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Constituents of *Quercus eduardii* leaf infusion: Their interaction with gut microbiota communities and therapeutic role in colorectal cancer

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ABSTRACT

The infusion of *Q. eduardii* leaves has been used ancestrally for medicinal purposes. We investigated the effect of *Q. eduardii* infusion intake on inflammatory and oncogenic biomarkers in animals induced to have colorectal cancer with DMH. We also investigated its influence on the microbiota, establishing the main microbial metabolites in the intestinal content of the experimental animals. The microbial metabolites associated with greater anti-inflammatory and anticarcinogenic activity were 3́-hydroxyphenylacetic acid (3HPA) and 6́-3-(3,4,5-trimethoxyphenyl)prop-2-enoic acid (TMCA). Finally, the intake of the *Q. eduardii* infusion significantly increased the abundance of the *Erysipelotrichaceae* and *Lachnospiraceae* families, that seems to have a great influence on the antitumor responses detected in the study.

1. Introduction

Colorectal cancer (CRC) is one of the leading causes of death worldwide, after lung and liver cancer, with more positive diagnoses in men than in women (Keum & Giovannucci, 2019). Various factors related to lifestyle have been linked to the development of CRC, among them, 75 % are associated with changes in feeding habits, overweight or obesity, physical inactivity, smoking, and alcohol consumption (Rawla et al., 2019; Balhareth et al., 2019). Additionally, 25 % are associated with the aging colonic mucosa (Noreen et al., 2014; Zhu et al., 2017), a personal history of inflammatory bowel disease (Ou et al., 2015), including ulcerative colitis or Crohn's disease, family history of colorectal cancer or adenomatous polyps and hereditary syndromes.

Most risk factors associated with a lifestyle that promotes CRC also modify the intestinal commensal microbiota being diet one of the most relevant. The microbiota can metabolize fiber and bioactive compounds such as polyphenols that can decrease the incidence of cancer but also protein and fat producing inflammatory and carcinogenic metabolites that promote cancer incidence (O'Keefe, 2016). Therefore, the microbiota and/or its metabolites are closely related to the risk of colon cancer. In fact, molecular mechanisms that link specific metabotypes with colon carcinogenesis have been identified in experimental animal models (Zhu et al., 2014). The heterogeneity of the cancer-associated microbiota may also reflect differences in the host immune-inflammatory response to cancer, which is known to influence prognosis (Flemer et al., 2017). Likewise, mutations in specific genes are associated with the regulation of cell proliferation or DNA repairing systems. Particularly, genes related to the wtn / β catenin signaling pathway are altered in colon cancer, with the consequent involvement in the homeostasis of the intestinal epithelium, regulation of cell differentiation and proliferation, as well as in apoptosis (Testa et al. 2018). On the other matters, alterations in the K-RAS pathway are associated with migration, apoptosis, differentiation and control of the cell cycle. This is the reason why K-Ras mutations are located in approximately 33 % of all cancers, including CRC, where they have been detected in >50 % of colorectal carcinomas and polyps. Specifically, K-Ras mutations occur in 42 % of CRC cases and more frequently in advanced adenomas (Andreyev et al., 2001).

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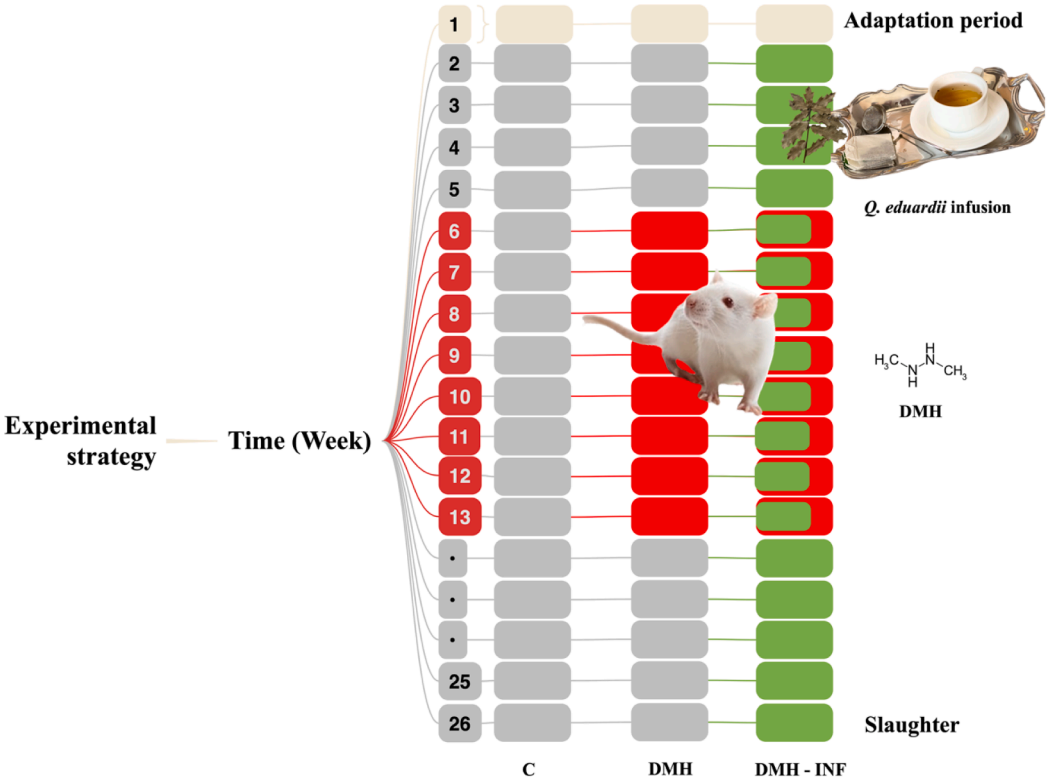

Fig. 1. Scheme of the experimental design.

Various epidemiological studies suggest the use of dietary phytochemicals for the treatment and prevention of CRC, due to their easy availability, low toxicity and ability to modulate signal transduction pathways and cellular processes (Zhao et al., 2018). Various phytochemicals that are constituents of herbal products have been attributed with chemoprotective, antioxidant, anti-inflammatory, cell cycle, autophagy and microbiota modulating properties (Zeng et al., 2016). In recent years, there has been an increase in publications related to these topics, proposing, most of them, the modulation of the microbiota as a new therapeutic target against CRC. Within this framework, the mechanisms by which dietary polyphenols and / or their microbial metabolites exert their action on the pathogenesis and prevention of CRC are highlighted.

In Mexico, infusions prepared from various ancestral resources have traditionally been used, including leaves of red oak species. A previous study has shown that the continued consumption of infusions from oak species, particularly *Quercus sideroxyla*, decreases various colorectal cancer biomarkers in animal models (Moreno-Jimenez et al., 2015). Furthermore, this study has shown that other oak species such as *Q. eduardii*, whose ethnobotanical information supports a medicinal use, are able to decrease intestinal tumorigenesis, however, their abuse of intake promotes adverse effects in the small intestine.

Since the beneficial and adverse effects depend on the dose consumed, in this study, we aimed to evaluate the effects of consuming *Q. eduardii* leaf infusion on colon carcinogenesis at a dose equivalent in humans to one cup of drink per day. To this end, in a murine model of colon carcinogenesis, the profile of the endogenous phenolic metabolites derived from the infusion polyphenols was characterized and the potential changes in the microbiota, as well as the interactions between them, were studied. Likewise, we also addressed the expression of biomarkers associated with the molecular mechanisms involved in the immuno-inflammatory responses associated with intestinal health.

2. Materials and methods

2.1. Reagents

Methanol, ethanol, acetone, acetonitrile, ethyl alcohol, formaldehyde, hematoxylin and eosin (J. Baker), apigenin, quercetin, isoquercetin, luteolin, procyanidin B2, catechin, (*epi*)-catechin, rutin, acacetin, and mangiferin (Sigma Chemical, St. Louis MO, USA). 1,2-dimethylhydrazine (DMH), polyclonal primary antibodies (Cox-1 and Cox-2) and monoclonal primary antibodies (TNF alpha-IgG, NF-κB, IL-8, IL-10, p21, K-Ras, β-Catenin, GADPH); secondary antibodies (mouse anti-goat IgG-HRP, mouse anti-rabbit IgG-HRP, bovine anti-mouse IgG-HRP from the commercial company Santa Cruz Biotechnology, Inc); The Clarity Western ECL immunodetection kit, glycine, tris base, tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS) and 0.45 μm nitrocellulose membrane from Bio-Rad Laboratories (Hercules, CA, USA), ammonium persulfate (Sigma Chemical, St. Louis MO, USA). Test strips for the dry chemistry photometer Spotchem II kidney-2 and Spotchem II Kenshin-2 (Arckray).

2.2. Plant material

The leaves of *Quercus eduardii* were collected on the Durango-Mazatlán free highway km 13.5, in July 2017, taxonomic identification was carried out by botanist Socorro González and the Boucher with number 48,468 of the specimen was placed in the herbarium of the Interdisciplinary Research Center for Integral Development of the Region of the National Polytechnic Institute (CIIDIR), Durango Unit. The leaves were dried at a temperature of 23 ± 2 °C and crushed in an IKA® MF10 knife mill until obtaining a flour with a particle size of 700 μm. The flour was kept in the dark to avoid the degradation of the phenolic components until its use.

2.3. Preparation of Q. eduardii infusion

The infusions were prepared at a concentration of 1 % (w / v), according to Rocha-Guzmán et al., (2012) with slight modifications. 10 g per L of water at a temperature of 80 °C were used, the infusion was subsequently filtered and lyophilized until its chemical characterization by UPLC-ESI⁻QqQ and its use in the colon carcinogenesis model.

2.4. Animals and ethical aspects

Four-week-old male Sprague-Dawley (SD) rats (Rismart S.A. de C.V. México) (135.03 ± 19.64 g) were organized in collective cages in groups of 4 animals per cage and were fed with standard food (Agribrands Purina México S. de R.L. de C.V.) and water *ad libitum*. The animals were kept in light–dark cycles (12 / 12 h) and kept at 24 ± 1 °C and relative humidity of 40–50 %, animals were acclimatized at least 1 week before the starting of the study.

2.4.1. Ethical aspects

The experiments were carried out in accordance with the protocol of use and animal care of the NOM-062-ZOO-1999 based on the federal law of animal health and attending the guidelines of the International Council for Laboratory Animal Science (ICLAS). The research protocol was authorized and supervised by the research committee of the Master of Science in Biochemical Engineering of TecNM/I.T.Durango.

2.4.2. Carcinogenesis model

2.4.2.1. Colon cancer induction and treatment administration. Four-week-old male SD rats (Rismart S.A. de C.V. México) were used. After one week of acclimatization, rats were randomly distributed into four experimental groups: the negative control group (C; n = 9), the positive control treated with DMH (DMH; n = 10), and the experimental group co-treated with infusion and DMH (DMH-INF; n = 10). All the experimental groups received food and water *ad libitum* during the 26 weeks of the experiment. The DMH and DMH-INF groups received alternately in the same week two intraperitoneal (i.p.) injections in the left and right flanks of DMH (21 mg kg of weight^{-1}) from the 6th to the 13th week (Fig. 1). The DMH solution was prepared at a concentration of 4 mg mL^{-1} in sterile distilled water containing EDTA (3.7 mg mL^{-1}) as a stabilizing agent and a pH of 6.5 (adjusted with a NaOH solution). Throughout the experiment, the DMH-INF group was administered by gavage with ~ 410 mg of lyophilized extract dissolved in water, this dose corresponds to the human equivalent dose for a 70 kg person that consumes a 240 mL cup of infusion per day (Shin et al., 2010) (Fig. 1). All animals were weighed once a week, water and food consumption were recorded daily. After a fast of 12 h at the end of the experiment period, animals were anesthetized with a mixture of ketamine and xylazine (100 mg kg^{-1} / 10 mg kg^{-1}) and sacrificed by cardiac puncture. Afterward, the colons of the rats were excised, and the colonic content was collected separately and immediately frozen in liquid nitrogen for metabolic and metagenomic analysis.

2.4.2.2. Serum biochemistry analysis. Blood was collected and injected on vacutainer tubes. To separate serum, blood was allowed to clot for 30 min and then centrifuged at 13000g for 15 min at 4 °C in a Thermo Scientific Labofuge 400R centrifuge (North Caroline, USA). The serum was immediately frozen at −84 °C for further analysis. Levels of aspartate aminotransferase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), cholesterol (*T*-Chol), triglycerides (TG), total protein (*T*-proteins), albumin (Alb), blood urea nitrogen (BUN), uric acid (UA), creatinine were measure on a Spotchem^{TM}-EZ SP-4430 chemistry analyzer (Illinois, USA).

2.4.2.3. Macroscopical and histopathology examination. Tumor classification and other injures found were established in accordance with Piñols (1995). Subsequently, colons were rinsed with sterile physiological saline, opened longitudinally and macroscopical lesions of distal colon section were cut and fixed in 4 % formaldehyde solution for 12 h, embedded in paraffin blocks, and microdissection (5 μm) were processed for subsequent hematoxylin and eosin staining. The histopathological examination was performed by two observers independently. For this analysis, the total sheets obtained were divided into 9 quadrants, to observe and categorize the areas of interest for the experiment. With this procedure, a database of 846 images was obtained with dimensions 1024x768 pixels. Using Motic Imagen Plus 2.0 software, images were taken using an exposure level of 1867.065 (CT-2.00, GCR (Gray component substitution) 0.91, BCR 60.00, GC 1.00, GCA 2.60, BCA 9.00, R 33/239, G 0.42/29.99), the images obtained served us to evaluate the following parameters: mild, moderate and severe inflammation, dysplasia, metaplasia and carcinoma. For the degree of dysplasia, the scale was performed according to the scale established by Riddell et al. (1983).

2.4.2.4. Immunodetection analysis. The distal colon section (50 mg) was dissected from each individual and macerated with liquid nitrogen. Subsequently, the sample was lysed in ultraturrax (IKA-T10basic) using phosphate buffer (1 mM EDTA, 50 mM Tris, 1 mM DTT, 100 mM CH_3CO_2K) and a 1 % protease inhibitor cocktail. The homogenate obtained was separated by centrifugation at 4 °C and 12,000 rpm for 15 min and supernatant was recovered for immunodetection assays.

The protein content of the homogenates was quantified using a modification of the colorimetric method of Bradford (1976). The low molecular weight proteins present in homogenates were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions with sodium dodecyl sulfate (SDS). Afterward, the protein profiles were electro-transferred for 1 h at 100 V to nitrocellulose membranes, which were blocked for 12 h with 10 mL of album solution (2.5 %) in TBS-T (Tris/HCl [20 mM], NaCl [100 mM] and Tween 20 [0.2 % v/v], pH 7.6; the membranes were incubated with the first antibody with a dilution prepared in 10 mL of TBS-T: for GADPH (1:800), COX-1 (1:800) and COX-2 (1:600), for TNF-alpha and NF-kβ (1:800), IL-8 and IL-10 (1:400), K-RAS, β-catenin and p-21 (1:800) for 12 h. Before incubating the secondary antibodies, additional washes were performed on the membranes with TBS-T. The antibodies were diluted at a ratio of 1:2000 in 10 mL of TBS-T, using mouse anti-goat IgG-HRP, anti-rabbit IgG-HRP or anti-mouse IgG-HRP according at primary antibody the membranes were incubated for 2 h. After incubation, the membranes were revealed by chemiluminescence Clarity Western ECL substrate kit (Bio-Rad), the chemiluminescence signal was recorded with the ChemiDoc MP Imaging System (Bio-Rad). The densitometric analysis was performed with TotalLab v8.3 software.

2.5. Determination of colonic microbial metabolites

Colonic content was powdered using liquid nitrogen, subsequently, 40 mg of sample were homogenized with 750 μL of MeOH/HCl/deionized water (79.9/0.1/20.0) in an ultraturrax at 24,000 rpm for 1 min. Suspensions were centrifuged at 13000 rpm for 15 min at 4 °C, the supernatant was recovered. After extraction, supernatant fractions containing metabolites were dried using a CentriVap Labconco (Kansas City, MO, USA) at a temperature of 37 °C, subsequently 200 μL of methanol were added to each sample and filtered, before analysis by UPLC-ESI⁻-QqQ.

2.6. UPLC-ESI⁻-QqQ

The analyses of colonic content samples were carried out by Ultra-High-Performance Liquid Chromatography (Waters Corp., Milford) coupled with a tandem ElectroSpray Ionization-Triple Quadrupole

Table 1
Colonic microbial metabolites explored in intestinal content, in the different experimental groups C, DMH and DMH-INF after of intervention study [assigned acronym].

No.	Compound	Retention time (min)	Transitions
1	2-Hydroxy-2-(3́-hydroxyphenyl)acetic acid [*HMA*]	3.17	167 > 121
2	3́,4-Dihydroxyphenylacetic acid [*DOPAC*]	3.19	167 > 123
3	3,4-Dihydroxybenzoic acid [*PCA*]	3.30	153 > 109
4	4-hydroxybenzoic acid [*PHBA*]	5.29	137 > 93
5	3-hydroxybenzoic acid [*mSA*]	5.92	137 > 93
6	4́-hydroxyphenylacetic acid [*4HPA*]	6.93	151 > 107
7	2-hydroxy-2-phenylacetic acid [*MA*]	6.96	151 > 107
8	3́-hydroxyphenylacetic acid [*3HPA*]	7.01	151 > 107
9	3-(3,4-dihydroxyphenyl)-propionic acid [*DHCA*]	7.09	181 > 137
10	4-Hydroxy-3-methoxybenzoic acid [*VA*]	7.49	167 > 152
11	3́,4́-Dihydroxycinnamic acid [*CA*]	7.56	179 > 137
12	5-(3́,4́,-dihydroxyphenyl)-γ-valerolactone [*DHPV*]	7.60	207 > 163
13	5-(4́-hydroxyphenyl)-γ-valerolactone [*5,4HPV*]	8.49	191 > 147
14	4́-hydroxy-3́-methoxyphenylacetic acid [*HVA]*	8.84	181 > 137
16	5-(3́-hydroxyphenyl)-γ-valerolactone [*5,3HPV*]	9.86	191 > 147
17	4́-Hydroxycinnamic acid [*CouA*]	9.90	163 > 119
19	3-(4́-Hydroxyphenyl)propanoic acid [*DAT*]	10.70	165 > 121
20	3,4-dimethoxyphenylacetic acid [*DMPA*]	10.91	195 > 136
21	4-hydroxy-5-(3́,4́-dihydroxyphenyl) valeric acid [*HDHPV*]	11.44	225 > 163
22	4́-Hydroxy-3́-methoxycinnamic acid [*FA*]	11.45	193 > 134
23	4-hydroxy-5-(3́-hydroxyphenyl)valeric acid [*HHPV*]	11.65	209 > 147
24	3́-Hydroxycinnamic acid [*m-CouA*]	11.88	163 > 119
25	3-(3́-hydroxyphenyl)propionic acid [*HPPA*]	12.01	165 > 121
26	4́-methoxyphenylacetic acid [*MPA*]	12.03	165 > 106
27	Benzoic acid [*BA*]	12.22	121 > 77
28	2-hydroxybenzoic acid [*SA*]	12.86	137 > 93
29	Ellagic acid [*EA*]	12.98	301 > 229
30	4-hydroxy-5-(3́,4́5-trihydroxyphenyl) valeric acid [*HTHPV*]	14.41	241 > 179
31	Iso Urolithins (6H-Dibenzo[*b,d*]pyran-6-one)3-Hydroxy-urolithin [*IsoUroB*]	14.75	211 > 167
32	5-(3́,4́,5́-trihydroxyphenyl)-γ-valerolactone [*THPV*]	15.11	223 > 179
33	4-Methoxybenzoic acid [*MBA*]	15.40	151 > 107
34	(*E*)-3-(3,4,5-trimethoxyphenyl)prop-2-enoic acid [*TMCA*]	15.91	237 > 103
35	Phenylpropionic acid [*PPA*]	15.92	149 > 105
36	5-(3́,4́-Dihydroxyphenyl)-γ -valerolactone -O-sulphate [*DHPV-sul*]	15.92	287 > 207
37	4-hydroxy-5-(phenyl)valeric acid [*HPVA*]	15.93	193 > 175
38	(*epi*)-catechin-O-glur [*EC glur*]	15.94	465 > 289
39	3,8,9-Trihydroxy-urolithin	15.94	243 > 171
41	5-(3́,4́-Dihydroxyphenyl)-γ -valerolactone -O-glur [*DHPV-glur*]	15.96	383 > 207
42	Urolithin-3-glucuronide [*UroBglur*]	15.97	387 > 211
43	3́-Hydroxy-4́-methoxycinnamic acid [*IsoFA*]	15.97	193 > 134
44	(*epi*)-catechin-O-sulphate [*EC sul*]	15.97	369 > 289
45	O-methyl-(*epi*)-catechin-O-glucuronide [*MEC glur*]	15.98	479 > 303
46	O-methyl-(*epi*)-catechin-O-sulphate [*MEC sul*]	15.99	383 > 303
47	5-(3́-Methoxyphenyl)-γ-valerolactone-O-glucuronide [*MHPV glur*]	16.00	397 > 221
48	3,8,9,10-Tetrahydroxy-urolithin [*UroM6*]	16.02	259 > 213
49	3-Hydroxy-urolithin [*UroB*]	16.03	211 > 167
50	5-(3́-Methoxyphenyl)-γ-valerolactone-O-sulphate [*MHPV sul*]	16.03	301 > 221
51	3,4,5-trimethoxybenzoic acid [*TMBA*]	16.09	211 > 167

*retention time in QqQ instrument.

(Xevo TQS, Waters Corp., Wexford). For the determination of colonic microbial metabolites an Acquity UPLC BEH-C18 column [2.1X100mm, 1.7 μm], (Waters Corp., Milford) with mobile phase A (2 % acetic acid in water) and B (2 % acetic acid in acetonitrile) was used and operated at 40 °C. The linear gradient at a flow rate of 350 μL/min was (% mobile phase B, time (min)): (0.10, 0.00); (0.10, 1.50); (16.30, 11.17); (18.40, 11.50); (18.40, 14.00); (99.90, 14.10); (99.90, 15.50); (0.10, 15.60); (0.10, 18.00). The sample volume injected was 5 μL and spectrometric conditions were as follows: capillary voltage, 3 kV; desolvation temperature, 400 °C; source temperature, 130 °C; cone gas flow, 60L/h; collision gas flow, 0.13 mL/min; MS mode collision energy, 5.0; and MS/MS mode collision, 20.0. For identification purposes, data were collected in the multiple reaction monitoring (MRM) mode. MRM transitions for colonic microbial metabolites are shown in Table 1.

2.7. Microbiota profiling bioinformatics analysis

Bacterial DNA was extracted from 100 mg of colonic content using the commercial E.Z.N.A® Stool DNA Mini Kit (Omega, Biotec, Madrid, Spain) and the Bullet Blender® sample homogenizer. The concentration and purity of the DNA will be measured using a Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) and an FP-8300 spectrofluorimeter (Jasco, Tokyo, Japan) according to Moreno-Pérez et al. (2018). For the C and INF groups samples were pooled in four batches (of two samples each one, n = 4) whereas for the DMH and DMH-INF eight samples of each group were analyzed (because not all animals had colon content). Metagenomic analysis of the microbiota was performed using 16 s rRNA sequencing. The variable regions V3-V4 of the rRNA were amplified with the primers specific for that region (Klindworth et al., 2013) and sequenced on an Illumina Miseq sequencer (Illumina, Inc. USA). Data quality filtering was performed according to Caporaso et al., (2010). The reads were assigned to operational taxonomic units (OTUs) using the QIIME 2.0 tool with which the sequences are checked against the SILVA132 database. The reads were assigned to an OTU when the identity to the sequence was greater than or equal to 97 %. Sequences that did not match any reference were discarded. The β-diversity microbiota indexes (Bray-Curtis and Jaccard) were analyzed by the Permutation Based Analysis of Variance (PERMANOVA). Alpha-diversity was analyzed using the Kruskal-Wallis test whereas Linear discriminant analysis (LDA) coupled with effect size (LEfSe) was performed to identify bacterial taxa differentially represented between groups (Segata et al., 2011).

2.8. Statistical analysis

The results were expressed as the mean and / or the median standard deviation. The data were subjected to one-way ANOVA, and the differences between the treatments were determined by comparing the mean values using ranges and the comparison of the median with the Kruskal-Wallis method of multiple comparisons with 95 % confidence, in the MetaboAnalyst 5.0 for statistical analysis [one factor].

3. Results and discussion

Regardless of the treatment, there was a 100 % of survival in all experimental groups. The evaluation of the clinical signs of the different groups did not show any alteration, being the behavior of the animals normal. The body weight gain recorded during the 26-week experimental period, shows that no significant difference is observed between C group (1.54 ± 0.2 g rat^{-1} day^{-1}) and DMH group (1.32 ± 0.1 g rat^{-1} day^{-1}), or between DMH group and DMH-INF group (1.24 ± 0.2 g rat^{-1} day^{-1}); however, a significant difference was recorded between C and DMH-INF groups.

In recent years, the consumption of water infused with herbs and leaves has gained interest among the population. Rocha-Guzmán et al., (2012) has reported that herbal infusions obtained from *Quercus eduardii*

Table 2
Serum biochemical parameters in SD rats treated with DMH and *Q. eduardii* leaf infusion.

Parameter	Reference value	C	DMH	DMH-INF
Hepatic enzymes				
AST (UI L^{-1})	(0.0 – 276.2)	22.0 – 216.0[a]	7.0 – 124.0[a]	42.0 – 110.0[a]
ALT (UI L^{-1})	(10.0 – 83.1)	10.0 – 50.0[a]	10.0 – 50.0[a]	11.0 – 34.0[a]
GGT (UI L^{-1})	(0.0 – 83.1)	10.0 – 159.0[a]	10.0 – 18.0[a]	10.0 – 10.0[a]
Lipid parameters				
T-Chol (mg dL^{-1})	(50.0 – 95.2)	50.0 – 63.0[a]	50.0 – 106.0[a]	50.0 – 68.0[a]
TG (mg dL^{-1})	(20.0 – 115.0)	25.0 – 55.0[ab]	25.0 – 74.0[b]	25.0 – 44.0[a]
Kidney parameters				
T-proteins (g dL^{-1})	(1.0 – 7.9)	2.0 – 3.8[a]	2.0 – 6.2[a]	2.0 – 6.1[a]
Alb (g dL^{-1})	(0.4 – 2.4)	1.0 – 2.8[a]	1.0 – 3.4[a]	1.0 – 3.4[a]
BUN (mg dL^{-1})	(4.0 – 22.0)	7.0 – 27.0[a]	5.0 – 19.0[a]	10.0 – 21.0[a]
UA (mg dL^{-1})	(1.0 – 3.7)	1.0 – 3.7[a]	1.0 – 1.6[a]	1.0 – 1.4[a]
Creatinine (mg dL^{-1})	(0.4 – 1.5)	0.3 – 0.6[a]	0.3 – 0.6[a]	0.4 – 0.8[b]

AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: Gamma-glutamyl transpeptidase; *T*-cho: total cholesterol; TG: triglycerides; Bun: blood urea nitrogen. Different line literals denote statistical differences. Statistical analysis was carried out using analysis of variance (ANOVA) by ranges, using the Kruskal-Wallis method with multiple comparisons test with 95% confidence, using Statistica v12.5 software (StatSoft, Tulsa, OK).

leaves exhibit antioxidant activity, while Moreno-Jiménez has shown an important anti-inflammatory effect in HT-29 cells. This source of oak has ethnobotanical antecedents that report its use for medicinal purposes (Luna-José et al., 2003). There are no antecedents that indicate adverse effects in humans, associated with the consumption of infusions made with this source. Although herbal remedies are perceived by consumers as harmless, some often cause liver damage (Teschke and Eickhoff, 2015) and kidney damage (Vanderperren et al., 2005), therefore, they are not completely harmless. For this reason, establishing those doses in which no adverse effects are observed is important in the consumption of infusions. In our study, the analysis of the biochemistry parameters (Table 2) indicated that there were no significant differences between the groups and that the observed variations in the lipid, hepatic and kidney-related parameters lack of pathological relevance since the results are within the normal ranges established for the SD strain (Alemán et al, 1998). In general, it can be stated that the consumption of *Q. eduardii* infusion in our experimental model in equivalent doses to the intake of one cup per day in humans, does not lead to modifications or alterations in the liver or kidney that could be interpreted as toxic or signs of damage, although in the DMH-INF group a tendency to increase in creatinine levels was detected, these are within the ranges reported for the SD strain. In the histopathological evaluation, the histological structure of the C group was established as a reference parameter. The colon structure has a normal cytoarchitecture, without alterations of the mucosa, submucosa, muscular and serous layers (Fig. 2.a). Fig. 2.a.1 shows in detail the integrity of the mucosa, while Fig. 2.a.2 shows the crypts of Lieberkühn. In contrast, treatment with the DMH carcinogen induced the presence of histological irregularities in the distal area of the colon, where areas with metaplasia, dysplasia and adenocarcinoma were identified (Fig. 2.b). Fig. 2.b.1, shows the presence of erosions and changes in the superficial epithelium of the mucosa accompanied with areas where cell proliferation is observed indicating metaplasia in the DMH group. The DMH treatment also induced dysplasia, with visible structural abnormalities and an atypical cytoarchitecture in the epithelium (Fig. 2.b.2) and several adenocarcinomas in which hyperproliferation and loss of cell differentiation are seen (Fig. 2.b.3).

Of particular importance is the colon histology detected in DMH-INF, in which areas with mild inflammation were observed (Fig. 2.c), with loss of normal architecture of the Lieberkühn crypts (Fig. 2.c.1), combined with normal segments (Fig. 2.c.2); additionally, we also detected the presence of polyps (Fig. 2.d) but in a lower proportion than those detected in the DMH group. The histopathological evaluation revealed that the colon of SD rats from the control group (C) had a normal histoarchitecture without signs of colitis.

Table 3
Percentage of incidence of mild, moderate, and severe inflammation found in colon samples of SD rats treated with DMH and *Q. eduardii* infusion.

Treatment	*n*	Mild inflammation	Moderate inflammation	Severe inflammation
C	9	10.5(20/189*)[a]	0.00[a]	0.00[a]
DMH	10	17.0(46/270*)[b]	12.5(34/280*)[b]	14.8(40/270*)[b]
DMH-INF	10	19.2(45/234*)[b]	0.00[a]	0.00[a]

Different literals in columns denote statistical differences. Statistical analysis was carried out using analysis of variance (ANOVA) by ranges, using the Kruskal-Wallis method with multiple comparisons test with 95% confidence, using Statistica v12.5 software (StatSoft, Tulsa, OK). * denotes the number of sheets.

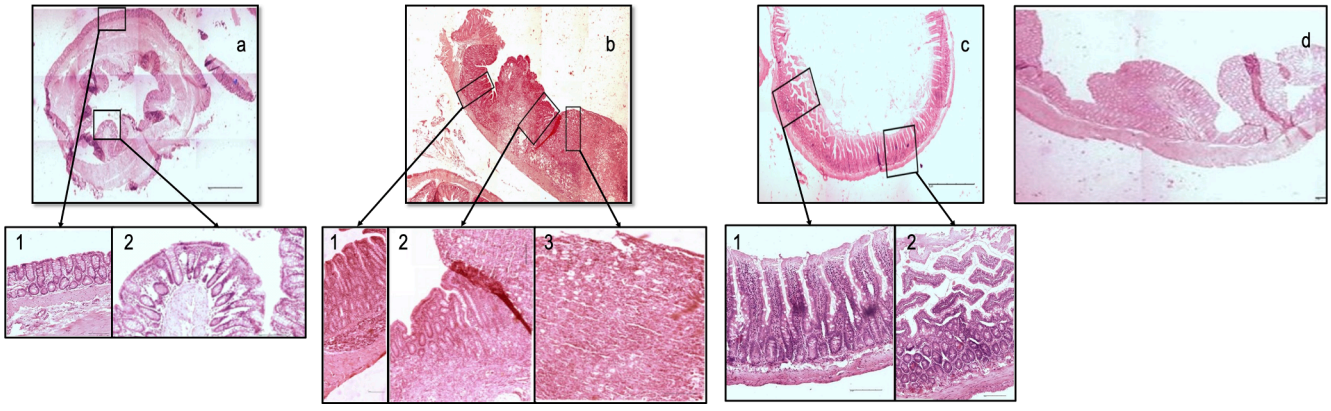

Fig. 2. Panoramic reconstruction of the longitudinal section of the colon of Sprague Dawley rats stained with hematoxylin and eosin technique observed with a 5x objective. a) C, b) DMH, c) and d) DMH-INF. Observations with 40x objective: a.1) histological image of normal epithelium in the distal colon; a.2) histological image of the four layers (mucosa, submucosa, muscular and serous characteristic of the normal cytoarchitecture of colon epithelium); b.1) metaplasia of the distal colon; b.2) dysplasia of the distal colon; b.3) distal colon carcinoma characteristic of the pathophysiology of colon cancer; c.1) normal epithelium of distal colon; c.2) inflammation characterized by the loss of the normal architecture of the crypts; d) polyps located in the apical zone of the epithelium developed towards the lumen.

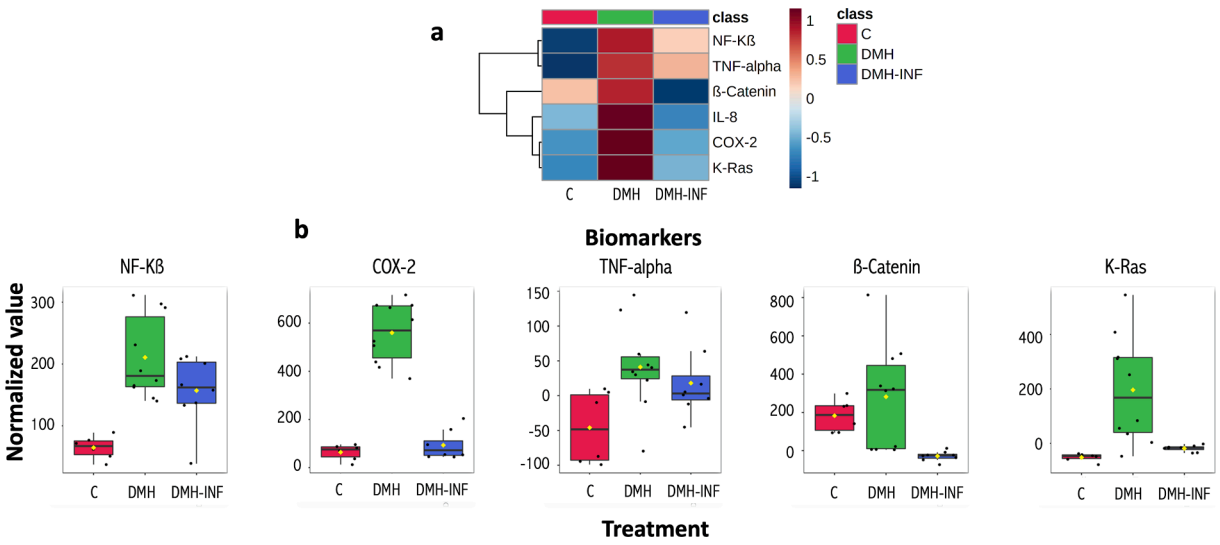

Fig. 3. a) Hierarchical clustering heatmaps and b) main inflammatory and oncogenic biomarkers identified whose relative protein level was significantly different between C, DMH and DMH-INF groups. Important features in biomarkers were established with the analysis of variance (ANOVA) by ranges and the comparison of the median, using the Kruskal-Wallis method of multiple comparisons with 95% confidence.

In summary, when examining the incidence of inflammation (Table 3), a level of mild inflammation is detected in the distal colon tissue of group C, in contrast, mild to severe inflammation levels were detected in the experimental group treated with the DMH carcinogen, being statistically different from the C group. It is important to highlight that the consumption of the infusion by the animals treated with the carcinogen (DMH-INF) completely reduces the inflammation levels detected in the DMH group.

In addition to this study, we explored agonist and antagonist effects associated with the treatment of the Q. *eduardii* leaf infusion, on biomarkers of inflammatory effect such as NF-kB, COX-2, TNF-alpha and IL-8. Fig. 3a shown the clustered heatmap analysis of relative protein expression of the inflammatory biomarkers, showing in general, a high agonist effect of DMH on biomarkers of inflammatory effect, a response that is attenuated in the DMH-INF group, demonstrating the antagonist effect of bioactive compounds present in the INF towards pro-inflammatory processes. Given that inflammation is fundamental to the carcinogenesis process, its role in the present study was evaluated the NF-kB signaling pathway, these regulates the expression of downstream mediators, including those encoding cyclooxygenase-2 (COX-2), cytokines and chemokines, and participates in inflammasome regulation a critical role in the regulation of cell survival and cell death (Man & Kanneganti, 2015).

In our study, the strongest agonist effect associated with the action of the DMH was generated in the expression of COX-2 ($\chi = 17.01$), an inducible enzyme with carcinogenic properties that is active in inflamed and transformed tissues. So, its increased levels are normally low in healthy individuals, but it tends to increase rapidly in response to inflammation processes, abnormal cell proliferation, angiogenesis or metastasis (Owczarek & Lewandowska, 2017). In this sense, the oral administration of extractables obtained from the equivalent of the consumption of a cup of INF, in animals treated with DMH results in a

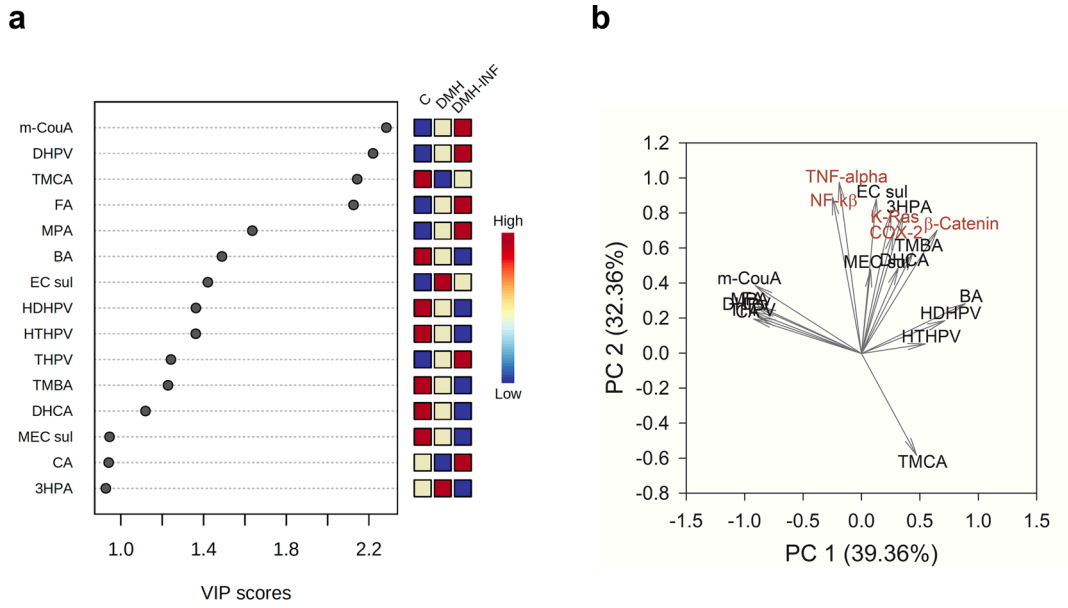

Fig. 4. Important features identified by a) partial least square-discriminant analysis (PLS-DA) of microbial metabolites (15 principal component score plots); b) Principal Component Analysis (PCA) of the interactions between the microbial metabolites and inflammatory and oncogenic biomarkers.

Table 4
Incidence percentages of polyps, metaplasia, dysplasia and carcinomas detected in the colon of SD rats treated with DMH and *Q. eduardii* infusions.

Treatment	*n*	Polyps (%)	Metaplasia (%)	Dysplasia (%)	Carcinoma (%)
C	9	0.00[a]	0.00[a]	0.00[a]	0.00[a]
DMH	10	37.0(28/270*)[b]	11.5(31/270*)[b]	5.5(15/270*)[b]	10.0(27/270*)[b]
DMH-INF	10	4.3(10/234*)[c]	0.00[a]	0.00[a]	0.4(1/234*)[c]

Different literals in columns denote statistical differences. Statistical analysis was carried out using analysis of variance (ANOVA) by ranges, using the Kruskal-Wallis method with multiple comparisons test with 95% confidence, using Statistica v12.5 software (StatSoft, Tulsa, OK). * denotes the number of sheets.

significantly antagonist effect towards COX-2 expression. An important feature identified by One-way ANOVA ($p < 0.05$) indicates a significant difference of protein expression relative of NF-kB ($\chi = 12.26$) and TNF-alpha ($\chi = 7.86$) biomarkers, without finding a significant difference in the level of IL-8 recorded in the different groups (Fig. 3b).

Based on our results we could postulate that phenolic-derived microbial metabolites produced by the gut microbiota might mediate anti-inflammatory responses. Principally flavonoids, as catechin and (*epi*)-catechin gallate and hydrolysable tannins, described with inhibitory effect on the NF-kB pathway and COX-2 expression, respectively (Sujuan et al. 2020). To evaluate the relevance of microbial metabolites in the experimental groups, the Fig. 4a shows important features identified by partial least square-discriminant analysis (PLS-DA), associated with the 15 main microbial metabolites detected by UPLC-ESI$^-$-QqQ. To discern the possible role of polyphenol metabolites in the observed inflammatory effects, a principal component analysis was performed. Fig. 4b shows the main interactions between these metabolites and the responses associated with biomarkers of inflammatory and oncogenic effect. So, the main agonist interaction observed, was related with the expression of COX-2 and 3HPA ($R^2 = 0.833$), while the main antagonist response of this inflammatory biomarker was with TMCA ($R^2 = -0.651$). Studies conducted by Karlsson et al. (2005) indicate that aqueous extractable metabolites obtained from human feces are abundant in 3HPA. These researchers showed that these aqueous extracts inhibit COX-2 protein levels in colonic HT-29 cells stimulated with TNF-alpha. It is convenient to note that the chemical composition of the *Q. eduardii* infusion has been documented by García-Villalba et al., (2017). Their studies indicate that the *Q. eduardii* infusion is a rich source of flavonols such as glycosylated derivatives of quercetin. Another important group in this beverage are the procyanidins, typically composed of catechin, gallocatechin, and epicatechin gallate. The catabolism of these intake biomarkers leads to the production of microbial metabolites such as 3HPA (Dias et al., 2022). Although the agonist responses found in our research are contradictory to the antagonist responses that 3HPA has shown in HT-29 cells induced to inflammatory processes with TNF-alpha (Karlsson et al., 2005), it must be taken into consideration that even when experimenting with transformed cells derived from human tissue, are important for establish the mechanisms of action of compounds with presumed bioactivity, they do not necessarily represent the responses that occur in animals.

In complement, in our study TMCA showed antagonist effects towards inflammatory biomarkers NF-kB ($R^2 = -0.825$) and TNF-alpha ($R^2 = -0.709$). TMCA is a cinnamic acid substituted by multi-methoxy groups and their esters and amide derivatives have been reported to show anti-inflammatory activity through the targets including TNF alpha and NF-kB (Zhao et al., 2019).

To determine the influence of treatment with *Q. eduardii* leaf infusion in animals induced to colorectal cancer with DMH, the incidence of polyps, metaplasia, dysplasia and carcinoma was analyzed in the distal colon and significant differences were detected between the different experimental groups (Table 4). Normal histoarchitecture was observed in the C group, in contrast, the experimental group DMH showed significant occurrence of polyps (10.3 %), metaplasia (11.5 %), dysplasia (5.5 %) and carcinoma (10.0 %). Of particular importance is the fact that consumption of the *Q. eduardii* infusion significantly reduces the presence of polyps (58.2 %) and carcinomas (96.0 %) in the DMH-INF group, in which the presence of metaplasia and dysplasia zones was no detected.

During the malignant transformation from adenoma to carcinoma, the Wnt/β-catenin pathway stimulates the transcriptional activity of the c-MYC oncogene. Fig. 3a shown the clustered heatmap analysis of relative protein expression of the oncogenic biomarkers (β-catenin and K-Ras), showing in general, a higher level of expression in the DMH group, a response that is attenuated in the DMH-INF group. In the Fig. 3b it is indicated that the relative protein levels of β-catenin ($\chi = 14.08$) is significantly different between experimental groups, with a significant decrease in the expression levels of this biomarker in the DMH-INF group. The β-catenin gene is mutated in most colon cancer cases (Shang et al., 2017). Similarly, RAS mutations are found in 50 % of colorectal cancer cases, leading to hyperplasia and dysplasia (Anwar et al., 2018). K-Ras mutations are associated with adenoma enlargement and dysplasia, suggesting that they may be predispose the early growth disorder in tumorigenesis. The results obtained in DMH group coincide with that reported in the literature that details that DMH treatment results in a high-frequency mutation (~60 %) of K-Ras in colorectal adenomas in the rat model. Our studies indicate that particularly, the effects of *Q. eduardii* infusion generated significant changes K-Ras expression ($\chi = 14.08$). The results indicate that the administration of the *Q. eduardii*

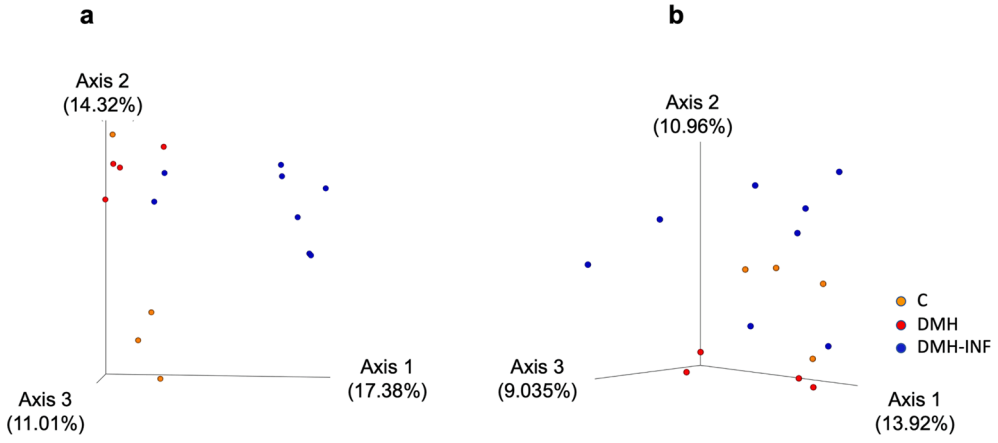

Fig. 5. Community beta-diversity indexes. A) Bray-Curtis index and b) Jaccard index.

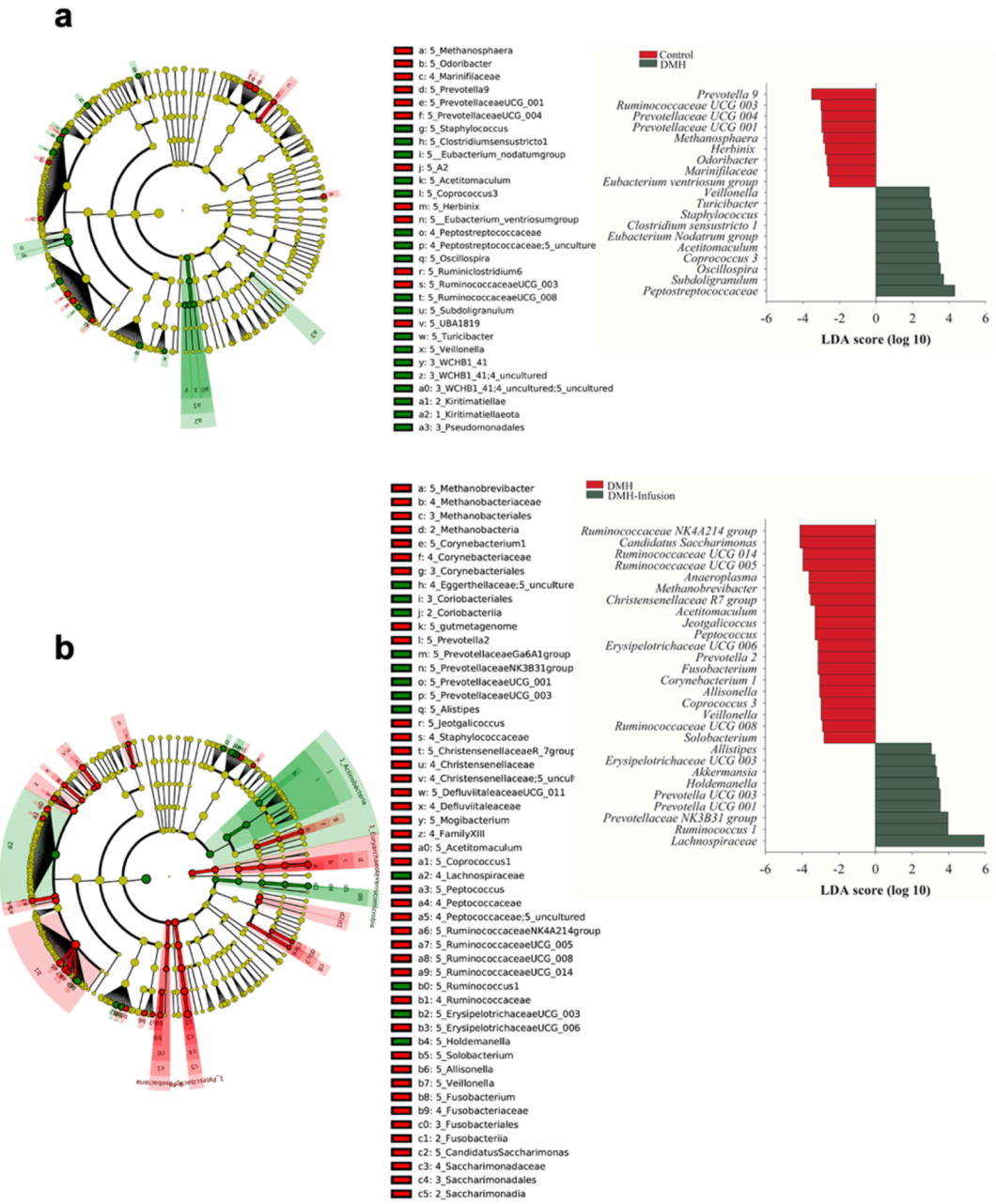

Fig. 6. LDA score generated by LefSe indicates differences in bacteria and Archae taxa between different treatments. a) represents the LDA score when comparing the group induced to tumorigenesis with DMH against the control and b) shown the LDA score when comparing experimental group treated with DMH and co-treated with infusion, compared to the group treated with DMH. Only the taxa meeting a significant LDA threshold value > 2.5 are shown.

infusion decreased the expression of β-catenin with a significant effect on K-Ras. These results are related to the cooperation described between both pathways (Moon et al., 2014), where it is established that the accumulation of β-catenin by mutation of APC, Axin-GSK3B in the WNT/β-catenin pathway generates the activation of Ras promoting the tumorigenesis process. Furthermore, like what was observed in anti-inflammatory responses, a high correlation of the 3HPA metabolite with relative protein levels for β-catenin ($R^2 = 0.906$) and K-ras ($R^2 = 0.984$) was detected.

One of the mechanisms that can be underlying the observed effects is the modification of the gut microbiota by the *Q. eduardii* infusion. Although herbal infusions could be a good option as adjuvants in cancer treatments, few studies have addressed their effects on the modulation of the microbiota and its production of polyphenol-derived metabolites. In our study microbiota beta-diversity analysis showed significant differences in the Bray-Curtis dissimilarity index ($p = 0.001$) showing that the microbiota of the INF-DMH group was significantly different from C ($q = 0.009$) and DMH ($q = 0.006$) groups and a trend but not significant between the C and DMH groups ($q = 0.107$) (Fig. 5). When the Jaccard index was analyzed, all groups were significantly different between them ($q = 0.046$) (Fig. 5). The alpha-diversity analyses (observed features, Shannon index, Pielou evenness index and Faith Phylogenetic Diversity index did not show any significant difference between groups. These results indicate that the infusion treatment produces changes in the structure of the microbiota, especially when we take into account the absence/presence of species (Jaccard index), but does not modify the diversity of species, with no increase or decrease in the number of species.

LEfSe was used to detect class discriminating OTUs between the different experimental groups at genus level. When C and DMH groups

were compared it can be observed that the DMH group showed a decreased abundance in several members of the *Prevotellaceae* family and in the *Prevotella 9* genus as well as in the *Ruminococcaceae UCG003* taxon (Fig. 6a). These observations are in line with those previously described in which the abundance of the *Prevotella* taxa have been considered a key phylotype that contributes to the dysbiosis of mucosa-associated microbiota in patients suffering colorectal cancer (Gao et al., 2015; Bundgaard-Nielsen et al., 2019; Zorron et al., 2020). Moreover, the lower abundance in the members of the *Ruminococcaceae* family has been also associated with colon cancer (Chen et al., 2012; Sheng et al. 2020). An unidentified genus of the *Peptostreptococcaceae* family (LDA score 4.352) and the *Subdoligranulum* genus (LDA score 3.704) were the most abundant taxa in the DMH group. These two phylotypes of the microbiota have previously been associated with colon cancer (Wang et al., 2018; Youssef et al., 2018).

A final comparison was made between the experimental group with DMH-induced colorectal cancer and the group induced with DMH and co-treated with oak infusion (Fig. 6b). The major increase detected in this analysis occurred in an identified genus of the *Lachnospiraceae* family (LDA score 5.918), this is a group that significantly is depleted in colorectal cancer (Park et al., 2021). According to the results observed in the INF group, genera classified within the *Erysipelotrichaceae* family were increased in the intestinal microbiota because of co-treatment with oak infusions, such as the case of *Erysipelotrichaceae UCG 003* (LDA score 3.259) and *Holdemanella* (LDA score 3.452), to this genus belong species such as *Holdemanella biformis* that are recognized as anti-tumor strains (Perillo et al., 2020).

Several genera within the Bacteroidetes phylum were meaningfully increased in comparison to the DMH group, among these, *Prevotellaceae UCG 003* (LDA score 3.250) and *UCG 001* (LDA score 3.534), followed by the predominant shifting of *Prevotellaceae NK3B31* group (LDA score 3.945), genera that are strongly and negatively correlated with colorectal cancer traits (Jiang et al, 2020). In this comparison, an increase in the *Akkermansia* genus was detected, as detected in the group that consumed the oak infusion. The most conspicuous decrease occurred in the *Ruminococcaceae* NK4A214 group (LDA score −4.110), which presence is increased in patients suffering from colorectal cancer (Kim et al., 2020).

It is well documented that the composition of the gut microbiota depends on various factors such as diet. Dietary polyphenols are usually present as glycosides. This characteristic influences their low bioavailability; however, this condition makes them reach the colon where they are transformed by the intestinal microbiota with consequent production of microbial metabolites through hydrogenation, dehydroxylation, demethylation reactions, among others that influence their bioactivity. For instance, in this study it has been established that the constant consumption of *Q. eduardii* infusion promotes the increase of the *Lachnospiraceae* family. This family is closely related to O-glycosidase activity, C-Ring cleavage, dehydroxylation and demethylation reactions within the phenolic family such as flavonols, flavanones, dihydrochalcones, flavan-3-ols, tannins, lignans and hydroxycinnamates (Cortés-Martín et al., 2020). Therefore, it is inferred that the main microbial metabolites with influence on inflammatory and oncogenic biomarkers could be derived from the catabolic activity of this family.

4. Conclusions

The role of polyphenols is still uncertain in the case of diseases associated with the colon since we can consider them as biomarkers of intake highly dependent on the action of the microbiota. Treatment in environments that promote colorectal cancer with the infusion of *Q. eduardii* promote the adaptation of *Lachnospiraceae* and *Erysipelotrichaceae*, families that are depleted in colorectal cancer, and that seems to have a great influence on the antitumor responses detected in the study.

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CRediT authorship contribution statement

M.R. Moreno-Jiménez: Funding acquisition, Methodology, Investigation, Writing – review & editing. **N.E. Rocha-Guzmán:** Supervision, Funding acquisition, Conceptualization, Validation, Formal analysis, Writing – original draft. **M. Larrosa:** Conceptualization, Methodology, Investigation, Writing – review & editing. **C. Bressa:** Formal analysis. **C. A. Segura-Sánchez:** . **A. Macías-Salas:** Methodology, Investigation. **J. O. Díaz-Rivas:** . **P. Flores-Rodríguez:** Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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