Development of an in vitro model to study digital dermatitis in cows

Abstract

Digital dermatitis (DD) is a common and significant condition in dairy cattle, characterized by painful skin lesions, reduced productivity, and substantial economic losses. Despite decades of research, the aetiology and pathogenesis of DD remain incompletely understood. This study aimed to develop an in vitro model of bovine foot skin to reduce reliance on live animal studies and facilitate research into DD pathogenesis and host-pathogen interactions. Therefore, three studies were conducted i.e. development of a bovine skin culture model, culturing of bacteria involved in the pathogenesis and development of an infection model.

Skin Culture

Protocols were established for the in vitro culture of bovine foot skin explants. Adult skin remained viable for up to 72 hours, while juvenile skin demonstrated extended viability up to 96 hours. Histological analysis revealed progressive degradation of skin structure over time, suggesting the need for improved culture conditions to extend explant longevity. The shorter viability of adult skin was attributed to its greater thickness and potential limitations in nutrient diffusion.

Bacterial Cultures

Efforts to isolate *Treponema spp.* and *Dichelobacter nodosus* from DD lesions studied confirmed their presence via PCR but unfortunately failed to produce isolated cultures. Mixed bacterial populations, suboptimal growth conditions, and insufficient sequential plating cycles likely contributed to these challenges. The difficulty in obtaining isolated colonies underscores the complexity of isolating fastidious anaerobes from polymicrobial environments.

Infection Model

The infection model involved exposing bovine foot skin explants to bacterial suspensions derived from DD lesions. Significant results were observed for incubation time, with both histological scores and *IL8* expression levels showing marked changes as time progressed. Longer incubation times were associated with increased degradation of skin integrity and heightened inflammatory responses. However, no significant differences were detected between control and infected groups for either histological scores or *IL8* expression. These findings suggest that observed changes of the histological score and the IL8 expression were primarily due to declining skin viability over time rather than infection-specific effects. Limitations such as small sample sizes, fungal contamination, and variability in inoculum composition likely influenced these outcomes.

Conclusion

This study has laid the groundwork for developing an in vitro model of DD, but achieving a fully functional and validated culture system requires the improvement of multiple factors contributing to the outcome of the model. Enhanced culture protocols, improved bacterial isolation methods, and better-controlled infection experiments are essential. Once established, such a model could significantly advance the understanding of DD pathogenesis, support the development of targeted therapies, and reduce the reliance on live animal research, contributing to both scientific progress and animal welfare.

Introduction

Digital dermatitis (DD) was first described by Cheli and Mortellaro in 1974 in Italy (Cheli, Mortellaro 1974). Worldwide, DD is a major economic problem in the dairy industry due to decreased milk production, increased reproductive intervals, increased culling rates, and increased costs associated with footbaths and other (veterinary related) treatments (Bruijnis, MRN, Hogeveen et al. 2010, Cha, Hertl et al. 2010). DD leads to severe lameness, which is a major welfare issue (Bruijnis, M. R. N., Beerda et al. 2012). Cha et al. estimated the total costs of an individual case of DD at around US $132.96 per cow (Cha, Hertl et al. 2010). Holzhauer described a prevalence of 21,2% in the population in the Netherlands and at 90,1% of the farms one or more cows were affected (Holzhauer, M., Hardenberg et al. 2006).

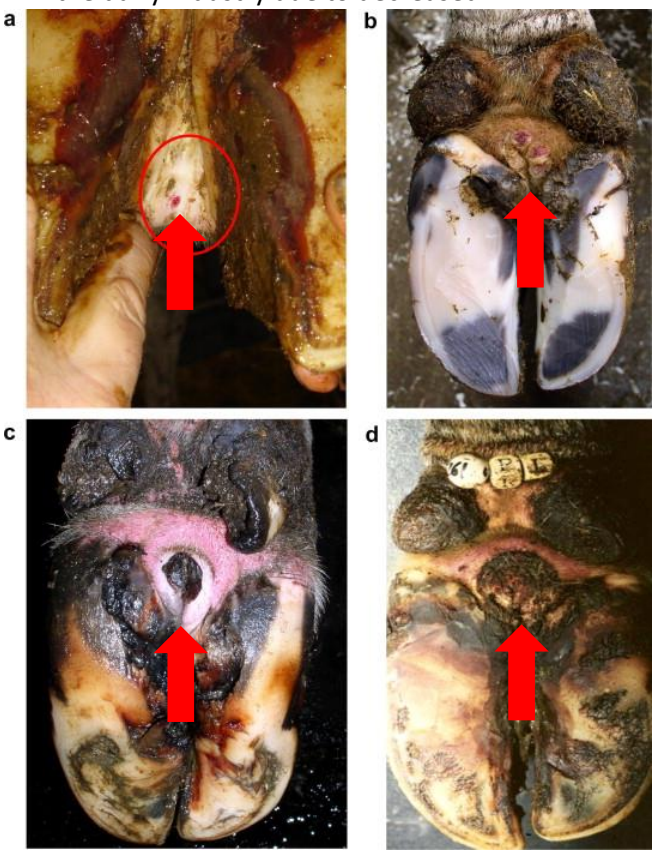

Figure 1 redrawn from Holzhauer et al. (2008) ***Clinical Presentation of Digital Dermatitis Stages***
(a) ***M1 (Early Stage):*** *Lesion within a red oval, typically 0–2 cm in diameter, and not painful on palpation.*
(b) ***M2 (Active Infection):*** *Classic ulcerative stage with a lesion diameter >2 cm, often painful on palpation.*
(c) ***M3 (Healing Stage):*** *Lesion covered by a scab following local therapy, generally not painful on palpation.*
(d) ***M4 (Chronic Stage):*** *Characterized by dyskeratosis or surface proliferation, typically not painful on palpation. (Holzhauer, Menno, Bartels et al. 2008).*

DD lesions occur at the plantar aspect of the foot, commonly at the hind feet, next to the interdigital cleft or the skin immediately above the coronary band at the heel bulbs (Holzhauer, Menno, Bartels et al. 2008). A lesion scoring system was proposed, whereas M0 has no skin lesions. M1 is an early stage of the DD lesions, presenting with an ulcer smaller than 2 cm. The classical ulcerated lesion, greater than 2 cm of diameter, near the coronary band is referred to as M2. The healing stage of M2 is referred to as M3, characterized by formation of scar tissue on top of the ulcer. M4 is a healed lesion with hyperkeratosis. The stage M4.1 is a M4 lesion

with a new active ulcer (Döpfer, Ter Huurne et al. 1997). Acute DD histological changes include suppurative inflammation of the epidermis characterized by infiltration of plasma cells and lymphocytes, with superficial necrosis and hyperkeratosis.

Currently, despite intensive research, the aetiology of the disease is not completely clear. Silver-stained sections of lesions showed infiltration of spirochetes within the stratum spinosum and reaching the stratum basale of the epidermis (Döpfer, Ter Huurne et al. 1997, Krull, Cooper et al. 2016). It has been suggested that DD is a polymicrobial disease and that *Treponema spp.* plays a key role in the lesion development. Phylogenetic analysis showed that three different phylogroups of *Treponema spp.* are associated with DD lesions; Group 1) *Treponema medium* and *Treponema vincentii*-like, group 2) *Treponema phagedenis*-like and group 3) *Treponema denticola* and *Treponema putidum*-like, respectively. Moreover, differences between the phylogroups and other *Treponema* species at the genotypic and phenotypic levels have been shown by the bacterial expression of messenger RNA coding for different enzymes (Evans, Brown et al. 2008).

Other bacteria that have been found in DD lesions are anaerobic bacteria of the genus *Peptostreptococcus, Peptococcus, Bacteroides, Campylobacter, Fusobacterium, Streptococcus* and *Clostridium. Guggenheimella spp*. and *Dichelobacter nodosus* were also isolated (Wilson-Welder, Alt et al. 2015). Rasmussen et al hypothesized that *Dichelobacter nodosus* has a specific role in damaging the skin using proteolytic enzymes, and creating a port of entrance for *Treponema spp.* These authors found *Dichelobacter nodosus* in 51% of the lesions sampled at Danish farms and in 100% of the lesions sampled at Norwegian farms (Rasmussen, Capion et al. 2012). Krull et al. showed *Dichelobacter nodosus* to be present specifically in the early stages of the DD infection(Krull, Shearer et al. 2014).

Recently, an in vivo model of DD has been proposed (Krull, Cooper et al. 2016) where DD lesions were successfully induced using isolated cultures of *Treponema spp., Dichelobacter nodosus, Bacterioides spp., P. levii* and *C. urealyticus*. The highest incidence of disease within the inoculated groups was observed after 7 days of incubation when a suspension of macerated lesions was inoculated (Krull, Cooper et al. 2016). This study used over 126 animals, raising a major animal welfare concern. Study subjects were treated until clinical signs disappeared. However, it is known that lesions may re-appear after treatment, raising a welfare and biosafety concern regarding the use of the proposed model for further studies on DD. In addition to welfare concerns, sampling in the early stages of infection is hindered by restricted access to the inoculation site. This limitation arises from experimental methodology in which the feet are wrapped with inoculum, thereby obstructing comprehensive investigations into the complete aetiology of DD.

In this study, we propose to develop an in vitro culture model for bovine foot skin to study the aetiology of DD gaining a full understanding of pathophysiology. At the moment of writing, no such in vitro culture protocol is known for bovine foot skin studies. In human medicine, authors have previously described a human skin explants in vitro model by using skin material derived from reconstructive surgery leftovers, they succeeded, culturing the skin explants up to four weeks (Yasuoka, Larregina et al. 2008, Steinstraesser, Rittig et al. 2009, Larregina, Watkins et al. 2001).

Objectives

1. To develop, optimize and validate a protocol for in vitro culture of bovine foot skin explants.
2. To optimize an isolation protocol for *Dichelobacter nodosus* and *Treponema spp.*
3. To develop an infection model of DD by exposing bovine skin explants to inoculum containing specific bacteria spp. causing DD.

Expected outcomes

1. We will develop a model for bovine foot skin culture in vitro. This culture system will contribute to reducing the number of research animals used in scientific experiments because the hypothesis can be tested first in a validated in vitro culture system.
2. After this study we will be able to produce isolated cultures of *Dichelobacter nodosus* and *Treponema spp.* using the isolation protocol. The cultures will be frozen in glycerol at -80˚C, enabling us to use them in future research.
3. We will have an in vitro model to study the host response after an in vitro infection. Finally, this model can be used to investigate different inoculums that consist of different pathogens which gained information will provide a better understanding of DD.

Material and methods

Experimental design

To create an in vitro infection model, a series of pilot experiments were conducted. The first 3 experiments performed in this study were to develop a culture system for bovine skin. After we were able to culture the skin, multiple experiments were conducted to set up the infection model. Finally, a working model was designed, and a trail was run to collect experimental data to investigate any significant differences between the tested groups.

During the pilot experiments, also attempts were performed to isolate *Dichelobacter nodosus* and *Treponema spp.* to subsequently utilize and study those bacteria in the developed infection model.

Skin culture

An initial protocol for bovine foot skin culture was based on the current literature (Yasuoka, Larregina et al. 2008, Steinstraesser, Rittig et al. 2009, Larregina, Watkins et al. 2001). Explants were collected from cows euthanized due to reasons unrelated to this study at the clinic of Farm Animal Health, FVM, UU. Samples were obtained from the hind feet, i.e. above the coronary band at the plantar side of the foot. Prior to sampling, target sites were cleaned with tap water, dried with paper towels, rinsed with 70% ethanol, and left to dry in the air. Samples were transported to the laboratory in refrigerated Phosphate-buffered saline (PBS, pH 7.4) for processing steps. Tissue processing comprises the use of forceps and scissors to remove the hypodermis, leaving explants with a thickness of approximately 0.5 cm. Next, the tissue samples were segmented into square pieces of 1 x 1 cm.

Petri dishes were loaded with 8mL of culture media (KBM Gold Bullet Kit, Lonza, Breda, Netherlands) supplemented with 0,15 mMol/l of $CaCl_2$ and a special Bullet kit containing BPE (bovine pituitary extract), hEGF, Insulin (recombinant human), Hydrocortisone, GA-1000 (gentamicin, amphotericin-B), Epinephrine and Transferrin. Blocks of approximately 2 cm x 2 cm of 1% technical agar No.3 (Oxoid microbiology products, Thermo Fisher Scientific, Bremen, Germany) was added to the petri dishes. Prepared skin explants were then placed on top of agar blocks and cultured at 37˚ Celsius on 95% air-5% CO2 gas mix. Explants were serially, every 24 hours, removed from the incubator and fixed in formalin. Fixed tissues were routinely processed and stained using H&E based on the protocols from Rutten et al. (Rutten, Bequet-Passelecq et al. 1990). Scoring of the sections was performed to evaluate the degree of epidermal health during the culture period. Scoring procedure was carried out by following the scoring system as shown in appendix I.

Infection model design

Prior to the harvesting of the skin samples, the plates used for the culturing were prepared. For this procedure, 6 wells culture plates (Thermo Fisher Scientific, Bremen, Germany) have been used to culture the explants separately. The wells were loaded with 1mL of culture media as described above (KBM Gold Bullet Kit, Lonza) and with blocks of approximately 1,5 cm x 1,5 cm of 1% technical agar No.3 (Oxoid microbiology products, Thermo Fisher Scientific, Bremen, Germany) and thereafter kept refrigerated until using. At a local slaughterhouse (Gosschalk, Epe, The Netherlands), skin samples were collected from cows with no macroscopical signs of DD using a sterile punch biopsy (1 cm diameter, Acuderm inc., Fort Lauderdale, FL). Before the sampling procedure, the feet have been cleaned using pressurized tap water and rinsed with 70% ethanol. Collected samples were transported to the laboratory for one hour in refrigerated Phosphate-buffered saline (PBS, pH 7.4) and after arrival at the lab placed in a biosafety cabinet. A polypropylene ring (5 mm in diameter by 5mm in height) was attached to the epidermal side of the tissue using innocuous tissue adhesive (Kwik-cast, World precision instruments Inc., Sarasota, Florida) to prevent inoculum leakage from the ring. The inoculation ring itself contains 100 µl of inoculum. The inoculum for the infection group consisted of the supernatant of macerated DD lesions suspended in OTEB; for the control group only OTEB was used as inoculum. The inoculation ring was closed by using a polypropylene cap with a rounded top of 7 mm in diameter which was glued to the top, attempting to create an anaerobic environment. After this procedure, the plates were cultured at 37˚ Celsius on 95% air 5% CO2 gas mix. This setup was tested in two trails which proved the concept worked. A schematic representation of the model is shown in figure 2 The results of these trails can be found in appendix II.

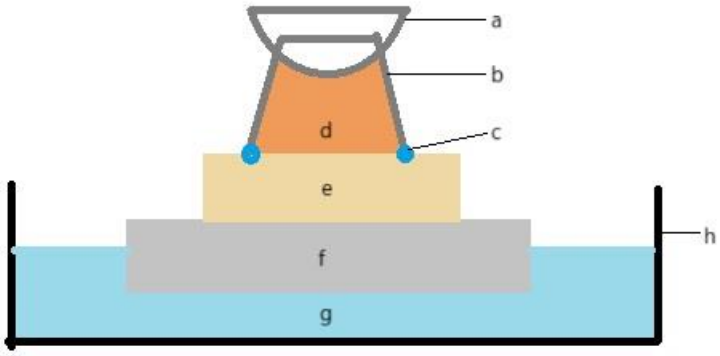

Figure 2 schematic representation of the infection model. Legend:
a. Ppolypropylene cap with a rounded top of 7 mm in diameter
b. A polypropylene ring 5 mm in diameter by 5mm in height
c. Innocuous tissue adhesive (Kwik-cast, World precision instruments Inc., Sarasota, Florida)
d. 100 µl of inoculum
e. skin sample 10 mm in diameter
f. 1,5 cm x 1,5 cm of 1% technical agar No.3 (Oxoid microbiology products, Thermo Fisher Scientific, Bremen, Germany)
g. 1 ml culture medium (KBM Gold Bullet Kit, Lonza, Breda, Netherlands) supplemented with 0,15 mMol/l of $CaCl_2$ and a special Bullet kit containing BPE (bovine pituitary extract), hEGF, Insulin (recombinant human), Hydrocortisone, GA-1000 (gentamicin, amphotericin-B), Epinephrine and Transferrin
h. walls of 1 well in the 6 well plate

Histological analysis

At 24-, 48- and 72-hours the samples were taken out and stored in 4% formalin for fixation. The fixated tissue was routinely processed, stained by H&E dye and scored according to the identified deviations in the normal alignment of the skin; the presence of the stratum corneum and level of degeneration in the stratum spinosum as well as the stratum granulosum was scored. The differences in the layers of the epidermis were scored based on the scoring system presented in appendix I. Also, the presence of bacteria on the skin was added to the scoring system. All scores have been analyzed using logistic regression and the fisher exact test.

Gene expression analysis

Next to the histological analysis the mRNA expression of skin samples analyzed for IL8 and GABDH, as an reference gene. Primers for IL8 and GAPDH were tested and the results can be found in appendix II. Therefore, samples were placed in RNA later at the same time points (24h, 48h and 72h). RNA extraction was done using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. After RNA extraction the total RNA content was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). An equalized amount of the total RNA was converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Nazareth, Belgium), following the manufacturer's instructions. Quantitative Real-time PCR was performed to detect the gene expression levels for Interleukin-8 (IL8) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the SYBR Green master Mix (Applied Biosystems, Ghent, Belgium)(Refaai, Ducatelle et al. 2013). Ct values were

subtracted to calculate the ΔCT. This value outcome was used in statistical analysis by using a linear regression model.

Trail setup

For the start of every trail, skin samples from 10 feet have been collected. Each individual foot provided 6 pieces of skin resulting in a total number of 60 samples which have been randomly distributed ingroups, a table is added in appendix III which shows how the randomization took place. The samples have been treated as stated above. The used inoculums consisted of PBS, DD lesion macerate and 1000ng/ml PAMP's (pathogen associated molecular patterns). Half of the samples were sanded prior to inoculation. At every time point (i.e. 24, 48 and 72h) samples were collected from every inoculum 6 of which 3 have been sanded and 3 not. After collecting the samples, each individual sample was cut in half at the inoculation site. One half of the sample was put in formalin and the other half in RNA later. A flowchart of the trail is added in appendix IV. The differences between groups were assessed by using both results derived from the histological scoring and the gene expression analysis as stated above. Statistical analysis was carried out using R studio.

Statistical analysis

Statistical analyses were conducted to evaluate differences in histological score and ΔCT values between the following experimental variables: condition (normal/sanded), incubation time (0h/24h/48h/72h), stimulus (experiment/negative control/positive control) and group (combination of all factors, for example 0h/n/exp). All statistical analyses were conducted using R studio version 4.4.1. A 95% confidence interval was used for statistical interpretation to provide additional context to the results by quantifying the precision and reliability of the estimates, and a significance threshold of $p < 0.05$ was applied.

Histological scores were analyzed using a logistic regression model to determine the relationship between the variables. Fisher's Exact Test was used to assess associations between categorical variables, histological score >2, while the logistic regression provided a framework for modeling the probability of specific outcomes.

For gene expression analysis, ΔCT values were calculated by subtracting the GABDH Ct value from the IL8 Ct value to determine de relative IL8 expression. Linear regression models were applied to evaluate the influence of experimental variables on the relative IL8 expression. All models included appropriate checks for assumptions, such as normality of residuals and homoscedasticity, using diagnostic plots.

Bacterial culture

Treponema spp.

Culture and isolation of *Treponema spp.* followed previously described guidelines (Clegg, Mansfield et al. 2015, Evans, Brown et al. 2008). DD lesions have been collected from cows euthanized for reasons other than this study. After cleaning of the affected feet by using pressurized water, selected M2 or M4.1 lesions were excised and placed in OTEB (oral treponeme enrichment broth, Anaerobe Systems, Morgan

Hill, CA, USA) supplemented with enrofloxacin (5µg/ml) and rifampin (5µg/ml). A piece of the biopsy was transferred to OTEB supplemented with the above cited antibiotics and 10% fetal calf serum and cultured at 37°C in an anaerobic jar (85% N2, 10% CO2, 5% H2 gas mix). The anaerobic environment was created using an Anoxomat (Mart Microbiology BV, Drachten, Netherlands). After 2-5 days, an aliquot from the broth was harvested using a sterile loop and then platted in fastidious anaerobe agar (FAA, Oxoid microbiology products, Thermo Fisher Scientific, Bremen, Germany) supplemented as described for OTEB. After 1-2 weeks, single colonies identified were harvested from FAA agar plates and inoculated into OTEB without antibiotics. Isolates have been typed using phase microscopy and a genus-specific PCR analysis. For this, DNA was extracted using InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA, USA), followed by sequencing of amplicons(Demirkan, Carter et al. 2001). Isolates of *Treponema spp.* were transferred to a 1:1 OTEB/glycerol mix for freezing at -80˚C.

Dichelobacter nodosus

Isolation of *Dichelobacter nodosus* has been attempted from feet with DD lesions. Only M2 and M4.1 lesions were selected and have been swabbed for this study. After sampling, swabs were placed in 15 ml tubes containing Eugon agar for transportation in a refrigerated container. In a biosafety hood environment, swabs were plated on 4% agarose Eugon agar in a capital E pattern penetrating the agar (BULLER, EAMENS 2014). Plates have been cultured in an anaerobic jar at 37˚C for 2-3 days. When 0,5 mm to 3 mm translucent, white to gray colored, colonies were visible the identified colonies were re-plated on a new Eugon agar dish, as described above. These plates also have been cultured in an anaerobic jar at 37˚C for 2-3 days. Presence of isolated colonies was assessed by phase microscopy and gram-staining. The colonies that only consisted of gram-negative rods have been inoculated into Eugon broth. After culturing for 24 hours, DNA was isolated using InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA, USA) and used as a template for a species specific 16S rRNA PCR protocol (Frosth, Slettemeås et al. 2012). Sequencing of amplicons was performed for further genotyping of the isolates. For this, isolates were transferred to a 1:1 eugon broth/glycerol mix for freezing at -80˚C.

Results

Skin culture

First trail

At the first trial, skin samples were cultured for 196 hours (about 1 week 1 day). Every 24 hours samples were taken, only at 168 hours no samples were taken. The results of the first trail are shown in Table 1. The HE histological slides showed a clear decline of the condition of the skin over time. At 24, 48 and 72 hours culturing the skin showed little signs of decline. After 72 hours the normal alignment of the epidermis began to disappear.

Table 1. Histological scores first trail skin culture

foot [a]	explant number [b]	group	time [c]	Histological score[d]
euthanized cow 1	MC 1.1	control	0	1
euthanized cow 1	MC 1.2	control	0	1
euthanized cow 1	MC 2.1	control	24	1
euthanized cow 1	MC 2.2	control	24	1
euthanized cow 1	MC 3.1	control	48	2
euthanized cow 1	MC 3.2	control	48	2
euthanized cow 1	MC 3.3	control	48	1
euthanized cow 1	MC 3.4	control	48	2
euthanized cow 1	MC 4.1	control	72	2
euthanized cow 1	MC 4.2	control	72	2
euthanized cow 1	MC 4.3	control	72	4
euthanized cow 1	MC 4.4	control	72	3
euthanized cow 1	MC 5.1	control	96	3
euthanized cow 1	MC 6.1	control	120	4
euthanized cow 1	MC 7.1	control	144	5
euthanized cow 1	MC 9.1	control	192	7

a. The skin was derived from a single euthanized cow for other reasons than this study.

b. The explants are numbered according to the collection day for fixation in formalin.

c. The explants were collected every 24 hours except for the 168 hours point.

d. The histological scores are based on the scoring system used for this study (appendix I)

Second trail

At the second trail the skin samples were cultured for 120 hours (about 5 days) because the results in the first trail showed a maximum lifetime in culture of 72 hours. The results were comparable to the results of the first experiment and are shown in table 2. The skin samples of the first 72 hours showed little decline (score 1 or 2), after 72 hours the samples scored above 2 and weren't viable anymore. The Figure 3 to 5 show the histological images of respectively 0 hours, 72 hours and 120 hours. At 0 hours and 72 hours of culture the normal alignment of the skin was visible while after 120 hours of culture the normal alignment completely disappeared, the stratum corneum was fully detached and a loss of nuclei was seen in the stratum spinosum, granulosum and basale.

Table 2. Histological scores second trail skin culture

Foot [a]	Explant number [b]	group	Time [c]	Histological score[d]
euthanized cow 2	SL 8-4	control	0	1
euthanized cow 2	SL 9-4	control	24	1
euthanized cow 2	SL 10-4	control	48	2
euthanized cow 2	SL 11-4	control	72	2
euthanized cow 2	SL 12-4	control	96	3
euthanized cow 2	SL 13-4	control	120	4

a. The skin was derived from a single euthanized cow for other reasons than this study.

b. The explants are numbered according to the collection day for fixation in formalin.

c. Due to the first trial, samples were collected for fixation in formalin till the 120 hours, as the explants were viable till 72 hours.

d. The histological scores are based on the scoring system used for this study (appendix I)

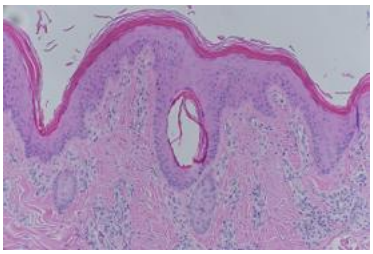

Figure 3: 0h second trail HE 20X

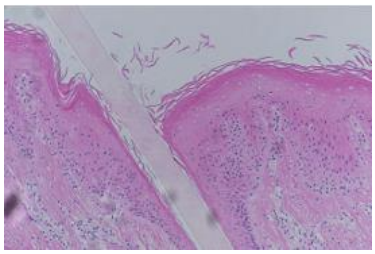

Figure 4: 72h second trail HE 20x

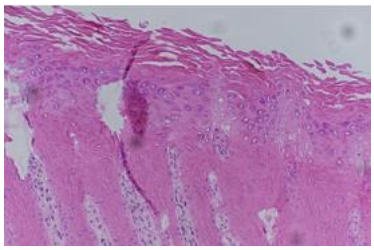

Figure 5: 120h second trail HE 20x

Calf skin trail

A study utilizing calf skin was conducted, as the skin of younger animals was considered to be more viable and capable of sustaining longer culture durations. Histological analysis revealed that calf skin viability in culture maintained for a longer period compared to skin derived from adult cows, i.e the skin of calves remained vital for up to 120 hours (approximately 5 days; Table 3).

Table 3: Histological scores calf skin trial

Foot [a]	Explant number [b]	group	Time [c]	Histological score [d]
euthanized calf 1	DD 1.1	control	0	1
euthanized calf 1	DD 1.2	control	48	1
euthanized calf 1	DD 1.3	control	72	1
euthanized calf 1	DD 1.4	control	96	2
euthanized calf 1	DD 1.5	control	120	2
euthanized calf 1	DD 1.6	control	144	3
euthanized calf 1	DD 1.7	control	168	3

a. The skin was derived from a single euthanized calf for other reasons than this study.

b. The explants are numbered according to the collection day for fixation in formalin.

c. Due to the expectation that the skin would be viable longer because of the use of skin of a younger animal the skin was cultured up to 168 hours.

d. The histological scores are based on the scoring system used for this study (appendix I).

Bacterial cultures

Dichelobacter culture

Approximately 20 different feet were sampled according to the protocol stated in the materials and methods section. The eugon plates showed a significant growth with *Dichelobacter* typical colonies. The colonies observed often included gram-negative rods under microscopy, though they rarely consisted exclusively of rods. Attempts were made to isolate isolated cultures by replating on new eugon plates. After replating isolated cultures at microscopy, as shown in figure 6, were used for PCR. None of the isolated cultures held *Dichelobacter nodosus* DNA. The PCR was also performed on direct samples of infected skin derived from slaughterhouse material, which showed to be positive on PCR for *Dichelobacter nodosus*. Moreover, sequencing of the samples from the slaughterhouse provided a 100% resemblance outcome to *Dichelobacter nodosus*.

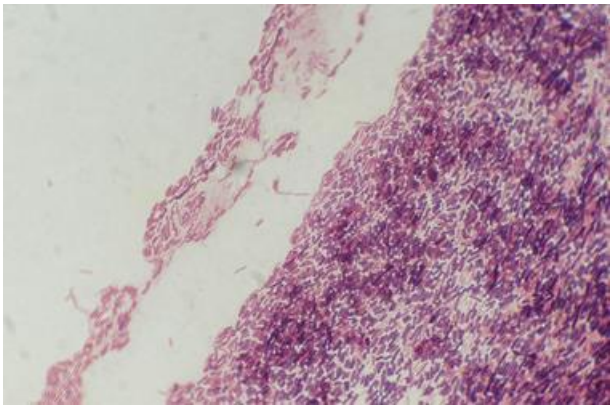

Figure 6: Example of a Gram staining culture in eugon broth. Contains only gram- rods as expected, but at PCR Dichelobacter nodosus wasn't found.

Treponema culture

The culturing of *Treponema* has proven to be difficult, none of the samples taken (n=12) resulted in a isolated culture consisting of spirochetes. After 3 to 5 days of culture in OTEB broth, spirochetes were observed by phase contrast microscopy in al samples. Also, in these cultures in OTEB broth PCR showed a positive result for *Treponema* in 9 of 12 samples. The cultures positive at PCR were plated out on FAA plates. After 2 weeks of culture several colonies were observed on the FAA plates. None of the colonies observed consisted of only spirochetes, all of the colonies were either contaminated with small rods (n=5) or no spirochetes (n=4) were seen at phase contrast microscopy DNA isolated from the colonies consisting of spirochetes and small rods was positive for *Treponema* at PCR. These colonies were replated which again resulted in colonies of rods and spirochetes at phase contrast microscopy. No further attempts of culturing were caried out.

Samples of lesions taken at the slaughterhouse where positive at PCR for *Treponema.* Moreover, Sequencing of these samples showed a 84% resemblance to *Treponema spp..* These samples where used in the infection model.

Infection model

Infection model trail

Microscopical testing

In this pilot the information gained over the pilots caried out before was merged into this concluding experiment. First the histological score was assessed. In the pilot three main variables were used, condition (normal, sanded), incubation time (0h, 24h, 48h, 72h) and stimulus (negative control (NC), experimental (exp), positive control (PC)).

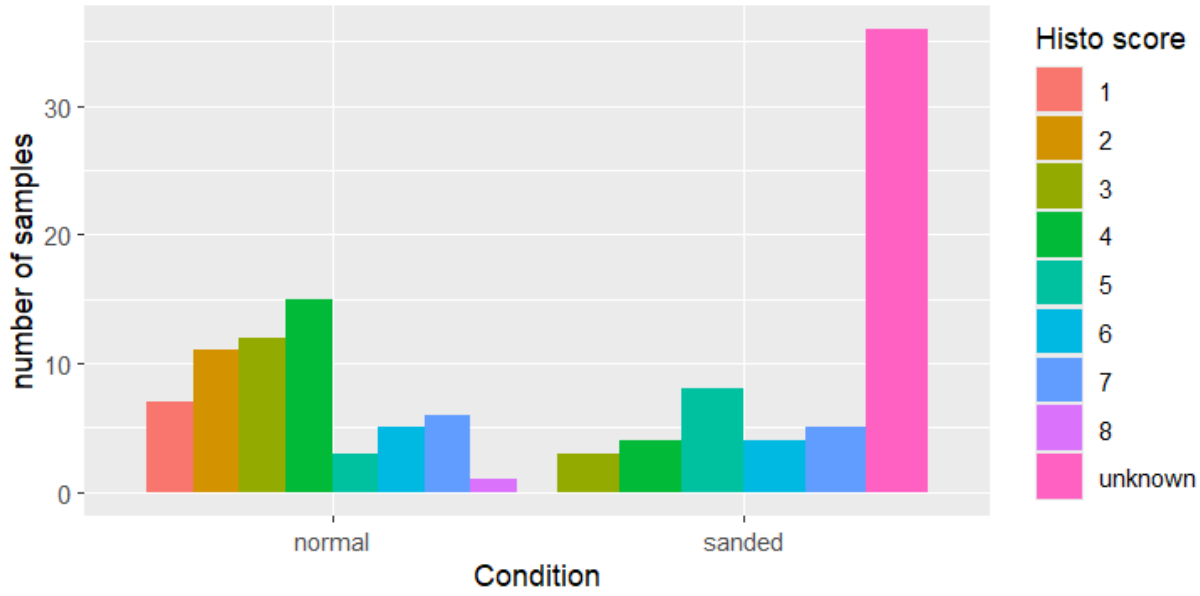

Figure 7 Bar plot of histoscore per condition, scores are based on scoring system found in appendix I, all samples were scored using microscopy (normal n=60, sanded n=60). Due to loss of epithelial layers and further damage in the process of sanding the samples there are 36 out of a total of 60 samples with an unknown score. Therefore, the condition "sanded" has been excluded in further analysis of the histological score.

Influence of condition on histological score

In figure 7 the histological score per condition is shown. In the condition sanded 36 of 60 of the samples are scored unknown. This is the result of loss of the epithelial layers due to the effect of the sanding. Therefore, the condition sanded was excluded in further analysis of the histological score.

Influence of incubation time on histological score

To investigate whether incubation time influences the histological score, the data was analyzed using a generalized linear model (GLM) with a binomial family. The null hypothesis assumed no difference in the proportion of samples with a histological score greater than 2 across the incubation times of 0h, 24h, 48h, and 72h. The alternative hypothesis posited significant differences between these time points.

A Fisher's Exact Test revealed a highly significant association between incubation time and the histological score (p-value = 3.771e-07), indicating a relationship between these variables. However, due to the limited sample size, the standard errors for individual comparisons were high, leading to non-significant results when comparing specific time points. For example, in the GLM analysis, the p-values for the pairwise comparisons of 0h vs. 24h, 48h, and 72h were all above 0.99.

In figure 8 the histological scores whit the variable incubation time is shown, the condition sanded was excluded due to the reason stated before. A summary of the data showed that for the 0h incubation time, all samples had a histological score of 2 or lower. For 24h, the number of samples with scores greater than 2 increased sharply (21 TRUE vs. 9 FALSE), with declining counts at 48h (12 TRUE) and 72h (9 TRUE).

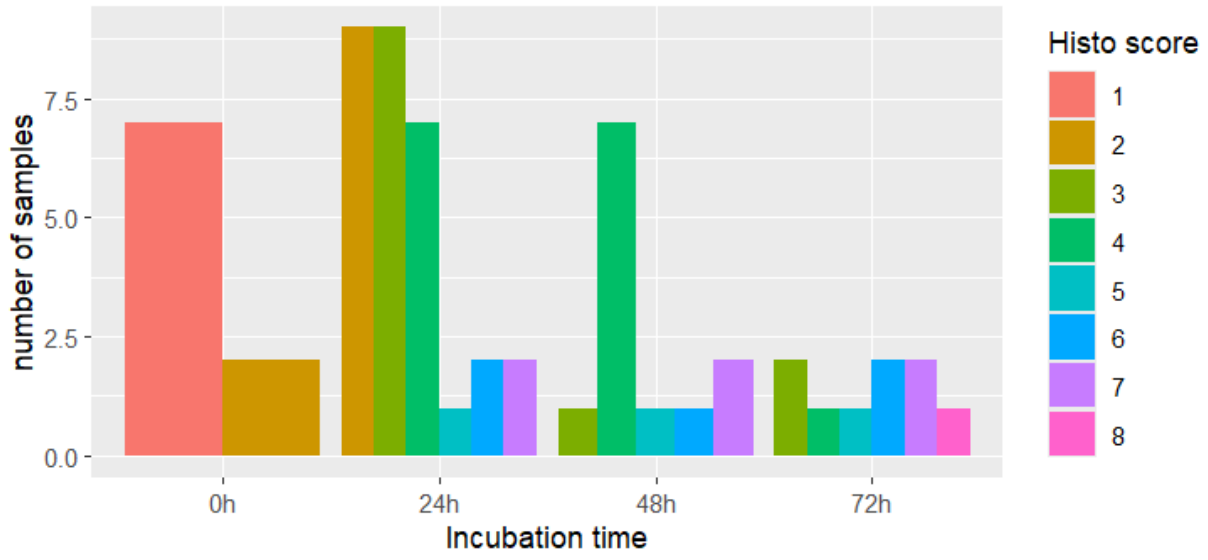

Figure 8 Bar plot od Histological score per incubation time, sanded condition excluded, scores are based on scoring system found in appendix I, al samples were scored using microscopy (0h n=9, 24h n=30, 48h n=12, 72h n=9). The statistical analysis investigated whether there was a significant difference for scores greater than 2. In the fisher exact test, a P value of 3.771e-07 was found, at logistic regression no significance was found due to high standard errors. This can be explained by the relatively low number of samples.

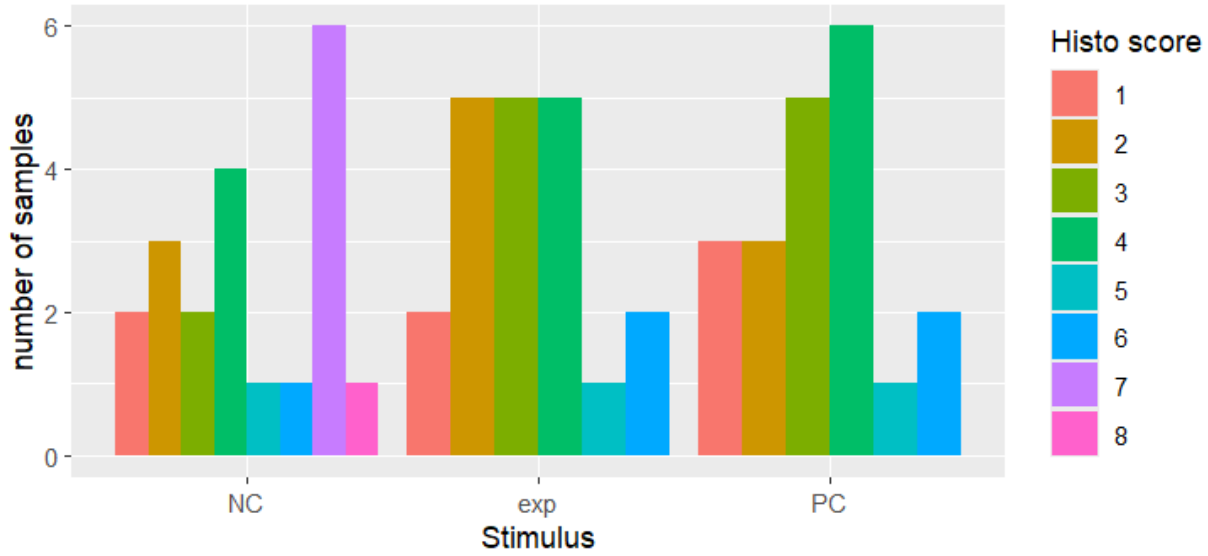

Figure 9 Bar plot of histological score per stimulus sanded condition excluded, scores are based on scoring system found in appendix I, all samples were scored using microscopy (NC n=20, exp n=20, PC n=20). The statistical analysis investigated whether there was a significant difference for scores greater than 2. Neither the Fisher's Exact Test nor the logistic regression analysis provided evidence for a significant influence of the stimulus type on the histological score.

Influence of Stimulus on histological score

To determine whether the type of stimulus influences the histological score, data was analyzed using a Fisher's Exact Test and logistic regression. The null hypothesis assumed no difference in the proportion of samples with a histological score greater than 2 across the stimulus conditions (experiment, negative

control, and positive control), while the alternative hypothesis posited significant differences between these groups.

A Fisher's Exact Test indicated no significant association between stimulus and histological score (p-value = 0.9371), suggesting that the distribution of histological scores greater than 2 does not differ substantially across the stimulus conditions.

A logistic regression model was used to further investigate whether the stimulus type predicts the probability of obtaining a histological score greater than 2. The results revealed that the overall effect of stimulus was not significant (LRT = 0.478, p-value = 0.7875). None of the individual stimulus types significantly influenced the likelihood of a histological score greater than 2:

- Negative control (NC) versus experiment: odds ratio (OR) = 0.619 (95% CI: 0.150–2.410, p = 0.4917).
- Positive control (PC) versus experiment: OR = 0.778 (95% CI: 0.186–3.150, p = 0.7235).

In Figure 9 a barplot of histological score per stimulus with the sanded condition excluded is shown. Visual inspection of the barplot revealed a relatively high number of samples with a histological score of 7 in the negative control group. Other differences between stimulus groups were not visually prominent.

Influence of Group on histological score

To evaluate whether group, the combination of all variables (Incubation time/ stimulus/ condition), influences the histological score, data were analyzed using Fisher's Exact Test and logistic regression. The null hypothesis assumed no difference in the proportion of samples with a histological score greater than 2 across the groups, while the alternative hypothesis posited significant differences between the groups.

Visual inspection of the barplot of the histological score per group, sanded condition excluded is shown in figure 10 revealed that groups with shorter incubation times (0h and 24h) contained the majority of scores of 2 or lower, while all other groups consistently had scores greater than 2.

A Fisher's Exact Test indicated a significant association between group and histological score (p-value = 0.0007064). This suggests that the distribution of histological scores differs significantly across the groups.

A logistic regression model was used to investigate whether group predicts the probability of a histological score greater than 2. The overall contribution of group to the model was significant, as indicated by the likelihood ratio test (LRT), with a deviance difference of 37.618 (p-value < 0.001). However, individual comparisons between groups were not significant, with p-values exceeding 0.99 for all group coefficients. This was likely due to extremely large standard errors.

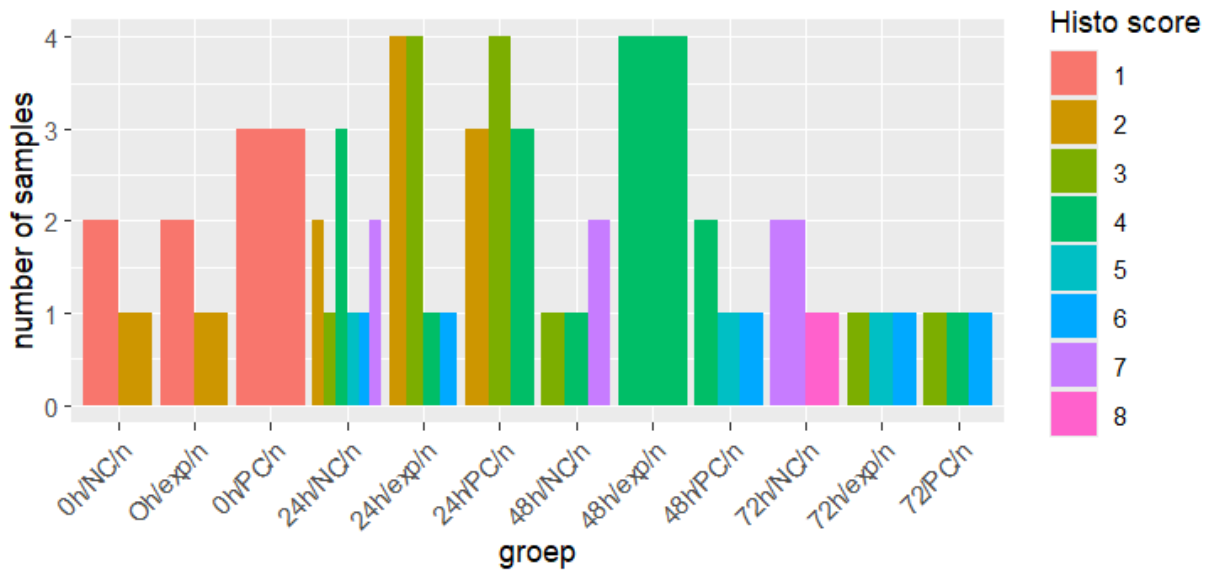

Figure 10 Barplot of histological score per stimulus sanded condition excluded, scores are based on scoring system found in appendix I, al samples were scored using microscopy. The statistical analysis investigated whether there was a significant difference for scores greater than 2. A Fisher's Exact Test indicated a significant association between group and histological score (p-value = 0.0007064). The overall contribution of group to the model was significant, as indicated by the likelihood ratio test (LRT), with a deviance difference of 37.618 (p-value < 0.001). However, individual comparisons between groups were not significant, with p-values exceeding 0.99 for all group coefficients.

PCR testing

To evaluate the immunological response in the samples ΔCT data was analyzed using descriptive statistics, boxplots, and a generalized linear model (GLM) with a Gaussian family. ΔCT = Ct value IL8 – Ct value GABDH. 4 samples had no measurable values at PCR for GABDH and are not used. In the pilot three main variables were used, condition (normal, sanded), incubation time (0h, 24h, 48h, 72h) and stimulus (negative control (NC), experimental(exp), positive control (PC)). These are combined in groups defined by the before mentioned variables.

Influence of Condition on the ΔCT

To evaluate whether the condition influences ΔCT, the data were analyzed using descriptive statistics, boxplots, and a generalized linear model (GLM) with a Gaussian family. The null hypothesis assumed no difference in ΔCT between the two conditions, while the alternative hypothesis posited a significant difference.

The mean ΔCT for the normal condition (n = 60) was 4.8 (SD = 1.4), while for the sanded condition (n = 56), the mean was 4.7 (SD = 0.9). The medians for both conditions were nearly identical (4.4 and 4.7, respectively). The ranges of ΔCT values were also similar: 2.4–9.2 for normal and 3.0–7.4 for sanded.

The boxplot of ΔCT by condition (figure 11) shows complete overlap between the ΔCT distributions for the two conditions, visually suggesting no significant difference.

A GLM was performed to test the effect of condition on ΔCT. The model results indicated that condition did not significantly predict ΔCT (p = 0.597). The confidence interval for the effect of condition (-0.56 to

0.32) included zero, further confirming the non-significance. The null deviance of the model (166.09) and residual deviance (165.68) showed minimal reduction, indicating that adding condition as a predictor did not meaningfully improve the model. The likelihood ratio test (LRT) for condition also showed no significant contribution to the model (p = 0.593). The diagnostic plots (QQ-plot and Residuals vs. Fitted Values) showed no violations of model assumptions, supporting the validity of the GLM.

The statistical analysis found no evidence to reject the null hypothesis, indicating that condition does not significantly influence ΔCT. This result was consistent with the descriptive statistics and boxplot, both of which show substantial overlap in ΔCT values between the conditions.

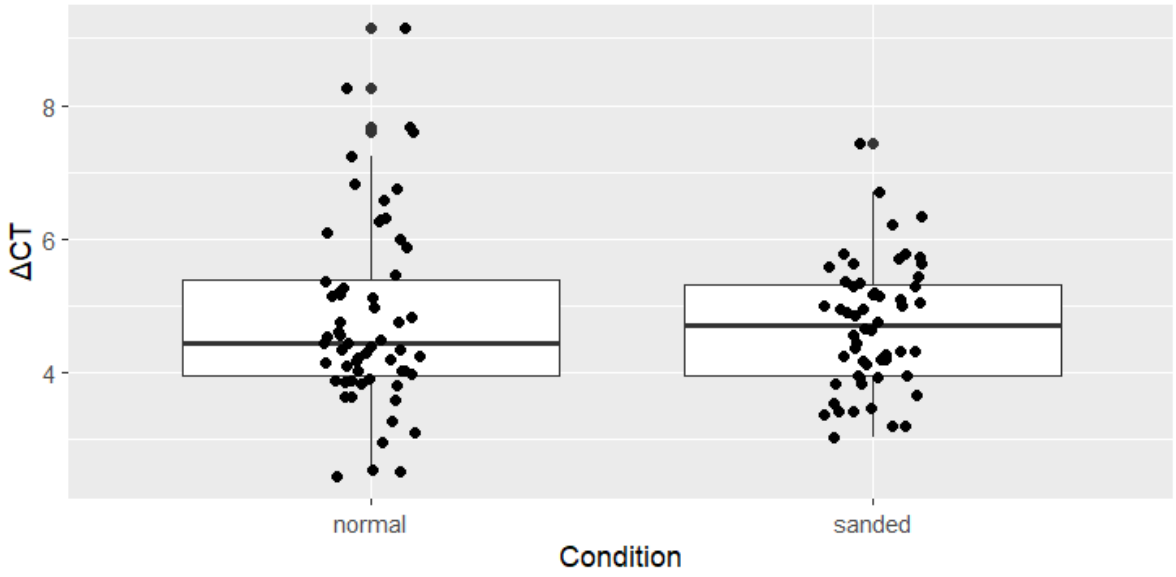

Figure 11 boxplot of ΔCT by condition. ΔCT = Ct value IL8 – Ct value GABDH. 4 samples had no measurable values at PCR for GABDH and are not shown. The mean ΔCT for the normal condition (n = 60) was 4.8 (SD = 1.4), while for the sanded condition (n = 56), the mean was 4.7 (SD = 0.9). A GLM was performed to test the effect of condition on ΔCT. The model results indicated that condition did not significantly predict ΔCT (p = 0.597). The likelihood ratio test (LRT) for condition also showed no significant contribution to the model (p = 0.593), indicating that condition does not significantly influence ΔCT.

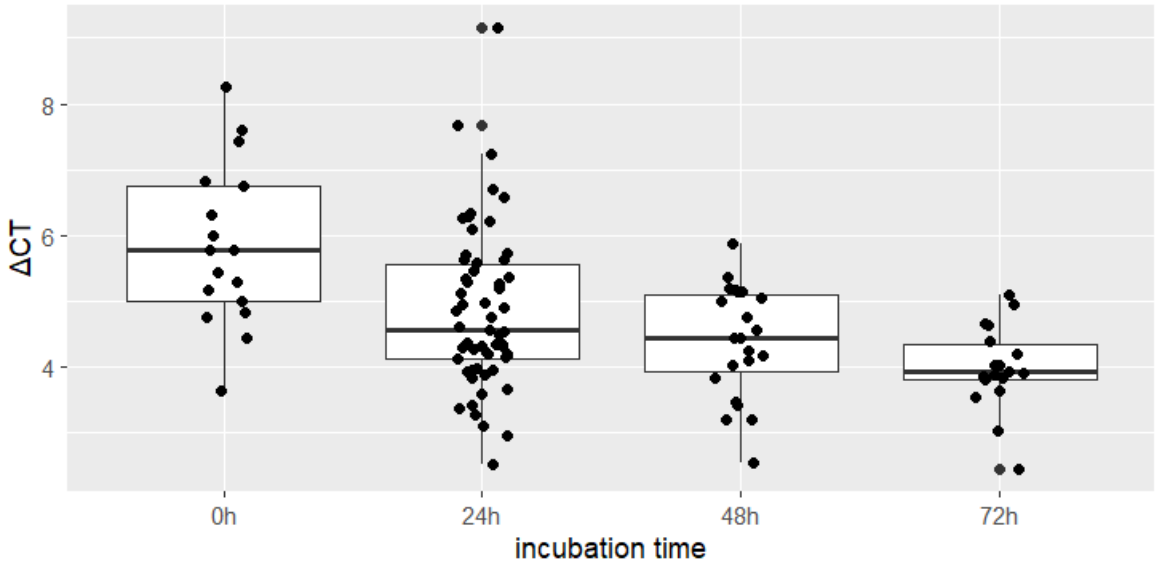

Figure 12 boxplot of ΔCT by incubation time. ΔCT = Ct value IL8 – Ct value GABDH. 4 samples had no measurable values at PCR for GABDH and are not shown. 0h: Mean = 5.8 (SD = 1.2), n = 17, 24h: Mean = 4.8 (SD = 1.2), n = 58, 48h: Mean = 4.4 (SD = 0.8), n = 23, 72h: Mean = 4.0 (SD = 0.6), n = 18. A linear regression model confirmed that incubation time significantly influenced ΔCT. Using 0h as the reference group: At 24h, ΔCT was reduced by 0.994 units (95% CI: -1.58, -0.41; p = 0.0012), At 48h, ΔCT was reduced by 1.471 units (95% CI: -2.15, -0.79; p < 0.001), At 72h, ΔCT was reduced by 1.851 units (95% CI: -2.57, -1.13; p < 0.001).

Influence of Incubation time on the ΔCT

To evaluate whether incubation time influences ΔCT, data were analyzed using descriptive statistics, boxplots, and a linear regression model. The null hypothesis assumed no difference in ΔCT across the incubation times (0h, 24h, 48h, and 72h), while the alternative hypothesis posited significant differences between the groups.

The mean ΔCT decreased with increasing incubation time:

- 0h: Mean = 5.8 (SD = 1.2), n = 17
- 24h: Mean = 4.8 (SD = 1.2), n = 58
- 48h: Mean = 4.4 (SD = 0.8), n = 23
- 72h: Mean = 4.0 (SD = 0.6), n = 18

This pattern of decreasing ΔCT values was consistent across the descriptive statistics and visualized in the boxplot (figure 12), where ΔCT appeared to decline steadily with longer incubation times.

A linear regression model confirmed that incubation time significantly influenced ΔCT. Using 0h as the reference group:

- At 24h, ΔCT was reduced by 0.994 units (95% CI: -1.58, -0.41; p = 0.0012).
- At 48h, ΔCT was reduced by 1.471 units (95% CI: -2.15, -0.79; p < 0.001).
- At 72h, ΔCT was reduced by 1.851 units (95% CI: -2.57, -1.13; p < 0.001).

The overall contribution of incubation time to the model was highly significant (scaled deviance = 26.971, $p < 0.001$). Diagnostic plots showed no violations of model assumptions, supporting the validity of the model.

This leads to the conclusion that the analysis demonstrated that ΔCT decreases significantly with increasing incubation time. Incubation times of 24h, 48h, and 72h were all associated with significantly lower ΔCT values compared to the 0h reference group. These findings suggest a clear influence of incubation time on ΔCT.

Influence of Stimulus on the ΔCT

To determine whether the type of stimulus influences ΔCT, data were analyzed using descriptive statistics, boxplots, and a linear regression model. The null hypothesis assumed no difference in ΔCT among the three stimuli, negative control, experiment and positive control (NC, exp, and PC), while the alternative hypothesis posited significant differences between the groups.

The mean ΔCT values for the three stimuli were as follows:

- Exp: Mean = 4.9 (SD = 1.3), n = 39
- NC: Mean = 4.8 (SD = 1.0), n = 40
- PC: Mean = 4.6 (SD = 1.3), n = 37

The ranges of ΔCT were similar across stimuli, with no notable differences in medians or spread. Visual inspection of the boxplot (figure 13) showed substantial overlap between the ΔCT distributions for all three stimuli, suggesting minimal differences.

A linear regression model was fitted to test the effect of stimulus on ΔCT. The model results indicated that stimulus type did not significantly predict ΔCT:

- NC versus exp: Estimate = -0.090 (95% CI: -0.62 to 0.44, $p = 0.741$)
- PC versus exp: Estimate = -0.297 (95% CI: -0.84 to 0.25, $p = 0.286$)

The overall contribution of stimulus to the model was also non-significant (scaled deviance = 1.229, $p = 0.541$). The confidence intervals for both NC and PC included zero, further confirming the non-significance of stimulus as a predictor of ΔCT. Diagnostic plots, including QQ-plots and Residuals vs. Fitted Values, showed no deviations from model assumptions, validating the use of this linear regression model.

The analysis found no evidence to reject the null hypothesis, indicating that stimulus does not significantly influence ΔCT. This finding was consistent with the descriptive statistics and boxplot, which show substantial overlap between the groups.

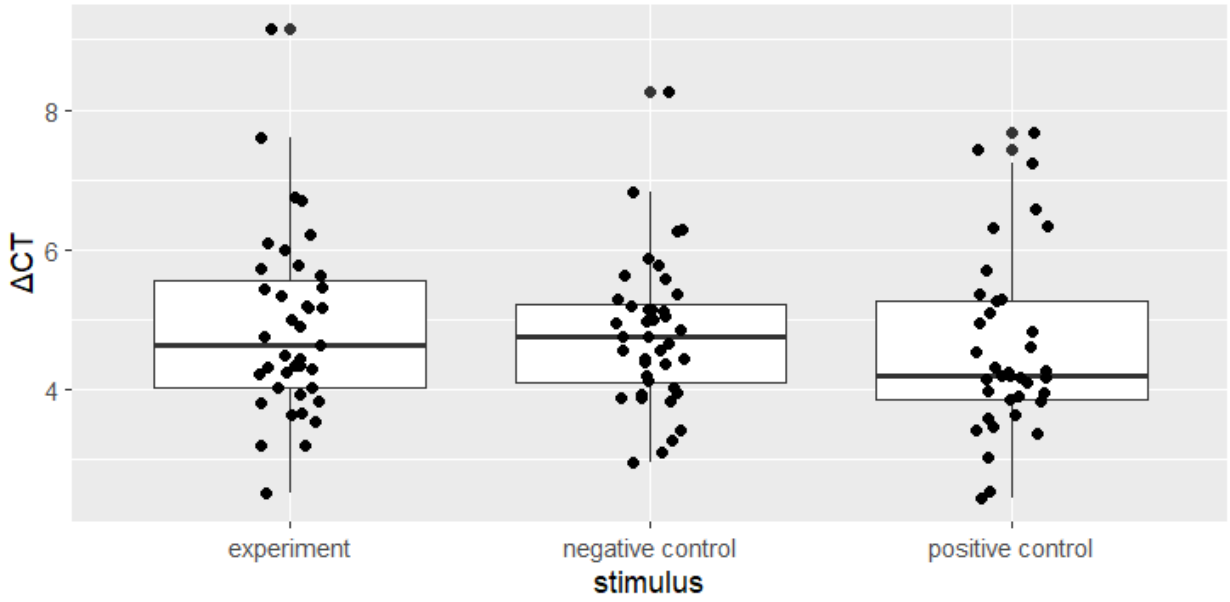

Figure 13 boxplot of ΔCT by stimulus. ΔCT = Ct value IL8 – Ct value GABDH. 4 samples had no measurable values at PCR for GABDH and are not shown. Exp: Mean = 4.9 (SD = 1.3), n = 39, NC: Mean = 4.8 (SD = 1.0), n = 40, PC: Mean = 4.6 (SD = 1.3), n = 37, NC versus exp: Estimate = -0.090 (95% CI: -0.62 to 0.44, p = 0.741), PC versus exp: Estimate = -0.297 (95% CI: -0.84 to 0.25, p = 0.286). The overall contribution of stimulus to the model was also non-significant (scaled deviance = 1.229, p = 0.541).

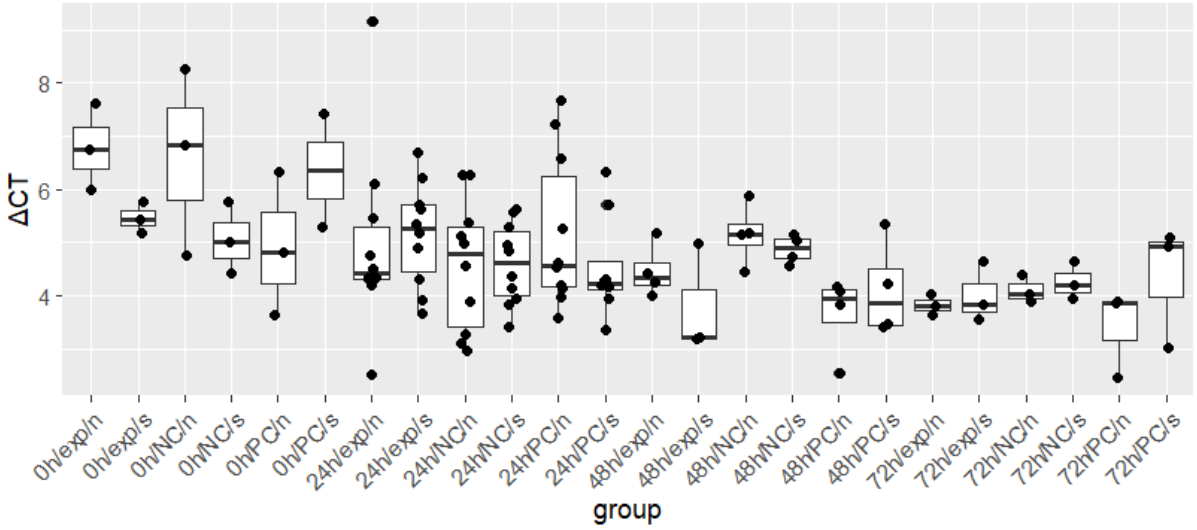

Figure 14 boxplot of ΔCT by stimulus. ΔCT = Ct value IL8 – Ct value GABDH. 4 samples had no measurable values at PCR for GABDH and are not shown. The groups are defined by combinations of incubation time (0h, 24h, 48h, 72h), stimuli (exp, NC, PC) and conditions (normal, sanded). A linear regression model confirmed significant effects of the groups on ΔCT, the model explained a substantial portion of the variability in ΔCT (null deviance = 166.09, residual deviance = 108.96, AIC = 371.94).

Influence of Group on the ΔCT

To evaluate whether the incubation group influences ΔCT, data were analyzed using descriptive statistics, boxplots, and a linear regression model. The null hypothesis assumed no difference in ΔCT among the groups defined by combinations of incubation time (0h, 24h, 48h, 72h), stimuli (exp, NC, PC)

and conditions (normal, sanded), while the alternative hypothesis posited significant differences between these groups.

The mean ΔCT values varied across the groups:

- 0h/exp/n: Mean = 6.8 (SD = 0.8, n = 3)
- 24h/NC/n: Mean = 4.6 (SD = 1.2, n = 10)
- 72h/PC/n: Mean = 3.4 (SD = 0.8, n = 3)

Visual inspection of the boxplot, shown in figure 14, suggested noticeable differences in ΔCT values between some groups, particularly between 0h and the later time points.

A linear regression model confirmed significant effects of the groups on ΔCT. Using 0h/exp/n as the reference group, several groups showed significant reductions in ΔCT:

- 24h/exp/n: Estimate = -1.82, 95% CI = [-3.22, -0.41], p = 0.013
- 48h/PC/n: Estimate = -3.13, 95% CI = [-4.76, -1.50], p < 0.001
- 72h/NC/s: Estimate = -2.52, 95% CI = [-4.26, -0.78], p = 0.006

Groups associated with longer incubation times (48h and 72h) consistently showed significant reductions in ΔCT compared to the reference group. Overall, the model explained a substantial portion of the variability in ΔCT (null deviance = 166.09, residual deviance = 108.96, AIC = 371.94). Diagnostic plots, including QQ-plots and Residuals vs. Fitted Values, showed no deviations from model assumptions, supporting the validity of the model.

The analysis demonstrated that ΔCT was significantly influenced by the incubation group, with longer incubation times generally associated with lower ΔCT values. These findings indicate that both time and condition combinations contribute to ΔCT variability.

Discussion

In this study three objectives were defined:

1. To develop, optimize and validate a protocol for the in vitro culture of bovine foot skin explants.
2. To optimize and validate an isolation protocol for *Dichelobacter nodosus* and *Treponema spp.*
3. To develop an infection model of digital dermatitis (DD) by exposing bovine skin explants to an inoculum containing specific bacteria spp. causing DD.

The objectives are discussed separately, and it was evaluated whether the expected outcome has been achieved. Upon this discussion, the used methodology as well as the limitations and hence consequences for the interpretation of the results will be reflected.

Skin culture

The first objective was to develop, optimize and validate a protocol for *in vitro* culture of bovine foot skin explants. The results showed that it was possible to culture adult bovine foot skin up to 72 hours, juvenile skin even up to 96 hours. Due to a lack of numbers of samples, the power of this study was too low and no significant conclusions were able to draw. In this respect, the majority of the samples was lost due to a detrimental fungal infestation and could not be used for the interpretation and analysis of this objective.

The expected outcome was as follows: a model for bovine foot skin culture in vitro was developed which was supposed to be used for performing validation experiments in vitro that in turn contribute to reducing the number of research animals used in scientific experiments. These expectations were not met due to the short live longevity of the explants of adult cows.

In contrast, the longer viability of calf skin suggests that younger animals skin was more resilient in vitro, which may be explained by the inherent differences in sample skin vitality and regenerative capacity during culture comparing young and adult animals. This finding is particularly relevant as it implies that calf skin could serve as a durable model for extended in vitro culture studies on DD, potentially reducing the number of animals needed for vivo experiments.

The decline in skin integrity observed after 72 hours of culture in adult cow skin indicates a limitation in the current culture conditions. The observed rapid degeneration beyond this time point suggests that while the initial culture conditions support short-term viability, further optimization of the culture system is needed to extend the culture period for adult bovine skin. Improvements for refining the culture medium may be involved, like adjusting gas exchange conditions, or incorporating additional growth factors or extracellular matrix components to better mimic the in vivo environment. Also, changing the setup using other supportive agents or, for example, changing the used technical agar No.3 for Millicell-HA insert as being used in Rutten et al (1990) or using an air liquid interface as used by Yasuoka et al and Steinstraesser et al. (2009) may be expected to improve the culture environment. Moreover, a stainless-steel setup system as described by Steinstraesser et al. could be used as they showed human skin explants were viable up to a period of 4 weeks.(Yasuoka, Larregina et al. 2008)(Steinstraesser, Rittig et al. 2009)(Rutten, Bequet-Passelecq et al. 1990).

Baumbach et al cultured cow skin collected from the exact same location of the cows' foot as used in this study, but he showed to be able to culture the skin up to 7 days. The main differences in Baumbach's' methodology compared to our study were the use of an air liquid interface and a different composition of the culture medium. Also, the collected skin explants were cut down thinner to approximal 2 mm thickness whereas the samples used in this study were 4-5 mm thick. This 4-5 mm procedure approach likely resulted in a distance to be too thick for the culture medium to diffuse into the skin tissue consequently resulting in a shorter viable time period of the skin cultured in this study (Baumbach, Anantama et al. 2024).

Similarly to this study Baumbach et al experienced fungal overgrow during their experiments as well, although it seemed to happen less frequently. This may be the result of the use of a different washing protocol, which showed to be beneficial for culturing bovine skin (Baumbach, Anantama et al. 2024).

Future research, in conclusion, has to focus on the optimization of the culture conditions for the skin in order to increase its viability over a longer period of time. It may be useful to focus more on using the skin of younger animals for the invitro culture model to increase the duration of viability during the culture. This optimization will contribute to the ultimate goal of this study, i.e. to create an in vitro model for culturing bovine skin to be used for studying claw tissues from cows that will lead to a reduction of research animals to perform in vivo experiments hence increasing animal welfare.

Bacterial cultures

The second objective of this study was to optimize and validate a culture protocol for *Dichelobacter nodosus* and *Treponema spp..* After this study, the goal, we will be able to produce isolated cultures of *Dichelobacter nodosus* and *Treponema spp.* using the isolation protocol. The harvested cultures will be frozen in glycerol at -80˚C, enabling us to use them in future research. The culture and isolation of *Treponema spp.* and *Dichelobacter nodosus* from DD lesions in cattle posed significant challenges, highlighting the complexities of working with these anaerobic bacteria. Consequently, this part of the study resulted in not being able to obtain isolated cultures of *Treponema spp.* and *Dichelobacter nodosus*, learning that the expected outcome was not met.

Treponema spp. Culture

The isolation of *Treponema spp.* proved to be challenging, consistent with previous reports that underscore the difficulty in cultivating these fastidious organisms (Evans, Brown et al. 2008). All the attempts performed in this study to culture the lesions collected failed to produce isolated colonies. Despite the presence of *Treponema spp. w*as proven by PCR analysis and phase contrast microscopy, the samples were always contaminated with so-called rod shaped bacteria. The observation of the presence of small rods alongside spirochetes in the mixed bacteria colonies suggests that the used culture conditions in this study may not have been optimal for gaining the selective growth of *Treponema spp.*.

The inability to culture isolated *Treponema* colonies might have been caused by the high presence of other bacteria species in the lesions collected as reported by Clegg et al. (Clegg, Mansfield et al. 2015). In the study of Clegg et al. and Evans et al., isolated colonies could be cultured, and since the exact same culture method and conditions were used, one expected to be able to culture isolated colonies as well in this study (Clegg, Mansfield et al. 2015, Evans, Brown et al. 2008). In Evans et al. was reported that the isolation of isolated colonies cultures required 2–3 sequential passages on supplemented FAA plates. In our study only a maximum of 2 sequential passages were carried out which could be one of the factors that contributed in the lack of isolated colonies (Evans, Brown et al. 2008). Moreover, Evans et al. also used both rabbit serum as well as fetal calf serum as an OTEB growth supplement which combination showed a better growth rate of several strains in rabbit serum supplemented OTEB. In this study only

fetal calf serum was used and one may conclude as a possibility that the samples used would have shown a better growth with rabbit serum (Evans, Brown et al. 2008).

This difficulty in isolated *Treponema spp.* cultures limits the ability to fully characterize the pathogenic mechanisms of *Treponema spp.* in DD. Future studies have to explore and test alternative culture conditions, such as modifications to the nutrient composition of the culture media or the used anaerobic environment system, to improve the isolation success as aimed.

Dichelobacter nodosus Culture

The isolation of *Dichelobacter nodosus* was similarly challenging compared to the *Treponema* culture. Although cultured colonies with characteristics typical of *Dichelobacter nodosus* were observed on Eugon agar, microscopy observations often revealed the presence of gram-negative rods alongside other forms, indicating either contamination or the presence of other, non-specified, bacterial species. Despite these observations, none of the cultures that appeared to have isolated colonies under microscopy tested positive for *Dichelobacter nodosus* DNA by PCR analysis.

The failure to obtain isolated cultures of *Dichelobacter nodosus* despite molecular evidence by PCR of its presence suggests that culture protocols used in this study need refinement. This optimization may involve adjusting the growth medium composition, incubation conditions, or exploring alternative co-culture techniques that do support the growth of *Dichelobacter nodosus* bacteria in the presence of other microbes.

Future research should focus on the optimizing culture conditions and further exploring the microbial ecology of DD lesions. A better understanding of the interactions between *Treponema spp.*, *Dichelobacter nodosus*, and other bacteria within the lesion microenvironment may provide new insights into their pathogenicity and persistence. Additionally, the development of more sensitive and specific culture techniques, possibly informed by genomic data, could enhance the ability to isolate and hence study these important pathogens in culture environments.

In conclusion, while this study confirms the presence of *Treponema spp.* and *Dichelobacter nodosus* in DD lesions, the experienced difficulties in obtaining isolated cultures limit our ability to fully understand and unravel their biology and pathogenic mechanisms. Continued efforts to refine culture methods will be essential for advancing our knowledge of these complex infections.

Infection model

The third objective of this study was to develop an infection model of DD by exposing bovine skin explants to an inoculum containing bacteria known causing DD. The expected outcome was to develop an in vitro model to study the host response after an induced in vitro infection. Finally, this model could be used to investigate different inoculums that consist of different pathogens which studies will gain extensive information that will provide a better understanding of DD pathogenesis.

The results of the trial revealed significant changes in both the histological score and ΔCT associated with incubation time and the group classification. However, since the tested stimulus did not have a significant effect on either the histological score or ΔCT, it was likely to conclude that the observed significance in group classification was solely attributable to incubation time. Consequently, this trial did not achieve the expected outcome of establishing a functional model for in vitro infections. For the model to be considered successful, the stimulus had to show a significant measurable effect on both the histological score and ΔCT.

The significance of the duration of the incubation time can be attributed to the observed decline in skin viability, a pattern that was also evident in previous tests conducted without the use of an inoculum as shown in the first part of this study and in Baumbach et al. (Baumbach, Anantama et al. 2024). Similarly, the histological score decreased over the course of the skin culture trial, indicating that a limited degree of deterioration was expected. This observed degradation in the skin integrity may also account for the relative measured increase of IL-8 expression, indicating that cellular damage likely stimulated an inflammatory response or relatively down regulated the expression of GAB DH.

For this study, one major factor contributing to the lack of significance was the limited sample size. This limitation was particularly evident in the analysis of the histological scores, where logistic regression was infeasible due to standard errors that exceeded 2000 for incubation time and even larger errors for group classifications. A power analysis, assuming a relative low effect size and a significance level of 0.05, indicated that a sample size of minimal 1000 observations is required to achieve a power value of 0.82. In this respect, the sample sizes used in the study trial, 60 for histological scoring and 120 for ΔCT analysis, respectively, were far of sufficient. Moreover, the effective sample size was further reduced by the loss of trail samples due to severe fungal contamination.

Furthermore, the reliability of the statistical analysis was undermined by the potential lack of randomization. Samples that were collected at the slaughterhouse, by which procedure the lower legs were detached from animals distal of the tarsal joint, without a means of identifying the source of those animals. Consequently, leading to a risk of clustering among the collected samples used for testing. Theoretically, it may be possible that all samples collected originated from cows originating from the same farm, which situation could introduce an unaccounted-for dependencies and biases in the data.

In this study, only the cytokine IL8 was utilized to assess the immunological response tested. In contrast, Evans et al. (Evans, Brown et al. 2014) reported the upregulation of a number of interesting genes, including those encoding RANTES/CCL5, MMP12, TNFα, TGFβ, and TIMP3, in bovine keratocyte and fibroblast cell lines stimulated with sonicated *Treponemes*. Additionally, genes such as A2ML1, IL17, PI3, CCL11, and the elafin-like protein were upregulated at various stages of DD. Conversely, genes associated with keratins and anti-inflammatory molecules, such as SCGB1D and MGC151921, were downregulated in the same study (Vermeersch, Geldhof et al. 2022). These findings suggest that evaluating a broader panel of relevant genes may be beneficial. However, IL8 remains a critical component of testing due to its crucial role in the innate immune response during an infection, as clearly has been demonstrated by Refaai et al. (Refaai, Ducatelle et al. 2013).

By addressing the aforementioned limitations and suggestions expanding the scope of analysis, future research can advance the development of a robust in vitro model. This model will enable to perform more accurate investigations into host-pathogen interactions and hence provides a valuable tool for testing various inoculums and therapeutic interventions.

Conclusion

While this study has laid the fundament for developing an in vitro model of DD, achieving a fully functional system requires addressing several challenges. Enhanced culture protocols, improved bacterial isolation methods, and better-controlled infection experiments are essential. Once established, such a model could significantly advance the understanding of DD pathogenesis, support the development of targeted therapies, and reduce the reliance on live animal research, contributing to both scientific progress and animal welfare.

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Further information

Appendix I

Scoring system digital dermatitis *in vitro*

1	Normal skin with attached stratum corneum, a stratum granulosum of 5 layers of thickness a stratum spinosum with a maximum of 5 layers of cells with normal nuclei and a clear stratum basale. Hair follicles are present.
2	Skin with lose flakes, more than 10 less than 40, of stratum corneum. Remaining layers as described at score 1
3	Skin with detached stratum corneum, moved for 0 to 20 µm. Remaining layers as described at score 1
4	Skin with detached stratum corneum, moved for 0 to 20 µm and vacuolisation holes in the stratum granulosum and spinosum, normal stratum basale.
5	Skin with detached stratum corneum, moved for 20 to 50 µm and vacuolisation holes in the stratum granulosum and spinosum, normal stratum basale.
6	Total loss of stratum corneum and vacuolisation holes in the stratum granulosum and spinosum, loss of normal alignment of stratum basale.
7	Total loss of stratum corneum and vacuolisation holes in the stratum granulosum and spinosum with necrotizing tissue in stratum granulosum an, loss of normal alignment of all the layers. Bacteria on top of the skin.
8	Total loss of stratum corneum. Thickening of stratum spinosum between 5 and 20 layers without normal alignment, thickening of stratum granulosum more than 5 layers without normal alignment and necrosis. Bacteria on top of the skin
9	Total loss of stratum corneum. Thickening of stratum spinosum between 20 and 30 layers without normal alignment, thickening of stratum granulosum more than 5 layers without normal alignment and necrosis. Infiltration of bacteria.
10	Total loss of stratum corneum. Thickening of stratum spinosum between 20 and 50 layers without normal alignment, thickening of stratum granulosum more than 5 layers without normal alignment and necrosis. Stratum basale consist of 2 or more layers. Infiltration of bacteria

Appendix II

Cell culture stimulated with PAMP

In an attempt to illustrate if IL 8 cytokine mRNA and GAP DH mRNA can be isolated from a lysate of keratocytes by PCR, cells derived from cell cultures were stimulated with different concentrations of PAMP, 1000ng/ml, 100ng/ml, 10ng/ml and 0 ng/ml. respectively.

The GAP DH primers worked at qPCR and at sequencing the product was proven to be Bovine GAB DH. The IL 8 primers didn't work. On the supernatant of the cells an ELISA for IL8 was performed. This gave a positive result for IL8. New primers were tested for IL8. These were tested in a PCR gradient. The optimal temperature which followed out of the gradient PCR was 59,0˚C. At sequencing the product was proven to be IL 8.

First infection trial

The first time an infection model was conducted was to test if the model created could work technically. The way of attaching the inoculation tube has proven itself over three days it could work. The samples were thrown out on the third day. There was no further testing done on these samples. The samples were inoculated with lesion macerate in which *Treponema* and *Dichelobacter* were present proven by PCR.

Second infection trial

At the second trail samples were taken from 5 feet at the slaughterhouse (Gosschalk, Epe, The Netherlands). Of this 1 foot was used for histology. The skin was cultured for 72 hours, inoculated with lesion macerate and a negative control of OTEB. The skin was histologically scored according to the scoring system shown in appendix I. The results are shown in table 4. At 24 hours there seems to be a difference between the samples but due to the low number of samples it isn't possible to draw any conclusions.

Table 4: Histological scores infection trail

Foot [a]	Explant number [b]	group	Time [c]	Histological score [d]
2	dd 01	control	0	1
2	dd02	infected	24	4
2	dd 03	control	24	2
2	dd 04	infected	48	5
2	dd 05	control	48	4
2	dd 06	infected	72	7
2	dd 07	control	72	6

a. The skin was derived from a single cow slaughtered at Godschalk, Epe.

b. The explants are numbered according to the order of collection for fixation in formalin.

c. Due to earlier trials, samples were collected for fixation in formalin till 72 hours.

d. The histological scores are based on the scoring system used for this study (appendix I).

The other feet collected at the slaughterhouse were treated the same as stated above for the exception that the samples were placed in RNA later. These were used to detect IL8 and GAP DH. After equalizing for the GAB DH there were no significant differences

Inoculum testing

A second trial was carried out using different inoculums: oteb+old lesion, oteb+new lesion, oteb, oteb fresh lesion+ 1000ng/ml pamps, 1000 ng/ml pamps, 100 ng/ml pamps and PBS. The samples containing these different inoculums were cultured for 24h. After culturing cut in half. This was used for RNA isolation and an Elisa on a filtrate of lysed tissue. ΔCT showed no differences at PCR.

Appendix III

Feet	Tissue	#		Random # Generator
1	1	1	1.1	36
	2	2	1.2	49
	3	3	1.3	12
	4	4	1.4	20
	5	5	1.5	39
	6	6	1.6	60
2	1	7	2.1	26
	2	8	2.2	48
	3	9	2.3	9
	4	10	2.4	15
	5	11	2.5	58
	6	12	2.6	43
3	1	13	3.1	5
	2	14	3.2	24
	3	15	3.3	34
	4	16	3.4	59
	5	17	3.5	57
	6	18	3.6	37
4	1	19	4.1	7
	2	20	4.2	27
	3	21	4.3	50
	4	22	4.4	1
	5	23	4.5	8
	6	24	4.6	4
5	1	25	5.1	32
	2	26	5.2	18
	3	27	5.3	33
	4	28	5.4	28
	5	29	5.5	46
	6	30	5.6	47

1

	PBS	PAMP2c	Lesion
healthy	36	49	12
Sanded	20	39	60

2

	PBS	PAMP2c	Lesion
healthy	26	48	9
Sanded	15	58	43

3

	PBS	PAMP2c	Lesion
healthy	5	24	34
Sanded	59	57	37

4

	PBS	PAMP2c	Lesion
healthy	7	27	50
Sanded	1	8	4

5

	PBS	PAMP2c	Lesion
healthy	32	18	33
Sanded	28	46	47

6

	PBS	PAMP2c	Lesion
healthy	38	2	31
Sanded	11	23	29

7

	PBS	PAMP2c	Lesion
healthy	13	22	41
Sanded	42	45	53

8

	PBS	PAMP2c	Lesion
healthy	55	44	52
Sanded	35	14	21

6	1	31	6.1	38
	2	32	6.2	2
	3	33	6.3	31
	4	34	6.4	11
	5	35	6.5	23
	6	36	6.6	29
7	1	37	7.1	13
	2	38	7.2	22
	3	39	7.3	41
	4	40	7.4	42
	5	41	7.5	45
	6	42	7.6	53
8	1	43	8.1	55
	2	44	8.2	44
	3	45	8.3	52
	4	46	8.4	35
	5	47	8.5	14
	6	48	8.6	21
9	1	49	9.1	17
	2	50	9.2	30
	3	51	9.3	51
	4	52	9.4	40
	5	53	9.5	3
	6	54	9.6	19
10	1	55	10.1	10
	2	56	10.2	56
	3	57	10.3	25
	4	58	10.4	16
	5	59	10.5	6
	6	60	10.6	54

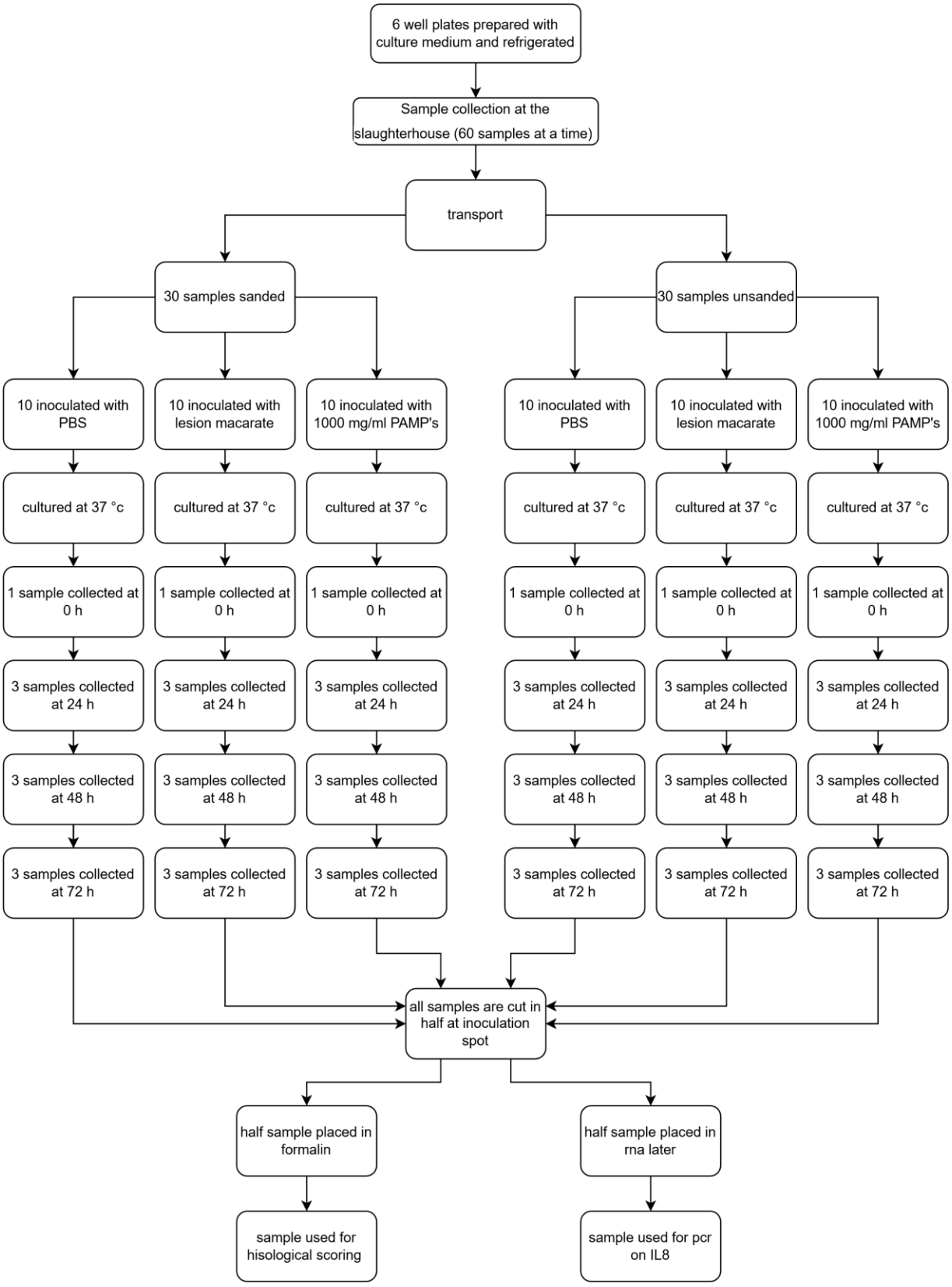
9

	PBS	PAMP2c	Lesion
healthy	17	30	51
Sanded	40	3	19

10

	PBS	PAMP2c	Lesion
healthy	10	56	25
Sanded	16	6	54

Appendix IV


6 well plates prepared with culture medium and refrigerated
Sample collection at the slaughterhouse (60 samples at a time)
transport
30 samples sanded
30 samples unsanded
10 inoculated with PBS
10 inoculated with lesion macarate
10 inoculated with 1000 mg/ml PAMP's
10 inoculated with PBS
10 inoculated with lesion macarate
10 inoculated with 1000 mg/ml PAMP's
cultured at 37 °c
cultured at 37 °c
cultured at 37 °c
cultured at 37 °c
cultured at 37 °c
cultured at 37 °c
1 sample collected at 0 h
1 sample collected at 0 h
1 sample collected at 0 h
1 sample collected at 0 h
1 sample collected at 0 h
1 sample collected at 0 h
3 samples collected at 24 h
3 samples collected at 24 h
3 samples collected at 24 h
3 samples collected at 24 h
3 samples collected at 24 h
3 samples collected at 24 h
3 samples collected at 48 h
3 samples collected at 48 h
3 samples collected at 48 h
3 samples collected at 48 h
3 samples collected at 48 h
3 samples collected at 48 h
3 samples collected at 72 h
3 samples collected at 72 h
3 samples collected at 72 h
3 samples collected at 72 h
3 samples collected at 72 h
3 samples collected at 72 h
all samples are cut in half at inoculation spot
half sample placed in formalin
half sample placed in rna later
sample used for hisological scoring
sample used for pcr on IL8