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Dairy bioactives and functional ingredients with skin health benefits

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ABSTRACT

The potential of whey from Italian cheese productions to support skin health and anti-ageing mechanism was studied. The effect of whey on dermal and epidermal cells was evaluated. Whey inhibited the activity of elastase and tyrosinase enzymes by 60% and 32%, respectively. Whey cytotoxicity against tested skin cell lines, human fibroblasts (HDFa) and keratinocytes (HaCaT) was not observed. Moreover, the antioxidant activity of the samples was noted, after treatment with whey intracellular ROS level was decreased by 87% in comparison with the hydrogen peroxide-treated cells. Fibroblasts produced a significant amount of extracellular matrix molecules, collagen I, elastin and glycosaminoglycans as a result of treatment with tested whey. In addition, the ability of samples to improve the cell barrier integrity of keratinocytes was proven. The obtained results indicate that pure whey supports skin health and shows potential to be used by the cosmetic industry.

1. Introduction

Cheese is the second most frequently consumed milk product, right after fresh dairy commodities. Each year, over 22 megatons of various types of cheese are produced worldwide. The large-scale production of cheeses is also associated with a large amount of generated by-products, whey. It is estimated that one kg of produced cheese generates 9–10l of whey. Globally each year 100 million tonnes of cheese whey are produced (Flinois et al., 2019; Buchanan et al., 2023) and this value is estimated to increase 1–2% annually due to the growing consumer demand (Buchanan et al., 2023). For instance, Italy, one of the biggest cheese industries in the European Union, produces at least 10 thousand tons of whey every year (Italy: Whey Powder from Cheese, 2023).

Whey is a threat to the natural environment and considered as a pollutant due to its high content of organic matter, high bio-chemical oxygen demand and the scale of its generation (Chatzipaschali & Stamatis, 2012). This is particularly the case if we consider that a huge amount of it is undeveloped and disposed of as wastewater, as only half of the global production of whey is reused in food sector (Macwan et al., 2016; Lavelli & Beccalli, 2022). Transforming our world: the 2030 Agenda for Sustainable Development adopted by all Member States of United Nations in September 2015, includes 17 main goals of sustainable development and 169 related targets. The objectives cover a wide range of challenges, including those related to waste management. The goal by 2030 is to substantially reduce waste generation through prevention, reduction, recycling and reuse. To achieve this goal, it is necessary to re-examine waste generated from various branches of industry, including the dairy processing. Industry and researchers have, therefore, started to look for new opportunities to use dairy waste, the largest percentage of which is whey. Development of value-added products from whey seems particularly advantageous from the point of view of small and medium enterprises (SMEs), comprising more than 80% of the total number of dairy companies in the European Union. As whey processing into value-added products is correlated with high energy consumption, it is only applicable in large size enterprises where the large scale of producing whey allows for cost compensation. For small-scale cheese producers, the cost associated with whey generation is between €3500 and €7000 each year. New applications of whey would therefore support SMEs in terms of whey recycling cost (Lavelli & Beccalli, 2022).

This clear liquid left over from curdling the milk during the production of cheese or casein is a rich source of nutritional values – proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins, lactoferrin, lysozyme), minerals (calcium, zinc, magnesium, phosphorus, copper, sodium, potassium, chlorine, iron), vitamins (especially B-complex vitamins), amino acids and lactose

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(Blažić et al., 2018; Ha & Zemel, 2003; Papademas & Kotsaki, 2020). The presence of these nutrients that are easily digestible for humans means that whey can be viewed as a value-added material and not merely a difficult to utilize waste from dairy processing. The health benefits of whey have been appreciated since ancient times. The Father of Modern Medicine, Hippocrates, prescribed whey to his patients to support their immune system. In the Middle Age whey was used as a medicine to cure various diseases, aphrodisiac to inspire vitality and a skin balm for soothing burns (Kosikowski, 1982; Smithers, 2008). Modern science also sees the advantages of using pure whey and its individual components. Cheese whey has the ability to maintain muscle health and reduce effects of sarcopenia (Kang et al., 2020; Chanet et al., 2017; Hou & Madden, 2022). The positive effects of whey on bone health were observed in both *in vitro* and *in vivo* studies (Langsetmo et al., 2018; Douglas et al., 2018). Research has shown that whey and whey proteins affect cardiovascular system through increase in blood pressure and reduction of total cholesterol (Fekete et al., 2018; Fekete et al., 2016).

Pure whey as well as its individual components (e.g. proteins) are gaining more and more interest in the scientific community. Due to its composition (i.a. lactose content), whey is an excellent medium for the growth of microorganisms, which makes it a substrate in the production of many valuable products. The use of whey-based medium for culture of different bacterial or yeast strains (e.g. *Lactobacillus casei*, *Lactobacillus plantarum*, *Bacillus coagulans*, *Lactobacillus delbrueckii*, *Lactococcus lactis*, *Rhodovulum sulfidophilum*, *Haloferax mediterranei*, *Pseudomonas hydrogenovora*, *Pseudomonas taetrolens*, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Kluyveromyces lactis, Yarrowia lipolytica*) resulted in production of lactic acid (Catone et al., 2021; Nagarajan et al., 2020; Olszewska-Widdrat et al., 2020; Sahoo & Jayaraman, 2019; Luongo et al., 2019), bioplastics and biopolymers (Carlozzi et al., 2021; Raho et al., 2020; Fradinho et al., 2019; Oliveira et al., 2018); polyhydroxyalkanoates (Koller et al., 2017), bioethanol (Gabardo et al., 2014; Sampaio et al., 2020; Beniwal et al., 2021; Tesfaw et al., 2021; Alves et al., 2019; Murari et al., 2019), single cell proteins (Yadav et al., 2016; Nayeem et al., 2017) and single cell oil (Taskin et al., 2015). Products made by whey utilization, as well as the recovered individual components, are not only of industrial importance, but are also important because of their biological activity. From a health benefit perspective, the most important component of whey are proteins. Scientific data indicates that whey-derived peptides possess a range of valuable biological properties for human health. Whole whey proteins and protein subfractions (α-lactalbumin, bovine serum albumin, lactoferrin) have anticancer activity, what was proven using different cancer cell line models, from both human and animals (Kennedy et al., 1995; See et al., 2002; Gillis et al., 2016; Madzima et al., 2017; Bumrungpert et al., 2018; Sternhagen & Allen, 2001; Laursen et al., 1990; Cheng et al., 2017; Attaallah et al., 2012; Xiao et al., 2006). Various *in vitro* methods were used to evaluate antioxidant potential of whey proteins- DPPH radical assay, ABTS assay, ferric-reducing antioxidant power, and oxygen radical absorbance capacity. The results indicate that whey-derived proteins have ability to reduce oxidative stress (Power-Grant et al., 2016; Ortega et al., 2015; Zhang et al., 2012; Kerasioti et al., 2014).

Another application for cheese whey is production of whey-based beverages. They consist of whey alone (processed and unprocessed), but might also be supplemented with fruit or vegetable juices, milk, probiotics or other additives (Özer & Evrendilek, 2022). Using whey as a raw material it is possible to manufacture a wide range of non-alcoholic and alcoholic beverages (whey wine, champagne, beer). Whey used for the beverage production can be fermented before the production process, and the resulted products are considered to be the main functional dairy-based beverages (Turkmen et al., 2019). In addition to the production of beverages, whey, by-product from cheese manufacturing, might be used as a starter for production of different types of cheese, called whey cheeses, such as Ricotta (Italy), Brunost and Primost (Norway), Anari (Cyprus) and many more. Whey derived from production of casein-based cheeses is thermally treated (90 °C) to coagulate whey proteins and mixed with organic acids, mineral salts (Smithers, 2008). Certain type of whey cheeses are produced through the evaporation of water for starting whey (Jelen & Zadow, 1992). The use of whey for production of cheeses increases the application repertoire for this dairy waste, but does not solve the issue related to the huge amount of this product generated from the industry (Farkye, 2017). There is a need to find new sustainable, environmentally and economic friendly applications for cheese whey.

There are not many studies assessing the potential of whey in the cosmetics industry, but the available results, from experiments performed on mice, indicate that whey might be considered as an anti-aging agent (Kimura et al., 2014). *In vivo* studies involving humans also showed a positive bioactivity of whey in the context of skin health (Sobkowska et al., 2021).

The objective of the present study was to investigate the effect of whey derived from production of three Italian cheeses (namely Caciotta, Squacquerone and Ricotta) on skin health. Tested samples were analysed for their cytotoxicity against dermal and epidermal cells (fibroblasts and keratinocytes), antioxidant, skin-whitening and anti-wrinkle properties.

2. Materials and methods

2.1. Materials

Whey, a by-product of production of three Italian cheeses, Caciotta, Ricotta and Squacquerone, were supplied by Mambelli srl (Bertinoro, FC, Italy). Samples were stored at 4 °C and filter sterilized (0.2 μm) before analysis.

2.2. Elastase inhibition assay

Elastase inhibition was measured using commercially available fluorimetric assay – The SensoLyte® Green Elastase Assay Kit, according to the manufacturer's instructions (AnaSpec, Inc., Fremont, CA, USA). Kit uses natural substrate elastin labelled with the 5-FAM fluorophore and the QXL™ 520 quencher. Proteolytic cleavage of labelled elastin yields brightly green fluorescence, which can be continuously monitored at excitation/emission = 488 nm/520 nm. Increase in fluorescence intensity is directly proportional to elastase activity. Tested concentrations of analysed whey samples were prepared in purified water.

2.3. Tyrosinase inhibition assay

Tyrosinase inhibition was measured according to the protocol described by Kang et al. (2012). Briefly, a reaction mixture (200 μl) containing phosphate buffer (50 mM, pH 6.5, 140 μl), L-tyrosine (1.5 mM, 40 μl), sample solution (10 μl), and mushroom tyrosinase solution (1000 units/ml, 10 μl) in a 96-well plate was incubated at 37 °C for 12 min. Then, the reaction was stopped by cooling the plate on ice for 5 min. The amount of dopachrome was measured at 490 nm using Thermo Scientific™ Varioskan™ LUX microplate reader. Purified water was used to prepare tested concentrations of whey samples.

2.4. Cell culture

Human Dermal Fibroblasts, adult (HDFa) were purchased from Thermo Fisher Scientific (C0135C). The cells were maintained in Medium 106 supplemented with 2% foetal bovine serum (FBS),

1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor and 10 μg/ml heparin (Gibco). Human Adult Low Calcium High Temperature Keratinocytes (HaCaT) were acquired from Cell Line Service GmbH (300493) and maintained in no calcium DMEM supplemented with 4.5 g/l glucose, 1% L-glutamine and 10% FBS. Both cell lines were cultured in a humidified incubator at

37 °C and 5% CO2.

2.5. Cytotoxicity assay

Fibroblasts and keratinocytes were seeded at a density of 10,000 cells per well in 96-well plates in 100 µl of complete culture media. Cells were allowed to adhere overnight. After 24 h the cell culture media was carefully removed and replaced with culture media supplemented with sterile-filtered tested samples at concentrations between 100% and 0.05%. Media only was used to treat control cells. After 24 h of incubation, PrestoBlue® Cell Viability Reagent (Thermo Fisher, Waltham, MA, USA) was added to each well of the 96-well plate to the final concentration of 10%. Plates were incubated in the dark for 2 h at 37 °C. Fluorescence was read using a 560 nm excitation/590 nm emission filter set (10 nm bandwidth) with a Thermo Scientific™ Varioskan™ LUX spectrophotometer. Fluorescence data in wells containing cells were corrected for background fluorescence using cell-free media control replicates.

2.6. Cellular antioxidant activity (CAA) assay

HDFa were seeded at a density of 25,000 cells per well in black, clear bottom 96-well plates in 100 µl of complete cell culture media and allowed to adhere overnight. Cell culture media was carefully removed, and the cells were washed twice with PBS. Cells were treated with the tested samples (sterile-filtered) in medium containing 50 µM 2′,7′-dichlorofluorescin diacetate (DCF-DA; Sigma Aldrich). After 60 min of incubation, 0.25 mM hydrogen peroxide was added to the wells for 60 min. Supernatants were removed from the wells, the cells were washed with PBS. Fluorescence was read using a 485 nm excitation/535 nm emission filter set with a Thermo Scientific™ Varioskan™ LUX spectrophotometer.

2.7. Determination of extracellular matrix molecule expression

2.7.1. Collagen I detection

HDFa were seeded at a density of 10,000 cells per well in 96-well plates in 100 µl of complete culture media. After 24 h of incubation, the cells were treated with the sterile tested samples for 48 h (37 °C, 5% CO_2). Supernatants were used to analyze the level of collagen I using Human Pro-Collagen I alpha 1 DuoSet ELISA kit according to the manufacturer's instruction (R&D Systems).

2.7.2. Elastin detection

Elastin was detected using Human ELN ELISA according to the manufacturer's instruction (Bioassay Technology Laboratory). HDFa were seeded at a density of 10,000 cells per well in 96-well plates in 100 µl of complete culture media. After 24 h of incubation, the cells were treated with the test samples for 24 h (37 °C, 5% CO2). Supernatants were used to analyse the level of elastin.

2.7.3. Glycosaminoglycans detection

Human dermal fibroblasts were seeded at a density of 60,000 cells per well in 12-well plates in 1 ml of complete cell culture media. After 24 h of incubation, the cells were treated with sterile tested samples for another 72 h. 20 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA, 2 mM dithiothreitol and 300 µg/mL papain was placed directly on the cell layer after removing of supernatants. The cells were incubated for 60 min at 60 °C. Glycoaminoglycans in papain-digested samples were measured by colorimetric assay with the metachromatic dye 1, 9-dimethylmethylene blue using commercially available kit - Proteoglycan Detection Kit (Amsbio, Cambridge, MA, USA) according to the manufacturer's instructions.

2.8. Transepithelial electrical resistance (TEER) measurement

HaCaT cells were cultured in 12-well cell culture plates using Corning® inserts (12 mm Transwell® with 0.4 µm Pore Polyester Membrane). When cells reached confluency, complete no calcium cell culture media was replaced with high calcium concentration (1.8 mM) DMEM with 10% FBS. After 10 days inserts containing the confluent differentiated keratinocytes were transferred to new 12-well plates. The cells were treated with sterile-filtered tested samples – 1 ml of treatment was added to the apical side and 2 ml to the basolateral side. EVOM2™ (Epithelial Volt/Ohm (TEER) Meter) with chopsticks electrode was used according to the manufacturer's instructions (Precision Instruments, Sarasota County, FL, USA) to measure TEER after 6 and 24 h.

2.9. Statistical analysis

All results were expressed as the means ± standard deviation from three independent experiments. GraphPad Prism 9 (GraphPad Software, CA, USA) and Dunnets's multiple comparison tests were used to analyze significant differences ($p < 0.05$) between the mean values of the individual group.

3. Results and discussion

3.1. Whey composition

The composition of four different batches of cheese whey is reported in the Table 1.

Ricotta whey was obtained from the production of Ricotta, a common whey cheese that can be made from sheep, cow, goat, or Italian water buffalo milk whey. The consumption of Ricotta in Italy was estimated to be more than 55 thousand tons/year (ISTAT, 2013). Caciotta whey derives from the production of Caciotta, a soft or semi-hard cheese with a short (15–20 days) or long (2–6 months) ripening time which includes also some specific Protected Designation of Origin (PDO) products. The production of Caciotta in Italy has been estimated to be 23 thousand tons per year (ASSOLATTE). Eventually, Squacquerone whey is the by-product obtained from Squacquerone cheese production. This is a fresh cheese that possess the PDO status and can be produced only in a specific area of Emilia-Romagna region of Italy. According to the Consultancy and Market Research Food and Dairy (CLAL), 2 thousand tons of Squacquerone were produced in 2021.

3.2. Elastase inhibition

Elastic fibers located in the dermal layer of the skin and consisted

Table 1
Composition of cheese whey used in the work.

	Caciotta whey	Ricotta whey	Squacquerone whey
Proteins (g/l)	11.8 ± 1.2	5.7 ± 0.5	11.5 ± 0.6
Fats (g/l)	5.3 ± 0.5	3.0 ± 0.1	3.7 ± 0.6
Glucose (g/l)	< 1	< 1	1.7 ± 0.6
Lactose (g/l)	47.3 ± 0.5	46.5 ± 0.6	49.2 ± 0.5
Phosphorus	476.2 ± 33.1	330.8 ± 37.6	487.7 ± 29.3
Phosphates as PO_4 (by calculation) (mg/kg)	1507. ± 98.7	1014.6 ± 115.3	1523.7 ± 85.6
Calcium (mg/kg)	404.2 ± 3.1	330.0 ± 18.6	339.3 ± 104.6
Iron (mg/kg)	< 2	< 2	< 2
Potassium (mg/kg)	1444.0 ± 61.9	1425.7 ± 50.7	1504.3 ± 126.3
Sodium (mg/kg)	3563.5 ± 92.3	2548.5 ± 262.1	2886.0 ± 112.9
Zinc (mg/kg)	< 2	< 2	< 2
Iodine (mg/kg)	0.51 ± 0.01	2.64 ± 0.01	0.52 ± 0.03

mainly of elastin (90%), are responsible for skin strength and elasticity (Mecham, 1991). Elastin is one of the longest-lived human proteins, with about 70-years half-life, showing remarkable resistance to internal and external stimuli (Shapiro et al., 1991). Even for a protein with such properties, multiple exposures to enzymatic processes are significant. Enzymes from the proteases class, elastases, are responsible for enzymatic cleavage of elastin and degradation of elastic fibers. Activity of the enzymes increases with aging and is also negatively influenced by the external environment, including exposure to UV radiation. Elastogenesis, a process of assembly of tropoelastin into elastin fibers, occurs during the foetal and early neonatal stage of skin and other organs development (Ozsvar et al., 2021; Wang et al., 2019; Swee et al., 1995). Multiple exposure of elastin to enzymatic degradation leaves traces visible on the skin in the form of wrinkles and sagging skin (Tsuji et al., 2001; Shin et al., 2019). Therefore, compounds that inhibit the action of elastase can improve skin condition and can be considered potential anti-wrinkle agents.

The ability of tested whey samples to reduce activity level of elastase was measured using commercially available fluorimetric assay. The obtained results are presented in Fig. 1.

Statistically significant inhibition of elastase activity was observed after using samples derived from all three types of cheeses. The highest elastase inhibitory effect was possessed by Squacquerone-derived whey used at concentration 22.5% where the activity of enzyme was inhibited by 60%. Anti-elastase activity of whey was observed previously by Benfeldt et al. (Benfeldt et al., 1998). The results obtained by the researchers from analysis of sweet whey enzyme inhibition potential indicated that rennet whey possess the inhibitory activity against trypsin, elastase, and plasmin. The methodology used in the study was not sufficient to identify the specific components of the whey responsible for the observed effect. Santos et al. (Santos et al., 2021) examined potential inhibitory activity of fermented cheese whey against two matrix metalloproteases, MMP-2 and MMP-9. Whey from the manufacturing of cheese from a milk of ewe, goat, and cow was fermented with lactic acid bacteria (LAB) consortium. As a result of the process, 11 polypeptides were obtained. The whey-derived compounds have been found to inhibit MMP-2 and MMP-9 activity. These two enzymes from the subgroup of gelatinases are characterized by broad substrate specificity and can degrade extracellular matrix molecules like collagen type IV, V, VII, X, fibronectin, laminin, entactin, aggrecan, and even elastin (Van den Steen et al., 2002; Pittayapruek et al., 2016). Reduction in the activity level of MMP-2 and MMP-9 was also reported by Kimura et al. (Kimura et al., 2014) in an *in vivo* mice study. Oral administration of whey peptides decreased activity of both enzymes and protected animal skin from harmful effect of UVB radiation – inhibition of skin thickness increased, wrinkle formation, pigmentation and a reduction in skin elasticity were observed. Besides the protein constituents of cheese whey, skin anti-aging properties were also reported for the lipid fraction constituting between 0.1% and 0.2% of the whey. The analysis of Swiss cheese whey composition revealed that the major phospholipid components of the lipid fraction were phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin (Kamath & Morr, 1997). The *in vivo* study of Higurashi et al. (Higurashi et al., 2015) investigated the effect of orally administered sphingomyelin-containing milk phospholipid concentrate on, among others, skin elasticity. The results showed a significant positive change in skin elasticity. The observed effect has been linked by researchers with the fact that sphingomyelin can promote synthesis of extracellular matrix component, ceramides (Haruta et al., 2008; Haruta-Ono et al., 2012; Duan et al., 2012).

3.3. Tyrosinase inhibition

Tyrosinase is an enzyme involved in the process of melanogenesis, converting tyrosine to dihydroxyphenylalanine (DOPA) and therefore it is responsible for skin pigmentation (Iwata et al., 1990). The increased synthesis and accumulation of skin pigment, melanin, occur in many types of skin disorders, including i.a. acanthosis nigricans, cervical poikiloderma, melasma, Riehl's melanosis, periorbital hyperpigmentation, lentigines, lichen planus, nevus of Ota (Cestari et al., 2014; Dorga & Sarangal, 2014). Compounds with ability to inhibit tyrosinase activity could be used as a skin-lightening agent. Cheese whey potential to influence melanogenesis through inhibition of tyrosinase enzymatic action was assessed using mushroom tyrosinase and L-tyrosine as a substrate.

As can be seen from Fig. 2, only one of three tested samples showed

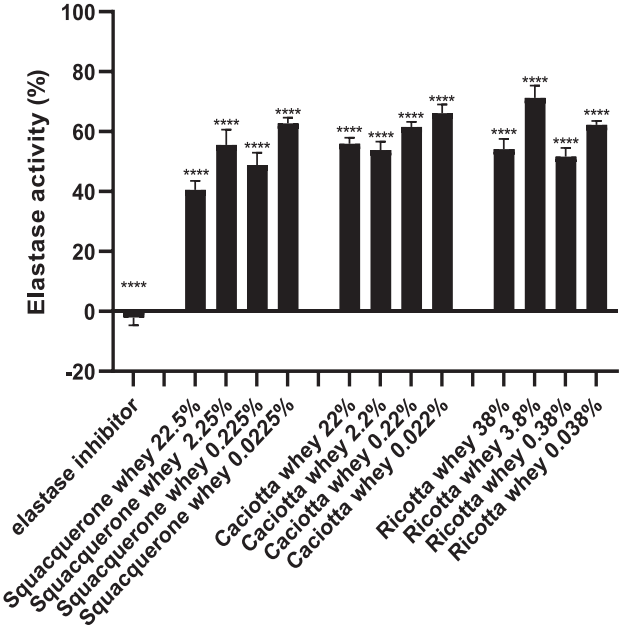

Fig. 1. Effect of Squacquerone, Caciotta and Ricotta-derived whey on elastase activity. Results are presented as a percentage of the elastase activity. 1 mM MeOSuc-Ala-Ala-Pro-Val-CMK was used as a positive control (elastase inhibitor). Bars correspond to mean ± SEM of three independent experiments. **** $p < 0.0001$ vs. negative control (elastase activity; 100%).

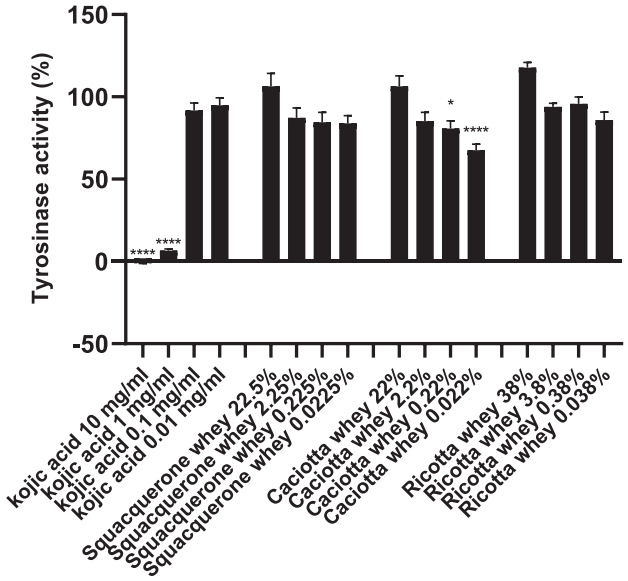

Fig. 2. Inhibitory effects of Squacquerone, Caciotta, and ricotta-derived whey on tyrosinase activity. Results are presented as a percentage of the untreated control. Bars correspond to mean ± SEM of three independent experiments. Kojic acid was used as positive control. * $p < 0.05$; **** $p < 0.0001$ vs. negative control (tyrosinase activity; 100%).

anti-tyrosinase activity: Caciotta-derived whey. Statistically significant reduction in tyrosinase activity was observed after using this sample at concentrations 0.22% and 0.022%, the inhibitory rates were 19.19% and 32.38%, respectively. The use of two other types of whey did not significantly affect the tyrosinase activity. The potential anti-tyrosinase activity of Ricotta-derived whey was examined by Monari et al. (Monari et al., 2019). The authors investigated ability of scotta, by-product of Ricotta production, to inhibit tyrosinase and the results were presented as grams of kojic acid (KA) equivalents per litre (gKA eq/L). The experiment was performed with pure whey and product after enzyme-based digestion (with pancreatin and pepsin). Obtained results show that anti-tyrosinase activity of digested samples was 2-times higher when compared to the non-digested sample. Low molecular weight peptides produced by papain treatment show anti-tyrosinase activity up to 0.14 gKA eq/L. Melanin formation occur in specific tyrosinase-containing melanocyte organelle, melanosomes (melanin granules) (D'Alba & Shawkey, 2019). *In vivo* study of Kimura et al. (Kimura et al., 2014) performed on UVB-irradiated mice, revealed that oral administration of whey proteins resulted in the reduction of melanin granules expression in the stratum basale. Whey produced by fermentation of reconstituted, pasteurized 9% (w/w) skim milk with *Lactobacillus helveticus* CM4 has been investigated *in vitro* as a potential anti-tyrosinase agent (Ikarashi et al., 2020). Mouse B16 melanoma cells were treated with α-melanocyte-stimulating hormone (α-MSH; inducer of tyrosinase expression) alone or simultaneously tested whey. The results indicated that the whey significantly suppressed tyrosinase expression at both protein and mRNA level.

3.4. Cytotoxicity assay

Cytotoxicity assessment is the most common method used to ensure the safety of every material tested for its biological activities. In the present study, human dermal fibroblasts and immortalized keratinocytes were treated with tested whey samples for 24 h and the viability of the cells was measured using fluorescent dye, PrestoBlue™. Tested cells reflect two of the three layers of skin- epidermis (keratinocytes) and dermis (fibroblasts). The choice of cell lines was dictated by the purpose of the study, the assessment of the whey effect on the human skin.

The results of the performed cytotoxicity experiment are displayed in Fig. 3.

As can be seen from the graphs, the observed dose–response effect was non-linear. This is not an uncommon phenomenon that obtained cellular response to the different concentrations of treatment is complex and non-linear. This is particularly noticeable when the tested sample is not pure, single compound but a mixture of many components. Various extracts of natural origin might be an example of such samples. Cheese whey can be also included in this group, as it is a combination of proteins, sugars, amino acids, minerals and vitamins.

In the case of human dermal fibroblasts, the only cytotoxic concentration of Ricotta-derived whey was the highest one (100%) and the cell viability was 24.51% (Fig. 2A). The other two tested whey, from Caciotta and Squacquerone, significantly decreased cell viability when

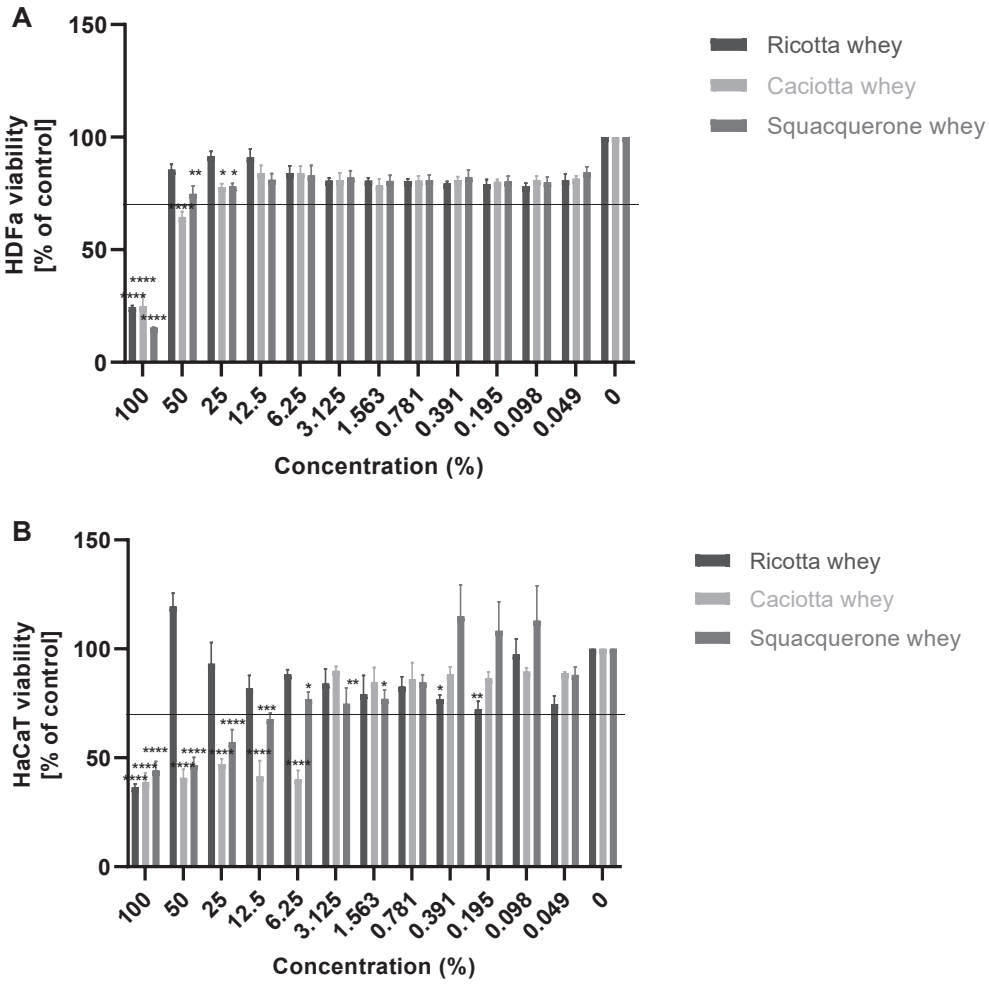

Fig. 3. Cytotoxicity evaluation of Ricotta, Caciotta and Squacquerone-derived whey with the PrestoBlue™ assay. HDFa (A) and HaCaT (B) were treated with different concentrations of whey for 24 h. Results are presented as a percentage of the untreated control. Bars correspond to mean ± SEM of two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs. untreated cells. The black line indicates the cytotoxicity limit according to ISO 10993–5.

used at the three highest concentrations, between 100% and 25%. There were no statistically significant changes in cell viability compared to untreated control cells for concentrations lower than or equal to 12.5%. Therefore, concentrations from 12.5% to 3.125% (for Caciotta and Squacquerone) and from 50% to 12.5% (for Ricotta) were used for further studies. Cellular response pattern of immortalized keratinocytes differed from that displayed by fibroblasts. Ricotta-derived whey significantly decreased cell viability not only at the highest concentration, 100%, but also at two lower concentrations, 0.391% and 0.195%. Both tested samples, Caciotta and Squacquerone whey, significantly reduced keratinocytes viability at concentrations between 100% and 6.25%. Moreover, the viability rate of HaCaT, after treatment with 3.125% and 1.563% Squacquerone-derived whey, was significantly decreased when compared to the control cells. Three doses of tested samples were chosen for further experiments, 3.125% and 1.563% (Caciotta whey), 25% and 12.5% (Ricotta whey), 0.781% and 0.391% (Squacquerone whey).

3.5. Cellular antioxidant activity (CAA) assay

Antioxidants are a group of chemical compounds with the ability to scavenge reactive oxygen species and stop or delay the oxidation of other molecules. The potential of dairy by-products to protect the cells from reactive oxygen species (ROS) and oxidative stress was the focus of a series of studies. Whey proteins have relatively a lot of sulfur-containing amino acids (cysteine, methionine) and such composition contributes to ROS scavenging. Moreover, one of the whey components, lactoferrin, has the property of chelating agent (Carthy et al., 2015). Lactoferrin increases bioavailability of iron by its chelation and inhibits pro-oxidant effects (Cutone et al., 2020). Another ingredient in whey, β-lactoglobulin, also owes its antioxidant effect to the presence of sulfur amino acids. It was found that a half of milk total antioxidant capacity derived from a presence of β-lactoglobulin (Liu et al., 2007). Whey proteins were also reported to increase level of glutathione peroxidase – a family of enzymes with peroxidase activity, with the main function of cell protection against oxidation by biochemical processes-derived peroxides (Clouatre, 1999).

In the present study, the ability of cheese-derived whey to protect the cells from oxidative stress by reducing the level of reactive oxygen species was measured using DCF assay. As evident in Fig. 4, two tested whey, derived from Caciotta and squacquerone, possess antioxidant capacity when used at all three tested concentrations. Intracellular reactive oxygen species level was significantly lower in comparison to the cells treated with 0.25 mM hydrogen peroxide. Ricotta whey had antioxidant property when used at the lowest concentration (12.5%). In fact, intracellular ROS level was decreased by 24 percentage points in relation to the fibroblasts treated with hydrogen peroxide. Caciotta-derived whey was the most effective antioxidant of all three tested samples. This was especially observed after using it at the two higher concentrations, 12.5% and 6.25% (obtained ROS level amounted to 12.90% and 38.11%, respectively). Antioxidant effect of 12.5% Caciotta whey activity was significantly better than the result obtained for positive control, 100 μg/ml ascorbic acid ($p < 0.0001$). The same experimental method, DCF-DA assay, was used by Kleekayai et al. (Kleekayai et al., 2020) to evaluate the antioxidant properties of whey protein hydrolysates using HepG2 cells. The results indicated that whey protein after hydrolyzation decreased the level of generated reactive oxygen species – the generation of ROS was between 20% and 78%.

3.6. Determination of extracellular matrix molecule expression

3.6.1. Collagen I detection

There are three basic layers in the human skin: the outer layer (epidermis), followed by dermis and hypodermis. Middle layer is composed of fibroblasts, blood and lymphatic vessels, nerve fibres and the network with macromolecules and minerals, extracellular matrix (ECM). This non-cellular component of skin consists of two main types of macromolecules, fibrous proteins (collagens, elastin, fibronectin and laminin) and proteoglycans. Collagens are the most abundant ECM proteins produced by fibroblasts. The ability of compound to stimulate expression of collagen in the dermal layer is related to anti-aging effect.

To assess potential anti-aging properties of tested whey samples, production of collagen I after fibroblasts treatment was assessed using enzyme-linked immunosorbent assay (ELISA). The results are presented in Fig. 5.

Two tested dairy by-products, derived from production of

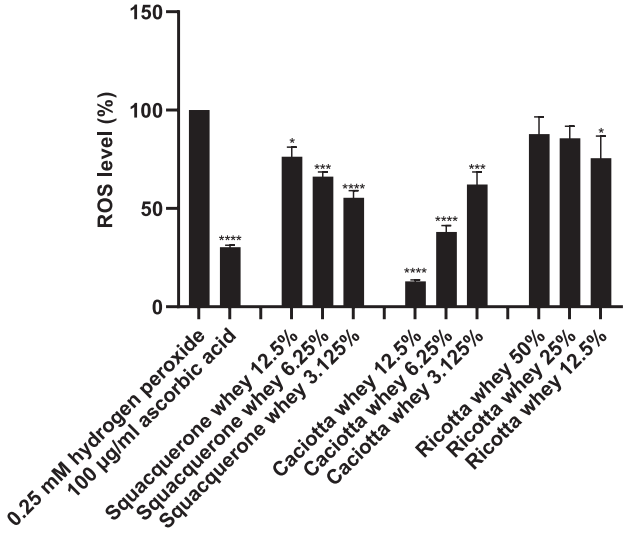

Fig. 4. Intracellular antioxidant activity of Squacquerone, Caciotta and Ricotta-derived whey measured by DCF assay. HDFa were treated with different concentrations of whey for 1 h. Results are presented as a percentage of the hydrogen peroxide-treated cells. Bars correspond to mean ± SEM of three independent experiments. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$ vs. hydrogen peroxide-treated cells.

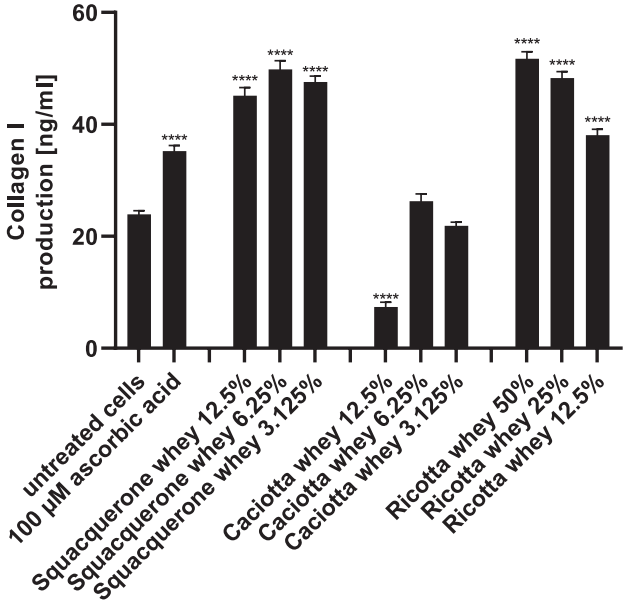

Fig. 5. Collagen I production measured by ELISA in HDFa after treatment with Ricotta, Caciotta and Squacquerone-derived whey. HDFa were treated with different concentrations of whey for 48 h. Results are presented as a percentage of the untreated cells. Ascorbic acid was used as positive control. Bars correspond to mean ± SEM of three independent experiments. **** $p < 0.0001$ vs. untreated cells.

Squacquerone and Ricotta strongly induced generation of collagen I by human dermal fibroblasts. Squacquerone whey, used at all three tested concentrations, was a significantly better inducer of collagen I expression than positive control, 100 μM ascorbic acid ($p < 0.0001$). Detected concentration of collagen I after treatment with squacquerone whey was 28%, 42% and 35% higher in comparison to positive control, for concentration 12.5%, 6.25% and 3.125%, respectively. In the case of Ricotta-derived whey, statistically significant difference in collagen I level in relation to the 100 μM ascorbic acid was observed for two higher concentrations, 50% and 25% - the obtained values were 47% and 37% higher, respectively ($p < 0.0001$). Caciotta whey used at concentration 12.5% significantly inhibited collagen I production and detected level of this protein was 69% lower when compared to the untreated fibroblasts. The other two concentrations of Caciotta whey did not significantly affect the level of collagen I produced by dermal fibroblasts. A compound that might be responsible for observed collagen synthesis and is present in cheese whey is transforming growth factor-β (TGF-β). It was proven that TGF-β can stimulate the transcription of collagen I gene (COL1A2) in dermal fibroblasts (Chen et al., 1999).

According to the study from 2021 performed by Katayoshi et al. (Katayoshi et al., 2021), observed increase in collagen I level after treatment with whey proteins relates to the activity of transforming growth factor β. Researchers showed that low-molecular whey proteins promote production of collagen I and III by human dermal fibroblasts. Obtained results revealed that the tested proteins acted through activation of transforming growth factor β receptor (TβR)/Smad signalling pathway. Low-molecular whey proteins were fractionated and two components, β-lactoglobulin and α-lactalbumin were identified as potential signalling inducer. Involvement of the Smad signalling molecules in the TGF-β-derived expression of collagen I was previously reported by Chen et al. (Chen et al., 1999). Smad 3 plays a role in transition signal of TGF- β from the receptor to the collagen gene promoter in human fibroblasts and is expected to stimulate COL1A2 promoter activity elicited by TGF-β (Chen et al., 1999).

The TGF-β signalling pathway was also linked with the whey activity in the study of Panahipour et al. (Panahipour et al., 2021). The obtained results showed that activity of TGF-β is maintained in oral fibroblasts even when milk is processed into casein and whey powder. Moreover, reduced expression of two transcriptional regulatory proteins, inhibitor of DNA-binding-1 (ID1) and inhibitor of DNA-binding-3 (ID3), was observed in HSC2 oral squamous carcinoma cells. ID1 and ID3 are known for downregulation of fibronection and collagen, extracellular components induced by TGF-β (Mody et al., 2017; Mody et al., 2021). Chen et al. (2021) investigated nerve regeneration in the wound repair process. Topical administration of ID1/ID3-overexpressing fibroblasts to the wound tissue increased nerve regeneration and improved healing process. Tested fibroblasts was characterized by upregulated expression of neural stem cell, the Schwann cell, the astrocyte, and mesoderm markers (including collagen 1A1). Decreased expression was observed only for mesoderm markers.

3.6.2. Elastin detection

Elastin is the primary component of the dermal elastic fibres, the extracellular matrix protein complexes produced by dermal fibroblasts. The main role of the complexes is to maintain the extensibility and elasticity of skin (Wang et al., 2021; Rossetti et al., 2011). With aging, due to the reduced synthesis and increased degradation, the amount of the elastic fibres in the skin decrease, the skin loses its firmness and wrinkles appear. The ability to promote elastin synthesis is a desirable feature of the compound for use as a cosmetic product.

The capacity of tested whey samples to stimulate dermal fibroblasts to produce one of the major components of the extracellular matrix, elastin, was analyzed using ELISA.

From Fig. 6 it can be seen that whey derived from production of all three types of cheeses did not promote elastin synthesis by HDFa. On the contrary, Caciotta-derived whey used at the highest concentration

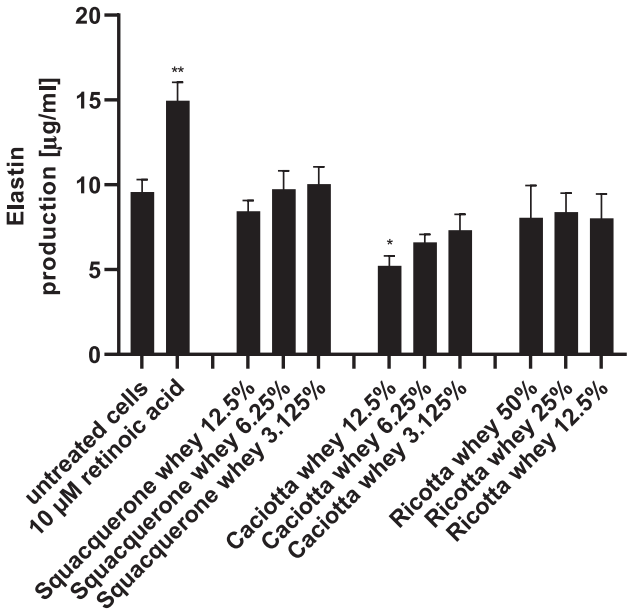

Fig. 6. Elastin production measured by ELISA in HDFa after treatment with Squacquerone, Caciotta and Ricotta-derived whey. HDFa were treated with different concentrations of whey for 24 h. Results are presented as a percentage of the untreated cells. Bars cor-respond to mean ± SEM of three independent experiments. * $p < 0.05$; ** $p < 0.01$ vs. untreated cells.

(12.5%) statistically significant decreased level of detected elastin. In fact, the obtained value was 45% lower in comparison to the untreated cells.

Transforming growth factor-β, the presence of which in the tested whey samples was associated with the increased collagen I expression, is known for his ability to stimulate other extracellular matrix components, including elastin (Davidson et al., 1993; Kähäri et al., 1992). It was reported that up-regulation of elastin expression by TGF-β occurs via stabilizing the elastin messenger RNA, not by modulation of elastin gene transcription (Kähäri et al., 1992; Kucich et al., 1997; Zhang et al., 1995). Study of Kucich et al. (1997) proven involvement of Smad signaling in the stabilization of elastin mRNA. It is suggested that Smads are required for the process, but not involved directly. In fact, 4 to 6 h lag period was observed before any change in the steady state level of elastin mRNA. It is probable that during this lag period Smad activity induces expression of proteins necessary for the mRNA stabilization.

The findings of other researchers are in contrast to the presented results. Taking into consideration obtained fibroblasts response regarding collagen I production, it was expected that similar increase in elastin expression would be observed. The reason for the difference might lie in the too short cell incubation period. In fact, before removing the supernatants for further assays, dermal fibroblasts were treated with tested whey samples for 24 h, not 48 h as in the case of collagen I analysis. Moreover, the analysis of elastin mRNA amount instead of the elastin protein, could provide a better picture of the whey effect on fibroblasts ability to produce elastin.

3.6.3. Glycosaminoglycans detection

Glycosaminoglycans (GAGs) are long, linear polysaccharides comprised of repeating disaccharide units characterized by high expression in the skin – occurring within the skin cells, on the surface and as a component of extracellular matrix. There are two types of GAGs present in the skin: the nonsulfated GAG, hyaluronic acid, and sulfated GAGs, dermatan sulfate, chondroitin sulfate, heparan sulfate, keratan sulfate, heparin. Level of activity of glycosaminoglycans as well as total GAGs level, similar to collagen or elastin, decreases with skin aging (Wang et al., 2021). Two strategies are used for targeting GAGs in the

skin, topical application of the product enriched in glycosaminoglycans or modulation of the GAGs content of the skin by using compound stimulating GAGs expression. Potential ability of cheese-derived whey samples to promote GAGs expression within the fibroblasts was performed using metachromatic dye 1, 9-dimethylmethylene blue.

As illustrated by Fig. 7, fibroblasts treatment with Ricotta derived whey did not cause significant change in the production of GAGs. Squacquerone-derived whey used only in one concentration (6.25%) statistically significant stimulated tested cells to produce glycosaminoglycans, and the obtained level was 56% higher in comparison to the untreated cells and similar to the result achieved by positive control, 100 μg/ml N-acetylglucosamine. The strongest ability to promote glycosaminoglycans production by tested cells was observed for Caciotta-derived whey sample and the concentration of detected GAGs increased in dose-dependent manner. In comparison to the untreated cells, the obtained values were increased by 170%, 101% and 68%, for the concentration of 12.5%, 6.25% and 3.125%, respectively. Treatment of HDFa with the highest concentration of Caciotta whey, 12.5%, resulted in a greater increase in GAGs production than that observed for the positive control (increase by 66%, $p = 0.0031$).

One of the components of tested whey that might be responsible for the observed results is lactic acid, a compound from the alpha hydroxy acids (AHA) group. Topical skin treatment with lotion containing 25% of AHA (glycolic, lactic, or citric acid) for skin peeling results in increasing total GAG content (Ditre et al., 1996).

3.7. Transepithelial electrical resistance (TEER) measurement

One of the most important functions of the human skin is to act as a barrier between the organism and the environment. This function can be performed mainly due to the presence of the stratum corneum. This outermost thin layer consists of the end-products of epidermal keratinocytes terminal differentiation, the dead corneocytes.

The ability of tested dairy by-products to improve the cell barrier integrity of epidermal cells, keratinocytes, was investigated by measure electrical resistance of the monolayer using EVOM2™ with chopstick electrodes, at the time 0 (baseline measurement) and after 6 and 24 h. The results are displayed in Fig. 8.

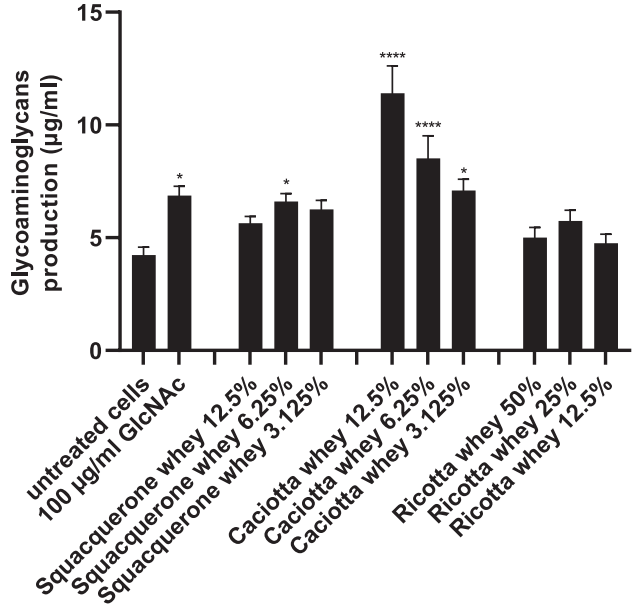

Fig. 7. Glycosaminoglycans production in HDFa after treatment with Squacquerone, Caciotta and Ricotta-derived whey. HDFa were treated with different concentrations of whey for 72 h. N-Acetylglucosamine (GlcNAc) was used as positive control. Results are presented as a percentage of the untreated cells. Bars correspond to mean ± SEM of three independent experiments. * $p < 0.05$; **** $p < 0.0001$ vs. un-treated cells.

Treatment of keratinocytes with all tested samples lasting 24 h, except for the lowest concentration of Caciotta whey, enhanced the barrier function of the keratinocyte monolayers, as evidenced by the significantly increased transepidermal electrical resistance value. Ricotta-derived whey used at two tested concentrations, 25% and 12.5%, caused a significant increase in TEER value after 6 h treatment, in comparison to the baseline (0 h). Moreover, when we present the obtained results as a difference to the baseline, Ricotta whey at concentration 25% induced statistically significant higher increase in TEER value between timepoints 0 and 6 h, than the positive control- cells stimulated with high calcium culture medium ($p = 0.0022$). After 24 h of incubation significant difference between activity of this sample and positive control was not observed.

Shinagawa et al. (2018) investigated the effects of by-product of rennet-type cheese, sweet whey, on primary keratinocytes. The influence of low-molecular fraction of whey on expression of aquaporin 3 (AQP3), and tight junction proteins claudin-1 (CLDN1), claudin-4 (CLDN4), and occludin (OCLN), was evaluated by Western blotting. It was found that cell treatment with low-molecular whey fraction caused significant increase in detected level of molecules of interest.

In vivo skin barrier effects of whey were investigated by Sobkowska et al. (2021). Thirteen Caucasian adult females, with no dermatological diseases, were treated with sweet whey test bath. Courage-Khazaka MPA-9 (Courage + Khazaka Electronic, Köln, Germany) device was used to evaluate the effects of the treatment on skin hydration, transepidermal water loss (TEWL), melanin and erythema index and pH level in human skin. It was found that bathing in the sweet whey solution significantly improved the skin's barrier function (in comparison with the tap water treated control). Tested whey acted as a moisturizer and significantly increased facial skin hydration.

Not only the full whey but also its components, such as lipid fraction, were analyzed for its effect on the skin barrier. Higurashi et al. (2015) examined the effect of 12 weeks of consuming sphingomyelin-containing milk phospholipids (SM-MPC) on the condition of skin in a double-blind, placebo-controlled, randomized trial. Researchers reported that the hydration of the skin was significantly increased after 9 and 12 weeks of supplementation in the low-SM group (5 mg/day) compared with the placebo group. Skin elasticity in the region below the eye was significantly increased at week 9 in the high-SM group (10 mg/day) versus placebo. Lee's research team, in turn, analyzed the effect of topical application of milk sphingolipid-enriched fraction (MSEF) cream (Lee et al., 2020). The treatment improved the water holding capacity and skin barrier recovery of damaged skin through increased transepidermsl water loss (TEWL).

4. Conclusions

Whey, the by-product of cheesemaking was investigated for its potential use by the cosmetic industry. The effect of whey on dermal and epidermal cell lines (fibroblasts and keratinocytes) was evaluated. The obtained results show that tested whey is not cytotoxic against used cell culture models. The antioxidant activity of tested samples was proven by using DCF-DA assay. Increased production of extracellular matrix molecules, such as collagen, elastin or glycosaminoglycans was observed when human fibroblasts were treated with whey. Tested samples improved epidermal barrier integrity. Moreover, the ability of whey to inhibit elastase and tyrosinase was demonstrated in non-cellular assays. It must be mentioned that the present results were obtained with samples collected from a specific season of the year (Nov 2020) and from one Italian cheesemaker. These two factors have a significant impact on the raw materials applied and therefore on the resulting whey composition and microbiota (Celano et al., 2022; Timlin et al., 2021). Factors such as milk origin, the type of produced cheese, as well as location, season and type and health of cattle can influence composition of whey and thus

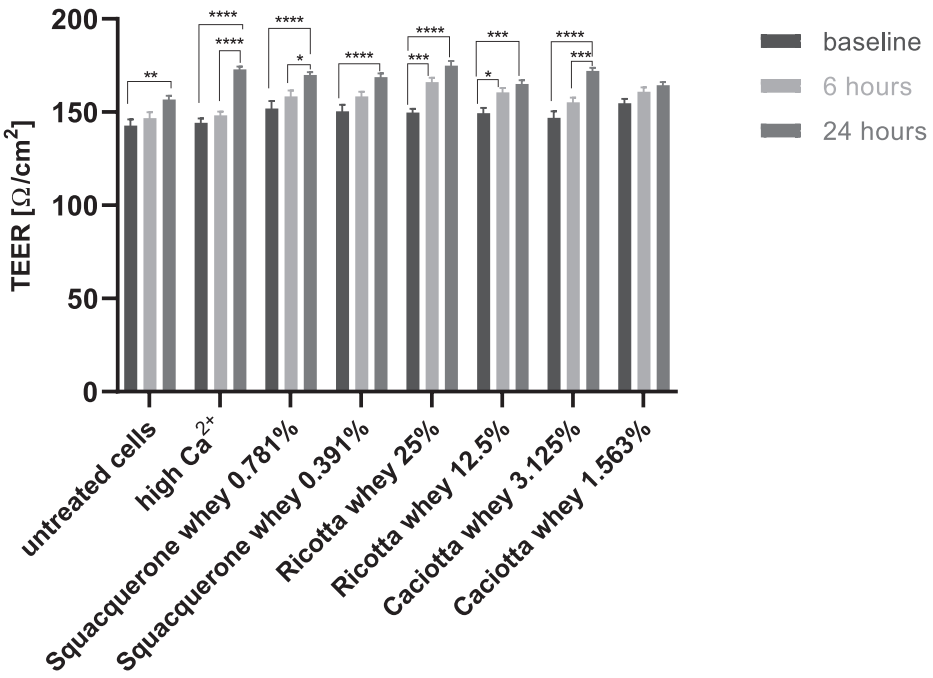

Fig. 8. Effect on the tight junction barrier function of HaCaT monolayer after treatment with Squacquerone, Caciotta and Ricotta-derived whey. Ca^{2+} was used as positive control. Bars correspond to mean ± SEM of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

affect the biological activity. However, as a preliminary screening of different types of whey produced on the same time of the year with the same milk, this could provide a first overview of the functional properties of the by-product. Although further trials may be performed for specific functions to evaluate seasonality, all the obtained results indicate that whey offer promising potential as cosmetic ingredient, because of its lack of toxicity, antioxidant, skin-whitening or anti-wrinkle properties.

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CRediT authorship contribution statement

Aleksandra Augustyniak: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Davide Gottardi:** Writing – review & editing. **Barbara Giordani:** Writing – review & editing. **James Gaffey:** Supervision. **Helena Mc Mahon:** Conceptualization, Validation, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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