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FAKULTÄT FÜR BIOWISSENSCHAFTEN;
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# Organotypic co-culture of bovine keratinocytes and fibroblasts as a 3D skin model for studying the pathogenesis of digital dermatitis.

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**Christina - Marie Baumbach**

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Gutachter: Prof. Dr. Magin
Gutachter: Prof. Dr. Mülling

## Acknowledgements

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Leipzig, den 08.12.2016

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## II. Summary

Bovine digital dermatitis (DD) is a worldwide occurring, infectious disease in cattle primarily affecting the plantar skin above the coronary band near the interdigital cleft on hind feet. Painful ulceroproliferative lesions with acute and chronic appearances lead to behavioral changes and lameness. Hence, DD has a major impact on animal welfare and performance. The disease is also associated with large economic losses because of reduced reproduction rates, decreased milk yields and high treatment costs.

Substantial efforts in investigating the etiology of the disease revealed a synergistic origin with evidence for a multibacterial infection and the strong involvement of bacteria from the genus *Treponema*. As the interaction between host, pathogen and environment is not negligible, surrounding circumstances such as housing, general hygiene and genetic predispositions have been investigated intensively. Nevertheless, infection reservoirs, transmission routes and pathomechanisms remain widely unclear. To better understand the cellular and molecular events during *Treponema*-infection of bovine skin, it was the specific aim of this study to establish an organotypic in vitro skin model, which could be challenged with the causative agent of the disease.

A technique to reliably and reproducibly isolate primary keratinocytes and fibroblasts from the site of infection was established. Appropriate cell culture media for the long-term cultivation and storage of bovine skin cells were identified. Two different methods to develop the skin model were compared. The second strategy in which keratinocytes were directly seeded on top of a dermal equivalent, i.e. a bovine collagen type I pad with embedded post-mitotic fibroblasts, gave rise to a promising organotypic skin equivalent. The incorporated post-mitotic fibroblasts showed a characteristic cell morphology with intact nuclei. The terminal differentiation of the keratinocytes on top of the dermal equivalent was shown with anti-K14 and anti-Dsg1 immunofluorescence stainings. The results of initial *Treponema*-experiments proved that the skin equivalent is a suitable model to investigate the underlying mechanisms during *Treponema*-infection of bovine skin and hence, the pathogenesis of DD.

Future co-infection experiments with other bacteria, the usage of cells from genetically predisposed animals or the manipulation of primary cells (e.g. enzymatic treatments) may also contribute to improved prevention and treatment strategies.

## III. Zusammenfassung

Bovine Dermatitis digitalis (DD) ist eine weltweit verbreitete Infektionskrankheit bei Rindern, die primär die plantare Haut über dem Kronenrand nahe des Zwischenzehenspalts der Hinterklauen betrifft. Schmerzhafte ulzero-proliferative Läsionen mit akuten und chronischen Erscheinungsformen führen zu Verhaltensänderungen und Lahmheit der Tiere. DD hat damit einen erheblichen Einfluss auf deren Wohl und ihre Leistungen. Durch erniedrigte Reproduktionsraten und Milcherträge sowie hohe Behandlungskosten betroffener Tiere entstehen große ökonomische Verluste.

Zahlreiche Untersuchungen zur Ätiologie der Krankheit ergaben, dass es sich um das Zusammenspiel verschiedener Ursachen handelt. Einer synergistischen multibakteriellen Infektion mit starker Beteiligung von Bakterien der Gattung *Treponema* kommt dabei besondere Bedeutung zu. Aspekte wie Tierhaltung, Hygienestandards und genetische Prädispositionen wurden ebenfalls intensiv untersucht. Nichtsdestotrotz bleiben Infektionsherde, Transmissionsrouten und Pathomechanismen weitgehend unklar.

Zum besseren Verständnis der Ereignisse, die zu DD-Läsionen führen, sollte im Zuge dieser Arbeit ein organotypisches Zellkulturmodell der bovinen Haut erstellt werden, welches in späteren Versuchen mit dem Krankheitserreger zum Einsatz kommen soll. Verlässliche und reproduzierbare Techniken zur Isolation und Kultur von bovinen primären Keratinozyten und Fibroblasten wurden etabliert; geeignete Zellkulturmedien für die Langzeitkultivierung und –aufbewahrung der Hautzellen wurden identifiziert. Zur Erstellung des Hautmodells wurden zwei verschiedene Ansätze miteinander verglichen. Der zweite Ansatz, bei dem Keratinozyten direkt auf ein dermales Äquivalent, d.h. ein Pad aus bovinem Kollagen I mit eingesäten post-mitotischen Fibroblasten, gesät wurden, brachte ein vielversprechendes Hautmodell hervor. Die inkorporierten post-mitotischen Fibroblasten wiesen eine charakteristische Zellmorphologie mit intakten Nuklei auf. Die terminale Differenzierung der Keratinozyten auf dem dermalen Äquivalent wurde mittels Immunfluoreszenzfärbungen mit Antikörpern gegen die Markerproteine Keratin 14 und Desmoglein 1 gezeigt. Die Ergebnisse erster Experimente mit *Treponema* spp. verdeutlichen, dass das Hautäquivalent ein geeignetes Modell zur Untersuchung der Pathogenese der DD darstellt.

Von zukünftigen Versuchen mit Zellen genetisch prädisponierter Tiere, mit manipulierten Zellen (z. B. nach enzymatischer Behandlung) oder Co-Infektionsversuchen mit anderen Bakterienarten könnten Maßnahmen zur Verbesserung der Behandlung der Symptome sowie zu einer kausalen Prävention des Auftretens der Krankheit abgeleitet werden.

## IV. Contents

Acknowledgements ..... 2
I. Erklärung ..... 4
II. Summary ..... 5
III. Zusammenfassung ..... 6
IV. Contents ..... 8
V. List of abbreviations ..... 10
VI. Units ..... 12
VII. List of Figures ..... 13
VIII. List of Tables ..... 15
1. Introduction – Specific aims of this thesis ..... 16
2. Literature review ..... 18
2.1 Digital dermatitis (DD) ..... 18
2.2 Relevant knowledge about human and bovine skin ..... 22
2.3 Causative agents of DD belong to the bacterial genus *Treponema* ..... 27
2.4 Skin Models in Human Cell Culture and Dermatology ..... 30
2.5 Development of a bovine skin model – comparison of two strategies ..... 32
3. Material and Methods ..... 34
3.1 Material ..... 34
3.1.1 Animal material ..... 34
3.1.2 Consumables, chemicals and devices ..... 34
3.1.3 Antibodies ..... 35
3.2 Cell culture media and solutions ..... 36
3.3 Methods ..... 40
3.3.1 Primary isolation and cultivation of bovine keratinocytes and fibroblasts 40
3.3.2 Skin equivalents ..... 42
3.3.2.1 Preparations ..... 42

3.3.2.1.1 Generation of post-mitotic fibroblasts (pmF) with Mitomycin C . 42
3.3.2.1.2 Coating of the underside of 12-well inserts with pmF ..... 42
3.3.2.1.3 Collagen pads as dermal equivalents ..... 42
3.3.2.2 Skin equivalents I: Two-step-model ..... 43
3.3.2.3 Skin equivalents II: direct model ..... 44
3.3.3 Sample preparation for standard histology and immunocytochemistry/ immunohistochemistry ..... 45
3.3.4 Histological staining and immunocytochemistry/ immunohistochemistry 46
3.3.5 Culture of *Treponema* spp. ..... 47
3.3.6 Experiments with *Treponema* spp. ..... 48
4. Results ..... 50
4.1 Primary isolation and cultivation of bovine keratinocytes and fibroblasts ..... 50
4.2 Skin equivalents ..... 54
4.2.1 Preparations: Post-mitotic fibroblasts (pmF), coating of cell culture inserts and dermal equivalents ..... 54
4.2.2 Skin equivalents I: Two-step-model ..... 57
4.2.3 Skin equivalents II: direct model ..... 59
4.3 Culture of and experiments with *Treponema* spp. ..... 62
5. Discussion ..... 66
6. Outlook ..... 70
7. References ..... 71
8. Appendix ..... 80

## V. **List of abbreviations**

| | |
|---|---|
| 6-well plate | cell culture dish with six wells (also available with 12, 24, 48 or 96 wells) |
| AB | antibiotics |
| ali | air-liquid-interface culture |
| ATMP | advanced therapy medicinal product |
| BM | basal membrane |
| BPE | bovine pituitary extract |
| $CO_2$ | carbon dioxide |
| CODD | contagious ovine digital dermatitis |
| cfu/ml | colony forming units per milliliter |
| (B)DD | (bovine) digital dermatitis |
| DMEM | Dulbecco´s modified Eagle medium |
| DMSO | dimethyl sulfoxide |
| Dsg1 | desmosomal protein desmoglein 1 |
| EGF | epidermal growth factor |
| equiv | equivalent |
| Exp | experiment |
| FCS | fetal calf serum |
| H&E | histological staining with hematoxylin and eosin |
| ICC | immunocytochemistry |
| IF | intermediate filament(s) |
| IgG | immunoglobulin class G |
| IHC | immunohistochemistry |
| K 1/5/10/14 | intermediate filament keratin 1/5/10/14 |
| K-SFM | keratinocyte – serum free medium |
| LPSN | List of Prokaryotic names with Standing in Nomenclature |
| NaOH | sodium hydroxide |
| MS | histological (modified) Steiner stain |
| ns | not specified |
| OCT compound | optimal cutting temperature compound |
| oN | over night |
| ORS | outer root sheath of hair follicles |

| | |
|---|---|
| OTEB | oral treponeme enrichment broth |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| Pen/Strep | penicillin/streptomycin |
| PFA | paraformaldehyde |
| pH | negative decimal logarithm of the hydrogen ion activity in a solution |
| pmF | post-mitotic fibroblasts |
| rh | relative humidity |
| rpm | rounds per minute |
| rRNA | ribosomal ribonucleic acid |
| RT-qPCR | real time quantitative polymerase chain reaction |
| RT | room temperature |
| SB | stratum basale of epidermis |
| SC | stratum corneum of epidermis |
| SG | stratum granulosum of epidermis |
| SS | stratum spinosum of epidermis |
| T25 flask | cell culture flask with a total growth area of 25 $cm^2$ (also available with a total growth area of 75 $cm^2$ (T75) and 175 $cm^2$ (T175)) |
| TEDTA | trypsin/ethylenediaminetetraacetic acid |
| TERT | telomerase reverse transcriptase |
| Trep | *Treponema* spp. |
| w/ | with |
| w/o | without |

## VI. Units

| | |
|---|---|
| cm | centimeter |
| $cm^2$ | square centimeter |
| l | liter |
| ml | milliliter |
| µl | microliter |
| µg | microgram |
| ng | nanogram |
| h | hour(s) |
| min | minute(s) |
| U | unit(s) |
| M | molar |
| mM | millimolar |
| °C | degree Celsius |

## VII. List of Figures


Figure 1 DD stages.....18

Figure 2 Micrograph of MS-stained paraffin section of a skin biopsy taken from an acute DD lesion.....19

Figure 3 Histological specimen (left) and schematic drawing (right) of human skin.....22

Figure 4 Skin sections from different animal body sites illustrating the varying manifestations of the epidermis and the dermo-epidermal junction.....25

Figure 5 Phylogenetic tree of the domain (eu)bacteria based on 16S rRNA comparative analyses.....27

Figure 6 Classification of the genus Treponema in the phylogenetic system according to LPSN.....28

Figure 7 Interpretative drawing of basic anatomic features of spirochetes.....28

Figure 8 *Treponema* spp. culture in liquid and on solid culture media.....29

Figure 9 Application and histological evaluation of EpiDex®.....32

Figure 10 Paraffin sections of normal human skin (left column) and skin equivalents (right column).....33

Figure 11 Diagram of experimental set up of experiments with *Treponema* spp.....49

Figure 12 Bovine skin cells in culture.....50

Figure 13 Cultured bovine keratinocytes.....52

Figure 14 Cultured bovine fibroblasts.....53

Figure 15 pmF-coated cell culture insert.....55

Figure 16 Collagen pads with embedded fibroblasts.....56

Figure 17 Post-mitotic fibroblasts in collagen pads of bovine collagen type I (d35 of air-liquid-interface culture).....56

Figure 18 Skin equivalents I: Two-step-model (keratinocytes stratify on a pmF-coated cell culture insert, macroscopic).....57

Figure 19 Skin equivalents I: Two-step-model (H&E-stained paraffin sections of keratinocytes on cell culture inserts at d26 (A), d36 (B) and d42(C) of air-liquid-interface culture).....58

Figure 20 H&E-stained paraffin sections of bovine foot skin (A) and the skin equivalent II (B)……59
Figure 21 Immunofluorescence stainings of native bovine skin (A) and the skin equivalents II (B) with anti-K14, a marker of basal keratinocytes in the epidermis……..60
Figure 22 Immunofluorescence stainings of native bovine skin (A) and the skin equivalents II (B) with anti-Dsg1, an antibody directed against a desmosomal protein mainly found in suprabasal epidermal strata……..61
Figure 23 Culture of *Treponema* spp……62
Figure 24 MS-stained paraffin section of dermal equivalent after 1h of incubation with a *T. phagedenis*-like CR2220RR CV suspension under anaerobic conditions……64
Figure 25 MS-stained paraffin section of a collagen pad after 4h of incubation with a *T. phagedenis*-like CR2220RR CV suspension under anaerobic conditions……..64
Figure 26 *T. phagedenis*-like CR2220RR CV were exposed to skin equivalents (d43 of ali)……..65
Figure 27 Amplification plot of RT-qPCR run……..83

## VIII. List of Tables


Table 1 Brief description of DD stages according to Döpfer (1994) and Berry et al. (2012)……..……18

Table 2 Overview of different commercially available skin replacement products and skin models……..31

Table 3 Specifications of primary antibodies and their application protocol…….35

Table 4 Specifications of secondary antibodies and Bisbenzimide Hoechst 33342 and their application protocols……...36

Table 5 Keratinocyte Growth Medium K-SFM+ (w/ AB)……..36

Table 6 Fibroblast Growth Medium DMEM+ (w/AB)……37

Table 7 Transport medium for skin samples……37

Table 8 Solutions that were used during sample processing, 2D and 3D cell culture and staining……38

Table 9 Materials that were used for culture of *Treponema* spp……...39

Table 10 Set up and results of experiments with *Treponema* spp……...63

Table 11 Devices that were used during culture of cells and bacteria……...80

Table 12 Protocol for paraffin embedding of tissue samples……...80

Table 13 Protocol for dewaxing of paraffin-embedded tissue samples……..81

Table 14 Staining protocol for standard histological H&E-stain……81

Table 15 DNA extraction protocol for suspensions containing *Treponema* spp……...81

Table 16 Details of primer probe used to detect *Treponema* spp……82

Table 17 RT-qPCR set up……..82

Table 18 Thermal profile of RT-PCR run……82

## 1. Introduction – Specific aims of this thesis

Claw disorders and claw lesions are by far the predominant reason for lameness and one of the three major reasons for premature culling in dairy cattle. Among infectious claw diseases, Digital dermatitis (DD), also known as “hairy heel warts” or Mortellaro disease, has the highest impact on animal welfare due to its high incidence, clinical occurrence and long duration (Bruijnis et al., 2012).

Digital dermatitis (DD) is a nowadays worldwide common, distinct, highly contagious dermatitis primarily affecting intensively managed cattle (Argáez-Rodríguez et al., 1997; Blowey and Sharp, 1988; Cheli and Mortellaro, 1974; Read and Walker, 1998). In the United States, the disease is referred to as papillomatous DD (PDD) because a proliferative form of the disease predominates. In Europe, where it was initially described in 1974 (Cheli and Mortellaro, 1974), an erosive form is more common (referred to as DD; Read and Walker, 1998). However, both terms describe painful ulceroproliferative lesions around the coronary band near the interdigital cleft of mostly hind feet. In the vast majority of the cases, these lesions imply behavioral changes of the animals, e.g. lameness, walking on toes, shifting of weight from one foot to another or avoidance of movement altogether. These symptoms of discomfort and pain often lead to a reduced ingestion accompanied by weight loss, reduced milk production, decreased fertility and prolonged calving intervals (Argáez-Rodríguez et al., 1997; Rebhun et al., 1980). Additionally, treatment costs for affected animals and the higher possibility of premature culling sum up to enormous financial losses of dairies worldwide (Zinicola et al., 2015). Hence, DD entails serious animal welfare problems and economic consequences.

DD seems to be not only a growing problem in the dairy industry but also in other livestock managing industries: *Treponema* spp., the bacteria considered to be the primary causative agent of DD, have also been identified in similar lesions in beef cattle (Brown et al., 2000), dairy goats (Sullivan et al., 2015) and sheep (Duncan et al., 2014). On top of that, treponematoses/spirochaetoses are not restricted to animal hosts: venereal and endemic syphilis, yaws and pinta are tissue-destructive infectious diseases that cause skin lesions (among other impairments) in humans (Antal et al., 2002). Certain strains of *Treponema* spp. isolated from DD lesions were found to be

very similar to those associated with human periodontitis (Choi et al., 1997; You et al., 2013). Another human dermopathy called “Morgellon´s Disease” shows striking similarities to DD in terms of resembling skin lesions and an unusual filament formation in infected and adjacent non-infected skin sites. The disease is poorly studied but the spiral bacterium *Borrelia burgdorferi,* which belongs to the same order as *Treponema* ssp., is thought to play a major role in the pathogenesis (Middelveen and Stricker, 2011; Middelveen et al., 2015).

Taken together these findings consolidate the ever-growing importance of research on DD and related diseases in terms of etiology, transmission, prevention and treatment for animals and humans equally.

To better understand the pathogenesis of DD, i.e. the bacterial and cellular mechanisms that lead to the characteristic lesions, it was the aim of this study to establish an organotypic in vitro model of bovine skin, which later on will be challenged with the causative agent of the disease. By studying the interaction of spirochetes with bovine skin cells, e.g. adhesion, invasion and encystation, this research wants to add essential information to the knowledge about DD.

The specific objectives of this study were:

1. Establishment of protocols for the isolation and cultivation of pure cell cultures of bovine primary keratinocytes and fibroblasts originating from the site of infection, i.e. the plantar aspect of the bovine hind claw.

2. Establishment of a reproducible three dimensional bovine skin model consisting of stratified, differentiated keratinocytes (≙ epidermal part) and collagen-embedded fibroblasts (≙ dermal equivalent) to mimic the in vivo microenvironment of the infection site as close as possible.

3. Conduct initial experiments as proof of principle in which this skin model is challenged with different spirochetal bacteria of the genus *Treponema*.

# 2. Literature review

## 2.1 Digital dermatitis (DD)

*Disease stages.* The clinical presentation of bovine digital dermatitis and accompanying symptoms are described above (see chapter 1. Introduction – Specific aims of this thesis). The course of a DD infection can be divided into five stages (M-stages for “Mortellaro”) according to the classification scheme of Döpfer (1994) and Berry et. al (2012). A brief description of the appearance of each stage and corresponding photographs of lesions can be found in Table 1 and Figure 1, respectively.

*Table 1 Brief description of DD stages according to Döpfer (1994) and Berry et al. (2012)*

| Stage | Macroscopic appearance |
|---|---|
| M1 | Small (<2cm), focal, active state; circumscribed lesion, moist surface, foul odor, ragged, mottled red-grey with scattered small (1mm) red foci |
| M2 | Classic large (>2cm) ulcerative, active stage; extensively mottled red-grey lesions, painful upon palpation |
| M3 | Healing stage; typically few days after antibiotic treatment, ulceration transformed to dry, brown, firm scab, no pain on manipulation |
| M4 | Chronic stage; papilliform to mass-like tan/brown/black irregular hyperkeratosis |
| M4.1 | Chronic stage with small active painful M1 lesion within it |
| M5 | No sign of pre-existing lesions, normal skin |


**Figure 1 DD stages.** A M0, i.e. intact skin. B Small, circumscribed M1 lesion (blue circle). C M2 ulcer. D Proliferative M2 ulceration with heel horn erosion in multiple layers. E Brown, healing scab (M3). F M4.1 chronic stage with small M1 lesions (blue circle). Photographs are kindly provided by Prof. D. Döpfer.

*Etiology and pathogenesis.* Substantial efforts in investigating the etiology of the disease revealed a multifactorial origin with strong involvement of different phylogenetic groups from the bacterial genus *Treponema* (Collighan and Woodward, 1997; Döpfer et al., 1997; Grund et al., 1995). Since animal infection models demonstrated that DD lesions are not reproduced when bovine feet are inoculated with cultured *Treponema* spp. solely, it is widely believed that the interaction of the causative agent, the host and the environment lead to the development of the disease (Gomez et al., 2012; Krull, 2015).

The exact course of events during DD manifestation, i.e. involved bacterial and cellular structures (receptors, surface proteins etc.) and processes (mechanisms, signaling, etc.), remain mostly speculative. The disruption of the intact epidermal barrier, either by mechanical damage or maceration, seems to be a prerequisite for *Treponema* spp. to be able to begin skin invasion (Krull, 2015). Bacterial translocation through the skin tissue is hypothesized to be a four-step process beginning with the adhesion of the bacteria via adhesins with different receptor specificities. Thereafter, the epidermis is invaded via the intercellular space by disruption and penetration of cell contacts (desmosomes, gap junctions; Figure 2).

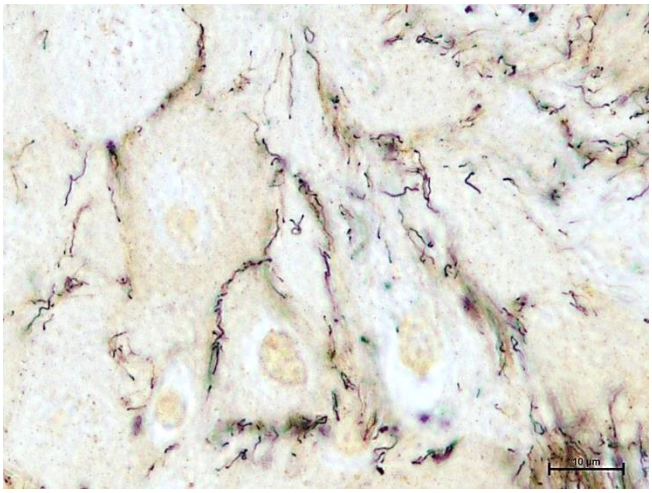

**Figure 2 Micrograph of MS-stained paraffin section of a skin biopsy taken from an acute DD lesion.** MS-stain reveals spiral organisms between the cells of the stratum spinosum of the epidermis (bar indicates 10µm; sample and micrograph are kindly provided by Profs. D. Döpfer and C. Mülling).

Subsequently, the basal membrane is partially or completely disintegrated thereby also disrupting dermal-epidermal cell signaling and finally causing uncontrolled proliferation and ulceration. A chronic state of the disease with frequent reoccurrences of symptoms is established by morphological changes and encystation of *Treponema* spp. in the dermis (personal communication with Profs. D. Döpfer and C. Mülling).

*Transmission and risk factors.* Infection reservoirs and transmission routes remain widely unclear as typical examples like the (usual) intestinal microbiota, environmental slurry or diptera do not seem to harbor DD-associated *Treponema* spp. (Evans et al., 2012).

Nevertheless, environmental factors (e.g. housing), physical and physiological properties (e.g. claw conformation, immune status), personality and behavior of the cattle (e.g. social hierarchy, coping strategies), biosecurity and farm management (e.g. buying in replacements, herd size and grouping, diet) seem to influence the occurrence and the course of DD substantially (Palmer and O'Connell, 2015).

Among the most important environmental factors are cleanliness in general and a proper foot hygiene in particular. Many studies revealed a higher prevalence of DD when moist, unhygienic conditions were found on the dairy (Argáez-Rodríguez et al., 1997; Blowey and Sharp, 1988; Döpfer et al., 1997; Relun et al., 2013). Textured concrete flooring for example seems more suited to reduce DD occurrence than solid, grooved concrete because the slurry is removed more efficiently. Wells et al. (1999) explain this with the exposure of the claws to manure, i.e. wet conditions and poor hygiene lead to softened claw tissue and worse skin integrity which is thought to facilitate infection. This is also in accordance with the finding that DD lesions are histologically similar to the human treponematosis yaws which is also promoted by poor hygiene, humid conditions and moist skin (Read et al., 1992). Likewise, cows that have full access to pasture and/or long and wide cubicles were found to be at lower risk to become diseased (Somers et al., 2005).

Cleanliness includes not only stables and animals but also all mechanical vectors that may be in contact with cattle such as rubber boots or hoof trimming equipment. According to Sullivan et al. (2014), DNA of DD-associated *Treponema* spp. was found on hoof trimming knifes and shears even after routine disinfection.

For a comprehensive risk factor assessment, biosecurity needs to be considered as well: introducing new animals to a herd, e.g. when buying in replacement heifers, increases DD prevalence compared to closed herds (Rodríguez-Lainz et al., 1996).

Age, parity and lactation state of each individual cow may also contribute to the DD prevalence. According to Somers et al. (2005), primiparous cows have a higher risk to develop DD lesions; this may be attributed to the altered metabolism and the nutritional and environmental change (due to herd management) after the first calving. Other intrinsic aspects may be genetic features and pre-dispositions: firstly, on herd level, as

different breeds seem to be more affected than others. And secondly, on cow level, seeing that some animals are recurrently and more severely infected whilst others, kept in the same herd and under the same conditions, are affected only once or never (Rodriguez-Lainz et al., 1999; Scholey et al., 2012).

*Prevention and Treatment.* To date, no reliable vaccine is available and many different treatment strategies are discussed controversially (Palmer and O'Connell, 2015). Common topical treatments include chemical disinfectants such as copper sulfate ($CuSO_4$) or formalin ($CH_2O$) applied under a bandage, as a spray or as footbath additives (Laven and Logue, 2006). However, since both substances are considered harmful and dangerous for the environment (EU regulation 1272/2008 (CLP)) special precautions in terms of dosage, application and disposal should be taken into account. As formalin is additionally considered toxic and carcinogenic, safety demands with respect to users, cattle and environment are not met at all and hence alternatives are required urgently (Speijers et al., 2010).

The off-label use of antimicrobials, e.g. oxytetracycline and erythromycin, is also widespread (Speijers et al., 2010). In contrast, systemic antibiotic treatments are rarely used due to their perceived ineffectiveness, high costs and the requirement for milk or meat withdrawal after treatment (Laven and Logue, 2006).

Lesions are often cured clinically but not bacteriologically, i.e. while clinical symptoms decline (disease stages M3 and M4), *Treponema* spp. still reside deep in the dermis of foot skin. Hence, the incomplete removal of the causative agents leads to frequently reoccurring acute lesions (M4.1 stage) and clinical symptoms (Berry et al., 2010; Döpfer et al., 1997). This puts an even stronger focus on constant control and prevention, i.e. "pro-active management approaches" are required (Laven, 2001). Establishment and maintenance of dry and hygienic living conditions and properly managed footbaths seem to be the most promising approaches to date (Rodríguez-Lainz et al., 1996). Routinely hoof trimming twice a year has also been reported to reduce DD occurrence. Whether this is because the animals are controlled and – in case of DD lesions – treated regularly or the trimming actually improves claw conformation is still a matter of debate (Laven and Logue, 2006; Rebhun et al., 1980; Relun et al., 2013; Somers et al., 2005).

## 2.2 Relevant knowledge about human and bovine skin

*Overview.* The skin is the largest organ of the human body (average: 16% of total body weight) and the outer most one making it the physical border between an organism and its direct environment (Wickett and Visscher, 2006). Accordingly, it plays a major role in the primary defense against external insults such as microbial infections, UV radiation, chemical impacts and mechanical stress.

However, next to this “outside-in” barrier function, the skin also establishes an “inside-out” barrier function by reducing dehydration and electrolyte loss (Natsuga, 2014).

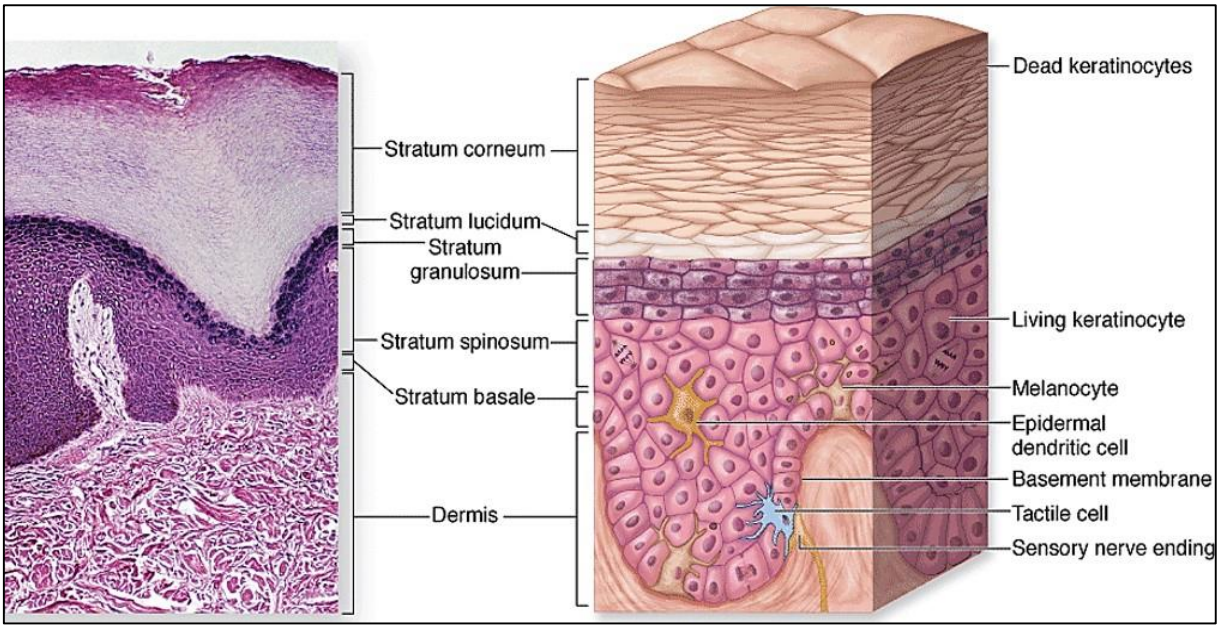


**Figure 3 Histological specimen (left) and schematic drawing (right) of human skin.** Shown are the uppermost layer of skin, the epidermis, and the underlying dermis (corium). The epidermis itself is also layered: the stratum basale (SB) is the innermost layer right above the basal membrane (BM), followed by stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC). The most abundant cell type of all epidermal strata is the keratinocyte. Interspersed are melanocytes, dendritic and tactile cells. The dermis mainly consists of collagen produced by fibroblasts (adapted from Junqueira´s Basic Histology; Text and Atlas, 12th Edition).

The skin consists of the epidermis and the dermis (together also termed cutis) and is associated with the subcutis. Dermis and subcutis mainly accomplish the mechanical support, nutritional supply, shock absorption and thermoregulation. Skin appendages such as hair follicles, sweat and sebaceous glands are found in the vascularized and innervated dermis (Dyce et al., 1987).

The aforementioned barrier function of the skin is primarily realized by its outermost layer, the epidermis (Fuchs and Raghavan, 2002). This avascular structure consists of

multiple layers, or strata, of keratinocytes which are connected by desmosomes and other cell-cell junctions (adherence, tight and gap junctions; Baroni et al., 2012).
85% of the epithelial cells are keratinocytes; other cell types are e.g. melanocytes (5-8%), Langerhans´ cells and Merkel´s disks (Jubb, 1985). Melanocytes reside in the stratum basale (SB). These cells produce melanin which is transferred (in so-called melanosomes) to up to 36 neighboring keratinocytes where it protects their DNA from the impact of UV radiation (Seiberg, 2001).
Interspersed in basal and suprabasal cell layers of the epidermis are Langerhans´ cells. These are the professional antigen-presenting cells of the skin and, accordingly, a part of the adaptive immune system (Chomiczewska et al., 2009).
At least four different cell types are known to transmit tactile perception to the brain, i.e. Pacinian and Meissner corpuscles, Merkel´s disks and Ruffini end organs, each of which being specialized in the sensation of a distinct magnitude of touch (McGlone and Reilly, 2010).

*Keratinocytes are the most abundant cell type of the epidermis.* Viable, proliferating keratinocytes make up the innermost layer of the epidermis, the stratum basale (SB), just above the basal membrane (BM). This germinative layer harbors stem cells and their progeny, i.e. transit amplifying cells (Fuchs, 1990), which enable the constant renewal of the skin by asymmetric cell division. The BM is known to be a “stabilizing as well as dynamic interface” (Breitkreutz et al., 2009) between dermis and epidermis. Its main components laminin, collagen type IV, nidogen and perlecan are secreted by epidermal keratinocytes and dermal fibroblasts equally. The BM plays a vital role in the control of traffic of cells and bioactive molecules from the dermis to the epidermis and vice versa (Breitkreutz et al., 2009).
The next upward epidermal layer is the stratum spinosum (SS). Post-mitotic but metabolically active keratinocytes herein undergo a complex differentiation program. Protein biosynthesis changes completely, e.g. the cytoskeleton is rearranged from keratin 5/14 intermediate filaments (mainly found in SB) to keratin 1/10 intermediate filaments (mainly found in the stratum corneum, SC; Fuchs, 1990). Lipid compounds and different hydrolyzing enzymes for both the lipid and the cornified envelope of the SC are produced (Candi et al., 2005).
In the stratum granulosum (SG), these lipid compounds, namely ceramides, cholesterols and fatty acids, are packed into granules (≙ lamellar bodies), and

extruded into the extracellular space. Here, they are metabolically modified by hydrolyzing enzymes such as β- glucocerebroside and phospholipase $A_2$. The lipids contribute to the lipid envelope which plays an important role in the formation and maintenance of the epidermal barrier (Candi et al., 2005). Tight junctions seal the keratinocytes in the SG together and control paracellular pathways of molecules (Baroni et al., 2012).

Concurrently, the production of structural proteins as involucrin, loricrin and small prolin-rich proteins (SPRs) as well as transglutaminases and profilaggrin takes place. Among the last steps of keratinocyte differentiation are:

- keratin filaments are aggregated into tight bundles by filaggrin
- involucrin, trichohyalin and other proteins are crosslinked by transglutaminases
- nuclei and mitochondria are depleted
- desmosomes are reorganized into corneodesmosomes (Candi et al., 2005; Wickett and Visscher, 2006).

The end product of this cornification process are dead, flat, anucleate corneocytes packed with keratin-protein-complexes and surrounded by insoluble lipids making the stratum corneum (compactum) an almost impassable physical barrier. The “live cycle” of all keratinocytes ends when corneodesmosomes are proteolytically degraded leading to the desquamation of the corneocytes approximately two weeks after leaving the SB (Fuchs, 1990).

*Comparison of human and bovine skin.* In general, bovine skin shows the typical morphology of mammalian skin, its overall composition and function (Netzlaff et al., 2006; Pitman and Rostas, 1982; Stahl et al., 2009).

Depending on the body site and different load situations, specific adaptations of the skin layers are observed in all mammalian species (see Figure 4). For instance, with increasing mechanical stress, the epidermis develops more and more cell layers and cornified layers become more pronounced. Elias et al. (1981) report a mean stratum corneum thickness for human abdominal skin of 21.8µm and for leg skin of 26.8µm, respectively. This load-related difference correlates with data from bovine skin: udder stratum corneum was found to be 10.1µm thick (Stahl et al., 2009) whereas heel skin stratum corneum, which is subjected to much more physical stress, was found to be on average 34.56µm thick (adapted from Palmer et al., 2013). Especially under

compression, tensile and/or torsion load, dermis and epidermis form interdigitating finger- or ridge-like projections (epidermal rete ridges, dermal papillae) that enlarge their connecting area and, hence, their adherence (Dyce et al., 1987). Figure 4 illustrates some of the different manifestations of the epidermis and the dermo-epidermal junction, not only in cattle but also in other mammalian species.

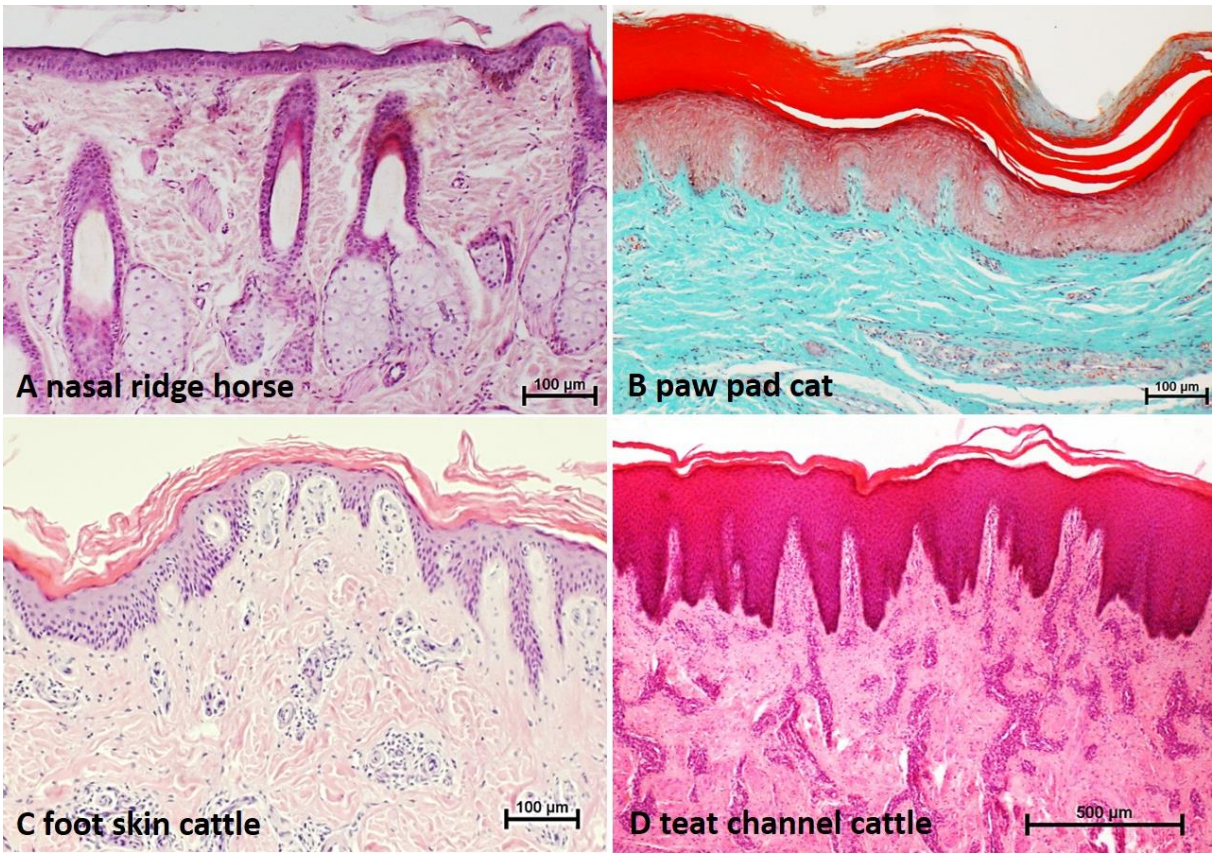


**Figure 4 Skin sections from different animal body sites illustrating the varying manifestations of the epidermis and the dermo-epidermal junction.** A Nasal ridge, thin epidermis (H&E, horse; bar ≙ 100µm). B Feline paw pad, pronounced stratum corneum (trichrome stain, cat; bar ≙ 100µm, fissures are embedding artefacts). C Bovine foot skin from the plantar aspect of the hind limb between main claws and dewclaws, distinct rete ridges and papillae (H&E, cattle; bar ≙ 100µm). D Teat channel, pronounced epidermal rete ridges and dermal papillae (H&E, cattle; bar ≙ 500µm). The histological specimens A, B and D were kindly provided by Profs Mülling and Seeger, the sample of native bovine skin was taken as part of the current project.

Differences between human and cattle skin are found e.g. in the epidermal lipid composition (Netzlaff et al., 2006; Stahl et al., 2009) and the pH (Meyer and Neurand, 1991). Meyer and Neurand (1991) found slightly acidic to neutral pH values of 6.49 to 8.8 in cattle skin depending on sex, physical stress and excitement of the animal whereas the normal human skin pH is around 5.5 (Lambers et al., 2006).

The number of hair follicles and other skin appendages also differs greatly: depending on the body site up to 900 hair follicles/$cm^2$ are found in bovine skin in contrast to 40 to 70 hair follicles/$cm^2$ in human skin (Pitman and Rostas, 1982). This large number of hair follicles might be of special importance in the context of DD since hair follicles were proposed to serve as an entrance route for pathogens to deeper skin layers and/or as bacterial reservoirs from which re-infections could emanate (Knorr et al., 2009; Palmer and O'Connell, 2015).

## 2.3 Causative agents of DD belong to the bacterial genus *Treponema*

With spirochete bacteria from the genus *Treponema* being found consistently in DD lesions and deep in underlying tissue, they are thought to be the primary causal agent of the disease (Choi et al., 1997; Dhawi et al., 2005; Döpfer et al., 1997; Evans et al., 2009; Walker et al., 1995).

*Phylogeny.* The phylum Spirochetes is one of 30 phyla in the domain (eu)bacteria according to the List of Prokaryotic names with Standing in Nomenclature (LPSN, accessed 10/26/2016; Euzéby, 1997; Parte, 2014).

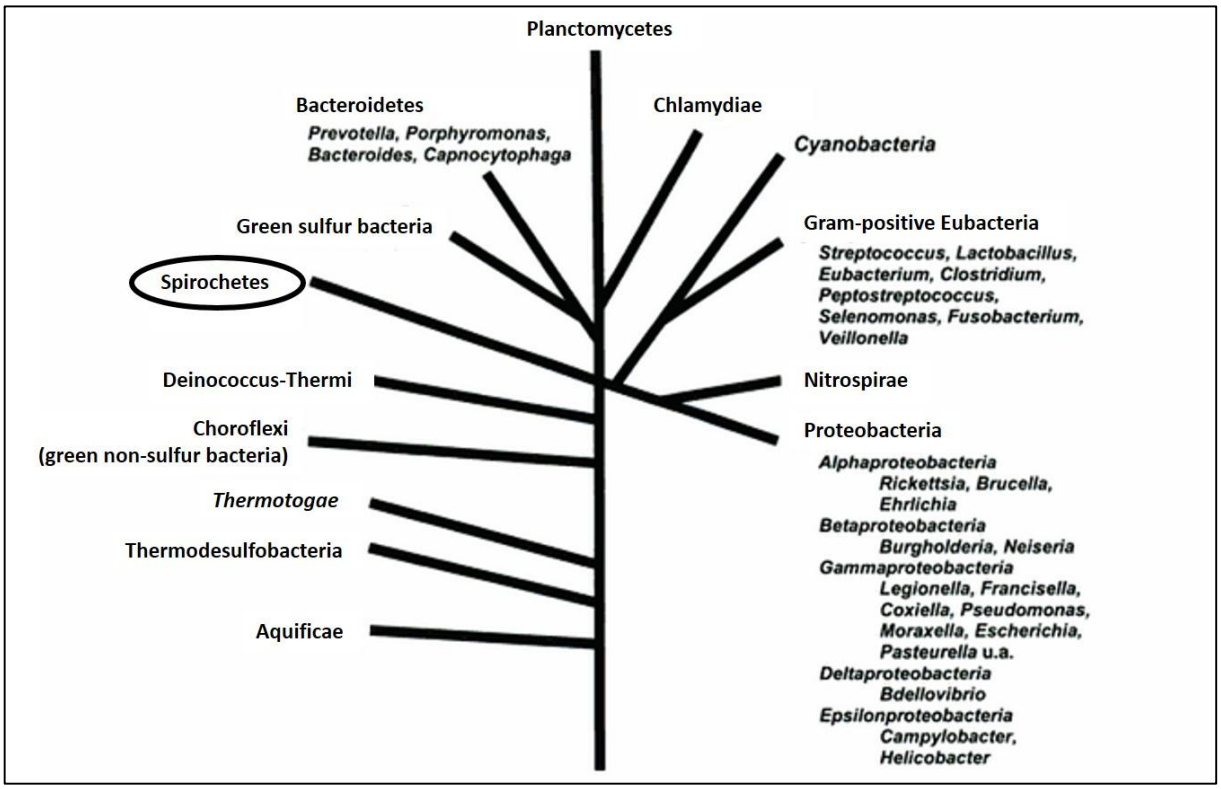


**Figure 5 Phylogenetic tree of the domain (eu)bacteria based on 16S rRNA comparative analyses**; not all of the 30 phyla that are currently accepted by the List of Prokaryotic names with Standing in Nomenclature (LPSN; October 2016) are shown. (Scheme adapted from LPSN and „Medizinische Mikrobiologie, Infektions- und Seuchenlehre", M. Rolle and A. Mayr, 8th edition, 2007)

Spirochetes are morphologically distinct from all other bacteria: they are helical-shaped with tight, loose, regular or irregular coils, remarkably thin (in terms of the ratio length to diameter), flexible, very motile and all possess a varying number of up to several hundred endo-flagella (Edwards et al., 2003; Rolle and Mayr, 2007).

*Treponema*. Treponema is one of the genera in the family Spirochaetaceae in the order Spirochaetales in the class Spirochaetes (LPSN, accessed 10/26/2016; Euzéby, 1997; Parte, 2014).

Domain: Eubacteria (next to Archaea and Eucaryota)
Phylum: Spirochaetes or Spirochaetae (next to Cyano-, Proteo-, Fusobacteria ...)
Class: Spirochaetes
Order: Spirochaetales
Family: Spirochaetaceae
Genera: Clevelandina, Diplocalyx, Hollandina, Pillotina, Salinispira, Spirochaeta, Treponema
Family: Borreliaceae
Genera: Borrelia, Borreliella, Cristispira
Family: *unassigned*
Genera: Exilispira

**Figure 6 Classification of the genus Treponema in the phylogenetic system according to LPSN** (http://www.bacterio.net/-classifphyla.html, accessed 10/26/2016).

Spiral treponemes range in diameter from 0.09 to 0.5µm and in length from five to 20µm; the cell poles are rounded or tapered. They possess up to ten endo-flagella (or axial fibrils, AF) that are situated between the protoplasmic cylinder and an outer sheath. One end of each flagellum is anchored at one cell pole; the other end is free in the periplasm enabling an active motility in a rotational manner around the longitudinal axis of the cell (Holt, 1978). Their metabolism is based on chemoheterotrophy and growth is restricted to anaerobic conditions (Demirkan et al., 1999; Walker et al., 1995).

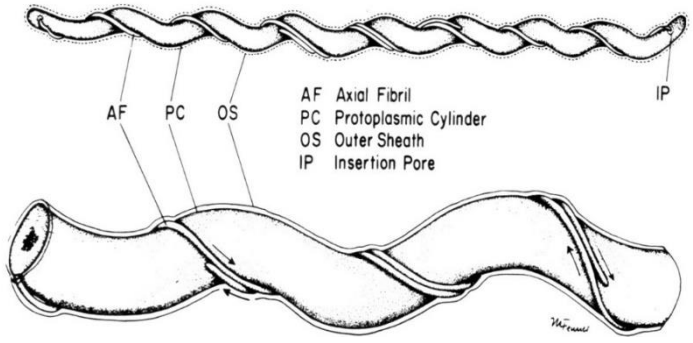


**Figure 7 Interpretative drawing of basic anatomic features of spirochetes.** AF- axial fibril; PC - protoplasmic cylinder; OS - outer sheath; IP - insertion pore of axial fibril (adapted from Holt, 1978).

During the exponential growth phase (4-7 days of culture depending on the strain), cells in broth culture look like “fine strands of cotton” (Demirkan et al., 1999) and are

very motile; circular forms and intermediate shapes appear after thawing from a frozen stock or in older cultures. For the spirochete *Borrelia burgdorferi* and other species under study was shown that spiral cells encyst in hostile environments and converse back into vegetative forms under more favorable conditions (Murgia and Cinco, 2004). In how far these encysted, presumably metabolically dormant forms of *Treponema* spp. contribute to the frequent reoccurrence of the clinical symptoms of DD needs to be further examined (Döpfer et al., 2012).

*Treponema* spp. colonies on fastidious anaerobe agar (FAA) are circular, translucent and up to 1mm in diameter (sub-surface growth is also possible). They are visible after 10 to 14d of anaerobic culture at 37°C. Depending on the strain, accelerated growth is seen on agar plates that contain 5% sheep blood. On blood agar plates, hemolysis was reported for some strains of *Treponema* (Walker et al., 1995).

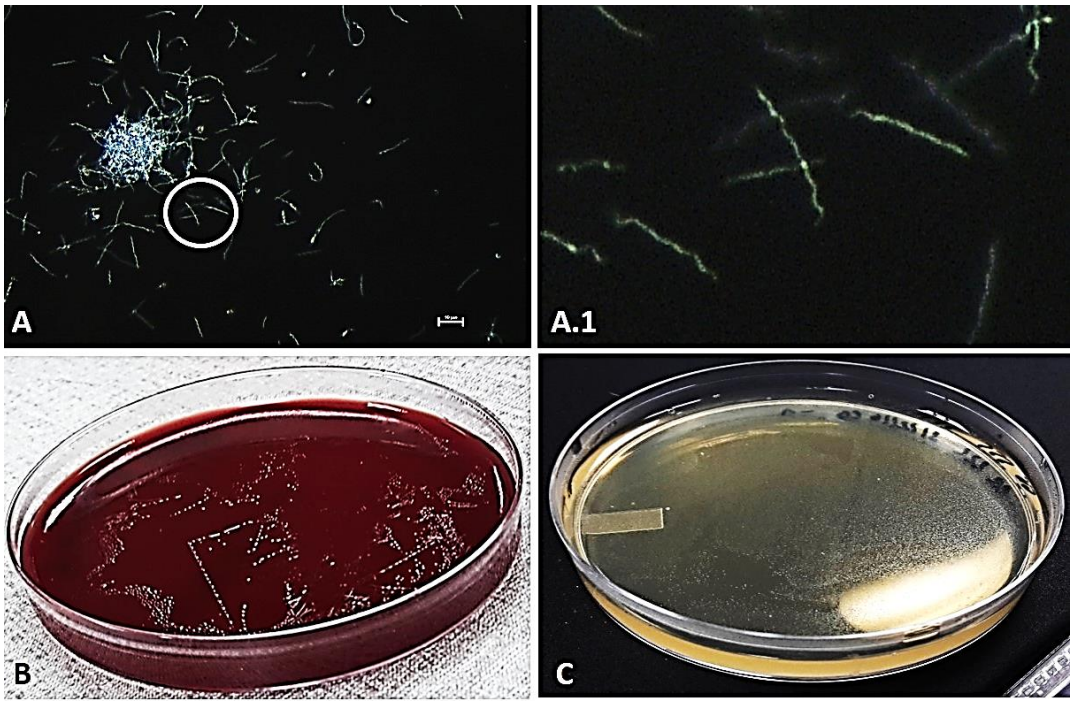


**Figure 8 *Treponema* spp. culture in liquid and on solid culture media.** A Dark field microscopy of *T. denticola*-like 1-9185MED in OTEB culture (bar indicates 10µm). A.1 Magnification of white circle in A; note the eponymic spiral shape.
Small, circular *T. denticola*-like 1-9185MED-colonies on solid sheep blood agar (B) and on fastidious anaerobe agar (FAA) plates (C).

Based on sequence identity screenings of 16s rRNA, isolates from DD-lesions usually belong to one of the following phylotypes or operational taxonomic units (OTU; Evans et al., 2008; You et al., 2013): *T. medium/vincentii*-like, *T. phagedenis*-like and *T. denticola/putidum-like.*

## 2.4 Skin Models in Human Cell Culture and Dermatology

It was the aim of the current thesis to establish an organotypic in vitro skin model to investigate the pathomechanisms of DD.

The theoretical basis for this skin model is provided by more than 100 years of clinical and experimental research in the field of human dermatology and cell culture. The first skin grafting experiments were conducted in 1871 by the French surgeon Jaques-Louis Reverdin (Reverdin, 1871). He described not only autologous but also allogenic (European donor/European recipient, African donor/European recipient) and xenogenic (rabbit skin) transplantations of skin pieces (Mudry, 2014). His early discoveries were further developed and nowadays autologous split-thickness skin grafts represent the “gold standard” of skin injury treatment (Kremer and Berger, 2000). Cultured and/or tissue-engineered skin substitutes were developed since extensive burn injuries and chronic non-healing wounds impose even higher requirements on skin grafts (Horch et al., 2005). This advanced phase of artificial skin development started undoubtedly with the pioneering work of Rheinwald and Green in 1975 about the serial cultivation of human keratinocytes on irradiated feeder cells (Rheinwald and Green, 1975).

To date, numerous skin replacement products for clinical applications and skin models for pharmaceutical safety assessments are available. They are very divers in composition (e.g. epidermal, dermal, composite with bioengineered scaffolds), durability (temporary or permanent) and manufacturing process (e.g. ad hoc transplantation, cell culture, refrigeration/cryopreservation etc.; Horch et al., 2005; Kagan et al., 2005). Table 2 describes selected skin substitutes and models in more detail.

*Table 2 Overview of different commercially available skin replacement products and skin models.*

| **Product** | **Composition** | **Indication/Purpose** | **Manufacturer/ Reference** |
|---|---|---|---|
| *Fully synthetic product* | | | |
| EpiGARD® | acellular, synthetic Teflon® matrix | temporal wound closure | Biovision GmbH www.biovison.de |
| *Epidermal replacement product* | | | |
| ReCell® (autologous) | keratinocyte suspension (not cultured) | deep partial thickness burns | Avita medical www.avitamedical.com (Gravante et al., 2007) |
| EpiCell® (autologous; considered xenotransplant due to manufacturing process) | autologous keratinocyte sheets | deep dermal/full thickness burn wounds | Vericel Corporation, USA www.epicell.com (Rheinwald and Green, 1975) |
| *Dermal replacement product (additional epidermal replacement needed)* | | | |
| AlloDerm® (allogenic) | decellularized human dermis | 3rd degree burn wounds, full-thickness skin damages | LifeCell Corporation www.lifecell.com |
| INTEGRA™ Bilayer Matrix Wound Dressing (allogenic) | bovine tendon collagen with chondroitin-6-sulfate (10-15%) and silicon membrane | 3rd degree burn wounds, scar corrections | Integra LifeSciences Corporation www.integralife.com |
| *Full-thickness skin graft* | | | |
| ApliGraf® (allogenic) | bovine collagen type I matrix with allogenic neonatal fibroblasts and epidermal layer (neonatal, differentiated keratinocytes) | application permit for venous leg and diabetic foot ulcers (USA) | OrganoGenesis Inc. www.apligraf.com |
| *Skin models for scientific purposes* | | | |
| epidermal equivalent | primary human keratinocytes on filter inserts | studies on bacterial adhesion, infection, biofilm formation etc. | Breij et al., 2012 |
| full-thickness skin equivalent | TERT-immortalized keratinocytes and fibroblasts | high throughput studies of products/drugs | Reijnders et al., 2015 |

## 2.5 Development of a bovine skin model – comparison of two strategies

*Skin equivalent I: Two-step-model.* The first strategy is based on EpiDex® (euroderm GmbH, Leipzig, Germany) which is a tissue-engineered skin replacement product resembling the human epidermis. Here, autologous keratinocytes are expanded from the outer root sheath (ORS) of human hair follicles. Follicles in the anagen growth phase are plucked and transferred onto 6-well cell culture inserts that carry a so-called feeder layer of post-mitotic human fibroblasts on their underside. A specially designed keratinocyte growth medium promotes the outgrowth of keratinocytes from the ORS. When reaching confluence, the cells are detached and transferred to a 12-well cell culture insert also carrying a fibroblast feeder layer. Cell differentiation and stratification, i.e. the formation of an epidermis-like cell sheet, is induced by a special culture method where the cells are cultivated at the interface between medium and air. After approximately two weeks of cultivation, a fully differentiated in vitro generated epidermis equivalent is ready to be shipped and transplanted (Limat et al., 1996; Ortega-Zilic et al., 2010; personal communication with co-workers). EpiDex® was an officially approved advanced therapy medicinal product (ATMP) in Switzerland (approved by Swiss Agency for Therapeutic Products; date of approval: 07/03/2013; Swissmedic Journal 07/2013, 08/2013, pp. 588/589).

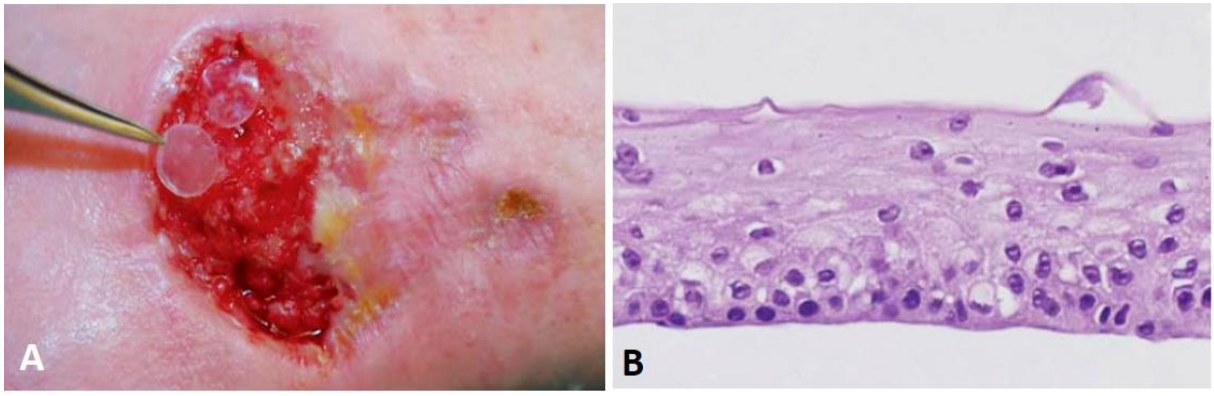


**Figure 9 Application and histological evaluation of EpiDex®**. A EpiDex® discs are directly placed on a chronic leg ulcer, the basal side facing the wound. B Histologic analysis of EpiDex® showing epidermal differentiation of the cultured keratinocytes, i.e. stratum basale, stratum granulosum and a faint stratum corneum (adapted from Ortega-Zilic et al., 2010).

In the bovine skin equivalent, keratinocytes stratify at the air-liquid-interface (ali) on a 12-well cell culture insert that is coated with growth-promoting post-mitotic fibroblasts (pmF) on the underside, as well. The resulting epithelium-like cell sheet is then

removed from the insert membrane and transferred to a readily prepared dermal equivalent to form a full thickness skin equivalent.

*Skin equivalent II: direct model.* The second strategy is a one-step model where keratinocytes are directly seeded onto prepared dermal equivalents and stratify during 21 days of air-liquid-interface culture without further manipulation. This method was pursued by Voersmann et al. (2013) who developed this full-thickness skin model for in vitro drug safety testing experiments.

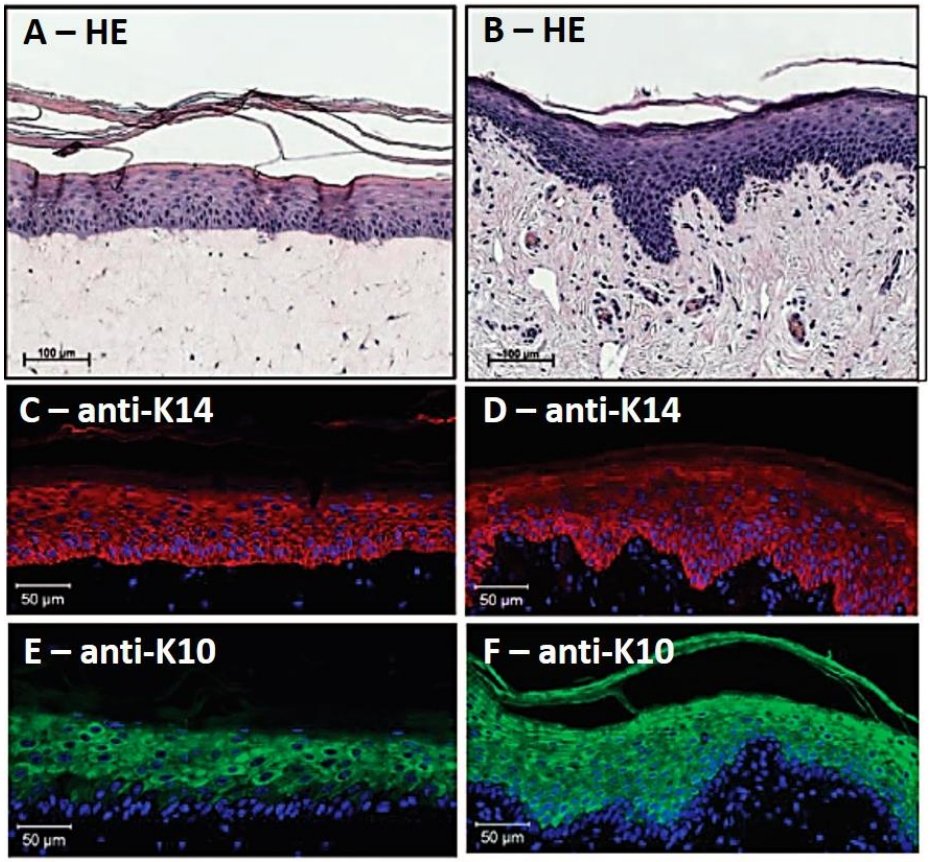


**Figure 10 Paraffin sections of normal human skin (left column) and skin equivalents (right column).** The artificial skin equivalent shows great similarity to normal human skin in terms of overall structure and differentiation status of the incorporated cells. First row: H&E-stained sections (bars indicate 100µm). Second row: immunofluorescence staining with anti-K14 directed against a basal keratinocyte marker (bars indicate 50µm). Third row: immunofluorescence staining with anti-K10 directed against K10 which is found in more differentiated keratinocytes (bars indicate 50µm; adapted from Vörsmann et al., 2013).

Following this method, the bovine keratinocytes are also seeded directly onto a dermal equivalent, which in this case is made from bovine collagen type I and contains bovine fibroblasts, and cultured at the air-liquid-interface.

# 3. Material and Methods

## 3.1 Material

### 3.1.1 Animal material

In total, 76 distal limbs of hind feet from adult dairy cows (mainly Holstein-Friesian) were obtained from a local slaughterhouse, transported to the Institute of Veterinary-Anatomy, University of Leipzig, Germany or the School of Veterinary Medicine, University of Wisconsin-Madison, USA and processed as soon as possible after slaughtering.

### 3.1.2 Consumables, chemicals and devices

Plastic ware and reaction tubes were obtained from TPP Techno Plastic Products (Trasadingen, Switzerland), Eppendorf AG (Hamburg, Germany), Corning (Corning, USA), Greiner Bio-One (Frickenhausen, Germany) and Sarstedt (Nuembrecht, Germany).

Chemicals were purchased from Carl Roth AG (Karlsruhe, Germany), Sigma Aldrich (Taufkirchen, Germany), Biochrom GmbH (Berlin, Germany), AppliChem (Darmstadt, Germany) and Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA).

Cell culture media, buffer solutions, antibiotics and antibodies were obtained from Invitrogen/Life Technologies & Gibco BRL (Karlsruhe, Germany), Capricorn Scientific GmbH (Ebsdorfergrund, Germany), Sigma Aldrich (Taufkirchen, Germany), Bethyl Laboratories Inc. (Montgomery, Texas, USA), Dianova (Hamburg, Germany), antibodies-online GmbH (Aachen, Germany) and Merck KGaA (Darmstadt, Germany).

For an overview over devices that were used during culture of cells and bacteria, see Table 11 (appendix).

### 3.1.3 Antibodies

*Table 3 Specifications of primary antibodies and their application protocol.*

| **Antigen** | **Species** | **Sub class** | **Sample pretreatment/ dilution (in PBS)/ incubation** | **Supplier (Cat. no.)** |
|---|---|---|---|---|
| anti-Desmoglein-1 | mouse (clone 32-2B; monoclonal) | IgG2 | IHC: paraffin/cryo sections, PFA-fix (Meth/Ac-fix also possible), 1:100, 4°C oN | Merck (MABT118) |
| anti-Keratin 14 | guinea pig (polyclonal) | ns | IHC: cryo sections, PFA-fix,1:50, 4°C oN<br>ICC: PFA-fix, 1:50, 4°C oN | Antibodies-online (ABIN113455) |
| anti-Keratin10 | mouse (monoclonal) | IgG1 | IHC: cryo sections, PFA-fix, 1:50, 4°C oN | Antibodies-online (ABIN1106899) |
| anti-Pan-CK | Mouse (C-11; monoclonal) | IgG1 | IHC: PFA-fix, 1:20, 4°C oN<br>ICC: PFA-fix, 1:20, 4°C oN | Sigma (C2931) |
| anti-Vimentin-Cy3 | Mouse (V9; monoclonal) | IgG | IHC: paraffin/cryo sections, PFA-fix,1:500, 4°C oN<br>ICC: PFA-fix,1:500, 4°C oN | Sigma (C9080) |
| anti-Ki67 | Mouse (Ki-67P; monoclonal | IgG1 | IHC: cryo sections, PFA-fix,1:50, 4°C oN<br>ICC: PFA-fix, 1:100, 4°C oN | Dianova (DIA-670-P1) |

*Table 4 Specifications of secondary antibodies and Bisbenzimide Hoechst 33342 and their application protocols.*

| **Name** | **Antigen** | **Species** | **Dilution (in PBS)/ incubation** | **Supplier (Cat. no.)** |
|---|---|---|---|---|
| goat@mouse488 | mouse | goat | 1:500, 2h RT (dark) | Bethyllab.inc (A90-516D2) |
| donkey@gp647 | guinea pig | donkey | 1:500, 2h RT (dark) | Merck (AP193SA6) |
| Bisbenzimide Hoechst 33342 | - | - | 1:1000, 2h RT (dark) | Invitrogen (H1399) |

## 3.2 Cell culture media and solutions

*Table 5 Keratinocyte Growth Medium K-SFM+ (w/ AB).*

| **Component** | **Supplier (Cat. no.)** | **Final concentration** | **Volume for 50ml [ml]** |
|---|---|---|---|
| K-SFM basal medium | Gibco (17005-034) | - | 43.5 |
| FCS | Gibco (10270-106) | 10% | 5 |
| BPE | Gibco (13028-014) | 10ng/ml | 0.05 |
| EGF | Gibco (10450-013) | 10ng/ml | 0.005 |
| *antibiotics (w/AB)* | | | |
| Penicillin/Streptomycin (Pen/Strep) | Gibco (15140-122) | 1% (100U/ml; 10-100µg/ml) | 0.5 |
| Amphotericin B (Fungizone) | Gibco (15290-026) | 1% (2.5-3µg/ml) | 0.5 |
| Gentamicin | Capricorn Scientific (GEN-10B) | 1% (15-50µg/ml) | 0.5 |

*Table 6 Fibroblast Growth Medium DMEM+ (w/AB).*

| **Component** | **Supplier (Cat. no.)** | **Final concentration** | **Volume for 50ml [ml]** |
|---|---|---|---|
| DMEM (4.5g/l Glucose) | Gibco (61965-026) | - | 43.5 |
| FCS | Gibco (10270-106) | 10% | 5 |
| EGF | Gibco (10450-013) | 10ng/ml | 0.01 |
| *antibiotics (w/AB)* | | | |
| Penicillin/Streptomycin (Pen/Strep) | Gibco (15140-122) | 1% (100U/ml; 10-100µg/ml) | 0.5 |
| Amphotericin B (Fungizone) | Gibco (15290-026) | 1% (2.5-3µg/ml) | 0.5 |
| Gentamicin | Capricorn Scientific (GEN-10B) | 1% (15-50µg/ml) | 0.5 |

*Table 7 Transport medium for skin samples.*

| **Component** | **Supplier (Cat. no.)** | **Final concentration** | **Volume for 50ml [ml]** |
|---|---|---|---|
| DMEM (4.5g/l Glucose) | Gibco (61965-026) | - | 48.5 |
| *antibiotics (w/AB)* | | | |
| Penicillin/Streptomycin (Pen/Strep) | Gibco (15140-122) | 1% (100U/ml; 10-100µg/ml) | 0.5 |
| Amphotericin B (Fungizone) | Gibco (15290-026) | 1% (2.5-3µg/ml) | 0.5 |
| Gentamicin | Capricorn Scientific (GEN-10B) | 1% (15-50µg/ml) | 0.5 |

*Table 8 Solutions that were used during sample processing, 2D and 3D cell culture and staining.*

| **Solution** | **Preparation** | **Supplier (Cat. no.)** |
|---|---|---|
| PBS w/o $CaCl_2$/$MgCl_2$ | ready-to-use | Gibco (14190-094) |
| PBS w/ $CaCl_2$/$MgCl_2$ | ready-to-use | Gibco (14040-133) |
| 10xDMEM | ready-to-use | Sigma (D5648) |
| Trypsin-EDTA 0.25% | ready-to-use [= stock] | Gibco (25200-072) |
| Trypsin-EDTA 0.05% | stock diluted with DMEM (4.5g/l Glucose) | made from Gibco (25200-072) |
| Trypan Blue | = stock, use 1:1 with sample | Biochrom AG (L6323) |
| Dimethyl sulfoxide (DMSO) | = stock, use 1:10 with FCS | MP Biomedical (196055) |
| Mitomycin C | prepare stock: 1mg/ml in PBS w/ $MgCl_2$/$CaCl_2$ | Sigma (M4287-2mg) |
| Collagen Type I (4mg/ml) | pads: 8:1 with buffering solutions | Merck (L7213) |
| Isopentane | ready-to-use; precool to -80°C | VWR (103614T) |
| Methanol | ready-to-use, precool to -20°C | Roth (4627.4) |
| Acetone | ready-to-use, precool to -20°C | Roth (9372.4) |
| Dispase II | prepare stock: 10mg/ml in PBS w/o $MgCl_2$/$CaCl_2$ | Gibco (17105-041) |
| Histogel™ | ready-to-use; liquefy in water bath at 100°C | ThermoScientific (HG-4000-012) |
| Donkey normal serum | = stock, use at 10% | Capricorn Scientific (DOS-F) |
| Goat normal serum | = stock, use at 10% | PAA (B11-035) |
| Triton® X-100 | = stock, use at 0.1% in PBS | AppliChem (A4975.0100) |
| Shandon Immu-Mount™ | ready-to-use | ThermoScientific (9990412) |
| O.C.T. compound | Ready-to-use | VWR (361603E) |

If not indicated differently, all media and solutions were prewarmed to 37°C in a water bath before usage.

Amphotericin B was added to the growth media only during the first week of cell culture due to its potential negative influence on cells as well (Harmsen et al., 2011). Additionally, penicillin and streptomycin were continuously reduced during cell culture and finally omitted (latest by week 3). Cell cultures that were contaminated with bacteria or fungi thereafter were discarded.

*Table 9 Materials that were used for culture of Treponema spp.*

| **Material** | **Supplier (cat. Nr.)** |
|---|---|
| Oral Treponeme enrichment broth (OTEB) in 7ml glass vials | Anaerobe Systems (AS-603) |
| Fastidious anaerobe agar (FAA) | Neogen (7531A) |
| Anaerobic blood agar plates: Remel™ (CSC formulation) | Thermo Fisher Scientific (R01040) |
| Fetal Calf Serum (FCS) | Gibco (10270-106) |
| Rifampicin (1µg/ml) | Sigma (1604202) |

## 3.3 Methods

### 3.3.1 Primary isolation and cultivation of bovine keratinocytes and fibroblasts

Bovine distal limbs were cleaned thoroughly with brushes under running tap water; adhering dirt had to be removed completely to minimize bacterial and/or fungal infection of the cell cultures. The area between heel bulbs and dewclaws was disinfected with 70% ethanol and iodine solution and shaved with a disposable razor. Skin samples of approximately 3x5 cm were cut out from this area using scalpel and hemostat. The specimens were transferred to a 50 ml falcon tube containing the transport medium and incubated for about one hour at room temperature (RT) with occasional shaking.

Subsequently, skin samples were transferred to 6 cm petri dishes in a sterile biosafety cabinet. For (immuno)histological preparations of native skin, samples of 1x1 cm were cut out and transferred to another 6 cm petri dish. Further treatment is described in chapter “3.3.3 Sample preparation for standard histology and immunocytochemistry/ immunohistochemistry”.

Dermal and fatty tissue were removed from the remaining specimen as much as possible and the specimens were cut into smaller cubes (2x2 mm) using scalpel and forceps. For washing purposes, these cubes were transferred successively to three 6 cm petri dishes containing fresh transport medium for 1 min each. Afterwards, the skin pieces were transferred to 15ml falcon tubes (2 per donor) with 3 ml of 0.25% Trypsin-EDTA solution (TEDTA) supplemented with Pen/Strep (100 U/ml / 100 µg/ml) and Amphotericin (2.5 µg/ml).

Following overnight incubation at 4°C, these 15 ml tubes were kept at room temperature for about one hour with occasional shaking. Two ml of fetal calf serum were added to stop the enzymatic digestion. The tubes were shaken vigorously and the cell suspension was microfiltered through cell strainers (pore size Ø100 µm) into 50 ml falcon tubes. The micro filter was rinsed twice with PBS w/o $MgCl_2$/$CaCl_2$. The tubes were centrifuged at 1500 rpm at RT for 4 min and the supernatant was aspirated. The cell pellet was re-suspended in 12 ml of the culture medium K-SFM+ w/AB and the cells were seeded into each well of two 6-well plates prepared with 1 ml K-SFM+ w/AB per well. The culture plates were incubated at 37°C, 5% $CO_2$ and 95% relative humidity (rh). The medium was changed every 2 to 3 days. If necessary, the cells were washed with PBS w/o $MgCl_2$/$CaCl_2$ before adding new medium.

K-SFM+ is a specially designed, commercially available culture medium for epithelial cells but it also promotes the growth of other, less demanding cell types such as fibroblasts. To establish pure cultures of both cell types, i.e. keratinocytes and fibroblasts, the primary cultures were differentially trypsinized. Under TEDTA-treatment, fibroblasts detach from cell culture dishes already after an incubation time of 3 min whereas keratinocytes detach only after 10 to 15 min. Hence, the primary cultures were incubated with 0.25% TEDTA for 3 min at RT after washing with PBS w/o $MgCl_2$/$CaCl_2$. The enzymatic reaction was stopped with 10% FCS in PBS w/o $MgCl_2$/$CaCl_2$; the cell suspension (containing mostly fibroblasts) was centrifuged. The cells were counted using a Neubauer chamber and re-seeded into an appropriate cell culture dish containing the fibroblast growth media DMEM+ w/AB (e.g., $0.2x10^6$ cells were seeded into a T25 cell culture flask). The remaining cells on the first cell culture plate (predominantly keratinocytes) were provided with K-SFM+ w/AB and further incubated as described above. This procedure was repeated every three days until keratinocyte cultures were free of fibroblasts (visual control).

When keratinocyte colonies reached confluency, they were sub-cultured using 0.25% TEDTA for 3 min and subsequently 0.05% TEDTA for 10 min at 37°. Gentle tapping at the side of the 6-well plate facilitated the detachment; rinsing each well with PBS w/o $MgCl_2$/$CaCl_2$ ensured the transfer of nearly all cells. The cell suspension was centrifuged (1500 rpm, RT, 4 min); the cell pellet was re-suspended in an appropriate volume of culture medium and re-seeded. Keratinocytes were split in a ratio of 1:2 and used for skin equivalents in passage 3 and 4.

Fibroblasts were sub-cultured equally using 0.25% TEDTA for 3 min at 37°C; they were split in ratios up to 1:6 and were used for skin equivalents from passage 5.

For cryo preservation in liquid nitrogen, cells were detached and counted as described earlier, centrifuged (1500 rpm, RT, 4 min) and re-suspended in FCS supplemented with 10% dimethyl sulfoxide (DMSO; $1x10^6$ cells/ml cryo solution). Fibroblasts were cryo preserved with 0.5 to $1x10^6$ cell per vial, keratinocytes with $2x10^6$ to $3x10^6$ cells per vial in the respective volume of FCS supplemented with DMSO.

### 3.3.2 Skin equivalents

#### 3.3.2.1 Preparations

##### 3.3.2.1.1 Generation of post-mitotic fibroblasts (pmF) with Mitomycin C

For the generation of post-mitotic fibroblasts pmF, they were seeded into T175 flasks and grown to 90% confluency. The Mitomycin C was reconstituted in PBS w/ $MgCl_2$/$CaCl_2$ (1mg/ml). 1.6 ml of this solution were added to 8.4 ml of DMEM+ w/o AB and prewarmed to 37°C. The growth medium in the culture flask was aspirated and the cells were washed three times with PBS w/ $MgCl_2$/$CaCl_2$. Subsequently, the Mitomycin C/DMEM-solution was added and the cells were incubated at 37°C for 3 to 5 h. Afterwards, the solution was aspirated and the cells were washed three times with PBS w/ $MgCl_2$/$CaCl_2$ again. DMEM+ w/o AB was added and the cells were cultured at usual conditions for at least two days before further manipulation was carried out, e.g. freezing.

##### 3.3.2.1.2 Coating of the underside of 12-well inserts with pmF

Post-mitotic fibroblasts were detached and counted in a Neubauer chamber as described above. The cell suspension was adjusted to $0.4x10^6$ cells/ml DMEM+ w/o AB. 12-well cell culture inserts (Greiner BioOne, Frickenhausen, Germany) were placed up-side-down in an extra deep petri dish and 0.3 ml of the aforementioned cell suspension were pipetted cautiously on top of the insert membrane (total: $0.12x10^6$ cells/insert). The cell culture inserts were incubated for 4 to 5 h at 37°C in this position to allow cell adherence to the underside of the insert membrane. Subsequently, the inserts were placed in the correct orientation in a corresponding 12-well dish provided with DMEM+ w/o AB. Coated cell culture inserts were prepared the day prior to keratinocyte seeding but could be stored in the incubator up to 2 weeks (37°C, 5% $CO_2$, 95% rh; medium was changed twice a week).

##### 3.3.2.1.3 Collagen pads as dermal equivalents

The dermal compartment (i.e., the collagen pad) of both skin models consists of pmF embedded in commercially available bovine collagen type I pads.

The collagen pads were prepared according to manufacturer´s instructions. Briefly, eight parts of chilled collagen type I (4 mg/ml; Biochrom GmbH, Berlin, Germany) were cautiously mixed with one part of chilled 10xDMEM. The pH was adjusted to 7.2 to 7.6 by dropwise addition of 0.7M NaOH. Post-mitotic fibroblasts were detached as described above, re-suspended at a density of $0.1x10^5$ cells/collagen pad in one part FCS and mixed with the collagen/10xDMEM-solution. About 1 ml of this solution was pipetted into 12-well cell culture inserts set into a corresponding 12-well plate. An incubation time of 45 min to 1 h at 37°C followed, i.e. until the collagen was completely polymerized. Subsequently, 0.5 ml of DMEM+ w/AB were pipetted into the lower cavity and on top of the collagen pad inside the cell culture insert. The collagen pads were kept at 37°C, 95% rh and 5% $CO_2$. Fibroblasts should be stretched out and returned to typical morphology by the next day.
The dermal equivalents were prepared the day prior to keratinocyte seeding but could be stored up to 5 days in the incubator (37°C, 5% $CO_2$, 95% rh; medium was changed twice a week).

#### 3.3.2.2 Skin equivalents I: Two-step-model

Keratinocytes stratify on a 12-well cell culture insert that is coated with growth-promoting post-mitotic fibroblasts (pmF) on the underside. The resulting epithelium-like cell sheet is then loosened from the insert membrane and transferred to the top of a dermal equivalent.

Readily prepared pmF-coated cell culture inserts (see chapter “3.3.2.1.2 Coating of the underside of 12-well inserts with pmF”) were provided with KSFM+ w/o AB, i.e. the DMEM+ w/o AB in the lower compartment was exchanged with K-SFM+ w/o AB.
Keratinocytes were detached and counted as described above. The cells were re-suspended in K-SFM+ w/o AB at a density of $1x10^6$ cells/ml; 0.65 ml of this suspension were pipetted into the upper cavity of the pmF-coated cell culture inserts. After 2 days, the keratinocytes were lifted, i.e. the medium in the upper cavity was aspirated (= air-liquid-interface culture). This exposition to the ambient atmosphere initiates the terminal differentiation process in keratinocytes concomitant with asymmetric cell division and stratification. This should be visible with the unaided eye 3 to 5 days later.

The air-liquid-interface culture was maintained for 14 days with a change of medium twice a week.

A reconstituted Dispase II solution was used to remove the resulting epithelium-like cell sheet from the insert membrane. The lower cavity was washed twice with PBS w/o $MgCl_2$/$CaCl_2$. 1 ml of Dispase II solution (0.05U/ml; in PBS w/o $MgCl_2$/$CaCl_2$) was added to the lower cavity and the plate was incubated at 37°C. After 5 to 10 min, the Dispase solution was aspirated and the cavity was washed twice with PBS w/o $MgCl_2$/$CaCl_2$. Using forceps, the epithelium-like cell sheets were transferred from the insert membrane onto sterile silicone membranes (Ø 1 cm; Polytech Health & Aesthetics GmbH, Dieburg, Germany). Cell sheet and silicone membrane were placed on top of dermal equivalents (note orientation: silicone membrane is facing apical surface of keratinocyte cell sheet, basal part of cell sheet is facing dermal equivalent). Two days later, the silicone membrane was removed cautiously with forceps and further experiments could be carried out.

#### 3.3.2.3 Skin equivalents II: direct model

Keratinocytes are directly seeded onto prepared dermal equivalents and stratify during 21 days of air-liquid interface culture without further manipulation.

Dermal equivalents were prepared as described above (see chapter “3.3.2.1.3 Collagen pads as dermal equivalents”). The growth medium on top and below the dermal equivalent was aspirated and the lower cavity was provided with 0.5 ml of K-SFM+ w/o AB.

Keratinocytes were detached from their initial cell culture vessel and counted as described above. The cells were re-suspended in K-SFM+ w/o AB at a density of $1x10^6$ cells/ml; 0.65 ml of this suspension were pipetted onto the collagen pad. The cells were incubated at 37°C, 5% $CO_2$ and 95% rh for 21 days with a change of medium in the lower cavity twice a week. The medium in the upper cavity, i.e. on top of the pad, was cautiously aspirated 2 days after seeding to induce keratinocyte terminal differentiation and stratification.

### 3.3.3 Sample preparation for standard histology and immunocytochemistry/ immunohistochemistry

*Native skin.* For paraffin embedding and microtome sectioning, the samples were washed with PBS w/o $MgCl_2$/$CaCl_2$ and transferred to a 12-well dish containing the fixative, i.e. paraformaldehyde (PFA; 4% in PBS) or Methanol/Acetone (1:1) depending on the subsequent staining method. The samples remained in the fixative (PFA; 4°C) or were transferred to PBS w/o $MgCl_2$/$CaCl_2$ (Methanol/Acetone; 4°C) until paraffin embedding was carried out. For a detailed embedding protocol, see Table 12 (appendix).
For snap freezing and cryo sectioning, the samples were washed (PBS w/o $MgCl_2$/$CaCl_2$) and transferred to cryo molds containing O.C.T. compound. The samples were covered with O.C.T. compound, snap frozen in precooled isopentane (-80°C) and stored at -80°C until sectioning was carried out.

*Primary cells.* For antibody staining, fibroblasts and keratinocytes were detached as described above, counted and pipetted into the wells of a 48-well plate ($0.02x10^6$ cells/well). The respective growth medium was added (0.2 ml/well) and the cells were incubated for 2 to 3 days as described above. Subsequently, cells were washed with PBS w/o $MgCl_2$/$CaCl_2$ and fixed with 4% PFA in PBS w/o $MgCl_2$/$CaCl_2$ for 10 min at RT. The cells were washed with PBS w/o $MgCl_2$/$CaCl_2$ three times for 5 min each and kept in this solution at 4°C until staining was carried out.
For alternative fixing, ice cold Methanol/Acetone-solution (1:1; -20°C) was added to the cells after washing. After 5 min of incubation at -20°C, the solution was aspirated, the cells were washed twice and kept in PBS w/o $MgCl_2$/$CaCl_2$ at 4°C until staining was carried out.
The choice of fixative depended on the subsequent staining method (see Table 3 for further details).

*Multilayered keratinocyte sheets.* Keratinocyte sheets were fixed with 4% PFA in PBS w/o $MgCl_2$/$CaCl_2$ for 10 min at RT and washed three times with PBS w/o $MgCl_2$/$CaCl_2$ for 5 min each time. To ensure specimen integrity, the fixed cell sheets on the insert membrane were embedded in HistoGel™ according to manufacturer`s instructions

prior to paraffin embedding. Paraffin embedding was carried out according to general protocols (see Table 12, appendix).
For snap freezing and cryo sectioning, the fixed cell sheets were washed (PBS w/o $MgCl_2$/$CaCl_2$) and transferred to cryo molds containing O.C.T. compound. The samples were covered with O.C.T. compound, snap frozen in precooled isopentane (-80°C) and stored at -80°C until sectioning was carried out.

*Skin equivalents.* For paraffin embedding and microtome sectioning, skin equivalents were PFA-fixed in the cell culture inserts as described above. They were removed from the insert, embedded in Histogel™ according to manufacturer`s instructions and placed in tissue embedding cassettes. Paraffin embedding followed according to general protocols (see Table 12, appendix).
For cryo sectioning, skin equivalents were embedded in O.C.T. compound and snap frozen in precooled isopentane (-80°C).

### 3.3.4 Histological staining and immunocytochemistry/ immunohistochemistry

Histological Hematoxylin and Eosin-staining (H&E) of dewaxed paraffin-embedded microtome sections (10µm) or cryotome sections (10µm) was carried out according to standard protocols. For details on the dewaxing protocol and the staining process, see Table 13 and Table 14, respectively (appendix).
*Treponema* spp. in dewaxed paraffin-embedded tissue samples and skin equivalents were detected with a modified Steiner stain (silver stain for spirochetes and non-filamentous bacteria in paraffin-embedded sections) that was performed by the Histology Laboratory at the School of Veterinary Medicine of the University of Madison.

Immunocytochemistry/ immunohistochemistry with proliferation and differentiation markers was conducted in two steps, i.e. indirect immunofluorescent stainings with fluorochrome coupled secondary antibodies were carried out (exception: anti-Vimentin was directly labeled with the fluorophore Cy3; Sigma, C9080). Briefly, the specimens were incubated with a diluted primary antibody detecting the desired antigen at 4°C over night in a humid atmosphere. After three washing steps, the incubation with the diluted fluorochrome labeled secondary antibody detecting the primary antibody for 2 h at RT in the dark (avoids fading of fluorochrome) in a humid atmosphere followed.

The secondary antibody was directed against the IgG of the host species of the primary antibody and was produced in a different species than the primary antibody. The nuclear counterstain with Bisbenzimide (Hoechst 33342) was included during the incubation with the secondary antibody.
This two-step procedure offers two advantages over directly labeled primary antibodies: firstly, the signal is amplified since several secondary antibodies can bind to one primary antibody. Secondly, many different antigens can be detected with the same secondary antibody (Mulisch and Welsch, 2015).
Evaluation of the stainings and taking photographs was carried out with the fluorescence microscope TE2000 (Nikon). For details on sample pretreatment after sectioning (i.e. antigen retrieval), incubation times and antibody dilutions, see Table 3 and Table 4 (see chapter “3.1.3 Antibodies”).

### 3.3.5 Culture of *Treponema* spp.

All work was carried out in an anaerobic chamber (Bactron Anaerobic/Environmental Chamber, Sheldon Manufacturing Inc., Oregon, USA) to ensure anaerobic conditions. Frozen aliquots of the following *Treponema* spp. strains were kindly provided by Prof. D. Döpfer, School of Veterinary Medicine, University of Madison, Wisconsin, USA:

- *T. denticola-like* 1-9185MED
- *T. phagedenis-like* CR2220RR CV.

Cells were transferred from the cryo vial to oral treponeme enrichment broth (OTEB, Anaerobe Systems, CA, USA) supplemented with 10% FCS and 1 µg/ml rifampicin and cultured under anaerobic conditions (85% $N_2$, 10% $H_2$, and 5% $CO_2$) at 37°C. After four to seven days of culture (depending on the strain), spiral and very motile cells were visible under dark field microscopy. Any further handling, e.g. plating, expansion of the culture (by addition of OTEB with 10% FCS) or adhesion/invasion experiments, were carried out with motile cells as only those were considered to be in the exponential growth phase. The cell number was determined by plate count. Bacterial identity was confirmed via real time quantitative polymerase chain reaction (RT-qPCR) with the QuantiTect Multiplex PCR Kit (Cat. Nr. 204543) from Quiagen (for further details on DNA extraction, primer sequences, master mix components and PCR protocol see Table 15, Table 16, Table 17 and Table 18, appendix).

### 3.3.6 Experiments with *Treponema* spp.

Bovine skin equivalents and *Treponema*-suspensions were prepared as described earlier (see chapters “3.3.2 Skin equivalents” and “3.3.5 Culture of *Treponema* spp.”) and transferred to the anaerobic chamber. The medium in the lower cavity of the 12-well plate of the skin equivalents was aspirated and replaced by OTEB supplemented with 10% FCS. 0.4ml of the prepared *Treponema* spp.-suspension were pipetted into each insert with a skin equivalent and 0.1 ml were serially diluted and plated on FAA and/or blood agar plates for the determination of colony forming units (cfu/ml). A wet mount of the inoculum was conducted in advance to confirm bacterial viability using dark field microscopy.

After incubation for different periods ranging from 0 min to 4 h, the OTEB underneath the cell culture insert was collected in a 1.5 ml reaction tube. This supernatant was divided into 3 fractions:

1. 150 µl were used for a dilution series (in OTEB) with dilutions of $10^0$, $10^{-1}$, $10^{-2}$ and $10^{-3}$; 0.1 ml of each dilution were plated on FAA plates and/or blood agar plates. The plates were incubated at 37°C in anaerobic atmosphere (85% $N_2$, 10% $H_2$, 5% $CO_2$) for 10 to 14 d; colonies were counted thereafter. The number of cfu was calculated according to the following equation:

   | cfu = (final dilution factor) x (No. of colonies) x (factor of volume plated) |
   |---|

2. 50 µl of the supernatant were used for an immediate visual control for spiral organisms under dark field microscopy (wet mount).
3. The remaining 300 µl of the supernatant were used for DNA extraction and RT-qPCR.

The skin equivalents were formalin-fixed (10% neutral buffered, RT), paraffin-embedded, microtome sectioned and histologically stained as described earlier (see chapter 3.3.4 Histological staining and immuno).

The experimental procedure is illustrated in Figure 11.

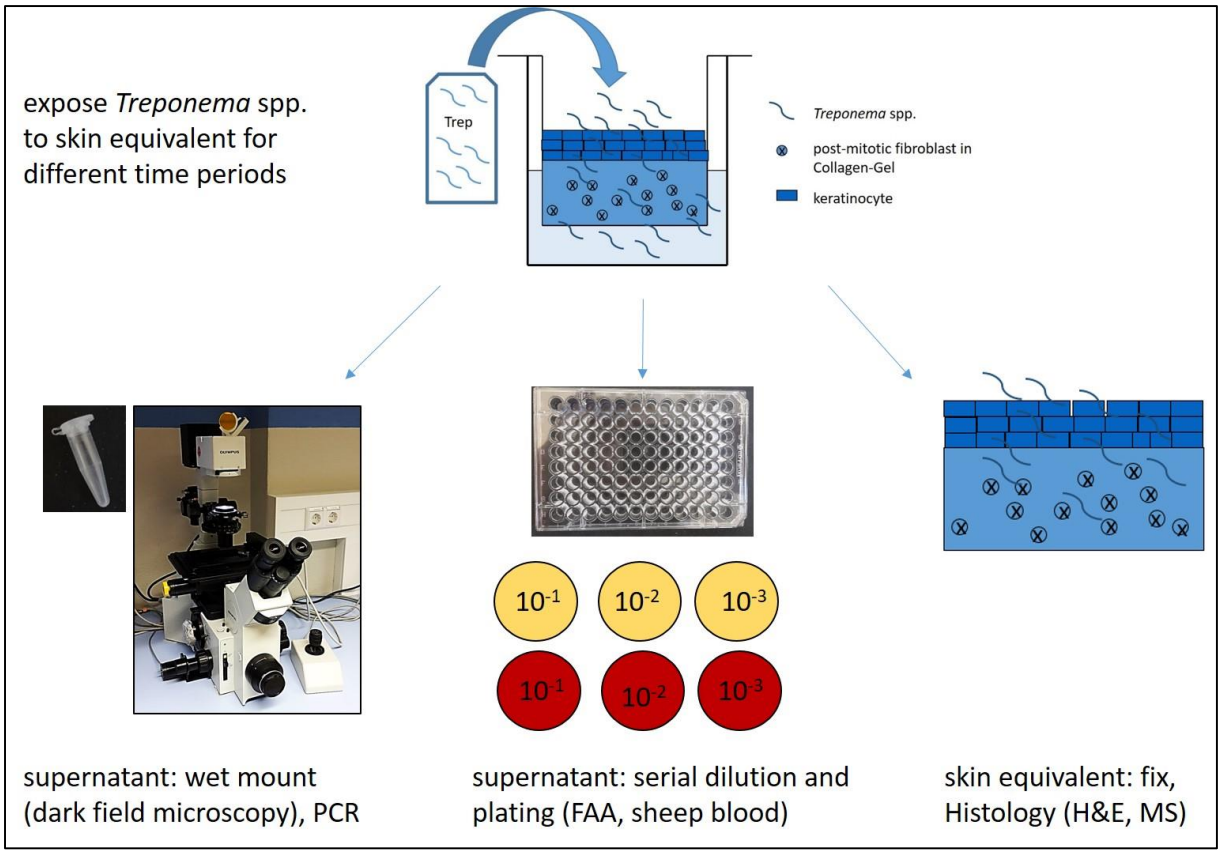


**Figure 11 Diagram of experimental set up.** At first, *Treponema* spp. are exposed to skin equivalents under anaerobic conditions and incubated for different periods. Afterwards, the supernatant underneath the cell culture insert is collected and divided into two fractions. Fraction 1 is used for a wet mount for immediate visual control and for PCR. Fraction 2 is serially diluted and plated onto FAA (yellow circles) and sheep blood agar plates (red circles). The skin equivalents are formalin-fixed and submitted to Histology for H&E and MS staining.

# 4. Results

## 4.1 Primary isolation and cultivation of bovine keratinocytes and fibroblasts

Initially, different isolation protocols and cultivation media were tested for their ability to promote growth and proliferation of bovine skin cells.

Various isolation methods are described in the literature: some focus on enzymatic digestion, others on mechanic disassembly of the different skin strata. In this study, a combination of mechanic removal of dermal sample parts and enzymatic overnight digestion gave rise to the highest number of bovine skin cells that were able to adhere to the cell culture vessel and proliferate (Figure 12, A).

Furthermore, different media and additives (growth factors, antibiotics etc.) were tested for their ability to support cell viability. A recently described formulation (K-SFM, FCS, EGF, antibiotics; Lübbe, 2015) was additionally supplemented with bovine pituitary extract (BPE). This growth medium gave rise to more than $1.5x10^6$ keratinocytes in 18 out of 22 isolations although it must be assumed that only the minor part of isolated epithelial cells are able to proliferate in vitro, namely those originating from the stratum basale (Figure 12, B).

Since fibroblast growth was also supported by this medium, a separate isolation protocol for fibroblasts was not necessary. Both skin cell types were successfully separated and sub-cultured in different cell culture vessels using 0.05% and 0.25% TEDTA, respectively. For economic reasons, the less demanding fibroblasts were further cultivated in a DMEM-based culture medium containing only EGF, FCS and antibiotics (Figure 12, C).

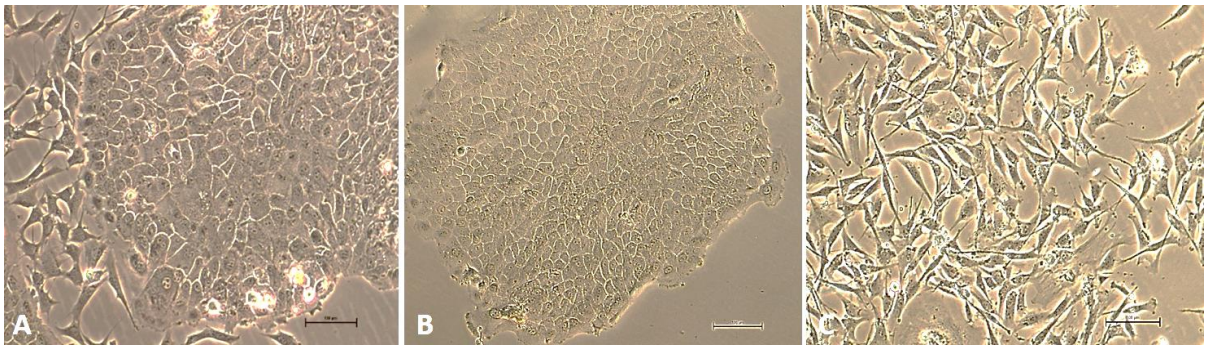


**Figure 12 Bovine skin cells in culture.** A Mixed culture of bovine skin cells, mainly keratinocytes and fibroblasts (p0; d6). B Keratinocyte colony with typical cobblestone-like morphology (p1; d11). C Elongated, spindle-shaped fibroblasts (p1; d10); bars indicate 100µm.

In summary, the isolation and cultivation of bovine skin cells, i.e. keratinocytes and fibroblasts, from 50 distal limbs in 22 isolations was carried out successfully as outlined above in chapter “3.3.1 Primary isolation and cultivation of bovine keratinocytes and fibroblasts”. Sixteen out of twenty-two isolations were completely free of bacterial and/or fungal contamination; in six isolations, contamination was limited to a few wells only. Additionally, cell culture supernatants from seven isolations were tested for mycoplasma contamination which were also negative (PCR test for *Mycoplasma* (M.) *bovis, M. californicum, M. canadense, M. agalactiae, M. fermentans*; data not shown). Cell identity and differentiation state could be verified by light microscopy and immunofluorescence staining for specific markers due to characteristic cell morphologies and distinct protein expression patterns, respectively.
Moreover, to get an impression of the growth fraction of cultured cells, i.e. the fraction of cells that were in active cell cycle phases (G1, S, G2, M), immunofluorescence stainings against the nuclear proliferation marker Ki-67 were conducted (Scholzen and Gerdes, 2000).

*Keratinocytes.* Cultured keratinocytes show a typical cobblestone-like morphology as can be seen in Figure 12 (B) and Figure 13 (A) and grow preferentially from cell clusters of about five cells to confluency. The necessary time span to reach confluency depended on the size of the cell culture vessel, on the presence of fibroblasts and the number of passages the cells had already undergone. The time interval between two passages ranged from one to three weeks. Generally, the presence of fibroblasts in the cell culture vessel accelerated keratinocyte growth and proliferation. On the contrary, the doubling time of keratinocytes increased with increasing number of passages.
Intermediate filaments (IF) form cytoskeletal compounds and are grouped into six types based on their amino acid sequence and protein structure. According to this classification scheme, acidic and basic-to-neutral keratins are type I and type II-IF, respectively (Steinert and Roop, 1988). Since keratins that form IF are only expressed in epithelial cells (Moll et al., 1982), antibodies against one or more keratin protein can be used to discriminate between epithelial and e.g. mesenchymal cells. Hence, keratinocyte identity of the cultured bovine skin cells was confirmed with an antibody that recognized various keratins, namely cytokeratins 4, 5, 6, 8, 10, 13 and 18 (anti-

Pan-cytokeratin, mouse monoclonal antibody, clone C-11, Sigma Aldrich; see Figure 13, C).

The acidic type I keratin K14, which forms tonofilaments with the type II IF keratin 5, is associated with mitotic activity and was also found to be expressed in cultured human keratinocytes (Bragulla and Homberger, 2009; Fuchs and Green, 1980).

Hence, this marker was used to confirm the basal differentiation state of cultured bovine keratinocytes (see Figure 13 D).

Ki-67, a nuclear transcription factor, is strongly associated with cell proliferation although its function during cell division is not yet fully understood (Scholzen and Gerdes, 2000). Since this marker was always found in a subset of the cultured bovine keratinocytes (see Figure 13, B), cell viability and their ability to proliferate was concluded.

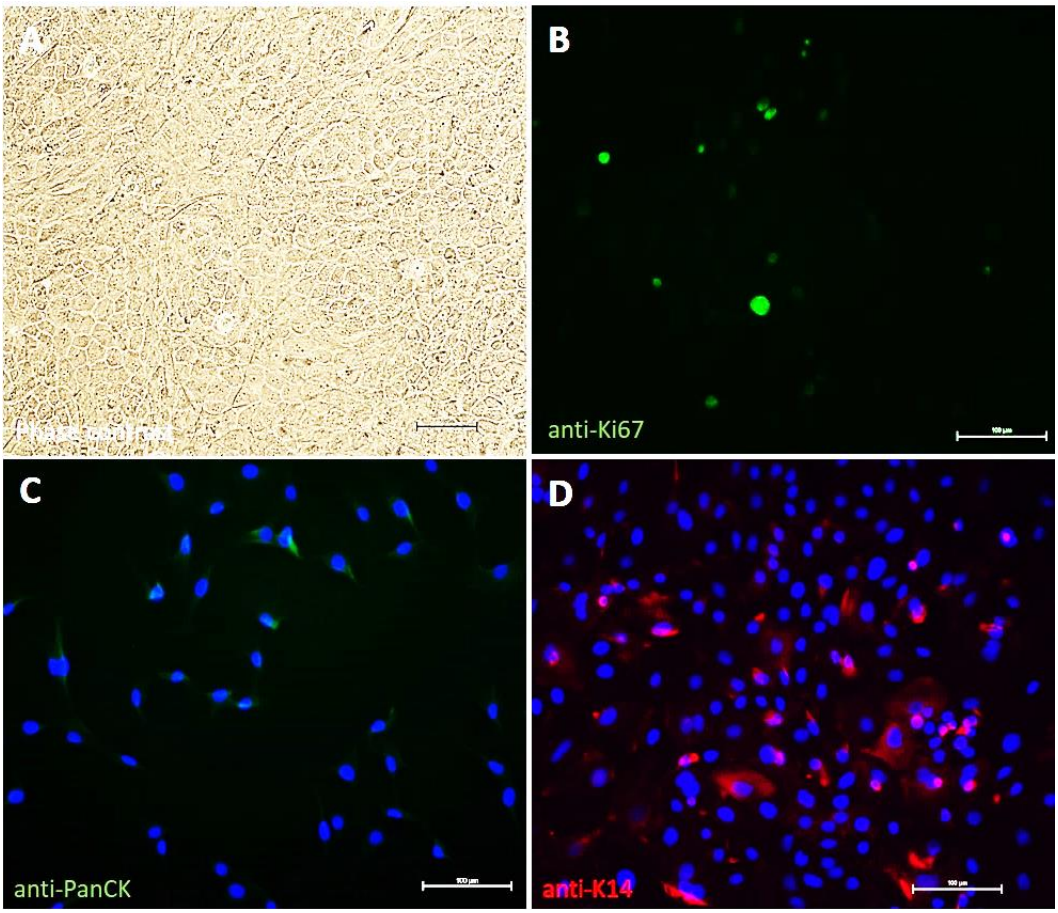


**Figure 13 Cultured bovine keratinocytes.** A Phase contrast micrograph of a confluent keratinocyte monolayer showing the typical cobblestone-like morphology (p0; d9). B Immunofluorescence staining with anti-Ki67, which detects the nuclear proliferation marker Ki-67. C Immunofluorescence staining with anti-PanCK Clone C-11, which recognized various cytokeratins (Note: less distinct cell morphology due to sub-confluency of cells). D Immunofluorescence staining with anti-K14, which detects K14 that is mainly found in basal keratinocytes (bars indicate 100µm).

*Fibroblasts.* It is not possible to separate dermis and epidermis completely. Therefore, dermal fibroblasts were always present in keratinocyte cultures. Nevertheless, the two cell types could be easily distinguished and separated (see chapter “3.3.1 Primary isolation and cultivation of bovine keratinocytes and fibroblasts”). Fibroblasts are thin, elongated, spindle-shaped cells that adhere quickly to cell culture vessels (see Figure 14, A).

After the morphological examination with the inverted microscope, cell identity was also proved using immunofluorescence stainings. At first, an antibody that detects the type III intermediate filament vimentin was used (see Figure 14, C). Vimentin is specific for mesenchymal cells; cytokeratins are not expressed by fibroblasts. Since some epithelial cells co-express keratins and vimentin in vitro (Biddle and Spandau, 1996), fibroblasts were also stained for keratins to exclude their presence (data not shown).

The ability to proliferate was confirmed with immunofluorescence stainings using an antibody that detects the nuclear, proliferation-associated Ki67-protein (see Figure 14, B).

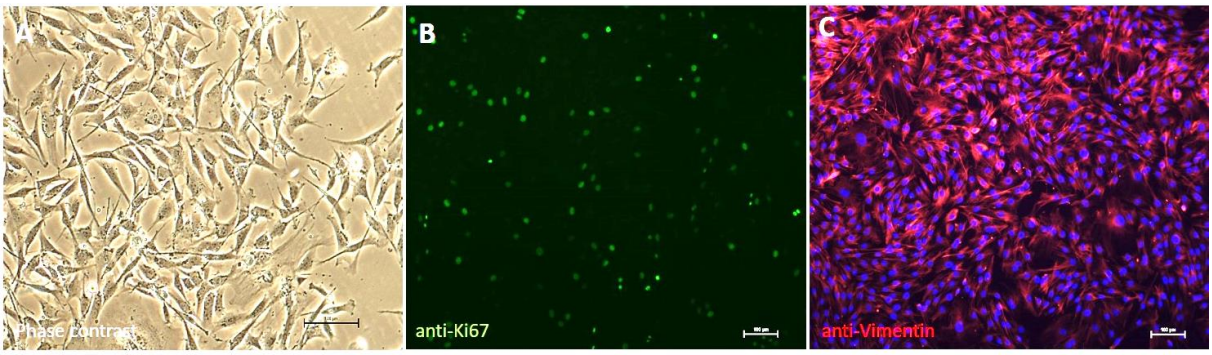


**Figure 14 Cultured bovine fibroblasts.** A Phase contrast micrograph of elongated, spindle-shaped bovine fibroblasts (p0; d10). B Immunofluorescence staining with anti-Ki67 to demonstrate cell viability. C Immunofluorescence staining with anti-vimentin, which detects the type III intermdiate filament vimentin that is specific for mesenchymal cells such as fibroblasts (bars indicate 100µm).

## 4.2 Skin equivalents

### 4.2.1 Preparations: Post-mitotic fibroblasts (pmF), coating of cell culture inserts and dermal equivalents

*Generation of post-mitotic fibroblasts (pmF).* In order to take advantage of the growth-promoting influence of fibroblasts upon keratinocytes (Limat et al., 1989), close proximity of both cell types is essential. However, since normal fibroblasts proliferate much faster than keratinocytes, the former will eventually overgrow the latter if cultured in the same cell culture dish. Consequently, post-mitotic fibroblasts – still exhibiting keratinocyte promoting features but not dividing – were chosen for the present studies (i.e. coating of insert membranes, embedding into collagen pads).

For the generation of post-mitotic fibroblasts the bifunctional alkylator Mitomycin C was used. It inhibits DNA synthesis by specifically crosslinking CpG sequences in the DNA of an organism. Accordingly, Mitomycin C-treated cells are cell-cycle arrested and will not divide any further (Tomasz, 1995).

Fibroblasts are very robust and less demanding cells, i.e. they grow in a relatively simple DMEM-based medium, do not require special cell culture vessels or levels of confluency and 30 passages and more are easily possible.

After Mitomycin C incubation, cells did not look any different from before, i.e. still elongated and spindle-shaped, and only a few cells detached from the cell culture dish. During cell culture after the treatment for recovery purposes, the cell number did not decrease significantly (personal impression). Even when post-mitotic fibroblasts were frozen for cryo preservation and thawed again, only a few cells were not able to re-adhere to the cell culture vessel. Most of the cells survived around three more weeks in routine cell culture before cytopathic effects were observed (e.g. rounding and swelling of cells, formation of inclusion bodies; data not shown).

*Coating of underside of cell culture inserts.* Coating of the underside of cell culture inserts with pmF was conducted successfully (see Figure 15).

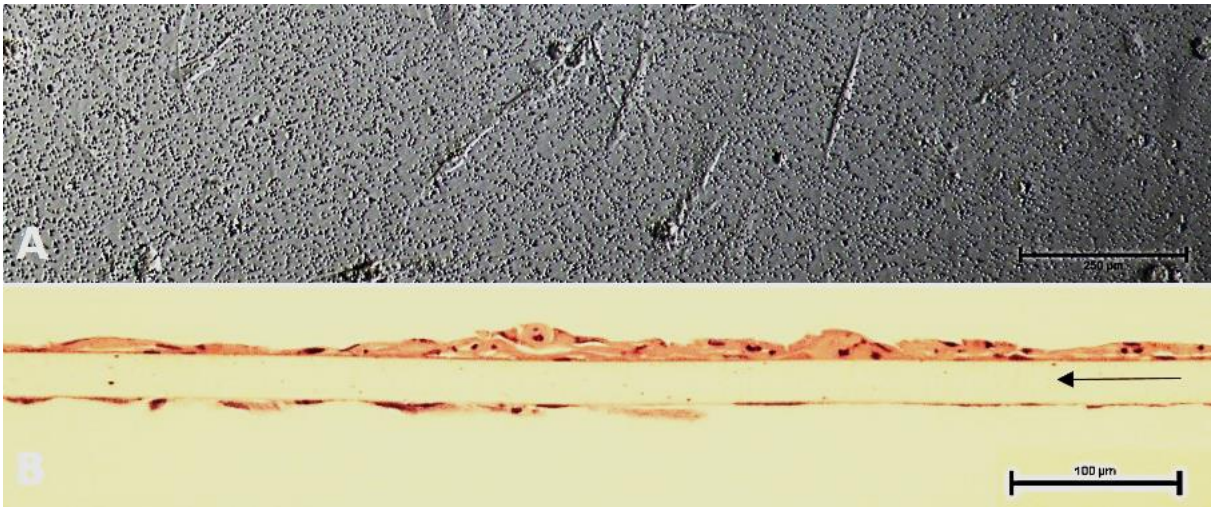


**Figure 15 pmF-coated cell culture insert.** A Phase contrast micrograph of fibroblasts attached to the underside of the membrane of the cell culture insert (bar indicates 250µm). B H&E-stained paraffin section of cell culture insert with keratinocytes on top and fibroblasts attached to the underside of the insert membrane (arrow indicates insert membrane; bar indicates 100µm).

*Dermal equivalent.* To mimic the in vivo situation as close as possible, the dermal compartment of the skin model was composed of fibroblasts that were embedded in commercially available bovine collagen type I (≙ collagen pad, 3D cell culture).

As can be seen in Figure 16 (A), the dermal equivalents were prepared in 12-well cell culture inserts, which facilitated further handling and processing.

In vivo, fibroblasts produce collagenases to maintain the integrity and functionality of the extracellular matrix during tissue development and remodeling (Bauer et al., 1975). The contraction of the collagen pads in 3D cell culture is therefore considered a sign of fibroblast vitality since it is attributed to the physiologic synthesis of collagenases. However, the pads were degraded immensely already after a short time of culture if regular fibroblasts were used (see Figure 16 B and C – note the color change of the culture medium due to the high metabolic activity of fibroblasts). When pmF were seeded into the pads, contraction was also observed but only on d14 of cell culture (see Figure 16 D, white arrow). This later time point was crucial, especially when keratinocytes were seeded directly on top of the pad (skin equivalents II: direct skin model), because the differentiation process of keratinocytes takes about three weeks (Baroni et al., 2012).

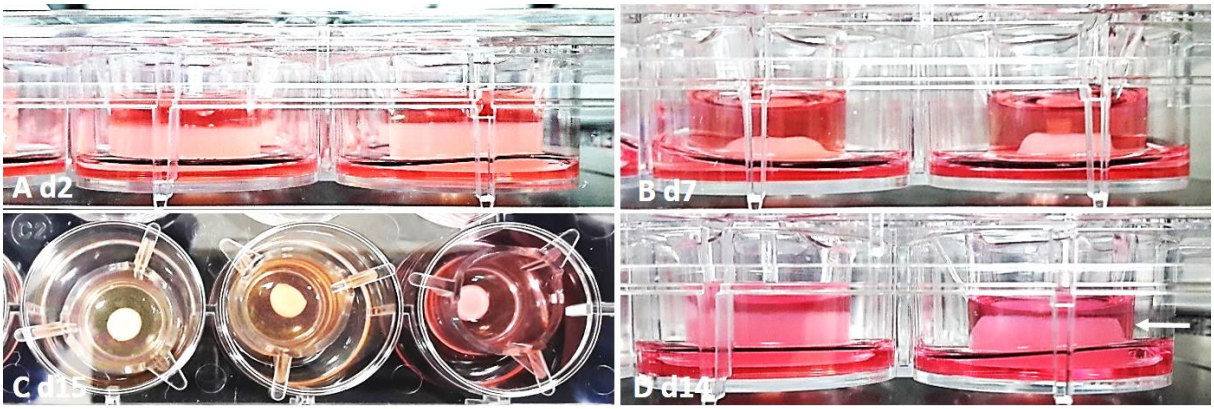


**Figure 16 Collagen pads with embedded fibroblasts two (A), 7 (B) and 15 days (C) after seeding.** Note the proceeding contraction of the collagen pads. D Collagen pads with embedded pmF; the white arrow indicates pad contraction but only on day 14 of 3D cell culture.

Post-mitotic fibroblasts in collagen pads showed the characteristic cell morphology (spindle-shaped, elongated; see Figure 17, A). The correct adjustment of the pH of the collagen pad was crucial for cells to stretch out and thrive inside the pad. The type III intermediate filament vimentin was equally demonstrated as in 2D cell culture (see Figure 17, B; note that keratinocytes also express vimentin but to a lesser extent).

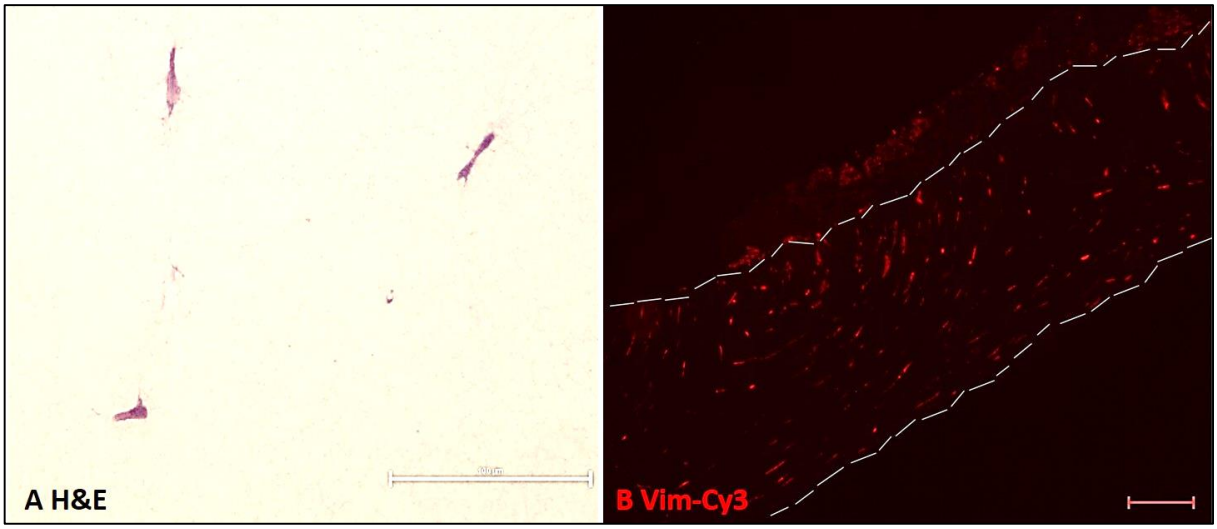


**Figure 17 Post-mitotic fibroblasts in collagen pads of bovine collagen type I (d35 of air-liquid-interface culture).** A H&E-stained paraffin section showing characteristic fibroblast morphology (bar indicates 100µm). B Paraffin section stained with anti-Vimentin-Cy3. The white lines highlight the contour of the dermal equivalent. Note that keratinocytes on top of the collagen pad also express vimentin but to a lesser extent (bar indicates 100µm).

### 4.2.2 Skin equivalents I: Two-step-model

During the first stage of the two-step skin model, keratinocytes were seeded onto 12-well cell culture inserts that were coated with pmF (underside). Stratification and terminal differentiation of keratinocytes was induced when the level of culture medium was lowered and the cells were further cultured at ambient atmosphere. As can be seen in Figure 18 (A), stratification was visible macroscopically. Here, this process was not concerted leading to areas of higher cell density (white arrow in Figure 18 A and A.2) and areas where the insert membrane was still visible through the cell monolayer.

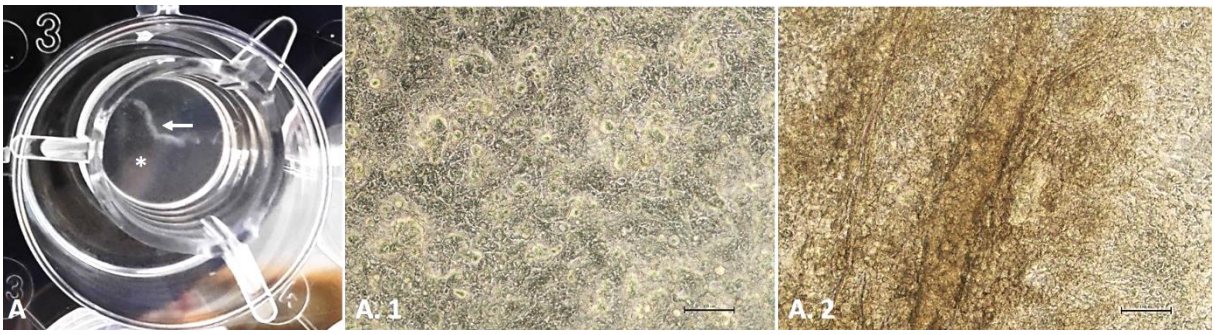


**Figure 18 Skin equivalents I: Two-step-model.** A During the first part of the two-step-model, keratinocytes stratify on a pmF-coated cell culture insert. Stratification is visible macroscopically (d16 of air-liquid-interface culture). A.1 and A.2 show phase contrast micrographs of keratinocytes in the same cell culture insert: A.1 corresponds to the position marked with * in A (monolayer), A.2 corresponds to the position indicated with the arrow in A (higher cell density; bars indicate 100µm).

To follow the stratification progress, keratinocytes grown on cell culture inserts were fixed and H&E-stained at different time points (see Figure 19). Stratification was observed, but did not yield more than 5 to 7 cell layers. Additionally, the morphological transformation from cuboidal keratinocytes with big, round, centric nuclei in the stratum basale to flat, anucleate corneocytes in the stratum corneum did not occur in the typical manner. Instead, flattened cells were found facing the insert membrane (= basal) rather than the apical part of the cell sheet.

To complete the skin equivalent, the epithelial cell sheet was to be transferred to the top of the dermal equivalent. This transfer was not possible because the detachment of the keratinocytes from the insert membrane as an intact cell sheet failed. Although the protease Dispase II, which is known to cleave cell-substrate contacts but leaves cell-cell contacts mostly intact (Wei et al., 2011), was used very cautiously (low concentration 0.05U/ml, incubation time 3 min), the cell sheet fell apart.

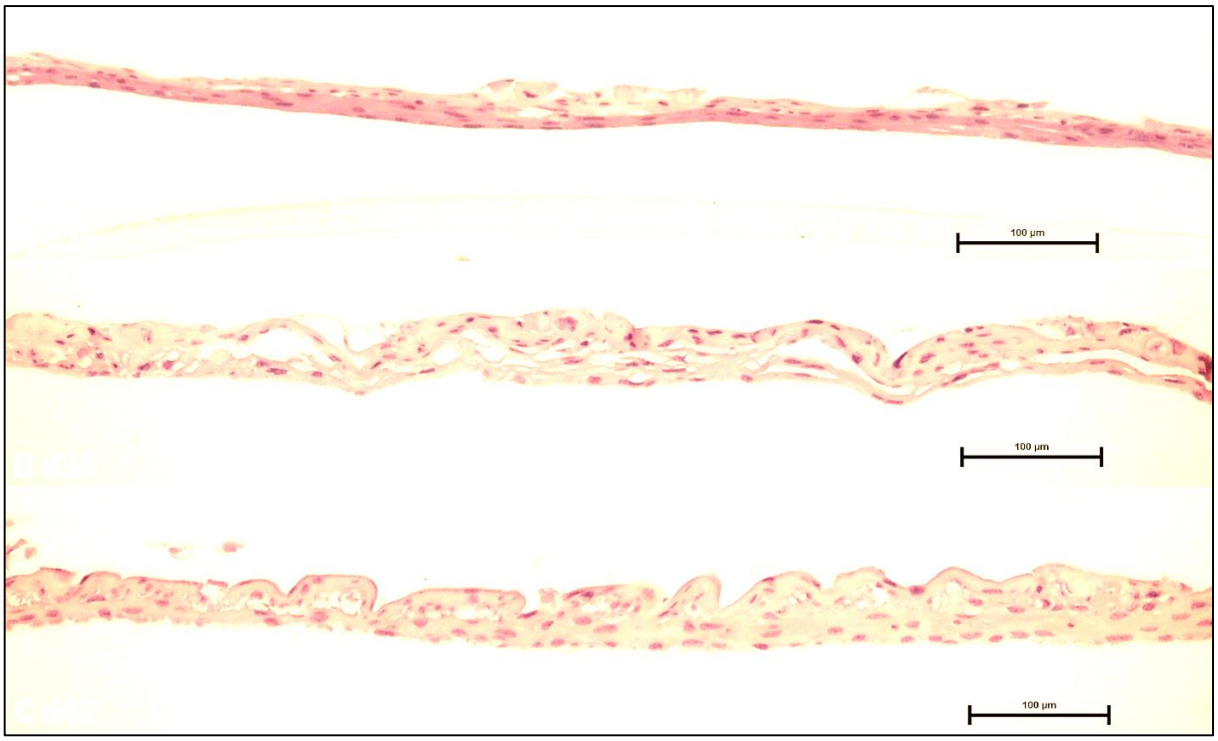


**Figure 19 Skin equivalents I: Two-step-model.** H&E-stained paraffin sections of keratinocytes on cell culture inserts at d26 (A), d36 (B) and d42 (C) of air-liquid-interface culture. Initial stratification of keratinocytes occurred but no more than seven cell layers were observed. Additionally, the typical morphological transformation of keratinocytes could not be seen. Note: pmF and/or insert membranes were lost during processing and staining; fissures between cell layers in B are processing artefacts (bars indicate 100µm).

### 4.2.3 Skin equivalents II: direct model

The direct skin model was composed of keratinocytes that were seeded on top of a readily prepared dermal equivalent with incorporated pmF. Keratinocyte stratification was also induced by the air-liquid-interface culture method.

Figure 20 displays H&E-stained sections of native bovine skin and the skin equivalent II. Native skin shows the typical layered structure of mammalian skin as described earlier (see chapter “2.2 Relevant knowledge about human and bovine skin”). Interdigitating dermal papillae and epidermal rete ridges were observed as well as a pronounced stratum corneum with desquamating layers of corneocytes (stratum corneum disjunctum). The skin equivalent II also consisted of two major layers: the dermis was resembled by fibroblasts embedded in the collagen pad; many layers of keratinocytes simulated the epidermis.
While fibroblasts in the dermal part exhibited the typical elongated cell morphology with intact nuclei, the characteristic morphological transformation from cuboidal basal keratinocytes to flat, anucleate corneocytes was found only in some regions of the epidermal part. Desquamating cell layers were missing due to the absence of physical stress. Skin appendages such as hair follicles and sweat glands are also not found in the skin equivalent because technically they cannot be included into skin replacement products so far.

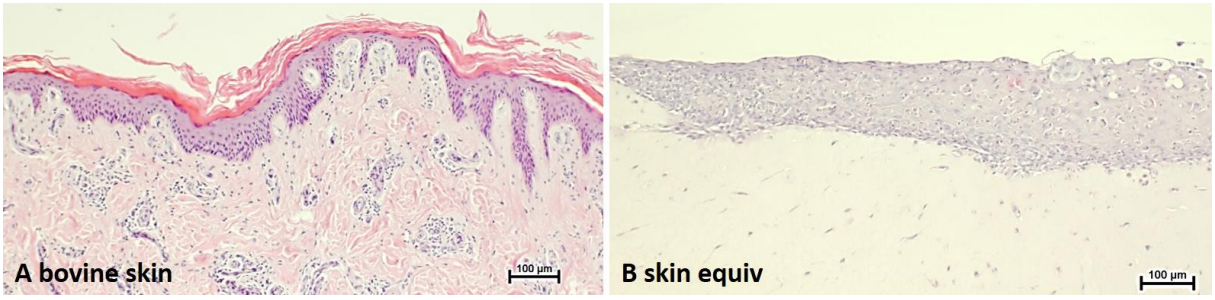


**Figure 20 H&E-stained paraffin sections of bovine foot skin (A) and the skin equivalent II (B).** Native skin shows the typical layered skin structure with epidermis and dermis and a pronounced epidermal stratum corneum. B The skin equivalent also consists of an epidermal and a dermal part. Fibroblasts embedded in bovine collagen (≙ dermal equivalent) exhibit the characteristic elongated cell morphology with intact cell nuclei. Many layers of keratinocytes resemble the epidermis. The characteristic morphological transformation of keratinocytes can only be observed in some areas of the skin equivalent. Due to the absence of physical stress, the stratum corneum is not desquamated. Skin appendages such as hair follicles and glands are not included in the skin equivalent.

The state of keratinocyte differentiation on top of the dermal equivalent was investigated using indirect immunofluorescence stainings with the epidermal differentiation marker keratin 14 (K14) and desmoglein 1 (Dsg1). As outlined earlier, K14 is considered a marker of basal epidermal cells (Fuchs and Green, 1980). This intermediate filament is expressed in the lower epidermal cell layers and around hair follicles. This was also demonstrated for bovine skin and can be seen in Figure 21 A. Since hair follicles were not included in the skin equivalent, K14 was found only in the lower cell layers of the epidermal equivalent (see Figure 21 B).

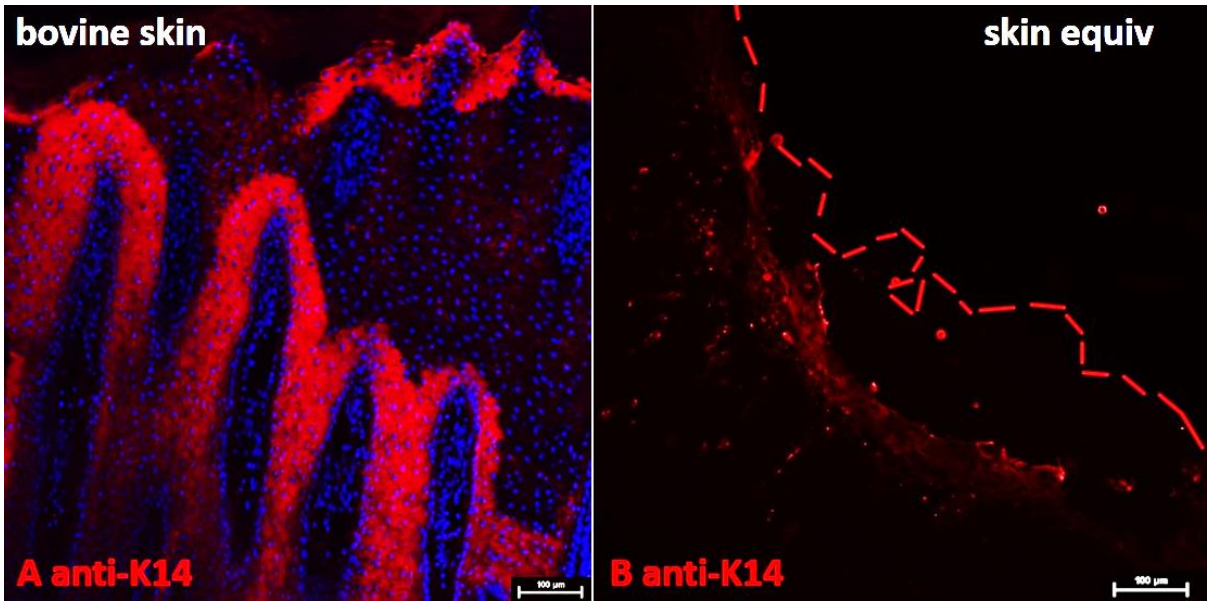


**Figure 21 Immunofluorescence stainings of native bovine skin (A) and the skin equivalent II (B) with anti-K14, a marker of basal keratinocytes in the epidermis.** Note the differentiation specific expression pattern of K14, i.e. K14 is found mainly in the basal epidermal cell layers. In native skin it is also found around hair follicles (red lines in B indicate upper edge of epidermal part of the skin equivalent; bars indicate 100µm).

Desmosomes are adhering junctions that connect neighboring cells. They provide not only mechanical strength to intercellular adhesion but also play a major role in the outside-in cell signaling (Waschke, 2008). To date, four different isoforms of desmoglein, which is a member of the cadherin protein superfamily, are known. Dsg1 (165kDa), Dsg2 (116kDa), Dsg3 (130kDa) and Dsg4 (108kDa) show typical expression patterns in different tissues, e.g. Dsg4 was shown to be the principal desmoglein of hair follicles (Whittock and Bower, 2003). Differentiation specific expression patterns are also seen, e.g. Dsg1 is most abundant in the superficial epidermal cell layers whereas Dsg3 is mainly found in the deeper, basal layers (Waschke, 2008). This holds equally true for bovine skin and is demonstrated in Figure 22 A. This expression pattern was also seen in the skin equivalent II: the immunofluorescence signal was strongest in suprabasal strata of the epidermal part (see Figure 22 B).

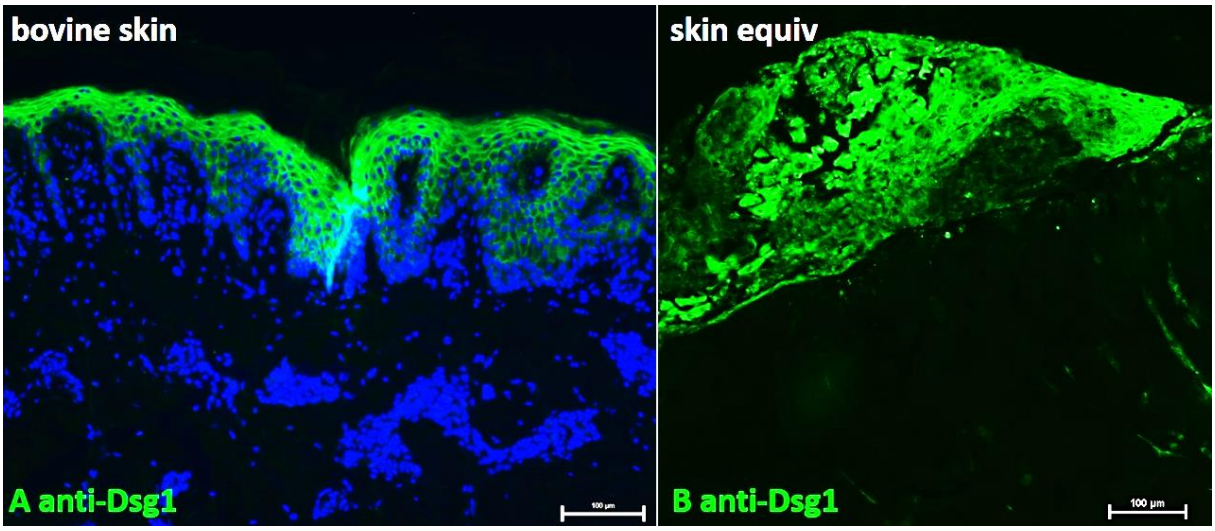


**Figure 22 Immunofluorescence staining of native bovine skin (A) and the skin equivalent II (B) with anti-Dsg1, an antibody directed against a desmosomal protein mainly found in suprabasal epidermal strata.** Note: in (A), cell nuclei were counterstained with Bisbenzimide Hoechst 33342. Bars indicate 100µm.

### 4.3 Culture of and experiments with *Treponema* spp.

*Culture.* As can be seen in Figure 23, culture and propagation of *Treponema* spp. was carried out successfully according to the procedure outlined earlier (see chapter “3.3.5 Culture of *Treponema* spp.”). Spiral bacteria of the strain *T. phagedenis-like* CR2220RR CV could be observed by d7 of anaerobic liquid culture in OTEB (Figure 23 A). Circular, translucent colonies of *T. phagedenis*-like CR2220RR CV and *T. denticola*-like 1-9185MED on FAA and sheep blood agar plates were observed between d8 and d10 of culture (Figure 23 B and C), respectively. Bacterial identity was confirmed using dark field microscopy and RT-qPCR (data not shown).

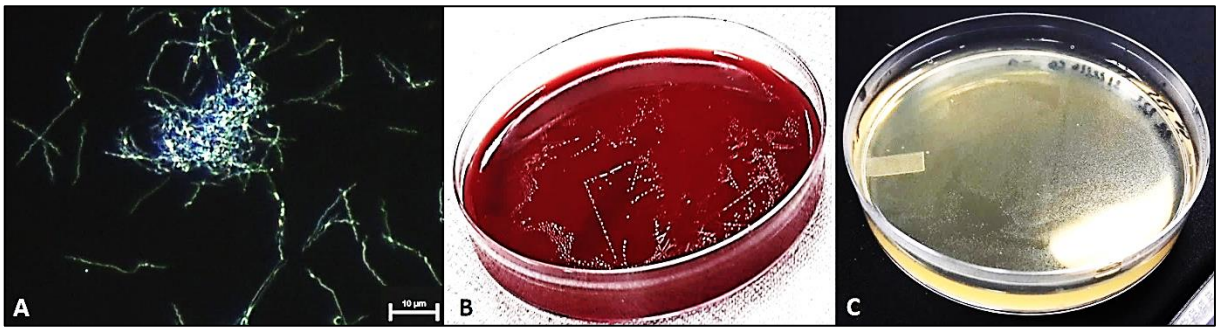


**Figure 23 Culture of *Treponema* spp.** A Dark field microscopy of *T. phagedenis*-like CR2220RR CV in liquid OTEB (bar indicates 10µm). B *T. denticola*-like 1-9185MED-colonies on solid sheep blood agar plates. C *T. phagedenis*-like CR2220RR CV colonies on fastidious anaerobe agar plates.

*Preliminary experiments.* The incubation experiment described in chapter “3.3.6 Experiments with *Treponema* spp.” was conducted as outlined.

*T. phagedenis*-like CR2220RR CV were exposed to collagen pads, keratinocytes grown on pmF-coated cell culture inserts and skin equivalents in separate trials. Table 10 summarizes the experimental set up and the results of each trial. Before each experiment, *T. phagedenis*-like CR2220RR CV were checked for cell viability and motility with dark field microscopy. The inoculum was plated on sheep blood agar/FAA plates as positive control for bacterial growth and to determine the number of cfu/ml. RT-qPCR was always conducted in duplicates with the inoculum as positive control and fresh OTEB as negative control. Histological staining was carried out according to standard protocols.

*Table 10 Set up and results of experiments with Treponema spp.*

| Exp | Set up | Results | | | |
|---|---|---|---|---|---|
| | | **wet mount** | **Histology** | **RT-qPCR** | **plates** |
| 1 | Trep on derm equiv; incubation:0, 10, 20, 30 min | - (inoculum: +; $8.32x10^6$ cfu/ml) | - | - (positive control: +) | - (inoculum: +) |
| 2 | Trep on derm equiv; incubation: 1, 2, 3, 4 h | contamination with spherical bacteria (inoculum: +; $6.45x10^6$ cfu/ml, no contamination) | technical problems with sections of 3 h; 1, 2 and 4 h + | - (positive control: +) | inoculum: +; other plates: contamination, 4 h also few spiral organisms |
| 3 | Trep on keratinocytes grown on pmF-coated inserts; incubation: 0, 10, 20, 30 min | - (inoculum: +; $7.8x10^6$ cfu/ml) | - | - (positive control: +) | - (inoculum: +) |
| 4 | Trep on skin equivalent d37; incubation: 1.5 h | - (inoculum: +; $6.96x10^6$ cfu/ml) | 1.5 h: + | not conducted | not conducted |

(Exp ≙ experiment; Trep ≙ *T. phagedenis*-like CR2220RR CV; derm equiv ≙ dermal equivalent; +/- ≙ positive/negative for *T. phagedenis*-like CR2220RR CV)

*T. phagedenis*-like CR2220RR CV were exposed to dermal equivalents twice. The immediate visual control (dark field microscopy) of the supernatant after all incubation periods (0, 10, 20 and 30 min; 1, 2, 3 and 4 h) did not reveal any spiral organisms. Bacterial growth was not observed on both types of agar plates with one exception: growth of bacterial colonies could be detected on the plates with the supernatant after 4 h of incubation. Unfortunately, growth of contaminating organisms was also observed on this plate and hindered the determination of cfu/ml.

Apart from the inoculum, RT-qPCR was unable to detect *Treponema* spp. DNA even in the sample after 4 h of incubation where spirochete colonies were observed on the corresponding agar plates (see Figure 27 as an example for an amplification plot, appendix).

*T. phagedenis*-like CR2220RR CV were also not found in collagen pads after up to 30 min of incubation. When incubated 1 h and longer, spiral organisms were observed throughout the complete dermal equivalent with a decreasing cell number towards the basal side of the collagen pad (see Figure 24 and Figure 25).

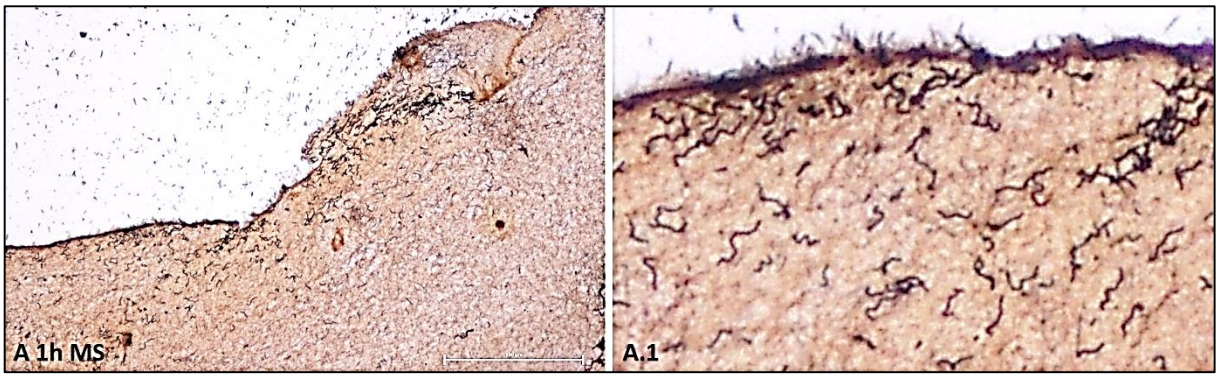


**Figure 24 MS-stained paraffin section of a dermal equivalent after 1h of incubation with a *T. phagedenis*-like CR2220RR CV suspension under anaerobic conditions.** Spiral organisms are found throughout the collagen pad (bar indicates 100µm). A.1 Magnification of A highlighting the spiral organisms in the collagen pad.

Besides the elongated, spiral organisms, small, circular, MS-stained forms were also found after 4 h of incubation under anaerobic conditions. These forms are thought to be encysted *T. phagedenis*-like CR2220RR CV (see Figure 25, black arrows; asterisks indicate spiral *T. phagedenis*-like CR2220RR CV).

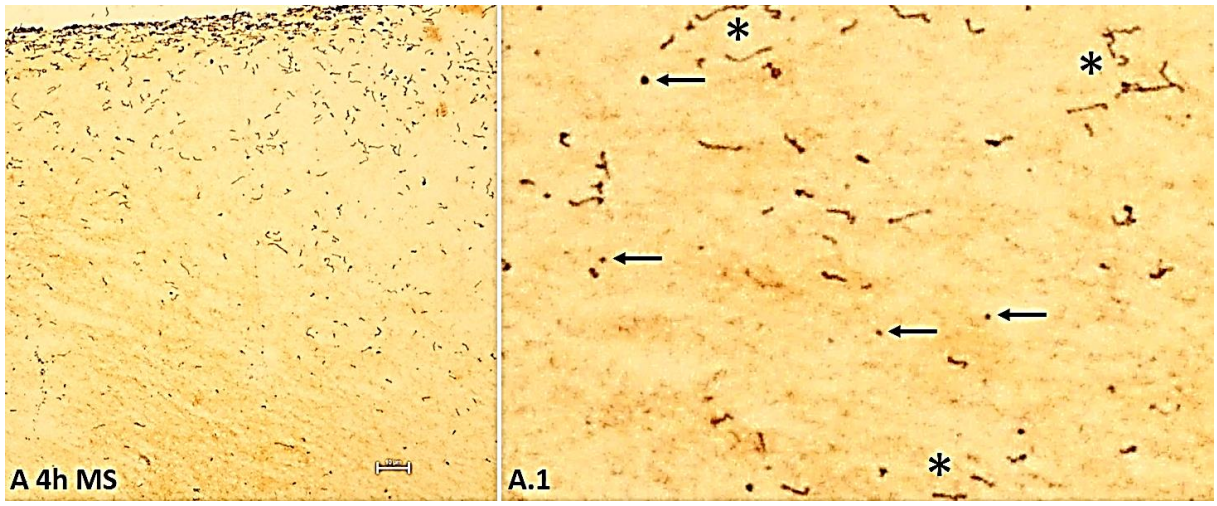


**Figure 25 MS-stained paraffin section of a collagen pad after 4h of incubation with a *T. phagedenis*-like CR2220RR CV suspension under anaerobic conditions.** A Modified Steiner stain revealed spiral bacteria throughout the whole collagen pad with a decreasing cell number towards the basal side of the dermal equivalent (bar indicates 10µm). A.1 Magnification of A demonstrating circular (black arrows) and spiral forms (asterisks) of *T. phagedenis*-like CR2220RR CV. This scenario closely resembles the in vivo situation.

*T. phagedenis*-like CR2220RR CV were also exposed to keratinocyte monolayers (0, 10, 20 and 30 min). They were not detectable in the supernatant visually by dark field microscopy, by PCR or by plating. Likewise, histological staining of the fixed cell monolayer did not reveal any attached or invading spiral organism (data not shown).

In the fourth trial of these initial experiments, *T. phagedenis*-like CR2220RR CV were incubated with full thickness skin equivalents II. Cells and bacteria were incubated for 1.5 h under anaerobic conditions. H&E-staining of the skin equivalent showed an uneven keratinocyte stratification on top of the dermal equivalent (see Figure 26 A). MS-stained paraffin sections revealed spiral organisms in the epidermal and the dermal part of the skin equivalent (see Figure 26 B).

Since the skin equivalent was not correctly differentiated and did not cover the whole insert membrane, PCR and plating of the supernatant were not conducted.

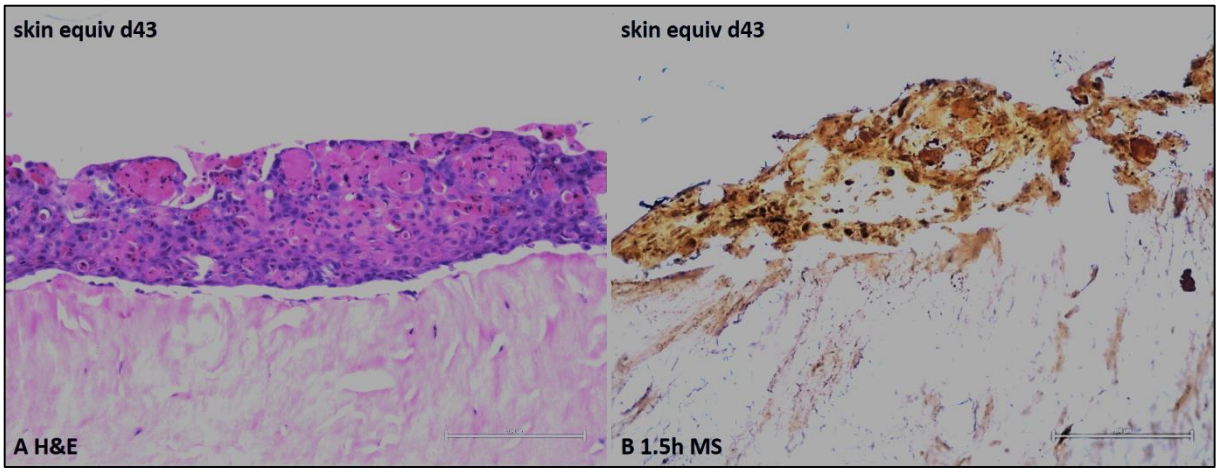


**Figure 26 *T. phagedenis*-like CR2220RR CV were exposed to skin equivalents (d43of ali).** A H&E-stained paraffin section of the bovine skin equivalent II. Keratinocytes stratified unevenly on top of the dermal equivalent. B MS-stained paraffin section of skin equivalent II, which was exposed to a *T. phagedenis*-like CR2220RR CV suspension for 1.5h under anaerobic conditions. Spiral organisms are found in the epidermal and the dermal part of the skin equivalent equally (note: fissures between cell layers and in the dermal equivalents are embedding artefacts, bars indicate 100µm).

## 5. Discussion

Digital dermatitis, a painful dermatitis of mainly bovine hind limbs, has developed into an ever-growing global threat to the cattle industry. Since its initial description in 1974 in Italy (Cheli and Mortellaro, 1974), diverse studies were conducted to shed light on the details of this disease, e.g.:

- histological and bacteriological examinations of intact and affected skin (Cruz et al., 2005; Döpfer et al., 1997),
- immunologic analyses on local and systemic immune responses to infection (Refaai et al., 2013)
- genetic investigations to identify predispositions and susceptibility factors (Relun et al., 2013; Rodriguez-Lainz et al., 1999)
- development of experimental in vivo infection models (Gomez et al., 2012),
- studies on host and environmental reservoirs of infection (Evans et al., 2012) and possible routes of transmission between animals and farms (Evans et al., 2009)
- field trials on different feeding strategies and food additives (Gomez et al., 2014), on housing (Ouweltjes et al., 2011; Somers et al., 2005) and hoof trimming (van der Tol et al., 2004).

It was the intention of the present study to use the numerous advantages of cell culture, e.g. a high reproducibility under standardized conditions, to establish an in vitro 3D skin model, which enables the detailed investigation of cellular and microbiological events during the infection of bovine skin by *Treponema* spp.

*Primary cell culture.* Initially, a technique to reliably and reproducibly isolate primary keratinocytes from the site of infection, i.e. the plantar aspect of the bovine distal limb, was established. Appropriate cell culture media for the long-term cultivation and storage of bovine skin cells were found.

During primary cell culture, the cell culture media should promote cell viability and especially proliferation. The addition of retinoic acid to the culture media, which is known to suppress keratinocyte differentiation (dose-dependent), might enlarge the number of obtained cells even more (Fuchs and Green, 1981; Ponec and Boonstra, 1987). Epidermal growth factor (EGF), which was added to the medium at a concentration of 10ng/ml, was shown to reduce the colony-forming efficiency of

cultured human keratinocytes when added right from the beginning of the culture (Rheinwald and Green, 1977). It might be worthwhile to add EGF after a few days of culture for the first time and evaluate the resulting effect on the final cell number.
Another aspect when it comes to improving primary cell culture is the nature of the surface of cell culture dishes. The (glyco)proteins laminin, fibronectin and collagen are components of basal membranes and the extra cellular matrix (Breitkreutz et al., 2009; Sechler et al., 1997). Cell attachment to culture dishes might be facilitated and/or accelerated when their surfaces are coated with one or more of these proteins.

*Skin equivalents.* The basis for the generation of organotypic bovine skin equivalents provided two approaches that were different in terms of handling and application. The in vitro generated autologous epidermis equivalent EpiDex®, which was intended as a medical product, served as a role model for the first approach. In this two-step-model, the dermal and the epidermal part were generated independently and then assembled to form the complete skin equivalent. The second approach was based on the work of Voersmann et al. (2013) who established a 3D skin model as a scientific drug testing system. Here, the skin equivalent was generated in a single step, i.e. keratinocytes were directly seeded on top of the dermal equivalent to grow into the complete skin equivalent.

*Skin equivalents I: two-step-model.* For the two-step-model, bovine keratinocytes were seeded into cell culture inserts that were coated with growth promoting but post-mitotic fibroblasts and cultured with the air-liquid-interface method. The resulting cell sheet consisted of several keratinocyte layers but - in contrast to EpiDex® - was not transferable to the dermal equivalent.
Besides the culture conditions such as temperature and atmosphere, the composition of the culture medium is crucial for cell viability. The culture medium that achieved best results during primary isolation and cultivation of bovine cells was also used for the production of the skin model. During the production of EpiDex®, the cell culture medium was also neither adapted nor changed, i.e. the exact same medium was used for outgrowth of keratinocytes from human hair follicles and the generation of epidermal discs (personal communication with co-workers). Concluding from the results of the present study for the bovine system, it is suggested to use different media for cell isolation/propagation and model generation. A culture medium for 3D cell

culture would have to promote the correct cell differentiation and sheet formation. Calcium ions are crucial for the induction of terminal differentiation and the formation of cell-cell-contacts, namely desmosomes (Windoffer et al., 2002). The concentration of calcium ions of the medium used for the present study was 0.35mM (data not shown; test carried out by Clinical Pathology Unit of UW Veterinary Care). Hennings et al. (1980) demonstrated for mouse epidermal cells that a calcium concentration of 1.2mM leads to the formation of dense, stratified cell sheets with desmosomes between the cells. Thus, different calcium concentrations could be tested to find the optimal concentration for bovine keratinocytes to form well-stratified and stable cell sheets. The glucocorticoid hydrocortisone is known to stimulate keratinocyte terminal differentiation (Ponec and Boonstra, 1987); it could be added to the culture medium to promote the development of the cornified envelope.

*Skin equivalents II: direct model.* The second approach in which keratinocytes were seeded on top of a dermal equivalent, i.e. a bovine collagen type I pad with embedded post-mitotic fibroblasts, gave rise to a promising organotypic skin reconstruction. The incorporated post-mitotic fibroblasts showed a typical cell morphology with intact nuclei (see Figure 17). The differentiation of keratinocytes on top of the dermal equivalent was demonstrated with anti-K14 and anti-Dsg1 immunofluorescence stainings (see Figure 22 and Figure 22). It is therefore concluded that the positive influence of fibroblasts on keratinocyte growth and differentiation, which has already been described in 1989 for 2D (Limat et al., 1989) and 3D cell culture with human cells (Coulomb et al., 1989), also applies to this bovine system. Furthermore, the cells in the model are viable for more than four weeks, which might be crucial for subsequent incubation experiments with *Treponema* spp.

Nevertheless, further improvement of the generation process and a more detailed characterization of the skin model as outlined below should be pursued. One aspect of 3D cell culture that needs revision is the continuous air exposure of the skin model after medium withdrawal. It was not always given with the current protocol and leaded to malformed skin equivalents (medium from lower compartment diffused into upper compartment). Vörsmann et al. (2013) suggest the transfer of the skin models to bigger culture dishes when introducing the air-liquid interface culture, i.e. 3 skin models (size of 24-well insert) were transferred to one well of a 6-well plate. Since the skin models of the current thesis are generated in 12-well inserts, they might be transferred to

smaller inserts after contraction. If the complete insert membrane is covered by the skin model, the diffusion of the culture media from the lower to the upper compartment should come to an end.

Further characterization could be done with more immunofluorescence stainings, e.g. with antibodies against the proteins laminin, involucrin and loricrin. The basal membrane physically separates epidermis and dermis and is made up of proteins synthesized and secreted by keratinocytes and fibroblasts equally (Marinkovich et al., 1993), as e.g. the glycoprotein laminin (Breitkreutz et al., 2009). Its presence in the skin equivalent would indicate a physiological cell metabolism of both cell types. The correct formation of the cornified envelope of the corneocytes in the stratum corneum could be demonstrated with anti-loricrin and anti-involucrin, antibodies that are directed against the two major structural cornified envelope proteins (Candi et al., 2005). The presence of both proteins could also be demonstrated by Western blotting.

*Experiments with Treponema spp.* The results of initial *Treponema*-experiments proved that the skin equivalent is a suitable model to investigate the underlying mechanisms during *Treponema*-infection of bovine skin. First evaluations of paraffin-embedded MS-stained sections of skin equivalents showed a very close-to-reality picture of invaded bovine skin (see Figure 24 and Figure 25).

However, the experiments should be repeated and refined in terms of handling and timing. The readily prepared skin equivalents should be transferred to smaller cell culture inserts before the incubation with the bacterial suspension starts. Since the contracted pads do not cover the whole 12-well insert membrane, it cannot be ensured that all *Treponema* spp. found in the pad or in the supernatant went all the way through the pad. In smaller inserts or other cell culture dishes, this insecurity could be eliminated.

So far, no spirochete bacteria were observed in the supernatant, could be detected by RT-qPCR or grown on different agar plates after the incubation experiment. This might be due to the encysted forms of the bacteria that were observed in the collagen pads (see Figure 25). Döpfer et. al (2012) also found a discrepancy between cell counting via hemocytometer and plate count which they explain with the fact that encysted bacteria do not form colonies on plates. Thinner collagen pads or longer incubation periods might allow the complete passage of the bacteria through the pad into the supernatant underneath the insert.

## 6. Outlook

The current project provides a suitable experimental set up to study the pathogenesis of digital dermatitis. Future in vitro studies on bacterial adhesion, invasion and encystation under standardized conditions might help to elucidate the pathomechanisms during lesion formation.

To investigate the nature of the interaction between skin cells and bacteria, the skin equivalents could be manipulated. After enzymatic treatment or incubation with specific antibodies, the skin equivalents could be exposed to *Treponema* spp. again. The results might give some indication of the cell surface molecules or receptors involved, e.g. when cell-cell-contacts are penetrated. Questions concerning the influence of the bacteria on the skin cells could be investigated, e.g. in how far the cytokine expression of keratinocytes is changed or even suppressed. Moreover, recent work has shown that cattle can be typed into three groups concerning the occurrence and the course of DD: type 1 animals do not develop acute M2 lesions, type 2 animals suffer from these lesions once and type 3 animals suffer from repeated episodes of M2 lesions (Döpfer et al., 2004). Skin models could be generated with primary cells from cattle of all three types and subjected to *Treponema*-exposure. The results of these experiments might elucidate some of the reasons for the different disease courses.

Selectively changed culture conditions might answer questions in the context of disease chronicity, e.g. which conditions lead to the encystation or the reactivation of the bacteria residing in the host tissue.

Once reliable data has been obtained for one *Treponema*-strain, others should also be tested to compare the behavior of different strains. Co-incubation experiments with other bacterial organisms commonly found in or near by DD lesions such as *Fusobacterium* spp. (Cruz et al., 2005), *Campylobacter* spp. (Döpfer et al., 1997) or *Dichelobacter nodosus* (Rasmussen et al., 2012) could be conducted as well to study bacterial interaction and the role of each species involved.

Anyway, in terms of animal health and welfare, it must be the goal of all the different efforts in research about digital dermatitis to reach at least the so-called “manageable state of the disease” (Döpfer and Bonino Morlán, 2008; Green and George, 2008) until a more detailed understanding enables its eradication.

## 7. References

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# 8. Appendix

*Table 11 Devices that were used during culture of cells and bacteria.*

| **Device** | **Model** | **Supplier** |
|---|---|---|
| Biosafety Cabinet | KS 12 | Heraeus |
| Centrifuge | 5804R | Eppendorf |
| Incubator | INCO 153 med | Memmert |
| Microscope (upright) | IX70 | Olympus |
| Microscope (inverse) | BX51 | Olympus |
| Fluorescence microscope (inverse) | TE2000 | Nikon |
| Water bath | WNB10 | Memmert |
| pH meter | pHenomenal 1000L | VWR |
| Anaerobic chamber | Bactron Anaerobic/ Environmental Chamber | Sheldon Manufacturing Inc., Oregon, USA |

*Table 12 Protocol for paraffin embedding of tissue samples*

| **Chemical** | **Time [min]** |
|---|---|
| EtOH 30% | 60 |
| EtOH 50% | 300 |
| EtOH 70% | 60 |
| EtOH 80% | 120 |
| EtOH 96% | 120 |
| Isopropyl | 120 |
| Isopropyl | 60 |
| Isopropyl | 120 |
| EBE | 180 |
| EBE | 60 |
| Paraffin | 120 |
| Paraffin | 120<br>→ embed |

*Table 13 Protocol for dewaxing of paraffin-embedded tissue samples*

| **Chemical** | **Time [min]** |
|---|---|
| Rotihistol | 10 |
| Rotihistol | 10 |
| EtOH 90% | 5 |
| EtOH 80% | 5 |
| EtOH 70% | 5 |
| EtOH 50% | 5 |
| $dH_2O$ | short wash<br>→ stain |

*Table 14 Staining protocol for standard histological H&E-stain*

| **Chemical** | **Time [min]** |
|---|---|
| Hematoxylin (1:1 $H_2O$) | 10 |
| tap water | short wash (2x) |
| tap water | 10 |
| Eosin | 5 |
| $dH_2O$ | 5 |
| EtOH 96% | 2 |
| EtOH 96% | 2 |
| Isopropyl | 2 |
| Isopropyl | 2 |
| Rotihistol | 2-5 |
| Rotihistol | 2-5<br>→ coverslip |

*Table 15 DNA extraction protocol for suspensions containing Treponema spp.*

| **Step** | **Details** |
|---|---|
| 1. centrifuge supernatant | 10.000rpm, 5min<br>→ remove supernatant |
| 2. add nuclease free water | 50µl |
| 3. vortex | 30sec |
| 4. boil | 100°C, 20min |
| 5. centrifuge | max. speed, 20min<br>→ transfer 30µl of supernatant to fresh reaction tube |

*Table 16 Details of primer probe used to detect Treponema spp.*

| **Target Gene** | **Primer/Probe Specificity** | **Primer/Probe Sequence (5'-3')** | **Amplicon [bp]** | **Position** |
|---|---|---|---|---|
| 16s rRNA | *Treponema* spp. | CAAGGCDWYGATGGGTAT | 119 | Varies |
| 16s rRNA | *Treponema* spp. | GTCAGACTTYCGTCCATTG | 119 | Varies |
| 16s rRNA | *Treponema* spp. | CGGACACATTGGGACTGAGATACG | 119 | varies |

*Table 17 RT-qPCR set up*

| **PCR component** | **Volume [µl]** |
|---|---|
| MMRox | 12.5 |
| AllTrepPP | 1.0 |
| NF $H_2O$ | 9.0 |
| Sample DNA | 2.5 |
| total | 25 |

*Table 18 Thermal profile of RT-PCR run*

| **Step** | **Temperature [°C]** | **Time [min]** |
|---|---|---|
| Initial denaturation | 95 | 10 |
| Denaturation | 95 | 1 |
| Elongation | 60 | 1 |
| | → repeat denaturation and elongation 40x | |

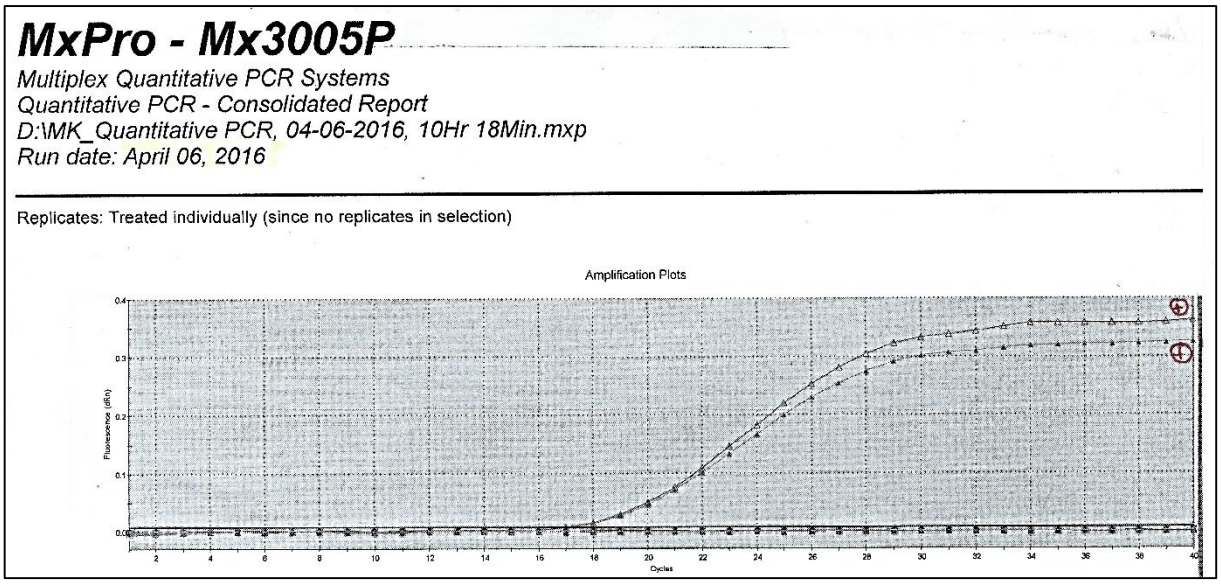


**Figure 27 Amplification plot of RT-qPCR run.** RT-qPCR of each sample was conducted in duplicates with the inoculum as positive control and fresh OTEB as negative control. *Treponema*-DNA was detected in the positive control only (indicated by +) but not in any of the samples (supernatant underneath the skin equivalents after inoculation with *T. phagedenis*-like CR2220RR CV).