ANDRESSA LADEIRA BERNARDES

EFFECTS OF HIBISCUS (*Hibiscus sabdariffa* L.) INTAKE ON THE DEVELOPMENT OF PRENEOPLASTIC LESIONS AND ON THE COMPOSITION OF THE INTESTINAL MICROBIOTA OF BALB/c MICE

Thesis submitted to the Universidade Federal de Viçosa, as part of the requeriments of Program in Science of Nutrition to obtaining the title of *Doctor Scientiae*.

Adviser: Maria do Carmo Gouveia Peluzio

Co-advisers: Lisiane Lopes da Conceição
Leandro Licursi de Oliveira
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To God for granting me life, health and faith to walk my path.

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BIOGRAPHY

Andressa Ladeira Bernardes, daughter of Lucenir de Fátima Ladeira and Romário Bernardes de Oliveira, was born on September 29, 1993 in the city of Cachoeiro de Itapemirim – Espírito Santo. In March 2011, he joined the Nutrition course at the Federal University of Espírito Santo, where he obtained the title of Nutritionist in August 2015. In the same month and year, he entered the Master's Degree in Food Science and Technology at the Federal University of Espírito Santo, and in August 2017 he obtained the title of Master.

ABSTRACT

BERNARDES, Andressa Ladeira, D.Sc., Universidade Federal de Viçosa, September, 2022. **Effects of hibisco (*Hibiscus sabdariffa* L.) intake on the development of preneoplastic lesions ando n the composition of the intestinal microbiota of Balb/c mice.** Adviser: Maria do Carmo Gouveia Peluzio. Co-advisers: Lisiane Lopes da Conceição, Leandro Licursi de Oliveira and Reggiani Vilela Gonçalves.

Colorectal câncer (CRC) includes morphological and pathological changes that affect the entire colon and rectum segment, with the appearance of aberrant crypt foci (ACF). ACFs are considered the first pre-neoplastic lesions that can progress to CRC. In addition to genetic factors, the development of CRC is also influenced by modifiable factors, that is, factors related to the individual's lifestyle. Therefore, changes related to lifestyle, such as the consumption of a diet rich in bioactive compounds, fibers, among others, contribute to reducing the risk of developing pre-neoplastic lesions and, consequently, CRC. *Hibiscus sabdariffa* L. is a medicinal plant belonging to the Malvaceae family, a source of bioactive compounds such as phenolic compounds, anthocyanins and dietary fiber. These compounds have antioxidant, anti-inflammatory, anti-proliferative properties, in addition to being hepatoprotective. Another possible mechanism of action of phenolic compounds in the body refers to the modulation of the intestinal microbiota, being metabolized by colonic bacteria producing metabolites and serving as a substrate for bacterial growth. In view of the above, the objective of the present study was to investigate whether the supplementation of 5% and 10% of dehydrated calyces of *H. sabdariffa* (DHSC) have the ability to attenuate the development of pre-neoplastic lesions, in addition to acting as prebiotics modulating the composition of the intestinal microbiota of mice. An *in vivo* experiment was carried out for 12 weeks, the animals were randomly divided into 3 experimental groups: the control group and the groups supplemented with 5% DHSC and 10% DHSC. During the first 8 weeks, the animals were induced to pre-neoplastic lesions with injection of the drug 1,2-Dimetilhidrazine (DMH). Manuscript 1: characterization of DHSC, determination of the activities of hepatic catalase and superoxide dismutase enzymes, ACF count, quantification of short-chain fatty acids, count of inflammatory infiltrates, goblet cells and leukocytes in the colonic mucosa. There was a significant reduction in ACF and in the presence of inflammatory infiltrates in the colon of animals in the 5DHSC and 10DHSC groups. The 10DHSC group showed an increase in the activity

of the catalase enzyme, in the production of butyrate and in the presence of NK cells in the colon, in addition to more hypertrophied goblet cells. Based on these findings, it is suggested that DHSC supplementation may be recommended to attenuate cellular responses in the early stage of pre-neoplastic lesions. Manuscript 2: A systematic review was performed to elucidate the interaction of flavonoids with the microbiota and hepatic alterations in obesity-induced rats/mice. Studies were searched in PubMed, ScienceDirect and LILACS databases. Experimental studies that evaluated the influence of flavonoids on the composition of the microbiota and on hepatic alterations were included. After applying all exclusion criteria, the review consists of 15 experimental studies. It was shown that obesity-induced and flavonoid-treated mice showed an increase in bacterial diversity, a reduction in the abundance of bacteria associated with the consumption of a high-fat diet, such as *Firmicutes*, *Proteobacteria*, and an increase in the abundance of bacteria such as *Bacteroidetes*, *Verrucomibrobia*, *Akkermansia*, and *Bifidobacterium*. associated with improved metabolic and pathological status. Furthermore, flavonoids are able to reduce oxidative stress and inflammation of liver tissue. Finally, an association was observed between the composition of the microbiota and hepatic cholesterol through the metabolism of bile salts. Therefore, positive effects are suggested through the administration of flavonoids. Manuscript 3: Serum AST and ALT markers, hepatic cytokine profile, intestinal microbiota composition and expression of p53, c-Myc, caspase-3 and PCNA genes were determined. There was no difference between the experimental groups for serum markers of ASL and ALT and hepatic cytokines. Supplementation with 5% and 10% DHSC altered the composition of the intestinal microbiota, increasing the abundance of the *Lachnospiraceae*, *Ruminococcaceae* and *Clostridiaceae* families and the *Clostridum* genus, important butyrate producers. Furthermore, supplementation with 5% and 10% DHSC increased caspase-3 and c-Myc expression, respectively. Therefore, phenolic compounds, anthocyanins and dietary fiber contributed to modulate the composition of the intestinal microbiota, in addition to influencing the expression of genes associated with cellular apoptosis.

Keywords: Colorectal Cancer. Microbiota. Hibiscus. Flavonoids. Anthocyanins.

RESUMO

BERNARDES, Andressa Ladeira, D.Sc., Universidade Federal de Viçosa, setembro de 2022. **Efeitos da ingestão de hibisco (*Hibiscus sabdariffa* L.) no desenvolvimento de lesões pré-neoplásicas e na composição da microbiota intestinal em camundongos Balb/c.** Orientadora: Maria do Carmo Gouveia Peluzio. Coorientadores: Lisiane Lopes da Conceição, Leandro Licursi de Oliveira e Reggiani Vilela Gonçalves.

O CCR inclui alterações morfológicas e patológicas que acometem todo o segmento do cólon e o reto, com aparecimento dos focos de criptas aberrantes (FCA). Os FCAs são considerados as primeiras lesões pré-cancerosas que podem progredir para o CCR. Além dos fatores genéticos, o desenvolvimento do CCR também é influenciado por fatores modificáveis, ou seja, relacionados ao estilo de vida dos indivíduos. Portanto, mudanças relacionadas ao estilo de vida como o consumo de uma alimentação rica em compostos bioativos, fibras, entre outros, contribuem para a redução do risco de desenvolvimento das lesões pré-neoplásicas e consequentemente do CCR. O *Hibiscus sabdariffa* L. é uma planta medicinal pertencente à família Malvaceae, fonte de compostos bioativos como os compostos fenólicos, antocianinas e fibra dietética. Estes compostos apresentam propriedades antioxidantes, anti-inflamatórias, anti-proliferativas, além de serem hepatoprotetores. Outro possível mecanismo de atuação destes compostos no organismo refere-se à modulação da microbiota intestinal, sendo metabolizados pelas bactérias colônicas produzindo metabólitos e servindo de substrato para o crescimento bacteriano. Diante do exposto, o objetivo do presente estudo foi investigar se a suplementação com 5% e 10% de cálices desidratados de *H. sabdariffa* atenua o desenvolvimento das lesões pré-neoplásicas, além de atuar como prebióticos alterando a composição da microbiota intestinal dos camundongos. Foi realizado um experimento in vivo durante 12 semanas, os animais foram divididos aleatoriamente em 3 grupos experimentais: o grupo controle e os grupos suplementados com 5% de DHSC e 10% de DHSC. Durante as 8 primeiras semanas os animais foram induzidos as lesões pré-neoplásicas com injeção da droga DMH. Manuscrito 1: caracterização de DHSC, determinação das atividades das enzimas catalase hepática e superóxido dismutase, contagem de ACF, quantificação de ácidos graxos de cadeia curta, contagem de infiltrados inflamatórios, células caliciformes e leucócitos na mucosa colônica. Houve

redução significativa do ACF e da presença de infiltrados inflamatórios no cólon dos animais dos grupos 5DHSC e 10DHSC. O grupo 10DHSC apresentou aumento na atividade da enzima catalase, na produção de butirato e na presença de células NK no cólon, além de células caliciformes mais hipertrofiadas. Com base nesses achados, sugere-se que a suplementação de DHSC possa ser recomendada para atenuar as respostas celulares no estágio inicial de lesões pré-neoplásicas. Manuscrito 2: Foi realizada uma revisão sistemática para elucidar a interação dos flavonóides com a microbiota e alterações hepáticas em ratos/camundongos induzidos a obesidade. Os estudos foram pesquisados nas bases de dados PubMed, ScienceDirect e LILACS. Foram incluídos estudos experimentais que avaliaram a influência dos flavonóides na composição da microbiota e nas alterações hepáticas. Após a aplicação de todos os critérios de exclusão, a revisão é composta por 15 estudos experimentais. Foi demonstrado que camundongos induzidos a obesidade e tratados com flavonóides mostraram aumento na diversidade bacteriana, redução na abundância de bactérias associadas ao consumo de uma dieta rica em gordura, como *Firmicutes*, *Proteobacteria* e aumento na abundância de bactérias como *Bacteroidetes*, *Verrucomibrobia*, *Akkermansia* e *Bifidobacterium* associados à melhora do estado metabólico e patológico. Ainda, os flavonóides são capazes de reduzir o estresse oxidativo e a inflamação do tecido hepático. Por fim, foi observada associação entre a composição da microbiota e o colesterol hepático através do metabolismo dos sais biliares. Portanto, são sugeridos efeitos positivos através da administração de flavonóides. Manuscrito 3: Foram determinados os marcadores séricos AST e ALT, perfil de citocinas hepáticas, composição da microbiota intestinal e expressão dos genes p53, c-Myc, caspase-3 e PCNA. Não houve diferença entre os grupos experimentais para marcadores séricos de ASL e ALT e citocinas hepáticas. A suplementação com 5% e 10% de DHSC alterou a composição da microbiota intestinal, aumentando a abundância das famílias *Lachnospiraceae*, *Ruminococcaceae* e *Clostridiaceae* e do gênero *Clostridum*, importantes produtores de butirato. Além disso, a suplementação com 5% e 10% de DHSC aumentou a expressão de caspase-3 e c-Myc, respectivamente. Portanto, compostos fenólicos, antocianinas e fibra alimentar contribuíram para modular a composição da microbiota intestinal, além de influenciar a expressão de genes associados à apoptose celular.

Palavras-chave: Câncer Colorretal. Microbiota. Hibisco. Flavonoides. Antocianinas.

LIST OF FIGURES

Manuscript 1

Manuscript 2

Manuscript 3

LIST OF TABLES

Manuscript 1

Manuscript 2

Manuscript 3

LIST OF ABBREVIATIONS

ACF Aberrant Crypt Foci

ALT Alanine Aminotransferase

AST Aspartate Aminotransferase

CAT Catalase

COX-2 Ciclooxigenase-2

CON Control

CRC Colorrectal Câncer

DHSC Dehydrated *H. sabdariffa* Calyces

DMH 1,2-Dimethylhydrazine

DPPH 2,2-Diphenyl-1-Picrylhydrazyl

HS *Hibiscus sabdariffa* L.

HDAC Histone Deacetylases

IFN-γ Interferon

IL-2 Interleucina-2

IL-4 Interleucina-4

IL-10 Interleucina-10

IL-17 Interleucina-17

MDA Malondialdehyde

NF-Kβ Nuclear Factor Kappa-β

NAFLD Non-Alcoholic Fatty Liver Disease

NK Natural Killer

PCNA Proliferating Cell Nuclear Antigen

$PPAR_{\gamma}$ Peroxisome Proliferator Activated Receptor $_{\gamma}$

SCFA Short Chain Fatty Acids

SOD Superoxide Dismutase

TNF-α Fator de Necroes Tumoral- α

SUMMARY

1. GENERAL INTRODUCTION

The occurrence of genetic and epigenetic changes in colonic epithelial cells can contribute to the development of a sporadic, hereditary or inflammatory bowel disease, and prolonged exposure to inflammatory processes can increase the risk of developing colorectal cancer (CRC) (FERLAY et al., 2015; CHO et al., 2019). On the other hand, the homeostasis of the intestinal epithelium, that is, the balance between proliferation, migration, differentiation and cell death, is associated with a reduced risk of cancer incidence (FUNG et al., 2013; SUBRAMANIAM; GENG; TAN, 2020).

CRC includes morphological and pathological changes that affect the entire segment of the large intestine (colon) and the rectum. This process involves the appearance of aberrant crypt foci (ACF) with subsequent formation of adenomatous polyps that can develop on the inner wall of the large intestine (FUNG et al., 2013). ACFs are considered the first pre-neoplastic lesions that can progress to CRC. These pre-neoplastic lesions can be identified microscopically on the surface of the entire colonic mucosa after staining with methylene blue, being considered a biomarker in the development of colon carcinogenesis (Kowalczyk et al., 2018; Perše & Cerar, 2011).

In addition to genetic factors, the development of CRC is also influenced by modifiable factors, that is, factors related to the individual's lifestyle, such as smoking, alcohol consumption, consumption of processed and ultra-processed foods, obesity and sedentary lifestyle (CHO et al., 2019). Thus, changes related to lifestyle, such as the consumption of a diet rich in bioactive compounds, fibers, among others, contribute to reducing the risk of developing pre-neoplastic lesions and, consequently, CRC (BEDEIR et al., 2011; LIN et al., 2012; BRAY et al., 2018).

Plants in general are sources of classes of bioactive compounds that are associated with reducing the risk and treatment of some chronic diseases, including hypertension, diabetes, cardiovascular diseases, cancer, among others (BARATA et al., 2016; JABEUR et al., 2017). *Hibiscus sabdariffa* L. (*H. sabdariffa*), also known as hibiscus, roselia, sorrel or sour okra, is a medicinal plant belonging to the Malvaceae family, being widely cultivated in regions such as Asia, Central America and Africa. *H. sabdariffa* is a source of bioactive compounds such as phenolic compounds. Among the phenolic compounds, flavonoids stand out, mainly anthocyanins, being the

pigments responsible for their red-purple color. Hibiscus consumption increases due to its concentration of bioactive compounds (BORRÁS-LINARES et al., 2015; JABEUR et al., 2017).

Anthocyanins act as potent antioxidants reducing the risk of damage to the DNA molecule. In addition, they have anti-inflammatory properties, through the modulation of inflammation, and anti-proliferative properties, being attributed to the reduction in the expression of genes related to cell proliferation such as Cyclin-D1, c-Myc, β-catenin, COX-2, and the increase of genes related to cellular apoptosis such as Bax. Furthermore, these compounds are hepatoprotective, promoting an increase in the activity of the antioxidant enzymes catalse (CAT) and superoxide dismutase (SOD) and a reduction in serum markers, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (REDAN et al., 2016; HE et al., 2017).

Another possible mechanism of action of phenolic compounds and anthocyanins in the body refers to the modulation of the intestinal microbiota. Only a fraction of these ingested compounds, about 5% to 10%, are absorbed from the upper gastrointestinal tract. The unabsorbed fraction reaches the colon, being metabolized by the bacteria that make up the intestinal microbiota, serving as a substrate for the growth of these bacteria. Therefore, these compounds can act as prebiotics influencing the bacterial composition of the colon (CHEN et al., 2018).

In view of the above, it is suggested that phenolic compounds and anthocyanins from *H. sabdariffa* have the ability to mitigate the development of pre-neoplastic lesions through their antioxidant and anti-inflammatory properties, in addition to acting as prebiotics changing the composition of the intestinal microbiota of mice.

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2. OBJECTIVES

2.1 General

To evaluate the effects of a diet supplemented with 5% and 10% dehydrated calyces of *H. sabdariffa* on the development of pre-neoplastic lesions, on the modulation of the intestinal microbiota composition and on the expression of markers involved in colorectal carcinogenesis in BALB/c mice.

2.2 Specifics

Manuscript 1

- To evaluate the proximate composition, the concentration of total fiber and soluble and insoluble fibers of DHSC;
- Determine the concentration of total phenolic compounds and total anthocyanins;
- To evaluate the antioxidant capacity (DPPH) in DHSC;
- To evaluate the activity of hepatic SOD and CAT antioxidant enzymes;
- Quantify foci of aberrant crypts (ACFs) in the colonic mucosa;
- Determine the concentration of short-chain fatty acids (acetic and butyric) in the feces of mice in the 1st, 5th and 13th week of the experiment;
- Quantify inflammatory infiltrates in the colonic mucosa;
- To evoluate area, diameter and volumetric density of goblet cells in the colonic mucosa;
- Quantify leukocytes in the colonic mucosa.

Manuscript 2

- To evaluate and discuss data from studies with obesity-induced rats/mice with a high-fat diet on the influence of flavonoids on the composition of the intestinal microbiota.
- Discuss results related to the hepatoprotective effects of flavonoids in obesity-induced rats/mice with a high-fat diet.

- Report variability between studies, types and duration of flavonoid treatments.

Manuscript 3

- To determine the serum markers of liver injury AST and ALT in mice induced to pre-neoplastic lesions;
- To evaluate the concentration of cytokines (IL-2, IL-4, IL-10, IL-17, TNF-α and IFN-γ) in the liver;
- To characterize the composition of the intestinal microbiota of mice;
- Avaliar a expressão gênica dos genes p53, c-Myc, Caspase-3 e PCNA no colon.

3. HYPOTESIS

- The supplementation of dehydrated *Hibiscus sabdariffa* calyces attenuates the development process of pre-neoplastic lesions, reducing ACFs and the infiltration of inflammatory cells in the colon.
- The supplementation of dehydrated *Hibiscus sabdariffa* calyces will modify the composition of the intestinal microbiota of mice induced to pre-neoplastic lesions attributed to the concentration of fibers and anthocyanins with prebiotic action.

4. RESULTS

4.1 MANUSCRIPT 1

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Hibiscus (Hibiscus sabdariffa L.) supplementation increases butyrate synthesis and reduces inflammatory cells, attenuating the formation of aberrant crypt foci in BALB/c mice induced to pre-neoplastic lesions.

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Abstract

The development of colorectal cancer involves some morphological changes, and in the initial stage, pre-neoplastic lesions called aberrant crypt foci (ACF) appear. Thus, an intervention with sources of bioactive compounds such as *Hibiscus sabdariffa* L., rich in phenolic compounds and anthocyanins, could attenuate the risk of developing these lesions due to its antioxidant, anti-inflammatory and anti-proliferative properties. Therefore, the aim of this study was to evaluate the effects of 5% and 10% supplementation of dehydrated *H. sabdariffa* calyces (DHSC) during the development of 1,2-dimethylhydrazine-induced preneoplastic lesions in male BALB/c mice. The

characterization of DHSC was carried out. The *in vivo* experiment lasted 12 weeks, and the animals were randomly divided into 3 experimental groups: the control group (CON) and the supplemented groups with 5% DHSC and 10% DHSC. The activities of liver enzymes catalase and superoxide dismutase were determined. In addition, ACF, short chain fatty acids (SCFA), presence of inflammatory infiltrates, goblet cells and leukocytes in the colonic mucosa were quantified. There was a significant reduction in ACF and the presence of inflammatory infiltrates in the colon of animals in groups 5DHSC and 10DHSC. In addition, the 10DHSC group showed an increase in the activity of the catalse enzyme, in the production of butyrate and in the presence of NK cells in the colon, in addition to more hypertrophied goblet cells. Based on these findings, it is suggested that DHSC supplementation may be recommended to attenuate cellular responses in the early stage of preneoplastic lesions.

Keywords: phenolic compounds, anthocyanins, antioxidants, pre-neoplastic lesions.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer and the second with the highest mortality rate among men and women worldwide, and in Brazil it is the second with the highest incidence accompanied by high mortality rates [1,2]. CRC is a neoplasm that includes morphological and pathological changes that affect the entire segment of the large intestine, and this process involves the appearance of pre-neoplastic lesions called aberrant crypt foci (ACF) [3-5]. ACF can be identified microscopically on the surface of the entire colonic mucosa, being considered a biomarker for evaluating the progression of CRC [6].

Colorectal carcinogenesis can be influenced by genetic factors and modifiable factors such as food intake, in which a greater consumption of processed and ultra-processed foods and a reduction in fresh foods is observed [1,3]. In this context, sources of bioactive compounds with antioxidant, anti-inflammatory, anti-carcinogenic properties, among others, are of great scientific interest, as they are related to reducing the risk of developing chronic diseases such as cancer. Among the sources of bioactive compounds, the *Hibiscus sabdariffa* L. (*H. sabdariffa)* of the Malvaceae family stands out, commonly known as hibiscus, roselia, sorrel or sour okra, and widely cultivated in Africa, Southeast Asia, and some countries in America. It is consumed in the form of

tea, jellies, fermented products, among others, due to its considerable concentration of phenolic compounds and anthocyanins [7-9].

Plant derived phenolic compounds include simple phenols, phenolic acids, tannins, flavonoids, among others.[10] Anthocyanins belong to the group of flavonoids and are the pigments responsible for the coloration of *H. sabdariffa*. These compounds play anti-inflammatory, anti-proliferative and hepatoprotective functions, in addition to a powerful antioxidant capacity, acting in the neutralization of free radicals, reducing the risk of damage to the DNA molecule, and the development of diseases or injuries associated with oxidative stress [11-14].

Given the information above, phenolic compounds and total anthocyanins from DHSC can be used to reduce oxidative stress, the inflammatory state, and consequently to suppress the carcinogenesis process [15-18]. Therefore, the aim of the study was to evaluate the effects of supplementation of 5% and 10% of DHSC during the development of pre-neoplastic lesions induced by 1,2-dimethylhydrazine (DMH) in BALB/c mice.

Methodology

Sample acquisition and preparation

Ten kg of *H. sabdariffa* calyces were originally obtained from a producer in Coimbra - Minas Gerais (latitude: -20,830,127, Longitude: -42,801,351) and purchased from a local market in Viçosa - Minas Gerais. *H. sabdariffa* calyces were then pulverized in a knife mill and stored at -20 °C.

DHSC characterization

Centesimal composition and dietary fiber

The determination of the proximate composition of the DHSC followed the methodologies proposed by the Association of Official Analytical Chemists – AOAC [19]. Proteins were determined by the Kjeldahl method. Lipids were quantified by extraction with ethyl ether in Soxhlet. Ashes were determined (fixed mineral residue) by a gravimetric process. The total fiber content was quantified by the gravimetric-

enzymatic method. Finally, carbohydrates were calculated by difference [100 – (moisture + protein + lipids + fiber + ash)].

Total phenolic compounds, total anthocyanins and antioxidant activity

The extraction of total phenolic compounds was performed according to Borrás-Linares et al. [8], in which 20 grams of DHSC was macerated in 100 mL of extraction solution (70% ethanol acidified with 0.1% HCl) at a ratio of 1:10 (p/v). The extraction took place for 24 hours, at refrigeration temperature (8 ± 2 ºC). Finally, the extract was filtered and concentrated in a rotary evaporator at 40 °C.

The determination of total phenolic compounds was performed according to the Folin-Ciocateu colorimetric method, and the results expressed in mg of Gallic Acid Equivalents / 100 grams of sample [20]. Total anthocyanins were quantified by the differential pH method, and the results expressed in mg of cyanidin-3-glucoside/100 grams of sample [21].

The scavenging capacity of the DPPH radical was determined using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) as previously described [8,22]. The antioxidant activity of DHSC was defined using a trolox standard curve, and the result expressed as µmol equivalent of trolox per gram of DHSC.

Experimental design

Forty-five male BALB/c mice, seven weeks old, initial weight between 20 - 40 grams, purchased from the Animal Facility of the Biological Sciences Center of the Universidade Federal de Viçosa were used. Animal protocol was approved by the Ethics Committee on Animal Experimentation from the Universidade Federal de Viçosa, Brazil, under the process number 10/2017. BALB/c mice were used because they develop neoplasms more easily, in addition, male mice were defined due to the hormonal variability of females. The sample calculation was performed according to Mera, Thompson and Prasad [23] who consider the effect of treatment between the experimental groups on the outcome variables of interest. The animals were housed in collective cages, with controlled temperature (22 ± 2 ° C), 12-hour photoperiod and ad libitum access to water and feed. After two weeks of acclimatization, the animals were randomized according to body weight and distributed in ascending order in the

cages in three experimental groups: control group - CON (diet AIN-93M, American Institute of Nutrition for Maintenance, n = 15), group 5DHSC (diet AIN - 93M + 5% DHSC, n = 15) and the 10DHSC group (diet AIN-93M + 10% DHSC, n = 15) (Table 1).

Table 1 – Composition of experimental diets AIM-93M* (g 100g-1).

Ingredients (g)	CON[1]	5HS[2]	10HS[3]
Cornstarch	39,75	39,75	39,75
Casein	20,00	20,00	20,00
Maltodextrin	13,20	13,20	13,20
Sucrose	10,00	10,00	10,00
Soybean oil	7,00	7,00	7,00
Cellulose	5,00	5,00	5,00
Mineral mix	3,50	3,50	3,50
Vitamin mix	1,00	1,00	1,00
L-Cystine	0,30	0,30	0,30
Choline bitartrate	0,25	0,25	0,25
t-butylhydroquinone	0,0014	0,0014	0,0014
Dietary DHSC	-	5g	10g

1 Control group (AIM-93M); **2** 5DHSC group (AIM-93M supplemented with 5% dietary DHSC); **3** 10DHSC group (AIM-93M supplemented with 10% dietary DHSC). *AIN-93M (American Institute of Nutrition for Maintenance).

The 5% and 10% DHSC supplementation was defined in order to provide about 100 - 200 mg of anthocyanins. Such amounts can be incorporated into the human diet. The difference in the average weights between the animals was not greater than 5% and the cages were properly identified. The experimental period was 12 weeks, and during the first eight weeks all animals received an intraperitoneal injection in a single dose per week of the drug DMH (20 mg / kg of body weight) for induction of pre-neoplastic lesions (Figure 1).

At the end of the experimental period, the mice were fasted for 12 hours, then anesthetized in the Experimental Nutrition Laboratory with 3% isoflurane (Cristália®, Brazil), followed by total exsanguination through the cervical retro-orbital sinus. The entire colon was dissected, washed with PBS buffer to remove luminal content, cut

along the mesenteric margin and then fixed in Karnovsky's solution for 24 hours for ACF analysis. Fecal samples were collected one week before euthanasia and used for SCFA analysis. For immunophenotyping, after dissection, the colon was washed with cold PBS buffer (pH 7.2), cut into small pieces and incubated in DMEN medium (Sigma-aldrich™) for 90 minutes at 37 °C. Another part of the colon was fixed in Carson's formalin solution for 24 hours for histological analyses. Livers were excised, weighed, immediately frozen in liquid nitrogen and stored at -80 °C until liver enzyme activity was determined.

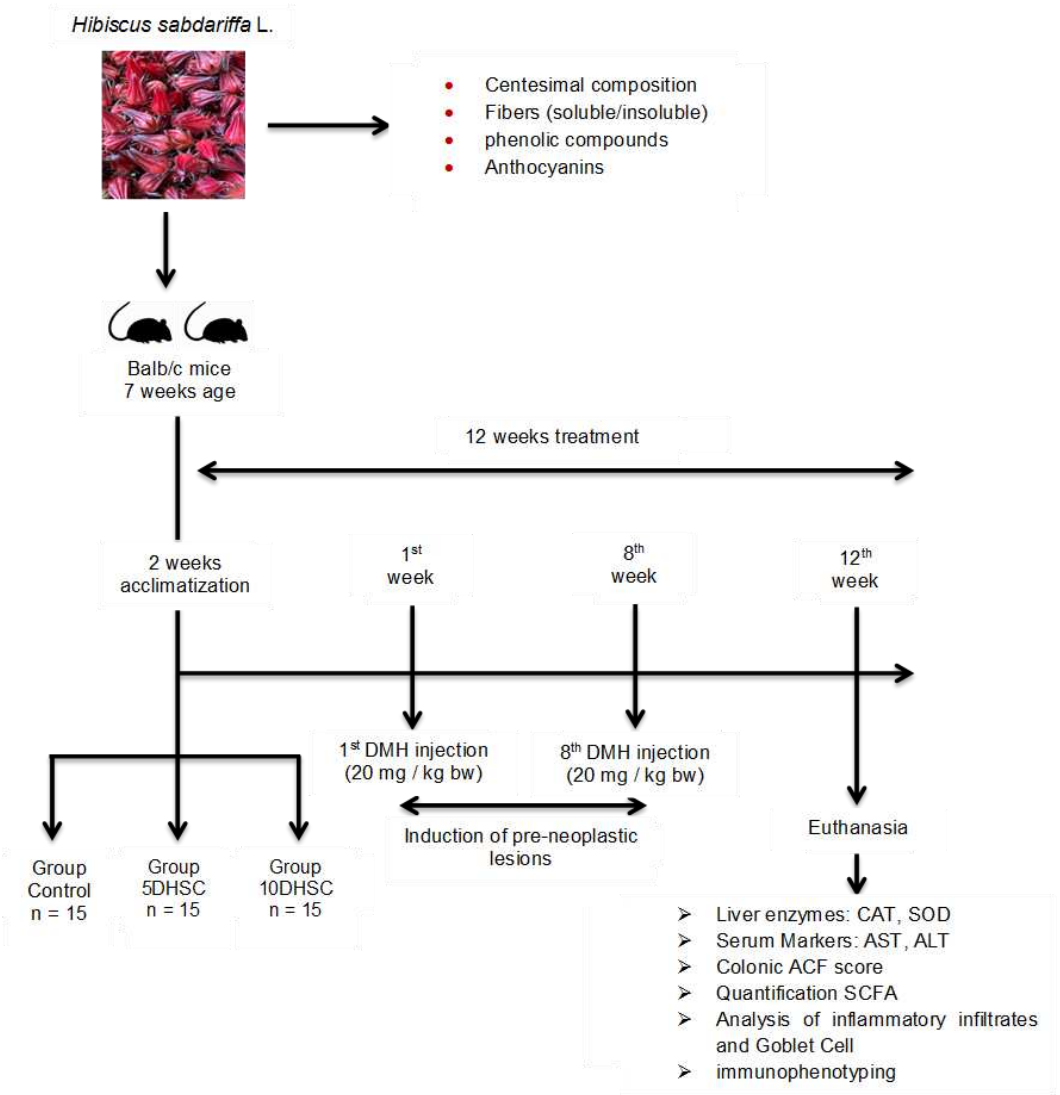

Figure 1. Flow diagram of the study steps.

Antioxidant Liver Enzymes

The activity of liver antioxidant enzymes was determined in liver homogenate. Catalase (CAT) concentration was determined according to Aebi [24], and superoxide dismutase (SOD) concentration was determined based on the ability of this enzyme to reduce pyrogallol auto-oxidation [25]. All readings were performed in a spectrophotometer (Thermo Scientific®, model Multiskan GO, Vantaa, Finland), and data were expressed in units (U) / mg protein. The determination of the concentration of proteins resident in the liver tissue used in the CAT and SOD analyzes was carried out according to Lowry et al. [26]. Readings were performed in a spectrophotometer (Thermo Scientific®, model Multiskan GO, Vantaa, Finland) at a wavelength of 700 nm.

Colonic ACF score

The colon was measured and divided into three equidistant segments, identified as proximal, medial, and distal in relation to the cecum. To count the ACF, the colon segments were stained with 0.1% methylene blue solution for two minutes. Counting was performed with the aid of an optical microscope (Olympus America InC., Model CBA, Pennsylvania, USA) at 100x magnification by two trained double-blind observers. The categorization of ACF was performed based on the number of aberrant crypts per focus, defined as foci with more than three (ACF > 3) or less than three aberrant crypts (ACF ≤ 3) [27].

Quantification of Fecal Short Chain Fatty Acids (SCFA)

SCFA extraction and quantification followed the Smiricky-Tjardeset et al. [28] and Marcon et al. [29]. Acetic and butyric acids were detected and quantified by an ultraviolet detector (model SPD-20A VP) at 210 nm. Results were expressed as µmol SCFA / g of feces.

Histological analyzes in the colon

The colon fragments were fixed in Carson's formalin and subsequently dehydrated in increasing concentrations of ethanol and cleared with xylene and

embedded in paraffin (Sigma Aldrich®, St. Louis, USA). 5 µm-thick cross sections were obtained on a rotating microtome (Olympus America InC., Model CUT 4055, Pennsylvania, USA). The slides were stained with hematoxylin and eosin (HE) to verify the presence of inflammatory infiltrates. For analysis of goblet cells, slides were stained with Alcian Blue (AB, pH 2.5) and periodic acid Schiff (PAS). All slides underwent a pre- and post-staining step and were then analyzed under an optical microscope (40x) (Leica Microsystems®, Inc.).

The counting of inflammatory infiltrates and the determination of goblet cells were performed with the aid of image analysis software (Image Pro Plus 4.5, Media Cybernetcs Inc, Rockville, USA).

Determination of leukocytes in the colon mucosa by immunophenotyping

Leukocytes were quantified and characterized in the colon mucosa as previously described Belkaid, Jouin e Milon [30] and Marcon et al. [29]. The colon was removed and washed in ice-cold PBS, cut into small fragments, and incubated in cell culture medium, DMEM, pH 7.2 (Sigma-aldrich™) for 90 minutes at 37°C. Thereafter, the suspension was centrifuged three times at 42 *g* for 5 minutes and the supernatant removed. Then it was centrifuged again at 543 *g* for 10 minutes. Afterwards, the remaining sediment was resuspended with PBS buffer (100 µL, pH 7.2). Cell viability was assessed using Trypan blue dye and, after staining, the cells were counted in a Neubauer chamber. The leukocytes obtained were incubated with the following antibodies, according to the manufacturer's instructions: anti-CD4 (PeCy5), anti-CD25 FITC-conjugate, anti-CD196 (anti-CRC6) PE-conjugate, anti-CD49b (anti- PanNK) APC-conjugated, PECy7-conjugated anti-CD8 (Biolegend, San Diego, CA, USA). Leukocytes (1x104 events) were acquired (FACSVerse™ and BD FACSuite software; BD Biosciences PharMingen San Jose, CA, USA) according to size (direct scatter) and granularity (side scatter). One or two stains were used to identify CD4 T lymphocytes (CD4+), CD8 T lymphocytes (CD8+), regulatory T cells (CD4+ CD25+), Th17 lymphocytes (CD4+ CD196+) and Natural Killer cells (CD49b +). Results are expressed as mean ± SD of the percentage of each subpopulation stained with the specific antibody within the blocked cells.

Statistical analysis

Results are presented as mean ± standard deviation. The means of the three groups (CON, 5DHSC and 10DHSC) were compared to each other by means of analysis of variance (ANOVA) complemented with Tukey's test, to verify the difference between the control group and the groups supplemented with *H. sabdariffa*. A significance level of 5% ($p < 0.05$) was considered. Data were analyzed using Graph Pad Prism 5 software, version 5.01.

Results and discussion

Centesimal characterization, dietary fiber, and phenolic compounds of DHSC

The chemical composition of the *H. sabdariffa* calyces in the present work showed a predominance of total dietary fiber, with the highest concentration of insoluble fiber, followed by proteins, ash, total phenolic compounds, lipids, carbohydrates, and anthocyanins (Table 2). Differences in the chemical composition of *H. sabdariffa* calyces between different studies are common and attributed to factors such as different cultivars, planting conditions, storage, climate type, soil type, harvest time, among other factors [8, 9,31].

Table 2. Characterization of the centesimal composition, dietary fiber, and phenolic compounds of DHSC.

Nutritional composition (g)	Nutritional value per 100 grams
Protein	3,61 ± 0,26
Lipids	0,66 ± 0,13
Ashes	2,69 ± 0,01
Carbohydrate	0,47 ± 0,32
Total Dietary Fiber	9,22 ± 2,79
Soluble Dietary Fiber	1,76 ± 0,14
Insoluble Dietary Fiber	7,46 ± 2,64
Total Phenolic Compounds	1,09 ± 0,02
Total Anthocyanins	0,22 ± 0,04
DPPH (µmol / g)	67,45 ± 0,11

Data are expressed as mean ± standard deviation of the analyzes in triplicate.

Differently of our results, Jabuer et al. [32], after to analyze the proximate composition of DHSC was observed higher concentrations of carbohydrates, followed by ash, proteins, and lipids. Regarding dietary fiber, in the present study, the concentration of soluble and insoluble fibers in the DHSC was higher than that reported by Kalla et al. [33], who found a total dietary fiber concentration of 6.15 g / 100 g, with 5.86 g / 100 g of insoluble fiber and 0.29 g / 100 g of soluble fiber. However, higher concentrations of dietary fiber were reported by Sáyago-Ayerdi et al. [31], with a total dietary fiber concentration ranging from 36 to 39 g / 100g, in which soluble fiber ranged from 7.06 to 8.32 g / 100 g and insoluble fiber from 13.0 to 17.08 g / 100 g among different cultivars of *H. sabdariffa* calyces whole flower stems analyzed in the study.

As for bioactive compounds, the concentration of total phenolic compounds in the present work was lower than that found by Maciel et al. [9] with a total concentration of phenolic compounds of 1.71 g in 100 grams, but the value of anthocyanins was like our study, with a concentration of 0.24 g in 100 grams. The antioxidant activity of DHSC in the present study was similar to that reported by Borrás-Linares et al. [8], with a concentration ranging from 27.4 to 112 µmol of trolox / gram of dehydrated calyx. The therapeutic potential of anthocyanidins already was demonstrated by Hemmati et al. [34] and Casao et al. [35], which relate to the action of this compound in inflammatory diseases due to its antioxidant and anti-inflammatory properties. It indicates that its therapeutic action is probably related to the high level of phenolic compounds associated with Anthocyanins.

Body weight and food intake of mice

Body weight did not differ between the groups (Figure 2-A). However, considering the total food consumption per week (Figure 2-B), all groups showed a reduction in diet intake during the second, third, and fourth experimental weeks. The control and the 5DHSC groups presented lower food intake compared to the 10DHSC group between the second and fourth week. Interestingly, such decrement in food consumption matches the beginning of DMH protocol. After the fifth week, no difference in food consumption was observed.

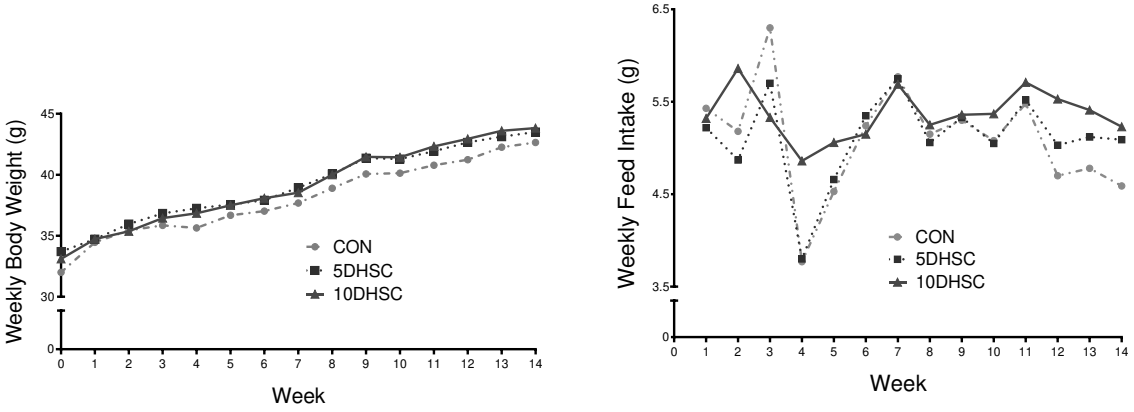

Figure 2. Effect of DHSC supplementation on body weight (A) and food intake (B) of mice induced to preneoplastic lesions with DMH.

According to the average DHSC consumption of the animals, the 5DHSC group consumed 250 mg of DHSC / day which provided 23.05 mg of total dietary fiber, with 4.4 mg of soluble fiber and 18.65 of insoluble fiber, in addition to 2.73 mg of total phenolic compounds and 0.55 mg of total anthocyanins. In contrast, the 10DHSC group was supplemented with twice the amount of DHSC in the diet, and this group consumed 530 mg of DHSC / day containing 48.87 mg of total dietary fiber, with 9.33 mg of soluble fiber and 39, 54 mg of insoluble fiber, in addition to 5.77 mg of total phenolic compounds and 1.17 mg of total anthocyanins. After, it was possible to calculate the quantity of DHSC possibly ingested by humans. This amount was determined through the method of normalization of the body surface area considering the mice's diet consumption. According to the calculations of Reagan-Shaw, Nihal and Ahmad [36], considering the average weight of an adult to be 70 kg, the equivalent ingestion of DHSC for a human would be approximately 36 g/day (5DHSC) and 75 g/day (10DHSC) incorporated in preparations such as cakes, jams, ice creams, and yogurts, for example [8,9].

Liver enzyme activity and serum markers in serum

No significant differences in SOD enzyme activity were observed between the experimental groups (Table 3). However, there was an increase in CAT enzyme activity in the group supplemented with 10% DHSC. The drug DMH is a powerful carcinogen that induces oxidative stress, in addition to hepatotoxicity through its hepatic

metabolism [37,38]. Therefore, phenolic compounds and anthocyanins, due to their antioxidant properties, can help reduce the risk of oxidative damage, in addition to contributing to hepatoprotective effects by increasing the activity of the antioxidant enzymes CAT and SOD as shown in the literature [39-42].

Table 3. Effects of DHSC supplementation on liver enzyme activity and liver serum markers in BALB/c mice.

Markers / Liver Enzymes	Groups		
	CON	5DHSC	10DHSC
CAT	12,89 ± 3,70[a]	22,24 ± 10,58[a]	33,46 ± 9,47[b]
SOD	10,80 ± 4,01[a]	14,57 ± 8,26[a]	17,85 ± 11,38[a]

Catalase (CAT), superoxide dismutase (SOD) (n=10); control (CON); 5% DHSC (5DHSC), 10% DHSC (10DHSC). Data are expressed as mean ± standard deviation. Different letters mean statistical difference according to ANOVA complemented with Tukey's test ($p < 0.05$).

These enzymes are extremely important in the fight against the generation of free radicals within the cell. When the tissue is DMH exposure, occur the generation of reactive oxygen species (ROS) usually increases, which changes the lipids, protein, and DNA of the cells, causing tissue stress and decreased cellular function [34,43]. Therefore, the formation of these radical species is controlled by the antioxidant enzymes, and it is desirable that occur the increased of these enzymes to protect the tissue from the activity of these radicals. We believed that the increase in the antioxidant system was stimulated by 10% DHSC was important to reduce oxidative damage and, consequently, to reduce the risk of liver disease. The mechanism of action of antioxidant enzymes begins with the action of the SOD enzyme, which converts the superoxide anion into hydrogen peroxide, then, by the action of the CAT enzyme, this peroxide is transformed into water, reducing oxidative damage [37, 44].

Effect of DHSC on the development of ACF in the colon

In the present study, it was observed that, in the proximal and medial segments of the colon, there was no difference in the number of ACF between the groups (Table 4). However, in the distal portion of the colon, the groups supplemented with 5% or 10% of DHSC demonstrated a reduction of 34% and 48%, respectively, in ACF

compared to the control group. Still, considering the entire portion of the tissue (proximal, medial, and distal), there is a reduction in ACF in the 5% and 10% DHSC groups compared to the control group. Similarly, studies in the literature have shown that phenolic compounds are able to attenuate the formation of ACF in animals induced to CRC, reducing the risk of progression of the carcinogenesis process [39,45-47]. ACFs can be observed in Figure S1 in the supplementary material.

Table 4. Effect of DHSC supplementation (5% and 10%) on ACF formation in mice with DMH-induced preneoplastic lesions.

Colon segments	Groups CON	5DHSC	10DHSC
Proximal			
ACF ≤ 3	32.83 ± 13.19[a]	25.57 ± 8.94[a]	29,00 ± 8.12[a]
Medial			
ACF ≤ 3	30.00 ± 11.06[a]	21.29 ± 6.67[a]	23,57 ± 4.86[a]
Distal			
ACF ≤ 3	40.33 ± 5.31[a]	26.43 ± 8.87[b]	21,00 ± 5.85[b]
Total ACF ≤ 3 *	103.2 ± 22.46[a]	73.29 ± 13.96[b]	73.57 ± 9.07[b]

Data are expressed as mean ± standard deviation (n=7). Different letters in each line mean statistical difference according to ANOVA complemented with Tukey's Test. * Total number of ACF in all colon segments (proximal, medial and distal).

Have been reported that DMH exposure causes deep modifications in tissue associated with inflammation and oxidative stress and consequently promotes intestinal crypt proliferation, and increase of ammonia concentration, which can damage healthy cells. The lesions induced by this drug, present epithelial origin, and histologic, morphologic, and anatomic characteristics considered highly reproducible for CRC studies. Furthermore, they are highly specific, leading to the initiation and promotion of carcinogenesis in a dose-dependent manner [6,48].

In our study, we believe that the reduction in ACF after DHSC exposure occurred because the DHSC interferes in the inflammatory and oxidative process, as well as promotes a regression in the morphologic alteration imposed by the carcinogenic process. Corroborating with our study, Chewonarin et al. [49], showed that chemopreventive effects are more effective in the early stages, in which the *H. sabdariffa* calyces extract reduced the formation of ACF in the early stage of carcinogenesis, but in the post-initiation stage the group treated with *H. sabdariffa*

showed a formation of ACF similar to the untreated group, especially the ACF that had four or more crypts / focus. Phenolic compounds in general contribute to attenuate the progression of colorectal carcinogenesis acting as natural chemopreventives, promoting inhibitory effects on the colorectal carcinogenesis process [50-52].

It is noteworthy that ACF with more than 3 aberrant crypts per focus (ACF > 3) are more likely to progress to tumor during colorectal carcinogenesis [52]. In the present study, no ACF > 3 crypts / focus were found. It is suggested that this result is associated with the antioxidant properties of phenolic compounds and anthocyanins present in DHSC, since the increase in antioxidant defenses helps to reduce the risk of developing pre-neoplastic lesions.

Effects of DHSC supplementation on butyrate production

SCFA (acetic, butyric, and propionic acids) are the main metabolites produced by the fermentation of dietary fibers through the bacteria that make up the intestinal microbiota [53]. It was observed that the concentration of acetic and butyric acid varied between the experimental groups (Figure 3-A,B). The diet supplemented with 5% or 10% DHSC did not significantly influence the concentration of fecal acetic acid (Figure 3-A), however, butyric acid was higher in the 10 DHSC group compared to the control group (Figure 3-B). Among the experimental weeks, a reduction in butyric acid is noted in the group supplemented with 10% DHSC from the 1st to the 12th week. However, it should be noted that all fatty acids showed a reduction in their concentrations from the beginning to the end of the experimental period, but this reduction, even if significant, was smaller in the 10DHSC group compared to the control and 5DHSC groups.

According to Liu et al. [54], animals treated with myrityl extract rich in anthocyanins showed a high representation of *Lachnospiraceae* and *Ruminococcaceae* in the microbiota, in which the presence of members of these families is considered an important producer of butyric acid. This result was consistent with the increase in butyric acid concentration in the supplemented group. These changes in the structure of the intestinal microbiome of animals treated with anthocyanins contribute to increased SCFA production, especially butyric acid.

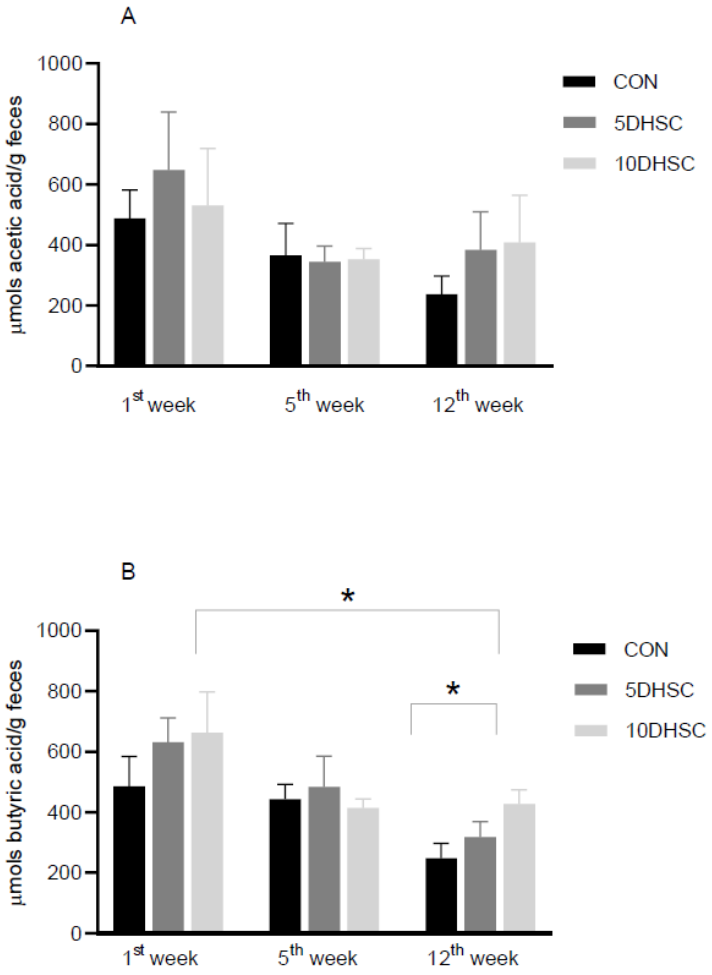

Figure 3. Fecal concentration of acetic and butyric acid (µmols SCFA/g feces) in mice induced to pre-neoplastic lesions with DMH and supplemented with DHSC. SCFA were quantified on weeks 1, 5 and 12 of the experiment. Data are expressed as mean ± standard deviation (n=5). * Means statistical difference according to ANOVA complemented with Tukey's Test ($p < 0.05$).

The ingestion of dietary fiber and its fermentation by the intestinal microbiota increases SCFA production. Fiber intake significantly alters the microbiota population, increasing SCFA-producing bacteria, their metabolite levels, and may play a protective role in colon cancer [55]. Therefore, it is suggested in the present study that such increase in butyric acid concentration in the 10DHSC group can be attributed to the higher consumption of DHSC and, consequently, fiber, phenolic compounds and anthocyanins. Acetic acid is absorbed in the colonic epithelium, transported and metabolized in the liver where it can be used by some ways such as lipogenesis,

ketogenesis, production of cholesterol, glutamine and glutamate. Another part of the acetic acid reaches the circulation, is captured and oxidized by the muscle for energy production [56]. Butyric acid, however, is absorbed in the colonic epithelium, so its bioavailability is mainly restricted to the colon. It is later metabolized to produce ATP, providing 70% of energy to colonocytes, in addition to playing other roles in maintaining colon homeostasis and epithelial integrity [56-59].

Butyric acid can exert immunomodulatory effects, such as the inhibition of histone deacetylases (HDAC), by inhibiting the activity of HDCA, butyric acid induces the expression of the $p21^{Waf1/Cip1}$ gene, which can interrupt cell proliferation [57,59]. The anti-inflammatory mechanisms of butyric acid involve the suppression of activation of the nuclear factor kappa-β (NF-Kβ). The inhibition of NF-Kβ by butyric acid can result in the reduction of myoperoxicities, COX-2, adhesion molecules and pro-inflammatory cytokines. In addition, butyric acid can inhibit interferon-$_\gamma$ production and regulate peroxisome proliferator activated receptor $_\gamma$ ($PPAR_\gamma$) signaling. Activation of this receptor on colonic epithelial cells can inhibit the production of inflammatory cytokines, promoting anti-inflammatory effects [56].

Intake of fiber and/or polyphenols has been associated with reduced inflammation and inflammatory diseases due to the production of metabolites from polyphenols (valerolactones, aromatics, among others) and fiber (SCFAs) [60,61]. Therefore, the increased production of butyric acid in the 10DHSC group in the present study may be associated with a reduction of ACF and the presence of inflammatory infiltrates in this group.

Histological analyzes of the distal colon

The groups treated with 5% and 10% DHSC in the diet showed a significant reduction in the percentage of inflammatory infiltrates compared to the animals in the control group (Figure 4).

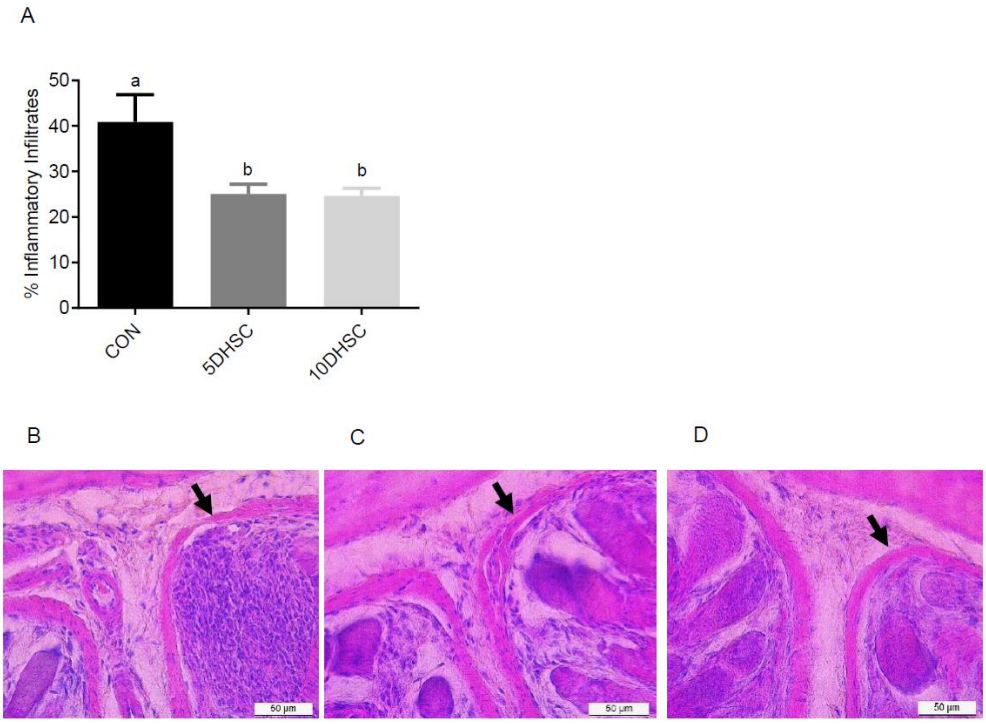

Figure 4. Percentage (%) of inflammatory infiltrates (A) in the control group (B), 5DHSC (C) and 10DHSC (D) in the distal colon portion of BALB/c mice induced to preneoplastic lesions with DMH and supplemented with DHSC. Arrows indicate inflammatory infiltrates. Data are expressed as mean ± standard deviation (n=7). Different letters mean statistical difference according to ANOVA complemented with Tukey's test ($p < 0.05$).

Possibly, the phenolic compounds present in the DHSC showed antioxidant and anti-inflammatory properties during the development of pre-neoplastic lesions. In our study, the inflammation observed in Control group probably is related to DMH exposure, since already been known that this drugs causes oxidative stress through the methylation of epithelial cell biomolecules resulting in an inflammatory state that contributes to the progression of these lesions [62]. To better understand the inflammatory process that occurred, it is known that, after the appearance of a tissue injury, the recruitment of inflammatory cells to the injury site begins. The infiltration and enhanced activation of inflammatory cells in the injured tissue can initiate and promote the progression of the carcinogenesis process, since these inflammatory cells can produce inflammatory cytokines, several of which play crucial roles during this process [63-65]. Furthermore, it is noteworthy that with the progression of the inflammatory

process, the intestinal mucosa is exposed to oxidative processes, which contributes to a greater risk of transformation of normal cells into malignant ones [66]. We believed that the reduction in inflammatory infiltrates after DHSC exposure occurred due the presence of phenolic compounds and anthocyanins may involve some mechanisms such as, the neutralization of free radicals, regulation of pro-inflammatory compounds and inflammation-associated cells [67]. Thus, the antioxidants present in the DHSC may be able to alleviate the damage to the colon caused by free radicals, with less infiltration of inflammatory cells in the tissue of the treated animals.

In relation to the goblet cells, based on area and diameter values (Figure 5-A,B), the group supplemented with 10% DHSC had more hypertrophied goblet cells compared to the CON group. In addition, the CON group demonstrated a reduction in the volume density of goblet cells per tissue area, that is, a depletion of these cells in relation to the 10DHSC group (Figure 5-C). The mechanisms that could explain this hypertrophy is that after the development of tissue inflammation, the immune system induces goblet cells to extensive mucus release to remove intruders, making them larger due to increased mucus production [68,69].

Bioactive compounds can help maintain the integrity of goblet cells in the colon as reported by Almagrami et al. [70], in which the authors observed that animals in the control group induced to CCR with Azoxymethane showed a significant reduction in the size of goblet cells and a depletion of mucin. On the other hand, the group of animals treated with *Acanthus ilicifolius Linn* in the diet (250 mg / kg of body weight) showed a slight reduction in these cells. *Acanthus ilicifolius Linn* is a prickly herb that is a source of flavonoids, terpenes, and alkaloids. Furthermore, Rehman et al. [71] demonstrated that prophylactic treatment for 14 days with the flavonone miracetin (25 and 50 mg / Kg of body weight) during cisplatin-induced colon toxicity was able to maintain the integrity of the goblet cells in addition to preventing the mucin depletion. Goblet cells perform necessary functions to combat oxidative damage, inflammatory disorders and intestinal infections, in addition to being part of the innate immune system of the mucosa to defend the host against possible pathogens [72].

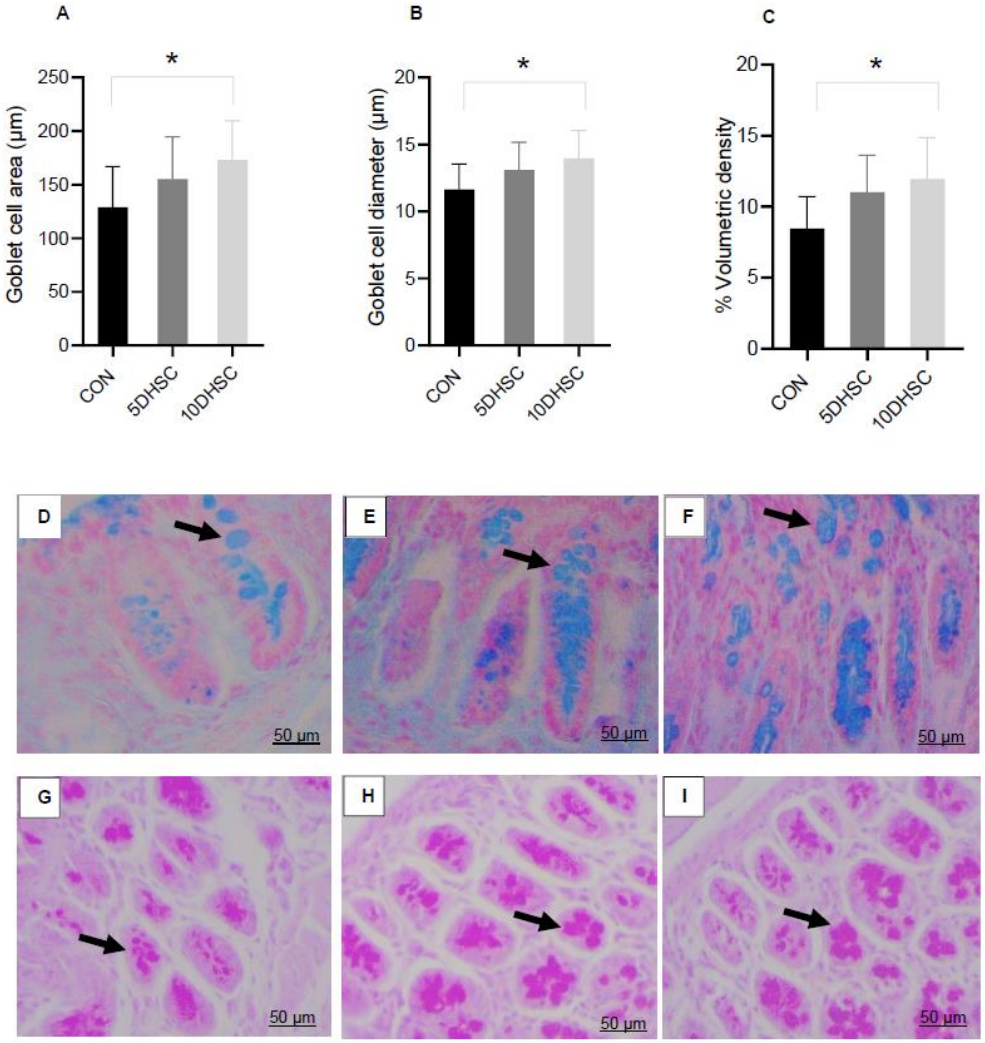

Figure 5. Area (µm), diameter (µm) and percentage (%) volumetric density of goblet cells present in the colonic mucosa of BALB/C mice induced to pre-neoplastic lesions with DMH and supplemented with DHSC. Staining was performed with Alcian Blue pH 2.5 (Control - D, 5DHSC - E, 10DHSC - F) and periodic acid Schiff (Control - G, 5DHSC - H, 10DHSC - I). Arrows indicate goblet cells. Data are expressed as mean ± standard deviation (n=10). * Means statistical difference according to ANOVA complemented with Tukey's Test ($p < 0.05$).

Determination of the presence of leukocytes in the colonic mucosa

No significant differences were observed in the percentage of CD4, CD8, Th17 and Treg cells between groups (Figure 6-A,C,D,E). However, the group that was supplemented with 10% DHSC showed an increase in NK cell infiltration into the colonic mucosa (Figure 6-B).

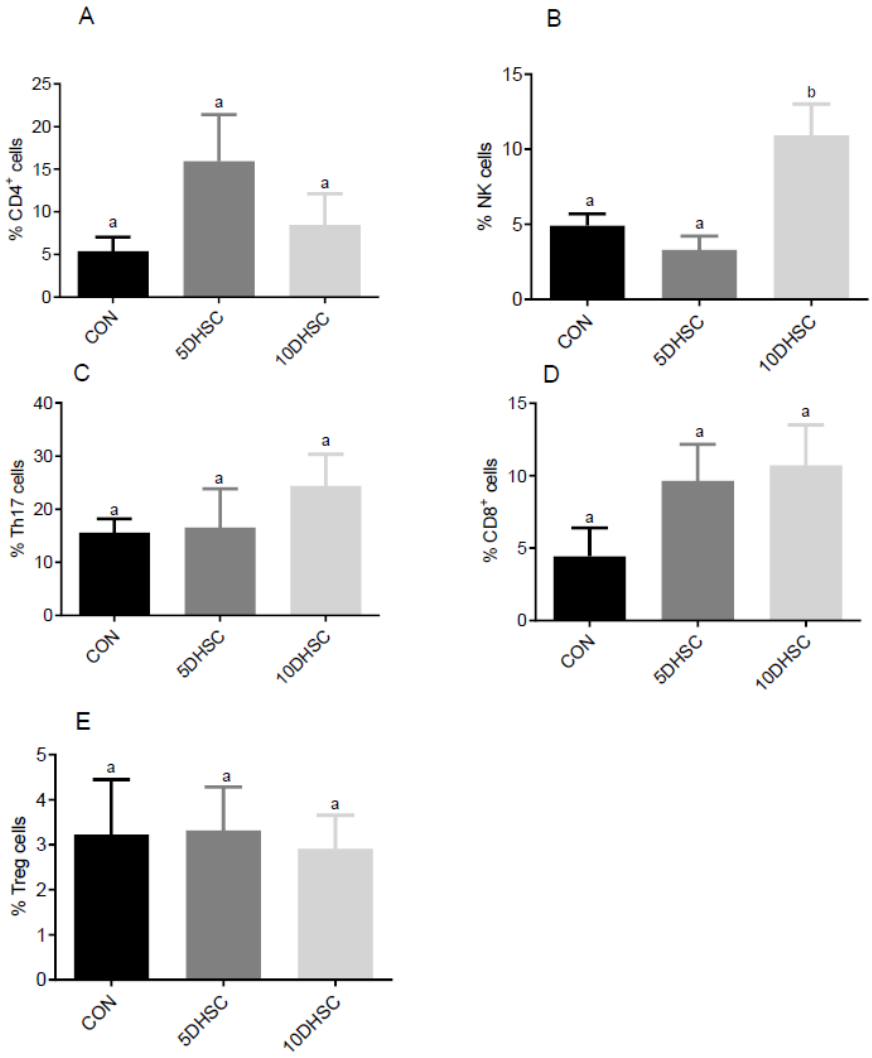

Figure 6. Leukocytes quantified in the colon mucosa of BALB /c mice induced to pre-neoplastic lesions with DMH and supplemented with DHSC. Data are expressed as mean ± standard deviation (n = 6). Different letters between bars mean statistical difference according to ANOVA complemented with Tukey's Test ($p < 0.05$).

NK cells are cytotoxic lymphocytes that play a role in immune defense. These cells through class I major histocompatibility complex (MHC) can distinguish between normal and altered cells, and can lyse malignant cells, acting against the formation and development of tumors [73,74].

Unlike normal cells, pre-neoplastic cells express specific receptors on the cell surface. These receptors are recognized by NK cells and then the immune response is initiated, through the release of granules containing perforin, a protein that breaks the cell membrane and induces cell apoptosis [75]. Cell death mediated by NK cells may be more effective in the early stage of differentiation of carcinogenic cells [76].

Thus, the greater infiltration of NK cells in the colonic mucosa of animals in the 10DHSC group may be associated with the reduction in ACF that was observed in this same group of animals.

Conclusion

Supplementation with 5% and 10% of DHSC promoted a significant reduction in ACF, and this result is consistent with the reduction in the presence of inflammatory infiltrates in these groups. Supplementation with 10% DHSC was more effective, as in addition to these changes in ACF and inflammatory infiltrates, there was an increase in hepatic CAT enzyme activity, butyrate production and a greater infiltration of NK cells in the colon. Therefore, it is suggested that the consumption of DHSC attenuates cellular responses in the early stage of development of pre-neoplastic lesions. Intervention in the initial phase is relevant as it can help reduce the risk of progression of the carcinogenesis process. Furthermore, oxidative stress can be attenuated due to the potential antioxidant capacity of phenolic compounds and anthocyanins present in the DHSC included in the diet. The reduction of the oxidative process is another important factor to reduce the risk of developing pre-neoplastic lesions.

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Supplementary Materials: Complete checklist according to ARRIVE guidelines and image of aberrant crypt foci. Study data are available upon request to the author.

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4.2 MANUSCRIPT 2

Manuscript submitted to the **Britsh Journal of Nutrition** (Impact Factor: 3,718)

Interaction of flavonoids with the intestinal microbiota and liver tissue of obesity induced animals: a systematic review.

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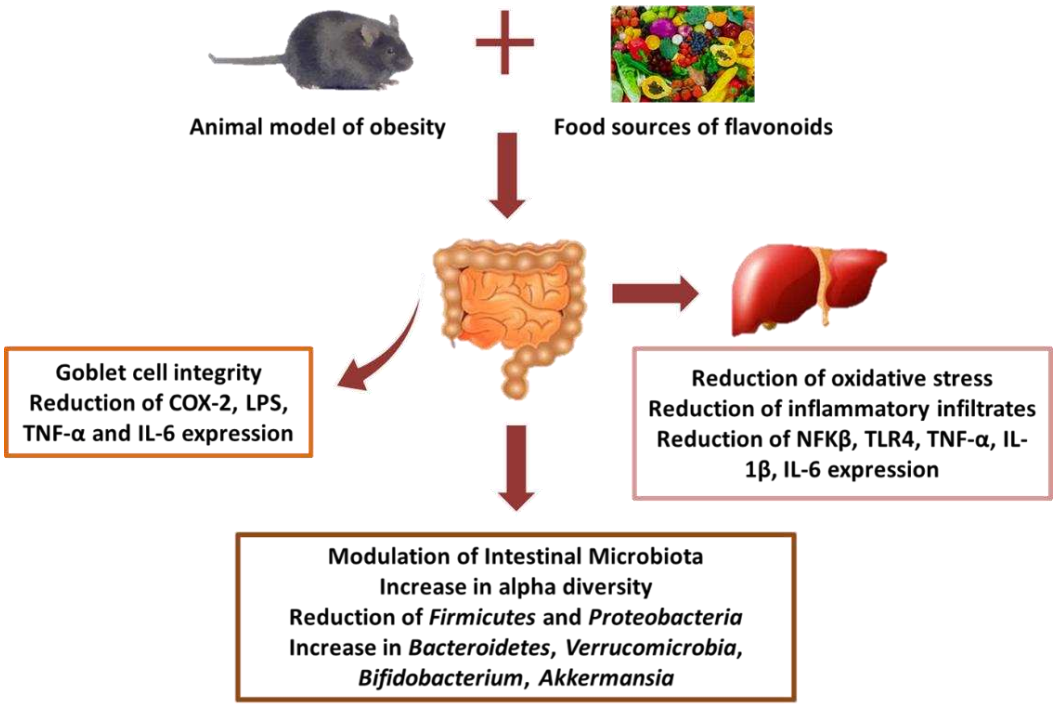
Abstract

The obesity process can promote changes in the composition of the intestinal microbiota and liver tissue. Currently, evidence suggests that flavonoids can modulate the composition of the microbiota through their metabolism by colonic bacteria. In addition, the microbiota is associated with liver changes due to the fact that an imbalance in the composition of the microbiota alters the function of the intestinal barrier, increasing its permeability to microbes and microbial products that reach the liver causing damage such as inflammatory processes. Therefore, this review aims to elucidate the interaction of flavonoids with the microbiota and hepatic alterations through the induction of the obesity process. The studies were searched in PubMed, ScienceDirect and LILACS databases. Experimental studies that evaluated the influence of flavonoids on the composition of the microbiota and on hepatic alterations were included. After applying all the exclusion criteria, this review is composed of 15 experimental studies. It was shown that mice that consumed a high-fat diet and treated with flavonoids showed an increase in bacterial diversity, *Bacteroidetes*, *Verrucomibrobia*, *Akkermansia* and *Bifidobacterium*, and a reduction in the abundance of *Firmicutes*, *Proteobacteria*. In addition, flavonoids are able to reduce oxidative stress

and inflammation of liver tissue. Finally, an association was observed between the composition of the microbiota and reduction hepatic cholesterol through the metabolism of bile salts. Therefore, positive effects are suggested through the administration of flavonoids.

Key words: obesity, microbiota, liver, flavonoids, lipids, cholesterol.



Graphic abstract. Reproduced with kind permission from Peluzio, Martinez & Milagro (2021).

Introduction

Obesity is a multifactorial condition influenced by a complex interaction of genetic, epigenetic, dietary, lifestyle and environmental factors. In addition, it is associated with important chronic metabolic disorders, including type II diabetes, non-alcoholic fatty liver disease (NAFLD), cardiovascular disease, and cancers[1]. Furthermore, in recent years it has been suggested that obesity alters the composition of the intestinal microbiota[2,3].

The gut microbiota plays an essential role in human health, regulating the normal physiological functions of the host, such as metabolism, nutrition and immunity

[4,5,6]. The composition of the microbiota can be influenced by genetic factors, drugs and food intake. Changes in its bacterial composition can be associated with inflammatory bowel disease, cancer, obesity, diabetes, among others[7,8,9]. In addition, changes in the abundance of certain bacteria can affect other organs, such as the liver, favoring the development of liver fat, liver cirrhosis, and changes in liver metabolism [4,9].

In this context, studies on the composition of the intestinal microbiota, mainly associated with food intake, are being increasingly explored. Currently, flavonoids have received attention because they can act as prebiotics modulating the composition of the intestinal microbiota[2,4,10]. The interest in studying the interaction of flavonoids with the microbiota is due to the fact that ingested flavonoids have low bioavailability, about 5% to 10% are absorbed in the upper gastrointestinal tract[11,12,13]. After absorption in the small intestine, the unabsorbed fraction undergoes biotransformation in the colon and hepatocytes through Phase I (oxidation, reduction, and hydrolysis) and Phase II (conjugation) reactions. These transformations produce water-soluble conjugated metabolites (glucuronides, sulfates and methyl derivatives). These metabolites are released into the systemic circulation to be transported to the organs or excreted in the urine. The microbiota promotes the hydrolysis of glycosylated flavonoids (anthocyanins, flavones, flavonols and isoflavones) into their respective aglycones, this hydrolysis is catalyzed by glycosidases produced by intestinal bacteria. It is noteworthy that some flavonoids, depending on their concentration, have the ability to bind to bacterial cell membranes, altering the functional aspects of the membrane and, thus, preventing its growth[14].

In view of the above, the objective of the review is to investigate the influence of flavonoids on the composition of the intestinal microbiota and on liver markers related to oxidative stress and inflammation of obesity induced mice.

Methodology

The writing of this systematic review followed the PRISMA-P recommendation protocol (Preferred Reporting Items for Systematic Reviews and Meta-Analyses)[15].

Literature search

Scientific articles were searched in the electronic databases PubMed, ScienceDirect and LILACS (Science direct and Latin American and Caribbean Health Sciences Literature) from 2015 to March 2022. The search terms were determined according to Health Science Descriptors (DeCS) and Medical Subject Headings (MeSH). The following search terms were used in English and Portuguese: flavonoids, flavonols, isoflavones, proanthocyanidins, anthocyanins, quercetin, gastrointestinal microbiome, bifidobacterium, lactobacillus, hepatocytes, antioxidants agents, anti-inflammatory agents, obesity. The logical operators “AND” or “OR” were used to combine the descriptors. Only original and experimental articles with rats and mice were considered, which investigated the interactions of flavonoids with the intestinal microbiota and liver tissue, and language restrictions were applied to select articles only in English and Portuguese. Also, the reference lists of selected articles were analyzed to identify other relevant studies that could be included in this review.

Screening and eligibility of studies

The inclusion of studies in this systematic review was according to the PICOS strategy (Table 1). After searching the databases, the initial selection was based on titles and abstracts. After screening, duplicate, *in vitro* or human studies were excluded. In addition, dissertations, theses, letters to the editor, reviews were also excluded. The eligibility criteria for the articles were restricted to original works, with rats or mice, which evaluated the interaction of flavonoids with the intestinal microbiota and/or liver tissue of animals induced to obesity published in the last seven years. Selected articles were read in full and evaluated for compliance with the established eligibility criteria.

Table 1. Definition of PICOS for inclusion and exclusion of studies.

Parameter	Definition
Population	Rodents
Intervention	Ingestion/supplementation of food sources of flavonoids or isolated extract of compounds belonging to the group of flavonoids.
Comparison	Control group (no intervention).
Results	Antioxidant activity; anti-inflammatory activity; changes in the composition of the intestinal microbiota; hepatoprotective effects, among others.
Study design	Experimental studies with rodents published in the last seven years. *In vitro* and human studies, dissertations and theses, letters to the editor, reviews, languages other than English and Portuguese were excluded.

PICOS: Population, Intervention, Comparison, *Outcomes*, Study Type.

Data extraction and synthesis

After the selection and reading of the articles in full, were extracted the general characteristics of the studies (title, authors, date of publication, country), the characteristics of the experimental model (lineage, age, sex, number and initial weight of the animals), the methodology of the studies (induction of changes in the microbiota or liver tissue, number of experimental groups, animals per group, type of intervention, dose and duration of intervention) and, finally, the main results found by the authors. All extracted information is presented in tables.

Risk-of-bias assessment

The experimental studies included in this review were analyzed for risk of bias according to the ARRIVE (Animal Research: Reporting of in Vivo Experiments) guidelines[16]. The guide's criteria are based on the description of important characteristics of experimental studies with animal models, such as the theoretical and methodological basis, research objectives, refinement of analytical methods, statistical analysis, sample calculation and measurement of results.

Results

Selected Studies

The flowchart (Figure 1) was developed according to PRISMA guidelines and presents the search and selection process in the literature. A total of 132 articles were found in the three databases, 72 studies in PubMed, 58 in Science Direct and 2 in Lilacs. Afterwards, 119 studies were excluded according to the following criteria: duplicate articles, title and abstracts, reviews, *in vitro* and human studies. Then, 2 studies were included after checking the reference lists of the 13 previously selected articles. Finally, the review comprises 15 experimental studies.

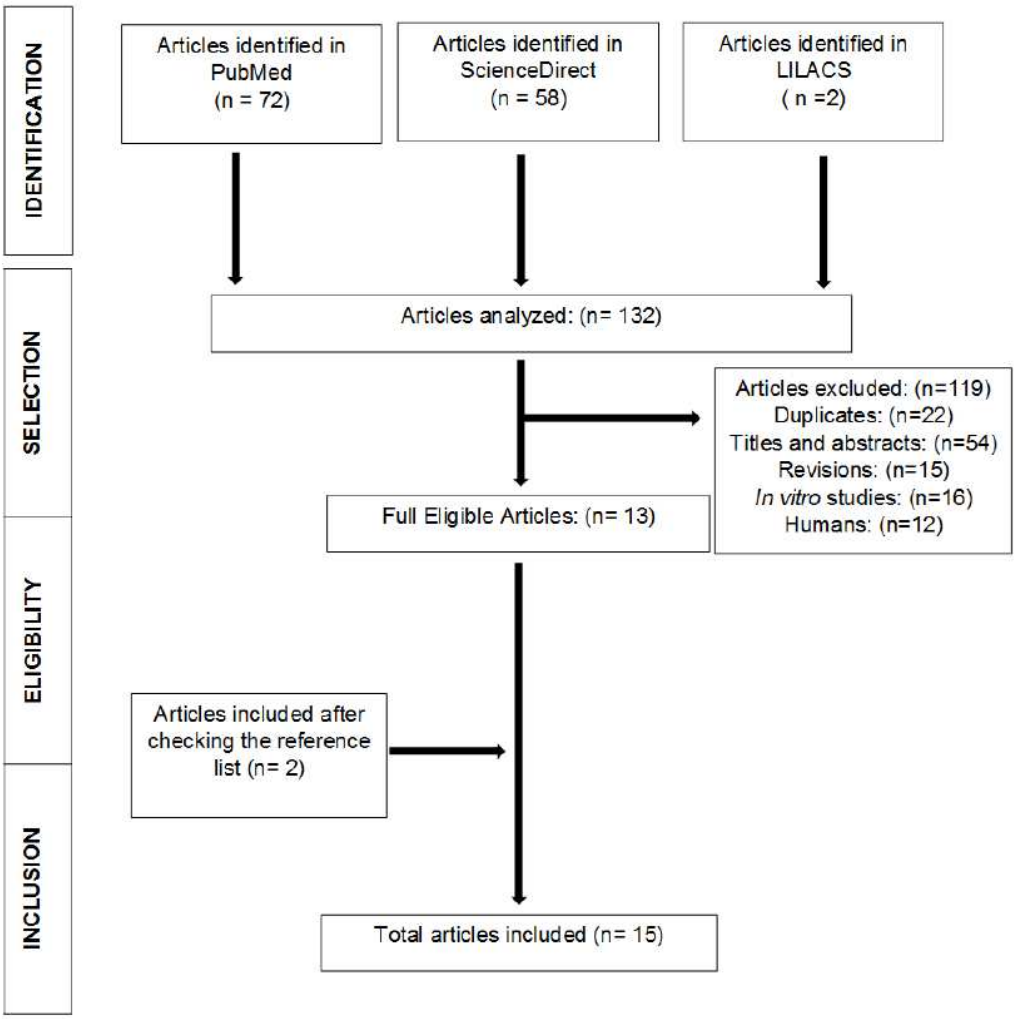


Figure 1. Flowchart of the study selection process.

Qualitative data

The studies included were conducted in five different countries (Table 2), with the majority conducted in China[20,23,24,26,27,29,30,31], followed by the United States[17,19] Japan[18,25] Spain[28] and Brazil[22]. The experimental models used were Wistar rats and mice (C57BL/6J, C57BL/6N) males. The age of the animals ranged from 1 to 9 weeks, and one study did not reported this information. The initial weight of animals was not reported in more than 70% of the studies[18,20-28,30].

Table 2. Characteristics of preclinical studies on the interactions of phenolic compounds with the intestinal microbiota and liver tissue.

Reference	Country	Animal Model	Number of animals	Sex/Age (weeks or days)	Starting weight (g)	Number of Groups	Animals/Group
Roopchand et al. (2015)[17]	USA	C57BL/6J mice	45	Males, 5 weeks	10 - 20	3	15
Masumoto et al. (2016)[18]	Japan	C57BL/6J mice	40	Males, 9 weeks	?	4	?
Lee et al. (2018)[19]	USA	Wistar rats	24	Males, ?	200 - 220	3	8
Zheng et al. (2018)[20]	China	C57BL/6J mice	18	Males, 3-4 weeks	?	3	6
Cheng et al. (2019)[21]	China	C57BL/6J mice	24	Males, 6 weeks	?	3	8
Araújo et al. (2019)[22]	Brazil	Wistar rats	24	Males, 11 weeks	?	3	8
Guo et al. (2019)[23]	China	C57BL/6 mice	48	Males, 1-3 weeks	?	4	9 a 12
Bai et al. (2019)[24]	China	C57BL/6J mice	36	Males, 8 weeks	?	3	12
Nakano et al. (2020)[25]	Japan	C57BL/6N mice	20	Males, 5 weeks	?	4	5
He et al. (2021)[26]	China	C57BL/6J mice	18	Males, 6-8 weeks	?	3	6
Song et al. (2021)[27]	China	C57BL/6J mice	36	Males, 4 weeks	?	3	12
Juárez-Fernández et al. (2021)[28]	Spain	Wistar rats	60	Males, 6 weeks	?	6	7-8
Sun et al. (2021)[29]	China	Wistar rats	36	Males, 8 weeks	160-200	6	6
Liu et al. (2021)[30]	China	C57BL/6J mice	?	Males, 5 weeks	?	3	6-8
Bian et al. (2022)[31]	China	C57BL/6J mice	22	Males, 8 weeks	20-23	3	6-8

Obesity was induced in all studies using a high-fat diet to investigate changes in gut microbiota and liver tissue (Table 3)[17-31]. The studies are composed of two control groups that received a standard diet[16-19,22-26,28-31] or a low-fat diet[20,21,27] and a high-fat diet[17-31]. The animals were treated with orally administered flavonoids incorporated into the diet[17-19,21-23,26,28-31] or gavage[20,24,27]. The flavonoids quantified in the treatments were anthocyanins (cyanidin-3-glycoside)[17,19,23,25,27], procyanidins[18,20,23], Kaempferol[21,31], 7-epiclosinone[22], moreflavone[22], naringin[24,26], neohesperidin[24,26], narirutin[24,26], hesperidin[24,26], quercetin[28], miracetin[29] and P3G (petunidin-3-O- [rhamnopyranosyl-(trans - p -coumaroyl)]-5-O-(β-D-glucopyranoside))[30]. The intervention period ranged from 8 to 20 weeks and only one study did not report this information[23].

Table 3. Methodologies used in preclinical studies on the interactions of phenolic compounds with the intestinal microbiota and liver tissue.

Reference	Induction	Untreated groups	treatment groups and dose	Quantified Flavonoids	Route of administration	Duration of Intervention
Roopchand et al. (2015)[17]	High fat diet (Obesity)	SPI (HFD diet + 10% SPI) HFD (Hyperlipid Diet)	HFD + 10% GP-SPI (grape flavonoids)	- anthocyanins (C3G)	Oral	13 weeks
Masumoto et al. (2016)[18]	High fat diet (Obesity)	Control (standard diet) HFHS (high fat and fructose diet)	HSHF + 0,5% OP HSHF + 0,5% PP	Procyanidins: OP (oligomeric) PP (polymeric)	Oral	20 weeks
Lee et al. (2018)[19]	High fat diet (Obesity)	Control (standard diet) HFD (Hyperlipid Diet)	HFD + BB (10 g blueberry powder / 100g HFD diet)	anthocyanins (213 mg)	Oral	8 weeks
Zheng et al. (2018)[20]	High fat diet (Obesity)	LFD (Hypolipid Diet) HFD (Hyperlipid Diet)	HFD + P (100 mg P / Kg body weight in the HFD diet)	Procyanidin (100 mg)	Oral gavage	12 weeks
Cheng et al. (2019)[21]	High fat diet (Obesity)	LFD (Hypolipid Diet) HFD (Hyperlipid Diet)	HFD + 0,1% w/w CPF (Cyclocarya paliurus flavonoids)	- kaempferol-3- O - β – glicuronídeo (472 mg)	Oral	8 weeks
Araújo et al. (2019)[22]	High fat diet (Obesity)	Control (standard diet) HFD (Hyperlipid Diet)	HFD + 300 mg/animal of EEB (flavonoid extract from the bark of *Garcinia brasiliensis*)	- 7-epiclusianone (42 mg/animal) - moreloflavone (10.76 mg/animal)	Oral	8 weeks
Guo et al. (2019)[23]	High fat diet (Obesity)	CHOW (standard diet) HFD (Hyperlipid Diet)	HFD + 0,5% m/v BE (blueberry extract)	- Anthocyanins (219 mg/g BE) -Proanthocyanidins (161 mg/g BE)	Oral	?

SPI (soy protein isolate); P3G (petunidina-3- *O*- [ramnopiranosil-(trans - *p* -coumaroil)]-5- *O*- (β-D-glicopiranosídeo)), PE: pomegranate extract; UROA: Urolitin-A; HFD: high fat diet; Va vanillin.

Table 3. Continued

Reference	Induction	Untreated groups	Treatment groups	Treatment dose	Route of administration	Duration of Intervention
Bai et al. (2019)[24]	High fat diet (Obesity)	Control (standard diet) HFD (high fat diet)	HFD + 300 mg/ kg bw of TFQ (flavonoid extract from *Quzhou Fructus Aurantii*)	- Naringin (122 mg/kg bw) - Neohesperidin (61,83 mg/kg bw) - Narirutin (13,71 mg/kg bw) - Hesperidin (7,89 mg/kg bw)	Oral gavage	12 weeks
Nakano et al. (2020)[25]	High fat diet (Obesity)	Control (standard diet) Western diet (WD)	WD + 2% BA (bilberry anthocyanins)	- Anthocyanins	Oral	18 weeks
He et al. (2021)[26]	High fat diet (Obesity)	Control (standard diet) HFD (high fat diet)	HFD + 50 mg / kg / day of PTFC (citrus flavonoids)	- Naringin (62,16 ug/ml) - Neohesperidin (49,28 ug/ml)	Oral	16 weeks
Song et al. (2021)[27]	High fat diet (Obesity)	LFD (Low Fat Diet) HFD (high fat diet)	HFD + 150 mg / kg bw of AEA (Açaí extract rich in anthocyanins)	- Anthocyanins	Gavage	14 weeks
Juárez-Fernández et al. (2021)[28]	High fat diet (Obesity)	Control (standard diet) HFD (high fat diet)	HFD + 37,5 mg / kg Q HFD + 2 × 10 8 UFC A	- Q (quercetin) - A *(Akkermansia m.)*	Q (oral) A (gavage)	9 weeks
Sun et al. (2021)[29]	High fat diet (Obesity)	Control (standard diet) HFD (high fat diet)	HFD + 0,5% myricetin	- Myricetin	Oral	12 weeks
Liu et al. (2021)[30]	High fat diet (Obesity)	Control (standard diet) HFD (high fat diet)	HFD + 100 mg/kg bw of P3G	- P3G	Oral	12 weeks
Bian et al. (2022)[31]	High fat diet (Obesity)	Control (standard diet) HFD (high fat diet)	HFD + 0,1% of kaempferol	- Kaempferol	Oral	16 weeks

Bw (body weight); P3G (petunidina-3- *O*- [ramnopiranosil-(trans - *p* -coumaroil)]-5- *O*- (β-D-glicopiranosídeo)).

Main results of individual studies

Studies have shown that flavonoids can both modulate the composition of the gut microbiota and contribute to improving oxidative stress and liver inflammation (Table 4). Regarding the composition of the intestinal microbiota of the animals, an increase in alpha diversity was observed according to the Shannon, Simpson and Chao1 indices[21,24,29]. Only one study analyzed beta diversity, and there was an increase according to the Bray-Curtis index[20].

The alterations observed in the bacterial composition of the microbiota were reduction of the phylum *Firmicutes* (Table 4)[17-25,27,29-31]. Within this phylum, the reduction occurred at the level of order (*Clostridiales* and *Lactobacillales*) [17,19,20,25], family (*Clostridiaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Eubacteraceae*, *Streptococeae*)[18,20,21,26,30,31] and genus (*Clostridium*, *Roseburia, Bacillus*, *Eubacterium*, *Blautia, Lactobacillus*, *Lactococcus*)[17,18,19,20,26,28]. On the contrary, there was an increase in the abundance of the phylum *Bacteroidetes*[18,20-24], this increase was also observed at the level of order (*Bacteroidales*)[21], family (*Bacteroidaceae*, *Rikenellaceae*, *Provetellaceae*)[20,21,31] and genus (*Bacteroides*, *Alistipes*, *Parabacteroides)*[17,18,24,25,26,31]. The reduction of *Firmicutes* and increase of *Bacteroidetes* contributed to the reduction of the *Firmicutes/Bacteroidetes* ratio[17,18,20,24,25,29,31].

The phylum *Proteobacteria* underwent changes such as increase[19,31] and decrease[21,22,27] according to the flavonoid used as treatment (Table 4). Within this phylum the class *Gamaproteobacteria*, the order *Pasteurellales* and the genus *Actinobacillus* were increased[19], while the genus *Desulfovibrio*[20] and the species *Bilophila*[23] were reduced. Similarly, the phylum *Actinobacteria* also showed an increase[29] and decrease[24,28] according to the treatment, among those belonging to this phylum, the families *Bifidobacteriaceae*[30] and *choriobacteriaceae*[29] and the genus *Bifidobacterium*[18,23] showed an increase in their abundance.

Other phyla that showed an increase were *Verrucomicrobia*[17,18,24,25,27], *Cianobacteria*[28] and *Fusobacteria*[19] (Table 4). However, only one study showed a reduction in *Verrucomicrobia*[29]. Within the phylum *Verrucomicrobia*, there was an increase in the order *Verrucomicrobiales*[27], in the family *Verrucomicrobiaceae*[27], and

in the genus *Akkermansia*[18,23-28,31]. In addition, the family *Fusobacteriaceae*[19] belonging to the phylum Fusobacteria showed an increase.

Table 4. Main results of preclinical studies on the interaction of phenolic compounds with the intestinal microbiota and liver tissue.

Reference	Microbial diversity	Bacterial Characterization	AGCC	liver changes	Others
Roopchand et al. (2015)[17]	-	↓ *Firmicutes/Bacteroidetes* porpotion ↓ *Clostridiales* ↑*Verrucomicrobia*, *Alistipes*	-	-	↓ TNF-α, IL-6 serum and colon ↑ occludin expression
Masumoto et al. (2016)[18]	-	↓ *Firmicutes/Bacteroidetes* porpotion ↓ *Firmicutes, Clostridium*, *Lachnospiraceae*, ↑ *Bifidobacterium, Bacteroidetes, Verrucomicrobia, Roseburia, Bacteroides, Akkermansia*	-	↑ *Pparα*, *Prkaa1*, *Cpt1a*	↓ TNF-α, IL-6, LPS serum ↓ p-cresol sulfate, phenol sulfate and indole urine derivatives
Lee et al. (2018)[19]	-	↓ *Firmicutes* ↑ *Bacillus, Lactobacillales, Proteobacteria, Gammaproteobacteria, Pasteurellales,* Actinobacillus, *Fusobacteria, Fusobacteriaceae*	↑ Acetate and propionate	↓ MDA	Restoration of villous length ↑ goblet cells and MUC2 expression
Zheng et al. (2018)[20]	↑ Bray-Curtis	↓ *Firmicutes/Bacteroidetes* porpotion ↓ *Lachnospiraceae, Clostridiales, Ruminococcaceae, Eubacterium, Desulfovibrio* ↑*Bacteroidetes, Rikenellaceae, Blautia*	-	-	-
Cheng et al. (2019)[21]	↑ Shannon and Simpson index	↑ *Bacteroidetes, Bacteroidales, Prevotellaceae*, *Bacteroidaceae* ↓ *Firmicutes*, *Proteobacteria, Eubacteriaceae*, *Lachnospiraceae*, *Ruminococcaceae, Clostridiaceae*	-	-	-

Pparα: Peroxisome proliferator activated receptors; *Prkaa1:* AMP-activated protein kinase; *Cpt1a:* carnitine palmitoyltansferase-1a; TNF-α: tumor necrosis fator-α; IL-6: interleukin-6; LPS: lipopolysaccharide; MDA: malondialdehyde; MUC2: mucin.

Table 4. Continued

Reference	Microbial diversity	Bacterial Characterization	Main metabolites	liver changes	Others
Araújo et al. (2019)[22]	-	↑ *Bacteroidetes* ↓ *Firmicutes*, *Proteobacteria*	↑ Propionic acid	↓ MDA and NO ↑ HSP72 and CAT expression. ↓ NF-Kβ expression	-
Guo et al. (2019)[23]	-	↑ *Bacteroidetes* ↓ *Firmicutes* ↑ *Akkermansia, Bifidobacterium* ↓ *Desulfovibrio*, *Bilophila*	-	-	↑MUC2, occludin, ZO-1 in the small intestine ↓ TLR4, IL-6, TNF-α in the colon
Bai et al. (2019)[24]	↑ Chao1 and Shannon Index	↓ *Firmicutes* / *Bacteroidetes* proportion ↑*Verrucomicrobia*, *Bacteroidetes, Akkermansia, Alistipes* ↓ *Actinobacteria*	-	↓ CT, TG, LDL-c, NEFA, inflammatory infiltrates, hepatic steatosis, NAFLD activity score	↑ expression of Cldn3 and Ocln Inhibition of NF-κB, TNF-α and COX-2 in the colon
Nakano et al. (2020)[25]		↓ *Firmicutes* / *Bacteroidetes* proportion ↑*Verrucomicrobia, Bacteroides, Parabacteroides, Akkermansia* ↓ *Lactobacillales, Clostridiales*		↓ liver fat, TBARS	↓ AST, ALT, LDL-c serum
He et al. (2021)[26]		↑ *Bacteroides, Akkermansia* ↓ *Streptococcaceae, Eubacterium*		↓ fat and inflammatory cells ↑FXR and TGR5 expression	↓ toxic bile acids

MDA: malondialdehyde; NO: Nitric oxide; HSP72: heat-shock protein; CAT: Catalase; NF-κB: nuclear factor kappa B; MUC2: Mucin; ZO-1: Zonula occludens-1; TLR4: Toll-like receptor 4; IL-6: interleukin-6; TNF-α: tumor necrosis fator-α; TG: triglycerides; CT: total cholesterol; NEFA: non-esterified fatty acids; NAFLD: Non-alcoholic fatty liver disease; Cldn3: Claudina; Ocln: Occludine; COX-2: Cyclooxygenase 2; TBARS: Thiobarbituric acid; α-SMA: Smooth muscle alpha-actin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; FXR: Farnesoid X receptor; TGR5: Takeda G-protein 5.

Table 4. Continued

Reference	Microbial diversity	Bacterial Characterization	Main metabolites	liver changes	Others
Song et al. (2021)[27]	-	↓ *Firmicutes, Proteobacteria* ↑ *Verrucomicrobia, Verrucomicrobiales, Verrucomicrobiaceae, Akkermansia.*	-	↓ Lipid accumulation, TC, TG and hepatic steatosis score ↑Cpt1a e Acox1	↓ TG, CT, NEFA, LDL-C, AST and ALT in serum
Juárez-Fernández et al. (2021)[28]		↑ *Cyanobacteria, Akkermansia, Roseburia* ↓ *Actinobacteria, Lactobacillus, Lactococcus, Blautia.*		↓ steatosis, triglycerides, IL-6 (Q) ↑ *Pparα* (Q)	↑ primary BAs
Sun et al. (2021)[29]	↑ Shannon index	↓ *Firmicutes / Bacteroidetes* proportion ↑ *Actinobacteria, Coriobacteriaceae*		↓ TC, TG ↓ hepatic steatosis and fibrosis ↓ IL-6, TNF-α	↓ TC, TG e LDL-c serum
Liu et al. (2021)[30]		↓ *Firmicutes, Lactobacillaceae, Streptococcaceae, Ruminococcaceae,* ↑ *Bifidobacteriaceae*		↓ liver fat ↓ PPARδ, Acox1 expression	↓ body weight, adipose tissue ↓ AST, ALT, TG, LDL-c serum
Bian et al. (2022)[31]		↓ *Firmicutes / Bacteroidetes proportion* ↑ *Proteobacteria, Akkermansia* ↓ *Verrucomicrobia, Rikenallaceae, Lachnospiraceae , Lactobacillaceae*, *Alistipes*			↓ TC, glucose serum

TG: triglycerides; TC: total cholesterol; *Cpt1a:* carnitine palmitoyltansferase-1a; NEFA: non-esterified fatty acids; AST: aspartate aminotransferase; ALT: alanine aminotransferase; IL-6: interleukin-6; *Pparα*: Peroxisome proliferator activated receptors; BAs: bile acids; TNF-α: Tumor necrosis fator; *Tlr2:* Toll-Like Receptor 2; PPARδ: Proliferator-activated receptor δ; Acox1: Acyl-coenzyme A oxidase 1.

The most relevant hepatic alterations (Table 4) were the reduction of oxidative stress by malondialdehyde analysis (MDA), reduction of lipid peroxidation (TBARS)[19,22,25], lower infiltration of inflammatory cells[24,26], reduction of fat [25,26,30] and lipid accumulation in the liver[27]. Regarding the markers involved in the inflammatory pathways, a reduction in the expression of NF-Kβ[22], TNF-α[29] and IL-6[28,29] was observed. Lipid levels were analyzed in liver tissue and was observed a reduction in total cholesterol[24,27,29], triglycerides[24,27-29], LDL-c and non-esterified fatty acids (NEFA)[24]. In addition, there was an increase in the expression of PPARα (peroxisome proliferator activated receptor alpha)[18,28], Prkaa1 (AMP-activated protein kinase)[18], CPT-1a (carnitine palmitoyltansferase 1a)[18,27] and Acox1 (acyl-coenzyme A oxidase 1)[23,30] involved in lipogenesis and fatty acid oxidation. Furthermore, the treatment increased the expression of FXR and TGR5 proteins[26], considered a therapeutic target for NAFLD. The expression of the protein HSP72 and the enzyme catalase were increased with treatment[22], playing anti-inflammatory and antioxidant functions, respectively.

Other results found in the studies (Table 4) were in the colon, which showed a reduction in the expression of inflammatory markers such as TNF-α[17,18,23,24], IL-6[17,18,23], COX-2[24] and LPS (lipopolysaccharide)[18]. In addition, an increase in goblet cells and restoration of the length of the colon villi is observed,[19] in addition to less infiltration of inflammatory cells. In the small intestine there was an increase in the expression of occludin[19], ZO-1[19] and MUC2[19,23].

Risk of bias

After evaluating the selected studies, it was observed that all had adequate summaries, sufficient scientific context and primary and secondary objectives (Supplementary Table 1). Ethical approaches were reported in all studies.

All studies described the number of animals used per experimental group, the type of intervention, the route of administration and the duration of treatment, only one studies did not report the duration of the intervention.

In relation to experimental animals, all studies reported the species, lineage and sex of the animals. As for housing and rearing, all studies described the type of cage, rearing program, light/dark cycle and temperature.

As for the sample size, no study presented the sample size used. Information on how animals were allocated to experimental groups, including randomization, was described in only 34% of studies. The statistical methods used for each analysis were described by all studies, as well as information on the unit of analysis of each study and on the mean and standard deviation. All studies interpreted the results taking into account the objectives and hypotheses, in addition to current theories and other relevant studies in the literature.

No studies reported adverse events regarding mortality and changes in the experimental protocol. Comments on study limitations, such as sources of bias, animal model and inaccuracy associated with the result, were reported by only three studies. Reports of possible new discoveries that could benefit other species or systems, including relevance to human biology, were described by 60% of the studies. Finally, 94% of studies reported funding sources and the role of funders in the studies.

Discussion

Influence of flavonoids on the composition of the intestinal microbiota

The distribution of different bacterial species in the intestinal microbiota is evaluated by alpha diversity, and in some pathologies such as obesity, inflammatory bowel disease, type II diabetes, among others, a reduction in bacterial diversity is observed[3,4,5]. In the present review, the studies reported an increase in the alpha diversity of animals treated with flavonoids even with the induction of the obesity process, suggesting a positive result of the administration of flavonoids[21,24,29].

The phyla *Firmicutes* and *Bacteroidetes* dominate the microbiota of mice (92.6%), and ob/ob mice (genetically obese) show a 50% reduction in *Bacteroidetes* compared to a higher abundance of *Firmicutes*[32]. The induction of the obesity process in animals is related to the reduction of bacterial diversity, reduction of the phylum *Bacteroidetes* and increase of *Firmicutes*, contributing to a higher *F/B* ratio[33,34]. Bacteria of the *Firmicutes* phylum metabolize sugars more efficiently compared to Bacteroidetes, converting them into absorbable energy products, favoring the reabsorption of calories and consequent weight gain and obesity[35,36]. Bacteria belonging to the phylum *Bacteroidetes* participate in the metabolism of flavonoids by

the microbiota, transforming them into short-chain fatty acids (SCFA) and phenolic acids, thus, these compounds can be used as an additional substrate for bacterial growth[1].

Other relevant modulations observed in the studies of this review were the reduction of the phylum *Proteobacteria* by flavonoids (Kaempferol, 7-epiclusianone, moreloflavone, anthocyanins and procyanidins), in addition to the reduction of the genus *Desulfovibrio* and the species *Bilophila* by anthocyanins and procyanidins. The reduction in the abundance of Proteobacteria may indicate a balance in the microbial community and improvement of pathological and metabolic states[1,37,38]. The genus *Desulfovibrio*, on the other hand, contributes to the inflammatory process, producing hydrogen sulfide that is potentially toxic, releasing sulfated mucin as a by-product of this metabolism[39]. Furthermore, the abundance of *Bilophila* species was associated with high-fat diets, which may increase the metabolic effects of the HFD diet in the animal[40]. Therefore, the reduction of *Desulfovibrio* and *Bilophila* may suggest improvement in the metabolic effects generated by the HFD diet[20,23].

Similarly, the *Actinobacteria* phylum increased and decreased according to treatment. However, the families *Bifidobacteriaceae* and *Coriobacteriaceae* and the genus *Bifidobacterium* showed an increase in their abundance by the administration of the flavonoids P3G, Myricetin and procyanidins/anthocyanins, respectively[18,23,29,30]. The greater abundance of the genus *Bifidobacterium* was associated with greater alpha diversity, through mechanisms that include starch degrading activity. In addition, this genus contributes to improving the resistance of the microbiota to factors such as HFD diet intake and antibiotics[41,42]. Furthermore, *Bifidobacteria* attenuate the metabolic disturbance of blood lipids caused by the HFD diet, in addition to reducing hepatic steatosis and inflammatory cell infiltration[43].

The phylum *Verrucomicrobia* and the genus *Akkermansia* showed an increase in their abundance with treatment with flavonoids[17,18,23-28,31], and this increase is usually accompanied by an improvement in host metabolism[44]. The *Akkermansia* species belonging to this phylum is a probiotic and its reduction is associated with metabolic syndrome and obesity, but the mechanisms are not fully elucidated. Obesity has been associated with a decrease in the thickness of the mucus layer, favoring increased intestinal permeability. The presence of *Akkermansia* is positively correlated with the restoration of the mucus layer. This genus uses mucins as an energy source,

stimulating goblet cells to produce mucus, increasing the thickness of the mucus layer and intestinal barrier, as well as reducing intestinal permeability and LPS leakage[17,36,44].

It is also noteworthy that interindividual differences in the composition of the microbiota can produce different flavonoid metabolites. Thus, in addition to the food matrix and the daily intake of these compounds, the composition of the microbiota can also influence the bioavailability and beneficial effects of flavonoids[19,22,26,27].

Influence of flavonoids on liver changes

According to the studies in the present review, flavonoids can modulate the hepatic changes induced by the HFD diet, since a reduction in oxidative stress was observed through the analysis of malondialdehyde (MDA)[19,22] and a reduction in aspartate aminotransferase (AST) and alanine aminotransferase markers (ALT) of liver injury, in addition to less infiltration of inflammatory cells in the liver[24,26]. MDA is the end product of lipid degradation and is considered a marker of oxidative stress reflecting the degree of liver damage[45,46]. In addition, it is known that high levels of AST and ALT are often associated with severe liver damage. Therefore, it is suggested that treatment with flavonoids attenuated the oxidative processes and the occurrence of liver damage induced by the HFD diet.

Regarding inflammatory markers, the studies in this review reported that flavonoid treatment was able to reduce the expression of nuclear factor-kappaB (NF-Kβ)[22], toll-like receptor (TLR4), tumor necrosis factor- α (TNF-α), Interleukin-1β (IL-1β) and Interleukin-6 (IL-6)[29]. The HFD diet correlates with changes in the expression of oxidative stress and inflammatory markers that play an important role in promoting inflammation, immune response, cell proliferation and apoptosis. However, flavonoids reversed this situation[22,33,29].

Hepatic synthesis and secretion of IL-6 is induced by inflammatory responses. Likewise, IL-1β is an inflammatory cytokine that can activate and recruit leukocytes, especially neutrophils, to the liver. Activated neutrophils act as effector cells and can induce hepatocyte necrosis by cytotoxicity[46,47]. Like these cytokines, TNF-α plays an important role in the development of liver injury, being able to induce necrosis and increase the production of inflammatory cytokines. In addition, TNF-α and inflammatory

cytokines can activate NF-Kβ, which, once activated, is translocated to the nucleus where it acts on target genes, therefore, inhibition of NF-Kβ reduces the production of inflammatory cytokines[47,48]. The effects of flavonoids against liver damage can be attributed to their potent antioxidant capacity, scavenging free radicals, in addition to their anti-inflammatory activity, regulating markers involved in inflammatory pathways[22-26].

Furthermore, an association between gut microbiota composition and lipid metabolism is suggested[13,29,49]. According to the studies in this review, animals treated with flavonoids showed increased bacterial diversity and greater abundance of *Bacteroidetes*, *Bacteroides*, *Verrucomicrobia*, *Akkermansia* and a consequent reduction in total cholesterol and triglycerides[24,27,29]. High levels of cholesterol and triglycerides are associated with lower microbial diversity and lower abundance of the phyla *Bacteroidetes* and *Proteobacteria*[50].

Still, in the study by Zhang et al.[51], the authors reported an association between the increase in the abundance of *Akkermansia* and *Bacteroides* with the reduction of total cholesterol and development of NAFLD. These bacteria have bile salt hydrolase (BSH) activity and act by deconjugating bile acids, that is, removing conjugated glycine or taurine, preventing their active reuptake in the small intestine. Subsequently, these acids are excreted in the feces or metabolized by intestinal bacteria. As a mechanism to compensate for the lower availability of bile acids, there is a new synthesis of these acids and, consequently, a reduction in total cholesterol (Figure 2). It is noteworthy that long-term high cholesterol induces the spontaneous and progressive development of NAFLD in mice, influencing the composition of the intestinal microbiota[52,53,54,55].

According to Jia, Xie & Jia[54], changes in the microbiota caused by the HFD diet can contribute to an increase in secondary bile acids in the intestine, in addition to greater intestinal reabsorption and return of these acids to the liver, increasing the occurrence of inflammation in the organ. Elevated levels of secondary bile acids in the colon harm colonic tissue through inflammation, NF-Kβ activation, oxidative DNA damage, and increased cell proliferation.

It is important to highlight that the association of intestinal microbiota and liver alterations is also due to the fact that an imbalance in the composition of the microbiota alters the function of the intestinal barrier, increasing its permeability to microbes and microbial products such as endotoxins, LPS and peptidoglycan. These, in turn, enter

the portal circulation, reach the liver and cause organ damage. The translocated bacteria or their products are potential causes of inflammatory processes by binding to TLRs receptors, initiating the inflammatory cascade with the production of inflammatory cytokines[55].

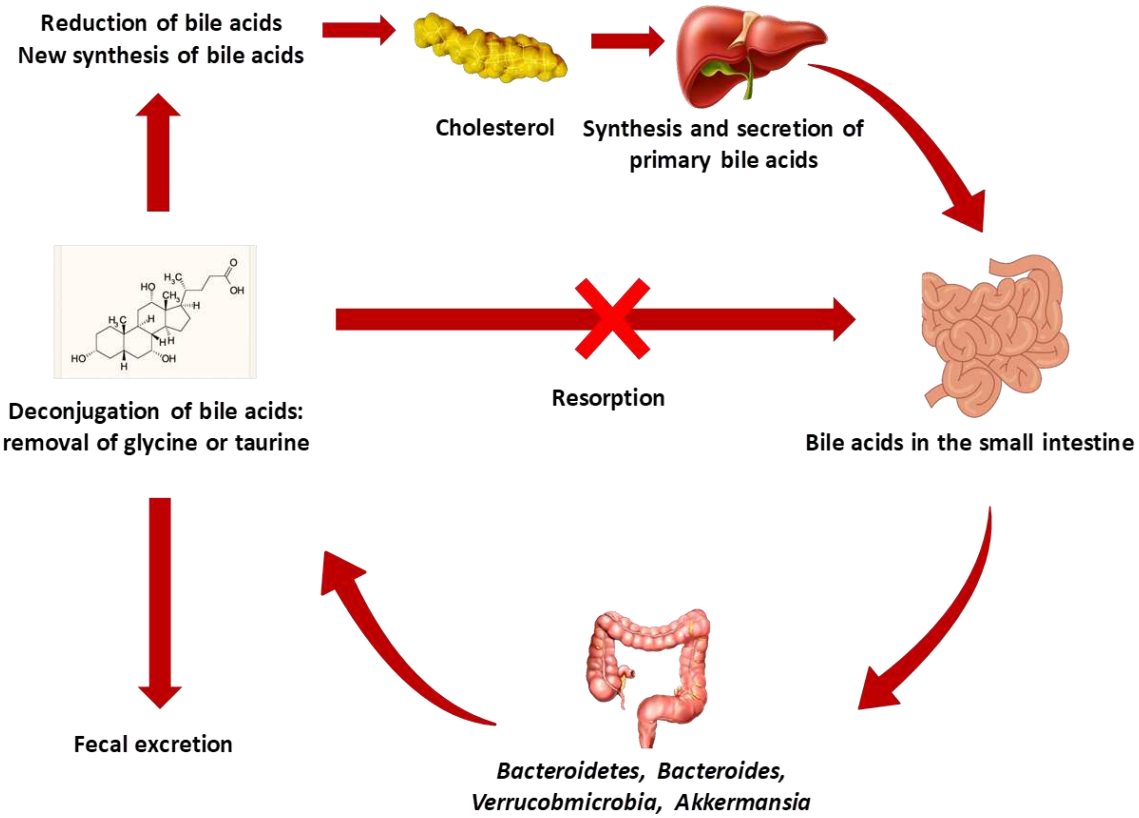


Figure 2. Bile acid synthesis in the liver and metabolism by the intestinal microbiota in obesity induced mice. Reproduced with kind permission from Peluzio, Martinez & Milagro (2021).

Changes in intestinal tissue by flavonoids

Treatment with flavonoids also appears to help maintain the integrity of goblet cells in the colon, in addition to preventing mucin degradation in mice[35,56]. Goblet cells play an important role in combating oxidative damage, inflammatory disorders and intestinal infections, in addition, these cells are part of the innate mucosal immune system to defend the host against possible pathogens[57]. Another observation was

about the reduced expression of inflammatory markers such as COX-2, LPS, TNF-α and IL-6 in the colon. Flavonoids are reported to be effective suppressors of TLR4 / NF-kβ inflammatory pathway signaling, by inhibiting TLR4 up-regulation and blocking NF-Kβ activation. Due to the central role of NF-Kβ in inflammation, it is believed that this blockade can inhibit the inflammatory signaling cascade, and consequently the production of inflammatory cytokines (TNF-α, IL-6)[36,58]. Therefore, dietary flavonoids may influence the expression of intestinal inflammatory markers.

Conclusion

The present review reports that the administration of flavonoids is able to influence the composition of the intestinal microbiota, increasing alpha diversity and reducing the abundance of bacteria associated with the obesity process in animals. In addition, flavonoids attenuated the oxidative and inflammatory processes in the liver triggered by the ingestion of HFD diet. Still, an association was observed between a greater abundance of *Verrucomicrobia*, *Akkermansia* and *Bacteroides* bacteria with the reduction of total cholesterol in the liver, suggesting that these bacteria can influence hepatic lipid metabolism, however more studies are needed to elucidate these mechanisms.

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Interest conflicts. The authors declare that there are no conflicts of interest.

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Supplemental Table S1 - Guia Animal Research: Reporting of in Vivo Experiments (ARRIVE).

	Roopchand et al. (2015)	Masumoto et al., 2016)	Lee et al. (2018)	Zheng et al. (2018)	Cheng et al. (2019)	Araújo et al. (2019)	Guo et al. (2019)	Bai et al. (2019)	Nakano et al. (2020)	He et al. (2021)	Song et al. (2021)	Juárez-Fernández et al. (2021)	Sun et al. (2021)	Liu et al. (2021)	Bian et al. (2022)	Percentage
Study design:																
For each experiment, provide brief details of study design including:																
a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
b. The experimental unit (e.g. a single animal, litter, or cage of animals).	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
Sample size:																
2.a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.	ok	-	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	94
b. Explain how the sample size was decided. Provide details of any a priori sample size calculation, if done.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Inclusion and exclusion criteria																
3.a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established a priori. If no criteria were set, state this explicitly.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
c. For each analysis, report the exact value of n in each experimental group.	ok	-	ok	-	ok	ok	ok	ok	ok	-	-	ok	ok	-	ok	67

Supplemental Table S1 – continued.

	Roopchand et al. (2015)	Masumoto et al., 2016)	Lee et al. (2018)	Zheng et al. (2018)	Cheng et al. (2019)	Araújo et al. (2019)	Guo et al. (2019)	Bai et al. (2019)	Nakano et al. (2020)	He et al. (2021)	Song et al. (2021)	Juárez-Fernández et al. (2021)	Sun et al. (2021)	Liu et al. (2021)	Bian et al. (2022)	Percentage
Randomisation:																
4.a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.	ok	-	-	ok	ok	ok	-	ok	-	-	-	-	-	-	-	34
b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
Blinding:																
5. Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Outcome measures:																
6.a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Statistical methods:																
7.a. Provide details of the statistical methods used for each analysis, including software used.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
7.b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100

Supplemental Table S1 – continued.

	Roopchand et al. (2015)	Masumoto et al., 2016)	Lee et al. (2018)	Zheng et al. (2018)	Cheng et al. (2019)	Araújo et al. (2019)	Guo et al. (2019)	Bai et al. (2019)	Nakano et al. (2020)	He et al. (2021)	Song et al. (2021)	Juárez-Fernández et al. (2021)	Sun et al. (2021)	Liu et al. (2021)	Bian et al. (2022)	Percentage
Experimental animals:																
8.a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	-	-	ok	-	-	-	-	ok	ok	ok	-	-	ok	ok	ok	47
Experimental procedures:																
9.a. For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
9.b. When and how often.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
9.c. Where (including detail of any acclimatisation periods).	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
9.d. Why (provide rationale for procedures).	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
Results:																
10. For each experiment conducted, including independent replications, report:																
a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range)	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
b. If applicable, the effect size with a confidence interval.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Supplemental Table S1 – continued.

	Roopchand et al. (2015)	Masumoto et al., 2016)	Lee et al. (2018)	Zheng et al. (2018)	Cheng et al. (2019)	Araújo et al. (2019)	Guo et al. (2019)	Bai et al. (2019)	Nakano et al. (2020)	He et al. (2021)	Song et al. (2021)	Juárez-Fernández et al. (2021)	Sun et al. (2021)	Liu et al. (2021)	Bian et al. (2022)	Percentage
Abstract:																
11. Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
Background:																
12.a. Include sufficient scientific background to understand the rationale and context for the study, and explain the experimental approach.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
b. Explain how the animal species and model used address the scientific objectives and, where appropriate, the relevance to human biology.	ok	-	ok	ok	ok	ok	ok	ok	ok	ok	-	ok	ok	ok	ok	94
Objectives:																
13. Clearly describe the research question, research objectives and, where appropriate, specific hypotheses being tested.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
Ethical statement:																
14. Provide the name of the ethical review committee or equivalent that has approved the use of animals in this study, and any relevant licence or protocol numbers (if applicable). If ethical approval was not sought or granted, provide a justification.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
Housing and husbandry:																
15. Provide details of housing and husbandry conditions, including any environmental enrichment.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100

Supplemental Table S1 – continued.

	Roopchand et al. (2015)	Masumoto et al., 2016)	Lee et al. (2018)	Zheng et al. (2018)	Cheng et al. (2019)	Araújo et al. (2019)	Guo et al. (2019)	Bai et al. (2019)	Nakano et al. (2020)	He et al. (2021)	Song et al. (2021)	Juárez-Fernández et al. (2021)	Sun et al. (2021)	Liu et al. (2021)	Bian et al. (2022)	Percentage
Animal care and monitoring:																
16.a. Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering and distress.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	-	ok	94
b. Report any expected or unexpected adverse events.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
c. Describe the humane endpoints established for the study, the signs that were monitored and the frequency of monitoring. If the study did not have humane endpoints, state this	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	100
Interpretation/ scientific implications:																
17.a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature.	ok	-	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	94
b. Comment on the study limitations including potential sources of bias, limitations of the animal model, and imprecision associated with the results.	ok	-	ok	-	-	-	ok	-	-	-	-	-	-	-	-	20
Generalisability/ translation:																
18. Comment on whether, and how, the findings of this study are likely to generalise to other species or experimental conditions, including any relevance to human biology (where appropriate).	ok	ok	ok	ok	ok	ok	-	ok	ok	ok	-	-	-	-	-	60
Protocol registration:																
19. Provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Supplemental Table S1 – continued.

	Roopchand et al. (2015)	Masumoto et al., 2016)	Lee et al. (2018)	Zheng et al. (2018)	Cheng et al. (2019)	Araújo et al. (2019)	Guo et al. (2019)	Bai et al. (2019)	Nakano et al. (2020)	He et al. (2021)	Song et al. (2021)	Juárez-Fernández et al. (2021)	Sun et al. (2021)	Liu et al. (2021)	Bian et al. (2022)	Percentage
Data access:																
20. Provide a statement describing if and where study data are available.	-	-	ok	-	-	-	-	-	-	ok	-	ok	ok	ok	ok	40
Declaration of interests:																
21.a. Declare any potential conflicts of interest, including financial and non-financial. If none exist, this should be stated.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	-	ok	ok	ok	ok	94
b. List all funding sources (including grant identifier) and the role of the funder(s) in the design, analysis and reporting of the study.	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok	-	ok	ok	ok	ok	94

4.2 MANUSCRIPT 3

The manuscript will be submitted to **The Journal of Nutritional Biochemistry** (Impact Factor: 6,117)

Intake of Hibiscus Sabdariffa L. alters the composition of the intestinal microbiota and influences the expression of c-Myc and caspase-3 genes in BALB/c mice induced to preneoplastic lesions.

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Abstract

The development of colorectal cancer includes the appearance of aberrant crypt foci, which are the first lesions of the initial stage of colorectal carcinogenesis. This process is influenced by genetic and environmental factors and by the composition of the intestinal microbiota. *Hibiscus sabdariffa* L. is a considerable source of phenolic compounds, anthocyanins and dietary fibers that may exert anti-inflammatory, antioxidant and prebiotic properties. Therefore, the aim of the study was to investigate whether supplementation with 5% and 10% dehydrated calyces of *H. sabadariffa* (DHSC) influences the composition of the intestinal microbiota and the expression of genes related to colorectal carcinogenesis in BALB/c mice. The *in vivo* experiment lasted 12 weeks, the animals were divided into 3 experimental groups: the control group and the supplemented groups (5% and 10% DHSC). Serum AST and ALT

markers, hepatic cytokine profile, intestinal microbiota composition and expression of p53, c-Myc, caspase-3 and PCNA genes were determined. Supplementation with 5% and 10% DHSC altered the composition of the intestinal microbiota, increasing the abundance of the *Lachnospiraceae*, *Ruminococcaceae* and *Clostridiaceae* families and the Clostridum genus, important butyrate producers. In addition, 5% and 10% DHSC supplementation increased caspase-3 and c-Myc expression, respectively, which may suggest apoptotic events. Therefore, the effects of phenolic compounds, anthocyanins and dietary fiber on the composition of the intestinal microbiota and on the expression of genes associated with cellular apoptosis may contribute to reducing the risk of developing pre-neoplastic lesions.

Keywords: colorectal cancer, hibiscus, flavonoids, anthocyanins, microbiota.

Introduction

Colorectal cancer (CRC) is a neoplasm that includes morphological and pathological changes that affect the entire segment of the large intestine and the rectum, and this process involves the appearance of aberrant crypt foci (ACF) that are the first lesions that appear at the beginning of colorectal carcinogenesis (Nguyen; Goel; Chung, 2020; Loke et al., 2020). This process of development of preneoplastic lesions and consequently of CRC is influenced by genetic factors and environmental factors such as smoking, alcohol consumption, physical inactivity, low fiber consumption, increased oxidative stress, among others. In addition, the imbalance between the microorganisms that make up the intestinal microbiota, that is, the process of intestinal dysbiosis, is being associated with the development of CRC (Schwabe & Jobin, 2013; Wang et al., 2022).

The gut microbiota is a complex community of microorganisms that inhabit the gastrointestinal tract and play important roles such as metabolism, immune system activation, and host health (Magne et al., 2020). The composition of the microbiota can be influenced by environmental factors such as infection, type of diet, use of drugs, and these factors can lead to an imbalance in the microbiota causing dysbiosis and promoting diseases such as cancer (Schwabe; Jobin, 2013; Lee, 2021; Wang et al., 2022). Some food components such as dietary fibers undergo the fermentation

process by the microbiota. Bacterial strains such as the genera of the *Lachnospiraceae*, *Ruminococcaceae* and *Clostridiaceae* families produce metabolites such as short chain fatty acids (SCFA), especially butyrate, exerting anticarcinogenic and anti-inflammatory functions (Encarnação; Abrantes; Pires, 2015; Bultman, 2017; Lee, 2021).

On the other hand, the relationship between the microbiota and the development of pre-neoplastic lesions may occur through the production of cancerous metabolites, by the action of pathogenic bacteria, inducing damage to the DNA molecule and activation of proinflammatory pathways, promoting inflammation and suppression of the immune system. However, the detailed mechanisms of these phenomena have not yet been fully elucidated (Lee, 2021; Wang et al., 2022).

In this context, *Hibiscus sabdariffa* L. (*H. sabdariffa*), belonging to the Malvaceae family, and commonly known as hibiscus, roselia, sorrel or sour okra, is a considerable source of bioactive compounds, such as phenolic compounds, anthocyanins, and fiber dietary (soluble and insoluble) (Borrás-Linares et al., 2015; Jabeur et al., 2017). These compounds are associated with a lower risk of developing chronic diseases, including cancer, through their potential antioxidant, anti-inflammatory and anti-carcinogenic capacity, which may reduce the activation of the metabolic pathways involved in colorectal carcinogenesis (Lin et al., 2012; Barata et al., 2016; Jabeur et al., 2017; Huang et al., 2018). Furthermore, anthocyanins and dietary fibers present in *H. sabadariffa* can act as prebiotics, being metabolized by the microbiota to produce metabolites, such as SCFA, in addition to influencing its bacterial composition (Chen et al., 2018).

Therefore, the aim of the study was to investigate whether the intake of 5% and 10% of dehydrated *Hibiscus sabdariffa* L. calyces (DHSC) in the diet could influence the composition of the intestinal microbiota, the expression of genes related to the initial stage of colorectal carcinogenesis and liver markers in BALB /c mice.

Methodology

Acquisition and characterization of raw material

Ten kilograms of *H. sabdariffa* calyxes were originally obtained from a producer in Coimbra - Minas Gerais (latitude: -20,830,127, Longitude: -42,801,351) and purchased at a local market in Viçosa - Minas Gerais. The *H. sabdariffa* calyxes were then pulverized in a knife mill and stored at -20°C. Our previous study carried out the characterization of the chemical composition (proteins, lipids, ash, carbohydrates), total fiber, soluble and insoluble fiber, in addition to the concentration of total phenolic compounds, total anthocyanins, and antioxidant capacity (DPPH) of *H. sabdariffa* (Bernardes et al., 2022).

Experimental design

Forty-five male BALB/c mice, seven weeks old, initial weight between 20 - 40 grams, purchased from the Animal Facility of the Biological Sciences Center of the Universidade Federal de Viçosa were used. Animal protocol was approved by the Ethics Committee on Animal Experimentation from the Universidade Federal de Viçosa, Brazil, under the process number 10/2017. BALB/c mice were used because they develop neoplasms more easily, in addition, male mice were defined due to the hormonal variability of females. The sample calculation was performed according to Mera, Thompson and Prasad (1998) who consider the effect of treatment between the experimental groups on the outcome variables of interest. The animals were housed in collective cages, with controlled temperature (22 ± 2 ° C), 12-hour photoperiod and *ad libitum* access to water and food. After two weeks of acclimatization, the animals were randomized according to body weight and distributed in ascending order in the cages in three experimental groups: control group - CON (diet AIN-93M, American Institute of Nutrition for Maintenance, n = 15), group 5DHSC (diet AIN - 93M + 5% DHSC, n = 15) and the 10DHSC group (diet AIN-93M + 10% DHSC, n = 15) (Table 1).

The 5% and 10% DHSC supplementation was defined in order to provide about 100 - 200 mg of anthocyanins. Such amounts can be incorporated into the human diet. The difference in the average weights between the animals was not greater than 5% and the cages were properly identified. The experimental period was 12 weeks, and during the first eight weeks all animals received an intraperitoneal injection in a single dose per week of the drug DMH (20 mg / kg of body weight) for induction of pre-neoplastic lesions. At the end of the experimental period, the mice were fasted for 12

hours, then anesthetized in the Experimental Nutrition Laboratory with 3% isoflurane (Cristália®, Brazil), followed by total exsanguination through the cervical retro-orbital sinus. The blood was taken and centrifuged for further analysis. The liver and colon were removed and immediately frozen in liquid nitrogen and stored at -80°C. The feces were immediately frozen until the time of analysis.

Table 1 – Composition of experimental diets AIN-93M* (g 100g-1).

Ingredients (g)	CON[1]	5DHSC[2]	10DHSC[3]
Cornstarch	39,75	39,75	39,75
Casein	20,00	20,00	20,00
Maltodextrin	13,20	13,20	13,20
Sucrose	10,00	10,00	10,00
Soybean oil	7,00	7,00	7,00
Cellulose	5,00	5,00	5,00
Mineral mix	3,50	3,50	3,50
Vitamin mix	1,00	1,00	1,00
L-Cystine	0,30	0,30	0,30
Choline bitartrate	0,25	0,25	0,25
t-butylhydroquinone	0,0014	0,0014	0,0014
Dietary DHSC	-	5g	10g

1 Control group (AIN-93M); **2** 5DHSC group (AIN-93M supplemented with 5% dietary DHSC); **3** 10DHSC group (AIN-93M supplemented with 10% dietary DHSC). *AIN-93M (American Institute of Nutrition for Maintenance).

Analysis of the composition of the intestinal microbiota

For DNA extraction and analysis, three pools of feces (3 animals/pool-group) were performed before and after the induction of pre-neoplastic lesions, that is, in the 1st and 13th week of the experiment, respectively. Total DNA was extracted from the fecal contents with the E.Z.N.A.® Stool DNA Kit according to the manufacturer's protocol. Briefly, DNA was isolated using a HiBind® Mini Column with spin column technology capable of eliminating humic acid, polysaccharides, phenolic compounds and enzyme inhibitors. DNA quality was checked by electrophoretic run and PCR

execution using primers for the 16s gene. DNA quantification was performed by Nanodrop. Then, the bacteria from the fecal content were identified by sequencing the V3/V4 regions of the 16S rRNA gene. Library preparation followed a proprietary protocol (Neoprospecta Microbiome Technologies, Brazil). Paired end amplification was performed with primers for the V3-V4 region of the 16S rRNA gene, 341F (CCTACGGGRSGCAGCAG, doi: 10.1371/journal.pone.0007401) and 806R (GGACTACHVGGGTWTCTAAT, doi: 10.1038/ismej.2012.8). Libraries were sequenced using the MiSeq Sequencing System (Illumina Inc., USA).

The quality of the sequences were evaluated by the FastQC package and the low quality sequences were trimmed using the Trimmomatic program version 0.36. The high quality sequences were analyzed in the DADA2 package implemented on the R platform and aligned to the Silva 16S rRNA database, and the levels of taxonomic interest were evaluated: phylum, family and genus.

Gene expression of p53, c-Myc, Caspase-3 and PCNA

RNA was extracted by colon homogenization with Trizol (Invitrogen™, Carlsbad, CA, USA) in a tissue homogenizer and incubated for 5 minutes at room temperature. Then, chloroform was added in a ratio of 0.2:10 v/v Trizol, and left to stand. Afterwards, the samples were centrifuged (12,000 g/ 15 minutes/ 4 °C), and the aqueous phase was removed to which 500 µL of isopropanol was added and left overnight at -80 °C. The following day, the samples were centrifuged (12,000 g/ 15 minutes/ 4 °C), the supernatant was discarded and 500 µL of 75% alcohol was later added to each sample. Subsequently, the samples were centrifuged (7500 g/ 5 minutes/ 4 °C), the supernatant was discarded and the samples were allowed to dry in the flow.

Total RNA was quantified on a Multiskan™ GO spectrophotometer (Thermo Fisher Scientifics; Waltham, MA, USA) and purity was determined by the absorbance ratio 260/280 nm. After quantification, the mRNA samples were treated with DNAse (DNAse I; Promega, Madison, WI, USA), for which the samples containing 2 µg of RNA were treated with 3 µL of DNAse buffer and 4.5 µL of DNAse. The final volume of 30 µL was made up with water. Subsequently, the samples were incubated at 37°C for 30 min and then at 70°C for 10 min. After the incubation step, the samples were placed

on ice for 5 min. Quantitative mRNA analyzes by real-time PCR were performed on an Illumina Eco Real-Time PCR system (Illumina, San Diego, CA, USA), using the GoTaq® 1-Step RT-qPCR system (Promega, Madison, WI, USA). 10 µL of GoTaq® qPCR Master Mix, 0.4 µL of Reverse Transcriptase, 0.4 µL of sense primer, 0.4 µL of antisense primer, sample (containing at least 100 ng of mRNA) and nuclease - free water was added at each reaction (final volume of 20 µL). The thermal cycles used were: 1 cycle of reverse transcription (37 °C, 15 min) and 1 cycle of inactivation of reverse transcriptase and activation of DNA polymerase GoTaq® (95 °C, 10 min), followed by 40 cycles of denaturation (95°C, 10 seconds), hybridization (60°C, 30 seconds) and extension (72°C, 30 seconds). The analysis was performed by the 2-ΔΔCt algorithm of the EcoStudy® software (Illumina), using the GAPDH gene as an endogenous control. Table 2 presents the primers used in the amplification of the p53, c-Myc, Caspase-3, PCNA (cellular proliferation nuclear antigen) and GAPDH genes.

Table 2 - Primers used in the amplification of p53, c-Myc, Caspase-3, PCNA genes.

Primer	Primer sequence	Reference
p53	F: GTATTTCACCCTCAAGATCC R: TGGGCATCCTTTAACTCTA	Zeineldin et al., 2012
c-Myc	F: TCCTGTACCTCGTCCGATTC R: GGAGGACAGCAGCGAGTC	Kiatpakdee et al., 2020
Caspase-3	F: ATGGGAGCAAGTCAGTGGAC R: CGTACCAGAGCGAGATGACA	Lin & Sun (2015)
PCNA	F: TAAAGAAGAGGAGGCGGTAA R: TAAGTGTCCCATGTCAGCAA	Mengying et al., 2017
GAPDH	F: CAACTCACTCAAGATTGTCAGCAA R: TACTTGGCAGGTTTCTCCAGGC	Piranlioglu et al., 2019

Determination of liver markers

After euthanasia, blood of the animals was collected from the retro-orbital sinus and centrifuged at 1190 *g* / 10 minutes/4°C. The serum markers: aspartate aminotransferase (AST) (u/L), alanine aminotransferase (ALT) (mg/L) were assessed

by specific colorimetric assays (Bioclin®, Brazil) using a clinical chemistry analyzer BS-200 (Mindray®).

Liver tissue (100 mg) was used to prepare a phosphate buffered homogenate containing EDTA at pH 7.0 (1:10) for malondialdehyde (MDA) and carbonyl protein analysis. The MDA concentration was determined to assess the occurrence of lipid peroxidation, and the results are expressed as nmol MDA/mg protein. To evaluate protein oxidation, carbonylated protein was measured, and the results are expressed in nanomoles per milligram of protein (nmol/mg). The determination of the concentration of proteins in the liver tissue was performed according to Lowry et al. (26), and spectrophotometer readings (Thermo Scientific®, model Multiskan GO, Vantaa, Finland) at a wavelength of 700 nm.

Hepatic cytokine profile

The determination of the concentration of cytokines IL-2, IL-4, IL-10, IL-17, TNF-α and IFN-γ were performed with the Cytometric Bead Array (CBA) kit (BD™) according to the protocol of the manufacturer with some modifications described below. The liver (100 to 200 mg) was ground with a tissue homogenizer (IKA®, model T10 basic) in pH 7 phosphate buffer and kept on ice. The samples were then centrifuged (10,000 *g*, 10 minutes, 4°C) and the supernatant was collected. The beads were diluted with the diluent solution and distributed in the microtubes. 25 µL of the sample and 17 µL of the detector solution were added to each microtube and incubated for 2 hours. Subsequently, 1 mL of the washing solution was added, followed by centrifugation (1800 *g*, 5 minutes, 4ºC). Part of the supernatant was discarded (about 800 µL), and the remaining volume was used for reading on the flow cytometer. Data were processed in FCAP Array Software v3.0 and results expressed in pg/g of tissue.

Statistical analysis

Results are expressed as mean ± standard deviation. The means of the three groups (CON, 5DHSC and 10DHSC) were compared using analysis of variance (ANOVA) complemented with Tukey's test, considering a significance level of 5% ($p < 0.05$). Metagenomics results are expressed as a relative percentage. The analyzes between samples and groups for richness and alpha bacterial diversity were performed

by Anova One-way, complemented with Tukey's test. (*) represents significant differences with p<005. Data were analyzed using Graph Pad Prism 5 software, version 5.01.

Results and discussion

Supplementation with DHSC alters the composition of the intestinal microbiota of animals induced to pre-neoplastic lesions

Our study explored the prebiotic effects of *H. sabadariffa*, sources of anthocyanins and dietary fiber, in composition of the intestinal microbiota of animals in the early stage of colorectal carcinogenesis. The analyzes to determine the composition of the microbiota of the animals were carried out at the beginning and at the end of the experimental period, that is, before and after the induction of carcinogenesis.

After amplicon sequencing of the 16S rRNA gene, the relative distributions of bacteria at phylum, family, and genus level were calculated (Figure 1A-F). At the phylum level, before the induction of preneoplastic lesions (Figure 1A), the control group had the highest abundance of *Firmicutes* (91.4%), followed by the presence of *Patescibacteria* (5.9%) and *Bacteroidetes* (2.7 %). In relation to the groups supplemented with DHSC, the 5DHSC group also showed a higher abundance of *Firmicutes* (77.5%) and *Bacteroidetes* (22.5%), and the 10DHSC group only showed *Firmicutes* (100%). Corroborating with our results, a reduction of *Bacteroidetes* and an increase of *Firmicutes* in the microbiota of animals were reported during the development of CRC associated with colitis (Wang et al., 2022).

However, after the process of inducing preneoplastic lesions (Figure 1B), this composition was changed in all groups, and it is possible to observe in the control group a reduction in *Firmicutes* (55.5%) and an increase in *Patescibacteria* (44 .5%). The groups supplemented with DHSC changed the number and abundance of phyla, in which the 5DHSC group showed a reduction in *Firmicutes* (39.9%) and an increase in *Bacteroidetes* (16.5%), *Patescibacteria* (36.1%) and *Actinobacteria* (7.5%). Similarly, the 10DHSC group reduced *Firmicutes* (50.9%) and increased abundance of *Bacteroidetes* (49.1%).

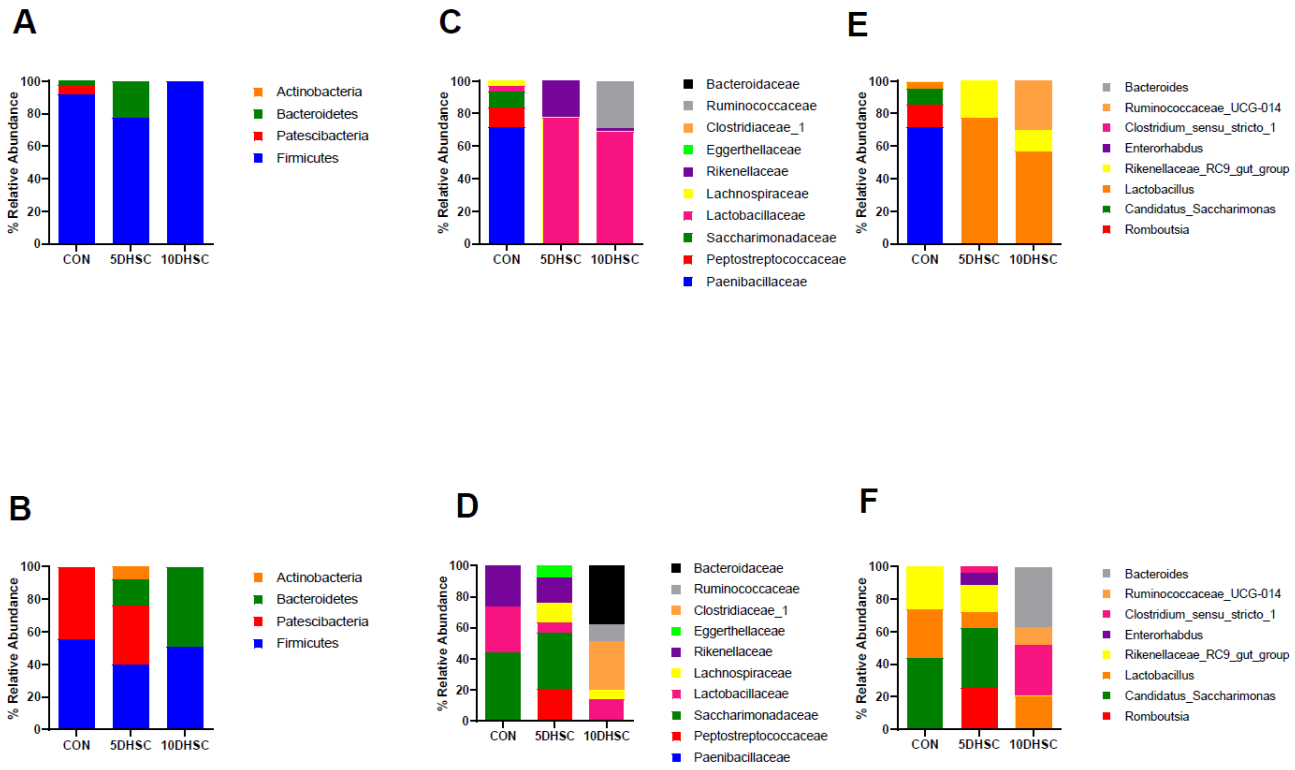

Figure 1. Relative abundance of the top 50 bacteria at phylum (A,B), family (C,D) and genus (E,F) level in control, 5DHSC and 10DHSC groups before (A,C,E) and after (B,D,F) the process of inducing pre-neoplastic lesions in Balb/c mice. The taxa were ordered in decreasing order of mean relative abundance (%).

Studies report an association of anthocyanin consumption with the reduction of the phylum Firmicutes (Roopchand et al., 2015; Lee et al., 2018; Guo et al., 2019; Song et al., 2021; Liu et al., 2021;) and an increase in Bacteroidetes (Guo et al., 2019), in addition to a reduction in the Firmicutes/Bacteroidetes ratio (Roopchand et al., 2015; Nakano et al., 2020). In the present study, we can say that supplementation with DHSC, rich in anthocyanins, exerted this effect on the abundance of these two phyla, even with the process of inducing pre-neoplastic lesions. The phyla Firmicutes and Bacteroidetes dominate about 90% of the intestinal microbiota of mice (Ley et al., 2005). Bacteria belonging to the phylum Bacteroidetes participate in the metabolism of flavonoids by the microbiota, transforming them into SCFA and phenolic acids, thus, these compounds can be used as an additional substrate for bacterial growth (Cheng et al., 2019; Bernardes et al., 2022).

At the family level, before the induction of preneoplastic lesions (Figure 1C), among the 50 most abundant families, the control group presented *Paenibacillaceae*

(71.8%) followed by *Peptostreptococcaceae* (12%), *Saccharimonadaceae* (9.5%), *Lactobacillaceae* (3.8%) and *Lachnospiraceae* (2.9%). In contrast, the 5DHSC group showed a predominance of only *Lactobacillaceae* (77.5%) and *Rikenellaceae* (22.5%), and the 10DHSC group showed an abundance of *Lactobacillaceae* (68.9%), *Ruminococcaceae* (29%) and *Rikenellaceae* (2 ,1%). However, after the lesion induction process (Figure 1D) there was a change in the profile of the families that make up the animals' intestinal microbiota. The control group started to present greater abundance only of the families *Saccharimonadaceae* (43.9%), *Lactobacillaceae* (29.7%) and *Rikenellaceae* (26.4%). On the other hand, the groups supplemented with DHSC increased the number of families present in the microbiota, so that the 5DHSC group showed greater abundance of *Saccharimonadaceae* (36.1%), *Peptostreptococcaceae* (20.4%), *Rikenellaceae* (16.4 %), *Lachnospiraceae* (12%), *Egggerthellaceae* (7.5%), *Lactobacillaceae* (6.9%) and *Clostridiaceae_1* (0.7%). On the other hand, the 10DHSC group exhibited greater abundance of *Bacteroidaceae* (37.7%), *Clostridiaceae_1* (31.5%), *Lactobacillaceae* (14.5%), *Ruminococcaceae* (10.8%) and *Lachnospiraceae* (5.5%).

The *Lachnospiraceae* and *Ruminococcaceae* families are related to higher butyrate production and its abundance is reduced in CRC and inflammatory bowel diseases (Wang et al., 2022), therefore, we can observe that DHSC supplementation was positive, since there was an increase in the abundance of these families in the supplemented groups. In our previous study (Bernardes et al., 2022) we observed a significant increase in butyrate concentration in the group supplemented with 10% DHSC, corroborating the results of the present study. In addition, it is known that the greater abundance of *Lachnospiraceae* and *Rikenellaceae* contributes to the improvement of the intestinal environment and promotes anticancer effects (Wang et al., 2022).

As for the genera, among the 50 most abundant genera before the induction of preneoplastic lesions (Figure 1E), the control group had the highest abundance of *Paenibacillus* (71.8%), *Romboutsia* (13.5%), *Candidatus_Saccharimonas* (9, 9%) and *Lactobacillus* (4.8%). The 5DHSC group showed greater abundance of *Lactobacillus* (77.5%) and *Rikenellaceae_RC9_gut_group* (22.5%), and the 10DHSC group of *Lactobacillus* (56.7%), *Ruminococcaceae_UCG-014* (30.2%) and *Rikenellaceae_RC9_gut_group* (13, 1%). After the lesion induction process (Figure

1F) there was a change in the profile of the genera present in the microbiota in all experimental groups. The control group started to present greater abundance of *Candidatus_Saccharimonas* (43.9%), *Lactobacillus* (29.7%) and *Rikenellaceae_RC9_gut_group* (26.4%). On the other hand, the supplemented groups increased the number of genera and it was observed that the 5DHSC group showed greater abundance of *Candidatus_Saccharimonas* (36.6%), *Romboutsia* (25.5%), *Rikenellaceae_RC9_gut_group* (16.4%), *Lactobacillus* (10%), *Enterorhabdus* (7.6%) and *Clostridium* (3.9%). The 10DHSC group showed an increase in the abundance of *Bacteroides* (37%), *Clostridium* (31.4%), *Lactobacillus* (20.4%) and *Ruminococcaceae_UCG-014* (11.2%).

The *Clostridium* genus belonging to the *Clostridiaceae* family is considered an important producer of butyrate (Chen et al., 2020; Wang et al., 2022). In the present study, this genus showed abundance in the microbiota of animals that were supplemented with DHSC for 12 weeks, and the percentage of relative abundance was higher in the group that received 10% of DHSC. This result is consistent with our previous study, in which we demonstrated that the animals in the group supplemented with 10% DHSC had a higher concentration of butyrate in the feces (Bernardes et al., 2022).

DHSC supplementation may have influenced the changes that occurred in the supplemented groups in which there was an increase in the number of phyla, families and genera. Anthocyanins have been reported to have a prebiotic action, increasing the abundance of the *Lachnospiraceae* and *Ruminococcaceae* families, and of the *Lactobacillus* genus, in addition to increasing the concentration of butyrate (Liu et al.; Marín et al., 2015). Furthermore, DHSC is a source of fibers that, when fermented by the microbiota, produce SCFA, such as butyrate (Bishehsari et al., 2018).

According to the Venn diagram (Figure 2), the Lactobacillus genus was present in the microbiota of the animals in the three experimental groups before (Figure 2A) and after (Figure 2B) the process of inducing pre-neoplastic lesions.

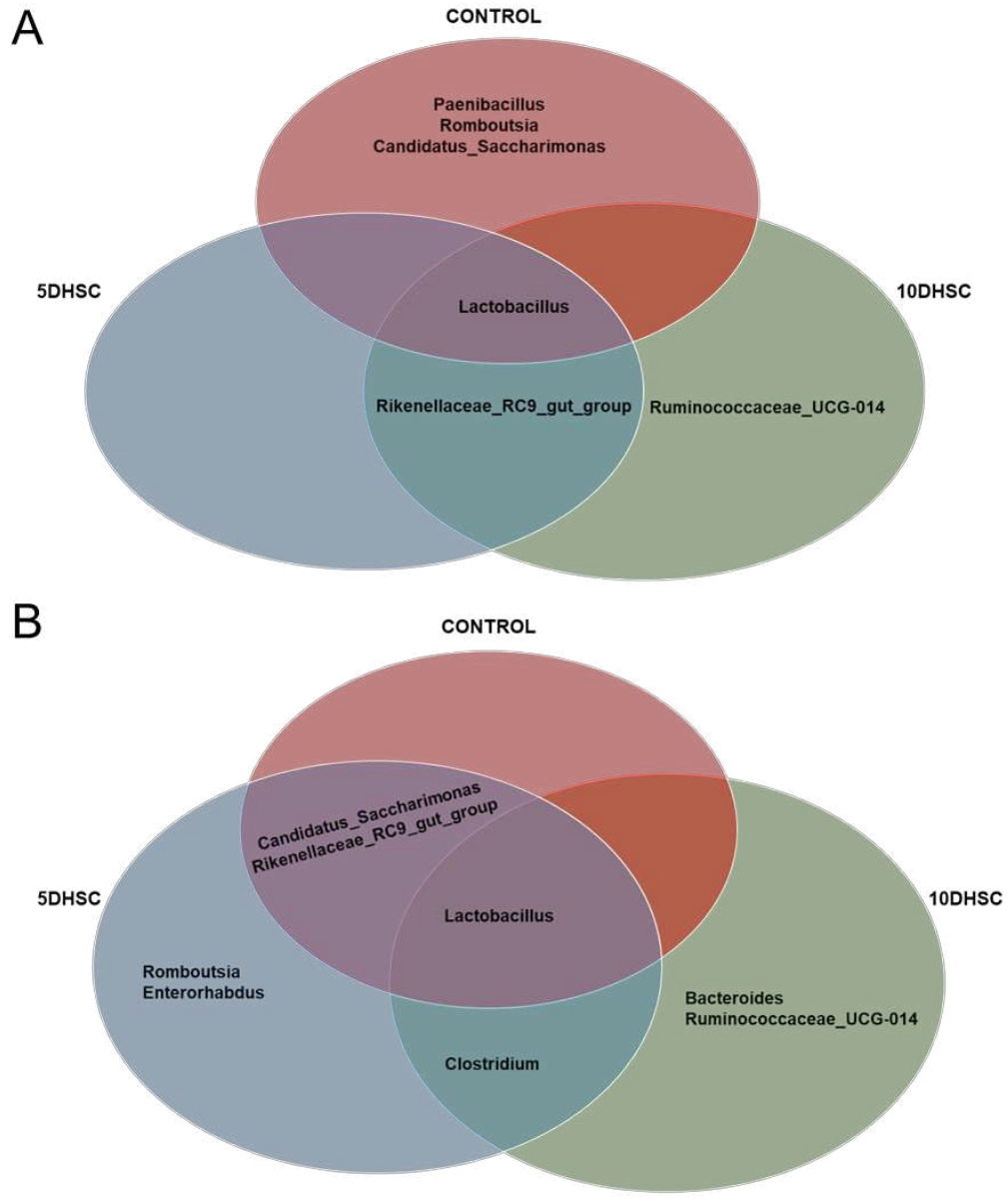

Figure 2. Venn diagram of the top 50 of the most abundant genera in the intestinal microbiota of Balb/c mice before (A) and after (B) the process of inducing preneoplastic lesions with DMH.

In addition, an abundance of the genus *Rikenellaceae_RC9_gut_group* was observed in the 5DHSC and 10DHSC groups before the induction of lesions, however, after the induction of lesions, this genus was present in the 5DHSC and control groups. The *Clostridum* genus showed abundance in the 5DHSC and 10DHSC groups only

after the process of induction of pre-neoplastic lesions and concomitant supplementation of DHSC.

Non-metric multidimensional scale analysis (NMDS) was performed to analyze the observed variability. According to the NMDS graph, before (Figure 3A) and after (Figure 4A) the induction of preneoplastic lesions, the samples from the three experimental groups showed similarity to each other, suggesting sample homogeneity.

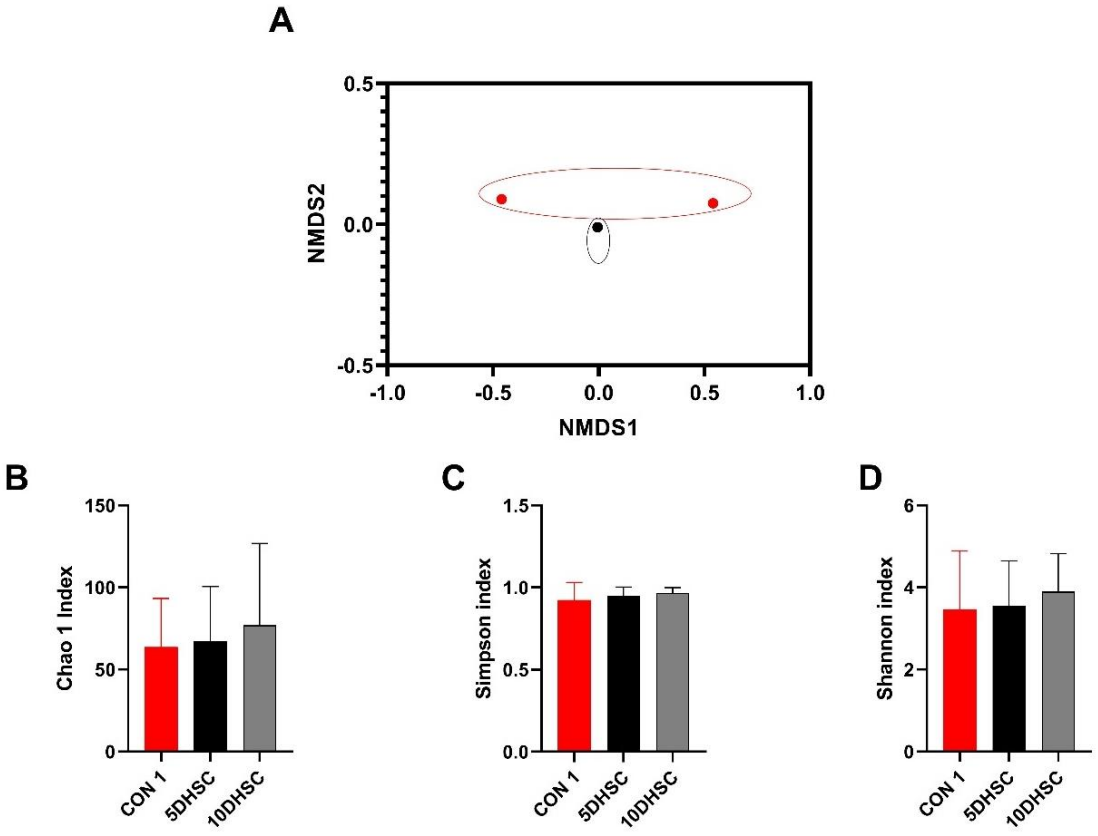

Figure 3. Non-metric multidimensional scale (NMDS) plot (A) of metagenomic sequencing data before induction of pre-neoplastic lesions. The black dot represents the supplemented groups (5DHSC and 10DHSC) and the red dot the control group. (B) Bacterial richness and (C, D) Alpha bacterial diversity. Statistical differences between groups were analyzed by the unpaired T test, in which (*) represents significant differences ($p < 0.05$).

Bacterial richness among the experimental groups was analyzed according to the Chao1 index (Figure 3B, 4B) and alpha diversities were compared between groups using the Shannon (Figure 3C, 4C) and Simpson index (Figure 3D, 4D). It is observed

that there was no statistical difference in terms of richness or alpha bacterial diversity between the animals before and after the induction of pre-neoplastic lesions ($p > 0.05$).

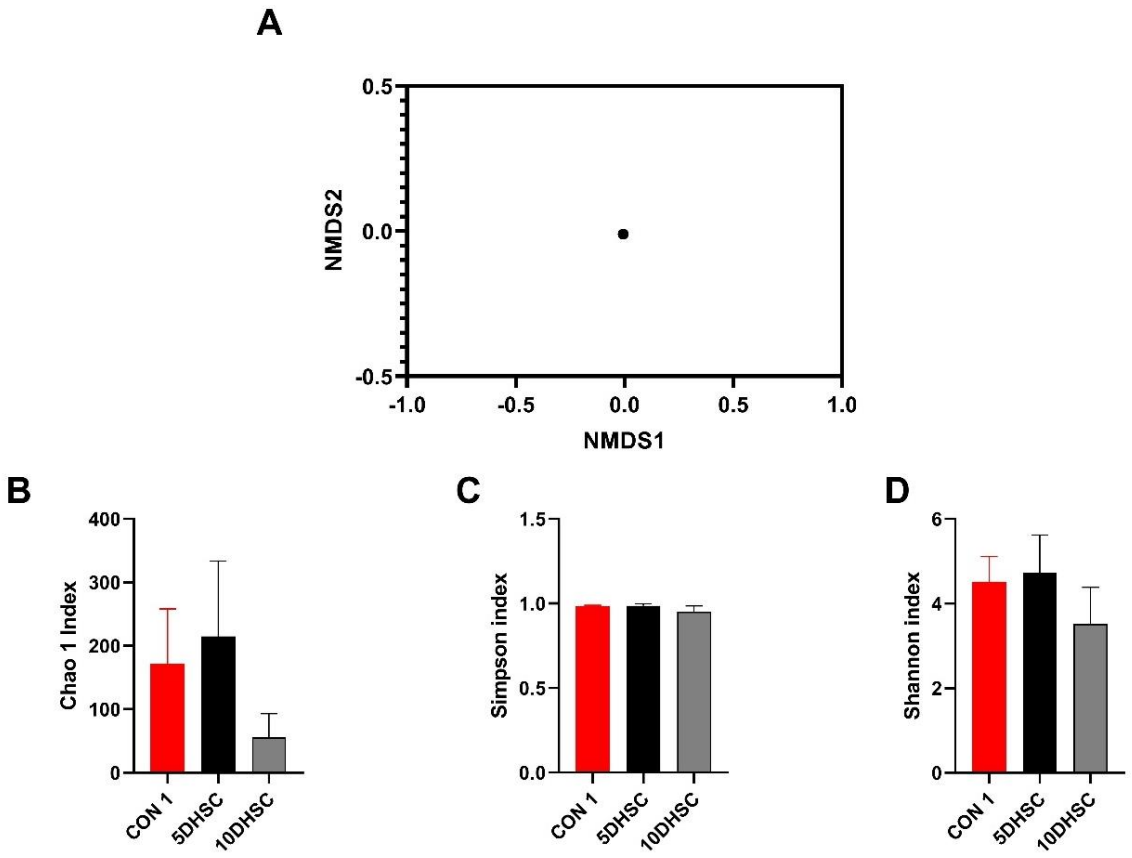

Figure 4. Non-metric multidimensional scale (NMDS) plot (A) of metagenomic sequencing data after induction of pre-neoplastic lesions. The black dot represents all treatments (control, 5DHSC and 10DHSC). (B) Bacterial richness and (C, D) Alpha bacterial diversity. Statistical differences between groups were analyzed by the unpaired T test, in which (*) represents significant differences ($p < 0.05$).

Effects of DHSC supplementation on p53, c-Myc, caspase-3 and PCNA gene expression

Data on gene expression of genes involved in carcinogenesis are shown in Figure 5. Supplementation with 5% or 10% DHSC did not significantly influence the expression of p53 and PCNA. However, supplementation with 10% DHSC induced higher expression of c-Myc compared to the control group. The c-Myc gene is a proto-

oncogene also involved in cell proliferation, differentiation and death (Liu et al., 2017; Sipos; Firneisz; Muzes, 2016). c-Myc target genes participate in cellular functions including cell cycle, survival, protein synthesis, cell adhesion and microRNA expression. Therefore, the final cellular response to elevated c-Myc values may correspond to increased proliferation and/or increased apoptosis (Sipos; Firneisz; Muzes, 2016).

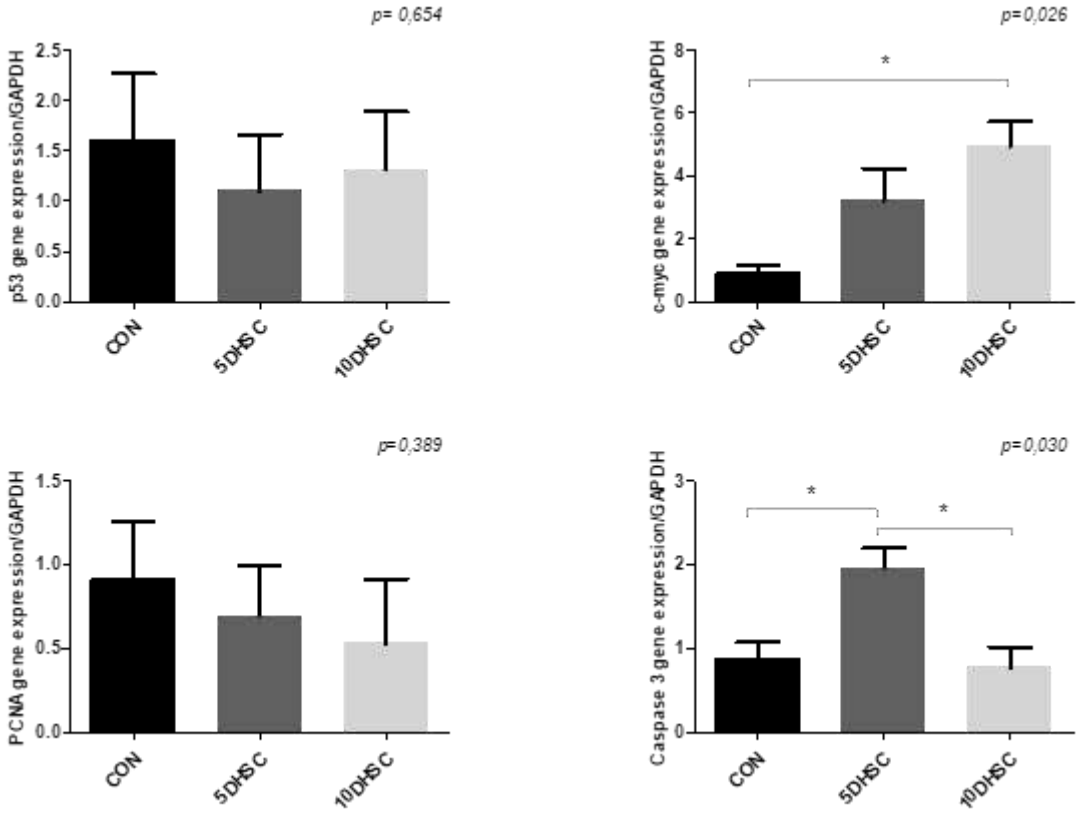

Figure 5. Gene expression of p53, PCNA, c-Myc and caspase 3 in the colonic mucosa of BALB/c mice induced to preneoplastic lesions with DMH and supplemented with 5% and 10% DHSC. Data are expressed as mean ± standard deviation (n = 6). * means statistical difference according to ANOVA supplemented with Tukey's test ($p < 0.05$).

Elevated levels of c-Myc genes can promote cell proliferation and transformation, in addition to tumorigenesis. However, this gene is also involved in the ARF/p53 and apoptosis tumor suppressor pathway, in which c-Myc overexpression induces ARF transcription and stability, by inhibiting Ubiquitin ligase ULF activity, promoting the activation of p53 and suppression of c-Myc oncogenic activity (Chen et

al., 2013). Activation of p53 results in the subsequent induction of p21 and other proteins involved in the p53-dependent apoptosis pathway (García-Gutiérrez et al. 2019). Furthermore, after damage to the DNA molecule, ARF is degraded by ULF, however, this degradation can be inhibited through the overexpression of c-Myc, and as a result cells are induced to p53-mediated apoptosis (Chen et al., 2013). In mice, the c-Myc gene can promote cellular apoptosis through the apoptotic protein Bax (Sipos, Firneisz, & Məzes, 2016). However, it is noteworthy that the mechanisms by which different levels of c-Myc can induce cell proliferation or apoptosis are not fully elucidated (Chen et al., 2013; Sipos, Firneisz, & Məzes, 2016).

As for caspase-3 expression, higher expression was observed in the group supplemented with 5% DHSC compared to the control group and the 10% DHSC group (Figure 5). The caspase family acts in the apoptotic process and consists of an initiation group and a cell death execution group. The beginning of the apoptotic pathway is mediated by caspase-9, followed by the execution of apoptosis by caspase-3 (Jing et al., 2020). An increase in caspase-3 expression in colon cells was observed in animals supplemented with 20% purple potato (200g/kg diet) rich in anthocyanins. This increase in caspase 3 expression could suggest a higher occurrence of cellular apoptosis events (Jiang et al., 2017), reinforcing our aforementioned hypothesis.

The mechanisms by which anthocyanins may help to reduce the risk of developing CRC may be related to the induction of apoptosis, cell cycle arrest, inhibition of proliferation and modulation of inflammation (Lim et al., 2013).

Effects of DHSC supplementation on liver markers

In the present study, all experimental groups showed an increase in serum markers ALT and AST (Table 3) according to the reference standards for the animal model (Petri et al., 2020), which may indicate hepatic alterations induced by the DMH drug. However, there was no significant difference between the groups. Also, in relation to oxidative stress markers, there was no difference between the groups for MDA and carbonyl protein, a result also found when analyzing the profile of hepatic cytokines IL-2, IL-4, IL-10, IL-17, TNF-α and IFN-γ , quantified in the liver where there was also no difference between groups.

Table 3. Effect of DHSC supplementation (5% and 10%) on liver markers in the liver of mice with DMH-induced preneoplastic lesions.

Liver markers	Groups CON	5DHSC	10DHSC
AST (U/L)	178,00 ± 35,86[a]	171,60 ± 27,52[a]	162,0 ± 30,38[a]
ALT (U/L)	71,20 ± 35,09[a]	55,80 ± 7,80[a]	48,00 ± 6,46[a]
MDA (nmol/mL)	0,579 ± 0,07[a]	0,099 ± 0,02[a]	0,072 ± 0,02[a]
carbonylated protein (nmol/mL)	4,56 ± 1,58[a]	2,04 ± 0,22[a]	2,33 ± 0,53[a]
IL-2 (pg/mg)	0,41 ± 0,006	0,28 ± 0,003	0,10 ± 0,005
IL-4 (pg/mg)	0,20 ± 0,001	0,20 ± 0,008	0,21 ± 0,009
IFN- γ (pg/mg)	0,54 ± 0,010	0,46 ± 0,017	0,50 ± 0,011
TNF-α (pg/mg)	0,92 ± 0,080	0,72 ± 0,017	0,71 ± 0,010
IL-17 (pg/mg)	0,40 ± 0,003	0,28 ± 0,035	0,35 ± 0,018
IL-10 (pg/mg)	0,28 ± 0,011	0,20 ± 0,080	0,19 ± 0,006

Data are expressed as mean ± standard deviation (n=5). Different letters in each line mean statistical difference according to ANOVA supplemented with Tukey's Test. Interleukin-2,4,10 and 17 (IL-2, IL-4, IL-10 and IL-17, respectively); Interferon- γ (IFN-γ); Tumor necrosis factor-α (TNF-α).

The DMH drug used in the present study can cause liver damage, as it is metabolized in the liver to produce methylazoxymethanol. This metabolite can cause damage to the liver tissue itself, increasing the release of aspartate and alanine aminotransferases, in addition to being transported to the colon where it will promote the development of pre-neoplastic lesions (Venkatachalam et al., 2020). Therefore, it became relevant to investigate liver damage in animals induced pre-neoplastic lesions with DMH.

Conclusion

Supplementation with DHSC did not influence serum markers (AST and ALT), oxidative stress (MDA and carbonyl protein) and cytokines (IL-2, IL-4, IL-10, IL-17, TNF-α and IFN- γ) in the liver. Bacterial diversity and richness did not differ between the experimental groups, however, DHSC supplementation increased the abundance of butyrate-producing families and genera, being attributed to the presence of fibers and anthocyanins in DHSC. Supplementation with DHSC did not influence the expression of PCNA and p53, however there was an increase in the expression of caspase-3 in the 5DHSC group and of c-Myc in the 10DHSC group, suggesting the

occurrence of apoptotic events in the initial stage of carcinogenesis. It is noteworthy that it is necessary to analyze other markers of the pathways in which these genes are involved to better elucidate the mechanisms of action of the compounds present in DHSC.

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5. GENERAL CONCLUSION

Supplementation with DHSC promoted positive results in the development of pre-neoplastic lesions as there was a significant reduction in ACFs and less infiltration of inflammatory cells in the supplemented groups. When comparing the dosages of DHSC used as treatment, the dosage of 10% of DHSC was more effective because in addition to reducing ACFs and inflammatory infiltrates, this group showed an increase in the activity of the hepatic antioxidant enzyme CAT, greater infiltration of NK cells in the colon and higher concentration of butyrate in feces. In addition, when analyzing the composition of the intestinal microbiota of the animals, greater abundance was observed in families and genera related to greater production of butyrate. This result justifies the increase in the concentration of this SCFA in the feces of animals supplemented with DHSC. Furthermore, DHSC supplementation promoted an increase in caspase-3 expression in the 5DHSC group and c-Myc in the 10DHSC group, which may indicate the occurrence of apoptotic events, however, further studies are needed to understand this mechanism.

In conclusion, it is suggested that the consumption of DHSC included in the diet attenuates cellular responses in the early stage of development of preneoplastic lesions through its antioxidant, anti-inflammatory and prebiotic properties. Intervention in the initial phase is relevant, as it helps to reduce the risk of carcinogenesis progression.