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Effects of Glucosinolate-Enriched Red Radish (*Raphanus sativus*) on In Vitro Models of Intestinal Microbiota and Metabolic Syndrome-Related Functionalities

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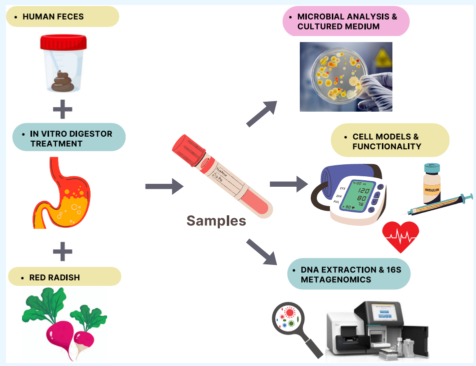

ABSTRACT: The gut microbiota profile is determined by diet composition, and therefore this interaction is crucial for promoting specific bacterial growth and enhancing the health status. Red radish (*Raphanus sativus L.*) contains several secondary plant metabolites that can exert a protective effect on human health. Recent studies have shown that radish leaves have a higher content of major nutrients, minerals, and fiber than roots, and they have garnered attention as a healthy food or supplement. Therefore, the consumption of the whole plant should be considered, as its nutritional value may be of greater interest. The aim of this work is to evaluate the effects of glucosinolate (GSL)-enriched radish with elicitors on the intestinal microbiota and metabolic syndrome-related functionalities by using an *in vitro* dynamic gastrointestinal system and several cellular models developed to study the GSL impact on different health indicators such as blood pressure, cholesterol metabolism, insulin resistance, adipogenesis, and reactive oxygen species (ROS). The treatment with red radish had an influence on short-chain fatty acids (SCFA) production, especially on acetic and propionic acid and many butyrate-producing bacteria, suggesting that consumption of the entire red radish plant (leaves and roots) could modify the human gut microbiota profile toward a healthier one. The evaluation of the metabolic syndrome-related functionalities showed a significant decrease in the gene expression of endothelin, interleukin IL-6, and cholesterol transporter-associated biomarkers (ABCA1 and ABCG5), suggesting an improvement of three risk factors associated with metabolic syndrome. The results support the idea that the use of elicitors on red radish crops and its further consumption (the entire plant) may contribute to improving the general health status and gut microbiota profile.

1. INTRODUCTION

The gut microbiota is closely linked with the host's health and has a key role in the development of several diseases, such as obesity. Its profile is determined by the diet and therefore this interaction is essential for synthesizing vitamins and beneficial bioactive molecules derived from polyphenols and fibers and postbiotic molecules released by bacteria following their death.[1] These compounds are capable of promoting specific bacterial growth and enhancing the health status because gut microbiota is also involved in the maintenance of intestinal functions, modulating the immune system response, and working as a barrier against certain pathogens. Many studies suggest a link between gut microbiota and obesity[2] and other interrelated metabolic diseases, including hyperglycemia, hyperlipidemia, insulin resistance, and hepatic steatosis,[3] that are defined as metabolic syndrome (MS). In humans, the development of obesity correlates with shifts in the relative abundance and diversity of bacterial phyla and species in the gut.[4] Current diet health standards include more vegetables and fruits daily.[5]

Radish (*Raphanus sativus L.*), belonging to the Brassicaceae family, is a major root vegetable crop that is widely cultivated and consumed. Radish plants consist of two main organs: a large, extended root, which acts as a storage organ, and the aboveground leaves. Radish roots are commonly harvested and consumed as vegetables.[6] They contain various minerals, nutrients, and bioactive compounds, including polysaccharides, organic acids, phenolic compounds, alkaloids, nitrogen compounds, and glucosinolates (GSLs).[7,8] Several studies have shown that radish contains several secondary plant metabolites that can exert protective effects on chemically induced carcinogenesis in animals and tumor growth and

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formation in humans.[9,10] Moreover, a study by Jung et al.[11] demonstrated that an extract of radish roots stimulated the spasmodic activity of the gastrointestinal tract through the regulation of post-junctional muscarinic receptors *in vitro* and *in vivo*. In the gastrointestinal tract, acetylcholine (ACh) receptors, metabotropic muscarinic (mAChR), and ionotropic nicotinic receptors (nAChRs) play important physiological roles in the stimulation of spontaneous phasic and tonic contraction in the fasting and postprandial states.[12] Unlike radish roots, radish shoots (green radish leaves) have received limited attention, and they are considered a byproduct of radish cultivation. However, recent studies have shown that the radish leaves have a higher content of major nutrients, minerals, and fiber than the roots.[13] The radish leaves, trimmed from the radish roots and dried, have garnered attention as a healthy food or supplement;[14] therefore, the consumption of the whole plant should be considered, as its nutritional value may be of greater interest.

Among the health-promoting biomolecules mentioned before, GSLs stand out due to their antifungal, antibacterial, antioxidant, antimutagenic, hepatoprotective, nephroprotective, antidiabetic, anti-inflammatory, and antiproliferative activities.[15,16] These sulfur-rich anionic secondary metabolites are classified into three groups based on their amino acid precursors: aliphatic, aromatic, and indolic GSLs.[17] GSLs usually undergo a process of degradation by the myrosinase enzyme (EC 3.2.1.147), giving the bioactive molecules isothiocyanates (ITCs).[18] These hydrolysis products have been demonstrated to have tumor cell inhibitory effects[19−21] and antioxidant properties.[22−24] Specifically, phenethyl isothiocyanate (PEITC) has been reported to be a bioactive ingredient of *R. sativus*.[25] However, GSL profiles and content vary considerably, depending on the radish cultivar,[26] tissue,[27] and storage duration.[28] GSL profiles of radish shoots are more diverse than those of radish roots, and these differences have been noted in the total GSL content between the two parts.[29] The GSL profiles also differ between plant varieties.[30] To increase the phytochemical content in the plant, elicitors have emerged as a practical tool[31] for altering the concentration of secondary metabolites in plants. For example, methyl jasmonate (MeJA) and salicylic acid (SA) have been widely used in cruciferous plants, such as red cabbage, reporting an increase in GSL concentration.[32] These treatments act as stressors in plants, activating defense response mechanisms against pathogen infections or environmental stimuli and enhancing the synthesis of GSLs.[33−35]

The purpose of this work is to study the effects of glucosinolate-enriched radish on the intestinal microbiota and metabolic syndrome-related functionalities by using an *in vitro* dynamic gastrointestinal system conditioned with gut microbiome from obese adults, as well as several cellular models. The derived plant products, under the use of elicitors and the samples obtained from the digestion system, will establish the foundations for the development of new food products for obesity and metabolic syndrome management.

2. MATERIALS AND METHODS

2.1. Plant Material and Treatments. 160 seeds from *R. sativus L.* kindly provided by SAKATA Seed Ibérica S.L.U. (Valencia, Spain) were prehydrated and aerated in deionized water for 24 h to induce germination. Then, seeds were transferred to a vermiculite substrate and kept in darkness for 2 days, with conditions of 60% relative humidity and 28 °C. Resultant seedlings were transplanted in a farm experimental soil under a semi-arid Mediterranean climate (37°47052.7″ N, 0°52000.7″ W, 15 m asl, Murcia, Spain). The radishes were grown for 25 days during August 2019 and drip-irrigated with a 1/4 Hoagland solution. Forty plants were allocated for each treatment with elicitors: (1) control, (2) 100 μM methyl jasmonate (MeJA), (3) 200 μM salicylic acid (SA), and (4) the combination (SA + MeJA). Elicitors were dissolved in 0.2% ethanol in water to increase their solubility. In addition, treatments were complemented with a patented concentration of the surfactant (Patent #PCT/ES2019/0704457), and their dosages were based on previous experiments.[34,36] Each treatment was applied twice, leaving two days in between treatments. Then, the red radishes were harvested two days after the last treatment. For the analyses, plants were randomly grouped in mixtures of 10, and the roots (edible part) from the shoots (aerial parts) were separated to obtain 4 technical replicates for further experiments. Samples were transferred to the lab and stored at −80 °C.

2.1.1. Glucosinolate and Isothiocyanate Quantitative Determination. Glucosinolates extracted from fresh radish were identified by HPLC−DAD-ESI-MS_n, according to their [M−H] and MS^2 fragmentation patterns. The conditions for analysis were the same as described in Baenas et al.[37] For the quantitative analysis of intact glucosinolates, 20 μL of extract were introduced into an Agilent 1100 HPLC−DAD system (Santa Clara, California). The glucosinolates were identified according to their UV spectra and elution order. Sinigrin and glucobrassicin were used as external standards (Phytochem, Neu-Ulm, Germany). Isothiocyanates were measured by a high throughput UHPLC-QqQ-MS/MS method, as described by Baenas et al.[37] The standards employed for quantification were sulforaphane, SFN-gluthatione (SFN-GSH), SFN-cysteine (SFN-CYS), SFN- *N*,acetylcysteine (SFN-NAC), iberin, and indole-3-carbinol (I3C) from Santa Cruz Biotech (California). Results were analyzed by the plant part and the sum of both to elucidate the concentration in the whole plant.

2.2. Dynamic Gastrointestinal and Colonic Fermentation Model Description, Sampling, and Analysis. The equipment simulates the entire gastrointestinal digestive process. It consists of a computer-assisted model of five interconnected compartments, double jacket vessels, that simulate the physiological conditions of the stomach (R1), small intestine (R2), and the three colonic sections: the ascending colon (R3), transverse colon (R4), and descending colon (R5). This model was developed by AINIA (Valencia, Spain)[38] based on van de Wiele et al.[39] and Marzorati et al.[40] R1 and R2 work semicontinuously, while the colon reactors (R3, R4, and R5) work continuously. A peristaltic bomb ensured the flow of the content from one reactor to the next. The system did not simulate water absorption.

The volumes and transit times for each region of the gastrointestinal tract were: 260 mL for 2 h in R1, 410 mL for 6 h in R2 and the colon, 1000 mL for 20 h in R3, 1600 mL for 32 h in R4, and 1200 mL for 24 h in R5.[41−43] The temperature was kept at 37 °C during the entire process. An anaerobic environment was maintained by flushing gaseous N2 for 15 min twice a day in all five reactors. During all of the processes, the system was hermetically closed. Gastric digestion was simulated by continuous addition of a 0.03% (w/v) pepsin solution (2100 units/mg) for 2 h (total volume of 60 mL). A typical gastric digestion pH curve (based on *in vivo* data) was simulated by adding a 1 M HCl solution. Digestive processes

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of the small intestine were mimicked by the continuous addition of a pancreatin solution (0.9 g/L), NaHCO3 (12 g/L), and oxgall dehydrated fresh bile (6 g/L) in distilled water (total volume of 440 mL), maintaining the intestinal content at pH 6.5.

2.2.1. Fecal Inoculum. Fresh feces were used to reproduce the gut conditions from four adult participants with pathologies associated with obesity and/or metabolic syndrome (BMI 30−40 kg/m^2; age 30−50 y.o.), nonsmokers, no history of antibiotic treatment in the last three months, and no intestinal disease background.[44] Fecal samples were collected and maintained in special anaerobic plastic bags (BD GasPak systems). Feces were pooled and diluted with thioglycolate 20% (w/v) and homogenized with a stomacher to obtain a fecal slurry. The fecal suspension was centrifuged at 3000*g* for 3 min, and the collected supernatant was immediately inoculated in the colon vessels (50, 80, and 60 mL for R3, R4, and R5, respectively), followed by culture medium up to a total volume of 1000, 1600, and 1200 mL, respectively. The composition of the culture medium followed by Molly et al.[45,46] provides the necessary nutritional components to simulate the conditions of the human colon and allows the intestinal microbiota to grow. Each reactor was maintained at different optimal pH levels. The bacteria present in each region of the colon have an optimal pH of action: pH 5.5−6 in the colon ascending (R3), pH 6−6.4 in the transverse colon (R4), and pH 6.4−6.8 in the descending colon (R5). To regulate the pH changes that occur during fermentation and maintain them in the optimal intervals for each region, an acid or a base was added.

2.2.2. Process Description and Duration. After fecal inoculation, a stabilization period of 12 days (T12) was required to allow bacteria to grow and reach stable levels.[47,48] During this period, 200 mL of cultured medium was added to the stomach (R1) three times a day. At this point, the system was ready to start the sample treatment period to study its modulation effects on the gut microbiota.[38,39] The treatment period was conducted for another 14 days (T26) (a total of 26 days of the experiment) by feeding the equipment with 25 g of crushed raw red radish with a regular hand blender once a day (in culture medium up to 200 mL) and with 200 mL of cultured medium twice a day. The maintenance of the microbial population during the stabilization and treatment period was checked by bacteria plate counts. The following bacterial groups were quantified by growth on specific media, expressing the result as CUF/mL of colonic media: acid−lactic bacteria (MRS agar, the matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) technique was employed to verify lactobacilli colonies), bifidobacteria (TOS-propionate agar enriched with MUP), enterobacteria (VRBD agar), clostridia (TSC agar enriched with cycloserin), and total anaerobic bacteria (agar Schaedler). Then, 10 mL of the samples was taken from each reactor (R3, R4, and R5) and serially diluted in a saline solution. Plates were inoculated with 1 mL of the sample of four serial dilutions by duplicate and incubated at 37 °C under aerobic or anaerobic conditions. In addition, samples were taken from each reactor at T12 and T26, 10 mL and 3 mL, respectively, and stored at −20 °C to determine the short-chain fatty acid (SCFA) content in the colonic media and to conduct the metabolomic analysis. In addition, for the metagenomic analysis, 1.5 mL of the inoculum, as well as samples from each reactor at the end of T12 and T26, were collected using OMNIgene GUT kits from DNA Genotek (Ottawa, ONT, Canada), according to the standard instructions provided by the company. Finally, for the functionality analysis, two samples were taken from the *in vitro* digester at two different stages:

(a) the sample taken at the end of T12 when the microbiota has adapted to the *in vitro* conditions (**Mbasal).** This sample is obtained from the mixture of each of the three reactors ("R3" ascending, "R4" transversal, and "R5" descending).

(b) the sample taken at the end of T26, after the addition of the radish whole plant treated with MeJA on the *in vitro* digester, and the reaction of the microbiota (**Mtt).** This sample is obtained from the mixture of each of the three reactors ("R3" ascending, "R4" transversal, and "R5" descending).

The samples were diluted 1/2 with Hank's balanced salt solution (HBSS), selected dilution 1/2.

2.2.3. SCFA Analyses. For the metabolomic analysis, these samples were centrifuged (15,000*g*, 15 min) and filtered through a 0.22-μm-Ø Millipore filter (Billerica, Massachusetts) into vials for UHPLC-ESI-QqQ-MS/MS analysis. Short- and medium-chain fatty acids were extracted from the sample using liquid−liquid extraction with diethyl ether. The resulting extract was analyzed using an AS 800 C.U. gas chromatograph (CE Instruments) equipped with an HP-FFAP 25 m × 0.2 mm × 0.33 mm column (Agilent Technologies) and a flame-ionization detector (FID). The samples were quantified by interpolation in the calibration curve with an internal standard. The concentration of the fatty acids was provided directly by the software using a 1 × linear regression. The results were expressed as mg of the compound per kg of colonic medium.

2.2.4. Microbiota Composition and 16S rRNA Analysis. DNA was extracted with the QIAamp DNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.[49] The microbiota composition was analyzed according to the 16S rRNA protocol with next-generation sequencing (NGS) using a MiSeq (Illumina, San Diego, California). A first PCR was performed using 12.5 ng of genomic DNA obtained from the samples and 16S-Fw and 16S-Rv primers. After that, a second PCR reaction was performed using 5 μL of DNA and the Nextera XT DNA Index kit (FC-131-1002, Illumina). Then, the process quality was verified using a Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). When all of the samples were obtained, they were multiplexed by mixing equimolar concentrations from each sample and the internal standard Phix. The mix was diluted until obtaining a concentration of 8 pM. The sequencing was performed in the MiSeq using a MiSeq Reagent v2 (MS-102-2003) kit (Illumina).

2.2.5. Bioinformatic Analysis. The 16S rRNA sequences obtained were curated following the quality criteria from the OTU processing protocol using the LotuS pipeline.[50] This protocol includes the clustering of de novo sequences by UPARSE and the deleting of chimeric and contaminant sequences for OTU identification. Also, this software generates the corresponding abundance matrix. An OTU is defined as organisms that are clustered according to the similarity of their DNA sequence. The taxonomy was assigned by using BLAST and HITdb, reaching a species sensitivity level. The abundance matrix was curated and normalized in R and Bioconductor. Global normalization was performed using the library size as a correction factor. Data were transformed to Log2.

2.2.6. Richness and Evenness. Richness was defined as the total of species. Evenness, defined as the α diversity index, was calculated using the Shannon index[51] according to the following formula: $H = -\text{sum}(\text{Pi}\ln[\text{Pi}])$.

2.3. Cell Models, Sampling, and Analysis. *2.3.1. Cell Models to Study Intestinal Transport and Absorption.* Intestinal transport has been studied using cellular lines from human enterocytes **CaCo-2** (ATCC HTB-37, ATCC, Manassas, VA, EEUU), as it is a cellular model commonly used since the 90s for intestinal absorption studies.[52] For the maintenance of the cellular lines, protocols from the ATCC (American Type Culture Collection) were used, keeping the cell cultures at 37 °C and 5% of CO_2.

2.3.2. Endothelial Model for the Study of Blood Pressure Modulation. The endothelial human cell line EA.hy926 (ATCC CRL-2922)[53] was used to study the effect of Mtt on blood pressure. The cells were seeded into a multiwell plate following the ATCC recommendations. After that, a hypertension context was created, inducing cell oxidation and treating them with the interest samples, in this case, the medium from the *in vitro* digestor. This allows us to determine how the different compounds from this medium affect the vasodilation and vasoconstriction metabolism of the endothelial cells, while checking on blood pressure biomarkers, such as reactive oxygen species (ROS) and endothelin-1 (ET-1). Both are increased with hypertension, being intrinsic vasoconstrictors of the endothelium.

2.3.3. Hepatic Model for the Study of Cholesterol Metabolism Modulation. Human epithelial liver cells HepG2 (ATCC-HB-8065) were used as a model for the evaluation of cholesterol metabolism in liver cells.[54] With the same simulation transwell plate system (Figure 1), the

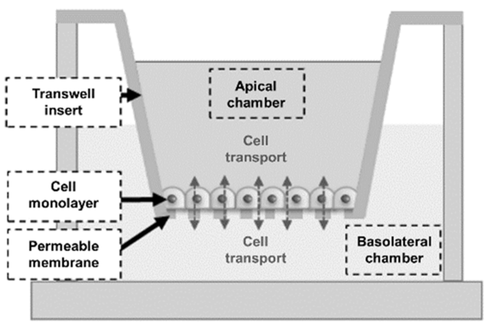

Figure 1. Design of the transwell plate system used in the hepatic models.

molecules pass through the intestinal barrier, allowing us to study the effects of these molecules on the hepatocytes. The intestinal cells (enterocytes) were treated with Mbasal and Mtt for 6 h, and later, these samples were directly exposed to the hepatocytes. After this period, samples were collected for molecular analysis. Biomarkers associated with the cholesterol pathway were studied, including the endogenous synthesis of cholesterol and ATP-independent cholesterol transporters, like ABCA1 and ABCG5. This *in vitro* model is an approach to simulate the access of colonic metabolites, considering risk factors and targeting metabolic syndrome-related tissues.

2.3.4. Hepatic Model for the Study of the Insulin Resistance Modulation. For the insulin resistance evaluation, HepG2 cells (ATCC-HB-8065) were used as a hepatic model, modifying their culture conditions to induce insulin resistance. The proposed model is based on cell stimulation with insulin and the measurement of the accumulated glycogen inside the cells to evaluate the response to this hormone. For this purpose, the hepatocytes were treated with 100 nM insulin for 48−72 h, which should induce a reduction in the intracellular glycogen uptake.[55]

2.3.4.1. Protein Concentration Quantification. In model 3.2.4, to evaluate changes in insulin resistance in hepatocytes, the results obtained for intracellular glycogen were normalized against total protein levels. For this, the Bradford method was used. The cells were lysed in cellytic buffer (CelLytic M Sigma-AldricH) and a 1.5% cocktail of protease inhibitors (Halt (100X) Thermo-fisher), and the amount of protein in the supernatant was quantified. The protein concentration in each sample was calculated, extrapolating the absorbance measured at 540 nm on a standard with a known protein concentration.

2.3.4.2. Cellular Glycogen Quantification. To study insulin sensitivity in model 2.7.4, after treatment with this hormone in the presence or absence of Mtt, the accumulation of glycogen in the cells was measured with the commercial kit MAK016-Glycogen Assay Kit (Sigma-Aldrich, Steinheim, Germany). The lysed cells, whose amount of total protein was previously quantified, were evaluated with the kit following the manufacturer's instructions. The results were extrapolated to a standard curve made with known concentrations of glycogen.

2.3.4.3. Determination of Fat Accumulation by Staining with "Oil Red". The detection of fat clusters was performed with a commercial kit (Enzo Life Sciences, Inc., Farmingdale, New York) following the recommended protocol. The cells were fixed with a solution of 3.7% formaldehyde in phosphate-buffered saline for 30 min. After the removal of the cell fixative, the adipogenesis dye (a solution of Oil Red O lipid dye in 60% isopropanol) was added for 30 min. After the removal of excess dye with distilled water, its intracellular content was extracted with an extraction solution (solution containing 60% isopropanol). Absorbance was measured on a 490 nm plate reader. The absorbance is proportional to the amount of lipid present in each well.

2.3.5. Model with Adipocytes to Study Adipogenesis or Fat Formation. 3T3-L1 fibroblasts (CL-173, ATCC) were used to study adipogenesis. For differentiation to adipocytes, cells were seeded in a multiwell plate with the standard growth medium, and when cells reached 80% confluence, this medium was replaced by Mtt for 2 days, changing it again for the differentiation medium and obtaining mature adipocytes at the end of the assay. Once the adipocytes were treated with the two different samples (Mbasal and Mtt), they were dyed with red oil for the quantification of fat accumulation. The gene expression of adiponectin, an adipokine expressed by the adipocytes with an insulin-sensitizing, anti-inflammatory, and antiatherogenic activity, and interleukin 6, an unspecific cytokine associated with the inflammatory process that occurs during adipogenesis, was analyzed by quantitative PCR.[56]

2.3.6. Concentration Quantification of Reactive Oxygen Species (ROS). The test used for ROS detection quantifies intracellular ROS. The assay uses a reagent permeable to cells, 2′,7′−dichlorofluorescine diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxyl, and other ROS activities within the cell. After the diffusion of DCFDA (a non-fluorescent compound) in the cells, it is oxidized by ROS into 2′,7′-dichlorofluorescein (DCF), a highly fluorescent compound that can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 and 529 nm

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respectively. The higher the absorbance, the more the ROS generated by the cells.

2.3.7. Gene Expression Analysis: Quantitative PCR. Gene expression analyses were performed after total RNA extraction from cells. The extraction was performed automatically with MAXWELL equipment (Promega, Madison, Wisconsin). cDNA was obtained from RNA using the high-capacity cDNA reverse transcription kit transcriptase enzyme (Applied Biosystems, Foster City, California). From cDNA, the real-time PCR was performed using the previously acquired primers of the selected biomarkers specified in Table 1.

Table 1. Specification of the Gene Primers Used in the Quantitative PCR for Each Functional Study

models	gene primers
2.3.2. Endothelial model to study changes in blood pressure	Endothelin-1 (**END-1**)
2.3.3. Liver model to study modulation of cholesterol metabolism	Cholesterol transporters (**ABCA1** and **ABCG5**)
2.3.5. Model with adipocytes to study adipogenesis or fat formation	Adiponectin (**ADIPOQ**) Interleukin 6 (**IL-6**)
Gene Primer References	
END-1: Hs00174961_m1 → Thermo-Fisher Scientific (Waltham, Massachusetts)	
ABCA1 and ABCG5: Hs01059137_m1 and Hs00223686_m1 → Thermo-Fisher Scientific (Waltham, Massachusetts)	
ADIPOQ and IL-6: Mm00456425_m1 and Mm00446190_m1 → Thermo-Fisher Scientific (Waltham, Massachusetts)	
Control Gene References	
Beta-actin (B-Act): 4326315E → Thermo-Fisher Scientific (Waltham, Massachusetts)	
GAPDH: Mm99999915_g1 → Thermo-Fisher Scientific (Waltham, Massachusetts)	

Beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as control genes, whose expression is constitutive in these cells. The amplification conditions in the thermocycler (7300 AppliedBiosystem) were the universal ones, and the quantification of gene expression was performed in a relative way so that the magnitude of the physiological changes in the biomarker gene is obtained in comparison with a reference gene. For the calculations, the formula $2^{-■\Delta\Delta Ct}$[50] was used.[57]

2.4. Data Analysis. Results are expressed as the mean and standard deviation of the mean of at least two to six replicates per study. Statistical analysis was usually carried out with the Student *t*-test to compare the results obtained between Mbaseline and Mtt or between the included controls (* $p < 0.05$; ** $p < 0.025$). For the field elicitation experiment, one-way analysis of variance (ANOVA) was performed using Tukey's HSD as a post-hoc test. For the SCFA production and the bacterial growth analysis, a Mann−Whitney *U* Test was applied for differences between two groups on a single, ordinal variable with no specific distribution.[58] These analyses were carried out using GraphPad Prism version 6.0c for MAC OS X, GraphPad Software, San Diego, California, www.graphpad.com (13/09/2021).

3. RESULTS AND DISCUSSION

3.1. Plant Material and Treatments. The influence of the elicitors on the fresh weight is a parameter used to determine its efficiency. Since these compounds simulate a defense reaction in the plant, at some dosage, they could cause a growth arrest, which would result in a lower field yield.[59] Table 2 (Figure 7 in complementary materials) shows the effect of these treatments on the whole plant, the aerial part, and the edible part of fresh red radish (g). When comparing the weight of the treated red radishes with the control plants, a decrease in biomass by 16% is shown when applying 100 μM MeJA and its combination with SA (SA + MeJA, $p < 0.05$). Nevertheless, no changes were observed with 200 μM SA alone, suggesting that the main effect could come from the MeJA application ($p < 0.05$). Furthermore, when analyzing the edible part and the aerial part separately, the main effect in biomass was observed in the bulb (edible part) after applying the treatments, which include MeJA ($p < 0.05$). However, since the total biomass only decreases by 16%, the treatment with these elicitors could achieve a good balance between plant yield and this enrichment in health-promoting metabolites.

3.1.1. Glucosinolates Concentration. The effect of the elicitors on the GSL content (μg mL^{-1}) is represented in Table 3, which varies between the plant parts (Figure 8 in complementary materials).

- Glucoraphanin levels decreased after the three treatments in the edible part but increased in the aerial part by 69% with MeJA ($p < 0.05$ for each of the comparatives).
- Dehydroerucin raised up to 130% in the edible part with SA and up to 140 and 180% in the aerial part with MeJa and SA + MeJA, respectively ($p < 0.05$).
- 4-Hydroxy-glucobrassicin (HGB) concentrations increased up to 130% in the edible part and up to 67% in the aerial part with MeJA ($p < 0.05$). With SA, concentrations increased by 35% in the edible part and 20% in the aerial part ($p < 005$ for both parts).
- Glucobrassicin (GB) showed the highest increase in both plant parts with MeJA—5 times higher in the edible part and 2 times in the aerial part.
- 4-Methoxy-glucobrassicin (MGB) levels increased in the aerial part with all of the treatments ($p < 0.05$), but none of them caused any increase in the edible part ($p > 0.05$).

In previous studies with brassica plants, elicitors like MeJA or combined with SA have already been seen to increase the total content of glucosinolates.[34,36] For example, with Bimi

Table 2. Study of the Elicitors' Effect on the Plant Biomass (g) of Red Radish. Data Show the Means ($n = 3$) Along with the Standard Deviation[a]

plant biomass (g)	control	200 μM SA	100 μM MeJA	SA + MeJA
whole plant	12.12 ± 0.83 a	10.40 ± 0.66 a	9.58 ± 0.65 b	8.13 ± 1.03 b
edible part	6.15 ± 0.29 a	5.38 ± 0.25 a	5.56 ± 0.22 a	5.23 ± 0.35 a
aerial part	5.75 ± 0.58 a	4.73 ± 0.49 ab	3.77 ± 0.47 bc	3.25 ± 0.67 c

[a]Different letters show statistically significant differences between samples ($p < 0.05$), using an HSD Tukey as a post-hoc test. The statistics were performed within each plant part. MeJA: methyl jasmonate and SA: salicilic acid.

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Table 3. Elicitors' Effect on Red Radish Glucosinolates Content[a]

glucosinolate	control	200 μM SA	100 μM MeJA	SA + MeJA
Edible Part				
glucoraphenin	1.62 ± 0.10[a]	0.95 ± 0.80[c]	0.91 ± 0.08[c]	1.16 ± 0.17[b]
4-hidroxy-glucobrassicin	0.82± ± 0.10[c]	1.11 ± 0.02[b]	1.89± ± 0.33[a]	1.25 ± 0.16[b]
dehydroerucin	2.43 ± 0.30[c]	4.33 ± 0.17[b]	5.70 ± 0.25[a]	5.1 ± 0.70[ab]
glucobrassicin	0.12 ± 0.01[c]	0.13 ± 0.02[c]	0.65 ± 0.05[a]	0.40 ± 0.06[b]
4-metoxy-glucobrassicin	0.97 ± 0.05[a]	0.76 ± 0.02[c]	0.78 ± 0.05[c]	0.83 ± 0.09[b]
Aerial Part				
glucosinolate	control	200 μM SA	100 μM MeJA	SA + MeJA
glucoraphenin	2.25 ± 0.05[b]	2.70 ± 0.70[b]	3.86 ± 0.63[a]	3.80 ± 0.20[a]
4-hidroxy-glucobrassicin	0.43 ± 0.09[d]	0.52 ± 0.12[c]	0.72 ± 0.07[a]	0.66 ± 0.04[b]
dehydroerucin	0.16 ± 0.05[c]	0.17 ± 0.03[c]	0.38 ± 0.13[b]	0.45 ± 0.16[a]
glucobrassicin	3.25± ± 0.71[b]	3.61 ± 0.24[b]	7.62 ± 0.33[a]	6.27 ± 1.20[a]
4-metoxy-glucobrassicin	0.55 ± 0.10[c]	0.78 ± 0.06[b]	0.85 ± 0.12[a]	0.80 ± 0.08[a]

[a]Average values per treatment (n = 4) ± standard error are shown. Different letters indicate statistically significant differences in the HSD Tukey test (p < 0.05). SA: salicylic acid and MeJA: methyl jasmonate.

inflorescences (the edible part of this brassica), both SA and MeJA treatments showed an increase of 5.5 and 7 times, respectively. However, in this study, SA elicitation alone does not seem to have any impact on GB, neither in the edible or the aerial part, suggesting that the influence of the elicitor in each type of glucosinolates has a strong link with their genotype and environmental conditions.

3.1.2. Extract Concentration. Quantification and identification of the isothiocyanates, the bioactive form of the glucosinolates present in the extract obtained from the edible and aerial part of red radish (μg/mL), are shown in Figure 2.

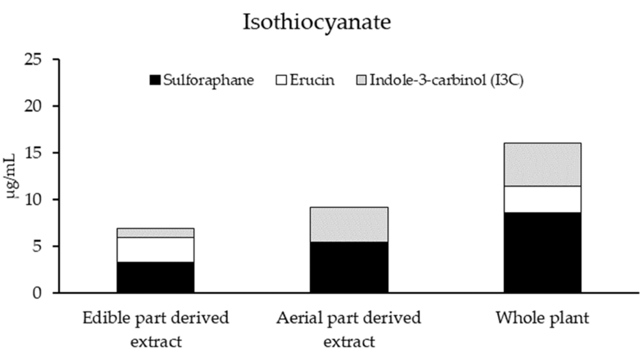

Figure 2. Elicitors' effect on isothiocyanates content (μg/mL) in the different red radish parts. The whole plant graph represents the sum of the values from the edible and the aerial part.

High concentrations of sulforaphane were obtained, although glucoraphanin was not detected in the red radish samples (Figure 2), so its presence might be due to a possible interconversion of glucoraphanin and erucin into sulforaphane.[60] The results indicated that the product with greater chances to have an impact on the gut microbiota was the whole radish plant treated with MeJA, which is the one used in the *in vitro* digestor.

3.2. Bacterial Behavior with the Colonic Fermentation. The results from the dynamic colonic model showed stabilization of the human fecal microbiota in R3, R4, and R5 after 12 days since the incorporation of culture medium. The recovered levels (per mL of culture medium) of total anaerobic microorganisms (indicators of the majority total intestinal flora) were greater than 10^7 cfu in the three portions of the colon (Table 4) (Figure 9 in complementary materials). During the treatment period, 25 g of fresh radish of the whole plant was added to the digester. During this period, total anaerobic microorganisms remained stable, even reaching a rise of 1 logarithm in R3, obtaining levels greater than 7 logarithmic units in the three sections of the colon (Table 4). It can be deduced that radish administration favored the development of total intestinal flora. Microorganisms of the *Clostridium* genus are part of the composition of the intestinal microbiota but are usually associated with potential detrimental effects on human health due to their metabolic activity and the pathogenic nature of some species that may be involved in inflammatory processes of intestinal diseases.[61,62] However other species of this genus are associated with SCFA production, known to have a beneficial impact on gut health.[63] During the treatment period, a significant increase of *Enterobacteria* and *Clostridium* levels was observed in the three sections of the colon (Table 4). The beneficial intestinal microbiota, represented mainly by the genera *Bifidobacterium* and *Lactobacillus*,[64] showed different growth patterns with the treatment. *Bifidobacterium* had a slight decrease, and

Table 4. Microbiological Count of the Three Colonic Reactors (R3, R4, and R5) during the Fermentation of Radish[a]

phase	*Bifidobacterium*	*Lactobacillus*	*Enterobacteria*	*Clostridium*	total anaerobic
R3T12	6.62 ± 0.03	6.72 ± 0.19	6.26 ± 0.03	1.50 ± 0.03	6.86 ± 0.11
R3T26	6.13 ± 0.08	5.29 ± 0.16	7.15 ± 0.02	3.51 ± 0.11	7.95 ± 0.14
R4T12	6.84 ± 0.02	7.17 ± 0.12	6.02 ± 0.13	1.25 ± 0.30	8.51 ± 0.09
R4T26	6.20 ± 0.07	7.53 ± 0.07	7.60 ± 0.04	4.41 ± 0.01	8.48 ± 0.15
R5T12	6.71 ± 0.00	6.62 ± 0.17	6.64 ± 0.10	2.54 ± 0.00	8.16 ± 0.23
R5T26	6.11 ± 0.09	7.45 ± 0.03	8.06 ± 0.07	3.89 ± 0.01	8.46 ± 0.01

[a]T12 represents the end of the stabilization period, and T26 is the end of the treatment period. Results are shown as log (CFU/mL).

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Lactobacillus also had a decrease in R3 but had an opposite response in R4 and R5 (Table 4). Total anaerobic bacteria did not have a clear response, as some genera increased and others decreased.

3.2.1. Bacterial Metabolome: SCFA. After treatment with fresh radish, there was a significant increase in acetic and propionic acid production in the three sections of the colon. Butyric acid only showed a significant increase in R3 but decreased in R4 and R5. These results are shown in Table 5 (Figure 10 in complementary materials). SCFA-producing bacteria have a positive impact on gut health, improving the integrity of the intestinal barrier.[65] Butyrate regulates cell apoptosis,[66] stimulates the production of anaerobic hormones, and through cell differentiation regulation,[67] suppresses allergic and inflammatory responses.[68] Reduced levels of butyrate have been related to diseases such as colon cancer and obesity,[69] so increased levels could be beneficial for health. Propionate is also considered to have health benefits, including the promotion of satiety, reduction of cholesterol, and adipogenesis inhibition. High levels of propionic acid could be related to a hypocholesterolaemic effect.[70] Acetate is a net fermentation product for most gut anaerobes that is also produced by reductive acetogenesis and, almost invariably, achieves the highest concentrations among the SCFA in the gut lumen.[71] Previous studies showed that the butyryl-CoA: acetate-CoA-transferase pathway is the main process for the biosynthesis of butyrate,[72,73] so acetate is utilized by butyrate producers to produce butyrate.[74] Thus, it seems logical to expect a butyric increase following the acetic increase; however, the results do not show that behavior. In the

Table 5. SCFA Production in the Three Reactors (R3, R4, and R5) after the Stabilization Period (T12) and the Treatment Period (T26)[a]

reactor/phase	acetic acid	butyric acid	propionic acid
R3T12	1667 ± 168	161 ± 15	178 ± 11
R3T26	2041 ± 202	440 ± 41	836 ± 64
R4T12	1841 ± 185	1165 ± 106	360 ± 28
R4T26	2657 ± 266	722 ± 66	784 ± 59
R5T12	1756 ± 171	1407 ± 130	382 ± 31
R5T26	3084 ± 309	915 ± 82	856 ± 64

[a]Data are expressed as mg/Kg of colonic medium.

Table 6. Bacterial Genus Abundance in the Three Colonic Sections (R3, R4, and R5)[a]

tax	inoculation	R3T12	R3T26	R4T12	R4T26	R5T12	R5T26
Acidaminococcus	0.00	3.58	5.02^	0.00	0.00	2.48	2.55^
Acinetobacter	0.00	0.00	0.00	1.46	8.82^	0.00	9.65^
Akkermansia	0.00	0.00	0.00	0.00	0.00	1.10	2.77^
Alistipes	1.40	0.00	0.00	0.00	0.00	8.29	8.43^
Anaerofustis	0.00	0.00	0.00	0.00	0.00	2.62	4.54^
Anaerotruncus	0.86	0.00	0.00	0.00	0.00	3.17	4.48^
Bacillus	0.00	3.17	8.29^	2.18	2.24^	0.00	0.00
Bifidobacterium	3.98	4.08	7.02^	0.00	0.00	0.00	0.00
Barnesiella	7.90	0.00	0.00	0.00	0.00	5.70	5.84^
Blautia	10.56	0.00	0.00	4.47	4.50^	5.67	5.82^
Catabacter	7.11	0.00	0.00	0.00	6.71^	3.08	6.48^
Christensenella	4.63	0.00	0.00	4.22	5.11^	5.58	5.69^
Cloacibacillus	4.97	0.00	0.00	0.00	1.69^	0.00	0.00
Clostridium	11.97	0.00	0.00	0.00	0.00	5.82	9.66^
Desulfovibrio	7.28	0.00	0.00	0.00	0.00	0.00	1.97^
Dialister	0.00	0.00	0.00	0.91	1.99^	0.00	1.56^
Dorea	8.22	0.00	0.00	7.39	7.75^	7.40	7.76^
Enterobacter	0.00	5.21	6.32^	0.00	0.00	3.88	5.00^
Enterococcus	1.40	0.00	0.00	0.00	11.76^	1.44	11.68^
Eubacterium	11.79	0.00	0.00	7.02	10.14^	6.89	8.91^
Faecalibacterium	5.50	0.00	0.00	0.00	0.00	6.78	7.43^
Holdemania	4.58	0.00	0.00	0.00	0.00	7.68	8.09^
Lachnoclostridium	11.66	0.00	0.00	0.00	0.00	9.50	9.99^
Lachnospira	10.56	0.00	0.00	8.77	10.43^	8.95	10.36^
Lactobacillus	4.93	5.90	12.11^	0.00	0.00	0.00	0.00
Lysinibacillus	0.00	2.00	2.05^	0.00	0.00	0.00	0.00
Melainabacter	7.45	0.00	0.00	0.00	11.86^	0.00	11.77^
Odoribacter	8.00	0.00	0.00	0.00	0.00	4.71	5.28^
Oscillibacter	11.37	0.00	2.87^	11.15	11.91^	11.08	11.21^
Parabacteroides	7.79	0.00	0.00	5.77	5.83^	5.65	6.02^
Propionibacterium	0.00	1.00	2.05^	0.00	0.00	1.95	1.97^
Ralstonia	2.35	2.32	4.53^	1.46	1.69^	0.00	0.00
Roseburia	11.27	0.00	3.76^	0.00	0.00	5.05	5.98^
Ruminococcus	11.00	0.00	2.87^	6.68	10.97^	7.22	10.50^
Victivallis	5.58	0.00	0.00	0.00	0.00	7.29	8.69^

[a]T12 represents the end of the stabilization period, and T26 is the end of the treatment period. Values show the relative abundance %. ^: Bacterial genus increase.

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Table 7. Bacterial Species Abundance in the Three Colonic Sections[a]

R3	T12	T26	R5	T12	T26
Acidaminococcus intestini	3.58	5.021	*Acidaminococcus intestini*	2.48	2.55
Bacteroides dorei	3.45	4.065	*Akkermansia muciniphila*	1.10	2.77
Bacteroides faecis	0.00	4.710	*Alistipes putredinis*	7.38	7.59
Bifidobacterium bifidum	4.08	6.914	*Anaerofustis stercorihominis*	2.62	4.54
Lactobacillus paracasei	3.16	6.068	*Anaerotruncus colihominis*	3.17	4.48
OTU1217|Roseburia inulinivorans|D = 96.6	0.00	3.764	*Bacteroides clarus*	2.75	3.13
OTU20|Bacteroides xylanisolvens|D = 96.4	2.58	5.154	*Bacteroides uniformis*	0.00	0.98
OTU815|Pseudomonas monteilii|D = 96.2	7.45	9.218	*Bifidobacterium adolescentis*	0.00	1.56
Propionibacterium acnes	0.99	2.052	*Christensenella minuta*	5.32	5.45
Ralstonia mannitolilytica	2.31	4.525	*Desulfovibrio piger*	0.00	1.97
R4	T12	T26	*Dorea longicatena*	7.40	7.76
Alistipes indistinctus	5.68	5.99	*Eubacterium eligens*	3.99	5.24
Bacteroides dorei	3.42	5.04	*Holdemania filiformis*	7.68	8.09
Bilophila wadsworthia	1.46	1.99	*Odoribacter splanchnicus*	4.71	5.28
Christensenella minuta	4.22	5.11	*Oscillibacter valericigenes*	1.95	2.29
Dorea longicatena	7.39	7.75	*Oscillospira plautii*	2.62	2.96
Enterobacter cancerogenus	0.00	0.80	OTU1096|Alistipes indistinctus|D = 96	4.04	4.77
Eubacterium eligens	3.83	6.28	OTU1103|Oscillospira[Pseudoflavonifractor]capillosus|D = 92.3	1.95	2.29
Oscillibacter valericigenes	1.86	1.99	OTU1151|Ruminococcus bromii|D = 89.6	0.00	1.97
Oscillospira plautii	0.91	2.45	OTU1209|Roseburia intestinalis|D = 93.1	6.12	6.99
OTU1096|Alistipes indistinctus|D = 96	3.29	3.69	OTU1217|Roseburia inulinivorans|D = 96.6	5.05	5.98
OTU1209|Roseburia intestinalis|D = 93.1	5.49	7.84	OTU1235|Parabacteroides distasonis|D = 96.7	4.86	5.45
OTU1251|Gemmiger formicilis|D = 94.7	2.84	6.02	OTU1251|Gemmiger formicilis|D = 94.7	4.68	5.63
OTU1262|Catabacter hongkongensis|D = 84.1	0.00	6.71	OTU1262|Catabacter hongkongensis|D = 84.1	2.32	6.30
OTU1572|Parabacteroides distasonis|D = 95.3	4.00	4.45	OTU132|Bacteroides vulgatus|D = 97	1.95	4.54
OTU719|Victivallis vadensis|D = 94.5	3.53	6.52	OTU1461|Desulfitobacterium frappieri|D = 84.5	3.76	3.87
Parabacteroides merdae	0.91	1.32	OTU1524|Anaerofilum pentosovorans|D = 90.6	0.00	1.97
Ralstonia mannitolilytica	1.46	1.69	OTU616|Turicibacter sanguinis|D = 87.1	0.00	7.02
Ruminococcus callidus	0.00	7.75	OTU719|Victivallis vadensis|D = 94.5	5.25	8.56
			OTU80|Eubacterium desmolans|D = 94.1	5.02	5.05
			OTU914|Ruminococcus bromii|D = 87.6	3.82	4.21
			OTU955|Barnesiella intestinihominis|D = 87	5.42	5.84
			Propionibacterium acnes	1.95	1.97
			Ruminococcus callidus	0.00	7.91

[a]T12 represents the end of the stabilization period, and T26 is the end of the treatment period. Values represent the relative abundance %.

following paragraph, the presence of butyrate-producing bacteria will be discussed; however, a significant modulation of the metabolic activity of the intestinal microbiota was found during the treatment period, assuming a potential positive influence of fresh radish.

3.2.2. Bacterial Genera and Species. During the *in vitro* simulation in the colonic digester, samples were taken from the three different reactors to compare differences in the bacterial microbiota due to the treatment with the red radish inoculation. Samples were analyzed by a 16s-metagenomics protocol, and the results are shown in Tables 6 and 7, classifying the bacterial growth by genus and species. As observed in the SCFA production, butyric acid does not show an increase in R4 and R5; however, the bacterial taxa in those reactors show an increase of the following butyrate-producing genera (Table 6) (Figure 11 in complementary materials): *Alistipes*,[75] *Anaerofustis*,[76] *Anaerotruncus*,[77] *Blautia*,[78] *Cloacibacillus*,[79] *Eubacterium*,[80] *Odoribacter*,[81] *Parabacteroides*,[82] *Roseburia*,[83] and *Ruminococcus*.[69] These results, together with the increase of acetate, which is the main resource for butyrate production,[84] suggest that ingestion of radish should stimulate the butyrate synthesis at some point. There is also a marked increase of benefits for the host genera in R3, such as *Bifidobacterium*,[85] and probiotic species such as *Lactobacillus paracasei*.[86] Many other species known to have a positive impact on health increase in R4 and R5: *Christensenella minuta* positively related with individuals with a lower body mass index,[87] *Oscillibacter valericigenes* a valeric acid producer,[88] another SCFA with potential to regulate the inflammatory state, as well as the emotional and cognitive status,[89] *Oscillospira plauti*, strongly related to elevated production of propionate, butyrate, and total SCFA, associated with thin phenotypes,[90] *Gemmiger formicilis*, another butyrate-producing bacteria,[91] and *Victivallis vadensis*, an acetate producer.[92]

R5 is the last reactor, representing the distal part of the colon, and is the one with the highest bacterial population and diversity (Table 8). There are exclusive bacterial species within this reactor, with a potential impact on gut health, that also shows an increased population after treatment: *Akkermansia muciniphila*, whose abundance is inversely correlated with

Table 8. Bacterial Diversity of the Inoculum and the Three Reactors (R3, R4, and R5) before (T12) and after the Treatment Period (T26) with Fresh Radish (g)

SW div	inoculation	R3	R4	R5
T12	3.575	1.516	2.822	3.015
T26		1.202	2.818	2.870

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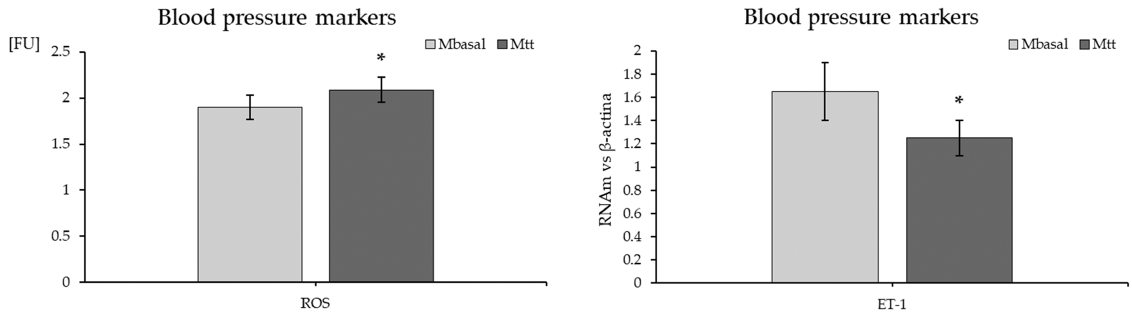

Figure 3. Blood pressure biomarker changes: reactive oxygen species (ROS) in fluorometric units [FU] on the left and endothelin-1 (ET-1) expressed in genic biomarker units on the right ($n = 6$). A Mann−Whitney U Test was applied for differences between two groups on a single, ordinal variable with no specific distribution. "*" indicates a very statistically significant difference ($p < 0.025$).

various diseases. It is located mainly in the mucosa of the colon, involved in maintaining intestinal integrity.[93] *Bacteroides clarus*[94] and *B. uniformis* can interact with the host modifying its immune cells and regulating metabolites. They can improve various intestinal and behavioral disorders, cardiovascular diseases, and cancer. The use of *Bacteroides* in food processing has been authorized by the European Commission after evaluating its safety. They are considered a source of next-generation probiotics.[95] *Desulfovibrio piger* uses lactate, pyruvate, ethanol, and hydrogen as electron donors for sulfate reduction. It also oxidizes lactate and pyruvate incompletely to acetate.[96] *Holdemania filiformis* is a carbohydrate-fermenting bacterium that produces acetic, lactic, and succinic acids.[97] *Turicibacter sanguinis* appears to have robust host effects impacting metabolism and overall gastrointestinal health.[98] *Barnesiella intestinihominis* is considered an "oncomicrobe" of great value since it improves the efficacy of alkaline immunomodulatory compounds for cancer treatment.[99] These results seem to indicate that the use of elicitors on red radish crops increases their glucosinolate content, enhancing the beneficial effect of this root (when consuming the entire plant) on the colon integrity and on the microbiota health status through an improved bacterial profile and SCFA production. This translates into a favored growth of several bacterial genera and species that are known to have a positive impact on the overall health status, working synergistically and improving the metabolome and therefore the intestinal functionality.

3.3. In Vitro Study of Functionalities Related to Metabolic Syndrome.

3.3.1. Endothelial Model to Analyze Blood Pressure Changes. Changes in the blood pressure markers ROS and END-1, after inducing a "hypertension" phenotype to the cell models, are represented in Figure 3. The treatment did increase ROS levels (Mbasal: 1.90 ± 0.13 vs Mtt: 2.09 ± 0.133 fluorometric units [FU]), but it decreased the gene expression of endothelin (END-1) (Mbasal: 1.65 ± 0.25 vs Mtt: 1.25 ± 0.15). Therefore, fresh radish may have a potential antihypertensive effect. These results are consistent with a previous study where an aqueous extract of radish seed showed a hypotensive effect in rats[100] and another where an ethyl acetate extract of radish leaves improved hypertension by increasing serum nitric oxide (NO) levels.[101] It seems to be a link between the increase of ROS post-treatment and the decrease of ET-1, as NO, in conjunction with other ROS, contributes to oxidative stress[102,103] and is also a potent vasodilating substance implicated in vasodilatation.[104]

3.3.2. Modulation of Cholesterol Metabolism in Hepatocytes. In the evaluation of biomarkers associated with cholesterol metabolism, the treatment induced a significant increase in the gene expression of the transporters ABCA1 (Mbasal = 1.00 ± 0.1 vs Mtt = 1.44 ± 0.07: relative units of biomarker mRNA vs beta-actin mRNA) and ABCG5 (Mbasal = 1.00 ± 0.09 vs Mtt = 2.08 ± 0.23: relative units of biomarker mRNA vs beta-actin mRNA), as shown in Figure 4. ABCA1 plays a key role in regulating cholesterol efflux. Reverse

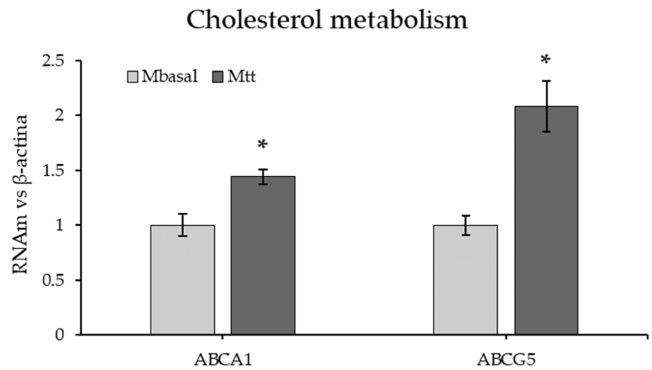

Figure 4. Changes in the gene expression of ABCA1 and ABCG5, associated with cholesterol metabolism, after being in contact with the red radish samples ($n = 4$). A Mann−Whitney U Test was applied for differences between two groups on a single, ordinal variable with no specific distribution. "*" indicates a very statistically significant difference ($p < 0.025$).

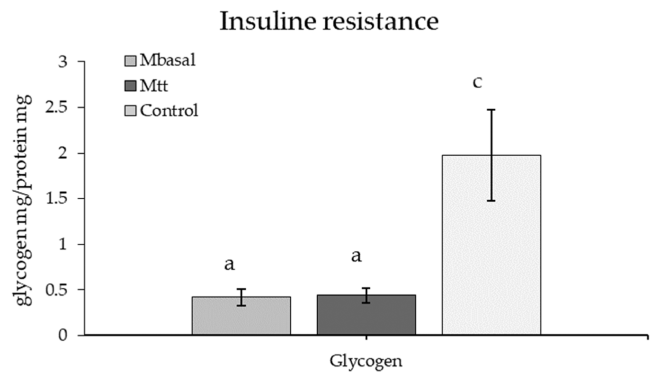

Figure 5. Changes in the intracellular glycogen associated with insulin resistance. The + control is an internal control that increases intracellular glycogen uptake ($n = 4$). Relative units: mg glycogen/mg protein. A Mann−Whitney U Test was applied for differences between two groups on a single, ordinal variable with no specific distribution. Different letters mean statistically significant differences: a, no significant difference ($p > 0.05$), and c, a very significant difference ($p < 0.025$).

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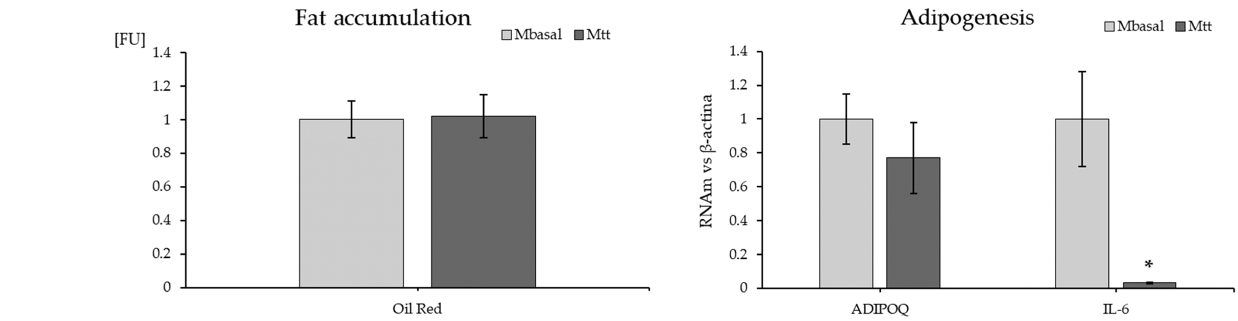

Figure 6. Changes in fat accumulation expressed in fluorometric units on the left and changes in adipogenesis markers ADIPOQ and IL-6 expressed in genic biomarker units on the right ($n = 3$). A Mann−Whitney U Test was applied for differences between two groups on a single, ordinal variable with no specific distribution. "*" indicates a very statistically significant difference ($p < 0.025$).

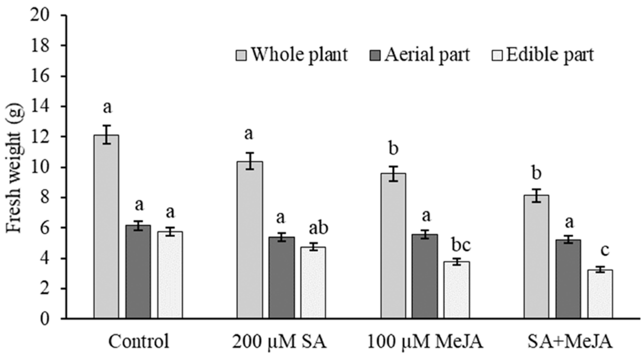

Figure 7. Representation of the plant growth with different elicitor treatments. Different letters indicate statistically significant differences in the HSD Tukey's test ($p < 0.05$).

cholesterol transport (RTC) is a multistep process that results in the movement of cholesterol from peripheral tissues to the liver through circulation. Lipidation of apolipoprotein A-I (apoA-I) by ABCA1 is the rate-limiting step in RTC and generates plasma HDL.[105] This is consistent with *in vivo* studies in rats,[106] suggesting a lipid-lowering and an antiatherosclerosis effect from radish, as it may help to reduce plasma cholesterol levels while increasing HDL.

3.3.3. Modulation of Insulin Resistance in Hepatocytes. Insulin resistance is one of the complications associated with metabolic syndrome. It is characterized by the fact that cells lose their normal response to insulin, having reduced glycogen storage. The HepG2 cells reproduce the scenario of insulin resistance, and results show (Figure 5) that the intracellular level of glycogen was not modified after treatment (Mtt = 0.44 ± 0.08 relative units) when compared to Mbasal (0.41 ± 0.09 relative units). This means that there is no improvement in the analyzed biomarker when compared to the control group (1.98 ± 0.5 relative units). However, *in vivo* studies with diabetic rats have shown improved blood glucose levels after the consumption of radish, in sprouts, or in juice.[107]

3.3.4. Study of Adipogenesis and Fat Formation in Adipocytes. Obesity is one of the traits usually involved in metabolic syndrome, and the adipocytes are the cells responsible for fat accumulation in the adipose tissue. To determine changes in adipogenesis and the general inflammatory status associated with obesity,[108] total fat accumulation

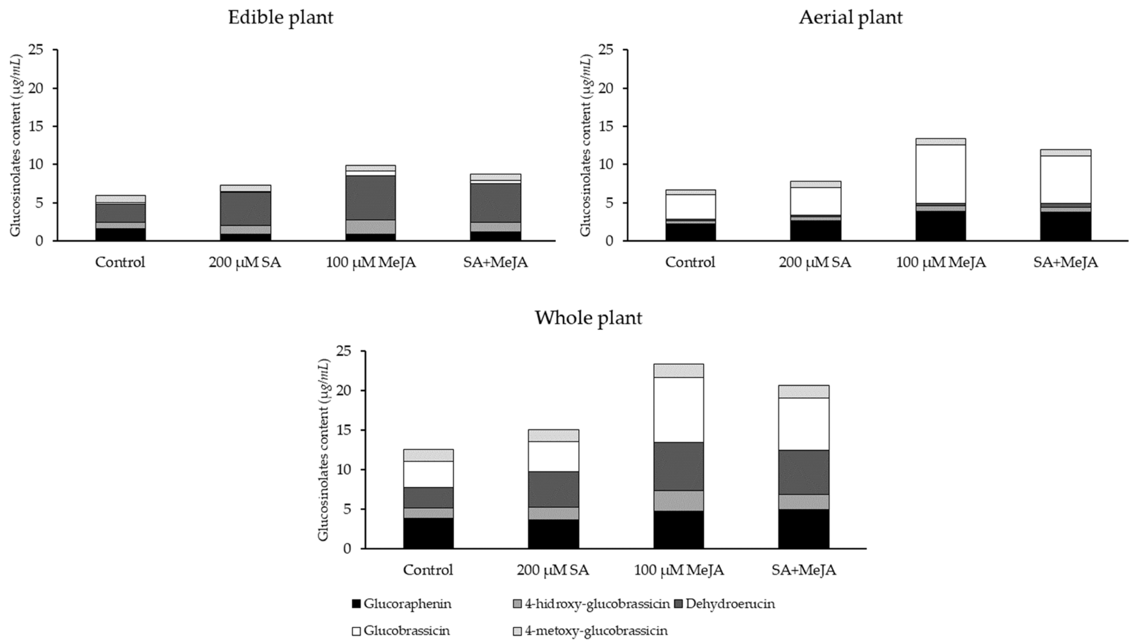

Figure 8. Representation of the elicitors' effect on glucosinolate content µg/mL on the different red radish parts. The whole plant graph represents the sum of the values from the edible and the aerial part.

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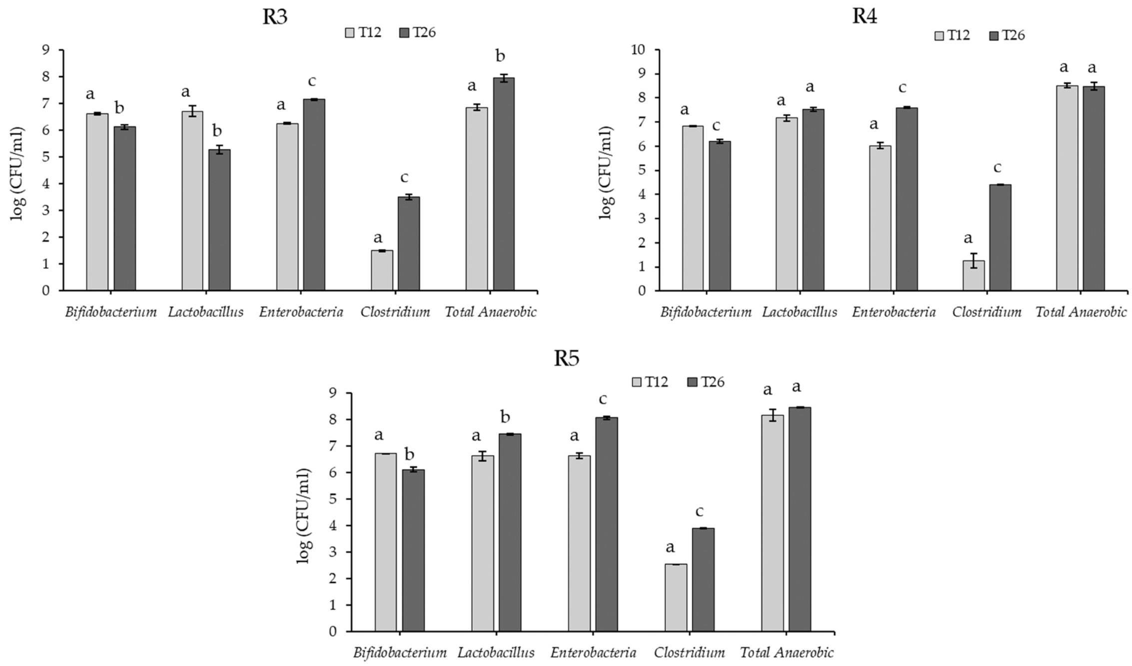

Figure 9. Microbiological count of **R3**, **R4**, and **R5** at T12 and T26 ($n = 2 \pm$ SD). The Mann−Whitney *U* test was applied for differences between two groups on a single, ordinal variable with no specific distribution. Different letters mean statistically significant differences: a, no significant difference ($p > 0.05$), and c, a very significant difference ($p < 0.025$). Results are shown as log (CFU/mL).

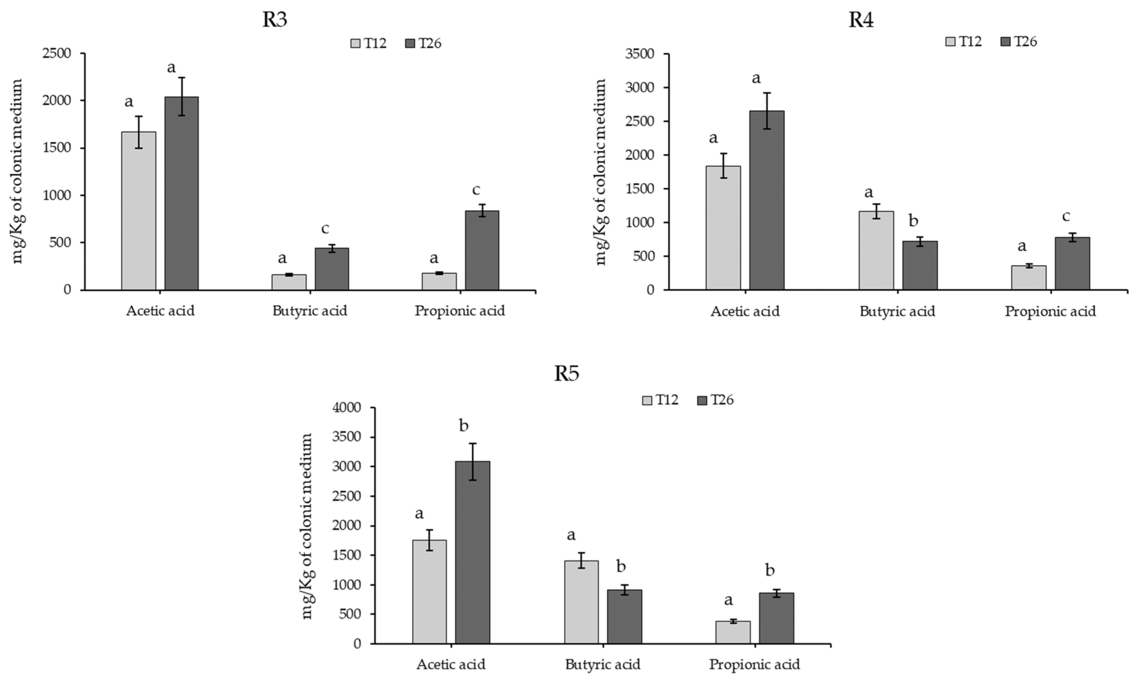

Figure 10. Representation of the SCFA production on **R3**, **R4**, and **R5** at T12 and T26 ($n = 2 \pm$ SD). The Mann−Whitney *U* test was applied for differences between two groups on a single, ordinal variable with no specific distribution. Different letters mean statistically significant differences: a, no significant difference ($p > 0.05$), and c, a very significant difference ($p < 0.025$).

and the gene expression of adiponectin (ADIPOQ) (an adipocyte-secreted factor, which is an insulin-sensitizing and anti-inflammatory adipokine, concentrations of which are decreased in obesity[109] and interleukin (IL)-6 cytokine (an inflammatory mediator) have been measured. The intracellular level of accumulated lipids was not modified with the treatment (Mbasal: 1.00 ± 0.11 vs Mtt: 1.02 ± 0.13); however, ADIPOQ showed a tendency toward reduced levels (Mbasal: 1.00 ± 0.15 vs Mtt: 0.77 ± 0.21), and IL-6 showed a significant reduction (Mbasal: 1.00 ± 0.28 vs Mtt: 0.031 ± 0.004) (Figure 6). *In vitro* and *in vivo* studies carried out with a radish extract alone or mixed with other vegetables have observed an improvement of some obesity-related parameters, such as the accumulation of fat or the modulation of adipokines such as adiponectin.[106,110] Thus, the results obtained regarding the decrease in interleukin IL-6 are consistent with the literature.

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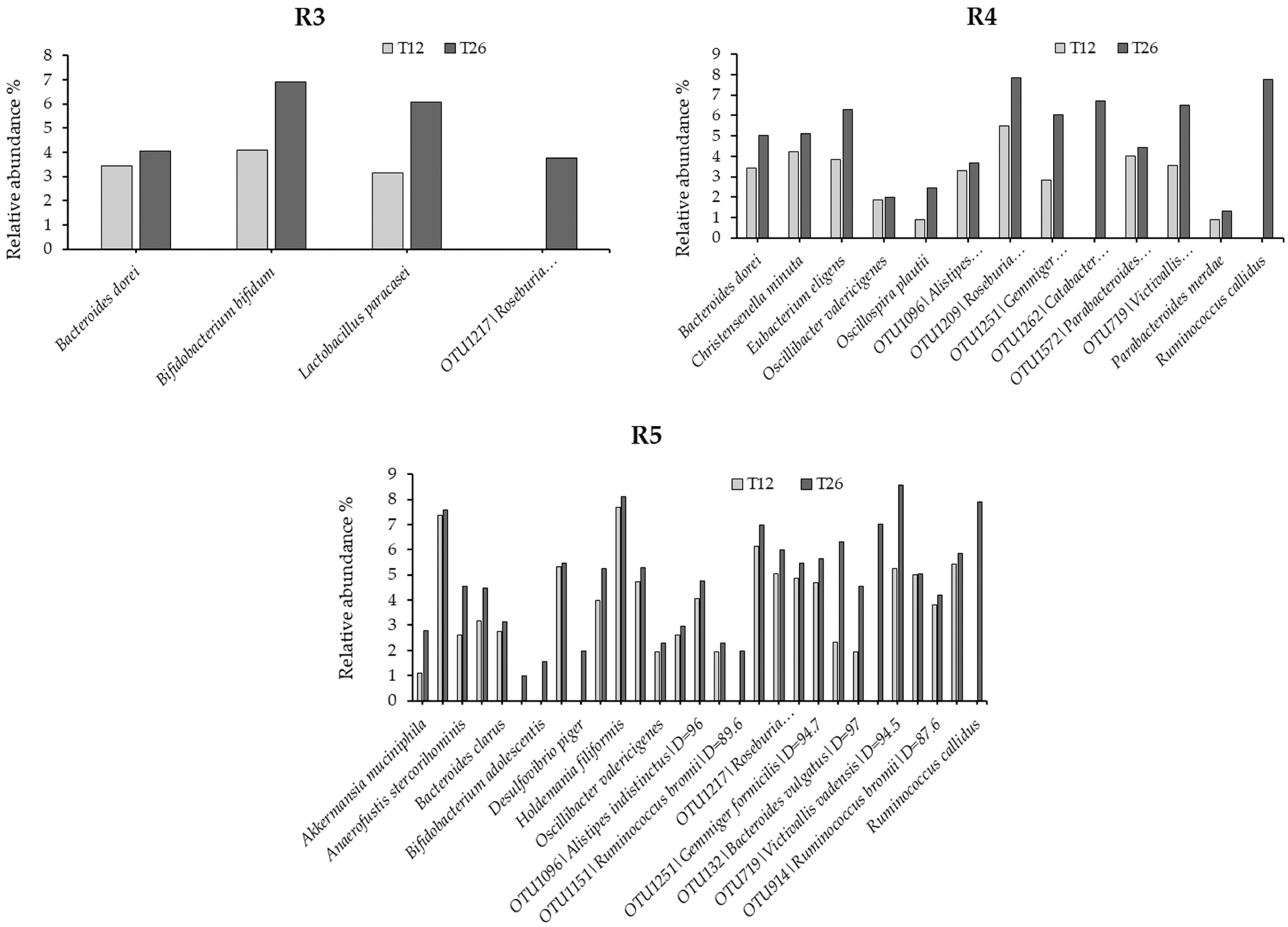

Figure 11. Representation of the relative abundance of bacterial species in the different reactors: (**R3**) ascending colon, (**R4**) transversal colon, and (**R5**) descending colon before (T12) and after the treatment period (T26) with fresh radish.

4. CONCLUSIONS

The use of elicitors on red radish crops seems to stimulate the GLS content of the plant, especially MeJA, which showed the highest increase. Therefore, the use of MeJA could be considered in crops to increase the nutritional quality of this food product. The analysis of isothiocyanate, the bioactive form of GLS, showed that the consumption of the whole red radish plant has a greater and more varied content of them, suggesting that adding the leaves of this root to the diet may have a healthier impact on health through these substances that exhibit powerful biological functions.

The SCFA analysis of the *in vitro* samples suggested that the treatment with red radish had an influence on SCFA production, especially on acetic and propionic acids. Although butyrate levels did not show any increase with the treatment, the general data suggest that the scenario has improved with the treatment. At some point, it would be coherent to perceive an increase of butyric acid, as many butyrate-producing bacteria increased with the treatment, and acetic acid, which is the main substrate for bacteria to produce butyrate. These results suggest that red radish consumption could modify the human gut microbiota profile toward a healthier one.

The evaluation of the metabolic syndrome-related functionalities of the bioactive compounds to modulate blood pressure, cholesterol metabolism, insulin resistance, and adipocyte metabolism showed a significantly reduced endothelin and IL-6 gene expression and cholesterol transporter-associated biomarkers (ABCA1 and ABCG5), suggesting that red radish consumption may improve some risk factors associated with metabolic syndrome.

Altogether, these results suggest that the use of elicitors on red radish crops may contribute to enhancing the beneficial impact of some food products toward a greater improvement of the general health status of individuals with metabolic syndrome, from the inside (gut microbiota) to the outside (risk factors such as blood pressure, cholesterol, and obesity).

■ COMPLEMENTARY MATERIALS

Figures 7−11.

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Notes

The authors declare the following competing financial interest(s): Conflicts of Interest: Agatha Agudelo is employee at Sakata Seeds Iberica SL., company. The grant obtained by the company from the Spanish Ministry of Economy, Industry and Competitiveness under the programme "Convocatoria de Programa Estratgico CIEN, Ref. IDI-20170842, supported this research.

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Navarra (protocol code 132/2015).

Informed consent was obtained from all subjects involved in the study.

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