



Digestive fate of betalains and phenolics from *Opuntia* fruits and their interaction with human colonic microbiota in a dynamic *in vitro* gut model

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ABSTRACT

Opuntia cactus fruits are abundant in betalains and phenolic compounds, widely recognized for their health-promoting potential, yet studies simultaneously addressing their digestive fate and interaction with human gut microbiota remain limited. This research investigated the gastrointestinal digestion and colonic transformation of phytochemical-rich extracts obtained from three *Opuntia* varieties (*Opuntia stricta* var. *dillenii* whole fruit, *Opuntia ficus-indica* var. *Colorada* pulp, and *Opuntia ficus-indica* var. *Blanca Buenavista* peel) in a dynamic *in vitro* gut simulator. Betalain and phenolic compound transformation, microbial composition, short-chain fatty acid (SCFA) and ammonium production, and predicted functional capacity were analyzed. Betacyanins were the most labile compounds, with betanin retaining 46% after gastrointestinal digestion and becoming undetectable from the transverse colon onwards. Betaxanthins showed greater persistence, with indicaxanthin reaching the descending colon (7.66%). Picrodonic acid was the most stable phenolic, retaining up to 73% after gastrointestinal digestion and remaining detectable throughout all colonic compartments. *Opuntia* extracts induced extract- and compartment-dependent modulation of gut microbiota, including increases in Actinobacteriota and Bifidobacteriaceae (up to 22.58 and 22.78%, respectively), and reductions in Desulfovibrionaceae (up to -4.45%). Shifts in *Megasphaera* and *Bacteroides* were observed (up to -10.52 and -23.48%, respectively), while key functional taxa remained stable. All extracts enhanced SCFA production, particularly acetate (13.57–26.12 mM), butyrate (1.82–5.3 mM) and valerate (1.4–5.18 mM), while ammonium levels (11.23–13.08 mM) remained within physiological ranges. Functional prediction analyses associated phytochemical-related pathways with core species, supporting functional microbiota resilience. These findings reinforce the potential of *Opuntia*-derived phytochemicals to modulate colonic metabolic activity, reinforcing their value as functional ingredients within sustainable food systems.

1. Introduction

Human gut microbiome is increasingly recognized as a dynamic and complex ecosystem, constituted by multiple microorganisms that establish symbiotic relationships within the host (Hertli & Zimmermann, 2022). It plays a key role in maintaining health and modulating diseases, and it is highly responsive to external factors, such as dietary components, which can influence both their composition and metabolic activity (Yang et al., 2025). However, the biological effects of dietary bioactive compounds are also strongly conditioned by their

digestive fate along the gastrointestinal tract, which determines their stability, bioaccessibility, and the fraction that ultimately reaches the colon available for microbial metabolism. Among these components, plant-derived bioactive compounds are specially relevant, as their digestive transformation determines both their bioavailability and their subsequent interactions with the gut microbiota (Zheng et al., 2024).

Phytochemicals such as polyphenols, betalains, carotenoids, and alkaloids, are secondary metabolites produced by plants that, despite not being considered essential nutrients, exert significant biological effects on the organism (Dingeo et al., 2020). During gastrointestinal digestion,

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these compounds may undergo structural modifications due to pH changes, digestive enzymes, and interactions with the food matrix, which can alter their stability and bioactivity. The fraction that escapes absorption in the upper gastrointestinal tract can reach the colon, where it interacts with the gut microbiota. These interactions are bidirectional: phytochemicals can modulate microbial growth and activity, while microbial enzymes can transform them into metabolites with altered or enhanced biological properties (Parralejo-Sanz, Requena & Cano, 2025; Read & Holmes, 2017; Ross et al., 2024). This dynamic interplay is now considered a key mechanism underlying the health benefits attributed to plant-based diets.

Among the wide diversity of plants, the genus *Opuntia*, which belongs to the botanical family of Cactaceae, has historically attracted scientific interest due to its unique phytochemical and bioactive profile and its traditional use in human nutrition and medicine (Kim et al., 2018; Madrigal-Santillán et al., 2022). *Opuntia* species, commonly known as cactus prickly pear or nopal, are native to Latin America but are now widely distributed and consumed in multiple regions of the world, particularly in arid and semi-arid areas where they are valued for their nutritional qualities and their ecological benefits such as degraded land rehabilitation, biodiversity, and desertification prevention (Cruz-Jiménez et al., 2023; Ramadan, Moussa Ayoub and Rohn, 2021). *Opuntia* spp. compositional profile includes betalains, phenolic compounds, carotenoids, vitamins, and significant amounts of dietary fiber (Rodríguez et al., 2023). Specifically, the present research is focused on betalains and phenolic compounds, as they are the most abundant bioactives in these species (Gómez-López et al., 2021; Gómez-Maqueo et al., 2020).

Betalains are nitrogen-containing molecules synthesized as secondary plant metabolites. They can be classified into two groups depending on their structural interaction with betalamic acid: betaxanthins, which are formed by conjugation with different amino acids and provide yellow-orange tones, and betacyanins, which are formed by condensation with cyclodopa or its glycoside derivatives and give rise to red-violet colourings (Gandía-Herrero et al., 2016; Slimen et al., 2017). On the other hand, phenolic compounds are a diverse group of molecules found in plants, characterized by one or more hydroxyl groups attached to aromatic rings, and are classified into different types attending to their chemical structure such as phenolic acids and flavonoids (Saltveit, 2017). Both families of compounds have been associated with multiple beneficial biological activities, including antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective, and lipid-lowering effects, among others (Lima et al., 2006; Parralejo-Sanz, Antunes-Ricardo, et al., 2025; Rodríguez-Rodríguez et al., 2015; Serrano-Sandoval et al., 2025).

Importantly, the health effects attributed to these compounds depend not only on their intrinsic bioactivity but also on their transformation during digestion. Betalains and phenolic compounds from *Opuntia* fruits may be degraded, released from the matrix, absorbed, or transformed into new metabolites during gastrointestinal digestion, while the non-absorbed fraction can reach the colon and undergo further microbial biotransformation. These processes may influence microbial community dynamics, metabolic activity, and the production of metabolites relevant to host physiology (Catalkaya et al., 2020; Dingeo et al., 2020; Selma et al., 2009). In addition, the antioxidant, anti-inflammatory, and antimicrobial properties reported for betalains and phenolic compounds indicate that these molecules may also modulate the gut environment itself, although the extent and mechanisms of these effects remain incompletely understood (Aruwa et al., 2018; Rodrigues et al., 2023).

To date, few studies have compared how distinct betalain- and phenolic-rich *Opuntia* matrices are transformed throughout gastrointestinal digestion and subsequent colonic conditions, and how these transformations relate to microbial community structure and metabolic activity. In this context, we aimed to investigate the effects of *Opuntia*-derived extracts obtained using environmentally friendly ('green')

extraction techniques (Parralejo-Sanz, Antunes-Ricardo, et al., 2025), from three distinct varieties: *Opuntia stricta* var. *dillenii* whole fruit extract, *Opuntia ficus-indica* var. *Colorada* pulp extract, and *Opuntia ficus-indica* var. *Blanca Buenavista* peel extract. These tissues were deliberately selected based on their distinctive phytochemical profiles: the whole fruit of var. *dillenii* is particularly rich in betacyanins (specially betanin, isobetanin, and neobetanin), while the pulp of var. *Colorada* is characterized by high levels of betaxanthins (mainly indicaxanthin and vulgaxanthins), and the peel of var. *Blanca Buenavista* is especially abundant in phenolic compounds (piscidic acid and flavonoid glycosides). Together, these matrices represent the *Opuntia* tissues with the highest concentrations and diversity of betalains and phenolics reported among their respective plant parts (Gómez-López et al., 2021; Gómez-Maqueo et al., 2020). The interaction between *Opuntia* extracts and human gut ecosystem was examined using a dynamic *in vitro* gut simulation model (BFBL gut simulator), allowing the assessment of phytochemical transformations during gastrointestinal digestion, as well as microbial-driven metabolism during colonic fermentation, together with extract-specific effects on microbial composition and metabolic function. Overall, this work aims to provide a comparative and mechanistic understanding of how *Opuntia* fruits derived phytochemicals are processed along the digestive tract.

2. Materials and methods

2.1. Plant material

Opuntia stricta var. *dillenii* purple fruits were collected from Tinajo (Tenerife, Canary Islands, Spain; 29° 3' N, 13° 4' W; 209 m above sea level), *O. ficus-indica* var. *Colorada* orange fruits from Colorada, Fasnia (Tenerife, Canary Islands, Spain; 28° 2' N, 16° 4' W; 446 m above sea level), and *O. ficus-indica* var. *Blanca Buenavista* green fruits from Buenavista del Norte (Tenerife, Canary Islands, Spain; 28° 2' N, 16° 5' W; 127 m above sea level). All fruits were grown under subtropical semi-arid climatic conditions and harvested at commercial ripening stage in November 2024. Fruits were selected based on maturity, size, and colour, and their physicochemical characteristics were determined directly over ten individual fruits of each variety. Apical and equatorial diameters (cm) and fresh weight (g) were individually measured for each fruit. Juice extracted from the fruits was used to measure pH (Metrohm, Madrid, Spain) and total soluble solids (°Brix) at 25 °C with a digital refractometer (Atago, Tokyo, Japan). Peel and pulp colour parameters were evaluated using the CIELAB colour space (L^* , a^* , and b^*) with a Konica Minolta CM-3500d spectrophotometer (Tokyo, Japan), where L^* represents lightness, a^* the green-to-red axis, and b^* the blue-to-yellow axis (Data shown in Table S1). Continuously, fruits were frozen, freeze-dried, pulverized, and stored at -20 °C until use. The tissues selected as study material were *O. stricta* var. *dillenii* whole fruit, *O. ficus-indica* var. *Colorada* pulp, and *O. ficus-indica* var. *Blanca Buenavista* peel due to their profiles in betalains and phenolic compounds, described in a previous study within our research laboratory (Parralejo-Sanz, Antunes-Ricardo, et al., 2025).

2.2. Obtention of *Opuntia* extracts

Extracts from the freeze-dried tissues were prepared using the procedure previously described by Gómez-López et al. (2021). Ethanol-water mixtures and pure ethanol (CAS: 64-17-5; ACS reagent) (Merck, Darmstadt, Germany) were used for extraction, with the assistance of an ultrasonic bath. Both methods considered environmentally friendly or 'green' approaches. In each extraction cycle, 1 g of lyophilized pulp was mixed with 5 mL of an ethanol-water solution (1:1, v/v), homogenized with vortex and treated with an ultrasonic water bath during 4 min (3,000,514, 50/60 Hz, 360 W, J.PSelecta S.A., Barcelona, Spain), and centrifuged at 10,000 ×g, 4 °C, 10 min (Eppendorf, Hamburg, Germany). This process was repeated thrice. An additional extraction was

then carried out using 3 mL of pure ethanol. All resulting supernatants were combined, and the solvents were removed using a rotary evaporator (Buchi, Flawil, Switzerland). The concentrated extracts were subsequently freeze-dried using a LyoBeta 15 (Azbil Telstar SL, Terrasa, Spain) and stored at -20°C until further use. The final extracts were labelled as follows: OD for *O. stricta* var. *dillenii* whole fruit extract, COL for *O. ficus-indica* var. *Colorada* pulp extract, and BB for *O. ficus-indica* var. *Blanca* peel extract.

2.3. Microbiota inoculum

Microbiota inoculum was performed following the study described by Barroso et al. (2015). Human faecal sample (10 g) was obtained the morning of the day starting the experiment from a healthy adult donor (protocol approved by the Ethics Committee of the Spanish Research Council (CSIC), code 051/2024, issued on February 26, 2024). The stool was transported into a bag with anaerobe-gas generation sachet (AnaeroGen, Oxoid, Thermo Fisher) and stored under refrigeration conditions until its use that same day. The sample was mixed with 10 mL of buffered solution for the inoculum (composition detailed in Table S2) and homogenized using a Stomacher® (VWR International, Worthing, West Sussex, UK) for 10 min at 300 rpm. This process allowed the separation of the liquid phase containing the gut microbiota from the solid residue. The resulting microbial suspension was then inoculated (10% v/v) into the nutritive medium used for the adequate development of gut microbiota (composition detailed in Table S2).

2.4. Gut dynamic simulation

The dynamic simulation of gut conditions was performed in the BFBL gut simulator (Martínez-Cuesta et al., 2019), a dynamic *in vitro* system composed of four sequential reactors, where the first one mimic the upper gastrointestinal transit (GI) (stomach and small intestine), and the other three mimic the colonic regions and their microbial environment: ascending (AC), transverse (TC) and descending colon (DC). A schematic representation of the experimental setup is shown in Fig. S1. The four reactors were continuously stirred (150 ± 5 rpm) with magnetic stirrers (Agimatic N, Selecta, Barcelona, Spain) and temperature was kept at $37 \pm 0.1^{\circ}\text{C}$. The first reactor (GI) simulated sequential gastric and small-intestinal conditions within a single vessel. Briefly, the nutritive medium and the *Opuntia* extracts were exposed to an initial acidic stage (gastric-like conditions; $\text{pH } 2 \pm 0.2$, 37°C) followed by an intestinal-like stage through the addition of pancreatic juice and bile salts ($\text{pH } 7 \pm 0.2$, 37°C) (Table S2). The GI simulation was carried out with a total residence time of 2 h, generating a pre-digested material, representing the whole chyme, that was subsequently transferred to the colonic compartments for microbial fermentation. This feeding regimen was applied thrice a day (every 8 h) corresponding to 'breakfast', 'lunch' and 'dinner' (composition detailed in Table S2) (Barroso et al., 2015; Bellanco et al., 2024). Overall, the GI reactors enabled partial digestion and physico-chemical transformation of the extracts prior to their exposure to the colonic microbiota. Continuously, the AC, TC and DC reactors operated under controlled anaerobic conditions with a permanent nitrogen flush, and compartment-specific pH values set to 5.8 ± 0.2 , 6.3 ± 0.2 and 6.8 ± 0.2 , respectively, reflecting the physiological pH gradient along the human colon. These values were maintained automatically by peristaltic pumps operating in continuous flow, dosing HCl 0.5 M and NaOH 0.5 M into the reactors in response to real-time pH monitoring. The residence time in the reactors was stated according to the volumes, which were controlled by level sensors at 250 ± 5 mL, 300 ± 5 mL and 350 ± 5 mL, for AC, TC, and DC, respectively.

The faecal inoculum was introduced the first day of the experiment into the colonic reactors (AC, TC, and DC), which contained the nutritive medium (Table S2) and, given the continuous dynamic nature of the BFBL gut simulator, the same microbial community was maintained within the colonic reactors throughout all three sequential assays,

ensuring consistent microbial exposure across treatments. After the inoculation, an overnight incubation in batch conditions was performed. An initial 7-day stabilization period of the microbial community was performed to reach the steady state prior to the extract supplementation. After this stabilization period, 1 g of *Opuntia* extract was added each day (for 7 days) to the GI reactor at the 'breakfast' stage. Finally, another 7-day wash-out period was included. This complete process was performed sequentially three times, one for each *Opuntia* variety. Each assay included independent stabilization and wash-out phases between treatments. Along the whole study, samples of GI, AC, TC and DC were collected at regular time points and stored at -20°C until further analyses. Even though each experimental phase lasted 7 days, to ensure that metabolic and microbial parameters reflected steady conditions, samples used for the subsequent analyses were only collected during the final three days of each phase.

2.5. Analysis of betalains and phenolic compounds

Individual betalains and phenolic compounds were simultaneously identified and quantified using HPLC-DAD-MS, following the method described by García-Cayuela et al. (2019). A 1200 Series Agilent HPLC with a C18 reverse phase column was used with gradient elution of Phase A, composed of 1% formic acid (CAS: 64-18-6; $\geq 95\%$; analytical grade) in water and methanol (CAS: 67-56-1; $\geq 99.9\%$; analytical grade) for 70 min. Detection was set at 535 (betacyanins), 480 (betaxanthins), 370 (flavonoids), and 280 (phenolic acids) nm using a photodiode array. The system was coupled to an LC/MS SQ 6120 with ESI in positive ion mode. Nitrogen was used as the drying gas, maintained at a flow rate of 3 L/min, a pressure of 137.9 kPa, and a temperature of 300°C , with an applied voltage of 3500 V. Mass spectra (m/z 100–1000) and UV/Vis data were compared to standards for identification and quantification was done by calibration curves. Betanin, betaxanthins, phylloactins, and piscidic acid were isolated in our laboratory as described in García-Cayuela et al. (2019). Standards for flavonoid glycosides were kindly provided by Dr. Serna-Saldivar's laboratory from Centro de Biotecnología FEMSA (Escuela de Ingeniería y Ciencias, Instituto Tecnológico de Monterrey, Mexico). All standards were verified for identity and purity by HPLC-ESI-MS-QToF. Data relative to betalains and phenolic compounds individual quantification is only presented during the *Opuntia* extracts' supplementation phase, as these compounds were not detectable in both stabilization and wash-out periods.

2.6. DNA extraction and 16S rRNA gene amplicon sequencing

DNA was isolated using the E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek) according to the manufacturer's instructions. Microbial cells were lysed by mechanical disruption with glass beads (0.1 mm zirconia/silica; Merck) using a FastPrep instrument (Bio 101 FastPrep FP120, Savant Instruments). DNA concentration was measured with a NanoDrop 1000 UV/VIS Spectrophotometer (Thermo Fisher, Massachusetts, USA). The extracted DNA underwent amplicon-based metagenomic sequencing targeting the full-length 16S rRNA gene that was sequenced by Novogene (Munich, Germany) using the PacBio Sequel II/Ile platform. Taxonomic classification of the resulting amplicon sequence variants (ASV) was performed using the QIIME2 pipeline. ASV tables were rarefied to the sequencing depth of the sample with the lowest read count to ensure comparability across samples. Comparison of relative abundances were presented as net changes during extract supplementation, calculated relative to the non-supplemented phases (stabilization and wash-out) with the aim of reflecting extract-induced shifts while accounting for baseline community structure. Net changes were calculated as the difference between the relative abundance during supplementation and the mean relative abundance of the stabilization and wash-out phases. Positive values indicate increases, whereas negative values indicate decreases in relative abundance. Additionally, functional potential was predicted using PICRUST2 based on 16S rRNA data using

KEGG Orthology (KO) annotations to infer the metabolic potential functions at the species level of the bacterial communities. ASV sequences were linked to functional orthologs, allowing the estimation of pathways related to the metabolism of betalains and phenolics. These predictions are inherently inferential and subject to the limitations of 16S rRNA-based taxonomic resolution and reference genome completeness and should therefore be interpreted as estimated metabolic potential rather than demonstrated functional activity.

2.7. Analysis of short-chain fatty acids and ammonium

Short-chain fatty acids (SCFAs) were analyzed by HPLC following the methodology described by Sanz et al. (2005). Briefly, SCFAs were separated using a Rezex ROA organic acid column (Phenomenex, California, USA) maintained at 56 °C, with a linear gradient of 5 mM sulfuric acid (CAS: 7664-93-9; 95–98%; ACS reagent) as the mobile phase at a flow rate of 0.6 mL/min. Their concentrations in the samples were determined by comparing to calibration curves prepared with known concentrations (0.5 to 100 mM) of acetic, propionic, butyric, and valeric acids. Ammonium concentration was measured using Nessler's reagent (Merck), following the method outlined by Doo et al. (2017). Data were expressed as net changes in SCFA production during supplementation to reflect extract-induced responses while accounting for baseline activity of the microbial community. Net values were calculated relative to the non-supplemented phases (stabilization and wash-out).

2.8. Statistical analysis

Samples were collected on each of the final three days of each experimental phase (stabilization, supplementation, and wash-out), once steady-state conditions had been reached, yielding three time-point replicates per condition ($n = 3$). Statistical analyses were selected according to the biological nature and temporal dynamics of each dataset. Individual betalains and phenolic compounds, SCFAs and ammonium data were analyzed using one-way ANOVA followed by Tukey's *post hoc* test or Student's *t*-test, considering a confidence level of 95%. Differences in microbial communities' composition and KEGG orthology functional predictions were analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparison *post hoc* test ($p < 0.05$). To minimize statistical noise and reduce the impact of rare or potentially artifactual sequences, taxa with relative abundances below 0.1% were excluded from the analysis. Additionally, only statistically significant microbial changes are discussed in the manuscript, while the complete raw datasets are publicly available at <https://digital.csic.es/handle/10261/420274>.

3. Results

3.1. Initial composition of *Opuntia* extracts

The characterization profile of the main betalains and phenolic compounds identified in *Opuntia stricta* var. *dillenii* whole fruit extract (OD), *Opuntia ficus-indica* var. *Colorada* pulp extract (COL), and *Opuntia ficus-indica* var. *Blanca* peel extract (BB) is shown in Table S3, and the quantification of each compound is shown in Table S4.

OD extract was characterized by a predominance of betacyanin-type betalains, with betanin as the major compound ($10,245 \pm 18 \mu\text{g/g}$ dry extract), followed by isobetanin ($5128 \pm 28 \mu\text{g/g}$) and neobetanin ($4072 \pm 135 \mu\text{g/g}$). In addition, piscidic acid was present at high levels ($12,154 \pm 579 \mu\text{g/g}$), together with flavonoid glycosides, including quercetin glycoside I (QG1), quercetin glycoside II (QG2), isorhamnetin glucosyl-rhamnosyl-rhamnoside (IG1), and isorhamnetin glucosyl-rhamnosyl-pentoside (IG2), although at substantially lower concentrations than betalains (44.3 ± 2.6 – $888 \pm 29 \mu\text{g/g}$) (Table S4).

On the other hand, COL extract exhibited a more diverse betalain composition dominated by betaxanthins rather than betacyanins.

Indicaxanthin was the most abundant pigment ($587 \pm 34 \mu\text{g/g}$), followed by portulacaxanthin III ($35 \pm 2.5 \mu\text{g/g}$) and vulgaxanthins I–III at lower concentrations. Piscidic acid was also present in considerable amounts ($3955 \pm 62 \mu\text{g/g}$), together with smaller quantities of flavonoid glycosides QG2 and IG2 (26.2 ± 3.1 and $37.4 \pm 5 \mu\text{g/g}$, respectively) (Table S4). This composition reflects a betaxanthin-rich matrix with moderate phenolic content.

BB extract differed markedly from OD and COL by lacking detectable betalains, but containing notably higher concentrations of phenolic compounds. Piscidic acid was the predominant compound ($53,354 \pm 173 \mu\text{g/g}$), representing the value among all extracts, followed by a broad diversity of flavonoid glycosides, including QG2 ($761 \pm 41 \mu\text{g/g}$), isorhamnetin hexosyl-rhamnoside (IG7) ($468 \pm 16 \mu\text{g/g}$), IG2 ($100.2 \pm 3.8 \mu\text{g/g}$), IG1 ($94 \pm 2.1 \mu\text{g/g}$), kaemferol glucosyl-rhamnoside (KG1) ($80.1 \pm 0.4 \mu\text{g/g}$), QG1 ($48.3 \pm 0.4 \mu\text{g/g}$), and isorhamnetin glucosyl-rhamnoside (IG5) ($39.5 \pm 4.2 \mu\text{g/g}$) (Table S4).

3.2. Progression of betalains and phenolic compounds during gastrointestinal stage and colonic simulation

Absolute concentrations of individual compounds in the raw extracts (previously described), and their evolution after simulated gastrointestinal (GI) phase and each colonic compartment (AC, TC, and DC) and their statistically significant differences ($p > 0.05$), expressed as μg of each compound per g of dry extract, are presented in Table S4. For clarity, these results are presented as percentages in Table 1 and were calculated using the initial extract composition as reference (100%), allowing the evaluation of compound stability and progression throughout digestion and fermentation.

Overall, GI stage caused major reductions in most compounds, highlighting its key role in determining phytochemical stability prior to colonic fermentation. Additional microbiota-driven transformations occurred along the colon, with betalains being the most labile, phenolic acids the most persistent, and flavonoid glycosides showing intermediate stability.

Supplementation with OD showed that, betanin, isobetanin, and neobetanin retained 45.58%, 24.95%, and 33.94% of their initial concentrations after the GI phase, respectively, indicating a significant susceptibility to digestive conditions (Table 1 and Table S4). Their levels decreased further in the AC, where only 33.52, 13.34, and 22.2% remained respectively, before becoming undetectable in the TC and DC, suggesting microbial transformation in proximal regions.

In contrast, betaxanthin-type betalains in COL extract displayed higher stability in comparison with betacyanins. After GI digestion, betaxanthins retained between 23.37 and 48.33% of their initial concentrations, in the AC between 12.62 and 35.27%, and in the TC between 9.9 and 28.41%. In the DC, vulgaxanthins I and II were already undetectable, while indicaxanthin, portulacaxanthin III, and vulgaxanthin III remained at 7.66, 11.13, and 33.54%, respectively.

Piscidic acid exhibited a consistent pattern across extracts. High proportions of this compound resisted the GI phase (65.83, 68.59, and 73.4% in OD, COL, and BB, respectively), followed by a gradual decline along the colonic compartments. In the AC, concentrations remained relatively high, accounting for 32.59, 43.62, and 53.77% of the initial levels in OD, COL, and BB, respectively, indicating limited early microbial utilization. Although further reductions were observed in the TC and DC, piscidic acid continued to be detectable in distal compartments, with values ranging from 16.88% to 47.74%.

Regarding flavonoids, quercetin glycosides showed moderate stability during GI digestion, retaining between approximately 23% and 49% of their initial concentrations depending on the extract, followed by substantial reductions in the AC, and disappearance in distal compartments. For instance, in OD, QG1 and QG2 decreased to 15.77% and 9.22% in the AC, respectively, and were no longer detected beyond the TC, while QG2 was retained at 25.78 and 16.96% in the AC with COL and BB, respectively, and not detected in subsequent compartments.

Table 1

Progression of individual betalains and phenolic compounds during supplementation of the BFBL gut simulator with *Opuntia* extracts, expressed as percentages relative to the initial composition in raw extracts (set as 100%). Values represent the evolution of each compound along the gastrointestinal phase and colonic compartments during the supplementation phase. Different lowercase superscript letters indicate statistically significant differences among columns for each individual compound (one-way ANOVA followed by Tukey's *post hoc* test or t-Student tests, $p < 0.05$).

<i>Opuntia stricta</i> var. <i>dillenii</i> , whole fruit extract (OD)				
Compound	Compartment			
	GI	AC	TC	DC
Piscidic acid	65.83 ± 5.66 ^a	32.59 ± 5.89 ^b	25.18 ± 3.97 ^{bc}	16.88 ± 4.49 ^c
Betanin	45.58 ± 2.5 ^a	33.52 ± 2.28 ^b	n.d.	n.d.
Isobetanin	24.95 ± 7.27 ^a	13.34 ± 0.14 ^b	n.d.	n.d.
Neobetanin	33.94 ± 10.35 ^a	22.20 ± 0.77 ^b	n.d.	n.d.
QG1	27.00 ± 9.71 ^a	15.77 ± 3.34 ^{ab}	4.60 ± 0.81 ^b	n.d.
QG2	28.96 ± 2.74 ^a	9.22 ± 2.00 ^b	3.95 ± 1.12 ^c	n.d.
IG1	21.54 ± 5.56 ^a	13.98 ± 3.48 ^{ab}	4.05 ± 2.49 ^b	n.d.
IG2	31.15 ± 7.66 ^a	18.30 ± 2.12 ^b	9.82 ± 0.88 ^b	10.15 ± 0.47 ^b
<i>Opuntia ficus-indica</i> var. <i>Colorada</i> pulp extract (COL)				
Compound	Compartment			
	GI	AC	TC	DC
Portulacaxanthin III	36.74 ± 4.22 ^a	32.10 ± 18.7 ^a	27.39 ± 4.20 ^a	11.13 ± 3.07 ^a
Vulgaxanthin III	48.33 ± 3.32 ^a	31.66 ± 16.20 ^a	28.41 ± 3.29 ^a	33.54 ± 15.81 ^a
Vulgaxanthin I	23.37 ± 0.33 ^a	12.62 ± 1.68 ^b	9.90 ± 2.66 ^b	n.d.
Vulgaxanthin II	31.51 ± 1.56 ^a	35.27 ± 2.12 ^a	n.d.	n.d.
Piscidic acid	68.59 ± 7.76 ^a	43.62 ± 4.92 ^a	41.59 ± 3.95 ^a	47.74 ± 18.55 ^a
Indicaxanthin	46.07 ± 4.35 ^a	25.71 ± 1.99 ^b	25.06 ± 6.80 ^b	7.66 ± 0.76 ^c
QG2	49.03 ± 6.72 ^a	25.78 ± 13.72 ^a	n.d.	n.d.
IG2	34.11 ± 5.15 ^a	n.d.	n.d.	n.d.
<i>Opuntia ficus-indica</i> var. <i>Blanca</i> peel extract (BB)				
Compound	Compartment			
	GI	AC	TC	DC
Piscidic acid	73.40 ± 6.68 ^a	53.77 ± 20.38 ^{ab}	38.08 ± 1.71 ^b	26.39 ± 3.15 ^b
QG1	45.25 ± 15.18 ^a	24.88 ± 4.79 ^a	n.d.	n.d.
QG2	23.01 ± 1.29 ^a	16.96 ± 2.83 ^b	n.d.	n.d.
IG1	46.01 ± 2.99 ^a	24.08 ± 3.17 ^b	n.d.	n.d.
IG2	29.66 ± 4.18 ^a	18.45 ± 1.73 ^b	n.d.	n.d.
IG7	39.52 ± 3.34 ^a	38.56 ± 1.83 ^a	n.d.	n.d.
KG1	61.06 ± 4.48 ^a	30.78 ± 5.24 ^b	n.d.	n.d.
IG5	52.00 ± 12.13 ^a	n.d.	n.d.	n.d.

GI: gastrointestinal phase, AC: ascending colon, TC: transverse colon, DC: descending colon, n.d.: non-detected, QG1: quercetin glycoside I, QG2: quercetin glycoside II, IG1: isorhamnetin glucosyl-rhamnosyl-rhamnoside, IG2: isorhamnetin glucosyl-rhamnosyl-pentoside, IG7: isorhamnetin hexosyl-rhamnoside, KG1: kaempferol glucosyl-rhamnoside, IG5: isorhamnetin glucosyl-rhamnoside.

Isorhamnetin glycosides displayed more variable behaviour depending on both the compound and extract. Supplementation with OD, showed a retention in IG1 and IG2 of 21.54 and 31.15% after GI and decreased to 4.05 and 9.82% in the TC, before becoming undetectable in the DC. In contrast, in COL there was a lower persistence of these compounds, with IG2 retaining 34.11% after GI and disappearing in the AC. BB showed retentions for isorhamnetin glycosides between 29.66 and 52% after GI, and between 18.45 and 38.56 in the AC, while all of them were undetectable in the TC.

3.3. Effects of *Opuntia* extracts on gut microbiota composition

To characterize the effects of *Opuntia* extract supplementation on colonic microbiota composition and diversity, microbial profiles were analyzed across stabilization, supplementation, and wash-out phases, and net changes attributable to supplementation were calculated. Microbial relative abundances at the phylum and family levels during supplementation are shown in Fig. 1, while statistically significant changes induced by the extracts relative to the non-supplemented phases (stabilization and wash-out) are summarized in Table 2. Given the high diversity at the genus level, only genera showing statistically significant differences between experimental periods within each colonic compartment are shown in Fig. S2, while net increments during supplementation relative to the non-supplemented periods (stabilization and wash-out), are exposed in Table 3.

At the phylum level, the community was dominated by Actinobacteriota (syn. Actinomycetota), Firmicutes (syn. Bacillota), and Bacteroidota, while Proteobacteria (syn. Pseudomonadota), Verrucomicrobiota, and Desulfobacterota were present at lower relative abundances (Fig. 1). Statistically significant differences attributed to the extract supply were only observed with COL, which induced a net increase of Actinobacteriota in the TC (15.25%) and DC (22.58%), concomitant with a net decrease in Bacteroidota in the AC (−17.68%) (Table 2). A progressive increase in Verrucomicrobiota was observed over time during the continuous operation of the system, regardless of extract supplementation.

At the family level, the baseline microbiota was mainly composed of Bifidobacteriaceae, Akkermansiaceae, Bacteroidaceae, and Enterobacteriaceae, with compartment-specific distributions (Fig. 1). Some statistically significant differences attributed to *Opuntia* extract supply were observed. OD induced a decrease in Rikenellaceae (−0.63%) in the AC. COL supplementation produced increases in Bifidobacteriaceae in the TC (15.3%) and DC (22.78%), and a decrease in Veillonellaceae in the AC (−1.39%). BB supplementation was associated with a net reduction of Desulfovibrionaceae in the DC (−4.45%). These variations were compartment-dependent and restricted to the supplementation phase (Table 2).

During supplementation with OD, net decreases in the relative abundance of several genera were observed in the AC. *Alistipes* showed a reduction of −0.69%, *Eubacterium* decreased by −0.15%, and *Megasphaera* by −9.69. No significant net changes were detected for these genera in the transverse colon (TC) or descending colon (DC). In the case of COL, a strong net reduction in *Bacteroides* was observed, reaching −23.48% in the AC and −15.37% in the TC. *Megasphaera* also showed consistent decreases along the proximal compartments, with −10.52% in the AC and −5.91 in the TC, and a smaller reduction of −1.55% in the DC. In addition, *Parabacteroides* exhibited a net decrease of −5.38% in the DC. Conversely, *Citrobacter* displayed an increase of +2.3% in the DC. Supplementation with BB resulted in a distinct pattern of net changes. In the AC, *Dialister* increased by +1.30%, *Lachnoclostridium* by +2.23%, and *Megasphaera* by +7.82%. In contrast, *Bilophila* showed a net decrease of −4.86% in the DC. Similarly to COL, *Citrobacter* exhibited a net increase of +2.34% in the DC. Interestingly, genera such as *Akkermansia*, *Bifidobacterium*, *Faecalibacterium*, *Lactobacillus* (*sensu lato*), and *Roseburia* did not show statistically significant variations during supplementation with any of the extracts (results not shown).

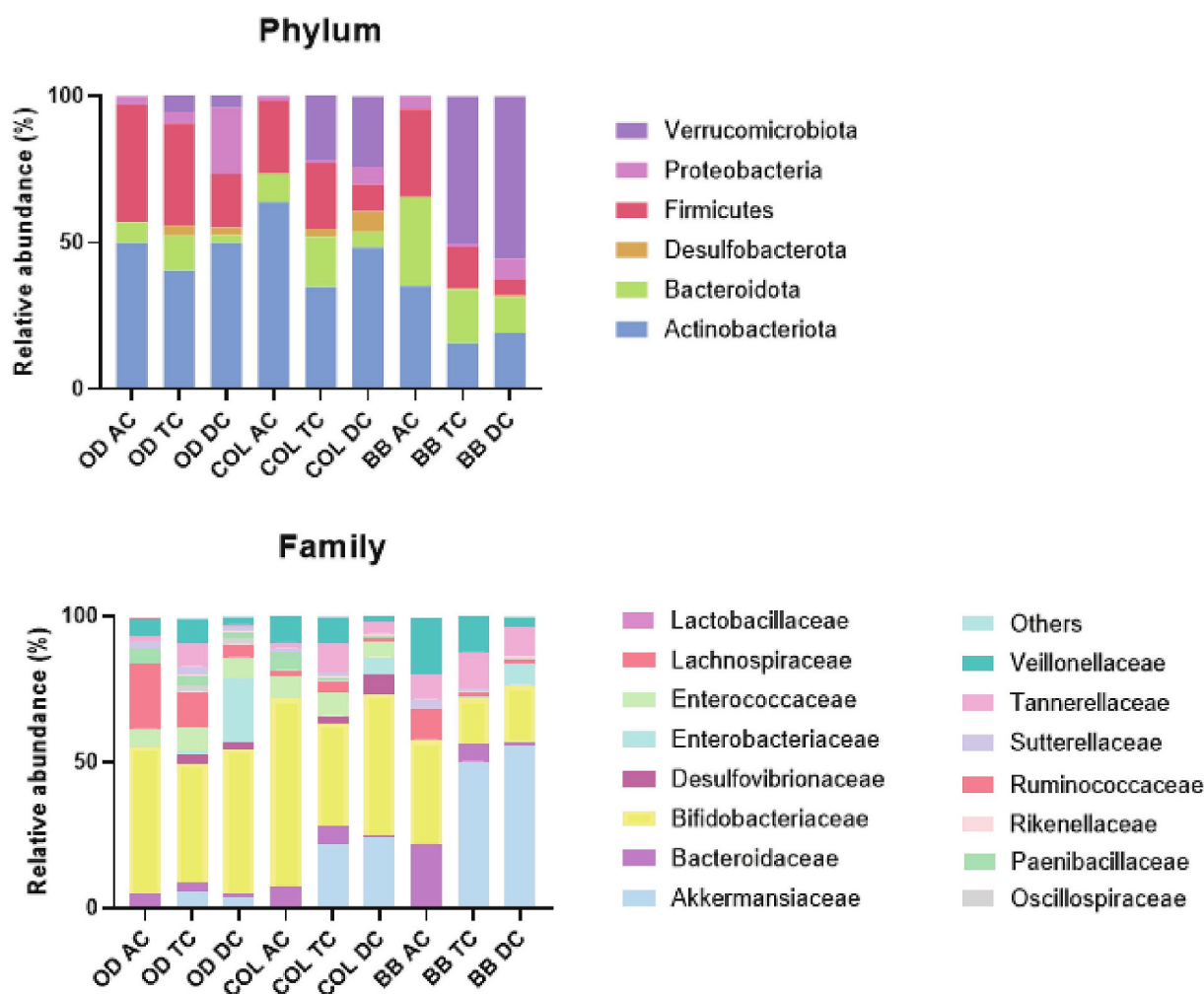


Fig. 1. Relative abundance (%) of gut microbiota at the phylum (a) and family (b) levels in the ascending (AC), transverse (TC), and descending (DC) colon compartments of the dynamic *in vitro* BFBL gut simulator during supplementation with *Opuntia* extracts: OD (*Opuntia stricta* var. *dillenii* whole fruit), COL (*Opuntia ficus-indica* var. *Colorada* pulp), and BB (*Opuntia ficus-indica* var. *Blanca* peel).

Table 2

Net variations in the relative abundance (%) at the phylum and family levels during supplementation with *Opuntia* extracts. Values are expressed as net increases or decreases in the relative abundance of each taxa calculated relative to the non-supplemented periods (stabilization and wash-out). Only taxa showing statistically significant differences according to the Kruskal–Wallis and Dunn's *post hoc* tests ($p < 0.05$) are presented. Results are shown as means $\pm$ standard deviation. Spaces labelled with ‘-’ mean there were no statistically significant changes between periods.

Taxa	<i>Opuntia</i> extract	AC	TC	DC
Phylum	COL	Actinobacteriota	–	15.25 $\pm$ 7.9
		Bacteroidota	–17.68 $\pm$ 8.09	–
	OD	Rikenellaceae	–0.63 $\pm$ 0.23	–
Family	COL	Bifidobacteriaceae	–	15.3 $\pm$ 7.89
		Veillonellaceae	–1.39 $\pm$ 0.54	–
	BB	Desulfovibrionaceae	–	–4.45 $\pm$ 2.02

OD: *Opuntia stricta* var. *dillenii* whole fruit extract; COL: *Opuntia ficus-indica* var. *Colorada* pulp extract; BB: *Opuntia ficus-indica* var. *Blanca* peel extract, AC: ascending colon, TC: transverse colon, and DC: descending colon.

3.4. SCFA and ammonium production

Changes in SCFA and ammonium concentrations were expressed as net increases (mM) induced by the supply of *Opuntia* extracts, calculated relative to the phases without extract (stabilization and wash-out). Data are shown in Table 4.

Acetic acid represented the highest net increase in SCFA across all *Opuntia* varieties. Its increase remained relatively stable across compartments, with no statistically significant differences between AC, TC, and DC within each extract. The highest increase was observed in the DC under BB supplementation (26.12 mM). Propionic acid displayed a more extract-dependent response. During exposure to OD, its net concentration increased progressively from the AC to the DC, reaching 2.45 mM net increase, with significantly higher levels in the DC compared with the AC and TC ($p < 0.05$). A similar trend was observed for BB, where propionic acid increased from 1.21 mM in the AC to 3.44 mM in the DC. In contrast, COL supplementation induced a decrease in the production of propionic acid in all three compartments, with values up to -1.33 mM but with no statistical differences between them. Butyric acid showed a clear net increase during supplementation with all *Opuntia* extracts. For OD, concentrations rose significantly in the DC compared to the AC and TC, whereas COL induced a marked increase already in the TC, reaching the highest net value in the DC (5.3 mM). BB also promoted butyric acid formation, with increasing concentrations along the colonic compartments. Valeric acid followed a similar behaviour, with net

Table 3

Net variations in the relative abundance (%) at the genus level during supplementation with *Opuntia* extracts. Values are expressed as net increases or decreases in the relative abundance of each genus calculated relative to the non-supplemented periods (stabilization and wash-out). Only taxa showing statistically significant differences according to the Kruskal–Wallis and Dunn's *post hoc* tests ($p < 0.05$) are presented. Results are shown as means $\pm$ standard deviation. Spaces labelled with ‘-’ mean there were no statistically significant changes between periods.

Net variations in relative abundance (%)				
Opuntia extract	Genus	Colonic compartment		
		AC	TC	DC
OD	<i>Alistipes</i>	-0.69 $\pm$ 0.22	-	-
	<i>Eubacterium</i>	-0.15 $\pm$ 0.03	-	-
	<i>Megasphaera</i>	-9.69 $\pm$ 3.68	-	-
	<i>Anaerostipes</i>	-0.41 $\pm$ 0.18	-	-
	<i>Bacteroides</i>	-23.48 $\pm$ 4.82	-15.37 $\pm$ 7.65	-
COL	<i>Citrobacter</i>	-	-	2.34 $\pm$ 0.74
	<i>Megasphaera</i>	-10.52 $\pm$ 2.76	-5.91 $\pm$ 2.32	-1.55 $\pm$ 0.82
	<i>Parabacteroides</i>	-	-	-5.38 $\pm$ 2.69
	<i>Bilophila</i>	-	-	-4.86 $\pm$ 1.83
BB	<i>Citrobacter</i>	-	-	2.34 $\pm$ 0.75
	<i>Dialister</i>	1.30 $\pm$ 0.12	-	-
	<i>Lachnoclostridium</i>	2.23 $\pm$ 1.16	-	-
	<i>Megasphaera</i>	7.82 $\pm$ 1.58	-	-

OD: *Opuntia stricta* var. *dillenii* whole fruit extract; COL: *Opuntia ficus-indica* var. *Colorada* pulp extract; BB: *Opuntia ficus-indica* var. *Blanca* peel extract, AC: ascending colon, TC: transverse colon, and DC: descending colon.

concentrations generally increasing from the AC to the DC for all extracts. The highest values were consistently detected in the DC, ranging from 3.39 to 5.18 mM. However, no statistically significant differences among extracts were observed within each compartment.

Regarding ammonium production, net concentrations did not differ significantly among extracts in the AC and TC, with increases ranging from 0.92 to 2.73 mM. In the DC, OD supply resulted in a further increase in ammonium concentration (2.97 mM), but COL and BB reduced their production in -0.76 and -3.04 mM, respectively.

3.5. Microbial functional prediction

The predicted KEGG orthology analysis (Kanehisa et al., 2017) identified several microbiota pathways potentially associated with the metabolism of phenolic compounds and betalains, including betalain biosynthesis (K00965), flavonoid biosynthesis (K00941), flavonoid degradation (K00946), aminobenzoate degradation (K00627), and tyrosine metabolism (K00350) at the species level. These predictions should be interpreted as estimated metabolic potential rather than demonstrated functional activity, as they are based on 16S rRNA compositional data and are subject to the inherent limitations of PICRUST2-based inference. Results are exposed in Fig. 2 and Fig. 3 as heatmaps performed associating the percentage contribution of each individual species to the total functional potential for the three assays conducted with OD, COL, and BB extracts at the different colonic compartments.

Across the three extracts, *Akkermansia muciniphila* was predicted to be a major contributor to flavonoid degradation and biosynthesis (accounting up to 83 and 79% of the functional assignment, respectively), as well as to aminobenzoate degradation (98%). *Bifidobacterium adolescentis*, *Parabacteroides distasonis*, and *Dialister* sp. were also predicted

Table 4

Net variations in the production of short-chain fatty acids (SCFAs) and ammonium during supplementation with *Opuntia* extracts. Values are expressed as net increase or decrease in concentrations (mM) calculated relative to the non-supplemented periods (stabilization and wash-out). Results are shown as means $\pm$ standard deviation. Superscript capital letters indicate statistically significant differences among *Opuntia* extracts within each compartment for a given compound, while superscript lowercase letters indicate statistically significant differences among colonic compartments within each extract (ANOVA followed by Tukey's *post hoc* test, $p < 0.05$).

Net variations in SCFA production (mM)				
Compound	Opuntia extract	Colonic compartment*		
		AC	TC	DC
Acetic acid	OD	15.55 $\pm$ 3.3 ^{Ab}	21.25 $\pm$ 2.13 ^{Aa}	22.44 $\pm$ 1.73 ^{Aa}
	COL	16.4 $\pm$ 3.42 ^{Aa}	18.36 $\pm$ 2.53 ^{Aa}	22.29 $\pm$ 4.05 ^{Aa}
	BB	13.57 $\pm$ 5.34 ^{Aa}	20.97 $\pm$ 8.39 ^{Aa}	26.12 $\pm$ 9.21 ^{Aa}
	OD	0.62 $\pm$ 0.16 ^{Ab}	1.39 $\pm$ 0.42 ^{Bb}	2.45 $\pm$ 0.34 ^{Aa}
	COL	-1.33 $\pm$ 0.71 ^{Ba}	-0.66 $\pm$ 0.33 ^{Ca}	-1.2 $\pm$ 0.5 ^{Ba}
Propionic acid	BB	1.21 $\pm$ 0.03 ^{Aa}	2.5 $\pm$ 0.44 ^{Aa}	3.44 $\pm$ 1.5 ^{Aa}
	OD	3.38 $\pm$ 0.59 ^{Aab}	2.96 $\pm$ 0.25 ^{Bb}	4.11 $\pm$ 0.91 ^{Aa}
	COL	3.35 $\pm$ 0.62 ^{Aa}	4.62 $\pm$ 1.2 ^{Aa}	5.3 $\pm$ 1.43 ^{Aa}
Butyric acid	BB	1.82 $\pm$ 0.84 ^{Bb}	3.19 $\pm$ 0.96 ^{Bab}	4.19 $\pm$ 0.81 ^{Aa}
	OD	1.4 $\pm$ 0.32 ^{Ab}	3.13 $\pm$ 0.16 ^{Aa}	3.39 $\pm$ 0.53 ^{Aa}
	COL	2.71 $\pm$ 0.77 ^{Ab}	4.87 $\pm$ 0.95 ^{Aa}	5.18 $\pm$ 0.76 ^{Aa}
Valeric acid	BB	2.98 $\pm$ 1.53 ^{Aa}	4.58 $\pm$ 1.96 ^{Aa}	4.61 $\pm$ 1.45 ^{Aa}
	OD	2.16 $\pm$ 0.89 ^{Aa}	2.73 $\pm$ 1.01 ^{Aa}	2.97 $\pm$ 1.34 ^{Aa}
	COL	1.32 $\pm$ 0.84 ^{Aa}	2.65 $\pm$ 0.89 ^{Aa}	-0.76 $\pm$ 0.23 ^{Bb}
Ammonium	BB	1.53 $\pm$ 0.42 ^{Aa}	0.92 $\pm$ 0.46 ^{Aa}	-3.04 $\pm$ 0.17 ^{Cb}

OD: *Opuntia stricta* var. *dillenii* whole fruit extract; COL: *Opuntia ficus-indica* var. *Colorada* pulp extract; BB: *Opuntia ficus-indica* var. *Blanca* peel extract, AC: ascending colon, TC: transverse colon, and DC: descending colon.

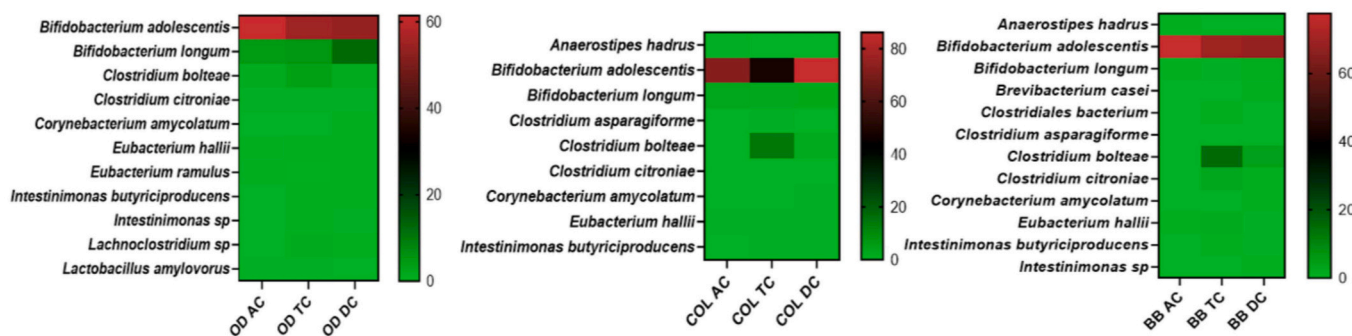
to contribute substantially to several of these pathways (with values up to 50, 16, and 14%, respectively), particularly those related to flavonoids metabolism. Regarding betalain biosynthesis and tyrosine metabolism functions, which participate in both the formation and breakdown of betalainic structures, *B. adolescentis* and *P. distasonis* were the main predicted contributors (with a functional contribution up to 98 and 71%, respectively). *Bifidobacterium longum*, *Clostridium bolteae*, *Bacteroides uniformis*, *Bacteroides caccae*, and *Bacteroides ovatus* were also predicted to contribute variably to these functions, although to a lesser extent (Fig. 2 and Fig. 3).

4. Discussion

The present study is focused on the comprehensive evaluation of the metabolic fate of extracts abundant in betalains and phenolic compounds from three *Opuntia* varieties: *Opuntia stricta* var. *dillenii* (OD), *Opuntia ficus-indica* var. *Colorada*, and *Opuntia ficus-indica* var. *Blanca* (BB) during gastrointestinal stage and colonic fermentation, together with their impact on gut microbiota composition, metabolic activity, and predicted microbial functional capacity.

The distinct initial phytochemical composition of OD, COL, and BB extracts reflects their variability among species, varieties, and fruit tissues within this genus. Taken together, OD was characterized by a

K00965 – Betalain biosynthesis



K00941 – Flavonoid biosynthesis

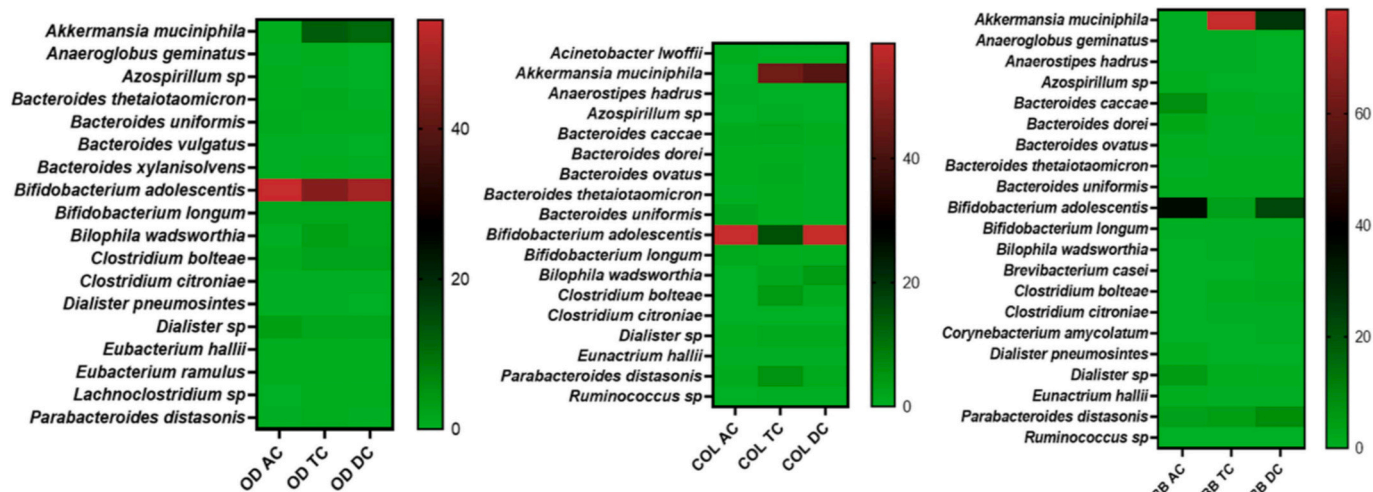


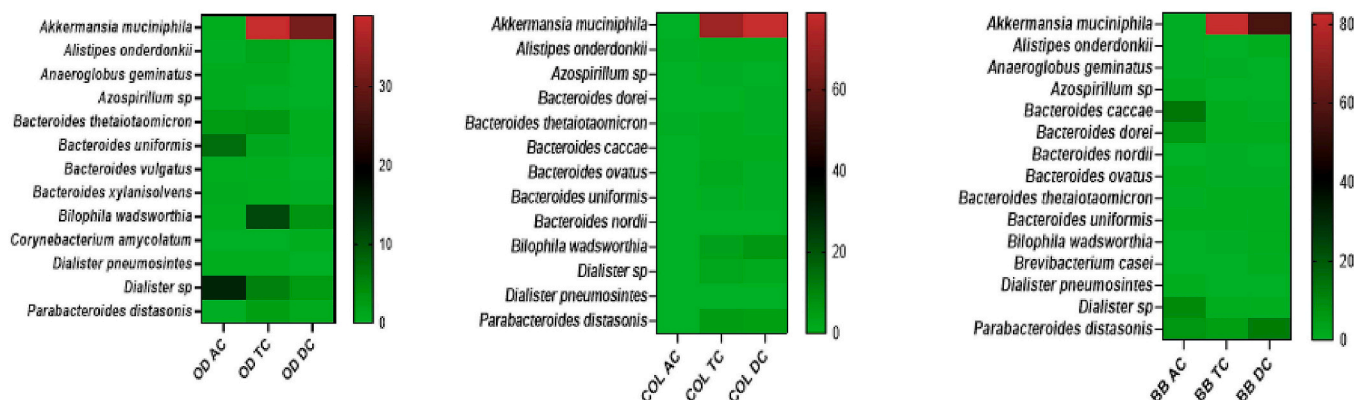
Fig. 2. Functional prediction for pathways K00965 – Betalain biosynthesis and K00941 – Flavonoid biosynthesis. Heatmaps were generated associating the contribution percentage of each individual species to the total functional potential within each assay. OD: *Opuntia stricta* var. *dillenii* whole fruit extract; COL: *Opuntia ficus-indica* var. *Colorado* pulp extract; BB: *Opuntia ficus-indica* var. *Blanca* peel extract, AC: ascending colon, TC: transverse colon, and DC: descending colon.

predominance of betacyanins, COL by a betaxanthin-rich composition, and BB by a phenolic-rich profile lacking betalains, providing a suitable framework to evaluate how matrix composition influences the digestive fate of *Opuntia* phytochemicals. Previous studies have reported similar patterns. For instance, Gómez-López et al. (2021) characterized extracts from different tissues of *O. dillenii* (whole fruit, peel, pulp, juice), showing high levels of betacyanins and their derivatives (Gómez-López et al., 2021), while yellow or orange varieties, such as COL are richer in betaxanthins (Castellanos-Santiago & Yahia, 2008; Gómez-Maqueo et al., 2020). In contrast, white cultivars such as BB, tend to contain very low or undetectable betalains but remain abundant in phenolic compounds such as piscidic acid and flavonoid derivatives (Gómez-Maqueo et al., 2020). The initial compositional differences in *Opuntia* extracts were reflected in the distinct stability patterns observed during gut simulation.

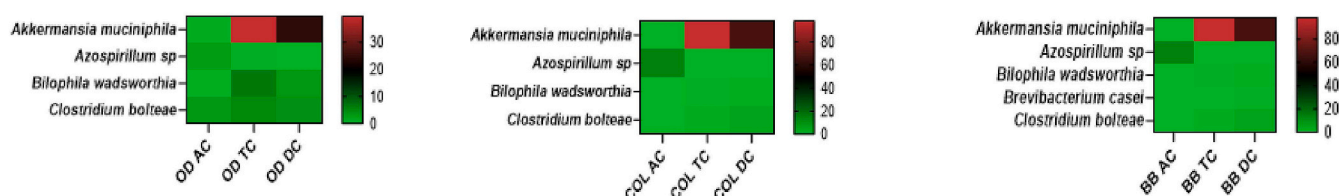
Analyses of individual betalains and phenolics showed that the GI phase emerged as the primary driver of compound degradation, as most betalains and flavonoids experienced substantial reductions before reaching the colon. This finding highlights the importance of physicochemical conditions during digestion, including pH transitions, enzymatic activity, bile salts, and oxidative processes, which can promote chemical instability independently of microbial action (Montiel-Sánchez et al., 2021). Betalains, particularly betacyanins, were especially susceptible to these conditions, showing pronounced losses already during the GI phase and becoming undetectable after the AC. This behaviour is consistent with the documented sensitivity of betalains to neutral or

slightly alkaline pH and oxidative environments, which can induce structural rearrangements and cleavage reactions (Garzón et al., 2025; Sadowska-Bartosch & Bartosz, 2021; Sawicki et al., 2018). In contrast, betaxanthins from COL extract followed a more moderate transformation along the colonic compartments. This behaviour may reflect differences in matrix composition between extracts, which can influence compound accessibility to microbial enzymes. In this context, betacyanins in OD may have been more readily available for microbial transformation, resulting in a faster apparent turnover despite their reported higher intrinsic stability (Azeredo, 2009; Herbach et al., 2006). Among phenolic compounds, piscidic acid displayed the most consistent behaviour across the studied extracts, characterized by high stability after GI and gradual decline along colonic compartments, but remaining detectable. In contrast, flavonoid glycosides underwent progressive disappearance along the colon, consistent with sequential microbial transformations such as deglycosylation, reduction, dehydroxylation, and aromatic ring cleavage (Serra et al., 2012), which may generate smaller phenolic metabolites not quantified in the present study. The identification and characterization of the potential secondary metabolites arising from colonic biotransformation of betalains and phenolic compounds warrants attention in future studies, given their potentially unexplored biological activity. Comparable results have been reported in other studies evaluating the metabolic fate of betalain- and phenolic-rich matrices. Previous studies have indicated that betalains are extensively degraded along the gastrointestinal tract, with approximately 60% of betanin being metabolized at the colonic level (Khan, 2016),

K00946 – Flavonoid degradation



K00627 – Aminobenzoate degradation



K00350 – Tyrosine metabolism

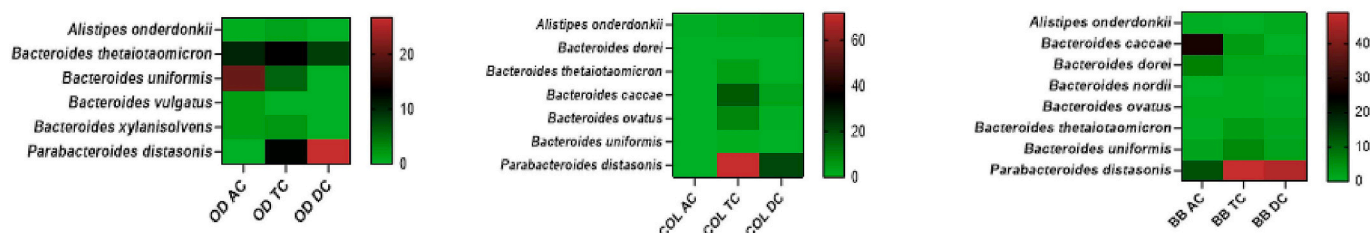


Fig. 3. Functional prediction for pathways K00946 – Flavonoid degradation, K00627 – Aminobenzoate degradation, and K00350 – Tyrosine metabolism. Heatmaps were generated associating the percentual contribution of each individual species to the total functional potential within each assay. OD: *Opuntia stricta* var. *dillenii* whole fruit extract; COL: *Opuntia ficus-indica* var. *Colorada* pulp extract; BB: *Opuntia ficus-indica* var. *Blanca* peel extract, AC: ascending colon, TC: transverse colon, and DC: descending colon.

which may partly explain the low recovery of intact betalains reaching the distal colon. Consistently, in a long-term intervention with healthy volunteers, Sawicki et al. (2018) described extensive degradation of native betalains into circulating metabolites after the consumption of fermented red beet juice. Serra et al. (2012) analyzed the colonic metabolism of individual flavonoids and phenolic acids, and reported extensive biotransformation of flavonoids, whereas phenolic acids were only slightly metabolized, in agreement with the patterns observed in the present study. Similarly, De Santiago et al. (2019) exposed *O. ficus-indica* cladodes to colonic fermentation and reported faster degradation of flavonoids rather than phenolic acids. In line with these observations, recent experiments from our research group in which *O. dillenii* pulp extracts were analyzed under batch fermentation conditions, also showed pronounced metabolism of both betalains and flavonoids, but a more moderate degradation pattern for piscidic acid (Parralejo-Sanz, Requena & Cano, 2025). Altogether, these findings reinforce that *Opuntia* phytochemicals are extensively transformed during digestion and colonic fermentation, reducing the concentration of native molecules reaching the distal colon, while likely generating secondary metabolites whose biological relevance remains to be clarified.

The baseline microbial community was characterized by a relatively high abundance of Actinobacteriota, particularly Bifidobacteriaceae, and a limited proportion of Bacteroidota, which should be interpreted as

representative of the single donor used in this study, as considerable interindividual variability in gut microbiota composition is well documented in the literature (Arbolea et al., 2016; Rinninella et al., 2019). Overall, the community exhibited high resilience, as only a limited number of taxa showed significant net changes attributable to extract supplementation. Notably, these modest compositional shifts contrasted with clear increases in SCFA production, suggesting that *Opuntia* extracts primarily influenced microbial metabolic activity rather than driving extensive community restructuring. All changes were resolved after wash-out, indicating reversible modulation dependent on the continuous phytochemical exposure, consistent with the capacity of gut microbial communities to maintain functional stability despite taxonomic fluctuations (Louca et al., 2018).

At the phylum and family levels, COL supplementation induced the most pronounced effects, characterized by an increase in Actinobacteriota in distal compartments driven by the expansion of Bifidobacteriaceae, a family widely associated with beneficial metabolic functions and considered a hallmark of prebiotic activity (Gibson et al., 2017). These findings suggest a selective bifidogenic effect with potential functional implications for gut health, although further investigation is warranted. A decrease in Bacteroidota in the AC was also observed, possibly reflecting competitive substrate utilization within the community, as taxa within this phylum are key degraders of complex

carbohydrates that adjust their abundance according to substrate availability (Shin et al., 2024). In contrast, OD supplementation produced minor effects, limited to a small decrease in Rikenellaceae in the AC. BB was associated with a selective reduction in Desulfovibrionaceae in the DC, a family of sulfate-reducing bacteria involved in hydrogen sulfide production, a metabolite that has been linked to intestinal inflammation (Carbonero & Gaskins, 2015). This reduction may represent a biologically relevant modulation of microbial taxa associated with inflammatory processes. A progressive increase in Verrucomicrobiota was observed over time independently of supplementation, likely reflecting adaptation to the continuous operation of the system. This increase occurred mainly in the distal compartments, consistent with their higher pH and the typical colonic distribution of this phylum (Geerlings et al., 2018).

At the genus level, OD supplementation was associated with decreases in *Alistipes*, *Eubacterium* and *Megasphaera* in the AC, genera associated with gut health maintenance through their roles in SCFA production (particularly butyrate and valerate) (Flint et al., 2014; Liu et al., 2022; Parker et al., 2020), likely reflecting the rapid degradation of betacyanins during digestion, which may have limited sustained microbial exposure despite their high initial content. However, SCFA production remained consistently elevated, suggesting that betacyanin degradation products retained metabolic activity.

COL induced the strongest reductions, particularly affecting polysaccharide-degrading taxa such as *Bacteroides* in the AC and TC, and *Parabacteroides* in the DC, as well as *Megasphaera* across all compartments (Flint et al., 2014), possibly related to the prolonged colonic persistence of betaxanthins and piscidic acid compared to the rapidly degraded OD extract. Despite these taxonomic shifts, SCFA production remained elevated, highlighting the functional resilience and redundancy of the gut microbial community.

BB supplementation increased fermentative genera associated with cross-feeding and SCFA-related pathways, including *Dialister*, *Lachnospirillum*, and *Megasphaera*, and decreased *Bilophila* in the DC, a genus frequently associated with pro-inflammatory profiles (Devkota et al., 2012; Fernandez-Jalao et al., 2025; Zhao et al., 2024). Together with the increased SCFA production and reduced ammonium accumulation, these findings suggest a shift towards a more saccharolytic microbial metabolism, potentially supported by the sustained availability of phenolic compounds throughout colonic fermentation.

These genus-level changes were transient and resolved after wash-out, further supporting reversible microbiota modulation dependent on continuous extract exposure. Additionally, the stability of key taxa associated with core gut functions (including *Akkermansia*, *Bifidobacterium*, *Lactobacillus*, and *Roseburia*) throughout all supplemented and non-supplemented periods further supports preservation of overall ecosystem functionality despite temporary compositional shifts (Hou et al., 2020; Martín et al., 2023; Saleena et al., 2024; Zhang et al., 2019). However, this stability should not only be attributed to intrinsic ecological resilience, as it can also be influenced by the experimental setup and the use of microbiota from a single donor.

With respect to the variations in SCFA production induced during *Opuntia* extracts supply, acetate showed the highest net increase across all treatments and compartments, with minimal regional differences, suggesting enhanced fermentative activity (Cuciniello et al., 2023). This pattern aligns with the stability of core fermentative genera and the increased abundance of *Dialister* and *Megasphaera*, observed particularly under BB supplementation, as they are commonly associated with cross-feeding interactions (Döring & Basen, 2024). Propionate production exhibited a more extract-dependent response, being enhanced under OD and BB but reduced under COL, consistent with the observed reduction of propionate-associated genera (*Megasphaera*, *Bacteroides*, and *Parabacteroides*) during COL dosing. Additionally, the increase in butyrate and valerate observed towards the DC for all extracts may reflect intensified secondary fermentation under conditions of limited readily fermentable carbohydrates and more neutral pH, which favour the

utilization of intermediate metabolites and secondary substrates (Flint et al., 2007). Notably, the increase in butyrate production is particularly relevant, given its role as the primary energy source for colonocytes and its contribution to epithelial barrier maintenance and immune homeostasis (Litvak et al., 2018). Collectively, the consistent increases in SCFA production induced by all three *Opuntia* extracts highlight the potential relevance of these plant matrices for gut health, given the central role of SCFAs in host physiology and immune regulation (Mukhopadhyay & Louis, 2025), though *in vivo* confirmation will be necessary to establish their physiological relevance.

Comparable responses have been observed in the literature for other phytochemical-rich matrices, both in terms of microbial modulation and SCFA production. For instance, Paterson et al. (2025) showed that colonic fermentation of *Nannochloropsis gaditana* microalgae digests, increased genera such as *Akkermansia*, *Butyrivibrio*, and *Lachnospirillum*, while concurrently reducing *Faecalibacterium*, *Roseburia*, *Agathobacter* and *Anaerostipes*, among others, together with enhanced production of butyric and valeric acids. Similarly, Bialonska et al., 2010 reported that fermentation of pomegranate polyphenols led to increased *Bifidobacterium* and *Lactobacillus* without influencing *Clostridium* and *Eubacterium*, while enhancing acetate and butyrate production. In the context of *Opuntia*, Guevara-Arauz et al. (2012) exposed *Opuntia* cladode extracts to microbial communities from the human colon, observing increased *Lactobacillus* and *Bifidobacterium*, and reduced *Enterococcus* and *Clostridium*, and an elevated propionate and butyrate production, while Zhang et al. (2022) also observed significant increases in the production of acetic, propionic, and butyric acids following exposure to *Opuntia ficus-indica* extracts. Together, these studies support selective microbiota modulation by phytochemical-rich plant matrices, consistent with the present findings.

Regarding proteolytic activity, moderate net increases were observed for all extracts in the AC and TC. Luminal ammonium concentrations in the human colon have been estimated to range approximately from 4 to 70 mM under normal physiological conditions (Fried et al., 2017). In this context, the ammonium concentrations measured during the supplementation phase with the different *Opuntia* extracts (11.23 mM for OD, 13.08 mM for COL, and 11.41 for BB) fell within the estimated physiological range, rather than reflecting excessive proteolytic fermentation. Exceptionally, a decrease in ammonium production was observed in the DC with COL and BB. This reduction, while remaining within physiological range, may indicate a partial decrease in proteolytic fermentation in the DC, a compartment in which protein-derived metabolites tend to accumulate (Diether & Willing, 2019). This observation may be biologically relevant given the association between excessive proteolytic metabolism and potentially detrimental colonic compounds.

Functional prediction analyses suggested that pathways related to flavonoid degradation, aminobenzoate degradation, betalain biosynthesis, and tyrosine metabolism were predicted to be mainly associated with the species *Akkermansia muciniphila*, *Bifidobacterium adolescentis*, *Parabacteroides distasonis*, and *Dialister* sp. Notably, the genera to which these species belong did not exhibit significant net variations during supplementation with *Opuntia* extracts (with the exception of *Parabacteroides*, which decreased during dosing with COL). These predictions suggest that the transformation of *Opuntia*-derived phytochemicals in the present system could rely primarily on stable metabolic functions of key gut microbial species. This interpretation is consistent with the broader concept of functional redundancy and resilience within gut microbial communities, whereby ecosystem-level functions are preserved despite taxonomic fluctuations (Kanehisa et al., 2017; Louca et al., 2018). However, direct validation through metatranscriptomic or targeted enzymatic approaches would be required to confirm these contributions.

Overall, the BFBL gut simulator enabled a controlled and compartment-resolved assessment of phytochemical transformation and microbiota responses under standardized conditions. In this context, the

use of a single-donor microbiota and a sequential experimental design provided reproducible mechanistic insights; nevertheless, future studies including multiple donors and *in vivo* validation would be required to confirm the broader applicability of these findings.

5. Conclusions

The present study demonstrates that *Opuntia*-derived betalains and phenolic compounds undergo extensive transformation during simulated digestion, with gastrointestinal conditions accounting for the major loss of native compounds and colonic fermentation contributing to further selective microbial metabolism. Betalains, especially betacyanins, were highly labile, while flavonoids showed intermediate persistence, and phenolic acids remained detectable throughout all the colonic compartments.

Regarding colonic microbiota, extract supplementation induced selective, extract- and compartment-dependent modulation of gut microbiota across taxonomic levels, but these changes were transient and resolved after wash-out phases. COL induced the most pronounced changes with a transient bifidogenic response and reductions in polysaccharide-degrading taxa, while BB reduced *Desulfovibrionaceae*, a family associated with hydrogen sulfide production and intestinal inflammation. In comparison, OD induced less compositional changes. Genus-level shifts in genera such as *Megasphaera* and *Bacteroides* were transient and did not disrupt key taxa associated with core gut functions, although the stability of these taxa cannot be unambiguously attributed to intrinsic ecological resilience given the single-donor, *in vitro* nature of the experimental system.

Functionally, all three extracts enhanced SCFA production, particularly acetate, butyrate, and valerate, whereas ammonium levels remained within physiological ranges, indicating no increase in proteolytic fermentation and an overall metabolic profile compatible with gut health maintenance. Interestingly, these metabolic changes were more pronounced than compositional shifts, suggesting that *Opuntia* phytochemicals primarily modulate microbial activity rather than community structure. Functional prediction analyses suggested that key species such as *Akkermansia muciniphila* and *Bifidobacterium adolescentis* were estimated to be major contributors to phytochemical transformation pathways; however, metatranscriptomic or enzymatic approaches would be required to confirm these predicted contributions.

Overall, *Opuntia*-derived extracts induced moderate but functional modulation of the colonic microbial activity while preserving ecosystem stability, supporting their potential as functional food ingredients. Nevertheless, further multi-donor *in vivo* studies are required to confirm the physiological relevance of these effects.

CRedit authorship contribution statement

Sara Parralejo-Sanz: Writing – original draft, Investigation, Formal analysis, Data curation. **Cristina Yépez-Notario:** Investigation, Formal analysis, Data curation. **M. Pilar Cano:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Teresa Requena:** Writing – review & editing, Visualization, Supervision, Funding acquisition, 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.foodres.2026.119953>.

Data availability

<https://digital.csic.es/handle/10261/420274>

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