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D-limonene supplementation does not alter postprandial metabolism of postmenopausal women challenged with a mixed macronutrient tolerance test: a pilot study

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Abstract

The hormonal decline during menopause increases women's risk of chronic diseases. D-limonene, a monoterpene found in the human diet, possesses biological properties related to hypolipemic, antioxidant, anti-inflammatory, and gut microbiota-modulating activities, primarily observed in preclinical studies. Postprandial responses include physiological adaptations to the stress of a nutrient surplus, providing an opportunity to assess metabolic resilience, being a suitable strategy for exploring post-menopause-associated metabolic alterations. Here, we investigated the effects of D-limonene supplementation (2 g/day) on postprandial metabolism in postmenopausal women ($n=9$) challenged with a standardized mixed meal in a 3-week single-arm clinical study. Our findings revealed that D-limonene did not induce marked differences in postprandial responses to the dietary challenge. The supplementation with D-limonene induced no alterations in serum lipid/lipoprotein profile or glycemia/insulinemia. D-limonene supplementation did not affect the transient postprandial inflammatory response regarding changes in gene expression of peripheral blood mononuclear cells (PBMC) and circulating inflammatory markers. Nevertheless, D-limonene reduced postprandial levels of lithocholic acid, a gut microbiota-derived bile acid, and regulated the plasma concentrations of selected amino acids, carbohydrate metabolism-derived metabolites, and organic acids. In conclusion, our data do not support the claim that short-term D-limonene supplementation beneficially affects the postprandial metabolism of postmenopausal women.

Keywords Monoterpenes, Inflammation, PBMC, Dyslipidemia, Obesity

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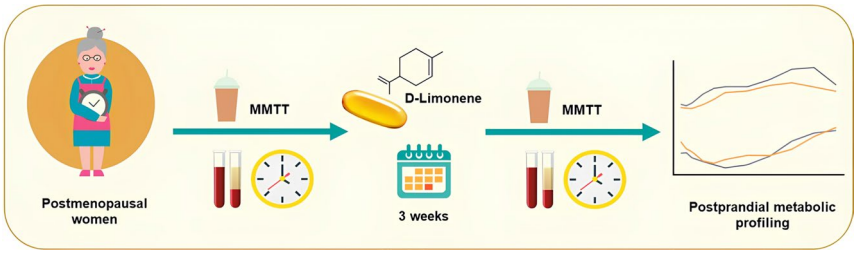
Graphical Abstract



Introduction

Menopause is a transition period of physiologic and psychosocial changes when women approach reproductive senescence (El Khoudary et al. 2020; Harlow et al. 2012). The aging process and decline in the secretion of sex hormones lead to long-term changes in metabolism, resulting in body weight changes, sexual dysfunction, urogenital and skin atrophy, and other metabolic disorders. The physiological basis of these manifestations is complex and linked, but not restricted, to hormonal deficiency (Monteleone et al. 2018). Metabolic alterations and the risk of noncommunicable chronic diseases emerge coincidently in the menopausal transition. Postmenopausal women experiment an increase in body fat mass associated with an insulin resistance scenario, dyslipidemia, risk of cardiovascular adverse events, and cancer incidence (Nappi et al. 2022; Pan et al. 2020).

Dietary components such as phytochemicals aid in ameliorating metabolic disorders in postmenopausal women (Ko & Kim 2020). Monoterpenes represent a class of metabolites produced by plants that contribute to the aroma and taste of fruits and vegetables, being D-limonene one of the most abundant monoterpenes in the human diet because of its high content in citrus fruit, especially oranges (de Alvarenga et al. 2021). D-limonene has been reported to exhibit a range of biological properties related to hypolipemic, antioxidant, immune-modulatory, anti-inflammatory, and microbiota-regulating effects that are frequently reported in animal studies, but very little studied in humans (Anandakumar et al. 2021). Considering the recently found role of gut microbiota-derived metabolites such as secondary bile acids (BA) in the regulation of different aspects of metabolism and inflammation, it is possible that the positive effects of D-limonene may be exerted through modulation of the gut microbiota and BA metabolism (Alemán et al. 2018).

Clinical research is regularly conducted in the fasted condition to evaluate metabolism in a "basal state" when the composition of a recent meal does not influence variability amongst subjects. However, for the purpose of risk assessment and diagnosis of postmenopause-associated metabolic alterations, dietary challenges may provide a helpful tool, as postprandial responses offer the possibility to assess phenotypic flexibility and markers of chronic diseases not observed in the fasting state (Fiamoncini et al. 2022a, 2022b; LaBarre et al. 2021). Metabolic adaptations in response to a dietary challenge involve the activation of interrelated pathways to maintain energy homeostasis. These adaptations lead to an increase in postprandial glycemia, insulinemia, lipemia, plasma levels of nutrients, signaling molecules (e.g., incretins and hormonal factors), as well as other endogenous metabolites. Ingesting an energy-dense meal rich in fat and/or simple carbohydrates can result in an acute inflammatory response in the first hours of the postprandial period (Herieka & Erridge 2014; Hoevenaars et al. 2019). Indeed, meals rich in saturated fat lead to an increased expression of proinflammatory genes in peripheral blood mononuclear cells (PBMC) from individuals with metabolic syndrome (Cruz-Teno et al. 2012).

The heterogeneity of postprandial responses and their involvement in the risk of chronic diseases is ignored, particularly in postmenopausal women. Understanding these phenomena could lead to the identification of markers to nutrient intake with the ability to predict health status in women of this age group, which are at a higher cardiovascular risk. Considering the potential of D-limonene as a health-promoting agent and the lack of human interventional studies, there are knowledge gaps in our understanding of D-limonene's role in metabolic processes triggered by food intake. From an interventional point of view, how D-limonene can affect systemic metabolic regulation during the postprandial phase is a critical question. Here, we investigated the effects of a short-term D-limonene supplementation on postprandial metabolic and inflammatory responses in postmenopausal women challenged with a standardized, energy-dense

mixed meal. Despite evidence from pre-clinical studies, D-limonene was not associated with changes in postprandial responses in terms of plasma levels of nutrients, signaling molecules, endogenous metabolites, or circulating inflammatory markers. Instead, we found that D-limonene induced alterations in selected carbohydrate metabolism-derived metabolites, amino acids, organic acids, and lithocholic acid – an important gut microbiota-derived BA.

Material and methods

Study population

The clinical trial was performed at the Clinical and Epidemiological Research Center located at the University Hospital of the University of São Paulo (São Paulo-SP, Brazil). Fourteen postmenopausal women (characterized by the absence of menstruations in the previous year) were included in the study according to the following criteria: 1) age between 50 and 65 years old, 2) BMI between 18.5 and 34.9 kg/m^2; 3) no history of surgeries in the gastrointestinal tract; 4) absence of inflammatory bowel diseases, celiac disease, gastric ulcers, liver diseases, cholecystectomy, diabetes, allergy to casein, lactose intolerance, and alcoholism, which were self-declared.

Experimental protocol

A single-arm, non-randomized, pre-, and post-supplementation study was conducted. Volunteers were assigned to a 2 g/day D-limonene supplementation for three weeks. D-limonene used in this study was provided by Citro Flavor (Catanduva, São Paulo, Brazil) and presented 94% of purity as characterized by gas-chromatography (Supplementary information 3). The supplementation was presented in the form of 1 g gelatin capsules produced by Soolis (São Paulo, São Paulo, Brazil). Volunteers were instructed to consume one capsule in the morning after breakfast and a second capsule after dinner. Compliance was monitored by weekly telephone contact or text messages during the intervention period. On the day before the visits to the study center, volunteers were instructed to refrain from physical activity, alcohol, caffeine, and food rich in citrus products (fruit, beverages, and confection).

To evaluate the postprandial response, before and after the supplementation period, the participants underwent a standardized mixed meal tolerance test (MMTT) containing 75 g glucose, 60 g canola oil, and 20 g micellar casein in a volume made up to 200 mL with water. The MMTT started at 08:00 am following a ~ 10-h fasting. Participants had a catheter placed in the antecubital vein by a trained nurse, and a fasting blood sample was obtained. Next, the liquid mixed meal was ingested within a 10-min interval, and postprandial blood sampling happened at 15, 30, 60, 90, 120, 240, 300, 360, and 420 min after the start of the meal intake. Cannula was flushed with a saline solution between blood collections, and the initial volume (~ 2 mL) of saline-diluted blood was discarded. Blood samples were collected in serum (CAT Serum Sep. Clot Activator, Vacuette® Greiner Bio-One) and plasma (K3E EDTA-treated, Vacuette® Greiner Bio-One) tubes. Serum and plasma were separated by centrifugation at 3500 rpm for 15 min at 4 °C and stored at -80 °C until analyses. Before and after the supplementation period, an anthropometric evaluation was assessed by BMI determination and body fat percentage by using a bioelectrical impedance (InBody 270, Ottoboni, Rio de Janeiro, Brazil).

Hematological evaluation, inflammatory and biochemical parameters

Blood samples collected in EDTA-treated tubes at 0, 120, 240, and 360 min were used for hematological parameters using a Sysmex XN 550 automated hematology analyzer (Sysmex America Inc., USA). Plasma insulin, gastric inhibitory polypeptide (GIP), peptide YY (PYY), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1/CCL2), and tumor necrosis factor-α (TNF-α) were assessed by Milliplex® MAP Human Metabolic Hormone Magnetic Bead Panel (HMHEMAG-34 K, Millipore) kit. Serum glucose, triglycerides, cholesterol, HDL-cholesterol, LDL-cholesterol, creatinine, urea, bilirubin, and albumin levels were analyzed by an automatized analyzer (Roche, Cobas® 6000, Switzerland) in the Clinical Analysis Laboratory of the University Hospital of Sao Paulo. Plasma non-esterified fatty acids (NEFA) levels were measured by an enzymatic method (Fujifilm, Osaka, Japan).

PBMC Isolation

PBMC were isolated from blood collected at 0, 120, 240, and 360 min, after centrifugation and separation of the plasma. The precipitated blood fraction was diluted 1:1 in PBS pH 7.4, and PBMC was isolated using Histopaque® 1077 (Sigma-Aldrich) according to the manufacturer's instructions. Diluted blood was slowly layered over Histopaque in a sterile 15 mL tube and centrifuged at 2000 rpm for 30 min at room temperature. The layer containing cells was carefully aspirated and washed twice with ice-cold PBS and centrifuged at 1500 rpm for 10 min. Lastly, PBMC was resuspended in 1 ml of PBS, and cell viability was examined by trypan blue dye exclusion using an automated cell counter (Countess 3, Invitrogen, Thermo Fisher Scientific).

RNA extraction and RT-qPCR

PBMC RNA was extracted using Trizol reagent (Life Technologies, Thermo Scientific) and purified by PureLink™ RNA Mini kit (Invitrogen, Thermo Fisher Scientific) following the manufacturer's instructions. The RNA concentration was measured on a NanoDrop spectrophotometer (Thermo Scientific) and reverse transcribed to cDNA using a High-Capacity Reverse Transcription kit (Applied Biosystems™, Thermo Fisher Scientific) in a Veriti 96-well Thermal Cycler (Applied Biosystems™, Thermo Fisher Scientific). cDNA was evaluated by quantitative real-time PCR using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma Aldrich) as a fluorescent dye as previously described (Donado-Pestana et al. 2018). Gene expression was performed using the ΔΔCt method and expressed relative to the GAPDH expression as a reference gene. Primer sequences are provided in Supplementary Table 1.

Bile acids analysis

Deuterated bile acid standards were purchased from CDN isotopes (Quebec, Canada) and Cambridge Isotope Laboratories (Tewksbury MA, USA). Fifteen labeled standards were used to quantitatively assess the plasma concentrations of bile acids: d_4-cholic acid, d_4-chenodeoxycholic acid, d_4-deoxycholic acid, d_4-ursodeoxycholic acid, d_4-lithocholic acid, d_4-glycocholic acid, d_4-glycochenodeoxycholic acid, d_4-glycodeoxycholic acid, d_4-glycoursodeoxycholic acid, d_4-glycolithocholic acid, d_4-taurocholic acid, d_4-taurochenodeoxycholic acid, d_4-taurodeoxycholic acid, d_4-tauroursodeoxycholic acid, d_4-taurolithocholic acid were used as standards for the quantitative analysis of their equivalent native compounds.

The procedure is an adaptation of a previously described method and used an LC–MS method to separate and quantify bile acids (Fiamoncini et al. 2017). Ten microliters (10 µl) of plasma were deproteinized by adding 100 µL ice-cold methanol containing 0.2 µmol/L deuterium-labelled bile acid standards. Samples were vigorously vortexed and centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants were dried using a vacuum concentrator (Centrivap, LabConco) and resuspended in 80 µL methanol:water (1:1). Ten microliters (10 µL) of the sample extracts were injected into an Agilent 1260 Infinity II UPLC system (Agilent Technologies, CA, USA) equipped with a Waters Acquity UPLC HSS T3 column (1.8 µm, 2.1 × 150 mm) kept at 40 °C. The mobile phases consisted of 10 mM ammonium acetate + 0.1% formic acid in MilliQ water (Mobile phase A) and 0.1% formic acid in LC–MS grade acetonitrile (Mobile phase B) flowing at 0.3 mL/min. The gradient started with solvent B at 2%, reaching 98% at 24 min. A triple quadrupole mass spectrometer (5500 Sciex, MA USA) was used in the negative mode to detect the compounds of interest through multiple reaction monitoring (MRM). The chromatograms were processed, and peak picking was performed using Analyst software (Sciex, MA, USA).

Gas chromatography-mass spectrometry-based plasma metabolite profiling

Metabolite extraction and GC–MS analysis were performed as per our previous publication (More et al. 2022). Briefly, 10 µL of plasma samples were extracted using 100 µL of the extraction fluid, consisting of methanol:H_2O (8:1) with a standard internal concentration of 2 µg/ml (U13C-Ribitol and D6-Glutaric acid). The dried metabolic extracts underwent two-step derivatization using an automated robot (Gerstel MPS). Methoxamine hydrochloride in pyridine was added and shaken for 90 min at 40 °C, followed by the addition of N-methyl-N-trimethylsilyl-trifluoroacetamide and shaking for 30 min at 40 °C. One microliter of the sample was injected into an SSL injector at 270 °C in spitless mode.

GC–MS analysis was conducted using an Agilent 7890A GC equipped with a 30 m DB-35MS + 5 m Duraguard capillary column (0.25 mm inner diameter, 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The GC oven temperature was ramped from 80 °C to 320 °C at a rate of 15 °C/min with an 8-min hold, resulting in a total run time of 25 min per sample. The GC was connected to an Agilent 5977B MSD. The transfer line temperature was set to 280 °C, and the MSD operated under electron ionization at 70 eV. The MS source was maintained at 230 °C, and the quadrupole at 150 °C. Full scan mass spectra were acquired from m/z 70 to m/z 800 at a scan rate of 5.2 scans/s. Pooled plasma samples were used to prepare QC samples, which were measured every eighth GC–MS measurement for quality control and data correction (Trezzi et al. 2017). Deconvolution of mass spectra, peak picking, integration, and retention index calibration was performed using our in-house software (Hiller et al. 2009). Compounds were identified using an in-house mass spectral library based on spectral and retention index similarity. The data were normalized to the quality control pool measurement and the peak area of the internal standard (U-^{13}C-Ribitol).

Statistical analysis

The distribution of variables was analyzed by the Shapiro–Wilk test. Log transformation was performed for variables with an abnormal distribution. A *t*-test was applied to compare the means of variables between D-limonene pre- and post-supplementation. Mixed-effects analysis was performed to determine the effects of D-limonene supplementation on the postprandial period.

Results were expressed as mean ± standard error and a p-value < 0.05 was set as being the statistical level of significance. Analysis was performed using GraphPad Prism software (GraphPad Software, version 9.0, La Jolla, CA, USA).

Results

Five participants withdrew partway through the supplementation period because of COVID-19 pandemic restrictions. Therefore, nine women completed the study. The anthropometric evaluation and clinical parameters are summarized in Table 1. D-limonene supplementation was generally well-tolerated by the participants. Some individuals reported gastrointestinal discomforts (mainly belching), but no serious adverse events were stated during the study. Following the 3-week D-limonene supplementation, there were no significant differences in the anthropometric measurements and clinical characteristics from the baseline (Table 1), except for serum creatinine levels that resulted in a significant decrease ($p < 0.001$) after the D-limonene supplementation when compared to baseline values.

Glucose and insulin plasma concentrations reached peak values 30 and 90 min after ingestion of the standardized mixed meal, respectively, returning to fasting values after 180 and 420 min, respectively. Both variables were not altered after D-limonene supplementation (Fig. 1a, b). A significant increase in plasma triglycerides levels was observed in the postprandial period, with a slight decrease after 7 h and the absence of response to the supplementation with D-limonene (Fig. 1c). NEFA plasma levels decreased after the ingestion of the mixed meal reaching nadir at 120 min, followed by a rebound to levels higher than those observed in fasting after 360 min of the postprandial period. No changes in NEFA plasma concentrations during the postprandial period were observed in response to D-Limonene supplementation (Fig. 1d). HDL-, LDL-, and total cholesterol levels were decreased during the postprandial period. Mean values to both HDL- and LDL-cholesterol tended to be higher and lower respectively, after D-Limonene supplementation, although this difference did not reach statistical significance (Fig. 1e-g). Serum levels of creatinine, urea, and bilirubin displayed a significant decrease in postprandial samples in comparison to their fasting values, without any effects in response to D-limonene supplementation (Fig. 1h-j).

Fasting and postprandial concentrations of incretins GIP and PYY were similar in participants before and after D-limonene supplementation. The postprandial appearance of GIP and PYY showed a remarkable increase ($p < 0.0001$ and 0.0071, respectively) between 30 and 240 min, with decrease at the end of the dietary challenge (Fig. 2a, b).

The number of circulating leukocytes increased during the dietary challenge at similar rates in both groups (Fig. 3d). The proportion of neutrophils tended to increase in the second hour of the dietary challenge, while eosinophil and basophil counts decreased steadily during the postprandial period ($p < 0.014$ and $p < 0.006$), respectively) (Fig. 3f, g). In turn, agranular leukocytes such as monocytes and lymphocytes decreased in the early phase of the dietary challenge, reaching a nadir at 120 min, followed by a rebound phase thereafter (Fig. 3h, i). Most importantly, there were no effects of the supplementation with D-limonene on the number of circulating leucocytes in blood. We also assessed the anti-inflammatory properties of D-Limonene on the transient inflammatory response to the meal, but our findings did not observe a modulatory effect on the plasma concentrations of proinflammatory cytokines including TNF-α, IL-6, and MCP-1 (Fig. 3a-c). Our analyses also did not detect changes in the expression levels of proinflammatory genes in PBMC isolated during the postprandial period. The expression levels of IL-6, TLR-4, TNF-α, and IL-1β were not influenced by the supplementation, nor the meal intake (Fig. 3j-m). Interestingly, the postprandial kinetics of TLR-4 expression in PBMC followed the response of circulating NEFA, well-recognized TLR-4 agonists. D-limonene supplementation showed a non-significant decreasing trend in the gene expression of IL-6 and TLR-4 ($p = 0.2312$ and 0.3141) when compared to the postprandial response at baseline.

The dietary challenge triggered a transitory increase in the plasma levels of bile acids (BA) reaching T_{max} 120 min after the intake of the meal. The range of

Table 1 Anthropometric evaluation and clinical parameters from postmenopausal women before and after D-limonene supplementation during a 3-week period

Variable	Before		After		*p*-value*
	Mean	SEM	Mean	SEM	
Age (years)	59.8 1.83				-
Weight (kg)	67.06	2.65	67.30	2.77	0.2947
BMI (kg/m^2)	26.44	1.15	26.48	1.22	0.7802
Body fat (%)	37.29	1.94	37.40	2.14	0.8154
Lean mass (kg)	22.76	1.04	22.77	1.06	0.9452
Visceral fat (%)	12.11	2.47	12.11	2.67	> .9999
Waist/hip ratio	0.98	0.02	0.98	0.02	0.9001
Creatinine (mg/dL)	0.77	0.03	0.70	0.03	0.0007
Bilirubin (mg/dL)	0.14	0.02	0.11	0.02	0.1005
Albumin (mg/dL)	4.39	0.07	4.40	0.09	0.9283

Values are means and SEM ($n = 9$)

* Student´s *t*-test

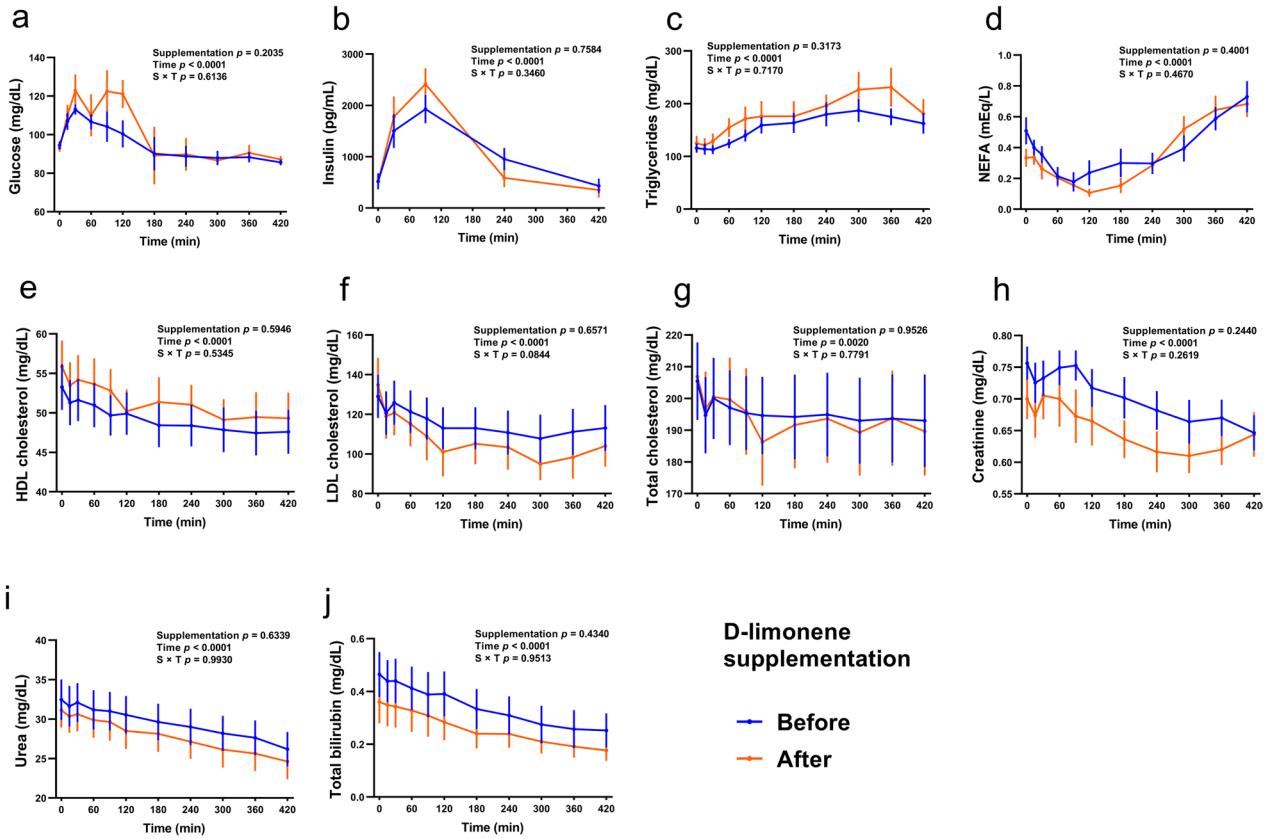


Fig. 1 Postprandial circulating biochemical markers from postmenopausal women during a mixed meal tolerance test before and after D-limonene supplementation. Data are means ± SEM ($n = 9$)

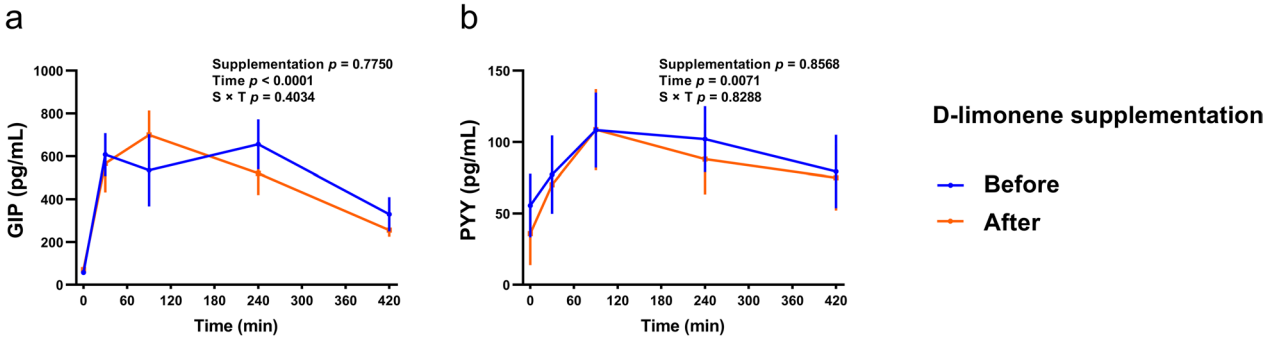


Fig. 2 Postprandial plasma incretin levels from postmenopausal women during a mixed meal tolerance test before and after D-limonene supplementation. Data are means ± SEM ($n = 9$)

increase varied between fivefold and tenfold for the different BA measured. Even at $t = 420$ min, plasma levels of most BA were still higher than in the fasting state, although significantly decreased, if compared to T_{max}. D-limonene supplementation did not cause differences in the plasma concentration of total (sum) BA during the postprandial period (Fig. 4a). Inspecting each BA class (sum of unconjugated, glycine- and taurine-conjugated species), we observed that D-limonene supplementation had distinct effects in different BA species: while causing a decrease ($p < 0.05$) in the postprandial levels of litho-cholic acid (LCA) (Fig. 4d), a major secondary BA, it did not induce changes in plasma concentrations of the other measured BA (Fig. 4e, f).

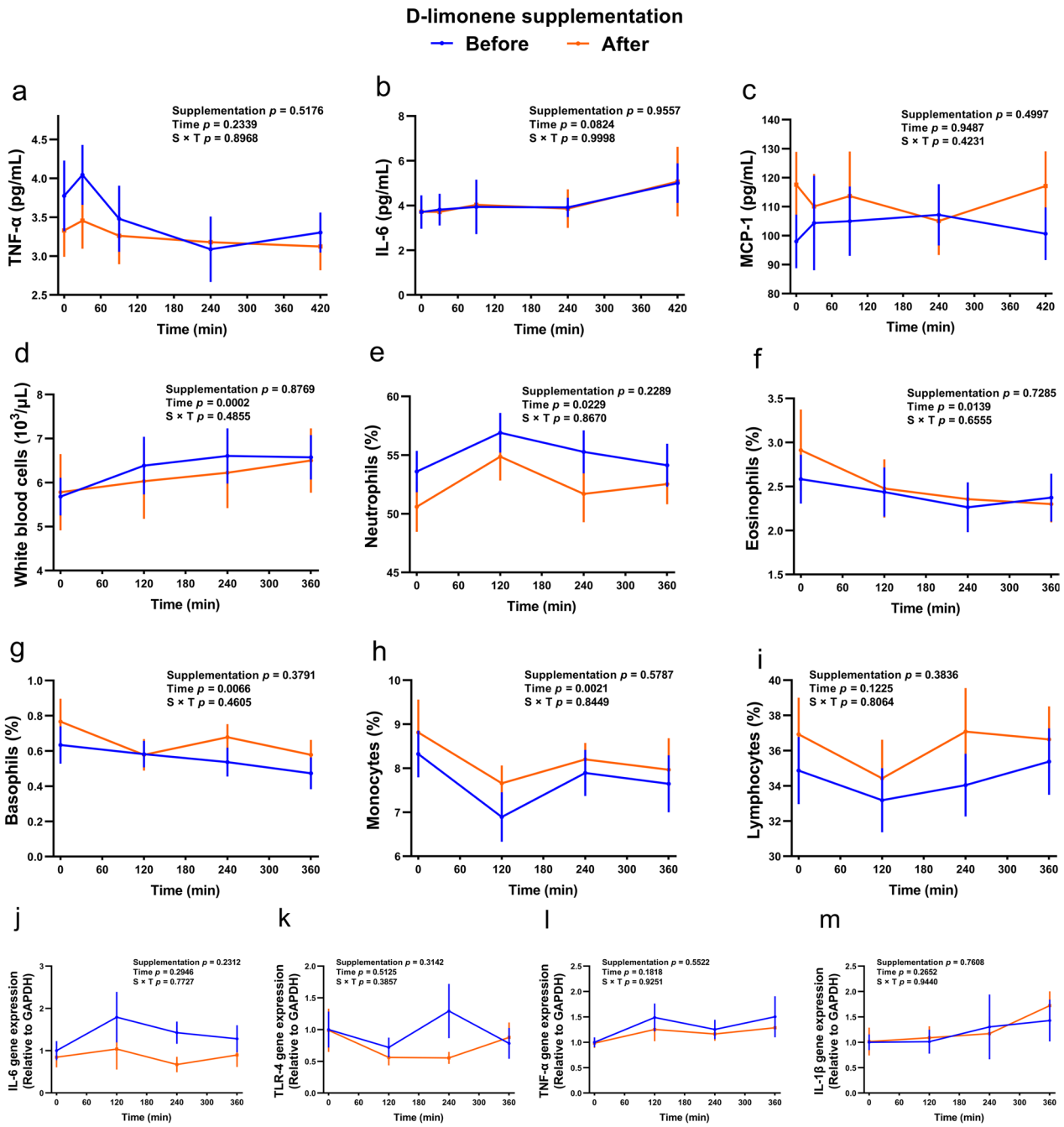


Fig. 3 Postprandial inflammatory markers from postmenopausal women during a mixed meal tolerance test before and after D-limonene supplementation. Data are means ± SEM ($n = 9$)

By considering the mean values of different metabolites in all plasma samples collected during the dietary challenge, the untargeted metabolomic analysis (Table 2) revealed that D-limonene supplementation was associated with decreasing levels of several amino acids and metabolites, including 2-hydroxybutyric acid, leucine, serine, and valine. In contrast, postprandial plasma concentrations of aspartic acid and glycine increased significantly after supplementation. Metabolites related to carbohydrate metabolism, such as erythronic acid, glycerol-3-phosphate, and lactic acid, also increased after D-limonene supplementation, whereas 1,5-anhydro-D-glucitol and myo-inositol plasma levels decreased after the supplementation with D-limonene.

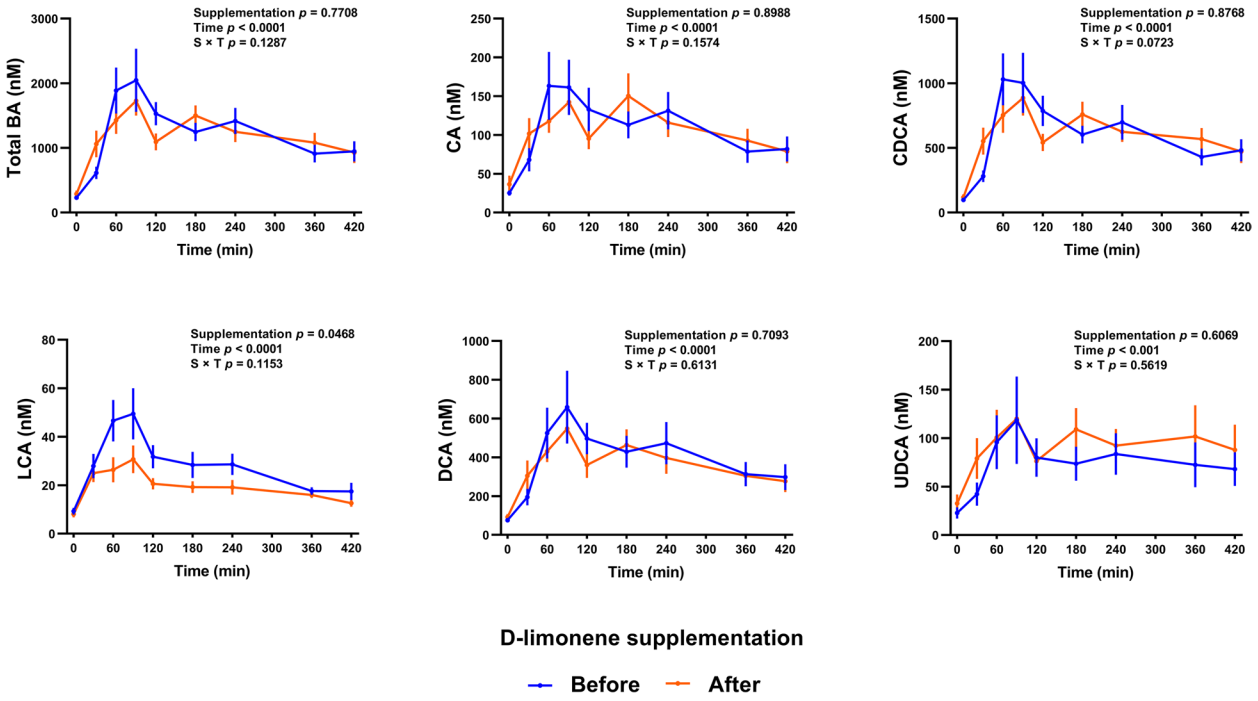


Fig. 4 Postprandial plasma bile acids from postmenopausal women during a mixed meal tolerance test before and after D-limonene supplementation. Data are means ± SEM ($n = 9$)

Discussion

In this pilot study, we investigated the potential effects of D-limonene supplementation on postprandial metabolism in postmenopausal women. Our findings showed that the ingestion of 2 g/day of D-limonene for three weeks did not induce marked differences in postprandial responses to the mixed meal in postmenopausal women. D-limonene did not induce alterations in circulating markers of lipid metabolism (LDL-, HDL- and total cholesterol, NEFA, triglycerides), glucose, or insulin. Furthermore, the postprandial inflammatory response was not regulated by D-limonene intake as observed in the plasma markers, including proinflammatory cytokines levels or PBMC IL-6, TLR-4, TNF-α, and IL-1β mRNA levels. Some plasma metabolites responded to D-limonene supplementation, including selected amino acids, carbohydrate metabolism-derived metabolites, organic acids, and lithocholic acid—a gut microbiota-derived BA.

After menopause, women commonly experience increased abdominal adiposity, dyslipidemia, and insulin resistance, all factors that increase the risk for cardiovascular diseases (Zore et al. 2018). Despite considerable knowledge about the health effects of D-limonene, that has shown positive modulation of glucose and lipids metabolism, with potential anti-obesogenic effects, mostly in animal or in vitro models (Anandakumar et al. 2021; Rinaldi de Alvarenga et al. 2022), in this study, the supplementation with the monoterpene did not induce alterations in body composition. Previous studies demonstrated that D-limonene displays anti-obesity effects by decreasing adipogenesis and lipogenesis and promoting lipolysis in diet-induced male obese rats (Huang et al. 2022; Liao et al. 2023). However, these beneficial effects were not reported in female obese mice receiving D-limonene and a high-fat diet (Jing et al. 2013). In the present study, we did not observe a beneficial effect of D-limonene on fasting or postprandial lipid, glucose, and insulin levels. Different reports have indicated that monoterpenes can induce positive modulations of glucose and lipid metabolism. Indeed, in animal models of diet-induced obesity, D-limonene administration was effective in inducing a decrease in total cholesterol, LDL-cholesterol, triglycerides, and an increase in HDL-cholesterol (Bacanlı et al. 2017; Jing et al. 2013).

In this study, we showed that the BA kinetics presented an increase in the first 2 h of the dietary challenge. A similar postprandial BA profile was observed in previous studies (Fiamoncini et al. 2022a, 2022b; Schmid et al. 2016), but it is different than what was previously published for a larger cohort, when the mean levels of total BA were sustained up to six hours after the intake of a meal with similar composition, replacing canola oil (used in the current study) with palm oil (Fiamoncini et al. 2017). Noteworthy, our experimental data revealed

Table 2 Mean plasma levels of metabolites during a mixed meal tolerance test before and after D-limonene supplementation during a 3-week period. *N* = 9. Results are expressed as chromatographic peak areas normalized by the internal standard

Variable	Before		After		*p*-value*
	Mean	SEM	Mean	SEM	
Amino acid metabolism					
2-Hydroxybutyric acid	1.101	0.044	0.917	0.066	0.0340
Alanine	1.121	0.032	1.174	0.046	0.3549
Aspartic Acid	0.882	0.025	0.996	0.017	0.0017
Glycine	1.010	0.025	1.275	0.017	< .0001
Isoleucine	1.153	0.028	1.108	0.027	0.2678
Leucine	1.196	0.024	1.092	0.022	0.0053
Serine	1.267	0.034	1.134	0.020	0.0041
Valine	1.161	0.018	1.101	0.020	0.0441
Threonine	1.316	0.035	1.052	0.020	< .0001
Carbohydrate metabolism					
1,5-Anhydro-D-glucitol	1.000	0.013	0.885	0.014	< .0001
Erythronic Acid	0.940	0.016	1.030	0.026	0.0097
Fructose	0.966	0.040	1.121	0.083	0.1134
Glycerol-3-phosphate	0.965	0.028	1.195	0.028	< .0001
Lactic Acid	0.969	0.053	1.161	0.034	0.0080
Myo-inositol	1.070	0.018	0.988	0.023	0.0145
Lipid metabolism					
3-Hydroxybutyric acid	1.067	0.323	1.025	0.385	0.9338
Glycerol	1.087	0.043	1.021	0.054	0.3493
Oleic acid	0.934	0.063	0.997	0.064	0.5014
Palmitic acid	0.963	0.087	0.957	0.092	0.9628
Stearic acid	1.055	0.196	1.017	0.264	0.9085
Organic acids					
Citric acid	1.088	0.037	0.997	0.041	0.1202
Malic acid	0.799	0.023	0.937	0.014	0.0001
Oxalic acid	0.913	0.027	0.973	0.023	0.1066
Pyroglutamic acid	1.172	0.021	1.259	0.026	0.5197

Values are means and SEM ($n = 9$)

* Student´s *t*-test

that D-limonene supplementation stimulated a decrease in postprandial levels of lithocholic acid (LCA) suggesting a microbiota-modulating effect by the monoterpene. A clinical trial identified LCA, the most prominent activator of G-protein-coupled receptor TGR5, as being an atherosclerosis risk factor (Duboc et al. 2012; Perino et al. 2021). Since LCA is a major gut microbiota-derived secondary BA, it is reasonable to hypothesize that the D-limonene supplementation during three weeks promoted an alteration in the gut microbiota of the subjects. In fact, previous studies have suggested that D-limonene might have an impact on intestinal microbiota composition. In diet-induced obese rats, D-limonene-rich orange essential oil increased the content of *Bifidobacterium*, protecting against endotoxemia and ameliorating low-grade inflammation (Li et al. 2018). An independent study reported that intragastric administration of D-limonene to mice increased the abundance of *Lactobacillus* and reduced the production of short-chain fatty acids in the cecum and colon of mice (Wang et al. 2019). Since this was not in the scope of the present study, further studies are required to evaluate the modulation of the gut microbiota of postmenopausal women in response to D-limonene intake and its effects on postprandial metabolism.

In the study reported here, we did not observe a favorable and clinically relevant anti-inflammatory effect of D-limonene supplementation as measured by the plasma concentrations of cytokines and their gene expression in isolated PBMC. It should also be noted that the meal used in the dietary challenge did not trigger an acute inflammatory response in the population of this study. Human trials investigating the effects of a single ingestion of a hypercaloric meal have reported increases in inflammatory biomarkers during the postprandial period promoting acute systemic inflammation (Emerson et al. 2017; Herieka & Erridge 2014). In fact, high-fat meals may induce an increase in postprandial plasma concentrations of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and C reactive protein (CRP) (Derosa et al. 2012). Consistent with these observations, increased circulating IL-6 levels were also observed after ingestion of meals high in either fat or carbohydrate, with high-carbohydrate meals leading to greater postprandial oxidative stress in healthy individuals (Gregersen et al. 2012). On the other hand, the intake of specific types of dietary fats demonstrated that saturated fatty acids induced an increase in TNF-α gene expression in PBMC of healthy men during the postprandial period when compared to mono- and poly-unsaturated fats (Jiménez-Gómez et al. 2009). Since we include canola oil, a rich source of mono- and polyunsaturated fats, we assume that the absence of a postprandial inflammatory response in the actual study may be linked, at least in part, to the use of the fat type in the mixed meal.

A study by Ghanim et al. (2010) found that a high-fat, high-carbohydrate meal increased TLR4 gene expression in PBMCs of normal-weight men and women. Previously, it was reported that fatty acids directly bind to TLR4 modulating the TLR4-mediated signaling pathway and consequent inflammatory response which are associated with the development and progression of many chronic diseases (Wang et al. 2017; Wong et al. 2009). Nuclear factor-κB (NF-κB) is a major downstream target of the TLR4 pathway and regulates the expression of

proinflammatory genes. During a postprandial challenge evaluating the intake of different fat types, it was observed that saturated fats increase NF-κB activation in patients with metabolic syndrome (Cruz-Teno et al. 2012).

An interesting observation in the present study is related to the effects of the D-limonene supplementation on the concentration of specific amino acids, glucose-related metabolites, and organic acids. Plasma levels of branched-chain amino acids (BCAA) including leucine and valine were decreased after D-limonene supplementation. Elevated plasma BCCA levels are associated with an increased risk of insulin resistance and diabetes, and have been reported in subjects with high levels of postprandial plasma glucose and exaggerated insulin response (Fiamoncini et al. 2022a, 2022b; Gar et al. 2018; Glynn et al. 2015; Wang et al. 2011). Another important consideration among postmenopausal women is that the plasma levels of glycine were elevated after D-limonene supplementation. Glycine has been associated with a lower risk of diabetic complications, and improved insulin sensitivity and glucose homeostasis (Adeva-Andany et al. 2018). An inverse association between glycine and BCAA levels has been observed, which is based on increased nitrogen load in tissues caused by increased BCAA concentration that is dissipated by using glutamate to form alanine from pyruvate. The decline of pyruvate in these pathways can be restored by glycine through enzymes of serine metabolism, thereby reducing glycine levels during the progression of insulin resistance (White et al. 2021). Moreover, our results related to the decreased levels of 2-hydroxybutyric acid and threonine after D-limonene supplementation may reflect a protective effect against an impaired and dysfunctional BCAA catabolism. 2-hydroxybutyric acid, a catabolite of methionine/threonine metabolism, has been shown to be elevated in individuals with reduced insulin sensitivity (Vanweert et al. 2022) and serves as a substrate in the BCAA synthesis (Pirro et al. 2022).

The increased plasma levels of erythronic acid, glycerol-3phosphate, and lactic acid after D-limonene supplementation are indicators of the regulation of carbohydrate metabolism by the monoterpene. Previous studies have demonstrated that plant-derived bioactive substances may have anti-diabetic effects by promoting alternative routes for the metabolism of glucose, including the pentose phosphate pathway, glycolysis, and TCA cycle (Hasanpour et al. 2020; Rahman et al. 2022). Koves et al. (2008) postulated that obesity-induced perturbations in mitochondrial fuel metabolism are characterized by impaired switching to carbohydrate substrate usage during the fasted-to-fed transition and depletion of organic acid intermediates of the tricarboxylic acid cycle.

There are several limitations to our study that should be noted. First, the limited sample size, as a consequence of the withdrawal of some volunteers during the supplementation period, is reflected in limited statistical power. Second, the absence of a placebo arm in the study design limits the establishment of a causal nature of the observations. Finally, the administration of a relatively low dose (2 g/day) of D-limonene for a short period of time (three weeks) may be related to a limited efficacy of the supplementation. The duration and dose used in this study were chosen in an attempt to avoid or reduce the incidence of adverse effects related to monoterpene consumption, such as gastrointestinal discomfort in the volunteers. Despite these limitations, we believe that this data will provide important outcomes for further studies to better evaluate D-limonene effects on postprandial metabolism.

Conclusions

Taken together, these results suggest that D-limonene supplementation does not influence the postprandial metabolism of postmenopausal women after a short-term supplementation. Although a mixed-meal triggers classical responses in intermediate metabolism and inflammatory response, our data indicate that supplementation with a feasible dose of D-limonene for 3 weeks does not provide protective benefits related to glucose and lipid metabolism in postmenopausal women. Furthermore, D-limonene did not alter the inflammatory profile evaluated by PBMC gene expression and circulating inflammatory markers. The potential anti-inflammatory properties of D-limonene remain to be established in further clinical studies. Despite the lack of major changes in postprandial responses, D-limonene altered the circulating levels of specific metabolites, including lithocholic acid, amino acids, glucose-related metabolites, and organic acids.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43014-023-00211-7.

Additional file 1.

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Authors' contributions

Amanda D. Vasconcelos: Methodology, Investigation, Formal analysis. *Carlos M. Donado-Pestana:* Methodology, Investigation, Formal analysis, Writing – original draft, review & editing. *Tushar H. More:* Investigation. *Graziela B. S. Duarte:* Investigation, Methodology, Formal analysis. *Stephany G. Duarte:* Investigation.

Caroline G. Dias: Investigation, Methodology. *Larissa Rodrigues:* Investigation. *Guilherme N. Hernandez:* Investigation. *Ricardo Fock:* Resources. *Karsten Hiller:* Investigation, Resources. *Jarlei Fiamoncini:* Project administration, Funding acquisition, Term, Conceptualization, Methodology, Formal analysis, Supervision, Writing – original draft, review & editing.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences (CAAE: 15438019.7.0000.0067) and by the Ethics Committee of the University Hospital of the University of São Paulo (CAAE: 15438019.7.3001.0076). It was registered at The Brazilian Registry of Clinical Trials (ReBEC) with the code RBR-3vgndbw. Written informed consent was obtained from all participants.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest.

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