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# A survey of foot disinfection practices for control of bovine digital dermatitis; evaluating solution depth, footbath hygiene, and the potential of footbaths as infection reservoirs for *Treponema* species.

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## ABSTRACT

Bovine digital dermatitis remains a widespread endemic disease of dairy cattle worldwide. Footbathing is commonly used as a control measure and has significant economic and environmental impact. There are few studies documenting footbathing practices on dairy farms, or evaluating their suitability for achieving foot disinfection. This study describes footbathing practices on 32 farms observed in the United Kingdom, Ireland, and the Netherlands. We measured solution depth throughout footbathing and observed levels below 7cm on 9/32 farms, which leads to inadequate foot coverage. Solution depth was associated with the number of cow passages, decreasing by 1.2cm for every 100 cow passages.

We also describe levels of organic matter content (g/L) throughout footbathing as a proxy for footbath hygiene. Our data indicates that almost half of footbaths (15/32) became contaminated above the 20g/L threshold to which veterinary biocides are tested for efficacy, and that organic matter content is associated with the number of cow passages per liter of footbathing solution provided. A multivariable mixed model predicted that one liter of footbathing solution per cow should be sufficient to prevent excess contamination.

As a further measure of hygiene, we tested a subset of footbath samples to quantify the amount of DNA present from the *Treponema* species which are considered instrumental in the etiology of digital dermatitis. We did not detect *Treponema* DNA in footbath samples, suggesting they are unlikely to act as infection reservoirs for this disease.

Multivariable mixed models including farm identity as a random effect demonstrated that for both change in solution depth and organic matter content the effect of farm-level factors was large. Because of the magnitude of this farm effect, applying model predictions will not translate to adequate solution depth and hygiene on all farms. Our data highlights the importance of footbath auditing on individual farms.
Key Words. biocide, dairy cow, digital dermatitis, quantitative PCR, questionnaire

## INTRODUCTION

Bovine digital dermatitis (BDD) is the most prevalent infectious foot disease of dairy cattle (Murray et al., 1996) and among the causes of cattle lameness it has been considered to have the greatest impact in terms of economics and animal welfare (Bruijnis et al., 2012). BDD is characterized by painful acute ulcerative lesions, most commonly seen between the heel bulbs on the hind feet (Murray et al., 2002). BDD has a polybacterial etiology, with pathogenic species from the *Treponema* genus regarded as most important for pathogenesis. These pathogenic species have been characterized and classified into 3 phylogroups: *Treponema medium, Treponema phagedenis,* and *Treponema pedis* (Evans et al., 2016). Their role in pathogenesis has been established using histological and serological evidence (Demirkan et al., 1998, 1999), and through the development of infection models for inducing skin lesions in cattle (Read & Walker, 1996; Gomez et al., 2012; Krull et al., 2016;).

Footbathing is routinely used for BDD control as it reduces the risk of BDD compared with no footbathing (Rodriguez-Lainz et al., 1999), and improves foot

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

hygiene, which also reduces the risk of BDD (Hultgren & Bergsten, 2001). Footbathing is likely to work by preventing the appearance of new acute lesions and preventing transition of chronic lesions back to acute lesions, resulting in a state of "manageable" endemic disease (Döpfer et al., 2012). Debate remains surrounding how frequently footbathing should be carried out, however 4 times weekly appears to be optimal (Speijers et al., 2012; Jacobs et al., 2019) and can be adjusted every 6–8 weeks for individual farms depending on BDD lesion prevalence and infection pressure. There is some concern that overzealous footbathing regimens in terms of frequency, biocide concentration, or both, may damage feet, causing more proliferative chronic lesions (Dopfer, 2022). There is also a need for more concise guidelines because the cost of footbathing can easily equate to the cost of all other animal medications and treatments per cow, as well as overuse of chemicals becoming an environmental problem (Cook, 2017).

There are few surveys describing footbathing practices in the dairy industry; those that have been done identified a wide variation in footbath design, frequency, and biocides used (Holzhauer et al., 2004; Cook et al., 2012; Solano et al., 2015). A footbath length of 3m is recommended to allow at least 2 immersions of each rear foot in disinfectant, and a width of 0.6m allows a single cow to pass through while minimizing the solution required (Cook et al., 2012). A footbath depth of 12cm is needed to immerse the whole foot including the skin-horn junction on the dorsal aspect (Agricultural and Horticultural Development Board (AHDB), 2019), however shallower depths of 7–9cm may reduce solution loss (Blowey, 2015) and are adequate to immerse the most susceptible heel bulb region.

There are 2 major sources of contamination in footbaths: dirt from cows' feet, and feces voided during footbathing (Ariza et al., 2019). Biocides approved for veterinary usage are required under EU Legislation (Regulation EU No 528/2012) to show efficacy against *Enterococcus hirae, Proteus vulgaris, Pseudomonas aeruginosa,* and *Staphylococcus aureus* after exposure to up to 20g/L of organic matter. It is assumed that the level of contamination would not normally exceed 20g/L if solution is changed at the rate of 1L per cow passage, however this threshold was exceeded on 2/6 farms observed in a recent study (Ariza et al., 2019). The same study reported that remaining footbath depth is likely to be inadequate before contamination levels become high, and therefore concluded that footbath renewal rates should be decided based on remaining footbath depth (Ariza et al., 2019).

BDD-associated *Treponema* spp. have been identified in the bovine gastrointestinal tract (Evans et al., 2012), and a metagenomic study identified *Treponema* spp. in slurry collected from dairy cattle housing in BDD-infected herds (Klitgaard et al., 2017). These findings raise concern that fecal and slurry contamination of footbaths could provide a source of pathogens, alongside BDD pathogens from infected feet washed into footbaths as diseased hosts pass through. In addition, high levels of slurry contamination may inactivate biocides.

In this study we surveyed 32 dairy farms to collect data describing current industry footbathing practices. We measured solution depth throughout footbathing and we translate this insight into practical advice regarding initial solution depth required to meet industry standards for all cows passaging through footbaths. In addition, we hypothesized that organic matter content (OMC) in footbaths frequently exceeds the biocide testing threshold, risking survival of BDD pathogens. Our objective was to measure OMC and pathogenic *Treponema* spp. content throughout footbathing.

## MATERIALS AND METHODS

A convenience sample of dairy herds using veterinary services from the University of Glasgow School of Biodiversity, One Health and Veterinary Medicine (UG), the University College Dublin School of Veterinary Medicine (UCD), and the Farm Animal Practice of Utrecht University (FAPU) were selected. Inclusion criteria were minimum herd size of 50 cows, Holstein-Friesian herds, BDD confirmed to be present in the herd by the herd veterinarian, and regular footbathing regimen already in place. FAPU dairy herds were selected for those using formalin in footbaths, as they were also simultaneously part of another study (Janssen et al., 2023). Ethical approval was obtained from individual universities with the following reference numbers: Glasgow EA28/19, Dublin AREC-E-21–28-McAloon, and Utrecht AVD 1080020209606. The farm visits with surveys and collection of measurements and footbath samples were carried out from May to September 2019 for UG dairy herds, November 2020 to April 2021 for FAPU herds, and January to November 2022 for UCD herds.

### Farmer Questionnaire

Researchers carried out a short interview-style questionnaire with the farmer or herd manager before sampling. The questionnaire is available in the Supplementary Information (10.6084/m9.figshare.24829833).

### Footbath Measurements and Samples

Footbathing was carried out according to the farms' normal routines. Measuring tapes were used to measure the dimensions of each footbath and the depth of the so-

lution. Footbaths were sampled before any cows walked through, after approximately every 50 cow passages, and at the end of the footbathing session to determine OMC (g/L) and for isolation of bacterial DNA. The researcher mixed the footbath contents by hand (wearing a protective arm-length glove) or using a large spoon in a zig-zag motion along its entire length to create a homogenous solution. Samples were collected from the center of the footbath in a 20mL universal container, stored on ice, and frozen at −20°C as soon as possible. Solution depth was re-measured at each sampling point, before mixing the footbath solution.

### Measuring Organic Matter Content in Footbath Samples

Each footbath sample was thawed, homogenized by vortexing, and the volume measured using a measuring cylinder. Pairs of gauze swabs (10x10, 8 layers, Covetrus or 7x10, 8 layers, Gauze™, Mölnlycke Health Care AB) were weighed and used to filter each sample, retaining the organic matter. Swabs were dried overnight and re-weighed to ascertain the weight of the organic matter. Results are expressed as grams of organic matter per liter of footbath solution [OMC (g/L)].

### Statistical Analysis

Data collected by researchers and from farmer questionnaires was collated and analyzed using R version 4.2.3 in R Studio, software version 2023.03.0 (R Core Team, 2020; RStudio Team, 2020).

First, footbath solution depth was assessed, as inadequate solution depth would lead to ineffective foot disinfection. The effect of number of cow passages and footbath dimensions (length, width, original solution depth, and the difference between solution depth and footbath depth) on the solution depth were assessed using univariable linear regression after using histograms to check for normal distribution of variables. Explanatory variables significantly associated with footbath solution depth ($P < 0.1$) were number of cow passages and footbath length. These 2 variables were offered to a final model with the random effect of farm included to give a multivariable mixed effects model. Residual plots were used to check for model bias.

Second, the values for footbath organic matter content (g/L) (OMC) were used as a measure of footbath hygiene; we interpret that higher levels of organic matter content equate to poorer footbath hygiene. Continuous variables were checked for normal distribution using histograms. The explanatory variables considered to affect footbath hygiene were: number of times herds were footbathed per month, footbath length, housing (housed, at pasture, or at pasture during the day and housed at night), and number of cow passages per liter of original footbath solution. Only number of cow passages per liter of original footbath solution was significantly associated with the outcome OMC ($P < 0.1$), and was therefore offered to a final multivariable mixed effects model together with the random effect of farm. The outcome OMC was log-transformed in the final model as it was not normally distributed. Residual plots were used to check for model bias.

### Footbath Samples Pathogen Load Measurements

***Treponeme qPCR Validation and Use.*** To validate *Treponema* qPCR protocols for detection of *Treponema* DNA in footbath samples, lesions from cows (n = 4) with M2 or M4 BDD lesions (Berry et al., 2012) were sampled using sterile viscose swabs (cat. No. 80.625, Sarstedt) to provide positive controls from field samples. Feces were also sampled, rectally or during spontaneous defecation.

The qPCRs were validated using DNA from the following samples: DNA isolated from cultured *T. medium* T19, *T. phagedenis* T320A, *T. pedis* T3552B, and *Fusobacterium necrophorum* as described by Staton et al. (2021); DNA isolated from 2 fecal samples from randomly selected cows, pure cultures of *Campylobacter fetus* and *Dichelobacter nodosus* from an in-house biobank of isolates (species confirmed by MALDI-TOF), and pure cultures of *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922.

To test how formalin treatment (the disinfectant in the majority of footbaths in this study) affects the qPCRs, resuspended BDD lesion swabs and feces were split in 2 and one duplicate treated with 5.4% formalin (2% formaldehyde, cat.no. 158127, Sigma-Aldrich) and the other with mock treatment (TheraPEAK™ PBS without Calcium or Magnesium, Lonza) at room temperature for one hour. To test how freezing of footbath samples affects the qPCRs, each sample was again split in 2, one half was frozen at −20°C and genomic DNA was isolated immediately from the other half. This process resulted in 4 treatments for each sample: mock treatment, formalin treatment, mock treatment and freezing, and formalin treatment and freezing.

***DNA Isolation.*** Before DNA isolation, footbath samples were thawed and vortexed, feces samples were mixed 1:1 with PBS, and BDD lesion swabs were vigorously resuspended in 700ul PBS. Genomic DNA from the different samples was isolated using the QIAamp Fast DNA Stool Mini Kit (Qiagen) according to the DNA from Stool for Pathogen Detection protocol, with the following modifications; after the addition of the InhibitEX buffer the samples were first heated at 70°C for 5 min and then at 95°C for 30 min (Weiss et al., 2011). After

addition of Buffer AL and vortexing the samples were incubated at 70°C for 20 min.

***qPCR.*** Footbath samples were subjected to qPCR to detect *Treponema* spp. DNA. The following selection of footbath samples (total n = 67) were tested: all samples from 5 farms with high bulk tank milk titers against *Treponema* spp. (Holzhauer et al., 2023) from the FAPU collection (n = 36); all samples from 4 farms that used disinfectants other than formalin from the UG collection (n = 16); the last footbath sample from all 11 farms from the UCD collection, and 4 start samples from randomly selected farms from the UCD collection (n = 15). This selection was targeted to include samples that were considered higher risk for contamination with appropriate low risk controls, and a range of different disinfectants.

A 16S rRNA gene qPCR targeting the V5V6 region was used to quantify the total bacterial DNA (Table 1). These qPCR assays were performed in 20µL comprising: 10µL iQ SYBR Green Supermix (BioRad), 0.5µM of each primer, and 1µL template. The cycling conditions consisted of a single activation step at 95°C for 5 min followed by 45 cycles of 95°C for 10 s, 56°C for 30 s, and 72°C for 30 s.

A general *Treponema* spp. qPCR targeted to the 16S rRNA gene was developed using previously described primers (Asai et al., 2002). To design a probe, regions in the product amplified by the primers that were conserved in the BDD associated species of treponeme, *T. medium, Treponema vincentii, T. phagedenis, Treponema putidum, Treponema denticola,* and *T. pedis* were analyzed using the PrimerQuest tool (IDT) and the probe with the best characteristics was ordered (Table 1) and validated as described below. The general *Treponema* qPCR assays were performed as described above for the total bacteria qPCRs, except for use of LC480 Probe Master mix (Roche) and a probe with a concentration of 62.5 nM instead of iQ SYBR Green Supermix (BioRad). The activation step was shortened to 3 min, and the annealing temperature raised to 64°C.

The protocols for the species-specific *Treponema* qPCRs which target the Recombinase A (RecA) genes of *T. medium* (accession number CP027017), *T. phagedenis* (accession number CP027018), and *T. pedis* (accession number CP045760), respectively, were previously described by Staton et al. (2021). These species-specific qPCR assays were performed in 20µL comprising: 10µL LC480 Probe Master mix (Roche), 0.5µM of each primer, 125 nM probe and 1µL template. The cycling conditions consisted of a single activation step at 95°C for 10 min followed by 45 cycles of 95°C for 10 s, 61°C for 30 s, and 72°C for 30 s.

All qPCRs were performed in duplicate on a Lightcycler 96 (Roche); primer and probe sequences for all qPCRs are given in Table 1.

**Table 1.** Primer and probe oligonucleotide sequences, product size, and annealing temperature for the qPCR assays

| Specificity | Sequence | Orientation | Product Size | Annealing Temperature |
|---|---|---|---|---|
| 16S rRNA gene v5v6 region (Total Bacterial) | 5′-ATTAGATACCCTGGTAGTCC-3′ | Forward | ±294 bp | 56°C |
| | 5′-TCACGACACGAGCTGACGACA-3′ | Reverse | | |
| *Treponema* spp.(General *Treponema* spp.) | 5′-TTACGTGCCAGCAGCCGCGGTAAC-3′ | Forward | 657 bp | 64°C |
| | 5′-GTCRYMGGCAGTTCCGCCWGAGTC-3′ | Reverse | | |
| | 5′-6FAM-TGGCGAAGGCGGGTTTCTGGCCGAT-BBQ-3′ | Probe | | |
| *Treponema medium* | 5′-CTACAAATCGAAAAGGAGTTTGGA-3′ | Forward | 255 bp | 61°C |
| | 5′-GGCATGTTCGGCATCCAC-3′ | Reverse | | |
| | 5′-6FAM-TAGAATTATCGAAATATTCGGCCCAGA-BBQ-3′ | Probe | | |
| *Treponema phagedenis* | 5′-GCCTTCAAATCGAAAAACAATTC-3′ | Forward | 235 bp | 61°C |
| | 5′-GCCGCAATGCCGCCGCG-3′ | Reverse | | |
| | 5′-6FAM-TAGATGAGGCACTGGGAATCGG-BBQ-3′ | Probe | | |
| *Treponema pedis* | 5′-AAATTGAAAAACAATTCGGACAG-3′ | Forward | 248 bp | 61°C |
| | 5′-GTGTTCGGCATCTATAAAAGCC-3′ | Reverse | | |
| | 5′-6FAM-ATACCCCAGAGGCCGTATTATCGAG-BBQ-3′ | Probe | | |

FAM - 6-carboxylfluorescein fluorophore.
BBQ - non-fluorescent Black Berry Quencher.

***Bacterial Culture.*** For footbath samples collected at FAPU, aliquots were thawed, homogenized by vortexing, and 100 µL was plated on a Columbia Agar plate supplemented with 5% Sheep Blood (BD). After incubation for 22–24 h at 37°C, colonies were counted.

## RESULTS

A total of 32 farms were recruited: 8 farms in Scotland, 13 in the Netherlands and 11 in Ireland. Relevant farm characteristics are available in Supplementary Table 1 (10.6084/m9.figshare.24829833).

### Farmer Questionnaire

The majority of farms (20/32, 63%) reported BDD as the main cause of lameness in the herd. Footbathing frequency ranged from once a month to twice a day (mean 9.5 times per month, SD 14.9). Nineteen farms (59%) were footbathing dry cows as well. The questionnaire did not specifically ask for the frequency of dry cow footbathing, however some farms volunteered information indicating it was less frequent than for milking cows.

The most frequently used footbath chemical was formaldehyde with almost half of the farms using this alone (15/32, 47%), 5 (16%) using a commercially available formaldehyde blend which also contained copper sulfate and zinc sulfate, 2 farms (6%) combining copper sulfate powder with formaldehyde, and one (3%) using a commercial product combining formaldehyde with peracetic acid. Five farms (16%) used copper sulfate alone, one (3%) used a commercial product combining copper with phosphoric and sulfuric acid, and one (3%) used a commercial product containing zinc and tea tree alongside copper. One farm (3%) used zinc sulfate alone, and one (3%) used a commercial product containing a quaternary ammonium compound.

Twenty-seven participants (27/32, 84%) replied to a question regarding how they decided on the quantities of water and footbathing solution they were using (Figure 1). Nine farmers reported using the product information or labeling (28%), 4 took advice from a product sales representative (13%), 3 used internet searches (9%), 3 followed recommendations from farmer discussion groups (9%), one followed veterinary advice (3%), one followed advice from farming publications (3%), and one followed advice from a foot trimmer (3%). Rather than citing a particular source for concentration recommendations, 4 farmers (13%) cited practical considerations or based their decisions on their experience of footbath efficacy, and one farmer (3%) reported there was no precise calculation (Figure 1). The remaining 5 respondents (16%) had calculated the quantities in the past, but could not remember how they were decided.

### Footbath Characteristics

The type of footbath used on each farm is described in Supplementary Table 1 (10.6084/m9.figshare.24829833) and footbath dimensions are reported in Table 2. Mean footbath length was 2.6m (SD 0.6m), and mean width was 1.0m (SD 0.5m). Mean depth was 0.15m (SD 0.03m) with mean solution depth 0.10m (SD 0.02m) at the start of footbathing. Most farms used a single footbath (24/32, 75%). Five farms (16%) used 2 footbaths positioned in series; of these, one farm used a water prewash in the first footbath while the rest used the same solution throughout. One farm (3%) used 3 consecutive footbaths, and one farm (3%) used 2 footbaths side-by-side.

Of the 21 participants asked, 20 (95%) were able to fully report the number of liters of water used per footbath and the volume or weight of the treatment product needed in this volume to reach their intended footbath concentration. Seven of these participants (33%) did not know what concentration they were aiming for.

Volume calculations carried out using measurements taken by researchers identified that mean measured footbath solution volume was 73L (37%) higher than farmers reported (272L compared with 199L), according to their answers regarding water used per footbath and the amount of product added. Researchers were not able to measure the amount of treatment product added to the footbaths as these were often prepared before arrival, however for 2 farms the increased measured footbath volumes may have resulted in concentrations of active ingredient below 2%. Conversely, one farmer who did not report footbath volume reported using enough copper sulfate to result in a 10.5% solution using researcher volume measurements.

Current industry recommendations on footbath dimensions advise they are at least 3m long with a solution depth of 12cm, providing a footbathing solution volume of at least 1L/cow. Only one farm met all of these recommendations before footbathing began. Just over half of footbaths were < 3m (18/32, 56%), and 10/32 (31%) of footbathing regimens were not providing at least 1L of footbathing solution per cow. A small number of farms met the optimal solution depth criterion of 12cm (6/32, 19%); almost all footbaths met the adequate solution depth criterion of 7cm (30/32, 94%). The depth of solution decreased throughout footbathing resulting in no footbaths remaining above 12cm depth and 9/32 (28%) having depths of < 7cm by the end of the session (Table 2).

### Footbath Solution Depth

Univariable linear regression determined that number of cow passages was associated with solution depth ($P <$

**Table 2.** Description of footbathing practices on 32 dairy cattle farms, including solution depth and organic matter content (g/L) at the end of footbathing

| Farm No. | No. of cow passages through footbath | Footbath dimensions | | | Depth of original solution (cm) | Calculated starting footbath solution volume (Liters) | Liters per cow | Depth of final solution (cm) | Final OMC (g/L) reading |
|---|---|---|---|---|---|---|---|---|---|
| | | Length (m) | Width (m) | Depth (m) | | | | | |
| 1 | 96 | 3.3 | 1.6 | 0.15 | 11.0 | 581 | 6.05 | 10.3 | 7.50 |
| 2 | 250 | 1.9 | 0.75 | 0.15 | 11.5 | 164 | 0.66 | 8.0 | 30.30 |
| 3 | 122 | 2.45 | 0.65 | 0.1 | 6.0 | 96 | 0.79 | 6.5 | 117.90 |
| 4 | 112 | 3.3 | 0.6 | 0.15 | 14.0 | 277 | 2.47 | 12.0 | 16.82 |
| 5 | 150 | 2.0 | 2.0 | 0.15 | 10.0 | 400 | 2.67 | 9.0 | 6.25 |
| 6 | 150 | 1.8 | 0.75 | 0.15 | 10.5 | 142 | 0.95 | 7.5 | 30.27 |
| 7 | 112 | 2.9 | 0.8 | 0.1 | 7.5 | 174 | 1.55 | 7.0 | 28.84 |
| 8 | 384 | 4.5 | 2.7 | 0.23 | 6.0 | 729 | 1.90 | 5.0 | 42.73 |
| 9 | 242 | 3.0 | 0.8 | 0.15 | 7.0 | 168 | 0.69 | 7.5 | 17.31 |
| 10 | 149 | 3.0 | 0.8 | 0.15 | 12.0 | 288 | 1.93 | 7.0 | 27.50 |
| 11 | 120 | 1.9 | 0.8 | 0.15 | 12.0 | 182 | 1.51 | 6.0 | 16.67 |
| 12 | 224 | 3.0 | 0.8 | 0.15 | 10.0 | 240 | 1.07 | 7.5 | 51.38 |
| 13 | 252 | 2.2 | 0.9 | 0.12 | 10.0 | 198 | 0.79 | 6.5 | 10.87 |
| 14 | 396 | 2.0 | 0.75 | 0.15 | 13.0 | 195 | 0.49 | 7.0 | 46.09 |
| 15 | 220 | 3.0 | 0.85 | 0.15 | 9.5 | 242 | 1.10 | 8.5 | 15.00 |
| 16 | 236 | 3.0 | 0.8 | 0.15 | 9.0 | 216 | 0.92 | 4.0 | 8.75 |
| 17 | 91 | 2.8 | 0.8 | 0.15 | 11.0 | 246 | 2.70 | 8.0 | 8.46 |
| 18 | 92 | 3.0 | 0.8 | 0.15 | 10.5 | 252 | 2.74 | 8.5 | 22.50 |
| 19 | 182 | 1.9 | 0.8 | 0.15 | 10.0 | 152 | 0.84 | 6.0 | 16.25 |
| 20 | 85 | 1.9 | 0.8 | 0.15 | 11.5 | 175 | 2.06 | 8.5 | 6.36 |
| 21 | 178 | 1.9 | 0.8 | 0.15 | 11.5 | 175 | 0.98 | 9.0 | 10.83 |
| 22 | 138 | 2.4 | 0.75 | 0.15 | 10.0 | 180 | 1.30 | 7.6 | 9.37 |
| 23 | 486 | 2.9 | 1.56 | 0.15 | 9.5 | 428 | 0.88 | 6.1 | 13.91 |
| 24 | 339 | 3.1 | 1.77 | 0.16 | 10.2 | 560 | 1.65 | 8.0 | 7.73 |
| 25 | 271 | 2.4 | 0.89 | 0.19 | 18.0 | 386 | 1.42 | 10.8 | 28.50 |
| 26 | 305 | 2.0 | 1.67 | 0.16 | 10.0 | 339 | 1.11 | 8.0 | 17.14 |
| 27 | 208 | 2.9 | 0.85 | 0.25 | 13.0 | 320 | 1.54 | 11.4 | 68.57 |
| 28 | 292 | 2.9 | 1.07 | 0.105 | 9.0 | 275 | 0.94 | 6.9 | 35.00 |
| 29 | 180 | 2.9 | 0.8 | 0.10 | 7.5 | 171 | 0.95 | 6. | 12.27 |
| 30 | 336 | 2.8 | 0.84 | 0.125 | 10.5 | 243 | 0.72 | 7.6 | 47.44 |
| 31 | 149 | 2.4 | 0.90 | 0.2 | 10.0 | 216 | 1.45 | 8.0 | 33.16 |
| 32 | 222 | 2.4 | 0.75 | 0.2 | 16.0 | 288 | 1.30 | 8.0 | 68.67 |

Shading denotes country: [INSERT Figure 001][INSERT Figure 002][INSERT Figure 003] = Scotland, = Netherlands, = Ireland.

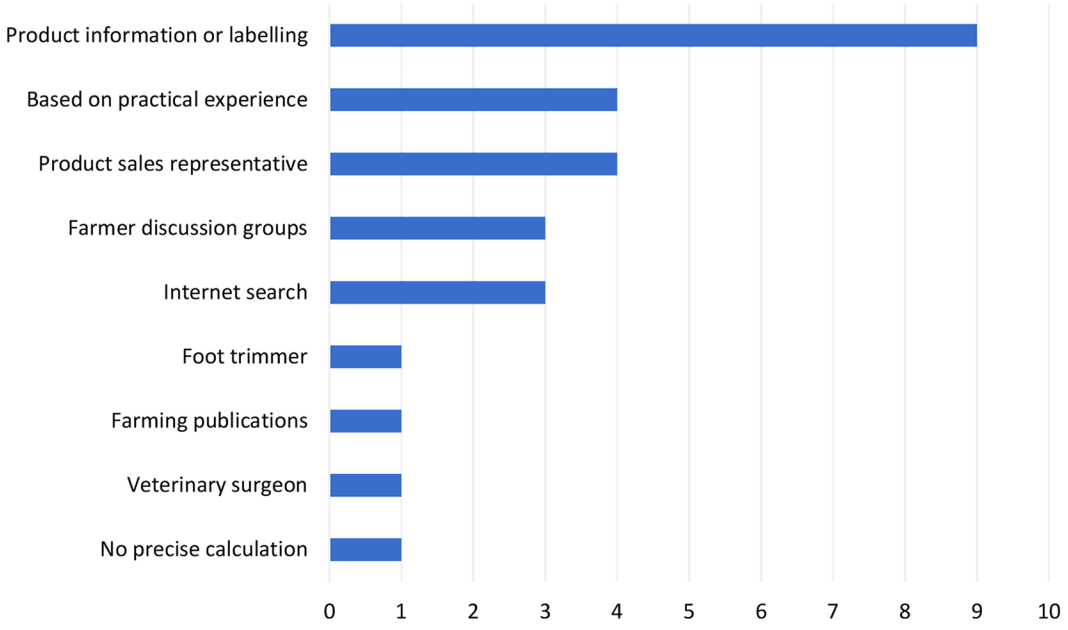


**Figure 1.** Bar chart with sources of information farmers used to decide on footbath concentration.

0.001), with the regression coefficient indicating a loss of 1cm for every 100 cow passages. This relationship is plotted in Figure 2, visualizing the distribution of study data in relation to the adequate and optimal industry recommendations for footbath solution depth of 7 and 12cm.

Solution depth was also related to footbath length ($P < 0.001$), therefore both footbath length and number of cow passages were included as fixed effects in a final multivariable mixed model with farm as a random effect. This demonstrated that farm identity was responsible for 76% of variation in footbath depth, but the association with number of cow passages remained ($P < 0.001$) with the coefficient suggesting 1.2cm loss in solution depth per 100 cow passages (Table 3). The model had a marginal $R^2$ value of 0.25, a conditional $R^2$ value of 0.83, and a mean absolute error of 0.67. After accounting for the number of cows passing through the footbath and the random effect of farm identity, the association with footbath length disappeared.

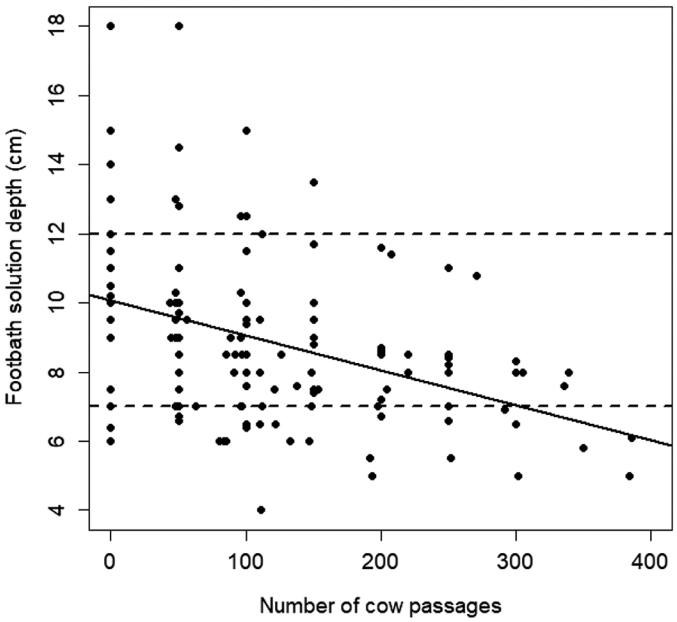


**Figure 2.** Change in footbath solution depth with number of cow passages from serial footbath samples on 32 dairy cattle farms. The solid line is the linear regression line visualizing the relationship between number of cow passages and footbath solution depth, regression equation $y = -0.010x+10.1$, $R^2 = 0.16$, $P < 0.0001$. The dashed lines represent the adequate and optimal industry recommendations of 7 and 12cm, respectively, for footbath solution depth.

## Footbath Hygiene

Organic matter content exceeded 20g/L during or at the end of footbath use on almost half of the farms (15/32, 47%). Median OMC at the end of footbathing was 20.4g/L (IQR 11.9–34.8), suggesting the biocide could have become ineffective for some of the cows passing through the solution. Univariable linear regression with the outcome OMC outlined in our data that 0.6 cow passages per liter of initial footbath solution kept OMC below the 20g/L threshold (Figure 3).

The outcome variable OMC was right skewed and therefore was log-transformed to produce log OMC before final modeling. Housing, frequency of footbathing, and footbath length were not associated with log OMC. Number of cow passages per liter of original footbath solution was associated with log OMC ($P < 0.001$). Subsequently this parameter was offered to a multivariable mixed linear regression model, which included the random effect of farm, with the outcome log OMC (Table 4). The model prediction plotted in Figure 4 identified that once the large variance attributable to the random effect of farm is accounted for, one cow passage per liter keeps OMC < 20g/L. The model had a marginal $R^2$ value of 0.25, a conditional $R^2$ value of 0.74, and mean absolute error of 0.14. After exponentiation, the mean absolute error between measured and predicted values for OMC was 1.37g/L.

Combining the 2 criteria of adequate solution depth of 7cm and OMC < 20g/L at the end of footbathing, 11/32 (34.4%) farms were compliant with recommended foot disinfection practices.

## Footbath Samples Pathogen Load

All samples were negative in the species-specific *Treponema* spp. qPCRs, while one duplicate from 2 of the 67 footbath samples were positive in the general *Trepo-*

**Table 3.** Results from the multivariable mixed effects model for footbath solution depth from serial footbath samples on 32 dairy cattle farms

| Fixed effects | Estimate | Standard error | T value | P value |
|---|---|---|---|---|
| (Intercept) | 11.4 | 1.6 | 7.2 | <0.0001 |
| Number of cow passages | −0.012 | 0.001 | −11.7 | <0.0001 |
| Footbath length | −0.48 | 0.59 | −0.82 | 0.421 |
| Random effects | Variance | Standard deviation | | |
| Farm identity | 3.5 | 1.9 | Farm variance as % of total variance = (3.5/4.6)*100 | |
| Residual | 1.1 | 1.0 | = 76% | |

Marginal $R^2 = 0.25$, Conditional $R^2 = 0.83$, Mean absolute error = 0.67.

**Table 4.** Results from the multivariable mixed effects model for organic matter content (OMC), using log OMC as the outcome variable from serial footbath samples on 32 dairy cattle farms

| Fixed effects | Estimate | Standard error | T value | P value |
|---|---|---|---|---|
| (Intercept) | 0.8 | 0.06 | 13.1 | <0.0001 |
| Number of cow passages per liter of footbath solution | 0.5 | 0.05 | 9.2 | <0.0001 |
| Random effects | Variance | Standard deviation | | |
| Farm identity | 0.077 | 0.28 | Farm variance as % of total variance = (0.077/0.12)*100 = 64% | |
| Residual | 0.043 | 0.21 | | |

Marginal $R^2$ = 0.25, Conditional $R^2$ = 0.74, Mean absolute error = 0.14.

*nema* spp. qPCR (Supplementary Table 2) (10.6084/m9.figshare.24829833). The negative qPCR results could be caused by non-detectable levels or absence of *Treponema* spp. in footbaths, or by freezing or formalin treatment resulting in killing of bacteria and protein-DNA cross linking and/or DNA degradation (Weiss et al., 2011). Testing of swabs from BDD lesions and feces from affected cows showed that freezing had little effect on the qPCR Ct values, whereas formalin treatment resulted in an increase of Ct values of approximately 10 (Supplementary Figure 1) (10.6084/m9.figshare.24829833). This equates to a 1000-fold decrease in amplifiable DNA, and after formalin treatment only one of the BDD lesion swabs was positive for one duplicate in the *T. phagedenis* qPCRs. Bacteriological cultures of the 36 FAPU footbath samples on sheep blood agar were all negative. Together these data indicate that the footbath samples did not contain viable BDD-associated *Treponema* spp..

## DISCUSSION

This study reports varied footbathing practices from 32 dairy farms where BDD is endemic. At the start of footbathing, only one farm met industry recommendations regarding optimal footbath dimensions and solution

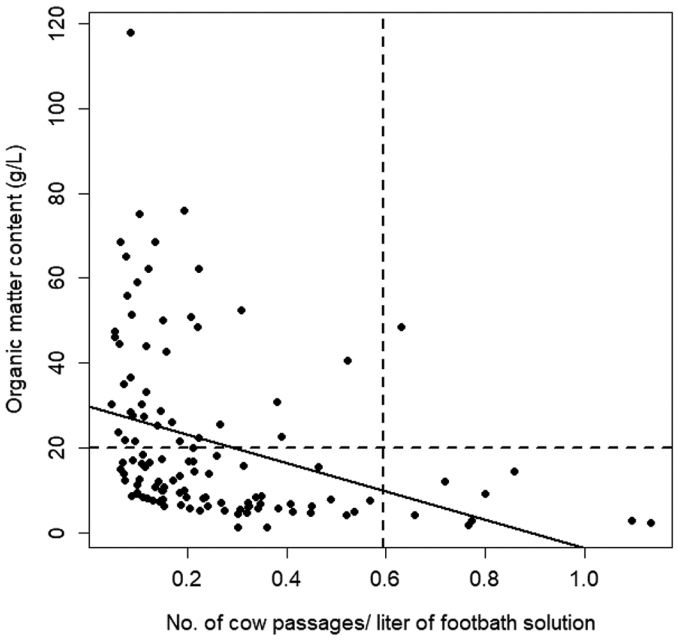


**Figure 3.** Number of cow passages per liter of footbath solution plotted against organic matter content (g/L) from serial footbath samples on 32 dairy cattle farms. The solid line is the linear regression line visualizing the relationship between number of cow passages per liter of footbath solution and the level of footbath contamination [OMC (g/L)]. Regression equation y = 19.2x+8.6, $R^2$ = 0.17, $P$ < 0.0001. The dashed lines represent the intersection where the regression equation describes the number of cow passages per liter of footbath solution which for our data maintains footbath contamination below 20g/L OMC.

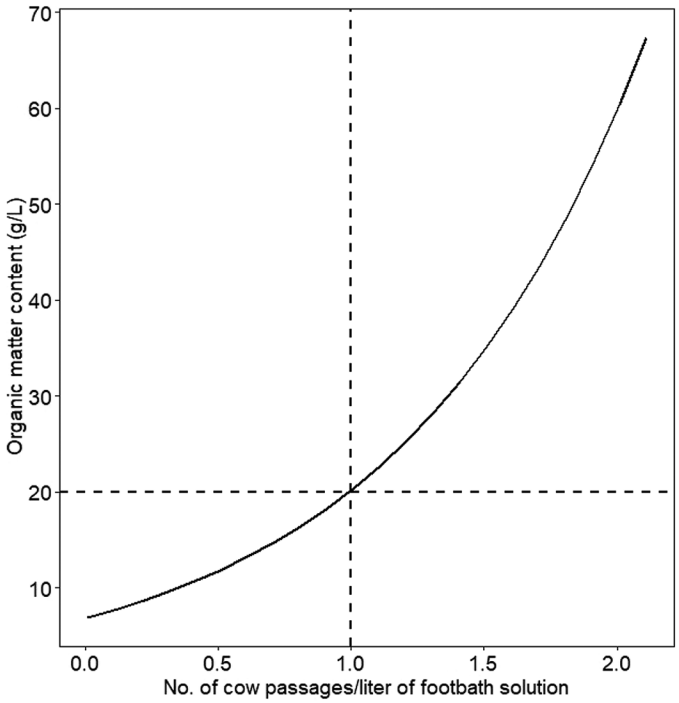


**Figure 4.** Mixed effects model prediction for the relationship between number of cow passages per liter of footbath solution and organic matter content (OMC) from serial footbath samples on 32 dairy cattle farms. The model outcome OMC is shown in g/L rather than log values for presentation purposes.

depth of 12cm. At the end of footbathing sessions, only 11/32 (34.4%) footbaths met the criteria regarding adequate solution depth of 7cm and adequate hygiene of < 20g/L OMC, indicating good foot disinfection practices. Multivariable mixed models for both criteria identified large variance attributable to farm-level factors not accounted for in our data. In addition, use of copper sulfate was common, which is not approved for veterinary hygiene purposes according to current EU legislation (Bell & Vanhoudt, 2020). Together with the finding that farmers rarely consult veterinary surgeons or foot trimmers regarding footbath concentration, our data indicates there is scope for veterinary surgeons and hoof health professionals to engage clients in footbathing audits to improve footbathing practices. Despite footbath samples frequently exceeding the 20g/L OMC biocide testing threshold, we obtained no evidence of footbaths acting as infection reservoirs for pathogens via aerobic culture or qPCR for BDD pathogenic *Treponema* spp..

### Questionnaire Answers

It is likely that there is selection bias in the study population as a result of a non-random sampling strategy and convenience sampling of clients using 3 university-associated clinical services. Our data, however, is consistent with previously published studies (Cook et al., 2012; Holzhauer et al., 2004; Solano et al., 2015), with the exception of an increase in median footbath length from 2.03m to 2.78m, which could be a response to advice published in 2012 suggesting longer footbaths were beneficial for increasing the number of foot immersions (Cook et al., 2012).

### Footbathing Solution Depth

Several footbaths (9/32, 28%) in our study did not meet a minimum solution depth of 7cm by the end of footbathing, risking inadequate contact with the BDD lesion predilection site. Our data suggests 1.2cm of depth is lost per 100 cows using the footbath, however there were marked differences in the volume of solution lost among the farms. Our observations suggest this is caused by differences in how quickly the cows walk though footbaths and how much competition there is between cows, although we were unable to quantify these differences. In 2 instances the depth of solution increased. This could be due to volume replaced by feces, urine, dirt, or a combination of these (Holzhauer et al., 2004). The influence of unmeasured farm factors on solution depth results emphasizes the need for individual farm footbath audits.

### Footbath Hygiene

We hypothesized that variation in footbath contamination levels (OMC g/L) between farms could be caused by differences in foot cleanliness before footbathing, and differences in the volume of feces voided by cows into the footbath. We did not record foot cleanliness, however since access to pasture is associated with improved foot cleanliness (Ellis et al., 2007; Nielsen et al., 2011), we assessed whether this had an effect on footbath OMC. It is often advised that increased footbathing frequency improves foot cleanliness (Cook, 2006), therefore we assessed the effect of footbathing frequency on footbath OMC. Neither of these parameters affected footbath OMC in our study, which lends support to the premise that footbath contamination is mainly through defecation, not through contamination of the feet (Ariza et al., 2019). We acknowledge, however, that the hygiene status of herds in this study may differ from the wider population due to our non-random sampling strategy.

Footbaths on 15/32 (47%) of the farms in our data exceeded 20g/L OMC by the end of footbathing. We used a simple method to measure OMC (Manning et al., 2017), which may tend to overestimate OMC as the mineral content of the sample is still present. The 'weight loss on ignition' method, which was used in a previous study of footbathing hygiene, may have been more accurate (Ariza et al., 2019).

The wide variation in OMC among individual farms and the prediction from the final model together highlight the importance of the farm effect, emphasizing the need for individual farm footbathing audits and advice.

### Treponema qPCR

Although BDD prevalences were not measured, samples from 5 farms with high bulk tank milk titers against *Treponema* spp. were included, therefore failure to demonstrate treponeme contamination of footbath samples was unexpected. The association between cow passages and contamination with *Treponema* spp. may be different from the overall dairy cattle population if BDD prevalence was significantly different for our study farms.

It is possible that *Treponema* spp. shed from infected cows were diluted in the footbath solution so that the presence of DNA was below the limit of detection of the qPCR, which is 32.5, 10.3 and 26.6 genome copies per µL for *T. medium, T. phagedenis,* and *T. pedis* respectively (Staton et al., 2021). Lack of bacterial growth on sheep blood agar plates indicates that biocides are likely to remain effective against *Treponema* spp., even at high OMC. In addition, testing the effects of formalin treatment and freezing of samples on detection of treponemal DNA by the qPCR showed that formalin degrades DNA

and decreased the sensitivity of the qPCR 1000-fold. This could explain the negative qPCR results, and also illustrates the effective disinfection by formalin.

## CONCLUSION

Footbathing solution depths frequently fell below 7cm, risking poor biocide contact with the highest BDD risk heel bulb region. Organic matter content in footbaths frequently exceeded biocide testing requirements, which raises concern regarding disinfectant efficacy, however, no BDD-pathogenic treponemes were detected using qPCR, and no aerobic bacteria could be cultured from a subset of footbath samples. Our data suggest that footbaths are not likely to be an infection reservoir for BDD-causative treponemes.

Footbathing practices vary among dairy farms, with only one third of the farms surveyed providing footbaths complying with recommended OMC and solution depth. Farm-level factors were responsible for 64% of variation in contamination levels and 71% of variation in solution depth. Farmers in this study were not typically using veterinary surgeons or hoof trimmers as a source of advice regarding footbathing; there is an opportunity for veterinary surgeons and hoof health professionals to audit footbaths in-person during use as part of lameness control planning.

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