.002314	-1.03097	2.91E-06	6.59E-05
	DYQ05_08565	CP045670.1(+)	11.23249	-1.03714	0.000157	0.001929
	DYQ05_12305	CP045670.1(+)	2.929884	-1.03965	0.005408	0.034639
	DYQ05_07910	CP045670.1(-)	6.52828	-1.04283	0.000249	0.002829
	DYQ05_12800	CP045670.1(-)	8.192734	-1.05908	1.64E-06	3.93E-05
	DYQ05_11905	CP045670.1(+)	5.468447	-1.06381	5.57E-05	0.000837
	DYQ05_08050	CP045670.1(-)	11.34165	-1.07209	0.000128	0.001658
	DYQ05_11925	CP045670.1(+)	8.789756	-1.08856	5.97E-06	0.00012
	DYQ05_05410	CP045670.1(+)	3.691279	-1.09047	0.001172	0.010394

	DYQ05_11480	CP045670.1(-)	6.276133	-1.10403	4.30E-05	0.000676
	DYQ05_13145	CP045670.1(-)	6.017417	-1.10466	4.22E-05	0.000667
	DYQ05_04415	CP045670.1(+)	8.808222	-1.10727	6.97E-06	0.000136
	DYQ05_03555	CP045670.1(+)	4.186372	-1.13877	0.000155	0.001917
	DYQ05_12970	CP045670.1(-)	5.333498	-1.16513	0.000192	0.002264
	DYQ05_08330	CP045670.1(-)	10.33922	-1.16558	6.24E-08	1.71E-06
	DYQ05_05480	CP045670.1(+)	2.54421	-1.17318	0.002616	0.019853
	DYQ05_08070	CP045670.1(-)	6.636821	-1.17418	1.38E-05	0.000251
	DYQ05_05445	CP045670.1(+)	10.24951	-1.18199	8.66E-09	2.89E-07
	DYQ05_11965	CP045670.1(-)	10.20037	-1.19132	7.84E-09	2.65E-07
	DYQ05_07890	CP045670.1(-)	8.365459	-1.22543	1.72E-05	0.000305
	DYQ05_12075	CP045670.1(-)	3.346004	-1.2771	0.000142	0.00181
	DYQ05_07930	CP045670.1(-)	2.490547	-1.28724	0.00648	0.039553
	DYQ05_08035	CP045670.1(-)	9.879947	-1.30657	2.78E-05	0.000473
	DYQ05_09040	CP045670.1(-)	6.712501	-1.30722	1.77E-06	4.20E-05
	DYQ05_04410	CP045670.1(+)	8.602775	-1.33578	1.21E-07	3.28E-06
	DYQ05_10865	CP045670.1(+)	2.883768	-1.35162	0.000152	0.001891
	DYQ05_07950	CP045670.1(-)	10.50031	-1.40618	2.99E-06	6.64E-05
	DYQ05_09585	CP045670.1(-)	7.408311	-1.45818	6.35E-11	2.77E-09
	DYQ05_09650	CP045670.1(+)	2.161352	-1.46421	0.002397	0.018414
	DYQ05_07920	CP045670.1(-)	7.822605	-1.60216	1.19E-06	2.87E-05
	DYQ05_00120	CP045670.1(-)	5.960301	-1.64236	5.82E-11	2.61E-09
	DYQ05_06160	CP045670.1(-)	1.303286	-1.65997	0.007344	0.043328
	DYQ05_01595	CP045670.1(-)	8.772079	-1.66876	1.23E-05	0.000226
	DYQ05_07940	CP045670.1(-)	5.504042	-1.71497	0.000544	0.00548
	DYQ05_07945	CP045670.1(-)	6.49731	-1.74306	2.72E-08	7.88E-07
	DYQ05_12080	CP045670.1(-)	3.83715	-1.81631	1.94E-08	5.91E-07
	DYQ05_07935	CP045670.1(-)	5.640405	-1.82348	3.97E-08	1.13E-06
	DYQ05_00665	CP045670.1(+)	5.491455	-1.85335	1.88E-05	0.000332
	DYQ05_00645	CP045670.1(+)	6.111615	-1.85389	8.36E-15	7.11E-13
	DYQ05_04850	CP045670.1(-)	1.054943	-1.86736	0.004625	0.030659
	DYQ05_00660	CP045670.1(+)	2.924894	-1.91348	0.000218	0.00252
	DYQ05_08040	CP045670.1(-)	6.535292	-1.95573	1.30E-14	1.03E-12
	DYQ05_00655	CP045670.1(+)	5.746226	-2.10167	0.002119	0.0168
	DYQ05_02250	CP045670.1(+)	6.693472	-2.23392	2.48E-07	6.64E-06
	DYQ05_01230	CP045670.1(+)	3.666711	-2.7882	9.67E-05	0.001325
	DYQ05_01235	CP045670.1(+)	5.608493	-3.91861	2.29E-08	6.87E-07
	DYQ05_04820	CP045670.1(-)	-2.02862	-4.62735	0.003858	0.026874
	DYQ05_01240	CP045670.1(+)	4.994071	-4.69168	1.25E-08	4.00E-07
	DYQ05_01245	CP045670.1(+)	8.330562	-4.70041	7.83E-10	3.02E-08
T19 + T320A + T3552B/ T19	C5N99_12200	CP027017.1(+)	7.83844	5.14384	5.64E-22	2.53E-19
	C5N99_00055	CP027017.1(-)	7.282489	4.639794	3.75E-17	9.35E-15
	C5N99_12195	CP027017.1(+)	10.53135	4.301677	3.37E-49	7.56E-46
	C5N99_10630	CP027017.1(+)	6.544864	3.910731	9.21E-07	7.66E-05
	C5N99_10625	CP027017.1(+)	7.510617	3.865719	1.81E-13	3.69E-11
	C5N99_10620	CP027017.1(+)	9.967245	3.794933	5.35E-39	6.01E-36

	C5N99_05410	CP027017.1(-)	5.230884	3.791166	6.24E-05	0.003693
	C5N99_06460	CP027017.1(-)	8.606693	3.702	2.39E-20	7.68E-18
	C5N99_07185	CP027017.1(-)	8.570135	3.586178	2.10E-25	1.18E-22
	C5N99_04990	CP027017.1(-)	4.453074	3.226957	0.001503	0.045637
	C5N99_05495	CP027017.1(+)	5.09888	3.078599	0.000374	0.016176
	C5N99_12205	CP027017.1(+)	8.36652	3.035806	8.41E-19	2.36E-16
	C5N99_10065	CP027017.1(+)	10.70677	2.872999	2.95E-21	1.11E-18
	C5N99_05075	CP027017.1(+)	12.63495	2.797458	4.20E-28	3.15E-25
	C5N99_02885	CP027017.1(+)	6.984262	2.740317	5.03E-09	6.65E-07
	C5N99_05280	CP027017.1(-)	5.828485	2.721183	0.000474	0.018349
	C5N99_09180	CP027017.1(+)	7.360735	2.5142	5.84E-09	7.29E-07
	C5N99_10070	CP027017.1(+)	9.022781	2.42396	2.54E-08	2.85E-06
	C5N99_11590	CP027017.1(-)	5.491929	2.272364	0.0006	0.02091
	C5N99_02890	CP027017.1(+)	9.130088	2.093782	7.28E-07	6.29E-05
	C5N99_08040	CP027017.1(+)	7.909278	2.048473	4.26E-10	6.38E-08
	C5N99_09175	CP027017.1(+)	6.814106	2.036174	4.42E-06	0.000318
	C5N99_10400	CP027017.1(+)	8.788846	2.03541	1.45E-07	1.48E-05
	C5N99_11755	CP027017.1(+)	10.14751	1.836555	1.13E-14	2.53E-12
	C5N99_11760	CP027017.1(+)	6.639381	1.829156	0.000804	0.026948
	C5N99_10150	CP027017.1(-)	9.127788	1.814432	4.08E-13	7.64E-11
	C5N99_00510	CP027017.1(-)	10.00588	1.777474	4.82E-13	8.34E-11
	C5N99_05270	CP027017.1(-)	11.17394	1.68394	3.26E-07	2.93E-05
	C5N99_11160	CP027017.1(+)	6.534321	1.619595	0.00169	0.048674
	C5N99_07955	CP027017.1(-)	10.30893	1.587649	1.72E-11	2.75E-09
	C5N99_10395	CP027017.1(+)	10.07842	1.575689	4.11E-09	5.77E-07
	C5N99_11750	CP027017.1(+)	7.518483	1.562933	4.53E-06	0.000318
	C5N99_00995	CP027017.1(+)	9.894745	1.485186	4.00E-05	0.002497
	C5N99_10060	CP027017.1(+)	7.055721	1.473745	0.000502	0.018783
	C5N99_09195	CP027017.1(+)	9.212961	1.439925	2.11E-06	0.000164
	C5N99_03640	CP027017.1(+)	7.396647	1.436609	4.23E-05	0.002571
	C5N99_03050	CP027017.1(-)	8.340268	1.428541	6.42E-05	0.003697
	C5N99_12135	CP027017.1(-)	9.395068	1.418996	2.86E-08	3.06E-06
	C5N99_03845	CP027017.1(-)	7.986664	1.396001	0.000519	0.019109
	C5N99_05045	CP027017.1(+)	9.954372	1.328208	0.000442	0.017936
	C5N99_10820	CP027017.1(-)	10.43113	1.24055	0.000125	0.006555
	C5N99_10445	CP027017.1(+)	8.601197	1.22104	0.000203	0.009697
	C5N99_08875	CP027017.1(+)	7.439664	1.178103	0.000605	0.02091
	C5N99_11835	CP027017.1(-)	14.47145	1.155179	0.00049	0.018658
	C5N99_06030	CP027017.1(-)	8.895724	1.058298	0.000121	0.00647
	C5N99_06950	CP027017.1(-)	7.962341	1.056769	0.000303	0.013873
	C5N99_08225	CP027017.1(+)	9.874318	1.023595	0.000276	0.012905
T19 + T320A + T3552B/ T320A	C5O78_00675	CP027018.1(-)	3.021179	8.649911	4.61E-05	0.001765
	C5O78_00680	CP027018.1(-)	0.978006	6.57969	0.001262	0.034676
	C5O78_13215	CP027018.1(-)	3.121794	5.678251	6.88E-10	5.20E-08
	C5O78_13220	CP027018.1(-)	5.852363	5.020915	2.27E-09	1.63E-07
	C5O78_13235	CP027018.1(-)	5.607418	4.945323	2.25E-10	1.80E-08

	C5O78_13225	CP027018.1(-)	6.174678	4.897317	1.25E-11	1.09E-09
	C5O78_12220	CP027018.1(-)	6.014056	4.841524	1.01E-21	3.94E-19
	C5O78_12225	CP027018.1(-)	6.615099	4.774239	1.96E-30	2.66E-27
	C5O78_12630	CP027018.1(+)	10.80472	4.588755	1.24E-17	2.25E-15
	C5O78_04840	CP027018.1(+)	13.02309	4.565728	4.56E-15	5.40E-13
	C5O78_12620	CP027018.1(+)	3.711107	4.4954	5.27E-13	5.74E-11
	C5O78_12615	CP027018.1(+)	5.279194	4.35108	5.52E-20	1.36E-17
	C5O78_09620	CP027018.1(+)	12.32338	4.256253	2.72E-13	3.08E-11
	C5O78_12610	CP027018.1(+)	4.665768	4.215718	2.94E-16	4.45E-14
	C5O78_12230	CP027018.1(-)	6.552747	4.206108	2.27E-21	7.73E-19
	C5O78_12625	CP027018.1(+)	8.338045	4.045667	5.03E-26	3.42E-23
	C5O78_12600	CP027018.1(+)	7.646576	4.026626	7.50E-36	2.04E-32
	C5O78_12605	CP027018.1(+)	5.006455	3.989131	1.28E-15	1.74E-13
	C5O78_13240	CP027018.1(-)	6.330563	3.914128	4.85E-12	4.89E-10
	C5O78_12635	CP027018.1(+)	6.709535	3.865292	3.35E-25	1.82E-22
	C5O78_09625	CP027018.1(+)	8.731795	3.838127	1.57E-15	2.04E-13
	C5O78_04910	CP027018.1(-)	8.385014	3.715573	3.55E-21	9.66E-19
	C5O78_02685	CP027018.1(-)	6.939273	3.66874	2.18E-17	3.71E-15
	C5O78_13070	CP027018.1(-)	9.604709	3.60359	9.27E-28	8.40E-25
	C5O78_02690	CP027018.1(-)	10.65216	3.563171	6.88E-16	9.85E-14
	C5O78_10250	CP027018.1(+)	8.130399	3.530975	1.74E-08	1.10E-06
	C5O78_05175	CP027018.1(+)	9.538843	3.518736	4.84E-19	1.01E-16
	C5O78_12235	CP027018.1(-)	8.560222	3.457922	1.70E-18	3.30E-16
	C5O78_08060	CP027018.1(+)	8.90822	3.345655	9.93E-20	2.25E-17
	C5O78_04915	CP027018.1(-)	8.835208	3.288488	7.99E-23	3.62E-20
	C5O78_01810	CP027018.1(-)	5.388644	3.18374	1.04E-11	9.73E-10
	C5O78_09630	CP027018.1(+)	6.097398	3.026676	4.37E-11	3.60E-09
	C5O78_13270	CP027018.1(+)	5.447356	3.023549	3.23E-12	3.38E-10
	C5O78_02680	CP027018.1(-)	5.022376	2.869958	4.24E-08	2.56E-06
	C5O78_10720	CP027018.1(+)	5.998709	2.744267	0.000647	0.020466
	C5O78_01815	CP027018.1(-)	7.859286	2.735185	3.89E-15	4.81E-13
	C5O78_10575	CP027018.1(-)	7.080642	2.432002	1.13E-11	1.02E-09
	C5O78_00015	CP027018.1(-)	6.336794	2.397585	1.41E-11	1.20E-09
	C5O78_08590	CP027018.1(+)	5.804351	2.28039	0.000547	0.017716
	C5O78_02275	CP027018.1(-)	10.17235	2.279195	6.84E-12	6.64E-10
	C5O78_05170	CP027018.1(+)	2.565042	2.270446	0.001001	0.029286
	C5O78_08055	CP027018.1(+)	7.858486	2.216925	4.62E-09	3.14E-07
	C5O78_13275	CP027018.1(+)	5.381328	2.081269	1.14E-06	5.27E-05
	C5O78_02675	CP027018.1(-)	5.790558	2.073199	1.88E-07	9.82E-06
	C5O78_11550	CP027018.1(+)	8.362911	2.059774	1.52E-08	9.87E-07
	C5O78_04860	CP027018.1(-)	6.475604	2.050817	1.58E-07	8.62E-06
	C5O78_04865	CP027018.1(-)	10.17359	2.024007	9.89E-09	6.56E-07
	C5O78_13975	CP027018.1(-)	4.76309	1.993793	6.57E-07	3.25E-05
	C5O78_05940	CP027018.1(+)	7.102766	1.990431	4.55E-10	3.53E-08
	C5O78_01605	CP027018.1(+)	5.863263	1.988189	1.44E-06	6.54E-05
	C5O78_00165	CP027018.1(-)	6.783119	1.987843	1.29E-09	9.48E-08

	C5O78_10580	CP027018.1(-)	5.739955	1.933189	8.06E-07	3.91E-05
	C5O78_03445	CP027018.1(+)	5.712579	1.895491	8.48E-07	4.05E-05
	C5O78_13265	CP027018.1(+)	8.188121	1.882731	3.12E-09	2.17E-07
	C5O78_13980	CP027018.1(-)	5.059195	1.876404	6.06E-07	3.05E-05
	C5O78_09140	CP027018.1(-)	6.656471	1.859613	1.52E-07	8.46E-06
	C5O78_10820	CP027018.1(+)	4.76201	1.804888	0.001517	0.039805
	C5O78_03595	CP027018.1(+)	6.790208	1.796063	0.000483	0.016211
	C5O78_13280	CP027018.1(-)	7.530614	1.795717	2.31E-06	0.000101
	C5O78_03450	CP027018.1(+)	4.367811	1.761594	0.000497	0.016418
	C5O78_08755	CP027018.1(+)	5.374196	1.725281	0.000131	0.004876
	C5O78_11595	CP027018.1(-)	7.502961	1.711581	6.45E-08	3.81E-06
	C5O78_13985	CP027018.1(-)	6.164202	1.658071	4.84E-06	0.0002
	C5O78_09500	CP027018.1(-)	8.267556	1.650495	1.80E-07	9.58E-06
	C5O78_01820	CP027018.1(-)	9.879651	1.643371	1.80E-06	8.03E-05
	C5O78_11305	CP027018.1(+)	9.805931	1.608903	3.56E-06	0.000151
	C5O78_04955	CP027018.1(-)	11.69894	1.582672	0.001385	0.037289
	C5O78_08890	CP027018.1(+)	6.919617	1.577578	1.06E-07	5.99E-06
	C5O78_11555	CP027018.1(+)	7.017154	1.576306	5.68E-06	0.00023
	C5O78_07410	CP027018.1(+)	9.06223	1.570403	1.98E-07	1.02E-05
	C5O78_03590	CP027018.1(+)	7.064994	1.559647	0.000744	0.022492
	C5O78_09505	CP027018.1(-)	6.849533	1.527579	3.54E-06	0.000151
	C5O78_00245	CP027018.1(+)	4.409962	1.516459	0.000221	0.008024
	C5O78_00480	CP027018.1(+)	7.771974	1.50012	1.07E-06	5.01E-05
	C5O78_10305	CP027018.1(-)	10.7598	1.490926	0.000723	0.02236
	C5O78_01465	CP027018.1(-)	7.664347	1.480962	6.71E-08	3.88E-06
	C5O78_11590	CP027018.1(-)	6.786267	1.439741	1.82E-05	0.000717
	C5O78_12590	CP027018.1(+)	6.508231	1.403458	0.001206	0.033462
	C5O78_10310	CP027018.1(-)	10.20929	1.399046	7.89E-06	0.000315
	C5O78_04875	CP027018.1(+)	4.05731	1.393513	0.001529	0.039805
	C5O78_13450	CP027018.1(+)	9.414965	1.355905	4.50E-06	0.000188
	C5O78_05920	CP027018.1(+)	6.539216	1.308046	0.000393	0.013521
	C5O78_01725	CP027018.1(-)	5.463738	1.295441	0.000417	0.014175
	C5O78_01770	CP027018.1(-)	5.041286	1.257282	0.000753	0.022493
	C5O78_08795	CP027018.1(-)	5.434891	1.225748	0.000615	0.019688
	C5O78_07000	CP027018.1(-)	5.926296	1.214935	0.000268	0.009455
	C5O78_03880	CP027018.1(-)	5.728235	1.195062	0.001085	0.031056
	C5O78_10500	CP027018.1(+)	9.622223	1.136796	3.56E-05	0.001381
	C5O78_07435	CP027018.1(+)	8.262691	1.113085	7.19E-05	0.002717
	C5O78_12095	CP027018.1(-)	9.173945	1.050526	0.000258	0.009241
	C5O78_08260	CP027018.1(+)	6.920548	1.04981	0.001134	0.032137
	C5O78_03890	CP027018.1(-)	8.208491	1.035271	0.000188	0.006905
T19 + T320A + T3552B/ T3552B	DYQ05_09285	CP045670.1(+)	0.788742	6.560326	0.002197	0.017158
	DYQ05_10360	CP045670.1(+)	3.642342	4.745541	1.41E-16	1.83E-14
	DYQ05_02270	CP045670.1(+)	7.45546	4.342051	1.70E-48	4.20E-45
	DYQ05_00630	CP045670.1(-)	4.848647	4.082785	2.90E-06	6.59E-05
	DYQ05_04155	CP045670.1(+)	3.867116	4.054061	2.07E-16	2.43E-14

	DYQ05_00625	CP045670.1(-)	2.903541	3.992826	2.69E-08	7.88E-07
	DYQ05_03760	CP045670.1(-)	8.766294	3.948539	9.87E-30	8.11E-27
	DYQ05_02280	CP045670.1(+)	5.074133	3.662297	2.14E-20	4.40E-18
	DYQ05_02275	CP045670.1(+)	5.341832	3.634351	4.95E-12	2.72E-10
	DYQ05_04160	CP045670.1(-)	1.230924	3.626577	8.99E-05	0.001246
	DYQ05_02775	CP045670.1(+)	9.445623	3.52321	1.35E-24	4.16E-22
	DYQ05_10365	CP045670.1(+)	1.153905	3.443935	0.000102	0.001378
	DYQ05_04440	CP045670.1(+)	10.2205	3.395691	1.84E-25	6.48E-23
	DYQ05_01975	CP045670.1(-)	9.945773	3.380724	3.54E-26	1.75E-23
	DYQ05_13320	CP045670.1(-)	10.9491	3.376127	2.60E-27	1.60E-24
	DYQ05_02635	CP045670.1(+)	11.63038	3.342183	2.93E-15	2.78E-13
	DYQ05_06975	CP045670.1(-)	3.845216	3.341471	1.13E-14	9.30E-13
	DYQ05_13315	CP045670.1(-)	8.670659	3.194513	2.18E-24	5.98E-22
	DYQ05_04130	CP045670.1(+)	6.13657	3.133103	1.91E-21	4.27E-19
	DYQ05_02285	CP045670.1(+)	6.347342	3.073966	1.99E-18	3.07E-16
	DYQ05_13415	CP045670.1(-)	9.759782	3.049253	1.78E-32	2.20E-29
	DYQ05_04570	CP045670.1(+)	1.618028	2.988603	3.08E-05	0.000516
	DYQ05_13165	CP045670.1(+)	4.838155	2.984652	7.09E-15	6.24E-13
	DYQ05_12840	CP045670.1(-)	8.747863	2.841093	2.48E-16	2.78E-14
	DYQ05_00770	CP045670.1(-)	3.42576	2.801347	8.71E-11	3.70E-09
	DYQ05_10355	CP045670.1(+)	6.449396	2.76146	4.14E-09	1.46E-07
	DYQ05_11880	CP045670.1(+)	4.772329	2.732603	4.74E-14	3.44E-12
	DYQ05_02490	CP045670.1(-)	4.05477	2.720026	0.000277	0.003052
	DYQ05_09830	CP045670.1(-)	9.077954	2.695608	5.51E-07	1.43E-05
	DYQ05_02690	CP045670.1(-)	7.487875	2.690211	1.63E-25	6.48E-23
	DYQ05_02265	CP045670.1(+)	7.94576	2.645928	1.87E-16	2.31E-14
	DYQ05_06970	CP045670.1(-)	5.750477	2.635634	1.15E-11	6.16E-10
	DYQ05_07410	CP045670.1(+)	9.209978	2.631984	1.25E-21	3.09E-19
	DYQ05_13305	CP045670.1(-)	4.715078	2.631084	1.37E-12	8.43E-11
	DYQ05_04870	CP045670.1(-)	1.605903	2.605633	0.00011	0.001468
	DYQ05_06520	CP045670.1(-)	5.14154	2.566689	6.02E-13	4.01E-11
	DYQ05_06485	CP045670.1(-)	5.645379	2.558667	9.77E-16	1.05E-13
	DYQ05_13310	CP045670.1(-)	7.040041	2.542677	1.53E-14	1.18E-12
	DYQ05_05125	CP045670.1(-)	10.65497	2.525972	6.10E-15	5.57E-13
	DYQ05_04125	CP045670.1(+)	6.473551	2.483018	1.54E-15	1.52E-13
	DYQ05_12835	CP045670.1(-)	4.595583	2.48205	3.47E-11	1.64E-09
	DYQ05_12520	CP045670.1(+)	12.96004	2.461882	1.19E-11	6.26E-10
	DYQ05_06530	CP045670.1(-)	6.336542	2.441886	7.18E-19	1.18E-16
	DYQ05_12830	CP045670.1(-)	4.295654	2.425869	2.91E-10	1.18E-08
	DYQ05_05260	CP045670.1(-)	7.312749	2.411395	4.00E-18	5.80E-16
	DYQ05_07415	CP045670.1(+)	10.29869	2.361507	9.28E-13	5.94E-11
	DYQ05_09735	CP045670.1(-)	10.48428	2.358899	3.68E-19	6.99E-17
	DYQ05_05265	CP045670.1(-)	5.962798	2.338741	3.90E-14	2.92E-12
	DYQ05_00300	CP045670.1(+)	12.81855	2.336406	4.62E-17	6.33E-15
	DYQ05_06525	CP045670.1(-)	3.169767	2.328753	3.49E-08	1.00E-06
	DYQ05_02780	CP045670.1(+)	7.103758	2.231709	2.68E-12	1.54E-10

	DYQ05_11055	CP045670.1(+)	6.235262	2.220818	1.47E-15	1.52E-13
	DYQ05_06930	CP045670.1(-)	13.75916	2.148058	1.34E-11	6.87E-10
	DYQ05_04565	CP045670.1(+)	2.932244	2.123598	1.98E-06	4.66E-05
	DYQ05_00835	CP045670.1(-)	9.322617	2.108161	1.74E-10	7.15E-09
	DYQ05_06490	CP045670.1(-)	10.80211	2.105699	5.12E-19	9.02E-17
	DYQ05_05585	CP045670.1(+)	1.085144	2.046715	0.007996	0.046285
	DYQ05_06965	CP045670.1(-)	5.665576	2.043619	1.43E-09	5.24E-08
	DYQ05_08525	CP045670.1(-)	7.776943	2.036201	6.41E-11	2.77E-09
	DYQ05_06980	CP045670.1(-)	7.037934	2.035446	4.15E-12	2.32E-10
	DYQ05_07420	CP045670.1(+)	8.106993	2.028802	9.39E-13	5.94E-11
	DYQ05_01810	CP045670.1(+)	6.029915	2.017964	3.12E-11	1.54E-09
	DYQ05_12465	CP045670.1(-)	9.385456	1.997958	4.28E-11	1.99E-09
	DYQ05_10570	CP045670.1(+)	7.838652	1.968456	1.49E-11	7.48E-10
	DYQ05_01815	CP045670.1(+)	5.217398	1.955848	5.13E-10	2.04E-08
	DYQ05_04120	CP045670.1(+)	5.997195	1.940235	6.84E-09	2.34E-07
	DYQ05_04145	CP045670.1(+)	5.10849	1.930778	4.32E-07	1.13E-05
	DYQ05_00325	CP045670.1(+)	3.705021	1.914587	5.90E-05	0.000872
	DYQ05_04560	CP045670.1(+)	3.437744	1.907922	7.40E-07	1.88E-05
	DYQ05_07510	CP045670.1(+)	4.23135	1.873095	2.33E-05	0.000405
	DYQ05_07875	CP045670.1(-)	5.159927	1.859951	4.74E-08	1.33E-06
	DYQ05_04165	CP045670.1(-)	3.424832	1.823182	0.001375	0.012067
	DYQ05_04555	CP045670.1(+)	5.823051	1.802858	4.69E-06	9.93E-05
	DYQ05_04925	CP045670.1(+)	6.831966	1.783654	5.71E-10	2.24E-08
	DYQ05_12335	CP045670.1(-)	8.231328	1.777822	9.96E-07	2.46E-05
	DYQ05_00305	CP045670.1(+)	8.492072	1.776494	1.50E-08	4.75E-07
	DYQ05_02175	CP045670.1(+)	8.408688	1.775177	7.88E-14	5.55E-12
	DYQ05_00495	CP045670.1(+)	3.648357	1.769572	2.64E-06	6.08E-05
	DYQ05_00845	CP045670.1(+)	7.163157	1.74865	1.29E-13	8.86E-12
	DYQ05_03190	CP045670.1(-)	5.504749	1.735229	0.000261	0.002912
	DYQ05_06765	CP045670.1(+)	7.356616	1.731695	2.59E-12	1.52E-10
	DYQ05_08900	CP045670.1(-)	2.644172	1.724284	0.000359	0.003817
	DYQ05_01805	CP045670.1(+)	9.310905	1.701253	4.89E-11	2.23E-09
	DYQ05_04575	CP045670.1(+)	1.373619	1.689589	0.008448	0.048041
	DYQ05_04220	CP045670.1(+)	9.61236	1.662937	1.68E-12	1.01E-10
	DYQ05_06480	CP045670.1(-)	6.705616	1.659161	1.16E-09	4.35E-08
	DYQ05_01800	CP045670.1(+)	6.320128	1.620824	2.42E-09	8.79E-08
	DYQ05_04580	CP045670.1(+)	4.472868	1.616394	8.13E-06	0.000154
	DYQ05_04135	CP045670.1(+)	8.494716	1.6162	6.20E-08	1.71E-06
	DYQ05_04255	CP045670.1(-)	2.168861	1.599426	0.003823	0.026781
	DYQ05_03350	CP045670.1(-)	3.402248	1.597747	0.000128	0.001658
	DYQ05_00105	CP045670.1(+)	6.269781	1.595846	1.54E-08	4.81E-07
	DYQ05_06500	CP045670.1(-)	7.550113	1.588797	3.42E-11	1.64E-09
	DYQ05_09165	CP045670.1(+)	3.070708	1.581855	0.005214	0.033745
	DYQ05_11195	CP045670.1(+)	11.70129	1.573513	1.09E-10	4.57E-09
	DYQ05_07495	CP045670.1(+)	2.213688	1.572055	0.006094	0.037854
	DYQ05_12500	CP045670.1(+)	8.76161	1.562187	8.66E-05	0.001207

	DYQ05_11190	CP045670.1(+)	5.002292	1.537379	0.000656	0.00637
	DYQ05_03050	CP045670.1(+)	5.148021	1.514976	0.000429	0.004468
	DYQ05_02575	CP045670.1(+)	9.407638	1.514518	5.94E-07	1.53E-05
	DYQ05_02180	CP045670.1(+)	8.272422	1.512821	8.43E-10	3.20E-08
	DYQ05_12330	CP045670.1(-)	6.24651	1.484269	3.47E-05	0.00057
	DYQ05_00765	CP045670.1(-)	9.686458	1.480158	3.01E-09	1.08E-07
	DYQ05_12560	CP045670.1(+)	3.527463	1.473702	0.001133	0.010119
	DYQ05_04245	CP045670.1(-)	7.748859	1.443843	1.68E-08	5.19E-07
	DYQ05_01670	CP045670.1(+)	7.278436	1.408249	9.28E-09	3.05E-07
	DYQ05_13430	CP045670.1(+)	11.67478	1.400882	2.69E-05	0.000463
	DYQ05_00840	CP045670.1(+)	12.57907	1.375853	5.23E-09	1.81E-07
	DYQ05_09565	CP045670.1(+)	11.53054	1.362547	8.38E-06	0.000158
	DYQ05_03670	CP045670.1(+)	8.479745	1.346122	0.002049	0.016459
	DYQ05_12905	CP045670.1(-)	3.689553	1.345824	0.000715	0.006892
	DYQ05_07955	CP045670.1(-)	6.186466	1.339716	6.05E-05	0.000883
	DYQ05_01830	CP045670.1(+)	10.00263	1.333905	1.20E-08	3.89E-07
	DYQ05_08450	CP045670.1(-)	11.60527	1.33173	2.42E-08	7.19E-07
	DYQ05_00310	CP045670.1(+)	8.443048	1.32583	6.08E-06	0.000121
	DYQ05_13225	CP045670.1(+)	8.745857	1.3179	3.95E-06	8.62E-05
	DYQ05_02510	CP045670.1(-)	3.595004	1.302182	0.000599	0.005864
	DYQ05_01665	CP045670.1(+)	10.516	1.293637	2.51E-07	6.67E-06
	DYQ05_01340	CP045670.1(-)	3.889657	1.271315	0.000563	0.005624
	DYQ05_01020	CP045670.1(+)	3.021548	1.269854	0.002735	0.020438
	DYQ05_10580	CP045670.1(+)	10.53174	1.267769	2.05E-06	4.78E-05
	DYQ05_04310	CP045670.1(+)	2.457412	1.255961	0.005742	0.036243
	DYQ05_05950	CP045670.1(+)	6.880863	1.242342	1.16E-06	2.82E-05
	DYQ05_01880	CP045670.1(-)	6.89107	1.239878	9.59E-07	2.39E-05
	DYQ05_10620	CP045670.1(-)	6.413022	1.231975	4.71E-06	9.93E-05
	DYQ05_11850	CP045670.1(+)	2.449559	1.231882	0.005708	0.036243
	DYQ05_07360	CP045670.1(-)	5.850095	1.216827	0.000224	0.002572
	DYQ05_09175	CP045670.1(-)	9.214526	1.214808	4.67E-06	9.93E-05
	DYQ05_02185	CP045670.1(+)	3.532209	1.202167	0.001066	0.009737
	DYQ05_06985	CP045670.1(-)	8.063167	1.194983	0.000292	0.003194
	DYQ05_00150	CP045670.1(+)	6.524959	1.194472	0.003126	0.022809
	DYQ05_03965	CP045670.1(+)	2.662799	1.191365	0.008196	0.047158
	DYQ05_12325	CP045670.1(-)	4.993559	1.186794	0.001486	0.012815
	DYQ05_09170	CP045670.1(-)	6.385573	1.186263	4.15E-06	8.98E-05
	DYQ05_10585	CP045670.1(+)	9.491548	1.185097	5.09E-06	0.000105
	DYQ05_02580	CP045670.1(+)	5.556698	1.173956	0.000165	0.002012
	DYQ05_11230	CP045670.1(-)	8.837444	1.173219	0.000123	0.001623
	DYQ05_06645	CP045670.1(+)	4.203214	1.17107	0.002432	0.018564
	DYQ05_03710	CP045670.1(-)	6.000623	1.169454	0.000255	0.002867
	DYQ05_01675	CP045670.1(+)	8.261644	1.168314	8.05E-07	2.02E-05
	DYQ05_01835	CP045670.1(+)	6.59665	1.163538	5.77E-06	0.000117
	DYQ05_01845	CP045670.1(+)	6.097463	1.161089	8.09E-06	0.000154
	DYQ05_01660	CP045670.1(+)	8.158898	1.160466	0.000182	0.002166

	DYQ05_01565	CP045670.1(+)	9.107911	1.151767	0.000108	0.001443
	DYQ05_04585	CP045670.1(+)	3.823865	1.13859	0.001677	0.014015
	DYQ05_12455	CP045670.1(-)	3.15437	1.130191	0.005757	0.036243
	DYQ05_03935	CP045670.1(+)	9.211473	1.121207	6.76E-06	0.000133
	DYQ05_00205	CP045670.1(+)	6.320882	1.113585	0.00108	0.009826
	DYQ05_06700	CP045670.1(-)	4.509647	1.110143	0.0043	0.028959
	DYQ05_07490	CP045670.1(+)	3.919294	1.104501	0.001406	0.012252
	DYQ05_08505	CP045670.1(-)	6.182732	1.103896	0.000151	0.001891
	DYQ05_09105	CP045670.1(+)	9.199012	1.102387	2.99E-06	6.64E-05
	DYQ05_12040	CP045670.1(-)	6.651307	1.097722	7.00E-06	0.000136
	DYQ05_13335	CP045670.1(+)	6.548373	1.091021	3.12E-05	0.00052
	DYQ05_05295	CP045670.1(+)	7.718955	1.085403	5.28E-06	0.000108
	DYQ05_08445	CP045670.1(-)	7.954597	1.076707	3.59E-06	7.91E-05
	DYQ05_03845	CP045670.1(+)	6.611967	1.069143	0.000578	0.005723
	DYQ05_03945	CP045670.1(+)	5.825926	1.068798	4.89E-05	0.000754
	DYQ05_01575	CP045670.1(-)	3.815351	1.066451	0.00192	0.015654
	DYQ05_00435	CP045670.1(+)	3.395084	1.061661	0.007972	0.046257
	DYQ05_03855	CP045670.1(+)	8.661215	1.060012	2.29E-05	0.0004
	DYQ05_01885	CP045670.1(-)	6.721275	1.055435	8.51E-05	0.001192
	DYQ05_08270	CP045670.1(+)	4.732319	1.049554	0.004158	0.028403
	DYQ05_03015	CP045670.1(-)	6.766372	1.037349	6.80E-05	0.000975
	DYQ05_01570	CP045670.1(-)	6.33471	1.036922	0.000144	0.001818
	DYQ05_08435	CP045670.1(-)	3.816287	1.034607	0.002161	0.017023
	DYQ05_07505	CP045670.1(+)	5.005823	1.034456	0.001503	0.012918
	DYQ05_01820	CP045670.1(+)	10.47061	1.031422	5.62E-06	0.000115
	DYQ05_01335	CP045670.1(-)	7.588374	1.017158	9.42E-05	0.001298
	DYQ05_07880	CP045670.1(-)	7.254476	1.01584	0.004107	0.028208
	DYQ05_03730	CP045670.1(+)	6.262909	1.011888	0.001639	0.013838
	DYQ05_01795	CP045670.1(+)	6.706948	1.009003	0.000862	0.008113
	DYQ05_05360	CP045670.1(+)	8.438201	1.007904	0.000325	0.003481

Supporting Publications

Gillespie, A., Carter, S. D., Blowey, R. W., & Evans, N. (2019). Survival of bovine digital dermatitis treponemes on hoof knife blades and the effects of various disinfectants. *Veterinary Record*, *186*, 67. https://doi.org/10.1136/vr.105406

Gillespie, A., & Evans, N. (2019). Infection reservoirs and transmission of digital dermatitis in the dairy herd. *Livestock*, *25*(3).

Gillespie, A. V., Carter, S. D., Blowey, R. W., Staton, G. J., & Evans, N. J. (2020). Removal of bovine digital dermatitis-associated treponemes from hoof knives after foot-trimming: a disinfection field study. *BMC Veterinary Research*, *16*(1), 330. https://doi.org/10.1186/s12917-020-02552-8

Gillespie, A. v., Carter, S. D., Blowey, R. W., Staton, G. J., Walsh, T. R., & Evans, N. J. (2021). Measuring the impact of bovine digital dermatitis research on knowledge and practice of biosecurity during cattle foot-trimming. *Journal of Dairy Research*, *88*(1), 60–63. https://doi.org/10.1017/S0022029921000170

Bay, V., Gillespie, A., Ganda, E. K., Evans, N., Carter, S., Lenzi, L., Lucaci, A., Haldenby, S., Barden, M., Griffiths, B. E., Sánchez-Molano, E., Bicalho, R., Banos, G., Darby, A., & Oikonomou, G. (2021). The Bovine Foot Skin Microbiota is Associated with Host Genotype and the Development of Infectious Digital Dermatitis Lesions. *Research Square*. https://doi.org/10.21203/rs.3.rs-650860/v1