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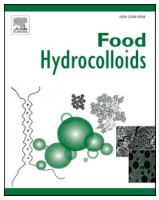

Novel rutin-casein composites as functional dry ingredients for the delivery of high concentration of rutin in dairy beverages: *in vitro* bioaccessibility, cytotoxicity, absorption, and intestinal barrier integrity

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ABSTRACT

Rutin, a flavonoid with antioxidant and anti-inflammatory properties, has poor solubility (highly hydrophobic) and is unstable during gastrointestinal digestion, limiting its use in functional foods. To overcome this challenge, we developed two rutin-caseinate composites (RCC1 and RCC2) as delivery vehicles for incorporation into functional foods/beverages. While both systems deliver rutin at high concentration, they differ in terms of methodology, loading capacity, and applications. The gastrointestinal stability, bioaccessibility, and antioxidant potential of these delivery systems, both alone and incorporated into a functional dairy beverage (flavoured milk), were assessed. We also examined the cytotoxicity, absorption, and intestinal barrier integrity of rutin using an intestinal epithelial cell model. The bioaccessibility of rutin from RCC1 and RCC2 was found to be 63 % and 45 %, respectively, compared to untreated rutin (UR), which was undetectable due to precipitation. Additionally, RCC2 exhibited superior intestinal barrier integrity with a trans-epithelial electrical resistance (TEER) value of 1655 Ω/cm^2 for 24 h , outperforming both RCC1 (1384 Ω/cm^2) and UR (915 Ω/cm^2). Intracellular antioxidant activity was significantly higher for both composites in terms of lower relative fluorescent units (RFU); 44 RFU for RCC1 and 42 RFU for RCC2, compared to 63 RFU for UR, demonstrating their enhanced protective effects. Caco-2 cell viability of the composite samples was higher, with no cytotoxicity observed compared to UR, confirming their safety. When incorporated into milk, both systems improved rutin bioaccessibility, with RCC1 showing a stronger antioxidant response (87 RFU) than RCC2 (100 RFU) and untreated rutin (140 RFU) during extended incubation. These findings suggest that both RCC1 and RCC2 are stable, soluble, and safe for physiological systems. Their incorporation into dairy matrices enhances rutin bioaccessibility and antioxidant potential, making them a promising approach for functional foods development.

1. Introduction

Chronic diseases such as type-2 diabetes, cardiovascular diseases, cancer, and neurodegenerative disorders are significant global health concerns, often driven by poor diet and lifestyle. Diets high in fats, sugars, and salts contribute to these conditions through mechanisms like oxidative stress, inflammation, and mitochondrial dysfunction (Frutos et al., 2019; Wu & Schauss, 2012). Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and the body's antioxidant defense system, plays a crucial role in disease progression by damaging cells and tissues (Sies et al., 2017). This has led to increased interest in functional foods containing various bioactive compounds, which can help modulate oxidative stress and inflammation, offering potential health benefits beyond basic nutrition (Obayomi et al., 2024).

Flavonoids, a class of polyphenolic compounds found in fruits and vegetables, have garnered attention for their potential to induce health-promoting biological actions, including antioxidant, anti-inflammatory, and anticancer effects (Lu et al., 2013; Zheng et al., 2025). Among these, rutin, a flavonoid glycoside stands out not only for its potent antioxidant and anti-inflammatory properties, but also for its widespread availability from cost-effective natural sources such as buckwheat, citrus peel, and Sophora japonica flowers (Ganeshpurkar & Saluja, 2017; Ghorbani, 2017). Rutin is also favoured for its relatively higher solubility in water compared to other flavonoids such as quercetin, which can

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enhance its potential for use in food systems (Gullon et al., 2017). Furthermore, rutin has demonstrated multi-functional effects, including protection of epithelial barrier integrity, vascular health (Carrasco-Pozo et al., 2013), and potent antioxidant effects by scavenging free radicals enhancing the body's antioxidant defence systems (Yang et al., 2008). These properties make rutin an attractive candidate for managing chronic conditions exacerbated by oxidative stress, including diabetes and cardiovascular diseases (Ghorbani, 2017; Lin et al., 2018; Wang et al., 2012) in functional food contexts. However, its low bioavailability, resulting from its hydrophobic nature and limited gastrointestinal absorption, restricts its therapeutic efficacy (Ben Sghaier et al., 2016) and its delivery via functional foods, especially when consumed orally at the recommended daily doses (above 500 mg) (Boyle et al., 2000; Forouzanfar et al., 2025).

To address these limitations, in previous works (Rashidinejad, 2020; Rashidinejad et al., 2019) we developed a rutin-casein composite (RCC), namely RCC1 in this paper and marketed and patented as FlavoPlus 1 (WO EP US CN JP AU EP3876754A1). In this work, we also investigated another composite (namely RCC2 in this paper and patented as FlavoPlus 2; WO EP CN WO 2022162565 A1). RCC2 differs in the amount of rutin delivered in the composites, physicochemical properties (e.g., higher dispersibility and solubility), and the delivery method/-technology (more suitable for beverage fortifications). Compared to the previous delivery systems for rutin, both composites exhibit high entrapment and loading capacity, alongside improved dispersibility in aqueous media. The enhanced dispersibility increases the stability of rutin and improves its compatibility with food matrices, facilitating its potential use in functional foods (Rashidinejad et al., 2019). However, the bioaccessibility, stability, and bioactivity of either of these composites still require further investigation to fully understand their practical applications in functional foods.

Accordingly, building on our previous works (Rashidinejad, 2020; Rashidinejad et al., 2022a, b; Rashidinejad et al., 2019; Thompson et al., 2020), this study advances the field by moving beyond formulation development to evaluate the biological performance and functional relevance of rutin-casein composites (RCC1 and RCC2). Specifically, we assessed their cytocompatibility, antioxidant efficacy, and *in vitro* bioaccessibility in a dairy matrix using a Caco-2 human intestinal epithelial cell model and a simulated gastrointestinal digestion system—approaches not covered in our earlier patented work.

While previous studies, including our own, have addressed the formulation and encapsulation of hydrophobic flavonoids like rutin, the current research introduces several key innovations. First, we integrate high-loading-capacity delivery systems into a realistic food matrix (milk) and evaluate their performance under physiologically relevant conditions. Second, we employ cell-based assays to assess intracellular antioxidant responses, providing a more accurate representation of bioactivity than chemical assays alone. Third, we address the gastrointestinal bioaccessibility of rutin, a critical factor influencing its therapeutic potential, which has not been previously explored in our patented systems. This work bridges the gap between formulation science and functional validation, enhancing the translational potential of these delivery systems for real-world food and nutraceutical applications. Furthermore, our use of food-grade ingredients and scalable processing techniques (e.g., spray drying) ensures industrial relevance and feasibility for large-scale production. By improving both the bioaccessibility and bioactivity of rutin, this study offers a novel and practical approach to advancing the use of hydrophobic flavonoids in functional foods.

2. Materials and methods

The products and methods discussed in this paper are covered by published patent applications, including WO EP US CN JP AU EP3876754A1 and WO EP CN WO2022162565A1. For the detailed information related to the properties of the delivery systems used in this experiment please refer to these patents, as well as our previous work published in this journal (Rashidinejad et al., 2019).

2.1. Chemicals, reagents, and cell lines

Rutin and quercetin standards were procured from Sigma-Aldrich. Sodium caseinate was from Fonterra Co-operative Ltd. (Auckland, New Zealand). Rutin hydrate (≥94 %; #207671-50-9) was procured from Thermo Fisher Scientific (Waltham, MA, USA). Ultrapure water (Millipore Corp., SAS, #67120, Bedford, MA, USA) was also procured. DCFH-DA (#4091-99-0, Sigma-Aldrich®, St. Louis, MO, USA), DMSO (#D4540, Sigma-Aldrich®, St. Louis, MO, USA), and 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) (#M6494, Invitrogen by Thermo Fisher Scientific™, Waltham, MA, USA) were procured as well. All chemicals and enzymes, including pepsin, pancreatin, and bile salts (Sigma-Aldrich) were of analytical grade. Pepstatin A (#ab141416, Abcam, UK) and a protease inhibitor cocktail (#S8820, Sigma-Aldrich®, St. Louis, MO, USA) were also procured. AAPH (#2997-92-4, Sigma-Aldrich, St. Louis, MO, USA), HBSS buffer (#14175095, Thermo Fisher Scientific™, Waltham, MA, USA), DPPH reagent (Sigma-Aldrich Co., Inc., Darmstadt, Germany), and Folin-Ciocâlteu (FC) reagent (Merck Co., Inc., New Jersey, USA) were also procured. Commercial banana-flavoured milk was purchased from a local supermarket (New World) in Palmerston North, New Zealand.

Caco-2 cells were procured from ATCC (American Tissue Culture Collection, #HTB-37). For the cell culture experiments, complete MEM was prepared by supplementing Gibco™ MEM (L-Glutamine; #1095080, Thermo Fisher Scientific™, Waltham, MA, USA) with 10 % Fetal Bovine Serum (FBS, Australian sourced, ɣ-radiated, Thermo Fisher Scientific™, Waltham, MA, USA), 1 % non-essential amino acids (MEM non-essential amino acids 100x solution; #11140-050), and 1 % penicillin-streptomycin (Pen-Strep, 10,000 units/mL), which were procured from Gibco, Invitrogen, MA, USA. Cells were sub-cultured every third day using trypsin (TrypLE™, Gibco, Invitrogen Corporation, Carlsbad, CA, USA) and Gibco PBS (Phosphate-buffered saline), pH 7.4 (#10010023).

2.2. Preparation and resuspension of bioactive compounds

2.2.1. Rutin stock solution

A stock solution of rutin hydrate was prepared by dissolving 1 mg of the powder in 0.3 % DMSO solution before further dilution in the treatment medium. The solution was syringe-filtered (0.22 μm) and stored at −20 °C until used.

2.2.2. Rutin-casein composites stock solutions

The composition of food-grade rutin caseinate composite 1 (RCC1) powder included: sodium caseinate (NaCas; 46 %), rutin (46 %), and dipotassium phosphate (8 %). This powder was developed and manufactured in our laboratory by Rashidinejad et al. (2019). The manufacturing procedure for RCC1 (FlavoPlus 1™) and RCC2 (FlavoPlus 2™) was described in a patent by Thompson et al. (2020). A stock solution was prepared by dissolving 2 mg of the powder in 1 mL Ultrapure water (Millipore Corp) before further dilution in the treatment medium. The solution was then syringe-filtered (0.22 μm) and stored at −20 °C until use. RCC2 was composed of NaCas (54.5 %), rutin (27.3 %), and dipotassium phosphate (18.2 %). This powder was previously developed and manufactured in our laboratory (Rashidinejad at al., 2022b). A stock solution was prepared by dissolving 2 mg of the powder in 1 mL Milli-Q (Millipore Corp) water before further dilution in the treatment medium. The solution was syringe-filtered (0.22 μm) and stored at −20 °C until use.

2.3. UV-spectrophotometric DPPH assay

The DPPH (2,2-diphenyl-1-picryl-hydrazyl) free radical scavenging method (Vogrincic et al., 2010) was employed to assess the antioxidant

capacity of RCC1, RCC2, and untreated rutin (UR) using a UV-spectrophotometric assay. A 100 μL sample of each was mixed with a 1:10 dilution of DPPH stock solution in a cuvette and incubated in the dark at 25 °C for 30 min. Absorbance was measured at 517 nm using a spectrophotometer (Multiskan™, ThermoFisher Scientific™). The control contained DPPH solution alone, and the blank contained water and DPPH solution. The reduction of DPPH radicals, indicated by a colour change from purple to yellow, was quantified as scavenging activity or inhibition (%) using the following equation:

$$\text{Scavenging activity (\%)} = \frac{(Absorbance\ of\ control – Absorbance\ of\ sample)}{Absorbance\ of\ control} \times 100 \quad \text{Eq. 1}$$

where, the absorbance of the control is at t = 0 min and the absorbance of the sample is at t = 30 min. All measurements were carried out at room temperature.

2.4. Total phenolic content estimation

The total phenolic content (TPC) of RCC1, RCC2, and UR was evaluated using the method of Gangwar et al. (2014) with Folin-Ciocâlteu (FC) reagent. A 0.1 mL sample aliquot was mixed with 2.5 mL of FC reagent and incubated for 10 min. Then, 2 mL of sodium carbonate (75 g/L) was added, vortexed, and incubated in the dark at room temperature for 2 h. Absorbance at 765 nm was measured using a spectrophotometer (Multiskan™, ThermoFisher Scientific™). Results were expressed as gallic acid equivalents (μg GAE) using a gallic acid standard curve (50–300 μg/mL) (Fig. S1).

2.5. Maintenance and culture of intestinal epithelial model Caco-2 cells

Caco-2 cells were cultured in complete MEM at 37 °C with 5 % CO_2 in a humidified incubator (Heracell™ VIOS 160i, Thermo Fisher Scientific™). Cell growth was monitored daily under an inverted microscope (Nikon Eclipse TS100, Japan) until 70 % confluency. Cells were subcultured every three days by washing with PBS (pH 7.4) and detaching with trypsin (Invitrogen, USA) for 4 min. After centrifugation at 110 RCF for 4 min (MegaFuge™, Thermo Fisher Scientific), cells were resuspended in MEM and seeded into 96-well plates for assays (Natoli et al., 2012).

2.5.1. Differentiation of Caco-2 cells

The cells were differentiated for absorption experiments and barrier integrity assessment. Cell viability was checked using trypan blue (#T10282, Thermo Fisher Scientific™) with a 1:1 cell-to-dye ratio. A 12 μL sample was loaded onto a Countess™ chamber slide and analysed using a Countess™ automated cell counter. Live cells appeared clear, while dead cells stained blue. Cells were seeded at 8 × 10^4 cells/Transwell (6.5 mm, polyester, 0.33 μm pores; Corning® #CLS3470) with 200 μL MEM in the apical and 810 μL in the basolateral compartment. Plates were incubated at 37 °C, 5 % CO_2, and the medium was refreshed every two days. Differentiation occurred after 15–17 days. TEER values were measured using the EndOhm TEER cup and EVOM2 voltohmmeter (World Precision Instruments) to confirm barrier integrity before experiments.

2.5.2. Cytotoxicity assessment via MTT assay

Caco-2 cells were treated with RCC1, RCC2, UR, and DMSO at four different concentrations (0.1, 0.5, 15 and 50 μg/mL) and incubated for 24 h. For RCC1 and RCC2 treatments, the concentrations were calculated based on the rutin content previously quantified in each composite formulation, ensuring equivalent rutin exposure across treatments. These doses were selected based on previous literature reporting non-cytotoxic and physiologically relevant concentrations for rutin in Caco-2 cells (Wan et al., 2015).

After treatment, cells were washed with phenol-red-free MEM and incubated with 0.5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye for 3–4 h. The formazan crystals formed were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader. Cell viability was calculated using the following equation, with the negative control being DMSO-treated cells, and the positive control being untreated cells as reported previously by Kuntz et al. (1999).

$$\text{\% Cell viability} = \frac{(Absorbance\ of\ sample\ –\ Absorbance\ of\ blank)}{(Absorbance\ of\ control\ –\ Absorbance\ of\ blank)} \times 100 \quad \text{Eq. 2}$$

2.6. Intracellular antioxidant activity (DCFH-DA assay)

Intracellular antioxidant activity was measured using the 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) assay, as described by Wan et al. (2015). Caco-2 cells were seeded at 6 × 10^5 cells/mL in 96-well plates and treated with three concentrations of 0.5, 25, and 50 μg/mL for 24 h. These concentrations were selected based on previous literature demonstrating rutin's antioxidant effects in Caco-2 and other epithelial models without inducing cytotoxicity, while capturing a range from low physiological medium and higher dose from pharmacological relevance (Kuntz et al., 1999; Wan et al., 2015; Zhang et al., 2013).

After discarding the medium and washing with PBS, the cells were treated with 100 μL of sample solutions along with 25 μM DCFH-DA dye. The cells were incubated for 1 h at 37 °C, allowing the dye to enter the cells and be hydrolyzed to DCFH. The cells were then washed three times, and 100 μL of 600 μM ROS inducer, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was added to induce ROS production. Fluorescence intensity, indicating ROS levels, was measured every at an excitation of 480 nm and emission at 530 nm using a fluorescence microplate reader. The control group had no sample, while the blank contained no AAPH or samples, only PBS. The fluorescence intensity was calculated using the following equation.

Fluorescence intensity % = $\frac{F_{Sample}}{F_{Control}} \times 100$ Eq. 3 where, F_{Sample} is the fluorescence of the sample and $F_{Control}$ is the fluorescence of the control.

2.7. Transepithelial electrical resistance assay (TEER)

Caco-2 cells were seeded at 8 × 10^4 cells/Transwell (6.5 mm, polyester, 0.33 μm pore size; Corning Inc.) in MEM medium, with 200 μL in the apical and 810 μL in the basolateral compartments. The plates were incubated at 37 °C, 5 % CO2, with medium changes every 2 days. Differentiation and monolayer formation were typically observed after 15–17 days. Transepithelial electrical resistance (TEER) was measured using an EndOhm chamber coupled with an EVOM2 Epithelial Voltohmmeter. Only cell monolayers that reached TEER values ≥ 500 Ω/cm^2 were considered suitable for downstream experiments, as this threshold is widely accepted to indicate intact tight junctions and adequate monolayer integrity (Antunes et al., 2013; Hubatsch et al.,

2007). Following confluency confirmation, TEER values were recorded every 2 h for 12 h and the final reading at 24 h. Resistance values were calculated using the following equation.

$$\text{TEER}\ (\Omega / \text{cm}^2) = \textit{Raw TEER value}\ (\Omega) \times \textit{surface area of insert}\ (cm^2) \quad \text{Eq. 4}$$

2.8. Preparation and characterisation of the flavoured milk fortified with rutin-casein composites

To prepare the fortified beverage, 500 mg of UR, or an equivalent amount of rutin delivered via RCC1 or RCC2, was added per 250 mL serving of commercial banana-flavoured milk, resulting in a final concentration of 7.32 g/L for RCC2. The amount of RCC incorporated was calculated based on the widely accepted daily intake of rutin (approximately 500 mg/day for adults), ensuring that each serving delivers a physiologically relevant dose. This dosage is consistent with previous clinical studies demonstrating the safety and tolerability of rutin supplementation at levels up to 500 mg/day (Boyle et al., 2000; Forouzanfar et al., 2025). Since RCC2 contains 27.3 % rutin by weight, 7.32 g of RCC2 per litre of milk is needed to achieve this target rutin concentration. This ensures that all fortified milk samples (UR, RCC1, and RCC2) deliver an equivalent rutin dose, despite differences in rutin loading between the formulations. Furthermore, the cytocompatibility of the RCC formulations was confirmed in this study using Caco-2 cell assays, supporting the safety of the selected fortification level. These considerations collectively support the suitability of RCC-fortified milk as a functional food product for routine consumption.

The powders were stirred at 1200x RPM into the milk for 15 min and the mixture was heated at 70 °C for 30 min and cooled before storage. Fortified milk was then bottled and stored at 4 °C until further use. The fortified milk was then subjected to *in vitro* digestion to assess rutin's release and its structural changes during digestion. Rutin release was quantified using high-performance liquid chromatography (HPLC) at 0-, 30-, 60-, and 120-min post-digestion. The rutin released during digestion was subsequently tested on a Caco-2 cell monolayer to evaluate cytotoxicity, barrier integrity, and antioxidant activity.

2.9. In vitro digestion of fortified milk

The *in vitro* digestion of banana-flavoured milk fortified with RCC1, RCC2, and UR was performed according to Minekus et al. (2014) with modifications. Freshly prepared fortified milk was used for the digestion study (in triplicates). Milk fortified with RCC1, RCC2, and UR was compared to unfortified milk, with Milli-Q water as the blank. Three aliquots were taken at each time point for further analysis, and two tubes were used per replicate. The digestion was carried out in a shaking water bath maintained at 37 ± 1 °C. A 10 mL volume of each sample was mixed with simulated gastric fluid (SGF) (5 μL $CaCl_2$, 8 mL SGF, 0.5 mL pepsin) at pH 3 ± 0.1 for the gastric phase. Samples were collected at 0, 30, 60, and 120 min. For the intestinal phase, samples were obtained from the remaining tubes in the water bath, which had a final volume of 20 mL from the gastric phase. The digesta was mixed with simulated intestinal fluid (SIF) to a final ratio of 1:1 containing 40 μL $CaCl_2$, 2.5 mL bile salts (at 10 mM), and 5 mL pancreatin (at a final 100 U/mL trypsin activity), and the pH of the tubes was adjusted to 7 ± 0.1 with regular stirring. During the intestinal phase, samples were collected at 0, 30, 60, and 120 min. To stop the enzymatic reactions, enzyme inhibitors were immediately added to the samples. 10 μL of Pepstatin A (#ab141416, Abcam, UK) in methanol (0.5 mg/mL) was added to every 1 mL of gastric digesta sample taken and 0.45 mL of the protease inhibitor cocktail (1 tablet in 50 mL Milli-Q water; #S8820, Sigma-Aldrich®, St. Louis, MO, USA) solution was added to every 1 mL of the intestinal digesta sample. All digesta samples were stored at −20 °C for further experimental analysis. For the complete information about salt conc in different phases please refer to Table S1.

The percentage bioaccessibility of rutin was calculated as the proportion of the soluble compound after digestion (accessible for absorption) relative to its initial concentration in the fortified milk samples, using the following formula:

$$\textit{Bioaccessibility}\ (\%) = (\textit{Amount of soluble rutin after digestion} / \textit{Initial amount of rutin before digestion}) \times 100 \quad \text{Eq. 5}$$

This calculation reflects the fraction of rutin potentially available for absorption following gastrointestinal digestion.

2.10. Quantification of rutin using high-performance liquid chromatography (HPLC)

Rutin and quercetin standards were analysed using an Agilent 1200 HPLC system with a UV/visible diode array detector and a Kinetex XB-C18 column. Rutin concentrations ranged from 105 to 421 ppm, and quercetin from 10 to 40 ppm. The mobile phases were acetic acid 0.5 % (A) and acetonitrile (B), with a flow rate of 1 mL/min and a sample injection volume of 5 μL. The column temperature was maintained at 26 °C, and the UV detector was set at 356 nm. Rutin was identified based on retention time and spectra compared to a calibration curve with standard concentration ranging from 105 ppm to 421 ppm.

2.11. Particle size and zeta potential measurements

Particle size was measured post-digestion using a Malvern Mastersizer Hydro 2000MU, with a refractive index of 1.460 for milk and 1.33 for water. A small amount (2–3 mL) of digested sample was added to 800 mL of water to maintain the obscuration level at 9.5 %.

Zeta potential was determined using a Malvern Zetasizer Nano ZS at 25 °C. Samples were diluted with deionized water to avoid multiple scattering before measurement.

2.12. Statistical analysis

Data were analysed using analysis of variance (ANOVA), followed by Tukey's multiple comparison post-hoc test to determine statistical significance between groups. All analyses were performed using GraphPad Prism 10 software, with significant differences determined at $p < 0.05$.

3. Results and discussion

3.1. Antioxidant activity of rutin-protein composites

The antioxidant capacity of RCC1 and RCC2 was assessed using both the DPPH free radical scavenging assay and TPC analysis. The DPPH assay is a standard method that evaluates antioxidant potential based on the ability of compounds to donate hydrogen atoms and neutralize free radicals (Pyrzynska & Pękal, 2013). TPC complements this assay by quantifying the total phenolic compounds, which are closely linked to antioxidant activity (Rashidinejad et al., 2014).

As shown inFig. 1A, both RCC1 and RCC2 significantly enhanced DPPH radical scavenging compared to unbound rutin (UR) ($p < 0.05$),

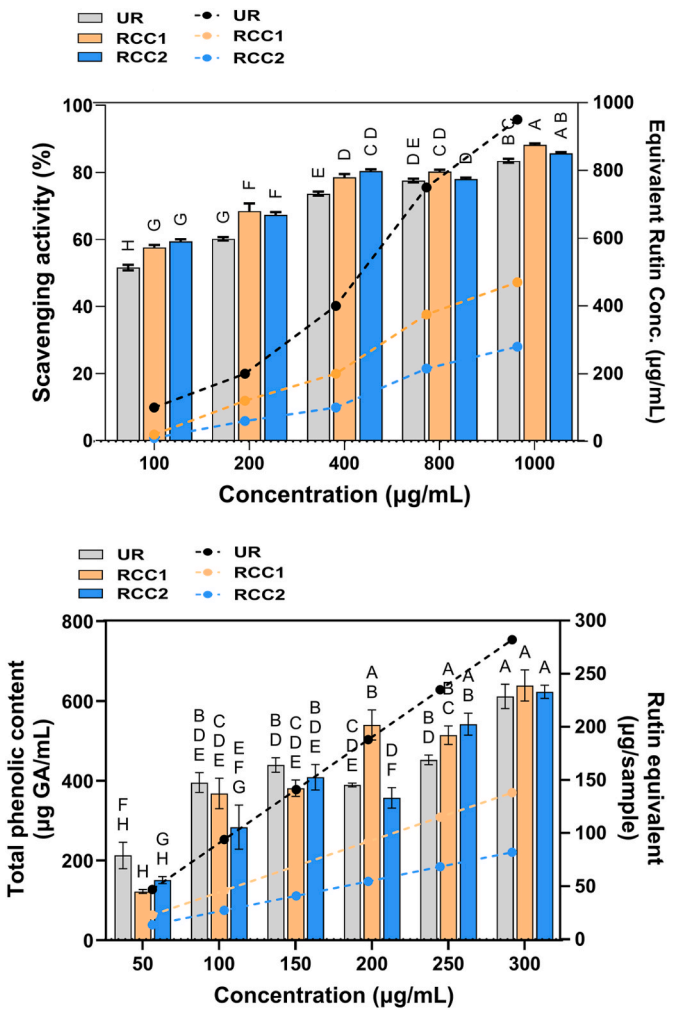

Fig. 1. Percentage DPPH radical scavenging activity (A) and total phenolic content (expressed as µg gallic acid equivalents [GA]/mL; B) for untreated rutin (UR) and rutin–caseinate composites (RCC1 and RCC2). In both panels, the right Y-axis represents the equivalent rutin concentration (µg/mL) for each sample. Data are presented as mean ± SEM (n = 3). Different letters denote statistically significant differences between groups ($p \leq 0.05$; two-way ANOVA followed by Tukey's multiple comparison test).

demonstrating the beneficial impact of casein complexation. RCC2 consistently exhibited slightly higher scavenging activity than RCC1, although the difference was not statistically significant at all concentrations. This minor difference is likely due to comparable casein interaction dynamics despite RCC1's higher rutin loading. These findings are consistent with studies showing that encapsulation/entrapment can increase rutin's solubility and stability in aqueous environments, enhancing its antioxidant performance (Celik et al., 2015; Jain et al., 2012).

UR displayed substantially lower antioxidant activity, which may be attributed to its high crystallinity, poor aqueous solubility, and limited availability of hydrogen donor atoms for free radical neutralization. Both composites demonstrated a dose-dependent increase in antioxidant activity, underlining the role of protein–polyphenol interactions particularly hydrophobic and electrostatic forces in stabilising and enhancing bioactive properties (Nguyen et al., 2013).

TPC results (Fig. 1B) mirrored the DPPH findings, with RCC1 and RCC2 showing higher phenolic content than UR, despite UR containing 94 % pure rutin, compared to lower rutin content in RCC1 (46 %) and RCC2 (27.3 %). This disparity is likely due to the poor solubility and stability of UR under assay conditions, limiting phenolic release. The improved TPC in RCC formulations supports the idea that protein conjugation enhances phenolic bioaccessibility, as also observed by Horincar et al. (2019).

A strong positive correlation ($R^2 = 0.98$) was observed between TPC and DPPH values (Figs. S2A and S1B), confirming the direct relationship between phenolic content and antioxidant activity. This is consistent with previous research on rutin–soy protein and flavonoid–protein complexes (Wei et al., 2015; Ye et al., 2021), reinforcing the advantage of protein conjugation in boosting the functional properties of bioactives.

In summary, both RCC1 and RCC2 outperformed UR in antioxidant assays, with RCC2 offering marginally better performance. The enhanced functional properties are attributed to improved solubility and stability conferred by protein encapsulation, supporting their application in functional food matrices.

3.2. *Effect of rutin-protein composites on cytotoxicity and cell viability*

Cytotoxic effects of unmodified rutin (UR) and the rutin-casein composites (RCC1 and RCC2) were evaluated on Caco-2 cells using the MTT assay at concentrations of 0.1, 0.5, 15, and 25 µg/mL. To ensure accuracy in cytotoxicity assessment, the concentration of dimethyl sulfoxide (DMSO) used for dissolving UR was optimised in preliminary experiments, since DMSO is known to affect cell viability (Da Violante et al., 2002). Caco-2 cells were exposed to DMSO concentrations ranging from 0 % to 5 %, and a marked decrease in viability was observed above 2 % (Fig. S3). As a result, all subsequent assays used DMSO at or below 2 %, ensuring that vehicle effects did not confound the cytotoxicity outcomes. Additionally, to confirm the biocompatibility of the delivery matrix, sodium caseinate alone (at concentrations equivalent to those used in RCC formulations) was also tested on Caco-2 cells viability. No significant differences in cell viability were observed compared to control media, indicating that sodium caseinate did not exhibit any cytotoxic effects under the experimental conditions (Fig. S4).

As shown in Fig. 2, both RCC1 and RCC2 significantly ($p < 0.05$)

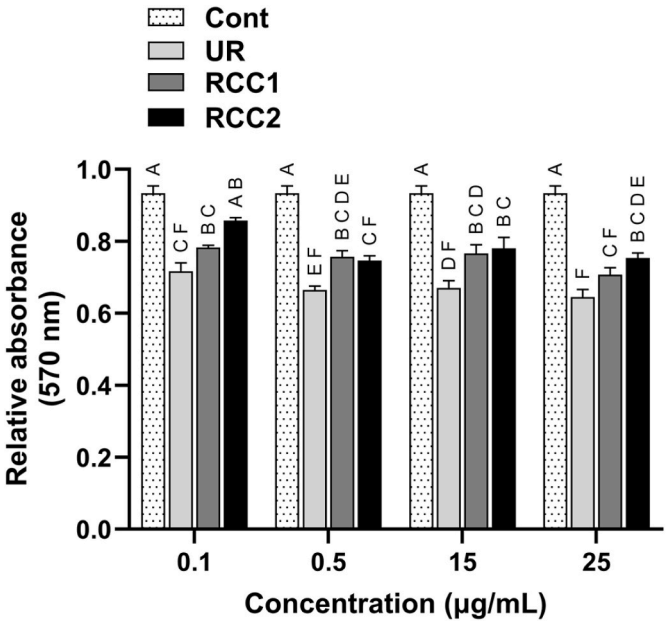

Fig. 2. Effect of untreated rutin (UR), rutin–caseinate composite 1 (RCC1), and rutin–caseinate composite 2 (RCC2) on Caco-2 cell viability at four concentrations: 0.1, 0.5, 15, and 50 µg/mL. Note: Data represent the mean of four biological replicates, each with three technical replicates. Error bars indicate the standard error of the mean. Columns not sharing the same letters differ significantly (pp ≤ 0.05one-way ANOVA followed by Tukey's multiple comparison test).

improved cell viability across all tested concentrations when compared to UR. These results demonstrate that protein conjugation effectively reduces the cytotoxicity associated with free rutin. Among the two formulations, RCC2 consistently showed the highest viability, especially at the elevated concentration of 25 μg/mL, suggesting superior biocompatibility and reduced cellular stress. While RCC1 also offered protection compared to UR, RCC2 provided notably better outcomes, which may be attributed to differences in protein-polyphenol interactions and the resulting structural stability of the matrix.

The enhanced cell viability observed for RCCs can be linked to their ability to mediate the controlled release of rutin. Entrapment of rutin within the sodium caseinate matrix helps regulate its release profile, reducing the likelihood of high local concentrations that might otherwise induce oxidative damage (Sak, 2014). Controlled delivery systems are essential for enhancing the bioavailability and physiological effectiveness of bioactive compounds like rutin. Sustained release maintains cellular integrity, improves uptake, and minimises oxidative stress. Casein-based encapsulation, in particular, has been shown to prolong antioxidant activity and stabilise phenolics under physiological conditions (Bazana et al., 2019; Rezagholizade-shirvan et al., 2024). RCC2's superior performance may stem from a more consistent interaction between rutin and the protein matrix, enabling slower release and greater cellular protection.

At the highest concentration tested (25 μg/mL), both RCCs maintained high cell viability without impairing proliferation, further confirming their low cytotoxicity. Although this study did not directly assess molecular mechanisms, existing literature suggests that rutin can activate endogenous antioxidant defense pathways, such as the Nrf2 signaling cascade, and upregulate enzymes like superoxide dismutase (SOD) and catalase (Anjum et al., 2022; Ganeshpurkar & Saluja, 2017; Pisoschi & Pop, 2015). These pathways are essential for mitigating reactive oxygen species (ROS) and maintaining redox homeostasis. In conclusion, the results demonstrate that RCC2 exhibits the most favourable cytotoxicity profile, followed by RCC1 and then UR. The reduced toxicity and enhanced cell viability observed with RCCs are attributed to the protective role of the protein matrix and its ability to modulate rutin release. It is important to note that although sodium caseinate is widely used in food applications and is generally recognized as safe (GRAS), it is also classified as a major food allergen. The formation of polyphenol–protein complexes, such as those between rutin and casein, may induce conformational changes in the protein structure, potentially modifying its immunogenic properties (Zhang et al., 2021, 2024). These structural alterations could either mask or expose allergenic epitopes, thereby influencing the sensitization potential of the resulting complexes. Therefore, future studies should incorporate comprehensive allergenicity assessments—such as IgE-binding assays, basophil activation tests, or in vivo models—to evaluate the immunological safety of rutin–casein composites, particularly for individuals with known milk protein allergies.

3.3. *In vitro intracellular antioxidant activity of rutin-protein composites*

Antioxidants play a crucial role in protecting cells from oxidative stress, which is linked to chronic diseases such as cancer, cardiovascular disorders, and neurodegenerative conditions (Zeb, 2020). This study examined the intracellular antioxidant activity of rutin, both as free unencapsulated rutin and in composite form with sodium caseinate (NaCas), using the DCFH-DA assay in Caco-2 cells under oxidative stress induced by AAPH-generated peroxyl radicals. ROS levels were measured at low (0.5 μg/mL) and high (50 μg/mL) concentrations over 60 min, with fluorescence intensity as relative fluorescence units (RFU).

At low concentrations (0.5 μg/mL), both rutin-caseinate composites (RCC1 and RCC2) showed significantly higher ($p < 0.05$) free radical-quenching activity compared to free rutin (UR), with the highest antioxidant activity observed at 30 min (Fig. 3). At high concentrations (50 μg/mL), all three forms (UR, RCC1, RCC2) demonstrated comparable

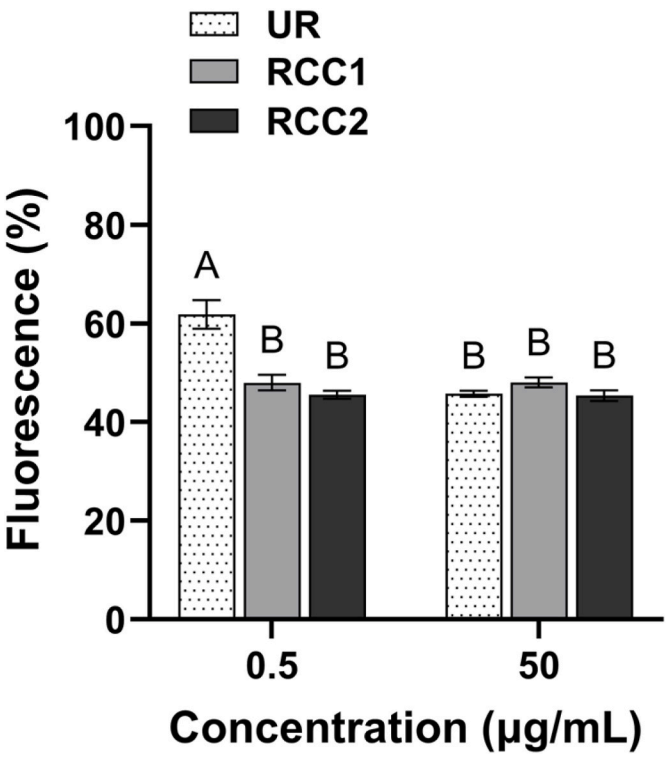

Fig. 3. Effect of untreated rutin (UR), rutin–caseinate composite 1 (RCC1), and rutin–caseinate composite 2 (RCC2) on intracellular antioxidant activity, expressed as the percentage fluorescence of reactive oxygen species (ROS) present in treated groups after 30 min of incubation. Intracellular antioxidant activity was quantified using 2′,7′-dichlorofluorescin diacetate (DCFH-DA). Mean ± SE; n = 3. Columns not sharing the same letters are significantly different ($p \leq 0.05$; one-way ANOVA followed by Tukey's multiple comparison test.

antioxidant efficacy. The enhanced antioxidant performance of RCCs can be attributed to the synergistic effects of rutin and NaCas, which significantly improve rutin's solubility, bioavailability, and cellular uptake factors critical for maximising antioxidant efficacy. Free rutin's poor solubility limits its cellular uptake and antioxidant performance efficacy (Rashidinejad et al., 2014; Wang et al., 2012). Encapsulation in NaCas improves the dispersibility of rutin, reduces its hydrophobicity, and enhances its ability to penetrate cell membranes, ultimately boosting ROS scavenging capacity. Although we did not test the efficacy of sodium caseinate alone in this study, previous research has shown that its antioxidant activity is minimal unless combined with polyphenols, where synergistic interactions significantly enhance bioactivity (Gong et al., 2022; Liao et al., 2022). These findings support our interpretation that the improved intracellular antioxidant activity of RCCs results from the synergistic interaction between rutin and NaCas, with rutin being the principal contributor to this enhancement.

Importantly, RCC2 exhibited the highest antioxidant potential, particularly at lower concentrations. This difference likely arises from the more efficient controlled release of rutin from RCC2 compared to RCC1, as indicated by its more effective radical-scavenging ability. The encapsulation of rutin within NaCas enhances the compound's stability through hydrogen bonding and hydrophobic interactions with the protein matrix (Gong et al., 2022). These interactions allow for better interaction with ROS, increasing the efficiency of free radical neutralization (Jiang et al., 2018; Yin et al., 2014; You et al., 2014).

Moreover, the structural arrangement of NaCas creates a favourable microenvironment for rutin, optimising its antioxidant action. This ensures that more rutin is available for ROS scavenging while minimising premature degradation (Czubinski & Dwiecki, 2017). The mild

antioxidant properties of NaCas further complement rutin's activity, leading to a stronger overall antioxidant response (Gong et al., 2022).

The controlled release mechanism within RCCs is crucial for minimising the cytotoxicity associated with free rutin. High concentrations of free rutin can lead to uncontrolled release, which contributes to oxidative stress. In contrast, the gradual release of rutin from RCCs ensures sustained antioxidant protection without the risks posed by high local concentrations of free rutin. This controlled release not only protects rutin from environmental degradation during storage and digestion but also enhances its bioactivity throughout gastrointestinal transit (Czubinski & Dwiecki, 2017; Tumilaar et al., 2024). Although free rutin exhibited cytotoxicity at high concentrations, likely due to uncontrolled release, RCCs maintained antioxidant protection while reducing the risk of oxidative stress. The superior performance of RCC2, particularly at lower concentrations, suggests that this formulation could be more effective for functional food applications aimed at mitigating oxidative stress-related damage.

In short, rutin-caseinate composites, particularly RCC2, demonstrate enhanced antioxidant potential compared to free rutin. By improving rutin's solubility, stability, and release profile, RCCs present a promising strategy for functional foods targeting oxidative stress. Further studies should explore the long-term bioavailability and efficacy of these composites in vivo to fully understand their antioxidant potential.

3.4. Physicochemical characterisation of rutin-protein composites incorporated into the functional milk product

The interactions between NaCas and rutin play a crucial role in determining the particle size and zeta potential of the system during digestion. Casein, a key protein in the milk matrix, has been widely studied for its ability to form complexes with bioactive compounds like polyphenols, including rutin, through electrostatic interactions, hydrophobic interactions, and hydrogen bonding (Ke et al., 2023; Rashidinejad et al., 2019; Sengupta et al., 2023). These interactions lead to the formation of stable complexes, which influence the particle size and charge during *in vitro* digestion. Understanding these physicochemical characteristics is essential for optimising the delivery and bioavailability of bioactive compounds like rutin in functional foods, such as the fortified dairy beverage developed in this study (Table S2).

3.4.1. Particle size

As shown in Table 1 and Fig. S2A, UR exhibited the largest initial particle size (274.61 ± 8.74 μm) during the gastric phase, attributed to its hydrophobic nature, which limits its dispersibility in aqueous systems such as milk matrices. This is consistent with previous findings that hydrophobic compounds like rutin tend to form poorly dispersed aggregates, resulting in large particles throughout digestion (Premathilaka et al., 2022; Rashidinejad et al., 2022a).

In comparison, RCC1 started with a significantly smaller particle size (38.95 ± 0.44 μm), increasing moderately to 49.42 ± 6.68 μm during gastric digestion and peaking at 264.48 ± 10.24 μm at 120 min. This trend reflects the dissociation of the NaCas-rutin complex under acidic gastric conditions, followed by reaggregation in the alkaline intestinal environment. Sodium caseinate is known to aggregate under acidic conditions and dissociate under alkaline pH, releasing rutin, which contributes to increased particle size (Hamzalioglu et al., 2023; Zhao et al., 2020). RCC2 followed a similar but more pronounced trajectory, with an initially higher particle size (369 ± 19.1 μm) and consistently larger values than RCC1 at all time points ($p < 0.05$). This difference is attributed to a higher degree of rutin entrapment and stronger intermolecular interactions within the NaCas matrix, resulting in slower release and greater aggregation. Notably, RCC2 showed approximately 54.5 % dissociation of NaCas from rutin during the. Gastric phase facilitating more extensive casein hydrolysis and particle enlargement. Overall, RCC2 demonstrated superior stabilization of rutin in the food matrix, indicating a more effective encapsulation system. The distinct physicochemical behaviour of RCC1 and RCC2 highlights the critical influence of formulation on digestion dynamics.

3.4.2. Zeta potential

The zeta potential of the formulations during digestion provided insights into colloidal stability and interactions. As shown in Fig. S2B, unencapsulated rutin (UR) exhibited a consistently negative surface charge throughout all digestion phases, ranging from −1.64 mV in the oral phase to −16.1 mV in the intestinal phase (Table 2). This trend reflects poor dispersibility and a high tendency for aggregation, consistent with the hydrophobic nature of free rutin in aqueous systems (Luo et al., 2024). In contrast, RCC1 and RCC2 exhibited significantly higher zeta potentials during the gastric phase, ranging from +4.23 to +3.53 mV and +4.30 to +2.39 mV, respectively ($p < 0.05$). These positive charges are indicative of electrostatically stabilized NaCas–rutin complexes. The higher surface charge of RCC1 suggests slightly improved stability under acidic conditions. However, during the intestinal phase, both formulations underwent a marked shift to negative potentials (RCC1: 18.35 mV; RCC2: 13.55 mV), suggesting enzymatic disruption of the protein–polyphenol complex and the subsequent release and aggregation of rutin. The more negative zeta potential of RCC1 during intestinal digestion may imply a more extensive exposure of charged residues upon proteolysis, potentially contributing to increased particle dispersion (Fig. S1B). These results highlight the

Table 1
The particle size, specific surface area, and zeta potential of digested fortified milk containing untreated rutin (UR). Data represent the mean of three replicates. Values in the same columns that do not share the same superscripted letters are significantly different ($p \leq 0.05$).

Sample	D [4,3] (μm)	Zeta potential (mV)	Specific surface area (m2/gm)
Gastric, 0 min	274.61 ± 8.74^{a}	−1.64 ± 0.15^{a}	0.13 ± 0.002^{d}
Gastric, 30 min	279.72 ± 8.322^{a}	−1.52 ± 0.12^{a}	0.16 ± 0.001^{d}
Gastric, 60 min	138.62 ± 32.05^{b}	−1.37 ± 0.14^{a}	0.22 ± 0.02^{c}
Gastric, 120 min	95.09 ± 18.14^{c,d}	−1.88 ± 0.15^{a}	0.21 ± 0.01^{c}
Intestinal, 0 min	73.04 ± 10.59^{d,e}	−15.88 ± 0.48^{b}	0.41 ± 0.02^{b}
Intestinal, 30 min	60.06 ± 2.68^{e}	−15.8 ± 0.61^{b}	0.43 ± 0.01^{b}
Intestinal, 60 min	104.22 ± 23.28^{c}	−15.2 ± 0.77^{b}	0.44 ± 0.03^{a,b}
Intestinal, 120 min	78.25 ± 15.33^{c,d,e}	−16.1 ± 0.41^{b}	0.48 ± 0.05^{a}

Table 2
The particle size, specific surface area, and zeta potential of digested fortified milk containing rutin-caseinate composite 1 (RCC1). Data represent the mean of three replicates. Values in the same columns that do not share the same superscripted letters are significantly different ($p \leq 0.05$).

Sample	D [4,3] (μm)	Zeta potential (mV)	Specific surface area (m^2/gm)
Gastric, 0 min	38.95 ± 0.44^{c}	4.23 ± 0.23^{a}	0.31 ± 0.001^{c}
Gastric, 30 min	43.38 ± 1.89^{c}	3.6 ± 0.80^{a}	0.32 ± 0.02^{c}
Gastric, 60 min	51.57 ± 14.75^{b,c}	4.91 ± 0.65^{a}	0.32 ± 0.01^{c}
Gastric, 120 min	49.42 ± 6.68^{b,c}	3.53 ± 0.27^{a}	0.29 ± 0.01^{c}
Intestinal, 0 min	64.39 ± 2.35^{b}	−18.35 ± 1.82^{c}	0.42 ± 0.02^{b}
Intestinal, 30 min	40.67 ± 9.02^{c}	−18.38 ± 1.70^{c}	0.45 ± 0.05^{a,b}
Intestinal, 60 min	61.87 ± 13.03^{b}	−13.01 ± 1.89^{c}	0.49 ± 0.04^{a}
Intestinal, 120 min	264.48 ± 10.24^{a}	−5.95 ± 0.14^{b}	0.10 ± 0.002^{d}

Table 3
The particle size, specific surface area, and zeta potential of the digested fortified milk containing rutin-caseinate composite 2 (RCC2). Data represent the mean of three replicates. Values in the same columns that do not share the same superscripted letters are significantly different ($p \leq 0.05$).

Sample	D [4,3] (μm)	Zeta potential (mV)	Specific surface area (m2/gm)
Gastric, 0 min	369.36 ± 19.12^{a}	4.30 ± 0.20^{a}	0.19 ± 0.01^{e}
Gastric, 30 min	181.39 ± 3.97^{b}	3.35 ± 0.13^{a}	0.28 ± 0.01^{a}
Gastric, 60 min	186.11 ± 9.93^{b}	2.66 ± 0.16^{a}	$0.23 \pm 0.01^{c,d}$
Gastric, 120 min	169.81 ± 2.74^{b}	2.39 ± 0.19^{a}	$0.26 \pm 0.003^{a,b}$
Intestinal, 0 min	371.68 ± 16.16^{a}	$-13.55 \pm 0.47^{b,c}$	$0.21 \pm 0.01^{d,e}$
Intestinal, 30 min	345.88 ± 32.11^{a}	-11.5 ± 0.29^{b}	$0.24 \pm 0.03^{b,c}$
Intestinal, 60 min	353.15 ± 10.48^{a}	-17.33 ± 2.14^{c}	0.27 ± 0.01^{a}
Intestinal, 120 min	369.59 ± 10.70^{a}	-11.32 ± 1.53^{b}	0.27 ± 0.02^{a}

dynamic nature of electrostatic interactions during digestion and support the role of NaCas in modulating rutin release, colloidal behaviour, and stability through gastrointestinal conditions.

It is important to note that the fortified banana-flavoured milk used in this study contains native milk proteins, which may interact with both unmodified rutin and the rutin–caseinate composites. These interactions could influence the observed zeta potential values, particularly under intestinal pH conditions. While the negative surface charge of RCCs in the intestinal phase was initially attributed to casein degradation and exposure of negatively charged residues, it is also plausible that the presence of other milk proteins, many of which have isoelectric points below intestinal pH, contributed to the net surface charge through ampholytic dissociation. Therefore, the zeta potential values observed in this study likely reflect the combined effects of casein–rutin complexation, protein digestion, and interactions with matrix proteins. Future studies using simplified or protein-free matrices may help isolate the specific contributions of RCCs versus native milk components.

3.4.3. Surface area

The surface area behaviour of the samples during digestion revealed important differences in the structural dynamics of free rutin (UR) compared to the rutin–caseinate composites (RCC1 and RCC2). As shown in Fig. S2C (see also Table 3), UR exhibited minimal surface area changes during digestion, primarily due to the large, poorly dispersed rutin crystals. The hydrophobic nature of UR promotes aggregation, which limits its dispersion within the milk matrix and may result in possible interactions with digestive enzymes and cellular membranes, restricting its absorption potential (Teng & Chen, 2019; Wojtunik-Kulesza et al., 2020).

In contrast, rutin-caseinate composite (RCC1) demonstrated a notable increase in surface area during the intestinal phase (Table 2). This increase is likely due to the dissociation of NaCas from rutin, leading to the formation of smaller protein aggregates with a larger surface area available for interaction. The enhanced surface area is critical as it facilitates greater exposure of rutin to digestive enzymes and the intestinal epithelium, potentially improving its solubility and absorption. The breakdown of the NaCas-rutin complex during digestion not only increases the surface area but also enhances the accessibility of rutin's reactive sites, which can be crucial for interactions with transport mechanisms in the intestinal cells (Fang et al., 2017; Martinez-Gonzalez et al., 2017).

RCC2 also showed a similar increase in surface area during digestion (Table 3). The higher initial particle size in RCC2, resulting from a stronger interaction between NaCas and rutin, contributed to a larger surface area upon dissociation. This greater surface area can facilitate more robust interactions with digestive enzymes, leading to improved enzymatic hydrolysis of the complex. As a result, the increased exposure of rutin may enhance its solubility in the gastrointestinal environment, further promoting its absorption through the intestinal lining.

The correlation between surface area changes and the potential for rutin absorption is significant. A larger surface area not only improves the physical accessibility of the bioactive compound but also promotes effective contact with digestive enzymes that facilitate its breakdown into smaller, more absorbable forms. This enhanced interaction can lead to increased transport of rutin across the intestinal membrane, significantly boosting its bioavailability. Studies have shown that enhanced surface area correlates with improved absorption rates of various nutrients and bioactive compounds, underscoring the importance of structural modifications during digestion in determining the efficacy of bioactive compounds like rutin (Manach et al., 2004; Martinez-Gonzalez et al., 2017; Zhao et al., 2019).

Moreover, the increase in surface area also aids in reducing the hydrophobic interactions that often limit solubility and bioavailability. By disrupting these aggregates and promoting a more homogeneous dispersion of rutin within the digestive milieu, the composites can maximise the delivery of rutin to the absorption sites in the intestine. Consequently, the structural dynamics of RCC1 and RCC2 during digestion not only improve their antioxidant capacity but also enhance their overall bioavailability, making them more effective in exerting their functionality.

In summary, UR exhibited the largest initial particle size and negative zeta potential, but its poor dispersibility and hydrophobic nature limited its surface area and bioavailability. RCC1 and RCC2 both showed an increase in particle size and a shift from positive to negative zeta potential during digestion, indicating the breakdown of the NaCas-rutin complex. These changes were accompanied by an increase in surface area, particularly in the intestinal phase, suggesting that the NaCas matrix helps modulate the release and bioavailability of rutin. RCC2, in particular, demonstrated a stronger interaction with NaCas, leading to a higher degree of rutin entrapment and more favourable physicochemical properties for bioactive compound delivery.

3.5. In vitro bioaccessibility of rutin

The bioaccessibility of rutin in the fortified beverage reflects the

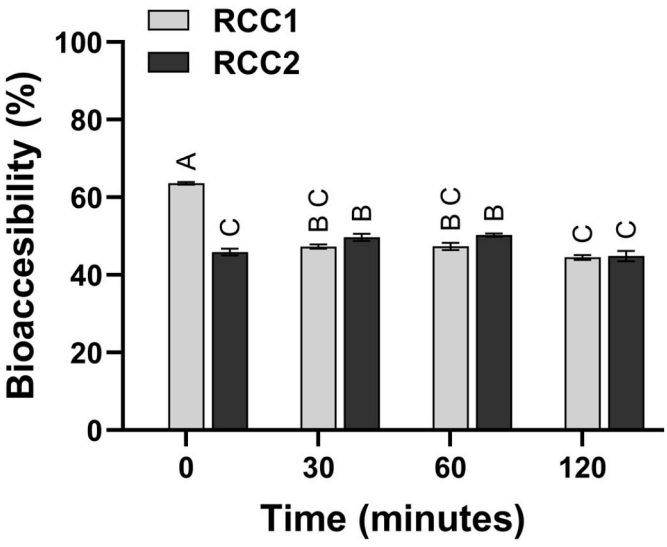

Fig. 4. Bioaccessible rutin in untreated rutin, rutin–caseinate composite 1 (RCC1), and rutin–caseinate composite 2 (RCC2) obtained during the *in vitro* intestinal digestion phase. Data represent the mean of three replicates, with error bars corresponding to the standard error of the mean. Columns not sharing the same letters are significantly different ($p \leq 0.05$one-way ANOVA followed by Tukey's multiple comparison test). Note: Untreated rutin was not detected.

amount of rutin available for absorption in the body, directly impacting its bioavailability and bioactivity, such as antioxidant potential. Even if rutin is ingested in substantial amounts, its bioavailability depends on how effectively it can be released and absorbed by the intestine. By increasing bioaccessibility, a larger proportion of rutin can be absorbed in the small intestine, where it can enter the bloodstream and exert its bioactive effects, such as antioxidant and anti-inflammatory properties (D Archivio et al., 2007; Stromsnes et al., 2021).

In this study, after gastric digestion, RCC1 and RCC2 underwent intestinal digestion, simulating the in vivo digestive process using pancreatin and bile salts. Rutin recovery was quantified using HPLC, allowing for comparison between the initial and digested samples to assess bioaccessibility. The results showed that the bioaccessibility of rutin in RCC1 during intestinal digestion was significantly ($p < 0.05$) higher (Fig. 4), likely due to a pH shift from acidic to alkaline conditions during digestion (Rashidinejad et al., 2022a).

The casein matrix in RCC1 and RCC2 played a protective role during the gastric phase, preventing rutin destabilisation. This protection likely stems from electrostatic interactions, hydrophobic binding, and steric hindrance, which together stabilise rutin under low pH conditions (Kamiloglu et al., 2021; Shahidi & Pan, 2022; Tarko & Duda-Chodak, 2020). The structure of casein can entrap rutin, reducing its exposure to gastric enzymes and acidic degradation, thus delaying its release into the intestinal phase where absorption occurs.

Milk is a complex matrix containing lipids, carbohydrates, and sugars, which have the potential to interact competitively with bioactive compounds like rutin, potentially influencing their binding and bioaccessibility (Lv et al., 2025; Qin, Chen, et al., 2025; Qin, Zhang, et al., 2025). In this study, rutin was entrapped within sodium caseinate to form composites (i.e., RCC1 and RCC2), aiming to reduce non-specific interactions and protect rutin from competitive binding by other milk constituents. The improved bioaccessibility of rutin observed in the composites compared to unmodified rutin suggests that casein entrapment may effectively mitigate the competitive binding effects of milk components, enhancing rutin stability and release. However, the specific roles of lipids, carbohydrates, and sugars in modulating rutin interactions within these composites requires further investigation to fully elucidate the mechanisms governing bioactive release and absorption in complex food matrices.Furthermore, the possible interactions in RCC1 and RCC2 may promote a slower, more controlled release of rutin in the intestine, increasing its chances of being absorbed efficiently (Fig. 4). This finding aligns with those reported in the studies of Lamothe et al. (2014), where the incorporation of polyphenols into a milk matrix significantly improved their bioaccessibility by 42 %, likely due to similar protective effects. The controlled release observed in RCC1 and RCC2 suggests that protein-based composites not only shield rutin during gastric digestion but also modulate its intestinal release, preventing premature degradation and enhancing absorption efficiency.

As seen in Fig. 4, rutin recovery in RCC1 ranged from 43 % to 48 % during intestinal digestion (30–120 min), while RCC2 demonstrated a consistent recovery of 42 %–45 % over the same period. These values represent time-specific recovery of rutin during the intestinal phase only. However, it is notable that these values are different than the overall bioaccessibility of rutin from RCC1, which is approximately 63 %. This higher value reflects the overall release, solubilization, and stability of rutin throughout the entire simulated gastrointestinal digestion process.

Although the bioaccessibility of rutin from UR was not directly assessed in this study due to its precipitation, the literature suggests that untreated/unencapsulated rutin typically suffers from extensive degradation in acidic gastric conditions, leading to significantly lower intestinal recovery rates (Acevedo-Fani et al., 2021; Chua, 2013; Gayoso et al., 2016). Studies on flavonoid solubility and stability also indicate that free (untreated) rutin often precipitates in aqueous environments with pH fluctuations, reducing its solubility and subsequent absorption (Pateiro et al., 2021; Rashidinejad et al., 2022a). This aligns with the observed behaviour of UR in our study, further highlighting the advantage of protein-based composite formulations in improving the stability and delivery of bioactive compounds.

After 120 min of intestinal digestion, both RCC1 and RCC2 showed similar bioaccessibility ranges of 45 %–47 %, suggesting a relatively stable rutin recovery over time. However, RCC1 experienced a 16 %–18 % loss in bioaccessibility during the intestinal phase, while RCC2 showed only a 3 %–4 % loss, indicating the higher stability of rutin in RCC2 throughout digestion (Fig. 4). The total bioaccessible rutin quantified from the digested samples (0.5 mL) was 641.31 ppm for RCC1 and 263.28 ppm for RCC2, demonstrating the superior bioaccessibility of rutin in composite formulations compared to free rutin. These concentrations were then evaluated for epithelial absorption through diffusion across polarised Caco-2 cell monolayers, an *in vitro* model for intestinal absorption (see next section).

As mentioned before, the bioaccessibility of rutin from UR was not assessed in this study, as UR precipitated when resuspended for the experiment, resulting in no measurable recovery of rutin. This behaviour further emphasises the challenges of using free rutin in functional food formulations, as its poor solubility and tendency to aggregate may hinder its bioaccessibility. Based on existing studies, free rutin typically exhibits a bioaccessibility of less than 20 % under simulated intestinal conditions, which is significantly lower than the levels observed for RCC1 and RCC2.

The enhanced bioaccessibility observed in RCC1 and RCC2 underscores the importance of formulation strategies, particularly the use of protein-based composites, in maximising the potential health benefits of bioactive compounds like rutin. Bioaccessibility is a critical factor influencing bioavailability because even if a compound is present in the food matrix, its bioavailability hinges on how much is released during digestion and absorbed by the intestines (Lorenzo et al., 2019). By protecting rutin from degradation and controlling its release, these composites ensure that more of the compound reaches its target sites in the body, thereby improving its bioavailability. Future studies should further explore the role of specific protein-polyphenol interactions and

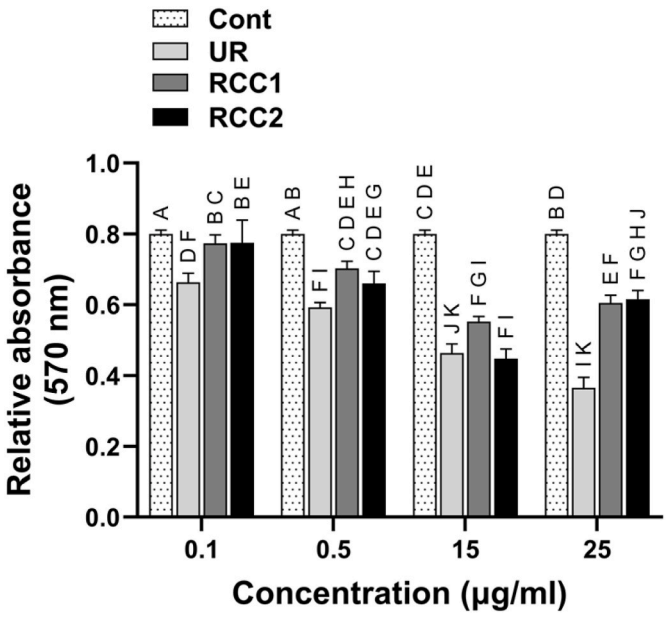

Fig. 5. Effect of digested banana-flavoured milk fortified with untreated rutin (UR) and its two caseinate composites (RCC1 and RCC2) on the viability of Caco-2 cells. Data represent different concentrations used in the assay. Values are expressed as the mean of four biological replicates, each with three technical replicates. Error bars indicate the standard error of the mean. Columns that do not share the same letters are significantly different($p \leq 0.05$one; way ANOVA followed by Tukey's multiple comparis

optimise composite formulations to enhance the absorption and functional efficacy of rutin in food applications.

3.6. Cytotoxicity and gut barrier integrity of bioaccessible fractions from digested banana milk containing rutin composites

3.6.1. Cell viability and cytotoxicity assessment

The bioaccessible fractions of rutin composites in the digested flavoured milk were evaluated for their cytotoxic effects on the intestinal epithelium, a key barrier before rutin absorption into the bloodstream. We utilised a Caco-2 differentiated cellular intestinal epithelial barrier to study the transport and absorption of untreated rutin and its caseinate composites. Initially, cell viability was assessed using the MTT assay (Fig. 5) across concentrations ranging from 0.1 μg/mL to 25 μg/mL.

Both RCC1 and RCC2 exhibited minimal cytotoxicity, maintaining significantly higher cell viability than UR ($p < 0.05$) at 0.1 μg/mL and similar to the control (UR) at lower concentrations. At 0.5 μg/mL, RCC1 and RCC2 still showed higher cell viability than UR. However, at concentrations of 15–25 μg/mL, both RCC1 and RCC2 demonstrated reduced cytotoxicity compared to UR, suggesting that NaCas encapsulation effectively mitigated the hydrophobicity and potential cytotoxic effects of rutin. In contrast, UR, due to its insoluble and aggregated nature during digestion, exhibited significantly lower cell viability (<70 %), likely limiting its protective effects on Caco-2 cells.

The reduced cytotoxicity observed in RCC1 and RCC2 can be attributed to NaCas serving as a protective carrier that modulates rutin's physicochemical properties. NaCas reduces rutin's hydrophobicity through protein-polyphenol interactions, forming a stable colloidal complex (Helal & Tagliazucchi, 2018; Rashidinejad et al., 2019, Rashidinejad et al., 2022a; Wei et al., 2015). This interaction enhances rutin solubility, preventing it from aggregating into large crystalline structures that could disrupt cellular membranes and induce cytotoxic effects. NaCas encapsulation ensures that rutin remains in a dispersed and bioavailable form, reducing excessive membrane perturbation and oxidative stress, thereby sustaining higher cell viability, even at elevated concentrations.

Additionally, the entrapment of rutin by NaCas alters its surface charge and dispersion during digestion, as reflected by changes in zeta potential observed in RCC1 and RCC2 (see Fig. S1B). The gradual dissociation of NaCas during digestion enhances rutin bioavailability by providing a controlled release mechanism, preventing localised concentrations of free rutin that could induce cytotoxicity. This controlled release was particularly evident at higher concentrations, where free rutin (UR) demonstrated significantly reduced cell viability compared to NaCas-entrapped rutin (Fig. 5). NaCas acts as a molecular buffer, ensuring rutin remains bioaccessible without reaching toxic levels, as previously noted for other polyphenol-protein delivery systems (Martinez-Gonzalez et al., 2017).

Furthermore, the protein-polyphenol interactions inherent in NaCas entrapment/encapsulation may contribute to reducing oxidative stress. Polyphenols, in their free form, can exhibit pro-oxidant effects at high concentrations (Araújo et al., 2013; Fang et al., 2017; Yang et al., 2014). By embedding rutin within NaCas, the reactive hydroxyl groups of rutin are partially shielded, minimising their direct interaction with cellular components and preventing oxidative stress-induced cytotoxicity. This controlled interaction ensures that rutin retains its beneficial antioxidant properties while reducing potential adverse effects. Taken together, RCC1 and RCC2 demonstrated reduced cytotoxicity and enhanced biofunctionality compared to UR. NaCas served as a stabilising carrier, improving rutin's solubility, cellular compatibility, and minimising cytotoxic risks.

3.6.2. Impact of bioaccessible rutin-protein composites on Caco-2 monolayer barrier integrity

The bioaccessible fractions of RCC1, RCC2, and UR were evaluated for their impact on the barrier integrity of Caco-2 monolayers by monitoring transepithelial electrical resistance (TEER). Differentiated cells with baseline TEER values above 500 Ω/cm^2 indicated intact tight junctions suitable for permeability studies (Fig. 6A–D). The concentrations (0.1, 10, 50, and 200 μg/mL) were selected to model a range of intestinal exposures, from typical dietary intake (low doses) to higher levels achievable through supplementation. The highest concentration (200 μg/mL) was used to assess barrier resilience under stress conditions, allowing evaluation of both physiological and supraphysiological effects on epithelial integrity.

At lower concentrations of 0.1, 10 and 50 μg/mL RCC1 and RCC2

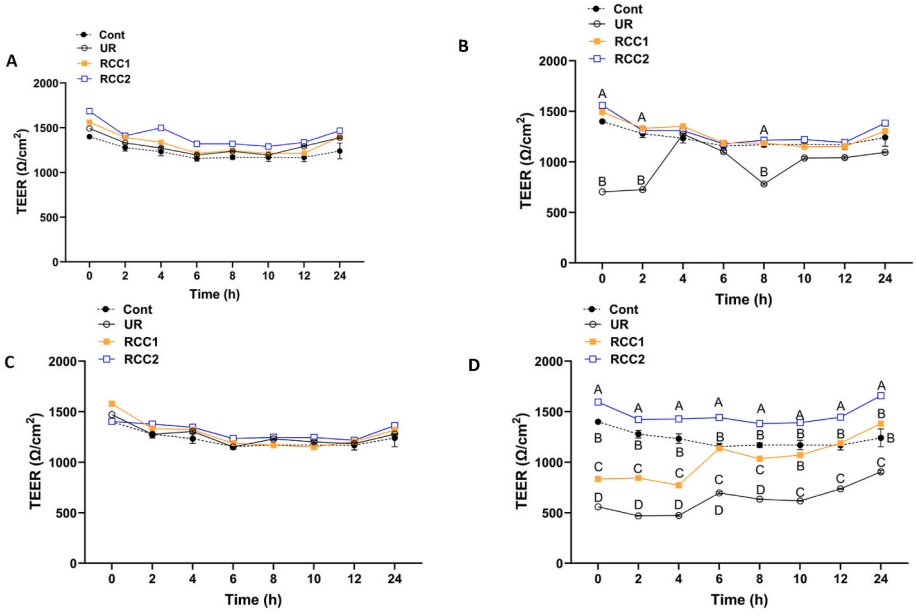

Fig. 6. Transepithelial electrical resistance (TEER) of differentiated Caco-2 cell monolayers measured every 2 h over a 24-h period following exposure to digested banana-flavoured milk containing control medium alone (Cont), untreated rutin (UR), rutin–caseinate composite 1 (RCC1), or rutin–caseinate composite 2 (RCC2) at concentrations of 0.1 μg/mL (A), 10 μg/mL (B), 50 μg/mL (C), and 200 μg/mL (D). Data are presented as mean ± standard error (SE), n = 3. Samples and time points not sharing the same letters are significantly different ($p \leq 0.05$; one-way ANOVA followed by Tukey's multiple comparison test).

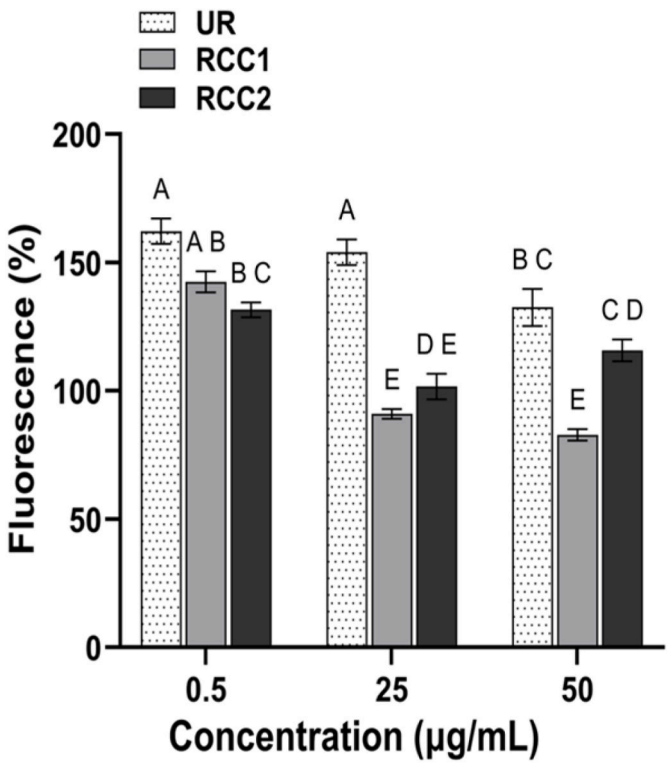

Fig. 7. Effect of digested banana-flavoured milk fortified with untreated rutin (UR), rutin–caseinate composite 1 (RCC1), and rutin–caseinate composite 2 (RCC2) on intracellular antioxidant activity in differentiated Caco-2 cells over a 60-min period. Antioxidant activity was quantified using the DCFH-DA assay and expressed as relative fluorescence units. Data represent the mean of three biological replicates, each with three technical replicates. Error bars indicate the standard error of the mean. Columns not sharing the same letters are significantly different ($p \leq 0.05$; one-way ANOVA followed by Tukey's multiple comparison test).

consistently maintained high TEER values (>1400 Ω/cm^2) over the course of 24 h reflecting robust barrier function (Fig. 7 A-D). However, at highest conc. of 200 µg/mL, RCC1 reduced TEER after 10 h, indicating potential cytotoxic effects and likely tight junction disruption (Fig. 6D). Intermediate concentrations (10 and 50 µg/mL) showed transient reductions, likely due to interactions between digestive enzymes, bile salts, and milk proteins in the digesta (Fig. 6B, C). Nevertheless, TEER values recovered by 24 h, suggesting that RCC1 caused minimal long-term disruption.

Overall, RCC2 demonstrated significant superior performance compared to RCC1 ($p < 0.05$), maintaining or even enhancing TEER values (>1500 Ω/cm^2) at higher concentrations (200 µg/mL) throughout the incubation period (Fig. 6D) suggesting its minimal cytotoxic effect on epithelium monolayer. This improved barrier protection is attributed to the strong encapsulation of rutin by NaCas, providing a stable protective matrix that enhances solubility, minimises cytotoxicity, and supports controlled release, preventing localised high concentrations that could disrupt cellular integrity. These results align with previous reports highlighting protein encapsulation as a strategy for increasing bioaccessibility and functionality (Dissanayake & Bandara, 2024; Rashidinejad et al., 2024).

In contrast, UR significantly compromised barrier integrity, with TEER dropping to 570–900 Ω/cm^2 at 200 µg/mL, and noticeable reductions even at 10 µg/mL when compared to RCC1 and RCC2 ($p < 0.05$; Fig. 6B). These adverse effects are likely due to UR's poor solubility and low stability during digestion, leading to cytotoxicity and likely disruption of tight junctions as reported elsewhere (Olejnik et al., 2016). At lower concentrations (0.1 and 50 µg/mL), UR showed better preservation of TEER, possibly due to rapid degradation by digestive enzymes reducing its impact on the epithelium monolayer.

The observed higher TEER values for RCC2 may arise from its structurally distinct formulation, characterized by a higher protein-to-rutin ratio that results in a denser, more compact sodium caseinate matrix compared to RCC1. This compact structure might facilitate a slower and more controlled release of rutin, potentially minimising epithelial stress and helping to maintain tight junction integrity. Additionally, RCC2's matrix may better shield cells from irritants and reactive oxygen species, thereby supporting improved barrier function. These observations align with previous findings suggesting that sustained flavonoid delivery can help preserve epithelial barrier integrity by stabilising tight junction protein expression (Amasheh et al., 2008; Suzuki & Hara, 2009).

These findings highlight the superior efficacy of RCC2 in preserving barrier integrity compared to RCC1 and UR. The NaCas-based encapsulation likely played a critical role in stabilising rutin, enhancing its bioavailability, and minimising cytotoxic effects on the Caco-2 monolayer. In contrast, UR's poor performance underscores the need for structural modifications to improve its solubility, stability, and reduce cytotoxicity. Thus, both composites demonstrated potential in maintaining barrier integrity and supporting rutin absorption, positioning them as promising candidates for functional food applications.

3.6.3. Intracellular antioxidant activity of digested rutin composites vs. untreated rutin on differentiated Caco-2 cell monolayers

The intracellular antioxidant activity of rutin-protein composites (RCC1 and RCC2) was assessed in differentiated Caco-2 monolayers at concentrations of 0.5 µg/mL, 25 µg/mL, and 50 µg/mL following a 30-min incubation. At the lowest concentration (0.5 µg/mL), RCC2 exhibited significantly higher antioxidant activity compared to untreated rutin (UR) ($p < 0.05$) (Fig. 7). At higher concentrations (25 µg/mL and 50 µg/mL), both RCC1 and RCC2 demonstrated stronger antioxidant activity than UR, which showed reduced antioxidant potential as concentration increased ($p < 0.05$ RCC1, RCC2 vs UR 25 µg/mL; $p < 0.05$ for RCC2 vs UR at 50 µg/mL). The diminished activity of UR at higher concentrations can likely be attributed to its poor dispersibility during milk fortification, leading to lower rutin availability after digestion and a corresponding decrease in its radical-scavenging capability.

The superior antioxidant potential of RCC1 and RCC2 compared to UR is attributed to their enhanced physicochemical properties and bioaccessibility. NaCas, used for the composite formulation, stabilises rutin during digestion, protecting it from degradation while enhancing its solubility in the intestinal environment. Bioaccessibility rates of RCC1 (63 %) and RCC2 (45 %) were significantly higher compared to UR ($p < 0.05$), which showed poor dispersibility and limited bioavailability. The colloidal matrix formed by NaCas likely shields rutin from oxidative and enzymatic degradation, improving its stability and bioactivity during digestion (Pateiro et al., 2021).

Additionally, the smaller particle size of the composites compared to the highly crystalline UR increases the surface area (Fig. S1A–C), improving interactions with reactive oxygen species (ROS) and facilitating more efficient radical scavenging. This improvement in surface area enhances the dispersion of rutin in the gastrointestinal tract, increasing its dissolution rate and enhancing cellular absorption. Smaller particles present a larger surface area-to-volume ratio, which facilitates increased molecular interactions with ROS and digestive enzymes (Tang et al., 2025) leading to enhanced intracellular antioxidant activity.

The higher antioxidant activity of RCC1 and RCC2, particularly at 25 µg/mL and 50 µg/mL, is also linked to their higher soluble rutin content (46 % for RCC1 and 27.3 % for RCC2.The sustained release of rutin from these composites enables prolonged antioxidant activity, whereas UR's poor dispersibility reduces its cellular availability and antioxidant potential, as reflected in its lower ROS scavenging ability at different

concentrations (Fig. 7).

Furthermore, TEER assay results indicated that both RCC ingredients, especially RCC2, were more effective in maintaining intestinal barrier integrity over 24 h compared to UR, with RCC2 showing the strongest ability to preserve epithelial barrier function, followed by RCC1 (Fig. 6A–D). UR was the least effective in this regard. This further supports our hypothesis that NaCas (used in the entrapment technology reported in this work) enhances rutin bioaccessibility, which in turn, may offer additional biofunctional benefits, such as promoting gut health and reducing oxidative stress. The enhanced stability, solubility, and sustained bioactivity of the composites in this study make them more effective than pure UR. These findings align with those reported in previous research (Sun et al., 2022), demonstrating that protein-polyphenol complexes improve antioxidant properties and gastrointestinal stability of hydrophobic flavonoids such as rutin.

4. Conclusions

This study demonstrates that both RCC1 and RCC2 enhance antioxidant activity, bioaccessibility, and cellular stability compared to free (untreated) rutin, retaining the ability to scavenge ROS even after digestion. RCC2, in particular, showed superior absorption across Caco-2 monolayers and greater support for intestinal barrier integrity. These findings highlight the potential of rutin–caseinate composites as effective delivery systems for hydrophobic bioactives in functional foods. For successful industrial application, several factors must be addressed. Large-scale production methods must be cost-effective, scalable, and capable of preserving the composites' stability and bioactivity. Ongoing work in our laboratories, in collaboration with industry partners, focuses on optimising manufacturing processes and evaluating the economic viability of incorporating RCCs into commercial food products. Formulation compatibility is also critical. The interaction of RCCs with various food ingredients must be examined to ensure product quality and consumer acceptance are maintained. Sensory properties, often overlooked in early-stage development, should be assessed alongside stability and bioactivity to determine real-world applicability. Based on the cytocompatibility results and the well-established safety profiles of both sodium caseinate and rutin, the recommended fortification level of RCCs in dairy products can be guided by the widely accepted daily intake of rutin, which is approximately 500 mg/day for adults. In this study, the amount of RCC incorporated into the fortified milk was calculated to deliver this target dose within a standard serving size (e.g., 250 mL). This dosage is consistent with previous clinical studies demonstrating the safety and tolerability of rutin supplementation at levels up to 500 mg/day. These findings support the feasibility of safely incorporating RCCs into functional dairy products for routine consumption. To ensure successful translation into commercial applications, further research is warranted. *In vivo* studies are needed to confirm the bioavailability and health benefits of RCC1 and RCC2 under physiological conditions. Long-term stability testing under varied storage environments will be essential to ensure sustained functional performance. Finally, clinical trials will be critical to establish the long-term safety, efficacy, and potential of these composites in reducing oxidative stress and preventing chronic diseases, thereby supporting regulatory approval and broader adoption in the functional food sector.

CRediT authorship contribution statement

Raise Ahmad: Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anubhavi Singh:** Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Ajitpal Purba:** Methodology, Investigation. **Ali Rashidinejad:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2025.111735.

Data availability

Data will be made available on request.

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