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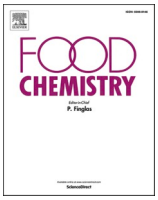

Improving fermented milk antioxidant properties by functional enrichment with *Vitis Vinifera* leaf extract cv Monica loaded in nanovesicles

Debora Dessì [a,1], Luca Casula [a,1], Giacomo Fais [b,c], Alessio Pittiu [a], Michele Schlich [a], Chiara Sinico [a], Francesco Lai [a,*], Giorgia Sarais [a,*]

[a] Department of Life and Environmental Sciences, University of Cagliari, Cagliari, Italy
[b] Interdepartmental Center of Environmental Science and Engineering (CINSA), University of Cagliari, Via San Giorgio 12, 09124 Cagliari, Italy
[c] Department of Mechanical, Chemical and Materials Engineering, University of Cagliari, Piazza d'Armi, 09123 Cagliari, Italy

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ABSTRACT

Kefir is increasingly appreciated for its probiotic-rich composition and health benefits, aligning with the growing demand for functional foods. This study introduces an innovative approach to fortify kefir with grapevine leaves extract, an underutilized viticultural by-product, thereby promoting circular economy practices. The extract, characterized by a high total polyphenol content and remarkable antioxidant capacity, was analysed by HPLC-DAD, which identified caftaric acid, quercetin-3-O-glucuronide, and peonidin-3-O-glucoside as major phenolic constituents. The extract was encapsulated in nanovesicles prepared via a gentle high-shear homogenization method, a milder alternative to sonication that preserves thermosensitive compounds. The resulting nanovesicles exhibited encapsulation efficiencies ranging from 28.8 % to 73.9 % and demonstrated good physical stability. Moreover, they effectively mitigated the degradation of the extract components in simulated gastrointestinal conditions and protected enterocytes from oxidative stress in vitro. Incorporation of the loaded nanovesicles into the fermented drink enhanced its antioxidant activity in a dose-dependent manner without altering rheological properties.

1. Introduction

Grapevine (*Vitis vinifera* L.) is among the most significant horticultural crops globally, with an estimated annual production of approximately 72 million tons, predominantly valued for grape and wine production (Zhou et al., 2022). With around 10,000 grape varieties, grapevine cultivation generates substantial amounts of by-products, particularly grape pomace, composed of skins, seeds, and pressing residues, which have long been recognized for their rich polyphenolic content (Kokkinomagoulos & Kandylis, 2023; Parihar & Sharma, 2021). In recent years, the growing focus on sustainable production systems and circular economy practices has sparked renewed interest in the valorization of these agro-industrial residues, promoting their transformation into high-value resources through innovative reuse strategies (Mamalis, 2019; Pal et al., 2024). Despite this, grapevine leaves, typically regarded as waste from routine pruning and vineyard management, represent a valuable yet largely underutilized resource that has received limited attention from both the agri-food and pharmaceutical sectors. Numerous studies have demonstrated that grapevine leaves, possess a diverse phytochemical profile rich in flavonols, anthocyanins, proanthocyanidins, and phenolic acids (Sousa et al., 2024). Specifically, grapevine leaf extracts and their constituents, that have long been recognized for their effectiveness in the symptomatic treatment of chronic venous insufficiency (Bencsik et al., 2024), exhibit a broad spectrum of biological activities, including antioxidant, anti-inflammatory (Sangiovanni et al., 2019), antidiabetic (Orhan et al., 2006), hepatoprotective (Paka et al., 2012), and gastroprotective properties (Saadaoui et al., 2020), suggesting numerous potential applications in health-oriented products. From this perspective, given the increasing consumer demand for health-promoting and naturally derived ingredients, grapevine leaves may be an excellent choice for the agri-food industry that is actively pursuing innovative ways to enrich food products with natural ingredients, including plant extracts (Mihai et al., 2025).

The aim of this practice, usually called food fortification, is not only to increase the nutritional and health benefits offered by the final

* Corresponding authors.
E-mail addresses: frlai@unica.it (F. Lai), gsarais@unica.it (G. Sarais).
[1] *These authors contributed equally*

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products, but also to improve their quality and, as a result, enhance the marketability (García-Burgos et al., 2020). In this sense, the reuse of vine leaves offers advantages to both the agri-food sector and the environment, as it contributes to supporting eco-sustainable viticulture by transforming waste into high-added-value products.

Since milk-based products, such as fermented dairy products, are among the most consumed foods worldwide, they may be an excellent delivery vehicle for natural bioactive molecules and plant extracts (Marsh et al., 2014; Nazhand et al., 2020). Moreover, fermentation processes usually enhance the food nutritional interest because of the beneficial effect on the bacterial microbiota, and they can also increase the bioavailability of nutrients (Shiby & Mishra, 2013).

Polyphenols are increasingly recognized for their ability to exert stimulatory or prebiotic-like effects on lactic acid bacteria, supporting their metabolism by providing additional substrates and antioxidant protection, and thereby contributing to the improvement of commensal bacterial populations and overall human health (Piekarska-Radzik & Klewicka, 2021). Polyphenol-rich plant extracts can be effectively incorporated into fermented dairy systems such as kefir without negatively affecting fermentation kinetics or viable microbial counts, while improving the functional and antioxidant profile of the final product (Hossein Marhamatizadeh et al., 2013; Kwon et al., 2019; Oh et al., 2016). In this context, the incorporation of grape-derived extracts into yogurt and other fermented milk matrices maintained viable counts of lactic acid bacteria within Codex Alimentarius requirements, and in some cases even promoted the growth of probiotic strains such as *Lactobacillus* and *Bifidobacterium* (Chouchouli et al., 2013; dos Santos et al., 2017; El-Sayed et al., 2022).

However, several fortifying agents, such as polyphenols, are chemically unstable and prone to degradation or oxidation, due to their sensitivity to environmental variations. In addition, flavonoids are highly water-soluble molecules, thus showing a poor absorption. Their inability to cross lipid membranes results in loss of bioavailability and efficacy (Maia et al., 2021).

Accordingly, different approaches have been developed and evaluated to overcome these limitations, including encapsulation in biocompatible micro- and nanocarriers, which can increase physical, chemical and biological stability as well as bioavailability of their payload. Among the various nanocarriers that have been developed as delivery systems, phospholipid vesicles have attracted increasing attention in recent years, because they are highly versatile in terms of composition and structure (Bilia et al., 2016). Considering the complexity of plant extracts, encapsulation must concurrently reduce the degradation process and mask unpleasant taste, while controlling release, absorption and biological response. This is an extremely important consideration for the success of a fortified food.

In previous works, our research group has successfully encapsulated in liposomes plant extracts and/or single molecules isolated from extracts (Ilgaz et al., 2025; Ravaghi et al., 2017; Schlich et al., 2020; Sinico et al., 2005; Sinico et al., 2008).

Although grapevine leaves are a rich source of polyphenols, their valorization in functional food formulations remains limited. In particular, the use of phospholipid vesicles to incorporate grape leaf extracts into fermented dairy matrices has not been thoroughly investigated. In this paper, a grape leaves extract, *Monica* cultivar (cv.), has been prepared, chemically characterized and finally encapsulated in phospholipid nanovesicles. Then, liposomes were also characterized in terms of mean size, size distribution and encapsulation efficiency. After evaluating the antioxidant properties of the extract both alone and encapsulated in liposomes, a fermented milk drink made of pasteurized goat's milk and probiotics has been fortified by the addition of the liposome-loaded extract. The final goal was to produce a fermented milk drink with enhanced antioxidant properties by adding a grape leaves extract.

2. Materials and methods

2.1. Materials

Soy phosphatidylcholine for vesicles preparation (Lipoid® S75, S75) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Triton X-100 was purchased from Sigma–Aldrich (Milan, Italy). Orthophosphoric acid 85 %, ethanol absolute (grade RPE ACS Reagent Ph Eur), and sodium acetate anhydrous ≥99.0 % were purchased from Carlo Erba (Val de Reuil Cedex, France). Acetonitrile (grade HPLC), sodium carbonate, Folin–Ciocalteau reagent and gallic acid, caftaric acid, quercetin-3-O-rutinoside, quercetin-3-O-galactoside, quercetin-3-O-glucoside, quercetin-3-O-glucuronide, kaempferol-3-O-glucoside, kaempferol-3-O-glucuronide, delphinidin 3-o-glucoside, cyanidin 3-o-glucoside, cyanidin 3-o-arabinoside, peonidin 3-o-glucoside, malvidin 3-o-glucoside standards were purchased from Extrasynthese (St. Louis, MO, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), ferric chloride, glacial acetic acid (grade ACS, ISO, Reag. Ph Eur) and ferrous sulfate were obtained from Sigma-Aldrich, Fluka (Milan, Italy). Ultrapure water (conductivity lower than 18.2 MΩ) was distilled and filtered with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Chemical and functional characterization of the extract

2.2.1. Preparation of extracts

The study was conducted on *Vitis Vinifera* L. leaves cv Monica sample collected at the end of the harvest season and provided by Argiolas SpA (Serdiana, Sardinia, Italy). For chemical analysis, the samples were lyophilized with a freeze dryer LIO 5P DGT (Cinquepascal, Trezzano s/ Naviglio (MI)). Lyophilized samples were finely ground in an electric coffee grinder until a homogeneous powder was achieved and preserved in a tightly closed tube in a dark and dry place until analysis. The sample (500 mg) was extracted with 10 mL of an ethanol/water solution (70:30, *v*/v). The suspension was sonicated for 1 h in an ultra-sonicator (040S model, AC220-240 V 50 Hz, heating power 200 W, frequency 40 KHz, time 0–30 min, Be-Right Medical Co., Ltd., Foshan, China) and subsequently centrifuged with an Eppendorf Centrifuge 5810 R (Eppendorf, Milan, Italy) for 30 min at 4000 rpm and temperature-controlled (4 °C). The supernatant was collected, and the extraction was repeated twice. Supernatants were pooled, and an aliquot was injected into HPLC-DAD for phenolic fraction characterization and analysed for antioxidant activity determination.

2.2.2. Total polyphenols content

The determination of total polyphenol content was carried out using the Folin-Ciocalteu colorimetric method, following a modified procedure based on Singleton, V. L. (1965) (Singleton & Rossi, 1965). In summary, 100 µL of either the sample extract or gallic acid standard were mixed with 500 µL of Folin-Ciocalteu reagent and allowed to stand for 5 min at room temperature. Subsequently, 3 mL of 10 % (*w/v*) sodium carbonate solution were added, and the mixture was brought to a final volume of 10 mL with ultrapure water. The reaction was incubated at room temperature for 90 min and then the absorbance was measured at 725 nm using a Varian Cary 50 UV–Vis Spectrophotometer (Agilent Technologies, Woodburn, Australia). Quantification was achieved via an external standard curve, and results were reported as milligrams of gallic acid equivalents (GAE) per gram of dry weight (DW) sample.

2.2.3. Ferric reducing antioxidant power assay

The antioxidant capacity was evaluated using the FRAP (Ferric Reducing Antioxidant Power) assay, as described by a slightly modified version of the method by Axelrod et al. (Axelrod et al., 1976). The FRAP reagent was freshly prepared by combining 10 mM TPTZ and 20 mM ferric chloride in an acetate buffer adjusted to pH 3.6. A 50 µL aliquot of

the extract (diluted 1:10, *v*/v) or ferrous sulfate standard was added to 2 mL of the reagent mixture. Following a 4-min incubation at ambient temperature, absorbance was recorded at 593 nm. The concentration of antioxidant compounds was determined using a calibration curve constructed with ferrous sulfate standards, and results were expressed as mmol of Fe^{2+} equivalents per gram of DW sample.

2.2.4. Radical scavenging assay

The DPPH radical scavenging activity was determined spectrophotometrically (Dessì et al., 2025). The extract (50 μL) properly diluted was mixed to 2 mL of DPPH (60 μM in methanol). The mixture was stored in the dark at room temperature for 1 h and UV–Vis readings were carried out with a spectrophotometer Varian Cary 50 at 517 nm. The antiradical activity was expressed as mM/g DW of TEAC (Trolox equivalent antioxidant capacity) using a Trolox calibration curve.

2.2.5. HPLC analysis of phenolic compounds

High-performance liquid chromatography (HPLC) analyses were conducted using an Agilent 1100 system, equipped with a Thermo Finnigan UV6000 DAD detector and controlled through ChromQuest software. The separations took place on a Kinetex C18 column (5 μm, 100 Å, 150 × 4.6 mm; Phenomenex). A sample volume of 10 μL was injected, and the compounds were eluted over a 120-min period at a flow rate of 0.4 mL/min, using a binary solvent system composed of 0.22 M ortho-phosphoric acid (solvent A) and acetonitrile (solvent B). The gradient program included the following steps: 95 % A/5 % B at 0 min, 90 % A/10 % B at 30 min, 85 % A/15 % B at 35 min, 70 % A/30 % B at 70 min, 10 % A/90 % B at 100 min, and 100 % B at 120 min. Following each run, the column was re-equilibrated for 15 min under the initial conditions (D'amelia et al., 2022). The detection wavelengths were chosen based on the chemical structures of the target phenolic compounds: 280 nm, 360 nm for caftaric acid, quercetin and kaempferol derivatives quantification, and 520 nm for anthocyanins. Peak identification was accomplished by comparing the retention times and UV–Vis spectra of the samples with those of authentic standards. Quantification utilised external calibration curves created by diluting standard stock solutions (1000 mg/L in methanol) in 0.22 M ortho-phosphoric acid.

2.3. Preparation of extract-loaded vesicles

Prior to the nanovesicles preparation, the extract was freeze-dried using the lyophiliser LIO 5P DGT. A stock solution was prepared by dissolving the obtained dry powder (2 mg/mL) in a water:ethanol mixture (70:30, *v*/v). S75 (50 mg/mL) was weighted in a glass vial and hydrated with the prepared stock solution. The dispersion was then homogenised using an Ultra Turrax T25 basic for 6 min at 8000 rpm, until a clear dispersion of loaded nanovesicles (LNV) was obtained. During the homogenization, the vial was kept in an ice bath to avoid overheating of the sample. Empty nanovesicles (ENV) were prepared as control using the same protocol described above, using pure water: ethanol mixture (70:30, v/v) instead of the extract stock solution.

2.3.1. Particle size analysis

The average diameter and Polydispersity Index (PdI, as a measure of the size distribution width) of the samples were determined using a Zetasizer nano (Malvern Instrument, Worcestershire, UK) through the Dynamic Light Scattering (DLS) technique. A helium-neon laser (633 nm) was used to backscatter samples at an angle of 173 and a constant temperature of 25 °C. The M3-PALS (Phase Analysis Light Scattering) technique was also used on the Zetasizer nano to estimate the Zeta potential value. The samples were diluted with distilled water just before the analysis. All measurements were made in triplicate. Additionally, the same parameters were monitored over 30 days of storage at 4 °C.

2.3.2. Determination of encapsulation efficiency

To evaluate the encapsulation efficiency of the extract, the nanovesicles dispersions were dialyzed against distilled water using dialysis tubing (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories, Inc., USA). Each sample (1 mL) was dialyzed in 1000 mL of water for 2 h to allow dissolution and subsequent removal of the non-entrapped compounds, while also avoiding destabilization of the vesicular suspension (i.e., osmotic swelling and vesicle fusion). After dialysis, the samples were treated with 0.1 % (*v*/v) Triton X-100 to disrupt the nanovesicles and then analysed using the HPLC method described above. The encapsulation efficiency (EE%) was evaluated by monitoring the amount of the main components of the extract, before and after dialysis.

2.3.3. LNV stability in gastrointestinal fluids

To evaluate the ability of the vesicles to protect the extract components from degradation under gastrointestinal conditions, both the LNV and the extract solution − at the same concentration of the nanovesicles (2 mg/mL) − were incubated (1:3) with Simulated Gastric Fluid (SGF) or Simulated Intestinal Fluid (SIF), and analysed via DLS and HPLC as described above. SGF was prepared by dissolving 200 mg NaCl in 100 mL of water, adjusting the pH to 1.2 with HCl 1 M. For SIF preparation, 680 mg KH_2PO_4 were dissolved in 100 mL of water and the pH was adjusted to 6.8 with NaOH 2 M (Ilgaz et al., 2025).

Briefly, 200 μL of each sample (LNV or extract solution) were mixed with 800 μL SGF or SIF and incubated in an orbital shaker (BioSan ES-20, BioSan, Latvia) at 37 °C and 100 rpm. At predetermined time points (2 h for SGF, 6 h for SIF), 200 μL of samples were withdrawn and diluted with Triton X-100 in order to achieve the same final concentration used for the encapsulation efficiency studies (2.3.2).

2.3.4. Antioxidant activity of LNV on intestinal cells in vitro

The antioxidant activity of LNV, ENV and the free extract (FE) was assessed in a cellular intestinal model using Caco-2 cells and the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay (Chiaradia et al., 2023; Lyublinskaya et al., 2017). Cell cultures were maintained in DMEM supplemented with 1 mM sodium pyruvate, 1 mM non-essential amino acids (NEAA), 1 % penicillin–streptomycin (P/S), and 10 % fetal bovine serum (FBS), and incubated at 37 °C with 5 % CO_2. For the experiments, 2×10^4 cells/well were seeded into a 96-well plate and incubated for 48 h. Subsequently, cells were treated for 4 h with FE and LNV (final extract concentration for both treatments: 25 μg/mL or 400 μg/mL) diluted in complete medium. Another group was treated with an equal volume of ENV diluted in medium as a control. After 4 h, the treatments were removed and DCFH-DA in PBS was added for 30 min. Oxidative stress was then induced by exposing cells to a hydrogen peroxide solution in PBS for 15 min. The probe oxidation by intracellular reactive oxygen species (ROS) was quantified by measuring its fluorescence (480–530 nm) using a Biotek Synergy LX plate reader. The percentage of intracellular ROS was derived using the following equation:

ROS (%) = (Ft-Fblank) × 100 / (Fut – Fblank).

Where Ft is the fluorescence of FE/ENV/LNV-treated cells, Fblank is the background fluorescence of cells not exposed to DCFDA and Fut is the fluorescence of untreated cells not exposed to hydrogen peroxide. ROS (%) was then normalized to the viability of cells exposed for the same time (4 h) to the same formulations and doses in identical conditions (2×10^4 cells/wells, allowed to attach for 48 h before treatments), determined by MTT assay in a preliminary experiment. Data is reported as the average ± standard deviation ($n = 5$).

2.4. Fortified drink preparation

A fermented goat milk drink was provided by a local dairy company (White Goat Kefir Drink, Latte Arborea s.c.a.p.a Soc. Benefit, Brand Girau, Arborea, Sardinia, Italy). Different volumes of the nanovesicles dispersion were added to the kefir drink to achieve concentrations of 5, 10 and 30 % (*v*/v). The obtained fortified drinks were manually stirred until the nanovesicles were homogenously dispersed and then tested to

evaluate the antioxidant activity and the rheological properties.

2.4.1. Rheological measurements

The rheological properties of the fermented milk drink with increasing concentrations of the nanovesicles were studied using a Kinexus rotational rheometer (Malvern Instruments, Worcestershire, United Kingdom) equipped with data acquisition and elaboration software rSpace; a cone-plate geometry CP1/60 (Cone Plate; 1° angle 60 mm diameter) was used. The samples were loaded on the lower plate and the analysis were carried out after 10 min of rest at the temperature of 25 ± 1 °C. The steady shear rate sweep was performed to measure the change of shear viscosity of fermented milk samples in the range of 0.1–1000.0 s^{-1}. An amplitude sweep test was performed to determine the to determine the linear viscoelastic region from 0.1 % to 100 % at 1.0 Hz frequency. The dynamic frequency sweep (0.1–10.0 Hz) were determined and the storage modulus (G′) and loss modulus (G″) of the samples were recorded. The fermented milk drink without the addition of the nanovesicles was used as control for all the experiments. Three replicates' tests were performed for each sample.

2.4.2. Assessment of antioxidant activity in the fortified fermented Milk drink

The analysis was carried out as previously described in paragraph 2.1.4. According to this protocol, the antiradical activity was expressed as % of inhibition and calculated as following:

% Inhibition = ((A_control - A_sample) / A_control) * 100.

A_control = Absorbance of the DPPH solution alone (control).

A_sample = Absorbance of the DPPH solution mixed with the sample.

2.5. Statistical analysis

Each experiment was repeated independently at least three times, with results expressed as mean % ± SD. Statistical analysis was performed using GraphPad PRISM 8.00 (GraphPad Software, San Diego, CA, USA). Means and standard deviations (SD) were calculated from three distinct experiments. Data analysis used ANOVA Tukey's test to compare multiple groups against the control while adjusting for cumulative alpha error. Significance levels, denoted by asterisks (*), are determined by *p*-values. An absence of an asterisk indicates a p-value >0.05; * signifies p-value <0.05; ** denotes p-value <0.01; *** indicates p-value <0.001; and **** indicates p-value <0.0001.

3. Results

3.1. Chemical and functional characterization of the extract

A preliminary determination of total polyphenol content (TPC) in *Vitis vinifera* cv. Monica leaf extract yielded a value of 19.60 ± 2.17 mg GAE/g DW (Table 1), highlighting a notable accumulation of phenolic compounds in this by-product. Importantly, the TPC observed in Monica leaves compares favorably with data reported by Šuković et al. for grapevine leaves, which reached 28.98 ± 0.61 mg/g DW in certain cultivars (Šuković et al., 2020). Similarly, Fernandes et al. reported high polyphenol concentrations in their analysis performed on multiple cultivars (Fernandes et al., 2013). Given the high polyphenol content of the extract, its antioxidant activity was assessed through DPPH and FRAP assays, which further substantiated its bioactive potential. The DPPH radical scavenging activity was 0.04 ± 0.00 mmol Trolox/g DW, while the FRAP reducing power reached 0.25 ± 0.04 mmol $FeSO_4$/g DW. When compared with the literature, the DPPH value obtained in this study is lower than those reported by Šuković et al., who found DPPH activities ranging from 0.14 ± 0.01 to 0.24 ± 0.01 mmol TE/g DW in different grapevine leaf varieties (Šuković et al., 2020). This discrepancy can be attributed to several factors, including cultivar, environmental conditions, leaf age, extraction method, and solvent polarity, which affect both yield and phenolic composition, and consequently, the antioxidant response, depending on compound structure and assay sensitivity. The FRAP assay yielded a result of 0.25 ± 0.04 mmol $FeSO_4$/g DW, which is comparable to that reported by Alijanipoor et al. (0.22 ± 0.03 mmol $FeSO_4$/g DW), indicating that the extract retains a significant electron-donating ability and iron-reducing potential (Alijanipoor et al., 2019).

Table 1
Total polyphenols, DPPH and FRAP concentrations in cv. Monica Leaf Extract. Values are expressed as mg/g DW (mean ± SD; $n = 3$) for total polyphenols; mmol/g DW (mean ± SD; n = 3) for DPPH and FRAP.

Total Polyphenols	DPPH	FRAP
mg GAE/g DW	**mmol Trolox/g DW**	**mmol $FeSO_4$/g DW**
19.60 ± 2.17	0.04 ± 0.00	0.25 ± 0.04

The high total polyphenol content and antioxidant activity observed in the hydroalcoholic extract of *Vitis Vinifera* cv. Monica leaves prompted an in-depth HPLC-DAD analysis to identify and quantify the main phenolic constituents (Table 2). Fig. 1 presents chromatograms acquired at 360 and 520 nm, with the main peaks numbered. No additional peaks were identified or quantified at the 280 nm chromatogram, so it is not reported. As shown, the extract revealed a diverse and rich phytochemical profile, primarily composed of hydroxycinnamic acids, flavonols, and anthocyanins. Among these, hydroxycinnamic acids and flavonols were detected at 360 nm, with caftaric acid being the only compound belonging to the hydroxycinnamic acid family and quercetin and kaempferol derivatives belonging to the flavonol group. Caftaric acid, whose presence is well-documented in grapes and wine and is known for its antioxidant capacity (Saima et al., 2023), in our extract has reached the concentration of 1.80 ± 0.13 mg/g DW. This result aligns with findings from Schoedl et al., who reported high caftaric acid levels in Pinot noir leaves across different developmental stages with an average concentration of around 7.32 mg/g (Schoedl et al., 2012). Similarly, Monagas et al. and Fernandes et al. emphasized the prominence of caftaric acid among phenolic acids in grapevine tissues with a wide range among 1.83 ± 0.10 mg/g and 14.1 ± 0.26 mg/g (Fernandes et al., 2013; Monagas et al., 2006). Among flavonols, quercetin-3-O-glucuronide was by far the most abundant (2.54 ± 0.31 mg/g DW), followed by quercetin-3-O-glucoside (1.11 ± 0.20 mg/g DW). Minor quercetin derivatives, including quercetin-3-O-galactoside (0.19 ± 0.02 mg/g DW) and quercetin-3-O-rutinoside (0.19 ± 0.02 mg/g DW), were also detected. The relevance of quercetin glycosides is widely supported in the literature. Kocsis et al. reported quercetin-3-O-glucuronide as the major flavonol in Gohér grapevine leaves, especially under full sunlight, where concentrations reached up to 18.52 ± 0.39 mg/g DW. These high concentrations may be related to the light-induced accumulation, reflecting the role of flavonols in photoprotection and oxidative stress

Table 2
Polyphenols content in cv. Monica Leaf Extract. Values are expressed as mg/g DW (mean + SD; $n = 3$).

	Retention time (min)	Compounds	Concentration (mg/g DW)
360 nm			
1	27.1	caftaric acid	1.80 ± 0.13
2	51.6	quercetin-3-O-rutinoside	0.19 ± 0.02
3	52.7	quercetin-3-O-galactoside	0.19 ± 0.02
4	53.2	quercetin-3-O-glucoside	1.11 ± 0.20
5	53.7	quercetin-3-O-glucuronide	2.54 ± 0.31
6	57.5	kaempferol-3-O-glucoside	0.15 ± 0.01
7	58.8	kaempferol-3-O-glucuronide	0.18 ± 0.01
520 nm			
8	38.9	delphinidin 3-O-glucoside	0.38 ± 0.04
9	41.2	cyanidin 3-O-glucoside	2.04 ± 0.14
10	42.5	cyanidin 3-O-arabinoside	0.24 ± 0.03
11	44.5	peonidin 3-O-glucoside	23.67 ± 2.20
12	45.5	malvidin 3-O-glucoside	1.39 ± 0.14

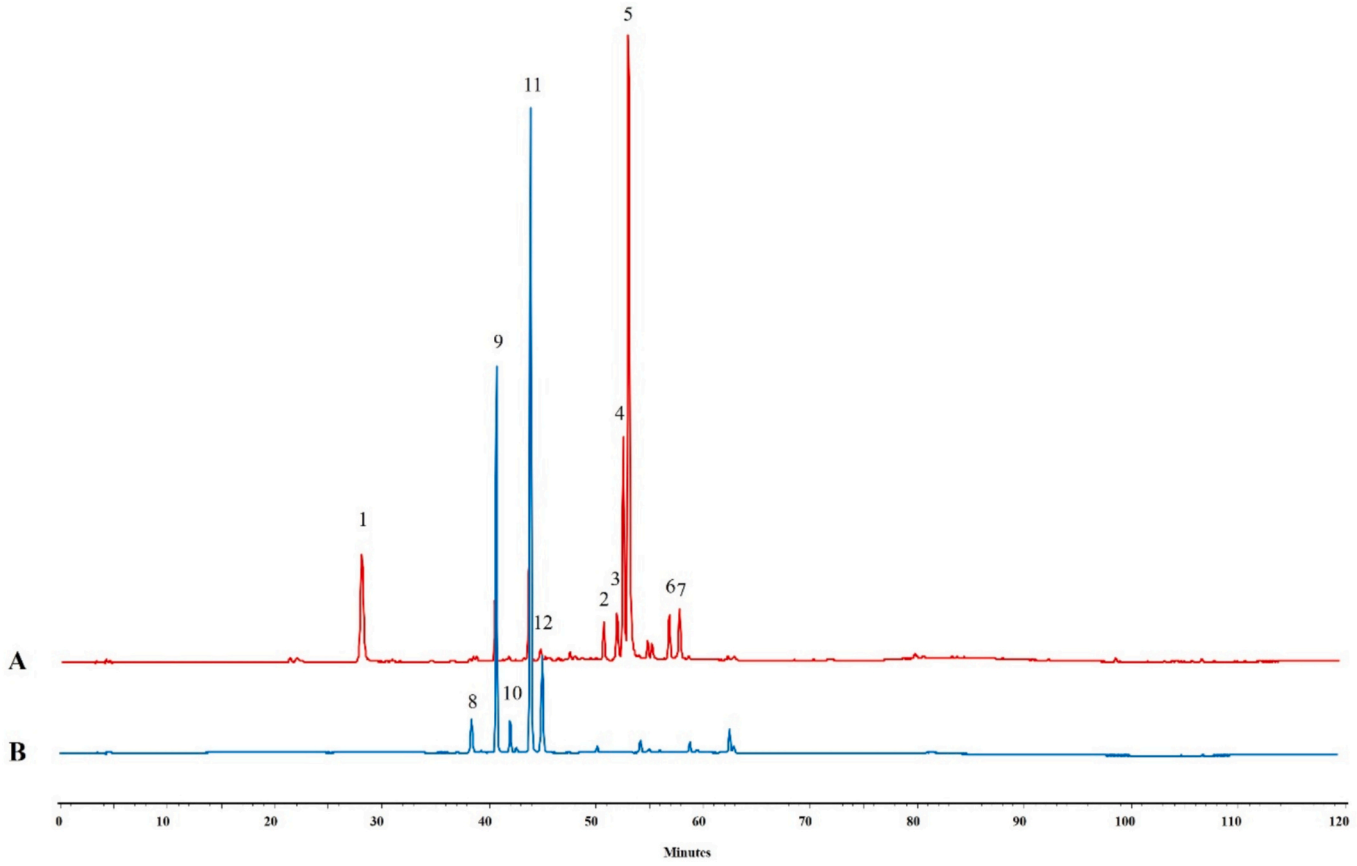

Fig. 1. HPLC-DAD chromatogram at 360 nm (A), and 520 nm (B) in cv. Monica Leaf Extract. (1) caftaric acid, (2) quercetin-3-O-rutinoside, (3) quercetin-3-O-galactoside, (4) quercetin-3-O-glucoside, (5) quercetin-3-O-glucuronide, (6) kaempferol-3-O-glucoside, (7) kaempferol-3-O-glucuronide, (8) delphinidin 3-o-glucoside, (9) cyanidin 3-o-glucoside, (10) cyanidin 3-o-arabinoside, (11) peonidin 3-o-glucoside, (12) malvidin 3-o-glucoside.

response (Kocsis et al., 2015). Similarly, Monagas et al. identified quercetin-3-O-glucuronide as the most abundant flavonol, with concentrations up to 9.92 ± 0.16 mg/g DW (Monagas et al., 2006). These flavonols, well-known for their health benefits, exert vasoprotective and tonic effects on the circulatory system, promoting microcirculation, preserving vascular wall integrity, and contributing to redox homeostasis (Ozorowski et al., 2025). In addition, two kaempferol derivatives were identified: kaempferol-3-O-glucoside (0.15 ± 0.01 mg/g DW) and kaempferol-3-O-glucuronide (0.18 ± 0.01 mg/g DW). These flavonols are known for their anti-inflammatory (Yeon et al., 2019), anticancer (Imran et al., 2019), and antioxidant activities (Kluska et al., 2022), and have been similarly detected in other cultivars by Fernandes et al. and Schoedl et al., with concentrations ranged from a minimum of 0.03 mg/g to a maximum of 6.20 ± 0.55 mg/g, supporting the consistency of their occurrence across different grapevine genotypes (Fernandes et al., 2013; Schoedl et al., 2012). At 520 nm, several anthocyanins were identified, which are flavonoid pigments that exhibit strong radical-scavenging activity. The most abundant was peonidin-3-O-glucoside (23.67 ± 2.20 mg/g DW), followed by cyanidin-3-O-glucoside (2.04 ± 0.14 mg/g DW) and malvidin-3-O-glucoside (1.39 ± 0.14 mg/g DW). Other detected anthocyanins included delphinidin-3-O-glucoside (0.38 ± 0.04 mg/g DW) and cyanidin-3-O-arabinoside (0.24 ± 0.03 mg/g DW), which may contribute synergistically to the antioxidant potential of the extract. Monagas et al. reported strong variability in anthocyanin content among grapevine leaf commercial dietary ingredients, with cyanidin-3-O-glucoside reaching up to 0.69 mg/g and peonidin-3-O-glucoside up to 0.57 mg/g.

3.2. Nano-encapsulation of the extract

After the characterization of the obtained extract, the dry powder was dissolved in a solvent mixture composed of water and ethanol (70:30, *v*/v) and used for the nano-vesicles preparation. Phytoextracts are often encapsulated in vesicles and nanocarriers to improve their bioavailability and increase their stability (Armendáriz-Barragán et al., 2016). Among the techniques for phospholipid vesicles preparation, the sonication method is one of the most used. However, it involves a high amount of energy delivered to the sample, which might cause the degradation of the thermosensitive compounds, especially with the probe tip sonicators (Uhumwangho & Okor, 2009). Here, in order to preserve the integrity of the highest number of natural compounds and to maintain their activity, the nanovesicles were prepared through a high-shear homogenization technique (Perrie et al., 2017), with the samples being constantly immersed in an ice-bath to avoid any possible heating. High-shear homogenization is also recognized for its scalability and reproducibility, which are key advantages for the potential industrial translation of functional formulations in the food sector (Patel et al., 2021). The reliability and applicability of this method are further supported by recent studies reporting similar liposomal systems prepared through high-shear mixing or rotor–stator devices (Beltrán et al., 2020; Lapčík et al., 2025; Shah et al., 2022). The composition and characterization of the prepared samples are indicated in the Table 3.

As shown by the DLS analysis, the nanovesicles were characterized by an average diameter of 256 nm and a proper size distribution. It is important to highlight that using a technique that lowers the amount of energy delivered to the samples might lead to a reduction in the sample homogeneity. Even though there is a lack of more specific standards and guidelines for the acceptability of PdI range for different applications (e.

Table 3
Composition and characterization of the prepared LNV.

Composition		
P90G (mg/mL)	Extract (mg/mL)	H_2O:EtOH (v/v)
50	2	70:30
Characterization		
D (nm)	PdI	ZP (mV)
256 ± 12	0.35 ± 0.03	−62 ± 4

g., food, cosmetic, etc.), a PdI around 0.3 is considered to be acceptable in drug delivery applications using lipid-based carriers (Danaei et al., 2018). Moreover, multiple studies report successful food-grade liposomal systems with PdI values in the 0.3–0.5 range, showing satisfactory performance and functional stability in complex matrices. For instance, Javadi et al. developed chitosan-coated nanoliposomes for the simultaneous encapsulation of caffeine and roselle anthocyanins in beverage fortification, achieving PDI values between 0.23 and 0.36, along with high encapsulation efficiencies and improved sensory acceptability of the fortified beverage (Javadi et al., 2024). Similarly, Seyedabadi et al. reported chitosan-coated nanoliposomes for caffeine delivery with PdI values ranging from 0.41 to 0.43, which maintained structural stability and demonstrated sustained release behaviour under simulated gastrointestinal conditions (Seyedabadi et al., 2021). In another study, Machado et al. formulated rice- and soybean-lecithin liposomes encapsulating Spirulina phenolic extracts, showing PDI values between 0.38 and 0.52 while maintaining high encapsulation efficiency and gastrointestinal stability, confirming their suitability for food applications (Machado et al., 2019). Collectively, these findings demonstrate that moderate PdI values are not uncommon in food-grade liposomal formulations and can still ensure good physical stability and functional efficacy when supported by appropriate encapsulation and storage conditions.

The Zeta potential, which predicts the strength of repulsion between charged particles in the dispersion, is a crucial parameter for predicting the stability of the system. Elevated values indicate highly charged particles and reduce the likelihood of electrostatic interaction-induced aggregation. Generally speaking, a sample is deemed highly stable if its zeta potential value is greater than |30| mV (Bhattacharjee, 2016). The obtained LNV were characterized by a highly negative zeta potential value around – 60 mV, which predicts a good stability of the colloidal dispersion. As a matter of fact, the monitoring of the above-mentioned parameter for 30 days (Fig. 2) showed that the nanovesicles retained the average diameter, with a slight variation of the PdI and zeta potential values at the end of the test.

To evaluate the proper encapsulation of the extract, the samples were dialysed against distilled water and then treated with 0.1 % Triton X-100, a nonionic polyoxyethylene surfactant that is commonly used to disrupt the vesicles after purification (Gibis et al., 2014; Miere (Groza et al., 2021).

Table 4 outlines the encapsulation efficiency (EE%) of various components within nanovesicles, assessed at distinct wavelengths (360 nm and 520 nm). Encapsulation efficiency is an essential parameter to evaluate, which measures the percentage of a specific component that is successfully encapsulated within the nanovesicles and thus indicates the effectiveness of the encapsulation process itself (Zahed et al., 2023).

At the wavelength of 520 nm, the encapsulation efficiency (EE%) showed considerable variability among the different compounds (28.8–73.9 %). Such variability is consistent with previous reports on nanovesicular systems loaded with polyphenols and can be mainly attributed to structural differences among the molecules (Zhao et al., 2014). For instance, delphinidin derivatives have been reported to achieve high EE% in nanoliposomes and hybrid lipo-fibrosomes, owing to strong hydrogen-bonding and electrostatic interactions between the positively charged flavylium cation and the polar head groups of

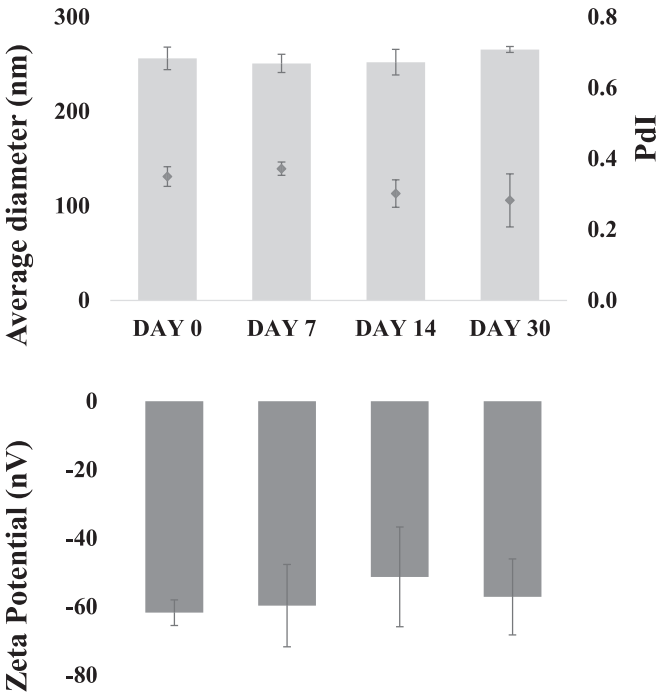

Fig. 2. Average diameter (nm). Polydispersity Index (PdI). and zeta potential (mV) of the LNV over 30 days of storage at 4 °C ($n = 3$; mean ± SD).

Table 4
Evaluation of the encapsulation efficiency of the different components of the extract loaded in the LNV.

Compounds	**EE%**
360 nm	
Caftaric acid	42.27 ± 4.75
quercetin-3-O-rutinoside	49.79 ± 4.28
quercetin-3-O-galactoside	50.01 ± 6.75
quercetin-3-O-glucoside	46.39 ± 5.95
quercetin-3-O-glucuronide	52.44 ± 3.55
kaempferol-3-O-glucoside	51.08 ± 6.68
kaempferol-3-O-glucuronide	51.20 ± 4.12
520 nm	
delphinidin 3-O-glucoside	73.90 ± 4.80
cyanidin 3-O-glucoside	36.75 ± 2.36
cyanidin 3-O-arabinoside	58.83 ± 4.48
peonidin 3-O-glucoside	39.37 ± 0.83
malvidin 3-O-glucoside	28.84 ± 2.79

phospholipids, which stabilize the compound within the vesicular membrane (Karim et al., 2023). In line with these observations, delphinidin-3-O-glucoside in our system exhibited the highest EE (73.90 %). Conversely, malvidin-3-O-glucoside showed the lowest EE (28.84 %). Malvidin is more *O*-methylated and therefore less polar than hydroxylated anthocyanins such as delphinidin. This altered distribution of methoxy and hydroxyl groups modulates both solubility and affinity toward the lipid bilayer (Castañeda-Ovando et al., 2009). Cyanidin 3-O-arabinoside (58.83 %) shows an intermediate value, followed by cyanidin 3-O-glucoside and peonidin 3-O-glucoside, 36.75 % and 39.37 %, respectively. Even subtle structural variations, such as the nature of the sugar moiety linked to the anthocyanidin, can markedly affect EE. It is well established that differences in the sugar residue alter steric hindrance, local polarity, and molecular conformation, thereby influencing the interaction with the vesicle surface and the aqueous core, with measurable consequences on the entrapment yield (Gupta et al., 2025).

At 360 nm, caftaric acid shows a moderate encapsulation efficiency of 42.27 %. Flavonols like quercetin-3-O-rutinoside, quercetin-3-O-galactoside, quercetin-3-O-glucoside, and quercetin-3-O-glucuronide

exhibit EE% values ranging from 46.39 % to 52.44 %. In particular, kaempferol-3-O-glucoside and kaempferol-3-O-glucuronide show an EE % of 51.08 % and 51.20 %, respectively, which aligns with the trend of efficient encapsulation among these phenolic derivatives. The consistently exhibited high efficiencies may indicate a pattern or similar encapsulation behaviour among these two related components. All things considered, the provided encapsulation efficiency data offers valuable insights into the nanovesicle formulation's effectiveness for the different compounds of the extract. Understanding these variations is pivotal for tailoring encapsulation strategies, developing optimized formulations for the delivery of the bioactive compounds.

3.3. *In vitro gastrointestinal stability*

To evaluate whether nanovesicles could protect the polyphenolic constituents of the grapevine leaf extract from degradation during gastrointestinal transit, the LNV formulation and the extract solution (2 mg/mL) were incubated in SGF and SIF for 2 h and 6 h, respectively, and then analysed by HPLC. The results (Fig. 3) are expressed as the percentage of each phenolic compound measured in the samples (LNV or extract incubated in SGF/SIF), relative to the same compound in the reference extract solution at the same concentration, which was set as 100 %.

After 2 h of exposure to SGF (Fig. 3A), caftaric acid, quercetin and kaempferol derivatives contained in the FE underwent substantial degradation, confirming their high susceptibility to acidic conditions. In contrast, nanovesicle encapsulation significantly attenuated the loss of caftaric acid, kaempferol-3-O-glucoside, and kaempferol-3-O-glucuronide relative to the free extract ($p < 0.05$). Anthocyanins did not undergo substantial degradation under these acidic conditions, maintaining values close to 100 %, in agreement with reported literature data (Liu et al., 2018). Incubation in SIF (pH 6.8) for 6 h produced the opposite trend observed in SGF. Flavonols, particularly caftaric acid and kaempferol-3-O-glucuronide, were only minimally degraded, whereas anthocyanins underwent significant breakdown, confirming their higher susceptibility to intestinal-like conditions. A similar pattern was

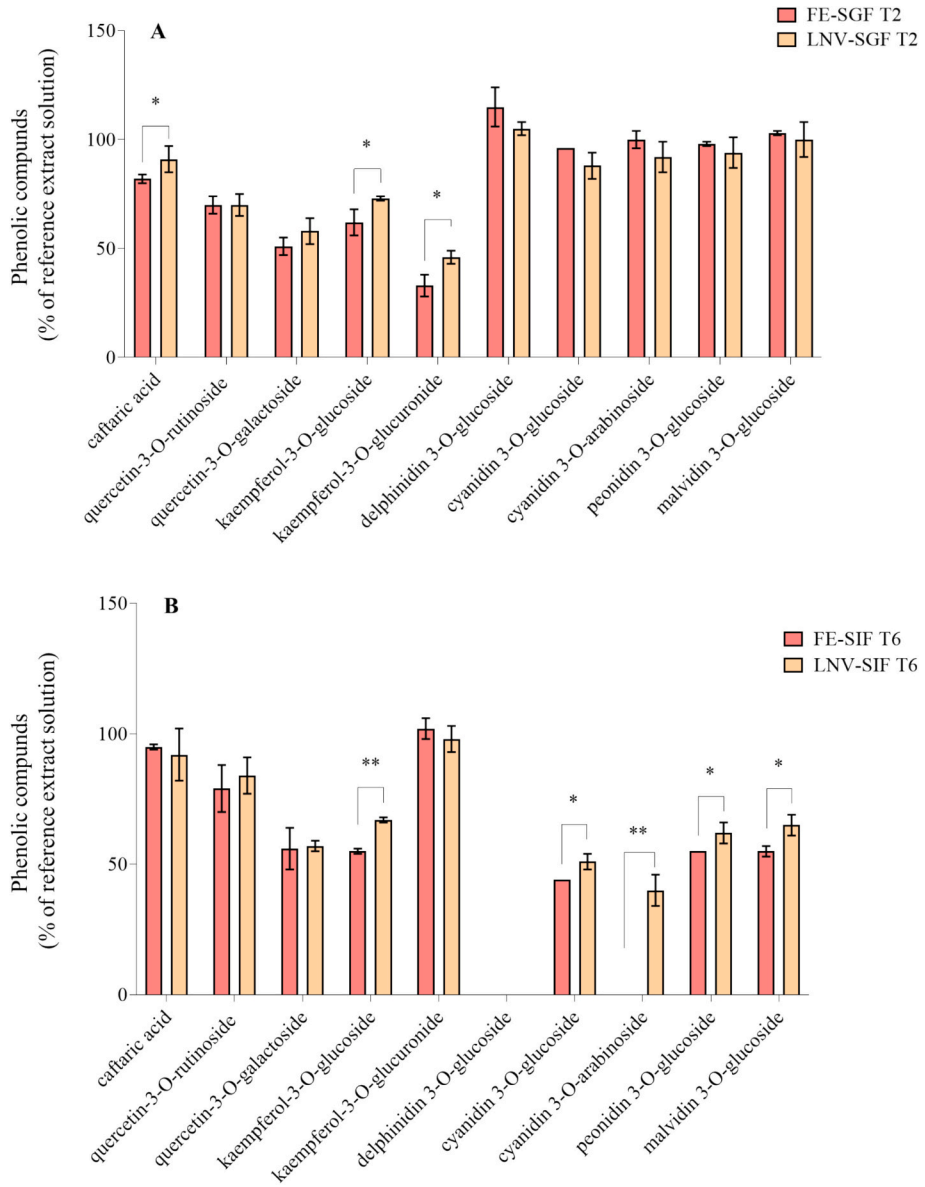

Fig. 3. Relative amount of extract components after incubation of the free extract (FE) or nanovesicles-encapsulated extract (LNV) with A) simulated gastric fluid (SGF) for 2 h or B) simulated intestinal fluid (SIF) for 6 h. Values are expressed as mean % ± SD; $n = 3$. Mean differences were compared using Student *t*-test (* $p < 0.05$; ** $p < 0.01$).

observed by Jiao et al., who evaluated the effect of gastrointestinal digestion on a blueberry extract, which contains polyphenols also present in our extract, highlighting the consistency of their behaviour under digestive conditions (Jiao et al., 2018). Nevertheless, nanovesicle encapsulation markedly improved the preservation of kaempferol-3-O-glucoside ($p < 0.001$) and all monitored anthocyanins ($p < 0.05$) compared to the free extract, with cyanidin-3-O-arabinoside being completely degraded in the free extract (0 %) but retaining approximately 40 % of its initial content when encapsulated ($p < 0.001$).

Overall, these findings demonstrate that nanovesicle encapsulation markedly enhances the gastrointestinal stability of the grapevine leaf extract, improving the persistence of its phenolic constituents under both gastric and intestinal conditions. This protective effect is expected to increase the bioavailability of the encapsulated compounds upon ingestion and further supports the suitability of the LNV formulation for the functional enrichment of kefir.

3.4. Fortified fermented milk drink

According to recent market research, the global kefir market is currently valued between 1.26 billion and 2.85 billion dollars, with expectations of continued expansion at a Compound Annual Growth Rate (CAGR) of approximately 6.40 % through 2030 (Mordor Intelligence, 2025). Its market has experienced robust growth in recent years, driven by consumer awareness of the positive impact of probiotics on gut health, immunity, and overall well-being (Azizi et al., 2021; Basnet et al., 2024). For this reason, Kefir has been chosen as the delivery vehicle for functional formulations, which, from a formulation perspective, represents an ideal matrix for incorporating bioactive compounds and innovative ingredients, thanks to its naturally probiotic-rich profile and fermentation process. In line with global trends, enriching kefir with plant-based and agro-food waste extracts can enhance its antioxidant and antimicrobial properties while preserving probiotic viability (Aiello et al., 2020). In particular, several studies have demonstrated that the incorporation of encapsulated plant extracts into fermented dairy systems does not negatively affect fermentation parameters or probiotic viability; on the contrary, encapsulation technologies can promote microbial growth and stability by improving the protection and release of bioactive compounds during fermentation (El-Sayed et al., 2022; Maleki et al., 2021; Robles-García, Del-Toro-Sánchez, Limón-Vargas, et al., 2025; Robles-García, Del-Toro-Sánchez, Tapia-Beiza, et al., 2025; Salama et al., 2020).

In our case, a fermented milk drink, produced by a local dairy company, was chosen. The obtained formulation was added to the fermented milk drink at different concentrations (5, 10 and 30 % (p/p)) to obtain fortified products (Fig. 4).

An important criterion for quality assessment of milk drinks is their rheological properties. Milk-based drinks are known to show a number of non-Newtonian properties, including yield stress, viscoelasticity, time dependence, and shear-thinning (pseudo-plastic) behaviour (Miocinovic et al., 2018; Vieira et al., 2022). The flow curves of the blank milk drink and the obtained fortified drinks are shown in the Fig. 5A.

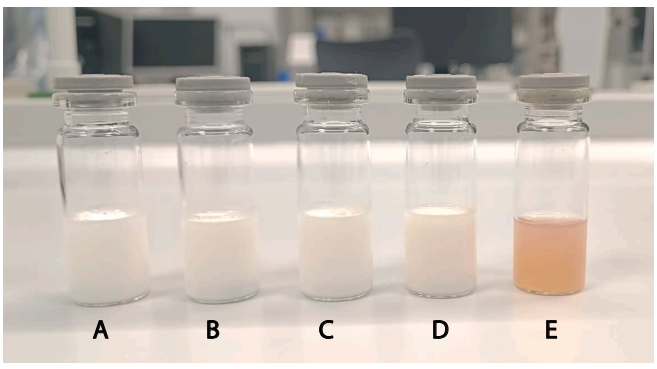

Fig. 4. Representative images of Kefir with different LNV addition – A) 0 %, B) 5 %, C) 10 %, D) 30 % *v*/v – and E) LNV.

In accordance with literature, all the tested samples show a non-Newtonian shear-thinning behaviour, becoming less viscous at increasing shear rate values (Ma et al., 2023). The highest viscosity is observed in the blank sample, whereas its value tends to decrease in a concentration-dependent manner in the fortified products. This suggests that the loaded nanovesicles might act as a thinning agent, improving the fluid flow under shear stress, and thus the pourability required for a milk-drink product (Wilbanks et al., 2022). Subsequently, an amplitude sweep test was carried out to determine the linear viscoelastic region and further analyse the samples. The mentioned region was found to be at shear strain values lower than 1, and thus an average value of 0.25 % was chosen to determine the storage modulus (G') and the loss modulus (G") (Fig. 5B-C).

The G′ value (storage or elastic modulus) is a measure of stored energy related to the stiffness of the network, representing the solid-like characteristics of the sample, whereas the G" value (loss or viscous modulus), represents a portion of the energy consumed by the sample during the deformation period, reflecting the liquid-like characteristics of the sample (Vieira et al., 2022).

Over the tested frequency range, the blank milk drink (0 % LNV) and all the fortified ones exhibit G' values that are greater than G", indicating a typical weak viscoelastic gel with an elastic structure. The addition of LNV affects the fermented milk's viscoelastic properties significantly, reducing both the rigidity and viscous behaviour. Potentially due to modifications on the fermented milk drink structure. Nevertheless, an increase of both G' and G" can be observed in all samples with the increase of frequency, confirming the physical gel behaviour as observed also by other authors(Du et al., 2021; Gonçalves et al., 2022; Zong et al., 2022). Designing milk drinks with the appropriate mechanical and textural qualities can help reach the consumer's preferences and processing efficiency.

3.5. Assessment of antioxidant activity in the fortified fermented Milk drink

Before evaluating the antioxidant activity of the enriched drink, comparative studies were conducted to assess whether the nano-vesicle formulation could influence the antioxidant activity of the grape leaf extract compared to its free form. Antioxidant activity was measured using the DPPH assay, which is widely employed to assess free radical scavenging activity. The results (Fig. 6) indicate substantial antioxidant activity in the grape leaf extract itself, exhibiting an inhibition percentage of approximately 83 %. Notably, the nano-vesicles incorporating the extract showed a similar antioxidant profile, maintaining high inhibitory activity (84 %), thereby demonstrating successful encapsulation without compromising the intrinsic antioxidant properties of the grape leaf bioactive compounds. This outcome is consistent with the use of a gentle mechanical homogenization method, carried out under continuous cooling in an ice bath, which effectively minimized the risk of thermal degradation typically associated with sonication-based techniques (Uhumwangho & Okor, 2009). In contrast, empty nano-vesicles displayed negligible antioxidant activity (2 %), confirming that the observed antioxidant potential derives mainly from the encapsulated extract. Furthermore, a significant difference (** $p < 0.01$) was found between the free extract and loaded nanovesicles, likely attributable to the slight antioxidant activity exhibited by the carrier itself. This result confirms that the encapsulation process preserves the antioxidant potential of the extract, supporting the suitability of the nano-vesicular system for the effective delivery of bioactive compounds without compromising their efficacy.

Then, the fermented milk drink (FMD) was enriched with the extract-loaded nanovescicles (FMD-LNV) at three different concentrations (5 %, 10 %, and 30 % *v*/v) to investigate their potential to enhance the

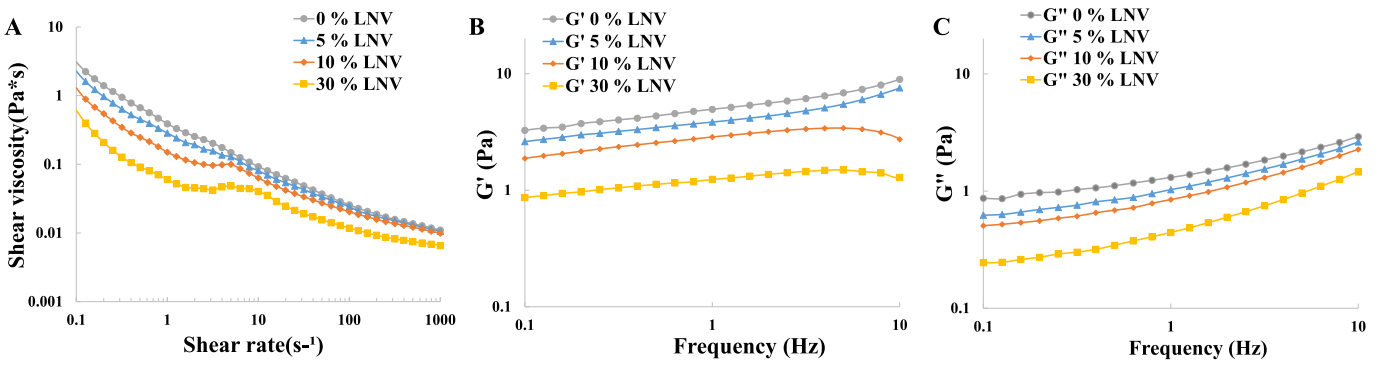

Fig. 5. Rheological analysis of the fermented milk drink with different concentrations of the Loaded Nanovesicels (LNV) (0, 5, 10, 30 % (p/p)) as (A) flow curve expressed as shear viscosity as a function of shear rate. (B) storage modulus (G') and (C) loss modulus (G") as a function of Frequency.

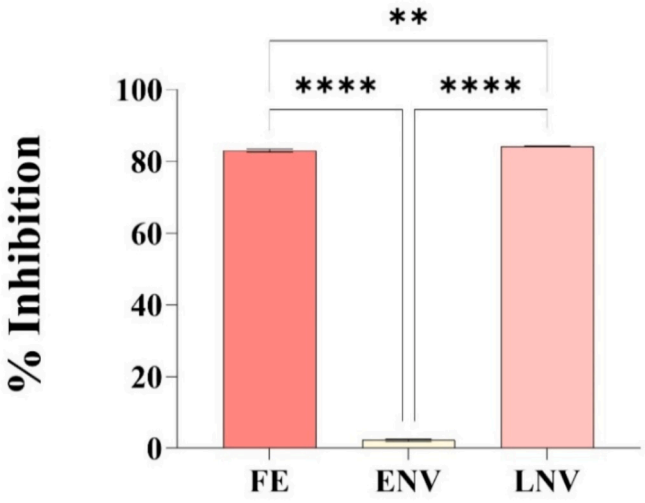

Fig. 6. Comparison of the antioxidant activity of free extract (FE), empty nanovesicles (ENV), and loaded nanovesicles with extract (LNV). Values are expressed as mean % + SD; $n = 3$. Mean differences were compared using one-way ANOVA with Tukey's multiple comparisons test (** $p < 0.01$; **** $p < 0.0001$).

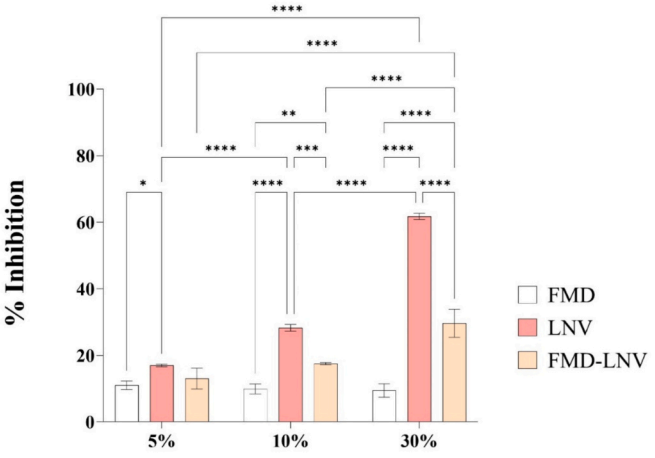

Fig. 7. Comparison of antioxidant activity of fermented milk drink (FMD), loaded nanovesicles (LNV), and fermented milk drink enriched with loaded nanovesicles (FMD-LNV). Values are expressed as mean % + SD; n = 3. Mean differences were compared using one-way ANOVA with Tukey's multiple comparisons test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

antioxidant properties of the final product (Fig. 7). ENV were not used for this test as they previously showed a low antioxidant activity. FMD was tested as a control and showed negligible antioxidant activity, confirming that the fermented milk drink matrix itself does not interfere with the assay and also supported the validity of the comparative analysis. The results show a clear increase in antioxidant capacity in FMD-LNV, with a direct correlation between the concentration of nanovesicles and the enhancement in antioxidant activity. Specifically, the 30 % concentration of FMD-LNV shows the most significant improvement compared to the controls and the other tested concentrations (5 % and 10 %), suggesting that increasing the amount of LNV allows for greater availability of bioactive compounds responsible for antioxidant activity. Despite their evident antioxidant activity, FMD-LNV still shows lower antioxidant activity compared to isolated LNV, suggesting that the effectiveness of the nanovesicles may be partially reduced by interactions with milk components during the determination of the antioxidant activity. As already proved, the biochemical environment of the dairy matrix introduces critical variables, particularly the interactions between polyphenols and milk proteins, which can influence the actual bioavailability and efficacy of the encapsulated compounds. As previously demonstrated, these interactions can occur through both non-covalent and covalent mechanisms, the latter typically involving oxidized quinone intermediates forming irreversible bonds with nucleophilic amino acid side chains (Yildirim-Elikoglu & Erdem, 2018; Zhang et al., 2024). Polyphenols derived from grapevine leaves, as seen particularly rich in flavonols and phenolic acids, have a high propensity to interact with milk proteins such as β-lactoglobulin and caseins (Mehranfar et al., 2013). Notably, β-casein, which contains a high proportion of proline residues, shows strong affinity for polyphenol hydroxyl groups (Bohin et al., 2014), leading to a masking effect that can reduce measured antioxidant activity in in vitro assays such as TEAC or DPPH. Arts et al. demonstrated that such interactions can decrease the antioxidant activity of catechins by up to 30 %, depending on both the protein and polyphenol structure (Arts et al., 2002). Similar evidence has been reported in other fortified dairy systems, where milk proteins play a crucial role in modulating the apparent antioxidant response. In coffee-fortified yogurt, Helal et al. observed that the total phenolic content represented only 38.9 % of that in coffee, attributed to the binding of hydroxycinnamic acids to caseins and whey proteins; however, after in vitro digestion, the yogurt matrix enhanced the bioaccessibility of phenolics and displayed the highest antioxidant activity, confirming a protective carrier effect of milk proteins (Helal et al., 2022). Likewise, Perna et al. found that fortification of goat milk yogurt with *Rhus coriaria* leaf powder significantly increased the total phenolic content and antioxidant capacity, although the extent of this increase depended on the αS1-casein genotype, indicating that casein–polyphenol complexation affects the fraction of free phenols contributing to measurable antioxidant capacity (Perna et al., 2018). A similar trend was previously described by the same authors in cow milk yogurt

fortified with chestnut honey, where the observed antioxidant response was influenced both by casein haplotype and by the nature of polyphenolic compounds, suggesting interactive protein–polyphenol effects on bioavailability (Perna et al., 2014). Despite these limiting interactions, the nanovesicle-enriched fermented milk drink resulted in a significant increase in antioxidant activity, with an inhibition capacity reaching 30 % compared to 9 % in the control drink milk, corresponding to an approximately threefold increase.

3.6. Determination of the antioxidant protective effect of LNV on intestinal epithelial cells in vitro

The potential of LNV as fortifying ingredient was further evaluated in a biologically relevant environment. Specifically, the antioxidant activity of LNV was determined in an in vitro intestinal epithelium model based on Caco-2 cells, employing the DCFH-DA assay to detect intracellular ROS (Eruslanov & Kusmartsev, 2010; Giacomazzo et al., 2023). In this work, the DCFH-DA assay was employed to measure the protective effect of the grape leaf extract from the oxidative damage triggered by hydrogen peroxide. As such, cells were initially treated with LNV (or ENV or FE as controls) to simulate the preventive consumption of the fortified drink and subsequently exposed to hydrogen peroxide. Cell fluorescence readings obtained 15 min after oxidative stress were normalized by cells viability in each condition and expressed as a % of the baseline ROS detected in control cells (without treatment and with no exposure to hydrogen peroxide (Fig. 8).

In good agreement with the results of the DPPH assay, both FE and LNV showed a marked cell protection against oxidative stress when employed at the highest concentration (400 μg/mL), reducing the intracellular ROS levels to baseline values, with no significant differences between the two treatments. Importantly, when lower concentrations were used (25 μg/mL), only the LNV were able to significantly reduce the intracellular ROS ($p < 0.01$), while the free extract only showed a mild, non-significant reduction compared to the untreated cells. This observation supports the hypothesis that nanoencapsulation enhances the extract's bioactive properties -specifically its antioxidant features- by protecting it from premature degradation in the cell culture medium and/or promoting the cellular internalization of its components. Lastly, and as expected due to the lack of antioxidant components, ENV did not have any impact on intracellular ROS.

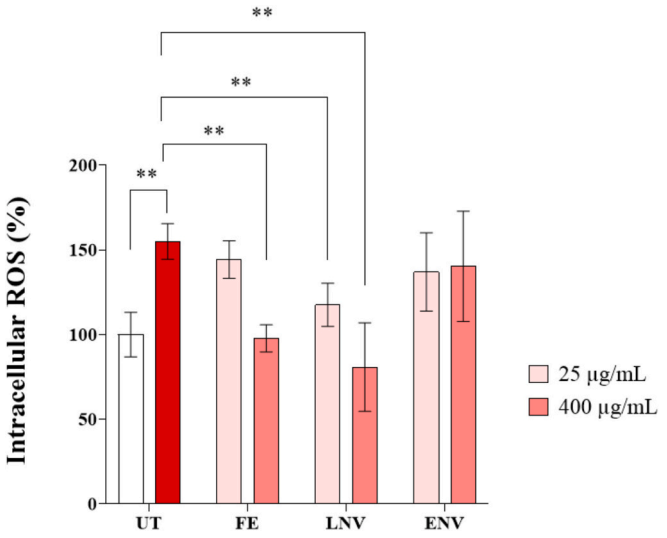

Fig. 8. Intracellular ROS (% of baseline, white bar), after pretreatment with FE or LNV at two different extract concentrations (25 and 400 μg/mL) or an equal dilution of ENV for 4 h and exposure to hydrogen peroxide for 15 min. The red bar represents ROS generated by cells exposed to hydrogen peroxide without any pretreatment. Data is reported as average ± SD ($n = 5$). Symbols indicate statistical difference with cells without pretreatment but exposed to hydrogen peroxide (red bar) (**$p < 0.01$, Student t-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

The increasing consumer demand for functional foods, along with the global focus on sustainability and circular economy, has sparked interest in the valorization of agro-industrial by-products. Among these, grapevine (*Vitis vinifera* L.) leaves, rich in polyphenols and possessing bioactive potential, offer promising opportunities as functional ingredients, particularly suited for fortifying widely consumed fermented dairy products. However, the inherent instability and limited bioavailability of polyphenols necessitate the use of appropriate delivery systems. Thus, encapsulation of grapevine leaf extract into phospholipid nanovesicles emerges as an innovative strategy to enhance stability, bioavailability, and ultimately the efficacy of polyphenols within food matrices, supporting the development of health-promoting functional products. In this study, a grapevine leaf extract (*Vitis vinifera* cv. Monica) was successfully developed and characterized, encapsulated within phospholipid-based nanovesicles, presenting an effective method to improve the stability and bioavailability of bioactive polyphenolic compounds. The extract demonstrated significant antioxidant properties, largely due to its rich phytochemical composition, which includes caftaric acid, quercetin derivatives, and anthocyanins.

Nanovesicles were produced using a gentle mechanical homogenization method with continuous cooling (ice-bath), which notably minimized the degradation of thermosensitive compounds when compared to conventional sonication techniques. These nanovesicles displayed excellent physicochemical characteristics, including appropriate size distribution, a high negative zeta potential indicating strong stability, and acceptable encapsulation efficiencies, especially for delphinidin-3-O-glucoside and quercetin derivatives. Importantly, the encapsulation of grapevine leaf extract preserved its antioxidant properties, showing similar inhibition percentages for both free and encapsulated extracts. Furthermore, incorporating these nano-formulations into a fermented goat milk drink significantly enhanced its antioxidant capacity in a concentration-dependent way, without negatively impacting its rheological and sensory qualities. Overall, the findings of this study highlight the promise of grapevine leaf nanovesicle formulations as potent functional ingredients, suggesting their application in developing innovative fermented dairy products.

CRediT authorship contribution statement

Debora Dessì: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Luca Casula:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Giacomo Fais:** Writing – review & editing, Investigation, Data curation. **Alessio Pittiu:** Data curation, Investigation. **Michele Schlich:** Writing – review & editing, Investigation, Data curation. **Chiara Sinico:** Writing – review & editing, Supervision, Methodology, Data curation. **Francesco Lai:** Writing – review & editing, Supervision, Project administration, Data curation, Conceptualization. **Giorgia Sarais:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

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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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Data availability

Data will be made available on request.

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