Highlights

- Granulometric fractionation (GF) and GF + micronization (GFM) reduce particle size
- GF and GFM increase olive pomace (OP) phenolic compounds (PC) that reach colon
- Tyrosol (tyr) and hydroxy-tyr (OH-tyr) were the major PC in colonic fermentation
- GFM released more tyr and OH-tyr from OP during colonic fermentation
- Only GFM-modified OP samples had oleuropein in the colonic phase of digestion

Effect of micronization on olive pomace biotransformation in the static model of colonic fermentation

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Abbreviations: Abbreviations: GF, granulometrically fractionated OP; GFM, granulometrically fractionated and micronized OP; IN: fraction that remains inside the dialysis tube and corresponds to the digesta that will reach the colon; IN-GF, IN-OP from

granulometrically fractionated OP; IN-GFM, IN-OP from granulometrically fractionated and micronized OP; IN-NF, IN-OP from non-fractionated OP; IN-OP: olive pomace fraction that was not bioaccessible after gastrointestinal digestion; NF, non-fractionated OP; OP, olive pomace; OUT, fraction that crosses the dialysis membrane and represents the bioaccessible fraction; UHPLC-MS/MS, ultra high-performance liquid chromatograph coupled to a triple-quadrupole mass spectrometer.

Abstract

The effect of granulometric fractionation and micronization of olive pomace (OP) on the biotransformation of phenolic compounds by intestinal microbiota was investigated *in vitro*. Three types of powdered OP samples were incubated with human feces to simulate colonic fermentation, after a sequential static digestion: non-fractionated OP (NF), granulometrically fractionated OP (GF) and granulometrically fractionated and micronized OP (GFM). GF and GFM favored the release of hydroxytyrosol, oleuropein aglycone, apigenin and phenolic acid metabolites in the first hours of colonic fermentation compared to NF (up to 41-fold higher). GFM caused higher release of hydroxytyrosol than GF. GFM was the only sample to release tyrosol and sustained tyrosol levels up to 24 h of fermentation. Micronization associated with granulometric fractionation was more efficient than granulometric fractionation alone to increase the release of phenolic compounds from the OP matrix during simulated colonic fermentation and can be further studied for nutraceutical purposes.

Keywords: phenolic compounds, *in vitro* digestion, gut phenolic metabolites, superfine grinding

1. Introduction

The extraction of olive oil generates olive pomace (OP) that contains pulp, peel and stones and amounts to 80% of the processed fruits. Due to its chemical characteristics and the great volume produced, OP stands out for its potential to pollute the environment. OP is rich in vitamins, residual lipids, and dietary fiber, but the high content of insoluble fiber such as lignin prevents its direct use in food (Dermeche et al., 2013; Speroni et al., 2019). Additionally, this residue contains significant amounts of phenolic compounds, about 95-96% of the phenolic compounds present in the olive remain in the residue after the extraction of olive oil (Rodríguez-López et al., 2020). Generally, the residue is destined for energy production or as a soil fertilizer, however, studies show that OP can be an interesting source of bioactive compounds for food application (Rocchetti et al., 2020).

Processes that modify the fibrous matrix and increase its functionality can be useful for adding value to agri-food by-products as demonstrated by Speroni et al. (2020) for the micronization of OP. This ultrafine milling method reduces particle size to less than 100 µm and improves food dispersibility. Furthermore, the increased solubility of nutritive components would likely improve their intestinal absorption (Chen et al., 2018; Shu et al., 2019). Our research group has recently demonstrated that short time micronization of OP (less than 30 min) is efficient to increase the extractability of phenolic compounds and antioxidant capacity (Speroni et al., 2019). In addition, long-time micronization of OP (5 h) has been shown to be efficient for reducing lignin content and increasing soluble fiber content (Speroni et al., 2020). Long-time micronization of OP has been also shown to increase the release of phenolic compounds in the salivary and gastric phases during simulated *in vitro* static digestion, resulting in increased antioxidant capacity (Speroni et al., 2021). Moreover, long-time micronization of OP has been shown

to increase the intestinal bioaccessibility of hydroxytyrosol, decarboxymethyl oleuropein aglycone, oleuropein, luteolin, and apigenin in a static digestion model. Thus, micronization can be potentially used for the transformation and reuse of food by-products by increasing the bioaccessibility of bioactive compounds, the soluble dietary fiber content and powder functional properties (Zhao et al., 2018; Li et al., 2022). An avenue of possibilities will be opened for industrial application of micronization, which is already used (through ball milling) in the food and pharmaceutical industries to improve the dispersibility of different powder ingredients/components (Shu et al., 2019; Dhiman & Prabhakar, 2021).

Phenolic compounds of OP are mostly composed by phenolic alcohols (hydroxytyrosol and tyrosol), secoiridoids (oleuropein and its derivatives), flavonoids (luteolin), and phenolic acids (caffeic acid and *p*-coumaric acid) (Malapert et al., 2018). These compounds have been described to reduce the risk of cardiovascular diseases and some types of cancer, by exerting an antioxidant effect, neutralizing reactive species and oxidative reactions (Conterno et al., 2019; Covas et al., 2006; Serreli & Deiana, 2018). However, a small fraction of phenolic compounds is absorbed intact up to the small intestine and can exert their systemic effects in the native form (Augusti et al., 2021). According to Rocchetti et al. (2020), the bioaccessibility of phenolic acids and secoiridoids in olive oil was low during digestion (10-25%), whereas phenolic alcohols, mainly hydroxytyrosol, was the only one that showed higher bioaccessibility (> 60%). Compounds with a complex structure resist to the acidic conditions of stomach and will be found at significant amounts in the non-absorbable fraction in the small intestine (Mosele et al., 2015). After reaching the colon, they become available for metabolism by intestinal microbiota (Rodríguez-López et al., 2020; Gil-Sánchez et al., 2018). The products formed during the biotransformation of phenolic compounds have low

molecular weight, resulting in greater availability for absorption and in some cases greater biological activity than their parent compounds (Augusti et al., 2021). In addition, phenolic compounds that reach the large intestine can reshape the intestinal microbiota exerting a prebiotic-like effect (Augusti et al., 2021; Ribeiro et al., 2021).

Studies on the biotransformation that micronized OP suffers during digestion and colonic fermentation can provide information about the bioaccessibility of phenolic compounds in the small intestine and the microbial-derived phenolic metabolites that are likely implicated in the bioactive properties of dietary phenolic compounds. The hypothesis of the study is that micronization of OP enhances the amount of phenolic compounds released during colonic fermentation by increasing the solubility of the food matrix and their interaction with gut microbiota. Thus, the objective of this study was to evaluate the effect of granulometric fractionation followed by micronization of OP on the biotransformation of phenolic compounds in an *in vitro* static model of colonic fermentation with human feces.

2. Materials and methods

2.1. Olive pomace

The OP from *Olea europaea* cv. 'Arbequina' was collected immediately after olive oil extraction by the two-phase continuous extraction process, in an extra virgin olive oil industry located in the city of Formigueiro, Rio Grande do Sul state, Brazil (29° 59′ 01″ S; 53° 21′ 50 ″ W).

2.2. Granulometric fractionation and micronization of olive pomace

The crude sample of OP was submitted to granulometric fractionation at 2-mm sieve, as described below, to obtain a fraction that was named granulometrically

fractionated OP (GF). GF was submitted to micronization to obtain a fraction that was named granulometrically fractionated and micronized OP (GFM), as described below. OP without physical modification was lyophilized in a freeze dryer (LS 3000, Terroni Equipamentos Científicos, SP, Brazil), crushed in a knife mill (MA 630, Marconi®, SP, Brazil) and identified as non-fractionated OP (NF).

Crude OP was subjected to granulometric fractionation with a 2-mm sieve as described by Speroni et al. (2019), and the fraction with particle size < 2-mm was centrifuged (1774 *x* g for 10 min), the sediment was collected, lyophilized in a freeze dryer (LS 3000, Terroni Equipamentos Científicos, SP, Brazil), crushed in a knife mill (MA 630, Marconi®, SP, Brazil) and degreased with *n*-hexane, according to Goulart et al. (2013). This fraction was identified as GF.

Thereafter, GF was micronized in a planetary ball mill (PM 100, Retsch Co., Haan, Germany), using a 250 mL container with six stainless steel balls (30 mm diameter each). The milling time was optimized by Speroni et al. (2020), with 15 g of sample ground at 300 r min^{-1} for 5 h, with a 2 min pause every 10 min of grinding. After micronization, the sample was identified as GFM.

2.3. Particle size analysis

Particle size was assessed in a Laser Diffraction Particle Size Analyzer (model LS 13320, Beckman Coulter, FL, USA) following the manufacturer instructions. Triplicate samples were added to the equipment until optimal obscuration was achieved in the software. Thereafter, the powders were dispersed in an aqueous medium and submitted to an ultrasound treatment for 1 min inside the equipment before analysis.

2.4. *In vitro* simulation of gastrointestinal digestion

Samples of NF, GF and GFM (proximate composition shown in Table S1, supplementary material) were submitted to a simulated gastrointestinal digestion based on the standardized INFOGEST 2.0 *in vitro* digestion method (Brodkorb et al., 2019). The samples are subjected to digestion under conditions that sequentially mimic the oral, gastric and intestinal stages (Fig. 1) with amounts of electrolytes, enzymes, bile, dilution, pH and digestion time based on the physiology of the gastrointestinal tract. The conditions were based on the study of Brodkorb et al. (2019) with the following modifications: the oral phase did not include amylase because OP is not a starchy food, lipase was not included in the gastric phase because OP was deffated before micronization, and the bioaccessible fraction was separated by dialysis during the intestinal phase.

For the oral phase, 4 g of each sample (NF, GF and GFM) were separately incubated (without amylase) with salivary fluid pH 7.0 at 37 °C for 2 min on a rotary shaker (AGROT-BI, IONLAB, PR, Brazil). Subsequently, for the gastric phase, the pH was corrected to 3, by adding gastric fluid and pepsin (2000 U/mL; P7000, Sigma-Aldrich, MO, USA) in a final volume of 20 mL, followed by incubation at 37 °C in the rotary shaker for 2 h. In the intestinal phase, the pH was adjusted to 7, the intestinal fluid, bile salts with sodium deoxycholate and sodium cholate (10 mM; C6750 and C1254, Sigma Aldrich, MO, USA) and pancreatin (100 U/mL; P7545, Sigma Aldrich, MO, USA) were added in a final volume of 40 mL. This solution was transferred to a dialysis membrane (12400 Da; D0530, Sigma Aldrich, MO, USA) that was placed in a beaker containing 200 mL of phosphate buffer (24.9 mM, pH 7.4) and intestinal incubation was performed for 2 h at 37 °C with sporadic shaking. The membrane was previously activated as described by Dutra et al. (2017). The fraction that remained inside the dialysis membrane after intestinal digestion (IN-NF, IN-GF and IN-GFM) represents the fraction that is not available for absorption, and will reach the colon, whereas the fraction that was

able to cross the dialysis membrane (OUT-NF, OUT-GF and OUT-GFM) represents the bioaccessible fraction. The OUT fraction was used for another study, whereas the IN fractions were lyophilized and used for colonic fermentation with human feces.

2.5 Static model of colonic fermentation *in vitro*

The protocol was approved by the Ethics Committee of the Federal University of Santa Maria (CAAE 20528819.4.0000.5346). Eighteen healthy volunteers (18-55 years, 2 males, 16 females) were included as fecal donors in this study. Exclusion criteria were chronic, infectious, or gastrointestinal diseases or those who had received antibiotic treatment in the last 6 months. Twenty stool samples were collected and used to perform five independent fermentation assays. Each assay was performed with a pool of feces from four donors. Each donor made a single fecal donation, except for two donors that donated twice. Feces were used within 2 h after defecation and kept at room temperature under anaerobic conditions (N_2) until the time of the experiment.

Colonic fermentation was mimicked using the nutrient base medium described by Ribeiro et al. (2021). Equal amounts of fecal samples from 4 donors were pooled and homogenized in the nutrient base medium at a ratio of 0.5:10 m/v. The fecal suspension was filtered through sterile gauze under anaerobic conditions (N_2) and then used for the fermentation assay. The fecal inoculum buffer was purged with N_2 for 30 s in each fermentation tube to remove O_2.

Samples (0.25 g of IN-NF, IN-GF and IN-GFM) were incubated with 25 mL of fecal inoculum buffer at 37 °C for 0, 2, 8, 24 and 48 h in autoclaved Falcon tubes closed with rubber stoppers that allow excess gas to escape (Ribeiro et al., 2021). Separate flasks were used for each fermentation time and the pH was determined at the end of each incubation time. During the adjustment of the experimental conditions for colonic

fermentation, the fecal inoculum was incubated with 0.25 g inulin as a positive control to prove the viability of fecal inoculum in the fermentation assay. Thereafter, during the experimental stage, two control fermentation runs were carried out in parallel with samples. Control 1 was then composed of 0.25 g of IN-NF, IN-GF and IN-GFM samples that were incubated with buffer solution, in the absence of feces and was used to evaluate the chemical degradation of phenolic compounds, independent of the microbiota. Control 2 was the fecal inoculum buffer without the inclusion of IN samples and was used to account for the phenolic compounds that were already found in the feces. The phenolic content found in control 2 was always subtracted from the phenolic content found in the fermentation assays of IN-NF, IN-GF and IN-GFM. After the end of fermentation, samples were centrifuged at 1,400 *x* g for 10 min and the supernatant was immediately frozen under liquid N_2 and stored at -20 °C.

2.6 pH analysis

The pH value was determined immediately after finishing colonic fermentation assays, using a digital potentiometer (P1000, PHOX Suprimentos Científicos, Paraná, Brazil).

2.7 Extraction of phenolic compounds from colonic fermentation assay

Aliquots of supernatant samples obtained after fermentation (6 mL) were extracted using an acidified acetone solution (0.35% formic acid, v/v; 7 mL) according to Quatrin et al. (2020). After vortex mixing for 1 min, samples were centrifuged at 1,100 *x* g for 10 min to collect the supernatant. The organic solvent was removed in a rotary evaporator (38 ± 2 °C) and the extract was filtered on a 0.22-µm polytetrafluoroethylene filter.

225

226 **2.8 Identification and quantification of phenolic compounds**

227 Phenolic compounds were identified and quantified using an ultra high-
228 performance liquid chromatograph (Nexera XR, Shimadzu, Kioto, Japan) coupled to a
229 triple-quadrupole mass spectrometer (UHPLC-MS/MS) (LCMS-8045, Shimadzu, Kioto,
230 Japan) equipped with a binary pump, degasser, communication module, oven column and
231 automatic injector. Samples were injected (10 μL) onto a Zorbax RRHD Eclipse XDB-
232 C18 analytical column (4.6 mm x 150 mm, 1.8 μm particle size; Agilent Technologies,
233 CA, USA) at 35 °C. The mobile phase was HPLC grade water obtained from a Direct-
234 Q® 3 UV equipment (Merck Millipore, Darmstadt, Germany) with 0.5% acetic acid
235 (eluent A) and acetonitrile (eluent B) at 0.2 mL min^{-1}. Chromatographic separation was
236 carried out in a reverse-phase mode according to the following multistep elution gradient,
237 adapted from Abu-Reidah, Arráez-Román, Al-Nuri, Warad, & Segura-Carretero, (2019):
238 0% B from 0 to 7 min; 10% B from 7 to 11 min; 14% B from 11 to 17 min; 18% B from
239 17 to 20 min; 20% B from 20 to 21 min; 27% B from 21 to 22 min; 29% B from 22 to 23
240 min; 30% B from 23 to 33 min; 36% B from 33 to 40 min. Phenolic compounds were
241 monitored in the multiple reaction monitoring spectrum mode at conditions optimized
242 using authentic phenolic standards. The equipment was operated with an electrospray
243 ionization source (ESI) under the following conditions: interface temperature at 350 °C,
244 heating gas flow at 6 L min^{-1}, nebulizing gas flow at 2 L min^{-1}, drying gas flow at 4 L
245 min^{-1}, interface voltage at -3.5 V. Analytical curves were constructed using commercial
246 standards of verbascoside (Chromadex, CO, USA), protocatechuic acid, 3-
247 hydroxytyrosol, 4-hydroxybenzoic acid, tyrosol, caffeic acid, vanillic acid, homovanillic
248 acid, *p*-coumaric acid, ferulic acid, oleuropein, luteolin and apigenin (Sigma-Aldrich,
249 MO, USA). Phenolic compounds were quantified using authentic reference standards

except for hydroxytyrosol-glycoside that was quantified as equivalents of hydroxytyrosol and oleuropein aglycone that was quantified as equivalents of oleuropein. Validation data for the analysis of phenolic compounds is shown in Table S2 (supplementary material).

2.9 Statistical analysis

All statistical evaluations were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, CA, USA). The data were expressed as mean ± standard error of the mean (S.E.M.). Data on the profile of phenolic compounds during fermentation were analyzed by factorial analyses of variance (5 fermentation times × 3 OP-IN samples) with the fermentation time treated as a repeated measure. Duncan's test was used for *post hoc* comparison when analysis of variance revealed a significant main effect or interaction between factors.

3. Results and discussion

3.1 Particle size

The average particle size of NF decreased after granulometric fractionation (GF) and micronization (GFM), as shown in Fig. S1 (supplementary material). Powdered samples obtained from OP without physical modification (NF) had a wide distribution range for the particle size, reaching values over 1800 μm, with an average size of 320.8 ± 195.0 μm (mean ± standard deviation). This occurred mainly due to the presence of large particles of stones and pulp that are difficult to grind in conventional knife milling devices.

The average particle size of powdered OP samples was reduced by granulometric fractionation using a 2-mm sieve (GF) (mean ± standard deviation: 143.0 ± 5.3 μm) and even more reduced when samples were subsequently micronized (GFM) (mean ±

standard deviation: 22.4 ± 0.9 μm). As previously demonstrated by our research group, long-time (5 h) micronization, as the one used in the present study, standardizes the particle size as indicated by the great decrease in the standard deviation of particle size values (Speroni et al., 2020).

3.2 pH and fermentation parameters

GFM samples had a small decrease in pH values during fermentation (pH 7.22 at 0 h vs. pH 6.95 at 48 h), with a significant difference from the Control 2 (fermentation of feces without OP) at 8, 24, and 48 h. The pH of GF was also reduced during fermentation (pH 7.21 at 0 h vs. pH 6.96 at 48 h) and significantly lower than Control 2 at 24 h and 48 h ($p<0.05$), as shown in Fig. S2 (supplementary material).

The reduction of pH is associated to the production of short chain fatty acids, which is expected to increase during intestinal fermentation with viable fecal microbiota and enough amount of fermentable substrates (Tejada-Ortigoza et al., 2022). The viability of fecal microbiota used in this study was demonstrated by the inclusion of a positive control that contained a high content of inulin (fermentable carbohydrate) and resulted in a pH drop from 8.29 to 5.15 (data not shown). Thus, the small pH drop observed during the final fermentation assay was not caused by low microbial viability but was rather related to the composition of the nutrient base medium (Ribeiro et al., 2021) and that of the OP samples (Table S1, supplementary material) which resulted in low amount of fermentable substrates. This low fermentative activity did not affect our results that were focused on the biotransformation of phenolic compounds.

3.3 Transformation of phenolic compounds during colonic fermentation

The concentration of phenolic compounds in OP samples during fermentation was corrected to eliminate the interference of phenolic compounds already present in the feces (Control 2). The sum of phenolic compounds was high in the start of colonic fermentation assay (time 0), especially in GFM (146.7 mg 100 g^{-1} d.b.) compared to NF and GF (31.1 and 85.5 mg 100 g^{-1} d.b.) (Table S3, supplementary material). The fractionation of OP followed by micronization resulted in lower particle size that likely facilitated the release of phenolic compounds bound to the matrix yielding greater amounts of phenolic compounds during the colonic fermentation of GFM than in GF and NF.

Hydroxytyrosol is the main phenolic alcohol found in OP (Ribeiro et al., 2020). Hydroxytyrosol has high bioaccessibility in the salivary, gastric and intestinal phases during *in vitro* digestion and it is still found in the insoluble fraction that reaches the colon (Speroni et al., 2021). Granulometric fractionation of OP followed by micronization significantly increased the amount of hydroxytyrosol released during *in vitro* gastrointestinal digestion (Speroni et al., 2021). At the start of fermentation (0 h) the concentration of hydroxytyrosol (mg 100 g^{-1} of sample d.b.) was higher in GFM (15.2 ± 2.0) and GF (8.1 ± 0.9) than in NF (3.0 ± 0.2) (Fig. 2A; Table S3, supplementary material). The concentration of hydroxytyrosol released during colonic fermentation increased for all OP samples during the first hours, reached a peak at 8 h, and thereafter decreased from 24 h onwards (p<0.05; Fig. 2A). GF and GFM released higher hydroxytyrosol levels than NF after 2 (4.8x and 7.9x, respectively), 8 (2.6x and 3.8x, respectively) and 24 h (2x and 1.6x, respectively) of fermentation (p<0.05; Fig. 2A). Additionally, hydroxytyrosol levels released by GFM were higher than GF at 2 and 8 h of fermentation (p<0.05; Fig. 2A). At 48 h of fermentation, small amounts of hydroxytyrosol were found regardless of the sample, demonstrating the complete

metabolism of the compound and in agreement with a recent study on OP fermentation (Ribeiro et al., 2021).

Hydroxytyrosol-glycoside represents 36 - 60% of the phenolic compounds found in the digested NF, GF and GFM samples that were used for colonic fermentation (time 0 h, Table S3, supplementary material). In addition, at the start of fermentation (0 h) there was a significant difference in the levels of hydroxytyrosol-glycoside among samples: GFM > GF > NF (p<0.05; Fig. 2B). This data corroborates the effectiveness of micronization to improve the release of phenolic alcohols and related compounds, and agrees with our previous study on the gastrointestinal digestion of OP up to the small intestinal phase (Speroni et al., 2021). However, hydroxytyrosol-glycoside was detected at trace levels after 2 h of fermentation. The parallel increase in the levels of hydroxytyrosol indicates a rapid and extensive metabolism of hydroxytyrosol-glycoside by fecal microbiota that removes the sugar moiety favoring the increase of aglycone levels (Fig. 2A).

The most remarkable effect of OP fractionation followed by micronization (GFM) was the significant increase in the release of tyrosol during colonic fermentation compared to NF and GF, in which the presence of this compound was not detected (p<0.05; Fig 2C). The increase in tyrosol levels of GFM from 0 to 2 h is likely related to the metabolism of hydroxytyrosol by intestinal microbiota (Fig. 2), which follows the biotransformation pathway depicted in Fig. 3. Although GFM keeps much higher levels of tyrosol than NF and GF up to 24 h (p<0.05), a progressive decrease was observed from 2 h onwards and culminates with the disappearance of tyrosol at 48 h (Fig. 2C). The main metabolites generated from the microbial transformation of phenolic alcohols, such as hydroxytyrosol and tyrosol, are homovanillic acid, phenylacetic acid and its derivatives, and vanillyl alcohol (Ribeiro et al., 2021; López de Las Hazas et al., 2016) (Fig. 3). Only

homovanillic acid was monitored in the present study. Its levels progressively increased during the colonic fermentation of OP samples (Fig. 2D), which agrees with the study by Ribeiro et al. (2021), but no difference was observed among NF, GF or GFM samples. The presence of this compound was also reported by Conterno et al. (2019), who quantified homovanillic acid in the plasma of volunteers after feeding with cookies made from OP.

Oleuropein is the main phenolic glycoside found in olive fruits, but it is extensively degraded during olive oil extraction generating phenolic alcohols that are the major phenolics in OP (Pedan et al., 2019). Thus, small amounts of secoiridoids, such as oleuropein and its derivatives, have been reported in OP (López de Las Hazas et al., 2016). In the present study, only trace amounts of these compounds were detected in NF samples at the start of fermentation (Fig. 4). Micronized OP has been shown to have a greater release of oleuropein during the salivary and gastric phases of digestion, but oleuropein was still found in the fraction that was not accessible for intestinal absorption, which is the one that will be available for biotransformation by the intestinal microbiota after reaching the colon (Speroni et al., 2021). In the present investigation, oleuropein was found only at the initial fermentation time (0 h) in GFM and it was completely degraded up to 2 h (p<0.05; Fig. 4A). The degradation of oleuropein follows the biotransformation pathway depicted in Fig. 3, leading to the increase of oleuropein aglycone, as depicted in Fig. 4B. Granulometric fractionation associated or not to micronization (GFM and GF), were effective to increase the release of oleuropein aglycone at the start of fermentation (up to 2 h) compared to NF (p<0.05), which released only trace amounts of this compound (Fig. 4B). After 2 h of fermentation, oleuropein aglycone was rapidly degraded in GF and GFM samples. After 8h of fermentation oleuropein aglycone was not detected, as observed by Mosele et al. (2014), who detected

elenolic acid and oleuropein aglycone at low amounts in the initial times decreasing to trace levels at 12 h of fermentation.

Luteolin and apigenin are the main flavonoids in OP. They show good stability during simulated *in vitro* digestion, which results in a quite high proportion of their intake dose reaching the colon (43.1% for luteolin and 85.2% for apigenin in GF) (Speroni et al., 2021). Luteolin concentration was high in the OP samples at the initial fermentation time (Fig. 5A), but it was rapidly reduced up to 8 h, when it was found at trace levels. No difference was observed in luteolin levels among OP samples. Apigenin had a similar time-course behavior compared to luteolin but GFM and GF released higher apigenin amounts than NF up to 2 h of fermentation ($p<0.05$, Fig. 5B). The rapid degradation of these flavonoids is likely related to their deglycosylation by microbial action (Mosele et al., 2015).

Among the phenolic acids found in fraction of OP that reaches the colon, the derivatives of hydroxybenzoic acid, namely protocatechuic, vanillic and 4-hydroxybenzoic acids, showed an increase up to 8 h of fermentation (Fig. 6A, B, C). Thereafter, the levels of protocatechuic and vanillic acid were decreased, whereas 4-hydroxybenzoic acid remained at a plateau up to 48 h of fermentation (Fig. 6). GF (at 2 h) and GFM (at 2, 8 and 24 h) had higher protocatechuic acid levels than NF during fermentation ($p<0.05$; Fig 6A). GF and GFM also had higher levels of vanillic acid than NF during fermentation (at 2 and 8 h, $p<0.05$; Fig. 6B). The levels of 4-hydroxybenzoic acid did not differ among OP samples during fermentation (Fig. 6C). These metabolites are described by Mosele et al. (2015), as end products of flavonoid fermentation. Accordingly, the increase in hydroxybenzoic acid derivatives up to 8 h of fermentation was associated with the degradation of luteolin and apigenin (Fig 5). Fractionation and micronization increased the release of these hydroxybenzoic acid derivatives which will

399 favor their absorption. The particle size reduction likely favored microbial metabolic
400 activity in the OP matrix.

401 Hydroxycinnamic acid derivatives, such as *p*-coumaric and caffeic acid, had
402 their levels decreased during fermentation being found at trace levels from 8 h onwards.
403 At 0 h, *p*-coumaric and caffeic acid levels were higher for GFM and GF than NF, whereas
404 at 2 h of fermentation the levels were higher for GFM than for GF and NF ($p<0.05$; Fig.
405 6D and 6E).

406 A major finding of the study is that GF and at a greater degree GFM increased
407 the amount of phenolic compounds released during the colonic fermentation and
408 sustained higher levels of phenolic compounds than NF up to 24 h of colonic
409 fermentation. It was remarkable that GF and GFM increased the colonic levels of
410 hydroxytyrosol, hydroxytyrosol glycoside, oleuropein aglycone, apigenin and phenolic
411 acid metabolites in the first hours of colonic fermentation compared to the non-modified
412 OP, whereas GFM caused an even greater increase of hydroxytyrosol and tyrosol
413 compared to GF. These findings are particularly relevant because they reveal that
414 granulometric fractionation followed by micronization is able improve the nutraceutical
415 potential of OP. In fact, hydoxytyrosol and tyrosol have been shown to exhibit
416 cardioprotective, anticancer and neuroprotective effects (Marković et al., 2019;
417 Rodríguez-López et al., 2020), whereas hydroxytyrosol has been demonstrated to
418 attenuate insulin resistance and obesity through the modulation of gut microbiota (Liu et
419 al., 2019).

420

421 **4. Conclusions**

422 Parallel to the reduction in particle size, granulometric fractionation followed by
423 micronization increased the amount of phenolic compounds in the fraction of OP that

reaches the colon. The increased surface area of the OP modified by granulometric fractionation or by granulometric fractionation followed by micronization favored the release of hydroxytyrosol, oleuropein aglycone, apigenin and phenolic acid metabolites in the first hours of colonic fermentation compared to the non-modified OP. Hydroxytyrosol (8 h) and tyrosol (2 h) were the compounds that reached the highest concentration and highest stability during simulated colonic fermentation. Granulometric fractionation followed by micronization of OP caused higher release of hydroxytyrosol than granulometric fractionation alone. Moreover, granulometric fractionation followed by micronization was the only process that resulted in the release of tyrosol during colonic fermentation and allowed relatively high tyrosol levels to be sustained up to 24 h of fermentation. Oleuropein was found only in the OP that was modified by granulometric fractionation followed by micronization being completely degraded into oleuropein aglycone within 2 h of fermentation. The final metabolites formed were homovanillic acid and hydroxybenzoic acids, such as vanillic, protocatechuic and hydroxybenzoic acid. These data confirm the hypothesis that micronization of OP enhances the amount of phenolic compounds released during colonic fermentation and support the conclusion that micronization associated to granulometric fractionation was more efficient than granulometric fractionation alone to increase the release of phenolic compounds from the OP matrix during simulated colonic fermentation. *In vivo* assays are needed to confirm the bioavailability of phenolic compounds and the effect on the gut microbiota to further corroborate the health benefits of micronized OP.

CRediT authorship contribution statement

Camila Sant'Anna Monteiro: Investigation, Methodology, Data curation, Conceptualization, Writing - original draft, Writing - review & editing, Visualization.

Paula Colpo Bortolazzo: Investigation, Methodology, Visualization, Data acuration. **Camila Araujo Amorim Bonini:** Investigation, Visualization, Methodology. **Luana Tamires Dluzniewski:** Investigation, Visualization. **Dariane Trivisiol da Silva:** Investigation, Methodology. **Julia Baranzelli:** Formal analysis, Visualization, Methodology. **Franciele Aline Smaniotto:** Investigation, Visualization, Methodology. **Cristiano Augusto Ballus:** Writing - review & editing, Methodology. **Jesús Lozano-Sánchez:** Writing - review & editing, Formal analysis. **Sabrina Somacal:** Investigation, Writing - review & editing, Data curation, Visualization. **Tatiana Emanuelli:** Conceptualization, Resources, Supervision, Writing – original draft, Writing - review & editing, Funding acquisition, Project administration.

Conflict of interest

Authors declare no conflict of interest.

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References

Abu-Reidah, I. M., Arráez-Román, D., Al-Nuri, M., Warad, I., & Segura-Carretero, A. (2019). Untargeted metabolite profiling and phytochemical analysis of *Micromeria fruticosa* L. (*Lamiaceae*) leaves. *Food Chemistry*, 279, 128–143.

https://doi.org/10.1016/j.foodchem.2018.11.144

Augusti, P. R., Conterato, G. M. M., Denardin, C. C., Prazeres, I. D., Serra, A. T., Bronze, M. R., & Emanuelli, T. (2021). Bioactivity, bioavailability, and gut microbiota transformations of dietary phenolic compounds: implications for COVID-19. *Journal of Nutritional Biochemistry*, 97, 108787. https://doi.org/10.1016/j.jnutbio.2021.108787

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … Recio, I. (2019). INFOGEST static *in vitro* simulation of gastrointestinal food digestion. *Nature Protocols*, 14, 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Chen, T., Zhang, M., Bhandari, B., & Yang, Z. (2018). Micronization and nanosizing of particles for an enhanced quality of food: A review. *In Critical Reviews in Food Science and Nutrition*, 58, 993-1001. https://doi.org/10.1080/10408398.2016.1236238

Conterno, L., Martinelli, F., Tamburini, M., Fava, F., Mancini, A., Sordo, M., Pindo, M., Martens, S., Masuero, D., Vrhovsek, U., Dal Lago, C., Ferrario, G., Morandini, M., & Tuohy, K. (2019). Measuring the impact of olive pomace enriched biscuits on the gut microbiota and its metabolic activity in mildly hypercholesterolaemic subjects. *European Journal of Nutrition*, 58, 63–81. https://doi.org/10.1007/s00394-017-1572-2

Covas, M. I., de la Torre, K., Farré-Albaladejo, M., Kaikkonen, J., Fitó, M., López-Sabater, C., Pujadas-Bastardes, M. A., Joglar, J., Weinbrenner, T., Lamuela-Raventós, R. M., & de la Torre, R. (2006). Postprandial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in humans. *Free Radical Biology & Medicine*, 40, 608–616. https://doi.org/10.1016/j.freeradbiomed.2005.09.027

Dermeche, S., Nadour, M., Larroche, C., Moulti-Mati, F., & Michaud, P. (2013). Olive mill wastes: Biochemical characterizations and valorization strategies. *Process Biochemistry*, 48, 1532–1552. https://doi.org/10.1016/j.procbio.2013.07.010

Dhiman, A., & Prabhakar, P. K. (2021). Micronization in food processing: A comprehensive review of mechanistic approach, physicochemical, functional properties and self-stability of micronized food materials. *Journal of Food Engineering*, 292, 110248. https://doi.org/10.1016/j.jfoodeng.2020.110248

505 Dutra, R. L. T., Dantas, A. M., Marques, D. A., Batista, J. D. F, Meireles, B. R. L. A.,
506 Cordeiro, A. M. T. M., Magnani, M., & Borges, G. S. C. (2017). Bioaccessibility and
507 antioxidant activity of phenolic compounds in frozen pulps of Brazilian exotic fruits
508 exposed to simulated gastrointestinal conditions. *Food Research International*, 100,
509 650–657. https://doi.org/10.1016/j.foodres.2017.07.047

510 Gil-Sánchez, I., Cueva, C., Sanz-Buenhombre, M., Guadarrama, A., Moreno-Arribas,
511 M. V., & Bartolomé, B. (2018). Dynamic gastrointestinal digestion of grape pomace
512 extracts: Bioaccessible phenolic metabolites and impact on human gut microbiota.
513 *Journal of Food Composition and Analysis*, 68, 41–52.
514 https://doi.org/10.1016/j.jfca.2017.05.005

515 Goulart, F. R., Speroni, C. S., Lovatto, N. D. M., Loureiro, B. B., Corrêia, V., Neto, J.
516 R., & Picolli, L. (2013). Activity of digestive enzymes and growth parameters of
517 juvenile jundiá (Rhandia quelen) fed linseed meal in nature and demucilaged. *Semina:*
518 *Ciências Agrárias*, 34, 3069–3080. https://doi.org/10.5433/1679-0359.2013v34n6p3069

519 Li, Y., Li, M., Wang, L., & Li, Z. (2022). Effect of particle size on the release behavior
520 and functional properties of wheat bran phenolic compounds during *in vitro*
521 gastrointestinal digestion. *Food Chemistry*, 367, 130751.
522 https://doi.org/10.1016/j.foodchem.2021.130751

523 Liu, Z., Wang, N., Ma, Y., & Wen, D. (2019). Hydroxytyrosol improves obesity and
524 insulin resistance by modulating gut microbiota in high-fat diet-induced obese mice.
525 *Frontiers in Microbiology*, 10, 390. https://doi.org/10.3389/fmicb.2019.00390

526 López de las Hazas, M. C., Piñol, C., Macià, A., Romero, M. P., Pedret, A., Solà, R.,
527 Rubió, L., & Motilva, M. J. (2016). Differential absorption and metabolism of
528 hydroxytyrosol and its precursors oleuropein and secoiridoids. *Journal of Functional*
529 *Foods*, 22, 52–63. https://doi.org/10.1016/j.jff.2016.01.030

530 Malapert, A., Reboul, E., Loonis, M., Dangles, O., & Tomao, V. (2018). Direct and
531 rapid profiling of biophenols in olive pomace by UHPLC-DAD-MS. *Food Analytical*
532 *Methods*, 11, 1001–1010. https://doi.org/10.1007/s12161-017-1064-2

533 Marković, A.K.; Torić, J.; Barbarić, M.; Brala, C. J. (2019). Hydroxytyrosol, tyrosol
534 and derivatives and their potential effects on human health. *Molecules*, 24, 2001.
535 https://doi.org/10.3390/molecules24102001

Mosele, J. I., Macià, A., & Motilva, M. J. (2015). Metabolic and microbial modulation of the large intestine ecosystem by non-absorbed diet phenolic compounds: A review. *Molecules*, 20, 17429–17468. https://doi.org/10.3390/molecules200917429

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, 1809–1819. https://doi.org/10.1002/mnfr.201400124

Pedan, V., Popp, M., Rohn, S., Nyfeler, M., & Bongartz, A. (2019). Characterization of phenolic compounds and their contribution to sensory properties of olive oil. *Molecules*, 24, 2041. https://doi.org/10.3390/molecules24112041

Quatrin, A., Rampelotto, C., Pauletto, R., Maurer, L. H., Nichelle, S. M., Klein, B., Rodrigues, R. F., Maróstica Junior, M. R., Fonseca, B. de S., de Menezes, C. R., Mello, R. de O., Rodrigues, E., Bochi, V. C., & Emanuelli, T. (2020). Bioaccessibility and catabolism of phenolic compounds from jaboticaba (*Myrciaria trunciflora*) fruit peel during *in vitro* gastrointestinal digestion and colonic fermentation. *Journal of Functional Foods*, 65, 103714. https://doi.org/10.1016/j.jff.2019.103714

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, 106312. https://doi.org/10.1016/j.foodhyd.2020.106312

Ribeiro, T. B., Oliveira, A., Campos, D., Nunes, J., Vicente, A. A., & Pintado, M. (2020). Simulated digestion of an olive pomace water-soluble ingredient: relationship between the bioaccessibility of compounds and their potential health benefits. *Food & Function*, 11, 2238–2254. https://doi.org/10.1039/C9FO03000J

Rocchetti, G., Senizza, B., Giuberti, G., Montesano, D., Trevisan, M., & Lucini, L. (2020). Metabolomic study to evaluate the transformations of extra-virgin olive oil's antioxidant phytochemicals during *in vitro* gastrointestinal digestion. *Antioxidants*, 9, 1–15. https://doi.org/10.3390/antiox9040302

Rodríguez-López, P., Lozano-Sánchez, J., Borrás-Linares, I., Emanuelli, T., Menéndez, J. A., & Segura-Carretero, A. (2020). Structure–biological activity relationships of

extra-virgin olive oil phenolic compounds: Health properties and bioavailability. *Antioxidants*, 9, 1–17. https://doi.org/10.3390/antiox9080685

Serreli, G., & Deiana, M. (2018). Biological relevance of extra virgin olive oil polyphenols metabolites. *Antioxidants*, 7, 11–13. https://doi.org/10.3390/antiox7120170

Shu, Y., Li, J., Yang, X., Dong, X., & Wang, X. (2019). Effect of particle size on the bioaccessibility of polyphenols and polysaccharides in green tea powder and its antioxidant activity after simulated human digestion. *Journal of Food Science and Technology*, 56, 1127–1133. https://doi.org/10.1007/s13197-019-03573-4

Speroni, C. S., Bender, A. B. B., Stiebe, J., Ballus, C. A., Ávila, P. F., Goldbeck, R., Morisso, F. D. P., Silva, L. P. da, & Emanuelli, T. (2020). Granulometric fractionation and micronization: A process for increasing soluble dietary fiber content and improving technological and functional properties of olive pomace. *LWT*, 130, 109526. https://doi.org/10.1016/j.lwt.2020.109526

Speroni, C. S., Guerra, D. R., Bender, A. B. B., Stiebe, J., Ballus, C. A., Silva, L. P. da, Lozano-Sánchez, J., & Emanuelli, T. (2021). Micronization increases the bioaccessibility of polyphenols from granulometrically separated olive pomace fractions. *Food Chemistry*, 344, 128689. https://doi.org/10.1016/j.foodchem.2020.128689

Speroni, C. S., Stiebe, J., Guerra, D. R., Bender, A. B. B., Ballus, C. A., dos Santos, D. R., Morisso, F. D. P., da Silva, L. P., & Emanuelli, T. (2019). Micronization and granulometric fractionation improve polyphenol content and antioxidant capacity of olive pomace. *Industrial Crops and Products*, 137, 347–355. https://doi.org/10.1016/j.indcrop.2019.05.005

Tejada-Ortigoza, V., Garcia-Amezquita, L. E., Campanella, O. H., Hamaker, B. R., & Welti-Chanes, J. (2022). Extrusion effect on *in vitro* fecal fermentation of fruit peels used as dietary fiber sources. *LWT*, 153, 112569. https://doi.org/10.1016/j.lwt.2021.112569

Zhao, G., Zhang, R., Dong, L., Huang, F., Tang, X., Wei, Z., & Zhang, M. (2018). Particle size of insoluble dietary fiber from rice bran affects its phenolic profile, bioaccessibility and functional properties. *LWT*, 87, 450-456. https://doi.org/10.1016/j.lwt.2017.09.016

598

599 **Legends**

600 **Figure 1. Scheme of *in vitro* gastrointestinal digestion (oral, gastric, and intestinal**
601 **phase) of OP samples, followed by *in vitro* colonic fermentation with fresh human**
602 **feces.** OP: olive pomace; IN: fraction that remains inside the dialysis tube and
603 corresponds to the digesta that will reach the colon; OUT: fraction that crosses the dialysis
604 membrane and represents the bioaccessible fraction; NF: IN from non-fractionated OP;
605 GF: IN from granulometrically fractionated OP; GFM: IN from granulometrically
606 fractionated and micronized OP; Control 1: IN samples incubated with nutrient base
607 medium, without feces; Control 2: fermentation with feces but without IN samples.

608

609 **Figure 2. Changes in phenolic alcohols and related compounds during the colonic**
610 **fermentation of IN-OP samples**. The samples of NF, GF and GFM that remained inside
611 the dialysis membrane (IN) after intestinal digestion, which correspond to the digesta that
612 will reach the colon, were used for the colonic fermentation assay. Data are presented as
613 mean ± SEM (n = 5). *Different from NF at the same time point (p<0.05). #Different
614 from GF at the same time point (p<0.05). NF: non-fractionated OP; GF: granulometrically
615 fractionated OP; GFM: granulometrically fractionated and micronized OP.

616

617 **Figure 3. Proposed biotransformation pathways for secoiridoids and phenolic**
618 **alcohols from olive pomace during colonic fermentation.**

619

620 **Figure 4. Changes in secoiridoids during the colonic fermentation of IN-OP samples.**
621 The samples of NF, GF and GFM that remained inside the dialysis membrane (IN) after
622 intestinal digestion, which correspond to the digesta that will reach the colon, were used

for the colonic fermentation assay. Data are presented as mean ± SEM (n = 5). *Different from NF at the same time point (p<0.05). #Different from GF at the same time point (p<0.05). NF: non-fractionated OP; GF: granulometrically fractionated OP; GFM: granulometrically fractionated and micronized OP.

Figure 5. Changes in flavonoids during the colonic fermentation of IN-OP samples. The samples of NF, GF and GFM that remained inside the dialysis membrane (IN) after intestinal digestion, which correspond to the digesta that will reach the colon, were used for the colonic fermentation assay. Data are presented as mean ± SEM (n = 5). *Different from NF at the same time point (p<0.05). #Different from GF at the same time point (p<0.05). NF: non-fractionated OP; GF: granulometrically fractionated OP; GFM: granulometrically fractionated and micronized OP.

Figure 6. Changes in hydroxybenzoic acids and hydroxycinnamic acids derivatives during the colonic fermentation of IN-OP samples. The samples of NF, GF and GFM that remained inside the dialysis membrane (IN) after intestinal digestion, which correspond to the digesta that will reach the colon, were used for the colonic fermentation assay. Data are presented as mean ± SEM (n = 5). *Different from NF at the same time point (p<0.05). #Different from GF at the same time point (p<0.05). NF: non-fractionated OP; GF: granulometrically fractionated OP; GFM: granulometrically fractionated and micronized OP.

Graphical Abstract

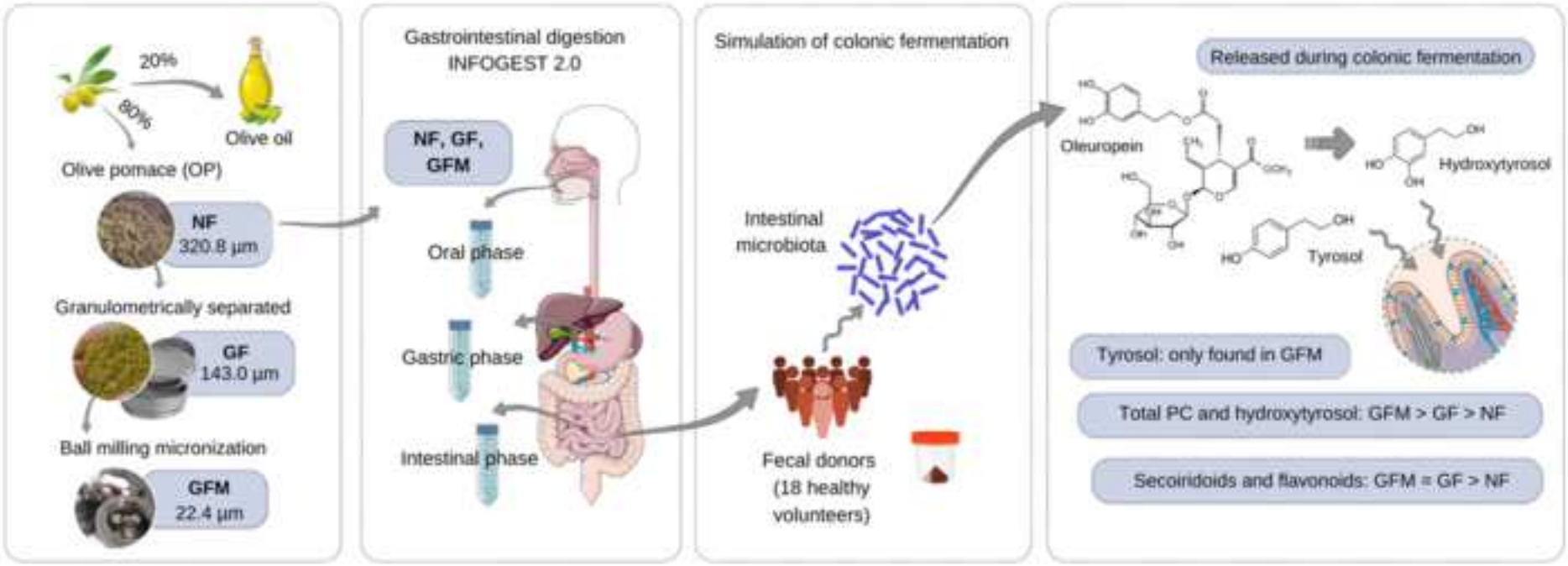

Figure 1

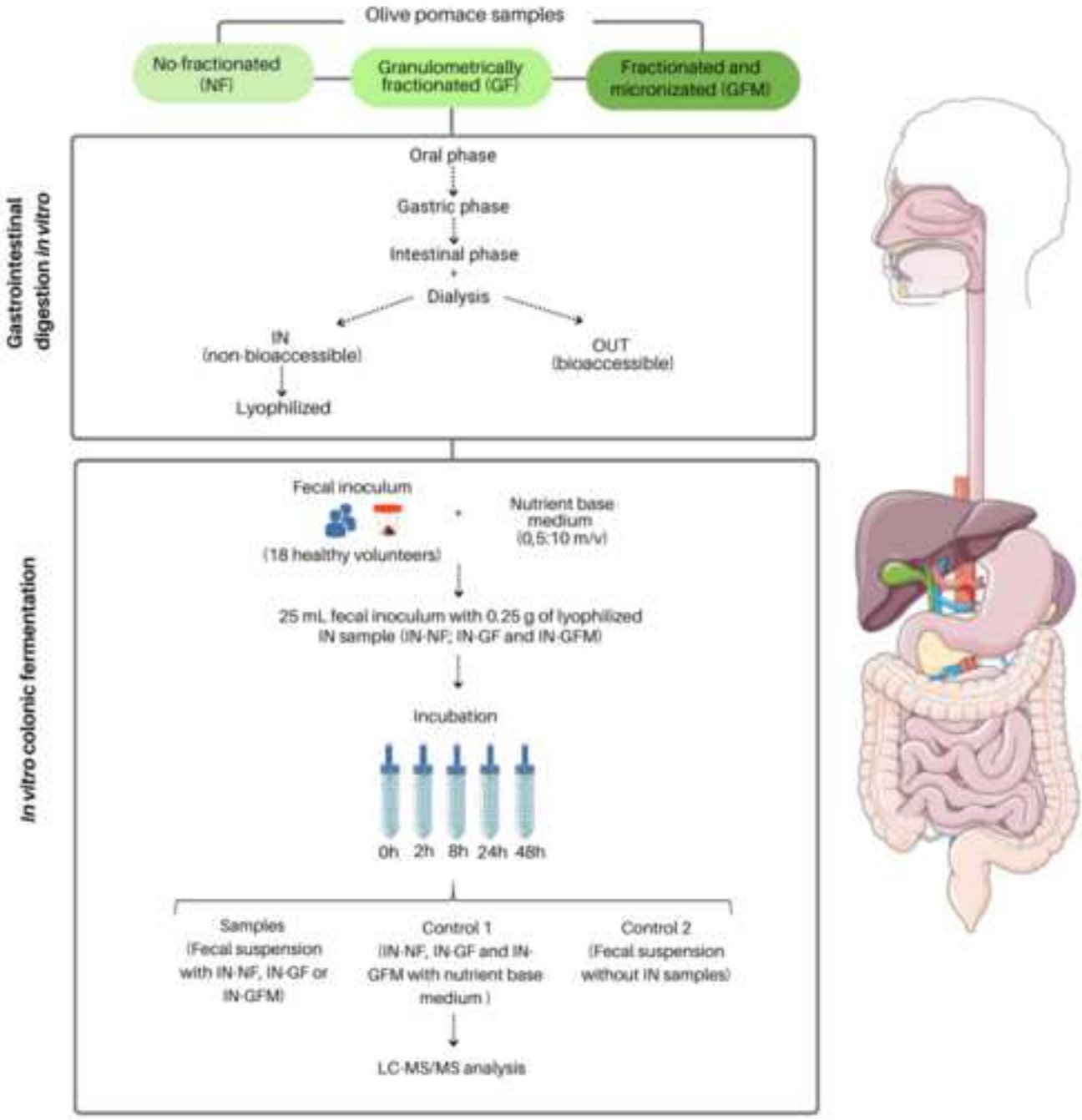

Figure 2

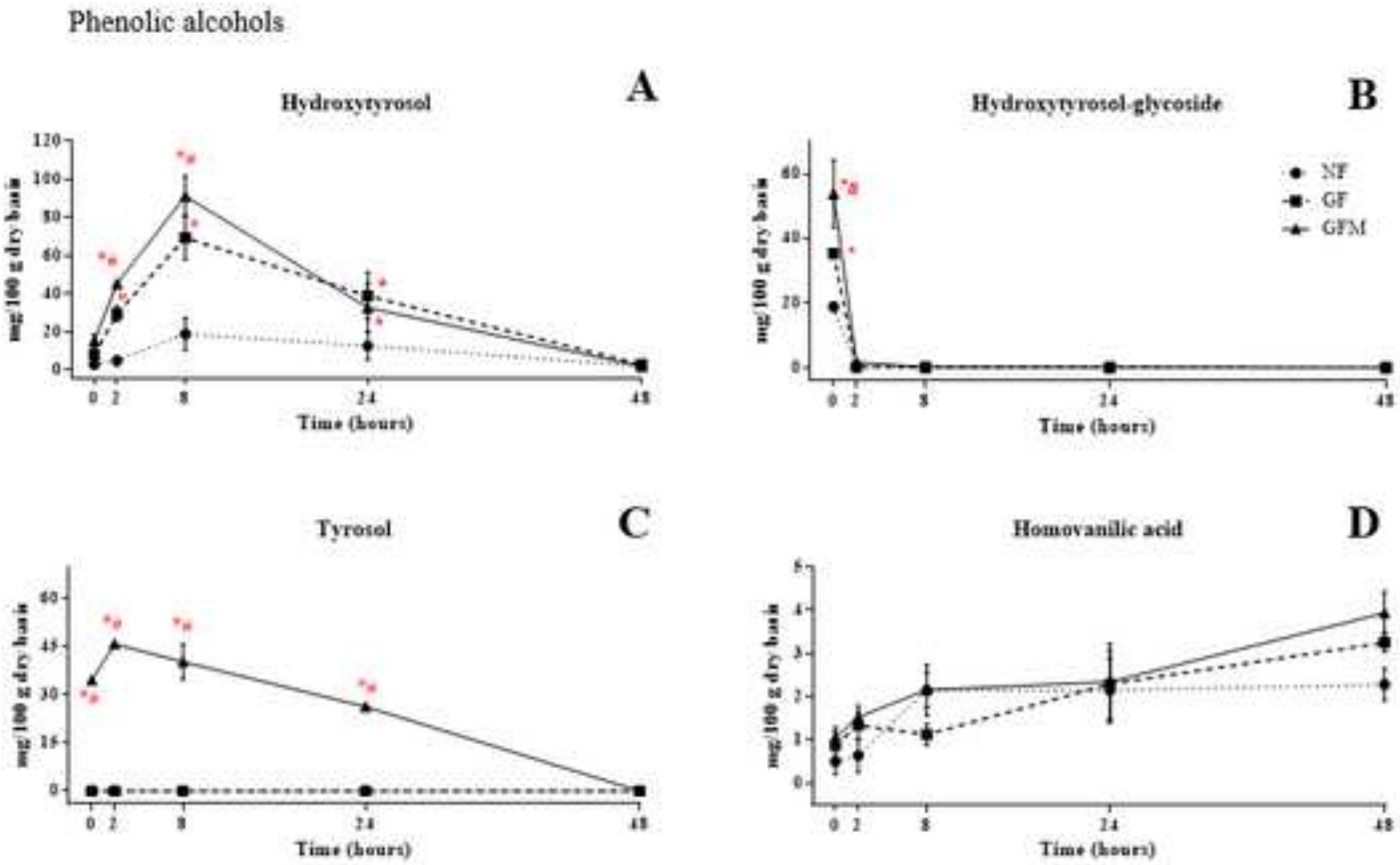

Figure 3

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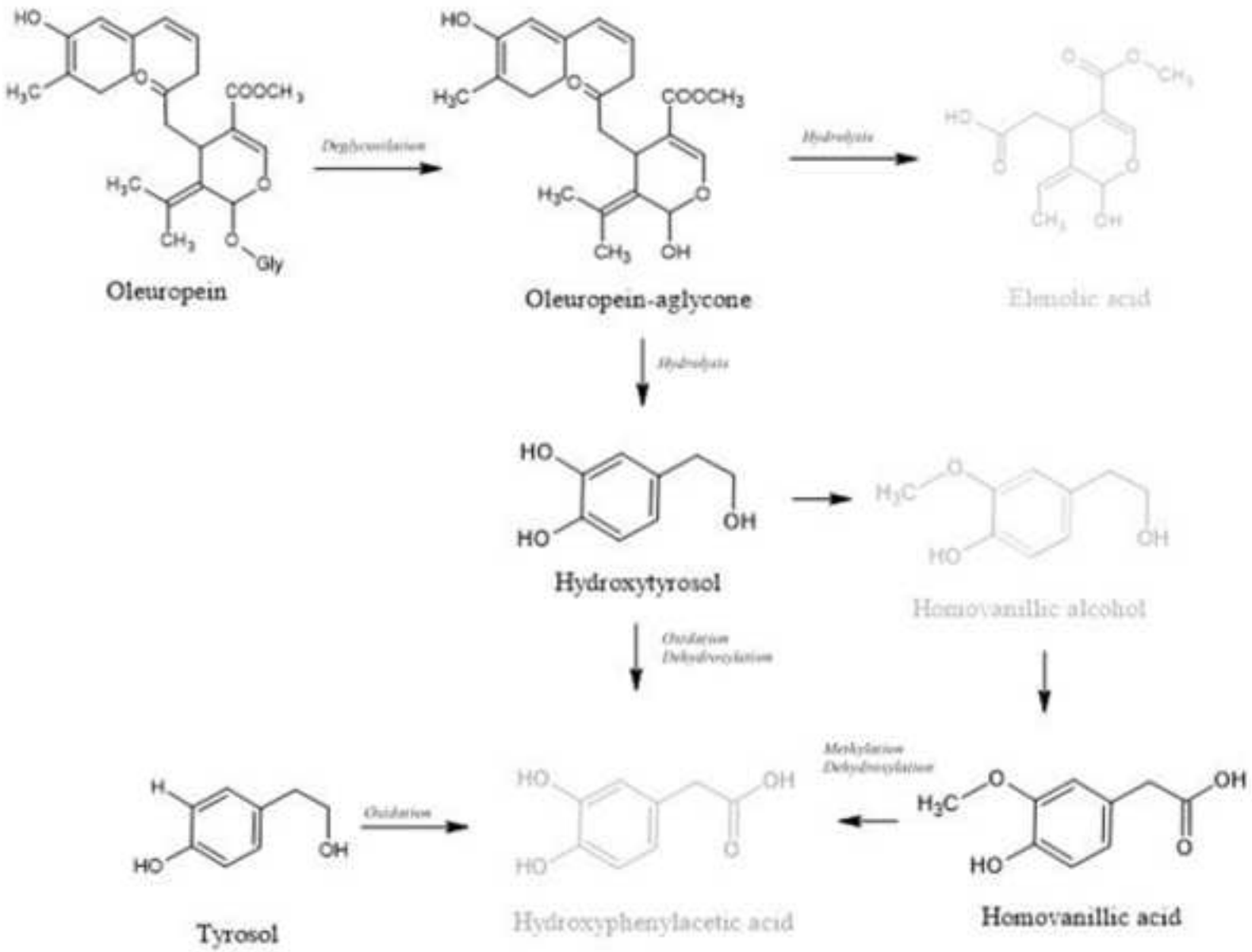

Figure 4

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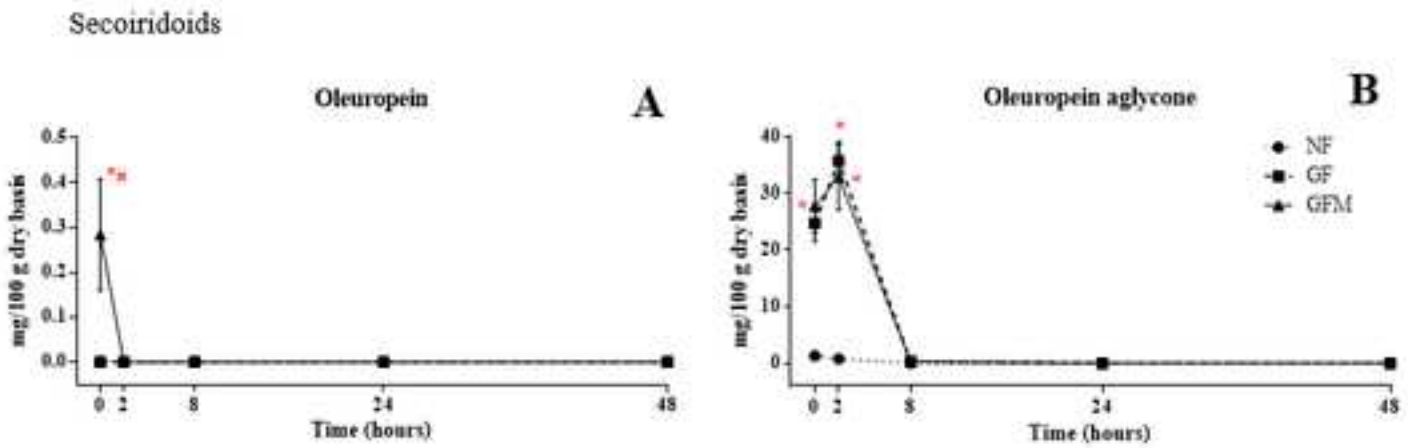


Figure 5

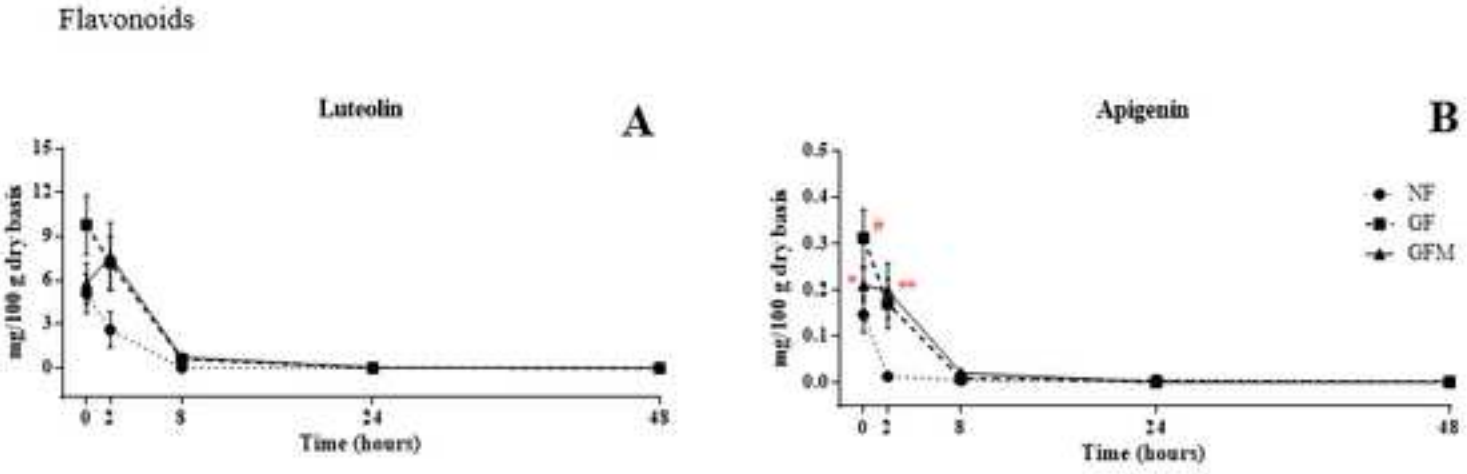
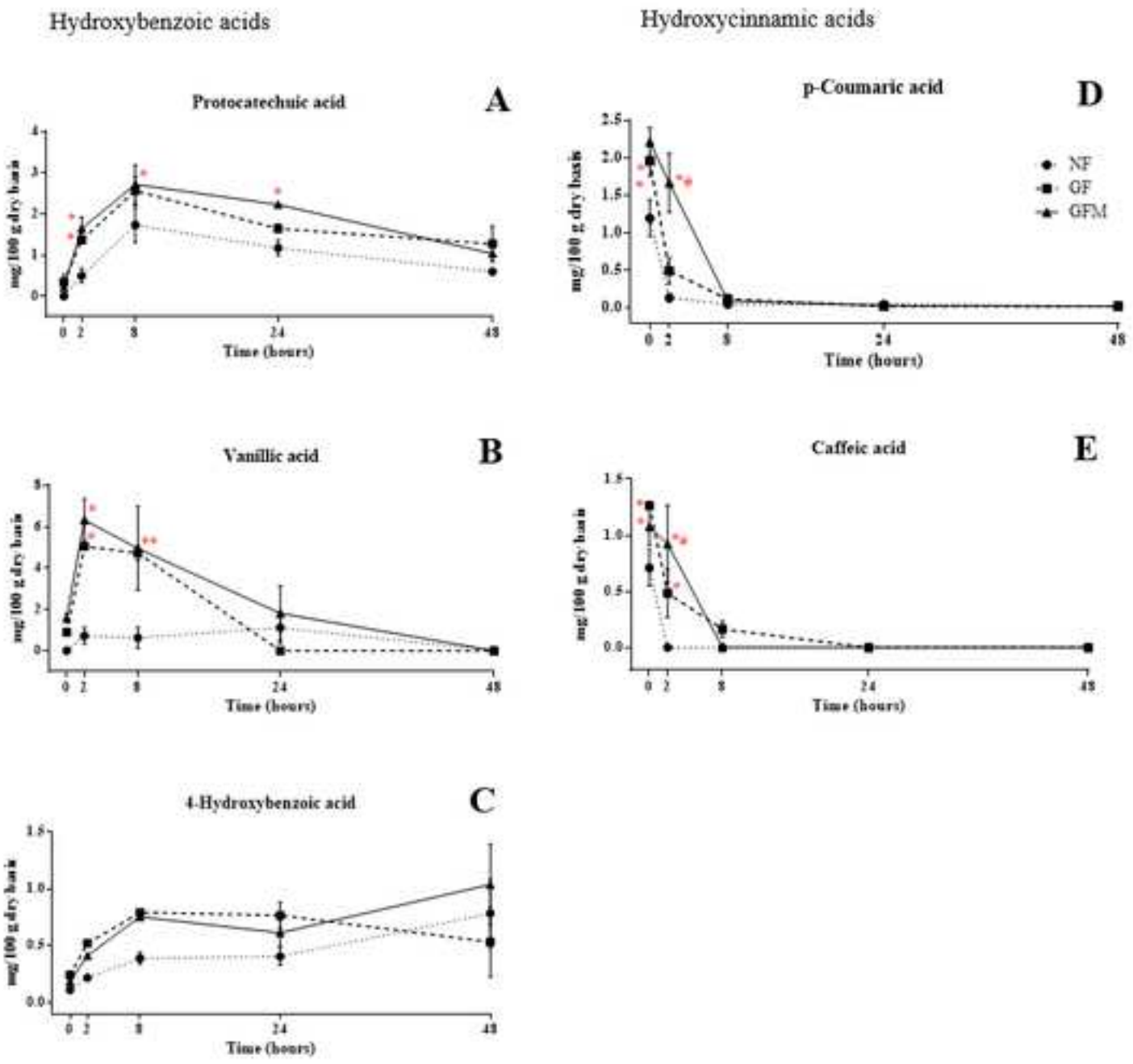


Figure 6



Supplementary material

Effect of micronization on olive pomace biotransformation in the static model of colonic fermentation

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Table S1. Proximate composition (g 100 g^{-1} dry mass. except for moisture) of samples of olive pomace.

	NF	GF	GFM
Moisture (%)	9.12±0.09 a	9.29±0.08 a	8.24±0.01 b
Fat	4.17±0.04 b	6.94±0.02 a	7.32±0.60 a
Ash	2.69±0.39 b	4.37±0.20 a	4.38±0.09 a
Protein	0.04±0.002 b	0.09±0.000 a	0.09±0.002 a
Total dietary fiber	68.08±1.54 a	52.74±0.85 b	44.25±0.98 c
Insoluble dietary fiber	65.76±4.39 a	49.44±2.51 b	37.62±0.70 c
Soluble dietary fiber	2.32±2.85 b	3.30±1.66 b	6.63±0.28 a

Data are presented as mean ± SD (n = 3). Lower case letters indicate differences among samples. NF= non-fractionated OP; GF= granulometrically fractionated OP; GFM= granulometrically fractionated and micronized OP.

Table S2. Validation data for the analysis of phenolic compounds by LC-MS/MS analysis.

Phenolic compound	*m/z* [M-H]⁻	T	CE (eV)	RT (min)	Linear range (mg L^{-1})	Regression equation	R^2	LoD (mg L^{-1})	LoQ (mg L^{-1})	Repeatability intra-day precision CV (%)						Intermediate (inter-day) precision CV (%) (n=10)	
										Low level (n=10)		Medium level (n=10)		High level (n=10)			
										RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area
Pro	153	109.05	15	9.01	0.018 - 2.30	y = 2E+07x + 447958	0.9971	0.6	1.8	0.44	5.13	0.20	0.91	0.32	0.81	0.18	1.90
HT	153	123.15; 95.00	16	9.35	0.019 - 2.30	y = 9E+06x + 60531	0.9987	0.0006	0.0019	0.51	7.04	0.25	0.97	0.29	0.95	0.19	1.90
HT-gly	315	153; 123	16; 20	9.36	7.80 - 3.40	y = 6E+06x + 5E+06	0.9982	0.0006	0.0019								
4-HBA	137.2	93.1	15	11.41	0.018 - 2.30	y = 3E+07x + 136172	0.9972	0.0006	0.0018	0.25	4.17	0.12	0.86	0.20	0.64	0.12	1.33
Tyr	137.2	106; 119.1	19	11.63	3.50 - 40	y = 7566.8x - 4864.8	0.9981	1.1607	3.5172	0.33	4.86	0.22	0.87	0.25	0.95	0.18	3.21
Van	167.15	152;108.1	16; 17	13.25	0.0035 - 2.30	y = 4E+06x + 561288	0.9772	0.0012	0.0035	0.27	3.57	0.09	0.80	0.16	1.08	0.12	2.80
Caf	179.2	135.05; 134.2	16; 24	13.37	0.0012 - 2.30	y = 4E+07x + 590172	0.9991	0.0004	0.0012	0.23	3.01	0.10	0.94	0.15	1.13	0.12	5.39
HVan	181.1	137.2; 122.1	11; 16	14.23	0.0032 - 2.30	y = 2E+06x + 75852	0.9972	0.0010	0.0032	0.32	5.84	0.07	0.74	0.14	1.26	0.09	3.11
Ole-aglyc	377	197; 153	22; 16	15	0.001 - 4.40	y = 2E+07x + 664497	0.9997	0.0004	0.0013								
p-Cou	163.2	119.1	15	17.15	0.0004 - 5.0	y = 4E+07x + 4E+06	0.9984	0.0001	0.0004	0.21	1.34	0.18	0.78	0.18	1.04	0.09	2.06
Ole	539.2	275.2; 377.2; 307.15	22; 16; 21	24.86	0.001 - 8.90	y = 2E+07x + 1E+06	0.9993	0.0004	0.0013	0.16	3.24	0.09	0.89	0.05	1.45	0.06	6.32
Lut	285	133; 151.05	33; 26	27.36	0.0003 - 2.30	y = 3E+07x + 1E+06	0.9986	0.0001	0.0003	0.13	2.42	0.09	1.41	0.11	1.63	0.07	7.00
Api	269	117.1; 151	35; 25	31.53	0.0003 - 2.30	y = 6E+07x + 2E+06	0.9982	0.0001	0.0003	0.11	0.49	0.08	0.90	0.16	1.29	0.08	6.07

T=transition; RT= retention time; CV= coefficient of variation; CE=collision energy Low level= LOQ of each compound; Medium level= 2.77 ppm for all compounds; High level=5 ppm for all compounds. Pro=protocatechuic acid; HT=3-Hydroxytyrosol; HT-gly=3-Hydroxytyrosol-glycoside; 4-HBA= 4-hydroxybenzoic acid; Tyr=tyrosol; Chlo=chlorogenic acid;Van=vanillic acid; Ole-agly=oleuropein aglycone; PhLac=3-Phenyllactic; Caf=caffeic acid; HVan=Homovanillic acid; p-Cou= p-coumaric acid; Verb=verbascoside; Ole=oleuropein; Lut=luteolin; Api=apigenin

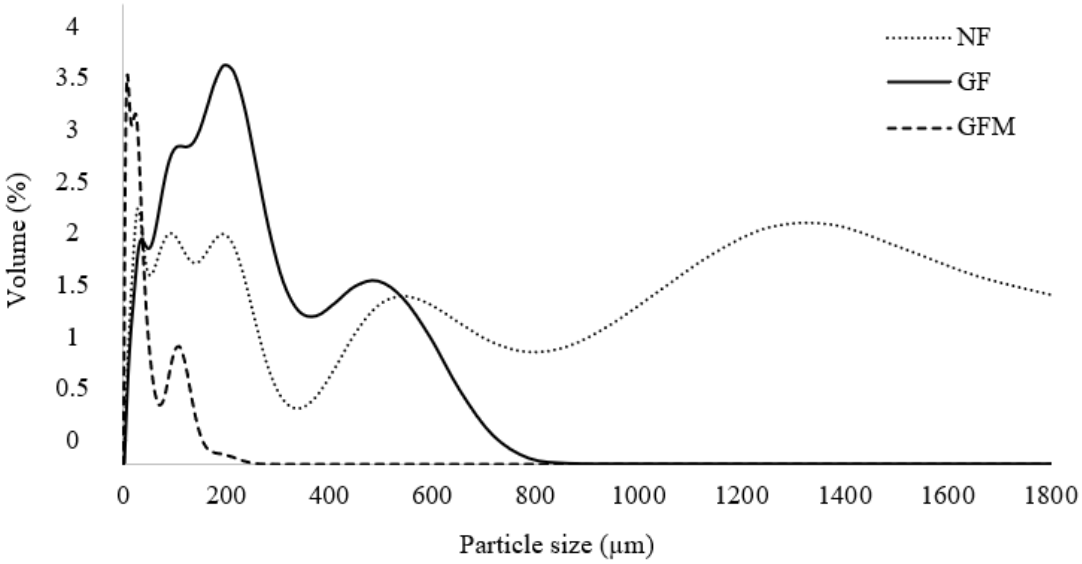

Figure S1. Particle size distribution of OP samples. NF= non-fractionated OP; GF= granulometrically fractionated OP; GFM= granulometrically fractionated and micronized OP. Results were obtained in triplicate.

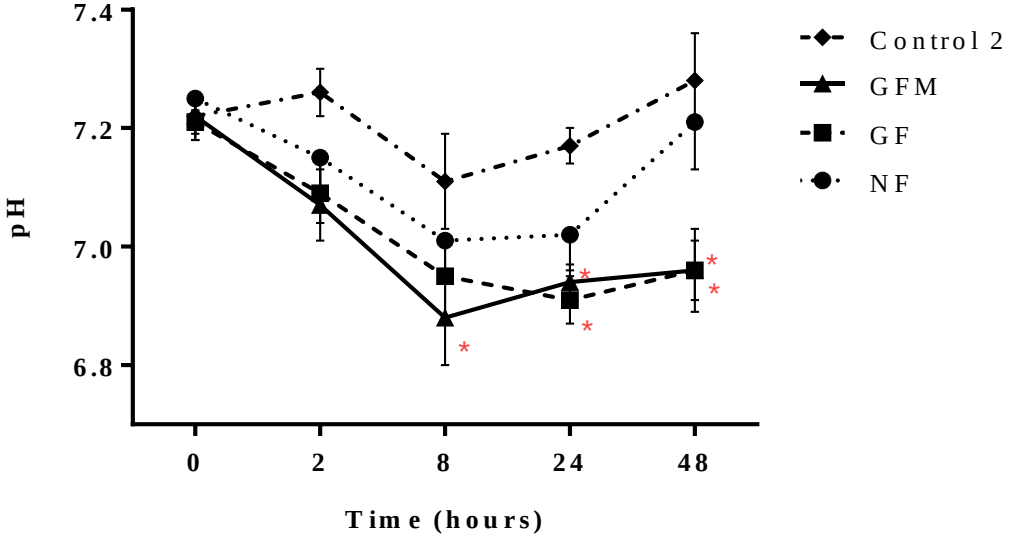

Figure S2. Changes in pH values during the colonic fermentation of IN-OP samples. The samples of NF, GF and GFM that remained inside the dialysis membrane (IN) after intestinal digestion, which correspond to the digesta that will reach the colon, were used for the colonic fermentation assay. OP: olive pomace. Control 2= fermentation with feces but without IN-OP; NF= non-fractionated OP; GF= granulometrically fractionated OP; GFM= granulometrically fractionated and micronized OP. Data are presented as mean ± SEM (n = 5). *Different from Control 2 at the same time point (p<0.05).

Table S3. Quantification of phenolic compounds during the colonic fermentation of IN-OP samples.

Time	0 h			2 h			8 h			24 h			48 h		
Samples	NF	GF	GFM	NF	GF	GFM	NF	GF	GFM	NF	GF	GFM	NF	GF	GFM
Phenolic Alcohols															
HT	3.0±0.18	8.1±0.94	15.21± 2.0	5.1±1.42 c	29.5±2.30 b	45.4±1.34 a	19.0±4.8 c	69.5±6.6 b	91.3±6.1 a	12.8±4.2 b	39.0±7.0 a	32.9±7.3 a	2.0±0.7	2.9±0.6	2.0±0.1
HT-gly	18.8±1.4 c	35.3±0.9 b	53.7±10.4 a	0.03±0.0	0.1±0.0	1.5±1.1	0.2±0.1	0.04±0.01	0.14±0.03	0.018±0.01	0.05±0.04	0.01±0.0	nd	nd	nd
Tyr	nd b	nd b	34.6±1.2 a	nd b	nd b	46.2±0.8 a	nd b	nd b	40.3±5.5 b	nd b	nd	26.3±1.2	nd	nd	nd
Secoiridoids															
Ole	nd b	nd b	0.3±0.12 a	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ole-agly	1.3±0.3 b	24.7±3.19 a	27.8±4.7 a	0.8±0.3 b	35.7±3.4 a	32.9±5.8 a	nd	0.3±0.05	0.32±0.26	nd	nd	nd	nd	nd	nd
Hydroxycinnamic acids															
Pro	nd	0.3±0.2	0.22±0.04	0.5±0.2 b	1.4±0.08 a	1.6±0.3 a	1.8±0.4 b	2.5±0.3 a	2.8±0.4 a	1.2±0.2 b	1.6±0.1 ab	2.2±0.05 a	0.6±0.05	1.28±0.4	1.0±0.2
Van	nd	0.9±0.08	1.6±0.22	1.2±0.5 b	5.0±0.0 a	6.3±1.0 a	1.0±0.8 b	4.8±0.3 a	4.9±2.0 a	nd	nd	4.5±2.2	nd	nd	nd
4-HBA	0.1±0.02	0.2±0.02	0.2±0.02	0.216±0.02	0.5±0.03	0.4±0.01	0.4±0.05	0.8±0.02	0.8±0.02	0.4±0.08	0.7±0.1	0.6±0.1	0.8±0.3	0.5±0.3	1.0±0.3
Hydroxybenzoic acids															
p-Cou	1.2±0.2 b	2.0±0.2 a	2.2±0.19 a	0.1±0.05 b	0.5±0.17 b	1.7±0.4 a	0.05±0.01	0.1±0.06	0.1±0.03	0.05±0.01	0.02±0.01	0.03±0.01	0.02±0.0	0.02±0	0.02±0.0
Caf	0.7±0.16 b	1.2±0.03 a	1.0±0.01 a	nd c	0.5±0.2 b	0.9±0.3 a	nd	0.2±0.07	nd	nd	nd	nd	nd	nd	nd
Flavonoids															

Lut	5.0±1.3	9.7±2.0	5.8±1.4	2.6±1.2	7.2±1.8	7.6±2.3	nd	0.6±0.2	0.7±0.1	nd	nd	nd	nd	nd	nd
Api	0.1±0.04 b	0.3±0.06 a	0.2±0.03 b	0.01±0.01 b	0.2±0.05 a	0.2±0.06 a	nd	nd	nd	nd	nd	nd	nd	nd	nd
Other compounds															
Verb	0.4±0.1	1.9±0.2	3.0±1.3	0.2±0.2	2.0±1.2	2.1±1.2	nd	nd	nd	nd	nd	nd	nd	nd	nd
HVan	0.5±0.3	0.9±0.3	1.0±0.24	0.6±0.4	1.3±0.3	1.5±0.3	2.1±0.6	1.1±0.2	2.1±0.4	2.1±0.7	2.3±0.8	2.4±0.9	2.3±0.4	3.3±0.2	4.0±0.5
Total	31.1	85.5	146.7	11.4	83.8	148.2	24.5	79.2	143.5	16.5	43.7	68.9	5.7	7.0	8.0

The samples of NF, GF and GFM that remained inside the dialysis membrane (IN) after intestinal digestion, which correspond to the digesta that will reach the colon, were used for the colonic fermentation assay. OP: olive pomace. NF= non-fractionated OP; GF= granulometrically fractionated OP; GFM= granulometrically fractionated and micronized OP. Data are presented as mean ± SEM (n = 5). ND = not detected. Lower case letters indicate differences among samples at the same fermentation time (p<0.05). Pro=protocatechuic acid; HT=3-Hydroxytyrosol; HT-gly=3-Hydroxytyrosol-glycoside; 4-HBA= 4-hydroxybenzoic acid; Tyr=tyrosol; Van=vanillic acid; Ole-agly=oleuropein aglycone; Caf=caffeic acid; HVan=Homovanillic acid; p-Cou= *p*-coumaric acid; Verb=verbascoside; Ole=oleuropein; Lut=luteolin; Api=apigenin.

Declaration of interests

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT authorship contribution statement

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