MIRIAN APARECIDA DE CAMPOS COSTA

EFFECTS OF GREEN AND BLACK TEA KOMBUCHAS CONSUMPTION ON THE BODY COMPOSITION, INTESTINAL HEALTH, AND MARKERS OF METABOLISM, OXIDATIVE STRESS, AND INFLAMMATION IN WISTAR RATS AND HUMANS

Thesis submitted to the Graduate Program in Food Science and Technology of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Advisor: Frederico Augusto Ribeiro de Barros

Co-advisors: Bruce R. Hamaker
Hercia Stampini Duarte Martino
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RESUMO

COSTA, Mirian Aparecida de Campos, M.Sc./D.Sc., Universidade Federal de Viçosa, junho de 2023. **Efeitos do consumo de kombuchas de chá verde e preto na composição corporal, saúde intestinal e em marcadores do metabolismo, estresse oxidativo e inflamação em ratos *Wistar* e seres humanos**. Orientador: Frederico Augusto Ribeiro de Barros. Co-orientadores: Bruce R. Hamaker, Hércia Stampini Duarte Martini e Josefina Bressan.

A kombucha é uma bebida obtida pela fermentação de chá verde ou preto por bactérias acéticas, bactérias láticas e leveduras, cujo consumo aumentou exponencialmente nos últimos anos. Estudos *in vitro* e *in vivo* sugerem um potencial benefício relacionado ao consumo de kombucha devido, principalmente, ao seu elevado teor de compostos fenólicos. Entretanto, ainda não há consenso na literatura, especialmente em relação à saúde humana. O objetivo deste estudo é avaliar o efeito do consumo regular de kombucha de chá verde e preto sobre a saúde intestinal de ratos *Wistar*, bem como o consumo regular de kombucha de chá preto sobre a saúde intestinal e marcadores metabólicos, de estresse oxidativo e inflamatórios em adultos com ou sem obesidade. O estudo com animais foi conduzido a partir de amostras obtidas em estudo prévio. Após serem alocados aleatoriamente em um dos grupos, os animais receberam uma das seguintes dietas por dez semanas: dieta padrão (AIN-93M) (n=10); dieta rica em gordura saturada e frutose (HFHF) (n=10); HFHF + kombucha de chá verde (n=10); HFHF + kombucha de chá preto (n=10). Amostras de fezes e do ceco foram utilizadas nas análises. Para o estudo com seres humanos, foram incluídos 23 indivíduos eutróficos e 23 indivíduos com obesidade. Durante oito semanas consecutivas, os participantes consumiram 200 mL de kombucha de chá preto/dia. Eles foram orientados a manter o consumo alimentar habitual e o mesmo padrão de atividade física ao longo do estudo. Amostras de sangue, fezes e urina foram coletadas antes (*baseline*) e após a intervenção. Em ratos *Wistar*, kombuchas de chá verde e preto foram capazes de modular a microbiota intestinal ao aumentar a produção de propionato e favorecer o crescimento de microrganismos benéficos como *Adlercreutzia* no grupo que consumiu kombucha de chá verde. Em humanos, houve diminuição das concentrações séricas de insulina e gama-glutamil transferase em indivíduos com obesidade, bem como marcadores de resistência (HOMA-IR) e sensibilidade à insulina (HOMA-β e índice QUICKI). Em indivíduos eutróficos, as concentrações plasmáticas de interleucina 13 e óxido nítrico aumentaram após a ingestão de kombucha. Houve ainda, aumento das concentrações séricas de colesterol total e fosfatase alcalina, embora esses resultados estivessem atrelados ao hábito alimentar dos indivíduos. A kombucha favoreceu microrganismos como Bacteroidota, Akkermanciaceae e Prevotellaceae e reduziu a abundância de microrganismos associados à obesidade, como

Ruminococcus e *Dorea*, especialmente no grupo obeso. Houve aumento na diversidade fúngica, maior abundância de *Saccharomyces* e diminuição de *Exophiala* e *Rhodotorula*. *Pichia* e *Dekkera*, dois dos principais microrganismos encontrados na kombucha e SCOBY, foram identificados como biomarcadores após a intervenção. Em relação às kombuchas utilizadas no estudo experimental, os microrganismos mais abundantes encontrados nos SCOBYs também foram encontrados nas bebidas, embora em maior diversidade. Em relação à kombucha de chá preto utilizada no estudo clínico, 145 compostos fenólicos foram identificados por UPLC-MS^E; a maioria flavonoides (81%) e ácidos fenólicos (19%). Lignanas, estilbenos e outros polifenois representam 1% do total. Nossos resultados sugerem desfechos positivos relacionados ao consumo regular de kombucha, embora enfatizemos a importância da alimentação saudável como um todo.

Palavras-chave: Antioxidantes; *Camellia sinensis*; Compostos bioativos; Microbiota intestinal; Polifenois; Obesidade.

ABSTRACT

COSTA, Mirian Aparecida de Campos, M.Sc./D.Sc., Universidade Federal de Viçosa, June, 2023. **Effects of green and black tea kombuchas consumption on the body composition, intestinal health, and markers of metabolism, oxidative stress, and inflammation in Wistar rats and humans**. Advisor: Frederico Augusto Ribeiro de Barros. Co-advisors: Bruce R. Hamaker, Hércia Stampini Duarte Martini, and Josefina Bressan.

Kombucha is a beverage obtained through the fermentation of green or black tea by acetic and lactic bacteria and yeast, whose consumption has increased exponentially in recent years. *In vitro* and *in vivo* studies suggest a potential benefit related to kombucha consumption mainly due to its high content of phenolic compounds. However, there is still no consensus in the literature, especially regarding human health. This study aims to evaluate the effect of regular consumption of green and black tea kombucha on the intestinal health of Wistar rats, as well as the regular consumption of black tea kombucha on intestinal health and markers of metabolism, oxidative stress, and inflammation in individuals with or without obesity. The animal study was conducted using samples obtained in a previous study. After being randomly allocated to the groups, the rats received one of the following diets for ten weeks: standard diet (AIN-93M) (n=10); a high-fat high-fructose diet (HFHF) (n=10); HFHF + green tea kombucha (n=10); HFHF + black tea kombucha (n=10). Stool and cecal samples were used in the analyses. For the clinical study, 23 eutrophic and 23 individuals with obesity were included. For eight consecutive weeks, participants consumed 200 mL of black tea kombucha/day. They were instructed to maintain their usual food consumption and the same pattern of physical activity throughout the study. Blood, stool, and urine samples were collected before (baseline) and after the intervention. In Wistar rats, green and black tea kombuchas could modulate the intestinal microbiota by increasing propionate production and favoring the growth of beneficial microorganisms such as *Adlercreutzia* in the group that consumed green tea kombucha. In humans, there was a decrease in serum concentrations of insulin and gamma-glutamyl transferase in obese individuals, as well as markers of resistance (HOMA-IR) and insulin sensitivity (HOMA-β and QUICKI index). In normal weight individuals, plasma concentrations of interleukin 13 and nitric oxide increased after kombucha ingestion. There was also an increase in serum concentrations of total cholesterol and alkaline phosphatase, although these results were associated with the individuals' eating habits. Kombucha favored microorganisms such as Bacteroidota, Akkermanciaceae, and Prevotellaceae and reduced the abundance of microorganisms associated with obesity, such as *Ruminococcus* and *Dorea*, especially in the obese group. There was an increase in fungal diversity, greater abundance of *Saccharomyces*,

and a decrease in *Exophiala* and *Rhodotorula*. *Pichia* and *Dekkera*, two of the main microorganisms found in kombucha and SCOBYs, were identified as biomarkers after the intervention. Regarding the kombuchas used in the experimental study, the most abundant microorganisms found in the SCOBYs were also found in the beverages, although in greater diversity. Regarding the black tea kombucha used in the clinical study, 145 phenolic compounds were identified by UPLC-MS^E. Most belong to the class of flavonoids (81%) and phenolic acids (19%). Lignans, stilbenes, and other polyphenols represent 1% of the total. Our results suggest positive outcomes related to regular kombucha consumption, although we emphasize the importance of a healthy dietary pattern.

Keywords: Antioxidants; Bioactive Compounds; *Camellia sinensis*; Intestinal Microbiota; Obesity; Polyphenols.

SUMMARY

1. INTRODUCTION

In recent decades, rapid economic development and industrialization have contributed to changes in the population's dietary pattern, with increased consumption of processed and ultra-processed foods rather than fresh or minimally processed ones (MONTEIRO et al., 2019; ROMIEU et al., 2017). Besides having a high caloric density, ultra-processed foods are rich in sugar, sodium, and trans-fat (MONTEIRO et al., 2019) and their consumption has been associated with weight gain (HALL et al., 2019), changes in the intestinal microbiota (LANE et al., 2020; ZINÖCKER; LINDSETH, 2018) and the incidence of chronic non-communicable diseases (NCDs) such as cardiovascular diseases, cancer, and obesity (MONTEIRO et al., 2019).

Obesity is currently considered one of the biggest public health problems in the world (WORLD HEALTH ORGANIZATION, 2021). Being overweight is associated with the development of several chronic diseases, including type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVD), and some types of cancer (BRAY; KIM; WILDING, 2017; WORLD HEALTH ORGANIZATION, 2021). The global prevalence of obesity nearly tripled between 1975 and 2016, reaching epidemic proportions (WORLD HEALTH ORGANIZATION, 2020). Thus, the search for effective solutions to reduce its prevalence has been a public health priority worldwide (WORLD HEALTH ORGANIZATION, 2018). It is known that diet, associated with genetic and environmental factors, plays a significant role in weight control, thus the inclusion of certain foods in the diet can be a useful strategy in the prevention of obesity (WORLD HEALTH ORGANIZATION, 2020).

In this context, kombucha has emerged as a potential strategy for the control of obesity and its associated comorbidities (COSTA et al., 2021). Kombucha is a fermented beverage obtained from a Symbiotic Culture of Bacteria and Yeast (SCOBY) that uses as a substrate the infusion of green or black tea and sugar (GAGGÌA et al. al., 2018; LEAL et al., 2018). Kombucha originates from China, where it has been consumed since 220 B.C. (JAYABALAN et al., 2014), although it has become popular only in the last decade due to evidence of its potential health benefits (KAPP; SUMNER, 2019).

Kombucha is rich in acetic bacteria, mainly those of the genus *Acetobacter* and *Gluconobacter*; lactic acid bacteria and yeast (GREENWALT; STEINKRAUS; LEDFORD, 2000; VILLARREAL-SOTO et al., 2018; VINA et al., 2014; WATAWANA et al., 2015). There are also organic acids, such as acetic, glucuronic, and gluconic, micronutrients (iron,

copper, manganese, nickel, and zinc), vitamins C and B complex, amino acids, and phenolic compounds (GAGGÌA et al., 2018; JAYABALAN et al., 2014). Regardless of the type of tea used – green or black –, kombucha has a high content of phenolic compounds, even higher than in the tea itself (BELLASSOUED et al., 2015; CARDOSO et al., 2020; VINA et al., 2014).

Recently, an article published by our research group demonstrated that green and black tea kombuchas have high antioxidant capacity and different profiles of phenolic compounds. We have identified 127 phenolic compounds in kombuchas, of which 103 were reported for the first time in the literature (CARDOSO et al., 2020). The presence of phenolic compounds in the blood helps to combat oxidative stress, since they act as antioxidant, anti-inflammatory, and anti-carcinogenic agents (LEAL et al., 2018; VILLARREAL-SOTO et al., 2018). Phenolic compounds can also modulate the intestinal microbiota, exerting anti-obesity, anti-diabetes, and anti-hypercholesterolemic effects (GOWD et al., 2019).

In vitro studies have shown that kombucha has antimicrobial (SREERAMULU; ZHU; KNOL, 2000), antioxidant (CHU; CHEN, 2006) and anti-carcinogenic (CARDOSO et al., 2020; JAYABALAN et al., 2011) properties. *In vivo* studies have shown that the consumption of green and black tea kombucha was able to reduce oxidative stress and inflammation (CARDOSO et al., 2021; BHATTACHARYA; GACHHUI; SIL, 2013; SAI RAM et al., 2000), improve the lipid profile (BELLASSOUED et al., 2015), and modulate the gut microbiota (COSTA et al., 2022; JUNG et al., 2019). Other benefits include hypoglycemic (SRIHARI et al., 2013), antidiabetic (BHATTACHARYA; GACHHUI; SIL, 2013), anti-obesity effects (YANG et al., 2009), and antimalarial potential (DE NORONHA et al., 2022). Despite evidence that kombucha consumption is beneficial to health, little is known about the effect of the bioactive compounds of this beverage on the organism. It is important to mention that, to date, no study has evaluated the effects of kombucha ingestion on human health.

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2. JUSTIFICATION

There is evidence that kombucha consumption is beneficial to health. The phenolic compounds and organic acids present in the beverage have anti-inflammatory and antioxidant properties, helping to combat oxidative stress and inflammation. In addition, it is likely that the microorganisms present in kombucha, in conjunction with the phenolic compounds, help modulate the intestinal microbiota, whose integrity is essential for the proper functioning of the organism. Thus, regular consumption of kombucha would benefit both normal weight and individuals with obesity.

However, most of the studies available in the literature were carried out *in vitro*, and, of the few studies carried out *in vivo*, none of them evaluated the effect of kombucha on human health. Thus, considering the exponential increase in kombucha consumption in recent years worldwide, it is necessary to investigate the effects of the beverage on animal and human health.

Thus, the present work is relevant for evaluating the possible benefits that the regular consumption of kombucha exerts on the intestinal health of Wistar rats; and in metabolic markers, oxidative stress, inflammation, and intestinal microbiota of individuals with or without obesity, contributing to the literature knowledge regarding kombucha consumption.

3. OBJECTIVES

3.1 General Objective

Evaluate the effect of chronic consumption of kombucha on the health of Wistar rats and adults with or without obesity.

3.2 Specific Objectives

- Carry out a physical-chemical characterization of the produced kombuchas.
- In Wistar rats, evaluate the consumption of green and black tea kombuchas with regards to:
  - Gut microbiota.
  - Histomorphometry of the intestine.
- In adults with or without obesity, evaluate the chronic consumption of black tea kombucha with regards to:
  - Body weight and composition.
  - Lipid profile.
  - Glucose, insulin, glycated hemoglobin, insulin resistance, and insulin sensitivity.
  - Liver enzymes.
  - Pro-inflammatory and oxidative stress markers on plasma.
  - Gut microbiota.
- In adults with obesity, evaluate the chronic consumption of black tea kombucha with regards to:
  - mRNAs associated with inflammation, and glucose and lipid metabolism.

4. HYPOTHESES

After regular consumption of green and black tea kombuchas for eight consecutive weeks, Wistar rats will improve their intestinal health, with modulation of the gut microbiota, including an increase in the production of short-chain fatty acids and greater microbial diversity, with an increase in the abundance of beneficial microorganisms and a reduction of the pathogenic ones.

After consumption of black tea kombucha for eight consecutive weeks, normal weight and obese individuals will present a general improvement of their health, which may include a reduction in the concentration of oxidative stress and inflammatory markers; improvement in the lipid profile (decrease in serum concentrations of total cholesterol, triglycerides, LDL-c, and an increase in serum concentration of HDL-c); reduced serum concentrations of liver enzymes (aspartate aminotransferase, alanine aminotransferase, and gamma-glutamyl transferase); lower glycemia; improvement of insulin resistance and sensitivity; intestinal microbiota modulation including reduced intestinal permeability; increased production of short-chain fatty acids and greater microbial diversity, with an increased abundance of beneficial bacteria and reduction of the pathogenic ones.

CHAPTER 1: LITERATURE REVIEW

5.1 Obesity

Obesity is defined as an excessive accumulation of adipose tissue that reduces the quality of life, impairing health (WORLD HEALTH ORGANIZATION, 2021a). It is considered by some countries and by the World Obesity Federation, as a chronic, relapsing, and progressive disease (BRAY; KIM; WILDING, 2017).

The global prevalence of obese adults nearly tripled between 1975 and 2016 (WHO, 2020b). This exponential increase makes obesity currently considered one of the biggest public health problems in the world, affecting individuals of all ages and socioeconomic conditions (WORLD HEALTH ORGANIZATION, 2021b). The World Health Organization (WHO) pointed out that more than 1 billion people were obese in 2022. Of these, approximately 63% are adults, 33% are adolescents, and 4% are children (WORLD HEALTH ORGANIZATION, 2022). In Brazil, recent data from VIGITEL indicate that 57.2% of the population is overweight and 22.4% is obese (BRASIL, 2021).

According to the Brazilian Association for the Study of Obesity and Metabolic Syndrome, 2.3 billion adults in the world will be overweight and more than 700 million will be obese in 2025 (ABESO, 2016). Thus, the WHO developed the "Global Action Plan for the Prevention and Control of Non-communicable Diseases 2013-2020", whose goals include establishing and strengthening initiatives for the surveillance, prevention, and control of non-communicable diseases, including obesity (WORLD HEALTH ORGANIZATION, 2013).

Since overweight causes dysregulation in blood pressure, lipid profile, and insulin resistance, obesity is one of the main risk factors for cardiovascular disease, type 2 diabetes mellitus (T2DM), and some types of cancer (WORLD HEALTH ORGANIZATION, 2021a). It is estimated that at least 2.8 million deaths per year worldwide are due to comorbidities related to overweight (WORLD HEALTH ORGANIZATION, 2021c).

The etiology of obesity is multifactorial; and involves genetic, environmental, and behavioral factors. None of these factors alone can explain the obesity pandemic. However, it is known that overweight occurs when there is an imbalance between the total energy consumed and the total energy spent (BRESSAN; VIDIGAL; HERMSDORFF, 2013). In this sense, diet plays a fundamental role in the control and prevention of obesity.

Recently, it was proposed the NOVA classification, in which foods are categorized into four groups: unprocessed and minimally processed foods; processed culinary ingredients;

processed foods; and ultra-processed foods (MONTEIRO et al., 2017). Among the ultra-processed foods are soft drinks, cookies, margarine, sausages, and other ready-to-eat foods (MONTEIRO et al., 2019).

In recent decades, rapid economic development and industrialization have contributed to changes in the population's dietary pattern, with increased consumption of processed and ultra-processed foods rather than fresh ones (MONTEIRO et al., 2019; ROMIEU et al., 2017). The processing of food itself is not a problem. However, besides having a high caloric density, ultra-processed foods are high in sugar, sodium, and trans-fat. In addition, it is common that those foods undergo chemical modifications such as hydrolysis and hydrogenation and are added to coloring, flavoring, emulsifying, and other additives to increase shelf life and make them convenient and highly attractive for the consumer (MONTEIRO et al., 2019).

Some studies suggest that the sensory characteristics of ultra-processed foods alter the brain-gut axis to stimulate food intake, resulting in abnormal eating behavior (SCHULTE; AVENA; GEARHARDT, 2015; SMALL; DIFELICEANTONIO, 2019). Thus, it is likely that the high consumption of ultra-processed foods – which today represent most of the calories consumed in the United States – has contributed to the exponential increase in obesity, although a causal relationship has not been established yet (HALL et al., 2019; STEELE et al., 2016).

A randomized cross-over study conducted by the National Institutes of Health (NIH) pointed to an association between ultra-processed food consumption and obesity and its associated comorbidities. Participants received, for two weeks, a diet rich in ultra-processed foods or a diet rich in fresh/minimally processed food. Meals were consumed *ad libitum* and matched in caloric value, energy density, and macronutrient content, including sugar, sodium, and dietary fiber. Participants allocated to the ultra-processed food group had an average increase of 508 kcal/day, resulting in a gain weight of 0.9 kg at the end of the two weeks. On the other hand, participants allocated to the fresh/minimally processed food group, lost approximately 0.9 kg/week. These results confirm an association between ultra-processed food consumption and weight gain, suggesting that limiting this type of food may be a useful strategy in the prevention and treatment of obesity (HALL et al., 2019).

5.2 Inflammation

Overeating leads to the accumulation of energy in the form of free fatty acids (FFA) by the adipose tissue (AT), causing adipocyte hypertrophy and hypoxia (HARFORD et al., 2011).

As a result, resident mast cells in AT stimulates angiogenesis to improve its vascularization (LYONS; KENNEDY; ROCHE, 2016). In turn, the overloaded tissue and hypoxia lead to adipocyte cell death, resulting in the infiltration of M1 macrophages (pro-inflammatory) from the peripheric tissues into the AT, initiating an inflammatory response (JACK et al., 2019; LYONS; KENNEDY; ROCHE, 2016). This process culminates in increased expression of pro-inflammatory genes and, consequently, hyperinsulinemia (LYONS; KENNEDY; ROCHE, 2016). Therefore, AT hypoxia seems to be the trigger for the inflammatory response cascade in obesity (FERNÁNDEZ-SANCHÉZ et al., 2011).

The inflammatory response is mediated by chemokines, small proteins secreted by adipocytes that attract macrophages to the AT. CCL2 (C-C motif ligand 2), also known as MCP-1 (monocyte chemoattractant protein-1), is produced by TA and its expression is increased in obesity. CCL2 and its CCR2 receptor play a key role in macrophage recruitment in the AT (HARFORD et al., 2011).

Immune cell infiltration into AT seems to be a defense mechanism. Its role is to clear the necrotic adipocytes, which are indicated through crown-like structures (CLS) around the cells (LYONS; KENNEDY; ROCHE, 2016). Indeed, more than 90% of AT macrophages from obese rats and humans are located around dead adipocytes, forming multinucleated giant cells (MGC) (CINTI et al., 2005). These cells remain in the AT for weeks or months, and, since there is no real threat to be eliminated, their presence leads to a state of chronic low-grade inflammation, which is characteristic of obesity (CINTI et al., 2005; LYONS; KENNEDY; ROCHE, 2016). Similarly to what happens in AT, in peripheral tissues occurs recruitment of immune cells in response to the lipotoxicity generated by excess FFA, culminating in metabolic dysfunction (JACK et al., 2019).

With the progression of obesity, the resident macrophage population in the AT increases from 10-15% to 45-60%. Those cells phenotype also change from M2 (anti-inflammatory) to M1 (pro-inflammatory). This change is caused by the inflammatory process resulting from obesity, but it can also be influenced by diet. A diet rich in saturated fatty acids, for example, activates M1 genes; on the other hand, a diet rich in monounsaturated fatty acids (MUFA) activates M2 genes (LYONS; KENNEDY; ROCHE, 2016).

M1, or classically activated macrophages, are stimulated by interferon-gamma (IFN-γ) and produce pro-inflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (HARFORD et al., 2011). IL-1β induces inflammatory activity in the pancreas by activating NF-κB in pancreatic islets, affecting insulin

production by β cells (GREGOR; HOTAMISLIGIL, 2011). IL-6 and TNF-α play a central role in insulin resistance by inhibiting the phosphorylation of insulin receptor substrate-1 (IRS-1), glucose transporter 4 (GLUT4), and other transcription factors (JACK et al., 2019; MARSEGLIA et al., 2015).

On the other hand, M2, also known as alternatively activated macrophages, produce anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor β (TGFβ). IL-10 increases insulin sensitivity and inhibits the production of pro-inflammatory cytokines such as TNF-α, IL-6, and chemokines (BALISTRERI; CARUSO; CANDORE, 2010; LYONS; KENNEDY; ROCHE, 2016). TGFβ, in turn, is part of a family of growth factors produced by TA whose expression is altered in obesity. It is involved in several cellular processes, such as cell proliferation and differentiation, playing an important role in adipogenesis (LEE, 2018).

The secretion of cytokines by adipocytes and macrophages plays a key role in the inflammatory process and the complement system is likely an important mediator in this process (RICKLIN et al., 2010). The main function of the complement system was thought to be a defense mechanism against pathogenic microorganisms; however, it is currently known that it is also involved in other processes such as lipid metabolism and angiogenesis (RICKLIN et al., 2010). Complement C3, for example, is involved in the synthesis and storage of triglycerides, and its concentration is significantly increased following the consumption of a diet rich in fatty acids (LOPES et al., 2018; RICKLIN et al., 2010). Since complement C3 plays a central role in the immune and inflammatory process (RICKLIN et al., 2010), it has been considered an inflammatory marker, and high concentrations are a risk factor for cardiometabolic diseases and other comorbidities associated with obesity (VAN GREEVENBROEK et al., 2014).

Some microRNAs (miRNAs) have also been reported in the literature as important regulators of biological processes (LOZANO-BARTOLOMÉ et al., 2018; ORTEGA et al., 2010, 2013; WANG et al., 2013). miRNAs are small non-coding RNA molecules of 18 to 25 nucleotides that regulate gene expression (GIARDINA et al., 2018; LANDRIER; DERGHAL; MOUNIEN, 2019. They bind to target mRNAs, being able to block their translation or degrade the transcript (GIARDINA et al., 2018). miR-210, for example, is an adipogenic miRNA involved in AT hypoxia (HUANG; LE; GIACCIA, 2010); miR-29a and miR-34a are involved in the development of insulin resistance (MEERSON et al., 2013; ROGGLI et al., 2010); while miR-132 induces the secretion of pro-inflammatory cytokines mediated by NF-κB activation

(STRUM et al., 2009). Thus, an increased gene expression of these and other miRNAs is associated with inflammation. In contrast, a decreased expression improves insulin sensitivity and decreases inflammation (GIARDINA et al., 2018).

A study carried out with 48 individuals with overweight or obesity evaluated the impact of different types of diet (low-glycemic index; high-glycemic index, and low-fat) on the miRNAs profile. Subcutaneous adipose tissue (SAT) samples were collected before and after the intervention. The authors concluded that diet was not capable of influencing the gene expression of the analyzed miRNAs. However, a positive correlation was found between microRNAs and anthropometric features such as body weight, BMI, waist circumference, and body fat, indicating that adiposity is the main determinant in the gene expression of miRNAs in the SAT (GIARDINA et al., 2018).

Another study evaluated the effect of resveratrol supplementation for 30 days on SAT in 11 obese men. The authors observed a decrease in the adipocyte size and an upregulation of genes involved in the cell cycle, suggesting an improvement in adipogenesis (KONINGS et al., 2014). However, the results are controversial (POULSEN et al., 2013; YOSHINO et al., 2013) and more studies are needed to elucidate the role of phenolic compounds in adipose tissue and the expression of genes related to obesity.

5.3 Oxidative Stress

Inflammation can be also a consequence of oxidative stress, which is usually increased in obese individuals (FERNÁNDEZ-SANCHÉZ et al., 2011). Oxidative stress is defined as an imbalance between oxidant and antioxidant compounds, resulting from an excessive production of free radicals and reactive oxygen species (ROS) (KHOSRAVI et al., 2019). Under physiological conditions, radicals play an important role in biochemical reactions as mediators in electron transfer and are essential in intracellular signaling and in the immune response against pathogens (BARBOSA et al., 2010; KHOSRAVI et al., 2019). However, the excessive accumulation of these substances leads to biomolecule oxidation and homeostatic imbalance, causing damage to various cellular structures, including membranes, proteins, and DNA (BARBOSA et al., 2010; MARSEGLIA et al., 2015).

The antioxidant defense system inhibits or reduces the damage caused by oxidative stress through three main mechanisms: preventing the formation of free radicals and ROS (prevention systems); preventing the action of these compounds in the body (scavenging

systems); or favoring the repair of damaged biological structures (repair systems) (BARBOSA et al., 2010). This process is carried out by enzymatic antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx); and non-enzymatic antioxidants from endogenous and dietary sources, especially vitamins A, C, and E; minerals such as copper, zinc, and selenium; and phytochemicals such as resveratrol, catechins, and phenolic acids (BARBOSA et al., 2010; DI DOMENICO et al., 2019). Adequate intake of vitamins and minerals is essential for the proper functioning of the antioxidant defense system. Furthermore, to act as antioxidant agents, enzymatic activity depends on the daily intake of these nutrients (DI DOMENICO et al., 2019).

M1 macrophages in the adipose tissue of individuals with obesity produce high amounts of ROS. Nitric oxide, for example, is increased due to the nitric oxide synthetase (NOS) activity, which, in turn, induces oxidative stress (HARFORD et al., 2011). Once ROS increase in the AT, they induce the secretion of pro-inflammatory cytokines and the expression of adhesion molecules and growth factors, such as IGF-1 (Insulin-like Growth Factor 1) and VCAM-1 (Vascular Cell Adhesion Molecule 1), aggravating the inflammatory condition. This process is mediated by transcription factors, especially by NF-κB and NADPH oxidase (NOX) (MARSEGLIA et al., 2015). NOX is a transmembrane enzyme complex whose main function is the electrons transference across cell membranes. Since the electron acceptor is usually oxygen, this process generates superoxide radicals, turning NOX into an important source of free radicals (BARBOSA et al., 2010). Superoxide radicals are later converted into hydrogen peroxide (H2O2), and despite not being a free radical, it has a high reactive potential capable of crossing cell membranes (BARBOSA et al., 2010; MARSEGLIA et al., 2015). Hydrogen peroxide also stimulates the secretion of IL-4 and IL-6 via APE1/Ref-1 (MARSEGLIA et al., 2015).

In addition to the mechanisms mentioned, chronic inflammation and oxidative stress in individuals with obesity overload the antioxidant defense system due to lower concentrations of carotenoids, vitamin C, and vitamin E, and to a down expression of antioxidant enzymes such as SOD and catalase (DI DOMENICO et al., 2019; MARSEGLIA et al., 2015). Indeed, some studies have shown that in individuals with obesity, SOD and GPx activities were significantly lower than in the normal weight (OLUSI, 2002; OZATA et al., 2002). Other oxidative stress biomarkers such as plasma malondialdehyde (MDA) and urinary 8-isoprostane had their concentrations increased in obesity (AMIRKHIZI et al., 2007; DAVI et al., 2008), while serum concentrations of FRAP (Ferric Reducing Ability of Plasma) and TAS (Total

Antioxidant Status) are reduced (LOPES et al., 2003). These studies suggest that obesity itself is related to increased oxidative stress and a decreased antioxidant capacity (AMIRKHIZI et al., 2007; OLUSI, 2002).

Several other conditions contribute to the development of oxidative stress in obesity, including hyperglycemia, dyslipidemia, hyperleptinemia, vitamin and mineral deficiency, diet, chronic inflammation, muscle overload caused by overweight, and others (MANNA; JAIN, 2015). This metabolic dysfunction, typical in obesity and mediated by oxidative stress, leads to the development of several chronic diseases, such as diabetes, cardiovascular diseases, renal and hepatic dysfunction, asthma, sleep disorders, and cancer (MANNA; JAIN, 2015). It should be mentioned that a minority of chronic diseases are due to genetic predisposition; most of them develop because of environmental and lifestyle factors. Oxidative stress is essential in the early stages of chronic disease development (BEETCH et al., 2020).

Weight loss and antioxidant intake, such as phenolic compounds, have been identified as efficient strategies against inflammation and oxidative stress since they cause a reduction in the concentrations of cytokines and pro-inflammatory biomarkers and improve the antioxidant defense system (JACK et al., 2019; MANNA; JAIN, 2015). Recent studies have shown that phenolic compounds are also capable of modulating gene expression, reversing epigenetic changes associated with inflammation and oxidative stress, therefore playing a role in the prevention and/or mitigation of chronic diseases (BEETCH et al., 2020). The link between oxidative stress and epigenetic changes, more specifically associated with DNA methylation, has not been fully clarified yet (DING et al., 2016, 2019).

5.4 Gut Microbiota

The gastrointestinal tract (GIT) is colonized by more than 100 trillion bacteria, which, together with archaea, fungi, and viruses, compose the gut microbiota (TILG; MOSCHEN, 2015; ZHUANG et al., 2019). Each bacterium contains thousands of genes, making this complex and dynamic ecosystem present extensive genetic information, even larger than the human genome (GÉRARD, 2016; GILBERT et al., 2018; TILG; MOSCHEN, 2015).

In adult individuals, a healthy intestinal microbiota contains more than 1000 species of bacteria (MU; YANG; ZHU, 2016). However, only 50 species belonging to five or six genera and two phyla correspond to 99% of the total (CONLON; BIRD, 2015). *Firmicutes* (gram-positive), *Bacteroidetes* (gram-negative), and *Actinobacteria* (gram-positive) are the

predominant phyla; comprise more than 90% of the bacteria (GÉRARD, 2016). Among the species, *Bifidobacterium, Lactobacillus, Bacteroides, Clostridium, Escherichia, Streptococcus,* and *Ruminococcus* are the most prevalent (CONLON & BIRD, 2015). However, bacterial composition and density are not stable and can be affected by several factors, such as genotype, pH, inflammation, and antibiotics intake (GÉRARD, 2016; REIJNDERS et al., 2016).

Regarding the fungal population, although it comprises only 0.1% of the gut microbiota, it is crucial for maintaining the body's homeostasis since it acts in synergism with the bacterial microbiota and the immune system (ZHANG et al., 2022). Imbalances in the fungal composition and/or morphology have been associated with immunological, gastrointestinal, and neurological disorders caused mainly by opportunistic species (BERNARDES et al., 2020; FORBES et al., 2019; RICHARD; SOKOL, 2019; ZHANG et al., 2022).

In general, the fungal microbiota shows greater variability among individuals and is more unstable than the bacterial microbiota, but both start to be modulated in childhood and are greatly influenced by food, environmental factors, and the gut microbiota itself. The metabolites generated by the fungal microbiota – alcohol, peptides, and other metabolites – influence the bacterial microbiota which, in turn, modulates the fungal microbiota by producing short-chain fatty acids (SCFA) (ZHANG et al., 2022). In a study carried out with germ-free rats, no pathophysiological alterations were observed when colonization was performed exclusively with fungi; however, the inflammatory response was exacerbated when done in conjunction with the colonization of bacteria, demonstrating a synergism between them (BERNARDES et al., 2020)

Diet plays a central role in the modulation of the intestinal microbiota, possibly being the main determinant of the dynamics and composition of microorganisms (MU; YANG; ZHU, 2016; TILG; MOSCHEN, 2015). Diets rich in fruits and vegetables favor homeostasis and have an anti-inflammatory effect. On the other hand, high-fat and high-carbohydrate diets have a negative effect on the taxonomy, genetics, and metabolism of the gut microbiota (TILG; MOSCHEN, 2015).

Differences in bacterial composition were demonstrated in a study that compared the gut microbiota of children in urban Italy with children in rural Africa. A fiber-rich diet, consumed by African children, showed a reduction in the phylum *Firmicutes* and the *Enterobacteriaceae* family (*Shigella* and *Escherichia*), in addition to an increase in the genus *Bacteroidetes* and the species *Prevotella* and *Xylanibacter*, and an increase in the SCFA concentrations when compared to the diet consumed by Italian children. The last one, rich in

animal protein and saturated fat, was associated with the predominance of the genus *Bacteroides* (DE FILIPPO et al., 2010).

The gut microbiota is involved in several essential functions for the organism's homeostasis, including non-digestible compounds fermentation such as fibers; synthesis of vitamins and other micronutrients; conversion of cholesterol to bile acids; metabolism of toxins and carcinogenic substances; maturation of the immune system; and protection against pathogens (GÉRARD, 2016). Since the gut microbiota exerts a symbiotic relationship with the organism, they are influenced by each other (WEGH et al., 2017).

Studies in the area have pointed out that the gut microbiota is related to the development of several metabolic disorders, including obesity (GÉRARD, 2016). The first evidence was a study carried out with germ-free rats. The researchers observed that these animals spent approximately 30% more calories to maintain body weight than animals that had an intact gut microbiota (WOSTMANN et al., 1983), suggesting that the gut microbiota would be involved in energy metabolism. In another study, researchers transplanted the cecum microbiota of eutrophic and obese ob/ob mice into germ-free mice. After two weeks, those mice that received the microbiota from the obese ones gained more weight than those that received the microbiota from eutrophic mice, suggesting a causal relationship between the intestinal microbiota and the development of obesity (TURNBAUGH et al., 2006).

An important mechanism that helps explain the relationship between intestinal microbiota and body weight concerns the energy expended by bacteria during fermentation (GÉRARD, 2016). Polysaccharides that escape digestion are used as substrates by bacterial fermentation generating monosaccharides and short-chain fatty acids, mainly acetate, propionate, and butyrate (DEN BESTEN et al., 2013; GÉRARD, 2016; HOLSCHER, 2017). SCFA play a key role in the integrity of the intestinal epithelium, glycemic homeostasis, lipid metabolism, and immune function. Most SCFA (90-99%) are absorbed in the intestine or used by the microbiota itself. A small part, mainly propionate and acetate, is found in the peripheral circulation (HOLSCHER, 2017). Butyrate, in turn, is the main energy source of colonocytes and enterocytes, thus helping to maintain the intestinal epithelium healthy (HOLSCHER, 2017; MASLOWSKI; MACKAY, 2011). Besides being used as substrates in energy metabolism, SCFA act in the activation of the hormones GLP-1 (glucagon-like peptide 1) and PYY (peptide YY). GLP-1 regulates appetite, inhibits gastric emptying, and, at the same time, stimulates insulin secretion. PYY, in turn, reduces appetite and inhibits gastric motility. Thus, SCFA act by regulating food consumption and satiety (DAHIYA et al., 2017)

Currently, it is well established that changes in the gut microbiota play an important role in the development of obesity, although the mechanisms by which it happens are still unclear (JACK et al., 2019). One hypothesis is that intestinal permeability is increased in obese individuals, which leads to increased translocation of bacteria and other substances, including lipopolysaccharides (LPS). Once these substances are released into the bloodstream, they stimulate an inflammatory response and induce the infiltration of immune cells in different areas, especially in the adipose tissue and the liver, stimulating the production of pro-inflammatory cytokines (JACK et al., 2019; TARANTINO; FINELLI, 2015). This mechanism highlights the importance of the gut-liver-adipose tissue axis in the pathophysiology of obesity and other metabolic changes (TARANTINO; FINELLI, 2015).

In obesity, it is also observed a decrease in bacterial richness and diversity, as well as changes in its composition (CONLON; BIRD, 2015; SUN et al., 2018). While in healthy individuals the *Bacteroidetes:Firmicutes* ratio is generally proportional, in obesity, it is observed an increase in *Firmicutes*, causing an imbalance in the community (CONLON; BIRD, 2015). Type 2 diabetic individuals also present alterations in this ratio, with a low concentration of *Firmicutes* and a high concentration of *Bacteroidetes* and *Proteobacteria* (LARSEN et al., 2010). Like in obesity, the imbalance between *Firmicutes* and *Bacteroidetes* in T2DM impairs the production of SCFA, increasing the production of acetate and decreasing the concentration of butyrate, which favors insulin resistance. In turn, decreased butyrate concentrations aggravate the chronic inflammation condition (PASCALE et al., 2018).

Probiotics have also been used to modulate the gut microbiota (GÉRARD, 2016). By definition, probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (HILL et al., 2014). Several clinical and experimental studies have demonstrated that probiotics act through several mechanisms, including improving immune function; increasing antibody production; strengthening the integrity of the intestinal epithelium; and competing with pathogenic microorganisms (CONLON; BIRD, 2015; GÉRARD, 2016). As an example, the *Bifidobacterium* genus has shown decreased inflammation, improved lipid profile, and increased insulin sensitivity in rats fed a high-fat diet (CANO et al., 2013; KONDO et al., 2010; YIN et al., 2010). Probiotics containing *Lactobacillus*, in turn, were able to reduce body weight, and improve lipid profile and glycemic homeostasis in obese rats and humans (KADOOKA et al., 2010; LEE et al., 2006).

5.5 Effect of Phenolic Compounds on Metabolic Markers and Modulation of the Gut Microbiota

Polyphenols are secondary plant metabolites, widely found in fruits, vegetables, cereals, nuts, and beverages such as tea, coffee, and wine. Its bioavailability depends on the physical structure of the plant, but in general, a large part is not absorbed by the small intestine (FAVA; RIZZETTO; TUOHY, 2019). In the large intestine, the microorganisms can metabolize high molecular weight phenolic compounds, making them more bioactive (GOWD et al., 2019). However, similarly to dietary fibers, phenolic compounds are part of a very heterogeneous group, requiring the presence of specific enzymes in cleavage, hydrolysis, and dihydroxylation to make them bioactive (FAVA; RIZZETTO; TUOHY, 2019).

Phenolic compounds modulate the gut microbiota, exerting an effect like prebiotics by favoring the growth of beneficial bacteria and inhibiting the growth of pathogenic ones (GOWD et al., 2019). This modulation seems to be influenced by the type of polyphenol (FAVA; RIZZETTO; TUOHY, 2019). In a study conducted with humans, the consumption of isoflavones, proanthocyanidins, ellagitannins, and stilbenes at a concentration of 100 – 1000 mg/day favored the growth of *Bifidobacterium* and *Lactobacillus* spp. (JIMÉNEZ-GIRÓN et al., 2013). The consumption of proanthocyanidin, ellagitannins, and isoflavones, in turn, was associated with the growth of *Ruminococcaceae*, *Akkermansia,* and *Clostridium cocoides* spp. (DUDA-CHODAK et al., 2015; SAUCEDA et al., 2018).

Polyphenols seem to attenuate the metabolic changes induced by a high-fat diet via activation of PPARα and GLUT4 (DING et al., 2013). In another study, polyphenols exerted an anti-obesity effect by modulating the gut microbiota and reducing the Firmicutes/Bacteroides ratio (RASTMANESH, 2011). There is evidence that the antioxidant and anti-inflammatory activities of polyphenols are related to anti-adipogenic, anti-diabetic, cardioprotective, neuroprotective, and anticarcinogenic properties via modulation of the gut microbiota (PANDEY; RIZVI, 2009).

5.6 Kombucha

Kombucha is a fermented beverage obtained from a symbiotic culture of bacteria and yeast (SCOBY) that uses tea infusion - usually green or black - and sugar as a substrate (GAGGÌA et al., 2019; LEAL et al., 2018). Other types of teas can be used (JAYABALAN et al., 2014); however, in Brazil, the Ministry of Agriculture, Livestock, and Food Supply

considers kombucha as a product obtained exclusively from *Camellia sinensis* leaves (BRASIL, 2019).

Kombucha is popularly known for its medicinal properties. It is originally from China, where it has been consumed since 220 B.C. (JAYABALAN et al., 2014), but only in the last 10 years, it has become popular in the rest of the world concomitantly with findings about the role of the gut microbiota in health (KAPP; SUMNER, 2019).

Traditionally, kombucha is prepared using an average of 5 grams of tea and 50 grams of sugar for each liter of water. Fermentation occurs between 7 and 10 days at room temperature (18-26°C). The low pH, in conjunction with the strong symbiosis exerted by bacteria and yeasts in the medium, prevents the growth of pathogenic microorganisms (JAYABALAN et al., 2014; VILLARREAL-SOTO et al., 2018).

Substrate degradation is performed in different ways by microorganisms. Yeasts hydrolyze sucrose to glucose and fructose and preferentially use fructose as a substrate for ethanol production via glycolysis. Bacteria use glucose and ethanol to produce gluconic and acetic acids (DUFRESNE; FARNWORTH, 2000).

5.6.1 Chemical Composition of Kombucha

Kombucha has organic acids in its composition, such as acetic, glucuronic, and gluconic acids; phenolic compounds; micronutrients (iron, copper, manganese, nickel, and zinc); vitamins C and B complex; amino acids; and substances with antibiotic properties (GAGGÌA et al., 2019; JAYABALAN et al., 2014; KAPP; SUMNER, 2019). The presence of these nutrients, especially organic acids and residual sugar, provides kombucha with a slightly sweet, acidic, and refreshing taste (JAYABALAN et al., 2014; SREERAMULU; ZHU; KNOL, 2001).

The chemical and nutritional composition of kombucha depends on several factors, including fermentation time and temperature, sugar concentration, and microorganisms present in the SCOBY (GAGGÌA et al., 2019; JAYABALAN et al., 2014). The quality of the tea is another important aspect since it directly interferes with the composition of phenolic compounds and, consequently, with the final product (GAGGÌA et al., 2019).

Some differences are observed between green and black tea kombuchas. In general, green tea kombucha has a lower pH and a higher acidity (CARDOSO et al., 2020; JAYABALAN; MARIMUTHU; SWAMINATHAN, 2007). This difference is probably due to the predominance of different acetic and lactic bacteria among kombuchas, influencing the

production of organic acids (COTON et al., 2017). Cardoso et al. (2020) observed that green tea kombucha had a lower concentration of sucrose and a higher concentration of glucuronic acid than black tea kombucha, which helps explain why green tea kombucha had higher acidity. Other compounds such as glucose, fructose, acetic acid, lactic acid, and ethanol did not differ significantly between kombuchas (CARDOSO et al., 2020).

The type of tea used in the preparation of kombucha also influences the polyphenols found in the beverage. In fresh leaves of *Camellia sinensis*, flavonols are the main polyphenols found, represented by catechins (LEAL et al., 2018). According to the monomeric structure, catechins are further categorized into epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). During the processing of *C. sinensis* leaves, occurs a change in the monomeric structure of catechins. In green tea, enzymes are inactivated immediately after harvesting to prevent oxidation. On the other hand, oxidation is desirable in black tea. Thus, during the production of black tea, catechins undergo enzymatic oxidation by polyphenol oxidase. As a result, the polyphenols in green tea resemble those of fresh leaves, while black tea has a high content of theaflavins and thearubigins, which are polymeric phenolic compounds generated during the oxidation process (LEAL et al., 2018; TANAKA; KOUNO, 2003).

Regardless of the type of tea used, kombucha has a high content of phenolic compounds, even higher than in the tea itself (BELLASSOUED et al., 2015; VINA et al., 2014). There is evidence that the concentration of total phenolics increases progressively until the 12th day of fermentation due to the enzymes released by the bacteria and yeasts present in the SCOBY, which degrade the more complex polyphenols into smaller molecules, enhancing the antioxidant potential of kombucha (BELLASSOUED et al., 2015; DE NORONHA et al., 2022).

Recently, an article published by our research group demonstrated that green and black tea kombuchas have high antioxidant capacity and different phenolic compound profiles. We identified 127 phenolic compounds in kombuchas, of which 103 were reported for the first time in the literature. Most correspond to flavonoids (70.2%), followed by phenolic acids (18.3%), polyphenols (8.4%), lignans (2.3%), and stilbenes (0.8%). Gallocatechin 3-O-gallate/epigallocatechin 3-O-gallate was the most abundant phenolic compound in both kombuchas (CARDOSO et al., 2020).

In that same study, black tea kombucha had a higher phenolic compound concentration (1.09 mg GAE/mL) in comparison with green tea kombucha (0.70 mg GAE/mL), as well as greater antioxidant capacity (65.32 %). As expected, the theaflavin and thearubigin content was

also higher in black tea kombucha compared to green tea kombucha. The phenolic compounds profile in green tea kombucha was very similar to its origin tea; verbascoside was the only one found exclusively in GTK but not in green tea. On the other hand, black tea kombucha had 28 phenolic compounds that were not found in black tea, including flavonoids, phenolic acids, and other phenolic compounds (CARDOSO et al., 2020). Some of these phenolic compounds, such as pelargonidin 3-O-glucoside, gardenin B, lithospermic acid, and oleuropein, have bioactive properties, acting in the modulation of the intestinal microbiota and the control of blood pressure, cholesterol, and oxidative stress (CABRERA et al., 2016; OMAR, 2010; SU et al., 2020).

Other authors also observed a higher concentration of phenolic compounds and greater antioxidant capacity in black tea kombucha compared to green tea kombucha (KALLEL et al., 2012; MALBAŠA et al., 2011). A possible reason is due to the concentration of polymeric phenolic compounds, such as theaflavins and thearubigins, which are higher in black tea. When degraded by SCOBY enzymes and/or low pH during fermentation, these compounds are broken down into several other phenolic compounds of lower molecular weight, thus increasing the diversity of phenolic compounds in black tea kombucha (CARDOSO et al., 2020; JAYABALAN; MARIMUTHU; SWAMINATHAN, 2007; DE NORONHA et al., 2022).

5.6.2 Microbiological Composition of Kombucha

Similarly to the chemical composition, the microbiological composition of kombucha is influenced by several factors, such as fermentation time and temperature, the origin of the SCOBY, and the type of tea used (COTON et al., 2017). Some studies have shown that the number of microorganisms in the beverage increases considerably in the first 15 days of fermentation (ARIKAN et al., 2020; DE NORONHA et al., 2022). However, fermentation depends on the availability of the substrates, and other authors have observed that microorganisms' growth occurs only up to the sixth or tenth day of fermentation (CHEN; LIU, 2000; COTON et al., 2017).

In general, the microorganism count of kombucha is, on average, in the range of 10^6 colony forming units/mL (CFU/mL) (CARDOSO et al., 2020; NEFFE-SKOCIŃSKA; SIONEK; ŚCIBISZ, 2017; ZHAO et al., 2018). In the study by Cardoso et al. (2020), the count of acetic, lactic, and mesophilic bacteria, as well as yeast, ranged from 10^5 to 10^6 CFU/mL, and no significant difference was observed between green and black tea kombuchas. Similar results were found in a study by Zhao et al. (2018), in which the counts of mesophilic bacteria and

yeast were 6.93 x 10^6 CFU/mL and 7.52 x 10^5 CFU/mL, respectively. In another study, the count of acetic bacteria and yeasts was in the range of 10^7 CFU/mL, however, the results were probably influenced by the greater amount of sugar used in tea preparation (100 g/L) (NEFFE-SKOCIŃSKA; SIONEK; ŚCIBISZ, 2017). In these studies, fermentation took place for 10 days and the temperature varied between 25-28°C (CARDOSO et al., 2020; NEFFE-SKOCIŃSKA; SIONEK; ŚCIBISZ, 2017; ZHAO et al., 2018).

Among the microorganisms present in kombucha, acetic bacteria are one of the most prevalent, especially those of the genus *Acetobacter* and *Gluconobacter* (*Acetobacter xylinum*, *A. aceti*, *A. pasteurianus*, *Gluconobacter oxydans*); lactic bacteria (*Lactobacillus spp*., *Lactococcus spp*.); and yeasts (*Zygosaccharomyces kombuchaensis*, *Z. bailii*, *Saccharomyces cerevisiae, Torulopsis sp., Pichia spp*., *Brettanomyces sp*.) (GREENWALT; STEINKRAUS; LEDFORD, 2000; VILLARREAL-SOTO et al ., 2018; VINA et al., 2014; WATAWANA et al., 2015).

Teoh et al. (2004) evaluated yeasts from black tea kombuchas that were prepared using SCOBYs from different locations. After 14 days of fermentation at controlled temperature (20-22°C), the number and microbial diversity varied among the beverages, however, a predominance of the following species was observed: *Zygosaccharomyces bailii*, *Schizosaccharomyces pombe*, *Torulospora delbreuckii*, *Rhodotorula mucilaginosa*, *Brettanomyces bruxellensis,* and *Candida stellata* (TEOH; HEARD; COX, 2004).

In a recent study, the authors evaluated the microbial diversity of kombucha whose fermentation time varied between 3 and 15 days. In all analyzed samples, the bacteria belonged to eight phyla: Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Deinococcus-Thermus, Firmicutes, Proteobacteria, and Verrucomicrobia. The phylum Proteobacteria was predominant, encompassing more than 99% of the species. Among the yeasts, a predominance of the genus *Zygosaccharomyce*s (>99%) was observed (ARIKAN et al., 2020).

In another study, green and black tea kombuchas produced on an industrial scale showed differences in microbiological composition. Lactic bacteria, especially *Oenococcus oeni*, was associated with the fermentation of green tea kombucha, while black tea kombucha showed a higher predominance of acetic bacteria. The presence of these bacteria was associated with a higher concentration of lactic acid in green tea kombucha and acetic acid in black tea kombucha. Yeast diversity was not influenced by the type of tea; in both kombuchas, the species were predominantly *Dekkera bruxellensis*, *D. anomala*, *Hanseniaspora valbyensis* and *Zygosaccharomyces bailli* (COTON et al., 2017).

5.6.3 Bioactive Properties of Kombucha

Kombucha has a high content of phenolic compounds, whose bioavailability is increased due to the presence of glucuronic acid. The phenolic compounds help to fight against oxidative stress, since they act as antioxidant, anti-inflammatory, and anticarcinogenic agents (LEAL et al., 2018; VILLARREAL-SOTO et al., 2018). Glucuronic acid also assists the liver in detoxification. By binding to toxic compounds such as drugs, pollutants, and toxins, for example, glucuronic acid increases their solubility and facilitates their excretion from the body (LEAL et al., 2018; VINA et al., 2014).

In some *in vitro* studies, the authors demonstrated that kombucha showed antiproliferative and anticarcinogenic activity in normal lung cells (IMR90) as well as in tumor cell lines such as colorectal (HCT8), epithelial (CACO-2) (CARDOSO et al., 2020), pulmonary (A549) (CARDOSO et al., 2020; DEGHRIGUE et al., 2013, and epidermoid (Hep-2) (DEGHRIGUE et al., 2013). In both studies, green tea kombucha showed greater antiproliferative activity than black tea kombucha. This is probably due to the high content of catechins in green tea kombucha, especially verbascoside (CARDOSO et al., 2020). Catechins and verbascoside exert antitumoral activity and protect against the development of some types of cancer by inhibiting cell proliferation and promoting apoptosis of tumor cells (YANG; WANG, 2016; ZHANG et al., 2018). The phenolic compounds present in black tea kombucha also demonstrated *in vitro* antimalarial activity, as recently published by our research group (DE NORONHA et al., 2022).

In vivo studies, including some published by our group, showed that green and black tea kombuchas were able to reduce the concentration of oxidative stress and inflammatory markers, reduce the degree of hepatic steatosis, and improve the glycemic response of Wistar rats fed a high-fat and high-fructose diet (CARDOSO et al., 2021). We also demonstrated how kombuchas were able to improve intestinal health by increasing propionate concentrations and favoring beneficial microorganisms in the gut microbiota (COSTA et al., 2022).

Similarly, Jung et al. (2019) demonstrated that black tea kombucha was able to reduce fat storage in the liver and modulate the gut microbiota of rats. The authors observed an increase in *Lactobacillus spp*. and the genus *Mucispirillum* in the animals' feces, which was related to the attenuation of non-alcoholic fatty liver disease (NAFLD) and the increase in circulating leptin, respectively (JUNG et al., 2019).

In a study carried out with albino rats, kombucha consumption was able to reverse the oxidative stress induced by sodium dichromate, thus demonstrating an antioxidant and

immunoprotective potential (SAI RAM et al., 2000). In another study carried out with diabetic rats, kombucha consumption exerted a protective role against oxidative stress in several organs, such as the pancreas, liver, kidneys, and heart. Furthermore, it was able to reverse pathophysiological changes caused by Alloxan administration for diabetes induction.

Kombucha consumption could also improve lipid profiles in rats. Compared with green tea, kombucha was able to reduce serum concentrations of total cholesterol, triglycerides, VLDL-cholesterol, LDL-cholesterol, and increase HDL-cholesterol. Such results were attributed to the phenolic compounds present in kombucha that reduced oxidative stress and, consequently, improved the lipid profile (BELLASSOUED et al., 2015).

Other benefits associated with kombucha consumption have been reported in animal studies, including a hypoglycemic effect (SRIHARI et al., 2013), and antidiabetic (BHATTACHARYA; GACHHUI; SIL, 2013) and anti-obesity potential (YANG et al., 2009).

We proposed a mechanism of action considering what is known about the *in vivo* effects of kombucha consumption so far, represented by **Figure 1**. It is important to mention that, to date, no study has evaluated the effect of kombucha consumption on human health.

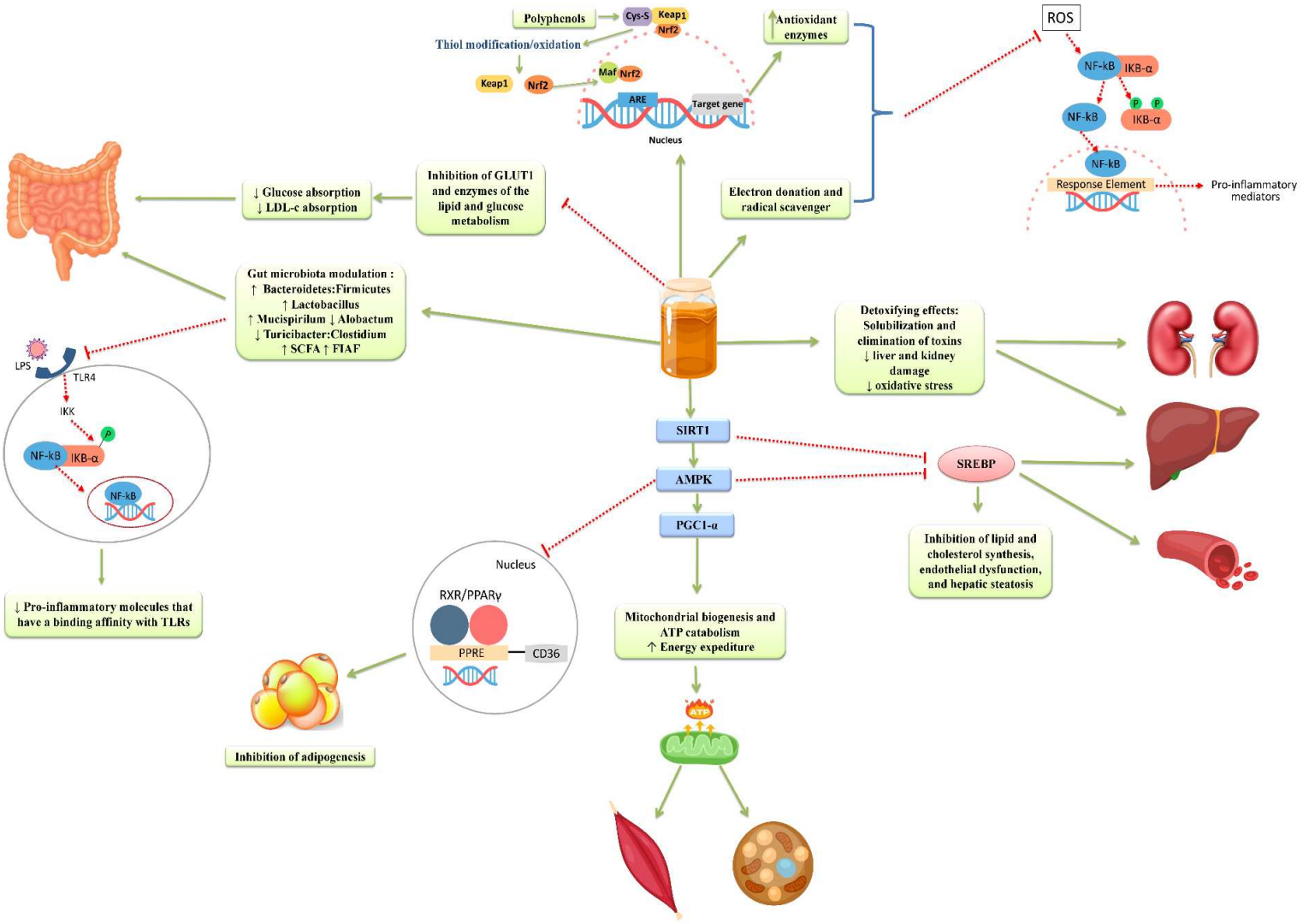

Figure 1. Proposed mechanism of action of kombucha. Adapted from Costa et al. (2021).

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CHAPTER 2: ANIMAL STUDY

This animal study was conducted using the biological samples collected in the Doctorate degree by Cardoso (2019). Our objective was to evaluate the effects of regular kombucha consumption on the intestinal health of Wistar rats. The results have been published in Nutrients 2022, 14(24), 5234; <https://doi.org/10.3390/nu14245234>.

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Kombuchas from Green and Black Tea Modulate the Gut Microbiota and Improve the Intestinal Health of Wistar Rats Fed a High-Fat High-Fructose Diet

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Abstract: The Western diet can negatively affect the gut microbiota and is associated with metabolic disorders. Kombucha, a tea fermented by a symbiotic culture of bacteria and yeast (SCOBY), is known for its bioactive properties and has become popular in the last years. In this study, we evaluated the effects of regular kombucha consumption on the gut microbiota and on outcomes related to the intestinal health of Wistar rats fed a high-fat high-fructose diet. After eight weeks receiving a standard diet (AIN-93M) (n = 10) or a high-fat and high-fructose diet (HFHF) (n = 30) to induce metabolic disorders, the animals were subdivided into four groups: AIN-93M (n = 10); HFHF (n = 10); GTK (HFHF + green tea kombucha (n = 10); and BTK (HFHF + black tea kombucha; n = 10) for 10 weeks. Although body composition did not differ among the groups, the HFHF diet was associated with metabolic alterations, and stimulated the growth of gram-negative bacteria such as *Proteobacteria* and *Bacteroides*. Kombucha ingestion could somewhat modulate the gut microbiota, attenuating the effects of a Western diet by increasing propionate production and favoring the growth of beneficial bacteria, such as *Adlercreutzia* in the GTK group. Our results suggest that regular kombucha consumption may be beneficial to intestinal health, which can be mostly attributed to its high content and diversity of phenolic compounds.

Keywords: experimental study; gut microbiome; intestinal permeability; obesity; polyphenols; probiotic; short-chain fatty acids

1. Introduction

The Western diet style, despite not having a specific definition, is an unhealthy diet generally composed of a high amount of saturated fat and fructose [1], which is involved in metabolic disorders such as obesity and metabolic syndrome [2,3], as well as in alterations in the gut microbiota profile [4]. Although the mechanisms by which dietary fat can modulate the gut microbiota are not completely understood yet, it is known that the small amount of this nutrient that is not absorbed in the small intestine can be fermented by the gut microbiota. Free fatty acids (FFA) resulting from the lipid metabolism can be utilized as substrates by the microorganisms present in the gut microbiota, influencing its composition [5] by increasing the *Bacteroidetes*:*Firmicutes* ratio [6] and the proportion of *Proteobacteria*, which are a major source of lipopolysaccharides [7]. Similarly, a high-fructose

diet has also been linked with alterations in the gut microbiota. Studies have suggested that it can affect the morphology and function of the intestine by altering the structure of the tight junction proteins, leading to increased intestinal permeability and inflammation [8,9].

Kombucha, a fermented beverage usually produced from green or black tea, has been highlighted as a promising alternative to minimize the impact of the Western diet or even for those who wish for a healthier lifestyle [10]. Kombucha presents in its composition several microorganisms as a result of the fermentation process performed by microorganisms known as SCOBY (symbiotic culture of bacteria and yeast) [11,12]. These microorganisms include lactic and acetic bacteria, particularly from the genera *Acetobacter* and *Gluconobacter*, and yeasts [12–15].

Beyond the microorganisms, kombucha also presents in its composition organic acids such as acetic, gluconic, and glucuronic; vitamins C and B complex; minerals; and amino acids [10,11,16]. However, it seems that the main benefits associated with kombucha intake are due to the presence of bioactive compounds. Previous studies conducted by our research group have shown that kombuchas from green and black tea present a high antioxidant capacity due to a high amount and diversity of phenolic compounds. Of the 127 phenolic compounds that we have identified, 103 were reported for the first time in the literature [17]. Nonetheless, its nutritional composition is influenced by many factors, including the tea type and quality, the amount of substrate, and the time and temperature used in the fermentation process [17–19]. SCOBYs also present differences in their composition and can influence the microorganism profile [20,21].

Regardless of the differences obtained in the manufacturing process, kombucha intake has been associated with health benefits through the modulation of the gut microbiota in mice. Black tea kombucha was associated with a decreased abundance of *Allobaculum*, *Turicibacter*, and *Clostridium* genera and an increase in *Mucispirillum*, a genus positively correlated to circulating leptin, which is a hormone involved in the regulation of appetite and food intake [22]. In another study, green tea kombucha supplementation was associated with an increase in alpha-diversity as well as favored the growth of bacteria involved in butyrate production [23]. Additionally, a recent systematic review has pointed out that kombucha consumption was able to reduce intestinal dysbiosis in vivo, being sug- gested as a potential alternative for the control and treatment of obesity and its associated comorbidities [24].

Although there is evidence that kombucha intake can bring benefits to health, there is still no consensus in the literature, especially when associated with the Western diet. Even though many commercial kombuchas have an appeal as a probiotic product, there is no evidence to support it so far. Thus, we aimed to investigate the effects of regular kombucha consumption on the gut microbiota and on outcomes related to the intestinal health of Wistar rats fed a high-fat high-fructose diet. Based on the chemical and microbiological composition of the beverages, as well as on previous studies [22,23], we hypothesized that green and black tea kombuchas would be able to modulate the gut microbiota and improve the intestinal health of those animals.

2. Material and Methods

2.1 Kombuchas Preparation

Kombuchas from green and black tea were prepared as previously described [17,25]. In summary, green tea (Lung Ching) and black tea (Darjeeling Gielle FTGFOP1) were obtained in a certified store (Tea Shop®) located in Belo Horizonte, Minas Gerais, Brazil.

Both beverages were prepared using 12 g of tea leaves and 50 g of sugar per liter of mineral water. Green tea infusion was performed at 75 °C for 3 min and the black tea at 95 °C for 5 min, according to the manufacturer. The beverages were cooled in an ice bath and when they reached room temperature, they were added to a SCOBY (3% *w*/*v*) (Enziquímica®, Gravataí, Brazil). A previously prepared kombucha (10% *v*/*v*) was also added to the beverages to decrease the pH and inhibit the proliferation of pathogenic microorganisms [11].

Fermentation occurred for ten days at 25 °C and then the SCOBY was removed and the kombuchas were filtered (Whatman #1 qualitative filter paper). The beverages were stored at 4°C for up to two weeks before being offered to the animals.

2.2 *Animal Study*

2.2.1 Study Design

Forty Wistar rats (*Rattus norvegicus*) aged between 45 and 50 days old were obtained at the Central Animal Facility from the Center of Biological Sciences and Health at Universidade Federal de Vicosa, Brazil. The animals were allocated to individual stainless-steel cages and kept in a light-dark cycle (12 h/12 h) at room temperature at 22±2 °C.

The experiment was divided into two phases. Phase I lasted eight weeks and the animals were separated into two groups: group 1 (n = 10); received a standard control diet (AIN-93M) [26] and group 2 (n = 30); received a high-fat and high-fructose (HFHF) diet to induce metabolic alterations [27]. In phase II, which lasted ten weeks, group 1 continued receiving a standard diet while the HFHF group was subdivided into three other groups: HFHF group (positive control) (n = 10); green tea kombucha (GTK group), which received HFHF diet + green tea kombucha diluted in water (30% *v*/*v*) (n = 10); and black tea kombucha (BTK group), which received HFHF diet + black tea kombucha diluted in water (30% *v*/*v*) (n = 10) (Figure 1). Both diets and water were consumed *ad libitum* during the whole experimental period.

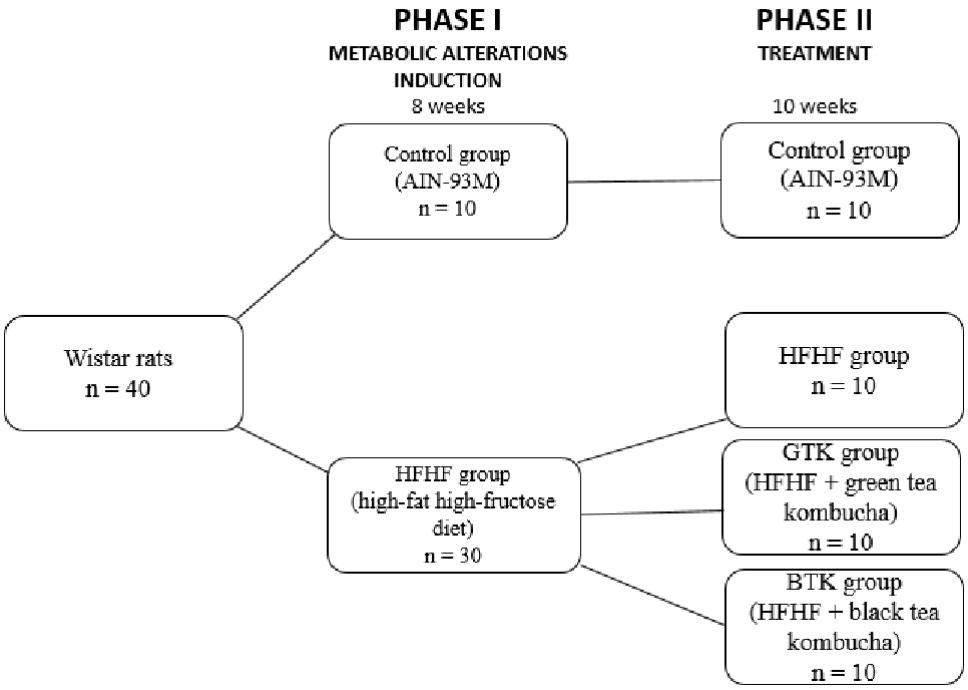

Figure 1. Experimental study design.

All procedures were performed following the ethical principles for animal use in experimental studies. The study protocol was approved by the Ethics Committee on Animal Use (CEUA—Universidade Federal de Vicosa, Protocol 06/2019; date of approval: 28 May 2019).

2.2.2 Kombuchas Characterization and Dosage

This study is a follow-up to our previous work and details about the experimental diet and the physical-chemical analyses of the kombuchas used in this experiment have been extensively described [17,25]. Briefly, sugars (sucrose, glucose, and fructose), organic acids (acetic, glucuronic, and lactic), and ethanol were identified and quantified by high-

performance liquid chromatography (HPLC) (Shimadzu, model LC-10A VP) coupledto a refractive index detector (RID 6A). The total acidity was determined according to the methodology proposed by IAL (2008) using phenolphthalein as an indicator and the results expressed as % acetic acid (w/v) [28]. The pH was measured by a calibrated pH meter (Kasvi, K-39,1014B, China). The concentrations of theaflavins and thearubigins were determined by a spectrophotometer according to the methodology proposed by Jayabalan et al. (2007), and the results were expressed as % (w/v) [29]. Regarding the microbiological characterization, serial kombucha samples were used to determine acetic bacteria, lactic bacteria, and yeasts using plates with GYC agar and ethanol, MRS, and PDA agar, respectively. The results were expressed as CFU/mL.

Kombucha dosage was determined according to preliminary tests that indicated the total phenolic content of beverages. Calculations were performed based on a previous study that recommends a daily total phenolic intake of 17 mg/kg/body weight [30].

2.2.3 Euthanasia and Samples Collection

As previously described [25], at the end of the experimental period, the animals were anesthetized by inhalation (Isoforine, Cristália®, São Paulo, Brazil), and euthanized by exsanguination by cardiac puncture. The tissues were immediately collected, weighted, and frozen in liquid nitrogen, and stored at −80 °C for further analysis. Feces were collected from the cecum, weighted, and stored at −80 °C for future analyses. Colon fragments were collected and fixed in formaldehyde (10% v/v) for the first 24 h and then stored in ethanol (70% v/v) and embedded in paraffin for histological analysis.

2.2.4 Histological Analysis

Serial sections of the colon, with a thickness of 5 µm, were collected and subsequently deparaffinized in xylene, rehydrated with different alcohol solutions, and stained with hematoxylin and eosin.

Histological sections were visualized in an Olympus AX70 photomicroscope, and the images were captured in a 20X objective with an AxioCam HRc—Zeiss digital camera. The following features were analyzed: crypt depth, crypt width, and the number of goblet cells. For that, we randomly selected six animals per group and twenty random fields per animal, analyzing one crypt per field. Only crypts with a well-defined and visible structure were used.

The measurements of the crypts were performed using the ImagePro-Plus® version 4.5 software (Media Cybernetics Inc., 1700 Rockville Pike, Suite 240, Rockville, MD 20852, USA) and the goblet cell count was performed using the Image J® 1.48v software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

2.2.5 Intestinal Permeability

Intestinal permeability analysis was performed at the end of the 10th week of treatment. After 12 h of fasting, the animals received 1 mL of a solution containing 100 mg lactulose and 50 mg mannitol by gavage. Then, they were kept in metabolic cages, fasting for 5 h. The urine was collected for 24 h and stored at −80 °C.

The urine was centrifuged (Hermle centrifuge, model Z326K, Wehingen, Germany), filtered on 0.45 µm membrane filters (Millipore, São Paulo, Brazil), and transferred to vials for high-performance liquid chromatography (HPLC). The mobile phase consisted of water in sulfuric acid (0.005 mM) with an injection volume of 20 µL and a mobile phase flow of 0.6 mL/min [31]. Lactulose® and Mannitol® were used as internal standards (Sigma-Aldrich, São Paulo, Brazil) and the concentrations were transformed to g/L to calculate the percentage of urinary excretion. The lactulose/mannitol ratio was calculated by dividing lactulose concentration by mannitol concentration [32].

2.2.6 pH and Short-Chain Fatty Acids Analysis

For pH analysis, approximately 1 g of cecum stool was homogenized in 10 mL of distilled water and vortexed with glass beads. Subsequently, the glass electrode of the pH meter was inserted, and the pH was measured in duplicate [33].

The short-chain fatty acids (SCFA) analysis was performed according to Siegfried et al. (1984) with modifications [34]. Briefly, approximately 500 mg of stool samples was homogenized in 1 mL of Milli-Q® water in a vortex and centrifuged at 12000 x *g* for 10 min. The supernatant was removed, and the samples were injected on a high-performance liquid chromatography (HPLC) (injection volume: 20 µL; Dionex Corporation, Sunnyvale, CA, USA). The SCFA were separated on a Phenomenex Rezex ROA ion exclusion column (300x 7.8 mm) (Phenomenex Inc. Torrance, CA, USA) coupled to a Shodex RI-101 refractive index (IR) maintained at 45 °C. Sulfuric acid 5 mM with a flow of 0.7 mL/min was used as a mobile phase. Stock solutions were prepared using acetic, propionic, and butyric acids as standards with a final concentration of 10 mmol/L (Sigma-Aldrich, Sao Paulo, Brazil). Stock solutions were diluted 2-, 4-, 8-, and 16-fold in 5 mmol/L^{-1} sulfuric acid (0.08–10 mM) to be used as standards in the HPLC analysis.

2.2.7 DNA Extraction and Microbiota Profile

DNA extraction of stool samples collected from the cecum of the animals was performed according to the methodology proposed by Steveron and Weimer (2007) [35]. Briefly, mechanical cellular lysis was performed using glass beads and phenol chloroform to promote the partitioning of lipids and cellular debris into the organic phase. After DNA extraction, a total of 39 samples encompassing all the animals of each experimental group were sequenced at the Argonne National Laboratory, Illinois, USA (AIN-93M n = 10; HFHF: n = 10; GTK: n = 9; and BTK: n = 10). The V4 region of the 16S rRNA genes was amplified by PCR using 515f/806r primers and amplicons sequenced using Illumina MiSeq desktop sequencer producing 150 bp paired-end (PE) reads.

The demultiplexed raw paired-end reads obtained after sequencing were uploadedand processed into QIIME2 (version 2020.2) via the Casava 1.8 paired-end pipeline [36]. DADA2, which allows improved taxonomic resolution based on the exact identification and error correction of sample sequences that differ as little as a single nucleotide, was chosen to assess the quality of the reads in sequential steps such as filtering, trimming, denoising, dereplicating, merging paired reads, as well as chimeric sequences removal [37]. Afterward, amplicon sequence variants (ASV) were forwarded to generate a phylogenetic tree using the align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin [38]. When convenient, samples were rarefied to an appropriate sampling depth of 15,349. Taxonomy was assigned to the 16S data using a Naïve Bayes pre-trained Greengenes 13_8 99% OTUs classifier [39].

With regards to the DNA obtained from the kombuchas and their respective SCOBYs, samples used during the experiment were mixed and lyophilized at - 62 °C for 24 h under a pressure of 35 uHg (Liotop, model L101, serial no. 01610, Liobras, São Carlos, Brazil). Microbial DNA was extracted from frozen pellets using the Qiagen Powersoil Pro kit with bead beating, according to the manufacturer's protocol. Then, the samples were forwarded to the company Molecular Research LP (MR DNA, Shallowater, TX, USA) where amplicon preparation and sequencing were performed considering the bacterial V4 region of the 16S rRNA gene (515f/806r primers) and the variable internal transcribed spacer (ITS)-1 of the fungal rRNA region (ITS1F and ITS2 reverse primers). Amplicon libraries were prepared and sequenced with the Illumina MiSeq desktop sequencer producing 250 bp paired-end (PE) reads. For microbiota profiling, sequence data were processed using an MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Briefly, sequences were joined, and short reads with ambiguous base calls were removed. Afterward, sequences were quality filtered using a maximum expected error threshold of 1.0, dereplicated, and denoised. Lastly, unique sequences identified with sequencing and/or PCR point errors were removed followed by chimera removal, thereby providing a denoised sequence or

zero-radius OTU (zOTU). Final zOTUs were taxonomically classified using BLASTn against a curated database derived from NCBI (http://www.ncbi.nlm.nih.gov; accessed on 10 July 2020). Raw reads were deposited in the Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra; deposited on 23 November 2022) under the BioProject PRJNA904803.

2.3 Statistical Analysis

Statistical analysis was performed using the software GraphPad Prism®, version 6.01. The normality of the data was tested by the Shapiro–Wilk test. Groups with parametric distribution were analyzed by one-way ANOVA followed by Tukey post-hoc. Non-parametric data were evaluated by the Kruskal–Wallis test followed by Dunn's post-hoc. Data were expressed as mean ± standard deviation (SD) and the values were considered significant when $p < 0.05$.

For statistical analysis of the gut microbiota, qiime artifacts were imported into R (R Core Team 3.6.2, 2019) with the qiime2R package v.099.20 (https://github.com/jbisanz/qiime2R; accessed on 10 July 2020). Significant differences in alpha-diversity among the four groups (AIN-93M, GTK, BTK, and HFHF) were determined using the alpha function in microbiome R package v.2.1.24 adopting Kruskal–Wallis as a statistical test followed up by Wilcoxon's test to calculate pairwise comparisons between groups. For beta-diversity, weighted and unweighted UniFrac distances were subjected to permutational multivariate analysis of variance (PERMANOVA) to assess significant differences (pseudo-F test) in bacterial community composition and structure among the groups with a permutation number of 999. Principal coordinates analysis (PCoA) was chosen to explore and visualize the clustering of groups. All graphs were constructed and visualized with RStudio (v. 1.2.5033) using one or the combination of the following R packages: MicrobiomeR, dplyr, ggplot2, phyloseq [40], tidyr, and vegan.

To determine which bacterial taxa were differentially abundant among groups, taxonomy was firstly collapsed to the genus level and then analyzed via linear discriminant analysis (LDA) effect size (LEFSe) [41] (p-value cut-off of 0.05 and log LDA score of 2.0).

3. Results

3.1 Kombucha Chemical Characterization and Consumption

The main compounds found in kombuchas used in this study are presented in Table 1. As previously demonstrated, both green and black tea kombuchas presented high content of phenolic compounds; however, black tea kombucha presented a higher antioxidant capacity, probably due to its higher phenolic compound concentration [17,25].

Among the 127 phenolic compounds identified in the kombuchas, 14 were more abundant: gallocatechin 3-O-gallate/epigallocatechin 3-O-gallate; gallocatechin isomer 2/epigallocatechin; catechin; quercetin 3-O-rhamnosyl-rhamnosyl-glucoside isomer 2; quercetin 3-O-glucosyl-rhamnosylgalactoside-isomer 2; gallocatechin isomer 1/epigallocatechin; quercetin 3-O-rhamnosyl-rhamnosylglucoside-isomer 1; quercetin 3-O-glucosyl-rhamnosyl-galactoside isomer 1; catechin 3-O-gallate; and catechin 5-O-gallate, which belong to the flavonoids class; and 5-O-galloylquinic acid; 3-[2-(carboxymethyl)-3,4-dihydroxyphenyl] prop-2-enoic acid; 4-coumaroylquinic acid isomer 2; and 1-O-caffeoylquinic acid isomer 2/3-caffeoylquinic acid, which belong to the phenolic acids class [17,25].

We did not observe a difference between the GTK and BTK groups regarding daily, weekly, and total kombucha consumption. However, the BTK group ingested a higher amount of phenolic compounds, which can be explained by its higher concentration in the black tea kombucha compared to green tea kombucha, as mentioned (Figure 2).

Table 1. Green and black tea kombuchas chemical characterization.

	Green Tea Kombucha	Black Tea Kombucha	*p*-Value
Chemical composition			
Sucrose (g/L)	19.30 ± 2.73 [b]	34.98 ± 1.42 [a]	0.0382
Glucose (g/L)	3.19 ± 0.15 [a]	2.45 ± 0.96 [a]	0.4690
Fructose (g/L)	0.15 ± 0.01 [a]	0.05 ± 0.02 [a]	0.0583
Ethanol (g/L)	7.23 ± 0.03 [a]	4.91 ± 0.35 [a]	0.0653
Theaflavin (g/L)	0.28 ± 0.03 [b]	1.51 ± 0.06 [a]	0.0066
Thearubigin (g/L)	13.30 ± 0.67 [b]	19.99 ± 0.10 [a]	0.0416
pH	3.2 ± 0.1 [b]	3.5 ± 0.1 [a]	0.0078
Total acidity (% *w*/*v*)	0.36 ± 0.01 [a]	0.32 ± 0.01 [b]	0.0100
Organic acids			
Acetic acid (g/L)	3.22 ± 0.39 [a]	2.78 ± 0.16 [a]	0.3336
Glucuronic acid (g/L)	1.17 ± 0.06 [a]	0.47 ± 0.02 [b]	0.0323
Lactic acid (g/L)	0.01 ± 0.00 [a]	0.02 ± 0.00 [a]	0.2604
Microbiological characterization			
Acetic bacteria (log CFU/mL)	6.0 ± 0.30 [a]	5.30 ± 0.10 [a]	0.1071
Lactic bacteria (log CFU/mL)	6.50 ± 0.20 [a]	5.90 ± 0.60 [a]	0.3959
Yeast (log CFU/mL)	6.30 ± 0.40 [a]	5.50 ± 0.10 [a]	0.1690

Values are expressed as mean ± SD. Different letters in the same row indicate a significant difference ($p < 0.05$) according to unpaired *t* test followed by Welch's correction. Details about the methodology used for the analyses are described in Cardoso et al. (2020).

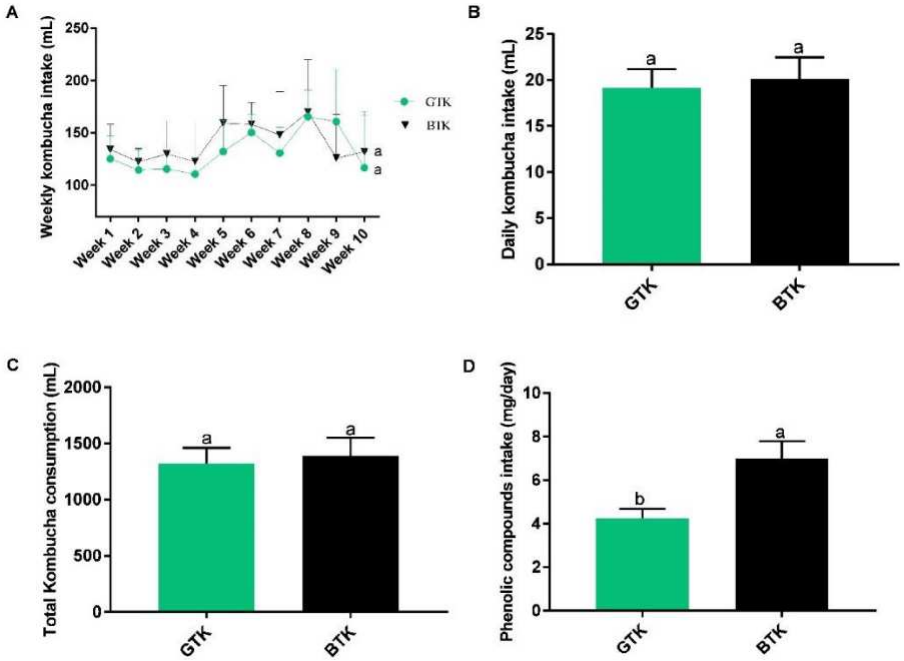

Figure 2. Weekly kombucha intake (**A**), daily kombucha intake (**B**), total kombucha consumption (**C**), and phenolic compounds intake (**D**) by the animals during the treatment. Data were expressed as mean ± SD. Different letters indicate a significant difference ($p < 0.05$) between groups according to t-test. GTK: HFHF diet + green tea kombucha diluted in water (30% *v*/*v*); BTK: HFHF diet + black tea kombucha diluted in water (30% *v*/*v*).

3.2 Biometric Parameters

Initial weight, final weight, weight gain, and BMI did not differ among groups, although some metabolic disorders were observed [25]. The group that received a standard diet (AIN-93M) presented a higher cecum weight than the HFHF and GTK groups, but no significant difference was observed compared to the BTK group. Regarding cecum weight:body weight ratio, the AIN93-M group presented a higher value when compared to the GTK group; however, this difference was not significant when compared to the other groups (Table 2).

Table 2. Body composition and intestinal parameters of the animals after 10 weeks of treatment.

Features	AIN93-M (n = 10)	HFHF (n = 10)	GTK (n = 9)	BTK (n = 10)
Body composition				
Initial weight (g)	349.90 ± 30.71 [a]	366.90 ± 36.90 [a]	370.40 ± 36.20 [a]	364.60 ± 36.05 [a]
Final weight (g)	415.00 ± 34.50 [a]	438.10 ± 66.65 [a]	415.30 ± 37.07 [a]	409.60 ± 50.08 [a]
Weight gain (g)	65.00 ± 22.70 [a]	71.25 ± 38.10 [a]	44.90 ± 30.69 [a]	44.90 ± 23.14 [a]
BMI (g/cm^2)	0.68 ± 0.08 [a]	0.61 ± 0.04 [a]	0.61 ± 0.08 [a]	0.61 ±0.08 [a]
Cecum weight (empty) (g)	1.01 ± 0.23 [a]	0.97 ± 0.13 [a]	0.96 ± 0.18 [a]	0.96 ± 0.04 [a]
Cecum weight (full) (g)	5.09 ± 1.15 [a]	3.92 ± 0.89 [b]	3.62 ± 0.75 [b]	4.03 ± 0.80 [ab]
Cecum weight:body weight ratio	1.23 ± 0.28 [a]	0.91 ± 0.24 [ab]	0.87 ± 0.17 [b]	0.98 ± 0.21 [ab]
Intestinal Permeability				
Lactulose:mannitol ratio	1.51 ± 0.57 [a]	1.56 ± 0.78 [a]	1.62 ± 0.94 [a]	2.17 ± 1.08 [a]
Histological Features				
Crypt depth (µM)	179.10 ± 43.20 [a]	223.10 ± 40.69 [a]	221.20 ± 24.92 [a]	209.30 ± 40.83 [a]
Crypt width (µM)	19.51 ± 2.66 [a]	18.85 ± 4.16 [a]	21.03 ± 1.46 [a]	19.46 ± 2.68 [a]
Number of goblet cells (units)	18.60 ± 3.27 [a]	16.59 ± 4.71 [a]	17.94 ± 2.93 [a]	22.38 ± 5.51 [a]
Fecal pH	9.01 ± 0.40 [a]	9.17 ± 0.25 [a]	9.27 ± 0.07 [a]	9.13 ± 0.13 [a]

Data are expressed as mean SD. Different letters in the same row indicate a significant difference ($p < 0.05$) according to one-way ANOVA followed by Tukey post-hoc (parametric data) or Kruskal–Wallis test followed by Dunn's post-hoc (non-parametric data). AIN-93M: standard diet (negative control group); HFHF: high-fat and high-fructose diet (positive control group); GTK: HFHF diet + green tea kombucha diluted in water (30% *v*/*v*);BTK: HFHF diet + black tea kombucha diluted in water (30% *v*/*v*).

3.3 Bioinformatics Analysis

To better comprehend the effects of the long-term intake of black tea kombucha (BTK) and green tea kombucha (GTK) on the gut microbiota of Wistar rats, we conducted a deep amplicon sequencing of the V4 region of the 16S rRNA genes. After the removalof low-quality and chimeric sequences from 39 datasets (AIN-93M, n = 10; BTK, n = 10; GTK, n = 9; HFHF, n = 10), a total of 1,004,357 high-quality reads, with an average of 25,752 (minimum: 15,349; maximum: 34,422) sequences for each sample, were obtained and assigned to 1218 predicted ASVs (99% similarity).

Regarding the sequences obtained from the kombuchas and their respective SCOBYs, 153,322 high-quality reads were obtained based on the amplification of the V4 region of the 16S rRNA genes (mean: 38,330; minimum: 36,957; maximum: 39,631) for bacterial community analysis, whereas 138,569 reads were forwarded for metataxonomic analysis based on the ITS1–2 regions of fungal ribosomal DNA (mean: 34,642; minimum: 34,566; maximum: 34,716).

3.4 Microbiota Profiling of GTK and BTK and Their Respective SCOBYs

In both kombuchas and their respective SCOBYs, the fungal community was greatly dominated by the species *Dekkera bruxellensis* (GTK SCOBY: 99.3%; BTK SCOBY: 99.6%; GTK: 99.6%; BTK: 99.9%) and, to a lesser extent, by the species *Saccharomyces bayanus* (GTKSCOBY: 0.7%; BTK SCOBY: 0.4%; GTK: 0.4%; BTK: 0.1%).

Regarding the bacterial community, microorganisms belonging to the phylum *Proteobacteria* dominated both tea and SCOBY samples (average of 77.07% considering the four groups); however, in the BTK group, this phylum accounted for only 37.24% of the sequences (Figure 3A). Notably, the phyla *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* also represented an important part of the bacterial community found in kombucha samples, but not in their related SCOBYs. At the family level (Figure 3B), *Acetobacteraceae* stands as the dominant taxon across the groups, corresponding to almost 100% of the samples in the GTK and BTK SCOBYs groups. For kombucha samples, *Acetobacteraceae*, *Erysipelotrichaceae*, *Porphyromonadaceae*, *Rikenellaceae*, and *Streptococcaceae* encompass the

top five families. Lastly, at the genus level (Figure 3C), *Gluconacetobacter* appears as the dominant taxon in samples obtained from both SCOBY samples, while *Acetobacter* comprehends the most abundant taxon in kombucha samples. In conjunction with the genus *Acetobacter*, *Allobaculum*, *Komagataeibacter,* and *Barnesiella*, they correspond to the top five genera. Interestingly, kombucha produced from black tea showed a higher number of taxa classified as low-abundant (13.05%), which is quite different from that obtained from the fermentation of green tea (6.85%), which may indicate greater bacterial diversity and richness in the GTK.

A

Phylum	SGTK	SBTK	GTK	BTK
Proteobacteria	99.67%	99.90%	71.46%	37.24%
Firmicutes	0.15%	0.04%	14.32%	32.85%
Bacteroidetes	0.12%	0.04%	8.16%	20.49%
Actinobacteria	0.04%	0.02%	2.37%	7.32%
Total	99.98%	99.99%	96.31%	97.90%

B

Family	SGTK	SBTK	GTK	BTK
Acetobacteraceae	99.38%	99.80%	66.96%	28.60%
Erysipelotrichaceae	0.06%	0.02%	7.57%	18.84%
Porphyromonadaceae	0.06%	0.03%	3.64%	10.93%
Rikenellaceae	0.03%	0.00%	2.32%	5.26%
Streptococcaceae	0.03%	0.00%	2.14%	3.81%
Bacteroidaceae	0.02%	0.01%	1.91%	3.76%
Clostridiaceae	0.02%	0.00%	1.61%	3.71%
Bifidobacteriaceae	0.01%	0.01%	1.02%	4.10%
Enterobacteriaceae	0.02%	0.00%	0.92%	2.63%
Lachnospiraceae	0.01%	0.00%	0.91%	1.86%
Oscillatoriales	0.02%	0.01%	2.66%	0.03%
Eubacteriaceae	0.01%	0.00%	0.77%	1.67%
Nocardiaceae	0.01%	0.00%	0.60%	1.50%
Helicobacteraceae	0.01%	0.00%	0.66%	1.40%
Pasteurellaceae	0.01%	0.00%	0.71%	1.06%
Xanthomonadaceae	0.01%	0.01%	0.41%	1.20%
Total	99.71%	99.87%	94.81%	90.35%

C

Genus	SGTK	SBTK	GTK	BTK
Gluconacetobacter	96.93%	94.21%	19.09%	9.97%
Acetobacter	1.67%	3.68%	38.18%	18.49%
Allobaculum	0.05%	0.01%	6.36%	15.92%
Komagataeibacter	0.79%	1.91%	9.89%	0.34%
Barnesiella	0.03%	0.01%	1.86%	5.53%
Bacteroides	0.02%	0.01%	1.92%	3.79%
Clostridium	0.02%	0.00%	1.62%	3.73%
Lactococcus	0.03%	0.00%	1.82%	3.44%
Bifidobacterium	0.01%	0.01%	1.03%	4.12%
Rikenella	0.01%	0.00%	1.61%	3.30%
Turicibacter	0.01%	0.01%	1.21%	2.87%
Odoribacter	0.01%	0.01%	0.59%	2.30%
Alistipes	0.02%	0.00%	0.71%	2.00%
Halospirulina	0.02%	0.01%	2.67%	0.03%
Eubacterium	0.01%	0.00%	0.78%	1.69%
Serratia	0.01%	0.00%	0.67%	1.74%
Rhodococcus	0.01%	0.00%	0.60%	1.51%
Helicobacter	0.01%	0.00%	0.66%	1.41%
Tannerella	0.02%	0.00%	0.54%	1.37%
Lachnoclostridium	0.01%	0.00%	0.62%	1.13%
Stenotrophomonas	0.01%	0.01%	0.41%	1.21%
Parabacteroides	0.00%	0.01%	0.31%	1.09%
Total	100%	100%	93.15%	86.95%

Figure 3. Heat map based on the relative abundance (>1.0% in at least one sample) of the most abundant bacterial taxa of phylum (**A**), family (**B**), and genus (**C**) identified in green tea kombucha(GTK), black tea kombucha (BTK), and their respective SCOBY (SGTK and SBTK). Green to red gradient indicates low to high relative levels of OTUs within the given taxonomic unit.

3.5 Alpha and Beta-Diversity Metrics of Gut Microbiota

The effects of the daily ingestion of green tea kombucha (GTK) and black tea kombucha (BTK) for 10 weeks on the gut microbiota of Wistar rats were firstly investigated using alpha- and beta-diversity indices and compared against the AIN-93M and HFHF groups. Considering the alpha-diversity analysis, the observed ASVs, Shannon, and Chao1 indices reached a plateau and are indicative that sequencing depth covered most of the microbial diversity and the majority of bacterial phylotypes were sampled (Figure 4).

A significant reduction in bacterial diversity, evaluated through Shannon's diversity index, was observed in the groups that received green and black tea kombucha when compared to both controls (Figure 4A). However, we did not observe significant differences among the control groups (AIN-93M vs. HFHF), as well as among the treatment groups (GTK vs. BTK) ($p > 0.05$). In terms of bacterial richness, a significantly lower Chao1 index was identified in the groups supplemented with GTK and BTK when compared to the AIN-93M group, but not when compared to the HFHF group (Figure 4B).

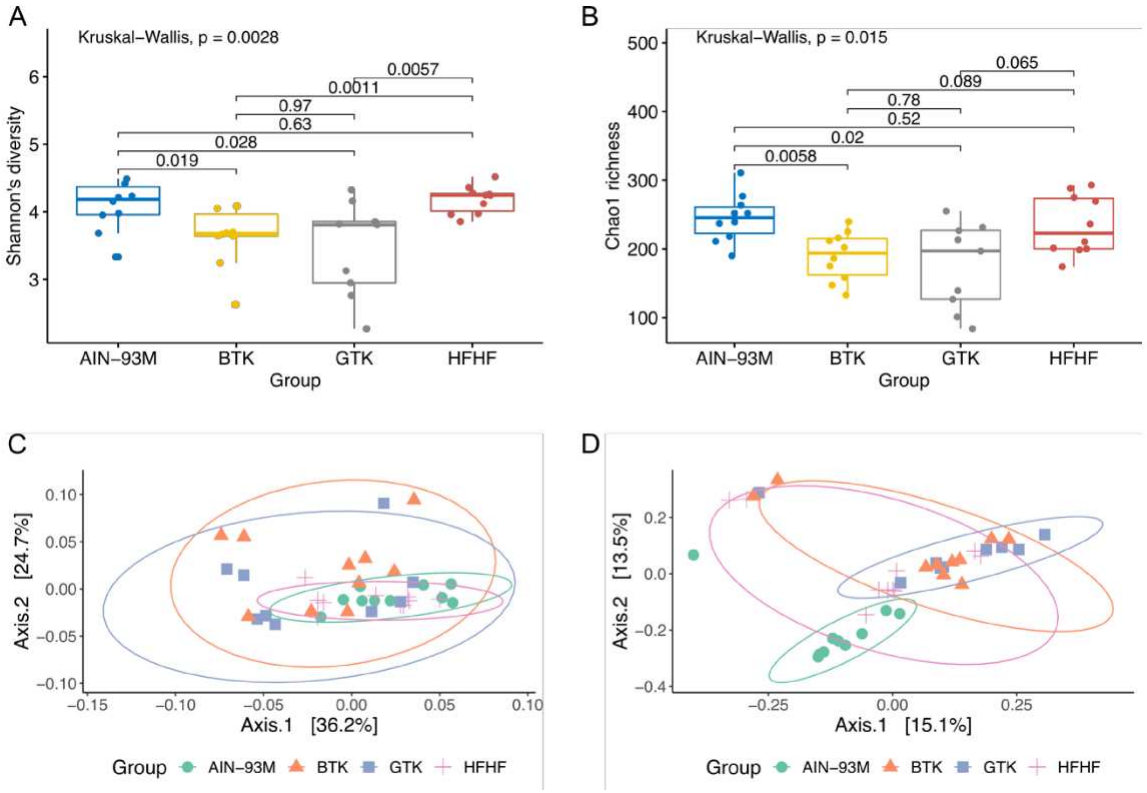

Figure 4. Box and whisker plots comparing species diversity (**A**) and richness (**B**) among the groups AIN-93M, BTK, GTK, and HFHF at the end of the experimental period. Horizontal bold lines show the median values. The bottom and top of the boxes show the 25th and the 75th percentiles, respectively. The whiskers extend up to the most extreme points within 1.5 times the interquartile ranges (IQR).Principal coordinate analysis (PCoA) based on weighted (**C**) and unweighted (**D**) UniFrac distances.PERMANOVA with 999 permutations was used to detect significant differences between microbialcommunities (dissimilarity) of different experimental groups. Standard error ellipses show 95% confidence areas.

Regarding the beta-diversity analysis, gut microbiota clustering on PCoA plots based on weighted and unweighted UniFrac distance metrics showed significant differences among the groups (Figure 4C,D). Pairwise comparisons using Qiime beta-group-significance command revealed that the gut composition of the groups that received the two different types of kombucha significantly differed from those animals receiving a standard (AIN-93M) or a high-fat and high-fructose (HFHF) diet. However, high similarity in terms of bacterial composition and abundance was observed among the GTK and BTK groups. Moreover, according to pairwise PERMANOVA results, the AIN-93M and HFHF groups differed only qualitatively regarding community dissimilarity (unweighted UniFrac: pseudo-F = 4.86, q = 0.0020). Taken together, alpha- and beta-diversity indices evidenced that after receiving an HFHF diet for eight weeks, long-term intake of green and blacktea kombuchas was not able to establish a high diversity bacterial community as that observed in both control groups as well as a community as rich as that observed in the AIN-93M group.

3.6 Taxonomic Assignment and Gut Bacterial Composition

The Linear discriminant analysis Effect Size (LEfSe) was adopted to better characterize the gut bacterial composition of each group, as well as identify the differently abundant taxa. Moreover, inter-group comparisons were conducted based on the relative abundance of interest taxa. At the phylum level, *Firmicutes* (69.09%), *Bacteroidetes* (15.82%), *Proteobac- teria* (8.13%), *Actinobacteria* (3.26%), and *Euryarchaeota* (2.15%) were the dominant taxa (Figure 5A).

The phylum *Firmicutes* showed higher relative abundance in the groups treated with kombucha prepared from black tea (BTK group, 75.21%) and green tea (GTK, 76.30%) when compared to both control groups (AIN-93M, 61.41%; HFHF, 63.45%), although a significant difference has been observed only between AIN-93M and BTK (p = 0.019). The same trend was observed for the phylum *Actinobacteria* (AIN-93M, 2.88%; BTK, 3.56%, GTK, 4.62%; HFHF, 1.97%), although no significant difference among the groups (p > 0.05) was observed. On the other hand, *Bacteroidetes* was significantly more abundant in the control groups AIN-93M (24.65%) and HFHF (22.20%) when compared to both kombucha groups (BTK, 8.72%; GTK, 7.69%), but not between AIN-93M and HFHF. Our results also indicate that the administration of both kombuchas increased the *Firmicutes*:*Bacteroidetes* ratio in these groups (BTK: F/B = 8.63; GTK: F/B = 9.93) when compared to the control groups AIN-93M (F/B = 2.49) and HFHF (F/B = 2.86). Regarding *Proteobacteria*, this phylum displayed the lowest proportion in the group GTK (5.38%), whereas its highest relative abundance was observed in the HFHF group (10.86%; Figure 6D, LDA > 3; p = 0.035). Interestingly, the phylum *Euryarchaeota* stands out in the BTK group (4.10%); it appears lessabundant in the AIN-93M (1.08%) and GTK (2.53%) groups, while a very little abundance was observed in the group HFHF (0.88%) (BTK vs. HFHF, p = 0.035).

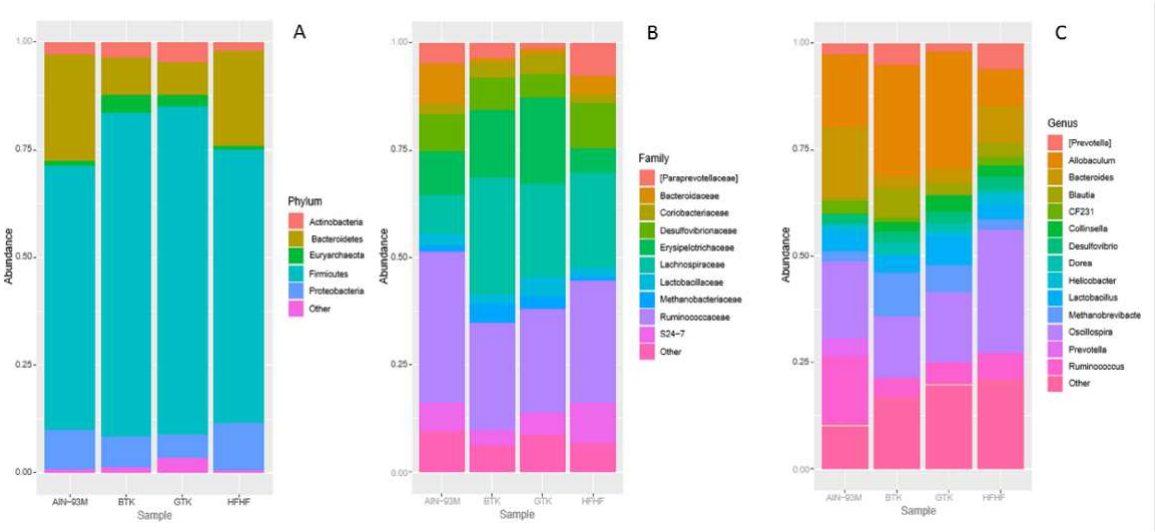

Figure 5. Stacked bar chart based on the relative abundance of major phyla (top-five) (**A**), families (top-ten) (**B**), and genera (top-fifteen) (**C**) across the groups AIN-93M, BTK, GTK, and HFHF.

At the family level, 12 taxa (relative abundance greater than 1%) accounted for approximately 95% of the total sequences in each group. Among them, *Ruminococcaceae* was the most abundant family in the AIN-93M, GTK, and HFHF groups, whereas *Lachnospiraceae*was the most prevalent in the BTK group (Figure 5B). Although *Lachnospiraceae* was the most abundant in the BTK group and differed from the AIN-93M group (p = 0.00049), this taxon appears as a biomarker of the HFHF group when compared to AIN93M (Figure 6A, LDA > 3, p = 00013). The third most abundant family among the groups, *Erysipelotrichaceae*, appears enriched in the groups that received both kombuchas (AIN-93M, 10.37%; BTK, 15.87%; GTK, 20.21%; HFHF, 5.74%) and was identified as a biomarker of the BTK group when compared to the group HFHF (Figure 6E LDA > 4; p = 0.0068). Interestingly, groups treated with green and black kombuchas showed a very low abundance (around 0.1%) of members belonging to the family *Bacteroidaceae*—a biomarker of the group HFHF—when compared to the group BTK (Figure 6E, LDA > 3), and when compared to both control groups (p < 0.05), which may justify the higher F/B ratio observed in kombucha treated groups. Positively correlated with diabetes and obesity [42,43], the families *S24–7* and *Desulfovibrionaceae* were significantly less abundant in the BTK and GTK groups, respec- tively, while in the HFHF group, these families reached their highest values. Indeed, LEfSe analysis revealed that the family *Desulfovibrionaceae*, in conjunction with the family *Paraprevotellaceae*, were detected as biomarkers of the HFHF group when compared to the group that received green tea kombucha (Figure 6D, LDA >3).

Lastly, as shown in Figure 6A–C, *Prevotellaceae* was identified as a biomarker of the AIN-93M group (LDA >3)when compared to the other experimental groups enrolled in this study (AIN-93M, 2.42%; BTK, 0.02%, GTK, 0.01%; HFHF, 0.004%).

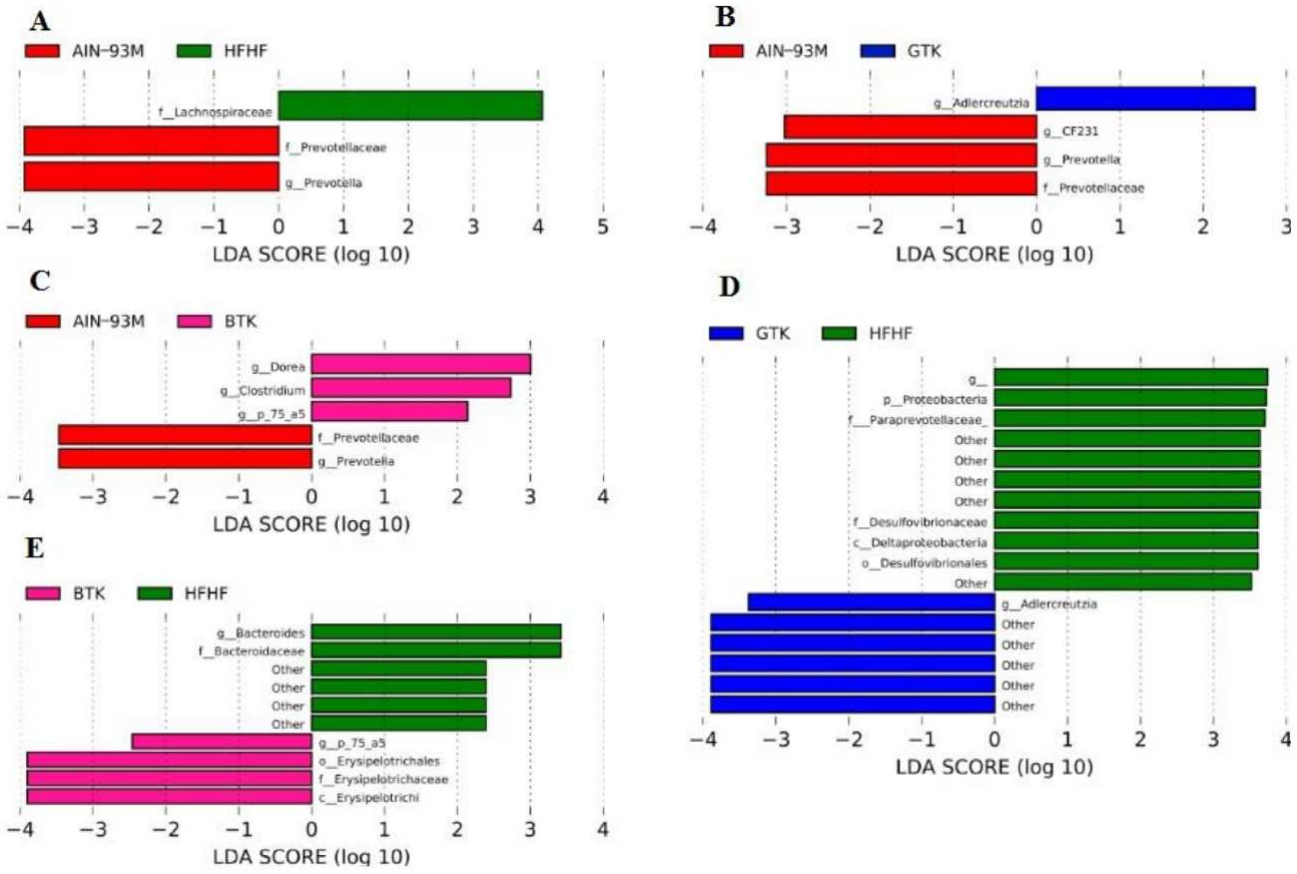

Figure 6. Differential abundance analysis was conducted with Linear discriminant analysis Effect Size (LEfSe) following the experimental period. Comparisons were made between AIN-93M with HFHF (**A**); GTK (**B**); and BTK (**C**) groups and between HFHF with GTK (**D**) and BTK (**E**) groups. Only biomarkers showing linear discriminant analysis (LDA) scores greater than 2.0 with a false discovery rate (FDR) $p < 0.05$ are depicted. Letters: p, phylum; c, class; o, order; f, family; g, genus.

After defining the most abundant genera, the top-ten taxa were selected and accounted for at least 70% of the total sequences in each group (Figure 5C). *Oscillospira* was the most abundant microorganism in the control group HFHF ($p < 0.05$). In the groups treated with kombuchas, *Allobaculum* was the dominant genus; however, this difference was significant just among the BTK and HFHF groups ($p = 0.0068$). Differential abundance analysis considering taxa at the genus level showed enrichment of *Prevotella* in the AIN-93M group (Figure 6A–C; LDA > 3). Although not considered biomarkers by LefSe analysis, but still included in the top-ten genera, it was possible to identify that *Bacteroides* and *Ruminococcus* are highly abundant in the group AIN-93M when compared to all other groups ($p < 0.05$). Regarding the BTK group, the genera *Dorea*, *Clostridium,* and *p-75-a5*, all of them belonging to the family *Erysipelotrichaceae*, appeared as biomarkers when compared to the AIN-93M group (Figure 6C, LDA > 3). However, when compared to the HFHF group, only the genus *p-75-a5* was identified as a biomarker in the BTK group (Figure 6E, LDA >3). Concerning the GTK, *Adlercreutzia* appeared as a biomarker in this group when compared to both control groups (Figure 6B,D; AIN-93M, LDA >2; HFHF, LDA >3). We did not identify biomarkers between the GTK and BTK groups regardless of the taxonomic level.

Lastly, we predicted and explored the structural basis (core taxa) of the bacterial communities of the groups enrolled in this study after the experimental period (Figure 7). Considering only ASVs with a prevalence of 75% across samples, the group AIN-93M contained 40 core taxa, while in the groups GTK, BTK, and HFHF, we noticed 14, 29, and 38 taxa, respectively. At a first inspection, five ASVs were identified as common to both groups and assigned to the following taxa: *Clostridiales, Allobaculum,* and *Oscillospira*. Secondly, considering only those ASVs specific to each group, 16 ASVs were identified in samples from the group AIN-93M and assigned to the following taxa: orders *Clostridiales* and *Bacteroidales*, class *Clostridia*, genera *Oscillospira*, *Bacteroides*, and *Helicobacter*, and species *Mucispirillum schaedleri*. Regarding the groups that underwent kombucha ingestion, only two ASVs stood out in the GTK group and were assigned to the genus *Lactobacillus* and the species *Ruminococcus flavefaciens*, whereas seven ASVs were typical for the BTK group and were assigned to the family *Lachnospiraceae*, genera *Dorea*, *Blautia*, *Allobaculum*, and *Mogibacteriaceae*, as well as the species *Collinsella stercoris*. Lastly, 12 ASVs were identified asspecifically present in the HFHF group and were taxonomically assigned to the following taxa: families *Desulfovibrionaceae*, *S24-7*, *Ruminococcaceae,* and *Lachnospiraceae*, in addition to the genera *Roseburia* and *Oscillospira*.

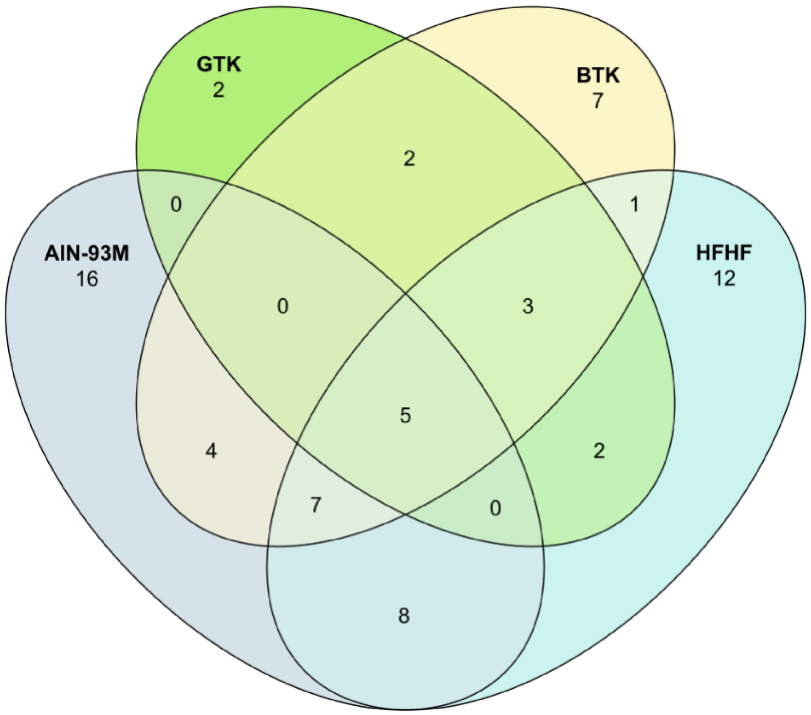

Figure 7. Venn diagram representing shared amplicon sequence variants (ASVs) of the core microbiome identified in the groups AIN-93M, GTK, BTK, and HFHF.

3.7 Fecal pH and Short-Chain Fatty Acids Content

We did not find a significant difference in fecal pH among the groups (Table 2). Regarding the SCFA, both treatment groups—GTK and BTK—presented a higher propionic acid concentration when compared to the AIN93-M and HFHF groups. Acetic acid concentration was significantly higher in the AIN93-M group when compared to the GTK and BTK groups, but no significant difference was noted when comparing the HFHF group to the other groups. Butyric acid concentrations did not differ significantly among groups (Figure 8).

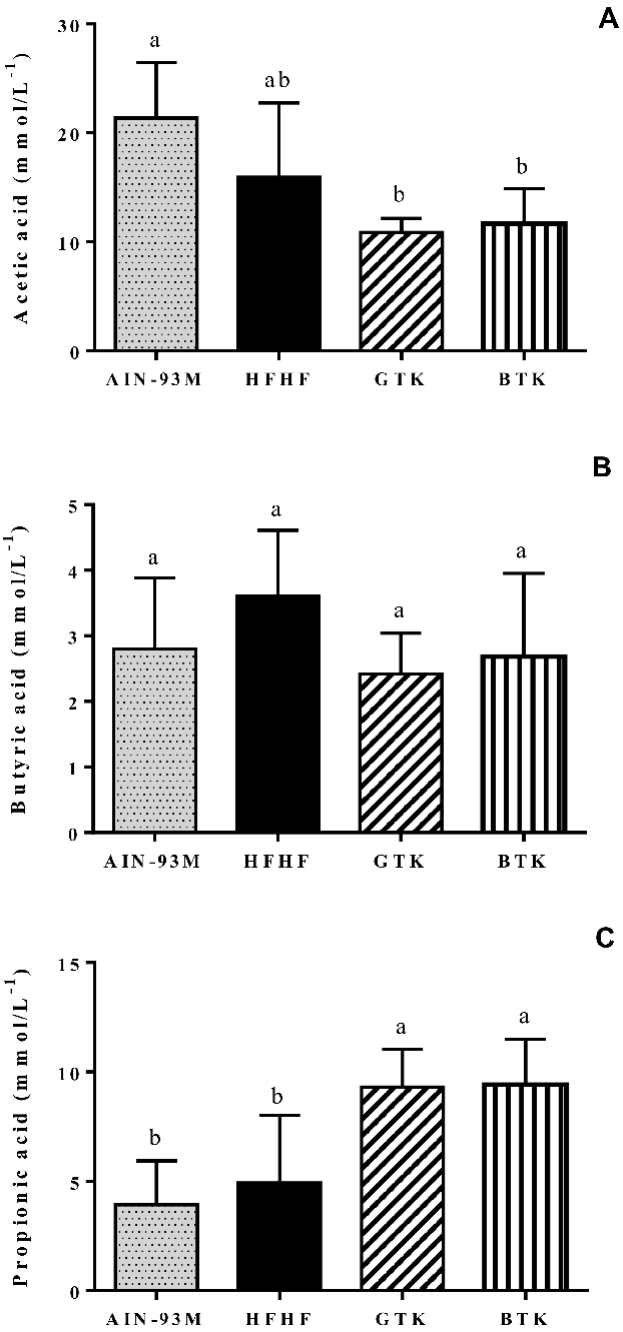

Figure 8. Acetic (**A**), butyric (**B**), and propionic (**C**) acids concentrations identified on stool samples from the animals. AIN-93M: standard diet (negative control group); HFHF: high-fat and high-fructose diet (positive control group); GTK: HFHF diet + green tea kombucha diluted in water (30% *v*/*v*); BTK: HFHF diet + black tea kombucha diluted in water (30% *v*/*v*). Values are expressed as means ± SD. Different letters indicate a significant difference between groups ($p < 0.05$) according to ANOVA one-way followed by Tukey post-hoc (acetic and butyric acids) and Kruskal–Wallis test followed by Dunn's post-hoc (propionic acid).

We also investigated whether there is an association between the SCFA content and the microorganisms found in the gut microbiota of the animals. For that, MaAsLin 2 [44] was performed to find significant multivariable associations between specific microbial genera, cecal SCFA (acetate, propionate, and butyrate), and phenolic intake. The compound Poisson linear model (CPLM) function was utilized on cumulative sum scaling (CSS) normalized data with minimum prevalence (1%). For analysis of changes across the different

interventions, samples obtained from the HFHF group were assigned as references. All *p*-values were false discovery rate-adjusted (Benjamini–Hochberg, q-values), and features with q < 0.25 were considered significant (Table S1).

3.8 Intestinal Permeability and Histological Analysis

There was no difference between groups regarding the excretion of lactulose and mannitol in the urine, which was expressed as lactulose/mannitol ratio (Table 2).

Histological features are demonstrated in Figure 9. We did not observe differences among the groups in terms of crypt depth, crypt width, and the number of goblet cells (Table 2).

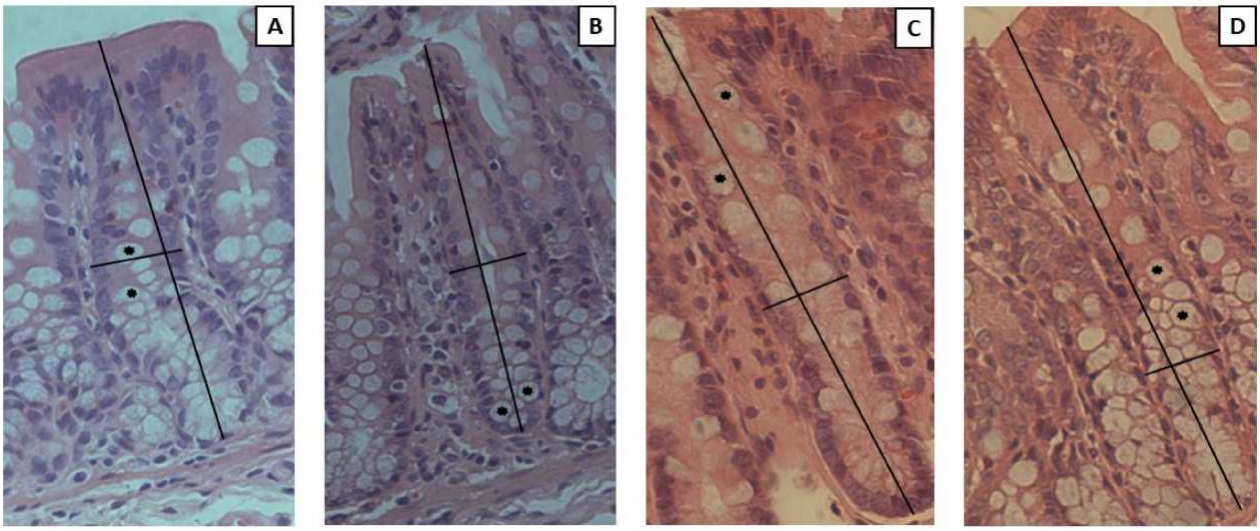

Figure 9. Representative photomicrographs of cecum sections after 10 weeks of treatment. (**A**) AIN- 93M; (**B**) HFHF; (**C**) GTK; (**D**) BTK. Vertical lines indicate crypt depth; horizontal lines indicate crypt width; asterisks indicate goblet cells. All images were captured in a 20X objective. AIN-93M: standard diet (negative control group); HFHF: high-fat and high-fructose diet (positive control group); GTK:HFHF diet + green tea kombucha diluted in water (30% *v*/*v*); BTK: HFHF diet + black tea kombuchadiluted in water (30% *v*/*v*).

4. Discussion

In this study, we evaluated the effects of regular kombucha consumption on the gut microbiota and on the intestinal health of Wistar rats fed a high-fat high-fructose diet. Our results show that both green tea (GTK) and black tea (BTK) kombuchas were able to modulate the gut microbiota, which corroborates our hypothesis.

We believe that our results can be attributed, in large part, to the high content and diversity of phenolic compounds present in kombuchas. In general, only a small portion—approximately 5–10%—of the dietary phenolic compound will be absorbed in the small intestine, mainly those with a monomeric or dimeric structure. The more complex ones—oligomeric and polymeric structures—reach the colon practically unchanged where they are metabolized by the gut microbiota, making them more bioactive [45–47].

Once biotransformed in less complex compounds such as phenolic acids, the generated metabolites and bioactive molecules will modulate the gut microbiota [45,47,48], exerting an effect similar to prebiotics by favoring the growth of beneficial bacteria and inhibiting the growth of pathogenic ones [47,49]. Studies have shown that the antioxidant and anti-inflammatory activities exerted by the polyphenols act against metabolic disorders such as cancer, obesity, and diabetes via modulation of the gut microbiota [24,48,50–52]. There is evidence that metabolic alterations induced by a high-fat diet can also be attenuated by polyphenols intake via activation of PPARα and GLUT4 [53].

Regarding the microbial composition of the kombuchas and their respective SCOBYs,we noticed that the most abundant microorganisms found in the SCOBYs were also found in the beverages, although the diversity was greater in the kombuchas. Interestingly, *Gluconacetobacter* was the predominant genus in both SCOBYs and was much less prevalent in the kombuchas. On the other hand, the genus *Acetobacter* was favored during the fermentation, reaching its maximum abundance in the GTK. Those differences can be explained by the metabolic adaptations of the microorganisms, which are capable of utilizing different substrates depending on the type of tea and consequently will generatedifferent metabolites [17,18,54]. The genus *Acetobacter*, for example, belongs to the group of acetic acid bacteria and has the ability to oxidize ethanol and sugar to acetic acid [55]. Itshigher prevalence in the GTK group is probably responsible for the lower pH observed inthis beverage.

Our results were partially similar to other studies. Recently, it was evaluated, through metagenomics analysis, the microbial diversity of kombuchas whose fermentation time varied between 3 and 15 days. In all analyzed samples, the bacteria belong to eight phyla, and, likewise to our study, *Proteobacteria* was the predominant one, encompass- ing more than 99% of the species. Among the yeasts, the genus *Zygosaccharomyces* was predominant (>99%) [20]. In another study, green and black tea kombuchas producedon an industrial scale presented differences in microbiological composition. Lactic acid bacteria, especially *Oenococcus oeni*, was associated with the fermentation of green tea kombucha, while black tea kombucha showed a greater predominance of acetic bacte- ria. The presence of these bacteria was associated with a higher concentration of lac- tic acid in green tea kombucha and acetic acid in black tea kombucha. Yeast diversity was not influenced by the type of tea; in both kombuchas, the authors observed a pre- dominance of the species *Dekkera bruxellensis*, D. anomala, Hanseniaspora valbyensis, and *Zygosaccharomyces bailli* [56].

The microbial composition of SCOBYs has also been investigated. In a recent study in which 103 samples obtained from commercial kombucha brewers were analyzed, the authors observed that the microorganisms' predominance changed according to their position at the SCOBY's surface. The fungi *Brettanomyces* and the bacteria *Gluconacetobacter*, which have a strong affinity for oxygen, were the main microorganisms found at the upper layer. On the other hand, a greater abundance of *Lactobacillus* was found at the bottom SCOBY side, which corroborates the fact that this genus prefers a low-oxygen environment [21].

Regarding the in vivo results, we should mention that although body composition was not significantly different among the groups, a high-fat and high-fructose diet was able to induce metabolic alterations, as previously reported [15]. As expected, the high-fat content stimulated the growth of gram-negative bacteria such as the phylum *Proteobacteria* and the genus *Bacteroides*, being a biomarker in the HFHF group when compared to the GTK and BTK groups, respectively. Gram-negative bacteria present lipopolysaccharides (LPS) in their cell wall [57], an endotoxin recognized by toll-like receptor 4 (TLR-4) that activates NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and induces the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β [21]. A higher intestinal permeability allows LPS to migrate into the bloodstream, triggering an inflammatory response in the organism [58]. Our previous work demonstrated that the HFHF diet promoted an increase in the levels of inflammatory markers in the liver (TNF-α) and blood (NLR—neutrophil/lymphocyte ratio) as well as reduced the total antioxidant capacity in plasma and liver and increased oxide nitric concentrations, which were reverted in the groups that consumed both kombuchas [25]. A decrease in the *Proteobacteria* and *Bacteroides* abundance noted in the treatment groups suggest that green and black tea kombuchas may present activity against gram-negative bacteria, which may explain the attenuation of the systemic inflammation and oxidative stress markers observed.

The HFHF diet also favored the *Lachnospiraceae* family, which was a biomarker in this group when compared to AIN93-M, but not when compared to both treatments. Although

bacteria that belong to the *Lachnospiraceae* family usually promote a good impact on the gut microbiota by being involved in SCFA production [59,60], it can also be a result of the bile acids' metabolism. The liver is the main organ responsible for lipid metabolism, where the primary bile acids are synthesized from cholesterol [61,62]. The liver–gut axis is not completely understood yet, but it is known that these bile acids can be used as substrates by the microorganisms in the colon and are converted into secondary bile acids, which are pro-inflammatory metabolites involved in steatosis and NAFLD (non-alcoholic fatty liver disease) [62,63]. Studies conducted with humans [64] and mice [65] have pointed out that the main microorganisms involved in this mechanism belong to the *Lachnospiraceae* family, particularly *Blautia* and *L. incertae sedis* [60,66], and elevated taxa of these bacteria in the gut microbiota have been related to liver diseases. Indeed, as reported in our previous work [25], the HFHF diet has induced liver steatosis in the animals, which was reverted from degree 2 to 1 in those treated with both kombuchas.

The genus *Prevotella* and its family *Prevotellaceae* were identified as biomarkers in the AIN -93M group when compared to the other groups, especially HFHF. They belong to the *Bacteroidetes* phylum and are involved in the metabolism of complex carbohydrates and cellulose. De Filippo et al. (2010) compared the gut microbiota of children living in a rural area in Africa who followed a low-fat and high-fiber diet versus children livingin an industrialized city in Europe, whose diet has a high content of fat and protein. The African children presented a higher abundance of the genus *Prevotella* and a higher content of SCFA, which were attributed to a healthier diet [67]. *Erysipelotrichaceae* species are also related to diets rich in carbohydrates and negatively correlated with *Prevotella*. The genera *Dorea*, *Clostridium*, and *p-75-a5*, all belonging to the family *Erysipelotrichaceae*, were reported as biomarkers in the BTK group when compared to the AIN-93M group. The genus *Dorea* has been positively correlated to a Western diet [68] and *p-75-a5* is involved in protein and lipid digestion [69]. Interestingly, when comparing the BTK and HFHF groups, the genus *p-75-a5* and its family *Erysipelotrichaceae* were negatively associated with the BTK group, suggesting that BTK consumption was able to attenuate the negative impact of a HFHF diet. The same biomarkers were not observed in the GTK group, although it also presented a high abundance of the *Erysipelotrichaceae* family.

Considering the treatment groups, we observed that *Actinobacteria* was present in a higher amount in both of them. This phylum is associated with short-chain fatty acids (SCFA) production [70], which are by-products derived from microbial fermentation that are used as an energy source by the enterocytes, favoring intestinal homeostasis and metabolism [70,71]. SCFA also act in the activation of the hormones GLP-1 (glucagon-like peptide 1) and PYY (peptide YY). GLP-1 regulates appetite by inhibiting gastric emptying and stimulating insulin secretion. PYY, in turn, is involved in appetite reduction and gastric motility inhibition. Thus, SCFA act by regulating food consumption and satiety [72], acting on obesity control. Recent studies have suggested that SCFA can modify the epigenome, acting on tissues and organs besides the intestine [71]. Beyond *Actinobacteria*, other microorganisms are pointed out as SCFA producers as those belonging to the *Bifidobacterium* and *Lactobacillus* genera. Both have shown an increased abundance in treatment groups, while *Lactobacillus* was especially higher in the GTK group.

Among SCFA, butyrate is considered the main energy source of colonocytes and enterocytes, thus favoring their growth [73,74]. In our study, butyrate concentrations did not differ significantly among the groups. On the other hand, we observed a higher acetate production in the AIN-93M and HFHF groups, although this last one was not significantly different from the treatments. Since acetate is a metabolite produced especially by bacteria from the *Bacteroidetes* phylum [71], it may explain its elevated abundance in those groups. Finally, we observed a higher concentration of propionate in both treatment groups. This SCFA is produced by a few bacteria, especially those from the genus *Akkermansia*. Phe- nolic compounds can induce changes in microbial composition, favoring the growth of *Akkermansia muciniphila* [75]. Since both kombuchas present high amounts of phenolic compounds [17,76], it may explain the increase in the *Akkermansia* abundance observed in the treatment

groups. The presence of *Akkermansia muciniphila* is associated with a decreasein intestinal permeability and beneficial effects on diabetes mellitus and obesity [77,78].

In both treatment groups, we also observed the presence of *Ocillospira*, a genus involved in glucuronic acid degradation that is positively associated with leanness and health [79]. However, its presence was predominant in the HFHF group, probably because *Ocillospira* can use metabolic products secreted by other microorganisms, including *Bacteroides*. Since a high-fat diet stimulates the growth of *Bacteroides*, it can indirectly favor *Ocillospira* [79].

Finally, when considering the LEfSe analysis, the genus *Adlercreutzia* was observed as a biomarker in the GTK group when compared to both controls. This genus, and more particularly, *Adlercreutzia equolifaciens*, exerts a fundamental role in the metabolism of polyphe- nols in conjunction with other bacteria such as *Flavonifractor plautii, Slackia equolifaciens, Slackia isoflavoniconvertens, Eubacterium ramulus, Eggerthella lenta,* and *Bifidobacterium* spp. [45].

Our study has several strengths. To our knowledge, this is the first that investigated the effects of regular kombucha consumption on the intestinal health of rats fed a high- fat high-fructose diet. Our methodology allowed us to compare both kombuchas and sequencing the beverages and their respective SCOBYs was crucial to analyze if those results reflect the ones found in vivo. The results will help on the understanding of the mechanisms involved in kombucha consumption, and certainly will contribute to filling out the lack of evidence about its impact on intestinal health. As the main limitation, we should mention that the gut microbiota analyses were performed using stool samples from the cecum, which probably has not allowed us to fully explore the results in the same way as if they were collected after undergoing the whole large intestine. The literature is still limited, and the results are controversial, so more studies are necessary to confirm those hypotheses.

5. Conclusions

Our results demonstrated that diets were able to modulate the gut microbiota in different ways. A high-fat high-fructose diet, as expected, was associated with the prevalence of pathobionts, such as *Proteobacteria* and *Bacteroides*. Even though a healthier diet will be always encouraged to prevent and attenuate metabolic disorders, we have noticed that kombucha intake could somewhat modulate the gut microbiota, mitigating the impair- ments provoked by a Western diet by increasing propionate production and favoring the growth of beneficial bacteria, such as *Adlercreutzia* in the GTK group. Thus, we conclude that regular kombucha intake may be beneficial to intestinal health, although more studies, especially clinical trials, are necessary.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14245234/s1. Table S1. Multivariable associations betweenspecific microbial genera, fecal short-chain fatty acids, and phenolic intake.

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CHAPTER 3: CLINICAL TRIAL

7.1 Kombucha Production

The kombucha used in this study was produced at the Laboratory of Bioactive Compounds and Carbohydrates, located in the Department of Food Science and Technology at UFV. The production followed the guidelines established by the Brazilian Ministry of Agriculture, Livestock, and Food Supply (MAPA) (BRASIL, 2019).

Kombucha was prepared using 12 grams of black tea leaves purchased locally (Vivenda Naturalles, Viçosa, MG, Brazil) and 50 grams of sugar per liter of mineral water. The infusion was performed at 95°C for 5 minutes, according to the manufacturer. Then, the beverage was cooled in an ice bath and added to a SCOBY (3% w/v) once it reached room temperature (Enziquímica®, Gravataí, Brazil). A previously prepared kombucha (10% v/v) was also added to the beverages to decrease the pH and inhibit the proliferation of pathogenic microorganisms (JAYABALAN et al., 2014).

Fermentation was carried out in polypropylene buckets kept in a biochemical oxygen demand incubator (BOD) for temperature control (25°C) for 7 days. After fermentation, the SCOBY was removed and kombuchas were transferred to plastic bottles and stored in a refrigerator to be distributed to participants.

Although we adopted the same time and temperature for the infusion, the kombucha used in the clinical trial differed from the black tea kombucha used in the experimental study (Chapter 2) regarding the fermentation time, which was 10 days in the previous one.

7.2 Kombucha Characterization

Total acidity, volatile acidity, and pH

Total acidity was determined by titration with a standard sodium hydroxide solution of 0.01M, using phenolphthalein as an indicator (IAL, 2008). The result was expressed as % acetic acid (w/v). The volatile acidity in acetic acid was determined following steam distillation and volumetric titrimetry, based on sample neutralization with a standardized 1M NaOH solution (IAL, 2008). The result was expressed as milliequivalent of acetic acid per liter of kombucha (meq/L). The pH was determined by a previously calibrated pHmeter (Kasvi, K39-1014B, China).

Acetic acid, sugars, and ethanol

The quantification of acetic acid, sugars (glucose, fructose, and sucrose), and ethanol were analyzed by high-performance liquid chromatography (HPLC) (Shimadzu, model LC-10A VP) coupled to a refractive index detector (RID 6A). Twenty μL of a previously filtered sample (0.45 μm filter) was injected into an HPX-87P column (BIORAD, 30 cm x 4.5 mm). Ultrapure water was used as a mobile phase. The flow was adjusted to 0.6 mL/min and the column temperature was 80°C. Standards of the analyzed compounds were used for identification (retention time) and quantification (external standard). The results were expressed in g/L.

Total phenolics, theaflavin, and thearubigin

The concentration of total phenolics in kombucha samples was determined by the colorimetric method of Follin-Ciocalteu, using gallic acid as a standard (SINGLETON & ROSSI, 1965). The absorbance was measured by a spectrophotometer at 760 nm and the results were expressed in mg of gallic acid equivalent per mL of kombucha (mg GAE/mL).

Theaflavin and thearubigin concentrations were determined by spectrophotometer according to the method proposed by Jayabalan et al. (2007) and the results expressed in % (w/v).

Phenolic Compounds Identification through UPLC-MS^E

Additionally, the phenolic compounds were identified through UPLC-MS^E (Ultra Performance Liquid Chromatography Mass Spectrometry). Kombucha samples were concentrated using an evaporator centrifuge (Savant, SpeedVac, Thermo). The concentrated extracts were then resuspended in a solution consisting of 1 mL of 2% methanol (LC-MS grade), 5% acetonitrile (LC-MS grade), and 93% Milli-Q water. The resulting resuspended extracts were filtered through a hydrophilic PTFE filter (Analytical) with a pore size of 0.22 μm and stored in vials.

A mixture of 33 phenolic compound standards (Sigma Aldrich, Brazil) was prepared by combining them to achieve a final concentration of 10 ppm. The standards included vanillic acid, p-coumaric acid, catechin, caffeic acid, ellagic acid, trans-ferulic acid, kaempferol, myricetin, pyrogallol, flavonone, quercetin, syringic acid, gallic acid, epicatechin, 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 4-hydroxyphenyl acetic acid, sinapinic acid, benzoic acid, quercetin 3-O-glucoside, 3,4-dihydroxyphenyl acetic

acid, epigallocatechin, epigallocatechin gallate, chlorogenic acid, 2,5-dihydroxybenzoic acid, p-anisic acid, 2-hydroxycinnamic acid, vanillin, trans-cinnamic acid, 3-methoxycinnamic acid, 4-methoxycinnamic acid, and L-(−)-3-phenylacetic acid. This standard solution was injected in triplicate before analyzing the samples, using the same parameters described, to ensure instrument reproducibility and serve as a confirmation of the identified phenolic compounds in the samples.

The identification of the phenolic profile was performed using mass spectrometry, following the method described by Santos et al. (2019) with modifications. Two microliters of each sample were injected into a UPLC Acquity system (Waters Co., USA) coupled with Xevo G2S Q-Tof (Waters Co., England) equipped with electrospray ionization (ESI) as the ionization source and quadrupole and time-of-flight (QTof) mass analyzer. Chromatographic separation was achieved using a UPLC HSS T3 C18 column (100mm×2.1 mm, 1.8 μm particle diameter; Waters), maintained at 30 °C, with a flow rate of 0.5 mL/min. The mobile phases used were as follows: mobile phase A (ultra-pure water containing 0.3% formic acid and 5 mM ammonium formate); and mobile phase B (LC-MS-grade acetonitrile containing 0.3% formic acid). The following gradient was applied: 0 min - 97% A, 11.80 min - 50% A, 12.38 min - 15% A, 14.23 min - 15% A, 14.70 min - 97% A.

Data acquisition was performed in MS^E mode using argon as the collision gas; low and high collision energy was applied with a ramp from 25 to 55 V. Acquisitions were conducted in negative and centroid mode within the *m/z* range of 50 to 1000. The ionization conditions included a cone voltage of 30 V, capillary voltage of 3.0 kV, desolvation gas (N2) flow rate of 1,200 L/h at 600 °C, cone gas flow rate of 50 L/h, and source temperature of 150 °C. Leucine enkephalin (Leu-Enk) with m/z 554 and 2615 $[M-H]^-$ was used for lock mass calibration.

Data processing was performed using Progenesis QI software (Waters). Identification of compounds was accomplished by comparing the acquired data with standard runs, utilizing parameters such as the isotope distribution of neutral mass, retention time, and MS/MS fragment spectra. Non-targeted identification was carried out using a database of phenolic compounds built from PubChem, with the aid of MetaScope, an integrated search tool enabling the loading of a custom database. The following parameters were applied in descending order of importance: exact mass (<10 ppm), isotopic similarity (>80%), score (>30), and fragmentation score, all generated by the software. Additionally, parameters from phenol explorer, data from the literature, and chemical characteristics of the molecule were considered as criteria for the possible identification of unknown compounds. Tentative identification and

annotation of metabolites followed the guidelines of the Metabolomics Standard Initiative (SUMNER et al., 2007). Only compounds present in all three technical replicates (3/3) and with a coefficient of variation (CV) of less than 30% were considered tentatively identified.

The processed data were exported to XLSTAT software (Addinsoft, France), where the ion mass spectra-derived abundance values were utilized for relative quantification and statistical evaluation of the data using one-way ANOVA with a post-test Tukey. A significance level of $p < 0.05$ was adopted for the analysis.

Microbiological characterization

Acetic bacteria count was determined through serial dilutions of kombucha samples plated in GYC agar (50 g/L glucose, 10 g/L yeast extract, 5 g/L calcium carbonate, and 20 g/L agar) and ethanol (70 mL/L). Lactic bacteria were counted in MRS agar (De Man, Rogosa & Sharpe; Merck; Germany), using bromocresol (0.004%) as an indicator. Mesophilic bacteria count was performed on PCA agar (Merck, Germany) and yeasts, on PDA agar (Merck, Germany).

The plates were incubated at 30°C for 3 days in aerobiosis, except for the lactic acid bacteria count, which was incubated in a microaerophilic medium. Microorganisms that were gram-positive, catalase-negative, and acid-producers were counted as colonies of lactic acid bacteria. The results were expressed in log colony-forming units per mL (log CFU/mL).

7.3 Subjects

Participants were recruited through advertising on social media and institutional email. Those interested in participating were asked to fill out an online questionnaire for pre-selection with questions about their medical and nutritional history (**APPENDIX A**). A screening was scheduled with people who met the established inclusion criteria. This evaluation was carried out at the Laboratory of Energy Metabolism and Body Composition (LAMECC), located in the Department of Nutrition and Health at Universidade Federal de Viçosa (UFV).

During the screening, a trained nutritionist performed an anthropometric and body composition assessment and evaluated the nutritional, medical, and familiar history of the individuals through a selection questionnaire (**APPENDIX B)**. We included men and women aged 18-45 years old. Normal weight individuals should present the following characteristics: body mass index (BMI) between 18.5 and 24.9 kg/m²; body fat percentage (BF): up to 25% for

men and up to 30% for women; waist circumference (WC) <88 cm for women and <102 cm for men. Obese individuals should present BMI ≥30 kg/m²; BF >27% for men and >34% for women; and WC >88 cm for women and >102 cm for men (WORLD HEALTH ORGANIZATION, 2008).

We did not include individuals with chronic diseases other than obesity, such as diabetes mellitus, cancer, cardiovascular disease; renal or hepatic dysfunction; anemia and/or inflammatory diseases; those who: have the habit of consuming kombucha; make regular use of anti-inflammatory drugs, corticoids or drugs that affect lipid or glucose metabolism; make use of antioxidant and/or vitamin supplements; used antibiotics 3 months before study; have had infectious or allergic episodes in the last month; were on weight loss diets; did not present a stable weight in the last 3 months before the study (±3 kg); have an aversion to kombucha; alcohol intake greater than 105 grams of ethanol/week for women and greater than 210 grams/week for men; smokers; pregnant and lactating women.

Those who met all the inclusion criteria were informed about the objectives, benefits, and potential discomforts of the study. Participants were informed that they could withdraw from the study at any time without any penalties. Written informed consent was obtained from all subjects (**APPENDIX C**).

7.4 Study Design

This is a clinical, chronic study, lasting eight consecutive weeks. Participants were allocated into two groups: normal weight + black tea kombucha; and obese + black tea kombucha. They were instructed to maintain their usual diet and physical activity pattern throughout the study to avoid any biases.

Once a week, participants stopped by the laboratory to pick up seven bottles of black tea kombucha, each one containing 200 mL. The volume of kombucha offered to participants was established following recommendations from the US Centers for Disease Control and Prevention (1995). Participants were asked to keep the bottles refrigerated and consume one per day for eight weeks, preferably during the morning. Those who eventually fail to consume kombucha for more than three consecutive days would be excluded from the study.

Participants were evaluated at the beginning of the study (baseline) and at the end of treatment (8^{th} week). In these two moments, we assessed body composition and collected blood, urine, and stool samples from all participants.

7.5 Sample Size Calculation

The variable malondialdehyde and hydroxynonenal from the study entitled "Green Tea Supplementation Affects Body Weight, Lipids, and Lipid Peroxidation in Obese Subjects with Metabolic Syndrome" (BASU et al. 2010) was used for calculations of sample size. This study was chosen because there were no previous clinical trials with kombucha published. The sample size was calculated on GPower software 3.1.9.7 version.

The sample size was estimated considering an effect size of approximately 1.66 μM (mean difference of green tea versus control treatments = -0.39 and standard error = 0.06; mean difference of green tea extracts versus control = -0.11 and standard error = 0.05), a statistical power of 95%, and two-sided α 5%. The estimated number of participants required in each group to answer our research question is at least 11 participants per group. An additional 30% was included considering eventual dropouts, which resulted in a sample size of 15 participants per group.

7.6 Measurements and Assessments

7.6.1 Weight and Body Composition

Assessment of weight and body composition was performed at LAMECC during the screening and in the 8th week of treatment. Individuals were weighed using a micro digital electronic scale (InBody®, model 230, BiospaceCo), capacity of 150 kg and accuracy of 100 grams, wearing light clothes. The same equipment was used to evaluate the body composition.

The height was determined using a vertical stadiometer fixed to the wall, with a length of 2.2 meters and a scale of 0.5 centimeters (SECA®, model 206, Hamburg, Germany). The evaluations were carried out with the individuals standing, barefoot, in a firm position, with relaxed arms, and the head in the horizontal plane, according to protocol (BRASIL, 2011). The body mass index (BMI) was calculated using the following formula: BMI = weight (kg) ÷ (height)^2 (m).

Waist and hip circumferences were measured using a flexible and inelastic measuring tape, following specific protocols (BRASIL, 2011; WORLD HEALTH ORGANIZATION, 2008). The data were also used to calculate the conicity index from the formula: waist circumference (cm)/0.109 x √weight (kg)/height (m) (VALDEZ et al., 1993). The waist/hip ratio – WHR and waist/height – WHR were also calculated.

7.6.2 Blood Collection and Metabolic Parameters

Blood samples were drawn in the morning after a 12-hour fasting period, at baseline, and at the 8th week of treatment. Half of the blood was added to tubes for serology and the remaining into tubes containing EDTA to obtain plasma. The tubes rested for 20 minutes at 4°C and then the blood was centrifuged at 2200 x g for 15 minutes at 4°C. Plasma and serum were aliquoted in microtubes and stored in an ultra-freezer at -80º C for further analyses.

The samples were analyzed in an auto-analyzer (Mindray/BS-200® Chemistry Analyzer) following the methodology of commercial kits (Quibasa - Química Básica). Serum concentrations of glucose, total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides were measured by enzymatic colorimetric test. Serum concentration of glycated hemoglobin (HbA1c) was measured by immunoturbidimetric method. Serum concentrations of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by a UV kinetic method. Serum insulin concentration was measured by electrochemiluminescence immunoassay (ECLIA). All analyses were performed at the Clinical Analysis Laboratory (LAC) at the Department of Nutrition and Health at UFV, except insulin, which was analyzed by a third party.

Insulin resistance was assessed by the Homeostasis Model Assessment (HOMA-IR) (MATTHEWS et al., 1985) and by the TyG index (Triglycerides-Glycemia) (GUERRERO-ROMERO et al., 2016) using the following formulas:

$$\text{HOMA-IR} = [(\text{blood glucose in mg/dL}) \times (\text{insulin in } \mu\text{U/mL})/405]$$

$$\text{TyG} = \text{Ln}\,[(\text{fasting triglycerides in mg/dL}) \times (\text{fasting blood glucose in mg/dL})/2]$$

where Ln is the neperian logarithm.

Insulin sensitivity was assessed by HOMA-beta (MATTHEWS et al., 1985) and by the QUICKI index (Quantitative Insulin Sensitivity Check Index) (KATZ et al., 2000) using the formulas:

$$\text{HOMA-beta} = 360 \times (\text{fasting insulin [IU/L]} / (\text{fasting glucose [mg/dL]} - 63))$$

$$\text{QUICKI} = 1 / (\log(\text{fasting insulin [IU/L]}) + \log(\text{fasting glucose [mg/dL]}))$$

7.6.3 Inflammatory Markers

Complement C3 was evaluated by turbidimetric method and high-sensitivity C-reactive protein (hs-CRP), by immunoturbidimetric method. Both analyses were performed in serum samples by an autoanalyzer (Mindray/BS-200® Chemistry Analyzer) following the manufacturer's methodology (Quibasa - Química Básica). This analysis was performed at LAC/UFV.

Pro- and anti-inflammatory markers were measured on plasma, in duplicate, using the V-PLEX Proinflammatory Panel 1 Human Kit (K15049D-1, Meso Scale Diagnostics, LLC, Rockville, MD, USA), including interferon-gamma (IFN-$^{\gamma}$), interleukins (IL)-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-1B, IL-12p70, and tumor necrosis factor-alpha (TNF-α). All assays were conducted according to the manufacturer's protocol. The analysis was performed at Rush University, USA.

7.6.4 Oxidative Stress Markers

The superoxide dismutase (SOD) activity was determined in triplicate on plasma samples. The method is based on the ability of this enzyme to catalyze the reaction of superoxide (O_2-) in molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) and thus reduce the pyrogallol oxidation rate (MARKLUND & MARKLUND, 1974). For the determination of enzymatic activity, 30 µL of plasma was added to microplates, followed by the addition of 95 µL of phosphate buffer, 10 µL of MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium) and 15 µL of pyrogallol. Then, the plate was incubated for 5 minutes at 37˚C. After that, the reaction was stopped by adding 150 µL of DMSO (dimethylsulfoxide). The plates were read in a spectrophotometer (Multiskan™ FC Microplate Photometer) at 570 nm and the values were expressed in U of SOD/mL plasma (DIETERISH et al., 2000).

Plasma malondialdehyde (MDA) dosage was performed in duplicate according to the method described by Buege & Aust (1978) with modifications. This method is based on the measurement of reactive species to thiobarbituric acid (TBARS), whose product is malondialdehyde (MDA). TBARS solution was prepared with 15% trichloroacetic acid (TCA), 0.375% thiobarbituric acid (TBA), and 0.25 M hydrochloric acid (HCl) diluted in distilled water in equal proportions. Then, 400 µL of TBARS solution was added to 200 µL of plasma in a microtube. The microtubes were vortexed for 10 seconds and then placed in a water bath for 40 minutes at 90°C. Following this, they were cooled in an ice bath for 5 minutes. Once reached room temperature, 600 µL of N-butanol was added to the microtubes and the mixture was

vortexed for 1 minute. Then, the samples were centrifuged for 5 minutes at 3000 rpm at 4°C. The supernatant was transferred to microplates to determine the amount of MDA in the samples. Readings were performed in a spectrophotometer (Multiskan™ FC Microplate Photometer) at 535 nm. The MDA concentration in the samples was determined by using a TMPO curve (1,1,3,3-Tetramethoxypropane) as standard. Final values were expressed in µmol/L.

The nitric oxide (NO) dosage method is based on the detection of nitrite used as an indicator of Nitric Oxide (NO) synthesis by the Griess Reagent (GRISHAM et al., 1996). For that, 50 µL of plasma and 100 µL of standard solution (Griess Reagent) were added to microplates and incubated for 10 minutes at room temperature. Readings were performed in a spectrophotometer (Multiskan™ FC Microplate Photometer) at 570 nm. Analyzes were performed in duplicate and final values were expressed in µmol/L.

The Ferric Reducing Ability of Plasma (FRAP) dosage was performed in duplicate to measure the antioxidant power following the protocol described by Benzie & Strain (1996). The method consists of adding 10 µL of plasma to 300 µL of FRAP solution (25 mL of acetate buffer; 2.5 mL of ferric tripyridyltriazine solution and 2.5 mL of iron trichloride solution). The microplates were incubated at 37°C for 4 minutes. The readings were performed in a spectrophotometer (Multiskan™ FC Microplate Photometer) at 595 nm and the results were expressed in µM per mL of plasma.

7.6.5 Barrier Function Proteins

Zonulin (a marker of intestinal barrier integrity; MBS706368, MyBioSource) and lipopolysaccharide-binding protein (LBP, a marker of bacterial translocation; HK315-01, Hycult Biotech) were evaluated by ELISA. Both analyses were performed using plasma, in duplicate, following the manufacturer's instructions, and were conducted at Rush University (USA).

7.6.6 Subcutaneous Adipose Tissue

Material collection

The collection of subcutaneous adipose tissue (SAT) was restricted to obese individuals and carried out by a surgeon. Samples were collected at baseline and in the 8th week of treatment, through needle aspiration biopsy. On the scheduled days, participants attended the procedure after 8 hours of fasting. The skin was sterilized with ethanol (70% v/v) before needle

insertion and then 5 mL of xylocaine (2% v/v) was applied as a local anesthetic. The surgeon then introduced a biopsy needle (Becton Dickinson & Company, Switzerland) coupled to an automatic trigger (Becton Dickinson & Company, Switzerland) into the participant's periumbilical region, making between 1 and 2 shots to take the sample.

The collected tissue was placed in a sterile recipient and washed with saline solution to remove blood residue. Then, the sample was immediately transferred to microtubes containing an RNA protective solution (RNAlater™ Stabilization Solution, Invitrogen®) for subsequent gene expression analyses. The microtubes were placed in an ice box and then transferred to an ultra-freezer where they were stored at -80°C until analysis.

RNA Extraction and Analysis

RNA extraction was performed with the Maxwell RSC microRNA Tissue Kit (Promega, AS1460). cDNA was then synthesized with the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, 4368813) and pre-amplified with the TaqMan PreAmp Master Mix (ThermoFisher, 4391128) for 14 cycles before performing real-time polymerase chain reaction (qPCR) with UltraPlex 1-Step ToughMix, Low ROX (QuantaBio, 95168).

The primers used for qPCR are described in Table 1. Quantification of gene expression was determined by the 2-ΔΔCt method and normalized by the mean expression of β-actin and PGK1 genes (TaqMan™ SNP Genotyping Assays, ThermoFisher). The analyses were performed in triplicate.

Table 1. Primers used for qPCR.

Gene	Commercial Name	Assay ID
ACTB	ACTB	Hs99999903_m1
SREBP-1c	SREBF1	Hs00231674_m1
PPARγ	PPARG	Hs01115513_m1
NF-κB	NFKB1	Hs00765730_m1
GLUT4	SLC2A4	Hs00168966_m1
IRS-1	IRS1	Hs00178563_m1
PGC-1α	PPARGC1A	Hs00173304_m1
PAI-1	SERPINE1	Hs00167155_m1
TNF	TNF	Hs00174128_m1
Leptin	LEP	Hs00174877_m1
Adiponectin	ADIPOQ	Hs00977214_m1
PGK1	PGK1	Hs99999906_m1
PPIA	PPIA	Hs99999904_m1

Abbreviations: ACTB: Beta-Actin; SREBP-1c: Sterol Regulatory Element-Binding Protein 1c; PPARγ: Peroxisome Proliferator-Activated Receptor Gamma; NF-κB: Nuclear Factor Kappa B; GLUT4: Glucose Transporter 4; IRS-1: Insulin Receptor Substrate 1; PGC-1α: Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha; PAI-1: Plasminogen Activator Inhibitor-1; TNF: Tumor Necrosis Factor; PGK1: Phosphoglycerate Kinase 1; PPIA: Peptidylprolyl Isomerase A.

7.6.7 Intestinal Permeability

Intestinal permeability was also assessed at baseline and the 8th week of treatment. The test consists of the administration of non-metabolizable substances of different molecular weights quantified in the urine.

On both test days, participants attended the LAMECC after an 8-hour overnight fast. Then, they ingested a solution containing lactulose (10 g) and mannitol (5 g) within 10 minutes.

Participants remained in the laboratory for 4 hours, and all urine was collected in a container during this period. No food or beverage was allowed in the first 60 minutes. Then, the participants were required to drink 150 mL water at 2 and 3 hours of testing.

Part of the collected urine was added with Thimerosal (0.23 g/L) and stored in an ultra-freezer (Thermo Scientific/Forma 900 Series®) at -80°C until the analysis.

For that, 10 µL of urine was filtered through 0.22 µm membrane filters (Millipore, São Paulo, Brazil) and transferred to vials for High-Performance Liquid Chromatography (HPLC) (SHIMADZU) on the following conditions: pump (LC-20AT); oven (CTO 20A); autoinjector (SIL-20A HT), RID detector - 10 A; degasser (DGU-20A 5R); HPX87N column (BIO-RAD;

mobile phase: ultrapure water, oven temperature: 70ºC; flow rate: 0.6 mL/min). Lactulose® and Mannitol® (Sigma-Aldrich, São Paulo, Brazil) were used as internal standards and the concentrations were transformed into g/L to calculate the percentage of urinary excretion. Mannitol was calculated by dividing the lactulose concentration by the mannitol concentration (SONG et al., 2011).

7.6.8 Gut Microbiota

Fecal Collection

Stools were collected at baseline and the 8th week of treatment. Participants were instructed to collect the samples as aseptically as possible, preferably in the morning, using a sterile container wrapped in aluminum foil to preserve the fatty acids. The samples were transported in an ice box to LAMECC. Once received, the samples were placed in microtubes and stored in an ultra-freezer at -80°C until analysis.

Short-Chain Fatty Acids Quantification

For the quantification of short-chain fatty acids (SCFA), the stool samples were weighed and homogenized in distilled water at a 1:10 ratio. The internal standard was prepared by mixing 157.5 µL of 4-methylvaleric acid ($C_6H_{12}O_2$), 1.47 mL of phosphoric acid (H_3PO_4) (85% v/v), and 39 mg of copper sulfate pentahydrate (CuSO4.5H2O) diluted in 25 µL of distilled water.

Stool samples (1:10) were centrifuged at 13000 rpm at 4°C for 10 minutes. Then, 400 µl of supernatant and 100 µl of the internal standard were homogenized and added to vials for gas chromatography (GC).

GC analysis was performed using a NukolTM column 30m x 0.25mm I.D., phase 0.25 µm (Cat # 24107) under the following conditions: injection: 4 µL; oven: 100°C-200°C at 8°C/min; carrier: helium at 0.75mL/min. Acetate (0.6M), propionate (0.15M), and butyrate (0.15M) (Sigma Aldrich, Germany) were used as external standards and the standard curve was used for quantification.

DNA Extraction and Sequencing

Stool samples were used for DNA extraction following the manufacturer's methodology (QIAamp® PowerFecal® Pro DNA Kit, Qiagen, USA).

For the analyses, approximately 300 mg of feces were solubilized in 700-800 µL of CD1 buffer solution (QIAamp® PowerFecal® Pro DNA Kit, Qiagen, USA). Then, the whole content

was transferred to microtubes containing microspheres (PowerBead Pro Tubes®, Qiagen, USA) and centrifuged 2 times at room temperature for 40 seconds, at intervals of 5 minutes between each cycle (FastPrep-24TM, MP Biomedicals, USA). This step precedes DNA extraction and aims to promote cell lysis.

Then, the microtubes were centrifuged for 1 minute at 15000 g at room temperature (Microfuge® 20R, Beckman Coulter, Germany). Six hundred µL of supernatant were transferred to a new microtube and followed for DNA extraction in an autoanalyzer following the manufacturer's protocol (QIAcube Connect, Qiagen, USA).

The amount of DNA obtained from the extraction was measured by a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Sample purity was estimated by the 260/280 and 260/230 nm ratios. Values between 1.8-2.0 and 2.0-2.2, respectively, were considered desirable.

The genetic material was kept in an ultra-freezer at -80°C until sequencing by a third-party company (Genome Research Center, University of Illinois at Chicago, USA). DNA sequencing was performed considering the bacterial regions V3 and V4 of the 16S rRNA gene (primers 341F/806R) and the internal spacers transcribed from the nuclear ribosomal DNA (ITS1 and ITS2) of the fungal rRNA region (primers ITS1F and ITS2R).

7.6.9 Eating Behavior Assessment

The eating behavior was evaluated during screening by evaluating if participants presented any signals of food restriction (conscious control of food intake), food disinhibition (excessive ingestion due to interruption of cognitive control), or hunger (constant desire to eat). For this, the participants answered the Three Factor Eating Questionnaire - R21, translated and validated for the Brazilian population by Natacci & Ferreira Júnior (2011) (**Annex 1**). The objective was to identify, through the questionnaire, whether the individual had any extreme behavior – compulsion or restriction – regarding their diet.

Furthermore, during the screening, a Food Frequency Questionnaire (FFQ) validated for the Brazilian population (SELEM et al., 2014) was applied to assess the individuals' eating habits (**Annex 2**). For each FFQ item, participants should inform the average frequency of habitual consumption (number of times) and the unit of time (daily, weekly, monthly, or annual) related to the last year, in addition to the size of the portion ingested (small, medium, large, or extra-large), which were later converted into quantity (grams) of food consumed per day.

Throughout the study, the food intake assessment was performed using food records, which were filled out by the participants. They were instructed by a nutritionist on how to complete food records properly and these were jointly reviewed to ensure accuracy. By the end of the study, they should fill out eight food records in total (1 per week); two of them on weekends (**Appendix E**).

7.6.10 Physical Activity Assessment

The physical activity pattern of the participants was evaluated during screening using the short version of the International Physical Activity Questionnaire (IPAQ), which was proposed in 1998 by the World Health Organization and validated for the Brazilian population (MATSUDO et al., 2001) (**Annex 3**). Through the IPAQ, it was possible to classify the participant as sedentary, irregularly active A, irregularly active B, active, or very active. Participants were instructed to maintain the same pattern of physical activity throughout the study to avoid any biases.

7.7 Adherence to the Study

Participants were instructed to fill out a form containing information about their daily kombucha intake (**Appendix E**). A failure to have kombucha intake for more than three consecutive days would result in exclusion from the study.

7.8 Ethical Aspects

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and the procedures were approved by the National Research Ethics Committee – CONEP/Brazil (registration no. 3.948.033) (**Annex 4**). The study was also registered on the Brazilian Clinical Trial Registry (ReBEC), available at <https://ensaiosclinicos.gov.br/rg/RBR-9832wsx> (UTN code U1111-1263-9550).

7.9 Feedback to Participants

All potential participants who were screened for the study received a body composition assessment. At the end of the study, the participants received the blood test results by e-mail. A 30-day nutritional service was also offered for all the participants who finished the study. They

received guidance on healthy eating habits, and, in the case of obese individuals, an individualized meal plan aimed at weight loss and health promotion.

7.10 Statistical Analyses

The Shapiro-Wilk test was performed to verify the normality of continuous variables. For parametric data, the paired t-test was applied to compare differences between treatment and baseline for the same individual. Non-parametric data was evaluated through the Wilcoxon test. Analyzes were performed using the GraphPad Prism® software (GraphPad Software Inc., version 6, USA). Data are expressed as mean ± SEM. A statistical significance criterion (α) of 0.05 was adopted for all analyses.

Based on the data generated by sequencing, a metagenomic study was carried out to analyze the structure of the microbial community (species richness and distribution) and its metabolic potential. Significant differences in alpha diversity between the four groups (normal weight T0 and T8; obese T0 and T8) were determined using the alpha function in the R microbiome package v.2.1.24 adopting Kruskal-Wallis as a statistical test followed by the Wilcoxon test to calculate paired comparisons between groups. For beta diversity, weighted and unweighted UniFrac distances were subjected to PERMANOVA (Permutational Multivariate Analysis of Variance) analysis to assess significant differences (pseudo-F-test) in bacterial community composition and structure between groups with a permutation number of 999. Principal coordinate analysis (PCoA) was chosen to explore and visualize the clustering of groups. All graphs were constructed and visualized by RStudio software (v. 1.2.5033) using one or a combination of the following R packages: MicrobiomeR, dplyr, ggplot2, phyloseq (MCMURDIE; HOLMES, 2013), alignr, and vegan.

To determine which bacterial taxa were significantly different between groups in terms of abundance, the taxonomy was first reduced to the genus level and then analyzed using LEfSe (Linear discriminant analysis Effect Size) (SEGATA et al., 2011), using a p-value of 0.05 and an LDA log score of 2.0.

7.11 RESULTS

7.11.1 Kombucha Characterization

The chemical characterization of the kombucha used in this study is shown in **Table 2**. The results meet the parameters established by the Brazilian Ministry of Agriculture, Livestock,

and Food Supply (MAPA) which establishes that the pH should be between 2.5 and 4.0, and the volatile acidity, 30 to 130 mEq/L (BRASIL, 2019).

Since no additional ingredients were added to the kombucha beyond the mandatory ones – tea, sugar, and SCOBY – it is classified as "original kombucha". According to the same criteria established by MAPA, it is a non-alcoholic beverage, since the ethanol content did not exceed 0.5% v/v (BRASIL, 2019).

As expected, we identified yeasts, acetic, and lactic bacteria in kombucha, which are responsible for fermenting the tea (**Table 2**). As a result, a low pH and an increased acidity add to kombucha an acidic and slightly sweet taste, which is from the residual sugar. The ethanol, although in a low concentration, is also produced during the fermentation process.

Table 2. Black tea kombucha characterization.

Parameters	**Mean ± SD**
pH	3.48 ± 0.05
Volatile acidity (meq/L)	30.00 ± 3.75
Total acidity (% acetic acid - g/100mL)	0.31 ± 0.009
Acetic acid (g/L)	0.99 ± 0.01
Total phenolics (mg/mL)	0.69 ± 0.02
Theaflavin (g/100mL)	0.12 ± 0.003
Thearubigin (g/100mL)	1.88 ± 0.07
Ethanol (g/L)	4.53 ± 0.06
Sucrose (g/L)	13.22 ± 0.22
Glucose (g/L)	4.24 ± 0.07
Fructose (g/L)	8.54 ± 0.18
Yeasts (log CFU/mL)	6.10 ± 0.21
Acetic bacteria (log CFU/mL)	5.80 ± 0.28
Lactic bacteria (log CFU/mL)	6.20 ± 0.14

The total phenolics, theaflavin, and thearubigin derive from the black tea used in kombucha production and are increased during the fermentation process. The presence of theaflavins and thearubigins has been pointed out as the reason why black tea kombucha has a greater phenolic compound diversity when compared to green tea kombucha. During the fermentation process, these compounds undergo biotransformation or degradation facilitated by enzymatic action and low pH. This process leads to the formation of several lower molecular weight phenolic compounds, thereby increasing its overall diversity in black tea kombucha (CARDOSO et al., 2020; DE NORONHA et al., 2022).

7.11.2 Phenolic Compounds Profile

Overall, 145 phenolic compounds were identified through UPLC-MS^E. The majority belongs to flavonoids (81%) and phenolic acids (19%). Lignans, stilbenes, and other polyphenols represent 1% of the total. This result is like our previous study in terms of phenolic compounds profile (127 phenolic compounds: 70.2% belong to flavonoid class, 18.3% phenolic acids, 8.4% other polyphenols, 2.3% lignans and 0.8% stilbenes) (CARDOSO et al., 2020).

Flavonoids, phenolic acids, and other polyphenols were also subdivided into classes, as represented in **Figure 1**. Flavonols, hydroxybenzoic acids, and hydroxycoumarins were the most abundant subclasses identified in those groups, respectively.

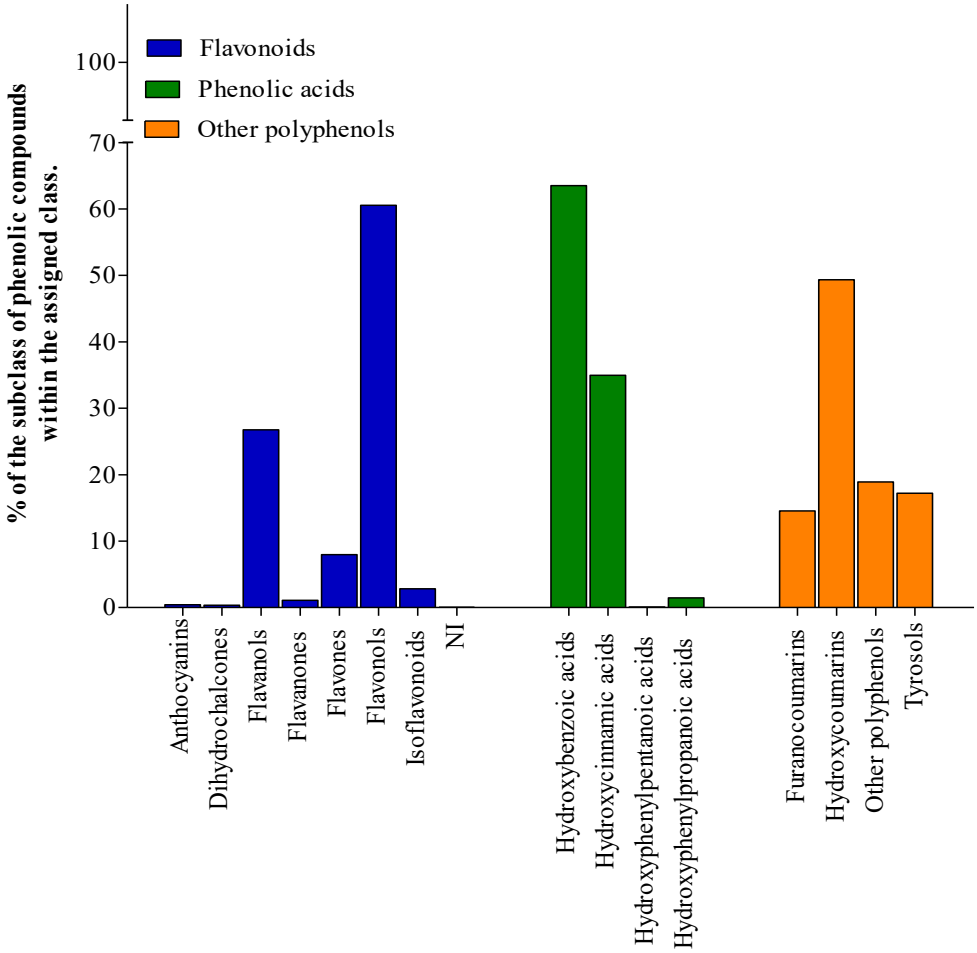


Figure 1. Subclasses of flavonoids, phenolic acids, and other polyphenols identified in black tea kombucha.

Regarding the 10 most prevalent phenolic compounds in the samples, 80% of them belong to the flavonoids and 20% belong to the phenolic acids class (**Table 3**). Among them, five were also identified in our previous work as the most abundant: epigallocatechin 3-O-

gallate; catechin 5-O-gallate; 5-O-galloylquinic acid; quercetin 3-O-glucosyl-rhamnosyl-galactoside; and 4-p-coumaroylquinic acid (CARDOSO et al., 2020, 2021).

Table 3. Top 10 phenolic compounds identified on black tea kombucha, from the most to the least abundant.

Compound Name	Molecular formula	*m/z*	RT (min)	Score (%)	FS (%)	Error (ppm)	IS (%)	Class	Abundance
Quercetin 3-O-rutinoside	**$C_{27}H_{30}O_{16}$**	**609.1454**	**8.09**	**48.5**	**44.7**	**-1.12**	**98.90**	**F**	**1.05E+07**
Quercetin 3-O-glucosyl-rhamnosyl-galactoside	$C_{33}H_{40}O_{21}$	771.1984	7.87	45.5	35.9	-0.70	92.72	F	6.74E+06
(+)-catechin 5-gallate	$C_{22}H_{18}O_{10}$	441.0808	8.23	44.8	31.9	-4.28	97.30	F	5.61E+06
Quercetin 3-O-glucoside	**$C_{21}H_{20}O_{12}$**	**463.0864**	**8.32**	**55.0**	**80.2**	**-3.88**	**99.27**	**F**	**5.00E+06**
Kaempferol 3-O-rutinoside	$C_{27}H_{30}O_{15}$	593.1485	8.57	47.3	44.1	-4.52	97.50	F	4.71E+06
(-)-epigallocatechin 3-O-gallate	$C_{22}H_{18}O_{11}$	457.0773	7.08	48.8	46.9	-0.82	98.34	F	4.01E+06
5-O-galloylquinic acid	$C_{14}H_{16}O_{10}$	343.0664	2.19	49.8	52.4	-1.95	98.84	PA	3.77E+06
Kaempferol 3-O-glucoside	$C_{21}H_{20}O_{11}$	447.0914	8.78	47.2	44.5	-4.11	96.43	F	2.68E+06
4-p-coumaroylquinic acid	$C_{16}H_{18}O_{8}$	337.0921	6.92	48.8	48.0	-2.35	98.92	PA	2.63E+06
Quercetin	**$C_{15}H_{10}O_{7}$**	**301.0340**	**10.23**	**53.7**	**75.2**	**-4.60**	**98.70**	**F**	**2.45E+06**

Abbreviations: m/z: mass/charge; RT: retention time; FS: fragmentation score; IS: isotope similarity; PA: phenolic acids; F: flavonoids. Bold compounds were confirmed through the mix of standards used in the analysis.

7.11.3 Participants

One hundred and ninety-three subjects answered the online pre-selection questionnaire. Among the 62 subjects who were screened at LAMECC, 46 met the established selection criteria and were allocated to the normal weight (n = 23) or obese (n = 23) group. Thirty-eight participants completed the study: 21 from the normal weight group and 17 from the obese group (**Figure 2**). Among them, 25 are female and 13 are male.

Participants in the normal weight group had a mean age of 26.5 years and a BMI of 21.64 kg/m² at baseline, while in the obese group, these values were 35.3 years and 34.47 kg/m², respectively.

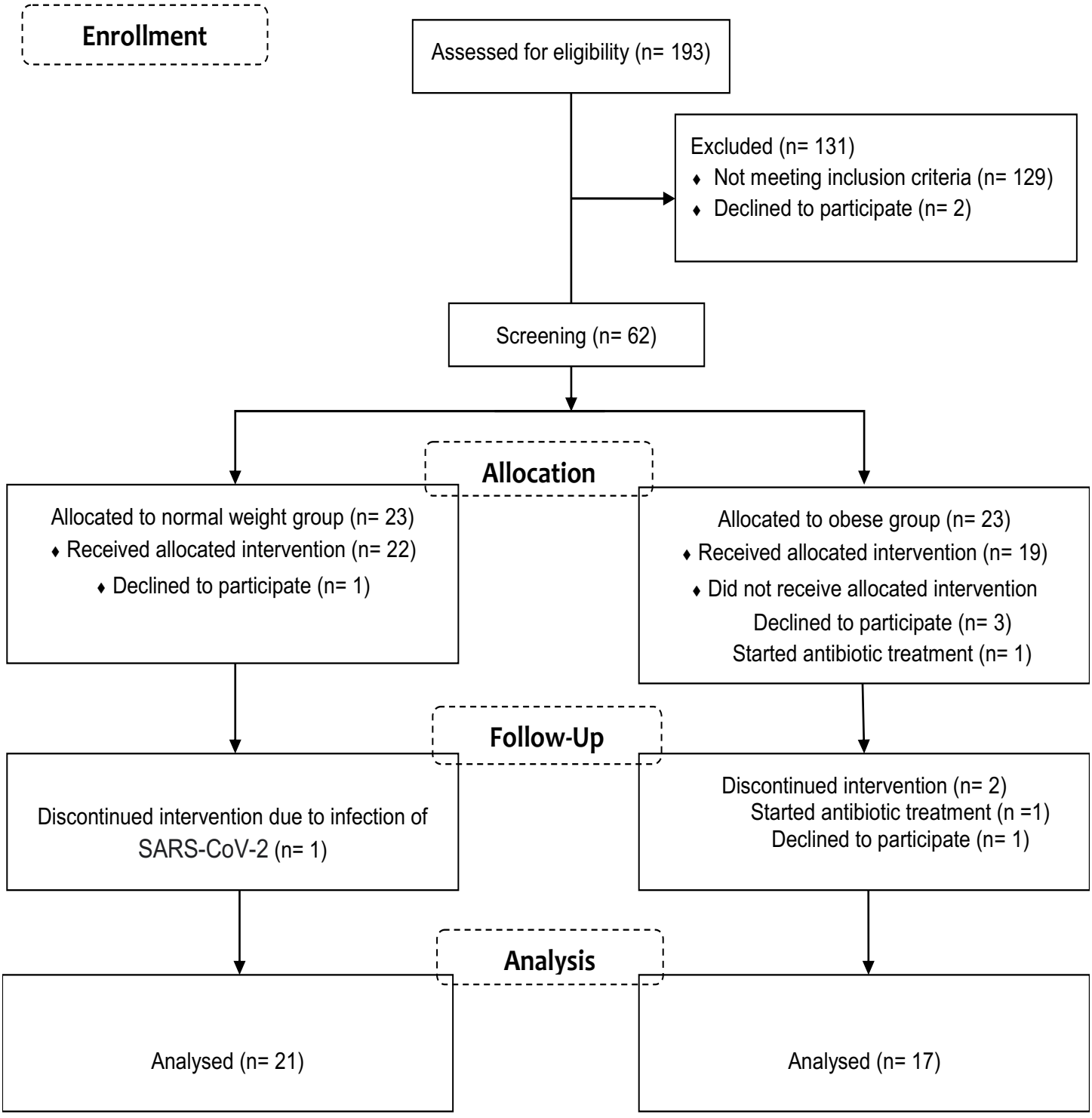


Figure 2. Participant selection flowchart.

No participants were excluded for non-adherence to the study, as assessed by the daily kombucha consumption registry (**Appendix F**). The dietary patterns and physical activity, assessed by dietary records and IPAQ, respectively, show no significant difference in the groups before and after the intervention (data not shown). As expected, no difference was noted regarding food consumption among the groups at the baseline and after 8 weeks of intervention, evaluated through the Food Frequency Questionnaire (FFQ) (**Table 4**).

Table 4. Energy and nutrient ingestion of participants, before and after intervention, evaluated through the Food Frequency Questionnaire (FFQ).

Consumption	Total (n=36)		Normal weight (n=20)		Obesity (n=16)		Baseline p-value	Between groups p-value
	Baseline	8th week	Baseline	8th week	Baseline	8th week		
Energy (kcal)	1985.52 ± 149.35	1879.15 ± 141.58	1804.25 ± 158.12[a]	1632.50 ± 122.22[b]	2212.11 ± 267.14	2187.45 ± 154.91	0.178	0.495
CHO (g)	230.45 ± 16.78	218.73 ± 18.70	207.72 ± 17.76	191.13 ± 15.42	258.86 ± 29.70	253.23 ± 36.30	0.132	0.672
Proteins (g)	83.00 ± 6.90	78.52 ± 6.55	76.00 ± 8.22a	67.52 ± 6.56[b]	91.75 ± 11.58	91.44 ± 10.26	0.263	0.425
Total fats (g)	78.52 ± 6.55	75.21 ± 5.53	72.24 ± 6.92[a]	64.56 ± 5.19[b]	86.38 ± 11.94	88.51 ± 9.85	0.291	0.341
TC (mg)	403.09 ± 39.16[a]	337.71 ± 31.87[b]	366.93 ± 49.08[a]	282.91 ± 31.13[b]	448.29 ± 63.17	406.20 ± 56.89	0.309	0.498
Fibers (g)	19.32 ± 1.14	18.22 ± 1.59	18.20 ± 1.38	16.45 ± 1.38	20.73 ± 1.90	20.44 ± 3.12	0.279	0.487

Values presented as mean ± SEM. Different letters on the same line indicate statistical difference within group according to Paired t-test ($p < 0.05$). Baseline and between groups p-value was obtained through Student t-test.

7.11.4 Effect of Black Tea Kombucha Consumption on Anthropometry and Adiposity

The anthropometric characteristics of the participants at baseline and after the intervention with kombucha are presented in **Table 5**. In the normal weight group, we observed a higher hip circumference after kombucha treatment. No other significant differences were observed among the groups regarding body weight, BMI, waist circumference, body fat, waist-hip ratio, waist-to-height ratio, and conicity index.

Table 5. Anthropometric characteristics of participants at baseline and after 8 weeks of intervention with black tea kombucha.

Variables	Total (n=36)		Normal weight (n=20)		Obesity (n=16)		Baseline p-value	Between groups p-value
	Baseline	8th week	Baseline	8th week	Baseline	8th week		
Body weight (kg)	78.98 ± 4.19	78.98 ± 4.21	60.31 ± 2.13	60.69 ± 2.24	102.33 ± 4.43	101.85 ± 4.76	**≤0.001**	**0.009**
BMI (kg/m^2)	28.67 ± 1.46	28.63 ± 1.45	21.64 ± 0.40	21.76 ± 0.41	37.47 ± 1.34	37.24 ± 1.41	**≤0.001**	0.123
Waist circumference (cm)	93.68 ± 4.02	93.77 ± 4.00	74.10 ± 1.47	74.53 ± 1.48	118.15 ± 3.13	117.84 ± 3.31	**≤0.001**	0.821
Hip circumference (cm)	106.45 ± 2.81	107.29 ± 2.79	93.78 ± 1.26^b	94.98 ± 1.26^a	122.31 ± 2.97	122.69 ± 3.17	**≤0.001**	0.524
Body fat (%)	32.66 ± 2.29	32.79 ± 2.28	21.95 ± 1.30	22.53 ± 1.53	46.06 ± 1.82	45.63 ± 1.96	**≤0.001**	0.116
Waist-hip ratio	0.87 ± 0.01	0.86 ± 0.01	0.79 ± 0.01	0.78 ± 0.01	0.96 ± 0.01	0.96 ± 0.01	**≤0.001**	0.097
Waist-to-height ratio	0.56 ± 0.02	0.56 ± 0.02	0.44 ± 0.007	0.44 ± .006	0.71 ± 0.01	0.71 ± 0.01	**≤0.001**	0.722
Conicity index	1.24 ± 0.02	1.24 ± 0.02	1.13 ± 0.01	1.13 ± 0.01	1.37 ± 0.01	1.37 ± 0.01	**≤0.001**	**0.030**

Values are presented as mean ± SEM. Different letters on the same line indicate statistical differences within the group according to the Paired t-test ($p < 0.05$). "Baseline p-value" was obtained through the student t-test. "Between groups p-value" was obtained through ANCOVA analysis adjusted by the baseline valor of the variable.

7.11.5 Effect of Black Tea Kombucha Consumption on Metabolic Markers

Regarding the metabolic markers, we observed that after 8 weeks of treatment with black tea kombucha, the normal weight group showed an increase in total cholesterol and

alkaline phosphatase concentrations, while the obese group showed a decrease in insulin and GGT concentrations. The obese group also presented an improvement in insulin resistance and sensitivity, as evaluated by HOMA-IR, HOMA-β, and QUICKI index, which reinforces the result.

No differences were observed in both groups regarding glucose, HDL-c, LDL-c, triglycerides, alanine aminotransferase, aspartate aminotransferase, creatinine, urea, and TyG index after treatment with kombucha (**Table 6**).

Table 6. Serum concentrations of metabolic markers at baseline and after 8 weeks of intervention with black tea kombucha.

	Normal weight (n=20)			**Obesity (n=16)**		
	Baseline	8th week	*p*-value	*Baseline*	8th week	*p*-value
Glucose (mg/dL)	88.5 ± 6.1	87.0 ± 5.7	0.372	91.0 ± 8.3	94.3 ± 9.7	0.340
Insulin (IU/L)	5.4 ± 1.6	6.0 ± 1.9	0.398	15.6 ± 6.0	12.4 ± 4.6	**0.002**
Total cholesterol (mg/dL)	166.4 ± 32.9	177.1 ± 34.8	**0.023**	189.9 ± 25.4	194.4 ± 22.1	0.201
HDL-c (mg/dL)	60.3 ± 13.8	61.6 ± 12.6	0.517	53.2 ± 15.7	48.4 ± 12.5	0.060*
LDL-c (mg/dL)	86.4 ± 26.6	90.8 ± 24.4	0.529	104.2 ± 18.2	106.3 ± 17.3	0.636
TG (mg/dL)	71.9 ± 29.9	85.1 ± 43.0	0.135*	149.6 ± 87.8	121.8 ± 67.2	0.103*
ALT (U/L)	17.0 ± 6.8	16.3 ± 6.5	0.605	20.1 ± 10.0	19.0 ± 7.5	0.396
AST (U/L)	14.8 ± 3.2	15.5 ± 4.4	0.285	16.7 ± 5.8	15.6 ± 6.4	0.082
GGT (U/L)	15.8 ± 7.2	16.7 ± 7.2	0.451*	24.6 ± 13.4	23.5 ± 14.9	**0.042***
Alkaline phosphatase (U/L)	71.1 ± 18.0	76.7 ± 15.9	**0.044**	82.4 ± 16.0	83.0 ± 21.1	0.891
Creatinine(mg/dL)	0.94 ± 0.09	0.92 ± 0.13	0.487	0.93 ± 0.14	0.94 ± 0.16	0.720
Urea (mg/dL)	24.3 ± 7.1	23.4 ± 5.3	0.772*	23.3 ± 4.9	23.5 ± 6.5	0.892
HOMA-IR	1.2 ± 0.4	1.3 ± 0.4	0.591	3.6 ± 1.6	2.7 ± 0.6	**0.016**
HOMA-β	79.4 ± 25.9	94.1 ± 34.2	0.164	220.2 ± 98.0	145.7 ± 75.7	**0.002***
QUICKI Index	3.3 ± 0.2	3.2 ± 0.2	0.745	2.8 ± 0.1	2.9 ± 0.1	**0.020***
TyG Index	4.3 ± 0.2	4.4 ± 0.2	0.235	4.6 ± 0.3	4.6 ± 0.2	0.469

Values are expressed as mean ± SD. Different letters on the same line indicate statistical differences between the baseline and the 8th week in the same group according to the t-test ($p < 0.05$). * Indicate non-parametric data and therefore evaluated by Wilcoxon test. *Abbreviations:* HDL-c: high-density lipoprotein; LDL-c: low-density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transferase; TG: Triglycerides; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; HOMA-β: Homeostasis Model Assessment of β-cell function; QUICKI: Quantitative Insulin Sensitivity Check Index**;** TyG: triglyceride-glucose index.

7.11.6 Effect of Black Tea Kombucha Consumption on Inflammatory and Oxidative Stress Markers

After 8 weeks of regular kombucha consumption, participants in the normal weight group showed increased concentrations of IL-13 and nitric oxide in plasma. No difference was observed in the obese group regarding the inflammatory (C3, hs-CRP, INF-γ, IL-1β, -2, -4, -6, -8, -10, -12p70, -13, TNF-α) and oxidative stress markers (FRAP, MDA, NO, SOD) evaluated.

When comparing obese x normal weight individuals, we observed that obese individuals presented greater concentrations of complement C3, hs-CRP, and IL-4 before and after the intervention. At the baseline, the normal weight group presented higher concentrations of IL-10 and SOD, whereas at T8, the obese group presented higher IL-12p70 concentration compared to the normal weight group (**Table 7**).

Table 7. Plasma concentrations of inflammatory and oxidative stress markers of participants at the baseline and after 8 weeks of intervention.

Markers	Total (n=36)		Normal weight (n=20)		Obesity (n=16)		Baseline p-value	Between groups p-value
	Baseline	8th week	Baseline	8th week	Baseline	8th week		
Inflammatory								
C3 (mg/dL)	125.12 ± 4.51	123.64 ± 4.35	106.83 ± 3.69	109.97 ± 3.34	147.98 ± 4.74	140.73 ± 6.85	**<0.001**	0.571
hs-CRP (mg/L)	2.89 ± 0.48	3.13 ± 0.49	1.25 ± 0.32	1.34 ± 0.34	4.97 ± 0.73	5.41 ± 0.68	**<0.001**	**0.022**
INF-γ (pg/mL)	4.04 ± 0.21	4.02 ± 0.22	4.01 ± 0.28	4.08 ± 0.36	4.10 ± 0.32	3.94 ± 0.19	0.498	0.704
IL-1β (pg/mL)	0.26 ± 0.07	0.29 ± 0.07	0.26 ± 0.11	0.34 ± 0.11	0.25 ± 0.09	0.21 ± 0.08	0.572	0.264
IL-2 (pg/mL)	0.58 ± 0.06	0.57 ± 0.07	0.52 ± 0.08	0.45 ± 0.08	0.64 ± 0.09	0.73 ± 0.12	0.370	0.206
IL-4 (pg/mL)	0.04 ± 0.005	0.05 ± 0.005	0.03 ± 0.006	0.04 ± 0.008	0.06 ± 0.007	0.06 ± 0.006	**0.010**	0.923
IL-6 (pg/mL)	1.78 ± 0.35	1.88 ± 0.30	1.31 ± 0.27	1.58 ± 0.31	2.37 ± 0.72	2.26 ± 0.57	0.186	0.180
IL-8 (pg/mL)	3.90 ± 0.25	4.04 ± 0.26	3.91 ± 0.34	3.80 ± 0.25	3.88 ± 0.41	4.36 ± 0.52	0.960	0.208
IL-10 (pg/mL)	0.24 ± 0.01	0.24 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.29 ± 0.02	0.29 ± 0.02	**0.007**	0.447
IL-12p70 (pg/mL)	0.25 ± 0.03	0.28 ± 0.02	0.18 ± 0.02	0.22 ± 0.02	0.33 ± 0.06	0.35 ± 0.05	0.058	0.890
IL-13 (pg/mL)	3.42 ± 0.42[a]	3.77 ± 0.45[b]	3.80 ± 0.57[b]	4.12 ± 0.59[a]	2.95 ± 0.64	3.33 ± 0.69	0.329	0.774
TNF-α (pg/mL)	1.52 ± 0.17	1.60 ± 0.16	1.38 ± 0.20	1.46 ± 0.19	1.69 ± 0.31	1.78 ± 0.27	0.400	0.965
Oxidative Stress								
FRAP (mmol/mL)	95.91 ± 4.76	100.88 ± 4.35	90.24 ± 5.32	94.74 ± 4.44	103.01 ± 8.27	108.55 ± 7.81	0.187	0.889
MDA (μM/mg)	4.52 ± 0.16	4.91 ± 0.18	4.38 ± 0.23	4.71 ± 0.26	4.67 ± 0.23	5.13 ± 0.25	0.891	0.782
NO (μM/mL)	168.59 ± 22.56[b]	225.75 ± 27.14[a]	117.41 ± 8.09[b]	184.44 ± 18.82[a]	234.39 ± 45.53	278.85 ± 55.08	**0.012**	0.626
SOD (U/mL)	373.28 ± 21.88	404.99 ± 28.51	315.05 ± 16.77	370.13 ± 36.34	443.16 ± 36.86	446.84 ± 43.98	**0.003**	0.802

Values presented as mean ± SEM. Different letters on the same line indicate statistical difference within group according to Paired t-test ($p < 0.05$). Baseline p-value was obtained through Student t-test. Between groups p-value was obtained through Student t test or ANCOVA adjusted by the baseline valor of the variable. *Abbreviations:* C3: complement C3; hs-CRP: high-sensitivity C-reactive protein; INF-γ: interferon-gamma; IL- interleukin; TNF-α: tumor necrosis factor alpha; FRAP: Ferric Reducing Ability of Plasma; MDA: malondialdehyde; NO: nitric oxide; SOD: superoxide dismutase.

7.11.7 Effect of Black Tea Kombucha Consumption on the Subcutaneous Adipose Tissue of Subjects with Obesity

After 8 weeks of regular kombucha consumption, no differences were observed in the relative expression of target genes related to inflammation, glucose, and lipid metabolism in the subcutaneous adipose tissue samples collected from the obese group (**Figure 3**).

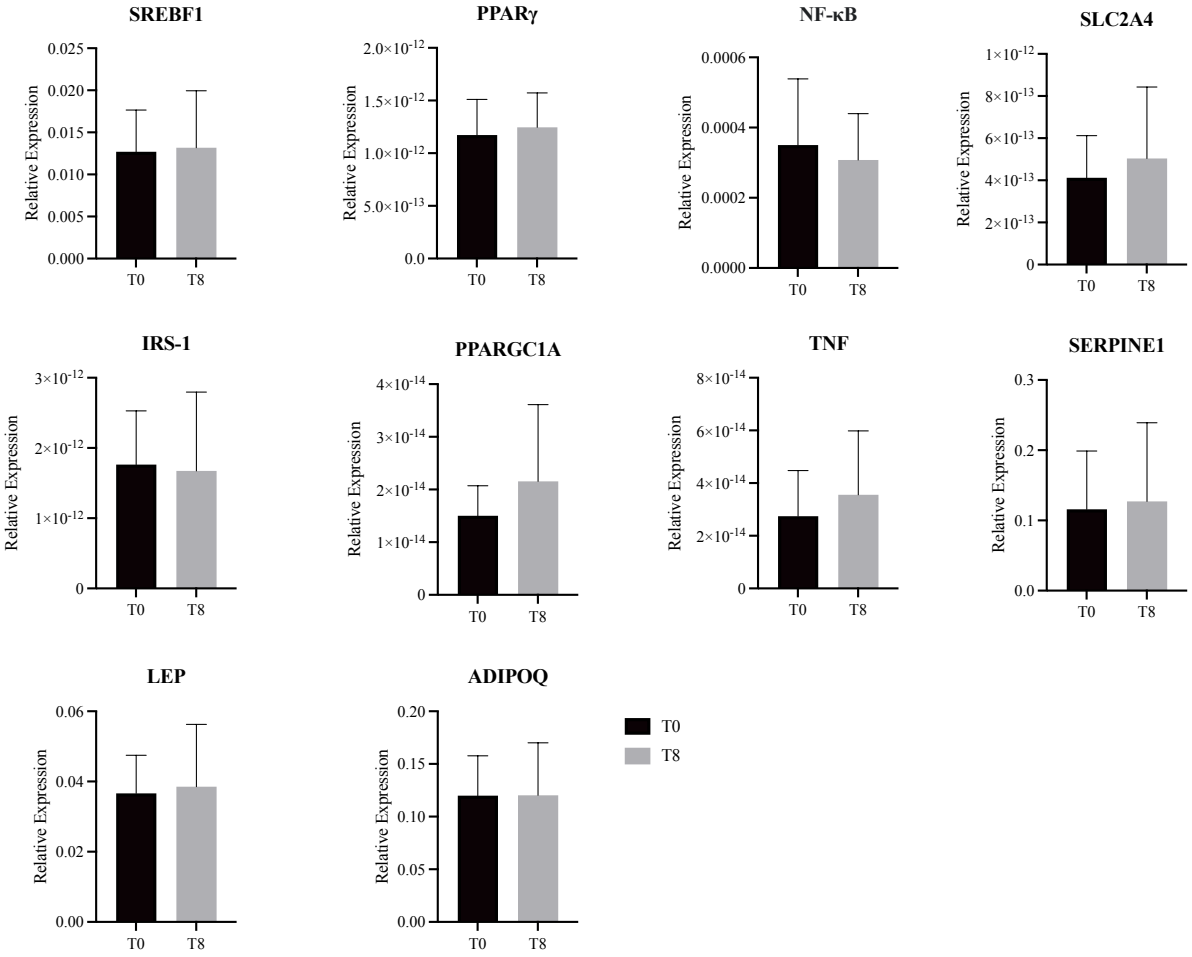


Figure 3. Relative expression of target genes in the subcutaneous adipose tissue of individuals with obesity evaluated through qPCR (n=16). No significant difference was observed between groups according to paired t test (parametric data) and Wilcoxon matched-pairs signed rank test (non-parametric data) ($p>0.05$). *Abbreviations:* ACTB: Beta-Actin; SREBP-1c: Sterol Regulatory Element-Binding Protein 1c; PPARγ: Peroxisome Proliferator-Activated Receptor Gamma; NF-κB: Nuclear Factor Kappa B; GLUT4: Glucose Transporter 4; IRS-1: Insulin Receptor Substrate 1; PGC-1α: Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha; PAI-1: Plasminogen Activator Inhibitor-1; TNF: Tumor Necrosis Factor; PGK1: Phosphoglycerate Kinase 1; PPIA: Peptidylprolyl Isomerase A.

7.11.8 Effect of Black Tea Kombucha Consumption on Intestinal Health

7.11.8.1 Intestinal Permeability and Integrity

Intestinal permeability was evaluated considering the relationship between lactulose and mannitol excretion in urine samples according to the described methodology. No significant differences were observed between baseline and treatment in both groups (**Figure 4**).

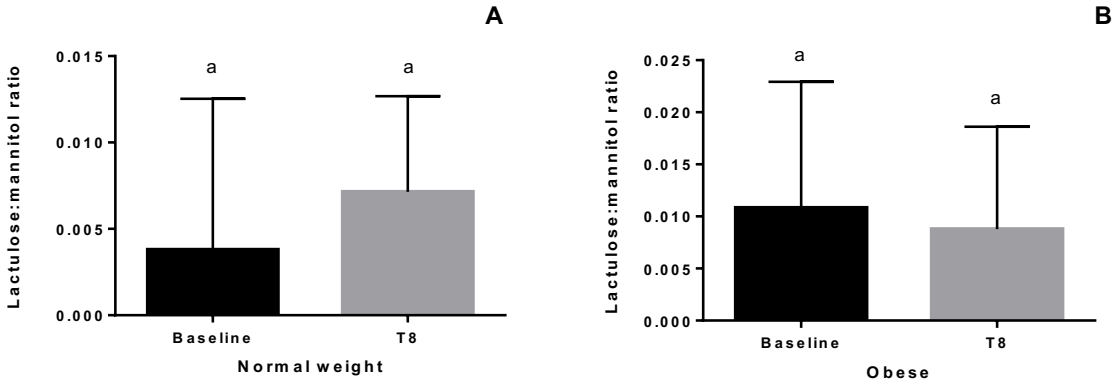


Figure 4. Relationship between lactulose and mannitol excretion in normal weight (A) and obese (B) individuals before (baseline) and after regular consumption of black tea kombucha for 8 weeks (T8). Same letters indicate that there was no significant difference between the groups according to the t test ($p > 0.05$).

Similar results were observed regarding lipopolysaccharide-binding protein (LBP) and zonulin concentrations in plasma. These proteins are present in the intestinal epithelium and are associated with its integrity (**Figure 5**).

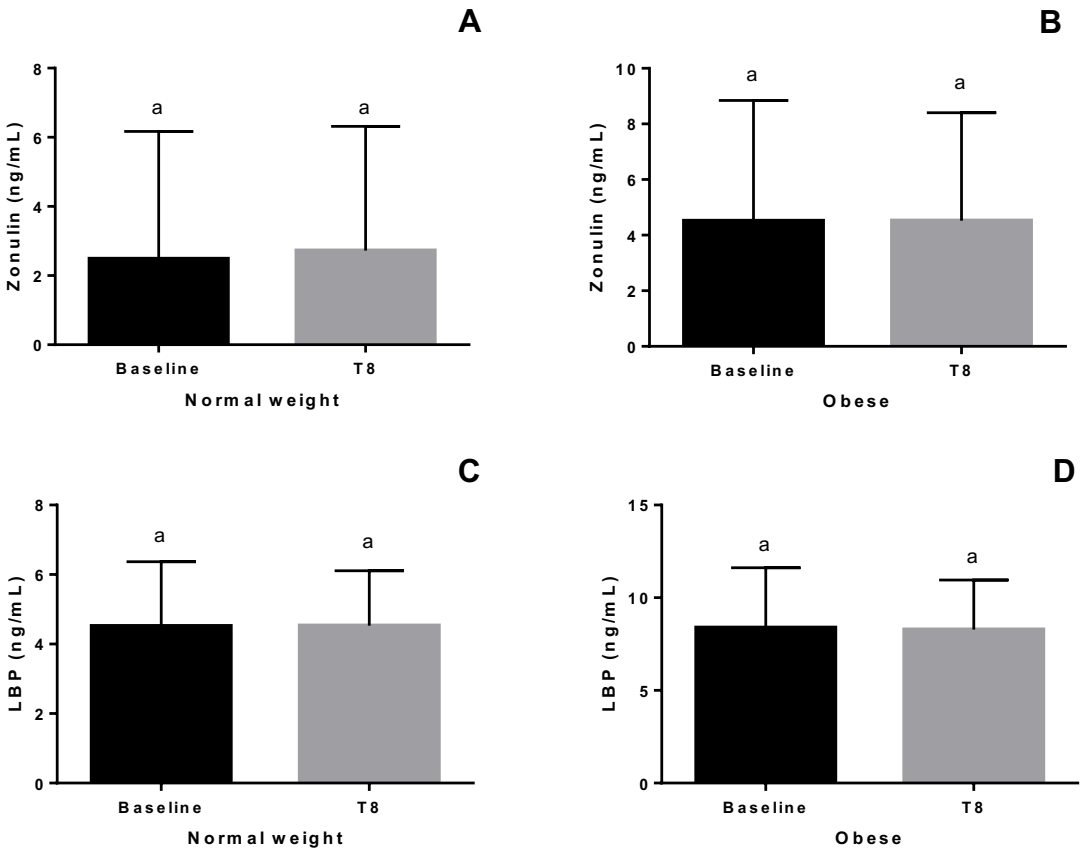


Figure 5. Plasma concentrations of zonulin in normal weight (A) and obese (B) and LBP in normal weight (C) and obese (D) individuals before (baseline) and after regular consumption of black tea kombucha for 8 weeks (T8). Same letters indicate that there was no significant difference between the groups according to the t test ($p > 0.05$).

7.11.8.2 Short-Chain Fatty Acids (SCFA)

No significant differences were observed in the concentrations of acetic, butyric, and propionic acids in the stool of participants in both groups before and after kombucha consumption for eight weeks (**Figure 6**).

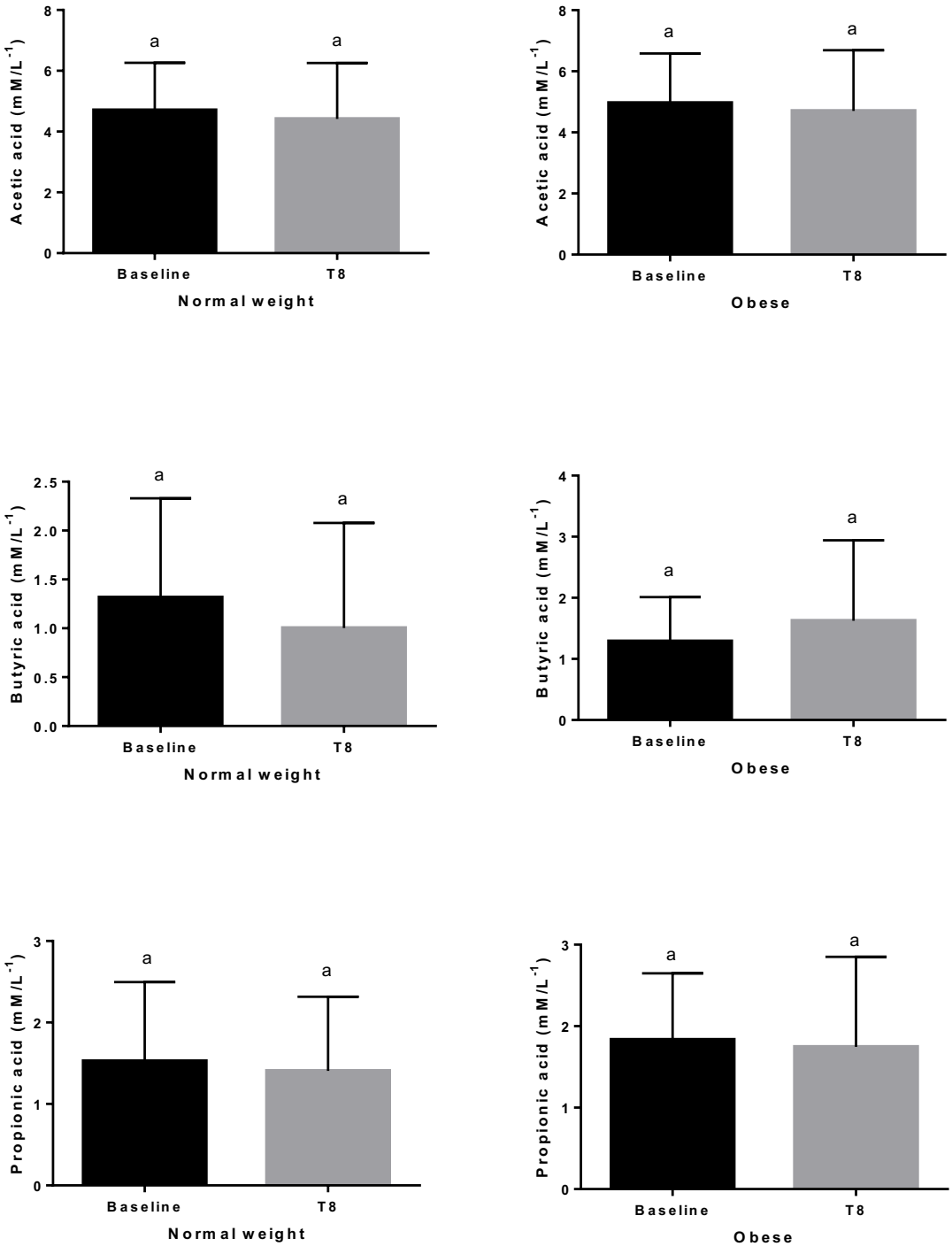


Figure 6. Concentrations of acetic (A), butyric (B), and propionic (C) acids measured in the stool samples of normal weight and obese individuals before (baseline) and after kombucha consumption for 8 weeks (T8). Same letters indicate that there was no statistical difference between the groups by the t-test ($p > 0.05$).

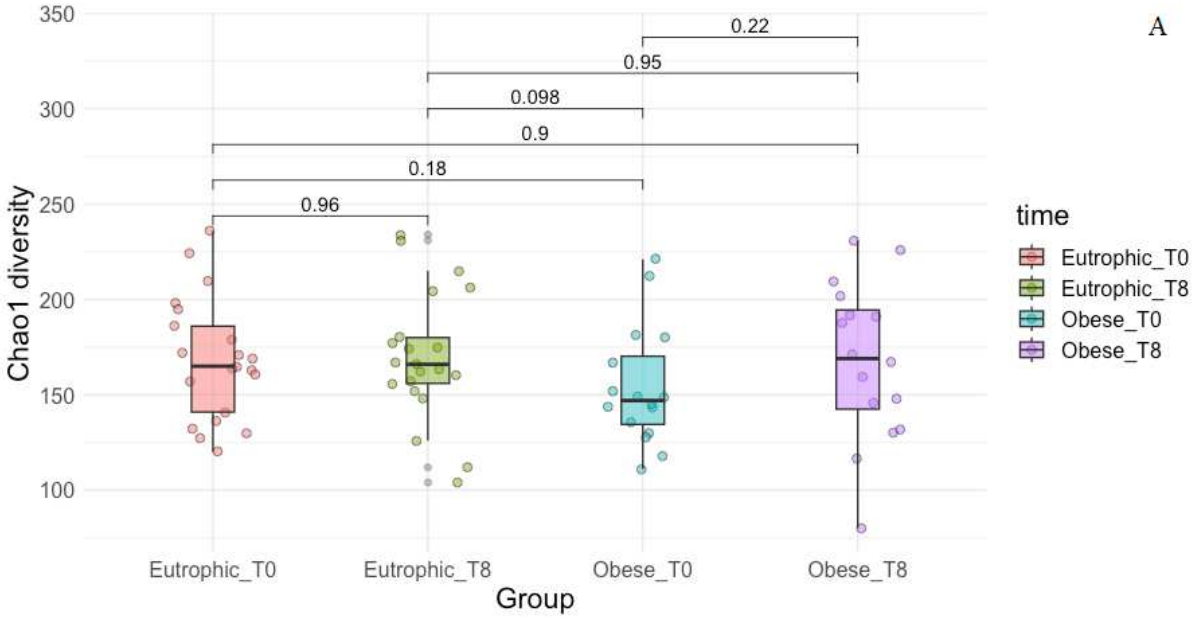
7.11.8.3 Gut Microbiota

Bioinformatics analysis

DNA sequencing was performed considering the bacterial regions V3 and V4 of the 16S rRNA gene (primers 341F/806R) and the internal spacers transcribed from the nuclear ribosomal DNA (ITS1 and ITS2) of the fungal rRNA region (primers ITS1F and ITS2R). After removing chimerical and low-quality sequences, 37 datasets (eutrophic, n=21; obese, n=16) were evaluated.

Bacterial Microbiota (16S rRNA)

The effects of daily intake of black tea kombucha for eight weeks on the gut microbiota were firstly investigated using alpha- and beta-diversity indices. Considering the alpha-diversity analysis, the observed Shannon and Chao1 do not indicate a significant difference among the groups before and after treatment, not even when comparing the normal weight and obese individuals (**Figures 7A, 7B**).



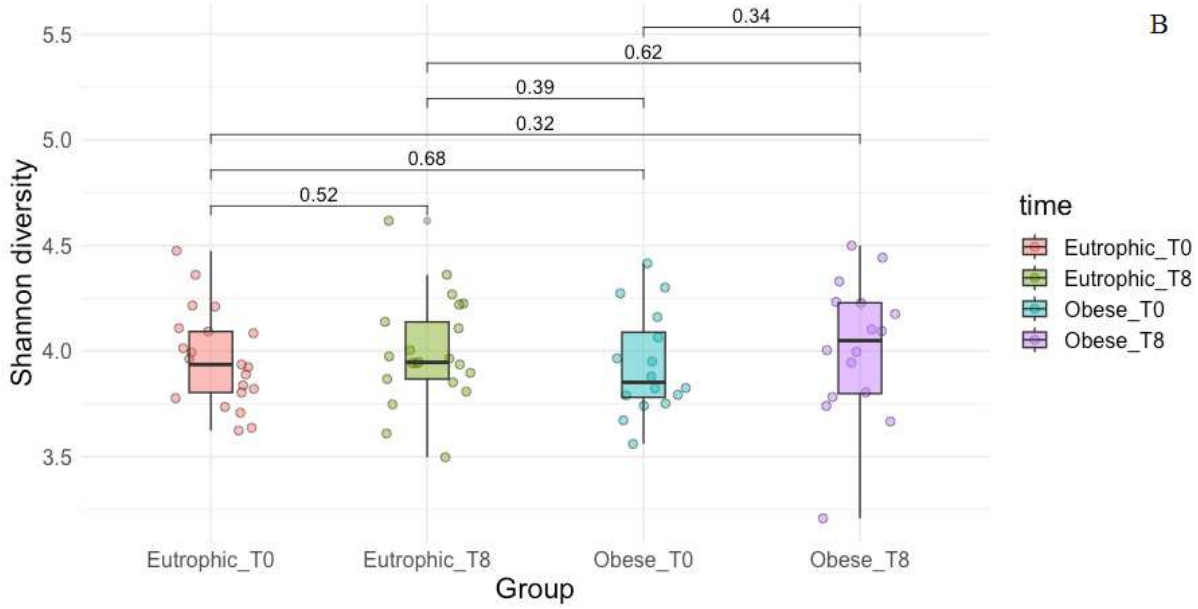


Figure 7. Box and whisker plots comparing species diversity (**A**) and richness (**B**) among the groups at baseline (T0) and at the end of the experimental period (T8). Horizontal bold lines show the median values. The bottom and top of the boxes show the 25th and the 75th percentiles, respectively. The whiskers extend up to the extreme points within 1.5 times the interquartile ranges (IQR).

Regarding the beta-diversity analysis, gut microbiota clustering on PCoA plots based on weighted and unweighted UniFrac distance metrics does not show significant differences among the groups (**Figures 8A, 8B**). Pairwise comparisons using Qiime beta-group-significance command revealed that the gut composition of the groups also did not differ among them. The same tendency was observed for the pairwise PERMANOVA results, whose groups did not differ regarding community dissimilarity.

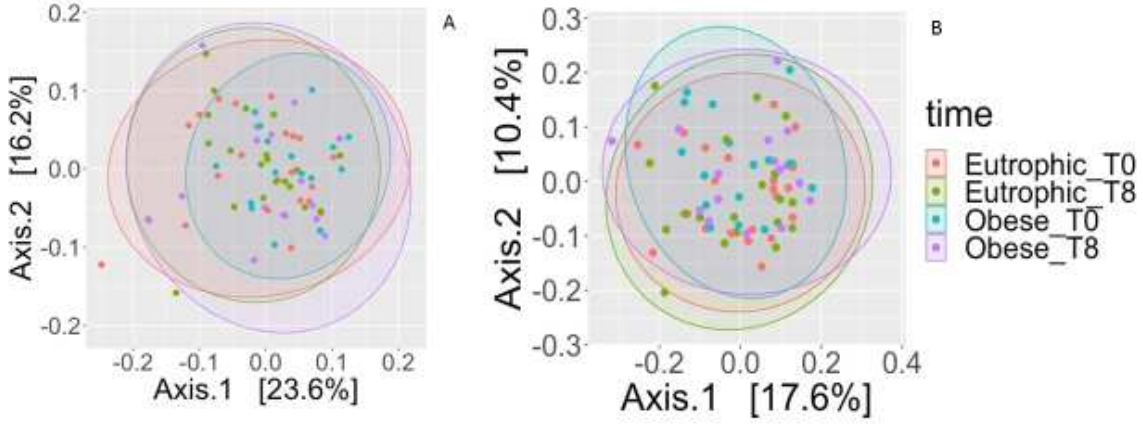


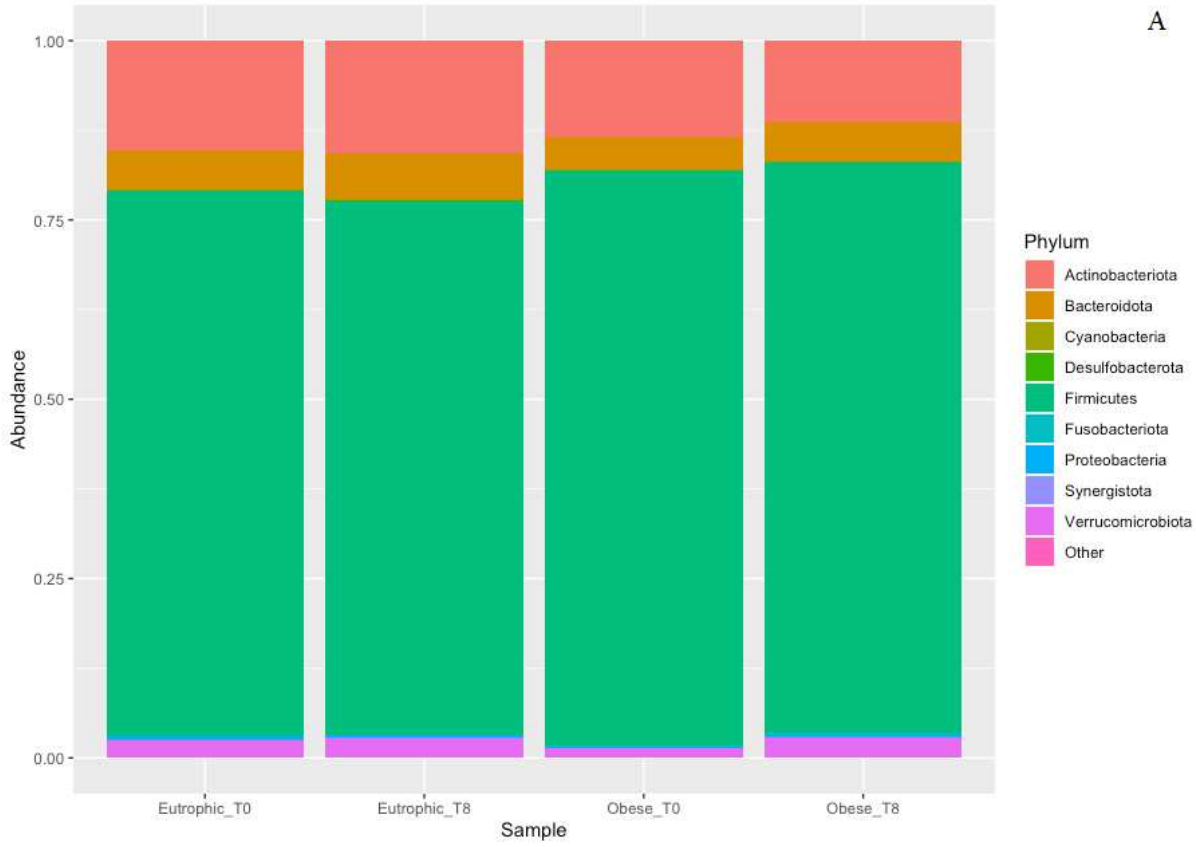
Figure 8. Principal coordinate analysis (PCoA) based on weighted (A) and unweighted (B) UniFrac distances. PERMANOVA was used to detect significant differences between microbial communities (dissimilarity) of different groups.

Regarding bacterial community, we made comparisons among the groups based on relative abundance. At the phylum level, Firmicutes was dominant in both normal weight and obese groups, followed by Actinobacteria and Bacteroidetes (**Figure 9A**). The obese group presented a higher Firmicutes:Bacteroidetes ratio compared to the normal weight group, which has been reported by other authors (CONLON; BIRD, 2014; STOJANOV; BERLEC; ŠTRUKELJ, 2020). Regular kombucha consumption favored a slight increase in Bacteroidota in both normal weight and obese groups, decreasing this ratio.

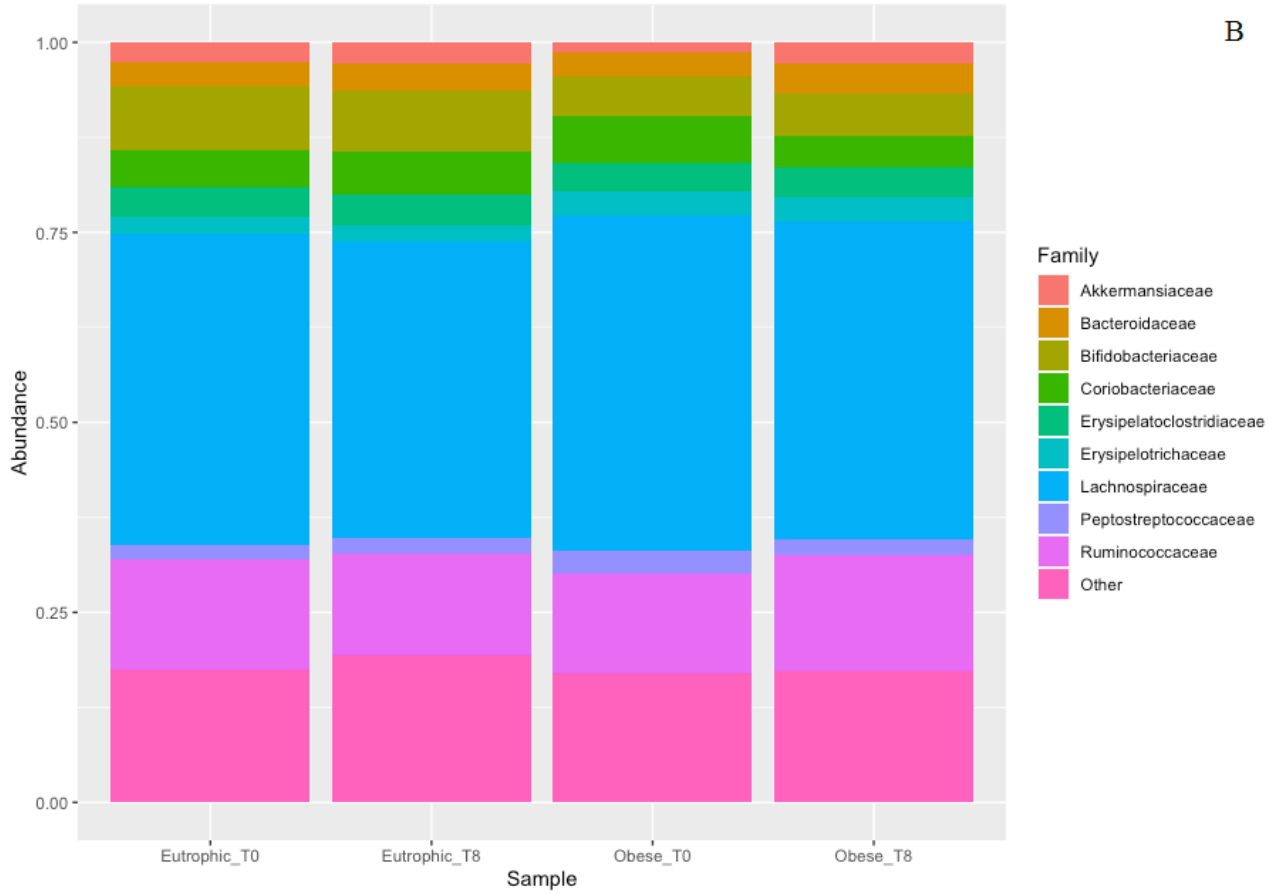
The 10 most abundant families among the groups are represented in **Figure 9B**. Lachnospiraceae is the most abundant among them, followed by Ruminococcaceae. Both belong to the order Clostridiales and are associated with the maintenance of intestinal health (BIDDLE et al., 2013). Akkermansiaceae was also abundant among the groups and increased after kombucha treatment, especially in the obese group. Species belonging to this family, especially *Akkermansia muciniphila*, are favored in the presence of phenolic compounds. They are one of the main producers of propionate and are associated with a decrease in intestinal permeability (ANHÊ et al., 2016; LIU et al., 2016; NAITO; UCHIYAMA; TAKAGI, 2018).

The most abundant genera among the groups are represented in **Figure 9C** (top 20) and **Figure 10** (top 9). Three of them presented a significant difference: the genera *Ruminococcus* and *Dorea* are increased in the obese group at baseline in the normal weight group before and after kombucha consumption ($p<0.05$); the genus *Subdoligranulum*, in turn, is increased in the obese group when compared to the normal weight group, both at T8.

A


1.00
0.75
0.50
0.25
0.00
Abundance
Eutrophic_T0
Eutrophic_T8
Obese_T0
Obese_T8
Sample
Phylum
Actinobacteriota
Bacteroidota
Cyanobacteria
Desulfobacterota
Firmicutes
Fusobacteriota
Proteobacteria
Synergistota
Verrucomicrobiota
Other

B


1.00
0.75
0.50
0.25
0.00
Abundance
Eutrophic_T0
Eutrophic_T8
Obese_T0
Obese_T8
Sample
Family
Akkermansiaceae
Bacteroidaceae
Bifidobacteriaceae
Coriobacteriaceae
Erysipelatoclostridiaceae
Erysipelotrichaceae
Lachnospiraceae
Peptostreptococcaceae
Ruminococcaceae
Other

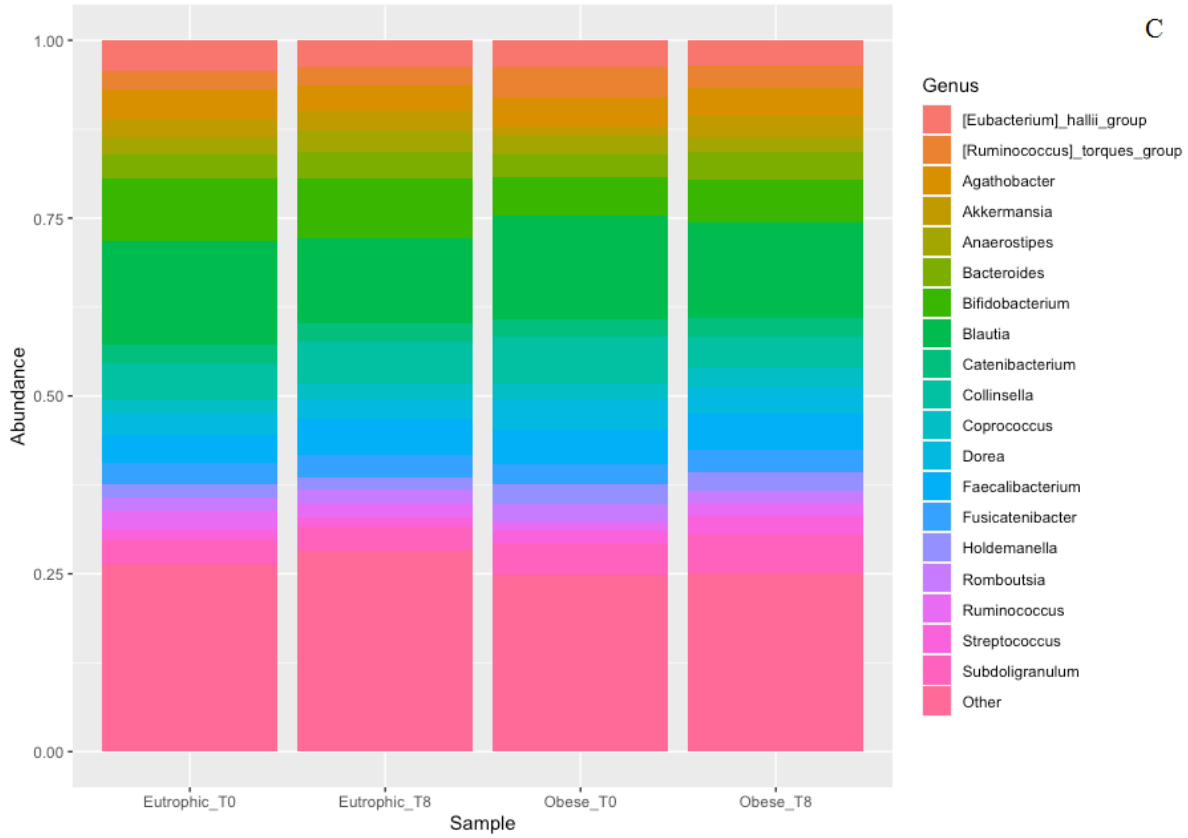


Figure 9. Relative abundance of major phyla (top 5) (A), families (top 10) (B) and genera (top 20) (C) in normal weight and obese individuals at baseline (T0) and after 8 weeks of regular consumption of black tea kombucha (T8).

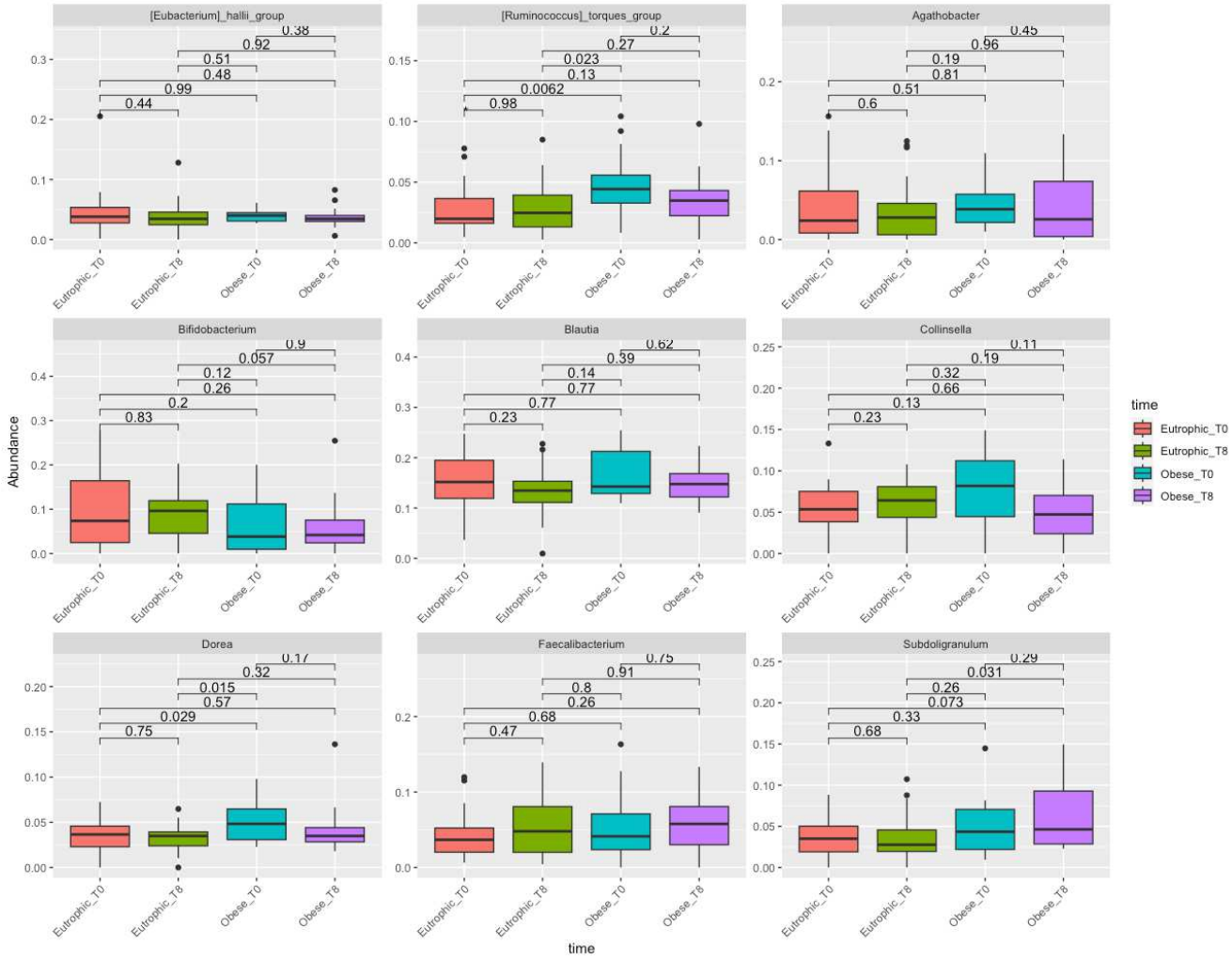


Figure 10. Box and whisker plots comparing the abundance of the main genera (top 9) observed in eutrophic and obese groups at baseline (T0) and the end of treatment (T8). The bold horizontal lines show the median values. The bottom and top portions of the boxes show the 25th and 75th percentiles, respectively. Vertical lines extend to the most extreme points within 1.5 times the interquartile ranges.

Finally, a LEfSe (Linear discriminant analysis Effect Size) analysis was performed to better characterize the gut bacterial composition of each group, as well as to identify the differently abundant taxa. Only taxa that presented an LDA>2 are represented (**Figure 11**).

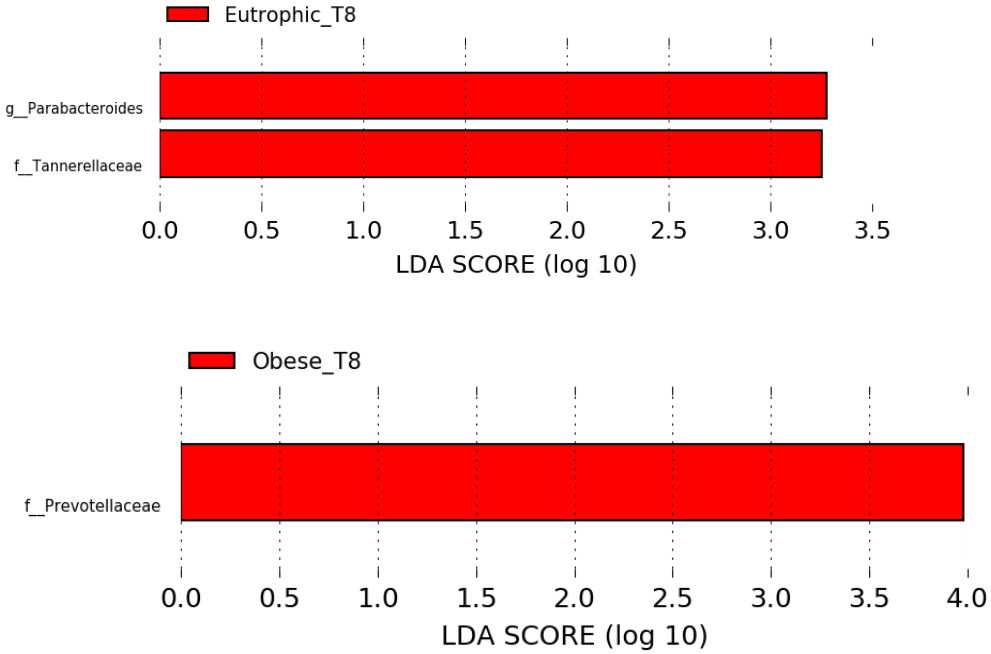


Figure 11. Differential abundance analysis conducted with linear discriminant analysis Effect Size (LEfSe) in normal weight and obese individuals after kombucha intake. Only biomarkers showing linear discriminant analysis (LDA) scores greater than 2.0 with a false discovery rate (FDR) $p<0.05$ are represented. Letters: f, family; g, gender.

Fungal microbiota (ITS1/ITS2)

Fungal richness and diversity were also evaluated by Chao1 and Shannon diversity index, respectively. As it was observed in the bacterial community, none of the indices indicated a significant difference among the groups before and after treatment, not even when comparing the normal weight and obese groups (**Figures 12A, 12B**).

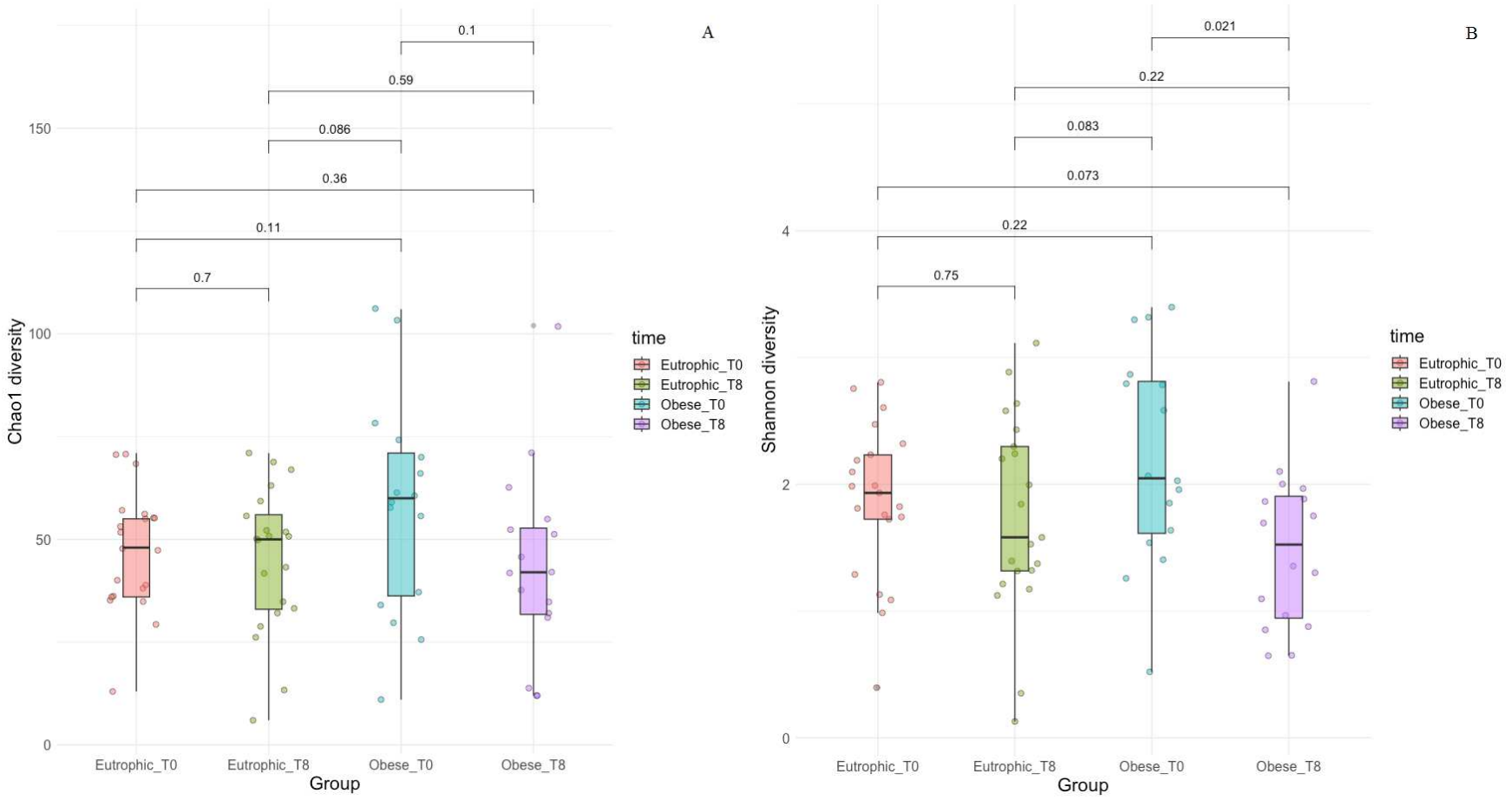


Figure 12. Box and whisker plots comparing bacterial richness (A) and diversity (B) between normal weight and obese groups at the baseline (T0) and at the end of the treatment (T8). Horizontal bold lines show the median values. The bottom and top of the boxes show the 25th and the 75th percentiles, respectively. The whiskers extend up to the extreme points within 1.5 times the interquartile ranges (IQR).

To better comprehend the beta-diversity of the fungal community, the Bray-Curtis index was used to measure the similarity in the community composition among the groups (**Figure 13A**), whereas the Jaccard index was used to quantify the presence/absence of similarity of the fungal species among the groups (**Figure 13B**). Contrary to the bacterial community, both indices indicated a difference in the beta-diversity of the fungal community among the groups, indicating that kombucha consumption was able to modify the fungal composition ($p<0.01$).

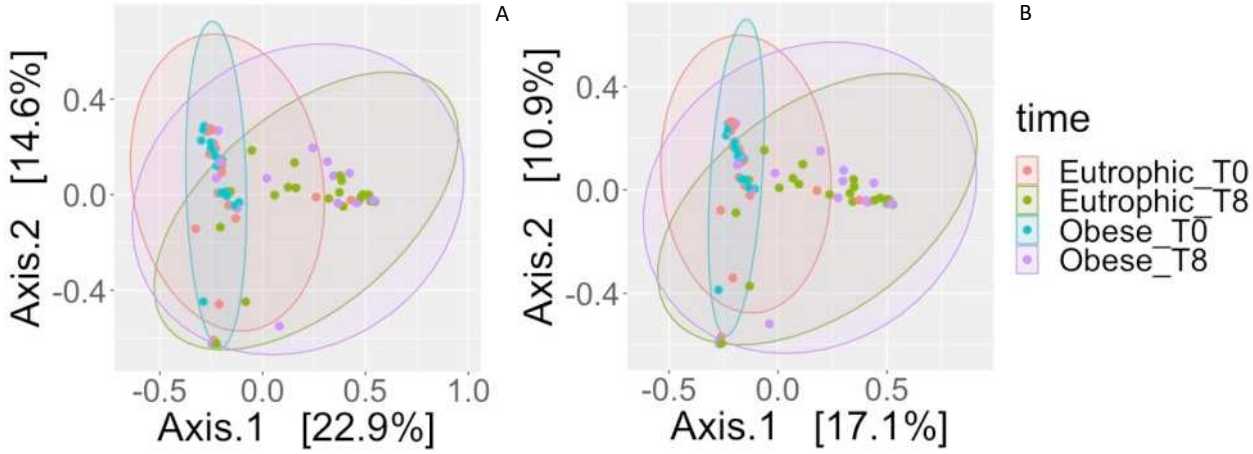


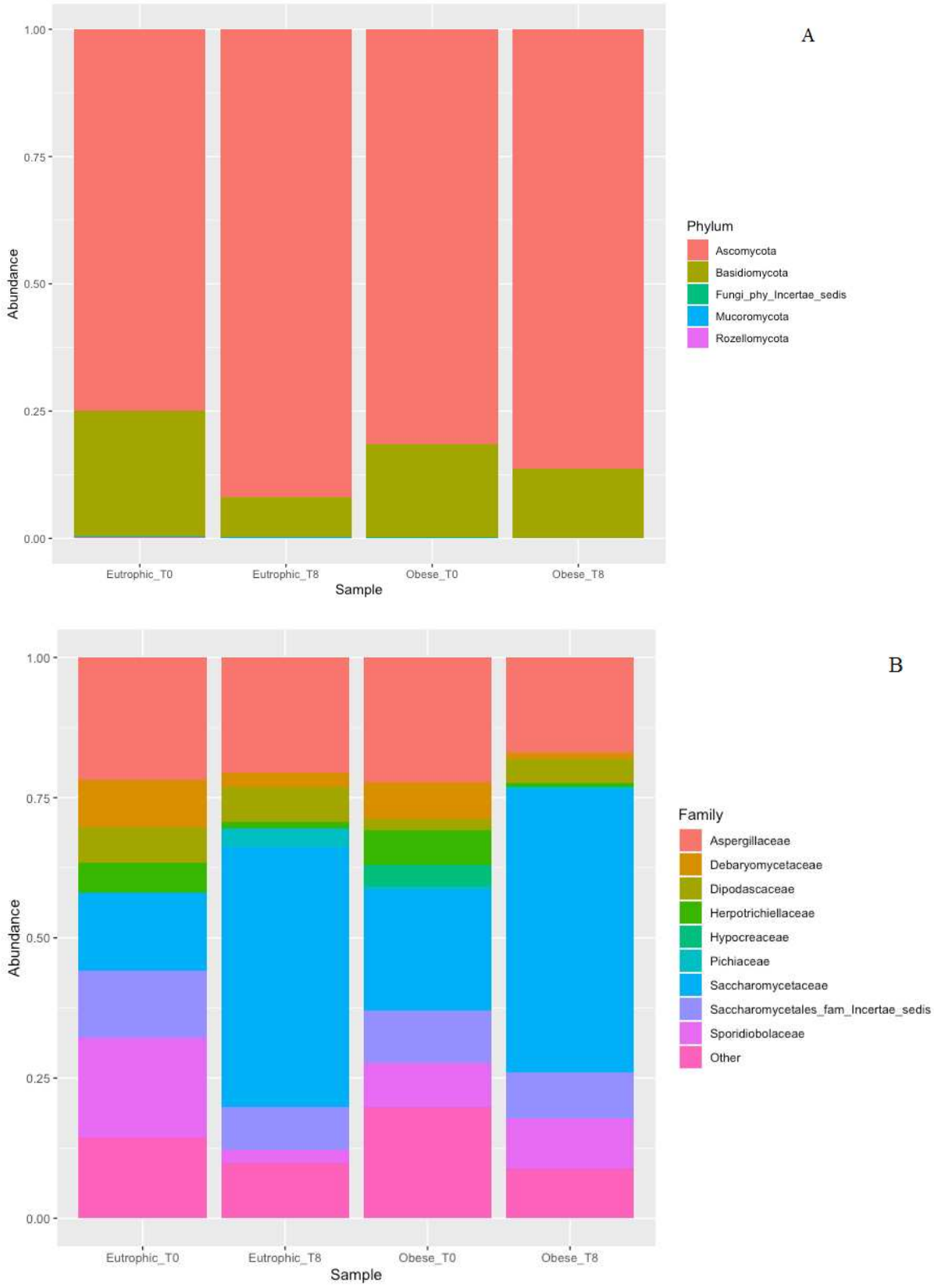
Figure 13. Beta-diversity of the fugal community measured by Bray-Curtis (A) and Jaccard (B) indices between normal weight and obese individuals before (T0) and after (T8) regular consumption of black tea kombucha.

The five most abundant phyla are represented in **Figure 14A**. Ascomycota is the predominant phylum in all groups, representing more than 75% of them. Basidiomycota appears next, accounting for approximately 24% in the normal weight group at the baseline and in smaller proportions in the other groups. We observed that kombucha intake favored the growth of Ascomycota in the normal weight and obese individuals, thus decreasing the abundance of Basidiomycota at T8. The other three phyla represented in the figure represent up to 1% of the total.

The kombucha treatment also favored the growth of Saccharomycetaceae, the predominant family in all groups (**Figure 14B**). On the other hand, other families that were numerous at baseline had their relative abundance decreased after kombucha consumption, such as Debaryomycetaceae and Herpotrichiellaceae. The Hypocreaceae family was observed only in obese individuals at baseline.

The predominant genera among the groups are represented by **Figure 14C** (top 20). *Saccharomyces* was favored by kombucha consumption and became the most abundant genus

in normal weight and obese groups at T8. On the other hand, other genera such as *Aspergillus* showed lower abundance at T8 when compared to baseline in both groups.



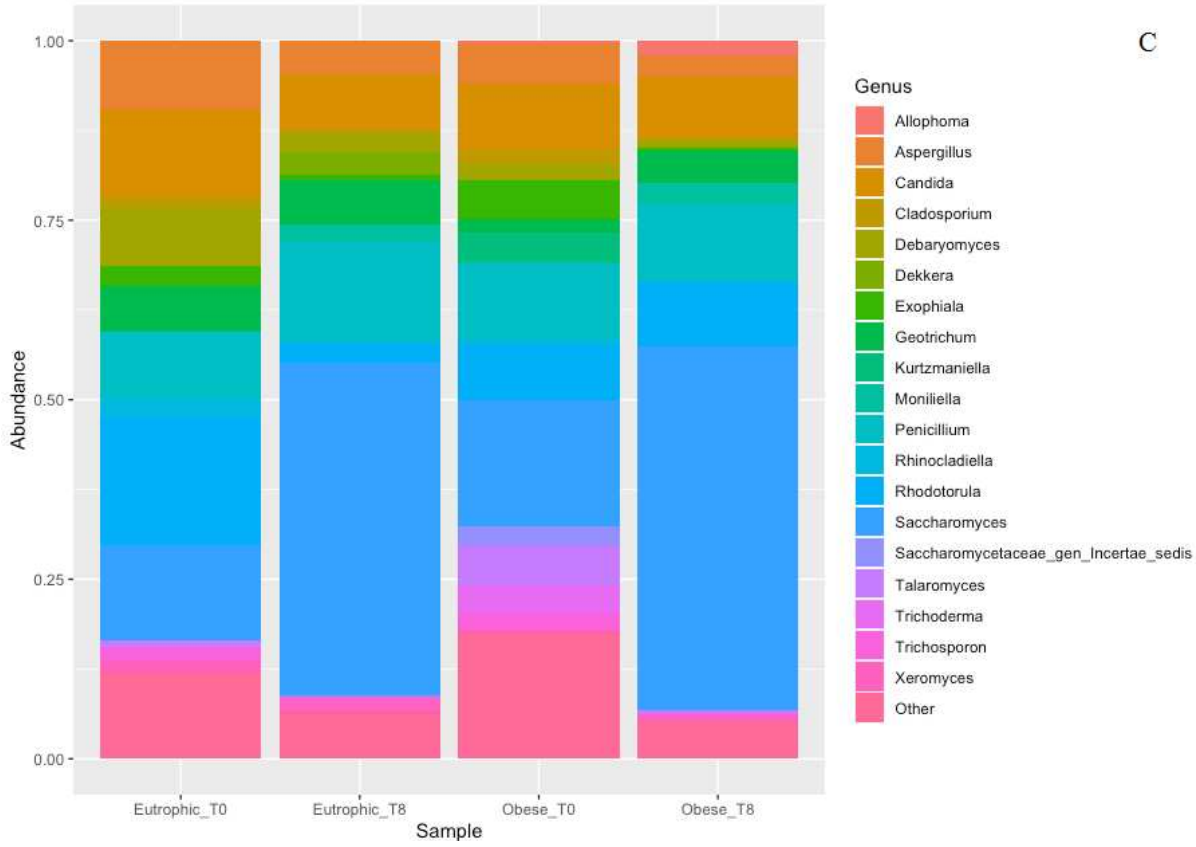


Figure 14. Relative abundance of major phyla (top 5) (A), families (top 10) (B), and genera (top 20) (C) in normal weight and obese individuals at baseline (T0) and after 8 weeks (T8) of regular kombucha consumption.

Considering the nine most abundant genera (**Figure 15**), we observed that the normal weight and obese individuals presented a greater abundance of the genus *Exophiala* at baseline, which was reduced after kombucha consumption. It was even more evident when comparing the groups obese T0 with the normal weight T8 ($p = 0.041$) and the obese T8 ($p = 0.012$). A similar trend was observed for the genus *Rhodotorula*, whose relative abundance was increased at baseline and drastically decreased after kombucha consumption in both groups ($p<0.05$).

The genus *Geotrichum* was more abundant in the normal weight individuals compared to the obese group, before and after kombucha consumption ($p<0.05$). However, no difference was observed when comparing baseline and T8 in the same group.

The genus *Saccharomyces*, as mentioned above, was greatly increased after kombucha consumption, as observed in Figures 13 and 14. The difference can be observed between T0 and T8, regardless of the group ($p<0.05$).

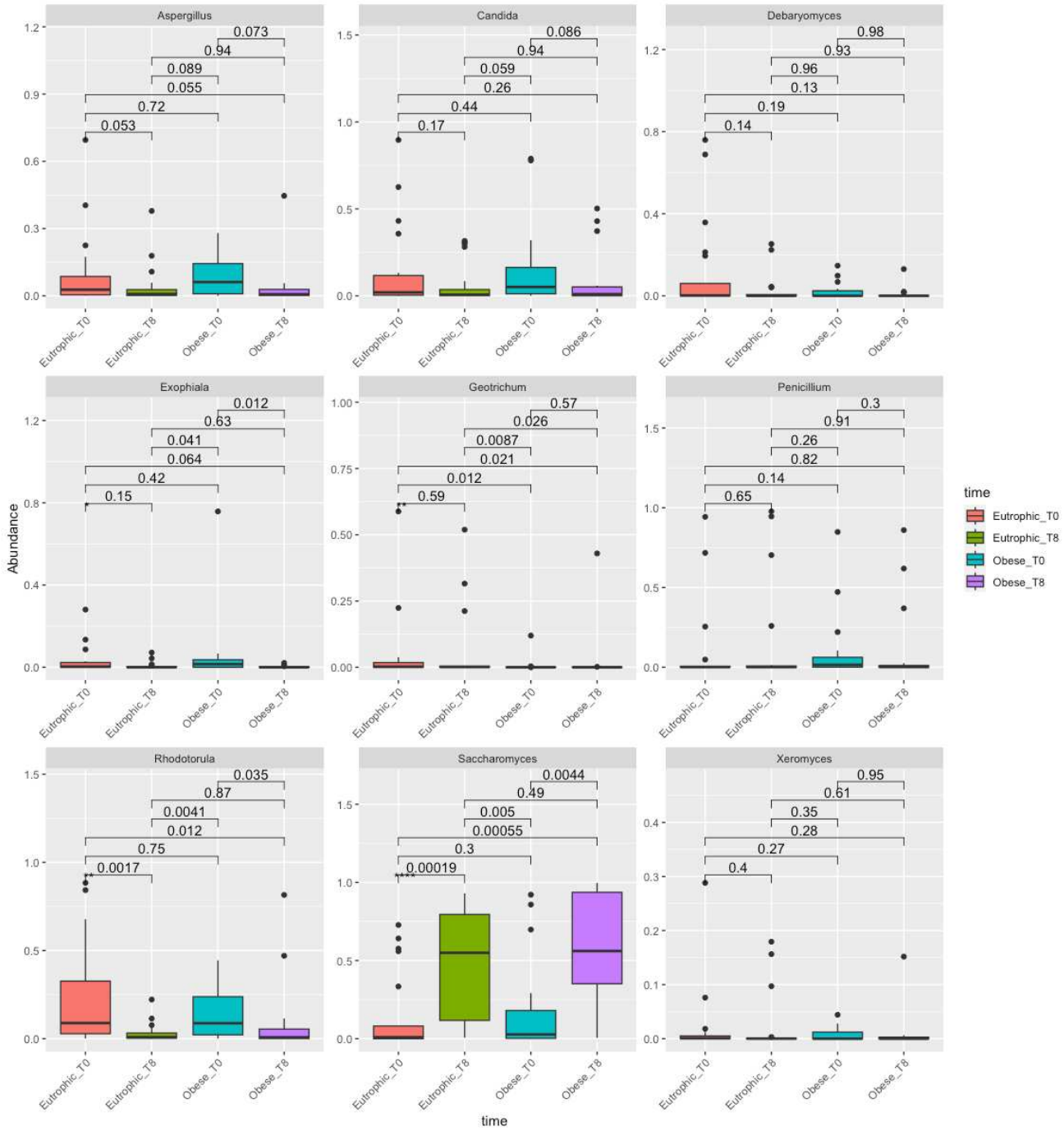
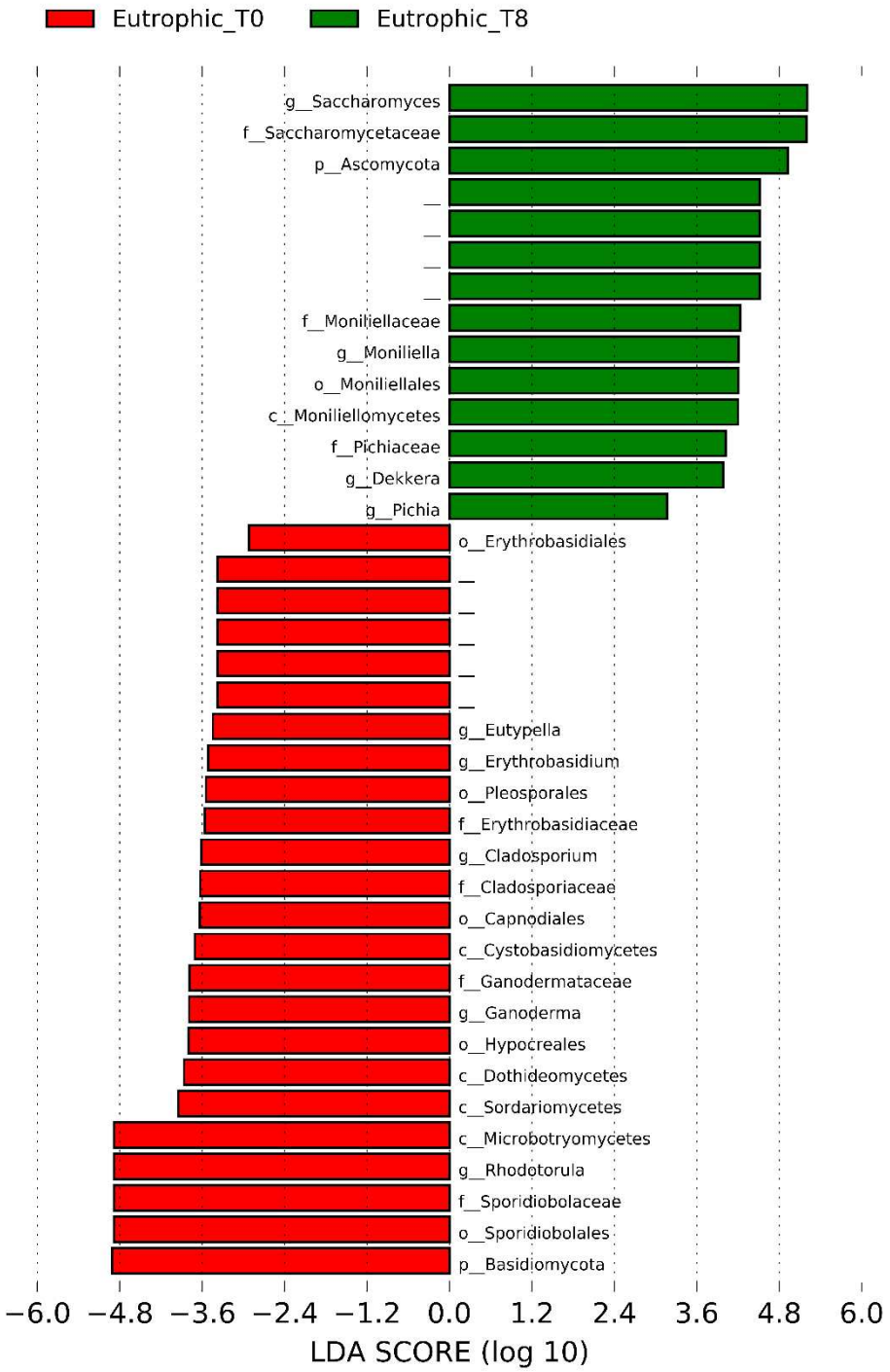


Figure 15. Box and whisker plots comparing the abundance of the main genera (top 9) observed in normal weight and obese individuals at baseline (T0) and the end of treatment (T8). The bold horizontal lines show the median values. The bottom and top portions of the boxes show the 25th and 75th percentiles, respectively. Vertical lines extend to the most extreme points within 1.5 times the interquartile ranges.

Finally, a LEfSe (Linear discriminant analysis Effect Size) analysis was performed to better characterize the composition of each group, as well as to identify the differently abundant taxa. Only taxa that presented an LDA>2 are represented (**Figure 16**).



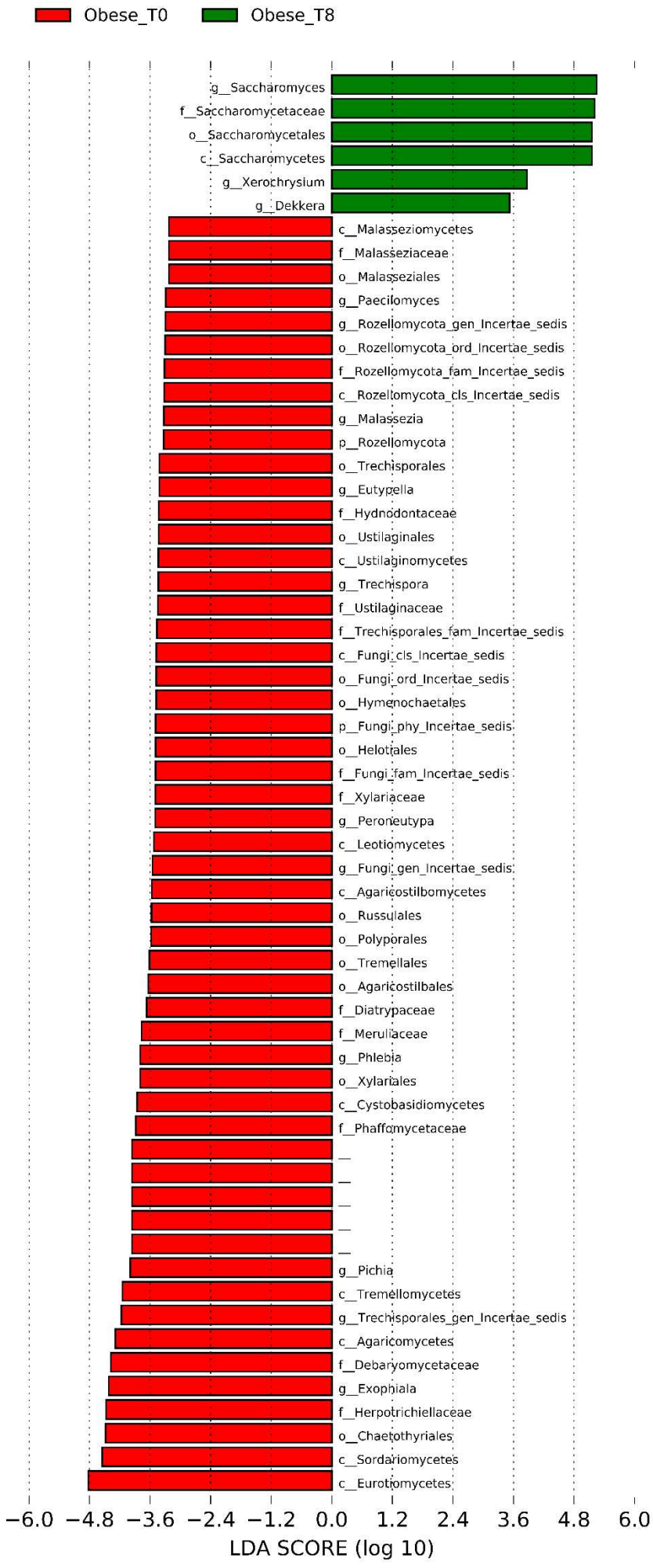

Obese_T0
Obese_T8
g__Saccharomyces
f__Saccharomycetaceae
o__Saccharomycetales
c__Saccharomycetes
g__Xerochrysium
g__Dekkera
c__Malasseziomycetes
f__Malasseziaceae
o__Malasseziales
g__Paecilomyces
g__Rozellomycota_gen_Incertae_sedis
o__Rozellomycota_ord_Incertae_sedis
f__Rozellomycota_fam_Incertae_sedis
c__Rozellomycota_cls_Incertae_sedis
g__Malassezia
p__Rozellomycota
o__Trechisporales
g__Eutypella
f__Hydnodontaceae
o__Ustilaginales
c__Ustilaginomycetes
g__Trechispora
f__Ustilaginaceae
f__Trechisporales_fam_Incertae_sedis
c__Fungi_cls_Incertae_sedis
o__Fungi_ord_Incertae_sedis
o__Hymenochaetales
p__Fungi_phy_Incertae_sedis
o__Helotiales
f__Fungi_fam_Incertae_sedis
f__Xylariaceae
g__Peroneutypa
c__Leotiomycetes
g__Fungi_gen_Incertae_sedis
c__Agaricostilbomycetes
o__Russulales
o__Polyporales
o__Tremellales
o__Agaricostilbales
f__Diatrypaceae
f__Meruliaceae
g__Phlebia
o__Xylariales
c__Cystobasidiomycetes
f__Phaffomycetaceae
g__Pichia
c__Tremellomycetes
g__Trechisporales_gen_Incertae_sedis
c__Agaricomycetes
f__Debaryomycetaceae
g__Exophiala
f__Herpotrichiellaceae
o__Chaetothyriales
c__Sordariomycetes
c__Eurotiomycetes
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

Figure 16. Differential abundance analysis was conducted with linear discriminant analysis effect size (LEfSe) in normal weight and obese individuals after kombucha treatment. Only biomarkers showing linear discriminant analysis (LDA) scores higher than 2.0 with a false discovery rate (FDR) $p<0.05$ are represented. Letters: p, phylum; c, class; o, order; f, family; g, gender.

7.12 DISCUSSION

Although consumed for at least two thousand years in China, kombucha became popular in the rest of the world about two decades ago, concurrently with the interest of the population in establishing a healthier diet (KAPP; SUMNER, 2019). However, there is still not enough scientific evidence to prove the benefits attributed to the consumption of kombucha on human health, since the studies carried out so far is restricted to *in vitro* and animal studies. Our study is, as far as we know, the first clinical study in the area. Our objective is to investigate whether the consumption of kombucha, by itself, would be able to modulate body composition, inflammation, and gut microbiota.

Our research group has shown several benefits associated with green and black tea kombuchas (CARDOSO et al., 2020, 2021; COSTA et al., 2021, 2022; DE NORONHA et al., 2022). Most of the functional claims are due to the high antioxidant content of the beverage resulting from the high amount and diversity of phenolic compounds whose composition depends on the tea of origin (LEAL et al., 2018). Tea leaves (C*amellia sinensis*) are rich in flavonoids, especially catechins such as epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (TANAKA; KOUNO, 2003). According to the degree of fermentation to which the leaves undergo after harvesting, they are categorized as green tea (unfermented tea), white tea and yellow tea (slightly fermented tea), oolong tea (semi-fermented tea), black tea (fermented tea), and Pu-erh tea (post-fermented tea) (UMESH, 2023). In addition to the organoleptic characteristics, the degree of fermentation will influence the phenolic compound profile – green tea leaves are like fresh *C. sinensis* leaves while black tea has a high content of theaflavins and thearubigins that are formed during processing (TANAKA; KOUNO, 2003). Other phenolic compounds are favored during kombucha fermentation, such as quercetin and kaempferol, which were identified among the 10 most abundant in this study.

In general, phenolic compounds act against oxidative stress and inflammation due to their ability to neutralize free radicals through three main mechanisms: 1) direct antioxidant action through electron donation; 2) stimulating the production of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (BARBOSA et al., 2010); and 3) regulating

cell signaling and gene expression by inhibiting the activity of inflammatory mediators such as cyclooxygenase as well as regulating transcriptional elements that act in antioxidant pathways such as NF-κB and Nrf-2 (RAHMAN et al., 2022a). In a previous study conducted with Wistar rats, we demonstrated that regular kombucha intake was able to attenuate the oxidative stress caused by a high-fat high-fructose diet by increasing the plasma antioxidant capacity and activating antioxidant enzymes in the liver such as superoxide dismutase and catalase (CARDOSO et al., 2021).

Among the inflammatory and oxidative stress markers evaluated in this study, we observed that regular consumption of black tea kombucha increased the plasmatic concentrations of interleukin 13 and nitric oxide of normal individuals. No difference was noted in obese individuals regarding the same inflammatory and oxidative stress markers. Interleukin 13 is a type II cytokine that plays an immunoregulatory role in the body (MEYER; GOLDENRING, 2018). It is produced by several cell types, including T cells, mast cells, and eosinophils; and is involved in the regulation of the immune response, particularly in the context of allergies, asthma, and certain autoimmune diseases (MOHNING et al., 2019). It also promotes the production of immunoglobulin E (IgE), which is involved in allergic reactions, and increases the survival and activity of eosinophils, which are involved in the inflammatory response (DORAN et al., 2017). Therapeutically, IL-13 has been targeted to treat asthma and other allergic diseases, but it also exerts effects beyond the immune system (MOHNING et al., 2019).

Since IL-13 is homologous to IL-4, both cytokines share several metabolic pathways and the same receptor on endothelial cells (O'SHEA et al., 2023). However, IL-13 seems to exert an even more pronounced effect in response to the organism's injury by inhibiting the production of inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, and INF-α) (DEMBIC, 2015), in addition to increasing the VCAM-1 expression (SAHINER; AKDIS; AKDIS, 2022). In studies conducted with mice, IL-13 played an important role in protecting against the nematodes *Nippostrongylus brasiliensis*, *Ticheris muri,* and *Trichinella spiralis*, inducing its expulsion from the gastrointestinal tract (DE VRIES; CARBALLIDO, 2003). Also in the gastrointestinal tract, IL-13 appears to play a key role in mucus secretion by goblet cells (MANNON; REINISCH, 2012; MEYER; GOLDENRING, 2018), adding a protective barrier to the gastric epithelium in response to pre-cancerous lesions in the mucosa (MEYER; GOLDENRING, 2018).

Nitric oxide, in turn, is a signaling molecule whose functions depend on the place and amount produced (LUBOS; HANDY; LOSCALZO, 2008). Although it is identified as one of the main molecules involved in oxidative stress, in normal doses it plays a key role in homeostasis due to its antithrombotic properties, favoring vasodilation and vascular permeability (LOSCALZO; JIN, 2010). Some authors suggested a value of 122.3 μmol/L as a cut-off; NOx<122.3 μmol/L was positively associated with BMI, waist, and hip circumference, and it was considered a predictor of cardiometabolic risk in black individuals (MAURER, 2015).

Studies show that phenolic compounds can stimulate nitric oxide production in endothelial cells, which may contribute to improved vascular function (APPELDOORN et al., 2009). This is because phenolic compounds can activate the enzyme nitric oxide synthase (NOS), which is responsible for producing nitric oxide (SERRELI; DEIANA, 2023). Phenolic compounds also inhibit cyclooxygenase 1 (COX-1), which increases the production of thromboxane A2 and induces vasoconstriction. In response, there is an increase in endothelial nitric oxide signaling, resulting in vasodilation (RUDRAPAL et al., 2022). Although there is no direct relationship between nitric oxide and IL-13, these molecules may interact indirectly in the body, since both are involved in inflammatory and immunological processes.

When the normal weight and obese groups were compared, we observed that overweight individuals had greater plasma concentrations of C3 complement and hs-CRP ($p<0.05$). Differences were observed before and after treatment, indicating that kombucha consumption, *per se*, was not able to attenuate these markers. Complement C3 and hs-CRP are two common markers in clinical practice, whose high concentrations indicate an increased risk for cardiovascular disease (BRATTI et al., 2017; ELLULU et al., 2017; GUPTA et al., 2014).

Although this is not our primary objective, comparing the two groups helps us to better understand the kombucha's mechanism of action in different conditions. Obesity is considered a chronic disease, whose pathophysiology is associated with the development of other metabolic disorders mediated by inflammatory cytokines such as interleukin 6, TNF-α, and C-reactive protein (CRP) (KURYŁOWICZ; KÓZNIEWSKI, 2020). These cytokines trigger a chronic low-grade inflammatory response in adipose tissue and other organs such as the liver, muscle, and vascular tissue. This process, known as meta-inflammation, leads to insulin resistance, endothelial dysfunction, dyslipidemia, and other metabolic changes often associated with obesity and the development of chronic diseases (LI et al., 2018; PEREIRA et al., 2014; RUSSO et al., 2021).

On the other hand, some anti-inflammatory markers such as interleukins 4 and 10 and superoxide dismutase were also higher in the obese group, before and/or after kombucha intake. It is known that the balance between pro- and anti-inflammatory cytokines is an important mechanism for maintaining the body's homeostasis in the immune response and inflammation. This balance will not be necessarily quantitative in terms of biomarker concentration, but qualitative in the sense of causing an equilibrium between activation and inhibition of cytokines and other molecules involved in the immune and inflammatory response (CICCHESE et al., 2018). Therefore, the increase in anti-inflammatory cytokines observed in this study may be an attempt by the body to maintain homeostasis in response to other inflammatory markers that were elevated, such as complement C3 and hs-CRP.

Regarding the gut microbiota, both groups showed an increase in the relative abundance of Bacteroidota after kombucha consumption. This phylum, also known as Bacteroidetes, is present in great abundance in the gut microbiota and comprises a variety of bacterial genera, including Bacteroides, Prevotella, and Alistipes. Although some Bacteridota species can be opportunistic pathogens, most are symbiotic species involved in the degradation of proteins and carbohydrates, generating, in this case, short-chain fatty acids (KIRBY; HENDRIX; OCHOA-REPÁRAZ, 2019). An imbalance between Bacteroidota and Firmicutes, another phylum that comprises a large part of the intestinal microbiota, has been observed in obesity (MAGNE et al., 2020; STOJANOV; BERLEC; ŠTRUKELJ, 2020). A decrease in the abundance of Bacteroidota in individuals with colorectal cancer has also been reported (SHEN et al., 2010).

The Akkermanciaceae family was also favored after kombucha consumption, especially in the obese group. In general, the genus *Akkermansia*, which belongs to this family, has been attributed to several health benefits. *Akkermansia muciniphila*, especially, exerts probiotic properties and has been identified as a potential microorganism in the prevention and treatment of obesity and diabetes, since it is related to insulin sensitivity and decreased inflammation in obese individuals (DAO et al., 2016; ZHOU, 2017). Indeed, we observed a decrease in the serum insulin concentration in the obese group after kombucha intake, as well as an improvement in the markers of insulin resistance (HOMA-IR) and sensitivity (HOMA-β and QUICKI index) evaluated.

Although the mechanism of action of *A. muciniphila* is not completely understood, it is known that it can modulate mucosal thickness and intestinal integrity. Intestinal mucus is synthesized and excreted by goblet cells, whose composition, mucin, is one of the major sources of nutrients for *A. muciniphila*. Mucin degradation generates SCFA that will be used as an

energy source by the intestinal epithelium which, in turn, will produce more mucin, thus generating a feedback mechanism (ZHOU, 2017). A study carried out with obese and type II diabetic mice demonstrated that supplementation with *A. muciniphila* was able to reduce the concentration of serum lipopolysaccharides (LPS), an indicator of intestinal permeability. In the same study, the authors observed an improvement in glucose tolerance and a decrease in endogenous hepatic glucose production, which may have occurred through an LPS-dependent mechanism (EVERARD et al., 2013). *A. muciniphila* is also favored in the presence of phenolic compounds (KEMPERMAN et al., 2013; LIU et al., 2019, 2016; USHIRODA et al., 2019), which may explain the increase in Akkermanciacea abundance observed after kombucha intake. One hypothesis is that phenolic compounds increase mucus secretion by goblet cells, thereby creating a suitable environment for the growth of goblet cells (ANHÊ et al., 2016).

The genera *Ruminococcus* and *Dorea*, in turn, were increased in the obese group at baseline when compared to the normal weight group (T0 and T8) ($p<0.05$). However, there was no difference between the normal weight and obese groups at T8, indicating that kombucha consumption decreased the abundance of both genera. A recent systematic review, which included data from 60 articles, pointed out that *Ruminococcus* and *Dorea* are positively associated with obesity and its comorbidities, while *Akkermansia* is associated with normal weight individuals (XU et al., 2022). Interestingly, the authors noticed that *Ruminococcus* is associated with obesity in the Western population, but not in the Eastern population. In fact, the Western diet has been associated with a greater abundance of *Ruminococcus* (NEWMAN et al., 2021) and *Dorea* (VILLAMIL et al., 2018), possibly due to the high-fat content, which is typical of this diet. In another study, *Ruminococcus torques* and *Ruminococcus gnavus* showed a strong correlation with visceral fat (YAN et al., 2022). *Ruminococcus* was also positively associated with colorectal cancer (FLEMER et al., 2017; SHEN et al., 2010), non-alcoholic fatty liver disease (NAFLD) (PONZIANI et al., 2019), and even autism in children (WANG et al., 2013). *Dorea* was also positively correlated with inflammatory markers in obese women such as hs-CRP (highly sensitive CRP) and CD14 (differentiation cluster 14) (BRAHE et al., 2015), whereas concentrations of IL-6 and CCL-2 decreased concomitantly with the decrease of this gender in the microbiota (BAILEY et al., 2011). Other studies point to a positive relationship between *Dorea* and gastrointestinal tract diseases such as inflammatory bowel disease (RAJILIĆ-STOJANOVIĆ et al., 2011) and colorectal cancer (SHEN et al., 2010).

The genus *Subdoligranulum* was favored in the group obese, especially when compared to the normal weight group, both at T8 ($p<0.05$). Although not fully understood yet, studies

have been suggesting a potential beneficial effect associated with this genus on health (CANI; DE HASE; VAN HUL, 2021). Higher levels of *Subdoligranulum* were associated with improved metabolic risk parameters in both mice (EVERARD et al., 2011) and obese subjects (DAO et al., 2016), being negatively associated with CRP, FLI index, and body weight (LOUIS et al., 2016). In contrast, individuals with NAFLD had reduced levels of this microorganism (LOUIS et al., 2016). A recent study evaluated metagenome data from 108 obese individuals and highlighted that, among the microorganisms associated with *A. muciniphila*, *Subdoligranulum* was the one that presented the strongest correlation. The authors noticed that, on several occasions, a high abundance of *A. muciniphila* occurred concomitantly with the presence of *Subdoligranulum sp*. (VAN HUL et al., 2020). As mentioned about *A. muciniphila*, *Subdoligranulum* has been associated with improved glycemic response in obese individuals, including HOMA-IR (LOUIS et al., 2016) and glycated hemoglobin (ZHANG et al., 2017). Antidiabetic drugs such as metformin and acarbose also increased the relative abundance of *Subdoligranulum* (FORSLUND et al., 2015; ZHANG et al., 2017). *S. variable*, the only known species of the genus, is a butyrate producer (HOLMSTRØM et al., 2004), whose abundance is generally reduced in several chronic diseases, including type II diabetes and obesity (COPPOLA et al., 2021).

Finally, when considering the LEfSe analysis, the genus *Parabacteroides* and its family Tannerellaceae were identified as biomarkers in the normal weight group at T8. Although most of the *Parabacteroides* species are pathogenic, some of them have been identified as potential probiotics, such as *P. diastanosis* and *P. goldsteinii* MTS01 (LAI et al., 2022; WU, 2023).

Prevotellaceae was the only biomarker identified in the group obese T8, indicating that kombucha consumption favored their growth. *Prevotella*, one of the four genera belonging to this family, has been associated with a healthy and fiber-rich diet since it participates in the metabolism of complex carbohydrates and cellulose (DE FILIPPO et al., 2010). Other positive outcome related to *Prevotella* includes an improved glucose metabolism in mice by promoting hepatic glycogen storage (KOVATCHEVA-DATCHARY et al., 2015), and it likely acts on insulin sensitivity in conjunction with the diet (STANISLAWSKI et al., 2019). In obese subjects, *Prevotella* was positively associated with HDL-c (LOUIS et al., 2016). However, this is the first time that we observed an increase in Prevotellaceae associated with kombucha consumption; in our study conducted with Wistar rats, Prevotellaceae and *Prevotella* were identified as biomarkers in the group that received a standard diet (AIN-93M), but not in the groups that received kombucha (COSTA et al., 2022).

Among the yeasts favored by kombucha consumption, Ascomycota and Saccharomyces are part of a healthy adult microbiota. Saccharomyces, especially, had their relative abundance greatly increased after kombucha consumption compared to baseline ($p<0.01$). Both microorganisms become abundant after solid food introduction in childhood, indicating that they are indeed influenced by diet.

Although the difference was not significant among the groups, the relative abundance of Debaryomycetaceae and *Candida* decreased after kombucha intake in both normal weight and obese subjects when compared to the same group on the baseline. *Debaryomyces* has been linked to the Western diet and gastrointestinal inflammation. *D. hansenii*, a fungus widely used in the food industry, has been linked to mucosal inflammation and Crohn's disease via CCL5 (JAIN et al., 2021). *Candida* is part of a healthy microbiota, but in abundance, has been identified as an opportunistic microorganism, causing a reduction in diversity, and unbalancing the gut microbiota. Studies show a relationship between *Candida spp.* and the Western diet, which, in turn, is related to the development of obesity and associated comorbidities (GARCÍA-GAMBOA et al., 2021; ZHANG et al., 2022).

Kombucha consumption was also associated with a decrease in *Exophiala*, whose abundance was especially increased in the group obese T0 ($p<0.05$). In general, *Exophiala spp*. has been associated with infectious and pulmonary diseases, especially cystic fibrosis (AYLING-SMITH et al., 2022; KONDORI et al., 2011; LEBECQUE et al., 2010). It seems that this is the first study in which *Exophiala* was associated with obesity.

Rhodotorula and *Aspergillus* showed the same tendency: both genders presented a high abundance at baseline and decreased after kombucha consumption, although a significant difference was observed only for *Rhodotorula* ($p<0.05$). *Rhodotorula spp*. were identified by some studies as an opportunistic fungus and have been related to obesity (GARCÍA-GAMBOA et al., 2021; HOF, 2019).

Both groups have several biomarkers, as shown in Figure 15. Most of them have already been highlighted in terms of relative abundance, such as *Saccharomyces* and *Ascomycota.* Others, despite not being as abundant, were identified as biomarkers after kombucha consumption in relation to the same group at baseline, such as *Dekkera* and *Pichia*. *Dekkera bruxellensis* was the main yeast found in kombuchas and SCOBYs analyzed by our research group, accounting for more than 99% in both (COSTA et al., 2022). *Pichia* has also been identified as one of the top seven yeasts in commercial kombucha and seems to be involved in the SCOBY structure (MAYSER et al., 1995; WANG et al., 2022). Thus, it is very likely that

these microorganisms found in the gut microbiota of the participants were favored by kombucha microbiota.

Although we noticed a modulation of the gut microbiota, there was no statistical difference regarding intestinal permeability and SCFA. Several hypotheses can be attributed to this, such as the fact that most SCFA is absorbed in the intestinal epithelium rather than being excreted on stool (RAHMAN et al., 2022b). Thus, we could notice an indirect association favoring microorganisms that benefit from the production of SCFA, such as the fungal community. In fact, a great impact was noticed on the fungal microbiota after kombucha consumption, which may have been generated not only by the kombucha itself but also by the competition for nutrients (for example, phenolic compounds), favoring certain groups according to the features that they present. Similar to our study, Kovatcheva-Datchary et al. (2015) observed changes in the composition of the gut microbiota, but not in the production of SCFA, attributing this to the inter-individual variability of the participants.

Finally, it is important to mention that the lack of consensus in the literature concerning the role of certain microorganisms in health and disease suggests a critical approach that considers not only the microorganisms themselves but the intra and interspecies relationship between bacteria and fungi, as suggested by Zhang et al. (2022). Equally important is to observe the results considering the general context of the diet and other factors that may interfere with the results. Although the participants were instructed to maintain the same pattern of eating and physical activity that they had before the study, we know that this could have been a challenge, especially during the SARS-CoV-2 pandemic, when this study was conducted.

In our first study in the area, we evaluated the impact of the diet, specifically the dietary inflammatory index (DII) on the results, since, as mentioned, the participants were not submitted to any dietary intervention. As expected, a diet with a high inflammatory index was associated with worse results, indicating that kombucha, at least at the dose and time administered, was not able to attenuate the negative effects of an unbalanced diet (data in publication phase). Future studies should evaluate other approaches such as intervention time and dosage since the only publication about it was made by the CDC in 1995 (CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC), 1995).

7.13 CONCLUSION

Regular consumption of black tea kombucha for eight consecutive weeks was able to modulate some of the metabolic markers evaluated in this study, such as insulin and GGT in the obese group and IL-13 and nitric oxide in the normal weight group. Regarding the gut microbiota, it was able to reduce the abundance of microorganisms associated with obesity such as *Ruminococcus* and *Dorea,* especially in the obese group. In addition, microorganisms related to insulin sensitivity such as *Akkermansia*, *Subdoligranulum,* and Prevotellaceae were favored, which may explain the positive outcomes that we observed in the obese group. Other studies are needed to confirm those results, as well as to test other dosages and intervention time, associated or not to dietary intervention.

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8. OVERALL CONCLUSION

In conclusion, the kombuchas analyzed in this study exhibited a significant antioxidant capacity, primarily due to their high content of phenolic compounds. These phenolic compounds likely work synergistically with the organic acids such as acetic, lactic, and glucuronic acids, as well as the microorganisms involved in the fermentation process. Both kombuchas and SCOBYs displayed a rich diversity of microorganisms, indicating the presence of a thriving microbial community. Some of them, such as *Pichia* and *Dekkera* were also found in the human stool samples, suggesting that they were able to reach the intestine and modulate the microbiota.

In Wistar rats, the regular consumption of green and black tea kombuchas for ten weeks was able to attenuate the impacts of a high-fat high-fructose diet. Both kombuchas improved intestinal health by increasing propionate production and favoring the growth of beneficial bacteria, such as *Adlercreutzia* in the group that consumed green tea kombucha.

In humans, the regular consumption of black tea kombucha for eight weeks was associated with some health outcomes. Obese individuals presented lower concentrations of gamma-glutamyl transferase and insulin, which was also reflected in the indexes of insulin resistance (HOMA-IR) and sensitivity (HOMA-β and QUICKI) evaluated. Normal weight individuals presented higher IL-13 and nitric oxide concentrations in plasma. Some negative outcomes were also noticed in the normal weight group, which presented an increase in total cholesterol and alkaline phosphatase concentrations. However, those results were associated with a pro-inflammatory diet, as evaluated in our previous study. Although kombucha consumption can attenuate the negative impact of a Western diet, as proved in our experimental study, we reinforce the importance of a balanced diet for overall health.

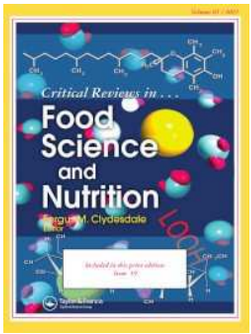
Kombucha could also modulate the human gut microbiota, favoring beneficial microorganisms such as *Parabacteroides* in the normal weight group and decreasing the relative abundance of microorganisms associated with obesity such as *Ruminococcus* and *Dorea,* especially in the obese group. In obese individuals, kombucha favored the growth of microorganisms involved in insulin sensitivity, such as Prevotellaceae, Akkermanciaceae, and *Subdoligranulum,* which may explain the positive outcomes on insulin markers. The fungal diversity was also increased after kombucha consumption and influenced the abundance of several fungi, especially an increased abundance of *Saccharomyces* and a decrease of *Exophiala* and *Rhodotorula*.

9. SUPLEMMENTARY MATERIALS

Part of the results generated through this study was also evaluated on the master's degree of Gabriela Macedo Fraiz, from the Department of Nutrition and Health at Universidade Federal de Viçosa. The paper entitled "Black tea kombucha consumption: effects on cardiometabolic parameters, considering the diet's quality of individuals with and without obesity", by Gabriela M. Fraiz, Mirian A. C. Costa, Rodrigo R. Cardoso, James R. Hebert, Longgang Zhao, Frederico A. R. Barros, and Josefina Bressan is currently under review.

In this paper, we investigated the impact of the diet on the metabolic parameters of the participants through the Dietary Inflammatory Index (DII) and the Total Antioxidant Capacity of the Diet (DTAC). We concluded that some of the negative outcomes noticed in the normal weight group, such as an increase in total cholesterol and alkaline phosphatase, were only observed in those individuals whose DII and DTAC scores were increased, i.e., those who have a pro-inflammatory and pro-oxidant diet. Those results reinforce the importance of allying kombucha supplementation with a healthy diet.

In addition, we also conducted a systematic review to investigate the effects of kombucha consumption on the gut microbiota and obesity-related comorbidities as part of the discipline "MBI 796 – Microbiology of Fermented Foods and Benefits to the Health", coordinated by Prof. José Guilherme Prado Martin. The paper was published in the journal Critical Reviews in Food Science and Nutrition, 2021, Oct 26; 1-16, doi: <10.1080/10408398.2021.1995321>.



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Effect of kombucha intake on the gut microbiotaand obesity-related comorbidities: A systematic review

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APPENDICES

APPENDIX A – PRE-SELECTION QUESTIONNAIRE (ON-LINE)

Olá!

Primeiramente, nós agradecemos o seu contato.

Você foi convidado (a) a participar de uma pesquisa do Departamento de Tecnologia de Alimentos em conjunto com o Departamento de Nutrição e Saúde da UFV. Nosso objetivo é investigar o efeito do consumo de kombucha na saúde humana. Por isso, gostaríamos de fazer algumas perguntas para conhecê-lo (a) melhor. Por favor, pedimos que você seja sincero (a) e que responda às questões com o máximo de detalhes possível, quando for o caso.

O preenchimento desse questionário leva cerca de 10 a 15 minutos. Garantimos que suas respostas serão tratadas de forma totalmente anônima e sigilosa e que nenhum dado será divulgado. Caso tenha alguma dúvida em relação ao questionário, envie um e-mail para projetokombuchaufv@gmail.com

Ressaltamos que este questionário se trata de uma pré-seleção e seu preenchimento não garante participação na pesquisa.

Bloco 1 – Dados pessoais

Nome completo* _____

Telefone* _____ Melhor horário para entrar em contato* _____

E-mail* _____

Idade (anos)* _____

Sexo* ( ) Masculino ( ) Feminino

Bloco 2 – Informações gerais

Você é fumante?* ( ) Sim ( ) Não

Para mulheres:

Você está grávida ou amamentando? ( ) Sim ( ) Não

Pretende engravidar nos **próximos 3 meses**? ( ) Sim ( ) Não

Seu peso* _____

Sua altura* _____

Nos últimos 3 meses, você ganhou ou perdeu peso?*

( ) Sim ( ) Não

Caso tenha respondido sim, quantos kg você ganhou ou perdeu **nos últimos 3 meses**? _____

Bloco 3 – Alimentação e estilo de vida

Atualmente você está seguindo dieta para perda de peso ou alguma outra finalidade?*

( ) Sim, estou seguindo dieta prescrita por nutricionista.

( ) Sim, estou fazendo dieta por conta própria.

( ) Não, estou me alimentando como de costume.

Nos últimos 3 meses, houve alguma alteração brusca no seu hábito alimentar? Por exemplo, você incluiu algum alimento no dia-a-dia ou excluiu algum alimento/nutriente da sua dieta?*

( ) Sim ( ) Não

Caso tenha respondido sim, explique o que mudou e o porquê.

Você tem o hábito de consumir kombucha?*

( ) Sim ( ) Não

Caso tenha respondido sim, especifique a frequência e quantidade. _____

Você faz uso de algum suplemento alimentar (vitaminas, ômega 3 ou outro)?*

( ) Sim ( ) Não

Caso tenha respondido sim, especifique qual suplemento, frequência e quantidade. _____

Nos últimos 3 meses, você mudou seu padrão de atividade física*?

( ) Sim, comecei a praticar algum esporte/entrei na academia.

( ) Sim, parei de praticar algum esporte/academia por vontade própria

( ) Sim, parei de praticar algum esporte/academia por motivo de doença ou lesão muscular.

( ) Não, não houve nenhuma mudança no meu padrão de atividade física nos últimos 3 meses.

Bloco 4 – Uso de medicamentos

No último mês você apresentou algum episódio alérgico ou infeccioso?*

( ) Sim ( ) Não

Caso tenha respondido sim, especifique. _____

Atualmente você está fazendo uso regular de algum medicamento?*

Caso tenha respondido sim, especifique qual(is) e o motivo. _____

Nos últimos 3 meses, você fez uso de algum antibiótico? Exemplo: Amoxicilina, Azitromicina, Cefalexina, Ciprofloxacino, Ampicilina, Tetraciclina ou outro(s).*

( ) Sim ( ) Não

Este é o fim do questionário. Obrigada por participar. Entraremos em contato em breve.

APPENDIX B – MEDICAL AND NUTRITIONAL QUESTIONNAIRE

Nome:_____ **ID:** _____

Data de nascimento: _____ **Data da entrevista:** _____

Tabagismo:

( ) não fumante

( ) fumante Cigarros/dia: _____ Fuma desde: _____ (ano)

( ) ex-fumante Cigarros/dia (antes): _____ Parou: _____ (ano)

Em caso de mulheres:

Gestante ou lactante? ( ) Sim ( ) Não

Pretende engravidar nos próximos 3 meses? ( ) Sim ( ) Não

Você já apresentou ou apresenta algumas destas doenças?

Doenças Pré-existentes	Não	Sim	Especificar (se necessário)	Se sim, data do diagnóstico
Hipertensão arterial sistêmica				
Doença cardiovascular (ex. insuficiência coronariana, insuficiência cardíaca congestiva, AVC, infarto agudo do miocárdio etc.)				
Dislipidemia				
Hipoglicemia				
Diabetes *mellitus*				
Tireoidopatias				
Gota				
Alergia				
Anorexia/Bulimia				
Doenças psiquiátricas (ex. esquizofrenia, transtorno de ansiedade, depressão)				
Anemia				

Osteoporose				
Doença renal				
Doença hepática				
Doença celíaca				
Doença intestinal crônica				
Câncer				
Alterações no TGI (ex. retirada de vesícula)				
Outras doenças *				

Histórico familiar (pais/irmãos)

Doença	Não	Sim
Hipertensão arterial sistêmica		
Doença cardiovascular (ex. insuficiência coronariana, insuficiência cardíaca congestiva, AVC, infarto agudo do miocárdio etc.)		
Dislipidemia		
Diabetes *mellitus*		

Atualmente, você faz uso de algum medicamento?

Medicamentos	Posologia (dose e frequência)	Tempo de uso	Motivo

Histórico alimentar

Tem o hábito de consumir kombucha?

( ) Sim ( )Não

Se sim, com que frequência? _____ Quantidade: _____

Tem aversão à kombucha? ( ) Sim ( )Não ( ) Não conhece a bebida

Você consome bebida alcoólica? ( ) Sim ( ) Não

Se sim, qual tipo e com que frequência?

Bebida	Quantidade	Frequência	g álcool
Cerveja			
Cachaça			
Caipirinha			
Rum/Vodca			
Ice			
Vinho			
Whisky			
Catuaba			
Licor			

Você tem alguma intolerância ou alergia alimentar? Favor excluir da resposta alimentos que você apenas não gosta. ( ) Sim ( ) Não

Quais e sintomas:

_____

_____

Existe algum alimento (s) que você não gosta ou não ingere por motivo religiosos/filosóficos?

( ) Sim ( ) Não Se sim, qual (is)?

_____

Indique as horas do dia em que você consome refeições e lanches. Coloque a letra R para refeições e L para lanches sob cada hora do dia.

Manhã e início da tarde

1	2	3	4	5	6	7	8	9	10	11	12
___	__	__	__	__	__	__	__	__	__	__	__

Tarde e noite

1	2	3	4	5	6	7	8	9	10	11	12
__	__	__	__	__	__	__	__	__	__	__	__

Você perdeu ou ganhou peso nos últimos 3 meses? ( ) Sim ( ) Não

( ) Perdeu ____Kg ( ) Ganhou ____Kg

Atualmente você está seguindo alguma dieta para perda de peso ou outra finalidade?

( ) Sim ( ) Não Especifique: _____

Nos últimos 3 meses, houve alguma mudança no seu hábito alimentar?

( ) Sim ( ) Não Especifique: _____

Nos últimos 3 meses, houve alguma mudança no seu padrão de atividade física?

( ) Sim ( ) Não Especifique: _____

Nos últimos 3 meses, você apresentou algum episódio alérgico ou infeccioso?

( ) Sim ( ) Não Especifique: _____

Você fez uso de algum antibiótico nos últimos 3 meses? (ex.: Amoxicilina, Azitromicina, Cefalexina, Ciprofloxacino, Ampicilina, Tetraciclina etc.)

( ) Sim ( ) Não Especifique: _____

Você utiliza alguma forma de suplemento alimentar? (ex: vitaminas, minerais, proteínas, ômega 3 etc.) ( ) Sim ( ) Não

Se sim, especifique:

Marca do produto	Tipo de suplemento	Dosagem	Frequência de uso

Dados socioeconômicos e demográficos

Escolaridade:

( ) Analfabeto
( ) Sabe ler e escrever
( ) Fundamental – Incompleto
( ) Fundamental – Completo
( ) Médio – Incompleto
( ) Médio – Completo
( ) Técnico
( ) Superior – Incompleto
( ) Superior – Completo
( ) Pós-graduação (Lato senso) – Incompleto
( ) Pós-graduação (Lato senso) – Completo
( ) Pós-graduação (Stricto sensu, nível mestrado) – Incompleto
( ) Pós-graduação (Stricto sensu, nível mestrado) – Completo
( ) Pós-graduação (Stricto sensu, nível doutor) – Incompleto
( ) Pós-graduação (Stricto sensu, nível doutor) – Completo

Ocupação: _____

Quantas pessoas vivem na sua casa (incluindo o participante): _____

Renda familiar: _____

Estado civil:

( ) Solteiro (a) ( ) Divorciado (a)
( ) Casado (a) ou união estável ( ) Viúvo (a)

Composição corporal

Antropometria		BIA	
Estatura (m)		Gordura (%)	
Peso (kg)		Gordura (kg)	
IMC		MM (kg)	
PC		TMB (kcal/dia)	
PQ		Água (% peso)	
Pressão Arterial			

APPENDIX C – CONSENT FORM

Nome: _____ Data: ____/____/_____

Você está sendo convidado (a) a participar como participante do estudo ***"Efeito do consumo de kombuchas de chás verde e preto na microbiota intestinal e nas alterações metabólicas de indivíduos eutróficos e obesos"****. Todas as informações necessárias sobre a pesquisa encontram-se descritas abaixo e em caso de dúvida, favor esclarecê-las antes da assinatura do presente termo.*

A kombucha é uma bebida fermentada a base de chá verde ou chá preto, consumida há milhares de anos. Estudos recentes têm relatado diversos efeitos benéficos associados à ingestão de kombucha para a saúde. Dessa forma, o objetivo dessa pesquisa é investigar se a ingestão de kombucha por 8 semanas consecutivas está associada a mudanças favoráveis no organismo de indivíduos adultos que se encontram dentro ou acima do peso.

O estudo será conduzido no Laboratório de Metabolismo Energético e de Composição Corporal (LAMECC) do Departamento de Nutrição e Saúde em conjunto com o Departamento de Tecnologia de Alimentos, ambos na UFV. Caso aceite participar do estudo, você deverá comparecer ao LAMECC em duas ocasiões diferentes (no início e ao final do estudo) para avaliação do peso, altura e medidas corporais. Na primeira visita, será solicitado a você o preenchimento de questionários com perguntas relacionadas à sua saúde, alimentação e estilo de vida. A avaliação nutricional e a entrevista têm duração média de 1 hora. Será realizada ainda coleta de sangue, urina e fezes de todos os participantes e biópsia do tecido adiposo subcutâneo (gordura localizada logo abaixo da pele) de indivíduos acima do peso. O sangue será utilizado em análises bioquímicas, como glicemia, colesterol e triglicerídeos, e em análises de marcadores do estresse oxidativo (situação em que há excesso de radicais livres no organismo). A urina também será utilizada em análises de marcadores do estresse oxidativo e para análise da permeabilidade intestinal (integridade da parede intestinal). As fezes serão utilizadas para análise de ácidos graxos de cadeia curta (gorduras presentes nas fezes) e avaliação da microbiota intestinal (bactérias que habitam o intestino). O tecido adiposo (gordura abaixo da pele) será utilizado em análises histológicas (análise detalhada do tecido com auxílio de um microscópio) e em análises de micro RNAs relacionados à obesidade (material genético extraído a partir do DNA que estão relacionados à obesidade). As análises citadas têm por objetivo avaliar sua saúde antes e após o consumo da kombucha.

Os procedimentos invasivos serão realizados por pessoas treinadas, minimizando ao máximo eventuais desconfortos. A coleta de sangue será realizada ao início e ao final do estudo por um técnico em enfermagem, utilizando apenas materiais descartáveis, sendo possível uma sensação incômoda ou dolorida na hora de inserir a agulha e formação de hematomas no local da entrada da agulha que desaparecerão espontaneamente. Será solicitado ao técnico ser o mais preciso possível para minimizar qualquer incômodo. No caso de eventuais complicações no momento da punção venosa, serão prestados os primeiros socorros pelo técnico em enfermagem. Em caso de quaisquer outras complicações decorrentes do procedimento, você receberá assistência médica imediata e integral cujos custos serão de responsabilidade dos pesquisadores.

A biópsia do tecido adiposo será realizada ao início e ao final do estudo por um médico cirurgião. Esse procedimento será restrito aos indivíduos acima do peso. O procedimento será realizado na Divisão de Saúde da UFV, utilizando apenas materiais descartáveis. Será administrada anestesia local antes do procedimento, sendo possível uma sensação incômoda ou dolorida no local de entrada da agulha. Será solicitado ao médico ser o mais preciso possível para minimizar qualquer incômodo. O médico irá coletar cerca de 1 a 3 gramas de tecido adiposo subcutâneo (camada de gordura localizada abaixo da pele) da região periumbilical (região dos "flancos") com uma agulha específica. Pode haver formação de hematomas que desaparecerão espontaneamente, e risco de infecção no local da biópsia que serão minimizados esterilizando a área antes do procedimento e fazendo curativo no local. Além disso, o médico dará orientações quanto aos devidos cuidados que você deverá tomar para minimizar o risco de infecção. Em caso de eventuais complicações relacionadas ao procedimento, o médico prestará assistência necessária e todos os custos serão de responsabilidade dos pesquisadores.

Os demais procedimentos do estudo não envolvem riscos à saúde.

A coleta da urina será realizada em duas ocasiões distintas, no começo e ao fim do estudo. Nos dias agendados, você deverá comparecer ao LAMECC após jejum noturno de 12 horas e deverá permanecer no laboratório durante 4 horas. Durante esse período, toda a urina será coletada em recipientes fornecidos pela nossa equipe.

A avaliação do peso, altura e medidas corporais serão realizadas por profissional treinada, em uma sala fechada e silenciosa, com o intuito de preservar sua privacidade e minimizar qualquer constrangimento no momento das aferições. Durante a resposta dos questionários, você poderá deixar de responder a uma ou a um conjunto de perguntas caso sinta-

se constrangido (a) sem que isso traga qualquer alteração de tratamento por parte dos pesquisadores.

As kombuchas fornecidas durante o estudo terão boa procedência e qualidade e serão devidamente acondicionadas visando a manutenção da qualidade nutricional e microbiológica. Ressalta-se que será uma bebida não-alcóolica.

Todos os indivíduos incluídos ou não no estudo receberão gratuitamente uma avaliação nutricional, antropométrica e de composição corporal. Os participantes incluídos no estudo receberão ainda uma cópia dos exames realizados durante a pesquisa e, caso seja de interesse, será oferecido também acompanhamento nutricional durante 30 dias ao final do estudo. Acreditamos ainda, com base em estudos preliminares, que a ingestão de kombucha por oito semanas consecutivas proporcionará benefícios à saúde dos participantes.

O material biológico coletado (sangue, urina, fezes e tecido adiposo) será armazenado em ultra freezers do Departamento de Nutrição e Saúde da UFV até o momento das análises. Dessa forma, ao assinar o presente termo, você autoriza a coleta, depósito, armazenamento e utilização do material biológico coletado (sangue, urina, fezes e tecido adiposo, quando for o caso) em posteriores análises. Ressaltamos que, a qualquer momento, você pode retirar seu consentimento para a guarda, utilização e/ou armazenamento do material biológico e essa atitude não lhe trará qualquer prejuízo. A solicitação deve ser feita por escrito e assinada, com validade a partir da data da comunicação de sua decisão ao pesquisador.

Os dados e instrumentos utilizados na pesquisa ficarão arquivados com o pesquisador responsável por um período de 5 (cinco) anos após o encerramento do estudo e depois desse tempo, serão destruídos. Nós trataremos sua identidade com sigilo e confidencialidade, atendendo à legislação brasileira, em especial, à Resolução 466/2012 do Conselho Nacional de Saúde e utilizaremos as informações somente para fins acadêmicos e científicos. Seus dados ficarão guardados em local seguro, onde apenas os pesquisadores terão acesso. Além disso, você será identificado pelas iniciais de seu nome e/ou número de registro a fim de garantir a confidencialidade dos dados.

Em caso de danos decorrentes da pesquisa, você receberá assistência integral e imediata pelo tempo que for necessário, cujos custos serão de responsabilidade dos pesquisadores. Destacamos que você terá direito à indenização caso ocorram eventuais danos, previstos ou não, decorrentes da pesquisa.

A equipe de trabalho não se responsabiliza por informações não prestadas por você, que possam interferir na sua saúde.

A decisão de participar desse estudo é completamente voluntária. Esse estudo não trará nenhum custo ou prejuízo financeiro e todos os materiais serão fornecidos pela equipe da pesquisa. Todos os gastos decorrentes à sua participação na pesquisa, incluindo gastos com transporte e alimentação para você e seu (s) acompanhante (s), quando for o caso, serão ressarcidos.

Você pode se recusar a participar ou poderá sair do estudo a qualquer momento, mesmo depois de dar o seu consentimento, e essa atitude não lhe trará prejuízos futuros. Em caso de interrupção do estudo, você receberá assistência de forma adequada, pelo tempo que for necessário, cujos custos serão de responsabilidade dos pesquisadores.

A qualquer momento você poderá fazer perguntas sobre o estudo ou esclarecer dúvidas. Para isso, poderá entrar em contato pelo telefone (31) 97524 8244.

Esse termo de consentimento encontra-se impresso em duas vias originais, sendo que uma será arquivada pelo pesquisador responsável e a outra será fornecida a você. Todas as páginas deverão ser rubricadas por você e pelo pesquisador responsável ou pela doutoranda da pesquisa.

Eu, _____, contato _____, fui informado (a) dos objetivos da pesquisa ***"Efeito do consumo de kombuchas de chás verde e preto na microbiota intestinal e nas alterações metabólicas de indivíduos eutróficos e obesos"*** de maneira clara e detalhada, e esclareci minhas dúvidas. Sei que a qualquer momento poderei solicitar novas informações e modificar minha decisão de participar se assim o desejar. Declaro que concordo em participar. Recebi uma via original deste termo de consentimento livre e esclarecido e me foi dada a oportunidade de ler e esclarecer minhas dúvidas.

Pesquisador Responsável: Professor Frederico Augusto Ribeiro de Barros
Endereço: Universidade Federal de Viçosa. Edifício Beck Andersen, 2° andar. Avenida PH Rolfs, s/n – Campus Universitário. CEP: 36570-000 Viçosa - MG
Telefone: (31) 3612-6803
E-mail: fredbarros@ufv.br

Em caso de discordância ou irregularidades sobre o aspecto ético dessa pesquisa, você poderá consultar:
Comitê de Ética em Pesquisa com Seres Humanos – CEP/UFV

Universidade Federal de Viçosa - Edifício Arthur Bernardes, piso inferior. Avenida PH Rolfs, s/n – Campus Universitário. CEP: 36570-000 Viçosa - MG
Telefone: (31) 3612-2316
E-mail: cep@ufv.br
Website: www.cep.ufv.br

Viçosa, _____ de _____ de _____.

_____	_____
Mirian Aparecida de Campos Costa Doutoranda PPGCTA/UFV	Participante (a)

APPENDIX D – FOOD RECORD

Orientações para realização do registro alimentar

- Quando você relatar um alimento ou bebida, seja o mais claro e preciso possível. Forneça o máximo de informações sobre os alimentos. Cite marcas em casos de produtos industrializados e tamanho das porções. No caso de receitas caseiras, cite os ingredientes utilizados, por exemplo, molhos e coberturas com leite, creme de leite, algum tipo de espessante (farinha de trigo, maisena etc.).
- Anote também o tipo de alimento (integrais ou não, diet ou light, integral, desnatado ou semidesnatado).
- Relate se as bebidas como café e suco são adoçadas com açúcar (refinado, cristal, mascavo) ou adoçante (marca) e indique a quantidade ou proporção.
- Cite a forma de preparo de carnes (assada, cozida, frita, grelhada, à milanesa) e hortaliças (crua, cozida, frita, ensopada, refogada) e se foram consumidas com azeite, maionese, margarina, *ketchup*.
- Anote tudo no momento em que estiver comendo. Não deixe para anotar depois que tiver acabado de comer. Observe quanto foi servido de cada alimento ou bebida e se consumiu tudo que foi servido ou se sobrou e anote a quantidade consumida.
- Anote todos os alimentos e bebidas que consumir o dia inteiro, incluindo até mesmo balas, doces, chicletes. Só não é necessário anotar a ingestão de água.
- Informe com precisão, sempre que possível, o peso dos alimentos e o volume dos líquidos ingeridos.
- Coloque o tamanho dos alimentos (pequeno, médio e grande) caso não saiba o peso. Exemplo: uma banana média; 1 fatia grande de bolo de chocolate sem cobertura e sem recheio.
- Descreva as quantidades em medidas caseiras, como colher de sopa, chá, sobremesa, ou de servir, se estava rasa ou cheia ou outras formas de medida utilizando utensílios domésticos. O mesmo vale para conchas, escumadeiras, copos (americano/requeijão), pratos (fundo/raso) e xícaras. Exemplo: 4 colheres de sopa de arroz branco cozido; 1 unidade de maçã pequena; 1 xícara de café adoçado com 1 colher de chá de açúcar.
- Qualquer dúvida entre em contato.

Registro alimentar de 24 horas

	ALIMENTOS	MEDIDA CASEIRA	QUANT (g)
Horário: **Local:**			
Horário: **Local:**			
Horário: **Local:**			
Horário: **Local:**			
Horário: **Local:**			
Horário: **Local:**			

APPENDIX E – DAILY KOMBUCHA CONSUMPTION REGISTRY

Nome: _____ **Início do estudo:** _____/_____/_____

Semana 1		
Dia	Consumo	Horário
Segunda-feira	( ) S ( ) N	
Terça-feira	( ) S ( ) N	
Quarta-feira	( ) S ( ) N	
Quinta-feira	( ) S ( ) N	
Sexta-feira	( ) S ( ) N	
Sábado	( ) S ( ) N	
Domingo	( ) S ( ) N	

Semana 2		
Dia	Consumo	Horário
Segunda-feira	( ) S ( ) N	
Terça-feira	( ) S ( ) N	
Quarta-feira	( ) S ( ) N	
Quinta-feira	( ) S ( ) N	
Sexta-feira	( ) S ( ) N	
Sábado	( ) S ( ) N	
Domingo	( ) S ( ) N	

Semana 3		
Dia	Consumo	Horário
Segunda-feira	( ) S ( ) N	
Terça-feira	( ) S ( ) N	
Quarta-feira	( ) S ( ) N	
Quinta-feira	( ) S ( ) N	
Sexta-feira	( ) S ( ) N	
Sábado	( ) S ( ) N	
Domingo	( ) S ( ) N	

Semana 4		
Dia	Consumo	Horário
Segunda-feira	( ) S ( ) N	
Terça-feira	( ) S ( ) N	
Quarta-feira	( ) S ( ) N	
Quinta-feira	( ) S ( ) N	
Sexta-feira	( ) S ( ) N	
Sábado	( ) S ( ) N	
Domingo	( ) S ( ) N	

Semana 5		
Dia	Consumo	Horário
Segunda-feira	( ) S ( ) N	
Terça-feira	( ) S ( ) N	
Quarta-feira	( ) S ( ) N	
Quinta-feira	( ) S ( ) N	
Sexta-feira	( ) S ( ) N	
Sábado	( ) S ( ) N	
Domingo	( ) S ( ) N	

Semana 6		
Dia	Consumo	Horário
Segunda-feira	( ) S ( ) N	
Terça-feira	( ) S ( ) N	
Quarta-feira	( ) S ( ) N	
Quinta-feira	( ) S ( ) N	
Sexta-feira	( ) S ( ) N	
Sábado	( ) S ( ) N	
Domingo	( ) S ( ) N	

Semana 7		
Dia	Consumo	Horário
Segunda-feira	( ) S ( ) N	
Terça-feira	( ) S ( ) N	
Quarta-feira	( ) S ( ) N	
Quinta-feira	( ) S ( ) N	
Sexta-feira	( ) S ( ) N	
Sábado	( ) S ( ) N	
Domingo	( ) S ( ) N	

Semana 8		
Dia	Consumo	Horário
Segunda-feira	( ) S ( ) N	
Terça-feira	( ) S ( ) N	
Quarta-feira	( ) S ( ) N	
Quinta-feira	( ) S ( ) N	
Sexta-feira	( ) S ( ) N	
Sábado	( ) S ( ) N	
Domingo	( ) S ( ) N	

ANNEXES

ANNEX 1 – THREE FACTOR EATING QUESTIONNAIRE - R21

QUESTIONÁRIO TFEQ-R21 - VERSÃO EM PORTUGUÊS

Esta seção contém declarações e perguntas sobre hábitos alimentares e sensação de fome. Leia cuidadosamente cada declaração e responda marcando a alternativa que melhor se aplica a você.

1. Eu deliberadamente consumo pequenas porções para controlar meu peso.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

2. Eu começo a comer quando me sinto ansioso.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

3. Às vezes, quando começo a comer, parece que não conseguirei parar.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

4. Quando me sinto triste, frequentemente como demais.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

5. Eu não como alguns alimentos porque eles me engordam.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

6. Estar com alguém que está comendo, me dá frequentemente vontade de comer também.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes

- ☐ Totalmente falso

7. Quando me sinto tenso ou estressado, frequentemente sinto que preciso comer.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

8. Frequentemente sinto tanta fome que meu estômago parece um poço sem fundo.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

9. Eu sempre estou com tanta fome, que me é difícil parar de comer antes de terminar toda a comida que está no prato.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

10. Quando me sinto solitário (a), me consolo comendo.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

11. Eu conscientemente me controlo nas refeições para evitar ganhar peso.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

12. Quando sinto o cheiro de um bife grelhado ou de um pedaço suculento de carne, acho muito difícil evitar de comer, mesmo que eu tenha terminado de comer há muito pouco tempo.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

13. Estou sempre com fome o bastante para comer a qualquer hora.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

14. Se eu me sinto nervoso (a), tento me acalmar comendo.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

15. Quando vejo algo que me parece muito delicioso, eu frequentemente fico com tanta fome que tenho que comer imediatamente.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

16. Quando me sinto depressivo (a), eu quero comer.

- ☐ Totalmente verdade
- ☐ Verdade, na maioria das vezes
- ☐ Falso, na maioria das vezes
- ☐ Totalmente falso

17. O quanto frequentemente você evita "estocar" (ou se aprovisionar de) comidas tentadoras?

- ☐ Quase nunca
- ☐ Raramente
- ☐ Frequentemente
- ☐ Quase sempre

18. O quanto você estaria disposto (a) a fazer um esforço para comer menos do que deseja?

- ☐ Não estou disposto (a)
- ☐ Estou um pouco disposto (a)
- ☐ Estou relativamente bem-disposto (a)
- ☐ Estou muito disposto (a)

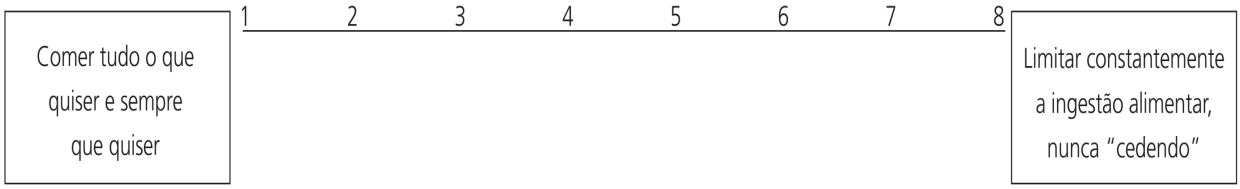
19. Você comete excessos alimentares, mesmo quando não está com fome?

- ☐ Nunca
- ☐ Raramente
- ☐ Às vezes
- ☐ Pelo menos 1 vez por semana

20. Com qual frequência você fica com fome?

- ☐ Somente nos horários das refeições
- ☐ Às vezes entre as refeições
- ☐ Frequentemente entre as refeições
- ☐ Quase sempre

21. Em uma escala de 1 a 8, onde 1 significa nenhuma restrição alimentar, e 8 significa restrição total, qual número você daria para si mesmo?



ANNEX 2 – FOOD FREQUENCY QUESTIONNAIRE (FFQ)

Setor: ___ ___ ___ Nº. do quest _____ __ __

QUESTIONÁRIO DE FREQÜÊNCIA ALIMENTAR ADULTO

☞ *PARA TODAS AS PESSOAS COM 20 ANOS OU MAIS*

Data da entrevista ____/___/_____ Hora de início: _____

Nome do entrevistador: _____

Nº de identificação: _____

Nome: _____ Sexo ( ) F ( ) M

Idade atual: _____ Data de nascimento: ____/___/_____

1. Você mudou seus hábitos alimentares recentemente ou está fazendo dieta para emagrecer ou por qualquer outro motivo?

(1) Não
(2) Sim, para perda de peso
(3) Sim, por orientação médica
(4) Sim, para dieta vegetariana ou redução do consumo de carne
(5) Sim, para redução de sal
(6) Sim, para redução de colesterol
(7) Sim, para ganho de peso
Outro motivo: _____

2 . Você está tomando algo para suplementar sua dieta (vitaminas, minerais e outros produtos)?

(1) não (2) sim, regularmente (3) sim, mas não regularmente

3. Se a resposta da pergunta anterior for sim, favor preencher o quadro abaixo:

SUPLEMENTO	MARCA COMERCIAL	DOSE	FREQUÊNCIA

4. As questões seguintes relacionam-se ao seu hábito alimentar usual no PERÍODO DE UM ANO. Para cada quadro responda, por favor, a frequência que melhor descreva QUANTAS VEZES você costuma comer cada item e a respectiva UNIDADE DE TEMPO (se por dia, por semana, por mês ou no ano). Depois responda qual a sua PORÇÃO INDIVIDUAL USUAL em relação à porção média indicada. ESCOLHA SOMENTE UM CÍRCULO PARA CADA COLUNA. Muitos grupos de alimentos incluem exemplos. Eles são sugestões e você pode pode consumir todos os itens indicados. Se você não come ou raramente come um determinado item, preencha o círculo da primeira coluna (N=nunca come). NÃO DEIXE ITENS EM BRANCO.

GRUPO DE ALIMENTOS	Com que frequência você costuma comer?		Qual o tamanho de sua porção em relação à porção média?	
	QUANTAS VEZES VOCÊ COME:	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Alimentos e preparações	Número de vezes: 1, 2, 3, etc. (N = nunca ou raramente comeu no último ano)	D=por dia S=por semana M=por mês A=por ano	Porção média de referência	P = menor que a porção média M = igual à porção média G = maior que a porção média E = bem maior que a porção média

SOPAS E MASSAS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Sopas (de legumes, canja, creme, etc)	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 concha média (150g)	P M G E
Salgados fritos (pastel, coxinha, rissólis, bolinho)	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 unidade grande (80g)	P M G E
Salgados assados (esfiha, bauruzinho, torta)	N 1 2 3 4 5 6 7 8 9 10	D S M A	2 unidades ou 2 pedaços médios (140g)	P M G E
Macarrão com molho sem carne	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 prato raso (200g)	P M G E
Macarrão com molho com carne, lasanha, nhoque	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 escumadeira ou 1 pedaço pequeno (110g)	P M G E
Pizza, panqueca	N 1 2 3 4 5 6 7 8 9 10	D S M A	2 fatias pequenas ou 2 unidades (180g)	P M G E
Polenta cozida ou frita	N 1 2 3 4 5 6 7 8 9 10	D S M A	2 colheres de sopa ou 2 fatias pequenas (70g)	P M G E

CARNES E PEIXES	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Carne de boi (bife, cozida, assada), miúdos, vísceras	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 bife médio ou 2 pedaços (100g)	P M G E
Carne de porco (lombo, bisteca)	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 fatia média (100g)	P M G E
Carne seca, carne de sol, bacon	N 1 2 3 4 5 6 7 8 9 10	D S M A	2 pedaços pequenos (40g)	P M G E
Linguiça	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 gomo médio (60g)	P M G E
Embutidos (presunto, mortadela, salsicha)	N 1 2 3 4 5 6 7 8 9 10	D S M A	2 fatias médias (30g)	P M G E
Frango (cozido, frito, grelhado, assado)	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 pedaço ou 1 filé pequeno (60g)	P M G E
Hambúrguer, nuggets, almôndega	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 unidade média (60g)	P M G E
Peixe (cozido, frito, assado) e frutos do mar	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 filé pequeno ou 1 posta pequena (100g)	P M G E

LEITE E DERIVADOS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Leite - tipo: ( ) integral ( ) desnatado ( ) semi-desnatado	N 1 2 3 4 5 6 7 8 9 10	D S M A	1/2 copo requeijão (125ml)	P M G E
Iogurte - tipo: ( ) natural ( ) com frutas	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 unidade pequena (140g)	P M G E

LEITE E DERIVADOS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Queijo mussarela, prato, parmesão, provolone	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	1 1/2 fatias grossas (30g)	P M G E ○ ● ○ ○
Queijo minas, ricota	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	1 fatia média (30g)	P M G E ○ ● ○ ○

LEGUMINOSAS E OVOS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Ovo (cozido, frito)	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	1 unidade (50g)	P M G E ○ ● ○ ○
Feijão (carioca, roxo preto, verde)	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	1 concha média (86g)	P M G E ○ ● ○ ○
Lentilha, ervilha seca, grão de bico, soja	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	1 colher de servir (35g)	P M G E ○ ● ○ ○
Feijoada, feijão tropeiro	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	1 concha média (210g)	P M G E ○ ● ○ ○

ARROZ E TUBÉRCULOS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Arroz branco ou integral cozido com óleo e temperos	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ● ○ ● ○ ○ ○ ○ ● ○	D S M A ○ ○ ● ○	2 escumadeiras médias (120g)	P M G E ○ ● ○ ○
Batata frita ou mandioca frita	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ● ○ ● ○ ○ ○ ○ ● ○	D S M A ○ ○ ● ○	2 colheres de servir cheias (100g)	P M G E ○ ● ○ ○
Batata, mandioca, inhame (cozida ou assada), purê	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ● ○ ● ○ ○ ○ ○ ● ○	D S M A ○ ○ ● ○	1 escumadeira cheia (90g)	P M G E ○ ● ○ ○
Salada de maionese com legumes	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ● ○ ● ○ ○ ○ ○ ● ○	D S M A ○ ○ ● ○	3 colheres de sopa (90g)	P M G E ○ ● ○ ○
Farinha de mandioca, farofa, cuscuz, aveia, tapioca	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ● ○ ● ○ ○ ○ ○ ● ○	D S M A ○ ○ ● ○	3 colheres de sopa (40g)	P M G E ○ ● ○ ○

VERDURAS E LEGUMES	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Alface	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	3 folhas médias (30g)	P M G E ○ ● ○ ○
Tomate	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	3 fatias médias (40g)	P M G E ○ ● ○ ○
Cenoura	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	1 colher de sopa (25g)	P M G E ○ ● ○ ○
Outros legumes (abobrinha, berinjela, chuchu, pepino)	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	1 colher de sopa cheia (30g)	P M G E ○ ● ○ ○
Outras verduras cruas (acelga, rúcula, agrião)	N 1 2 3 4 5 6 7 8 9 10 ○ ○ ○ ○ ● ○ ● ○ ○ ● ○	D S M A ○ ○ ● ○	1 prato de sobremesa (38g)	P M G E ○ ● ○ ○

VERDURAS E LEGUMES	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Outras verduras cozidas (acelga, espinafre, escarola, couve)	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 colher de servir (30g)	P M G E
Brócolis, couve-flor, repolho	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 ramo ou 2 colheres de sopa (30g)	P M G E

MOLHOS E TEMPEROS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Óleo, azeite ou vinagrete para tempero de salada	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 fio (5ml)	P M G E
Maionese, molho para salada, patê, chantilly	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 colher de chá (4g)	P M G E
Sal para tempero de salada	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 pitada (0,35g)	P M G E
Condimentos	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 pitada (0,35g)	P M G E

FRUTAS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Laranja, mexerica, abacaxi	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 unidade média ou 1 fatia grande (180g)	P M G E
Banana	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 unidade média (86g)	P M G E
Maçã, pêra	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 unidade média (110g)	P M G E
Melão, melancia	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 fatia média (150g)	P M G E
Mamão	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 fatia média ou ½ unidade média (160g)	P M G E
Goiaba	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 unidade grande (225g)	P M G E
Abacate	N 1 2 3 4 5 6 7 8 9 10	D S M A	2 colheres de sopa cheias (90g)	P M G E

BEBIDAS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Suco natural	N 1 2 3 4 5 6 7 8 9 10	D S M A	1/2 copo americano (80ml)	P M G E
Suco industrializado	N 1 2 3 4 5 6 7 8 9 10	D S M A	1 copo de requeijão (240ml)	P M G E
Café ou chá sem açúcar	N 1 2 3 4 5 6 7 8 9 10	D S M A	2 xícaras de café	P M G E

BEBIDAS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
	O O O O O O O O O O O	O O O O	(90ml)	O O O O
Café ou chá com açúcar	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 xícaras de café (90ml)	P M G E O O O O
Refrigerante ( ) comum ( ) diet/light	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 copo de requeijão (240ml)	P M G E O O O O
Cerveja	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 latas (700ml)	P M G E O O O O

PÃES E BISCOITOS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Pão francês, pão de forma, integral, pão doce, torrada	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 unidade ou 2 fatias (50g)	P M G E O O O O
Biscoito sem recheio (doce, salgado)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	4 unidades (24g)	P M G E O O O O
Biscoito recheado, waffer, amanteigado	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	3 unidades (41g)	P M G E O O O O
Bolo (simples, recheado)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 fatia média (60g)	P M G E O O O O
Manteiga ou margarina passada no pão ( ) comum ( ) light	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	3 pontas de faca (15g)	P M G E O O O O
Sanduíche (cachorro-quente, hambúrguer)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 unidades simples (220g)	P M G E O O O O

DOCES E SOBREMESAS	QUANTAS VEZES VOCÊ COME	UNIDADE	PORÇÃO MÉDIA (M)	SUA PORÇÃO
Chocolate, bombom, brigadeiro	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 barra pequena (25g)	P M G E O O O O
Achocolatado em pó (adicionado ao leite)	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	2 colheres de sopa (25g)	P M G E O O O O
Sobremesas, doces, tortas e pudins	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1 pedaço ou 1 fatia média (60g)	P M G E O O O O
Açúcar, mel, geléia	N 1 2 3 4 5 6 7 8 9 10 O O O O O O O O O O O	D S M A O O O O	1/2 colher de sopa (6g)	P M G E O O O O

5 . Por favor, liste qualquer outro alimento ou preparação importante que você costuma comer ou beber pelo menos UMA VEZ POR SEMANA que não foram citados aqui (por exemplo: leite-de-coco, outros tipos de carnes, receitas caseiras, creme de leite, leite condensado, gelatina e outros doces etc.).

ALIMENTO	FREQUÊNCIA POR SEMANA	QUANTIDADE CONSUMIDA

6 . Quando você come carne bovina ou suína, você costuma comer a gordura visível?

(1) nunca ou raramente (2) algumas vezes (3) sempre (9) não sabe ☐

7 . Quando você come frango ou peru, você costuma comer a pele?

(1) nunca ou raramente (2) algumas vezes (3) sempre (9) não sabe ☐

Hora do Término da entrevista _____

ANNEX 3 – INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (IPAQ)

Nome: _____

Data: _____/_____/_____ **Idade:** _____ **Sexo:** ( ) F ( ) M

Você trabalha de forma remunerada? ( ) Sim ( ) Não

Quantas horas você trabalha por dia? _____

Quantos anos completos você estudou? _____

De forma geral, sua saúde está:

( ) Excelente ( ) Muito boa ( ) Boa ( ) Regular ( ) Ruim

Nós estamos interessados em saber que tipos de atividade física as pessoas fazem como parte do seu dia-a-dia. As perguntas estão relacionadas ao tempo que você gasta fazendo atividade física em uma semana **NORMAL, USUAL** ou **HABITUAL.** As perguntas incluem as atividades que você faz no trabalho, para ir de um lugar a outro, por lazer, por esporte, por exercício ou como parte das suas atividades em casa ou no jardim. Suas respostas são MUITO importantes. Por favor, responda cada questão mesmo que considere que não seja ativo.

Para responder as questões, lembre-se que:

- Atividades físicas **VIGOROSAS** são aquelas que precisam de um grande esforço físico e que fazem respirar MUITO mais forte que o normal.
- Atividades físicas **MODERADAS** são aquelas que precisam de algum esforço físico e que fazem respirar UM POUCO mais forte que o normal.

Para responder as perguntas pense somente nas atividades que você realiza **por pelo menos 10 minutos** contínuos de cada vez.

1a. Em quantos dias de uma semana normal, você realiza atividades **VIGOROSAS** por pelo menos 10 minutos contínuos, como por exemplo correr, fazer ginástica aeróbica, jogar futebol, pedalar rápido na bicicleta, jogar basquete, fazer serviços domésticos pesados em casa, no quintal ou no jardim, carregar pesos elevados ou qualquer atividade que faça você suas **BASTANTE** ou aumentem **MUITO** sua respiração ou batimentos cardíacos?

_____ dias por **SEMANA** ( ) Nenhum

1b. Nos dias em que você faz essas atividades vigorosas por pelo menos 10 minutos contínuos, quanto tempo no total você gasta fazendo essas atividades **por dia**?

Horas: _____ Minutos: _____

2a. Em quantos dias de uma semana normal, você realiza atividades **MODERADAS** por pelo menos 10 minutos contínuos, como por exemplo pedalar leve na bicicleta, nadar, dançar, fazer ginástica aeróbica leve, jogar vôlei recreativo, carregar pesos leves, fazer serviços doméstico na casa, no quintal ou jardim como varrer, aspirar, cuidar do jardim, ou qualquer atividade que faça você suar leve ou aumentam **MODERADAMENTE** sua respiração ou batimentos cardíacos? (POR FAVOR, NÃO INCLUA CAMINHADA)

_____ dias por **SEMANA** ( ) Nenhum

2b. Nos dias em que você faz essas atividades moderadas por pelo menos 10 minutos contínuos, quanto tempo no total você gasta fazendo essas atividades **por dia**?

Horas: _____ Minutos: _____

3a. Em quantos dias de uma semana normal você caminha por pelo menos 10 minutos contínuos em casa ou no trabalho, como forma de transporte para ir de um lugar para outro, por prazer ou como forma de exercício?

_____ dias por **SEMANA** ( ) Nenhum

3b. Nos dias em que você caminha por pelo menos 10 minutos contínuos quanto tempo no total você gasta caminhando **por dia**?

Horas: _____ Minutos: _____

4a. Estas duas últimas perguntas são em relação ao tempo que você gasta sentado ao todo no trabalho, em casa, na escola ou faculdade e durante o tempo livre. Isso inclui o tempo que você gasta sentado no escritório ou estudando, fazendo lição de casa, visitando amigos, lendo e sentado ou deitado assistindo televisão.

Quanto tempo **por dia** você fica sentado em um dia da semana?

Horas: _____ Minutos: _____

4b. Quanto tempo **por dia** você fica sentado no final de semana?

Horas: _____ Minutos: _____

ANNEX 4 – PROJECT APPROVAL BY THE NATIONAL COMMISSION ON RESEARCH ETHICS (CONEP)

COMISSÃO NACIONAL DE ÉTICA EM PESQUISA

Plataforma Brasil

PARECER CONSUBSTANCIADO DA CONEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito do consumo de kombuchas de chás verde e preto na microbiota intestinal e nas alterações metabólicas de indivíduos eutróficos e obesos

Pesquisador: Frederico Augusto Ribeiro de Barros

Área Temática:

Versão: 3

CAAE: 25880819.3.0000.5153

Instituição Proponente: Departamento de Tecnologia de Alimentos

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE MINAS GERAIS
CONSELHO NACIONAL DE DESENVOLVIMENTO CIENTIFICO E TECNOLOGICO-CNPQ

DADOS DO PARECER

Número do Parecer: 3.948.033

Apresentação do Projeto:

As informações elencadas nos campos "Apresentação do Projeto", "Objetivo da Pesquisa" e "Avaliação dos Riscos e Benefícios" foram retiradas do arquivo do arquivo de Informações Básicas (PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1473587.pdf, de 16/03/2020)

INTRODUÇÃO

Considerada pela Organização Mundial de Saúde (OMS) como um dos maiores problemas de saúde pública do mundo (WHO 2019a), a obesidade está associada ao desenvolvimento de diversas doenças crônicas, dentre elas, diabetes mellitus tipo 2 (DM2), doenças cardiovasculares (DCV) e alguns tipos de câncer (BRAY et al., 2017; WHO 2019b). Sua prevalência quase triplicou entre 1975 e 2016, alcançando proporções epidêmicas (WHO, 2019b). Dessa forma, a busca por soluções efetivas para reduzir o número de casos da doença tem sido prioridade de saúde pública em todo o mundo (WHO, 2018a). Na obesidade, o excesso de tecido adiposo promove um estado de inflamação crônica de baixa intensidade, com produção excessiva de radicais livres e de substâncias pró-inflamatórias como fator de necrose tumoral (TNF), proteína C reativa (PCR) e interleucinas (IL), levando ao estresse oxidativo (AL-DOMI & AHMAD, 2017; PETELIN et al., 2017). O excesso de peso está associado, ainda, à resistência à insulina, desregulação do perfil lipídico e

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TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_modificado_versao3_limpa.docx	16/03/2020 15:37:37	Frederico Augusto Ribeiro de Barros	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_modificado_versao3.docx	16/03/2020 15:37:09	Frederico Augusto Ribeiro de Barros	Aceito
Folha de Rosto	Folha_de_rosto_modificada.pdf	16/03/2020 15:35:18	Frederico Augusto Ribeiro de Barros	Aceito
Outros	Questionario_de_selecao_online_modificado.pdf	20/12/2019 14:34:39	Mirian Aparecida de Campos Costa	Aceito
Outros	Autorizacao_DSA.pdf	20/12/2019 14:33:53	Mirian Aparecida de Campos Costa	Aceito
Cronograma	Cronograma.pdf	19/11/2019 12:54:11	Mirian Aparecida de Campos Costa	Aceito
Outros	Questionario_Internacional_de_Atividade_Fisica.pdf	18/11/2019 18:05:26	Mirian Aparecida de Campos Costa	Aceito
Outros	Questionario_de_Frequencia_Alimentar.pdf	18/11/2019 18:04:32	Mirian Aparecida de Campos Costa	Aceito
Outros	ThreeFactor_R21.pdf	18/11/2019 18:03:45	Mirian Aparecida de Campos Costa	Aceito
Outros	Registro_consumo_de_kombucha.pdf	18/11/2019 18:02:53	Mirian Aparecida de Campos Costa	Aceito
Outros	Registro_alimentar.pdf	18/11/2019 18:02:15	Mirian Aparecida de Campos Costa	Aceito
Outros	Questionario_de_selecao_triagem.pdf	18/11/2019 18:01:32	Mirian Aparecida de Campos Costa	Aceito
Outros	Questionario_de_selecao_online.pdf	18/11/2019 17:59:55	Mirian Aparecida de Campos Costa	Aceito
Declaração de Instituição e Infraestrutura	Autorizacao_LAFA.pdf	18/11/2019 17:59:16	Mirian Aparecida de Campos Costa	Aceito
Declaração de Instituição e Infraestrutura	Autorizacao_LAMECC.pdf	18/11/2019 17:59:00	Mirian Aparecida de Campos Costa	Aceito
Declaração de Instituição e Infraestrutura	Autorizacao_LAC.pdf	18/11/2019 17:58:09	Mirian Aparecida de Campos Costa	Aceito

Situação do Parecer:
Aprovado