A green and cost-effective approach for the efficient conversion of grape byproducts into innovative delivery systems tailored to ensure intestinal protection and gut microbiota fortification

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Abstract
According to circular economy, wine-making by-products represent a fascinating biomass, which can be used for the sustainable exploitation of polyphenols and the development of new nanotechnological health-promoting products. In this study, polyphenols contained in the grape pomace were extracted by maceration with ethanol in an easy and low dissipative way. The obtained extract, rich in malvidin-3-glucoside, quercetin, procyanidin B2 and gallic acid, was incorporated into phospholipid vesicles tailored for intestinal delivery. To improve their performances, vesicles were enriched with gelatine or a maltodextrin (Nutriose®), or their combination (gelatine-liposomes, nutriosomes and gelatine-nutriosomes). The small (~147 nm) and negatively charged (~ –50 mV) vesicles were stable at different pH values mimicking saliva (6.75), gastric (1.20) and intestinal (7.00) environments. Vesicles effectively protected intestinal cells (Caco-2) from the oxidative stress and promoted the biofilm formation by probiotic bacteria. A preliminary evaluation of the vesicle feasibility at industrial levels was also performed, analysing the economic and energetic costs needed for their production.

Keywords: grape pomace extract; phospholipid vesicles; Caco-2 cells; probiotic bacteria; manufacturing costs; antioxidant activity.

1. Introduction

Grape pomace is the main residue remaining after wine-making process. It is composed of grape skin, seeds and stalks and accounts for about 60% of the total winery solid waste (Jin et al., 2018). This large amount generated is an effective cost for producers and its management needs to find sustainable strategies to preserve and protect the environment (Jin et al., 2021). Deriving from grape, pomace represents a rich source of valuable compounds with multiple applications in cosmetics, pharmaceuticals, and foods industries. Unfortunately, despite its useful content, it is still considered as a by-product and is mainly used in distilleries or simply discarded in the fields causing relevant environmental problems, when incorrectly disposed (Ferrer et al., 2001). Consequently, the sustainable valorisation of grape pomace, as an opportunity feedstock in a valuable circular loop, can open new horizons capable of promoting economic growth by marketable bio-based products (Lee et al., 2020). Previous studies proposed the conversion of grape waste in biogas by means of biological fermentation of carbohydrates. This process did not affect the carbohydrate content, especially if during the extraction process the use of water is avoided, and it should be performed after the extraction of polyphenols (François et al., 2021). The pauperization of pomace from polyphenols by their extraction and the subsequent distillation or biotechnological gasification of residual matrix can become a multiple-complementary process, comprising different steps, specifically aimed at promoting the circular bioeconomy of the wine-chain (Cho et al., 2020; Sharma et al., 2021). It was also demonstrated that this whole biorefinery producing multiple products is technically and economically feasible at a commercial scale and can generate optimal economic performances (Jin et al., 2021). Driving from waste to health, in the present study, the extraction of polyphenols by a simple and low dissipative method, along with the conversion of the extract in innovative nanotechnological delivery systems tailored for the promotion of intestinal health, is proposed. The use of nanosystems is known to improve the biological efficacy of synthetic and natural molecules, and it seems especially effective for the delivery of extracts, since they are composed of several kinds of phytochemicals with different structures and chemical characteristics and capable of exerting a synergic activity responsible for their beneficial properties. Nanocarriers are among the most innovative tools in modern pharmaceutical, cosmetic and food research and are expected to become a driving economic force in the near future. Thus, the main components of the obtained extract were identified and loaded in liposomes, composed of naturally occurring and safe ingredients (phospholipids) and prepared by an easy and scalable method. These vesicles were enriched with gelatin or with a soluble maltodextrin (Nutriose®), or with a combination of the two additives. Gelatin is a protein-polymer widely used in food, pharmaceutical and cosmetic industries, thanks to its gelling effect associated with high biocompatibility and biodegradability. Nutriose® is a soluble maltodextrin with prebiotic effect on intestinal flora and if added to phospholipid vesicles can improve their performance after oral administration (Allaw et al., 2020; Catalán-Latorre et al., 2018). Indeed, phospholipids are sensitive to pH variations and gastro-intestinal enzymes, especially lipases, and the addition of polymers and fibres to the vesicles improved their resistance and ability to protect the payload (Whateley, 2002)(Coma-Cros et al., 2018). To confirm the effectiveness or prepared vesicles, their main physico-chemical characteristics (size and size distribution, zeta potential and incorporation efficiency) along with their stability at high ionic strength and different pH values simulating saliva (6.75), stomach (1.20) and intestine (7.00), were investigated. The ability of the formulations to scavenge free radicals and protect intestinal cells (Caco-2) from damages

induced by hydrogen peroxide were evaluated. Moreover, the ability of the vesicles to stimulate the biofilm formation of commercial probiotic bacteria (i.e., *Streptococcus salivarius* strain K12 and *Lactobacillus reuteri*) (Orrù et al., 2017) along with their prebiotic potential on the microbiota of the oral and gastro-intestinal environments were investigated as well. Finally, in order to evaluate the feasibility of the formulations and their potential commercialization, the economic and energetic costs needed for their production were preliminarily estimated, along with the possible profit to be obtained.

2. Material and methods

2.1. Materials

Lecinova® granular soy lecithin (Céréal, Nutrition & Santé Italia S.p.A., Origgio, Italy), was purchased in a local pharmacy. Nutriose® FM06 was kindly supplied by Roquette (Lestrem, France). Gelatin, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and all the other products of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). The standards of phenolic compounds were purchased from Extrasynthese (Genay, France). The grape extract was obtained from red grape cv. Cannonau pomace provided by a local winery (Argiolas srl, Serdiana, Italy). Cell medium, foetal bovine serum, penicillin, and streptomycin and all the other reagents for cell studies were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

2.2. Extraction method

The dry pomace (100 g) were left to macerate in ethanol (500 mL) at room temperature without external inputs for 24 h. At the end of the extraction process, the extractive dispersion was centrifuged (15 min, 4032 relative centrifugal force) using a NEYA 8 centrifuge (Neya 8, Remi Elektrotechnik Ltd., Vasai, India) to separate the coarse fractions. The ethanol was removed from the extract by evaporation at low pressure using a rotavapor (RII, BÜCHI Labortechnik AG, Flawil, Switzerland) coupled to a vacuum pump (V-700, BÜCHI Labortechnik AG, Flawil, Switzerland) and recovered for further extractions. A dark brown-purple powder highly hygroscopic was obtained and maintained under vacuum and in the dark until use.

2.3. HPLC analysis of phenolic compounds

The phenolic compounds contained in the grape pomace extract were identified and quantified by using a Varian ProStar HPLC-DAD (High-performance liquid chromatography-diode-array detector) system fitted with a 230 pump module, a 410 autosampler module with a 10 μL loop, and a SpectraSystem UV 6000LP photodiode array detector (ThermoSeparation, San Jose, CA, US). Separation was obtained with a Kinetex PFP C18 column (150×4.60 mm, 5 μm, Phenomenex, Casalecchio di Reno, Italy) using 0.22 M phosphoric acid (solvent A) and acetonitrile (solvent B) as mobile phase, at a constant flow rate of 1.0 mL/min. A gradient was generated by keeping 100% of solvent A for 5 min, then decreasing the solvent A to 85% for 15 min, to 50% for 20 min, to 10% for 10 min, and returning to 100% for 5 min. The chromatograms were elaborated with ChromQuest software V. 2.51 (ThermoQuest, Rodano, Italy), working at 520 nm for anthocyanins, 360 nm for flavonols, and 280 nm for the other phenolics. The standard solutions were prepared in methanol and the working standards in ultrapure water. The calibration curves were obtained by means of the external standard method, correlating the area of the peaks with the concentration. Correlation values ranged from 0.9991 to

0.9999 in the range of 0.5-20 mg/L. The pomace extract was solubilized in ethanol, filtered through a GD/X cellulose acetate membrane (0.45 µm, Ø 25 mm; Whatman, Milan, Italy), and injected into the HPLC system without further purification (n = 3) (Manca, Marongiu, et al., 2016; Manconi et al., 2017; Tuberoso et al., 2011, 2013).

2.4. Vesicle preparation

To prepare liposomes, Lecinova® (240 mg/mL) and the grape pomace extract (40 mg/mL) were weighed in a glass vial and hydrated with 1 mL of water. Gelatin (10 mg/mL) or Nutriose® FM06 (50 mg/mL), or both, were added to the lipid components to obtain gelatin-liposomes, nutriosomes and gelatin-nutriosomes. All the aqueous dispersions were sonicated (10+10+10 cycles, 5 sec on and 2 sec off, allowing the cooling of the samples between each sonication step) by using a Soniprep 150 ultrasonic disintegrator (MSE Crowley, London, UK) to obtain homogeneous dispersions with small vesicles. Empty formulations (i.e., without the extract) were also prepared and used as references. Composition of vesicles is reported in Table 1 of Supplementary Materials.

2.5. Vesicle characterization

Vesicle formation and morphology were evaluated by cryogenic transmission electron microscopy (cryo-TEM) analysis. A thin aqueous film was formed by placing 5 µl of the vesicular dispersion on a glow-discharged holey carbon grid, and then blotting the grid against filter paper. The resulting thin sample film spanning the grid holes was vitrified by plunging the grid (kept at 100% humidity and room temperature) into ethane, maintained at its melting point with liquid nitrogen, using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitreous film was transferred to a Tecnai F20 TEM (FEI Company) using a Gatan cryo-transfer (Gatan, Pleasanton, CA), and the sample was observed in a low-dose mode. Images were acquired at 200 kV, at a temperature of –170/–175 °C, using low-dose imaging conditions not exceeding 20 $e^{-}/Å^2$, with a 4096 × 4096 pixel CCD Eagle camera (FEI Company).

The average diameter, polydispersity index (a measure of the width of size distribution) and zeta potential of the vesicles were determined by dynamic and electrophoretic light scattering using a Zetasizer Ultra (Malvern Instruments, Worcestershire, UK). Samples were diluted with water (1:100) and analysed at 25 °C.

Each vesicle dispersion (2 mL) was purified from the non-incorporated components by dialysis (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands) against water (4 L) for 2 h, changing the water every hour. The antioxidant activity of the samples (see Section 2.6 for the method used) was measured before and after dialysis, and the entrapment efficiency was calculated as the percentage of the antioxidant activity of dialysed samples versus those non-dialysed.

2.6. DPPH assay

The samples (25 µL) were dissolved in 1975 µL of DPPH methanolic solution (40 µg/mL), and incubated for 30 min at room temperature, in the dark. 25 µL of samples were chosen to avoid the overlap of the colours and to ensure the rupture of the vesicles and the release of the phytocomplex. Thereafter, the absorbance (ABS) was measured at 517 nm against blank. All the experiments were performed in triplicate. Antioxidant activity was calculated according to the following formula:

Antioxidant activity (%) = $[(ABS_{DPPH} - ABS_{sample})/ABS_{DPPH}] \times 100$ (Manca et al., 2015).

2.7. Vesicle behaviour at salivary and gastro-intestinal pHs

A solution simulating saliva was prepared by dissolving 2.38 g of Na_2HPO_4, 0.19 g of KH_2PO_4, and 8 g of NaCl in 1 L of water. The pH was adjusted at pH 6.75 with phosphoric acid (Khiang Peh & Fun Wong, 1999). Acidic medium simulating gastric fluid (pH 1.2) was prepared by dissolving 1.75 g of NaCl in a mixture consisting of 94 mL of water and 6 mL of 1 M HCl according to USP XXIV (US. Pharmacopeia XXIV, 2006) and PhEur X (European Pharmacopoeia X, 2020), but without enzymes (Roger et al., 2009). Neutral medium simulating intestinal fluid (pH 7.0) was prepared by dissolving 7.26 g of Na_2HPO_4, 3.56 g of NaH_2PO_4 and 17.54 g of NaCl in 1 L of water (Manconi et al., 2013, 2016)

The vesicles were diluted (1:500) with the fluids, thermostated at ∼37 °C and maintained for 10 min at pH 6.75, 2 h at pH 1.2, or 6 h at pH 7.0. The mean diameter, polydispersity index and zeta potential of the vesicles were measured at the end of each incubation period (Catalán-Latorre et al., 2018)

2.8. Biocompatibility of formulations

Caco-2 cells were grown as monolayers in 75-cm^2 flasks incubated in 100% humidity and 5% CO_2 at 37 °C. Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 10% foetal bovine serum and 1% penicillin/streptomycin was used to culture the cells. Cell viability was measured using the MTT (3-[4, 5-dimethylthiazol-2-yl]-3, 5 diphenyl tetrazolium bromide) test (Manca, Cencetti, et al., 2016; Manca et al., 2013). The cells (7.5×10^3 per well) were seeded into 96-well plates. After 24 h, 25 µl of each sample (grape pomace extract in aqueous dispersion or loaded in vesicles), previously diluted to reach the desired concentrations of the extract (0.4, 4, 40 and 400 µg/mL), was applied to the cells. After 48 h of incubation, MTT solution (0.5 mg/mL, final concentration) was added to each well removed 3 h later and replaced with dimethyl sulfoxide. The absorbance of the solubilized dye was measured at 570 nm with a microplate reader (Multiskan EX, Thermo Fisher Scientific Inc., Waltham, MA, US). The percentage of viability of treated cells in comparison with untreated control cells (100% cell viability) was calculated (Manca et al., 2014).

2.9. Evaluation of the protective effect of the formulations against cell damages induced by hydrogen peroxide

Caco-2 cells were seeded in 96-well plates and incubated at 37 °C in 5% CO_2 for 24 h. The cells were stressed with hydrogen peroxide (30% diluted 1:40000) and straightaway incubated for 4 h with the grape pomace extract in aqueous dispersion or loaded in the vesicles, diluted with the medium to reach 100 µg/mL of extract in each well. Cells not stressed with hydrogen peroxide and untreated (100% of viability) were used as a positive control, while those exposed to hydrogen peroxide and untreated were used as a negative control. After 4 h, the cells were washed with phosphate buffered saline, pH 7.4 (PBS), and the viability was measured by the MTT assay (Manconi et al., 2018).

2.10. Prebiotic activity of formulations

Lactobacillus reuteri (*L. reuteri*, Reuflor®, DSMZ-German Collection of Microorganisms and Cell Cultures strain DSM 17938), and *Streptococcus salivarius* (*S. salivarius*, Bactoblis®, American Type Culture Collection strain BAA-1 024), were used (Orrù et al., 2017). Prior to use, the probiotic bacteria, in exponential growth phase, were stored at –80 °C in plastic tubes containing 20% of glycerol and Brain Heart Infusion (BHI) broth for *L. reuteri* or Schaedler Broth for *S. salivarius*. The biofilm formation

assay was performed by using the modified protocol described by Montana University's Centre for Biofilm Engineering (di Petrillo et al., 2017). Briefly, a 96-well microplate containing serial dilutions (125, 250, 500 and 1000 µg/mL) of each sample (the extract in dispersion or loaded in the vesicles), and the selected bacterial strains were incubated at 37 °C for 6 days to allow the formation of the microbial biofilm. The plate samples were gently washed three times with PBS solution (GIBCO-PBS®, Thermo Fisher Scientific Inc, Waltham, MA, US) to eliminate planktonic cells then, the biofilm was stained with 100 µl of a crystal violet solution (0.1% w/v, Microbial, Uta, Italy) for 10 min at 25 °C. After three washes with PBS solution, 200 µl of acetic acid solution (30% v/v) was added to each well to solubilize the dye from the bacterial biomass. The biofilm growth was measured at 620 nm by using a microplate reader spectrophotometer (SLT-Spectra II, SLT Lab Instruments, Crailsheim, Germany). Experiments were performed in triplicate. The increase in the absorbance values in comparison with the negative control (untreated bacteria) indicated a promotion of the biofilm growth.

2.11. Economic cost and carbon dioxide emission

The complete process from phytocomplex extraction to formulation in vesicles can be divided into three main steps: (1) extraction of phytocomplex (2) separation of phytocomplex (3) formulation of phytocomplex in vesicles. The timeline for processing 1 batch is reported in Table 2 of Supplementary Materials.

2.12. Statistical analysis of data

Results are expressed as the mean ± standard deviation. Statistically significant differences among samples were determined by 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 statistical analysis was performed by using the Excel software package (Microsoft Corp, Redmond, USA) equipped with a tool for statistical analysis. The minimum level of significance chosen was $p < 0.05$.

3. Results and discussion

The use of wine-making waste has raised great interest in recent years, mainly because of its high content in bioactive compounds, such as polyphenols, and their suitable conversion in health-promoting products capable of driving the wine-chain toward a circular and sustainable bioeconomy (Chowdhary et al., 2021). According to this, in the present study, the pomace of Cannonau red grape was used to extract the bioactive components still present after vinification. The qualitative and quantitative composition of the extract was assessed, along with its antioxidant power.

3.1. Extraction and characterization of the grape pomace extract

The extraction of the bioactive compounds from grape pomace was performed by maceration in ethanol, which was recovered after extraction, by evaporation under vacuum, to be used in further extraction processes. From 100 g of pomace 4.8 ± 0.7 g of extract was obtained. The qualitative and quantitative composition of extract was detected by HPLC-DAD (Table 1).

The extract components were separated and quantified at three different wavelengths, 520, 360, 280 nm (Figure 1 of Supplementary Materials). Procyanidin B2 (98.1 mg/100 g of extract), quercetin (67.0 mg/100 g of extract), malvidin-3-glucoside (55.8 mg/100 g

of extract), and gallic acid (43.2 mg/100 g of extract) were the major bioactive compounds found in the extract, within the chemical classes analysed.

3.2. Characterization of vesicles loading the grape pomace extract

The physico-chemical properties of gelatin-liposomes, nutriosomes and gelatin-nutriosomes were evaluated and compared with those of liposomes used as a reference. Soy lecithin and the grape pomace extract were the basic components of all the formulations. Furthermore, gelatin, a protein polymer produced from collagen, with gelling properties, was added to increase the stability of the vesicles, and Nutriose®, a dextrin from maize that resists to digestion in the small intestine and slowly ferments in the colon, was included in the formulations to beneficially alter the human gut microbiota. Cryo-TEM analyses were performed to evaluate the morphology of the vesicles (Figure 1). The microscopic observation disclosed that all the vesicles were spherical in shape, liposomes were mainly uni- and bilamellar, while some oligolamellar and multicompartment vesicles were detected in nutriosome dispersions. The addition of gelatin did not significantly affected the morphology of the vesicles.

The average diameter, the polydispersity index and the surface charge of empty (i.e., without the extract) and extract loaded vesicles were measured to determine the influence of additive components and payloads on the assembly and structure of the vesicles. Empty liposomes were small (~63 nm), homogeneously dispersed (~0.22) and negatively charged (~–46 mV). The addition of Nutriose®, or gelatin, or their combination, did not significantly modify the vesicle features (Table 2). As already reported, the incorporation of an extract in vesicles may cause a significant increase or decrease in size, due to a modification of the phospholipid packing, and thus may affect the assembly of the vesicles. The loading of grape pomace extract into the vesicles caused a marked increase of the mean diameter of liposomes, up to ~140 nm, and nutriosomes, up to ~175 nm (Table 2). The presence of gelatin mitigated such vesicle enlargement in both liposomes and nutriosomes, which were sized ~128 and ~146 nm, probably due to an electrostatic interaction between the polymer and the phospholipid that modifies the vesicle assembly (Manca et al., 2014). All the formulations were homogeneously dispersed, as polydispersity index was ≤0.27 irrespective of the composition of vesicles (Table 2).

The zeta potential was highly negative, ~–50 mV, mainly due to the negative group of phosphatidylcholine at the pH of formulations (~5.5) (Zhou & Raphael, 2007). Only gelatin-liposomes had a zeta potential less negative (~–38 mV), probably because gelatin mainly consists of proteins containing cationic and anionic groups and the amide groups can electrostatically interact with the negative surface charge of vesicles decreasing their negative zeta potential (Duconseille et al., 2015). The extract loaded vesicles were able to scavenge free radicals, probably due to its main components: procyanidin B2, quercetin, malvidin-3-glucoside and gallic acid. Indeed, the antioxidant activity of the extract measured by the DPPH assay was ~83% and it was not significantly modified by its loading in the vesicles, confirming that the activity of the extract was not altered (Table 2).

All the formulations were capable of loading a high amount of extract, as the entrapment efficiency of liposomes was ~71% and that of nutriosomes increased up to ~86%, likely due to the ability of Nutriose®, as well as other fibres, to non-covalently interact with polyphenols (Jakobek & Matić, 2019). This binding process usually starts on the fibre surface and continues with diffusion into the fibre shell, improving the binding of polyphenols contained in the extract with the dextrin solubilized in the water compartment of vesicles (Liu et al., 2017).

3.3. Stability of extract loaded vesicles in fluids simulating oral, gastric and intestinal environments

Oral administration is the route of choice for many drugs, as it is easy to practice, cheap, and safe, thus improving patient compliance (Günday Türeli & Türeli, 2020). The main problem of oral administration is the poor stability of the active compounds or their carriers under the different pHs encountered in the gastro-intestinal tract. When the formulation arrives in the mouth, it is mixed with saliva, which has a slightly acidic pH; then, it reaches the stomach where it encounters a highly acidic environment, to continue into the intestines, where there is neutral pH (Fallingborg, 1999). To study how the pH variations and high ionic strength influence the main physico-chemical properties of the vesicles, the formulations were incubated at 37 °C for 10 min at pH 6.75 (saliva), 2 h at pH 1.2 (stomach), or 6 h at pH 7.0 (intestine) (Table 2). Incubation with the simulating saliva medium did not significantly modify the mean diameter of vesicles, as only a slight increase of gelatin-nutriosome diameter was detected (from ~129 to ~184 nm). The saliva solution also affected the homogeneity of the dispersions, which slightly decreased, and the surface charge as well, which was significantly reduced due to the presence of the salts in the medium. A different behaviour was observed following incubation of samples in acidic solution (Table 2): the vesicles became larger and highly polydispersed, with surface charge values approaching zero. To better evaluate these results, the size distribution of the particles as a function of their volume and number was considered. These distributions express the percentage that each size class occupies in the overall distribution, when calculated as a percentage of either the total volume or the number of particles. Both ways of expressing the data can provide different information about particle populations (Figure 2).

In the dispersion of nutriosomes, the larger vesicles (around 4000 nm) occupied a larger total volume than the smaller ones (~190 nm). Furthermore, they scattered more the light, which resulted in small particles being hidden, with a consequent increase in apparent average size and polydispersity. When the number distribution was considered, it revealed that the larger particles represent less than 0.5% of the nutriosomes' population, while the predominant size was ~190 nm. Similar results were observed for gelatin-nutriosomes. On the contrary the distribution of liposomes and gelatin-liposomes, as a function of the particle number, indicated that the main vesicles (60%) were sized ~1800 nm (data not shown). Therefore, results point to a minor destabilization and enlargement of these enriched vesicles, nutriosomes and gelatin-nutriosomes, under acidic conditions. The dilution and incubation with medium at pH 7.0 did not significantly affect the size and size distribution of the vesicles, given the small increase of size and polydispersity index detected, while the surface charge was significantly reduced, irrespective of the formulation tested, due to the presence of the salts in the medium (Table 2).

These results suggest that nutriosomes and gelatin-nutriosomes are potential delivery systems for the supplementation of grape pomace extract, since they can protect it from the oral and gastro-intestinal environments and facilitate its absorption at intestinal level (Allaw et al., 2020; Manconi et al., 2019).

3.4. Biocompatibility of the extract loaded vesicles against Caco-2 cells

The biocompatibility of grape pomace extract in dispersion or loaded in vesicles was evaluated using Caco-2 cells, as a model of human epithelial cells of the colon-rectal tract of the intestine (Lea, 2015). The cells were incubated for 48 h with samples at different dilutions, corresponding to 0.4, 4, 40 and 400 μg/mL of the extract. Previous studies confirmed that at this time it is possible to adequately evaluate the cell viability while

longer time (72 hours) of exposition, without changing the medium, is not reproductive of the in vivo real conditions (Castangia et al., 2022). The viability of Caco-2 cells treated with the grape pomace extract in aqueous dispersion was ~80%, irrespective of the concentration tested ($p<0.05$; Figure 3A). The loading of the extract in the vesicles increased cell viability, which was ~100%, or even higher (up to ~120%), indicating a stimulation of cell growth, especially when nutriosomes were used.

3.5. Protective effect of extract loaded vesicles against damages induced by hydrogen peroxide in Caco-2 cells

Hydrogen peroxide is the most widely used apoptosis inducer in cells; it causes cell damage and death in time- and concentration-dependent manners. The ability of grape pomace extract formulations to prevent such damages was evaluated using Caco-2 cells (Figure 3B). The stress induced by hydrogen peroxide significantly reduced cell viability up to ~60% (Ido et al., 2015). The simultaneous treatment of cells with the extract in dispersion or loaded in liposomes prevented oxidative damage, as the viability was ~80% ($p>0.05$ between values obtained using dispersion and liposomes, $p<0.05$ versus viability values of cells stressed with hydrogen peroxide and untreated). The incorporation of the extract into gelatin-liposomes, nutriosomes and gelatin-nutriosomes further improved the protection of the cells, as the viability was ~100%, irrespective of the formulation tested ($p>0.05$ among the values obtained using these vesicles, $p<0.05$ versus viability values of cells stressed and untreated or treated with the extract in dispersion). These results confirmed that the extract when loaded in vesicles is safe and capable of efficiently protecting the intestinal cells from damages and death caused by oxidative stress.

3.6. Prebiotic effect of extract loaded vesicles

The prebiotic effect of the grape pomace extract in aqueous dispersion or loaded in vesicles was evaluated measuring its ability to promote biofilm formation by the probiotic strains *S. salivarius* and *L. reuteri*. The former is a microorganism normally present in the bacterial flora of the human oral cavity, and the latter is a microorganism present in the intestinal microbiota that contributes to the promotion of intestinal health (Miyoshi et al., 2006). Biofilm formation by probiotic bacteria is considered beneficial because it can promote colonization and longer permanence in the oral-intestinal mucosa of the host, avoiding colonization by pathogenic bacteria (Nagata & Saito, 2006). In this study, the biofilm formation by the two selected probiotic bacteria was effectively stimulated by the extract-loaded vesicles especially at the higher concentrations (250, 500 and 1000 µg/mL; Figure 3C1 & 2). In particular, vesicles enriched with gelatin stimulated the proliferation of *L. reuteri* to a greater extent, even at the lower concentrations (125 and 250 µg/mL; Figure 3C1). Considering that the gelatin is obtained by partial hydrolysation of collagen, it probably promotes the binding of vesicles with the *L. reuteri* surface, which expresses collagen-binding proteins (Hsueh et al., 2010). The proliferative effect was further improved by the treatment with vesicles enriched with Nutriose®, a soluble fibre with prebiotic effect in the gut microbiota (Hobden et al., 2013).

The biofilm growth of *S. salivarius* was especially improved by using liposomes and gelatin-liposomes (Figure 3C2) while the presence of the maltodextrin (Nutriose®) was less effective in this strain. The different behaviour of the vesicles towards these two probiotic strains may be related to their different cell surface composition (e.g., extracellular matrix, surface electrical charge, lipophilicity, etc.).

The performed study confirmed that the extraction of a phytocomplex rich in antioxidant polyphenols is a promising strategy that can be carried out through an easy and low

dissipative process. The resulting extract can be converted into valuable nanotechnological products using naturally occurring and inexpensive components, such as soy lecithin, gelatin and maltodextrin. In addition, the obtained products are optimal systems suitable for protecting and maintaining the human intestine in a healthy condition.

3.7. Costs and possible profits

The challenges faced to manufacture sustainable products are linked not only to the type of the resources exploited, but mainly to the nature of the process through which these resources are generated from energetic, environmental, and economic points of view. The exploitation of industrial by-products in combination with an efficient process improved by the use of nanotechnological methodologies can engender innovative, sustainable and economically profitable products.

Based on that and given the biological effects of grape pomaces and its derivatives, the economic and environmental issues connected with the production of grape pomace extract loaded in vesicles were taken into consideration, in order to evaluate their impact and feasibility. Some important evaluations regarding the costs of production (equipment and process) and carbon dioxide emission were analysed for the main experimental steps and the required equipment, processes, and hours of utilization have been estimated (Table 3) (Dassey & Theegala, 2013). The expenses related to the extraction of the phytocomplex (1 kg) and to its formulation in gelatin-nutriosomes (1 L), considered the most suitable and complex formulation, were reported.

The carbon dioxide emission was calculated based on the Italian emission factor of national electricity production and consumption in 2019 (Caputo, 2020). The cost of electricity was calculated using 0.06 €/kWh as the power supply unit cost for electricity (Trucillo et al., 2020). The cleaning of equipment was calculated considering 0.0003 €/l as the price of water (Jin et al., 2021). Finally, the cost of maceration of biomass was calculated by dividing by 5 the mean price of ethanol in the Italian market (20 €/l), because after the evaporation it was recycled for at least five more extractions. The detailed costs related to the materials used to formulate the vesicles was calculated using the Italian market values (Table 3).

The cost of producing the extract was mainly related to the solvent evaporation process, which represents the most expensive and energy consuming step also connected with the highest level of carbon dioxide emission (Table 3). However, it is a necessary step to obtain the extract in powdered form and the costs connected with the process can be significantly reduced because, as reported above, according to the circular economy principles, ethanol can be recovered and recycled for further extractions. Actually, the price of the obtained extract is around 400 €/kg, which is not very competitive, because a wide range of vine leaf extracts are already present in the international market and sold at lower prices. However, this extract is different from others since obtained from grape pomace (a by-product) and in addition, when loaded in gelatin-nutriosomes, it becomes unique and very competitive. Indeed, manufacturing extract loaded gelatin-nutriosomes is an inexpensive step of the whole process and the cost of this formulation was low (39.96 €/l) and competitive with respect to that of the raw extract. The low cost of this formulation was related to the easy and low dissipative method applied and the use of natural components, which were accurately selected, according to the purpose of the study.

At the moment, no formulations of grape extract loaded in nanovesicles are present on the market, thus preventing comparison of the price of the obtained product. However,

some nutraceutical products containing nanocarriers loading other natural antioxidants, such as resveratrol (Liposomal OPC by ActiNovo®, Liposomal Resveratrol by CureSupport® and Liposomal C by Da Vinci® Laboratories of Vermont) are already present on the market and their price can be used as a reference. They are usually very expensive, and their indicative market value estimated by the mean selling prices is around 215.95 €/l. Hence, the cost of the gelatin-nutriosomes formulation reported here (39.96 €/l) seems to be adequate to allow the manufacture of a profitable product. In addition, the proposed formulation proved to be eco-friendly, because the dioxide carbon emission was low. Considering the sustainability of the preparation method and cost, these gelatin-nutriosomes can be proposed as additive to produce enriched food as reported in previous studies, that confirmed the possibility to mix lamellar vesicles with various matrix food (i.e., yogurt and milk among others) as their structure was not modified by the components (Díaz-Ruiz et al., 2021; Hasibi et al., 2020; Machado et al., 2021).

4. Conclusion

An effective/sustainable extract, rich in antioxidant molecules, was obtained from red grape pomace by maceration, and its efficacy was improved by its loading into phospholipid vesicles tailored for intestinal delivery. Vesicles were capable of protecting Caco-2 cells from oxidative stress and promoting the biofilm formation by *S. salivarius* and *L. reuteri*. Prepared formulations represent a promising natural delivery system that can be easily added to foods/beverage, to improve their health-promoting properties. Moreover, the conversion of grape pomace into intestinal health-promoting products represents a profitable step capable of enriching the grape value chain and improving environmental and human health.

CRediT authorship contribution statement

Matteo Perra: Investigation, Formal analysis, Data curation, Writing- Original draft preparation. **Maria Letizia Manca**: Investigation, Data curation, Writing- Original draft preparation. **Carlo I.G. Tuberoso**: Methodology, Formal analysis, Investigation. **Carla Caddeo**: Methodology, Investigation. **Francesca Marongiu**: Investigation. **Josè Esteban Peris**: Methodology, Formal analysis, Investigation. **Germano Orrù**: Methodology, Investigation. **Antonella Ibba**: Methodology, Investigation. **Xavier Fernàndez-Busquets**: Methodology, Formal analysis, Investigation. **Sami Fattouch:** Review & Editing. **Gianluigi Bacchetta**: Supervision, Project administration, Writing - Review & Editing. **Maria Manconi**: Supervision, Project administration, Writing - Review & Editing.

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Conflict of interest statement

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

Data access statement

The authors declare that this manuscript do not includes a statement on how/if data supporting the research is available.

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Figure Captions

Figure 1. Representative cryo-TEM images of grape pomace extract-loaded liposomes (A), gelatin-liposomes (B), nutriosomes (C) and gelatin-nutriosomes (D).

Figure 2. Representative images of the size distribution by volume and by number of nutriosomes following dilution and incubation in acidic solution (pH 1.2).

Figure 3. Viability of Caco-2 cells incubated for 48 h with the grape pomace extract in aqueous dispersion or loaded in liposomes, gelatin-liposomes, nutriosomes and gelatin-nutriosomes. Mean values ± standard deviations are reported (**A)**.
Viability of Caco-2 cells stressed with hydrogen peroxide and treated with grape pomace extract in aqueous dispersion or loaded in vesicles. Mean values ± standard deviations are reported (**B)**.
Effect of the grape pomace extract-loaded vesicles on the proliferation of *L. reuteri* (1). and *S. salivarius* (2). The maximum standard deviation was assessed as ±15% of the mean (**C)**.

Table 1. Bioactive compounds identified and quantified in grape pomace extract. Average content (mg/100 g of extract) ± standard deviation is reported (n=3). Tr was reported when sample was identified by comparison with standard; UV-Vis was reported when sample was identified by comparison of spectra with similar molecules and literature data.

		Identification method*	mg/100 g
Anthocyanins			
Delphinidin-3-glucoside	1	tr, UV-Vis	2.1±0.0
Cyanidin-3-glucoside	2	tr, UV-Vis	2.2±0.1
Petunidin-3-glucoside	3	tr, UV-Vis	4.5±0.1
Peonidin-3-glucoside	4	tr, UV-Vis	9.6±0.3
Malvidin-3-glucoside	5	tr, UV-Vis	55.8±1.4
Delphinidin-3-acetylglucoside	6	UV-Vis	2.7±0.1
Malvidin-3-acetylglucoside	7	UV-Vis	2.9±0.0
Cyanidin-3-*p*-cumaroylglucoside	8	UV-Vis	2.4±0.1
Petunidin-3-*p*-cumaroylglucoside	9	UV-Vis	2.6±0.1
Peonidin-3-*p*-cumaroylglucoside	10	UV-Vis	3.1±0.1
Malvidin-3-*p*-cumaroylglucoside	11	UV-Vis	19.6±0.8
Total			**107.6±6.3**
Flavonols			
Quercetin-3 glucoside	12	tr, UV-Vis	19.6±0.7
Quercetin	13	tr, UV-Vis	67.0±2.5
Others		UV-Vis	66.7±3.5
Total			**153.4±6.7**
Flavanols			
Procyanidin B1	14	tr, UV-Vis	47.4±3.4
(+) Catechin	15	tr, UV-Vis	51.3±3.5
Procyanidin B2	16	tr, UV-Vis	98.1±7.8
(–) Epicatechin	17	tr, UV-Vis	89.0±5.7
Others		UV-Vis	167.8±18.7
Total			**453.6±39.1**
Hydroxy cinnamic acids			
trans-caftaric acid	18	tr, UV-Vis	27.51.0±1.0
Hydroxy benzoic acids			
Gallic acid	19	tr, UV-Vis	43.2±1.1

Table 2. Characterization of vesicles. Mean values ± standard deviations are reported (n≥6) (Upper part).
Mean diameter, polydispersity index and zeta potential of the extract loaded vesicles incubated at 37 °C for 10 min at pH 6.75; 2 h at pH 1.2; or 6 h at pH 7.0. Mean values ± standard deviations are reported (n=3), (Lower part).

	Mean diameter (nm)	Polydispersity index	Zeta Potential (mV)	Entrapment efficiency (%)	Antioxidant Activity (%)
Empty liposomes	63±4	0.22	–46±3	-	
Empty gelatin-liposomes	69±8	0.23	–41±4	-	
Empty nutriosomes	70±2	0.22	–47±2	-	
Empty gelatin-nutriosomes	77±4	0.22	–39±3	-	
Extract liposomes	140±45	0.23	–55±5	65±16	82±3
Extract gelatin-liposomes	128±13	0.24	–38±3	76±10	78±3
Extract nutriosomes	175±53	0.24	–60±12	85±5	80±5
Extract gelatin-nutriosomes	146±32	0.27	–48±6	88±13	68±11

	pH	Time (min)	Mean diameter (nm)	Polydispersity index	Zeta Potential (mV)
Liposomes	6.75	10	129±4	0.18	–23±1
	1.2	120	1386±256	0.89	–2±1
	7.0	360	197±48	0.25	–18±3
Gelatin-liposomes	6.75	10	134±15	0.27	–20±2
	1.2	120	1051±279	0.59	+1±1
	7.0	360	149±14	0.17	–11±2
Nutriosomes	6.75	10	134±3	0.16	–23±1
	1.2	120	1474±103	0.70	+0±3
	7.0	360	193±43	0.32	–16±4
Gelatin-nutriosomes	6.75	10	184±16	0.19	–18±4
	1.2	120	5703±214	0.59	+0±1
	7.0	360	193±52	0.28	–14±1

Table 3. Main expenses and carbon dioxide emission related to the use (as a function of time) of the main instruments and processes needed to obtain 1 kg of Cannonau grape pomace extract and 1 L of vesicle formulation (Upper part).
Costs related to the materials needed to manufacture 1 litre of gelatin-nutriosomes (Lower part).

Main step	Procedure	kW/h	CO_2 emission	Electricity Cost	Material Cost (€)	References

			(kg)	(€)		
Extraction	Cleaning of equipment	--	--	--	0.01	(Jin et al., 2021)
	Loading of biomass	--	--	--	--	
	Maceration of biomass	8.64	2.39	0.52	400.00	(Caputo, 2020; Trucillo et al., 2020)
Separation						
	Transferring of dispersion	--	--	--	--	
	Centrifugation of dispersion	2.48	0.68	0.15	--	(Caputo, 2020; Trucillo et al., 2020)
	Solvent evaporation	132	36.47	7.92	--	(Caputo, 2020; Trucillo et al., 2020)
Total			39.52	8.59	400.01	
Formulation						
	Weighting of components	0.006	0.002	0.0004	39.71	(Caputo, 2020; Trucillo et al., 2020)
	Blending of components	4	1.11	0.24	--	(Caputo, 2020; Trucillo et al., 2020)
	Recovery of formulation	--	--	--	--	
	Cleaning of equipment	--	--	--	0.01	(Jin et al., 2021)
Total				0.24	39.72	

	Cost (€/kg)	Amount (kg)	Total cost (€)
Lecinova®	25.8	0.24	6.19
Cannonau pomace extract	408.60	0.04	16.34
Dextrin from maize	228	0.05	11.40
Gelatin	176	0.01	1.76
Distilled water	4.02	1	4.02
Total			**39.71**