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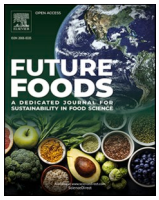

Impact of *Quercus ilex* leaves by-products on *in vitro* human colonic fermentation and microbiota composition

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

[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, España, Spain*

[b] *Grupo Biopren (RNM940), Área 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, España, 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*

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ABSTRACT

The leaves of *Quercus ilex*, rich in phenolic compounds and dietary fibre, represent a promising source of bioactive substances with potential health benefits. *In vitro* human colonic fermentation was performed to evaluate the prebiotic effect of ground *Q. ilex* leaves. Prior to fermentation, (–) epicatechin, catechin, and rutin were identified as the predominant phenolic compounds that reached the colon after gastrointestinal digestion, with (–) epicatechin present at the highest concentration (74 mg/100 g DM). Among then, rutin undergoing the most extensive metabolization during fermentation, decreasing from 22 mg/100 g DM at baseline to below the detection limit after 48 h of fermentation. Compared with negative control, *Q. ilex* leaves increased the abundance of *Bifidobacterium* spp. and *Prevotella* spp. by 46% and 40%, respectively, after 24 h of fermentation, whereas fructooligosaccharides (FOS) increased these bacterial groups by 31% and 16%, respectively. In addition, ground *Q. ilex* leaves enhanced the production of short-chain fatty acids (SCFAs), particularly acetate and propionate, which reached their highest concentrations after 12 h of fermentation. Moreover, *Q. ilex* leaves tend to maintain a Firmicutes:Bacteroidetes ratio closer to 1 than FOS throughout fermentation (1,31 vs. 1,82), suggesting a more balanced gut microbiota composition. Overall, the observed effects of *Q. ilex* leaves support their potential use as a natural and currently undervalued source of bioactive compounds with prebiotic properties. Considering the growing interest in sustainable, low-impact and locally sourced food ingredients, *Q. ilex* by-products may represent a promising alternative for the development of new functional foods and nutraceutical formulations designed to promote gut health.

1. Introduction

Environmentally friendly and sustainable food production methods have attracted increasing attention over recent decades. By emphasizing resource efficiency and waste reduction, this transition has encouraged a movement away from linear to circular manufacturing approaches (Cano-Gonzalez et al., 2025; Pascoalino et al., 2026; Silvestri et al., 2026). Nevertheless, the agri-food sector continues to generate millions of tons of residues and non-edible by-products each year, posing a major challenge by increasing environmental degradation and adding economic burdens to the industry (Cárdenas-Hernández et al., 2024; Maqsood et al., 2025; Pascoalino et al., 2024).

In this context, the bioeconomy has emerged as a production model aimed at addressing key challenges of modern society. Forest biomass represents a valuable resource that can be promoted both as a green energy source and as a reservoir of functional compounds (Cañadas et al., 2023; Kaul et al., 2024; Lozano et al., 2025). In this regard, pruning of *Quercus ilex* constitute abundant forestry by-products in the *dehesa* ecosystem that pose risks related to vegetation fires and disease spread (Malico et al., 2016). With an annual production of approximately 2.4 million tonnes in Andalusia, these by-products are currently used primarily as firewood, a practice considered unsustainable and

* Corresponding author.
E-mail address: v02sagum@uco.es (M. Sánchez-Gutiérrez).

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generating little or no added value. Their efficient collection and utilization, with low energy consumption, would therefore contribute to reducing both environmental impact and economic costs (Agencia Andaluza de la Energía, 2020).

The *dehesa* of the Iberian Peninsula is a Mediterranean silvopastoral forest ecosystem covering approximately 2.4 million hectares in Spain, among which 1.2 million are located in Andalusia (Life bioDehesa, 2018). It consists mainly of pastures and tree species of the genus *Quercus*, with *Quercus ilex* subsp. *ballota* being the most representative specie in the area (Guerrero-Sánchez et al., 2021; Parra-López et al., 2023; Tienda-Parrilla et al., 2025). Within this ecosystem, Iberian pigs graze in the last fattening stage of the extensive outdoor system known as *montanera* in the *Dehesa*, consuming acorns from *Q. ilex* as their primary feed (Horrillo et al., 2022; Rodríguez-Estévez et al., 2009), rich in monounsaturated fatty acids and phenolic compounds (Hernández-Lao et al., 2025; Reyes-Palomo et al., 2023), thus contributing to the high nutritional and functional value of meat and cured products derived from acorn-fed Iberian pigs (García-Gudiño et al., 2026; López-Hidalgo et al., 2021).

The polyphenolic profile of *Q. ilex* leaves (QIL), which consists primarily of ellagic acid, catechin, (-)-epicatechin, quercetin, hydroxytyrosol, rutin, p-coumaric, gallic acid, luteolin-7-O-glycoside and kaempferol, has been investigated due to its bioactive potential (Hadidi et al., 2017; Sánchez-Gutiérrez et al., 2022). QIL is particularly abundant in soluble dietary fibres (hemicellulose), insoluble dietary fibres (cellulose and lignin), as well as in phenolic compounds, which are closely linked to a wide range of health benefits (Tarasov et al., 2018). Owing to their high phenolic content, extracts from different parts of *Q. ilex* have been shown to possess antioxidant, antibacterial, anti-inflammatory, anticancer, gastroprotective, and cardioprotective properties (Gonzalez-Iglesias et al., 2025; Karioti et al., 2011; Paz-Arteaga et al., 2023). Indeed, various portions of the *Quercus* tree have long been used in the treatment of inflammation and gastrointestinal disorders (Berahou et al., 2007; López-Hidalgo et al., 2021; Rtibi et al., 2017).

Dietary fibre and phenolic compounds can beneficially modulate gut microbiota composition and exert prebiotic effect by acting as substrates for microbial fermentation (Moorthy et al., 2020). These indigestible polysaccharides and polyphenols are partially absorbed in the small intestine or reach the colon unaltered, where they undergo microbial fermentation and biotransformation (Mithul Aravind et al., 2021; G. S. Silva et al., 2025)(G. S. Silva et al., 2025). This process is closely linked to the generation of short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate, as well as to the transformation of high molecular-weight polyphenols into smaller, more physiologically active metabolites (Z. Liu et al., 2022; Luzardo-Ocampo et al., 2025; Rezende et al., 2021).

Although the growing interest in sustainable and functional foods has promoted research on the relationship between diet and health, the potential of QIL as a functional ingredient with prebiotic activity remains unexplored (Clímaco et al., 2026). Given its high bioactive potential, QIL represents a promising candidate for the development of innovative, high-value products (Ahmad et al., 2024).

In light of these considerations, the present study aimed to evaluate, for the first time, the prebiotic potential of QIL compounds on *in vitro* human colonic fermentation.

2. Materials and methods

2.1. Chemicals

Sigma-Aldrich (Sintra, Portugal) provided the calcium chloride dihydrate ($CaCl_2 \bullet 2H_2O$), magnesium chloride hexahydrate ($MgCl_2 \bullet 6H_2O$), potassium dihydrogen phosphate (KH_2PO_4), and sulfuric acid (H_2SO_4), while Fischer Scientific (Oeiras, Portugal) provided the formic acid and methanol. Beneo(Oreye, Belgium) supplied the FOS (Orafti®P95) standard for fructooligosaccharides (Fructan-type non-available carbohydrate).

Individual phenolic compounds (rutin, gallic acid, catechin, p-cumaric, (-) epicatechin, and catechin) were identified using standards from Sigma-Aldrich (Sintra, Portugal), whereas hydroxytyrosol and luteolin-7-O-glycoside were provided by Extrasynthese (Lyon, France). Phenol content was measured using a calibration curve for each compound (0.005 – 0.25 mg/mL).

Individual organic acids (lactic, acetic, succinic, butyric, and propionic acids) were detected using standards that were acquired from Sigma-Aldrich (Sintra, Portugal), and calibration curves ranging from 0.05 to 2 g/L was developed for every organic acid.

2.2. Raw material and sample preparation

The leaves of *Q. ilex* were gathered at random from the *dehesa* area in Los Pedroches Valley (Córdoba, southern Spain; latitude 38.2967, longitude 4.4539) after the "montanera" season ended and during the pruning period that was permitted. Leaves from at least ten distinct trees (minimum of 25 g of leaves per tree) were collected to make up a pooled sample of 250 g. The leaves were cleaned by hand using tap water and then allowed to dry in the dark and open air for 7 ± 2 days, until the leaf moisture was below 8%. They were then ground into powder in a Retsch SM2000 automatic grinder (Restsh GmbH, Haan, Germany) and sieved manually with an ASTM No 10 sieve to obtain particles with a diameter of <2 mm. QIL, with a moisture content of $6.54 \pm 0.05\%$, were stored in a dark and dry place at room temperature (about 25 °C) until examination (Sánchez-Gutiérrez et al., 2022).

2.3. In vitro simulated colon fermentation

The procedure previously described by Sánchez-Gutiérrez et al. (2025a) was followed for the *in vitro* gastrointestinal digestion of QIL. To summarize, two grams of QIL were dissolved in twenty milliliters of ultrapure water and then sequentially digested using the appropriate enzymes for each phase: α-amylase with starch hydrolysis activity for the oral phase, pepsin with proteolytic activity for the gastric phase, and pancreatin with bile salts with protease, lipase, and amylase activities for the intestinal phase. Each step involved adjusting the pH and simulating physiological conditions of agitation and temperature (37 °C). To simulate intestinal absorption and differentiate between both absorbed and non-absorbed fraction (available for the colon), dialysis using a 3 kDa membrane was finally carried out. Simulated *in vitro* gastrointestinal digestion was carried out in triplicate using three independent experimental runs on separate aliquots of the homogenised sample.

Following digestion simulation, the colon's accessible fraction was subjected to *in vitro* colon fermentation to assess QIL's prebiotic activity. Five healthy human donors provided fresh faecal samples, which were then placed in sterile plastic vases and stored anaerobically for up to two hours from collection. These samples were anonymously collected with non-invasive techniques. Donors provided informed consent and reported no gastrointestinal or metabolic disorders, no antibiotic intake in the previous three months, and no use of prebiotic or probiotic supplements. All experiments were performed in compliance with relevant law and institutional guidelines (Gullón et al., 2014; Sánchez-Gutiérrez et al., 2025b).

The basal medium, described by Madureira et al. (2016), consisted of 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. After being dispensed into airtight glass anaerobic bottles and sealed with aluminium caps, the basal medium was sterilized. Yeast Nitrogen Base (YNB) stock solutions were added after filter

sterilization (0.2 μm) (Chromafils, Macherey-Nagel, Düren, Germany). A final concentration of 2% (w/v) of QIL was added to serum bottles and were inoculated with 2% (v/v) faecal slurry for 48 h at 37 °C without shaking. Additionally, fructooligosaccharides (FOS) 2% (w/v) was used as positive control, while basal medium inoculated with faecal slurry without added substrate served as the negative control. Samples were collected at 0, 12, 24, and 48 h of fermentation, which was performed in an anaerobic cabinet with 5% H_2, 10% CO_2 and 85% N_2.

2.4. Characterization of gut microbiota

2.4.1. DNA isolation

To extract and purify genomic DNA from faecal samples (Madureira et al., 2016), the NZY Tissue gDNA Isolation Kit (Nzytech, Lisbon, Portugal) with some modifications, was used. In short, samples were centrifuged for 10 min at 11,000 g, to separate the supernatant from the pellet. At 0, 12, 24, and 48 h of fermentation, faecal pellets (about 170–200 mg) were extracted from the control and test samples, homogenized in TE buffer (10 mM Tris–HCl; 1 mM EDTA, pH 8.0), and centrifuged at 4000 g for 15 min. The pellet was resuspended in 350 μL of buffer NT1, incubated for 10 min at 95 °C, and centrifuged for 1 min at 11,000 g after which the supernatant was discarded. 200 μL of supernatant was then mixed with 25 μL of proteinase K, and the mixture was incubated for 10 min at 70 °C. A NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to measure the purity and quantity of the DNA.

2.4.2. DNA amplification by quantitative real-time polymerase chain reaction (qPCR)

In sealed 96-well microplates, qPCR was performed using a LightCycler FastStart DNA Master SYBR Green kit and a Light Cycler device (Roche Applied Science, Indianapolis, ID, USA), as previously was reported by Madureira et al. (2016). 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) were all included in qPCR mixes with a total volume of 10 μL. The different bacterial groups were quantified by qPCR using specific primers targeting the 16S rRNA gene (Sigut microbiota-Aldrich, St. Louis, MO, USA). Primer sequences and amplification conditions are detailed in Table 1. Amplicon specificity was confirmed by melting curve analysis, monitoring SYBR Green fluorescence over a temperature range from 60 to 97 °C. Data acquisition and analysis were carried out using LightCycler software (Roche Applied Science). Standard curves were created using repeated tenfold dilutions of bacterial genomic DNA. Bacterial genomic DNA used as a standard (Table 1) was obtained from DSMZ (Braunschweig, Germany). Genome size and 16S rRNA gene copy number for each reference strain were retrieved from the NCBI Genome database (http://www.ncbi.nlm.nih.gov) to ensure accurate quantification. Results are expressed as the mean values of duplicate qPCR analyses.

The number of copies of Firmicutes phylum was divided by the number of copies of Bacteroidetes phylum to determine the so-called F:B ratio. Eq. (1) was also used to determine the relative differences to the negative control % (only faeces fermentation).

$$\text{Relative difference to control } (\%) = \frac{SMC - CMC}{CMC} * 100 \quad (1)$$

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 negative control sample at the same time as SMC. Positive percentage numbers indicate a higher number of copies at a given time compared to the control sample (Gómez-García et al., 2022).

Table 1
Primer sequence and real-time quantitative PCR conditions applied for gut microbiota analysis.

Target group	Primer sequence (5′–3′: F- forward, R-reverse)	Genomic DNA standard	Target length (bp)	AT (°C)
Universal	**F:** AAA CTC AAA KGA ATT GAC **R:** GG CTC ACR RCA CGA GCT GAC	*Bacteroides vulgatus* ATCC 8482 (DSMZ 1447)	180	45
Firmicutes	**F:** ATG TGG TTT AAT TCG AAG CA **R:** AGC TGA CGA CAA CCA TGC AC	*Lactobacillus gasseri* ATCC 33,323 (DSMZ 20,243)	126	45
Clostridium leptum	**F:** GCA CAA GCA GTG GAG T **R:** CTT CCT CCG TTT TGT CAA	*Clostridium leptum* ATCC 29,065 (DSMZ 753)	239	45
Lactobacillus spp.	**F:** GAG GCA GTA GGG AAT CTT C **R:** GGC CAG TTA CTA CCT CTA TCC TTC	*Lactobacillus gasseri* ATCC 33,323 (DSMZ 20,243)	126	55
Bacteroidetes	**F:** CAT GTG GTT TAA TTC GAT **R:** AGC TGA CGA CAA CCA TGC AG	*Bacteroides vulgatus* ATCC 8482 (DSMZ 1447)	126	45
Bacteroides spp.	**F:** ATA GCC TTT CGA AAG RAA **R:** GAT CCA GTA TCA ACT GCA ATT TTA	*Bacteroides vulgatus* ATCC 8482 (DSMZ 1447)	495	45
Bifidobacterium spp.	**F:** CGC GTC YGG TGT GAA AG **R:** CCC CAC ATC CAG CAT CCA	*Bifidobacterium longum* subsp. infantis ATCC 15,697 (DSMZ 20,088)	244	50

bp: base pairs; AT: annealing temperature.

2.5. Characterization of biocompounds by HPLC

2.5.1. Phenolic identification and quantification

High Performance Liquid Chromatography, employing a Diode-Array Detector (HPLC-DAD), was used to determine the polyphenolic profile of QIL. This procedure was modified from that described by Campos et al. (2020). Samples were injected into an HPLC-DAD-interfaced Waters Series e2695 Separation Module System (Mildford, MA, USA). Two mobile phases, consisting of mobile phase A (water:methanol:formic acid, 92.5:5:2.5%, (v/v/v)) and mobile phase B (methanol:water:formic acid, 92.5:5:2.5%, (v/v/v)), were used to perform separation in a reverse-phase column (COSMOSIL 5C1 8-AR-II Packed Column – 4.6 mm I.D. × 250 mm, Dartford, UK). The conditions and gradient were as follows: 50 μL of sample was injected; the flow was continuous at 0.5 mL/min; the gradient elution began at 100% mobile phase A for 50 min, then was reset at 45% A and 55% B between 50 and 55 min; it then returned to 100% mobile phase A and remained for 4 min (until 59 min). To collect and analyse data, Empower 3® software was used. A calibration curve with pure standards was used to identify and quantify different compounds, such as catechins or procyanidins (280 nm), phenolic acids (320 nm), and flavanols (330 nm), using retention times, UV absorption spectra, and peak areas at

maximum absorption wavelengths. The detection was conducted at wavelengths ranging from 200 to 600 nm. All determination was made three times. The findings were reported as milligrams of phenolic compounds per 100 *g* of DM.

2.5.2. Short chain fatty acid identification and quantification

A Beckman Coulter HPLC system connected to an IR (K-2301) and UV detector (K-2501) (Knauer, Berlin, Germany) was used to carry out the chromatographic separation. Samples taken at 0, 12, 24, and 48 h during the fermentative process were filtered through a 0.45 μm cellulose acetate membrane. Following this, 20 μL aliquots were examined using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) running at 40 °C with 5 mM H_2SO_4 as the mobile phase at a steady flow of 0.6 mL min−1 for 30 min. An ultraviolet detector was used to identify organic acids, and calibration curves from standards were used for quantification. Clarity® software was used for data collection and processing. All determination was made three times, and the outcomes were reported in milligrams of short-chain fatty acid per mL (Gómez-García et al., 2022).

2.6. Statistical analysis

IBM® SPSS® Statistics software Version 25 (IBM Corporation, New York, NY, USA) was used for statistical analysis. Data from the five donors were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey's post-hoc for pairwise multiple comparison tests were used to evaluate differences between fermentation intervals at a significance level of $p < 0.05$.

3. Results and discussion

3.1. Biocompounds during in vitro colon fermentation

3.1.1. Phenolic compounds

During QIL fermentation, nine phenolic compounds were identified, as can be seen in Table 2, arranged from highest to lowest concentration: (-) epicatechin, catechin, rutin, (-) epicatechin derivate 2, (-) epicatechin derivate 1, luteolin-7-O-glycoside, gallic acid and hydroxytyrosol. The concentration of p-cumaric was under the limit of detection.

Except for rutin, whose concentration did not decrease significantly ($p > 0.05$), notable changes were observed in the levels of phenolic compounds after 12 h of fermentation compared to the beginning of fermentation. After 24 and 48 h, all phenolic compounds showed a considerable overall decline, particularly rutin and luteolin-7-O-glycoside, which exhibited an even more pronounced reduction after 48 h of fermentation.

Table 2
Bioactive compounds (mg/ 100 g DM) identified and quantified by HPLC throughout in vitro colon fermentation.

RT (min)	Phenolic compounds	Time (h)			
		0	**12**	**24**	**48**
10.38	Gallic acid	2.27 ± 0.78^{a}	UD	UD	ND
18.946	Hydroxytyrosol	0.77 ± 0.49^{a}	UD	UD	UD
23.983	Catechin	27.23 ± 5.59^{a}	15.62 ± 6.25^{a,b}	7.89 ± 2.08^{b}	4.99 ± 0.52^{b}
24.714	*p*-cumaric	UD	UD	UD	ND
31.492	(-) Epicatechin	37.02 ± 5.07^{a}	24.39± 2.52^{b}	15.96 ± 0.84^{c}	12.79 ± 0.21^{c}
34.803	(-) Epicatechin derivative	18.10 ± 1.27^{a}	14.92 ± 0.64^{b}	12.80 ± 0.21^{c}	12.00 ± 0.05^{c}
36.537	(-) Epicatechin derivative	19.16 ± 1.66^{a}	15.45 ± 0.83^{b}	12.98 ± 0.28^{c}	12.05 ± 0.07^{c}
46.23	Rutin	22.20 ± 8.05^{a}	19.10 ± 8.53^{a}	4.12 ± 0.94^{b}	UD
53.022	Luteolin-7-*O*-Glycoside	12.34 ± 2.84^{a}	6.23 ± 2.32^{b}	2.74 ± 0.77^{c}	ND

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, according to Tukey's Test. RT: Retention time. ND: not detected. UD: under the limit of detection.

Numerous studies have documented that gut microbiota activity promotes the release of polyphenols reaching the colon and their subsequent transformation into smaller metabolites, thereby influencing their bioaccessibility, absorption, and physiological effects. Structural modifications occurring during fermentation may alter the biological activity and availability of these compounds, and their concentration generally decreases over time as a result of microbial metabolism. The gradual reduction in phenolic compounds observed during fermentation is likely to reflect their progressive conversion into metabolites produced by microorganisms (S. Li et al., 2026; Y. Li, Chen, et al., 2025; Y. Li, Nie, et al., 2025; Petersen and Mansell, 2025; Tang and Peng, 2025).

In addition, some research indicates that polyphenols may help prevent colon cancer, inhibit pathogenic intestinal bacteria such as *Escherichia coli, Salmonella typhimurium*, L. *monocytogenes, Yersinia enterocolitica* or *S. aureus*, and promote the growth and proliferation of beneficial bacteria by serving as a substrate for microorganisms such as *Bacteroides, Clostridium* and *Eubacterium* (Tang and Peng, 2025).

In fact, and consistent with our results, several authors have reported that phenolic compounds such as gallic acid, catechin, epicatechin, hydroxytyrosol and rutin progressively decrease throughout fermentation, likely as a result of their extensive metabolism and bioconversion by the gut microbiota (de Oliveira et al., 2023; Mosele et al., 2014; Ribeiro et al., 2021).

In the colon, phenolic compounds are not primarily used as fermentative substrates but undergo specific microbial metabolic pathways that convert them into more bioactive and absorbable metabolites (B. Cheng et al., 2025; H. Cheng et al., 2023; S. Liu et al., 2019; Petersen and Mansell, 2025). Glycosylated flavonoids, such as rutin and luteolin-7-O-glucoside, are hydrolysed by α-rhamnosidase and β-glucosidase activities mainly from *Lactobacillus* spp. and *Bifidobacterium* spp., releasing quercetin or other aglycones that are subsequently degraded into smaller phenolic acids (Amaretti et al., 2015; Huang et al., 2022). Flavanols, including catechin and (−)-epicatechin, follow microbial pathways of C-ring cleavage and A-ring fission, mediated by *Lactobacillus* spp. and *Bifidobacterium* spp., producing γ-valerolactones and phenylvaleric acids that are further transformed into low-molecular-weight phenolic acid (Cano et al., 2024; Xu et al., 2024). Simple phenolic acids, such as gallic and p-coumaric acids, are degraded through microbial enzymatic routes, for example by gallate decarboxylase or hydroxycinnamate esterases in lactic acid bacteria, yielding pyrogallol, phenylpropionic, and benzoic derivatives (Rodríguez-Daza et al., 2021).

Following microbial conversion, colon-derived metabolites of QIL phenolics exert significant health benefits. Metabolites of catechin and epicatechin, such as γ-valerolactones, have been associated with improvements in endothelial function, blood pressure regulation, and antioxidant and anti-inflammatory effects contributing to cardiovascular protection (Bernatova, 2018; Schön et al., 2021). Hydroxytyrosol has been reported to promote the growth of beneficial bacteria (*Lactobacillus* spp. and *Bifidobacterium* spp.), and increase short chain fatty acids (SCFAs), thereby indirectly modulating the intestinal ecosystem. Once in the colon, it also enhances gut barrier integrity by upregulating tight junction proteins (e.g., occludin) and mucins (MUC2), while reducing oxidative stress and inflammation in the intestinal epithelium. (Garrido-Romero et al., 2025). Additionally, quercetin, released from rutin through microbial degradation, exhibits anti-inflammatory, antioxidant, anti-obesity and anti-hypertensive effects. This compound promotes intestinal microbial diversity and improves obesity outcomes by regulating gluconeogenesis and reducing hepatic fat accumulation. (Bai et al., 2025).

Together, these metabolites derived from QIL phenolic compounds that reach the colon support cardiovascular health, anti-inflammatory responses, and intestinal barrier protection, highlighting their potential role in preventing cardiometabolic and gastrointestinal disorders, while also indicating that the activity of human gut microbiota can be modulated. All in all, these findings support the use of QIL as functional ingredient, suggesting that the phenolic compounds are transformed in the colon into low molecular weight metabolites with greater bioavailability and potential health benefits.

3.1.2. Short-chain fatty acids (SCFAs) production

QIL exhibit a composition characteristic of lignocellulosic biomass, being rich in dietary fibre fractions such as cellulose, hemicellulose, and lignin. In the proximal colon, gut bacteria ferment the prebiotic dietary fibre producing SCFAs, mainly acetate, propionate, and butyrate, which are commonly considered markers of a healthy gut flora (Rezende et al., 2021; Zhang et al., 2025).

In the presence of QIL (2% (w/v)), negative control (C-) and FOS (2% (w/v)), the synthesis of SCFAs during the *in vitro* fermentation of colonic microbiota was assessed at 0, 12, 24, and 48 h. The fermentation process revealed the presence of five organic acids, with acetate and succinate at the highest levels in QIL and FOS, followed by lactate, propionate and butyrate acids (Fig. 1). After 12 h of fermentation, the highest levels of total SCFAs were found in FOS and QIL, with 7.53 and 6.92 mg/mL, respectively. At 24 and 48 h of fermentation, butyric, acetic and propionate acids concentrations in QIL were greater than in FOS (positive control), although only the two formers presented statistical differences ($p < 0.05$). Moreover, lactate and succinate did not change significantly during the fermentation process ($p > 0.05$).

The progressive reduction in SCFAs concentrations after 12 h of fermentation may be explained by the depletion of highly fermentable substrates, resulting in a lower rate of microbial fermentation and SCFA production over time (Wang et al., 2019).

In agreement with our findings, acetate has been described as the predominant short-chain fatty acid produced during the *in vitro* colonic fermentation of plant-derived substrates. Similar results have been observed for mulberry leaves (Zhao et al., 2023), litchi pomace (X. Chen et al., 2025), Chinese cabbage outer leaves (Jin et al., 2026) and artichoke by-products (Holgado et al., 2022), where acetate was identified as the most abundant SCFA generated during fermentation.

The most notable SCFA in this work, acetate, is primarily produced by three metabolic pathways: the Wood–Ljungdahl pathway, which converts hydrogen and carbon dioxide or formic acid into acetyl-CoA and contributes approximately one-third of colonic acetate, and the pyruvate acetyl-CoA and succinate pathways, the major pathways for acetate production through carbohydrate fermentation (Ríos-Covián et al., 2016). Bacteria from the genera *Lactobacillus, Bifidobacterium, Akkermansia, Bacteroides, Prevotella, Ruminococcus*, and *Streptococcus* are the primary producers of acetate in the gut (Cunningham et al., 2021a). By activating G-protein-coupled receptors, acetate reduces inflammation and modifies immunological responses, both of which have major positive health consequences. Additionally, it enhances metabolic homeostasis, fortifies intestinal barrier integrity, and controls appetite through both central and peripheral pathways, all of which help to prevent inflammatory and metabolic diseases (Du et al., 2024). Compared to the starting values, acetate showed a substantial increase at 12 h of fermentation ($p < 0.05$), and then it decreased at 24 and 48 h. In spite of this decrease, the concentration in QIL samples was more than twice that in FOS samples at 24 h and six times higher at 48 h ($p < 0.05$).

The gut microbiota produces propionic acid primarily by genera such as *Phascolarctobacterium, Bacteroides, Dialister, Megasphaera, Veillonella, Coprococcus, Roseburia, Ruminococcus* and *Salmonella* though the succinate, acrylate, and propylene glycol pathways (Al-Lahham et al., 2010; Cunningham et al., 2021a). It improves β-cell function, insulin production, and defense against inflammatory stress by modifying host metabolism through FFAR2/FFAR3 signaling and HDAC inhibition (Kasubuchi et al., 2015; Pingitore et al., 2017). Likewise, propionate exhibits cardioprotective and immunoregulatory effects. Its effects vary depending on context, as high systemic levels might lead to neurological or metabolic disorders (Bartolomaeus et al., 2019; Haghikia et al., 2022). Throughout fermentation, propionate values in QIL exhibited a similar pattern to succinate, showing a substantial increase at 12 h ($p < 0.05$) and a subsequent decline at 24 and 48 h. Although not statistically significant, the concentration of propionate in QIL samples was higher than in FOS at 24 and 48 h (0.27 and 0.13 mg/mL; and 0.12 and 0.06 mg/mL, respectively).

Butyrate, a SCFA generated by the human gut Firmicutes phylum, serves as the primary carbon source for colonocytes. *Clostridium leptum* and *Clostridium coccoides* are among the most significant Firmicutes bacteria found in the gut (Karim et al., 2024). The pyruvate acetyl-CoA route is the primary source of butyrate, while succinate, 4-aminobutyrate, lysine, and glutarate also contribute to its production to a lesser extent (Anand et al., 2016). In addition to its anti-inflammatory effects through HDAC inhibition and G-protein-coupled receptor activation, butyrate promotes regulatory T cell differentiation, intestinal barrier integrity through tight-junction protein regulation, and protect against inflammatory, metabolic, and neoplastic disorders (Kalkan et al., 2025). At 12 and 24 h, butyrate concentration in QIL raised to comparable levels of around 0.50 mg/mL; however, after 48 h, it dropped to one-third. At 24 h, the butyrate content in QIL was noticeably higher than in FOS, approximately sixfold.

Similarly, SCFAs appear to be involved in cholesterol metabolism. Acetate promotes cholesterol synthesis, whereas propionate inhibits it, highlighting the importance of the acetate/propionate ratio in maintaining metabolic balance in the liver and gut (Sáyago-Ayerdi et al., 2019). This ratio was 3.04 for FOS and 2.76 for QIL at 12 h. As it has been associated with a reduction in blood lipids, this lower acetate/-propionate ratio is considered beneficial. Consistent with the present results, similar acetate-to-propionate ratios have been observed during in vitro fermentation of human faecal microbiota using plant-derived by-products such as pineapple by-product flours, melon peel flour, and olive pomace (Campos et al., 2020; Gómez-García et al., 2022; Ribeiro et al., 2021).

Through dietary fibre fermentation, gut bacteria produce succinic acid, mainly via by the succinate pathway, which converts glucose to pyruvate before it enters the tricarboxylic acid cycle (TCA). Although succinate is largely considered a key intermediate in propionate synthesis, it is also an end-product in some bacterial phyla, including Actinobacteria, Bacteroidetes and Firmicutes. Succinic acid supports metabolism and intestinal health, maintaining intestinal barrier integrity, regulating microbiota composition, and providing energy to colonocytes (Fernández-Veledo and Vendrell, 2019). Succinate has also been linked to immune response regulation and systemic metabolism (Connors et al., 2019). Furthermore, studies have demonstrated that succinate enhances energy metabolism and glucose homeostasis, suggesting potential therapeutic application for metabolic diseases (De Vadder et al., 2016). Succinate was found in QIL prior to fermentation, and by 24 h, it drastically decreased to 0.06 mg/mL ($p < 0.05$), while propionate increased to 0.27 mg/mL at 24 h. This suggests that microorganisms may have used succinate to produce propionate, highlighting the diversity of gut microbial metabolic pathways.

Lactate is produced primarily via microbial fermentation of carbohydrates. Key lactate producers, which frequently acts as a bridge in cross-feeding interactions with other gut microorganisms, are members of the genera *Lactobacillus, Bifidobacterium*, and *Streptococcus*. These pathways illustrate how the intestinal microbiota collectively influences the host's biochemical environment (Louis et al., 2022). Lactate has several positive impacts on human health in addition to its function as a microbial metabolite, contributing to epithelial barrier integrity, anti-inflammatory immune responses, and local pH modulation to prevent pathogen proliferation (Li et al., 2024; Zapaśnik et al., 2022). Additionally, butyrate-producing bacteria utilise lactate as a substrate,

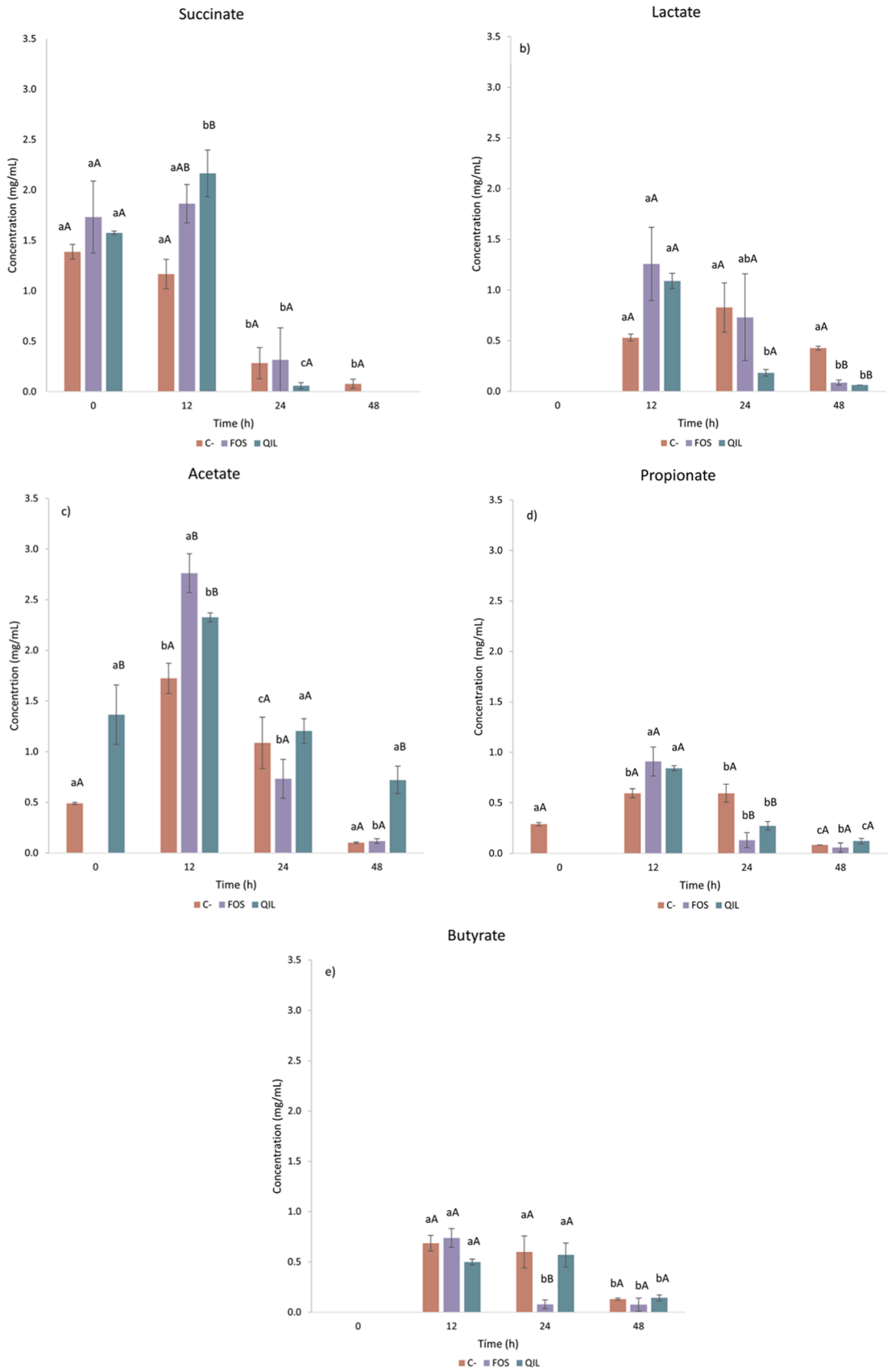

Fig. 1. Organic acids concentration through the in vitro colon fermentation a) succinate; b) lactate; c) acetate; d) propionate; e) butyrate; C-: negative control; FOS: positive control (2% (w/v)); QIL: *Quercus ilex* leaf (2% (w/v)). Results are expressed as means of five determinations ± standard deviation.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

connecting its synthesis to downstream pathways that provide cardiovascular and metabolic benefits (Silva et al., 2020). Following 12 h of fermentation, lactate was produced in QIL; however, levels declined markedly after 24 and 48 h ($p < 0.05$).

As mentioned above, SCFAs help maintaining a low colonic pH, promoting healthy bacteria growth and inhibiting harmful bacteria. During *in vitro* fermentation with QIL, pH decreased slightly, while FOS showed a significant drop ($p < 0.05$) (Fig. 2). Similar observations have been made when comparing FOS and inulin, which are rapidly fermentable short-chain fructans, with higher-molecular-weight molecules (Koecher et al., 2014). Nevertheless, QIL fermentation produced a SCFA profile with comparable or higher concentrations of acetate, propionate, and butyrate throughout fermentation than in FOS. And, as a global result, it was observed that the amounts of SCFAs produced by gut microbiota in the presence of QIL were similar to those produced in the case of FOS, suggesting that QIL may be a beneficial prebiotic agent for the gut and other organs.

3.2. Modulation of faecal microbiota during in vitro fermentation

Following GIT simulation, the QIL samples were freeze-dried and exposed to a 48-hour simulated fermentation in the colon with human faeces. To assess the impact on the human gut microbiota and understand the growth pattern of the microorganisms during the fermentative process, aliquots were collected at 0, 12, 24, and 48 h. A sample without an additional carbon source was employed as a negative control during intestinal fermentation, whereas FOS, a well-established and widely recognised prebiotic agent, served as a positive control (Pedrosa et al., 2024). To further assess the effect of QIL on the gut microbiota of the human donors, the abundance of selected bacterial groups was determined by qPCR targeting the 16S rRNA gen.

The phyla *Firmicutes* (*Clostridium* spp. and *Lactobacillus* spp.) and *Bacteroidetes* (*Prevotella* spp.) dominate the human gut microbiota, representing approximately 90% of its composition in healthy adults, while *Actinobacteria* (*Bifidobacterium* spp.), *Proteobacteria, Fusobacteria*, and *Verrucomicrobia* account for the remaining 10% (Patloka et al., 2024). The copy numbers of the dominating phylum in the human gut were compared to those in the faeces of healthy volunteers, and the F:B ratio, together with the average abundance of the main microbial groups determined by qPCR, is presented in Table 3. Based on this approach, three of the four dominant phyla in the human gut, *Firmicutes, Bacteroidetes, and Actinobacteria*, were evaluated. Among the analysed groups, *Lactobacillus* spp. exhibited the lowest 16S rRNA gene copy numbers, consistent with its typically low abundance in the normal gut microbiota. Accordingly, *Clostridium* spp. and *Prevotella* spp. were identified as

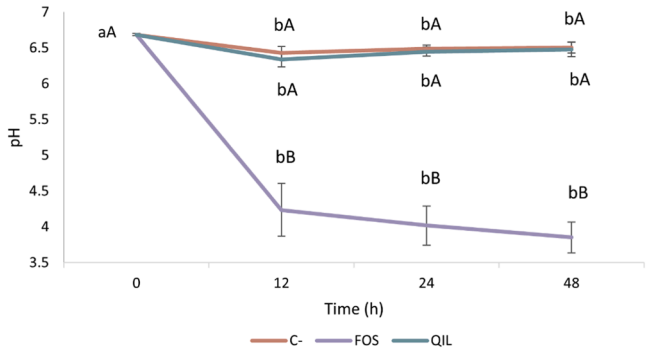

Fig. 2. . Evolution of pH values throughout the in vitro colon fermentation. C-: negative control; FOS: positive control (2% (w/v)); QIL: *Quercus ilex* leaf (2% (w/v)). Results are expressed as means of five determinations ± standard deviation. Different lowercase letters indicate significant differences between sampling times (0, 12, 24 and 48 h) within type of sample, while uppercase letters indicate significant differences between types of samples (negative control, FOS, and QIL) within sampling time, at $p < 0.05$.

Table 3
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
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 log 16S rRNA copies per 20 ng of DNA. Fecal microbiota composition was determined by qPCR.

the most abundant genera, whereas *Lactobacillus* spp. showed the lowest levels in our results (Gómez-García et al., 2022; Salsinha et al., 2025).

The bacterial groups' relative abundance differences (%) at 0, 12, 24, and 48 h of fermentation are displayed in Fig. 3. In the case of FOS, the most prevalent bacterial group was *Bifidobacterium* spp. (50%), followed by *Lactobacillus* spp. (49%), *Prevotella* spp. (48%), and *Clostridium leptum* (20%). In contrast, QIL enhanced the growth of *Prevotella* spp. (62%), *Bifidobacterium* spp. (47%), and *Clostridium leptum* (17%). In summary, it was found that during the faecal fermentation test, QIL had a higher stimulatory effect than FOS on *Bifidobacterium* and *Prevotella* species, a comparable effect on *Clostridium leptum*, and a lower effect on *Lactobacillus* species ($p > 0.05$). In terms of fermentation duration, *Bifidobacterium* spp. and *Clostridium leptum* fermented QIL equally (increasing at 24 and decreasing at 48 h), but *Prevotella* spp. decreased from 12 to 48 h. The observed similarity in the composition of the microbiota, with no significant differences compared to the positive control, suggest that QIL, primarily because of its high dietary fibre and phenolic content, greatly promotes the development of a healthy microbiota and may thus be regarded as a prebiotic agent. In this sense, the observed effects are likely related not only with the phenolic composition of QIL but also with the dietary fibre fractions naturally present in the lignocellulosic matrix of *Q. ilex* leaves, particularly non-starch polysaccharides such as cellulose and hemicellulose derived compounds, which may serve as fermentable substrates for gut microorganisms (Rezende et al., 2021). Moreover, phenolic compounds found and released during fermentation in the colon in QIL, including quercetin and hydroxytyrosol, have been shown to have prebiotic effects by promoting the development of beneficial gut flora (Marano et al., 2023). Therefore, the modulation of gut microbiota observed in this study may result from the combined and potentially synergistic action of phenolic compounds and fermentable dietary fibre fractions present in QIL.

Bifidobacterium spp. play a key role in modulating gut microbial composition, maintaining gastrointestinal homeostasis through the fermentation of complex carbohydrates into short-chain fatty acids (SCFAs) and supporting of mucosal barrier integrity (K. Chen et al., 2025; Kamlárová et al., 2025). Beyond their gastrointestinal functions, *Bifidobacterium* spp. modulate host metabolism, reduce low-grade inflammation, and influence the gut–brain axis (Marano et al., 2023; Neyrinck et al., 2021; Yassin et al., 2025). Evidence shows benefits in alleviating symptoms of irritable bowel syndrome, improving metabolic parameters in metabolic syndrome, lowering circulating lipids, and attenuating depressive symptoms (Li et al., 2025; Niu et al., 2024; Xiao et al., 2024; Zhao et al., 2024).

As predicted, during the *in vitro* colonic fermentation process, the negative control did not lead to bacterial growth in terms of DNA copy number ($\log_{10}$ 16SrRNA gene copies/ng of DNA), with all microbial groups under study showing a general decline, primarily in the first hours (Fig. 4). Except for *Lactobacillus* species, where the copy number at 48 h in the FOS sample was almost eight times higher than in the QIL sample, the bacterial responses in FOS and QIL were quite similar for all phyla. QIL and FOS seemed to promote the growth of *Bifidobacterium*

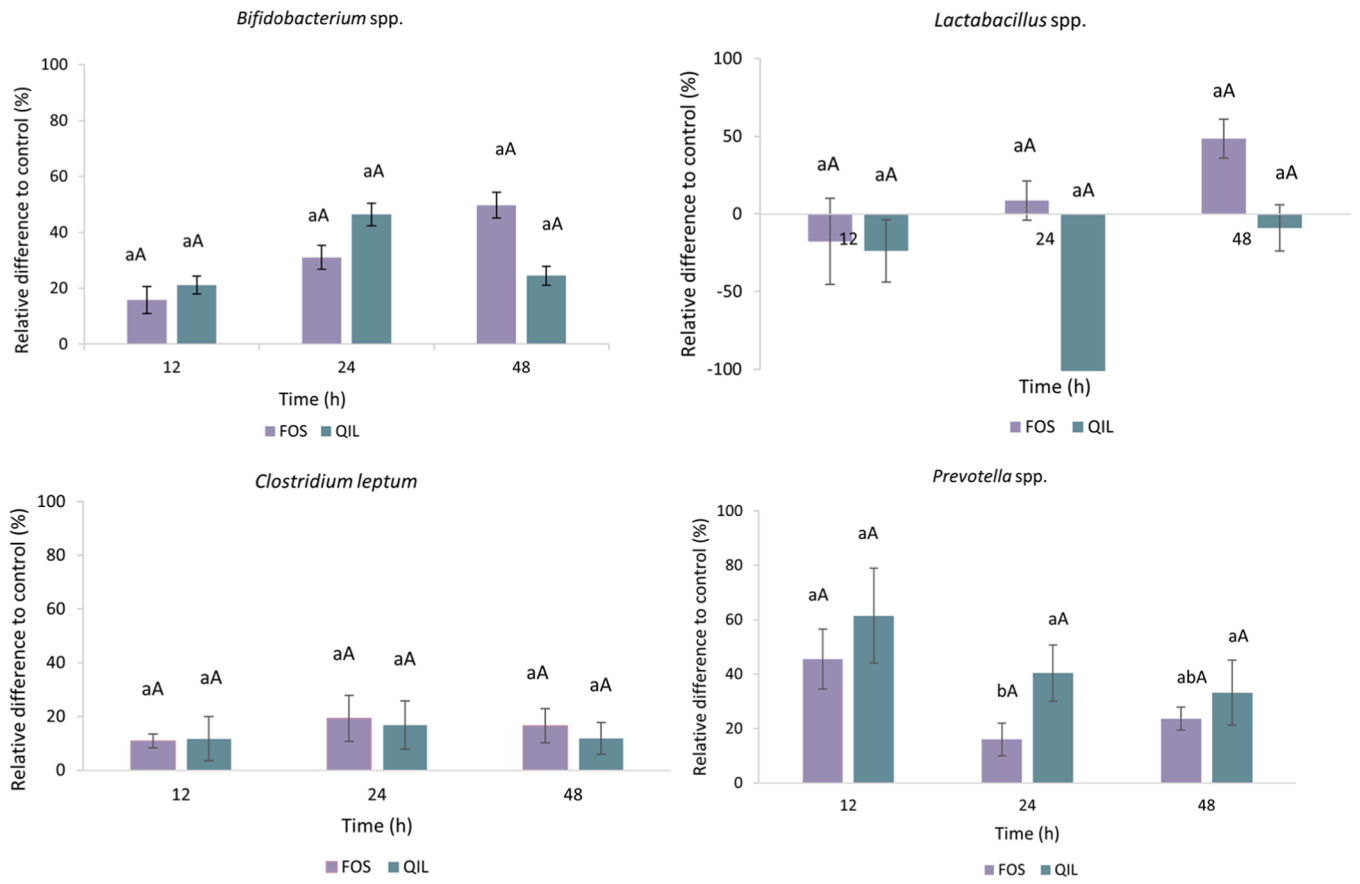

Fig. 3. Relative differences to negative control through in vitro colon fermentation. FOS: positive control (2% (w/v)); QIL: *Quercus ilex* leaf (2% (w/v)). Results are the means of five determinations ± standard deviation. Different lowercase letters indicate significant differences between sampling times (0, 12, 24 and 48 h) within type of sample, while uppercase letters indicate significant differences between types of samples (negative control, FOS, and QIL) within sampling time, at $p < 0.05$.

spp. and *Lactobacillus* spp. As can be seen in Fig. 4, the highest values for both microorganisms were 4.17 for FOS at 48 h and 3.93 for QIL at 24 h for the first group, and 1.9 for FOS at 48 h and 1.03 for QIL at 24 h for the second. Conversely, it was found out that QIL had no noticeable effects on the growth of *Prevotella* spp., while FOS had lesser stimulating effects on the growth of *Clostridium leptum* or *Prevotella* spp. As observed in the present study, previous research on brewer's spent grain flour (Bonifácio-Lopes et al., 2022), pineapple by-product flours (Campos et al., 2020) and melon peel flour (Gómez-García et al., 2022) suggest that *Bifidobacterium* species are selectively stimulated by FOS, whereas *Clostridium leptum* and *Prevotella* spp. show a limited ability to use them as an energy source. This differential response can be attributed to the composition of the substrates, which are rich in dietary fibre and simple sugars, favouring the growth of certain microbial groups whilst restricting that of others (Respondek et al., 2013).

One of the major genera in the phylum *Bacteroidetes, Prevotella* spp., has been revealed to possess a crucial role in the regulation of body weight and glucose metabolism. Particularly in people who consume high-fibre diets, recent research has shown enhanced insulin sensitivity and glucose tolerance. *Prevotella* spp. may also help manage obesity because it has been linked to decreased visceral fat and improved hepatic glycogen storage (Christensen et al., 2019; Kovatcheva-Datchary et al., 2015; Pedersen et al., 2016). *Clostridium* spp., a key player in preserving gut health and reducing systemic inflammation, is one of the most significant genera of the phylum *Firmicutes*. Improvements in metabolic profiles and a decrease in inflammation related to obesity have been associated with *Clostridium leptum*. Early-life exposure increases regulatory T cells and inhibits pro-inflammatory responses in chronic inflammatory respiratory conditions such asthma (Huang et al., 2022; Li et al., 2024).

Two of the most prevalent phyla in the human gut microbiota are included in the Firmicutes:Bacteroidetes (F:B) ratio, which is frequently used to determine the composition of the gut microbiota and has been linked to metabolic health. Although considerable inter-individual variability exists, healthy individuals typically exhibit F:B ratios close to 1:1 (Demirci et al., 2020; Magne et al., 2020). In contrast, higher F:B ratios have often been reported in individuals with obesity, type 2 diabetes, and metabolic disorders, suggesting a potential association between gut microbial imbalance and metabolic dysfunction (Bannazadeh Baghi et al., 2022; D. Chen et al., 2026; Crovesy et al., 2020; Cunningham et al., 2021b; Mi et al., 2024; Salamat et al., 2024). Conversely, lower F:B ratios have been observed in inflammatory bowel disease, including both Crohn's disease and ulcerative colitis (Tsai et al., 2025). Recent studies have also shown that dietary interventions, probiotics, and prebiotics can modulate the F:B ratio, thereby influencing host metabolism, immune function, and systemic inflammation, highlighting its potential relevance in the management of metabolic and inflammatory disorders (Meiners et al., 2025; Shan et al., 2025).

With average F:B ratios of 1.28 for QIL and 1.81 for FOS throughout the fermentation period, QIL tend to maintain values closer to 1 than those observed for FOS (Fig. 5). Furthermore, the F:B ratio remained relatively stable in the QIL treatment from 12 to 48 h of fermentation. While, no statistically significant differences were detected between treatments ($p > 0.05$), the values obtained are within the range (0.8–1.80) described above for plant-based by-products with prebiotic potential (Bonifácio-Lopes et al., 2022; Campos et al., 2020; Gómez-García et al., 2022; Ribeiro et al., 2021).

In general, the phyla under evaluation were enhanced by the digested QIL, indicating its potential application as a functional component with a prebiotic impact that promotes health. These findings

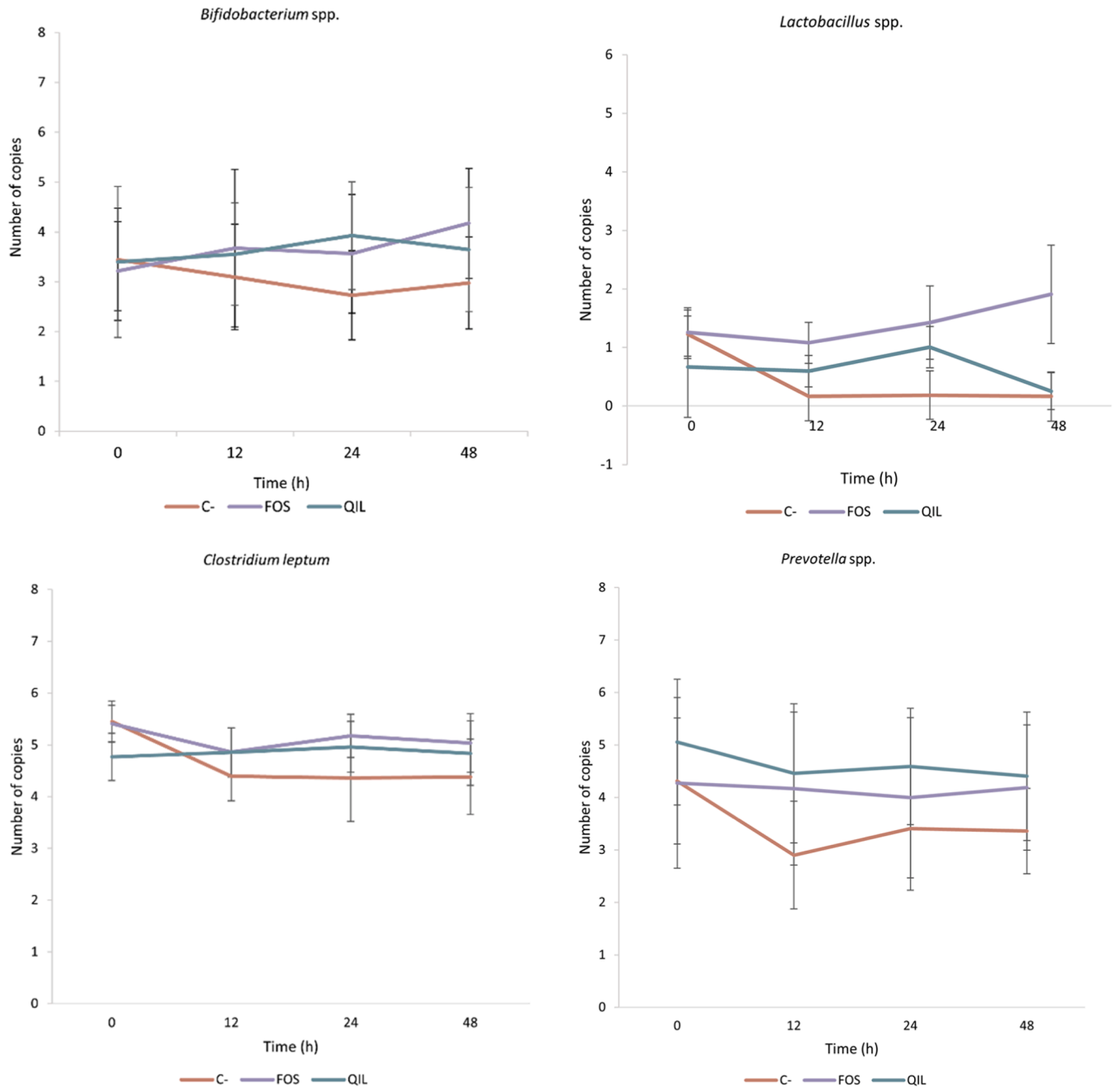

Fig. 4. Number of DNA copies measured by qPCR through in vitro colon fermentation of *Quercus ilex* leaf (QIL: 2% (w/v)), positive control (FOS: 2% (w/v)) and negative control (C-). Results are expressed as means of five determinations ± standard deviation.

are consistent with previous reports suggesting that polyphenols can modulate gut microbiota metabolism, promoting the growth of beneficial bacteria while reducing potentially harmful populations (Duda-Chodak et al., 2015). A substantial proportion of phenolic compounds that escape absorption in the small intestine reach the colon, where they are metabolised by the gut microbiota. This bidirectional interaction leads not only to the formation of bioavailable metabolites but also to shifts in microbial community composition. Through these processes, microbial activity influences both the metabolism and bioavailability of phenolic compounds, generating metabolites associated with various health benefits (de Paulo Farias et al., 2025). Nevertheless, the observed modulation of gut microbiota composition and activity is likely not attributable solely to phenolic compounds, as other dietary constituents present in QIL, particularly fermentable fibre fractions and non-starch polysaccharides, may also contribute to these effects.

4. Conclusions

The prebiotic potential of bioactive compounds derived from *Quercus ilex* ground leaf (QIL) was evaluated during *in vitro* fermentation. The findings indicate that QIL constitutes a valuable source of fermentable bioactive compounds that can be utilised by the gut microbiota. The main phenolic compounds reaching the colon after gastrointestinal digestion, such as rutin, catechin, and (-) epicatechin, suffered progressive microbial transformation during fermentation. These changes were accompanied by beneficial modification in gut microbiota composition, including increased abundances of *Bifidobacterium* spp.

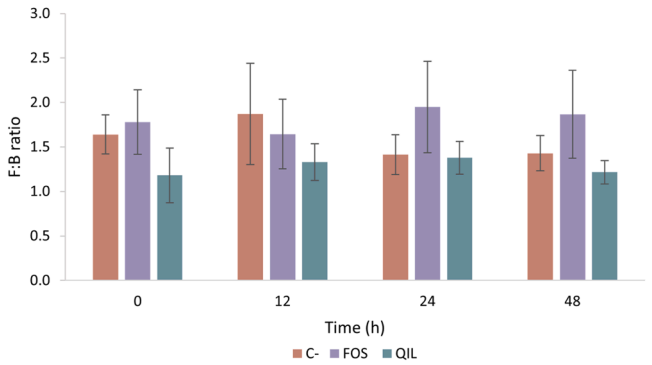

Fig. 5. *Firmicutes:Bacteroidetes* (F:B) ratio evaluation during in vitro colon fermentation. FOS: positive control (2% (w/v)); QIL: *Quercus ilex* leaf (2% (w/v)). Results are expressed as a ratio over fermentation time. Values represent the means of five determinations ± standard deviation. No significant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

and *Prevotella* spp., as well as higher SCFA production, particularly acetate. Collectively, these results suggest that phenolic compounds and other fermentable constituents naturally present in *Q. ilex* leaves contribute to gut microbiota modulation and the production of health-promoting metabolites, supporting the prebiotic potential of QIL.

While this study offers valuable insights into the prebiotic effects of QIL, the inter-individual variability of the human faecal microbiota and the limited number of donors (n = 5) should be considered when interpreting the results. Therefore, the results should be evaluated considering the study design and sample size. Future studies involving larger cohorts and complementary metagenomic approaches are warranted to further elucidate the impact of QIL on gut microbiota composition, diversity, and functionality. Overall, the present findings suggest that QIL is a promising source of phenolic compounds and fermentable carbon substrates with potential prebiotic properties. As a natural by-product, QIL can offer opportunities for the development of functional foods and bioactive ingredients within the framework of a circular economy, contributing both to the promotion of human health and to the sustainable use of plant resources.

Statement for studies in humans and animals

All procedures involving human participants were conducted in compliance with relevant laws and institutional guidelines. Five healthy human donors provided fresh fecal samples, which were collected anonymously using non-invasive techniques and placed in sterile plastic vases for anaerobic storage for up to two hours. Donors provided informed consent and reported no gastrointestinal or metabolic disorders, no antibiotic intake in the previous three months, and no use of prebiotic or probiotic supplements.

No animals were used in this study.

CRediT authorship contribution statement

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

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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