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Bridging biotechnology and nanomedicine to produce biogreen whey-nanovesicles for intestinal health promotion

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Abstract

New intestinal health-promoting biotechnological nanovesicles were manufactured by combining the main environmental pollutant generated from the cheese-making process, well known as whey, with phospholipid, sodium hyaluronate and dextrin, thus overcoming environmental and medical challenges. An efficient, consolidated and eco-friendly preparation method was employed to manufacture the vesicles and the bioactive whey was obtained by mesophilic dark fermentation without external inoculum through a homolactic pathway, which was operated in such a way as to maximize the production of lactic acid. The biotechnological whey-dextrin liposomes and hyalurosomes were relatively small (~100 nm) and characterized by a net negative surface charge (> -30 mV). The addition of maltodextrin to the liposomes and especially to the hyalurosomes significantly stabilized the vesicles under acidic conditions, simulating the gastric environment, as their size and polydispersity index were significantly lower ($p<0.05$) than those of the other formulations. The vesicles were effectively internalized by the intestinal Caco-2 cells and protected them against oxidative stress. Nutriosomes promoted the proliferation of *Streptococcus salivarius,* a human commensal bacterium, in a better extent ($p<0.05$) than liposomes and hyalurosomes, as a function of the concentration tested. These findings could open a new horizon in intestinal protection and health promotion by integrating biotechnology, nanomedicine, sustainability principles and bio-circular economy.

Keywords: Liposome; Caco-2 cells; Whey; Oxydative stress; Dark fermentation; *Streptococcus salivarius.*

1. Introduction

In recent decades, it has been recognized that lactate is involved in different host mechanisms and may contribute to the maintenance of human health status, shaping the gut environment and influencing the physiology of the colon and acting as a source of energy for both host cells and beneficial bacteria of the intestinal microbiota (Ríos-Covián et al., 2016). Lactate is the conjugate base and the main form of lactic acid in aqueous solution and physiological conditions and the two names can be considered interchangeable (Phypers and Pierce, 2006). The latter can protect the intestinal mucosa from inflammation via several mechanisms such as stimulating the anti-inflammatory cytokines, thus exerting interesting pharmaceutical properties (Sittipo et al., 2019). Lactic acid may be chemically or biologically synthesized and it is produced on an industrial scale mostly through pure Lactobacilli fermentation of carbohydrates (Ghaffar et al., 2014).

Whey is a green-yellowish watery portion formed by the coagulation and removal of the milk casein during the cheese-making process. It is considered a major pollutant waste stream in cheese production not only for the high volume generated (80-90% of processed milk, by volume, and 127-143 million tonnes/year) but also for its high organic load, characterized by 50–102 g/L of chemical oxygen demand and 27–60 g/L of biological oxygen demand (Carvalho et al., 2013; Ryan and Walsh, 2016; Slavov, 2017). Furthermore, cheese whey contains salts such as sodium chloride, potassium chloride and calcium (0.46 – 10%), and has a pH ranging from 3.8 to 6.5 (Prazeres et al., 2012). Its improper disposal management results in severe environmental problems, such as dissolved oxygen depletion and consequent rising of the eutrophication and toxicity for aquatic animals in surface water bodies, ground-water contamination, and impairment of soil characteristics. Moreover, the high contents of lactose and minerals in whey can cause concern issues for farms when it is used as animal feed. Therefore,

77 proper treatment of cheese whey is mandatory, and traditional approaches have been
78 employed (Ahmad et al., 2019). However, the implementation of the sustainability
79 principles and circular economy requires a step forward in whey waste management as
80 compared to the mere treatment aimed at controlling the impacts (Asunis et al., 2020;
81 Rezvani et al., 2022). The prevention of environmental impacts must be associated with
82 the recovery of resources that must be characterized as much as possible by high added
83 value on the market. In this respect and as an example, ultrafiltration, microfiltration,
84 reverse osmosis, and ion exchange have been studied and applied for the recovery of
85 whey protein concentrate and whey protein isolate (Carvalho et al., 2013). Another
86 interesting option, which was considered in the present work, is the employment of
87 bioreactors to treat the cheese whey and produce compounds to be used in the
88 pharmaceutical, cosmetic, nutritional or other industrial fields (Amoabediny et al.,
89 2010). Dark fermentation is a promising approach for the valorisation of residual
90 organic substrates that may be converted to gaseous biohydrogen and soluble volatile
91 fatty acids in bioreactors. Lactic, acetic, propionic, butyric and other organic acids can
92 be produced from the dark fermentation of organic residues (De Gioannis et al., 2014).
93 High levels of lactose and nutrients in cheese whey along with the need to treat it
94 properly have attracted many interests in its use as an initial substrate in the
95 fermentation process. Besides, the association of lactic acid and whey proteins seems
96 to be virtuous (Luongo et al., 2019). In fact, whey proteins have been described to be
97 precursors of the antioxidant glutathione that is known to constrain the adverse effects
98 of the stressors provided by modern processed foods, environmental pollutants,
99 smoking, alcohol and radiation, which in turn generate high level of oxygen species
100 (ROS), a redox imbalance and chronic disorders in the digestive tract (Rezvani et al.,
101 2019b). Given that, the use of whey proteins may exert a potent prebiotic effect on the

oral cavity and gut (Sánchez-Moya et al., 2017). On the other hand, a part of the whey proteins, especially the most abundant such as beta-lactoglobulin, are degraded by the bacterial strains as they represent the main nitrogen source during the fermentation performed to produce lactic acid, bioactive peptides and branched-chain amino acids (Daliri et al., 2018; Mazzoli et al., 2014; Pescuma et al., 2010). Therefore, developing a whey-derived substrate, enriched in lactic acid and containing its proteins, bioactive peptides and amino acids, may promote the growth of useful bacteria, and reduce the oxidative status boosting gut health and modulating dysbiosis and inflammatory disorders. Moreover, due to its high instability under external (light, heat and radiations) and internal (pH variation, enzymes etc) conditions, its formulation in nanovesicles can improve its beneficial effect and the sensory characteristics masking the sour taste of the lactic acid, and the bitter taste of the protein residues produced during fermentation (Rezvani et al. 2022). Its administration in humans contained in ad-hoc formulated vesicles could promote the stability protecting it against the gastro-intestinal harsh conditions; control the release; prevent the rapid clearance and reduce side effects, such as acidosis and short bowel syndrome; improve their shelf life and local biodistribution (Rao et al., 2018; Rezvani et al. 2019a; Rezvani et al., 2018).

Considering these promising challenges, for the first time in this study, ovine fermented whey enriched in lactic acid was loaded in modified phospholipid vesicles and their in vitro beneficial effect on intestine was evaluated. The biotechnological, nanotechnological and green approaches were combined to covert a potential pollutant, whey, into an efficient bioactive product for intestinal health promotion. Ovine cheese whey was fermented in dark conditions to recover the whey enriched lactic acid, which was combined with phospholipid, hyaluronan and maltodextrin (Glucidex) to obtain innovative biotechnological whey-nanovesicles. Liposomes, hyalurosomes,

nutriosomes and hyalonutriosomes were prepared using an easy, green and organic solvent-free method and their physico-chemical properties and biological performances were evaluated as functional delivery systems. The mean diameter, size distribution, zeta potential and lactic acid content of vesicles were measured along with their morphology and stability in acidic and neutral media to select the most suitable formulations for intestinal delivery. Furthermore, their biocompatibility and ability to counteract the oxidative stress and internalization in intestinal epithelial cells (Caco-2) along with the prebiotic effect on *S. salivarius*strain *K12*, were evaluated as well.

2. Material and methods

2.1. Materials

Fresh raw ovine whey was collected from local Sardinian dairy industries (Italy). Samples of raw ovine whey were stored in 1 L containers at -15°C until use. Before feeding the bioreactor, the whey was thawed at 25 °C. Lactic acid, bovine serum albumin, Folin-Ciocalteu, 1,2-dioleoyl-sn glycero-3-phosphoethanolamine-N-lissamine-sulfo-rhodamine B, 5(6)-carboxyfluorescein, Hoechst 33342 (Trihydrochloride, Trihydrate) and all other reagents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Lipoid S75 (S75), a soybean phospholipids mixture (70% phosphatidylcholine, 3% lysophosphatidylcholine and 9% phosphatidylethanolamine), was provided by a local supplier for Lipoid GmbH (Ludwigshafen, Germany), AVG S.r.l. (Garbagnate Milanese, Milan, Italy). Glucidex, a natural maltodextrin with a dextrose equivalent of 9 (molecular weight ~80 kDa) was kindly provided by Roquette® Italia s.p.a (Cassano Spinola, Alessandria, Italy). Sodium hyaluronate low molecular weight (200-400 kDa) was obtained from DSM Nutritional Products AG Branch Pentapharm (Switzerland). Plastics and reagents for cell culture were acquired from Life Technologies Europe (Monza, Italy). *S. salivariusK12* was

isolated from a commercial product (Bactoblis®), corresponding to the American Type Culture Collection (ATCC) BAA-1024 strain. Under sterile conditions, a pill was inserted into a Falcon® tube containing 50 µl of Schaedler Broth (Microbiol, Uta, Cagliari) with 20% glycerol. The culture at the mid-lag phase was stored at -80 °C until use.

2.2. Experimental set-up

Batch dark fermentation of raw ovine whey was performed in a 2 L-bioreactor (working volume = 1.8 L) equipped with an automatic system for pH control and acquisition data (BIOFLO 110-New Brunswick Scientific; BioCommand Lite software). The raw ovine whey was directly used as the feed material without any additional external inoculum. The operating temperature, pH and stirring frequency were adjusted at 39 ± 1 °C, 5.5 and 150 rpm, respectively. The sodium hydroxide (5 M) and hydrochloric acid (1M) solutions were used to control the pH, continuously. A eudiometer was used to measure the biogas production based on the displacement principle using a saturated sodium chloride solution at pH 2. To prevent the gas dissolution and visual estimation of the liquid level in the eudiometer, sulphuric acid and methyl orange were used, respectively. The measured gas volume was standardized to 273.15 °K and 10^5 Pa. A black plastic film and a nitrogen gas flushing were used to prevent photo-fermentation and ensure air drive off from the headspace of the bioreactor, respectively. Sampling was done at the scheduled time intervals up to 80 h and the contents of lactose, lactic acid and organic acids were measured.

2.3. Analytical methods

The analytical parameters were determined in raw ovine whey and fermented ovine whey. The total organic carbon concentration and its dissolved fraction (0.45 µm-

filtered samples) were measured using a Shimadzu analyzer (TOC-VCSN, Shimadzu, Japan). Soluble carbohydrate concentration (0.45 µm-filtered samples) was estimated as described by DuBois colorimetric phenol-sulphuric acid method (DuBois et al., 1956). The content of soluble protein was determined spectrophotometrically at 750 nm, based on the Lowry alkaline copper method (Lowry et al., 1951). Bovine serum albumin (BSA) and lactose were used as the standard in Lowry and DuBois methods, respectively. The concentration of lactic acid was measured using a spectrophotometric method (HITACHI U-200), detecting the colored product of the reaction between iron (III) nitrite and lactate at 390 nm (Borshchevskaya et al., 2016). The concentration of volatile fatty acids (acetic, propionic and butyric) was determined using a gas chromatograph with flame-ionization detection (model 7890B, Agilent Technology) equipped with a capillary column (HP-FFAP, 25 m, inner diameter 0.32 mm, Agilent Technology). The 0.45 µm-filtered samples were acidified by phosphoric acid (pH<3) and injected (0.6 µl) into the instrument. The temperature of the injector was 230 °C and that of the detector was 300 °C. The oven temperature was initially set at 60 °C (3-minutes holding time), followed by a ramp of 10 °C/minutes up to 160 °C. Helium (1.6 ml/min, splitless) was used as the carrier gas. The gas produced during the dark fermentation was sampled periodically from the reactor headspace with a 1 ml gastight syringe and injected through a valve in a gas chromatograph (model 7890B, Agilent Technology) equipped with a thermal conductivity detector and two connected stainless columns in series packed with Shincarbon ST (50/80 mesh) and HayeSep N (80/100 mesh). The operating temperatures of the detector and the valve were 200 °C and 90 °C, respectively, and Helium was used as the carrier gas with the pressure of 8 and 25 psi in the HayeSep N and Shincarbon ST columns, respectively. The oven temperature was set initially at 70 °C (3-min holding time), followed by a ramp of 10 °C/minutes

up to 160 °C (3-minutes holding time). All analyses were performed in triplicate for both raw ovine whey and fermented ovine whey.

2.4. Preparation and characterization of biotechnological whey-nanovesicles

Sterilized and unsterilized fermented ovine whey were used and tested for the preparation of the nanovesicles. The fermented ovine whey was sterilized at 121 °C for 30 minutes and filtered through 0.2 μm filters. In a typical preparation of liposomes and nutriosomes, S75 (240 mg) and maltodextrin (200 mg, when necessary) were weighed in glass vials and hydrated with 2 mL of fermented ovine whey (unsterilized and sterilized). To prepare hyalurosomes and hyalonutriosomes, firstly, sodium hyaluronate (0.1 mg/mL, 10 mL) was dispersed in fermented ovine whey and sonicated (99 cycles, 5 seconds on and 5 seconds off), subsequently, an aliquot of it (2 mL) was used as hydrating medium of S75 (240 mg) and maltodextrin (200 mg, when necessary). The formulations were sonicated for 25 cycles (5 seconds on and 5 seconds off) by Soniprep 150 ultrasonic disintegrator (MSE Crowley, London, UK) (Castangia et al., 2013).

2.5. Characterization of biotechnological whey-nanovesicles

Cryogenic transmission electron microscopy (FEI Company, Eindhoven, Netherlands) using a low-dose mode, at 200 kV and −173 °C was employed to evaluate the formation and morphology of nanovesicles (Rezvani et al., 2019a).

The average diameter, polydispersity index and zeta potential of each sample were measured by using a Zetasizer Ultra (Malvern Panalytical, Worcestershire, United Kingdom) (Manca et al., 2014a).

Vesicle dispersions were purified by dialysis to eliminate the unloaded lactic acid. Each formulation (1 ml) was transferred in a dialysing bag (Spectra/Por® membranes;

12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., USA) and dialyzed in 2 L of distilled water for 2 h at 25 °C (Rezvani et al., 2019b). The distilled water was refreshed a 1 h to allow the complete removal of unloaded lactic acid. The acid lactic content before and after dialysis was measured and the percentage of lactic acid inside the vesicles was calculated as its amount after dialysis versus that before dialysis.

2.6. Stability of biotechnological whey-nanovesicles in acidic and neutral media with high ionic strength

The formulations were diluted (1:100 v/v) with simulated gastric (pH 1.2) and intestinal (pH 7) fluids and incubated at 37 °C for 2 and 6 h, respectively. Then, their mean diameter, polydispersity index and zeta potential were determined. The acidic solution at pH 1.2 was prepared by mixing hydrochloric acid 1 M (6% v/v) and sodium chloride (20 mg/mL) in distilled water. The solution at pH 7 was prepared by dissolving sodium phosphate dibasic (7.26 mg/mL) and sodium chloride (17.54 mg/mL) in distilled water (Manconi et al., 2017).

2.7. Biocompatibility and protective effect of biotechnological whey-nanovesicles against oxidative stress-induced in Caco-2 cells with hydrogen peroxide

Caco-2 cells (1 x 10^5 cells/well) were seeded in 96-well plates using Dulbecco's Eagle Medium (DMEM) high glucose, enriched with foetal bovine serum, L-glutamine, fungizone, penicillin and streptomycin as growth medium. The cells were incubated at 37 °C in 5% carbon dioxide for 24 h. Then, fermented whey or whey-nanovesicles, properly diluted with cell medium (1:10, 1:100, 1:1000, 1:10000 and 1:100000 v:v), were added to each well. After incubating for 48 h, the medium was removed from each well and replaced with 100 µL of MTT (0.5 mg/mL in PBS, final concentration). After

3 h of incubation at 37 °C the MTT was removed, replaced with 100 µL of dimethyl sulfoxide and the absorbance of the solubilized formazan crystals was read at 570 nm using a microplate reader (Multiskan EX, Thermo Fisher Scientific, Inc., Waltham, MA, USA) (Rezvani et al., 2019b). Untreated cells were used as negative controls (100% viability).

The ability of the formulations to protect the Caco-2 cells against oxidative stress was tested measuring their survival after treatment with hydrogen peroxide (Manconi et al., 2017). 1 x 10^5 cells/well were grown in 96-well plates at 5% carbon dioxide and 37 °C in complete Dulbecco's Eagle Medium (as described above). After 24 h of incubation, the cells were stressed with hydrogen peroxide (1:50000 v/v dilution in PBS), immediately after were treated with fermented ovine whey or biotechnological whey-nanovesicles (1:10 and 1:100 v/v dilutions) and finally incubated for 4 h. The cell viability was measured by means of MTT colorimetric assay as described above. The untreated cells and hydrogen peroxide-stressed cells without treatment were used as positive and negative control, respectively.

2.8. Uptake of fluorescent biotechnological whey-nanovesicles by Caco-2 cells

The biotechnological whey-nanovesicles were labelled with a lipophilic fluorescent marker (1, 2-dioleoyl-sn glycero-3-phosphoethanolamine-N-lissamine-sulfo-rhodamine B; Rho-PE, 0.025 mg/mL; red) and loaded with a hydrophilic fluorescent marker (5(6)-carboxyfluorescein; CF, 0.025 mg/mL; green). The Caco-2 cells were incubated at 37 °C with different formulations for 2, 4 and 8 h. Hoechst 33342 (10 µg/ml), was used as the cell nucleus marker and the internalization of fluorescent vesicles by Caco-2 cells was detected using a confocal microscopy (Olympus, Barcelona, Spain) (Manca et al., 2014b). Fluorescence excitation/emission wavelengths

of phosphoethanolamine-N-lissamine-sulfo-rhodamine B was 559/578 nm, that of 5(6)-carboxyfluorescein was 470/535 nm and that of Hoechst was 360/460 nm.

2.9. Prebiotic effect of the biotechnological whey-nanovesicles on S. salivariusK12

A microplate containing *S. salivariusK12* (*S. salivarius*) and different dilutions of the fermented ovine whey or biotechnological whey-nanovesicles (50, 25, 12.5, 6.3, 3.1, 1.6 0.8, 0.4%) were incubated at 37 °C for 48 h to allow the formation of a structured microbial biofilm. Alternatively, the same samples were added 24 h after the incubation of *S. salivarius* and left for 48 h at 37 °C. At the end of this growth phase, a crystal violet staining protocol described by Montana University Center for Biofilm Engineering was used to determine the biofilm amount as a function of the detected optical density at 550 nm. The wells were washed with 0.9% sodium chloride solution, crystal violet solution (0.1 ml, 0.4%) was added and discarded, the wells were gently washed with 0.9% sodium chloride, air-dried for 15 min, finally, acetic acid solution (0.25 ml, 30%) was added and the optical density was read at 550 nm using a microplate reader (Multiscan FC, Thermo Fischer Scientific, USA) (Orrù et al., 2017). The untreated bacteria were considered as positive control and higher values of optical density were considered as improvement of biofilm forming capacity.

2.10. Statistical analysis of data.

The results were expressed as mean value ± standard deviation. Statistically significant differences among samples were determined using variance analysis. The post-hoc Tukey-Kramer t-test was used to substantiate a significant difference between the means of two specific groups. The Excel software package (Microsoft Corp, Redmond, USA) was used for the statistical analysis, considering $P < 0.05$ as the minimum level of significance.

297 **3. Results**

298 *3.1. Results of the dark fermentation process*

299 The organic content of raw ovine whey is mainly associated with carbohydrates
300 (57% of total organic carbon content) and soluble proteins (14% of total organic carbon
301 content), Table 1. In the present study, all the raw ovine whey carbohydrates were
302 assumed as lactose, whilst to estimate the protein presence, the carbon content was
303 considered as 0.46 g/g BSA.

304 The lactic acid content increased almost linearly during the dark fermentation
305 process (Fig. 1A). The maximum concentration of lactic acid (47.9 g/L) was obtained
306 at 53 h of fermentation (Table 1 and Fig. 1), while propionic, butyric and acetic acids
307 were detected at extremely low or undetectable concentrations. The trends of gaseous
308 metabolites production are reported too as specific cumulative hydrogen and carbon
309 dioxide generation per unit of lactose consumed (Fig. 1B). No appreciable gaseous
310 production was observed in this phase to underline that lactic acid production was
311 associated with the homolactic fermentation pathway. The second phase of the dark
312 fermentation process started when lactic acid production peaked and was characterized
313 by lactate-consuming pathways with an accompanied production of volatile fatty acids,
314 hydrogen and carbon dioxide up to 80 h (Fig. 1). For this reason, in the present study,
315 the dark fermented whey accumulated up to 53 h was characterized (Table 1) and then
316 used for nanovesicles preparation to have the highest concentration of lactic acid. The
317 characterization of the soluble metabolites showed that most of the carbohydrates were
318 consumed during dark fermentation (87% up to 53 h), while the protein content was the
319 same in the raw cheese whey and in the fermented one.

320 *3.2. Preparation and characterization of biotechnological whey-nanovesicles*

The ovine whey enriched in lactic acid obtained by dark fermentation (fermented ovine whey) was combined with phospholipid, hyaluronan and dextrin to produce innovative biotechnological whey-nanovesicles. Specifically, the fermented ovine whey was used as the hydrating medium for phospholipid and dextrin mixture, producing the so called nutriosomes (Catalan-Latorre et al. 2018). Alternatively, hyaluronan was initially dispersed in the fermented ovine whey, which in turn was used to obtain hyalonutriosomes. For comparison, whey liposomes and whey hyalurosomes without dextrin were prepared as well. The peculiar structure of hyalurosomes and hyalonutriosomes is mainly due to the interactions that may occur between their components. Indeed, the headgroup of phosphatidylcholine, which is a zwitterionic molecule, may be differently oriented as a function of the binding or adsorption of negatively charged molecules. In this case, it can interact with sodium hyaluronate on the bilayer surface leading the formation of immobilized vesicles, as previously reported (Scherer et al. 1989; Manca et al. 2015). On the contrary, the negatively charged maltodextrin may interact with phosphatidylcholine headgroup reinforcing the structure and giving more stable and resistant systems (Abramović et al., 2008; Scherer and Seelig, 1989). The most important physico-chemical properties of nanovesicles were evaluated by measuring their mean diameter, polydispersity index and zeta potential. The mean diameter of whey liposomes was 189±23 nm and equal to that of nutriosomes (180±29 nm) and hyalonutriosomes (179±31 nm, $p>0.05$ among the three values) while that of hyalurosomes was significantly larger (331±60 nm, $p<0.05$ versus previous values). Results disclosed that the addition of dextrin also in association with hyaluronan did not change the size of whey vesicles, while hyaluronan addition allowed a statistically significant increase of the mean diameter of whey vesicles, only when combined with the dextrin. All formulations were slightly polydispersed as the

346 polydispersity index was ≥0.32 (Table 2). The zeta potential of all the nanovesicles was
347 strongly negative. However, the dispersions were not stable and after few days, they
348 aggregated and formed yellow pellets, indicating the occurrence of additional
349 fermentation and vesicle disruption. To stop the fermentation process and eliminate
350 microbial presence, the whey was sterilized at 121 °C for 30 minutes and then filtered
351 through 0.2 µm filters. Thus, a transparent solution was obtained while insoluble lipid
352 aggregates remained in the filters. The main proteins of the whey (i.e. beta-
353 lactoglobulin) was previously degraded by the bacterial strains in the whey during the
354 fermentation to produce lactic acid, bioactive peptides and amino acids. The remain
355 proteins were partially hydrolysed in polypeptides and amino acids during the
356 sterilization by autoclaving. It was previously demonstrated that the total amino acids
357 were essentially unaffected and can exert their beneficial effect (Ford 1976). The same
358 biotechnological whey-nanovesicles were prepared using this sterilized whey as
359 hydrating medium. The obtained vesicles were significantly smaller, less polydispersed
360 and more stable (Table 3) than the corresponding vesicles prepared with unsterilized
361 fermented whey as the mean diameter of liposomes and hyalurosomes was ~133 nm
362 ($p>0.05$ between the two values, $p<0.01$versus the mean diameter of vesicles prepared
363 with unsterilized whey) and that of nutriosomes and hyalonutriosomes was smaller
364 (~116 nm, $p<0.05$ versus the mean diameter of nutriosomes and hyalonutriosomes
365 prepared with sterilized and unsterilized whey). The small size of vesicles prepared
366 without maltodextrin indicated its contribution in the vescicle packing, probably due to
367 their emulsifier properties, hydrophilic or lipophilic affinity, which depends on the
368 number of glucose residues. Therefore, they can facilitate the interaction among the
369 whey components, the phospholipid bilayer and/or the vesicle surface (Shogren and
370 Biresaw, 2007). The zeta potential of all the biotechnological whey nanovesicles

prepared with sterilized whey was highly negative. More precisely, that of whey liposomes and whey nutriosomes was ~-47 mV ($p>0.05$ between the values of the two samples) and that of nutriosomes and hyalonutriosomes was even more negative (~-61 mV), supporting the occurrence of negatively charged hyaluronan on the vesicle surface (Cosco et al., 2017). Among the studied biotechnological nanovesicles, the hyalonutriosomes held the highest amount of lactic acid (~58%, $p<0.05$).

No separation or sedimentation were observed in the vesicle dispersions during three months of storage at 4 °C, as they kept unchanged their physico-chemical properties.

Fermented whey liposomes and fermented whey nutriosomes were mainly spherical and uni- or bi-lamellar, while the presence of hyaluronan in whey hyalurosomes and hyalonutriosomes allowed the formation of oligolamellar and multicompartment vesicles (Fig. 2).

3.3. Stability of whey-nanovesicles in acidic and neutral media characterised by high ionic strength

After oral administration, the vesicles have to pass through the destabilizing environment of the stomach to reach the intestine and exert their beneficial effects. During this passageway, first they go through the high ionic strength and acidic environment of the stomach and then they meet the neutral environment of the intestine. Therefore, the incubation of vesicles at 37 °C, at hight ionic strength and pH 1.2 and 7, is a suitable test, which permits to check their stability under these harsh conditions and could predict the in vivo delivery effectiveness (Catalan-Latorre et.al 2018; Parekh et al. 2022). In order to confirm this, vesicles were diluted with the acidic (pH 1.2) or neutral (pH 7) medium at high ionic strength, and their mean diameter, polydispersity index and zeta potential were monitored for 2 h at pH 1.2 and 6 h at pH 7 (Table 4). At pH 1.2 and high ionic strength, fermented whey liposomes (~130 nm and ~0.27) and

fermented whey nutriosomes (~117 nm and ~0.26) underwent a strong increase in size, up to ~1500 nm ($p<0.01$ versus the starting values), and polydispersity index, up to ~0.62 ($p<0.01$ versus the starting values), indicating the possible aggregation and fusion of original vesicles to form new larger vesicles. The presence of hyaluronan in fermented whey hyalurosomes (~136 nm and ~0.27) was able to attenuate this phenomenon, addressing a moderate increase of their size, up to 325±43 nm ($p<0.05$ versus the starting value), and polydispersity index, ~0.39 ($p<0.05$ versus the starting value). It could be related to the special structure of hyalurosomes, where hyaluronan stays inside and outside, immobilizing the vesicles, protecting them, and avoiding their destabilization and rupture (Catalan-Latorre et al. 2018; Catalan-Latorre 2016). As previously reported for nutriosomes, the addition of maltodextrin to hyalurosomes seemed to further stabilize the hyalonutriosomes (~115 nm and ~0.19) in the acidic medium, indeed they had the lowest mean diameter, 217±29 nm ($p<0.05$ versus the starting value) and polydispersity index 0.35 ($p<0.05$ versus the starting value). Results confirmed the ability of dextrin and hyaluronan to protect the phospholipid vesicles from external environmental (Catalan-Latorre et al. 2018; Catalan-Latorre et al. 2016).

At neutral pH, the mean diameter of fermented whey liposomes and nutriosomes increased less than that in the acidic medium (up to 500 nm). The behaviour of fermented whey hyalurosomes and hyalonutriosomes was similar to that observed in acidic conditions. The mean diameter of fermented whey hyalurosomes was 332±58 nm and that of fermented whey hyalonutriosomes was 170±32 nm, confirming once again the positive effect of the combination of polymer and dextrin on vesicle stability.

These results disclosed that the combination of lactic acid-enriched whey with maltodextrin, hyaluronan and phospholipid led to the formation of performing biotechnological whey-nanovesicles that are stable at pH 1.2 and 7 and characterised

421 by high ionic strength. The same vesicles prepared without hyaluronan and dextrin did
422 not have the same resistance under the adopted harsh conditions.

423 *3.4. Evaluation of in vitro biocompatibility of whey-nanovesicles against Caco-2*
424 *cells*

425 Considering the composition of the prepared whey-nanovesicles and their ability to
426 maintain unchanged their structure in the acidic environment, their safety was evaluated
427 using Caco-2 as a model of intestinal cells. These cells share some similarities with
428 intestinal enterocytes and can be a useful tool for *in vitro* studies (Sambruy et al., 2001).
429 The fermented ovine whey at the lowest dilution (1:10 v/v) caused ~25% of cell
430 mortality (viability ~75%, $p<0.05$ versus other values except that obtained using whey
431 liposomes at lower dilution) and at the highest dilution (1:100000 v/v) did not address
432 toxic effect, as the viability was ~95%, $p<0.05$ versus the values obtained using other
433 vesicles except whey hyalurosomes at lower dilution (Fig. 3A). Similarly, the
434 fermented whey liposomes disclosed ~25% of toxicity (viability ~75%, $p>0.05$ versus
435 values obtained using fermented whey) when used at the lowest dilution (1:10 v/v),
436 whilst at the higher dilutions the toxic effect was eliminated (~95% viability, $p>0.05$
437 versus the values obtained using whey hyalurosomes at lower dilution and whey
438 nutriosomes at all the dilutions). No significant toxicity (viability ~95%) was observed
439 when the cells were treated with the fermented whey nutriosomes, irrespective of the
440 dilution, and with the fermented whey hyalurosomes at the higher dilutions. Moreover,
441 fermented whey hyalurosomes (at all dilutions except the lowest) and fermented whey
442 hyalonutriosomes (at all dilutions) improved the cell viability up to ~110% ($p<0.05$ in
443 comparison values obtained using liposomes and nutriosomes).

444 Results indicated that, except whey liposomes at the lower dilution, the
445 biotechnological whey-nanovesicles were compatible with Caco-2 cells at all the tested

446 dilutions, and that the association with dextrin, hyaluronan and phospholipid eliminated
447 the toxic effect of whey and promote cell proliferation, probably because of the
448 beneficial effects of these components (Hamann et al., 1995; Mohamed Amin et al.,
449 2015).

450 *3.5. Protective effect of biotechnological whey nanovesicles against oxidative*
451 *stress-induced in Caco-2 cells*

452 To evaluate the protective effect of the produced formulations against oxidative
453 stress, Caco-2 cells were stressed with hydrogen peroxide and simultaneously treated
454 with the biotechnological whey-nanovesicles (Fig. 3B). Considering the low toxicity of
455 these vesicles, the lower dilutions (1:10 and 1:100 v/v) were used to evaluate their
456 protective effect against oxidative stress. Stress induced by hydrogen peroxide reduced
457 the cell viability up to ~60% ($p<0.05$ versus the viability of cells treated with fermented
458 whey or whey vesicles). The simultaneous treatment with the fermented ovine whey
459 reduced the damaging effect as the viability increased up to ~80% ($p<0.05$ versus
460 viability of cells treated with whey-nanovesicles). The formulation of fermented whey
461 in phospholipid vesicles effectively counteracted the toxicity induced by hydrogen
462 peroxide as the healthy conditions of cells were completely restored (viability ~100%,
463 $p>0.05$ among the viability of cells treated with the whey-nanovesicles, irrespective of
464 their composition).

465 *3.6. Caco-2 cellular uptake of fluorescently labelled vesicles*

466 Bioactive molecules usually exert their activity inside the cells, so that an efficient
467 cellular uptake is one of the major requirements of nanocarriers. To track the pathway
468 of biotechnological whey-nanovesicles and their interactions with the cells, the vesicles
469 were functionalized with a lipophilic fluorescent probe (Rho-PE, 0.025 mg/ml) and

loaded with a hydrophilic fluorescent probe (CF, 0.025 mg/ml). Cells were observed using a confocal microscope at different time points (2, 4 and 8 h), Fig. 4. The localization and intensity of the rhodamine-phosphoethanolamine are displayed in red, those of carboxyfluorescein in green and those of the nucleus in blue. The orange areas resulted from the superposition of rhodamine and carboxyfluorescein. At 2 and 4 h using fermented whey liposomes and fermented whey hyalurosomes, the green fluorescence of the hydrophilic probe was well visible in the background and only a few red spots, due to the lipophilic probe, were detectable around the cells. The distribution of the hydrophilic probe in the background may be due to the unentrapped carboxyfluorescein, which diluted in the cell medium regained the fluorescence due to the inhibition of its auto-quenching (Chen et al 1988). After 8 h, an orange fluorescence (suggesting the superposition of both probes) was visible around the cell nucleus, indicating a tardive and moderate internalization of the vesicles, which are supposed to remain intact, as the probes were superposed. Using the fermented whey nutriosomes and fermented whey hyalonutriosomes, the internalization was more evident and occurred faster, probably thanks to their better ability to interact with the cell membrane, as the orange colour was visible around the cell nucleus and in the cytoplasm even at 4 h. These results suggested that the addition of dextrin could promote the internalization of vesicles inside the cells, which is in accordance with previous studies (Manconi et al., 2018).

3.7. Prebiotic effect of whey-nanovesicles on the biofilm formation of S. salivarius

The capability of biotechnological whey-nanovesicles to promote the growth of intestinal *S. salivarius* was evaluated *in vitro* by simultaneously adding the bacterial strain and vesicle dispersions at different dilutions to the culture medium. The samples were incubated for 48 h and a crystal violet staining protocol was used to determine the

biofilm formation capacity as a function of the optical density at 550 nm (Fig. 5A). In the case of simultaneous addition, the fermented ovine whey inhibited the grown of *Streptococcus salivarius,* regardless of the used concentrations and incubation time. Indeed, when the *S. salivarius* was incubated with the fermented whey, the optical density of samples was always <0.3, lower than that of the positive control (untreated bacteria), which was ~1.5 ($p<0.01$ versus the values of treatments with fermented whey). In the same way, the whey liposomes, hyalurosomes and hyalonutriosomes when added simultaneously to the bacterial strain inhibited the biofilm formation as the optical density of samples was <1.2 ($p<0.05$ versus the values of positive control). The decrease of the optical density was concentration-depending, as at lower concentrations the biofilm capacity formation was higher. On the contrary, an improvement of optical density up to ~2 ($p<0.05$ versus the values of positive control) was detected when nutriosomes at 6.3 and 3.1% were used. To better evaluate the promising effect of whey nutriosomes and whey hyalonutriosomes, the test was repeated adding the samples 24 h after the seeding of *S. salivarius* (Fig. 5B). In this case, the biofilm formation was concentration-depending; using whey hyalonutriosomes at concentrations from 50 to 6.3%, the biofilm formation exceeded the positive control reaching ~2 (discontinuous rectangle in Fig. 5B), confirming a possible contribution of the carriers, which was in turn affected by the dilution. The growth of *S. salivarius* treated with fermented whey was lower to that of positive control (untreated bacteria) and concentration-depending, indicating no contribution of this sample on bacterial proliferation. The fermented whey nutriosomes at concentrations 3.1, 1.6 and 0.8% provided an effect similar to that of the control, while at the other dilutions a reduction of the growth of *S. salivarius* was detected. The effect of fermented whey hyalonutriosomes at higher concentrations was the most evident and confirmed the beneficial effect addressed by this formulation,

probably due to the fruitful association of the four functional and structural components: fermented whey, maltodextrin, hyaluronan and phospholipids. In addition, the ability of fermented whey hyalonutriosomes to overstep the bacterial external surface (bacterial capsule) was underlined (Piletti et al., 2017).

4. Discussion

As the first remarkable result of this study, the production of lactic acid was achieved by means of homolactic dark fermentation avoiding preliminary treatment of raw ovine whey and the addition of any inoculum, relying on the indigenous biomass of cheese whey. Dark fermentation is a complex green process strongly governed by numerous substrate and operation factors, addressing the evolution towards different metabolic pathways and, in turn, final products. With adequate control, this process yields valuable chemicals and appears as an important sustainable alternative to traditional industrial production (Asunis et al., 2020). In this respect, the operating pH and the fermentation time are known to be among the most relevant parameters to attain a significant conversion of the original organic load into exploitable by-products such as and volatile fatty acids, lactic acid in particular. In case of cheese whey fermentation, the autochthonous lactic acid bacteria are capable of spontaneous lactic acid production (Asunis et al., 2019). Therefore, the sheep chese whey is an ideal substrate for lactic acid production since it is rich in carbohydrates and contains lactic acid bacteria that are added as starter cultures during the cheese-making process (Pescuma et al., 2008). In homolactic fermentation, these bacteria only provide lactic acid via the Embden-Meyerhof-Parnas pathway, generating pyruvate from glucose glycolysis and reducing it to lactate (Castillo et al., 2013). Conversely, in heterolactic fermentation one mole of pyruvate is converted to lactate and one mole is converted to ethanol (or acetate) and carbon dioxide via the phosphoketolase pathway. In the present study, the mesophilic

545 fermentation tests were performed by adopting operating pH conditions (5.5) such as
546 to favour homolactic fermentation, and by carefully observing the evolution of the
547 process over time in order to stop it before (53 h) the lactic acid was converted to other
548 soluble (acetic, butyric, proponic acid) and gaseous (hydrogen and carbon dioxide)
549 compounds. The resulting fermentation broth did not require any purification from
550 ethanol (or acetate) as it would have been necessary in the case of heterolactic
551 fermentation. Furthermore, it deserves to be underlined that the production of lactic
552 acid was obtained without the need to pre-treat or sterilise the cheese whey or to use a
553 specific inoculum. Therefore, the characteristics of the applied dark fermentation
554 process denote it as green, economical, and relatively simple to manage, in turn
555 promising to be scale up, also in consideration of the fact that the type of plant required
556 is very similar to that used for the more traditional anaerobic digestion.

557 For the first time in this study, the resulted green fermented sheep cheese whey was
558 combined with phospholipids, hyaluronan and maltodextrin to produce
559 biotechnological whey-nanovesicles. According to green chemistry, vesicles were
560 prepared by using a consolidated, easy, solvent-free and low-dissipative method, which
561 involved the direct dispersion of solid components in the fermented whey and the
562 subsequent sonication. Natural and low-purified components were selected to
563 manufacture the vesicles. The phospholipid used, Lipoid S75, is a mixture of unpurified
564 soybean phospholipids, triglycerides and fatty acids (Otto et al., 2018), the dextrin is a
565 natural fibre obtained from the enzymatic controlled hydrolysis of malt starch (Castro
566 et al., 2016) and hyaluronan is a constituent of different human tissues, cartilage and
567 synovial fluid, which can be extracted from animal or human tissues or alternativly
568 produced by fermentation of *S. zooepidemicus* (Amado et al., 2016).

569 The prepared vesicles were specifically tailored for treating intestinal disorders, in
570 order to this, their stability during the passage through the gastro-intestinal tract was
571 improved adding maltodextrin and hyaluronan to phospholipid. Indeed, liposomes are
572 usually destroyed after oral administration, especially upon exposure to the acidic
573 conditions of the stomach. The dextrin was added due to its thickener and prebiotic
574 properties (Singh et al., 2019) and hyaluronan was added aiming at enhancing the
575 bilayer integrity, adjusting the structural rigidity, controlling the bilayer fluidity and
576 promoting the adhesion of the carrier to the intestinal mucosa (Lee, 2020). As expected,
577 hyalurosomes were more stable under acidic and basic conditions in comparison with
578 liposomes and their stability significantly increased in association with the dextrin
579 (hyalonutriosomes). The combination of lactic acid-enriched whey with maltodextrin,
580 hyaluronan and phospholipid provided the formation of promising biotechnological
581 vesicles stable at pH 1.2 or 7 and high ionic strength. From the technological point of
582 view, fermented whey hyalonutriosomes appeared as effective and green carriers for
583 oral and intestinal protection and their suitability was confirmed by the evaluation of
584 their efficacy in vitro, by means of tests performed using Caco-2, as a model of
585 epithelial cells of the digestive tract. Results disclosed that all the whey-nanovesicles
586 inhibited the damages caused by hydrogen peroxide in cells and especially
587 hyalonutriosmes were better and faster internalized inside them. In addition, these
588 vesicles favoured the proliferation and biofilm formation of *S. salivarius.* In humans,
589 *S. salivarius* exhibits immunomodulatory properties, inhibitory effect on pathogen-
590 induced inflammatory pathways in epithelial cells, anti-adhesion and anti-colonizing
591 activity against the pathogens and anti-microbial effect against virulent streptococci
592 (Patras et al., 2015; Santagati et al., 2012). Therefore, biofilm formation by *S. salivarius*
593 and its adhesion to intestinal mucosa are of vital importance in maintaining

gastrointestinal health and microbiota homeostasis (Chaffanel et al., 2018). A well-balanced and harmonious relationship between the host and intestinal microbes, driven by the diet, is of great importance for host health protection and prevention of obesity (Ridaura et al., 2013), inflammatory diseases (Morgan et al., 2012), neurological disorders (Scheperjans et al., 2015) and metabolic syndromes (Vijay-Kumar et al., 2010). The whey alone did not promote the growth of the *S. salivarius* but this effect was obtained when fermented whey hyalonutriosomes were used, though the results were concentration-dependent. The results confirmed the positive effect provided by the combination of phospholipid, hyaluronan and dextrin in biotechnological whey-nanovesicles. Fermented whey hyalonutriosomes seemed to be able to overstep the bacterial capsule, probably thanks to the adhesiveness of hyaluronan (Piletti et al., 2017). Overall, the observed results suggested that the green, safe and effective delivery systems obtained in this study may represent a powerful alternative for the treatment of disorders affecting the digestive tract and associated with oxidative stress and inflammatory conditions, contributing to the maintenance of human health.

5. Conclusions

This study fits perfectly into the perspective of applying the principles of the circular economy: a residual stream can be managed in such a way as to avoid environmental impacts, but also to recover resources characterized by high added value on the market. Achieving results of this type requires the interaction between different expertises and economic sectors, sometimes very distant from each other. In the present case study, the waste of an agro-industrial sector has allowed the recovery of compounds that can be used in an extremely profitable sector such as the medical-pharmaceutical one. More precisely, it has been demonstrated that an efficient and effective biotechnological

nanoformulation can be obtained by combining advanced and sustainable bio- and nano-technologies, promising in terms of scale-up.

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.

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837
838 **Figures captions**
839 **Fig. 1.** Production of lactic and volatile fatty acids (A) and lactose degradation and gas
840 generation (B) during 80 h of dark fermentation at pH 5.5 and 39 °C. Mean values ±
841 standard deviations are reported (n = 3).

842 **Fig. 2.** Representative cryogenic transmission electron microscopy images of
843 fermented whey liposomes (A), fermented whey hyalurosomes (B), fermented whey
844 nutriosomes (C) and fermented whey hyalonutriosomes (D).

845 **Fig. 3**. Bioavailability of Caco-2 cells incubated with (upper panel) fermented ovine
846 whey, whey liposomes, whey hyalurosomes, whey nutriosomes, whey
847 hyalonutriosomes or (lower panel) stressed with hydrogen peroxide and protected with
848 fermented ovine whey, whey liposomes, whey hyalurosomes, whey nutriosomes, whey
849 hyalonutriosomes. Results are expressed as mean ± standard deviations (error bars), n
850 = 8. Each symbol indicates the same value ($p>0.05$ versus others).

851 **Fig. 4.** Confocal laser scanning microscopy images of living Caco-2 cells incubated for
852 2, 4 and 8 h with fluorescently labelled biotechnological whey-nanovesicles.

853 **Fig. 5.** Biofilm forming capacity evaluated as a function of the optical density at 550
854 nm (OD 550) of *S. salivarius* untreated (positive control) and treated for 48 h (upper
855 panel) with whey and whey-nanovesicles at different dilutions (50, 12.5, 6.3, 3.1, 1.6,
856 0.8, 0.4%) or alternatively treated for 48, but 24 h after the seeding (lower panel), with
857 whey, whey nutriosomes, whey hyalonutriosomes at different dilutions (50, 12.5, 6.3,

858 3.1, 1.6, 0.8, 0.4%). Results are expressed as mean ± standard deviations (error bars).
859 Each symbol indicates the same value ($p>0.05$ versus others).

860 **Table 1.** Main components of raw ovine whey and fermented ovine whey after 53 h of
861 fermentation. Mean values ± standard deviations are reported. a indicates values
862 expressed as a function of lactose; b indicates values expressed as a function of bovine
863 serum albumin; c indicates not assessed values; d indicates values below detection
864 limit.

865

Parameter	Content (g/L)	
	Raw whey	**Fermented whey**
Total solid	7.7 (as %)	N.A. [c]
Volatile solid	7.3 (as %)	N.A. [c]
Total organic carbon	28.8±5.3	23.9±2.3
Soluble total organic carbon	24.1±2.3	17.7±2
Carbohydrates [a]	38.8±1.9	4.9±1
Proteins [b]	9±0.3	8±0.4
Lactic Acid	1.4±0.5	48±0.2
Acetic acid	0.3±0.01	< D.L. [d]
Propionic acid	0.1±0.01	0.6±0.01
Butyric acid	0.3±0.01	1.2±0.2
Fe	0.6±0.1 (as mg/L)	N.A. [c]
Mg	0.1±0.01	N.A. [c]
K	1.2±0.2	N.A. [c]
Na	0.6±0.1	N.A. [c]
Ca	0.3±0.1	N.A. [c]

866

867 **Table 2.** Mean diameter (MD), polydispersity index (PI) and zeta potential (ZP) of
868 whey liposomes, whey hyalurosomes, whey nutriosomes, whey hyalonutriosomes.
869 Results are expressed as mean ± standard deviations (n=6). Each symbol indicates the
870 same value ($p<0.05$ versus others).

Samples	MD (nm)	PI	ZP (mV)
Whey liposomes	189±23*	0.32°	-31±3
Whey hyalurosomes	331±60	0.55	-53±5§
Whey nutriosomes	180±29*	0.32°	-47±4§
Whey hyalonutriosomes	179±31*	0.34°	-46±6§

871

872 **Table 3.** Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and lactic
873 acid content (LAC) of whey liposomes, whey hyalurosomes, whey nutriosomes, whey
874 hyalonutriosomes prepared with sterilized fermented whey. Results are expressed as
875 mean ± standard deviations (n=6). Each symbol indicates the same value ($p<0.05$
876 versus others).

Samples	MD (nm)	PI	ZP (mV)	LAC (%)
Whey liposomes	130±5*	0.27§	-45±6#	14±8¥
Whey hyalurosomes	136±9*	0.27§	-62±4+	34±5¥
Whey nutriosomes	117±6°	0.26§	-50±4#	23±9¥
Whey hyalonutriosomes	115±9°	0.19	-60±4+	58±6£

877

878 **Table 4.** Mean diameter (MD), polydispersity index (PI) and zeta potential (ZP) of
879 whey liposomes, whey hyalurosomes, whey nutriosomes, whey hyalonutriosomes
880 diluted with a high ionic strength solution and incubated at 37°C for 2 at pH 1.2 or 6 h
881 at pH 7. Results are expressed as mean ± standard deviations (n = 3). Each symbol
882 indicates the same value ($p<0.05$ versus others).

Samples	Time	pH 1.2			pH 7		
		MD (nm)	PI	ZP (mV)	MD (nm)	PI	ZP (mV)
Whey liposomes	$t_{2/6h}$	1384±94§	0.63	0±3	461±68^{+}	0.32	-14±3
Whey hyalurosomes	$t_{2/6h}$	325±43$^{\#}$	0.39	5±2	332±58$^{\#,+}$	0.47	-4±1
Whey nutriosomes	$t_{2/6h}$	1603±75$^{@}$	0.61	0±4	546±89^{+}	0.45	-12±2
Whey hyalonutriosomes	$t_{2/6h}$	217±29*	0.35	5±1	170±32*	0.23	-3±2

883