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Concentrated Buffalo Whey as Substrate for Probiotic Cultures and as Source of Bioactive Ingredients: A Local Circular Economy Approach towards Reuse of Wastewaters

Alberto Alfano [1], Sergio D'ambrosio [1], Antonella D'Agostino [1], Rosario Finamore [1], Chiara Schiraldi [1,†] and Donatella Cimini [2,*,†]

[1] Department of Experimental Medicine, Section of Biotechnology, Medical Histology and Molecular Biology, University of Campania L. Vanvitelli, Via de Crecchio 7, 80138 Naples, Italy; alfano84@libero.it (A.A.); sergio.dambrosio@unicampania.it (S.D.); antonella.dagostino@unicampania.it (A.D.); rosario.finamore@unicampania.it (R.F.); chiara.schiraldi@unicampania.it (C.S.)

[2] Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania L. Vanvitelli, Via Vivaldi 43, 81100 Caserta, Italy

* Correspondence: donatella.cimini@unicampania.it; Tel.: +39-081-566-7686

† These authors equally contributed to the work.

Abstract: Waste reduction and reuse is a crucial target of current research efforts. In this respect, the present study was focused on providing an example of local investment in a simple process configuration that converts whey into value-added compounds and allows recovery of a clean water stream. In particular, buffalo milk whey obtained during mozzarella manufacturing was ultrafiltered in-house on spiral membrane modules (20 kDa), and the two obtained fractions, namely the retentate and the permeate, provided by the dairy factory, were further processed during this work. The use of an additional nanofiltration step allowed the recovery of high-quality water to be reused in the production cycle (machine rinsing water within the facility) and/or in agriculture, also reducing disposal costs and the environmental impact. The ultrafiltration retentate, on the other hand, was spray-dried and the powder obtained was used as the main substrate for the cultivation of *Lactobacillus fermentum*, a widely studied probiotic with anti-inflammatory, immunomodulatory and cholesterol-lowering properties. In addition, the same sample was tested in vitro on a human keratinocytes model. Resuspended concentrated whey powder improved cell reparation rate in scratch assays, assisted through time-lapse video-microscopy. Overall these data support the potential of buffalo whey as a source of biologically active components and recyclable water in the frame of a local circular economy approach.

Keywords: whey product; proteins; ultrafiltration; nanofiltration; keratinocytes scratch assay; mozzarella cheese manufacturing

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1. Introduction

Whey is the main and most polluting by-product obtained from cheese manufacturing processes due to its organic load consisting of lactose, lactic acid, proteins and salts. However, the substantial production of whey worldwide, estimated to be around 180–190 $\times$ 10^6 ton/year [1], and the consideration that 1 or 2 kg of cheese yields 8 to 9 kg of whey [2] is fostering its valorization. In fact, the discovery of its potential use as a functional food with nutritional applications is transforming it from a waste [3] into an added value product. Numerous studies have attributed several biological actions to these by-products which are important in the medical, pharmaceutical and food industries for their properties with potential benefits to human health [4]. The biological components of whey, including lactoferrin, beta-lactoglobulin, alpha-lactalbumin, glycomacropeptide, and immunoglobulins, demonstrate a range of immune-enhancing properties [5]. In addition, whey has the ability to act as an antioxidant, antihypertensive, antitumor, hypolipidemic

and chelating agent. A number of clinical trials have successfully been performed using whey in the treatment of cancer, HIV and hepatitis B [6–8]. Moreover, today, whey is a popular dietary protein supplement that may provide antimicrobial activity, immune modulation, improved muscle strength and body composition, and prevent cardiovascular disease and osteoporosis [8,9]. The commercial success of whey proteins has led to the development of high-quality protein-based supplements manufactured as primary products, and not as a by-product, of cheese manufacturing. In fact, currently, proteins are processed with less aggressive treatments, for example under low temperatures and controlled pH, to avoid denaturing their native structures [5].

Due to the high lactose content in addition to proteins, whey, very often in combination with other medium components, was also used as a substrate for the cultivation of diverse microorganisms [10]. Whey-based media were in fact investigated for the production of value-added chemicals (e.g., succinic acid, lactic acid), antimicrobial peptides and probiotic biomasses [11–16].

The use of membrane technologies in order to obtain bioactive molecules from whey is a topic of growing interest. The main advantages are low energy requirement, no need for additives, separation efficiency and easy scale-up, and temperature control that prevents denaturation of recoverable added value products [5]. Several treatment technologies based on membranes were proposed. In particular, four basic types of membrane filtrations present potential applications for the dairy industry, i.e., microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) [17,18]. The application of filtration processes to produce clean effluents thereby reducing wastewater and generating a purified stream (e.g., machinery washing, irrigation) transforms a difficult to manage and polluting effluent into a resource. Moreover, the use of membrane devices with different cut-offs allows the separation of compounds (proteins, peptides and lactose) present in whey into differentially enriched fractions that are, therefore, suitable for different applications.

Cheese manufacturing is one of the main industrial activities in the food sector present in the Campania region. The scope of this study was to promote a locally integrated bio refinery approach fully exploiting discarded whey. Therefore, the permeate and retentate of ultrafiltered whey, both provided by a local dairy factory, were evaluated in this work. The permeate was further processed to investigate a potential downstream approach to obtain reusable water with a low organic load. The retentate was evaluated to identify other potential biotechnological applications of whey from buffalo milk. In particular, it was investigated as the main substrate for the growth of *Lactobacillus fermentum*, a probiotic with several potential biomedical usages [19]. Moreover, it was also assessed for the presence of molecules active on tissue repair induction by using wound healing assays on mammalian cells.

2. Materials and Methods

2.1. Materials

The buffalo whey used in this work was provided by the dairy factory "La Perla del Mediterraneo" (Battipaglia, Italy) in the framework of research and development processes in collaboration with the Dept. of Experimental Medicine, Bioteknet and the manufacturing facility. The company performed ultrafiltration on polyethersulphone 20 kDa spiral membranes (filtering surface 40 m^2) and provided (i) a sample (15–20 L) of the retentate concentrated fraction that was spray-dried in the framework of this research and used as substrate for the growth of *L. fermentum* and for wound healing assays (ii) a permeate that was nanofiltered and characterized.

L. fermentum was isolated from buffalo milk (data not shown). All salts and medium components for bottle and fermentation experiments were supplied by Sigma-Aldrich (St. Louis, MO, USA). Yeast extract was furnished by Organotechnie (La Corneuve, France), while sulphuric acid was purchased by Biochem s.r.l. (Turin, Italy). Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum(FBS), penicillin–streptomycin, Phosphate Buffer Solution, (PBS) and Trypsin are provided by Gibco Invitrogen (Milan, Italy). HaCaT

cells, a spontaneously transformed non-tumorigenic human keratinocytes cell line, were provided by the Zooprophylactic Institute (Brescia, Italy).

2.2. Downstream Process

NF processes were performed using a polyethersulfone spiral membrane with a nominal cut-off of 150–200 Da, respectively, with a total filtering area of 0.3 m^2 (Fluxa Filtri, Milano, Italy). The system used for the membrane process was a UF-NF system equipped with a 10 L volume steel tank, pressure gauges on the inlet and retentate lines, and a thermostatic bath to keep the temperature constant (Idea 3 Engineering, Lessona, Italy). The whey fraction provided by the dairy factory had been ultrafiltered in site on 20 kDa polyethersulfone membranes with a filtering surface of about 40–50 m^2 and flux of 2500–3000 L/h. The concentration factor reported was 13–14 fold, on the basis of 10,000 L used at the inlet for each treatment. In this study, 10 L of the permeate of the above-mentioned UF on 20 KDa were nanofiltered and concentrated. All recovered fractions were characterized by analyzing lactate, lactic acid and total proteins as described in the following paragraph.

2.3. Spray Drying

Spraying was carried out on the UF retentate sample (UF_Ret20) provided by dairy company. The spray drier used was a Mobile MinorTM (GEA Process Engineering, Danimarca). Samples were loaded into the specific chamber by means of a peristaltic pump with a flow rate of 2–2.5 Kg/h, the internal temperature of the chamber (in which the sample remained in contact with the gas for drying for about 10 s) was of about 160–170 °C, the outlet temperature 80–85 °C; compressed air was used as the drying gas. One g of spray-dried sample indicated as UF_Ret20Pow was dissolved in 100 mL of sterile bi-distilled water and characterized to determine the protein, sugars, lactic acid and insoluble solid content. Water content was determined by drying 100 mg of powder on a thermobalance (Mettler Toledo HR 83 Halogen). The sample was heated at 105 °C and temperature was maintained for 6 h until a constant weight was recorded.

For ashes determination, 10 g of sample were placed in a tared crucible. The crucible was placed in a muffle furnace for 18 h at about 550 °C. When the temperature dropped below 250 °C the sample was quickly transferred to a desiccator until cooled before weighing. Calculation for dry ash content [20]:

$$\% \text{ ash (dry basis)} = (\text{wt after ashing} - \text{tare wt of crucible})/(\text{original sample wt} \times \text{dry matter coefficient}) \times 100.$$

2.4. Analytical Methods

The various fractions and samples were analyzed for lactose, galactose, glucose, lactic acid, acetic acid and ethanol content using a UHPLC Dionex Ultimate 3000+ chromatograph (Thermofisher, Italy) equipped with a UV/Vis and RI detector. The standards and the samples, previously ultrafiltered on Centricon systems with 3 kDa cut-off, were injected in a Shodex sugar SH1011 300 × 8 mm, 6 µ column according to the following operating conditions: isocratic elution with 0.1% sulfuric acid in water at a flow rate of 0.8 mL/min; temperature: 40 °C; concentration range: 20–0.01 mg/mL; acquisition time 25 min [21].

2.5. Total Protein Quantification

The total protein content was obtained by analyzing the samples by UV/Vis spectrophotometry at 595 nm with a colorimetric method by using the Kit Protein assay Biorad (Bio-Rad Laboratories Inc., Hercules, CA, USA) [22] and the bovine serum albumin (BSA) as standard (Bio-Rad Laboratories Inc., Hercules, CA, USA). A Beckmann DU-800 spectrophotometer was used.

2.6. Bottle and Bioreactor Experiments

Frozen stocks of *L. fermentum* were prepared from cells growing exponentially on Mann, Rogosa and Sharpe (MRS) broth and stored at −80 °C after the addition of a 20% v/v glycerol solution. Bottle experiments were performed in 100 mL screw-cap bottles with a working volume of 90 mL and incubated at 37 °C and 150 rpm in a rotary shaker incubator (model Minitron, Infors, Bottmingen, Switzerland) for 24 h. Modified semi-define medium SGSL [23] contained the following per liter: 10 g yeast extract; 10 g soy peptone; 2 g $Na_3C_6H_5O_7$; 0.5 g L-ascorbic acid; 0.5 mL Tween80; 0.25 g $MgSO_4 \cdot 7\,H_2O$; 0.2 g NaCl; 0.05 g $MnSO_4 \cdot H_2O$. The concentrated and spray-dried UF retentate (UF_Ret20Pow) was used for growth experiments. In particular, the medium consisted of 1 ± 0.1, 2 ± 0.2 and 4 ± 0.2% UF_Ret20Pow reconstituted in SGSL medium. UF_Ret20Pow 4 ± 0.2% reconstituted in distilled water was also tested. Control experiments on SGSL supplemented with 30 g/L glucose or lactose were also performed. Samples were withdrawn at time 0, and after 8 and 24 h of growth to analyze optical density (600 nm) carbon sources consumption, and acid and ethanol production. All bottle experiments were performed in triplicate. Viability was evaluated by serially diluting the samples and plating on MRS-agar medium. Plates were incubated at 37 °C for 36 h before counting viable cells. Each sample was analyzed in triplicate.

Bioreactor experiments were performed in a Biostat CT plus (Sartorius Stedim, Gottingen, Germany) bioreactor with a working volume of about 2.2 L. Temperature was controlled at 37 °C, pH at 6.1 and agitation was fixed at 150 rpm. Fermentation medium containing UF_Ret20Pow reconstituted in water or in water supplemented with salts present in the SGSL medium and 2 g/L of yeast extract and soy peptone (1/5th of that present in SGLS) were used for bioreactor growth. Before each experiment, a concentrated stock solution of *L. fermentum* was inoculated in 0.25 L of SGSL medium at 37 °C and 150 rpm and grown for 8 h. The pre-culture was then transferred to the bioreactor with a peristaltic pump (model 313 U, Watson-Marlow, England) to reach up to 10% (v/v) of the working volume inside the fermenter. Stirring was set to 150 rpm and air was sparged at a constant flow of 0.44 vvm. A constant pH of 6.1 was maintained by addition of NaOH 10 M and 30% v/v H_2SO_4 solutions. Experiments lasted up to 24 h. Samples were withdrawn during the experiments to analyze cell density (600 nm), cell viability, carbon source consumption, and acid and ethanol production. Viability was evaluated as previously described. Batch experiments on UF_Ret20Pow only were repeated 4 times whereas those on UF_Ret20Pow supplemented with salts and complex nitrogen sources were performed in duplicate.

2.7. Cell Cultures and Treatments

Human Keratynocytes cell lines (HaCat) were grown in Dulbecco's Modified Eagle Medium DMEM, supplemented with 10% (v/v) heat-inactivated FBS, penicillin 100 U/mL and streptomycin 100 µg/mL (Sigma Aldrich, MI, USA). The cells were grown on tissue culture plates (BD Bioscience-Falcon, San Jose, CA, USA), in a humidified atmosphere (95% air and 5% CO_2, v/v) at 37 °C.

UF_Ret20Pow whey powder was dissolved in ultrapure (bi-distilled) water at a concentration of about 20 g/L (w/v) and sterilized by microfiltration on 0.22 µm filter devices. The sample was diluted with PBS to a final titer of 2 and 4 g/L before being added to the medium. Opportunely diluted PBS was used as control in the medium. During the wound healing assay, the concentration of FBS in the medium was reduced to 1% v/v to slow down the migration phenomena that could then be better evaluated.

2.8. In Vitro Scratch Test and Time-Lapse Video Microscopy (TLVM)

Briefly, HaCaT cells were seeded in 12-wells until complete cellular confluence was reached. Successively the confluent monolayer was scratched with a sterile tip (Ø = 0.1 mm) and the diluted UF_Ret20 samples were added to the medium.

The in vitro cell migration was analyzed by a video microscopy time-lapse station (TLVM) (OKOLAB, Pozzuoli, Italy), assembled with an inverted microscope (AxioVision200, Zeiss, Germany), a CCD-gray-camera (ORCA ER, Hamamatsu Photonics, Hamamatsu City, Japan) that records the images, a motorized stage incubator that logs the position and maintains the in vitro condition of the cell culture (37 °C, 5% CO_2 in humidified air) and the custom-tailored software OKO-Vision 4.3 software that follows the overall process and allows image analysis. The TVLM tracks the wound repair process in real-time for 48–72 h, due to the selection and recording of representative images of the experiments. The quantitative analysis of wound closure rates is calculated as [(Area t0 − Area t)/Area t0] × 100 directly by the software or alternatively by manual mode tracking for each image of the wound area over time. For each well a minimum of 5 fields of view was used for deriving the overall averaged curves of wound closures (%) as a function of the time, thus ensuring the statistical significance of the experiment.

2.9. Statistical Analysis

All data were analyzed by means of two-tailed non homoscedastic Student's *t*-test, and $p < 0.05$ was considered as statistically significant.

3. Results and Discussion

In line with current circular economy approaches, a waste material from one of the production sectors of excellence of the Campania region, namely the dairy industry, was processed and recovered to obtain on the one side purified water for cleaning use within the manufacturing facility, and on the other a spray-dried concentrate that could be tested as substrate for the growth of *L. fermentum* and as bioactive compound useful in promoting tissue regeneration. An overview of the process approach is presented in Figure 1.

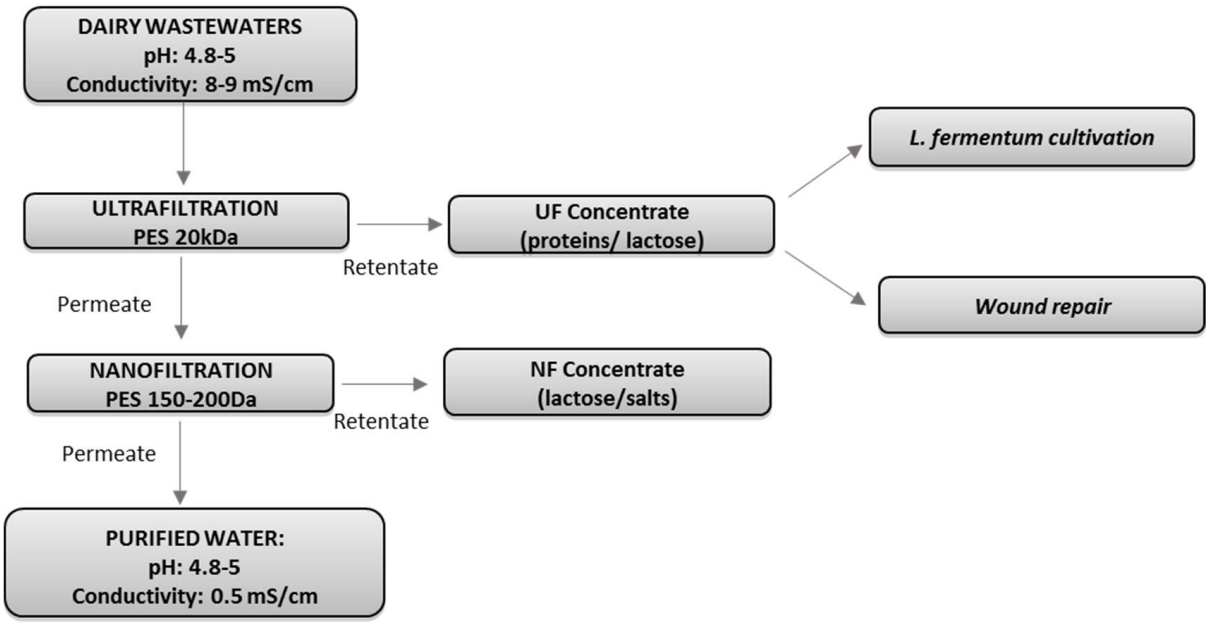

Figure 1. Overall downstream process of discarded whey and obtainment of added value fractions.

3.1. Clean Water Generation, Spray Drying of Concentrated Ultrafiltered Whey and Characterization of the Obtained Fractions

The first part of the project regarded the evaluation of a simple membrane-based process based on sequential UF and NF steps for the reduction of the organic load (BOD and COD) of whey that represents a substantial pollution source. The use of filtration is easily applicable in small/medium-sized companies that can not only reduce costs due to the disposal of numerous tons of discarded whey produced daily, but also reuse it for the

separation of fractions enriched in lactose, proteins and peptides, and for the recovery of clean water. Results of the NF process are reported in Table 1a,b.

Table 1. Downstream processing of whey (**a**) Nanofiltration on 150–200 Da membranes of buffalo whey previously ultrafiltered on 20 kDa membranes. TMP, transmembrane pressure; LMH, L/m^2·h; (**b**) Composition change of buffalo whey during ultrafiltration and nanofiltration. * Indicates the volume of permeate used for the nanofiltration experiments in the present study.

(a)	Nanofiltration Parameters	Initial Vol. (L)	Final Vol. (L)	Concentration Factor	Initial Flux (LMH)	Final Flux (LMH)	Initial TMP (bar)	Final TMP (bar)
	150–200 Da cut-off	10	1.25	8	33	28	10	12

(b)	Sample	Protein (g/L)	Lactose (g/L)	Lactic Acid (g/L)	Vol (L)
	Buffalo whey	1.10	29.8	5.0	10,000
	UF ret	5.19	42.9	6.7	800
	UF per	0.05	31.7	5.5	9200 (10 *)
	NF ret	0.09	120.3	10.9	1.25
	NF per	n.d.	0.03	2.3	8

Thirteen liters of UF_Ret20 were spray-dried in 6 h and resulted in the recovery of about 525 g of powder (Table 2). The residual water present in the sample resulted equal to 3.50 ± 0.50%. The powder was of thin and palpable grain size and contained prevalently lactose (Table 2). When suspended at 20 g/L, pH in bidistilled water was equal to 5.15 ± 0.05 and a conductivity of 3.18 ± 0.10 mS/cm was measured. Spray drying of the volume used in this work was affected by the void volume within the equipment, thus the yield was lower than 70%. However, the treatment of greater volumes on an industrial scale typically improves process yields, as the amount of solids lost in the spray dryer remains constant once a steady-state is achieved, and only the very fine powder that cannot be separated in the cyclone defines the actual process yield on solids.

Table 2. Characterization of spray-dried powder UF_Ret20Pow. Lac, lactose; Gal, galactose; Glu, glucose; LA, lactic acid. Ins. Solids, insoluble solids.

Sample	Protein *w/v* (%)	Lac *w/v* (%)	Gal *w/v* (%)	Glu *w/v* (%)	LA *w/v* (%)	Ins. Solids *w/v* (%)	Water *w/w* (%)	Ash *w/w* (%)
Dried powder	6	44	8	6	5	7	4	11

3.2. Evaluation of Ultrafiltered Spray-Dried Whey as Substrate for the Growth of L. fermentum

The use of whey proteins for the growth of biotechnologically interesting microorganisms is well established [16]. For example, the probiotic strain *L. casei* is a well-known case study for the production of biomass [11] and other antimicrobial products such as nisin and bacteriocins [12,13]. Since lactic acid bacteria present specific and critical nutritional requirements, often supplementation with growth factors, vitamins and amino acids is necessary. *L. fermentum* DSM 20,049 was previously grown on whey with the addition of hydrolyzed lupin flour as an auxiliary nitrogen source in flask experiments, showing a shorter lag phase and a 70% higher biomass yield as compared to growth on MRS in the same conditions [24].

In the present study, buffalo milk-derived whey was evaluated as a substrate for the growth of an *L. fermentum* strain isolated from buffalo milk. In particular, the powder obtained from the spray-dried ultrafiltered retentate (UF_Ret20Pow) was used in fermentation experiments to evaluate its potential as a one-pot medium; this would in fact strongly

simplify cultivation medium preparation and overall upstream processes. Since this fraction was not diafiltered it contained a large amount of sugars, in particular 44%, 8% and 6% of lactose, glucose and galactose, respectively, and about 6% of protein, a necessary nitrogen source for bacterial growth. Strain viability, sugars consumed and metabolic products produced (lactic acid, acetic acid and ethanol) were initially evaluated in bottle experiments. *L. fermentum* was cultivated on semi defined SGSL medium supplemented with different amounts of UF_Ret20Pow. SGLS supplemented with glucose, the carbon source most efficiently used by *L. fermentum* [25], or lactose, the main sugar present in whey powder, were used as controls. As shown in Figure 2, a higher concentration of viable cells and related metabolic products (e.g., lactic acid, ethanol) were observed in relation to higher initial concentrations of ultrafiltered whey; in particular, in the presence of 4% UF_Ret20Pow, the final average concentration of viable cells (8.85 ± 0.17 Log10 CFU/mL) was comparable to that obtained in control experiments on glucose (8.97 ± 0.14 Log10 CFU/mL), whereas it was significantly higher compared to results obtained in control experiments with lactose (8.43 ± 0.17 Log10 CFU/mL). Additional experiments on UF_Ret20Pow dissolved in water (in the absence of SGLS medium components) showed that growth was still supported by the organic compounds present in the ultrafiltered whey fraction, although a significantly lower sugar consumption and biomass concentration were achieved (Figure 2), probably due to the lower amount of nitrogen source compared to that present in SGSL medium [25].

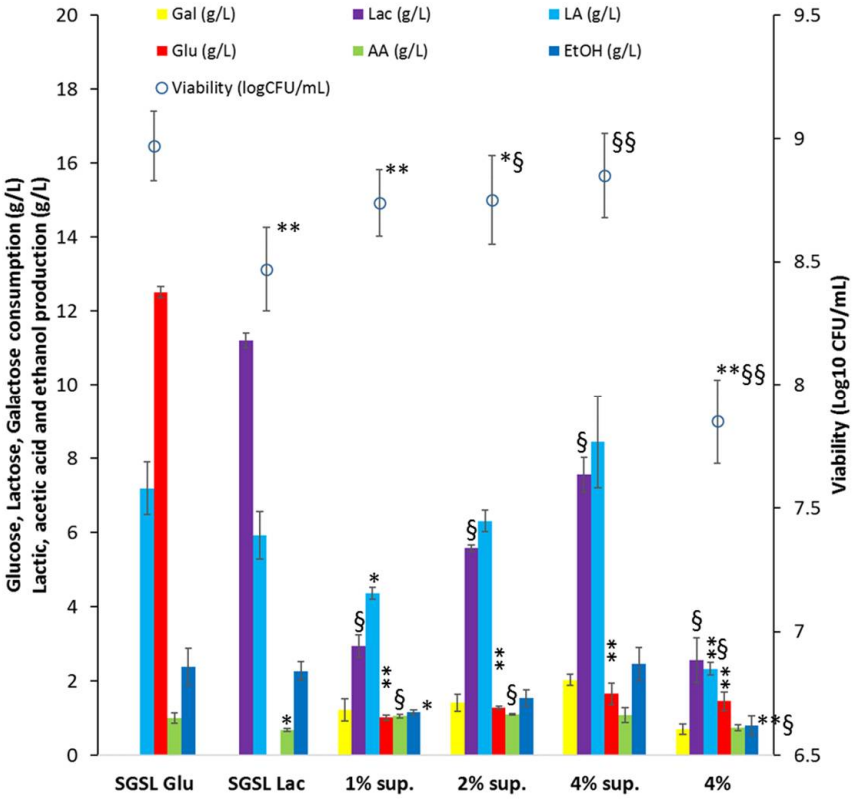

Figure 2. Small-scale experiments performed in 100 mL bottles at 37 °C and 150 rpm. Gal, galactose; Lac, lactose; Glc, glucose; LA, lactic acid; AA, acetic acid; EtOH, ethanol. Sup indicates media in which UF_Ret20Pow was reconstituted in SGLS medium. Data were analyzed by two-tailed non homoscedastic Student's *t*-test. * indicates $p < 0.05$ compared to results obtained on SGLS Glu; ** indicates $p < 0.01$ compared to results obtained on SGLS Glu; § indicates $p < 0.05$ compared to results obtained on SGLS Lac; §§ indicates $p < 0.01$ compared to results obtained on SGLS Lac.

With the aim of simplifying upstream and downstream procedures on an industrial scale by using buffalo milk waste as the only component of fermentation media, bioreactor experiments in controlled conditions were performed. *L. fermentum* probiotic biomass

production was therefore investigated on UF_Ret20 as a one-pot medium (powder reconstituted in water) and on UF_Ret20 supplemented with SGSL salts, yeast extract and soy peptone (1/5th of that present in SGLS). Each experiment was performed at least in duplicate. Table 3 shows the results obtained. Controlled pH and constant air sparging improved viability that reached 8.1 ± 0.2 Log10 CFU/mL on the medium containing concentrated whey only. The addition of salts and of low amounts of complex N sources (soy peptone and yeast extract), yielded similar results, indicating an impact only on sugar consumption and LA production which increased (Table 3); LA, in particular, showed a 3.3 fold titer increase with a final concentration of about 10.0 ± 0.3 g/L and a final yield of 0.61 ± 0.03 g/g. Apparently, due to the lower amount of nitrogen source, the sugars were addressed to acid instead of biomass production [26].

Table 3. Data obtained by growing *L. fermentum* in batch in a Biostat CT plus (3 L) bioreactor reported as mean ± s.d. UF_Ret20Pow indicates that the fermentation medium was obtained by reconstituting the UF_Ret20Pow spray-dried powder in water; UF_Ret20Pow sup indicates the additional presence of SGLS salts, yeast extract and soy peptone (2 g/L) in the medium. Lac, initial lactose; Gal, initial galactose; Glu, initial glucose; LA, lactic acid; AA, acetic acid; EtOH, ethanol. Data were analyzed by two-tailed non homoscedastic Student's *t*-test: * $p < 0.05$; ** $p < 0.01$.

	Viability (Log10 CFU/mL)	Lac (g/L)	Glu (g/L)	Gal (g/L)	Sugars Cons. (g/L)	LA (g/L)	AA (g/L)	EtOH (g/L)	$Y_{LA/s}$ (g/g)
UF_Ret20Pow	8.1 ± 0.2	3.3 ± 0.8 **	2.5 ± 0.8 *	1.5 ± 0.2	7.2 ± 1.4 **	3.0 ± 0.6 **	1.3 ± 0.4	1.4 ± 0.5	0.42 ± 0.01
UF_Ret20Pow sup	8.0 ± 0.2	9.4 ± 0.3	5.1 ± 0.3	2.0 ± 0.2	16.4 ± 0.3	10.0 ± 0.3	1.5 ± 0.4	1.9 ± 0.2	0.61 ± 0.03

3.3. Whey Valorization as Wound Repair Agent

Buffalo milk whey has been shown to contain various proteins with immunomodulatory and antitumor activity, and a series of bioactive peptides with antimicrobial, antioxidant, antihypertensive and remineralizing properties [5]. For example, the effect of whey proteins in improving the inflammatory status during wound healing in diabetic rats, in particular by reducing the expression of specific cytokines involved in the reparation, was previously reported [27]. Kalinina and collaborators [28] identified whey proteins, and in particular WFDC12, as a specific marker for the last stage of keratinocytes differentiation, probably enhancing the occurrence of epidermal homeostasis.

Our research aimed to evaluate the ability of ultrafiltered and concentrated whey from buffalo milk, to prompt cell migration and regeneration of the human keratinocyte monolayers by using a scratch assay [29]. This assay is generally run to obtain a preliminary but robust evaluation of the regenerative potential of biomolecules.

The sprayed powder was diluted in the cell growth medium and tested by an in vitro wound healing assay, as reported in the material and methods section. Representative fields of view of wound closure reported in Figure 3a are clearly showing that the addition of UF_Ret20Pow prompts keratinocytes migration, confirming the role of whey proteins in dermal repair. Quantitative analyses (Figure 3b) indicate that in the presence of the sprayed powder at a concentration of 4 g/L, 40% wound closure occurred within 15 h, as compared to the control that, at the same time, reached about 20% of repair. Moreover, the sample induced complete healing within 30 h, whereas the closure area of the control was lower than 40%. Data analysis by means of a two-tailed non homoscedastic Student *t*-test indicates that the wound closure rate significantly improves ($p < 0.05$) in the supplemented sample from 9 h onwards, as compared to the control (Figure 3b). The treatment with a less concentrated sample (2 g/L) did not improve significantly the natural occurrence of wound healing. Overall these data clearly establish a beneficial effect of diluted fractionated whey powder, suggesting a potentially promising effect in topical products for skin treatments.

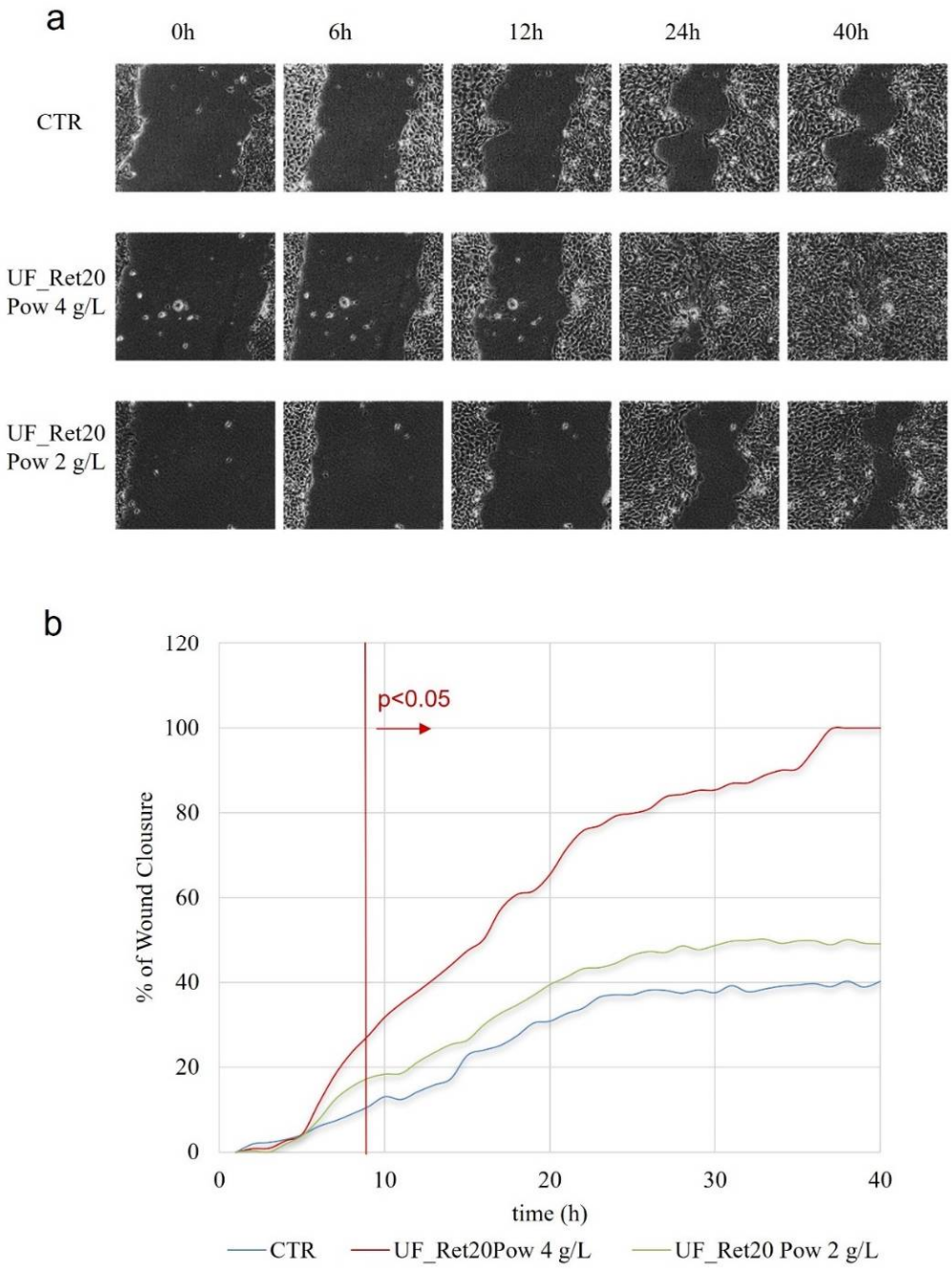

Figure 3. Wound healing assay (**a**) Representative field of view of HaCat scratch assays during time course of the experiments. At least five fields of view for each sample were analyzed; (**b**) Quantitative analyses of wound closure as percentage over time. Data significance was analyzed by two-tailed non homoscedastic Student *t*-tests. The vertical line in the graph indicates the time point from which a significant difference of wound closure % was observed between the CTR and the UF_Ret20Pow 4 g/L treated sample. CTR, control.

4. Conclusions

Overall, the present study provides a small local circular economy example in which one of the most abundant industrial wastes produced by small and medium regional companies could be easily valorized by configuring processes that generate value-added products using a bio-refining concept.

In particular, the implementation of a membrane-based process involving a ultrafiltration and a nanofiltration step was demonstrated to be sufficient to reduce the organic load of whey resulting in a clean and reusable effluent.

Maybe even more importantly, this downstream process generates fractions differentially enriched with potentially interesting compounds. The concentrated UF retentate was investigated here and demonstrated (i) to be by itself a sufficient source of sugars and proteins to support the growth of a probiotic strain with known biomedical applications, and (ii) to stimulate epidermis (keratinocyte) regeneration and therefore meaning potential applicability as an ingredient in skincare products.

Author Contributions: C.S. conceived the study; D.C. and C.S. drafted the manuscript; A.A. conducted downstream processes; S.D. conducted bottle and fermentation experiments; A.D. conducted time-lapse experiments; R.F. conducted HPLC analyses. All authors have read and agreed to the published version of the manuscript.

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