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TECHNOLOGY REVIEW

Studying the Human Skin Microbiome Using 3D *In Vitro* Skin Models

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Abstract

Labskin is a commercial 3D *in vitro* skin model based on the original Leeds Model specifically developed for studying interactions between the skin and its microbiome. Further development of the microbiology protocols for growth, preparation, inoculation, and recovery have enabled Labskin to be used for a wide variety of applications in skin microbiology. The method of construction of the model using a fibrin rather than collagen based dermal matrix also provides for a more robust physical structure, which may have benefits for using Labskin in a wide variety of testing applications.

Key words: skin microbiome, antimicrobials, cosmetics, Labskin, skin barrier.

Introduction

THREE-DIMENSIONAL (3D) HUMAN SKIN MODELS are an accepted part of the testing and regulatory landscape in skin-related industries[2,3,4] and their development, commercialization, and uses have been well documented.[5,6] At present, there are several established commercial model systems and a multitude of academic lab-specific 3D *in vitro* reconstructed human skin models, all with different characteristics, strengths, and weaknesses.[7,8,9] Some are simple and better suited to applications such as higher throughput screening,[10] while others are more sophisticated and capable of complex responses.[11,12] In all cases the primary objective is to produce a layered cellular structure with functional barrier qualities similar to human skin. However, in no way can we claim that modern 3D *in vitro* reconstructed human skin models of any type provide the perfect solution to all the research requirements of skin scientists, and their limitations need to be appreciated, as no single model is ubiquitous.

Over the last two decades a variety of skin models have been validated to replace certain types of animal experimentation, and their use to fulfill the regulatory requirements of Registration, Evaluation, Authorisation and Restriction of Chemicals, the European Union Cosmetics Directive, the Classification, Labelling, and Packaging Regulation, etc. is now well established.[10,13] More recently the focus has shifted from producing skin models that reliably and predictably fulfill regulatory requirements (albeit for limited assay endpoints in relatively artificial ways) to the development of skin models that are able to provide more complex behaviors and reactivity. As we develop more complex models than the simple reconstructed human epidermis models beloved by regulatory authorities, in an attempt to replicate the structure and functions of human skin, we trade off stability, reproducibility, reliability, and low cost for a better understanding of skin as a functional unit.

Increasing the complexity of 3D *in vitro* reconstructed human skin models, raises a number of issues that affect their reliability and predictability. While constructing a model of layered living fibroblasts and keratinocytes is a relatively straightforward, if rather complex, technical operation, it is actually very difficult to get near to reproducing the barrier properties of living human skin.[14] And even at this level, a range of complex techniques and skilled technical ability are required to consistently produce correctly differentiated skin models with acceptable permeability characteristics, strength, resilience, and surface water activity. We are able to measure a whole host of physical and biochemical parameters of these reconstructed ''full thickness'' skin models, but unsurprisingly, not all measurements come within what we would consider the ''normal'' range *in vivo.*[15] Consequently, we have to define and accept the limitations of such models.

The compromises and limitations suddenly start to escalate once we try to elaborate further beyond the standard ''full thickness'' model of dermal and epidermal layers constructed from fibroblasts and keratinocytes respectively. The introduction of other cell types such as melanocytes substantially increases the demands on the skill of the tissue culture technician, simply because of the differing complex

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nutritional and physical requirements of each cell type. While there has been some success in constructing complex models containing fibroblasts, keratinocytes, and melanocytes,[16] there is an inherent level of inconsistency and atypical behavior in the end product, which may be unacceptable for certain uses and applications. Likewise, the incorporation of Langerhan's cells into 3D skin models, although a Holy Grail of sorts for skin biologists, is inevitably pushing our technical capability too far at the moment,[17,18] at least until we understand more about the interactions between cell types and the biochemical and physical environment of the skin. Therefore, currently a keratinocyte and fibroblast 3D *in vitro* reconstructed human skin model provides the basic stable and predictable test platform.

While our natural direction of travel in pursuing more representative models of human skin is to attempt to incorporate all the relevant cell types, we often overlook the fact that not all interactions are restricted to human skin cell and immune cell types. The skin surface and associated appendages are home to a significant biomass of colonizing microorganisms.[19,20,21,22] It is relatively easy to isolate, identify, and enumerate the common microbial species from the skin and we know that each square centimeter of skin is home to up to one million metabolically active microbes.[23] So when we investigate the biological responsiveness of skin using models in the lab, why do we always use skin models that are sterile? It is obvious that such a large, mostly benign microbial load must play a significant role in supporting skin health.[24,25] Indeed, just as we now realize the huge value of a healthy gut microflora and the consequences of disturbing that balance, a healthy skin microflora demonstrates the same beneficial effects, while a disturbance in the ecological balance leads to skin irritation and infections.[23,26,27] Therefore, a more achievable goal in the short term—and possibly of more significant value in the long term—is the development of techniques to reliably colonize 3D *in vitro* reconstructed human skin models with microorganisms, thus establishing a more representative model of the skin ecosystem.

A surprisingly small number of investigations have been undertaken to study microbial interactions using 3D *in vitro* reconstructed human skin models, mainly due to the incompatibility of established models with bacterial colonization.[28,29,30,31,32] A significant change occurred with publication of a model system specifically devised for the purpose of studying the skin microflora by the Skin Research Centre at the University of Leeds.[1,33] This model was based on a polymerized fibrin dermal equivalent instead of the more usual collagen-based dermal matrix. The fibrin method had been demonstrated previously to produce a better differentiated epidermis,[34] and the Leeds model was shown to have good barrier function and an acidic surface, all in keeping with a highly differentiated epidermal layer.[1] Further characterization demonstrated that it was able to support the growth of skin microorganisms to densities more in keeping with *in vivo* situations than with other models[1] and reacted differently to the presence of commensal and pathogenic microbes.[33] The Leeds model was subsequently commercialized as Labskin and found niche applications in testing antimicrobials. The aim of this paper is to summarize the existing knowledge on this product and present supporting data to demonstrate the uses of Labskin primarily as a test platform for skin microbiology, but also to illustrate how the different construction process gives it additional characteristics that may be useful in a wide range of testing scenarios.

Materials and Methods

Characteristics of the Labskin 3D in vitro *skin model*

Labskin is categorized as a full thickness living skin equivalent model, consisting of a dermal equivalent of polymerized fibrin containing primary adult human fibroblasts and a well differentiated, air-exposed epidermis composed of neonatal foreskin primary human keratinocytes (Fig. 1). The use of fibrin to construct the dermal matrix imparts a robust structure to the model, which enables a more intuitive approach to testing and the use of product application, physical sampling, and testing procedures which are not practical with other, less structurally robust models (Fig. 2). The highly structured architecture of Labskin results in a surface that is relatively dry compared to other 3D skin models, making it better suited to growing skin microorganisms on the surface (Fig. 3). The Labskin 3D *in vitro* skin model was used for all experimentation in this study. Labskin (Innovenn Ltd.) was produced using the methods of Holland et al. (2008).[1] All cells, dermal equivalents, skin equivalents, and microbial colonization experiments were incubated at 37°C in 5% (v/v) carbon dioxide and air.

Skin microorganisms

All microbial strains were cultured in the appropriate growth medium and growth conditions to late exponential phase of growth as previously described.[1] Inocula were prepared by harvesting the cells by centrifugation and resuspending immediately prior to inoculation in GS-24 (Innovenn Ltd.) to give the appropriate density of cells in a 20-μL aliquot.

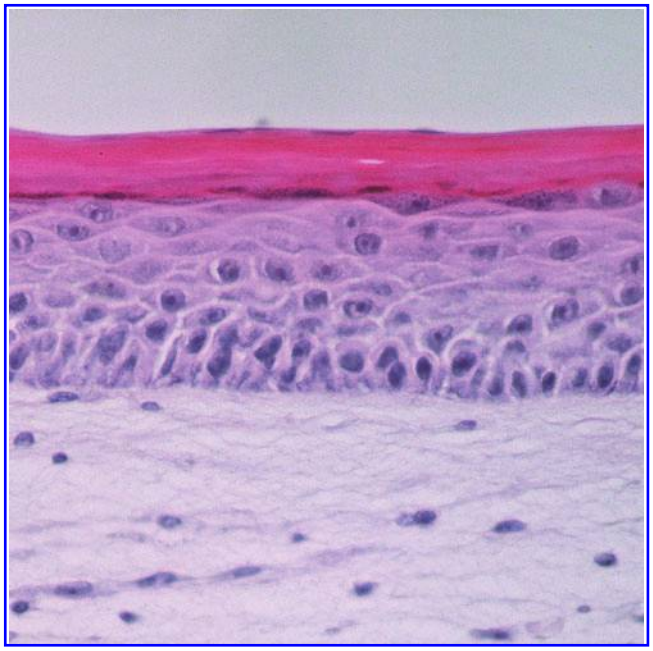

FIG. 1. Hematoxylin and eosin staining of paraffin-embedded sections of the Leeds Skin Model (Labskin).

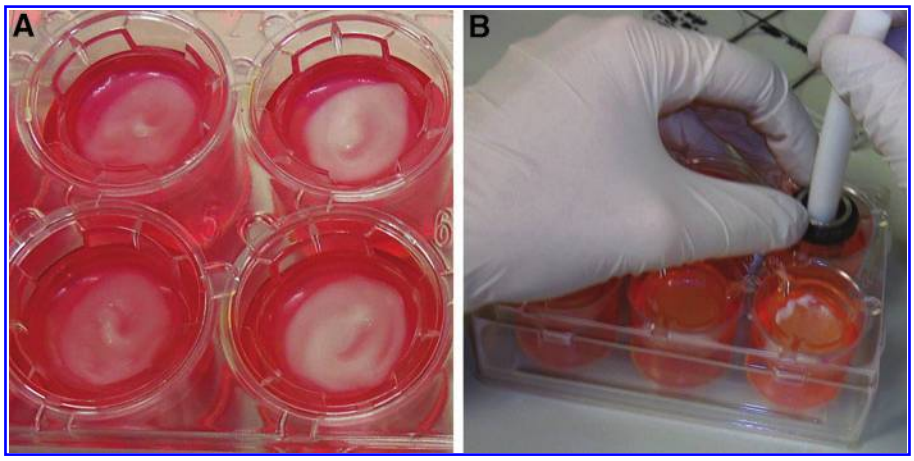

FIG. 2. The Leeds Skin Model (Labskin) in use. The 24-mm diameter three-dimensional (3D) skin equivalents are constructed from a dermal equivalent (fibrin matrix and primary human fibroblasts) supporting primary neonatal keratinocytes to form a structured epidermis after 14 days at the air–liquid interface. The large diameter and robust structure allows easy access for **(A)** the application of test materials (in this case a topical cream) and **(B)** the use of conventional sampling techniques, including multiple samples for different assay protocols.

Harvesting of the skin microbiome

The skin microbiome was recovered from the cheeks of volunteers by a modified version of the scrub wash technique used in previous studies[1] using GS-24 (Innovenn Ltd.) as the wash fluid. The suspended microbiome was applied directly to the surface of the skin models.

Results and Discussion

Microbial colonization of Labskin

The initial experiments with the Leeds Model demonstrated that the structural refinements of the model allowed the controlled surface growth of skin microorganisms.[1] Further refinement of the microbial preparation and inoculation techniques to utilize the proprietary GS-24 medium (Innovenn Ltd.) results in reliable growth of a range of skin-relevant microorganisms on Labskin (Fig. 4). Of particular interest is the survival and controlled growth of *Malassezia* spp. on the model using these methods, as these are notoriously difficult to culture using conventional techniques.[35] Previous to the availability of Labskin, it can only be assumed that the lack of success with collagen-based models was the result of their weaker barrier properties with the associated water activity.

Our initial experiments with laboratory type-strains (Fig. 4) indicated that the process of isolation, purification, and

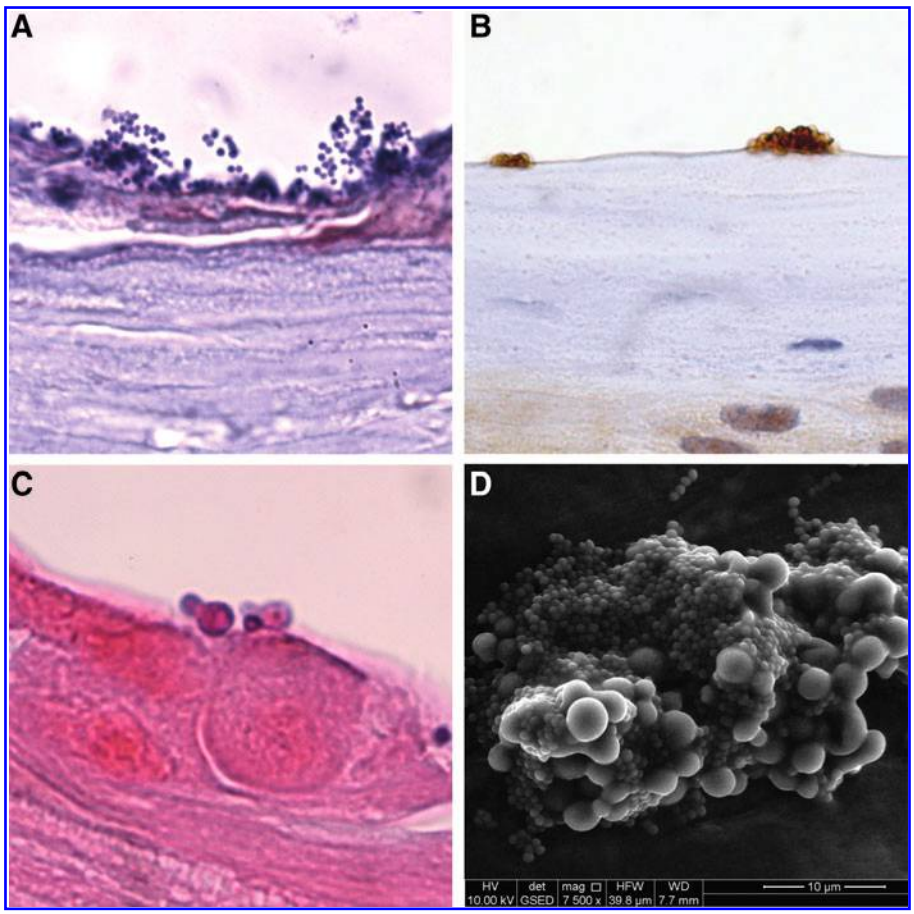

FIG. 3. Microbial colonization of the surface of the Leeds Skin Model (Labskin) demonstrating **(A)** surface growth of *Staphylococcus epidermidis*; **(B)** micro colony formation by *S. epidermidis*; **(C)** budding of the yeast *Malassezia globosa*, and **(D)** formation of a biofilm 48 hours after inoculation with *Propionibacterium acnes*, *S. epidermidis*, and *M. globosa.*

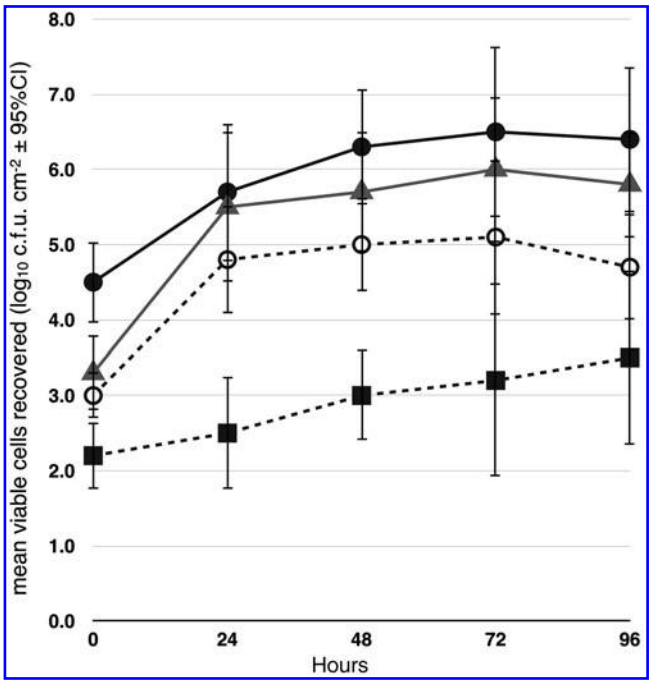

FIG. 4. Colonization kinetics of the Leeds Skin Model (Labskin, mean ± 95% confidence limits, $n = 4$) by reference microbial strains *P. acnes* ATCC 25746 (●), *S. epidermidis* NCTC 11047 (▲), *M. globosa* CBS 7874 (■), and *Staphylococcus aureus* ATCC 29213 (○). Abbreviations: c.f.u., colony forming units.

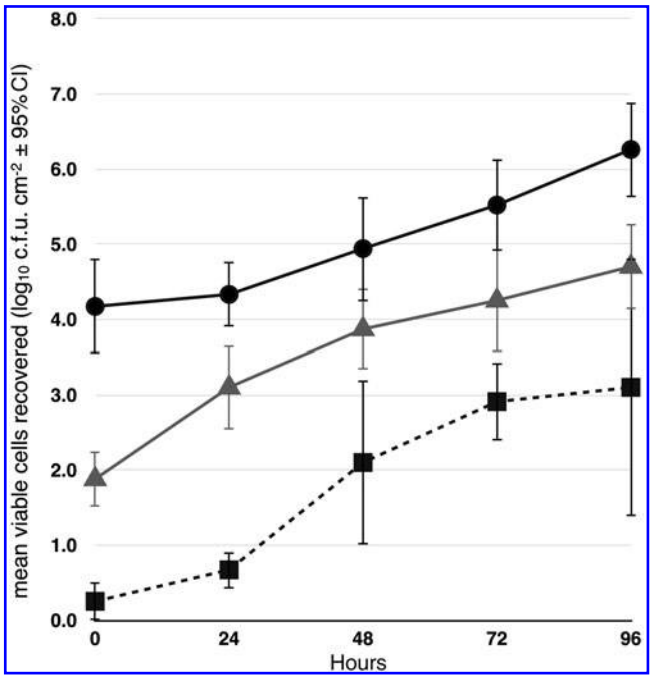

FIG. 5. Colonization kinetics of the Leeds Skin Model (Labskin, mean ± 95% confidence limits, $n = 4$) by a native skin washing from the cheek of a human volunteer, using selective growth media for the enumeration of *Propionibacterium* spp. (●), *Staphylococcus* spp. (▲), and *Malassezia* spp. (■).

storage unsurprisingly resulted in the gradual loss of the natural phenotype in laboratory-trained skin, resulting in a reduced ability to reliably colonize the skin model. We were able to devise two methods to improve the reliability of the colonization process. First, when working with laboratory type-strains, we encouraged the skin phenotype by sequential subculturing onto Labskin, using the skin model as a direct replacement for agar as the growth medium. In the short term, this produced more reliable colonization and can be recommended for routine use. A second method was devised to overcome altogether the shortcomings of using of purified, lab-trained microorganisms. By careful recovery of the skin surface microbiome from volunteers by washing and the exclusion of any selective isolation or growth prior to use, we have been able to directly reapply the mixed microflora (which also contains additional epidermal lipids, proteins, nutrients, etc.) to the model surface and perform experiments on this unselected microbiome (Fig. 5).

The data available from these preliminary experiments is currently limited by our restricted recovery and analysis tools. However, with the correct expertise in analyzing the total microbiome combined with methods to measure the reaction of the skin model, the Labskin/microbiome system would a provide powerful tool for understanding the dynamics of populations on the skin. Thus far, the historical reliance on recovery using viable counting, which naturally selects only those strains which can be grown on our preselected growth media, has somewhat skewed our understanding. It is easy to envisage that this method of direct application of the natural skin microbiome could be expanded to include specific clinical microbial populations from disease states (i.e. acne, eczema, psoriasis, etc.) and also developed to provide ''standard mixes'' for reproducible testing. With our capacity to also manipulate the skin model to produce a range of environmental changes (i.e., pH, water activity, nutrient availability, barrier, AMPs, etc.), the combination of skin model and microbiome will allow us to investigate in detail the ecological balance between the skin and its microbial occupants.

The use of Labskin as a test system for skin microbiology

It was always envisaged that the Leeds Skin Model (Labskin) would find suitable applications for testing topical antimicrobials, hygiene agents, prebiotic materials, probiotic concepts, etc., and our investigations have demonstrated that Labskin can be successfully utilized in a range of useful applications in this area. These include conventional antimicrobial hygiene assays against common pathogens found on the skin (Fig. 6) and the testing skin-specific antimicrobials such as those for use in antidandruff shampoos with activity against *Malassezia* spp. (Fig. 7). As Labskin provides a ''blank canvas'' for antimicrobial testing, it also allows methods to be developed that would be difficult and possibly unethical to perform on human volunteers (i.e., purposely inoculating the skin with pathogens such as *Staphylococcus aureus*). The balance between *S. aureus* and the usually harmless commensal *S. epidermidis* is of interest in a variety of dermatoses, as are methods to differentially manipulate the balance between the two species. Unfortunately, any treatment strategy that uses antimicrobials will equally affect

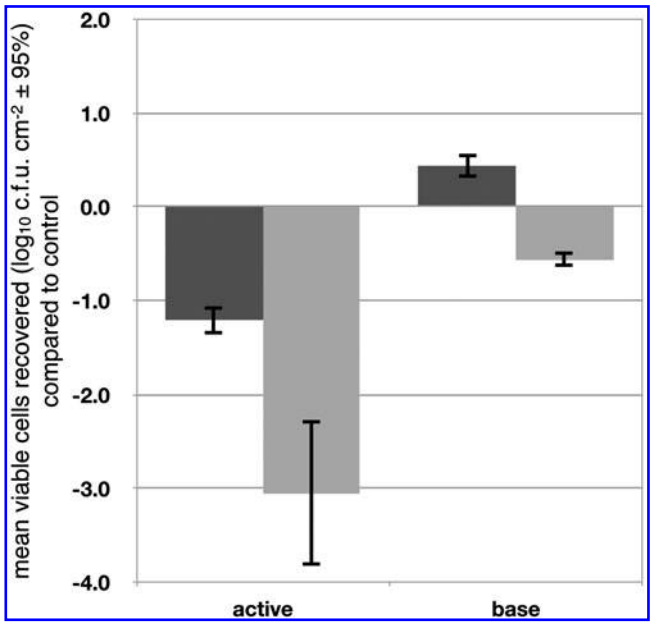

FIG. 6. The Leeds Skin Model (Labskin) as a test system for the antimicrobial activity of liquid hand soap formulations. The effect of a test liquid soap formulation and the nonactive base soap on *Escherichia coli* ATCC 25922 (■) and *S aureus* ATCC 29213 (■) colonized on the surface of the Leeds Skin Model (Labskin) is shown. Viable cells were recovered 3 hours post application of the test materials and compared with the control (water only).

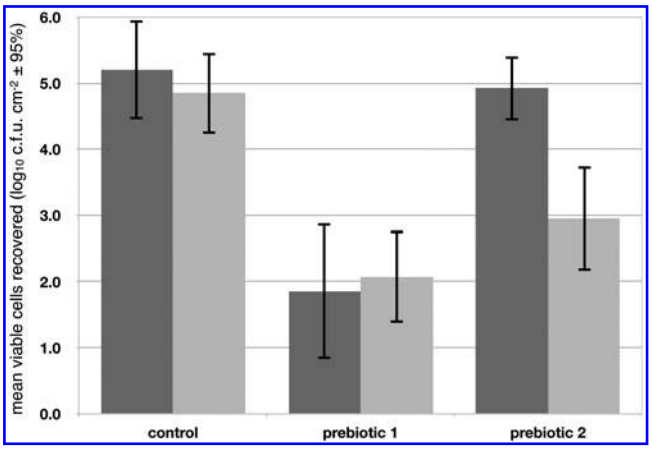

FIG. 8. The Leeds Skin Model (Labskin) as a test system for active agents to encourage skin colonization by commensal microorganisms. The bar graph shows the effect of prebiotic test formulations on the survival of *S. epidermidis* NCTC 11047 (■) and *S. aureus* ATCC 29213 (■) on the surface of the Leeds Skin Model (Labskin) 24 hours after colonization with 10^3 viable cells per cm^2.

both species due to their close genetic relationship, and therefore novel approaches such as prebiotic nutrients which differentially select the growth/colonization of one species over the other provide an interesting alternative (Fig. 8). A similar prebiotic approach can be utilized to shift the balance of microbial species in conditions such as underarm odor, where a complex and poorly understood relationship between *Staphylococcus* spp. and Corynebacteria can be manipulated without the use of antimicrobial agents (Fig. 9). Importantly, it would be very difficult to attempt such studies in human volunteers due to the background microbiology and skin physiology, as the simpler model system provides a more controllable environment. With many of these applications, the colonized skin model provides an important test platform for proving concepts, acting as interim step between discovery and clinical product testing.

Conclusions

The original objectives driving the development of the Leeds Skin Model (Labskin) were that it could be used to investigate interactions between the resident microbial species, to identify the genes of the resident microorganisms responsible for successful colonization, to determine the interactions between microorganisms and the skin to help understand the innate responses of skin, and as a highly versatile test system.

The initial investigations with Labskin indicated that keratinocytes and fibroblasts interact within the model as would be expected in a metabolically active, growing tissue.[1,33] But in addition, it was evident that the model was

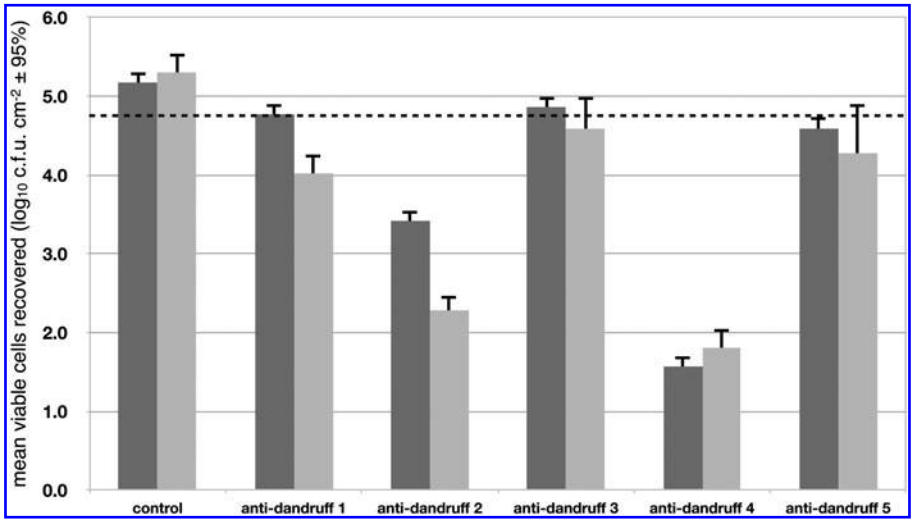

FIG. 7. The Leeds Skin Model (Labskin) as a test system for active agents against dandruff. The effect of 10-minute exposure to antidandruff test formulations on the survival of *M. globosa* CBS 7874 colonized on the surface of the Leeds Skin Model (Labskin) after 12 hours(■) and 24 hours (■) post recovery is shown. Colonization with 5×10^4 viable cells per cm^2 is indicated by the dashed line (- - -).

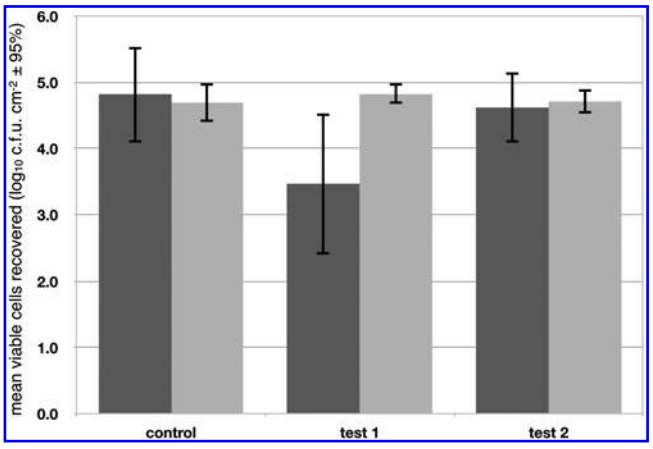

FIG. 9. The Leeds Skin Model (Labskin) as a test system for active agents against underarm odor. The effect of prebiotic test formulations on *S. epidermidis* NCTC 11047 (■) and *Corynebacterium jeikeium* NCTC 11915 (■) colonized on the surface of the Leeds Skin Model (Labskin) is shown. Viable cells were recovered 48 hours post application.

also interacting with and responding to the presence of microorganisms on the surface. While the concept of colonizing a skin model with microorganisms may initially appear straightforward, it is evident that the underlying interactions are potentially extremely complex and the rapid pace of development of gene array assay technologies in recent years opens up the intriguing possibility that we may at last be in a position to start unraveling the skin/microbiome complex. The 3D *in vitro* reconstructed human skin model and a single microbial species are a practical starting point, but we must also bear in mind that the skin microbiome is bewilderingly complex and we must surely take this into account when looking to develop more representative model systems.

While the focus of this investigation has been to better define the use of Labskin as a test system for microbial control strategies, it has become apparent that Labskin has many other applications in skin research resulting from the improved barrier function that makes the model so well suited to skin microbiome investigations. It is hypothesized that the improved physical robustness and barrier function of the model is in part due to the increased activity of fibroblasts in remodeling the fibrin dermal matrix by laying down native collagens compared to collagen-based models. Therefore, Labskin may also find much wider use than simply as a model for skin microbiology. It is also evident that the extremely close relationship and interaction between our skin and the skin microbiome begs the question as to why we continue to regard a sterile model as the standard, when in fact, colonized models should be the natural starting point.

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