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Microbiota modulation and effects on metabolic biomarkers by orange juice: a controlled clinical trial

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The impact of habitual orange juice consumption on microbiota, lipid and sugar metabolism was investigated in a controlled clinical trial. The clinical procedure is as follows: ten women who had a regular diet without orange juice for 30 days (OJ-free diet), followed by a regular diet plus 300 ml d^{-1} orange juice for 60 days (OJ-Diet), and 30 days with a regular diet without orange juice (Washout). Biochemical and dietary parameters were monitored, and blood, urine and stool samples were collected every 30 days until the end of the study. Hesperidin and naringin metabolites in the urine were identified by UHPLC, and the microbiota composition of the feces was determined by 16S rRNA. At the end of the OJ-Diet, there was a reduction in glucose (–6.5%), insulin (–33%), insulin resistance (–44%), LDL-C (–16%) and triglycerides (–30%). After the washout, these parameters returned to their initial values. There were no changes in the body weight or fat during the experimental time. The intestinal bacteria, *Lactobacillus* spp., *Akkermansia* spp., and *Ruminococcus* spp., increased after the intervention with orange juice. In addition, an inverse correlation was detected between these bacteria and glycemia, insulin, HOMA-IR, triglycerides, total cholesterol and LDL-C, but a direct correlation with HDL-C. In conclusion, orange juice showed a prebiotic effect, modulating the intestinal microbiota while improving the glycemia and lipid profiles.

Introduction

Nutrition experts recognize that pure fruit juices help to meet nutritional needs of valuable nutrients such as vitamin C, folate and potassium.[1] Additionally, bioactive compounds in fruit juices, such as citrus flavonoids, may improve the antioxidant and anti-inflammatory status, and lower blood pressure and cholesterol in normal and dyslipidemic subjects.[2–4] Only a small fraction of deglycosylated flavonoids by intestinal bacteria are absorbed, while most of them are degraded to short-chain fatty acids (SCFA), which help to maintain microbiota homeostasis, in addition to other beneficial effects on human metabolism.[5]

The gastrointestinal tract is inhabited by 10^{11} cells per mL of luminal content, and the main microbiota phyla are Bacteroidetes, Firmicutes and Actinobacteria, with lower abundances of Proteobacteria.[6] The microbiota is responsible for protecting the intestinal mucosa against pathogenic microorganisms, modulating the immune system and synthesizing some vitamins.[7] It is known that the metabolism and intestinal microbiota composition can be influenced by dietary habits, probiotic and prebiotic ingredients, and bioactive compounds.[6,7] Evidence from human studies has shown that some polyphenols may contribute to the maintenance of the intestinal health, preserving microbial balance by stimulating the growth of beneficial bacteria and inhibiting pathogenic bacteria.[8]

A previous study with "Human Intestinal Microbial Ecosystemic Simulator" (SHIME®) showed that orange juice had a selective prebiotic effect on the intestinal mucosa, reducing pathogenic microorganisms and increasing others with positive effects in the intestine.[9] A recent clinical trial by our group showed that the administration of orange juice for two months improved blood levels of cholesterol and glucose and insulin sensitivity and positively modulated the microbiota composition and metabolic activity of the microorganisms, detected by the increase of *Bifidobacterium* spp. and *Lactobacillus* spp., and SCFA production. These results showed that the consumption of orange juice could improve the microbial composition, with positive consequences on the body composition and biochemical parameters.[10]

Based on the cited evidence, the hypothesis of this study is that the habitual consumption of orange juice improves

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microbial colonization, in addition to promoting positive changes in lipid and glucose metabolism. Therefore, this study aims to explore the impact of the daily intake of orange juice on the intestinal microbiota and metabolic markers in women with healthy eating habits.

Materials and methods

Trial design

A controlled clinical study with temporal series intergroup design is carried out, which includes (1) ***O*range Juice-free Diet** (**OJ-free Diet**): 30 days with a habitual food pattern, without restriction of energy, avoiding rich sources of flavonoids, probiotics and prebiotics, and alcoholic beverages (1^{st} to 30^{th} day), (2) **Diet plus Orange Juice** (**OJ-Diet**): intervention of 60 days consuming 300 mL d^{-1} of orange juice (31^{st} to 90^{th} day) and (3) **Orange Juice-free Diet** (**Washout**): 30 days under a regular diet without orange juice (91^{st} to 120^{th} day) (Fig. 1). The sample size was calculated based on previous data from urine hesperetin after intake of 100% pure orange juice[11] with a confidence level of 95% (α = 5%) and a power of 80%. All participants maintained the standardized diet and dietary restrictions during the 120 days.

Subjects

Ten healthy female volunteers were selected from among students of the Pharmacy School, UNESP, in Araraquara, Brazil, and all gave their informed consent prior to their inclusion in the study. Inclusion criteria were aged 20–35 years, non-smokers and not vegetarians, without food allergy or intolerance and exclusion criteria were hormone treatments, dietary supplements and medication for gastrointestinal or metabolic disease, probiotics or prebiotics in the last 3 months, antibiotics in the last 6 months, heavy alcohol drink consumption or intensive physical exercise. Their usual diets were based on a variety of healthy foods from all food groups, with low levels of unhealthy fats, salt and added sugar. The Ethical Board of the Pharmacy School, UNESP (protocol 1644906) approved the study, and enrolled in the Clinical Trial Protocol Registration and Results System (ID: NCT03032861).

Orange juice

Commercial 100% pure orange juice made with Pera-Rio variety oranges was provided by a local producer (Citrosuco S.A., Matao, SP, Brazil). Orange juice physicochemical characteristics were determined according to AOAI.[12] Flavanones were extracted from homogenized orange juice, and triplicate samples were centrifuged and supernatants were collected and were spiked with internal standard 7-crystalline ethoxycoumarin (IS) and analyzed without further processing. The pellets were extracted with 80% dimethyl sulfoxide (DMSO) and placed in an ultrasonic bath. The supernatant was filtered, and the precipitate was extracted again. The supernatants were combined and the flask was filled to 25 mL with 80% (v/v) DMSO. The samples were cleared by passing through a C18-E Sep Pac cartridge Strata® (50 mg per 1 mL) preconditioned with DMSO and Milli-Q water. Flavanones were eluted from the cartridge with DMSO solution, and 400 μl was filtered through a 0.22 μm syringe filter (Allcrom, Sao Paulo, BR) for ultra-high performance liquid chromatography with a visible ultraviolet detector (UHPLC-UV-VIS).

Interventions

The body weight (kg), height (m), lean mass (kg), fat mass (kg) and body fat percentage were measured by using bioimpedance (Inbody 720®) at the basal period (0 day) and every 30 days until the end of the trial. The body mass index (BMI) was interpreted according to the World Health Organization.[13] Food intake was estimated using 3-day food records of non-consecutive days, applied at the beginning of the experiment (Basal period) and subsequently every 15 days until the end of the trial. The nutritional analyses of food intake were performed using the NUTRILIFE® Software based on the Brazilian Food Composition Table.[14]

Fasting blood samples from participants were collected at the baseline and before each experimental period: **OJ-free Diet** (30 days), **OJ-Diet** (60 days) and **Washout** (30 days). All samples were collected in a local clinical laboratory, and they were analyzed for total cholesterol, HDL-C, LDL-C, triglycerides, glucose and insulin. The glycemic and insulinemic curves were recorded after glucose loading at 0, 30, 60, 90 and 180 min, scheduled twice, at the end of the **OJ-free Diet** and after 60 days of the **OJ-Diet**. Insulin resistance was estimated by using HOMA-IR.[15] Lipid peroxidation was evaluated by the thiobarbituric acid reactive substance test[16] and the total antioxidant capacity (TAC) was evaluated by the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) radical assay.[17]

24-hour urine samples from each participant were collected at the beginning of basal, **OJ-free Diet**, **OJ-Diet** and **Washout** periods. Urine was maintained at 4 °C and the total volume was recorded. Homogenized urine samples were stored in sterile tubes and kept at −80 °C until analysis. For the extraction of flavanone metabolites in urine, the pH was adjusted, and then the sample was filtered in a C18-E Sep Pac cartridge Strata® (50 mg per 1 mL) preconditioned with methanol and water (1 : 2, v/v), and eluted in methanol. This extract was filtered and centrifuged and the supernatant was collected. The procedure was repeated twice and the organic phases containing the metabolites were combined and dried under vacuum using a 40 °C centrifugal evaporator (SpeedVac SVC 200H, Savant, Holbrook, NY, USA). The resulting extracts were re-dissolved in 400 μl of DMSO (containing IS) and filtered through a 0.22 micron syringe filter for UHPLC-UV-VIS analysis.

24-hour total feces were collected and kept at 4 °C until delivery. Samples were obtained on the eve of the 30^{th} day of the **OJ-free Diet**, **OJ-Diet** and **Washout**. They were homogenized and a portion was stored in a sterile plastic tube (10 g) and held at −80 °C until analysis. Subsequently, the samples were lyophilized and sent for microbiological analysis employing the 16S rRNA gene sequencing. To start the DNA isolation, 700 μL of bead solution was added to the freeze-dried sample,

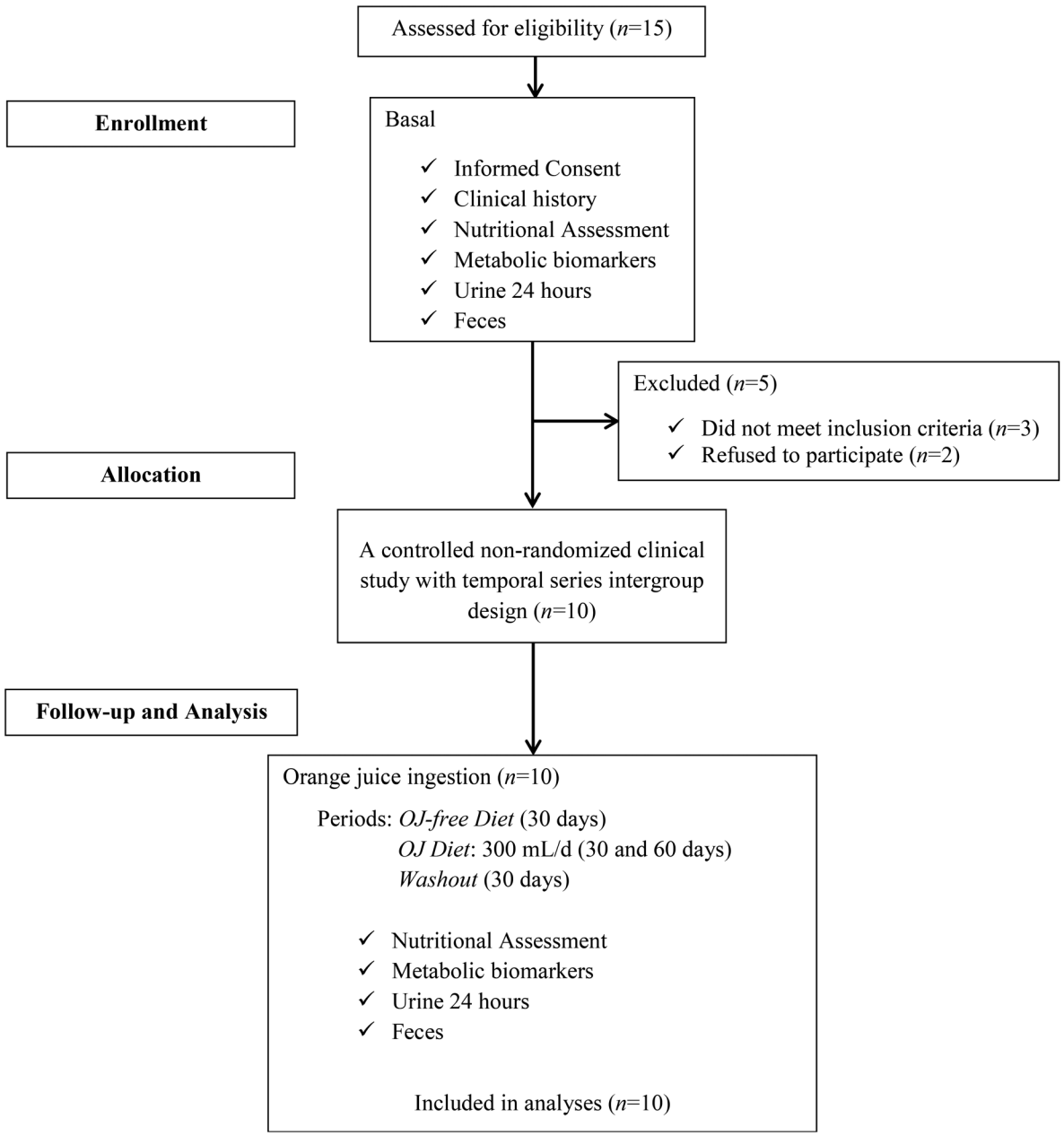


Fig. 1 Schematic of study design. Each participant took part in this controlled clinical study with temporal series intergroup design for 120 days.

and the next steps were performed according to the manufacturer's manual. DNA isolation of each sample was performed using the "PowerLyzer@PowerSoil DNA Isolation Kit" (Qiagen, Valencia, USA) and primer pairs 319F/806R were used to amplify the V3–V4 domains of the 16S rRNA using a two-step PCR procedure. The library was quantified *via* qPCR followed by 300 bp paired-end sequencing using an Illumina MiSeq instrument in the Genome Center DNA Technologies Core at the University of California, Davis. Demultiplexing of the Raw FASTQ files and adapter trimming of sequences were performed using dbcAmplicons v. 0.8.5. The unmerged forward and reverse reads were imported into QIIME2 version 2017.12, and sequence variants were determined following the DADA2 analysis pipeline. Measures of β-diversity were generated with weighted UniFrac analysis, and the resulting distance matrix was used to perform principal coordinate analysis (PCoA). Taxonomic classification was assigned using a Naive Bayes filtered classifier trained on the 99% identity Green genes database, version 13.8. The metagenomic project has been submitted to the NCBI-SRA database under accession number PRJNA552993.

UHPLC-UV-VIS-PDA analysis

Analysis of orange juice flavonoids: hesperidin (HSP), naringin (NRG), hesperetin (HSPT) and naringenin (NRGN) metabolites (hesperetin-7-glucuronide and hesperetin-3-glucuronide and

naringenin-7-*O*-glucuronide) in the urine of all subjects was performed by UHPLC, adapted for maximum sensitivity of flavanones.[18] Analyses were performed on a Shimadzu® High Efficiency Liquid Chromatograph – Nexera X2 system connected in parallel with a SPD-M20A UV-VIS PDA detector. Separation of compounds was performed on a Shim-pack XR-ODS III C18 column (2.0 × 200 mm), using linear gradients of acetonitrile/0.5% formic acid, initially composed of 10 : 90 (v/v) and increased in acetonitrile content to 30 : 70, 55 : 45 30 : 70, 55 : 45, and then decreased to 10 : 90 (v/v) at 10, 17 : 30 and 20 min, respectively, at a flow rate of 0.40 mL min^{-1} and 0.10 mL injected sample. Data handling was performed by using LabSolutions software (Shimadzu Corporation, Japan). Detection and identifications of the metabolites were based on their characteristic UV spectra (285 nm).

Statistical analysis

The data distributions were tested for normality, and subsequently, analyzed by One Way Repeated Measures Analysis of Variance when normal or Friedman Repeated Measures Analysis of Variance when not normal, with a significance level of $p < 0.05$, using Sigma Stat version 3.11 (Systat Software Inc., USA). A simple correspondence analysis was used to test the correlation between periods with and without orange juice and the microbiota composition using the Minitab Software (State College, USA) (Minitab 2010). Correlation analyses were performed to correlate the changes of metabolic biomarkers with specific groups of bacteria using the Spearman correlation test. The Spearman correlation test was conducted using the open-source RStudio software program (RStudio 2017).

Results

Subjects

The mean baseline characteristics of female participants were 26.8 ± 4.6 years, body weight of 63.4 ± 9.6 kg and BMI of 24.1 ± 3.3 kg m^{-2} (Table 1). Eight participants were eutrophic, and two were overweight. Food intakes were nutritionally balanced before (**OJ-free Diet**), during (**OJ-Diet**) and after the supplementation with orange juice (**Washout**). During all periods, vitamin C intake was above the daily recommendation.[19]

Orange juice composition

Nutritional composition and physicochemical characteristics of orange juice were within the stipulated standards (Table 2). Flavanone content found in the pellets and supernatants, along with percent averages, is listed in Table 3. The flavanone glycosides, HSP and NRG, were identified at UPLC based on the standard elution times and UV characteristics in comparison with the authentic standards.

Nutritional assessment

The body composition was not changed throughout the experimental period, and on average, the participants maintained a eutrophic nutritional status (Table 4). Dietary assessment showed that macronutrient intakes, such as carbohydrates, proteins and lipids, were according to the Acceptable Macronutrient Distribution Range.[19] Supplementation with orange juice showed an increase in vitamin C intake by 75% in relation to the **OJ-free Diet**. After the **Washout**, there was a reduction of 39% of vitamin C intake (Table 5).

Metabolic biomarkers

All biochemical measures were within the reference values for all periods of the experiment, compatible with the volunteers' apparent good health (Table 1). During the orange juice period, there was a reduction of glycemia (−6.3%), insulin (−33%), HOMA-IR (−44%), triglycerides (−30%), total cholesterol (−14%) and LDL-C (−16%) compared to the values of the

Table 1 Baseline characteristics of female volunteers: body composition, metabolic biomarkers, and urinary flavanones (n = 10)

Variables	Baseline
(A) Body composition	
Weight (kg)	63.0 ± 9.13
BMI (kg m^{-2})	23.9 ± 3.13
Lean muscle mass (kg)	38.4 ± 4.03
Body fat mass (kg)	22.9 ± 4.96
% Body fat	34.8 ± 5.67
(B) Metabolic biomarkers	
Glucose (mg dL^{-1})	79.1 ± 6.28
Insulin (μU mL^{-1})	11.5 ± 1.87
HOMA-IR	2.41 ± 0.37
Triglycerides (mg dL^{-1})	125 ± 38.9
Total cholesterol (mg dL^{-1})	198 ± 27.8
HDL-C (mg dL^{-1})	59.6 ± 7.70
LDL-C (mg dL^{-1})	114 ± 25.5
Antioxidant capacity (mM)	1.94 ± 0.02
Lipid peroxidation (mM)	1.3 ± 0.7
(C) Urinary metabolites	
Hesperetin-3-*O*-glucoronide (mg)	2.68 ± 0.31
Hesperetin-7-*O*-glucoronide (mg)	2.35 ± 0.29
Naringenin-7-*O*-glucuronide (mg)	0.23 ± 0.06
(D) **Bioavailability %**[a]	
HSP in 24 h (dose 72.9 mg)	6.89 ± 0.82
NRG in 24 h (dose 14.6 mg)	1.57 ± 0.42

[a] Bioavailability = ($AUC_{0-24\ h}$ urine/oral dose) × 100.

Table 2 Nutritional and physicochemical composition in 300 mL of commercial processed 100% pure orange juice

Nutritional composition of orange juice[a]	
Energy (kcal)	144.0
Total carbohydrate (g)	33.9
Fiber, total dietary	0.84
Protein (g)	2.0
Lipids (g)	0.4
Ascorbic acid (mg)	98.7
Physicochemical composition[b]	
pH	4.4
Total soluble solids (°Brix)	14.4
Titratable acidity (g citric acid per 100 mL)	1.8
Ratio (soluble solids/titratable acidity)	19.8
Total antioxidant capacity (μmol Trolox per 100 mL)	4.3 ± 0.4

[a] USDA: National Nutrient Database for Standard Reference, Release 21. NDB 09209. [b] Determined according to AOAC International.[12]

Table 3 Flavanones in the pellets and supernatant fractions in 300 mL of commercial processed 100% pure orange juice measured by UPLC-UV-VIS-PDA

	Pellet		Supernatant		Total	
Orange juice	Mg	%	mg	%	mg	%
Hesperidin	52.1 ± 1.2	71.5	20.8 ± 0.9	28.5	72.9 ± 1.6	100
Naringin	5.5 ± 0.2	37.5	9.1 ± 0.5	62.5	14.6 ± 0.5	100
Total flavanones	57.6 ± 0.8	65.8	29.9 ± 0.7	34.2	87.5 ± 1.0	100

Table 4 Body composition and metabolic biomarkers of women during the clinical trial in the **OJ-free Diet**, **OJ-Diet** and **Washout** for 120 days (n = 10)

			OJ-Diet (300 mL d^{-1})		
Variables	Diet Timeline	OJ-free Diet 30th day	60th day	90th day	Washout 120th day
(A) Body composition					
Weight (kg)		63.4 ± 9.64	63.5 ± 9.23	63.6 ± 9.51	63.7 ± 9.01
BMI (kg m^{-2})		24.1 ± 3.29	24.1 ± 3.13	24.1 ± 3.26	24.2 ± 3.05
Lean muscle mass (kg)		38.7 ± 4.31	39.3 ± 4.45	38.8 ± 4.52	39.6 ± 4.65
Body fat mass (kg)		23.0 ± 5.09	22.7 ± 5.03	23.3 ± 5.23	22.4 ± 4.85
% Body fat		34.5 ± 5.13	33.9 ± 4.75	34.6 ± 5.13	33.5± 5.25
(B) Metabolic biomarkers					
Glucose (mg dL^{-1})		80.1 ± 4.77^{a}	76.7 ± 7.30^{ab}	75.0 ± 8.01^{b}	79.9 ± 6.92^{a}
Insulin (μU mL^{-1})		11.9 ± 2.17^{a}	10.5 ± 2.82^{ab}	8.07 ± 1.96^{b}	11.2 ± 2.69^{ab}
HOMA-IR		2.42 ± 0.55^{a}	1.88 ± 0.50^{ab}	1.36 ± 0.30^{b}	2.06 ± 0.33^{a}
Triglycerides (mg dL^{-1})		128 ± 30.7^{a}	115 ± 28.8^{ab}	88.9 ± 20.7^{b}	108 ± 25.6^{ab}
Total cholesterol (mg dL^{-1})		201 ± 28.4^{a}	182 ± 24.4^{ab}	173 ± 28.8^{b}	194 ± 22.1^{a}
HDL-C (mg dL^{-1})		55.5 ± 9.40	60.1 ± 10.3	59.8 ± 12.9	58.7 ± 13.6
LDL-C (mg dL^{-1})		114 ± 27.9^{a}	97.1 ± 24.1^{b}	95.4 ± 19.6^{b}	100 ± 24.1^{ab}
Antioxidant capacity (mM)		1.93 ± 0.02^{b}	1.93 ± 0.02^{b}	1.94 ± 0.06^{a}	1.93 ± 0.03^{b}
Lipid peroxidation (mM)		1.4 ± 0.7^{b}	1.3 ± 0.6^{b}	1.2 ± 0.6^{a}	1.3 ± 0.7^{b}

Different letters represent the significant difference (p < 0.05) between periods.

Table 5 Intake of energy, macronutrients and micronutrients of women during the clinical trial in the **OJ-free Diet**, **OJ-Diet** and **Washout** for 120 days (n = 10)

		OJ-free Diet			OJ-Diet (300 mL d^{-1})				Washout	
Nutrients	Diet Days	0 day	15th day	30th day	15th day	30th day	45th day	60th day	15th day	30th day
Energy (kcal)		1937 ± 193	1920 ± 273	2048 ± 156	2046 ± 340	1993 ± 253	1887 ± 109	2046 ± 350	2046 ± 350	2033 ± 250
Carbohydrate (g)		254 ± 29.5	265 ± 36.0	266 ± 30.6	271 ± 39.5	260 ± 38	265 ± 30.9	264 ± 35.6	274 ± 35.3	275 ± 37.5
Fiber (g)		27.6 ± 6.8	24.3 ± 4.5	22.9 ± 5.24	24.8 ± 4.7	22.6 ± 5.6	25.8 ± 4.8	25.9 ± 5.6	26.8 ± 2.99	25.1 ± 6.1
Protein (g)		96.7 ± 19.1	94.3 ± 12.1	95.3 ± 14.3	92.4 ± 16.1	92.1 ± 22.9	92.9 ± 20.9	92.2 ± 22.8	93.6 ± 6.5	89.8 ± 20.3
Total fat (g)		65.4 ± 4.5	66.5 ± 2.8	64.6 ± 5.5	66.6 ± 3.6	66.3 ± 3.6	65.6 ± 4.3	65.8 ± 6.5	66.7 ± 3.9	65.9 ± 5.0
Cholesterol (mg)		253 ± 61.93	242 ± 38.3	238 ± 59.3	249 ± 58.4	236 ± 54	266 ± 31.3	236 ± 58.2	247.8 ± 58.2	268 ± 51.1
Saturated FA (g)		16.18 ± 3.8	17.2 ± 3.4	17.8 ± 4.1	17.7 ± 2.0	16.5 ± 4.3	16.8 ± 3.6	16.5 ± 2.8	18.4 ± 1.9	17.86 ± 1.92
Vitamin C (mg)		148 ± 36^{a}	146 ± 26.3^{a}	145 ± 28.5^{a}	250 ± 39.9^{b}	252 ± 29.9^{b}	256 ± 32.9^{b}	254 ± 35.5^{b}	151 ± 23.9^{a}	155 ± 36.9^{a}

Different letters represent the significant difference (p < 0.05) between periods.

OJ-free Diet (Table 4). After the **Washout**, there was an increase in glycemia (6.5%), insulin (38%), and HOMA-IR (51%), triglycerides (21%), total cholesterol (12%) and LDL-C (4.9%).

The antioxidant capacity increased 0.5% and lipid peroxidation increased by 14% in the **OJ-Diet** compared to values of the **OJ-free Diet**. The area under the curve (AUC) and the maximum concentration (C_{max}) of the glycemic and insulin curves are presented in Fig. 2. The AUC and C_{max} of insulin decreased by 16% (p < 0.01) in the **OJ-Diet** (Fig. 2).

Urine metabolites

Urine samples showed similar amounts of hesperetin-3′-*O*-glucoronide, hesperetin-7-*O*-glucuronide and naringenin-7-*O*-glucuronide at the two sampling times during the **OJ-Diet** (60th and 90th day). Metabolites were identified as hesperetin-7-*O*-glucuronide and hesperetin-3′-*O*-glucuronide, respectively, by comparison with authentic standards. The percentages of excretion/absorption estimated were within the values found

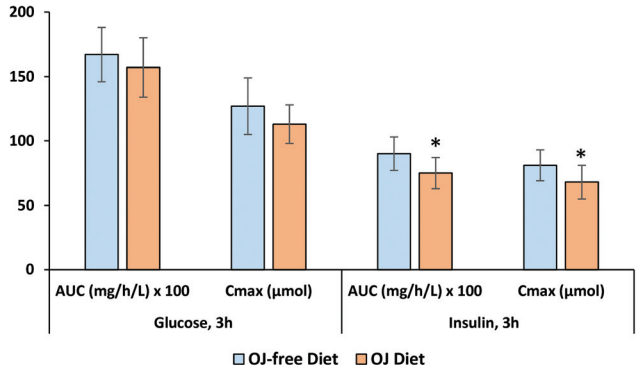


Fig. 2 Area under the curve (AUC) and maximum concentration (C_{max}) of the glycemic and insulin curves in the **OJ-free Diet** and **OJ-Diet**, *p < 0.01 (n = 10).

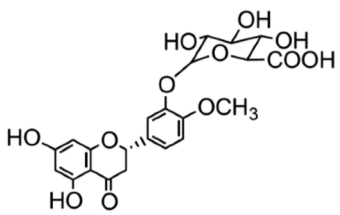
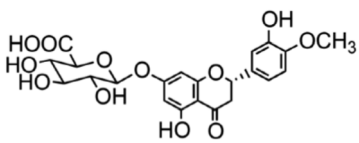
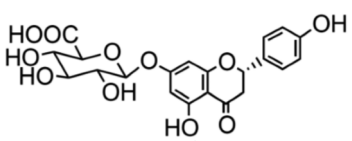
in the literature. However, once the volunteers stopped eating or drinking any source of citrus fruits, no metabolites were detected in the urine of any subject (Table 6). Bioavailability was estimated as the ratio of 24-hour urinary excretion to the oral dose, expressed as a percentage of the oral dose of parental flavanones.[20]

Microbiota composition

Sequencing readings of 16 100 (**OJ-free Diet**) and 43 859 (**OJ-Diet**) 16S rRNA were obtained from twenty fecal samples. Most of the OTU sequences were attributed to nine major phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Cyanobacteria, Synergistetes, Fusobacteria, Tenericutes and Verrucomicrobia. Comparing the abundance of the volunteer's microbiota in the **OJ-free Diet** with the **OJ-Diet** we observed two distinct groupings of the samples, as shown in Fig. 3. These results imply a significant effect of the regular intake of orange juice on the relative abundance of the microorganisms.

In both periods, **OJ-free Diet** and **OJ-Diet**, the most abundant phyla found at volunteers' feces were Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria (Fig. 4). A significant increase in the level of Actinobacteria (p = 0.003) was observed after the **OJ-Diet** mainly due to the abundance of *Bifidobacteriaceae* (p = 0.002), *Atopobiaceae* (p = 0.001), *Coriobacteriaceae* (p = 0.005), and *Eggerthellaceae* families (p = 0.002). There was a decrease in Bacteroidetes after the **OJ-Diet** with greater contribution of the *Bacteroidaceae*, *Barnesiellaceae*, *Muribaculaceae*, *Prevotellaceae*, *Rikenellaceae* and *Tannerellaceae* families. Firmicutes were decreased in the **OJ-Diet** with greater

Table 6 Metabolites of hesperetin (HSPT) and naringenin (NRGN) in the 24 h urine of women during the clinical trial in the **OJ-free Diet**, **OJ-Diet** and **Washout** for 120 days (n = 10)

	Diet	OJ-free Diet	OJ-Diet (300 mL d^{-1})		Washout
Urinary metabolites	Timeline	30th day	60th day	90th day	120th day
HSPT-3-O-Glucoronide (mg)		n.d.[a]	2.68 ± 0.19	2.73 ± 0.29	n.d.
					
HSPT-7-O-Glucoronide (mg)		n.d.	2.39 ± 0.24	2.41 ± 0.28	n.d.
					
Total (mg)		n.d.	5.07 ± 0.29	5.13 ± 0.42	n.d.
Bioavailability HSPT[b] (%) (HSPT oral dose = 72.9 mg)		n.d.	6.95 ± 0.39	7.04 ± 0.57	n.d.
NGRN-7-O-Glucuronide (mg)		n.d.	0.24 ± 0.11	0.29 ± 0.10	n.d.
					
Bioavailability NGRN[b] (%) (NGRN oral dose = 14.6 mg)		n.d.	1.65 ± 0.73	2.00 ± 0.68	n.d.

[a] n.d., not detected. [b] Bioavailability = ($AUC_{0-24\ h}$ urine/oral dose) × 100.

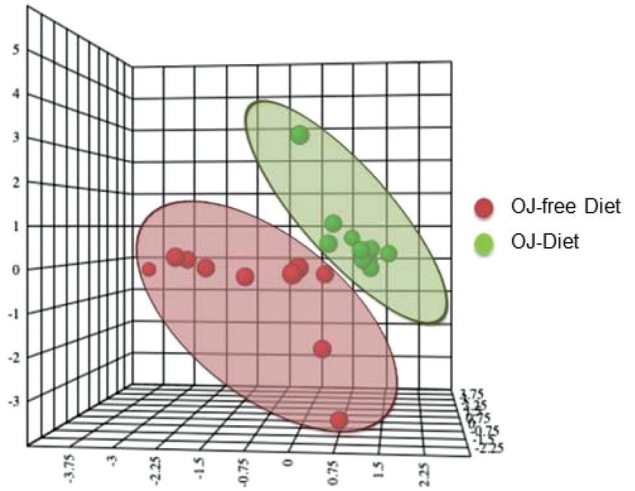


Fig. 3 Principle coordinate analyses of the intestinal microbiota of healthy women in the **OJ-free Diet** and **OJ-Diet** (n = 10).

contribution of the *Lactobacillaceae*, *Leuconostocaceae*, *Clostridiaceae* 1, *Lachnospiraceae*, *Peptococcacea* and *Ruminococcaceae* families. There was a small increase in the *Clostridiales* Family XIII and *Streptococcaceae*. Proteobacteria decreased in the **OJ-Diet** by a decrease of their only *Enterobacteriaceae* family. *Verrucomicrobia* (p = 0.002) increased mainly for the *Akkermansiaceae* family (Fig. 5a).

Comparison of the microbiota of healthy women in the **OJ-free Diet** and the **OJ-Diet** showed two different clusters suggesting that orange juice changed the intestinal bacterial community (Fig. 5b). For the *Akkermansiaceae* family it is interesting to highlight a significant increase (p = 0.04) of *A. muciniphila* abundance after supplementation with the juice (Fig. 6).

Correlation analysis was performed to identify the bacteria related to changes in the biochemical parameters (Fig. 7). The major findings showed negative correlations with glucose and relative abundance of *Lactobacillus* spp., (p = 0.001) and, *Akkermansia* spp. with cholesterol (p = 0.002), triglycerides

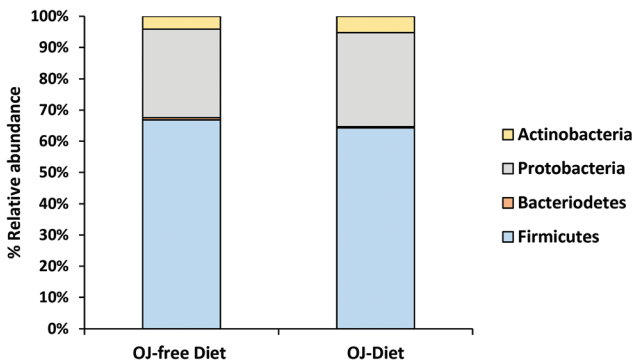


Fig. 4 The main bacterial phyla determined in the microbiota of healthy women in the **OJ-free Diet** and **OJ-Diet** (n = 10).

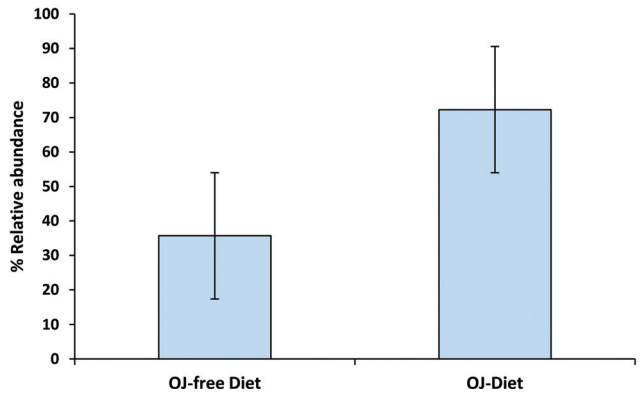


Fig. 6 Relative abundance (%) of *Akkermansia muciniphila* in the microbiota of healthy women in the **OJ-free Diet** and **OJ-Diet** (n = 10).

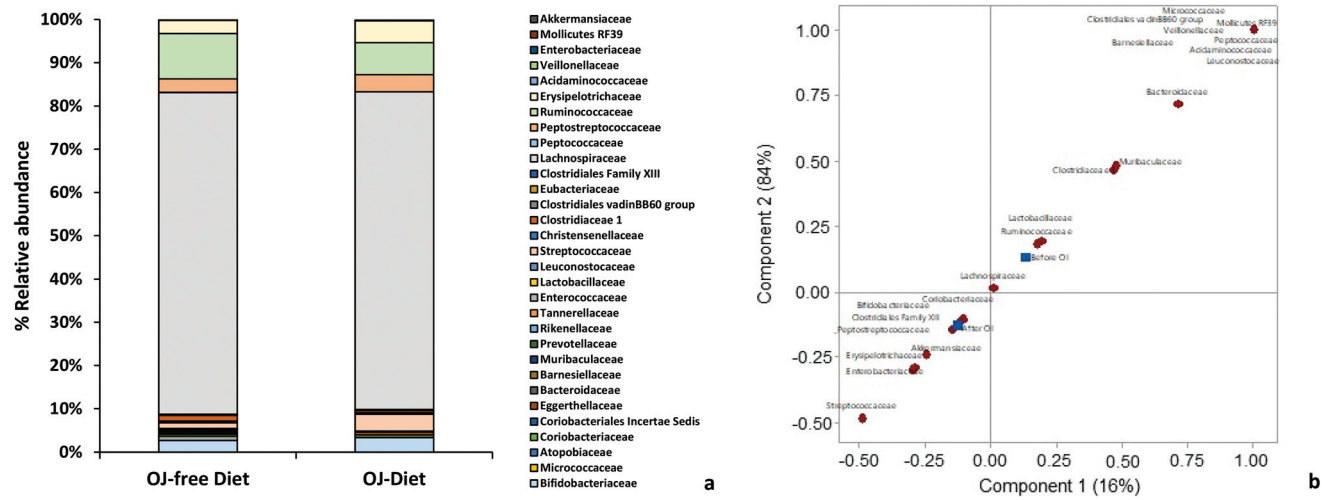


Fig. 5 Microbiota composition at a family level in the **OJ-free Diet** and **OJ-Diet**. (a) The main bacterial family determined before and after orange juice ingestion in fecal samples of volunteers (n = 10); (b) simple correspondence analysis showing a relationship between the before and after orange juice ingestion and bacterial families (n = 10).

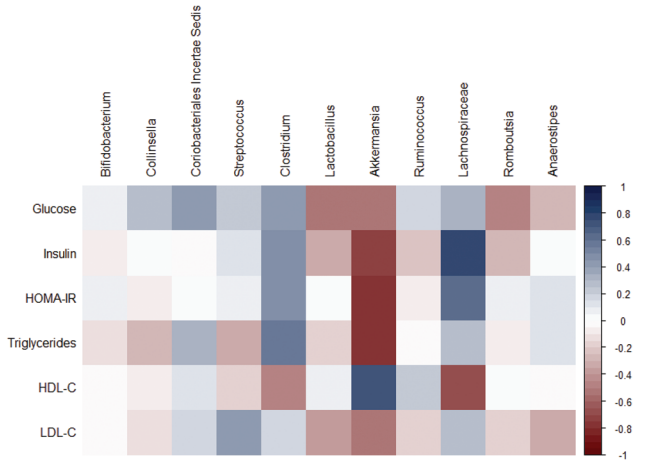


Fig. 7 Correlation between glucose (mg dL^{-1}), insulin (μU mL^{-1}), HOMA-IR, triglycerides (mg dL^{-1}), HDL-C (mg dL^{-1}) and LDL-C (mg dL^{-1}) and bacterial genera, after 60 days of orange juice consumption (**OJ-Diet**).

(p = 0.001), LDL-C (p = 0.0001), insulin (p = 0.005) and HOMA-IR (p = 0.002), and a negative correlation with HDL-C (p = 0.001), while a positive correlation of *Ruminococcus* spp. with HDL-C was detected (p = 0.001).

Discussion

The impact of daily consumption of orange juice on the intestinal microbiota, and its effect on body weight and fat mass, and lipid and glycide metabolism were investigated in a controlled clinical trial of healthy young women. We detected that regular consumption of 100% orange juice had a prebiotic effect by improving microbial colonization and modulating the hosts' metabolic profiles. In addition, orange juice increased antioxidants in the diet, such as citrus flavonoids and vitamin C, but did not change the macronutrient or energy balance, body weight or body composition.

Bioavailability of flavanones was estimated as 7% for HSP and 1.5% for NRG after daily orange juice supplementation, which was associated with beneficial effects on the lipid and glucose metabolism. In addition, regular consumption of orange juice promoted changes in microbiota patterns, as indicated by an increase of the phylum Actinobacteria and Proteobacteria and a decrease of Firmicutes and Bacteroidetes. The study of the association of metabolic biomarkers and microbiota showed an inverse correlation among biochemical parameters, such as glucose, insulin, HOMA-IR, triglycerides, total cholesterol and LDL-C, and *Arkkermansia* spp. The last results pointed out the prebiotic effect of orange juice on the microbiota and on the improvement of lipid and glucose metabolism.

Analysis of the women's diet showed a balanced energy and macronutrient intake at the basal time, with appreciable amounts of fruits and vegetables. Therefore, no specific dietary advice was needed to improve the dietary pattern. After 120 days of follow-up, no changes were observed in proteins, carbohydrates, total lipids, cholesterol, saturated fatty acids and fibers. As expected, consumption of orange juice increased the vitamin C intake by 1.7 times, and 3.4 times above the daily recommendation (RDA) without exceeding the upper limit (UL).[19] Previously it was shown that 100% pure orange juice improves dietary quality, helping to achieve and maintain caloric balance.[21] In addition, an improvement of insulin sensitivity with reduction in blood glycemia was observed in this trial after the **OJ-Diet**. After the **Washout**, the blood serum glycemia and insulin, and HOMA-IR returned to the values of the **OJ-free Diet** period. These effects on metabolite parameters may be due to orange juice bioactive compounds such as flavonoids and pectin, as well as beneficial changes in the intestinal microbiota.[22–24]

HSP and NRGN in diabetic rats attenuated gluconeogenesis, raised insulin sensitivity and improved glycemic control.[22] NRGN also showed an antihyperglycemic effect *in vivo* inhibiting digestion and absorption, and delayed glucose uptake by the intestinal brush border membrane.[25] Blood glucose levels may also be affected by soluble dietary fibers, such as pectin from orange juice, which may decrease intestinal transit time, leading to more gradual absorption of nutrients and prolonging satiety.[26] Another clinical study from our group showed that although orange juice has a small amount of pectin, it still helps to lower the glycemic peak compared to an isocaloric drink.[24] In addition, intestinal fermentation of pectin and flavanone metabolism stimulate microbiota growth, increasing the production of AGCC (propionate, butyrate and acetate), which may improve insulin and liver sensitivity and glucose absorption in the muscle.[20,27] We also observed a reduction in the blood concentrations of total cholesterol, triglycerides and LDL-C after the consumption of orange juice (**OJ-Diet**). Moreover, in the **Washout** an increase in all these variables was observed. Indeed, the lipid-lowering effect of orange juice consumption has been demonstrated in humans, and several mechanisms have been attributed to citrus flavonoids to explain the decrease in plasma lipids.[2,4]

An experimental study of the metabolic syndrome model in mice showed that supplementation with NRG activated hepatic AMPK leading to an increase of PPARα gene expression. In turn, PPARα reduced the synthesis of fatty acids and glucose, and the production of apoB and VLDL and inhibited the hepatic activity of HMG-CoA reductase.[28] In hepatocytes, HSP and NRG decreased the monoacylglycerol transfer protein (MTTP) and the enzyme acyl-CoA cholesterol acyltransferase (ACAT), which work together to assemble VLDLs.[29] Moreover, NRGN increased the expression and activity of the LDL receptor (LDL-R), involved in the clearance of plasma lipoproteins. These mechanisms underlay the capacity of flavanones to reduce the hepatic volume of triglycerides, and decrease the secretion of VLDLs and, consequently, the amount of circulating LDL-C.[30]

In the present study, there was a gradual enhancement of antioxidant status in the volunteers' blood, reaching a significant effect two months after the daily intake of orange juice.

Indeed, we have previously shown a positive effect on the antioxidant capacity after regular intake of orange juice.[3,4] These effects have been attributed to the high concentration of vitamin C and HSP in the juice that results in direct elimination of free radicals and increases the Nrf2-ARE pathway, which is related to the control of antioxidant genes.[31] On the other hand, NRGN can reduce free radicals increasing the antioxidant activity of superoxide dismutase, catalase and glutathione.[32]

Commercial processing of orange juice influences the concentration and solubility of citrus flavonoids. In the processed juice, some of the extracted flavanones remain soluble, while others precipitate, becoming part of the cloud part of the juice. *In vitro* studies have shown that soluble fraction flavanones are readily available to be absorbed.[33] However, flavanones that precipitated in the cloud require enzymatic actions in the gastrointestinal tract to be absorbed.[33,34] The chemical analysis of orange juice performed in this study showed a higher percentage of HSP (71.5%) in the cloud, and a higher percentage of NRG in the supernatant (62.5%) or soluble fraction.

An increase in urinary flavanones was also observed after 30 and 60 days of consumption of orange juice (**OJ-Diet**); in contrast, no flavanones were detected prior to consumption (**OJ-free Diet**) or after volunteers stopped drinking orange juice (**Washout**). These findings are in line with previous studies showing that flavanone metabolite levels in the bloodstream and urine are related to flavanone concentration in the diet.[35] Urinary recovery of metabolites of HSPT and NRGN, at the basal period, during orange juice supplementation, and afterwards, is in agreement with the literature.[11,18,34,35] In our study, the bioavailability of HSPT was estimated at 7%, while NRGN was 1.8%. The total bioavailability for both flavanones was 8.8% relative to the ingested dose.

Considering that orange juice has a HSP to NRG ratio of 5 to 1, and the bioavailability ratio was 3.8 to 1 among these compounds, a higher bioavailability of NRGN to HSPT was suggested. This assumption is consistent with a higher amount of NRGN in the soluble fraction of orange juice than in the precipitate and otherwise for HSPT. These results reflect the specific variations in the flavanones' bioavailability and metabolism, and some of the differences in the colon microbiota, which are essential for the absorption of flavanones.[11,34]

Most orange juice HSP and NRG glycosides are deconjugated by the action of rhamnosidase and beta-glucosidase, synthesized by Bifidobacterium strains, before being absorbed into the distal colon. Within the enterocyte cell, aglycone compounds are converted into glucuronides and secreted into the portal circulation. In the liver, the glucuronide metabolites are conjugated to phase II metabolites by glucuronidation, sulfation, or methylation, and excreted in the systemic circulation. These metabolites reach organs such as pancreas, liver, spleen, brain, adipose tissue, muscle, and finally kidneys, where they are excreted in the urine.[36] The colonic microbiota is also responsible for the fission of aromatic rings of polyphenols, producing SCFA and greatly promoting the absorption of acetate, propionate and butyrate.[11,35] In summary, the addition of 16% of the intact aglycones to 88% of the biotransformed flavonoids in SCFA equals almost 100% of the ingested dose, showing a high absorption of the flavonoids.[37]

In the present study, visualization, clustering, and modeling of the diversity in microbiota showed that the supplementation with orange juice changed the microbiota profile. Recently, it has been reported that the proportion of intestinal bacteria species may change in response to the addition of novel diet foods, although this may not constitute a change in enterotypes.[38] Enterotypes were associated with long-term diets, as Bacteroides with protein and animal fat, and Prevotella with carbohydrates. A controlled-feeding study of 10 subjects showed that the microbiome composition changed detectably within 24 hours after high-fat/low-fiber or low-fat/high-fiber diet, but that enterotype identity remained stable during the 10-day study, showing that the enterotypes need a longer period to present changes.[38]

In the **OJ-Diet** we observed an increase of Actinobacteria and Proteobacteria and a decrease of Firmicutes and Bacteroidetes. In fact, the relative abundance of *Bifidobacterium* spp. (Actinobacteria phylum) was higher after consumption of orange juice, and this fact may play a prominent role in host metabolism. This species of bacteria is commercially applied as a probiotic due to its production of acetate and lactate as the main products of sugar fermentation.[39] Acetate plays a central role in appetite regulation, and an increase in its production in the distal colon promotes fat oxidation and improves glucose homeostasis and inflammatory status.[40]

On the other hand, *Lachnospiraceae* (Firmicutes) remained stable in the **OJ-free Diet** and **OJ-Diet**. Members of this family are associated with the production of butyrate, which is consumed by gut epithelial cells as a source of energy contributing to energy homeostasis and lowering cholesterol synthesis, with anti-inflammatory effects.[41] A recent study showed that after the intake of two different cultivars "Cara Cara" and "Bahia" Citrus sinensis Osbeck orange juice, there was an increase in the abundance of a network of *Clostridia* OTUs from *Mogibacteriaceae*, *Tissierellaceae*, *Veillonellaceae*, *Odoribacteraceae*, and *Ruminococcaceae* families.[42]

In the present study, we detected a negative correlation between the *Akkermansia* family and biochemical parameters such as glucose, insulin, HOMA-IR, triglycerides, cholesterol and LDL-C, and observed a significant increase of *A. muciniphila*. This microorganism is inversely associated with obesity, diabetes, cardiometabolic diseases, and low-grade inflammation, and therefore *A. muciniphila* is considered a next generation of beneficial microbes.[43] Besides the numerous correlations observed, a large body of evidence has demonstrated the causal beneficial impact of this bacterium in a variety of preclinical models.[44] The higher abundance of *A. muciniphila* at the baseline was associated with greater improvement in glucose homoeostasis, blood lipids and body composition after calorie restriction.[45] Recently, *A. muciniphila* has been associated with metabolic health for the first time in

a Greek urban population.[46] In addition, *A. muciniphila* produces several fermentation products, such as SCFA, through mucin degradation. These substrates may serve as energy sources for other bacteria and the host. Through this cross-feeding, *A. muciniphila* may contribute to the expansion of other beneficial species, while it may itself have a direct effect on host metabolism.[43]

A negative correlation between *Lactobacillus* spp. and glycemia was also shown in our data. Orange juice may stimulate the increase of *Lactobacillus* spp. in the intestinal microbiota and improve glucose metabolism, which agrees with previous studies that showed that probiotic lactobacilli improved glucose metabolism.[47] On the other hand, a positive correlation of *Ruminococcus* spp. with HDL-C was observed, however, after the **OJ-Diet.** The abundance of this genera decreased (p = 0.09). Interestingly, a recent study conducted with obese Mexicans adolescents showed a positive association between *Ruminococcus* spp. and cholesterol.[48] In addition, the significance of this correlation and its health benefits can be understood as a combination of two main factors: dirst, the increase of SCFA produced by microorganisms during orange juice supplementation,[12,39] which influence glucose homeostasis and energy,[49] and second, the effect of flavanone metabolites of orange juice that have the potential to reduce cholesterol and triglycerides by inhibiting synthesis or increasing catabolism, and improve insulin sensitivity that lowers blood glucose.[24,26,29]

This study showed several strong aspects, such as a very homogeneous group of volunteers, multiple evaluations throughout the experiment, and high adherence to the following imposed dietary restrictions. Some limitations were also observed, such as the duration of the study (120 days), and lack of previous studies demonstrating the biological plausibility between the metabolic parameters and microbiota. Despite the limitations, we were able to conclude that the dynamic view between biochemical parameters and microbiome provided relevant insights into the interaction between orange juice consumption and intestinal and metabolic health.

Conclusions

The results indicated a prebiotic effect of orange juice on intestinal microbiota and a positive influence on metabolic biomarkers of adult women. However, it also suggested that in order to achieve these benefits, orange juice should be consumed regularly and in moderate amounts along with a balanced diet.

Abbreviations

SCFA	Short-chain fatty acid
UHPLC-UV-VIS	Ultra-high performance liquid chromatography with a visible ultraviolet detector
OJ-free Diet	Orange juice-free diet
OJ-Diet	Diet plus orange juice
Washout	Orange juice-free diet
IS	Internal standard 7-crystalline ethoxycoumarin
DMSO	Dimethyl sulfoxide
HSP	Hesperidin
NRG	Naringin
HSPT	Hesperetin
NRGN	Naringenin

Conflicts of interest

There are no conflicts to declare.

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