TALITHA SILVA MENEGUELLI

EFFECT OF CASHEW NUT (*Anacardium occidentale* L.) AND ITS BY-PRODUCTS (OIL AND SOLUBLE EXTRACT) ON BIOMARKERS OF CARDIOMETABOLIC RISK AND GUT HEALTH

Dissertation submitted to the Nutrition Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Helen Hermana Miranda Hermsdorff

Co-advisers: Josefina Bressan
Hércia Stampini Duarte Martino
Elad Tako

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Data: 20/05/2024 12:26:05-0300
Verifique em https://validar.iti.gov.br

Talitha Silva Meneguelli
Author

gov.br Documento assinado digitalmente
HELEN HERMANA MIRANDA HERMSDORFF
Data: 20/05/2024 11:32:58-0300
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Helen Hermana Miranda Hermsdorff
Advisor

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ABSTRACT

SILVA MENEGUELLI, Talitha, D.Sc., Universidade Federal de Viçosa, February, 2024. **Effect of cashew nut (*Anacardium occidentale* L.) and its by-products (oil and soluble extract) on biomarkers of cardiometabolic risk and gut health**. Adviser: Helen Hermana Miranda Hermsdorff. Co-advisers: Josefina Bressan, Hércia Stampini Duarte Martino, and Elad Tako.

Obesity is a global public health issue that triggers chronic low-level inflammation, and dysbiosis, which can lead to increased intestinal permeability. This contributes to various chronic conditions like liver fat accumulation, type 2 diabetes, and cardiovascular disease. Therefore, effective nutritional strategies to combat obesity, lower cardiovascular risk factors, and enhance gut health are essential. Cashew nut is one of the most consumed nuts and contain nutrients that can promote overall health. Thus, the objective of this dissertation was to evaluate the effects of cashew nut soluble extract (CNSE) in the microbiota, intestinal morphology and functionality, and inflammatory markers in an experimental model (*Gallus gallus*), and cashew nut and cashew nut oil consumption in weight, body composition, cardiometabolic and inflammatory markers, and intestinal permeability in humans. For the experimental study developed at Cornell University-USA, CNSE was assessed *in vivo* via intra-amniotic administration in intestinal brush border membrane (BBM) morphology, functionality, and gut microbiota. Four groups were evaluated: no injection (control); H_2O injection (control); 10 mg/mL CNSE (1%); and 50 mg/mL CNSE (5%). The clinical trial developed at Universidade Federal de Viçosa - Brazil is an 8-wk randomized controlled three-arm dietary intervention, in which subjects were assigned to receive control (CT, free-nuts), cashew nut (CN, 30 g/day) or cashew nut oil (OL, 30 mL/day) plus an energy-restricted diet (-500 kcal). During initial and final days were measured anthropometry and body composition, collected blood, and performed the intestinal permeability test. Also, a 24-hour dietary recall was applied. Statistical analysis was conducted using SPSS (p-value <0.05). Related to experimental study, CNSE on duodenal morphological parameters showed, in both concentrations, higher Paneth cell numbers, goblet cell (GC) diameter in villi, and mixed GC per villi, while only in the 5% concentration showed higher GC diameter in crypt, depth crypt, and villi surface area. Further, both concentrations decreased GC number and acid and neutral

GC. Also, treatment with CNSE at 5% showed a lower abundance of Bifidobacterium, Lactobacillus, and E. coli, which was statistically significant. Moreover, in intestinal functionality, CNSE upregulated aminopeptidase (AP) gene expression at 5% compared to 1% CNSE. In the intervention study, all three groups reduced body fat and weight, waist and hip circumferences. The CN-group had reduction in liver serum markers and in the number of individuals with obesity, as well as an improvement in the lipid profile intensified by the improvement in intestinal permeability, while OL-group had LDL-c, atherogenic index, and IL-1β reductions. Both intervention groups reduced neck circumference and apo B levels. However, it is important to highlight that there was no difference in adiposity, biochemicals, intestinal permeability and inflammatory markers between the three groups. In conclusion, cashew nut and cashew nut oil associated with weight loss and CNSE demonstrated beneficial potential for health. However, the hypothesis of the intervention study was denied since when comparing the three groups there was no statistically significant difference in the markers evaluated.

Keywords: cashew nut; *Anacardium occidentale* L; clinical trial; obesity; cardiometabolic markers; intestinal permeability; inflammatory markers; experimental model; gut health.

RESUMO

SILVA MENEGUELLI, Talitha, D.Sc., Universidade Federal de Viçosa, fevereiro, 2024. **Efeito da castanha de caju (*Anacardium accidentale* L.) e seus subprodutos (óleo e extrato solúvel) em biomarcadores de risco cardiometabólico e saúde intestinal**. Orientador: Helen Hermana Miranda Hermsdorff. Coorientadores: Josefina Bressan, Hércia Stampini Duarte Martino, e Elad Tako.

A obesidade é um problema de saúde pública e induz inflamação crônica de baixo grau e disbiose, o que leva ao aumento da permeabilidade intestinal. Tais condições favorecem o acúmulo de gordura no fígado, diabetes tipo 2, e doenças cardiovasculares. Estratégias nutricionais para combater a obesidade, reduzir os fatores de risco cardiovascular e melhorar a saúde intestinal são essenciais. A castanha de caju é uma das oleaginosas mais consumidas e contém nutrientes essenciais para a saúde. O objetivo dessa tese foi avaliar os efeitos do extrato solúvel da castanha de caju (CNSE) na microbiota, morfologia e funcionalidade intestinal, e marcadores inflamatórios em modelo experimental (*Gallus gallus*), e da castanha de caju e do óleo da castanha de caju no peso, na composição corporal, nos marcadores cardiometabólicos e inflamatórios, e na permeabilidade intestinal em humanos. No estudo experimental desenvolvido na Universidade de Cornell - EUA, o CNSE foi avaliado *in vivo* via administração intra-amniótica na morfologia (membrana da borda em escova do intestino), funcionalidade e microbiota intestinal, e marcadores inflamatórios em quatro grupos: sem injeção (controle); injeção de H_2O (controle); 10 mg/mL CNSE (1%); e 50 mg/mL de CNSE (5%). O ensaio clínico desenvolvido na Universidade Federal de Viçosa - Brasil é um estudo de intervenção randomizado de 8 semanas, que teve grupo controle (CT), castanha de caju (CN, 30 g/dia) ou óleo da castanha de caju (OL, 30 mL/dia) associada à restrição calórica (-500 kcal). Nos dias inicial e final foram avaliadas antropometria e composição corporal, coletado sangue, realizado o teste de permeabilidade intestinal, e aplicado um recordatório 24 horas. A análise estatística foi realizada no SPSS (p-valor <0,05). No estudo experimental, o CNSE aumentou o número de células de Paneth, diâmetro de células caliciformes (GC) em criptas e vilosidades, profundidade de cripta, GC misto por vilosidades e área de superfície de vilosidades. Além disso, diminuiu o número de GC e GC ácido e neutro. Na microbiota, o tratamento com

CNSE apresentou menor abundância de Bifidobacterium, Lactobacillus e E. coli. No que diz respeito a funcionalidade intestinal, o CNSE regulou positivamente a expressão do gene aminopeptidase em 5% comparado com 1% do CNSE. No estudo de intervenção, todos os três grupos reduziram gordura e peso corporais, perímetros da cintura e quadril. O grupo CN apresentou redução nos marcadores séricos hepáticos e no número de indivíduos com obesidade, bem como melhora no perfil lipídico intensificada pela melhora da permeabilidade intestinal. Enquanto o grupo OL apresentou redução do LDL-c, do índice aterogênico, e da IL-1β. Ambos os grupos intervenção reduziram o perímetro do pescoço e os níveis de apo B. Entretanto é importante destacar que não houve diferença dos marcadores de adiposidade, bioquímicos, permeabilidade intestinal e inflamação entre os três grupos. Conclui-se que, a castanha de caju e o óleo associados a perda de peso e o CNSE demonstraram potencial benéfico para a saúde. Entretanto, a nossa hipótese em relação ao estudo de intervenção foi negada haja vista que quando comparado os três grupos não houve diferença estatisticamente significativa nos marcadores avaliados.

Palavras-chave: castanha de caju; *Anacardium occidentale* L; ensaio clínico. obesidade; marcadores cardiometabólicos; permeabilidade intestinal; marcadores inflamatórios; modelo experimental; saúde intestinal.

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LIST OF ACRONYMS AND ABBREVIATIONS

SCFA: short chain fatty acids
FFA: free fatty acids
MUFA: monounsaturated fatty acid
PUFA: polyunsaturated fatty acid
SFA: saturated fatty acid
ANGPTL2: angiopoietin-like protein 2
AP: aminopeptidase
CN: cashew nut
CT: control group
CVD: cardiovascular disease
DXA: dual emission X-ray densitometry
T2DM: type 2 diabetes mellitus
DNS: Department of Nutrition and Health
EMBRAPA: Brazilian Agricultural Research Corporation
FDA: Food and Drug Administration
GAE: gallic acid
SAH: systemic arterial hypertension
HDL: high density lipoproteins
HOMA-IR: homeostasis model assessment
IBGE: Brazilian Institute of Geography and Statistics
ICAM-1: intercellular adhesion molecule-1
DF: international diabetes federation
IFN-Y: Interferon gamma
IKK: Iκ kinase
IL: interleukin
BMI: body mass index
IPAQ: international physical activity questionnaire
IRAK: IL receptor-associated kinase
IRS: insulin receptor substrates
IκB: B cell inhibitor
JNK: N-terminal c-Jun kinase

LAMECC: Laboratory of Energy Metabolism and Body Composition

LAP: leucine aminopeptidase

LCC: cashew nut shell liquid

LDL: low density lipoproteins

LPS: lipopolysaccharide

MAL/TIRAP: MyD88-like adapter protein

MCP: monocyte chemotactic protein

MyD88: myeloid differentiation factor 88 adapter protein

OL: cashew nut oil

WHO: world health organization

PAI-1: plasminogen activator type 1 inhibitor

PC: waist circumference

CRP: C-reactive protein

POF: family budget survey

WHtR: waist/height ratio

WHR: waist/hip ratio

REBEC: Brazilian registry of clinical trials

IR: insulin resistance

SFRP5: frizz-related secreted protein 5

MS: metabolic syndrome

TAK1: kinase 1 associated with transforming growth factor beta

TLR4: toll like receiver 4

TMAO: trimethylamine oxide

TNF: tumor necrosis factor

TRAF6: factor 6 associated with the TNF receptor

TSH: thyroid stimulating hormone

TYG: triglyceride-glucose index

UFV: Federal University of Viçosa

VCAM-1: vascular cell adhesion molecule-1

VLDL: very low density lipoproteins

ZO: occlusive zonula

SUMÁRIO

1. INTRODUCTION

Obesity is a global public health issue, affecting 800 million people worldwide (Chong *et al.*, 2023), and is also recognized as a major risk factor for other noncommunicable diseases (NCDs) such as type 2 diabetes, cardiovascular disease and certain types of cancer (Chong *et al.*, 2023). In 2019, approximately 5 million deaths were from cardiovascular diseases, diabetes, cancers, neurological disorders, chronic respiratory diseases, and digestive disorders (Chong *et al.*, 2023). Additionally, the medical costs associated with obesity are projected to exceed US$1 trillion by 2025 (Chong *et al.*, 2023).

In Brazil, 96 million individuals had overweight and 41 million had obesity, according to 2019 data (IBGE, 2020). This indicates annual growth, as the proportion of Brazilians with obesity doubled between 2003 (12.2%) and 2019 (26.8%) (IBGE, 2020). Moreover, obesity places a significant burden on the Brazilian health system, with total costs related to hospitalizations and outpatient expenses reaching R$ 1.39 billion in 2018 (Nilson *et al.*, 2020).

Obesity is characterized by chronic low-grade inflammation, driven by changes in the size and number of adipocytes. Adiposity when accentuated prompts the production of pro-inflammatory cytokines and chemokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1, IL-1β, as well as adipokines like leptin and resistin. Visceral fat plays a key role in regulating chronic inflammation and is more strongly associated with the risk of CVD (Cartier *et al.*, 2008).

In this context, cardiometabolic alterations, such as elevated fasting glycemia and dyslipidemia characterized by increased levels of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL), triglycerides (TG), and decreased high-density lipoproteins (HDL), are prevalent in individuals with obesity (Vekic *et al.*, 2019). Metabolic disorders encompass a range of conditions where multiple interconnected pathological factors, including obesity, non-alcoholic steatohepatitis (NASH), dyslipidemia, glucose intolerance, insulin resistance (IR), hypertension, and diabetes converge (Agus, Clément and Sokol, 2021). When these conditions coexist, they significantly elevate the incidence of CVD and mortality. Therefore, effective nutritional interventions that address not

only obesity, but also cardiovascular risk factors are essential for reducing cardiovascular events and, consequently, overall mortality.

Also, individuals with obesity often have increased intestinal permeability and a distinct microbiota composition compared to those of normal weight. The gut plays a significant role in regulating cardiometabolic function by influencing levels of bioactive metabolites in the bloodstream, thus promoting overall health (Heianza *et al.*, 2017). Intestinal permeability refers to the intestine's ability to control what is absorbed into the bloodstream (Schoultz and Keita, 2020). On the other hand, the intestinal microbiota plays a crucial role in intestinal and overall health (El-Sayed, Aleya e Kamel, 2021). Its functions include digestion, metabolism, protection against pathogens, immune system regulation, body weight control, and production of bioactive substances such as short-chain fatty acids (SCFA) (Croci *et al.*, 2021; Jia *et al.*, 2021; Mirzaei *et al.*, 2021; Ruff, Greiling e Kriegel, 2020). Dysbiosis, an imbalance in the microbiota, can increase intestinal permeability, reducing barrier protection and increasing the presence of bacteria and toxins such as lipopolysaccharide (LPS), which can trigger inflammation (Velasco, de *et al.*, 2018). Moreover, obesity is associated with increased intestinal fatty acid production and enhanced chylomicron release, leading to heightened fatty acid transport to the liver (Dash *et al.*, 2015; Xiao *et al.*, 2016), increased triglyceride synthesis, and greater formation and secretion of VLDL (Xiao *et al.*, 2016).

Not only do intestinal bacteria contribute to intestinal homeostasis, but various parameters are also associated with gut health and are crucial for the proper functioning of the intestine. These parameters encompass histomorphological aspects such as villi, crypts, goblet cells, and Paneth cells, as well as factors related to the functionality of the brush border membrane (BBM). This functionality includes the expression of genes responsible for intestinal absorption and digestion, such as sucrase-isomaltase (SI), peptide transporter 1 (PEPT1), sodium-glucose cotransporter (SGLT-1), and aminopeptidases (AP), as well as those responsible for maintaining tight junctions such as occlusion zone (OZs), claudin, and occludin (Husein *et al.*, 2022; Sano, Shinozaki e Ohta, 2020; Taylor, 1993). Therefore, while the microbiota is crucial in evaluating gut

health, consideration of intestinal morphology and functionality is equally important.

When these elements are in homeostasis, overall health is maintained. In this regard, weight loss and the adoption of healthy eating habits can impact the mechanisms associated with these pathways, contributing to the reduction of metabolic changes and improvement of gut health (Nascimento *et al.*, 2018). Thus, studies have highlighted the positive effects of nuts in this context (Casas *et al.*, 2014; Estruch *et al.*, 2006). Cashew nut is native of Brazil, rich in monounsaturated fatty acids (MUFA), particularly oleic acid (60.7%). They constitute 48.3% of total fat, with 61.8% being MUFA, 17.9% polyunsaturated fatty acids (PUFA), 20.1% saturated fatty acids (SFA), and 0.19% trans fats (Rico, Bulló e Salas-Salvadó, 2016). Beyond their nutritional content and bioactive compounds (e.g., MUFA, Vitamin E, phytosterols and polyphenols), cashew nut also exhibits prebiotic properties. An in vitro study showed that defatted cashew nut flour had a prebiotic effect, leading to an increase in beneficial bacteria (*Bifidobacteria*) (Sisconeto Bisinotto *et al.*, 2021).

Despite cashew nut being among the most consumed (ranking third) and containing nutrients and bioactive compounds with health benefits that act through various mechanisms, there is a lack of studies evaluating the effects of this food. The findings from these studies indicate a reduction in systolic blood pressure, with no significant changes observed in the lipid profile and serum antioxidant markers. Results regarding fasting glucose levels are conflicting (Damavandi *et al.*, 2019; Davis *et al.*, 2007; Jalali *et al.*, 2020; Mohan *et al.*, 2018; Morvaridzadeh *et al.*, 2020; Mukuddem-Petersen *et al.*, 2007). In animal studies, the consumption of cashew nut resulted to reduction in visceral fat (Dias, C. C. Q. *et al.*, 2019), oxidative stress and inflammatory markers (Fusco *et al.*, 2020; Siracusa *et al.*, 2020), and intestinal permeability (Fusco *et al.*, 2020).

This study holds the potential to encourage the optimal utilization of cashew nut based on its findings. The profitability of cashew nut relies on extracting them without damage. As an alternative, by-products such as flour, oil, and butter have been explored for a more lucrative use of broken nuts in food applications (Sisconeto Bisinotto *et al.*, 2021). This research represents the first attempt to assess the health effects of cashew nut oil, a product currently under

development by Brazilian Agricultural Research Corporation (EMBRAPA) - Fortaleza, Brazil, and not yet available in the market. Moreover, the dissertation innovates by conducting an unprecedented evaluation of the soluble fraction of cashew nut on gut health, achieved by creating a soluble extract through freeze-drying.

Overall, there are not conclusive results regarding the impact of cashew nut on metabolic and inflammatory markers, with no *in vivo* studies (human and animal models) evaluating its effect on gut health. Similarly, there are no studies assessing the effects of cashew nut oil and cashew nut soluble extract on health. Consequently, this dissertation makes a groundbreaking contribution by elucidating which components or fractions present in cashew nut can deliver health benefits and specifying the nature of these benefits.

2. HYPOTHESIS

The hypothesis of this study proposes that the inclusion of both cashew nut and cashew nut oil in an energy-restricted diet could yield health benefits extending beyond mere enhancements in body composition. These dietary components hold the potential to positively influence various cardiometabolic factors, inflammatory status, and gut health. Cashew nut besides a substantial content of monounsaturated fatty acids (MUFA) has essential non-lipid nutrients such as proteins, dietary fiber, total phenolics, and antioxidants. This nutrient profile suggests that cashew nut may contribute to weight loss, reduce body fat accumulation, improve intestinal permeability and inflammatory markers. Similarly, cashew nut oil, owing to its lipid profile and higher concentrations of MUFA and fat-soluble vitamins, is anticipated to have a positive impact on cardiovascular markers as improvements in the lipid profile and vascular health. Furthermore, the soluble extract derived from cashew nut flour could potentially enhance gut health through its ability to modulate the microbiota and improve intestinal morphology and functionality *in vivo*.

3. OBJECTIVES

3.1 General objective

To evaluate the effects of cashew nut and its oil consumption for health in humans and cashew nut soluble extract in an experimental model (*Gallus gallus)*.

3.2 Specific objectives

- **Systematic review**

- To investigate the effects of carotenoids on gut microbiota, gut barrier, and inflammatory status on healthy animals.

- **Study in experimental model – Cornell University**

- Differentiate fibers and protein content of cashew nut soluble extract and partially defatted cashew nut flour;
- Evaluate the effect of administering the soluble extract from cashew nut flour partially defatted *in ovo* (*Gallus gallus*) on:
    I) Microbiota;
    II) Inflammatory markers;
    III) Intestinal morphology and functionality.

- **Study with humans - UFV**

- Differentiate the centesimal composition, minerals, fatty acid profile and phenolic compounds of cashew nut and cashew nut oil;
- Evaluate the effect of consuming cashew nut and oil extracted from cashew nut, associated with an energy-restricted diet, for 8 weeks, on changes in:
    I) Weight and body composition;
    II) Cardiometabolic markers;
    III) Inflammatory markers;
    IV) Intestinal permeability.

4. LITERATURE REVIEW

4.1 Obesity and adiposity indicators

Obesity is considered a global public health problem, more than 1.9 billion adults are overweight (WHO, 2018). In Brazil, recent data from the IBGE reveals that 96 million individuals (60.3%) are classified as overweight, with 41 million falling into the obese category. The prevalence of obesity has more than doubled between 2003 (12.2%) and 2019 (26.8%) (IBGE, 2020). This complex issue stems from a myriad of factors, including individual, environmental, and social elements. The pathophysiological contributors encompass both endogenous factors such as genetics, psychology, and changes in the neuroendocrine-metabolic axis, as well as exogenous factors like dietary patterns, sedentary lifestyles, and sleep quality (IBGE, 2020).

According to the World Health Organization (WHO), obesity is defined by excess body fat deposited in different parts of the body, which can generate a low degree of inflammation (WHO, 2018). The diagnosis is based on nutritional assessment by calculating body mass index (BMI) (i.e., BMI ≥ 30 kg/m²) (WHO, 2011). Although BMI serves as a good indicator, it does not fully distinguish between fat mass and lean mass.

Obesity can be classified as android (central obesity), where fat predominantly accumulates in the upper part of the body, or gynoid (peripheral obesity), where fat accumulation is primarily in the lower part, such as the hips, buttocks, and thighs (Walker *et al.*, 2014). Android obesity stands out as the primary independent risk factor for obesity-related metabolic and cardiovascular disorders, particularly IR and dyslipidemia (Walker *et al.*, 2014). In this sense, to enhance the accuracy of obesity diagnosis, additional measurements and index can be employed, especially those able to identify an elevated risk of associated conditions, such as cardiometabolic diseases (ABESO, 2016).

Increased waist circumference (WC), for example, indicates an increase in subcutaneous and visceral adipose tissue. It is regarded as a straightforward and cost-effective method, constituting one of the essential components for diagnosing Metabolic Syndrome (MS) and serving as a potential underlying pathophysiological marker contributing to an increase in cardiovascular risk

factors (Bays et al., 2010). Various cutoff points exist to assess an elevated WC. WHO utilizes values of ≥ 94 cm for men and ≥ 80 cm for women to indicate high risk, with very high risk designated as ≥ 102 cm for men and ≥ 88 cm for women (WHO, 2011). Conversely, the International Diabetes Federation (IDF) takes ethnicity and gender into account (**Table 1**) (Alberti *et al.*, 2009).

Table 1. Abdominal obesity by WC according to ethnicity.

Ethnicity	Cutoff points
Europeans, South Africans, Western Mediterranean and Middle East	≥ 94 cm (M); ≥ 80 cm (W)
South Asians, Chinese, South Americans and Central Americans	≥90 cm (M); ≥80 cm (W)
Japaneses	≥90 cm (M); ≥85 cm (W)

M (men); W (women); WC (waist circumference).

In addition to waist circumference, waist/hip ratio (WHR) and waist/height ratio (WHtR), which are employed to assess central obesity, providing insights into the distribution of abdominal body fat and establishing a closer link to CVD risks (WHO, 2011), a more recent method gaining widespread use for obesity diagnosis is neck circumference (NC) (Hu *et al.*, 2022; Kroll *et al.*, 2017). NC serves as a stable marker for determining the distribution of subcutaneous adipose tissue in the upper body (Hu *et al.*, 2022). The method offers several advantages, being simple, reliable, practical, cost-effective, and a time-saving anthropometric parameter designed to indicate central obesity, while also remaining stable throughout the day, unlike waist circumference, which can be affected by distension and breathing (Kamarli Altun and Suna, 2022). Furthermore, NC is associated with cardiometabolic risk factors such as hyperlipidemia, hyperglycemia, blood pressure, IR, and obstructive sleep apnea, independently of BMI and WC (Zanuncio *et al.*, 2022). Moreover, research using cross-sectional and prospective cohort studies has indicated a potential association between elevated NC values and cardiovascular risk factors, as well as a correlation with all-cause mortality in both males and females (Wan *et al.*, 2020).

Besides body circumferences, the body composition can be assessed by ultrasound, bioimpedance analysis, dual-energy X-ray absorptiometry (DEXA) and imaging techniques such as magnetic resonance imaging and computed

tomography (Achamrah *et al.*, 2018; SISVAN, 2011). DEXA offers a fast and non-invasive way to evaluate FM (fat mass), FFM (fat-free mass), and bone mineral density, making it the preferred technique for clinical research (Achamrah *et al.*, 2018). However, its application in routine clinical practice is limited due to the requirement for specialized radiology equipment and its high cost (Andreoli *et al.*, 2009). Studies have indicated that, particularly among individuals with obesity, the DEXA method exhibits significant accuracy in measuring body composition and could detect changes in body composition which were paralleled in part by changes in biochemical indices compared to alternative approaches (Ponti *et al.*, 2019).

4.2 Obesity, inflammation and cardiometabolic risk

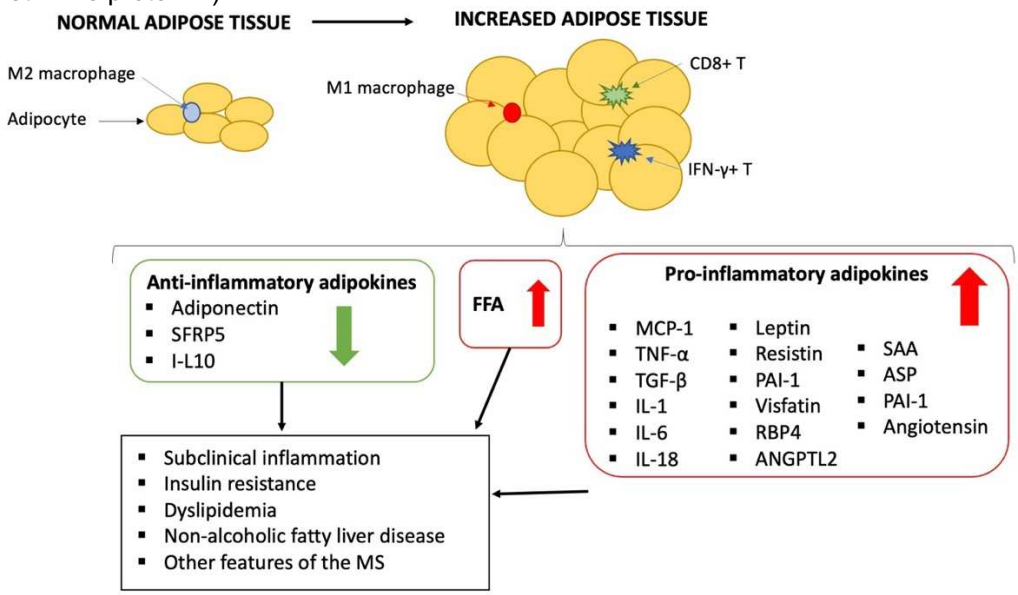
Obesity, particularly the accumulation of excess abdominal fat, triggers macrophage infiltration and disrupts the balance between pro- and anti-inflammatory factors released by adipose tissue. This process results in inflammation, compromised insulin sensitivity, and dysregulation of lipid metabolism, thereby fostering the development of various cardiometabolic risk factors. These include atherosclerosis, dyslipidemia, hypertension, IR, type 2 diabetes mellitus (T2DM), and the onset of other conditions such as cancer and cardiovascular disease (CVD) (ANS, 2017; Haslam e James, 2005).

In obesity, changes occur in the size (hypertrophy) and number (hyperplasia) of adipocytes (Pradhan, Zachara e Deplancke, 2017). These changes dictate the extent to which adipose tissue contributes to metabolic shifts. Adipocyte dysfunction can impede adipogenesis, limiting energy storage in adipose tissue and resulting in an elevation of free fatty acids (FFA) in the bloodstream. This, in turn, leads to lipotoxicity and the development of metabolic disorders. Elevated FFA levels can directly enter in the liver through the portal circulation, triggering increased lipid synthesis, gluconeogenesis, and IR in the tissue (Jung e Choi, 2014). Furthermore, FFAs act as ligands for the toll-like receptor complex 4 (TLR4), stimulating the production of pro-inflammatory cytokines and macrophages. This modulation of adipose tissue inflammation contributes to the metabolic complications associated with obesity (Shi *et al.*, 2006; Suganami, Nishida e Ogawa, 2005).

Adipose tissue serves a crucial endocrine function, secreting numerous adipokines, including chemokines, cytokines, and hormones, many of which play roles in energy homeostasis and inflammation (Jung e Choi, 2014). In the obese state, adipocytes play a pivotal role in the development of obesity-induced inflammation by increasing the secretion of various pro-inflammatory chemokines and cytokines (Jernås *et al.*, 2006). Additionally, the content of adipose tissue macrophages positively correlates with adipocyte size and body mass, and the expression of pro-inflammatory cytokines, such as TNF-α, primarily originates from macrophages rather than adipocytes (Weisberg *et al.*, 2003).

As obesity progresses, there is an increased presence of macrophages in adipose tissue, coupled with a phenotypic shift in these cells from an anti-inflammatory M2 polarization state to a pro-inflammatory M1 polarization state (Lumeng, Bodzin e Saltiel, 2007). The accumulation of M1 macrophages in adipose tissue has been demonstrated to lead to the secretion of various pro-inflammatory cytokines and chemokines, potentially contributing to obesity-related IR (Xu *et al.*, 2003) (**Figure 1**).

Figure 1. Schematic drawing of FFA and inflammatory adipokine secretion from adipose tissue in obesity leading to metabolic disturbances. IFN-γ (Interferon gamma). SFRP5 (Curly-related Secreted Protein 5). FFA: free fatty acid. MCP-1 (monocyte chemoattractant protein 1). TNF-α (tumor necrosis factor-alpha). TGF-β (Transforming growth factor beta). IL (Interleukin). PAI-1 (Plasminogen Activator Type 1 Inhibitor). RBP4: retinol-binding protein-4. ANGPTL2 (angiopoietin-like protein 2).



Source: Adapted from Jung and Choi, 2014.

A meta-analysis revealed that CRP concentrations exceeding 3 mg/L were associated with a 60% increased risk of coronary heart disease compared to those with CRP below 1 mg/L (Buckley *et al.*, 2009). Thus, in addition to obesity, the accumulation of visceral fat may play a crucial role in regulating chronic low-grade inflammation (Cartier *et al.*, 2008). Moreover, the connection between obesity and CVD risk becomes more pronounced when considering abdominal obesity (Janssen, Katzmarzyk e Ross, 2002; Rexrode *et al.*, 1998). A study demonstrated that, in men with abdominal obesity, the mortality rate for myocardial infarction increased by 23% (Kragelund *et al.*, 2005). In addition to triggering the inflammatory process and affecting insulin sensitivity, abdominal obesity can also lead to endothelial dysfunction and stimulate proliferative responses in the vascular wall, thereby elevating the risk for the development of type 2 diabetes mellitus (T2DM) and CVD (Vieira, Freitas e Volp, 2014).

Another condition linked to cardiometabolic risk is nonalcoholic fatty liver disease (NAFLD), encompassing dyslipidemia, IR, inflammation, oxidative stress, and endoplasmic reticulum (ER) stress, which share commonalities with cardiometabolic syndrome (Ristic-Medic, Vucic e Bajerska, 2022). NAFLD refers to a persistent condition characterized by the accumulation of fat in the liver, accompanied by low-grade inflammation in the hepatic tissue (Lim, Taskinen e Borén, 2019). The development of NAFLD involves a complex interplay of factors, including diet, physical activity, obesity, alterations in the microbiota, and genetic predisposition (Lim, Taskinen e Borén, 2019). An elevated flow of fatty acids originating from augmented visceral fat and de novo lipogenesis can trigger NAFLD and hinder insulin sensitivity (Shoelson, Lee e Goldfine, 2006). As a result, individuals grappling with obesity, IR, and dysregulated lipid levels are at the highest susceptibility to NAFLD (Stefan, Kantartzis e Häring, 2008). The distinction between NAFLD and NASH lies in the fact that NAFLD encompassing various stages of liver conditions, being a more general term (Carpi *et al.*, 2022). NAFLD begins with the accumulation of fat in the liver, a condition known as hepatic steatosis. In some cases, this fat accumulation can trigger inflammation in the liver (Younossi *et al.*, 2023). In contrast, NASH specifically denotes the inflammatory manifestation of this disease, characterized by hepatic steatosis, inflammation, and potential fibrosis (Carpi *et al.*, 2022).

NASH initiates cardiometabolic risk by releasing inflammatory, prothrombotic, and oxidative stress agents into the body's systems. Key intracellular pathways, including nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK), underlie the mechanistic connection between NAFLD and inflammation (S. Hamirani, 2014; Tarantino e Caputi, 2011). Activation of NF-κB in the livers of NAFLD patients prompts steatosis, heightening gene expression for molecules like intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1), associated with atherosclerosis progression (Tarantino e Caputi, 2011). Separately, JNK exacerbates IR by affecting insulin receptor substrate-1 (IRS-1) and interfering with downstream insulin signaling (Tarantino e Caputi, 2011; Tefan *et al.*, 2002). Elevated liver enzyme levels may reflect inflammation, disrupting insulin signaling locally and throughout the body. Recent research has revealed new roles for inflammatory agents in the NAFLD/NASH-inflammation nexus. Immune receptors known as Toll-like receptors (TLRs) activate proinflammatory pathways (Wei *et al.*, 2023). Notably, TLR2 and TLR4 induce IR, a pivotal factor in NAFLD, obesity, and cardiometabolic risk development (Lim, Taskinen e Borén, 2019; S. Hamirani, 2014). TLR4 is triggered by fatty acids from diet, visceral adipose tissue influx, and endotoxemia, culminating in NF-κB activation and heightened release of inflammatory cytokines such as IL-6, IL-1β, TNF-α, and MCP-1, which are crucial in the pathophysiology of cardiometabolic risk (S. Hamirani, 2014). The global occurrence of NAFLD is estimated to range from 25% to 30%, with its most substantial prevalence in the Middle East and South America, while Africa exhibits the lowest occurrence rates (Lim, Taskinen e Borén, 2019). Currently, NAFLD stands as the prevalent chronic liver disease in the Western hemisphere and is recognized as an archetypal metabolic disorder at the crossroads of obesity, MS, and T2DM (Vos, De *et al.*, 2022).

4.3 Gut health

The intestine serves as the body's largest immune organ. The term gut health is extensive and encompasses more than just the modulation of microbiota; it also involves macro and microstructural integrity, such as effective

digestion and absorption, a balanced gut microbiota, and a robust immune status (Zheng, Liwinski e Elinav, 2020). The absorption process occurs in the brush border membrane (BBM), covered by a mucus layer (Smirnov *et al.*, 2005). Goblet cells secrete this mucous gel, forming a protective barrier against intraluminal components like mechanical forces during digestion, enzymes, and intestinal bacteria (Smirnov *et al.*, 2005). Thus, the mucus layer serves as the innate first line of defense in the gastrointestinal tract (GIT), acting as a protective physical barrier preventing the translocation of pathogenic bacteria and the passage of toxins. Pathogens must traverse this mucosal layer before adhering to and invading epithelial cells (Duangnumsawang, Zentek e Goodarzi Boroojeni, 2021). Several mucins are expressed in the intestine (MUC1, MUC2, MUC3, MUC4 and MUC6) (Klinken *et al.*, 1995), with MUC2 being the primary mucin in the colon and small intestine, predominantly expressed in goblet cells (Chang *et al.*, 1994; Klinken *et al.*, 1995).

In addition to goblet cells responsible for mucus production, the enterocytes crucial for nutrient digestion and absorption, and the enteroendocrine cells, the intestine also includes Paneth cells located in the crypts (Gassler, 2017). The primary role of Paneth cells is to secrete antimicrobial peptides (AMPs), with defensins being the predominant ones. Consequently, Paneth cells play a vital role in forming a mucus layer enriched with AMPs, serving as a crucial defense mechanism in the interaction between the host and the microbiome (Gassler, 2017).

Like enterocytes, intestinal villi also play a crucial role in nutrient absorption, as greater surface area of villi (including increased length and width of villi) enhances nutrient absorption efficiency (Choct, 2009). Biomarkers, such as the expression of genes related to BBM functionality (SI, PEPT1, SGLT1, and AP), are used to evaluate nutrient absorption and digestion capabilities, along with overall intestinal functionality. Sucrase-isomaltase (SI) is the main disaccharidase in the small intestine, responsible for hydrolyzing disaccharides or oligosaccharides into monosaccharides. Alterations in this gene can indicate inadequate digestion of sucrose and starch (Husein *et al.*, 2022). Peptide transporter (PEPT1), located in the intestine, transports peptides from the intestinal lumen to enterocytes, playing a crucial physiological role in absorbing

small peptides resulting from food protein digestion in the small intestine (Shen, Smith e Iii, 2001). Sodium-glucose cotransporter (SGLT-1), like the glucose transporter (GLUT), is a glucose transporter expressed in the BBM, facilitating the transport of both glucose and sodium against the gradient into enterocytes (Sano, Shinozaki e Ohta, 2020). Among SGLTs, SGLT-1 is extensively investigated for its key role in the BBM of intestinal cells (Sano, Shinozaki e Ohta, 2020). Glucose influx into epithelial cells is catalyzed by apical membrane-located SGLT-1, and glucose exits into the circulation through basolateral membrane-located GLUT2 (Sano, Shinozaki e Ohta, 2020). Aminopeptidases (AP) are situated in the BBM, with their function being to catalyze amino acids from the amino terminal of proteins or peptides (Taylor, 1993). Thus, the intestinal barrier comprises a physical barrier, consisting of cellular components like vascular endothelium, epithelium, and mucus layer, and a chemical barrier, consisting of digestive secretions, immune molecules, antimicrobial peptides (mainly produced by Paneth cells in small intestine crypts), and other cellular products such as cytokines and inflammatory mediators. The intestinal barrier plays a vital role in absorbing nutrients and electrolytes once food is digested, while also preventing pathogens and toxins from entering the bloodstream, thereby helping to maintain body homeostasis (Gasmi *et al.*, 2020).

On the other hand, intestinal permeability is a measure indicating the passage of different absorbable substances from the intestinal barrier into the bloodstream (Bischoff, 2011). Tight junctions are crucial for maintaining the integrity of this intestinal barrier (Gasmi *et al.*, 2020). They possess a complex structure, connecting adjacent epithelial cells and controlling the selective movement of molecules through the paracellular pathway. Key proteins like Zona-occludens family (ZO-1, 2, and 3), Claudins, Occludins, Zonulin, and Junction Adhesion Molecules are involved in tight junction formation (Bischoff *et al.*, 2014). Additionally, factors like mucus from goblet cells, AMPs from Paneth cells, and immunoglobulin secretions from immune cells also contribute to maintaining the barrier (Cox, West e Cripps, 2015). When occurs disruption of the tight junctions it leads to a compromised intestinal barrier, resulting in increased intestinal permeability (Gasmi et al., 2020). This allows bacterial pathogens and high molecular weight antigens to pass from the gut into the

bloodstream, triggering an immune response and disturbing the body's homeostasis (Gasmi *et al.*, 2020).

Therefore, the preservation of intestinal homeostasis involves the integration of several factors, including the intestinal microbiota (bacteria residing in the intestine); histomorphological parameters (surface area of villi, depth and diameter of crypts, number and diameter of goblet and Paneth cells), along with the production of antimicrobial peptides and the mucus layer; and functional parameters such as modulation in the expression of proteins related to the absorptive capacity of the intestine and intestinal permeability (tight junctions). When all these factors are in balance, they collectively contribute to gut health.

Furthermore, the intestinal microbiota is involved in metabolic processes and modulates the barrier, although it does not represent a direct function of the intestinal barrier. Intestinal bacteria also play a vital role in the production of short-chain fatty acids (SCFA), including butyrate, propionate, acetate, and lactate, which serve as primary sources of energy for colonic epithelial cells and are essential for the development of intestinal villi (Liao *et al.*, 2020). Both bacteria and SCFA act as significant modulators of the gastrointestinal tract, influencing the digestion and absorption of nutrients (Liao *et al.*, 2020). The gut microbiota comprises Bacteria, Archaea, and Eukarya, being Bacteria the most prevalent gut microorganisms, with the phyla Bacteroidetes, Firmicutes, and Actinobacteria being the predominant numerical contributors (Ragonnaud e Biragyn, 2021). Numerous members of these phyla have been associated with the emergence of metabolic disorders (Myhrstad *et al.*, 2020). Thus, maintaining a harmonious bacterial composition is crucial for sustaining intestinal immunity and stability, as an imbalance of the gut microbiota is referred to as dysbiosis and has metabolic consequences (Myhrstad *et al.*, 2020).

4.4 Microbiota in the context of obesity, inflammation, and intestinal permeability

The intestinal microbiota plays a crucial role in the development of obesity, evidenced by differences in the microbiota of individuals with healthy weight and those with obesity (Xu *et al.*, 2022). Gut dysbiosis, commonly observed in obesity, entails a bacterial imbalance that diminishes the diversity and richness of the gut microbiome (Hamjane *et al.*, 2024). The gut bacteria community significantly

influences various aspects of metabolic disorders, with this control relying on factors such as the microbiota generating a diverse range of metabolites and their interactions with receptors on host cells (Xu *et al.*, 2022). These interactions can activate or inhibit signaling pathways, potentially impacting the host's well-being in both positive and negative ways. To elucidate the role of gut dysbiosis in obesity-related inflammation, two distinct mechanisms have been proposed: the release of bacterial components such as LPS and microbial metabolites such as SCFA (Vos, De *et al*., 2022). These mechanisms are described below (**Figure 2**).

In individuals with obesity, dysbiosis and the presence of gram-negative bacteria lead to intestinal barrier dysfunction, leading to increased intestinal permeability (Hamjane *et al.*, 2024). This results from the diminished expression of proteins such as zonula occludens (ZO)-1, claudin, and occludin, which constitute the tight junctions essential for preventing the translocation of bacteria and intestinal products to the bloodstream (Suzuki, 2020). The breakdown of these junctions allows the translocation of lipopolysaccharide (LPS), an endotoxin present in the membranes of gram-negative bacteria, triggering an inflammatory response (Mohammad e Thiemermann, 2021). Metabolic endotoxemia, primarily associated with alterations in gut microbiota and increased intestinal permeability, is the primary cause of chronic low-grade inflammation, while elevated plasma lipopolysaccharide-binding protein (LBP) levels significantly contribute to its pathogenesis (Gonzalez-Quintela *et al.*, 2013).

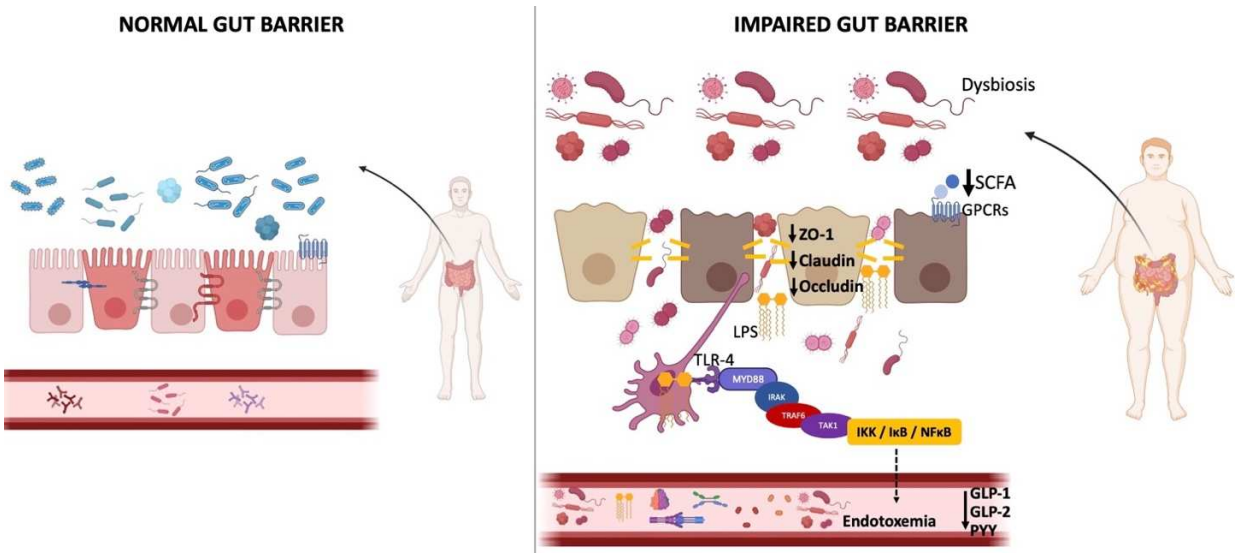
LBP recognizes the LPS, activating the TLR4 receptor and initiating a cascade of inflammatory events by forming a complex with myeloid differentiation factor 2, leading to the activation of both MyD88-dependent and non-MyD88-dependent downstream signaling pathways (Gonzalez-Quintela *et al.*, 2013). This results in the activation of kinases such as IRAK, TRAF6, TAK1, and IKK, which converge on NF-κB, a regulator of the inflammatory response. Additionally, the activation of IKK can impact insulin signaling, contributing to insulin resistance. Thus, any disruption in tight junctions can compromise the integrity of the intestinal barrier, increasing permeability and triggering an inflammatory process associated with metabolic disturbances in obesity.

Compared to other acute-phase reactants, the increase in LBP levels occurs relatively slowly, making it a valuable indicator to monitor the ongoing interaction between bacterial compounds, especially LPS, and the body's innate immune cells over time (Barchetta *et al.*, 2023). While measuring bacterial products like LPS in biological fluids has limitations, serum LBP levels have been proposed as a clinical marker for effective endotoxemia (Kopp, Kupsch e Schromm, 2016). In a selected group of apparently healthy Chinese individuals, serum LBP concentrations were associated with obesity and related metabolic disorders (Sun *et al.*, 2010). Contrary, LBP appears to act in the opposite way in the liver. In patients with obesity, serum LBP levels were related to lower non-alcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) prevalence and visceral adipose tissue inflammation (Barchetta *et al.*, 2023). This occurs because LBP possesses the ability to bind and transport LPS to the liver (Barchetta *et al.*, 2023). Specifically, in addition to initiating the CD14/TLR4 downstream pathway, which initiates inflammation, LBP can transfer LPS onto lipoproteins, aiding in their transportation to the liver for elimination (Barchetta *et al.*, 2023). A study demonstrated that a specific subtype of intestine-derived HDL, HDL3, travels through the portal vein accompanied by LBP, preventing LPS from binding to and activating liver macrophages (Han *et al.*, 2021). This process promotes the deactivation of LPS outside cells, thus mitigating the inflammatory response. Consequently, these alternative pathways for clearing bacterial toxins do not induce significant inflammation, ultimately providing defense against metabolic disorders.

Dysbiosis also reduces the abundance of SCFA-producing bacterial species in the gut, reinforcing the connection between SCFA and intestinal permeability (Gasmi *et al.*, 2020). SCFAs play a protective role in maintaining the intestinal barrier by inhibiting the activity of the NLRP3 inflammasome and autophagy (Feng *et al.*, 2018). Additionally, they impede the function of histone deacetylases (HDACs), which activate the NLRP3 inflammasome (Feng et al., 2018; Harbison et al., 2019). Consequently, SCFAs prevent the disruption of the intestinal barrier induced by LPS (Gasmi *et al.*, 2020). On the other hand, in a healthy state, bacteria producing SCFAs contribute to reducing intestinal permeability and inflammation by generating these beneficial metabolites (Gasmi

et al., 2020). SCFAs, serving as an energy source for colon cells, enhance the integrity of the intestinal barrier by upregulating the expression of ZO-1 and claudin-1, crucial components for forming tight junctions (Gasmi *et al.*, 2020). This reinforced barrier integrity minimizes LPS leakage, resulting in decreased insulin resistance and improved metabolic function (Vos, De *et al.*, 2022). Furthermore, SCFAs stimulate the secretion of gut peptides by interacting with specific G-protein-coupled receptors (GPCRs) on the surface of enteroendocrine L-cells predominantly located in the terminal ileum and colon (Vos, De *et al.*, 2022). Activation of these receptors triggers the release of gastrointestinal hormones such as Glucagon-like Peptide 1 (GLP-1), crucial for insulin release by the pancreas and reduced gastric emptying, contributing to appetite control; Glucagon-like Peptide 2 (GLP-2), a key protein regulating gut barrier function; and Peptide YY (PYY), vital for appetite suppression, decreased gastrointestinal motility, and reduced gastric secretion, promoting feelings of fullness after meals (Brooks *et al.*, 2017; Rastelli, Knauf e Cani, 2018).

Figure 2. Difference between normal gut barrier in a healthy state and impaired gut barrier in obesity condition. SCFA: short chain fatty acids; GPCRs: G-protein-coupled receptor; ZO-1: zonula occludens 1; LPS: lipopolysaccharide; TLR-4: Toll-Like Receptor 4; MYD88: Myeloid Differentiation Primary Response 88; IRAK: Interleukin-1 Receptor-Associated Kinases; TRAF6: TNF Receptor-Associated Factor 6; TAK1: Transforming Growth Factor-β-Activated Kinase 1; IKK: IκB Kinase; IκB: kappa B inhibitor; NFκB: Nuclear Factor kappa B; GLP: Glucagon-Like Peptide; PYY: Peptide YY.



Source: Own elaboration.

4.5 Nutritional intervention for obesity (and its comorbidities) and gut health

Food represents the primary modifiable factor for weight loss, and an energy-restricted diet stands as the initial choice in treating obesity. According to ABESO, the recommended treatment involves making lifestyle changes, engaging in regular physical exercise, and adopting a healthy diet that creates an energy deficit to promote weight loss (ABESO, 2022). Studies comparing various weight loss diets have demonstrated comparable outcomes over a 12-month period, suggesting that the impact of energy restriction is more significant than specific macronutrient composition (ABESO, 2022). A reduction of 5% in body weight has demonstrated positive effects on health outcomes and is currently considered a typical target in weight loss interventions (Varkevisser *et al.*, 2019). Despite this, a significant number of individuals struggle to sustain weight loss achieved through energy reduction (Varkevisser *et al.*, 2019). The lack of variety and monotony in the diet pose challenges to prolonged adherence. This trend of weight regain tends to intensify over time, accompanied by the reemergence of metabolic risks and psychosocial challenges (Wei *et al.*, 2022).

A meta-analysis showed that individuals with overweight/obesity and T2DM with a weight loss of at least 5% improved glucose, lipids, and blood pressure over 12 months (Franz *et al.*, 2015). Others demonstrated that, inflammation is reduced by an energy restriction of about 30% of calories, low fat, moderately high protein intake and carbohydrates until equilibrium (Bianchi, 2018), and a 25% energy-restricted diet of daily energy needs reduce weight loss (-4.94 kg), and improves cardiometabolic markers such as WC (−5.02 cm), body fat (−2.79), free fat mass (−1.61 kg), TG (−0.21 mmol/L), SBP (−5.59 mmHg), DBP (−4.87 mmHg), HC (−4.10 cm), TC (−0.31 mmol/L), LDL-c (−0.17 mmol/L), fasting insulin (−0.32 μmol/L), and increased HDL-c (0.05 mmol/L) (Xu *et al.*, 2023).

When assessing the impact of energy restriction on gut microbiota, a systematic review demonstrated a decrease in total bacterial abundance in three trials (60%), while no effect on bacterial abundance was observed in the remaining two trials (40%) (Seganfredo *et al.*, 2017). Results regarding microbial richness (alpha diversity) were inconsistent. Concerning phylum composition, five trials showed no impact, two displayed an increase in *Bacteroidetes* and a

decrease in *Firmicutes*, and one reported the opposite effect on these phyla. In terms of species assessment, six trials employing similar interventions (hypocaloric, low carbohydrate, high protein) revealed a reduction in certain species like *Roseburia* spp., *Eubacterium rectale*, *Clostridium Cluster XIVa*, and *Bifidobacterium* sp. Notable changes in *Lactobacillus* sp., *A. muciniphila*, and *F. prausnitzii* were observed, but not consistently across studies. When comparing individuals with a healthy weight and those with obesity, differences were noted in microbial community fingerprints. In 75% of cases, higher levels of the Bacteroidetes phylum (Prevotella sp.) were found in individuals with obesity, and 50% reported higher Archaea levels. Conversely, one study found a reduction in Bacteroidetes in patients with obesity, along with lower A. muciniphila abundance and increased SCFA production. They highlighted that baseline gut microbiota composition influenced responses to weight-loss interventions. Also, comorbidities and the presence of T2DM were potential confounders.

The implementation of energy restriction alongside the consumption of certain foods with properties conducive to weight loss, improvement of metabolic and inflammatory markers, and modulation of intestinal microbiota may represent a notably efficacious approach toward enhancing overall health. In this regard, nuts exhibit properties that contribute to the improvement of these markers as they have fiber, monounsaturated fats, and bioactive compounds with antioxidant and anti-inflammatory properties, such as β-carotene, vitamin E, minerals like selenium, phytochemicals, and phenolic compounds (Tuttolomondo *et al.*, 2019).

Contrary to the belief that nuts, being high in caloric density (> 4 kcal/g) due to their fat content, would lead to weight gain, studies show an inverse association between nut consumption and the prevalence of obesity and metabolic syndrome (Ibarrola-Jurado *et al.*, 2013; Li *et al.*, 2018; Silva, da *et al.*, 2022; Souza Silveira *et al.*, 2024). This can be explained by the increase in satiety caused by nuts. However, a meta-analysis investigating the effect of nuts on hunger, satiety, and caloric intake in randomized clinical trials found that while nuts increased daily calorie intake and suppressed hunger, they had no effect on feelings of fullness or weight (Akhlaghi *et al.*, 2020). This can be explained by the incomplete digestion of nuts, as their cell walls remain intact during the digestion process and pass undigested through the gastrointestinal tract (Mandalari *et al.*,

2014). In vitro studies have shown that even prolonged digestion times are unable to release encapsulated lipids from intact cells, and in some cases, the slow penetration of lipase into cell walls can lead to the release of fat in the ileum or colon, where fat absorption sites have already passed (Grundy *et al.*, 2015, 2016).

Furthermore, nuts have the potential to increase energy expenditure. Peanuts and walnuts, for instance, have demonstrated the ability to raise resting energy expenditure and body fat oxidation (Alper e Mattes, 2002; Tapsell *et al.*, 2009). Mono- and polyunsaturated fatty acids present in nuts have been shown to increase postprandial oxidation (Bonifácio et al., 2023; Zhang et al., 2010). Subgroup analyses have revealed that overweight individuals, in comparison to eutrophic individuals, experience less sensation of satiety, suppressed hunger to a lesser extent, have greater weight, and an increased caloric intake after consuming nuts. These weight-dependent effects of nuts can be attributed to the altered appetite sensitivity and regulation observed in individuals with obesity. When compared to eutrophic individuals, those with overweight are less adept at perceiving signals of hunger and satiety (Caldas *et al.*, 2022; Herbert e Pollatos, 2014). However, research on the effect of nuts on hunger and satiety is limited, and results are still inconclusive.

A systematic review, including prospective cohort studies, investigated the relationship between the consumption of total nuts and/or nut subtypes with changes in weight, BMI, and WC, as well as the risk of overweight/obesity, with a follow-up duration of ≥1 year (Eslami, Shidfar e Dehnad, 2019). Among the six studies conducted, four indicated a negative correlation between consuming nuts (typically at quantities of ≥1 to 2 servings per week) and the likelihood of gaining weight or facing overweight/obesity issues. The other two studies assessed the association between nut intake and changes in WC. From these two studies, only one reported a significant inverse association. Taken together, the evidence derived from a restricted number of cohort studies suggests that incorporating nuts into one's diet over the long term is linked to lower weight gain and a decreased risk of becoming overweight or obese.

Benefits have also been identified in reducing cardiovascular events. A meta-analysis of prospective cohort studies, including those evaluating a mix of

nuts, peanuts, and tree nuts, revealed that nut consumption was significantly associated with a 19% lower risk of all-cause mortality, a 44% lower risk of cardiovascular disease (CVD), a 30% lower risk of coronary heart disease mortality, and a 47% lower risk of sudden cardiac death (Mayhew *et al.*, 2016). Furthermore, nuts have demonstrated efficacy in lowering the risk of coronary heart disease and hypertension (Billingsley e Carbone, 2018; Chen *et al.*, 2017; Estruch *et al.*, 2006). These benefits are attributed not only to the high content of unsaturated fatty acids but also to the presence of polyphenols, which have the ability to reduce inflammatory markers, blood pressure, weight, and the incidence of diabetes (Billingsley e Carbone, 2018). This polyphenol-rich dietary pattern has been shown to influence the expression of genes involved in vascular inflammation, foam cell formation, and thrombosis (Llorente-Cortés *et al.*, 2010). Indeed, polyphenols appear to play a key role, as individuals in the highest tertile of total polyphenol excretion in urine had lower plasma levels of inflammatory biomarkers, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), IL-6, TNF-α, and MCP-1 when compared to the lowest tertile. This demonstrates a dose-dependent anti-inflammatory effect of polyphenols (Martínez-González, Gea e Ruiz-Canela, 2019).

These findings align with studies showing that individuals with higher nut consumption had the lowest concentrations of VCAM-1, ICAM-1, IL-6, CRP, P-selectin, LDL-c, and systolic and diastolic blood pressure (Arpón *et al.*, 2018; Casas *et al.*, 2014). In a study involving healthy individuals, the consumption of 20 or 50 grams of Brazil nuts led to a reduction in IL-1, IL-6, TNF-α, and IFN-γ, and an increase in IL-10 (Gobbo, Del *et al.*, 2015). The mechanisms by which nuts act on inflammatory markers are not yet well understood. One explanation is that nuts reduce total cholesterol and LDL-c, which are involved in the oxidized-LDL process and play a key role in inflammatory processes in atherosclerotic lesions (Gobbo, Del *et al.*, 2015; Guasch-Ferré *et al.*, 2018). Additionally, nuts contain antioxidants such as flavonoids, polyphenols, and tocopherols that are important in reducing oxidized LDL and act on signal transduction pathways such as NF-kB, consequently regulating inflammatory genes in macrophages and endothelial cells (Rahman, Biswas e Kirkham, 2006). Nuts, especially walnuts, contain high amounts of α-linolenic acid, which significantly improves arterial

compliance and decreases serum concentrations of pro-inflammatory cytokines. Other components of nuts, like magnesium, fiber, and L-arginine, are also protective against inflammation (Bode-Böger *et al.*, 1996; Galisteo *et al.*, 2010; Paolisso e Barbagallo, 1997).

A meta-analysis investigated the impact of nut consumption on weight outcomes, considering the type of instructions provided (Guarneiri e Cooper, 2021). The findings revealed that dietary interventions incorporating nuts did not result in changes in body weight (BW), body mass index (BMI), or waist circumference (WC) in studies conducted with or without substitution instructions (Guarneiri e Cooper, 2021). However, there is a possibility of slight reductions in body fat percentage (BF%) when substitution instructions are implemented. Another meta-analysis focused on the effects of tree nuts and peanuts on body weight (BW), body mass index (BMI), waist circumference (WC), and body fat percentage (BF%) (Fernández-Rodríguez *et al.*, 2021). The analysis revealed that, when comparing nut consumption with adherence to a control diet, significant increases in adiposity-related indicators were generally absent. However, hazelnut-enriched diets showed an increase in waist circumference (WC), while almond-enriched diets led to a notable decrease in WC compared to both the control diet and diets enriched with pistachios, mixed nuts, and hazelnuts (Fernández-Rodríguez *et al.*, 2021). Subgroup evaluations, specifically focusing on randomized controlled trials (RCTs) designed to explore the impact of nut consumption on weight loss, indicated that almonds were associated with diminished BMI, while walnuts were linked to a reduction in body fat percentage (%BF) (Fernández-Rodríguez *et al.*, 2021). In another meta-analysis that assessed body weight and adiposity in patients with T2DM, no significant effects of nut-enriched interventions were found for body weight, body mass index, WC, or body fat percentage (Fernández-Rodríguez *et al.*, 2022).

When considering gut health, nuts can exert beneficial influences. Beyond the fibers that stimulate the growth and metabolism of beneficial bacteria, leading to the production of SCFA, other constituents such as phenolic compounds and monounsaturated fatty acids have demonstrated their ability to modify the microbiota and influence the *Bacteroides/Firmicutes* ratio (Sánchez-Tapia, Tovar e Torres, 2019). Polyphenols play a role in shaping the composition of the

microbiota by acting as prebiotics, inhibiting the growth of pathogenic bacteria such as *E. coli*, and promoting the growth of probiotic bacteria like *Bifidobacterium* (Deiana, Serra e Corona, 2018). Simultaneously, unsaturated fatty acids contribute to altering microbiota composition, not only by increasing the proportion of *Bacteroidetes* to *Firmicutes* but also by stimulating the growth of species such as Actinobacteria and Proteobacteria—all of which are associated with greater SCFA production (i.e., acetate, propionate, and butyrate) (Machate *et al.*, 2020). A prebiotic effect of nuts on the intestinal microbiota has been proposed owing to their physical structure and constituents (i.e., fiber, unsaturated fatty acids, and polyphenols).

A meta-analysis of eight studies did not reveal any significant impact of nut consumption (e.g., walnuts, almonds, or pistachios) at the phylum level or in terms of bacterial diversity. However, certain genera experienced significant alterations, including *Lachnospira*, *Clostridium*, and *Roseburia*, all of which are known producers of butyrate. The influence of *Clostridium* and *Roseburia* was contingent on the type of nut, as their significance diminished when nuts were excluded from the analyses. The latter was also affected by the dose and duration of the intervention; higher doses and longer interventions lost significance, suggesting an adaptation of the microbiota over time. However, it's important to note that the authors highlighted the low quality of the included studies and the weak strength of evidence in the meta-analysis (Creedon *et al.*, 2020). As for intestinal morphology and functionality, no studies have been identified to date that have assessed the effects of nuts.

Currently, it is evident that nuts, owing to their properties, offer various health benefits. However, the effects on individual markers and their respective benefits vary based on the type of nut, the dosage administered, the specific target population, and the duration of the study. In this context, cashew nut stands out as one of the most widely consumed, despite being among the least studied. This underscores the necessity for additional research on cashew nut, primarily due to their potential beneficial effects, due to the lipid profile similarity observed with olive oil.

4.6 Cashew nut and health

The cashew tree, scientifically known as *Anacardium occidentale L.*, is a plant belonging to the *Anacardiaceae* family predominantly found in tropical regions (Elaine e Lomonaco, 2009) **(Figure 3).** Native to Brazil and thriving in the Cerrado biome, the cashew nut holds significant agricultural, economic, and social importance for the country. According to data from 2022/23, cashew nut rank third in global nut production (20%), trailing behind almonds (27%) and walnuts (22%) (INC, 2023). In terms of consumption, cashew nut also takes the lead, being one of the most consumed worldwide (19%, equivalent to 965,490 tons). The two most consumed nuts are almonds (31%, totaling 1,570,589 tons) and walnuts (19%, amounting to 977,272 tons) (INC, 2023).

Figure 3. Cashew tree.

Source: (Embrapa, 2020)

Cashew nut contain 48.3% of total fat, with 61.8% being monounsaturated fat, 17.9% polyunsaturated, 20.1% saturated fat, and 0.19% trans-fat (Rico, Bulló e Salas-Salvadó, 2016). Table 2 illustrates other nutrients and bioactive compounds present in cashew nut (Kornsteiner-Krenn, Wagner e Elmadfa, 2013; Rico, Bulló e Salas-Salvadó, 2016).

Table 2. Nutrients and bioactive compounds present in cashew nut according to literature findings.

Nutrients	g/ 100 g of food
Protein	21,3 g
Carbohydrate	20,5 g
Lipid	48,3 g
Total fiber	3,6 g
Fatty acids	
Oleic acid	60,7 g
Linoleic acid	17,8 g
Palmitic acid	10,2
Stearic acid	8,9 g
Amino acids	
Glutamic acid	4,6 g
Arginine	2,2 g
Minerals	
Iron	5,7 mg
Magnesium	248,8 mg
Phosphor	502,5 mg
Zinc	5,3 mg
Manganese	0,826 mg
Potassium	622,5 mg
Copper	2220 mg
Bioactive compounds	
β-carotene	7,63 µg
Lutein + zeaxanthin	23 µg
α-tocopherol	0,453 mg
γ-tocopherol	5,07 mg
Thiamine	0,477 mg
Phytosterols	158 mg

Source: Kornsteiner-krenn, Wagner and Elmadfa, 2013; Rico, Bulló and Salas-Salvadó, 2015.

Flavonoids such as catechin, epicatechin and epigallocatechin are also present in cashew nut. Additionally, cashew nut stands out as one of the primary and cost-effective sources of non-isoprenoid phenolic lipids, found in cashew nut shell liquid (LCC). These lipids include cardanol, cardol, 2-methyl cardol, and anacardic acid, known for their significant antioxidant capacity. Anacardic acid constitutes over 80% of the total extracted solvent (Philip *et al.*, 2007). This liquid, considered a renewable agricultural by-product, consists of monomers that facilitate the synthesis of polymers and bioactive compounds. The biological activities of this liquid have garnered interest in various areas, including its potential as an antitumor agent, antimicrobial substance, inhibitor of tyrosinase and xanthine oxidase, inducer of uncoupling effects in oxidative phosphorylation within liver mitochondria, and promoter of antioxidant activity (Gaitán-Jiménez *et al.*, 2022; Souza *et al.*, 2022).

While the nutritional composition of cashew nut may vary based on their origin, one study conducted a comparative analysis of cashew nut from six different regions, namely India, Brazil, Côte d'Ivoire, Kenya, Mozambique, and Vietnam. The study revealed that the variations were minimal, and these differences were ascribed to the distinct soil compositions, climatic conditions, and genetic evolution of the trees in each respective region (Rico, Bulló e Salas-Salvadó, 2016). Other differences may be related to the storage and manipulation processes and the methods used for lipid extraction (Rico, Bulló e Salas-Salvadó, 2016).

While it is known that nuts, in general, offer health benefits, there is a scarcity of intervention studies specifically examining the impact of cashew nut on health. Existing studies on these subject present conflicting results regarding the influence of cashew nut on lipid profiles, inflammatory markers, and blood pressure improvement (Damavandi *et al.*, 2019; Mah *et al.*, 2017; Mohan *et al.*, 2018). Moreover, there is limited evidence suggesting any notable changes in body composition related to the consumption of cashew nut (Jamshidi *et al.*, 2021). Some meta-analyses have shown that cashew nut does not significantly improve the lipid profile (Jalali *et al.*, 2020; Morvaridzadeh *et al.*, 2020) and showed reduction in systolic blood pressure, but no effect on diastolic (Jalali *et al.*, 2020). It is worth noting that the authors explicitly highlight the limited number

of studies included in the meta-analysis -only three- attributed to the scarcity of published studies involving cashew nut. Furthermore, there was considerable variation among the included studies in terms of intervention duration (ranging from 4 to 12 weeks), dosage administered (28 – 108 g/day), and the characteristics of the studied population (including healthy individuals, those with T2DM, or MS) (Jalali *et al.*, 2020; Morvaridzadeh *et al.*, 2020). Another meta-analysis also showed no difference in effect on body composition and glycemic indices (fasting blood glucose, insulin, and HOMA-IR) (Damavandi *et al.*, 2019). Other studies using cashew nut showed that people with T2DM consuming this nut (28 g/day) for 8 weeks had reductions in serum insulin and LDL-c / HDL-c rates (Damavandi *et al.*, 2019). Also, individuals with MS who were divided into three groups (walnuts, cashews and control diet) for 8 weeks had increased dietary antioxidant capacity in the nut groups, however no difference was observed in serum antioxidant capacity between groups (Davis *et al.*, 2007) (**Table 3**).

Other studies have been conducted using animal models. One of these studies demonstrated that the consumption of cashew nut by rats subjected to intestinal ischemia/reperfusion significantly reduced mortality rates, lowered blood pressure, mitigated oxidative stress, and restored antioxidant enzyme activities. These effects were attributed to mechanisms involving NRF2 and NF-kB. NRF2 plays a pivotal role as a transcription factor in orchestrating the cellular response to oxidative stress and other cellular stresses (Baird e Yamamoto, 2020). Typically, it remains inactive in the cytoplasm through its interaction with Keap1. However, upon exposure to stressors, NRF2 becomes stabilized and migrates to the nucleus (Baird e Yamamoto, 2020). There, it binds to Antioxidant Response Elements (AREs) situated in the promoters of target genes. This prompts the transcription of antioxidant and cellular defense genes, including SOD and GPx, crucial for mitigating oxidative and other cellular stresses (Baird e Yamamoto, 2020). NF-κB is activated by stimuli including pro-inflammatory cytokines, growth factors and bacterial LPS (Lai *et al.*, 2017). After the extracellular stimulus, intracellular signaling cascades activate NF-κB. After being active, typically as a dimer (usually a p65/p50 dimer), it translocates to the nucleus and binds to DNA at specific sequences known as κBREs (Lai *et al.*,

2017). This action modulates gene expression, influencing processes such as inflammatory response, apoptosis and cell proliferation (Lai *et al.*, 2017). Additionally, cashew nut consumption led to a decrease in plasma cytokine levels, nitrotyrosine, Poly (ADP-Ribose Polymerase, PARP) expression, and adhesion molecule expression. Moreover, it alleviated dysfunction of the intestinal barrier, minimized mucosal damage, and hindered the translocation of toxins and bacteria. Ultimately, these findings contributed to the mitigation of systemic inflammation and the prevention of organ damage (Fusco *et al.*, 2020). In another rat study, the consumption of cashew nut was associated with reduced food and caloric intake in comparison to the two other groups (one with dyslipidemia induction but no nut consumption, and a control group). Furthermore, the study found that cashew nut led to a reduction in visceral and retroperitoneal fat deposition and reversed low levels of HDL-c. However, the study also noted an impairment in glycemic metabolism and an increase in fat deposition in hepatic tissue (Dias, C. C. Q. *et al.*, 2019).

Table 3. Human intervention studies that evaluated the effect of cashew nut on health.

Author / year	Study design / duration	Participants	Groups	Intervention results
PIETERS *et al.,* 2005	Randomized, parallel, and controlled trial 8 weeks	Men and women with MS (21-65 years)	Control (n=22) Intervention (n=21): Cashew nut (63 – 108 g/day) Intervention (n=21): Walnut (63 – 108 g/day)	↔ BP, glucose, uric acid, fructosamine, hs-CRP, HDL-c, TG, LDL-c, TC (all groups) ↑ fasting insulin (all groups)
SCHUTTE *et al.,* 2006	Randomized, parallel, and controlled trial 8 weeks	Men and women with MS (21-65 years)	Control (n=21) Intervention (n=21): Cashew nut (63 – 108 g/day) Intervention (n=20): Walnut (63 – 108 g/day)	↔ WC, BMI, TG, HDL-c, SBP, and DBP ↑ fasting glucose and BRS (cashew nut) ↓ BRS (walnut)
DAVIS et al., 2007	Randomized, parallel, and controlled trial 8 weeks	Men and women with MS (21-65 years)	Intervention (n=21): walnuts (63-108 g/d) Intervention (n=21): unsalted cashew nut (63-108 g/d) Control (n=22)	↔ Serum antioxidant markers
MUKUDDEM-PETERSEN et al., 2007	Randomized, parallel, and controlled trial 8 weeks	Men and women with MS (21-65 years)	Intervention (n=21): walnuts (63-108 g/d) Intervention (n=21): unsalted cashew nut (63-108 g/d) Control (n=22)	↔ HDL-c, TG, CT, LDL-c, frutosamina sérica, PCR, PA e ácido úrico ↑Glucose (castanha de caju)
MAH et al., 2017	Randomized, crossover and controlled trial 2 periods of 28 days	Men and women (21-79 years)	Intervention: Cashew nut salted and rosted (28-64 g) Control: Baked potato chips (32-64 g)	↓TC e LDL-c ↔ HDL-c

			(n total = 51)	
MOHAN et al., 2018	Randomized, parallel, and controlled trial 12 weeks	Men and women with T2D (30-65 years)	Intervention (n=150): Cashew nut (30 g/d) Control (n=150): Standard diet for diabetics	↓ SBP ↑ HDL-c ↔ BMI, weight, lipid profile and glucose
BAER; NOVOTNY, 2019	Randomized, crossover and controlled trial 2 periods of 4 weeks	Healthy adults	Intervention: Cashew nut (42 g/d) Control (n total = 42)	↔ Lipid profile, BP, glucose, endothelin, adhesion molecules or clotting factors ↑ TNF-a ↓ PCSK9
DAMAVANDI et al., 2019	Randomized, parallel, and controlled trial 8 weeks	Men and women with T2D (30-75 years)	Intervention (n=25): cashew nut (28 g/d) Control (n=25)	↔ Weight, BMI, WC ↓Insulin and LDL-c/HDL-c

TC: Total cholesterol; WC: waist circunference; MS: metabolic syndrome; BP: blood pressure; SBP: systolic blood pressure; DBP: diastolic blood pressure; TG: triglycerides; CRP: C-reactive protein; BMI: Body mass index; BRS: baroreflex sensitivity; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TNF: tumor necrosis factor; PCSK9: Proprotein convertase subtilisin/kexin type 9; T2D: type 2 diabetes.

Some beneficial effects of cashew nut can be attributed to their high proportion of oleic acid. Epidemiological studies have shown that diets rich in MUFA, specifically in the form of oleic acid, such as the Mediterranean diet, have the potential to lower the risk of CVD (Becerra-Tomás et al., 2020); Garg et al., 1988). Though to a lesser extent, cashew nut also contains polyunsaturated fatty acids (PUFA), and both types of fatty acids are easily oxidizable. This oxidative quality may enhance the thermogenic effect, potentially contributing to weight loss (Flores-Mateo *et al.*, 2013). Beyond the contributions arising from the lipid profile, non-lipid components of cashew nut also appear to play a role in reducing serum lipids (Mah *et al.*, 2017). Phytosterols influence the metabolism of intestinal cholesterol through various mechanisms, including competition with cholesterol, thereby promoting its reduction (Mah *et al.*, 2017). Arginine, the second most abundant amino acid in cashew nut, serves as a precursor to nitric oxide, an endogenous vasodilator (Huynh e Chin-Dusting, 2006). Moreover, it possesses significant antioxidant potential attributed to the presence of phenolic compounds and flavonoids, which play a crucial role in preventing oxidation and safeguarding unsaturated fatty acids (Dias, D. M. *et al.*, 2019). In this way, cashew nut contains nutrients and compounds that contribute to various health benefits.

The quality of dietary fat is known to have a substantial impact on health. As there is a shift from animal fat to vegetable fat, promoting the beneficial effects of this lipid source, and considering the high lipid profile inherent in nuts, recent efforts have focused on extracting oil from these sources. Studies have been conducted to evaluate the health benefits associated with such oil extraction. Similarly, the analysis of bioactive compounds present in these oils has also been undertaken. Oil extraction typically involves three methods: pressing, extraction using a soxhlet apparatus, and a combination of pre-pressing and solvent extraction (Juhaimi, Al *et al.*, 2018; Özcan *et al.*, 2013). Considering that cold-pressed oils are preferred for preserving bioactive compounds that contribute to health benefits (Bail *et al.*, 2008). While variations exist based on the extraction method employed, oils obtained from various nuts, such as almond, hazelnut, peanut, pecan, walnut, pistachio, and cashew nut, were found to contain

substantial quantities of bioactive compounds, fatty acids like oleic and linoleic acid, as well as tocopherols and phenolic compounds (Juhaimi, Al *et al.*, 2018).

There have been limited studies evaluating the impact of consuming oil extracted from nuts on human health, and to date, no study has been identified that specifically utilized cashew nut oil. Among the studies examining nut-derived oil, two focused on almond oil. One study, conducted on individuals with hyperlipidemia, administered 10 ml of almond oil twice a day for 30 days. The results revealed a significant decrease of 16.26 mg/dl in total cholesterol and 7.68 mg/dL in LDL-c. However, no observable effects were noted on triglycerides and HDL-c. In the control group, there was an increase in LDL-c (Zibaeenezhad *et al.*, 2019). In another study spanning 12 weeks and involving normolipidemic men and women, participants were divided into two groups. For the initial 6 weeks, one group consumed 66.5 g of whole almonds, while the other ingested 35 g of almond oil. After an exchange between the groups, the evaluation continued for another 6 weeks. The results indicated reductions of 4% in total cholesterol, 6% in LDL-c, 14% in triglycerides, and a 4% increase in HDL-c, irrespective of whether the individuals consumed almonds or almond oil (Hyson, Schneeman e Davis, 2002). A study involving hemodialysis patients who were supplemented with 5 g of baru oil capsules for 12 weeks demonstrated a decrease in hs-CRP concentrations (-1.2 ± 0.2 mg/L) compared to the placebo group supplemented with 5 g capsules of mineral oil (+0.8 ± 0.2 mg/L). However, supplementation with baru oil did not prove effective in improving body composition, lipid profile, and oxidative stress (Schincaglia *et al.*, 2020a).

Other studies involving nuts have been conducted using animal models or *in vitro*. In one such study, hamsters were initially fed a high-cholesterol diet for 4 weeks to induce hyperlipidemia. After this period, they were divided into two groups: one receiving a low-cholesterol diet (5%) and the other a high-cholesterol diet (10%), both enriched with hazelnut oil, for 12 weeks. In comparison to the group on the high-cholesterol diet, the groups receiving hazelnut oil exhibited improvements in their lipid profile (including reductions in triglycerides, total cholesterol, LDL-c, and VLDL-c) and demonstrated enhanced liver function. Furthermore, hazelnut oil supplementation led to improvements in lipid peroxidation (measured by malondialdehyde levels) and the concentration of liver

antioxidant enzymes (SOD, GPx, and GST) (Lu *et al.*, 2019). A study conducted with male Wistar rats assessed the impact of a high-fat diet supplemented with pecan oil (HF+PO), polyphenols extracted from pecan nuts (HF+PP), or whole pecan nuts (HF+WP). The diet incorporating whole pecan nuts proved more effective in improving several markers, including hyperleptinemia, total cholesterol, and liver expression. On the other hand, the diet incorporating pecan oil was effective in reducing triacylglycerols (Domínguez-Avila *et al.*, 2015). Another study in male C57BL/6 mice found that macadamia oil supplementation for 8 weeks attenuated adipocyte hypertrophy and inflammation in adipose tissue and macrophages (Lima *et al.*, 2014). A study conducted in rats examined the effects of macadamia oil rich in oleic acid, safflower oil rich in linoleic acid, and flaxseed oil rich in α-linolenic acid on changes in adiposity, cardiovascular and hepatic structure and function, and fatty acid compositions in tissues. The study observed that only oleic acid increased adiposity but concurrently reduced total cholesterol and markers of liver damage. Linoleic and α-linolenic acids decreased triglycerides and non-esterified fatty acids, with the latter improving left ventricular structure and function, diastolic stiffness, systolic blood pressure, and glucose tolerance. Oleic and linoleic acids increased basal plasma glucose concentrations, while oleic and α-linolenic acids normalized blood pressure (Poudyal *et al.*, 2013). An *in vitro* study demonstrated that pistachio oil effectively decreased inflammatory markers, including Ifit-2, TNF-α, and IL-6. Ifit genes serve as inflammation markers induced by LPS or IFN stimulation. As suggested by the authors, pistachio oil seems to exert beneficial effects on the regulation of inflammation-related genes (Zhang, Z. *et al.*, 2010).

In conclusion, there are currently limited intervention studies assessing the impact of cashew nut, and further research is warranted to thoroughly evaluate the benefits of this nut in mitigating cardiometabolic risk. While studies on oils extracted from various nuts are scarce, the available ones have indicated several positive effects on certain cardiometabolic markers, such as lipid profile, inflammation, and blood pressure, depending on the specific type of nut. Moreover, notwithstanding the fact that cashew nut is rich in essential nutrients and bioactive compounds with overall health significance, a recent study has identified prebiotic effects in cashew nut flour (Sisconeto Bisinotto *et al.*, 2021).

4.7 Animal model *Gallus gallus* and intra-amniotic administration of plant-based foods

The animal model *Gallus gallus* has currently been used as a technique to assess the effect of nutrients and bioactive compounds, mainly plant-derived compounds on gut health (i.e., BBM functionality, morphology, and microbiota) (Hou e Tako, 2018). This animal model has been widely used due to several advantages. *Gallus gallus* possesses a complex and dynamic intestinal microbiota that is significantly influenced by host genetics, environment, and diet. At the phylum level, it shares similarities with the human microbiota, featuring Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria. Moreover, it exhibits over 85% homology in its intestinal gene sequences with human intestinal genes, demonstrating substantial genetic conservation with humans. The genetic mechanisms regulating the development of these animals are also relevant to human biology. Additionally, the *Gallus gallus* model undergoes rapid maturation, displays a well-characterized phenotype, exhibits fast growth, and is highly responsive to dietary manipulations (Hou e Tako, 2018; International Chicken Genome Sequencing Consortium, 2004). Consequently, it is recognized as a model for human nutrition (Qin *et al.*, 2010; Yegani e Korver, 2008; Zhu *et al.*, 2002). Furthermore, it is a cost-effective technique (Hou e Tako, 2018). The *in ovo* intra-amniotic administration of the food solution under evaluation should be implemented between days 17-18 of embryo incubation. This timeframe is selected because it corresponds to the period when the embryo is transferred from the incubator to the incubation basket. Also, these days precede the 19th day when amniotic fluid ingestion occurs, reaching enteric tissues such as enterocytes and other cells of the intestinal mucosa (Hou e Tako, 2018; Uni, Noy e Sklan, 1999). This technique provides a significant advantage by enabling the assessment of specific foods or nutrients on overall gut health without the interference of external factors. The food compound in question is the only factor under evaluation (Hou e Tako, 2018).

Previous studies involving edible plant-based nutrients/foods, such as lentils and chickpeas (Pacifici *et al.*, 2017), duck egg white peptides (Hou *et al.*, 2017), wheat bran (Wang *et al.*, 2019), beans (Dias, D. M. *et al.*, 2019), chia seed

(Silva, da *et al.*, 2019), yacon (Martino, Kolba e Tako, 2020), grapes (Gomes *et al.*, 2021), catechins (Kolba *et al.*, 2022), quinoa (Agarwal, Kolba, Khen, *et al.*, 2022), saffron flower (Agarwal, Kolba, Jung, *et al.*, 2022), genistein (Cheng *et al.*, 2022), apple (Jackson *et al.*, 2022) and black corn (Hamjane *et al.*, 2024), due to their properties such as soluble fraction and/or bioactive compounds were evaluated by *in ovo* intra-amniotic administration. Although the results varied according to the nutrient/food evaluated and the concentrations used, the studies, in general, demonstrated several beneficial effects on intestinal health. These effects included improvements in functionality, as measured by absorption and digestion capacity through biomarkers like the expression of genes related to BBM functionality (AP, SI, SGLT1 and PepT1) (Hou *et al.*, 2017; Kolba *et al.*, 2022; Pacifici *et al.*, 2017) and markers of intestinal permeability, such as those related to tight junctions (OCLN) and those related to mucus production (MUC2) (Agarwal, Kolba, Jung, *et al.*, 2022).

Additionally, positive effects were observed on morphological parameters, including cell proliferation, increases in length, width, and surface areas of intestinal villi, and parameters related to the number, diameter and type of goblet cells (acidic, neutral and mixed) (Agarwal, Kolba, Khen, *et al.*, 2022; Agarwal, Shukla, *et al.*, 2022; Agrizzi Verediano *et al.*, 2022; Cheng *et al.*, 2022; Jackson *et al.*, 2022; Kolba *et al.*, 2022). The studies also identified positive effects on the microbiota, such as an increase in beneficial bacterial populations (*Bifidobacterium, Lactobacillus,* and *L. plantarum*) (Agarwal, Kolba, Khen, *et al.*, 2022; Agarwal, Shukla, *et al.*, 2022; Cheng *et al.*, 2022; Gomes *et al.*, 2021; Hamjane *et al.*, 2024; Hou *et al.*, 2017; Kolba *et al.*, 2022; Martino, Kolba e Tako, 2020; Wang *et al.*, 2019). This resulted in an increase in the production of short-chain fatty acids (SCFA), a reduction in intestinal pH, and consequently, an increase in mineral bioavailability, as evidenced by markers related to the expression of genes such as zinc transporters (ZnT1, ZnT7, ZIP4, and ZIP9), iron metabolism (DMT-1, ferroportin, DcytB, and hepcidin), and proteins related to calcium metabolism (PMCA1b and NCX1) and magnesium (TRPM6) (Agarwal, Kolba, Jung, *et al.*, 2022; Dias, D. M. *et al.*, 2019; Gomes *et al.*, 2021; Hamjane *et al.*, 2024; Hou *et al.*, 2017; Jackson *et al.*, 2022; Martino, Kolba e Tako, 2020; Pacifici *et al.*, 2017; Silva, da *et al.*, 2019; Wang *et al.*, 2019). Furthermore, these

studies indicated other beneficial effects on the body's immune response and inflammation-related genes (Agarwal, Kolba, Jung, *et al.*, 2022; Agrizzi Verediano *et al.*, 2022; Gomes *et al.*, 2021; Hamjane *et al.*, 2024) (**Table 4**).

Table 4. Studies on the effect of plant-based foods on intestinal health and mineral bioavailability in the Gallus gallus model by intra-amniotic administration in ovo.

Author / year	Food	Groups	Main results
PACIFICI et al., 2017	Raffinose and stachyose (lentils and chickpeas)	1) No injection 2) 18 Ω H2O 3) Raffinose (100 mg/ml) 4) Raffinose (50 mg/ml) 5) Stachyose (100 mg/ml) 6) Stachyose (50 mg/ml)	**Groups 3,4,5 and 6:** ↑ Bifidobacterium and Lactobacillus; expressions of AP, SI and SGLT1; surface areas of villi; goblet cell diameters ↓ Clostridium; expressions of DcytB, DMT1 and ferroportin
HOU et al., 2017	Chickpeas, lentils, and duck egg white peptides	1) No injection 2)18 MΩ H2O 3) Ca (4 mmol/L) 4) Chickpea (50 mg/mL) + Ca (4 mmol/L) 5) Lentils (50 mg/mL) + Ca (4 mmol/L) 6) DPs (40 mg/mL) + Ca (4 mmol/L) 7) Val-Ser-Glu-Glu (VSEE) + Ca (4 mmol/L) 8) Chickpea (50 mg/mL) 9) Lentils (50 mg/mL) 10) DPs (40 mg/mL) 11) VSEE (5 mg/mL)	**Groups 4 - 11:** ↑ serum bone alkaline phosphatase content; villous surface areas and goblet cell diameter **Groups 8 and 11:** ↑ *Bifidobacterium* and *Lactobacillus* **Group 5:** ↑ expression of SI **Group 8:** ↓ *E. coli* **Group 9:** ↑ *Lactobacillus*; expression of Zip 4 **Groups 3, 4 and 5:** ↑ gene expression of CalbindinD9k
WANG et al., 2019	Wheat bran	1) No injection 2)18 Ω H2O 3) Wheat bran (100 mg/ml) 4) Wheat bran with cellulase (100 mg/ml)	**Group 3:** ↑ AP; SGLT-1; Bifidobacterium; goblet cell diameter **Groups 3 and 4:**

		5) Arabinose (50 mg/ml)	↑ DMT1; DcytB; ferroportin; ZnT1; Zip4; MT4; villus heights and number of goblet cells (per villus)
DIAS et al., 2019	Bean	1) No injection 2) 18 MΩ H2O 3) Inulin (40 mg/mL) 4) BRS Perola (standard carioca) (50 mg/mL) 5) BRS Cometa (carioca, Fe biofortified) (50 mg/mL) 6) BRS Esteio (standard black) (50 mg/mL) 7) SMN 39 (black, Fe biofortified) (50 mg/mL) 8) BRS Artico (standard white) (50 mg/mL)	**Group 5**: ↑ Expression of ZnT1, ferroportin, Dcytb e AP ↓ Hepcidin expression **Group 6**: ↑ Expressão de hepcidina **Groups 7 e 8**: ↓ Expressão de ZnT1
SILVA et al., 2019	Chia seed	1) No injection 2) 18 Ω H2O 3) Inulin (40 mg/mL) 4) ESSC (5 mg/mL) 5) ESSC (10 mg/mL) 6) ESSC (25 mg/mL) 7) ESSC (50 mg/mL)	**Group 4:** ↑ Bifidobacterium and Lactobacillus **Group 6:** ↓ DMT1 gene expression. **Group 7:** ↑ SI gene expression (compared to group 6) ↓ *Bifidobacterium, Lactobacillus* and *Clostridium* **Groups 5,6 and 7:** ↑ Relative expression of DcytB, hepcidin and ZnT1 ↓ E. coli; ferroportin gene expression **Groups 4 - 7**: ↑ villus surface areas, villus length, width, number and diameter of goblet cells
		1)Sem injeção	**Group 4:**

MARTINO, KOLBA E TAKO, 2020	Yacon	2) 18 Ω H2O 3) Inulina 4) Yacon (1%)	↑ Glycogen in the pectoral muscle; Lactobacillus, Bifidobacterium, Clostridium and E. coli; expression of ferroportin, DcytB; expression of zinc transporters (ZnT1, ZnT7 and ZIP9); crypts depth; diameter and number of goblet cells in the crypt and diameter of goblet cells in the villi; expression of SI, TNF-α, IL1β and PCK1 ↓ Liver Fe, hepcidin; villus length, delta-6-desaturase; IL1β and SGLT1 gene expression
GOMES et al., 2021	Grape	1) No injection 2)18 MΩ H2O 3) Inulin (5%) 4) Resveratrol (5%) 5) Pterostilbene (5%) 6) Synergists: resveratrol (4,75%) + pterostilbene (0,25%)	**Group 4:** ↓ Relative expression of DMT1 **Group 5:** ↓ IL-6 **Groups 4 and 5:** ↓AP **Groups 4, 5 and 6:** ↑ Delta-6-desaturase; SGLT1; Goblet cell diameter; Bacteroidetes and Firmicutes ↓ DcytB and ferroportin expression; inflammatory cytokines IL-1β and TNF-α; Proteobacteria, Verrucomicrobia, Actinobacteria, Deferribacteres and Cyanobacteria **Groups 4 and 6:** ↓ SI and AT1R expression. **Groups 5 and 6:** ↑ Proteins related to calcium (PMCA1b and NCX1) and magnesium (TRPM6) metabolism, increased villus lengths and diameters, and increased crypt depths **Group 6:** ↓ ECA expression; n° of goblet cells in the crypt; α-diversity
		1) No injection	**Groups 2-7:**

KOLBA et al., 2022	Catechin	2) 18 MΩ H2O 3) Emulsifier (0.004 mg/ml dose) 4) Inulin (50 mg/mL dose) 5) Catechin (6.2 mg/mL dose) 6) Catechin pentaacetate (10 mg/mL dose) 7) Catechin pentabutanoate (12.8 mg/mL dose)	↓ Expression of ferroportin, ZIP9, n° of GC in villi **Groups 3-7:** ↓ Neutral and mixed GC in villi, neutral GC in crypts **Group 4:** ↑ Clostridium, SGLT1 expression (compared to group 3), villus GC diameter, acid GC in villi ↓ NF-κβ1 expression (compared to group 1), TNF-a (compared to groups 1, 3 and 6) **Group 5:** ↑ SI compared to group 2, diameter of GC in crypts **Group 4 and 7:** ↓ ZnT7 compared to group 1 **Groups 4-7:** ↓ AP and MUC2 compared to group 1 **Groups 6 and 7:** ↑ Bifidobacterium **Groups 3, 5, 6 and 7:** ↓ Lactobacillus **Groups 5-7:** ↓ E. coli, n° and diameter of the Paneth cell ↑ Surface area of villi **Group 7:** ↓ ZNT1 compared to groups 1 and 2
			Group 5: ↑ GC diameters in villi and crypts **Group 6:** ↓ Expression of DMT1 and E. coli

AGARWAL et al., 2022	Quinoa (*Chenopodium quinoa Willd*)	1) No injection 2) 18 MΩ H2O 3) 5% inulin 4) 1%Q3G 5) 5% quinoa soluble fiber 6) 1%Q3G + 5% quinoa soluble fiber	↑ TNF-α compared to group 4 **Groups 4 and 5:** ↑Bifidobacterium **Groups 3-6:** ↑ n° of GC in villi and crypts ↑ surface area of the villi ↑ Clostridium **Groups 4-6:** ↑ n° of Paneth cells **Group 4:** ↑ Paneth cell diameter
AGARWAL et al., 2022	Saffron flower (*Crocus sativus L.*)	1) No injection 2) 18 MΩ H2O 3) CFWE (1%) 4) CFWE (2%) 5) CFWE (5%) 6) CFWE (10%)	**Groups 3 and 6:** ↓ n° of GC in villi and crypts, depth of crypts, surface area of villi, diameter of GC in villi **Groups 4:** ↑ DcytB gene expression ↓ Expressions of ferroportin, ZnT1, NF-κβ and TNF-α **Groups 5 and 6:** ↑ MUC2 gene expression. **Group 6:** ↓ DMT1 and IL8 gene expression. ↑ Ferroportin expression and number and diameter of Paneth cells **Groups 3,4,5 and 6:** ↓ Lactobacillus and Clostridium
		1) No injection	**Group 3:** ↑ n° of GC in crypts, acidic, neutral, and mixed GC in crypts

AGARWAL et al., 2022	Grape (*Vitis labrusca L.*)	2) 18 MΩ H2O 3) Grape juice (6%) 4) Grape pomace (6%) 5) Grape puree (6%)	**Group 4:** ↑ CRBP2, VDAC2 gene expression, n° of GC in villi, neutral GC ↓ Villus surface area, crypt depth, GC diameter in villi and crypts, acid GC in villi **Group 5:** ↑ Mixed GC, Clostridium ↓ Bifidobacterium
CHENG et al., 2022	Genistein	1) No injection 2)18 MΩ H2O 3) 5% inulin 4)1,25% genistein 5) 2,5% genistein	**Groups 2-5:** ↑ Lactobacillus **Groups 3-5:** ↑ Neutral GC, L.plantarum, Clostridium **Groups 4 and 5:** ↓ DcytB gene expression and GC mix (acidic and neutral), crypt depth, E.coli ↑ diameter and n° of GC in villi, acid GC and Paneth cells, hepcidin gene expression. **Group 5:** ↓ Gene expression of DMT1, ZIP6 ↑ Surface area of villi, Bifidobacterium
JACKSON et al., 2022	Empire apple (*Malus domestica*)	1) No injection 2) 18 MΩ H2O 3) Apple juice (6%) 4) Apple pomace (6%)	**Group 3:** ↓ DMT1 expression (compared to group 1), OCLN, Bifidobacterium ↑ Hepcidin expression (compared to group 1), RBP4 (compared to group 1), GC diameter in villi (compared to groups 2, 4 and 5), Lactobacillus **Group 4:** ↓ Diameter of the GC in the crypts **Groups 3 and 4:** ↓ DcytB Expression **Groups 4 and 5:**

		5) apple pulp (6%)	↑ Villus surface area, number of Paneth cells **Groups 3-5:** ↑ Clostridium ↓ Crypt depth, neutral GC (compared to group 2) **Group 5:** ↑ n° of GC in the crypts, acidic and mixed GC
AGRIZZI VEREDIANO et al., 2022	Black corn	1) No injection 2) 18 MΩ H2O 3) BCSE (5 mg/mL) 4) BCSE (15 mg/mL)	**Groups 3 and 4:** ↑ E. coli and Clostridium; CDX2 and SGLT1 gene expression; pectoral and hepatic glycogen; n° of goblet cells in the crypt, diameter of goblet cells in the villi, n° of GC in the villi, n° of Paneth cells in the crypt ↓ Gene expression of MUC2, AP, TNF-α, IL-6 and NF-κB **Group 3:** ↑ AMPK gene expression. **Group 4:** ↑ PCK1 gene expression; surface area and length of villi ↓ Paneth cell diameter
AGRIZI VEREDIANO et al., 2022	Black corn	1) No injection 2) 18 MΩ H2O 3) Black corn extract (5%) 4) C3G (0.38%)	**Groups 2-4:** ↓ CDX2 Expression **Group 3:** ↑ Clostridium ↓ Acidic GC **Groups 3 and 4:** ↑ Bifidobacterium, Mixed GC in the villi ↓ E. coli, n° of GC in crypts, n° of GC acid in the villi **Group 4:** ↓ Lactobacillus, TNF- α compared to groups 3 and 2

	↑ Villus surface area, crypt depth, number of Paneth cells, GC diameter (villi and crypt), and number (villi) (compared to group 3)

ESSC (soluble chia seed extract); BCSE (black corn extract); CFWE (saffron flower soluble extract); GC: *goblet cells*; SGLT1 (glucose-1 sodium cotransporter); DMT1 (divalent metal transporter 1); Dcytb (duodenal cytochrome); Znt (zinc transporter); Zip 4 (zinc transporter 4); MT4 (metallothionein 4); MUC 2 (mucin 2); AP (amino peptidase); PCK1 (phosphoenolpyruvate carboxykinase); CalbidinD9k (Ca transporter protein); SI (sucrose isomaltase); NF-κβ (nuclear factor kappa beta); TNF-α (tumor necrosis factor-alpha); Q3G (quercetin 3-glucoside); CRBP2 (cellular retinol-binding protein); VDAC2 (voltage-gated anion channel); C3G (cyanidin-3-glucoside); CDX2 (caudal related homeobox transcription factor 2); RBP4 (retinol acyltransferase).

5. METHODOLOGY

This research project encompasses the following components:

1. A systematic review investigating the effects of carotenoids on gut microbiota, gut barrier, and inflammation in healthy animals.

2. An animal experiment utilizing the *Gallus gallus* model, carried out at the Department of Food Science at Cornell University in the USA.

3. A parallel randomized controlled clinical trial for individuals with overweight/obesity, carried out at the Laboratory of Energy Metabolism and Body Composition (LAMECC) in the Department of Nutrition and Health at Universidade Federal de Viçosa (UFV).

5.1 *Gallus gallus* study – Cornell University (USA)

The intervention involved the intra-amniotic administration of the soluble extract derived from cashew nut flour, employing established methodologies previously utilized in studies involving extracts from various foods, such as black corn (Hamjane *et al.*, 2024), carioca beans (Dias, D. M. *et al.*, 2019), grape (Gomes *et al.*, 2021), duck egg white (Hou *et al.*, 2017), yacon flour (Martino, Kolba e Tako, 2020), chia seed (Silva, da *et al.*, 2019), and wheat bran (Wang *et al.*, 2019). *Gallus gallus* eggs were incubated under optimal conditions in the Animal Science poultry hatchery at Cornell University.

5.1.1 Extraction of the soluble fraction of cashew nut flour

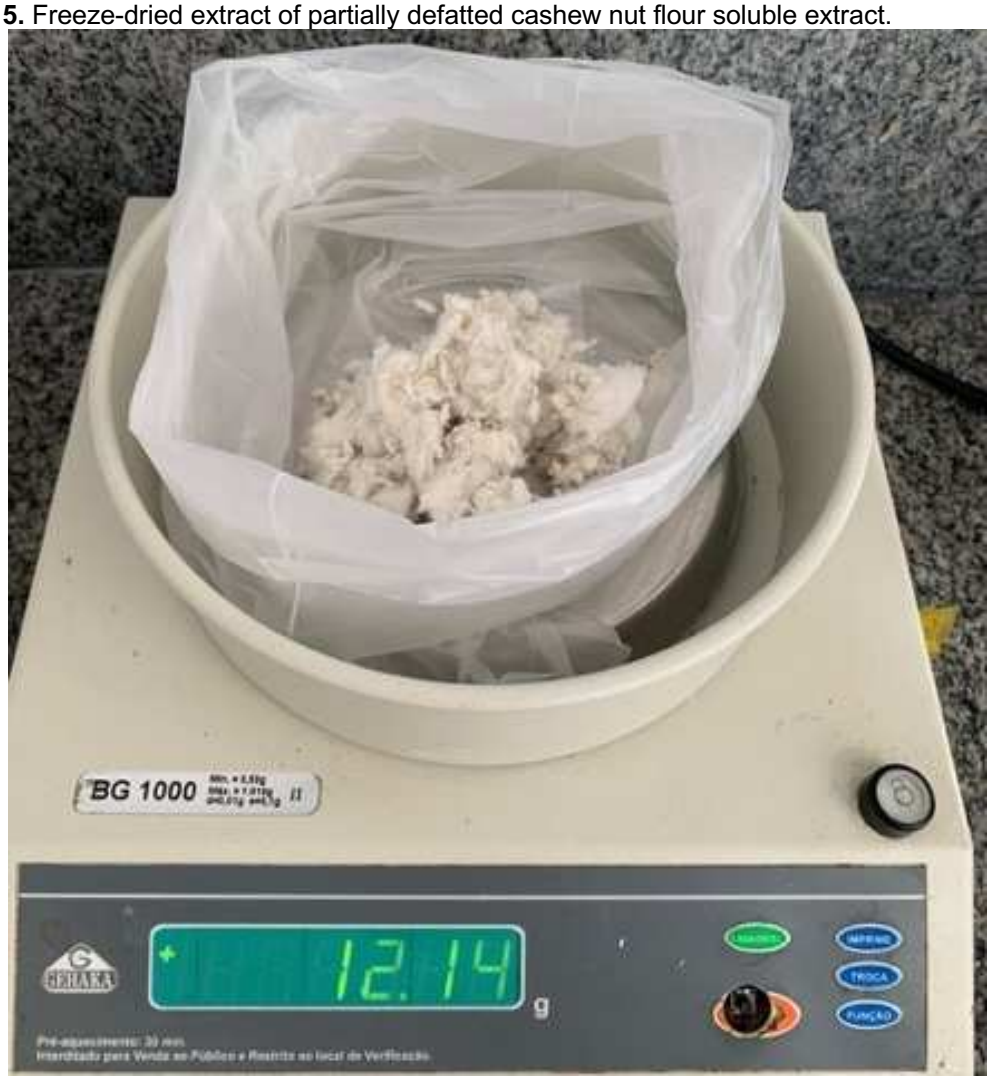
The soluble fraction was extracted using partially defatted cashew nut flour, specifically, the partially defatted cake (**Figure 4**). The partially defatted cake was acquired through a process previously specified by the Embrapa team on a pilot scale, as outlined in project activities SEG 20.18.03.059.00.00. In this process, cashew nut pieces were roasted at 110°C for 15 minutes in an oven with forced air circulation. Subsequently, these pieces were placed in a stainless-steel cylinder, specially designed by the Embrapa Agroindústria Tropical team, and pressed at room temperature (30 °C) through a batch process, using 1 kg of cashew nut per process. A hydraulic press (Marconi MA098/50A/1, Brazil) was employed, applying pressure up to 100 kgf/cm². The resulting cake underwent

processing in a processor equipped with knife blades (Robot Coupe R502V.V, Vincennes, France) and was stored in plastic packages under freezing conditions (-10 °C) until needed.

Figure 4. Partially defatted cashew nut flour cake.

The extraction of the soluble fraction of the cashew nut flour was conducted at UFV, following established methodologies (Tako *et al.*, 2014; Vidanarachchi *et al.*, 2009). Partially defatted cashew nut flour samples, also known as partially defatted cashew nut cake, were dissolved in water (50 g/L) at 60 °C for 60 minutes using a magnetic stirrer. Subsequently, the mixture underwent centrifugation at 4,000 rpm (4 °C) for 15 minutes, and the supernatant was collected. This supernatant was then dialyzed in distilled water for one week using a membrane with a molecular weight cutoff (MWCO) of 12–14 kDa. The resulting dialysate was collected and subjected to freeze-drying, producing a fine powder known as cashew nut soluble extract (**Figure 5**).

Figure 5. Freeze-dried extract of partially defatted cashew nut flour soluble extract.



5.1.2 Protein and fiber analysis (total, soluble, and insoluble) of cashew nut soluble extract

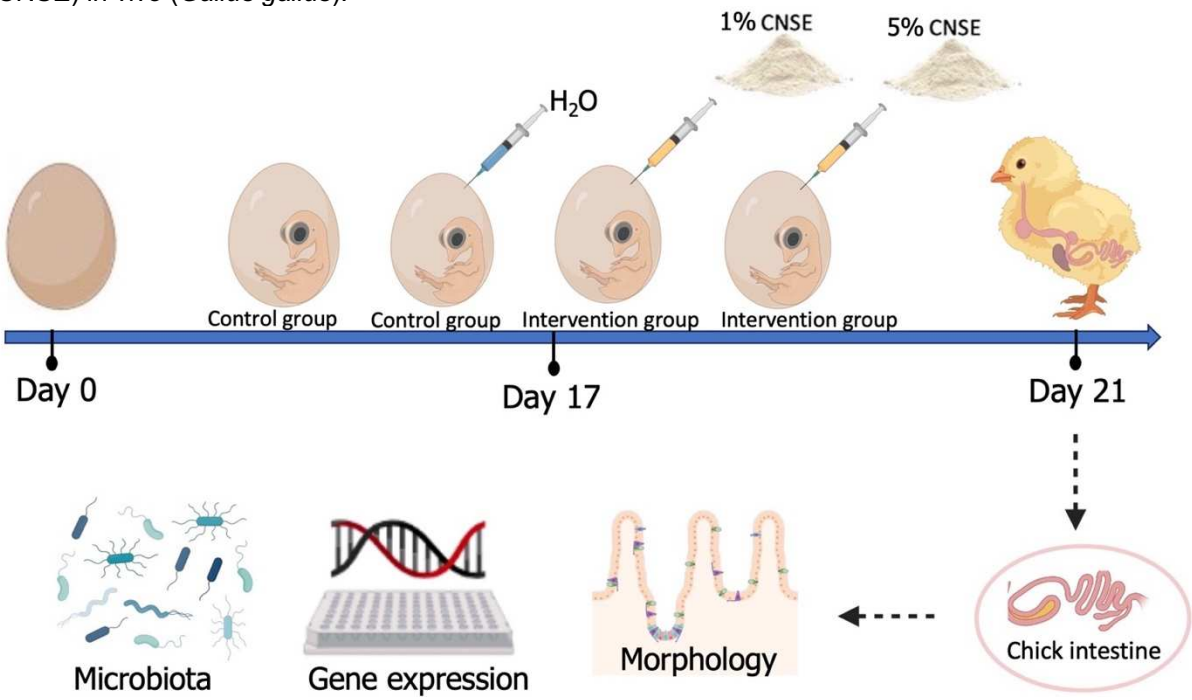
From the cashew nut soluble extract, the protein and fiber contents (total, soluble, and insoluble) were assessed. Total protein and both soluble and insoluble fibers were determined using the gravimetric-enzymatic method by AOAC International, employing the Total Dietary Fiber Assay Kit from Sigma®, San Luis, Missouri, USA (AOAC, 2012). The analysis of protein content was conducted in triplicate, while the determination of total dietary fiber was performed in duplicate. The total dietary fiber was calculated as the sum of the soluble and insoluble fractions.

5.1.3 Animals and Study design

The protocol carried out was approved by the Cornell University Institutional Animal Care and Use Committee (IACUC #2020-0077). All procedures were conducted in compliance with the pertinent regulations and guidelines. Approximately 40 fertile Cornish-cross broiler eggs were acquired from a commercial hatchery (Moyer's Chicks, Quakertown, PA, USA). These eggs underwent incubation under optimal conditions (37 ± 2 °C and 89.6 ± 2% humidity) until hatching at the Cornell University Animal Science poultry farm. The hatchability rate for the experiment was 34/40 eggs (85% survival). Therefore, for the in vivo administration, 34 fertile eggs were weighed and randomly assigned to 4 groups (no injection, H2O, 1% cashew nut soluble extract, and 5% cashew nut soluble extract).

The soluble extract of cashew nut flour was diluted in 18 ohms (Ω) H_2O to establish concentrations with osmolarity not exceeding 320 OSM, preventing embryo dehydration post-injection. Subsequently, the concentrations selected for this study adhered to an osmolarity limit of ≤320 OSM. The injections were administered on the 17th day of embryonic incubation. Each group received a specific solution via injection (1 mL per egg) using a 21-gauge needle into the amniotic fluid, identified by light. Injection sites were sealed with cellophane tape post-procedure, and eggs were evenly distributed in incubator baskets, ensuring each treatment was equally represented in each incubator location. Chicks were humanely euthanized through exposure to CO_2 shortly after hatching (21 days), and the small intestine and cecum were collected for the analysis of intestinal morphology, intestinal barrier gene expression, inflammatory gene expression, and intestinal microbiota (**Figure 6**).

Figure 6. Applied methodology for intra-amniotic administration of cashew nut soluble extract (CNSE) in vivo (*Gallus gallus*).



Source: Own elaboration.

5.1.4 Extraction of total RNA from duodenum tissue

Total RNA was extracted from 30 mg of duodenal tissue (n=5) under RNase-free conditions using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA), following the manufacturer's protocol. The tissues, suspended in buffer RLT® containing β-mercaptoethanol, were disrupted and homogenized with a rotor–stator homogenizer. The lysate was then centrifuged at 8000*g* for 3 minutes in a microcentrifuge (C2400-R, Labnet International Inc, Edison, NJ, USA). The supernatant was transferred to another tube containing 70% ethanol and mixed immediately. Subsequently, 700 µL of the sample was applied to a RNeasy mini column, centrifuged for 15 seconds at 8000*g*, and the flow-through material was discarded.

The RNeasy columns were then transferred to new collection tubes (2-mL), and 500 µL of buffer RPE® was pipetted onto the RNeasy column, followed by centrifugation for 15 seconds at 8000*g*. An additional 500 µL of buffer RPE was pipetted onto the RNeasy column, and centrifugation was performed at 8000*g* for 2 minutes. The elution of total RNA was carried out using 50 µL of RNase-free water. RNA concentration was determined by absorbance at A

260/280. The integrity of the 18S ribosomal RNAs was verified through 1.5% agarose gel electrophoresis followed by ethidium bromide staining. Any DNA contamination was eliminated using a TURBO DNase treatment and removal kit from AMBION (Austin, TX, USA).

5.1.5 Real-Time Polymerase Chain Reaction (RT-PCR)

cDNA was created from extracted RNA in a 20 µL reverse transcriptase (RT) reaction applying BioRad C1000 touch thermocycler using the Improm-II Reverse Transcriptase Kit (Catalog #A1250; Promega, Madison, WI, USA). For that, 1 µg of total RNA template, 2 mM of oligo-dT primers, and 10 µM of random hexamer primers were added to the vial. The ideal annealing temperature was 94 °C (5 min); followed by amplification at 60 min (42 °C), and heat inactivation at 70 °C (15 min). The cDNA obtained was analyzed by Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) or stored at −80 °C until analysis. Concentration was determined by measuring the absorbance at 260 nm and 280 nm, using an extinction coefficient of 33 for single-stranded DNA. Genomic DNA contamination was assessed by an RT-PCR assay (real-time) for the reference gene samples.

5.1.6 Primer design

The Real-Time Primer Design Tool software (IDT DNA, Coralvilla, IA, USA) was utilized to design the primers based on six gene sequences from the Genebank database. The primer sequences (17-25-mier), amplicon length (restricted to 90–150 bp), and gene ID can be found summarized in Table 5. BLAST searches verified primer specificity against the genomic National Center for Biotechnology Information (NCBI) database. The *Gallus gallus* primer 18S rRNA served as a reference gene.

Table 5. DNA sequences of primers used.

Analyte	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Base Pair	GI Identifier
		Intestinal Barrier		
AP	CGTCAGCCAGTTTGACTATGTA	CTCTCAAAGAAGCTGAGGATGG	138	45,382,360
SGLT1	GCATCCTTACTCTGTGGTACTG	TATCCGCACATCACACATCC	106	8346783
MUC2	CCTGCTGCAAGGAAGTAGAA	GGAAGATCAGAGTGGTGCATAG	155	423,101
OCLN	GTCTGTGGGTTCCTCATCGT	GTTCTTCACCCACTCCTCCA	124	396,026
		Inflammatory Response		
NF-κβ	CACAGCTGGAGGGAAGTAAAT	TTGAGTAAGGAAGTGAGGTTGAG	100	2,130,627
IL-1	TCATCCATCCCAAGTTCATTCA	GACACACTTCTCTGCCATCTT	105	395,872

AP: amino peptidase; SGLT1: sodium-glucose transport protein 1; MUC2: mucin 2; OCLN: L; NF-κB: nuclear factor kappa beta; IL1: interleukin 1.

5.1.7 Real-time qPCR design

cDNA (2 µL) was pipetted into a 96-well plate with 2× Bio-Rad SSO Advanced Universal SYBR Green Supermix (8 µL) (Cat #1725274, Hercules, CA, USA), containing buffer, dNTPs, Taq polymerase and dye. Both forward and reverse primers and cDNA (or water as control) were added to each PCR reaction. Each run had duplicates of 7 standard curve points. To prevent and eliminate any potential DNA contamination, a 'no-template control' containing nuclear-free water was incorporated. DNA amplification was performed under the following conditions: an initial denaturation step at 95 °C for 30 seconds, 40 cycles of denaturation at 95 °C for 15 seconds, annealing temperatures varying according to Integrated DNA Technologies (IDT) for 30 seconds, and extension at 60 °C for 30 seconds, using a Bio-Rad CFX96 Touch (Hercules, CA, USA). Gene expression data were obtained by measuring the lowest cyclic product (Cp) values using the automated 'second derivative maximum method.' The results were quantified against the standard curve, diluted at 1:10, and the reaction for each gene was run in duplicates.

A graph illustrating the correlation between the Cq and log (10) concentrations was generated by the software, and the efficiencies were determined using the equation 10 (1/slope). The specificity of the amplified real-time RT-PCR products was confirmed by analyzing the melting curves (ranging from 60 to 95 °C) after 40 cycles, revealing distinct products with specific melting temperatures (Dias, D. M. *et al.*, 2019; Hamjane *et al.*, 2024).

5.1.8 Collection of microbial samples and intestinal contents DNA extraction

Under sterile conditions, cecum samples (n=5) were weighed (0.2 ± 0.02 g) and transferred to a 15 mL tube containing 9 mL of PBS (pH 7.4). Plastic beads were added to the tube, and the mixture was vortexed for 3 minutes. The tube was then centrifuged at 1000× *g* for 5 minutes, and the supernatant was collected and subjected to a second centrifugation step at 4000 *g* for 10 minutes. The buffer was discarded, and the pellet was washed twice with 1 mL of PBS before being stored at -20°C until DNA purification. For purification, the pellet was treated with 50 mM EDTA (pH 8) and lysozyme (Sigma Aldrich CO., St. Louis, MO, USA) (10 mg/mL) at 37 °C. Bacterial DNA isolation was performed using a Wizard Genomic DNA purification kit according to the manufacturer's protocol (Promega Corp., Madison, WI, USA).

5.1.9 Primers design and PCR amplification of bacterial 16S rDNA analysis

Primers for Lactobacillus, Bifidobacterium, Clostridium, Escherichia coli and L. plantarum were used (Tako et al., 2008). The universal primers were designed with the invariant region in the 16S rRNA of bacteria and were used as internal standards. The PCR products were separated by 2% agarose gel, stained with ethidium bromide, and quantified by Quantity One 1-D analysis software (Bio-Ra, Hercules, CA, USA). Each bacterium's relative abundance was evaluated as previously described (Dias, D. M. et al., 2019; Tako et al., 2014). All products were expressed close to the content of the universal 16 s rRNA primer product and proportions of each examined bacterial product.

5.1.10 Morphological examination of duodenal tissue

The duodenum samples per treatment group (n=5) were preserved in 4% (v/v) formaldehyde solution, stabilized with phosphate buffer, dehydrated, cleaned, and embedded in paraffin. Four 5 µm sections of each sample were obtained and placed on a glass slide. The sections were deparaffinized in xylene, and the sample was rehydrated using a series of graded alcohol. The Alcian blue/periodic acid-Schiff (PAS) staining was applied in the slides, and the sample

was assessed under a light microscope (BX3M series, Olympus Waltham, MA, USA) using the CellSens Standard Software. Paneth cells were stained a light purple color. The number and diameter of Paneth cells were recorded, and the following morphometric measurements were evaluated: villus height and width; goblet cells number and diameter in villi and crypt; crypt depth; and goblet cell type (acid, neutral, and mixed) per villi and crypt.

5.2 Brazilian Nut Study – UFV (Brazil)

The Brazilian Nuts study aims to assess the health benefits of native Brazilian nuts on individuals with obesity and overweight through an energy-restriction intervention. The Brazilian Nut Study project has included three clinical trials to date:

- Clinical trial 1: 8-wk randomized controlled intervention, with 40 overweight women meeting at least one additional metabolic syndrome criterion or women with obesity and two parallel groups receiving energy restriction (-500 kcal/d): the control free-nut group (n=19) and the intervention group (n=21), which received Brazilian nuts (30 g of cashew nut, and 15 g of Brazil nut/day). Randomization used a method stratified by blocks, considering BMI, body fat percentage, and age as variables. The computerized randomization system WinPepi, version 11.65, was employed for this purpose. The research adhered to the principles outlined in the Declaration of Helsinki and obtained approval from the local ethics committee (Ethics Committee for Research with Human Subjects at Universidade Federal de Viçosa; CAAE: 92004818.0.0000.5153). Furthermore, it was registered in the Brazilian Registry of Clinical Trials, ReBEC (Trial Registry: https://ensaiosclinicos.gov.br/; identifier: RBR-3ntxrm).
- Clinical Trial 2: 8-wk non-randomized, controlled clinical study, with 56 overweight women meeting at least one additional criterion of the metabolic syndrome or women with obesity and two parallel groups receiving energy restriction (-500 kcal/d): the control free-nut group (n=29) and the intervention group (n=27), which received Brazilian nuts (30 g of cashew nut, and 15 g of Brazil nut/day). In this trial, 10 additional volunteers were assigned to the control group, and another 19 volunteers who participated in phase 1 were included in the control group of this trial, resulting in a total of 29 volunteers in this group.

Therefore, the second stage of the research was conducted in a controlled manner, but not at random, due to logistical constraints in data collection for a new control group. It was also conducted in compliance with the Declaration of Helsinki. It received approval from the local ethics committee (Ethics Committee for Research with Human Subjects at Universidade Federal de Viçosa; CAAE: 92004818.0.0000.5153; N° 2.832.601/2018) and was registered in the Brazilian Registry of Clinical Trials, ReBEC (Trial Registry: https://ensaiosclinicos.gov.br/; identifier: RBR-3ntxrm).

The present study, which encompasses Clinical Trial 3, is an 8-week parallel randomized controlled clinical trial, including men or women with overweight associated with at least one more criterion of metabolic syndrome or with obesity. Three groups receiving energy restriction (-500 kcal/d) were utilized for this trial: the control free-nut group (n=19), the cashew nut group receiving a single dose of cashew nut (30g/day) (n=24), and the cashew nut oil group (30 ml/day) (n=25). The MinimPy 3.1 software, a computerized randomization system, was employed for this trial. The procedures outlined in the Brazilian Nuts Clinical Trial 3 study adhere to Resolution CNS/466 of 2012 and the Declaration of Helsinki, which address ethical principles in clinical research. The same project underwent review by the Human Research Ethics Committee of the Universidade Federal de Viçosa (N° 4.543.541/CEPH) and was submitted to the Brazilian Registry of Clinical Trials (ReBEC) (RBR-8xzkyp2). Selected individuals received information about the study's objectives and procedures, and those who accepted the conditions, terms, and objectives, as well as agreed to the collection of biological materials (blood, and urine), signed the Informed Consent Form in duplicate (APPENDIX A).

5.2.1 Study design

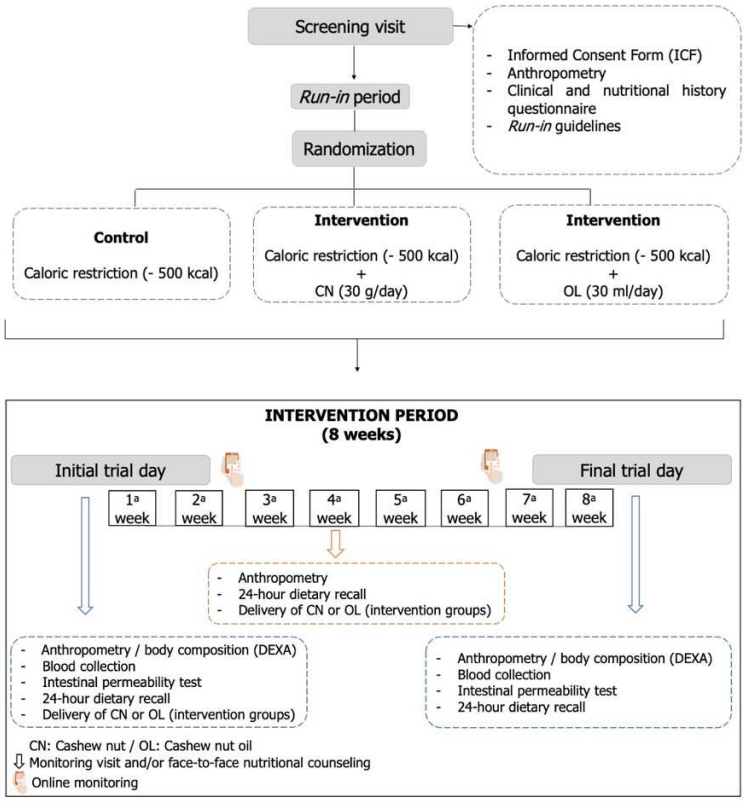
This study comprises a controlled, randomized, three-arm study involving the control group, cashew nut group, and cashew nut oil group. As part of clinical trial 3 within the Brazilian Nuts Study, participants attended the Laboratory of Energy Metabolism and Body Composition at the Department of Nutrition and Health at Universidade Federal de Viçosa (LAMECC/UFV) on three occasions during the intervention: two collection days (initial and final) and in the middle of

the study, specifically in the fourth week (30 days) for a face-to-face monitoring visit.

On the initial and final days at LAMECC, measurements of weight, anthropometry, and body composition were conducted. Additionally, blood samples were collected to assess cardiometabolic and inflammatory markers, along with urine collection for the analysis of intestinal permeability and completion of a food questionnaire. During the mid-study visit, only weight information and anthropometric measurements was collected. Furthermore, participants visited the Health Division (DSA/UFV) on the initial and final days for a body composition test using DEXA.

In addition to the face-to-face visits, participants received online monitoring for any clarifications or doubts. Researchers contacted participants to inquire about their ability to adhere to the food plan, whether any dietary changes were necessary, and if there were any adverse effects. Information about healthy eating and the benefits of cashew nut and oil on health was also provided to the intervention groups through folders, aiming to stimulate and enhance participant adherence to the study (**Figure 7**).

Figure 7. Study flowchart.

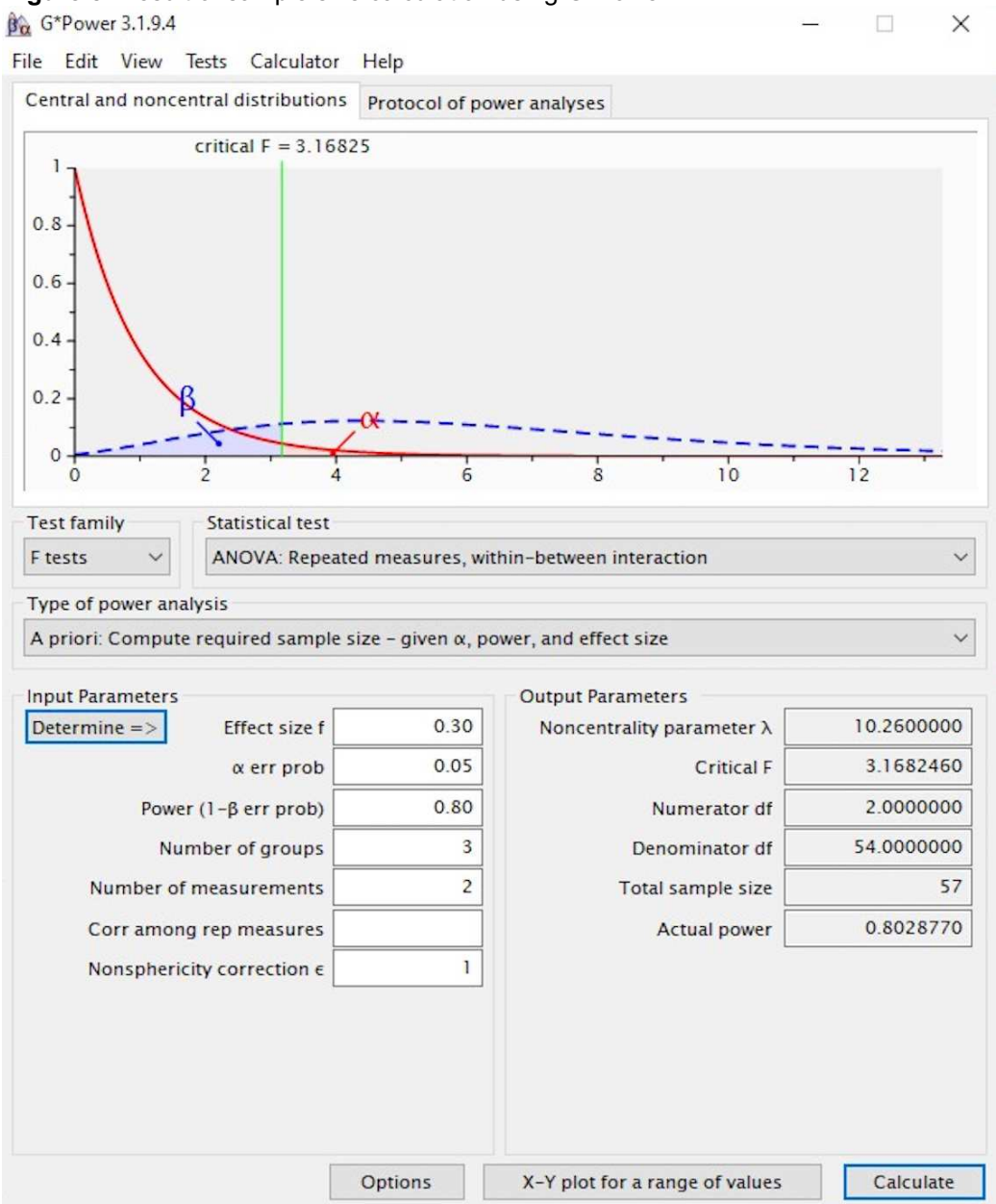


Source: Own elaboration.

5.2.2 Sample size

The sample size was determined a priori using the G*Power 3.1 program. For this calculation, a total of 57 volunteers were determined, based on an average estimated effect size derived from clinical studies (0.30), considering statistical analyses for three groups, two intervention points (baseline and endpoint), an alpha value set at 0.05, and a power of 0.80. By adding 20% as a result of losses during follow-up, the total sample size was determined to be 68 participants (**Figure 8**).

Figure 8. Result of sample size calculation using G*Power.



5.2.3 Inclusion and non-inclusion criteria

These are the eligibility criteria for the study:

- Men and women between 20 and 55 years old;
- Excess weight (27 – 29.9 kg/m²), high waist circumference (WC) (≥ 80 cm for women; ≥ 90 cm for men), and excess body fat (> 30% for women and > 20% for men), associated with at least one other MS component: triglycerides ≥150 mg/dL; blood pressure ≥ 130/85 mmHg) or fasting blood glucose ≥100 mg/dL or taking medication to control these markers; or;
- Obesity (BMI ≥ 30 kg/m²), high WC (≥ 80 cm for women; ≥ 90 cm for men), and excess body fat (> 30% for women and > 20% for men), regardless of the presence of other components of MS.

The non-inclusion criteria are:

• Being pregnant, lactating, or women in menopause;

• Be athletes;

• Be vegan;

• Having a diagnosis of insulin-dependent diabetes;

• Have a diagnosis of HIV, digestive, hepatic, renal, cardiovascular, thyroid disease or alterations, cancer, inflammatory diseases and eating disorders;

• Having a history of drug and/or alcohol abuse;

• Having an aversion or allergy to nuts;

• Having an infection in the last month;

• Habitually consume nuts above 30 g/day;

• Using drugs such as anti-inflammatories, corticoids, and antibiotics, capable of causing biochemical changes;

• Having problems that may interfere with chewing;

• Indicate weight instability (5% of usual weight) in the last 3 months;

• Consume alcohol > 21 units (≈168g) per week;

• Consume vitamin, mineral, and omega 3 supplements.

5.2.4 Recruitment and screening

Study participants were recruited in Viçosa, Minas Gerais, Brazil, through the dissemination of the study on radio stations, social networks, and the UFV network platform, with email and telephone contacts provided. Alongside the online platform promotion, a link to an online form with questions determining individuals' eligibility was made available. After interested individuals completed this online form, those meeting the inclusion criteria in the study underwent a first visit to confirm eligibility through a semi-structured face-to-face selection questionnaire (APPENDIX B). This questionnaire included inquiries about clinical and dietary history, sociodemographic and anthropometric data, body composition, blood pressure, and biochemical tests from the last three months presented by the volunteer (when available). During this visit, participants were informed about all the procedures to be performed. They also received a booklet containing guidelines about the study and were instructed to record any changes in the type and/or dosage of medications for continuous use, as well as changes in general health status (APPENDIX C).

5.2.5 Run-in

A one-week run-in period was implemented to identify and exclude participants likely to deviate from the research protocol. During this phase, volunteers adhered to their usual diet with restrictions on consuming any type of nut, dried fruits (such as berries, cranberry, blueberry, gojiberry, raisins), açaí, cocoa, cinnamon, olive oil, and alcoholic beverages. After run-in period, participants were weighed, and those exhibiting a body weight variation exceeding ± one kg or reporting the consumption of any restricted foods and/or beverages were deemed poor responders and subsequently excluded from the study.

5.2.6 Randomization

After completing the run-in period, participants were randomly allocated using the stratified minimization method, considering gender, age, and BMI as

factors with three levels per factor. This approach ensured a balanced distribution of potential factors that could influence the outcome variables. Randomization was executed using the MinimPy 0.3 program.

5.2.7 Intervention

The initial experimental day coincided with the first day of intervention. The intervention involved daily consumption of an energy-restricted eating plan without nut consumption (control), or the same plan with either cashew nut (30 g, CN group) (APPENDIX D) or cashew nut oil (30 ml/day, OL group) (APPENDIX E), depending on the intervention group, for eight weeks. For the oil group, participants were provided with a dosing cup to standardize and ensure the exact recommended amount was consumed. At the end of the data collection on day 0 of the study, each participant received a food plan with guidelines and was released. All participants were given five menu options to follow during the eight weeks of the study. Energy restriction was calculated by subtracting 500 kcal from the daily caloric requirement, estimated using the Mifflin formula (Muffin *et al.*, 1990). The diets were isocaloric across groups, respecting individual participant needs and considering their level of physical activity. All menus were calculated using the Brazilian Institute of Geography and Statistics (IBGE) table in an Excel® spreadsheet (IBGE, 2011).

The meal plans were structured into five meals: breakfast, morning snack, lunch, afternoon snack, and dinner. Regarding macronutrients, the intervention groups received approximately 27-32% of their total caloric intake from lipids, while the control group obtained around 21% of ingested calories (**Table 6**). The objective was that both nuts and oil would be an additional food in the diet of participants who consume these, and for this reason, naturally resulting in a higher lipid supply in these groups. Therefore, no other food with similar characteristics was included, and there was no adjustment for macronutrient distribution balance in the control group. Both cashew nut and oil were provided at two points: on the initial day of data collection and during the on-site monitoring visit (4th week). The amount delivered on the first day was adequate for daily consumption until the first monitoring visit, and the quantity given in the 4th week was enough for consumption until the end of the study.

It is important to emphasize that, after the study, all participants in the control group were given cashew nut. Additionally, all care was given to this group. Participants present in the laboratory during the morning period were individually attended to in separate rooms, where they received their meal plans and were provided with all the instructions. This approach ensured that other participants present on those days remained unaware of the specific guidance given to each individual.

Table 6. Distribution of macronutrients among study groups.

Macronutrients (%)	Control	Cashew nut	Cashew nut oil
Total fat	21.19 ± 1.84	27.04 ± 2.49	31.83 ± 3.87
Saturated Fat	*8.63 ± 1.97*	*8.96 ± 1.45*	*7.78 ± 1.47*
Monounsaturated Fat	*6.14 ± 1.58*	*9.90 ± 1.79*	*14.29 ± 2.32*
Polyunsaturated Fat	*3.28 ± 0.86*	*4.43 ± 1.19*	*5.54 ± 1.33*
Carbohydrates	54.82 ± 3.51	48.06 ± 4.17	48.47 ± 5.55
Proteins	23.54 ± 2.74	24.89 ± 3.14	20.99 ± 3.15

5.2.8 Monitoring and adherence to the study protocol

Adherence to the protocol was monitored through follow-up visits, with online check-ins at 15 and 45 days after the start of the intervention conducted via WhatsApp or video call. The 30-day follow-up was conducted face-to-face at LAMECC/DNS/UFV, lasting 30 minutes. During these visits, participants' nut/oil consumption, potential adverse effects, weight, and body composition were recorded. A 24-hour recall was administered, nutritional guidelines were reinforced, and additional cashew nut/oil were provided. Participants who did not adhere to the intervention protocol or experienced adverse effects were excluded. The control group also attended the monitoring visit and underwent the same evaluations as the other intervention groups but did not receive nuts/oil.

To enhance control over nut and oil consumption, participants in the CN and OL groups returned any unconsumed nut packages and the cashew nut oil bottle during monitoring visits. Additionally, to promote better adherence among oil consumers, various recipes utilizing cashew nut oil, such as shakes, salad dressings, and chickpea paste to be consumed with toast, were provided (APPENDIX E).

Throughout the eight-week intervention period, participants displaying poor adherence to the study protocol or experiencing adverse reactions to nut/oil consumption were excluded from the research. Additionally, women who became pregnant or exhibited menopausal symptoms (as diagnosed by a physician) discontinued their participation in the study.

5.2.9 Test foods and microbiological quality

Cashew nut (*Anacardium occidentale* L.) and cashew nut oil were sourced from Brazil, specifically from the Brazilian Agricultural Research Corporation (Embrapa) Agroindústria Tropical- Fortaleza-CE (**Figure 9**). The oil samples were extracted through centrifugation. To prepare the samples, the cashew nut was roasted at 110 °C for 15 minutes; subsequently, the cashew nut was ground in a food processor. Water was added to the cashew nut (in a ratio of 4:1 cashew nut to water, w/w), and the mixture was homogenized in a processor at 90 °C for 10 minutes. This mixture underwent centrifugation for 1 hour at 4,500 rpm at room temperature. Following centrifugation, the oil was heated in an oven at 105 °C for 1 hour.

Figure 9. Cashew nut and cashew nut oil provided from EMBRAPA.

Source: Own elaboration.

The raw materials were obtained from the same harvest, and their microbiological quality was analyzed and ensured through reports provided by the supplier company until they were delivered to LAMECC. The nuts were sent already portioned in laminated packages, sealed under a vacuum, similar to how the oil was fractionated into 250 mL amber glass bottles. Both products were stored in a freezer at -20 ºC until the moment of their distribution to the study participants to prevent nutrient loss, oxidation, sensory alterations, and microbiological contamination. All materials for consumption were handled in the LAMECC kitchen, and the handlers adhered to all hygienic-sanitary standards, including the use of clean lab coats, caps, masks, and disposable gloves.

5.2.10 Proximate composition of minerals, fatty acids, amino acids, vitamin E and its fractions, total phenolics, and antioxidant capacity of cashew nut and cashew nut oil

The centesimal characterizations of cashew nut and cashew nut oil were conducted by Embrapa in Fortaleza, CE, and the samples underwent determinations in quintuplicate. Regarding cashew nut, moisture and ash contents were determined according to the AOAC methodology (2016). Protein content was determined by combustion using the DUMAS method in a Nitrogen/Protein Analyzer NDA 701 Dumas, with EDTA as a standard, based on the AOAC method (AOAC, 2016). Lipid content was determined by the method no. Am 5-04 of the American Oil Chemists' Society (AOCS, 2004), employing a high-pressure and high-temperature extraction system in Ankom XT-15 equipment (ANKON Technology Corporation, 2009). Carbohydrate content was calculated as the difference of 100 minus the sum of values obtained for moisture, ash, proteins, and lipids. The energy value per 100 g of each product was calculated using the Atwater system: Caloric value = (g of protein × 4) + (g of lipids × 9) + (g of carbohydrates × 4). Amino acids (including aspartic acid, glutamic acid, serine, glycine, histidine, taurine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, cystine, isoleucine, leucine, phenylalanine, lysine, hydroxyproline, tryptophan, and the total sum of amino acids) were determined using the MA-009 R0 method (Hagen *et al.*, 1993; White, Hart e Fry, 1986). Tryptophan was specifically analyzed using method MA-010 R.1 (Lucas e Sotelo, 1980), and crude protein was quantified through the MA-001 R4 method.

In vitro digestibility was assessed following the procedure outlined by Akeson and Stahmann (1964) (Akeson e Stahmann, 1964). The determination of total dietary fiber (soluble and insoluble fiber) was carried out at the Experimental Nutrition Laboratory of the Department of Nutrition and Health at UFV through the non-enzymatic gravimetric method, utilizing a commercial kit (Total Dietetic Fiber Assay Kit, Sigma®, San Luis, Missouri, USA) (AOAC, 2016).

Concerning cashew nut oil, the following analyses were conducted by Embrapa using the specified methodologies: the acidity index (Ca 5a-40 Method) and peroxides were determined under AOCS (2004) guidelines. For fatty acid analysis, previously extracted oil samples were transformed into methyl esters (FAMEs) following the procedure outlined by Hartman and Lago (1973). Subsequently, the materials were examined using a gas chromatograph equipped with a flame ionization detector (GC2010; Shimadzu, Kyoto, Japan) and a stationary-phase biscyanopropyl-polydimethylsiloxane capillary column (SP2560, 100m×0.25 mm, df 0.20; Supelco®, Bellefonte, PA, USA).

Regarding both, cashew nut and cashew nut oil, the analysis of total phenolics and antioxidant capacity was conducted at the Experimental Nutrition Laboratory of the Department of Nutrition and Health at UFV, following the methodology outlined below. The determination of total phenolic compounds, obtained from absorbance readings, was performed using a spectrophotometer (Thermo Scientific, Evolution 606, USA) at 765 nm. An analytical curve of gallic acid (0.005–0.10 mg/mL) was employed for compound quantification, with results expressed in mg of gallic acid equivalents per gram of cashew nut flour or cashew nut oil (mg GAE/g). Antioxidant activity was assessed through the DPPH (2,2-diphenyl-1-picryl-hydrazyl) free radical scavenging ability, as previously described (Bloor, 2001). Mineral analyses (including phosphorus, potassium, calcium, magnesium, selenium, sodium, copper, iron, zinc, and manganese) were conducted in accordance with the Food and Drug Administration (FDA) methodology (Fda *et al.*, 2010, 2015).

The analysis of vitamin E isomers (α-, β-, γ- and δ-tocopherol and tocotrienol) in cashew nut and cashew nut oil was conducted at the Vitamin Analysis Laboratory of the Department of Nutrition and Health at UFV, following the methodology described by PINHEIRO-SANT'ANA et al. (2011) (Pinheiro-Sant'Ana *et al.*, 2011). For analysis, the HPLC system (Shimadzu, model SCL

10AD VP) was employed, consisting of a high-pressure pump with a valve for low-pressure quaternary gradient (model LC-10AD VP), autosampler with loop of 50 µL (model SIL-10AF), helium degassing mobile phase system (Shimadzu, model DGU-2 A) and fluorescence detector (model RF-10A XL). Qualitative identification of the compounds was performed by comparing the retention times obtained for standards and samples analyzed under the same conditions. The isomers identified in the cashew nut and cashew nut oil were quantified by external standard curves. The real concentration was calculated based on the performed dilutions. The quantification of the compounds was performed from calibration curves and regression equations with R2 greater than 0.997. The final concentration was obtained by corrected calculations for the dilutions and concentrations performed during the extraction and analysis procedures. During the analyses, the samples were protected from sunlight and artificial light by means of amber glassware, aluminum foil and blackout curtains, and protected from oxygen using lids and environments with nitrogen gas in glass flasks.

5.2.11 Variables and measurement instruments

5.2.11.1 Collection and separation of biological samples

Biochemical determinations in the present study were conducted by the Hemolab Clinical Analysis Laboratory (Viçosa-MG), the contracted facility responsible for blood collection and analysis of biochemical markers. On the day preceding the collection (both at the beginning and end of the intervention period), participants fasted for 10 to 12 hours to perform the blood collection and intestinal permeability at LAMECC. Nursing technicians, specifically employed for this project, conducted the blood collection, obtaining samples ranging from 20 to 30 mL via vacuum. Following collection in suitable tubes, centrifugation was performed at 3500 rpm for 10 minutes at 4˚C. Subsequently, biological samples (serum and plasma) were isolated and stored in an ultra-freezer at -80 ºC until analysis.

For the intestinal permeability test, participants submitted all collected fasting urine, which was stored in a 2 L of a plastic container under refrigeration. Subsequently, participants underwent the procedure for collecting postprandial urine. Upon arriving at the laboratory, they consumed, within 10 minutes, a

solution containing lactulose (10 g), mannitol (5 g), and sucrose (20 g). Throughout the next 4 and a half hours spent in the laboratory, participants collected their entire urine volume in a container designated for postprandial urine storage. During the initial 60 minutes, no food or water intake was permitted. Subsequently, participants were allowed to consume only water (150 mL after 2 and 3 hours of testing). The total volume of urine collected during fasting (night urine) and postprandial phases was aliquoted (30 mL), treated with thimerosal (0.007 g) to inhibit microbial growth, and preserved at -80 ºC until analysis.

5.2.11.2 Anthropometric and body composition measurements

Anthropometric measurements, including weight, height, and circumferences of the waist, hip, and neck, were acquired with participants dressed in light clothing and barefoot, adhering to standardized guidelines. The hip circumference was measured at the most prominent region, waist circumference at the umbilical height, and neck circumference at the midpoint between the cervical spine and the anterior neck. Inelastic tape with a precision of 0.1 cm was utilized for all measurements. Participants stood upright with legs firmly grounded and shoulder-width apart, head in the Frankfurt plane, and arms crossed, ensuring hands touched the shoulders. Waist measurement was taken during expiration. Additionally, waist-to-height ratio (WHtR) and waist-to-hip ratio (WHR) were calculated.

Body composition was evaluated using the Dual-energy X-ray Absorptiometry (DEXA) technique employing fan-beam technology (Lunar Prodigy Advance DXA System, version 13.31, GE Lunar), following the manufacturer's recommended protocol. Dual-energy X-ray absorptiometry provided assessments of fat mass (FM), fat-free mass (FFM), lean mass (LM), and total mass, both for the entire body and specific regions such as trunk, android, and gynoid. The android area spans between the ribs and pelvis, while the gynoid region encompasses the hips and upper thighs, overlapping with the leg and truncal areas. Percentage-based calculations for body composition were derived from total body measurements. As an alternative method, weight and body composition were also measured using tetrapolar electrical bioimpedance (InBody, model 230, Biospace Co., Ltd), with a maximum capacity of 250 kg, adhering to the manufacturer's protocol. Height was measured with a wall

stadiometer (Seca, model 206, Hamburg, Germany), with a maximum length of 2 meters and one-millimeter precision. BMI was calculated as weight divided by height squared (kg/m^2), and classifications were based on World Health Organization (WHO, 2011) cutoff points.

All anthropometric and body composition measurements were taken at the beginning and at the end of the intervention.

5.2.11.3 Cardiometabolic and liver function markers

After a 12-hour fasting period, samples were collected to assess cardiometabolic markers, including triglycerides (≥150 mg/dL), total cholesterol (≥ 240 mg/dL), LDL-c (≥ 160 mg/dL), HDL-c (<40 or <50 mg/dL for men and women, respectively), and VLDL-c (≥30 mg/dL). Additionally, apolipoprotein-A-1 (APO-A-1), apolipoprotein-B (APO-B), cortisol, liver markers such as AST transaminase, gamma GT, ALT transaminase, and alkaline phosphatase were compared as mean and standard deviation between groups. Atherogenic indices, namely total cholesterol:HDL-c and LDL-c:HDL-c, proposed by Castelli (1988), were also calculated (Castelli *et al.*, 1988).

5.2.11.4 Inflammatory markers

The evaluation of serum CRP concentration was performed by the Hemolab laboratory using the immunoturbidimetry technique on an autoanalyzer (Mindray / BS-200® Chemistry Analyzer). Interleukins (IL-6, TNF-a, IL-10, IL-1β, IL-18, and IL-12p70) were analyzed via flow cytometry in serum or plasma samples, employing commercial kits (BD Biosciences®), and the data were analyzed using FCAP Array Software v3.0 (BD Biosciences®).

5.2.11.5 Biomarkers of intestinal permeability

For the intestinal permeability analyses, thawed urine was homogenized using a vortex, and 2 mL of urine were withdrawn with a sterile syringe. The syringe was connected to a polyethersulfonic (PES) microporous membrane (0.22 µm × 13 mm), allowing the sample to be filtered directly into vials for HPLC. The final volume of urine filtered into the vials was 600 µl. Lactulose and mannitol excretion in urine were measured by HPLC using a Dionex Ultimate 3000 Dual

coupled to a Refractive Index (IR) Detector (Shodex RI-101) maintained at 40˚C. Analytes were separated on an ion exclusion column Phenomenex Rezex ROA (300 × 7.8 mm) maintained at 40 °C. 5 mM sulfuric acid (H2SO4) served as the mobile phase with a flow rate of 0.7 mL/min. Internal lactulose and mannitol standards were used to establish the normalization curve. The total volume of urine collected was multiplied by the concentration of each sugar to determine the total amount excreted in the urine. Results in the spreadsheet were expressed as the percentage excretion of mannitol (% M) and lactulose (% L) and then calculated the lactulose/mannitol ratio (L/M).

LBP and zonulin in EDTA plasma were measured by human Enzyme-Linked Immunosorbent Assay (ELISA) kits (LBP, catalogue n° MBS704355; Human zonulin, catalog n° MBS167049). All the measurements were conducted with a Thermo Multiskan™ FC Microplate Photometer, following the manufactures' instructions.

5.2.11.6 Physical activity

The level of habitual physical activity practice was estimated using the International Physical Activity Questionnaire (short IPAQ) (ANNEX A), at the beginning and at the end of the intervention.

5.2.11.7 Food consumption

Food consumption was assessed at three time points: during the initial collection, after 30 days, and on the day of the final collection, using the 24-hour recall method. The Erica-REC24h software (Barufaldi *et al.*, 2016) was employed for quantifying food intake, featuring an extensive database with nearly two thousand registered food items derived from the 2008-2009 Family Budget Survey (POF) conducted by the Brazilian Institute of Geography and Statistics (IBGE). Any foods not present in the Erica database were added by the researchers. The quantity of food consumed was converted into grams and/or milliliters to correlate with a nutritional composition table (IBGE, 2011) using the Statistical Package for the Social Sciences (SPSS® Inc. version 25.0). Subsequently, energy and nutrient intake were calculated.

5.2.11.8 Return to study' participants

At the end of the intervention, each participant received a personalized report detailing the results of anthropometric and body composition analyses, as well as biochemical and blood pressure assessments. Furthermore, they were guided on the significance of maintaining a healthy diet and ensuring adequate intake of beneficial lipid sources. Participants who were excluded either during the run-in period or throughout the intervention were also offered nutritional feedback, accompanied by advice on healthy eating, and received a report containing the information collected up to the point of their withdrawal from the study.

5.3 Statistical analyzes

For all statistical analyses, the Shapiro–Wilk test was conducted to assess data normality, and IBM SPSS Statistics®, version 22, was utilized, adopting a p-level < 0.05. In the clinical trial study, data were expressed as mean values and standard deviation. Variable changes among groups were compared by one-way ANOVA followed by Tukey's post hoc test or using the non-parametric Kruskal–Wallis test followed by Dunn's post hoc test. One-way repeated measures ANOVA with group and time interaction followed by post-hoc was used to evaluate the effect of time (initial, return, and final) on the following variables: weight, waist, hip, and neck circumferences in the three groups. To compare differences between baseline and post-intervention within the groups, pairwise tests were performed (paired t-test or Wilcoxon). Independent samples *t*-test was employed to compare independent groups. For categorical variables, the chi-square test was used, and McNemar's test was employed to analyze paired nominal data. Pearson and Spearman correlations were used to investigate the association between intestinal permeability, inflammatory, cardiometabolic, and adiposity markers. Multiple linear regression models were performed to analyze the association between inflammatory markers by using IL-1β marker (dependent variable), and adiposity, inflammatory, and intestinal permeability (LBP) markers, and also consumption of cashew nut and cashew nut oil (independent variables). In the animal model study, data were presented as mean values ± standard error. Experimental treatments and controls for intra-amniotic administration were

randomly assigned after ensuring weight distribution across all groups. One-way Analysis of Variance (ANOVA) or Kruskal-Wallis was employed to analyze the results, followed by post-hoc Duncan or Dunn's test.

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6. RESULTS

6.1 Article 1

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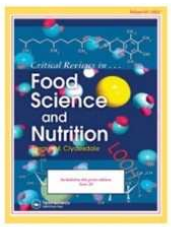

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Effect of carotenoids on gut health and inflammatory status: A systematic review of *in vivo* animal studies

Talitha Silva Meneguelli, Marcella Duarte Villas Mishima, Helen Hermana Miranda Hermsdorff, Hércia Stampini Duarte Martino, Josefina Bressan & Elad Tako

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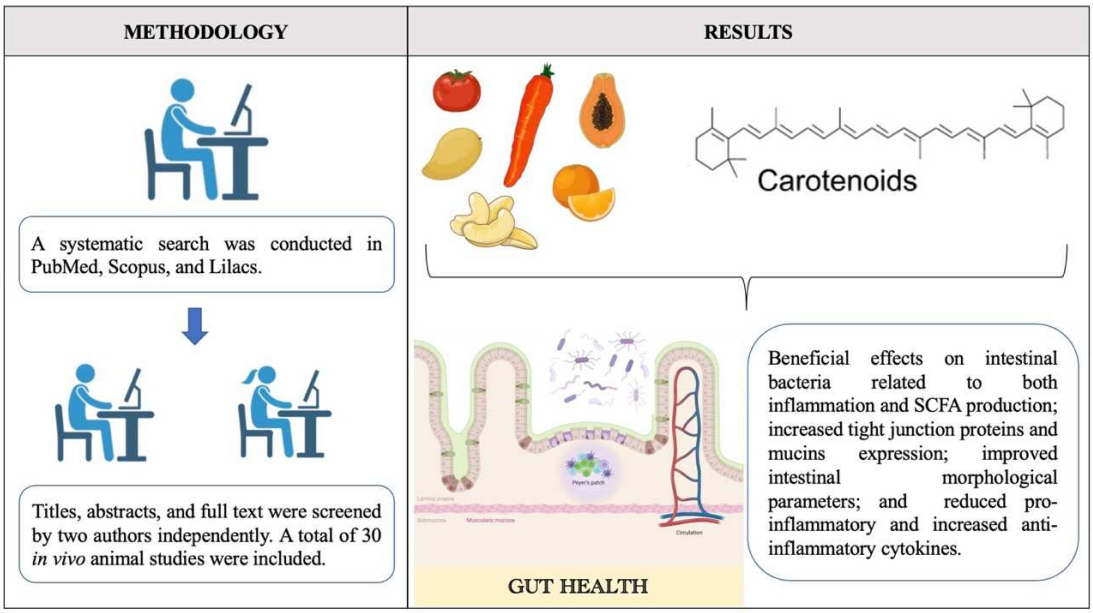
Abstract

Carotenoids have anti-inflammatory and antioxidant properties, being a potential bioactive compound for gut health. The objective of this systematic review was to investigate the effects of carotenoids on gut microbiota, gut barrier, and inflammation in healthy animals. The systematic search from PubMed, Scopus, and Lilacs databases were performed up to March 2023. The final screening included thirty studies, with different animal models (mice, rats, pigs, chicks, drosophila, fish, and shrimp), and different carotenoid sources (β-carotene, lycopene, astaxanthin, zeaxanthin, lutein, and fucoxanthin). The results suggested that carotenoids seem to act on gut microbiota by promoting beneficial effects on intestinal bacteria related to both inflammation and SCFA production; increase tight junction proteins expression, important for reducing intestinal permeability; increase the mucins expression, important in protecting against pathogens and toxins; improve morphological parameters important for digestion and absorption of nutrients; and reduce pro-inflammatory and increase anti-inflammatory cytokines. However, different carotenoids had distinct effects on gut health. In addition, there was heterogeneity between studies regarding animal model, duration of intervention, and doses used. This is the first systematic review to address the effect of carotenoids on gut health. Further studies are needed to better understand the effects of carotenoids on gut health.

Keywords: Carotenoids; xanthophylls; animal model; gut health; inflammation

GRAPHICAL ABSTRACT



Introduction

Gut health is influenced by many factors such as normal and stable gut microbiota composition, effective digestion, and absorption of food in the brush border membrane (BBM), and a solid gut barrier which consists of physical and chemical protection against pathogens and toxins (Bischoff, 2011; Gilbert *et al.*, 2018; Huttenhower *et al.*, 2012; Picariello, Ferranti e Addeo, 2016; Silva, Da, Martino e Tako, 2021; Solis *et al.*, 2020). When there is an imbalance in the intestinal microbiota, known as dysbiosis, there is an increase in intestinal permeability, which causes weaknesses in gut barrier function, dysregulation of the innate and adaptive immune response, and an increase in inflammatory process which can lead to inflammatory bowel disease (IBD) and various metabolic disorders (Bischoff *et al.*, 2014; Boulangé *et al.*, 2016).

In this regard, several environmental factors may influence gut health, being diet one of the main (Gentile e Weir, 2018). Several bioactive compounds and their role in gut health have been investigated in recent years, among them carotenoids have received great attention. Carotenoids are fat-soluble pigments which provide red, orange, or yellow color for the foods and are found in the plant kingdom (algae, fungi, fruits and vegetables, such as carrots, pumpkins, sweet potatoes, tomatoes and dark leafy greens, in addition to nuts (Mandalari *et al.*, 2013), especially cashew nuts (which has β-carotene, lutein and zeaxanthin) (Trox *et al.*, 2010), and animal products (eggs and fish) (Toti *et al.*, 2018). They are classified as provitamin A (β-carotene, α-carotene, β-cryptoxanthin,), and non-provitamin A or xanthophylls (zeaxanthin, lutein, astaxanthin, cantaxanthin, fucoxanthin, lycopene), and have antioxidant and anti-inflammatory properties (Claudia Stange *et al.*, 2016). As mammals are not capable of synthesizing carotenoids from endogenous precursors, their acquisition is necessary through the diet, and the major site of their absorption and metabolism is the small intestine (Lintig, von *et al.*, 2020).

When ingested, carotenoids are mainly absorbed in the small intestine, by their incorporation into mixed micelles (containing bile salts, cholesterol, fatty acids, monoacylglycerides and phospholipids). Then, these are transported to intestinal cells by passive diffusion and/or via a transporter-dependent process by lipid membrane transporters as the cluster determinant 36 (CD36) (Borel et al., 2013),

NPC1-like transporter 1 (NPC1L1), and the scavenger receptor class B type 1 (SRB1) in the BBM. SRB1 is regulated by two carotenoid cleavage enzymes, one is the beta-carotene oxygenase 1 (BCO1) that cleaves pro-vitamin A carotenoids resulting in a production of vitamin A and converted into retinoic acid, and another is the beta-carotene oxygenase 2 (BCO2) that cleaves all carotenoids into apocarotenals (Reboul, 2019). As fat-soluble bioactive compounds bound to plants, in crystalline form or dissolved in oil, the bioavailability of carotenoids can be low and variable, depending on food sources and processing, which can vary between 10-40%(Palm *et al.*, 2014). In this way, a significant part is not absorbed and reaches the colon intact, where it is metabolized and fermented by the intestinal microbiota (Eroglu *et al.*, 2023). Carotenoids have been widely studied owing to their beneficial properties on health such as diabetes (Hamer e Chida, 2007), overweight and obesity (Yao *et al.*, 2021), cardiovascular risk factor (Ming e Lara, [s.d.]), coronary artery disease (Osganian *et al.*, 2003), and total mortality (Buijsse et al., 2005), due to their anti-inflammatory and antioxidant properties (Krinsky e Johnson, 2005). However, carotenoid metabolism in the gut and their impacts on gut microbiome remain unclear (Bohn, 2017), and to the best of our knowledge, there is no systematic review that has addressed the effect of carotenoids on gut health (microbiota, morphology and functionality).

Thus, the objective of this systematic review was to describe the effects of carotenoids on gut health by evaluating the gut microbiota, gut barrier, and its role in inflammation in healthy animals that have not received any intervention other than dietary intake of carotenoids.

Materials and methods

This systematic review was conducted according to Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (Page et al., 2021). The study is registered on International Prospective Register of Systematic Reviews (PROSPERO) (n°CRD42023410227).

Eligibility criteria

To identify the studies and evaluate the inclusion and exclusion criteria, PICOS anagram was used (P=Population; I=Intervention; C=Control; O=Outcomes; S=Study design) (Table 1), and the question was: “How carotenoids can act on

gut health with regard to intestinal morphology and functionality, microbiota and inflammation?"

In order to obtain the largest number of articles and evidence, we carefully include articles that, even if they evaluated their effect on some pathology or dysfunction, if at least one group was healthy and did not receive any other type of intervention besides carotenoid ingestion, these articles were included.

Search strategy and data collection process

The following databases were used: PubMed, Scopus, and Lilacs (Scientific and Technical Literature of Latin America and the Caribbean/VHL—Virtual Health Library). There was no date restriction, and no filters were used. The descriptors used were defined from the Medical Subject Headings (MeSH), using the Boolean operators "OR" and "AND" on searches to associate one term with another. The search strategy used the following indexed terms: #1 "carotenoids" OR "carotenoid" OR "tetraterpenes" OR "tetraterpene derivatives" OR "derivatives, tetraterpene" OR "carotenes" OR "carotene" OR "xanthophylls" OR "xanthophyll carotenoids" OR "carotenoids, xanthophyll" AND #2 "intestines" OR "intestine" OR "intestinal" OR "gut". The last search was performed in March 2023. The results found in each database were submitted into Rayyan QCRY Software® (Ouzzani *et al.*, 2016). After deleting duplicates, the study selection process was conducted by two authors independently first by screening titles and abstracts, then by reading the entire text. In case of disagreement during the evaluation process of the articles, the two authors discussed the topic, and in case of disagreement, a third author also evaluated it.

For each included study, the following characteristics of the studies were extracted: authors; year of publication; country in which the study was conducted; animal characteristics; number of animals per group; type of carotenoid; time of intervention; and administration of the carotenoid. Also, we extracted information about the groups (control and intervention), dose of the carotenoid used, and main results of the outcomes of interest. All this information was summarized in a data extraction template by using Microsoft Excel®. Also, information was obtained about the amounts of each type of carotenoid (β-carotene, lycopene, astaxanthin, zeaxanthin, lutein, and fucoxanthin) found in the included articles. All these data were presented on tables and figures.

Risk of bias assessment

The methodological quality and risk of bias of the studies included in this systematic review were assessed using the Systematic Review Center for Laboratory Animal Experimentation Risk of Bias (SYRCLE). This tool takes into account six types of bias such as, selection bias, performance bias, detection bias, attrition bias, reporting bias and other. For each question there is a classification considering the risk of bias in the article as low (−), unclear (?), and high (+) (Hooijmans *et al.*, 2014).

Results

Study selection and characteristics

Flow diagram was made according to the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) guideline. From our initial screening in three selected databases, 8124 articles were found (Scopus= 2754, PubMed= 3562, Lilacs= 1808), 4947 of which were duplicates. All titles and abstracts were assessed, as a result, 3049 articles were excluded. From the reports assessed for eligibility, 128 articles were evaluated, of which 96 were excluded for the following reasons: outcome of interest not measured (n= 28), in vitro studies (n= 4), another component that not carotenoid was used (n= 2), carotenoid associated with other foods or components (n= 4), carotenoid was not administered orally (n= 4), conference abstracts (n= 4), language other than English (n=1), and animals with disease or that have received any intervention other than the use of carotenoids (n=51). Therefore, 30 articles were included for this review (Figure 1).

All characteristics of the studies included in this systematic review are shown in table 2. The studies took place in different countries, most of them in China (n=15), others in Japan (n=3), Turkey (n=2), India (n=2), USA (n=2), Russia (n=1), Egypt (n=1), Argentina (n=1), Hungary (n=1), Poland (n=1), and Australia (n=1). Among the animal models, the most used was mice (n=11), followed by rats (n=8), piglets (n=3), chicks (n=2), carps (n=2), drosophila (n=1), fish (n=1), sows (n=1), and shrimp (n=1). Among the carotenoids, the most used was β-carotene (n=9), followed by lycopene (n=7), astaxanthin (n=5), zeaxanthin (n=4), lutein (n=3), and fucoxanthin (n=3) (Figure 2). One study used carotenoids in general, which was the Hungarian sweet red pepper (Capsicum annuum) extract.

Furthermore, some studies used xanthophylls (which means both lutein and zeaxanthin), another used Alfafa protein-xanthophyll concentrate (PXC) (which had lutein, zeaxanthin, violaxanthin, and neoxanthin).

Quality assessment

The studies included in the systematic review did not have a high risk of bias. Although most studies performed randomization, the majority did not make it clear which variable was used for randomization and which method was used, such as computer random number generator or random number table. Most did not present all the baseline characteristics of the animals such as sex, age and weight. Details about randomly housed and blinding during the experiment were also not detailed in the studies. Most studies did not include all animals in the analyzes and did not make it clear why. All studies were apparently free of other problems that could result in high risk of bias (Figure 3).

Carotenoids

There are many in vivo (animal models) studies (n=50) that evaluated the effects of dietary carotenoids under various disease conditions (Figure 1), but studies evaluating the effect of dietary carotenoids on gut health and inflammation in healthy animal models are fewer. The form of carotenoids administration was oral in all included studies, with some added to the diet (n= 17), others by gavage (n= 12), and one added to water (n= 1). The doses of each carotenoid varied between studies, and the studies varied in relation to the animal model, number of animals per group, and as well as the intervention time (Table 2). The results found in this systematic review, and in reference to each type of carotenoid, will be described by topics below.

β-carotene

Regarding the outcomes of interest in this systematic review, which are gut microbiota; functionality (gene expression of intestinal barrier); intestinal morphology and inflammation, six studies evaluated the microbiota, while none evaluated short chain fatty acid (SCFA). Among these, some showed that supplementation with β-carotene did not change α-diversity (Honarbakhsh *et al.*, 2022; Li *et al.*, 2021; Zhu *et al.*, 2021), one increased (Yuan *et al.*, 2020) and other decreased (Rossi *et al.*, 2020). Regarding bacteria, one study reported that β-carotene did not change the abundance of Actinobacteria, Firmicutes,

Bacteroidetes, and Firmicutes/Bacteroidetes ratio (Honarbakhsh *et al.*, 2022). Others β-carotene increased Bilophila and Sutterella (belonging to the Proteobacteria) (Yuan *et al.*, 2020), Firmicutes (Li et al. 2021, Yuan et al. 2020), Corynebacterium (belonging to the Actinobacteria) (Yuan *et al.*, 2020), Bacteroidetes (Zhu *et al.*, 2021) and Lactobacillus (Hui *et al.*, 2020). Others showed a reduction in Lachnospiraceae and Ruminococcaceae (belong to the Firmicutes) (Yuan *et al.*, 2020), Prevotellaceae (belong Bacteroidete) (Yuan *et al.*, 2020), Firmicutes (Yuan *et al.*, 2020), Actinobacteria (Yuan *et al.*, 2020), Bacteroidetes (Li *et al.*, 2021), Prevotella (Li *et al.*, 2021), and E.coli (Hui *et al.*, 2020). Only two studies evaluated gene expression of intestinal barrier and β-carotene increased mRNA ZO-1, ZO-2, occludin, and also mRNA of IgA and MUC-2 (Hui *et al.*, 2020), on the other hand in another study gene expression of MUC-2 was maintained, but increased gene expression of MUC-3 (Honarbakhsh et al., 2022). Two evaluated intestinal morphology, and supplementation with β-carotene increased villus height, crypt depth, villi height/crypt depth ratio, and villus area (Hui *et al.*, 2020), but in another at higher doses reduced crypt depth (Li *et al.*, 2021). Finally, six studies evaluated inflammation, in some β-carotene did not alter inflammation (neither gene expression nor serum levels) (Trivedi e Jena, 2015; Ünal et al., 2020; Yang et al., 2021). While in others there was a reduction in expressions of NF-κβ, IL-6, IL-1β, IL-22 e IL-17, TNF- α, phosphorylation of IκBα, and LPS, (Honarbakhsh *et al.*, 2022; Li *et al.*, 2021a; Zhu *et al.*, 2021) (Table 3).

Lycopene

Only two studies evaluated the microbiota. Lycopene did not change Bifidobacteria, Bacteroidetes, Firmicutes, Roseburia spp, and Akkermensia muciniphila, and α-diversity but when added to a high fat diet lycopene increased Lactobacillus and SCFA, on the other hand lycopene reduced Enterobacteriaceae, and at genes level Treponema_2, Prevotellaceae_unclassified, and members of Treponema and Prevotella (Meng *et al.*, 2022; Singh *et al.*, 2016). Four studies evaluated gene expression of intestinal barrier. There were no differences in the expression of these tight junction genes (mRNA claudin 3, claudin 4, and ZO-2) (Liu et al., 2022; Meng et al., 2022; Wang, Y. et al., 2021), but there was an increase in the expression of

mRNA claudin-1, occludin, and ZO-1 (Wang, Y. et al., 2021). Five studies evaluated intestinal morphology, in one study HFD significantly altered the normal morphological features of ileum (reduction of villus and crypts length), but supplementation of lycopene (10 mg/kg) significantly prevented these changes (Singh *et al.*, 2016). In three studies, lycopene did not alter crypt depth (Liu *et al.*, 2022; Meng *et al.*, 2022; Rajput *et al.*, 2021), and in one study decreased (Lin *et al.*, 2022). Regarding villus height, two studies showed that the lycopene did not change (Lin *et al.*, 2022; Liu *et al.*, 2022), and in two others there was an increase (Meng *et al.*, 2022; Rajput *et al.*, 2021). All studies showed an increase in villus height/crypt depth ratio (Lin *et al.*, 2022; Liu *et al.*, 2022; Meng *et al.*, 2022; Rajput *et al.*, 2021). Five studies evaluated the effect of lycopene on inflammation. In one, IL-1β, and TNF-α in the serum decreased, e IL-6 in the serum increased in high fat diet + lycopene (HFD+Lyc) compared to HFD, but these same markers had no differences in diet normal diet (NPD)+Lyc compared to NPD (Singh *et al.*, 2016). In some studies, lycopene did not alter the expression of TNF-α e IL-1β, IL-6, IL-8, IL-17, NF-κβ (Meng *et al.*, 2022; Wang, Y. *et al.*, 2021; Yucel *et al.*, 2016), although at higher doses of lycopene there was an increase in mRNA IL-1β in jejunum (Lin *et al.*, 2022) in contrast, lycopene up-regulated the expression of IL-10 and IL-22 (Lin *et al.*, 2022)(Table 3).

Astaxanthin

Four studies evaluated the effect of astaxanthin on gut microbiota and showed no difference in bacterial diversity (Pratap *et al.*, 2022; Wang, W. *et al.*, 2021; Wu *et al.*, 2020; Yasuda *et al.*, 2022). The studies varied greatly in the genus and phylum of bacteria analyzed. Two studies evaluated SCFAs, one study showed no difference (Wu *et al.*, 2020), and another showed a reduction in acetic acid and butyric acid (Yasuda *et al.*, 2022). One study showed that astaxanthin increased the abundance of Cyanobacteria and Gastranaerophilales, and decreased the abundance of Firmicutes and Spirochaetes, and Parabacteroides distasonis (Wu *et al.*, 2020). Another study showed that astaxanthin did not alter Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Clostridium, Ruminococcus, Bacteroides, Bifidobacterium, Escherichia– Shigella, Klebsiella, Sutterella, Prevotella, Eubacterium, Lachnospiraceae, Rikenellaceae, Blautia, Haemophilus, and Lactobacillus (Wang, W. *et al.*, 2021). In addition, Yasuda et

al. (2022) showed reductions in Clostridiales, Lactobacillales, Coprococcus and Butyricicoccus; and increase in Verrucomicrobiales, while Pratap et al. (2022) showed reduction in abundance of bacteria from the families Akkermansiaceae and Ruminococcaceae, and from the genus Akkermansia. No study evaluated gene expression of intestinal barrier and morphology. One study evaluated inflammation and showed that there was no change in mRNA TNF-a, IL-1β, IL-6, IL-36a, and IL-36γ (Shigeki Sakai *et al.*, 2019) (Table 3).

Other carotenoids (zeaxanthin, lutein, and fucoxanthin)

One study evaluated the effect of xanthophylls (40% lutein and 60% zeaxanthin) in two different kinds of experiments. Results from experiment 1 showed that, compared to the control, hens who received 20 or 40 mg/kg of xanthophylls decreased mRNA IFN-γ and IL-1β in jejunum, and there was no difference in IL-4 mRNA and IL-6 levels among treatments. Results from the experiment 2 shows that *in ovo*, xanthophylls decreased mRNA IL-6 (in duodenum and jejunum), mRNA IL-1β (jejunum and ileum) and mRNA IFN-γ (duodenum, jejunum, and ileum), and increased mRNA IL-4 (jejunum) and IL-10 (jejunum and ileum), there was no affect in mRNA IL-4 (in duodenum, and ileum) and IL-10 (duodenum) (Gao *et al.*, 2012). In another study, Wistar rats were supplemented with 1.5% or 3% of alfafa protein-xanthophyll concentrate (which has lutein, zeaxanthin, violaxanthin, and neoxanthin), the results showed no difference in SCFAs (acetic, propionic, isobutyric, butyric, isovaleric, valeric), crypt depth, MUC-2 and MUC-3 gene expression, but there was a reduction in mucus layer thickness (Barszcz *et al.*, 2021) (Table 3).

Two studies evaluated the effect of zeaxanthin alone. One used 50 mg/kg/day of zeaxanthin during 15 days in sprague dawley rats and evaluated only inflammatory markers and there were no differences in gene expression of TNF-α, IFN-γ, IL-1β, IL-6, NF-κβ, and COX-2 (El-Akabawy e El-Sherif, 2019). The other used 20 mg/kg/day during 22 weeks in C57BL/6N mice and evaluated only microbiota and did not show differences in α-diversity, Firmicutes, Proteobacteria, Clostridia, and Desulfovibrio (Xie *et al.*, 2021). No studies evaluating zeaxanthin alone have evaluated intestinal morphology and functionality (Table 3).

Regarding fucoxanthin, three studies evaluated this isolate. One used 1 μM of fucoxanthin in drosophilas for 63 days and showed decreased intestinal permeability in males and increased expression of antimicrobial peptide genes (drosomycin, drosocin, and diptericin) (Moskalev *et al.*, 2018). Another used 150 mg/kg of fucoxanthin for 12 weeks in C57BL/6J mice and showed decrease in SGLT1 expression (Cao *et al.*, 2018). And the other study used 125 mg/kg for 4 weeks in BALB/c mice, when supplemented with fucoxanthin related to phyla there was a reduction in Proteobacteria, Actinobacteria, Deferribacteres, Verrucomicrobia and increase of Bacteroidetes in cecal microbiota and Firmicutes in fecal microbiota. Regarding bacteria classes, there was a reduction in Epsilonproteobacteria and Verrucomicrobiae and increase of Bacteroidia in cecum and increased in Clostridia and reduction in Bacteroidia in fecal. Related to bacteria families, there was an increase in Ruminococcaceae and S24-7 in cecum, but reduction in S24-7 in fecal, in addition to increase in Oscillospira and Prevotella in fecal. Related to genus, there was an increase in Bacteroides and Oscillospira and decrease in Akkermansia in cecum. Although those who received a high fat diet and supplemented with fucoxanthin had different results (Table 3). No difference between TNF-α, IFN-γ, IL-1β, IL-6, NF-κ β, COX-2, histological score, and inflammatory response scores were observed (Guo *et al.*, 2019). No studies have evaluated the effect of fucoxanthin on intestinal morphology and data regarding inflammation and gene expression on intestinal functionality are scarce. Regarding the microbiota, fucoxanthin seems to act beneficially on bacteria that produce SCFA, since it increased Bacteroidetes, Bacteroidia, Clostridia, Oscilospira, Prevotella, and Ruminococcaceae which are bacteria with an important role in the digestion and absorption of nutrients and in the production of SCFA (Broadfield *et al.*, 2022; Portincasa, Bonfrate, Vacca, *et al.*, 2022; Tomova *et al.*, 2019) (Table 3).

Only one study evaluated lutein in isolation, and there were no changes in TNF-a, IL-1β, LBP, and LPS in the serum, proteins expression levels of NF-κβ, IkBa, TLR4 and MyD88, length of duodenum, jejunum and ileum, villi length, villi lenght/crypt depth ratio, protein expression levels and mRNA of the claudin1, occludin, ZO-1, alpha diversity, but had reductions in crypt depth and increase in Akkermansia, Phascolarctobacterium,and Incertae sedis (Zhao *et al.*, 2023). A study evaluated carotenoids in general, using sweet red pepper pulp extract e

there was an increase in class Bacteroidia, order Bacteroidales, families Bacteroidaceae, Rhodospirillaceae, and the genera Chryseobacterium, Enterococcus, and Acidovorax, Fusobacteria, Cetobacterium, and decreased in Proteobacteria, P/F ratio, F/B ratio, Acinetobacter, Bacillus, and Pseudomonas (Feher *et al.*, 2021) (Table 3).

Discussion

The present systematic review evaluated the effects of different carotenoids on gut health. Only healthy animals were included, because the objective was to evaluate how carotenoids can act in a physiologically stable environment, and because it is difficult to compare the effects of such intervention on various markers under different disease circumstances. Among the carotenoids, the most studied were β-carotene (n=9), lycopene (n=7) and astaxanthin (n=5).

β-carotene had a beneficial effect in the digestion and absorption of nutrients by acting on the intestinal morphology, increasing villus height, villi height/crypt depth ratio and villus area, and in the regulation of the intestinal barrier by modulating the gene expression of proteins present in tight junctions, although only two studies evaluated these markers. Also, β-carotene showed beneficial effects on the immune system by increasing IgA and sIgA (produced in the mucous membranes) (Hui *et al.*, 2020). Regarding the gut microbiota, in general, β-carotene acted mostly on gram-negative bacteria and/or bacteria that are related to various inflammatory processes (including inflammatory diseases) such as Bilophila, Sutterella, Sedimentbacter, Prevotellaceae, Prevotella, E.coli, Bacteroidetes, Firmicutes, and Ruminococcaceae, although this is gram-positive, some species are associated with inflammatory bowel disease. In this way, β-carotene reduced LPS, transcription factor NF-κβ and inflammatory cytokines.

Lycopene seems to have beneficial effects on gut microbiota by increasing SCFA, and acting on dysbiosis, as it increased Lactobacillus (gram-positive bacteria, which can ferment sugars and produce lactic acid) and reduced gram-negative bacteria (Enterobacteriaceae and Prevotella). Dysbioses of Prevotella have been associated with inflammatory bowel disease (IBD). Lycopene has also shown beneficial effects on the intestinal barrier by increasing protein expression of claudin-1, occludin, and ZO-1. In morphology, the beneficial effects were mainly in the villus height/crypt depth ratio. On the other hand, in general, the

studies did not show an effect of lycopene on pro-inflammatory markers, but on anti-inflammatory by up-regulating IL-10 and IL-22, that have immunomodulatory activity. However, further studies are needed, as only one study evaluated these markers.

Astaxanthin had beneficial effects on intestinal microbiota, increasing Gastranaerophilales (investigated as a possible probiotic) and Verrucomicrobiales, since some species such as Akkermansia muciniphila is able to feed on intestinal mucus and produce beneficial metabolites such as propionic acid, in addition to helping to reduce intestinal permeability (Roussel *et al.*, 2022). Also, astaxanthin reduced both gram-negative bacteria (e.g., Firmicutes, Spirochates, Parabacteroides distasonis, Akkermansia), and gram positive (e.g., Firmicutes, Clostridiales, Coprococcus, Butyricicoccus, and Ruminococcaceae). Only one studied evaluated inflammation and did not show that astaxanthin altered inflammatory markers. Furthermore, the effect of this carotenoid on intestinal morphology, and relevant gene expression to gut health has not been report yet. So, more studies are needed to evaluate the effects of astaxanthin on gut health.

As indicated in the results section, the studies showed different carotenoid doses and intervention time. Studies using the following concentrations of β-carotene: 40, 50, or 80 mg/kg were able to reduce inflammatory markers, whereas a dosage of 20 mg/kg was not able to reduce them. Most of the studies that used lycopene used a concentration of 10 mg/kg, in this concentration and even in a higher concentration (50 mg/kg) there were no changes in inflammatory markers, regardless intervention time (10 days, 28 days, 30 days, or 12 weeks). Thus, at a concentration of 50 mg/kg, β-carotene could reduce inflammatory markers, but lycopene at the same concentration could not. However, higher concentration (200 mg/kg) of lycopene was able to reduce mRNA IL-1β. Regarding astaxanthin, the studies used the same concentration 0.04%, but different intervention times (10 days and 8 weeks), and both did not change inflammatory markers, except for IL-1β, which was reduced in the longer intervention time. Other carotenoids, such as zeaxanthin and fucoxanthin, it was not possible to compare the effects of dosages and intervention time because the studies evaluated different outcomes, and lutein had only one study using it isolated (without being

associated with another carotenoid). Another key point to consider, and in the context of the carotenoids dietary class (not type specific), is that carotenoids at dosages of between 20 - 80 mg/kg were able to reduce inflammatory markers (Gao *et al.*, 2012; Honarbakhsh *et al.*, 2022; Li *et al.*, 2021; Zhu *et al.*, 2021). While the increase in expression of mucins and tight junctions proteins were observed in higher doses (between 50 mg/kg and 200 mg/kg) (Honarbakhsh *et al.*, 2022; Hui *et al.*, 2020), and villi height/crypt depth ratio was increased in lower doses (from 10 mg/kg) of carotenoids (Hui *et al.*, 2020; Li *et al.*, 2021; Lin *et al.*, 2022; Liu *et al.*, 2022; Meng *et al.*, 2022; Rajput *et al.*, 2021). However, it is important to emphasize that it is difficult to make comparisons regarding the doses of carotenoids used and the time of intervention, as the reviewed studies utilized different animal models, and as a result, different biomarkers were evaluated, in each study. In addition, there is a limited number of studies that investigated the link between dietary carotenoids (dosage), and time of intervention.

Related to the outcomes evaluated in this systematic review, different types of carotenoids, were demonstrated to have different effects on gut health, such as gut microbiota, intestinal morphology and functionality, and inflammation. Specifically, β-carotene, fucoxanthin, astaxanthin and an extract rich in carotenoid (sweet red pepper pulp extract) acted on the microbiota by increasing or decreasing the abundance of Firmicutes and Bacteroidetes (Feher et al., 2021; Guo et al., 2019; Li et al., 2021; Wu et al., 2020; Zhu et al., 2021). High Firmicutes to Bacteroidetes ratio are associated with obesity (Sekirov *et al.*, 2010), while a low proportion may be related to inflammatory bowel disease (IBD) (Stojanov, Berlec e Štrukelj, 2020). Both diseases are related to inflammation. Other bacteria like Ruminococcaceae, Proteobacteria and/or Prevotella that are also associated with inflammation were modified by carotenoids (β-carotene, astaxanthin, fucoxanthin, and/or an extract rich in carotenoids) (Feher *et al.*, 2021; Guo *et al.*, 2019; Li *et al.*, 2021; Lucas López *et al.*, 2017; Yuan *et al.*, 2020). A reduction in Ruminococcaceae was associated with several diseases such as IBD (L. Wang et al. 2021) Also, Proteobacteria is considered as an indicator of microbiome imbalance and is associated with inflammation and damage to the mucosal epithelial cells and increased permeability in the intestine

(Lupp *et al.*, 2007; Nagalingam, Kao e Young, 2011). Prevotella is associated with inflammation and appears to be related to mucin degradation in pigs (Wright, Rosendale e Roberton, 2000).

Both Proteobacteria and Prevotella are gram-negative bacteria that have LPS in their membranes (Aroutcheva, Ling e Faro, 2008; d'Hennezel *et al.*, 2017; Kim, S. J. *et al.*, 2007; Larsen, 2017; Rizzatti *et al.*, 2017). Almost all gram-negative bacteria have LPS which is a surface molecule derived from the outer membrane and can induce systemic inflammation by activating receptors present in the intestinal barrier such as TLR4 and subsequently activates NF-κB leading to the translocation of this from the cytoplasm to the nucleus. Thereby, NF-κB contributes to various inflammatory processes due to its ability to transcriptionally activating pro-inflammatory cytokines (Cheng *et al.*, 2021). The studies included in the present review showed that the β-carotene reduced LPS, NF-κB, and inflammatory cytokines. The same way, combination of lutein and zeaxanthin was able to reduce mRNA IFN-γ and mRNA IL-1β levels. As carotenoids are lipid-soluble molecules, they play important role within biological membranes, and can interact with the NF-κB pathway (Kaulmann e Bohn, 2014). NF-κB is responsible for the transcription of a variety of genes that regulate inflammatory responses. By blocking the activation of the NF-κB p65 subunit, carotenoids inhibit the downstream production of inflammatory cytokines, such as IL-1β, IL-6 and TNF-a (Kaulmann e Bohn, 2014). Among the studies included, one that combined lutein and zeaxanthin; astaxanthin; β-carotene; and lycopene were able to reduce these inflammatory cytokines (Gao *et al.*, 2012; Li *et al.*, 2021b; Liu *et al.*, 2022; Wu *et al.*, 2020; Zhu *et al.*, 2021). These are cytokines that affect the barrier function of the intestinal mucosa causing disruption of the intestinal TJ barrier resulting in a further increase in TJ permeability as TNF-α induces apoptosis and alter the tight junctions (Al-Sadi, Boivin e Ma, 2009). Also, IL-1β increases intestinal tight junction permeability through the reduction in occludin and cytoskeletal rearrangement, and IL-6 can induce the stimulation of NF-κB (Al-Sadi, Boivin e Ma, 2009). In this way, by acting to reduce inflammation, carotenoids can consequently improve the intestinal barrier.

The intestinal barrier consists of a physical protective barrier against the entry of pathogens and other harmful substances into the body (Camilleri *et al.*, 2012;

Keita e Söderholm, 2010). The intracellular and paracellular mechanisms of the intestine work together to maintain the integrity of the barrier and the breakdown of this barrier can lead to various metabolic changes and diseases (Suzuki, 2020). When the tight junction barrier is damaged and the paracellular permeability is increased, it can lead to persistent inflammation and tissue damage (Turner, 2009). The results of this review showed that carotenoids positively altered the intestinal barrier as they increased the expression of ZO-1, ZO-2, occludin, and claudin-1. The intestinal cells are joined by the tight junctions, as ZOs, occludin and claudins which are transmembrane proteins that form the intercellular junctions of intestinal epithelial cells. This physical barrier prevents increased intestinal permeability, and consequently the passage of pathogens and other harmful substances from the intestinal lumen (Suzuki, 2020). In addition to the beneficial effects on tight junctions, this review also showed that carotenoids increased the expression of MUC-2 and MUC-3. MUC-2 acts preventing the penetration of microorganisms and other harmful agents, while MUC-3 contributes to the prevention of inflammation (Gendler e Spicer, 1995; Seregni1 *et al.*, 1997). Both are mucin proteins involved in the production of mucus, which is produced by goblet cells and functions as a protective barrier against pathogens in the intestine (Seregni1 *et al.*, 1997). This layer of mucus is essential to maintain the integrity of the epithelial cells layer which is essential for gut barrier function (Seregni1 *et al.*, 1997).

SCFA are also important in producing mucus and preventing the growth of pathogenic bacteria by lowering intestinal pH, thus maintaining intestinal integrity (Luu e Visekruna, 2019; Xiao *et al.*, 2020). Also, SCFA can provide anti-inflammatory effects in the gut (Singh *et al.*, 2017). Few studies have evaluated the effect of carotenoids on SCFA, (Barszcz *et al.*, 2021; Singh *et al.*, 2016; Wu *et al.*, 2020), and only one showed an increase (Singh *et al.*, 2016). Some SCFA-producing bacteria such as Lactobacillus, Bacteroidetes, Firmicutes, Oscilospira, Prevotella, and Ruminococcaceae were modulated by β-carotene, fucoxanthin, astaxanthin, Sweet red pepper pulp extract, or lycopene (Feher *et al.*, 2021; Guo *et al.*, 2019; Hui *et al.*, 2020; Jiao *et al.*, 2018; Li *et al.*, 2021b; Tomova *et al.*, 2019; Wu *et al.*, 2020; Zhu *et al.*, 2021). Altogether, these results suggest that carotenoids may be promising in the production of SCFA. Among these bacteria, some play an important role in the digestion and absorption of nutrients (Jin *et*

al., [s.d.]; Krajmalnik-Brown *et al.*, 2012; Zhang *et al.*, 2022). Other important factors for the digestion and absorption of nutrients are the morphological parameters of the intestinal barrier (Choct, 2009; Sobolewska *et al.*, 2017). In this regard, carotenoids may also be able to improve digestion and nutrient absorption as lycopene, β-carotene and lutein increased villi height and villi height/crypt depth ratio. Both the increase in villi and the reduction in crypts lead to greater digestion and absorption of nutrients (Sobolewska *et al.*, 2017).

In this way, carotenoids seem to act on gut microbiota by promoting beneficial effects on intestinal bacteria related to both inflammation and SCFA production. This is because the studies included in this review showed that carotenoids were able to modulate (increasing or reducing) Firmicutes, Bacteroidetes, Ruminococcaceae, and Prevotella. All these bacteria are involved both in inflammatory processes and production of SCFA (Hu *et al.*, 2020). The distinction between these two outcomes depends on a variety of factors such as the presence of different subspecies or strains within the same bacterial species, as well as the environmental conditions in which it is found. As different animal models were evaluated in this study, this may have interfered in environmental-related conditions.

Related to inflammation, carotenoids may affect gut microbiota by reducing bacterial populations that are associated with inflammation, as gram-negative bacteria which possess LPS (e.g., Proteobacteria and Prevotella), as shown in the results, thus blocking the cascade effects of inflammatory processes related to NF-κB, thereby inhibiting the production of pro-inflammatory cytokines, as IL-1β, IL-6 and TNF-α (as shown in most studies), and increasing anti-inflammatory cytokines (IL-10 and IL-22), which reduce intestinal permeability by increasing the expression of proteins present in tight junctions such as ZOs, occludin, and claudin.

Considering the foregoing, a schematic design was proposed showing the effects of carotenoids on gut health according to the main findings of this systematic review (Figure 4). However, any statement in regard to the mechanism and effects of carotenoids on intestinal health is still incipient due to several factors. Although carotenoids have acted in the reduction of inflammatory markers as demonstrated by many studies, and in bacteria such as Proteobacteria and

Prevotella that have LPS in their cell wall, only four studies evaluated LPS (Singh *et al.*, 2016; Trivedi e Jena, 2015; Zhao *et al.*, 2023; Zhu *et al.*, 2021), and one study evaluated TLR4 and MyD88 (Zhao *et al.*, 2023), which makes it difficult to state any hypothesis regarding this mechanism. However, as a study with β-carotene showed LPS reduction (Zhu *et al.*, 2021), this may be a possible mechanism to understand the role of carotenoids in inflammatory process. Related to SCFA, as the number of studies evaluating this was scarce, one showing increase (Singh *et al.*, 2016), other reduction (Yasuda *et al.*, 2022), and others two no change (Barszcz *et al.*, 2021; Wu *et al.*, 2020), further studies are needed to find out whether in fact carotenoids can act by increasing their production. Although SCFA-producing bacteria have been modulated by carotenoids, many of these, as previously discussed, also act in inflammatory processes, so assessing SCFA levels would be important to understand whether in fact these bacteria would be acting in inflammatory processes or in the production of SCFA. In addition, few studies have evaluated outcomes such as intestinal functionality and morphology, compared to those that evaluated microbiota and inflammation, and there is no study on the effect of astaxanthin or zeaxanthin alone evaluating intestinal morphology and functionality. Also, no studies have evaluated goblet cells, which have an important role in mucus production. Thus, more studies evaluating the effects of carotenoids on different intestinal markers of gut such as microbiota, inflammation, but mainly functionality, morphology, and levels of SCFA and LPS, as well as TLR4 and MyD88 are needed for a better understanding of the effects and mechanisms of carotenoids in gut health.

Conclusion

According to the results that are included in this systematic review, carotenoids were demonstrated to have beneficial effects on gut microbiota, intestinal functionality and morphology, and inflammation. Specifically, carotenoids promoted beneficial effects on intestinal bacteria related to both inflammation and SCFA production; increased the expression of tight junction proteins, important for reducing intestinal permeability; increased the expression of mucins, which are mucus-producing and important in protecting against pathogens and toxins; improved morphological parameters important for digestion and absorption of

nutrients; and reduced pro-inflammatory cytokines and increased anti-inflammatory cytokines. However, further studies are needed to confirm the effects and mechanisms of carotenoids on intestinal health, since depending on the type of carotenoid, they acted differently on gut health and inflammatory status. Thus, any conclusion about the effects and mechanisms of carotenoids on intestinal health is still incipient.

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Declaration of interest statement:

The authors declare no conflict of interest.

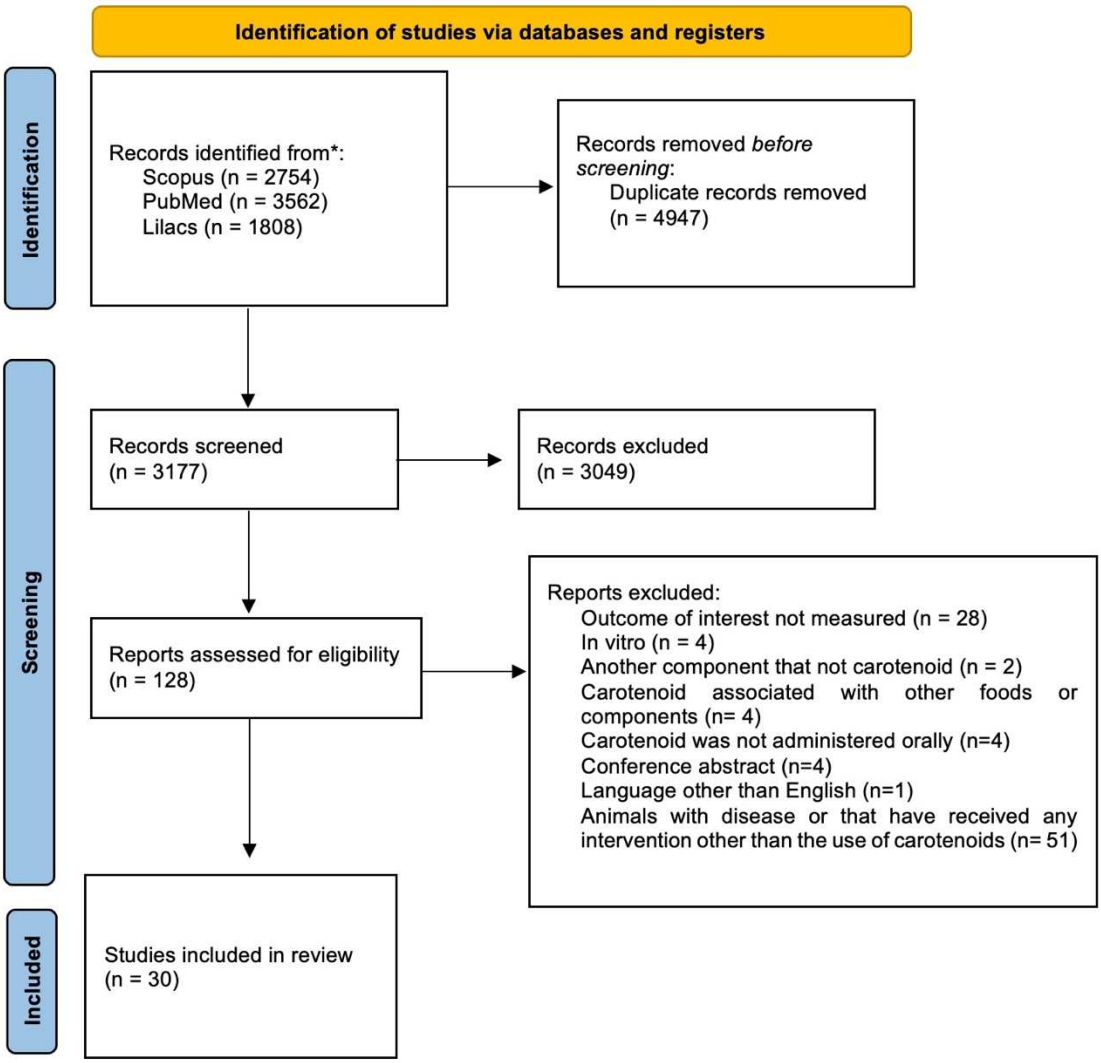

Figure 1. PRISMA flow diagram for the included studies.

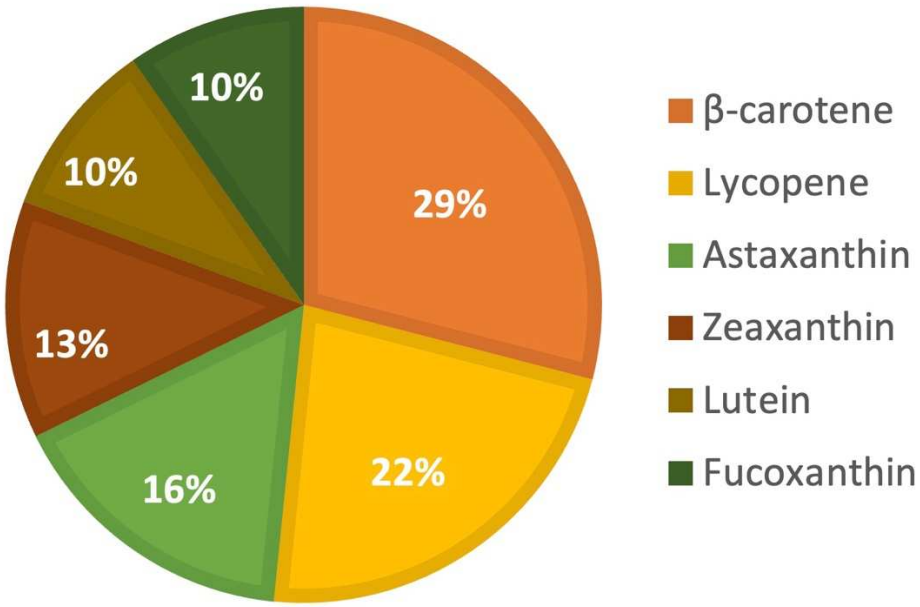

Figure 2. Percentage of different types of carotenoids used in included studies.

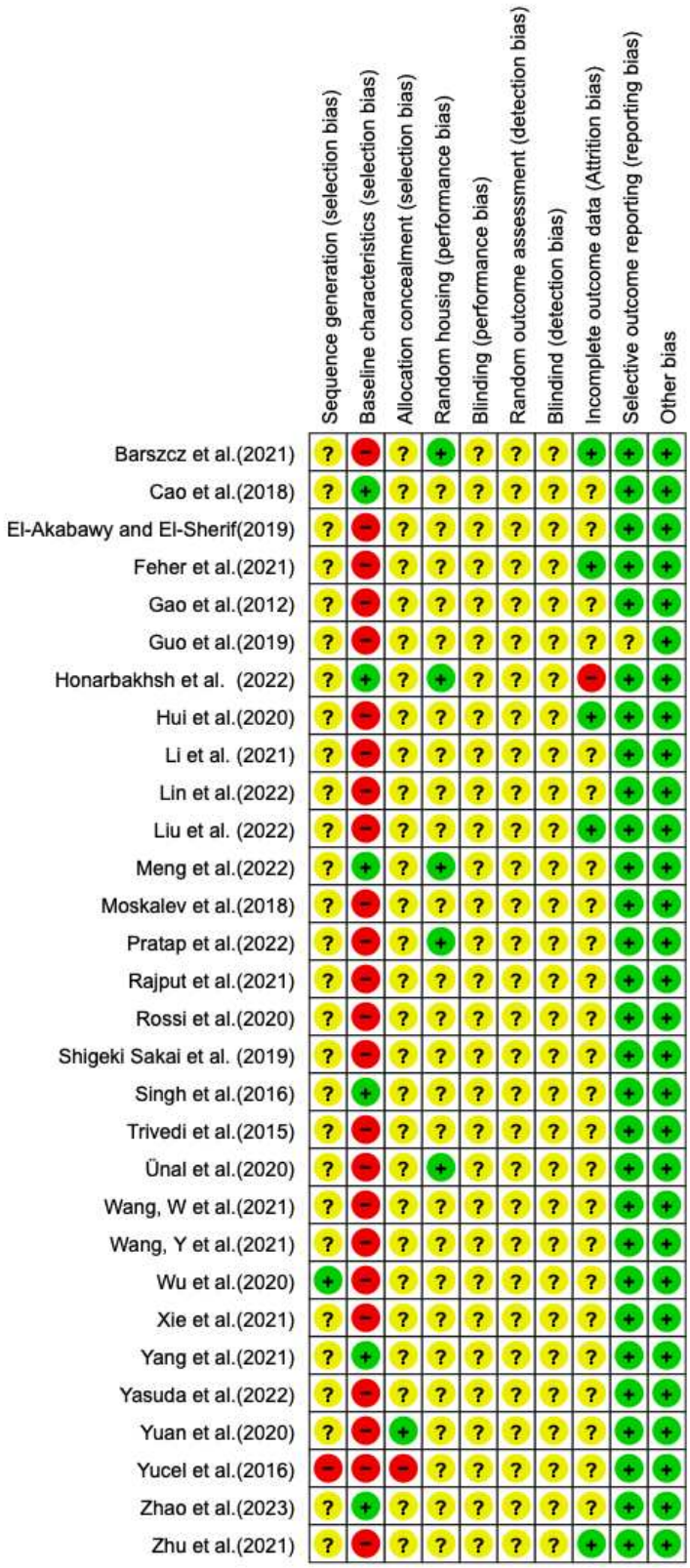

Figure 3. Risk of bias of each item assessed in the studies included.

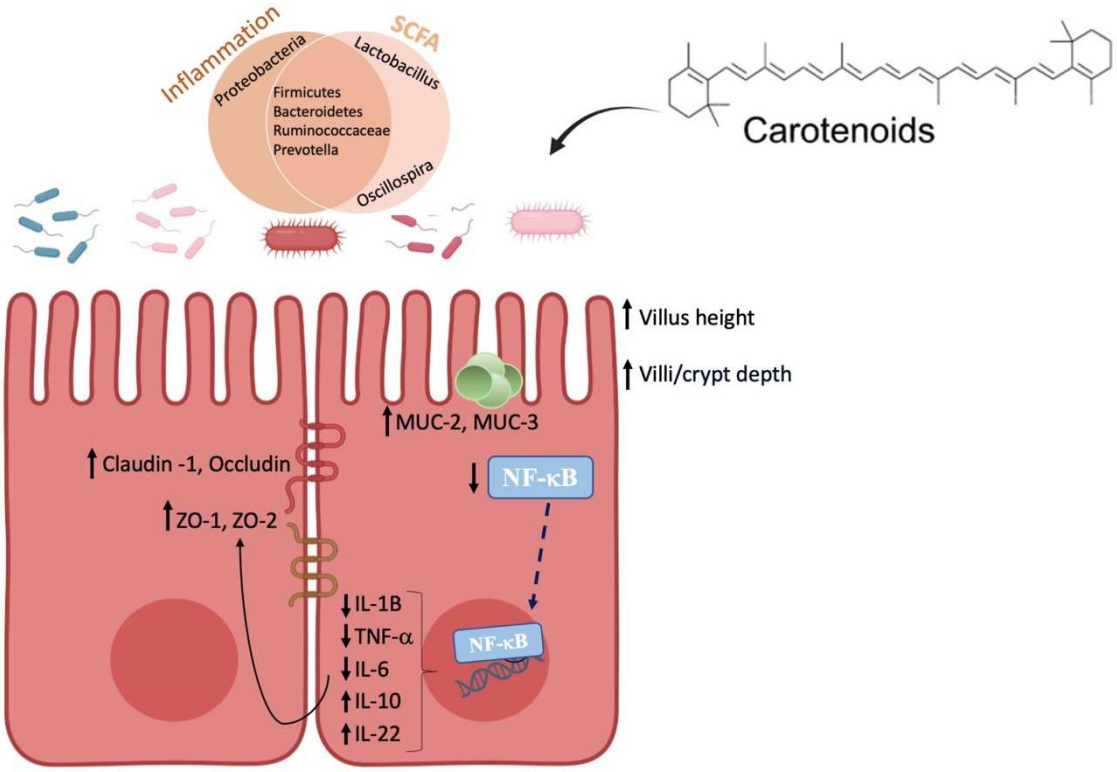

Figure 4. Schematic figure showing the main results on the effects of carotenoids on gut health according to findings from this systematic review. Source: Elaborated by the authors.

Table 1. Inclusion and exclusion criteria based on PICOS anagram.

PICOS	Inclusion criteria	Exclusion criteria
Population	At least one group with healthy animal or that received only carotenoid without any other intervention	If all groups have no healthy animal
Intervention	Dietary intake, supplementation, or extract of carotenoids	Carotenoids associated with other components or not measured or not been administered orally or intra-amniotic, or if all groups received any type of intervention other than diet
Control	Without the carotenoid intervention	No control group
Outcome	Microbiota composition, SCFA, gene expression of tight junction and brush border membrane, plasm endotoxin, histological parameters of small and large intestine, and inflammatory markers	Outcomes related to the bioavailability and accessibility of carotenoids or Outcomes evaluated with scores in general, without evaluated specific markers, for example, inflammation score or histological score.
Study	Experimental controlled studies	Review, clinical studies, theses, dissertation, book chapters, *in vitro* experiments, studies published in other languages than English, editorial, opinions, letter, unpublished manuscripts, conferences abstracts

Table 2. Characterization of the included studies.

Authors/year	Country	Animal characteristics	Animals/group	Carotenoid/time of intervention	Administration
Gao et al.(2012)	China	**Expt 1**: 432 hens (34 week-old, similar weight) **Expt 2**: 360 healthy male chicks	144 90	Xanthophylls (20 and 40 mg/kg): Lutein (40%) and Zeaxanthin (60%) / 35 days Xanthophylls (40 mg/kg): Lutein (40%) and Zeaxanthin (60%) / 21 days	Oral (diet) *In ovo* and oral
Trivedi et al.(2015)	India	Male Swiss Albino mice (25–28 g)	8	B-carotene (20 mg/kg) / 28 days	Oral (diet)
Singh et al.(2016)	India	Male Swiss albino mice (6–8 weeks-old, 18 ± 2 g)	8 a 10	Lycopene / 12 weeks	Oral (diet)
Yucel et al.(2016)	Turkey	Adul male Sprague Dawley rats (200–275g)	7	Lycopene / 10 days	Oral (gavage)
Moskalev et al.(2018)	Russia	Drosophila melanogaster wild type Canton-S line (first day of life)	315-323	Fucoxanthin / 63 days	Oral (diet)
Cao et al.(2018)	China	Male C57BL/6J mice (4-week-old, 18-20 g)	8	Fucoxanthin / 12 weeks	Oral (gavage)
Shigeki Sakai et al. (2019)	Japan	Females C57BL/6J mice (6-8 weeks-old)	5	Astaxanthin / 10 days	Oral (diet)
Guo et al.(2019)	China	Male BALB/c mice (6-week-old)	10	Fucoxanthin / 4 weeks	Oral (gavage)
El-Akabawy and El-Sherif(2019)	Egypt	Male Sprague Dawley rats (200–250 g)	10	Zeaxanthin / 15 days	Oral (gavage)
Ünal et al.(2020)	Turkey	Male and female Wistar albino rats (290 ± 10 g)	10	Extract of Momordica charantia (bitter melon) (300 mg/kg); carotenoid	Oral (water)

				(11.73μg/g); B-carotene (1.57 mg/g) / 7 weeks	
Wu et al.(2020)	USA	Male and female KO and the genetic background WT mice (6-week-old)	10	Astaxanthin / 8 weeks	Oral (diet)
Hui et al.(2020)	China	Male New-born Hyline Brown chicks with similar weight	90	β-carotene (60 mg/kg) / 28 days	Oral (diet)
Rossi et al.(2020)	Argentina	Juvenile Pacú (Piaractus mesopotamicus) (12.4 ± 2.8 g)	105	β-carotene (225 g/kg) / 62 days	Oral (diet)
Yuan et al.(2020)	Japan	Hybrid pregnant sows (Landrace × Yorkshire)	16	β-carotene (30 or 90 mg/kg)/ from day 90 of gestation until parturition	Oral (diet)
Yang et al.(2021)	China	Male Wistar rats (21 days-old, ≅ 150 g)	10	β-carotene (50 mg/kg) / 15 days	Oral (gavage)
Wang, Y et al.(2021)	China	Healthy grass carps (98.23 ± 3.53 g)	30	Lycopene / 30 days	Oral (diet)
Feher et al.(2021)	Hungary	Common carp juveniles (7 months (30 weeks), 123.45 ± 0.37 g)	33	1% carotenoid provided by Hungarian sweet red pepper (Capsicum annuum) extract / 42 days	Oral (diet)
Wang, W et al.(2021)	China	Shrimp (Penaeus monodon. Juveniles (3.15 ± 0.12 g)	150	Astaxanthin / 56 days	Oral (diet)
Barszcz et al.(2021)	Poland	Male Crl:W (Han) Wistar rats (4-week-old)	8	Alfafa protein-xanthophyll concentrate (PXC) (xanthophylls (lutein, zeaxanthin, violaxanthin, and neoxanthin)) / 28 days	Oral (diet)
Zhu et al.(2021)	China	Male specific pathogen-free SD rats (200 ± 20 g)	6	β-carotene (50 mg/kg) / 7 days	Oral (gavage)
Rajput et al.(2021)	China	Healthy C57BL/6 mice	12	Lycopene / 12 days	Oral (gavage)
Xie et al.(2021)	China	Male C57BL/6N mice (4-week-old)	12	Zeaxanthin / 22 weeks	Oral (gavage)

Li et al. (2021)	China	Male Jun Mu No. 1 white piglets (Sanjiang White Pig×Seghers hybrid)	6	β-carotene (40 or 80 mg/kg) / 26 days	Oral (diet)
Yasuda et al.(2022)	Japan	Male Wistar rats (5-week-old)	6	Astaxanthin / 4 weeks	Oral (diet)
Liu et al. (2022)	China	Healthy Duroc x Landrace x Yorkshire (DLY) barrows (63.89 ± 1.15 kg)	6	Lycopene / 10 weeks	Oral (diet)
Meng et al.(2022)	China	Castrated male piglets (Duroc × (Lan- drace × Yorkshire) (21 day-old, 5.48 ± 0.10 kg)	12	Lycopene / 28 days	Oral (diet)
Lin et al.(2022)	China	Male specific-pathogen-free ICR mice (6-week-old)	20	Lycopene / 14 days	Oral (gavage)
Pratap et al.(2022)	Australia	Female BALB/c mice (6–8-week-old)	5	Astaxanthin from *H. pluvalis* / 28 days	Oral (gavage)
Honarbakhsh et al. (2022)	USA	Female WT mice (6-week-old, ≅ 18 g)	3 a 4	β-carotene (50 mg/kg) / 6 weeks	Oral (gavage)
Zhao et al.(2023)	China	Male Wistar rats (140–160 g, 6-week-old)	10	Lutein / 14 weeks	Oral (gavage)

*WT: Wild Type

Table 3. Main results of the studies included in this systematic review.

Authors/year	Intervention / control	Results (intervention x control)
Gao et al. (2012)	**Expt 1:** Control diet Intervention 1: Diet supplemented with 20 mg/kg xanthophylls Intervention 2: Diet supplemented with 40 mg/kg xanthophylls **Expt 2:** Control diet Intervention: Diet supplemented with 40 mg/kg xanthophylls	**Expt 1**: ↔ mRNA IL-4 and IL-6 in duodenum, jejunum and ileum Both interventions: ↓ mRNA IL-1β and IFN-γ in jejunum Intervention 2: ↓ mRNA LITAF in duodenum **In ovo:** ↔ mRNA LITAF, IL-4, and IL- 10 in the duodenum ↓ mRNA IL-6, IFN-γ and LITAF in duodenum; mRNA IL-1β and IL-6 in jejunum; mRNA IL-1β and IFN-γ in ileum ↑ mRNA IL-4 and IL-10 in jejunum; mRNA IL-10 in ileum **Expt 2**: ↔ mRNA IL-4 in duodenum, jejunum and ileum. ↓ mRNA IL-6 and IFN-γ in duodenum; mRNA IL-1β in jejunum; and mRNA IFN-γ in ileum ↑ mRNA IL-10 in jejunum
Trivedi et al. (2015)	Control diet Intervention: β-carotene (20 mg/kg)	↔ colon length; inflammatory markers (MPO acitivity; IL-17, IL-6, and TNF-α levels in the colon; expression of NF-kβ, COX-2, STAT3 in the colon); histological score; LPS, IL-6 and TNF-a levels on plasma.
Singh et al. (2016)	Control diet 1: NPD (10% kcal from fat) Control diet 2: HFD (60% kcal from fat)	Between groups: ↔ Bifidobacteria, Bacteroidetes, Firmicutes, Roseburia spp., Akkermensia muciniphilia, and serum LPS

	Intervention 1: HFD+Lycopene (10 mg/kg) Intervention 2: NPD+Lycopene (10mg/kg)	NPD+L10 compared to other groups: ↔ Lactobacilli and Enterobacteriaceae NPD+L10 compared to NPD: ↔TNF-a, IL-1β, and IL-6 in the serum HFD+L10 compared to HFD: ↔ propionic acid; ↑ Lactobacilli; total SCFA, acetic acid, and butyric acid; serum IL-6; villus height and crypts length; ↓ Enterobacteriaceae; TNF-a and IL-Iβ in the serum
Yucel et al. (2016)	Control diet Intervention: Lycopene (10 mg/kg)	↔ histopathological; TNF-α and IL-1β in small intestine tissues
Moskalev et al. (2018)	Control diet Intervention: yeast paste with fucoxanthin in concentration of 1 μM	↓ intestinal permeability in males ↑ antimicrobial peptide gene expression (drosomycin (Drs), drosocin (Dro), and diptericin (Dpt))
Cao et al. (2018)	Control diet: 10% calories in fat, 7% calories in sugar + 200 ul of saline Intervention: high-fat high-sugar diet (HFS; 45% calories in fat, 17% calories in sugar) + fucoxanthin (150 mg/kg) Solvent group: HFS + soybean oil (200 μL) Model group: HFS + 200 ul of saline	Intervention: ↓ SGLT-1 gene expression compared to solvent group
Shigeki Sakai et al. (2019)	Control diet Intervention 2: astaxanthin 0.04% group	↔ mRNA TNF-a, IL-1β, IL-6, IL-36a and IL-36γ in mucosal epithelial cells; histological score

Guo et al. (2019)	Control diet 1: NCD (~4% fat) Intervention 1: NCD + fucoxanthin (125 mg/kg) Control diet 2: HFD (17.9% fat) Intervention 2: HFD + fucoxanthin (HFDF, 125 mg/kg)	↔ α-diversity (Simpson index) in cecum, and α-diversity (Shannon, Simpson, and Observed_species) fecal; F/B ratios in fecal between groups Intervention 1: ↑ α-diversity (Chao 1, Shannon, and Observed_species indexes) in cecum; and α-diversity (Chao 1) fecal compared to NCD. Intervention 2: ↑ alpha-diversity (Shannon index) in cecum compared to HFD **Related to phyla:** Cecal microbiota: ↓ Firmicutes; ↑Actinobacteria, Deferribacteres and Proteobacteria in HFDF compared to HFD Cecal microbiota: ↔ Firmicutes; ↓Proteobacteria, Actinobacteria and Deferribacteres in NCDF compared to NCD Cecal microbiota: ↓Verrucomicrobia with fucoxanthin Cecal microbiota: ↑Bacteroidetes in NCDF and HFDF compared to their controls (higher in NCDF compared to HFDF) Fecal microbiota: ↑ Firmicutes in NCDF and HFDF (higher in NCDF); Bacteroidetes (↔ in HFDF, ↓in NCDF) **Related to class:** Cecal microbiota: ↔ Clostridia and Deferribacteres, ↓ Epsilonproteobacteria in NCDF compared to NCD Cecal microbiota: ↑ Deferribacteres, Epsilonproteobacteria, and Bacilli, ↓Clostridia in HFDF compared to HFD Cecal microbiota: ↓ Verrucomicrobiae; ↑Betaproteobacteria Fecal microbiota: Clostridia (↔ in HFDF, ↑ in NCDF), Bacteroidia (↑ in HFDF, ↓ in NCDF) **Related to families:** Cecal microbiota: ↔ Lachnospiraceae by fucoxanthin

		Cecal microbiota: ↑ S24-7 in NCDF and HFDF (but higher in NCDF) compared to their controls Cecal microbiota: Ruminococcaceae: ↑ in NCDF and ↓ in HFDF Cecal microbiota: ↑Paraprevotellaceae in HFDF Fecal microbiota: S24-7 (↔ in HFDF, ↓ in NCDF), ↑Oscillospira and Prevotella in NCDF **Related to genus level:** Cecal microbiota: ↔ Bacteroides, ↑ Parabacteroides, Rikenella, Bilophil, Helicobacter, Mucispirillum and Octoribacter in HFDF compared to HFD Cecal microbiota: ↑Bacteroides in NCDF compared to NCD Cecal microbiota: ↑ Oscillospira, ↓ Akkermansia in NCDF and HFDF
El-Akabawy and El-Sherif (2019)	Control diet: received 2 ml of saline transrectally Intervention: Zeaxanthin (50 mg/kg) + 2 ml of saline transrectally	↔ gene expression of TNF-α, IFN-γ, IL-1β, IL-6, NF-κB in the colon; COX-2, histological score, inflammatory response scores of colon
Ünal et al. (2020)	Control diet Bitter melon extract (300 mg/kg); carotenoid (11.73μg/g); β -carotene (1.57 mg/g)	↔ IL-17 and TNF- α both in colon and serum; IL-10 in colon ↑ IL-10 in serum
Wu et al. (2020)	KO-CONT (BCO2 KO mice fed CONT): AIN93M diet (10% kcal from fat) KO-ASTX (BCO2 KO fed ASTX): AIN93M diet + 0.04% astaxanthin (wt/wt) ad libitum	↔ α-diversity and richness; SCFAs; TNF-a, IL-6, and IL-17A in plasma AST: ↑ Cyanobacteria, Gastranaerophilales and ↓Firmicutes, Spirochaetes, Spirochaetales

	WT-CONT (WT fed CONT): AIN93M diet (10% kcal from fat) WT-ASTX (WT fed ASTX): AIN93M diet + 0.04% astaxanthin (wt/wt) ad libitum	KO-ASTX: ↑ F/B ratio in male compared to KO-CONT WT-ASTX: ↓ F/B ratio in female mice; and IL-1β in plasma compared to WT-CONT WT-ASTX: ↓ Parabacteroides distasonis; IL-1β in plasma compared to WT-CONT
Hui et al.(2020)	Control diet: regular cockerel diet Intervention: regular cockerel diet + 60 mg/kg of β-carotene	↑villus height (duodenum, jejunum and ileum), serum sIgA, jejunal mucosa IgA and sIgA, Lactobacillus, ↓Escherichia coli; ↔ serum IgA, Bifidobacteria Duodenum and Jejunum: ↑ crypt depth, villi height/crypt depth ratio, villus area Ileum: ↔ crypt depth, villi height/crypt depth ratio, villus area; ↑ mRNA IgA, ZO-1, ZO-2, occludin, and MUC-2
Rossi et al.(2020)	Control diet: basal diet (based on vegetable flours) β-carotene group: basal diet supplemented with β-carotene (225 g/kg)	↔ Lactic acid bacteria; ↑ Enterobacteria, Psychrotrophs, and total aerobic Cetobacterium somerae, Candidatus Arthromitus, Clostridium paraputrificum, Porphyromonas catoniae and Cetobacterium somerae, Uncultured Bacteroides, Bacteroides oleiciplenus, Parabacteroids sp, and Cetobacterium somerae ↓ Lactic acid bacteria /Enterobacteria, Shannon's diversity index and Margalef's richness index
Yuan et al. (2020)	Control diet: basal diet (meets the nutritional requirements of pigs according to NRC (2012)) Intervention 1: β -carotene low dose (30 mg/kg) Intervention 2: β -carotene high dose (90 mg/kg)	β-carotene (30 mg/kg): ↔ Corynebacterium 1†, ↑Shannon index†, Ruminococcaceae UCG-008, Sutterella†, Corynebacterium†

		↓Lachnospiraceae AC2044†, Lachnospiraceae NK4B4†, Sedimentibacter†, Bilophila†. β-carotene (90 mg/kg): ↑Shannon index, Sedimentibacter, Sutterella, Corynebacterium 1, Bilophila, Corynebacterium, ↓Lachnospiraceae NK4B4, Lachnospiraceae AC2044, Ruminococcaceae UCG-008 β-carotene dose-dependently: ↑ Proteobacteria, ↓Prevotellaceae UCG-001
Yang et al. (2021)	Control diet β -carotene group: (50 mg/kg)	↔ TNF-α and IL-1β in the serum; and mRNA TNF-α, IL-1β, NF-kB, and phosphorylation of NF-kβ in jejunal tissue
Wang, Y et al. (2021)	Control diet: normal commercial diet containing corn oil Intervention: Lycopene (10 mg/kg)	↔mRNA claudin 3, claudin 4, ZO-2, TNF-a, IL-1β, IL-6, IL-8, IL-10, TGF-B in intestinal tissue ↑mRNA claudin 1, occludin, ZO-1 in intestinal tissue
Feher et al. (2021)	Control diet BD + 1% carotenoids provided by sweet red pepper pulp extract	↑ class *Bacteroidia,* order *Bacteroidales,* families *Bacteroidaceae*, *Rhodospirillaceae*, and the genera *Chryseobacterium*, *Enterococcus*, and *Acidovorax, Fusobacteria*, Cetobacterium ↓ Proteobacteria, P/F ratio, F/B ratio, Acinetobacter, Bacillus, Pseudomonas
Wang, W et al. (2021)	Control diet: 0 mg/kg diet of AST	↔ Shannon and Chao 1 indexes, operational taxonomic units, Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Clostridium, Ruminococcus, Bacteroides, Bifidobacterium, Escherichia– Shigella, Klebsiella, Sutterella, Prevotella,

		Eubacterium, Lachnospiraceae, Rikenellaceae, Blautia, Haemophilus, and Lactobacillus among all the treatments.
	Intervention 1: 20.5 mg/kg diet of AST Intervention 2: 41 mg/kg diet of AST Intervention 3: 61.5 mg/kg diet of AST Intervention 4: 82 mg/kg diet of AST Intervention 5: 102.5 mg/kg diet of AST	
Barszcz et al. (2021)	Control diet: commercial, cereal-based diet Intervention 1: commercial, cereal-based diet + 1.5% PXC Intervention 2: commercial, cereal-based diet + 3% PXC	↔ SCFA: Acetic, Propionic, Isobutyric, Butyric, Isovaleric, Valeric, and total SCFA; crypt depth or myenteron thickness in caecum and colon; and MUC-2 and MUC-3 gene expression in the colon between groups ↓ Mucus layer thickness in both intervention groups compared to control
Zhu et al. (2021)	Control diet: normal standard growth feed Intervention: β- carotene (50 mg/kg)	↔ Alpha diversity indexes in fecal microbiota, Proteobacteria ↑ colon length; protein expressions of TNF-a, IFN-γ in the serum; mRNA TNF-a and IFN-γ in the colon; Bacteroidetes ↓Protein expressions of IL-6, IL-1β, LPS and D-lactate in the serum; mRNA IL-6 and IL-1β in the colon; Firmicutes; Actinobacteria
Rajput et al. (2021)	Control diet: corn oil Lycopene: (10 mg/kg)	↔ duodenum, jejunum and ileum weights; crypt depth; trans-epithelial electrical resistance (TEER) in the jejunal tissue; diamine oxidase (DAO) activity in the serum; claudin-1, occludin, and MUC-2 protein expression in the jejunum ↑ villus height; villus/crypt

Xie et al. (2021)	Control diet: standard diet (STD) with a vehicle (0.5% CMC-Na) Intervention 1: STD with ZEA (STDZEA, 20 mg/kg)	↔α-diversity, Firmicutes, Proteobacteria, Clostridia, Desulfovibrio
Li et al. (2021)	Control diet: normal suckling Weaning group (WG, piglets weaned on d 21) Intervention 1: Weaning+ β-carotene (40 mg/kg) Intervention 2: Weaning+β-carotene (80 mg/kg)	↔ mRNA TNF-α and IL-1β in the jejunum; mRNA IL-1β and IL-6 in the colon, richness and diversity between groups Intervention 1 and 2: ↑ villus height; villus height/crypt depth ratio; Firmicutes; Clostridiales; Parabacteroides; ↓ IL-1β, IL-6, and TNF-α in the serum; mRNA TNF-α in the colon; phosphorylation of IκBα and NF-κB p65 proteins in the colon; Bacteroidetes, Bacteroidales, Bulleidia, Bacteroides, Fibrobacter, Prevotella, Blautia, Eubacterium, Clostridium compared to WG group Intervention 2: ↓ crypt depth; mRNA IL-6 in jejunum; and phosphorylation of IκBα and NF-κB p65 proteins in jejunum compared to WG group
Yasuda et al. (2022)	Control diet: commercial animal diet (injected with saline) Intervention: astaxanthin (0.02% (w/w)) (injected with saline)	↔ Bacteroidales; α-diversity ↑ Verrucomicrobiales ↓ Clostridiales; Lactobacillales; Coprococcus and Butyricicoccus; acetic acid and butyric acid
Liu et al. (2022)	Control diet: corn and soybean meal (0 mg/kg LYC) Intervention 1: supplemented with 100 mg/kg LYC Intervention 2: supplemented with 200mg/kg LYC	↔ villus height and crypt depth of the small intestine; IL-6 (pg/mg prot) in duodenum, jejunum and ileum; IL-10 (pg/mg prot) in duodenum, jejunum and ileum; IL-1β (pg/mg prot) in ileum; TNF-α (pg/mg prot) jejunum and ileum; NF-kB (pg/mg prot) in jejunum and ileum; mRNA IL-1β in duodenum and ileum, mRNA IL-6 in duodenum, jejunum and ileum; mRNA

		TNF-α in duodenum, jejunum and ileum; mRNA NF-kB in duodenum, jejunum and ileum; mRNA IL-10 in ileum; mRNA and protein expression of ZO-1 and occludin in jejunum between groups Intervention 2: ↑ Villus height/crypt depth in jejunum; mRNA IL-10 in duodenum and jejunum; mRNA and protein expression of claudin-1 in jejunum; ↓IL-1β (pg/mg prot) duodenum and jejunum; TNF-α (pg/mg prot) duodenum, and NF-kB (pg/mg prot) in duodenum; mRNA IL-1β in jejunum.
Meng et al. (2022)	Control diet: corn and soybean meal Intervention: supplemented with 50 mg/kg of lycopene	↔ Crypt depth; mRNA ZO-1, occludin, MUC-2, NF-κB, TNF-α, TGFB1, IL-1β, IL-6, IL-8, IL-10, and IL-17 in the jejunum; Simpson, Shannon and Chao1 indices ↑Villus height; villus/crypt ratio in the jejunum; mRNA occludin-1 and IL-22 in the jejunum, Phascolarctobacterium, Solobacterium, Dorea, Catenisphaera, and Parasutterella ↓Schwartzia, Treponema, Moraxella, Roseburia, Agathobacter, Alloprevotella, Selenomonas, Lachnospira, Megasphaera, Acidaminococcus, Bacteroidales_RF16_group_unclassified, Ruminococcaceae_UCG_014, and Prevotellaceae_unclassified
Lin et al. (2022)	Control diet Intervention: Lycopene (10 mg/kg)	↔ villus height; ↓crypt depth; ↑ villus height/crypt depth in the jejunum

Pratap et al. (2022)	Control diet Intervention: astaxanthin doses (1 mg/mouse)	↔ diversity and richness between the fecal and caecum samples ↓ *Akkermansiaceae and Ruminococcaceae* family, Akkermansia genus
Honarbakhsh et al. (2022)	Control diet: vehicle (corn oil) Intervention: β-carotene in corn oil (50 mg/kg)	↔ α-diversity indexes (Shannon Index and Faith's PD); Actinobacteria, Firmicutes, Bacteroidetes, Firmicutes/Bacteroidetes ratio, Paraprevotella sp., Alistipes sp., Millionella sp., Parabacteroides sp., Lachnoclostridium sp., Marvinbryantia sp., Desulfovibrio sp., and Brachyspira sp; gene expression of MUC-2, IL-6, IL-23 in the colon ↑ expression of MUC-3 in the colon ↓TNF-α, IL-1β, IL-22, IL-17 in the colon
Zhao et al. (2023)	Control diet Intervention: Lutein (24 mg/kg)	↔ TNF-a, IL-1β, LBP, and LPS in the serum; length of duodenum, jejunum and ileum; villi length; villi length/crypt depth ratio; protein expression levels and mRNA of the claudin1, occludin, ZO-1 in the ileum; alpha diversity ↓ crypt depth in small intestine ↑ Akkermansia, *Phascolarctobacterium*,and Incertae sedis

*IL: interleukin; LITAF: Lipopolysaccharide Induced TNF Factor; IFN-γ: *Interferon-gamma; LPS: lipopolysaccharide; TNF-α: Tumor necrosis factor alpha; NPD: normal diet; HFD: high fat diet; HFDF: high fat diet + fucoxanthin; NCDF: normal diet + fucoxanthin; SCFA: short chain fatty acids; IgA: Immunoglobulin A; MUC: mucin; ZO:* Zonula occludens. †It was not statistically significant.

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6.2 Article 2

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Article

Intra-Amniotic Administration of Cashew Nut (*Anacardium occidentale* L.) Soluble Extract Improved Gut Functionality and Morphology In Vivo (*Gallus gallus*)

Talitha Silva Meneguelli [1], Nikolai Kolba [1], Arundhati Misra [1], Ana Paula Dionísio [2], Ana Claudia Pelissari Kravchychyn [3], Bárbara Pereira Da Silva [3], Hercia Stampini Duarte Martino [3], Helen Hermana Miranda Hermsdorff [3] and Elad Tako [1,*]

[1] Department of Food Science, Cornell University, Ithaca, NY 14850, USA; talithasilvameneguelli@gmail.com (T.S.M.); nk598@cornell.edu (N.K.); am2657@cornell.edu (A.M.)
[2] Embrapa Agroindústria Tropical—CNPAT, Fortaleza 60511-110, CE, Brazil; ana.dionisio@embrapa.br
[3] Department of Nutrition and Health, Universidade Federal de Vicosa, Viçosa 36570-900, MG, Brazil; ana.pelissari@ufv.br (A.C.P.K.); barbara.p.silva@ufv.br (B.P.D.S.); hercia@ufv.br (H.S.D.M.); helenhermana@ufv.br (H.H.M.H.)
* Correspondence: et79@cornell.edu

Abstract: Cashew nuts are rich in dietary fibers, monounsaturated fatty acids, carotenoids, tocopherols, flavonoids, catechins, amino acids, and minerals that offer benefits for health. However, the knowledge of its effect on gut health is lacking. In this way, cashew nut soluble extract (CNSE) was assessed in vivo via intra-amniotic administration in intestinal brush border membrane (BBM) morphology, functionality, and gut microbiota. Four groups were evaluated: (1) no injection (control); (2) H_2O injection (control); (3) 10 mg/mL CNSE (1%); and (4) 50 mg/mL CNSE (5%). Results related to CNSE on duodenal morphological parameters showed higher Paneth cell numbers, goblet cell (GC) diameter in crypt and villi, depth crypt, mixed GC per villi, and villi surface area. Further, it decreased GC number and acid and neutral GC. In the gut microbiota, treatment with CNSE showed a lower abundance of *Bifidobacterium*, *Lactobacillus*, and *E. coli*. Further, in intestinal functionality, CNSE upregulated aminopeptidase (AP) gene expression at 5% compared to 1% CNSE. In conclusion, CNSE had beneficial effects on gut health by improving duodenal BBM functionality, as it upregulated AP gene expression, and by modifying morphological parameters ameliorating digestive and absorptive capacity. For intestinal microbiota, higher concentrations of CNSE or long-term intervention may be necessary.

Keywords: cashew nut; *Anacardium occidentale* L.; *Gallus gallus*; gut health

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1. Introduction

The nutritional benefits of nuts to human health are well documented, primarily in the context of cardiovascular health [1,2], glycemic control [3], lipid metabolism [4–6], and antioxidants effects, as nuts provide numerous nutrients that play a key role in the possible prevention of cardiometabolic diseases, including favorable fatty acid (FA) profiles such as unsaturated FAs, dietary fiber, antioxidants, minerals (magnesium, calcium, manganese, selenium, and potassium), vitamins E, K, and B-vitamins, and phytonutrients (lutein, phytosterols, and flavonoids) [7,8]. The link between health, disease, and diversity of the gut microbiome is known since disruption of the gut microbiome, or dysbiosis, is associated with an increased risk of cardiometabolic diseases, such as metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM), development of systematic inflammation, and cardiovascular disease (CVD) [9–12]. However, there are only a few studies that discuss the impact of nuts (only including almonds, walnuts, hazelnuts, and pistachio) on the intestinal microbiota [13,14]. The beneficial modification of microbiota composition is a promising approach for improving intestinal and overall health, as dietary fibers and phytochemicals that

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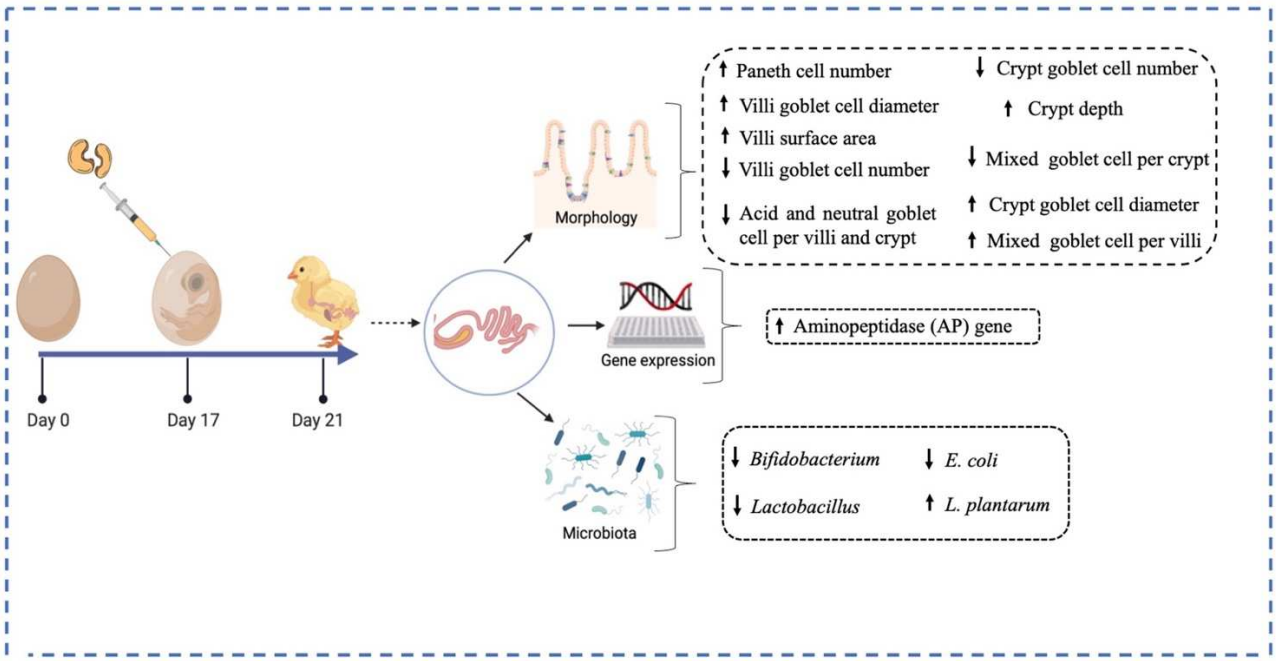
Keywords: cashew nut; *Anacardium occidentale* L.; dietary fiber; *Gallus gallus*; gut health

GRAPHICAL ABSTRACT



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In 2020/2021 (5.3 million tons), the global production of nuts grew 15% compared to 2011/2012 and 65% more than the previous decade. Furthermore, the global production of cashew nuts almost doubled in 2020/2021 (836,940 tons) compared to 2010/2011 (469,079 tons) [19], occupying the third position (16%), only behind almonds (31%), walnuts, and pistachios (19% each) [19]. Moreover, cashew nuts are the third most consumed (18%) nuts in the world, behind only almonds (30%) and walnuts (20%) [19]. The cashew tree (*Anacardium occidentale* L.) belongs to the *Anacardiaceae* family and is mainly in tropical regions [20]. The cashew nut is native to Northeast Brazil, present in the Cerrado biome [21–23]. It is cultivated primarily in India, Vietnam, Côte d'Ivoire, Guinea-

Bissau, Tanzania, Benin, Brazil, and other countries in East and West Central Africa and Southeast Asia [23].

Anacardium occidentale L. contains various nutrients and bioactive compounds, including monounsaturated fatty acids, β-carotene, lutein, zeaxanthin, vitamin E as tocopherols, dietary fibers, flavonoids, catechins, amino acids, minerals, anacardic acids, cardanols, cardols, and phytosterols [24–26]. Most of these bioactive compounds present in cashew nuts play an important role in gut health as demonstrated by studies; catechins can modulate microbiota [27], selenium can affect gut microbial colonization avoiding health damage associated with dysbiosis [28], β-carotene can increase tight junctions expression (ZO-1, ZO-2, occludin), MUC-2 and MUC-3 expression, and increase villus height and villi height/crypt depth ratio [29,30], and lutein can decrease crypt depth [31]. Furthermore, flavonoids can modulate biomarkers relevant to intestinal inflammation [32]. In addition, cashews appear to have prebiotic properties, as an in vitro study demonstrated that defatted cashew nut flour had a prebiotic effect, increasing beneficial bacteria such as *Bifidobacterium* [33]. Non-bio-accessible nutrients from cashew nuts, such as polyphenols, polysaccharides, and dietary fiber, have prebiotic properties [34,35]. The fermentation of prebiotics produces short-chain fatty acids (SCFA) with numerous positive functions, including stimulating intestinal epithelial tissue, nourishing the intestinal cells, mucus production and secretion, increasing gut microbiome, and affecting their proper maturation and differentiation. Thus, it is believed that cashew nuts may offer the potential to modulate the microbiota composition as well as improve intestinal morphology and functionality [36–39]. However, current knowledge on the effect of cashew nuts on intestinal functionality, morphology, and microbiome is lacking [40–48].

Gallus gallus is an animal model widely used in scientific studies due to its biological and physiological characteristics that resemble those of humans, being recognized as an in vivo model of human nutrition [49–51]. This animal model has been applied to evaluate the functional effects of foods, mainly plant-based compounds, on gut health. Specifically, brush border membrane (BBM) functionality, prebiotic properties, and interaction with microbial population, as well as dietary mineral bioavailability [51,52]. This model has been widely used as it has a complex and dynamic intestinal microbiota influenced by genetics,

environment, and the host's diet. Its phylum-level microbiota is similar to that of humans (*Bacteroidetes*, *Firmicutes*, *Proteobacteria,* and *Actinobacteria*)—more than 85% of its intestinal gene sequences are homologous to human intestinal genes [53–55]; therefore, it shares significant genetic conservation with humans. It has rapid maturation, a well-characterized phenotype, and is very receptive to dietary manipulations [51,55]. In addition to these characteristics and advantages, *Gallus gallus* is one of the most studied models in biomedical research, occupying the third position, behind only rats and mice [56]. With all this, we have decided to use *Gallus gallus* as an animal model. Therefore, this study aimed to evaluate the effects of cashew nut soluble extract (CNSE) on intestinal BBM morphology, which means morphological parameters as Paneth cells, crypts, villi, and goblet cells, intestinal barrier as gene expression of occludin, AP, sodium-glucose transport protein 1 (SGLT-1), and mucin 2 (MUC2), inflammatory response as gene expression of NFκβ and IL-1β, and cecal bacteria populations, using the embryonic phase of *Gallus gallus* as the only external variable for the injected CNSE. Current investigation may contribute to the further understanding of the nutritional benefits of cashew nuts on gut health.

2. Materials and Methods

2.1. Proteins and Dietary Fiber in Cashew Nut Flour and Cashew Nut Soluble Extract (CNSE)

Proteins and soluble and insoluble fibers were determined by the gravimetric-enzymatic method, AOAC International, using a total dietary fiber assay kit, Sigma®, San Luis, MO, USA [57]. More specifically, the analysis of protein content was performed in three repetitions and determined by the micro-Kjeldhal method, while total dietary fiber was performed in two repetitions.

2.2. Extract Preparation

The Brazilian Agriculture Research Corporation (EMBRAPA), Fortaleza-CE, Brazil, provided the cashew nut flower. The soluble extract was extracted as previously described [58,59]. To prepare the cashew nut extract, cashew nut flour samples were homogenized in distilled water in a concentration of 50 g of cashew nut flower for 1 L of distilled water at 60 °C, for 60 min, using a magnetic stirrer. The solution was filtered through a 600 µm screen to remove particulate matter. Then, the solution was placed in falcon tubes and centrifuged at 4000× *g* rpm (4 °C) for 15 min. The supernatant collected was dialyzed (MWCO 12–14 kDa,

Medicell International Ltd., London, U.K.) for 7 weeks against distilled water. The dialysate was lyophilized, which resulted in a fine off-white powder (cashew nut soluble extract).

2.3. Animals and Study Design

The protocol carrier out was approved by the Cornell University Institutional Animal Care and Use Committee (IACUC #2020-0077). The protocols were carried out in accordance with the relevant regulations and guidelines. About 40 fertile Cornish-cross broiler eggs were purchased from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA). The eggs were incubated under ideal conditions (37 ± 2 °C and 59.6 ± 2% humidity, embryonic days 0–18, 72.5 ± 1.8% hatch phase) until hatched at the Cornell University Animal Science poultry farm. CNSE in powder form was diluted in 18 Ω H_2O to determine the concentrations necessary to maintain an osmolarity value (Osm) of less than 320 Osm to ensure that the viable embryos would not be dehydrated upon injection of the tested solution. The hatchability for the experiment was 34/40 eggs (85% survived). So, for the in vivo administration, 34 fertile eggs were weighed and randomly into 4 groups. On day 17 of incubation, candling was used to check fertilization and establish the injection spot. The spot was then sanitized, followed by 1 mL injected with a 21-gauge needle of the CNSE extracts and control (H_2O). The four treatments were (1) no injection (n = 6), (2) 18 Ω H_2O (n = 9), (3) 1% (10 mg/mL) CNSE (n = 10), and (4) 5% (50 mg/mL) CNSE (n = 9). After intra-amniotic administration, the injection spot was sealed with cellophane tape, and the eggs were incubated until hatch (day 21) [51]. Then, the birds were euthanized in a CO_2 chamber, and the samples were collected. The duodenum and cecum were removed and placed in liquid nitrogen. The samples were then transferred to a −80 °C incubator until analysis.

2.4. Gene Expression Analysis

2.4.1. Extraction of Total RNA from Duodenum Tissue

Total RNA was extracted under RNase-free conditions from 30 mg of the duodenal tissue (n = 5). For that, we used Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. The tissues in buffer RLT®, containing β-mercaptoethanol, were disrupted and homogenized with a rotor–stator homogenizer. The lysate was centrifuged at 8000× *g* (3 min) in a microcentrifuge (C2400-R, Labnet International Inc, Edison, NJ, USA). The supernatant was transferred to another tube with 70% ethanol and

mixed immediately. A total of 700 µL of the sample was applied to an RNeasy mini column, centrifuged for 15 s at 8000× *g*, and the flow-through material was discarded. Then, the RNeasy columns were transferred to new collection tubes (2 mL), and 500 µL of buffer RPE® was pipetted onto the RNeasy column, followed by centrifugation for 15 s at 8000× *g*. An additional 500 µL of buffer RPE was pipetted onto the RNeasy column and centrifuged at 8000× *g* (2 min). Total RNA was eluted in 50 µL of RNase-free water. RNA was quantified by absorbance at A 260/280. The integrity of the 18S ribosomal RNAs was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. DNA contamination was eliminated using a TURBO DNase treatment and removal kit from AMBION (Austin, TX, USA).

2.4.2. Real-Time Polymerase Chain Reaction (RT-PCR)

cDNA was created from extracted RNA by a 20 µL reverse transcriptase (RT) reaction applying BioRad C1000 touch thermocycler using the Improm-II Reverse Transcriptase Kit (Catalog #A1250; Promega, Madison, WI, USA). For that, 1 µg of total RNA template, 2 mM of oligo-dT primers, and 10 µM of random hexamer primers were added to the vial. The ideal annealing temperature was 94 °C (5 min), and amplification was 60 min (42 °C)**,** followed by heat inactivation at 70 °C (15 min). The cDNA obtained was analyzed by Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) or stored at −80 °C until analysis, and the concentration was determined by measuring the absorbance at 260 nm and 280 nm with an extinction coefficient of 33 (for single-stranded DNA). The extent of genomic DNA contamination was estimated by an RT-PCR assay (real-time) for the reference gene samples.

2.4.3. Primer Design

The Real-Time Primer Design Tool software (IDT DNA, Coralvilla, IA, USA) was utilized to design the primers based on six gene sequences from the Genebank database. The primer sequences (17–25-mier), amplicon length (restricted to 90–150 bp), and gene ID can be found summarized in Table 1. BLAST searches verified primer specificity against the genomic National Center for Biotechnology Information (NCBI) database. The *Gallus gallus* primer 18S rRNA served as a reference gene.

Table 1. DNA sequences of primers used.

Analyte	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Base Pair	GI Identifier
Intestinal Barrier				
AP	CGTCAGCCAGTTTGACTATGTA	CTCTCAAAGAAGCTGAGGATGG	138	45,382,360
SGLT1	GCATCCTTACTCTGTGGTACTG	TATCCGCACATCACACATCC	106	8346783
MUC2	CCTGCTGCAAGGAAGTAGAA	GGAAGATCAGAGTGGTGCATAG	155	423,101
OCLN	GTCTGTGGGTTCCTCATCGT	GTTCTTCACCCACTCCTCCA	124	396,026
Inflammatory Response				
NF-κβ	CACAGCTGGAGGGAAGTAAAT	TTGAGTAAGGAAGTGAGGTTGAG	100	2,130,627
IL-1β	TCATCCATCCCAAGTTCATTCA	GACACACTTCTCTGCCATCTT	105	395,872

AP: amino peptidase; SGLT1: sodium-glucose transport protein 1; MUC2: mucin 2; OCLN: occludin; NF-κ β: nuclear factor kappa beta; IL-1 β: interleukin 1 beta.

2.4.4. Real-Time qPCR Design

cDNA (2 µL) was pipetted into a 96-well plate with 2× Bio-Rad SSO Advanced Universal SYBR Green Supermix (8 µL) (Cat #1725274, Hercules, CA, USA), followed by buffer, dNTPs, Taq polymerase, and dye. Both forward and reverse primers (Table 1) and cDNA (or water as control) were added to each PCR reaction. Each run had duplicates of 7 standard curve points. To prevent and eliminate any potential DNA contamination, a “no-template control” containing nuclear-free water was incorporated. DNA amplification was performed under the following conditions: an initial denaturation step at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 15 s, annealing temperatures varying according to Integrated DNA Technologies (IDT) for 30 s, and extension at 60 °C for 30 s, using a Bio-Rad CFX96 Touch (Hercules, CA, USA). Gene expression data were obtained by measuring the lowest cyclic product (Cp) values using the automated “second derivative maximum method”. The results were quantified against the standard curve, which was diluted at 1:10, and the reaction for each gene was run in duplicates.

A graph showing the correlation between the Cq and log (10) concentrations was generated by the software, and the efficiencies were calculated as 10 (1/slope) (10) . The specificity of the amplified real-time RT-PCR products was confirmed

by analyzing the melting curves (ranging from 60 to 95 °C) after 40 cycles, which revealed several distinct products with specific melting temperatures [60,61].

2.5. Collection of Microbial Samples and Intestinal Contents DNA Extraction

Under sterile conditions, cecum samples (n = 5) were weighed (0.2 ± 0.02 g) and transferred to a 15 mL tube containing 9 mL of PBS (pH 7.4). Plastic beads were added to the tube, and the mixture was vortexed for 3 min. The tube was then centrifuged at 1000× *g* for 5 min, and the supernatant was collected and subjected to a second centrifugation step at 4000× *g* for 10 min. The buffer was discarded, and the pellet was washed twice with 1 mL of PBS before being stored at −20 °C until DNA purification. For purification, the pellet was treated with 50 mM EDTA (pH 8) and lysozyme (Sigma Aldrich CO., St. Louis, MO, USA) (10 mg/mL) at 37 °C. Bacterial DNA isolation was performed using a Wizard Genomic DNA purification kit according to the manufacturer's protocol (Promega Corp., Madison, WI, USA).

2.6. Primers Design and PCR Amplification of Bacterial 16S rDNA Analysis

Primers for *Lactobacillus*, *Bifidobacterium*, *Clostridium, Escherichia coli,* and *L. plantarum* were used [62]. The universal primers were designed with the invariant region in the 16S rRNA of bacteria and were used as internal standards. The PCR products were separated by 2% agarose gel, stained with ethidium bromide, and quantified by Quantity One 1-D analysis software (Bio-Ra, Hercules, CA, USA). Each bacterium's relative abundance was evaluated as previously described [52,61]. All products were expressed close to the content of the universal 16 s rRNA primer product and proportions of each examined bacterial product.

2.7. Morphological Examination of Duodenal Tissue

The intestinal morphology examination was conducted as previously described [53,63–66]. The duodenum samples per treatment group (n = 5) were preserved in 4% (*v*/*v*) formaldehyde solution, stabilized with phosphate buffer, dehydrated, cleaned, and embedded in paraffin. Four 5 µm sections of each sample were obtained and placed on a glass slide. The sections were deparaffinized in xylene, and the sample was rehydrated using a series of graded alcohol. The Alcian blue/periodic acid-Schiff (PAS) staining was applied in the slides, and the sample was assessed under a light microscope (BX3M series, Olympus, Waltham, MA, USA) using the CellSens Standard Software. Paneth cells were stained a light

purple color. The number and diameter of Paneth cells were recorded, and the following morphometric measurements were evaluated: villus height and width; goblet cells number and diameter in villi and crypt; crypt depth; and goblet cell type (acid, neutral, and mixed) per villi and crypt.

2.8. Statistical Analysis

The data in this paper are depicted as their mean values ± standard error. Experimental treatments and controls for intra-amniotic administration were assigned randomly after ensuring weight distribution to all groups. The Shapiro–Wilk test was used to assess data normality. One-way analysis of variance (ANOVA) or Kruskal–Wallis was used to analyze the results and *p*-values ($p < 0.05$), followed by post hoc Duncan or Dunn's test. Statistical software IBM SPSS Statistics®, v25, was used for all analyses.

3. Results

3.1. Concentration of Proteins, Total Dietary Fiber, and Fractions

The cashew nut soluble extract (CNSE) had a higher amount of proteins (41.65%) compared to cashew nut flour (21.50%) but a lesser amount of total fiber (9.82% vs. 22.36%), insoluble (9.29% vs. 21.33%), and soluble (0.53% vs. 1.03%) compared to the cashew nut flour (Table 2).

Table 2. Content of proteins, total dietary fiber, and fractions in cashew nut flour and soluble extract.

	Proteins	**Fibers**		
		Total	**Insoluble**	**Soluble**
Cashew nut flour (g/100 g)	21.50 ± 0.51	22.36 ± 0.05	21.33 ± 0.40	1.03 ± 0.45
Cashew nut soluble extract (g/100 g)	41.65 ± 0.18	9.82 ± 0.42	9.29 ± 0.47	0.53 ± 0.05

Data are presented as mean and standard deviation.

3.2. Body Weight

The body weight (g) was similar between groups with no statistical difference (*p*-value = 0.316) (Figure 1).

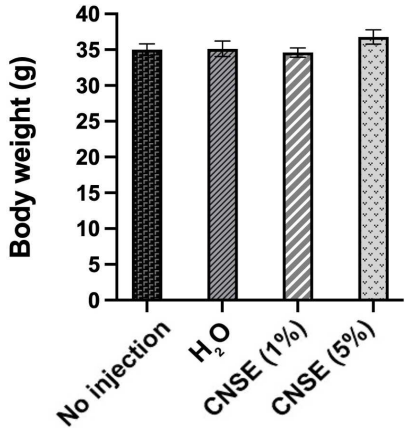

Figure 1. Body weight differences *between* groups. Mean and standard error of the mean (SEM) were evaluated using one-way ANOVA followed by Duncan test. CNSE: cashew nut soluble extract.

3.3. Effect of Cashew Nut Soluble Extract (CNSE) on Duodenal Morphological Parameters

CNSE promoted improvements in the morphometric parameters of the duodenum. At 5% and 1% CNSE, the Paneth cell number was higher compared to the controls (no injection and H_2O) (Table 3). No difference was observed in Paneth cell diameter between groups (Table 3). In the crypts, the number of goblet cells (GCs) was smaller in CNSE treatments (1% and 5%), while GC diameter and depth were higher at 5% CNSE, all these compared to no injection (Table 4). In the villi, GC numbers were smaller in 1% and 5% concentrations compared to H_2O injection; however, 1% was higher compared to no injection, and GC diameter was higher in both intervention groups compared to controls (no injection and H_2O) (Table 4). Further, at a higher concentration of CNSE (5%), the villus surface area was larger compared to other groups (Table 4).

Related to the type of GC (acid, neutral or mixed) in villi, the acid was smaller in treatment groups compared to control groups (no injection and H_2O), the neutral was smaller in all groups compared to no injection, and the mixed type was higher in CNSE treatments (being higher at 1% compared to 5%) compared to the control groups (no injection and H_2O) (Table 5). Regarding the type of GC in the crypt, neutral and mixed were smaller at 5% compared to H_2O and all other groups, respectively (Table 5). No difference was observed for acid GC per crypt (Table 5).

Table 3. Effect of intra-amniotic administration of CNSE on Paneth cell between groups.

Groups	Paneth Cell	
	Number	**Diameter (μM)**
No injection	1.94 ± 0.08 [bc]	1.45 ± 0.04 [a]
18 Ω H_2O injection	1.68 ± 0.06 [b]	1.40 ± 0.04 [a]
CNSE (1%)	2.43 ± 0.11 [a]	1.42 ± 0.03 [a]
CNSE (5%)	2.39 ± 0.12 [ac]	1.34 ± 0.04 [a]

Superscript alphabets ([a–c]) not indicated by the same letter in the same column means that there was a statistical difference between groups ($p < 0.05$) according to one-way ANOVA or Kruskal–Wallis followed by post hoc tests. When there is a statistical difference between groups, the superscript alphabets ([a–c]) are represented in descending order of the values (letter "a" represents the highest value, while letter "c" is the lowest); CNSE: cashew nut soluble extract.

Table 4. Effect of intra-amniotic administration of CNSE on crypt and villi measurements between groups.

Groups	Crypt		
	GC Number	**GC Diameter (μM)**	**Depth (μM)**
No injection	12.67 ± 0.55 [a]	2.92 ± 0.05 [b]	14.02 ± 0.50 [b]
18 Ω H_2O injection	10.95 ± 0.62 [b]	3.13 ± 0.05 [a]	16.00 ± 0.65 [ab]
CNSE (1%)	9.72 ± 0.41 [b]	3.12 ± 0.06 [ab]	15.80 ± 0.56 [ab]
CNSE (5%)	9.39 ± 0.42 [b]	3.23 ± 0.06 [a]	17.10 ± 0.69 [a]
	Villi		
	GC number	**GC diameter (μM)**	**Surface area (mm²)**
No injection	24.68 ± 0.74 [c]	2.46 ± 0.06 [b]	11668.81 ± 446.06 [b]
18 Ω H_2O injection	38.38 ± 0.91 [a]	2.20 ± 0.05 [c]	8248.42 ± 364.05 [c]
CNSE (1%)	33.41 ± 0.81 [b]	2.81 ± 0.07 [a]	8341.78 ± 342.77 [c]
CNSE (5%)	25.59 ± 0.82 [c]	2.79 ± 0.10 [a]	14491.08 ± 505.97 [a]

Superscript alphabets ([a–c]) not indicated by the same letter in the same column means that there was a statistical difference between groups ($p < 0.05$) according to one-way ANOVA or Kruskal–Wallis followed by post hoc tests. When there is a statistical difference between groups, the superscript alphabets ([a–c]) are represented in descending order of the values (letter "a" represents the highest value, while letter "c" is the lowest). GC: goblet cell. CNSE: cashew nut soluble extract.

Table 5. Effect of intra-amniotic administration of CNSE on GC type in villi and crypt between groups.

Groups	GC per Villi			GC per Crypt		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
No injection	15.28 ± 0.71 [b]	0.79 ± 0.13 [a]	8.68 ± 0.57 [c]	8.53 ± 0.46 [a]	0.41 ± 0.06 [ab]	3.73 ± 0.27 [a]
18 Ω H_2O injection	26.71 ± 1.12 [a]	0.10 ± 0.04 [b]	11.57 ± 0.66 [b]	7.88 ± 0.51 [a]	0.50 ± 0.07 [a]	2.58 ± 0.21 [bc]
CNSE (1%)	12.13 ± 0.54 [c]	0.24 ± 0.09 [b]	21.03 ± 0.75 [a]	6.93 ± 0.30 [a]	0.29 ± 0.06 [ab]	2.63 ± 0.17 [ab]
CNSE (5%)	10.87 ± 0.66 [c]	0.57 ± 0.14 [b]	14.16 ± 0.72 [b]	7.34 ± 0.35 [a]	0.30 ± 0.07 [b]	1.76 ± 0.14 [c]

Superscript alphabets ([a–c]) not indicated by the same letter in the same column means that there was a statistical difference between groups ($p < 0.05$) according to one-way ANOVA or Kruskal–Wallis followed by post hoc tests. When there is a statistical difference between groups, the superscript alphabets ([a–c]) are represented in descending order of the values (letter "a" represents the highest value, while letter "c" is the lowest). GC: goblet cell. CNSE: cashew nut soluble extract.

When evaluating the villi height/crypt depth ratio, the value was higher in 5% CNSE (16.89 ± 0.76) than in other groups (no injection: 15.46 ± 0.81; H_20: 13.76 ± 1.04; 1% CNSE: 10.55 ± 0.56) (Figure 2).

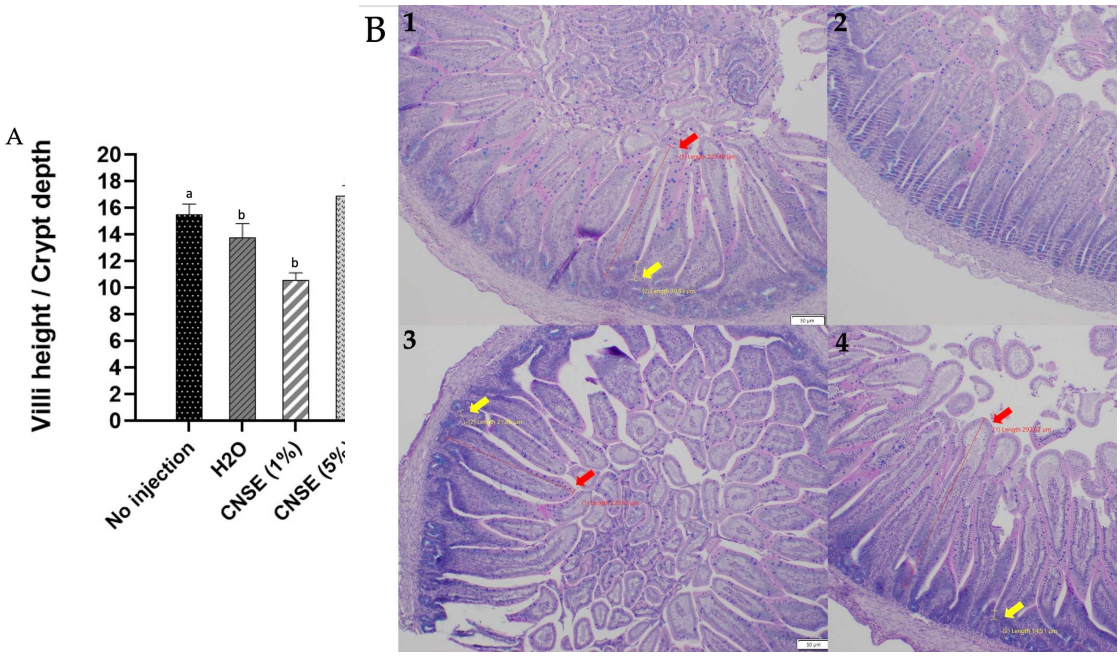

Figure 2. (**A**) Villi height/crypt depth ratio between groups. Mean and standard error (SEM) were used. Superscript alphabets (a,b) not indicated by the same letter in the figure means that there was a statistical difference between groups

($p < 0.05$) according to one-way ANOVA with post hoc Duncan test, letter "a" represents the highest value. (**B**) Villi height (red arrow) and crypt depth (yellow arrow) in each group is represented through the intestinal morphology analysis. 10× sizing was used in each measurement. 1: No injection; 2: H_2O; 3: 1% cashew nut soluble extract (CNSE); 4: 5% CNSE.

3.4. Effect of Cashew Nut Soluble Extract on the Abundance of Intestinal Bacterial Populations

In relation to bacterial population in cecum contents, the intervention groups that received the CNSE at a concentration of 50 mg/mL (5%) had a lower abundance of *Bifidobacterium* compared to control groups and *Lactobacillus* compared to all other groups. For *E. coli*, the group that received 5% of the CNSE had a lower abundance than the group that received H_2O. For *Clostridium* spp., there was no statistical difference between groups. Regarding *L. plantarum*, the intervention groups (1% and 5% of the CNSE) had higher abundance when compared to the group that received H_2O and lower than the no-injection group (Figure 3).

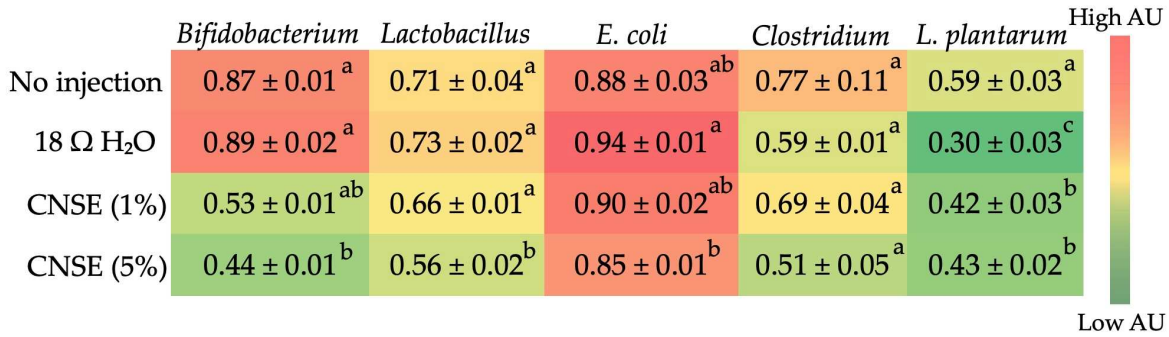

Figure 3. Heatmap of the difference between the bacterial population in cecum by intra-amniotic administration of injected CNSE in different concentrations (10 mg/mL; 50 mg/mL).Superscript alphabets ($^{a–c}$) not indicated by the same letter in the same column means that there was a statistical difference between groups ($p < 0.05$) according to one-way ANOVA or Kruskal–Wallis, followed by post hoc tests. When there is a statistical difference between groups, the superscript alphabets ($^{a–c}$) are represented in descending order of the values (letter "a" represents the highest value, while letter "c" is the lowest). CNSE: cashew nut soluble extract.

3.5. Effect of Cashew Nut Soluble Extract on Gene Expression of Intestinal Barrier Proteins and Inflammatory Biomarkers

NFκβ1 and IL-1β are pro-inflammatory genes, and no statistical difference was identified in these genes between groups. Regarding those genes related to the intestinal barrier, which are occludin (OCLN), mucin 2 (MUC2), aminopeptidase (AP), and sodium-glucose cotransporter 1 (SGLT-1), no difference was observed between groups for OCLN and SGLT1. In contrast, for MUC2, there was a reduction in the groups that received H_2O, 1%, and 5% CNSE compared to the no-injection group. Besides this, an upregulation in the AP in 5% CNSE compared to the 1% was observed (Figure 4).

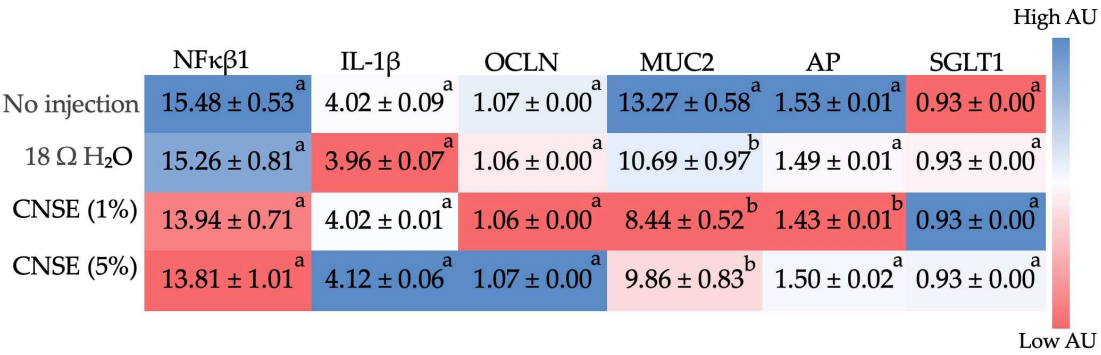

Figure 4. Heatmap of the difference between duodenal gene expression by intra-amniotic administration of injected CNSE in different concentrations (10 mg/mL; 50 mg/mL). Superscript alphabets (^{a,b}) not indicated by the same letter in the same column means that there was a statistical difference between groups ($p < 0.05$) according to one-way ANOVA or Kruskal–Wallis, followed by post hoc tests. When there is a statistical difference between groups, the superscript alphabets (^{a,b}) are represented in descending order of the values (the letter "a" represents the highest value). NFκβ1: nuclear factor kappa beta; IL-1β: interleukin 1 beta; OCLN: occludin; MUC2: mucin 2; AP: aminopeptidase; SGLT1: sodium-glucose cotransporter 1; CNSE: cashew nut soluble extract.

4. Discussion

Although cashew nut is an excellent dietary source of dietary fiber [16], studies describing the dietary effects of cashew nuts on intestinal functionality, morphology, and microbiome, are scarce. To our knowledge, the current study is the first to investigate this.

The microbial analysis indicated that the groups that received CNSE had decreased abundance of *Bifidobacterium*, *Lactobacillus*, and *E. coli* compared to the control groups. Further, there was no statistical difference between treatment groups for *Clostridium* spp. In the case of *L. plantarum*, there was a lower abundance in the intervention groups compared to the no-injection group and a greater abundance when compared to the H_2O injection. *Lactobacillus* and *Bifidobacterium* are known as probiotics, whereas *Clostridium* is potentially pathogenic, and *E. coli* depending on the strain, can be either pathogenic or beneficial[51].

Of the total dietary fiber that was measured in the CNSE used in this study, 9.29% were insoluble dietary fibers, while 0.53% were soluble dietary fibers. Soluble dietary fibers are rapidly fermented, while insoluble dietary fibers are slowly or only partially fermented [67]. This may explain the reduced fermentation in the CNSE treatment groups. *Lactobacillus*, *Bifidobacterium,* and *L. plantarum* belong to the lactic acid bacteria groups and produce lactate through fermentation [68]. Similarly, in a study that assessed the soluble extract of chia seed, assessed concentrations of 1% and 5% of the soluble section of chia also showed a reduction in *Bifidobacterium* and *E. coli*, and at a 5% concentration, there was a *Lactobacillus* reduction [69]. Chia flour (2.89 g/100 g soluble; 30.47 g/100 g insoluble)[70] and cashew nut flour (1.03 g/100 g soluble; 21.33 g/100 g insoluble) have similar contents of fiber fractions, but when we compare the fiber fractions of the soluble extract, the CNSE presents much fewer amounts of soluble fibers (0.53 g/100 g vs. 19.68 g/100 g) and insoluble (0.47 g/100 g vs. 23.53 g/100 g) compared to the soluble extract of chia [69]. Previously, Dias et al. (2019) used 5% of the soluble extract of carioca beans and documented a reduction in *Bifidobacterium* at this concentration compared to the control groups [54]. On the other hand, a study that used a soluble extract of chickpeas and lentils showed an increased abundance of *Bifidobacterium* and *Lactobacillus* in the 5% extraction treatment groups, compared to the control groups ($p < 0.05$) [59]. However, it is important to note that the soluble/insoluble dietary fiber ratios in chickpeas (0.22) and lentils (0.23) are higher compared to the soluble/insoluble dietary fiber ratios in cashew nuts (0.05) and chia (0.09). Further, chickpea has 3.5 g/100 g of soluble fiber and 15.8/100 g of insoluble, and lentil has 3.1 g/100 g of soluble and 13.6 g/100 g of insoluble [71]. This might be a possible

explanation of why the amount of 5% of chickpea and lentil extracts was sufficient to increase the relative abundance of these beneficial bacteria, given that these foods have a higher soluble/insoluble dietary fiber ratio. Wang et al. (2019) evaluated wheat bran, which is rich in insoluble dietary fiber (35–48.4 g/100 g) and arabinoxylan (22–30 g/100 g) [72], and showed that higher amounts of wheat bran extract (10%) promote higher abundance of *Bifidobacterium* and *Lactobacillus* compared to a lower concentration (5%) [73]. Altogether, these findings further reinforce the hypothesis that a higher amount of cashew nut soluble extract may be necessary to increase the concentration of these beneficial bacterial populations.

It is important to emphasize that *L. plantarum* competes with Gram-negative or other potential pathogens (i.e., *E.coli*) on receptor sites at the mucosal cell surfaces in the gastrointestinal tract (GIT) [74]. In the present study, the 5% CNSE treatment group had an increased abundance of *L.plantarum* (compared to the H_2O injection group) and a reduced abundance of *E.coli*, which can perhaps be explained by the competition of these bacteria on the same receptor. Another possible hypothesis to explain the reduction in pathogenic bacterial abundance is associated with the increased number of Paneth cells. Paneth cells are found in the intestinal villi crypts and produce and secrete various antimicrobial peptides (AMPs), which are essential for defense against intestinal pathogens [75]. In the current study, it was demonstrated that in treatment groups where the Paneth cell number increased, the *E. coli* and *Clostridium* decreased. It is, therefore, possible that the increased production of AMPs by Paneth cells generated by the CNSE contributed to the reduced abundance of bacterial populations. In addition, a study that used inulin (which is a soluble fiber) showed that, unlike cellulose (which is insoluble), inulin increased the level of Paneth cells [76]. In addition to fibers, CNSE showed high concentrations of proteins, and these also exert effects on the composition of the microbiota. The protein source is a very important determinant for the utilization of intestinal bacteria. However, compared to proteins from plant-based food, those from animal-based food sources seem to have better effects on the microbiota, first because protein digestibility from animal sources is higher, and second, because digestion of plant proteins may be limited by the presence of antinutritional factors found in plants [77]. As demonstrated in a study, rats fed with protein from animal-based food

increased *Lactobacillus* and/or *Bifidobacterium* compared to those fed from plant-based food [78–81]. Thus, despite the high protein content found in the CNSE, as the source of this protein is a plant-based food, this may also have contributed to the non-growth of beneficial bacteria.

Regarding gene expression, although there were no statistical differences in investigated inflammation-related genes, the results suggested a trend toward a reduction in NFκβ1 gene expression in the CNSE groups compared to the control groups. It is possible that the lower abundance of beneficial bacteria (*Lactobacillus* and *Bifidobacterium*) in the CNSE groups has not been able to generate enough anti-inflammatory effects to be significant. This is because these bacterial populations produce lactate, which is often associated with immunomodulating properties via suppression of the LPS/Toll-like receptor 4 signaling pathway [82]. Lactate and acetate can also be converted to butyrate by intestinal bacteria*,* which supplies energy to colonic epithelial cells, maintaining gut barrier functions and modulating the immune system in an anti-inflammatory manner [83]. Thus, these metabolites have anti-inflammatory action. Therefore, less concentrated bacteria and low fermentation and production of these metabolites were insufficient to make this anti-inflammatory effect significant in the CNSE groups compared to the control. In contrast, the upregulation in IL-1β gene expression at the 5% CNSE group compared to the control groups may have been due to competition between *L.plantarum* and *E.coli*, producing toxins that are responsible for generating inflammation [84,85]. This hypothesis is confirmed by the increased number of Paneth cells in this group since these cells are needed to produce antimicrobials [86]. Further, this is supported by the fact that IL-1β is produced by immune cells (i.e., monocytes and macrophages) and non-immune cells (i.e., endothelial and epiderm cells) in response to different stimuli*,* including microbes, bacterial lipopolysaccharides (LPS), and cytokines [87].

Regarding the BBM functional proteins, MUC2 is expressed and secreted by GC, and it is the prominent intestinal gel-forming mucin of the mucus layer of the small intestine, creating a protective mucus layer against bacterial invasion [88,89]. They are also involved in the immunoregulation and intestinal digestive and absorptive capabilities[90]. In this study, there were fewer GC numbers in the intervention groups, which may have impacted mucin production and secretion and

consequently downregulated the MUC2 gene; since there were a small number of cells, there would be less production of mucins. In addition, intestinal bacteria play an essential role in mucin secretion, as *Lactobacillus*, *Bifidobacterium*, and *L. plantarum* species could increase the synthesis and secretion of mucins [85,91,92]. Duangnumsawang et al. (2021) showed that the lack of gut bacteria in germ-free chickens led to a reduction in the number and density of GC, as well as a decrease in MUC2 expression [91]. Other studies showed that germ-free animals exhibited a decrease in GC size and number with a consequent reduction in mucus layer thickness, indicating a reduction in mucus production [93,94]. The lower concentration of bacteria (*Lactobacillus*, *Bifidobacterium*, and *L. plantarum*) in the CNSE groups may also have contributed to a lower number of GC, consequently lower mucin secretion and reduced MUC2 gene expression.

The expression of the amino peptidase (AP) gene was dose-dependently upregulated in CNSE. AP is involved in protein and peptide degradations; it cleaves amino acids from the N-terminus of peptides and provides substrates for the amino acid transporters. Proteins are soluble in water. As demonstrated in the Results section, the amount of protein in CNSE was much higher (41.65%) than in cashew nut flour (21.50%). Therefore, AP could act in the hydrolysis of these proteins and peptides, increasing the AP gene expression[95]. One of the most abundant amino acids in cashew nuts is glutamic acid [96], which is the primary energy source for intestinal epithelial cells [97]. No differences were found in OCLN and SGLT1 expression, as the values were practically the same between groups. OCLN is regarded as one of the most critical tight junction-associated structural proteins in the intestine and plays a vital role in keeping the physical barrier of the intestinal mucosa [98]. Similar results were described in previous studies using the same in vivo model [54,60,99,100]. The authors highlighted that the *Gallus gallus* model has a limited ability to digest and absorb nutrients before hatch[75,101], as AP and SGLT1 are biomarkers of BBM digestive and absorptive functions; perhaps for that reason, a longer intervention time would be necessary to be able to observe more significant differences in BBM functionality.

The intestinal morphology results showed an increase in the crypt GC diameter and depth in the group that received 5% CNSE compared to the no-injection group. These results may be associated with a reduction in GC number to maintain homeostasis. As this group had few numbers of GC, there were an

increase in the diameter and depth of these existing cells. Deeper crypts may indicate a renewal of intestinal epithelial cells, positively affecting intestinal absorption and secretion function [102]. Dietary fibers mechanically stimulate the intestinal epithelium to secrete mucus, thus stimulating the secretion of water and mucous as a defense mechanism to protect [74]. Zou et al. (2018) demonstrated that inulin (fermentable dietary fiber), but not cellulose (not fermentable fiber), was able to reverse reductions in colon mass and crypt length, gut atrophy, reduced enterocyte proliferation, and microbiota encroachment from a high-fat diet (HFD) [62]. In addition, the high concentration of protein present in the extract may also have affected the number of goblet cells. One study showed that higher amounts of protein (53%) compared to lower doses (14%) showed a lower number of GC at the epithelial surface [103].

Regarding the villi, the present study showed an increase in the villi surface area in the 5% CNSE treatment group compared to others. The longer the length of villi, the greater the absorption of nutrients, and this is dependent on the diet provided. This may have been attributed to high protein concentrations in the 5% CNSE as amino acids, mostly glutamine (which comes from glutamic acid), one of the main fuels for the small intestine mucosa, as they provide the energy required for intestinal ATP-dependent metabolic processes, such as active nutrient transport and high rates of intracellular protein turnover [104]. Besides the protein, this finding could also be attributed to soluble dietary fibers that caused an increase in the proliferation of enterocytes, which leads to hyperplasia/hypertrophy of these cells and, consequently, growth in villi surface area. Thus, improving the absorptive and digestive capacity of the villi [69]. This is supported by current findings, as the 5% CNSE group showed higher values in the villi height/crypt depth ratio relative to other groups. The relationship between these two markers indicates intestinal health, as the appropriate proportion between villus height and crypt depth are important indicators of gut development and animal health and, as such, influence nutrient digestion and absorption [102]. Shorter villi reduce the surface area as well as deeper crypt lead to a higher secretion of digestive enzymes, which consequently decreases nutrient absorption [102,105,106].

Related to the distribution of mucin types (acid and neutral) in GC, they can be affected by host factors (inflammatory markers, neurotransmitters, or hormones)

and external factors (commensal bacteria, pathogens, dietary nutrients, or pre/probiotics) [107]. In this study, we demonstrated less acid and neutral GC per villi (where the mucus is produced) in CNSE groups. This result is because these groups had less abundance of bacteria compared to the control groups. This hypothesis can be reinforced based on a study that compared conventionally raised (CR) animals and germ-free animals (GF) and showed that GF animals displayed less neutral mucin and sulfated (i.e., acid) mucin compared to CR [108]. Lastly, it is essential to highlight that this was the first study to evaluate the cashew nut soluble extract on intestinal health in vivo, and subsequent studies are needed. However, the results demonstrated in this study contribute to a better understanding of cashew nuts' benefits on intestinal health through their action on different parameters such as morphology, functionality, and microbiota. Understanding all these factors contributes to differentiation in future studies, mainly clinical trials.

5. Conclusions

Cashew nut soluble extract improved gut health by promoting benefits on morphological parameters as it increased villi surface area, villi height/crypt depth ratio, and intestinal functionality by upregulating AP gene expression. All these parameters are essential for improving digestive and absorptive capacity. In addition, cashew nut soluble extract was beneficial for acting in defense against intestinal pathogens by increasing Paneth cell number. Thus, serving as a preliminary step to provide a greater understanding of the potential of cashew nuts to promote gut health. As this was the first study to assess the effects of cashew nuts on gut health in vivo*,* more studies are needed, mainly using higher concentrations of the extract and long-term studies to elucidate additional potential health benefits and mechanisms of action. As well, future studies using next-generation sequencing (NGS) methods can also help to identify greater bacteria diversity from the consumption of cashew nut soluble extract.

Author Contributions: T.S.M.: Methodology; formal analysis; investigation; data curation; writing—original draft preparation. N.K.: Methodology; formal analysis; investigation; data curation; writing—original draft preparation. A.M.: Methodology; formal analysis; investigation; data curation; writing—original draft preparation. A.P.D.: Supervision; resources. A.C.P.K.: Methodology. B.P.D.S.:

Methodology; data curation; writing—original draft preparation. H.S.D.M.: Methodology; data curation; writing—original draft preparation; supervision. H.H.M.H.: Methodology; formal analysis; investigation; data curation; writing—original draft preparation; supervision. E.T.: Methodology; formal analysis; investigation; data curation; writing—original draft preparation; supervision; resources. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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6.3 Article 3

Cashew nut (*Anacardium occidentale* L.) and cashew nut oil improve atherogenic and liver function biomarkers in adults on weight-loss treatment: a randomized controlled three-arm trial (Brazilian Nuts Study)

Talitha Silva Meneguelli[1]; Ana Claudia Pelissari Kravchychyn[1]; Aline Lage Wendling[1]; Ana Paula Dionísio[2]; Josefina Bressan[1]; Hércia Stampini Duarte Martino[1]; Elad Tako[3]; Helen Hermana Miranda Hermsdorff[1]

[1] Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil
[2] Embrapa Agroindústria Tropical - CNPAT - (Fortaleza - CE), Brazil
[3] Department of Food Science, Cornell University, Ithaca, NY 14850, USA

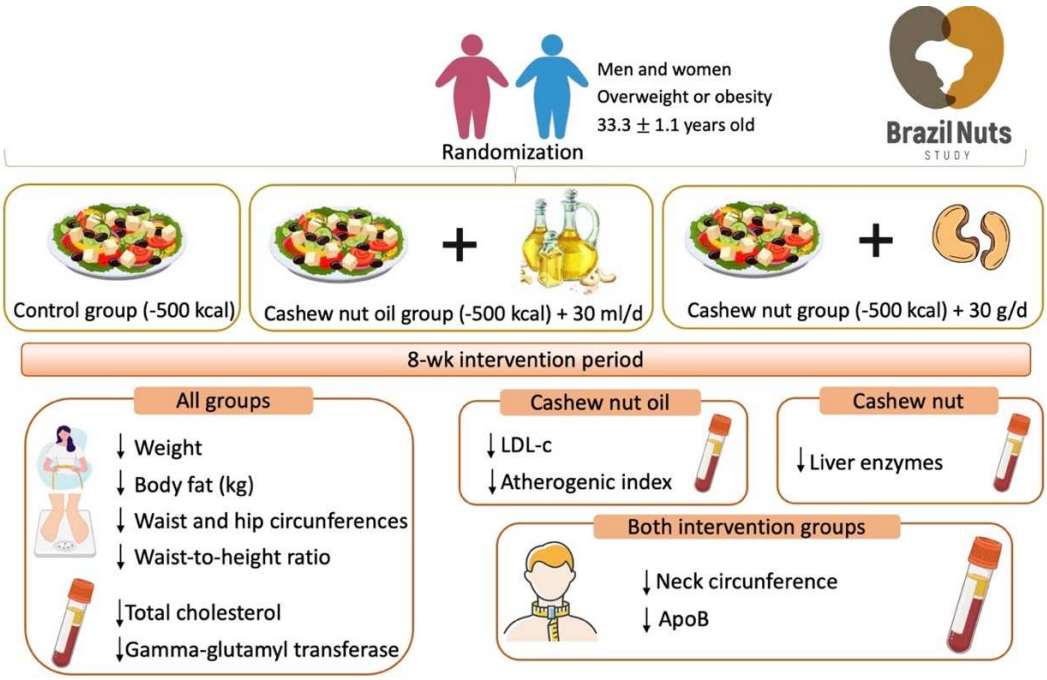
Corresponding Author: Helen Hermana Miranda Hermsdorff, Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, Brazil. Address: Av. PH Rolfs, s/n, Viçosa, Minas Gerais, 36570-000, Brazil. E-mail: helenhermana@ufv.br

Abstract

Introduction: Cashew nut contains bioactive compounds that modulate satiety and food intake, but its effects on body fat during energy restriction remains unknown. This study aimed to assess the effects of cashew nut and cashew nut oil on body fat (primary outcome) as well as adiposity, cardiometabolic and liver function markers (secondary outcomes). **Methods:** An eight-week (8-wk) randomized controlled-feeding study involved 68 adults with overweight/obesity (40 women, BMI: 33 ± 4 kg/m^2). Participants were randomly assigned to one of the energy-restricted (-500 kcal/d) groups: control (CT, free-nuts), cashew nut (CN, 30 g/d), or cashew nut oil (OL, 30 mL/d). Body weight, body composition, and blood collection were assessed at the baseline and endpoint of the study. **Results:** After 8-wk, all groups reduced significantly body fat (CT: -3.1 ± 2.8 kg; CN: -3.3 ± 2.7 kg; OL: -1.8 ± 2.6 kg), body weight (CT: -4.2 ± 3.8 kg; CN: -3.9 ± 3.1 kg; OL: -3.4 ± 2.4 kg), waist (CT: -5.1 ± 4.6 cm; CN: -3.9 ± 3.9 cm; OL: -3.7 ± 5.3 cm) and hip circumferences (CT: -2.9 ± 3.0 cm; CN: -2.7 ± 3.1 cm; OL: -2.9 ± 2.3 cm). CN-group reduced liver enzymes (AST: -3.1 ± 5.3 U/L; ALT: -6.0 ± 9.9 U/L), while the OL-group reduced LDL-c (-11.5 ± 21.8 mg/dL) and atherogenic index (-0.2 ± 0.5). Both intervention groups decreased neck circumference (CN: -1.0 ± 1.2 cm; OL: -0.5 ± 1.2 cm) and apo B (CN: -6.6 ± 10.7 mg/dL; OL: -7.0 ± 15.3 mg/dL). **Conclusion:** After an 8-wk energy-restricted intervention, all groups had reductions in body fat (kg), weight, and some others adiposity indicators, with no different effect of cashew nut or cashew nut oil. However, participants in the intervention groups experienced additional reductions in atherogenic marker, liver function biomarkers, and cardiovascular risk factors (e.g, neck circumference and apo B levels), with these effects observed across the cashew nut oil, cashew nut, and both intervention groups, respectively.

Keywords: Clinical trial, Cashew nut, *Anacardium occidentale* L., Obesity, Anthropometry, Cardiometabolic markers

GRAPHICAL ABSTRACT



Introduction

Obesity is a multifactorial and complex disease characterized by excessive adiposity. It is linked to an elevated risk of developing other chronic conditions, including type 2 diabetes mellitus (T2DM), hypertension, dyslipidemia, cardiovascular diseases (CVDs), and some types of cancer (Wharton *et al.*, 2020). This condition represents a burgeoning global pandemic, with estimates indicating that by 2030, over 1 billion people worldwide will be affected by obesity (body mass index (BMI) ≥ 30 kg/m²). This projection translates to approximately one in five women and one in seven men (Barata Cavalcanti *et al.*, 2022). In 2019, obesity played a contributing role in around 5 million deaths attributed to cardiovascular diseases, diabetes, cancers, neurological disorders, chronic respiratory diseases, and digestive disorders (Chong et al., 2023).

One potential strategy for mitigating obesity involves dietary approaches aimed at achieving an optimal energy balance and energy-restriction as treatment (Canuto *et al.*, 2021; Chopra *et al.*, 2021). Besides, a growing body of evidence from epidemiological studies and clinical trials supports the potential benefits of nuts. Not only do they avoid causing weight gain, but they also seem to contribute to improved body composition and reduced cardiometabolic risk through favorable effects on lipid profiles (Eslami *et al.*, 2022; Flores-Mateo *et al.*, 2013; Guarneiri e Cooper, 2021; Razquin *et al.*, 2017; Schlesinger *et al.*, 2019).

Among all nuts, cashew nut is one of the most produced and consumed globally, ranking third in both categories (INC, 2021). In addition to their unsaturated fat content, cashew nut are whole foods that offer supplementary non-lipid nutrients, including proteins, dietary fiber, and phenolic compounds (Kornsteiner-Krenn, Wagner e Elmadfa, 2013). Furthermore, a derivative of cashew nut, the oil extracted from these nuts, shows promise for promoting health. Cashew nut oil contains high content of monounsaturated fatty acids (MUFA) (Leal *et al.*, 2023), α-linolenic acids, tocopherols, phytosterols, and phenolic compounds (Zanqui *et al.*, 2020). Hence, the oil can be positioned as a new product with enhanced value, attributable to its distinctive sensory characteristics, substantial nutritional advantages, and chemical stability (Leal *et al.*, 2023). However, the combined effects of an energy-restricted diet and the dietary intake of cashew nuts has been not reported, nor has the effect of cashew nut oil on human health.

Thus, we hypothesized that cashew nut and cashew nut oil could contribute to body fat loss and further improvements in body composition, cardiometabolic and liver function markers. The objective of this study was to assess the effects of both cashew nut and cashew nut oil over an 8-week energy restriction on body fat (primary outcome) and other adiposity indicators, cardiometabolic, and liver function markers among adults with overweight/obesity. Furthermore, the study analyzed the proximate composition, minerals, fatty acid profile, and phenolic compounds of both cashew nut and cashew nut oil.

METHODS

Cashew nut and cashew nut oil

Cashew nut (*Anacardium occidentale* L.) and cashew nut oil were produced in Brazil, coming from donation of the Brazilian Agricultural Research Corporation (Embrapa), Agroindústria Tropical, Fortaleza (Brazil).

All procedures described next were carried out at the Embrapa. Oil samples were extracted by centrifugation. For sample preparation, the cashew nut was roasted at 110 °C for 15 min; cashew nut was ground in a food processor; adding water to the cashew nut (4:1 cashew nut: water w/w) and the mixture was homogenized in a processor at 90 °C for 10 min. This mixture was centrifuged for 1 hour at 4,500 rpm at room temperature. After centrifugation, the oil was heated in an oven at 105 °C for 1 hour (Leal *et al.*, 2023). The raw material was obtained from the same crop, and its microbiological quality was analyzed and assured via reports by the supplier company until they were delivered to the Laboratory of Energy Metabolism and Body Composition of the Universidade Federal de Viçosa (LAMECC/ UFV).

For the intervention, cashew nuts were portioned into laminated and vacuum-sealed packages (30 g), while cashew nut oil was fractionated and stored in 250 mL amber glass bottles. Both foods were stored in a freezer at -20ºC until distribution to participants to avoid nutrient oxidation, sensory changes, and microbiological contamination. All material for consumption was handled following hygienic-sanitary standards, including the use of clean lab coats, caps, masks, and disposable gloves.

Regarding nutrients and bioactive compounds of cashew nut, moisture, ash, protein, lipids, carbohydrates, dietary fibers, amino acids, and in vitro digestibility

were evaluated. The moisture, ash, and protein contents were performed according to the methodology indicated by the AOAC (2016), the last one was obtained by combustion in the Nitrogen/Protein Analyzer equipment. Carbohydrate content was calculated by the difference of 100 and the sum of the values obtained for moisture, ash, proteins, and lipids. The energy value per 100 g of each product was calculated using the Atwater system: Caloric value = (g of protein × 4) + (g of lipids × 9) + (g of carbohydrates × 4). Total dietary fiber (soluble and insoluble fiber) was determined by the gravimetric non-enzymatic method, using the commercial kit (Total dietary fiber assay kit, Sigma®, San Luis, Missouri, EUA) (AOAC, 2016). The amino acid contents (aspartic acid, glutamic acid, serine, glycine, histidine, taurine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, cystine, isoleucine, leucine, phenylalanine, lysine, hydroxyproline, tryptophan and the sum of total amino acids) were performed based on the MA-009 R0 method(Hagen et al., 1993; White, Hart e Fry, 1986), and tryptophan by the MA-010 R.1 method (Lucas e Sotelo, 1980). *In vitro* digestibility was analyzed by the previously reported method (Akeson e Stahmann, 1964).

Both cashew nut and cashew nut oil underwent analysis for minerals, vitamin E and its derivatives, total phenolics, and antioxidant capacity. Mineral analyzes (phosphorus, potassium, calcium, magnesium, selenium, sodium, copper, iron, zinc, and manganese) were performed according to the methodology of the Food and Drug Administration (FDA) (Fda *et al.*, 2010). The preparation and analysis of the vitamin E isomers (α-, β-, γ-, δ- tocopherols and tocotrienols) were extracted according to Pinheiro-Sant'Ana et al. (2011), and performed in five replicates by High-Performance Liquid Chromatography (HPLC). During analysis, the samples were protected from sunlight and artificial light using amber glassware, aluminum foil, and blackout curtains, and protected from oxygen by using lids and environments with nitrogen gas in glass bottles. The total phenolic compound content was obtained from reading of absorbance in a spectrophotometer (Thermo Scientific, Evolution 606, USA) at 765 nm. Analytical curve of gallic acid (0,005–0,10 mg/mL) was used to quantify the compounds. The results were expressed in mg of gallic acid equivalents/g of cashew nut (mg GAE/g). The antioxidant activity was determined by the sequestering capacity of

free radical DPPH (2,2-diphenyl-1-picryl-hydrazil) as described before (Bloor, 2001).

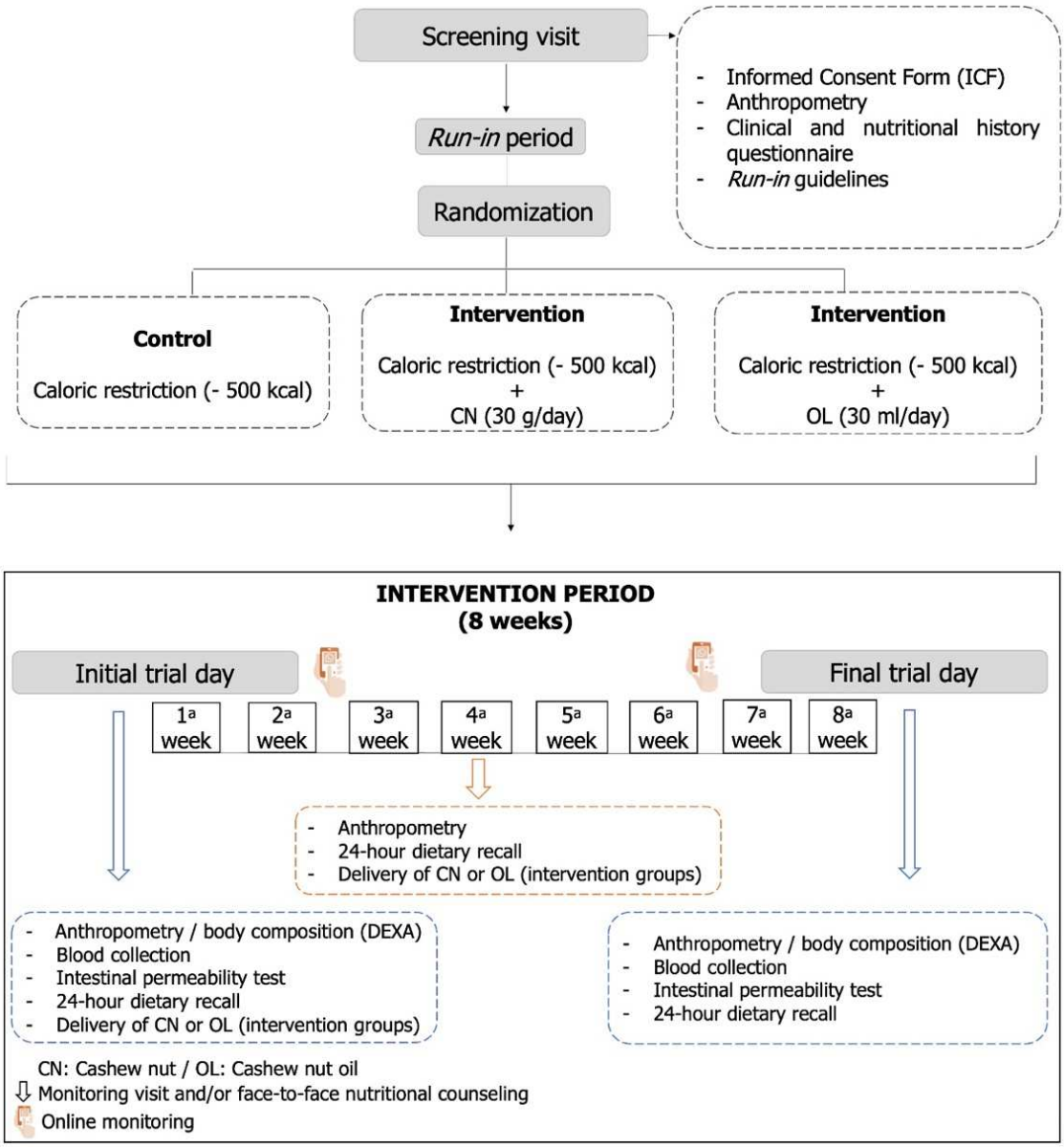
Additionally, we also performed analysis of fatty acids, acidity level, and peroxide index in cashew nut oil. Lipids were obtained using the high-pressure, high-temperature extraction system in Ankom XT-15 equipment according to the American Oil Chemists' Society (AOCS, 2004). The fatty acid profile was determined using the procedure described by Hartman and Lago (1973). The determinations of acidity and peroxides were performed according to AOCS (2003).

Study Design

This is an 8-wk randomized controlled three-arm dietary intervention, in which subjects were assigned to receive control (CT), cashew nut (CN) or cashew nut oil (OL) plus an energy-restricted diet. This study was conducted at the Department of Nutrition and Health of the Universidade Federal de Viçosa (UFV), Brazil, between January 2022 and July 2022, according to the guidelines laid down in the Declaration of Helsinki. All procedures involving human subjects were approved by the Ethics Committee in Research with Human Experimentation of the Universidade Federal de Viçosa (N° 4.543.541/CEPH). Written informed consent was obtained from all subjects/patients. The study is registered at the Brazilian Registry of Clinical Trials (ReBEC) with ID number RBR-8xzkyp2.

During the intervention, participants attended on three occasions at the LAMECC/ UFV: initial and final days for blood sample collection, anthropometry, body composition evaluation and fill out questionnaires about physical activity practice and food record, and in the fourth week (30 days) for a face-to-face monitoring visit and anthropometric measurements. Between face-to-face visits, participants received online monitoring (Figure 1).

Figure 1. Study design flowchart.



Source: Own elaboration.

Study participants

Study participants were recruited in Viçosa, Minas Gerais, Brazil, via radio announcements, social media, and the UFV network platform. An online form assessed individuals' eligibility, with eligible candidates undergoing a face-to-face selection questionnaire to confirm eligibility. This questionnaire covered clinical, dietary, sociodemographic, and anthropometric data, along with body composition, blood pressure, and recent biochemical test results. Participants

received a booklet containing guidelines about the study and were instructed to report any changes in medication or health status.

The inclusion criteria for participants in the study consisted of men or women (20-55 y); with overweight (27 – 29.9 kg/m²), waist circumference (WC) ≥ 80 cm for women and ≥ 90 cm for men and with body fat percentage > 30% for women and > 20% for men associated with at least another component of metabolic syndrome (MS): triglycerides (TG) ≥150 mg/dL; blood pressure ≥ 130/85 mmHg) or fasting blood glucose ≥100 mg/dL or who uses medication to control these markers; or men or women with obesity (BMI ≥ 30 kg/m²), WC ≥ 80 cm for women and ≥ 90 cm for men, and body fat percentage > 30% for women and > 20% for men with or without metabolic complications.

The non-inclusion criteria included pregnant, lactating, or menopausal women; athletes; vegans; or have a diagnosis of insulin-dependent diabetes; diagnosis of HIV, digestive, hepatic, renal, cardiovascular, thyroid, cancer, inflammatory diseases and eating disorders; history of drug and/or alcohol abuse; have an aversion or allergy to nuts; present infection in the last month; habitually consume nuts above 30 g/day; use drugs such as anti-inflammatories, corticosteroids, and antibiotics, capable of biochemical alterations; chewing difficult; weight instability (5% of usual weight) in the last 3 months; alcohol consumption > 21 units (≈168g) per week; and intake of vitamin, mineral, and omega 3 supplements.

Run-in

One week before intervention, the participants participated in a run-in period. During run-in the subjects were instructed to consume their habitual diets without nuts, dried fruits like berries (cranberry, blueberry, goji berry and raisins), açaí, cocoa, cinnamon, olive oil and alcoholic beverages, and to maintain their usual activities. Following the run-in period, individuals whose body weight fluctuated beyond ±1 kg or who consumed prohibited foods or beverages were categorized as "poor responders" and excluded from the study.

Intervention

All participants received an energy-restricted (-500 kcal/d) diet. In addition, the cashew nut group received 30 g/d of vacuum-sealed cashew nut to be consumed

daily, and the cashew nut oil group received 250 mL amber glass bottles of oil along with a measuring cup to standardize the amount to 30 mL/d of cashew nut oil. All dietary advice was individualized and provided by dietitians. At the beginning, five energy-restricted diet options for all groups were designed and divided into five meals: breakfast, morning snack, lunch, afternoon snack, and dinner. All menus were calculated in an Excel spreadsheet using the Brazilian Institute of Geography and Statistics (IBGE) table. Energy requirements were calculated according to the Mifflin's formula (Muffin *et al.*, 1990). For everyone, 500 kcal were reduced from the total calculated energy requirement, considering the level of physical activity of each participant. For the interventional groups, a daily cashew nut (30 g/d) or cashew nut oil (30 mL/d) was added to the individual meal plans, and the percentage of energy from total fat was around 27% for cashew nut group, 32% for the cashew nut oil group, while the control group had around 21% (Table 1). This amount of cashew nut was based on previous studies that have used similar amounts, the PREvención con DIeta MEDiterránea (PREDIMED), which demonstrated beneficial effects in the improvement of blood pressure, lipid profile, lipoprotein particles, inflammation, oxidative stress, and carotid atherosclerosis (Martínez-González *et al.*, 2015; Salas-Salvadó *et al.*, 2011). For the cashew nut oil group, 30 mL/d was calculated to reach similar amounts of lipid between the two intervention groups. There was no statistical difference in energy calculated between the groups (p=0.959).

Table 1. Macronutrients, dietary fiber, and energy distribution among groups

Nutrients	Control	Cashew nut	Cashew nut oil	p-value
Total fat (%)	21.19 ± 1.84 [c]	27.04 ± 2.49 [b]	31.83 ± 3.87 [a]	**<0.001**
Saturated Fat (%)	*7.87 ± 1.97 [b]*	*10.96 ± 1.51 [a]*	*7.78 ± 1.47 [b]*	**<0.001**
Monounsaturated Fat (%)	*5.97± 1.58 [c]*	*17.38 ± 2.71 [a]*	*14.52 ± 2.32 [b]*	**<0.001**
Polyunsaturated Fat (%)	*3.57 ± 0.86 [b]*	*6.41 ± 1.31 [a]*	*6.01 ± 1.33 [a]*	**<0.001**
Carbohydrates (%)	55.26 ± 3.51 [a]	48.06 ± 4.17 [b]	47.17 ± 5.55 [b]	**<0.001**
Proteins (%)	23.55 ± 2.74 [a]	24.89 ± 3.14 [a]	20.99 ± 3.15 [b]	**<0.001**
Dietary fiber (g)	25.90 ± 7.69	22.62 ± 7.02	22.64 ± 8.49	0.065
Energy (kcal)	1600.83 ± 318.29	1607.40 ± 307.82	1618.05 ± 317.15	0.959

Superscript alphabets [(a-c)] not indicated by the same letter means statistical difference between groups (p<0.005) according to one-way ANOVA or Kruskal-Wallis followed by post hoc tests. Letter a represents the highest value, while letter c is the lowest.

Participants were instructed to incorporate cashew nut as a mid-morning snack, while those assigned to the cashew nut oil group were provided with recipes for incorporating the oil into shakes and salad dressings. Members of the cashew nut and cashew nut oil groups (intervention) were explicitly directed not to use the cashew nut or their oil for cooking, roasting, or frying purposes. Additionally, they were advised against consuming olive oil, avocado, or any other nuts aside from the allocated quantity of cashew nut, as well as any other foods with high unsaturated fat content. Control group participants were similarly instructed to refrain from consuming any type of nuts, olive oil, avocado, or other foods high in unsaturated fat.

Outcomes

The primary outcome of the trial was a change in body fat. Secondary outcomes were changes in the values of body weight, BMI, waist, hip and neck circumferences, waist-to-hip ratio (WHR), waist-to-height ratio (WHtR), cardiometabolic (TG, total cholesterol, LDL-c, HDL-c, VLDL-c, ApoA1, ApoB, cortisol, total cholesterol:HDL-c, LDL-c:HDL-c) and liver function markers (AST, ALT, GGT, alkaline phosphatase) after 8 weeks of follow-up.

Body composition was assessed by dual-energy X-ray absorptiometry (Lunar Prodigy Advance DXA System, GE Lunar) and provided fat mass (FM), fat-free mass (FFM), lean mass (LM), and total mass were obtained from the total body and regions, such as trunk, android, and gynoid. The android area is between the ribs and the pelvis, while the gynoid region includes the hips and upper thighs and overlaps the leg and truncal regions. The body composition in percentages was calculated in relation to total body measurements. Body weight was assessed by a bioelectrical impedance analysis device (Inbody 230, Biospace Corp.). Height (meters), waist, hip, and neck circumferences (centimeters) were measured according to standard protocols. BMI was calculated as weight divided by squared height (kg/m^2). WHR was calculated as waist divided by hip circumference, and WHtR was calculated as waist divided by height.

Fasting (10-12 h) venous whole blood samples were collected by a registered nurse at baseline and the end of the study (8-wk) into vacuum tubes containing EDTA as an anticoagulant. Then, blood samples were centrifugated (3500 r.p.m,

10 min, 4°C), separated in aliquots and stored until analysis. The biochemical determinations were performed by the Hemolab clinical analysis laboratory (Viçosa-MG, Brazil). Trained nursing technicians, specifically employed for this project, conducted the blood collection, obtaining samples ranging from 20 to 30 mL via vacuum. Samples were collected for evaluation of cardiometabolic risk as TG (≥150 mg/dL), total cholesterol (≥ 240 mg/dL), LDL-c (≥ 160 mg/dL), HDL-c (<40 or <50 mg/dL for men and women, respectively), and VLDL-c (≥30 mg/dL). Also, apolipoprotein-A-1 (APO-A-1), apolipoprotein-B (APO-B), liver markers such as AST transaminase, gamma GT, ALT transaminase, and alkaline phosphatase were compared as mean and standard deviation between groups. Besides, the atherogenic indices, total cholesterol:HDL-c and LDL-c:HDL-c proposed by Castelli (1988) were calculated.

Dietary assessments

At baseline and the end of the study, we applied a 24-hour recall (24HR) to monitor food consumption during the intervention. The reported intake was analyzed using the 24HR- ERICA software, adapted for the Brazilian population, and the IBGE table (Barufaldi *et al.*, 2016; Belchior Sé Marcia Maria Melo Quintslr *et al.*, 2011).

Compliance

Compliance was evaluated during the monitoring visit to determine whether participants were adhering to the intervention. We tracked the consumption of cashew nut and cashew nut oil from the initial day of the intervention until their return. If participants in the intervention group still possessed cashew nuts or oil from the beginning of the study upon returning, they were required to surrender them for assessment, verifying whether they had indeed consumed the prescribed quantity. Additionally, during the application of the return questionnaire, we evaluated whether participants had commenced any new medication or developed any illnesses. The participants who started taking any drug or developed any disease listed in the non-inclusion criteria was excluded from the study. Furthermore, compliance was gauged at the study's conclusion by monitoring weight gain. Since the intervention aimed to body fat loss by energy restriction, participants were expected to experience weight loss. Consequently,

individuals who exhibited weight gain were excluded from the study due to non-compliance.

Sample size and study power

The sample size and study power were determined using the G*Power 3.1 program. For this calculation, a total of 57 volunteers were determined, based on an average estimated effect size derived from clinical studies (0.30), considering statistical analyses for three groups, two intervention points (baseline and endpoint), an alpha value set at 0.05, and a power of 0.80. By adding 20% as a result of losses during follow-up, the total sample size was determined to be 68 participants (Supplementary figure 1).

For the power of the study, the effect size of 0.28 was calculated from the Eta squared (0.074) based on the values of body fat from our database, an α of 0.05 was used, three groups, two intervention points (baseline and endpoint), and the total sample size of 68 individuals, whom we have information on body fat data. The calculation revealed a study power of 0.94 (Supplementary figure 2).

Randomization

To initiate the intervention, after the run-in period, we performed the randomization using MinimPy 0.3 program (Saghaei e Saghaei, 2011). This was achieved through the stratified minimization method, accounting for sex, age, and BMI, with three levels per factor. This approach ensured a well-balanced distribution of potential factors that could interfere with the outcome variables.

Statistical analysis

Statistical analysis was conducted using SPSS version 22.0 (SPSS, Inc.), and figures displaying statistical analysis were produced using Microsoft Excel. A p-value <0.05 was considered statistically significant. The Shapiro–Wilk test was performed to check the normality of variables. Data are expressed as mean values and standard deviation. Among groups, variable changes were compared by one-way ANOVA followed by Tukey's post hoc test or using the non-parametric Kruskal–Wallis test followed by Dunn's post hoc test. To compare differences between baseline and post-intervention within the groups, pairwise tests were performed (paired *t*-test or Wilcoxon). McNemar's test was employed to analyze paired nominal data.

RESULTS

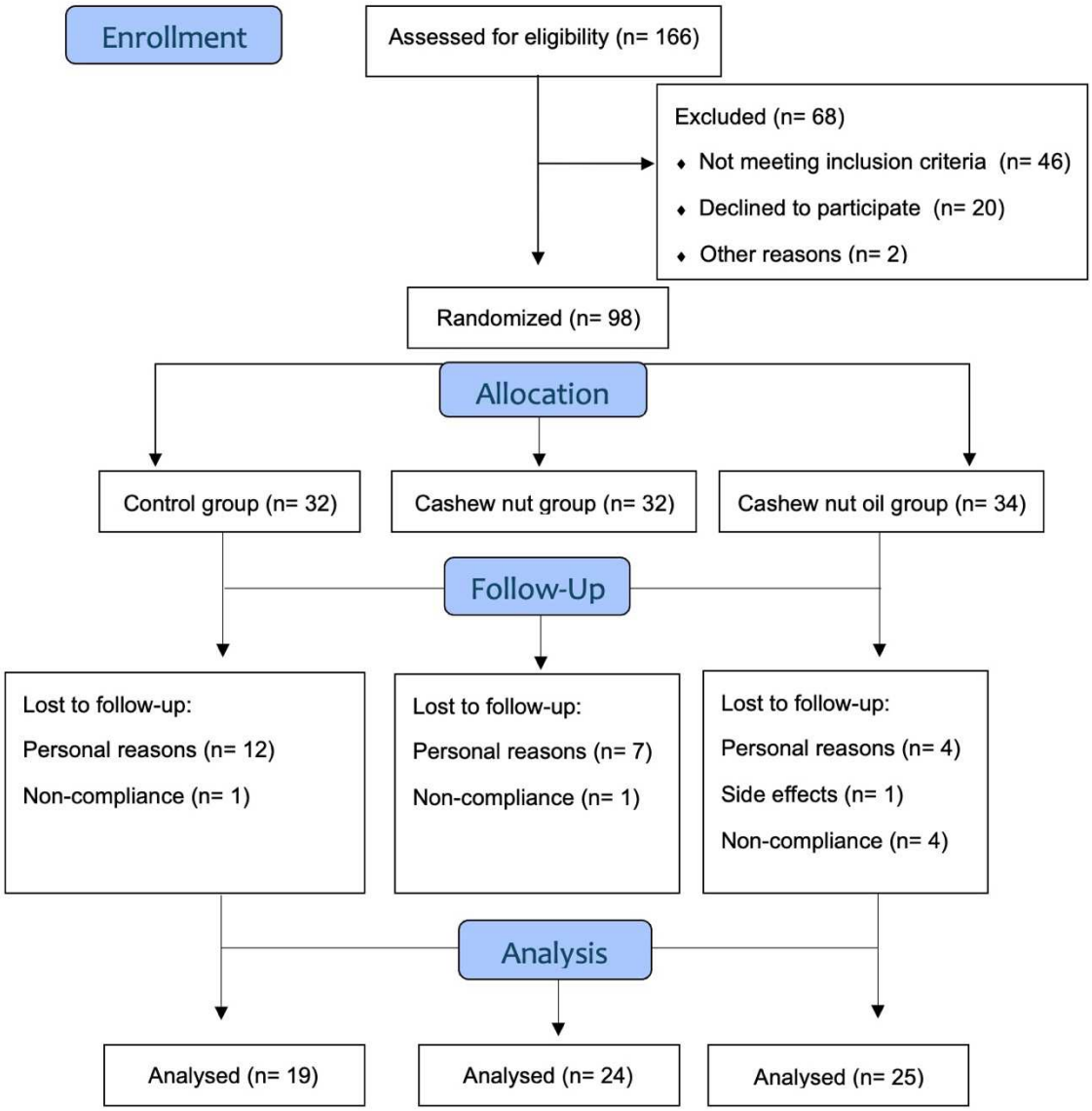
Cashew nut and cashew nut oil composition

Regarding minerals, the content of calcium (CN: 0.37 g/kg *vs.* OL: 0.01 g/kg) and iron (CN: 64.00 mg/kg *vs.* OL: 6.10 mg/kg) was higher in cashew nut compared to the oil. Other minerals were not detected in the oil. The oil demonstrated elevated amounts of vitamin E (OL: 2225.93 µg/100g *vs.* CN: 1334.02 µg/100g) and γ tocopherol (OL: 2055.12 µg/100g *vs.* CN: 1334.02 µg/100g) compared to cashew nut. Additionally, β tocopherol, γ tocotrienol, and δ tocotrienol, which were not present in cashew nut, were found in the oil. Conversely, cashew nut exhibited higher levels of total phenolics (CN: 60.45 *vs.* OL: 2.25 mg GAE (gallic acid equivalent) / 100 g) and antioxidant capacity (CN: 15.99 *vs.* OL: 9.18 uM TE /g sample) in comparison to their oil. (Supplementary Tables 1 and 2).

Participants and compliance

Among the participants initially assessed for study eligibility (n=166), 98 were included and randomly assigned to the following groups: CT (n=32), CN (n=32) and OL (n=34). Of these, 74 participants completed the 8-wk intervention, allocated as follows: CT (n=20), CN (n=25) and OL (n= 29). Of these 74 participants, six participants were subsequently excluded due to non-compliance with the prescribed diet as they gained weight. Since all participants were on a low-energy diet, weight loss was expected. Consequently, those who concluded the study with weight gain were excluded due to non-compliance, resulting in the following numbers for analysis in each group: CT (n=19), CN (n=24) and OL (n= 25) (Figure 2).

Figure 2. Consort statement of participants flow diagram.



The study population predominantly comprised females (n= 40), individuals with a completed college education/incomplete postgraduate (n= 27), white (n= 33), self-reported single marital status (n= 39), and a family income between 2 and 3 minimum wages (n= 23). Regarding lifestyle habits, the majority did not smoke (n = 65) and did not engage in regular physical activity (n = 43) (Table 2). Concerning cardiometabolic risk at baseline, 19 (79.2%), 15 (78.9%), and 23 (92%) individuals had obesity, while 13 (54.2%), 11 (57.9%), and 13 (52%) had dyslipidemia in the CN, CT, and OL groups, respectively (data not shown).

Table 2. Sociodemographic and behavioral characteristics of the total participants according to the control and intervention groups (cashew nut and cashew nut oil).

Variables	Total (n=68)	CT (n= 19)	CN (n= 24)	OL (n= 25)	p-value
Age (years)	33.31 ± 8.75	34.68 ± 9.65	33.79 ±8.39	31.80 ± 8.50	0.53
Sex:					
Male	28	10	9	9	0.49
Female	40	9	15	16	
Smoking					
Yes	3	1	1	1	0.98
No	65	18	23	24	
Physically active					
Yes	25	5	10	10	0.53
No	43	14	14	15	
Schooling					
Complete primary education / Incomplete high school	2	0	0	2	0.09
Complete high school / Incomplete college education	23	7	6	10	
Complete college education / Incomplete postgraduate	27	10	8	9	
Complete postgraduate	16	2	10	4	
Family income					
1 minimum wage	3	2	0	1	0.55
1 to 2 minimum wages	16	4	3	9	
2 to 3 minimum wages	23	7	9	7	
3 to 5 minimum wages	15	4	6	5	
5 to 10 minimum wages	7	1	3	3	
> 10 minimum wages	3	1	2	0	
Race					
White	33	8	12	13	0.92
Black	15	4	6	5	
Pardo	20	7	6	7	
Marital status					
Single	39	9	11	19	**0.02**

Married/stable partnership	27	10	13	4
Divorced	2	0	0	2

CT: control group; CN: cashew nut group; OL: cashew nut oil group.

Following the 8-wk intervention, all participants in the study demonstrated a reduction in energy intake (-205 kcal; p=0.026), indicating adherence to energy-restriction (data not shown). However, this reduction was not as substantial as expected (-500 kcal). When examining the groups individually, the control group exhibited a reduction in the intake of saturated fat (SFA), while the cashew nut group experienced a decrease in polyunsaturated fat (PUFA) and α-linolenic acid (Table 3).

Table 3. Food consumption according to 8-wk energy-restricted intervention groups

Daily Nutrient Intake	Control (n=17)			Cashew nut (n=20)			Cashew nut oil (n=23)		
	Baseline	Δ	p-value	Baseline	Δ	p-value	Baseline	Δ	p-value
Energy intake (kcal)	1667.7 ± 560.3	-301.3 ± 692.8	0.114	1663.3 ± 592.6	-32.5 ± 685.4	0.838	1670.9 ± 671.8	-284.6 ± 660.6	0.051
Protein (% EI)	20.1 ± 6.9	-1.2 ± 5.9	0.455	20.60 ± 6.3	0.7 ± 6.4	0.640	17.7 ± 6.7	1.8 ± 8.2	0.312
Carbohydrate (% EI)	44.2 ± 10.5	6.4 ± 13.1	0.078	46.1 ± 10.3	-0.3 ± 13.5	0.920	47.2 ± 8.6	-2.4 ± 12.5	0.378
Lipids (% EI)	35.8 ± 8.8	-4.1 ± 11.4	0.186	34.1 ± 8.2	-0.2 ± 13.2	0.957	35.7 ± 8.8	1.4 ± 11.7	0.572
SFA (g)	13.4 ± 4.8	-2.5 ± 4.2	**0.037**	12.4 ± 3.00	0.1 ± 6.1	0.959	11.9 ± 3.5	-0.3 ± 5.6	0.819
MUFA (g)	12.3 ± 3.7	-0.9 ± 5.8	0.540	11.5 ± 3.9	1.6 ± 5.8	0.249	12.7 ± 4.4	1.3 ± 6.2	0.332
PUFA (g)	5.6 ± 1.3	-0.1 ± 2.9	0.892	6.7 ± 3.1	-2.0 ± 3.4	**0.019**	6.2 ± 3.00	-0.2 ± 3.8	0.799
LA (C18:2n6) (g)	9.2 ± 4.2	-1.9 ± 6.3	0.240	10.3 ± 6.7	-2.7 ± 6.7	0.100	10.1 ± 6.5	-1.7 ± 8.8	0.374
ALA (C18:3n3) (g)	0.6 ± 0.9	-0.1 ± 1.1	0.696	1.2 ± 1.7	-0.9 ± 1.8	**0.037**	0.9 ± 1.1	-0.3 ± 1.2	0.324
Cholesterol (mg)	387.8 ± 276.5	-137.3 ± 262.7	0.062	341.2 ± 293.8	-31.3 ± 296.4	0.651	328.6 ± 226.5	9.7 ± 304.5	0.880
Fiber (g)	17.6 ± 11.1	1.8 ± 10.9	0.522	17.3 ± 7.2	0.9 ± 8.4	0.644	15.6 ± 7.7	0.4 ± 6.7	0.786

Δ = endpoint – baseline assessment. Values are mean ± SD (standard deviation). P-value = intra-group comparison (paired t-test). According to one-way ANOVA or Kruskal–Wallis followed by post hoc tests there was no statistical difference between groups. EI: energy intake. SFA: saturated fatty acid. MUFA: monounsaturated fatty acid. PUFA: polyunsaturated fatty acid. LA (C18:2n6): linoleic acid. ALA (C18:3n3): α-linolenic acid.

Body fat and adiposity indicators

After 8-wk of intervention, all groups presented significant reduction in body fat (kg), with consequent weight-loss (CT: -4.4%; CN: -4.1%; OL: -3.5%). The participants also had significant reduction in other adiposity indicators as: weight-loss, WC (cm), hip circumference (HC) (cm), and WHtR. Concerning WHR and body fat (%), significant losses were observed only in the control and cashew nut groups. Both intervention groups (cashew nut and oil) exhibited a significant reduction in neck circumference. No differences were found between groups, except for android fat in the endpoint between CT and OL groups (CT: 9.5 ± 1.3 %; OL: 8.1 ± 1.5 %) (Table 4). Additionally, after the intervention, there was a decrease in the number of individuals with obesity among those who consumed cashew nut (p=0.032) (Supplementary figure 3).

Table 4. Change in body fat and other adiposity indicators according to 8-wk energy-restricted intervention groups.

Outcomes		Baseline (n=68)	Endpoint (n=68)	Δ	p-value (intraindividual)
Body fat (kg)	CT	38.5 ± 7.7	35.4 ± 8.1	-3.1 ± 2.8	**< 0.001**
	CN	41.5 ± 8.9	38.2 ± 9.1	-3.3 ± 2.7	**< 0.001**
	OL	41.1 ± 7.9	39.3 ± 8.9	-1.8 ± 2.6	**0.002**
p-value (interindividual)		0.357	0.345	0.106	
Body fat (%)	CT	40.1 ± 7.8	38.5 ± 8.2	-1.6 ± 1.6	**< 0.001**
	CN	43.6 ± 7.6	41.8 ± 8.4	-1.9 ± 1.9	**< 0.001**
	OL	43.8 ± 7.6	43.2 ± 8.4	-0.7 ± 2.0	0.106
p-value (interindividual)		0.307	0.206	0.085	
Android fat (%)	CT	9.7 ± 1.3	9.5 ± 1.3 [a]	-0.2 ± 0.6	0.233
	CN	9.4 ± 3.4	8.7 ± 1.7 [ab]	-0.8 ± 2.6	0.169
	OL	8.5 ± 1.4	8.1 ± 1.5 [b]	-0.4 ± 1.1	0.072
p-value (interindividual)		0.122	**0.022**	0.579	
Gynoid fat (%)	CT	17.2 ± 1.8	17.3 ± 1.8	0.1 ± 0.7	0.463
	CN	18.9 ± 3.6	18.2 ± 1.5	-0.7 ± 3.5	0.360
	OL	17.8 ± 2.3	18.2 ± 1.9	0.3 ± 1.2	0.182
p-value (interindividual)		0.353	0.214	0.372	
Muscle mass (kg)	CT	54.3 ± 13.0	53.8 ± 12.5	-0.5 ± 1.8	0.943
	CN	50.9 ± 11.3	50.4 ± 11.4	-0.4 ± 1.7	0.241
	OL	50.2 ± 12.1	48.9 ± 11.6	-1.2 ± 2.0	**0.005**
p-value (interindividual)		0.753	0.472	0.582	
Body weight	CT	95.4 ± 17.2	91.2 ± 15.9	-4.2 ± 3.8	**< 0.001**

(kg)	CN	96.1 ± 14.8	92.2 ± 13.8	-3.9 ± 3.1	**< 0.001**
	OL	95.6 ± 14.3	92.2 ± 13.8	-3.4 ± 2.4	**< 0.001**
p-value (interindividual)		0.982	0.944	0.852	
BMI	CT	33.7 ± 3.7	32.3 ± 3.7	-1.4 ± 1.2	**< 0.001**
(kg/m²)	CN	34.1 ± 4.9	32.7 ± 4.8	-1.4 ± 1.0	**< 0.001**
	OL	33.9 ± 3.6	32.8 ± 3.8	-1.2 ± 0.8	**< 0.001**
p-value (interindividual)		0.978	0.804	0.833	
WC	CT	109.5 ± 9.5	104.4 ± 7.7	-5.1 ± 4.6	**< 0.001**
(cm)	CN	109.3 ± 11.7	105.4 ± 11.7	-3.9 ± 3.9	**< 0.001**
	OL	107.7 ± 12.1	103.9 ± 10.5	-3.7 ± 5.3	**0.002**
p-value (interindividual)		0.838	0.884	0.612	
HC	CT	113.8 ± 5.7	110.8 ± 7.0	-2.9 ± 3.0	**< 0.001**
(cm)	CN	116.6 ± 7.3	113.9 ± 7.4	-2.7 ± 3.1	**< 0.001**
	OL	116.8 ± 6.6	113.8 ± 6.2	-2.9 ± 2.3	**< 0.001**
p-value (interindividual)		0.283	0.271	0.902	
	CT	0.9 ± 0.0	0.9 ± 0.1	-0.01 ± 0.0	**0.040**
WHR	CN	0.9 ± 0.1	0.9 ± 0.1	-0.01 ± 0.0	**0.010**
	OL	0.9 ± 0.1	0.9 ± 0.1	-0.01 ± 0.0	0.264
p-value (interindividual)		0.186	0.323	0.552	
	CT	0.7 ± 0.1	0.6 ± 0.1	-0.03 ± 0.0	**0.003**
WHtR	CN	0.7 ± 0.1	0.6 ± 0.1	-0.02 ± 0.0	**< 0.001**
	OL	0.6 ± 0.1	0.6 ± 0.1	-0.02 ± 0.0	**0.003**
p-value (interindividual)		0.837	0.887	0.591	
Neck circumference (cm)	CT	40.6 ± 3.5	39.6 ± 3.5	-0.9 ± 2.5	0.104
	CN	39.9 ± 4.5	38.9 ± 3.8	-1.0 ± 1.2	**< 0.001**
	OL	38.9 ± 4.4	38.4 ± 4.1	-0.5 ± 1.2	**0.038**
p-value (interindividual)		0.438	0.555	0.238	

BMI: body mass index; HC: hip circumference; WHR: Waist-to-hip ratio; WHtR: waist-to- height ratio; CT: control; CN: cashew nut; OL: cashew nut oil. Paired t test or Wilcoxon test ($p < 0.05$ within-group); Superscript alphabets ($^{a-b}$) not indicated by the same letter in the same column means that there was a statistical difference between groups ($p < 0.05$) according to one-way ANOVA or Kruskal-Wallis followed by post hoc tests.

Cardiometabolic and liver function markers

After 8-wk intervention, all groups reduced total cholesterol and GGT. In the intervention groups, both the cashew and oil groups had reductions in apo B, while those consuming only the oil experienced reductions in LDL-c and the atherogenic index (total cholesterol/HDL-c). The control and cashew nut groups observed reductions in TG and VLDL-c. In terms of liver enzymes, the cashew nut group demonstrated reductions in AST and ALT. No differences were found between groups (Table 5).

Table 5. Change in cardiometabolic and liver function markers according to 8-wk energy-restricted intervention groups.

Biomarkers		Baseline (n=68)	Endpoint (n=68)	Δ	p-value (intra)
Cardiometabolic markers					
Triglycerides (mg/dL)	CT	176.4 ± 98.4	124.1 ± 79.2	-52.3 ± 46.8	**<0.001**
	CN	127.9 ± 58.8	96.9 ± 45.6	-30.9 ± 46.2	**0.003**
	OL	142.2 ± 89.2	120.9 ± 74.9	-21.3 ± 48.9	0.055
p-value (interindividual)		0.119	0.284	0.052	
Total cholesterol (mg/dL)	CT	194.3 ± 30.9	181.6 ± 37.2	-12.7 ± 23.9	**0.046**
	CN	186.2 ± 31.6	172.4 ± 32.4	-13.8 ± 24.4	**0.011**
	OL	207.2 ± 46.7	190.8 ± 39.8	-16.4 ± 30.4	**0.007**
p-value (interindividual)		0.133	0.266	0.996	
LDL-c (mg/dL)	CT	103.1 ± 30.3	101.6 ± 31.6	-1.5 ± 25.4	0.616
	CN	105.9 ± 30.1	100.1 ± 30.9	-5.8 ± 18.5	0.141
	OL	121.4 ± 34.7	109.9 ± 30.3	-11.5 ± 21.8	**0.016**
p-value (interindividual)		0.119	0.357	0.316	
HDL-c (mg/dL)	CT	56.1 ± 11.1	55.1 ± 12.2	-1.0 ± 6.3	0.636
	CN	54.3 ± 9.8	52.9 ± 9.1	-1.5 ± 6.7	0.216
	OL	57.4 ± 11.5	56.8 ± 13.5	-0.6 ± 5.4	0.464
p-value (interindividual)		0.735	0.694	0.895	
VLDL-c (mg/dL)	CT	35.3 ± 19.7	24.8 ± 15.8	-10.5 ± 9.7	**0.002**
	CN	25.8 ± 11.4	19.4 ± 9.1	-4.3 ± 9.8	**0.002**
	OL	28.4 ± 17.8	24.2 ± 14.9	-3.7 ± 10.2	0.055
p-value (interindividual)		0.119	0.284	0.050	
ApoA1 (mg/dL)	CT	127.9 ± 18.8	123.8 ± 17.9	-4.1 ± 10.1	0.111
	CN	120.8 ± 12.1	117.2 ± 14.4	-3.6 ± 15.7	0.123
	OL	127.0 ± 19.6	124.6 ± 22.7	-2.4 ± 12.1	0.368
p-value (interindividual)		0.583	0.543	0.912	
ApoB	CT	87.8 ± 15.1	84.4 ± 22.2	-3.4 ± 13.1	0.103

(mg/dL)	CN	84.7 ± 17.4	78.1 ± 18.3	-6.6 ± 10.7	**0.003**
	OL	93.7 ± 25.1	86.6 ± 21.3	-7.0 ± 15.3	**0.020**
p-value (interindividual)		0.291	0.322	0.980	
Cortisol (mcg/dL)	CT	13.0 ± 3.3	13.7 ± 4.7	0.7 ± 4.7	0.546
	CN	14.9 ± 6.892	14.9 ± 5.570	0.02 ± 6.4	0.753
	OL	14.4 ± 5.9	15.0 ± 5.6	0.67 ± 5.8	0.502
p-value (interindividual)		0.899	0.664	0.909	
Atherogenic indices					
Total cholesterol:HDL-c	CT	3.6 ± 0.8	3.4 ± 0.8	-0.2 ± 0.4	0.091
	CN	3.5 ± 0.7	3.4 ± 0.9	-0.1 ± 0.4	0.076
	OL	3.7 ± 0.9	3.5 ± 0.7	-0.2 ± 0.5	**0.026**
p-value (interindividual)		0.833	0.742	0.741	
LDL-c:HDL-c	CT	1.9 ± 0.6	1.9 ± 0.6	0.02 ± 0.5	0.968
	CN	1.9 ± 0.6	1.9 ± 0.7	-0.03 ± 0.4	0.394
	OL	2.2 ± 0.7	2.0 ± 0.6	-0.2 ± 0.4	0.072
p-value (interindividual)		0.297	0.757	0.310	
Liver markers					
AST (U/L)	CT	26.7 ± 12.4	22.5 ± 7.5	-4.3 ± 11.7	0.098
	CN	24.7 ± 6.5	21.5 ± 5.5	-3.1 ± 5.3	**0.007**
	OL	25.5 ± 8.9	26.5 ± 14.9	1.0 ± 11.7	0.945
p-value (interindividual)		0.884	0.825	0.400	
ALT (U/L)	CT	25.7 ± 9.7	24.1 ± 12.6	-1.6 ± 11.7	0.314
	CN	26.8 ± 14.2	20.8 ± 8.7	-6.0 ± 9.9	**<0.001**
	OL	25.9 ± 12.4	25.0 ± 14.4	-0.9 ± 7.8	0.375
p-value (interindividual)		0.924	0.671	0.240	
GGT (U/L)	CT	39.8 ± 14.8	32.8 ± 13.8	-7.0 ± 10.7	**0.005**
	CN	42.9 ± 30.9	31.4 ± 16.4	-11.5 ± 20.3	**<0.001**
	OL	39.0 ± 19.1	31.4 ± 13.9	-7.1 ± 10.8	**0.005**
p-value (interindividual)		0.746	0.690	0.902	
Alkaline phosphatase (U/L)	CT	79.2 ± 21.0	79.1 ± 17.9	-0.1 ± 7.1	0.679
	CN	81.4 ± 23.5	82.9 ± 25.1	1.5 ± 12.1	0.661
	OL	76.9 ± 21.5	79.1 ± 19.5	2.2 ± 11.1	0.277
p-value (interindividual)		0.781	0.561	0.784	

LDL-c: low-density lipoprotein-cholesterol; HDL-c: high-density lipoprotein cholesterol; VLDL-c: very-low-density lipoprotein; AST: aspartate aminotransferase; ALT: alanine transaminase; GGT: gamma-glutamyl transferase; ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; CT: control; CN: cashew nut; OL: cashew nut oil. Paired t-test or Wilcoxon test ($p < 0.05$ within-group); Superscript alphabets ($^{a-b}$) not indicated by the same letter in the same column means that there was a statistical difference between groups ($p < 0.05$) according to one-way ANOVA or Kruskal-Wallis followed by post hoc tests.

DISCUSSION

In this clinical trial, all groups demonstrated a reduction in body fat (kg) and other total adiposity (body weight and BMI) and central adiposity indicators (WC, HC and WHtR), as well as in total cholesterol and GGT. Additionally, both intervention groups (CN and OL) experienced a decrease in neck circumference and apo B, but not control group. Cashew nut group reduced liver enzymes (AST and ALT), while cashew nut oil group reduced LDL-c and atherogenic index. Furthermore, there was a reduction in the number of individuals with obesity in the group consuming cashew nut. However, no differences were found between groups.

We expected that the presence of cashew nut or cashew nut oil would exert a greater reduction in body fat, and other adiposity indicators, as well as cardiometabolic markers compared to control group. Thus, the results of this study were not consistent with our hypothesis. Several factors are crucial for contributing to weight loss, with chewing time playing a pivotal role in satiety due to its impact on neural and endocrine mechanisms. The effort involved in oral consumption and the duration spent chewing whole nuts have been linked to significant effects on satiety, the presence of fat in meals, and the stimulation of postprandial hormones such as insulin, ghrelin, CCK, PYY, and GLP-1 (Guarneiri, Paton e Cooper, 2022), which has previously been discussed by our research group (Costa *et al.*, 2021). As oil has a liquid form, its digestion and absorption are quicker, abbreviating the duration of satiety. A study showed that satiety increased after chewing whole walnuts compared to walnut butter, although gut peptide concentrations remained unchanged (McArthur, Mattes e Considine, 2018). Nonetheless, although we standardized the timing of cashew nut consumption among all participants, we did not regulate the duration of chewing, which made a detailed discussion on this aspect impossible. Thus, to gain a comprehensive understanding of the effects of cashew nut consumption on satiety in future studies, it is important to incorporate a protocol that specifies chewing duration. Previous findings from our research group indicated a decrease in ghrelin hormone levels among those who consumed a mix of nuts (30 g of cashew nuts + 15 g of Brazil nuts) compared to the control group. (Mayumi Usuda Prado Rocha *et al.*, 2023).

Our previous research supports the findings of this study concerning adiposity indicators and other cardiometabolic markers. We demonstrated that both the

control group and the group consuming a mix of nuts (30 g of cashew nuts + 15 g of Brazil nuts), alongside an energy-restricted diet for 8 weeks, experienced reductions in total and central adiposity indicators and other cardiometabolic markers, with no statistically significant differences between the groups. However, exceptions were observed in body fat (%) and VCAM-1 levels, where a statistically significant difference emerged, indicating a reduction in the group that consumed the mix of nuts compared to an increase in the control group (Caldas *et al.*, 2022).

Nuts appear to not promote an increase in adiposity markers, while the reduction of these markers is still controversy, depending on the type of nut and intervention design (Fernández-Rodríguez *et al.*, 2021; Guarneiri e Cooper, 2021). A meta-analysis has shown that almonds were able to reduce body weight and fat mass, but not waist circumference (Eslampour *et al.*, 2020). On the other hand, walnuts and cashews did not significantly modify adiposity indicators (Fang *et al.*, 2020; Jamshidi *et al.*, 2021). However, it is important to highlight that there are relatively few studies evaluating the health effects of cashew nuts compared to other nuts such as almonds, walnuts, pistachio, and peanuts (Fernández-Rodríguez *et al.*, 2021, 2022; Jamshidi *et al.*, 2021; Mejia *et al.*, 2014). Despite this, a meta-analysis presented an interesting result when comparing the duration of nut intake interventions (< 12 weeks *vs.* ≥ 12 weeks), showing sustained significance in reductions of body weight, BMI, and WC in individuals with overweight and obesity when the intervention duration was ≥ 12 weeks, in contrast to durations of < 12 weeks (Fernández-Rodríguez *et al.*, 2021). This result leads us to consider that perhaps if the duration of our study were ≥ 12 weeks, we could find differences between the intervention groups compared to the control group, especially considering our target population (individuals with overweight and obesity), since the result demonstrated by this meta-analysis was for this specific group.

While our study did not uncover any statistically significant differences among the three groups, both cashew nuts and cashew nut oil demonstrated a potential in improving cardiovascular risk. This was evidenced by a statistically significant reduction in neck circumference and apo B levels observed in both intervention groups, which was not observed in the control group. The neck circumference is an indicator of subcutaneous fat distribution (Luo *et al.*, 2017). A larger neck

circumference is suggestive of higher levels of body fat, including visceral adipose tissue, which poses a risk for cardiovascular disease (Dai *et al.*, 2016). Other studies found that daily nut consumption led to decreases in LDL-c by 4.2 mg/dL and apo B levels by 4.1 mg/dL (4% and 6% reduction in coronary events, respectively) (Gobbo, Del *et al.*, 2015). Both pistachios and almonds reduced apo B levels (Gebauer *et al.*, 2008; Jalali-Khanabadi, Mozaffari-Khosravi e Parsaeyan, 2010), while pistachios also lowered LDL-c levels (Gebauer *et al.*, 2008). A meta-analysis involving twenty-five randomized controlled trials (RCTs) along with four newer RCTs and a controlled parallel trial showed that reducing SFA intake while increasing MUFA intake leads to a reduction in plasma apoB and LDL-C. However, the findings are less consistent concerning to plasma TAG, HDL-C, apoA1, and the apoB:apoA1 ratio (Lamantia, Sniderman e Faraj, 2016).
Another important outcome of this study was the significant reduction in LDL concentrations and the atherogenic index (total cholesterol:HDL) observed in the OL-group. These markers are closely associated with cardiovascular risk(Ivanova *et al.*, 2017). LDL-c, recognized as an atherogenic lipoprotein, plays a pivotal role in the development and progression of atherosclerosis. The TC:HDL-C ratio is considered a more valuable marker for determining Coronary Heart Disease (CHD) risk, being more sensitive and specific than total cholesterol as a risk predictor (Mensink *et al.*, 2003; Stampfer *et al.*, 1991).
Cashew nut, with their high content of MUFA and PUFA and low levels of SFAs, have previously been associated with LDL-lowering effects(O'Neil, Fulgoni e Nicklas, 2015). Our study found a PUFA/SFA ratio of 1.04 ± 0.01 (Table 1). This ratio indicates the potential of a food to contribute to fat accumulation in body tissues when consumed. The Department of Health and Social Security advises avoiding edible oils with a PUFA/SFA ratio below 0.45 (Zanqui *et al.*, 2020). Therefore, the cashew nut oil used in our clinical trial is considered beneficial for the human diet and could help reduce cardiometabolic markers.
Moreover, oleic acid, the main type of MUFA present in cashew nut, is associated with better cardiovascular health, and may have contributed to the reduction of these markers. Oleic acid exhibits several protective mechanisms in vascular cells (Gómez-Hernández *et al.*, 2014). Firstly, it increases the levels of uncoupling proteins-2 (UCP-2), which are associated with vascular cell protection, preventing atherosclerosis development(Kim, H. S. *et al.*, 2007). Also,

oleic acid reduces the activation of JNK1/2, crucial for cardiovascular cells, through its anti-inflammatory action. Unexpectedly, oleic acid has been found to possess anti-inflammatory properties by preventing NLRP3 inflammasome activation (L'homme *et al.*, 2013). Furthermore, oleic acid protects against vascular smooth muscle cell (VSMC) proliferation stimulated by TNF-α, Ang II, or palmitate, thereby contributing to the prevention of atherosclerotic plaque growth (Perdomo *et al.*, 2015).

Consistent with our findings, the PREDIMED study revealed that the consumption of olive oil, particularly the extra-virgin variety, was associated with reduced risks of cardiovascular disease and mortality in individuals at high cardiovascular risk. The authors attribute these benefits to components present in extra virgin olive oil (EVOO), such as the high content of MUFAs, which are less susceptible to oxidation than other types of fatty acids. Additionally, they point to other minor components with significant biological properties, including phenolic compounds, vitamin E, and other lipid derivative molecules (such as squalene, tocopherols, and triterpene alcohols), particularly abundant in EVOO (Guasch-Ferré *et al.*, 2012). Cashew nut oil had significant amounts of both MUFA and vitamin E, whereas cashew nuts were rich in phenolic compounds. It is plausible that these components contributed to the reduction of markers associated with cardiovascular risk.

Another noteworthy outcome of the present study was the reductions in liver enzymes observed in the group that consumed cashew nut. Abnormal levels of liver enzymes have been linked to metabolic disorders such as insulin resistance and diabetes (Koike, Miyamoto e Oshida, 2010). Cashew nut contained higher amounts of magnesium, selenium, and phenolic compounds. The combination of these elements in cashew nut appears to have potentiated these findings, as some studies indicate that supplementation with magnesium, and selenium can be beneficial to the liver (Guilestad *et al.*, 1992; Liu *et al.*, 2024; Poikolainen e Alho, 2008; Sodhi, Sharma e Brar, 2006). Moreover, a study demonstrated that pecan shells contain higher levels of phenolic compounds making them an important source of antioxidants (Villarreal-Lozoya, Lombardini e Cisneros-Zevallos, 2007). The beneficial actions of phytochemicals are acknowledged for their biologically active polyphenols, such as flavonoids and phenolic acids, which

exhibit potent antioxidant activities, including the reduction of lipid peroxidation observed in liver tissue (Müller *et al.*, 2013).

Cashew nut and cashew nut oil have the potential to improve cardiometabolic markers. However, the energy-restricted diet alone has also demonstrated substantial health benefits, including weight loss, improved body composition, and lowered levels of total cholesterol and triglycerides. Therefore, when aiming to enhance health, incorporating these two foods should be complemented by a comprehensive and well-balanced eating plan, taking into account the overall nutritional quality of the diet and the bioactive compounds present in the foods. Other studies from our laboratory also demonstrated beneficial effects of nuts on health. The consumption of the mix of nuts enhanced the intestinal microbiota correlating with body fat reduction (Souza Silveira *et al.*, 2024). In this way, our research group has demonstrated some beneficial effects of Brazilian nuts (Brazil nut and cashew nut) during energy-restriction treatment. Furthermore, when we evaluated the acute effects of these nuts, we observed a reduction in oxidative stress, as evidenced by a decrease in malondialdehyde levels, which was positively correlated with the concentrations of TG, VLDL, TG/HDL, and blood pressure (Bonifácio *et al.*, 2023).

The study has some limitations. First, we find a discrepancy between the planned (-500 kcal) and reported calorie restriction (-205 kcal). This variance is a common challenge in human intervention studies, especially those in free-living condition, when individuals maintain their daily life patterns, in contrast to controlled studies conducted in laboratory settings. Additionally, during the follow-up period, some participants discontinued their participation, a common occurrence in dietary intervention studies due to challenges in altering lifestyle habits and adherence difficulties, as we can see in other randomized controlled trials (Bischof *et al.*, 2024; Choo *et al.*, 2021; Crichton *et al.*, 2024; Duarte *et al.*, 2019; Fildes *et al.*, 2014; Nijssen *et al.*, 2023).

The study's strengths include its randomized controlled design, ensuring groups with similar characteristics and reducing selection bias, thereby enhancing the study's representativeness for the target population. This design also significantly improves the study's external validity. Adherence to the study protocol was diligently monitored through regular online and face-to-face visits conducted every 15 days. The inclusion of both men and women in the study enhances the

extrapolation of results to real-life scenarios, increasing the applicability and relevance of the findings.

Our study contributes to the literature since there are few studies evaluating cashew nut compared to other nuts (e. g. almonds, walnuts, pistachio, and peanuts). Also, this was the first study to evaluate cashew nut oil on health, discerning the benefits of cashew nut arising from its lipid fraction or other non-lipid constituents.

CONCLUSIONS

Individuals in all three groups experienced reduced body weight and other indicators of adiposity over an 8-week period, with no differences between the three groups. Thus, our hypothesis regarding the potential benefits of cashew nut and cashew nut oil on body fat loss, improvements in body composition and cardiometabolic risk has not been confirmed. However, cashew nut group reduced liver enzymes, while cashew nut oil group reduced LDL-c and atherogenic index, and both the group consuming cashew nut or cashew nut oil experienced reductions in neck circumference and apo B after intervention. All these reductions were not statistically significant in the control group. Thus, the study's findings support the incorporation of cashew nut and cashew nut oil, along with an energy-restricted diet, to have a potential to improve atherogenic and liver function biomarkers in individuals with overweight or obesity. To see differences in body fat and other adiposity as well as cardiometabolic markers between the intervention and control groups, it may be necessary to provide guidance to participants on chewing time and extend the study duration to at least 12 weeks. Since this was the first study to evaluate the impact of cashew nut oil on health, further investigations, particularly focusing on the oil, are needed.

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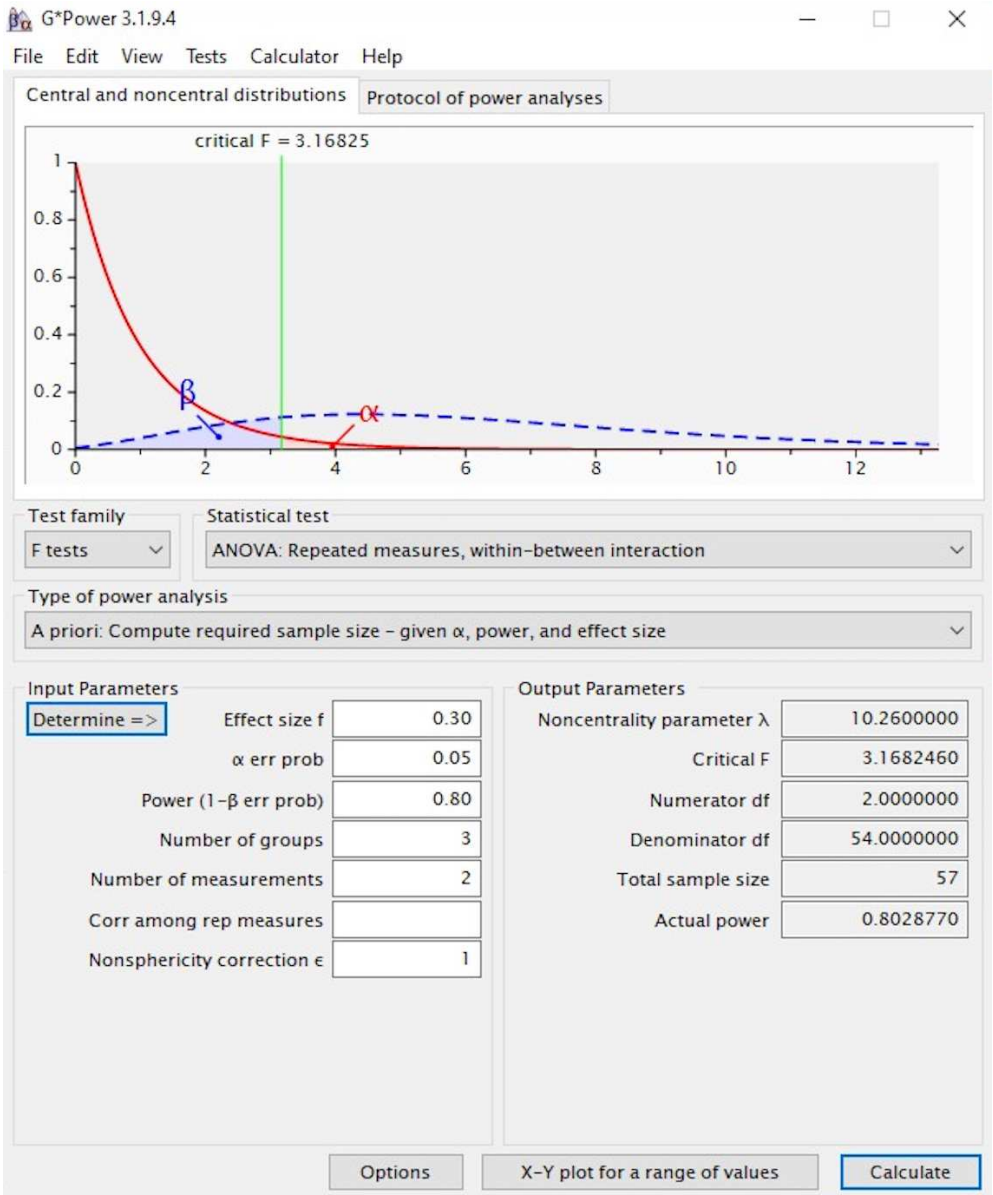
Conflict of interest

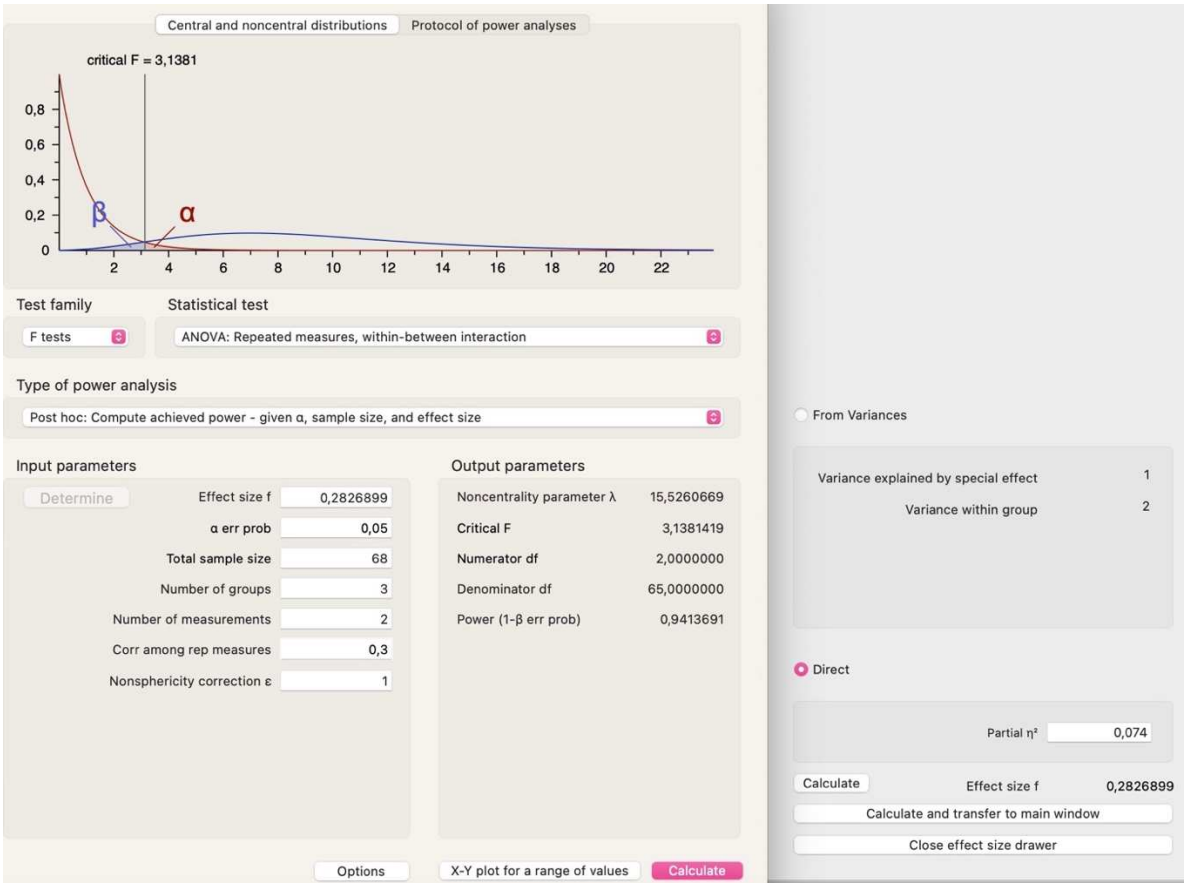
The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

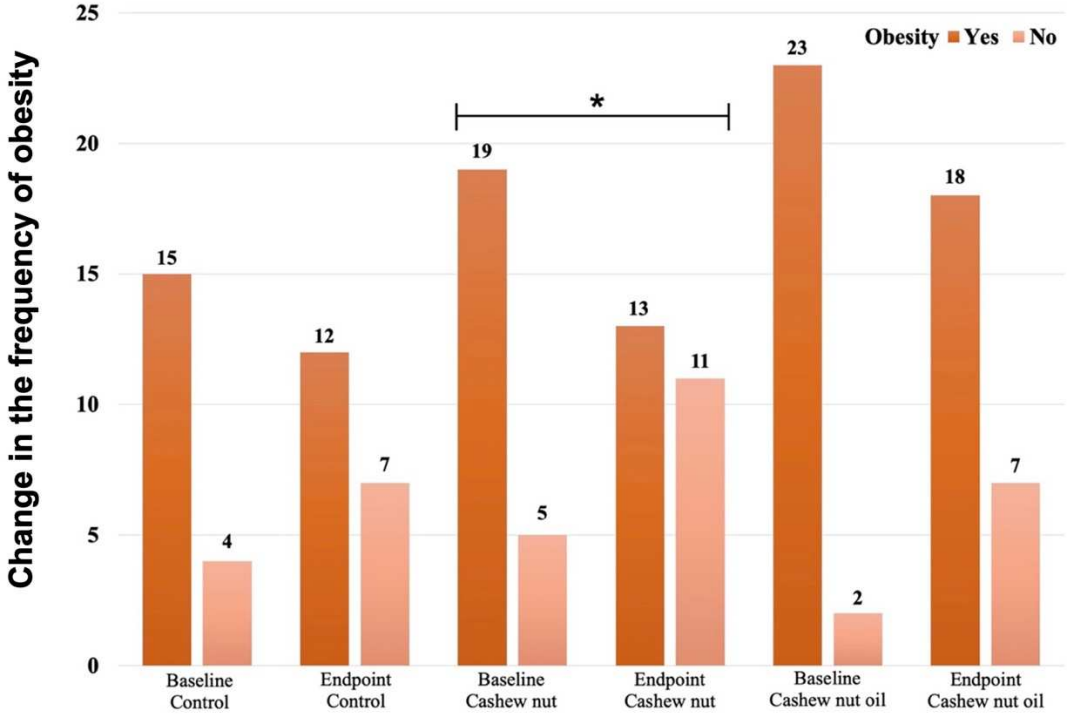
Supplementary Figure 1. Result of sample size calculation according to G*Power 3.1 program.



Supplementary figure 2. Study power calculation according to G*Power 3.1 program.



Supplementary Figure 3. Individuals with obesity according 8-wk energy-restricted groups (cashew nut, control, cashew nut oil). McNemar's test ($p < 0.05$ within-group).



Supplementary Table 1 Chemical composition, dietary fiber, minerals, amino acids, digestibility, total phenolics and antioxidant capacity of the cashew nut

Cashew nut parameters	Values
Centesimal composition	
Moisture (%)	3.18 ± 0.01
Ashes (%)	2.69 ± 0.02
Lipidis (%)	41.98 ± 0.22
Protein (%)	21.50 ± 0.51
Carbohydrates (%)	30.65 ± 0.47
Energetic value (kcal/100 g)	586.41 ± 1.11
Fibers (%)	
Total	22.36 ± 0.05
Insoluble	21.33 ± 0.40
Soluble	1.03 ± 0.45
Minerals	
Phosphorus (g/kg)	5.36 ± 0.43
Potassium (g/kg)	7.01 ± 0.43
Calcium (g/kg)	0.37 ± 0.08
Magnesium (g/kg)	2.79 ± 0.35
Selenium (g/kg)	1.71 ± 0.16
Sodium (g/kg)	0.18 ± 0.02

Copper (mg/kg)	17.33 ± 1.15
Iron (mg/kg)	64.00 ± 6.00
Zinc (mg/kg)	54.00 ± 4.00
Manganese (mg/kg)	17.33 ± 1.15
Vitamin E total (µg/100g)	1334.02 ± 150.51
γ tocopherol (µg/100g)	1334.02 ± 150.51
Amino acids (%)	
Aspartic Acid	1.99
Glutamic Acid	4.7
Serine	1.18
Glycine	0.96
Histidine	0.47
Taurine	LQ
Arginine	2.49
Threonine	0.8
Alanine	0.88
Proline	0.79
Tyrosine	0.72
Valine	1.18
Methionine	0.41
Cystine	0.42
Isoleucine	0.87
Leucine	1.57
Phenylalanine	0.99
Lysine	1.01
Hydroxyproline	LQ
Tryptophan	0.3
Sum of Total Amino Acids	21.73
Crude Protein	23.24
In vitro digestibility	86.72 ± 0.68
Total phenolics (mg GAE /100 g sample)	60.45 ± 0.86
Antioxidant capacity (DPPH) (uM TE /g sample)	15.99 ± 0.45

GAE: Gallic Acid Equivalent; TE: Trolox Equivalent. α tocopherol, β tocopherol, α tocotrienol, β tocotrienol, γ tocotrienol, and δ tocotrienol were not detected. Amounts of taurine and hydroxyproline were identified below the quantification limit.

Supplementary Table 2. Acid and peroxide index, fatty acids, total phenolics and antioxidant capacity of cashew nut oil

Cashew nut oil parameters	Values
Minerals	
Calcium (g/kg)	0.01
Iron (mg/kg)	6.1
Vitamin E (µg/100g)	2225.93 ± 234.20
β tocopherol (µg/100g)	48.92 ± 11.57
γ tocopherol (µg/100g)	2055.12 ± 195.41
γ tocotrienol (µg/100g)	26.24 ± 3.68
δ tocotrienol (µg/100g)	95.64 ± 23.54
Fatty acids (%)	
Mystic (C14:0)	0.02 ± 0.00
Palmitic (C16:0)	9.22 ± 0.01
Palmitoleic (C16:1)	0.28 ± 0.00
Margaric (17:0)	0.12 ± 0.00
Heptadecenoic (17:1)	0.05 ± 0.00
Stearic (C18:0)	8.59 ± 0.01
Oleic (C18:1n-9)	67.85 ± 0.06
Linoleic (C18:2n-6)	17.90 ± 0.02
Arachidonic (C20:4n-6)	0.73 ± 0.00
Gondoic (C20:1n-9)	0.13 ± 0.00
γ-Linolenic (C18:3n-6)	0.22 ± 0.00
Heneicosanoic (C21:0)	0.04 ± 0.00
Behenic (C22:0)	0.08 ± 0.03
Unidentified	0.35 ± 0.00
SFA	18.05 ± 0.02
MUFA	68.31 ± 0.06
PUFA	18.85 ± 0.02
PUFA/SFA	1.04 ± 0.01
Acidity level (mg KOH/g)	0.37 ± 0.03
Peroxide Index (meq/Kg)	0.83 ± 0.16
Total phenolics (mg GAE / 100 g sample)	2.25 ± 0.35
Antioxidant capacity (DPPH) (uM TE /g sample)	9.18 ± 0.81

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; GAE: Gallic Acid Equivalent; TE: Trolox Equivalent. α tocopherol, δ tocopherol, α tocotrienol, and β tocotrienol were not detected. Phosphorus, potassium, magnesium, selenium, sodium, copper, zinc, and manganese were identified below the quantification limit.

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6.4 Article 4

Consumption of cashew nut (*Anacardium occidentale* L.) improved lipid profile related with intestinal permeability, and cashew nut oil reduced an inflammatory marker in adults undergoing energy-restricted diet: a randomized controlled three-arm trial (Brazilian Nuts Study)

Talitha Silva Meneguelli[1]; Aline Lage Wendling[1]; Ana Claudia Pelissari Kravchychyn[1]; Daniela Mayumi Usuda Prado Rocha[1]; Ana Paula Dionísio[2]; Josefina Bressan[1]; Hércia Stampini Duarte Martino[1]; Elad Tako[3]; Helen Hermana Miranda Hermsdorff[1]

[1] Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil
[2] Embrapa Agroindústria Tropical - CNPAT - (Fortaleza - CE), Brazil
[3] Department of Food Science, Cornell University, Ithaca, NY 14850, USA

Corresponding Author: Helen Hermana Miranda Hermsdorff, Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, Brazil. Address: Av. PH Rolfs, s/n, Viçosa, Minas Gerais, 36570-000, Brazil. E-mail: helenhermana@ufv.br

Abstract

Individuals with obesity experience increased intestinal permeability, leading to inflammation. Although cashew nuts contain bioactive compounds capable of modulating intestinal permeability and inflammation, there is no clinical trial assessing their impact on intestinal permeability and inflammation. This 8-week randomized controlled trial aimed to evaluate the effect of cashew nut and its oil, associated with an energy-restricted diet, in intestinal permeability and inflammatory markers, as well as the relationship between change in these biomarkers. Sixty-four adults (39 women) with overweight or obesity were allocated into three groups receiving energy restriction (- 500 kcal/ day): control (CT, free-nuts), cashew nuts (CN, 30 g/d), or cashew nut oil (OL, 30 mL/d). Urine lactulose and mannitol, plasma zonulin and lipopolysaccharide-binding protein (LBP), plasma interleukins (IL-6, TNF-α, IL-10, IL-1β, IL-18 and IL-12p70) and C-reactive protein were analyzed. Increased intestinal permeability was identified as high LPB (> percentile 75) of the sample. All groups reduced adiposity indicators, without difference among groups. Only control group increased LBP after 8-wk intervention. The cashew nut group had a more significant reduction in triglycerides, VLDL, and IL-1β among those who had reduction in LBP compared to those who also consumed cashew nut but had an increase in LBP, while an increase in IL-1β was noted among those with elevated LBP levels. Additionally, the cashew nut oil group reduced IL-1β levels by 0.65 pg/mL. When compared between the groups (p-interindividual) there was no statistically significant difference between the groups regarding inflammation and intestinal permeability markers. In conclusion, as there was no difference between the groups, we cannot assert that incorporating cashew nut or cashew nut oil into an energy-restricted diet enhanced inflammatory markers and intestinal permeability. However, cashew nut exhibited potential for enhancing the lipid profile, which was amplified by improved intestinal permeability, while cashew nut oil showed a potential in reducing an inflammatory marker. Further research is needed, as studies evaluating cashew nut in these markers remain scarce.

Keywords: *Anacardium occidentale* L.; Cashew nut oil; Intestinal permeability; Inflammation; Energy-restricted diet.

Introduction

Intestinal permeability, a functional aspect of the intestinal barrier, is influenced by the integrity of the epithelial layer and it is measured by analyzing flux rates across the intestinal wall. Epithelial cells are connected by junction complexes, including tight junctions, adherents' junctions, and desmosomes (Martínez Velasco *et al.*, 2023). Tight junctions, characterized by proteins like occludins, claudins, and zonulin regulate the passage of molecules through the paracellular space (Zeisel, Dhawan e Baumert, 2019). Elevated intestinal permeability occurs when tight junctions between enterocytes fail to effectively prevent the entry of microbes or toxins into the systemic circulation (Li *et al.*, 2015).

Individuals with obesity may experience increased intestinal permeability, allowing toxins and bacteria to easily enter the bloodstream (Portincasa, Bonfrate, Khalil, *et al.*, 2022). Multiple factors, including chronic inflammation and changes in gut microbiota composition, may contribute to altered intestinal permeability among individuals in this condition (Cox, West e Cripps, 2015; Vincenzo, Di et al., 2023). The increase in intestinal permeability can lead to "metabolic endotoxemia," where bacterial components like lipopolysaccharides (LPS) bind to the lipopolysaccharide-binding protein (LBP), triggering an inflammatory response (Koutoukidis *et al.*, 2022). Interestingly, while directly measuring bacterial products like LPS in biological fluids has limitations, LBP levels are recommended as a clinical marker for evaluating "effective endotoxemia" (Gonzalez-Quintela *et al.*, 2013). Unlike other acute phase reactants, LBP increases gradually, enabling monitoring of the interaction between bacterial components, particularly LPS, and innate immune cells over time (Gonzalez-Quintela *et al.*, 2013).

Furthermore, systemic inflammation is a recognized link between obesity and conditions like insulin resistance, type 2 diabetes, cardiovascular diseases, and metabolic disorders (Koutoukidis *et al.*, 2022).

In turn, weight reduction is one of the most effective treatments for obesity (Recchia *et al.*, 2023), in addition to improving metabolic disturbances, and decreasing the systemic inflammatory tone (ClÉment *et al.*, 2004; Cottam *et al.*, 2004). Furthermore, cashew nuts have properties that contribute to weight loss and improve intestinal permeability. They are particularly abundant in monounsaturated fatty acids (MUFA), with oleic acid constituting the majority at

61.8%, in addition to containing a diverse array of essential nutrients and bioactive compounds, including dietary fibers, minerals, carotenoids, phytosterols, amino acids, and flavonoids like catechin, epicatechin, and epigallocatechin (Gonçalves *et al.*, 2023; Rico, Bulló e Salas-Salvadó, 2016; Sruthi e Naidu, 2023). Moreover, cashew nut oil appears to be promising for health, as it shares properties akin to extra virgin olive oil for being rich in MUFA, primarily oleic acid.

However, few studies have evaluated whether an energy-restricted diet also affects intestinal permeability (Netto Cândido *et al.*, 2021; Ott *et al.*, 2017). Only one study, from our research group, was found in the literature evaluating the effect of nuts on intestinal permeability (Souza Silveira *et al.*, 2024). Others from our group have evaluated the effects of these foods on body composition, endothelial function, and satiety (Bonifácio *et al.*, 2023; Caldas *et al.*, 2022; Mayumi Usuda Prado Rocha *et al.*, 2023). Besides that, there is currently no study assessing the impact of cashew nut on intestinal permeability, neither clinical trial evaluating their effects in inflammation.

Thus, this study aims to evaluate the effect of consuming cashew nut (*Anacardium occidentale* L.) and its oil, associated with an 8-wk energy-restricted diet in intestinal permeability and inflammatory markers. As a secondary outcome, we evaluated the relationship of intestinal permeability with adiposity indicators and cardiometabolic and inflammatory markers. Our hypothesis suggests that both cashew nuts and cashew nut oil have the potential to enhance intestinal permeability and inflammation in these individuals. Moreover, changes in adiposity indicators, cardiometabolic factors, and inflammatory markers after energy-restriction intervention may also contribute to the improvement of intestinal permeability, and vice versa.

Methods

Study Design

This is an 8-wk randomized controlled three-arm dietary intervention, performed at the Department of Nutrition and Health of the Universidade Federal de Viçosa (UFV), Brazil, between January 2022 and July 2022. Participants received nutritional intervention with energy restriction (-500 kcal/ day) and were allocated to one of three groups: control (CT; free nuts), cashew nuts (CN; 30 g/d), and

cashew nut oil (OL, 30 ml/d). During the intervention period, participants attended the LAMECC/ UFV on three occasions: initial and final days for blood collection, anthropometry, body composition, intestinal permeability test, and in the fourth week (30 days) for a face-to-face monitoring visit and anthropometric measurements. Between face-to-face visits, participants received online monitoring.

Ethical Aspects

The research adhered to the principles outlined in the Declaration of Helsinki, and the protocols involving human participants received approval from the Ethics Committee in Research with Human Experimentation at Universidade Federal de Viçosa (Approval No. 4.543.541/CEPH). Written informed consent was obtained from all individuals involved. The study was registered with the Brazilian Registry of Clinical Trials (ReBEC) under the ID number RBR-8xzkyp2.

Study Participants

Inclusion criteria encompassed both men and women aged 20-55, with overweight (BMI 27–29.9 kg/m²), specific waist circumference (WC) measurements (≥ 80 cm for women; ≥ 90 cm for men), and body fat percentages (> 30% for women and > 20% for men). These criteria were associated with at least one other component of metabolic syndrome (MS), such as elevated triglycerides (TG ≥ 150 mg/dL), blood pressure (≥ 130/85 mmHg), fasting blood glucose (≥ 100 mg/dL), or the use of medication to manage these markers. Alternatively, individuals with obesity (BMI ≥ 30 kg/m²), elevated WC, and body fat (%), with or without metabolic complications, were also included.

Exclusion criteria comprised pregnant, lactating, or menopausal women; athletes; vegans; those with insulin-dependent diabetes; HIV diagnosis; digestive, hepatic, renal, cardiovascular, thyroid, cancer, inflammatory diseases, and eating disorders; a history of drug and/or alcohol abuse; aversion or allergy to nuts; recent infection; habitual consumption of nuts exceeding 30 g/day; use of drugs like anti-inflammatories, corticosteroids, and antibiotics capable of causing biochemical alterations; issues interfering with chewing; weight instability (5% of usual weight) in the last 3 months; alcohol consumption exceeding 21 units (≈168 g) per week; and the consumption of vitamin, mineral, and omega-3 supplements.

Randomization

Following the initial run-in period, participants were randomly assigned through the stratified minimization method, considering gender, age, and BMI, with three levels for each factor. This approach aimed to achieve an even distribution of potential influencing factors on performance outcome variables. The randomization process utilized the MinimPy 0.3 program (Saghaei e Saghaei, 2011).

Sample size and study power

The sample size and study power were determined using the G*Power 3.1 program. For this calculation, a total of 57 volunteers were determined, based on an average estimated effect size derived from clinical studies (0.30), considering statistical analyses for three groups, two intervention points (baseline and endpoint), an alpha value set at 0.05, and a power of 0.80. By adding 20% as a result of losses during follow-up, the total sample size was determined to be 68 participants (Supplementary figure 2).

For the power of the study, the effect size of 0.27 was calculated from the Eta squared (0.068) based on the values of intestinal permeability from our database, an α of 0.05 was used, three groups, two intervention points (baseline and endpoint), and the total sample size of 64 individuals, whom we have information on intestinal permeability data. The calculation revealed a study power of 0.90 (Supplementary figure 3).

Dietary intervention

At the beginning, five energy-restricted diet options for all groups were designed and divided into five meals: breakfast, morning snack, lunch, afternoon snack, and dinner. All menus were calculated in an Excel spreadsheet using the Brazilian Institute of Geography and Statistics (IBGE) table. Energy requirements were calculated according to the Mifflin's formula (Mifflin et al., 1990). For everyone, 500 kcal were reduced from the total calculated energy requirement, considering the level of physical activity of each participant. For the interventional groups, a daily cashew nut (30 g/d) or cashew nut oil (30 ml/d) was added to the individual meal plans, and the percentage of energy from total fat was around 27-32% of the total caloric intake, while the control group had around 21%. This

quantity of cashew nut was based on previous studies that have used similar amounts, the PREvención con DIeta MEDiterránea (PREDIMED), which demonstrated beneficial effects in the improvement of blood pressure, lipid profile, lipoprotein particles, inflammation, oxidative stress, and carotid atherosclerosis (Martínez-González et al., 2015; Salas-Salvadó et al., 2011). For the cashew nut oil group, 30 ml/d was calculated to reach similar amounts of lipid between the two intervention groups.

Cashew nuts (*Anacardium occidentale* L.) and cashew nut oil were produced in Brazil, coming from the Brazilian Agricultural Research Corporation (Embrapa) Agroindústria Tropical- Fortaleza-CE (Leal et al., 2023). The nuts were sent to LAMECC already portioned in laminated packages, sealed under vacuum, and the oil was fractionated into 250 mL amber glass bottles. Both products were stored in a freezer at -20ºC until the moment of their distribution to the study participants to prevent nutrient loss, oxidation, sensory alterations, and microbiological contamination.

Outcomes

The primary outcome of the trial was body fat loss, as previously reported (paper 1). For this study, we reported the changes in intestinal permeability and inflammatory markers (secondary outcome), and their association with body composition, cardiometabolic and inflammatory markers.

Intestinal permeability markers

LBP and zonulin in EDTA plasma were assessed using human Enzyme-Linked Immunosorbent Assay (ELISA) kits (LBP, catalog n° MBS704355; Human zonulin, catalog n° MBS167049). All the measurements were conducted with a Thermo Multiskan™ FC Microplate Photometer, adhering to the manufactures' instructions. We considered increased intestinal permeability as values of LBP higher than percentile 75 of the sample, since there is not defined cut-off.

Also, the participants collected urine samples before the test, comprising all urine excreted from the last meal of the day until the test. This urine served as the control. On the subsequent day, after an overnight fast, participants consumed a solution containing lactulose (10 g), mannitol (5 g), and sucrose (20 g) in a 200 ml volume. After drinking this solution, urine was collected over a period of four

hours and thirty minutes. Participants refrained from food intake during the test, and water consumption was regulated, 150 mL after 2h and 3h of testing. The total volume of urine excreted the night before the test (control urine) and during the test (test urine) was documented, thimerosal was added (4:1, mg:mL), and the samples were stored at -20 °C until analysis. For analyses, urine was thawed, homogenized in a vortex and 2 mL of urine was withdrawn using a sterile syringe. The syringe was connected to a polyethersulfonic (PES) microporous membrane (0.22 µm × 13 mm), allowing the sample to be filtered directly into vials for HPLC. The final filtered urine volume in the vials amounted to 600 µL.

Lactulose and mannitol excretion in urine was measured using HPLC on a Dionex Ultimate 3000 Dual coupled to a Refractive Index (IR) Detector (Shodex RI-101) maintained at 40˚C. Analytes were separated on an ion exclusion column (Phenomenex Rezex ROA, 300 × 7.8 mm) at 40 °C. The mobile phase consisted of 5 mM sulfuric acid (H_2SO_4) with a flow rate of 0.7 mL/min. Internal lactulose and mannitol standards were employed to establish the normalization curve. The total urine volume collected was multiplied by the concentration of each sugar to determine the overall amount excreted in the urine. Results in the spreadsheet were expressed as the percentage excretion of mannitol (% M) and lactulose (% L), and then calculated the lactulose/mannitol (L/M) ratio.

Anthropometry and body composition

Anthropometric evaluation was conducted on the initial, midway, and final days of the intervention, while body composition was obtained at the initial and final days of the intervention. Weight was determined through the bioelectrical impedance (Inbody 230, Biospace Corp.) with a capacity of 250kg and an accuracy level of 100 g. Height was measured using a vertical millimeter stadiometer extending 2.2 meters, with a precision of 0.5 cm. Hip (HC), neck, and waist circumferences (WC) were measured using an inelastic tape with a precision of 0.1 cm. The waist circumference was measured at the umbilical height. Dual-energy X-ray absorptiometry (Lunar Prodigy Advance DXA System, GE Lunar) was employed for body composition assessment. The android region spans between the ribs and the pelvis, while the gynoid region encompasses the hips and upper thighs, overlapping with both leg and truncal regions.

Food assessment

To monitor food intake throughout the intervention, a 24-hour recall (24HR) was administered at the study's commencement and conclusion. The 24HR-ERICA software, adapted for the Brazilian population, was employed to quantify the reported intake, while nutrient analysis utilized the IBGE table (Barufaldi *et al.*, 2016; Belchior Sé Marcia Maria Melo Quintslr *et al.*, 2011).

Blood samples

Fasting (10-12 h) venous whole blood samples were collected by a registered nurse at baseline and the end of the study (8-wk) into vacuum tubes containing EDTA as an anticoagulant. Then, blood samples were centrifugated (3500 rpm, 10 min, 4°C), aliquoted and stored until analysis. The biochemical determinations were performed by the Hemolab clinical analysis laboratory (Viçosa-MG, Brazil). Then biological samples were separated and stored in an ultra-freezer at -80 ºC until the time of analysis.

Cardiometabolic markers

Blood samples were collected for evaluation of cardiometabolic risk as glucose, insulin, triglycerides, total cholesterol, LDL-c, HDL-c, VLDL-c. Also, apolipoprotein-A-1 (APO-A-1), apolipoprotein-B (APO-B); hepatic markers such as AST transaminase, gamma GT, ALT transaminase, alkaline phosphatase.

Inflammatory markers

The ultrasensitive method for assessing plasma C-reactive protein (CRP) was determined by the Hemolab clinical analysis laboratory using immunoturbidimetry, following established protocols. Interleukins (IL-6, TNF-α, IL-10, IL-1β, IL-18 and IL-12p70) were analyzed by flow cytometry in serum or plasma samples, using commercial kits (BD Biosciences®) and the data analyzed in FCAP Array Software v3.0 (BD Biosciences®).

Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (SPSS, Inc.), considering p-value < 0.05 as statistically significant. The Shapiro–Wilk test was performed to check the normality of variables. Data were presented as mean values and standard deviation. Comparisons between groups were conducted

using either one-way ANOVA with Tukey's post hoc test or the non-parametric Kruskal–Wallis test with Dunn's post hoc test. Within-group differences between baseline and post-intervention were evaluated through pairwise tests, utilizing either the paired t-test or Wilcoxon test, while independent samples t-test was employed to compare independent groups. One-way repeated measures ANOVA with group and time interaction followed by post-hoc was used to evaluate the effect of time (initial, return and final) on the following variables: weight, waist, hip and neck circumferences in the three groups. To analyze paired nominal data McNemar's test was used.

Pearson and Spearman correlations were used to investigate the association between changes (Δ) in intestinal permeability, inflammatory, cardiometabolic, and adiposity markers. Multiple linear regression models were performed to analyze the association between changes in intestinal permeability by using an LBP marker (dependent variable), and changes in both adiposity and inflammatory markers (independent variables).

It is important to highlight that throughout the entire paper, we adopted normal permeability as having LBP values below the 75th percentile (p75), while increased permeability as having LBP values above the p75.

Results

Based on the baseline characteristics of the individuals, the average age was 33.47 y ± 1.15. At the end of the study, 64 individuals completed the intervention (25 men and 39 women). There were no statistically significant differences in any variables between the groups at baseline, which demonstrates homogeneity between the groups before intervention (Table 1). There was no statistically significant difference in the number of people with normal and increased permeability before and after the intervention (Supplementary figure 1). All participants exhibited a reduction in energy intake (-205 kcal; $p = 0.026$) (data not shown). Based on the R-24h, the control group ended the study with an intake of 16.91 ± 7.31 for MUFA, 7.95 ± 4.01 for PUFA, and 16.44 ± 7.17 for SFA. In contrast, the cashew nut group consumed 24.88 ± 12.18 for MUFA, 8.59 ± 2.89 for PUFA, and 24.27 ± 14.61 for SFA. The cashew nut oil group had intakes of 21.16 ± 12.78 for MUFA, 9.34 ± 6.33 for PUFA, and 18.05 ± 11.51 for SFA (data not shown).

Table 1. Baseline characteristics of sample between groups

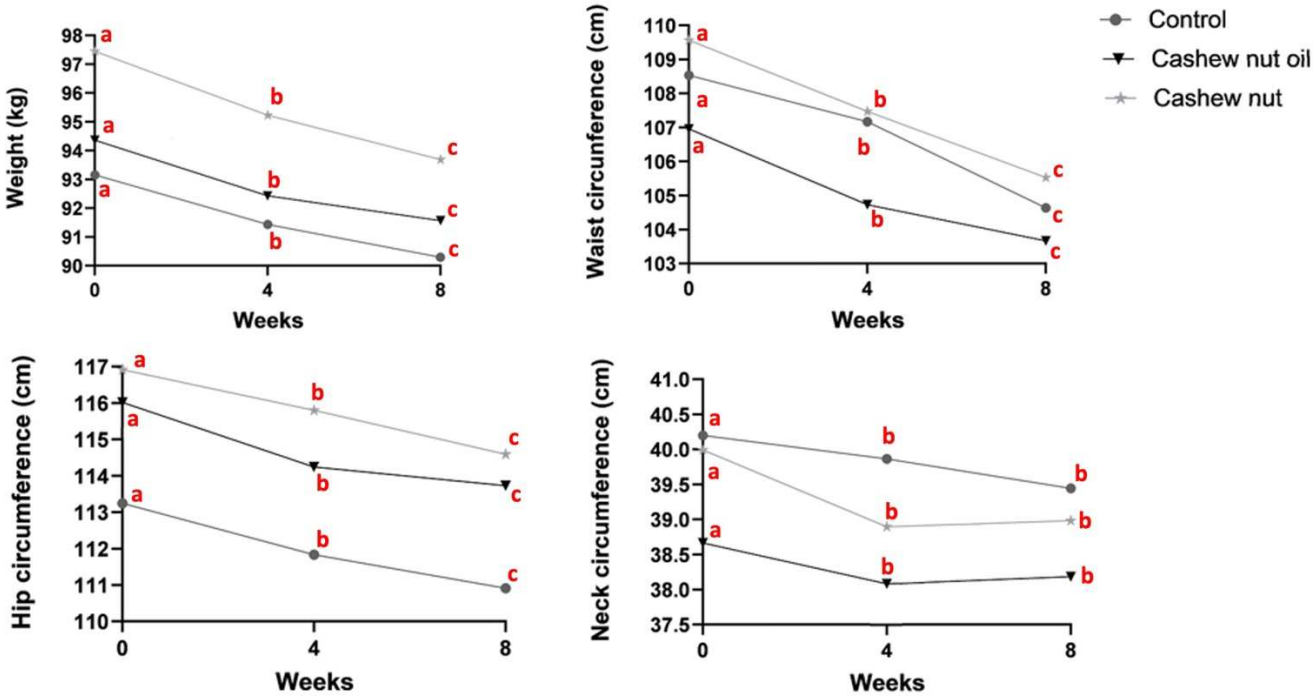
Variables	Total	CT	CN	OL	p-value
Age (years)	33.47 ± 1.15	34.18 ± 10.01	33.79 ± 8.39	32.48 ± 8.53	0.810
Sex:					
Man	25	8 (12.5%)	9 (14.1%)	8 (12.5%)	0.720
Woman	39	9 (14.1%)	15 (23.4%)	15 (23.4%)	
Smoking:					
Yes	3	1 (1.6%)	1 (1.6%)	1 (1.6%)	0.96
No	61	16 (25%)	23 (35.9%)	22 (34.4%)	
Physically active:					
Yes	22	4 (6.3%)	10 (15.6%)	8 (12.5%)	0.48
No	42	13 (20.3%)	14 (21.9%)	15 (23.4%)	
Adiposity markers:					
Weight (kg)	95.14 ± 1.89	92.63 ± 14.93	96.13 ± 14.78	95.26 ± 13.96	0.742
HC (cm)	116.21 ± 0.82	113.49 ± 5.88	116.56 ± 7.28	116.89 ± 6.24	0.226
WC (cm)	108.64 ± 1.42	108.09 ± 8.52	109.27 ± 11.74	107.64 ± 12.06	0.875
NC (cm)	39.56 ± 0.56	40.15 ± 3.47	39.89 ± 4.53	38.89 ± 4.56	0.613
Body fat (%)	43.42 ± 0.94	40.91 ± 8.18	43.63 ± 7.58	43.67 ± 7.85	0.471
Android (%)	50.69 ± 0.78	47.55 ± 7.46	48.06 ± 7.64	48.23 ± 8.47	0.965
Gynoid (%)	48.36 ± 1.04	42.95 ± 9.37	46.44 ± 9.10	48.70 ± 9.45	0.173
Android/Gynoid	1.06 ± 0.02	1.12 ± 0.16	1.06 ± 0.12	1.02 ± 0.11	0.055
Cardiometabolic markers:					
Glucose (mg/dL)	94.58 ± 1.71	97.06 ± 18.05	91.83 ± 9.15	94.83 ± 12.50	0.449
Insulin (µUI/mL)	15.44 ± 1.02	16.76 ± 6.85	17.11 ± 10.16	12.25 ± 4.96	0.073
Triglycerides (mg/dL)	146.83 ± 11.08	175.65 ± 100.15	127.88 ± 58.85	145.83 ± 91.28	0.203
Cholesterol (mg/dL)	196.42 ± 5.08	195.12 ± 31.16	186.17 ± 31.58	207.30 ± 47.81	0.172
LDL-c (mg/dL)	111.31 ± 4.26	104.58 ± 29.67	105.92 ± 30.13	120.13 ± 35.49	0.216
HDL-c (mg/dL)	55.63 ± 1.30	55.53 ± 10.90	54.33 ± 9.83	57.99 ± 11.72	0.506
VLDL-c (mg/dL)	29.47 ± 2.20	35.13 ± 20.03	25.83 ± 11.36	29.17 ± 18.25	0.215
Apo A (mg/dL)	124.35 ± 2.11	126.88 ± 18.45	120.75 ± 12.10	128.09 ± 20.03	0.299
Apo B (mg/dL)	88.93 ± 2.66	88.71 ± 15.52	84.71 ± 17.38	93.04 ± 25.22	0.373
Liver markers:					
AST (U/L)	25.77 ± 1.25	27.41 ± 12.94	24.67 ± 6.52	25.78 ± 9.21	0.663
ALT (U/L)	26.10 ± 1.63	26.47 ± 9.98	26.79 ± 14.18	25.65 ± 12.82	0.952
GGT (U/L)	39.73 ± 3.08	39.76 ± 15.68	42.92 ± 30.97	37.96 ± 19.43	0.770
Alkaline phosphatase (U/L)	79.48 ± 2.91	80.24 ± 21.95	81.38 ± 23.48	78.22 ± 21.71	0.889
Intestinal permeability markers:					
LBP	7.63 ± 0.78	6.96 ± 3.83	8.69 ± 7.62	7.39 ± 5.66	0.629
L/M	0.02 ± 0.006	0.04 ± 0.08	0.02 ± 0.02	0.02 ± 0.02	0.215

Inflammatory markers:					
CRP (mg/L)	3.02 ± 0.37	2.25 ± 1.80	3.06 ± 2.95	3.52 ± 3.49	0.396
IL-12p70 (pg/mL)	1.85 ± 0.16	1.70 ± 0.76	1.98 ± 1.62	2.00 ± 1.29	0.746
TNF-a (pg/mL)	0.41 ± 0.18	0.27 ± 1.07	0.71 ± 1.98	0.48 ± 1.67	0.712
IL-10 (pg/mL)	2.91 ± 0.32	2.58 ± 0.49	3.33 ± 3.79	2.76 ± 0.97	0.578
IL-6 (pg/mL)	4.05 ± 0.33	3.92 ± 2.15	4.15 ± 3.24	4.21 ± 2.16	0.938
IL-1β (pg/mL)	2.28 ± 0.18	2.68 ± 1.75	2.30 ± 1.46	2.16 ± 1.12	0.521
IL-8 (pg/mL)	10.84 ± 1.34	8.53 ± 3.09	13.63 ± 15.64	9.25 ± 3.56	0.203

CT: control group; CN: cashew nut group; OL: cashew nut oil group; LBP: Lipopolysaccharide-binding protein; HC: Hip circumference; WC: Waist circumference; NC: Neck circumference; LDL-c: Low-density lipoprotein-cholesterol; HDL-c: High-density lipoprotein-cholesterol; VLDL-c: Very low-density lipoprotein-cholesterol; AST: Aspartate transferase; ALT: Alanine transaminase; GGT: Gamma-glutamyl transferase; Apo: Apolipoprotein; CRP: C-reactive protein; TNF: Tumor necrosis factor; IL: Interleukin. Independent samples t-test was used for quantitative variables and chi-square for the qualitative variable.

Figure 1 illustrates the impact of three different time points: time 1 (initial), time 2 (return), and time 3 (final) on anthropometric variables such as weight, WC, HC, and neck circumference. Concerning weight, WC, and HC variables, a notable effect of the three time points (time 1, 2, and 3) was observed across all groups (control, nuts, and oil) ($p<0.001$). However, for the neck circumference there was a significant difference between time 1 to time 2 ($p < 0.001$) and time 1 to time 3 ($p < 0.001$), but not between time 2 to time 3 ($p = 0.483$).

Figure 1. Anthropometric differences between initial, middle, and final intervention days. Different letters indicate statistical significance resulting from the One-Way Repeated Measures ANOVA with group and time interaction.



When comparing outcomes related to intestinal permeability and inflammatory markers before and after the intervention, participants in the control group increased LBP after 8-wk intervention ($p = 0.045$). No other changes were statistically significant for other intestinal permeability and inflammation markers in any of the three groups, nor between groups (Table 2).

Table 2. Change in intestinal permeability and inflammatory markers according to 8-wk energy-restricted intervention groups.

Outcomes		Baseline	Endpoint	Δ	p-value (intraindividual)
LBP (ng/ml)	CT	6.96 ± 3.84	9.28 ± 5.30	2.32 ± 4.41	**0.045**
	CN	8.69 ± 7.62	8.38 ± 4.84	-0.31 ± 6.39	0.813
	OL	7.40 ± 5.66	7.77 ± 9.43	0.38 ± 8.02	0.823
p-value (interindividual)		0.629	0.795	0.447	
Zonulin (ng/ml)	CT	8.75 ± 8.51	6.53 ± 6.76	-2.22 ± 6.21	0.160
	CN	10.64 ± 8.85	8.97 ± 7.85	-1.67 ± 8.24	0.332
	OL	11.81 ± 8.30	10.79 ± 9.45	-1.02 ± 5.55	0.389
p-value (interindividual)		0.539	0.276	0.857	
	CT	0.32 ± 0.38	0.17 ± 0.17	-0.15 ± 0.42	0.140

Lactulose excretion (%)	CN	0.24 ± 0.20	0.28 ± 0.41	0.04 ± 0.47	0.698
	OL	0.33 ± 0.33	0.19 ± 0.19	-0.14 ± 0.36	0.068
p-value (interindividual)		0.559	0.465	0.248	
L/M	CT	0.04 ± 0.07	0.01 ± 0.01	-0.03 ± 0.08	0.170
	CN	0.02 ± 0.02	0.03 ± 0.06	0.01 ± 0.06	0.328
	OL	0.02 ± 0.02	0.02 ± 0.02	-0.003 ± 0.03	0.630
p-value (interindividual)		0.277	0.247	0.105	
IL-12P70 (pg/mL)	CT	1.65 ± 0.73	1.68 ± 1.11	0.02 ± 1.31	0.948
	CN	1.98 ± 1.62	2.29 ± 1.32	0.32 ± 1.19	0.206
	OL	2.09 ± 1.27	2.17 ± 1.39	0.08 ± 1.11	0.715
p-value (interindividual)		0.543	0.266	0.688	
TNF-α (pg/mL)	CT	0.24 ± 1.01	0.05 ± 0.17	-0.19 ± 1.04	0.457
	CN	0.71 ± 1.98	1.07 ± 3.08	0.36 ± 1.45	0.233
	OL	0.47 ± 1.60	0.45 ± 1.10	-0.02 ± 1.42	0.949
p-value (interindividual)		0.647	0.228	0.391	
IL-10 (pg/mL)	CT	2.87 ± 1.08	3.56 ± 3.99	0.69 ± 3.12	0.362
	CN	3.33 ± 3.79	2.82 ± 1.21	-0.51 ± 3.28	0.454
	OL	2.77 ± 0.95	2.68 ± 0.87	-0.09 ± 1.05	0.401
p-value (interindividual)		0.694	0.409	0.343	
IL-6 (pg/mL)	CT	3.72 ± 2.14	3.34 ± 1.92	-0.39 ± 2.39	0.504
	CN	4.15 ± 3.24	4.42 ± 2.87	0.27 ± 3.23	0.687
	OL	4.22 ± 2.08	4.11 ± 1.89	-0.11 ± 2.75	0.847
p-value (interindividual)		0.803	0.292	0.756	
IL-1β (pg/mL)	CT	2.75 ± 1.66	2.26 ± 1.44	-0.49 ± 1.01	0.056
	CN	2.30 ± 1.46	2.33 ± 1.41	0.03 ± 1.21	0.899
	OL	2.21 ± 1.09	2.03 ± 0.85	-0.17 ± 0.98	0.401
p-value (interindividual)		0.422	0.680	0.307	
IL-8 (pg/mL)	CT	8.70 ± 3.18	8.67 ± 2.26	-0.02 ± 3.63	0.980
	CN	13.64 ± 15.64	8.59 ± 3.16	-5.05 ± 15.63	0.127
	OL	9.30 ± 3.42	8.57 ± 3.05	-0.73 ± 4.05	0.376
p-value (interindividual)		0.186	0.988	0.189	

L/M: lactulose/mannitol ratio; LBP: Lipopolysaccharide-binding protein; CT: Control group; CN: Cashew nut group; OL: Cashew nut oil group. P-values were from paired t-test to compare before and after intervention and a one-way ANOVA to compare between groups.

We assessed changes in adiposity, inflammatory and cardiometabolic variables among individuals who experienced a decrease or increase in LBP following an

8-week intervention. The cashew nut group decreased triglycerides (-53.50 ± 55.95 mg/dL *vs.* -14.79 ± 30.61 mg/dL; p = 0.040) and VLDL (-11.30 ± 10.13 mg/dL *vs.* -2.96 ± 6.12 mg/dL; p = 0.020), being these reductions greater in individuals who decreased LBP compared to those who increased LBP. Regarding IL-1β, individuals in the cashew nut group experienced a reduction in this inflammatory marker among those who decreased LBP, whereas increased it among those who increased LBP (-0.59 ± 1.09 pg/mL *vs.* 0.48 ± 1.12 pg/mL; p = 0.029). Conversely, control group demonstrated a more substantial reduction in body fat (-1.98 ± 1.25 % *vs.* -0.61 ± 0.45 %; p = 0.018), android fat (-3.40 ± 1.51 % *vs.* -1.04 ± 0.81 %; p = 0.002), and CRP (-1.84 ± 1.94 mg/L *vs.* 1.37 ± 2.89 mg/L; p = 0.016) in the group that increased LBP compared to those with a decrease in LBP. No statistically significant difference was observed for the cashew nut oil group (Table 3).

Table 3. Changes in adiposity indicators, inflammatory and cardiometabolic markers after 8-wk according to changes in intestinal permeability

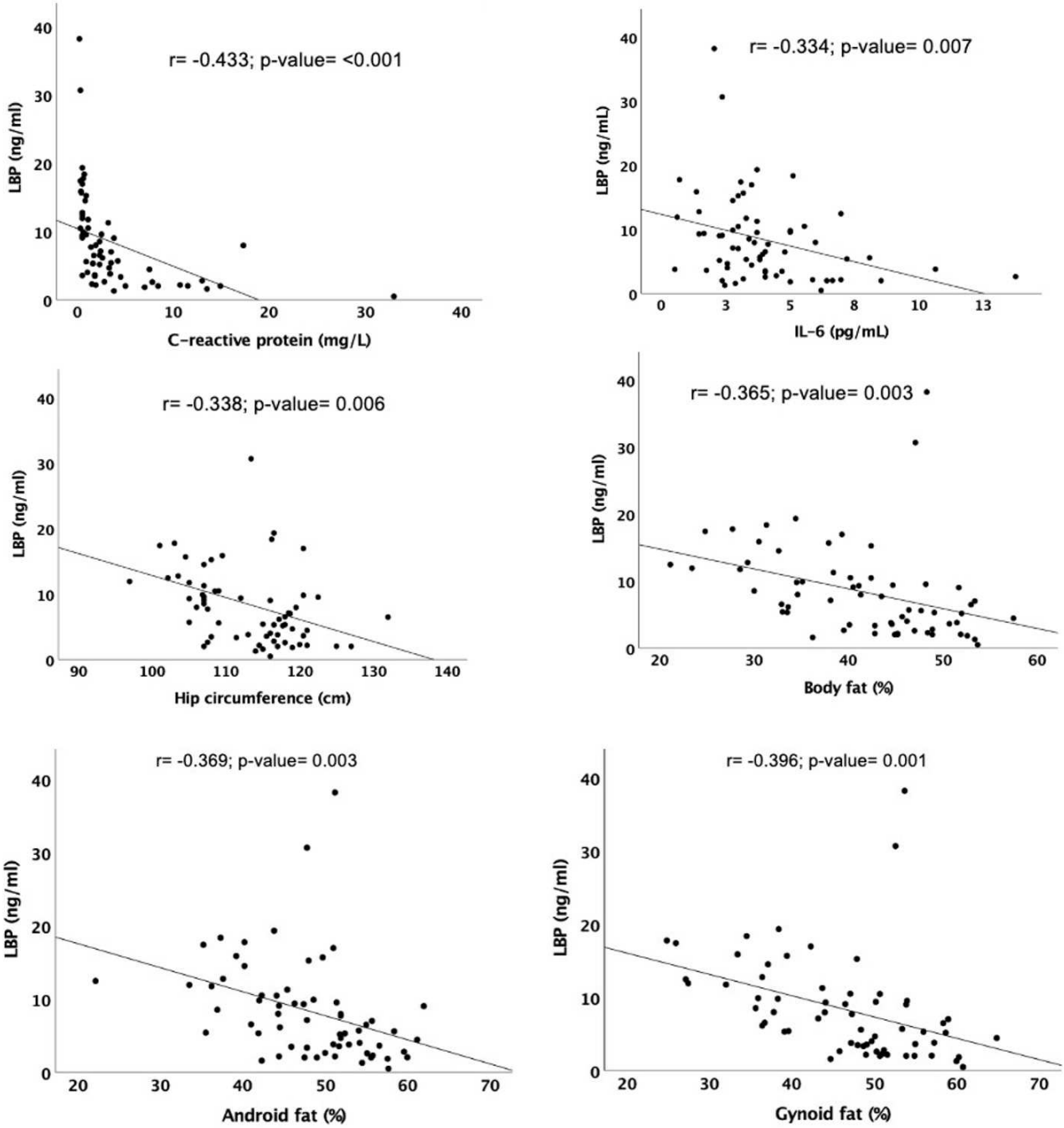
Variables	Control			Cashew nut			Cashew nut oil		
	LBP Reduction	LBP Increase	p-value	LBP Reduction	LBP Increase	p-value	LBP Reduction	LBP Increase	p-value
Δ Weight (kg)	-2.71 ± 1.74	-3.49 ± 2.32	0.452	-4.88 ± 3.07	-3.33 ± 3.01	0.238	-2.96 ± 2.04	-3.05 ± 2.22	0.923
Δ HC (cm)	-1.94 ± 1.88	-3.06 ± 3.25	0.407	-3.21 ± 3.32	-2.25 ± 3.06	0.472	-2.54 ± 2.36	-3.52 ± 1.49	0.262
Δ WC (cm)	-4.38 ± 3.50	-4.24 ± 4.77	0.950	-4.01 ± 4.75	-3.80 ± 3.36	0.900	-3.22 ± 5.98	-2.98 ± 1.92	0.925
Δ NC (cm)	0.13 ± 1.06	-1.51 ± 3.14	0.182	-1.33 ± 0.82	-0.81 ± 1.37	0.302	-0.19 ± 1.11	-1.08 ± 1.16	0.114
Δ Body fat (%)	-0.61 ± 0.45	-1.98 ± 1.25	**0.018**	-1.93 ± 2.46	-1.82 ± 1.69	0.899	-0.58 ± 2.43	-0.75 ± 0.83	0.808
Δ Android (%)	-1.04 ± 0.81	-3.40 ± 1.51	**0.002**	-3.52 ± 3.92	-2.39 ± 1.62	0.406	-1.43 ± 3.89	-0.50 ± 2.35	0.595
Δ Gynoid (%)	-1.00 ± 1.18	-2.69 ± 2.02	0.061	-1.83 ± 2.30	-2.16 ± 2.22	0.724	-0.31 ± 2.64	-0.75 ± 2.32	0.725
Δ Android/Gynoid	-3.26 ± 7.98	2.17 ± 1.63	0.098	0.38 ± 7.54	1.70 ± 2.31	0.541	2.31 ± 4.83	1.46 ± 1.67	0.681
Δ Glucose (mg/dL)	-2.50 ± 5.53	-6.11 ± 9.88	0.376	-4.90 ± 8.67	-0.86 ± 8.57	0.269	-2.35 ± 15.81	-3.17 ± 7.28	0.905
Δ Triglycerides (mg/dL)	-26.25 ± 39.97	-66.56 ± 42.06	0.062	-53.50 ± 55.95	-14.79 ± 30.61	**0.040**	-26.88 ± 51.94	-20.00 ± 44.35	0.776
Δ Cholesterol (mg/dL)	-6.00 ± 18.81	-12.33 ± 26.50	0.583	-15.20 ± 28.64	-12.71 ± 22.06	0.812	-18.00 ± 32.30	-12.83 ± 23.57	0.724
Δ LDL-c (mg/dL)	-1.88 ± 21.97	1.27 ± 29.07	0.807	-0.80 ± 19.28	-9.36 ± 17.73	0.273	-11.35 ± 21.14	-9.83 ± 18.90	0.878
Δ HDL-c (mg/dL)	0.75 ± 3.54	-0.44 ± 6.69	0.648	-3.00 ± 6.97	-0.36 ± 6.55	0.353	-1.28 ± 6.19	1.17 ± 3.43	0.372
Δ VLDL-c (mg/dL)	-5.25 ± 7.99	-13.31 ± 8.41	0.062	-11.30 ± 10.13	-2.96 ± 6.12	**0.020**	-5.38 ± 10.39	-4.00 ± 8.87	0.776
Δ AST (U/L)	-9.38 ± 16.50	0.22 ± 4.63	0.114	-4.30 ± 4.06	-2.29 ± 6.11	0.374	0.53 ± 9.79	1.83 ± 17.81	0.824
Δ ALT (U/L)	-7.13 ± 9.28	4.22 ± 12.56	0.054	-5.40 ± 7.82	-6.5 ± 11.42	0.795	0.53 ± 8.19	-5.50 ± 5.54	0.111

Δ GGT (U/L)	-5.88 ± 5.14	-6.67 ± 14.76	0.888	-6.00 ± 5.48	-15.43 ± 25.82	0.271	-5.76 ± 12.15	-11.50 ± 11.43	0.325
Δ Alkaline phosphatase (U/L)	2.50 ± 8.65	-1.22 ± 5.47	0.300	5.70 ± 11.89	-1.50 ± 11.77	0.155	4.35 ± 11.11	-4.00 ± 10.94	0.127
Δ Insulin (µUI/mL)	-5.34 ± 4.71	-2.09 ± 4.14	0.151	-1.34 ± 7.01	-3.29 ± 7.66	0.532	1.25 ± 8.87	-2.08 ± 5.33	0.399
Δ Apo A (mg/dL)	0.63 ± 7.89	-4.22 ± 8.38	0.240	-7.00 ± 13.55	-1.14 ± 17.20	0.381	-4.18 ± 12.15	1.83 ± 12.70	0.315
Δ Apo B (mg/dL)	-2.50 ± 8.73	-1.11 ± 15.95	0.830	-6.20 ± 12.88	-6.93 ± 9.42	0.874	-7.00 ± 16.62	-6.83 ± 7.70	0.982
Δ CRP (mg/L)	1.37 ± 2.89	-1.84 ± 1.94	**0.016**	0.18 ± 3.34	-0.04 ± 4.72	0.901	3.36 ± 7.55	-1.87 ± 4.51	0.128
Δ IL-12p70 (pg/mL)	0.48 ± 1.42	-0.37 ± 1.33	0.241	0.55 ± 0.99	0.15 ± 1.32	0.422	-0.01 ± 1.21	-0.04 ± 0.51	0.947
Δ TNF-α (pg/mL)	-0.52 ± 1.68	0.030 ± 0.09	0.423	0.00 ± 0.00	0.62 ± 1.88	0.238	-0.18 ± 1.52	0.54 ± 1.33	0.314
Δ IL-10 (pg/mL)	0.44 ± 0.53	-0.14 ± 0.91	0.157	-0.05 ± 0.71	-0.84 ± 4.29	0.572	0.12 ± 1.06	-0.70 ± 1.08	0.121
Δ IL-6 (pg/mL)	0.49 ± 3.02	-1.23 ± 1.82	0.179	0.34 ± 2.20	0.22 ± 3.88	0.927	0.30 ± 2.93	-1.55 ± 1.94	0.169
Δ IL-1β (pg/mL)	-0.52 ± 0.87	-0.15 ± 0.98	0.449	-0.59 ± 1.09	0.48 ± 1.12	**0.029**	-0.25 ± 1.10	0.07 ± 0.77	0.526
Δ IL-8 (pg/mL)	-0.47 ± 4.91	0.69 ± 2.44	0.546	-4.11 ± 6.18	-5.72 ± 20.11	0.809	-1.05 ± 4.71	0.69 ± 1.91	0.396

CN: Cashew nut; CT: Control; OL: Cashew nut oil. LBP: Lipopolysaccharide-binding protein. HC: hip circumference; WC: waist circumference; NC: neck circumference. CRP: C-reactive protein. LDL-c: Low-density lipoprotein-cholesterol; HDL-c: High-density lipoprotein-cholesterol; VLDL-c: Very low-density lipoprotein-cholesterol; AST: Aspartate transferase; ALT: Alanine transaminase; GGT: Gamma-glutamyl transferase; Apo: Apolipoprotein. TNF: Tumor necrosis factor; IL: Interleukin. It was used independent samples t-test or Mann-Whitney.

Correlation analyses were found between LBP, inflammatory markers (CRP and IL-6) and adiposity markers (hip circumference, body fat, android fat, and gynoid fat). LBP were negatively correlated with CRP ($r = -0.433$; $p < 0.001$), IL-6 ($r = -0.334$; $p = 0.007$), HC ($r = -0.338$; $p = 0.006$), body fat ($r = -0.365$; $p = 0.003$), android fat ($r = -0.369$; $p = 0.003$), and gynoid fat ($r = -0.396$; $p = 0.001$) (Figure 2). There was no statistically significant difference observed in LBP concerning other inflammatory, cardiometabolic, and adiposity markers (data not shown). Despite the correlation between LBP and the variables mentioned above, we performed regression analysis to evaluate causality. The results revealed no statistically significant difference between LBP and these variables: CRP ($\beta = -0.300$; $p = 0.056$), IL-6 ($\beta = -0.588$; $p = 0.084$), HC ($\beta = -0.121$; $p = 0.371$), body fat ($\beta = 1.443$; $p = 0.062$), android fat ($\beta = -0.612$; $p = 0.070$), and gynoid fat ($\beta = -1.003$; $p = 0.051$). This shows that LBP correlated with these variables, but neither the inflammatory nor adiposity markers evaluated caused a reduction or increase in LBP in our study (Supplementary table1).

Figure 2. Correlation analysis between LBP and inflammatory and adiposity markers. r = Pearson or Spearman correlation coefficient.



Another linear regression model was used to analyze the potential associations between dependent variable (IL-1β) and independent variables (body fat, LBP, some inflammatory markers, and cashew nut/cashew nut oil consumption). In this case, among all inflammatory markers, the selection of IL-1β as dependent variable was made based on the model that best fulfilled all the prerequisites of linear regression. These prerequisites included Durbin-Watson (1.607), ANOVA ($p<0.001$), which suggests that the inclusion of these predictors enhanced the model's quality, and the fulfillment of multicollinearity criteria, as indicated by tolerance > 0.1 and VIF < 10 for all variables included. Thus, each increase of one unit in the inflammatory markers TNF-α and IL-12p70 results in an IL-1β increase of 0.221 pg/mL and 0.400 pg/mL, respectively. As well as those who consumed cashew nut oil reduced IL-1β by 0.648 compared to the variable used as a reference, in this case the control group. Contrary, among those consuming cashew nuts, there was no statistically significant difference compared to the reference group (control group) (Table 4).

Table 4. Multiple regression analysis showing the contributions of body fat, LBP, and some inflammatory markers to the IL-1β (pg/mL) (dependent variable).

Variables	β coefficient	95% Confidence interval	P-value
Body fat (%)	0.004	-0.028; 0.035	0.819
LBP (ng/ml)	-0.006	-0.045; 0.032	0.741
TNF-α (pg/mL)	0.221	0.072; 0.371	**0.004**
IL-12p70 (pg/mL)	0.400	0.161; 0.638	**0.001**
CN	-0.551	-1.191; 0.090	0.090
OL	-0.648	-1.288; -0.009	**0.047**

LBP: lipopolysaccharide-binding protein; TNF: Tumor necrosis factor; IL: interleukin; CN: cashew nut group; OL: cashew nut oil group.

Discussion

In this trial, we investigated whether the consumption of cashew nuts (30g/d) or cashew nut oil (30 mL/d) has additionally benefitted changes in intestinal permeability and inflammatory markers compared to only the energy-restriction intervention for adults with overweight/obesity, and how these outcomes are related.

In this context, LPB significantly increased in the control group following an 8-wk energy-restriction intervention, whereas no significant difference was observed in the groups consuming cashew nut or cashew nut oil. Additionally, other intestinal permeability markers (zonulin, lactulose (%), and L/M ratio), as well as inflammatory markers, showed no significant differences before and after intervention. Moreover, there were no statistical differences in intestinal permeability and inflammatory markers between the groups (CT, CN, OL).

LBP is regarded as a reliable indicator of intestinal barrier dysfunction and bacterial translocation because it binds to LPS and is more stable compared to other markers, making it easier to detect (Kopp, Kupsch and Schromm, 2016). The presence of LBP in the bloodstream suggests that bacterial translocation has occurred due to a compromised intestinal barrier (Kopp, Kupsch and Schromm, 2016), which was the case of control group. Moreover, the detection of LBP in the blood may indicate not only intestinal barrier dysfunction but also the increase in inflammation, as LBP facilitates the binding of LPS with CD14 e MD-2, triggering an inflammatory response and consequently the potential for the development of chronic inflammatory conditions (Lim, Chang and Wu, 2019; (Mazgaeen and Gurung, 2020). Hence, LBP holds significant importance as a marker in assessing intestinal permeability, as its presence in the blood can offer insights into intestinal health and the risk of developing diseases linked to intestinal barrier dysfunction and systemic inflammation.

Only one clinical trial addressing the effect of nuts on intestinal permeability (by lactulose and L/M ratio) was identified in the literature, conducted by our research group as previously described (Souza Silveira *et al.*, 2024). However, no study specifically evaluating LBP or zonulin was found in the literature. We have also examined the impact of cashew nut soluble extract on gut health using an animal model, *Gallus gallus* (Meneguelli *et al.*, 2023). This study revealed no alterations in inflammatory markers (NF-κB and IL-1β), occludin (a tight junction protein), or

mucin 2 (a gene responsible for mucus production) (Meneguelli *et al.*, 2023). Nevertheless, improvements in intestinal morphological parameters and functionality were observed due to the upregulation of aminopeptidase (AP) gene expression (Meneguelli *et al.*, 2023).

Specifically, the group consuming cashew nuts exhibited decreased levels of TG and VLDL, with a more significant reduction observed in individuals who lowered their LBP levels compared to those who had an increase. LBP plays a crucial role in lipid transportation in the bloodstream, particularly for triglycerides and other lipoproteins (Hudgins *et al.*, 2003). Cashew nuts possess beneficial properties for improving lipid profiles, as evidenced by MUFA's ability to reduce triglycerides (Cao *et al.*, 2022), and have beneficial impact in lipoprotein levels, thereby reducing VLDL (Mah *et al.*, 2017). Moreover, a higher intake of monounsaturated fats can indirectly influence LBP activity by modulating its expression or efficiency in lipid transport, further contributing to the reduction of circulating TG and VLDL (Enkhmaa *et al.*, 2020). These elucidates the greater reduction in these lipids (TG and VLDL) in the group consuming cashew nuts, which was related with a greater decrease in LBP levels. Also, the group consuming cashew nuts experienced a reduction in IL-1β levels among participants who decreased LBP. LBP is involved in the inflammatory response, particularly in regulating inflammation linked with lipid metabolism and obesity (Gonzalez-Quintela *et al.*, 2013). However, there was an observed increase in IL-1β levels among participants in cashew nut group who experienced elevated LBP. Nonetheless, regression analysis indicated that LBP did not significantly influence IL-1β. Another finding was the negative correlation observed between LBP and some inflammatory variables (CRP and IL-6), as well as adiposity markers (HC, body fat, android fat, and gynoid fat). However, despite these correlations, regression analysis did not establish causality between these variables and LBP. In other words, while they are correlated, we cannot conclude that these variables directly influenced an increase or decrease in LBP, at least during the 8-wk intervention. A prolonged study duration may be necessary to observe these intrinsic changes, encompassing both the reduction in body composition and inflammatory markers associated with obesity in LBP, as well as the reciprocal relationship between LBP and inflammation.

Of particular interest was the significant reduction in IL-1β observed in the group consuming cashew nut oil, while the group that consumed cashew nuts had no statistically significant difference regarding regression analysis. Some studies have investigated the impact of walnuts and walnut oil on intestinal permeability related with inflammation. One study showed that walnut oil improved the damage score in inflamed tissue and restored colonic wall permeability in mice exposed to dextran sulfate sodium (DSS) (Bartoszek *et al.*, 2020). Another study showed that walnut supplementation protected the colonic mucosa after injury (Nakanishi *et al.*, 2019). Also, researchers analyzed fecal and colonic samples finding that the metabolic changes induced by walnut consumption may play a role in protecting against DSS-induced inflammatory tissue injury (Nakanishi *et al.*, 2019).

Some major and minor components rich in cashew nut oil may explain this reduction in IL-1β. Oleic acid, the main MUFA present in cashew nut oil, appears to have an anti-inflammatory effect. One study showed that while SFA induced the NLRP3 inflammasome activation and subsequent IL-1β release in macrophages, the oleic acid inhibited these (Karasawa et al., 2018). Furthermore, oleoylethanolamide is a bioactive lipid produced postprandially from dietary oleic acid in the small intestine and has anti-inflammatory properties (Santa-María et al., 2023), including acting in the reduction of IL-1β (Antón et al., 2017). Vitamin E, abundant in cashew nut oil, also appears to have anti-inflammatory actions, including reducing IL-1β (Lewis, Meydani e Wu, 2019). While the precise mechanisms through which dietary fatty acids inhibit cytokine production remain unclear, they may involve the suppression of the inflammatory process at the cyclooxygenase (COX) and lipoxygenase (LOX) levels (Fiorucci *et al.*, 2001).

Given the scarcity of studies examining nuts oil, making comparisons with literature findings is challenging, but data from the literature show that the effects of nuts on inflammatory markers are still contradictory, as discussed below.

When evaluating the impact of nuts on inflammatory markers in clinical trials, the majority of studies lasting 8 weeks or less did not show any changes in these markers, regardless of nut type or dosage (39-60 g/d of mixed nuts for 8-wk ; 1 unit/d of Brazil nut for 8-wk; 42 g/d of cashew nuts for 4-wk; 56 g/d of almonds for 8-wk; 30 g/d of mixed nuts for 6-wk) (Baer e Novotny, 2019; Bowen *et al.*, 2019;

Duarte *et al.*, 2019; Ghanavati e Nasrollahzadeh, 2023; Lee *et al.*, 2014). Only one 8-wk study reduced inflammation, but it used higher doses (39 - 60 g/d of mixed nuts) compared to those in our study (Ghanavati *et al.*, 2021). Studies also demonstrated reductions in some inflammatory markers in longer durations, such as 12 weeks (Corella *et al.*, 2009; Liu *et al.*, 2013; Rajaram, Connell e Sabaté, 2010; Schincaglia *et al.*, 2020b; Stockler-Pinto *et al.*, 2014), or 12 months (Casas *et al.*, 2014). However, some 12-week studies also failed to alter inflammatory markers, despite varying nut types and dosages (30 g/d or 60 g/d of hazelnuts; 59-128 g of pistachios; 30 g/d of pecan nut or 30 ml/d of extra-virgin olive oil; 30 g/d of mixed nuts; 45 to 60 g/d of peanut or almond) (Casas-Agustench *et al.*, 2011; Hou *et al.*, 2018; Sauder *et al.*, 2015; Tey *et al.*, 2013; Weschenfelder *et al.*, 2022). Similarly, a 16-week study utilizing 42.5 g/d of mixed nuts did not result in inflammation changes (Nora *et al.*, 2023). Notably, two exceptions were observed in studies evaluating Brazil nuts, where one 6-wk trial revealed reductions in inflammatory markers among individuals aged 52-75 years at risk for colorectal cancer (Hu *et al.*, 2016), and another indicated reductions after 30 days of single intake (20 or 50 g) among healthy volunteers (Colpo *et al.*, 2014). Although the studies mentioned above varied in target populations and types of nuts, it's plausible that a longer study duration or higher doses of cashew nuts could produce different results compared to those observed in our study. Therefore, extending the study period may be interesting to explore whether the cashew nut can impact inflammatory markers and alter intestinal permeability.

Our study has some limitations. Firstly, it relied only one 24-hour recall to assess food consumption. Additionally, the study participants, overweight and obese individuals, typically underestimate their food intake, further complicating the accuracy of the 24-hour recall method. Moreover, the relatively short duration of the study may have constrained our findings. A longer study duration could potentially reveal the effects of cashew nuts and cashew nut oil on inflammatory and intestinal permeability markers that were not detected in 8-wk.

As strengths, we emphasize our monitoring with the participants throughout the study. In addition to conducting in-person and online visits, we maintained an open channel for participant inquiries and regularly provided booklets and information on healthy lifestyle habits to enhance adherence. Notably, this study marks the first evaluation of a nut's impact on LBP and zonulin, with no prior

research exploring nuts' effects on these markers of intestinal permeability. Additionally, our research group is the first to investigate the health effects of consuming cashew nut oil. Finally, the inclusion of both men and women allows for the extrapolation of our study findings to an adult population grappling with overweight and obesity.

Conclusions

Incorporating cashew nut oil into an energy-restricted diet enhanced an inflammatory marker. Also, cashew nut enhanced lipid profile, which was amplified by improved intestinal permeability, while also altering inflammation in response to changes in intestinal permeability. However, our hypothesis that cashew nut and cashew nut oil would improve inflammation and intestinal permeability was denied, as there was no statistically significant difference between the three groups (p-interindividual). We believe that a longer study period could show benefits of cashew nuts and cashew nut oil compared to control in improving intestinal permeability and inflammation.

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Conflict of interest

The authors declare no conflict of interest.

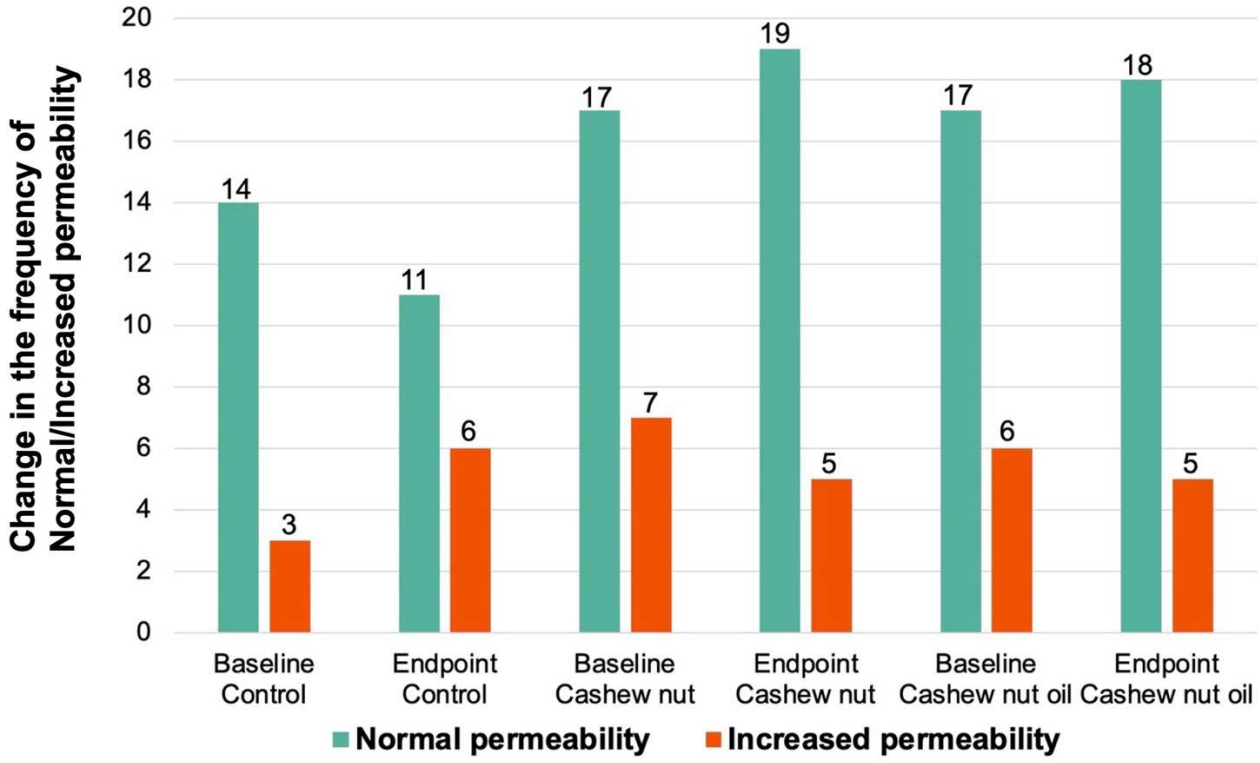
SUPPLEMENTARY MATERIAL

Sumplemmentary table 1. Multiple regression analysis showing the contributions of some inflammatory and adiposity markers to the LBP (ng/ml) (dependent variable).

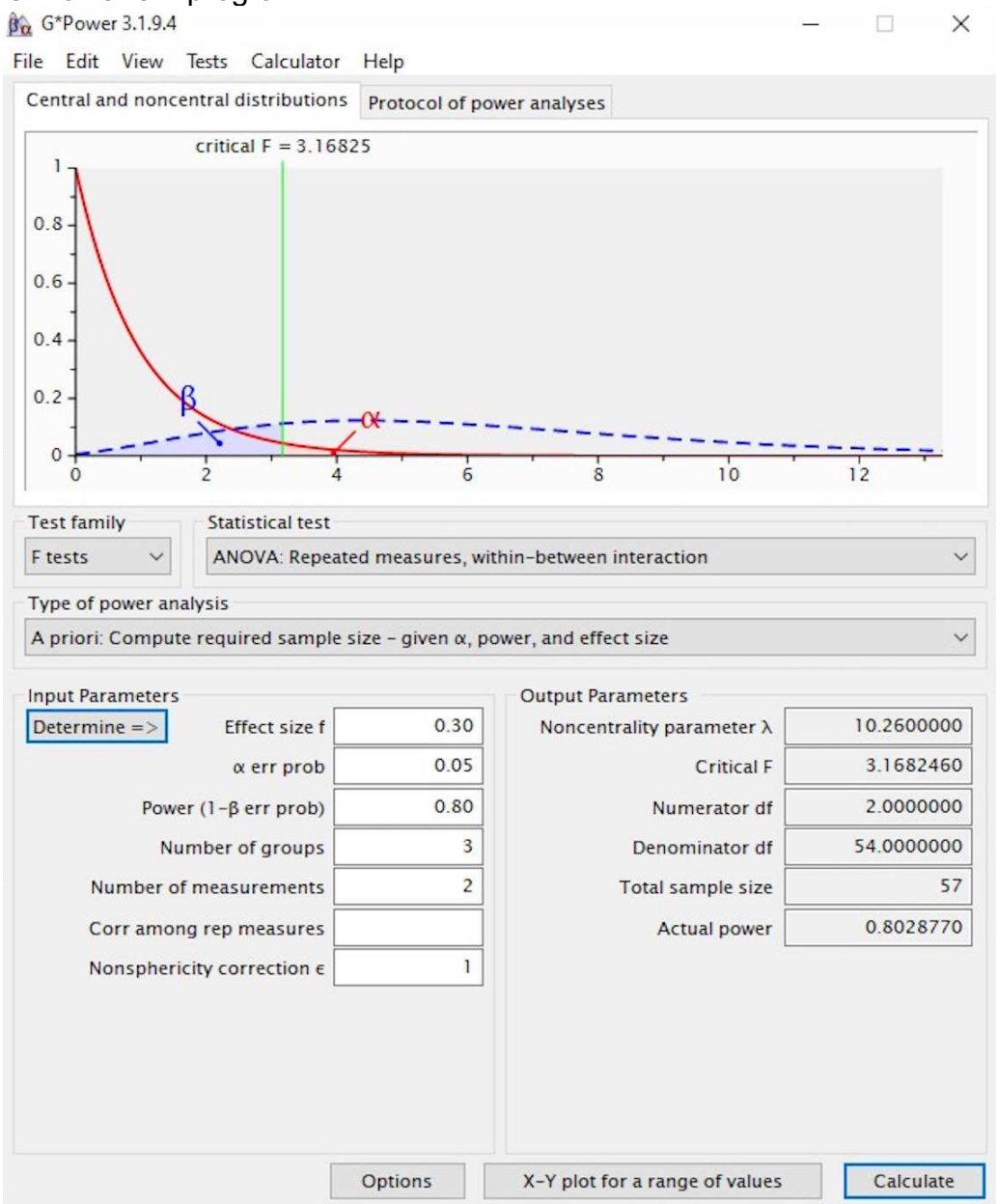
Variables	β coefficient	95% Confidence interval	P-value
CRP (mg/L)	-0.300	-0.608; 0.008	0.056
IL-6 (pg/mL)	-0.588	-1.256; 0.080	0.084
HC (cm)	-0.121	-0.391; 0.148	0.371
Body fat (%)	1.443	-0.074; 2.960	0.062
Android fat (%)	-0.612	-1.277; 0.053	0.070
Gynoid fat (%)	-1.003	-2.010; -0.004	0.051

CRP: C-reactive protein; IL: interleukin.

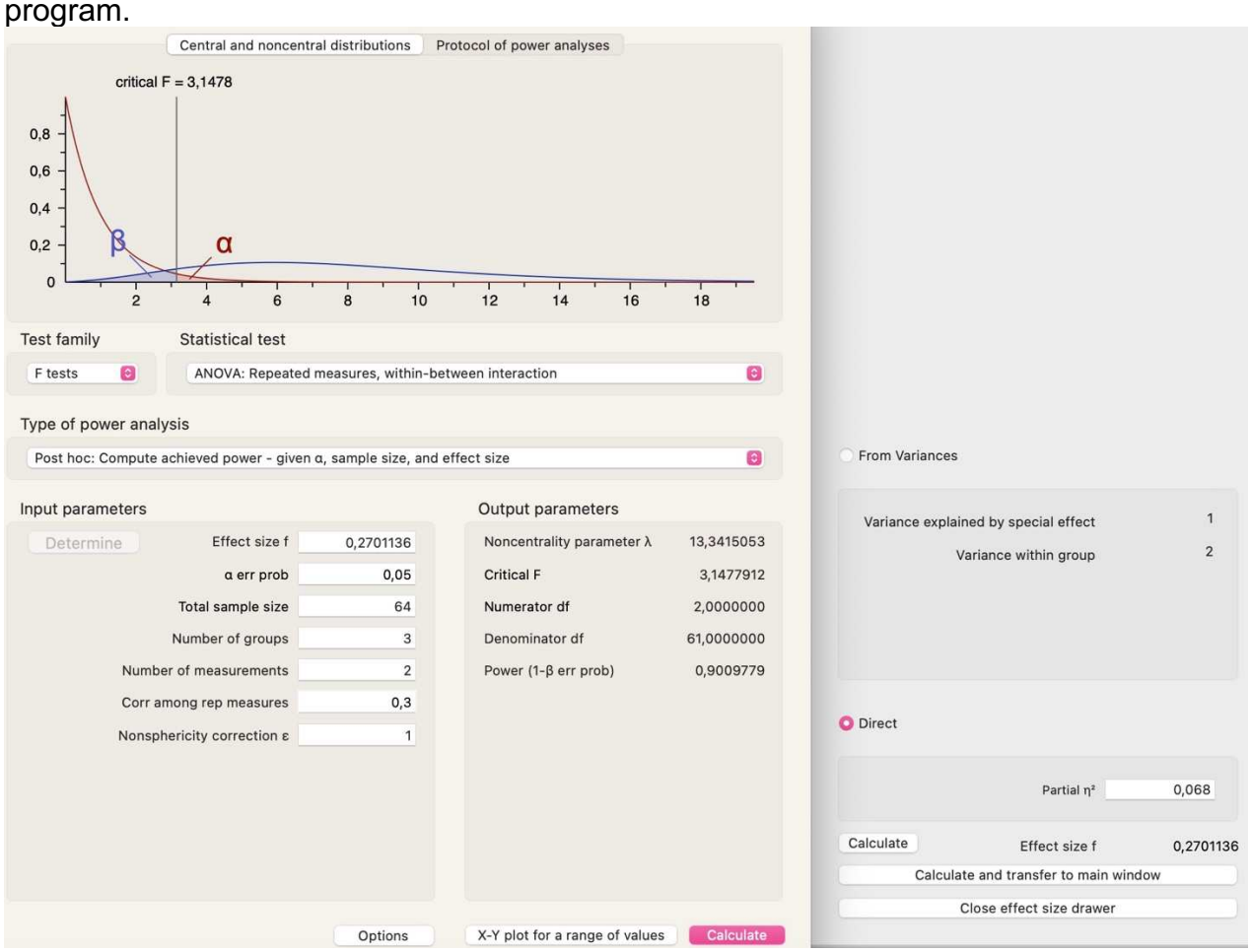
Supplementary figure 1. Individuals with normal and increased intestinal permeability according 8-wk energy-restricted groups (control, cashew nut, and cashew nut oil). McNemar's test ($p < 0.05$ within-group). Normal and increased intestinal permeability according to the p75 of LBP (values at baseline and endpoint: 9.47 and 11.09 ng/ml).



Supplementary Figure 2. Result of sample size calculation according to G*Power 3.1 program.



Supplementary figure 3. Study power calculation according to G*Power 3.1 program.



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7. DISSERTATION CONCLUSIONS

According to the experimental study (*Gallus gallus* model), we can conclude:

1. CNSE improved duodenal morphological parameters. Related to microbiota, a 5% concentration of CNSE showed lower abundance of *Bifidobacterium* compared to control groups, *E. coli* compared to the H_2O group, and *Lactobacillus* compared to all other groups. For *L. plantarum*, the intervention groups (1% and 5%) had higher abundance compared to the H_2O group and lower abundance than the no-injection group. Regarding the gene expression of intestinal barrier proteins (OCLN, MUC2, AP and SGLT-1) and inflammatory biomarkers (NFκβ1 and IL-1β), there was a reduction in MUC2 in the H_2O, 1% and 5% CNSE groups compared to the no-injection group, and an upregulation in AP in the 5% CNSE group compared to the 1% CNSE group. CNSE had a higher protein content compared to cashew nut flour but a lower amount of total fiber, insoluble, and soluble compared to cashew nut flour. Hence, the 5% concentration demonstrated superior efficacy, particularly concerning the expression of genes associated with the brush border membrane and intestinal morphology. However, there is a compelling rationale to explore concentrations exceeding 5%, given that concentrations surpassing this threshold would likely remain within the acceptable limits of osmolarity.

According to the clinical trial developed in this dissertation, we can conclude:

1. Our hypothesis regarding the potential benefits of cashew nut and cashew nut oil on body fat loss, improvements in body composition and cardiometabolic risk has not been confirmed since there was no difference between the groups (control, cashew nut, and cashew nut oil). However, the group that consumed cashew nut reduced the number of individuals with obesity and serum liver enzymes levels (AST and ALT). The group consuming cashew nut oil improved markers related to cardiovascular risk by reducing LDL-c and the atherogenic index. Both intervention groups, cashew nuts and cashew nut oil, reduced neck circumference and apoB levels. In terms of physical-chemical composition, cashew nut presented higher amounts of total phenolics and antioxidant capacity, while cashew

nut oil presented greater amounts of vitamin E and its fractions (tocopherol and tocotrienol).

2. Our hypothesis regarding the potential benefits of cashew nut and cashew nut oil on intestinal permeability and inflammation has not been confirmed since there was no difference between the groups. However, cashew nut oil demonstrated a reduction in an inflammatory marker through a linear regression, while cashew nut consumption improved the lipid profile, which was further enhanced by enhanced intestinal permeability. Additionally, the group that consumed cashew nut showed a decrease in IL-1β levels among those with reduced LBP levels, whereas an increase in this cytokine was observed among those whose LBP levels increased. Notably, only the control group exhibited increased intestinal permeability, as measured by LBP, which was not observed in the intervention groups.

Therefore, although our research has already revealed a health promising potential related to cashew nut and cashew nut oil consumption regarding certain markers as mentioned above, no statistically significant differences were observed between the groups (p-interindividual), which meant that our hypothesis was denied. Thus, it remains premature to say that the incorporation of cashew nut and cashew nut oil yields significant benefits for body composition, cardiometabolic markers, gut health, and inflammation. Therefore, further studies are necessary to validate this claim. We propose that an extended study duration, lasting at least 12 weeks, may provide additional benefits. While our investigation delved into various fractions of cashew nut, including their by-products such as soluble extract and oil, more studies are necessary to investigate the mechanisms related to aqueous or lipid fraction. Thus, our study stands as a pioneering in evaluating these cashew nut fractions in terms of health, laying the groundwork for future research endeavors that continue to delve into these byproducts. This approach will allow for a deeper comprehension of the benefits and mechanisms linked to each fraction of cashew nuts across diverse health parameters.

APPENDIX A: INFORMED AND VOLUNTARY CONSENT FORM

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

O (a) Sr. (a) está sendo convidado(a) como voluntário(a) a participar da pesquisa “Efeito da amêndoa da castanha de caju e seu óleo sobre marcadores de risco cardiometabólico, genéticos e da microbiota intestinal em indivíduos com excesso de peso”. Nesta pesquisa nós pretendemos avaliar o efeito de uma dieta reduzida em calorias associada ao consumo diário de amêndoas da castanha de caju (um pacote de 30 g/dia) ou do seu óleo (600mL/semana) sobre a perda de peso e potenciais benefícios à saúde em indivíduos com excesso de peso e risco cardiometabólico. O motivo que nos leva a estudar as amêndoas da castanha de caju e o seu óleo é o valor nutricional desses alimentos e seus potenciais benefícios para a saúde. Esse estudo terá duração de nove semanas e a coleta dos dados será realizada ao início, durante e ao final do estudo.

O (a) Sr. (a) será orientado quanto ao preenchimento de três registros alimentares de 24 horas (R24h), em dois dias da semana e um dia de final de semana, para avaliação do consumo habitual. Em seguida, o (a) Sr. (a) participará de um período de avaliação com duração de uma semana para a avaliação dos possíveis participantes e sua capacidade de adesão ao protocolo de pesquisa. Nesta fase o (a) Sr. (a) deverá manter sua dieta habitual com restrição ao consumo de qualquer tipo de amêndoa, frutos secos tipo berries (cranberry, blueberry, gopjiberry e uvas passas), açaí, cacau, canela, azeite de oliva e bebidas alcoólicas. Ao final deste período, o(a) Sr.(a) será pesado. Caso haja variação no peso corporal superior a ± um kg ou caso haja o consumo de algum dos alimentos e/ou bebidas restringidos, o(a) Sr.(a) não poderá dar seguimento à pesquisa.

Para participar do estudo, o(a) Sr.(a) deverá comparecer ao Laboratório de Metabolismo Energético e Composição Corporal (LAMECC-UFV) do Departamento de Nutrição e Saúde da UFV em duas ocasiões, nas quais permanecerá no local de 6:30 horas às 12 horas, que ocorrerão no início do estudo e após oito semanas de seguimento da dieta. Os procedimentos da pesquisa para esses dois dias serão descritos a seguir, bem como os seus

respectivos riscos e desconfortos e as medidas a serem tomadas para minimizar tais:

I) **Dieta e consumo de amêndoa da castanha de caju ou do seu óleo:**

Procedimento: Ao longo desse período o (a) Sr. (a) seguirá uma dieta reduzida em calorias prescrita por nutricionista, a qual poderá ou não ser acompanhado da ingestão da amêndoa da castanha de caju ou do seu óleo, de acordo com sorteio realizado pelos pesquisadores.

Possíveis riscos: Contaminação pela ingestão dos alimentos.

Medidas a serem tomadas para redução dos riscos: Para minimizar qualquer tipo de contaminação por microrganismos das amostras fornecidas serão realizadas a avaliação da qualidade microbiológica, sendo analisadas e asseguradas via laudos pela empresa fornecedora. Após serem entregues as amêndoas estas serão imediatamente porcionadas em embalagens adequadas e armazenadas em freezer a -20˚C até o momento de sua distribuição aos voluntários, evitando assim perda e oxidação de nutrientes, alterações sensoriais e contaminação microbiológica. Além disso, todo o material para consumo será manipulado na cozinha do LAMECC, e os manipuladores seguirão todas as normas higiênico-sanitárias, incluindo uso de jaleco limpo, toucas, máscara e luvas descartáveis.

II) **Coleta de material biológico:**

Procedimento: Nas duas visitas (início e final), o (a) Sr.(a) comparecerá ao laboratório às 6:30h após realizar jejum noturno de 10 a 12 h, trazendo uma amostra de urina e uma de fezes. Um técnico de enfermagem capacitado coletará uma amostra do seu sangue (20 mL) e aferirá sua pressão arterial por meio de monitor de braço automático Omron. Em seguida, o (a) Sr. (a) fará o teste de permeabilidade intestinal, que consiste na ingestão de uma bebida contendo açúcares e a coleta de toda a urina até 12h.

Possíveis riscos: O(a) Sr.(a) poderá sentir uma sensação incômoda ou dolorida na hora de inserir a agulha, bem como náuseas e tonturas, ou ainda poderá formar hematomas no local da entrada da agulha algumas horas após o teste.

Medidas a serem tomadas para redução dos riscos: a coleta de sangue será realizada por um técnico em enfermagem ou enfermeiro experiente contratado pelo estudo, utilizando apenas materiais descartáveis e estéreis. Ademais, caso

sinta qualquer desconforto, o(a) Sr.(a) receberá assistência imediata e adequada pelos pesquisadores.

III) **Avaliação da composição corporal**:

Procedimento: Após a coleta do material biológico, será realizada medida de composição corporal no aparelho de bioimpedância e DEXA (absorção de raios-X de dupla energia).

Possíveis riscos: O uso da bioimpedância elétrica para avaliação da gordura corporal é desaconselhado a pessoas que possua marca-passo ou quaisquer aparelhos eletrônicos semelhantes, pinos, placas metálicas e gestantes. Em relação ao DEXA é desaconselhado a realização em gestantes. Devido ao jejum prolongado poderá haver risco de mal-estar, como fraqueza, letargia, dores de cabeça, tontura ou náuseas.

Medidas a serem tomadas para redução dos riscos: Caso o(a) Sr.(a) possua algumas dessas restrições descritas acima não será realizado os exames de composição corporal. Na ausência dessas restrições, a realização desses exames não está associada a prejuízos à saúde. Em caso de qualquer mal-estar devido ao jejum o (a) Sr. (a) deverá reportar imediatamente a equipe qualquer indisposição e imediatamente o procedimento será interrompido e a nossa equipe prestará socorro. No entanto, para minimizar este risco, estes exames serão realizados em sequência um após o outro para que o (a) Sr.(a) não fique muito tempo em jejum e imediatamente após a realização desses exames será oferecido um lanche.

IV) **Avaliação antropométrica:**

Procedimento: Em seguida, o (a) Sr.(a) realizará medidas do peso, altura, circunferência da cintura, do quadril e do pescoço.

Possíveis desconfortos: Durante a aferição das medidas de cintura, quadril, pescoço e peso corporal, o(a) Sr.(a) poderá se sentir constrangido ou desconfortável.

Medidas a serem tomadas para redução de qualquer desconforto: a avaliação antropométrica será realizada em uma sala fechada, silenciosa, na presença do pesquisador devidamente treinado.

V) **Aferição da pressão arterial pela MAPA:**

Procedimento: Após as medidas antropométricas, será colocado um aparelho de monitorização ambulatorial da pressão arterial (MAPA) no braço do(a) Sr.(a), e deverá utilizá-lo durante as 24 horas seguintes, ininterruptamente, sem retirar para tomar banho ou dormir. Após estas 24 horas, o(a) Sr.(a) deverá comparecer novamente ao LAMECC para retirar o aparelho MAPA, o que levará apenas 5 minutos. O aparelho MAPA será programado para fazer medições da pressão arterial a cada 20 minutos durante o dia, e a cada 30 minutos durante o período da noite. Para que a medida seja feita, é necessário que o braço esteja parado e relaxado ao longo do corpo.

Possíveis desconfortos: Em algumas pessoas, em função da repetição das medidas nas 24 h, a região onde o manguito será colocado poderá ficar arroxeada ou um pouco irritada.

Medidas a serem tomadas para redução de qualquer desconforto: será realizada medição do perímetro do braço para que seja fornecido o tamanho adequado do manguito para cada indivíduo. Ademais, não há contraindicações para a realização deste exame.

Ainda, durante as oito semanas do estudo, o(a) Sr.(a) deverá comparecer ao LAMECC a cada quinze dias para visitas de monitoramento (pré-agendadas), totalizando 3 visitas de 20 minutos cada.

I) Questionários:

Procedimento: Durante as visitas de monitoramento, como parte dos procedimentos da pesquisa, o(a) Sr.(a) responderá a questionários estruturados contendo perguntas sobre sua história clínica e socioeconômica, hábitos alimentares, nível de atividade física, comportamento frente ao alimento e qualidade do sono, com tempo de aplicação previsto em uma hora.

Possíveis desconfortos: Durante a aplicação dos questionários, o (a) Sr. (a) poderá se sentir entediado ou constrangido com alguma pergunta.

Medidas a serem tomadas para redução de qualquer desconforto: o(a) Sr. (a) poderá deixar de responder qualquer pergunta caso sinta-se constrangido, sem que isso traga qualquer alteração na relação de tratamento por parte dos pesquisadores, e realizar pausas entre um questionário e outro.

Quanto aos benefícios da pesquisa, o(a)Sr. (a) receberá gratuitamente a avaliação do seu estado nutricional e um plano alimentar individualizado por um

nutricionista, visando a redução de peso corporal. Durante o estudo (8 semanas), o(a) Sr.(a) receberá consultas individuais (20 minutos) com nutricionista para monitoramento e ajustes na dieta. Além disso, o(a) Sr.(a) poderá receber amêndoas/óleo de amêndoas para consumir em casa e esses alimentos estão associados a benefícios à saúde e prevenção de doenças. Ainda, o(a) Sr.(a) terá acesso aos seus dados de avaliação antropométrica, composição corporal e exames bioquímicos ao finalizar o estudo. Ao final do estudo, o Sr. (a) receberá um novo plano alimentar individualizado, visando a manutenção de hábitos alimentares saudáveis e a adequação dos marcadores bioquímicos que se apresentarem fora dos níveis de normalidade. Caso necessário, os pesquisadores irão ressarcir todos os gastos que o (a) Sr. (a) tiver com deslocamento necessários aos dias de comparecimento ao LAMECC, referentes a pesquisa. O valor total será ressarcido ao final do estudo, e, ou, até final da participação no estudo e será equivalente ao número de visitas realizadas multiplicado por dois para corresponder ao trajeto de ida e volta, multiplicado pelo valor do transporte coletivo (ônibus) vigente no período da pesquisa.

Para participar deste estudo o (a) Sr. (a) não terá nenhum custo, nem receberá qualquer vantagem financeira. Apesar disso, diante de eventuais danos, identificados e comprovados, decorrentes da pesquisa, o a) Sr.(a) tem assegurado o direito à indenização. O(a) Sr.(a) tem garantido plena liberdade de recusar-se a participar ou retirar seu consentimento, em qualquer fase da pesquisa, sem necessidade de comunicado prévio. A sua participação é voluntária e a recusa em participar não acarretará qualquer penalidade ou modificação na forma em que o (a) Sr. (a) é atendido pelos pesquisadores.

Os resultados da pesquisa estarão à sua disposição quando finalizada. O (a) Sr. (a) não será identificado em nenhuma publicação que possa resultar desse estudo. Seu nome ou material que indique sua participação não serão liberados sem a sua permissão.

Este termo de consentimento encontra-se impresso em duas vias originais, sendo que uma será arquivada pelo pesquisador, no LAMECC-UFV e a outra será fornecida ao (à) Sr. (a). Os dados e instrumentos utilizados na pesquisa ficarão arquivados com o pesquisador por um período de “cinco anos” após o término da pesquisa. Depois desse tempo, os mesmos serão destruídos. Os pesquisadores tratarão a sua identidade com padrões profissionais de sigilo

e confidencialidade, atendendo à legislação brasileira, em especial, à Resolução 466/2012 do Conselho Nacional de Saúde, e utilizarão as informações somente para fins acadêmicos e científicos. Eu,_____,contato_____,fui informado (a) dos objetivos da pesquisa **"Efeito da amêndoa da castanha de caju e seu óleo sobre marcadores de risco cardiometabólico, genéticos e da microbiota intestinal em indivíduos com excesso de peso"** 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.

Pesquisadores:

Dra. Helen Hermana Miranda Hermsdorff (Coordenadora) – 31 3612-5195

Dra. Josefina Bressan – 31 3612-5611

Brenda Kelly Souza Silveira - 31 3612-5220

Alessandra da Silva - 31 3612-5220

Talitha Silva Meneguelli - 31 3612-5220

Endereço: Av. P.H. Rolfs s/n. Laboratório de Metabolismo Energético e Composição Corporal (LAMECC), Departamento de Nutrição e Saúde, Centro de Ciências Biológicas II, sala 50, 6˚ andar. Campus Universitário. Viçosa/MG.

Telefone: (31) 3612-5220

E-mail: castanhasbrasileiras@gmail.com

Em caso de discordância ou irregularidades sob o aspecto ético desta pesquisa, o (a) Sr. (a) poderá consultar:

CEP/UFV – Comitê de Ética em Pesquisa com Seres Humanos
Universidade Federal de Viçosa
Edifício Arthur Bernardes, piso inferior
Av. PH Rolfs, s/n – Campus Universitário
Cep: 36570-900 Viçosa/MG
Telefone: (31) 3612-2316
E-mail: cep@ufv.br
www.cep.ufv.br

Viçosa,_____de_____de 20____.

_____ _____

Assinatura do Participante Assinatura do Pesquisador

APPENDIX B: PRESENTIAL SCREENING QUESTIONNAIRE

ESTUDO
castanhas brasileiras - Fase 3

QUESTIONÁRIO: TRIAGEM PRESENCIAL (Folha 1/3)

I. IDENTIFICAÇÃO

Data de aplicação: ____/____/____ Pesquisador responsável: _____

Nome:_____ Data de nascimento: ____/____/____

Idade: _____ **Se idade < 20 anos ou > 55 anos, exclusão**

E-mail: _____ Endereço: _____

Complemento: _____ CEP: _____ Fone: ( )_____/ ( ) _____

II. ANTROPOMETRIA E COMPOSIÇÃO CORPORAL

Peso usual (kg): _____	Peso atual (kg): _____	Ganho/perda 5% peso (3 m): ( ) NÃO**( ) SIM - exclusão**
Altura (m): _____	IMC (kg/m2): _____	27 kg/m^{2}I< IMC: ( ) NÃO **( ) SIM - exclusão**
PC umbilical (cm): _____	PQ (cm): _____	PC < 80 cm M / < 90 cm H: ( ) NÃO **( ) SIM - exclusão**
PPescoço (cm): _____		
Gordura (%): _____	Gordura (kg): _____	GC ≤ 30% M / ≤ 20% H : ( ) NÃO **( ) SIM - exclusão**
TMB (Kcal/dia): _____	MM (kg): _____	
Água (L): _____	Água (% peso): _____	

III. PRESSÃO ARTERIAL E EXAMES BIOQUÍMICOS

PAS (mmHg): _____	PAD (mmHg): _____	>130 e/ou >85 mmHg ou medicação:
Medicamento para HAS: ( ) NÃO ( ) SIM		( ) NÃO **( ) SIM - tem fator de risco**
Triglicerídeo (mg/dL): _____	Medicamento (sinvastatina): ( ) NÃO ( ) SIM	≥150 mg/dL ou medicamento: ( ) NÃO **( ) SIM - tem fator de risco**
Glicemia (mg/dL): _____	DM: ( ) NÃO **( ) SIM, tipo 1 ou tipo 2 - exclusão** Glicemia > 100 mg/dL? ( ) NÃO **( ) SIM - tem fator de risco**	

Apresenta pelo menos mais um fator de risco além do excesso de peso?

( ) NÃO - EXCLUIR ( ) CONFIRMAR, **solicitar exames**, e continuar triagem

IV. HISTÓRIA CLÍNICA

1.	Você fuma ou já fumou?
	( ) Não fumante
	Ex Fumante há:
	( ) mais de 1 ano **( ) < 1ano - exclusão**
	( ) Fumante - exclusão
2.	Você é atleta, pratica crossfit ou faz atividade física intensa/vigorosa? ( ) NÃO **( ) SIM - exclusão**
3.	Você é gestante ou está amamentando ou está na menopausa? ( ) NÃO **( ) SIM - exclusão**
4.	Você planeja engravidar num futuro próximo (nos próximos 6 meses)? ( ) NÃO **( ) SIM - exclusão**
5.	Você tem algum problema de dentição ou mastigação (próteses, aparelho)? ( ) NÃO **( ) SIM - exclusão**
6.	Pretende fazer alguma cirurgia nos próximos 3 meses, mesmo que seja (ex: retirar amídalas ou dente siso, etc)? ( ) NÃO **( ) SIM - exclusão**

ESTUDO

castanhas brasileiras - Fase 3

QUESTIONÁRIO: TRIAGEM PRESENCIAL (Folha 2/3)

7. Atualmente, você faz uso de algum medicamento (incluindo anticoncepcionais)? Qual (is)?Liste também os medicamentos

Medicamentos	Posologia		Tempo de uso	Razão para o uso
	Dosagem	Frequência		

8. Você já apresentou ou têm alguma das seguintes doenças?

Doenças/Alterações	NÃO	**SIM**
Alergia (ex. cutânea, respiratória, alimentar, outras)		
Anorexia/Bulimia		
Bronquite/Asma		
Câncer		
Doença cardiovascular (ex. ataque cardíaco, derrame, insuficiência coronariana, insuficiência cardíaca congestiva, AVC, infarto agudo do miocárdio, etc)		
Doença celíaca, doença intestinal crônica, alterações no TGI (ex. retirada de vesícula)		
Doença renal		
Doenças/ alterações neurológicas ou psiquiátricas (ex. esquizofrenia, transtorno de ansieda-de, depressão)		
Hipoglicemia		
HIV/Aids		
Tireoidopatias (ex. hipo ou hipertireoidismo)		
Outras doenças crônicas (que afetem o estudo)		

V. HISTÓRIA DIETÉTICA

1. Você apresenta alergia, intolerância ou aversão a alimentos como, castanhas, leite e derivados, como creme de leite, macarrão e outros: ( ) NÃO **( ) SIM - exclusão**

2. Você pratica algum tipo de restrição alimentar de cunho religioso (promessa): ( ) NÃO **( ) SIM - exclusão**

3. Você consome castanhas: : ( ) NÃO ( ) SIM, esporadicamente **( ) SIM, todos os dias (>30g) - exclusão**

4. Você está seguindo alguma dieta, restrição ou faz acompanhamento nutricional: ( ) NÃO **( ) SIM - exclusão**

5. Você faz uso de suplemento (vitaminas, minerais, ômega 3, proteína ou outros?): ( ) NÃO **( ) SIM - exclusão**

6. Qual quantidade e frequência do consumo de bebidas alcóolicas?

Bebida	**Unidade (s) / Volume (mL)**	**Frequência**	**g álcool**
Cerveja pilsen (3,6%)			
Cachaça (31,1%)/Caipirinha (16%)			
Vinho (14%)			
Whisky (40%)			
Vodka (40%)			
Outro? Qual?			

( ) Consumo de álcool < 23g/dia **( ) Consumo de álcool >= 23g/dia - exclusão**

ESTUDO

castanhas brasileiras - Fase 3

QUESTIONÁRIO: TRIAGEM PRESENCIAL (3/3)

7.	**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.**											
	AM (manhã e início da tarde)											
	1	2	3	4	5	6	7	8	9	10	11	12
	___	___	___	___	___	___	___	___	___	___	___	___
	PM (dia e noite)											
	1	2	3	4	5	6	7	8	9	10	11	12
	___	___	___	___	___	___	___	___	___	___	___	___

II. DADOS SOCIOECONÔMICOS E DEMOGRÁFICOS

1. Escolaridade:

( ) Analfabeto - exclusão	( ) Sabe ler e escrever
( ) Fundamental Incompleto	( ) Fundamental Completo
( ) Médio Incompleto	( ) Médio Completo ( ) Técnico
( ) Superior Incompleto	( ) Superior Completo
( ) Pós-graduação Incompleto	( ) Pós-graduação Completo

( ) Profissão: _____

2. Raça

( ) Branca ( ) Preta ( ) Parda ( ) Amarela ( ) Indígena ( ) Outro

3. Estado civil:

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

4. Renda Familiar	( ) R$ 3.300,01 –5.500,00 (3 a 5 salários)
( ) Até R$ 1.100,00 (1 salário)	( ) R$ 5.500,01 – 11.000,00 (5 a 10 salários)
( ) R$ 1.100,01 – 2.200,00 (1 a 2 salários)	( ) R$ 11.000,01 –22.000,00 (10 a 20 salários)
( ) R$ 2.200,01 – 3.300,00 (2 a 3 salários)	( ) R$ 22.000,01 ou mais (> 20 salários)

Quantas pessoas dependem dessa renda (incluindo o voluntário)? _____

5 Controle Menstrual

Qual foi a data da sua última menstruação? ____/____/_____ Sua menstruação é regular? ( ) Sim ( ) Não

APPENDIX C: BOOKLET

ESTUDO

castanhas brasileiras

Equipe de Nutricionistas:

Dr. Josefina Bressan (Coordenadora)

Dr. Helen Hermana Miranda Hermsdorff (Coordenadora)

Talitha Silva Meneguelli (Doutoranda)

Aline Lage Wendling (Doutoranda)

CONTEÚDO

Você agora faz parte do ESTUDO CASTANHAS BRASILEIRAS

Durante esse período é importante que você siga algumas orientações:

- Mantenha o mesmo nível de atividade física habitual, incluindo atividades domésticas.
- Utilize óleo de soja no preparo das suas refeições (com moderação).
- Sucos, chás, cafés devem ser adoçados com o adoçante de sua preferência (não utilize açúcar nessas preparações).

Ainda, alguns alimentos NÃO DEVEM SER CONSUMIDOS durante esse período:

- Abacate.
- Azeite de oliva, óleo de dendê, óleo de coco.
- Bebidas alcoólicas de qualquer tipo.
- Castanhas, nozes e amendoim (somente se a equipe do estudo orientá-lo sobre esse consumo).
- Suplementos vitamínicos e minerais ou ômega 3.

Durante esse período, também, não fume.

Sabemos da importância, mas, nesse período não é aconselhável a doação de sangue.

Em caso de alteração na sua alimentação, atividade física e hábitos diários, por favor, nos avise. Também nos comunique em caso de surgimento de doenças, alergias e gripes, e mudanças na medicação.

ANOTAÇÕES:

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COMO FUNCIONA O ESTUDO CASTANHAS BRASILEIRAS

DURAÇÃO: 8 SEMANAS

Durante esse período, você será acompanhada por seu Nutricionista com objetivo de promover a perda de peso. **Serão três tipos de contatos:**

Consultas com Nutricionista	**Contatos não presenciais** por e-mail, SMS, *Whatsapp*, telefone	**Visita ao laboratório**: Coleta de urina; Coleta de sangue; Preenchimento de questionários
		

Durante o estudo, você comparecerá ao laboratório nas seguintes ocasiões:

Dia 1: Coleta de sangue e Teste de permeabilidade intestinal. Nesse dia, você comparecerá ao laboratório LAMECC em jejum e fará a coleta de sangue, após permanecerá por 4 horas e 30 minutos coletando urina e respondendo questionários. Também será aferido o peso e as medidas de cintura, quadril, pressão arterial, etc. Ao final da manhã, será fornecida uma refeição.

Dia 2: Exame de Densitometria Óssea (DEXA). Nesse dia, você comparecerá em jejum na Divisão de Saúde-UFV para a realização do exame DEXA (previamente agendado). O exame tem duração aproximada de 20 minutos.

Dia 3: Consulta de monitoramento. Após 30 dias, você deverá comparecer ao LAMECC em horário previamente agendado para consulta nutricional que tem duração de 15 a 30 minutos.

Dias 4 e 5: Ao final das 8 semanas, serão repetidos os dias 1 e 2, para realização de um novo teste de permeabilidade intestinal, nova coleta de sangue e novo exame DEXA.

EM TODAS AS OCASIÕES, LEMBRE DE TRAZER ROUPAS LEVES PARA REALIZAÇÃO DA PESAGEM!

NA NOITE ANTERIOR AOS DIAS DE TESTE DE PERMEBILIDADE INTESTINAL E COLETA DE SANGUE, VOCÊ DEVERÁ CONSUMIR REFEIÇÕES EQUILIBRADAS:

Escolha uma das três opções para o jantar (20h):

1) Sanduíche de Frango com Queijo

2 Fatias de Pão de forma OU 1 Pão Francês

3 Colheres de sopa de peito de frango OU atum

À gosto: alface, tomate, cenoura, beterraba, pepino, cebola, etc.

1 Fatia de queijo muçarela ou 2 Colheres de sopa de queijo cottage OU ricota

Acompanhamento: água (com ou sem gás) com limão espremido.

2) Sopa ou Caldo de Legumes com Carne

2 Conchas médias de Carne cozida em pedaços

2 Cenouras

2 Batatas inglesa pequenas

1 Abobrinha

1 Cebola

1 Tomate

Salsinha e cebolinha à gosto

Preparo: Lave os vegetais, corte em cubos e adicione em uma panela com água já fervendo, até cobrir. Quando os vegetais estiverem cozidos, amasse um pouco com o garfo para encorpar o caldo. Acrescente a carne e a cebola, deixe ferver mais 2 minutos. Desligue e sirva com salsinha e cebolinha à gosto. Rende 2 a 3 porções

3) Arroz, feijão, vegetais e proteínas

1 Colher de servir de arroz (80g)

1 Concha P de feijão (100g)

1 Concha de legumes cozidos

1 Pegador de legumes crus

1 Bife grelhado OU 1 omelete com 2 ovos

⇨Monte o prato equilibrado, conforme a imagem. Use o mínimo de óleo no preparo.

Ao final dessa cartilha, você encontra receita para o preparo do arroz, do feijão e da carne.

ESTUDO
castanhas brasileiras

ORIENTAÇÕES PARA COLETA DE SANGUE

JEJUM (10—12 HORAS)

- ☑ Você deverá comparecer ao laboratório em jejum de 10 a 12h.
- ☑ Nesse período será permitido consumir apenas água e/ou remédios.
- ☑ Recomendamos que sua **última refeição** seja consumida entre **20:00 - 22:00 horas**.
- ☑ OBS.: NÃO ULTRAPASSE MAIS DE 14 HORAS DE JEJUM.

VOCÊ NÃO DEVE CONSUMIR NAS ÚLTIMAS 48 HORAS (2 DIAS) ANTES:

- ☑ Açaí
- ☑ Bebidas alcoólicas
- ☑ Cafeína (café, chás de topos os tipos, refrigerante, chocolate, achocolatado)
- ☑ Frutas secas (damasco, ameixa seca, uva passas, cranberry e outros), uva, laranja e suco de frutas natural
- ☑ Castanhas (nozes, amêndoas, castanha do caju, amendoim e outros)
- ☑ Termogênicos (pimenta, canela)
- ☑ Suplementos vitamínicos e minerais

ATIVIDADE FÍSICA NAS ÚLTIMAS 24 HORAS (1 DIA) ANTES:

- ☑ Você não deverá realizar atividade física ou esforço físico intenso no dia anterior (24 horas) antes da coleta de sangue.

PERÍODO MENSTRUAL:

- ☑ Você não poderá estar no período menstrual na data da coleta de sangue.

ROUPAS LEVES:

- ☑ Por favor, venha ou traga roupas leves (short/bermuda e camiseta) no dia da coleta de sangue.

*** EM CASO DE IMPREVISTOS, POR FAVOR, NOS AVISE COM MÁXIMA ANTECEDÊNCIA PARA AGENDARMOS COM OUTRAS VOLUNTÁRIAS.**

ORIENTAÇÕES PARA COLETA DE URINA

VOCÊ DEVERÁ SEGUIR OS SEGUINTES PASSOS PARA COLETA DE URINA:

- ☑ Primeiro, esvazie a bexiga, mesmo que não esteja com vontade, imediatamente após o consumo da sua última refeição. Essa urina deverá ser desprezada (não será coletada).
- ☑ A partir daí, toda vez que for ao banheiro, coletar a urina em copo descartável e armazenar na garrafa fornecida pela nossa equipe. (Se você levantar a noite para ir ao banheiro, lembre-se que essa urina também deverá ser coletada).
- ☑ No dia seguinte, continuar a coleta de urina, incluindo a primeira urina do dia e as subsequentes até a chegada ao laboratório.
- ☑ **IMPORTANTE: armazenar a urina na geladeira.**
- ☑ Lembre-se de trazer a garrafa contendo a urina para o laboratório no dia da coleta de sangue.

ORIENTAÇÕES PARA COLETA DE FEZES

- ☑ Antes de coletar as fezes, se necessário, urinar no vaso sanitário para evitar a contaminação do material.
- ☑ Evitar também a contaminação com a água do vaso sanitário.
- ☑ Coletar uma amostra das fezes, em recipiente fornecido pela nossa equipe (pote estéril com tampa).
- ☑ Armazenar as amostras de fezes congeladas (caixa de isopor com gelo) até o momento da entrega no laboratório - LAMECC
- ☑ A entrega das amostras de fezes no laboratório deverá ser feita em até 24 horas.

OBS: horário de funcionamento do laboratório: 08:00—11:40h e de 14:00 às 17:40h. Se necessário, agende com nossa equipe um melhor horário para entrega do material.

ANOTAÇÕES:

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LIVRO DE RECEITAS

ESTUDO CASTANHAS BRASILEIRAS

Receitas do dia a dia para um consumo equilibrado e saudável de sal (sódio) e gorduras nas suas refeições

ARROZ BRANCO

Ingredientes:

Alimento	Quantidade
Arroz Cru	1 xícara (180g)
Água	1 ½ xícara (360mL)
Alho	3 dentes (6g)
Sal	2 colheres de café rasas (2g)
Óleo Vegetal	½ colher de sopa (4g)

Modo de preparo:

1) Ferva a água em uma panela separada.
2) Refogue o alho no óleo.

3) Junte o arroz e o sal e refogue mais um pouco.

4) Junte a água fervendo e deixe em fogo baixo, em panela tampada por aproximadamente 10 minutos.

Rendimento: 3 porções

FEIJÃO COZIDO

Ingredientes:

Alimento	Quantidade
Feijão Cozido com Caldo	2 conchas médias (180g)
Água	½ xícara (120mL)
Cebola	1 colher de sopa (10g)
Alho	2 dentes (4g)
Sal	2 colheres de café rasas (2g)
Óleo Vegetal	½ colher de sobremesa (2mL)

Modo de preparo:

1) Cozinhe o feijão sem tempero, e reserve
2) Refogue cebola, alho e sal no óleo .

3) Junte o feijão cozido .

4) Acrescente água e deixe ferver até engrossar .

Rendimento: 2 porções

CARNE GRELHADA

Ingredientes:

Alimento	Quantidade
Carne Magra sem Gordura	1 filé médio (120 g)
Cebola	½ colher de sobremesa (5g)
Alho	1 dente (2 g)
Sal	1 colheres de café rasas (1g)
Óleo Vegetal	1 colher de sobremesa (5mL)

Modo de preparo:

1) Tempere a carne com sal .

2) Refogue no óleo as cebolas picadas e o alho .

3) Junte o filé, cozinhe e sirva .

Rendimento: 1 porção

LEGUMES COZIDOS

Ingredientes:

Alimento	Quantidade
Batata Crua em Cubos	1/3 xícara (50g)
Cenoura Crua em Cubos	1/3 xícara (40g)
Vagem Crua	3 unidades (30g)
Água	Suficiente para cobrir
Sal	1 colheres de café rasas (1g)

Modo de preparo:

1) Lave os legumes em água corrente.

2) Descasque a batata e a cenoura. Pique os legumes.

3) Coloque na panela com água fervente, acrescentando sal.

4) Cozinhe até os legumes ficarem al dente.

5) Escorra a água e sirva.

Rendimento: 2 porções

UFV
Universidade Federal de Viçosa

PPGCN

LAMECC
Laboratório de Metabolismo Energético e Composição Corporal

APPENDIX D: MENU MODEL FOR CASHEW NUT GROUP

ESTUDO
castanhas brasileiras

PLANO ALIMENTAR

DIETA 01

Valor calórico total: 1500Kcal

Nome: _____

REFEIÇÃO	ALIMENTOS	MEDIDA CASEIRA	QUANTIDADE
Café da manhã	Pão integral	1 fatia	25g
	Requeijão Light	1 ponta de faca	6g
	Queijo minas	1 fatia	45g
	Café sem açúcar	1 xícara de café	50ml
	Mamão	1 banda	155g
Colação	Banana	1/2 unidade	37,5g
	Leite de vaca desnatado	1/2 copo americano	75ml
	Castanha de caju	**1 porção Individual**	**30g**
Almoço	Arroz	3 col de sopa	75g
	Feijão	2 col de sopa	34g
	Peito de frango sem pele grelhado	1/2 unidade média	90g
	Abobrinha cozida	2 col de sopa cheia picada	60g
	Tomate	4 fatias	60g
	Alface	3 folhas	30g
	Couve-flor cozida	2 ramos pequenos	120g
	Cenoura cozida	1 unidade pequena	55g
	Beterraba	1 col de sopa	16g
	Abacaxi	1 fatia	75g
Lanche da tarde	Torrada	2 unidades	16g
	Manteiga	2 pontas de faca	10g
	Pêra Grande	1 unidade	130g
Jantar	Sopa de abóbora com carne **(Ver receita)**	1 prato fundo cheio	450,75g
	Pão de sal	1/2 Unidade	25g

APPENDIX E: RECIPES TESTED IN THE LAB WITH CASHEW NUT OIL AND MENU MODEL FOR CASHEW NUT OIL GROUP

ESTUDO
castanhas brasileiras

PLANO ALIMENTAR

RECEITA DE SHAKE DE BANANA COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	**Quantidade**
Leite desnatado	1 Copo americano (150 ml)
Banana prata	1 Unidade (75 g)
Óleo de castanha de caju	1 Medidor ou 5 col sopa (30 ml)

Modo de preparo:

1) Misture todos os ingredientes em um liquidificador.

PLANO ALIMENTAR

RECEITA DE SHAKE DE MAÇÃ COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	Quantidade
Leite desnatado	1 copo americano (150 ml)
Maçã	1 Unidade (150 g)
Óleo de castanha de caju	1 Medidor ou 5 col sopa (30 ml)

Modo de preparo:

1) Misture todos os ingredientes em um liquidificador.

PLANO ALIMENTAR

RECEITA DE SHAKE DE MANGA COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	**Quantidade**
Leite desnatado	1 Copo americano (150 ml)
Manga	2 Lascas grandes (44 g)
Óleo de castanha de caju	1 Medidor ou 5 col sopa (30 ml)

Modo de preparo:

1) Misture todos os ingredientes em um liquidificador.

PLANO ALIMENTAR

RECEITA DE MOLHO DE IOGURTE COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	**Quantidade**
Iogurte desnatado	1/2 pote de iogurte natural (85 g)
Óleo castanha de caju	1 Medidor ou 5 col sopa (30 ml)
Alho	1 dente pequeno
Cebolinha	À gosto
Hortelã	À gosto
Suco de limão	1/2 limão
Sal e pimenta	À gosto

Modo de preparo:

1) Descasque o dente de alho e bata no pilão até formar uma pastinha (se preferir, pique bem fino numa tábua). Transfira para uma tigela
2) Com o óleo de castanha de caju, misture o iogurte com a cebolinha e o hortelã, ambos frescos e bem picados

3) Depois de misturar bem esses ingredientes, acrescente sal e uma pitada de pimenta à gosto

4) Por fim, coloque uma colher de sopa de suco de limão e mexa bem.

Rendimento: 1 porção

PLANO ALIMENTAR

RECEITA DE MOLHO CEASAR COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	**Quantidade**
Iogurte desnatado	2 colheres de sopa
Óleo castanha de caju	1 Medidor ou 5 col sopa (30 ml)
Mostarda	1 colher de sopa
Suco de limão	½ colher de sopa)
Queijo parmesão ralado	2 colheres de sopa
Pimenta do reino	À gosto

Modo de preparo:

1) Misturar todos os ingredientes em uma tijela e mexer com uma colher até ficar homogêneo.

Rendimento: 1 porção

PLANO ALIMENTAR

RECEITA DE MOLHO CHIMICHURRI COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	Quantidade
Chimichurri	1 colher de sopa
Óleo castanha de caju	1 Medidor ou 5 col sopa (30 ml)
Vinagre	1 colher de chá

Modo de preparo:

1) Numa tigela coloque o chimichurri e adicione o óleo de castanha e o vinagre e misture bem.

Rendimento: 1 porção

PLANO ALIMENTAR

RECEITA DE MOLHO VERDE COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	Quantidade
Suco de limão	1/2 limão
Óleo castanha de caju	1 Medidor ou 5 col sopa (30 ml)
Mostarda	1 colher de sopa
Orégano	1 colher de chá
Alho	2 dentes médios
Sal	À gosto

Modo de preparo:

1) Em um recipiente coloque o suco do limão, a mostarda, o óleo da castanha, o orégano e o alho amassado e sal a gosto, mexa bem.

Rendimento: 1 porção

PLANO ALIMENTAR

RECEITA DE MOLHO ITALIANO COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	Quantidade
Maionese	2 colheres de sopa
Óleo castanha de caju	1 Medidor ou 5 col sopa (30 ml)
Cebola	1/2 Unidade pequena
Vinagre branco	1/2 colher de sopa
Alho	1 dentes médio
Mel ou açúcar	1 colher de sopa
Sal	1 Colher de chá
Pimenta do reino	À gosto
Orégano	1 colher de sopa

Modo de preparo:

1) Descasque a cebola e corte em pedacinhos para facilitar e descasque o alho.

2) Misture todos os ingredientes em um pirex, utilizando uma colher.

3) Após homogeneizar os ingredientes, servir no próprio pirex.

4) Caso tenha dificuldade de misturar os ingredientes, utilize o mixer vertical.

Rendimento: 1 porção

PLANO ALIMENTAR

RECEITA DE MOLHO DE MOSTARDA E MEL COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	Quantidade
Vinagre de maçã	½ colher de sopa
Mostarda à gosto	2 colheres de sopa
Mel	2 colheres de sopa
Óleo de castanha de caju	1 Medidor ou 5 col sopa (30 ml)
Sal	1/2 colher de sobremesa
Pimenta-do-reino moída	À gosto

Modo de preparo:

1) Misture todos os ingredientes em um pirex, utilizando uma colher.

2) Após homogeneizar os ingredientes, servir no próprio pirex.

3) Caso tenha dificuldade de misturar os ingredientes, utilize o mixer vertical.

Rendimento: 1 porção

ESTUDO
castanhas brasileiras

PLANO ALIMENTAR

RECEITA DE MOLHO LEVEMENTE PICANTE COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	**Quantidade**
Suco de limão	1 Colher de sopa
Maionese	1 Colher de sopa
Catchup	1/2 Colher de sopa
Óleo de castanha de caju	1 Medidor ou 5 col sopa (30 ml)
Mostarda	1 Colher de sopa
Alho	1 Unidade média
Pimenta do reino	À gosto
Órégano	À gosto

Modo de preparo:

1) Misture todos os ingredientes em um pirex, utilizando uma colher.

Rendimento: 1 porção

PLANO ALIMENTAR

RECEITA DE PASTA DE GRÃO DE BICO COM ÓLEO DE CASTANHA DE CAJU:

Ingredientes:

Alimento	Quantidade
Grão de bico	½ xícara de chá cozidos = 1 xícara de chá crus
Alho	½ dente
Água	½ colher de sopa
Óleo de castanha de caju	1 Medidor ou 5 col sopa (30 ml)
Salsinha	4 Ramos
Sal e pimenta-do-reino moída	À gosto

Modo de preparo:

1) Sobre uma tigela passe os grãos-de-bico por uma peneira e deixe escorrer bem a água.

2) Com a lateral da faca, amasse o dente de alho e descarte a casca. Lave, seque e pique a salsinha grosseiramente.

3) No liquidificador, coloque o grão-de-bico, o alho, a água e 2 colheres (sopa) de óleo de castanha de caju. Bata bem até formar uma pasta lisa – esse passo é importante não só para bater o grão-de-bico,

4) Misture a salsinha, prove e tempere com sal e pimenta. Sirva a seguir com torradas.

Rendimento: 1 porção

ESTUDO
castanhas brasileiras

PLANO ALIMENTAR

DIETA 01

Valor calórico total: 1500 Kcal

Nome: _____

REFEIÇÃO	ALIMENTOS	MEDIDA CASEIRA	QUANTIDADE
Café da manhã	Pão integral	1 fatia	25 g
	Requeijão Light	1 ponta de faca	6 g
	Café sem açúcar	1 xícara de café	50 ml
	Mamão	1/2 Unidade	155 g
Colação	Shake de Banana (**ver receita**):		
	Banana	1 Unidade	75 g
	Óleo castanha de caju	**1 Medidor ou 5 col sopa**	**30 ml**
	Leite de vaca desnatado	1 Copo americano	150 ml
Almoço	Arroz	5 colheres de sopa	125 g
	Feijão	2 colheres de sopa	34 g
	Peito de frango grelhado sem pele	1/2 Unidade média	90 g
	Abobrinha cozida	1 colher de sopa cheia picada	30 g
	Tomate cru	2 Fatias médias	30 g
	Alface	3 folhas	30 g
	Couve-flor cozida	2 ramos pequenos	120 g
	Abacaxi	1 Fatia	75 g
Lanche da tarde	Pêra Grande	1 Unidade	130 g
	Torrada	2 Unidades	16 g
	Requeijão Light	2 Pontas de faca	12 g
Jantar	Sopa de abóbora com carne (**ver receita**)	1 prato fundo cheio	450,75 g
	Maçã	1 Unidade	150 g

ANNEX A: INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (IPAQ)

ESTUDO
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I. IDENTIFICAÇÃO

Data de aplicação: ___/___/___ Pesquisador responsável: _____ Tempo: ( ) Início ( ) Final

Iniciais (nome): _____ Grupo:_____ N° ID:_____

Nós estamos interessados em saber que tipos de atividade física as pessoas fazem como parte do seu dia a dia. Este projeto faz parte de um grande estudo que está sendo feito em diferentes países ao redor do mundo. Suas respostas nos ajudarão a entender quão ativos nós somos em relação à pessoas de outros países. As perguntas estão relacionadas ao tempo que você gastou fazendo atividade física na **ÚLTIMA** semana. 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. Obrigado pela sua participação!

Para responder as questões lembre 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 da última semana você **CAMINHOU** por pelo menos 10 minutos contínuos em casa ou no trabalho, como forma de transporte para ir de um lugar para outro, por lazer, por prazer ou como forma de exercício?

_____ dias por **SEMANA** ( ) Nenhum

1b Nos dias em que você caminhou por pelo menos 10 minutos contínuos quanto tempo no total você gastou caminhando **por dia**?

horas: _____ Minutos: _____

2a. Em quantos dias da última semana, você realizou 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 domésticos na casa, no quintal ou no jardim como varrer, aspirar, cuidar do jardim, ou qualquer atividade que fez aumentar **moderadamente** sua respiração ou batimentos do coração **(POR FAVOR NÃO INCLUA CAMINHADA)**

_____ dias por **SEMANA** ( ) Nenhum

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

horas: _____ Minutos: _____

ESTUDO
castanhas brasileiras

I. IDENTIFICAÇÃO

Data de aplicação: ____/____/____ Pesquisador responsável: _____

Iniciais (nome): _____Grupo:_____ Nº ID:_____

3a Em quantos dias da última semana, você realizou 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 cavoucar no jardim, carregar pesos elevados ou qualquer atividade que fez aumentar **MUITO** sua respiração ou batimentos do coração.

_____ dias por **SEMANA** ( ) Nenhum

3b Nos dias em que você fez essas atividades vigorosas por pelo menos 10 minutos contínuos quanto tempo no total você gastou fazendo essas atividades **por dia**?

horas: _____ Minutos: _____

Estas últimas questões são sobre o tempo que você permanece sentado todo dia, no trabalho, na escola ou faculdade, em casa e durante seu tempo livre. Isto inclui o tempo sentado estudando, sentado enquanto descansa, fazendo lição de casa visitando um amigo, lendo, sentado ou deitado assistindo TV. Não inclua o tempo gasto sentando durante o transporte em ônibus, trem, metrô ou carro.

4a. Quanto tempo no total você gasta sentado durante um **dia de semana**?

horas: _____ Minutos: _____

4b. Quanto tempo no total você gasta sentado durante em um **dia de final de semana**?

horas: _____ Minutos: _____

8. OTHER WORKS DEVELOPED DURING THE Ph.D. PROGRAM

8.1 Systematic Review Article

CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
https://doi.org/10.1080/10408398.2023.2245469

Taylor & Francis
Taylor & Francis Group

REVIEW

Check for updates

Effect of food derived bioactive peptides on gut health and inflammatory mediators in vivo: a systematic review

Marcella Duarte Villas Mishima[a], Hércia Stampini Duarte Martino[b], Talitha Silva Meneguelli[a] and Elad Tako[a]

[a]Department of Food Science, Cornell University, Ithaca, NY, USA; [b]Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Brazil

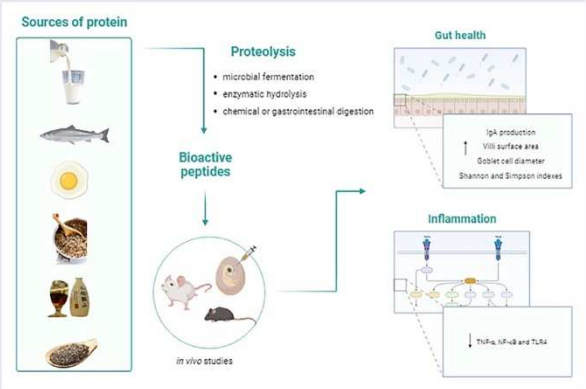
ABSTRACT

Dietary proteins serve as sources of exogenous peptides, after being released from the protein and absorbed, the bioactive peptides can perform several functions in the body. The objective of the current systematic review is to answer the question "How does food derived bioactive peptides can impact on gut health and inflammatory mediators in vivo?" The search was performed at PubMed, Cochrane, and Scopus databases for experimental studies, and the risk of bias was assessed by the SYRCLE tool. The data analysis was conducted following the PRISMA guidelines. Eleven studies performed in animal models evaluating bioactive peptides derived from animal and plant sources were included and evaluated for limitations in heterogeneity, methodologies, absence of information regarding the allocation process, and investigators' blinding. The bioactive peptides demonstrated potential positive effects on inflammation and gut health. The main results identified were a reduction in TNF-α, NF-κB, and TLR4, an improvement in IgA production and in intestinal morphology, with an increase in villi surface area and goblet cell diameter, and Shannon and Simpson indexes were also increased. However, more in vivo studies are still necessary to better elucidate the anti-inflammatory activity and mechanisms by which peptides regulate gut health. PROSPERO (CRD42023416680).

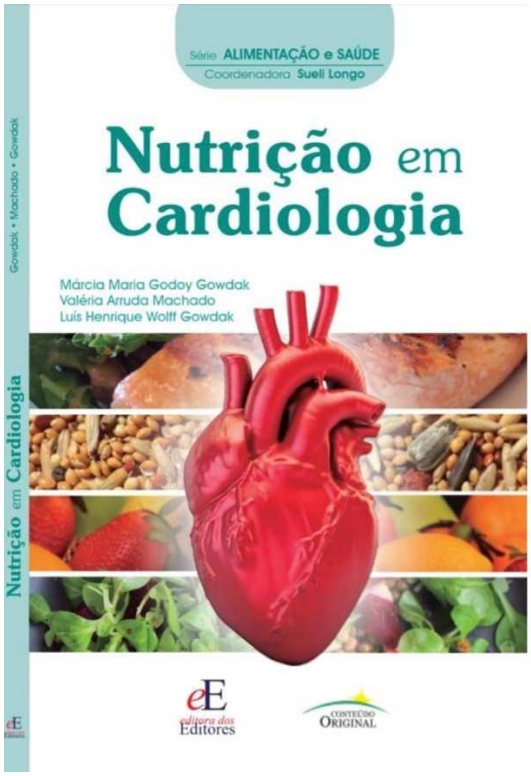
KEYWORDS

Peptides from food; intestinal health; anti-inflammatory mechanisms; inflammation; intestine; dietary protein

GRAPHICAL ABSTRACT



8.2 Book Chapter



CAPÍTULO 19

Talitha Silva Meneguelli
Helen Hermana Miranda Hermsdorff

Dieta do Mediterrâneo e DASH

INTRODUÇÃO

Padrão alimentar é definido por um conjunto de alimentos que geralmente são consumidos por indivíduos ou populações, o que permite avaliar a qualidade de uma dieta como um todo e não o consumo de determinados nutrientes ou alimentos de forma isolada. Esse conceito parte da premissa de que os alimentos interagem entre si, exercendo efeitos sinérgicos ou antagônicos. Assim, o efeito de um nutriente isolado pode ser pequeno em desfechos clínicos, ao passo que, em conjunto, o efeito na promoção da saúde ou no tratamento de doenças pode ser mais facilmente constado clínica e epidemiologicamente.

Nesse contexto, existem alguns padrões que são consistentes em relação às recomendações nutricionais para a saúde cardiovascular, além de serem palatáveis à mesa. Esses são caracterizados, em sua maioria, por elevado consumo de cereais integrais, frutas, hortaliças, legumes, leguminosas, oleaginosas, peixes, aves, consumo moderado de produtos lácteos e baixo em carboidratos simples, açúcares de adição, gordura trans, bebidas açucaradas e carnes vermelhas e processadas. Os padrões alimentares das dietas do Mediterrâneo e a *Dietary Approaches to Stop Hypertension* (DASH) destacam-se no meio acadêmico devido ao maior número e à qualidade de estudos científicos sobre os efeitos nos fatores de risco e eventos cardiovasculares. Desta forma, este capítulo tem o objetivo de apresentar esses dois padrões alimentares do ponto de vista nutricional, apontar seus benefícios potenciais no controle cardiometabólico e discutir os mecanismos pelos quais os alimentos e seus respectivos nutrientes impactam na diminuição do risco cardiovascular.

DIETA DO MEDITERRÂNEO E SAÚDE CARDIOVASCULAR

A dieta do Mediterrâneo representa um modelo de dieta tradicional que surgiu nas áreas de olivicultura na região da bacia do Mediterrâneo, berço

8.3 Abstract presented at National Conference - ABESO

Certificado

Certificamos que o trabalho ***PT.184***, "***Effects of cashew nut and cashew oil on adiposity markers in men and women with obesity in energy-restricted condition: a randomized controlled trial (Brazilian Nuts Study)***", de autoria de ***Talitha SM, Aline LW, Ana CPK, Mizaele GML, Polimar FF, Geovana MLS, Larissa PL, Nayara NG, Dionísio AP, Josefina B, Helen HMH***, foi apresentado como **PÔSTER** no **XX Congresso Brasileiro de Obesidade e Síndrome Metabólica**, realizado de 8 a 10 de junho de 2023 no Hotel Windsor Barra, Rio de Janeiro, RJ.

Bruno Halpern
Presidente ABESO

Amélio F. de Godoy-Matos
Presidente do XX CBOSM

ABESO
Associação Brasileira para o Estudo da Obesidade e da Síndrome Metabólica

8.4 Abstract presented at International Conference - NUTRITION

Current Developments in Nutrition
Volume 7, Supplement 1, July 2023, 100584

Dietary Bioactive Components

P03-073-23 Effects on Intestinal Health Following Intra-Amniotic Administration (Gallus gallus) of Cashew Nut Soluble Extract

Talitha Silva Meneguelli, Nikolai Kolba, Arundhati Misra, Helen Hermana Miranda Hermsdorff, Ana Paula Dionísio, Ana Claudia Pelissari Kravchychyn, Hércia Stampini Duarte Martino, Bárbara Pereira Da Silva, Elad Tako

Available online 27 July 2023, Version of Record 27 July 2023.

https://doi.org/10.1016/j.cdnut.2023.100584

8.5 Co-advisor of Undergraduates' Final Projects

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Certificado

Certificamos que

Talitha Silva Meneguelli

participou como **Coorientadora** da Banca de Defesa de Trabalho de Conclusão de Curso, da(o) estudante Viviane Maria de Oliveira, realizada e aprovada no dia **08 de julho de 2020**, pelos Membros: Dra. Leidjaira Juvanhol Lopes (UFV/DNS - Presidente), Dra. Helen Hermana Miranda Hermsdorff (UFV/DNS) e Ma. Talitha Silva Meneguelli (UFV/PPGCN), tendo como título "**Descenso noturno da pressão arterial e sua relação com indicadores antropométricos e de consumo alimentar: um estudo com indivíduos com risco cardiovascular (PROCARDIO-UFV)**".

Viçosa, 6 de outubro de 2020.

ELIANA CARLA GOMES DE SOUZA
Coordenadora da disciplina NUT 368 – Trabalho de Conclusão de Curso

RAQUEL MARIA AMARAL ARAÚJO
Chefe do Departamento de Nutrição e Saúde

Certificado registrado às 10:58 de 06/10/2020

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participou como **Coorientadora** da Banca de Defesa de Trabalho de Conclusão de Curso, da(o) estudante Renata Conceição Pimentel de Lima, realizada e aprovada no dia **12 de maio de 2021**, pelos Membros: Dra. Helen Hermana Miranda Hermsdorff (UFV/DNS - Presidente), Dra. Luiza Carla Vidigal Castro (UFV/DNS) e Ma. Talitha Silva Meneguelli (UFV/PPGCN), tendo como título "**Ingestão alimentar, risco cardiovascular e função renal em pessoas com risco cardiometabólico (PROCARDIO-UFV)**".

Viçosa, 9 de junho de 2021.

ELIANA CARLA GOMES DE SOUZA
Coordenadora da disciplina NUT 368 – Trabalho de Conclusão de Curso

RAQUEL MARIA AMARAL ARAÚJO
Chefe do Departamento de Nutrição e Saúde

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Talitha Silva Meneguelli

participou como **Coorientadora** da Banca de Defesa de Trabalho de Conclusão de Curso, da(o) estudante Lara Souza Fernandes, realizada e aprovada no dia **31 de março de 2022**, pelos Membros: Dra. Leidjaira Juvanhol Lopes (UFV/DNS - Presidente), Dra. Helen Hermana Miranda Hermsdorff (UFV/DNS) e Ma. Talitha Silva Meneguelli (UFV/PPGCN), tendo como título "**Consumo alimentar de acordo com o grau de processamento, obesidade e vitamina D sérica: um estudo com indivíduos em risco cardiovascular (PROCARDIO-UFV)**".

Viçosa, 13 de abril de 2022.

RAQUEL MARIA AMARAL ARAÚJO
Chefe do Departamento de Nutrição e Saúde
DNS/UFV

ELIANA CARLA GOMES DE SOUZA
Coordenadora da disciplina NUT 368 – Trabalho de Conclusão de Curso
DNS/UFV

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8.6 Participation as a Seminar Committee Member

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DEPARTAMENTO DE NUTRIÇÃO E SAÚDE / PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA DA NUTRIÇÃO

Certificado

Certificamos que

Talitha Silva Meneguelli

Participou como **Membro** da Banca de Avaliação na disciplina obrigatória NUT 797 - Seminário I durante a apresentação do seminário de **Jéssica Benatti Ribeiro**, Mestrando no Programa de Pós-Graduação em Ciência da Nutrição da Universidade Federal de Viçosa. Sua participação ocorreu no dia 19 de novembro de 2020, perfazendo um total de 1 hora.

Vicosa, 19 de novembro de 2020.

Certificado registrado às 11:43 de 19/11/2020

Código de registro:

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Certificado

Certificamos que

Talitha Silva Meneguelli

Participou como **Membro** da Banca de Avaliação na disciplina obrigatória NUT 797 - Seminário I durante a apresentação do seminário de **Gilmara Alves Zanirate**, Mestranda no Programa de Pós-Graduação em Ciência da Nutrição da Universidade Federal de Viçosa. Sua participação ocorreu no dia 12 de maio de 2021, perfazendo um total de 1 hora.

Vicosa, 18 de junho de 2021.

Certificado registrado às 15:49 de 18/06/2021

Código de registro:

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Certificado

Certificamos que

Talitha Silva Meneguelli

Participou como **Membro** da Banca de Avaliação na disciplina obrigatória NUT 797 - Seminário I durante a apresentação do seminário de **Ângela Maria Natal de Souza**, mestranda no Programa de Pós-Graduação em Ciência da Nutrição da Universidade Federal de Viçosa. Sua participação ocorreu no dia 01 de julho de 2020, perfazendo um total de 1 hora.

Vicosa, 18 de junho de 2021.

Certificado registrado às 15:06 de 18/06/2021

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Certificado

Certificamos que

Talitha Silva Meneguelli

Participou como **Membro** da Banca de Avaliação na disciplina obrigatória NUT 798 - Seminário II durante a apresentação do seminário de **Arieta Carla Gualandi Leal**, doutoranda no Programa de Pós-Graduação em Ciência da Nutrição da Universidade Federal de Viçosa. Sua participação ocorreu no dia 13 de outubro de 2021, perfazendo um total de 1 hora.

Vicosa, 21 de outubro de 2021.

Certificado registrado às 09:45 de 21/10/2021

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