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Fermented dairy beverage added with clove essential oil modulates oxygen reactive species (ROS) levels: an *in silico*, *in vitro,* and *in vivo* approach

Bebida láctea fermentada adicionada com óleo essencial de cravo da índia modula os níveis de espécies reativas de oxigênio (ERO): uma abordagem *in sílico, in vitro* e *in vivo*

La bebida láctea fermentada agregada con aceite esencial de clavo modula los niveles de especies reactivas al oxígeno (ERO): un enfoque *in silico, in vitro* e *in vivo*

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Abstract
This study aimed at investigating the effect of fermented dairy beverage with clove essential oil (CEO) on the levels of reactive oxygen species (ROS) through endogenous enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). For this, bioinformatic analysis was performed using the Genecards and String platforms. Headspace was used to analyze and confirm the compounds at low concentrations. Antioxidant activity was analyzed using the DPPH free radical sequestration method. For analysis of antioxidant activity, twenty-four male Swiss mice were divided into three groups and submitted histopathological analysis, analysis of CAT, SOD,GPx transcripts, and CAT and SOD enzyme activity in visceral adipose tissue were evaluated.The leader genes found were PIK3CD, PIK3CB, AKT1, and PIK3CA, as they had the highest WNL values. The beverage containing CEO showed higher antioxidant activity, with free radical scavenging capacity above 80%. In the *in vivo* analyses, it was possible to verify a reduction in the average adipocyte area size (μm2) between the groups that received fermented dairy beverage. Although functional studies have shown that the antioxidant enzymes, CAT and SOD, showed similar concentrations in visceral adipose tissue of the three groups, the expression levels of GPx1, CAT, and SOD were higher in group 2. Surprisingly, the group 3, that received the fermented dairy beverage with CEO had the lowest SOD concentration ($p < 0.05$). Therefore, the antioxidant mechanism of the CEO can be mediated by the activation of the cell survival pathway PI3K/Akt and modulation of SOD1 and CAT enzymes by means of ROS reduction.
Keywords: *Syzygium aromaticum*; Visceral adipose tissue; Superoxide dismutase; Catalase; Glutathione peroxidase.

Resumo
Este estudo teve como objetivo investigar o efeito da bebida láctea fermentada com óleo essencial de cravo da índia (OEC) nos níveis de espécies reativas de oxigênio (ERO) por meio das enzimas endógenas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). Para isso, foram realizadas análises bioinformáticas nas plataformas Genecards e String. Headspace foi usado para analisar e confirmar os compostos em baixas concentrações. A atividade antioxidante foi analisada usando o método de sequestro de radicais livres DPPH. Para análise da atividade antioxidante, vinte e quatro camundongos Swiss machos foram divididos em três grupos e submetidos a análise histopatológica, análise de CAT, SOD, transcritos de GPx e atividade das enzimas CAT e SOD no tecido adiposo visceral, PIK3CB, AKT1 e PIK3CA, pois apresentaram os maiores valores de WNL. A bebida contendo OEC apresentou maior atividade antioxidante, com capacidade de eliminação de radicais livres acima de 80%. Nas análises *in vivo*, foi possível verificar uma redução no tamanho médio da área de adipócitos (μm2) entre os grupos que receberam a bebida láctea fermentada. Embora estudos funcionais tenham mostrado que as enzimas antioxidantes, CAT e SOD, apresentaram concentrações semelhantes no tecido adiposo visceral dos três grupos, os níveis de expressão de GPx1, CAT e SOD foram maiores no grupo 2. Surpreendentemente, o grupo 3, que recebeu a bebida láctea fermentada com OEC teve a menor concentração de SOD ($p < 0,05$). Portanto, o mecanismo antioxidante do OEC pode ser mediado pela ativação da via de sobrevivência celular PI3K / Akt e modulação das enzimas SOD1 e CAT por meio da redução de ROS.
Palavras-chave: *Syzygium aromaticum*; Tecido adiposo visceral; Superóxido dismutase; Catalase; Glutationa peroxidase.

Resumen
Este estudio tuvo como objetivo investigar el efecto de la bebida láctea fermentada con aceite esencial de clavo (AEC) sobre los niveles de especies reactivas de oxígeno (ERO) a través de las enzimas endógenas superóxido dismutasa (SOD), catalasa (CAT) y glutatión peroxidasa (GPx). Para ello, se realizó un análisis bioinformático utilizando las plataformas Genecards y String. Se utilizó espacio de cabeza para analizar y confirmar los compuestos a bajas concentraciones. La actividad antioxidante se analizó utilizando el método de secuestro de radicales libres DPPH. Para el análisis de la actividad antioxidante, veinticuatro ratones suizos machos se dividieron en tres grupos y se sometieron a análisis histopatológico, análisis de CAT, SOD, transcripciones GPx y se evaluaron la actividad de las enzimas CAT y SOD en tejido adiposo visceral. Los genes líderes encontrados fueron PIK3CD, PIK3CB, AKT1 y PIK3CA, ya que tenían los valores de WNL más altos. La bebida que contenía el AEC mostró una mayor actividad antioxidante, con una capacidad de eliminación de radicales libres superior al 80%. En los análisis *in vivo*, fue posible

verificar una reducción en el tamaño medio del área de adipocitos (μm2) entre los grupos que recibieron bebida láctea fermentada. Aunque los estudios funcionales han demostrado que las enzimas antioxidantes, CAT y SOD, mostraron concentraciones similares en el tejido adiposo visceral de los tres grupos, los niveles de expresión de GPx1, CAT y SOD fueron mayores en el grupo 2. Sorprendentemente, el grupo 3 que recibió La bebida láctea fermentada con AEC tuvo la concentración más baja de SOD ($p < 0.05$). Por tanto, el mecanismo antioxidante del AEC puede estar mediado por la activación de la vía de supervivencia celular PI3K / Akt y la modulación de las enzimas SOD1 y CAT mediante la reducción de ROS.
Palabras clave: *Syzygium aromaticum*; Tejido adiposo visceral; Superóxido dismutasa; Catalasa; Glutatión peroxidasa.

1. Introduction

The fermented dairy beverage consists of a mixture of milk and whey and has at least 51% of milk base. It may or may not have added food products or substances, such as selected dairy yeasts, vegetable fat, fermented dairy, and other dairy products. For preserving fermented dairy beverage s, potassium sorbate, calcium sorbate, sorbic acid, and sodium sorbate can be used (Brasil, 2005).

At the moment there is a high demand from consumers for healthy natural products that can help prevent diseases (Baldissera et al., 2011).

Thinking about that, Farias (2016), in his study he sought to prioritize the use of natural preservatives in the manufacture of fermented dairy beverage. These beverages, were prepared by adding essential oils of Syzygium aromaticum, Lippia alba, and Cymbopogon citratus, evaluating the preservative potential of these oils, and the nutritional and quality parameters provided for in the legislation. Among the essential oils studied, the Syzygium aromaticum showed better antioxidant and antimicrobial results without changing the amount of desirable dairy bacteria.

Due to the scarcity of work using fermented dairy beverage with clove essential oil, studies of the antioxidant activity in the dairy matrix are necessary to verify the functional potential of the beverage.

The clove (*Syzygium aromaticum*) is a native spice from East Indonesia, widely used in cooking and extensively cultivated in African and Asian countries (Baldissera et al., 2011; Farias, 2016). The biological activities attributed to it draw attention to this spice and include: antioxidant activity, fungicide, acaricide, insecticide, antimicrobial, antiseptic, among others (Darvishi et al., 2013; Kamatou et al., 2012; Le Lay et al., 2014).

Antioxidants in foods retard oxidative changes, curbing it from deteriorating upon contact with air (Prakash et al., 2015). In vivo, this action is related to the control of the levels of reactive oxygen species (ROS) in the body through endogenous enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) as the main involved and exogenous enzymes that are those you get for food (Darvishi et al., 2013; Kamatou et al., 2012).

Changes involving these antioxidant enzymes or low production along with increased production of ROS can alter the body's balance and cause oxidative stress (Darvishi et al., 2013). This is a consequence of the imbalance between oxidant production and antioxidant enzymes, promoting the increase of ROS considered harmful to health and is associated with diseases, such as cancer, diabetes, and atherosclerosis (Le Lay et al., 2014). Since studies that address the molecular mechanism of antioxidant action of fermented dairy beverage added with clove essential oil are few, the present study investigated its effect on the levels of reactive oxygen species (ROS) through endogenous enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx).

2. Methodology

Research on genes involved in the antioxidant action of the clove, network construction method, and protein-protein interaction data

We collected different genes associated with the antioxidant action of Indian clove from Genecards (http://www.genecards.org), a database of genes, their products, and biomedical applications maintained by Israel's Weizmann Institute of Science. We searched the databases using the keywords "*Syzygium aromaticum*," "antioxidant," and "oxidative stress."

All the networks were created utilizing the network visualization string database. String (http://string-db.org/, ver. 10) is a database of known and forecasted protein–protein interactions. Inputting the targets and the data of the antioxidant action of clove into String constructs different networks based on this research of Genecards. Network construction was performed as shown in Table 1.

Table 1 – Parameters used in network construction.

Parameters	Basic Settings
Meaning of network edges	Molecular action
Active interaction sources	Textmining, Experiments, Databases, Co-expression, Neighborhood, Gene Fusion, Co-occurrence
Minimum required interaction score	Medium confidence (0.400)
Max number of interactors to show	1st shell: no more than 10 interactors 2nd shell: no more than 10 interactors

Source: Authors.

The data of other human protein and protein–protein interactions (the antioxidant action of clove) came from String, with the species limited to "Homo sapiens" and a confidence score >0.4.

From the sum of active interaction sources obtained from the String database, it was possible to calculate the weighted number of links (WNL) and consequently obtain the leader genes. These were the ones that presented the highest values of WNL. The genes were ranked according to this parameter in clusters by the K-means clustering method. To evaluate the differences among various classes based on WNL, an ANOVA test was used. Statistical significance was set at $p < 0.001$.

From the analysis of the String database, data for the ontological analysis were also extracted. The data sources were based on biological processes, molecular functions, and cellular components.

Characterization of fermented dairy beverage and chemical composition of clove essential oil

Fermented Dairy Beverage (FDB) were produced at the Food Technology Laboratory of the Institute of Agrarian Sciences (ICA) at the Federal University of Minas Gerais (UFMG) in compliance with current legislation (Brasil, 2005). To produce the fermented dairy beverage with the synthetic preservative, potassium sorbate (0.003 g/mL) was added and to the fermented dairy beverage (2 μL/mL) of the essential oil (EO) was added.

The elaborated beverages were in accordance with the standards established by the current legislation, obtaining a result of 0.70 to 0.74 Kcal/mL for fermented dairy with the addition of clove essential oil and 0.68 to 0.72 Kcal/mL for potassium sorbate fermented dairy beverage. The chemical composition of the clove essential oil was obtained by gas chromatography. In a previous study by Farias (2016) where eugenol (79.4%) was found to be the major component and to a lesser extent, (E)-caryophyllene, humulene, caryophyllene oxide, and δ-cadinene, respectively.

Headspace was used to analyze and confirm the compounds at low concentrations (Aguiar et al., 2014). One milliliter of the beverages was added to headspace vials, then these vials were placed on an autosampler (HS combi-PAL) for proper homogenization (500 rpm) and then incubated at 75°C for 5 minutes, where the volatile substances were extracted by static headspace. The syringe was heated to 75°C and the injection volume was 1000 µL. The volatile substances were identified by Agilent Technologies (7890A) coupled to a mass spectrometer (MS 5975C), using a temperature of 60ºC °C to 240ºC °C, with an increment of 3ºC °C · min-1. The entire procedure was operated in scan mode with an electronic impact at 70 eVwithin the range of 45 to 550 (mz). The data obtained were analyzed using the MSD Chemstation software and the National Institute of Standards and Technology library.

Antioxidant activity analysis by free radical sequestration method – DPPH

Antioxidant activity analyses were performed using the DPPH free radical sequestration method (2,2-diphenyl-1-picrihidrazil, Sigma-Aldrich) using a methodology adapted from Najgebauer-Lejko et al., (2011). For the analyses, three types of FMD (with clove essential oil (BO), potassium sorbate (BSO), and preservative free (BP) were used.

The beverage were diluted in methanol in a ratio of 1: 0.5. Then, 0.1 mL aliquots of this dilution were transferred to properly labeled transparent conical propylene tubes. Soon after, 3.9 mL of DPPH reagent was added and placed in a dark room for 30 minutes. After that, the samples were centrifuged (Centribio) at 4000 rpm for 10 minutes. The sequestration consumption of the DPPH radical was measured in the supernatant using a spectrophotometer (Micronal B-582) with a wavelength of 515 nm. Solutions containing 3.9 mL DPPH (0.277g / mL) and 100 µL methanol were used as negative controls. Methanol was used as a white solid. For the percentage of free radical sequestration (SRL), the following equation was used (Teixeira et al., 2013):

% FRS = ((Negative Control Abs - Reaction Mix Abs) / Negative Control Abs) x 100

Where:
FRS: free radical sequestration
Negative control Abs: absorbance of DPPH solution
Reaction mix Abs: DPPH reaction absorbance with 0.1mL of samples

To determine the EC_{50} values, which is the minimum concentration required for BLF to reduce the DPPH radical to 50%, to formulate the reduction curve, concentrations of 0.05, 0.25, 0.5, 0.75, 1.0, and 1.25 µL / mL of BO were chosen. Butylated hydroxytoluene (BHT) was used as a positive control at concentrations of 0.000125, 0.00025, 0.0005, 0.001, and 0.002 g/mL. To verify the antioxidant potential of the samples, the formula proposed by Scherer & Godoy (2009) was used, which classifies as: low antioxidant activity (AAI <0.5), moderate (0.5 <AAI <1.0), strong (1.0 <AAI <2.0) and very strong (AAI> 2.0).

$$AAI = \frac{CDPPH}{CE_{50}}$$

Analysis of antioxidant activity in an animal model

We used 24-week-old male Swiss mice. The study was carried out at the Animal Experimentation Center of the Montes Claros State University - Unimontes. It was approved under protocol number 231/2017 by the Animal Experimentation

Ethics Committee of the Federal University of Minas Gerais (CEUA/UFMG) and by the Animal Experimentation and Welfare Ethics Committee of the Montes Claros State University - Unimontes, under case number 149/2017.

To start the experimental period, the animals had a 7-day period of adaptation with the labina® commercial ration containing 23.3% protein, 2.6% lipids, 55.6% carbohydrates, and 4.0 kcal/g of total energy and water ad libitum. After four weeks of life, the animals were randomly divided into three groups post adaptation period using a completely randomized design. Weight was a division parameter to obtain homogeneous samples between groups. The division occurred according to the daily food offered by gavage for 30 days: control group (G1) fed only with ration; group two (G2) fed ration plus 2 mL of fermented dairy beverage with synthetic preservative (potassium sorbate), and group three (G3) received ration and 2 mL of the fermented dairy beverage with the addition of clove essential oil.

The diet provided was isocaloric, that is, each group was restricted to the ration so that all experimental animals received the same amount of Kcal per day. Thus, the diets were adjusted weekly according to the average weight of the animals belonging to each group. The animals were weighed weekly. To obtain the isocaloric diet, calorimetric analyses were performed according to the methodology described by Souza et al., (2018), obtaining the result of calories per gram of beverage.

The animals were euthanized after 12 hours of fasting. The collected visceral adipose tissue was divided and stored in separate Eppendorf tubes for later real-time PCR (qRT-PCR) and histological analysis.

Histopathological analysis of visceral adipose tissue

For the histopathological analysis of the visceral adipose tissue, it was immersed in 10% formaldehyde. Subsequently, tissue dehydration was performed in a growing alcohol series (70%, 80%, 90%, absolute).Then the samples were placed in xylol and paraffin impregnation, and the slides were stained with hematoxylin and eosin (Martins et al., 2018).We used 5 different fields on each slide containing 100 adipocytes for evaluation, and the images of each animal were captured. An Olympus FSX 100 microscope and Image Pro-Plus software (Media Cybernetics, USA) were used. The average adipocyte area (μm2) was calculated using Image J software (National Institute of Health, Bethesda, Maryland, USA) (Zicker et al., 2019).

Analysis of CAT, SOD, and GPx transcripts in visceral adipose tissue

RNA extraction from visceral adipose tissue was performed by homogenizing the tissue in Trizol, as specified by the manufacturer. Sample RNA quantification and purity were performed using a NanoDrop (Thermo Scientific®). After quantitation of the samples, they were treated with DNase and reverse transcribed with Moloney murine leukemia virus reverse (M-MLV RT) (Ludwig Biotec®) and OligodT.

The cDNA obtained was used for qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous primer to normalize mRNA gene expression. Reactions were performed using the SYBR Green Master Mix on the PlusOne platform (Applied Biosystems ™ QuantStudio ™ 6 Flex Real-Time PCR System®) (Andrade et al., 2019).

The following primers were used to quantify antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathioneperoxidase (GPx) (Table1). Expression levels were measured by relative comparison method by CT using equation $2 - \Delta\Delta CT$ (Sordella, 2004).

Evaluation of CAT and SOD enzyme activity in visceral adipose tissue

To analyze the activity of antioxidant enzymes, the enzymes catalase (CAT) and superoxide dismutase (SOD) were evaluated and 100 mg of visceral adipose tissue samples were taken separately to make the homogenate of each analysis.

To determine catalase activity, the methodology described by (Aebi, 1984) was used, using the reading times of 0, 20, 40.60, and 90 s at a wavelength of 340 nm. For the activity of superoxide dismutase, a methodology similar to that described by Bannister & Calabrese, (2006) was used, using the times of 0, 1, 2, 3, 4, and 5 minutes for reading at a wavelength of 420 nm. A spectrophotometer Amersham bioscience ultrospec 1100 pro was used. Results were expressed as enzymatic activity per mg protein. The values were obtained according to the following equation:

Antioxidant enzyme (U/mg protein) = sum of results obtained ((final abs. - Initial abs.) / 2) x V

Where:
Final Abs. Results of the last absorbance reading of the solution.
Initial Abs. Results of the first absorbance reading of the solution.
V: sample volume (mL).

Statistical analysis

The one-way analysis of variance (ANOVA) test was used subjected to specific tests with a statistical confidence of 95% ($p < 0.05$). Statistical significance of differences in mean values between groups of mice was assessed by the Tukey test. The results of the evaluation analyses of the activity of antioxidant enzymes in the visceral adipose tissue and the adipocyte diameter were submitted to the Bonferroni multiple comparison test to evaluate the statistical significance of the differences. All analyses were performed using GraphPad Prism (version 7.0 ©, San Diego, California, USA). For the values obtained in the EC50, Microsoft® Office Excel was used to perform the regression analysis.

3. Results

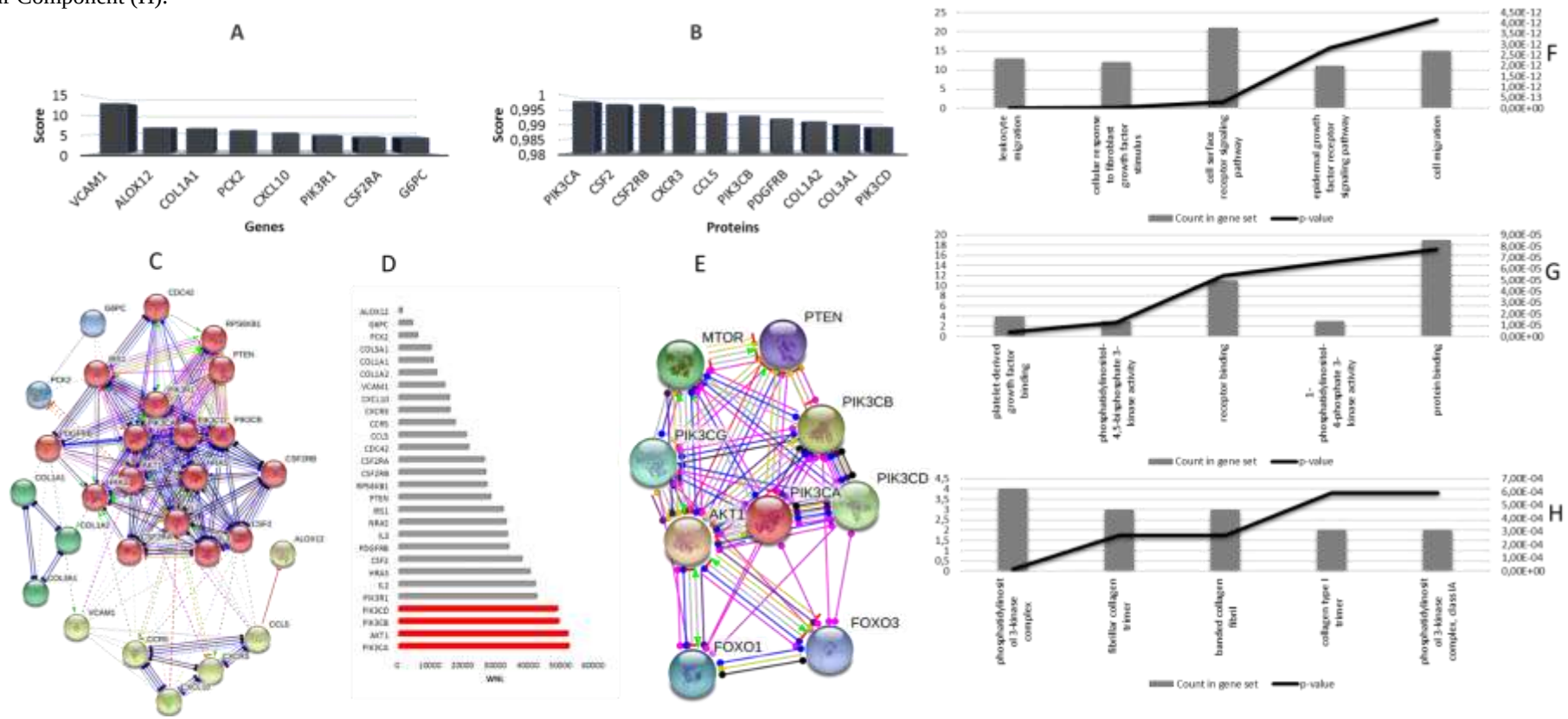
Investigation of molecular mechanisms involved in the antioxidant action of *Syzygium aromaticum* by *in silico* analysis

From the surveys conducted at Genecards, we obtained eight genes in total. The details are described in Figure 1A. From the searches made in the String database, it was possible to obtain the predicted functional partners. The details are described in Figure 1B.

The protein interaction network formed from genes extracted from Genecards is shown in Figure 1C. There was a large cluster of interactions around the AKT and PIK3 genes. Consequently, based on the WNL values, the leader genes found were PIK3CD, PIK3CB, AKT1, and PIK3CA, as they had the highest WNL values (Figure 1D). This analysis highlights the great importance of these genes in the antioxidant action of Syzygium aromaticum, as they directly or indirectly modulate a large amount of proteins. In the interaction network presented in figure 1E, it is possible to observe the main proteins modulated by the leader genes, indicating that mTOR is a candidate to be part of the molecular mechanism of the antioxidant action of *Syzygium aromaticum*.

The representation of gene ontologies consists of a controlled vocabulary that describes the role of genes and their products. Thus, the bioinformatics initiative was also used to define GO terms in three different domains: cellular component, molecular function, and biological process. The cellular component indicates the location of the gene product inside and/or outside the cell, which is a clue to where protein RNA acts in the cell, which in turn is a clue to its function. In the present study, we highlighted the phosphatidylinositol 3-kinase complex, with a large number of genes involved and a significant p-value ($6x10^{-4}$) (Figure 1H). The biochemical molecular function of a gene and its product (RNA and or protein) is indicated by protein binding, with more than 15 genes involved ($p = 8x10^{-5}$) (Figure 1G). The biological process, a set of well-defined beginning and end molecular events pertinent to the functioning of integrated life units, was the cell migration (15 genes involved and $p = 4x10^{-12}$) (Figure 1F).

Figure 1 - Target genes involved in the antioxidant action of the Indian clove obtained from Genecards (A) and predicted functional partners obtained from the String database (B). Network showed "gene-gene interaction" based on the data of antioxidant action of Indian clove. Network Stats - number of nodes: 28, number of edges: 162, average node degree: 11.6, avg. local clustering coefficient: 0.825, expected number of edges: 70, PPI enrichment p-value: < 1.0e-16 (C). Strength of interaction between the genes of the network. Leader genes are shown in red (D). Network showed "gene-gene interaction" based on the data of leaders genes (E). Gene Ontology (GO). Biological Process (F). Molecular Function (G). Cellular Component (H).



Source: Authors.

In vitro* functional assays to evaluate the antioxidant activity of *Syzygium aromaticum

Headspace analysis did not detect and identify compounds that could have antioxidant activity in the BP and BSO samples (Figure 2).

The BO samples (Figure 2A) showed similar results to those found for the pure oil (Figure 2B). The compounds Eugenol, Cariophilene, and α-humulene were identified in both samples. Thus, it can be noted that there was no change in oil composition even after being added to the milk matrix. The results obtained in the headspace analysis are similar to those found in the analysis of the chemical composition of the EO, where a higher peak of eugenol abundance was observed and caryophyllene and α-humulene compounds were identified in the samples.

The fermented dairy beverage containing clove EO showed higher antioxidant activity than BSO and BP ($p < 0.05$) with free radical scavenging capacity above 80% (Table 2).

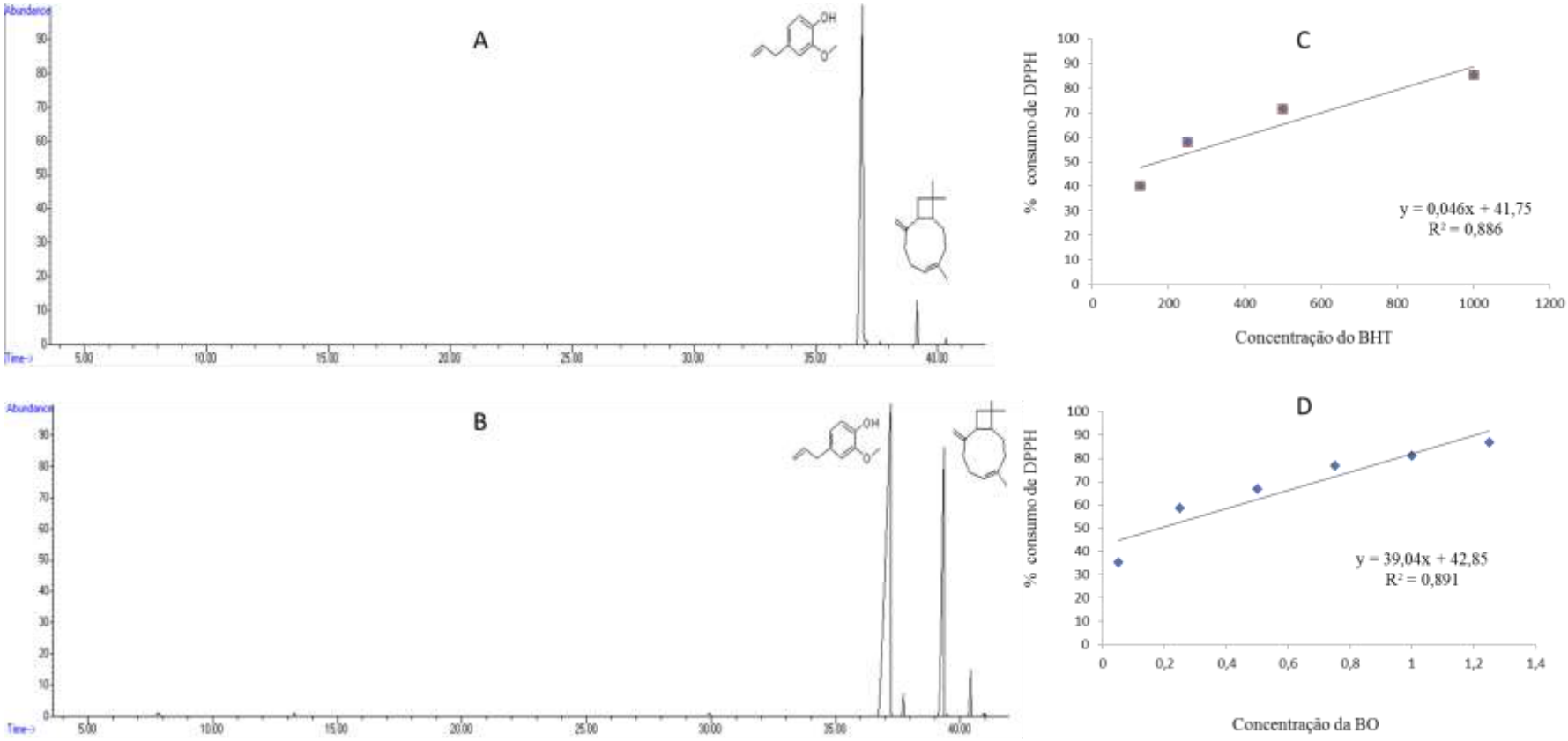
Antioxidant activity observed in the beverages remained unchanged during the storage period for 14 days ($p > 0.05$).

In evaluating the results, it was found that BO obtained the highest percentage of free radical sequestration by the DPPH method. These results indicate a greater capacity of BO to sequester free radicals in relation to BSO and BP.

When evaluating the ability of BO to reduce the DPPH radical by 50%, using a minimum concentration we found that the concentration of 0.25 µL / mL obtained a value of 58.64% SRL in the sample (Table 3). Regarding BHT at a concentration of 2 µg/mL, which is proportional to the addition of EO in the beverage, it was observed that there was no free radical sequestration. In addition to this concentration, concentrations of 0.02 g BHT / 100 mL methanol) and 0.0002 g/mL were tested, which did not result in free radical sequestration. Only at a concentration of 0.002 g/mL did the BHT-positive control present free radical sequestration (Table 3).

The EC_{50} values were 0.1831 for BO and 179.0 for BHT (Fig. 2). Due to these results, the formula proposed by Scherer and Godoy (2009) is inapplicable because the values obtained were discrepant. The percentage consumption of DPPH was proportional to the concentration of BHT and BO ($R^2 = 0.886$ and 0.891, respectively).

Figure 2 - Headspace analysis of fermented dairy beverage with clove essential oil (A) and pure clove essential oil (B). Regression analysis of the relationship between DPPH consumption and sample concentration and EC50 obtained: Fermented dairy beverage with the addition of clove essential oil (C); Fermented dairy beverage with the addition of butylated hydroxytoluene (D).



Source: Authors.

Table 2 - Antioxidant analyzes using the DPPH method of fermented dairy beverages.

	PRODUCTION 01		PRODUCTION 02		PRODUCTION 03		PRODUCTION 04	
	1° Sem.	2° Sem.	1° Sem.	2° Sem.	1° Sem.	2° Sem.	1° Sem.	2° Sem.
BO	92,0% ^{Aa} ± 5,11	86,6%^{Aa} ± 7,59	93,4%^{Aa} ± 4,37	90,3%^{Aa} ± 3,87	91,3%^{Aa} ± 1,91	82,7%^{Aa} ± 3,30	92,2^{Aa} ± 1,1	86.5^{Aa} ± 3,8
BSO	44,8%^{Ba} ± 12,09	12,7%^{Ab} ± 1,63	37,14% ^{Ca} ± 13,6	5,72%^{Bb} ± 2,02	45,1%^{Ca} ± 14,6	5,60%^{Bb} ± 4,74	42,3^{Ba} ± 4,5	8,9^{Bb} ± 4,6
BP	42,3%^{Ba} ± 1,71	8,75%^{Ab} ± 1,31	52,5%^{Ba} ± 3,47	4,86%^{Bb} ± 0,07	61,1%^{Ba} ± 11,38	15,1%^{Bb} ± 5,44	52,0^{Ba} ± 9,4	10,4^{Bb} ± 6,0

Values expressed by fermented dairy beverage as mean and standard deviation.
BO - Fermented dairy beverage with the addition of clove essential oil; BSO - Fermented dairy beverage with potassium sorbate; BP- Fermented dairy beverage without preservative.
By the Tukey test at 5% significance, means followed by the same capital letter in the same column in the row do not differ.
Source: Authors

Table 3 - Relationship between the concentration of antioxidants under study and the percentage of free radical scavenging.

Amostras	Concentração	%SRL
BO	1,25 µL/mL	86,98030635
BO	1 µL/mL	80,96280088
BO	0,75 µL/mL	76,80525164
BO	0,5 µL/mL	66,84901532
BO	0,25 µL/mL	58,64332604
BO	0,05 µL/mL	35,2297593
BHT	0,002 g/mL	85,33916849
BHT	0,0005 g/mL	71,5536105
BHT	0,00025 g/mL	57,9868709
BHT	0,000125 g/mL	40,15317287

BO: fermented dairy beverage with the addition of clove essential oil; BHT: Butylated hydroxytoluene; % SRL: percentage of free radical scavenging.
Source: Authors

In vivo* functional assays to evaluate the antioxidant activity of *Syzygium aromaticum

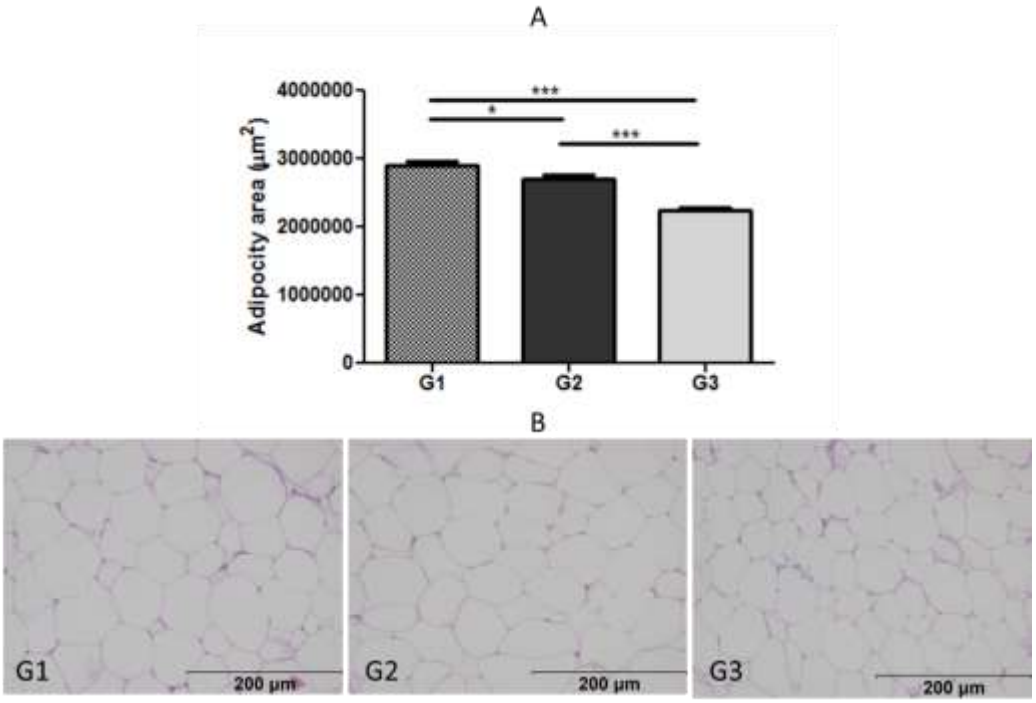
In the *in vivo* analyses, it was possible to verify a reduction in the average adipocyte area size (μm^2) between the groups that received the fermented dairy beverage s (G2 and G3) in relation to the control group (G1) that had only feed and water *ad. libitum*, ($p < 0.05$), and G3 presented smaller adipocytes than G2 (Figure 3A). In the evaluation of the histological slides, no alteration in the animal tissues was observed (Figure 3B).

When quantifying the antioxidant enzymes present by qRT-PCR analysis, it was found that the enzymes Glutathione Peroxides (GPx1) and Catalase (CAT) were statistically different ($p < 0.05$) for G2 in relation to G1 and G3, and they did not present statistical difference between them ($p > 0.05$) (Figure 6). For superoxide dismutase (SOD), G2 showed higher expression ($p < 0.05$) in relation to G1 and G3, and these groups presented statistical differences ($p < 0.05$) among themselves (Fig 4A).

The activity of antioxidant catalase (CAT) and superoxide dismutase (SOD) activity in adipose tissue (Figure4B) was similar for the groups ($p > 0.05$).

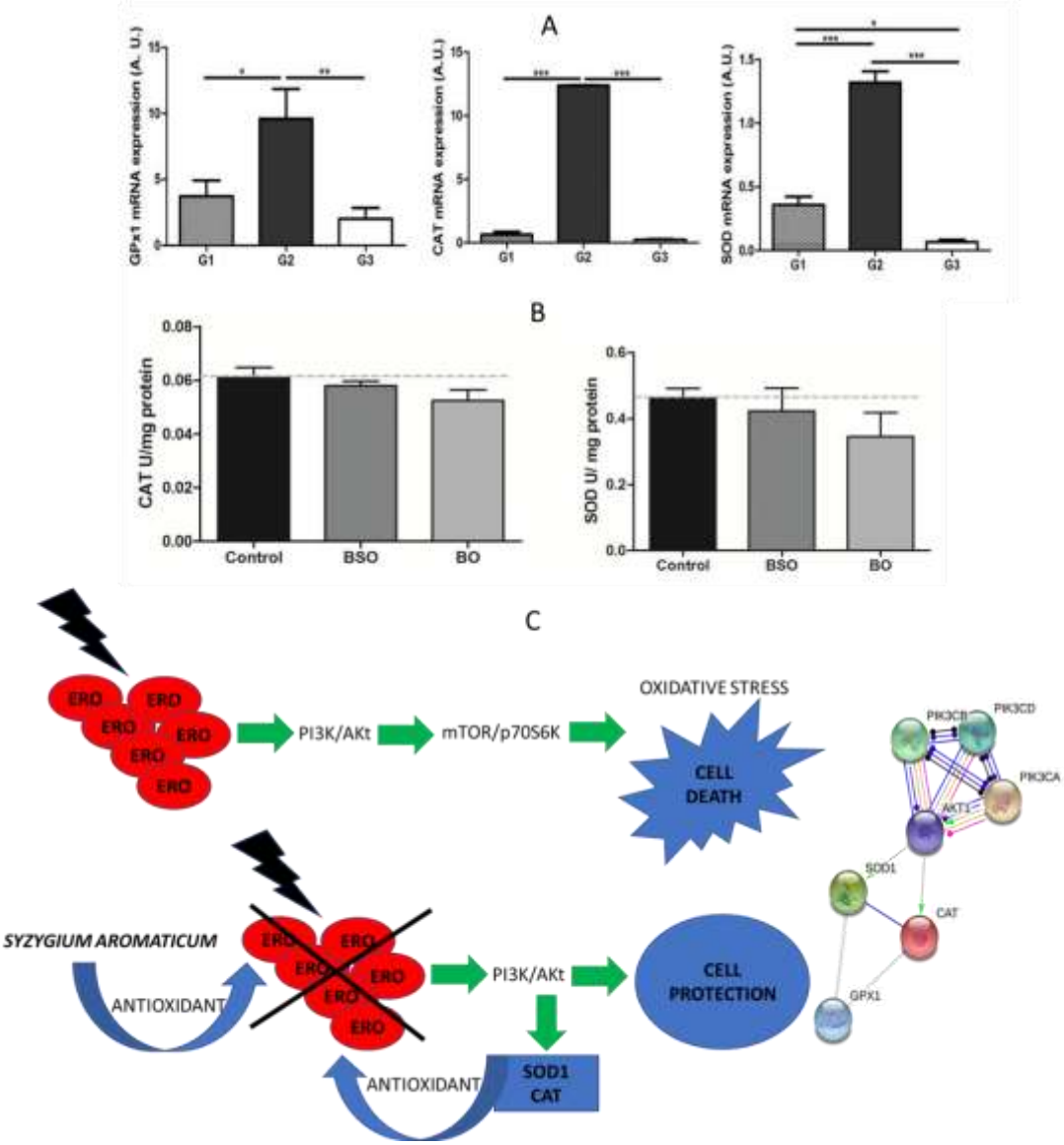
Based on the results, a scheme for the antioxidant action of cloves added to the fermented dairy beverage was developed. It was verified that the molecular mechanism of these activities goes through the leader genes and the enzymes that were studied (Figure 4C).

Figure 3 – Evaluation of the area of adipocytes in the visceral adipose tissue of mice after eating the evaluated diets (A). Representation of the histopathological evaluation of the visceral adipose tissue of male Swiss mice according to the different diets offered (B).



G1 - Control: diet with only feed; G2 - G1 isocaloric group: diet: feed + fermented dairy beverage with synthetic preservative (potassium sorbate); G3 - G1 isocaloric group: diet: feed + fermented dairy beverage with clove essential oil. Values were considered significant for * $p < 0.05$.
Source: Authors

Figure 4 – Quantification of mRNA expression of antioxidant enzymes in visceral adipose tissue of male Swiss mice (A). Evaluation of the activity of antioxidant enzymes in visceral adipose tissue of mice in the control group and those who received an elaborate diet (B). Schematic diagram of indian clove antioxidant activity in normal cells (C). Control (G1): diet with only feed; BSO (G2) - G1 isocaloric group: diet: feed + fermented dairy beverage with synthetic preservative (potassium sorbate); BO (G3) - G1 isocaloric group: diet: feed + fermented dairy beverage with clove essential oil.



ROS: species reactive to oxygen; CAT: Catalase; SOD: Superoxide dismutase; mTOR / p70S6K: Target rapamycin route in mammals (mTOR) / ribosomal p70 S6 protein kinase (p70S6K); PI3K: Phosphatidylinositol 3-kinases; AKT: Serine-threonine protein kinase. Source: Authors.

4. Discussion

Reactive oxygen species (ROS) can cause mutations in the DNA, silencing important proteins or inducing the synthesis of undesirable proteins, which can lead to cell death via apoptosis. On the other hand, oxidative stress can induce the emergence of a group of uncontrolled, independent, and indefinite growth cells called neoplastic cells (Zhou et al., 2014). Therefore, to reduce the possibility of the cell passing through these processes, evolutionary mechanisms of control were developed to allow the survival of living beings in the face of the threat posed by oxygen free radicals generated by cellular metabolism. In addition to the antioxidant cellular mechanisms that evolved evolutionarily, the antioxidants present in the

plants were also investigated. The clove has been pointed out as a powerful natural antioxidant, being necessary studies to elucidate the molecular mechanism of the antioxidant action of this plant.

Ontological analysis reinforces the importance of the leader genes in the antioxidant performance of clove through the PI3K/AKt signaling pathway. The phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin signaling pathway is a major signal transduction cascade involved in cell proliferation, survival, and metabolism (Porta et al., 2014; Shimizu et al., 2012).

Antioxidant substances, such as some clove compounds act by inhibiting the surplus production of reactive oxygen species, eliminating free radicals and consequently decreasing apoptosis. ROS inactivate the PI3K / Akt signaling pathway, which in turn activates the target pathway of mammalian rapamycin (mTOR) / p70 ribosomal S6 protein kinase (p70S6K). Activation of the mTOR / p70S6K pathway induces oxidative stress and elevates the expression of the enzyme acetylcholinesterase (Liu et al., 2011). However, many of the antiapoptotic effects of growth factors can be attributed in part to the activation of this pathway, which was first demonstrated in PC12 cells by Yao and Cooper (Yao & Cooper, 1995). Some studies have identified the PI3K / Akt signaling pathway as a key factor in protection against cell death (Lawlor & Alessi, 2001).

In view of this, it seems possible that the clove inhibits the production of ROS and activates the PI3K / AKt cell protection pathway. In this study, bioinformatic analysis has hypothesized that clove components play a protective role in cells by inhibiting apoptosis and oxidative stress. Therefore, the antioxidant mechanism of the clove can be mediated by the activation of the cell survival pathway PI3K / Akt and modulation of SOD1 and CAT enzymes by means of ROS reduction, as shown in figure 4C.

Although functional studies have shown that the antioxidant enzymes CAT and SOD had similar concentrations in visceral adipose tissue in G1, G2, and G3, the expression levels of GPx1, CAT, and SOD were higher in G2. Surprisingly, G3 had the lowest SOD concentration ($p < 0.05$). This fact may be related to the intake of BO, which due to its antioxidant potential may have caused a reduction in ROS, reducing the need for increased production of antioxidant enzymes, making the levels obtained close to G1 or lower. Studies have shown that phenolic compounds can increase the efficiency of antioxidant enzymes (Gokila Vani et al., 2013; Liu et al., 2011; Yao & Cooper, 1995), reduce ROS levels, and alter adipose tissue mass by different metabolic pathways (Le Lay et al., 2014). The antioxidant defense system is active in fat cells, where there may be concentration of these enzymes and limited bioavailability (Le Lay et al., 2014). This factor may contribute to higher levels of SOD and CAT in G1 and G2 compared to G3.

However, activation of the PI3K / Akt pathway is not always desirable. Under certain conditions, increased cell survival during the activation of Akt could foster the growth of neoplastic cells. A recent study identified Akt as a potential target to be blocked during treatment of cancer cells that contain mutations in epidermal growth factor (EGF) mutations (Sordella, 2004). Therefore, depending on activation time, cell compartment and cell type, variations in the action of each of the clove-modulated enzymes on the ability to cause cell destruction or protection in stress responses can be observed. For example, contrary to what we deduced in normal cells, Silva et al., (2018) hypothesized, through bioinformatic analysis, that the herbal carnation of India and more precisely the compound eugenol, can sensitize neoplastic cells to radiotherapy by activating CASP3 and inactivating CYP1A1.

BO presented a higher percentage of SRL than BP and BSO, but it is noted that these beverages also presented antioxidant potential. Antioxidants are important for combating oxidative stress that can cause different types of health damage. The results obtained in the analysis of EC_{50} indicate that BO showed better results than the synthetic compound BHT. Given the results, it can be said that a lower concentration of the essential oil of *Syzygium aromaticum* is required to reduce the DPPH radical by 50% in relation to the concentration required to obtain this result using BHT. Hence, the clove-fermented

milk beverage produced better results than the synthetic compound BHT, which is commonly used in industry when evaluating the results obtained in the EC_{50} analysis.

The results obtained in the adipocyte area evaluation showed that G3 that received the BLF diet with *Syzygium aromaticum* presented a smaller adipocyte area compared to the animals of G1 and G2. Increased adipocyte size may be related to the storage of ROS. According to this study's results, G3, which presented a smaller adipocyte size, may be the group with lower levels of oxidative stress.

Histological analysis showed no changes in visceral adipose tissue. According to (Liu et al., 2011), the concentration of essential oil used does not cause toxic effects, thus presenting no histopathological changes.

5. Final Considerations

Thus, understanding the molecular actions of the clove is intended to contribute to a better understanding of the protective and toxic role of the phytotherapeutic in the human organism.

Moreover, plants like cloves and others, which have antioxidant potential already proven *in vitro*, in the near future may become sustainable regional alternatives for natural therapies to control the deleterious effects of oxidative stress.

Given the antioxidant potential of this formulation, there is the possibility of the functional capacity of this beverage, thus generating a new product with natural preservative and functional potential in the market.

The beverage formulated in the present study can be considered safe, using the concentration of *Syzygium aromaticum* described. However, future studies are needed to evaluate possible actions of the elaborated beverage to prevent obesity.

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References

Aebi, H. (1984). *[13] Catalase in vitro* (pp. 121–126). https://doi.org/10.1016/S0076-6879(84)05016-3

Aguiar, M. C. S., Silvério, F. O., de Pinho, G. P., Lopes, P. S. N., Fidêncio, P. H., & Ventura, S. J. (2014). Volatile compounds from fruits of Butia capitata at different stages of maturity and storage. *Food Research International*, *62*, 1095–1099. https://doi.org/10.1016/j.foodres.2014.05.039

Andrade, J. M. O., Barcala-Jorge, A. S., Batista-Jorge, G. C., Paraíso, A. F., Freitas, K. M. de, Lelis, D. de F., Guimarães, A. L. S., de Paula, A. M. B., & Santos, S. H. S. (2019). Effect of resveratrol on expression of genes involved thermogenesis in mice and humans. *Biomedicine & Pharmacotherapy*, *112*, 108634. https://doi.org/10.1016/j.biopha.2019.108634

Baldissera, A. C., Betta, F. Della, Penna, A. L. B., & Lindner, J. D. D. (2011). Alimentos funcionais: uma nova fronteira para o desenvolvimento de bebidas protéicas a base de soro de leite. *Semina: Ciências Agrárias*, *32*(4), 1497–1512. https://doi.org/10.5433/1679-0359.2011v32n4p1497

Bannister, J. V., & Calabrese, L. (2006). *Assays for Superoxide Dismutase* (pp. 279–312). https://doi.org/10.1002/9780470110539.ch5

Brasil. (2005). *Ministério da Agricultura Pecuária e Abastecimento. Instrução Normativa n.º 16, de 23 de agosto de 2005. Aprova o regulamento Técnico de Identidade e Qualidade de Bebidas Láctea*. Diário Oficial Da República Federativa Do Brasil. https://www2.cead.ufv.br/sgal/files/apoio/legislacao/legislacao6.pdf

Darvishi, E., Omidi, M., Bushehri, A. A. S., Golshani, A., & Smith, M. L. (2013). The Antifungal Eugenol Perturbs Dual Aromatic and Branched-Chain Amino Acid Permeases in the Cytoplasmic Membrane of Yeast. *PLoS ONE*, *8*(10), e76028. https://doi.org/10.1371/journal.pone.0076028

Farias, P. K. S. (2016). *Elaboração de bebida láctea fermentada com adição de óleo essencial*. Instituto de Ciências Agrárias - Universidade Federal de Minas Gerais(ICA-UFMG).

Gokila Vani, M., Kumar, K. J. S., Liao, J.-W., Chien, S.-C., Mau, J.-L., Chiang, S.-S., Lin, C.-C., Kuo, Y.-H., & Wang, S.-Y. (2013). Antcin C from Antrodia cinnamomea Protects Liver Cells Against Free Radical-Induced Oxidative Stress and Apoptosis In Vitro and In Vivo through Nrf2-Dependent Mechanism. *Evidence-Based Complementary and Alternative Medicine*, *2013*, 1–17. https://doi.org/10.1155/2013/296082

Kamatou, G. P., Vermaak, I., & Viljoen, A. M. (2012). Eugenol—From the Remote Maluku Islands to the International Market Place: A Review of a Remarkable and Versatile Molecule. *Molecules*, *17*(6), 6953–6981. https://doi.org/10.3390/molecules17066953

Lawlor, M. A., & Alessi, D. R. (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *Journal of Cell Science*, *114*(Pt 16), 2903–2910. http://www.ncbi.nlm.nih.gov/pubmed/11686294

Le Lay, S., Simard, G., Martinez, M. C., & Andriantsitohaina, R. (2014). Oxidative Stress and Metabolic Pathologies: From an Adipocentric Point of View. *Oxidative Medicine and Cellular Longevity*, *2014*, 1–18. https://doi.org/10.1155/2014/908539

Liu, X.-M., Peyton, K. J., Shebib, A. R., Wang, H., & Durante, W. (2011). Compound C stimulates heme oxygenase-1 gene expression via the Nrf2-ARE pathway to preserve human endothelial cell survival. *Biochemical Pharmacology*, *82*(4), 371–379. https://doi.org/10.1016/j.bcp.2011.05.016

Martins, L. B., Oliveira, M. C. de, Menezes-Garcia, Z., Rodrigues, D. F., Lana, J. P., Vieira, L. Q., Teixeira, M. M., & Ferreira, A. V. M. (2018). Paradoxical role of tumor necrosis factor on metabolic dysfunction and adipose tissue expansion in mice. *Nutrition*, *50*, 1–7. https://doi.org/10.1016/j.nut.2017.07.006

Najgebauer-Lejko, D., Sady, M., Grega, T., & Walczycka, M. (2011). The impact of tea supplementation on microflora, pH and antioxidant capacity of yoghurt. *International Dairy Journal*, *21*(8), 568–574. https://doi.org/10.1016/j.idairyj.2011.03.003

Porta, C., Paglino, C., & Mosca, A. (2014). Targeting PI3K/Akt/mTOR Signaling in Cancer. *Frontiers in Oncology*, *4*. https://doi.org/10.3389/fonc.2014.00064

Prakash, B., Kedia, A., Mishra, P. K., & Dubey, N. K. (2015). Plant essential oils as food preservatives to control moulds, mycotoxin contamination and oxidative deterioration of agri-food commodities – Potentials and challenges. *Food Control*, *47*, 381–391. https://doi.org/10.1016/j.foodcont.2014.07.023

Scherer, R., & Godoy, H. T. (2009). Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. *Food Chemistry*, *112*(3), 654–658. https://doi.org/10.1016/j.foodchem.2008.06.026

Shimizu, T., Tolcher, A. W., Papadopoulos, K. P., Beeram, M., Rasco, D. W., Smith, L. S., Gunn, S., Smetzer, L., Mays, T. A., Kaiser, B., Wick, M. J., Alvarez, C., Cavazos, A., Mangold, G. L., & Patnaik, A. (2012). The Clinical Effect of the Dual-Targeting Strategy Involving PI3K/AKT/mTOR and RAS/MEK/ERK Pathways in Patients with Advanced Cancer. *Clinical Cancer Research*, *18*(8), 2316–2325. https://doi.org/10.1158/1078-0432.CCR-11-2381

Silva, J. M. de A., Santos, E. M. S., Almeida, A. C. de, Santos, H. O., Farias, P. K. S., Souza, K. S. S., Oliveira, S. P. de, & Santos, P. H. de A. S. (2018). Action of indian cloves (Syzygium aromaticum) in the sensitization of neoplastic cells for radiotherapy: a systematic review of literature. *Revista Unimontes Científica*, *20*, 39–57.

Sordella, R. (2004). Gefitinib-Sensitizing EGFR Mutations in Lung Cancer Activate Anti-Apoptotic Pathways. *Science*, *305*(5687), 1163–1167. https://doi.org/10.1126/science.1101637

Souza, K. S. S., Oliveira, S. P., Duarte, S. M., Brandi, I. V., Santos, S. H. S., Santos, E. M. S., Farias, P. K. S., Santos, H. O., & Almeida, A. C. (2018). Standardization of the methodology for calorimetric analysis of a fermented milk drink. *Caderno de Ciências Agrárias*, *10*(2), 61–63.

Teixeira, B., Marques, A., Ramos, C., Serrano, C., Matos, O., Neng, N. R., Nogueira, J. M. F., Saraiva, J. A., & Nunes, M. L. (2013). Chemical composition and bioactivity of different oregano (Origanum vulgare) extracts and essential oil. *Journal of the Science of Food and Agriculture*, *93*(11), 2707–2714. https://doi.org/10.1002/jsfa.6089

Yao, R., & Cooper, G. (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science*, *267*(5206), 2003–2006. https://doi.org/10.1126/science.7701324

Zhou, D., Shao, L., & Spitz, D. R. (2014). *Reactive Oxygen Species in Normal and Tumor Stem Cells* (pp. 1–67). https://doi.org/10.1016/B978-0-12-420117-0.00001-3

Zicker, M. C., Silveira, A. L. M., Lacerda, D. R., Rodrigues, D. F., Oliveira, C. T., de Souza Cordeiro, L. M., Lima, L. C. F., Santos, S. H. S., Teixeira, M. M., & Ferreira, A. V. M. (2019). Virgin coconut oil is effective to treat metabolic and inflammatory dysfunction induced by high refined carbohydrate-containing diet in mice. *The Journal of Nutritional Biochemistry*, *63*, 117–128. https://doi.org/10.1016/j.jnutbio.2018.08.013