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Untargeted metabolomics of gut-derived metabolites from *in vitro* colonic fermentation of garambullo (*Myrtillocactus geometrizans*)

Edelmira Sánchez-Recillas [a,1], Enrique Almanza-Aguilera [b,1], Elisa Dufoo-Hurtado [c], Ivan Luzardo-Ocampo [d,e], Rocio Campos-Vega [c], Haydé Azeneth Vergara-Castañeda [a,*]

[a] *Advanced Biomedical Research Center, School of Medicine, Universidad Autónoma de Querétaro, Querétaro Qro. 76140, México*
[b] *Unit of Nutrition and Cancer, Cancer Epidemiology Research Program, Catalan Institute of Oncology (ICO), Bellvitge Biomedical Research Institute (IDIBELL), Barcelona 08908, Spain*
[c] *Research and Graduate Studies in Food Science, School of Chemistry, Universidad Autónoma de Querétaro, Querétaro 76000, Mexico*
[d] *Tecnologico de Monterrey, The Institute for Obesity Research, Ave. Eugenio Garza Sada 2501 Sur, Monterrey 64849, Mexico*
[e] *Tecnologico de Monterrey, School of Engineering and Sciences, Campus Guadalajara, Av. General Ramon Corona 2514, Zapopan 45201, Mexico*

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ABSTRACT

Garambullo is a polyphenols and fiber-rich fruit, but its performance during *in vitro* fermentation and potential derived health benefits have yet to be explored. This study aimed to perform untargeted metabolomics with enrichment analysis of metabolites derived from the *in vitro* colonic fermentation of Garambullo. Additionally, short-chain fatty acids (SCFAs), were identified and quantified due to their biological relevance. The fermented extract (0, 6, and 24 h-fermentation) was analyzed through untargeted metabolomics. A total of 50 metabolites, such as benzene, indoles, phenols, and fatty acids were identified. Butyric acid, one of the produced SCFAs, was increased ($p < 0.05$) after 24 h. Metabolomic enrichment analysis from colonic metabolites indicated the potential modulation of several conditions. These results suggested that garambullo colonic metabolism generates bioactive molecules that might beneficially impact intestinal and systemic health. Although results from enrichment analysis should be interpreted cautiously, they warrant further research on garambullo's health benefits.

1. Introduction

The colonic microbial metabolism of non-digestible food components produces large amounts of metabolites with important effects on host physiology. Thus, while some metabolites protect the colonic mucosa, others are proinflammatory or pro-carcinogenic (Appunni et al., 2021). More interestingly, some of these components have been proven to serve as modulators of the so-called "gut-brain axis", as exhibit the potential to enter the circulation and act directly on the brain via paracrine actions or acting on afferent neurons related to the gut (Wachsmuth et al., 2022). The type and number of microbial-derived metabolites largely depend on the microbiota composition, transit time, and the available substrates (Hamer et al., 2008).

Among substrates, dietary fiber and polyphenols are considered some of the primary modulators of colonic microbiota (Bedu-Ferrari et al., 2022; Wang et al., 2010). Dietary fiber impacts the gut microbiota ecosystem due to its biological properties as a fermentative substrate for microorganisms. The main end-products deriving from microbial fermentation of dietary fiber are short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, among others, and gasses (H_2 and CO_2) (Vinelli et al., 2022). Together with several biotransformations of polyphenols, among other components, these metabolites collectively exert health benefits, promoting proper gastrointestinal function and reduce the risk of several non-communicable diseases (Bouyahya et al., 2022).

Untargeted metabolomics is an analytical approach that allows data processing of several metabolites based on their overall abundance rather than their particular identification, and has proven to be useful in several matrices, particularly fermented foods, as microbial metabolism generates an extensive amount of compounds that are not commercially available for quantification (Gao et al., 2021). Hence, a metabolomic signature can be obtained for a further association of the identified

* Corresponding author.
E-mail address: hayde.vergara@uaq.mx (H.A. Vergara-Castañeda).
[1] These authors equally contributed to this manuscript.

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metabolites with several disease conditions (Daliri et al., 2021), with high sensitivity and reproducibility (Gao et al., 2022; Gertsman & Barshop, 2018).

Garambullo (*Myrtillocactus geometrizans*) is an endemic Mexican fruit rich in bioactive compounds such as dietary fiber and polyphenols (Guzmán-Maldonado et al., 2010; Ramírez-Rodríguez et al., 2020). Recently, Montiel-Sánchez et al. (2021) and Sánchez-Recillas et al. (2022) conducted *in vitro* digestion experiments of Garambullo to characterize the gastrointestinal fate of their bioactive compounds. As a result, many phenolic compounds, betalains, phytosterols, and tocopherols were identified from the different digested fractions, suggesting a nutraceutical potential for consumer health. Remarkably, our research group incorporated the *ex vivo* everted sac technique into the intestinal digestion model and found that several compounds are retained in the non-digestible fraction (Sánchez-Recillas et al., 2022). However, the resulting metabolites from the colonic fermentation of the non-digestible fraction, which can be valuable in exhibiting health-derived benefits, have not yet been characterized through analytical methods such as untargeted metabolomics or chromatography.

As garambullo is an underutilized fruit with limited explored beneficial health effects, metabolomic analysis of derived beneficial metabolites is of utmost importance to add value to the fruit, which is currently consumed only in some regions of Mexico. Therefore, this study aimed to conduct untargeted metabolomics and enrichment analysis of metabolites derived from the *in vitro* colonic fermentation of Garambullo. Additionally, SCFAs were identified and quantified due to their biological relevance in producing health benefits.

2. Materials and methods

2.1. Materials

Unless indicated otherwise, all reagents used in this research were acquired from J. T. Baker (Mexico City, Mexico) or Sigma-Aldrich (St. Louis, MO, USA).

2.2. Biological samples

The non-digestible fraction of garambullo (NDFG) used in this study was obtained from a previous *in vitro* gastrointestinal digestion experiment (Sánchez-Recillas et al., 2022). Fresh and ripe garambullo fruits collected in June 2020 in Garabatillo (Guanajuato, Mexico) were selected, cleaned, and disinfected (200 ppm sodium hypochlorite solution). Once sanitized, the samples (1 g) were chewed by 4 healthy participants 15 times for 15 s, expectorated the samples into 250 mL beakers, and rinsed their mouths with 5 mL distilled water for 60 s to remove any sample residue in their teeth. The participants previously consented to the study and did not present any buccal conditions.

For the gastric stage, all samples were collected into a single beaker and immediately pH-adjusted (2.0) using a 1 M solution. Then, a pepsin mixture made of 0.055 g pepsin (≥2500 units/mg, Sigma-Aldrich, St. Louis, MO, USA) with 0.94 mL of a 150 mM HCl solution, was added to the samples, and the blends were incubated (2 h, 37 °C) in a shaking water bath (80 cycles/min). For the intestinal stage, the gastric samples were pH-adjusted (7.2–7.4) and mixed with an intestinal solution made with 3 mg of pancreatin (8XUSP, Sigma-Aldrich), 2.6 mg of bile bovine (Sigma-Aldrich), and 5 mL of a CO_2-gasified Krebs-Ringer solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, and 2.5 mM $CaCl_2$, pH: 6.8). An everted excised rat gut sac was prepared from male Wistar (250–300 g) rats, acquired in the Instituto de Neurobiología from the National Autonomous University of Mexico (UNAM). The animals were maintained in proper conditions, fasted 12 h before the experiment, anesthetized with CO_2 and pentobarbital sodium (60 mg/kg body weight), euthanized by cervical dislocation, and the jejunum was excised, rinsed with CO_2-gasified Krebs-Ringer buffer, tied in one end, filled with the same buffer (1 mL), and tied in the other end. The sacs were placed in the previously prepared intestinal solution and incubated under continuous shaking in a water bath (37 °C, 2 h, 80 cycles/min). After incubation, the remaining sample outside the sac was collected and referred to as the non-digestible fraction from garambullo (NDFG). The *in vitro* digestion experiments were carried out in duplicate. Distilled water subjected to the same *in vitro* gastrointestinal digestion condition was used as a blank. All procedures were approved by the Human Internal Committee of the Universidad Autónoma de Querétaro (approval ID: 11926) and complied with regulations from the National Institutes of Health's Guide for Care and Use of Laboratory Animals.

2.3. In vitro colonic fermentation

The *in vitro* colonic fermentation of the NDFG was carried out according to Campos-Vega et al. (2009). Briefly, two healthy donors (1 woman and one man, 26 and 27 years old, respectively) provided fresh fecal samples as a source of human colonic microbiota. Donors were selected if they did not use antibiotics for at least three months before the sample donation, had no recent history of gastrointestinal diseases, and followed an omnivorous diet. Before the experiment, each fecal sample was previously gasified with an anaerobic gas mixture (10:10:80, H_2:CO_2:N_2) and maintained at 37 °C to ensure microbial viability. The fecal inoculum was prepared from a pool of fecal samples (three grams), diluted with 27 mL of 0.1 M phosphate buffer (pH: 7.0), and homogenized. The NDFG (100 mg) from the simulated intestinal digestion described above was mixed with the fecal inoculum and was placed in sterile centrifuge tubes (15 mL) containing sterile basal nutrient medium adjusted to pH 7 made from peptone water (2 g/L), yeast extract (2 g/L), K_2HPO_4 (0.04g/L), NaCl (0.1 g/L), KH_2PO_4 (0.1g/L), $MgSO_4$ 7H2O (0.01 g/L), $CaCl_2{\cdot}2H_2O$ (0.01 g/L), $NaHCO_3$ (0.01 g/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L), Tween-80 (2 mL/L), and porcine hematin (0.2 g diluted in 5 mL of 1 M NaOH). Afterward, the tubes were introduced in fermentation equipment with an anaerobic atmosphere of H_2:CO_2:N_2, in a 10:10:80 v/v/v ratio. To simulate human physiologic conditions, the temperature was set at 37 °C during all experiments, whereas the starting pH was set at 7.0. Once the fermentation time was completed (0, 6, and 24 h), the samples were immediately frozen at −70 °C until their analysis. On the day of analyses, samples were thawed and centrifugated (1500× *g*, 10 min, 4 °C), and the supernatants were collected into sterile centrifuge tubes (15 mL). Each supernatant was referred to as garambullo-fermented NDFG extract (FE-NDFG). Raffinose (100 mg) (R0514, Sigma Aldrich) and 100 μL of the vehicle of the fermented extract (a pool of saliva from participants subjected to the same *in vitro* fermentation conditions), both mixed with the sterile basal nutrient medium and the fecal inoculum, were used as positive and negative controls, respectively. The pH was measured after each fermentation time as a secondary indicator of the fermentation development (Supplementary Table S1). Samples subjected to *in vitro* fermentation (FNDG, control, and blank) were performed in duplicate in two independent experiments.

2.4. Untargeted metabolomics of FE-NDFG extracts

An untargeted metabolomics analysis for FE-NDFG extracts was conducted after subjecting the samples to a gas chromatography analysis coupled to mass spectrometry (GC–MS), with a previous solid-phase microextraction (SPME) procedure Zamora-Gasga et al. (2015), with slight modifications. Briefly, FE-NDFG samples were centrifuged at 5000× g at 4 °C for 10 min. One gram of supernatant was weighed into a 20 mL GC–MS vial, placed into a Gerstel MPS2 XL autosampler (Gerstel, Luzern, Switzerland), and subjected to SPME using a 2 cm-polydimethylsiloxane-divinylbenzene-carboxene fiber. The extraction conditions used were 45 °C, 5 min incubation, 250 rpm stirrer speed, and an extraction time of 2 h. The desorption temperature was set at 240 °C for 10 min. Two independent replicate extractions were performed for each

sample. The GC–MS analysis was carried out on an Agilent 7820A GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent 5977B mass detector. The sample was separated using a DB-FATWAX UI capillary column (30 mm, 0.25 mm, 0.25 μm of granule size). Helium was used as carrier gas at a 1 mL/min flow rate. The gas injector was maintained at 250 °C, the mass spectrometry source at 230 °C, and the quadrupole at 150 °C. The oven temperature started at 40 °C for 5 min and was programmed at 5 °C/min in a range from 40 °C to 200 °C; then 20 °C/min at 230 °C, and remained at 230 °C for 15 min. The internal MSD ChemStation software v. E02.00.493 was used for FE-NDFG-derived metabolites identification. Metabolites were tentatively identified and selected for further analysis based on their similarity with compounds with similar molecular masses (90 %), confirmed by NIST/EPA/NIH Mass Spectra Library v. 1.7 (NIST, USA). Representative chromatograms for each fermentation time are presented in Supplementary Fig. S1 and representative mass spectra from selected identified compounds can be found in Supplementary Figs. S2-S5.

2.5. Identification and quantification of SCFAs amounts and their fermentability index

The identification of SCFAs such as acetic, propionic, and butyric acids was confirmed by comparing their retention times with those of chemical standards (Sigma-Aldrich) from results obtained in GC–MS. Once identified, these SCFAs were quantified using calibration curves of their corresponding chemical standards of acids, and the results were expressed in mmol/L. Metabolites present in both blank samples before and after *in vitro* digestion and FE-NDFG samples at any fermentation time were removed for further analysis. Fermentability index (FI, %) was calculated as [SCFAs sample/SCFAs raffinose] × 100 % for each fermentation time. The molar mass ratio (MMR) was calculated as n SCFAs sample/n SCFAs total, where "n" represents the molar mass of either the sample or the total SCFAs, calculated for each SCFA (acetic, propionic, and butyric acids).

2.6. Metabolite set enrichment analysis

The complete set of metabolites identified from the FE-NDFG was subjected to a metabolite set enrichment analysis using the MetaboAnalyst 5.0 platform with the "disease signature in feces" parameter (Pang et al., 2021). Diseases were considered potentially associated with the determined subset of metabolites when their adjusted p-value was < 0.05. The rationale for using this parameter was that in the current study, we simulated a colonic human fermentation and wanted to explore potential major health effects of the NDFG fermentation. The results of this analysis, especially the relationship between specific metabolites and diseases, were interpreted based on a subsequent literature revision.

2.7. Statistical analysis

Before the statistical analysis, normality was checked by homoscedasticity verification and the Shapiro-Wilk parameter. Then, a one-way analysis of variance (ANOVA), followed by Tukey-Kramer's test as a *post hoc* analysis was performed to test significant differences in selected variables among different times of *in vitro* fermentation and the type of samples. A $p \leq 0.05$ was considered statistically significant in all tests. Principal component analysis (PCA) was performed for the identified metabolites using JMP v. 17.0 (SAS, Cary, NC, USA). Unless indicated, the results were expressed as the mean ± standard deviation of two independent experiments in triplicate. All analyses were performed using JMP software version 17.0 (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. pH evolution during in vitro colonic fermentation

No changes were observed for pH at the beginning (Supplementary Table S1). During the first 6 h, the control (100 mg raffinose) showed the lowest values, and the increase from 6 to 12 h was also presented in FE-NDFG. A similar performance was obtained from Andean berry (*Vaccinium meridionale* Swartz) under the same procedure (Agudelo et al., 2018). pH variations are a result of rapid consumption of substrates (e.g., polysaccharides and oligosaccharides) during the first 6 h (Ding et al., 2017), where pH < 5.7 selectively favors acetic acid-producing bacteria, while propionic-producing Bacteroidota (formerly: Bacteroidetes) species decrease (Oba et al., 2020). As long as pH increases (6.5–6.8), Bacillota (formerly Firmicutes) phylum also augments, resulting in higher butyric acid production (Firrman et al., 2022).

3.2. Untargeted GC–MS metabolomics and PCA analysis of FE-NDFG metabolites

GC–MS analysis of metabolites using SPME has proven to be a proper method to identify and quantify SCFAs and volatiles as the low intrinsic boiling point of SCFAs and their presence in aqueous samples such as FE-NDFG might interfere with the signals given by commonly used derivatization agents (Rohde et al., 2022). Moreover, the amount of identified SCFAs and volatiles through SPME-GC–MS (Luzardo-Ocampo et al., 2018) and using a prior derivatization method before GC–MS analysis have proven to be similar in samples coming from *in vitro* colonic fermentation (Luzardo-Ocampo et al., 2020).

The tentative metabolite annotations, their chemical classes, and their occurrence at the different times of *in vitro* colonic fermentation are shown in Table 1. Out of 50 compounds from FE-NDFG, their main classifications were: benzene and substituted derivatives (6 compounds), fatty acids and conjugates (7), phenols (2), fatty alcohols (4), organooxygen (4), esters (1), carboxylic acid and derivatives (3), alkanes (5), fatty aldehydes (4), indoles and derivatives (1), fatty esters (1), organic disulfides and trisulfides (2), monoterpenoids (1), and 11 additional compounds that were not classified into any of this groups.

In the present study, indole and *p*-cresol values increased after 6 and 24 h (Fig. 1). Indole has not been previously reported for garambullo fermentation and represents an outstanding molecule derived from the bacterial tryptophanase-mediated metabolism of tryptophan of *Clostridium* and *Lactobacillus* species (Tennoune et al., 2022). Indole has been linked to controversial roles in the gut-brain axis: while it has been found to increase in children with autism spectrum disorder (ASD), can also stimulate enterochromaffin cells to release serotonin (Ahmed et al., 2022).

Tentatively identification of 2-hydroxy,andelic acid during all fermentation times could indicate gut microbiota metabolism of polyphenols, and some derived metabolites (e.g., 3′,4′-dihydroxyphenylacetic, 4′-hydroxyphenylacetic, and 3′-methoxy-4′-hydroxyphenylacetic acids) have been reported in human urine samples after a single consumption (300 g) of raspberries (González-Barrio et al., 2011) or after 4 weeks-administration of grape juice and red wine (van Dorsten et al., 2010). Benzoic acid and its derivatives were also present after garambullo fermentation, agreeing with reported concentrations of these compounds in strawberry fermentation *in vitro* (Diotallevi et al., 2022).

The principal components analysis (PCA) results are shown in Fig. 2. Three components explain more than 80 % of the total variation (Fig. 2A). Fig. 2B and C show the scatter plot and variable factor map of PC1 and PC2. As shown in the analysis, most metabolites were produced in 6 and 24 h (Fig. 2B and C), where a high relationship is shown between three distinctive groups of metabolites: Group 1: [(E)-2-nonenal], 13 (4,4-dipropylheptane), 20 (octamethyl-cyclotetrasiloxane), 30 (heptyl-ester-formic acid), 23 (2,4,6-trimethyl-decane), and 39 (octanal); Group 2: 4 (1-nonanol), 7 (2-ethylhexan-1-ol), 17 [1,3-bis(1,1-

Table 1
Occurrence of metabolites after different *in vitro* fermentation times.

PubChem CID	Metabolite	Time occurrence/ fermentation (h)		
		0	6	24
Benzene and substituted derivatives				
136,810	1,3-di-*tert*-butylbenzene	+	+	+
7311	2,4-di-*tert*-butylphenol	+	+	+
240	Benzaldehyde	+	+	
7501	Styrene			+
7150	Methyl ester benzoic acid			+
Fatty acids and conjugates				
6490	3-hydroxy-2,2,4-trimethylpentyl isobutyrate	+	+	+
1032	Propionic acid		+	+
5,364,506	Methyl ester-13-octadecenoic acid		+	+
264	Butyric acid		+	+
7755	3-methylpentanoic acid			+
8181	Methyl hexadecanoic acid			+
8892	Hexanoic acid			+
Phenols				
2879	*p*-cresol	+	+	+
996	Phenol	+	+	+
Fatty alcohols				
8914	1-Nonanol	+	+	+
8174	1-decanol		+	+
7720	2-ethylhexanol		+	+
8193	1-Dodecanol	+		
Organooxygen compounds				
8194	Dodecanal	+	+	+
6276	1-Pentanol			+
7410	Acetophenone			+
Esters				
622,433	di-TMS ethyl ester-2-hydroxymandelic acid	+	+	+
Carboxylic acids and derivatives				
176	Acetic acid		+	+
23,284	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	+		
8169	Heptyl ester formic acid		+	
Alkanes				
537,327	2,6,8-trimethyldecane	+	+	+
11,233	3,3-dimethylhexane		+	+
12,389	Tetradecane		+	+
8182	Dodecane		+	
12,398	Heptadecane	+		
Fatty aldehydes				
454	Octanal	+	+	
8175	Decanal			+
5,283,335	2-Nonenal	+		
31,289	Nonanal		+	
Fatty alcohol esters				
8176	Octyl formate	+	+	+
Indoles and derivatives				
798	Indole	+	+	+
Fatty esters				
10,882	Ethyl pentanoate			+
Organic disulfides				
12,232	Dimethyl disulfide		+	+
Organic trisulfides				
19,310	Dimethyl trisulfide		+	
Monoterpenoids				
637,531	Isobornyl acetate	+		
Unknown				
11,169	Octamethyl-cyclotetrasiloxane	+	+	+
7346	Isobutyric anhydride		+	+
10,914	hexamethyl-cyclotrisiloxane,			+
5,375,215	4-(2,5,6,6-tetramethyl-1-cyclohexen-1-yl)-3-buten-2-one			+
519,401	4,4-dipropylheptane		+	
609,653	4-methyl-2,4,6-tri-t-butylcyclohexa-2,5-dienone	+		
6937	6-*tert*-butyl-m-cresol			+
19,773	2,6,10-trimethyl-dodecane		+	
9,602,988	Methoxy-phenyl-oxime			+
528,938	TMS-4-*tert*-octyl-phenol	+		

Metabolites are shown in descending order according to the number of times they occur (i.e., from 3 to 1) within each class.

dimethylethyl)-benzene], 31 (octyl-ester-formic-acid), 34 (3,3-dimethyl-hexane), and 50 (tetradecane); and Group 3: 5 (1-pentanol), 21 (hexamethyl-cyclotetrasiloxane), 22 (decanal), 28 (dodecane), 33 (methyl-ester-hexadecanoic acid), 35 (hexanoic acid), 42 (3-metyl-pentanoic acid), and 49 (styrene).

Although untargeted metabolomics was the approach used in this article, which is suitable for fermented food matrices exhibiting a complex range of metabolites (Gao et al., 2021), these results should be validated through a directed study using high-purity-grade chemical standards. Besides, GC–MS analyses can be integrated with LC-MS for untargeted metabolomics profiling to increase the number of metabolites detected without losing sensitivity and considering a wide range of physicochemical properties, stationary phases, more metabolites-phase interactions, and polarities (Zeki et al., 2020).

3.3. SCFAs quantification in FE-NDFG and their fermentability index (%)

SCFAs (acetic, propionic, and butyric acids) were identified at 6 and 24 h in FE-NDFG, with butyric acid the highest relative abundance (Fig. 1). High dietary fiber contents in garambullo (about 35 g/kg fresh weight) have been linked to high SCFAs production (Guzmán-Maldonado et al., 2010). The same SCFAs have been reported in mulberry (*Morus alba*) and blackberry (*Rubus ursinus* × *Rubus idaeus*) after simulated colonic fermentation, associated with increased Bacteroidota and deceased Bacillota levels (Chen et al., 2016; Dou et al., 2022).

Table 2 shows the SCFAs concentrations for the FE-NDFG and the positive control (raffinose, 100 mg) at different *in vitro* fermentation times. FE-NDFG displayed higher levels of acetic acid but lower of propionic and butyric acids compared to the positive control, a trend shown for the amount and MMR. From 0 to 24 h, acetic acid decreased, butyric acid increased, and propionic acid remained the same. The positive control displayed a similar trend, except for propionic acid. These results partially agreed with the increase in butyric acid levels between 6 and 24 h of fermentation of other berries, such as blueberries (*Vaccinium ashei* Radei) (Wu et al., 2020) and Syrah grape products (Aura, 2008) for different *in vitro* colonic procedures. Typically, SCFAs production during the anaerobic metabolism of fiber by gut microbiota results in high proportions of acetic (60 %), followed by propionic (25 %) and butyric acids (15 %) (Wang et al., 2019), but it is important to

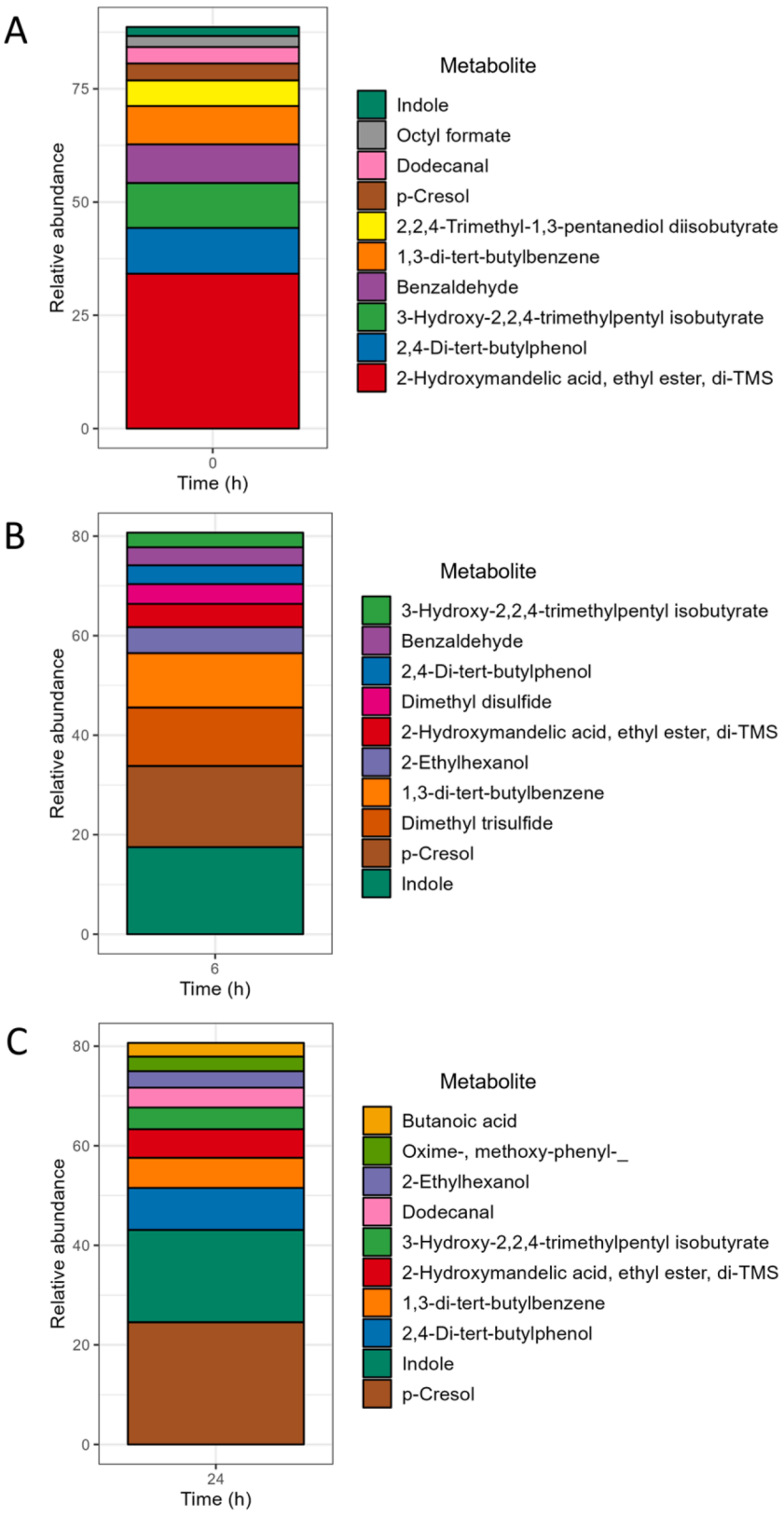

Fig. 1. Relative abundance of the top 10 metabolites produced in each time-point of *in vitro* fermentation. Metabolites were identified in the fermented extract of the non-digestible fraction of garambullo (FE-NDFG) at **(A)** 0 h, **(B)** 6 h, and **(C)** 24 h. The selected metabolites accounted for up to 80 % of the identified metabolites in FE-NDFG.

A

Principal Component (PC)	Eigenvalue	Participation (%)	Cummulative participation (%)
1	19.64	39.28	39.28
2	17.63	35.25	74.53
3	7.39	14.78	89.31
4	2.68	5.37	94.68
5	1.32	2.63	97.31
6	0.90	1.80	99.11
7	0.44	0.89	100

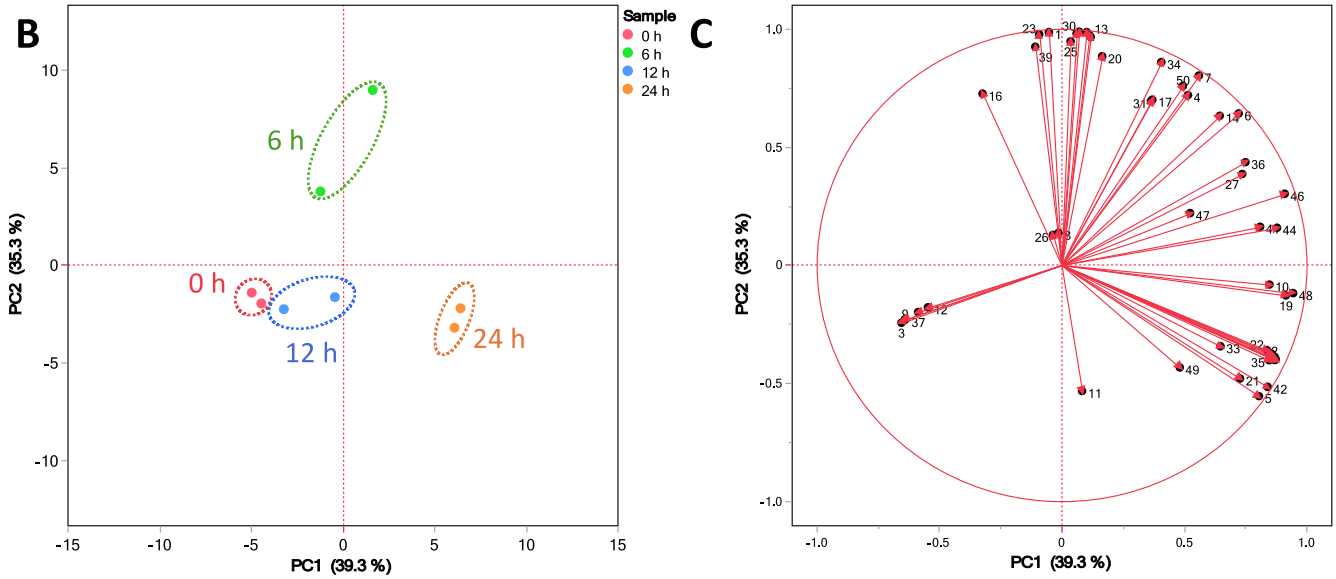

Fig. 2. Principal components analysis (PCA) of the quantified variables for the samples of each fermentation time. **(A)** Eigenvalues and their percentual participation within the total variation; **(B)** Scatter plot; **(C)** Factor map. The numbers in the variables (1–50) indicate the compounds identified by GC–MS: **1:** (E)-2-nonenal; **2:** 1-decanol; **3:** 1-dodecanol; **4:** 1-nonanol; **5:** 1-pentanol; **6:** methyl-ester-13-octadecenoic acid; **7:** 2-ethylhexan-1-ol; **8:** di-TMS-ethyl-ester-2-hydroxymandelic acid; **9:** 2,2,4-trimethyl-1,3-pentanediol-diisobutyrate; **10:** 2,4-di-*tert*-butylphenol; **11:** 2,4,6-tris(1,1-dimethylethyl)-4-methylcyclohexa-2,5-dien-1-one; **12:** TMS-derivative 4-*tert*-octylphenol; **13:** 4,4-dipropylheptane; **14:** acetic acid; **15:** acetophenone; **16:** benzaldehyde; **17:** 1,3-bis(1,1-dimethylethyl)-benzene; **18:** methyl-ester-benzoic acid; **19:** butanoic acid; **20:** octamethyl-cyclotetrasiloxane; **21:** hexamethyl-cyclotetrasiloxane; **22:** decanal; **23:** 2,4,6-trimethyl-decane; **24:** 3,4,6-trimethyl-decane; **25:** dimethyl-trisulfide; **26:** dimethyl-disulfide; **27:** dodecanal; **28:** dodecane; **29:** 2,6,10-trimethyl-dodecane; **30:** heptyl-ester-formic acid; **31:** octyl-ester-formic acid; **32:** heptadecane; **33:** methyl-ester-hexadecanoic acid; **34:** 3,3-dimethyl-hexane; **35:** hexanoic acid; **36:** indole; **37:** isobornyl-acetate; **38:** nonanal; **39:** octanal; **40:** methoxy-phenyl-oxime; **41:** *p*-cresol; **42:** 3-methyl-pentanoic acid; **43:** ethyl-ester-pentanoic acid; **44:** phenol; **45:** 2-(1,1-dimethylethyl)-5-methyl-phenol; **46:** propanoic acid; **47:** 2-methyl-3-hydroxy-2,2,4-trimethylpentyl-ester-propanoic acid; **48:** 2-methyl-propanoic acid anhydride; **49:** styrene; **50:** tetradecane. **PC:** principal component.

indicate that additional metabolites are produced after the fermentation of polysaccharides. However, SCFA proportions may change depending on the chemical composition of the fermented dietary fiber (Tan et al., 2014). Raspberries (*Rubus idaeus*) and blackcurrants (*Ribes nigrum*) administration produces higher acetic but lower butyric and propionic concentrations *in vivo* (Jakobsdottir et al., 2013). Changes in microbial diversity could be linked to butyric acid occurrence in the FE-NDFG at 6 h, and its rise after 24 h, since 24 h-dragon fruit (*Hylocereus undatus*) fermentation was attributed to Bifidobacterium and Lactobacillus population increase (Dasaesamoh et al., 2016).

Butyric acid displayed the highest molar ratio after 24 h of fermentation in both FE-NDFG and the control samples (Table 2). Although there are no previous reports in berries, pistachio nut (*Pistacia vera*) fermentation displayed increased butyric acid MMR at 4, 6, and 12 h (Dufoo-Hurtado et al., 2021). Our reported FI values at 24 h are similar to those reported by Zamora-Gasga et al. (2015) in agave (*Agave tequilana* Weber)-derived foods, where FI was close to 100 % after 12 and 24 h, suggesting that, as garambullo, a proper gut microbiota accessibility to fiber substrates (Hamaker & Tuncil, 2014).

Table 2
SCFAs quantification and fermentability index estimations during the colonic *in vitro* fermentation of the FE-NDFG.

Sample	Time (h)	Acetic acid		Propionic acid		Butyric acid		FI (%)
		Quantity (mM)	MMR	Quantity (mM)	MMR	Quantity (mM)	MMR	
FE-NDFG (100 μg)	0	n. d.	–	n. d.	–	n. d.	–	–
	6	3.51±0.12 [a]	29.00±2.05 [a]	4.53±0.69[c]	37.25±1.83[b]	4.08±0.45[c]	33.65±0.21[b]	50.98±3.31[b]
	24	2.73±0.15[b]	14.41±0.65[b]	4.87±1.11[c]	25.41±3.32[c]	11.40±0.64 [a]	60.18±2.66 [a]	95.15±6.54 [a]
Control (100 mg raffinose)	0	n. d.	–	n. d.	–	n. d.		–
	6	3.29±0.31 [a]	13.84±0.76[b]	13.20±0.35 [a]	55.64±0.71 [a]	7.24±0.27[b]	30.51±0.06[b]	–
	24	1.30±0.08[c]	6.54±0.70[c]	6.12±1.05[b]	30.40±0.14[c]	12.70±2.23 [a]	63.06±0.56 [a]	–

Data are the mean ± standard deviation of three independent experiments by triplicate. Different letters within the same column indicate significant differences ($p < 0.05$) by Tukey-Kramer's test. **n. d.:** not detected; **FE-NDFG:** Fermented non-digestible fraction of garambullo extract; **FI:** fermentability index (%); **MMR:** Molar mass ratio. No SCFAs were detected for the fermented saliva of the participants (negative control).

3.4. Enrichment analysis of FE-NDFG

F ig. 4 shows a list of the top 25 diseases presumably associated with the occurrence of 36 metabolites recognized by MetaboAnalyst 5.0 software, but only 17 reached statistical significance ($p < 0.05$). Although the conducted analysis did not allow us to determine if the resulting associations are positive or negative, results should be cautiously interpreted. Several studies have associated the consumption of berries with improvements in inflammatory diseases such as Crohn's disease and ulcerative colitis (Kang et al., 2017) or the improvement of neurological disorders (Imran et al., 2021) due to their polyphenolic composition, dietary fiber content, and the potential to modulate gut microbiota, among other mechanisms. Compounds such as 2,4-di-ter-butylphenol are recognized for their antioxidant and chemopreventive or chemoprotective activities in colorectal cancer (Song & Chan, 2018).

SCFAs are well-known anti-inflammatory compounds with significant roles in the colonic metabolism, preventing a wide range of inflammatory-related diseases, together with the participation of

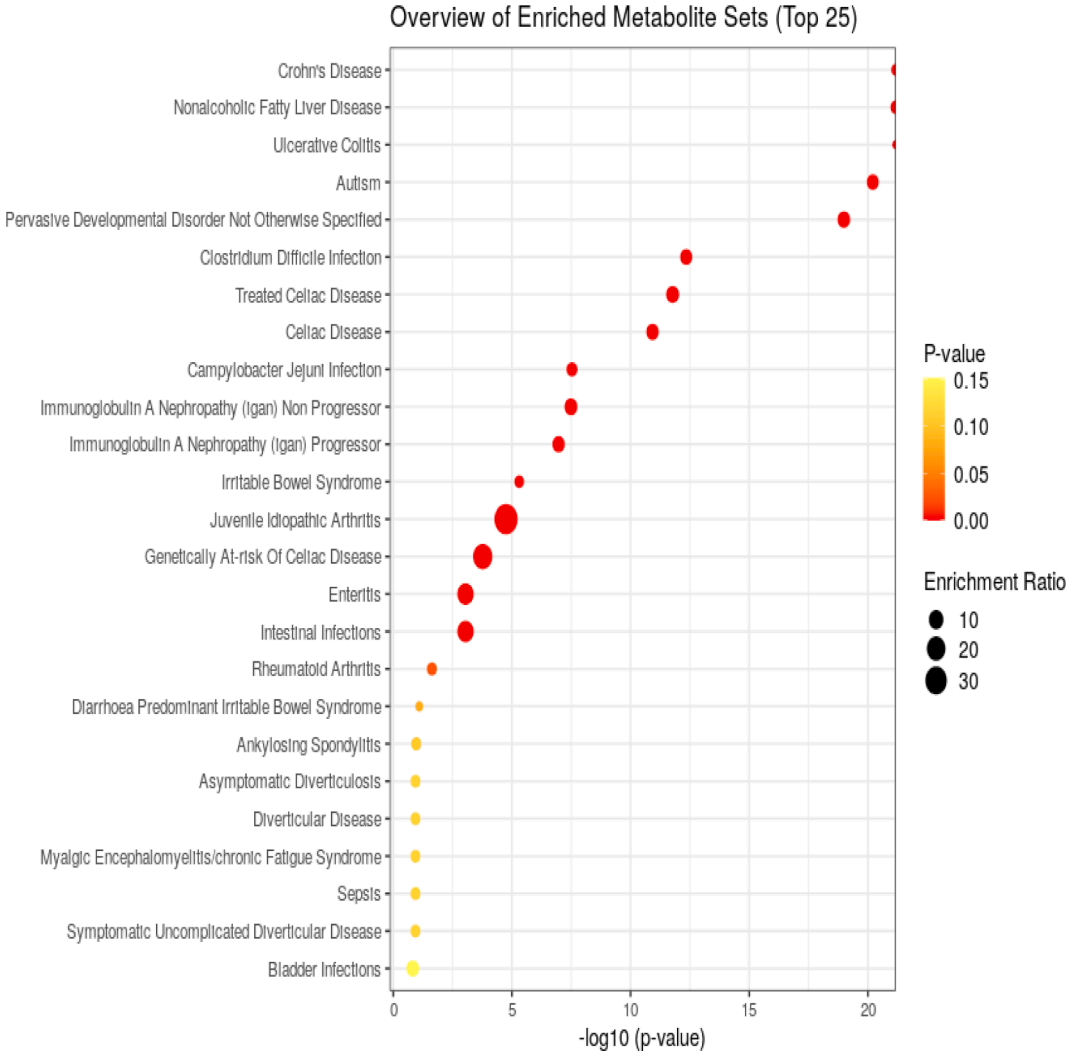

Fig. 3. Dot plot showing the top 25 diseases modulated by the occurrence of a set of metabolites produced between 0 and 24-h of *in vitro* fermentation of the FE-NDFG.

butyrate-producing species like *Faecalibacterium prausnitzii*, restoration of the mucosal layer, and optimization of energy metabolism of colonocytes (Machiels et al., 2014). SCFAs also activate the hydroxycarboxylic acid receptors GPR109A and GPR43 receptors and modulate inflammation, promoting colonic epithelial repair, induce expression of the interleukin 10 (IL-10) anti-inflammatory cytokine, block the Toll-like receptors TLR2-TLR1 proinflammatory signaling pathway (Parada Venegas et al., 2019) and butyrate inhibits histone deacetylase (HDAC), thereby reducing the severity of experimental colitis in mice and inhibiting proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin-6 (IL-6) (Glauben et al., 2006; Luzardo-Ocampo et al., 2020).

Rheumatoid arthritis (RA), one of the highlighted diseases in Fig. 3, has been recently linked with gut dysbiosis and SCFAs production, as these components reduce pro-arthritogenic biomarkers such as monocyte chemoattractant protein-1 (MCP-1/CCL2), IL-18, and IL-33 (Ferro et al., 2021). In addition, propionate supplementation restored the lactobacilli (*L. acidophilus, L. intestinalis,* and *L. amylovorus*) profile in patients with RA, suggesting its potential use as a supportive treatment in this pathology (Fan et al., 2021).

Interestingly, the presence of metabolites derived from the anaerobic fermentation of garambullo has been associated with disorders unrelated to the gut, such as ASD, supporting their role as mediators in the gut-brain axis. It is well known that dysbiosis at a young age can affect an individual's health later in life, a condition that might be exacerbated when a previous health condition exists in the mother, such as non-communicable diseases, causing a reduced microbiota composition, immune and endocrine factors, and nutrients, in the maternal milk (Luzardo-Ocampo et al., 2023). Children with ASD exhibit dysbiosis with lower bacterial diversity, and gastrointestinal symptoms are four times more common than the standard population (Srikantha & Mohajeri, 2019). Bacterial diversity may be related to the severity of gastrointestinal symptoms in this disorder, leading to lower SCFAs production in children with autism (Adams et al., 2011). Inflammation and immune system activation in the brain are characteristic of patients with ASD, leading to synaptic changes. SCFAs produced in the gut are transported to the central nervous system, cross the blood–brain barrier, and are taken up by glia and neurons involved in early brain development and modulation of neurotransmitter production. Although inconclusive, current evidence suggests that increasing SFCAs concentrations helps reduce the pathophysiology of ASD (MacFabe, 2012; Srikantha & Mohajeri, 2019). This background and the association of garambullo metabolites with ASD suggest that consumption of the fruit may, to some extent, promote neurochemistry of the disorder and improve gut health for ASD patients.

4. Conclusion

The present study shows, for the first time, a series of products formed during colonic metabolism of the non-digestible fraction of garambullo in an *in vitro* model. The identified metabolome from the FE-NDFG, which included SCFAs, would exert significant human health effects, including high antioxidant capacity and the predictive modulation of chronic diseases. Future research, however, should be conducted to confirm these health effects in experimental models and humans. Moreover, a targeted metabolomics approach would be suitable to quantify metabolites from FE-NDFG, giving an accurate approach to their quantitative levels in association with health-derived benefits.

CRediT authorship contribution statement

Edelmira Sánchez-Recillas: Writing – original draft, Formal analysis, Data curation. **Enrique Almanza-Aguilera:** Writing – original draft, Formal analysis, Data curation. **Elisa Dufoo-Hurtado:** Writing – review & editing, Validation, Methodology. **Ivan Luzardo-Ocampo:** Writing – review & editing, Validation, Methodology, Data curation. **Rocio Campos-Vega:** Writing – review & editing, Validation, Methodology. **Haydé Azeneth Vergara-Castañeda:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2024.106063.

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