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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1 Dasatinib inhibits viability of cSCC cells

Figure S2 Effect of dasatinib and imatinib on viability of normal human epidermal keratinocytes (NHEKs)

Figure S3 Dasatinib induces activation of procaspase-3 in cSCC cells

Figure S4 Dasatinib inhibits phosphorylation of Src, ERK, and p38 in cSCC cells

Table S1 Origin of cSCC cell lines used in the study

Data S1 Supplementary Materials and Methods

Data S2 Supplementary References

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Bifidobacterium longum extract exerts pro-differentiating effects on human epidermal keratinocytes, in vitro

1 | BACKGROUND

The term "probiotic" was popularised more than 20 years ago,[1] and more recently defined as "living microorganisms which, when administered in adequate amounts, confer a health effect on the host."[2] Traditionally, probiotics are shown to be beneficial in numerous inflammatory and allergic, mostly gastrointestinal, diseases.[3] However, orally consumed probiotics were also found to improve conditions of other organs, skin included[s1,s2,4]

Importantly, recent intriguing data suggest that microorganism-based topical formulations are also effective in various skin conditions. Indeed, locally applied living *Lactobacillus plantarum* suppressed inflammation and accelerated wound healing in patients with chronic infected leg ulcers.[5] Topical formulation containing non-pathogenic Gram-negative bacterium *Vitreoscilla filiformis* lysates improved symptoms and promoted the repair of the skin barrier in atopic dermatitis patients.[6] Of further importance, a specific *Bifidobacterium longum* (*B. longum*) extract from CLR Richter laboratories (Berlin, Germany), when applied as a cream to volunteers with reactive skin, decreased cutaneous sensitivity, increased skin resistance, and decreased skin dryness.[7] In addition, this *B. longum* extract inhibited neuropeptide-induced inflammatory cutaneous response in organ-cultured human skin and inhibited the release of neuropeptides from capsaicin-stimulated cultured sensory neurons.[7]

2 | QUESTIONS ADDRESSED

Epidermal keratinocytes are key players in establishing and maintaining the complex cutaneous barrier homoeostasis[s3]. However, very limited information is available on the direct actions of beneficial microorganisms on these cells. Therefore, in the current study, as a continuation of previously published work,[7] the effects of various preparations of *B. longum*, one of the most widely used probiotics, were investigated on cellular functions of primary normal human epidermal keratinocytes (NHEKs).

3 | EXPERIMENTAL DESIGN

In this study, the following *B. longum* preparations (BL) were used to examine their effect on the viability, differentiation, antimicrobial peptide production and calcium homoeostasis:

No 64, the non-replicative bacteria (heat-treated) from Nestle research center; *B. longum* NCC3001 (BL/64)
No 81, the non-replicative bacteria (heat-treated) from Nestle research center; *B. longum* NCC2705 (BL/81)
No 84, the *B. longum reuter* extract by sonication from Richter (INCI Bifida ferment lysate, 7; BL/84)

4 | RESULTS

First, the effect of the various BL preparations on the growth of the keratinocytes was assessed. Treatment of preconfluent (i.e. proliferating) NHEKs with various concentrations of BL for up to 72 hours did not modify the proliferation (as assessed by MTT assay) of the cells (Fig. S1). In addition, as MTT assay determines the viable cell number, it can also be concluded that none of the *BL extract* affected the viability of the NHEKs. Therefore, throughout

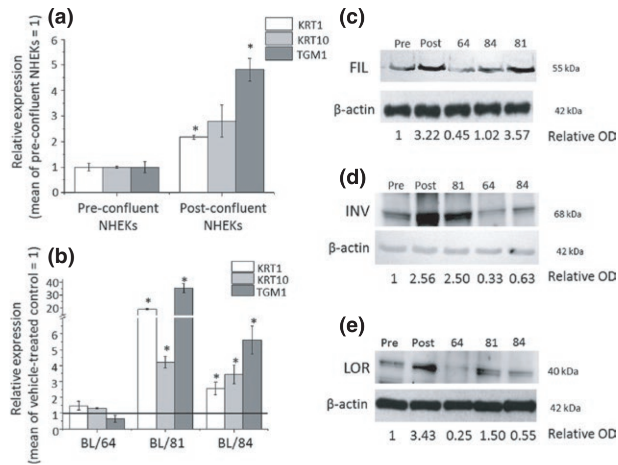

FIGURE 1 Effects of probiotics on differentiation of normal human epidermal keratinocytes (NHEKs). (a) QPCR evaluation of differentiation markers (cytokeratin KRT1, KRT10 and transglutaminase TGM1) in preconfluent (proliferating) and postconfluent (differentiating) NHEKs. Data are calculated using the ΔΔCT method using β-actin as an internal housekeeping gene. Results are expressed as mean±SD of three independent determinations relative to the preconfluent cultures (defined as 1). *$P<.05$ compared with expression determined in the preconfluent cultures. (b) Similar QPCR evaluation of the above differentiation markers after treatment of preconfluent NHEKs with various BLs (3%, 48 hours). *$P<.05$ compared with expression determined in the vehicle-treated cultures (defined as 1, solid line). (c–e) Western blot analysis of filaggrin (FIL), involucrin (INV) and loricrin (LOR) in the pre and postconfluent NHEKs as well as after the above probiotics treatment. Relative optical density (OD) values were determined by normalising the intensity of each band to the OD of their respective β-actin control; expression in the preconfluent vehicle-treated cultures was defined as 1. Two-three additional experiments yielded similar results

the experiments, the highest BL extract concentrations were employed (3 v/v %).

Next, the effects of BL preparations on the epidermal differentiation process were investigated. For this, the expression of various differentiation markers[s3] was compared in proliferating (pre-confluent) and differentiating NHEKs; the latter was achieved by culturing the cells for 2 days after reaching confluence (high cell density-induced differentiation model;[s4]. As expected (Fig. 1a), the expression of differentiation markers (KRT1, TGM1) was significantly higher in the postconfluent cultures (when compared with the pre-confluent ones).

Importantly, treatment of preconfluent NHEK with BL/81 increased the expression of all differentiation markers (Fig. 1b–e) to much higher levels than seen in the control (untreated) postconfluent cells (Fig. 1a,c–e).

BL/84 also up-regulated the levels of keratin (KRT) 1, KRT10, and transglutaminase 1 (TGM1). However, it did not induce an increase in filaggrin, loricrin and involucrin. Interestingly, BL/64 was ineffective in these assays.

The effects of BL on the expression of certain antimicrobial peptides (AMP) (β defensin 1 –BDEF- and Cathepsin S) and molecules with pivotal role in wound healing (Cathepsins B, D and H)[s5,s6,8] were

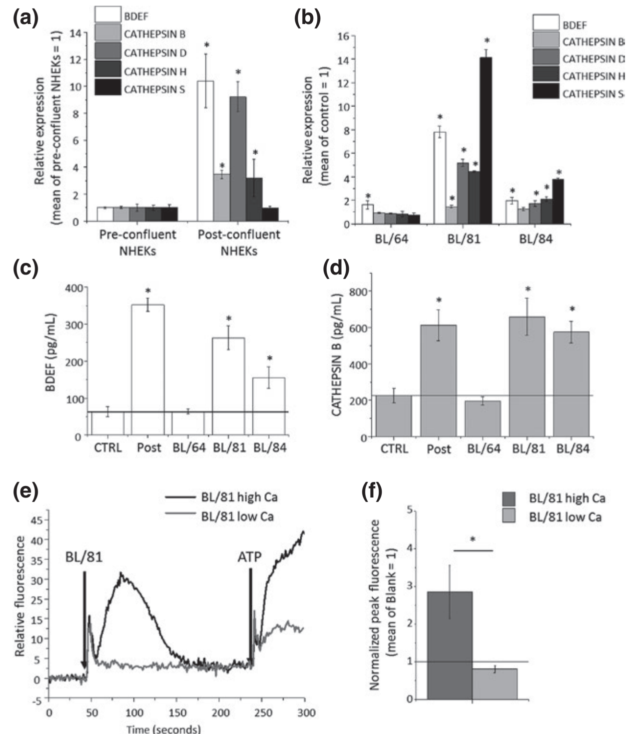

FIGURE 2 Effect of probiotics on the expression of antimicrobial peptides (AMP) and wound healing markers and on the intracellular calcium levels of normal human epidermal keratinocytes (NHEKs). (a) QPCR evaluation of AMP (β defensin 1 [BDEF], Cathepsin S) and molecules pivotal in wound healing (Cathepsins B, D and H) in preconfluent (proliferating) and postconfluent (differentiating) NHEKs. Data are calculated using the ΔΔCT method using β-actin as an internal housekeeping gene. Results are expressed as mean±SD of three independent determinations relative to the preconfluent cultures (defined as 1). *$P<.05$ compared with expression determined in the preconfluent cultures. (b) Similar QPCR evaluation of the above markers after treatment of preconfluent NHEKs with various BL extracts (3%, 48 hours). *$P<.05$ compared with expression determined in the vehicle-treated cultures (defined as 1). (c, d) ELISA analysis of the released BDEF and Cathepsin B in the pre and postconfluent NHEKs as well as after the above probiotics treatment. *$P<.05$ compared with levels determined in the preconfluent cultures. (e) Fluorimetric Ca^{2+} imaging using Fluo-4. BL/81 (3%) was applied as indicated by the arrow; as a positive control, ATP (180 µmol/L) was administered. Experiments were performed in media with high (1.8 mmol/L) and low (0.1 mmol/L) Ca^{2+} concentration. (f) Statistical analysis of Ca^{2+}-signals measured in the two media in four independent determinations. Results are expressed as mean±SD. *$P<.05$

also examined. Interestingly, levels of most of these markers (the only exception was Cathepsin S) were significantly elevated in postconfluent differentiating NHEKs (Fig. 2a,c,d).

Therefore, in the subsequent experiments, only preconfluent NHEKs were treated by BL. As shown in Fig. 2, BL/81 and BL/84 (with BL/81 being more efficient) markedly elevated the levels of mRNA transcripts and released amount of all (BL/81) or most (BL/84) molecules, BL/64, again, proved to be almost ineffective significantly up-regulating BDEF-specific mRNAs albeit not causing the release of the AMP.

Although the exact signalling pathway(s) that mediate the above effects remain to be determined, we have preliminarily attempted to assess the effect of BL extracts on the intracellular calcium levels, the elevation of which is one of the key factors that induce differentiation in NHEKs[s7]. Importantly, only BL/81 (but not BL/84 or BL/64) was effective in elevating the calcium concentration of the cells. This effect was completely absent when calcium was removed from the medium suggesting that BL/81 induces a calcium influx from the extracellular medium (Fig. 2e,f).

5 | CONCLUSIONS

The intriguing findings of this study provide the first evidence that certain *B. longum* extract preparations exhibit significant pro-differentiating and potentially pro-regenerating (cathepsins' expression) actions in NHEKs, together with an increased expression of BDEF, one key actor of cutaneous innate immune response. In the single available previous study, various probiotics (certain *B. longum* and *Lactobacillus* strains) were shown to increase the expression of multiple tight-junction proteins of NHEKs and enhanced barrier function (a decreased transepidermal water loss).[9] These data, therefore, collectively suggest that the beneficial effects of topical microbial formulations derived from bacteria may be mediated, at least in part, by direct modulation of epidermal keratinocytes' differentiation, most probably via inducing Ca^{2+} influx.

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AUTHOR CONTRIBUTION

AGS, JSP and NV performed the experiments, AG, OJ, CB, LB and TB participated in designed the research study and evaluation and interpretation of the obtained data. MA and IJ contributed human samples for the experiments. AGS, AG and TB also contributed to writing the manuscript.

CONFLICT OF INTEREST

The authors have declared no conflicting interests.

Keywords
antimicrobial peptide, barrier, innate immunity, probiotic, wound healing

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1 Effects of bacterial extract on proliferation and viability of NHEKs

Data S1 Supplementary Materials and Methods

Data S2 Supplementary References