Impact of simulated *in vitro* gastrointestinal digestion on bioactive compounds, bioactivity and cytotoxicity of melon (*Cucumis melo* L. i*nodorus*) peel juice powder

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A B S T R A C T

The objectives of this research work were to evaluate the effect of *in vitro* gastrointestinal digestion (GIT) on melon peel juice (MPJ) powder from fruit processing industry by-products, considering (i) the recovery and accessibility indexes, (ii) the changes on antioxidant activity, and (iii) the prebiotic effect. Throughout exposition to GIT conditions a decrease on the total phenolic content (TPC = 65.31%) and antioxidant activity by ABTS = 39.77% and DPPH = 45.91% were observed. However, these both parameters exhibited stable accessibility, accounting with 81.89%, 76.55%, and 54.07% for TPC, ABTS and DPPH, respectively. After gastrointestinal digestion, the non-absorbed fraction exhibited a positive impact on the growth of *Bifidobacterium* and *Lactobacillus* strains, possibly associated with the high content of simple sugar (glucose and fructose). This fraction also showed to be safe on Caco-2 intestinal cells. These findings suggest that MPJ might be used as a potential food functional ingredient.

1. Introduction

Functional food ingredients/additives have been stated as food stuffs, which can exert diverse benefits on human health, helping in the prevention or reduction of certain diseases as cancers, cardiovascular and inflammatory disorders and diabetes (Silva et al., 2018). Currently, consumers are demanding for natural and clean label food ingredients, leading to an increase in the search and development of plant-based products as rich sources of compounds useful in medicine, food and nutraceuticals with the objective to avoid side negative effects of synthetic compounds (Stefanucci et al., 2020). In this regard, food waste and by-products are recognized to be excellent sources of high content of value-added bioactive compounds (BCs), including fibres, vitamins, sugars, organic acids, carotenoids and phenolics (among others) with health promoting properties including antimicrobial, antioxidant and prebiotic activities (Oliveira et al., 2021; Vilas-Boas et al., 2021). Thus, bearing these facts in mind, these by-products may be used as natural food ingredients or additives for the preparation of innovative functional food products, which in turn responds to the current trends in

clean label and safe food products and increase food supply products (Gómez-García et al., 2022a).

In this context, fruit processing industries are confronted with the lack of efficient and proper management of foods before and after food processing, generating great amount of fruit waste and by-products, having environmental and economic negative impacts due to their null applications being discarded in landfills (Campos et al., 2020). EU commission launched new directives that industries must accept and implement by 2030 within their processing facilities and encourage them to shift from a linear to a circular economy, which in turn improve profitability and valorisation of their food by-products, while environmental pollution is avoided (Imbert, 2017). Currently, the circular economy encompasses outstanding strategies for food waste management and valorisation as a sustainable approach to use these organic biomasses as novel resources for human purposes, since the current strategies for its treatment will not be further accepted due to different disadvantages (large landfill spaces and high transportation costs) (Ghisellini et al., 2016; Gómez-García et al., 2021a). On the other hand, in order to feed the estimated 9 billion people in 2050, FAO forecasts a

20% increase in food production, so in a near future, it is expected an increasing industrial processing and generation of food waste and by-products (Trigo et al., 2019).

Nevertheless, it should be considering that the beneficial effects and effectiveness of food ingredients depend not only on their intake and BCs composition, but also on the changes (negative or positive) produced during each phase of the gastrointestinal digestion. To understand the health promoting effect of functional ingredients a static *in vitro* gastrointestinal digestion system appears to provide a useful alternative to animal and human models for screening of food ingredients; additionally *in vitro* techniques are ethically superior, faster and less expensive than in vivo techniques (Campos et al., 2020; Madureira et al., 2011). Thus, *in vitro* gastrointestinal digestion followed by the determination of bioactive compound concentrations and bioactivities as well as the stability and accessibility are the most common and accepted methodologies to create key markers, which help to identify and describe better patterns when compared to the real or in vivo models (Gullon et al., 2015; Lucas-Gonzalez et al., 2016).

In this scenario, the industrialization and commercialization of melon (*Cucumis melo* L.) fruit is taking part of the great amount of food waste and by-products generation currently released (ca. 1.3 billion tonnes/year). The high industrial melon processing activities have been carried out since it is one of the most important fruits in the world, thanks to its consumer acceptance for its sweet flavour, attractive fragrance, colour and nutritional and medicinal properties. Traditionally, melon fruit was used in the treatment of some common illness well-justified by its high content of BCs (Gómez-García et al., 2020; Rolim et al., 2020). One of the principal by-products generated during melon processing are the peels, which are constituted principally by around 70–75% of water and still keep BCs such as, organic acids, proteins and polyphenols and sugars, which have been shown some interesting beneficial effects on human health. Moreover, great attention has been raised for valorisation of melon by-products with the purpose to avoid or decrease the negative impact related to the environmental contamination and economic losses in order to develop suitable strategies to exploit these materials toward their potential application as functional ingredients/additives with beneficial properties. (Dahiya et al., 2018; Esparza et al., 2020). Presently, the food industries are interested in modernization toward innovations and developments aimed to mitigate their economic losses, while zero-waste approach is achieved, meeting with the future European directives, where the food by-products generated within their facilities is used as raw material for new food supplies and applications. Melon peels utilization and valorisation could lead to an effective way to not only for the promotion of human health and well-being, but also for their commercial viability by the incorporation into the food value chain (Chiappetta Jabbour et al., 2020).

However, the development of food functional ingredients from melon by-products has not been extensively studied nor the bioacessibility of biomolecules during the GIT. Therefore, owing to the richness in BCs, the specific aims of this research work were to evaluate the stability of sugar, organic acids and phenolics as well as antioxidant activity throughout the GIT, and the potential prebiotic activity and safety in Caco cells.

2. Material and methods

2.1. Chemicals and reagents

ABTS diammonium salt (2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), anhydrous sodium carbonate (Na_2CO_3), Folin-Ciocalteu's reagent, hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchase from Merck (Algés, Portugal). α-amylase, bile salts pancreatin, calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), pepsin and sodium hydrogen carbonate (NaHCO$_3$) and standards of gallic acid, caffeic acid, β-carotene (HPLC ≥95%, synth., cryst.), ferulic acid, 4-hydroxybenzoic,

protocatechuic acid, vanillic acid and ammonium acetate, *D*-(+)-glucose, potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), sulphuric acid (H_2SO_4), as well as the solvents have been used for HPLC analysis (formic acid, hexane and methanol) which were purchased from Sigma-Aldrich (Sintra, Portugal) while hydroxytyrosol, luteolin, luteolin-7-glycoside, luteolin-6-glycoside, naringenin-7-glycoside, naringenin, tyrosol, from Extrasynthese (Lyon, France).

2.2. Preparation of melon peel juice (MPJ)

Melon peels were processed as described previously by Gómez-García et al. (2021b) and split in two fractions, fresh solid fraction (SF) and liquid fraction (LF), employing a commercial juice machine (HR1869/8, 900 W, Philips). The SF fraction was manually pressed to recover the liquid excess, such liquid was mixed with the initial LF and then centrifuged (11469 *g*, 15 min, 4 °C). The clarified supernatant represented at least 76.78% on a fresh weight, this fraction was called as melon peels juice (MPJ). The MPJ was collected and stored at −80 °C until its draying process by lyophilisation, obtaining a fine powder with a yield of 6.00% on a dry weight.

2.3. In vitro simulated gastrointestinal digestion

To study the impact of gastrointestinal tract (GIT) upon the stability of bioactive compounds of melon peel extract, *in vitro* simulation of GIT was performed according to the method previously described by Madureira et al. (2011) with some modifications. Samples were prepared in three independent experiments by mixing 1 g of lyophilised MPJ in 20 mL of ultra-pure water. Digestion was simulated by the action of different enzymes, while for the intestinal absorption process was simulated by dialysis. All enzyme and reagents solutions were prepared immediately before usage and kept in an ice bath during gastrointestinal digestion. A water bath at 37 °C was used to simulate the temperature of the human body whereas peristaltic movements were imitated by mechanical agitation using intensities similar to those observed in vivo at each digestive phase. At the end of each GIT phase (oral, stomach and small intestine), aliquots of the digestion mixtures were taken and frozen stored until their analysis of bioactive compounds and antioxidant activity. The TPC, individual phenolics, sugars and organic acids as well as antioxidant activity (ABTS and DPPH) were measured before and after exposure to the simulated digestion conditions using the methodologies described above.

2.3.1. Mouth simulated digestion

The initial pH of the samples was adjusted between 5.6 and 6.9 using NaOH 0.1 M. The oral digestion was performed with 0.6 mL of α-amylase solution (100 U/mL) and the incubation took place for 1 min at 37 °C and 180 rpm.

2.3.2. Stomach simulated digestion

For gastric digestion, the pH of the samples was adjusted to 2.0 using HCl 1M. Pepsin at 25 mg/mL was added at a rate of 0.05 mL/mL of sample to simulate the gastric juice. The mixture was incubated during 1 h in a shaking bath at 37 °C and 130 rpm.

2.3.3. Gut simulated digestion

Small intestinal digestion was performed by adjusting pH to 6.0 with NaHCO$_3$ 1 M. The intestinal juice was simulated by dissolving and mixing 2 g/L of pancreatin and 12 g/L of bile salts; the mixture was added at a concentration of 0.25 mL/mL of sample. The solution was then incubated for 2 h, at 37 °C and 45 rpm, to mimic a long intestine digestion.

2.3.4. Small intestine absorption – dialysis

In the last phase of intestinal digestion, the sample solution was transferred into a cellulose acetate dialysis tube with a molecular weight

cut-off of 3 kDa (Spectra/Pro, Spectrum Lab, Breda, Netherlands) to reproduce the natural absorption step in the small intestine. Then, the membranes were immersed in a regularly renewed water container at room temperature, during 24 h and stirred at 1000 rpm to maintain homogeneity. At the end of the process, the solution that managed to diffuse the dialysis tubing represents the sample that is available for absorption (blood-available), and the solution left inside the dialysis tubing represented the non-absorbable sample (colon-available).

2.4. Accessibility and stability of melon bioactive compounds through in vitro gastrointestinal digestion

2.4.1. Recovery and accessibility index

To analyse the effect on the MPJ powder sugar, organic acid and phenolic contents along *in vitro* digestion, two different percentage indexes were studied: recovery (RI%), accessibility indexes (ACI%). The % RI measures the amount of phenolic compounds present in the digested food material after mouth, gastric and intestinal digestion, according to the equation (1):

$$Recovery\ index\ (RI\%) = \frac{BC_{DS}}{BC_{BD}} * 100 \qquad (1)$$

where, BC_{DS} is the bioactive content (mg/100 g DM) in the digested sample (DS) and BC_{BD} is the bioactive content (mg/100 g DM) quantified in the sample before digestion (BD) (undigested). Bioactive compounds, to exert their effects must be accessible and released from the food matrix and maintain their bioactive form, despite the reactions that might take place in the GIT. Accessibility is defined as the percentage of the bioactive compound solubilized after intestinal dialysis step; this index is the fraction of the bioactive compound that could become available for absorption into the blood system (equation (2)):

$$Accessibility\ index\ (ACI\%) = \frac{BC_{BS}}{BC_{DS-IP}} * 100 \qquad (2)$$

where BC_{BS} is the bioactive content (mg/100 g DM) at the intestinal absorption phase (bloodstream (BS)) and BC_{DS-IP} is the bioactive content (mg/100 g DM) in the digested sample at the intestinal phase (DS-IP).

2.5. Total phenolic content (TPC)

The total phenolic content of samples were determined by the Folin-Ciocalteu method with some modifications (Singleton & Rossi, 1965). In a 96-well plate, aliquots of 20 µL of samples were mixed with 80 µL of Folin–Ciocalteu reagent previously diluted 1:10 (v/v) in water and 100 µL of 7.5% (w/v) sodium carbonate. After 1 h of incubation at room temperature in darkness the absorbance was measured at 750 nm using a microplate reader (Synergy H1, Vermont, USA). Gallic acid was used as standard and results were expressed as mg gallic acid equivalents (GAE)/100 g of dry matter (DM). All measurements were performed in triplicate for each experiment.

2.6. Antioxidant activity determination by ABTS and DPPH

The ABTS scavenging activity of samples were determined as described by Arnao, M., Cano, A., and Acosta (2001). Briefly, the ABTS●+ radical stock solution was prepared by mixing ABTS●+ (7 mM) with potassium persulfate (2.5 mM) in ultra-pure water and kept in stirring at room temperature for 16 h. The solution ABTS●+ was diluted with water until reach an absorbance of 0.700 ± 0.020 at 734 nm (Synergy H1 microplate reader, Vermont, USA). After, in a 96-well plate 15 µL of the sample extract were allowed to react with 200 µL of ABTS●+ solution for 6 min in the dark and the absorbance was immediately recorded at 734 nm. The standard curve was made with L-ascorbic acid (AA) (0.05–0.5 mg/mL). All experiments were in triplicate and expressed in mg ascorbic acid equivalents (AAE)/100 g DE. The

DPPH●+ assay was performed according to the method of Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995) with some modification. A stock solution (600 µM) was prepared by dissolving 24 mg of DPPH in 100 mL of pure methanol. The DPPH solution was diluted until a concentration of 60 µM and adjusted to a final absorbance of 0.600 ± 0.100 at 515 nm (Synergy H1 microplate reader, Vermont, USA). After, in a 96-well plate 25 µL of sample were mixed with 175 µL of DPPH solution. The reaction mixture was kept at room temperature for 30 min in the dark and the absorbance was then measured at 515 nm. Trolox was used as a standard for the preparation of a calibration curve (0.005–0.08 mg/mL). The results were triplicated and expressed in µM of trolox equivalents (TE)/100 g DE.

2.7. Identification and quantification of polyphenols by HPLC

Polyphenolic profile of melon peel extract was acquired by High Performance Liquid Chromatography coupled to diode-array detector (HPLC-DAD), according to the method described by Campos et al. (2020) with some modifications. Samples were injected into Waters Series e2695 Separation Module System (Mildford MA, USA) interfaced with the UV/Vis photodiode array detector (PDA 190–600 nm). Separation was performed in reverse-phase column (COSMOSIL 5C1 8-AR-II Packed Column – 4.6 mm I.D. × 250 mm: Dartford, UK). Chromatographic separation of phenolic compounds was carried out with mobile phase A – water/methanol/formic acid (92.5:5:2.5, v/v/v) – and mobile phase B – methanol/water/formic acid (92.5:5:2.5, v/v/v) under the following conditions: 50 µL were injected at continuous flow of 0.5 mL/min, gradient elution starting at 100% mobile phase A for 50 min, then gradient reset at 45% A and 55% B between 50 and 55 min. Finally, the mobile phase A returns to 100% and remains at this percentage for 4 min (until 59 min). Detection was achieved at wavelengths ranging from 200 to 600 nm and, data acquisition and analysis were accomplished with Software Empower 3. Polyphenolic compounds were identified and quantified by external calibration curve by comparison with pure standards, comparing retention times, UV absorption spectra and peak areas. All determinations were made in triplicate and the results were expressed as mg of phenolic compounds/100 g of DE.

2.8. Identification and quantification of sugars and organic acids by HPLC

The chromatographic separation was carried out using a Beckman Coulter HPLC equipment coupled to IR (K-2301) and UV detector (K-2501) (Knauer, Berlin, Germany). The aliquots taken from the GIT process at before mouth, mouth, stomach, small intestine and after dialysis were filtered (0.45 µm cellulose acetate membrane) and then 20 µL of sample were analyzed using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) operated at 40 °C with 5 mM H_2SO_4 as mobile phase at constant flow of 0.6 mL/min. Data acquisition and analysis were accomplished using Clarity software. The detection of simple sugars and organic acids was obtained by the IR and UV detectors, respectively, as reported previously by Gómez-García et al. (2022b). The peaks from each sample were identified and quantified by comparison of retention time and by using calibration curves of each standard of lactic, formic, acetic, succinic, and propionic acids. All determinations were made in triplicate.

2.9. Prebiotic potential

The MPJ powder before and after GIT passage was tested for potential of prebiotic activity using the *in vitro* methods reported by Gullon et al. (2015) and Costa et al. (2019) using pure probiotic through the evaluation of growth curves using microplate assay.

2.9.1. Growth curves via microplate assay

Potential prebiotic effect of MPJ powder was determined for

Bifidobacterium animalis Bo (CSK, Ede, Netherlands), *Bifidobacterium animalis* spp. *lactis* Bb12, *Lactobacillus casei* 01 (Chr. Hansen, Hørsholm, Denmark) and *Lactobacillus acidophillus* LA-5 (Lallemand, Montreal, Canada). Strains were stored at −80 °C in de Man–Rogosa–Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) with 30% (v/v) glycerol. *L. casei* 01 and *L. rhamnosus* R11 inoculum were prepared by suspending each bacterial colony into De Man, Rogosa and Sharpe (MRS) broth, achieving a turbidity equivalent to 0.5 McFarland standard, and then diluting to reach the recommended concentration of probiotic bacteria in wells, 5×10^5 CFU/mL. Twenty microliters of inoculum was transferred to 96-well microplate and a final volume of 200 μL completed with MRS broth without glucose with MPJ at concentrations of 1 and 2% (w/v). Microplate was incubated at 37 °C for 24 h with agitation. *B. animalis* Bo and *B. lactis* BB12 inoculuns were prepared under anaerobic atmosphere, in MRS broth supplemented with 0.05% (v/v) L-cysteine-HCl, achieving a final turbidity equivalent to 0.5 McFarland standard (1×10^8 CFU/mL), and then diluted to reach the recommended concentration of probiotic bacteria in wells (5×10^5 CFU/mL). Twenty microliters of each inoculum were transferred to a 96-well microplate and every well was fulfilled (to final volume of 200 μL) with the MPJ diluted in basal MRS broth without glucose at concentrations of 1 and 2% (w/v). Microplates was sealed with paraffin and incubated at 37 °C for 48 h with agitation, with absorbance measurements at 660 nm registered every hour. Three samples were performed: i) Positive control, containing inoculum and MRS broth, ii) Sample containing the solubilized MPJ in MRS broth without glucose and iii) Negative control; containing only MRS broth.

2.10. Evaluation of cytotoxicity: metabolic activity assay

2.10.1. Cell line growth conditions

Human epithelial cells obtained from European Collection of Authenticated Cell Cultures (ECACC), Caco-2 (ECACC 86010202) were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, as monolayers using Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine without pyruvate (Gibco, Thermo Scientific, Waltham, MA, USA) containing 10% (v/v) of fetal bovine serum (FBS, Biowest, Nuaillé, France), 1% (v/v) of Penicillin-Streptomycin-Fungizone (Lonza, Verviers, Belgium) and 1% (v/v) of non-essential amino acids (Gibco, Thermo Scientific, Waltham, MA, USA.

2.10.2. Prestoblue assay

Prestoblue assay was performed according to the Thermo scientific guidelines. In brief, cells were seeded at 1.0×10^5 cells/mL into wells of 96-well tissue culture plates. After 24 h, the media was removed and replaced by 90 μL of the sample in the correspondent dilution, DMSO (dimethyl sulfoxide) was used as a negative control, the fresh media is used as a positive control (cell in normal growth conditions). After 24 h, 10 μL of prestoblue reagent was added, and the plates were incubated for 1h in the dark at 37 °C. The plate content was transferred to a black microplate and the fluorescence was read. The metabolic inhibition was calculated according to the equation (3):\

$$\% \text{ Metabolic Inhibition} = \frac{\text{Absorbanse positive control} - \text{Absorbanse sample}}{\text{Absorbanse positive control}} * 100$$

(3)

2.11. Statistical analysis

SPSS Statistics (version 23) was used to carry out the statistical analyses. All expriments were carried out in triplicates, and data were reported as mean ± standard deviation. Shapiro - Wilk test tested the normality of data distribution ($p < 0.05$). The differences of mean values among a concentration of bioactive compounds or bioactivities obtained in the each phase of the *in vitro* GIT were analyzed by one-way analysis of variance (ANOVA). The homogeneity of variances was assessed by Levene's test, and the multiple comparisons were made at those statistically significant variables using the Tukey's posthoc test at the $p < 0.05$ significance level.

3. Results and discussion

3.1. Stability and accessibility of bioactive compounds of melon peel juice during in vitro GIT digestion

3.1.1. Soluble sugars

Results of soluble sugars of MPJ powder through GIT simulation are depicted in Fig. 1a. Soluble sugars changed after the GIT digestion, where fructose (47.70 g/100 g DM) was the most representative sugar, followed by glucose (33.12 g/100 g DM) and sucrose (3.22 g/100 g DM). In the mouth, the content of fructose, glucose and sucrose was not significantly effected (p < 0.05), exhibiting a stable recovery index (RI %) (98.22%, 94.19% and 73.09%, respectively) then in stomach, fructose and glucose surfed a substantial decrease of 72.61% and 68.23%. All the sugars, fructose, glucose and sucrose exhibited a significant reduction in the small intestine (47.14%, 43.46% and 37.28%, respectively) and then reaching 42.67%, 37.66% and 35.39%, respectively, during the simulated intestinal absorption (bloodstream) (Table 1). On the other hand, the accessibility index (%ACI) for all three sugars was around 85–90% (ACI), becoming accessible for absorption to be used by the body (Table 1). Carbohydrates ingested can be absorption in intestinal mucosas, metabolized by the liver and, finally transported to working muscles for oxidation (Malone et al., 2021). The changes in the RI% values could be explained due to several factors, including enzymatic action and pH changes which can cause chemical modifications in the carbohydrates. The isomerisation of glucose into fructose also could justify the higher RI% of fructose than glucose (Ribeiro et al., 2020). Similar decrease behaviours during GIT simulation were observed on sugars (fructose and glucose) from olive pomace powder, starting from 59 to 62% in the mouth, then 36–47% at stomach and 30–40% in the small intestine, respectively (Ribeiro et al., 2020). Despite the significant variations of sugar contents of MPJ powder throughout the GIT phases, these compounds still exhibited good percentages of recovery and accessibility, which could remark their importance as natural resource of bioactive compounds with great stability after their consumption. In this regard, some available reports highlighted that fructose reduces post-prandial peak blood glucose, especially overweight or obese people, and in people with impaired glucose tolerance (type 1 diabetes, and type 2 diabetes) and also combinations of glucose and fructose intake have indeed been reported to increase performance during exercise (Zafar et al., 2021). Traditionally, the effects of ingested carbolydrates on exercise capacity have been attributed to the prevention of hypoglycaemia, muscle oxiation and central stimulation (Rosset et al., 2017). Usually, only about of 5–30% of the ingested sugars and sweeteners reaching the large intestine due to the absorption of these molecules in the small intestine through sugar transporters. Hence, compared to the large intestine, the small intestinal is enriched ten times higher in sugars and sweeteners. These sugars are important subtracts for microbes as they possess carbohydrate uptake and utilization genes and transcripts with respect to microbes in the large intestine (Di Rienzi & Britton, 2020). Fructose, sugar alcohols, and some sweeteners (e.g., sucralose) are passively, slowly, or very poorly absorbed in the small intestine. Up to 30–90% of these sugars and sweeteners pass into the large intestine. The exact amounts of sugars/sweeteners that reach the large intestine, however, is difficult to generalize across individuals because between females and males display considerable variation in their absorptive capacity for a sweetener or sugar. Thus, assessing simple sugars behaviors from MPJ at each stage of the GIT phases could allow to find significant indicators to be exploited as prototype of functional powder rich in natural compounds, which have beneficial effects to the human health.
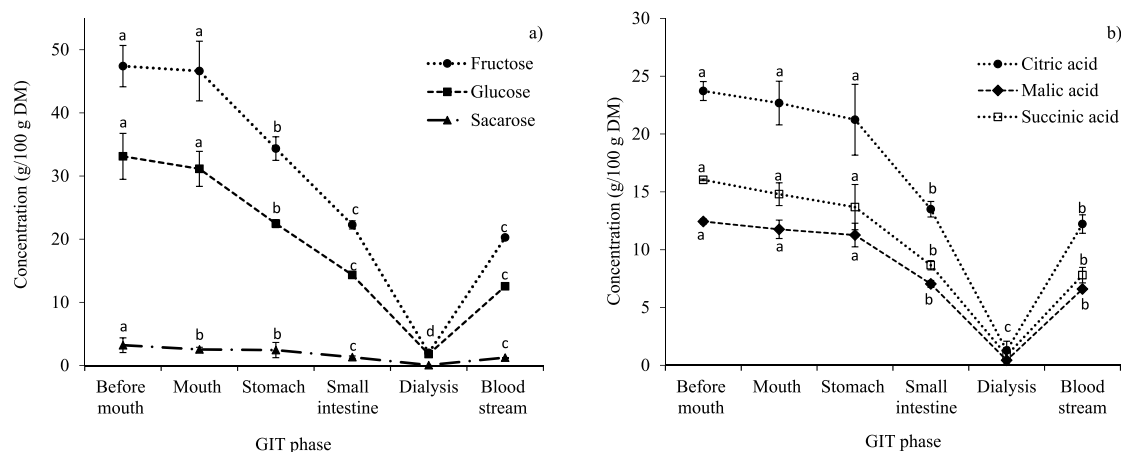
Fig. 1. Concentrations of a) soluble sugars and b) organic acids obtained after each phase of the *in vitro* gastrointestinal tract simulation (GIT). DM: dry matter. Results are the means of three independent determinations ± standard deviation. Values with different letters indicate significant differences between each GIT stage, as determined by one-way ANOVA test and Tukey's test ($p < 0.05$).

Table 1
Recovery index (RI %) and Accessibility index (ACI%) of phenolic compounds and antioxidant activity throughout GIT simulation from melon peel juice (MPJ). Results are the means of three independent determinations ± standard deviation. Values with different letters in the same line to RI% and the same column to BI% indicate significant differences between each GID stage, as determined by one-way ANOVA test and Tukey's test ($p < 0.05$).

Bioactive compound	Recovery index (RI %)					Accessibility index (ACI %)
	Mouth	Stomach	Small Intestinal	Dialysis	Blood stream	
Sugars						
Fructose	98.22 ± 3.15[a]	72.61 ± 3.55[b]	47.14 ± 3.79[c]	4.47 ± 0.87[d]	42.67 ± 3.26[c]	90.56 ± 1.48[a]
Glucose	94.19 ± 3.71[a]	68.23 ± 5.38[b]	43.46 ± 4.73[c]	5.98 ± 2.14[d]	37.66 ± 2.97[c]	86.51 ± 3.59[a]
Sucrose	73.09 ± 7.31[a]	71.01 ± 5.70[a]	37.28 ± 2.68[b]	1.88 ± 0.16[c]	35.39 ± 3.74[b]	91.91 ± 0.81[a]
Organic acids						
Citric	95.82 ± 4.02[a]	89.44 ± 9.98[a]	57.00 ± 1.66[b]	5.36 ± 0.82[c]	51.63 ± 2.46[b]	90.56 ± 1.73[a]
Malic	94.76 ± 4.20[a]	90.62 ± 6.49[a]	56.66 ± 1.18[b]	3.56 ± 0.64[d]	53.10 ± 0.55c	93.73 ± 1.01[a]
Succinic	92.51 ± 5.74	85.29 ± 11.02	54.02 ± 0.82	5.19 ± 3.73	52.11 ± 11.61	92.95 ± 10.79[a]
Phenolics						
Gallic acid	103.72 ± 1.92[a]	101.09 ± 1.18[a]	91.48 ± 0.69[a]	18.17 ± 1.98[c]	73.30 ± 1.58[b]	80.14 ± 2.07[a]
4-hydroxybenzoic	91.08 ± 11.58[a]	89.56 ± 15.36[a]	68.32 ± 7.54[ab]	13.22 ± 1.68[c]	55.10 ± 8.85[b]	80.35 ± 4.52[a]
Luteolin-6-glycoside	97.77 ± 1.95[a]	101.30 ± 0.74[a]	76.54 ± 1.42[b]	57.20 ± 1.00[d]	57.20 ± 1.00[c]	74.74 ± 0.07b
p-Coumaric acid	92.99 ± 2.85[b]	130.85 ± 10.76[a]	39.28 ± 3.35[c]	UQ	15.96 ± 1.06[d]	40.84 ± 4.65[c]
Ferulic acid	106.41 ± 10.71[a]	104.58 ± 9.40[a]	72.67 ± 5.26[b]	52.50 ± 4.73[c]	16.60 ± 2.81[d]	22.74 ± 2.27[d]
TPC (mg GAE/100 g DM)	94.56 ± 7.51[a]	93.99 ± 2.22[a]	65.31 ± 1.60[b]	12.54 ± 2.20[c]	55.35 ± 3.43[b]	81.89 ± 2.36[a]
DPPH (μM TE/100 g DM)	62.71 ± 18.15[a]	59.65 ± 11.69[a]	45.91 ± 14.77[ab]	17.31 ± 4.51[b]	36.02 ± 11.07[ab]	54.07 ± 6.96[b]
ABTS (mg AAE/100 g DM)	75.26 ± 8.31[a]	58.67 ± 7.90[a]	39.77 ± 7.67[b]	12.20 ± 0.64[c]	44.32 ± 18.73[b]	76.55 ± 3.62[a]

UQ: under quantification limit. All determinations were carried out in triplicate and results are shown as mean value ± standard deviation. Results are the means of three independent determinations ± standard deviation.

3.1.2. Organic acids

Results of the organic acids profile of MPF through GIT simulation are depicted in Fig. 1b. Three organic acids were identified by HPLC-UV, where citric acid was the most prominent organic acid with a value of 23.72 g/100 g DM followed by succinic (16.04 g/100 g DM) and malic acid (12.44 g/100 g DM). The content of all these organic acids decreases significantly during the GIT digestion (Fig. 1b). The highest reduction of organic acids took place in the stomach and small intestine because of severe acidic conditions, reaching RI% values of 89.44%, 90.62% and 85.29%, following a decrease in the small intestine of 57.00%, 56.66% and 54.02%, and at the end, having values for intestinal absorption of 51.63%, 53.10% and 52.11% for citric, malic and succinic acids, respectively (Table 1). These results are in concordance with previous reports indicating that melon fruit and its parts possess organic acids, highlighting their relevant presence for fruit sweetness and quality at sugar levels ranging from 1.5 to 3.5 g/100 g Fresh Weight (FW), as well as 0.25–9.89 g/100 g FW for organic acids contents (Huang et al., 2017; Obando-Ulloa et al., 2009; Tang et al., 2010). Despite the significant changes of the organic acids contents of MPJ

powder during the GIT phases, these compounds still exhibited stable percentages of recovery and accessibility, which could remark melon peels as an important natural resource of bioactive compounds with great stability after their intake. For example, citric acid is a natural antioxidant with high industrial value, which around 70% of its total production is used in the food and beverage industry for various purposes, 12% in pharmaceuticals and about 18% for other industrial uses (Di Rienzi & Britton, 2020). Moreover, citric acid extract from *Garcinia cambogia* was used as a plant-based drug to treat non-alcoholic fatty liver, which is the most common liver disease worldwide, which has the potential to progress to fibrosis cirrhosis and hepatocellular carcinoma (Nomi-Golzar et al., 2021). Also, succinic acid and its salts (succinates) are important organic compounds that have been shown several health benefits that positively affect the oxygenation of the internal environment, stabilize the structure and functional activity of mitochondria, and normalize the ion metabolism in the cell and having low toxicity succinic acid has well-manifested antioxidant, immunostimulating, adaptogenic properties (Lieshchova et al., 2020). Hence, measuring organic acids stability and accessibility from MPJ at the different

physiological phases of the GIT could allow the identification of key markers for the evaluation as a prototype of functional ingredient rich in molecules that promote health and well-being.

3.1.3. Polyphenols and antioxidant activity during in vitro gastrointestinal digestion

To evaluate the stability of polyphenols of MPJ powder throughout the digestive tract, the sample was submitted to a simulated gastrointestinal (GIT) digestion and evaluated for its total phenolic content (TPC) and antioxidant activity after each gastrointestinal phase. The results presented in Fig. 2a demonstrate that the TPC of the MPJ progressively decreased after each step from the GIT simulation. The TPC of MPJ was slightly kept from before mouth until the stomach with RI of 90–95% and then a significant decrease was observed after the small intestine phase ($p < 0.05$), exhibiting RI of 65%. On the other hand, the TPC showed to be more accessible for absorption in the bloodstream (80.14 %ACI) (Table 2). Similar negative decreases were observed on olive pomace TPC when exposed to GIT digestion at gastric and intestinal phases (Ribeiro et al., 2020). Additionally, during GIT conditions, pH has a key role as a protector of polyphenols against degradation in the stomach (acidic conditions) and as a promotor of degradation in the small intestine (mild alkaline conditions). On the other hand, the antioxidant activity determined by ABTS (r = 0.85) and DPPH (r = 0.80) was correlated with the TPC and significatively decreased from initial value of 738.18 to the lowest value 287.73 mg AEE/100 g DM and 1098.27 to 431.59 µM TE/100 g DM at small intestine phase, respectively (Fig. 2a.). These behaviours are in agreement with the TPC decrease in this phase where the conditions are more susceptible to polyphenols degradation and chemical modifications, losing the potential bioactivity. Likewise, some reports on TPC and antioxidant activity decreases have been associated a direct connection between phenolics inhibition/degradation during GIT and loss of their bioactive properties (Sánchez-Gutiérrez et al., 2022). For example, *F. vesiculosus* phlorotannins extracts exhibited a significative decrease of the TPC from the undigested sample to the small intestine (9.93–5.15 mg PGE/g extract, respectively) (Catarino et al., 2021); Date pits and apple bagasse flours also showed decreases in their TPC, obtaining 80.49% and 89.64% at mouth phase followed by 66.90% and 83.38% at gastric phase and finally after intestinal 46.02% and 80.05% of recovery index, respectively (Gullon, Pintado, Barber, et al., 2015). Regarding antioxidant properties, the same authors reported a slight increase (higher than undigested sample) at mouth step for ABTS (10.1% and 1.09%) and DPPH (16.4 and 2.8%), but a significative decrease (below than initial value) were observed at gastric (ABTS = 18.5% and 4.72%; DPPH =

8.2% and 6.27%) and intestinal digestion (ABTS = 38.5 and 36.8%; DPPH = 5.6% and 2.7%) for date pits and apple bagasse, respectively. The drop in TPC of the samples after the mouth digestion could be explained by possible interactions occurring between polyphenols and the salivary proteins. Indeed, such interactions are very well described for plant polyphenols and very relevant for the development of important sensory characteristics of certain foods and beverages (Wojtunik-Kulesza et al., 2020). Furthermore, these decreasing behaviours could be explained as well due to in the case melon peel powder polyphenols are not protected or mixed within a complex matrix, being more exposed and therefore more susceptible to modification, losing their exerting beneficial bioactive properties. Similar behaviours have been previously reported for phenolics and are on the basis of the delivery strategies in which phenolic compounds are loaded withing solid micro- or nano-carriers (encapsulation) in order to resist the gastrointestinal conditions and reach intact without modifications for intestinal absorption (Campos et al., 2015; Madureira et al., 2016). On the other hand, individual phenolic compounds of MPJ powder before and after *in vitro* GIT digestion were identified and quantified by HPLC-DAD (Fig. 2b). In this context, ten individual phenolic compounds were identified and at least five were quantified, where *p*-Coumaric acid, 4-hydroxybenzoic and luteolin-6-glycoside were the most noticeable compounds found in MPF (42.27, 35.02 and 24.09 mg/100 g DM, respectively) (Table 2). In addition, only *p*-Coumaric showed a substantial increment until 55.2 mg/100 g DM (130% RI) at the stomach phase, being more accessible for absorption in the bloodstream and exert its antioxidant properties, reaching 80.35% ACI, while the others phenolics were decreased throughout the GIT (Table 1). Similar results were described in coffee phenolic extracts where chlorogenic and cryptochlorogenic acids exhibited 50% and 80%, respectively, and Di-O-caffeoylquinic acids derivatives at intestinal phase exhibited very low RI % (less than 10%) (Vilas-Boas et al., 2020). Moreover, phenolics such as gallic, *p*-Coumaric acid, 4-hydroxybenzoic, ferulic acids have been widely studied due to their high associated antioxidant activity feature: (i) ability to scavenge free radical, (ii) reduction or chelation of transition metal ions or (iii) inhibition of lipid peroxidation (Govea-Salas et al., 2016; Lourenço et al., 2021; Olubunmi et al., 2019). Also, gallic, 4-hydroxybenzoic, luteolin-6-glycoside ferulic acids presented stable RI% at intestinal absorption (blood stream) (73.30%, 55.10%, 57.20% and 16.60%, respectively) and ACI% (80.14%, 80.35%, 74.75% and 22.74%, respectively) (Table 2). The present findings could suggest that most of the phenolics and their related antioxidant activity present in MPJ powder decreased throughout the GIT process, however, they kept stable percentages of ACI (50–80%), which could reinforce its
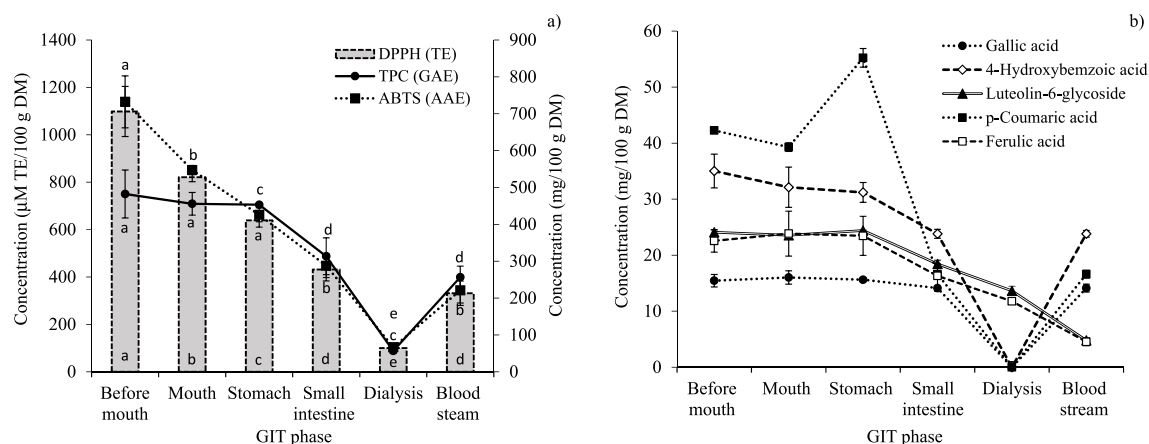
Fig. 2. a) Total phenolic content (TPC) and antioxidant activity behaviours and b) concentrations of the main individual phenolic compounds identified by HPLC after each phase of the *in vitro* gastrointestinal tract (GIT). DM: dry matter; TPC: total phenolic content; GAE: gallic acid equivalents; AAE: ascorbic acid equivalents; TE: trolox equivalents. Results are the means of three independent determinations ± standard deviation. Values with different letters indicate significant differences between each GIT stage, determined by one-way ANOVA test and Tukey's test ($p < 0.05$).

Table 2

Main polyphenols identified and quantified by HPLC-DAD and antioxidant activity during the *in vitro* gastrointestinal tract simulation of melon peel juice (MPJ).

| No.	Phenolic compound	Gastrointestinal phase (concentration mg/100 g DM)					
		Undigested sample	Mouth	Stomach	Small Intestinal	Dialysis	Blood stream
1	Gallic acid	15.45 ± 0.11[a]	16.02 ± 0.20 [a]	15.62 ± 0.28 [a]	14.13 ± 0.01[b]	2.81 ± 0.29[d]	11.03 ± 0.29[c]
2	Hydroxytyrosol	UQ	UQ	ND	UQ	UQ	UQ
3	Tyrosol	UQ	UQ	ND	UQ	UQ	UQ
4	4-hydroxybenzoic	35.02 ± 3.01[a]	32.13 ± 6.59[ab]	31.21 ± 4.94[ab]	23.81 ± 1.77[bc]	4.63 ± 0.75[c]	17.18 ± 2.37[d]
5	Luteolin-6-glycoside	24.09 ± 0.39[a]	23.55 ± 0.11 [ab]	24.41 ± 0.57[b]	18.44 ± 0.11[c]	13.78 ± 0.07[d]	1.13 ± 0.68[d]
6	7-hydroxycoumarin	NQ	NQ	NQ	NQ	NQ	NQ
7	*p*-Coumaric acid	42.27 ± 1.36[b]	39.29 ± 0.77[b]	55.23 ± 3.05[a]	16.62 ± 1.67[c]	UQ	9.74 ± 0.26[d]
8	Luteolin-7-glycoside	NQ	NQ	NQ	NQ	NQ	NQ
9	Ferulic acid	22.56 ± 2.16[a]	23.85 ± 0.23[a]	23.46 ± 0.27[a]	16.32 ± 0.50[b]	11.77 ± 0.08[c]	3.78 ± 0.31[d]
10	Apigenin	UQ	UQ	ND	ND	NQ	NQ
Total by HPLC		139.39 ± 4.97	134.84 ± 7.42	149.92 ± 7.77	89.32 ± 3.26	32.99 ± 1.18	54.73 ± 4.73
TPC (mg GAE/100 g DM)		482.27 ± 65.32	455.70 ± 30.79	453.15 ± 20.83	313.37 ± 51.64	56.74 ± 6.87	256.63 ± 25.46

DM: dry matter; NQ: non-quantified; ND: non-detected; UQ: under quantification limit. Results are the means of three independent determinations ± standard deviation.

application as functional powder with health stimulating molecules.

3.2. Prebiotic potential

The prebiotic potentiality of MPJ powder was studied on four strains in basal MRS medium without glucose, at concentrations of 1 and 2% w/v (10 and 20 mg/mL, respectively). Farther, fructooligosaccharides (FOS) and glucose (Glu) were used as positive controls during 48 h of incubation. Fig. 3 reflects the growth profile of *Bifidobacteria* and *Lactobacillus* strains as measured by turbidity at 660 nm of optical density (OD), increasing their biomass concentrations along the first 20 h of incubation in most cases. The maximum OD at 660 nm is described in Fig. 4, and the top growth rates are listed in Table 3. As expected, Glu demonstrated to be the best carbon source for both *Bifidobacteria* and *Lactobacilli* strains, showing the maximum μ_{max} values among all the strains followed by FOS (see Fig. 3 and Table 3). Moreover, the MPJ powder was capable to influence the growth of all the probiotic bacteria positively as compared to FOS and Glu, increasing their growth (OD at

660 nm) along the first 12–15 h of incubation for *Lactobacilli* and 10–12 h for *Bifidobacteria*, achieving very similar growth rates when compared to Glu and FOS (p < 0.05). The MPJ at 2% (w/v) stimulated higher growth effects on tested bacteria than 1% (w/v). Additionally, there were no significant differences on the maximum growth of both *Bifidobacteria* strains used when the MRS medium was supplemented with Glu and higher growth values were obtained when compared to the FOS, except for both *Lactobacilli* strains that exhibited lower growth profiles (p < 0.05). In this regard, the growth of *L. casei* 01 on MPJ powder (2%) showed a maximum OD value at 27 h, lower than Glu, but higher value than FOS (1.30, 2.26 and 0.92, respectively) and lower growth rates (0.09 h^{-1}) than most of the tested substrates. The strain L. *acidophillus* LA-5 was also able to ferment all the substrates tested, showing higher maximum OD values on MPJ than FOS (0.93 and 0.80, respectively). As stated in Fig. 4, using MPJ as substrate *B. animalis* spp. *lactis* Bb12 and *B. animalis* Bo cell density reached maximum OD values of 2.24 and 2.16 at 29 and 32 h, respectively, but showed lower value than Glu (2.36) and higher than FOS (1.03), obtaining very similar μ_{max} of 0.18 h^{-1} for both
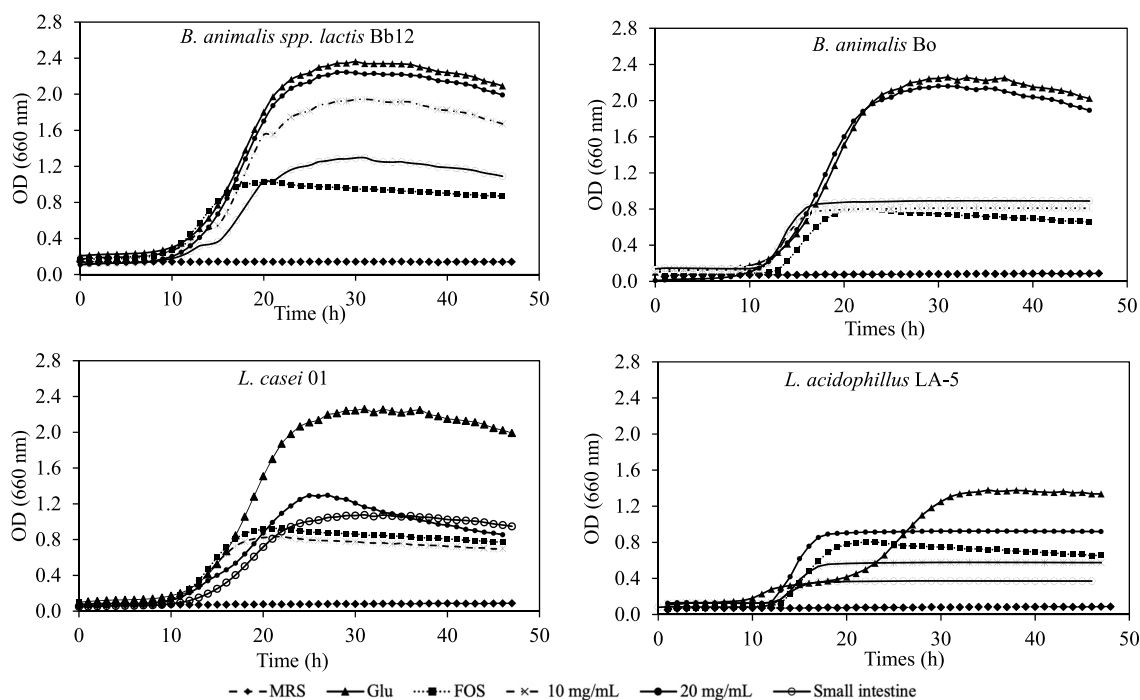
Fig. 3. Growth curves of *Bifidobacterium* and *Lactobacillus* probiotic strains in MRS media containing glucose (Glu), fructooligosaccharides (FOS) or melon peels juice (MPJ) at 10 and 20 mg/mL (1 and 2% w/v, respectively) during 48 h.
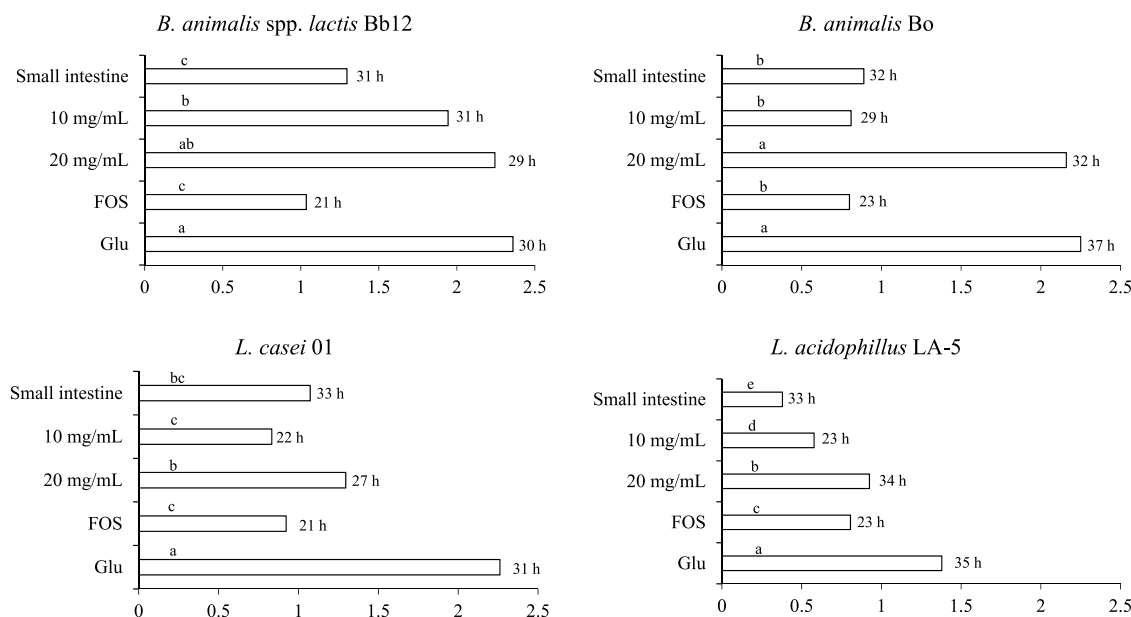
Fig. 4. Maximum optical densities (OD at 660 nm) at the corresponding incubation times in experiments with *Bifidobacterium* and *Lactobacillus* probiotic strains grown in media containing glucose (Glu), fructooligosaccharides (FOS) or melon peels juice (MPJ) at 10 and 20 mg/mL (1 and 2% w/v, respectively) during 48 h. Values with different letters in each line indicate significant differences between substrates within each microorganism determined by one-way ANOVA test and Tukey's test ($p < 0.05$).

Table 3
Maximum growth rates (μ_{max}, h^{-1}) of the tested probiotic bacteria strains grown in media containing MRS media containing glucose (Glu), fructooligo-saccharides (FOS) or melon peels juice (MPJ) at 10 and 20 mg/mL (1 and 2% w/v, respectively).

Probiotic strains	Maximum growth rate (μ_{max}, h^{-1})				
	Positive Control (w/v)		MPJ (w/v)		
	GLU 2%	FOS 2%	2%	1%	SI
Bifidobacterium animalis ssp. *lactis* Bb12	0.18	0.18	0.18	0.14	0.08
Bifidobacterium animalis Bo	0.18	0.11	0.18	0.13	0.10
Lactobacillus casei 01	0.18	0.10	0.09	0.09	0.08
Lactobacillus acidophilus LA-5	0.10	0.11	0.15	0.09	0.06

The expressed values are the equation slope, which (m) means maximum growth rate.

strains which means that MPJ at 2% (w/v) could be used as carbon source and therefore as a prebiotic enhancer. Beyond, to evaluate the suitability of MPJ as food ingredient rich in beneficial compounds such as fermentable carbohydrates for probiotic bacteria growth, it was assessed the stability of its prebiotic effect upon gastrointestinal physiological conditions, most specifically after small intestine. Results (depicted in Fig. 3 - continuous line with a circle in the middle) indicated that the gastrointestinal conditions negatively affect the prebiotic effect of MPJ when compared to the non-digested/intact melon sample (20 mg/mL), however, despite the negative decrease of its prebiotic potential, it still exhibited stable growth values for the four probiotic strains, achieving similar growth rates (μ_{max}, h^{-1}) when compared to FOS (Table 3). This prebiotic effect of MPJ exhibited for *Bifidibacterium* and *Lactobacillus* strains could be attributed to the content of simple sugars such as glucose and fructose (as described previously in section 3.1.1), which could be used as a carbon source by the probiotic bacteria for their metabolism and growth enhancement. These probiotic bacteria are heterofermentative, which can ferment monosaccharides such as glucose and fructose (among other carbohydrates) for their metabolism/

growth through the conversion of these simple sugars into intermediates of the hexose fermentation pathway, also called fructose-6-phosphate shunt or 'bifid' shunt and subsequently converted to short-chain fatty acids (SCFAs), which may be beneficial to the host (Pokusaeva et al., 2011; Zareba et al., 2012). These results indicated that melon substrates can promote the growth of all tested strains, with the exception of LA-5. The results obtained for the LA-5 are somehow different from the results obtained for *L. acidophilus* La3 using glucose and citric pectin, having similar OD (1.78 and 1.27, respectively) and μ_{max}, h^{-1} (0.21 and 0.09, respectively) (Hurtado-Romero et al., 2021). Overall, the results here discussed indicate that MPJ can stimulate a fast growth of different probiotic strains, possibly due to the presence of glucose and other compounds. These growth enhancement effects of MPJ on probiotic bacteria are related to the improvement of gastrointestinal functions as well as increase or change in the composition of short-chain fatty acids, increased faecal weight and mineral absorption, immune stimulation and decreased colonic pH values.

3.3. Cytotoxicity

In order to obtain a cytotoxic profile of the MPJ and fulfil with the trend and demand of non-toxic and safe plant-based ingredients, the toxicity of MPJ powder before and after intestinal digestion was evaluated through cell proliferation assay upon Caco-2 intestinal cells, using extracts concentrations of 1 and 2% (w/v), as these were the concentrations that showed potential bioactivities. The Fig. 5 presents the metabolism inhibition of Caco-2 cells in the presence of MPJ and the sample from intestinal digestion. The prestoblue cell proliferation assay effectively measures cell growth and drug sensitivity in tumor cell lines. The sample of MPJ at 1% promoted Caco-2 cells metabolism by 12.34 ± 2.45%, while for 2% by 17.53 ± 3.12%. After the digestion of MPJ powder, it is possible to observe a slightly inhibition of cell metabolism, corresponding to 5.36 ± 1.97% and 8.09 ± 0.76% for samples after intestinal digestion at concentrations of 1% and 2%, respectively. In a recent work carried out by Placines et al. (2020) was highlighted the importance to assess the potential toxic effects of a vegetable product to assure its safety for human consumption to increase industrial and
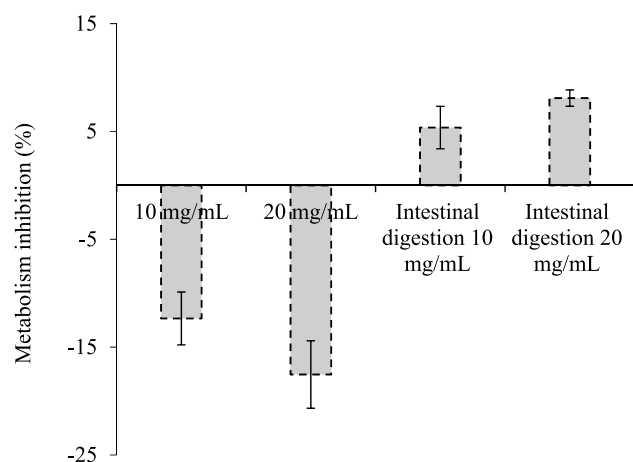
Fig. 5. Metabolism of Caco-2 intestinal cells upon the presence of MPJ extract at concentrations of 10 and 20 mg/mL (1% and 2% (w/v), respectively) and after intestinal digestion.

biotechnological interest. They reported a very low cytotoxicity on *in vitro* models using mammalian cell lines of novel natural extracts developed from *Cakile maritima* Scop. Della-Valle et al. (2020) remarked the importance to assess cytotoxic activity tests against prostate cancer line 3 cells of natural extracts containing fatty acids, phenolics and flavonoids from dried pepper (*Capsicum annuum* L.) and evaluate their protective effect on human health, showing good inhibitory values on cell growth assay with a rate of survival cells of 20%–40%. Therefore, it is possible to conclude that MPJ powder rich sugars, organic and phenolics acids is safe to be tested as vegetable food ingredient/additive at concentrations up to 2% w/v (20 mg/mL) but being limited to 3.5% w/v, where toxicity was observed (data not shown). It is also important to correlate the Caco-2 cells viability with the prebiotic potential when 2% (w/v) of MPJ powder was enough to proliferate probiotic bacteria strains as described in section 3.2.

4. Conclusions

These findings suggest that the melon peels juice powder might be used as potential food additive due to its high contents of bioactive compounds, including sugars, organic acids and phenolic acids as well as antioxidant properties that despite they decrease during the gastrointestinal tract (GIT) digestion, they showed good accessibility index 85–90% (sugars), 90–93% (organic acids) 80% (phenolics) and 54–76% (antioxidant activity), which allow to exert their related health benefits in the different steps of GIT for preventing some diseases caused by oxidative stress. In addition, this melon powder could be used as a prebiotic ingredient at 2% (w/v) establishing a potential carbon source that can be fermented by *Bifidobacterium* and *Lactobacillus*. However, profound studies are necessary to determine the possible synergic interactions of these melon by-products components, which could improve their related health benefits.

CRediT author statement

Ricardo Gómez- García: Roles/Writing - original draft; Visualization; Data curation.
Manuela Machado: Methodology; Validation; Data Curation.
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Débora A. Campos: Methodology; Visualization; Resources.
Cristóbal N. Aguilar: Supervision; Validation.
Ana R. Madureira: Resources; Supervision; Validation.
Manuela Pintado*: Conceptualization; Supervision; Investigation.

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