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Method For Preparing a Skin Repairing Ingredient from Cheese Whey Following *Lactobacillus* Fermentation

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Abstract

In case of impairments in skin integrity, the use of dermatological treatments may prevent the outbreaks of diseases, by restoring the physiological homeostasis of the injured tissue. Against this background, a method for preparing an active ingredient effective on wound healing has been developed, starting from sweet whey, a by-product of Fontina PDO cheese processing. Following the fractionation of whey constituents by ultrafiltration processes, the obtained permeate underwent controlled fermentation by a newly characterized *Lactobacillus delbrueckii* MF-20/7A/24 (BCCM accession number LMG P-31789) isolated in alpine pasture of Aosta Valley, a little region in the Northwest of Italy. The ability of the native bacterial strain was exploited to ferment the whey permeate fraction, in order to enhance its potentially bioactive components. In particular, the whey-based fermented product was rich in biomolecules with a potentially beneficial effect on human skin, including galacto-oligosaccharides and organic acids. The prepared ingredient, subject of a specific Italian patent (N. 102021000011006), showed the ability in vitro to induce skin wound healing, due to the presence of galacto-oligosaccharides (GOS) and butyric acid. As evidenced by the tests carried out, no cytotoxic reactivity or interferences with cell growth and cytoplasmatic metabolism were found out. Moreover, data from patch test on adult volunteers with sensitive skin confirmed the absence of irritating properties of the preparation.

Introduction

Cheese whey is a by-product of the dairy industry that causes serious environmental problems[1]. It is strained from the milk during the coagulation of caseins, with the addition of calf rennet, and retains about 55% of the milk's nutrients. It is composed mostly of 93-94% water and 6-7% dry matter. Whey components basically are lactose (approximately 70%), proteins (approximately 10%), minerals (approximately 15%), principally NaCl, KCl and calcium salts, and other minor constituents, such as organic acids, fat, non-protein nitrogen compounds (amino acids, urea and uric acid) and B group vitamins[2]. These nutrients make whey a high-value product that has benefits for human health.

Since ancient times whey was used as a nutrient with high digestibility, able to support the immune system, due to the presence of specific bioactive proteins and peptides with anti-microbial and anti-viral activities. Whey proteins promote muscle and bone health, display anti-oxidant activity and affect cardiovascular system through anti-hypertensive effect. All these features are directly related to their amino acid profiles[3,4].

In addition to the protein fraction, cheese whey contains prebiotic molecules, mainly non-digestible oligosaccharides,

able to specifically modify the intestinal microbiota and maintain the gut health[5]. Scientific reports indicate that whey has bioactive galacto-oligosaccharides (GOS) that originate from the bovine mammary gland and partly from microorganisms employed in cheese making processes, expressing *β*-galactosidase (EC 3.2.1.23)[5-7]. GOS are molecules able to mimic the functions of human milk oligosaccharides. They are widely studied for their role as prebiotics on the intestinal bacterial flora and as immune system modulators at the level of the intestinal epithelial cells[6].

The combination of different processes of bio-conversion reduced milk whey's environmental impact, while also providing an economical alternative to transform whey into high-value products[8]. By the application of the ultrafiltration process, it is possible to recover proteins from whey, without losing their functional properties[9]. This fraction is notably used as a food ingredient. The whey permeate instead represents the most voluminous part and is rich in lactose. This makes it an ideal medium for the growth of microorganisms, which means it can be used as a substrate for the production of many fermented products[1]. First of all, the production of organic acids can be obtained from whey fermentation. Several studies have reported that the action of lactic acid bacteria (LAB) on dairy products may contribute to the formation of free fatty acids, which have attracted much attention as a novel type of beneficial functional lipid[10]. In particular, scientists' attention focused notably on butyrate, which affects morphology, growth rate, and gene expression in mammalian cells and is involved in the regulation of the vitamin D signaling pathway[11-13]. In addition to its dietary use, whey was employed in the past as skin balm with soothing properties in case of burns. These properties also derived from the milk from which it originates and can be attributed to the protein fraction and derivatives[14]. However, whey is not currently widely used by the cosmetics industry, although some studies suggest it is a potentially anti-aging agent[15]. The effects of whey components on the skin are well known when taken orally, but there is little evidence about their direct benefit on keratinocytes[16,17]. Whey permeate in particular can be considered to be a carrier of prebiotics and various bioactive compounds, including peptides, poly- and oligosaccharides and organic acid that may play a functional role for skin care[3].

Given the above, the aim of this study was to develop an active ingredient, from Fontina cheese whey, which was rich in molecules with a high biological activity on human skin. The ability of a microorganism isolated in Aosta Valley, a small region in the Northwest of Italy, was exploited to ferment the whey permeate fraction, in order to enhance its potentially bioactive components, particularly GOS molecules and butyric acid.

Materials And Methods

Reagents

Unless otherwise specified, reagents were purchased from Sigma-Aldrich (Milan, Italy).

Experimental Design and Sweet Whey Sampling

Sweet whey was supplied by the *Institut Agricole Régional* (IAR) farm located in Aosta, Italy. It resulted from the production of Fontina, a traditional Italian PDO cheese typically produced in Aosta Valley. A dedicated Consortium supervises the cheesemaking process, which follows the specifications provided by the EU guidelines for PDO products[18,19]. Full cream raw cow's milk was added with autochthonous strains of *Streptococcus thermophilus* and *Lactococcus lactis* and was coagulated at 36°C with calf rennet. Curd was finely cut and gradually heated up to 46–48°C under gentle stirring, then transferred into round molds and pressed, before the maturation process. The residual whey, which strain from the curd during this process, is the liquid part of milk. It was not subjected to high temperature treatments, which could alter the properties of its components, and, in addition, it derived from a single milking of after-calving cows. During this stage of lactation, milk is produced in the highest quantity and of the best possible quality, to ensure adequate feeding of calves[20,21]. A single sample of 10 L of whey was collected directly from the copper vats in which the Fontina PDO is produced. Whey was immediately refrigerated (without any preservative) at 4°C in a temperature reduction system to suppress microbial proliferation. An aliquot of 100 ml was taken, and pH measurement, at ambient temperature, was carried out in triplicate with the pH-Meter BASIC 20 (Crison Instruments, Alella, Spain). The rest of the whey was divided into 2-litre portions and frozen at -20°C until further analysis.

Given its potentially beneficial components for skin health (organic acids, GOS and peptides, in particular), it was decided to use the non-protein fraction of whey to develop a cosmetic active ingredient. First, whey was subjected to ultrafiltration processes to remove fat and proteins. After purification, this part of the whey, called whey permeate, was fermented to enhance its biological properties.

Whey Permeate Extraction

The process of clarification by thermocalcic precipitation, followed by microfiltration, was used to remove the non-centrifugeable residual lipids present in whey, composed mainly by phospholipoproteins from the fat globule membrane[22]. This pre-treatment was applied to each aliquot of Fontina cheese whey, previously thawed. It's a process based on the formation of insoluble lipid-calcium phosphate

aggregates and was performed according to the method of Fauquant et al.[23], modified by Pereira et al.[24]. The level of Ca^{2+} present in whey was initially quantified by means of a complexometric titration with ethylenediamine tetraacetic acid (EDTA) and subsequently increased to 1,2 g/L with the addition of $CaCl_2$. The pH was adjusted to 7,5 with NaOH 10 N and an incubation at 55°C was conducted for 30 min, to obtain a prior physical separation. The whey was cooled and kept at 4°C for 72 hours for fatty sediment forming. After this time, the supernatant was twice centrifuged at 1600 xg, 4°C for 15 min. To achieve a progressive purification, clarified samples underwent vacuum filtration by using cellulose and glass microfibers filters (Whatman®, from 22 to 1,5 µm pore size) and vacuum microfiltration with cellulose esters (MCE) or polyethersulfone (PES) filters (Millipore, from 0,8 to 0,22 µm pore size). This pretreatment procedure was carried out to improve ultrafiltration (UF) rates, avoiding membrane fouling[25]. UF was performed in batch conditions, using a Cogent µScale tangential flow filtration (TFF) system (Merck Millipore), equipped with a Pellicon XL cassette made of PES, with a nominated membrane area of 0,005 m^2 and 10 kDa molecular weight cut-off. The flow rate was set to 25 mL/L, with transmembrane pressure of 20 psi and temperature ranging from 23,6 to 24,4°C. The deproteinized whey fraction, defined as permeate (PERM), was divided in 1-liter aliquots and kept to -20 °C until subsequent analysis.

Whey Based Preparations

Fermentation Process

The native strain of *Lactobacillus delbrueckii* subsp. unknown was designated as MF-20/7A/24. It was deposited at the Belgian Coordinated Collection of Microorganisms (BCCM, Ghent University, Belgium) with the accession number LMG P-31789. This strain was isolated in alpine pasture of Aosta Valley (Italy), in which no commercial starter cultures were used for cheese-making. Firstly, frozen strain was regenerated in liquid growth medium MRS broth (Biolife Italiana, Milan, Italy) for 24 hours at 37°C. A 0,1 mL aliquot of the broth culture was subsequently withdrawn and transferred into vials containing 10 mL of fresh MRS broth and allow to incubate 24 hours at 37°C. Lastly, the resulting bacterial culture was plate seeded in MRS agar (Biolife Italiana, Milan, Italy) in order to define the initial microbial charge. The clarified whey permeate (obtained as explained in Section 2.2), formerly autoclaved at 90°C for 20 min, underwent controlled fermentation by adding LMG P-31789 inoculum 1:100 (%*v/v*). The fermentation was conducted at 37°C for 24 hours, after which the fermented product (designated as LMGferm24) was subjected again to autoclave (at 100°C for 15 min) to stop the microbial activity and was subsequently microfiltrated (0,22 µm) for removing bacterial residues. Before samples pasteurization, microbial count was performed on MRS agar to track strain development. The bacterium's growth and metabolism can be influenced by the fermentation parameters, so values that favour it have been chosen. The optimal growth temperature for most LAB ranges from 30 to 45°C[26], therefore the average value of this range was selected. Instead, regarding the time of the fermentation, the process was carried out in order to increase the concentration of whey bioactive components. However, it was not further extended to avoid the complete catabolism of the same compounds. Unfermented whey permeate was pasteurized under the same conditions and was used to evaluate whether the fermentation process was able to enrich, or not, the sample in functional biomolecules.

LMG P-31789 Metabolic Profile

Lactic acid bacteria (LAB), such as LMG P-31789, belonging to the species *L. delbrueckii*, are characterized by an energy metabolism based mainly on carbohydrates fermentation, with lactic acid as principle end product[27,28]. Moreover, all described strain of *L. delbrueckii* subsp. *bulgaricus* are unable to metabolize galactose, whose accumulation in fermented products has negative implications for manufactures and consumers[29-31]. In order to investigate the metabolic profile of the strain taken into account and have an indication of the possible fermentation end-products to be detected in the fermented whey, different screening test were performed. Ortho-nitrophenyl-galactopyranoside (ONPG) rapid test (Liofilchem, Milan, Italy) for the detection of the bacterial β-galactosidase was performed according to provider specifications. The test is based on the formation of the yellow compound o-nitrophenol from the colorless ONPG, a specific substrate of the enzyme β-galactosidase. The objective of this test is to determine the ability of a microorganism to produce β-galactosidase. The provided test tubes, containing dehydrated ONPG medium, were left for a few minutes at room temperature and then reconstituted with 0,2 ml of physiological solution (Liofilchem, Italy). Subsequently, a 24-hour old well isolated bacterial colony was suspended in the culture medium and incubated at 37°C for a maximum of 24 hours. A test tube with only 0,2 ml of physiological solution was used as a negative control, indicating the absence of β-galactosidase activity. For the purpose to characterize, from a biochemical point of view, the sugar metabolism operated by the LMG P-31789 strain, the anaerobe system test and the specific fermentation test for galactose (both from Liofilchem, Milan, Italy) were performed following supplied instructions. The anaerobe system allowed the identification of bacteria by means of a 24-wells system containing different desiccated biochemical substrata. Briefly, the pure strain was first isolated in MRS agar culture medium, incubated at 37°C for 24 hours and then resuspended in a pH 7,1 ± 0,2 solution consisting of enzymatic digested casein (2,5 g/L), enzymatic digested soy flour (2,5 g/L), yeast extract (5

g/L), L-tryptophan (0,2 g/L), L-cystine (0,4 g/L), emina (5 mg/L), vitamin K1 (10 mg/L), sodium sulfate (0,1 g/L), and 1000 ml of distilled water, until final turbidity equivalent to 1 McFarland is obtained. A 0,2 suspension of the LMG P-31789 microorganism was inoculated in each well and incubated at 37°C for 24 hours. The positive test results were recorded by evaluating the color change, from blue to yellow; no color change indicated negative reaction. A provided identification table allowed to classify the bacterium depending on its metabolic abilities. In order to confirm the anaerobe system test results, another biochemical characterization test, more specific for galactose, was carried out. Tubes used for this test contained a dried medium consisting of a protein mixture, galactose and purple bromocresol as a pH indicator. Briefly, test tubes were left for a few minutes at room temperature and then reconstituted with 0,3 ml of a physiological solution (Liofilchem, Italy) and a galactose disc. A 24-hour old well isolated colony of the strain to be tested was suspended in the culture medium and incubated at 37°C for 24 hours. Positivity for sugar fermentation was highlighted by the color change, from purple to yellow, due to the pH indicator present in the medium; no color change indicated negative reaction.

Characterization of Whey and Fermented Ingredient

Chemical Analyses

The pH value was measured immediately after dairy collection as regard for sweet whey (as specified Section 2.2), and before and after the brew cycle for the permeate fraction. The obtained fermented preparation was characterized to determine the presence of molecules that may be potentially active on skin. Particularly, the milk solids non-fat components (MSNF) were quantified using milk AOAC protocols adapted to whey: AOAC 990.20 for total solids, AOAC 991.21 for non-protein nitrogen, and AOAC 945.46 for mineral salts[32]. The lactate amount and the carbohydrate fraction (lactose, glucose, and galactose) were quantified using specific enzymatic assay kits (Megazyme from Astori Tecnica, Poncarale, Italy). From the difference between total solids and the components listed above, it was possible to indirectly measure the amount of GOS available before and after fermentation. The same characterization was carried out on the experimental control PERM.

Butyrate Quantification

According to literature, bacterial metabolism can increase the release of healthy components, in particular butyric acid[33]. For this reason, qualitative and quantitative evaluation of the volatile fraction of the fermented whey was carried out by a SPME-GC/MS chromatographic method, using an 85 µm SPME Carboxen/PDMS StableFlexTM fiber and a DB-Wax column (30 m x 0,25 mm x 0,25 µm film thickness), according to Pan et al.[34], with some modification. Briefly, 3 g of the sample, with 50 µL of the internal standard methyl isobutyl ketone (0,04 g/L in water) was placed in a 20 mL vial sealed with a polytetrafluoroethylene septum and bathed at 55°C for 10 min to equilibrate. The septum was pierced with the SPME needle and the fiber was exposed to the sample headspace for 45 min. The GC oven temperature program was held at 40°C for 4 min and increased from 40 to 6°C at a rate of 5°C/min, 60 to 120°C (6°C/min), and 120 to 230°C (10°C/min). The GC-MS transfer line was maintained at 250°C. The helium flow rate was 0,8 mL/min. Mass spectra were collected in electron ionization mode. The concentration of butyric acid was calculated considering the chromatographic peak area of the selected standard and was expressed as milligrams per liter (mg/L) concentration. All data obtained were compared to those from non-fermented whey.

Statistical Analyses

Comparison of results was done with the one-way analysis of variance (ANOVA), using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). All data were expressed as the mean ± standard deviation (SD) of three observations, except for the amount of GOS available before and after fermentation, which was indirectly obtained for both samples. Differences between the mean values of groups were tested and were considered significant at $p < 0,05$.

Results

Characterization of the Whey Based Preparations

The sweet whey derived from the production of the Fontina PDO cheese was used as starting matrix for the preparation of a dermatologically active ingredient with beneficial function for the skin. The whey sample strained during cheese making had a pH value of 6,41 and the amount of calcium amounted to 0,52 g/L. After fat removing, the ultrafiltration process was applied. Whey proteins were retained by the filtering membrane, while substances of a molecular weight lower than 10 kDa, passed through the filter and became the permeate stream. The protocol applied allowed to recover about 70% of the initial volume. Whey permeate obtained by ultrafiltration had a yellowish colour similar to that of starting sweet whey, but clearer, having been deprived of the lipid fraction; at the end of ultrafiltration, its pH was 5,63, while after the fermentation process, this value decreased to 4,73.

Chemical characterization was carried out, in parallel, on whey permeate fermented (LMGferm24) and not fermented (PERM), in order to assess the possible modification and enrichment of the whey components by the action of the employed native bacteria. The concentration of the main solid whey permeate components, expressed in terms of

Table 1: Solid components characterizing whey samples.

Whey Type	TS*		Ash*		Lactose***		Galactose**		Glucose*		TN	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PERM	6,71	0,0232	1,14	0,0780	1,98	0,0118	0,25	0,0004	0,004	0,0006	0,038	0,0011
LMGferm24	6,36	0,0321	1,54	0,0334	1,62	0,0031	0,24	0,0011	0,001	0,0001	0,034	0,0050

Values, expressed in terms of percent weight by volume (% *w/v*), are averages ± SD = standard deviation; TS = total solids; TN = total nitrogen; GOS = galacto-oligosaccharides; PERM = whey permeate; LMGferm24 = fermented whey permeate with *Lactobacillus* LMG P-31789. * $p < 0,05$; ** $p < 0,005$; *** $p < 0,0001$ compared to PERM.

percent weight by volume (%*w/v*), is shown in Table 1; each value shown is the mean of a triplicate measurement. LMGferm24 sample had statistically different results from PERM one, in particular for total solids, minerals and glucose ($p < 0,05$), galactose ($p < 0,005$) and lactose($p < 0,0001$). The permeate composition depends on milk source and cheese processing conditions, but its main ingredient is lactose[2]. The solid composition of the whey permeate and the fermented one was similar, with small differences that could be directly attributable to the fermentation. The total solid was lower after the fermentation, due to the reduction in the quantity of sugars and nitrogen. These latter compounds, specifically lactose and peptides, were the substrate promoting the growth of Lactic Acid Bacteria (LAB)[35,36]. The metabolic capability of LAB to ferment lactose resulted in a decrease in its amount, and in an increase of galactose and glucose. However, the amount of galactose remained almost constant, while glucose was reduced. In the process of lactic acid fermentation, LAB use glucose as a carbon source to produce lactic acid[28]. As expected, the whey permeate fermentation with the LMG P-31789 strain got to a decrease of this monosaccharide. In relation to galactose, LMG P-31789 strain showed the capacity to use also galactose residues as highlited by the chemical analysis of fermented and unfermented permeate which did not reveal any increase in this sugar, despite the breakdown of lactose. Normally, bacteria belonging to the species of *L. delbrueckii*, like LMG P-31789, are not able of using galactose, as evidenced by scientific studies[29]; so, as a rule, galactose increase during fermentation. However, some LAB have developed two different strategies to metabolize it, the tagarose-6P pathway and the Leloir pathway[30,37]. Probably, LMG P-31789 strain has developed one of these metabolic capacities that enables it to use at least some of the galactose found in whey. Microbiological tests conducted confirm this hypothesis. While the ONPG rapid test showed the ability to produce *β*-galactosidase for the strain examined, through the anaerobe system test it was demonstrated that LMG P-31789 strain was able to ferment galactose. In addition, this last aspect was confirmed with the fermentation test specific for galactose (data not shown). In order to completely characterize sugar metabolism of the LMG P-31789 strain an Anaerob system test was conducted. The native strain showed the ability to ferment all the following compounds: glucose, mannitol, lactose, sucrose, maltose, salicin, xylose, arabinose, cellobiose, mannose, melezitose, raffinose, sorbitol, rhamnose, trehalose, fructose and galactose. It possessed also the capacity of utilization of glycerol, hydrolysis of esculine, indole formation. However, the native strain did not show N-acetyl-*B*-glucosaminidase, urease and catalase activities and nitrate reduction skills.

Finally, through the quantification of solids, it was possible to determine the amount of GOS available in the fermented ingredient and the original whey permeate. An indirect quantification was carried out from the difference between the mean values of the total solids and the carbohydrate components, mineral and ash. Values, expressed in percent weight in volume (% *w/v*), amounted to 3,29 and 2,93 % respectively for non-fermented and fermented whey permeate. The data indicated that the fermentation did not result in any increase in GOS amount, but probably a structural change in these components, since they probably came from different enzymes. As a matter of fact, compositional and structural differences have been reported regarding GOS originating from bovine mammary gland, compared to those derived from various microorganisms[3,5]. In addition, the amount of GOS may have decreased after fermentation, because it could be a source of short-chain fatty acids, such as butyrate[38, 39].

Monitoring the fermentation process

The LMG P-31789 bacterium turned out to be able to grow in the whey permeate, as evidenced by the increase in the bacterial load from 10^6 to 10^8 CFU/mL (CFU= colony forming units), respectively at the inoculum and after 24 hours. These data evidenced that this unknown subspecies possessed the ability to use the whey permeate as single growth substrate, in view of its metabolism. Compared to milk, the whey permeate fraction can be considered a nutrient-limited source, having been deprived of the majority of its solid fraction (fat and proteins) and, actually, a survival of this bacterial strain was reflected in the use of available compounds found in it.

Monitoring the fermentation process can be done by observing an increase in the organic acids content, such as changes in pH value and carbohydrate fraction. Organic acids derived from milk production and milk fat hydrolysis during cheese processing and storage. They are also produced during LAB fermentation and had a significant impact on the characteristics of the product that contains

them[10,34]. The amount of lactic acid was quantified as indicator of the bacterial activity. As shown in Figure 1A, the lactic acid content changed from 0,58 ± 0,053, up to 1,03 ± 0,001 g/L after 24 hours of fermentation. Data were highly statistically different ($p < 0,001$). An increase in its amount can be considered an advantage for a topical preparation with dermatological action. Indeed, lactic acid is one of the most popular alpha hydroxy acids (AHAs) available on the market. It is naturally found in dairy products, which have been used in cosmetic treatments for centuries by people across the world. It has been known to benefit our skin by removing dead skin cells, lightening dark spots, and improving the appearance of wrinkles on all skin types, including sensitive skin[40]. It is one of the gentler AHAs used for exfoliation, and it can create real skin turnover, if used regularly[41]. In addition, lactic acid is a natural moisturizing factor that helps the skin stay hydrated[42]. Another quantified biomolecule useful for defining the course of the fermentation process was the butyric acid. Its amount was 1,53 ± 0,12 mg/L in the unfermented whey permeate, and, following the fermentation, a maximum of 5,14 ± 0,93 mg/L was achieved. Fermentation has enabled butyric acid to increase similarly to lactic acid and its increase resulted statistically significative ($p < 0,05$) in fermented whey sample respect to unfermented one. Its enrichment in the fermented whey-based preparation could be correlated with a beneficial effect on the skin, where its production by the bacterial flora is minimal[43]. As a matter of fact, recent studies have reported the role of butyric acid in skin diseases. Specifically, it plays a role in inhibiting the tissue inflammatory response, promoting mitochondrial metabolism, immune system regulation and keratinocyte differentiation[44]. In addition, butyric acid is an interesting skin-lightening molecule that finds application in both cosmetic and dermatological fields. It is widely used to improve skin appearance, but also for the treatment of hyperpigmentation disorders[45].

Discussion

As already mentioned, whey is a by-product of relative importance in the dairy industry due to the large volumes produced and its nutritional composition. From a valorization point of view, the method described in this study represents an innovative option for cheese whey management. It is based on the application of the ultrafiltration technology to recover approximately 70% of the initial volume of the raw material. This process is able to preserve all natural components of whey, which are characterized by a safety biological profile. The second aspect relies on the application of a fermentation process to enrich the components of the previously obtained permeate and develop an added value product, rich in molecules with high biological activity on human skin, like GOS and organic acids. For this reason, the whey-based preparation represents a potentially active ingredient for a topical preparation (cosmetic or medical product) with dermatological action. Skin mediates between the organism and the external world as it represents a protective barrier against potentially dangerous substances and prevents the loss of water and solutes[46]. In case of impairment in skin integrity, the use of dermatological treatments may prevent the outbreaks of local or systemic diseases and can be useful in treating them, by restoring the physiological homeostasis of the injured tissue[47,48].

Given its features just summarized, the whey-based preparation was tested on HaCat keratinocyte cell line, about whom biomolecular pathways are known[49]. This cell line comes from mature skin that is spontaneously immortalized but still exhibits basal cell properties and holds epidermal differentiation abilities, representing the tissue on which could be applied the dermatological preparation. According to the information published so far, it was assumed that the product LMGferm24 would be able to stimulate the Toll-like receptors (TLR), inducing

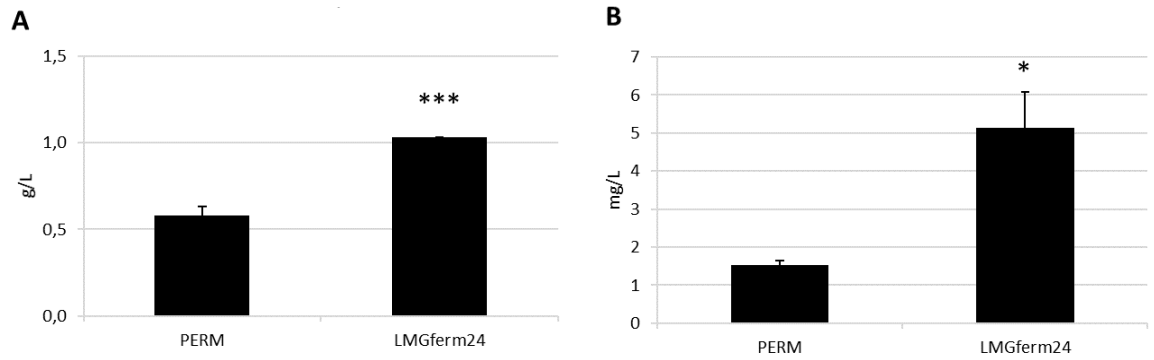

Figure 1. Selected organic acids content.
Lactic acid (A) and butyric acid (B) amounts before (PERM) and after whey fermentation with *Lactobacillus* LMG P-31789 (LMGferm24). Results are presented, respectively, in grams per liter and milligrams per liter of whey. Data, obtained in triplicate, are averages ± SD = standard deviation. * $p < 0,05$; *** $p < 0,001$ compared to PERM.

inflammatory responses, mediated by a molecular pathway that depends on the presence of GOS and butyric acid[50-52]. As shown for the first time in this study, previously published by the same authors[53], the components present in the whey-based preparation were able to exert a direct effect on human keratinocytes, demonstrating an interesting potential in the context of skin health. Specifically, several mechanisms of the wound closure process were explored: LMGferm24 started an inflammatory signal and induced cells migration, maintaining at the same time the state of differentiation necessary for proper skin barrier restoring. In contrast, the only whey permeate and purified solutions of GOS and butyrate showed completed different results, proving that the developed method object of study could enhance cheese whey bioactivity. Lastly, the fermented product was tested on 20 adult volunteers with sensitive skin, by means of a patch test, to assess the possible irritative power of the sample. The active ingredient created turned out to be particularly suitable for sensitive skin, which is very vulnerable and prone to adverse reactions. Indeed, no pathological manifestations have been shown following 48 hours contact with the fermented whey permeate.

The results obtained using the native LMG P-31789 strain for the fermentation showed that it possesses particular metabolic abilities, being a stable galactose-fermenting microorganism. A complete utilization of galactose is a desirable property in dairy fermentations. Lower levels of this sugar, generally secreted into the medium by LAB in equimolar amounts with lactose uptake[54], can be beneficial for human health. It has been established that D-galactose mediates skin cell senescence and cytotoxicity, through a molecular mechanism of non-enzymatic glycation[55,56]. Moreover, its build-up, due to several enzymatic impairments, induces severe problems in galactosemic individuals[57]. These aspects make the bacterium used interesting for its application in food and cosmetics. As evidenced by scientific studies, bacteria belonging to the species of *L. delbrueckii* subsp. *bulgaricus* usually do not possess the ability to ferment galactose[29]. In the future, it may be interesting to compare the effect of the fermentation with LAB strains belonging to the same species, as well as evaluating a possible extension of the fermentation times and the repeatability of the entire process. GOS and organic acids were identified as being particularly important in terms of components quantified in the obtained preparation. GOS can have beneficial effects on the skin when introduced through diet, but there is no information about their direct non-prebiotic effect[16-17]. The results obtained in this study therefore represent a novelty for the scientific community. On the other hand, the comparison with published data for butyrate role in wound healing shows correspondence[43]. Indeed, treatments based on this compound are able to di increase the rate of re-epithelization and repair in case of skin disorders.

Talking about skin aging and health, peptides play an important role. They are widely used as topical skin care ingredients with moisturizing, repairing and antioxidant action[58]. For this reason, the authors' future objectives include the characterization of the peptide fraction of the LMGferm24 sample. Lastly, given the particularly interesting composition of this preparation, it is planned to study further beneficial effects for the skin. The presence of different organic acids, indeed, suggests a potential inhibitory effect of the tyrosinase enzyme (EC 1.14.18.1)[41,45,59].

Conclusion

With a view to circular economy, the smart management of agro-food by-products has become an area of major environmental and economic importance worldwide. This research showed a novel and alternative approach that can provide the opportunity to produce an active substance starting from dairy wastewater. It is an extremely innovative process, taking advantage of specific microbial strain fermentation, which could find application in the cosmetics industry to obtain biomolecule-rich active ingredients. The obtained composition has proven to be safe and suitable for topical application. For such proposal, the preferred pharmaceutical dosage forms are lotions, adhesive plasters or impregnated gauze pads. This product could be applicable in the clinical setting, with reference to skin diseases or, alternatively, in cosmetology, with a view to solving unsightly skin features. Further investigations are needed to assess the involvement of other molecules in the preparation that have not yet been tested and that could act in skin health. For this reason, future developments in the field of medical devices cannot be excluded.

Funding And Patent

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Conflicts of Interest

The authors declare no conflict of interest.

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