Advanced strategy to exploit wine-making waste by manufacturing antioxidant and prebiotic fibre-enriched vesicles for intestinal health

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Abstract

Grape extract-loaded fibre-enriched vesicles, nutriosomes, were prepared by combining antioxidant extracts obtained from grape pomaces and a prebiotic, soluble fibre (Nutriose®FM06). The nutriosomes were small in size (from ~140 to 260 nm), homogeneous (polydispersity index <0.2) and highly negative (~ −79 mV). The vesicles were highly stable during 12 months of storage at 25 °C. When diluted with warmed (37 °C) acidic medium (pH 1.2) of high ionic strength, the vesicles only displayed an increase of the mean diameter and a low release of the extract, which were dependent on Nutriose concentration. The formulations were highly biocompatible and able to protect intestinal cells (Caco-2) from oxidative stress damage. *In vivo* results underlined that the composition of mouse microbiota was not affected by the vesicular formulations. Overall results support the potential application of grape nutriosomes as an alternative strategy for the protection of the intestinal tract.

Keywords: grape pomace; phospholipid vesicles; nutriosomes; antioxidant activity; prebiotic activity; gut microbiota; intestinal cells; *in vivo* studies.

Introduction

Recent evidence suggest that intestinal microbiota contribute to modulating immune response and protecting from gut inflammatory diseases [1]. The supplementation of exogenous bacteria can be useful to maintain or promote the health of local microbiota. However, the beneficial effect of probiotic bacteria may be transient, while the use of prebiotics that stimulate the growth and activity of the microbiota may ensure a more stable welfare [2]. Prebiotics are non-digestible but fermentable oligosaccharides that act as food supplements that feed gut microbiota promoting the proliferation and activity of bacteria (especially bifidobacteria and lactobacilli), with consequent impacts on host wellbeing [3]. In particular, prebiotics are not digested in the upper gastrointestinal tract and are selectively fermented into short chain fatty acids and lactate by resident bacteria in the colon [4]. The short chain fatty acids, such as acetate, propionate and butyrate, serve as fuel in different tissues and may play a role in the regulation of cellular processes [5]. Nowadays, only few dietary non-digestible oligosaccharides fulfil the criteria for the classification as prebiotic. Many food components, including fibres, have claimed prebiotic activity, even though they do not meet the required criteria, such as resistance to gastric acidity, hydrolysis by mammalian enzymes, absorption in the upper part of the gastrointestinal tract, fermentation by beneficial bacteria in the intestine, and selective stimulation of growth or activity of colonic microbiota toward a healthier composition [6]. Prebiotics are often found in plants (e.g., agave and chicory), which are important sources of carbohydrates, and their intake with the diet can provide benefits to human health [7,8]. As an example, dietary prebiotics can prevent enteric inflammatory disorders by promoting the growth of beneficial bacterial communities within the large intestine, which results in colitis reduction. Recent findings have shown how prebiotics increase the number of beneficial bacteria, such as lactobacilli and bifidobacteria, while decreasing the number of disease-causing bacteria in both animal models and human clinical studies [9]. Moreover, prebiotics can also provide resistance to the colonization of pathogenic bacteria by inhibiting the adherence of pathogens to gut

epithelium. For instance, non-digestible oligosaccharides were found to inhibit the intestinal colonization by *Clostridium difficile in vitro* [10,11].

Nutriose®FM06 prebiotic fibre

Nutriose®FM06 is a dextrin obtained from maize. Differently from maltodextrins, it is not completely hydrolysed and absorbed, and is resistant to digestion in the small intestine, while it is largely fermented in the colon because of a high percentage of α-1,6 linkages and the presence of non-digestible glucoside linkages (e.g., α-1,2 and α-1,3) [12]. Further, Nutriose is a soluble fibre that, if daily consumed, may reduce blood glucose and improve gut health as prebiotic supplement [13].

In a previous study, nutriosomes, that is phospholipid vesicles containing a high amount of Nutriose, were developed for the protection of the intestinal tract by taking advantage of the prebiotic properties of Nutriose and the antioxidant properties of a natural polyphenol, curcumin [14].

In the present study, the active components of grape pomaces were extracted by maceration, first in ethanol and propylene glycol, then in extra virgin olive oil. The two extracts were incorporated into phospholipid vesicles containing Nutriose at increasing concentrations (20, 40 and 60% w/v), thus producing grape nutriosomes. Vesicles without Nutriose were also prepared and used as a reference. All the vesicles were fully characterized for morphology, size, zeta potential and entrapment efficiency. The antioxidant activity of grape nutriosomes was evaluated *in vitro* in intestinal cells, and the prebiotic activity was assessed *in vivo* by the examination of faecal microbiota.

Material and methods

Materials

Lipoid S75 (S75), a mixture of soybean phospholipids (~70% phosphatidylcholine, 9% phosphatidylethanolamine and 3% lysophosphatidylcholine), triglycerides and fatty acids, was purchased from Lipoid (Ludwigshafen, Germany). Nutriose®FM06, a soluble dextrin from maize,

was kindly provided by Roquette (Lestrem cedex, France). Ethanol, propylene glycol and all other products of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Reagents for cell culture were purchased from Life Technologies Europe (Monza, Italia).

Extraction process

Pomaces were obtained from *Vitis vinifera* cultivar *Cannonau*. Even if they are considered a grape by-product, they are still rich in chemical components, such as water (~60%), neutral polysaccharides (~30%), pectic substances (~20%), insoluble proanthocyanidins, lignin, structural proteins, and phenols (~15%) [15]. Grape pomaces (100 g) were dispersed in 500 ml of a mixture of ethanol and propylene glycol (1:1 ratio, v/v) and kept under constant stirring for 48 h, at room temperature (25 °C) [16]. Thereafter, the dispersion was centrifuged twice (30 min, 8000 rpm). The extractive solution was diluted with water and lyophilized to reduce the volume to 100 ml. The pomace pellet obtained from the extraction with ethanol/propylene glycol was dispersed in 500 ml of extra virgin olive oil and kept under constant stirring for 48 h, at room temperature (25 °C). Then, the dispersion was centrifuged twice (30 min, 8000 rpm).

The characterization of the two extracts was previously reported [16].

Vesicle preparation and characterization

For the preparation of nutriosomes, S75 (120 mg/ml), the grape pomace oily extract (50 mg/ml) and Nutriose®FM06 (200, 400, 600 mg/ml) were weighed in a glass vial and dispersed in a mixture of grape pomace ethanol-propylene glycol extract and water (1:1 v/v). The dispersions were sonicated (40 cycles, 5 s on and 2 s off) with a Soniprep 150 ultrasonic disintegrator (MSE Crowley, London, UK). Vesicles without Nutriose, that is Penetration Enhancer-containing Vesicles (PEVs), were also prepared and used as a reference. The composition of the formulations is reported in Table 1S (Supplementary Materials).

Empty nutriosomes and PEVs were prepared by using extra virgin olive oil and a mixture of ethanol-propylene glycol and water (1:1 v/v) instead of the two grape pomace extracts.

Vesicle formation and morphology were evaluated by cryogenic transmission electron microscopy (cryo-TEM). Sample (5 μl) was applied on an EM grid Lacey carbon film (Electron Microscopy Science, Hatfield, PA, USA). The grid was mounted on an automatic plunge freezing apparatus (Vitrobot FEI, Eindhoven, The Netherlands) to control humidity and temperature, immersed in liquid ethane, fast cooled from outside by liquid nitrogen, avoiding the formation of ice crystals. Observation was made at −170 °C in a Tecnai F20 microscope (FEI, Eindhoven, The Netherlands) operating at 200 kV, equipped with a cryo-specimen holder Gatan 626 (Warrendale, PA, US). Digital images were recorded with an Eagle FEI camera, 4098×4098 pixels. Magnification between 20,000-30,000× and a defocus range of 2-3 μm was used.

The average diameter and polydispersity index of each formulation were determined by Photon Correlation Spectroscopy by using a Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK). The zeta potential was measured by means of M3-PALS method (electrophoretic light scattering) by using the Zetasizer nano ZS. All the measurements were performed at 25 °C after dilution of the samples with water (1:100).

The above parameters were measured during 12 months of storage at room temperature (25 °C) to evaluate the stability of the formulations.

Entrapment efficiency measurement

Grape nutriosomes and PEVs (2 ml) were purified from the non-incorporated extract bioactives by dialysis (Spectra/Por® membranes: 12-14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, Netherlands) against water (2 l) for 2 h, refreshing water every 30 min. The used water (8 l) was appropriate to allow the dissolution and consequent removal of the non-incorporated extract bioactives. The entrapment efficiency (EE) of the vesicles was expressed as the percentage of the antioxidant activity of the dialysed and non-dialysed samples. The antioxidant activity was measured by means of the DPPH assay. Briefly, the samples (25 μl) were dissolved in 1975 μl of a DPPH methanolic solution (40 μg/ml) and incubated for 30 min at room temperature, in the dark.

144 Then, the absorbance was measured at λ=517 nm against blank. All the experiments were
145 performed in triplicate. The antioxidant activity was calculated according to the following formula:
146 $AA\% = [(ABS_{DPPH} - ABS_{sample})/ABS_{DPPH}] \times 100$.

147 *Stability studies as a function of pH, ionic strength, and temperature*

148 The effect of pH (1.2 or 7.0), ionic strength (0.3 M NaCl), and temperature (37 °C) on the vesicles
149 was evaluated by measuring their average diameter, polydispersity index, zeta potential and amount
150 of grape extract released under these conditions. The vesicle formulations were diluted (1:5 v/v)
151 with a solution at pH 1.2 or pH 7.0 and high ionic strength, loaded into a dialysis bag and put in a
152 vessel containing the same solution maintained at 37 °C under constant stirring (100 rpm). After 2 h
153 at pH 1.2 and 6 h at pH 7.0, the above-mentioned parameters were measured [17]. The grape extract
154 released by the vesicles was estimated by means of the DPPH assay (see paragraph 2.4) performed
155 on the formulations before (t_0) and after (t_{2h} or t_{6h}) the experiments, and the amount of extract
156 released was expressed as a percent difference between the amounts found at t_0 and t_{2h} or t_{6h}.

157 *Cell viability and protection from oxidative stress*

158 Human intestinal epithelial cells (Caco-2) were grown as monolayer in 75 cm^2 flasks, incubated at
159 37 °C in 100% humidity and 5% CO_2. Dulbecco's Modified Eagle Medium with high glucose and
160 L-glutamine, supplemented with 20% foetal bovine serum, 10% penicillin and streptomycin and 1%
161 fungizone, was used as growth medium.

162 The cells were seeded into 96-well plates at a density of 7.5×10^3 cells/well. After 24 h of
163 incubation, the cells were treated with the grape ethanol/propylene glycol extract in aqueous
164 solution or loaded in PEVs and nutriosomes properly diluted with DMEM to achieve the desired
165 extract concentrations (6, 12, 60, 120 μg/ml). After 48 h, MTT [3(4,5-dimethylthiazolyl-2)-2, 5-
166 diphenyltetrazolium bromide] (100 μl, 0.5 mg/ml final concentration) was added to each well. After
167 3 h, the formed formazan crystals were dissolved with DMSO, and the absorbance was measured at
168 570 nm with a microplate reader (Synergy 4 Reader, BioTek Instruments, AHSI S.p.A,

169 Bernareggio, Italy). The experiment was repeated at least three times, each time in triplicate.
170 Results are shown as a percentage of cell viability in comparison with untreated control cells (100%
171 viability).
172 The protective effect of the grape ethanol/propylene glycol extract in aqueous solution or loaded in
173 PEVs and nutriosomes against oxidative damage induced in Caco-2 cells by using hydrogen
174 peroxide was also evaluated. The cells were seeded into 96-well plates at a density of 7.5×10^3
175 cells/well. After 24 h of incubation, the cells were treated with hydrogen peroxide (1:30000) and
176 simultaneously with the samples diluted to achieve the desired extract concentration (12 μg/ml).
177 The cells treated with hydrogen peroxide only and untreated cells were used as positive and
178 negative controls, respectively. After 4 h of incubation, the cells were washed with fresh medium
179 and their viability was determined by the MTT assay. The results are reported as the percentage of
180 untreated cells (100% viability).
181 *Evaluation of prebiotic activity in vivo*
182 Female BALB/c mice (5–6 weeks old, 25–35 g) were obtained from Harlan Laboratories
183 (Barcelona, Spain) and acclimatized for 1 week before use. All studies were performed in
184 accordance with European Union regulations for the handling and use of laboratory animals. The
185 protocols were approved by the Institutional Animal Care and Use Committee of the University of
186 Valencia (2016/VSC/PEA/00178 type 2, 9 September 2016).
187 Animals were fed rodent pellets (Global diet 2014, Harlan Teklad, Barcelona, Spain) *ad libitum*
188 with free access to water, and maintained in a room with controlled temperature (~20 °C), 60%
189 relative humidity, and a 12-h light/dark cycle (light from 8:00 a.m. to 8:00 p.m.). The mice were
190 divided into five groups (six animals per group): group 1 received only water (control); group 2 the
191 grape ethanol/propylene glycol extract; group 3 grape PEVs; group 4 grape 20nutriosomes; group 5
192 grape 40nutriosomes. The samples (200 μl) were administered intra-gastrically three times per week

during 4 weeks. The faeces were recovered prior to the treatment (day 0) and 28 days after the treatment, and stored at -80 °C.

Characterisation of faecal microbiota

In order to analyse the taxonomic structure of the gut microbiota of mice, a whole-community shotgun sequencing strategy was followed. Three faecal pellets per mouse were used to extract DNA by using the QIAamp Fast DNA Stool Mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The concentration of the extracted genomic DNA was measured by Qubit fluorometric quantification method (Thermo Fisher Scientific, Carlsbad, CA, US), and shotgun libraries were constructed using the Nextera XT DNA Library Prep kit (Illumina, CA, US). Paired-end sequencing was run in a MiSeq Desktop Sequencer (Illumina) by the Sequencing and Bioinformatics Service in the facilities of FISABIO (Valencia, Spain).

The resulting reads were filtered by length and quality, trimmed, and low-complexity sequences and Ns-proteins were removed by using Prinseq-lite (v0.20.4) [18]. Forward and reverse sequences were joined using *FLASH* program [19] by applying default parameters. Next, files were filtered from mouse reads using the end-to-end and very sensitive options implemented by Bowtie2 version 2.3.4.1 [20] against the reference mouse genome database GRCm38.p6 (September 2017). Taxonomic annotation was implemented on mice-free sequences with Kaiju v1.6.2 [21]. Lineage information was added, taxa were counted, and the abundance matrix was constructed for all samples, using R 3.1.0 statistical package [22]. Finally, the contingency table created was used for the ecological analyses with QIIME version 1.9.0 [23]. Those analyses included absolute and relative abundance bar plots, alpha diversity analysis by calculation of the Shannon index, beta diversity analysis using Principal Coordinates analysis (PCoA) on a Bray Curtis dissimilarity matrix, Adonis non-parametrical test for group and time comparisons, and finally Kruskal-Wallis and Wilcoxon–Mann–Whitney non-parametrical tests for discovery of differential taxa.

Statistical analysis of data

Results are expressed as the mean ± standard deviation. Analysis of variance (ANOVA), Tukey's test and Student's t-test for multiple comparison of means were performed using XLStatistics for Windows, if not ptherwise specified. Differences were considered significant at 0.05 level of probability (p).

Results and discussion

Vesicle characterization

The grape pomace oily extract and the ethanol-propylene glycol extract were successfully incorporated in phospholipid vesicles, namely Penetration Enhancer-containing Vesicles (PEVs), prepared by a simple and scalable method. The PEVs were previously proved effective for the delivery of grape pomace extracts to the skin [16]. In the present work, the PEVs were used as a reference to assess the potential of novel vesicles obtained by modifying the PEVs with the addition of Nutriose®FM06 (20, 40 and 60% w/v), thus combining the penetration enhancing properties of PEVs with the prebiotic and protective properties of Nutriose-enriched vesicles, namely nutriosomes, which were previously proved effective for the delivery of natural compounds to the intestines [13].

The prepared 20, 40 and 60nutriosomes appeared as homogeneous, viscous dispersions: the viscosity increased as the concentration of Nutriose increased. The vesicle formation and morphology were evaluated by cryo-TEM observation (Figure 1). 20 and 40nutriosomes were spherical and mostly oligolamellar; 60nutriosomes were oligolamellar as well, but more irregularly shaped and larger.

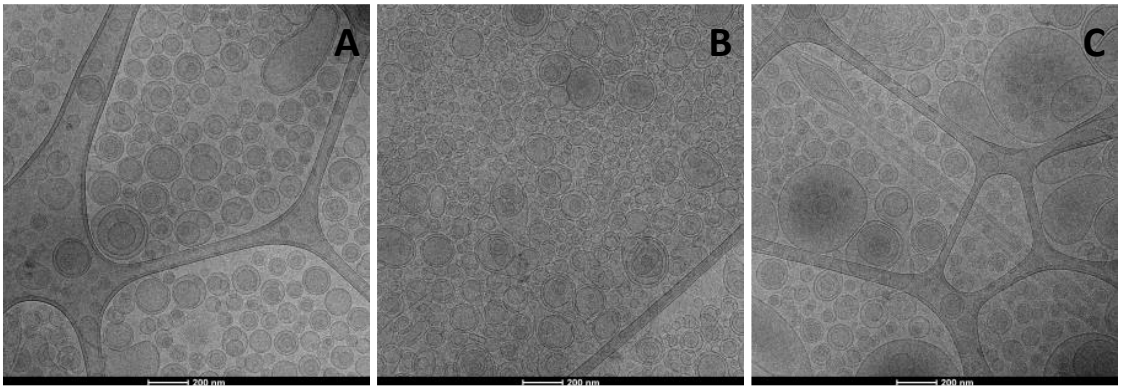


Figure 1. Representative Cryo-TEM micrographs of grape 20nutriosomes (A), 40nutriosomes (B) and 60nutriosomes (C).

The mean diameter, polydispersity index and zeta potential of grape PEVs and nutriosomes were measured, (Table 1). Empty vesicles were also prepared and characterized to evaluate the effect of the bioactives contained in the grape pomace extracts on the vesicle features.

Table 1. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (EE) of empty and grape PEVs and nutriosomes. Each value represents the mean ± standard deviation (SD) of at least six determinations.

	MD (nm±SD)	**PI**	**ZP (mV±SD)**	**EE (%±SD)**
Empty PEVs	150±25	0.11	-68±5	--
Empty 20nutriosomes	131±16	0.12	-64±4	--
Empty 40nutriosomes	140±7	0.15	-59±5	--
Empty 60nutriosomes	141±8	0.17	-62±3	--
Grape PEVs	167±27	0.17	-75±5	98±6
Grape 20nutriosomes	144±9	0.09	-77±4	95±7
Grape 40nutriosomes	173±15	0.11	-81±8	87±11
Grape 60nutriosomes	261±26	0.19	-79±9	85±13

All empty vesicles showed similar diameter (~143 nm), irrespective of their composition ($p>0.05$). The addition of extracts affected the vesicle features, as grape vesicles were larger than the corresponding empty ones. The difference in size between empty and grape vesicles was more marked for 40 and 60nutriosomes. Furthermore, an increase in size was observed as a function of the increase in the concentration of Nutriose: from 144 to 173 and 261 nm for grape 20, 40 and 60nutiosomes, respectively. It can be speculated that Nutriose competes with the hydrophilic components of the extracts, distributing both in the external and internal aqueous media.

The polydispersity index values were always ≤ 0.19, which indicates that the vesicle dispersions were highly homogeneous.

The values of zeta potential were highly negative for all the vesicles, with a statistically significant difference between empty and grape vesicles (~ -63 and -78 mV, respectively; $p<0.05$). Such high surface charge ensures a good repulsion among the vesicles in dispersion and is predictive of long-term stability. As a confirmation, no significant changes in the physico-chemical features of grape PEVs and nutriosomes were detected during 12 months of storage at 25 °C (Figure 2). Some fluctuation was observed for 60nutriosomes at the end of the monitoring period (Figure 2).

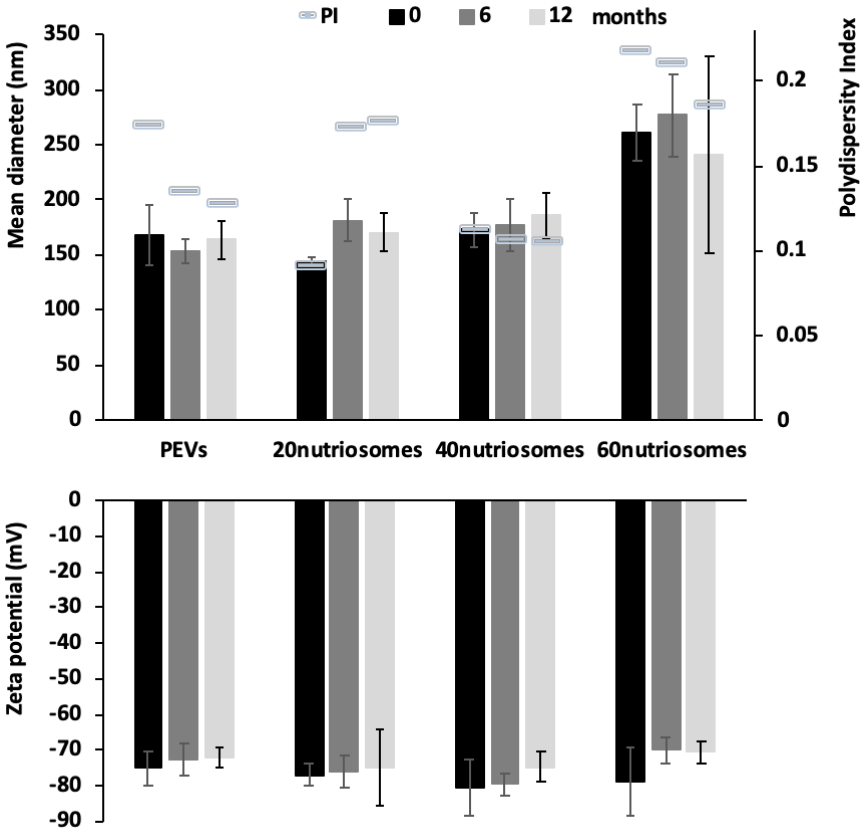


Figure 2. Mean diameter, polydispersity index and zeta potential of grape PEVs and nutriosomes stored for 12 months at 25 °C. Each value represents the mean ± standard deviation of at least six determinations.

Vesicle stability as a function of pH, ionic strength and temperature

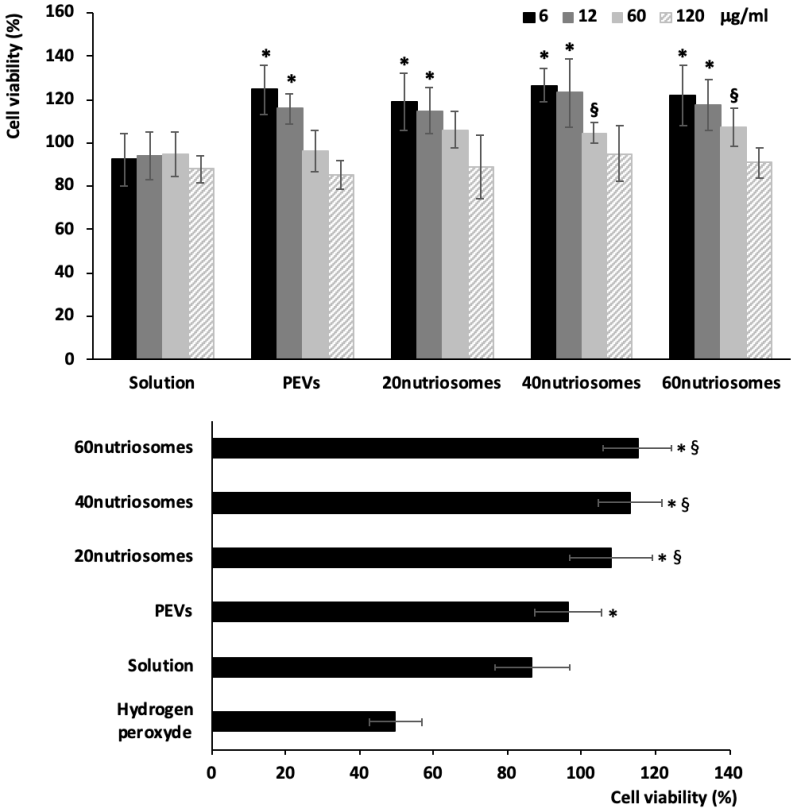
Aiming at mimicking the destabilizing conditions of the gastric and intestinal environments, the vesicles were diluted with solutions at pH 1.2 or 7.0 with high ionic strength and maintained at 37 °C for 2 or 6 h, respectively. The mean diameter, polydispersity index, and zeta potential of grape PEVs and nutriosomes, and the percentage of extract released from the vesicles were measured to evaluate the ability of these systems to preserve their structure, avoiding disruption and loss of their content under extreme conditions.

Table 2. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and released extract (RE) of grape extract loaded PEVs and nutriosomes. Each single value represents the average ± standard deviation of at least six determinations.

	pH - time	MD (nm±SD)	PI	ZP (mV±SD)	RE (%±SD)
Grape PEVs	pH 1.2 – 2 h	1946±219	0.59	+10±2	23±5
	pH 7.0 – 6 h	828±345	0.60	-7±2	57±25
Grape 20nutriosomes	pH 1.2 – 2 h	617±190	0.67	+12±3	5±13
	pH 7.0 – 6 h	150±37	0.59	-24±2	15±5
Grape 40nutriosomes	pH 1.2 – 2 h	318±140	0.54	+12±2	4±15
	pH 7.0 – 6 h	180±16	0.35	-21±8	31±12
Grape 60nutriosomes	pH 1.2 – 2 h	255±44	0.40	+11±2	21±5
	pH 7.0 – 6 h	198±20	0.38	-21±2	37±18

As can be seen in Table 2, at pH 1.2 PEVs underwent a strong increase in size (~2000 nm) and polydispersity index (0.59), which may be due to a fusion of the vesicles, which resulted in a 23% loss of grape extracts. Under the same conditions, the alterations in size were less pronounced in nutriosomes: an increase in size and polydispersity index was observed, but the higher the concentration of Nutriose®, the lower the impact of pH, ionic strength and temperature. Indeed, 20nutriosomes were around 600 nm, 40nutriosomes around 300 nm, and 60nutriosomes were the least affected (~250 nm). The same trend was observed for the polydispersity index. These results can be explained by a partial disassembly and re-assembly of nutriosomes, leading to the formation of clusters of vesicles, which resulted in size inhomogeneity (PI ≥0.4), and the loss of vesicle content. Nevertheless, the amount of grape extract released from 20 and 40nutriosomes was very low (~5%), while 60nutriosomes released a higher amount (21%), similar to that released from PEVs.

296 At acidic pH, the zeta potential was reversed, becoming positive (~ +11 mV) for all the
297 formulations, irrespective of their composition, due to the presence of a high concentration of
298 solvated positive ions in solution [24].
299 At pH 7.0, the average size of PEVs increased approximately 5 times (828 nm; see Table 2 *vs.* 1),
300 and the amount of extract released increased as well (57%), while the mean diameter of nutriosomes
301 remained almost constant and the amount of extract released was 15% for 20nutriosomes and ~30%
302 for 40 and 60nutriosomes. The zeta potential values remained negative, approaching neutrality in
303 the case of PEVs and being around -20 mV in the case of nutriosomes. Overall results disclosed that
304 nutriosomes, especially 20 and 40nutriosomes, were more stable than PEVs in both acidic and
305 neutral environments, and may allow a pH-dependent release of the grape extract bioactives.
306 *Vesicle biocompatibility and antioxidant efficacy*
307 Caco-2 cells from human colon adenocarcinoma are extensively used as a model of intestinal
308 barrier [25]. These cells differentiate into a monolayer of polarized cells, joined by tight junctions,
309 which express many morpho-functional characteristics of the absorbing epithelium of the small
310 intestine [26]. The biocompatibility of grape vesicles was evaluated by treating Caco-2 cells with
311 the formulations at different concentrations of the grape extract for 48 h (Figure 3, upper panel). An
312 aqueous solution of the grape ethanol-propylene glycol extract was used as a reference to evaluate
313 the effect of the carriers (i.e., the vesicles) on cell viability.
314



315

316 **Figure 3.** *Upper panel*: Viability of Caco-2 cells treated with different concentrations of grape
317 ethanol-propylene glycol extract in aqueous solution or grape PEVs, 20nutriosomes, 40 nutriosomes
318 and 60 nutriosomes (6, 12, 60, 120 µg/ml). Each value represents the mean ± standard deviation of
319 at least six determinations.
320 *Lower panel*: Viability of Caco-2 cells treated with hydrogen peroxide and grape ethanol-propylene
321 glycol extract in aqueous solution or grape PEVs, 20nutriosomes, 40nutriosomes and
322 60nutriosomes (12 µg/ml of grape extract). Each value represents the mean ± standard deviation of
323 at least six determinations.
324 * symbol indicates values statistically different from extract solution; § symbol indicates values
325 statistically different from liposomes.
326
327 When the grape extract aqueous solution was used, the cell viability was ~90%, irrespective of the
328 concentration tested, confirming its high biocompatibility. Similarly, when the grape vesicles were

used at the higher concentration (120 μg/ml), cell viability was ~90% ($p>0.05$ *vs.* solution). The cell viability was found to increase in a concentration-dependent manner, reaching ~120% ($p<0.05$ *vs.* solution) when the lower concentration was applied (6 μg/ml). Hence, not only the vesicle formulations were biocompatible, but they were also capable of stimulating the proliferation of intestinal cells, regardless of the presence and concentration of Nutriose.

The protective effect of the grape extract against oxidative stress was evaluated. Caco-2 cells were exposed to hydrogen peroxide, which is known to cause cell death [27,28], and simultaneously treated with the grape extract containing samples at a non-toxic concentration (12 μg/ml of grape extract; Figure 3 lower panel). The cells stressed with hydrogen peroxide, underwent a significant reduction in viability (~50%).

The treatment of stressed cells with the grape extract in aqueous solution reduced the damaging effect of hydrogen peroxide, as indicated by a ~87% viability ($p<0.05$ *vs.* hydrogen peroxide). The incorporation of the extract into the vesicles, further improved the protection provided to the cells, preventing cell death and even stimulating cell proliferation (viability up to ~115%; $p<0.05$ *vs.* hydrogen peroxide). More specifically, a composition-dependent protective effect was observed for the vesicles. The presence of Nutriose (but not the concentration) affected cell viability favouring cell growth. Indeed, the viability was ~96% for cells treated with grape PEVs and ≥108% for cells treated with grape nutriosomes ($p<0.05$).

Effect of grape extract on in vivo faecal microbiota

Considering the promising results *in vitro*, the effect of the oral administration of the grape pomace extracts was evaluated *in vivo* in mice. To reduce the number of treated animals, only the most promising formulations (i.e., 20 and 40nutriosomes) were tested. The grape ethanol-propylene glycol extract in aqueous solution and grape PEVs were tested as references to evaluate the effect of the carrier and Nutriose, respectively.

The microbiota composition was determined by means of the analysis of the DNA of the faecal pellets of the animals.

Shotgun sequencing generated a total of 384,906 reads (19,245.3 ± 8,210.4 reads per sample), after bioinformatic processing and taxonomical assignment of 20 samples. Overall, the most abundant phyla (Figure 4A upper panel) were Firmicutes (70.15%), Bacteroidetes (15.85%), and Proteobacteria (12.95%), with the remaining phyla (Actinobacteria, Spirochaetes, Tenericutes, and not assigned) accounting altogether for the remaining 1% of the reads. At genus level, the 5 most abundant taxa accounted for more than 85% of the reads were *Lachnoclostridium* (27.66%), *Blautia* (16.78%), *Bacteroides* (15.22%), *Lactobacillus* (13.53%), and *Vibrio* (12.27%).

No significant differences were detected among the sample at phylum level (Figure 4B Upper panel).

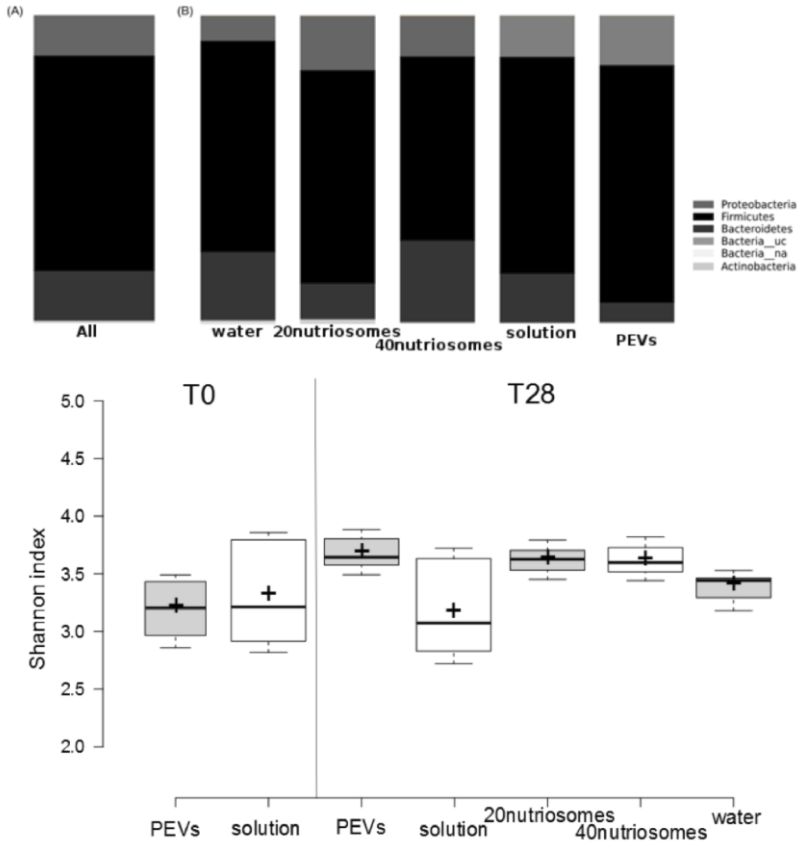


Figure 4: *Upper panel***:** Bacterial relative abundance, at phylum taxonomic level, of all samples (A) and samples grouped by treatment (B). Bacteria__uc: unclassified taxon (i.e., more than one candidate phylum matching a read with the same probability); Bacteria__na: not assigned (i.e., taxon with no name in taxonomy database).

Lower panel: Bacterial alpha diversity, measured by Shannon diversity index, of the samples grouped by treatment. Values for grape ethanol-propylene glycol extract in aqueous solution or grape PEVs, 20nutriosomes and 40nutriosomes are presented at two different times: t_0 and t_{28} (day 0 and day 28, respectively).

Similarly, Shannon index did not show significant differences ($p>0.05$) among samples as a function of treatment type, time or the combination of both (Figure 4 lower panel).

Results obtained by the Adonis test are reported in Table 2S (Supplementary Material). In this case, significant differences ($p<0.05$) were detected as a function of the treatment regardless of the time ($p=0.025$), but also at the same time ($p=0.048$).

The search for differential taxa between pairs of treatments at the same time, and, for those treatments for which two times were available, carried out by Wilcoxon–Mann–Whitney non-parametrical tests, did not detect genus with significant differences. This could be due to the low number of samples (only two or three) per subgroup, which led to a poor statistical signal. Actually, if several subgroups were collapsed, thus increasing the number of samples per group, e.g. by time-only (regardless of the treatment), or by treatment-only (regardless of the time), some taxa were identified as significantly different (data not shown).

Discussion

Intestinal microbiota composition is closely correlated with host's health status, and their alteration may cause not only local dysbiosis and intestinal problems, but also a wide range of metabolic diseases [29]. Therefore, it is important to explore novel protective and therapeutic systems capable

391 of preventing or counteracting intestinal dysbiosis [30]. To this purpose, in the present study new
392 phospholipid vesicles enriched with a dietary fibre, Nutriose®FM06, were proposed as carriers for
393 antioxidant extracts obtained from grape pomaces. The use of fibres has attracted the interest of the
394 scientific community. Fibres are plant polysaccharides, lignin and other molecules resistant to
395 hydrolysis by human digestive enzymes, which can exert beneficial effects on human health,
396 especially if consumed in high amounts, reducing the incidence of disorders that affect developed
397 countries, such as chronic bowel disorders, obesity, diabetes, cardiovascular disease and cancer
398 [31–33]. The beneficial prebiotic properties of the fibre used in this work, a soluble dextrin from
399 maize, can be complementary in potentiating the antioxidant power of the bioactives contained in
400 the extracts obtained from grape pomaces. Indeed, it is well known that grape pomaces are rich in
401 antioxidants, which can reverse the increase in oxidative radicals in villi and crypts of the small
402 intestinal mucosa, avoiding its damage and the destruction of the beneficial microbiota [34].
403 Additionally, the fibre can exert a technological function by improving the resistance of
404 phospholipid vesicles to the harsh conditions of the gastro-intestinal environment, thus ensuring the
405 delivery of the payload in the intestine [34]. Aiming at identifying the most suitable concentration
406 of Nutriose, increasing amounts were used to prepare grape nutriosomes (20, 40, 60% w/v). As a
407 comparison, grape PEVs (without Nutriose) were prepared and tested, as well. Nutriosomes were
408 highly viscous, oligolamellar and fairly spherical, except for 60nutriosomes, which were irregularly
409 shaped and larger (~260 *vs.* 150 nm). This may be due to the high amount of Nutriose, which
410 modifies the assembly of the different vesicle components, especially the hydrophilic ones
411 distributing in the aqueous compartments, causing an enlargement of the vesicles. All the vesicles
412 were highly negatively charged (~78 mV), which is predictive of a good stability over time.
413 Stability studies performed for 12 months at room temperature (25 °C) confirmed the good stability
414 of the vesicles, especially 20 and 40nutriosomes, as no significant changes in size, size distribution
415 and surface charge were detected. Additionally, the stability of the vesicles was evaluated under the

harsh conditions of the gastro-intestinal environment. Again, Nutriose was crucial to improving the stability of the vesicles, especially 20 and 40nutriosomes, which underwent a disassembly/re-assembly process without a massive leakage of the loaded extracts at acidic pH. A similar trend was observed at neutral pH mimicking the intestinal environment: the mean diameter of nutriosomes did not change significantly, and the amount of extract released increased as the amount of Nutriose increased (15, 31 and 37% for 20, 40 and 60nutriosomes, respectively).

All the vesicles, irrespective of their composition were highly biocompatible, as demonstrated in vitro by using Caco-2 cells as a model of intestinal cells. Regarding the ability of the vesicles to protect the cells from oxidative stress, nutriosomes, irrespective of the amount of Nutriose used, were more effective than PEVs and the raw grape extract. Moreover, not only nutriosomes were able to prevent cell death, but they were also able to stimulate cell growth. Presumably, Nutriose improved the ability of the carrier to interact with the cells, favouring the release of the grape extract bioactives within the cells.

In light of the obtained physico-chemical, technological and *in vitro* results, 20 and 40nutriosomes were considered the most promising systems to be tested *in vivo*. When administered orally, they are expected to have a dual benefit: the first one is connected to their ability to incorporate a high amount of bioactives contained in the grape pomace extracts, deliver them to the intracellular environment and exert an antioxidant effect; the second one is connected to their ability to incorporate a high amount of Nutriose, which is a prebiotic fibre that can improve the gut microbiota composition and the health of the intestinal tract [35,36].

The *in vivo* studies, performed to confirm the prebiotic effect of nutriosomes, were carried out for 4 weeks during which the intestinal microbiota was analysed in terms of bacterial abundance at phylum taxonomic level and bacterial alpha diversity. The composition of the microbiota was not affected by the vesicle composition or time of treatment, as no significant differences in bacterial composition and relative abundance were detected. Probably these results are due to the low

number of samples associated with the choice of using the lowest number of animals as recommended by the European Union regulations for the handling and use of laboratory animals and the ethical committee. Although differences in the bacterial composition and relative abundance associated to the different treatments and times were not detectable, we can suppose that the inclusion of a higher number of samples per subgroup (treatment and time) could increase the chances to detect differential taxa.

Conclusion

In this paper, aiming at developing a novel protective and therapeutic system based on natural functional components with antioxidant and prebiotic activities, phospholipid vesicles loaded with two extracts from grape pomaces and enriched with Nutriose were formulated. Grape PEVs and nutriosomes were prepared and their physico-chemical features, and *in vitro* and *in vivo* efficacy were evaluated. Nutriosomes were formulated by using increasing amounts of Nutriose®FM06, which led to the formation of viscous, stable formulations. The best results were obtained for 20nutriosomes and 40nutriosomes, which were more stable on storage and under the harsh conditions mimicking the gastro-intestinal environment, avoiding a massive release of the incorporated extracts, especially at acidic pH. *In vitro* studies confirmed the superior ability of nutriosomes to counteract the oxidative damages induced in Caco-2 cells, thanks to their ability to promote the release of the payload inside the cells. The oral administration of these vesicular formulations to mice did not significantly alter the microbiota composition. Overall, the results are encouraging and support the potential use of grape nutriosomes for the prevention or treatment of intestinal dysbiosis.

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