Contagious Ovine Digital Dermatitis: A Novel Bacterial Aetiology and Lesion Pathogenesis

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

JD, DGW, NJE, SDC co-designed, obtained financial support and co-supervised the study. JA, JD, SC collected the samples. SC, GS, NJE performed laboratory analyses. DGW performed data analysis. JD wrote the manuscript. GS, JA, SC, NJE, SDC, DGW critically evaluated the manuscript

Keywords

Sheep, lameness, CODD, Footrot, Treponema medium, Treponema phagedenis, Treponema pedis, Dichelobacter nodosus, Fusobacterium necrophorum

Abstract

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Contagious ovine digital dermatitis (CODD) is a severe and common infectious foot disease of sheep and a significant animal welfare issue for the sheep industry. The aetiology and pathogenesis of the disease are incompletely understood. In this longitudinal, experimental study, CODD was induced in 18 sheep, and for the first time, the clinical lesion development and associated microbiological changes in CODD affected feet are described over time, resulting in a completely new understanding of the aetiopathogenesis of CODD. The majority of CODD lesions (83.9%) arose from pre-existing interdigital dermatitis (ID) and footrot (FR) lesions. All stages of foot disease were associated with high levels of poly-bacterial colonisation with five pathogens which were detected by qPCR; Treponema medium, Treponema phagedenis, Treponema pedis, Dichelobacter nodosus and Fusobacterium necrophorum. Temporal colonisation patterns showed a trend for early colonisation by Treponema phagedenis, followed by Fusobacterium necrophorum and Dichelobacter nodosus, Treponema medium and then Treponema pedis. Dichelobacter nodosus was present at significantly higher predicted mean log10 concentrations in FR lesions compared to both ID and CODD whilst Treponema species were significantly higher in CODD & FR lesions compared to ID lesions ($P < 0.001$). Treatment of CODD affected sheep with 2 doses of 10mg/kg amoxicillin resulted in a 91.7% clinical cure rate by 3 weeks post treatment, however a bacteriological cure was not established for all CODD affected feet. The study found that in an infected flock healthy feet, healed CODD feet, and treated CODD feet can be colonised by some or all of the 5 pathogens associated with CODD and therefore could be a source of continued infection in flocks. The study is an experimental study and the findings require validation in field CODD cases. However, it does provide a new understanding of the aetiopathogenesis of CODD, further supportive evidence for the importance of current advice on the control of CODD; which is ensuring optimum flock control of footrot and prompt isolation and effective treatment of clinical cases.

Contribution to the field

Contagious ovine digital dermatitis is a newly emerging infectious lameness of sheep reported in many sheep producing countries across the world. Due to the severity of the foot lesions associated with CODD it is considered the most serious animal welfare issue in affected flocks. However, a lack of knowledge of its aetiology and pathogenesis are a significant barrier to effective disease control. The aim of this study was to determine the microbial aetiology and pathogenesis of CODD by undertaking a study of a naturally occurring outbreak of CODD in an experimental flock. In this longitudinal, study CODD was induced in the sheep, and for the first time, the clinical lesion development and associated microbiological changes in CODD affected feet are described over time, resulting in a completely new understanding of the aetiopathogenesis of CODD. The majority of CODD lesions developed from pre-existing footrot lesions and were associated with high levels of poly-bacterial colonisation with five pathogens which were detected by qPCR; Treponema medium, Treponema phagedenis, Treponema pedis, Dichelobacter nodosus and Fusobacterium necrophorum. Furthermore, healthy feet, healed CODD feet, and treated CODD feet can be colonised by some or all of the 5 pathogens associated with CODD. Finally, a highly effective antibiotic treatment protocol is described. Therefore, to control CODD increased efforts must be directed at controlling pre-existing footrot disease in flocks and identifying effective biosecurity and hygiene measures targeted against these 5 pathogens.

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Contagious Ovine Digital Dermatitis: A Novel Bacterial Aetiology and Lesion Pathogenesis.

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Key words

Sheep, Lameness, CODD, Footrot, *Treponema medium, Treponema phagedenis, Treponema pedis, Dichelobacter nodosus and Fusobacterium necrophorum*

Abstract

Contagious ovine digital dermatitis (CODD) is a severe and common infectious foot disease of sheep and a significant animal welfare issue for the sheep industry in the UK and some European countries. The aetiology and pathogenesis of the disease are incompletely understood. In this longitudinal, experimental study, CODD was induced in 18 sheep, and for the first time, the clinical lesion development and associated microbiological changes in CODD affected feet are described over time, resulting in a completely new understanding of the aetiopathogenesis of CODD. The majority of CODD lesions (83.9%) arose from pre-existing interdigital dermatitis (ID) and/or footrot (FR) lesions. All stages of foot disease were associated with high levels of poly-bacterial colonisation with five pathogens which were detected by qPCR; *Treponema medium*, *Treponema phagedenis, Treponema pedis, Dichelobacter nodosus* and *Fusobacterium necrophorum.* Temporal colonisation patterns showed a trend for early colonisation by *Treponema phagedenis*, followed by *Fusobacterium necrophorum* and *Dichelobacter nodosus*, *Treponema medium* and then *Treponema pedis*. *Dichelobacter nodosus* was present at significantly higher predicted mean $\log_{10}$ genome copy numbers in FR lesions compared to both ID and CODD whilst *Treponema* species were significantly higher in CODD & FR lesions compared to ID lesions ($P < 0.001$). Treatment of CODD affected sheep with 2 doses of 10mg/kg long acting amoxicillin resulted in a 91.7% clinical cure rate by 3 weeks post treatment, however a bacteriological cure was not established for all CODD affected feet. The study found that in an infected flock, healthy feet, healed CODD feet, and treated CODD feet can be colonised by some or all of the 5 pathogens associated with CODD and therefore could be a source of continued infection in flocks. The study is an experimental study and the findings require validation in field CODD cases. However, it does provide a new understanding of the

aetiopathogenesis of CODD and further supportive evidence for the importance of current advice on the control of CODD; namely ensuring optimum flock control of footrot and prompt isolation and effective treatment of clinical cases.

Introduction

Contagious ovine digital dermatitis (CODD) is a relatively new infectious foot disease of sheep, first recorded in the UK in 1997 (Harwood, Cattell et al. 1997). It is now widespread in the UK, affecting an estimated 35% to 58% of sheep flocks (Angell, Duncan et al. 2014, Dickins, Clark et al. 2016) and has also been reported in Ireland (Sayers, Marques et al. 2009), Germany (Tegtmeyer, Staton et al. 2020) and Sweden (personal comm). CODD is the most severe form of sheep lameness recorded (Angell, Blundell et al. 2015), and coupled with challenges around disease control in infected flocks (Duncan, Angell et al. 2014), CODD has a substantially negative impact on sheep welfare and is a priority issue for the sheep industry.

The severity of lameness in CODD affected sheep is a consequence of the extensive foot pathology caused by the disease (Figure 1). CODD is a progressive infectious foot disease which begins with an inflammatory lesion on the dorsal coronary band of the hoof and culminates in avulsion of the entire hoof capsule leaving highly sensitive underlying hoof lamellae tissue exposed (Figure1). Furthermore, radiographic investigation of affected feet revealed periosteal inflammatory changes in the pedal bone in most stages of the disease, indicating extensive internal damage to the structure of the sheep's foot and a further source of pain and functional compromise (Angell, Blundell et al. 2015). Histopathological examinations of early stage lesions are described as lymphoplasmacytic infiltration of the distal digital skin with suppurative coronitis and intracorneal pustules; in the more advanced stages of

disease there is complete separation of the dorsal wall of the hoof with a necrotizing and fibrinosuppurative exudate and dermatitis (Angell, Crosby-Durrani et al. 2015).

The aetiology of CODD is considered to be bacterial. From the earliest microbiology studies, species of the *Treponema* genus of bacteria have been associated with CODD lesions (Naylor, Martin et al. 1998, Collighan, Naylor et al. 2000, Demirkan, Carter et al. 2001, Sayers, Marques et al. 2009), in particular one of the three treponemal species, that are considered causal for bovine digital dermatitis (BDD), are consistently found in CODD lesions. These are *Treponema medium*, *Treponema phagedenis* and *Treponema pedis* (Sullivan, Clegg et al. 2015) (Angell, Crosby-Durrani et al. 2015) (Evans, Brown et al. 2009) (Sayers, Marques et al. 2009). Therefore, it is hypothesised that these three digital dermatitis (DD) associated treponemes may have crossed species from cattle to sheep to initiate the emergence of CODD in sheep (Sullivan, Evans et al. 2015) . Immunohistochemical analysis of CODD lesions also clearly demonstrate large numbers of unspecified *Treponema*-like organisms closely associated with the histopathological changes of CODD lesions, thus, providing further evidence for the role of treponeme bacteria in the aetiology of CODD (Angell, Crosby-Durrani et al. 2015).

However, a single pathogen aetiology for CODD has not been wholly established. Other bacterial species have also been repeatedly isolated and identified in CODD lesions, namely *D. nodosus* and *F. necrophorum* (Moore, Woodward et al. 2005) (Sullivan, Clegg et al. 2015). *D. nodosus* is the causal agent of footrot in sheep whilst *F. necrophorum* is considered a secondary invading pathogen in footrot lesions (Beveridge 1941, Witcomb, Green et al. 2014) as well as other diseases. Furthermore, epidemiological evidence has repeatedly demonstrated strong associations between

95 footrot and CODD (Angell, Duncan et al. 2014, Angell, Grove-White et al. 2015), and
96 vaccinating animals against *D. nodosus* does provide some protection against CODD
97 infection (Duncan, Grove-White et al. 2012). Therefore, it is possible that either of
98 these two known foot pathogens may play a role in the pathogenesis of CODD. They
99 may indeed be the primary pathogens of CODD, they may provide initiating skin/horn
100 damage allowing secondary treponemal invasion, or they may be secondary
101 pathogens of already established CODD lesions. They could also be contaminants of
102 no pathogenic significance at all. Consequently, the precise aetiology of CODD
103 remains unproven

104 There are several important limiting factors to previous research on the aetiology of
105 CODD which need to be overcome in order to clarify its aetiology. These include:-

106 *1) Sampling strategy*: To date, studies so far have employed a cross-sectional
107 sampling study design. Such methods provide information on the presence of bacteria
108 in foot lesions at a single time point. However, they provide limited evidence of disease
109 causality in terms of bacterial initiation and lesion progression.

110 *2) Culture method bias*: It is well recognised that the culture method used in a
111 bacteriological study will strongly influence the bacterial organisms identified from a
112 sample, meaning that many bacteria which may well be significant in a disease
113 process may not be detected (Steen, Crits-Christoph et al. 2019). Bacteria associated
114 with ovine foot disease are noted for their particularly fastidious nature and therefore
115 false negative culture results are likely (Moore, Woodward et al. 2005). Despite these
116 limitations, culturing bacteria from a sample does indicate the organisms are present
117 and viable in the tissue, which is one of Koch's original postulates for determining

disease causality (Koch 1893). Furthermore, isolating bacteria from lesions allows for further biochemical and molecular characterization of the organisms (Segre 2013).

3) Interpretation of PCR data: Routine diagnostic PCR data is limited to providing binary information on the presence or absence of a bacterial species in a sample. Quantitative molecular data on whether the organism is multiplying in a lesion is helpful in supporting causality and can help to distinguish between the inevitable environmental contamination of bacteriological samples collected from sheep's feet and pathogens that are actively multiplying in a lesion.

Therefore, bearing these previous study limitations in mind, the aim of the current study was to investigate the bacterial aetiology of CODD in a longitudinal, experimental study of naturally occurring CODD lesions; employing a quantitative PCR methodology to determine the temporal associations of previously identified bacterial species of *T. medium*, *T. phagedenis, T. pedis, D. nodosus* and *F. necrophorum* in the aetiology of CODD.

Material and Methods

Experimental Design

The project was carried out under UK Animal Scientific Procedure Act (ASPA) 1986; Home Office Project License PPL 708756 and University of Liverpool Ethics VREC417. The experimental study was supervised at all times by a Named Animal Care and Welfare Officer and a team of three veterinary surgeons. The reporting of the experiment is in accordance with the ARRIVE guidelines (Percie du Sert, Ahluwalia et al. 2020) (supplementary file 1).

The study design was an observational study of an experimentally induced outbreak of CODD in housed sheep whereby 30 healthy, 18-month-old, Texel cross-bred ewes were housed with 10 sheep of mixed age and breed, affected by CODD. Inclusion criteria for healthy ewes were acquisition from a single flock with no known history of CODD and same sex, breed and age. Inclusion criteria for infected sheep were that they were sourced from farms with a history of CODD in the flock and should have a confirmed veterinary diagnosis of an active, untreated CODD lesion in one foot. At study start all infected sheep were PCR positive (Evans, Brown et al. 2009) for at least one of the hypothesised causal pathogens of CODD (*T. medium*, *T. phagedenis, T. pedis)*. Sample size power calculations were not made for due to lack of data on expected variation in the microbiological consortium; however, sample sizes were consistent with other similar studies (Modric, Webb et al. 1998, Witcomb, Green et al. 2014). The observational design of the experiment meant that it did not require blinding or randomizing.

The sheep were housed in a Home Office Designated Building (according to UK Animal Scientific Procedures Act, Code of Practice for Care and Accommodation of Animals) on deep litter straw bedding at a stocking rate of 1.9m^2/sheep. Sheep were fed a maintenance ration of *ad libitum* hay. A footbath was placed under the feed racks which contained damp straw, water, and contaminated hoof clippings from a CODD infected farm to simulate naturally occurring risk factors for CODD. Sheep welfare was monitored by daily inspection of demeanour and feed intake, twice weekly locomotion (Angell, Cripps et al. 2015) and body condition scoring (Russel 1984) and weekly veterinary clinical examination. Humane endpoints were set (inappetence, recumbency or non-weight bearing lameness on any limb) and if an animal reached these predetermined points the animal was withdrawn from study. When half of the

166 sheep in the flock had developed CODD lesions, all sheep with any foot lesion was
167 treated with 2 doses, 48 hours apart, of a long acting amoxicillin (Betamox LA
168 150mg/ml, Norbrook, Northern Ireland, UK) at dose rate of 10mg/kg administered by
169 intramuscular injection. In addition, all bedding was removed from the housing, all
170 flooring and fitments cleaned by power washing with water and disinfectant (FAM 30,
171 Evans Vanodine plc, Preston, UK), and fresh straw bedding provided.

172 ***Animal Sampling***

173 At the start of the project and during every week of the study, the following data and
174 samples were collected from each sheep: a locomotion score (Angell, Cripps et al.
175 2015), a body condition score (Russel 1984), a foot lesion score of each foot (Angell,
176 Blundell et al. 2015) and foot skin swab (Copan, Italy) from each foot. When a foot
177 lesion was present a swab was applied to the entire surface of the visible lesion.
178 Collected swabs were immediately stored at -80°C until DNA extraction. Animal
179 metadata was stored on an Access Database (Microsoft, Washington USA).
180

181 ***Foot Lesion Classification***

182 All locomotion scoring and lesion scoring observations were performed by one of two
183 experienced observers (JA and JD). Sheep were locomotion scored using a four-
184 point ordinal locomotion scoring system that measured the degree of lameness
185 exhibited by the sheep from sound (score 0), mild (score 1), moderate (score 2) and
186 severe (score 3) (Angell, Cripps et al. 2015). Foot lesions were classified on the
187 basis of their clinical appearance as interdigital dermatitis (ID) or footrot (FR) as per
188 published descriptions (Egerton and Roberts 1971) and CODD (Angell, Blundell et
189 al. 2015). ID was defined as any degree of inflammation of the interdigital skin only,

whilst FR was defined as the presence of underrunning of the horn of the heel and sole (Figure 2). CODD lesions were graded (G#) as follows: G#1 is described as a focally extensive erosive or ulcerative lesion affecting the digital skin and coronary band; a G#2 lesion is reported as separation between the hoof capsule and the hoof lamellae affecting up to 50% of the dorsal and abaxial hoof wall; a G#3 lesion is described as separation between the hoof capsule and lamellae affecting greater than 50% of the dorsal and abaxial hoof wall; a G#4 lesion is described where there is evidence of horn regrowth on the surface of the lamellae but not over entire surface (lamellar tissue remains exposed); in G#5 lesions horn regrowth is apparent over the entire surface of the lamellae, although the horn surface maybe smooth and distorted by circumferential ridges and the affected digit may be wider and shortened in comparison to the unaffected digit (Figure 1).

In some cases, feet could be considered to have features of both footrot and CODD, or interdigital dermatitis (scald) and CODD. In these cases, the combination of lesions was recorded.

Isolation of foot swab DNA

Genomic DNA was isolated from foot swab samples using the DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK), according to manufacturer's instructions, as described previously (Duncan, Angell et al. 2021). Extracted DNA was stored at -80^{o}C until analysis.

qPCR

Primer and probe design

Previously described Taqman qPCR primer and probes targeting a 61 bp sequence within the RNA polymerase sigma-70 factor gene (*rpoD*) of *Dichelobacter nodosus* (Calvo-Bado, Oakley et al. 2011) and an 86 bp sequence within the β-subunit of

RNA polymerase gene (*rpoB*) of *Fusobacterium necrophorum* (Witcomb, Green et al. 2014) were employed. In addition, three novel Taqman qPCR assays were designed to individually target 254, 234 and 247 bp sequences within the Recombinase A (RecA) genes of *Treponema medium* (Accession number CP027017), *T. phagedenis* (Accession number CP027018) and *T. pedis* (Accession number CP045760), respectively. The RecA gene was selected because of its singular occurrence in the genomes of *Treponema* spp. and its relatively low within-species diversity across a global panel of digital dermatitis-associated treponeme isolates (Clegg, Carter et al. 2016). RecA gene sequences were extracted from the sequenced genomes, aligned with all available *recA* alleles from the previous multi locus sequence typing study and subjected to primer design using Mega X (Kumar, Stecher et al. 2018)and Primer 3 (Untergasser, Cutcutache et al. 2012). The quality of the primer and probe sequences were analysed using OligoCalc (Kibbe 2007). The primer and probe sequences for the *T. medium*, *T. phagedenis* and *T. pedis* qPCR assays are shown in table 1. ~~were as follows: *T. medium* forward primer 5'-CTACAAATCGAAAAGGAGTTTGGA-3', reverse primer 5'-GGCATGTTCGGCATCCAC-3' and Taqman probe 5'-TAGAATTATCGAAATATTCGGCCCAGA-3'; *T. phagedenis* forward primer 5'-GCCTTCAAATCGAAAAACAATTC-3', reverse primer 5'-GCCGCAATGCCGCCGCG-3' and Taqman probe 5'-TAGATGAGGCACTGGGAATCGG-3'; *T. pedis* forward primer 5'-AAATTGAAAAACAATTCGGACAG-3', reverse primer 5'-GTGTTCGGCATCTATAAAAGCC-3' and Taqman probe 5'-ATACCCCAGAGGCCGTATTATCGAG-3'.~~ All probes were labelled with the fluorophore, 6-carboxyl-fluorescein (FAM), at the 5'-end and the non-fluorescent

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241 Black Berry Quencher (BBQ) at the 3'-end. Primers and probes were synthesised
242 and purified commercially (TIB MOLBIOL, GmbH, Berlin, Germany).
243 *Standard curve preparation*
244 A 61 bp *rpoD* gene fragment from *Dichelobacter nodosus* strain VCS1703A was
245 commercially synthesised and inserted into a pGM plasmid (GeneMill, University of
246 Liverpool, UK). The plasmid was propagated in Top10 *Escherichia coli* (Life
247 Technologies, Paisley, UK) according to manufacturer's instructions and purified using
248 the Plasmid Mini Kit (Qiagen, Manchester, UK). *Fusobacterium necrophorum* subs.
249 *funduliforme*, previously isolated from a bovine digital dermatitis lesion by our
250 laboratory, was grown in oral treponeme enrichment broth (OTEB, Anaerobe Systems,
251 Morgan Hill, CA). *T. medium* T19 was cultured in OTEB supplemented with 10 % (v/v)
252 rabbit serum, whilst *T. phagedenis* T320A and *T. pedis* T3552B^T were cultured in
253 OTEB supplemented with 10 % (v/v) fetal calf serum as previously described (Evans,
254 Brown et al. 2008). Chromosomal DNA was extracted using the Wizard kit (Promega,
255 Southampton, UK) according to manufacturer's instructions and quantified using the
256 Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Loughborough,
257 UK). For each bacterial DNA preparation, genome copy numbers were calculated
258 using the following equation: number of copies of DNA template per µl = (DNA
259 concentration (ng/µl) x Avogadro's number) / (length of template (bp) x conversion
260 factor to ng x average weight of a base pair (Da)). Serial dilutions of the purified
261 bacterial DNA were subsequently prepared in 10 mM Tris-Cl, pH 8.5, to yield
262 estimated genome copy number of 3.25×10^6 to 3.25×10^{-1}, 1.03×10^6 to 1.03×10^{-1},
263 2.66×10^6 to 2.66×10^{-1}, 6.31×10^8 to 6.31×10^{-1} and 4.37×10^6 to 4.37×10^{-1} for *T.*
264 *medium*, *T. phagedenis*, *T. pedis*, *D. nodosus* and *F. necrophorum*, respectively.
265 *qPCR Cycling conditions*

All qPCR assays were performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and each 25 µl reaction comprised of the following: 5 µl 5 x HOT FIREPol ®Probe qPCR Mix Plus (ROX) (Solis Biodyne, Tartu, Estonia), 1 µl [0.4 µM] forward primer, 1 µl [0.4 µM] reverse primer, 0.625 µl [0.25 µM] probe, 16.375 µl PCR grade water and 1 µl of DNA template. All reactions were performed in triplicate. The *F. necrophorum* (Witcomb, Green et al. 2014) and *D. nodosus* (Calvo-Bado, Oakley et al. 2011) qPCR assays were performed using the cycling conditions previously described. Optimal qPCR cycling conditions for the three *Treponema* qPCR assays were empirically determined and comprised of a single activation step of 95ºC for 12 m followed by 40 cycles of 95ºC for 15 s and 61ºC for 15 s and 72ºC for 30 s. Analytical specificity and sensitivity were empirically defined for each assay. The baseline fluorescence signal was automatically calculated from the first 3 to 15 qPCR cycles. Where no increase in fluorescence signal was detected after 40 cycles, the bacterial load was classified as 'undetectable' i.e. below the limit of detection.

~~*Assay performance*~~

qPCR analytical specificit[*ies*](~~y~~) *and* [*limits of detection*](~~sensitivity.~~)

Analytical specificity of the *D. nodosus* and *F. necrophorum* qPCR assays have been previously described (Calvo-Bado, Oakley et al. 2011, Witcomb, Green et al. 2014). The specificity of the *F. necrophorum rpoB* assay was verified by analyzing the genomic DNA of *Fusobacterium varium*, a species closely related to *F. necrophorum*, whilst the genomic DNA of *Fusobacterium necrophorum* subsp. *necrophorum* (DSM21784) (DSMZ, Germany) was used as a positive control. The specificity of the *D. nodosus rpoD* assay was verified by analyzing the genomic DNA of the two closest relatives of *D. nodosus*: *Suttonella indologenes* (DSM8309) and *Cardiobacterium*

hominis (DSM8339), whilst using the genomic DNA of *D. nodosus* (DSM23057) as a positive control. The specificities of the three novel pathogenic *Treponema* qPCR assays were individually established by analyzing the genomic DNA of *T. medium*, *T. phagedenis* and *T. pedis*, to confirm the absence of cross-reactivity between the three assays, and by analyzing the genomic DNA of two commensal treponemes, *T. ruminis* (DSM 103462) and *T. rectale* (DSM 103679). The lower limits of detection for the *T. medium*, *T. phagedenis*, *T. pedis*, *D. nodosus* and *F. necrophorum* assays were 32.5, 10.3, 26.6, 63.1~~43.7~~ and 43.7~~63.1~~ genome copies/µl template, respectively; below these concentrations, DNA detection failed. The calibration standards for the *T. medium*, *T. phagedenis*, *T. pedis*, *D. nodosus* and *F. necrophorum* assays generated R2 values of >0.99 and mean slopes of −3.5 (SEM ± 0.04), −3.5 (SEM ± 0.01), −3.5 (SEM ± 0.07), −3.4 (SEM ± 0.07) and −3.7 (SEM ± 0.06), respectively, indicating that the amplification efficiency of these assays was >85%.

Each sample assay was performed in triplicate and the mean genome copy number (MGCN) calculated from the 3 assay results.

Statistical Analysis

All data was recorded in Access database (Microsoft, Washington, USA) spreadsheets. Following manual cleaning data was exported to Stata 16 (StataCorp, Texas, USA) for further cleaning, data checking, appropriate recoding where required and statistical analysis. Binary variables based on MGCN values were produced for all bacterial species using the respective lower limits of detection defined above with values above these values indicating presence of the bacterial species. MGCN data was $\log_{10}$ transformed for analysis. Mean values and

proportions were estimated with 95% confidence intervals where appropriate. Comparison of mean log_{10} MGCN values were performed using one-way ANOVA with a Bonferroni correction for multiple comparisons. Comparison of proportional data was performed using Fisher's Exact Test. Survival analysis with production of Kaplan Meyer plots was performed to investigate time to first recording of a specific foot lesion and time to first colonization by a specific bacterial species. Mixed effects linear regression models with random effects at sheep and foot level were fitted to investigate associations between lesion or CODD grade and log_{10} MGCN. Predicted marginal mean estimates were obtained from models and presented graphically.

Results

Clinical findings

Thirty, non-lame experimental sheep were recruited to the study. The duration of the study was 26 weeks. A total of 2,392 recordings of digit lesions were made during the study period. During the study period 24 sheep were recorded as having a foot lesion associated with lameness in one or more feet on at least one occasion with only 6 sheep showing no lesions throughout the study period. A total of 59 feet (49.2%) were recorded as having a lesion on at least one occasion. The distribution of lesions recorded at foot level is as follows; no lesions (86.29%), ID (3.64% of recordings), FR (2.68% of recordings), CODD (5.85% of recordings) and other lesions (1.55% of recordings).

Feet were affected by different lesions throughout the study period but there appeared to be a broad temporal trend in the distribution of lesions with ID and FR lesions representing the greatest proportion of lesions initially with an increasing proportion of CODD lesions observed as the study progressed (Figure 3).

Survival analysis was performed to investigate time to first recording of a lesion in a foot and Kaplan-Meyer survival curves plotted together with median survival times (95% CI) (Figure 4). These suggest that ID lesions were first recorded (median survival time 88 days, 95% CI 74-109 days), followed by FR (median survival time 103 days, 95% CI 95-116 days) then CODD (median survival time 116 days, 95% CI 103-123 days). The confidence intervals are large and overlap.

There was considerable heterogeneity in lesions recorded with multiple different lesions recorded on the same foot during the study period. For analysis purposes, CODD was assumed to be the final outcome in terms of progression severity and feet were then classified according to the final outcome. ID was recorded as the final outcome in 19 feet (15.8%), FR 9 feet (7.5%) and CODD was recorded as the final outcome lesion in 31 feet (25.8%) .

The majority of CODD lesions appeared to arise following previous ID and/or FR lesions. Only 5/31 (16%) of CODD lesions developed *de novo* whilst 21/31 (64%) were preceded by lesions of FR (with or without ID) and 6/31 (19%) were preceded by lesions of ID only.

A total of 31 feet were recorded with a CODD lesion on at least one occasion with the following CODD grade distribution: G#1 n=25 feet; G#2 n=14 feet; G#3 n=7; G#4 n=6; G#5 n=16. Whilst the majority of feet with CODD lesions had prior lesions of either ID (n = 6) or FR (n = 3) or both ID & FR (n = 17), CODD arose *de novo* in 5 feet (16%). It is worthy of note that of the 5 feet in which CODD arose *de novo*, all were recorded with G#1 lesions, of which only one progressed directly to G#5, while the remaining 4 feet resolved spontaneously without further progression. Overall, this suggests that CODD lesions arising *de novo* did not progress further to lesions

366 of “active CODD” but either resolved spontaneously or were recorded as G#5
367 (“healed lesions”).
368 Of the 26 feet which developed CODD subsequent to either ID, FR, or FR & ID,
369 there was a trend for lesions to progress in severity through the CODD disease
370 process (CODD G#1 – G#5). However, there was temporal “waxing and waning” in
371 lesion severity, although the overall trend was one of increasing lesion severity.
372 There were 12 CODD lesions (G#1 – G#4) present in 9 sheep at the time of treatment
373 of the flock. The treatment was (2 doses, 48 hours apart, of a long acting amoxicillin
374 (Betamox LA 150mg/ml, Norbrook, Northern Ireland, UK) at dose rate of 10mg/kg by
375 intramuscular injection together with environmental decontamination. One week
376 following completion of treatment, 100% of the CODD foot lesions had resolved to
377 CODD 5 or healthy status. However, by 3 weeks post treatment 1 CODD foot lesion
378 (8.33%) was noted to have clinically recurred (CODD G#1).

379 ***Microbiological Findings***

380 *Microbiology of ID, FR and CODD*

381 At the start of the study foot swab samples were collected from all the 10 source
382 sheep (with a veterinary clinical diagnosis of CODD, and the 30 experimental study
383 sheep (with a veterinary clinical diagnosis of healthy). All 5 target bacterial
384 pathogens were present in the source sheep, confirming the introduction of these
385 pathogens into the experimental flock as anticipated. *F. necrophorum, D. nodosus,*
386 *T. medium,* and *T. phagedenis* were also detected in the healthy study sheep
387 despite these sheep showing no clinical signs of disease in their feet (Figure 5).
388 However, the foot level mean $\log_{10}$ MGCN for *D. nodosus, T. medium, T. pedis* and
389 *T. phagedenis* were all significantly lower in the study sheep compared with the
390 source sheep and the proportion of colonised feet was also significantly lower in the

study compared to the source sheep (Table 21). The degree of colonisation of the feet by *F. necrophorum* in the source and study sheep was the same (Table 21).

A total of 2,320 samples were collected throughout the study period from the 30 sheep, with 877 samples (37.8%) yielding a positive qPCR result for at least one of the 5 target pathogens. There was considerable heterogenity in qPCR results, with positive results for all bacteria, namely *F. necrophorum, D. nodosus, T. medium, T. pedis* and *T. phagedenis* being recorded in both healthy and diseased feet, albeit with differing frequencies and pathogen loads (Figure 6). There was a tendency for both FR and CODD lesions have higher treponeme mean $\log_{10}$ MGCN compared to ID and other lesions (Figure 6).

Whilst all micro-organisms were detected on all sampling occasions during the study period, there were apparent broad temporal trends in detection frequency with all organisms being identified with greater frequency as the study progressed, up until commencement of treatment (Figure 7).

Survival analysis was performed to investigate time to first identification of an organism in a foot and Kaplan-Meyer survival curves plotted (Figure 8). These suggest an apparent trend for early colonisation by *T. phagedenis* (median survival time 39 days, 95% CI 32-36 days) followed by *F. necrophorum* and *D. nodosus* (median survival time 81 days, 95% CI 74-88 days), *T. medium* (median survival time 88 days, 95% CI 81-95 days) and *T. pedis* (median survival time 116 days, 95% CI 95-123 days).

As can be seen from Figure 6, there was considerable heterogeneity in bacterial mean $\log_{10}$ MGCN by recorded lesion type (ID, FR, and CODD). To further examine associations between each of the target bacterial species and lesion type (healthy, ID, FR and CODD), two sets of mixed linear regression models were fitted with

outcome variable being the specific bacterial species log_{10} MGCN and explanatory variables being lesion type or CODD grade. Model outputs are in Appendix 1 and predicted marginal means estimated from the models are displayed graphically in Figures 9 &14. Whilst all bacterial species were present in ID, FR and CODD, there were some trends apparent. At lesion level (Figure 9) *D. nodosus* was present at a significantly higher predicted mean log_{10} MGCN in FR lesions compared to both ID and CODD whilst *Treponema* species were significantly higher in CODD & FR lesions compared to ID lesions ($P < 0.001$) although there was no statistically significant difference between predicted mean log_{10} MGCN in FR and CODD lesions ($P = 0.47$).

Microbiology of CODD Lesion Grades

A total of 139 samples were collected from lesions described as CODD, from 31 affected feet. As described earlier, in the case of 5 feet, lesions of Grade 1 CODD arose *de novo,* with no prior foot disease recorded. In 26 feet, G#1 lesions were recorded, following prior lesions of ID and/or FR (progressive CODD lesions). Bacterial colonisation (as a binary event) and mean bacterial log_{10} counts were compared within Grade 1 lesions by origin i.e. arising *de novo* or progressive. In the case of all bacterial species, a high proportion (> 86%) of progressive G#1 lesions were colonised compared to the CODD lesions which arose *de novo.* Furthermore, in the *de novo* CODD lesions the qPCR signal for all bacterial species was totally absent, or insufficient as to indicate colonisation, suggesting an absence of all bacterial species tested for in lesions that arose *de novo* (Table 3~~2~~).

Within CODD lesions, there was considerable heterogeneity in both proportion and mean log_{10} genome copy number by CODD grade (Figure 10).

Whilst all bacterial species were present in all CODD lesions irrespective of grade (Figure 11), all predicted log_{10} mean genome copy numbers were significantly lower in Grade 5 lesions compared to other grades. Both predicted mean log_{10} MGCN for *Fusobacterium necrophorum* and *Dichelobacter nodosus* were significantly higher ($P < 0.001$) in G#1 lesions compared to all other grades. No other clear apparent trends in colonisation could be observed within CODD grade.

Effect of Antibiotic Treatment on Microbiology of CODD Lesions

All 12 CODD lesions (G#1 – G#4), present in the flock at the time of the treatment intervention, were colonized by the 5 target pathogens (Figure 12). One week following completion of treatment, when all of the CODD foot lesions had resolved to G#5 or healthy status, bacterial colonisation and the number of colonized feet had reduced substantially in the treated animals, however their feet remained colonized albeit at much lower levels. By three weeks post treatment, when one CODD foot lesion (8.33%) had clinically recurred, the level of colonisation remained at a low level and was broadly similar for *Fusobacterium necrophorum, Dichelobacter nodosus and Treponema phagedenis,* whilst *Treponema medium* and *Treponema pedis* were now absent from the treated feet (Table 4~~3~~).

Discussion

Main findings

The principle findings of the study are that CODD foot lesions can be induced experimentally in sheep by reproducing the known risk factor conditions for a naturally occurring out-break of disease. Secondly, two distinct patterns of CODD lesion pathogenesis were observed. The majority of CODD lesions emerged subsequent to pre-existing ID and/ or FR foot lesions in the same digit. These

lesions progressed from the pre-existing ID/FR to CODD G#1 and thence through the grades to CODD G#5 and were colonized by the 5 target bacterial species. The second pattern was the spontaneous appearance of *de novo* G#1 CODD lesions. Here, there was no pre-existing digital pathology. This apparent G#1 lesions did not progress to CODD G#2 – G#3, and were observed to spontaneously self-cure, typically within one week. Bacterial colonisation of these lesions by the 5 target pathogens (as measured by qPCR) was very low or absent (log_{10} MGCN < 1.303) compared to the progressive CODD lesions. This suggests that these *de novo* lesions were not, in fact, CODD but were due to other causes e.g. trauma. This would suggest that care must be taken in ascribing such lesions to CODD in the absence of further evidence in the flock such as presence of Grade 2 – 5 lesions. Thirdly, the study of the microbial colonisation of the different foot lesion types (ID, FR, CODD) observed and CODD lesion grades (1-5) by the 5 target bacterial species showed no particular association between any individual pathogen or grouping of pathogens and a disease state. This indicates, that in this experimental study, CODD had a poly-bacterial, rather than single pathogen aetiology.

Experimental Induction of CODD

In order to understand, as closely as possible, the aetiopathogenesis of CODD as it would occur in natural field cases of the disease, the experimental study was designed to transmit and induce CODD, by mixing healthy and CODD infected sheep in a typical, UK, indoor, sheep husbandry environment. Therefore, in this study, CODD infected sheep were introduced to a naïve flock (Angell, Duncan et al. 2014); animals were housed with exposure to wet underfoot conditions (Angell, Grove-White et al. 2015); and there was regular close gathering of sheep for sampling (Duncan, Grove-White et al. 2012). Transmission of CODD is

hypothesised to occur between sheep indirectly through the underfoot environment or via fomites such as gloves and hoof trimming equipment (Sullivan, Blowey et al. 2014, Angell, Clegg et al. 2017). However, to avoid cross-contamination of foot lesions for microbiological sampling no hoof trimming was carried out in the group and samplers' gloves were changed between handling each sheep.

The conditions described were sufficient to induce CODD lesions in 18 of the 30 sheep (60%) and 31 of the 120 feet (25.7%). The median survival time taken for the lesions to develop was 116 days (95%CI,103-123 days). There are no other CODD experimental transmission studies for direct comparison, however, anecdotal evidence on CODD transmission in the field and experimental studies of induction of FR and BDD suggest time to disease induction in our study was surprisingly long. For example in a study where BDD lesion material was directly inoculated onto abraded and wet bandaged sheep's feet (Wilson-Welder, Nally et al. 2017), lesions consistent with BDD were observed after 28 days in the sheep. Experimental induction of FR in other studies report a lag of seven days to the onset of FR when scarification of feet was combined with continued exposure to wet bedding and accumulation of faecal matter (Grogono-Thomas, Wilsmore et al. 1994). Following continued exposure to a wet underfoot environment, wet bandaging of feet and direct inoculation of lesions with *D. nodosus*, FR lesions developed after 10 days (Egerton and Roberts 1971). Similarly, recent, pasture transmission studies of induction of footrot by direct inoculation of foot skin and pasture contamination with *D. nodosus*, induced FR lesions in the feet typically by 8 days post exposure (McPherson, Whittington et al. 2021). This compares with a median survival time (in the present study) for ID of 88 days, (95%CI,74-109 days) and for FR 108 days, (95% CI 95-116 days). Therefore, it is apparent that foot disease induction in our experimental model

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was much slower than previously reported. This may be because we did not directly damage the integrity of the foot through scarification or bandaging or directly inoculate pathogens or infected material directly onto the feet~~wounds~~. It may also reflect the time taken for the relevant pathogens in the environment to reach infective levels. It is also highly likely that the environmental conditions of the experimental model may have affected the rate of CODD disease expression in the sheep. E~~; and although the straw under the feed troughs was kept wet and the bedding deep littered, the sheep were not continuously exposed to wet underfoot conditions.~~nvironmental conditions of moisture and temperature are known to affect the expression of footrot in sheep (McPherson, Whittington et al. 2021) and seasonal trends in field case occurrence of footrot and CODD have also been observed (Angell, Grove-White et al. 2015, Angell, Grove-White et al. 2018). In our experimental study, the straw under the feed troughs was kept wet and the bedding deep littered, however, the sheep were not continuously exposed to wet underfoot conditions, as this would not be consistent with the management of typical UK housed sheep which the study aimed to replicate. Environmental temperature and moisture parameters were not recorded in this study and are an area of important further work. A f~~F~~inal reason for the slow expression of CODD in our study could be ~~ll~~ythat~~,~~ there was no opportunity for ~~indirect~~ transmission of bacterial pathogens between sheep's feet via fomites from manual handling of the feet or foot trimming equipment which would be expected to occur in a typical farm environment.~~.~~ All progressive CODD lesions (i.e. not the *de novo* ones) did develop from ID and/or FR lesions. Therefore, consistent with field evidence ((Duncan, Grove-White et al. 2012, Angell, Duncan et al. 2014, Angell, Grove-White et al. 2015) and the experimental FR studies (Egerton and Roberts 1971, Grogono-Thomas, Wilsmore et

al. 1994), pre-existing damage to the foot was a prerequisite for CODD lesions to occur in this study, and CODD lesions represented the end stage of an infectious foot disease process which began with ID/FR.

Phenotypic CODD Lesion Development

Two distinct patterns of CODD lesion development were observed; progressive and *de novo* CODD lesions. The majority (83%) of CODD lesions in the study were progressive CODD lesions which developed from pre-existing ID and/or FR lesions; they progressed through stages of CODD lesions 1 to 5, (though there was a degree of waxing and waning of lesion grades over time); and were all heavily colonized by all 5 pathogens; *T. medium*, *T. phagedenis, T. pedis, D. nodosus* and *F. necrophorum*. A smaller number of *de novo* CODD G#1 lesions were observed to develop from clinically healthy sheep's feet, these did not progress beyond G#1. Colonisation of these *de novo* CODD lesions by the 5 foot pathogens, was substantially less than observed with the progressive CODD lesions. Therefore, it is likely that in the *de novo* foot lesions, there was insufficient initial damage to the foot (by ID or FR) to allow effective colonisation of the skin by the pathogenic bacteria in order for CODD lesions to fully develop.

Microbial Aetiology of CODD Lesions

In this experiment, CODD was a disease of polymicrobial aetiology. All 5 bacterial species, *T. medium*, *T. phagedenis, T. pedis, D. nodosus* and *F. necrophorum*. were identified in all CODD lesion stages, however the proportion of colonized samples, and mean $\log_{10}$ MGCN of all 5 bacteria species, were substantially lower in healthy feet, healed CODD grade 5, and treated CODD foot lesions, compared with feet affected by an active CODD lesion (grades 1 to 4). Thus, a clear association between bacterial colonisation and foot pathology is observed, and further evidence

for disease causality for the five pathogens consortium is demonstrated. This polymicrobial aetiology is consistent with previous cross-sectional studies of field cases of CODD aetiology where the same three DD treponeme bacteria were found in all CODD lesions; *D. nodosus* and *F. necrophorum* were also identified, but to a lesser extent (Sullivan, Clegg et al. 2015). Metagenomic analysis of the foot microbiome of during the development of CODD (from the present study) confirmed the polymicrobial aetiology of CODD and clear associations between the same bacterial consortium and ID, FR and CODD (Duncan, Angell et al. 2021).

Whilst it is clear that CODD is polymicrobial in nature, there were apparent trends in the data regarding the temporal development of CODD. As foot pathology progressed from ID to FR to CODD (grades1-4) linear regression models exploring bacterial colonisation suggested that *Dichelobacter nodosus* was present at significantly higher mean log_{10} concentrations in FR lesions compared to both ID and CODD (Figure 9); the treponeme bacteria colonised FR and CODD lesions at a higher mean log_{10} concentrations compared to the ID lesions (Figure 9); and finally *D. nodosus* and *F. necrophorum* counts were significantly higher ($P < 0.001$) in the earlier G#1 CODD lesions compared to the later ones (G#2 – G#4) (Fig 14).The survival analysis is not entirely consistent with the modelling data as early colonisation by *T. phagedenis* is then followed by *F. necrophorum* and *D. nodosus* and then colonisation by *T. medium* and *T. pedis* is observed.

In the pathogenesis FR the strain of *D. nodosus* present affects the ability of the bacteria to invade the epidermis and cause clinical disease (due to the expression of the $AprV_2$ gene) (Kennan, Wong et al. 2010). In the current study the strain of *D. nodosus* was not examined, but now we have shown a clear link in the pathogenesis of FR and CODD, this is an important area of future work.

590

591 Biosecurity measures and practices to prevent introduction of disease-causing
592 agents into a population are an essential component of almost all infectious disease
593 control strategies. The findings of the current study have important implications for
594 flock biosecurity. Current advice for prevention of the introduction of CODD into
595 naïve flocks is that all feet of all brought on sheep should be examined upon arrival
596 at the farm for CODD lesions (Duncan, Grove-White et al. 2018). However, this may
597 not be sufficient alone to prevent the introduction of CODD. At the start of the study
598 there was evidence of low level colonisation of the healthy experimental study
599 sheep's feet with *T. medium* (0.83% of feet), *T. phagedenis* (13.3% of feet), *D.*
600 *nodosus* (0.83% of feet) and *F. necrophorum* (4.17% of feet) (but not *T. pedis*). The
601 flock, from which the sheep were sourced, did have a history of infection with ID and
602 FR but had never observed CODD. It would be expected to find low level
603 colonisation with *F. necrophorum* in healthy sheep's feet and *D. nodosus*, as
604 reported in studies of FR in sheep (Clifton and Green 2016). However, it was
605 surprising to find low levels of CODD and DD associated treponemal species in the
606 healthy feet from a farm with no known history of CODD in sheep or DD in the beef
607 cattle on the farm. This contrasts with previous reports, employing routine gel based
608 PCR techniques which demonstrated an absence of CODD/BDD treponemes in
609 healthy versus infected CODD foot biopsy tissue (Sullivan, Clegg et al. 2015). The
610 present study offers no evidence that the bacterial load detected in healthy feet is
611 sufficient to initiate infection or was viable or may even be an artefact associated
612 with the chosen qPCR cut off values. Alternatively, given that *T. phagedenis* has
613 been previously reported as a commensal of the GI tract in humans, chimpanzees
614 (Wallce, Harris et al. 1967) and healthy cattle (Evans, Timofte et al. 2012) it's

presence on healthy feet could in fact represent commensal contamination from the GI tract and not be associated with foot disease *per se*. Indeed, recent work comparing bovine DD and human non-pathogenic *T. phagedenis* demonstrates the presence of unique gene clusters encoding key survival factors and a putative secretion system within the disease associated bovine *T. phagedenis* strains (Staton, Clegg et al. 2021). Using qPCR assays to target such virulence factors may enable better pathogen load determination to better describe disease progression in the future. However, based on the current data we have presented here, whilst the current advice regarding the examination of sheep feet at entry should serve to reduce the risk of introducing CODD – given that diseased sheep have a much greater bacterial load - it may not totally eliminate all risk.

Effect of Treatment on CODD Lesions

There was 100% clinical cure rate of CODD lesions (CODD G#5), observed one week after the treatment intervention of two doses of long acting amoxicillin and environmental decontamination. However, 1 lesion (8.3%) was observed to recur two weeks later. The microbiology of the feet also clearly demonstrates substantial reduction in the mean $\log_{10}$ MGCN of all five bacterial species one week and three weeks post treatment. Importantly, bacterial cure was not established in all feet. 16.7% of feet remained colonized by *D. nodosus* and 8.3% with *F. necrophorum*, and *T. phagedenis*, although bacteriological cure was achieved for *T. pedis* and *medium*. The antibiotic therapy chosen (amoxicillin) was selected based on its' previously reported field clinical cure rates for CODD (71%) (Duncan, Grove-White et al. 2012) and *in vitro* bacterial sensitivity assays for treponeme bacteria (Angell, Clegg et al. 2015) Therefore, the results of the current study are consistent with these data, and, emphasize that treated animals may still be infectious to the flock.

Study Limitations

The principle limitation of the study is that is an experimental study and the results require validation in the field. However, every effort was made to mimic known field conditions and the data is consistent with previous field studies. Furthermore, a dual sampling strategy of swabs and different biopsy depths in the field together with fluorescent in situ hybridization of key pathogens may help confirm and dissect findings in the future. Here, we used housekeeping genes as targets of qPCR, a well-established methodology, however in future studies if these were to be supplemented with qPCR assays targeting recently identified pathogenic determinants this may enable better associations of pathogen presence with disease progression.

Conclusions

1. This study presents a new understanding of the aetiopathogenesis CODD in sheep, whereby the majority of CODD lesions developed from pre-existing ID/and or FR lesions
2. CODD has a polymicrobial aetiology associated with infection with the bacteria *T. medium*, *T. phagedenis, T. pedis, D. nodosus* and *F. necrophorum*
3. As foot pathology progressed chronologically from ID to FR to CODD (grades1-4) *D. nodosus* was found to be highest concentrations in FR lesions compared with ID and CODD lesions, whilst *T. medium*, *T. phagedenis* and *T. pedis* were present in higher concentrations in the later stages of lesions (FR and CODD).
4. 91.7% clinical cure rate of CODD lesions was achieved with the amoxicillin-based treatment intervention by three weeks post treatment, however, some

feet remained colonized at a low level by *D. nodosus*, *F. necrophorum* and *T. phagedenis.*

5. Healthy, healed CODD, and antibiotic treated CODD feet, may all be colonized by one or more of the 5 pathogens associated with CODD infection *T. medium*, *T. phagedenis, T. pedis, D. nodosus* and *F. necrophorum,* and could therefore be a source of infection for between and within flock CODD disease spread.

Abbreviations

CODD (contagious ovine digital dermatitis); FR (footrot); ID (interdigital dermatitis); UK (United Kingdom of Great Britain and Northern Ireland); qPCR (quantitative polymerase chain reaction);MGCN (mean genome copy number); ARRIVE (Animal Research: Reporting of In Vivo Experiments);

Ethics approval and consent to participate

The project was carried out under UK Animal Scientific Procedure Act (ASPA) 1986; Home Office Project License PPL 708756 and University of Liverpool Ethics VREC417. Consent was obtained from all participating farms prior to the start of the study. All authors consent to the submission and publication of the manuscript.

Availability of data and material

The data sets generated and analysed during the study are available as supplementary file 2.

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

JD, DGW, NJE, SDC co-designed, obtained financial support and co-supervised the study. JA, JD, SC collected the samples. SC, GS, NJE performed laboratory analyses. DGW performed data analysis. JD wrote the manuscript. GS, JA, SC, NJE, SDC, DGW critically evaluated the manuscript.

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872

Target species	Oligonucleotide	Sequence (5'-3')
T. medium	Forward primer	CTACAAATCGAAAAGGAGTTTGGA
	Reverse primer	GGCATGTTCGGCATCCAC
	Probe	TAGAATTATCGAAATATTCGGCCCAGA
T. phagedenis	Forward primer	GCCTTCAAATCGAAAAACAATTC
	Reverse primer	GCCGCAATGCCGCCGCG
	Probe	TAGATGAGGCACTGGGAATCGG
T. pedis	Forward primer	AAATTGAAAAACAATTCGGACAG
	Reverse primer	GTGTTCGGCATCTATAAAAGCC
	Probe	ATACCCCAGAGGCCGTATTATCGAG

Table 1: Primer and probe oligonucleotide sequences used in the Taqman qPCR assays targeting the *RecA* gene of *T. medium*, *T. phagedenis* and T. *pedis*.

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Bacterium	Source sheep (n = 40 feet)		Study sheep (n = 120 feet)		
	Mean log_{10}MGCN	95% CI	Mean log_{10}MGCN	95% CI	P
F.necrophorum	0.358	0.026 - 0.689	0.341	0.225 - 0.457	0.90
D. nodosus	0.463	0.140 - 0.786	0.075	0.007 - 0.144	< 0.001
T. medium	1.918	1.230 - 2.607	0.087	0.028 - 0.147	< 0.001
T. pedis	0.914	0.437 - 1.392	0		< 0.001
T. phagedenis	1.508	0.959 - 2.056	0.350	0.263 - 0.436	< 0.001

Table 21: Mean log_{10} MGCN at foot level of study sheep compared to source sheep (Student t test) (source sheep, n=10 sheep, n=40 feet; study sheep n=30 sheep, n=120 feet).

	Pre-treatment			1 week post-treatment			3 weeks post-treatment		
Bacterium	**% feet**	**Mean log_{10} MGCN**	**95% CI**	**% feet**	**Mean log_{10} MGCN**	**95% CI**	**% feet**	**Mean log_{10} MGCN**	**95% CI**
F. necrophorum	33.3	1.25	0.73 - 1.78	6.2	0.33	0.05 - 0.62	8.3	0.48	0.15 - 0.81
D. nodosus	47.2	2.03	1.29 - 2.77	12.5	0.52	0.04 - 1.01	16.7	0.67	0.02 - 1.33
T. medium	44.2	1.74	1.05 - 2.43	0	0.05	- 0.05 - 0.14	0	0.00	0.00 - 0.00
T. pedis	33.3	1.46	0.92 - 1.99	0	0.07	- 0.01 – 0.15	0	0.01	- 0.01 - 0.03
T. phagedenis	44.4	1.53	0.93 - 2.13	6.2	0.17	- 0.02 - 0.37	8.3	0.21	- 0.01 - 0.43

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Table 32. Bacterial colonisation of G#1 CODD lesions by previous lesion history.

	Pre-treatment			1 week post-treatment			3 weeks post-treatment		
Bacterium	**% feet**	**Mean log_{10} MGCN**	**95% CI**	**% feet**	**Mean log_{10} MGCN**	**95% CI**	**% feet**	**Mean log_{10} MGCN**	**95% CI**
F. necrophorum	33.3	1.25	0.73 - 1.78	6.2	0.33	0.05 - 0.62	8.3	0.48	0.15 - 0.81
D. nodosus	47.2	2.03	1.29 - 2.77	12.5	0.52	0.04 - 1.01	16.7	0.67	0.02 - 1.33
T. medium	44.2	1.74	1.05 - 2.43	0	0.05	-0.05 - 0.14	0	0.00	0.00 - 0.00
T. pedis	33.3	1.46	0.92 - 1.99	0	0.07	-0.01 – 0.15	0	0.01	-0.01 - 0.03
T. phagedenis	44.4	1.53	0.93 - 2.13	6.2	0.17	-0.02 - 0.37	8.3	0.21	-0.01 - 0.43

Table 43: Percentage of feet colonised, mean (95% CI) foot level mean log_{10} MGCN before and after treatment (n=12 feet).

Figure and Table Legends

Figure 1: CODD G#1 (A), CODD G#2 (B), CODD G#3 (C), CODD G#4 (D), CODD G#5 (E).

Figure 2: Healthy sheep's foot (A), ID lesion (B), FR lesion (C), arrow pointing to under run horn tissue.

Figure 3: Clinical lesions observed during study period for the experimental sheep (n=30 sheep, n=120 feet).

Figure 4. Kaplan Meyer survival curves and median times to first observation of a clinical lesion for the experimental sheep (n=30 sheep, n=120 feet).

Figure 5. Proportion of colonized feet of study and source sheep and mean log_{10} MGCN at foot level of source and study sheep (source sheep, n=10 sheep, n=40 feet, study sheep n=30 sheep, n=120 feet).

Figure 6. Proportion of colonised samples and mean log_{10} MGCN by recorded clinical lesion (n=30 sheep, n=120 feet).

Figure 7. PCR positive results, by bacterial species, per week of study period (n=30 sheep, n=120 feet).

Figure 8. Kaplan Meyer survival curves for time to first bacterial colonisation of a foot (n=30 sheep, n=120 feet).

Figure 9. Predicted (95% CI) mean log_{10} MGCN by lesion type (n=30 sheep, n=120 feet).

Figure 10. Proportion of colonised samples and mean log_{10} MGCN by CODD grade (n=15 sheep, n=26 feet).

Figure 11. Predicted (95% CI) mean log_{10} MGCN by CODD grade (n=15 sheep, n=26 feet).

Figure 12. Number of colonised feet and mean log_{10} MGCN of CODD feet by pre-treatment, 1 week and 3 weeks post treatment (n=12 feet).

Table 1: Primer and probe oligonucleotide sequences used in the Taqman qPCR assays targeting the *RecA* gene of *T. medium*, *T. phagedenis* and T. *pedis*.

Table 2. Mean (95% CI) log_{10} MGCN at foot level of study sheep compared to source sheep (Student t test) (source sheep, n=10 sheep, n=40 feet; study sheep n=30 sheep, n=120 feet).

Table 3. Bacterial colonisation of Grade 1 CODD lesions by previous lesion history.

Table 4: Percentage of feet colonised, mean log_{10} MGCN before and after treatment (n=12 feet).

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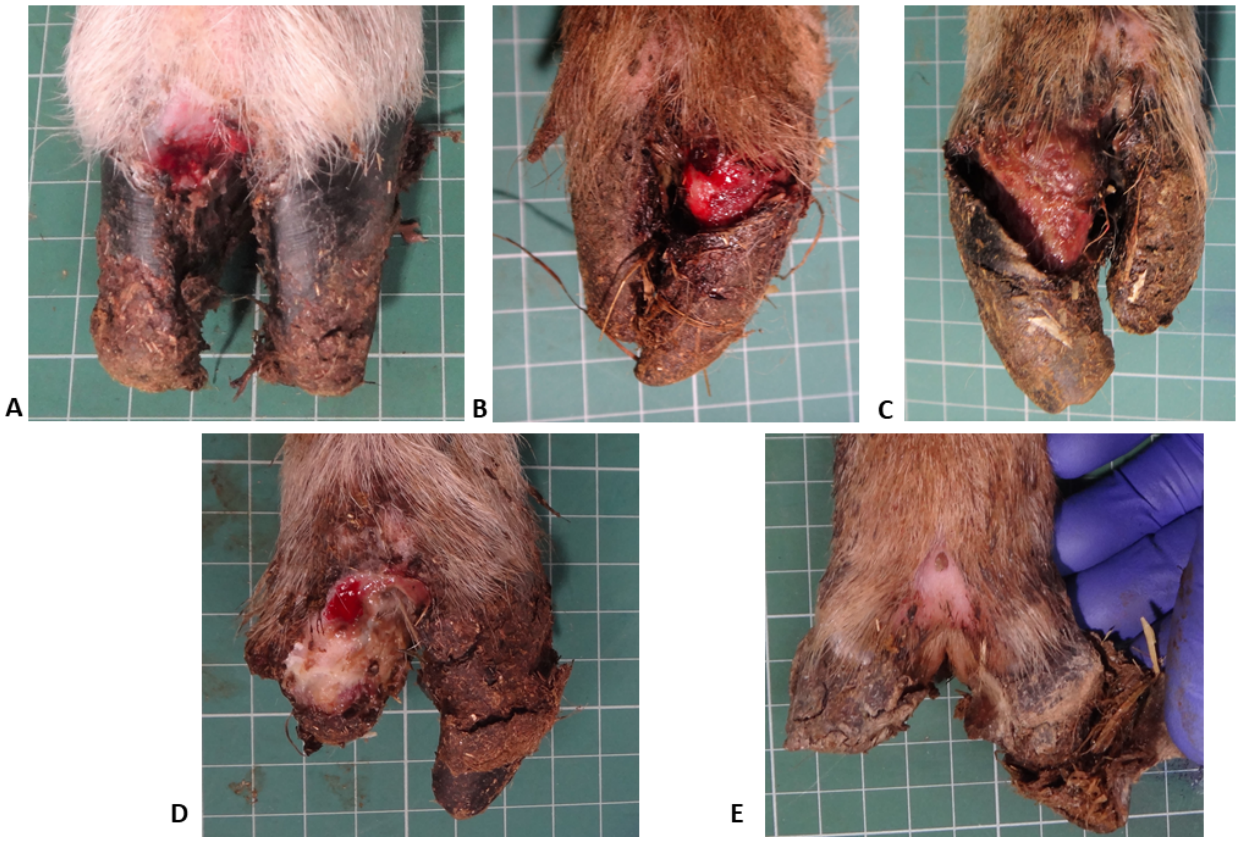

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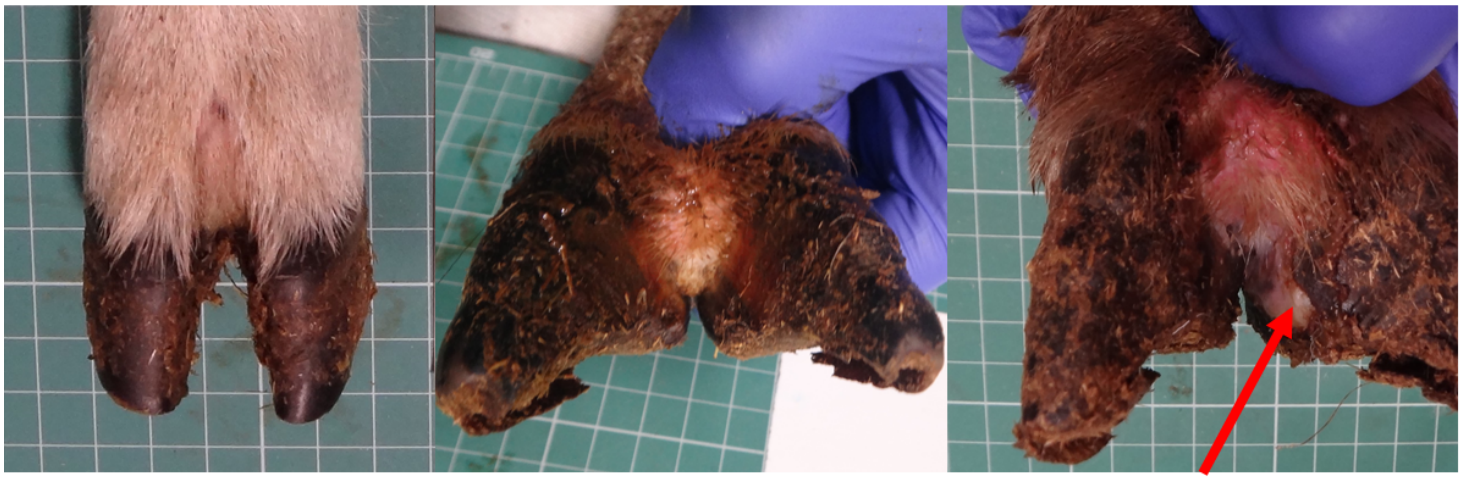

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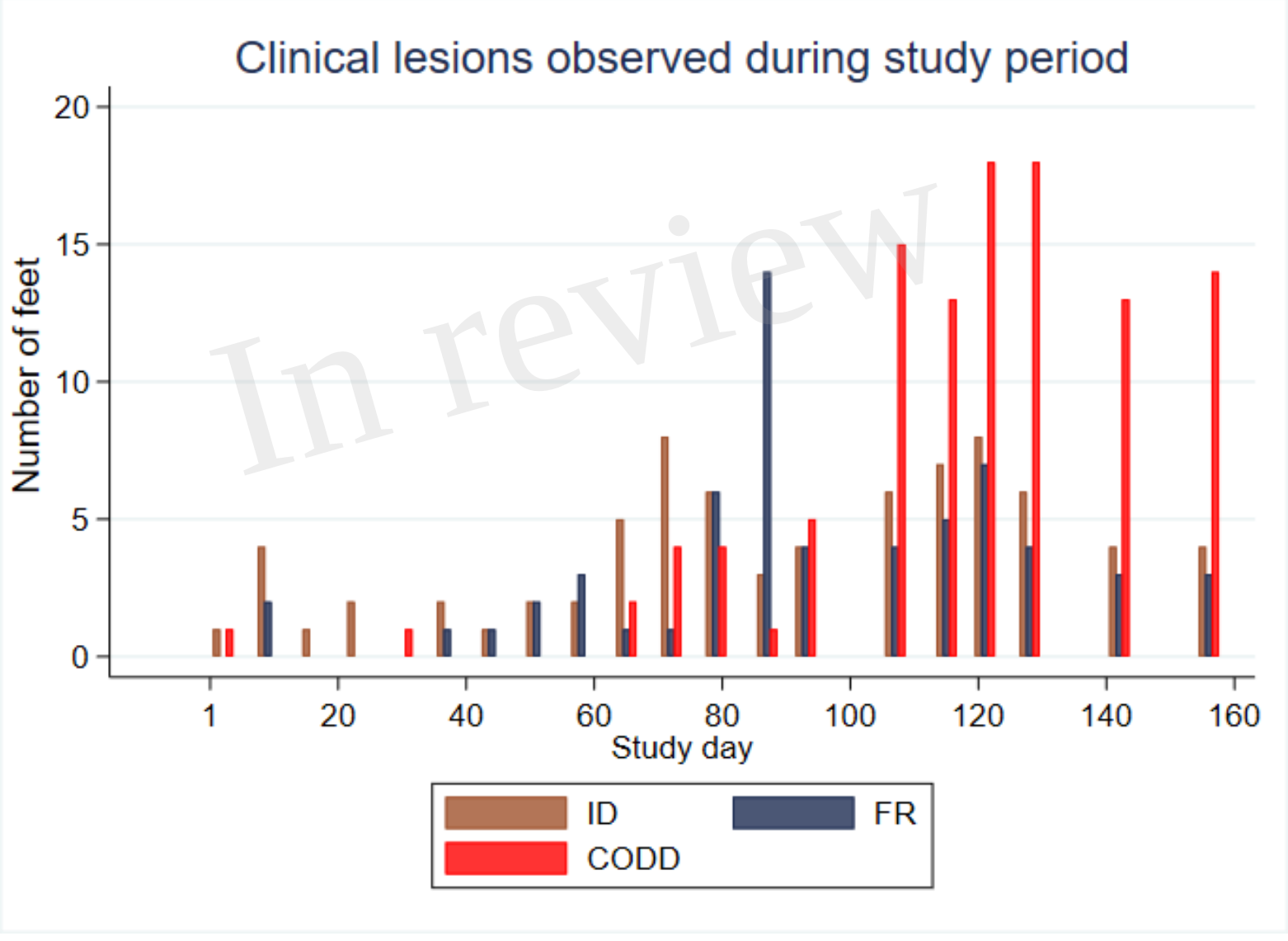

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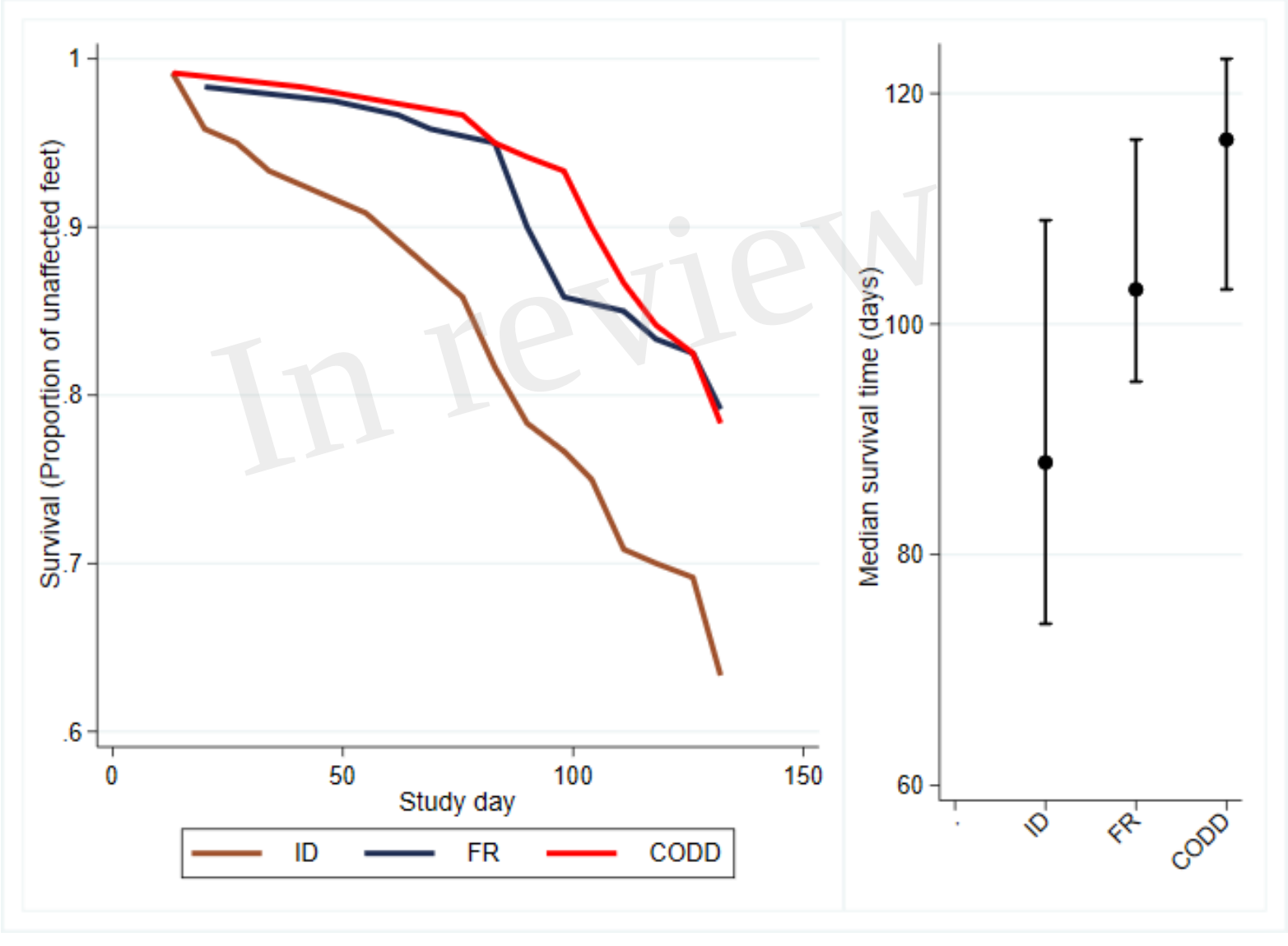

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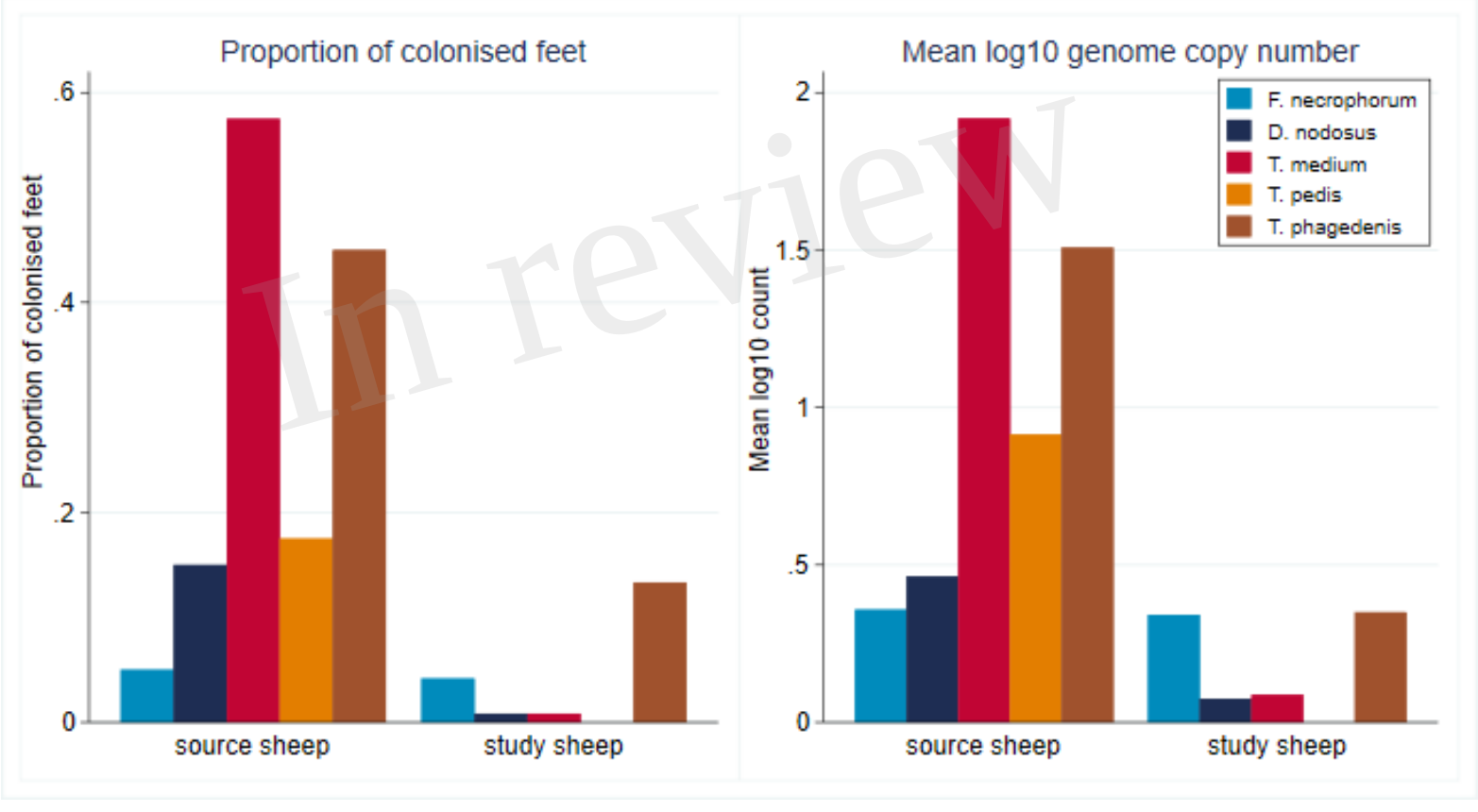

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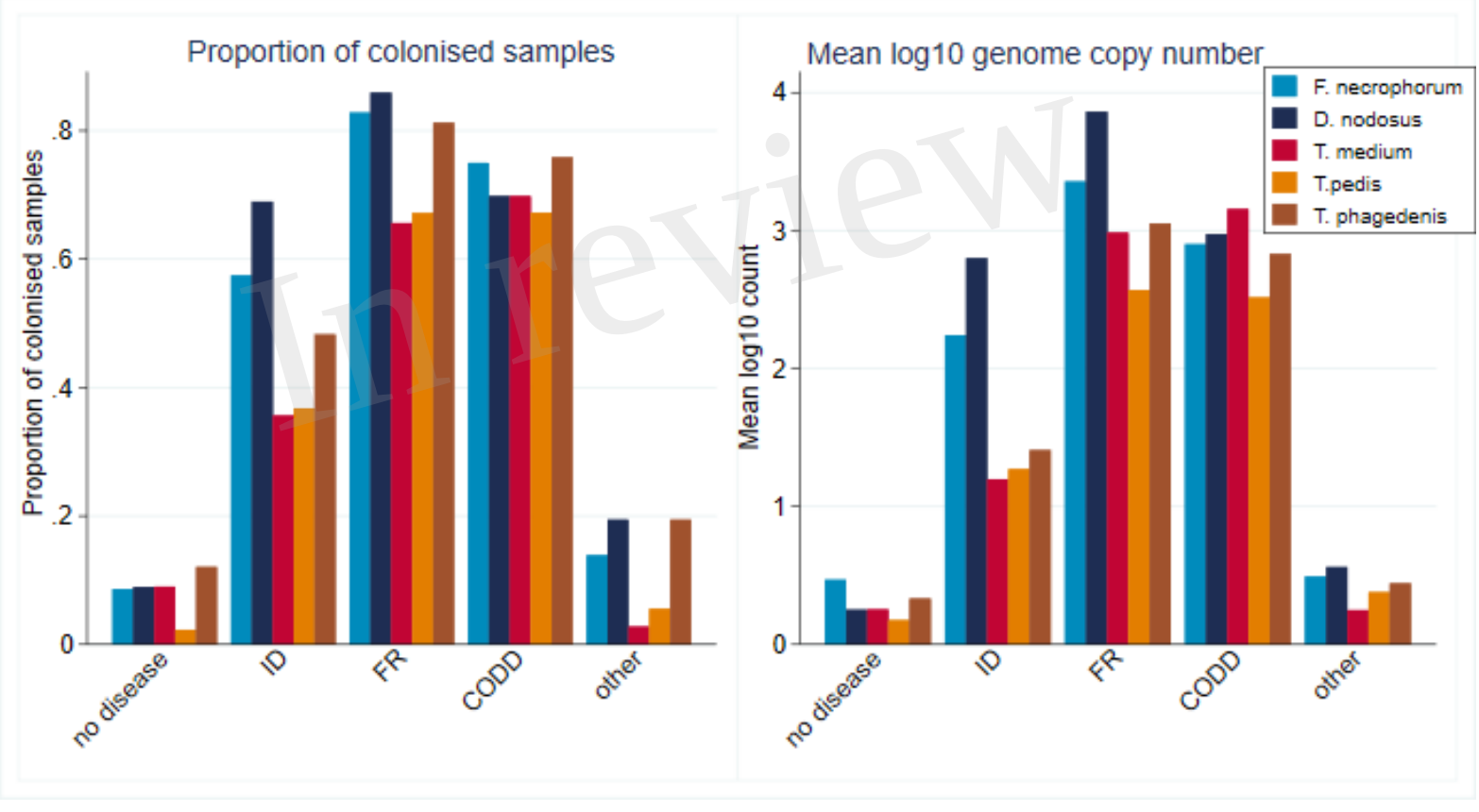

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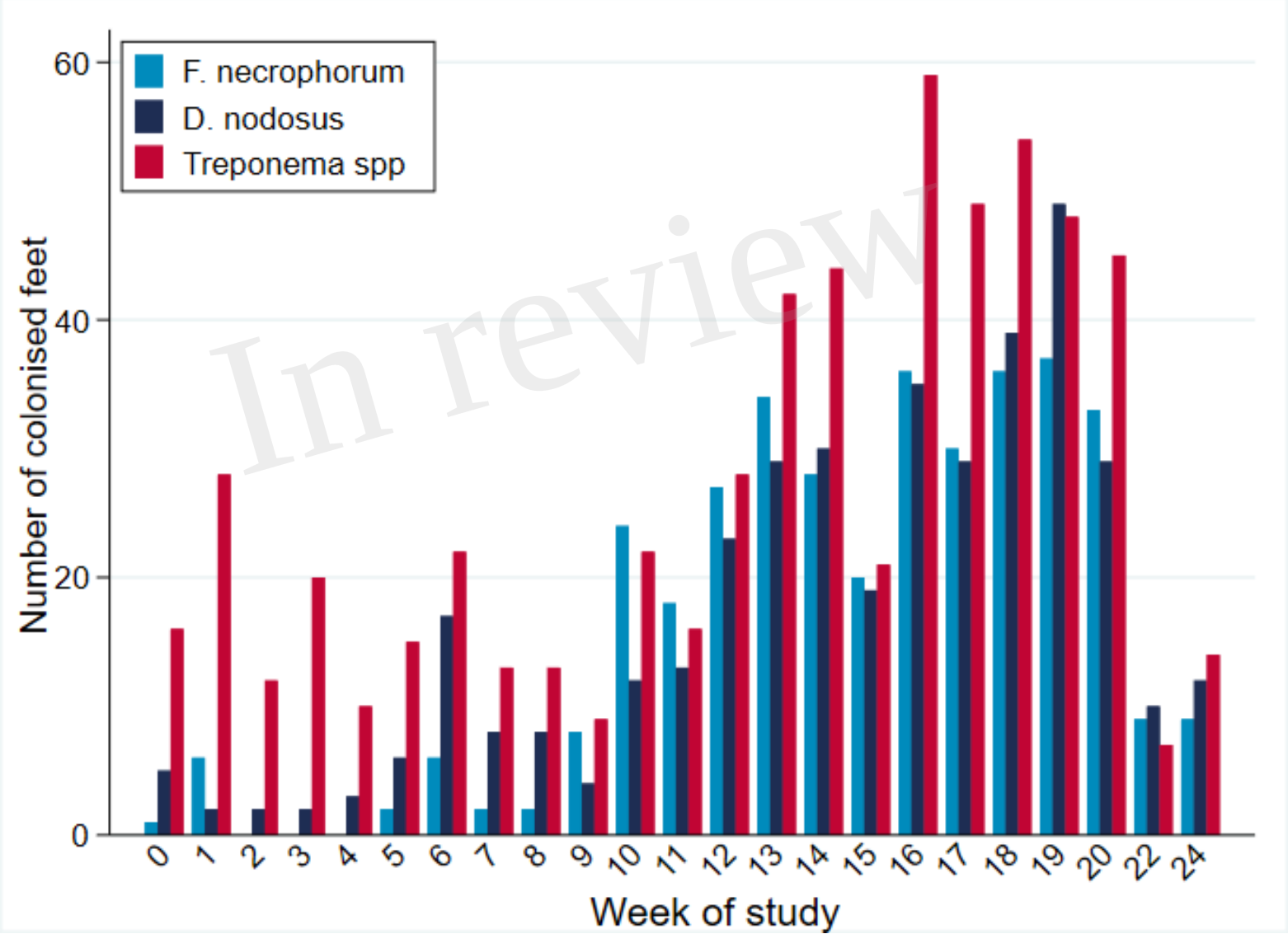

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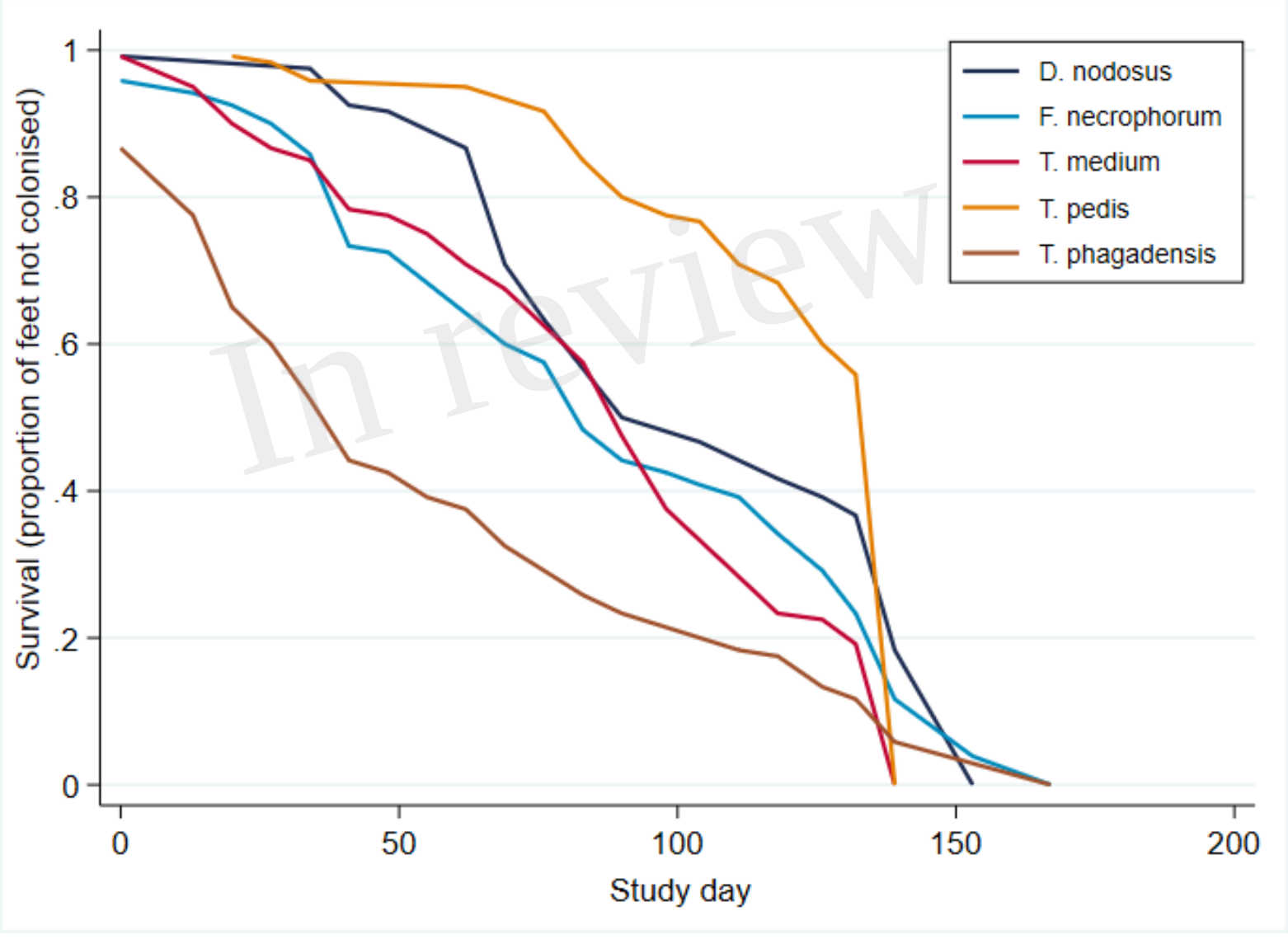

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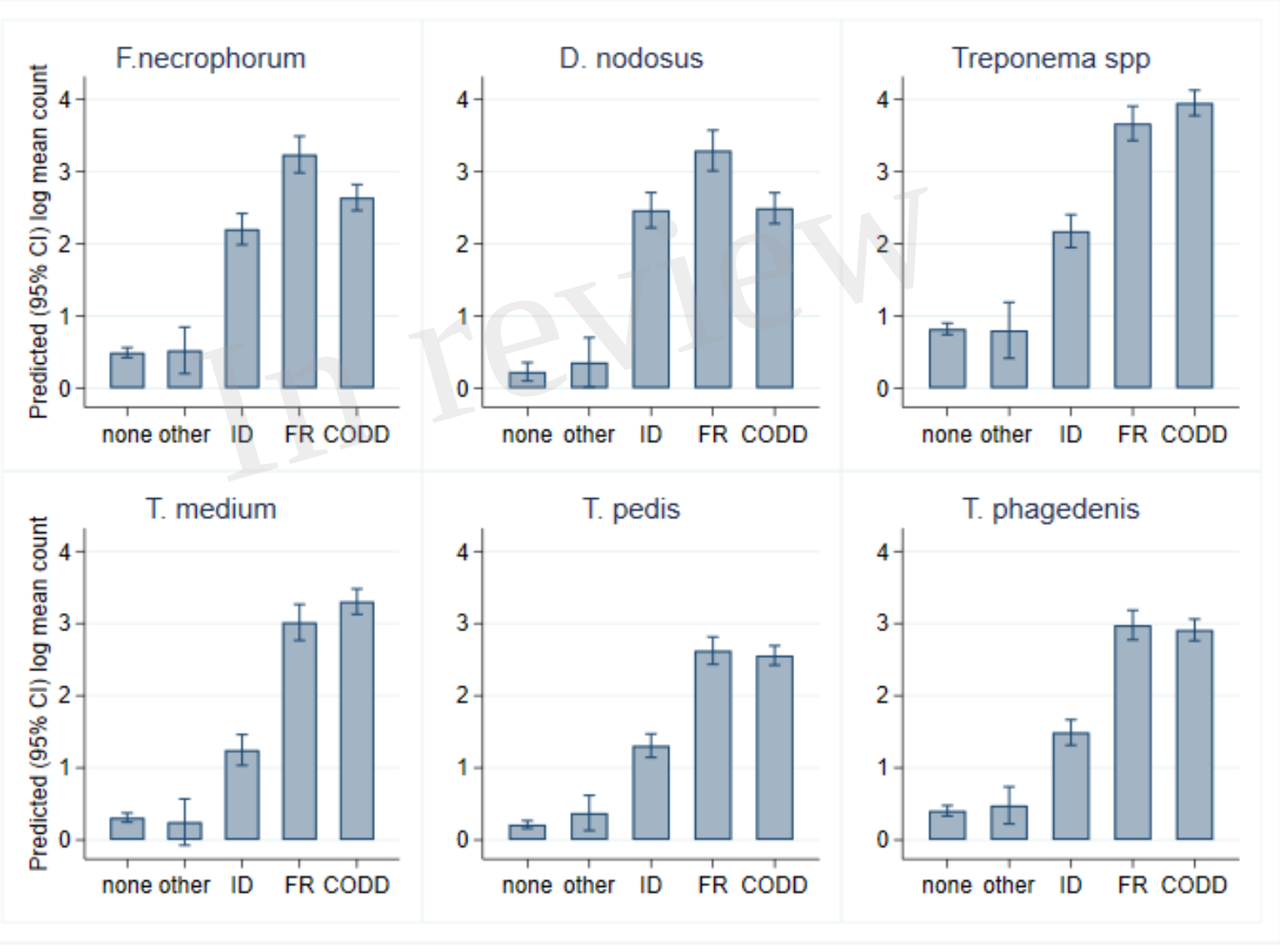

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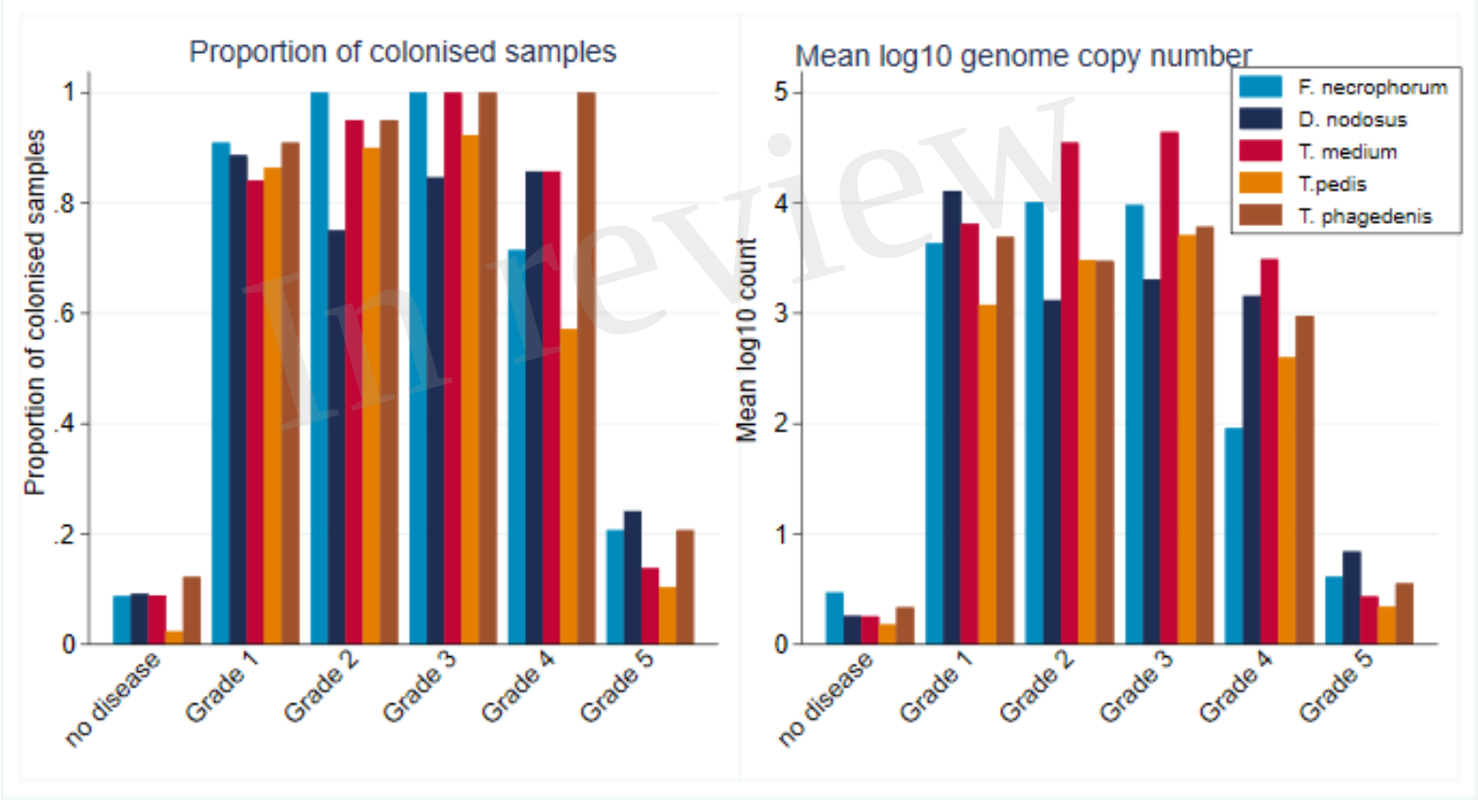

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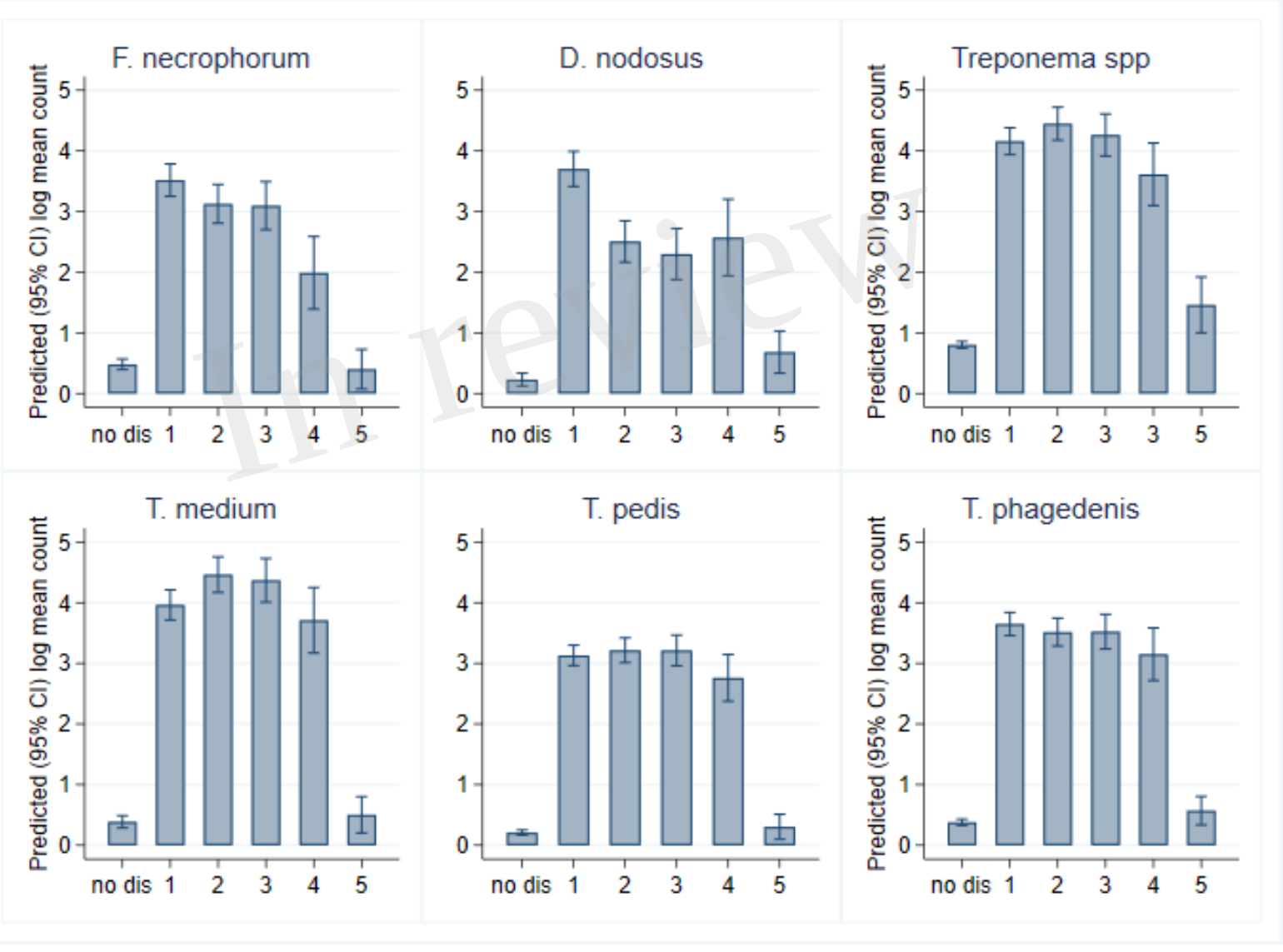

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