

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

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Keywords:	Probiotic, innate immunity, barrier, wound healing, antimicrobial peptide

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

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Background

The term “probiotic” was popularized more than twenty 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 highly 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 nonpathogenic 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, significantly decreased cutaneous sensitivity, increased skin resistance, and decreased skin dryness, ~~and strengthened the cutaneous barrier~~ (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).

Questions Addressed

Epidermal keratinocytes are key players in establishing and maintaining the complex cutaneous barrier homeostasis (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).

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 homeostasis:

- N° 64, the non-replicative bacteria (heat-treated) from Nestle research center; *B. longum* NCC3001 (BL/64)
- N° 81, the non-replicative bacteria (heat-treated) from Nestle research center; *B. longum* NCC2705 (BL/81)
- N° 84, the *B. longum reuter* extract by sonication from Richter (INCI Bifida ferment lysate, 7; BL/84)

Results

First, the effect of the various BL preparations on the growth of the keratinocytes was assessed. Treatment of pre-confluent (i.e. proliferating) NHEKs with various concentrations of BL for up to 72 hrs did not modify the proliferation (as assessed by MTT assay) of the cells (Supplementary Figure S1 online). 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 the experiments, the highest BL extract concentrations were employed (3 v/v %).

Next, the effects of BL preparations on the epidermal differentiation process was 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 (Figure 1A), the expression of differentiation markers (KRT1, TGM1) was significantly higher in the post-confluent cultures (when compared to the pre-confluent ones).

Importantly, treatment of pre-confluent NHEK with BL/81 increased the expression of all differentiation markers (Figure 1B-E) to much higher levels than seen in the control (untreated) post-confluent cells (Figure 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.

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5 The effects of BL on the expression of certain antimicrobial peptides (AMP) (β
6 defensin 1 –BDEF- and Cathepsin S) and molecules with pivotal role in wound
7 healing (Cathepsins B, D and H) (s5; s6; 8) was also examined. Interestingly, levels
8 of most of these markers (the only exception was Cathepsin S) were significantly
9 elevated in post-confluent differentiating NHEKs (Figure 2A, C, D).

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14 Therefore, in the subsequent experiments, only pre-confluent NHEKs were treated
15 by BL. As shown in Figure 2, BL/81 and BL/84 (with BL/81 being more efficient)
16 markedly elevated the levels of mRNA transcripts and released amount of all (BL/81)
17 or most (BL/84) molecules, BL/64, again, proved to be almost ineffective significantly
18 up-regulating BDEF-specific mRNAs albeit not causing the release of the AMP.
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24 Although the exact signaling pathway(s) that mediate the above effects remain to be
25 determined, we have preliminarily attempted to assess the effect of BL extracts on
26 the intracellular calcium levels, the elevation of which is one of the key factors that
27 induce differentiation in NHEKs (s7). Importantly, only BL/81 (but not BL/84 or BL/
28 64) was effective in elevating the calcium concentration of the cells. This effect was
29 completely absent when calcium was removed from the medium suggesting that
30 BL/81 induces a calcium influx from the extracellular medium (Figure 2E-F).
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38 Conclusions

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

The authors state no conflict of interest.

Acknowledgement

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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.

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Figure legends

Figure 1. Effects of probiotics on differentiation of NHEKs

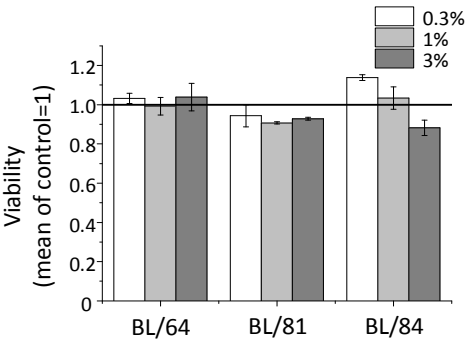
(A) QPCR evaluation of differentiation markers (cytokeratin KRT1, KRT10, and transglutaminase TGM1) in pre-confluent (proliferating) and post-confluent (differentiating) NHEKs. Data are calculated using the $\Delta\Delta CT$ method using β -actin as an internal housekeeping gene. Results are expressed as mean \pm SD of three independent determinations relative to the pre-confluent cultures (defined as 1). *P<0.05 compared to expression determined in the pre-confluent cultures. (B) Similar QPCR evaluation of the above differentiation markers after treatment of pre-confluent NHEKs with various BLs (3%, 48 hrs). *P<0.05 compared to 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 post-confluent NHEKs as well as after the above probiotics treatment. Relative optical density (OD) values were determined by normalizing the intensity of each band to the OD of their respective β -actin control; expression in the pre-confluent vehicle-treated cultures was defined as 1. Two-three additional experiments yielded similar results.

Figure 2. Effect of probiotics on the expression of antimicrobial peptides and wound healing markers and on the intracellular calcium levels of NHEKs

(A) QPCR evaluation of antimicrobial peptides (β defensin 1 [BDEF], Cathepsin S) and molecules pivotal in wound healing (Cathepsins B, D and H) in pre-confluent (proliferating) and post-confluent (differentiating) NHEKs. Data are calculated using the $\Delta\Delta CT$ method using β -actin as an internal housekeeping gene. Results are expressed as mean \pm SD of three independent determinations relative to the pre-confluent cultures (defined as 1). *P<0.05 compared to expression determined in the pre-confluent cultures. (B) Similar QPCR evaluation of the above markers after treatment of pre-confluent NHEKs with various BL extracts (3%, 48 hrs). *P<0.05 compared to 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 post-confluent NHEKs as well as after the above probiotics treatment. *P<0.05 compared to levels determined in the pre-confluent cultures. (E) Fluorimetric Ca^{2+} imaging

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3 using Fluo-4. BL/81 (3%) was applied as indicated by the arrow; as a positive
4 control, ATP (180 μ M) was administered. Experiments were performed in media with
5 high (1.8 mM) and low (0.1 mM) Ca^{2+} concentration. (F) Statistical analysis of Ca^{2+} -
6 signals measured in the two media in four independent determinations. Results are
7 expressed as mean \pm SD. *P<0.05
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Supplementary Figure 1

SUPPLEMENTARY INFORMATION

Supplementary Materials and Methods

Cell culturing

Human skin samples were obtained after written informed consent from healthy individuals undergoing surgery, adhering to Helsinki guidelines, and after obtaining Institutional Research Ethics Committee's permission (protocol No.: DE OEC RKEB/IKEB 3724-2012; document No.: IX-R-052/01396-2/2012). NHEKs were isolated after overnight dermal-epidermal separation in 2.4 U ml⁻¹ dispase (Roche Diagnostics, Berlin, Germany) by short trypsin (0.05%, Sigma-Aldrich, St. Louis, MO) digestion. Cells were cultured in EpiLife serum-free media supplemented with Human Keratinocyte Growth Supplement (both from Life Technologies, Carlsbad, CA).

Assessment of viability

Cells were plated in 96-well plates (20,000 cells per well density, which corresponded to approximately 70–80% confluence) in quadruplicate, treated for two days with the given concentration of probiotics. The number of viable cells was determined by measuring the conversion of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) to formazan by mitochondrial dehydrogenases. In all cases, experiments were repeated at least three times.

Quantitative RT PCR

Q-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) by using the 5' nuclease assay. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). One µg of total RNA were then reverse-transcribed into cDNA by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. PCR amplification was performed by using the TaqMan primers and probes (assay IDs: Hs00608345_m1 for human BDEF; Hs00947433_m1 for Cathepsin B, Hs00157201_m1 for Cathepsin D, Hs00544778_m1 for Cathepsin H, for Hs00175403_m1 Cathepsin S, Hs00196158_m1 for KRT1, Hs00818825_m1 for KRT7, Hs00413861_m1 for KRT9, Hs00166289_m1 for KRT10, Hs00165921_m1

for TGM1) using the TaqMan universal PCR master mix protocol (Applied Biosystems). As internal controls, transcripts of β -actin were determined (assay ID, Hs99999903_m1).

Western blotting

Keratinocytes were harvested and lysed in ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 5 mM EGTA, protease inhibitor cocktail 1:100, all from Sigma-Aldrich). After determining the protein content, 100 μ g protein from each sample was subjected to SDS-PAGE, transferred to BioBond nitrocellulose membranes (Whatman, Maidstone, UK), and then probed with antibodies against filaggrin, loricrin and involucrin (all used in a ratio of 1:250, all from Sigma-Aldrich). A horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1,000, Bio-Rad, Hercules, CA) was used as a secondary antibody, and the immunoreactive bands were visualized by a SuperSignal West Pico Chemiluminescent Substrate-Enhanced Chemiluminescence kit (Pierce, Rockford, IL) using LAS-3000 Intelligent Dark Box (Fuji, Tokyo, Japan). To assess equal loading, membranes were re-probed with an anti- β -actin antibody (1:1000, Sigma-Aldrich) and visualized as described above. Semiquantitative densitometric analysis of the signals was performed by using Image J software (National Institutes of Health, Bethesda, MD, USA).

ELISA

Secreted levels of β -defensin (Uscn Life Science Inc, Houston, TX) and Cathepsin B (Biorbyt LLC, San Francisco, CA) were determined from supernatants of probiotic treated NHEKs using pre-coated ELISA plates according to the manufacturer's protocol. Briefly, the supernatants were centrifuged for 10 minutes at 300 gs to remove bacterial debris, and subsequently diluted 100-fold.

Calcium measurement

Changes in intracellular Ca^{2+} concentration upon probiotic application were detected by fluorimetric Ca^{2+} imaging. Cells were seeded in 96-well black-well/clear-bottom plates (Greiner Bio One, Frickenhausen, Germany) at a density of 10,000 cells per well and then were incubated with culturing medium containing the cytoplasmic calcium indicator 2 μ M Fluo-4 AM (Life Technologies, Waltham, MA) at 37 °C for 40 minutes. The cells were washed four times with and finally cultured in Hank's

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3 balanced salt solution containing 1% bovine serum albumin and 2.5 mM probenecid
4 (both from Sigma-Aldrich) for 30 minutes at 37 °C. The plates were then placed to a
5 FlexStation 3 FLIPR (Molecular Devices, San Francisco, CA) and changes in
6 intracellular Ca^{2+} concentration (reflected by changes in fluorescence; IEX=494 nm,
7 IEM=516 nm) induced by various concentrations of the drugs were recorded in each
8 well (during the measurement, cells in a given well were exposed to only one given
9 concentration of the agent).
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16 *Statistical evaluation*

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18 When applicable, data were analyzed using a two-tailed un-paired *t*-test and $P <$
19 0.05 values were regarded as significant differences.
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24 **Supplementary Figure legends**

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26 *Supplementary Figure 1. Effects of bacterial extract on proliferation and viability of*
27 *NHEKs*
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29 Proliferation and viability of pre-confluent NHEKs were assessed by MTT assay after
30 48 hrs treatment with various concentrations of BLs. Results are expressed as
31 mean \pm SD of four independent determinations (untreated control was defined as 1).
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33 Two additional experiments yielded similar results.
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Supplementary Discussion

The upregulation of AMPs and Cathepsins in addition to their putative effects on wound healing, may also influence the local inflammatory milieu. Human beta defensin-1 for example has been shown (S8) to be anti-inflammatory in macrophages, where their upregulation resulted in decreased inflammatory cytokine expression (TNF- α , IL-6 and CXCL2). Cathepsin B has been shown to be instrumental for the development of the inflammatory response to mercury-induced autoimmunity (S9), and is also important in the immune response in inflammatory myopathies alongside Cathepsin H (S10). Cathepsin S has been shown to be instrumental in the innate immune response by regulating IL-1 β , since extracellular Cathepsin S is required for the maturation of the cytokine (S11). It is also highly expressed in macrophages (S12), takes part in MHC class II antigen presentation (S13) and toll-like receptor signalling (S14). Whether the combination of these factors results in pro- or anti-inflammatory effects requires further investigation.

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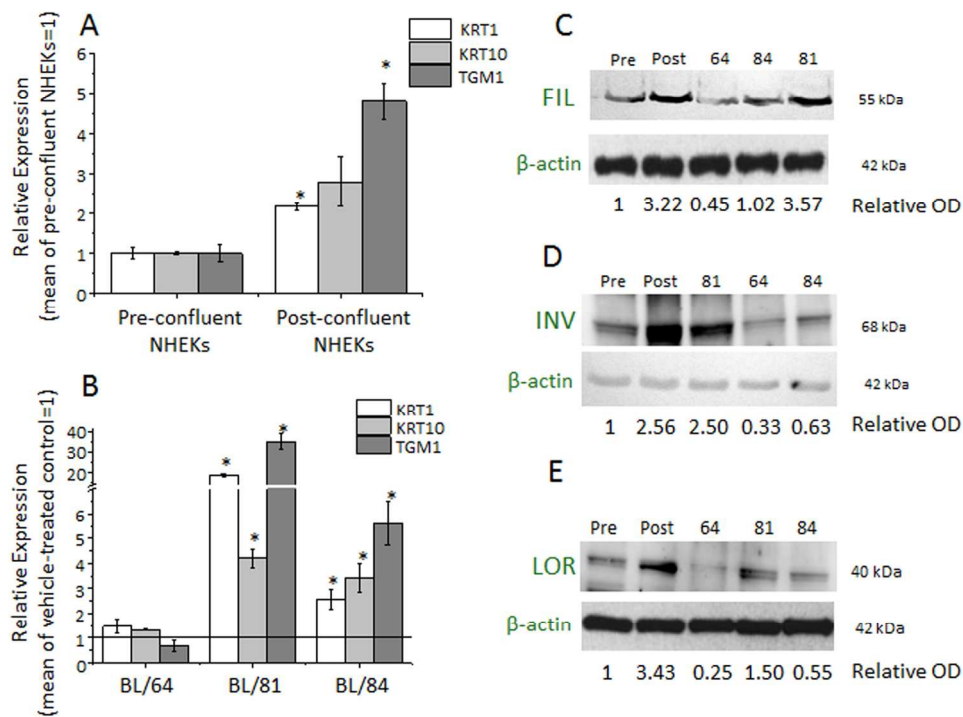


Figure 1

Figure 1. Effects of probiotics on differentiation of NHEKs

(A) QPCR evaluation of differentiation markers (cytokeratin KRT1, KRT10, and transglutaminase TGM1) in pre-confluent (proliferating) and post-confluent (differentiating) NHEKs. Data is calculated using the $\Delta\Delta CT$ method using β -actin as an internal housekeeping gene. Results are expressed as mean \pm SD of three independent determinations relative to the pre-confluent cultures (defined as 1). *P<0.05 compared to expression determined in the pre-confluent cultures. (B) Similar QPCR evaluation of the above differentiation markers after treatment of pre-confluent NHEKs with various BLs (3%, 48 hrs). *P<0.05 compared to 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 post-confluent NHEKs as well as after the above probiotics treatment. Relative optical density (OD) values were determined by normalizing the intensity of each band to the OD of their respective β -actin control; expression in the pre-confluent vehicle-treated cultures was defined as 1. Two-three additional experiments yielded similar results.

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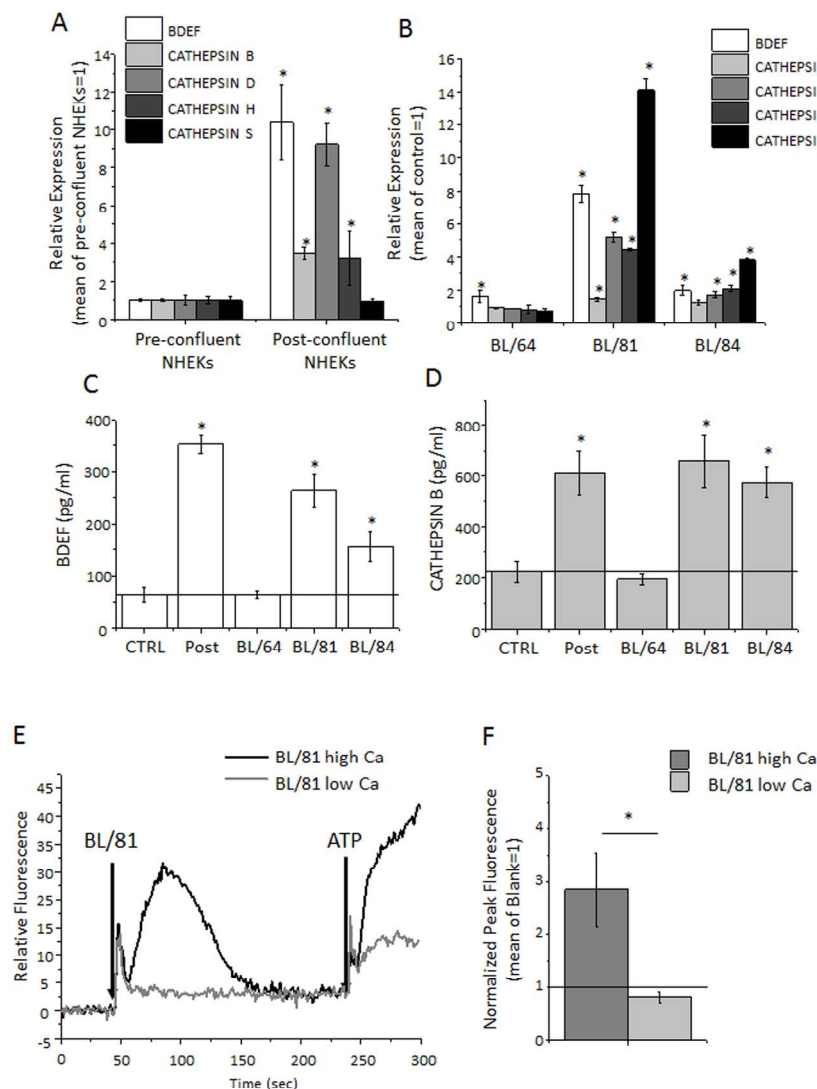


Figure 2

Figure 2. Effect of probiotics on the expression of antimicrobial peptides and wound healing markers and on the intracellular calcium levels of NHEKs

(A) QPCR evaluation of antimicrobial peptides (β defensin 1 [BDEF], Cathepsin S) and molecules pivotal in wound healing (Cathepsins B, D and H) in pre-confluent (proliferating) and post-confluent (differentiating) NHEKs. Data is calculated using the $\Delta\Delta CT$ method using β -actin as an internal housekeeping gene. Results are expressed as mean \pm SD of three independent determinations relative to the pre-confluent cultures (defined as 1). * $P < 0.05$ compared to expression determined in the pre-confluent cultures. (B) Similar QPCR evaluation of the above markers after treatment of pre-confluent NHEKs with various BL extracts (3%, 48 hrs). * $P < 0.05$ compared to 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 post-confluent NHEKs as well as after the above probiotics treatment. * $P < 0.05$ compared to levels determined in the pre-confluent 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 μM) was administered. Experiments were performed in media with high (1.8 mM) and low

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(0.1 mM) Ca²⁺ concentration. (F) Statistical analysis of Ca²⁺-signals measured in the two media in four independent determinations. Results are expressed as mean±SD. *P<0.05

195x261mm (300 x 300 DPI)

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