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Ginkgo biloba extract (GbE) restores gut microbiota dysbiosis in a rat model of lard-rich diet-induced obesity

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ABSTRACT

Background: Gut microbiota (GM) modulation has been considered a nutritional approach to manage obesity. Reduced Firmicutes/Bacteroidetes ratio (F/B) is associated with reduced energy harvesting capacity from the diet, ameliorates endotoxemia and inflammation, and restores gut hormone signaling related to hypothalamic control of energy homeostasis. As anti-obesogenic and anti-inflammatory properties have been attributed to *Ginkgo biloba* extract (GbE), the present study investigated whether GbE supplementation for two weeks modulates the GM composition of obese rats.

Method: Fifty-six 2-month-old male *Wistar* rats were submitted to a lard-rich diet-induced obesity protocol for 60 days (high-fat diet, HFD). Following the obesity-inducing period, rats were gavaged daily with GbE at 500 mg/kg (HFD+*G* group), or saline (HFD group), for 14 days. A 3rd group (pair-fed group, PF) was performed by mimicking the HFD group (saline administration) but with its food intake matched to the HFD+*G* group. Rats were euthanized on the 14th supplementation day. Stool DNA was extracted and amplified with V3–V4 region primers of the 16S rRNA gene.

Results: In comparison to both HFD and PF groups, GbE supplementation increased the number of Bacteroidetes colon community and concomitantly reduced the abundance of Firmicutes, reducing the F/B ratio. Hierarchical clustering showed that communities of the HFD+*G* group were less likely related to HFD and PF groups.

Conclusion: As GbE modulated the GM structure and diversity in GbE-supplemented obese rats, our results show that GbE possesses phytotherapeutic potential to modulate obesity by improving GM and lessening the consequences of obesity-related GM dysbiosis.

Introduction

As obesity is considered a multifactorial disease rather than a simple consequence of the imbalance between energy intake and energy expenditure, the mechanisms associated with the establishment of a pro-obesogenic profile are yet to be fully elucidated. The gut microbiota (GM) acts as a modulator of energy homeostasis, and its composition may contribute not only to the development of obesity, but also to its maintenance (Cani and Delzenne, 2011).

It has been proposed that the GM profile is strongly modulated by diet. High-fat diets (HFD) modify GM diversity and composition, resulting in an increased *Firmicutes/Bacteroidetes* ratio (F/B) (Ley et al., 2005), a profile frequently observed in obese individuals (Muscogiuri et al., 2019). Moreover, obesity-related dysbiosis is known to impair

Abbreviations: CHO, carbohydrate; F/B, *Firmicutes/Bacteroidetes* ratio; GbE, *Ginkgo biloba* extract; GLP-1, Glucagon-Like Peptide 1; GM, gut microbiota; HFD, high-fat diet; HFD+*G*, GbE-supplemented group; LPS, lipopolysaccharide; OTUs, Operational Taxonomic Units; PCA, Principal Component Analysis; PCoA, Principal Coordinate Analysis; PCR, polymerase chain reaction; PF, Pair-fed group; PYY, Peptide YY; QIIME, Quantitative Insights into Microbial Ecology; SCFA, short chain fatty acids.

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appetite regulation via gut-derived hormones, enhance the energy harvesting from food, increase intestinal permeability and lipopolysaccharide (LPS) circulating levels (Turnbaugh et al., 2006). These dysbiosis-related manifestations have been correlated with low chronic inflammation and metabolic disturbances, which further increase the risks for co-morbidities (Scheithauer et al., 2020). Considering that the prevalence of obesity has reached epidemic levels, and that most pharmacological agents currently available for obesity are associated with undesirable side effects (Tremmel et al., 2017), novel strategies which modulate the GM dysbiosis to a healthier profile may be promising therapeutic approaches to ameliorate obesity-related disorders.

Both probiotics – live microorganisms that colonize the colon – and prebiotics – indigestible carbohydrates that through bacterial fermentation induce selective changes in GM – have been employed to restore GM composition in obesity (Dahiya et al., 2017). Prebiotic-containing foods, such as Jerusalem artichokes and chicory, have been described as GM regulators due to their activities on stimulating the growth of a limited number of microorganisms (Shao et al., 2020). Another nutritional strategy involves dietary polyphenolic compounds, which exert a modulatory action on intestinal inflammation by improving the GM profile and re-establishing the *Bacteroidetes* and *Firmicutes* balance (Espley et al., 2014).

Standardised *Ginkgo biloba* extract (GbE) has been identified as a GM modulator, as observed in studies evaluating atherosclerosis and depression (Chen et al., 2019; Lv et al., 2021). The standardised GbE composition, with 24% flavonoids and 6% terpenoids, has shown antioxidant, anti-inflammatory and anti-obesogenic properties (Hirata et al., 2015; 2019a; 2019b). Combined, the properties attributed to GbE may offer a potential therapeutic approach to address obesity and related disorders.

Considering the involvement of GM composition in the control of energy homeostasis as well as GM alterations triggered by HFD intake, the present study investigated the effects of GbE supplementation upon the GM of lard-enriched diet-induced obese rats. As GbE has previously been found to influence food intake, the present study also investigated whether the modulation of GM associated with GbE supplementation was a specific effect attributed to the actual supplementation or a secondary effect associated with reduced food intake.

Material and methods

Source and chemical composition of GBE

GbE standardized extract was obtained from Huacheng Biotech Inc. (Hunan, China). Its major bioactive compounds were flavones (25.21%), terpenoids (6.62%), ginkgolides A, B, C (3.09%), and bilobalides (2.73%), as assessed and described in a previous study by our group (Machado et al., 2021).

Ethics statement

The Committee on Animal Research Ethics of the *Universidade Federal de São Paulo* approved all procedures for the care of the animals used in this study (Process number: 8,700,110,814). Every effort was made to reduce animal suffering, following the principles of the Brazilian law for the use of animals in research (Arouca Law - number 11.794 / 08). ARRIVE 2.0 guidelines were followed. Throughout the experimental period, animals were maintained under controlled temperature (23±1 °C) and light/dark cycle (lights on from 6:00 to 18:00) with free access to water and food, except for the pair-fed group, as detailed later.

Experimental design and sample collection

Fifty-six male *Wistar* rats aged 2 months, with average body weight between 350 and 400 g, were obtained from the Multidisciplinary Center for Biological Investigation in Laboratory Animals Sciences (CEMIB, Campinas, Brazil). After one week of habituation in the new animal house facilities at the Sao Paulo Federal University Department of Physiology, all rats were submitted to a diet-induced obesity protocol, with free access to a HFD as previously detailed by our group (Hirata et al., 2015). The diet offered was enriched with 28% lard, providing 19.5% of energy from carbohydrates, 23.2% from protein and 57.3% from fat, as previously described (Hirata et al., 2019a). We have demonstrated that this diet induces obesity in rats (Banin et al., 2021; Hirata et al., 2015; 2019a).

After a 60-day period of obesity induction, the animals were randomly allocated into three groups: (i) GbE supplemented (HFD+*G, n* = 19); (ii) placebo supplemented (HFD, $n = 18$); and (iii) pair-fed (PF, $n = 19$). The HFD+*G* group was daily gavaged with GbE 500 mg/kg for 14 days, as described previously (Hirata et al., 2015). The HFD and PF groups received 1 ml of 0.9% saline by gavage. The amount of food offered to the PF group was matched to that consumed by the HFD+*G* group. The PF group was intended to elucidate whether any GM modulation was attributed to the actual GbE supplementation rather than to a secondary effect associated with reduced food intake.

Animals from different groups were housed in separate cages, avoiding crossed coprophagia. Animal handling took place between 9:00 and 12:00, and the order in which the rats were gavaged was randomized daily, with each animal being handled at a different time each day.

Food intake was quantified throughout the study period and calculated as the difference between the food amount offered and the leftover 24 h later. Food consumption is presented as daily food and energy intake during the supplementation period (13 days, excluding the last day due to fasting). Body weight gain was calculated by the difference between the last and first days of supplementation. At the end of the two-week supplementation, animals were fasted for 8 h and anaesthetised (barbiturate 80 mg/kg intraperitoneally). After the confirmation of anesthesia, a midline incision was made, abdominal organs were visualised, and venous blood collected from the inferior vena cava. Animals were euthanized by decapitation, stool samples were harvested from the colon and placed in sterile microtubes.

A blinding protocol was adopted in this study, where the first investigator was responsible for the supplementation administration based on a randomization table, and the second investigator was responsible for the anesthetic procedure and sample collection, randomizing the sequence of sample outcome. The investigator responsible for metagenomics data was not aware of which specific supplement each rat received.

Results are presented as mean ± SEM. There were no sample exclusions. One-way ANOVA followed by Tukey's test was employed to assess the effects of supplementation among HFD, PF and HFD+*G*. A p-value of < 0.05 was considered statistically significant. Individual rats were the experimental unit for body weight gain and metagenomics data. Analyses were performed using R free software environment (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 9.0 (GraphPad Software, Boston, MA, USA).

Bacterial DNA extraction and 16S metagenomic library preparation

Total genomic DNA was extracted from stool samples with QIAamp DNA Stool minikit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The quality/quantity of extracted DNA was measured using a Thermo Nano-Drop 2000C (Thermo Fisher Scientific, Waltham, MA, USA), with A260/A280 ratio between 1.8 and 2.0 as criterion for quality control. Agarose gel electrophoresis were run, and DNA genomic bands were clearly defined. DNA was stored at 4 °C prior to PCR amplification. Fragments encompassing V3–V4 (450 bp) of the 16S rDNA hypervariable regions were PCR amplified from each of the 29 DNA samples (HFD=9; HFD+*G* = 10; PF=10), according to Illumina's protocols (Illumina Inc., USA). The full-length primer sequences for V3 and V4 16S with Illumina adapter overhang nucleotide sequences were: forward 5′

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGC AG and reverse = 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGGACTACHVGGGTATCTAATCC. Each sample was appended to a dual-index barcode. The PCR conditions were as follows: 95 °C for 3 min, 25 cycles at 95 °C for 30 s, 55 °C for 30 s (annealing) and 72 °C for 30 s (extension), 72 °C for 5 min (final extension) and holding at 4 °C. The PCR products were purified using AMPure XP beads (AGENCOURT®AMPur-e®XP) and NanoDrop® quantified. A 1.5% agarose gel was run to check the amplified products. Subsequently, the amplicon target was attached to dual indices Illumina sequencing adapters with the Nextera XT Index Kit (Illumina Inc.). The conditions were 95 °C for 3 min (denaturation), 12 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, 72 °C for 5 min. The products were purified using beads and a second agarose gel was subsequently run to verify the quality of the products obtained. The V3–V4 PCR products were sequenced by Illumina MiSeq using 2 × 250 pb (Illumina).

Bioinformatics analysis

The forward and reverse reads were merged and processed using Quantitative Insights Into Microbial Ecology – QIIME analysis pipeline and clustered using USEARCH sequence analyses. Paired-end joined sequences were grouped into operational taxonomic units (OTUs) with Green Genes database and UPARSE algorithm with a 97% threshold of pairwise identity. The Shannon Diversity Index, a measurement of within-sample (alpha diversity) community diversity, as well as the Chao1 Richness Estimator and the Simpson Diversity Index were used to ascertain differences within the three experimental groups. Differences between microbiota profiles within samples were assessed using: (i) a principal component analysis (PCA) based on OTU composition; (ii) a principal coordinate analysis (PCoA) based on a weighted UniFrac distance; and (iii) a clustering analysis performed using the Ward's minimum variance method together with the UniFrac distance. All multivariate analyses, as well as visual assessments of GM composition, were performed in R statistical software (R Core, 2013) together with ggplot2 pheatmap, ggtree and ape packages.

Results

Ginkgo biloba reduced food and energy intake but did not influence body weight gain

Average body weight was similar among the three groups at the start of the study. GbE supplementation, however, reduced food and energy intake by 5.8% ($p = 0.02$) in the HFD+*G* group in comparison to the HFD group, as depicted in Fig. 1A and 1B. Despite the changes observed in energy intake, no differences were observed in body weight gain (Fig 1C) within the two-week supplementation period.

Metagenomic analysis

The results of the bacterial 16S rRNA V3–V4 regions of the colon samples sequenced by the Illumina MiSeq platform were presented as operational taxonomic units (OTUs) using a 97% homology cut-off

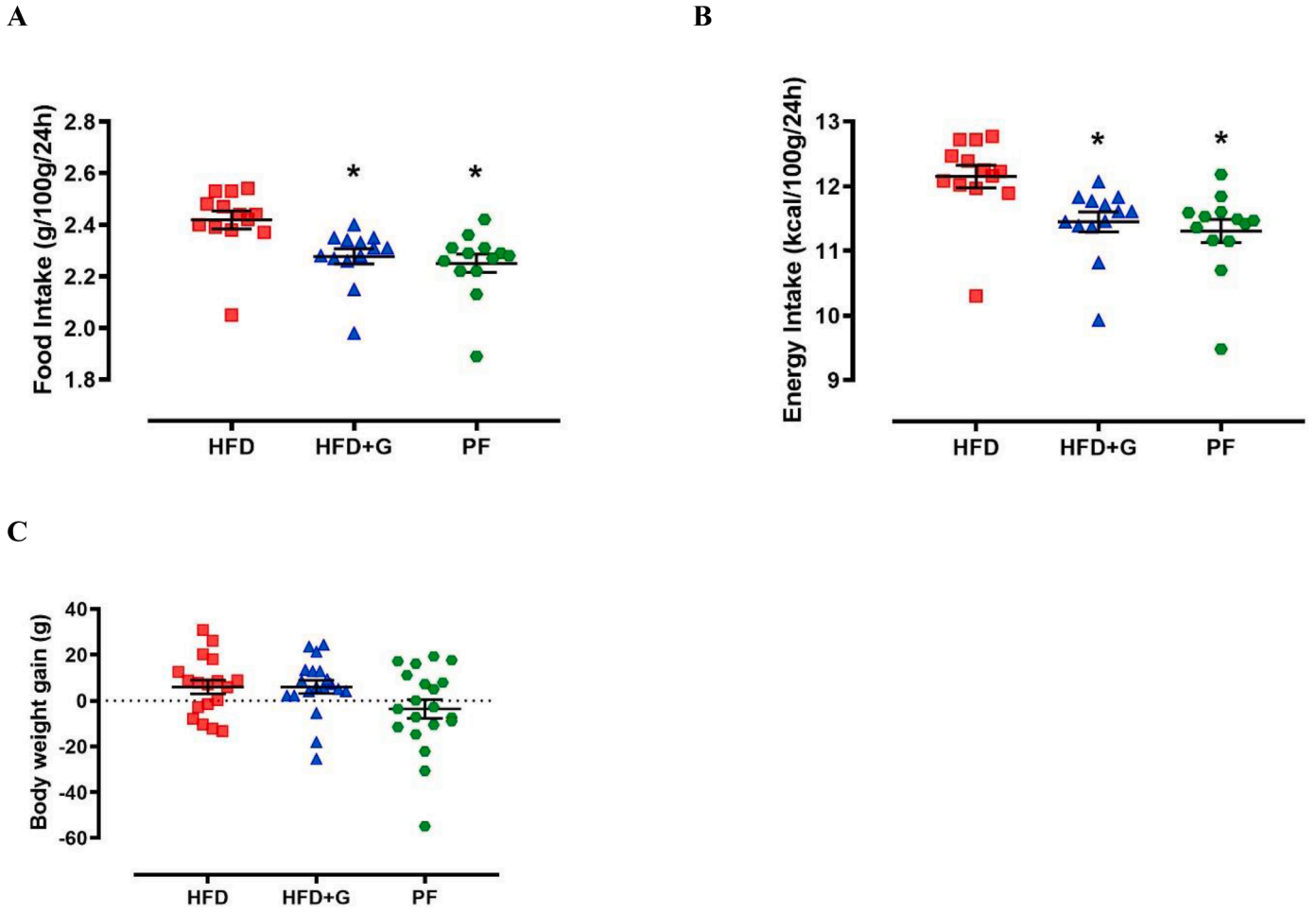


Fig. 1. ***Ginkgo biloba*** **extract supplementation reduced food and energy intake bud did not alter body weight gain:** Fig 1A. Food intake (kcal/100 g/24 h). Fig 1B Energy intake (kcal/100 g/24 h). Fig 1C. Body weight gain of high-fat diet (HFD), HFD+Ginkgo (G), and pair-fed (PF) groups. *$p < 0.05$ *vs.* HFD group. HFD $n = 18$; HFD+*G* $n = 19$; PF, $n = 19$.

value. The taxon abundance was generated before filtering, generating 1952 OTUs, 70 genera, 41 families, 25 orders, 20 classes and 12 phyla. After filtering, the data set was reduced to 61 genera, 43 families, 20 orders, 18 classes and 11 phyla. For statistical comparisons, we started from genus level and only considered genera with at least 75% of abundance in all obtained samples.

In Figs. 2A and 2B, the Principal Component Analysis (PCA) and Principal Coordinate Analysis (PCoA) show that most of the variation found within the samples were not related to the supplementation; however, differences in the abundance profile were observed within groups. The HFD+*G* group presented a GM composition that clustered separately from the HFD group. In Fig 2C, hierarchical clustering identified two main clusters, showing a clear distinction of microbial communities of the HFD group, more closely related to those of PF than of HFD+*G*. This finding is supported by PERMANOVA test values indicating differences in overall composition (genus level $p = 0.011$, family $p = 0.011$, order $p = 0.007$, class $p = 0.009$ and phyla $p = 0.012$).

The alpha diversity was calculated to investigate the effect of GbE on microbial diversity, corresponding to the analysis of within-sample bacterial diversity. Additionally, the Shannon and Simpson diversity indices consider the species richness as well as the community evenness of the GM species. Richness measures were calculated to assess intestinal microbial community structure using the Chao1 estimator (Fig 3), with no statistically significant differences found. Diversity measured by the Shannon's richness index decreased in HFD+*G* in comparison to HFD. The PF group had increased values of Shannon's index compared to HFD+*G* but did not reach the same level as HFD. Diversity measured by the Simpson's evenness index showed decreased values in HFD+*G* compared with HFD. The Simpson's index in PF was lower than in HFD but did not reach the same level as in HFD+*G*. Fig 3 also depicts the ratio of *Firmicutes* to *Bacteroidetes*, where HFD+*G* showed a noteworthy reduction of approximately 37% in comparison to HFD, and approximately 30% in comparison to PF.

Specific GM changes were assessed by comparing the relative abundance of the predominant taxa identified through sequencing (Fig 4). For all taxonomic levels with significant differences ($p<0.05$), heat maps were drawn to better illustrate differences in GM composition.

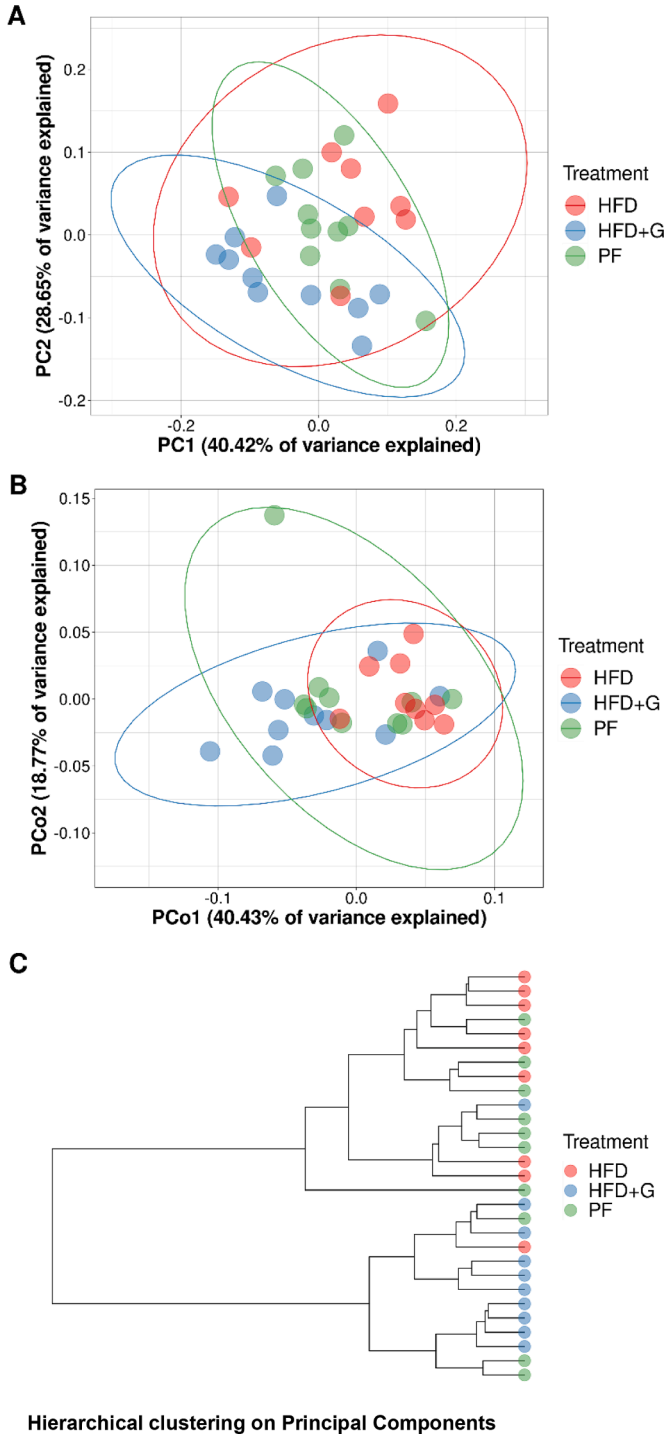


Fig. 2. *Ginkgo biloba* extract supplementation modulated the gut microbiota structure and diversity. Fig 2A Principal Component Analysis (PCA). Fig 2B Principal Coordinate Analysis (PCoA). Fig 2C. Sample clustering results of the UniFrac distances and Ward's method of microbial 16S rRNA sequences from the V3-V4 region in colon contents. High-fat diet (HFD), $n = 9$; HFD+Ginkgo (G), $n = 10$; pair-fed (PF), $n = 10$.

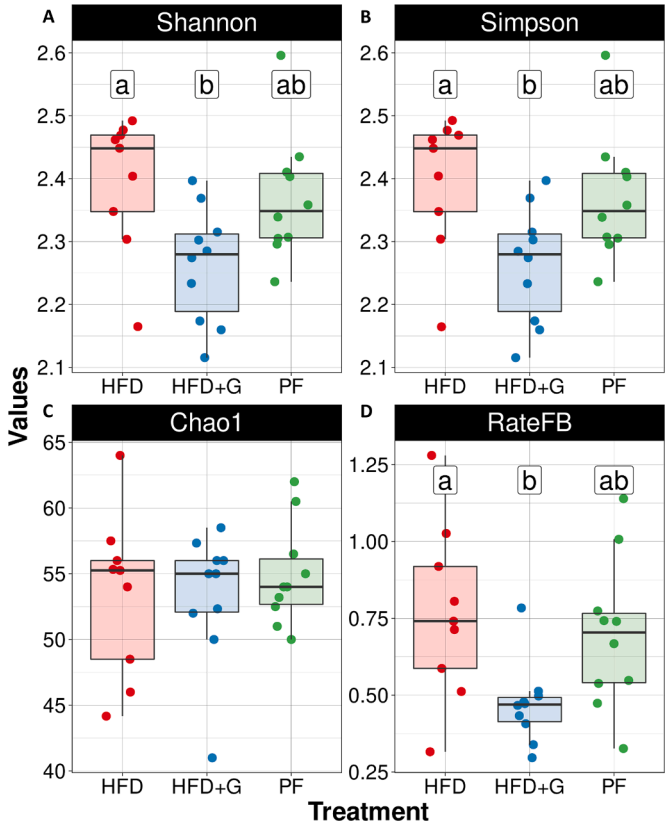
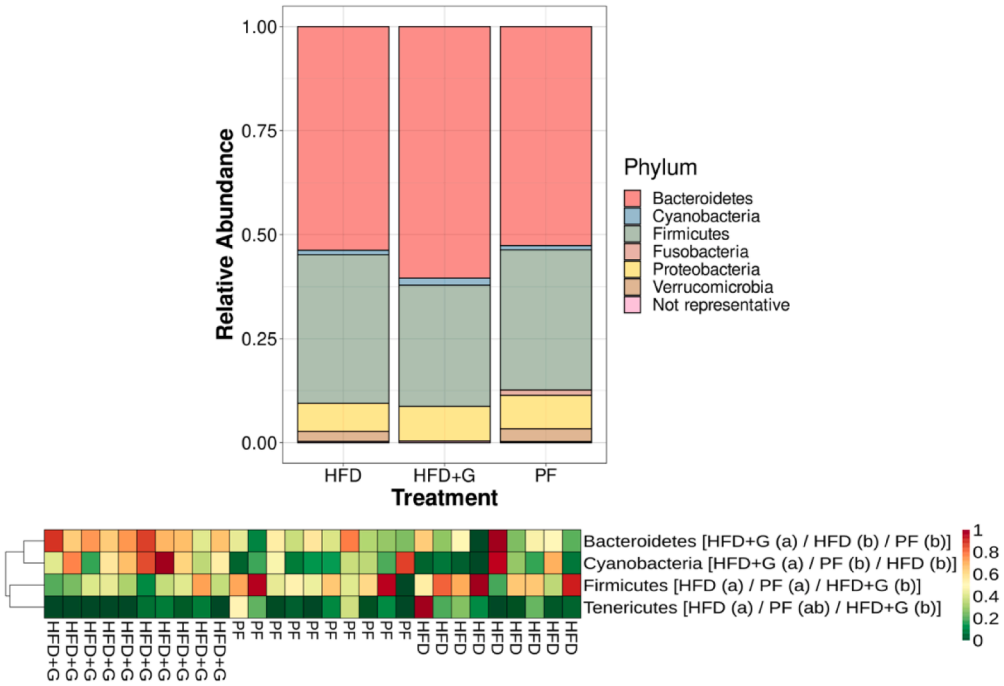


Fig. 3. *Ginkgo biloba* extract supplementation decreased the Firmicutes to Bacteriodetes ratio. Fig 3A. Shannon diversity index. Fig 3B Simpson's index. Fig 3 C. Chao1 estimator. Fig 3D. *Firmicutes* to *Bacteroidetes* ratio. 'b' is significantly different from 'a'. High-fat diet (HFD), $n = 9$; HFD+Ginkgo (G), $n = 10$; pair-fed (PF), $n = 10$.

A – phyllum



B – Class

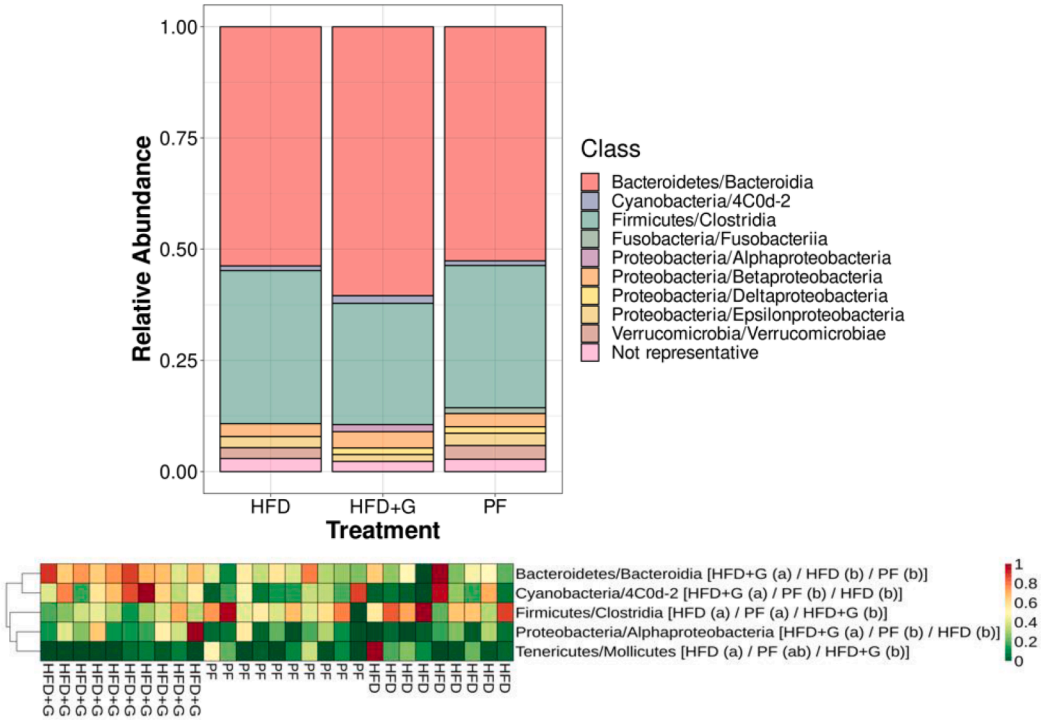
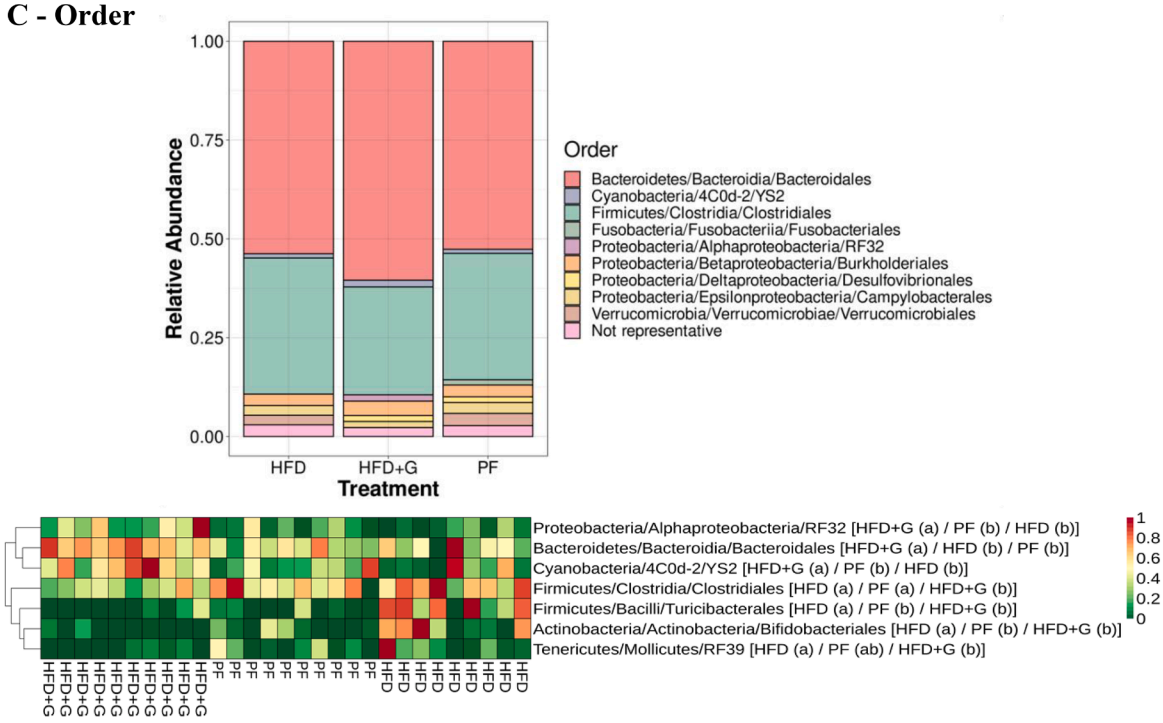
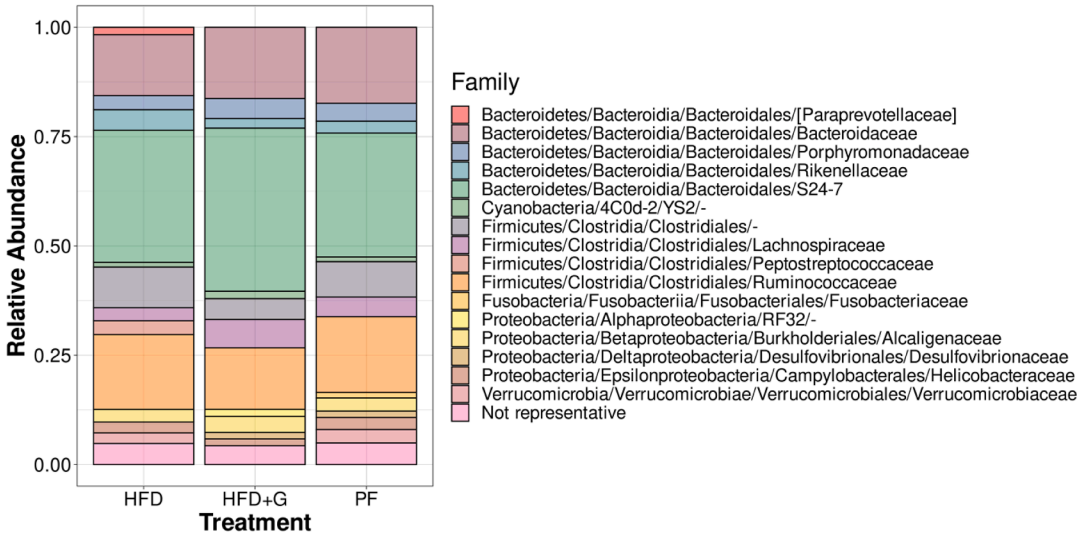


Fig. 4. ***Ginkgo biloba*** **extract supplementation modulated gut microbiota composition.** Fig 4A: Phylum-level, Fig 4B: class-level, Fig 4C: order-level and Fig 4D: family-level taxonomic distributions of the microbial communities in colon content determined by next generation sequencing. High-fat diet (HFD), $n = 9$; HFD+Ginkgo (G), $n = 10$; pair-fed (PF), $n = 10$.

C - Order



D - Family



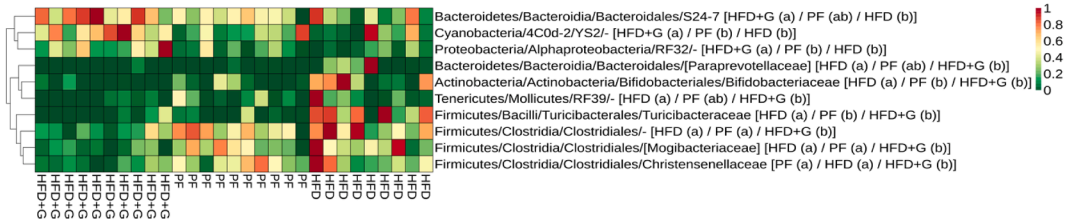


Fig. 4. (*continued*).

I Phylum level: HFD+*G* associated colon community showed a slight decrease in the *Firmicutes* relative abundance and an increase in the *Bacteroidetes* relative abundance compared with HFD, which is corroborated by the F/B ratio (Fig 4A), while PF showed a profile similar to HFD. HFD+*G* presented a higher relative abundance of *Cyanobacteria* and lower relative abundance of *Tenericutes* in comparison to HFD and PF.

II Class level: the HFD colon microbiota was dominated by *Clostridia*, whereas *Alphaproteobacteria* and *Bacteroidia* dominated in HFD+*G* (Fig 4B). HFD+*G* also presented increased levels of *4C0d-2*, and PF showed a pattern of relative abundance similar to all classes in HFD.

III Order level: HFD presented elevated *Clostridiales, Bifidobacteriales,* and *Turicibacterales*, with reductions in *RF32, Bacteroidales* and *YS2* (Fig 4C). In HFD+*G*, the opposite profile was observed, showing decreased levels of *Clostridiales* and *Bifidobacteriales* and increased levels of *Bacteroidales*. PF exhibited intermediated levels of *Clostridiales*, while *Bifidobacteriales, Turicibacterales* and *RF32* were similar to HFD+*G*, and *Bacteroidales* and *YS2* were similar to HFD.

IV Family level (Fig 4D): HFD+*G* showed higher levels of *S24–7* with reduction in the relative abundance of *Paraprevotellaceae, Mogibacteriaceae, Christensenellaceae, Rikenellaceae, Bifidobacteriaceae* and *Turicibacteraceae* in comparison to HFD. PF also had lower levels of *Paraprevotellaceae, Bifidobacteriaceae* and *Turicibacteraceae*, but greater abundance of *Mogibacteriaceae, Christensenellaceae* and *Rikenellaceae* in comparison to HFD.

Discussion

The detrimental relationship between gut dysbiosis and the deleterious outcomes of obesity has been documented (Scheithauer et al., 2020). The GM profile is an interface of the nutritional state of the host, with high fat and high refined carbohydrate intake, combined with low soluble fiber intake, inducing gut dysbiosis and exacerbating metabolic disorders such as obesity and type II diabetes (Dahiya et al., 2017). GM modulation through nutritional interventions shows potential to prevent weight gain and to manage weight loss (Dewulf et al., 2011). In this context, the present study aimed to investigate the effects of GbE supplementation upon the GM of HFD-induced obese rats.

In the present study, HFD+*G* showed reduced food intake in comparison to HFD (Fig. 1). This result has been previously reported by our group (Banin et al., 2021; Hirata et al., 2015; 2019a; 2019b). In order to confirm that the modulatory changes induced by GbE found in the present study were not a secondary effect attributed to reduced energy intake, a pair-fed group was also assessed, in which its calorie intake was matched to the GbE-receiving group, but under saline supplementation. As gut dysbiosis has been reported to disrupt the hypothalamic control of energy homeostasis through impairment of gut hormone signaling (Amabebe et al., 2020), the inhibitory effect of GbE on food intake herein observed may have occurred, at least indirectly, due to modulation of gut hormone signaling systems, which suggests a possibly anorexigenic effect for GbE.

In the present study, the 16S rRNA sequencing revealed that GbE exerted a strong modulatory effect upon GM composition, with different parameters used to confirm this finding. Our results show a clearly distinct pattern among the three groups, with the hierarchical clustering of HFD+*G* bacterial community less likely related with those of PF and HFD (Fig. 2). The three experimental groups were fed the same lard-enriched high calorie diet throughout the experimental protocol, but the microbiota composition pattern was similar between PF and HFD, and different in HFD+*G* (Fig. 2A and 2B). Our results show that the GM modulation herein observed in the HFD+*G* group was directly promoted by GbE, a finding that is not attributed to reduced energy intake. To this effect, PF and HFD+*G* consumed the same number of calories and yet their GM composition were different.

Furthermore, in comparison to both HFD and PF, HFD+*G* presented reduced relative abundance of *Firmicutes* with concomitantly higher abundance of *Bacteroidetes* colon communities (Fig. 3). This particular bacterial composition – lower *Firmicutes,* higher *Bacteroidetes* – is associated with a healthier GM profile, even when considering the lower values observed for richness and diversity, as evidenced by lowered Shannon and Simpson indices (Fig. 3). Such lower indices were found in HFD+*G* but not in HFD, showing that the reduction in richness and diversity observed represents, in fact, a reduction in deleterious bacterial strains.

Previous studies corroborate our findings in which gut dysbiosis in diet-induced obesity is characterised by greater abundance of *Firmicutes* and lower abundance of *Bacteroidetes* (Castaner et al., 2018). At the same time, the reduced Firmicutes/Bacteroidetes ratio herein observed after GbE supplementation corroborates other studies that evidenced a beneficial effect of nutraceuticals on GM (Li et al., 2016; Zhang et al., 2017).

Hildebrandt et al. (2009) observed in non-obese mice the same microbiota profile as in our study when their standard chow was replaced by a HFD, demonstrating a specific effect of nutrient intake on GM modulation. Thus, it is reasonable to suggest that alterations in the relative amount of *Firmicutes* and *Bacteroidetes*, as observed in HFD, may intensify the metabolic manifestations observed in obesity, especially those related with an exacerbated inflammatory response.

Further changes observed in HFD+*G* include lower amounts of *Mollicutes, Clostridiales, Bifidobacteriales* and *Turicibacterales*, followed with increased *Alphaproteobacteria* and *Bacteroidales* amounts in comparison to HFD, while PF presented a pattern that was intermediary between HFD+*G* and HFD (Fig. 4). In alignment with our findings, it has been reported in a rodent study that a HFD offered for three months increased *Clostridiales* and reduced *Bacteroidales* abundances (Hildebrandt et al., 2009). Moreover, greater proportions of *Mollicutes* were observed in a mouse model of obesity associated with a Western diet (Turnbaugh et al., 2008).

Firmicutes, shown to be elevated in obesity (Crovesy et al., 2020), have a greater propensity for dietary carbohydrate fermentation into short chain fatty acids (SCFA) (Ibrahim and Anishetty, 2012), providing an additional energy source for the body. It is known that a westernized HFD may contribute an additional 10% overall energy supply in the form of microbiota-derived SCFA. Such outcomes influence the levels of appetite-regulating hormones, such as glucagon, glucagon-like peptide 1 (GLP-1), peptide YY (PYY), and leptin (Nagpal et al., 2018), in accordance with intestinal energy availability.

Chronically elevated SCFA levels may lead to resistance of its effects on the anorexigenic hormones GLP-1, PYY, and leptin, negatively affecting satiety and energy expenditure, and favouring energy accumulation (Goffredo et al., 2016; Nagpal et al., 2018). Previous studies have also shown that obesity-related GM communities that favor a higher *Firmicutes* to *Bacteroidetes* ratio have greater propensity for higher SCFA plasma levels (Goffredo et al., 2016; Turnbaugh et al., 2006). Excess SCFA are transported to the liver where their metabolites (acetyl-CoA and propionate to propionyl-CoA) feed into the tricarboxylic acid cycle for *de novo* lipogenesis and gluconeogenesis (Solinas et al., 2015).

Interestingly, germ-free mice that experience reduced fermentation and SCFA energy absorption from the intestine are protected, via a GLP-1R-dependent mechanism, from HFD-induced obesity and associated hypothalamic inflammation, leptin resistance and weight gain (Heiss et al., 2021). Similarly, SCFA deficiency in germ-free mice increases colonic glucagon expression and plasma GLP-1 levels, which are restored by a HFD or SCFA supplementation. That same study has also shown that germ-free mice exhibit enhanced leptin sensitivity, independent of body weight and circulating leptin levels (Heiss et al., 2021).

Our findings agree with previous studies that have identified a modulatory effect of *G. biloba* on murine GM. Chen and colleagues (Chen et al., 2019) identified in a murine model of stress-induced

depression a beneficial effect of a water-soluble polysaccharide from *G. biloba* leaves on gut dysbiosis, increasing the richness of *Lactobacillus* species. That study also showed that mice treated with GbE presented increased microbiota diversity and lower abundance of *Proteobacteria* and *Deferribacteres*. Lv and colleagues (Lv et al., 2021) reported that Ginkgolide B, a GbE terpenoid, modulated GM, increasing *Bacteroides* and reducing *Helicobacter* abundances in a murine model of HFD-induced atherosclerosis. Another study showed that both extract and fruit of *G. biloba* modulated rumen fermentation in an in vitro model, improving propionate production and thus mitigating methane and acetate proportions via microbial selection (Oh et al., 2017). Moreover, other bioactive compounds present in GbE are likely to play a role within the effects so far identified, since flavonoids were also reported to modulate GM (Espley et al., 2014).

Our study is the first to report that the effects of GbE supplementation on F/B ratio of HFD-obese rats were not explained by reduced energy intake, but instead an actual effect of GbE, specifically. PF presented a microbiota pattern similar to HFD but an energy intake similar to HFD+*G*. In addition, although we did not find changes in body weight gain after GbE supplementation, our study involved a 2-week supplementation protocol, which is probably too brief to cause an effect, as well as a relatively small sample size. Previous GbE studies featuring larger sample sizes have identified a significant effect on body weight gain (Hirata et al., 2015; 2019a; 2019b).

Anti-obesogenic properties of GbE have been confirmed in different animal models. In ovariectomized rats, GbE restored the serotonin hypophagic response through the reestablishment of hypothalamic serotonergic levels, as well as attenuation of obesity and anxious/depressive-like behaviours (Banin et al., 2017; 2021). Moreover, in diet-induced obese male rats, GbE reduced food intake, visceral adiposity and body weight gain, and improved insulin signaling and sensitivity in white adipose tissue and gastrocnemius muscle (Hirata et al., 2015). Our research group has also observed a direct effect of GbE on white adipose tissue remodeling by modulation of proteins related to adipogenesis, mitochondrial activity, synthesis of triacylglycerol, along with the reduction on adipocyte volume (Hirata et al., 2019a; 2019b). Hypertrophic adipocytes contribute to the maintenance of a chronic low-grade inflammation through the release of pro-inflammatory cytokines (Choe et al., 2016). We have also previously identified anti-inflammatory activities for GbE, as evidenced by the reduction of NF-kB pathway activation and TNF-α levels in retroperitoneal adipose tissue of obese rats (Hirata et al., 2015).

Conclusion

In summary, GbE supplementation for two weeks modulated the GM of lard-rich diet-induced obese rats, reducing the *Firmicutes/Bacteroidetes* ratio. As an elevated *Firmicutes/Bacteroidetes* ratio has been associated with obesity, our findings support previous rat studies that have reported anti-obesogenic properties attributed to GbE. If similar effects can be observed in humans, GbE supplementation could be particularly helpful for obese individuals who are resistant to changes in their eating and lifestyle habits. Future human studies and clinical trials will clarify whether GbE may or may not be a promising alternative to improve the metabolic profile of obese patients, particularly those who are resistant to changes in diet and lifestyle.

Ethical approval

The Committee on Animal Research Ethics of the Universidade Federal de São Paulo approved all procedures for the care of the animals used in this study (Process number: 8,700,110,814).

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Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs

CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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