Food Bioscience 68 (2025) 106659

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Prebiotic potential of olive leaf by-product throughout *in vitro* human colon fermentation

Sánchez-Gutiérrez Mónica [a,*], Gómez-García Ricardo [c], Pintado Manuela [c], Rodríguez Alejandro [b], Carrasco Elena [a,**]

[a] Departamento de Ciencia y Tecnología de los Alimentos, UIC Zoonosis y Enfermedades Emergentes (ENZOEM), Campus de Excelencia Internacional en Agroalimentación (CeiA3), Universidad de Córdoba, Campus Rabanales, Edificio Darwin-Anexo, E-14071, Córdoba, Spain
[b] Grupo Biopren (RNM940), Departamento de Ingeniería Química, Instituto Químico para la Energía y el Medioambiente (IQUEMA), Facultad de Ciencias, Campus de Excelencia Internacional en Agroalimentación (CeiA3), Universidad de Córdoba, 14014, Córdoba, Spain
[c] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005, Porto, Portugal

ARTICLE INFO

Keywords:
Functional ingredient
Phenolic compounds
Colonic fermentation
Human microbiota

ABSTRACT

Olive leaf represents a promising source of novel ingredients with potential health benefits, being rich in dietary fiber and phenolic compounds. This study aimed to evaluate the prebiotic effects of olive leaf bioactive compounds through *in vitro* human colonic fermentation. The phenolic compounds identified prior to fermentation included hydroxytyrosol, vanillin, oleuropein, and tyrosol. Olive ground leaf significantly promoted the fermentation of colonic microbiota with respect to positive control (FOS) by enhancing the growth of probiotic strains, such as *Bifidobacterium* spp. or *Clostridium leptum*, and producing short-chain fatty acids, such as acetate, butyrate and propionate, related to health benefits. Furthermore, olive leaf showed a lower Firmicutes:Bacteroidetes ratio compared to FOS, resulting in a beneficial effect on gut microbiota composition. These findings support the potential of olive leaf as a functional ingredient with prebiotic properties, offering promising applications in the development of novel foods or ingredients aimed at improving human health.

1. Introduction

In recent decades, there has been a significant increase in interest in more sustainable and environmentally friendly food production systems. This shift has driven the transition from a linear to a circular production model, with an emphasis on efficient use of resources and minimisation of waste generation (Khatami et al., 2024). Despite these advances, agriculture and the agri-food industry continue to produce large quantities of non-edible by-products, generating millions of tonnes of waste each year. This biomass represents not only a significant economic burden on industry, but also a major source of environmental pollution (Wang & Qi, 2024).

In the context of the Spanish agri-food sector, olive oil production plays a particularly important role. Andalusia, the world's leading olive oil producing and exporting region, generates revenues of up to 3500 million € per year (Junta de Andalucia, 2023). However, this activity is associated with the generation of around 5 million tonnes of by-products each year (Consejería de de Agricultura, n.d.). Among them, olive leaves can be highlighted due to their promising applications in the food, pharmaceutical and nutraceutical industries (J. Madureira et al., 2022).

Olive leaves have been used for their health benefits since ancient times and have remarkable antioxidant, antimicrobial and prebiotic properties (Borges et al., 2020; Giacometti et al., 2018; Žugčić et al., 2019). As a rich source of bioactive compounds, including insoluble dietary fibres (such as cellulose and lignin), soluble dietary fibres (such as hemicellulose) and phenolic compounds, the latter have been associated with a wide range of beneficial health effects, such as antihypertensive, hypocholesterolemic, hypoglycaemic, cardioprotective and anti-inflammatory activities (Demirer & Samur, 2024).

In this context and given the increasing demand for more sustainable food products and the greater awareness of the relationship between diet and health, there is a growing interest in natural and functional foods.

This article is part of a special issue entitled: Food bioactives & gut health published in Food Bioscience.

* Corresponding author.
** Corresponding author.
E-mail addresses: v02sagum@uco.es (S.-G. Mónica), bt2cajie@uco.es (C. Elena).

https://doi.org/10.1016/j.fbio.2025.106659
Received 9 January 2025; Received in revised form 16 April 2025; Accepted 21 April 2025
Available online 24 April 2025

Functional foods are defined as foods that, in addition to providing basic nutritional value, confer additional health benefits, either by improving specific physiological functions or by contributing to disease prevention (Alongi & Anese, 2021). Although the composition and bioactive potential of olive leaf extracts have been extensively studied, limited attention has been focused on the potential application of ground olive leaves as a functional ingredient in foods. In this sense, the valorisation of ground olive leaves, rich in polyphenols and fibre, becomes essential to promote a green solution for the olive industry, since the development of new functional ingredients from these residues is a key challenge for modern society (Ribeiro et al., 2021).

Recent studies suggest that bioactive compounds and nutrients derived from agriculture and agri-food industry by-products influence the gut microbiota. Currently, a prebiotic is defined as a substrate that the gut microorganisms are able to selectively utilise, conferring a health benefit (Moon & Kim, 2024; Thorman et al., 2024). In this regard, dietary fibre and phenolic compounds confer health benefits as they are able to exert a prebiotic effect by positively modulating the beneficial microbial composition (Núñez-Gómez et al., 2024). During the passage through the GIT, these non-digestible polysaccharides and polyphenols may remain unchanged or not be absorbed in the small intestine, reaching the colon where they can be fermented and biotransformed by the gut microorganisms. This prebiotic activity is related to the positive modulation of the beneficial bacteria population, the production of short-chain fatty acids (SCFA) such as acetate, butyrate and propionate, and the ability of the gut microbiota to biotransform high molecular-weight polyphenols into their more biologically active metabolites (Ribeiro et al., 2021; Santana Andrade et al., 2022). Up to now, studies on olive leaf have considered isolated oligosaccharide fractions or polyphenol extracts to assess their prebiotic potential (Pérez-Burillo et al., 2020; Rocchetti et al., 2022a, 2022b; Ruiz et al., 2017), while the activity of olive ground leaf has not been assessed so far. In relation to the evaluation of a potential prebiotic effect on the gut microbiota modulation and SCFA production, *in vivo* experiments would the more accurate tests; however, they entail high costs and ethical restrictions, making *in vitro* fermentation systems the option of choice (Sáyago-Ayerdi et al., 2019).

In view of the above, the aim of this research was to evaluate the prebiotic *in vitro* potential of olive ground leaf compounds by *in vitro* colon fermentation.

2. Materials and methods

2.1. Chemicals and reagents

Calcium chloride dihydrate ($CaCl_2 \bullet 2H_2O$), magnesium chloride hexahydrate ($MgCl_2 \bullet 6H_2O$), potassium dihydrogen phosphate (KH_2PO_4), and sulphuric acid (H_2SO_4) were acquired from Sigma-Aldrich (Sintra, Portugal), whereas formic acid and methanol were supplied by Fischer Scientific (Oeiras, Portugal). FOS (Orafti®P95) – frutooligosaccharides (Fructan-type non-available carbohydrate) standard was obtained from Beneo (Oreye, Belgium).

The standards used for the detection and identification of individual phenolic compounds (gallic acid, caffeic acid, 4-hydroxybenzoic acid, protocatechuic acid and vanillic acid) were purchased from Sigma-Aldrich (Sintra, Portugal), while hydroxytyrosol, vanillin, oleuropein, luteolin-7-O-glycoside, and tyrosol from Extrasynthese (Lyon, France). A calibration curve for each compound (0.005–0.25 mg/mL) was used to quantify the content of phenols.

The standards employed for the detection of individual organic acids (lactic, acetic, succinic, butyric and propionic acid) were obtained from Sigma-Aldrich (Sintra, Portugal). For each organic acid, a calibration curve was prepared with a range of 0.05–2 g/L.

2.2. Olive leaf material

Olive leaves of the "Hojiblanca" variety were collected from an olive grove located in the province of Córdoba, (Spain, Latitude 37.9321, Longitude −4.8002), shortly after the olive harvesting season concluded, in mid-March. The prunings were promptly transported to the laboratory, where the leaves were separated from the branches, manually washed, and air-dried in the absence of light for 5 ± 2 days, until the leaf moisture was below 8 %. Once dried, the leaves were ground in a Retsch SM2000 automatic grinder (Restsh GmbH, Haan, Germany) and sieved with an ASTM No. 10 to obtain particles smaller than 2 mm in diameter. The processed ground olive leaves (OL), with a moisture content of 5.17 ± 0.02 %, were stored at room temperature in a dry, dark environment until further use.

2.3. In vitro colon fermentation assay

In vitro simulated gastrointestinal digestion of OL was followed by the method previously described by Sánchez-Gutiérrez et al. (2022). In brief, 2 g of OL were dissolved in 20 mL of ultrapure water and subjected to sequential digestion with specific enzymes for each phase: α-amylase for the oral phase, pepsin for the gastric phase, and pancreatin with bile salts for the intestinal phase. The pH was adjusted at each stage, and physiological conditions of temperature (37 °C) and agitation were replicated. Finally, dialysis with a 3 kDa membrane was performed to simulate intestinal absorption, distinguishing between the absorbed fraction and the non-absorbed fraction (available for the colon). The experiments were conducted in three biological replicates.

After digestion simulation, the fraction available for the colon was submitted to *in vitro* colon fermentation to evaluate the prebiotic activity of OL. Fresh faeces samples were collected from each donor into sterile plastic vases and kept under anaerobic conditions until further use (maximum of 2 h after collection). The samples were obtained from five healthy human donors and were collected anonymously by non-invasive procedures. All volunteers gave informed consent for the use of their samples in this research. They also declared not to have any metabolic or gastrointestinal disorders, not to have consumed any probiotic or prebiotic supplements, as well as any form of antibiotics during the 3 months prior to the assay (Gullon et al., 2014). The use of faecal material from healthy volunteers did not require human ethics review under applicable Regulations. All experiments were performed in compliance with relevant law and institutional guidelines.

The basal medium was prepared as described by Madureira et al. (2016). It contained 5.0 g/L trypticase soya broth (TSB) without dextrose (BBL, Lockeysville, USA), 5.0 g/L bactopeptone (Amersham, Buckinghamshire, UK), 0.5 g/L cysteine-HCl (Merck, Germany), 1.0 % (v/v) of salt solution A (100.0 g/L NH_4Cl, 10.0 g/L $MgCl_2 \cdot 6H_2O$, 10.0 g/L $CaCl_2 \cdot 2H_2O$), a trace mineral solution, 0.2 % (v/v) of salt solution B (200.0 g/L $K_2HPO_4 \cdot 3H_2O$) and 0.2 % (v/v) of 0.5 g/L resazurin solution, prepared in distilled water and adjusted at pH 6.8. The basal medium was dispensed into airtight glass anaerobic bottles, sealed with aluminum caps and sterilized in autoclave. Stock solutions of Yeast Nitrogen Base (YNB) were sterilized with 0.2 μm syringe filters (Chromafils, Macherey-Nagel, Düren, Germany) and inserted into the bottles. OL was incorporated to serum bottles at a final concentration of 2 % (w/v) and inoculated with faecal slurry (2 % v/v) at 37 °C for 48 h without shaking. Also, a positive control was prepared by supplementing the human faeces of the five donors with fructooligosaccharides (FOS) at a final concentration of 2 % w/v. Samples were taken at 0, 12, 24 and 48 h of fermentation. All experiments were carried out inside an anaerobic cabinet with 5 % of H_2, 10 % of CO_2 and 85 % of N_2.

2.4. Gut microbiota evaluation

2.4.1. DNA extraction

Genomic DNA was extracted and purified from faecal samples using

NZY Tissue gDNA Isolation Kit (Nzytech, Lisbon, Portugal) with some modifications (A. R. Madureira et al., 2016). In brief, samples were centrifuged at 11 000 *g* during 10 min. Faecal pellets (around 170–200 mg) were taken from control and test samples at times 0, 12, 24 and 48h of fermentation, homogenized in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) and centrifuged at 4000 *g* for 15 min. After discarding the supernatant, the pellet was resuspended in 350 μL of buffer NT1, incubated at 95 °C for 10 min and centrifuged at 11 000 *g* for 1 min. Next, 25 μL of proteinase K was added to 200 μL of supernatant for incubation at 70 °C for 10 min. The following steps were carried out according to the manufacturer's instructions. The DNA purity and quantification were determined with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.4.2. Real-time PCR for microbial analysis in faeces

Real-time PCR was carried out as previously described by; Madureira et al. (2016), employing a LightCycler FastStart DNA Master SYBR Green kit and a Light Cycler instrument (Roche Applied Science, Indianapolis, ID, USA) in sealed 96-well microplates. PCR mixtures with a total volume of 10 μL contained 5 μL of 2 × Faststart SYBR Green (Roche Diagnostics Ltd), 0.2 μL of each primer (final concentration of 0.2 μM), 3.6 μL of water and 1 μL of DNA (equilibrated to 20 mg). Primer sequences (Sigut microbiota-Aldrich, St. Louis, MO, USA) were used to target the 16S rRNA gene of the bacteria. To verify the specificity of the amplicon, a melting curve analysis was performed via monitoring SYBR Green fluorescence in the temperature ramp from 60 to 97 °C. Data were processed and analysed using the LightCycler software (Roche Applied Science). Standard curves were constructed using serial tenfold dilutions of bacterial genomic DNA, according to the following webpage http://cels.uri.edu/gsc/cbdna.html. Bacterial genomic DNA used as a standard was obtained from DSMZ (Braunschweig, Germany). Genome size and the copy number of the 16S rRNA gene for each bacterial strain used as a standard was obtained from NCBI Genome database (http://www.ncbi.nlm.nih.gov). Data are presented as the mean values of duplicate PCR analyses. The so-called F:B ratio was obtained by dividing the number of copies of *Firmicutes* divisions by the number of copies of *Bacteroidetes* divisions. In addition, the relative differences to negative control percentage (only faeces fermentation) were calculated using Equation (3). Positive % values mean an increase in the number of copies relative to the control sample at a certain time.

$$\text{Relative difference to control } (\%) = \frac{SMC - CMC}{CMC} * 100 \quad (3)$$

where SMC is the mean copy numbers of the sample at a certain time (12, 24 and 48 h) and CMC is the mean copy numbers of the control sample at the same time as SMC.

2.5. Identification and quantification of phenolics by HPLC

Polyphenolic profile of OL were determined by High Performance Liquid Chromatography, using a Diode-Array Detector (HPLC-DAD), according to the method described by Campos, Ribeiro, et al. (2020) with some modifications. Samples were injected into Waters Series e2695 Separation Module System (Mildford MA, USA) interfaced with HPLC-DAD. Separation was performed in a reverse-phase column (COSMOSIL 5C1 8-AR-II Packed Column – 4.6 mm I.D. × 250 mm, Dartford, UK), using two mobile phases composed by mobile phase A – water:methanol:formic acid (92.5:5:2.5, %v/v/v) – and B – methanol: water:formic acid (92.5:5:2.5, %v/v/v) with the following gradient and conditions: injection volume of 50 μL of sample; continuous flow of 0.5 mL/min; gradient elution starting at 100 % mobile phase A for 50 min, then gradient reset at 45 % A and 55 % B between 50 and 55 min; return to 100 % mobile phase A, remaining at this percentage for 4 min (until 59 min). Data acquisition and analysis were carried out using Empower 3 software. Detection was carried out at wavelengths ranging from 200 to 600 nm to investigate different compounds like catechins or procyanidins (280 nm), phenolic acids (320 nm) and flavanols (330 nm), which were identified and quantified through a calibration curve with pure standards in terms of retention times, UV absorption spectra and peak areas at maximum absorption wavelength. All determinations were made in triplicate. Results were expressed as mg of phenolic compounds per 100 g DM.

2.6. Identification and quantification of short chain fatty acids by HPLC

Chromatographic separation was performed with a Beckman Coulter HPLC equipment coupled to IR (K-2301) and UV detector (K-2501) (Knauer, Berlin, Germany). Samples collected during the fermentative process at 0, 12, 24 and 48 h, were filtered (0.45 μm cellulose acetate membrane) and then, aliquots of 20 μL were analysed using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) operated at 40 °C with 5 mM H_2SO_4 as mobile phase at constant flow of 0.6 mL/min during 30 min. Data acquisition and analysis were carried out using Clarity software. Detection of organic acids was performed with an UV detector, and were quantified through calibration curves built for standards. All determinations were made in triplicate and results were expressed as mg of short chain fatty acid per mL.

2.7. Statistical analysis

Statistical analysis was carried out through IBM® SPSS® Statistics software Version 25 (IBM Corporation, New York, NY, USA). Data were reported as mean ± standard deviation of the five donors. Differences between the different times of faecal fermentation were analysed by one-way analysis of variance (ANOVA), with the application of the Tukey's post-hoc test for pairwise multiple comparison. Significant differences were considered at a level of $p < 0.05$.

3. Results and discussion

3.1. Bioactive compounds during in vitro colon fermentation

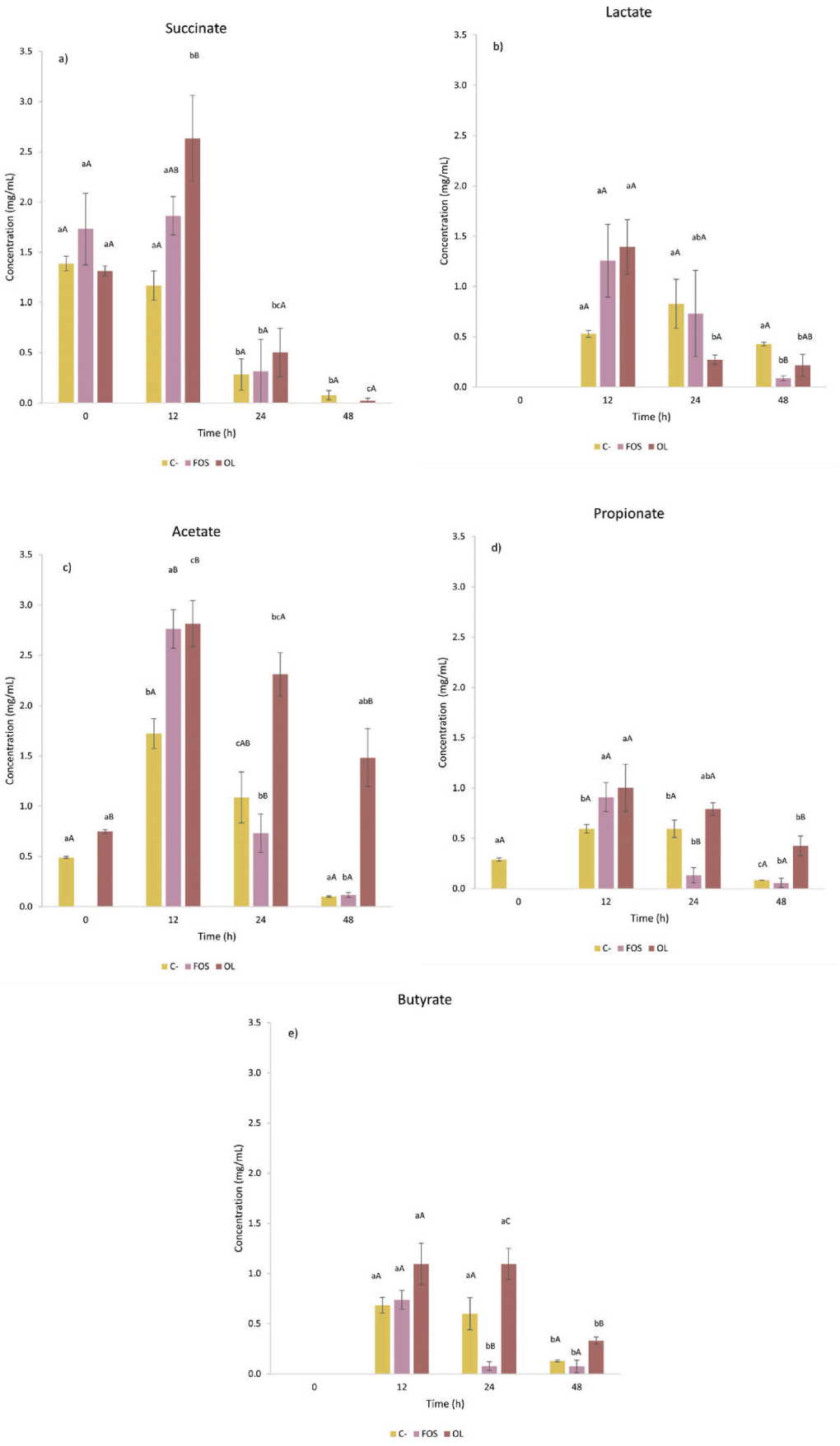
3.1.1. Short-chain fatty acids (SCFAs) production

SCFAs production during *in vitro* fermentation of colonic microbiota was measured at 0, 12, 24 and 48 h in the presence of OL (2 %), negative control (C-) and FOS (2 %), whose results are presented in Fig. 1. Five organic acids were identified during fermentation, with the highest concentration for acetate and succinate acids in OL and FOS, followed by lactate, butyrate and propionate acids.

SCFAs are metabolites of the fermentation of food components by the gut microbiota in the proximal colon. It is widely accepted that the presence and increase of SCFAs are indicators of a healthy gut microbiome (Baenas et al., 2020). Acetate, propionate and butyrate acid are the most common SCFAs. Acetate could be produced by metabolic cross-feeding via lactate consumption or have an endogenous origin, while butyrate and propionate are exclusively derived from bacterial metabolism (Ribeiro et al., 2021). Furthermore, succinate and lactate are formed as intermediate metabolites (Sáyago-Ayerdi et al., 2019).

Statistical analysis of SCFAs content showed that acetate, propionate and butyric acid concentrations in OL were significantly higher than in the positive control (FOS) at 24 and 48 h of fermentation ($p < 0.05$). However, they were similar at 12 h. Also, similar contents were found for lactate and succinate throughout the fermentation ($p > 0.05$). The maximum content of total SCFAs was obtained for OL after 12 h of fermentation, reaching around 9 mg/mL.

Succinate production is related to the same microorganisms that produce acetate and propionate (Campos, Coscueta, et al., 2020). In fact, succinate is a metabolite of bacterial polysaccharide fermentation, an intermediate in the microbial production of propionate, which has been shown to be effective against obesity-associated metabolic disorders (Gómez-García et al., 2022). Succinate was detected in OL before



(caption on next page)

Fig. 1. Organic acids concentration through the *in vitro* colon fermentation a) succinate; b) lactate; c) acetate; d) propionate; e) butyrate; C-: negative control; FOS: positive control (2 % w/v); OL: olive ground leaf (2 % w/v). Results are the means of five determinations ± standard deviation. Different letters indicate significant differences ($p < 0.05$). Small letters indicate differences between sampling times (0, 12, 24 and 48 h) within type of sample, and capital letters indicate differences between types of samples (negative control, FOS, and OL) at the same sampling time.

fermentation began, and showed a drastic reduction at 48 h with a concentration of 0.02 mg/mL ($p < 0.05$). While succinate was reduced to 0.50 mg/mL at 24 h, propionate increased to 0.79 mg/mL, suggesting, on one hand, that succinate may have been utilized by microorganisms in favour of propionate production, and on the other hand, it is possible that the heterogeneous gut microbiota shall use different propionate and energy production pathways.

According to literature, the main organic acid produced during carbohydrate metabolism by *Lactobacillus* spp. is lactic acid (Ribeiro et al., 2021). Lactate in OL was produced after 12 h of fermentation through carbohydrate metabolism and was significantly reduced after 24 and 48 h ($p < 0.05$). Elevated concentrations of this organic acid in faeces could be associated with individuals who have bowel syndrome or suffer from ulcerative colitis. Nevertheless, as an intermediate molecule for the production of butyrate, acetate and propionate, the presence of high levels of lactic acid is positive (Ruiz et al., 2017).

Acetate, the most remarkable SCFA in this work, has been reported to stimulate cholesterol synthesis, and is able to enter the systemic circulation, reduce appetite and inhibit enteropathogenic bacteria (Andrade et al., 2020; Gómez-García et al., 2022; Sáyago-Ayerdi et al., 2019). At 12 h of fermentation, it showed a significant increase with respect to the initial values ($p < 0.05$), decreasing later at 24 and 48 h. Despite this reduction, the concentration in OL samples was over three times higher than in FOS samples at 24 h and twelve times higher at 48 h ($p < 0.05$). Acetate is primarily produced through the fermentation of indigestible fibres such as oligosaccharides and phenolic compounds like hydroxytyrosol. It is synthesized via two key metabolic pathways: acetogenesis, where homoacetogenic bacteria produce acetate from H_2 and CO_2, and carbon fixation, which utilizes CO_2 as a precursor in the Wood-Ljungdahl pathway. These pathways not only promote acetate production but also contribute to an increase in the *Firmicutes/Bacteroidetes* ratio, enhancing cross-feeding mechanisms. For example, the activation of pyruvate fermentation to acetate and lactate by *Lactobacillus reuteri* supports the metabolic activity of beneficial gut microbiota (Facchin et al., 2024; Miao, 2022).

Propionic acid is generally produced by the Bacteroides genus (Ribeiro et al., 2021). Propionate plays an important role in hepatic gluconeogenesis, contributes to the reduction of cholesterol synthesis and lipogenesis, and is involved in the release of satiety hormones (Andrade et al., 2020; Sáyago-Ayerdi et al., 2019). Propionate values in OL showed a similar behaviour to succinate throughout fermentation, with a significant increase at 12 h ($p < 0.05$) followed by a decrease at 24 and 48 h. The concentration in OL samples relative to FOS was significantly higher at 24 h (0.79 vs. 0.13 mg/mL, respectively) and at 48 h (0.42 vs. 0.06 mg/mL, respectively). Propionate is mainly produced through two key pathways in the gut. The succinate pathway ferments hexose and pentose sugars to produce propionate, while the propanediol pathway generates propionate from the fermentation of fructose and rhamnose. The succinate pathway is primarily associated with *Bacteroidetes* and the *Negativicutes* (Firmicutes), which play a significant role in propionate production during the fermentation of dietary carbohydrates (Facchin et al., 2024).

The main source of carbon for colonocytes is butyrate, a SCFA produced in the human gut by the *Firmicutes* phylum (Ribeiro et al., 2021). Butyrate is known to have an anticancer effect by promoting cancer cell apoptosis; it also reduces inflammation and plays a key role in the maintenance of the mucosal barrier, contributing to the preservation of the integrity of the intestinal epithelium (Bussolo de Souza et al., 2019; Campos, Coscueta, et al., 2020; Ruiz et al., 2017). The concentration of butyrate in OL increased, reaching similar values of around 1.10 mg/mL at 12 and 24 h; subsequently, it fell to one third after 48 h. As evidenced for succinate and propionate, butyrate concentration in OL was significantly greater than in FOS at 24 and 48 h (between 14 and 4.5 times higher, respectively). The metabolic pathways involved in butyrate production are primarily driven by butyrate-producing bacteria, whose growth and metabolic activity are enhanced by phenolic compounds like hydroxytyrosol found in olive leaves. These compounds promote the activity of these bacteria, which in turn supports the production of butyrate (Miao, 2022). Butyrate is synthesized from butyryl-CoA through two distinct enzymatic pathways: one catalyzed by butyrate kinase and the other by butyryl-CoA:acetate CoA transferase (BCoAT). While both enzymes mediate the conversion of butyryl-CoA into butyrate, they operate via different biochemical mechanisms. However, within the human colonic environment, BCoAT is recognized as the predominant enzyme driving this transformation (Facchin et al., 2024).

As mentioned above, SCFAs appear to be involved in cholesterol metabolism. While acetate stimulates cholesterol synthesis, propionate inhibits it, demonstrating the importance of the acetate/propionate ratio in maintaining metabolic balance in both the liver and the gut (Sáyago-Ayerdi et al., 2019). This ratio for OL was 2.81 versus 3.04 for FOS at 12h. This low acetate/propionate ratio has been associated with a decrease in blood lipids and thus, it is considered a positive indicator. Similar acetate/propionate ratio was previously reported during *in vitro* fermentations of human faeces using pineapple by-products flours (Campos, Coscueta, et al., 2020), melon peel flour (Gómez-García et al., 2022) and olive pomace (Ribeiro et al., 2021).

In addition, SCFAs are essential for maintaining a low pH in the colon, which helps prevent colonisation and infection by pathogenic bacteria, favouring the growth of beneficial bacteria (Bussolo de Souza et al., 2019). As can be seen in Fig. 2 the pH after *in vitro* fermentation with OL showed a slight decrease, while a marked fall was found for FOS ($p < 0.05$). Koecher et al. (2014) also found a rapid decrease of the pH in the case of FOS and inulin, rapidly fermented, short chain fructans, in comparison with other higher molecular weight molecules like gum acacia or pea fibre. However, the OL fermentation resulted in a profile of SCFAs with a higher concentration of acetate, propionate and butyrate throughout the fermentation compared to FOS. This suggests that OL provides a source of nutrients for the gut microbiota, leading to a higher production of SCFA compared to FOS and C-. Given the role of SCFA in gut health and systemic metabolism, OL could be considered a prebiotic

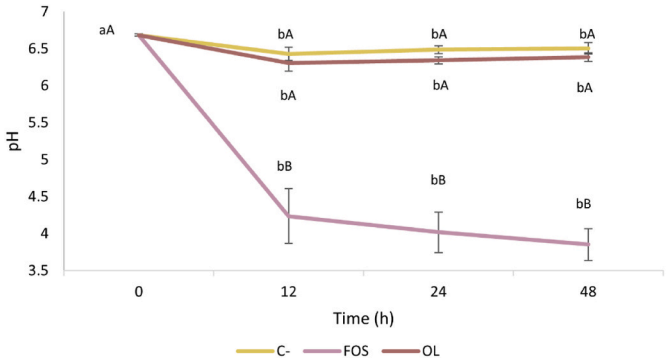


Fig. 2. Evolution of pH values throughout the *in vitro* colon fermentation. C-: negative control; FOS: positive control (2 % w/v); OL: olive ground leaf (2 % w/v). Results are the means of five determinations ± standard deviation. Different small letters within the type of sample indicate differences between sampling times (0, 12, 24 and 48 h) and different capital letters within the sampling time indicate differences between samples (C-, FOS and OL).

agent with potential benefits not only for the gut, but also for other organs, such as the liver and muscles This higher SCFA production can be attributed to the unique composition of OL, which is rich in both dietary fibre and phenolic compounds. Unlike FOS, OL fermentation promotes a more sustained release of SCFA, especially in later stages. In addition, OL polyphenols can facilitate microbial interactions and stimulate metabolic pathways involved in SCFA synthesis (Simonelli et al., 2024). In this context, polyphenols play a crucial role in modulating the gut microbiota by selectively enhancing the growth and metabolic activity of bacterial populations responsible for SCFA production (Meiners et al., 2025). Our results reinforce this idea, showing that OL fermentation significantly increases SCFA production, further highlighting the influence of polyphenols on gut flora metabolism.

3.1.2. Phenolic compounds and associated metabolites

The individual phenolic compounds were evaluated by HPLC during OL fermentation at 0, 12, 24 and 48 h (Table 1), identifying 10 phenolic compounds, ranked from the highest to the lowest concentration, were: hydroxytyrosol, vanillin, oleuropein, tyrosol, protocatechuic acid, lueolin-7-O-glycoside and caffeic acid. Gallic, 4-hydroxybenzoic and vanillic acids concentrations were below the detection limit.

After 12 h of fermentation, no significant differences were observed in the content of phenolic compounds compared to the start of fermentation, except for oleuropein, whose concentration was significantly reduced ($p < 0.05$). However, at 24 h, a general decrease was observed (significant only for protocatechuic acid), being still more remarkable at 48 h of fermentation, with only hydroxytyrosol and tyrosol detected with a non-significant decrease from 24 h ($p > 0.05$).

During OL fermentation, protocatechuic acid levels increase within the first 12 h, likely due to the microbial hydrolysis of complex polyphenols. The gut microbiota, with its specialized enzymatic capacity, facilitates this process by breaking down flavonoids and hydroxycinnamic acids, releasing protocatechuic acid as a degradation product (Xie et al., 2023). Notably, the microbial conversion of vanillic acid into protocatechuic acid has been reported, which may contribute to this early increase (Yang et al., 2024).

The subsequent abrupt decline in protocatechuic acid levels after 24 h can be attributed to microbial catabolism, where gut bacteria further transform it through decarboxylation, dehydroxylation, or conversion into short-chain fatty acids (SCFAs), which are then utilized for energy production or other metabolic functions (Yuan et al., 2025; Zhang et al., 2024). This process reflects the broader role of the gut microbiota in polyphenol metabolism, as several studies have shown that polyphenols reaching the colon undergo microbial transformation, becoming more bioaccessible in the distal part of the gastrointestinal tract.

In this context, dietary fibre plays a relevant role in this process by increasing the amount of polyphenols reaching the colon, acting as a carrier that enhances their bioaccessibility during fermentation (Jakobek & Matić, 2019). As an example, it was observed that caffeic acid increased in the intestinal step, as observed also by different authors, who stated that caffeic acid bound to insoluble fibre is released during *in vitro* fermentation by the action of the intestinal microbiota (Gómez-García et al., 2022).

Furthermore, some studies suggest that OL polyphenols may help prevent colon cancer, stimulate the growth and proliferation of beneficial bacteria by acting as a substrate for microorganisms such as *Bacteroides*, *Clostridium* and *Eubacterium* and inhibit intestinal pathogenic bacteria such as *E. coli*. *S.* Typhimurium, *L. monocytogenes*, *Y. enterocolitica* and *S. aureus* (Jakobek & Matić, 2019; Sánchez-Gutiérrez et al., 2021; Žugčić et al., 2019). In the case of oleuropein, the fraction available in the colon can be fermented by various bacterial strains such as *Lactobacillus*, *Bifidobacteria* and *Enterococcus*, yielding hydroxytyrosol as the final fermentation product (Rocchetti et al., 2022a, 2022b; Žugčić et al., 2019). In fact, and consistent with our results, several authors have found that, from the main phenolic compounds in olive leaf (oleuropein, hydroxytyrosol and tyrosol), hydroxytyrosol and tyrosol remain stable throughout *in vitro* fermentation using human faecal microbiota, while oleuropein undergoes a high degradation (Mosele et al., 2014; Rocchetti et al., 2022a, 2022b). It has been reported that a moderate intake of olive oil increases the amount of free hydroxytyrosol in human faeces, producing a reduction of adipocyte size, plasma glucose and insulin concentration, and levels of certain inflammatory markers in plasma (Rocchetti et al., 2022a, 2022b).

Overall, these results point out that OL provides phenolic compounds in the large intestine, whose analysis indicates that the activity of human gut microbiota can be modulated, thus supporting the implementation of OL as functional ingredient.

Table 1
Bioactive compounds (mg/100 g DM) identified and quantified by HPLC throughout *in vitro* colon fermentation.

Phenolic compounds	Time (h)			
	0	12	24	48
Gallic acid	UD	ND	ND	ND
Protocatechuic acid	4.59 ± 1.22^{a}	6.43 ± 1.09^{a}	0.68 ± 0.21^{b}	ND
Hydroxytyrosol	7.22 ± 0.14^{a}	5.69 ± 0.37^{a,b}	4.24 ± 1.08^{b,c}	1.92 ± 0.55^{c}
4-hydroxybenzoic	UD	UD	UD	ND
Tyrosol	4.70 ± 0.93^{a}	4.43 ± 0.91^{a,b}	2.58 ± 0.14^{a,b}	1.86 ± 0.19^{b}
Vanillic acid	UD	UD	UD	ND
Caffeic acid	3.07 ± 0.05^{a}	3.75 ± 0.67^{a}	4.55 ± 0.80^{a}	UD
Vanillin	5.83 ± 1.01^{a}	6.86 ± 1.53^{a}	UD	ND
Luteolin-7-O-Glycoside	3.88 ± 0.42^{a}	3.69 ± 0.63^{a}	3.18 ± 1.22^{a}	UD
Oleuropein	5.56 ± 0.72^{a}	3.08 ± 0.08^{b}	1.86 ± 0.09^{b}	UD

Values are expressed as mean of five determinations ± standard deviation. Values in the same row with different superscript letters indicate significant differences ($p < 0.05$) between fermentation times. ND: not detected. UD: under the limit of detection.

3.2. Changes in faecal microbiota during in vitro fermentation

After GIT simulation, the OL samples were freeze-dried, and the solid particles were subjected to a simulated fermentation in the colon with human faeces for 48 h. In order to evaluate the effect on human gut microbiota and to understand the microorganism's growth behaviour during the fermentative process, aliquots were taken at 0, 12, 24 and 48 h for further analysis. Fructooligosaccharides (FOS) were used as a positive control during intestinal fermentation, as it is a well-documented and accepted as a prebiotic agent, whereas a sample with no added carbon source was used as a negative control (Mao et al., 2019). The intestinal microbiota includes a variety of bacteria, of which about 85–90 % of the species belong to the phyla *Firmicutes, Bacteroidetes and Actinobacteria* (Nehmi-Filho et al., 2023). These dominant phyla in the human gut were evaluated matching the copy numbers with those found in the faeces of healthy human volunteers. Table 2 presents the average of copy number of the main groups of the human gut microbiota obtained by real-time PCR, as well as the F:B ratio. In a healthy adult, *Clostridium leptum* and *Lactobacillus* spp. (Gram + *Firmicutes*), and *Prevotella* spp. and *Bifidobacterium* spp. (Gram- *Bacteroridetes*) are the main phyla of the human gut microbiome, with *Firmicutes* and *Bacteroidetes* being present in greater numbers, whereas *Lactobacillus* spp. is found in fewer quantities in the normal intestinal microbiota, according to our results (Gómez-García et al., 2022; Guarner et al., 2003).

The relative differences (%) in the abundance of the microbiota groups evaluated at 0, 12, 24 and 48 h of fermentation are shown in Fig. 3. OL promoted the growth of *Prevotella* spp. (70 %) > *Bifidobacterium* spp. (61 %) > *Clostridium leptum* (33 %), while in the case of FOS,

Table 2
Fecal microbiota composition of volunteer donors.

Division (Genus)	Number of Copies (n = 5)
Universal	7.24 ± 0.9
Firmicutes	6.48 ± 1.3
Clostridium leptum	5.40 ± 0.4
Lactobacillus spp.	1.26 ± 0.9
Bacteroidetes	6.94 ± 0.9
Prevotella spp.	4.27 ± 1.6
Actinobacteria	
Bifidobacterium spp.	3.22 ± 1.0
F:B ratio	0.93 ± 1.5

Values are presented as mean of five determinations ± standard deviation and expressed as log10 16S rRNA copies per 20 ng of DNA.

the most abundant bacterial group was *Bifidobacterium* spp. (50 %), followed by *Lactobacillus* spp. (49 %), *Prevotella* spp. (48 %) and lastly *Clostridium leptum* (20 %). In overall terms, it can be observed that throughout the faecal fermentation assayed, OL showed a greater positive effect than FOS on all the microbial phyla studied, except for *Lactobacillus* spp. ($p > 0.05$). The lower growth of *Lactobacillus* spp. in OL sample compared to FOS suggests that while phenolic compounds in olive leaves may selectively promote the growth of beneficial bacteria (Žugčić et al., 2019), they may also exert antimicrobial effects that inhibit the growth of certain probiotic bacteria, such as *Lactobacillus* spp. In fact, OL polyphenols have been shown to specifically target *Lactobacillus* spp. with antimicrobial activity, highlighting the complex dual role of these compounds in both promoting and suppressing the growth of different bacterial strains within the microbiota (Chen et al., 2024; Sudjana et al., 2009).

Regarding the time of fermentation, OL was fermented similarly by *Prevotella* spp. and *Clostridium leptum* (12 h) and faster by *Bifidobacterium* spp. (24 vs. 48 h). *Bifidobacterium* spp. is one of the most abundant and critical bacteria species living in the gut, related to health-promoting functions such as immune system support, treatment of respiratory infections and decrease of risk of allergies, atopic diseases and different types of cancer (Li et al., 2023). These results indicate that OL would promote the growth of healthier microbiota in a high extent, and thus, it may be used as prebiotic agent, mainly due to the high content of dietary fibre on olive leaf and phenolic compounds (Borgonovi et al., 2022; Rocchetti et al., 2022a, 2022b). It has been reported that phenolic compounds such as oleuropein, hydroxytyrosol, tyrosol, and hydroxytyrosol acetate found on olive oil and also in OL (Mosele et al., 2014), are linked to prebiotic effects by promoting the growth of healthy gut microbiota, intestinal permeability, as well as inhibiting microorganisms (food pathogens) that cause intestinal diseases (Ribeiro et al., 2021; Sánchez-Gutiérrez et al., 2021; Silva et al., 2022).

In terms of DNA copy number (log_{10} 16SrRNA gene copies/ng of DNA), as expected, the negative control did not result in bacterial growth during the *in vitro* colonic fermentation process, observing a general decrease, mainly in the first hours, in all the microbial groups studied. FOS and OL showed rather similar bacterial response for all phyla, except for *Lactobacillus* spp. where copy number at 48 h in the FOS sample was nearly double that of the OL sample. In the case of *Bifidobacterium* spp. and *Lactobacillus* spp., OL and FOS control appeared to support their growth, being the highest values found for both microorganisms 4.17 for FOS at 48 h and 4.11 for OL at 24 h for the former, and 1.9 for FOS at 48 h and 1.13 for OL at 24 h for the latter (Fig. 4). In contrast, it was found that FOS had fewer promoting effects on the growth of *Clostridium leptum* or *Prevotella* spp., and OL did not show significant changes on the growth of *Prevotella* spp. These results are in agreement with previously reports on overlooked food by-products

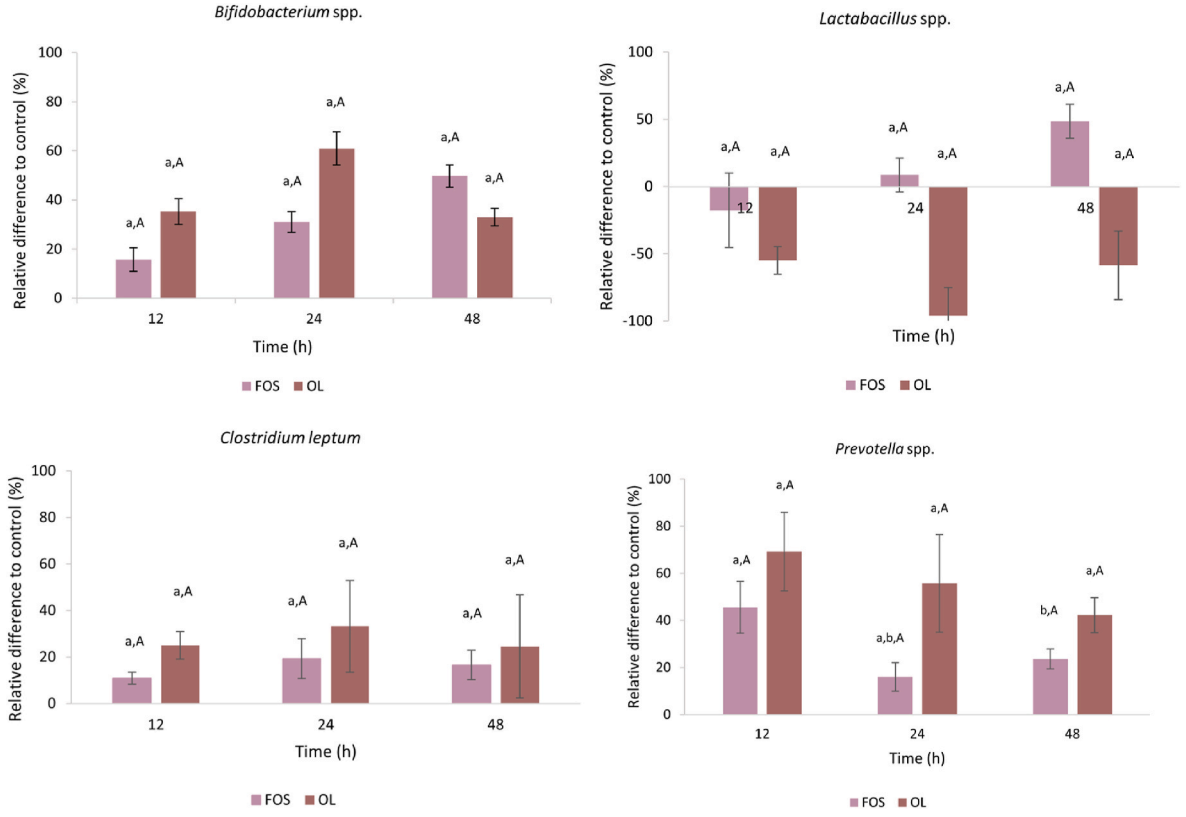


Fig. 3. Relative differences to negative control through *in vitro* colon fermentation. FOS: positive control (2 % w/v); OL: olive ground leaf (2 % w/v). Results are the means of five determinations ± standard deviation. Different letters indicate significant differences ($p < 0.05$). For each microorganism, small letters indicate differences between sampling times (12, 24 and 48 h) within type of sample, while capital letters indicate differences between types of samples (FOS and OL) at the same sampling time.

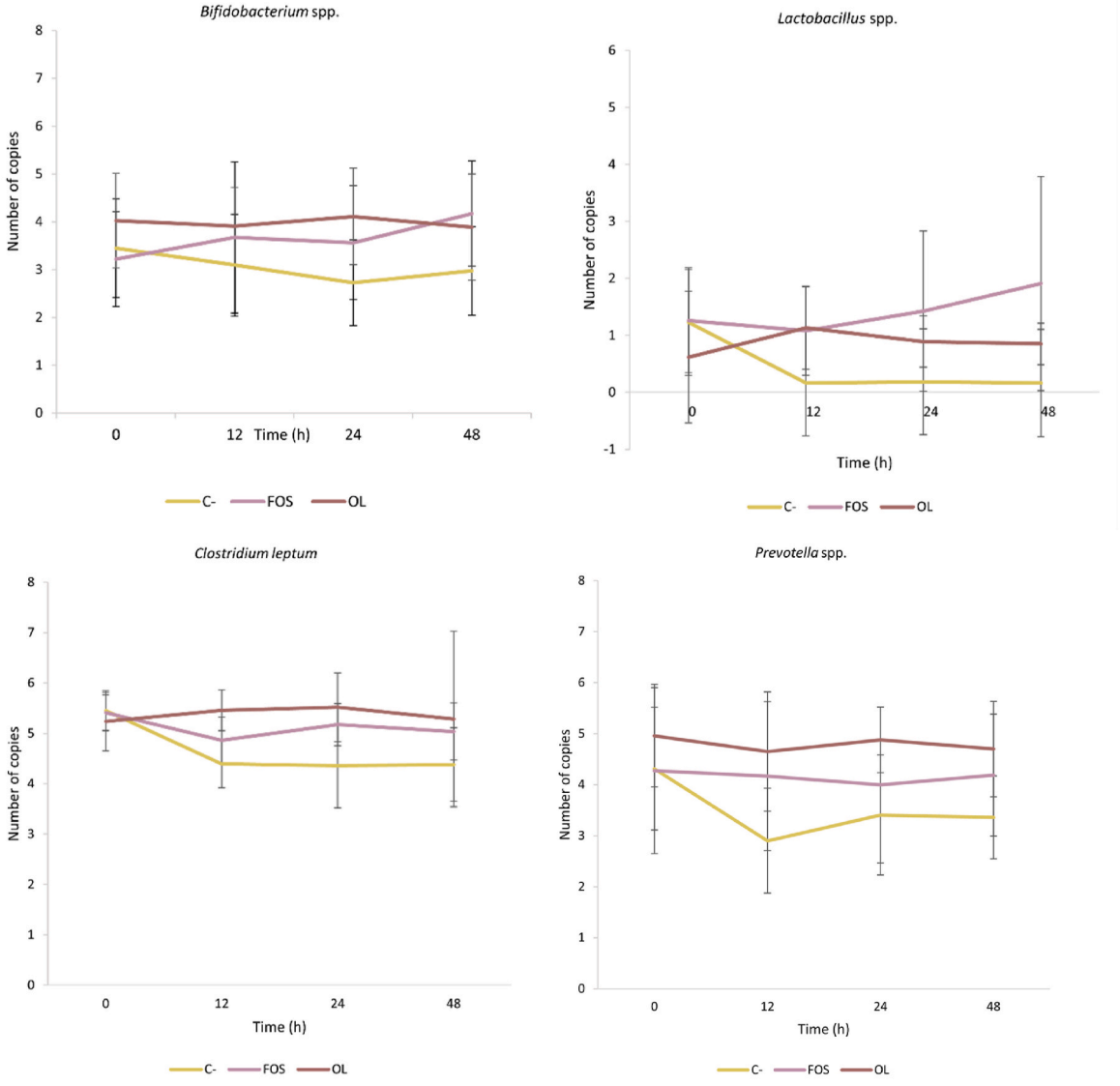


Fig. 4. Number of DNA copies measured by PCR through *in vitro* colon fermentation of olive ground leaf (OL: 2 % w/v), positive control (FOS: 2 % w/v) and negative control (C-). Results are the means of five determinations ± standard deviation.

(Bonifácio-Lopes et al. 2022; Campos, Coscueta, et al., 2020; Gómez-García et al. 2022; Ribeiro et al. 2021), suggesting that *Clostridum leptum* and *Prevotella* spp. do not have much affinity for FOS as an energy source, while beneficial bacteria are promoted due to the raw materials' composition rich in dietary fibre and simple sugars.

The relative abundance measure between *Firmicutes* and *Bacteroidetes* (F: B ratio) involves two of the most abundant phyla in the human gut microbiota and its changes can be related to microbiota dysbiosis. The F:B ratio is acknowledged as a possible biomarker for predicting the risk of developing metabolic disorders. For this reason, unhealthy people with obesity, type II diabetes or metabolic diseases tend to have a higher F:B ratio than healthy people, where it is normally reported around a ratio of 1:1 (Demirci et al., 2020; Salamat et al., 2024). *Prevotella* spp. is one of the main genera found belonging to the phylum *Bacteroidetes* associated with the improvement of glucose metabolism and obesity prevention (Christensen et al., 2019; Kovatcheva-Datchary et al., 2015), while *Clostridium* spp. is one of the important genera of the phylum *Firmicutes* with a protective effect against colorectal cancer (Elahi et al., 2023). As can be seen in Fig. 5, OL presented the lowest F:B ratios during the entire experiment, compared to FOS ($p > 0.05$), with an average ratio of 1.37 for OL throughout the experiment, and an average

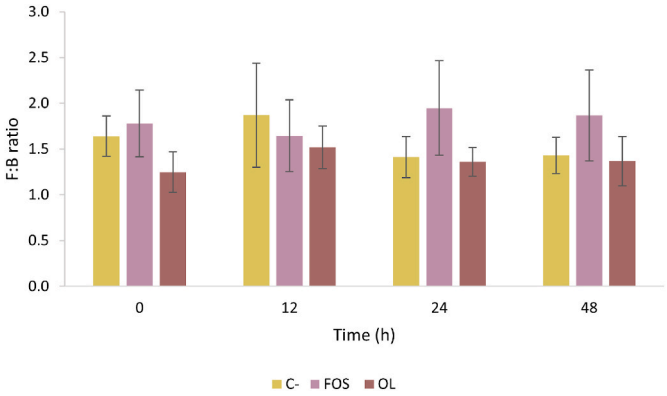


Fig. 5. *Firmicutes:Bacteroidetes* (F:B) ratio evaluation during *in vitro* colon fermentation. FOS: positive control (2 % w/v); OL: ground olive leaf (2 % w/v). Results are expressed as a ratio over fermentation time. Values represent the means of five determinations ± standard deviation. No significant differences were found between types of samples (FOS and OL) nor between sampling times (0, 12, 24 and 48 h) ($p > 0.05$).

of 1.81 for FOS. Through gut fermentation, OL showed similar F:B ratios at 24 and 48 h, reaching values close to one and with no statistical difference ($p < 0.05$) with respect to FOS (1.36 and 1.37 vs. 1.95 and 1.87, respective time). Similar F:B ratio values were previously reported by other authors using pineapple by-products flours (0.8–1.40), melon peel flour (1.10–1.80), olive pomace powder (1–1.60) and Brewer's spent grain (1.10–1.20) (Bonifácio-Lopes et al., 2022; Campos, Coscueta, et al., 2020; Gómez-García et al., 2022; Ribeiro et al., 2021). Overall, the digested OL allowed the enhancement of the phyla evaluated, highlighting its potential use as a functional ingredient with a health-promoting prebiotic effect. These findings reinforce the idea that the significant increase in beneficial bacteria is driven by the influence of polyphenols on gut microbiota metabolism. In contrast to FOS or C-, OL contains phenolic compounds that selectively stimulate the growth of beneficial bacteria while reducing the prevalence of harmful ones (Rodríguez-Daza et al., 2021). These polyphenols undergo extensive metabolism in the large intestine, facilitating interactions with gut microorganisms. This interaction works in both directions: polyphenols modulate the gut microbiota by promoting populations of beneficial bacteria and suppressing harmful ones, while microorganisms modulate the activity of the phenolic compounds. Through this dynamic process, microbial transformations regulate the metabolism and bioavailability of polyphenols, generating metabolites with diverse health effects (Alves-Santos et al., 2020; Moorthy et al., 2020).

4. Conclusions

In this study, the prebiotic effect of bioactive compounds from ground olive leaf (OL) during *in vitro* fermentation were studied. The results showed that *in vitro* colon fermentation of OL resulted in higher concentrations of acetate, propionate and butyrate acids than FOS, indicating that OL biocompounds can be utilized by the human gut microbiota to generate beneficial SCFA. Similarly, OL maintained and enhanced the growth of probiotic bacteria phyla and modulated their metabolism, with *Bifidobacterium* spp. and *Clostridium leptum* showing the highest levels. Also, hydroxytyrosol and tyrosol were the most relevant phenolic compounds identified after fermentation, whose presence may be related to growth promotion of the colon microbiota.

Although this study provides valuable information on the prebiotic effects of ground olive leaves, several limitations must be acknowledged. The lack of comparison between different extraction methods, combined with the possible influence of different modes of OL consumption (e.g. tea or extracts), limits our understanding of how alternative approaches could optimise the functional potential of OL bioactive compounds. Furthermore, although significant changes in specific bacterial populations and SCFA production were observed, further metagenomic analyses are needed to fully assess the global impact on gut microbiota diversity. Addressing these issues will be crucial to further our understanding of the prebiotic effects of OL and its potential as a functional food ingredient in future research.

Overall, our results suggest that ground olive leaf constitutes a good source of phenolic compounds and other sources of carbon (such as sugars or acids) with health-exerting prebiotic effects, that, together with fibre present in OL, could be applied for the development of functional foods or ingredients under the circular economy approach.

CRediT authorship contribution statement

Sánchez-Gutiérrez Mónica: Writing – original draft, Investigation, Formal analysis, Data curation. **Gómez-García Ricardo:** Writing – review & editing, Investigation, Data curation. **Pintado Manuela:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization. **Rodríguez Alejandro:** Writing – review & editing, Validation, Resources, Funding acquisition. **Carrasco Elena:** Writing – review & editing, Supervision, Resources, Funding acquisition, Formal analysis.

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.

Acknowledgments

This work was supported by Portuguese Funds from FCT - Fundação para a Ciência e a Tecnologia through project UID/Multi/50016/2019, by the project 202099901921213-R of the Consejería de Transformación Económica, Industria, Conocimiento y Universidades de la Junta de Andalucía and by the Andalusian knowledge transfer project AT17-5927 co-funded by the European Regional Development Fund instrument (ERDF Andalusian Operative Programme 2014–2020). Additionally, the authors wish to acknowledge the support of the Foundation for Science and Technology-FCT through the individual contract awarded to Ricardo Gomez-Garcia (reference 2023.08203.CEECIND).

Data availability

Data will be made available on request.

References

Alongi, M., & Anese, M. (2021). Re-thinking functional food development through a holistic approach. *Journal of Functional Foods, 81*(March), Article 104466. https://doi.org/10.1016/j.jff.2021.104466

Alves-Santos, A. M., Sugizaki, C. S. A., Lima, G. C., & Naves, M. M. V. (2020). Prebiotic effect of dietary polyphenols: A systematic review. *Journal of Functional Foods, 74* (June), Article 104169. https://doi.org/10.1016/j.jff.2020.104169

Andrade, A. C., Marinho, J. F. U., de Souza, A. C., de Sousa Tavares, T., Dias, D. R., Schwan, R. F., Nunes, C. A., & Bastos, S. C. (2020). Prebiotic potential of pulp and kernel cake from Jerivá (Syagrus romanzoffiana) and Macaúba palm fruits (Acrocomia aculeata). *Food Research International, 136*(March), Article 109595. https://doi.org/10.1016/j.foodres.2020.109595

Baenas, N., Nuñez-Gómez, V., Navarro-González, I., Sánchez-Martínez, L., García-Alonso, J., Periago, M. J., & González-Barrio, R. (2020). Raspberry dietary fibre: Chemical properties, functional evaluation and prebiotic in vitro effect. *Lwt, 134* (September). https://doi.org/10.1016/j.lwt.2020.110140

Bonifácio-Lopes, T., Catarino, M. D., Vilas-Boas, A. A., Ribeiro, T. B., Campos, D. A., Teixeira, J. A., & Pintado, M. (2022). Impact of circular Brewer's spent grain flour after in vitro gastrointestinal digestion on human gut microbiota. *Foods, 11*(15). https://doi.org/10.3390/foods11152279

Borges, A., José, H., Homem, V., & Simões, M. (2020). Comparison of techniques and solvents on the antimicrobial and antioxidant potential of extracts from acacia dealbata and olea europaea. *Antibiotics, 9*(2). https://doi.org/10.3390/antibiotics9020048

Borgonovi, T. F., Virgolin, L. B., Janzantti, N. S., Casarotti, S. N., & Penna, A. L. B. (2022). Fruit bioactive compounds: Effect on lactic acid bacteria and on intestinal microbiota. *Food Research International, 161*, Article 111809. https://doi.org/10.1016/J.FOODRES.2022.111809

Bussolo de Souza, C., Jonathan, M., Isay Saad, S. M., Schols, H. A., & Venema, K. (2019). Degradation of fibres from fruit by-products allows selective modulation of the gut bacteria in an in vitro model of the proximal colon. *Journal of Functional Foods, 57* (April), 275–285. https://doi.org/10.1016/j.jff.2019.04.026

Campos, D. A., Coscueta, E. R., Vilas-Boas, A. A., Silva, S., Teixeira, J. A., Pastrana, L. M., Manuela, M., & Pintado, M. M. (2020). Impact of functional fl ours from pineapple by-products on human intestinal microbiota. *Journal of Functional Foods, 67* (103830), Article 103830. https://doi.org/10.1016/j.jff.2020.103830

Campos, D. A., Ribeiro, T. B., Teixeira, J. A., & Pastrana, L. (2020b). Integral valorization of pineapple (ananas comosus L .) by-products through a green chemistry approach towards added value ingredients. *Foods, 9*(60), 22.

Chen, X., Lan, W., & Xie, J. (2024). Natural phenolic compounds: Antimicrobial properties, antimicrobial mechanisms, and potential utilization in the preservation of aquatic products. *Food Chemistry, 440*(November 2023), Article 138198. https://doi.org/10.1016/j.foodchem.2023.138198

Christensen, L., Vuholm, S., Roager, H. M., Nielsen, D. S., Krych, L., Kristensen, M., Astrup, A., & Hjorth, M. F. (2019). Prevotella abundance predicts weight loss success in healthy, overweight adults consuming a whole-grain diet Ad libitum: A post hoc analysis of a 6-Wk randomized controlled trial. *The Journal of Nutrition, 149*(12), 2174–2181. https://doi.org/10.1093/JN/NXZ198

Consejería de de Agricultura, P. y desarrollo R. J. de A. Más de ocho millones de toneladas de residuos vegetales para generar biomasa - Portavoz del Gobierno Andaluz. (n.d.) Retrieved December 18, 2024, from https://www.juntadeandalucia.es/presidencia/portavoz/tierraymar/135692/Olivar/ConsejeriadeAgricultura/pescayDesarrolloRural.

Demirci, M., Bahar Tokman, H., Taner, Z., Keskin, F. E., Çağatay, P., Ozturk Bakar, Y., Özyazar, M., Kiraz, N., & Kocazeybek, B. S. (2020). Bacteroidetes and Firmicutes levels in gut microbiota and effects of hosts TLR2/TLR4 gene expression levels in adult type 1 diabetes patients in Istanbul, Turkey. *Journal of Diabetes and its Complications, 34*(2), Article 107449. https://doi.org/10.1016/J.JDIACOMP.2019.107449

Demirer, B., & Samur, G. (2024). Health benefits of olive leaf: The focus on efficacy of antiglycation mechanisms. *Nutrition Reviews, 00*(0), 1–11. https://doi.org/10.1093/nutrit/nuae162

Elahi, Z., Shariati, A., Bostanghadiri, N., Dadgar-Zankbar, L., Razavi, S., Norzaee, S., Vazirbani Arasi, S., & Darban-Sarokhalil, D. (2023). Association of Lactobacillus, firmicutes, Bifidobacterium, Clostridium, and Enterococcus with colorectal cancer in Iranian patients. *Heliyon, 9*(12), Article e22602. https://doi.org/10.1016/J.HELIYON.2023.E22602

Facchin, S., Bertin, L., Bonazzi, E., Lorenzon, G., De Barba, C., Barberio, B., Zingone, F., Maniero, D., Scarpa, M., Ruffolo, C., Angriman, I., & Savarino, E. V. (2024). Short-chain fatty acids and human health: From metabolic pathways to current therapeutic implications. *Life, 14*(5), 1–44. https://doi.org/10.3390/life14050559

Giacometti, J., Bursać Kovačević, D., Putnik, P., Gabrić, D., Bilušić, T., Krešić, G., Stulić, V., Barba, F. J., Chemat, F., Barbosa-Cánovas, G., & Režek Jambrak, A. (2018). Extraction of bioactive compounds and essential oils from mediterranean herbs by conventional and green innovative techniques: A review. *Food Research International, 113*(June), 245–262. https://doi.org/10.1016/j.foodres.2018.06.036

Gómez-García, R., Sánchez-Gutiérrez, M., Freitas-Costa, C., Vilas-Boas, A. A., Campos, D. A., Aguilar, C. N., Madureira, A. R., & Pintado, M. (2022). Prebiotic effect, bioactive compounds and antioxidant capacity of melon peel (Cucumis melo L. inodorus) flour subjected to in vitro gastrointestinal digestion and human faecal fermentation. *Food Research International*. , Article 111045. https://doi.org/10.1016/j.foodres.2022.111045

Gómez-García, R., Sánchez-Gutiérrez, M., Freitas-Costa, C., Vilas-Boas, A. A., Campos, D. A., Aguilar, C. N., Madureira, A. R., Pintado, M., Gómez-García, R., Sánchez-Gutiérrez, M., Freitas-Costa, C., Vilas-Boas, A. A., Campos, D. A., Aguilar, C. N., Madureira, A. R., & Pintado, M. (2022). Prebiotic effect, bioactive compounds and antioxidant capacity of melon peel (Cucumis melo L. inodorus) flour subjected to in vitro gastrointestinal digestion and human faecal fermentation. *Food Research International, 154*(October 2021), Article 111045. https://doi.org/10.1016/j.foodres.2022.111045

Guarner, F., & Malagelada, J. R. (2003). Gut flora in health and disease. *The Lancet, 361*(9356), 512–519. https://doi.org/10.1016/S0140-6736(03)12489-0

Gullon, P., Tavaria, F., Pintado, M., Maria, A., Gullo, B., Alonso, L., & Parajo, J. C. (2014). Structural features and assessment of prebiotic activity of refined arabinoxylooligosaccharides from wheat bran. *Journal of Functional Food, 6*, 438–449. https://doi.org/10.1016/j.jff.2013.11.010

Jakobek, L., & Matić, P. (2019). Non-covalent dietary fiber - polyphenol interactions and their influence on polyphenol bioaccessibility. *Trends in Food Science and Technology, 83*(October 2018), 235–247. https://doi.org/10.1016/j.tifs.2018.11.024

Junta de Andalucia. (2023). *Aforo de producción de olivar en andalucía*.

Khatami, F., Cagno, E., & Khatami, R. (2024). Circular economy in the agri-food system at the country level—evidence from European countries. *Sustainability, 16*(21), 1–16. https://doi.org/10.3390/su16219497

Koecher, K. J., Noack, J. A., Timm, D. A., Klosterbuer, A. S., Thomas, W., & Slavin, J. L. (2014). Estimation and interpretation of fermentation in the gut: coupling results from a 24 h batch in vitro system with fecal measurements from a human intervention feeding study using fructo-oligosaccharides, inulin, gum acacia, and pea fiber. *Journal of Agricultural and Food Chemistry, 62*(6), 1332–1337. https://doi.org/10.1021/jf404688n.

Kovatcheva-Datchary, P., Nilsson, A., Akrami, R., Lee, Y. S., De Vadder, F., Arora, T., Hallen, A., Martens, E., Björck, I., & Bäckhed, F. (2015). Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of Prevotella. *Cell Metabolism, 22*(6), 971–982. https://doi.org/10.1016/J.CMET.2015.10.001

Li, L. Q., Chen, X., Zhu, J., Zhang, S., Chen, S. Q., Liu, X., Li, L., & Yan, J. K. (2023). Advances and challenges in interaction between heteroglycans and Bifidobacterium: Utilization strategies, intestinal health and future perspectives. *Trends in Food Science & Technology, 134*, 112–122. https://doi.org/10.1016/J.TIFS.2023.02.018

Madureira, A. R., Campos, D., Gullon, B., Marques, C., Rodríguez-Alcalá, L. M., Calhau, C., Alonso, J. L., Sarmento, B., Gomes, A. M., & Pintado, M. (2016). Fermentation of bioactive solid lipid nanoparticles by human gut microflora. *Food & Function, 7*(1), 516–529. https://doi.org/10.1039/c5fo01004g

Madureira, J., Margaça, F. M. A., Santos-Buelga, C., Ferreira, I. C. F. R., Verde, S. C., & Barros, L. (2022). Applications of bioactive compounds extracted from olive industry wastes: A review. *Comprehensive Reviews in Food Science and Food Safety, 21*(1), 453–476. https://doi.org/10.1111/1541-4337.12861

Mao, W., Han, Y., Wang, X., Zhao, X., Chi, Z., Chi, Z., & Liu, G. (2019). A new engineered endo-inulinase with improved activity and thermostability: Application in the production of prebiotic fructo-oligosaccharides from inulin. *Food Chemistry, 294*, 293–301. https://doi.org/10.1016/J.FOODCHEM.2019.05.062

Meiners, F., Kreikemeyer, B., Newels, P., Zude, I., & Walter, M. (2025). Strawberry dietary intervention influences diversity and increases abundances of SCFA producing bacteria in healthy elderly people. *13*(group 2), 1–31.

Miao, F. (2022). Hydroxytyrosol alleviates dextran sodium sulfate–induced colitis by inhibiting NLRP3 inflammasome activation and modulating gut microbiota in vivo. *Nutrition, 97*, Article 111579. https://doi.org/10.1016/j.nut.2021.111579

Moon, H., & Kim, M. (2024). Enzymatic synthesis of Artemisia capillaris-derived prebiotic oligosaccharide using Leuconostoc mesenteroides MKSR dextransucrase and its potential health functional effects. *Food Bioscience, 59*, Article 103873. https://doi.org/10.1016/J.FBIO.2024.103873

Moorthy, M., Chaiyakunapruk, N., Jacob, S. A., & Palanisamy, U. D. (2020). Prebiotic potential of polyphenols, its effect on gut microbiota and anthropometric/clinical markers: A systematic review of randomised controlled trials. *Trends in Food Science and Technology, 99*(February), 634–649. https://doi.org/10.1016/j.tifs.2020.03.036

Mosele, J. I., Martín-Peláez, S., Macià, A., Farràs, M., Valls, R. M., Catalán, Ú., & Motilva, M. J. (2014). Faecal microbial metabolism of olive oil phenolic compounds: In vitro and in vivo approaches. *Molecular Nutrition & Food Research, 58*(9), 1809–1819. https://doi.org/10.1002/mnfr.201400124

Nehmi-Filho, V., Alves de Freitas, J., Augusto Moysés Franco, L., Vanessa da Silva Fonseca, J., Cristina Ruedas Martins, R., Boveto Santamarina, A., Masahiro Murata, G., Cerdeira Sabino, E., Souza, E., Thomas Ferreira, M., Pinhata Otoch, J., & Flávia Marçal Pessoa, A. (2023). Novel Nutraceutical (silymarin, yeast β-glucan, prebiotics, and minerals) shifts gut microbiota and restores large intestine histology of diet-induced metabolic syndrome mice. *Journal of Functional Foods, 107*, Article 105671. https://doi.org/10.1016/J.JFF.2023.105671

Núñez-Gómez, V., Jesús Periago, M., Luis Ordóñez-Díaz, J., Pereira-Caro, G., Manuel Moreno-Rojas, J., & González-Barrio, R. (2024). Dietary fibre fractions rich in (poly) phenols from orange by-products and their metabolisation by in vitro digestion and colonic fermentation. *Food Research International, 177*, Article 113718. https://doi.org/10.1016/J.FOODRES.2023.113718

Pérez-Burillo, S., Hinojosa-Nogueira, D., Pastoriza, S., & Rufián-Henares, J. A. (2020). Plant extracts as natural modulators of gut microbiota community structure and functionality. *Heliyon, 6*(11), Article e05474. https://doi.org/10.1016/j.heliyon.2020.e05474

Ribeiro, T. B., Costa, C. M., Bonifácio - Lopes, T., Silva, S., Veiga, M., Monforte, A. R., Nunes, J., Vicente, A. A., & Pintado, M. (2021). Prebiotic effects of olive pomace powders in the gut: In vitro evaluation of the inhibition of adhesion of pathogens, prebiotic and antioxidant effects. *Food Hydrocolloids, 112*(April 2020), Article 106312. https://doi.org/10.1016/j.foodhyd.2020.106312

Rocchetti, G., Callegari, M. L., Senizza, A., Giuberti, G., Ruzzolini, J., Romani, A., Urciuoli, S., Nediani, C., & Lucini, L. (2022). Oleuropein from olive leaf extracts and extra-virgin olive oil provides distinctive phenolic profiles and modulation of microbiota in the large intestine. *Food Chemistry, 380*, Article 132187. https://doi.org/10.1016/J.FOODCHEM.2022.132187

Rocchetti, G., Luisa Callegari, M., Senizza, A., Giuberti, G., Ruzzolini, J., Romani, A., Urciuoli, S., Nediani, C., & Lucini, L. (2022). Oleuropein from olive leaf extracts and extra-virgin olive oil provides distinctive phenolic profiles and modulation of microbiota in the large intestine. *Food Chemistry, 380*(September 2021), Article 132187. https://doi.org/10.1016/j.foodchem.2022.132187

Rodríguez-Daza, M. C., Pulido-Mateos, E. C., Lupien-Meilleur, J., Guyonnet, D., Desjardins, Y., & Roy, D. (2021). Polyphenol-mediated gut microbiota modulation: Toward prebiotics and further. *Frontiers in Nutrition, 8*(June). https://doi.org/10.3389/fnut.2021.689456

Ruiz, E., Gullón, B., Moura, P., Carvalheiro, F., Eibes, G., Cara, C., & Castro, E. (2017). Bifidobacterial growth stimulation by oligosaccharides generated from olive tree pruning biomass. *Carbohydrate Polymers, 169*, 149–156. https://doi.org/10.1016/j.carbpol.2017.04.014

Salamat, S., Tabandeh, M. R., Jahan-Mihan, A., & Mansoori, A. (2024). The effect of supplementation with a multi-species synbiotic on serum lipid profile, abundance of beneficial gut bacteria and firmicutes to bacteroidetes ratio in patients with dyslipidemia; a randomized, double-blind, placebo-controlled, clinical trial. *PharmaNutrition, 27*, Article 100367. https://doi.org/10.1016/J.PHANU.2023.100367

Sánchez-Gutiérrez, M., Bascón-Villegas, I., Rodríguez, A., Pérez-Rodríguez, F., Fernández-Prior, Á., Rosal, A., & Carrasco, E. (2021). Valorisation of olea europaea L . Olive leaves through the evaluation of their extracts : Antioxidant and antimicrobial activity. *Foods, 10*(966), 20. https://doi.org/10.3390/foods10050966

Sánchez-Gutiérrez, R. G.-G., Carrasco, E., Bascón-Villegas, I., & Alejandro Rodríguez, M. P. (2022). Quercus ilex leaf as a functional ingredient : Polyphenolic profile and antioxidant activity throughout simulated gastrointestinal digestion and antimicrobial activity. *Journal of Functional Food, 91*. https://doi.org/10.1016/j.jff.2022.105025

Santana Andrade, J. K., Chagas Barros, R. G., Pereira, U. C., Nogueira, J. P., Gualberto, N. C., Santos de Oliveira, C., Shanmugam, S., & Narain, N. (2022). Bioaccessibility of bioactive compounds after in vitro gastrointestinal digestion and probiotics fermentation of Brazilian fruits residues with antioxidant and antidiabetic potential. *Lwt, 153*. https://doi.org/10.1016/j.lwt.2021.112469 (October 2020).

Sáyago-Ayerdi, S. G., Zamora-Gasga, V. M., & Venema, K. (2019). Prebiotic effect of predigested mango peel on gut microbiota assessed in a dynamic in vitro model of the human colon (TIM-2). *Food Research International, 118*(October 2017), 89–95. https://doi.org/10.1016/j.foodres.2017.12.024

Silva, F. A., de Souza, E. L., Queiroga, R. C. R. E., Voss, G. B., Pintado, M. M. E., & Vasconcelos, M. A. S. (2022). A fibre and phenolic-rich flour from Isabel grape by-products with stimulatory effects on distinct probiotics and beneficial impacts on human colonic microbiota in vitro. *Letters in Applied Microbiology, 75*(2), 249–260. https://doi.org/10.1111/lam.13723

Simonelli, N., Abraham, A. G., & Medrano, M. (2024). Stimulation of microbial butyrate synthesis through prebiotics. *Food Bioscience, 62*(May), Article 105329. https://doi.org/10.1016/j.fbio.2024.105329

Sudjana, A. N., D'Orazio, C., Ryan, V., Rasool, N., Ng, J., Islam, N., Riley, T. V., & Hammer, K. A. (2009). Antimicrobial activity of commercial Olea europaea (olive) leaf extract. *International Journal of Antimicrobial Agents, 33*(5), 461–463. https://doi.org/10.1016/j.ijantimicag.2008.10.026

Thorman, A. W., Morrow, A. L., Groeneveld, A., Nauta, A., & Newburg, D. S. (2024). Validation of collection and anaerobic fermentation techniques for measuring prebiotic impact on gut microbiota. *Pharmacological Research, 203*, Article 107169. https://doi.org/10.1016/J.PHRS.2024.107169

Wang, Y., & Qi, H. (2024). Waste to wealth: Bioprocessing methods for the conversion of food byproducts into value-added products: A mini-review. *Current Opinion in Food Science, 60*, Article 101215. https://doi.org/10.1016/j.cofs.2024.101215

Xie, F., Yang, W., Xing, M., Zhang, H., & Ai, L. (2023). Natural polyphenols-gut microbiota interactions and effects on glycolipid metabolism via polyphenols-gut-brain axis: A state-of-the-art review. *Trends in Food Science and Technology, 140* (August), Article 104171. https://doi.org/10.1016/j.tifs.2023.104171

Yang, R., Gong, L., Li, Y., & Wang, J. (2024). Role of the gut microbiota in determining the metabolic fate of free and bound phenolic compounds in wheat bran. *Lwt, 210* (September). https://doi.org/10.1016/j.lwt.2024.116892

Yuan, H., Huang, H., Du, Y., Zhao, J., Yu, S., Lin, Y., Chen, Y., Shan, C., Zhao, Y., Belwal, T., & Fu, X. (2025). Sea buckthorn polyphenols on gastrointestinal health and the interactions with gut microbiota. *Food Chemistry, 469*(September 2024), Article 142591. https://doi.org/10.1016/j.foodchem.2024.142591

Zhang, Q., Xu, Y., Bukvicki, D., Peng, Y., Li, F., Zhang, Q., Yan, J., Lin, S., Liu, S., & Qin, W. (2024). Phenolic compounds in dietary target the regulation of gut microbiota: Role in health and disease. *Food Bioscience, 62*(September), Article 105107. https://doi.org/10.1016/j.fbio.2024.105107

Žugčić, T., Abdelkebir, R., Alcantara, C., Collado, M. C., García-Pérez, J. V., Meléndez-Martínez, A. J., Režek Jambrak, A., Lorenzo, J. M., & Barba, F. J. (2019). From extraction of valuable compounds to health promoting benefits of olive leaves through bioaccessibility, bioavailability and impact on gut microbiota. *Trends in Food Science and Technology, 83*(August 2018), 63–77. https://doi.org/10.1016/j.tifs.2018.11.005