RESEARCH LETTER

Microbial colonization of an *in vitro* model of a tissue engineered human skin equivalent – a novel approach

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Received 30 July 2007; accepted 5 November 2007.
First published online December 2007.

DOI:10.1111/j.1574-6968.2007.01021.x

Editor: Jan-Ingmar Flock

Keywords
skin equivalent model; colonization; cutaneous microorganisms.

Abstract

This was a preliminary investigation to define the conditions of colonization of a human skin equivalent (SE) model with cutaneous microorganisms. SEs of 24 mm diameter were constructed with a dermal matrix of fibrin containing fibroblasts and a stratified epidermis. Microbial colonization of the SEs was carried out in a dry environment, comparable to '*in vivo*' skin, using a blotting technique to remove inoculation fluid. The microbial communities were sampled by scrub washing and viable cells enumerated on selective growth medium. *Staphylococcus epidermidis*, *Propionibacterium acnes* and *Malassezia furfur* (human skin commensals) and *Staphylococcus aureus* (transient pathogen) were colonized at inoculum densities of 10^2–10^6 CFU SE^{-1} on the surface of replicate SEs. Growth of all species was supported for upto 72–120 h, with recovery densities of between 10^4–10^9 CFU SE^{-1}. A novel, real-time growth monitoring method was also developed, using *S. aureus* containing a *lux* cassette. Light output increased from 20 to 95 h, and colonization increased from 10^2 to 10^8 CFU SE^{-1}, as confirmed by conventional recovery. Thus, the SE model has potential to investigate interactions between resident and transient microbial communities with themselves and their habitat, and for testing treatments to control pathogen colonization of human skin.

Introduction

A range of microorganisms are found on normal human skin, some of which are present as part of the resident commensal flora. The dominant microbial groups include staphylococci, propionibacteria and *Malassezia* spp. (Bojar & Holland, 2002). On normal undamaged skin these resident species are considered to be nonpathogenic. However, under certain conditions, which are not fully understood, they can cause opportunistic infections that may present serious medical problems. It is important therefore, to examine what factors control the colonization of these microorganisms, either as commensals or pathogens, in the skin environment. Because ethical considerations prevent the deliberate inoculation of microorganisms onto human skin, and animal models are neither appropriate, because the natural microbial communities are different to that of human skin, or desirable, because of increasing ethical constraints, a tissue engineered human skin equivalent model with similar properties to human skin has been developed to help understand cutaneous microbial interactions.

Laboratory production of differentiated skin using human skin cells is an established technology. Living skin equivalents (SE) have been widely used as wound coverings (Boyce, 2001; Horch *et al.*, 2005) and as a research tool (Stark *et al.*, 2006). The living SE model used in this study was composed of a dermal equivalent (DE) made up of a fibrin matrix containing viable fibroblasts and a stratified epidermis generated by seeding keratinocytes on top of the DE. Fibrin was chosen as the dermal matrix, rather than collagen, the more commonly used substrate, because it was found to support a better differentiated epidermis (El Ghalbzouri *et al.*, 2004).

Many previous studies have used isolated skin cells, such as primary human keratinocytes or HaCaTs, a keratinocyte cell line to examine microbial interactions with skin (Jugeau *et al.*, 2005; Kisich *et al.*, 2007). Few investigations have reported the use of SEs. Bhattacharyya *et al.* (1998) were the first to use SEs to study the early events in stratum corneum colonization by *Malassezia* yeasts and Lerebour *et al.* (2004) examined the adhesion of *Staphylococcus epidermidis* and *Staphylococcus aureus* to Episkin®, a reconstructed epidermis

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without a dermal layer, produced by L'Oreal. However, in both studies the microorganisms were present as high-density cell suspensions on the SE surface, which in reality does not occur on human skin *in vivo*, because under normal conditions skin is not overtly hydrated.

Therefore, the primary aim of this study was to develop a model system more comparable to the *in vivo* situation in which microbial colonization occurred in a relatively dry environment, without the presence of free water and where the inoculum density was low and similar to that found on human skin. In addition, it was aimed to develop methods that accurately monitored bacterial growth on the surface of the SEs, which ideally were noninvasive and gave real time assessment.

Materials and methods

Cell culture

Cryopreserved adult human dermal fibroblasts were purchased from Cascade Biologics and cultured in Dulbecco's modified Eagle medium (DMEM: Invitrogen) supplemented with 10% (v/v) foetal calf serum (FCS) (Biosera), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Sigma), 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Gibco). Fibroblasts were used in the construction of DEs between passages two and five.

Cryopreserved primary human keratinocytes, extracted from neonatal foreskins, were purchased from Invitrogen. The keratinocytes were cultured on a collagen type IV (Sigma) coated surface (0.67 μg cm^{-2}), in keratinocyte serum free medium (KSFM) containing insulin, fibroblast growth factor and epidermal growth factor (Invitrogen) with the addition of penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}). For construction of the SEs, keratinocytes were used at passage three or four.

DEs

DEs were constructed with a fibrin matrix containing viable fibroblasts. A modified method of El Ghalbzouri *et al.* (2004) was used to prepare the fibrin gel. Human fibrinogen, depleted of plasminogen, von Willebrand factor and fibronectin (Enzyme Research Laboratories, Swansea) was dissolved in phosphate-buffered saline (PBS with Ca^{2+} and Mg^{2+}: Invitrogen) to a concentration of 4 mg mL^{-1}. Each gel was prepared by mixing 2.1 mL of fibrinogen solution with 157 μL PBS and 265 μL of a fibroblast suspension (3.0×10^5 cells mL^{-1}). Bovine thrombin (Biopool) was dissolved in PBS to a concentration of 5 NIH U mL^{-1} and 107 μL was added to the fibrinogen mixture. The mixture was immediately poured into a Transwell® cell culture insert (Corning, six well plates, Transwell® inserts, 24 mm diameter, with polyester membrane, 3 μm pore size) and polymerization occurred within 10 min. After polymerization, the DEs were submersed in DMEM supplemented with 10% (v/v) FCS, L-glutamine (2 mM) sodium pyruvate (1 mM), penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}). The culture medium was changed daily and the DEs were incubated for 6/7 days before keratinocyte seeding.

SEs

The DEs were washed (× 2) in PBS (PBS without Ca^{2+} and Mg^{2+}: Invitrogen), before seeding 1.0×10^6 keratinocytes in KSFM (1 mL) onto each DE. The constructs were cultured under immersed conditions in KSFM for 2 days and the medium was changed twice daily. Aprotinin, a protease inhibitor, was added at 200 U mL^{-1} to the KSFM in the culture dish, to prevent fibrinolysis by either the fibroblasts or keratinocytes. After 2 days, when the keratinocytes were confluent, the constructs were raised to the air/liquid interface. The culture medium was also changed to DMEM and Ham's F12 medium (Gibco) in a 3 : 1 ratio (v/v) supplemented with 2 mM L-glutamine (Gibco), 10 ng mL^{-1} epidermal growth factor (Sigma), 0.4 μg mL^{-1} hydrocortisone (Calbiochem), 1.8×10^{-4} M adenine (Sigma), 5 μg mL^{-1} insulin (Sigma), 5 μg mL^{-1} transferrin (Sigma), 2×10^{-7} M triiodothyronine (Sigma), 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Gibco), 50 μg mL^{-1} vitamin C (Sigma) and 200 U mL^{-1} aprotinin (Nordic, Pharma). The medium was renewed twice daily for 10 days. The Transwell inserts were then transferred to deep well plates (BD Biosciences) and antibiotics and aprotinin were omitted from the culture medium. The medium was renewed daily for 4 days, to remove residual antibiotics/aprotinin from the SEs, after which they were used for microbial colonization.

Incubation conditions

All cells, DEs, SEs and microbial colonizations were incubated at 37 °C in 5% (v/v) CO_2 and air, unless otherwise stated. Four replicate SEs were used in all colonization experiments when different inoculum densities and/or incubation times were tested.

Trans epidermal water loss (TEWL) and surface pH measurements

The SEs were validated by examining barrier function integrity of the stratum corneum using a tewometer (AquaFlux, Biox Systems, London) to measure TEWL and by surface pH measurements using a flat headed electrode.

Histology and immunohistochemistry

Further characterization of the SEs was by examination of their morphology and epidermal differentiation. The SEs were fixed in 10% (v/v) normal buffered formalin for 24 h followed by paraffin embedding. Morphology of the SEs was

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assessed by standard haematoxylin and eosin staining (6 μm sections) and for immunohistochemical analysis, the SE sections (6 μm) were unmasked using antigen retrieval methods and then stained with a Vectastain Elite ABC kit and mouse monoclonal antibodies (Vector Laboratories) against filaggrin and keratins 1, 6, 10 and 16 (markers of epidermal differentiation) and collagen type IV (a basement membrane marker). The immunohistochemical methods were carried out according to the manufacturer's instructions.

Bacterial strains

Staphylococcus epidermidis (strain S9) was isolated from the volar forearm (Farrell *et al*., 1993): *Propionibacterium acnes* (type strain KPA 171202) was obtained from the German Type Culture Collection: *Malassezia furfur* (type strain CBS 1878) from the Centraal Bureau voor Schimmel Cultures, the Netherlands and *S. aureus* (strain SH 1000) was donated by Prof. Simon Foster, Sheffield University, UK.

Preparation of microbial suspensions for inoculation

Staphylococcus epidermidis and *S. aureus* were grown overnight in 2% (w/v) Tryptone at 37 °C to late exponential phase of growth. The inoculum density was obtained by dilution of the culture to $OD_{600\,nm} = 0.25$, precalibrated to be equivalent to 10^8 CFU mL^{-1}. *Propionibacterium acnes* was grown in tryptone (1% w/v), yeast extract (0.5% w/v) and glucose (0.25% w/v) in an anaerobic cabinet for 24 h at 34 °C to mid-exponential phase of growth. *Malassezia furfur* was grown in modified Leeming & Notman (1987) for 25 h at 34 °C, to early exponential phase of growth. The inoculum densities for *P. acnes* and *M. furfur* were estimated by cell counts under phase contrast microscopy.

Construction of *S. aureus* with a *lux* cassette

Staphylococcus aureus SH1000 (*sigB*+) was constructed with a *lux ABCDE* Km cassette, which was transduced from *S. aureus* Xen 29 (*sigB* −) (from Xenogen) using ϕ85phage (Foster, 1998). The *S. aureus* SH1000 *lux* strain hosted the cassette at ORF SA2154 and the growth curves were similar for the *S. aureus* strains with and without the cassette. There was a positive correlation between the cell numbers and luminescence during exponential growth of *S. aureus* SH1000 *lux* when the specific luminescence remained constant.

Statistics

Colonization of SEs by test microorganisms was calculated from replicates as $\log_{10}$ CFU SE^{-1} ± 95% confidence limits.

Results and discussion

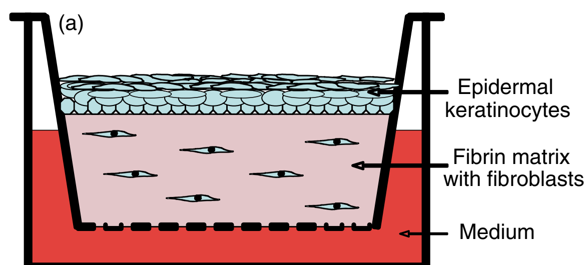
Characterization of SEs

After growth at the air/liquid interface for 14 days, the entire surface of the SE was complete and dry (Fig. 1a and b). The SE model has characteristics close to those of normal skin with a dermal matrix containing viable fibroblasts, an organized stratified epidermis and stratum corneum (Fig. 2a), a partially differentiated epidermis (Fig. 2b), a continuous basement membrane (Fig. 2c), an acidic surface pH 6.29 (human skin pH 5.4) and barrier integrity (TEWL value 60 g m^{-2} h^{-1} as compared with values for human skin of 10–45 g m^{-2} h^{-1}). The SEs thus provide a functional model to use for microbial colonization.

Monitoring of microbial colonization on the surface of the SEs

A modified Williamson and Kligman scrub technique was used to recover microorganisms from the surface of SEs. Stainless steel rings (18 mm diameter) were placed on the SE and the surface scrubbed with 500 μL wash fluid for 1 min (× 2) using a Teflon rod. Viable microorganisms were recovered using a Whitely Automatic Spiral Plater (WASP) and enumerated using a ProtoCOL automated colony counter (Synbiosis).

Homogenization (MSE blade homogenizer) and emulsification (polytron lipid emulsifier) methods of sampling of SEs were also investigated but were unsuitable as the homogenate contained visible clumps, which could not be processed through the WASP. Aerosols were also produced, making the techniques unsuitable for use with *S. aureus*.



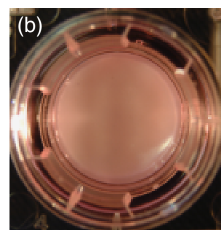


Fig. 1. SE structure. (a) Schematic of a SE in a cell culture insert with a polyester membrane (3 μm pores). The surface keratinocytes are exposed to the air, while the construct is fed by medium from below. (b) SE (24 mm diameter) after incubation for 14 days at the air/liquid interface showing an intact and dry surface, ready for microbial colonization.

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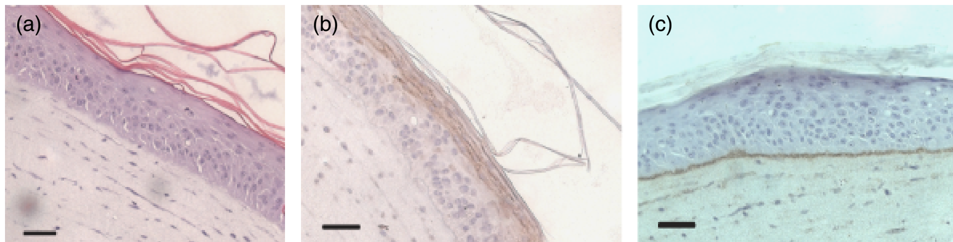

Fig. 2. Morphology and immunohistochemistry of paraffin embedded sections of a SE. (a) Haematoxylin and eosin staining showed fibroblasts evenly distributed throughout the dermal matrix and basal, spinous and granular cells and a stratum corneum in the epidermis. (b) Keratin1, a keratinocyte differentiation marker, was expressed in the upper epidermal layers. (c) The continuous deposition of collagen type IV at the junction of the epidermis and dermis indicates that a basement membrane was laid down. Scale bar: 50 μm.

Colonization of microorganisms on the SE model in dry conditions

Normal human skin is not overtly hydrated and it was considered important that the SEs should be colonized without free water on the surface of the stratum corneum. This was achieved on the SE model using a blotting technique. After inoculating the surface of replicate SEs with *S. epidermidis*, at a density of 10^4 CFU in 100 μL of water, incubation was carried out for either 2 or 4 or 6 h. These incubation times allowed the microorganisms to settle on the surface corneocytes. The SEs were then carefully blotted dry with strips of sterile filter paper avoiding damaging the surface. Immediately afterwards the microorganisms were recovered using the scrub technique. No increase in growth, compared with the inoculum density occurred with blotting after 2 h incubation. After blotting at 4 h there was a doubling in microbial numbers and after 6 h there were seven doublings (Table 1). The shortest time after incubation for blotting was therefore taken to be 2 h.

The colonization kinetics of *S. epidermidis* on SEs was determined over a period of 120 h after inoculation with 10^4 CFU in 100 μL of water and blotting after either 2 or 6 h. Owing to the destructive nature of sampling, replicate SEs were incubated for 24 or 48 or 72 or 96 or 120 h. The SEs were able to support the colonization of *S. epidermidis* on a dry surface for up to 120 h (Fig. 3), although the surface integrity was affected by 96 h incubation. After blotting at 6 h, the recovery density reached a maximum of 10^8 CFU by 24 h, whereas after a 2 h blot maximum colonization density was not reached until 72 h. The slower rate of microbial growth represents a more useful model and could be used to study interactions between staphylococci and keratinocytes of the SE. The faster colonization of SEs by *S. epidermidis* when blotted at 6 h compared with 2 h may be due to higher hydration of the stratum corneum in the former.

Table 1. Recovery densities of *Staphylococcus epidermidis* after colonization on replicate (× 4) SEs and blotting at 2 or 4 or 6 h of incubation

Incubation time (h)	Time SE surface blotted (h)	Inoculum density ($\log_{10}$)	Mean recovery density ($\log_{10}$) ± 95% confidence limits
2	2	3.95	3.93 ± 0.28
4	4	3.95	4.25 ± 0.23
6	6	3.83	6.10 ± 0.29

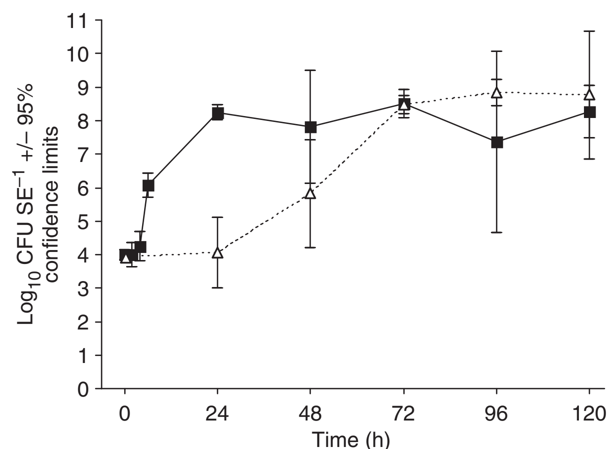

Fig. 3. Colonization kinetics. *Staphylococcus epidermidis* was inoculated at a density of 10^4 CFU SE^{-1}. Replicate SEs (× 4) were used for each incubation time point up to 120 h. Blotting was after 2 h (△) or 6 h (■) of incubation. Recovery densities were determined using automated colony counting on Isosensitest agar. The scale bars represent the ± 95% confidence limits.

Colonization by other resident cutaneous microorganisms on SEs

In addition to *S. epidermidis*, the commensal microbial communities of normal human skin contain high levels of *Propionibacterium* spp. and *Malassezia* spp. which are predominantly found on lipid-rich areas of the skin such as the face, back and chest (Bojar & Holland, 2002). In addition to their roles as commensals, *Propionibacterium* spp. are associated with inflammatory acne (Farrar & Ingham, 2004) and *Malassezia* spp. are involved in seborrheic dermatitis and pityriasis versicolor (Ashbee, 2006). In the laboratory, both are slow growing, fastidious microorganisms and therefore

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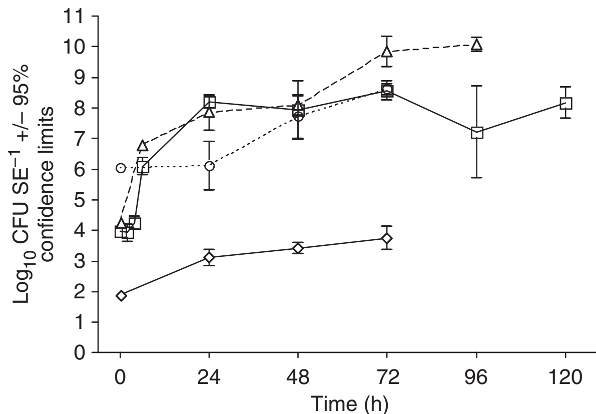

Fig. 4. Colonization kinetics of *Staphylococcus epidermidis* (□), *Staphylococcus aureus* (△), *Propionibacterium acnes* (○) and *Malassezia furfur* (◇) were compared on separate skin equivalents using inoculation densities of 10^4, 10^4, 10^6 and 10^2 CFU SE^{-1}, respectively. Replicate (× 4) SEs were used for each incubation time point up to 120 h for *S. epidermidis*, 96 h for *S. aureus* and 72 h for *P. acnes* and *M. furfur*. Blotting was after 6 h of incubation. Recovery densities were determined by automated colony counting on selective growth medium i.e. IsoSensitest agar for staphylococci, Reinforced Clostridial agar for *P. acnes* and Leeming and Notman agar for *M. furfur*. The scale bars represent the ± 95% confidence limits.

it was essential to determine whether the SEs would support their colonisation. *Malassezia furfur* was inoculated at a density of 10^2 CFU in 100 μL water and *P. acnes* at 10^6 CFU per 100 μL water. These densities were chosen to reflect the recovery densities of these microorganisms on normal human skin. *Malassezia furfur* and *P. acnes* were colonized individually on SEs for 24, 48 or 72 h, when the experiment was terminated, at 34 °C with surfaces blotted dry after 6 h.

Malassezia furfur produced slow and steady growth up to a maximum of 10^4 CFU after 72 h incubation (Fig. 4). The growth of *P. acnes* was slow at the beginning, with a substantial lag, but increased to 10^8 CFU after 72 h (Fig. 4). In laboratory growth medium *M. furfur* requires a supply of long chain fatty acids, which are not available in the culture medium used to grow the SEs and experimentation demonstrated that the SE culture medium alone did not support the growth of any of the major resident microrganisms. Therefore, these results indicate that additional, essential, nutrients are being provided by the SE itself.

Colonization of SEs by *S. aureus* (SH1000)

Staphylococcus aureus is not a natural resident of healthy human skin but it is frequently involved in many skin and soft tissue infections and so it was important to determine whether the SE model would sustain the growth of *S. aureus*, particularly if mixed colonization with commensal microorganisms was to be attempted. *Staphyolococcus aureus* (SH1000) was inoculated at 10^4 CFU in 100 μL water for either 6, 24, 48, 72 or 96 h, with blotting after 6 h. Recovery densities were consistent between replicates, showing that SEs supported colonization of *S. aureus* on a dry stratum corneum for 96 h (Fig. 4). However, the longer incubation times were not practical as surface damage to the SEs was evident at 72 h, presumably due to the activities of *S. aureus*. In contrast, during colonization with *S. epidermidis* the surface integrity remained intact for 96 h. Thus, the model was able to distinguish between the virulence potential of an opportunistic pathogen (*S. aureus*) and a closely related skin commensal (*S. epidermidis*).

Real-time monitoring of colonization of SEs

A method was developed for monitoring growth in real-time without the necessity for microbial recovery from the SE surface and destruction of the SE. *Staphylococcus aureus* (SH1000) was constructed with a *lux* cassette (SH1000 *lux*) and its colonization kinetics on SEs determined by measurement of light output in a Xenogen Imager. SH1000 *lux* was inoculated on replicate SEs (× 4) at a density of 10^2 CFU in 100 μL of water and blotted dry after 2 h. Light output increased from 20 to 95 h (Fig. 5) and viable cell recovery using the modified Williamson and Kligman technique confirmed that growth had increased from 10^2 CFU at inoculation to 10^8 CFU after 95 h. The results indicate that it may be possible to develop two complementary methods for monitoring colonization of the SE model, providing options for a more diverse range of future experimentation. For example, scrub wash sampling could be used to determine the interactions between mixtures of cutaneous microorganisms, whereas real time analysis of microorganisms

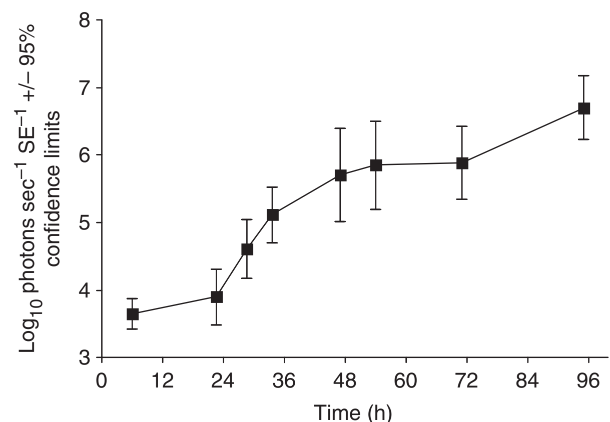

Fig. 5. Colonization kinetics of *Staphylococcus aureus* (SH1000 *lux*) using an inoculum density of 10^2 CFU SE^{-1} as measured by light output in a Xenogen Imager. Replicate (× 4) SEs were used for each incubation time point up to 96 h. Blotting was after 2 h incubation. The scale bars represent ± 95% confidence limits.

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hosting *lux*-reporters could be used to compare the differential colonization of wild type and isogenic mutants or to test the efficacy of topically applied antimicrobial agents on colonization.

Conclusions

In this preliminary investigation an *in vitro* model of human skin was developed and methods for microbial colonization and retrieval were established and optimized. In addition the model was shown to support colonization of the predominant human skin commensal species (*S. epidermidis*, *P. acnes* and *M. furfur*) and a transient bacterial pathogen (*S. aureus*) in a dry environment with acceptable variability. In future, this model could be used to investigate interactions between the resident microbial species: identifying the genes of the resident microorganisms responsible for successful colonization of the habitat; interactions between microorganisms and the host tissue by comparative transcript analysis of SEs with and without colonization to understand the innate responses of skin, which protect it from colonization by opportunistic pathogens: to test antimicrobials and topical agents. Thus, this novel approach has the potential for experimentation, which cannot readily be carried out *in vivo* due to ethical constraints.

Acknowledgements

The authors acknowledge financial support for this research from the Leeds Foundation for Dermatological Research and 3M Healthcare, USA.

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