CÍNTIA TOMAZ SANT' ANA

CHEMICAL CHARACTERIZATION AND FUNCTIONAL PROPERTIES *IN VITRO* AND *IN VIVO* OF MACAUBA (*Acrocomia aculeata*)

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

Advisor: Frederico Augusto Ribeiro de Barros

Co-advisors: Hércia Stampini Duarte Martino
Neuza Maria Brunoro Costa
Kee-Hong Kim

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ABSTRACT

SANT' ANA, Cíntia Tomaz, D.Sc., Universidade Federal de Viçosa, February, 2023. **Chemical characterization and functional properties *in vitro* and *in vivo* of macauba (*Acrocomia aculeata*)**. Advisor: Frederico Augusto Ribeiro de Barros. Co-advisors: Hércia Stampini Duarte Martino, Neuza Maria Brunoro Costa and Kee-Hong Kim.

Macauba (*Acrocomia aculeata*) is a Brazilian palm tree, and its fruit is formed by shell, pulp, endocarp and kernel. This fruit has several bioactive compounds such as carotenoids, tocopherol, oleic acid, proteins, fibers and phenolic compounds. However, macauba is still little used in human food, being mostly used in the biodiesel industry and animal feed. In this way, as macauba has great potential for use in human food, conferring health benefits due to its good composition, this thesis aimed to investigate: (I) complete composition of all parts of macauba and its co-products, and the potential action of macauba oils in the reduction of reactive oxygen species (ROS) in cells (lung adenocarcinoma, colorectal adenocarcinoma, hepatocarcinoma, lung fibloblast); (II) effect of macauba pulp oil on obesity and oxidative stress in mice fed a high-fat diet; (III) effect of macauba pulp oil on the intestinal microbiota of mice fed a high-fat diet; (IV) effect of macauba pulp oil on lipid metabolism and longevity in *Caenorhabditis elegans*. The methods used were: (I) analysis of moisture, ash, lipids, proteins, dietary fiber, total phenolics, phytic acid, tannins, minerals, carotenoids, tocopherol, fatty acid, and analysis of cell viability and formation of reactive oxygen species in cell lines; (II) C57BL/6 mice were fed for 8 weeks with a control diet, a high-fat diet, a high-fat diet with macauba pulp oil, and biochemical (cholesterol and fractions, superoxide dismutase, catalase, malondialdehyde, total antioxidant capacity, nitric oxide), histological and gene expression analyzes were performed in adipose tissue and liver (genes of the inflammation, adipogenesis, synthesis and oxidation of fatty acids pathway); (III) C57BL/6 mice were fed for 8 weeks with a control diet, a high-fat diet, a high-fat diet with macauba pulp oil, and short-chain fatty acid analysis, fecal pH, colon histology (crypt width and depth, goblet cells number, thickness of the circular and longitudinal muscle layer) and microbiota sequencing were performed using the 16S rRNA method; (IV) *Caenorhabditis elegans* was used as an experimental model and kept in the presence of macauba pulp oil for 4 days and was performed analysis of lipid, glycerol, gene expression, fatty acids, and lifespan was performed. Among the results obtained, it was found that shell showed high amount of dietary

fiber, minerals, and bioactive compounds, whereas its pulp and kernel oils were rich in oleic acid and lauric acid, respectively. Both pulp and kernel oil extraction coproducts (pulp and kernel press-cakes) showed high contents of dietary fiber, proteins, and bioactive compounds, and macauba pulp and kernel oils were able to reduce the ROS in cell lines. Consumption of macauba pulp oil increased antioxidant capacity and prevented oxidative stress, inflammation and adipogenesis in mice fed a high-fat diet. Macauba pulp oil changed the profile of gut microbiota, promoted short-chain fatty acid production, enhanced intestinal barrier integrity, and increased goblet cells in mice fed a high-fat diet. In addition, macauba pulp oil reduced the fat accumulation and increased lifespan in *C. elegans* in low temperature, and these results were associated by fat mobilization and unsaturated fatty acid biosynthesis. Our results demonstrate that macauba and its co-products has great potential to be included in human food because it is a source of bioactive compounds and the macauba pulp oil has positive effects on metabolic changes, demonstrating great potential to contribute to public health.

Keywords: Macauba. Bioactive compounds. Co-products. Gut microbiota. *Caenorhabditis elegans*.

RESUMO

SANT' ANA, Cíntia Tomaz, D.Sc., Universidade Federal de Viçosa, fevereiro de 2023. **Caracterização química e propriedades funcionais *in vitro* e *in vivo* da macaúba (*Acrocomia aculeata*)**. Orientador: Frederico Augusto Ribeiro de Barros. Coorientadores: Hércia Stampini Duarte Martino, Neuza Maria Brunoro Costa e Kee-Hong Kim.

Macaúba (*Acrocomia aculeata*) é uma palmeira brasileira, e seu fruto é formado por casca, polpa, endocarpo e amêndoa. Este fruto possui diversos compostos bioativos como carotenoides, tocoferol, ácido oleico, proteínas, fibras e compostos fenólicos. No entanto, a macaúba é ainda pouco usada na alimentação humana, sendo principalmente usada na indústria de biodiesel e alimentação animal. Desta maneira, como a macaúba tem um grande potencial para uso na alimentação humana, conferindo benefícios a saúde devido a sua boa composição, esta tese objetivou investigar: (I) composição completa de todas as partes da macaúba e seus co-produtos, e a potencial ação dos óleos da macaúba na redução de espécies reativas de oxigênio (ROS) em células (adenocarcinoma de pulmão, adenocarcinoma de colorectal, hepatocarcinoma, fibroblasto de pulmão); (II) efeito do óleo da polpa da macaúba na obesidade e estresse oxidativo em camundongos alimentados com dieta com alto teor de gordura saturada; (III) efeito do óleo da polpa da macaúba na microbiota intestinal de camundongos alimentados com dieta com alto teor de gordura saturada; (IV) efeito do óleo da polpa da macaúba no metabolismo lipídico e longevidade em *Caenorhabditis elegans*. Os métodos utilizados foram: (I) análise de umidade, cinzas, lipidios, proteínas, fibra alimentar, fenólicos totais, ácido fítico, taninos, minerais, carotenoides, tocoferol, ácido graxo, e análise de viabilidade celular e de formação de espécies reativas de oxigênio em linhagens celulares; (II) camundongos C57BL/6 foram alimentados por 8 semanas com dieta controle, dieta alta em gordura saturada, dieta alta em gordura saturada adicionada de óleo da polpa da macaúba, e realizado análises bioquímicas (colesterol e frações, superóxido dismutase, catalase, malondialdeído, capacidade antioxidante total, oxido nítrico), histológicas e expressão gênica no tecido adiposo e fígado (genes da via de inflamação, adipogênese, síntese e oxidação de ácidos graxos); (III) camundongos C57BL/6 foram alimentados por 8 semanas com dieta controle, dieta alta em gordura saturada, dieta alta em gordura saturada adicionada de óleo da polpa da macaúba, e realizado análise de ácidos graxos de cadeia curta, pH fecal, histologia do cólon (largura e profundidade das criptas, número de células

caliciformes, espessura da camada muscular longitudinal e circular) e sequenciamento de microbiota usando o método 16S rRNA; (IV) *Caenorhabditis elegans* foi utilizado como modelo experimental e mantido na presença do óleo da polpa da macaúba por 4 dias e foi realizado análise de lipídio, glicerol, expressão gênica, ácidos graxos, e vida útil. Entre os resultados obtidos, foi encontrado que a casca mostrou alto conteúdo de fibra dietética, minerais, e compostos bioativos, enquanto os óleos da polpa e amêndoa eram ricos em ácido oleico e ácido láurico, respectivamente. Ambos co-produtos da extração do óleo da polpa e da amêndoa (torta da polpa e da amêndoa) mostraram alto conteúdo de fibra dietética, proteínas, e compostos bioativos, e o óleo da polpa e amêndoa foram capazes de reduzir ROS em linhas celulares. O consumo do óleo da polpa da macaúba aumentou a capacidade antioxidante e preveniu o estresse oxidativo, inflamação e adipogênese em camundongos alimentados com dieta com alto teor de gordura. O óleo da polpa da macaúba mudou o perfil da microbiota intestinal, promoveu a produção de ácidos graxos de cadeia curta, melhorou a integridade da barreira intestinal, e aumentou células caliciformes em camundongos alimentados com dieta com alto teor de gordura. Ainda, o óleo da polpa da macaúba reduziu a acumulação de gordura e aumentou a expectativa de vida em *C. elegans* em baixa temperatura, e esses resultados foram associados pela mobilização de gordura e biossíntese de ácidos graxos insaturados. Nossos resultados demonstram que a macaúba e seus co-produtos tem grande potencial para ser incluída na alimentação humana pois é uma fonte de compostos bioativos e o óleo da polpa da macaúba tem efeitos positivos nas alterações metabólicas, demonstrando grande potencial de contribuição para a saúde publica.

Palavras-chave: Macaúba. Compostos bioativos. Co-produtos. Microbiota intestinal. *Caenorhabditis elegans*.

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LIST OF ABBREVIATIONS

aak-2; Functional homolog of AMP-activated protein kinase.

ABTS; 2,2'-azino-di-[3-etilbenztiazolina sulfonate.

ACAT; Acyl-CoA: cholesterol acyltransferase.

ACC-1; Acetyl CoA carboxylase 1.

acs-2; Functional homolog of fatty acid-CoA synthetase.

act-1; Functional homolog of Actin.

ADIPOR2; Adiponectin receptor 2.

ALT; Alanine aminotransferase.

AMPK; AMP-activated protein kinase.

ANOVA; Analysis of variance.

AOCS; American oil chemists society.

AST; Aspartate aminotransferase.

atgl-1; Functional homolog of adipose triglyceride lipase.

A549; Lung adenocarcinoma epithelial cells.

BMI; Body mass index.

Caco-2; Colorectal adenocarcinoma epithelial cells.

CE; Catechin equivalent.

CML; Circular muscle layer.

CPT-1α; Carnitine palmitoyl transferase 1 alpha.

daf-2; Functional homolog of insulin/IGF receptor family.

daf-16; Functional homolog of FOXO protein family.

DCFH-DA; Dichlorofluorescein diacetate.

DMSO; Dimethylsulfoxide.

DPPH; 2,2-diphenyl-1-picrylhydrazyl.

FAMEs; Fatty acid methyl esters.

fat; Functional homolog of fatty acid desaturase.

FER; Feed efficiency ratio.

GAE; Galic acid equivalent.

GAPDH; Glyceraldehyde-3-phosphate dehydrogenase.

GC-FID; Gas chromatograph equipped with a flame ionization detector.

GC-TOF/MS; Gas chromatography-time-of-flight/mass spectrometry.

HDL-c; High-density lipoprotein cholesterol.

HepG2; Human hepatocarcinoma cells.

HFD; High-fat diet.

hosl-1; Functional homolog of Hormone-sensitive lipase.

HPLC; High-performance liquid chromatography.

Ig A; Immunoglobulin A.

IL-10; Interleukin 10.

IMR90; Human lung fibroblast.

KEGG; Kyoto encyclopedia of genes and genomes.

LDL-c; Low-density lipoprotein cholesterol.

LEfSe; Linear discriminant analysis effect size.

LML; longitudinal muscle layer.

LPL; Lipoprotein lipase.

LPS; Lipopolysaccharide.

MDA; Malondialdehyde.

mdt-15; Functional homolog of the human mediator complex subunit 15.

mRNA; Messenger RNA.

MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

NCBI; National center for biotechnology information.

NF-κB; Nuclear factor kappa B.

NGM; Nematode growth media.

nhr-49; Functional homolog of peroxisome proliferator-activated receptor-α.

nhr-80; Functional homolog of Nuclear hormone receptor family.

OASIS; Online application of survival analysis.

OTU; Operational taxonomic unit.

PCoA; Principal coordinate analysis.

PERMANOVA; Permutational multivariate analysis of variance.

PGC-1α; Peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

pod-2; Functional homolog of acetyl-CoA carboxylase.

PPARγ; Peroxisome proliferator-activated receptor γ.

ROS; Reactive oxygen species.

RT-qPCR; Reverse transcriptase quantitative polymerase chain reaction.

sbp-1; Functional homolog of Sterol regulatory element-binding protein 1.

SCFAs; Short chain fatty acids.

SciELO; Scientific electronic library online.

SOD; Superoxide dismutase.

SRA; Sequence read archive.

SREBP; Sterol regulatory element-binding proteins.

TAC; Total antioxidant capacity.

TC; Total cholesterol.

TEAC; Trolox equivalent antioxidant activity.

TGL; Triacylglycerides.

TLR-4; Toll-like Receptor 4.

TNF-α; Tumor necrosis factor alpha.

UCP; Uncoupling protein.

VLDL; Very low-density lipoprotein.

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1. GENERAL INTRODUCTION

Macauba (*Acrocomia aculeata*) is a palm tree widely present in Brazil, and its fruit is composed of different parts: shell, pulp, endocarp and kernel (VIANNA et al., 2017). The shell can be used in handicrafts, from the pulp can be obtained oil for the biodiesel manufacture, and the pulp press-cake, a pulp co-product, used in animal feed, the endocarp used in the production of charcoal, and from the kernel can be obtained oil to be used in cosmetic products (LIMA et al., 2018). Among the products obtained from macauba, the oil stands out. Kernel oil has a high profile of lauric, while pulp oil has high oleic acid content and carotenoids (COIMBRA; JORGE; 2011; LIEB et al., 2019). However, macauba is still not used on a large scale, and its use is concentrated in small producers.

Current dietary patterns, characterized by high consumption of foods with high energy density, low nutritional quality, associated with a reduced level of physical activity, are one of the main causes of the high prevalence of metabolic changes (LE et al., 2020). Among the metabolic changes generated are changes in lipid metabolism, inflammation, oxidative stress, dysbiosis, and obesity, which have a great impact on the public health of the population (WHO, 2018). Regarding the strategies for preventing or treating these alterations, diet has a great impact. Thus, foods rich in bioactive compounds that can provide health benefits become extremely important to be included in the diet and associated with this, it is encouraged its insertion in the formulation of products in the food industry sector, with the aim of contributing to the prevention or treatment of these changes (WOLFENDEN et al., 2019).

Taking into account that macauba is a Brazilian fruit and is available and the current scenario with a high prevalence of metabolic changes, macauba can present itself as a promising alternative in this context. Macauba fruit has bioactive compounds of interest to health, such as carotenoids, dietary fiber, proteins and oleic acid, and the consumption and inclusion of macauba and its co-products in the diet can possibly act in a beneficial way to improve health (COIMBRA; JORGE; 2011; ALMEIDA et al., 2019). To date, there are no studies in the literature evaluating the effect of macauba consumption in relation to metabolic changes, however, research shows that the bioactive compounds present in macauba can positively modulate these changes. Oleic acid is related to the decreased expression of transcription factors related to the adipogenesis signaling pathway, such as PPARγ (*peroxisome proliferator-activated receptor gamma*) and SREBP (*sterol regulator element-binding protein*), which when at reduced levels causes less accumulation of fat in adipocytes and inhibition of lipogenic pathways, triggering less weight

gain, contributing to the reduction of obesity (DÍAZ et al., 2017; VENTURINI et al., 2015). In addition, adequate consumption of dietary fiber is highly associated with reduced weight gain and improved integrity of the intestinal barrier by producing short-chain fatty acids, which consequently favors the control of dysbiosis (MISKI et al., 2020; SANCHEZ-RODRIGUEZ et al., 2019; WADDELL et al., 2022). Carotenoids can act to reduce inflammation by modulating the NF-κB (*nuclear factor kappa B*) pathway, reducing the production of inflammatory cytokines (BONET et al., 2015).

The justification for this study is based on the fact that there are no studies evaluating the entire composition of macauba co-products and the effect of macauba pulp oil on health. It is expected, as a result of this study, that the macauba co-products may have a good nutritional composition and that the macauba pulp oil may trigger benefits in relation to metabolic changes in mice fed a high-fat diet and provide an increase in the lifespan and modulation of lipid metabolism in *Caenorhabditis elegans*.

Thus, the macauba, a fruit present in the Brazilian flora and still little explored, arouses the interest of research, with the possibility of including consumption in the population's diet and, consequently, the development of new products based on its oils and co-products, showing that an expressive alternative for the food, pharmaceutical and agricultural industries, associated with its possible health benefits.

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2. LITERATURE REVIEW

1. Macauba (*Acrocomia aculeata*)

Macauba is a palm tree with a wide geographic distribution in the Americas, and in Brazil it is considered as the one with the greatest dispersion with natural occurrence in almost the entire territory. However, the highest concentrations are located in Minas Gerais, Goiás, Mato Grosso and Mato Grosso do Sul, being widely spread across cerrado areas (LIMA et al., 2018). Macauba reaches a stem of 10 to 15 m in height and 20 to 30 cm in diameter. The node region is covered with dark, pointed spines about 10 cm long, and the green leaves are 4 to 5 meters long and thorns in the central region. The fruits are arranged in bunches and have a spherical or slightly flattened shape, with a diameter ranging from 2.5 to 5.0 cm (SANJINEZ-ARGANDONA; CHUBA, 2011).

Fruiting occurs throughout the year and the fruits ripen mainly between September and January and their exploitation is still rudimentary, traditionally linked to community extractivism (PIRES et al., 2013). The fruit is composed of four parts: shell (epicarp), pulp (mesocarp), endocarp and kernel (Figure 1). The epicarp breaks easily when mature. The mesocarp is fibrous, mucilaginous, yellow in color. The endocarp has a hard consistency, and the kernel are white in color and covered with a thin layer of integument. Each fruit usually contains a kernel surrounded by a hard and dark endocarp (VIANNA et al., 2017).

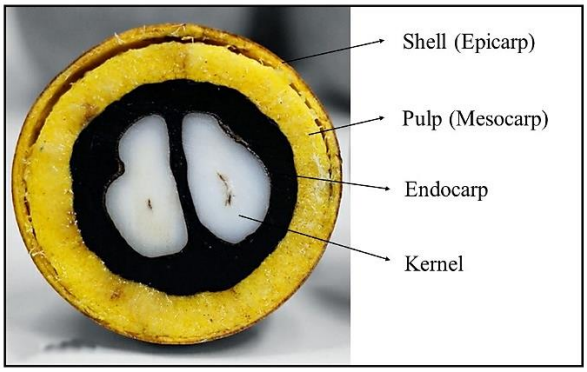

Figure 1. Longitudinal section of macauba and its parts.
Source: portalmacauba.com.br

The macauba crop stands out with its high productive potential, being able to supply the need and demand of vegetable oils and providing economic, social and nutritional advantages, since, according to estimates, macauba can produce 4.500 liters of oil per hectare/year. This value is higher than other crops, such as soybean, which yields 420 liters, sunflower 890 liters, castor

bean 1.320 liters and even palm with 3.000 liters of oil per hectare/year (COLOMBO et al., 2018). Economically, macauba exploration is interesting, given its use in general, and all its parts can be used in some industrial segment, associated with high oil production. The full use of co-products can make the macauba industrial complex more efficient, competitive and sustainable. From a social point of view, the insertion of macauba in the market could generate more jobs and increase the income of regional workers (CICONINI et al., 2013).

There is great variation in the composition of the fruit of different species of macauba, presenting a color from yellow to green, in addition to different flavors, from sweeter fruits to some with greater acidity, in addition to a difference in the oil content present in the fruit. These differences may be related to genetic and environmental factors, such as climate and soil (VIANNA et al., 2017). It is extremely important to know and characterize this variability, so that plants with greater potential and targeted for specific purposes can be identified depending on their characteristics (VIANNA et al., 2017; CICONINI et al., 2013).

2. Macauba chemical composition

Variations in composition are reported in the literature, which reveal that oil productivity, as well as fruit composition, is directly related to soil fertility, location, season and type of harvest (LIEB et al., 2019, CICONINI et al., 2013). The composition of the fruit (Table 1) is related to its application and use by the industry, and its components of interest and each part of macauba can be used in a certain segment, according to the components of its matrix (EVARISTO et al., 2016).

Table 1. Macauba chemical composition (%) (wet base).

	Macauba		
	Pulp	Kernel	Shell
Moisture	4.20 – 5.98	2.52 – 4.97	5.00 – 5.43
Ash	1.50 – 2.17	1.86 – 2.08	1.50 – 1.70
Protein	5.31 – 6.72	16.44 – 28.61	1.00 – 1.06
Lipid	23.00 – 42.00	46.00 – 65.00	5.29 – 6.87
Total dietary fiber	13.89 – 20.26	12.49 – 15.81	27.4 – 29.87
Carbohydrate	6.22 – 6.92	5.81 – 6.06	7.45 – 8.27

Source: Adapted from Lescano et al. (2015); Evaristo et al. (2016); Coimbra; Jorge (2011); Zanatta (2015).

The low moisture content provides benefits in the stability of the oil present both in the almond and in the pulp, and the low content in the kernel favors that it has a more rigid consistency, compared to other types of kernels (HIANE et al., 2006; RENCORET et al., 2006; RENCORET et al. al., 2018). The great highlight related to macauba is based on the expressive content of lipids, both related to the pulp and the kernel. This high lipid content places macauba in the industrial scenario, with oils with chemical characteristics of interest in health and commercialization (LIEB et al., 2019). Another positive point related to macauba focuses on the dietary fiber content, mainly related to pulp, which may favor the insertion of these products in the diet, due to the health benefits related to the consumption of fibers and the interest of the industry in the incorporation of this compound in foods (EVARISTO et al., 2016).

The macauba kernel has a higher protein content than the pulp, and protein consumption is essential, since they are related to vital functions of the body, such as maintenance of the immune system, energy source, oxygen transport, muscle mass, among others (KITADA et al., 2019, HIANE et al., 2006). The carbohydrate content has reduced values in macauba, both in the pulp, kernel and shell. In the study developed by Ramos et al. (2008) obtained for macauba pulp 12% of starch, 9% of glucose and 0.07% of sucrose in relation to the carbohydrate content.

2.1. Fatty acid profile of macauba oils

Two types of oils can be obtained from macauba, pulp oil and kernel oil (Figure 2).

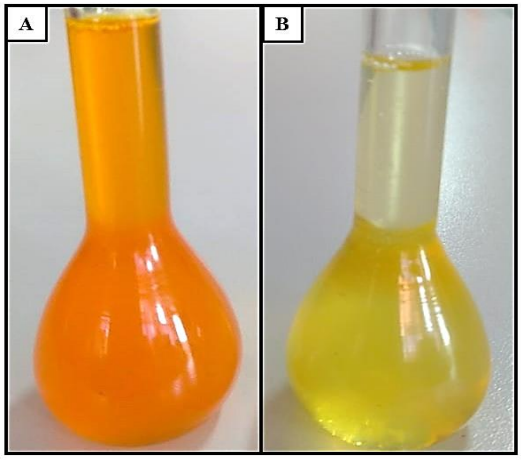

Figure 2. (A) Macauba pulp oil; (B) Macauba kernel oil.

Source: Author

Pulp and kernel oils have different lipid profile and composition (Table 2). The pulp oil has an orange color due to the presence of carotenoids, being an oil of the oleic/palmitic type composed mainly of oleic acid (C18:1). Kernel oil is light in color, with a high content of saturated fatty acids, predominantly lauric acid (COIMBRA; JORGE, 2012; LIEB et al., 2019).

Table 2. Fatty acid profile of macauba pulp and kernel oils (%).

Fatty acid	Macauba	
	Pulp oil	Kernel oil
Caprylic (C8:0)	-	3.67 – 4.90
Capric (C10:0)	-	2.79 – 3.27
Lauric (C12:0)	0.39 – 0.74	39.02 – 43.75
Mystic (C14:0)	0.38 – 0.41	9.21 – 13.40
Palmitic (C16:0)	19.10 – 24.70	8.25 – 9.20
Palmitoleic (C16:1)	3.75 – 4.28	-
Stearic (C18:0)	1.62 – 1.70	2.24 – 2.93
Oleic (C18:1)	52.57 – 64.90	26.27 – 32.70
Linoleic (C18:2)	5.54 – 13.80	3.08 – 3.82
Linolenic (C18:3)	1.40 – 2.26	-
Saturated	21.40 – 27.10	59.91 -62.84
Unsaturated	72.90 – 78.60	36.14 – 40.09

Source: Adapted from Coimbra; Jorge (2011), Lieb et al. (2019), Munhoz et al. (2018).

Kernel oil is similar to coconut oil, but it has the important characteristic of not only having a high content of lauric acid, but also having an outstanding percentage of oleic acid, which are important characteristics related to health (LESCANO et al., 2015, RÍO et al., 2016). Pulp oil has been popularly known as “cerrado olive oil”, because its characteristics are similar to olive oil (COIMBRA, JORGE, 2011). Although there is a great diversity of oleaginous plant species used to obtain oils for food purposes, among which soybean, corn, sunflower and canola stand out, none is capable of reaching the productivity of macauba or providing an oil that reproduces the same qualities. nutritional and sensory properties of olive oil (CICONINI et al., 2013; PIRES et al., 2013).

The high proportion of oleic acid present in the pulp oil, close to that found in olive oil, raises the hypothesis of a similar biological action on the lipid metabolism alleged to this traditional oilseed (TOME-CARNEIRO et al., 2020). Oleic acid is a monounsaturated fatty acid that has 18 carbon atoms in its composition and only one unsaturation. This molecular configuration gives it important characteristics related to the reduction of low-density lipoprotein (LDL-c) synthesis, increased levels of high-density lipoprotein (HDL-c), and inhibition of lipogenic pathways (VENTURINI et al., 2015). Because of the high oleic content, olive oil has gained much attention due to its beneficial implication on human health, playing a protective role in cardiovascular diseases (DAMGAARD et al., 2013; EDWARDS et al., 2000).

Unlike the olive tree, macauba can be cultivated in practically the entire Brazilian territory, due to its adaptability to various environmental conditions (PIRES et al., 2013). Considering the high cost of olive oil practiced in the Brazilian market and the possible availability of an alternative from the Brazilian flora itself, an important strategy is the inclusion of macauba pulp oil as a component of the daily diet. To date, there is no information on the effects of macauba oils on lipid metabolism, and this knowledge is essential to establish guidelines for the production and consumption of this oil.

2.2. Amino acid profile of macauba

Proteins are characterized by being of vital importance to living beings. Its functions range from catalysis of chemical reactions, transport of other molecules, transmission of nerve impulses, immune protection, hormonal function, among others (KITAD et al., 2019). Proteins of animal origin have greater biological value compared to plant proteins due to the fact that they contain all essential amino acids, while proteins of plant origin usually have a deficiency of one or more essential amino acids (KITAD et al., 2019). However, low-income populations have limited access to animal proteins, as well as people with a vegan food habit, so plant-based proteins become extremely important for these groups (HERTZLER et al., 2020). Thus, the identification of protein-rich plant species and incentives for the cultivation and consumption of these species can contribute to reducing nutritional deficiencies for the general population, especially those with different dietary habits and diets (ORTOLÁ et al., 2019).

The different parts of macauba present diversified protein content, being the kernel with higher protein content compared to the pulp (COIMBRA; JORGE, 2011). A study evaluating the

amino acids present in macauba kernel showed that glutamic acid and arginine are in higher concentrations, while threonine and lysine are the limiting amino acids, not having, therefore, all essential amino acids (HIANE et al., 2006).

2.3. Carotenoid content of macauba

Carotenoids are hydrophobic compounds, soluble in solvents such as acetone, alcohol, ether and chloroform. They have a wide distribution in nature, with different chemical structures and biological activities, and give a yellow to orange or red color to many fruits, vegetables, egg yolks, crustaceans and some fish (MAOKA, 2020). Carotenoids are also bioactive substances, with beneficial health effects, some of which have pro-vitamin A activity (RODRIGUEZ-AMAYA et al., 2019). The role of carotenoids in human health is related to provitamin A and antioxidant activity. They function as regulators of the immune system response and reduce the risk of chronic non-communicable diseases such as cancer, cardiovascular diseases, cataracts, macular degeneration and oxidative stress (JOHNSON et al, 2018; WANG et al., 2013).

The orange color of macauba pulp is due to high concentrations of carotenoids, with β-carotene corresponding to the highest composition of carotenoids (Table 3). Other carotenoids are also found in smaller amounts, such as γ-carotene, zeaxanthin, β-cryptoxanthin and lutein (COIMBRA; JORGE, 2011; COIMBRA; JORGE, 2012, MUNHOZ et al., 2018).

Table 3. Carotenoids in macauba pulp and kernel oil (μg g^{-1}).

	Macauba	
	Pulp oil	Kernel oil
Total Carotenoids	300 – 348	1.25 – 1.82
β-carotene	49 – 59	0.28 – 0.30
Lutein	12 – 26	0.19 – 0.27
Zeaxanthin	14 – 22	0.21- 0.25

Source: Adapted from Coimbra; Jorge (2011); Munhoz et al. (2018); Schex et al. (2018)

The high concentration of carotenoids present in crude macauba pulp oil can function as a natural antioxidant, capable of reducing the rate of lipid oxidation reactions. This functionality is

important to help maintain the oxidative stability of the oil during storage and possibly play health-related benefits (COIMBRA; JORGE, 2011).

2.4. Tocopherol content of macauba

Macauba has a tocopherol content in its composition, which possibly plays an important role in the stability of the oil and which may be related to health benefits (COIMBRA; JORGE, 2012).

Table 4. Tocopherols in macauba pulp and kernel oil (mg kg^{-1}).

	Macauba	
	Pulp oil	Kernel oil
Total tocopherols	212.95	23.10
α-tocopherol	143.70	14.35
β-tocopherol	3.25	0.85
γ-tocopherol	57.85	ND
δ-tocopherol	8.15	7.90

Source: Adapted from Coimbra; Jorge (2012). ND: not detected.

Tocopherols are monophenolic compounds that occur naturally in most vegetable oils. They consist of a basic nucleus consisting of two rings, one phenolic and the other heterocyclic, linked to a saturated side chain formed by 16 carbons. Depending on the number and position of methyl groups attached to the aromatic ring, tocopherols are presented as four homologous compounds, called α, β, γ and δ-tocopherol (SINGH et al., 2017). In macauba oils, α-tocopherol stands out in greater proportion (COIMBRA; JORGE, 2012).

Tocopherols have antioxidant activity *in vivo* and *in vitro*. In vegetable oils, they protect unsaturated fatty acids from lipid oxidation, and in the human body they present biological activity of vitamin E (AGGARWAL et al., 2010). The main group of natural antioxidants present in vegetable oils are tocopherols and one of the ways in which they act is by scavenging free radicals. Tocopherols are able to donate phenolic hydrogens to peroxyl radicals, originated by lipid oxidation, forming a hydroperoxide and a tocopheroxyl radical. The tocopheroxyl radical has a lower ability to propagate lipid peroxidation compared to the peroxyl radical. The

tocopheroxyl radical reacts with another peroxyl or tocopheroxyl, forming more stable products (BELO et al., 2017).

2.5. Phenolic compounds of macauba

Phenolic compounds originate from the secondary metabolism of plants, being essential for their growth and reproduction, in addition to acting as an antipathogenic agent and contributing to pigmentation. In foods, they are responsible for color, astringency, aroma, antioxidant activity and oxidative stability (IGNAT et al., 2011).

The antioxidant activity of phenolic compounds is mainly due to their reducing property and chemical structure. These characteristics play an important role in the neutralization or scavenging of free radicals and chelation of transition metals, acting both in the initiation stage and in the propagation of the oxidative process (ALBUQUERQUE et al., 2020). In addition, phenolic compounds can act as protectors and regenerators of primary antioxidants such as ascorbic acid, tocopherol and β-carotene (BABBAR et al., 2011). This mechanism of action of antioxidants plays an important role in reducing lipid oxidation in tissues, both plant and animal, because when incorporated into human food, they not only preserve the quality of the food, but also reduce the risk of developing pathologies, such as atherosclerosis and dyslipidemia (GAVAHIAN et al., 2019).

Among the parts that make up macauba, the pulp has a higher phenolic content when compared to macauba kernel (COIMBRA; JORGE, 2011). Study carried out by Oliveira et al. (2017) evaluating the content of total phenolics present in macauba oils showed 3.9 mg galic acid/100g in pulp oil and 0.70 mg galic acid/100g in kernel oil.

3. Potential use of macauba

Despite its abundant fruiting and other characteristics, macauba is a wild palm tree and in almost the entire national territory it has been exploited domestically, well below its economic potential, and its exploitation is still carried out in a rudimentary way, traditionally associated with to community extractivism (COLOMBO et al., 2018). Due to this, alternatives for its better use have been pointed out and researched in recent years, with the objective of allowing its introduction into the national agribusiness scenario (EVARISTO et al., 2016).

The use of macauba resources is very diverse, and all its parts can be used in different sectors. The shell can be used as wood, the leaves serve as raw material for crafts and animal fodder. The mesocarp (pulp) is generally used in human food, used in the manufacture of liqueurs, ice cream, sweets and obtaining oil. The pulp press-cake, a co-product of the extraction of oil from the pulp, has a high production and can be used as a component of animal feed or transformed into ingredients for human consumption, due to its high composition in dietary fiber, carotenoids and oil with a high oleic content (EVARISTO et al., 2016). The endocarp can be used as charcoal and in the replacement of concrete gravel, or even as craft material. The kernel, in addition to its consumption in natura, has great potential for use due to the protein content and can be used to obtain oil, and be used as an ingredient in the manufacture of soap and cosmetics (CÉSAR et al., 2015; EVARISTO et al., 2016; RAMOS et al., 2008).

Macauba emerged as a promising raw material for the production of biodiesel due to its expressive oil content, high productivity, and good adaptation to different climatic conditions. Another relevant aspect for the use of macauba in the production of biofuels is due to the fact that its fruit generates a high number of co-products that can be used (CÉSAR et al., 2015). Macauba pulp press-cake can be used in animal feed, especially for ruminants, as a substitute for traditionally used foods. This substitution triggers positive points related to cost reduction, given the high yield of this co-product and its low cost, associated with nutritional benefits for animals due to its fiber content (RIQUEIRA et al., 2017).

In human food, the uses of macauba and its co-products are also being applied, due to the nutritional value added to these products. A study with the development of alfajor using macauba pulp flour proved to be well accepted by children and even added a higher fiber and lipid content to the product, improving the nutritional profile of the product when compared to the control product (RODRIGUES et al., 2017).

The great potential of macauba use is based on the production of oil, presenting great productivity and oils with characteristics desired by the industrial sectors, being able to be used both in the food area, as in the formulation of biodiesel and also in the pharmaceutical and cosmetic sector (PIRES et al.; 2013, CÉSAR et al., 2015).

Brazil is a major importer of olive oil, as the country has low cultivation of olive trees (INTERNATIONAL OLIVE OIL COUNCIL, 2018). In contrast, macauba is a native plant and is found practically throughout the entire national territory (LIMA et al., 2018). Equating the issue

of processing and demonstrating similar health benefits between both oils, one can motivate the planting and industrialization of macauba oil, as well as its co-products. Potentially, the final cost of pulp oil should be lower than that practiced for olive oil, making it more accessible to the Brazilian population.

Thus, macauba presents itself as a palm tree with extensive use, and all its parts can be used, whether for use for food purposes or subsidies for agriculture and other sectors, demonstrating the importance of studies and research on the fruit of this palm.

4. Potential functional properties associated with macauba consumption

Several diseases, such as obesity, diabetes, metabolic syndrome, have a great impact on public health, and therefore strategies that can contribute to the reduction or prevention of these diseases are of great importance, and among these strategies, food is a key point (APOVIAN et al., 2015). Metabolic changes, such as excess body fat, inflammation, oxidative stress, changes in lipid metabolism and intestinal microbiota modulation are closely related (BONOMINI et al., 2015). The accumulation of body fat is characterized by the process of adipogenesis, which is mainly controlled by the transcription factors PPAR-γ (*peroxisome proliferator-activated receptor gamma*) and SREBP-1c (*sterol regulatory element-binding protein*) (LOWE et al., 2011). PPAR-γ is responsible for inducing the pre-adipocyte differentiation step into a mature adipocyte, and SREBP-1c induces the expression of critical genes for lipid biosynthesis in adipose tissue. This increase in adipogenesis is related to an increase in inflammation, since hyperplasia and hypertrophy of adipocytes induce a greater production of pro-inflammatory cytokines and adipokines, favoring the phosphorylation of IkB, which is linked to NF-κβ (*nuclear factor kappa B*), and with that, the translocation of NF-κβ to the nucleus occurs, increasing the inflammation cascade (WLODARCZYK et al., 2019). With increased inflammation, there is a disturbance in the oxidant and antioxidant balance in the body, generating excessive amounts of reactive oxygen species, characterizing a framework of oxidative stress, which causes cell damage (BONOMINI et al., 2015). Furthermore, all these metabolic changes may also be associated with the composition of the intestinal microbiota, since the intestinal microbiota is characterized by constant dynamism and is influenced by numerous factors, such as diet and metabolic changes, as verified by the association between obesity and increased of specific genes in the gut microbiota (CUNNINGHAM et al., 2021).

In this sense, macauba, due to its composition, has great potential to develop health benefits. Oleic acid, present in large proportions in macauba pulp oil, has a positive effect on the prevention of obesity and associated diseases. Oleic acid can reduce the amount of free cholesterol and consequently induce the activity of LDL receptors in the liver, since oleic acid is the preferred substrate of ACAT (*acyl-CoA: cholesterol acyltransferase*) (RUMSEY et al., 1995). In addition, it has the ability to inhibit the nuclear translocation of SREBP-2, which is related to the transcription of enzymes involved in the endogenous synthesis of cholesterol. It also reduces the expression of SREBP-1c, which controls the transcription of lipogenic enzymes in the liver, such as FAS and glycerol-3-phosphate acyltransferase (OU et al., 2001). Oleic acid can contribute to reducing inflammation by blocking the NF-κβ complex, reducing the expression of inflammatory genes and activating genes responsible for the IL-10 protein, which is an anti-inflammatory molecule (NI et al., 2015). Also, oleic acid demonstrates action on the intestinal microbiota, increasing the diversity of bacteria and favoring the proliferation of beneficial bacteria and reduction of pathogenic ones (ZHAO et al., 2019).

Carotenoids, present mainly in the macauba pulp, have been shown to act in the adipose tissue, interfering with the differentiation of adipocytes, increasing thermogenesis and contributing to the reduction of adipogenesis, which is an important factor in the process of obesity development (BONET et al., 2015). Carotenoids can interact with the PPARγ transcription factor, responsible for adipocyte differentiation, favoring less adipose tissue gain (BONET et al., 2015; RIBOT et al., 2012). Also, carotenoids improved the integrity of the intestinal barrier, reducing inflammation and other diseases related to the maintenance of the intestinal barrier (LYU et al., 2018). Moreover, carotenoids have been shown to inhibit the production of inflammatory cytokines (interleukin-8 and prostaglandin E2) by interacting with NF-kB, contributing to the inhibition of inflammation, and can also reduce oxidative stress through the activation of antioxidant enzymes (glutathione-*S*-transferase), resulting from the interaction of carotenoids with the nuclear factor erythroid 2–related factor 2 pathway (KAULMANN et al., 2015).

Proteins, which are present in all parts of the macauba, especially in the kernel, are essential compounds in several vital functions of the body (KITADA et al., 2019, HIANE et al., 2006). Although proteins of plant origin have less biological value than those of animal origin, they become important for populations with low purchasing power, who have limited access to

animal proteins, as well as people with vegan eating habits (ORTOLÁ et al., 2019). Adequate protein intake is associated with LDL reduction, contributing to the prevention of cardiovascular diseases (WU, 2006). Moreover, inadequate protein consumption can favor changes in the intestinal microbiota, stimulating the excessive growth of pathogenic bacteria and microbial metabolites, increasing the risk of disease (ZHAO et al., 2019). A study evaluating the protein quality of macauba kernel in an experimental model of *Wistar* rats concluded that macauba kernel is a good source of protein, and its consumption reduced bloods lipid and increased short chain fatty acids content (DUARTE et al., 2022).

Fibers, which make up the entire macauba fruit, are related to satiety and consequently obesity control (EVARISTO et al., 2016). In addition, they have been shown to modify the intestinal microbiota through fermentation and thereby promote the production of short-chain fatty acids, which result in positive effects on glucose and lipid metabolism (WADDELL et al. 2022). Fiber fermentation decreased intestinal barrier defects and inflammation in colitic mice through the production of SCFA, which protected the tight junctions and suppressed inflammatory responses in the colon (HUNG et al., 2016).

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3. GENERAL METHODOLOGY

1. Material

The macauba used in this experiment was collected in March 2019, in the city of Araponga, Minas Gerais, Brazil. The macauba was in the mature stage, and after harvesting the macauba was stored in room temperature for 8 days to provide better oil extraction. After this period, the macauba was peeled and pulped manually. The endocarp was broken, and the kernels were extracted. The macauba pulp was subjected to drying to reduce the moisture content and obtain oil with better quality. After, pulp and kernel were subjected to oil extraction using a manual hydraulic press (Laboratory Press, Fred S. Carver Inc- Summit, New Jersey-USA). The oil was centrifuged at 5000 rpm for 15 minutes. Shell, pulp press-cake, kernel press-cake and oils were stored in an ultrafreezer -80°C until use.

2. Experimental design

The experiments that make up the thesis were carried out with the following parts of macauba: shell, kernel press-cake, pulp press-cake, kernel oil and pulp oil. Shell, kernel press-cake, pulp press-cake, pulp oil and kernel oil were used to carry out the chemical and bioactive composition experiment, kernel oil and pulp oil for the experiment with cells, and pulp oil for the *in vivo* experiment with animals experimental (mice and *Caenorhabditis elegans*) (Figure 1).

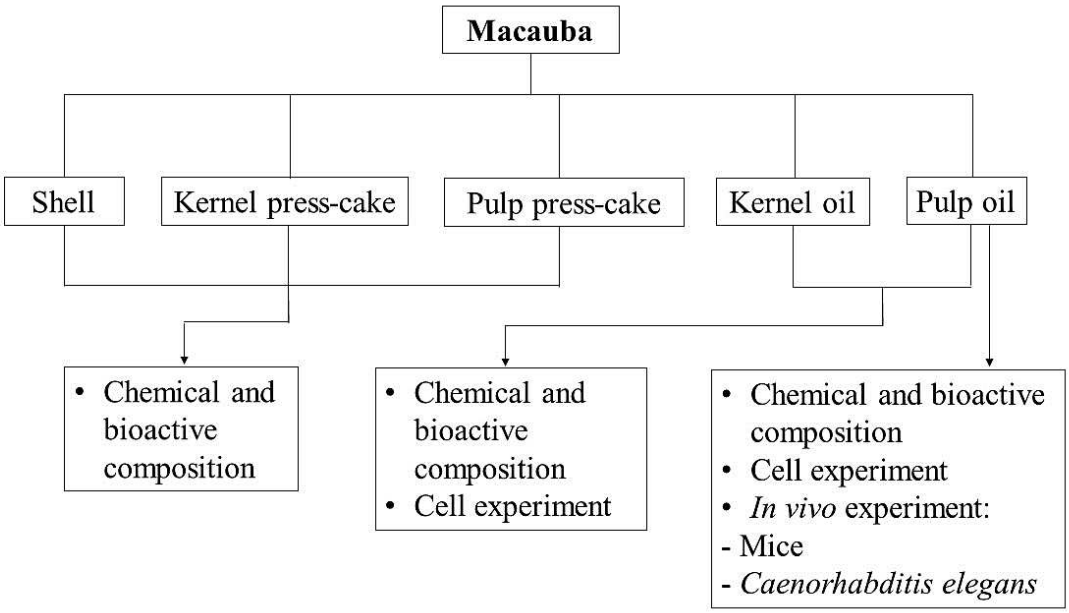

Figure 1. Experimental design of studies.

4. CHAPTER 1: MACAUBA (*Acrocomia aculeata*): PROMISING SOURCE OF NUTRIENTS AND ASSOCIATION WITH HEALTH BENEFITS, A REVIEW

MACAUBA (*Acrocomia aculeata*): PROMISING SOURCE OF NUTRIENTS AND ASSOCIATION WITH HEALTH BENEFITS, A REVIEW

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Abstract

Macauba is widely distributed in Brazilian territory. In addition to being used in the biodiesel production and animal food, it is also rich in bioactive compounds. Therefore, the objective of this review is to present the nutritional composition, the main bioactive compounds, and the potential health benefits of macauba. Macauba has high content of carotenoids, tocopherols, proteins, phenolic compounds, oleic acid, and fiber. Due the presence of bioactive compounds, macauba have the potential to promote health and can be used to prevent or treatment chronic non-communicable disease. Furthermore, this fruit and their by-products can be used in the development of new food and pharmaceutical products, promoting benefit family farming and contribute to the health benefits.

Keywords: macauba; bioactive compounds; health benefits.

1. Introduction

Changes related to food consumption patterns, the food production chain and environmental factors make it necessary to look for food sources that contribute to meet the industry's demand, associated with health benefits and environmental preservation (Le et al. 2020). In this sense, the appreciation of native cultures is of great importance to contribute to these issues and provide economic and public health benefits.

Macauba (*Acrocomia aculeata*) is a palm tree present in great extension in the Brazilian territory, present in the states of Minas Gerais, São Paulo, Rio de Janeiro, extending through the center-west, north and northeast of Brazil. Its fruit is composed of distinct parts: shell, pulp, endocarp and kernel. The shell can be used in handicrafts, the pulp in obtaining oil for the manufacture of biodiesel and the pulp press-cake, its co-product, in animal feed, the endocarp used in the production of charcoal and the kernel in obtaining oil used in the cosmetic industry (Vianna et al. 2017). However, macauba is also used in human food and its co-products have great potential for application in the food industry (Lima et al., 2018).

Macauba pulp and kernel are consumed in natura or in regional culinary preparations, and present compounds of interest to health, such as carotenoids, dietary fiber and proteins (Coimbra; Jorge 2011). Among the products obtained from macauba, oil stands out, with two types, pulp and kernel, with different compositions. Kernel oil stands out for its high fatty acid profile in lauric and oleic acids, while pulp oil stands out for its expressive content of oleic and carotenoids and tocopherol (Coimbra; Jorge 2011; Lieb et al. 2019).

Due to its chemical composition, with a high content of bioactive compounds, such as carotenoids, tocopherol, dietary fiber, proteins, oleic acid, macauba has great potential to act in the prevention or reduction of several pathologies that have a great impact on health. Thus, as macauba is a fruit present in the Brazilian flora and still little explored and with possible health potential, the objective of this review is to present the physical-chemical and nutritional aspects, the main bioactive compounds, and the association of health benefits attributed to consumption of macauba.

2. Material and Method

This work was carried out through a narrative literature review, according to Brizola; Fantin (2016). For the construction of this work, scientific articles were searched in the following

databases: Scientific Electronic Library Online (SciELO), National Library Medicine (NIH), PubMed, ScienceDirect, Elsevier.

The bibliographic research was carried out based on the search for the following keywords: macauba, *acrocomia aculeata*, bocaiuva, carotenoids, oleic acid, phenolic compounds, fiber, bioactive compounds, proteins, tocopherol. Articles published from 2000 to 2022, in English and Portuguese, were delimited with inclusion criteria. The articles resulting from the research were evaluated and those of greater importance for the proposed questioning were included.

3. Results and Discussion

3.1. Botanical description and chemical composition of macauba

Macauba is a palm tree with a wide geographic distribution in the Americas, and in Brazil it is considered as the one with the greatest dispersion with natural occurrence in almost the entire territory. However, the highest concentrations are located in Minas Gerais, Goiás, Mato Grosso and Mato Grosso do Sul, being widely spread across cerrado areas (Lima et al. 2018). Macauba reaches a stem of 10 to 15 m in height and 20 to 30 cm in diameter. The node region is covered with dark, pointed spines about 10 cm long, and the green leaves are 4 to 5 meters long and thorns in the central region. The fruits are arranged in bunches and have a spherical or slightly flattened shape, with a diameter ranging from 2.5 to 5.0 cm (Sanjinez-Argandona; Chuba 2011).

Fruiting occurs throughout the year and the fruits ripen mainly between September and January and their exploitation is still rudimentary, traditionally linked to community extractivism (Pires et al. 2013). The fruit is composed of four parts: shell (epicarp), pulp (mesocarp), endocarp and kernel. The epicarp breaks easily when mature. The mesocarp is fibrous, mucilaginous, yellow in color. The endocarp has a hard consistency, and the oilseeds are white in color and covered with a thin layer of integument. Each fruit usually contains a seed surrounded by a hard and dark endocarp (Vianna et al. 2017).

Macauba kernel stands out for its high protein and dietary fiber content, and macauba pulp also has a significant content of protein and dietary fiber (Table 1), and a high content of carotenoids, with a content of 300 – 348 μg g^{-1} of total carotenoids and 49 – 59 μg g^{-1} of β-carotene (Hiana et al. 2006; Coimbra; Jorge 2012). However, the great highlight related to macauba is based on the expressive content of lipids, both related to the pulp and the kernel (Lieb

et al. 2019). Pulp and kernel oils have different lipid profile and composition (Table 2). The pulp oil has an orange color due to the presence of carotenoids, being an oil of the oleic/palmitic type composed mainly of oleic acid (C18:1). Kernel oil is light in color, with a high content of saturated fatty acids, predominantly lauric acid (Coimbra; Jorge 2012; Lieb et al. 2019). Kernel oil is similar to coconut oil, but it has the important characteristic of not only having a high content of lauric acid, but also having an outstanding percentage of oleic acid, which are important characteristics related to health (Lescano et al. 2015; Río et al. 2016). Pulp oil has been popularly known as "cerrado olive oil", because its characteristics are similar to olive oil (Coimbra; Jorge 2011).

Other bioactive compounds are present in macauba, such as tocopherols and phenolic compounds. Macauba pulp has a higher value of tocopherol when compared to kernel, 212 mg kg^{-1} and 23 mg kg^{-1}, respectivelly (Coimbra; Jorge 2012; Schex et al. 2018). Study carried out by Oliveira et al. (2017) evaluating the content of total phenolics present in macauba oils showed 3.9 mg GAE/100g in pulp oil and 0.70 mg GAE/100g in kernel oil.

Table 1. Macauba chemical composition (%) (wet base).

	Pulp	Kernel
Moisture	4.20 – 5.98	2.52 – 4.97
Ash	1.50 – 2.17	1.86 – 2.08
Protein	5.31 – 6.72	16.44 – 28.61
Lipid	23.00 – 42.00	46.00 – 65.00
Total dietary fiber	13.89 – 20.26	12.49 – 15.81
Carbohydrate	6.22 – 6.92	5.81 – 6.06

Source: Adapted from Lescano et al. (2015); Evaristo et al. (2016); Coimbra; Jorge (2011).

Table 2. Fatty acid profile of macauba pulp and kernel oils.

Fatty acid	Pulp oil	Kernel oil
Caprylic (C8:0)	-	3.67 – 4.90
Capric (C10:0)	-	2.79 – 3.27
Lauric (C12:0)	0.39 – 0.74	39.02 – 43.75

Mystic (C14:0)	0.38 – 0.41	9.21 – 13.40
Palmitic (C16:0)	19.10 – 24.70	8.25 – 9.20
Palmitoleic (C16:1)	3.75 – 4.28	-
Stearic (C18:0)	1.62 – 1.70	2.24 – 2.93
Oleic (C18:1)	52.57 – 64.90	26.27 – 32.70
Linoleic (C18:2)	5.54 – 13.80	3.08 – 3.82
Linolenic (C18:3)	1.40 – 2.26	-
Saturated	21.40 – 27.10	59.91 -62.84
Unsaturated	72.90 – 78.60	36.14 – 40.09

Source: Adapted from Coimbra; Jorge (2011), Lieb et al. (2019), Munhoz et al. (2018).

3.2. Macauba and its potential use in human food

Despite its abundant fruiting and other characteristics, macauba is a wild palm tree and in almost the entire national territory it has been exploited domestically, well below its economic potential, and its exploitation is still carried out in a rudimentary way, traditionally associated with to community extractivism (Evaristo et al. 2016). The use of macauba resources is very diverse, and all its parts can be used in different sectors. The shell can be used as wood, the leaves serve as raw material for crafts and animal fodder. The press-cake, a co-product of the extraction of oil, has a high production and can be used as a component of animal feed. The endocarp can be used as charcoal and in the replacement of concrete gravel, or even as craft material. The kernel can be used to obtain oil and be used as an ingredient in the manufacture of soap and cosmetics (César et al. 2015; Evaristo et al. 2016; Ramos et al. 2008).

Macauba emerged as a promising raw material for the production of biodiesel due to its expressive oil content, high productivity, and good adaptation to different climatic conditions (Evaristo et al. 2016). Another relevant aspect for the use of macauba in the production of biofuels is due to the fact that its fruit generates a high number of co-products that can be used (César et al. 2015). Macauba pulp press-cake can be used in animal feed, especially for ruminants, as a substitute for traditionally used foods. This substitution triggers positive points related to cost reduction, given the high yield of this co-product and its low cost, associated with nutritional benefits for animals due to its fiber content (Riqueira et al. 2017).

Despite its prominent use in the production of biodiesel and animal feed, the uses of macauba and its co-products in human food are present great potential, due to the nutritional value. A study with the development of alfajor using macauba pulp flour proved to be well accepted by children and even added a higher fiber and lipid content to the product, improving the nutritional profile of the product when compared to the control product (Rodrigues et al. 2017). The press-cake, a co-product of the extraction of oil, can be transformed into ingredients for human consumption, due to its high composition in dietary fiber, carotenoids and oil with a high oleic content, and be used in the formulation of cakes, breads and cookies (Dessimoni-Pinto et al. 2010).

The great potential of macauba use is based on the production of oil, presenting great productivity and oils with characteristics desired by the industrial sectors. Brazil is a major importer of olive oil, as the country has low cultivation of olive trees (International Olive Oil Council 2018). In contrast, macauba is a native plant and is found practically throughout the entire national territory (Lima et al. 2018). Equating the issue of processing and demonstrating similar health benefits between both oils, one can motivate the planting and industrialization of macauba oil, as well as its co-products. Potentially, the final cost of pulp oil should be lower than that practiced for olive oil, making it more accessible to the Brazilian population. Unlike the olive tree, macauba can be cultivated in practically the entire Brazilian territory, due to its adaptability to various environmental conditions (Pires et al. 2013). Considering the high cost of olive oil practiced in the Brazilian market and the possible availability of an alternative from the Brazilian flora itself, an important strategy is the inclusion of macauba pulp oil as a component of the daily diet.

Thus, macauba presents itself as a palm tree with extensive use, and all its parts can be used, whether for use for food purposes or subsidies for agriculture and other sectors, demonstrating the importance of studies and research on the fruit of this palm. In addition, the insertion of macauba in the food sector favors the generation of jobs and appreciation of family farming.

3.3. Correlations between main properties and potential health benefits

Non-communicable chronic diseases such as obesity, diabetes, metabolic syndrome, among others, are currently a major public health problem and have a great impact at an

economic and social level (Apovian et al. 2015). With this, it becomes important, strategies that aim to contribute to the prevention and treatment of these pathologies. Among them, food and compounds present in foods that can trigger benefits in these pathological conditions stand out (WHO 2018). In this sense, macauba has beneficial potential in the treatment and prevention of diseases, due to the content of protein, fiber, oleic acid, carotenoids, phenolic compounds, and tocopherol.

The high protein content present in macauba, especially in kernel, may favor health promotion and adequate consumption of this extremely important macronutrient. Proteins are characterized by being of vital importance to living beings. Its functions range from catalysis of chemical reactions, transport of other molecules, transmission of nerve impulses, immune protection, hormonal function, among others (Shevkani et al. 2021). It has been suggested that the possible association between cardiovascular disease and protein intake is caused by the effect of protein intake on plasma LDL and weight maintenance (Wu 2016). A recent study using macauba kernel as a protein source improved the lipid profile and short-chain fatty acid content in rats (Duarte et al. 2022). In addition to proteins, as a significant source of dietary fiber, the consumption of macauba can be considered an important functional food in clinical conditions such as preventing obesity, reducing blood glucose, and also playing an important role in modifying the intestinal microbiota. There is a high association between the consumption of dietary fibers and the modification of the intestinal microbiota, resulting from the production of short chain fatty acids (SCFAs) by the fermentation of dietary fibers, culminating in positive effects on lipid and glucose metabolism (Barber et al. 2020). In addition, the consumption of dietary fiber helps reduce weight gain and control obesity through the greater satiety mechanism promoted by fiber (Waddell et al. 2022).

The high proportion of oleic acid present in the macauba pulp oil, close to that found in olive oil, raises the hypothesis of a similar biological action on the lipid metabolism alleged to this traditional oilseed (Guasch-Ferre et al. 2020). Oleic acid play a protective role in cardiovascular diseases, it is the preferred substrate of ACAT (acyl-CoA:cholesterol acyltransferase) and thus helps the formation of cholesteryl esters, reducing the amount of free cholesterol in the cell, which induces the activity of LDL receptors in the liver, triggering, therefore, neutral action on cholesterolemia when compared to the consumption of saturated fats (Damgaard et al. 2013; Edwards et al. 2000; Rumsey et al. 1995). Macauba, because it has a lipid

composition similar to olive oil, with a high oleic content, may favor the control of obesity and oxidative stress, through the inhibition of lipogenic pathways, with the ability to inhibit the nuclear translocation of sterol-regulatory element-binding protein 2 (SREBP-2) which is related to the transcription of enzymes involved in the endogenous synthesis of cholesterol (Hernandez-Rodas et al. 2017). Furthermore, oleic acid induces beta-oxidation of fatty acids through peroxisome proliferator-activated receptor gamma (PPARγ), contributing to lower synthesis of triacylglycerols and lower hepatic secretion of VLDL (very low-density lipoprotein) (Ou et al. 2001). Oleic acid is related to reduced inflammation due to the ability to block the NF-κβ complex through the activation of genes responsible for the IL-10 protein, which is a potent anti-inflammatory molecule and lower expression of inflammatory genes (Ni et al. al. 2015).

An important bioactive compound present in macauba pulp are carotenoids, which have important antioxidant activity. They function as regulators of the immune system response and reduce the risk of chronic non-communicable diseases such as cancer, cardiovascular diseases, cataracts, macular degeneration and oxidative stress (Johnson et al. 2018; Wang et al. 2013). The high concentration of carotenoids present in macauba pulp oil can function as a natural antioxidant, capable of reducing the rate of lipid oxidation reactions (Coimbra; Jorge 2011). Carotenoids, in addition to being essential compounds for health maintenance and acting to reduce inflammation by modulating the nuclear factor kappa B (NF-κβ) pathway, are associated with effects on adipose tissue, interfering with adipocyte hypertrophy and differentiation, fatty acid oxidation and thermogenesis, leading to the darkening of white adipose tissue (Bonet et al. 2015). Carotenoids have been shown to affect adipocyte function through interaction with the transcription factor PPARγ, interfering with adipocyte differentiation, as demonstrated in a study using experimental animals and finding an association between carotenoids and lower adipose tissue gain, related to lower PPARγ expression (Bonet et al. 2015; Ribot et al. 2012).

The antioxidant activity of phenolic compounds is mainly due to their reducing property and chemical structure. These characteristics play an important role in the neutralization or scavenging of free radicals and chelation of transition metals, acting both in the initiation stage and in the propagation of the oxidative process (Babbar et al. 2011). This mechanism of action of antioxidants plays an important role in reducing lipid oxidation in tissues, because when incorporated into human food, they reduce the risk of developing pathologies, such as atherosclerosis and dyslipidemia (Toma et al. 2020). Phenolic compounds from food have been

the focus of many studies about their anti-inflammatory, antimicrobial and antilipidemic properties. These compounds prevent peroxidation and oxidative modifications of LDL-c through their antioxidant activities (Gavahian et al. 2019). Additionally, tocopherols also have antioxidant activity and are related to attenuation of oxidative stress and inflammatory response, associated with the effect on thermogenesis with increased expression of the UCP1 gene (gene expressed in brown adipose tissue) in white adipose tissue (Tanaka-Yachi et al. 2018).

4. Conclusion

Macauba presents itself as a promising source of nutrients and bioactive compounds, with great potential for application in the food industry, and also shows a great association with health benefits, and may be a promising functional food, contributing to the prevention of chronic non-communicable diseases. Thus, research that helps to clarify the impact of inserting macauba in human food is of great importance.

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5. CHAPTER 2: MACAUBA (*Acrocomia aculeata*) OILS AND CO-PRODUCTS: CHEMICAL CHARACTERIZATION AND BIOACTIVE PROPERTIES

MACAUBA (*Acrocomia aculeata*) OILS AND CO-PRODUCTS: CHEMICAL CHARACTERIZATION AND BIOACTIVE PROPERTIES

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Abstract

Macauba (*Acrocomia aculeata*) is a palm tree native from Brazil, and its products and co-products have great potential to be used in human food. The objective of the study was to evaluate the chemical characterization of macauba oils and co-products (shell, pulp and kernel press-cakes), and the *in vitro* effect of macauba oils on cell viability and the production of reactive oxygen species (ROS). Macauba co-products showed higher concentrations of dietary fiber (shell 25.59%, pulp press-cake 25.41% and kernel press-cake 20.13%). Kernel press-cake stood out for its high concentration of protein and pulp press-cake high iron. Macauba kernel oil had a higher content of lauric acid (45.10%) and tocopherol (45.22 μg/g), and macauba pulp oil had higher content of oleic acid (49.32%) and carotenoids (207.52 μg/g). Macauba oils were able to reduce the formation of ROS in cell lines (HepG2, A549 and IMR90). Macauba oils and co-products are rich in bioactive compounds and nutrients, and is a promising raw material for to be used in the human food.

Keywords: macauba pulp oil; macauba kernel oil; bioactive compounds; co-products.

1. Introduction

Macauba (*Acrocomia aculeata*) is a Brazilian palm tree, it has a height of 10 to 15 meters, and its fruits are formed in clusters and have a spherical shape.[1] Macauba fruits consist of epicarp (shell), mesocarp (pulp), endocarp and kernel, and it is economically interesting to explore macauba, given its general use, and the fact that all its parts can be used in some segment.[2] The shell can be used in handicrafts, the pulp oil for the biodiesel production, the pulp-press cake in animal feed, the endocarp used in the production of charcoal and the kernel oil in the cosmetic industry. Macauba pulp and kernel are consumed *in nature* or in regional culinary preparations, however, macauba is still little utilized in the human food.[2,3]

Among the products obtained from macauba, the oil stands out, with two types, pulp and kernel oils, with different compositions.[4] Lauric acid is the major fatty acid present in kernel oil, followed by oleic acid, whereas pulp oil has expressive content of oleic acid.[3,5] In relation to macauba co-products, there are no studies on chemical composition and application in human food of shell. Moreover, there is little research on macauba pulp and kernel press-cakes, and their potential applications in human foods.[6]

The complete use of a given raw material has advantages at an economic, environmental and social level, and has become a focus in recent years due to the need for alternative sources of food and nutrients in order to meet the consumption demand of the population, associated with a reduction in the environmental damage generated by large-scale production.[7] In this sense, macauba stands out as promising, given the potential indication of the use of its main products and its co-products in human food. Macauba is a fruit rich in bioactive compounds and nutrients, which can contribute to the prevention or reduction of diseases and confer health benefits related to its consumption.[3]

Previous studies have determined the chemical composition of macauba pulp and kernel, however this is the first study to perform characterization of macauba co-products (shell, pulp and kernel press-cakes), and to evaluate the intracellular antioxidant activity and cytoprotective effects of macauba oils.[4,5] Thus, the objective of the study was to evaluate the chemical characterization of macauba oils and co-products (pulp and kernel press-cakes), and the *in vitro* antioxidant and cytotoxic activity of macauba oils.

2. Material and methods

2.1. Material and experimental design

Macauba fruits were harvested, peeled and pulped (Araponga - Minas Gerais/Brazil). Pulp and kernel oils were extracted using manual hydraulic press (Laboratory Press, Fred S. Carver Inc- Summit, New Jersey-USA). After extraction, the oils were centrifuged at 5000 rpm for 20 minutes. Shell, pulp press-cake, kernel press-cake and the oils were stored in a freezer at -80°C until use for analysis. The experimental design was followed according to Fig. 1.

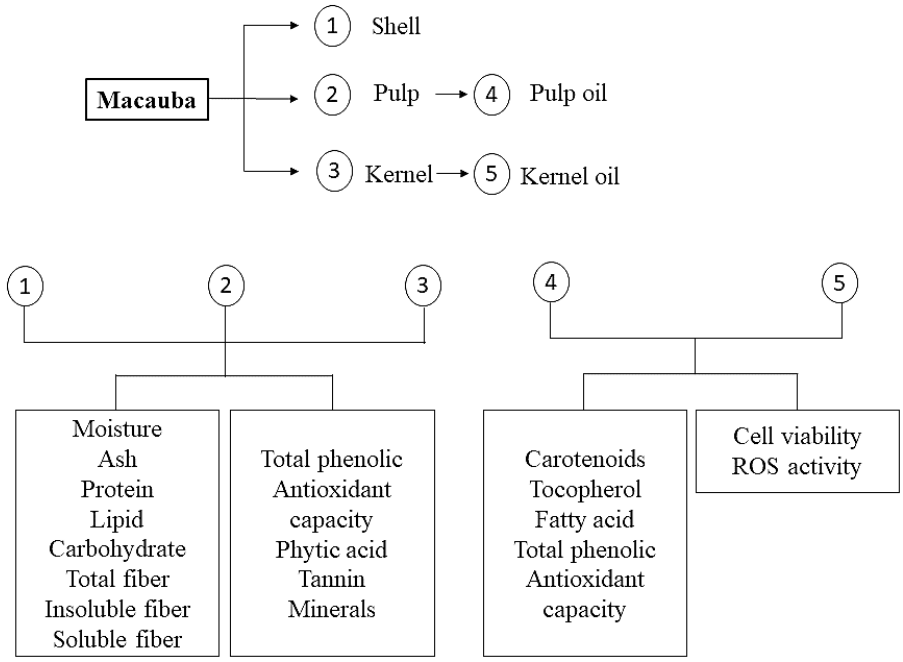

Fig. 1 Experimental design.

2.2. Macronutrients, moisture, ash, total dietary fiber, and minerals analysis of macauba co-products

The analysis of content of moisture, ash, proteins, lipids, and total dietary fiber were performed in three repetitions. Moisture was determined using an oven (Nova Ética®) at 105 °C and ash was quantified using a muffle furnace (Quimis, Q320 M) at 550 °C. Protein content was determined though micro-Kjeldhal method, total dietary fiber was determined by the gravimetric non-enzymatic method and lipids were determined by Soxhlet method.[8] Carbohydrates were calculated as the difference, using the following equation: [100 - (% moisture + % lipids + % proteins + % total dietary fiber + % ash)]. Concentrations of minerals were determined according to the methodology proposed by Gomes (1996).[9]

2.3. Bioactive compounds composition of macauba oils and co-products

Total phenolic compounds were determined spectrophotometrically, using Folin-Ciocalteu reagent and a standard curve of gallic acid. Results were expressed as milligrams gallic acid equivalents per gram of sample (mg GAE g^{-1}).[10]

Antioxidant capacity was assessed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The absorbance reading at 517 nm was performed on a spectrophotometer. The results of DPPH assay were expressed as μM TEAC (Trolox equivalent antioxidant activity) per g sample.[11]

Carotenoid analysis was carried out by high-performance liquid chromatography (HPLC) with detection of 450 nm, using the chromatographic conditions: HPLC system (Shimadzu, SCL 10AT VP, Japan); chromatographic column Phenomenex Gemini RP-18 (250mm×4.6mm, 5 mm), fitted with a guard column RP-18 Phenomenex ODS column (4mm×3mm). The mobile phase consisted of methanol:ethylacetate:acetonitrile (70:20:10, v/v/v) with a flow of 2.0 $mL \cdot min^{-1}$ and a run time of 15 min.[12,13]

Analysis of tocopherol and tocotrienols were determined by AOCS method by HPLC with fluorescence detection of 450 nm, using the chromatographic conditions: silica column 4.6×250 mm with pore of 5 μm, flow of 1.0 mL min^{-1}, and as mobile phase the mixture of 99.5% of *n*-hexane and 0.5% of isopropanol. The concentration values were calculated with the assessment of the peak area of excitation and expressed as separate values for each isomer. The concentration of total tocopherols (μg/g) was expressed as a sum of the major tocopherols.[14]

Tannins were analyzed according to the methodology by Price et al (1978)[15], and phytic acid content was analyzed with kit Phytic acid (phytate)/total phosphorus (K-PHYT 05/17 – Magazyme®).

2.4. Fatty acids composition of macauba oils

The oil was converted to fatty acid methyl esters (FAMEs) to obtain the fatty acid profile.[16] Samples were injected in a gas chromatograph equipped with a Flame Ionization Detector (Shimadzu, GC-2010, Japan) and a capillary column of 100 m x 0.25 mm (SP-2560, Sigma-Aldrich, USA). The analysis was performed by direct injection of 1 μl of the sample. Helium gas was used as the dragging gas and maintained at a constant flow rate of 363 kPa. The FAMEs were separated using a linear heating ramp from 100 °C to 270 °C, at a heating rate of 20

°C mim^{-1}, and high linear velocity for better peak resolution. Peak identification was confirmed by comparison with the standard FAME mix (Supelco 37 FAME mix, Sigma-Aldrich, USA).

2.5. *In vitro* assay on cell lines of macauba oils

2.5.1. Cell viability

The *in vitro* cytotoxic/cytoprotective effect of the macauba pulp and kernel oils were evaluated in relation to A549 (lung adenocarcinoma epithelial cells), Caco-2 (colorectal adenocarcinoma epithelial cells), HepG2 (human hepatocarcinoma cells) and IRM90 (human lung fibroblast) cell lines. The cells were kept in Dulbecco's Modified Eagle's Medium (DMEM/Ham-F12 – Sigma-Aldrich) supplemented with 10% FBS (fetal bovine serum). All culture medium was added with 100 μg/mL penicillin and streptomycin (Sigma-Aldrich). The cell lines were incubated in humidified atmosphere containing 5% CO_2, 5% O_2 and 95% N_2 at 37º C. Cell viability was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich). In brief, the cells were plated into 96-well plates at a density of 10000 cells/well (A549 and Caco-2), 20000 cells/well (HepG2) and 5000 cells/well (IRM90) (100 μL/well). For oil solubilization, dimethylsulfoxide (DMSO - 40%) and Tween (1%) were used and the same was used in the control cells. After 24 hr, the cells were treated with 100 μL serial concentrations (50, 250, 500, or 1000 μg/mL) of the oils. After 48 hr treatment, 10 μL of MTT solution (0.5 mg/mL in milliQ water) was added into each well, and after 4 hr incubation at 37 °C, the supernatant was replaced by 100 μL of dimethyl sulfoxide (DMSO).[17] The absorbance was measured at 570 nm using a microplate reader (Synergy™ H1, Biotek) and Gen5TM data analysis software. The following parameter was determined: IC50: the concentration of the agent that inhibits growth by 50% is the concentration at which (T/C) × 100 = 50, where T = number of cells at time *t* of treatment; C = control cells at time *t* of treatment.[18] The experiments were conducted in quadruplicate.

2.5.2. Intracellular reactive oxygen species activity

Generation of intracellular reactive oxygen species (ROS) was measured by a ROS assay with dichlorofluorescein diacetate (DCFH-DA).[19] Briefly, the cell lines Caco-2, HepG2, A549 and IMR90 cells (60000 cell/well) were treated with different concentrations (100 and 1000 μg/mL) of each oil, which were diluted in DCFH-DA solution (25 mmol/L). The cells were

incubated at 37 °C for 1 hr with the treatments and washed with PBS. For the positive control, the cells were treated with 15 μmol/L H_2O_2, and for the negative control, the cells were only treated with culture medium. Before the measurement, H_2O_2 at 15 μmol/L was in all wells and the fluorescence intensity was measured at an excitation wavelength of 485 nm and at an emission wavelength of 538 nm. The data were expressed as the percentage of fluorescence intensity relative to the control group.

2.6. Statistical analysis

Data were submitted to analysis of variance (ANOVA) and analyzed using a t-test to verify the difference between two groups, and Tukey test was performed to investigate differences between three or more groups. p-value ≤ 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism® version 8.0 (GraphPad Software, USA).

3. Results

3.1. Proximate composition of macauba co-products

The co-products of oil extraction (pulp and kernel press-cake) had high lipid content, even after oil extraction. About protein content, kernel press-cake showed higher content. All macauba co-products have a high content of total dietary fiber, and the pulp press-cake has a higher content of soluble fiber, while the shell and kernel press-cake have more of the insoluble fiber (Table 1).

Table 1 Proximate composition of macauba co-products (wet basis) (g 100 g^{-1}).

	Shell	Pulp press-cake	Kernel press-cake
Moisture	5.82 ± 0.90^{b}	8.49 ± 0.84^{a}	9.02 ± 0.97^{a}
Ash	2.02 ± 0.10^{c}	2.37 ± 0.27^{b}	4.75 ± 0.61^{a}
Lipids	4.58 ± 0.23^{c}	20.44 ± 0.39^{b}	21.47 ± 0.26^{a}
Protein	3.85 ± 0.01^{c}	5.98 ± 0.26^{b}	19.94 ± 0.69^{a}
Carbohydrate	58.14 ± 0.98^{a}	37.31 ± 0.98^{b}	24.69 ± 0.59^{c}
Total dietary fiber	25.59 ± 0.08^{a}	25.41 ± 0.17^{a}	20.13 ± 0.04^{b}
Soluble fiber	7.50 ± 0.04^{b}	14.19 ± 0.16^{a}	2.88 ± 0.03^{c}
Insoluble fiber	18.09 ± 0.06^{a}	11.21 ± 0.03^{c}	17.25 ± 0.11^{b}

Data expressed as mean ± standard deviation (n=3). Different letters on the same line mean statistical difference by Tukey test ($p \leq 0.05$).

Among the minerals presented in macauba shell, potassium, phosphorus and sulfur were highlights, while the kernel press-cake presented highest magnesium, phosphorus, and potassium content, and pulp press-cake presented highest potassium, magnesium and calcium content, with emphasis on the iron content in this co-product (Table 2).

Table 2 Mineral composition in macauba co-products (mg/kg).

Mineral	Shell	Pulp press-cake	Kernel press-cake
Phosphorus	1,164.2 ± 21.8[b]	1,171.0 ± 25.7[b]	3,267.1 ± 32.5a
Potassium	16,340.3 ± 270.6[a]	13,995.1 ± 22.6[b]	2,779.3 ± 86.3[c]
Calcium	649.5 ± 8.9[c]	1,654.5 ± 7.2[a]	953.6 ± 10.41[b]
Magnesium	592.4 ± 11.7[c]	2,200.5 ± 34.5[a]	1,558.3 ± 10.9[b]
Sulfur	785.8 ± 6.7[b]	1,061.1 ± 19.1[a]	1,094.3 ± 14.2[a]
Copper	9.01 ± 0.18[a]	9.60 ± 0.11[a]	4.89 ± 0.06[b]
Iron	41.28 ± 1.05[b]	92.14 ± 1.67[a]	31.43 ± 0.06[c]
Zinc	9.02 ± 0.20[b]	8.36 ± 0.13[c]	17.91 ± 0.25[a]
Manganese	4.26 ± 0.03[c]	6.38 ± 0.14[b]	34.75 ± 0.15[a]
Sodium	54.99 ± 1.1[a]	42.49 ± 0.98[b]	25.95 ± 0.17[c]
Boron	6.84 ± 0.74[a]	6.83 ± 0.53[a]	2.03 ± 0.37[b]

Data expressed as mean ± standard deviation (n=2). Different letters on the same line mean statistical difference by Tukey test ($p \leq 0.05$).

3.2. Bioactive compounds composition of macauba oils and co-products

Macauba oils showed the highest level of phenolic compounds (Figure 2 – A) and antioxidant capacity (Fig. 2 – B), highlighting the macauba pulp oil. Higher tannin content was found in the shell (Figure 2 – C) and the phytic acid content was similar in the macauba co-products (Fig. 2 – D).

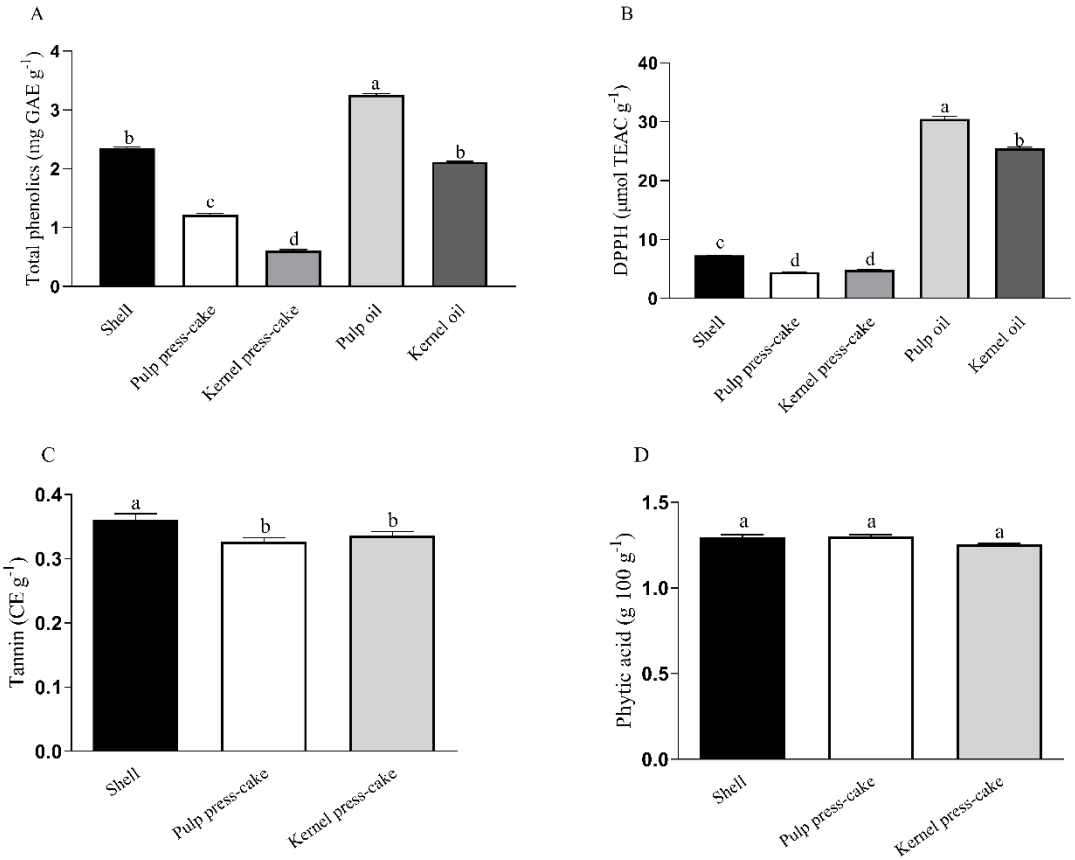

Fig. 2 Total phenolics (A), Antioxidant (B), Tannin (C) and Phytic acid (D) of macauba co-products and oils. Different letters mean statistical difference by Tukey test ($p \leq 0.05$). Data expressed as mean ± standard deviation (n=3). GAE: galic acid equivalent; TEAC: Trolox equivalent antioxidant capacity; CE: catechin equivalent.

Macauba pulp oil showed a high carotenoids content compared to kernel oil. Still, it stood out in high content of β-carotene, representing 78% of the total. Total tocopherol was higher in macauba kernel oil, and α-tocopherol and γ-tocotrienol were predominant isomers. In pulp oil, in addition to α-tocopherol, α-tocotrienol showed high values (Table 3).

Table 3 Carotenoids and tocopherol of macauba oils.

	Macauba pulp oil	Macauba kernel oil
Total carotenoids (µg/g)	207.52 ± 1.02^{a}	1.90 ± 0.07^{b}
β-carotene (µg/g)	163.63 ± 2.05^{a}	1.52 ± 0.79^{b}

α-carotene (μg/g)	21.03 ± 0.34[a]	0.27 ± 0.06[b]
Lutein (μg/g)	8.75 ± 0.10[a]	0.11 ± 0.02[b]
Lycopene (μg/g)	14.11 ± 1.07[a]	ND[b]
Total tocopherol (μg/g)	40.80 ± 0.09[b]	45.22 ± 1.04[a]
α-tocopherol (μg/g)	4.95 ± 1.12[b]	22.77 ± 1.01[a]
β-tocopherol (μg/g)	ND	ND
γ-tocopherol (μg/g)	ND	ND
δ-tocopherol (μg/g)	ND	ND
α-tocotrienol (μg/g)	30.87 ± 1.24[a]	ND[b]
β-tocotrienol (μg/g)	ND[b]	2.02 ± 0.05[a]
γ-tocotrienol (μg/g)	ND[b]	12.01 ± 1.03[a]
δ-tocotrienol (μg/g)	4.98 ± 0.89[b]	8.42 ± 0.13[a]

Different letters on the same line mean statistical difference by t-test ($p \leq 0.05$). Data expressed as mean ± standard deviation (n=3). ND: not detected.

3.3. Fatty acids composition of macauba oils

Macauba pulp oil showed a high content of monounsaturated fatty acids (55%), with 49.32% of oleic acid, and macauba kernel oil high saturated fatty acids (70%), with 45.10% of lauric acid and 26.27% of oleic acid (Table 4).

Table 4 Fatty acid composition of macauba oils (g.100 g^{-1}).

Fatty acids	Macauba pulp oil	Macauba kernel oil
Caprylic (C8:0)	ND	4.99
Capric (C10:0)	ND	3.92
Lauric (C12:0)	ND	45.10
Myristic (C14:0)	ND	8.69
Palmitic (C16:0)	22.84	5.57
Palmitoleic (C16:1)	5.93	ND
Stearic (C18:0)	1.23	2.42
Oleic (C18:1n9c)	49.32	26.27
Linoleic (C18:2n6c)	19.63	3.04

Linolenic (C18:3n3c)	1.05	ND
Saturated	24.07	70.69
Monounsaturated	55.25	26.27
Polyunsaturated	20.68	3.04

ND: not detected.

3.4. Cell viability of macauba oils

The macauba pulp and kernel oil showed a similar IC_{50} value in all cell lines, there was no statistical difference in cell viability between the doses tested (Fig. 3).

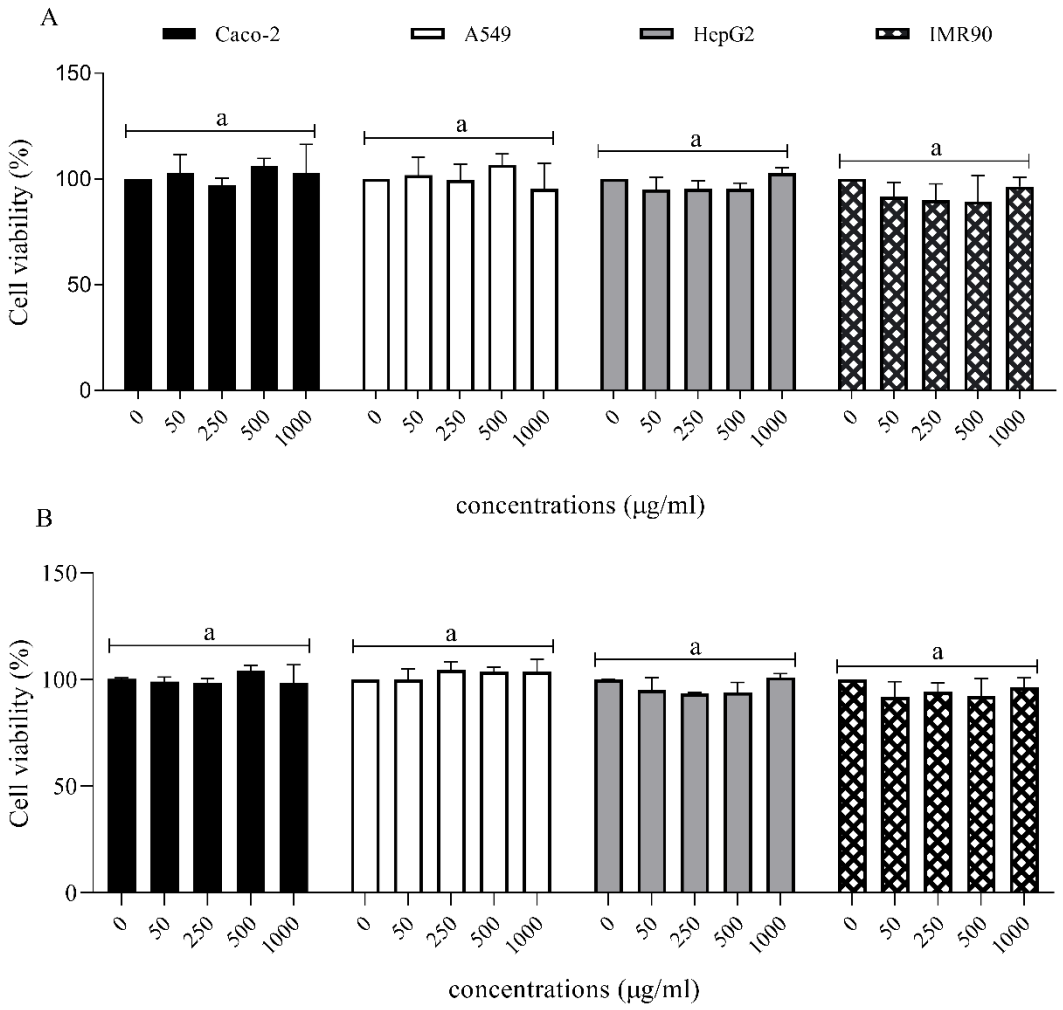

Fig. 3 Cell viability of colorectal adenocarcinoma epithelial (CACO-2), human lung adenocarcinoma epithelial (A549), human hepatoma carcinoma cells (HepG2) and normal lung

cell (IMR90) after 48 h exposure to macauba pulp oil (A) and macauba kernel oil (B). Different letters mean statistical difference by Tukey test ($p \leq 0.05$). Data expressed as mean ± standard deviation. There was no significant difference ($p>0.05$).

3.5. Effect of macauba oils on the formation of intracellular reactive oxygen species

Macauba pulp and kernel oils, in the two doses tested (100 and 1000 μg/mL) did not increase the formation of reactive oxygen species (ROS), maintaining levels similar to spontaneous production (negative control) in all analyzed cells (Fig. 4). In addition, when testing these two doses in the presence of H_2O_2, the oils were able to reduce the formation of reactive oxygen species at levels statistically lower than the group with H_2O_2 (positive control) in A549, HepG2 and IMR90 cells, showing the protective and beneficial effect of these oils in reducing the formation of reactive oxygen species, which is harmful to the cell. Furthermore, in the presence of H_2O_2, at a dose of 1000 μg/mL, in A549 and HepG2 cells, the reduction was comparable to the negative control group. In CACO-2 cell, it maintained ROS levels similar the negative control group, causing no greater increase in the formation of ROS (Fig. 4).

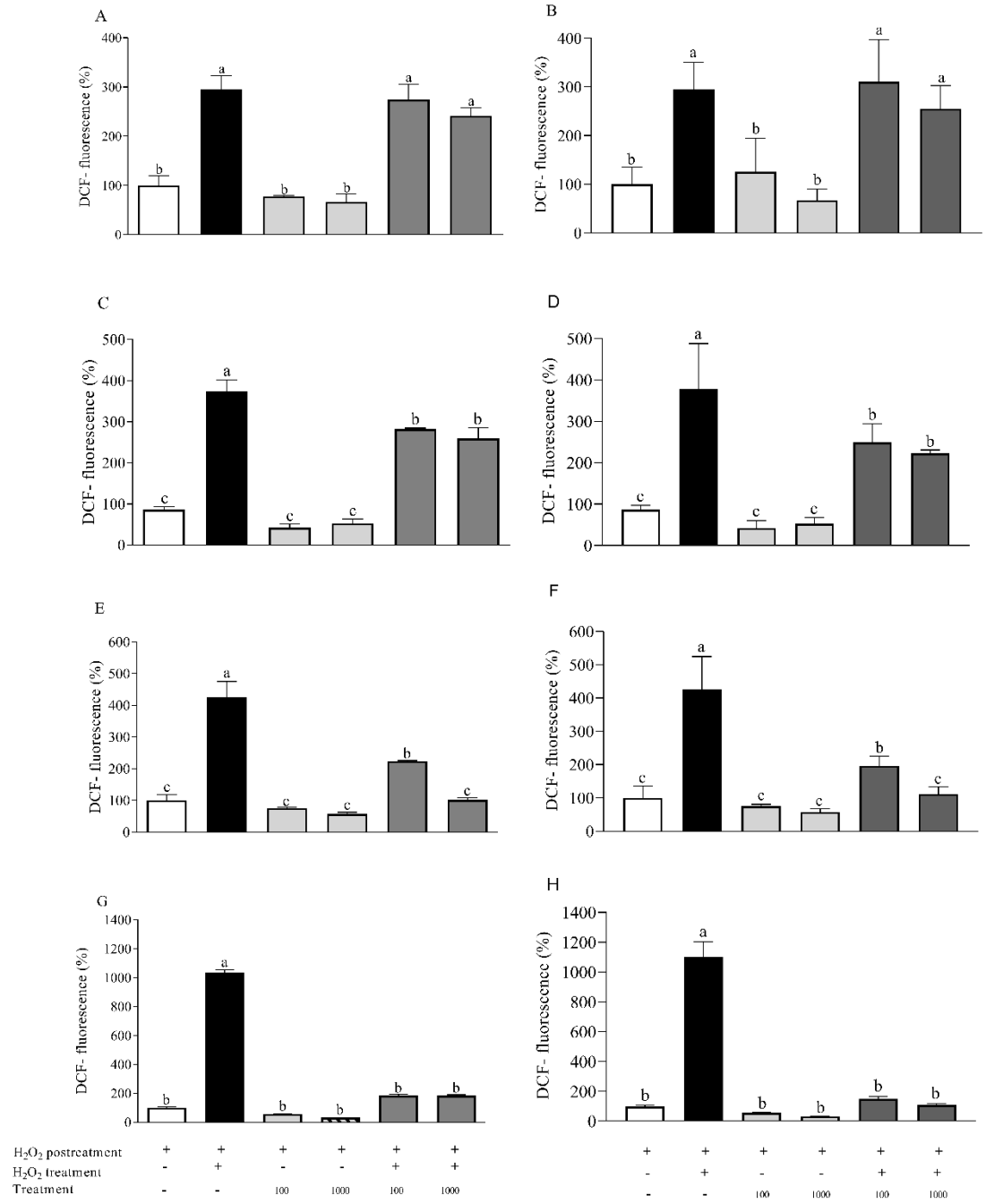

Fig. 4 Effect of macauba pulp and kernel oil on the formation of reactive oxygen species (ROS). (A) Pulp oil - CACO-2; (B) Kernel oil – CACO-2; (C) Pulp oil - IMR90; (D) Kernel oil – IMR90; (E) Pulp oil - A549; (F) Kernel oil – A549; (G) Pulp oil - HepG2; (H) Kernel oil – HepG2. Treatment = macauba pulp and kernel oil at 100 and 1000 µg/mL. Different letters mean statistical difference by Tukey test ($p \leq 0.05$). Data expressed as mean ± standard deviation.

4. Discussion

The present study focused on the characterization of macronutrients and micronutrients of macauba oils and co-products. Knowledge of the composition of macauba co-products can favor the insertion of this in the human food and determine its application in different sectors and implications for health, since macauba is still little explored in relation to human food.

The protein content found in macauba co-products demonstrate that the co-product of kernel oil extraction is a rich source of protein, higher than others vegetable protein sources, such as peanuts and soybean.[20] The protein content present in macauba co-products encourages its application in the human food, because it can be an important alternative related to health since the adequate consumption of protein sources is related to a lower incidence of some diseases.[21] Additionally, it can bring positive points for the food industries, given the current market that seeks sources of vegetable proteins for its application in the formulation of new products, aimed mainly at the vegan public and to supply market demand.[22] Previous studies have also determined protein content of macauba pulp and kernel, however this is the first study that evaluated the protein content of the macauba shell, which is important since this macauba part has not been used.[3,4]

The consumption of 100 g of pulp press-cake accounts for approximately 100% of the total amount of fiber consumption indicated per day, which is about 25 g, while the consumption of 100 g of each kernel press-cake accounts for around 80% of daily needs.[23] Changes in eating habits, with less dietary fiber intake has refocused the food industry on the benefits of incorporating different dietary fiber in the foodstuff, thus, foods that add dietary fiber to formulations are desired by industries.[24] The macauba shell, which is a generally discarded co-product, showed a high dietary fiber content, with an emphasis on the insoluble fraction, with this part of the macauba demonstrating a potential application, becoming a new source of dietary fiber, besides low cost, a point of interest for companies. The results of this study are innovative, as it is the first to determine the content of soluble and insoluble dietary fibers in the macauba co-products. The co-product of extracting the oil from the pulp (pulp press-cake) is noteworthy for its high soluble fiber content. Soluble fiber is fermented by the intestinal microbiota, producing short chain fatty acid, which are an important source of energy for intestinal epithelial cells and contribute to strengthening the intestinal barrier function, and consequently reducing immune responses related to inflammatory diseases.[25]

Macauba co-products showed significant levels of several minerals, which participate in important metabolic functions, corroborating the importance of the intake of these nutrients.[26] The iron content present in the pulp press-cake stands out, with values higher than that of beans, a traditional food source of iron.[27] Iron deficiencies have a great impact on the health of the population, with serious consequences on human health, therefore, the insertion of about 100 grams of macauba pulp press-cake per day can meet the need for this mineral.[28] In this context, this study provides information that enhances the prospects of consumption of macauba fruits, in addition supports and enables future studies for prevention of malnutrition and degenerative diseases.

The values found for some bioactive compounds in the macauba co-products show the potential that macauba parts that would be discarded has. The phenolic content in the macauba co-products allows the obtaining of phenolic extract, with potential for application as a natural antioxidant and as an ingredient in the development of products. There has recently been growing interest in research into the role of plant-derived antioxidants in food because they can protect the human body from free radicals and the effects of ROS (reactive oxygen species) on human health.[29] Regarding tannins and phytates, this is the first study that evaluated the content of these compounds in the macauba. The levels obtained are considered low when compared to other foods, for example beans (phytic acid 2.0 g/100g and tannin 0.50 mg/g) and other Brazilian fruits, as pequi (phytic acid 3.0 g/100g and tannin 0.35 mg/g).[30] Thus, it can be said that these co-products, presents advantages and potential for the sustainable industrial use, favoring its use from a nutritional and industrial point of view.

In relation to macauba oils, these presented specific characteristics that arouse interest in relation to health. Pulp oil with a high oleic fatty acid content is similar to the lipid profile of olive oil, so its applicability in health can trigger benefits, the same already consolidated to olive oil associated with high oleic content.[5,6] In addition, the final cost of macauba pulp oil should be lower than that practiced for olive, making it more accessible to the Brazilian population, triggering gains in greater input in micronutrients and, in the long run, reduction of diseases. Macauba kernel oil, on the other hand, has a lipid profile similar to coconut oil, with a high content of lauric acid, however, it has a content considered oleic acid, which coconut oil does not contain.[3] Thus, macauba kernel oil with a more balanced lipid profile from the nutritional point of view can also be related to health benefits, and for this reason it can is used in the food industry

and in food. The demand for vegetable oils special composition has increased in recent years, due to the change in the dietary pattern of the population, who are looking for foods considered healthier.[31] Thus, macauba is a promising alternative for insertion in the food industry, providing oils with components desirable by the food industry and of interest for health benefits. Therefore, macauba oils prove to be a more economical alternative and may be associated with health benefits and can become an accessible source for the low-income population, due to their high productivity. In addition, the use of these oils will trigger the appreciation of small producers and thereby create jobs and encourage the consumption of a national product.

In addition to a good lipid profile, the pulp oil has a high carotenoid content, with the content obtained greater than consolidated sources of this bioactive compound. By comparison, other studies have found a β-carotene content of 62 μg/g in carrots and 57 μg/g in pumpkins.[32] Vitamin A recommendations for adult individuals are 700 to 900 μg per day, and the consumption of 100 g of macauba pulp oil represents 100% of this recommendation.[33] Important health benefits have been attributed to the carotenoids, can act as antioxidants, due to the ability to sequester and inactivate free radicals, and act against cardiovascular conditions, certain cancers, neurological disorders, strengthen the immune system, macular degeneration, gene activation and inflammatory processes.[7,32] Ramos et al showed that the β-carotene from the macauba pulp is highly bioavailable, when compared to pure β-carotene, and in the case of oil consumption, this further increases bioavailability.[34]

Macauba oils have higher tocopherol content than other oils, such as olive oil, soybeans, and cotton.[3] This is the first study that analyzed the tocotrienols profile of these oils. In the human body, tocopherol exhibits biological activity vitamin E and antioxidant action, acting in several diseases, such as cancer, inflammation and neurodegenerative diseases, and in vegetable oils act protecting the unsaturated fatty acids from lipid oxidation and in the food industry they are used as natural antioxidants in foods.[35] Thus, macauba oils can be used in several applications in the food industry, where tocopherol and tocotrienol play important roles, such as being applied in foods and active packaging, preventing lipid oxidation in products.[35,36] The recommended intake of Vitamin E for adults is 15 mg of tocopherol equivalent, thus, 100 g of macauba pulp and kernel oil corresponds to about 3% and 15% of this recommendation, respectively.[23]

The composition of macauba oils can is related to their cytotoxic and antiproliferative action in cell lines observed in this study. The high IC_{50} value observed indicate that it is

necessary to use higher concentrations to inhibit the growth of half of the cells, that is, macauba oils have low cytotoxicity. Study evaluating the acute toxicity of macauba pulp oil, demonstrated that the LD_{50} is greater than 2000 mg/kg, demonstrating the low toxicity of this oil.[37] Silva et al (2019) demonstrated that the macauba pulp oil did not show cytotoxic activity in cells treated with doses 5 to 500 μg/mL, similar to the current study that evaluated an even higher dose (1000 μg/mL).[38]

The effects of reducing the ROS formation in cell lines may be related to the carotenoids, tocopherol and phenolic compounds present in the oils of macauba. The role of ROS in the pathogenesis of many human diseases is becoming increasingly recognized, and however, an increase in ROS formation in the human body can be blocked by bioactive compounds and antioxidants.[39] Increased ROS levels can cause cellular damage, however, tumor cells can readjust with adequate adaptations to conditions, including hypoxia and increased antioxidant activity, to remove excessive ROS while maintaining protumorigenic signaling, and if ROS levels increase dramatically to toxic concentrations, the c-Jun N-terminal kinase (JNK) pathway can be activated, resulting in apoptosis.[40] However, cell death was not observed in the present study, showing that macauba oils were able to overcome the toxic effects of ROS. The hepatoprotective effect of carotenoids is related to their ability to reduce oxidative stress caused by excess ROS, since carotenoids can be absorbed and accumulated in the liver, and thus, through their mechanism of antioxidant capacity, protect or treat cancer cells in the liver, since their conjugated double bonds allow them to accept electrons from reactive species and thus neutralize ROS.[41] In lung cancer cells, the beneficial effect observed with the reduction of ROS may be associated with tocopherols and carotenoids, based on in the strong correlation between the consumption of these bioactive compounds and the reduction in the incidence of lung cancer, closely related to the antioxidant and anti-inflammatory characteristics of these compounds.[42]

The current scenario aims to make full use of agricultural resources, by minimizing waste and using the potential of the generated co-products, meeting the growing demand for raw material, associated with sustainability.[24] In this sense, the use of co-products, with potential sources of biomolecules, with high added and nutritional value, can be used in food formulation and also as a potential use for extracting bioactive compounds.[20,24] The measure is sustainable against food waste and may add nutritional and environmental benefits, while also stimulating economic growth, from the lower cost of food purchases and the possibility of creating new

jobs.[26] Thus, macauba have great potential for use from nutritional, economic and sustainable point of view.

5. Conclusion

It was demonstrated that macauba oils and co-products has a great potential to be used in the human food. Macauba co-products (shell, pulp and kernel press-cakes) showed high contents of dietary fiber, proteins, minerals, and bioactive compounds. Pulp oil showed rich in oleic acid and carotenoids, and kernel oil in lauric acid and tocopherol, and these oils were able to reduce the formation of reactive oxygen species (ROS) in cell lines (HepG2, A549 and IMR90).

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6. CHAPTER 3: MACAUBA (*Acrocomia aculeata*) PULP OIL PREVENTS ADIPOGENESIS, INFLAMMATION AND OXIDATIVE STRESS IN MICE FED A HIGH-FAT DIET

MACAUBA (*Acrocomia aculeata*) PULP OIL PREVENTS ADIPOGENESIS, INFLAMMATION AND OXIDATIVE STRESS IN MICE FED A HIGH-FAT DIET

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Abstract

Macauba is a palm tree native from Brazil, whose fruits are rich in oil. Macauba pulp oil has high content of oleic acid, carotenoids and tocopherol, but its effect on health is unknown. We hypothesized that macauba pulp oil would prevent adipogenesis and inflammation in mice. Thus, the purpose of this study was to evaluate the effects of macauba pulp oil on metabolic changes in C57Bl/6 mice fed a high-fat diet. Three experimental groups were used (n=10): control diet (CD), high-fat diet (HFD) and high-fat diet with macauba pulp oil (HFM). HFM reduced malondialdehyde, and increased SOD activity and antioxidant capacity (TAC), showing high positive correlations between total tocopherol, oleic acid and carotenoids intake with SOD activity ($r = 0.9087$, $r = 0.8770$ and $r = 0.8585$, respectively). Animals fed the HFM had lower levels of PPAR-γ and NF-κB, which were negatively correlated with the oleic acid intake ($r = -0.7809$ and $r = -0.7831$, respectively). Moreover, consumption of macauba pulp oil reduced inflammatory infiltrate, adipocyte number and length, (mRNA) *TNF-α* and (mRNA) *SREBP-1c* in the adipose tissue, and increased (mRNA) *Adiponectin*. Therefore, macauba pulp oil prevents oxidative stress, inflammation, adipogenesis and increases antioxidant capacity, which highlights its potential against metabolic changes induced by HFD.

Keywords: bioactive compounds; metabolic changes; oleic acid; carotenoid; tocopherol.

1. Introduction

Current eating habits characterized by elevated consumption of saturated fats and simple carbohydrates, and low vitamins are one of the most important causes for the emergence of chronic non-communicable diseases, such as obesity and nonalcoholic fatty liver disease [1, 2]. Obesity is characterized by adipogenesis, which favors the induction of metabolic changes, including changes in cytokine concentrations, activation of inflammatory pathways, and lipotoxic effects in tissues as the liver. As a consequence of these changes, reactive oxygen species (ROS) production may increase, and thus result in exacerbation of inflammation, oxidative stress and cell alterations [3].

Regulation and control of adipogenesis and metabolic changes are performed by specific transcriptional regulators such as peroxisome proliferator activated receptor gamma (PPAR-γ), sterol regulatory element binding protein 1 (SREBP-1), nuclear factor kappa B (NF-κB) [4]. SREBP-1controls fatty acid biosynthesis by favoring the transcription of specific enzymes and activate the PPAR-γ, which controls the expression of genes that regulate adipocyte differentiation, and NF-κB controls the expression of inflammatory genes. In obesity there is an increased in these transcription factors, resulting in increased lipogenesis, resulting in the increase of triacylglycerol and reduction of lipolysis, favoring the development of inflammation and oxidative stress [3, 4]. This way, researches that demonstrate new dietary strategies with the purpose to prevent or control obesity and metabolic alterations become very important, and dietary fatty acid composition demonstrates a significant impact on the diseases development. [5]. Thus, nutritional strategies that aim to treat or prevent these metabolic alterations are of great importance.

Macauba (*Acrocomia aculeata*) is a palm tree present naturally in almost all Brazilian territory and it is considered a promising alternative of vegetable oil for fuel, cosmetics and to the food industry, due to its high oil production and its specific characteristics [6]. Two types of oils are obtained from macauba: pulp and kernel oils, both with important chemical and economical characteristics, highlighting their nutritional action and applications in the food industry [7]. As in olive oil, macauba pulp oil is rich in oleic acid [6]. Oleic acid has been shown to reduce the expression of transcription factors related to the adipogenic signaling pathway, such as PPAR-γ and reduction of oxidative stress markers [8]. Moreover, macauba pulp oil has a high content of carotenoids, which can act to reduce inflammation through NF-κB modulation [6, 9].

Additionally, this oil has tocopherol, which is an important antioxidant, which has been shown to act by improving inflammation and oxidative stress [10]. Thus, macauba pulp oil consumption may be related to improvement in metabolic changes associated with bioactive compounds in its composition [6].

We hypothesized that macauba pulp oil would prevent adipogenesis and inflammation in mice, however, to our best knowledge, no research has been performed to provide evidence of the health benefits of macauba pulp oil. Thus, the objective of this work was to evaluate the effect of macauba pulp oil on the adipogenesis pathways and metabolic changes in mice fed a high-fat diet, this being the first work to explore the health benefits of this promising vegetable oil.

2. Materials and methods

2.1. Materials

Macauba fruits were harvested in Araponga – Minas Gerais (Brazil) in the mature stage and then they were peeled and pulped to obtain the macauba pulp. The pulp was dried at 65°C (CIENLAB CE220, Brazil) for 15 hours. Oil was extracted using a manual hydraulic press (Laboratory Press, Fred S. Carver Inc- Summit, New Jersey, USA), centrifuged (5000 rpm/20 minutes) and then placed in a freezer (-80°C).

2.2. Chemical characterization of macauba pulp oil

To determine the macauba pulp oil fatty acid profile, the oil was converted to fatty acid methyl esters (FAMEs). Samples were injected in a gas chromatograph equipped with a Flame Ionization Detector (GC-FID) (Shimadzu, GC-2010, Japan) and a capillary column of 100 m x 0.25mm (SP-2560, Sigma-Aldrich, USA). The analysis was performed by direct injection of 1 µl of the sample. Helium gas was used as the dragging gas and maintained at a constant flow rate of 363 kPa. The FAMEs were separated using a linear heating ramp from 100 °C to 270 °C, at a heating rate of 20 °C mim^{-1}, and high linear velocity for better peak resolution. Peak identification was confirmed by comparison with the standard FAME mix (Supelco 37 FAME mix, Sigma-Aldrich, USA). In addition to the fatty acid profile, the oleic acid content (mg/g) of the oil was also determined, using an oleic acid standard (Sigma-Aldrich) [11].

Carotenoids analysis was carried out by high-performance liquid chromatography (HPLC) with detection of 450 nm, using the chromatographic conditions: HPLC system (Shimadzu, SCL

10AT VP, Japan); chromatographic column Phenomenex Gemini RP-18 (250mm×4.6mm, 5 mm), fitted with a guard column RP-18 Phenomenex ODS column (4mm×3mm). The mobile phase consisted of methanol:ethylacetate:acetonitrile (70:20:10, v/v/v) with a flow of 2.0 $mL \cdot min^{-1}$ and a run time of 15 min. Total carotenoid content (μg/g) was expressed as a sum of the major carotenoids present in the macauba pulp oil [12].

Total tocopherols content was determined following the AOCS method, using a HPLC with fluorescence detection of 450 nm, using the chromatographic conditions: silica column 4.6×250 mm with the pore of 5 μm, flow of 1.0 mL min^{-1}, and as mobile phase the mixture of 99.5% of *n*-hexane and 0.5% of isopropanol. The concentration of total tocopherols (μg/g) was expressed as a sum of the major tocopherols present in the macauba pulp oil [13].

2.3. Animals and experimental design

Black male mice C57BL/6 (30 animals), 8 weeks old, and with an average weight of 24.34 ± 0.18 g, were allocated into 3 groups with 10 animals each, based to the homogeneity of body weight. The sample calculation equation determined how many animals should be in each group, using the variables: α-error type I = 1.96, α-level = 5%, and data of fat mass mean from Schoemaker et al., 2017 [14, 15]. Individual stainless steel cages were used to keep the animals in a temperature-controlled room (light-dark cycles of 12 hours and temperature of 22 ± 2 °C). Water and respective experimental diets were supplied *ad libitum*.

Experimental diets were formulated according to AIN-93M and high-fat diet, using lard in the high-fat diet [16]. Each experimental group consumed the following diets: control diet – AIN93M (CD); high-fat diet (HFD); high-fat diet with macauba pulp oil (HFM). In the HFM, macauba pulp oil was added in the proportion of 40 g/kg (4%), replacing the soybean oil used in the AIN-93M diet (Table 1). The objective was to verify the effect of macauba pulp oil as a replacement of soybean oil, which is commonly used control diets, and not to perform supplementation. The formulated diets were stored at low temperature (-20°C) and offered to the animals every day.

At the end of 8 weeks, animals with 12 hours of fasting were anesthetized using isoflurane (Isoforine, Cristália), in accordance with the bodyweight of the mice. Using the methodology of cardiac puncture the blood was collected and centrifuged (4 °C at 800×g for 10 min -Fanem-204, São Paulo, Brazil), and the serum was collected and stored at −80 °C. Liver and adipose tissue

(epididymal and subcutaneous) were extracted and stored at (−80 °C) until analysis, and another part was fixed in formaldehyde (10%) for analysis of histological markers. Bodyweight gain and feed consumption were measured on a weekly basis throughout the experiment to calculate the Feed efficiency ratio (weight gain/consumption x 100), and the percentage of adiposity was measured by the weight of the adipose tissue (g) in relation to the total body weight. Body mass index (BMI) was measured by the ratio between weight and naso-anal length (cm) squared [17]. The hepatosomatic index was also determined (liver weight/body weight x 100) [18]. Carotenoid, oleic acid and tocopherol intake were determined by the total amount of diet consumed by the mice. Ethical principles for animal experimentation have been implemented for all processes performed on the animals [19]. Ethics Committee of the Federal University of Viçosa approved this research (Protocol 09/2019; date of approval: May 28th 2019).

Table 1. Composition of experimental diets (g/kg of diet).

Ingredients (g/kg)	CD	HFD	HFM
Albumin*	179.71	179.71	179.71
Dextrinized starch	155	155	155
Sucrose	100	100	100
Soybean oil	40	40	-
Lard	0	312	312
Cellulose	50	50	50
Mineral mix	35	35	35
Vitamin mix	10	10	10
L-cystine	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5
Corn starch	425.99	113.99	113.99
Macauba pulp oil	-	-	40
Carbohydrate (%)	76.9	44.1	44.1
Protein (%)	18.9	18.9	18.9
Lipids (%)	4.20	37	37
Caloric density (kcal g^{-1})	3.85	5.41	5.41

*Purity of 78%. CD: control diet (AIN93M); HFD: high-fat diet; HFM: high-fat diet with macauba pulp oil.

2.4. Biochemical analysis

Biochemical parameters were determined in the serum. Glucose concentration, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), triacylglycerides (TGL), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined based on colorimetric method with commercial kits (Bioclin®, Brazil).

2.5. Homogenate preparation and oxidative stress levels

Liver homogenate was prepared with 200 mg of the liver. The liver was mixed with 1 mM EDTA (pH 7.4), 1000 µL of phosphate buffer (50 mM). The content was macerated and centrifuged (1200×g/8 min/4°C), and the supernatant was collected for analysis of antioxidant enzymes.

For quantification of the enzyme superoxide dismutase (SOD), to the aliquoted homogenate were mixed 249 µL of 50 mM Tris-HCl buffer (pH 8.2) (1 mM EDTA, 6µl MTT (1.25 mM), 15 µl of pyrogallol (10 mM) and 279 µl of buffer). To determine the blank, 6 µl of MTT and 294 µl of buffer were added to the wells, that were incubated for 5 minutes at 37°C and performed the reading on a spectrophotometer at 570 nm (Multiskan GO Thermo Scientific). SOD quantification was expressed as units of SOD/mg protein [20].

Malondialdehyde (MDA) was determined on samples of the homogenate. 400 µL of trichloroacetic acid solution (15%) and thiobarbituric acid (0.375%) was added in 400 µL of the sample. It was placed in a water bath (90 °C/40 min) and then added 600 µL of n-Butanol, and centrifuged (3500 rpm/ 5 minutes). The supernatant was removed and absorbance read at 535 nm (Multiskan GO – *Thermo Scientific*). MDA level was expressed as MDA/mg protein [21].

Catalase was performed on samples of the homogenate as described above. At 0, 30, and 60 s after the reaction was initiated, the absorbance was determined at 240 nm (T70 + UV/VIS Spectrometer). Enzyme activity was reported as µmol per mL of sample data were expressed in U of catalase/mg protein. Catalase activity was calculated according to Lambert Beer's law [22].

For quantification of the nitric oxide 50 µL of the homogenate was used. 1% sulfanilamide solution and 0.1% nafityl ethylene amide dihydrochloride were added. A 0.025 M sodium nitrite standard curve was used, and the absorbance was determined at 570 nm (Multiskan GO – *Thermo Scientific*) [23].

2.6. Total antioxidant capacity of liver and serum

The total antioxidant capacity (TAC) of serum and liver was determined with an Antioxidant Assay Kit (Cayman Chem Corp, MI) Sigma Aldrich®. The absorbance reading was performed at 405 nm (Multiskan GO – *Thermo Scientific*).

2.7. PPAR-γ, PPAR-α, NF-κB and TLR-4 quantification

Adipose tissue and liver samples were homogenized using the NE-PER™ Nuclear and Cytoplasmic Extraction Kit reagents (Thermo Scientific Fisher, USA). The nuclear fractions were assessed by immunoassay using the Mouse PPAR-γ (Peroxisome Proliferator Activated Receptor Gamm – E-EL-M0893, Elabscience, USA), Mouse NF-κB p65 (Factor Nuclear Kappa B – E-EL-M0838, Elabscience, USA), Rat PPAR-α (Peroxisome Proliferator Activated Receptor Alfa – E-EL-R0725, Elabscience, USA), Rat NF-κB p65 (Factor Nuclear Kappa B – E-EL-R0674, Elabscience, USA) and Rat TLR-4 (Toll-like Receptor 4 – E-EL-R0990, Elabscience, USA), ELISA kits, respectively. The microplates that were provided in the ELISA kits were respectively precoated with anti-PPAR-γ, anti-NF-κB p65, anti-PPAR-α, and anti-TLR-4 antibodies. The concentrations were calculated by comparison to the corresponding standard curves.

2.8. Determination of gene expression in adipose tissue and liver by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

TRIzol reagent (Invitrogen, CA, USA) was used to extract total RNA from the liver and a specific kit (mirVana™ miRNA Isolation Kit, Life Technologies) was used to extract RNA from the adipose tissue, according to the manufacturer's protocols. RNA concentration and purity were evaluated by Microdrop plate spectrophotometer Multiskan™ GO (Thermo Scientific, DE, USA). To create cDNA synthesis was used the M-MLV Reverse Transcriptase Kit (Invitrogen, USA). RT-qPCR was used to the gene expression relative quantification using AB StepOne Real Time PCR System equipment and Fast SYBR Green Master Mix (Applied Biosystems, CA, USA) reagent. The initial parameters used were: 20 s at 95 °C and then 40 cycles at 95 °C (3 s), 60 °C (30 s) followed by melting curve analysis. A melting point analysis was performed to improve the specificity and sensitivity of amplifications reactions detected. All primers were designed by using Primer 3 Plus program and obtained from Sigma-Aldrich Brazil Ltda (Table 2). 2-Delta-

Delta C (T) method was used to calculate the gene expression, by using GAPDH and β-actin as a reference and high-fat diet group as control, which as normalized to 1 [24].

Table 2. Sequence of primers used in the RT-qPCR analyses.

Genes	Forward	Reverse
SREBP-1c	CGC TAC CGT TCC TCT ATC AAT GAC	AGT TTC TGG TTG CTG TGC TGT AAG
ADIPOR2	CAT GTT TGC CAC CCC TCA GTA	ATG CAA GGT AGG GAT TCC A
ACC-1	TCA AGA CGG CTC AGG TCA TCA	AGG CGC CAA ACT TCA GCA TC
CPT-1α	GTA AGG CCA CTG ATG AAG GAA GA	ATT TGG GTC CGA GGT TGA CA
LPL	TCA ACC ACA GCA GCA AGA	CCG ATA CAA CCA GTC TAC TAC AA
Adiponectin	ATG AGT ACC AGA CTA ATG AGA C	GGC AGG ATT AAG AGG AAC A
TNF-α	TAT GGC TCA GGG TCC AAC TC	GCT CCA GTG AAT TCG GAA AG
SREBP-1c	GCC GAG ATG TGC GAA CTG	GGA AGT CAC TGT CTT GGT TGT T
β-actin	TTC GTT GCC GGT CCA CC	GCT TTG CAC ATG CCG GAG CC
GAPDH	AGG TTG TCT CCT GTC ACT TC	CTG TTG CTG TAG CCA TAT TC

SREBP-1c: Sterol regulatory element binding proteins 1c; *ADIPOR2*: adiponectin receptor 2; *ACC-1*: acetyl CoA carboxylase 1; *CPT-1α*: carnitine palmitoyl transferase 1 alpha; *LPL*: Lipoprotein lipase; *TNF-α*: Tumor necrosis factor alpha; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

2.9. Histomorphometric analysis of adipose and liver tissue

Paraffin was used to fix samples of adipose tissue and liver.10 cuts per animal were performed (3 μm thick), mounted on glass slides, and stained with hematoxylin and eosin. Analyzes were performed under a light microscope (Leica DM750®). The histological sections images were captured in a 20× objective. Inflammatory infiltrate, number and length of adipocytes were evaluated in the adipose tissue (Image-Pro Plus® 4.5). Liver cellular

components (fat vesicles, inflammatory infiltrate, cytoplasm, and nucleus), for 10 histological fields per animal, were analyzed using a test system with 266 points, obtaining 2660 total points for each animal analyzed (Image J®, Wayne Rasband). The following formula was used to calculate the parameters: $V_v = P_p/P_T$ (P_p=number of points located on the interest structure, $P_{T=}$ total test points in the histological area) [25]. The steatosis degree was determined semi quantitatively according to 5° scale, and fat percentage: degree 0 (< 5%), grade 1 (≥5% and < 25%), grade 2 (≥25% and < 50%), grade 3 (≥50% and < 75%), grade 4 (≥75%) [26].

2.10. Statistical analysis

Data were initially submitted to a Kolmogorov-Smirnov normality test and then analysis of variance (ANOVA) test was applied, followed by the Newman-Keuls test for parametric variables. For correlation analysis, Pearson's Correlation was used. Data with a p-value ≤ 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism® version 8.0 (GraphPad Software, USA).

3. Results

3.1. Chemical characterization of macauba pulp oil

Macauba pulp oil showed a high content of monounsaturated fatty acids (55%), with significant oleic acid content (49.32%), as shown in Table 3. In addition, it had high content of carotenoids and tocopherol (Table 3).

Table 3. Fatty acid profile, carotenoids, and tocopherol contents in macauba pulp oil.

Components	
Palmitic (C16:0)	22.84%
Palmitoleic (C16:1)	5.93%
Stearic (C18:0)	1.23%
Oleic (C18:1n9c)	49.32%
Linoleic (C18:2n6c)	19.63%
Linolenic (C18:3n6c)	1.05%
Oleic acid (mg/g)	199.00

Tocopherol (μg/g)	40.80
Total carotenoids (μg/g)	209.00
β-carotene (μg/g)	163.63
α-carotene (μg/g)	21.03
Lutein (μg/g)	8.75
Lycopene (μg/g)	14.11

Caprylic, capric, lauric and myristic acid are not detected.

3.2. Effect of macauba pulp oil on biometric measures, food intake and lipid profile

Weight gain, body mass index (BMI), food efficiency ratio (FER) did not differ among the experimental groups (p>0.05; Table 4). The CD group had higher food consumption compared to the HFD and HFM groups due to lower caloric density of the AIN93M diet (p<0.0001; Table 4). The CD group had lower percentage of adiposity compared to the HFD and HFM groups (p=0.0018; Table 4).

The group that consumed macauba (HFM) did not differ to the HF group in relation to glucose, triglycerides, TC, LDL, HDL values, hepatic enzymes AST and ALT, and hepatosomatic index (p>0.05; Table 4).

Table 4. Biometric measures, food intake and serum biochemical values of the mice after consuming the experimental diets for 8 weeks.

	CD	HFD	HFM
Weight gain (g)	4.01 ± 1.74^{a}	4.14 ± 2.23^{a}	3.66 ± 2.23^{a}
BMI (g/cm^2)	0.33 ± 0.02^{a}	0.34 ± 0.01^{a}	0.34 ± 0.02^{a}
Adiposity (%)	0.71 ± 0.24^{b}	2.43 ± 1.28^{a}	2.26 ± 1.25^{a}
Food consumption (g/day)	4.07 ± 0.16^{a}	2.53 ± 0.41^{b}	2.63 ± 0.42^{b}
Food efficiency (%)	1.69 ± 0.60^{b}	2.67 ± 1.46^{a}	2.42 ± 1.55^{a}
Hepatosomatic index (%)	3.61 ± 0.29^{a}	3.72 ± 0.28^{a}	3.48 ± 0.29^{a}
Oleic acid intake (mg/day)	-	-	0.52 ± 0.08
Carotenoid intake (μg/day)	-	-	21.96 ± 4.11
Tocopherol intake (mg/day)	0.46 ± 0.01^{a}	0.29 ± 0.03^{b}	0.31 ± 0.03^{b}

Total cholesterol (mg dL^{-1})	151.48 ± 13.79[b]	166.49 ± 15.51[a]	169.91 ± 6.87[a]
Total triglycerides (mg dL^{-1})	79.91 ± 4.71[a]	84.83 ± 5.63[a]	83.06 ± 5.09[a]
HDL-c (mg dL^{-1})	38.13 ± 4.29[a]	37.35 ± 5.79[a]	43.07 ± 5.64[a]
LDL-c (mg dL^{-1})	12.80 ± 2.11[b]	20.64 ± 5.25[a]	20.80 ± 5.20[a]
Glucose (mg dL^{-1})	160.67 ± 44.23[a]	182.58 ± 30.09[a]	177.31 ± 36.68[a]
AST (mg dL^{-1})	88.14 ± 21.88[a]	71.66 ± 21.30[a]	73.39 ± 19.04[a]
ALT (mg dL^{-1})	18.74 ± 9.88[a]	15.71 ± 5.63[a]	18.84 ± 9.26[a]

Data are expressed as the mean ± standard deviation (n = 10). Different lowercase letters in the same row indicate a statistical difference by Newman-Keuls test *($p \leq 0.05$)*. CD: control diet – AIN93M; HFD: high-fat diet; HFM: high-fat diet with macauba pulp oil; BMI: body mass index; HDL-c: high-density lipoprotein; LDL-c: low-density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

3.3. Total antioxidant capacity and oxidative stress markers levels in mice

The HFM group had high SOD (p=0.0078; Figure 1A) and showed positive correlation with carotenoid (r = 0.8585, p=0.004), oleic acid (r = 0.8770, p=0.009) and tocopherol (r = 0.9087, p=0.0003) intake (Figure 1B, C, and D). Macauba pulp oil decreased malondialdehyde (p=0.0057; Figure 1E), showing negative correlation between this parameter and oleic acid (r = -0.9401, p<0.001) and tocopherol (r = -0.9317, p<0.0001) (Figure 1G, and H). Catalase and nitric oxide did not differ among the experimental groups (p>0.05; Figure 1M and N).

The HFM group had higher serum TAC compared to the HFD and CD groups (p=0.0058; Figure 1I), showing positive correlation between serum TAC and oleic acid (r = 0.8967, p=0.005) and tocopherol (r = 0.8430, p=0.013) intake from macauba pulp oil (Figure 1K and L). Liver TAC did not differ between the experimental groups (p>0.05; Figure 1O).

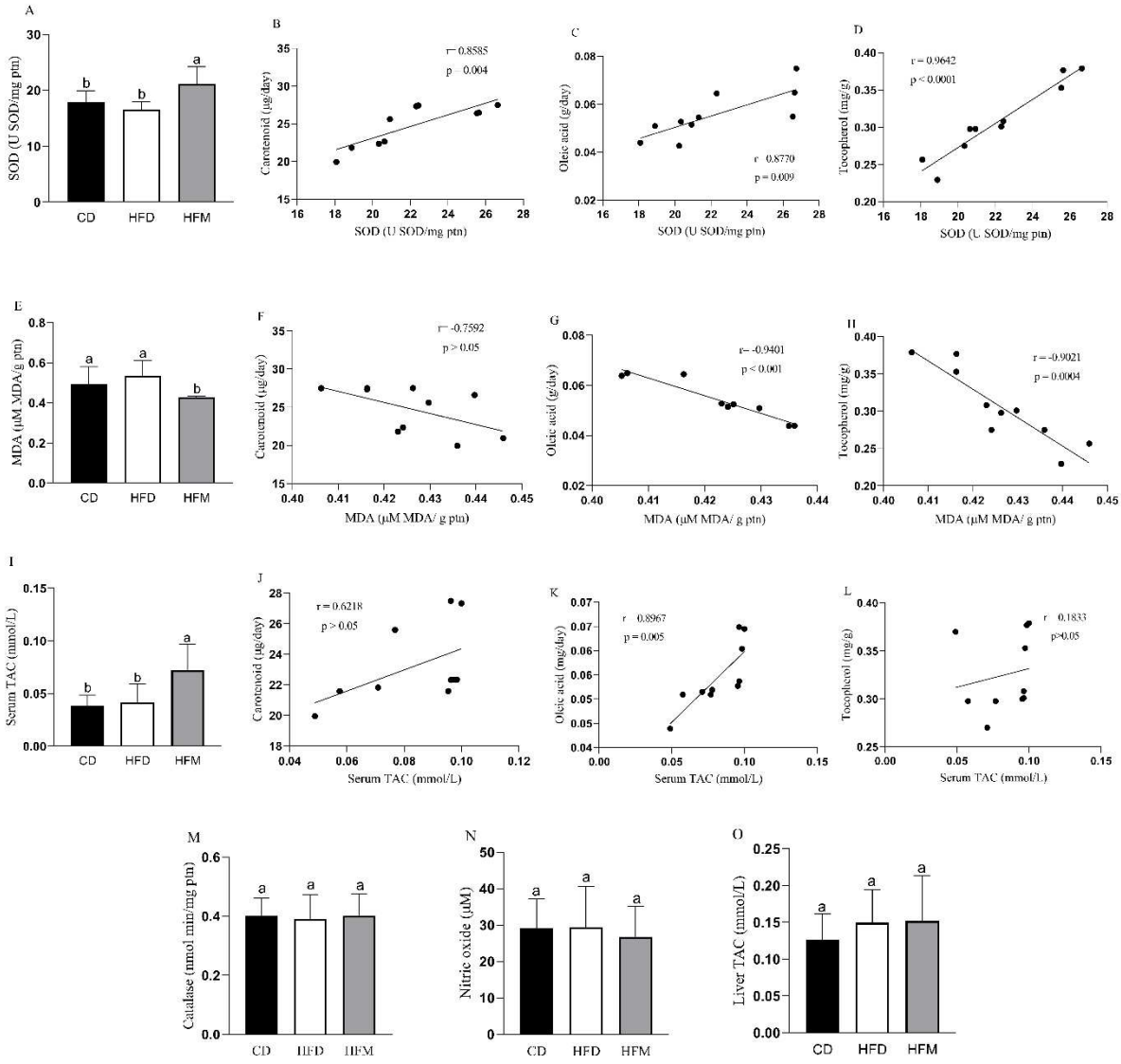

Figure 1. Oxidative stress level and antioxidant capacity of mice after consuming the experimental diets for 8 weeks and correlation with carotenoid, oleic acid and tocopherol intake. Data are expressed as the mean ± standard deviation (n = 10). Different letters indicate a statistical difference by Newman-Keuls test ($p \leq 0.05$). CD: control diet – AIN93M; HFD: high-fat diet; HFM: high-fat diet with macauba pulp oil; SOD: superoxide dismutase; MDA: malondialdehyde; TAC: total antioxidant capacity.

3.4. Effects of macauba pulp oil on NF-κB, TLR-4, and PPAR-(α, γ) quantification

The HFM group had lower nuclear quantification of NF-κB in the adipose tissue compared to the HFD and CD groups (p=0.0179; Figure 2A), showing negative correlation with oleic acid (r = -0.7831, p=0.037) and tocopherol (r = - 0.8144, p=0.035) intake (Figure 2C and D). Macauba pulp oil reduced PPAR-γ quantification (p=0.056; Figure 2E), showing negative

correlation with carotenoid (r = - 0.7301, *p*=0.021) and oleic acid (r = - 0.7809, *p*=0.022) (Figure 2F and G).

NF-κB, PPAR-α, and TLR-4, present in the nuclear fraction in the liver did not differ among the experimental groups (*p*>0.05; Figure 2I, J, and K).

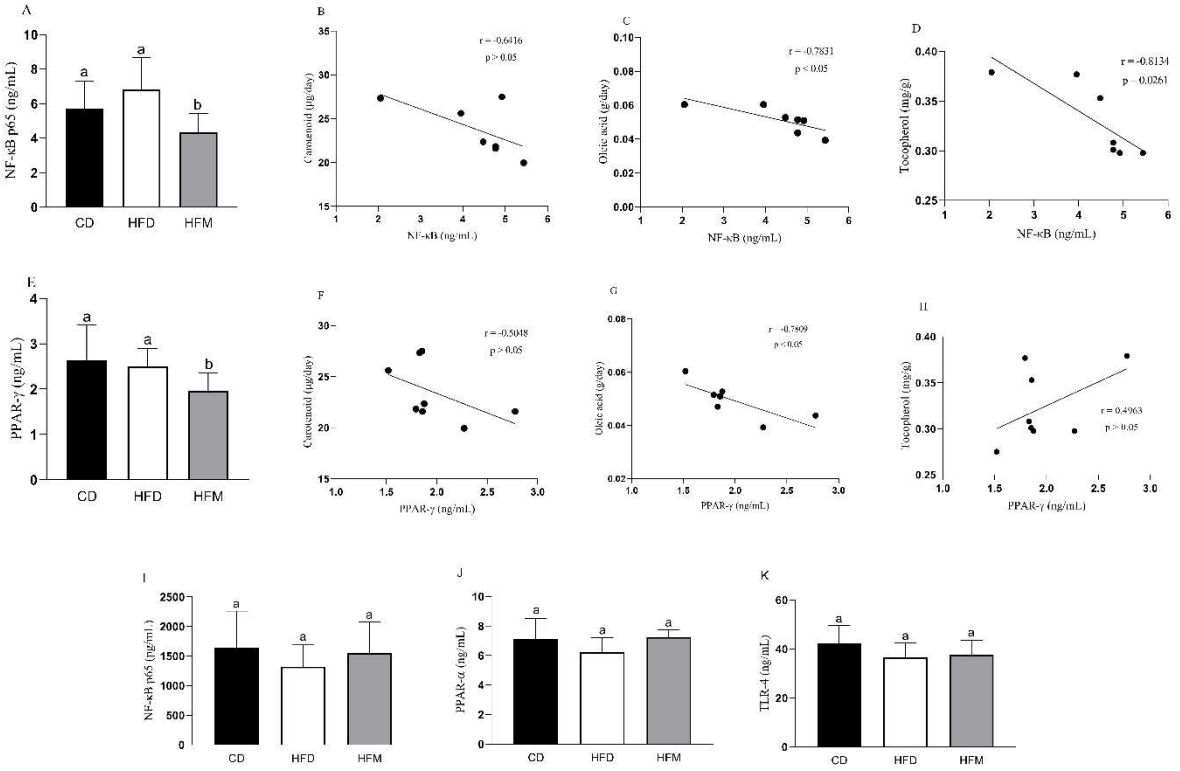

Figure 2. Levels of proteins in the adipose tissue (A – H) and liver (I – K) of mice after consuming the experimental diets for 8 weeks and correlation with carotenoid, oleic acid and tocopherol intake. Data are expressed as the mean ± standard deviation (n = 8). Different letters indicate a statistical difference by Newman-Keuls test (*p*≤0.05). CD: control diet – AIN93M; HFD: high-fat diet; HFM: high-fat diet with macauba pulp oil; NF-κB: nuclear factor kappa B; PPAR: Peroxisome proliferator activated receptor; TLR-4: Toll-like receptor 4.

3.5. Effects of macauba pulp oil on the gene expression in adipose and hepatic tissue

In the liver, in the HFM group, the mRNA expression of *SREBP-1c* was significantly increased compared to control groups (*p*<0.0001; Figure 3A), and (mRNA) *CPT-1α* was decreased (*p*=0.0031; Figure 3B). mRNA expression of *ACC-1α* and *AdipoR2* did not differ in relation to HFD (*p*>0.05; Figure 3C and D).

In the adipose tissue, in the HFM group, the mRNA expression of *SREBP-1c* (p<0.0001; Figure 3E) and (mRNA) *TNF-α* (p<0.0001; Figure 3H) were significantly decreased compared to HFD, and mRNA expression of *Adiponectin* was similar between HFM and CD groups (p>0.05; Figure 3G). mRNA expression of *LPL* was similar among groups (p>0.05; Figure 3F). Correlation analysis showed negative correlation with mRNA *SREBP-1c* and carotenoid intake (r = -0.8991, p=0.012), positive correlation with mRNA *Adiponectin* and carotenoid intake (r = 0.9130, p<0.001), and negative correlation with mRNA *TNF-α* and oleic acid intake (r = -0.9057, p=0.0009).

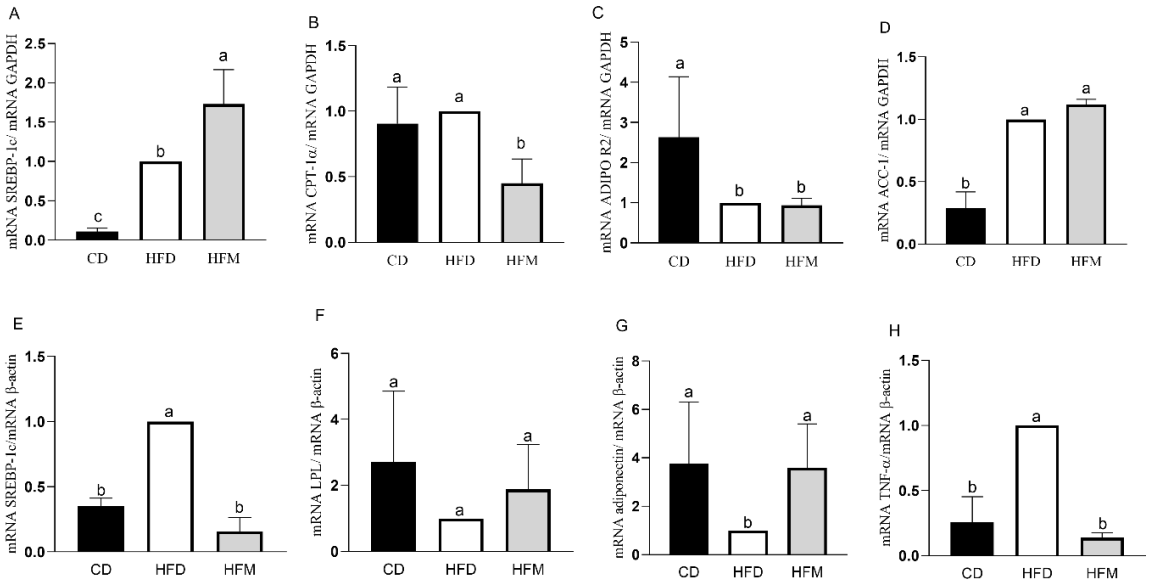

Figure 3. Gene expression in the liver (A – D) and adipose tissue (E – H) of mice after consuming the experimental diets for 8 weeks. Dates are expressed as the mean ± standard deviation (n = 8). Different letters indicate a statistical difference by Newman-Keuls test (p≤0.05). CD: control diet – AIN93M; HFD: high-fat diet; HFM: high-fat diet with macauba pulp oil; SREBP-1c: Sterol regulatory element binding proteins 1c; ADIPOR2: adiponectin receptor 2; ACC-1: acetyl CoA carboxilase 1; CPT-1α: carnitine palmitoyl transferase 1 alpha; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; LPL: Lipoprotein lipase; TNF-α: Tumor necrosis factor alpha.

3.6. Effects of macauba pulp oil on histological morphometrics of liver and adipose tissue

The percentage of the nucleus, cytoplasm, inflammatory infiltrate, and fat deposition in the hepatocytes did not differ among the groups (p>0.05, Figure 4A). The control group was identified as steatosis grade 0, the HFD and HFM group increased the steatosis to grade 1 and had similar values between them (Figure 4B). The HFM group had lower inflammatory infiltrate

(p<0.0001), adipocyte number (p=0.0027) and length (p=0.0088) in the adipose tissue compared to the HFD group and similar to CD group (Figure 4C and D).

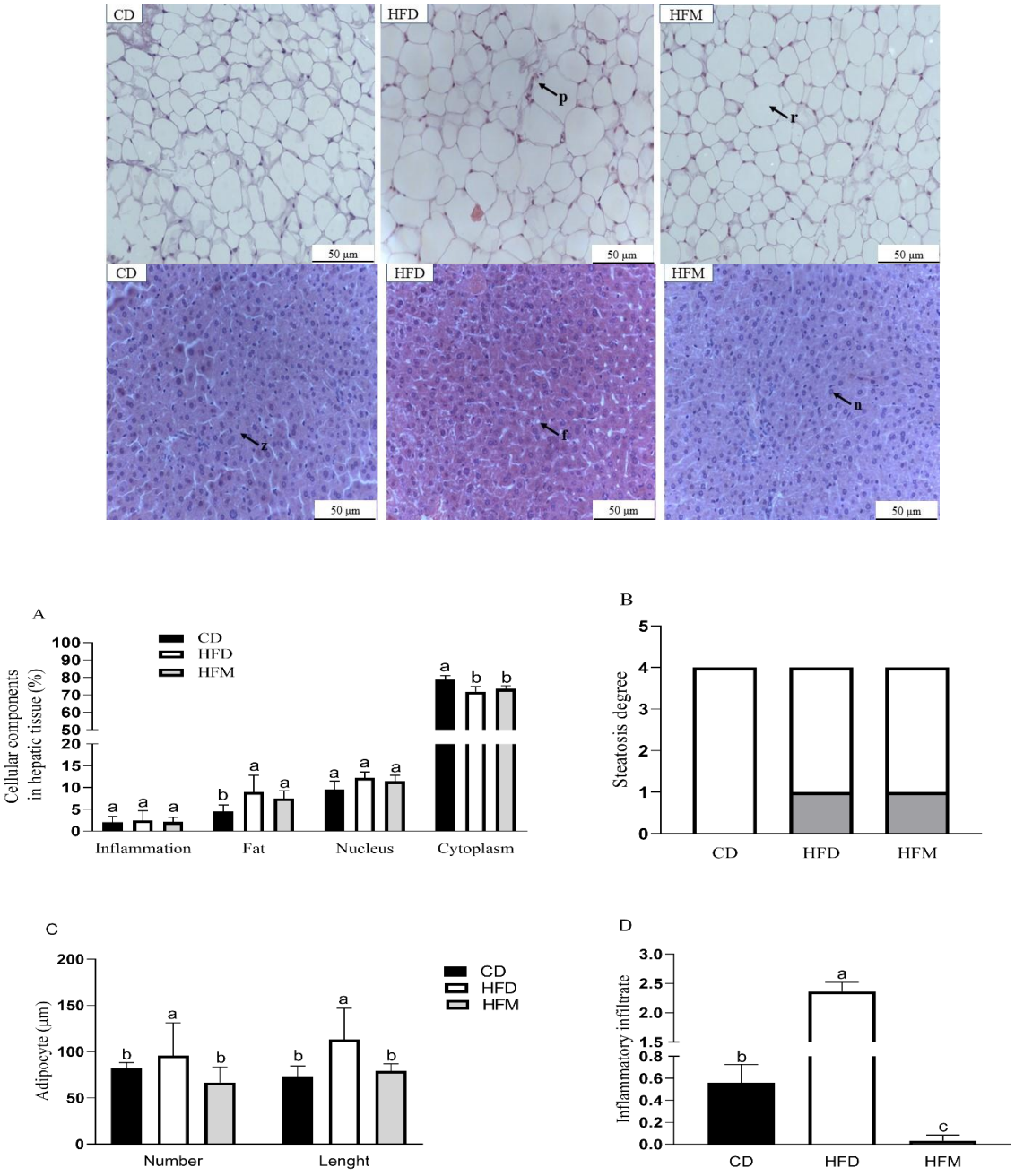

Figure 4. Cellular components percentage in hepatic tissue (A), steatosis degree (B), number and length adipocyte (C) and inflammatory infiltrate (D). Black arrows represent: z: cytoplasm, f: fat vesicles, n: nucleus, p: inflammatory infiltrate, r: adipocity. Data are expressed as the mean ± standard deviation (n = 10). Different letters indicate a statistical difference by Newman-Keuls test (p≤0.05). CD: control diet; HFD: high-fat diet; HFM: high-fat diet with macauba pulp oil.

4. Discussion

This is the first work that evaluated the influence of macauba pulp oil on undesirable metabolic changes in mice fed a high-fat diet. The present research focused on the effects of macauba pulp oil due to evidence that the oleic acid, carotenoid, and tocopherol present in this oil would trigger anti-inflammatory, anti-obesity, and antioxidant effects [27, 28]. In this study, macauba pulp oil intake prevented the adipogenesis pathway, inflammation and oxidative stress in mice fed a high-fat diet. In order to stimulate metabolic changes in animals, high-saturated fat diet consumption is extensively applied. In this case, the time to verify the effect of specific food or compound in the metabolic changes begins after 7 or 8 weeks of receiving the diet. In a different way, in our study, to determine the macauba pulp oil effects as a preventive treatment, macauba pulp oil was added in the diet since the beginning of the experimental along with the high-fat diet, to examine its mechanism of action and metabolic alterations.

In the present study, the consumption of macauba pulp oil reflected in higher total antioxidant capacity, which may be associated to the oleic acid content and tocopherol, confirmed by the correlation analysis that demonstrated a significant positive correlation between these compounds consumption and TAC. Oleic acid is well documented for its anti-inflammatory properties, possibly associated with the chemical configuration, with a double bond, causing less chance of oxidation and favor the antioxidant property against a high oxidative load, and tocopherol has potent antioxidant activity [10,29]. In addition, higher SOD activity and lower malondialdehyde levels were observed with the macauba pulp oil consumption. SODs are oxidoreductase enzymes that have the role of protecting cells against superoxide anions, performing the dismutation of $O_2^{\bullet-}$ into oxygen and H_2O_2, providing the antioxidant defense of the organism, and malondialdehyde is an important marker of the lipid peroxidation [30, 31]. In this way, it was shown that macauba pulp oil consumption can improve the antioxidant defenses, these results being attributed to the oleic acid, carotenoids, and tocopherol present in the macauba pulp oil, as demonstrated in other studies the relationship of these components and the improvement of the body's antioxidant defense [32, 33, 34]. Additionally, there was a positive correlation between SOD and these compounds and a negative correlation between MDA and oleic acid and tocopherol.

The consumption of macauba pulp oil prevented the adipogenesis pathway by decreasing the expression of PPAR-γ and (mRNA) *SREBP-1c*, and increasing of (mRNA) *Adiponectin* in the

adipose tissue. This effect is confirmed by the result of the histomorphometric analysis, which demonstrated that the animals that consumed the macauba pulp oil had smaller adipocyte number and length even high-fat diet consumption, that is, the macauba pulp oil caused less hypertrophy and hyperplasia of the adipocytes. Thus, the lower translocation of PPAR-γ in the present research can be associated to the high content of oleic acid and carotenoids in the macauba pulp oil and confirmed by the significant negative correlation between consumption of these compounds and PPAR-γ quantification. Oleic acid has been shown to act in PPAR-γ repression, resulting in less differentiation of pre-adipocytes in mature adipocytes, reducing adipogenesis [3, 35]. Similar to our results, were found a relationship between oleic acid consumption and reduction of PPAR-γ and (mRNA) *SREBP-1c* in obese animal model [35]. Research shows that carotenoids can affect adipocyte function through interaction with PPAR-γ, interfering with adipocyte differentiation, as demonstrated in a study using experimental animals and finding an association between carotenoids and lower adipose tissue gain, related to lower PPAR-γ expression [36]. Still, the result found is related to the increased expression of adiponectin, since PPAR-γ is tightly regulated by adiponectin [37]. Moreover, the observed results of reduction of genes related to the adipogenesis pathway, with concomitant reduction of histological markers of adipose tissue, could be related to the presence of β-carotene, which was the main carotenoid found in the macauba pulp oil, that can suppress PPAR-γ, resulting in lower total lipid in adipocytes [38,39].

Associated with this, macauba pulp oil was efficient in reducing inflammation in adipose tissue, since it reduced NF-κB in the nuclear fraction and this indicates a reduction in the inflammation cascade, showing significative reduction in (mRNA) *TNF-α* gene expression. Corroborating this result, the histomorphometric analysis of the adipose tissue showed less inflammatory infiltrate related to the consumption of macauba pulp oil. Hypertrophy of adipose tissue initiates the emission of chemotactic signals that recruit immune cells and lead to the infiltration of macrophages in adipose tissue, contributing to systemic subclinical inflammation [3]. This result may be associated with a lower amount of PPAR-γ and higher adiponectin, since PPAR-γ interferes with the differentiation of adipocytes and is consequently related to the inflammatory process, since obesity is already an inflammatory condition, as one of the complications related with obesity is the development of reactive oxygen species (ROS), and adiponectin is an anti-inflammatory adipokine with a negative correlation between the degree of

obesity and the level of this adipokine [40, 41]. These results are supported, since there was a positive correlation between the carotenoids consumption and the increase in the expression of adiponectin, indicating that the macauba pulp oil, high in carotenoids, may contribute to the reduction of inflammation. Additionally, there was a significant negative correlation between oleic acid and tocopherol and NF-κB, that is, the increase in oleic acid and tocopherol consumption is correlated with the decrease in the quantification of NF-κB. The study by Rosillo et al with mouse model, also demonstrated that administration of oleic acid is able of suppressing NF-κB activation [42]. Oleic acid is able to activate PGC-1α by forming a dimer with the protein called c-MAF, migrating to the nucleus and transcribing the gene responsible for IL-10, which dismantles the activation signaling of NF-κB, due to its potent anti-inflammatory action [43]. Tocopherol can act by blocking NF-kB activation through action on enzymes that regulate the NF-kB signaling pathway [44]. Despite not showing a correlation between the reduction of NF-κB and the consumption of carotenoids in the present study, this compound presents interference with the NF-κB pathway, resulting to the modulation of their interacting proteins, interacting with the cysteine residues of IκB kinase, thereby suppressing NF-κB activation or inhibiting of IκBα degradation [45, 46].

Although macauba pulp oil prevented the adipogenesis pathway and inflammation in the adipose tissue, significant effects in the hepatic markers were not observed after 8 weeks of high-fat diet. The current study was carried out as a prevention model, and for this reason it may not have verified alterations in the liver. Thus, in the current research, the consumption of diets for 8 weeks, even with a high concentration of saturated fats, was not able to do metabolic changes yet. These results are confirmed by histomorphometric analyzes that showed that there was no alteration of the cellular components evaluated, such as fat and inflammation in the liver.

Despite the decreased expression of (mRNA) *CPT-1α* gene, the quantification of PPAR-α did not change with the consumption of macauba pulp oil, which might be explained due the ADIPOR2 did not change either. The increase in the sensitization of ADIPOR2 receptor triggers activation of the PPAR-α, which regulates the fatty acid oxidation [47]. Moreover, the high traffic of free fatty acids by high-fat diet has the ability to trigger the SREBP-1c, which controls the enzymes expression essential in triacylglycerol synthesis and storage, and restricts lipogenic genes, such as ACC-1, responsible for the transformation of ACC-1 to malonyl CoA [48]. However, despite the overexpression of the gene in the fatty acid synthesis pathway, there was no

change in the proportion of fat and in the steatose degree in the liver, this may be due to the increased antioxidant capacity, decreasing the action of expression of this gene related to fatty acid synthesis.

The strain of mice used in this study was chosen since they are prone to metabolic disturbances generated by high-fat diet. However, it is known that experiments with mice do not fully reflect the effects in humans, due to differences in the organism and metabolism of these two species. However, taking into account the macauba pulp oil intake/animals weight, a human with 70 kg needs a consumption of a small amount per day (approximately 8 g/day of macauba pulp oil – similar 1 teaspoon) to have the same improvements observed in this research to prevent metabolic changes. Thus, further studies are suggested to verify the real effect of macauba pulp oil in human.

The influence on the body of the high-fat diet, and how the macauba pulp oil can act, which was demonstrated in our study, are summarized in Figure 5. The consumption of macauba pulp oil prevented inflammation and adipogenesis, as demonstrated by the reduction of expression of PPAR-γ, (mRNA) *SREBP-1c*, NF-κB and (mRNA) *TNF-α*, and increase of adiponectin in adipose tissue. In the liver, despite triggering an addition in *SREBP-1c* expression and a lower (mRNA) *CPT-1α* level, this did not lead to liver changes, according to histomorphometric analysis, due to increased antioxidant capacity. These modes of action may be related to macauba pulp oil, with a good composition of carotenoids, oleic acid and tocopherol, which improved the total antioxidant capacity, holding the adipogenesis even to a high level of saturated fat consumption.

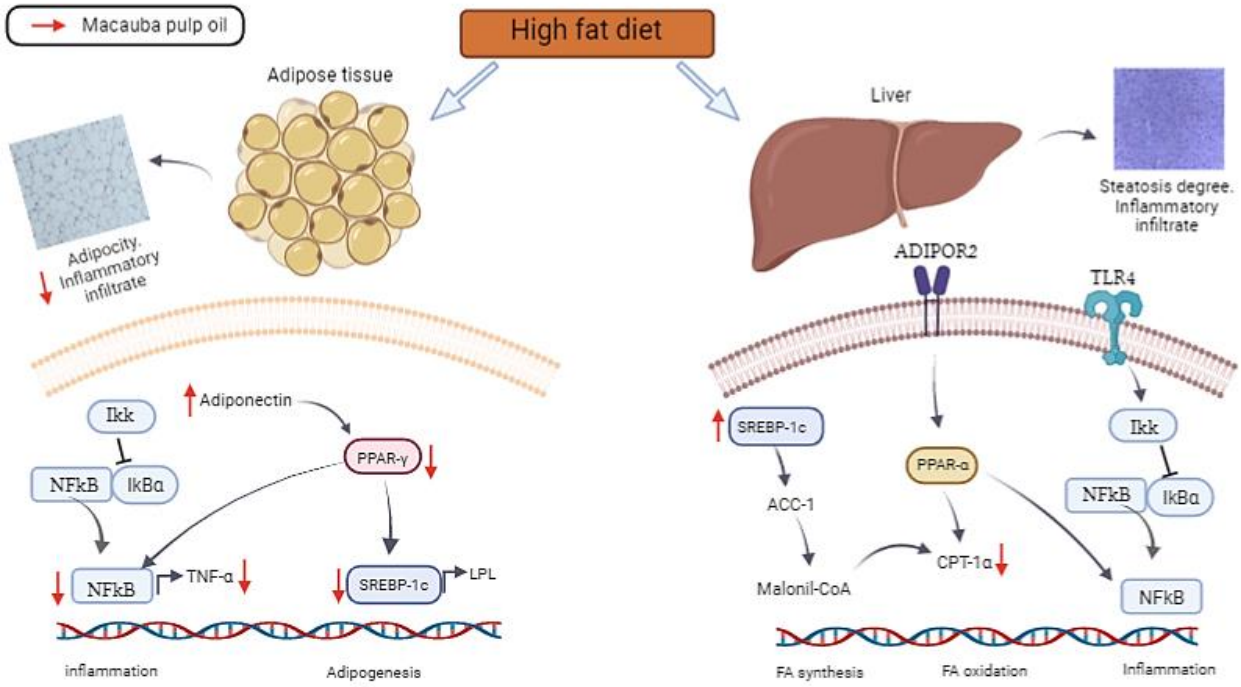

Figure 5. Effects of the high-fat diet on the adipose tissue and liver and potential action mechanism of macauba pulp oil. TLR-4: toll like receptor 4; NF-κB: nuclear factor kappa B; SREBP-1c: sterol regulatory element-binding proteins; ACC-1: acetyl-CoA carboxylase 1; ADIPOR2: adiponectin receptor 2; CPT-1α: carnitine palmitoyl transferase 1 alpha; PPAR: peroxisome proliferator-activated receptor; LPL: lipoprotein lipase; TNF-α: tumor necrosis factor alpha; IL-1β; FA: fatty acid.

5. Conclusions

Consumption of macauba pulp oil increased antioxidant capacity and prevented oxidative stress, inflammation and adipogenesis pathway. Therefore, macauba pulp oil shows to have a great potential for insertion in human foods to improve health, assisting in the prevention of reducing risk factors for chronic non-communicable diseases.

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7. CHAPTER 4: MACAUBA (*Acrocomia aculeata*) PULP OIL HAS THE POTENTIAL TO ENHANCE THE BARRIER MORPHOLOGY, GOBLET CELL PROLIFERATION AND GUT MICROBIOTA COMPOSITION IN MICE FED A HIGH-FAT DIET

MACAUBA (*Acrocomia aculeata*) PULP OIL HAS THE POTENTIAL TO ENHANCE THE INTESTINAL BARRIER MORPHOLOGY, GOBLET CELL PROLIFERATION AND GUT MICROBIOTA COMPOSITION IN MICE FED A HIGH-FAT DIET

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Abstract

Purpose: Macauba (*Acrocomia aculeata*) is a palm tree native from Brazil, who pulp is rich in oil that has a high content of oleic acid and carotenoids. Macauba pulp oil (MPO) can bring health benefits due to its bioactive compounds, however, its effects on gut health are unknown. Thus, the objective of this study was to evaluate the effect of MPO in the intestinal health in mice fed a high-fat diet.

Methods: Male mice were randomly divided into 3 groups (10 animals/group): control diet (CD), high-fat diet (HF) and high-fat diet with MPO (HFM). Short-chain fatty acid, fecal pH, and histomorphometric analysis of the colon were performed. Content of colon samples were used on microbiome analysis using 16S rRNA amplicon sequencing.

Results: Animals from the HFM group had higher butyric acid content and goblet cells number, greater circular and longitudinal muscle layer, and higher alpha-diversity compared with the HF group. Moreover, consumption of MPO reduced *Desulfobacterota* and increased *Actinobacteriota* phylum, *Ruminococcaceae*, *Oscillospiraceae*, *Prevotellaceae*, *Bifidobacteriaceae* family, *Faecalibacterium*, *Prevotella*, *Ruminococcus* and *Enterorhabdus* genus.

Conclusions: Therefore, macauba pulp oil was able to modulate the gut microbiota and enhance intestinal barrier morphology, showing preventive effects on gut dysbiosis in mice fed a high-fat diet.

Keywords: HPLC; butyrate; oleic acid; carotenoids; gut microbiota, goblet cell.

1. Introduction

The role of gut microbiota in the development of diseases has received increased attention from researchers worldwide [1]. These diseases share a common mechanism because the activation of the immune system leads to greater inflammation, and components originating from gut microbiota, such as lipopolysaccharide (LPL), peptidoglycan, flagellin and bacterial DNA, can cause immune system activation [2]. People with obesity generally have a lower abundance of beneficial gut microbiota, and studies have found that people with obesity have different gut microbiota compositions compared with lean people [3, 4].

Diet has a marked influence on the intestinal microbiota, generating different enterotypes with a predictable composition according to the type of diet consumed [5]. Compared with diets enriched with saturated fatty acids, diets with a high percentage of unsaturated fatty acids have been associated with a lower stimulatory effect on weight gain and hepatic lipid accumulation, related to the diet-induced changes observed in gut microbiota [6]. Furthermore, bioactive compounds such as carotenoids are capable of modulating the intestinal microbiota, providing positive beneficial effects on health [7, 8].

To date, however, little is known about the potential impact of specific plant-derived dietary oils on the composition of gut microbiota and host metabolic health. Macauba (*Acrocomia aculeata*) is a palm tree found naturally in almost all Brazilian territory and it is considered a promising alternative source of vegetable oil [9]. The oil extracted from the pulp is predominantly composed of monounsaturated fatty acids, oleic acid being its main constituent. Furthermore, it also presents compounds that play an important role in the health benefits, such as carotenoids [10].

We hypothesized that macauba pulp oil would modulate the microbiota composition, and improve the intestinal morphology, alleviating the disorder caused by high-fat diet consumption. Thus, considering the impact of metabolic diseases on public health and the association between these diseases and change microbiota, and the influence of diet on this parameter, this work focused on the effect of macauba pulp oil in gut microbiota modulation of mice fed a high-fat diet.

2. Material and methods

2.1. Materials

Macauba fruits utilized in this study were harvested in Araponga – Minas Gerais (Brazil) in mature stage, and then, they were peeled and pulped. Macauba pulp oil was extracted using a manual hydraulic press (Laboratory Press, Fred S. Carver Inc- Summit, New Jersey, USA), centrifuged at 5000 rpm for 20 minutes and stored in a freezer at -80°C until use.

2.2. Carotenoids and fatty acid composition of macauba pulp oil

Carotenoid analysis was carried out by high-performance liquid chromatography (HPLC) with detection of 450 nm, using the chromatographic conditions: HPLC system (Shimadzu, SCL 10AT VP, Japan); chromatographic column Phenomenex Gemini RP-18 (250mm×4.6mm, 5 mm), fitted with a guard column RP-18 Phenomenex ODS column (4mm×3mm). The mobile phase consisted of methanol:ethylacetate:acetonitrile (70:20:10, v/v/v) with a flow of 2.0 $mL \cdot min^{-1}$ and a run time of 15 min [11, 12]. In the fatty acid analysis, the oil was converted to fatty acid methyl esters (FAMEs) to obtain the fatty acid profile [13]. Samples were injected in a gas chromatograph equipped with a Flame Ionization Detector (Shimadzu, GC-2010, Japan) and a capillary column of 100 m x 0.25 mm (SP-2560, Sigma-Aldrich, USA). The analysis was performed by direct injection of 1 µl of the sample. Helium gas was used as the dragging gas and maintained at a constant flow rate of 363 kPa. The FAMEs were separated using a linear heating ramp from 100 °C to 270 °C, at a heating rate of 20 °C mim^{-1}, and high linear velocity for better peak resolution. Peak identification was confirmed by comparison with the standard FAME mix (Supelco 37 FAME mix, Sigma-Aldrich, USA).

2.3. Animals and diets

C57BL1/6 mice, male, lineages Inbred, with 08 weeks old were used in this study. Mice were obtained from the Center for Reproductive and Biology (Federal University of Juiz de Fora, Minas Gerais, Brazil). Animals were kept in a temperature-controlled room (22 ± 2 °C), with automatically controlled light-dark cycles of 12 hours, and in individual stainless-steel cages, with water and respective experimental diets supplied *ad libitum*. All experimental procedures with animals were performed in accordance with the ethical principles for animal experimentation and Animal Research guidelines: the ARRIVE Guidelines [14], and the study was approved by the Ethics Committee of the Federal University of Viçosa (Protocol 09/2019; date of approval: May 28th 2019).

The animals were randomly divided by body weight into 3 groups (10 animals/group): control diet – AIN93M (CD); high-fat (HF); and high-fat diet with macauba pulp oil (HFM). The number of animals per group was calculated based on the sample calculation equation, and were considered α-level = 5%, α-error type I = 1.96, and data of fat mass mean from Schoemaker et al., 2017 [15]. Experimental diets were based on AIN-93M and high-fat diet [16]. In diets with macauba pulp oil, this was added in the proportion of 4%, replacing the soybean oil used in the AIN-93M diet (Table 1). The diets were kept under freezing temperature (-20°C) and were placed daily for the animals. After the intervention period (8 weeks), animals were anesthetized with isoflurane (Isoforine, Cristália), according to the bodyweight of the animal. Colon and its content were collected and stored in a freezer at -80 °C until further analysis. For histological analysis, part of the colon was fixed in 10% formaldehyde.

Table 1. Composition of experimental diets (g/kg of diet).

Ingredients (g/kg)	CD	HF	HFM
Albumin*	179.71	179.71	179.71
Dextrinized starch	155	155	155
Sucrose	100	100	100
Soybean oil	40	40	-
Lard	0	312	312
Cellulose	50	50	50
Mineral mix	35	35	35
Vitamin mix	10	10	10
L-cystine	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5
Corn starch	425.99	113.99	113.99
Macauba pulp oil	-	-	40
Carbohydrate (%)	76.9	44.1	44.1
Protein (%)	18.9	18.9	18.9
Lipids (%)	4.20	37	37

*Purity of 78%. CD: control diet -AIN93M; HF: high-fat; HFM: high-fat diet with macauba pulp oil.

2.4. Fecal pH

Newly excreted feces were weighed, diluted in ultrapure water, and homogenized by vortexing for 15 seconds. The pH readings were then performed using a pH meter (Kasvi®) [17].

2.5. Short-chain fatty acid (SCFA) measurement

The SCFA analysis was performed in the feces from cecum (n=5 animals/group) following the methodology proposed by Siegfried et al. (1984) with modifications [18]. Throughout the analysis, the samples remained under low temperature. Briefly, 100 mg of cecum feces were homogenized in MiliQ water following a Vortex shaking protocol with calcium hydroxide and cupric sulfate to extract the short-chain fatty acids. The quantification of SCFA was performed by high-performance liquid chromatography (HPLC). The SCFA were determined in a Dionex Ultimate 3000 Dual detector HPLC apparatus (Dionex Corporation, Sunnyvale, CA, USA) equipped with a refractive index detector Shodex RI-101 maintained at 40 °C. The SCFA were separated on a Bio-Rad HPX-87H column (300 × 4.6 mm) (Phenomenex Inc. Torrance, CA, USA) maintained at 45 °C. Analyses were performed isocratically under the following conditions: mobile phase sulfuric acid 5 mmol l-1, flow rate 0.7 ml min^{-1}, column temperature 45 °C, and injection volume 20 µl. Stock solutions of the standards were prepared using the acetic, propionic, and butyric acid. All SCFA were prepared with a final concentration of 10 mmol/L.

2.6. Histomorphometric analysis of the colon

The colon samples were fixed in 10% formaldehyde, dehydrated, cleared, and embedded in paraffin. Sections were cut at 3 µm thick, mounted on glass slides, and stained with hematoxylin and eosin. Analyzes were performed under a photomicroscope (Leica DM750®, Germany). The histological sections images were captured in a 10× objective. Crypt width and depth, goblet cells number, thickness of the circular and longitudinal muscle layer was evaluated [19]. Scale of 50 µm was used. Twenty random fields per animal were selected. The images were processed using the ImagePro-Plus software version 4.5 (Media Cybernetics, Rockville, USA).

2.7. Gut microbiota analysis by 16S rRNA gene sequencing

The genomic DNA was extracted from approximately 100 mg of cecal content (n= 10 animals/group) following a mechanical disruption by beat-beating and phenol/chloroform

extraction protocol [20. The concentration and quality of DNA were determined spectrophotometrically by measuring the A260/280. Amplicons of the 16S rRNA V3-V4 region were generated using forward primer 341F (5'-CCTAYGGGRBGCASCAG-3') and reverse primers 806R (5'-GGACTACNNGGGTATCTAAT-3') and a barcoded primer set adapter for the Illumina NovaSeq platform (Illumina, San Diego, California, USA) [21]. Samples were loaded onto an Illumina flow cell for paired-end sequencing reactions using the Illumina NovaSeq PE250 platform in the Novogene Corporation at the University of California at Davis campus (Sacramento, California, USA). Amplicons were sequenced on a 2x250bp NovaSeq run using customized sequencing primers and procedures [21]. The sequences obtained for all samples were submitted to Sequence Read Archive (SRA) database on the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA906643.

2.7.1. 16S rRNA sequence processing

Data processing and analysis were performed using the software Mothur (version v.1.44.3). The R1 and R2 paired-end reads were joined, and sequences smaller than 380 or greater than 440 bp were removed. Sequences were discarded if they had homopolymers with at least eight nucleotides or contained ambiguous base pairs. Chimera sequences were detected and filtered with a reference-based approach using UCHIME version 4.2 [21]. After cleaning the sequences, they were aligned with the 16S rRNA gene using the SILVA database v.138 [23]. Taxonomic classification was performed using SILVA database v.138, and the Operational Taxonomic Units (OTUs) were grouped with a 97% sequence similarity cutoff. The coverage of all samples was assessed by Good's coverage estimator (Bacteria>97%). To correct for sampling bias due to unequal amplicon library sizes, the samples were normalized for the lowest number of sequences produced from any sample.

2.7.2. Gut microbiota diversity and composition analysis

The normalized data table was used for calculating alpha and beta diversity and the relative abundance of OTU (Operational Taxonomic Units). All the analysis were performed considering the taxonomic classification at genus level [23. The alpha-diversity of each sample was analyzed using the Chao1, Shannon and Simpson index for microbial community

composition. Beta-diversity metrics were calculated using the Jaccard dissimilarity index. Principal Coordinate Analysis (PCoA) plots were performed on calculated distance matrices in the bacterial communities. The statistical significance of β-diversity across sample groups was assessed with the non-parametric Permutational Multivariate Analysis of Variance (PERMANOVA, Monte Carlo permutations) test using the Past software (version 4.05). The predictions of metagenome functionality, grouped by experimental diets, was carried out using the PICRUSt 2.0 software [24]. Relative frequencies of different taxonomic categories obtained were calculated using the Statistical Analysis of Metagenomic Profiles program (STAMP v.2.1.3). LEfSe analysis was performed to identify the functional microbial pathways that were differentially expressed in the different experimental groups using the Galaxy website [25].

2.8. Statistical analysis

The results are expressed as the mean standard deviation. The results were analyzed by Analysis of Variance (ANOVA) followed by post hoc Duncan test. The significance of differences was defined at the $p \leq 0.05$ level. SPSS software version 26.0 was used to carry out the statistical analysis. For the microbiome results, the Chao 1, Shannon and Simpson indexes alpha-diversity metric was utilized to determine the bacterial richness in the samples, and the differences among the groups were analyzed by ANOVA. For assessing β-diversity, the Jaccard distances were calculated by the pairwise PERNOVA test. For correlation between cecal microbiota and intestinal parameters was used Spearman's correlation. SAS version 9.3 (SAS Institute, Cary, NC, USA) was used for the microbiota analyses statistics.

3. Results

3.1. Carotenoids and fatty acid composition of macauba pulp oil

The macauba pulp oil used in this study contained high content of monounsaturated fatty acids (55%), with 49.32% of oleic acid, and 207.52 μg/g of total carotenoids.

3.2. Macauba pulp oil promotes increased short-chain fatty acid production, enhances intestinal barrier morphology, and increased goblet cells

The group that consumed macauba pulp oil did not differ in relation to fecal pH ($p>0.05$; Table 2). After HFM intervention, the butyric acid was higher to than in the HF group ($p\leq0.05$;

Table 2). However, the HFM group did not differ in comparison to other groups for acetic acid and propionic acid contents (p>0.05; Table 2).

Table 2. Short-chain fatty acid and fecal pH of the colon of mice after consuming the experimental diets for 8 weeks.

	CD	HF	HFM
Acetic acid (Mmol/L)	2.12 ± 0.37[a]	2.36 ± 0.82[a]	2.17 ± 0.47[a]
Propionic acid (Mmol/L)	1.13 ± 0.95[a]	2.19 ± 1.05[a]	2.26 ± 0.70[a]
Butyric acid (Mmol/L)	1.27 ± 0.46[a]	0.18 ± 0.04[b]	0.44 ± 0.04[a]
Fecal pH	8.3 ± 0.08[a]	8.0 ± 0.20[a]	8.2 ± 0.42[a]

Dates are expressed as the mean ± standard deviation. Different letters indicate a statistical difference by Duncan test *(p*≤0.05). CD: control diet – AIN93M; HF: high-fat diet; HFM: high-fat diet with macauba pulp oil.

The animals that consumed macauba pulp oil showed an increase in the goblet cells number compared to control CD and HF groups (p>0.05; Fig. 1A). Furthermore, HFM showed greater circular and longitudinal muscle layer then HF group (*p*≤0.05; Fig. 1A), which were similar to CD group. Crypt length and depth showed no statistical difference between groups (p>0.05; Fig. 1B).

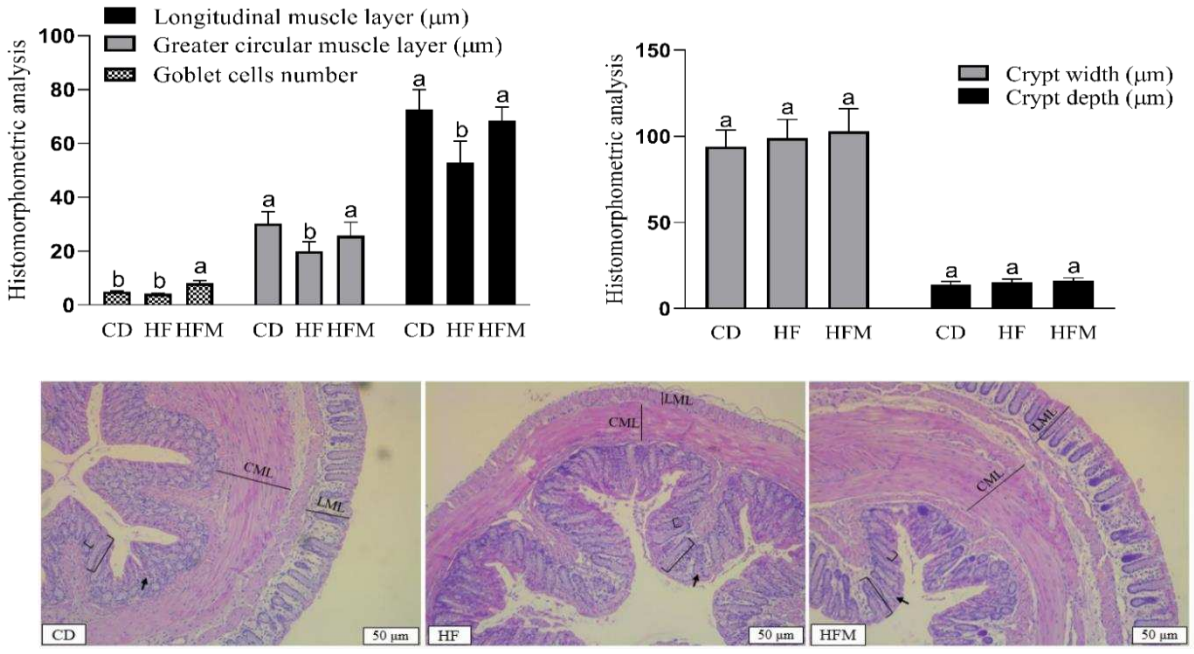

Fig. 1. Effects of macauba pulp oil consumption in colonic histomorphometric characteristics in mice fed a high-fat diet. Dates are expressed as the mean ± standard deviation (n=6 animals/group). Different letters indicate a statistical difference by Duncan test ($p \leq 0.05$). CD: control diet – AIN93M; HF: high-fat diet; HFM: high-fat diet with macauba pulp oil; CML: circular muscle layer; LML: longitudinal muscle layer. Black arrows represent goblet cells in the crypt. Black brackets represent the crypt depth and width.

3.3. Macauba pulp oil alters the diversity of gut microbiota

The sequencing of the 16S rRNA gene from the fecal samples generated 4381792 raw sequences. After filtering and cleaning the sequences, 726539 sequences of good quality were obtained. The Good's coverage obtained in the samples was > 99%, indicating good coverage of the sequencing. The summary of sample sequencing data is presented in Supplementary material (Table S1).

Alpha diversity is measuring by Chao1, Shannon and Simpson index. The abundance and uniformity of species is indicated by Chao 1 index, and compared with that in the HF group, the Chao 1 index was significantly increased (Fig. 2A). In the HFM group Shannon index was significantly increased (Fig. 2B), and the Simpson index was reduced (Fig. 2C). These results indicate that the alpha diversity increased in the HFM group.

In the β-diversity analysis, the PCoA revealed that there is a difference in the overall gut bacterial microbiota in animals fed the macauba pulp oil. Animals in the HFM group exhibited a distinct cluster compared with animals in the HF and NC groups, showing an evident distinction in bacterial communities of HFM group. Moreover, we observed that animals from the HF and NC groups showed low dissimilarity in the bacterial community (Fig. 2D).

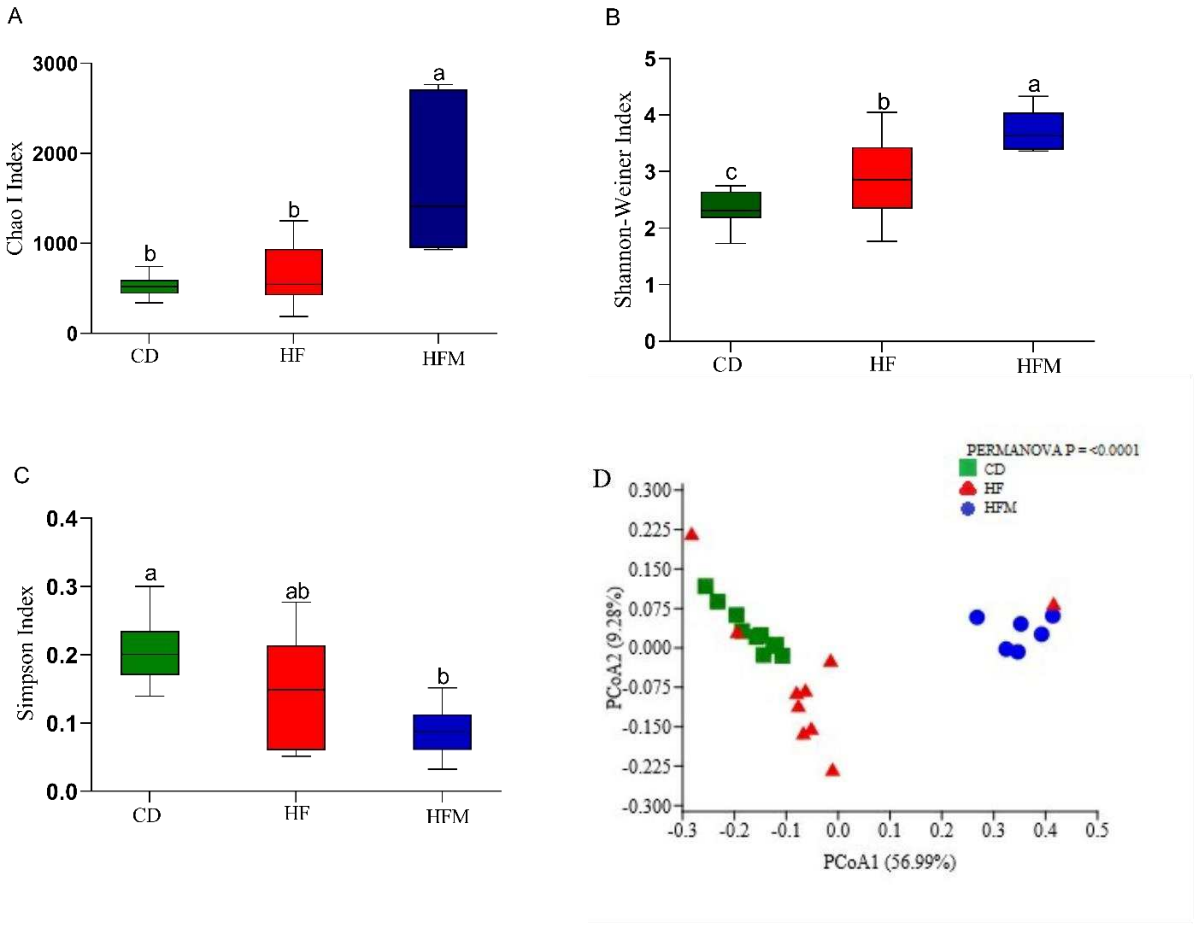

Fig. 2. Microbial diversity of the cecal microbiome after the consumption of macauba pulp oil for 8 weeks. Measure of α-diversity using the (A) Chao 1, (B) Shannon and (C) Simpson Indexes. (D) Principal coordinate analysis (PCoA) based on Jaccard similarity distance of cecal microbial communities. Each dot represents 1 animal, and the colors represent the experimental groups. CD: control diet– AIN93M, HF: high-fat diet, HFM: high-fat diet with macauba pulp oil. Different letters indicate a statistical difference by Duncan test *(*$p \leq 0.05$). PERMANOVA, permutational multivariate analysis of variance.

3.4. Macauba pulp oil shapes gut microbiota in different taxonomic levels

The taxonomic classification of samples showed 13 phyla, 20 classes, 50 orders, 89 families and 224 genera. At the phylum level, the relative abundance of *Desulfobacterota* in the HFM group was significantly reduced, while that of *Actinobacteriota* was increased ($p \leq 0.05$, Fig.

3A and B), however, there was no significant difference in *Firmicutes*, *Bacteroidota* and *F/B* ratio (p>0.05, Fig. 3C).

The relative abundance of *Bacilli*, *Desulfovibrionia*, and *Gammaproteobacteria* class in the HFM group were significantly reduced, while those of *Clostridia*, and *Coriobacteria* were significantly increased (p≤0.05, Supplementary material – Fig. S1). The relative abundances of *Oscillospirales* and *Clostridiales* order were higher in the HFM group than in the HF group. After HFM intervention, the abundance of *Bifidobacteriales* and *Christensenelales* were similar to than in the CN group (p≤0.05, Supplementary material – Fig. S1).

At the family level, the relative abundances of *Desulfovibrionaceae* were significantly reduced in the HFM group compared to those in the HF group, while those of *Ruminococcaceae, Oscillospireceae, Bifidobacteriaceae, Prevotellaceae* and *Eggerthellaceae* were significantly increased (p≤0.05, Fig. 3D and E).

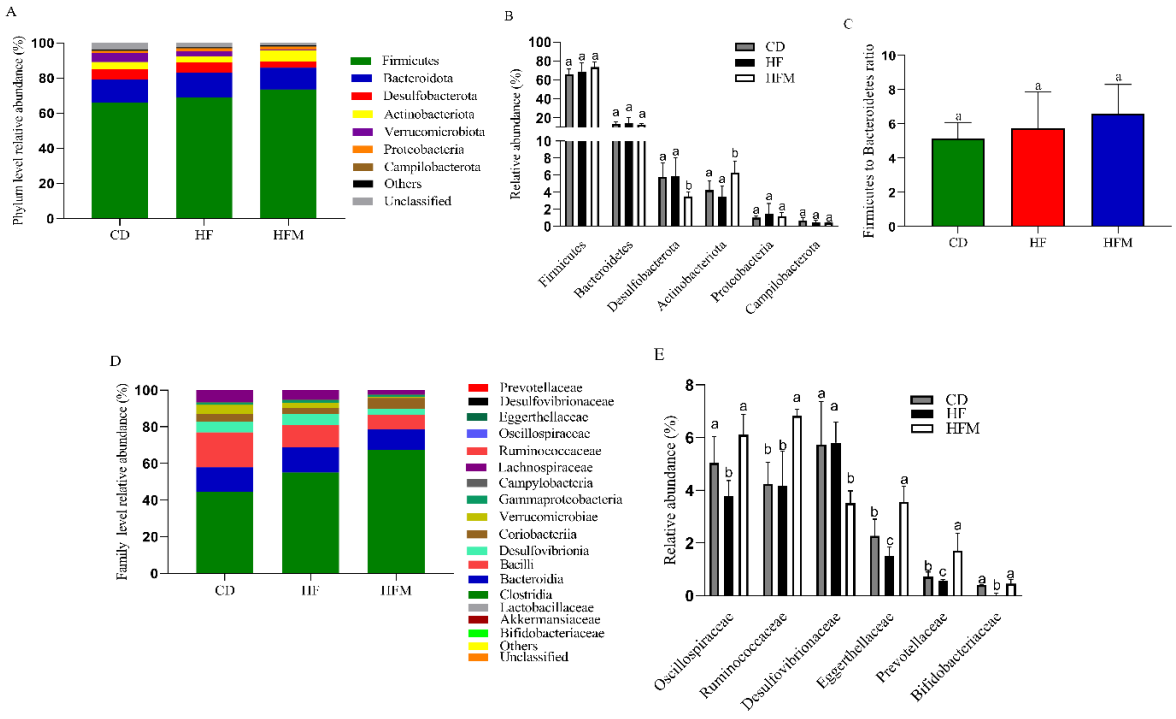

Fig. 3. Gut microbiota at phylum and family classification levels. (A) Distribution of mice gut microbiota at the level of phylum classification; (B) Relative abundance of the gut microbiota at the level of phylum classification; (C) Firmicutes/Bacteroidetes ratio; (D) distribution of mice gut microbiota at the level of family classification; (E) Relative abundance of the gut microbiota at the level of family classification. CD: control diet – AIN93M, HF: high-fat diet, HFM: high-fat

diet with macauba pulp oil. Different letters indicate a statistical difference by Duncan test ($p \leq 0.05$).

At the genus level, the relative abundances of *Ruminococcus*, *Prevotella*, *Faecalibacterium*, and *Enterorhabdus* were significantly increased in the HFM group, while those of *Akkermansia* were significantly reduced ($p \leq 0.05$, Fig. 4A and B). There was a significant correlation between *Ruminococcus*, *Enterorhabdus*, *Faecalibacterium* and circular muscle layer, and between *Enterorhabdus*, *Faecalibacterium* and goblet cell number, and between *Prevotella* and butyric acid ($p \leq 0.05$, Fig. 4C).

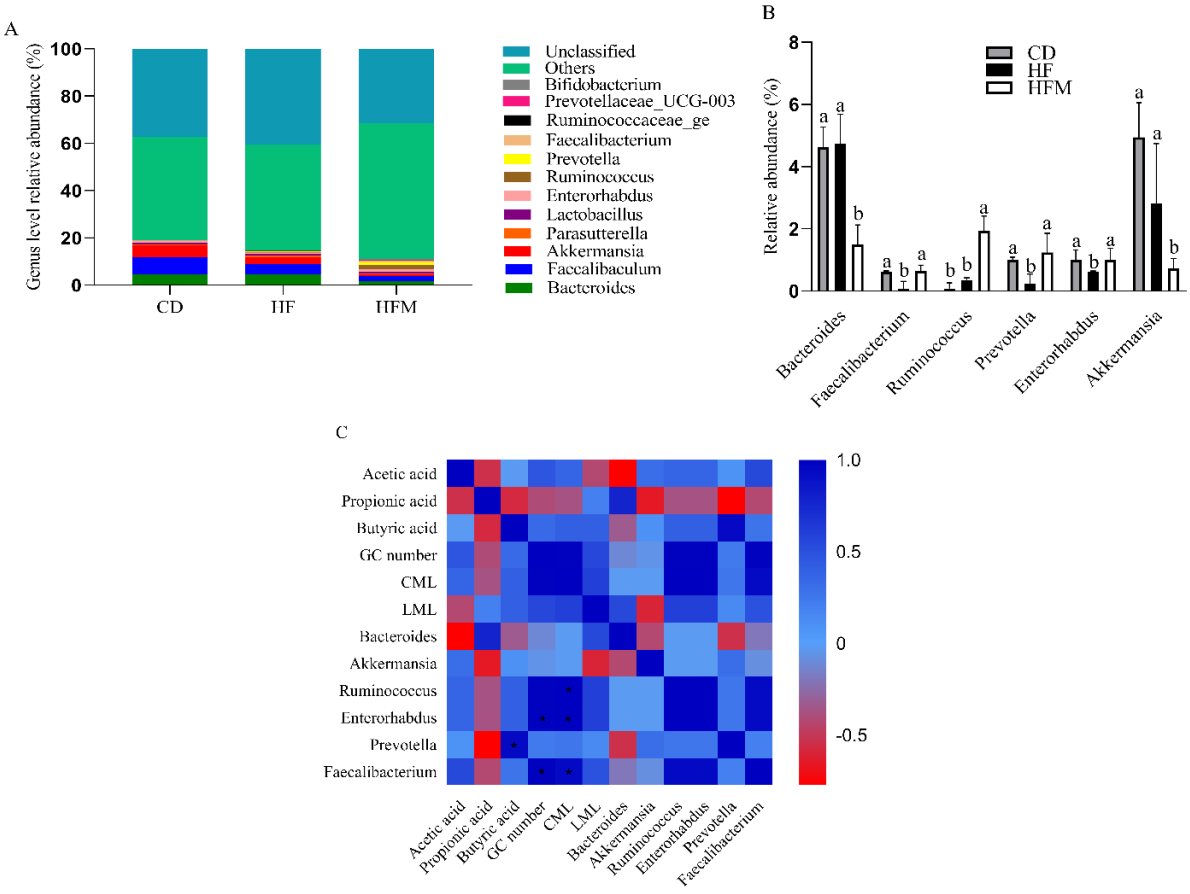

Fig. 4. Gut microbiota at genus class classification levels. (A) Distribution of mice gut microbiota at the level of genus classification; (B) Relative abundance of the gut microbiota at the level of genus classification; (C) Heatmap of Spearman's correlation between cecal microbiota and intestinal parameters. CD: control diet – AIN93M, HF: high-fat diet, HFM: high-fat diet with

macauba pulp oil; GC: goblet cell, CML: circular muscle layer; LML: longitudinal muscle layer. Different letters indicate a statistical difference by Duncan test *(p*≤0.05).

3.5. Effect of macauba pulp oil on the dominant cecal microbiota

The linear discriminant analysis effect size method (LEfSe) was used to investigate the bacterial biomarkers and isolate gut microbiome differences between the treatment groups. It was identified 28 dominant OTUs with effect size >3, with higher number of dominant taxa in CD group (n=15) when compared with HF (n=5) and HFM (n=8). In the macauba pulp oil group, we observed that the most differentially enriched taxa were related to members of the Clostridia and Coriobacteriia class. In CN group the dominant community was Faecalibaculum, Dubosiella and Akkermansia. Sutterellaceae family were the dominant in the microbiota of the HF group, showing a larger effect size (Fig. 5).

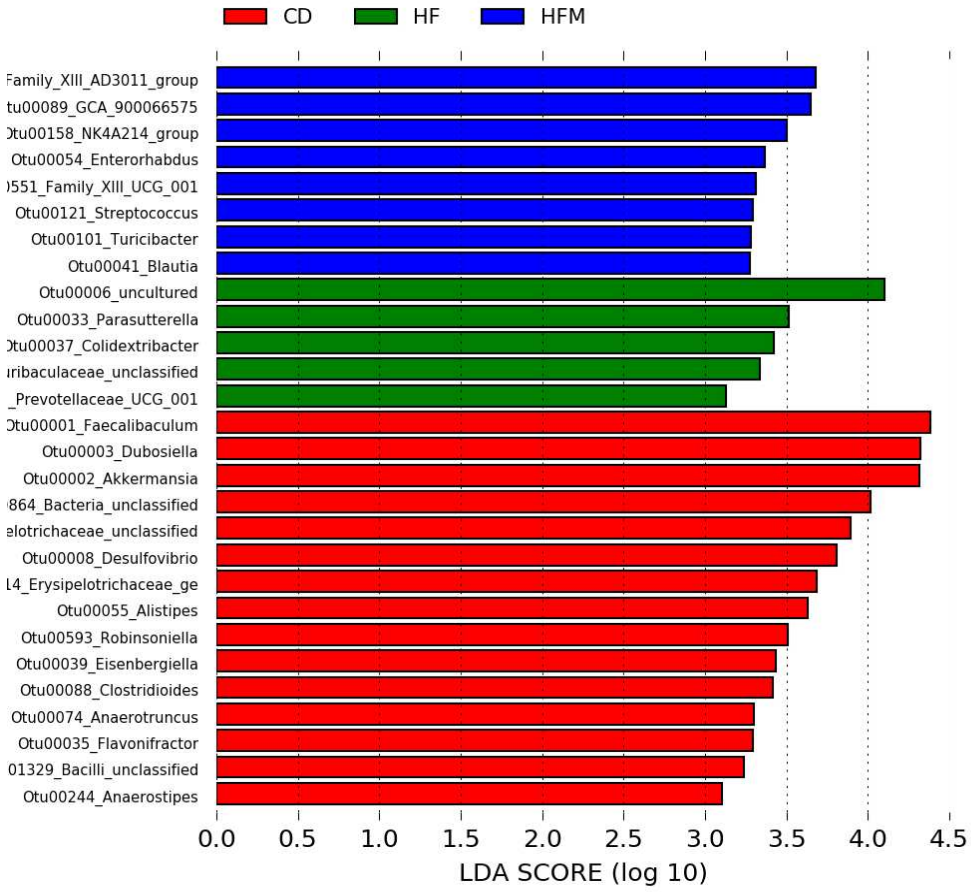

Fig. 5. Effect of macauba pulp oil in difference in dominant microorganisms between groups. CD: control diet – AIN93M, HF: high-fat diet, HFM: high-fat diet with macauba pulp oil.

4. Discussion

In this study, macauba pulp oil changed the gut microbiota profile, increased short-chain fatty acid production and goblet cells, enhanced intestinal barrier morphology in mice fed a high-fat diet, and these results can be associated with the bioactive compounds in macauba pulp oil, such carotenoids and oleic acid. The intestinal microbiota has been identified to be a potentially important player in the development, exacerbation, and/or alleviation of diseases, and has a beneficial role in the maintenance of physiological homeostasis, and the diet is an important environmental factor in modulating the gut microbiota, which is closely associated with individual health [1, 4, 2].

In the current study, consumption of macauba pulp oil resulted in an increase in the butyrate level when compared with high-fat diet. This SCFA can reduce translocation of bacteria in the intestine epithelium and increase epithelial integrity and mucus secretion [26]. SCFAs are metabolites of beneficial bacteria colonized in the colon and are closely related to the positive effect in metabolic diseases, inhibiting the proliferation of harmful bacteria and protect intestinal environmental homeostasis, and it is an indirect index reflecting whether the structure of the intestinal flora is normal [27, 28]. The high concentration of carotenoids in macauba pulp oil may have contributed to the increased in SCFAs (butyric acid), since carotenoids are fat-soluble bioactive compounds, so they have low bioavailability in the blood, and can probably be fermented in the intestine by the microbiota [29]. Gut microbes that synthesize more butyrate relative to acetate or propionate may decrease food intake or energy harvest, thereby decreasing hepatic glucose production, *de novo* lipid synthesis and adipogenesis and increasing lipolysis in white adipose tissue [30].

Complementing, the consumption of macauba pulp oil improved intestinal barrier morphology, showing higher circular and longitudinal muscle layer and increased in the goblet cells number. The intestinal tract is essential to maintaining intestinal homeostasis, through the intestinal barrier system that depends on interactions among several barrier components, including mucus layer, epithelial layer, and intercellular tight junctions [31]. The improving of intestinal barrier morphology can be associated with the level of carotenoids and oleic acid in the

macauba pulp oil. Carotenoids can improve gut barrier integrity, regulating proliferation and differentiation in the intestinal epithelium, which is crucial to the maintenance of the gut barrier, and can regulates the levels of secretory immunoglobulin A (IgA), which also influences the microbial composition in the gut [7]. Oleic acid can act to improve intestinal morphology by reducing bacteria that can reduce disulfide bonds in the mucus, which leads to the lysis of the MUC2 protein network (oligomeric mucus gel-forming), which are secreted by goblet cells, and with this improves intestinal morphology [32].

Macauba pulp oil consumption increased alpha-diversity, that is a marker most common indicator for assessing gut microbiota health. Low bacterial species diversity is closely associated with the disease status, and in general, a high diversity provides the ecosystem with strong stability [33]. Previous study showed association between oleic acid consumption and increased in the intestinal microbiota diversity, similar to current work with the consumption of macauba pulp oil, that is high in oleic acid [34].

At the phylum level, macauba pulp oil increased potentially beneficial bacteria phylum, such *Actinobacteriota*, and decreased potentially harmful bacteria phylum *Desulfobacterota*. The *Actinobacteriota* phylum also includes *bifidobacterial*, and some of these bacteria are known to have a positive effect on the health of the host, and a decrease in the content of *bifidobacterial* is associated with inflammatory gut disease [35]. Moreover, *Desulfobacterota* phylum consists of various organisms capable of reducing sulfur compounds, followed by butyrate degradation via the butyrate beta-oxidation pathway, which is not beneficial, as butyrate is a short-chain fatty acid that is very important for intestinal health [36]. At the current study, the abundance of *Firmicutes* and *Bacteroidetes*, and *Firmicutes*:*Bacteroidetes* ratio were not significative difference, but this ratio still cannot be considered parameter by gut health, because there are many unconfirmed issues about this possible biomarker [37]. It is important to keep in mind that changes more expressive is verify at lower taxonomic levels, such family, genus or species level, and not only phylum level.

When examining the macauba pulp oil effect at the family level, we demonstrated that macauba pulp oil increased the relative abundance of potentially beneficial bacteria. *Ruminococcaceae* may be involved in intestinal epithelium maintenance as it is inversely correlated with intestinal permeability and is closely related to the production of butyrate [38]. This association with maintenance of the intestinal epithelium is confirmed by the results of colon

histology which showed that macauba pulp oil increased muscle layer. We found that the consumption of macauba pulp oil reduced the abundance of LPS-producing and sulfate/sulfite-reducing bacteria, *Desulfovibrionaceae* family. LPS is a major component of the outer cell membrane of gram-negative bacteria, and the increase the concentration of LPS in the blood causes low-grade inflammation and, ultimately, obesity and related metabolic diseases, and this bacteria family produce hydrogen sulfide by sulfate/sulfite-reducing, that is a genotoxic gas that causes barrier dysfunction and endotoxemia, impairing barrier function [35, 30].

The present study showed several positive changes in the gut microbiota at the genus level, with the increased of *Faecalibacterium, Prevotella, Ruminococcus,* and *Enterorhabdus*, bacterial genus with potential healthy metabolic. The *Prevotella*, which belong to the phylum of *Bacteroides*, are anaerobic bacteria which produce acetate and succinate through fermentation and were positively associated with glucose, obesity and metabolic syndrome improvement [39, 40]. *Ruminococcus* are involved in SCFAs production, and consequently, involved in intestinal barrier integrity by modulating the mucin expression and the glycosylation level in goblet cells [41]. This corroborates with the data from SCFAs and histomorphometric analysis, which showed an increase in butyrate and goblet cells with the consumption of macauba pulp oil. *Enterorhabdus* and *Faecalibacterium* genus are indigenous butyric acid-producing bacteria in the gastrointestinal tract, and this SCFA has influence on the gut-brain axis, suppressive effects on excessive inflammatory response and enhancing effects in intestinal barrier function [42, 43]. This is confirmed by the correlation analysis that showed an association between these bacterial genera and an increase in the longitudinal muscle layer, which results in an improvement in the intestinal barrier.

After eight weeks of macauba pulp oil consumption, we observed a decrease in the relative abundance of *Akkermansia* genus, that is a genus involved in maintaining the mucus thickness. Despite the reduction of this important bacterium, we observed an increase in the number of goblet cells in the group that consumed macauba pulp oil. In this sense, the increased of goblet cells observed due the macauba pulp oil consumption might be due other factors besides the relative abundance of *Akkermansia* genus, because as demonstrated, there was no significant correlation between the relative abundance of *Akkermansia* and histological parameters. Further these results can be explained due the fact that *Akkermansia* are competitive exclusion bacteria, and in this case, we hypothesize that as the macauba pulp oil increased the relative abundance of

Rumminococus, *Prevotella*, *Enterorhabdus*, *Faecalibacterium* it could have promoted an environment of competition, thus resulting in the inhibition of *Akkermansia* [44].

Metabolic and physiological differences exist between the experimental model used and humans, but considering the consumption of macauba pulp oil per animal weight, this value would correspond to the consumption of approximately 8 g/day by a human weighing 70 kg. In this case, this small intake of macauba pulp oil can result in positive changes in the microbiota of humans, similar to what has been demonstrated in this study, with mice.

5. Conclusion

The consumption of macauba pulp oil for 8 weeks changed the profile of gut microbiota, increased butyric acid production, enhanced intestinal barrier morphology, and increased goblet cells in mice fed a high-fat diet. Therefore, macauba pulp oil has the potential to alleviate HFD-induced disorder of gut microbiota, and can be a promising functional food, promoting intestinal benefits.

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Supplementary material

Table S1. Summary of sequencing data at the end of 8 weeks of treatment.

Treatment	Good's coverage	Raw sequences	Filtered reads		Normalized reads	
		Reads	Reads	OTUs	Reads	OTUs
CD	0.9931 ± 0.0012	170640.1 ±11239.63	102676.3±7532.09	507.5±108.58	28376.8 ±96.5215	157.4±21.9959
HF	0.9966 ± 0.0028	167168.2±15164.01	87476.2±23744.40	570.6±162.70	28027.4 ±665.6092	162.5 ±28.2262
HFM	0.9931 ± 0.0012	167168.2±15164.01	38307.5±3678.70	678.20±110.10	27082.8±509.7903	200.16 ±10.3424

Values expressed as mean ± standard deviation. n=10 animals/group. CD: control diet– AIN93M, HF: high-fat diet, HFM: high-fat diet with macauba pulp oil; OTUs: Operational Taxonomic Units.

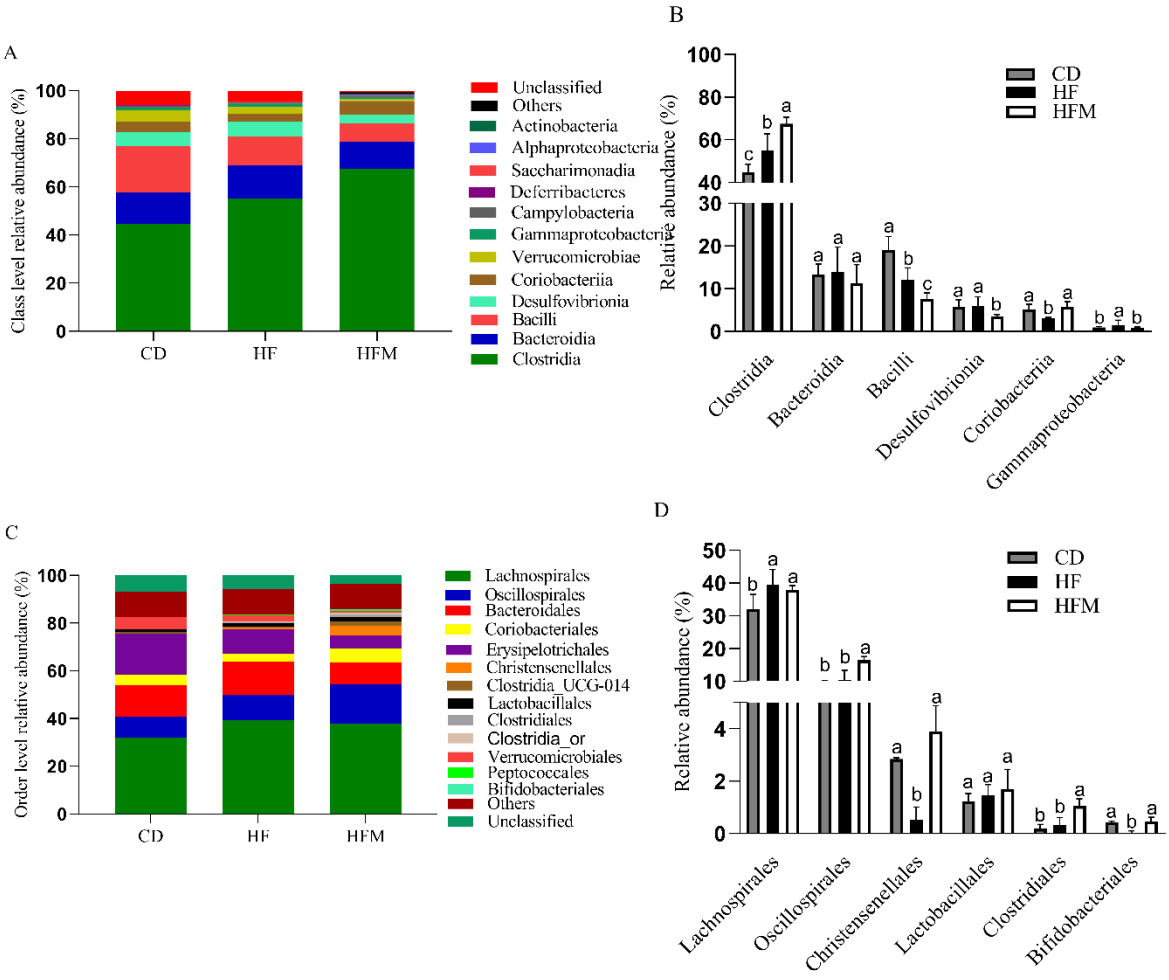

Fig. S1. Gut microbiota at Class and Order classification levels. (A) Distribution of mice gut microbiota at the level of Class classification; (B) Relative abundance of the gut microbiota at the level of Class classification; (C) Distribution of mice gut microbiota at the level of Order classification; (D) Relative abundance of the gut microbiota at the level of Order classification. CD: control diet– AIN93M, HF: high-fat diet, HFM: high-fat diet with macauba pulp oil. Different letters indicate a statistical difference by Duncan test ($p \leq 0.05$).

8. CHAPTER 5: MACAUBA (*Acrocomia aculeata*) PULP OIL REDUCES FAT ACCUMULATION, AND PROMOTES FAT MOBILIZATION, UNSATURATED FATTY ACID BIOSYNTHESIS AND LIFESPAN IN *CAENORHABDITIS ELEGANS* AT LOW TEMPERATURES VIA *fat-7*-DEPENDENT PATHWAY

MACAUBA (*Acrocomia aculeata*) PULP OIL REDUCES FAT ACCUMULATION, AND PROMOTES FAT MOBILIZATION, UNSATURATED FATTY ACID BIOSYNTHESIS AND LIFESPAN IN *CAENORHABDITIS ELEGANS* AT LOW TEMPERATURES VIA *fat-7*-DEPENDENT PATHWAY

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Abstract

Macauba (*Acrocomia aculeata*) is a Brazilian palm tree and its oil in the pulp is rich in oleic acid, carotenoids and tocopherol. However, its physiological function remains unknown. The objective of this study was to investigate the effects of macauba pulp oil (MPO) on the metabolic link between lipid metabolism and lifespan using *Caenorhabditis elegans*. MPO significantly suppressed fat accumulation and increased glycerol (a lipolysis index) and lifespan of *C. elegans* at low temperature (4°C). This was accompanied by decreased mRNA levels of the genes involved in lipogenesis (*spb-1* and *pod-2*) and increased levels of the genes involved in fatty acid β-oxidation (*acs-2*, *tub-1* and *nhr-49*). Additionally, MPO treatment modulated fatty acid pools in *C. elegans* at low temperature in that MPO treatment resulted in a decrease in saturated fatty acid levels and an increase in polyunsaturated fatty acid levels. Moreover, such effects of MPO on fat accumulation and lifespan at low temperature were abolished in *fat-7* mutants. Collectively, our results suggest that MPO promotes the lifespan of *C. elegans* at low temperature by increasing *fat-7*-mediated fat mobilization and shifting fatty acid profile to long-chain fatty acids.

Keywords: macauba; lifespan; low temperature; unsaturated fatty acids; *fat-7*; fat mobilization; *C. elegans*.

1. Introduction

Macauba (*Acrocomia aculeata*) is a palm tree native to Brazil and it is considered a promising alternative source of vegetable oil, due to its high oil production and its specific characteristics.[1] The oil extracted from the pulp is predominantly composed of monounsaturated fatty acids (56-69%), oleic acid being its main constituent (52-65%).[2,3] Furthermore, it also presents high concentration of carotenoids, which is known to protect lipids from oxidation, as well as various health benefits.[4] Macauba pulp oil (MPO) contains relatively high content compared to other foods known to have carotenoids.[4,5]

It is believed that MPO brings health benefits, such in anti-obesity, anti-oxidative stress, and anti-inflammation, largely due to its content of oleic acid and carotenoids.[6,7,8] For instance, oleic acid is known to a reduced expression of transcription factors involved in the adipogenesis pathway, such as peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element-binding proteins (SREBPs), and to reduce oxidative stress markers.[6,7] Carotenoids are reported to reduce inflammation and oxidative stress by modulating nuclear factor κB (NF-κB).[8] in this sense, macauba pulp oil shows strong evidence of positively contributing to health benefits.

Caenorhabditis elegans is a free-living nematode, which is widely used as an invertebrate animal model of obesity, aging, development, moving behavior, and neuro-degenerative disorders. Compared to other animal models, it possesses many advantages in research, with more than 65% of the human disease-related genes conserved.[9] *C. elegans* has conserved fat and energy regulatory pathways, and its known-whole genome sequence makes this model useful for determining mechanisms.[10,11] The goal of the current study was to investigate the role of MPO in fat metabolism and longevity in *C. elegans* as a model system and its underlying mechanisms of action.

2. Materials and Methods

2.1. Materials

Escherichia coli OP50 and *C. elegans* strains were obtained from *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA): N2, Bristol (wild-type), *fat-1* (wa9) IV, and *fat-7* (wa36) V. All chemicals used were purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA). Household bleach (The Clorox Company, Oakland, CA, USA)

was used for bleaching the worms. Macauba fruits were harvested in Araponga - Minas Gerais (Brazil) in mature stage, and then, they were peeled and pulped. MPO was extracted using a manual hydraulic press (Laboratory Press, Fred S. Carver Inc-Summit, New Jersey – USA), centrifuged at 5000 rpm for 20 minutes and stored in freezer at -80°C until further analysis.[12]

2.2. Chemical characterization of macauba pulp oil

To determine the macauba pulp oil fatty acid profile, the oil was converted to fatty acid methyl esters (FAMEs). Samples were injected in a gas chromatograph equipped with a Flame Ionization Detector (GC-FID) (Shimadzu, GC-2010, Japan) and a capillary column of 100 m x 0.25mm (SP-2l560, Sigma-Aldrich, USA). The FAMEs were separated using a linear heating ramp from 100 °C to 270 °C, at a heating rate of 20 °C mim^{-1}, and high linear velocity for better peak resolution. Peak identification was confirmed by comparison with the standard FAME mix (Supelco 37 FAME mix, Sigma-Aldrich, USA). In addition to the fatty acid profile, the oleic acid content (mg/g) of the oil was also determined, using an oleic acid standard (Sigma-Aldrich).[13]

Carotenoid analysis was carried out by high-performance liquid chromatography (HPLC) with detection of 450 nm, using the chromatographic conditions: HPLC system (Shimadzu, SCL 10AT VP, Japan); chromatographic column Phenomenex Gemini RP-18 (250mm×4.6mm, 5 mm), fitted with a guard column RP-18 Phenomenex ODS column (4mm×3mm). The mobile phase consisted of methanol:ethylacetate:acetonitrile (70:20:10, v/v/v) with a flow of 2.0 $mL \cdot min^{-1}$ and a run time of 15 min.[14]

2.3. Nanoemulsion preparation

Nanoemulsion was prepared as previously described.[15] Briefly, we used Tween-80 as an emulsifier at surfactant to oil ratios of 1:1 and then sonicated (Sonicator 505, Fisher Scientific) for 6 min.

2.4. *C. elegans* culture

Worms were cultured according to established protocols.[10,16] Worms were cultured on nematode growth media (NGM) plates (1.7% agar, 2.5 g/L peptone, 51 mM NaCl, 25 mM KPO_4 buffer pH 6.0, 5 μg/L cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$) and incubated at 25 °C, on an incubator (model DT2-MP-47, Tritech Research Inc., Los Angeles, CA). To obtain a

synchronized population of worms, eggs were collected using bleaching solution.[16] M9 buffer (41 mM Na_2HPO_4, 15 mM KH_2PO_4, 8.6 mM NaCl, 19 mM NH_4Cl) was used to wash the eggs, and then they were incubated overnight with S-complete (100 mM NaCl, 5.7 mM K_2HPO_4, 44 mM KH_2PO_4, 5 µg/L cholesterol, 0.1 M $C_6H_5K_3O_7$ pH 6.0, 3 mM $CaCl_2$, 3 mM $MgSO_4$, 50 µM disodium EDTA, 2.5 µM $FeSO_4 \cdot 7\ H_2O$, 1 µM $MnCl_2 \cdot 4\ H_2O$, 1 µM $ZnSO_4 \cdot 7\ H_2O$, 0.1 µM $CuSO_4 \cdot 5\ H_2O$). Finally, MPO (5.0 mg/mL) was added to the media and incubated for 4 days at 18 °C, 37 °C or 4 °C, depending on the proposed analysis. Tween-80 was added in the control group in the same concentration to the MPO added in the treatment group.

2.5. Triglycerides and glycerol assay

Worms were collected and washed with M9 buffer 3 times. The samples were homogenized with 0.05% PBST. The homogenized samples were subjected to triglyceride, glycerol and protein measurement using Infinity™ Triglycerides Reagent (Thermo Fisher Scientific Inc., Middletown, VA), Free-glycerol reagent (Sigma-Aldrich Co., St. Louis, MO), and Bio-Rad DC protein assay kit (Bio-Rad Co., Hercules, CA), respectively. Triglycerides and glycerol content were normalized by protein concentration.

2.6. Lifespan analyses and stress assay

C. elegans were cultured on a standard food source of *E. coli* OP50 and synchronized, as previously described. Adult worms (L4) were loaded into microfluidic chips (Infinity Chips, NemaLife Inc., TX, USA) with 20 mg/mL of *E. coli* OP50 and 5.0 mg/mL of MPO. The lifespan assay was initiated by loading day 1 adult into the microfluidic chip. For lifespan, cold and heat stress the chips were kept at 18°C, 4°C or 37°C, respectively. The percentage of worms survivals was counted every other day. Death was counted if the worms did not move.

2.7 Paraquat stress resistance assay

Resistance to lethal oxidative stress by paraquat (Sigma-Aldrich, Germany) was assessed as previously described.[17,18] Briefly, worms were treated with 5.0 mg/mL of MPO for 4 days after the L4 stage. Afterward, we transferred worms into 96-well plates: 10 worms in 100 µl of S-buffer, containing freshly dissolved 50 mM paraquat. Dead worms were scored every hour until all control worms were dead.

2.8. Quantitative Reverse-Transcription PCR (qRT-PCR)

The total RNA was extracted from the synchronized L4 worms using TRIzol reagent according to the manufacturer`s instruction. Reverse transcription was carried out by SuperScript II kit, and qRT-PCR was carried out using iTaq Universal SYBR Green Supermix according to the manufacturer`s instruction. Data are normalized to *act-1* and analyzed by $2^{-\Delta\Delta Ct}$ method.[19] Primers sequences are present in supplementary material (Table S1).

2.9. Fatty acids analysis by GC-TOF/MS

Fatty acid analysis was performed by gas chromatography-time-of-flight/mass spectrometry (GC-TOF/MS). First, fatty acids were extracted, for that the worms samples were mixed with methanol containing 2.5% sulfuric acid in solution after being spiked with 100 µl of an internal standard compound [100 ppm nonadecanoic acid (w/v) in methanol], and after the samples were then heated for 1 hour at 70 °C in a benchtop vacuum concentrator (Labconco Corp., Kansas City, MO). Hexane/water was used for to extract the fatty acids, and the hexane layers were then concentrated to 0.3 mL using nitrogen gas. After the extraction of fatty acids, the solution was subjected to GC-TOF/MS with a 6890 N GC (Agilent Technologies, Palo Alto, CA) equipped with a DB-5MS column (J&W Scientific, Folsom, CA) and a PegasusIII TOF/MS (Leco, St. Joseph, MI) (flow rate of helium: 0.8 ml/min; data acquisition rate: 20 scans/s in the mass range 35-500 m/z) with an initial oven temperature to 40 °C for 5 min and 190 °C for 10 min at 10 °C/min rate followed by raising to 280 °C for 5 min at 10 °C/min. 230 °C and 250 °C, respectively, was the temperature of the injector and detector. Fatty acid methyl esters (FAMEs) were identified based on their mass spectral data and retention index (RI) values, and their comparison with authentic standard compounds. When the authentic standard compounds were unavailable, FAMEs were tentatively identified based in MS search Program V.2.0d (NIST, 2005), NIST05 MS Library, and Wiley 7n mass spectral data base (Hewlett-Packard, Palo Alto, CA, 1995). N-alkanes C 7 -C 30 were used as external standards for the calculation of the RI of each compound. The relative peak areas of the FAMEs for each sample were assessed by comparing with those of the internal standard compound [100 ppm nonadecanoic acid (w/v) in methanol].[20]

2.10. Statistical analysis

The results are expressed as the mean standard deviation. Statistical analyses were conducted using Graph Pad Prism (SAS Institute, Cary, NC). Differences between groups were determined by one-way ANOVA followed by Tukey's multiple-range test. For lifespan assay, Online application of survival analysis (OASIS) was used.[21,22] The significance of differences was defined at the $p \leq 0.05$ level.

3. Results

3.1. Chemical characterization of MPO

Macauba pulp oil showed a high content of monounsaturated fatty acids (55%), with high oleic acid content (49%). In addition, it demonstrated high content of carotenoids (Table 1).

Table 1. Chemical characterization of macauba pulp oil

	Macauba pulp oil
Fatty acid (g.100 g^{-1})	
Saturated	24.07
Monounsaturated	55.25
Oleic acid (C18:1n9c)	49.68
Polyunsatured	20.68
Total carotenoids (μg/g)	207.52

3.2. MPO decreased fat accumulation and increased fat mobilization in C. elegans at low temperature

In order to determine the function of MPO on lipid metabolism, we examined the effect of MPO treatment on fat accumulation in C. *elegans*. As shown in Fig. 1, MPO treatment showed a 23.64% decrease in fat accumulation when compared to control group as judged by triglyceride assay ate 18 °C ($p<0.05$, Fig. 1A). The inhibitory effect of MPO was further augmented at 4 °C (-25.78%) when compared to control group ($p<0.05$, Fig. 1B). Since low temperature promotes fat mobilization in *C. elegans* through activation of lipolysis and fatty acid β-oxidation[23], we further determined the effect of MPO treatment on the level of glycerol, a lipolysis index, in *C. elegans*

at 4 °C. MPO treatment resulted in an increase in glycerol content (13.48% on day 7 and 11.32% on day 14) (p<0.05, Fig. 1C).

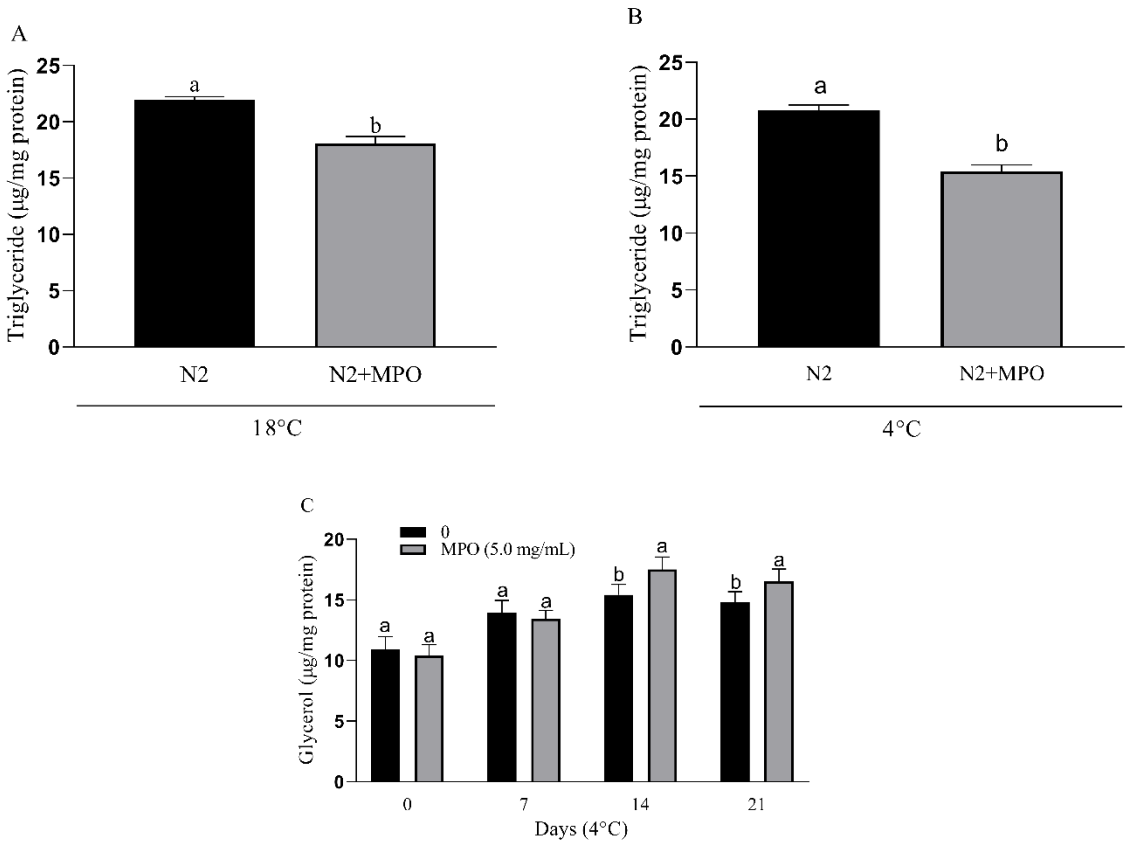

Fig. 1. Effect of macauba pulp oil (5.0 mg/mL) on the fat accumulation of *C. elegans*. (A) 37 °C; (B) 4 °C; (C) Glycerol. n > 1000 worms per group. Means with different letters are significantly different by the Tukey test ($p \leq 0.05$). MPO: macauba pulp oil.

3.3. MPO enhances the lifespan of C. elegans at low temperatures

Macauba pulp oil increased the survival of *C. elegans* (p<0.05, Fig. 2A, Table 2). For heat stress, MPO group showed the same maximum lifespan when compared to those of the control group (28 days) (p<0.05, Fig. 2B, Table 2). Macauba pulp oil resulted in significant improvement in the survival under cold stress by increased maximum lifespan (32 days) and mean lifespan by 25.71%, when compared to those of the control group (p<0.05, Fig. 2C, Table 2). For the oxidative stress response, macauba pulp oil administration resulted in a marginal extension of

mean lifespan when compared to the control group under paraquat treatment (p<0.05, Fig. 2D, Table 2).

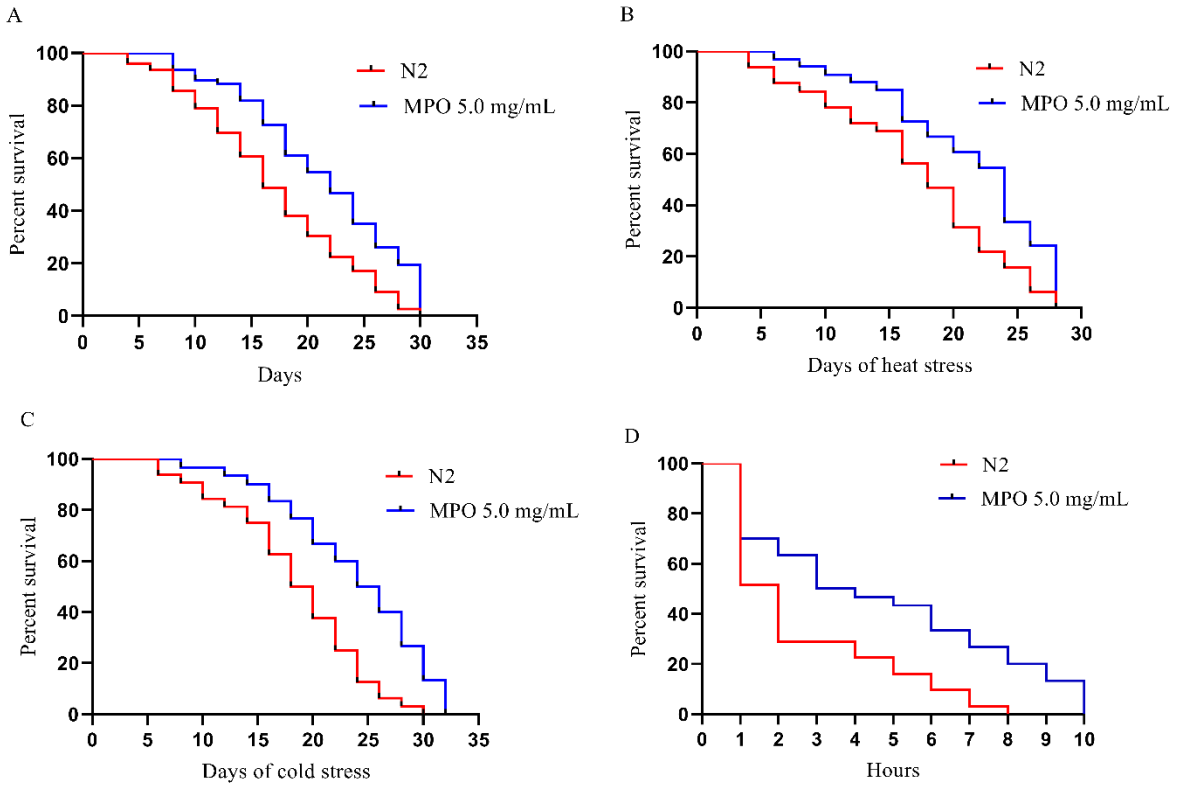

Fig. 2. Effect of stress conditions on the survival of N2 *C. elegans* by macauba pulp oil (5.0 mg/mL) treatment. (A) 18°; (B) 37°C; (C) 4°C; (D) oxidative stress with paraquat. MPO: macauba pulp oil.

Table 2. Lifespan of N2 and mutants *C. elegans*.

Strain	MPO (mg/mL)	Condition	Mean lifespan in days ± SD	Maximum lifespan (days)	*p*-value versus control	Number of nematodes
N2	0	18°C	17.05 ± 0.79	30	-	75
N2	5.0	18°C	22.44 ± 0.83	30	0.0000007	75
N2	0	37°C	17.25 ± 1.22	28	-	62
N2	5.0	37°C	21.13 ± 1.10	28	0.0175	62

N2	0	4°C	18.77 ± 1.06	30	-	65
N2	5.0	4°C	25.71 ± 1.24	32	0.000013	65
N2	0	Paraquat	2.63 ± 0.40	8	-	60
N2	5.0	Paraquat	4.67 ± 0.61	10	0.0036	60
N2	0	4°C	19.2 ± 0.89	30	-	75
fat-7	0	4°C	12.69 ± 0.85	24	0.0000001	75
fat-7	5.0	4°C	15.81 ± 0.90	28	0.0016	75
N2	0	4°C	18.93 ± 0.90	30	-	75
fat-1	0	4°C	15.71 ± 0.79	26	0.0007	75
fat-1	5.0	4°C	17.95 ± 0.85	30	0.2695	75

The mean lifespan was referred to the exact time when the survival rate dropped to 50%, which was generated by the OASIS application. For oxidative stress analysis, data are presented in hours. *p*-values were analyzed by log-rank test. Significant differences were defined at $p \leq 0.05$. MPO: macauba pulp oil.

3.4. MPO promotes the synthesis of unsaturated fatty acids and fat mobilization in C. elegans at low temperature

Macauba pulp oil, in low temperature increased the fatty acids desaturation genes (*fat-1*, *fat-5*, *fat-7*) and induced expressions of fat mobilization related genes (*aak-2*, *hosl-1*). In addition, MPO increased oxidative genes (*acs-2*, *tub-1*, *nhr-49*), decreased lipogenesis genes (*spb-1*, *pod-2*), and increased the *daf-16* and decreased *daf-2* gene ($p < 0.05$, Fig. 3).

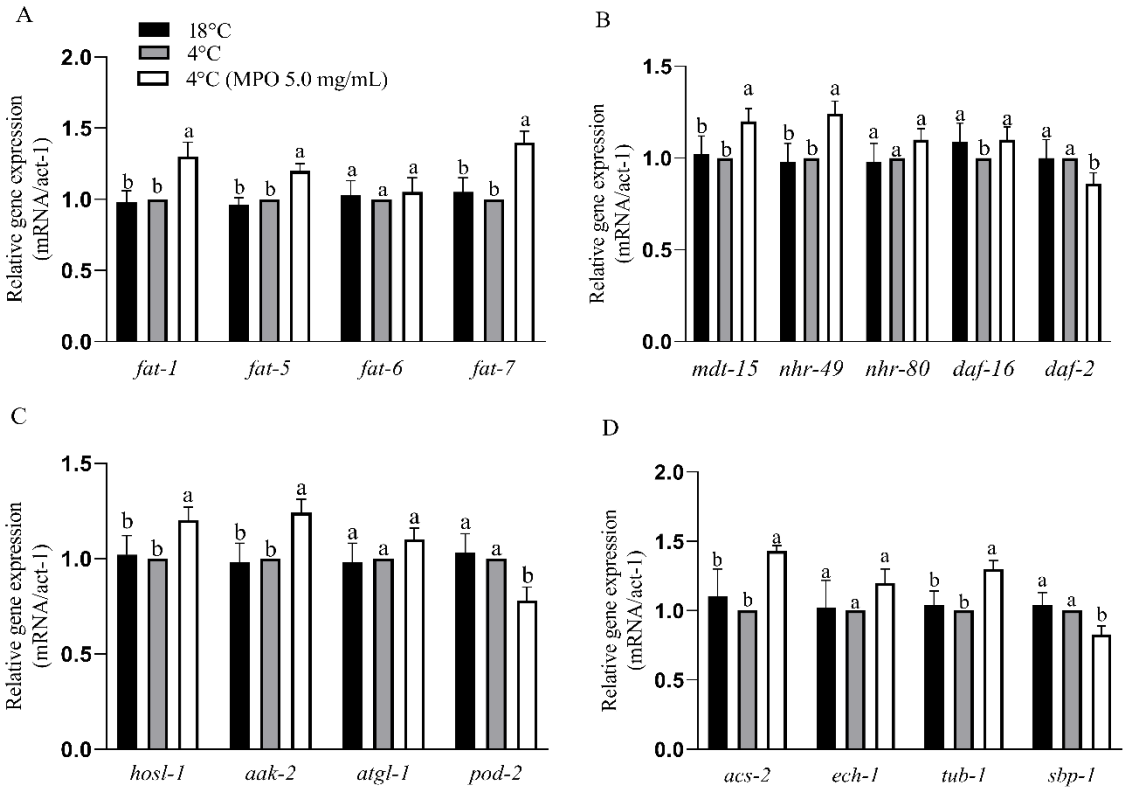

Fig. 3. Effects of macauba pulp oil (5.0 mg/mL) on genes in *C. elegans*. $n > 2000$ worms per group). Data are expressed as mean ± SEM (n=3). The presence of different letters indicates a statistical difference at $p \leq 0.05$ between bars. MPO: macauba pulp oil.

Macauba pulp oil in low temperature decreased saturated fatty acids (C16:00 and C18:00) ($p < 0.05$, Fig. 4A) and increased the levels of polyunsaturated fatty acids (PUFAs) (C18:1 n-9, C18:3 n-3 and C20:5 n-3) ($p < 0.05$, Fig. 4B).

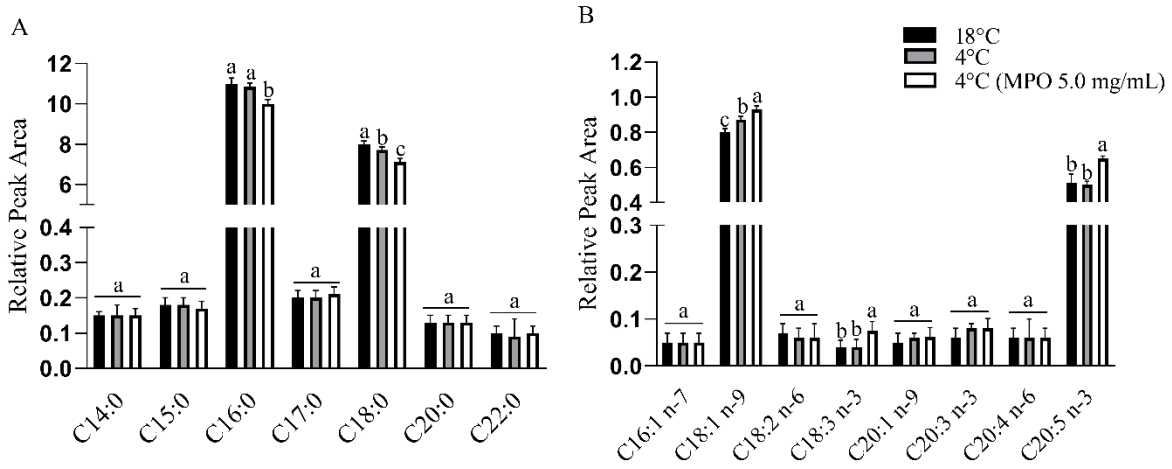

Fig. 4. Effect of macauba pulp oil (5.0 mg/mL) on the profile of saturated (A) and unsaturated (B) fatty acid in *C. elegans*. n > 5000 worms per group. Data are expressed as mean ± SEM (n=3). The presence of different letters indicates a statistical difference at $p \leq 0.05$ between bars. MPO: macauba pulp oil.

3.5. Desaturase genes (fat-1 and fat-7) is required for MPO extended survival under cold condition

In *fat-1* and *fat-7* mutant worms, macauba pulp oil showed decrease in the fat accumulation with 11.96% and 3.73% when compared to mutant worm groups, respectively (p<0.05, Fig. 5A). *fat-7* and *fat-1* mutant exhibited a reduction of survival at low temperature, demonstrating that these desaturase genes are very important in cold adaptation of *C. elegans*. The macauba pulp oil increased the survival at low temperature in the *fat-7* and *fat-1* mutant worms (p<0.05, Fig. 5B and C, Table 2). These results implicate that the desaturase gene *fat-7* and *fat-1* were required for macauba pulp oil extended survival under cold conditions.

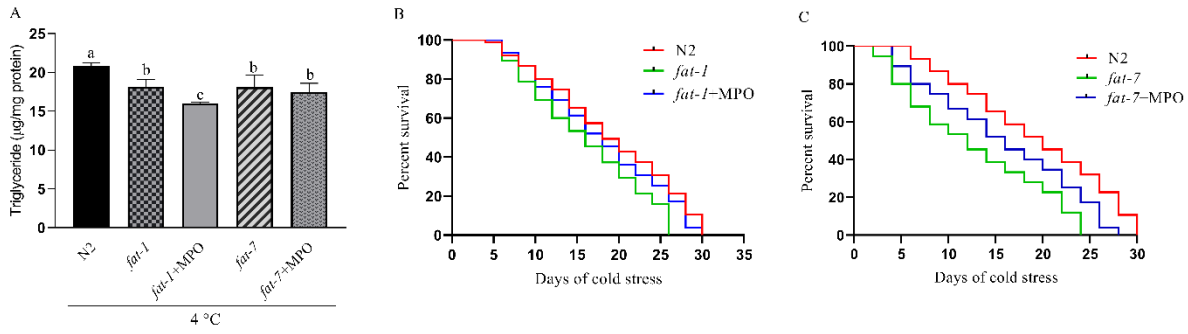

Fig. 5. Effect of macauba pulp oil (5.0 mg/mL) on the TG content and survival in mutant *C. elegans*. (A) TG content; (B) Survival of *fat-1* mutant; (C) Survival of *fat-7* mutant. For triglyceride measurement, data were from n > 1000 worms per group. Data are expressed as mean ± SEM (n=3). The presence of different letters indicates a statistical difference at $p \leq 0.05$ between bars. MPO: macauba pulp oil.

4. Discussion

The current study is the first to report the influence of macauba pulp oil on lipid metabolism and its potential role in the development of *C. elegans*. Macauba pulp oil is rich in oleic acid and carotenoids, and other studies reported the influence of this bioactive compounds in fat metabolism and oxidative stress.[6,7,8] In this study, we have demonstrated that MPO extended lifespan in *C. elegans* in low temperature through fat mobilization and unsaturated fatty acid biosynthesis (Fig. 6).

Macauba pulp oil increased the lifespan of *C. elegans* in normal condition (18°C), but more expressive results were verified at cold stress condition (4°C). These results can be explained by the increased expression levels of *fat-1*, *fat-5* and *fat-7* at low temperature. Previous study showed that *C. elegans* can adapt to the cold stress for survival through modulating membrane fluidity by altering the ratio between saturated and unsaturated fatty acids.[24] Unsaturated fatty acid has been known to increase membrane fluidity particularly at low temperature because the presence of the cis double bonds reduces the packing of phospholipids, thereby lowering its melting temperature and promoting survival.[24] Thus, the genes *fat-1*, *fat-5* and *fat-7*, required for polyunsaturated fatty acid biosynthesis in *C. elegans*, play an essential role in protecting worms from cold stress via modulating membrane fatty acid composition and its fluidity.[24,25]

Corroborating these results, in low temperature, MPO changed the fatty acid profile, with decrease in saturated fatty acid (C16:00 and C18:00) and increase in unsaturated (C18:1 n-9, C18:3 n-3 and C20:5 n-3). To confirm the relationship between the results obtained and the desaturases gene, we used *fat-1* and *fat-7* mutant worms, and our results about survival with mutant worms confirmed that the *fat-7* gene is essential for the lifespan increase generated by MPO at low temperature, since the effects of lifespan increase in the presence of MPO were abolished in *fat-7* mutant worms. Collaborating with these results, macauba pulp oil increased the

transcription factor *nhr-49* (functional homolog of PPAR-α), which resulted in the increase expression of *fat-1*, *fat-5* and *fat-7*, since *nhr-49* is cytosolic transcription factors, which are known to regulate the desaturase genes, entering the nucleus and bind specific promoters to activate transcription of *fat-1*, *fat-5*, *fat-6* and *fat-7*.[26, 27] The positive effects of macauba pulp oil observed in the present study may be associated with the high content of oleic acid and carotenoids present in this oil. Previous studies have shown that oleic acid and carotenoids increased lifespan in *C. elegans*.[28, 29]

Our study shows that MPO in low temperature induces expressions of fat mobilization related genes (*aak-2* and *hosl-1*). Fat mobilization is beneficial to cold resistance due to the production of glycerol, which functions as a cryoprotectant.[30] Consistently, MPO increased glycerol level, that is an end-product of fat mobilization. Associated with increased glycerol, MPO decreased fat accumulation, verified through TG level, and this may be associated with the decreased expression of *sbp-1* and *pod-2* genes, which are involved in lipogenesis pathway. *pod-2* encodes the acetyl-CoA carboxylase (ACC) homolog responsible for catalyzing the first step of de novo fatty acid biosynthesis and can be regulated by some factors, such as *sbp-1*(SREBP-1) and *aak-2* (AMPK).[31] Thus, increased *aak-2*/AMPK and decreased *sbp-1*/SREPB-1 might play a role in the regulation of *pod-2* by macauba pulp oil treatment. This is consistent to previous reports that oleic acid and carotenoids reduce fat accumulation by regulating lipogenesis-related molecules, such as SREBP and ACC.[8,32] Associated with the reduction of fat accumulation, macauba pulp oil increased fat oxidation-related genes (*acs-2* - fatty acid-CoA synthetase, *nhr-49* - functional homolog of PPAR-α, and *tub-1*).[11] Since *nhr-49*, *tub-1* and *acs-2* are both able to modulate fatty acid β-oxidation, we speculate that macauba pulp oil may regulate β-oxidation, which is consistent to fat reduction effect observed in this work. Additionally, the effect of MPO on reducing fat accumulation was found to be associated with the *fat-7* gene, as seen by blocking the effect of the MPO in *fat-7* mutant worms.

In addition, the gene *mdt-15*, homolog of the human mediator complex subunit 15, showed to be essential for maintaining a low temperature-induced longevity, interacting with transcription factors such as *nhr-49*, *daf-16* and *sbp-1*, and in this current work, macauba pulp oil increased *mdt-15* in low temperature.[23,33,34] Previous study showed that *mdt-15* is required to extend lifespan at low temperature by increasing the fatty acid saturated/unsaturated ratio through increased *fat-7* desaturase, similar to our present study.[35] Additionally, MPO increased *daf-16*, a

forkhead class transcription factor homologous to the mammal FOXO protein family, which is regulated by the insulin/IGF-1 signaling (IIS) pathway.[36] The ISS pathway is an evolutionary conserved pathway that regulates aging, longevity, and stress response in mammals and *C. elegans*.[37] Under normal conditions, the IIS pathway downregulates the *daf-16* activity by preventing its translocation into the nucleus and environmental condition such stress and nutrient depletion stimulate *daf-16* translocation into the nucleus, activating the expression of genes involve in the oxidative stress resistance and longevity, indicating that MPO can favor longevity through modulating *daf-16* pathway.[38]

It is very important to remember that despite the high similarity between *C. elegans* and humans, there are aspects of metabolism that are not fully conserved in this nematode. However, the study using *C. elegans* as a model showed a great indication of the potential beneficial effect of macauba pulp oil in relation to health.

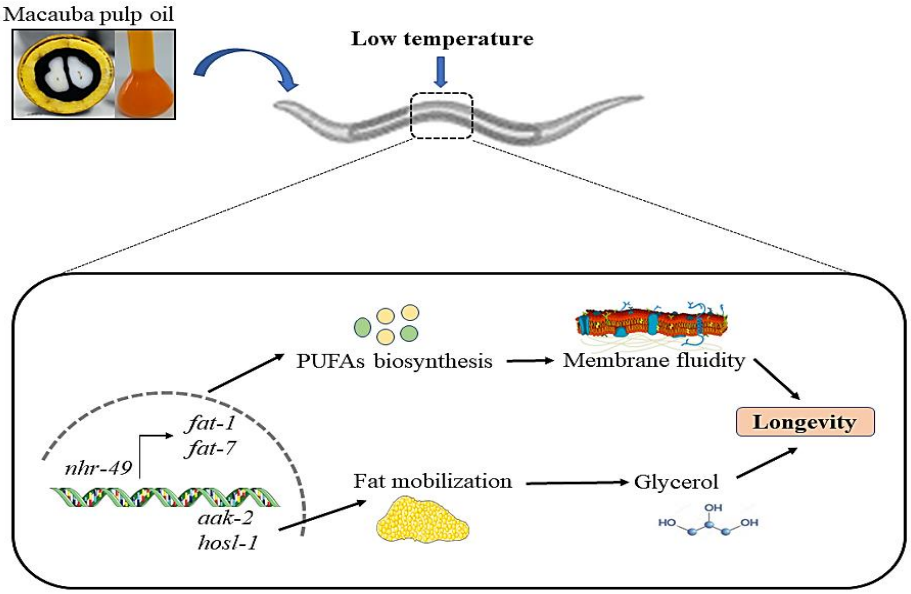

Fig. 6. Schematic model for the MPO mediated longevity at low temperature. At low temperature, the MPO upregulates the expression of *fat-1* and *fat-7*, which are encodes desaturase genes, by the nuclear receptor *nhr-49* in worms. *fat-1* and *fat-7* promotes the biosynthesis of PUFAs, which in turn induce membrane fluidity. MPO downregulates fat mobilization genes (*aak-2* and *hosl-1*), increasing the glycerol level. Membrane fluidity and glycerol promote longevity.

5. Conclusion

Our current data shows that macauba pulp oil significantly reduced the fat accumulation and increased lifespan in *C. elegans* in low temperature, and these results are associated with fat mobilization and unsaturated fatty acid biosynthesis. These effects are probably attributed to bioactive compounds present in macauba pulp oil, such carotenoids and oleic acid.

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Supplementary material

Table S1. Primer sequences for the genes in *C. elegans*.

Genes	Forward sequences (5'-3')	Reverse sequences (5'-3')
fat-1	TTGAAGCTTCATGGGAGG	TTCTGAAGCTGAACATCC
fat-5	CGCTCATATGGGATGGTTGT	CAGGGCGAAGCAGAAGATT
fat-6	GCCCAGAGACGCAATATCTC	CAGCAAAGAGAGCCACGTTA
fat-7	CAACAGCGCTGCTCACTATT	CACCAACGGCTACAACTGTG
sbp-1	CATGAATTCATTCGAGGGAGACGT CCC	CATGAATTCCTGATGTGGAGTCATC GC
daf-16	CCAGACGGAAGGCTTAAACT	ATTCGCATGAAACGAGAATG
daf-2	GGATAAAGGCGAATCAAAGTGTC	CGATACACTTTCCCTTGTGATAGAC
nhr-49	GTCGTTATTGTCGCTTTCAA	TCCGACACCGTTGCTGTTTC
nhr-80	TGAGGTTCAGGAGCCAAATAG	GAAGGAGGTGGACGATGAGA
atgl-1	GACTGGGACAGTCGAAGCAT	CTGGCTCATTTCGATCAATC
aak-2	TCTTCCGCCATCCGCATATC	CCTCTTCATCGGGTCTACGC
hosl-1	GGCTCGCTCATCAACACTGG	CACCATTTCTCCACTCTTCC
acs-2	AAGGAGATGAGAATGACTGAT	GTTCCGACATGGTGACTA
ech-1	CGAATGTAACTATCAATAAGG	ATGGCGGAATAATCAATT
tub-1	CCATGGGAGAGTCGCAGCAT	GAGGCTCATCTCGATCAATC
pod-2	ACTTGCGCCATCGGCATATC	TCGCTTCATCCGGTCTACGC
mdt-15	TCAACGTGGGCAACGATTTC	AAGGGCAAGCTGATGATAGG
act-1	TCGGTATGGGACAGAAGGAC	CATCCCAGTTGGTGACGATA

9. GENERAL CONCLUSION AND PERSPECTIVES

Macauba is an excellent source of nutrients and bioactive compounds, both in its normally consumed parts and in its co-products, presenting great potential for insertion in the food industry. Additionally, macauba pulp oil showed important health benefits, being indicative of beneficial potential in relation to obesity, inflammation, longevity and improved intestinal health. Our present work showed that macauba is rich mainly in dietary fiber, proteins, oleic acid, and carotenoids, and the adequate consumption of these compounds are related to health benefits. This is the first study that evaluated the effects of macauba pulp oil in relation to metabolic changes, and we found positive effects, such as preventing oxidative stress, inflammation and adipogenesis, modulating the intestinal microbiota and improving the integrity of the intestinal barrier in mice with a high-fat diet, providing the extension from lifespan and reducing fat accumulation in *C. elegans*. Therefore, macauba presents an important alternative for health promotion, as well as a healthy alternative for the food industry.

This work presents impacts at an academic, social, economic and environmental level. The scientific articles resulting from this work will result in the dissemination of knowledge about macauba and a better understanding of its composition and its potential use, and thus subsidize new studies with macauba. The appreciation of consumption and insertion of macauba in the food industry benefits the health of the population, as well as the appreciation of a Brazilian product, generating income for small producers and creating new jobs. Also, macauba pulp oil is a more economical alternative to olive oil consumption, making it an important food strategy for low-income families. The total utilization capacity of macauba (products and co-products) generates environmental impacts, reducing waste.

Despite the numerous positive points observed with this study, we have to keep in mind the limitations present in this work. It is important to remember that the benefits observed in relation to the consumption of macauba pulp oil were verified in experimental animal models (mice and *C. elegans*) and cells, and that there are metabolic differences between these models and humans. Thus, as this is the first work, more studies are needed, with different doses, and mainly experiments with human beings to faithfully prove the benefits found in this study.

It is hoped that this work is just the beginning of research on macauba and that other works can further explore the beneficial potential of macauba consumption and thus generate the appreciation of this Brazilian product and consequently the generation of jobs, local income and helth benefits.

10. ATTACHMENTS

Attachment 1. Certificate of approval of the Ethics Committee in Animal Use

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 09/2019, intitulado "**Avaliação do efeito do óleo da polpa de macaúba (*Acrocomia aculeata*) na obesidade, inflamação e estresse oxidativo em modelo animal alimentado com dieta hiperlipídica**", coordenado pelo professor Frederico Augusto Ribeiro de Barros do Departamento de Tecnologia de Alimentos, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 28/05/2019, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 09/2019, named **"Evaluation of the effect of oil of macaúba pulp (*Acrocomia aculeata)* on obesity, inflammation and oxidative stress in an animal model fed with a hyperlipidic diet"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/ MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on May 28, 2019 valid for 12 months.

Prof.ª Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV