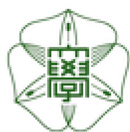

HOKKAIDO UNIVERSITY

Title	Potential of Gouda cheese whey to improve epidermal conditions by regulating proliferation and differentiation of keratinocytes
Author(s)	Shinagawa, Futa; Takata, Sayuri; Toba, Yu; Ikuta, Minoru; Hioki, Shoji; Suzuki, Takahiro; Nishimura, Takanori; Nakamura, Ryosuke; Kobayashi, Ken
Citation	International dairy journal, 87, 100-106 https://doi.org/10.1016/j.idairyj.2018.07.016
Issue Date	2018-12
Doc URL	http://hdl.handle.net/2115/76230
Rights	© 2018. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	http://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	International Dairy Journal_HUSCAP Figures_Potential of Gouda cheese whey improve epidermal conditions.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Potential of Gouda cheese whey to improve epidermal conditions by regulating proliferation and differentiation of keratinocytes

Futa Shinagawa[a], Sayuri Takata[a], Yu Toba[b], Minoru Ikuta[b], Shoji Hioki[b], Takahiro Suzuki[a], Takanori Nishimura[a], Ryosuke Nakamura[c] and Ken Kobayashi[a, *]

[a] Laboratory of Cell and Tissue Biology, Research Faculty of Agriculture, Hokkaido University, North 9, West 9, Sapporo 0608589, Japan.

[b] Field Science Center for Northern Biosphere, Hokkaido University, North 11, West 10, Sapporo 0600811, Japan.

[c] Department of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 6068507, Japan

[*]**Corresponding author:** Ken Kobayashi, Ph.D. Laboratory of Cell and Tissue Biology, Research Faculty of Agriculture, Hokkaido University, North 9, West 9, Sapporo 060-8589, Japan. Phone: +81-11-706-2540, E-mail: kkobaya@anim.agr.hokudai.ac.jp

Abstract

Sweet whey is a by-product of rennet-type cheese and contains abundant physiologically active substances. In this study, we investigated the effects of sweet whey on keratinocytes in mouse back skins using the warm-bathing model and using human keratinocyte culture model. The low-molecular-weight fraction (less than 3 kDa) of the whey was used for human keratinocyte culture because hydrophilic low molecular weight components can penetrate into the epidermis. The two experimental models revealed that whey treatment activated the proliferation of keratinocytes. Whey treatment also up-regulated the expression of CK10, a marker for differentiated keratinocytes. The expression of epidermal tight junction proteins and aquaporin 3 (AQP3) was also activated by whey treatment expression. Whey contains abundant lactose and calcium. However, neither lactose nor calcium affected proliferation activity and AQP3 expression in cultured keratinocytes. These findings suggest that cheese whey may have potential as a cosmetic ingredient to improve epidermal conditions.

1. Introduction

Sweet whey is a by-product produced from the manufacture of rennet-type cheese. This type of cheese whey comprises 85 to 90% of the total volume of the original milk and retains soluble whey proteins, lactose, growth factors, and minerals with microbial fermentation metabolites (Smithers, 2008; Yadav, et al., 2015). Such nutrient-rich whey is used as an additive in many processed foods, including breads, meat fish products, and infant formula. This type of cheese whey also contains physiologically active substances such as bioactive peptides and fermentation metabolites that are produced during the process of the enzymatic rennetting and fermentation in milk.

Lactoferrin is a whey protein that shows anti-bacterial, anti-inflammatory and immunomodulatory functions (Mayeur, Spahis, Pouliot, & Levy, 2016). Fragmented lactoferrin also retains its physiological functions (Jiang, Zhang, Xie, & Wang, 2016; Yamauchi, Tomita, Giehl, & Ellison, 1993). Transforming Growth Factor-β contained in whey strengthens the intestinal barrier by upregulating tight junction (TJ) proteins *in vitro* (Hering, et al., 2011). Glycomacropeptide (GMP) is released from κ-casein by enzymatic cleavage with rennet-chymosin during cheese making, and it attenuates inflammation via blockade of NF-κB activation (Cheng, Gao, Chen, & Mao, 2015; Yamauchi, et al., 1993). Exopolysaccharides, which are fermentation metabolites produced by lactic acid bacteria, attenuate UV-B-induced erythema formation and dryness in mouse skin (Morifuji, Kitade, Fukasawa, Yamaji, & Ichihashi, 2017). Furthermore, crude whey peptides improve skin wound healing (Wang, et al., 2010). Therefore, several cosmetic companies have incorporated cheese whey components for skin benefits. However, how cheese whey influences epidermal keratinocytes remains unclear.

Epidermal keratinocytes proliferate in the basal layer and then migrate towards the surface of the epidermis to form a multi-layered structure. The multi-layered epidermis protects the body from exogenous stimuli and restricts water loss. Such epidermal barrier functions are mainly maintained by TJs and the stratum corneum (Svoboda, Bilkova, & Muthny, 2016). The superbasal keratinocytes express claudin-1 (CLDN1), CLDN4, and occludin (OCLN) to form TJs, which restrict fluid flux through the paracellular space in the granular layer (Langbein, et al., 2002). The stratum corneum consists of highly keratinized cells embedded in a lipid matrix to block physical stimuli and restrict the fluid flux. Epidermal keratinocytes also express aquaporin 3 (AQP3), AQP9 and AQP10 (Jungersted, et al., 2013; Sugiyama, et al., 2014). In particular, undifferentiated basal keratinocytes regulate epidermal water and glycerol contents and elasticity by expressing AQP3 (Hara-Chikuma & Verkman, 2008).

Oral intake of fermented milk has been reported to improve the epidermal barrier in mice and keratinocyte proliferation (Baba, et al., 2010; Kimoto-Nira, et al., 2014). Fermented milk also regulates the appropriate differentiation of cultured normal human epidermal keratinocytes (Baba, Masuyama, & Takano, 2006). Cheese whey is recovered over when casein and fat in fermented milk are coagulated during the process of cheese production. Therefore, cheese whey contains similar components to fermented milk, excluding casein and fat (Yadav, et al., 2015). Furthermore, Gouda cheese whey contains the components that are produced during the rennetting process such as GMP. In this study, we investigated the influence of whey components in keratinocytes using the mouse warm-bathing model in addition to the human keratinocyte culture model. These

two experimental models were used to evaluate the potential of whey to improve epidermal conditions.

2. Materials and methods

2.1. Preparation of fermented cheese whey

The cheese whey was produced from the processing of Gouda type cheese using cow milk, multiple strain starter culture (CH-N-01; Chr. Hansen Laboratories, Copenhagen, Denmark) and calf rennet (Chr. Hansen Laboratories) at the Agri-Food Center in Hokkaido University (Sapporo, Japan). The cheese whey was collected after curd cutting and stored at -20°C until use for experiments. The intact whey was used for the warm-bathing model. The low-molecular-weight fraction (less than 3 kDa) of the whey (LMF-whey) was subsequently used for human keratinocyte culture because topically applied hydrophilic components of a low molecular weight can penetrate into the living epidermis although the epidermal barrier significantly prevents the penetration hydrophilic components of the high molecular weight (more than 3 kDa) (Magnusson, Anissimov, Cross, & Roberts, 2004). The low-molecular-weight fraction was recovered by centrifugation at 7,500 × g for 30 min at 4°C using an Amicon Ultra dialysis spin column (Ultra-4 3000; Merck Millipore, Burlington, MA, U.S.A.) followed by filter sterilization with a syringe-type filter (0.22 µm, Saltrius AG, Göttingen, Germany).

The measurement of lactose in LMF-whey was performed using the F-kit for lactose/galactose (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's protocol. The measurement of calcium in LMF-whey was performed using Naphthylazo-Naphthoic acid (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol.

2.2. Animals

Female C57BL/6N mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained under conventional conditions at 22–25°C. At 8 weeks of age, when the back skin was at the telogen stage of the hair cycle, mice were anaesthetized by intraperitoneal administration of sodium pentobarbital and then shaved. The back skin of the mice was immersed in a warm water or undiluted intact whey for 10 min at 38°C once daily for 5 days without a washout treatment. The treated back skin of mice was excised 2 h after bathing treatment and immersed in 4% formaldehyde in phosphate-buffered saline (PBS) for paraffin embedding and in RNAlater (Sigma-Aldrich, St. Louis, MO, U.S.A.) for quantitative PCR. All experimental procedures in this study were approved by the Animal Resource Committee of Hokkaido University (approval number: # 14-0005) and were conducted in accordance with Hokkaido University guidelines for the care and use of laboratory animals.

2.3. Human keratinocyte culture

Normal human epidermal keratinocytes (second passage) were obtained from Kurabo Industries (Osaka, Japan). Keratinocytes were seeded at $1 \times 10^3/cm^2$ on a dish coated with collagen type 1A (Nitta Gelatin Inc, Osaka, Japan) and cultured in HuMedia-KB2 (Kurabo) supplemented with HuMedia-KG (Kurabo) containing epidermal growth factor, insulin, hydrocortisone, and bovine pituitary extract at 37°C and under 5% CO_2 in a CO_2 incubator (Sanyo, Tokyo, Japan). Keratinocytes were then cultured with the culture medium containing 0.1, 0.33, and 1.0% LMF-whey (v/v) for 4 days and used for measurement of the proliferation activity before keratinocytes reach confluence. For

immunostaining and quantitative PCR, keratinocytes were cultured with the culture medium containing LMF-whey for 3 days after keratinocytes reached confluence.

2.4. Immunofluorescence staining

The back skins were embedded in paraffin after fixation in 4% PFA/PBS for 1 day and then sliced into 5-μm sections using a RM 2025 microtome (Leica Microsystems Inc., Wetzlar, Germany). The sections were deparaffinized and rehydrated, and then they were heated in a microwave in antigen retrieval buffer consisting of 10 mM Tris-HCl (pH 9.0) and 0.5 mM ethylene glycol tetra-acetic acid (Sigma-Aldrich). The cultured keratinocytes were fixed with methanol for 10 min at -20°C followed by 1% formaldehyde in PBS for 10 min at 4°C.

The paraffin sections and fixed cells were incubated in 5% bovine serum albumin (BSA) in PBS as the blocking solution to preclude nonspecific interactions. The sections were then incubated overnight at 4°C with antibodies against cytokeratin 10 (CK10; Progen, Heidelberg, Germany) and cytokeratin 14 (CK14; Sigma-Aldrich); a rabbit antibody against AQP3 (Alomone Laboratories, Jerusalem, Israel); or a rat antibody against Ki-67 (Dako Cytomation, Glostrup, Denmark) diluted in blocking solution. After the sections were washed with PBS containing 0.05% Tween 20 (PBST), they were exposed to Alexa Fluor 488-conjugated goat anti-rabbit, Alexa Fluor 546-conjugated goat anti-mouse, Alexa Fluor 488-conjugated donkey anti-mouse, or Alexa Fluor 546-conjugated goat anti-rat antibodies (Cell Signaling Technologies, Danvers, MA, U.S.A.) diluted in blocking solution for 1 h at room temperature. Controls for autofluorescence and nonspecific binding of secondary antibody were treated in the same manner, but without primary antibodies. Images of the stained

sections were obtained using a confocal laser-scanning microscope (TCS SP5; Leica).

2.5. Measurement of the proliferation activity

Proliferation activity of keratinocytes was evaluated using a WST-8 cell counting kit (Dojindo). The keratinocytes were seeded in 96-well plates and cultured for 1 d in HuMedia-KB2 without LMF-whey. Subsequently keratinocytes were then cultured for 4 days with or without 0.1, 0.33 and 1% LMF-whey. The proliferation activity of these keratinocytes was evaluated using a WST-8 Cell Counting Kit (Dojindo, Osaka, Japan) with a microplate reader (iMark; Bio-Rad Laboratories, Hercules, CA, U.S.A.) at 450 nm.

2.6. Quantitative PCR

For quantitative PCR, total RNA from mouse skin and human keratinocytes was extracted using an RNeasy® Mini Kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. Reverse transcription was performed using ReverTraAce® qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative real-time PCR was performed in a Light Cycler® 480 (Roche Applied Science, Indianapolis, IN, U.S.A.) using THUNDERBIRD® SYBR® qPCR Mix (Toyobo). The following amplification programme was used: 95°C for 1 min, followed by 40 cycles at 95°C for 15 s and 58°C for 1 min. The primer information is listed in Supplemental Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and β-actin (*ACTB*) were used as internal control.

2.7. *Western blotting*

The samples of keratinocytes were electrophoresed using a 10% or 12.5% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked for 1 h with PBST containing 3% nonfat dried milk and then incubated overnight at 4°C with primary antibodies diluted in PBST containing 5% BSA. Subsequently, the membranes were washed in PBST and incubated for 30 min at room temperature with the appropriate secondary horseradish peroxidase-conjugated antibody diluted in each blocking solution. The immunoreactive bands were detected using Luminata Forte Western HRP substrate (Millipore, Billerica, MA, U.S.A.). The images of the protein bands were obtained with a Bio-Rad ChemiDoc™ EQ densitometer and Bio-Rad Quantity One® software (Bio-Rad).

2.8. Statistical analysis

Statistical analyses were performed using the Analyse-It™ add-in (version 1.73, Analyse-it Software, Ltd., Leeds, United Kingdom) for Microsoft Excel (Microsoft Corporation, Redmond, WA, U.S.A.). For *in vivo* experiments, differences between whey and water groups were analysed using Student's unpaired *t*-test. For *in vitro* experiments, statistical analyses were performed using a two-tailed multiple *t*-test with Bonferroni correction. Differences were considered significant at a *p*-value of <0.05, as indicated herein by an asterisk. All experiments were performed a minimum of three times using different samples to ensure reproducibility.

3. Results

3.1. Whey treatment in the warm bath model facilitates mouse keratinocyte proliferation

The histological influence of whey on the epidermis was investigated using the *in vivo* mouse warm-bathing model. Haematoxylin and eosin (H&E) staining did not reveal any detectable morphological differences between the epidermis in whey-treated and water-treated mice (Fig. 1A, B). Proliferating keratinocytes were detected by immunostaining of Ki-67 in the basal layer of the epidermis in both whey-treated and water-treated skins (Fig. 1C, D). The ratio of Ki-67-positive cells to total epidermal cells stained with DAPI significantly increased in epidermis treated with whey for 1 and 5 days (Fig. 1E).

CK5/CK14 and CK1/CK10 are specific markers of undifferentiated and differentiated epidermal keratinocytes, respectively (Bragulla & Homberger, 2009). CK14-positive keratinocytes lined the basal layers, whereas CK10-positive cells were observed in the suprabasal layer both in the vehicle-and whey-treated skins (Fig. 1F-I). The qPCR analysis showed a significant increase in the mRNA expression of *Ck5* in response to whey treatment, whereas *Ck14*, *Ck1*, and *Ck10* showed no significant differences between the epidermis in whey-treated and water-treated skins (Fig. 1J, K).

3.2. Whey treatment in the warm-bathing model up-regulates AQP3 and TJ proteins

Epidermal keratinocytes expresses AQP3, which is a water and glycerol channel. AQP3 is involved in the moisture-retaining properties of skin (Hara-Chikuma & Verkman, 2008). AQP3-positive keratinocytes were observed in the basal layer of the epidermis in both whey-treated and water-treated skins (Fig. 2A, B). AQP3 localization in the cell membrane was more distinct in whey-treated than in water-treated skin. The qPCR analysis showed a significant increase in the mRNA expression of *Aqp3* (by approximately 1.5-fold) in response to whey treatment (Fig. 2C).

CLDN1, CLDN3 and OCLN are TJ proteins expressed in epidermal keratinocytes and form less-permeable TJs in the epidermal granular layer (Langbein, et al., 2002). The mRNA expression of *Cldn4* and *Ocln* was up-regulated in whey-treated skin (Fig. 2D). In particular, the expression of *Ocln* in whey-treated skin was two-fold higher than that in water-treated skin. In contrast, sphingomyelin synthase 1 (*Sgms1*), β-glucosidase (*Gba*), and filaggrin (*Flg*), which are involved in barrier formation of the stratum corneum (Gallala, Macheleidt, Doering, Schreiner, & Sandhoff, 2004), showed no significant difference between whey- and water-treated skins (Fig. 2E).

3.3. The low-molecular-weight fraction of whey facilitates human keratinocyte proliferation

The influence of LMF-whey on the proliferation of epidermal keratinocytes was examined by culturing human epidermal keratinocytes. Human keratinocytes exhibited a cobblestone-like shape regardless of the concentration of LMF-whey, whereas flat and large cells were observed in keratinocytes treated with 0.33% and 1% LMF-whey (Fig. 3A). The proliferation activity of keratinocytes was evaluated before reaching confluence. The proliferation activity of human keratinocytes significantly increased when they were treated with 0.1% and 0.33% LMF-whey, compared with the vehicle control (Fig. 3B).

Effect of LMF-whey on the differentiation of confluent keratinocytes was investigated by immunostaining of CK14 and CK10. In control, human keratinocytes were positive for CK14, whereas CK10-positive cells were hardly observed (Fig. 4C, E). In keratinocytes treated with 0.33% LMF-whey, weak positive reactions to CK14 were partially observed and some flat and large cells showed CK10-positive (Fig. 4D, F). The

qPCR analysis revealed significantly increased mRNA expression of *CK10* in response to LMF-whey treatment, but significantly decreased expression of *Ck5* (Fig. 4G, H).

3.4. The low-molecular-weight fraction of whey up-regulates AQP3 and TJ proteins

AQP3 localization in the lateral membrane was more distinct AQP3 in human keratinocytes treated with 0.33% LMF-whey compared with the control (Fig. 4A, B). The influence of LMF-whey treatment on AQP3 was evaluated by Western blotting (Fig. 4C). The amount of AQP3 significantly increased in keratinocytes treated with 0.33% LMF-whey compared with that in control (Fig. 4D). The mRNA expression of *Aqp3* tended to be moderately elevated in the presence of 0.1% and 0.33% LMF-whey compared with the control (Fig. 4E). In addition, Western blotting analysis showed the significant increase in TJ proteins CLDN1, CLDN4, and OCLN in response to LMF-whey treatment (Fig. 4C, D). The mRNA expression of *CLDN1* and *OCLN* were up-regulated in the presence of 1% LMF-whey (Fig. 4F). In contrast, *SGMS1*, *GBA*, and *FLG*, which are involved in the formation of the stratum corneum, didn't show significant response to LMF-whey (Fig. 4G).

3.5. Influence of lactose and calsium on keratinocyte behaviour

Whey is known to contain abundant levels of lactose and calcium (Yadav, et al., 2015). LMF-whey, which was used for the human keratinocyte culture model, contained 9.8 mM calcium and 39.4 mg/ml lactose. It was hypothesized that lactose and calcium in LMF-whey was responsible for regulating keratinocyte growth behavior. Therefore, the influence of lactose and calcium on keratinocytes was examined using human a keratinocyte culture model.

The proliferation activity of human keratinocytes was hardly changed in the presence of 0.04, 0.13, and 0.4 mg/ml lactose or 0.01, 0.03 and 0.1 mM calcium, which were equivalent to levels contained in 0.1%, 0.33%, and 1.0% LMF-whey, respectively (Fig. 5A). AQP3 and OCLN were detected by Western blotting in keratinocytes cultured in medium containing 0.33% whey, 0.13 mg/ml lactose and 0.33 mM calcium (Fig. 5B). AQP3 and OCLN significantly increased in keratinocytes treated with 0.33% LMF-whey (Fig. 5C). In contrast, lactose or calcium did not significantly affect changed the relative amount of AQP3 or OCLN compared to those in control.

4. Discussion

The multi-layered structure of the epidermis is maintained by the active proliferation and functional differentiation of keratinocytes. The multi-layered epidermis provides a barrier against physiological and physical stimuli. Cheese whey contains multiple bioactive peptides and microbial fermentation products (Yadav, et al., 2015). In this study, we investigated the influence of the topical application of whey in keratinocytes by using the warm-bathing model because warm temperature facilitates the permeation of hydrophilic components through the epidermis (Blank, Scheuplein, & MacFarlane, 1967; Peck, Ghanem, & Higuchi, 1995). In addition, human keratinocytes were cultured in the presence of a low-molecular-weight fraction of the whey (LMF-whey) because the epidermal barrier restricts the penetration of hydrophilic components depending on their molecular weight (Magnusson, et al., 2004). The results showed that whey components regulated the proliferation, differentiation, and expression of barrier-related genes in mouse and human keratinocytes, suggesting that topical application of whey could improve the condition of the epidermis by regulating keratinocyte behaviour.

The whey treatment increased keratinocyte proliferation in the basal layer in the warm-bathing model. Additionally, whey treatment induced the up-regulation of *Ck5* in the warm-bathing model. CK5 is a cytokeratin that is expressed in undifferentiated keratinocytes in the basal layer (Bragulla & Homberger, 2009). Whey treatment may increase the relative expression level of CK5 in association with the active proliferation of basal keratinocytes. Proliferation activity was also up-regulated in cultured human keratinocytes before confluency by treatment with LMF-whey, which is the whey fraction less than 3 kDa. The epidermal barrier allows the penetration hydrophilic components of the low molecular weight (Magnusson, Anissimov, Cross, & Roberts, 2004). These observations suggest that whey contains small-sized physiological substances that can activate keratinocyte proliferation. In contrast, confluent keratinocytes treated with 1% LMF-whey in the cell culture model showed an induction of CK10 expression, a marker for differentiated keratinocytes (Bragulla & Homberger, 2009). Baba and colleagues have reported that *Lactobacillus helveticus*-fermented milk facilitates the differentiation of cultured human epidermal keratinocytes with Ck10 up-regulation (Baba, et al., 2006). Fermented whey may also contain components to facilitate keratinocyte differentiation in addition to the components facilitating keratinocyte proliferation.

In this study, whey treatment activated the expression of CLDN1, CLDN4 and/or OCLN in keratinocytes *in vivo* and *in vitro.* In the epidermis, CLDN1, CLDN4 and OCLN form TJs in the epidermal granular layer to restrict fluid flux via the paracellular pathway (Furuse, et al., 2002; Morita & Miyachi, 2003). Additionally, TJ proteins are involved in keratinocyte differentiation and epidermal structure formation. For example, mice lacking CLDN1 display abnormalities in the stratum corneum, in addition to

weakened TJ barrier formation (Morita, Miyachi, & Furuse, 2011). Additionally, whey treatment facilitated AQP3 expression in the lateral membrane of the basal keratinocytes. AQP3 functions as a water and glycerol channel and maintains hydration in the epidermis (Boury-Jamot, et al., 2006). Conversely, whey treatment did not influence the expression of *Sgms1*, *Gba* or *Flg*, which are involved in the formation of the corenocytes/ceramide complex in the stratum corneum. These results supports that the topical application of the cheese whey positively affects hydration in the epidermis by regulating the expression of TJ proteins and AQP3.

Cheese whey contains the various physiological substances of high molecular weight such as lactoferrin, GMP, and exopolysaccharides. For example, lactoferrin stimulates epidermal keratinocyte growth and wound healing in the epidermis in the swine burn wound model (Tang, et al., 2010). Exopolysaccharides originating from *Streptococcus thermophilus* prevent ultraviolet-induced skin damage in hairless mice (Morifuji, et al., 2017). However, LMF-whey used for human keratinocyte culture model did not contain components with molecular weights higher than 3 kDa. Furthermore, LMF-whey didn't contain detectable proteins or protein fragments by silver staining after SDS electrophoresis in 15% polyacrylamide gels (data not shown). On the other hands, whey contains a high concentration of calcium and lactose of the low molecular weight. Therefore, we investigated the effects of calcium and lactose on human cultured human keratinocytes. However, under the conditions studied, neither calcium nor lactose individually influence in proliferation or expression of TJ proteins and AQP3 in this study. Cheese whey may contain bioactive peptides of low molecular weight produced during the process of the rennet enzyme reaction and fermentation in milk (Madureira, Tavares, Gomes, Pintado, & Malcata, 2010). Whey also contains abundant

phospholipids (Vaghela & Kilara, 1995). We have previously reported that topical application of phospholipids to murine dorsal skin facilitates Active keratinocyte proliferation and differentiation (Kumura, Sawada, Oda, Konno, & Kobayashi, 2012). Bioactive peptides and phospholipids may regulate keratinocyte behaviour after penetrating the epidermis. LMF-whey probably contains both bioactive peptides and phospholipids with molecular weights less than 3 kDa. However, it remains unclear which whey components regulate the proliferation and functional differentiation of keratinocytes. Further experiments are required to understand the regulatory effects of whey components on keratinocytes.

5. Conclusions

Fermented cheese whey facilitated the proliferation, differentiation, and expression of TJ proteins and AQP3 in keratinocytes both in the mouse warm-bathing model (undiluted) and the human cell culture model, following dilution. TJ proteins and AQP3 positively affect the barrier functions and hydration in the epidermis, respectively. These findings suggest that unknown compounds in cheese whey may have potential utility as a cosmetic ingredient to improve epidermal conditions, possibly as a bathwater additive.

Acknowledgements

This work was supported by the NOASTEC foundation from the Northern Advancement Center for Science & Technology. We are grateful to Prof. Fumio Nakamura, Hokkaido University, Japan for his helpful research advice.

References

Baba, H., Masuyama, A., & Takano, T. (2006). Short communication: Effects of Lactobacillus helveticus-fermented milk on the differentiation of cultured normal human epidermal keratinocytes. *J Dairy Sci, 89*, 2072-2075.

Baba, H., Masuyama, A., Yoshimura, C., Aoyama, Y., Takano, T., & Ohki, K. (2010). Oral intake of Lactobacillus helveticus-fermented milk whey decreased transepidermal water loss and prevented the onset of sodium dodecylsulfate-induced dermatitis in mice. *Biosci Biotechnol Biochem, 74*, 18-23.

Blank, I. H., Scheuplein, R. J., & MacFarlane, D. J. (1967). Mechanism of percutaneous absorption. 3. The effect of temperature on the transport of non-electrolytes across the skin. *J Invest Dermatol, 49*, 582-589.

Boury-Jamot, M., Sougrat, R., Tailhardat, M., Le Varlet, B., Bonte, F., Dumas, M., & Verbavatz, J. M. (2006). Expression and function of aquaporins in human skin: Is aquaporin-3 just a glycerol transporter? *Biochim Biophys Acta, 1758*, 1034-1042.

Bragulla, H. H., & Homberger, D. G. (2009). Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. *J Anat, 214*, 516-559.

Cheng, X., Gao, D., Chen, B., & Mao, X. (2015). Endotoxin-Binding Peptides Derived from Casein Glycomacropeptide Inhibit Lipopolysaccharide-Stimulated Inflammatory Responses via Blockade of NF-kappaB activation in macrophages. *Nutrients, 7*, 3119-3137.

Furuse, M., Hata, M., Furuse, K., Yoshida, Y., Haratake, A., Sugitani, Y., Noda, T., Kubo, A., & Tsukita, S. (2002). Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol, 156*, 1099-1111.

Gallala, H., Macheleidt, O., Doering, T., Schreiner, V., & Sandhoff, K. (2004). Nitric oxide regulates synthesis of gene products involved in keratinocyte differentiation and ceramide metabolism. *Eur J Cell Biol, 83*, 667-679.

Hara-Chikuma, M., & Verkman, A. S. (2008). Roles of aquaporin-3 in the

epidermis. *J Invest Dermatol, 128*, 2145-2151.
Hering, N. A., Andres, S., Fromm, A., van Tol, E. A., Amasheh, M., Mankertz, J., Fromm, M., & Schulzke, J. D. (2011). Transforming growth factor-beta, a whey protein component, strengthens the intestinal barrier by upregulating claudin-4 in HT-29/B6 cells. *J Nutr, 141*, 783-789.
Jiang, Q., Zhang, H., Xie, Y., & Wang, Y. (2016). Recombinant expression of porcine lactoferrin peptide LF-6 with intein technology and its immunomodulatory function in ETEC K88-infected mice. *Int Immunopharmacol, 39*, 181-191.
Jungersted, J. M., Bomholt, J., Bajraktari, N., Hansen, J. S., Klaerke, D. A., Pedersen, P. A., Hedfalk, K., Nielsen, K. H., Agner, T., & Helix-Nielsen, C. (2013). In vivo studies of aquaporins 3 and 10 in human stratum corneum. *Archives of Dermatological Research, 305*, 699-704.
Kimoto-Nira, H., Nagakura, Y., Kodama, C., Shimizu, T., Okuta, M., Sasaki, K., Koikawa, N., Sakuraba, K., Suzuki, C., & Suzuki, Y. (2014). Effects of ingesting milk fermented by Lactococcus lactis H61 on skin health in young women: a randomized double-blind study. *J Dairy Sci, 97*, 5898-5903.
Kumura, H., Sawada, T., Oda, Y., Konno, M., & Kobayashi, K. (2012). Potential of polar lipids from bovine milk to regulate the rodent dorsal hair cycle. *Journal of Dairy Science, 95*, 3629-3633.
Langbein, L., Grund, C., Kuhn, C., Praetzel, S., Kartenbeck, J., Brandner, J. M., Moll, I., & Franke, W. W. (2002). Tight junctions and compositionally related junctional structures in mammalian stratified epithelia and cell cultures derived therefrom. *Eur J Cell Biol, 81*, 419-435.
Madureira, A. R., Tavares, T., Gomes, A. M. P., Pintado, M. E., & Malcata, F. X. (2010). Invited review: Physiological properties of bioactive peptides obtained from whey proteins. *Journal of Dairy Science, 93*, 437-455.
Magnusson, B. M., Anissimov, Y. G., Cross, S. E., & Roberts, M. S. (2004). Molecular size as the main determinant of solute maximum flux across the skin. *J Invest Dermatol, 122*, 993-999.
Mayeur, S., Spahis, S., Pouliot, Y., & Levy, E. (2016). Lactoferrin, a Pleiotropic Protein in Health and Disease. *Antioxid Redox Signal, 24*,

813-836.
Morifuji, M., Kitade, M., Fukasawa, T., Yamaji, T., & Ichihashi, M. (2017). Exopolysaccharides Isolated from Milk Fermented with Lactic Acid Bacteria Prevent Ultraviolet-Induced Skin Damage in Hairless Mice. *Int J Mol Sci, 18*.
Morita, K., & Miyachi, Y. (2003). Tight junctions in the skin. *J Dermatol Sci, 31*, 81-89.
Morita, K., Miyachi, Y., & Furuse, M. (2011). Tight junctions in epidermis: from barrier to keratinization. *Eur J Dermatol, 21*, 12-17.
Peck, K. D., Ghanem, A. H., & Higuchi, W. I. (1995). The effect of temperature upon the permeation of polar and ionic solutes through human epidermal membrane. *J Pharm Sci, 84*, 975-982.
Smithers, G. W. (2008). Whey and whey proteins - From 'gutter-to-gold'. *International Dairy Journal, 18*, 695-704.
Sugiyama, Y., Yamazaki, K., Kusaka-Kikushima, A., Nakahigashi, K., Hagiwara, H., & Miyachi, Y. (2014). Analysis of aquaporin 9 expression in human epidermis and cultured keratinocytes. *Febs Open Bio, 4*, 611-616.
Svoboda, M., Bilkova, Z., & Muthny, T. (2016). Could tight junctions regulate the barrier function of the aged skin? *J Dermatol Sci, 81*, 147-152.
Tang, L., Wu, J. J., Ma, Q., Cui, T., Andreopoulos, F. M., Gil, J., Valdes, J., Davis, S. C., & Li, J. (2010). Human lactoferrin stimulates skin keratinocyte function and wound re-epithelialization. *Br J Dermatol, 163*, 38-47.
Vaghela, M. N., & Kilara, A. (1995). Quantitative-Analysis of Phospholipids from Whey-Protein Concentrates by High-Performance Liquid-Chromatography with a Narrow-Bore Column and an Evaporative Light-Scattering Detector. *Journal of the American Oil Chemists Society, 72*, 729-733.
Wang, J., Zhao, M., Liang, R., Zhang, Z., Zhao, H., Zhang, J., Li, T., & Li, Y. (2010). Whey peptides improve wound healing following caesarean section in rats. *Br J Nutr, 104*, 1621-1627.
Yadav, J. S., Yan, S., Pilli, S., Kumar, L., Tyagi, R. D., & Surampalli, R. Y. (2015). Cheese whey: A potential resource to transform into bioprotein, functional/nutritional proteins and bioactive peptides. *Biotechnol Adv, 33*, 756-774.

Yamauchi, K., Tomita, M., Giehl, T. J., & Ellison, R. T., 3rd. (1993). Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect Immun, 61*, 719-728.

Figure captions

Fig. 1.

Effects of undiluted whey on keratinocyte proliferation and differentiation in the warm-bathing model.

Images show haematoxylin and eosin (H&E; A, B) staining and immunofluorescence detection of Ki67 (red; C, D). The immunostaining images were merged with the bright field and nuclear staining images with DAPI (blue). Bars = 20 µm. (E) The graph shows the percentage of Ki-67-positive cells per total number of keratinocytes stained with DAPI. The data are presented as the mean (S.D.; n=12). Images show immunofluorescence detection of basal cell marker CK14 (green, F, G) and suprabasal cell marker CK10 (red; H, I) merged with the bright field and nuclear staining images with DAPI (blue). Bars = 20 µm. (J, K) The graphs show the relative expression levels of undifferentiated (J; *Ck5* and *Ck14*) and differentiated (K; *Ck1* and *Ck10*) cell cytokeratin by quantitative PCR. The data are presented as the mean (S.D; n = 4). Asterisks show significant differences between skins treated with water and whey. *$p < 0.05$.

Fig. 2.

Effects of undiluted whey on expression of AQP3, TJ- and stratum corneum-related genes in the warm-bathing model.

(A, B) Images show immunofluorescence detection of AQP3 (green) merged with the bright field and nuclear staining images with DAPI (blue). Bars = 20 μm. (C-E) The graphs show the relative expression levels of *Aqp3* (C), TJ- (D; *Cldn1*, *Cldn4*, *Ocln*) and stratum corneum-related genes (E; *Sgms1*, *Gba*, *Flg*) by quantitative PCR. The data are presented as the mean (S.D; n = 4). *$p < 0.05$.

Fig. 3.

Effects of LMF-whey on keratinocyte proliferation and differentiation in the human cell culture model.

(A) The images show representative phase-contrast images of human epidermal keratinocytes cultured for 4 d in the presence of 0.1, 0.33 and 1% (v/v) LMF-whey. Bars = 20 μm. (B) The graph shows the proliferation activity of human keratinocytes cultured for 5 d in the presence of LMF-whey by the WST-8 assay. The data are presented as the mean (S.D.; n=40-48). *$p < 0.05$. (C-F) Images show immunofluorescence detection of CK14 (green, C, D) and CK10 (red; E, F) merged with nuclear staining images with DAPI (blue) in human keratinocytes cultured for 4 d in the presence of 0.33% (v/v)

LMF-whey. Bars = 20 μm. (G, H) The graphs show the relative expression levels of undifferentiation (G; *CK5* and *CK14*) and differentiation marker genes (H; *CK1* and *CK10*) in human keratinocytes cultured for 4 d in the presence of 0.1, 0.33 and 1% (v/v) LMF-whey by quantitative PCR. The data are presented as the mean (S.D; n = 8). *$p < 0.05$.

Fig. 4.

Effects of LMF-whey on AQP3, TJ- and stratum corneum-related genes in the human cell culture model.

(A, B) Images show immunofluorescence detection of AQP3 (green, A, B) merged with nuclear staining images with DAPI (blue) in human keratinocytes cultured for 4 d in the presence of 0.33% (v/v) LMF-whey. Bars = 20 μm. (C) AQP3, CLDN1, CLDN4, and OCLN were detected by Western blotting in human keratinocytes cultured for 4 d in the presence of 0.1, 0.33 and 1% (v/v) LMF-whey. (D) Graphs show the corresponding results of densitometry analysis. Beta-actin was used as an internal control (S.D; n = 5). *$p < 0.05$. (E-G) The graphs show the relative expression levels of *AQP3* (E), TJ- (F; *CLDN1*, *CLDN4*, *OCLN*) and stratum corneum-related genes (G; *SGMS1*, *GBA*, *FLG*) by quantitative PCR in human keratinocytes cultured for 4 d in the presence of 0.1, 0.33

and 1% (v/v) LMF-whey. The data are presented as the mean (S.D; n = 8). *$p < 0.05$.

Fig. 5.

Effects of lactose and calcium on keratinocyte behaviour in the human cell culture model.

(A) The graph shows the proliferation activity of human keratinocytes cultured for 5 d in the presence of lactose and calcium by the WST-8 assay. The data are presented as the mean (S.D.; n=7). (B) AQP3 and OCLN were detected by Western blotting in human keratinocytes cultured for 4 d in the presence of 0.33% LMF-whey, 0.13 mg/ml lactose and 0.03 mM calcium (C) Graphs show the corresponding results of densitometry analysis. Beta-actin was used as an internal control (S.D; n = 4). *$p < 0.05$.

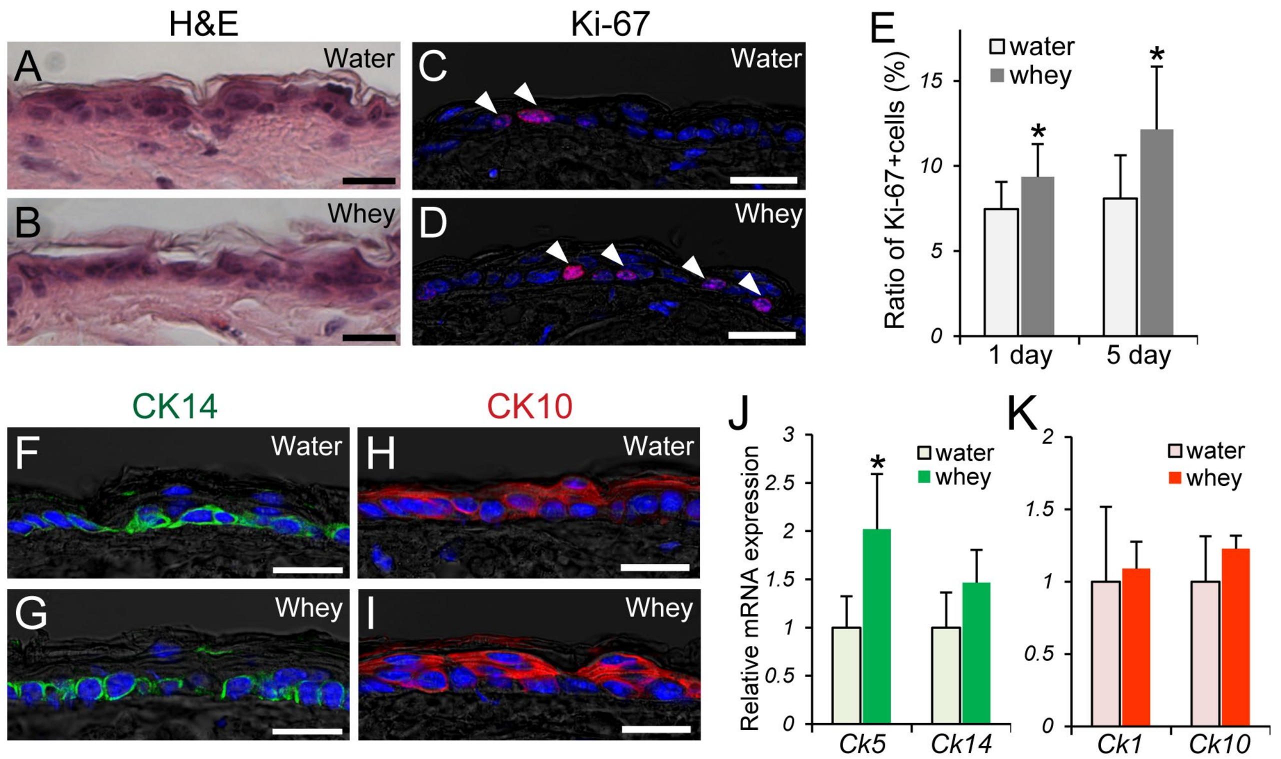

Fig. 1.

Effects of undiluted whey on keratinocyte proliferation and differentiation in the warm-bathing model. Images show haematoxylin and eosin (H&E; A, B) staining and immunofluorescence detection of Ki67 (red; C, D). The immunostaining images were merged with the bright field and nuclear staining images with DAPI (blue). Bars = 20 µm. (E) The graph shows the percentage of Ki-67-positive cells per total number of keratinocytes stained with DAPI. The data are presented as the mean (S.D.; n=12). Images show immunofluorescence detection of basal cell marker CK14 (green, F, G) and suprabasal cell marker CK10 (red; H, I) merged with the bright field and nuclear staining images with DAPI (blue). Bars = 20 µm. (J, K) The graphs show the relative expression levels of undifferentiated (J; *Ck5* and *Ck14*) and differentiated (K; *Ck1* and *Ck10*) cell cytokeratin by quantitative PCR. The data are presented as the mean (S.D; n = 4). Asterisks show significant differences between skins treated with water and whey. $*p < 0.05$.

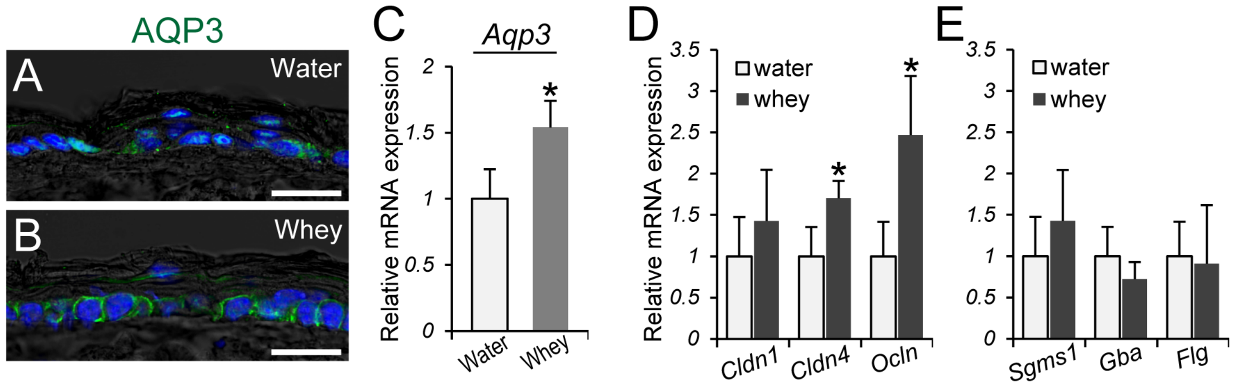

Fig. 2.
Effects of undiluted whey on expression of AQP3, TJ- and stratum corneum-related genes in the warm-bathing model.
(A, B) Images show immunofluorescence detection of AQP3 (green) merged with the bright field and nuclear staining images with DAPI (blue). Bars = 20 µm. (C-E) The graphs show the relative expression levels of *Aqp3* (C), TJ- (D; *Cldn1*, *Cldn4*, *Ocln*) and stratum corneum-related genes (E; *Sgms1*, *Gba*, *Flg*) by quantitative PCR. The data are presented as the mean (S.D; n = 4). *$p < 0.05$.

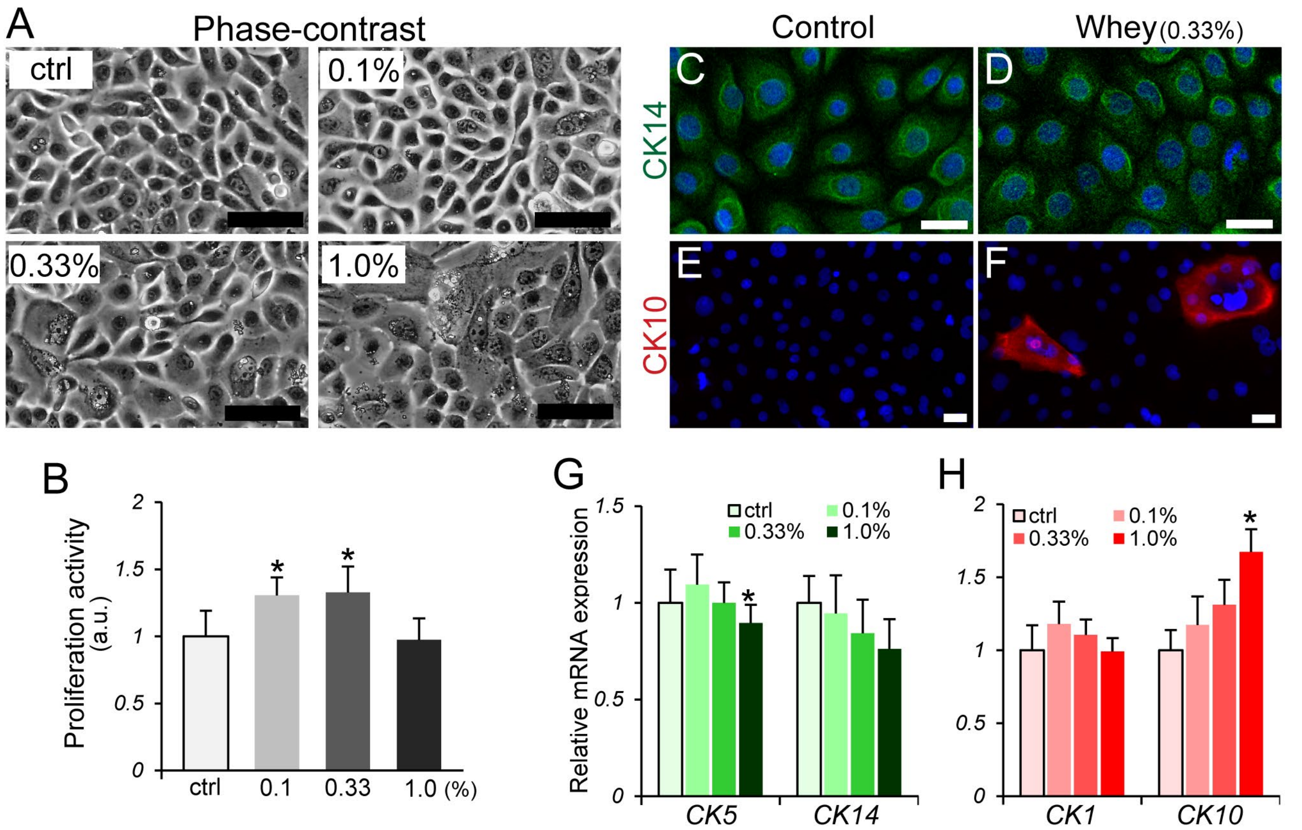

Fig. 3.

Effects of LMF-whey on keratinocyte proliferation and differentiation in the human cell culture model.

(A) The images show representative phase-contrast images of human epidermal keratinocytes cultured for 4 d in the presence of 0.1, 0.33 and 1% (v/v) LMF-whey. Bars = 20 µm. (B) The graph shows the proliferation activity of human keratinocytes cultured for 5 d in the presence of LMF-whey by the WST-8 assay. The data are presented as the mean (S.D.; n=40-48). *$p < 0.05$. (C-F) Images show immunofluorescence detection of CK14 (green, C, D) and CK10 (red; E, F) merged with nuclear staining images with DAPI (blue) in human keratinocytes cultured for 4 d in the presence of 0.33% (v/v) LMF-whey. Bars = 20 µm. (G, H) The graphs show the relative expression levels of undifferentiation (G; *CK5* and *CK14*) and differentiation marker genes (H; *CK1* and *CK10*) in human keratinocytes cultured for 4 d in the presence of 0.1, 0.33 and 1% (v/v) LMF-whey by quantitative PCR. The data are presented as the mean (S.D; n = 8). *$p < 0.05$.

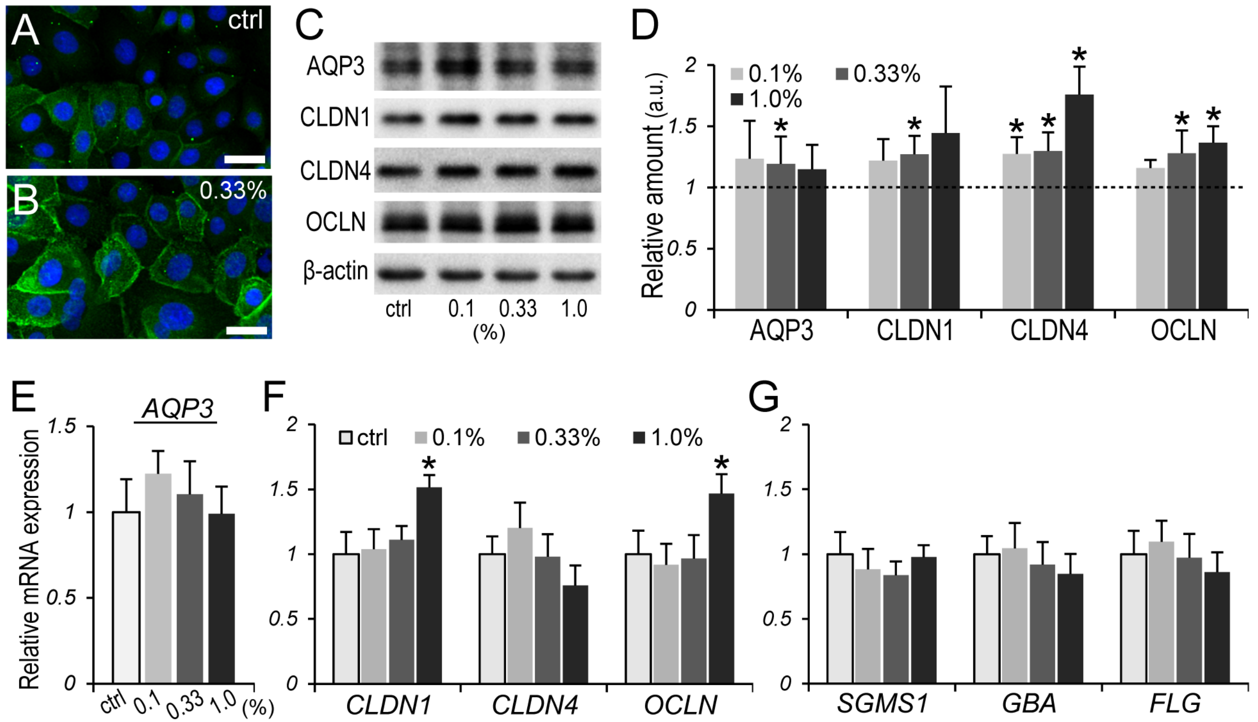

Fig. 4.

Effects of LMF-whey on AQP3, TJ- and stratum corneum-related genes in the human cell culture model.

(A, B) Images show immunofluorescence detection of AQP3 (green, A, B) merged with nuclear staining images with DAPI (blue) in human keratinocytes cultured for 4 d in the presence of 0.33% (v/v) LMF-whey. Bars = 20 µm. (C) AQP3, CLDN1, CLDN4, and OCLN were detected by Western blotting in human keratinocytes cultured for 4 d in the presence of 0.1, 0.33 and 1% (v/v) LMF-whey. (D) Graphs show the corresponding results of densitometry analysis. Beta-actin was used as an internal control (S.D; n = 5). *$p < 0.05$. (E-G) The graphs show the relative expression levels of *AQP3* (E), TJ- (F; *CLDN1*, *CLDN4*, *OCLN*) and stratum corneum-related genes (G; *SGMS1*, *GBA*, *FLG*) by quantitative PCR in human keratinocytes cultured for 4 d in the presence of 0.1, 0.33 and 1% (v/v) LMF-whey. The data are presented as the mean (S.D; n = 8). *$p < 0.05$.

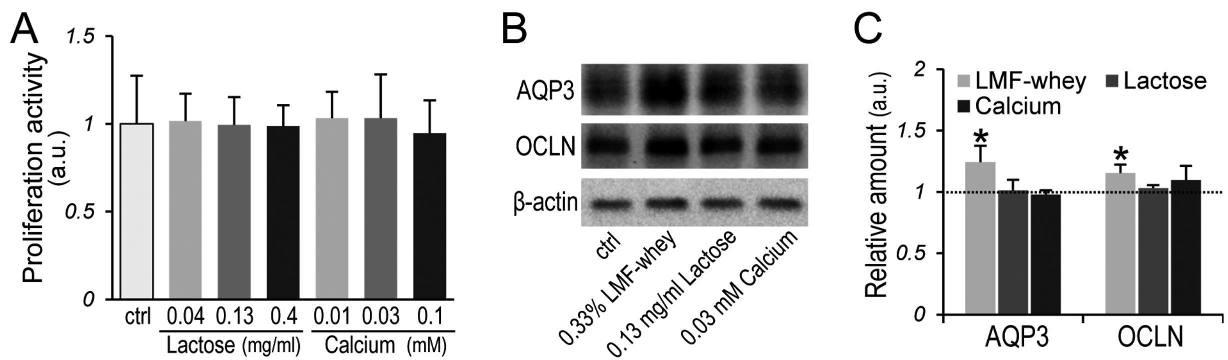

Fig. 5.

Effects of lactose and calcium on keratinocyte behaviour in the human cell culture model.

(A) The graph shows the proliferation activity of human keratinocytes cultured for 5 d in the presence of lactose and calcium by the WST-8 assay. The data are presented as the mean (S.D.; n=7). (B) AQP3 and OCLN were detected by Western blotting in human keratinocytes cultured for 4 d in the presence of 0.33% LMF-whey, 0.13 mg/ml lactose and 0.03 mM calcium (C) Graphs show the corresponding results of densitometry analysis. Beta-actin was used as an internal control (S.D; n = 4). *$p < 0.05$.