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In vitro colonic fermentation of orange peel fibres: Effect on microbial modulation, SCFAs production and carotenoid degradation

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ABSTRACT

Orange peel by-products are valuable for their content of dietary fibre and bioactive compounds that offer health benefits, such as the production of short-chain fatty acids (SCFAs) and modulation of gut microbiota. The aim of this study is to evaluate the prebiotic effect of three fibre-rich fractions of orange peel (orange peel extract, OP; insoluble fibre fraction, IFF; and water-soluble extract, WSE) by means of *in vitro* fermentation. Degradation of carotenoids during fermentation was examined to explore their interaction with the microbiota. The results indicate that *in vitro* fermentation of fibre-rich ingredients increased *Lactobacillus* and *Bifidobacterium* populations. WSE, rich in bioactive compounds, increased *Bifidobacterium* by 2.5 times compared with the other fractions, and gave a 1.5-fold higher total SCFAs production and a noteworthy evolution of butyrate. Moreover, although carotenoids decreased during fermentation, they remained detectable at the end of the process, suggesting that they are not efficiently metabolized by microbiota and could persist in the colon for a longer period. These findings open up new avenues of research in the use of by-products to develop novel ingredients. In addition, future research should focus on investigating the behaviour of carotenoids during digestion, as well as their antioxidant, anti-inflammatory, and microbiota-modulating effects.

1. Introduction

Fruit and vegetable industries generate huge amounts of by-products rich in compounds with potential health benefits, including different parts of plants such as pulp, peel, seeds, skin, pomace, husks, pods and stems. In general, these by-products contain significant amounts of dietary fibre and various bioactive compounds, such as (poly)phenols, carotenoids and glucosinolates (Reguengo et al., 2022). For this reason, the valorisation of by-products can be attractive for the food industry to obtain ingredients of nutritional interest, which can be reintroduced in the food chain (Schulz & Slavin, 2022).

Oranges constitute over half of global citrus fruit production, with Spain contributing more than half of the European production. Furthermore, approximately one-third of fresh oranges are used for industrial processing (FAO, 2021). In the case of the orange juice industry, the orange peel is a by-product rich in dietary fibre, (poly)phenols mainly from flavedo, and carotenoids from albedo. Although orange peel has historically been used in industry for the extraction of pectin and essential oils, to make orange liqueur or as comminute for soft drinks (Berk, 2016), the substantial volume of the by-products generated (approximately 70% of the initial fresh fruit) and their nutritional attributes are progressively underscoring the importance of exploring new applications (Casas Cardoso, Cejudo Bastante, Mantell Serrano, & Martínez de la Ossa, 2022; Pineda-Lozano et al., 2022).

Regarding their main components, dietary fibre provides several benefits to human health, depending on its composition and solubility characteristics, which determine the viscosity and fermentability in the gut (Gibson et al., 2017). Dietary fibre is categorised into soluble and insoluble fractions based on their solubility in water. Soluble fibres, such as pectin and soluble hemicellulose, exert positive effects on glucose and lipid metabolism by increasing viscosity and reducing absorption in the large intestine. In contrast, insoluble fibres, represented by cellulose and insoluble hemicellulose, promote satiety and decrease transit time (Slavin, 2013). Furthermore, dietary fibre exhibits a notable prebiotic

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effect by serving as a substrate for the gut microbiota, leading to the production of short-chain fatty acids (SCFAs) including acetic, propionic, and butyric acids (Gibson et al., 2017). SCFAs confer various health benefits to the host, such as alleviating constipation and exerting anti-inflammatory, immunoregulatory, anti-diabetic, hepatoprotective, and neuroprotective effects (Xiong et al., 2022). Additionally, SCFAs possess the capacity to modulate the composition of the microbiota, promoting the proliferation of beneficial bacterial families such as *Lactobacillus* and *Bifidobacterium* (Yegin et al., 2020). Alongside indigestible carbohydrates, the fibre-rich fraction obtained from orange peel contains (poly)phenol compounds that are associated with cell wall polysaccharides, forming the non-extractable (poly)phenols (NEPP). In oranges, more than half of the (poly)phenols exist as NEPP (Martínez-Meza, Reynoso-Camacho, and Pérez-Jiménez, 2021), which resist digestion in the large intestine and reach the colon intact, where they are released from the dietary fibre matrix and become available for microbial metabolization. Our recent findings indicate that NEPP from the fibre-rich fraction of orange peel can be metabolized by the microbiota during *in vitro* fermentation, resulting in the production of various catabolites (Núñez-Gómez et al., 2024).

In addition to the content of (poly)phenols, orange peels also have different carotenoids, which are tetraterpenoid pigments responsible for the orange colour. Carotenoids are absorbed partially during digestion, but a variable proportion can reach the colon during the digestive process, particularly those that are not incorporated into micelles during digestion (Böhm et al., 2021; Periago et al., 2016). Nevertheless, previous studies have shown the accumulation of carotenoids in the intestinal lumen, increasing the antioxidant content which could have beneficial effects on both the gut microbiota and intestinal epithelial cells (Böhm et al., 2021; Periago et al., 2016). However, limited research has been conducted on the metabolization of carotenoids by the microbiota, resulting in a lack of knowledge about the potential catabolites formed (Núñez-Gómez et al., 2023).

Evaluating the prebiotic effect of dietary fibre involves a multidisciplinary approach, including *in vitro* fermentation, animal studies, human clinical trials, microbiota analysis, SCFA measurement, gut health markers, and bioinformatics to assess the impact of dietary fibre on gut microbiota and overall host health. Among these, *in vitro* fermentation models stand out as particularly effective tools for the study of gut microbiota in a controlled environment, where factors such as pH, temperature, and medium composition can be accurately regulated (Pérez-Burillo et al., 2021). These models provide valuable insights into the prebiotic effects of specific ingredients or foods, including fibres and bioactive compounds (Poeker et al., 2018; Slavin, 2013).

To this end, the aim of this study was to determine the prebiotic effect of fibre-rich fractions isolated from orange peel by the evaluation of their impact on human microbiota modulation through the use *in vitro* colonic fermentations in a bioreactor simulating the proximal colon environment. In addition, the degradation of carotenoids during *in vitro* fermentation was also evaluated to elucidate the interaction between orange carotenoids and the microbiota.

2. Materials and methods

2.1. Samples

Oranges (variety Navel Late, produced in Cieza, Murcia, Spain) were purchased from a local supermarket and the peels were obtained after juice extraction with a home juicer. Three different fractions were obtained by using an environmentally friendly method previously published by Núñez-Gómez et al. (2024). Briefly, dried orange peel (OP) was subjected to solid/liquid extraction using distilled water (proportion 2.5/1, v/w) and after centrifugation two more fractions were obtained: the upper phase or supernatant representing the soluble fraction enriched with (poly)phenols, predominantly flavanones (referred to as WSE, water-soluble extract), and the lower phase or the precipitate containing the insoluble fibre fraction (IFF). More details of the extraction process, the proximate composition and the chemical characterisation of the three fractions are available in the study carried out by Núñez-Gómez et al. (2024).

2.2. Analysis of carotenoids in the fractions by HPLC-DAD

Carotenoids were quantified in the three fractions, with a previous saponification step described by Nan et al. (2012). The extraction procedure followed the protocol outlined by González-Barrio et al. (2018) with some modifications. Briefly, 0.1 g of the sample was mixed with $NaHCO_3$ and a mixture of ethyl acetate, methanol, and petroleum ether (1/1/1, v/v/v) containing 0.1% butylated hydroxytoluene. The samples were then sonicated for 5 min in an ultrasonic bath (Branson Digital model 250; Branson, Danbury, USA). The resulting mixture was subjected to centrifugation in a Beckman J2-21 centrifuge (Beckman, Indianapolis, USA), and the supernatant was collected. This process was repeated twice. Subsequently, the supernatants were washed with a 5% NaCl solution and evaporated using a Laborota-4002 rotary evaporator (Heidolph, Schwabach, Germany) at room temperature. The resulting residue was resuspended in diethyl ether and mixed with 30% KOH, followed by an 8-h stirring period in darkness. Afterward, the samples were washed with a 5% NaCl solution until a neutral pH was reached. The supernatant was evaporated at room temperature and the residue resuspended in a mixture of methyl tert-butyl ether and methanol (1/1, v/v). The extracts were subjected to analysis using HPLC-DAD on an Agilent 1100 system (Agilent Technologies, Germany). A C30 column (250 × 4.6 mm, 5 μm i. d.) (Trentec, Gerlingen, Germany) was employed for chromatographic separation at a temperature of 17 °C. Methyl tert-butyl ether (A) and methanol (B) were used as mobile phases, with a flow rate of 1 mL/min. The gradient began with 2% A, reaching 35% at 35 min and, 60% at 45 min; this was maintained until 55 min, followed by a washing step. Chromatograms were recorded at 472 and 450 nm. Carotenoids were identified according to their spectral characteristics, comparing them with those of available authentic standards and those previously reported in the literature (Meléndez-Martínez et al., 2005; Murador et al., 2019). The quantification was according to their chromatographic peak areas recorded at 450 nm, being expressed as lutenin equivalents in mg/kg of dry weight (d.w.).

2.3. In vitro faecal fermentation process

Three independent colonic fermentations were conducted for each condition tested (three different samples as substrate) using an automatically controlled Minifors 2 bioreactor (Infors HT, Surrey, UK). Batch experiments were conducted in a 0.6 L working volume with adapted Simulated Ileal Environment Medium (SIEM) (Table S1) continuously stirred at 300 rpm. The temperature was set at 37 °C and the pH was maintained in a range of 5.8–6.2 with automatic injection of 25% NH_3 and 20% H_3PO_4. The PO_2 level was set at 1 ppm by automatic control. The foam level was also controlled, by automatic dispensing of SE-15 antifoam (A8582, Sigma-Aldrich, Germany). Faecal samples were collected from six healthy women who had not taken antibiotics or probiotics for at least 6 months prior to the experiment (all donors provided informed consent before the faecal sample was delivered). Samples were collected at home, in a biological sample container with anaerobic sachets (Anaerogen Compact, ThermoFisher Scientific, San José, CA, USA) within the 12 h before the experiment, and were then kept under refrigeration (4–8 °C). Equal amounts of faecal material from each donor were pooled in an anaerobic workstation (Whitley A85 TG, Don Whitley Scientific Limited, England). Two grams of the faecal mixture were combined with 10 mL of a NaCl-glycerol solution (0.9% NaCl, 10% glycerol) and stored at −80 °C until inoculation in the bioreactor. For inoculation, preserved faecal inoculum was thawed in a water bath at 37 °C for 5 min and centrifuged at 100 rpm to precipitate big particles; 6 mL of the supernatant were injected in the bioreactor as

initial inoculum. Microbial biomass increased until stabilisation 18 h after inoculation. After biomass stabilisation, 6 g of the sample being tested were added (OP, IFF, WSE). Colonic fermentation was carried out for 24 h, and 10-mL samples were taken at 0, 4, 8, and 24 h to study the evolution of bacterial growth, SCFAs production and carotenoid degradation.

2.4. Bacterial growth determined by plate counting during in vitro fermentation

Six different culture media were used to determine the bacterial growth: BSM (*Bifidobacterium* selective medium) agar for Bifidobacterias (Neogen, Lansing, MI, USA), BBE (*Bacteroides* bile esculin) agar for the *Bacteroides fragilis* group (Sigma Aldrich, St. Louis, MO, USA), SDA (sabouraud dextrose) agar for moulds and yeasts (Neogen, Lansing, MI, USA), VRBG (violet red bile glucose) agar for the Enterobacteriaceae family (Neogen, Lansing, MI, USA), PCA (plate count agar) agar for total aerobes and total anaerobes bacteria (Sigma Aldrich, St. Louis, MO, USA), and LAMVAB (*Lactobacillus* anaerobic MRS with vancomycin and bromocresol green) agar (Neogen, Lansing, MI, USA), which was prepared according to Hartemink et al. (1997), for the growth of *Lactobacillus*. The growth dynamics of the selected bacterial groups were investigated throughout the colonic fermentation, after collecting samples at different time points (0, 4, 8, and 24 h). Decimal dilutions of the samples were prepared using peptone water prior to plating into specific agars in Petri dishes. The samples were incubated under different conditions of aerobiosis/anaerobiosis, temperature and time according to the requirements of the microorganism under investigation (Table S2). Bacterial counts were expressed as Log CFU/mL of sample.

2.5. SCFAs analysis during in vitro fermentation

The SCFAs (acetate, propionate, and butyrate) were assessed by GC-FID following the method previously published by Baenas et al. (2020). The samples obtained during the fermentation of the three fractions (OP, IFF and WSE) in the bioreactor were mixed with a 20% formic acid/-water solution, methanol, and 2-ethyl butyric acid (used as an internal standard at a concentration of 2 mg/mL in methanol) (1/4.5/1, v/v/v). GC-FID analysis was performed using an Agilent 7890A GC system equipped with a flame ionization detector (FID) and a 7683B automatic injector, both manufactured by Agilent Technologies (CA, USA). The separation of SCFAs was achieved using a fused-silica capillary column (Nukol TM, Supelco, Taufkirchen, Germany) under the conditions described by Baenas et al. (2020). The identification of SCFAs was conducted by comparing their retention times with those of authentic standards, and their quantification was based on calibration curves constructed using a Volatile Free Acid mix (Supelco, Taufkirchen, Germany). The concentrations of acetate, propionate, butyrate, and total SCFAs were expressed in mM.

2.6. Carotenoid analysis during in vitro fermentation

The samples collected at different time points during the *in vitro* colonic fermentation were examined to assess the degradation of carotenoids throughout the process. For the analysis, 0.8 mL of the sample was combined with 10 mL of hexane/acetone/ethanol (2/1/1, v/v/v). The mixture was vortexed for 1 min, and then the upper phase was evaporated using a Laborota-4002 rotary evaporator (Heidolph, Schwabach, Germany) at room temperature. The resulting residue was dissolved in 0.25 mL of methyl tert-butyl ether and methanol (1/1, v/v). The analysis was conducted using an HPLC-DAD on an Agilent 1100 system (Agilent Technologies, Germany), with the method described above for carotenoid analysis in the fibre samples. The results were expressed as ng of carotenoids/mL of sample.

2.7. Statistical analysis

The statistical analysis was conducted using R Studio, version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). Normality was assessed using the Shapiro-Wilk test. Homogeneity of variances was examined using the Bartlett test. One-way analysis of variance (ANOVA) was performed to identify significant differences among the analysed parameters across the different fractions and time points. Post-hoc analysis was conducted using Tukey's test to determine the specific differences between means. Differences were considered significant at a $p\text{-value} < 0.05$.

3. Results and discussion

3.1. Carotenoid content in the orange peel fractions

The carotenoid analysis by HPLC-DAD revealed the presence of 20 peaks, 10 of them being tentatively identified ten of them (Table 1). Among the identified compounds, sintaxanthin exhibited significantly higher levels in OP and IFF compared to WSE, whereas violaxanthin and lutein isomer I were notably higher in WSE (2-fold) and IFF (1.6-fold) compared to OP. Additionally, IFF showed the highest concentration of α-carotene and antheraxanthin isomer, whereas OP displayed the highest content of β-cryptoxanthin.

Thus, the differences observed were more pronounced at the quantitative than at the qualitative level. This indicates that the fractions had similar proportions of the identified carotenoids, although their concentrations varied among the fractions. The predominant carotenoid in the fibre samples (IFF and WSE) was violaxanthin. This observation is consistent with previous scientific literature, which identified violaxanthin and its isomers as the primary carotenoids in orange flavedo (Escobedo-Avellaneda et al., 2014; Ma et al., 2015; Rodrigo & Zacarias, 2007). Conversely, β-cryptoxanthin, the second most abundant carotenoid in our samples, was identified as the predominant carotenoid in orange juice (Escobedo-Avellaneda et al., 2014). Additionally, β-carotene was found in lower proportions in our samples, consistent with previous results for orange flavedo (Escobedo-Avellaneda et al., 2014).

In terms of the overall carotenoid content, representing the sum of all individual carotenoids (Table 1), both IFF (120.5 mg/kg) and OP (111.6 mg/kg) showed significantly higher contents compared to the WSE fraction (91.1 mg/kg). The OP consisted of the dried peel, while IFF represented the insoluble compounds of the orange peel, and both fractions contained the carotenoids responsible for the orange colour. The WSE fraction predominantly consisted of soluble compounds extracted from the orange peel during the water extraction process. Although this fraction also contained carotenoids, their concentration was relatively low, resulting in a lighter orange colour. The values observed in our fractions are higher than those previously reported: from 60 to 100 mg/kg (Escobedo-Avellaneda et al., 2014; Murador et al., 2019).

3.2. Bacterial growth modulation

Fig. 1 shows the effect of the three orange peel fractions on microbial growth, which was determined by evaluating the growth of *Lactobacillus*, *Bifidobacterium*, Enterobacteriaceae family, and the *Bacteroides fragilis* group, as well as total aerobes and anaerobes bacteria (Fig. 1). From a general perspective, the bacterial growth differed among the three fractions, which could be attributed to the interactions between the microbiota and the substrates of varying composition.

Regarding *Lactobacillus*, the fermentation of the WSE and IFF fractions resulted in an increase in this bacterial group of almost two logarithmic units at 24 h compared to OP, with significant differences between the samples. However, the potential prebiotic behaviour of the samples varied over the course of the fermentation; WSE gave a greater increase at 8 h than IFF and OP for both *Lactobacillus* and

Table 1
Spectral characteristics and content (mg/kg of d.w.) of the carotenoids detected by HPLC-DAD in the orange peel fractions obtained from orange by-products.

Peak	Carotenoid	t_R (min)[a]	λ_{max} (nm)[b]	Content (mg/kg d.w.)		
				OP	IFF	WSE
1	Luteoxanthin	5.7	400, 422, 447	8.3 ± 1.2	8.1 ± 0.8	5.2 ± 1.8
2	Sintaxanthin	6.3	419, 438, 466	10.4 ± 1.3[a]	8.1 ± 0.8[a]	6.6 ± 0.8[b]
3	Carotenoid-like spectrum	6.9	380, 402, 424, 434	10.0 ± 0.9[a]	9.3 ± 0.9[ab]	7.4 ± 0.8[b]
4	Carotenoid- like spectrum	7.5	426, 442, 460	13.1 ± 1.0[a]	11.4 ± 0.4[b]	9.7 ± 0.3[c]
5	Violaxanthin	7.9	418, 438, 465	6.9 ± 0.7[b]	13.2 ± 1.6[a]	15.3 ± 1.9[a]
6	Carotenoid- like spectrum	8.1	398, 418, 442	6.0 ± 0.6[b]	6.9 ± 0.4[a]	3.4 ± 0.4[c]
7	Carotenoid- like spectrum	8.3	400, 418, 444	1.8 ± 3.1[b]	9.1 ± 1.0[a]	n.d.
8	Carotenoid- like spectrum	8.7	380, 428, 446	3.8 ± 0.4	n.d.	n.d.
9	Lutein	9.1	423, 444, 468	5.3 ± 0.6	5.5 ± 0.9	4.3 ± 0.5
10	Lutein isomer I	9.4	418, 444, 466	2.9 ± 0.4[b]	4.9 ± 0.8[a]	4.0 ± 0.3[ab]
11	Lutein isomer II	10.2	418, 442, 468	3.3 ± 0.5	2.7 ± 0.5	2.3 ± 0.7
12	Carotenoid- like spectrum	11.4	404, 426, 450	6.1 ± 0.6	5.7 ± 1.1	4.8 ± 0.4
13	Carotenoid- like spectrum	11.8	398, 424, 450	9.7 ± 0.9	10.5 ± 0.9	8.4 ± 0.6
14	Carotenoid- like spectrum	15.7	412, 444, 470	1.6 ± 0.2	1.4 ± 0.4	1.2 ± 0.2
15	Carotenoid- like spectrum	17.9	406, 432, 450	3.3 ± 0.2[a]	3.3 ± 0.0[a]	2.3 ± 0.3[b]
16	α-carotene	19.4	414, 444, 470	2.3 ± 0.2[b]	3.1 ± 0.1[a]	2.4 ± 0.2[b]
17	β-cryptoxanthin	21.1	424, 450, 476	12.4 ± 0.1[a]	11.1 ± 0.3[b]	8.8 ± 0.9[c]
18	Antheraxanthin isomer	22.5	414, 436, 468	1.4 ± 0.0[b]	2.0 ± 0.1[a]	1.4 ± 0.2[b]
19	Carotenoid- like spectrum	29.9	414, 430, 450	1.2 ± 0.1[c]	1.9 ± 0.0[a]	1.5 ± 0.1[b]
20	β-carotene	31.5	436, 452, 476	2.0 ± 0.1	2.3 ± 0.2	2.1 ± 0.1
	Total			**111.6 ± 3.3[a]**	**120.5 ± 3.5[a]**	**91.1 ± 8.4[b]**

Values are expressed as mean ± SD (n = 3). Different letters (a–c) indicate significant differences ($p < 0.05$) among the fractions (OP, IFF and WSE). [a] Retention time on the C_{30} column; [b] λ_{max}, maximum absorption wavelength (nm); n.d., not detected; orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE).

Bifidobacterium. Conversely, OP did not influence significantly the *Lactobacillus* counts over time. In relation to *Bifidobacterium* growth, it is noteworthy that the WSE fraction demonstrated a potential microbial modulation effect, increasing the population of this bacterial group by one logarithmic unit after 24 h of fermentation. During the fermentation of OP and IFF, a decrease in *Bifidobacterium* growth was observed, although this change was not significant over time. Regarding the Enterobacteriaceae, all three samples gave a decrease at the end of the fermentation period, but only WSE exhibited significant differences over the 24-h period, achieving a reduction of more than one logarithmic unit. This effect was also observed for the *Bacteroides fragilis* group for both the WSE and IFF fractions, with a significant decrease only in the IFF. This suggests that other microorganisms, such as lactic acid bacteria, may have replaced them, likely favoured by the presence of dietary fibre (Wang et al., 2021). In contrast, an increase in growth of *B. fragilis* was observed at 24 h for the OP fraction. Total aerobes exhibited a decrease of almost one logarithmic unit for both the WSE and IFF fractions. Anaerobes increased for IFF and remained stable for the other two fractions, IFF showing the highest value at 24 h of fermentation.

These findings indicate a potential prebiotic effect, as evidenced by the increased populations of *Lactobacillus* and *Bifidobacterium* due to the fibre samples (WSE and IFF). These results align with previously published studies where the incubation of garlic fructans led to an increased population of *Bifidobacterium* (Zhang et al., 2013). Similarly, another study reported significant increases in *Bifidobacterium* and *Lactobacillus* following *in vitro* fermentation of whole oat flour and oat bran fractions (Kedia et al., 2009). However, unlike our results, this study did not show a notable impact on the presence of the Enterobacteriaceae (Kedia et al., 2009). Soluble dietary fibres might have also stimulated the growth of B*ifidobacterium*, while (poly)phenols promoted the proliferation of *Lactobacillus* and *Bifidobacterium* (Abreu y Abreu et al., 2021; Dueñas et al., 2015; Jaquet et al., 2009). These findings are consistent with the results observed for the IFF and WSE samples, where the former exhibited the highest dietary fibre content and the latter the highest (poly)phenol content.

3.3. SCFAs production

The SCFAs results for the analysed samples are shown in Fig. 2. As previously reported in the literature, acetate was found to be the major component, followed by propionate and butyrate (Blaut, 2018). Notably, in the WSE fraction, butyrate levels exceeded those of propionate at 4 and 8 h, which coincided with increased carotenoid degradation and high glucose content during that fermentation period. These two factors might explain the enhanced fermentability observed in this fraction (Blaut, 2018; Núñez-Gómez et al., 2024; Valdes et al., 2024). Regarding acetate, the levels progressively increased over time for all samples, reaching the highest values at 24 h. The WSE fraction exhibited the highest production at 24 h of fermentation, including the highest production of butyrate, potentially attributable to its greater hemicellulose content (Núñez-Gómez et al., 2024), as has been previously reported (Deloule et al., 2020; Lindstad et al., 2021). Regarding propionate, which is more associated with insoluble fibre fermentation (Widaningrum et al., 2020), the OP fraction showed higher production at 24 h compared to IFF, while the concentrations for IFF and WSE remained relatively constant over time. Lastly, the total production of SCFAs significantly increased throughout the fermentation process for the samples of all fractions; this increase was in concordance with the growth of *Lactobacillus* and *Bifidobacterium*, suggesting the potential prebiotic effect of the fractions. At 24 h, the WSE fraction exhibited higher production of total SCFAs compared to IFF and OP, likely due to its higher presence of (poly)phenols, glucose and hemicellulose, which have been reported previously to increase the production of SCFAs (Blaut, 2018; Rodríguez-Daza et al., 2021; Tomás-Pejó et al., 2023). Samples of the same fractions underwent batch fermentation in a previous study (under static conditions), and the results revealed similar

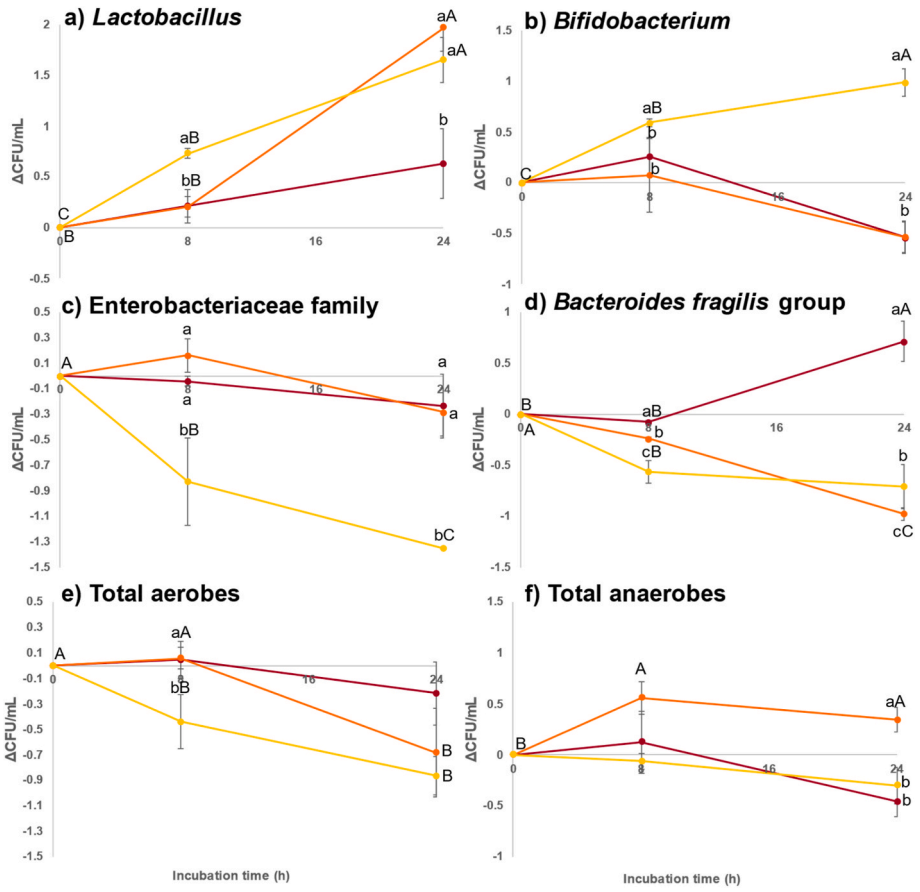

Fig. 1. Changes in bacterial growth (ΔCFU/mL of sample) of different groups ((a) *Lactobacillus*, (b) *Bifidobacterium*, (c) Enterobacteriaceae family, (d) Bacteroides fragilis group, (e) total aerobes and (f) total anaerobes) during *in vitro* fermentation of orange peel samples at 0, 8 and 24 h of incubation. Orange peel extract (red ●, OP) and the fibre fractions: insoluble fibre fraction (orange ●, IFF) and water-soluble extract (yellow ●, WSE). Values are expressed as mean ± SD (n = 3). Lowercase letters (a–c) indicate significant differences ($p < 0.05$) among the fractions at the same incubation time. Capital letters (A–D) indicate significant differences ($p < 0.05$) among the fermentation times for the same fraction.

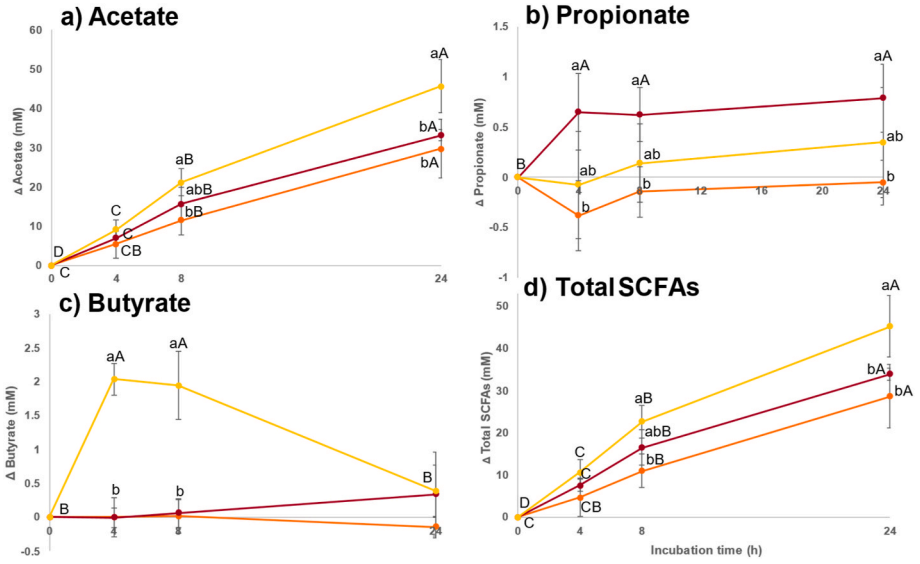

Fig. 2. Increment of SCFAs (ΔSCFAs) production ((a) acetate, (b) propionate, (c) butyrate and (d) total SCFAs) (mM) during *in vitro* fermentation of orange peel samples at 0, 4, 8 and 24 h of incubation. Orange peel extract (red ●, OP) and the fibre fractions: insoluble fibre fraction (orange ●, IFF) and water-soluble extract (yellow ●, WSE). Values are expressed as mean ± SD (n = 3). Lowercase letters (a–c) indicate significant differences ($p < 0.05$) among the fractions at the same incubation time. Capital letters (A–D) indicate significant differences ($p < 0.05$) among the fermentation times for the same fraction.

patterns (Núñez-Gómez et al., 2024). For instance, the WSE fraction exhibited the highest production of butyrate, indicating the reproducibility of the methods employed in this study. Additionally, continuous systems employing colonic fermentation of other soluble fibres, like lactulose, have demonstrated higher butyrate production (Bothe et al., 2017). Furthermore, continuous fermentation methods involving other insoluble fibres, such as chitin glucan and insoluble dietary fibre from soy hulls, have shown increased production of acetate, propionate and butyrate (L. Li et al., 2022; Marzorati et al., 2017), consistent with the data reported in our study.

3.4. Carotenoid degradation during in vitro fermentation

The concentration of carotenoids during the *in vitro* fermentation process was determined in this study, as it has been reported that a portion of the ingested carotenoids remained intact upon reaching the colon (Böhm et al., 2021; Periago et al., 2016). Within the colon, they have the potential to generate certain catabolites, including apo-carotenoids and fucoxanthinol, although these substances have not been extensively investigated (Liu et al., 2019; Steiger et al., 2012). Although sintaxanthin, an apo-carotenoid, was identified in the fibre samples, its presence was not observed during the fermentations. This implies that it might have been swiftly degraded along the fermentation process as previously reported (Guo et al., 2019; Z. Li et al., 2023) or formed complexes with the fermentation medium components, such as protein, impeding its extraction with conventional methods (Babu et al., 2008).

The results revealed the presence of four identified carotenoids in the fermentation samples: β-carotene, β-cryptoxanthin, lutein, and α-carotene (Fig. 3). It is worth noting that the most notable differences among the fermented samples were observed for lutein. The IFF fraction consistently displayed the highest concentration throughout the fermentation process, while OP gave the lowest concentration compared to the other two samples. Significant degradation was observed for lutein in all cases, with respective reductions of 48%, 55%, and 51% for OP, IFF, and WSE (Table 2). In contrast, significant degradation was only observed in the WSE fraction for α-carotene (17%), and in both fibre fractions (IFF and WSE) for β-cryptoxanthin (46% and 61%, respectively) and β-carotene (40% and 53%, respectively). Similar findings have been reported by other researchers, with degradation rates of around 40% for fucoxanthin during faecal fermentation (Guo et al., 2019). Our findings suggest that carotenoid degradation was more pronounced during the first 8 h of fermentation, particularly in the WSE fraction. This might have been due to the higher solubility of this fraction, which likely enhanced the bioavailability of carotenoids. Moreover, this was consistent with the levels of SCFAs, whose production could be enhanced by the presence of carotenoids as previously described (Li et al., 2023). These findings are significant due to the apparent degradation of carotenoids, which could have been linked to the environmental conditions in the medium or to microbial metabolism. However, the persistence of these compounds after a 24-h

Table 2
Percentage (%)carotenoids degradation (lutein, α-carotene, β-cryptoxanthin and β-carotene) during *in vitro* fermentation of orange peel fractions at 4, 8 and 24 h of incubation. Orange peel extract (OP) and the fibre fractions: insoluble fibre fraction (IFF) and water-soluble extract (WSE).

Sample	Time (h)	Lutein	α-Carotene	β-Cryptoxanthin	β-Carotene
OP	4	25.6 ± 4.6^{bB}	11.3 ± 4.1	20.3 ± 14.6^{bB}	14.8 ± 12.0^{C}
	8	44.5 ± 11.4^{A}	22.4 ± 8.3	34.5 ± 4.8^{bB}	27.7 ± 11.8^{BC}
	24	48.4 ± 2.3^{bA}	14.8 ± 2.0	44.3 ± 4.4^{bA}	34.6 ± 1.6^{bA}
IFF	4	44.3 ± 5.0^{aC}	13.7 ± 11.0	40.2 ± 1.9^{a}	29.8 ± 4.1
	8	47.3 ± 0.5^{BC}	14.9 ± 2.6	42.9 ± 4.9^{ab}	35.8 ± 6.1
	24	54.6 ± 4.2^{aA}	13.2 ± 0.8	46.1 ± 5.1^{b}	40.2 ± 8.8^{ab}
WSE	4	27.7 ± 1.6^{bB}	14.3 ± 2.6^{B}	28.1 ± 8.1^{abC}	30.9 ± 16.1
	8	48.6 ± 2.3^{A}	23.3 ± 3.2^{A}	46.9 ± 4.0^{aB}	44.4 ± 10.9
	24	50.6 ± 1.3^{abA}	17.1 ± 3.5^{AB}	61.3 ± 7.1^{aA}	52.9 ± 9.2^{a}

Values are expressed as mean ± SD (n = 3). Lowercase letters (a–c) indicate significant differences ($p < 0.05$) among the fractions (OP, IFF and WSE) at the same incubation time. Capital letters (A-D) indicate significant differences ($p < 0.05$) among the fermentation times for the same fraction.

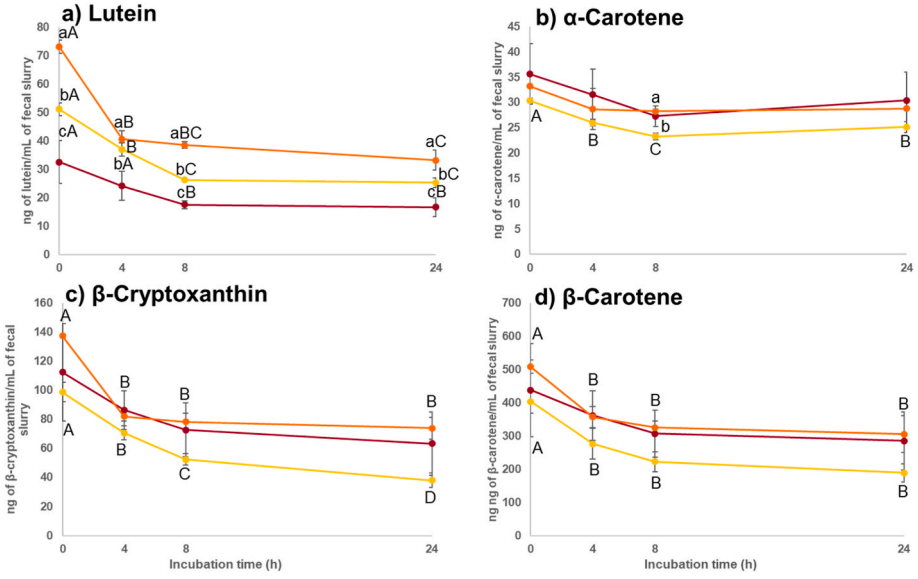

Fig. 3. Carotenoid degradation ((a) lutein, (b) α-carotene, (c) β-cryptoxanthin and (d) β-carotene) (ng of carotenoids/mL of sample) during *in vitro* fermentation of orange peel samples at 0, 4, 8 and 24 h of incubation. Orange peel extract (●OP) and the fibre fractions: insoluble fibre fraction (●IFF) and water-soluble extract (●WSE). Values are expressed as mean ± SD (n = 3). Lowercase letters (a–c) indicate significant differences ($p < 0.05$) among the fractions at the same incubation time. Capital letters (A–D) indicate significant differences ($p < 0.05$) among the fermentation times for the same fraction.

fermentation period implies their potential presence within the colonic environment under *in vivo* conditions. This underscores the bioavailability of carotenoids in different fractions of orange peels for utilization by the microbiota, offering a diverse array of advantages, encompassing antioxidant effects and microbial modulation (Böhm et al., 2021; Periago et al., 2016). In a previous study, these fractions exhibited antioxidant capacity, with FRAP values ranging from 41 to 105 μmol Trolox equivalents/g and ORAC values between 455 and 685 μmol Trolox equivalents/g, highlighting their antioxidant potential (Núñez-Gómez et al., 2024).

The carotenoid degradation data indicate that α-carotene was the least degraded carotenoid throughout the fermentation process. In contrast, the other three identified carotenoids exhibited degradation levels ranging from 35% to 61% after 24 h of fermentation. Notably, the degradation was highest in the IFF and WSE fibre fractions. This finding suggests that carotenoids were more prone to degradation in samples subjected to an extraction process compared to when they remained within the intact matrix (OP). Furthermore, our results for β-carotene degradation align with findings from other studies that evaluated the *in vitro* fermentation degradation of pure β-carotene. These studies reported degradation rates of 60%–80% over a 24-h fermentation period, indicating that the ingredient matrix might have offered a protective effect against degradation (Z. Li et al., 2023).

4. Conclusion

In summary, the analysed samples exhibited a substantial carotenoid content that remained during the fermentation process, suggesting the ability of carotenoids to remain in the colon after their metabolism. These findings open new avenues for investigating potential metabolites originating at the colonic level and their specific effects on the certain bacterial groups. Furthermore, fibre fermentation generated SCFAs with potential health benefits. Plate cultures demonstrated the positive relationship between the SCFAs and two key communities, *Lactobacillus* and *Bifidobacterium*, which are known to confer significant health benefits. These findings demonstrated that the utilization of by-products, such as orange peels in this research work, has the potential to create novel market niches for product development. Such products could find applications as nutraceuticals or innovative ingredients, to produce foods with enhanced health attributes, although further studies are needed to develop their potential applications.

CRediT authorship contribution statement

Vanesa Núñez-Gómez: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Carlos Gómez-Gallego:** Writing – review & editing, Resources, Methodology, Investigation. **Marjukka Kolehmainen:** Writing – original draft, Resources. **Rocío González-Barrio:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Funding acquisition, Data curation, Conceptualization. **María Jesús Periago:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2025.117536.

Data availability

Data will be made available on request.

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