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Original Research

A novel polyphenol-rich combination of 5 plant extracts prevents high-fat diet-induced body weight gain by regulating intestinal macronutrient absorption in mice

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ABSTRACT

Global prevalence of obesity and type 2 diabetes are rapidly increasing to pandemic proportions. A novel supplement composed of 5 plant extracts from olive leaf, bilberry, artichoke, chrysanthellum, and black pepper was designed to prevent type 2 diabetes development in people at risk. It was previously shown to improve body weight and glucose control in preclinical rodent models, with these effects being accompanied by increased fecal energy excretion and in vitro inhibition of several digestive enzymes. Thus, we hypothesized that, in mice fed a high-fat diet (HFD), a single dose of this botanical supplementation would decrease the responses to oral fat and carbohydrate tolerance tests, and that chronic supplementation would result in increased fecal triglyceride content. We showed that acute administration in HFD-fed mice (1.452 g/kg body weight) markedly reduced circulating triglycerides following an oral lipid gavage, whereas glycemic responses to various carbohydrate tests were only mildly affected. When incorporated into the food (2.5%) of HFD-fed mice, chronic supplementation prevented body weight gain and improved glucose homeostasis and lipid tolerance. Fecal free fatty acid content, but not triglyceride, was significantly increased in supplemented animals, suggesting reduced lipid absorption in the digestive tract. Congruently, this botanical supplementation downregulated several genes associated with fatty acid transport whose expression was increased by HFD, principally in the jejunum.

Abbreviations: AEAE, apparent energy assimilation efficiency; ANOVA, analysis of variance; AUC, area under the curve; CE, cholesterylester; FA, fatty acid; FFA, free fatty acid; HFD, high-fat diet; HFD-T63, High-fat diet + Totum-63; LFD, low-fat diet; T2D, type 2 diabetes; TG, Triglycerides.

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This study provides novel insights as for the mode of action behind the antiobesity effect of this plant-based supplementation, in HFD-fed mice.

1. Introduction

Type 2 diabetes (T2D) global prevalence is reaching pandemic proportions, with an estimated 537 million people affected by the disease [1]. Because obesity is widely recognized as the principal independent risk factor for developing T2D [2,3], the recommendations from major health organizations worldwide have now included lifestyle modifications to induce weight loss as first-line interventions for management of T2D [2,4–6]. In practice, albeit effective, this strategy faces poor adherence rates among individuals with T2D [7,8] and is associated with failure to meet weight loss and glycemic control objectives [9]. Hence, the development of complementary tools has become essential to help achieve the objectives associated with lifestyle modifications. In this context, the variety of bioactive compounds naturally found in plants, such as polyphenols, represent an attractive perspective, whose potential is now recognized by major international health institutions [10,11]. Totum-63 is a blend of 5 plant extracts selected for their abundance in polyphenols and combined to reduce T2D risk factors. In the framework of clinical trials, Totum-63 was previously shown to be safe and well-tolerated and to elicit antiobesity and antidiabetic effects in subjects with overweight [12] and in subjects with prediabetes or early-stage newly diagnosed T2D [13]. Preclinical studies in various rodent models of obesity and T2D have also confirmed the beneficial effects of Totum-63 supplementation on obesity and whole-body metabolic homeostasis [12,14–16]. Interestingly, the impact of the supplementation was found to be enhanced when associated with physical activity in high-fat diet (HFD)-fed rats [17], highlighting its potential benefits as part of a lifestyle modification strategy for T2D prevention. Several mechanisms related to the improvement of glucose homeostasis were evidenced in these studies, notably via ameliorations of certain HFD-induced impairments in different organs (e.g., organ-specific insulin resistance and associated inflammation, fibrosis, oxidative stress, intestinal dysbiosis). However, the mode of action by which Totum-63 compounds exert their antiobesity effects has yet to be elucidated. Recently, our group has published data showing an inhibitory action of Totum-63 components in vitro on major enzymes involved in macronutrient breakdown, with a moderate affinity for pancreatic α-amylase and lipase, and a higher affinity for α-glucosidase [15]. In parallel, quantitative and qualitative analyses of feces in HFD-fed mice have shown in vivo an increase of fecal energy excretion in supplemented mice [12,14]. Taken together, these results could indicate that Totum-63 active molecules operate in vivo by inhibiting the breakdown of lipids and complex carbohydrates, ultimately reducing their absorption. In line with this, recent reviews on polyphenols have highlighted their possible interactions with nutrients resulting in modulation of intestinal uptake [18,19] and interactions with uptake membrane transporters were reported [20]. Therefore, we hypothesized that a single dose of Totum-63 would decrease glucose responses to oral carbohydrate tolerance tests and blunt triglyceride (TG) response to an oral fat tolerance test, and that chronic Totum-63 supplementation would result in increased fecal TG content, resulting from inhibited enzymatic hydrolyzation of TG into free fatty acids (FFA). Thus, we investigated the acute effects of a single dose of Totum-63 in nonsupplemented HFD-fed mice on the glycemic and triglyceridemic response to various carbohydrate and fat tolerance tests and the consequences of chronic Totum-63 supplementation distributed throughout the day in fecal lipid profile and gene expression of intestinal macronutrient enzymes and transporters in a context of HFD, in mice.

2. Material and methods

2.1. *Characterization of Totum-63*

Totum-63 is a patented blend of 5 plant extracts designed to act in combination to target the risk factors of T2D. The mixture contains extracts from olive leaf *(Olea europaea)*, bilberry *(Vaccinium myrtillus)*, artichoke *(Cynara scolymus)*, chrysanthellum *(Chrysanthellum indicum subsp. afroamericanum B.L. Turner)*, and black pepper *(Piper nigrum)*. Total phenolic compound levels, total sugar level, and total fat level were assessed using the Folin-Ciocâlteu method [21], the Dubois colorimetric method [22], and the sulfo-phospho-vanillin colorimetric assay [23,24], respectively. Protein content was quantified with a fluorometric method using fluoraldehyde o-phthaldialdehyde reagent [25] and dietary fibers were quantified by the Prosky method [26]. Minerals and trace elements were assessed by ash analyses. A more precise characterization was performed by high-performance liquid chromatography-ultraviolet/Visible-mass spectrometry with a C18 column (250 × 4.6 mm, 5 µm). The entire biochemical characterization is presented in Table 1.

2.2. *Animals*

All animal procedures were approved by the local ethics committee (C2E2A, Auvergne, France, authorization no. 18588-2019012216136952-v3 on March 29, 2019) and comply with the ARRIVE guidelines. All experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments. A total of 116 male C57BL6JRj mice aged 6 weeks were used for the various procedures of this study (Janvier Labs, France). The study was conducted in male only to match the conditions of our previous experiments on this plant extract combination [12,14,16]. All mice were housed in our animal facility center (Valbiotis R&D Center, Riom, France) at 22 °C under a standard 12-hour light, 12-hour dark cycle, in individual cages. On arrival, all mice were fed a commercially available purified ingredient-based low-fat diet (LFD; D12450H, Research Diets, USA; see Table 2 for complete composition) for

Table 1 – Biochemical characterization of Totum-63.

Compound types (sorted by families)	Extract content (g/100 g)
Total lipids	6.9
Total proteins	1.9
Total sugars	31.3
Fibers	4.4
Ashes (minerals)	15.0
Total phenolic compounds (gallic acid equivalent)[a]	9.1
Total anthocyanins	0.964
Monocaffeoylquinic acids	1.073
Chlorogenic acid	0.645
Other monocaffeoylquinic acids	0.428
Dicaffeoylquinic acids	0.917
Cynarin	0.112
Other dicaffeoylquinic acids	0.805
Caffeic acid	0.019
Oleuropein	3.645
Oleuropein isomers	0.519
Hydroxytyrosol	0.454
Luteolin	0.030
Luteolin-7-O-glucoside	0.656
Luteolin-7-O-glucuronide	0.440
Apigenin	0.016
Apigenin-7-O-glucoside	0.093
Apigenin-7-O-glucuronide	0.324
Apigenin-6-C-glucoside-8-C-arabinoside (Shaftoside)	0.029
Apigenin-6,8-C-diglucoside (Vicenin 2)	0.060
Eriodictyol	0.008
Eriodictyol-7-O-glucoside	0.590
Marein and flavanomarein	0.318
Maritimein	0.129
Rutin	0.014
Verbascoside	0.046
Terpenes and terpenoids	
Oleanolic acid	2.004
Saponins	
Chrysanthellin A	0.553
Chrysanthellin B	0.507
Iridoids	
Oleoside	0.290
Alkaloids	
Piperine	0.007

[a] Colorimetric Folin-Ciocalteu method.

2 weeks of acclimatization until the age of 8 weeks. Sample size was estimated based on our previous experiments in HFD mice [12,14] and the exact number of mice per group is given thereafter for each experiment.

2.3. *Acute administration of Totum-63*

2.3.1. *Diet and test items*

Following habituation, a group of 50 nonsupplemented (Totum-63 naïve) mice was placed on a commercially available purified ingredient-based HFD (45% kcal from fat; D12451, Research Diets; see Table 2 for complete composition) for 2 weeks before being submitted to various oral tolerance tests. Totum-63 was suspended in Tween-20 1% (290.4 mg/mL) to allow for gastric gavage and distributed right before the test (Fig. 1). The dose given for acute tests was 1.452 g/kg, based on previous dose-response experiments (unpublished data).

2.3.2. *Oral carbohydrate and fat tolerance tests*

Tests were performed in 6-hour fasted mice. Twenty-eight mice were divided into 2 groups: Totum-63 ($n = 14$) and vehicle ($n = 14$). A single dose of Totum-63 or an equivalent volume of vehicle (Tween 20, 1%) was administered orally right before an oral gavage of a starch, maltose, or glucose solution (2 g/kg of body weight). Volume for each gavage was 5 mL/kg of body weight (10 mL/kg of body weight in total). Glycemia was recorded before (0), 15, 30, 60, 90, and 120 minutes after gavage out of a drop of whole blood from the tail of the mice with a glucometer (Stat Strip Xpress, Nova Biomedical, UK). Total glucose area under the curve (AUC) was determined using the trapezoidal method. One olive oil tolerance test was performed in overnight fasted mice. A single dose of Totum-63 or an equivalent volume of vehicle (Tween 20, 1%) was administered orally right before an oral gavage of olive oil (5 mL/kg of body weight [approximately 150 µL]). Volume for each gavage

Table 2 – Composition of experimental diets.

	D12450H	D12451	D12451 + Totum-63
	% kcal	% kcal	% kcal
Protein	20	20	20
Carbohydrate	70	35	35
Sucrose	17	17	17
Fat	10	45	45
Caloric value	3.8 kcal/g	4.7 kcal/g	4.6 kcal/g
Ingredient	g/kg diet	g/kg diet	g/kg diet
Casein	189.6	233.1	227.2
L-cystine	2.8	3.5	3.4
Corn starch	428.6	84.8	82.7
Maltodextrin 10	71.1	116.5	113.6
Sucrose	163.8	201.4	196.3
Cellulose	47.4	58.3	56.8
Soybean oil	23.7	29.1	28.4
Lard	19.0	206.9	201.7
Mineral mix S10026	9.5	11.7	11.4
Dicalcium phosphate	12.3	15.1	14.8
Calcium carbonate	5.2	6.4	6.2
Potassium citrate $1H_2O$	15.6	19.2	18.7
Vitamin mix V10001	9.5	11.7	11.4
Choline bitartrate	1.9	2.3	2.3
Totum-63	0	0	25

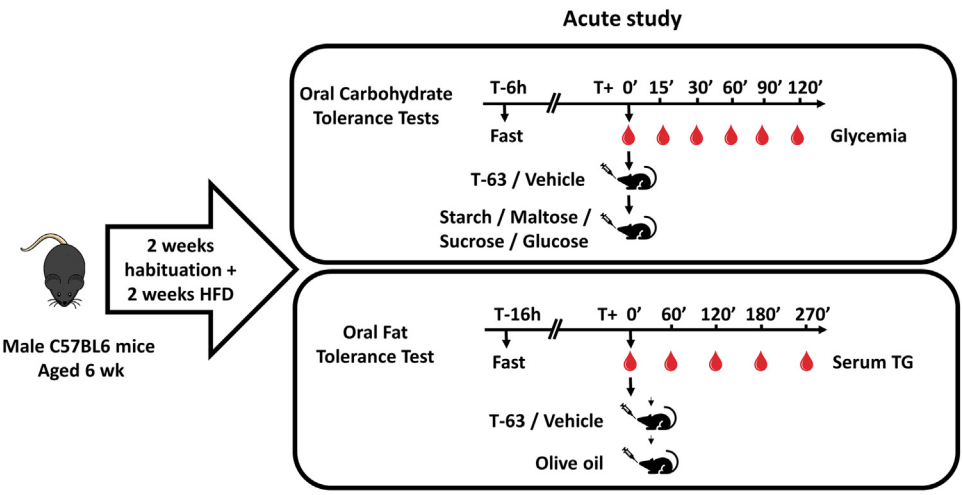

Fig. 1 – Schematic representation of the acute study. Following habituation, mice were placed on HFD for 2 weeks before being submitted to various carbohydrate and fat oral tolerance tests. Totum-63 was distributed right before each test. HFD, high-fat diet; T-63, Totum-63.

was 5 mL/kg of body weight (10 mL/kg of body weight in total). Approximately 20 µL of blood was collected from the tip of the tail during the oral fat tolerance test (before [0], 60, 120, 180, and 270 minutes after gavage) in an EDTA-treated microvette tube (#20.1288, Starstedt, Germany) which allowed preparation of approximately 5 to 10 µL of plasma by centrifugation (2000*g*, 10 minutes, 4 °C). Plasma was aliquoted and stored at –80 °C after preparation. Plasma TG were assessed as described in Section 2.6.1. Animals were given at least 14 days between each test for recovery and washout.

An oral sucrose tolerance test was performed in a separate group of 22 mice. Mice were fasted for 6 hours and then divided into 2 groups (Totum-63, $n = 14$ and vehicle, $n = 8$). The test was run as described previously except a solution of sucrose (2 g/kg of body weight) was distributed instead of glucose, maltose, or starch.

2.4. *Chronic administration of Totum-63*

Following habituation, 36 mice belonging to a different group were placed for 16 weeks either on LFD (D12450H, $n = 12$), HFD (D12451, $n = 12$), or the same HFD supplemented with Totum-63 (HFD-T63, $n = 12$; D12451 + 2.5% Totum-63 w/w, manufactured by Research Diets, USA; see Table 2 for complete composition). Totum-63 dose was chosen based on our previous studies in HFD-fed mice [12,14]. This HFD has been successfully

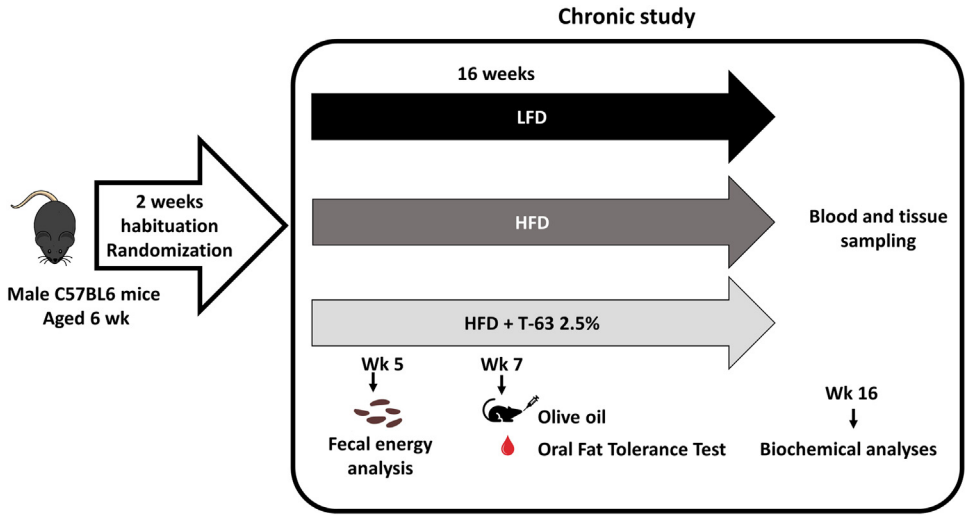

Fig. 2 – Schematic representation of the chronic study. Following habituation, mice were placed either on LFD, HFD, or the same HFD supplemented with Totum-63 (2.5%) for 16 weeks. Feces were collected at week 5. An oral fat tolerance test was performed at week 7. An FITC-dextran test was run at week 16 and blood and organs were collected at week 16 after euthanasia. FITC, 4-kDa fluorescein isothiocyanate-conjugated; HFD, high-fat diet; LFD, low-fat diet; T-63, Totum-63.

used to induce obesity and glucose intolerance in mice [14,27]. Mice were housed individually, and food and water were supplied ad libitum (Fig. 2).

2.4.1. *Body weight, energy intake, and food efficiency*

Mice were weighed every week. Fresh food was distributed every 2 to 3 days and daily food intake was measured by subtracting the weight of leftover food to that of distributed food, divided by the number of days. Whenever food grinding was reported, making it impossible to assert reliable weighing of leftover food, this value was excluded. Energy intake was estimated by multiplying food intake (in grams) to the energy density of each diet (in kilocalories/gram). Assimilable energy density of the diets provided by the manufacturer (Research Diets) was 3.82 kcal/g (LFD), 4.7 kcal/g (HFD), and 4.6 kcal/g (HFD-T63), assuming a null energy value of Totum-63. Food efficiency was calculated as body weight gain normalized by the amount of kcal ingested over the study period (in grams of body weight gained per kcal).

2.4.2. *Fecal energy analysis*

Feces were collected in the cage bedding during the fifth week of experiment and dried out at 60 °C for 72 hours. Fecal energy density was determined by direct calorimetry in a bomb calorimeter (IKA C200, Germany) in approximately 300 mg of dried feces in duplicate, in groups HFD and HFD-T63 only. Briefly, ignition was initiated via an electric resistance linked to the fecal sample by a cotton thread, in a vessel immerged in tap water in presence of ~30 bars of oxygen. The increase of water temperature was recorded during combustion for 7 minutes. Calorific value (CalV) was calculated as follows: $CalV = (C \times DT - QExt1)/m$; where C is a constant related to the heat capacity of the calorimeter, DT is the increase in water temperature, QExt1 is the correction value for the heat energy generated by the cotton thread, and m is the mass of the fecal sample.

Thereafter, apparent energy assimilation efficiency (AEAE) was calculated as fecal energy density × average daily feces production/average daily energy intake over the collection period [28].

2.4.3. *Fasting glycemia, insulinemia, and Homeostatic Model Assessment for Insulin Resistance score*

Fasting glucose and insulin were assessed in serum collected during euthanasia (after 16 weeks of experiment) using colorimetric assays, as described below. Homeostatic Model Assessment for Insulin Resistance score was calculated from serum insulin and glucose levels according to Matthews et al. [29].

2.4.4. *Euthanasia and tissue sampling*

Mice were fasted for 6 hours, then anesthetized with isoflurane, and blood was collected by intracardiac puncture before being centrifuged for 10 minutes at 2000*g*, at 4 °C. Serum was then collected and stored at –80 °C until analysis. Anesthetized mice were euthanized after blood collection by cervical dislocation; duodenum, jejunum, and ileum were carefully sampled out and snap frozen into nitrogen for further analyses.

2.5. *Oral fat tolerance test*

Oral fat tolerance test was performed in a separate group of 30 mice fed LFD (D12450H, $n = 6$), HFD (D12451, $n = 12$), or HFD-T63 ($n = 12$) for 7 weeks. During the seventh week of study, a gavage of olive oil (5 mL/kg of body weight [approximately 150 µL]) was administered in all mice. Oral fat tolerance test was then performed the same way as described in Section 2.3.2). Plasma TG were assessed as described in Section 2.6.1.

2.6. Biochemical analyses

2.6.1. Plasma TG

Plasma TG were assessed for each timepoint with a colorimetric assay (#10010303 Cayman Chemical, USA). Plasma was diluted 10 times in Standard Diluent Assay Reagent (1X) provided in the TG kit before assaying. Total TG AUC was determined using the trapezoidal method.

2.6.2. Glucose

Serum glucose level was estimated using a mouse glucose assay kit (#81692 Crystal Chem, USA), following the manual's instructions.

2.6.3. Insulin

Serum insulin level was estimated using a mouse ultrasensitive insulin enzyme-linked immunosorbent assay kit provided by Alpco (80-INSMSU-E01, Alpco Diagnostics, USA), following the manual's instructions.

2.6.4. Fecal lipidomics

Lipids were extracted from 50 mg of desiccated feces by the methyl-tert-butylether method and analyzed using the Lipidyzer, a direct infusion-tandem mass spectrometry–based platform (Sciex, Redwood City, USA), as previously described [14] . Lipid concentrations are expressed as pmol/mg of dry feces.

2.6.5. Quantitative real-time polymerase chain reaction

Total RNA was extracted from snap-frozen duodenum, jejunum, ileum, or colon samples using TRIzol (#15596026 ThermoFisher Scientific, USA). cDNA was synthesized from 2 µg RNA with the High-Capacity cDNA transcription kit (#4368814 Applied Biosystems, Life Technologies). Polymerase chain reaction amplification was carried out using the CFX Bio-Rad system with Taqman probes sets for mice (ThermoFisher Scientific) and the ΔΔCt method was used to quantify mRNA levels. The Taqman gene expression assay references used in this work are available in Table 3. Gene expression was normalized using *B2m, Ppia, Gapdh*, or *Polr2a* as a housekeeping gene. Samples were taken out when the measurement difference between duplicates exceeded 0.5. Data are represented using the Rq, which is normalized to the control group as $Rq = 2^{-\Delta\Delta Ct}$ [ΔCt = Ct (target) – Ct (*Housekeeping gene*); ΔΔCt = ΔCt (sample) – ΔCt (control)].

2.7. Statistical analyses

Prism V 9.2.0 (GraphPad Software, USA) was used to run statistical tests and draw figures. A Shapiro-Wilk normality test was used to determine whether the data are consistent with a Gaussian distribution. If data were not distributed according to the normal distribution, a Mann-Whitney test (when 2 groups were compared) or a Kruskal-Wallis test (when 3 groups were compared) followed by a Dunn test for post hoc comparison were used. When normal distribution was assumed, measures were subjected to a Student *t* test for unpaired samples (2 groups), 1-way analysis of variance (ANOVA) (3 groups) followed by the Tukey post hoc test, or repeated measures 2-way ANOVA (for repeated measurements over time) followed by the Sidak post hoc test. If a piece of data was missing, making it impossible to run a repeated measures 2-way ANOVA, a mixed-effects analysis was used instead. For comparison of fecal lipid classes, multiple Student *t* tests were used. Values are presented as the mean ± standard error of the mean. The differences were considered statistically significant at $P < .05$.

Table 3 – References of Taqman gene expression assays.

Target	Taqman gene expression assay
B2M	Mm00437762_m1
Gapdh	Mm99999915_g1
Ppia	Mm02342430_g1
Pol2ra	Mm00839502_m1
Cd36	Mm00432403_m1
Fabp2	Mm00433188_m1
Fabp6	Mm00434316_m1
Slc27a1	Mm000449511_m1
Slc27a4	Mm013227405_m1
Abcg8	Mm00445980_m1
Abcg5	Mm00446241_m1
Npc1l1	Mm01191973_m1
Mttp	Mm00435015_m1
Slc2a2	Mm00446229_m1
Slc5a1	Mm00451203_m1
Mgam	Mm01163791_m1
Sis	Mm01210305_m1

3. Results

3.1. Acute administration of Totum-63 improves lipid tolerance and affects glycemic responses following various carbohydrate ingestion

To assess its acute effects on macronutrient absorption in vivo, Totum-63 (or an equivalent volume of vehicle) was administered orally in nonsupplemented mice immediately before a gavage of starch, maltose, sucrose, or glucose. Although circulating glucose levels were not affected by Totum-63 during the starch tolerance test (Fig. 3A and 3B), we found transient improvements in glycemic responses to gavage with maltose (15-minute peak, $P < .001$; Fig. 3C), sucrose (15- and 30-minute peak, $P < .01$ and $P < .05$, respectively; Fig. 3E), and glucose (15-minutes peak, $P < .01$; Fig. 3G) following Totum-63 gavage. However, no significant effect was observed in AUC. We then assessed plasma TG response to an oral fat tolerance test following Totum-63 gavage (or equivalent volume of vehicle) and observed a significant decrease in plasma TG 120 minutes and 180 minutes after olive oil gavage, when Totum-63 had been administered beforehand ($P < .001$ and $P < .05$, respectively; Fig. 3I) as well as in the AUC ($P < .01$; Fig. 3J).

3.2. Chronic supplementation with Totum-63 prevents body weight gain and improves both whole-body metabolic homeostasis and lipid tolerance

We next investigated the impact of chronic supplementation for 16 weeks with Totum-63 in HFD-fed mice (Fig. 2). All ani-

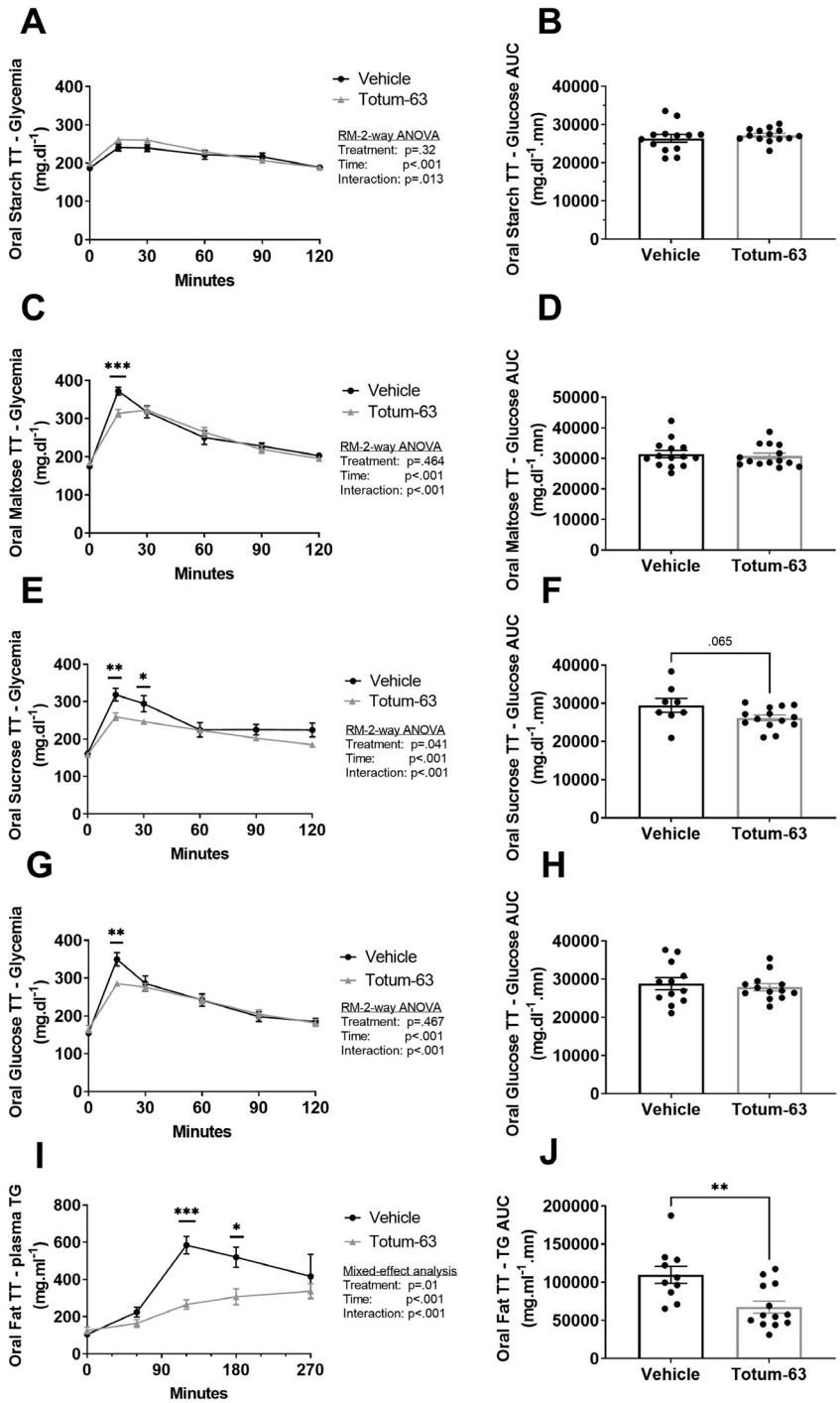

Fig. 3 – Acute administration of Totum-63 improves lipid tolerance and affects glycemic responses following various carbohydrate ingestion. Nonsupplemented mice were fasted for 5 hours and submitted to an oral gavage of a suspension of Totum-63 (in Tween 20 1%; 1.452 g/kg of body weight) or an equivalent volume of vehicle (Tween 20 1%) before receiving a solution of carbohydrate (2 g/kg of body weight) or olive oil (5 mL/kg, ~150 µL). (A) Blood glucose response following an oral starch tolerance test. (B) Glucose AUC following starch tolerance test. (C) Blood glucose response following an oral maltose tolerance test. (D) Glucose AUC following maltose tolerance test. (E) Blood glucose response following an oral sucrose tolerance test. (F) Glucose AUC following sucrose tolerance test. (G) Blood glucose response following an oral glucose tolerance test. (H) Glucose AUC following glucose tolerance test. (I) TG response following an oral fat (olive oil) tolerance test. (J) TG AUC following fat tolerance test. Repeated measures 2-way ANOVA or mixed model followed by Sidak post hoc test for multiple comparisons (curves). Unpaired *t* test or Welch corrected unpaired *t* test or Mann-Whitney test (AUC histograms), *P < .05; **P < .01; ***P < .001. Values as mean ± standard error of the mean. N = 8-14 animals per group for all experiments. ANOVA, analysis of variance; AUC, area under the curve; TG, triglyceride; TT, tolerance test.

mals completed the study. However, 2 animals were excluded in group HFD-T63: (1) an animal displaying significantly lower average food intake, resulting in lower Totum-63 intake, compared with the group average (–16%). The main effects were likely to be caused by lower energy intake rather than the effect of supplementation. (2) One animal displaying very low body weight (15.93 g at the end of the study, –50% compared with the group average) despite normal energy intake and no associated adverse clinical signs. Food grinding was reported 20 times over the study (of 310 measurements), leading to the exclusion of 6% of food intake data in HFD-T63. No significant effect of Totum-63 on average daily energy intake was observed compared with HFD (Fig. 4A, and B). Totum-63 intake is presented in Fig. 4C, averaging over study period at approximately 63 mg/d. Body weight was significantly reduced in HFD-T63 mice compared with HFD mice (Fig. 4D and E; $P < .01$). Subsequently, food efficiency was significantly lower in the HFD-T63 group compared with the HFD group (Fig. 4F; $P < .001$). After 16 weeks, although no significant differences were observed between groups in fasting serum plasma glucose levels (Fig. 4G), both fasting plasma insulin levels and calculated associated Homeostatic Model Assessment for Insulin Resistance score were significantly reduced in the HFD-T63 vs. HFD groups (Fig. 4H, $P < .01$ and Fig. 4I, $P < .05$, respectively). An oral fat tolerance test was performed during the seventh week of study. Serum TG levels after olive oil gavage are presented in Fig. 4J. Mixed-effect analysis revealed significant time ($P < .001$) and group ($P < .001$) effects, but no significant interaction ($P = .291$). TG AUC was increased in HFD compared with LFD ($P < .001$; Fig. 4K). TG AUC was significantly lower in HFD-T63 compared with HFD ($P < .001$ vs. HFD; Fig. 4K).

3.3. *Chronic supplementation with Totum-63 increases fecal energy density and FFA content*

Although the amount of feces produced over 24 hours was not significantly different between HFD and HFD-T63 (Fig. 5A), we observed that Totum-63–supplemented animals displayed higher fecal energy density (Fig. 5B; $P < .05$) and, consequently, lower AEAE (Fig. 5C). Consistent with this finding, broad-spectrum lipidomics analysis revealed increased fecal levels of FFA and cholesterylesters (CE) in HFD-T63 vs. HFD (Fig. 5D; $P < .05$). The difference in other lipid classes did not reach statistical significance threshold. FFA were found to be the most abundant class in the fecal lipidome (>99% of all species) in both groups. Feces were further investigated for individual lipid species within the FFA and CE classes. Mice in group HFD-T63 had significantly higher fecal abundance in 3 species of the FFA class (Fig. 5E) and 17 species of the CE class (Fig. 5F).

3.4. *Chronic supplementation with Totum-63 decreases the expression of genes associated with lipid and cholesterol transport*

Gene expression of several fatty acid (FA) and cholesterol transporters was investigated in mouse duodenum, jejunum, and ileum harvested after 16 weeks of study. In the duodenum, mice belonging to group HFD-T63 displayed increased expression of *Abcg5* and *Abcg8*, 2 genes coding for transporters involved in cholesterol efflux into the intestinal lumen (Fig. 6A). The expressions of genes involved in FA uptake or glucose transport and catabolism were not different between groups HFD and HFD-T63 (Fig. 6B). In the jejunum, the gene expression of *Cd36, Fabp2, Slc27a4*, and *Mttp* was increased in HFD compared with LFD. *Cd36, Fabp2* (statistical trend only, $P = .098$) and *Slc27a4* gene expressions were reduced in HFD-T63 compared with HFD, and we found decreased *Slc27a1* and *Npc1l1* gene expression in HFD-T63 vs. LFD and HFD (Fig. 6C). HFD induced downregulation of genes associated with carbohydrate catabolism (*Mgam, Sis*) and transport (*Slc5a1*) compared with LFD. The expression of *Slc5a1* and *Mgam* was significantly lower in HFD-T63 compared with HFD (Fig. 6D). Finally, in the ileum, *Fabp2* gene expression was increased in HFD vs. LFD and significantly decreased in HFD-T63 vs. HFD (Fig. 6E). To assess whether these effects were the result of a chronic exposure or of a more direct effect of Totum-63, we measured the expression of the same genes in jejunum (where most of the effects on the gene expression of FA and cholesterol transport occurred) and duodenum (the most proximal part of the intestine where most of glucose absorption takes place), following a single gavage with Totum-63 in naïve mice. No effect was observed on the expression of genes associated with FA uptake. The effects of that associated with cholesterol excretion and uptake were inconsistent as *Abcg5* and *Npc1l1* were found to be downregulated while *Mttp* was upregulated (Supplemental Fig. S1).

4. Discussion

This work aimed to investigate the effects of Totum-63 supplementation on macronutrient absorption, 1 of the main explanatory hypotheses for its antiobesity and associated antidiabetic effects. We confirmed our hypothesis that acute Totum-63 administration would reduce glycemic and triglyceridemic responses to oral carbohydrate and fat tolerance tests in HFD-fed mice; however, the hypothesis that chronic Totum-63 supplementation would increase fecal TG content was rejected.

Acutely, in HFD-fed nonsupplemented mice, a single bolus of a blend of 5 plant extracts, Totum-63, distributed right before a gavage of olive oil markedly reduced serum TG response, suggesting a reduction of lipid absorption. The effect on glucose response to oral carbohydrate tolerance test was modest and transient, with only early glucose peaks being blunted when Totum-63 was administered before the glucose, sucrose, and maltose tests. The response to a starch tolerance test was not altered. Studies have suggested that postprandial hyperglycemia is an independent risk factor for diabetic complications [30,31], possibly via induction of oxidative stress and inflammation [32]. Therefore, although glucose AUC in response to carbohydrate tests was not significantly affected by acute Totum-63 administration, the reduction of glucose spikes could constitute an advantage in a context of T2D.

Complex sugars are hydrolyzed by various glucosidases in the digestive tract into monosaccharides, which in turn are absorbed in the small intestine [33]. In this study, we found a modest inhibition of glucose response to a monosaccharide (glucose) tolerance test that, by definition, involves no enzyme

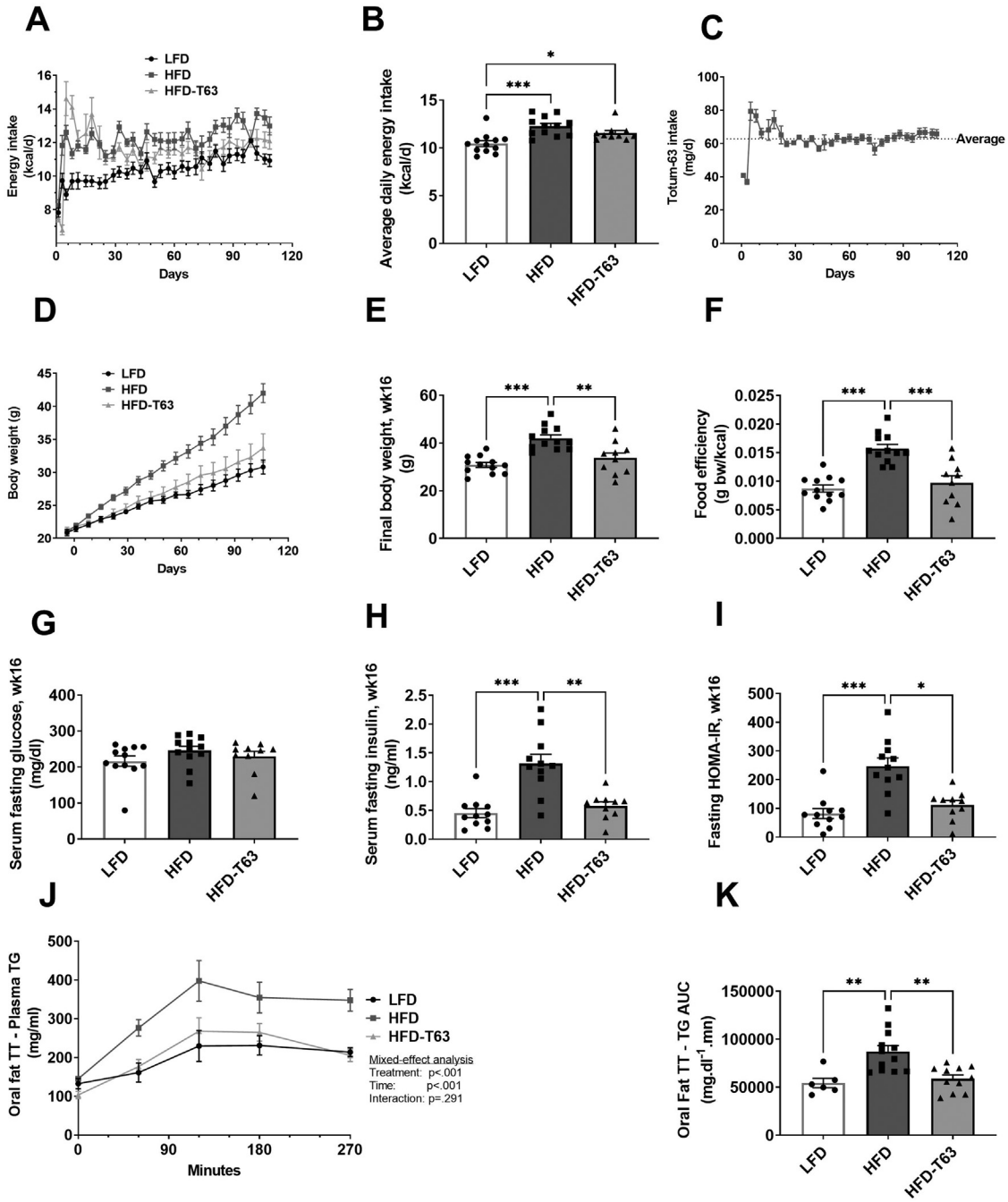

Fig. 4 – Chronic supplementation with Totum-63 prevents body weight gain and improves both whole-body metabolic homeostasis and lipid tolerance. (A) Daily energy intake over 16 weeks. (B) Average daily energy intake. (C) Daily Totum-63 intake over 16 weeks. The dotted line shows average Totum-63 over study period. (D) Body weight over 16 weeks. (E) Final body weight. (F) Food efficiency. (G) Serum fasting glucose at the end of the study. (H) Serum fasting insulin at the end of the study. (I) Homeostatic Model Assessment for Insulin Resistance score. (J) Serum TG of mice submitted to an oral gavage of 150 µL of olive oil, after 7 weeks of study. (K) Serum TG AUC following oral fat tolerance test. One-way ANOVA followed by Tukey post hoc test or Kruskal-Wallis followed by Dunn post hoc test, *P < .05; **P < .01; ***P < .001. Values as mean ± standard error of the mean. N = 6-12 animals per group for all experiments. ANOVA, analysis of variance; AUC, area under the curve; HFD, high-fat diet; HFD-T63, high-fat diet + Totum-63; LFD, low-fat diet; TG, triglyceride; TT, tolerance test.

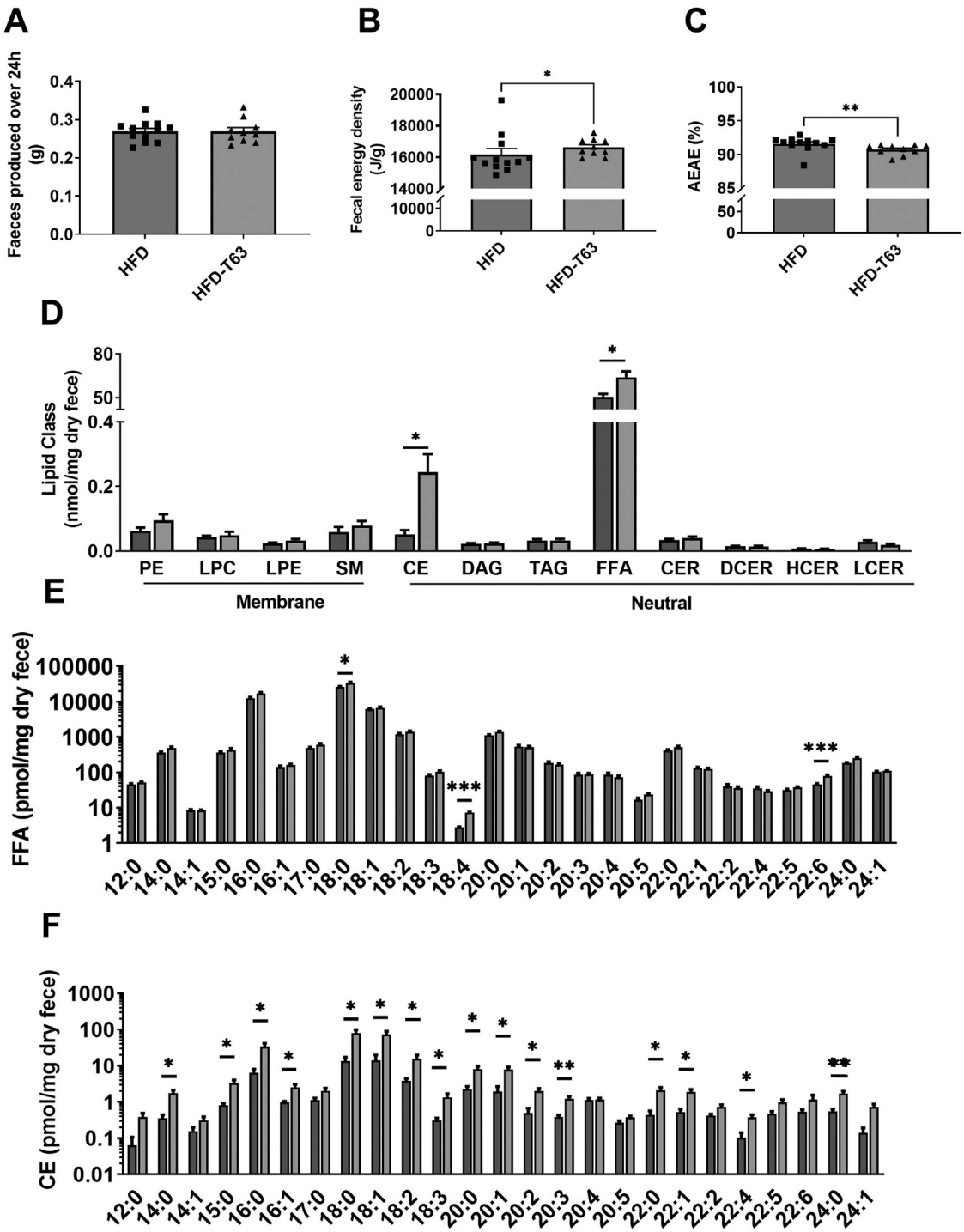

Fig. 5 – Chronic supplementation with Totum-63 increases fecal energy density and FFA content. Analyses were conducted in dried feces collected in the animals' bedding over the fifth week of the study, for groups HFD and HFD-T63. (A) Daily amount of feces produced. (B) Fecal energy density. (C) AEAE. Unpaired *t* test or Mann-Whitney test, *P < .05; **P < .01. (D) Lipid classes present in feces. (E) FFA individual subclasses. (F) CE individual subclasses. Multiple unpaired *t* tests, *P < .05; ***P < .001. Values as mean ± standard error of the mean. *N* = 10-12 animals per group for all experiments. AEAE, apparent energy absorption efficiency; CE, cholesterylester; CER: ceramides; DAG, diglycerides; DCER, dihydro-ceramides; FFA, free-fatty acids; HCER, hexosyl-ceramides; HFD, high-fat diet; HFD-T63, high-fat diet + Totum-63; LCER, lactosyl-ceramides; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; SM, sphingomyelin; TAG, triacylglycerol.

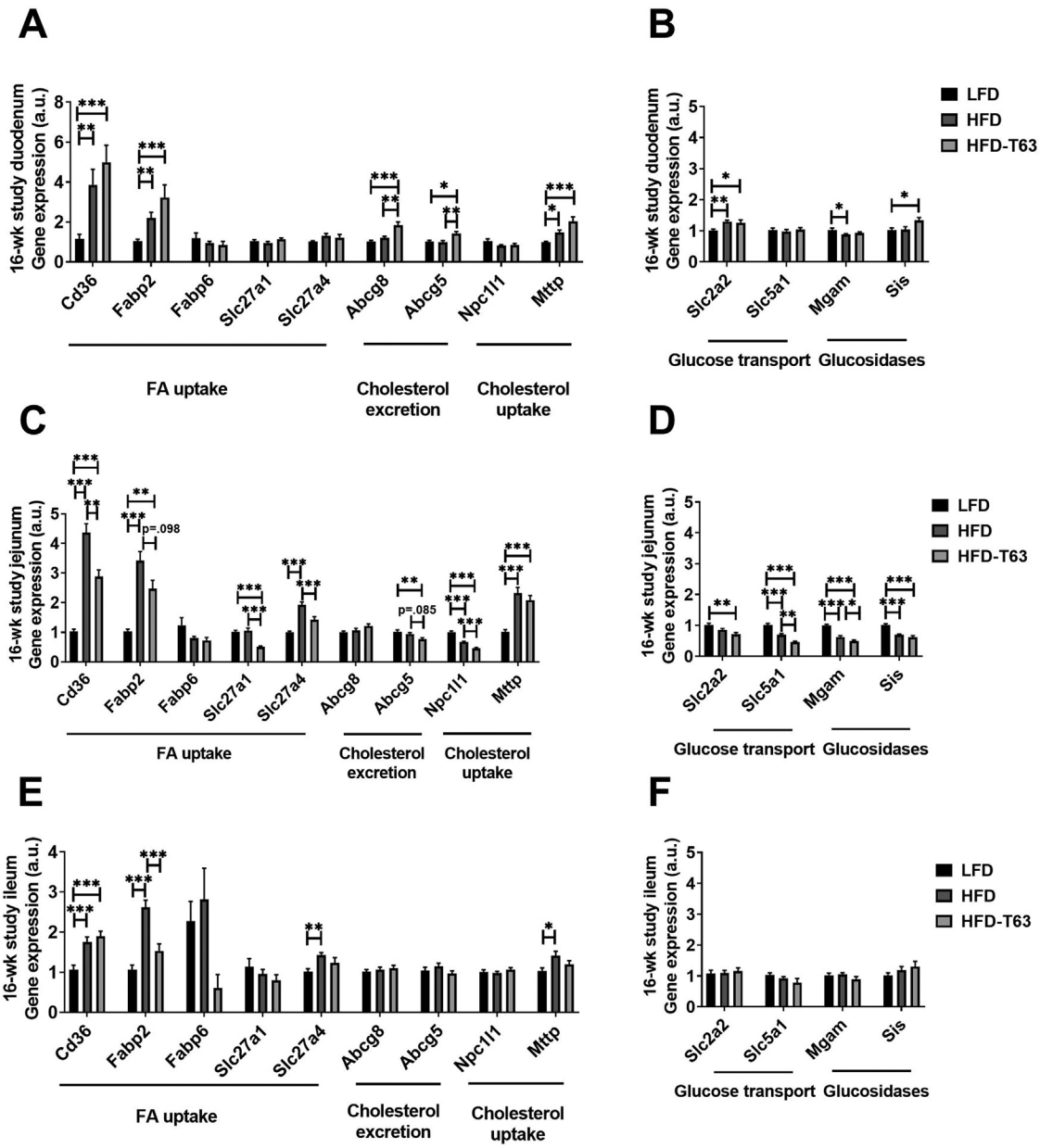

Fig. 6 – Chronic supplementation with Totum-63 decreases the expression of genes associated with lipid and cholesterol transport. Gene expression was analyzed by real-time quantitative polymerase chain reaction in tissues collected after 16 weeks of study. (A) Expression of genes associated with lipid transport in the duodenum. (B) Expression of genes associated with glucose transporters or glucosidases in the duodenum. (C) Expression of genes associated with lipid transport in the jejunum. (D) Expression of genes associated with glucose transporters or glucosidases in the jejunum. (E) Expression of genes associated with lipid transport in the ileum. (F) Expression of genes associated with glucose transporters or glucosidases in the ileum. One-way ANOVA followed by Tukey post hoc test or Kruskal-Wallis followed by Dunn post hoc test, *P < .05; **P < .01; ***P < .001. Values as mean ± standard error of the mean. N = 10-12 animals per group for all experiments. ANOVA, analysis of variance; FA, fatty acid; HFD, high-fat diet; HFD-T63, high-fat diet + Totum-63; LFD, low-fat diet.

hydrolyzation. This may suggest an interaction between the compounds present in Totum-63, glucose, and its membrane transporters (Glut 2 and SGLT-1), resulting in partial inhibition of glucose uptake. Interestingly, certain polyphenols found in Totum-63, such as apigenin, apigenin-7-O-glucoside, luteolin, or oleuropein have demonstrated such inhibition of glucose transporters in vitro [34].

That using disaccharides (sucrose and maltose) did not add any further benefits on blood glucose response may inform that Totum-63 compounds did not inhibit disaccharide hydrolases in this model (or very weakly), contrary to the findings of a previous in vitro study from our team that demonstrated a strong inhibitory effect of Totum-63 components on α-glucosidase, compared with reference drugs [15]. As already

postulated by other authors [35], in vitro inhibitory enzymatic activities are not always maintained in vivo, especially with natural extracts confronted with proteolytic enzymes and low pH in the stomach. That starch tolerance was not affected is intriguing, although it supports the hypothesis that Totum-63 molecules did not inhibit carbohydrate hydrolases. One putative explanation could be the complexity of starch as a carbohydrate, whose hydrolyzation involves several enzymes and makes it more slowly available than monosaccharides, thereby delaying its absorption and making it occur further downstream in the digestive tract [33,36]. It can be reasonably speculated that the molecules in Totum-63 responsible for inhibiting glucose transport are less active after a while, with these acute alterations being probably reversible. Finally, it should be kept in mind that the effects on glucose response were relatively mild compared with those observed on lipid absorption.

Chronically, we confirmed in this work the reduction of HFD-induced body weight gain and hyperinsulinemia in Totum-63 supplemented mice, in line with previous studies [12,14,17]. Because HFD-induced intestinal permeability has been associated with systemic endotoxemia, a triggering factor for insulin resistance [37], we indirectly investigated intestinal barrier function with a 4-kDa fluorescein isothiocyanate-conjugated-dextran assay. We examined the expression of several genes associated with inflammation and junction protein but no adverse effect of HFD was found, in our experimental setup (Supplemental Fig. S2).

On the other hand, the effects on body weight and glucose homeostasis were concomitant with a reduction of plasma TG response in fasted mice (vs. HFD) following an oral fat tolerance test (without any acute Totum-63 administration before the test), suggesting that chronic exposure to Totum-63 blunted HFD-induced exacerbated lipid absorption capacity. Although fat tolerance tests such as those in this study have been extensively used for assessment of lipid absorption in rodents [38], 1 of their limitations is that the resulting circulating TG response is a combination of the effects of lipid absorption and lipid storage metabolism. Once recombined in the intestinal epithelium, circulating TG can be hydrolyzed by the lipoprotein lipase to form FFA, making them available for cellular uptake and thereby reducing circulating TG levels. Using a chemical lipolytic enzyme inhibitor such as Tyloxapol [39] would have allowed us to block TG hydrolyzation in the circulation and therefore measure fat absorption only, following gavage. Nevertheless, although we cannot rule out with this test that Totum-63 components did not affect systemic lipid metabolism, that fecal FFA levels were increased in animals chronically supplemented with Totum-63 corroborates the hypothesis that fatty acid absorption is in supplemented animals compared with HFD.

Consistent with earlier studies from other teams [40,41], the increased lipid absorption capacity in group HFD was associated with upregulation of the gene expression of several long-chain and very-long-chain fatty acids binding and transport proteins, principally in the jejunum. Interestingly, Totum-63–supplemented animals displayed downregulated expression of several of these genes, namely *Cd36*, *Fabp2*, and *Slc27a4* (vs. HFD), as well as *Slc27a1* (vs. HFD and LFD). Cd36 is a membrane scavenger receptor, highly expressed in the proximal small intestine, and an important contributor of FA uptake by enterocytes [42]. In line with our findings, the exposure to a HFD in mice was previously shown to stimulate its expression [41,43]. However, other teams have demonstrated that FA uptake is not impaired in HFD-fed Cd36 deficient (knockout) mice [44]. Putatively, in this rather extreme model of complete Cd36 deletion, it has been speculated that FA uptake could be compensated for in other parts of the intestine or by other transport/binding proteins [45]. Because no compensation is observed in T63-supplemented mice in this work (whether in the other subparts of the intestine or in the other genes involved in FA uptake), the decrease in *Cd36* expression is likely to have played a role in the effects observed. *Slc27a4* codes for the overexpression of transcription of FA transport protein 4, which is thought to be 1 of the major regulators of FA uptake in the enterocyte [46], was shown to increase FA uptake, and, conversely, the reduction its gene expression led to FA uptake inhibition [47]. The role of *Slc27a1* (coding for the transcription of FA transport protein 1, Fatp1), an insulin-activated FA transporter, has mostly been studied in insulin-sensitive tissues (adipose tissue, skeletal muscle), where it stimulates FA uptake [46]. Therefore, the implication of its modest repression observed in the jejunum following Totum-63 supplementation remains to be clarified. Finally, Fabp2, a cytosolic protein involved in carrying FA to the intracellular TG biosynthesis site, was shown to reduce body-weight gain and increase fecal excretion when knocked out in a recent study in mice [48]. Together, these regulations constitute a plausible explanatory mechanism for the reduced TG response following a fat tolerance test and the lower body-weight gain compared with HFD in the chronic study, with the limitation that only the gene expression of theses transporters was assessed, as opposed to protein levels or activity of the corresponding enzymes. In addition, we could not assess in this work gut microbiome composition. The impact of HFD on the microbiome and subsequent dysbiosis is well-established in scientific literature and some studies have suggested that certain species could be involved in the enhanced lipid absorption following HFD exposure, via increased lipid transporter expression [49], as we observed in the present work. Other authors have found that Celastrol, a compound extracted from a plant belonging to the Celastraceae family, inhibits intestinal lipid absorption by modulating gut microbiota [50]. Because chronic Totum-63 supplementation was previously found to alter fecal microbiota composition in mice, it is possible that changes in gut microbiome induced by Totum-63 compounds play a role in the observed downregulation of lipid transporter gene expression.

In addition, we confirmed in this work the effects on AEAE previously observed [12,14]. Furthermore, mass spectrometry analysis of feces revealed that fecal FFA and CE content was higher in chronically supplemented animals, with FFA representing more than 99% of all lipids in both groups, whereas no differences were observed in fecal TG. This point is particularly relevant because it suggests that, contrary to our initial hypothesis, the mechanism by which Totum-63 components blunt HFD-induced enhanced lipid absorption during chronic use is an action on lipid uptake rather than a putative inhibitory action on pancreatic lipase, which is responsible for hydrolyzation of TG into FFA (this would have resulted

in increased fecal TG). Interestingly, this assumption may be corroborated by the downregulated expression of genes coding for FA transporter and binding proteins, observed in the jejunum of chronically supplemented mice, as developed previously. Contrary to the lipid transporters in jejunum, we found no consistent effect of Totum-63 supplementation on the expression of genes coding for carbohydrate transporters or glucosidases.

Unlike in the chronic study, mice belonging to the acute study had not received any supplementation beforehand, allowing for comparison of TG response in animals with equivalent lipid transport capacity, presumably. Because a single dose was successful at decreasing lipid absorption, it is plausible that an interaction with FA-binding proteins or transporters exists, in addition to the chronic effect on gene expression, making them less available for FA uptake.

Although this experiment does not allow discrimination of the compounds responsible for this effect, it can be speculated that the fibers present in Totum-63 could have contributed, as demonstrated in vitro in a study in which beta-glucan (a soluble fiber) was able to inhibit intestinal uptake of FA [51]. Besides, although fecal lipidomics analysis previously led us to rule out the hypothesis of lipase inhibition following chronic supplementation, we cannot exclude that this effect participated acutely, especially because the gavage represented a dose of 1.452 g/kg, indicating an average 49 mg in 1 shot (for a 33-g mouse, to be compared to the chronic dose of 63 mg/d in average in the chronic study, distributed via food all throughout the day). This is supported by the fact that, as mentioned before, a moderate inhibitory effect of Totum-63 molecules on pancreatic lipase has already been reported in vitro [15]. Here again, we cannot single out in this study which specific component is responsible for this effect, but it can be hypothesized that anthocyanins, luteolin, and rutin present in Totum-63 could have played a role, based on their inhibitory activity demonstrated in vitro [52,53].

In conclusion, we present in this work insights about the mechanisms underlying the effects of Totum-63 supplementation on body weight and glucose homeostasis, principally by tempering the HFD-induced exacerbated lipid uptake and by modestly reducing the glucose response to a carbohydrate load. The analysis of the present product composition revealed the presence of fibers and high levels of phenolic compounds, notably chlorogenic acid, oleuropein, luteolin derivatives, and apigenin derivatives. Other families such as terpenoids and alkaloids were also detected, notably including chrysanthellins and piperine, respectively. We postulate that the lowering effects of Totum-63 supplementation on body weight could be explained mainly by decreased lipid absorption in the intestine, resulting from a combination of downregulated FA transporters genes following chronic exposure and direct inhibition of lipid absorption, possibly involving pancreatic lipase inhibition with higher doses of Totum-63, and competitive interaction with lipid uptake proteins. The mild reduction of glucose peaks to monosaccharide and disaccharide tolerance tests could also participate in the antidiabetic effects of Totum-63 and, to a lesser extent, its antiobesity effects. Concomitant to the effects on body weight, the benefits of Totum-63 supplementation on glucose control have already been reported in former studies using various models [12,14,17], and, importantly, these improvements were shown to translate into humans [12,13]. The substantial improvements in fasting blood glucose 2-hour oral glucose tolerance test shown in these previous works would thus position Totum-63 as a potential tool for prevention of T2D as a first-line therapy (as part of lifestyle modifications).

Body weight management is an integral part of therapeutic strategies in the context of T2D, as mentioned earlier, and major health associations recognize antiobesity drugs, notably inhibitors of fat absorption, as promising strategies to help achieve weight management objectives, along with lifestyle modifications [4,54]. The results obtained in this study add to the understanding of Totum-63 effects in vivo. Tat an acute effect was observed in mice could indicate that the supplement should be taken before meals to exert optimal antiobesity effects in a target population. The present findings were used as a reference to define the research hypotheses and the study layout of a clinical mode of action trial (ClinicalTrials.gov ID: NCT05369585) designed to elucidate whether the effects observed in the present work, notably on macronutrient absorption, translate to humans. If so, Totum-63 should be considered as part as a comprehensive nutritional approach, just like any other weight loss intervention, and be used in a context of a well-balanced dietary plan, nutritional counseling, and possibly other nutritional supplements that ensure adequate intake of essential nutrients.

Author declarations

VC, CL, AM, DR, YO, PS, and FLJ are Valbiotis employees. SLP is Valbiotis CEO. TM and BG are members of the Valbiotis scientific board. MG declares no conflict of interest.

CRediT authorship contribution statement

Vivien Chavanelle: Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Cédric Langhi:** Investigation, Methodology, Project administration. **Arnaud Michaux:** Investigation, Methodology, Project administration, Writing – review & editing. **Doriane Ripoche:** Investigation, Methodology, Project administration. **Yolanda F. Otero:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Florian Le Joubioux:** Methodology, Formal analysis, Investigation, Visualization. **Thierry Maugard:** Supervision, Project administration, Funding acquisition. **Bruno Guigas:** Conceptualization, Resources. **Martin Giera:** Conceptualization, Resources, Investigation, Formal analysis. **Sébastien Peltier:** Supervision, Project administration, Funding acquisition. **Pascal Sirvent:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nutres.2023.07.010.

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