Preparation of reconstituted tissues

Ing. Kristýna Valášková, Ph.D.

Doctoral Thesis Summary

Tomas Bata University in Zlín

Tomas Bata University in Zlín
Centre of Polymer Systems

Doctoral Thesis Summary

Preparation of reconstituted tissues

Příprava rekonstituovaných tkání

Author: **Ing. Kristýna Valášková, Ph.D.**

Degree programme: P0711D130023 Biomaterials and Biocomposites

Supervisor: doc. Ing. Zdenka Víchová, Ph.D.

Consultant: prof. Ing. Petr Humpolíček, Ph.D.

Reviewers: doc. RNDr. Jitka Vostálová, Ph.D.
Mgr. Jiří Pacherník, Ph.D.

Zlín, September 2025

© Kristýna Valášková

Published by **Tomas Bata University in Zlín** in the Edition **Doctoral Thesis Summary**.
The publication was issued in the year 2025.

Keywords: *tissue engineering, three-dimensional skin model, organotypic culture, biomaterials, cell-assembled extracellular matrix, co-culture*

Klíčová slova: *tkáňové inženýrství, trojrozměrný model kůže, organotypická kultura, biomateriály, extracelulární matrix sestavená z buněk, kokultivace*

Full text of the Doctoral thesis is available in the Library of TBU in Zlín.

ISBN 978-80-7678-363-8

ACKNOWLEDGEMENT

I would like to express my deepest thanks to my doctoral thesis supervisor, doc. Ing. Zdenka Víchová Ph.D., from the Centre for Polymer Systems at Tomas Bata University in Zlín, for her professional guidance, constructive criticism, time sacrifice, and kind attitude. Furthermore, I would like to thank prof. Ing. Petr Humpolíček Ph.D., for his valuable advice during my academic journey.

Finally, I would like to express my sincere gratitude to all my colleagues at the Centre of Polymer Systems. Their support, collaboration, and shared experiences have significantly contributed to creating a supportive workplace that has greatly enriched my work.

Not least, I would like to thank my family for their unceasing support during my PhD studies.

ABSTRACT

Living tissues are highly dynamic systems in which continuously changing structural, mechanical, biochemical, and biological cues are intrinsically combined within three-dimensional structures. Cells and tissue scaffolds (extracellular matrix) play a critical and synergistic role, not only in the formation of tissue architecture but also in the maintenance of its physiological function. Therefore, both the role of cells and scaffolds are required to play integral roles in the construction of reconstituted tissues. This is a dynamic and multidisciplinary area of research. Models are finally applicable to tissue engineering and regenerative medicine, disease modeling, and *in vitro* experiments. Within the thesis, the main attention is paid to *in vitro* reconstituted three-dimensional (3D) models of skin. Models should allow the reconstitution of a skin architecture, and micro-environment, to enable the investigation of cell-matrix and cell-cell interactions between different cell types, and finally even mimic the function and physiology of their *in vivo* tissue counterparts. The work aims to clarify the preparation of the collagen layer and the subsequent provision of an appropriate fibroblast-keratinocyte co-culture resulting in the functional skin equivalent *in vitro*.

Regardless of this, the skin tissue cells, respectively, dermal fibroblasts, can be used as cell-assembled extracellular matrix (CAM) bio-scaffolds. Instead of using synthetic or natural materials as scaffolds, this approach harnesses the inherent ability of cells to produce their own ECM. The 3D architecture of CAM sheet can be formed and controlled by external factors such as mechanical stimulation. In our research, human dermal fibroblasts were cultured *in vitro* with ascorbic acid to promote cell-made scaffold assembly and deposition, resulting in intact ECM that preserves tissue composition and architecture, with enhanced cell-instructive properties like anisotropy. While CAM replicates biochemical and some structural ECM features, it lacks full hierarchical complexity and mechanical properties of native tissue. These cell-instructive properties influence CAM quality and functionality, shaped by cell-ECM interactions and modifiable through bio- physical/chemical cues for tissue engineering applications. To study this, a co-culture platform of human dermal fibroblasts and H9c2 cardiomyoblasts was established at varying ratios. This approach aligns with findings from decellularised CAM scaffolds, which preserve key ECM components critical for cell adhesion and growth. CAM scaffolds similarly support H9c2 proliferation, providing a fully cellular, immunogenically safe, biologically native substrate that enhances cell signalling and enables physiologically relevant tissue models.

Keywords: *tissue engineering, three-dimensional skin model, organotypic culture, biomaterials, cell-assembled extracellular matrix, co-culture*

ABSTRAKT

Živé tkáně jsou vysoce dynamické systémy, v nichž neustále probíhá a mění se řada strukturálních, mechanických, biochemických a biologických podnětů v rámci trojrozměrné struktury. Buňky a tkáňové scaffoldy (extracelulární matrix) hrají rozhodující a synergickou roli nejen při tvorbě architektury tkáně, ale také při udržování její fyziologické funkce. Proto musí být při přípravě rekonstituovaných tkání splněna úloha jak buněk, tak i scaffoldů. Jedná se o dynamickou, multidisciplinární oblast výzkumu, kde jsou modely v závěru použitelné pro tkáňové inženýrství a regenerativní medicínu, modelování nemocí a případné experimenty *in vitro*. V rámci práce je hlavní pozornost věnována rekonstituovaným trojrozměrným (3D) modelům kůže. Modely by tedy měly umožnit rekonctituci architektury kožní tkáně a jejího mikroprostředí, umožnit zkoumání interakce buňka-matrix a mezi různými typy kožních buněk, a koneckonců i funkci a fyziologii kožní tkáně *in vivo*. Cílem práce je objasnit přípravu kolagenové vrstvy a následné zajištění správné ko-kultivace fibroblasty-keratinocyty s výsledkem vytvořeného plně funkčního kožního ekvivalentu *in vitro.*

Bez ohledu na to mohou být buňky kožní tkáně, respektive dermální fibroblasty, použity jako scaffoldy extracelulární matrix samosestavené z fibroblastových buněk (CAM). Tato metodika využívá vlastní schopnost buněk produkovat vlastní extracelulární matrix (ECM), místo použití syntetických nebo přírodních materiálů jako scaffold. CAM scaffold může být vytvořen a řízen i vnějšími faktory, jako je mechanická stimulace. V této práci byly kultivovány lidské kožní fibroblasty za podmínek *in vitro* s kyselinou askorbovou, podporující tvorbu ECM. Výsledkem je neporušená ECM, která zachovává složení a strukturu původní tkáně a zároveň vykazuje vylepšené vlastnosti pro řízení buněčného chování, například anizotropii. Přestože CAM dokáže věrně napodobit biochemické a základní strukturu ECM, postrádá plnou hierarchickou složitost a mechanické vlastnosti přirozené tkáně. Tyto instruktivní vlastnosti buněk poté ovlivňují kvalitu a funkčnost CAM scaffoldů a jsou utvářeny interakcemi mezi buňkami a ECM, které lze modulovat pomocí biochemických/biofyzikálních signálů pro aplikace tkáňového inženýrství. Pro studium těchto interakcí jsme vytvořili ko-kultivační platformu lidských dermálních fibroblastů a H9c2 kardiomyoblastů v různých poměrech. Tento přístup je v souladu s výsledky studií na decelularizovaných CAM, které zachovávají klíčové složky extracelulární matrix zásadní pro buněčnou adhezi a růst. CAM scaffoldy obdobně podporují proliferaci buněčné linie H9c2, poskytují plně buněčný, imunologicky bezpečný a biologicky přirozený substrát, který zlepšuje buněčné signály a umožňuje tvorbu fyziologicky relevantních tkáňových modelů.

Klíčová slova: *tkáňové inženýrství, trojrozměrný model kůže, organotypická kultura, biomateriály, extracelulární matrix sestavená z buněk, kokultivace*

TABLE OF CONTENT

1. INTRODUCTION

Tissue engineering and regenerative medicine (TERM) represent an interdisciplinary field that seamlessly marries principles from biology, engineering, and medicine to innovate therapeutic strategies capable of restoring, repairing, or even replacing damaged tissues (Auxenfans *et al.* 2009). In this realm, TERM applications commonly encompass 3D scaffolds for providing a suitable microenvironment for the incorporation of cells and supporting factors to regenerate damaged tissues/organs. These scaffolds, in the 3D scale, can serve as architectural frameworks providing suitable microenvironments with mechanical support, and physical, or biochemical stimuli for optimal cell growth and function. It facilitates the organization and growth of cells, ultimately leading to the formation of functional tissues (Loh and Choong 2013; Choudhury and Das 2020). The development of in vitro reconstituted skin models to mimic living tissue is important for investigating therapeutic approaches. It can also mitigate animal testing and bridge the inter-species translational gap. Reconstituted tissue models from cells and extracellular matrix (ECM) simulates natural tissues. It provides simplified biological systems to study cell-matrix interactions in tissue development and wound healing (Watakatsuki *et al.* 2000).

Skin is an essential complex organ of the human body. Skin structure consists of two components. It is the multistratified epidermis consisting of the basal, spinous, granular, and cornified strata, and the second one is the dermis. It is the upper-most part of the skin which can be very interesting for the study of barrier properties (Gangatikar *et al.* 2007). The first developed skin co-cultures used two-dimensional cultivation as a feeder layer for seeding the cells, concretely - the combination of keratinocytes and fibroblasts. The problematic part of this skin tissue cultivation is the limitation of the stratification and partial differentiation of the keratinocytes and overall, the complex cellular response due to the lack of 3D tissue architecture. Accordingly, developments over the last decade have allowed construction of the tissue-engineered models that can simulate native skin. This model is known as skin equivalent (SE) (Fell 2016; Garlick 2006; Laurent and Denesvre 2021).

The knowledge of the SE has an important impact on facilitating the experimental system's progression to study skin biology and its potential diseases. Historically, various skin cell types have been studied using two-dimensional (2D) cultivation, but still individually as a monolayer system. The cultivation did not capture the complexity of the in vivo microenvironment and possible cell-cell interactions. Besides, it was found that there were significant differences in cell migration, proliferating, extracellular matrix synthesis, cellular signaling, and responses to various stimuli in the case of the cultivation of the same skin tissue

cells under 2D or 3D culture conditions. Therefore, it is important to embrace the complex 3D environment for spreading and migration of the skin cells to create the appropriate SE (Eberlin *et al.* 2020; Stark *et al.* 2004; Choudhury and Das 2020).

In this thesis, we employed the representatives of the traditional scaffold-free Transwell chamber system approach. The experiment was designed to evaluate possible interactions between the collagen layer and cells for the development of a highly adaptable skin organotypic culture model. In addition, a possible method for gaining knowledge about the dynamic skin equivalent interactions in the combination of the resident microbes is to develop a polymicrobial skin model. The scaffold-free 3D skin model was then used for cell cytotoxicity and biochemical analysis, skin irritation testing with high-throughput cell function.

Moreover, SE and other skin tissue engineering-related products have breakthrough potential and are currently the focus of extensive worldwide research. A breakthrough technology can be a cell-assembled extracellular matrix (CAM) produced *in vitro* by normal adult skin fibroblasts (Potart *et al.* 2023). Dermal fibroblast cells for CAM sheet production are readily available in the skin, making them easily accessible for isolation and culture. In addition, the dermal fibroblast cells are well-known for their role in producing ECM components in the body. Overall, the use of dermal fibroblast cells for CAM production is a strategic choice that is consistent with mimicking the native tissue environment. These cells deposit ECM components in a manner that closely mimics the structure and composition of healthy tissue. The versatility of the CAM material allows it to be applied across a broad range of regenerative medicine applications, without being restricted to a specific tissue or organ system (Kawecki *et al.* 2024; Borchiellini *et. al.* 2023).

In a recent experiment, we obtained a biological ECM-enriched scaffold made of CAM material, which favorably preserves the composition and architecture of the original tissue in a controlled manner. Moreover, we enriched this experiment by performing a co-culture of human dermal fibroblasts with cardiomyoblasts in defined ratios, thereby creating a novel type of CAM sheet. This long-term culture system resulted in tissue-like structures with enhanced cell-instructive properties. The CAM sheet material was finally analyzed using biochemical and molecular analysis confirming the structural and functional properties of the CAM scaffold.

2. FUNDAMENTALS OF SKIN TISSUE ENGINEERING

2.1 Anatomy and Physiology of the Skin

The skin is the largest vital and the most visible organ of the human body. The main function of the skin is to act as a barrier, protecting the internal environment from the essentially hostile, external environment. As a total barrier, it can be also permeable to many substances including chemicals, drugs, perfumes, or dyes with which we may be in frequent contact. Apart from protection, the skin plays a critical role in the control of body functions, such as thermoregulation and assistance in the control of blood pressure. It contains numerous sensory receptors and has endocrine functions or synthesis of vitamin D possibility (Casey 2002).

The integumentary system of skin is accounting for about 15 % of the total adult body weight and is formed by the skin and its derivative structures, as seen in Figure 1 (Kolarsick P., Kolarsick A. and Goodwin 2011).

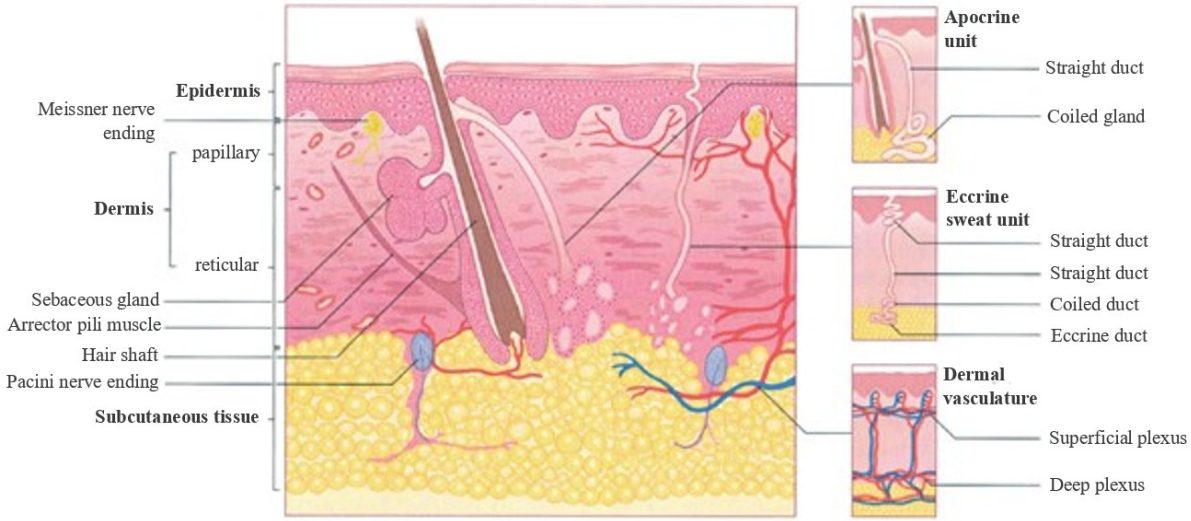


Figure 1: Structure of the skin (Kolarsick P., Kolarsick A. and Goodwin 2011)

Human skin is a complex organ made up of 3 layers (epidermis, dermis, and hypodermis), cell layers, and cell types whose primary function is to act as a barrier preventing dehydration and keep most chemicals, toxins, bacteria, and viruses out of the body. Skin mainly consists of dermal and epidermal tissues (Eberlin *et al.* 2020).

Embryonic skin development begins during the first two months from two germ layers—the ectoderm and mesoderm. The epidermis originates from the ectoderm, while the dermis and subcutaneous tissues derive from the mesoderm and mesenchyme (Koster and Roop 2007). After gastrulation, the ectoderm differentiates under the influence of signaling molecules including Wnt/β-catenin („Wingless-related integration site"), fibroblast growth factor (FGF), and bone morphogenetic protein (BMP) (Hu *et al.* 2018; Böttcher and Niehrs 2005; Stern 2005; Fuchs 2007). The signaling steps are visible in Figure 2.

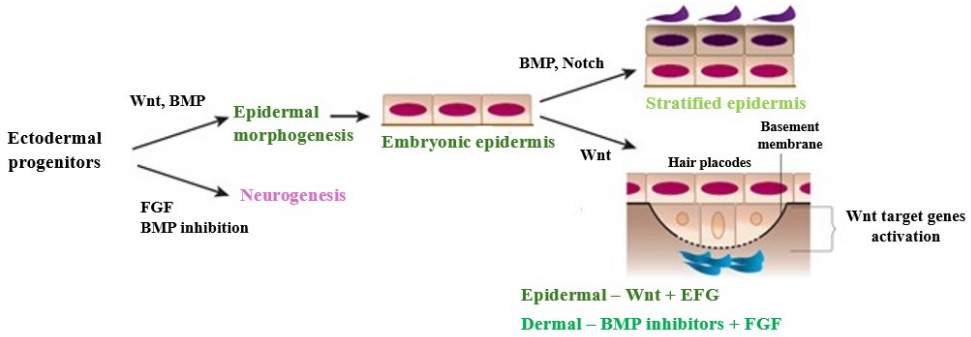


Figure 2: Signalling steps in the specification of embryonic skin (Fuchs 2007)

During embryonic skin organogenesis, both the epidermal and dermal skin layers are formed simultaneously. The skin is entered by melanocyte and Langerhans cell precursors, and between the subcutaneous layers and the dermis, initial vascular networks are established (Liu *et al.* 2013; Hoath *et al.* 2014). In the subsequent period of histogenesis, there is a stratification of the epidermis, dermal differentiation, and the formation of skin appendages such as glands, hair follicles, and nails. In this stage, melanocytes and specialized nerve endings also develop, giving rise to the skin's sensory and pigmentation functions (Pispa and Thesleff 2003; Mikkola 2007; Ness *et al.* 2013).

The last phase of development - maturation takes place after birth under the direction of a series of external environmental conditions such as temperature, humidity, and exposure to pathogens (Pispa and Thesleff 2003; Mikkola 2007; Ness *et al.* 2013). During this period in embryonic development, the interfollicular epidermis (IFE) matures. The basal layer of the stratified epidermis, which constitutes the embryonic epidermis, is developed from the multipotent cells of epithelium in the surface ectoderm. It expands to occupy all the layers of the prospective epidermis (Lawron and Kaur 2015). The epidermal basal cells proliferate and differentiate to establish layers. Above the basal layer lies the outer squamous layer, the so-called periderm, as can be seen in Figure 3 (Hu *et al.* 2018). Differentiation and stratification are regulated by proteins like p63 and Notch, which are essential in forming the spinous, granular, and cornified layers of the epidermis. Keratinocytes express basic structural proteins - keratins K -

intermediate filaments of epithelia at this period. Notch signaling, specifically, induces terminal differentiation of embryonic skin, ensuring proper epidermal barrier function (Kim *et al.* 2006; Hu *et al.* 2018).

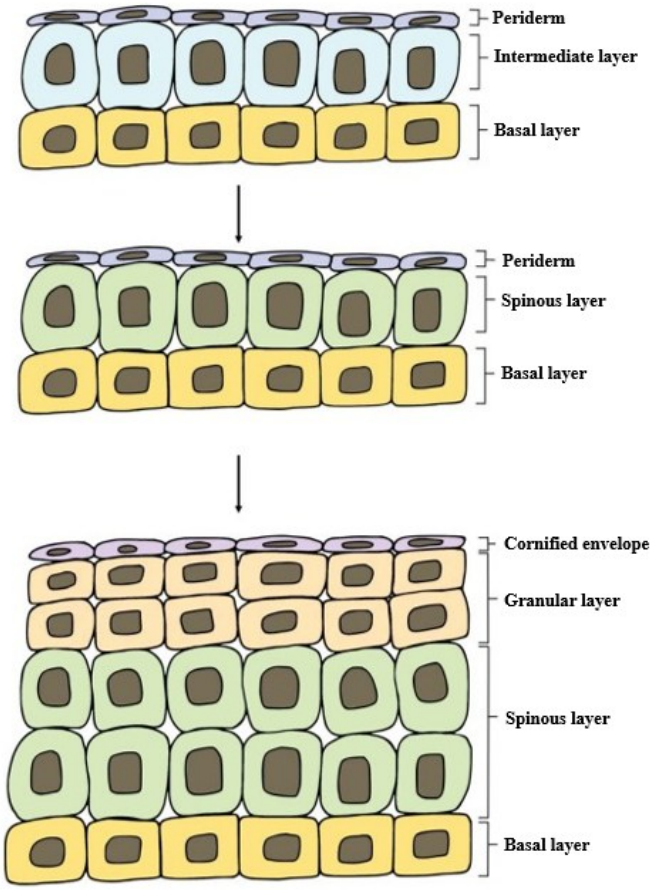


Figure 3: Embryonic skin maturing (adapted from Hu et al. 2018)

2.1.1 Epidermis

The epidermis is defined as the outermost layer of the skin and consists of a basal layer, which is at the bottom of the epidermis, and a suprabasal layer (the upper part of the epidermis). The epidermis is commonly divided into four layers according to the morphology and position of the keratinocytes. Based on the differentiation of the keratinocytes, they can be referred to as the basal cell layer (*stratum germinativum*), the squamous cell layer (*stratum spinosum*), the granular cell layer (*stratum granulosum*), and the cornified or horny cell layer (*stratum corneum*) (James *et al.* 2006).

This part of the skin morphology performs several functions. The cell layers of the epidermis are derived from germinative cells in the basal layer. This part contains the skin´s stem cell-generating the daughter cells. The daughter cells emerge from the basal layer and mature. Eventually, these cells develop protein

bridges to each other, creating cell-cell communication. This connection gives the layer a typical spiny contour - *stratum spinosum* (Kenneth and Roberts 2019). The spinous cell layer is located between the *stratum granulosum* and the *stratum basale* of the epidermis. It consists of a single layer of columnar or cuboidal cells with a large nucleus that is constantly dividing by mitosis to replenish the upper layers of the epidermis. Each cell is connected to the others through desmosome and hemidesmosome connections (Yadav *et al.* 2019). This layer is involved in providing strength and support to the epidermis. Cells in the *stratum spinosum* also begin to produce keratin, a tough fibrous protein that contributes to the structural integrity of the skin. This protein is produced by keratinocytes (Lai-Cheong and McGrath 2017). The keratinocytes differ from the "clear" dendritic cells by possessing intercellular bridges and ample amounts of stainable cytoplasm. Located in the basal layer of the epidermis, these types of keratinocytes can retain a stem cell-like property and thus support continuous cell division. Figure 4 shows the initiation of a differentiation program, as keratinocytes gradually exit the cell cycle and enter a terminally differentiated state. The differentiation process occurs during cell migration from the basal layer to the surface of the skin (Choi and Lee 2015; Roger *et al.* 2019).

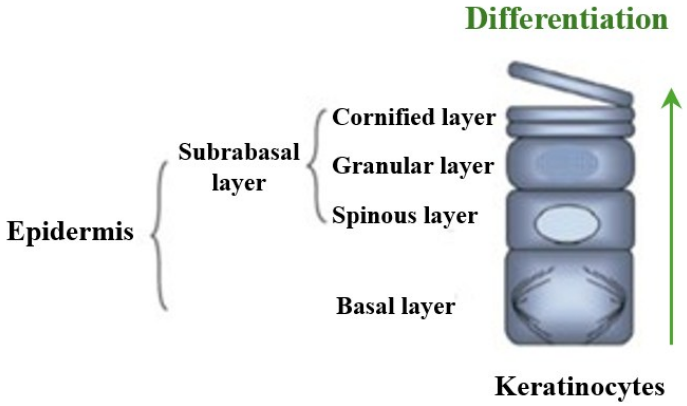


Figure 4: Schematic diagram of normal keratinocytes in different layers of the epidermal tissue. An arrow indicates the direction and levels of epithelial differentiation (Choi and Lee 2015)

As the keratinocyte cells move from the *stratum basale* to the *stratum spinosum*, their shape becomes more rounded and they can synthesize differentiation-specific keratins and other early differentiation markers (e.g. involucrin) (Lai-Cheong and McGrath, 2017).

The following structure is the granulosum layer. The cells in this layer are characterized by containing granules filled with keratohyalin, a protein that helps to bind keratin filaments together and form keratin fibers. These cells are undergoing apoptosis, and the cytoplasm is starting to fill with keratin. (Yadav *et*

al. 2019;). Keratohyalin secretes the keratin proteins tonofilaments and filaggrin which together form pre-keratin structures called tonofibrils. These structures play an important role in keratinization (Bragulla and Falabella 2001).

The outermost layer of the epidermis, *stratum corneum*, is made up of several layers of flattened, dead cells called corneocytes. These cells are filled with keratin. They lose their cellular organelles, and the contents of the cell are consolidated into a mixture of filaments and amorphous cell envelopes. The process of maturation leading to cell death is known as terminal differentiation (Montagna 1974; Yadav *et al.* 2019). The *stratum corneum* acts as a barrier to protect the body from external threats such as pathogens, UV radiation, and water loss. The key aspect of the epidermal permeability barrier function is provided by lipid lamellae. It is formed by cholesterol, ceramides, and free fatty acids that surround the protein components of the cornified envelope. Each layer of the epidermis plays a vital role in maintaining the integrity and function of the skin, ensuring that it remains resilient and able to fulfill its protective functions (Roger *et al.* 2019).

The epidermis is a continually renewing layer. It also arises derivative structures, such as the pilosebaceous apparatuses, nails, and sweat glands from here. The basal cells of the epidermis undergo cycles of proliferation that can provide renewal of the outer epidermis. It is a dynamic structure in which cells are constantly in unsynchronized motion, as differing individual cell populations not only pass each other but also move towards the skin surface (melanocytes, Langerhans cells) (Kolarsick P., Kolarsick A. and Goodwin 2011).

2.1.2 Dermis

The dermis is a critical component of the body, providing the nutritive, immune, and other support systems for the epidermis, through a thin papillary layer adjacent to the epidermis. The dermis comprises the bulk of the skin and provides pliability, elasticity, and tensile strength. It protects the body from mechanical injury and binds water. It also plays a role in regulating temperature, pressure, and pain. The dermis interacts with the epidermis to maintain the properties of both tissues (dermal-epidermal junction).

The dermis is composed of collagen fibers (70 %), which provide a scaffold for support and cushioning. There is also elastic connective tissue which provides elasticity in a semi-gel matrix of mucopolysaccharides. The main cells present are the fibroblasts (connective tissue components of collagen, laminin, fibronectin). The next cell type is mast cells, which are involved in the immune and inflammatory responses, and melanocytes, which are involved in the production of the pigment melanin (Walters and Roberts 2002; Kolarsick P., Kolarsick A., and Goodwin 2011).

2.2 The Role of Fibroblasts and Keratinocytes in Skin Tissue Dynamics

2.2.1 Role of Fibroblasts within Skin Architecture and the 3D Matrices

Fibroblasts can be defined as mesenchymal cells that create and maintain an anatomically diverse array of ECM-rich connective tissues. However, fibroblasts exhibit a high degree of heterogeneity. They are found in different tissues at different stages of development. Moreover, fibroblasts serve essential organ functions, like resistance to injuries, especially in the skin, or stretching/elastic recoiling of the organs. It provides positional information for neighboring cells using microarchitectural, biomechanical, and biochemical cues in the ECM, including the regulated secretion of soluble mediators such as cytokines, growth factors, and metabolites (Driskell and Watt 2014; Plikus *et al.* 2021).

In the case of skin tissue, fibroblasts are spindle-shaped cells found primarily in the dermis, the second layer of the skin. Their main function is the synthesis and deposition of collagen, the primary structural protein in connective tissue. Through their robust production of collagen, fibroblasts contribute significantly to the tensile strength and resilience of the skin, giving it the ability to withstand mechanical stress and maintain its structural integrity. (Wong *et al.* 2007; Watt and Fujiwara 2011).

In addition to collagen, fibroblasts secrete various other components of the ECM, including elastin, fibronectin, and proteoglycans. Fibroblasts can create a foundation that supports the epithelial keratinocytes of the outward-facing stratified epidermis and its numerous appendages, primarily hair follicles and sweat glands. Fibroblast´s ECM creation as a connective tissue serves to deposit fiber-and sheet-forming collagens, proteoglycans, elastin, fibronectin, microfibrillar proteins, and laminins, which together form the microsome. Fibroblasts actively shape dermal microarchitecture dynamically through modulated ECM remodeling and deposition. By regulating matrix-modifying enzymes such as lysyl oxidase, matrix metalloproteinases (MMPs), and their inhibitors, fibroblasts dynamically alter ECM structure to impart mechanical strength and facilitate cellular communication (Sorell and Caplan 2009; Plikus *et al.* 2021).

In particular, fibroblasts form discrete anatomical layers in the dermis—the papillary dermis, reticular dermis, and dermal white adipose tissue (dWAT)—

with unique cellular and structural properties, as illustrated in Figure 5 (Driskell and Watt 2014).

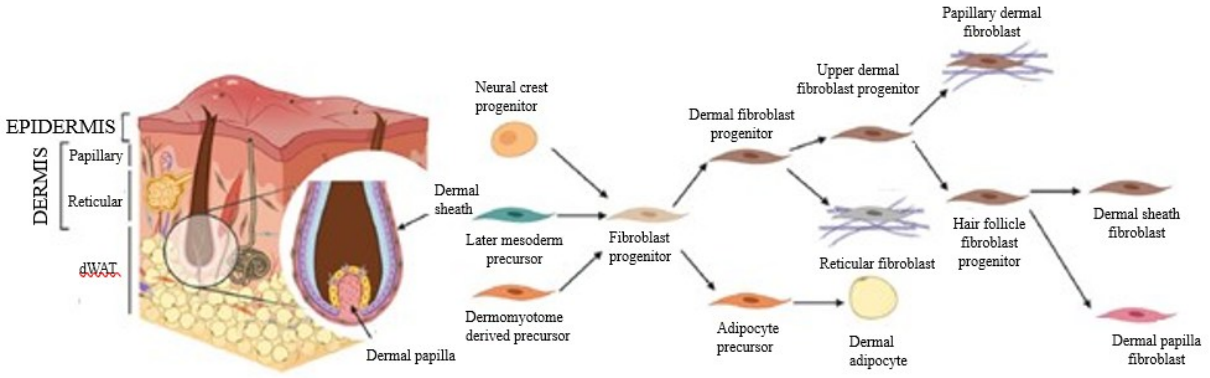


Figure 5: Skin-specific fibroblast organization and lineage relationships (Plikus et. al. 2021)

Apart from structural roles, fibroblasts are significantly involved in immune modulation and tissue repair. Fibroblasts secrete cytokines, chemokines, and growth factors, thereby modulating immune responses through the regulation of macrophages, neutrophils, and lymphocytes in the dermal microenvironment. Fibroblasts rapidly mobilize, proliferate, and migrate to the wound site upon damage, inducing tissue restoration and provisional scarring, which acts as a scaffold for further tissue remodeling (Wong *et al.* 2007; Bello *et al.* 2001).

Skin fibroblasts contribute significantly to the formation, organization, and function of skin-like structures within 3D matrices. 3D culture systems are typically used to mimic the natural ECM features found in vivo. They are manufactured to provide a tissue-like environment for cells cultured under in vitro conditions. Fibroblasts form dermal structures in 3D collagen-based matrices by synthesizing, organizing, and remodeling ECM components. These engineered microenvironments model physiological interactions leading to bidirectional mechanical entanglement between fibroblasts and collagen fibers to maintain tensile homeostasis and structural integrity in vitro (Grinnell 2003; Joe *et al.* 2022). Through active remodeling of the ECM and regulated biochemical signaling, fibroblasts in 3D skin models replicate important dermal function, indicating their significance in skin tissue engineering applications (Rhee 2009).

2.2.2 Keratinocytes´s Functions and 3D Skin Modeling

Keratinocytes are the predominant cell type in the epidermis constituting the outermost layer of the skin. Located in the stratified epidermal layers, keratinocytes experience a highly ordered process of differentiation known as keratinization, evolving from proliferative basal cells to completely differentiated

corneocytes that make up the stratum corneum. They are responsible for forming the epidermal barrier, which serves as the first line of defense against environmental insults, pathogens, and UV radiation (Gutowska-Owsiak *et al.*2020; Komine *et al.*2021).

Keratinocyte differentiation is a process of morphological, molecular, and biochemical alterations, as reviewed in Figure 6. During differentiation, keratinocytes synthesize and accumulate keratin proteins, transforming into flattened, dead corneocytes trapped within lipid-filled extracellular lamellae, which are central to the waterproof character of the barrier (Gutowska-Owsiak *et al.* 2020; Komine *et al.* 2021).

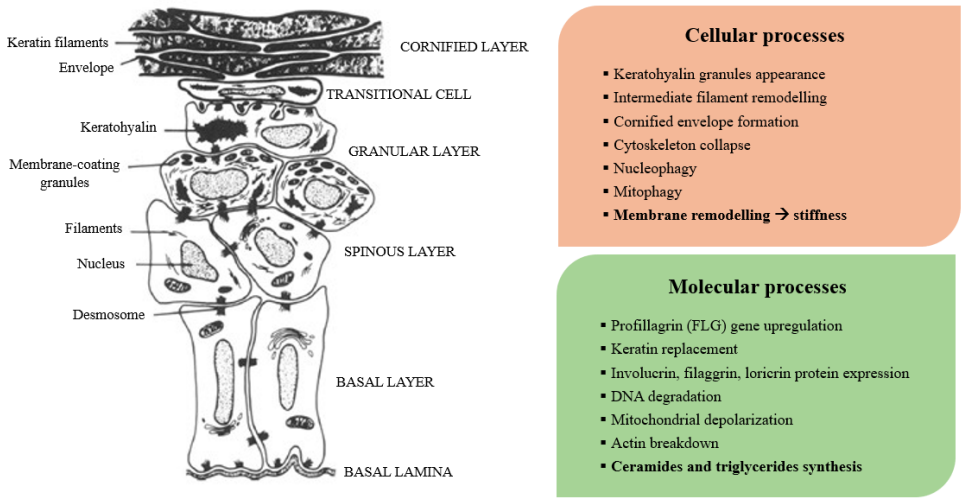


Figure 6: Keratinocytes differentiation process
(adapted from Eckert and Rocke 1989; Gutowska-Owsiak et al.2020)

Molecularly, differentiation is marked by sequential expression of specialized proteins such as involucrin, loricrin, filaggrin, and transglutaminase, all of which comprise the insoluble cornified cell envelope (CCE). The structure enhances the mechanical strength and elasticity of the epidermis, a role that is essential for protection from environmental damage and skin homeostasis maintenance (Ipponjima *et al.* 2020; Kirfiel and Herzog 2004). Keratinocytes also participate in tissue regeneration and repair processes. In wound healing, activated keratinocytes migrate from the periphery of the wound to the wound bed, proliferate, and secrete proteases like MMPs to regulate extracellular matrix (ECM) interactions and enable re-epithelialization. This process includes extensive interaction with fibroblasts, ECM molecules, and immune cells, which together enable wound closure and epidermal regeneration (Randall *et al.* 2008; El Ghalbzouri *et al.* 2004).

In 3D skin models, as well as in native skin, keratinocytes play essential roles in simulating the epidermal layer and thus contribute to the structural integrity, barrier function, and differentiation of the engineered skin tissue. These models

enable epidermal physiology investigations, dermal toxicity assays, and drug development. In these engineered environments, keratinocytes grow, differentiate, and form stratified epidermal layers, closely approximating native tissue behavior. The contact with fibroblast-populated dermal matrices sustains epidermal integrity, marking an important contribution of keratinocytes to the imitation of physiological conditions and the improvement of engineered skin construct validity. (Randall *et al.* 2008; Cai *et al.* 2023; Tahri *et al.* 2023).

3. THREE-DIMENSIONAL SKIN MODELS

Understanding the structure and composition of the skin is crucial in various fields including dermatology, cosmetics, and medical research. This includes cytotoxicity studies, drug targeting, testing testing of new therapeutics and treatment strategies (Bell *et al.* 1983, Reijnders *et al.* 2005). These areas of research require appropriate test systems. However, animal models are not ideal for these applications. Except for the ethical issue, the economical relevance is also important. Although some inexpensive species, such as rodents, are used, their skin composition is too different from human skin to be comparable (Mukherjee *et al.* 2022).

While traditional 2D cultivation provides valuable insights, a 3D model of native skin provides a more complete understanding of its intricate layers and components. This 2D culture environment lacks the natural 3D architecture of skin tissue. Cell-cell and cell-ECM interactions in 2D cultures are limited compared to 3D cultures, where cells typically form monolayers and may not fully recapitulate the complex cellular interactions found in native skin tissue. A 3D model more closely represents the complexity of the skin's structure, and the structure typically represents the epidermis, dermis and subcutaneous tissue (Carlson *et al.* 2008; Saji *et al.* 2019). In the case of the skin equivalent, it is composed of fibroblasts, collagen, and keratinocytes, and, like the native skin, the epithelium is stratified and differentiated (Vaughan *et al.* 2009).

3.1 Overview of 3D Skin Models

Understanding healthy and diseased skin conditions is a significant advancement in many areas of basic and applied skin research from risk assessment of chemicals (including cosmetics) to offering versatile platforms for studying skin biology, disease mechanisms, and therapeutic interventions. Animal models are currently used extensively only in the preclinical phase of drug development for risk assessment and identification of the mode of action of drugs. The weakness of these tests is that they are poor predictors of human response due to differences in skin physiology and immunity. Moreover, the pharmaceutical and cosmetic industries are calling for the reduction, refinement, and replacement of animal testing, in particular the 7th Amendment to the EU Cosmetics Directive 76/768/EEC (European Commission (European Commission [online]; Idress *et al.* 2021; Jang *et al.* 2023). As a replacement can serve as human *ex vivo* skin explants and can be used for risk assessment and drug testing. These include spheroids as classic 3D models for skin research, 3D *in vitro* skin models or the development of 3D skin-on-chips and potential 3D skin

bioprinting (Figure 7) (Mathes *et al.* 2014; van den Broek *et al.* 2017; Klicks *et al.* 2017). For this thesis summary, 3D skin models were described.

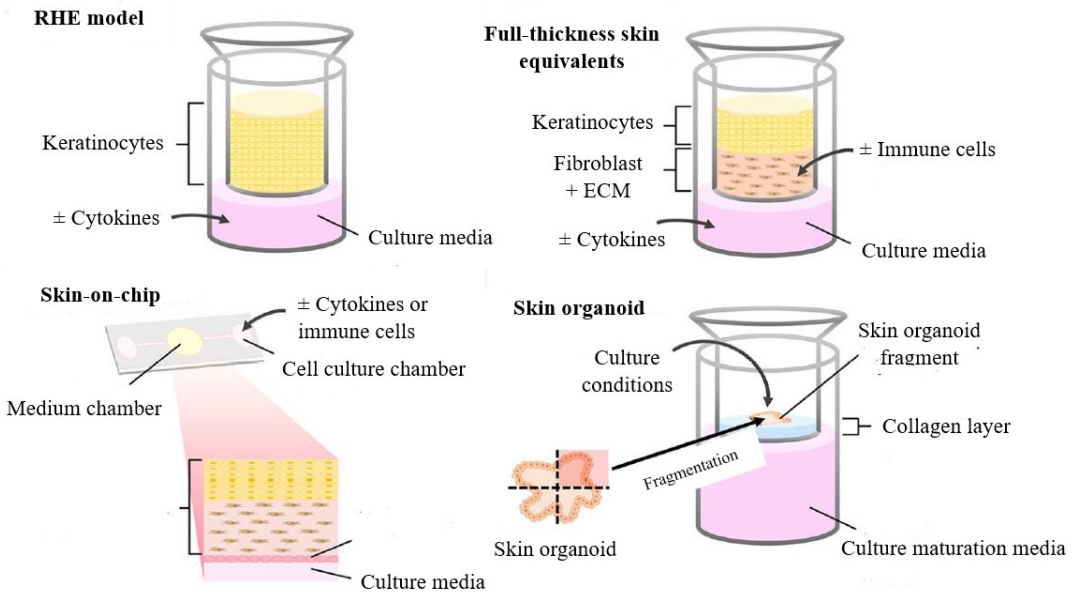


Figure 7: An overview of 3D skin models (Jang et al. 2023)

3.1.1 3D Skin Models

As we mentioned above, the great challenge in the field of skin tissue engineering has been to create physiologically relevant *in vitro* skin models comprising all skin layers – epidermis, dermis, and subcutis. 3D *in vitro* skin models represent an important advancement in skin research, offering a more sophisticated platform compared to traditional 2D cell cultures, which have limitations such as the inability to develop a stratified epidermis and the absence of 3D multicellular interactions. Although the skin can be viewed as a 2D surface composed primarily of keratinocytes, 3D skin reconstitution is essential to accurately reflect the physiology of multi-layered human skin tissue (Choudhury *et al.* 2020; Jang *et al.* 2023). Therefore, these models aim to replicate the complex architecture and cellular interactions of native human skin, providing valuable tools for studying skin biology, disease mechanisms, and drug responses. Two advanced in vitro skin models have been developed – reconstructed human epidermis (RHEs) and full-thickness human skin equivalents (HSEs). A RHEs model consists only of keratinocytes, whereas the HSEs model possesses dermal and epidermal layers (Zhang and Michniak-Kohn 2012; Hoffmann *et. al.* 2023).

Reconstructed Human Epidermis Model (RHEs)

The RHE model is an advanced *in vitro* system designed to mimic the structure and function of the human epidermis, the outermost layer of the skin. Unlike the full-thickness skin model, the RHE consists of primary human keratinocytes

isolated from skin biopsies or commercially available cell lines. The keratinocytes are seeded onto a 3D scaffold or matrix support made of synthetic/bio-materials such as collagen, fibrin, or agarose. This scaffold provides a framework for cell attachment and supports the keratinocytes' growth and differentiation. It only simulates the human epidermis (the epidermal substitutes) (Pedrosa *et al.* 2017). The RHE model exhibits a normal development into a stratified epithelium consisting of multiple layers of keratinocytes. These layers typically include the basal layer, spinous layer, granular layer, and cornified layer (*stratum corneum*), which closely resemble the layers of the native epidermis. The key feature of RHE models is their ability to form a functional barrier similar to the stratum corneum of native skin. Several commercially available skin models of RHE are SkinEthicTM or SkinEthic RHE 2.0 (EpiSkin, France), EpiDerm™ (MatTek Corporation, USA) or ZenSkin RHE-24 (ZenBio, USA). These RHE models serve as valuable human-relevant platforms for replacing and testing the efficacy and safety of topical medications (applied to the skin) compared to animal models. RHEs can be used for drug testing and to assess the safety and potential irritation of cosmetic products (Netzlaff *et al.* 2005).

Human Skin Equivalents (HSEs)

Full-thickness HSEs are *in vitro* models that closely mimic the structure and function of native human skin. In contrast to simpler epidermal models RHE, HSEs contain layers simulating the dermis and epidermis, facilitating the complex interactions between these crucial skin components. HSEs are typically constructed using a combination of keratinocytes, which are responsible for forming the epidermis, fibroblasts that create the dermal layer's supportive structure, and finally ECM as a network of proteins and molecules providing the structural foundation for the skin layers. ECM can be either natural (e.g. collagen) or synthetic (Rossi *et al.* 2015; Niehues *et al.* 2018).

First HSEs model, cultured human epidermal cells were seeded on a decellularized de-epidermized dermis (DED). The DED was suitable to induce epidermal cell differentiation and substituted the basal lamina. However, the key point was to develop a composite skin reconstruction using the dermis and epidermis with cell cultivation on hydrogel systems. Employing hydrogels for creating the HSE model is the most dominant technique. Hydrogel systems serve as a scaffold for dermal fibroblasts, which are then co-cultured with keratinocytes on their surface. Collagen is the typical hydrogel material thanks to its inclusion in native ECM and dermal fibroblast cells are usually distributed within the collagen gel to mimic the dermal layer. Adding a suspension of keratinocytes on top generates the epidermis layer. The HSE is raised to the air-liquid interface, enabling the top layer of cells to be exposed to air. This triggers essential differentiation processes. (Bell *et al.* 1981; El Ghalbzouri *et al.* 2009) According

to this collagen-cell combination model, several commercially available 3D skin models are put on the market, such as EpiDermFT™ (MatTek, USA), Episkin (L´Oreal; SkinEthic, France), or Labskin (Innovenn, Ireland) (Schmidt *et al.* 2020; Idrees *et al.* 2021).

The second approach combines the cell with chemical compounds and growth factors to develop viable biological substitutes. It is designing an HSE exclusively from cultured fibroblasts and keratinocytes without using any hydrogel/synthetic system. The well-organized dermal layer can be created by self-assembling fibroblasts´ extracellular matrix. Fibroblast ECM contains collagen as a major protein, thus it can be feasible with the addition of ascorbic acid for fibroblast metabolism support in long-term cultivation. This dynamic interaction leads to the formation of a structured platform for keratinocytes that form a stratified epithelium when raised to an air-liquid (ALI) interface (Auxenfans *et al.* 2009; Hayden and Harbell 2021).

The HSE model allows a suitable cellular environment for cell metabolism and function as they do in the native tissue. This microenvironment mimics critical aspects of the *in vivo* setting through the biological and chemical milieu to which the cells are exposed. The HSE model can be a backbone approach of any tissue-engineered skin substitute (Savoji *et al.* 2018).

3.2 Polymicrobial Skin Models

3.2.1 Microorganisms and Polymicrobial Iinteraction

Microorganisms play a crucial role in maintaining human skin health. The skin is home to a diverse community of microorganisms, including bacteria, fungi, and viruses, collectively known as the skin microbiota (Figure 8). The microbial composition is strikingly different on different parts of the skin and is determined by their physical, chemical, and biological characteristics. For instance, sebaceous regions create anaerobic and lipid-rich niches that promote the growth of *Propionibacterium*, whereas drier areas support the growth of the genera *Staphylococcus*, *Micrococcus*, *Corynebacterium*, and *Streptococcus*. Additionally, gram-negative bacteria, such as members of *Enterobacteriaceae*, albeit less well-characterized, also temporarily inhabit the skin surface (Grice and Segre 2011; Byrd *et al.* 2018; Sfriso *et al.* 2020; Boxberger *et al.* 2021).

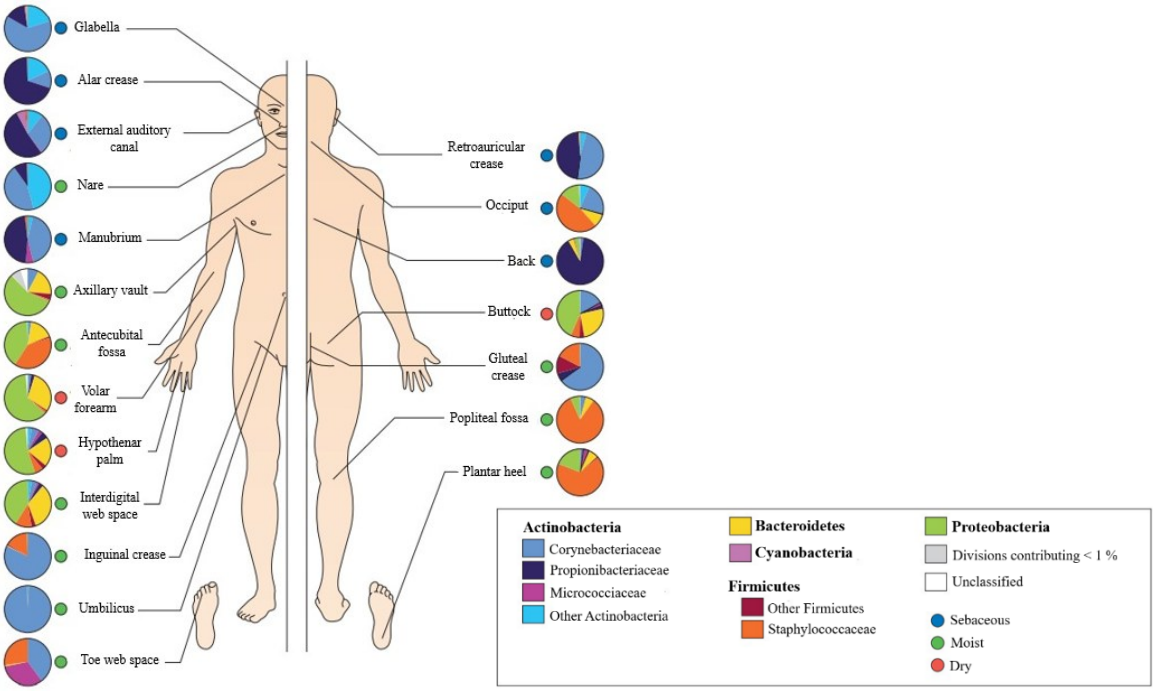


Figure 8: Bacteria distribution on the skin sites (Grice and Segre 2011)

The epidermal skin layer offers a nutritionally limited microenviroment, therefore forcing the resident microbes to adapt using the substrates present, including sphingolipids, amino acids, peptides, and sebum and sweat components, such as fatty acids, sterols, and wax esters (Grice and Segre 2011; Patra *et al.* 2020). In addition, eukaryotic species, including fungi, or yeast (e.g., *Malassezia*, *Cryptococcus*, *Rhodotorula*, *Candida*) and mites (*Demodex folliculorum*), are key contributors to the skin microbiome. *Malassezia* species occupy lipid-rich areas like the scalp and central body regions, but the surfaces of the feet host greater fungal diversity (Byrd *et al.* 2018).

The study of the human microbiome is diverse at an interpersonal or intrapersonal level. Moreover, the structure of the skin microbiome depends on the environment, gender, race, or age. Thus, skin health reflects the balanced skin microbiome, and any alterations lead to the overgrowth of pathogenic strains linked to various skin diseases (Hwang *et al.* 2021).

In the case of polymicrobial interactions, it represent a spectrum of relationships among various microorganisms (bacteria, fungi, viruses, and other microbes) existing together in the same microenvironment. These microorganisms are in constant competition for space and enabling dynamic control. This process is essential for skin health, as it keeps harmful microorganisms under control and supports skin homeostasis. Interactions among a collection of microorganisms in a polymicrobial community play a crucial role in skin homeostasis (Claesen and Fischbach 2015; Anju *et al.* 2022).

The interactions among microbial species range from mutualism and commensalism to antagonism and competition. Mutualistic activity by the commensal bacterium *Staphylococcus epidermidis* illustrates the defensive role played by microbes. *S. epidermidis* secretes antimicrobial peptides and proteolytic enzymes that inhibit the growth of pathogenic bacteria like *Staphylococcus aureus*. Further, its short-chain fatty acids also possess inhibitory activity against *Cutibacterium acnes* under anaerobic conditions. By contrast, competitive interactions characterized by coagulase-negative *Staphylococcus* species that produce bacteriocins (e.g., epidermin, Pep5, Epilancin K7) directly target gram-positive pathogens, including MRSA, emphasizing their pivotal role in microbial defense mechanisms (Chen *et al.* 2022; Caballero *et al.* 2023; Joshi *et al.* 2023; Glatthardt *et al.* 2024).

Moreover, polymicrobial interactions extend beyond bacterial-bacterial relationships. Yeast, such as *Malassezia*, and bacteriophages are significant in the complex interactions of the skin microenvironment, influencing microbial competition and host immune response. Disruptions of these interactions, broadly termed dysbiosis, have been observed in skin diseases like atopic dermatitis and psoriasis. Within a wide range, polymicrobial interactions in the skin are intricate and essential for health-maintaining processes such as colonisation resistance, immune modulation, and pathogen suppression. The application of a skin microbial network, along with the molecular mediators and dynamics involved, can lead to the establishment of novel therapeutic strategies in the field of tissue engineering, particularly concerning *in vitro* skin tissues (Flowers and Grice 2020; Wu and Yao 2023; Bényei *et al.* 2024)

3.2.2 Challenges in Polymicrobial Skin Model Development

Increasing the complexity of 3D *in vitro* reconstructed human skin models raises several issues in pursuing more representative models with their reliability and predictability. Construction of the full-thickness skin model contains relatively straightforwardly layered living fibroblasts and keratinocytes. Thus, the research attempts to incorporate all the relevant skin cell types, such as melanocytes or Langerhans cells, and the interaction between them. Nevertheless, all interaction is not only between cell types, but the skin surface can play a significant role in supporting skin health (Bojar 2015). The skin microbiota plays a crucial role in developing 3D skin models due to its considerable influence on various skin processes and barrier functions. First and foremost, the skin microbiota is involved in maintaining the physical barrier of the skin. (Galvan *et al.* 2024; Kim 2023).

A representative model of the skin ecosystem could be the colonization of 3D *in vitro* reconstructed human skin models with microorganisms. It is designed to

mimic the complex microbial communities present on human skin. The first research was based on the 2D culturing of human skin cells - keratinocytes or sebocytes, with bacteria or their metabolites. Polymicrobial biofilms, which are used for experiments, are a combination of microorganisms to represent the complex microbial community found on human skin. It is a mixture of commensal strains, such as *Staphylococcus epidermidis* and *Micrococcus luteus,* and pathogens, especially *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This interaction between skin cells and bacteria ensured the understanding of the pathways involved in pathogen infections or commensal benefits for the skin. In more detail, the keratinocytes were usually incubated with sterile filtered bacteria medium, such as *S. aureus* medium. Several studies showed that keratinocyte cultivation in these conditions increased the production of proteolytic enzymes, followed by the degradation of skin barrier proteins, such as desmoglein or filaggrin. This pathogen strain also induced cell cytotoxicity and increased the production of pro-inflammatory cytokines in skin cells. To compare, commensal *S. epidermidis* increased the keratinocyte production of antimicrobial peptides (Poppov *et al.* 2014; Rademacher *et al.* 2018; Jordana-Lluch *et al.* 2020).

The development of 3D skin models, such as RHE or HSE, colonized with bacteria could reproduce the skin barrier to study long-term interactions between the skin and its microbiota. Each selected microorganism is cultured under conditions that mimic the skin environment, including temperature, pH, and nutrient availability. These conditions are optimized to promote the formation of biofilms. An assembling polymicrobial skin model is considered when the bacteria are introduced onto a substrate that mimics the surface of a 3D skin model. Then they are adhered to the surface and begin to proliferate, eventually forming a polymicrobial biofilm. This biofilm structure allows for interactions between different microorganisms and facilitates their colonization of the keratinocyte layer (Rademacher *et al.* 2018; Emmert *et al.* 2020; Lizardo *et al.* 2022). Modeling of polymicrobial skin models using the biofilm-keratinocyte is analyzed to gain insights into the complex interactions between microorganisms and host cells, as well as the effects of antimicrobial interventions and understanding of skin microbiota dynamics, host-pathogen interactions, and the development of new strategies for managing skin infections and promoting skin health (van Belkum *et al.* 2023).

4. CELL-ASSEMBLED EXTRACELLULAR MATRIX (CAM) PRODUCTION

4.1 Extracellular Matrix Composition and Function

The ECM defined as a complex 3D network of macromolecules that provides structural support and biochemical cues to cells within tissues and organs. Initially viewed as a passive, inert scaffold providing mechanical support, the ECM is now recognized as a dynamic, 3D structures critical for cell survival, physiology, development of tissue etc. Thus, it patronizes the architectural structure/strength, and regulates biological processes, offering physical protection and signaling cues (Rozario *et al.* 2010). The ECM influences cell behavior, including proliferation, orientation, gene expression, migration, and differentiation (Muncie *et al.* 2018; Karamanos *et al.* 2021).

The ECM is comprised of multiple components that interconnect to form a sophisticated network of molecules within a 3D framework (Figure 9). These ECM constituents exhibit diverse characteristics in terms of size, morphology, and arrangement. The unique composition of ECM in various tissues and organs is a result of differential gene expression related to ECM synthesis. Processes such as post-transcriptional splicing and post-translational modifications also play a key role in this expression. This intricate interplay of ECM elements contributes to the structural integrity and functional diversity observed in different biological systems (Dzobo *et. al.* 2023). In general, the ECM is composed of two primary domains: the basement membrane, a condensed layer adjacent to various cell types such as epithelial, mesothelial, meningothelial, synovial, muscle, Schwann cells, adipocytes, and then the interstitial matrix. While both domains share a collagen scaffold as their basic structure, the composition and 3D architecture of the collagens differ significantly between them (Bosman and Stamenkovic 2003). There are various proteins (glycoproteins, proteoglycans) and polysaccharides, that are organized in a highly dynamic and intricate manner. Glycosaminoglycans (GAGs) and proteoglycans (PGs) form a hydrated gel-like substance that resists compressive forces and allows rapid diffusion. Adhesive glycoproteins, including laminin, tenascin, and proteoglycans typically adhere to collagen scaffold and interact with cells within or in proximity to the matrix. These interactions are mediated by matrix receptors, with integrins representing the most significant class of receptors involved. Other ECM proteins are fibrous proteins that provide strength and elasticity to the matrix. These insoluble components are synthesized by fibroblasts and other cells residing within connective tissue. (Frantz *et al.* 2010; Theocharis *et al.* 2016).

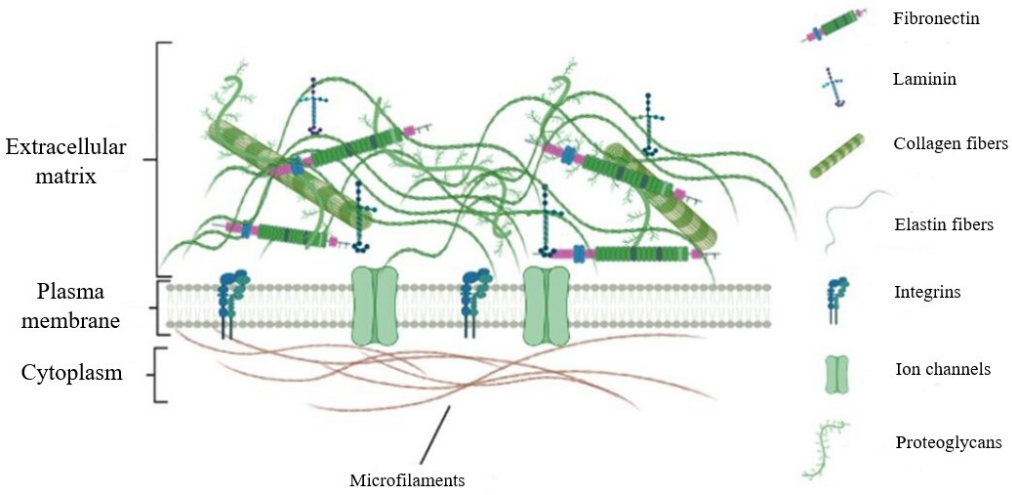


Figure 9: Extracellular matrix composition (Dzobo et al. 2023)

In addition to structural proteins, a group of ECM molecules known as matricellular proteins (e.g., thrombospondin-1 and -2, tenascin-C, or osteopontin) play a pivotal role in modulating cell-matrix interactions and cellular functions. Although they do not directly contribute to the structural framework of the ECM, matricellular proteins exert a significant influence on cellular behavior (Järveläinen *et al.* 2009). The ECM also undergoes constant turnover and remodeling in response to various signals. This process is particularly enhanced during inflammatory responses or wound repair. Key enzymes involved in ECM remodeling include MMPs and urokinase-type plasminogen activators (uPAs) (Leight *et al.* 2017; Marangio *et al.* 2022).

4.2 Techniques for Cell-Assembled ECM generation

In tissue engineering, an optimal scaffold should possess structural, physicochemical, and mechanical properties that are conducive to remodeling the implant into functional tissue by the host body. In addition, the biochemical properties of the scaffold are important to consider due to their significant role in cell-to-matrix interactions. Various biomaterials have been developed to fulfill these criteria and can be broadly categorized into two subtypes: naturally occurring and synthetic materials (Kawecki *et al.* 2020). Traditionally, tissue engineering has relied heavily on synthetic biomaterials to shape and provide mechanical properties to engineered tissues. These can include biodegradable synthetics (e.g., polylactic acid, polyglycolic acid, and polycaprolactone) or biologics (e.g., chitosan, alginate, and collagen). Synthetic scaffolds offer advantages in cost-effectiveness and precise control over composition, geometry, and structure. For instance, synthetic materials can be manufactured precisely and consistently, resulting in minimal variability. Furthermore, their properties, including mechanical strength and degradation profile, are easily modifiable,

multiple polymers can be seamlessly integrated within a single material can be easily modified, and multiple polymers can be seamlessly integrated into a single material. However, they are often hindered by drawbacks such as unpredictable degradation rates, chronic inflammation, and limited biological activity. In contrast, naturally occurring materials are typically derived from whole ECMs or purified individual ECM components, such as collagen, laminin, fibronectin, and silk. They provide inherent physiological cell-to-matrix interactions that facilitate tissue regeneration. Thus, synthetic scaffolds could be replaced with ECMs synthesized by cells cultured *in vitro*. These efforts culminated in the development of relevant tissue-engineered implants resulting in scaffolds with suitable mechanical properties (Hussey *et al.* 2018; Mangan *et al.* 2020).

Cell-derived ECM (CAM) partially mirrors the intricate biological machinery of native tissue. Derived from human cell cultures by gentle decellularisation, bioactivity is preserved while immunogenic components are removed. *In vitro* derivation permits the selection of appropriate ECM-producing cell types, genetic modification, and exposure to specific stimuli, facilitating the creation of ECM with desired properties. Consequently, CAM gained significant interest in TERM due to its potential to create more biomimetic and functional tissue constructs. Cell-derived ECM can be generated by culturing cells scaffold-free in 2D and 3D cultures, where the cells can self-assemble into 3D spheroids through hanging drop culture, non-adherent plates, or micro-molded wells. Alternatively, cells can also be seeded within hydrogels or scaffolds, forming hybrid ECM-based materials (Lee *et al.* 2007; Nicolas *et al.* 2020; Assunção *et al.* 2020).

The CAM can assist in the construction of 3D tissue without the use of other materials. This innovative approach exploits on the inherent ability of mesenchymal cells, such as fibroblasts, smooth muscle cells, adipose-derived stem cells, and bone marrow-derived stem cells, to assemble a completely biological and internally secreted ECM in vitro. Generally, cells are the traditional ECM builders and create tissue modules with precision and stoichiometric competence. This ability can lead to the development of cell-assembled prototype scaffolds, held together by cell-cell and cell-ECM junctions used for skin, blood vessels, and corneas. The CAM exhibits remarkable mechanical strength and has emerged as a promising form of potential biomaterial scaffold (Kumar *et al.* 2015; Mangan *et al.* 2018; Oliveira *et al.* 2021).

4.3 Co-cultivation Systems for Enhanced ECM Production

Cell co-culture refers to the cultivation of two or more different types of cells—either from the same or different specimens—within a shared culture system. It is a foundational tool in biological research, widely used to investigate natural and synthetic interactions between cell populations. Since the 1980s, cell co-culture technology has attracted increasing attention for its broad applications, including inducing stem cell differentiation, enhancing metabolite production, improving cell viability and function, and constructing tissue-like environments *in vitro* (Nakazawa *et al.* 1998; Goers *et al.* 2014). Compared with monoculture techniques, co-cultures provide a more physiologically relevant model by better mimicking the *in vivo* cellular microenvironment. This allows to observe not only natural cell-to-cell interactions but also interactions between cells and their surrounding environment (Figure 10) (Goers *et al.* 2014; Vis *et al.* 2020).

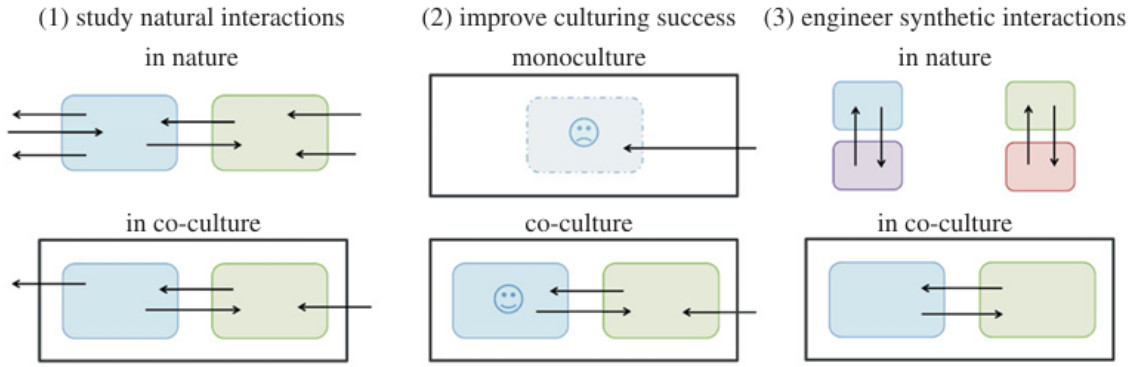


Figure 10: Overview of co-culture systems (Goers et al. 2014)
(1) Natural interactions between cell lines. (2) Improving cultivation success for certain cell lines. (3) Establishing synthetic interactions between cell lines.

Cell co-culture systems are generally categorised based on the degree of contact between the cultured cell types. These include direct contact co-culture, indirect contact co-culture and finally 3D cell co-culture. For this study, only direct co-culture is described. In the direct contact co-culture method, different cell types are grown together in direct physical contact, enabling juxtacrine communication through surface receptors and gap junctions. This method is easy to perform, requires minimal equipment, and closely mimics native tissue by maintaining both cytokine exchange and direct cell-to-cell interactions (Vis *et al.* 2020; Liu *et al.* 2022; Aydin *et al.* 2022).

This method is, in our experiments, particularly well-suited for applications involving the co-cultivation of human dermal fibroblasts (HDFs) and H9c2 cardiomyoblasts, as it enables direct cell–cell interactions that are critical for cardiac tissue engineering. This co-culture setup allows HDFs to provide not only paracrine support but also rich extracellular matrix (ECM) components that enhance cell adhesion, viability, organisation, and functional maturation—key

factors in the successful formation of scaffold-free cardiac cell sheets. The ECM itself is a complex and dynamic network composed of collagens, adhesion molecules, proteoglycans, growth factors, and cytokines. Its unique architecture plays a vital role in regulating cell behavior, including proliferation, differentiation, polarity, and migration. Compared to traditional ECM coatings such as fibronectin, gelatin, or collagen, increasing evidence supports the superior biological relevance of ECM derived from in vitro cultured cells. In this context, fibroblast-derived matrix, as a type of cell-derived matrix, is being explored as a promising ECM platform for cardiac tissue engineering. It provides a highly biomimetic microenvironment that supports cell adhesion, growth, and cardiomyogenic differentiation (Suhaeri *et al.* 2015; Rother *et al.* 2015; Iwamiya *et al.* 2016; Suhaeri *et al.* 2017).

4.4 Applications in Tissue Engineering

The ECM is a sophisticated and distinctive complex framework comprising structural proteins and glycosaminoglycans crucial for the precise transmission of intrinsic physical and chemical cues. These signals profoundly influence cellular behaviors, tissue regeneration, revascularization, and homeostatic regulation. Thus, the application of a cell-assembled extracellular matrix involves harnessing the innate ability of cells to construct and organize their surrounding ECM. This approach emphasizes utilizing the synergistic interactions between cells and their secreted ECM components to fabricate biomimetic tissue constructs. By cultivating cells in a controlled environment conducive to ECM production, CAM facilitates the creation of tissue-like structures that closely resemble native tissues in terms of architecture and function. The promising capacity of cell-assembled extracellular matrix biomaterials is not limited to tissue engineering but also has broader biomedical applications (Yao *et al.* 2019; Assunção *et al.* 2020). More specifically, the utilization of CAM can be either in its pure form or integrated into biomaterials. 3D scaffolds composed of cell-assembled ECM are developed through various methods, such as decellularizing stacked cell sheets/pellets or depositing ECM within materials - tubes/foams. A newly used approach involves using only the CAM sheets (Hussey *et al.* 2018; Kawecki *et al.* 2022). As mentioned, the cell line of the connective tissues (fibroblasts) can synthesize ECM components, in particular collagen. Furthermore, the combination of cell culture with ascorbic acid and its derivatives can stimulate collagen production. In general, the CAM is then investigated and utilized for skeletal and cardiovascular repair (cartilage, biological tissue-engineered vascular grafts, cardiac patches for cell delivery), as well as other applications such as in skin (skin patches for drug delivery) or peripheral nerve repair (Mangan *et al.* 2018; Torres *et al.* 2021; Kawecki *et al.* 2022).

5. AIM OF DOCTORAL THESIS

Skin, with its outermost layer called the *stratum corneum*, acts as the primary barrier against externally applied compounds. This barrier function is in the centre of attention once the safety/risk assessment of materials/compounds is considered. I*n vitro prepared* monolayer or two-dimensional keratinocyte cultures lack this crucial barrier function. Thus, the development of advanced in vitro skin-reconstituted tissues focuses on creating of complex model mimicking *in vivo* skin tissue functions. The remarkable adaptability of these 3D skin equivalents extends their utility and are precious in all branches of science, ranging from research on skin diseases to cosmetics, providing deep insights into human skin physiology. Recently, the additional part of skin, microbiome, has come into the centre of our attention. For this purpose, the doctoral thesis is aimed:

- To achieve a thorough understanding of the methodology for in vitro formation of reconstituting full-thickness skin tissue model, and to master the practical skills required for their preparation.
- To establish the protocols for the preparation of the reconstituted full-thickness skin tissue model in the laboratory.
- To **integrate** advanced reconstituted full-thickness skin tissue model models with the **microbiome to further enhance their scientific value**, particularly in the context of wound healing research.

Additionally, the ability of skin tissue cells, particularly dermal fibroblasts, to produce an extracellular matrix by self-assembling (CAM) can create an attractive biomaterial. This CAM scaffold resembles native tissue ECM in composition and architecture, offering enhanced cell-instructive properties for tissue regeneration. These “cell-made scaffolds” exhibit excellent biocompatibility, biodegradability, and tissue-specific cues, making them ideal for tissue repair and regeneration applications. Thanks to the findings, the doctoral thesis is also aimed:

- To master culturing techniques enabling the self-assembly of three-dimensional multilayered fibroblast structures, thereby generating extracellular matrix (ECM) with native-like architecture and composition.
- To investigate the modifiability of structural architecture in cell-made scaffolds through external stimuli (e.g., mechanical, biochemical), and evaluate their functional utility as bioactive scaffolds post de-/re-cellularization.

Last, but not least – the skills in advanced cell/biology and microbiology laboratory techniques allow me to support the studies of other colleagues, where my expertise help to provide a specific type of experiments..

6. EXPERIMENTAL PART

6.1 Cell and Bacterial Culture Techniques

6.1.1 Human Dermal Fibroblast

Human Dermal Fibroblasts HDF (Cat. N.: 106-05n, Cell Sigma-Aldrich) were routinely cultivated in T75 flasks (TPP, Trasadingen, Switzerland) in Basal Medium Eagle (BME, Sigma Aldrich, USA) with 10% fetal bovine serum (FBS, BioSera, France) and 1% Penicillin/Streptomycin (GE Healthcare HyClone, United Kingdom). BME medium was changed every other day and passaged when 80-85% confluent.

6.1.2 Spontaneously Transformed Human Keratinocyte

Spontaneously Transformed Human Keratinocyte HaCaT (Cat. N.: 102-05a, PromoCell, Sigma-Aldrich) cultivated in T75 flasks in Dulbecco's Modified Essential Medium DMEM (Biosera, France) with 10% FBS and 1% Penicillin/Streptomycin (GE Healthcare HyClone, United Kingdom). This medium was pre-supplemented with stabilized 1 mM Sodium Pyruvate (Life Technologies Limited, UK). Cells were maintained between 80–85% confluence.

6.1.3 Embryonic Rat Cardiomyocytes

Embryonic Rat Cardiomyocytes H9c2 (CRL-1446, ATTC, USA) were grown routinely in T75 flasks (TPP, Trasadingen, Switzerland) in Dulbecco's Modified Essential Medium DMEM (Biosera, France) with 10% FBS and 1% Penicillin/Streptomycin (GE Healthcare HyClone, United Kingdom). This medium was pre-supplemented with stabilized 2 μM L-glutamine (Biosera, France).

All cells were maintained at 37 °C in a 95% humidified atmosphere and 5% CO_2.

6.1.4 Bacterial strains

Staphylococcus epidermidis CCM 4418, *Micrococcus luteus* CCM 732 and *Staphylococcus aureus* CCM 4516, *Pseudomonas aeruginosa* CCM 1961 were obtained from the Czech Collection of Microorganisms (CCM).

Prior to commencing the experimental procedures, it was essential to revive the bacteria from gelatinous discs, which required culturing them on tryptone soy agar (TSA; MP290, HiMedia Laboratories, Mumbai, India) in Petri dishes at 37 °C in aerobic conditions. Following the formation of colonies, the bacteria were moved

to tryptone soy broth (TSB; M077-500G, HiMedia Laboratories, Mumbai, India), where they were cultivated for 24 hours for additional recovery.

All media for bacterial cultures were prepared according to the manufacturer's instructions and sterilized by autoclaving at 121 °C for 15 minutes.

6.1.5 Preparation of bacteria supernatants

Bacterial cultures were adjusted to 0.5 McFarland standard as inoculum. The inoculum was then diluted 1:10 in fresh TSB and incubated at 37 °C for 24 hours. For subsequent experiments, samples of bacterial supernatant were collected following 24 hours of incubation to ensure complete growth. After collection, bacterial cultures were centrifuged at 3000 RPM for 10 minutes to isolate the cultures and washed twice in sterile DPBS. Bacterial cell wall digestion was initiated using lysozyme (20 µg/ml) at 37 °C for 1 hour, followed by sonication on ice (10 cycles of 30 s with 30 s cooling intervals) to ensure complete lysis. The supernatants were cleared of cell debris by high-speed centrifugation and sterilized by filtration through a 0.22 µm membrane filter.

To verify the absence of live bacteria, culture samples (30 µl) were placed onto Petri agar plates containing PCA agar and incubated for 24 hours at 37 °C and 95% relative humidity to confirm there were no viable bacteria present. Bacterial supernatants were stored at -20 °C for later experiments. Throughout all experiments, the concentration of cultures in the culture medium was maintained at 10 %.

6.2 Construction of 3D Normal Skin Tissue Models

3D human skin models enable the investigation of cell-matrix and cell-cell interactions between different cell types. These cultures consist of two components. The first is a dermal equivalent consisting of a mixture of collagen matrix and fibroblasts. Collagen provides scaffolding, nutrient delivery, and potential for cell-to-cell interaction. The second is an epidermis that develops from keratinocytes plated on the dermal equivalent. e coculture system is thus grown at the air-liquid interface on collagen matrices. Finally, the overall procedure can be completed in 3 weeks (Figure 11). (Gangatikar *et al.* 2007; Fell 2016)

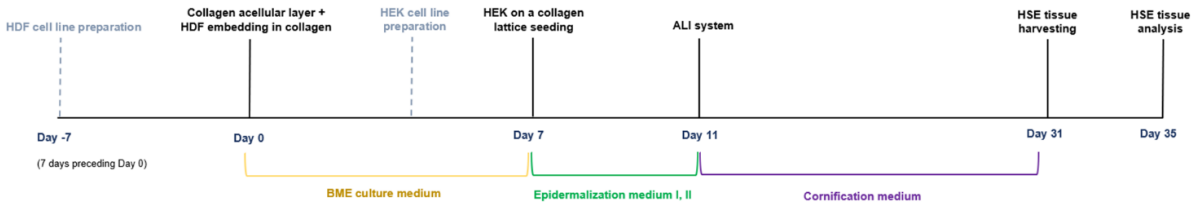


Figure 11: 3D skin model condition

6.2.1 Preparation of the Acellular Collagen Hydrogel

For the acellular collagen layer, rat tail collagen I (Corning® Collagen I, High Concentration, 100 mg; Corning, USA) was mixed with the neutralization buffer (NB), containing 10× PBS, HEPES, 7.5% sodium bicarbonate, 1M NaOH and dH_2O for the final volume. The NB buffer was prepared for this purpose before gelation to ensure a final pH of 7.4 for the hydrogel.

To prepare 1 ml of neutralized acellular collagen layer at a final concentration of 1 $mg \cdot ml^{-1}$, 750 µl NB buffer was first added to a test tube placed on ice, followed by 250 µl of collagen stock solution was added to the tube. The next step was to homogenize the mixture by slow and repeated pipetting, taking care to avoid air bubbles during pipetting. The NB-collagen mixture (1 ml) was added to a 12well plate in quadruplicate – 250 µl collagen mixture per 1 insert. The plate was incubated at 37 °C for 45 minutes to generate a clear hydrogel.

6.2.2 Fabrication of the Cellular Part of 3D Skin Equivalents

HDF was cultured and passaged the day before incorporation at a split ratio of 9:10. The pre-mix solution was then prepared according to protocol and containing 10× DMEM, L-glutamine, FBS, 7.5% $NaHCO_3$. This solution was mixed on ice, filtered, and sterilized with a 0.22 mm filter before use (without cell component and collagen). The HDF cell line suspension was resuspended to a final concentration of 3×10^5 cells·ml^{-1} and then was supplemented to the neutralized collagen solution. The HDF-collagen mixture was pipetted into inserts (Millicell® 12-well hanging cell culture insert; Sigma Aldrich, USA) with 750 µl in each insert on top of the gelled acellular collagen matrix. The inserts were then transferred to an incubator for 60 min to allow complete polymerization of the collagen-fibroblast gels.

When the cellularized collagen was completely gelled, the DMEM/F12 was pipetted as a feeding medium by adding 1.6 ml of to the well around the insert and 0.4 ml of directly onto the insert. This HDF cell layer was then incubated for 5-7 days to allow complete gel contraction.

For epidermal development, the HEK cell line was trypsinized with trypsin/EDTA for 5 min at 37 °C to obtain a single-cell suspension and then resuspended in a volume of epidermalization I (EP I) medium in a total volume of 5×10^5 cells per 50 µl for one insert. Before HEK seeding, the fibroblast culture medium was removed so that HEK suspension was seeded onto a moist collagen gel. The following step was to incubate for 45 minutes at 37 °C without any additional medium to allow complete attachment of the HEK suspension. Finally, EP I medium was added in the same volume as in the previous steps.

The different skin-equivalent culture media were used to generate a confluent cellular monolayer that will initiate tissue stratification – epithelialization. The 3D skin model was cultured and changed every other day with appropriate skin equivalents culture media as follows: 2 ml of EP I medium was added for the first 4 days and then changed to EP II medium. From day 6 until the termination of the experiment, the cornification (C) medium was pipetted to the bottom of the well so that the insert just touched the C medium. Whole skin equivalents were cultured in the air-liquid interface (ALI).

6.3 Histological analysis

For the histological analysis of the 3D skin model, the skin equivalents on the insert membrane were first washed in PBS and then cut out of the insert with a scalpel. They were then transferred to a disposable embedding mold and fixated in 4% paraformaldehyde (PFA) for 1 hour at room temperature. After fixation, the skin equivalent tissues were processed in ethanol in the following order: 30–

90 % EtOH for 30 minutes each. After ethanol, the xylene was used as an agent for paraffin infiltration. Xylene is changed three times, each time for 30 minutes. Finally, the skin equivalents are submerged in paraffin - twice for 1 hour and then overnight. The PFA-fixed paraffin-embedded skin equivalents were trimmed using the microtome Panthome LS-2064A and 5 μm thin sample sections were prepared on the microscope slides. The microscope slide containing samples was dried and fixed overnight in an oven at 40 °C. The next day, the hematoxylin-eosin (H&E) staining was performed according to the standard protocol.

6.4 *In vitro* Skin Irritation Test Using 3D Normal Skin Model

The *in vitro* 3D Skin Irritation Test is a method used to evaluate the potential irritancy of chemicals, cosmetics, pharmaceuticals, and other substances without the need for animal testing. During the 3-day test, the substance of interest is applied to the surface of the reconstructed epidermis. After application, the tissue is typically incubated for a period of time to allow any potential reactions to occur. Following the incubation period, the tissue is evaluated for signs of irritation. The severity of the irritation is assessed on the basis of predetermined criteria, often using a scoring system. The topical exposure of the test chemical to the 3D skin model is followed by a cell viability test and the metabolic activity of cells within tissue-like structures. The MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) assay is a widely used colorimetric assay for measuring the viability, proliferation, and cytotoxicity of cells *in vitro* (*In Vitro* EpiDerm™ Skin Irritation Test (EPI-200-SIT), MatTek [online]; Protocol Guide: MTT Assay for Cell Viability and Proliferation. [online]).

All reagents were prepared prior to the skin irritation test. Pre-warmed sterile DPBS was used as the negative control and 5% sodium dodecyl sulphate (SDS) as the positive control. The MTT stock solution 5 $mg.ml^{-1}$ in DPBS was also prepared and can be stored frozen at -20 °C (2 months). The MTT stock solution must be filtered and diluted in the assay medium to a final concentration of 1 $mg.ml^{-1}$.

The *in vitro* 3D skin model irritation test involves several steps to assess the potential irritation caused by substances. The general outline of the procedure can be seen in Figure 12. A 3D skin tissue model was prepared for testing according to the manufacturer's instructions. Finally, for each 3D skin model in the insert, the quadruplicate of 200µl aliquots of the blue formazan solution was pipetted into a 96-well microtiter plate. The DMSO solution was used as a blank. The optical density (OD) was read using an Infinite M200 PRO (Tecan, Switzerland) at 570 nm, without the use of a reference filter.

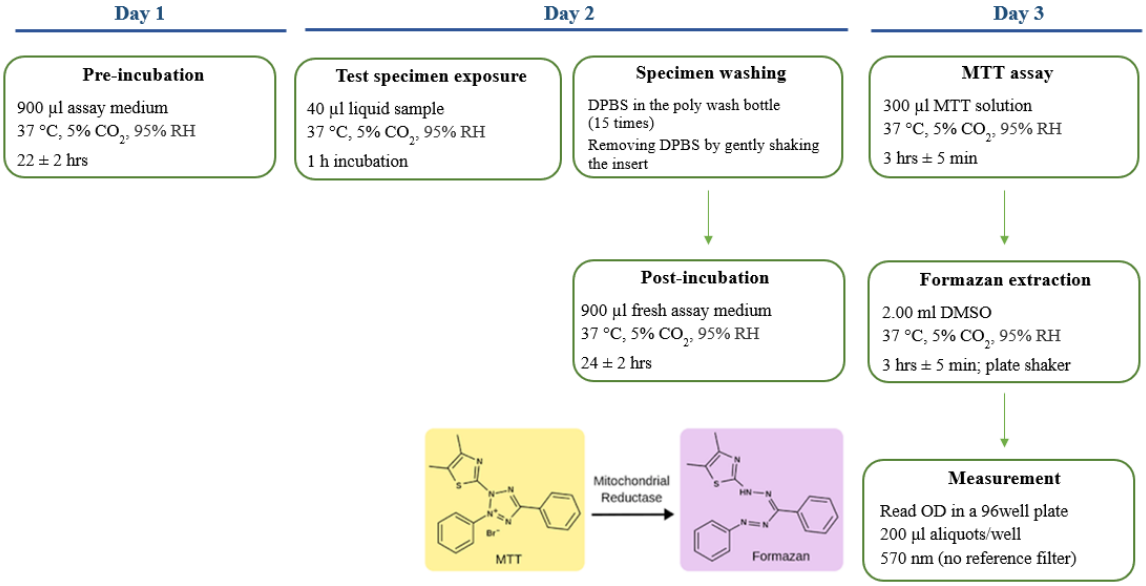


Figure 12: Diagram of in vitro 3D skin irritation test

6.5 The influence of the microbiome on skin cell lines

6.5.1 Trypan Blue staining viability test

The trypan blue exclusion test is among commonly used standard techniques to determine cell viability. It is a vital stain in the field of biology to selectively color necrotic (dead) cellswith microscopic examination (Strober 2015). Trypan blue is an azo dye derived from toluidine and is unable to penetrate the membranes of living cells. This assay operates on the principle that viable cells possess intact membranes that block the entry of trypan blue, thereby preventing the dye from entering. Conversely, trypan blue can infiltrate the membranes of necrotic cells, which have compromised membrane integrity, permitting the dye to access the cytoplasm (Avelar-Freitas *et al.* 2014; Abhishek *et al.* 2018).

The procedure for trypan blue viability testing involved preparing the cell suspension with 0.25% Trypsin-EDTA and then resuspending it in the cultivation medium. The resulting mixture was allowed to incubate for about 3 minutes at room temperature, after which the stained cell suspension was loaded into the Bürker chamber to count the stained/ unstained cells using a light microscope.

6.5.2 HaCaT proliferation assay

A HaCaT proliferation assay is used to evaluate the growth rate of keratinocytes, which are the primary cells found in the epidermis. These assays are crucial in dermatological and pharmaceutical research, especially in studying wound healing and skin regeneration. The keratinocyte proliferation assay using the MTT colourimetric assay is a widely used method to assess cell growth by measuring metabolic activity (Mosmann 1983; Sylvester 2011).

In this study, the MTT assay was performed in 96-well plates to assess the proliferation of HaCaT keratinocytes after treatment with bacteria and their supernatants, respectively *Staphylococcus epidermidis*, *Micrococcus luteus* as commensals, and *Stapyhlococcus aureus*, *Pseudomonas aeruginosa* as pathogens for 24 and 48 hours. Bacterial supernatants were used at 10% v/v in the cultivation DMEM medium. HaCaT cells were seeded at a density of 1×10^5 cells per well and incubated overnight at 37°C in a 5% CO_2 environment. The HaCaT cells were then washed with DPBS, and experimental treatments including bacterial suspensions and their supernatants were added to the wells. The DMEM cultivation medium was used as a control. After incubation time 0, 24 and 48 hours, an MTT assay was conducted to evaluate cell proliferation. Finally, the absorbance was measured at 570 nm using a spectrophotometer Infinite M200 PRO (Tecan, Switzerland), with the use of the reference at 630 nm.

Data analysis involved normalizing the absorbance values to untreated control cells, with relative proliferation calculated as a percentage. The absorbance of formazan produced by keratinocytes at time zero (at 70% confluency) was considered the normalized control, and any other time point was compared to this baseline. The percentage of the proliferation was measured using the Equation 1:

$$\text{Proliferation } \% = \frac{\text{The absorbance of formazan at any time point}}{\text{The absorbance of formazan at time zero}} \cdot 100 \qquad (1)$$

6.5.3 HaCaT co-culture with bacteria cells

To prepare the bacterial suspensions for co-culture with HaCaT cell line, overnight cultures of *Staphylococcus epidermidis* CCM 4418, *Micrococcus luteus* CCM 732, *Staphylococcus aureus* CCM 4516 and *Pseudomonas aeruginosa* CCM 1961 were centrifuged at 3.000 RPM for 5 minutes. The resulting pellets were washed twice with DPBS, and the optical density was adjusted to 0.5 McFarland in DPBS. The suspensions were then centrifuged again, after which the pellets were resuspended in 10 ml of DMEM medium without antibiotics.

The confluent HaCaT cell line at a density of 5×10^5 cells/ml was incubated for 30 minutes before the experiments in 1 ml of DMEM cultivation medium devoid of antibiotics within a 12well microtiter plate. For co-treatment, the HaCaT cells were exposed to 100 µL of the prepared bacterial cultures, achieving a multiplicity of infection (MOI) of approximately 1:20 for 2 hours. Additionally, the cells were treated with Staphylococcus epidermidis supernatant (SES). Following the incubation, the HaCaT cells were rinsed three times with DPBS to eliminate non-adherent bacterial cells. The cells were then detached using 0.25% trypsin-EDTA and gathered in 1 ml of cultivation medium. Subsequently, the cell suspension was serially diluted in PBS and spot-plated onto PCA agar plates to determine the count of adherent cells expressed as CFU/ml.

6.6 *In vitro* mixed infection 3D skin model

6.6.1 3D Skin Model

A 3D skin model (24 inserts) was constructed according to the protocol described in Section 6.3, Construction of 3D normal skin tissue models under laboratory conditions.

6.6.2 Bacteria colonization of the 3D Skin Model

For the bacteria colonization of the 3D skin model, *Staphylococcus aureus* and *Staphylococcus epidermidis* were streaked onto Tryptone Soya Agar (TSA) plates and cultured overnight at 37°C. For each distinct strain, a single colony was carefully transferred to a Tryptone Soya Broth (TBS) and subsequently incubated overnight at 35°C. The bacterial cells were then centrifugated at 5000 ×g for 5 minutes, followed by two washing steps to effectively remove any residual medium. The concentration of bacteria cells has been adjusted and diluted to obtain the initial inoculum concentration by measuring the optical density using McFarland densitometr (DEN-1B Grant-Bio; Grant Instruments, United Kingdom). The culture of each bacteria strain was adjusted to a 0.5 McFarland standard as the inoculum, which corresponds to a concentration of 6×10^6 CFU in the 30 µl of the applied suspension. Following inoculation, the 3D epidermal tissues were incubated at 37 °C in a humidified atmosphere supplemented with 5% CO_2 for a duration of 24 hours for bacterial adhesion.

6.6.3 *In vitro* infection 3D Skin model cytotoxicity

Following the bacteria colonization, the in vitro 3D skin model was then processed to quantitatively determine the lactate dehydrogenase (LDH) value, which serves as an indicator of cytotoxicity, and to assess the extent of colonisation of the 3D conjugate model by both *S. epidermidis* and *S. aureus* under conditions both in the absence and presence of the potential tested samples. This assay include the collecting of culture media at the time point (24 h) and the cytotoxicity was determined using the The Cytotoxicity Detection KitPLUS (LDH) (Merck, Germany). The resulting color intensity, measured spectrophotometrically at approximately 490 nm, is directly proportional to the amount of LDH released and thus the extent of cell death. By comparing the absorbance readings of treated samples to those of untreated controls (Equation 3). The degree of cytotoxicity induced by the experimental conditions can be determined. This method provides a quantitative and reliable measure of cell damage based on the extracellular presence of LDH (Cytotoxicity Detection KitPLUS (LDH) [online]; Kohda *et al.* 2021).

$$\text{Cytotoxicity } \% = \frac{(A_{490}\ \text{sample} - A_{490}\text{negative control})}{(A_{490}\ \text{positive control} - A_{490}\text{negative control})} \cdot 100 \qquad (3)$$

6.6.4 Determination of 3D skin model bacteria colonization

In the context of this study, the primary purpose of employing the bacteria adhesion test is to investigate the intricate interactions occurring between the 3D skin model and microbial cells. Within broader biological contexts, these adhesion tests are instrumental in elucidating the mechanisms by which bacteria adhere to host tissues during processes such as potential infection establishment or wound healing (Kohda *et al.* 2021; Villata *et al.* 2024). The bacterial cells present in each insert carefully collected by thoroughly washing the surface of the 3D skin model with 1 mL of normal saline solution supplemented with 0.1% Triton X-100 (ThermoFisher Scientific, USA). Following the detachment of adhered bacteria from the surface, the released bacteria were serially diluted and cultured on selective agar plates, specifically designed for the isolation and differentiation of pathogenic staphylococci (NutriSelect® Plus 70193; Merck, Germany). For the precise assessment of bacterial adhesion in our experiments, the Colony Forming Unit (CFU) counting method was employed.

6.7 CAM sheet production

The extracellular matrix (ECM) plays a pivotal role in the formation of tissue architecture and orchestrating cellular phenotype. These properties can be mimicked by in vitro cell-assembled extracellular matrix (CAM) sheets, as an innovative form of biomaterial in tissue engineering and regenerative medicine. This cellular self-assembly approach has evolved from the inherent ability of cells to organise and deposit ECM components. It results in the ability of cells to actively participate in mimicking the native surrounding microenvironment and architecture of anisotropic tissues. (Assunção *et al.* 2020; Xing *et al.* 2020)

6.7.1 Human dermal fibroblast culture and CAM production

The aim of CAM sheet production is to obtain an intact ECM with preserved composition and architecture of the original tissue in a controlled manner, potentially leading to the creation of tissue-like structures with enhanced cell-instructive properties. For the production of CAM sheets, the HDF cell line was cultured in DMEM/F12 medium containing 20% FBS and 1% penicillin/streptomycin. Briefly, the HDF cell line was seeded at a density of 1×10^4 cells·cm^2 in T-25 flasks with the addition of 500 μM of sodium L-ascorbate (Sigma Aldrich, USA) to ensure the formation of ECM components, including collagen. To produce the CAM sheets, the HDF cell line was cultured for 6–12 weeks, and the culture medium was changed three times per week.

6.7.2 CAM devitalization/decellularization

After providing the CAM sheets, the devitalization and decellularization processes were carried out. After providing the CAM sheets, the devitalization and decellularization processes were carried out. To devitalize the tissue, CAM were quickly rinsed in distilled water and dried at room temperature in a laminar flow cabinet. Dried CAM was stored at −80 °C. The decellularization process was realized on the devitalized CAM sheets. Chemical methods of decellularization contain detergents, such as sodium dodecyl sulfate (SDS) or Triton X-100 which are used to solubilize cell membranes and nuclear material (Schmidt *et al.* 2017; Neishabouri *et al.* 2022). Devitalized CAM sheets were rehydrated in UPW for 1 hour, rinsed with PBS, and then replaced with 5 ml of 0.66% SDS for 60 min. After the perfusion time, the SDS was removed, and the PBS was pipetted onto the CAM sheets. The perfusion time of the PBS was 10 min, and it was 5 times replaced for the whole process. Before further experiments, the CAM sheets were rehydrated in UPW and at 4 °C for 24 h.

6.7.3 Assessing H9c2 cell line proliferation using CAM sheets

CAM sheets can be used as a model scaffold for the deployment and proliferation of other cell lines, providing them with a support structure for growth and possibly orientation. The ECM complexity is not only typical for natural tissue but also essential for CAM and its possible biological functions. Thus, the cell-CAM scaffold interaction plays a prominent role in determining proliferation and testing biocompatibility. The acellular CAM sheets obtained by decellularisation were used to support the adhesion and growth of H9c2 myoblasts. First, the acellular CAM sheets were washed with sterile PBS and then placed into a 6well microtitrate plate. Subsequently, a prepared H9c2 cell line was then pipetted onto the surface of the CAM sheets ($1\times 10^{5}\cdot ml^{-1}$). The seeded CAM sheets were placed in the incubator under appropriate conditions. The H9c2 cell line was allowed to adhere and proliferate on the CAM sheet for the desired period of 3 and 7 days. After a period of time, fixation, permeabilization, and fluorescence staining of the cell culture were performed compared to control groups (H9c2 cells cultured on a 6-well microtitrate plate). ActinRed^{TM} 555 dye and Hoechst 33258 in PBS were chosen as fluorophores and evaluated using an Olympus IX81 microscope.

6.7.4 Histological analysis of CAM sheets

Histology of CAM sheets was performed as described in Chapter 6.3. CAM sheets were fixed in 4% PFA overnight at room temperature, dehydrated in ethanol (30-90 % EtOH) for 30 minutes each, and embedded in paraffin. Five-µm-thick sample sections were cut on the microtome Panthome LS-2064A and finally stained hematoxylin and eosin.

6.7.5 Preparation of CAM co-culture platform

To establish the CAM co-culture platform, HDF cell line and H9c2 cardiomyoblasts were seeded into T-25 culture flasks. HDF and H9c2/AC16 were cultured together at varying density ratios of 1:1, 1:2, and 2:1, respectively. The co-culture process was done in complete DMEM/F12 medium with the addition of 500 µM of sodium L-ascorbate to ensure the formation of ECM components under standard conditions. To produce the CAM co-cultivation sheets, it was cultured for 6–12 weeks, with the culture medium being refreshed every 2–3 days.

6.8 Investigating the CAM sheet components

6.8.1 RNA isolation and qRT-PCR reaction

Total RNA was extracted from the CAM sheet samples utilizing the Total RNA Mini Kit (cat. n.: 031-100; A&A Biotechnology, Poland), adhering to the protocol outlined by the manufacturer. (Total RNA Mini Manual [online]) To assess the concentration and purity of the extracted RNA, absorbance measurements were taken at wavelengths of 260 nm and 280 nm, respectively, using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

For the synthesis of complementary DNA (cDNA), the NG dART RT Kit (E0801) was utilized, again following the manufacturer's instructions. NG dART RT kit is first-strand cDNA synthesis kit convenient for two step RT-PCR. Kit is based on modified reverse transcriptase with improved thermostability up to 65 °C (NG dART RT Kit [online]).

In the next step of quantitative real-time PCR (qRT-PCR), the RT PCR Mix SYBR® kit (2008-100; A&A Biotechnology, Poland) was chosen. It is a ready-to-use, master mix designed for real-time PCR applications utilizing SYBR® Green dye for DNA detection. Each reaction was carried out in a total volume of 20 µl (2× RT PCR Mix SYBR®, forward/reverse primer mix, variable volume of template cDNAm and nuclease-free water to adjust the final volume). Amplification was conducted on a real-time PCR thermal cycler under the following cycling conditions for analyzed genes. The analysed genes were selected based on their relevance to the formation of ECM, CAM scaffold, respectively. A melt curve analysis was performed at the end of the amplification protocol to confirm the specificity of the PCR products. All reactions were run in triplicate, and non-template controls were included in each run to monitor for potential contamination.

6.8.2 SDS-PAGE electrophoresis

Preparation for SDS–PAGE electrophoresis

Prior to gel casting, it is essential to thoroughly clean all the component for the electrophoresis chamber with a detergent solution, followed by rinsing them with both tap and distilled water. Additionally, treating them with 70% ethanol can be done to guarantee maximum cleanliness. Next, the resolving and stacking gels are prepared according to the standart protocol.

CAM sheet sample preparation and SDS-PAGE electrophoresis

The prepared gel plates are placed into the slots of the electrophoresis module, ensuring that the shorter plate is oriented inward to create a buffer chamber. The

plates are fastened with clamps, and the entire assembly is positioned into the electrophoresis tank. The inner chamber is filled to the top with 1× TGS buffer. The CAM sheet samples were rehydrated and washed with PBS overnight before being lysed with ice-cold RIPA buffer containing protease and phosphatase inhibitors. Lysis of the CAM sheet was performed (1 h, 4 °C), followed by centrifugation for 10 minutes at 12,000x g, the removal of the supernatant, and dilution in 1x Loading Protein Buffer (E0269-01, EURx, Poland) along with a 5minute heating at 95 °C. A molecular weight marker (Perfect^{TM} Tricolor Protein Ladder; E3210-01, EURx, Poland) is loaded next to the CAM samples to determine the molecular weights of the resolved protein bands. Each well receives between 5-30 ng of total protein. After loading the samples, the electrophoresis tank is sealed with the electrode lid and connected to a power source. An initial voltage of 100 V is applied for 15 minutes, after which the voltage is raised to 150 V. Finally, the resolving gel after process is prepared for subsequent western blot analysis.

6.8.3 Semi-dry Western Blotting

For subsequent semi-dry western blotting, the Trans-Blot Turbo™ RTA Transfer Kit (Bio–Rad, USA) was utilized. This kit includes a 5× transfer buffer and Trans-Blot Turbo Mini-size membranes and filter stacks. However, the PVDF membrane provided was replaced with a nitrocellulose membrane with 0.22 µm pore size (Bio–Rad, USA). The assembly was sealed with the cathode tray, and the standard transfer program (Trans-Blot Turbo™ Transfer System, Bio-Rad, USA) was run for 7 minutes. Transfer efficiency was verified by staining the membrane with Ponceau S for 5 minutes, followed by washing with distilled water. The membrane was then subjected to immunodetection carried out using the Syngene G: Box Chemi XT 4 Chemiluminescence and Fluorescence Imaging System (Syngene, USA). Prior to imaging, the membranes were incubated for 5 minutes with WesternBright Quantum Kit (K-12042-D10, Advansta, USA), a chemiluminescent detection reagent.

7. SUMMARY OF RESULTS

All of the studies detailed in the practical section of this dissertation are the result of extensive, long-term experimental work. The very nature of these experiments demanded sustained attention to detail and a high degree of perseverance. Even the smallest mistake, or an unexpected contamination event, often meant that entire experimental series had to be repeated from the very beginning. Such setbacks were not only discouraging but also resulted in the loss of considerable amounts of time, energy, and resources. The process required constant vigilance, adaptability, and a willingness to learn from each challenge, no matter how minor it may have seemed at the outset.

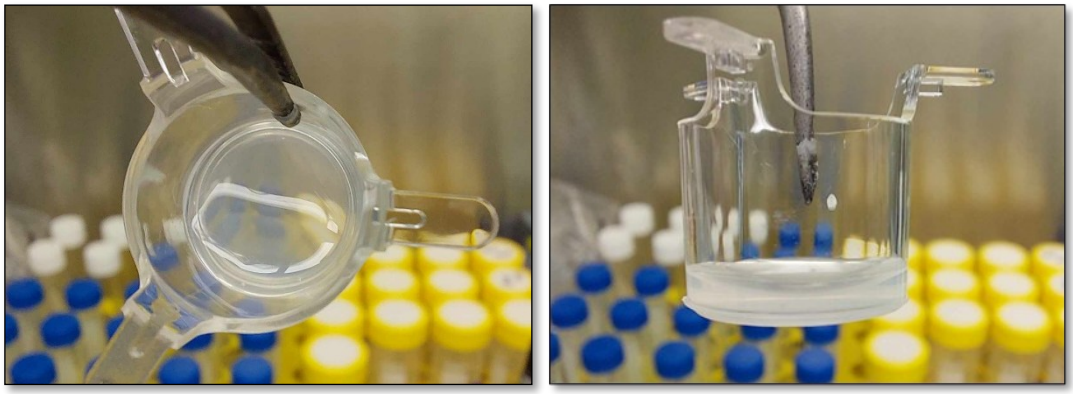
When this research project was initiated, the world was still grappling with the far-reaching consequences of the COVID-19 pandemic. This global crisis had a profound impact on scientific work, particularly in terms of the availability of laboratory reagents and materials. Many essential reagents were difficult to obtain, with supply chains disrupted and delivery times uncertain. These shortages necessitated a great deal of flexibility and creativity in the laboratory. Instead of following established protocols, it became necessary to adapt and optimize experimental procedures using whatever reagents were available at the time. This process of adaptation was often time-consuming and required repeated rounds of troubleshooting and optimization to achieve reliable and reproducible results.

Once the appropriate methods and experimental models had been successfully validated, these newly established approaches could be gradually implemented for the systematic measurement of the properties of various substances. This, in turn, enabled the progressive publication of the resulting data, contributing valuable new insights to the scientific community. However, it is important to note that many of the experimental series initiated during this research are still ongoing.

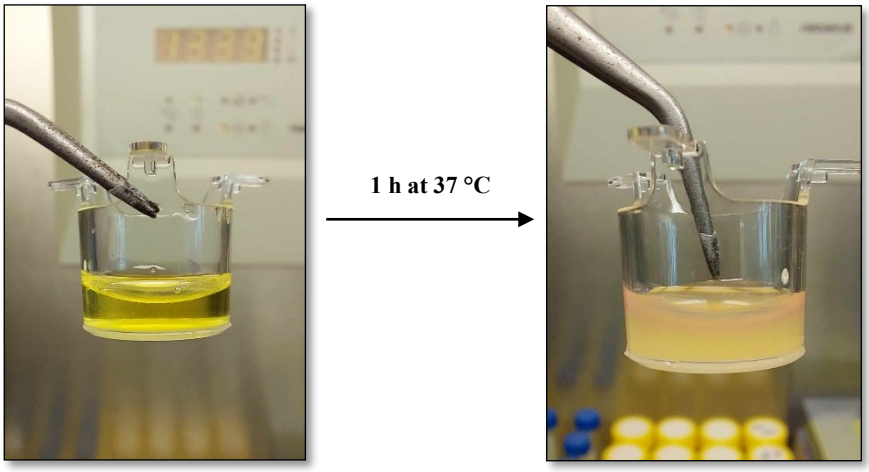
7.1 Establishment of an *in vitro* 3D Skin Culture Model

The first necessary step of the study was to identify a suitable protocol for developing a 3D skin model of human dermal fibroblast and keratinocyte. One approach regarding the supporting collagen matrix and the cell lines was used to produce a viable *in vitro* skin equivalent according to the modification to the protocols of Carlson *et al.* 2008 and Gantatirkar *et al.* 2007.

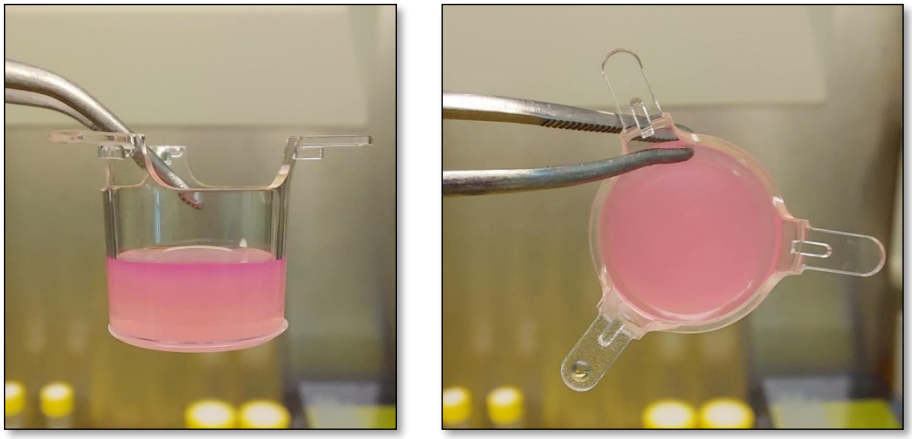
The stages of *in vitro* 3D skin model development are shown in Figure 13, alongside a representative scheme.



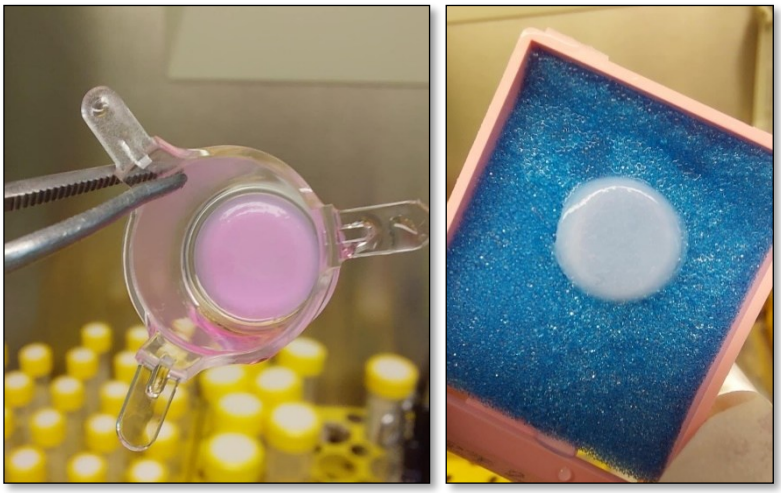
13A: Acellular collagen layer after 1h incubating at 37 °C



13B: Cellularized collagen (HDF cell line)



13C: Cellularized collagen after 7 days cultivation



13D: 3D Skin model - ALI system cultivation

Figure 13: Preparation of collagen-based 3D skin model
A: The preparation of acellullar collagen-based layer for the cell line seeding. B: Collagen-fibroblast layer od 3D skin model development. C: Collagen- fibroblast layer after 7 days of culture. D: Fully mature in vitro 3D skin model (ALI system).

In this Figure 13, it is shown that the collagen gel was used as a substrate for cell cultivation. According to the manufacturer Corning®, this collagen can form a three-dimensional gel on the 3 µm porous polycarbonate membranes as collagen support. Solutions of collagen was solidified into a stable gel by neutralizing with NB puffer and warming to 37 °C (incubation time: 45 min). After this polymerization, the collagen can form a stable acellular layer as a support of the skin model (Figure 13A). Simultaneously, the 3D skin cultures of preparation of the cellularized collagen with HDFs were assembled (Figures 13B). The cellular part consisting of the collagen matrix embedded with HDFs actively contracts over one week of culture in cultivation medium (Figure 13C). Subsequently, on the cellularized collagen, HaCaT were seeded. Finally, HSEs consist of a stratified epithelium grown at an air-liquid interface on a collagen matrix populated with HDFs (Figure 13D).

The established *in vitro* 3D skin model, which employs a collagen gel incorporated with HDF, and overlaid with HaCaT at the air-liquid interface (ALI), exhibits structural and functional attributes that are analogous to recognised *in vitro* skin equivalents. The documented contraction of the collagen matrix over a week of cultivation is consistent with the findings of Bell *et al.* 1981, who indicated that fibroblast-mediated contraction is a prevalent occurrence in collagen-based dermal equivalents, facilitating tissue maturation and enhancing mechanical stability. Furthermore, the development of a stratified epithelium by HaCaT cells on the cellular layer imitates dermal part mirrors the epidermal

differentiation documented in other investigations utilising comparable co-culture systems (Souren *et al.* 1989; Joshi *et al.* 2023).

The *in vitro* 3D skin model was developed to mimic the fundamental architecture of human skin, comprising a dermal equivalent composed of a collagen matrix populated with HDFs, and an epidermal layer formed by HaCaT seeded onto this dermal equivalent. This bilayered structure is evident in the histological image presented in Figure 14, which showcases a cross-section of the model stained with hematoxylin and eosin (H&E).

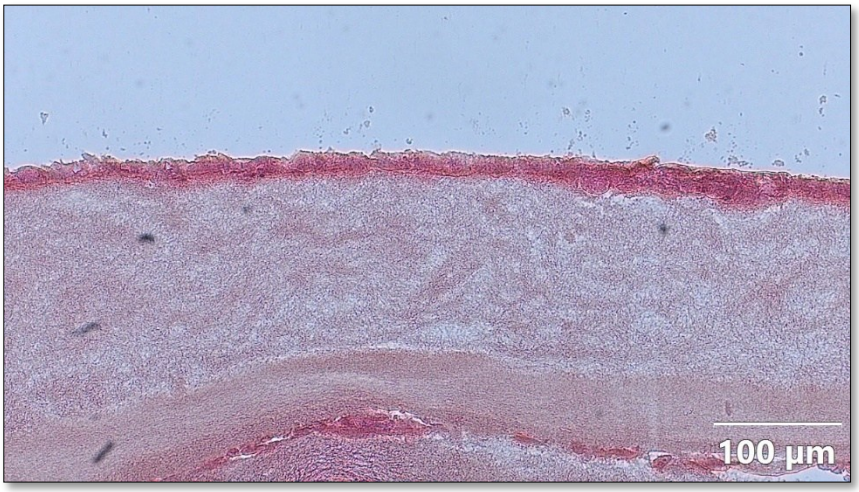


Figure 14: H&E stained cross-section of a in vitro 3D skin equivalent

Upon examination of the H&E-stained section (Figure 14), a distinct layered organization is observable. The lower region, characterized by lower cellular density and a lighter pink staining pattern, likely represents the collagen matrix containing the HDFs, thus forming the dermal compartment. The collagen appears as a lighter pink with the fibrillar network. The cellular density of this dermal equivalent can vary depending on the initial seeding conditions and the duration of the culture period.

The utilization of *in vitro* 3D skin models as valuable tools for investigating skin biology, drug testing, and tissue engineering is well-established in the scientific literature. Previous studies have demonstrated the potential of these models to form a stratified epidermis with specific characteristics, when HaCaT are cultured on a fibroblast-containing dermal equivalent (Stark *et al.* 2004; Wong *et al.* 2019; Imran *et al.* 2024).

To further standardize the *in vitro* skin equivalent model and mitigate variability, the spontaneously transformed human keratinocyte cell line HaCaT was employed instead of primary keratinocytes. HaCaT cells exhibit sustained genetic alterations indicative of transformed but non-tumorigenic cells (Boukamp *et al.* 1997). When cultured on cultivation flasks, HaCaT cells demonstrate continuous proliferation, independence from feeder cells, and a typical epithelial

morphology with the expression of a broad range of keratins. In 3D skin model of HaCaT cells with HDF, well-structured and differentiated stratified epithelia have been observed to develop, although the epdiermal part often remains parakeratotic, indicating incomplete keratinization.

In conclusion, the reproducible production and quality of HDF-HaCaT 3D co-cultures render them an excellent tool for large-scale investigations into the mechanisms regulating skin re-epithelialization and homeostasis within a tissue-like context. However, it is important to acknowledge that these *in vitro* models, including the one presented here, have limitations compared to native human skin. Aspects such as full epidermal barrier function, the presence of skin appendages (hair follicles, sweat glands), and complex immune cell interactions are typically not fully recapitulated in these simplified systems (Niehues *et al.* 2018). Therefore, the degree of stratification and differentiation observed in this image should be interpreted considering the specific culture conditions, the passage number of the cell lines used, and the duration of the experiment, all of which can significantly influence the final morphology of the reconstructed skin (Pampaloni *et al.* 2009).

Following the histological analysis of the 3D skin model, which showed light on its structural integrity and cellular arrangement, an additional tissue viability was evaluated using the MTT viability assay. This quantitative approach enables the assessment of metabolic activity in the cells of the reconstructed *in vitro* skin model. The outcomes of this viability and cytotoxicity assay are illustrated in Figure 15.

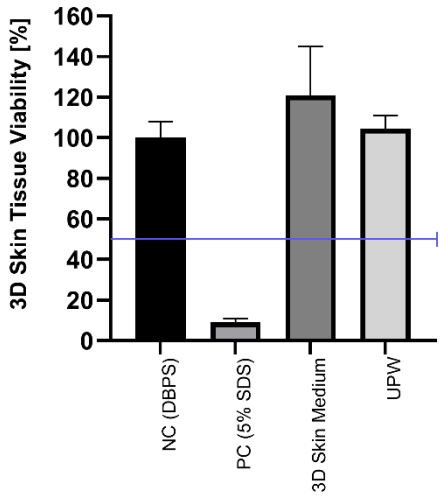


Figure 15: Viability of the 3D reconstructed skin model

Evaluating cell viability is a vital step in confirming the functionality and appropriateness of a reconstructed skin model for *in vitro* studies. The bar graph represents the mean viability of the 3D skin tissue for a treatment, and the error bars indicate the standard deviation. A blue line is drawn at the 50% viability threshold. A high baseline viability in the untreated control group is crucial to ensure the model retains its physiological traits and reactivity. A high viability in the negative control is crucial. It confirms that the vehicle or the basal conditions of the experiment do not negatively impact the skin tissue's health. (*In Vitro* EpiDerm™ Skin Irritation Test (EPI-200-SIT), MatTek [online]; OECD Test Guideline 439 *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method [online]).

On the other hand, treatments with non-irritating substances, such as the cell culture medium itself or ultrapure water under specific exposure conditions, should ideally reflect high tissue viability, signifying the model's compatibility with standard laboratory practices and the lack of inherent cytotoxic effects from these substances. The high viability observed in the 3D skin tissue treated with medium alone is expected, as it provides the necessary nutrients and environment for cell survival. The high viability with UPW suggests that short-term exposure to ultrapure water under these experimental conditions does not significantly compromise the viability of the 3D skin model. Finally, the results presented in the graph are generally consistent with expectations for a skin irritation test using a 3D skin model. These viability findings, when taken alongside histological results, provide a thorough assessment of the reconstructed skin model's reliability and its applicability for future experimental studies.

Moreover, I have fully validated the above-mentioned 3D skin models for laboratory practice at Tomas Bata University in Zlín. The validity of these models is demonstrated by the results presented desribed earlier, which provide strong evidence of their reliability and applicability in practical laboratory settings. The practical use of these validated models is currently exemplified in the SurfToGreen project (HORIZON-JU-IA-101157688), where we are investigating the effects of various types of surfactants. This ongoing work highlights the relevance and usefulness of the validated models in addressing real-world scientific questions and advancing our knowledge in the field of surfactant chemistry. However, due to the ongoing nature of the project and the intended use of the results within the framework of SurfToGreen, it is not possible to publish these findings at this time. We are committed to ensuring that the results are utilized effectively within the project before making them publicly available, in order to maximize their impact and maintain the integrity of our research process.

7.2 Bacterial behavior on HaCaT monolayers as a foundation for 3D skin model experiments

Before transitioning to complex bacteria-enriched *in vitro* 3D skin models, it is essential to understand the basic interactions between skin keratinocytes and representative bacterial strains under simplified conditions. To this end, a series of preliminary experiments were conducted using HaCaT monolayers exposed to both commensal and pathogenic bacterial species. These included the skin commensals *Staphylococcus epidermidis* and *Micrococcus luteus*, as well as the opportunistic pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are commonly implicated in skin infections and chronic wounds (Grice and Segre 2011; Sachdeva *et al.* 2022).

This part of study aimed to elucidate bacterial behavior and its impact on keratinocyte viability and proliferation, thereby providing a reference framework for subsequent integration of bacterial representatives into *in vitro* 3D skin constructs. Understanding whether commensals like *S. epidermidis* or *M. luteus* exert protective effects against pathogens such as *S. aureus* or *P. aeruginosa* may offer insights into microbiota-driven modulation of infection and tissue repair processes. Indeed, some probiotic and commensal bacteria have been shown to inhibit the adhesion and internalisation of *S. aureus* into keratinocytes, thereby reducing its pathogenic potential (Prince *et al.* 2012; Kiousi *et al.* 2024)

Three assays were performed, i. e. trypan blue viability assays, where the viability of HaCaT was evaluated following exposure to both whole bacterial cultures and their cell-free supernatants (lysates). To investigate whether bacterial lysates affect HaCaT regenerative potential, cell proliferation assay was monitored. And finally, since colonisation of epithelial surfaces often begins with adhesion, the capacity of bacterial strains to adhere to HaCaT monolayers was measured using adhesion test.

7.2.1 HaCaT viability and proliferation in the presence of microbiome

To assess the potential cytotoxic or protective effects of bacterial supernatants on HaCaT cells, the Trypan Blue exclusion assay was employed. This widely used method is based on the principle that viable cells possess intact cell membranes that exclude the membrane-impermeable dye Trypan Blue, while non-viable cells with compromised membranes allow the dye to enter, resulting in blue staining of the cytoplasm. In this assay, HaCaT cells were exposed to supernatants derived from cultures of *S. epidermidis*, *M. luteus*, *S. aureus* and *P. aeruginosa* + their lysates for specific time intervals (0, 2, 8, 24, and 48 hours), seen in Figure 16 and

17. Following incubation, the cells were harvested and incubated with Trypan Blue solution and quantified under a light microscope.

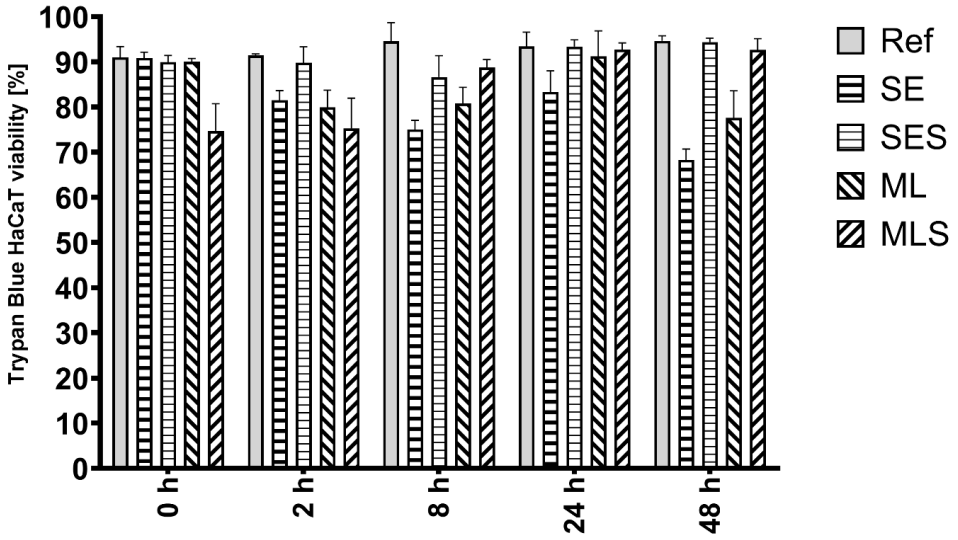


Figure 16: Effects of commensal bacteria/supernatants on HaCaT cell viability.

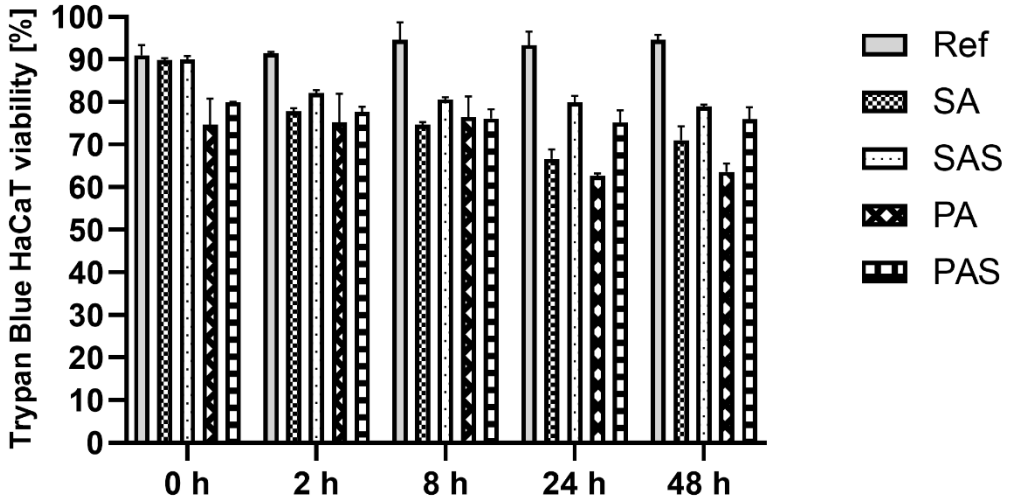


Figure 17: Effects of pathogenic bacteria/supernatants on HaCaT cell viability.

The bar graphs illustrate the Trypan blue HaCaT cell viability (%) at the specified time intervals. At the outset of the experiment (0 h), the viability of HaCaT cells is notably high, approximately 90-95 %, across all conditions, which is expected for healthy cells. The reference condition consistently shows high viability, remaining above 90 % throughout the 48h duration. The presence of both live (SE) and killed (SES) *S. epidermidis* supernatants seems to have a minimal effect on HaCaT cell viability over the 48 hours (Figure 16). The viability stays relatively high, generally above 85 %, with minor fluctuations. This indicates that soluble factors released by *S. epidermidis*, even when the bacteria

are inactivated, do not significantly damage HaCaT cells in this experimental context. In the case of *M. luteus*, similarly to SA, both ML and MLS do not markedly reduce HaCaT cell viability over the 48h timeframe. The viability typically remains above 80 %, with some variation noted across time points.

Comparable testing was conducted with the pathogenic bacteria (Figure 17). The SE and SAS appear to adversely affect HaCaT cell viability over time. At 2 hours, the viability in both SA and SAS conditions is significantly lower than the reference. This decline becomes more pronounced at subsequent time points (24 h and 48 h), with viability decreasing to about 65–75 %. Notably, the SAS displays a similar trend to the live bacteria, indicating that the secreted factors or cellular components of *S. aureus* play a role in the decreased cell viability. PA and PAS also show a decline in HaCaT cell viability throughout this testing period, with viability dropping to roughly 60-70 %. Similar to *S. aureus*, the PAS shows a comparable effect to the PA, suggesting that the stable components of PA contribute to the observed cytotoxicity.

To evaluate the impact of bacterial components on keratinocyte proliferation, a metabolic MTT assay was conducted on HaCaT cells exposed to 10% sterile-filtered bacterial lysates of both commensal and pathogenic species. Formazan production was measured at 0, 24, and 48 hours and normalized to the 0hour reference to assess relative changes over time (Figure 18).

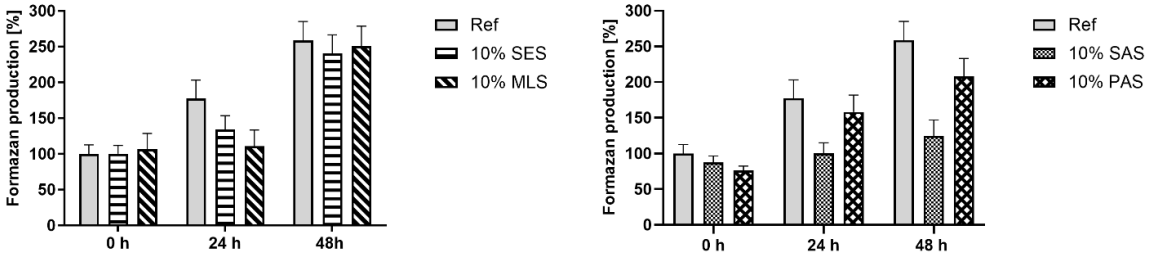


Figure 18: MTT proliferation assay in HaCaT monolayers exposed to bacterial supernatants over 0, 24, and 48 hours (expressed as % of time 0 reference). Left: Commensal bacteria (S. epidermidis – SES, M. luteus – MLS). Right: Pathogenic bacteria (S. aureus – SAS, P. aeruginosa – PAS).

Exposure of HaCaT keratinocytes to bacteria and their lysates revealed distinct effects on cell viability and proliferation, reflecting the differential impact of commensal versus pathogenic microorganisms. Supernatants from *Staphylococcus epidermidis*, and *Micrococcus luteus* did not induce any significant deviations from the reference proliferation pattern, as indicated by comparable increases in formazan production at both 24 and 48 hours. This suggests that these commensal strains do not negatively affect HaCaT cell metabolic activity or viability (Cogen *et al.* 2010; Lai *et al.* 2009). *S. epidermidis*

is known to support cutaneous homeostasis by promoting the production of AMPs and modulating host immune responses without harming keratinocytes. Although less is known about *M. luteus* in this context, its non-cytotoxic profile in the assay supports the hypothesis of its neutral or even beneficial interaction with host cells. Nevertheless, it is known to possess plant growth-promoting properties, indicating it may not be inherently toxic to mammalian cells (Ahmad *et al.* 2008). In contrast, supernatants from pathogenic bacteria, namely *Staphylococcus aureus* and *Pseudomonas aeruginosa*, significantly reduced HaCaT cell proliferation. SAS exposure led to a marked decrease in formazan production at both time points, consistent with the cytotoxic properties of *S. aureus*, which secretes numerous virulence factors such as alpha-toxin and phenol-soluble modulins that compromise epithelial barrier integrity and trigger inflammatory damage (Otto 2014; Mohammedsaeed *et al.* 2014). Similarly, PAS exposure resulted in reduced metabolic activity, albeit to a lesser extent. *P. aeruginosa* is recognised for its cytotoxic effects on keratinocytes, driven by a variety of virulence factors, including exotoxins, proteases, and components of biofilms. However, this variation may be attributed to strain-specific virulence or the timing of supernatant collection, which influences the concentration of cytotoxic compounds like pyocyanin and elastase (Ichikawa *et al.* 2000; Gellanty and Hancock 2013).

These data contribute to the pre-validation of microbial effects using a 2D HaCaT monolayer system and provide a robust foundation for follow-up studies employing 3D *in vitro* skin models that better recapitulate *in vivo* microbe-host interactions.

7.2.2 The influence of bacteria adhesion on HaCaT cell line

An adhesion test was conducted to evaluate the adherence capacity of selected bacterial strains to HaCaT keratinocytes. The procedure involved incubating confluent monolayers of HaCaT cells with bacterial suspensions, both individual bacteria and in co-culture with HaCaT keratinocytes. Briefly, HaCaT cells were seeded in 12well microtiter plates and allowed to proliferate.

As depicted in Figure 19, the results demonstrated that SE exhibited a high level of adhesion to HaCaT. The presence of ML in co-culture with SE (SE + ML) did not significantly alter SE adhesion. Moreover, cultivation of ML alone did not result in detectable adhesion of this bacterium to keratinocytes, suggesting a limited role in epithelial colonisation. The high adhesion of SE to HaCaT aligns with its role as a commensal bacterium frequently colonising the skin surface (Scharschmidt and Fischbach 2013). The lack of significant impact of ML on SE adhesion suggests a neutral interaction in this context, indicating non-competitive or even synergistic relationships between skin commensals (Yang *et al.* 2022; Glatthardt *et al.* 2024).

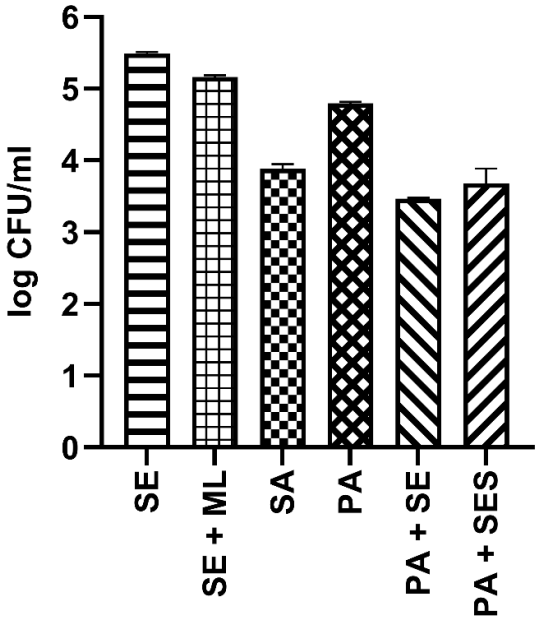


Figure 19: Adhesion profiles of skin-associated bacteria

In contrast, pathogen SA showed considerably lower adhesion. The lower adhesion of SA compared to SE in this assay is interesting, given its potential as a skin pathogen. This could reflect differences in specific adhesins and host cell receptors utilised by these two staphylococcal species (Mempel *et al.* 1998; Geoghegan and Dufrene 2018). PA displayed a moderate level of adhesion. Notably, the presence of SA, either live bacteria or in the form of lysate, reduced the adhesion of PA to keratinocytes, indicating a potential protective effect. This observation demonstrates that commensal bacteria can inhibit pathogen adherence through competitive exclusion mechanisms or the production of inhibitory substances. The fact that even the SE lysate exhibited this inhibitory effect suggests the involvement of secreted factors or cell wall components in this interaction. Further investigation into the specific molecules responsible for this anti-adhesive activity could lead to novel strategies for preventing *P. aeruginosa* infections (Prince *et al.* 2012; Alves *et al.* 2018).

7.3 Fabrication of CAM sheets as cell-made scaffolds

7.3.1 CAM sheets generation

During my dissertation research, I focused on the development and comprehensive characterization of cell-assembled matrix (CAM) scaffolds that closely mimic the structure and function of the native ECM. To produce structurally like ECM-based scaffolds, human dermal fibroblasts (HDFs) were cultured for extended periods, leading to the formation of homogeneous CAM sheets composed entirely of cell-secreted matrix components (Figure 20).

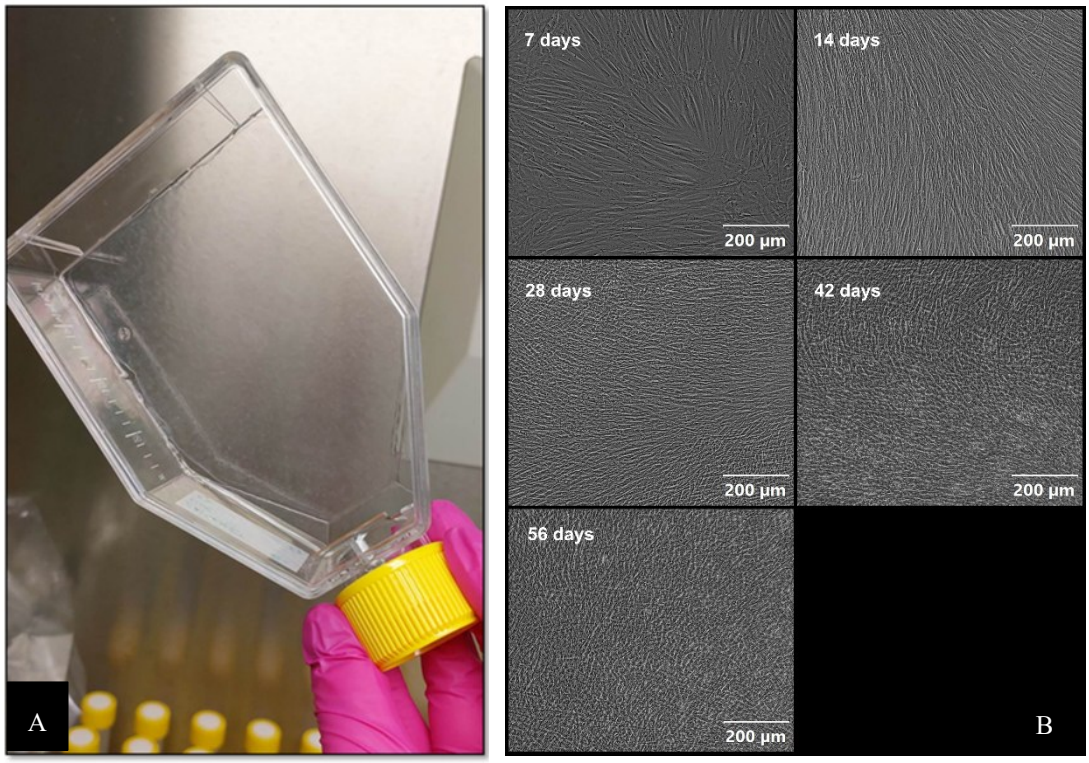


Figure 20. Generation and morphology of CAM sheet scaffold
A. Fully developed, CAM scaffold. B. In vitro formation of CAM sheet captured through photodocumentation.

To enhance their suitability as scaffolds, the CAM sheets underwent devitalization and decellularization, processes that effectively removed cellular material while preserving the extracellular matrix structure. Histological analyses confirmed that these procedures maintained tissue integrity and that essential matrix proteins, such as collagen, remained present. These results are in agreement with previous findings demonstrating that SDS-based decellularization protocols effectively eliminates immunogenic material while retaining the ECM framework necessary to support cell adhesion, migration, and tissue regeneration (Gilbert *et al.* 2006; Smitt *et al.* 2017). Overall, the CAM decellularization protocol satisfies key histological benchmarks, resulting in cell-free yet

structurally preserved scaffolds appropriate for both tissue engineering and in vitro model development.

The biological performance of the decellularized CAM scaffolds was evaluated by reseeding them with rat heart myoblasts, and fluorescence microscopy showed that these scaffolds promoted cell alignment and proliferation more effectively than standard culture surfaces (Figure 21) Therefore, the CAM sheet represents a completely cellular matrix produced by prolonged in vitro culture, offering a biologically native substrate. Similar to the other type of decellularized tissue, the CAM sheet, by its native cellular derivation, facilitate enhanced cell-mediated signalling. This, therefore, renders it more physiologically appropriate for tissue modeling and regenerative medicine (Badylak *et al.* 2009; Chen *et al.* 2024).

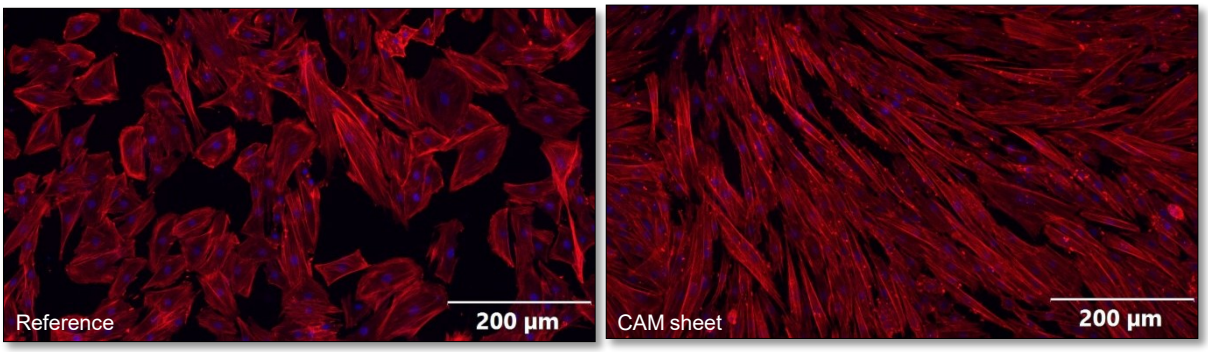


Figure 21: Fluorescence microscopy of H9c2 myoblast proliferation on standard culture flask and decellularized CAM scaffold. Fluorescent staining highlights actin filaments (red) and nuclei (blue), revealing improved cellular alignment and proliferation on the decellularized CAM scaffold compared to the standard culture surface

7.3.2 Impact of HDF-H9c2 co-culture on CAM scaffold generation and biochemical and molecular evaluation of CAM

The influence of different co-culture ratios on scaffold morphology and organization was systematically investigated. Thus, the composition and structural arrangement of cell-assembled matrix (CAM) scaffolds are significantly influenced by the types and ratios of cells used during their fabrication. In this part of study, we investigated how varying co-culture ratios of HDFs and H9c2 cardiomyoblasts influence ECM formation, cytoskeletal structure, and overall CAM scaffold architecture (Figure 22). CAM sheets were generated under each experimental condition described, and cytoskeletal (F-actin) and nuclear staining were performed to examine cellular activity and matrix formation over time (3rd and 7th day of cultivation).

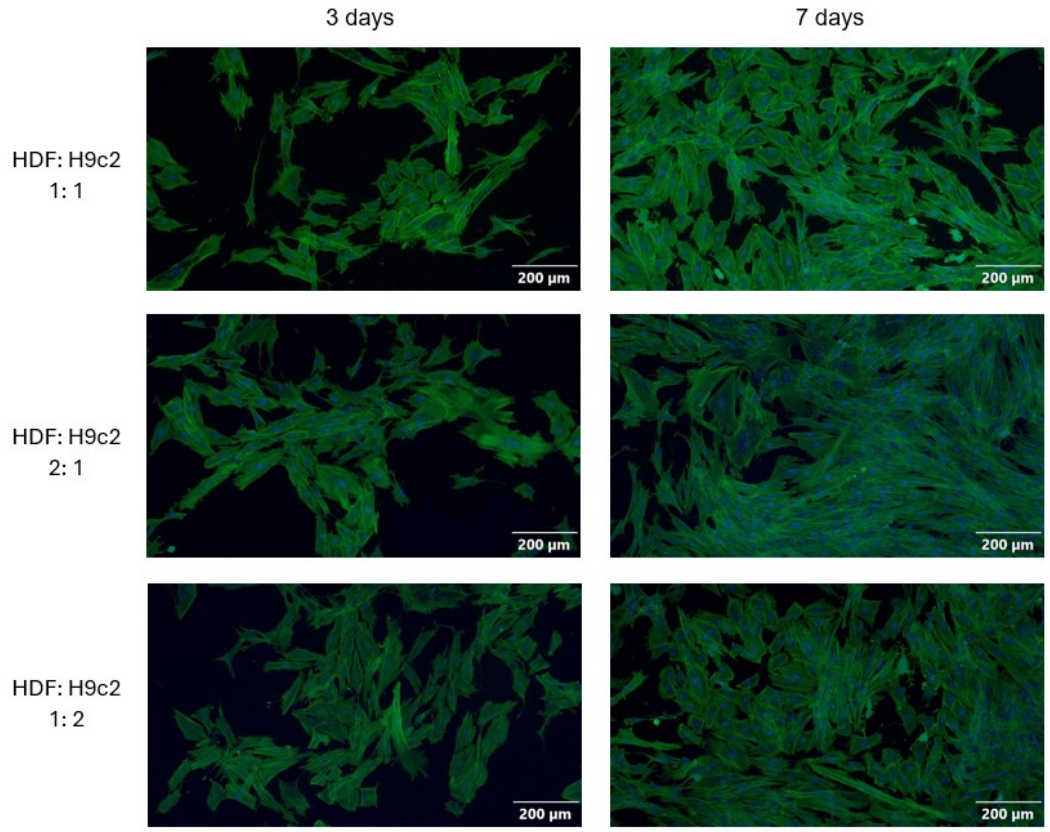


Figure 22: Fluorescence microscopy of CAM sheets formed from HDF:H9c2 co-cultures at varying ratios (3rd and 7th day proliferation). F-actin stained with ActinGreen 488 (green) and nuclei labeled with Hoechst 33258 (blue).

Balanced ratios of fibroblasts and cardiomyoblasts resulted in well-organized cellular networks and matrix structures, while other ratios led to less optimal scaffold architecture. These findings confirm that co-culture ratio plays a vital role in determining the structural and functional properties of engineered tissues, particularly regarding ECM dynamics and functional tissue replication. The balance between fibroblasts and cardiomyoblasts appears critical in promoting optimal ECM assembly, structural alignment, and finally biomimetic tissue development (Suhaeri *et al.* 2015; Brauer *et al.* 2023). Thus, properly optimized co-culture conditions are essential for generating physiologically relevant microenvironments, particularly in cardiac or vascular tissue models, where the dynamic interaction between stromal and parenchymal cells governs successful cell-made scaffold formation and integration (Zhang *et al.* 2012; Bartes *et al.* 2014).

The collagen content in the CAM sheets was assessed by quantifying hydroxyproline (Hyp), a major amino acid component of collagen, through a standard colourimetric assay. The measurement of hydroxyproline provides an indirect but reliable estimate of total collage. The conversion factor of 13 % is most commonly used to convert hydroxyproline to collagen (Edwards and O´Brien 1980; Reddy and Enwemeka 1996; Shoulders and Raines 2009). This

assay revealed that co-cultures of fibroblasts and cardiomyoblasts—especially at balanced or fibroblast-enriched ratios—resulted in higher collagen deposition than monocultures. This finding underscores the importance of optimizing cell ratios to achieve robust matrix assembly, while also recognizing that excessive ECM production could hinder tissue remodeling if not properly controlled (Medugorac 1980; Susic and Frohlich 2011; Iwamiya *et al.* 2016; Jang *et al.* 2020; Mierke 2024). Despite all this, these types of CAM sheets could be promising candidates for tissue engineering applications requiring durable, bioactive matrices that can mimic or exceed the collagen content of native tissues.

To determine the biochemical stability and retention of key proteins in the CAM sheets following processing, SDS-PAGE electrophoresis and subsequent western blotting were performed. These procedures are standard in protein analysis, enabling the separation and immunodetection of target proteins based on molecular weight and antigenic determinants. In the next step of gene expression evaluation, the quantitative real-time PCR (qRT-PCR) using the RT PCR Mix SYBR® kit was chosen.

Evaluating protein preservation ang gene expression after devitalization and decellularisation is of essential importance, as these processes aim to remove cellular content while leaving ECM components that play a significant role in cell-made scaffold bioactivity and re-cellularisation intact. The analysis was performed on HDF, H9c2 cell line, and their co-culture of CAM sheets, giving insight into how matrix quality and scaffold suitability for tissue engineering applications are determined by cell composition and processing. The area of protein analysis and gene expression analysis was conducted during an international internship at the Biotechnology Centre of the Silesian University in Gliwice, where advanced molecular techniques were applied to further characterize the CAM scaffolds and their cellular interactions.

This experiments demonstrated that key extracellular matrix proteins like laminin were best preserved in scaffolds derived from fibroblast-dominated cultures (Gauvin *et al.* 2011; Mouw *et al.* 2014). It also confirmed the biochemical stability of the scaffolds after processing, supporting their suitability for reseeding and tissue engineering applications (Jourdan-Lesaux *et al.* 2010; Yang *et al.* 2022; Golebiowska *et al.* 2023).

Due to the intention to publish these results in a scientific journal, it is not possible to include the detailed findings directly in the dissertation. However, for the purposes of the dissertation committee, the results are provided as a separate article attached to the thesis.

7.4 Characterization and biological evaluation of advanced polymeric materials

In addition to the primary focus of my doctoral thesis, various collaborative research initiatives were performer that have been published, particularly highlighting antimicrobial assessments of functional polymeric materials or their cytotoxicity. These investigations sought to evaluate the antibacterial characteristics of sophisticated nanocomposites and surface-modified substrates, employing standardized methods to guarantee reproducibility and comparability. Antimicrobial efficacy was measured by ISO 22196: Measurement of antibacterial activity on plastics and other non-porous surfaces, and ISO 20743: Textiles — Determination of antibacterial activity of textile products, which are internationally acknowledged standards for evaluating non-porous surfaces and textile materials (see List of publications). Following the ISO 22196 standard, conductive composite hydrogels featuring covalently bonded polypyrrole (PPy) nanoparticles (Káčerová *et al.* 2024), alongside crosslinked chitosan/PPy nanofibers with covalently anchored PPy (Muchová *et al.* 2025), were analyzed. The typical procedure includes inoculating the test surface with bacterial suspensions (e.g., *Staphylococcus aureus* and *Escherichia coli*), applying a film, incubating under regulated humidity and temperature, and quantifying viable bacteria after 24 hours to ascertain antibacterial effectiveness. For textile materials, the ISO 20743 standard was employed to evaluate cotton fabrics that had undergone plasma-assisted cationization with chitosan and quaternized poly[bis(2-chloroethyl) ether-alt-1,3-bis[3-(dimethylamino)propyl]urea], assessing bacterial reduction upon direct contact (Hamida *et al.* 2024). In addition to these antimicrobial evaluations, minimum inhibitory concentration (MIC) tests were conducted on colloidal systems, such as colloidal PPy (Káčerová *et al.* 2023) and composite colloids consisting of polyaniline (PANI) mixed with cellulose nanocrystals (CNC) or nanofibrils (CNF) (Korábková *et al.* 2024). The MIC assay identified the lowest concentration of the colloids necessary to prevent visible bacterial growth in broth culture, offering quantitative insights into the antimicrobial effectiveness of the dispersed nanomaterials. Furthermore, these contributions yielded valuable knowledge regarding the antimicrobial capabilities of innovative polymer-based substances and facilitated the advancement of multifunctional systems for biomedical and environmental purposes.

A series of cytotoxicity evaluations, conducted in line with ISO 10993-5, which delineates the procedure for *in vitro* cytotoxicity assessment of medical devices, were also carried out. This standard delineates protocols for evaluating the potential toxic effects of materials on cultured mammalian cells, typically utilizing assays such as MTT, which assess cell viability, metabolic activity, or membrane integrity following exposure to material extracts or direct contact with

test substances. In this context, several novel biomaterials, including a magneto-responsive hyaluronan-based hydrogel, developed for potential applications in mild hyperthermia and 3D bioprinting (Vítková *et al.* 2023), or alongside a collection of tritopic guest molecules (compounds 6a–6e) and cyclodextrin-modified hyaluronan polymers (CD-HA 12 and 14) (Jurtík *et al.* 2023), were evaluated. The findings provided crucial information on cell compatibility and material safety, which are vital parameters for subsequent in vivo testing or clinical application. They contributed to a broader understanding of how structural modifications of biopolymers and supramolecular assemblies affect cellular responses in tissue engineering and therapeutic environments.

8. CONTRIBUTION TO SCIENCE

Advancements *in vitro* skin tissue reconstitution techniques have resulted from diligent research, improved technological capabilities, and, in particular, deeper insights into focusing on 3D skin equivalents with more (patho)physiological functions to overcome the challenges of resembling the complex human skin. Various types of in vitro test skin models are being developed to assess the safety of drug testing or skin care products. Evaluating the toxicity of these substances applied directly to the skin depends not only on their chemical composition but also on their percutaneous absorption and xenobiotic metabolism (Choudhury and Das 2021; Hofmann *et al.* 2023). One such system, the full-thickness skin tissue model, consists of a human dermal fibroblast with epidermal-derived keratinocytes. These cells differentiate to form an epidermal-like structure, complete with basal, spinous, granular, and stratum corneum layers. In the preliminary studies of this doctoral thesis, the preparation of the given type of model, i.e. RHE, was accomplished. The preparation of RHE consisted of a dermal equivalent with human fibroblasts overlaying a stratified, well-differentiated epidermis derived from normal human keratinocytes cultured on an inert polycarbonate insert at an air-liquid interface. The *in vitro* skin irritation test and histological analysis of these 3D *in vitro* skin cultures demonstrated similarities in differentiation and metabolic cell viability properties to the skin. The established protocols significantly improve the potential of cell-biology laboratory at TBU in Zlín in providing of advanced experiments. Moreover, the initial experiments in bacteria/lysate enriched skin models were performed which is a state of art techniques opening a new horizon for wound healing experiments. Those models are the basis for highly in vivo mimicking experiments the laboratory will do in future.

Simultaneously with the previous study, the cell-assembled extracellular matrix (CAM) sheets, derived from human dermal fibroblast, were produced. Theoretically, CAM sheets refer to a method where cells are actively involved in the assembly of their extracellular matrix into sheet-like structures. It allowed the fibroblast cell line to produce and organize its own ECM components in a controlled manner (Fonseca *et al.* 2023). The CAM sheet production led to the creation of tissue-like structures with enhanced biomimicry and functionality thanks to the specific cultivating combination of an appropriate fibroblast density and culturing with the adding ascorbic acid as a collagen stimulant. Moreover, the decellularized process ensured the fabrication of acellular CAM scaffold for assessing cell line proliferation. Preliminary experiments have showcased the capacity of CAM sheets to facilitate the attachment and proliferation of various cell types, particularly demonstrating above-average growth and orientation of H9c2 myoblasts. Subsequently, the produced CAM sheets were scrutinized using

fluorescence microscopy and histological examination, affirming both their structural integrity and functional properties.

The foundation was established for inducing modifications in the internal architecture and composition of cell-made scaffolds by applying external stimuli (e.g., mechanical forces), which, through mechanotransduction, modulate cellular behavior and thereby drive scaffold remodeling. The knowledge and experience gained during these studies will facilitate further advancement in the field of tissue engineering by enabling the development of improved techniques, innovative models, and more effective therapeutic strategies. These insights will contribute to the ongoing progress and practical applications of tissue engineering in regenerative medicine and related disciplines.

REFERENCES

1. ABHISKEK, K. J.; DIVYA, S.; KAVITA, D.; RENUKA, M.; SANDEEP, M. and K. P. ALOK., 2018. Models and Methods for In Vitro Toxicity. In: DHAWAN, A., SEOK, K. In Vitro Toxicology [online], Academic Press, chapter 3, p. 45-65. Available from: https://doi.org/10.1016/B978-0-12-804667-8.00003-1. ISBN 9780128046678.
2. AHMAD, F., AHMAD, I. and M.S. Khan, 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research* [online], vol. 163, iss. 2, p. 173-181. Available from: https://doi.org/10.1016/j.micres.2006.04.001.
3. ALVES, P.M., AL-BADI, E., WITHYCOMBE, C., JONES, P.M., PURDY, K.J., MADDOCKS, S.E., 2018. Interaction between Staphylococcus aureus and Pseudomonas aeruginosa is beneficial for colonisation and pathogenicity in a mixed biofilm. *Pathogens and Disease* [online], vol. 76, no. 1. Available from: doi: 10.1093/femspd/fty003. PMID: 29342260.
4. ASSUNCAO, M., DEHGHAN-BANIANI D., YIU CH.K., SPÄTER T., BEYER S., BLOCKI A., 2020. Cell-Derived Extracellular Matrix for Tissue Engineering and Regenerative Medicine. *Frontiers in Bioengineering and Biotechnology* [online], vol. 3, no. 8: 602009. Available from: doi: 10.3389/fbioe.2020.602009.
5. AUXENFANS C., FRADETTE J., LEQUEUX C., GERMAIN L., KINIKOGLU B., BECHETOILLE N., BRAYE F., AUGER F.A., DAMOUR O., 2009. Evolution of three-dimensional skin equivalent models reconstructed in vitro by tissue engineering. *European Journal of Dermatology* [online], vol. 19 no.1, p. 107-13. doi: 10.1684/ejd.2008.0573.
6. AYDIN, O., PASSARO, A.P., RAMAN, R., SPELLICY, S.E., WEINBERG, R.P., KAMM, R.D., SAMPLE, M., TRUSKEY, G.A., ZARTMAN, J., DAR R.D., PALACIOS, S., WANG, J., TORDOFF, J., MONTSERRAT, N., BASHIR, R., SAIF, M.T.A., WEISS, R., 2022. Principles for the design of multicellular engineered living systems. *APL Bioengineering* [online], vol. 6. no. 1:010903. doi: 10.1063/5.0076635.
7. AVELAR-FREITAS, B.A.; ALMEIDA, V.G., PINTO, M.C.; MOURAO, F.A.; MASSENSINI, A.R.; MARTINS-FILHO, O.A.; ROCHA-VIEIRA, E.; BRITO-MELO G.E., 2014. Trypan blue exclusion assay by flow cytometry. *Brazilian Journal of Medical and Biological Research* [online], vol. 47, no. 4, p. 307-15. Available form: doi: 10.1590/1414-431X20143437.

8. BADYLAK, S.F., 2002. The extracellular matrix as a scaffold for tissue reconstruction. Seminars in Cell and Developmental Biology [online], vol. 13, no. 5, p. 377-83. Available from: doi: 10.1016/s1084952102000940.
9. BADYLAK, S.F., FREYETES, D.O., GILBERT, T.W, 2009. Extracellular matrix as a biological scaffold material: Structure and function. *Acta Biomaterialia* [online], vol. 5, no. 1, p. 1-13. Available from: doi: 10.1016/j.actbio.2008.09.013.
10. BELL, E., EHRLICH, H. P., BUTTLE, D. J. & NAKATSUJI, T, 1981. Living tissue formed in vitro and accepted as skin-equivalent tissue of full thickness. Science, [online], vol. 211, p. 1052-1054. Available from: doi: 10.1126/science.7008197
11. BELLO Y.M., FALABELLA A.F., EAGLSTEIN W.H, 2001. Tissue-engineered skin. Current status in wound healing. *American Journal of Clinical Dermatology* [online], vol. 2, no. 5, p. 305-13. Available from: doi: 10.2165/00128071-200102050-00005.
12. BÉNYEI, É.B., NAZEER, R.R., ASKENASY, I., MANCINI, L., HO, P.M., GAC, S., SWAIN, J.E.V., WELCH, M, 2024. The past, present and future of polymicrobial infection research: Modelling, eavesdropping, terraforming and other stories *Advances in Microbial Physiology* [online], vol. 85, p. 259-323. Available from: doi:https://doi.org/10.1016/bs.ampbs.2024.04.002.
13. BISHOP, J.E., GREENBAUM, R., GIBSON, D.G., YACOUB, M., LAURENT, G.J, 1990. Enhanced deposition of predominantly type I collagen in myocardial disease. *Journal of Molecular and Cellular Cardiology* [online], vol. 22, no. 10, p. 1157-65. Available from: doi: 10.1016/0022-2828(90)90079-h.
14. BOJAR Richard A, 2015. Studying the Human Skin Microbiome Using 3D In Vitro Skin Models. *Applied In Vitro Toxicology* [online], vol. 1 no. 2, p. 165-171. Available from: http://doi.org/10.1089/aivt.2015.0002
15. BOSMAN, F.T. and I. STAMENKOVIC, 2003. Functional structure and composition of the extracellular matrix. *The Journal of Pathology* [online], vol. 200, no. 4, p. 423-428. Available from: https://doi.org/10.1002/path.1437
16. BOUKAMP, P., POPP, S., ALTMEYER, S., HULSEN, A., FASCHING C., CREMER, T., FUSENING N.E, 1997. Sustained nontumorigenic phenotype correlates with a largely stable chromosome content during long-term culture of the human keratinocyte line HaCaT. *Genes Chromosomes Cancer* [online], vol. 19, no. 4, p 201-214. Available from: 10.1002/(sici)1098-2264(199708)19:4<201::aid-gcc1>3.0.co;2-0
17. BRAUER, J., LANGE, T., KELLER, D., GÖRLITZ, S., CHO, S., KEYE, J., GOSSEN, M., PETERSEN, A., KORNAK, U, 2023. Dissecting the

influence of cellular senescence on cell mechanics and extracellular matrix formation in vitro. *Aging Cell* [online], vol. 22, no. 3:13744. Available from: doi: 10.1111/acel.13744.
18. BARTHES, J., ÖZCELIK, H., HINDIE, M., NDREU-HALILI, A., HASAN, A., VRANA, N.E, 2014. Cell microenvironment engineering and monitoring for tissue engineering and regenerative medicine: the recent advances. *BioMed Research International* [online]; 921905. Available from: doi: 10.1155/2014/921905.
19. BRAGULLA H.H. and D.G. HOMBERGER, 2009. Structure and functions of keratin proteins in simple, stratified, keratinized, and cornified epithelia. *Journal of Anatomy* [online], vol. 214, no. 4, p. 516-59. Available from: doi: 10.1111/j.1469-7580.2009.01066.x.
20. BÖTTCHER R.T. and C. NIEHRS, 2005. Fibroblast growth factor signaling during early vertebrate development. *Endocrine Reviews* [online], vol. 26, no.1, p. 63-77. Available from: doi: 10.1210/er.2003-0040. PMID: 15689573.
21. BYRD A.L., BELKAID Y., SEGRE J.A, 2018. The human skin microbiome. *Nature Reviews Microbiology* [online], vol. 16, no. 3, p. 143-155. Available from: doi: 10.1038/nrmicro.2017.157.
22. BORCHIELLINI, P., RAMES, A., ROUBERTIE, F., L´HEREUX, N. and F. KAWECKI, 2023. Development and characterization of biological sutures made of cell-assembled extracellular matrix. *Biofabrication* [online], vol. 15, no. 4. Available from: doi: 10.1088/1758-5090/acf1cf.
23. BOXBERGER, M., CENIZOV., CASSIR N. *et al,* 2021. Challenges in exploring and manipulating the human skin microbiome. *Microbiome* [online], vol. 9, no. 125. Available from: https://doi.org/10.1186/s40168-021-01062-5
24. CABALLERO-FLORES, G., PICKARD, J.M., NÚNEZ, G, 2023. Microbiota-mediated colonization resistance: mechanisms and regulation. *Nature Reviews Microbiology* [online], vol. 21, no. 6, p. 347-360. Available from: doi: 10.1038/s41579-022-008
25. CAI, R., GIMENEZ-CAMINO, N., XIAO, M., BI S. and K. A. DIVITO, 2023. Technological advances in three-dimensional skin tissue engineering. *Reviews of Advanced Material Science* [online], vol. 62, no. 1, pp. 20220289. Available from: https://doi.org/10.1515/rams-2022-0289
26. CARLSON, M.W., ALT-HOLLAND, A., EEGLES, C., GARLICK, J.A., 2008. Three-dimensional tissue models of normal and diseased skin. *Current Protocols in Cell Biology* [online], ch. 19, u. 19.9. Available from: doi: 10.1002/0471143030.cb1909s41.

27. CHEN, Z., DU, CH., LIU, S., LIU, J., YANG, Y., DONG, L., ZHAO, W., HUANG, W., LEI, Y, 2024. Progress in biomaterials inspired by the extracellular matrix. *Giant* [online], vol. 19:100323. Available from: https://doi.org/10.1016/j.giant.2024.100323
28. CHEN, H., ZHAO, Q., ZHONG, Q. *et al,* 2022. Skin Microbiome, Metabolome and Skin Phenome, from the Perspectives of Skin as an Ecosystem. *Phenomics* [online], vol. 2, p. 363–382. Available from: doi:https://doi.org/10.1007/s43657-022-00073-y
29. CHOI, M. and CH. LEE, 2015. Immortalization of Primary Keratinocytes and Its Application to Skin Research. *Biomolecules and Therapeutics* [online], vol. 23, no. 5, p. 391–399. Available from: doi: 10.4062/biomolther.2015.038
30. CHOUDHURY, S. and A. DAS, 2021. Advances in the generation of three-dimensional skin equivalents: pre-clinical studies to clinical therapies. *Cytotherapy.* [online], vol. 23 no. 1, p. 1-9. Available from: doi: 10.1016/j.jcyt.2020.10.001.
31. COGEN, A.L., YAMASAKI, K., MUTO, J., SANCHEZ, K.M., CROTTY, A. L., TANIOS J., LAI, Y., KIM, J.E., NIZET, V., GALLO, R.L, 2010. Staphylococcus epidermidis antimicrobial delta-toxin (phenol-soluble modulin-gamma) cooperates with host antimicrobial peptides to kill group A Streptococcus. *PLoS One* [online] vol. 5. no.1. Available from: doi: 10.1371/journal.pone.0008557.
32. DRISKELL, R.R. and F.M. WATT, 2015. Understanding fibroblast heterogeneity in the skin. *Trends in Cell Biology* [online], vol. 25, no. 2, p. 92-9. Available from: doi: 10.1016/j.tcb.2014.10.001.
33. DZOBO, K.; DANDARA, C., 2023. The ExtracellularMatrix: Its Composition, Function, Remodeling, and Role in Tumorigenesis. *Biomimetics* [online], vol. 8, no. 146. Available from: https://doi.org/10.3390/biomimetics8020146
34. EBERLIN, S., da SILVA, M.S., FACCHINI, G., da SILVAi, G.H.; PINHERO, A.L., 2020 The ex vivo Skin Model as an Alternative Tool for the Efficacy and Safety Evaluation of Topical Products. *Alternatives to Laboratory Animals* [online], vol. 48, no. 1, p. 10-22. Available from: doi: 10.1177/0261192920914193
35. EDWARDS, C.A. and W. D. O'BRIEN, Jr., 1980. Modified assay for determination of hydroxyproline in a tissue hydrolyzate. *Clinica Chimica Acta* [online], vol.104, no. 2 p. 161-7. Available from: doi: 10.1016/0009-8981(80)90192-8.
36. EL GHALBZOURI, A., HENSBERGEN, P., GIBBS, S., KEMBENAAR, J., van der SCHORS R., PONEC, M., 2004 Fibroblasts facilitate re-epithelialization in wounded human skin equivalents. *Laboratory*

Investigation [online], vol. 84, no. 1, p. 102-12. Available from: doi: 10.1038/labinvest.3700014. PMID: 14631386.
37. EL GHALBZOURI, A., COMMANDEUR, S., RIETVELD, M.H., MULDER, A.A., WILLEMZE, R., 2009. Replacement of animal-derived collagen matrix by human fibroblast-derived dermal matrix for human skin equivalent products. *Biomaterials*. [online], vol. 30, no. 1, p. 71–8. Available from: doi: 10.1016/j.biomaterials.2008.09.002
38. EMMERT, H., RADEMACHER, F., GLÄSER, R., HARDER, J., 2020. Skin microbiota analysis in human 3D skin models-"Free your mice". *Experimental Dermatology* [online], vol. 29, no. 11, p. 1133-1139. Available from: doi: 10.1111/exd.14164.
39. FELL, B., 2016. Organotypic Human Skin Disease Models for the Assessment of Novel Therapeutic Approaches. Doctoral Thesis. Centre for Cutaneous and Cell Biology, The Blizard Institute. Barts and the London School of Medicine and Dentistry. Queen Mary University London.
40. FLOWERS, L., GRICE, E.A., 2020 The Skin Microbiota: Balancing Risk and Reward. *Cell Host Microbe.* [online], vol. 28, no.2, p. 190-200. Available from: doi: 10.1016/j.chom.2020.06.017.
41. FONSECA, V. C. and V.V.C BLANCHE., 2023. Primary Human Cell-Derived Extracellular Matrix from Decellularized Fibroblast Microtissues with Tissue-Dependent Composition and Microstructure. *bioRxiv*, [online], preprint August 17. Available from: doi: https://doi.org/10.1101/2023.08.15.553420.
42. FRANTZ, C., STEWART, K.M., WEAVER, V.M., 2010. The extracellular matrix at a glance. *Journal of Cell Science* [online], vol. 15, no. 123, p. 4195-200. Available from: doi: 10.1242/jcs.023820.
43. FUCHS, E. Scratching the surface of skin development. *Nature* 2007, vol. 445, no. 7130, p. 834-42. Available from: doi: 10.1038/nature05659.
44. GALVAN, A., PELLICCIARI, C. and L. CALDERAN, 2024. Recreating Human Skin In Vitro: Should the Microbiota Be Taken into Account? *International Journal of Molecular Sciences* [online], vol 25, no. 2, p. 1165. Available from: https://doi.org/10.3390/ijms25021165
45. GANGATIKAR, P., PAGUET-FIFIELD, S., LI, A., ROSSI, R., KAUR, P., 2007. Establishment of 3D organotypic cultures using human neonatal epidermal cells. *Nature Protocols*. [online], vol. 2, p. 178-186. Available from: doi: 10.1038/nprot.2006.448.
46. GAUVIN, R., PARENTEAU-BAREIL, R., LAROUCHE, D., MARCOUX, H., BISSON, F., BONNET, A., AUGER, F.A., BOLDUC, S., GERMAIN, L, 2011. Dynamic mechanical stimulations induce anisotropy and improve the tensile properties of engineered tissues

produced without exogenous scaffolding. *Acta Biomaterialia* [online], vol. 7, no. 9, p. 3294-301. Available from: doi: 10.1016/j.actbio.2011.05.034.
47. GARLICK, J. A., 2006. Engineering Skin to Study Human Disease – Tissue Models for Cancer Biology and Wound Repair. In: LEE, K., KAPLAN, D. Tissue Engineering II. Advances in Biochemical Engineering/Biotechnology. [online], Springer, Berlin, Heidelberg, p. 207-239. Available from: doi: https://doi.org/10.1007/b137206. eISBN: 978-3-540-36186-2
48. GELLANTLY, S.L. and R.E. HANCOCK, 2013. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. *Pathogens and Disease* [online], vol. 3, p. 159-73. Available from: doi: 10.1111/2049-632X.12033.
49. GEOGHEGAN, J.A., DUFRENE Y.F, 2018. Mechanomicrobiology: How Mechanical Forces Activate Staphylococcus aureus Adhesion. *Trends in Microbiology* [online] vol. 26, no. 8, p. 645-648. Available from: doi: 10.1016/j.tim.2018.05.004.
50. GILBERT, T.W., SELLARO, T.L., BADYLAK, S.F, 2016. Decellularization of tissues and organs. *Biomaterials* [online], vol. 27, no. 19, p. 3675-83. Available from: doi: 10.1016/j.biomaterials.2006.02.014.
51. GLATTHARDT, T., LIMA, R.D., de MATTOS, R.M., FERREIRA, R.B.R., 2024 Microbe Interactions within the Skin Microbiome. *Antibiotics (Basel)*. [online], vol. 13, iss.1:49. Available from: doi: 10.3390/antibiotics13010049.
52. GOERS, L., FREEMONT, P., POLIZZI, K.M., 2014. Co-culture systems and technologies: taking synthetic biology to the next level. *The Journal of the Royal Society Interface* [online], vol. 11, no. 96:20140065. Available from: doi: 10.1098/rsif.2014.0065.
53. GOLEBIOWSKA, A.A., INTRAVAIA, J.T., SATHE, V.M., KUMBAR, S.G., NUKAVARAPU, S. P., 2023. Decellularized extracellular matrix biomaterials for regenerative therapies: Advances, challenges and clinical prospects. Bioact Mater. [online], vol. 4, no. 32, p. 98-123. Avaialable from: doi: 10.1016/j.bioactmat.2023.09.017.
54. GRICE, E. A., SEGRE, J. A., 2011. The skin microbiome. *Nature Reviews Microbiology* [online], vol. 9, no. 4, p. 244–253. Available from: doi:10.1038/nrmicro2537
55. GRICE, E. A., KONG, H.H., RENAUD, G., YOUNG, A.C., BOFFARD, G.G., *et al.*, 2008. A diversity profile of the human skin microbiota. *Genome Research*. [online], vol. 18, no. 7, p. 1043–50. Available from: doi: 10.1101/gr.075549.107.

56. GRINNELL, F., 2008. Fibroblast mechanics in three-dimensional collagen matrices. *The Journal of Bodywork and Movement Therapies* [online], vol. 12, no. 3, p. 191-3. Available from: doi: 10.1016/j.jbmt.2008.03.005.
57. GRINNELL, F., 2003. Fibroblast biology in three-dimensional collagen matrices. *Trends in Cell Biology* [online], vol. 13, no. 5, p. 264-9. Available from: doi: 10.1016/s0962-8924(03)00057-6.
58. HAYDEN, P.J., HARBELL, J.W., 2021. Special review series on 3D organotypic culture models: Introduction and historical perspective. *In Vitro Cellular & Developmental Biology - Animal* [online], vol. 57, p. 95–103. Available from: https://doi.org/10.1007/s11626-020-00500-2
59. HOATH, S. B., MAURO, T. Fetal skin development. In: Eichenfeld, L, Frieden, I., Mathes, E. *et al.*. Neonatal and Infant Dermatology. 3rdeEdition, London, WB Saunders, 2014. [online]. eISBN: 9781455726394
60. HOFMANN, E., SCHWARZ, A., FINK, J., KAMOLZ, L-P., KOTZBECK, P., 2023. Modelling the Complexity of Human Skin In Vitro. *Biomedicines*. [online], vol. 11, no. 2, p. 794. Available from: https://doi.org/10.3390/biomedicines11030794
61. HU, M. S., BORELLI, M. R., HONG, W. X., MALHORTA, S., CHEUNG, A. T. M., RANSOM, R. C., RENNERT, R.C., MORRISON, S. D., LORENZ, H. P. and M. T. LONGAKERA., 2018. Embryonic skin development and repair. *Organogenesis*, [online], vol. 14, no. 1, p. 46–63. Available from: doi: 10.1080/15476278.2017.1421882
62. HUSSEY, G.S., DZIKI, J. L. and S.F. BADYLAK., 2018 Extracellular matrix-based materials for regenerative medicine. *Nature Reviews Materials* [online], vol. 3, p. 159–173. Available from: https://doi.org/10.1038/s41578-018-0023-x
63. HWANG, B.K., LEE, S., MYOUNG, J., HWANG, S.J., LIM, J.M., JEONG, E.T., PARK, S.G., YOUN, S.H., 2021. Effect of the skincare product on facial skin microbial structure and biophysical parameters: A pilot study. *Microbiology open* [online], vol. 10, no. 5, p. 1236. Available from: doi: 10.1002/mbo3.1236.
64. ICHIKAWA, J.K., NORRIS, A., BANGERA, M.G., GEISS, G.K., van 't WOUT, A.B., BUMGARNER, R.E., LORY, S., 2000. Interaction of pseudomonas aeruginosa with epithelial cells: identification of differentially regulated genes by expression microarray analysis of human cDNAs. *Proceedings of the National Academy of Sciences* [online], vol. 97, no. 17, p. 9659-64. Available from: doi: 10.1073/pnas.160140297.
65. IDREES, A., SCHMITZ, I., ZOSO, A., GRUHN, D., PACHARRA, S., SHAH, S., CIARDELLI, G., VIEBAHN, R., CHIONO, V., SALBER, J., 2021. Fundamental in vitro 3D skin equivalnet tool development for assessing biological safety and biokompatibility – towards alternativ efor

animal experiments. *4open* [online], vol. 4, no. 1, p. 21 Available from: https://doi.org/10.1051/fopen/2021001
66. IMRAN, M., MOYLE, P. M., KAMATO, D. and Y. MOHAMMED., 2024. Advances in, and prospects of, 3D preclinical models for skin drug Discovery. *Drug Discovery Today* [online], vol. 29, iss. 12. p. 104208. Available from: https://doi.org/10.1016/j.drudis.2024.104208
67. In Vitro EpiDerm™ Skin Irritation Test (EPI-200-SIT). For use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm™ (EPI-200-SIT). MatTek [online] [cit. 2024-03-13]. Available from: https://www.mattek.com/wp-content/uploads/EPI-200-SIT-Skin-Irritation-MK-24-007-0023.pdf
68. IPPONJIMA, S., UMINO, Y., NAGAYAMA, M., and M. DENDA 2020. Live imaging of alterations in cellular morphology and organelles during cornification using an epidermal equivalent model. *Scientific Reports* [online], vol. 10, a.n. 5515. Available from: doi: 10.1038/s41598-020-62240-3¨
69. IWAMIYA, T., MATSUURA, K., MASUDA, S., SHIMIZUh, T., OKANO, T., 2016. Cardiac fibroblast-derived VCAM-1 enhances cardiomyocyte proliferation for fabrication of bioengineered cardiac tissue. *Regenerative Therapy* [online], vol. 1, no. 4, p. 92-102. Available from: doi: 10.1016/j.reth.2016.01.005. JAMES, W. D., BERGER, T. G., ELSTON, D. M., 2006. Andrews' Diseases of the Skin. *Clinical Dermatology* [online], 10th ed. Saunders/Elsevier Inc., Philadelphia, p. 231-250.
70. JANG, H.J., LEE, J.B. and J. K. YOON., 2023. Advanced In Vitro Three-Dimensional Skin Models of Atopic Dermatitis. *Tissue Engineering and Regenerative Medicine* [online], vol. 20, no. 4, p. 539–552. Available from: https://doi.org/10.1007/s13770-023-00532-1
71. JÄRVELÄINEN, H., SAINIO, A., KOULU, M., WIGHT, T.N., PENTTINEN, R., 2009. Extracellular matrix molecules: potential targets in pharmacotherapy. *Pharmacological Reviews* [online], vol. 61, no. 2, p. 198-223. Available from: doi: 10.1124/pr.109.001289.
72. JOE, P., WOODLEY, D. W., LAMBERT, and I. O. ASENCIO, 2022. Understanding Fibroblast Behavior in 3D Biomaterials. *Tissue Engineering Part B: Reviews* [online], vol. 28, no. 3, p. 569-578. Available from: http://doi.org/10.1089/ten.teb.2021.0010
73. JOURDAN-LESAUX, C., ZHANG, J., LINDSEY, M.L., 2010. Extracellular matrix roles during cardiac repair. *Life Science* [online], vol. 87, no. 13-14, p. 391-400. Available from: doi: 10.1016/j.lfs.2010.07.010.
74. JOSHI, A.A., VOCANSON, M., NICOLAS, J-F., WOLF, P. and V. PATRA, 2023. Microbial derived antimicrobial peptides as potential

therapeutics in atopic dermatitis. *Frontiers in Immunology* [online], vol. 14:1125635. Available from: doi: 10.3389/fimmu.2023.112563533-7.

75. JORDANA-LUCH, E., GARCIA, V., KINGDON, A.D.H., SINGH, N., ALEXANDER, C., WILLIAMS, P. and K. R. HARDIE, 2020.A Simple Polymicrobial Biofilm Keratinocyte Colonization Model for Exploring Interactions Between Commensals, Pathogens and Antimicrobials. *Frontiers in Microbiology* [online], vol. 11, p: 291. Available from: doi: 10.3389/fmicb.2020.00291
76. KARAMANOS, N.K., THEOCHARIS, A.D., PIPERIGKOU, Z., MANOU, D., PASSI, A., SKANDALIS, S.S., VYNIOS, D.H., ORIAN-ROUSSEAU, V., Ricard-BLUM, S., SCHMELZER, C.E.H., DUCA, L., DURBEEJ, M., AFRATIS, N.A., TROEBERG, L., FRANCHI, M., MASOLA, V. and M. ONISTO 2021. A guide to the composition and functions of the extracellular matrix. *FEBS J*, [online], vol. 288, no. 24, p. 6850-6912. Available from: https://doi.org/10.1111/febs.15776
77. KAWECKI, F., GLUAIS, M., CLAVEROL, S., DUSSERRE, N, McALLISTER, T., L'HEUREUX, N., 2022. Inter-donor variability of extracellular matrix production in long-term cultures of human fibroblasts. *Biomaterial Science* [online], vol. 10, no. 14, p. 3935-3950. Available from: doi: 10.1039/d1bm01933c.
78. KIM, R., 2023. Advanced Organotypic In Vitro Model Systems for Host–Microbial Coculture. *BioChip Journal* [online], vol. 17, p. 147–173. Available from: https://doi.org/10.1007/s13206-023-00103-5
79. KIM, S., WONG, P. and P COULOMBE, 2006. A keratin cytoskeletal protein regulates protein synthesis and epithelial cell growth. *Nature* [online], vol. 441, no. 7091, p. 362–365. Available from: https://doi.org/10.1038/nature04659
80. KIOUSI, D.E., PANOPOULOU, M., PAPPA, A. and A. GALANIS, 2024. Lactobacilli-host interactions inhibit Staphylococcus aureus and Escherichia coli-induced cell death and invasion in a cellular model of infection. Fronties Microbiology [online]. Available from: doi: 10.3389/fmicb.2024.1501119.
81. KOLARSICK, P., KOLARSICK, A. and C. GOODWIN, 2011. Anatomy and Physiology of the Skin. Journal of the Dermatology Nurses' Association, [online], vol. 3, no. 4, p. 203–213. Available from: doi:10.1097/JDN.0b013e3182274a98.
82. KOSTER, M.I. and D. R. ROOP, 2007. Mechanisms regulating epithelial stratification. *Annual Review of Cell and Developmental Biology* [online], vol. 23, p. 93-113. Available from: doi: 10.1146/annurev.cellbio.23.090506.123357.

83. KUMAR, P., SATYAM, A., FAN, X., COLLIN, E., ROCHEV, Y., RODRIGUEZ, B.J., GORELOV, A., DILLON, S., JOSHI, L., RAGHUNATH, M., PANDIT, A., ZEUGOLIS, D.I., 2015. Macromolecularly crowded in vitro microenvironments accelerate the production of extracellular matrix-rich supramolecular assemblies. *Scientific Reports* [online], vol. 4, no. 5, p. 8729. Available form: doi: 10.1038/srep08729.
84. LAI, Y., Di NARDO, A., NAKATSUJI, T. *et al.*, 2009. Commensal bacteria regulate Toll-like receptor 3–dependent inflammation after skin injury. *Nature Medicine* [online], vol. 15, p. 1377–1382. Available from: ttps://doi.org/10.1038/nm.2062
85. LAI-CHEONG, J. E., and J. A. MCGRATH, 2017. Structure and function of skin, hair, and nails. *Medicine* [online], vol. 45, p. 347-351. Available from: doi: 10.1016/j.mpmed.2017.03.004
86. LAURENT, S., DENESVRE, C., 2021. 3D skin models in domestic animals. Veterinary Research. *BioMed Central* [online], vol. 52, no.1, p. 15. Available from: doi:10.1186/s13567-020-00888-5.
87. LAWLOR, K.T. and P. KAUR, 2015. Dermal Contributions to Human Interfollicular Epidermal Architecture and Self-Renewal. *International Journal of Molecular Sciences* [online], vol. 16, no. 12, p. 28098-107. Available from: doi: 10.3390/ijms161226078
88. LEE, D.Y., YANG, J.M., Park, K.H., 2007. A dermal equivalent developed from fibroblast culture alone: effect of EGF and insulin. *Wound Repair and Regeneration* [online], vol. 15, no.6, p. 936-9. Available from: doi: 10.1111/j.1524-475X.2007.00310.x.
89. LEIGHT, J.L., DRAIN, A.P. and V.M. WEAVER, 2017. Extracellular Matrix Remodeling and Stiffening Modulate Tumor Phenotype and Treatment Response. *Annual Review of Cancer Biology* [online], vol. 1, p. 313–334. Available from: doi: https://doi.org/10.1146/annurev-cancerbio-050216-034431
90. LIU, C., PEI, M., LI, Q., ZHANG, Y., 2022. Decellularized extracellular matrix mediates tissue construction and regeneration. *Front Med.* [online], vol. 16, no. 1, p. 56-82. Availabl from: doi: 10.1007/s11684-021-0900-3.
91. LIU, R., MENG, X., YU, X., WANG, G., DONG, Z., ZHOU, Z., QI, M., YU, X., JI, T., WANG F., 2022. From 2D to 3D Co-Culture Systems: A Review of Co-Culture Models to Study the Neural Cells Interaction. *International Journal of Molecular Sciences* [online], vol. 23, no. 21:13116. doi: 10.3390/ijms232113116.
92. LIU, S., ZHANG, H. D. E., 2013. Epidermal development in mammals: key regulators, signals from beneath, and stem cells. *International Journal*

of Molecular Sciences [online], vol. 14, no. 6, p. 10869-95. Available from: doi: 10.3390/ijms140610869.
93. LIZARDO, M., MAGALHAES, R.M., TAVARIA, F.K., 2022. Probiotic Adhesion to Skin Keratinocytes and Underlying Mechanisms. *Biology* [online], vol.11, no. 9, p. 1372. Available from: doi: 10.3390/biology11091372.
94. LOH, Q.L., CHOONG, C., 2013. Three-dimensional scaffolds for tissue engineering applications: role of porosity and pore size. *Tissue Engineering Part B: Reviews* [online], vol. 19, no. 6, p. 485-502. Available from: doi: 10.1089/ten.TEB.2012.0437.
95. MANGAN, L., LABRUNIE, G., MARAIS, S., REY, S., DUSSERRE, N., BONNEU, M., LACOMME, S., GONTIER, E., L'HEUREUX, N., 2018. Characterization of a Cell-Assembled extracellular Matrix and the effect of the devitalization process. *Acta Biomaterialia* [online], vol. 82, p. 56-67. Available from: doi: 10.1016/j.actbio.2018.10.006.
96. MANGAN, L., LABRUINE, G., FÉNELONé, M., DUSSERRE, N., FOULC, M.P., LAFOURCADE, M., SVAHN, I., GONTIER, E., VÉLEZ, V.J.H., McALLISTER, T.N, L' HEUREUX, N., 2020. Human textiles: A cell-synthesized yarn as a truly "bio" material for tissue engineering applications. *Acta Biomaterialia* [online], vol. 5, no. 105, p. 111-120. Available from: doi: 10.1016/j.actbio.2020.01.037.
97. MARANGIO, A., BICCARI, A., D'ANGELO, E., SENSI, F., SPOLVERATO, G., PUCCIARELLI, S., AGOSTINI, M., 2022. The Study of the Extracellular Matrix in Chronic Inflammation: A Way to Prevent Cancer Initiation? *Cancers* [online], vol. 14, no. 23, p. 5903. Available from: doi: 10.3390/cancers14235903.
98. MEDUGORAC, I., 1980. Collagen content in different areas of normal and hypertrophied rat myocardium, *Cardiovascular Research* [online], vol. 14, i. 9, p. 551–554. Available from: https://doi.org/10.1093/cvr/14.9.551
99. MEMPEL, M., SCHMIDT, T., WEIDINGER, S., SCHNOPP C., FOSTER, T., RING J., ABECK D., 1998. Role of Staphylococcus aureus surface-associated proteins in the attachment to cultured HaCaT keratinocytes in a new adhesion assay. *Journal of Investigative Dermatology* [online], vol. 111, iss. 3, p. 452-6. Available from: doi: 10.1046/j.1523-1747.1998.00293.x.
100. MIERKE, C.T., 2024. Extracellular Matrix Cues Regulate Mechanosensing and Mechanotransduction of Cancer Cells. *Cells* [online], vol. 13, no. 1:96. Available from: doi: 10.3390/cells13010096.

101. MIKKOLA, M.L., 2007. Genetic basis of skin appendage development. *Seminars in Cell & Developmental Biology* [online], vol. 18, no. 2, p. 225-36. Available from: doi 10.1016/j.semcdb.2007.01.007. PMID: 17317239.
102. MOHAMMEDSAEED, W., McBAIN, A.J., CRUICKSHANK, S.M., O'NEIIL C.A., 2014. Lactobacillus rhamnosus GG inhibits the toxic effects of Staphylococcus aureus on epidermal keratinocytes. *Applied and Environmental Microbiology* [online], vol. 18, p. 5773-81. Available from: doi: 10.1128/AEM.00861-14.
103. MONTAGNA W., 1974. The Structure and Function of Skin. 3rd Edition. ©Academic Press, pages: 448. ISBN: 9780323138697.
104. MOSMANN T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* [online], vol. 65, iss. 1–2, p. 55-63. Available from: https://doi.org/10.1016/0022-1759(83)90303-4.
105. MOUW, J.K., OU, G., WEAVER, V.M., 2014. Extracellular matrix assembly: a multiscale deconstruction. *Nature Reviews Molecular Cell Biology* [online], vol. 15, no. 12, p. 771-85. Available from: doi: 10.1038/nrm3902.
106. MURPHY, G., 1997. Histology of the Skin. In: ELDER D. E., *et al.* Lever's Histopathology of the Skin. Lippincott-Raven, Philadelphia. Chapter 3, pages: 5-50. ISBN: 0-397-51500-6.
107. MUKHERJEE, P., ROY, S., GHOSH, D. *et al.*, 2022. Role of animal models in biomedical research: a review. *Laboratory Animal Research* [online], vol.38, no. 1, p. 18. Available form: https://doi.org/10.1186/s42826-022-00128-1
108. MUNCIE, J.M., WEAVER, V.M., 2018. The Physical and Biochemical Properties of the Extracellular Matrix Regulate Cell Fate. *Current Topics in Developmental Biology* [online], vol. 130, p. 1-37. Available from: doi: 10.1016/bs.ctdb.2018.02.002.
109. NAKAZAWA, K., KALASSY, M., SAHUC, F., COLLOMBEL, C., DAMOUR, O., 1998. Pigmented human skin equivalent--as a model of the mechanisms of control of cell-cell and cell-matrix interactions. *Medical and Biological Engineering and Computing* [online], vol. 36, no. 6, p. 813-20. Available from: doi: 10.1007/BF02518888.
110. NEIL, J. E., BROWN, M. B. and A. C. WILLIAMS 2020. Human skin explant model for the investigation of topical therapeutics. *Scientific Reports* [online], vol. 10, no. 1, p. 21192. Available from: DOI: 10.1038/s41598-020-78292-4
111. NEISHABOURI, A., SOLTANI, K.A., DAGHIGH, F., KAJBAFZADEH, A-M. and M. MAJIDI ZOLBIN, 2022. Decellularization in Tissue Engineering and Regenerative Medicine: Evaluation, Modification, and

Application Methods. *Frontiers in Bioengineering and Biotechnology* [online], vol. 10, p. 805299. Avaialble from: doi: 10.3389/fbioe.2022.805299
112. NESS, M.J., DAVIS, D.M., CAREY W.A., 2013. Neonatal skin care: a concise review. *International Journal of Dermatology* [online], vol. 52, no. 1, p. 14-22. Available from: doi: 10.1111/j.1365-4632.2012.05687.x.
113. NETZLAFF, F., LEHR, C.M., WERTZ, P.W., SCHAEFER. U.F., 2005. The human epidermis models EpiSkin, SkinEthic, and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. *The European Journal of Pharmaceutics and Biopharmaceutics* [online], vol. 60, no. 2, p. 167-78. Available from: doi: 10.1016/j.ejpb.2005.03.004. PMID: 15913972.
114. NG dART RT Kit [online] [cit. 2024-05-26]. Available from: https://eurx.com.pl/docs/specs/en/e0801.pdf
115. NIEHUES, H., BOUWSTRA, J.A., GHALBZOURI, A.E., BRANDER, J.M., ZEEUWEN, P.J.L., van den BOGAARD, E.H., 2018: 3D skin models for 3R research: The potential of 3D reconstructed skin models to study skin barrier function. *Experimental Dermatology* [online], vol. 27, no. 5, p. 501-511. Available from: doi: 10.1111/xd.13531
116. NICOLAS, J., MAGLI, S., RABBACHIN, L., SAMPAOLESI, S., NICOTRA, F., RUSSO, L., 2020. 3D Extracellular Matrix Mimics: Fundamental Concepts and Role of Materials Chemistry to Influence Stem Cell Fate. *Biomacromolecules* [online], vol. 21, no. 6, p. 1968-1994. Available from: doi: 10.1021/acs.biomac.0c00045.
117. OECD Test Guideline 439 In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method [online] [cited 2025-04-29]. Available from: https://www.oecd.org/content/dam/oecd/en/publications/reports/2021/06/test-no-439-in-vitro-skin-irritation-reconstructed-human-epidermis-test-method_g1g59b2f/9789264242845-en.pdf
118. OLIVEIRA, H., MÉDINA, C., LABRUINEa G., DUSSERRE N., CATROS, S., MANGAN, L., HANDSCHIN, C., STACHOWICZ, M.L., FRICAIN, J.C., L'HEUREUX, N., 2021. Cell-assembled extracellular matrix (CAM): a human biopaper for the biofabrication of pre-vascularized tissues able to connect to the host circulationin vivo. *Biofabrication* [online], vol. 14, no. 1 Available from: doi: 10.1088/1758-5090/ac2f81.
119. OTTO M., 2014. Staphylococcus aureus toxins. *Current Opinion in Microbiology* [online], vol. 17, p. 32-7. Available from: doi: 10.1016/j.mib.2013.11.004.
120. PAMPALONI, F., STELZER E. H., and A. MASOTTI, 2009. Three-dimensional tissue models for drug discovery and toxicology. *Recent*

Patents on Biotechnology [online], vo. 3, no. 2, p. 103-17. Available from: doi: 10.2174/187220809788700201. PMID: 19519566.

121. PAPPINEN, S., PRYAZHNIKOV, E., KHIROUG, L., ERICSON, M.B., YLIPERTTULA, M., URTTI, A., 2012. Organotypic cell cultures and two-photon imaging: tools for in vitro and in vivo assessment of percutaneous drug delivery and skin toxicity. *The Journal of Controlled Release* [online], vol. 161, no. 2, p. 656-67. Available from: doi: 10.1016/j.jconrel.2012.03.005.
122. PATRA, V., GALLAIS SÉRÉZAL, I., WOLF, P., 2020. Potential of Skin Microbiome, Pro- and/or Pre-Biotics to Affect Local Cutaneous Responses to UV Exposure. *Nutrients* [online], vol. 12, no. 6, p. 1795. Available from: doi: 10.3390/nu12061795.
123. PISPA, J., THESLEFF, I., 2003. Mechanisms of ectodermal organogenesis. *Developmental Biology* [online], vol. 262, no. 2, p. 195-205. Available from: doi: 10.1016/s0012-1606(03)00325-7.
124. PLIKUS, M.V., WANG, X., SINHA, S., FORTE, E., THOMPSON, S.M., HERZOG, E.L., DRISKELL, R.R., ROSENTHAL, N., BIERNASKIE, J., HORSLEY, V., 2021. Fibroblasts: Origins, definitions, and functions in health and disease. *Cell Press* [online], vol. 184, no. 15, p. 3852-3872. Available from: doi: 10.1016/j.cell.2021.06.024.
125. POPOV, L., KOVALSKI, J., GRANDI, G., BAGNOLI, F., AMIEVA, M.R., 2014. Three-Dimensional Human Skin Models to Understand Staphylococcus aureus Skin Colonization and Infection. *Frontiers in Immunology* [online], vol. 6, no. 5, p. 41. Available from: doi: 10.3389/fimmu.2014.00041.
126. Protocol Guide: MTT Assay for Cell Viability and Proliferation. [online] [cit. 2024-03-11]. Available from: https://www.sigmaaldrich.com/technical-documents/protocols/biology/roche/cell-proliferation-kit-i-mtt.html
127. PEDROSA, T.D.N., CATARINO, C.M., PENNACCHI, P.C., ASSIS, S.R., GIMENES, F., CONSILARO, M.E.L., BARROS, S.B.M., MARIA-ENGLER, S.S., 2017. A new reconstructed human epidermis for in vitro skin irritation testing. *Toxicology in Vitro* [online], vol. 42, p. 31-37. Available from: doi: 10.1016/j.tiv.2017.03.010.
128. POTART, D., GLUAIS, M., GAUBERT, A., DA SILVA, N., HOURQUES, M., SARRAZIN, M., IZOTTE, J., CHARROT, L. M., L´HEREUX, N., 2023. The cell-assembled extracellular matrix: A focus on the storage stability and terminal sterilization of this human "bio" materiál. *Acta Biomaterialia* [online], vol. 66, p. 133-146. Available from: https://doi.org/10.1016/j.actbio.2023.05.002.

129. PRINCE, T., McBAIN, A.J., O'NEILL, C.A., 2012. Lactobacillus reuteri Protects Epidermal Keratinocytes from Staphylococcus aureus-Induced Cell Death by Competitive Exclusion. *Applied and Environmental Microbiology* [online], vol. 78, no. 15. Available from: https://doi.org/10.1128/AEM.00595-12
130. RADEMACHER, F., SIMANSKI, M., GLÄSER, R., HARDER, J., 2018. Skin microbiota and human 3D skin models. *Experimental Dermatology* [online], vol. 27, no. 5, p. 489-494. Available from: doi: 10.1111/exd.13517.
131. RANDALL, M. J., ASTRID, J., and M. RIMANN., 2018. Advances in the Biofabrication of 3D Skin in Vitro: Healthy and Pathological Models. *Frontiers in Bioengineering and Biotechnology* [online], vol. 6, p. 412822. Available from: https://doi.org/10.3389/fbioe.2018.00154.
132. REDDY, G.K., ENWEMEKA, C.S., 1996. A simplified method for the analysis of hydroxyproline in biological tissues. *Clinical Biochemistry* [online], vol. 29, no. 3, p. 225-9. Available from: doi: 10.1016/0009-9120(96)00003-6. PMID: 8740508.
133. ROGER, M., FULLARD, N., COSTELLO, L., BRADBURY, S., MARKIEWICZ, E., O'REILLY, S., DARLING, N., RITCHIE, P., MÄÄTTÄ, A. KARAKESISOGLOU, I., 2019. Bioengineering the microanatomy of human skin. *Journal of Anatomy* [online] vol. 234, no. 4, p. 438-455. Available from: doi: 10.1111/joa.12942.
134. ROTHER, J., RICHTER, C., TURCO, L., KNOCH, F., MEY, I., LUTHER, S., JANSHOFF, A., BODENSCHATZ, E., TARANTOLA, M., 2015. Crosstalk of cardiomyocytes and fibroblasts in co-cultures. *Open Biology Journal* [online], vol. 5, no. 6:150038. doi: 10.1098/rsob.150038.
135. ROSSI, A., APPELT-MENZEL, A., KURDYN, S., WALLES, H., GROEBER, F., 2015. Generation of a three-dimensional full thickness skin equivalent and automated wounding. *JoVE* [online], vol. 26, no. 96, p. 52576. Available from: doi: 10.3791/52576.
136. ROZARIO, T., DeSIMONE, D.W., 2010. The extracellular matrix in development and morphogenesis: a dynamic view. *Developmental Biology* [online], vol. 341, no. 1, p 126-40. Available from: doi: 10.1016/j.ydbio.2009.10.026.
137. SACHDEVA, C., SATYAMOORTHY, K., MURALI, T.S., 2022. Microbial Interplay in Skin and Chronic Wounds. *Current Clinical Microbiology Reports* [online], vol. 9, p. 21–31. Available from: https://doi.org/10.1007/s40588-022-00180-4
138. SAJI, J. J., TEBOGO, M. S., and M. NTWASA., 2019. Two-Dimensional (2D) and Three-Dimensional (3D) Cell Culturing in Drug Discovery. *Cell Culture* [online], Available from: doi:10.5772/intechopen.81552

139. SAVOJI, H., GODAU, B., HASSANI, M.S. and M. AKBARI., 2018. Skin Tissue Substitutes and Biomaterial Risk Assessment and Testing. *Frontiers in Bioengineering and Biotechnology* [online], vol. 6, p. 86. Available from: doi: 10.3389/fbioe.2018.00086
140. SFRISO, R., EGERT, M., GEMPELER, M., VOEGELI, R., CAMPECHE, R., 2020. Revealing the secret life of skin - with the microbiome you never walk alone. *International Journal of Cosmetic Science* [online], vol 42, no. 2, p.116–26. Available from: doi: 10.1111/ics.12594.
141. SCHMIDT, F.F., NOWAKOWSKI, S. and P.J. KLUGER, 2020. Improvement of a Three-Layered in vitro Skin Model for Topical Application of Irritating Substances. *Frontiers in Bioengineering and Biotechnology* [online], vol. 8, p. 388. Available from: doi: 10.3389/fbioe.2020.00388
142. SCHMITT, A., CSIKI, R., TRON, A., SALDAMLI, B., TÜBEL, J., SIEBENLIST F.K., BALMAYOR, E., and R. BURGKART 2017. Optimized protocol for whole organ decellularization. *European Journal of Medical Research* [online], vol. 22, no. 31. Available from: https://doi.org/10.1186/s40001-017-0272-y.
143. SCHARSCHMIDT, T.C., FISCHBACH, M.A., 2013. What lives on our skin: Ecology, genomics and therapeutic opportunities of the skin microbiome. *Drug Discovery Today: Disease Mechanisms* [online], vol. 10, no. 3-4, p. e83-e89. Available from: doi: 10.1016/j.ddmec.2012.12.003.
144. SHOULDERS, M.D., RAINES, R.T., 2009. Collagen structure and stability. *Annual Review of Biochemistry* [online], vol. 78, p. 929-58. Available from: doi: 10.1146/annurev.biochem.77.032207.120833.
145. SORRELL, J.M., CAPLAN, A.I., 2009. Fibroblasts-a diverse population at the center of it all. *International Review of Cell and Molecular Biology* [online], vol. 276, p. 161-214. Available from: doi: 10.1016/S1937-6448(09)76004-6.
146. SOUREN, J.M., PONEC, M., van WIJK, R., 1989. Contraction of collagen by human fibroblasts and keratinocytes. *In Vitro Cellular & Developmental Biology – Animal* [online], vol. 25, no. 11, p. 1039-45. Available from: doi: 10.1007/BF02624138.
147. STARK, H.J., SZABOWSKI, A., FUSENIG, N.E.; MASS-SZABOWSKI, N., 2004. Organotypic co-cultures as skin equivalents: A complex and sophisticated in vitro system. *Biological Procedures Online*. [online], vol. 6, p. 55-60. Available from: doi: 10.1251/bpo72.
148. STERN, C.D., 2005. Neural induction: old problem, new findings, yet more questions. *Development.* [online], vol. 132, no. 9, p. 2007-21. Available from: doi: 10.1242/dev.01794.

149. STROBER, W., 2015. Trypan Blue Exclusion Test of Cell Viability. *Current Protocols in Immunology* [online]. Available from: doi: 10.1002/0471142735.ima03bs111.
150. SUHAERI, M., SUBBIAH, R., KIM, S.H., KIM, C,H,, OH, S.J., KIM, S.H., PARK, K., 2017. Novel Platform of Cardiomyocyte Culture and Coculture via Fibroblast-Derived Matrix-Coupled Aligned Electrospun Nanofiber. *ACS Applied Materials & Interfaces* [online], vol. 9, no. 1, p. 224-235. doi: 10.1021/acsami.6b14020.
151. SUHAERI, M., SUBBIAH, R., VAN, S.Y., DU, P., KIM, I.G., LEE, K., PARK, K., 2015. Cardiomyoblast (H9c2) differentiation on tunable extracellular matrix microenvironment. *Tissue Engineering Part A* [online], vol. 21, p. 11-12:1940-51. Available from: doi: 10.1089/ten.TEA.2014.0591.
152. SUHONEN, T.M., PASONENS-SEPPANEN, S., KIRJAVAINEN, M., TAMMI, M., TAMMI, R., and A. URTTI, 2003. Epidermal cell culture model derived from rat keratinocytes with permeability characteristics comparable to human cadaver skin. *Eur J Pharm Sci* [online], vol. 20, no. 1, p. 107-113. Available from: doi: 10.1016/s0928-0987(03)00176-3.
153. SUSIC, D., FROHLICH, E.D., 2011: Increased collagen, per se, may not affect left ventricular function in spontaneously hypertensive rats. *Ochsner Journal* [online], vol. 11, no. 3, p. 241-5. PMID: 21960757; PMCID: PMC3179202.
154. SYLVESTER P.W., 2011. Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. *Methods in Molecular Biology* [online], vol. 716, p. 157-168. Available from: doi: 10.1007/978-1-61779-012-6_9.
155. Total RNA Mini Manual. [online] [cit. 2024-05-26]. Available from: https://www.aabiot.com/en/download?code=f4db219a4dcb85f5df40ed091532f7956cde12d3
156. TORRES, Y., GLUAIS, M., Da SILVA, N., REY, S., GRÉMARE, A., MANGAN, L., KAWECKI F., L'HEUREUX N., 2021. Cell-assembled extracellular matrix (CAM) sheet production: Translation from using human to large animal cells. *Journal of Tissue Engineering and Regenerative Medicine* [online], vol. 12: 2041731420978327. Available from: doi: 10.1177/2041731420978327.
157. van BELKUM, A., LISOTTO, P., PIROVANO, W., MONGIAT, S., ZORGANI, A., GEMPELER, M., BONGONI, R. and E. KLAASSENS 2023. Being friendly to the skin microbiome: Experimental assessment. *Frontiers in Microbiomes* [online], vol. 1, p. 1077151. Available from: doi: 10.3389/frmbi.2022.1077151

158. VIS, M.A.M., ITO, K., HOFMANN, S., 2020. Impact of Culture Medium on Cellular Interactions in in vitro Co-culture Systems. *Frontiers in Bioengineering and Biotechnology* [online], vol. 4, no. 8:911. Available from: doi: 10.3389/fbioe.2020.00911.
159. WALTERS, K. W., and M. S. ROBERTS. Skin Morphology, Development and Physiology. In: BENSON, H.A.E., BENSON, H., ROBERTS, M. S., LEITE-SILVA V. R. and K. WALTERS, 2019. Cosmetic Formulation. Principles and Practice. [online] 1st Edition. 514 pages Boca Raton. Available from: doi: https://doi.org/10.1201/9780429190674. eISBN:9780429190674.
160. WASAKATSUKI, T., KOLODNEY, M.S. ZAHALAK, G.I. and E.L. ELSON, 2000. Cell Mechanics Studied by a Reconstituted Model Tissue. *Biophysical Journal* [online], vol. 79, no. 5, p. 2353–2368. Available from: doi: 10.1016/S0006-3495(00)76481-2
161. WATT, F.M., FUJIWARA, H., 2011. Cell-extracellular matrix interactions in normal and diseased skin. *Cold Spring Harbor Perspectives in Biology* [online], vol. 3, no. 4, p. a005124. Available from: doi: 10.1101/cshperspect.a005124.
162. WONG, T., McGRATH, J. A., and H. NAVSARIA, 2017. The role of fibroblasts in tissue engineering and regeneration. *British Journal of Dermatology* [online], vol. 156, no. 6, p. 1149–1155. Available from: doi:10.1111/j.1365-2133.2007.07914.x
163. WONG, C.W., LeGRAND, C.F., KINNEAR, B.F., 2019. *In Vitro* Expansion of Keratinocytes on Human Dermal Fibroblast-Derived Matrix Retains Their Stem-Like Characteristics. *Scientific Reports* [online], vol. 9, no. 18561 Available from: https://doi.org/10.1038/s41598-019-54793-9
164. WU, M-Y. and X. YAO, 2023. Skin Microbiota and the Skin Barrier. *International Journal of Dermatology and Venereology* [online], vol. 7, no. 1, p. 18-26. Available from: doi: 10.1097/JD9.0000000000000334
165. XING, H., LEE, H., LUO, L., KYRIAKIDES, T.R., 2020. Extracellular matrix-derived biomaterials in engineering cell function. *Biotechnology Advances* [online], vol. 42, p. 107421. Available from: doi: 10.1016/j.biotechadv.2019.107421.
166. YADAY, N., PARVEEN, S., CHAKRAVATY, S. and M. BANERJEE. Skin Anatomy and Morphology. In: YADAY, N., PARVEEN, S., CHAKRAVATY, S. and M. BANERJEE, 2019. Skin Aging & Cancer. Ambient UV-R Exposure. Springer Singapore. [online]. ISBN: 978-981-13-2541-0. Available from: doi: https://doi.org/10.1007/978-981-13-2541-0

167. YANG, J., DANG, H., XU, Y, 2022. Recent advancement of decellularization extracellular matrix for tissue engineering and biomedical application. *Artif Organs*. [online], vol. 46, no. 4, p. 549-567. Available from: doi: 10.1111/aor.14126.
168. YANG, S., HU, H., KUNG, H., ZOU, R., DAI, Y., HU, Y., WANG, T., LV, T., YU, J., LI F, 2023. Organoids: The current status and biomedical applications. *MedComm (2020),* [online], vol. 4, no. 3, p. e274. Available from: doi: 10.1002/mco2.274.
169. YANG, Y., QU, L., MIJAKOVICi, I. *et al.*, 2022. Advances in the human skin microbiota and its roles in cutaneous diseases. *Microbial Cell Factories* [online], vol. 21, no. 176. Available from: https://doi.org/10.1186/s12934-022-01901-6
170. YAO, Q., ZHENG, Y.W., LAN, Q.H., KOU, L., XU, H.L., ZHAO Y.Z., 2019. Recent development and biomedical applications of decellularized extracellular matrix biomaterials. *Materials Science & Engineering C-Materials for Biological Applications* [online], vol. 104 p. 109942. Available from: doi: 10.1016/j.msec.2019.109942.
171. ZHANG, Z., MICHNIAK-KOHN, B.B., 2012. Tissue-engineered human skin equivalents. *Pharmaceutics.* [online], vol. 4, no. 1, p. 26-41. Available from: doi: 10.3390/pharmaceutics4010026.
172. ZHANG, P., SU, J., MENDE, U., 2012. Cross talk between cardiac myocytes and fibroblasts: from multiscale investigative approaches to mechanisms and functional consequences. *American Journal of Physiology-Heart and Circulatory Physiology* [online], vol. 303, no. 12, p. H1385-96. Available from: doi: 10.1152/ajpheart.01167.2011.

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ALI	Air-liquid interface
BMP	Bone morphogenetic protein
CAM	Cell-assembled extracellular matrix
DED	Decellularized de-epidermized dermis
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EVs	Extracellular vesicles
FGF	Fibroblast growth factor
GAGs	Glycosaminoglycans
HSE	Full-thickness human skin equivalents
LDH	Lactate dehydrogenase
MMPs	Matrix metalloproteinases
MTT	(3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide)
NB	Neutralization buffer
PFA	Paraformaldehyde
PGs	Proteoglycans
RHE	Reconstructed human epidermis
SDS	Sodium dodecyl sulfate
SE	Skin equivalent
TERM	Tissue engineering and regenerative medicine

CURRICULUM VITAE

Personal information	Valášková Kristýna, Ing. Contact: k1_valaskova@utb.cz Sex: Female | Date of birth: 12/4/96 | Nationality: Czech
Education	**2021 – present** *Doctoral Degree* The Centre of Polymer Systems, Tomas Bata University in Zlín (Czech Republic) **Field of study: Biomaterials and Biocomposites** **Thesis: Preparation of reconstituted tissues** **2019 – 2021** *Master´s degree* Faculty of Technology, Tomas Bata University in Zlin (Czech Republic) **Field of study: Biomaterials and cosmetics** **Thesis: Scaffolds in tissue engineering** **2016 – 2019** *Bachelor´s degree* Faculty of Science, Palacký University Olomouc (Czech Republic) **Field of study: Molecular and cell biology** **Thesis: Development of CRISPR/Cas9-based reporter cell**
Education internships	**12 May – 10 June 2025** Biotechnology Centre, Silesian University of Technology; Gliwice, Poland **September 2024** 3D Cell Culture, practical course Institute of Biophysics, The Czech Academy of Sciences; Brno, Czech Republic **August 2023** Institute of Animal Physiology and Genetics CAS, The Czech Academy of Sciences; Brno, Czech Republic

	November 2022 (1 week) MatTek In Vitro Life Science Laboratories; Bratislava, Slovak Republic **October 2021 (1 week)** Institute of Biophysics, The Czech Academy of Sciences; Brno, Czech Republic
Conferences	**5 – 6 June 2025** XIV Ogólnopolska Konferencja Studentów Slaskiej Wyzszej Szkoly Medycznej w Katowicach Katowice, Poland Presentation type: Oral **30 June – 3 July 2024** ESB 2024, 29th Congress of the European Society of Biomechanics, Edinburgh, Scotland Poster presenter
Work on projects	**2025 – present** SurfToGreen – 101157688, Horizone-JU-CBE-2023 Member of the team **2025 – present** GAČR 25-17718S, Ex vivo tissue models for small intestine tissue Member of the project **2025 – present** GAČR 23-07425S, Anisotropic and Electro-Conducting Biomaterials Member of the project team **2024** IGA/CPS/2024/007, Designing Smart Biomaterials for Tissue Engineering Leader of the project team

2023 – 2024
GAČR 19-16861S, Interaction of biomaterials with stem cells under *in vivo* simulated conditions
Member of the project team

2023 - 2024
GAČR 22-33307S, Development of novel 3D hierarchically structured polysaccharides and proteins porous systems
Member of the project team

2023
IGA/CPS/2023/001, Development of Advanced Biomaterials and Their Future Applications
Member of the project team

September 2022 – January 2023
JUNG-2020-007, Development of biopolymer-based hydrogels for microextrusion
Member of the project team

2022
IGA/CPS/2022/001, Preparation of advanced biomaterials and their application
Member of the project team

2021 – 2022
GAČR 20-28732S, Colloidal systems for topical formulations. Pickering emulsions and polymer-based colloids.
Member of the project team

LIST OF PUBLICATIONS

Articles published in journals indexed on Web of Science:

1. Muchová, M.; Münster, L.; **Valášková, K.**; Lovecká, L.; Víchová, Z.; Osicka, J.; Kaspárková, V.; Humpolícek, P.; Vasícek, O.; Vícha, J. One-step fabrication of chitosan/dialdehyde cellulose/polypyrrole composite nanofibers with antibacterial, antioxidant, and immunomodulatory effects. *International Journal of Biological Macromolecules* 2025, vol. 308, part 1. Available from: doi: 10.1016/j.ijbiomac.2025.142105

2. Hamida, S.B.; Stepánová, V. ; Zahedi, L.; Kovácová, M.; Nasadil, P.; **Valášková, K**.; Humpolícek, P.; Kovácik, D. Enhancement of the dyeability and antibacterial properties of cotton fabric by plasma assisted cationization using chitosan and quaternized poly[bis(2-chloroethyl) ether-alt-1,3-bis[3-(dimethylamino)propyl]urea. *Cellulose* 2024, vol. 31, iss. 11, p. 7119-7136. Available from: doi: 10.1007/s10570-024-06027-z.

3. Kácerová, S.; Muchová, M.; Doudová, H.; Münster, L.; Hanulíková, B.; **Valášková, K.**; Kaspárková, V.; Kuritka, I.; Humpolícek, P.; Víchová, Z.; Vasícek, O.; Vícha, J. Chitosan/dialdehyde cellulose hydrogels with covalently anchored polypyrrole: Novel conductive, antibacterial, antioxidant, immunomodulatory, and anti-inflammatory materials. *Carbohydrate Polymers,* 2024, 327. Available from: doi:10.1016/j.carbpol.2023.121640.

4. Korábková, E.; Kaspárková, V.; Vasícek, O.; Víchová, Z.; Kácerová, S.; **Valášková, K.**; Urbánková, L.; Vícha, J.; Münster, L.; Skopalová, K.; Humpolícek, P. Pickering emulsions as an effective route for the preparation of bioactive composites: A study of nanocellulose/polyaniline particles with immunomodulatory effect. *Carbohydrate Polymers*, 2024, 323. Available from: doi:10.1016/j.carbpol.2023.121429.

5. Kácerová, S.; Víchová, Z.; **Valášková, K.**; Vícha, J.; Münster, L.; Kaspárková, V.; Vasícek, O.; Humpolícek, P. Biocompatibility of colloidal polypyrrole. *Colloids and Surfaces B-Biointerfaces*, 2023, 232. Available from: doi:10.1016/j.colsurfb.2023.113605.

6. Vítková, L.; Kazantseva, N.; Musilová, L.; Smolka, P.; **Valášková, K.**; Kocourková, K.; Humeník, M.; Minarík, A.; Humpolícek, P.; Mrácek, A.; Smolková, I. Magneto-responsive hyaluronan hydrogel for hyperthermia and bioprinting: Magnetic, rheological properties and biocompatibility. *APL Bioengineering*, 2023, 7(3). Available from: doi:10.1063/5.0147181.

7. Jurtík, M.; Gresková, B.; Prucková, Z.; Rouchal, M.; Dastychová, L.; Vítková, L.; **Valášková, K.**; Achbergerová, E.; Vícha, R. Assembling a supramolecular 3D network with tuneable mechanical properties using adamantylated cross-linking agents and β-cyclodextrin-modified hyaluronan. *Carbohydrate Polymers*, 2023, 313. Available from: doi: 10.1016/j.carbpol.2023.120872

Ing. Kristýna Valášková, Ph.D.

Preparation of reconstituted tissues

Příprava rekonstituovaných tkání

Doctoral Thesis Summary

Published by: Tomas Bata University in Zlín,
nám. T. G. Masaryka 5555, 760 01 Zlín

Edition: published electronically

Typesetting by: Kristýna Valášková

This publication has not undergone any proofreading or editorial review.

Publication year: 2025

First Edition

ISBN 978-80-7678-363-8