# The impact of glutaraldehyde based footbaths on *Dichelobacter nodosus* prevalence and the antimicrobial resistant community of the ovine interdigital skin

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**Abstract**

Ovine footrot, is a highly contagious polymicrobial bacterial infection, primarily caused by *Dichelobacter nodosus.* Preventative bactericidal footbaths are commonly used in the sheep industry to reduce the spread of bacteria. However, their effect on the bacterial community is poorly understood. This is the first study to investigate the impact of ProGiene dairy hygiene footbath on the bacterial community of the ovine interdigital skin following a common UK footbathing routine. Swab samples were analysed by qPCR to determine prevalence and load of *D. nodosus* and numerated on MacConkey agar in the presence or absence of tetracycline and ampicillin to determine phenotypic antimicrobial resistance. Metagenomics were used to determine the impact of a single footbath on the bacterial community and genotypic antimicrobial resistance. The results suggest ProGiene dairy hygiene is ineffective at reducing the load of *D. nodosus* when applied as a one off or weekly footbath, however sheep may act as a reservoir for multi-drug resistant bacteria creating opportunities to spread antimicrobial resistance to other sheep and their environment.

**Key words:** Polymicrobial; *Dichelobacter nodosus;* Glutaraldehyde; Antimicrobial Resistance

## 1. Introduction

Ovine footrot is a highly contagious, polymicrobial bacterial infection of the interdigital skin, primarily caused by the Gram-negative bacterium *Dichelobacter nodosus* (*D. nodosus*) (Egerton et al., 1969). Current best practice for the treatment of footrot is to catch and treat individuals with systemic antibiotics within three days (Kaler et al., 2010). This, along with changes to husbandry practices, such as no longer routinely hoof trimming, has reduced the point prevalence of lameness from 10.6% in 2004 to 3.2% in 2019 (Best et al., 2020). It is therefore unsurprising that a recent study of 24 commercial flocks found lameness accounted for >65% of their total antibiotic use, with tetracyclines and penicillins being the most regularly used antibiotic classes (Davies et al., 2017). The Responsible Use of Medicines in Agriculture Alliance (RUMA) identified lameness as a key area for targeted and coordinated responsible antibiotic use within the sheep industry(RUMA., 2019). Therefore, it is important to explore preventative alternatives such a footbathing for the prevention on infection.

In the UK, footbaths are an integral part of infectious lameness control, with >58% of sheep farmers using them in 2015 (Prosser et al., 2019). Despite their widespread use, there is very little evidence of footbathing efficacy. Additionally, the two most common active ingredients formaldehyde and zinc sulphate (Winter et al., 2015), are associated with negative side-effects. Repeated exposure to formaldehyde can lead to non-infectious lameness caused by granulomas or shelly-hoof (Reeves et al., 2019) and zinc sulphate is a heavy metal and environmental contaminate. A recent study found 6% Desintec (0.36% glutaraldehyde) to be as effective as 4% formaldehyde in reducing the load of *D. nodosus*, when tested under in-vitro conditions (Hidber et al., 2020). This is the first study to investigate the impact of single or repeated application of 2% ProGiene dairy hygiene on the composition of the microbial community, the load of *D. nodosus* and the antimicrobial resistance carried by bacteria on the ovine interdigital skin.

## 2. Materials & Methods

### 2.1. Footbath regimes & sample collection

The impact of 2% ProGiene dairy hygiene, Digicur Footbath, (glutaraldehyde <0.40% and chlorhexidine digluconate <0.02% ) was investigated using single or repeated footbathing application.

**Single Footbath study**: The impact of a single footbath protocol was investigated twice using the University of Nottingham's outdoor flock. Six ewes were randomly selected during summer 2017 and autumn 2017 where each hoof was swabbed at nine time points over 29 days (one pre-footbath and eight post) and 34 days respectively. In both cases, sheep were walked through a 2.3m footbath, resulting in hoof submersion three times. The sheep were returned to the same pasture after each swab, and at time point 0 (immediately post-footbath) sheep were stood on a hard clean surface for a minimum of 15 minutes before swabs were taken. **Weekly footbath study**: Twelve sheep, were selected from the University of Nottingham's flock, housed indoors on straw over the winter 2017/2018. Each sheep interdigital skin was swabbed at baseline and assigned to a group (control & footbath) using stratified randomisation, dependent on age and foot score. The foot scoring system was adapted from Maboni et al (2016) (Table S1). The footbath group was footbathed weekly for six weeks. 24 hours after footbathing all sheep were swabbed. After each footbath, sheep were stood on a hard clean surface for a minimum of 15 minutes before being returned to their pen. All twelve sheep were housed together.

### 2.2. Sample processing and bacterial culture and exclusions

Swab samples (E-swabs 480CE, Appleton Woods Ltd) taken from the ovine interdigital space were agitated at 800rmp for 10 min. 100µl were removed from each of the four individual hoof samples and pooled. 10-fold serial dilutions were plated on MacConkey agar (Oxoid, CM007) in the presence or absence of antibiotics (tetracycline 32µg/ml, (PanReac AppliChem), ampicillin 64µg/ml, (Fisher BioReagents)). For the tetracycline analysis, one sheep from each of the single footbath studies was excluded (N=5 summer & autumn). For the ampicillin analysis, one sheep was excluded from the

single footbath autumn study only (N=6 summer & N=5 autumn). In all cases this was due to a processing error, resulting in the loss of the samples. For the repeated footbath study, two sheep, who became lame during the study, were treated with systemic antibiotics, and excluded from both *D. nodosus* and antibiotic resistance analysis. Finally, the samples for time point week two were not processed for the antibiotic resistance proportions only, as the samples were compromised during storage in the freezer and consequently could not be used.

### 2.4. DNA isolation and quantitative PCR assays

DNA was isolated using the QIAamp Cador Pathogen Mini Kit (Qiagen). DNA was quantified using the Qubit 3.0 fluorometer (Invitrogen). Bacterial load was quantified by qPCR using primers and TaqMan probe, targeting the 16S rRNA gene for *D. nodosus* and eubacteria (Maboni et al., 2016), using the PCR Lightcycler 480 (Roche Applied Science, Penzberg, Germany).

### 2.5 Metagenomics

DNA was sent to The Leeds NGS Facility and sequenced at 2x150bp on an Illumina HiSeq. Raw reads were assessed for quality and adaptors using Skewer (Jiang et al., 2014). The high-quality filtered reads were parsed through KrakenUniq (Breitwieser et al., 2018) for taxonomic assignment. The assignments were analysed using BinTaxaAssigner (https://github.com/shekas3/BinTaxaAssigner). Antimicrobial resistances were determined using DeepARG (Arango-Argoty et al., 2018). Diversity and richness were calculated using vegan (Oksanen et al., 2018) in R (Ihaka and Gentleman, 1996). Differences were calculated using Mann-Whitney U tests in Prism 8.01 (Graphpad Software Inc. USA). Correlation of the microbiota with external factors, was determined on trimmed mean of M-values (TMM) using the R package EdgeR (Robinson et al., 2009) only using genera detected in at least two samples. Bacterial genera were distinguished using Burata (Kursa and Rudnicki, 2010). Heatmap clustering was performed with "ward. D2" based on Euclidean distances. Differentially abundant ARGs calculation was completed

on 16S normalised relative abundance, (Segata et al., 2011) with the following settings: α value for factorial Kruskal-Wallis test among classes = 0.05; logarithmic LDA score (log 10) threshold = 0.2.

### 2.6 Other statistical analysis

One-way analysis of variance followed by Dunn's multiple comparisons test was applied for percentage of antimicrobial resistance under single footbath application. *D. nodosus* proportion and load over time were analysed using one-way ANOVA with mixed effect model. Comparison between control and footbath groups were analysed using Pearson or Spearman correlation dependent on normality of data distribution. GraphPad Prism Version 8.1.2 for Windows (Graphpad Software, La Jolla California USA).

## 2. Results

### 3.1. Single footbath with ProGiene dairy hygiene transiently increased the proportion of *D. nodosus* but reduced the diversity of the bacterial community on the ovine interdigital skin.

Low levels of *D. nodosus* were detected on healthy skin by qPCR throughout the study. A single application of ProGiene dairy hygiene led to a transient 10-fold (p=0.101) decrease of the mean total bacterial load immediately post-footbath (Day 0) (Fig.S1). This was accompanied by a transient increase in the mean load of *D. nodosus* (p=0.003) on day 1 post-footbath, resulting in a transient 10-fold increase (p=0.03) in the proportion (%) *of D. nodosus* from 0.003% ±0.001 before to 0.041% ±0.046 after footbath on day 0. The proportion of *D. nodosus* returned to similar levels compared to pre-footbath on day 7 at 0.002 % ±0.002 (Fig 1a-b).

Using metagenomics, a significant increase was observed by the Shannon diversity index (p=0.05) between day -1 (Minus one) and immediately post-footbath (day zero), and between day -1 and day 7. Similarly, the inverse Simpsons index showed a significant increase (p=0.05) between day -1 and directly after the footbath (day 0, FigS5). This is reflected in the taxonomic assignment of these data with an increase in diversity prior to the footbath, which declines over the remaining time points. For

two time points (-4 and -1 day) of the three pre-footbath samples, *Ralstonia pickettii* was the highest abundant bacterial species (54% and 67%) of the top 30 most abundant bacteria. The next most abundant bacteria prior to the footbath were *Escherichia coli* (15% and 4%), *Acholeplasma liadlawii* (3.7% and 0.4%), *Acinetobacter baumannii* (3.1% and 2.2%), *Variovorax paradoxus* (2.9% and 3.3%), *Arthrobacter arilaitensis* (2.9% and 3.2%) and Flavobacteriaceae 3519-10 (2.3% and 1.1%), with the remaining top 30 most abundant bacteria account for less than 1% each. Surprisingly, a shift in the bacterial community was observed immediately before the footbath (day -0.25), with the highest abundant bacterial species changed to *Escherichia coli* (25%), *Streptococcus uberis* (9.6%), *Arthrobacter arilaitensis* (9.2%), Flavobacteriaceae 3519-10 (6.9%), *Acinetobacter baumannii* (6.4%), *Solibacillus silvestris* (6.1%), *Methylobacterium radiotolerans* (5.6%), *Mannheimia haemolytica* (5.4%), with the remaining top 30 most abundant bacteria account for less than 5% each. Immediately after footbath treatment (day 0) the overall number of different bacterial species identified from the metagenomics reduced. The most abundant bacteria were *Methylobacterium radiotolerans* (43%), followed by Flavobacteriaceae 3519-10 (9.4%), *Escherichia coli* (7.9%), *Propionibacterium acnes* (5.8%) (Fig S5).

During the following three time points the diversity gradually increased. At the final time point (day 7) the most abundant bacteria were *Escherichia coli* (14%), Flavobacteriaceae 3519-10 (13%), *Acinetobacter baumannii* (11%), *Methylobacterium radiotolerans* (9.6%), *Arthrobacter arilaitensis* (7.6%), *Acholeplasma liadlawii* (6.3%), *Clostridium sticklandii* (5.2%), and *Porphyromonas asaccharolytica* (FigS5).

### 3.2. Correlation of microbiota with external factors

Investigation of the bacterial community structure change and opposing factors, revealed limited change at phylum level and genus level. Most genera did cluster into pre-footbath and post-footbath clades, apart from day seven, which may suggest the overall community was returning to a pre-footbath state. Identifying genera that vary the most by a) time point b) pre vs post treatment and c)

sheep ID, some more patterns emerged between time point and community structure at genus level. A selection of *Proteobacteria*, *Firmicutes* and *Bacteriodetes* that were abundant prior to footbath exposure significantly decreased immediately after treatment, and a portion did not recover (mostly *Proteobacteria*). In addition, a sub cluster that consisted of Proteobacteria and Firmicutes started to recover by day seven. Another cluster dominated by *Actinobacter* appeared to be negatively affected immediately after footbath exposure, temporarily benefited from a gain in abundance as it recovered. No other correlations were identified with individual sheep ID or time point (Fig S3).

### 3.3. Study 2: Repeated footbathing with ProGiene dairy hygiene has no significant impact on the load or proportion of *D. nodosus* on the ovine interdigital skin after six weeks.

Weekly exposure to ProGiene dairy hygiene footbathing led to a marginal reduction in *D. nodosus* load from 5.349 fg/µl ±3.369 prior to footbathing to 4.026 fg/µl ±4.021 after six weekly footbaths (Spearman correlation r=-0.64, p=0.14). In contrast, *D. nodosus* load on sheep not footbathed but housed in the same environment, marginally increased from 9.196 fg/µl ± 9.161 week 0 to 15.85 fg/µl ± 15.83 (r= 0.14, p=0.78) week 6. However, due to the high variability in the samples no statistically significant differences were observed (Fig 2c-d). The proportion (%) of *D. nodosus* in total bacterial DNA marginally reduced in both the footbathed (Pearson correlation r=-0.57, $R^2$= 0.32, p=0.18) and the not footbathed sheep (r=-0.066., $R^2$=0.004, p=0.89) from time point 0 to week 6.

### 3.4. Single footbath with ProGiene dairy hygiene transiently increased the mean proportion of aerobic antimicrobial resistant bacteria on the ovine interdigital skin.

For the summer cohort, at day -0.25 all six sheep carried a proportion of ampicillin resistant anaerobic bacteria (mean 0.12 % ±0.03) and the majority (4/5) carried tetracycline resistance (0.02 % ±0.01). The application of footbath had a transient effect on the proportion of tetracycline resistant bacteria; significantly increasing from (0.03 % ±0.03) at day 0 (immediately post-footbath) to (0.22 % ±0.05) day 3 post-footbath (P<0.01), with the proportion of resistant bacteria returning to similar pre-footbath levels day 7 post-footbath (0.04 % ±0.01) (Fig. 2A). The ampicillin resistant proportions

were also affected, significantly decreasing from (0.12% ±0.03) day -0.25 to (0.01 % ±0.001) day 3 post-footbath ($P<0.05$) (Fig. 2b).

The autumn cohort also showed a proportion of tetracycline and ampicillin resistance bacteria (mean 0.06 % ±0.02 & 5.20 % ±1.39), at the beginning of sampling (Fig. 2c&d). The mean proportion (%) of tetracycline resistant bacteria increased 5-fold post-footbath, from 0.6 ±0.6 immediately before to 3.0 % ±3.0 immediately after footbath. This increase, however, was not significant due to large variability between individual sheep, with one showing as much as 15% of the population to be tetracycline resistant, while the remaining sheep carried no tetracycline resistant bacteria at day 0 (Fig 2c). In contrast, the proportion of aerobic ampicillin resistant bacteria was more consistent between sheep. However, this was disturbed after the footbath (day 0), with two individuals carrying no resistant bacteria on their skin, two maintaining consistent levels and one sheep increasing the proportion of resistant bacteria to >15%. The mean proportion of ampicillin resistant bacteria transiently increased approximately 7-fold (from 0.46% ±0.06 to 3.17% ±3.05) as the total aerobic counts decreased (Fig 2d, Fig S4). Ampicillin resistant proportion of the population returned to similar pre-footbath levels at day 3 post-footbath ($P= 0.02$) (Fig. 2d).

Genotypically the most abundant antimicrobial gene classes found in the pre-footbath metagenomics data were encoding multi-drug resistance (MDR) (47%), MLS (macrolide, lincosamide and streptogramin, 12%) and tetracycline resistance (10%) (Fig S6). After the footbath treatment, the most abundant resistance genes were the same, multi-drug (51%), MLS (11%) and tetracycline resistance (9%). Overall, the resistance gene classes remained consistent across all sample time points (Fig S6). There were 12 ARGs that were part of three antimicrobial classes (Tetracycline resistance, peptide target alteration, efflux pumps and ATP-binding cassettes) which were significantly changed following footbaths and increased in abundance post-footbath (*bcaA efrB, lmrD, lsaA, pmrF, tet32* and *vgaB)* whilst *acrB, emrB, mexC, rosA, tetA* decreased post-footbath (Table S2).

### 3.5. Repeated footbathing with ProGiene dairy hygiene has no significant impact on the proportion of tetracycline resistant bacteria on the ovine interdigital skin after six weeks.

The proportion of tetracycline resistant bacteria varied between sheep for both, control, and footbath groups, prior to footbathing (week 0) ranging from 0 to 1.94 % and 0 to 1.02 %, respectively (Fig. 3). The proportion (%) of tetracycline resistant bacteria increased significantly over the seven-week for the footbathed group (Pearson correlation r=0.84, $R^2$= 0.70, p=0.04) but only marginally for the non-footbathed control (r=0.67, $R^2$= 0.45, p=0.15). There was no significant difference between the groups. In contrast, there were only two ampicillin resistant bacterial colonies isolated (carried by one sheep from each group), both cultured at week 4.

## 3. Discussion

The aim of this study was focused on the use of footbaths as a preventative management tool rather than a treatment. The data in this study suggest footbathing either once or weekly has no negative impact on *D. nodosus* load, consistent with a recent intervention study that found routine footbathing, irrespective of the chemical used, was neither detrimental nor beneficial for the control of footrot (Witt and Green, 2018). Footbathing had a transient impact on the interdigital microbiome. Proteobacteria, Firmicutes and Bacteriodetes were the most abundant phyla found on healthy skin samples, which is consistent with other studies (Maboni et al., 2016; Shaw et al., 2019 ).

Winter housing is common practice in the UK as it prevents damage to pasture and allows farmers closer control over the condition of their ewes when approaching lambing. However, this is a challenging environment for disease control, as stocking rates are increased and damp, warm straw around communal areas are an ideal environment for bacterial growth (Clifton et al., 2019) and many studies report an increase in the prevalence of lameness at this time (Winter et al., 2015; Witt and Green, 2018). The results of this study suggest there is no significant impact of weekly footbathing on the proportion of *D. nodosus* carried on housed sheep feet over the six-weeks, with both groups (footbathed and non-footbathed) maintaining similar levels. However, there are limitations to this

study; these sheep were housed indoors in the same pen, and therefore in the same environment, aiding the re-colonisation of the general bacterial community as well as by *D. nodosus*. Although not practical on this farm, future studies should be designed with sheep housed in separate pens, and the frequency with which the footbaths are applied altered to understand the true effect ProGiene dairy hygiene has on *D. nodosus* during indoor housing.

Antimicrobial resistance is a natural process and an inevitable consequence of the use of antimicrobials. However, the misuse and overuse of antibiotics in humans and animals has accelerated the development of resistance. Only one peer reviewed quantitative assessment of antibiotic usage in sheep (Davies et al., 2017) supports the perception that the sheep farming industry uses fewer antibiotic classes and lower quantities than other livestock sectors, such as dairy cattle and poultry There has been greater emphasis on improved hygiene to reduce the reliance on antibiotics within the livestock industry (Lovatt et al., 2019); and the use of non-antibiotic antimicrobials is essential to control disease outbreaks. In this study, the most abundant antimicrobial gene classes encoded for MDR (47%), MLS (12%) and tetracycline resistance (10%). The use of a one-off application of ProGiene dairy hygiene had no impact on ARG abundance. There was also no evidence of aldehyde resistant genes in the metagenomic analysis, but there was an increase in the abundance of *A. baumannii,* and *E. coli* maintained a high abundance. Both bacterial species are associated with multi drug resistance, and *A. baumannii* was found to carry glutaraldehyde resistant genes (AdeABC efflux systems) in hospital isolates (Dijkshoorn et al., 2007). This suggests footbathing may increase the abundance of those species most associated with multiple antibiotic resistance and could aid the spread of these bacteria between sheep, as they gather and pass through the footbath and potentially into the environment, including watercourses. In this study, we identified that the bacterial communities found on the interdigital skin from healthy sheep harboured resistances to both, tetracyclines and penicillins, when on pasture, the two most used antibiotic classes in the UK sheep industry (Davies et al., 2017), and tetracycline resistance when housed indoors. These results mirror that of other UK studies, which found *E. coli* isolated from diseased sheep were resistant to as many

as four antibiotic classes, including tetracyclines and penicillins (Cheney et al., 2015). During six weeks of repeated footbathing, the levels of tetracycline resistant bacteria increased in both, the footbath and control group. The presence of tetracycline resistance was not surprising given the use of oxytetracycline for historic lameness and mastitis within the flock. Individuals within the main flock were last treated with tetracycline two months prior to the summer sampling, and four months before the autumn sampling. As a significant proportion of unmetabolized antibiotic is excreted through faeces and urine (Kumar et al., 2005) it is likely that all flock members were exposed to the antibiotics. Furthermore, our data provide further insight into antimicrobial resistant genes classes in the microbiota carried by the UK sheep population, with similar results to that of our preliminary and only other study investigating ARG's and bacterial populations from the ovine interdigital skin (Shaw et al., 2019).

## Conclusion

These data suggests ProGiene dairy hygiene is ineffective at reducing the load of *D. nodosus* when applied either singularly or repeatedly, once weekly. Furthermore, ProGiene dairy hygiene increases the abundance of species most associated with MDR, such as *E. coli* and *A. baumannii* and therefore creates opportunities for the spread of antimicrobial resistance to other sheep and to the environment.

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## Ethical statement

Ethical approval was obtained from the School of Veterinary Medicine and Science Ethics committee, University of Nottingham (ethical approval references: 1831 160808, 1961 170202).

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**Fig. 1. Impact of footbath on *Dichelobacter nodosus* load.** Load of *Dichelobacter nodosus* (a,c), Proportion (%) of *D. nodosus* in total eubacteria DNA (b, d) after single footbath (a&b) and repeated weekly footbath (c&d). a,c: Swab samples taken before and after 2% ProGiene dairy hygiene footbath (vertical dashed line) with time points -4, -1 days and -0.25 (immediately before footbath) are classed as pre-footbath. Time point 0 is directly after footbath and time points 1 - 29 are days post-footbath. Data were analysed by Dunnett's multiple comparison test. *P≤0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001. (#P≤0.05 compared to day 0). c, d: Swab samples taken at baseline and 24 hours post-footbath of 2% ProGiene dairy hygiene for both groups. Minimum to maximum box plot, mean depicted by central line. Data were analysed by Spearman correlation (c) and Pearson correlation (d).

**Fig. 2**. **Proportion of resistant bacteria on the ovine interdigital skin of healthy sheep.** Proportion (%) of tetracycline (a, *c*), and ampicillin (b, d) resistant aerobic bacteria in the summer (a, b) and autumn cohort (c, d); Swab samples taken before and after footbath of 2% ProGiene dairy hygiene. Footbath is indicated by vertical dashed line. Time points -4, -1 days and -0.25 (immediately before footbath) are classed as pre-footbath. Time point zero is directly after footbath and time points one, to twenty-nine are days post-footbath. Each coloured circle represents an individual sheep. Although a different flock of sheep were used for summer and autumn cohorts, the same colours represent sheep 1-6 in their respective flocks (1=blue, 2=red, 3=green, 4=pink, 5=purple, 6=orange). Sheep 2 from the summer tetracycline (a) and sheep 4 from autumn tetracycline and ampicillin (c, d) were removed due to sample processing error. Mean depicted by central black line. Y-axis at 0.001 depict negative results. Data were analysed by Dunn's multiple comparison test. *P≤0.05, ** P≤0.01 ** P≤0.001.

**Fig. 3. Proportion (%) of tetracycline resistant bacteria on the ovine interdigital skin.** Swab samples taken at baseline and 24 hours post-footbath of 2% ProGiene dairy hygiene for both non-footbath (control) and footbath groups. Minimum to maximum box plot, mean depicted by central

line. Arrow indicates application of footbath. Y-axis at 0.001 depict negative results. Data were analysed by Pearson correlation *P=0.04. Therefore, week 2 is illustrated with NA.