THAISA AGRIZZI VEREDIANO

EFFECT OF BLACK CORN (*Zea mays* L.) ON ADIPOGENESIS, INFLAMMATION, OXIDATIVE STRESS, AND INTESTINAL HEALTH *IN VIVO*

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

Adviser: Hércia Stampini Duarte Martino

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ABSTRACT

VEREDIANO, Thaisa A, D.Sc., Universidade Federal de Viçosa, February, 2023. **Effect of black corn (*Zea mays* L.) on adipogenesis, inflammation, oxidative stress and intestinal health *in vivo***. Adviser: Hércia Stampini Duarte Martino. Co-advisers: Maria Cristina Dias Paes, Elad Tako, Mariana Grancieri and Barbara Pereira da Silva.

Black corn (*Zea mays* L.) is a type of colored corns, which shows different colors depending on its chemical composition. Over the past years, colored corn has been targeted for investigation due its bioactive components. Black corn is a source of anthocyanins, which is a water-soluble pigment responsible to the black-purple color. Anthocyanin is a subclass of flavonoids, and its consumption is associated with health promoting effects. Anthocyanin exerts antioxidant and anti-inflammatory effects, besides to modulate the intestinal function. However, anthocyanin shows a different effect depending on its origin, food matrix, or if it is isolated as an extract. The black corn TO 002 belongs to the Germplasm Activate Bank from Embrapa and showed a promising composition of phenolic compounds and anthocyanin. However, the biological effects of this genotype *in vivo* have never been tested. Therefore, we hypothesized that the black corn have a health-promoting effect in adipogenesis, inflammation, oxidative stress and on intestinal function. **Objectives:** To critically review the effects of anthocyanin supplementation on intestinal parameters in animal studies (Paper 1); To evaluate the effects of black corn whole flour associated with a high-fat diet in the adipogenesis, inflammation, oxidative stress and on the modulation of intestinal function (Paper 2 and 3); To investigate the effects of black corn soluble extract on the intestinal functionality, morphology and microbiota *in vivo* (Paper 4); To analyze the effects of black corn anthocyanin-rich extract on intestinal functionality, morphology and microbiota *in vivo* (Paper 5). Therefore, the general objective of this work was to investigate the effect of black corn (*Zea mays* L.) on the adipogenesis, inflammation, oxidative stress and on the intestinal health in animal models. **Methods:** *Paper 1*: For the systematic review, articles were reviewed following the PRISMA guidelines. The data search was performed at PubMed, Cochrane and Scopus for experimental studies, which evaluated the supplementation of anthocyanin on intestinal parameters. For the original studies: *Paper 2 and 3:* thirty adult male C57BL/6 mice were randomly divided into 3 groups (n=10/group): NC: normal control – AIN-93 M diet; HF: high-fat diet; HFC: high-fat diet + black corn whole flour (20%), receiving the diets for 8 weeks. The intestinal function was assessed in the content from cecum at the end of the experiment, and gut microbiota

composition was evaluated by 16S ribosomal gene sequencing. *Paper 4*: The biological experiment was conducted by the intra-amniotic approach (*Gallus gallus*). For this, the eggs were divided into 4 groups (n= 6 - 10/group): No Injection; 18 MΩ H_2O Injection; 5% black corn soluble extract (BCSE); 15% BCSE. The intra-amniotic administration was performed at the day 17 of embryonic incubation. Sample collection and evaluation of intestinal functionality, morphology and gut microbiota were performed after the hatching (day 21). *Paper 4:* The biological experiment was conducted by the intra-amniotic approach (*Gallus gallus*). For this, the eggs were divided into 4 groups (n= 8/group): No Injection; 18 MΩ H_2O Injection; 5% black corn anthocyanin-rich extract (BCE); cyanidin-3-glucoside (C3G). The intra-amniotic administration was performed at the day 17 of embryonic incubation. Sample collection and evaluation of intestinal functionality, morphology, and gut microbiota were performed after the hatching (day 21). **Results:** *Paper 1:* 27 studies in animal's models were included in the systematic review. The anthocyanin supplementation promoted an enrichment of Bacteroidetes abundance and a decrease of Firmicutes. Further, it improved short chain fatty acids (SCFA) production, which resulted in a lower pH and intestinal permeability, and an increase of goblet cell (GC), tight junction protein expression, and improvements at the villi morphology. *Paper 2 and 3:* HFC animals improved the total antioxidant capacity and demonstrated lower adipogenesis by showing a decrease at adipocyte number and length by downregulating sterol regulatory element-binding protein-1c (SREBP-1c) and peroxisome proliferator-activated receptor-γ (PPARγ). The consumption of HFC demonstrated to promote hepatic β-oxidation by downregulating the hepatic gene expression of SREBP-1c and acetyl CoA carboxylase 1. Further, the HFC intake prevented disorder at the intestinal function promoted by a high-fat diet. Animals fed a HFC diet had higher abundance of the genus *Ruminococcus*, *Roseburia*, and *Provotellacea_UCG-001*, and lower abundance of the genus *Bacteroides* and *Faecalibaculum*. These changes were observed with no changes at SCFA concentration, and with an increase at goblet cell (GC) proliferation. *Paper 4:* The intra-amniotic administration of BCSE (5 and 15%) demonstrated an anti-inflammatory effect by downregulating the gene expression of duodenal inflammatory biomarker: tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL6), and the transcriptional nuclear factor kappa beta (NF-κB). Further, the BCSE increased the relative abundance of cecal *E. coli* and *Clostridium* and improved the duodenal morphology by increasing GC number and size and Paneth cell number. Further, the BCSE demonstrated potential to improve the physical barrier integrity by upregulating the gene expression of AMP- activated protein kinase (AMPK) and caudal-related

homeobox transcriptional factor 2 (CDX2). *Paper 5:* The intra-amniotic administration of BCE demonstrated to increase the relative abundance of the genus *Bifidobacterium* and *Clostridium* and to decreased *E coli* abundance. The BCE maintained biomarkers of intestinal inflammation and functionality similar to the control group. Further, the duodenal morphology was maintained similar to the control after the intra-amniotic administration of BCE. **General Conclusion:** The black corn demonstrated to exert a beneficial effect *in vivo*, by acting as an anti-inflammatory and antioxidant component, and by modulating the intestinal function. Thus, the black corn should be considered as a potential source of anthocyanin and phenolic components with proved beneficial effect.

Keywords: Anthocyanin. High-fat. Intestinal microbioma. Soluble extract. Whole flour. Anthocyanin rich-extract

RESUMO

VEREDIANO, Thaisa A, D.Sc., Universidade Federal de Viçosa, fevereiro de 2023. **Effect of black corn (*Zea mays* L.) on adipogenesis, inflammation, oxidative stress and intestinal health *in vivo***. Orientador: Hércia Stampini Duarte Martino. Coorientadores: Maria Cristina Dias Paes, Elad Tako, Mariana Grancieri e Barbara Pereira da Silva.

Milho preto (*Zea mays* L.) é considerado um tipo de milho colorido o qual possui distintas colorações dependendo da sua composição química. Durante os últimos anos, milhos coloridos tem sido alvo de pesquisas cientificas devido a presença de compostos bioativos. O milho preto é considerado fonte de antocianinas, as quais são pigmentos solúveis em água responsáveis pela coloração preta/roxa. Antocianinas são consideradas uma subclasse do grupo dos flavonoides, e seu consumo é associado com efeitos benéficos a saúde, uma vez que demonstram efeito antioxidante e anti-inflamatório, além de modular a função intestinal. No entanto, as antocianinas apresentam um efeito distinto dependendo de sua origem, da matriz alimentar ou se for isolada na forma de extrato. O milho preto TO 002 pertence ao Banco de Germoplasma Ativo da Embrapa e apresentou composição promissora de compostos fenólicos e antocianinas. No entanto, os efeitos biológicos deste genótipo *in vivo* ainda não foram testados. Assim, nossa hipótese considera que o milho preto teria efeito promotor de saúde na adipogênese, inflamação, estresse oxidativo e na função intestinal. **Objetivos:** Revisar de maneira critica os efeitos da suplementação de antocianinas nos parâmetros intestinais em estudos com animais (*Artigo 1*); Avaliar os efeitos da farinha integral de milho preto associada a uma dieta hiperlipídica na adipogênese, inflamação, estresse oxidativo e na modulação da função intestinal (*Artigos 2 e 3*); Investigar os efeitos do extrato solúvel de milho preto na funcionalidade intestinal, morfologia e microbiota *in vivo* (*Artigo 4*); Analisar os efeitos do extrato rico em antocianinas de milho preto na funcionalidade intestinal, morfologia e microbiota *in vivo* (*Artigo 5*). Assim, o objetivo geral deste trabalho foi investigar o efeito do milho preto (*Zea mays* L.) na adipogênese, inflamação, estresse oxidativo e na saúde intestinal em modelo animal. **Métodos:** *Artigo 1*: Para a revisão sistemática, os artigos foram analisados seguindo o protocolo PRISMA. A busca de dados foi realizada no PubMed, Cochrane e Scopus para estudos experimentais, que avaliaram a suplementação de antocianina em parâmetros intestinais. Para os estudos originais: *Artigos 2 e 3:* trinta camundongos C57BL/6 machos adultos foram divididos randomicamente em 3 grupos (n=10/grupo): NC: controle normal – dieta AIN-93 M; HF: dieta hiperlipídica; HFC: dieta hiperlipídica + farinha integral de milho preto (20%), recebendo as dietas por 8

semanas. A função intestinal foi avaliada no conteúdo do ceco ao final do experimento, e a composição da microbiota intestinal foi avaliada pelo sequenciamento do gene ribossômico 16S. *Artigo 4*: O experimento biológico foi conduzido pela técnica metodológica intra-amniótica (*Gallus gallus*). Para isso, os ovos foram divididos em 4 grupos (n= 6 - 10/grupo): Sem administração; Administração de 18 MΩ H_2O; 5% de extrato solúvel de milho preto (BCSE); 15% BCSE. A administração intra-amniótica foi realizada no dia 17 da incubação embrionária. A coleta de amostras e avaliação da funcionalidade intestinal, morfologia e microbiota intestinal foram realizadas após a eclosão (dia 21). *Artigo 5*: O experimento biológico foi conduzido pela técnica metodológica intra-amniótica (*Gallus gallus*). Para isso, os ovos foram divididos em 4 grupos (n= 8/grupo): Sem administração; Administração de 18 MΩ H_2O; 5% de extrato rico em antocianinas de milho preto (BCE); cianidina-3-glicosideo (C3G). A administração intra-amniótica foi realizada no dia 17 da incubação embrionária. A coleta de amostras e avaliação da funcionalidade intestinal, morfologia e microbiota intestinal foram realizadas após a eclosão (dia 21). **Resultados:** *Artigo 1*: 27 estudos em modelos animais foram incluídos na revisão sistemática. A suplementação com antocianinas promoveu um enriquecimento da abundância de Bacteroidetes e uma diminuição de Firmicutes. Além disso, melhorou a produção de ácidos graxos de cadeia curta (AGCC), o que resultou em um menor pH e permeabilidade intestinal, e um aumento de células caliciformes (CC), expressão de proteínas de junção e melhorias na morfologia das vilosidades. *Artigo 2 e 3*: Animais alimentados com a dieta HFC melhoraram a capacidade antioxidante total e demonstraram menor adipogênese, mostrando uma diminuição no número e comprimento de adipócitos pela menor expressão de sterol regulatory element-binding protein-1c (SREBP-1c) e do receptor ativado por proliferador de peroxissoma-γ (PPARγ). O consumo de HFC demonstrou promover a β-oxidação hepática, por meio da redução da expressão gênica hepática de SREBP-1c e acetil CoA carboxilase 1. Além disso, a ingestão de HFC preveniu alterações na função intestinal ocasionados pela dieta hiper lipídica. Animais alimentados com a dieta HFC apresentaram maior abundância dos gêneros *Ruminococcus*, *Roseburia* e *Provotellacea_UCG-001*, e menor abundância dos gêneros *Bacteroides* e *Faecalibaculum*. Estas modificações foram observadas sem alterações na produção de AGCC, e com um aumento na proliferação de células caliciformes. *Artigo 4*: A administração intra-amniótica de BCSE (5 e 15%) demonstrou um efeito anti-inflamatório, pela menor expressão gênica do biomarcador inflamatório duodenal: fator de necrose tumoral alfa (TNF-α), interleucina 6 (IL6) e o fator nuclear transcricional kappa beta (NF-κB). Além disso, o BCSE aumentou a abundância relativa de *E. coli* e *Clostridium*

cecal e melhorou a morfologia duodenal aumentando o número e tamanho de GC e o número de células de Paneth. Ainda, BCSE demonstrou potencial para melhorar a integridade da barreira física regulando positivamente a expressão gênica da proteína quinase ativada por AMP (AMPK) e do fator de transcrição caudal-related homeobox 2 (CDX2). *Artigo 5*: A administração intra-amniótica de BCE demonstrou aumentar a abundância relativa dos gêneros *Bifidobacterium* e *Clostridium* e diminuir a abundância de *E coli*. O BCE manteve inalterado biomarcadores de inflamação intestinal e funcionalidade. Além disso, a morfologia duodenal foi mantida semelhante ao controle após a administração intra-amniótica de BCE. **Conclusão**: O milho preto demonstrou exercer um efeito benéfico *in vivo*, atuando como componente anti-inflamatório e antioxidante, e modulando a função intestinal. Assim, o milho preto deve ser considerado como uma fonte potencial de antocianinas e componentes fenólicos com comprovado efeito benéfico.

Palavras-chave: Antocianinas. Obesidade. Dieta hiper lipídica. Microbioma intestinal. Extrato solúvel. Farinha integral. Extrato rico em antocianina

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LIST OF ACRONYMS AND ABBREVIATIONS

^{0}C	Celsius
18S rRNA	18S ribosomal subunit
ABTS	2,2`-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid
ACC-1	Acetyl CoA carboxilase 1
ADIPOR2	Adiponectin receptor 2
ADP	Adenosine diphosphate
ALT	Alanine aminotransferase
AMDR	Acceptable Macronutrient Distribution range
AMPK	Adenosine monophosphate protein kinase
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AP	Amino peptidase
Apc	Adenomatous polyposis coli
AST	Aspartate aminotransferase
AU	Arbitrary units
BCE	Black corn extract
BCSE	Black corn soluble extract
BMI	Body mass index
BW	Body weight
C/EBPs	CCAAT/enhancerbinding proteins
C3G	Cyanidin-3- glucoside
CAPES	Coordination for the Improvement of Higher Education Personnel (Brazil)
CAT	Catalase
CD	Crypt depth
CDX2	Caudal-related homeobox transcriptional factor 2
CML	Circular muscle layers
CNPq	National Council of Technological and Scientific Development (Brazil)
Cp	Cyclic product
CRBP2	Cellular retinol-binding protein-2
CW	Crypt width

DAD	Diode array detector
EMBRAPA	Brazilian Agriculture Research Corporation
F/B	Firmicutes/Bacteroidetes
F6P	Fructose 6-phosphate
FDR	False discovery rate
g	Grams
G1P	Glucose-1-phosphate
G6PD	Glucose-6 phosphatase
GAE	Gallic acid equivalent
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal
GC	Goblet cell
GRP43	G protein-coupled receptor
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
HCl	Hydrochloric acid
HDACs	Histone deacetylases
HDL-c	High-density lipoprotein-cholesterol
HF	High-fat diet
HFC	High fat diet with black corn whole flour
HPLC	High-performance liquid chromatography
IFNγ	Interferon gamma
IkBα	I-Kappa-B-alpha
IL	Interleukin
JAM-1	Junctional adhesion molecule
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDL-c	Low-density lipoprotein-cholesterol
LML	Longitudinal muscle layers
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
LRAT	Lecithin: retinol acyltransferase
Lyz1	Lysosome-1

MDA	Malondialdehyde
MeSH	Medical Subject Headings
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MUC2	Mucin 2
MyD88	Myeloid differentiation primary response 88
n	Number
NC	Normal control diet
NCBI	National Center for Biotechnology Information
ND	Not detectable
NF-κβ	Factor nuclear kappa β
NLRP6	Inflammasome nucleotide-oligomerization domain-like receptor 6
Nrf2	Erythroid-2-related factor 2
NS	Not specified
OCLN	Occludin
Osm	Osmolarity value
OTUs	Operational Taxonomic Units
PBS	Phosphate-buffered saline
PCK1	Phosphoenolpyruvate carboxykinase
PCoA	Principal Coordinate Analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
PFK	Phosphofructokinase
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
PKF	Phosphofructokinase 1
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor-γ
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SCFA	Short chain fatty acids
SD	Standard deviation

SED	Standard error deviation
SGLT1	Sodium-glucose transport protein 1
SI	Sucrose isomaltase
sIgA	Secretory immunoglobulin A
SOD	Superoxide dismutase
SRA	Sequence read archive
SREBP-1c	Sterol regulatory element-binding protein-1c
STAMP	Statistical Analysis of Metagenomic Profiles
STAT3:	Signal transducers and activator of transcription 3
SYRCLE RoB	Systematic Review Centre for Laboratory Animal Experimentation Risk of Bias
TAC	Total antioxidant capacity
TC	Total cholesterol
TG	Total triglycerides
TLR-4	Toll-like receptor 4
TNFR-1	Receptor tumor necrosis factor receptor 1
TNF-α	Tumor necrosis factor alpha
U.S.A.	United States of America
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VDAC	Voltage-dependent anion channel
ZnT1	Zinc transporter 1
ZO-1	Zonula occludents 1

SUMMARY

1. INTRODUCTION

Corn, also known as maize (*Zea mays* L.), is one of the major cereals produced and consumed worldwide (FAO, 2023). Corns are naturally found in many colors due the presence of secondary metabolites as phenolic acids, carotenoids, and flavonoids. The difference in the expression of these metabolites results in corn tissues with colors varying from yellow orange to dark/black- purple-blue, cream and ivory colors (COLOMBO et al., 2021). The color can be expressed in the pericarp, aleurone and endosperm (SALVADOR-REYES, CLERICI, 2020). Over the past years, the scientific community have been investigating colored corns due the potential healthy properties associated to their composition (SHENG et al., 2018).

Black-purple corn was originated in the Andean region at South America, and until today it is traditionally consumed in countries as Peru, Chile, Mexican as a culinary ingredient for traditional foods. Although in a lower scale, purple corn is also used in industries as a natural coloring ingredient (SALVADOR-REYES, CLERICI, 2020). This variety of corn is composed of carbohydrates, protein, dietary fiber and lipids. However, the special attention to black corn is due the significant concentration of a bioactive components called anthocyanin, which accumulates at the pericarp and are responsible for its dark/purple color (SALVADOR-REYES, CLERICI, 2020) . The amount of anthocyanin in purple corn varies from 220 mg (BHASWANT, SHAFIE et al., 2017) to 310 mg / 100 g (RANILLA et al., 2017), although, it is known that the concentration can widely vary over different genotypes (ZHANG et al., 2019). Besides anthocyanin, other phenolic compounds as ferulic, chlorogenic, and caffeic acid are also observed at purple corn composition, thus contributing to its healthy properties (SALVADOR-REYES, CLERICI, 2020). Over the last years, studies have investigated some potential beneficial effects of black corn or its extract on diabetes (HUANG et al., 2015), obesity (WU et al., 2017) metabolic syndrome (BHASWANT et al., 2017), colorectal cancer (MAZEWSKI et al., 2017), glucose metabolism (LUNA-VITAL, MEJIA, 2018), and inflammation (LUNA-VITAL et al., 2017). Furthermore, black corn consumed a whole flour has the advantage of maintaining the original components to preserve the bioactive components in its original matrix.

Anthocyanins are bioactive water-soluble component and one of the most important subgroups at the flavonoid class. In the nature, anthocyanin can be found in several fruits, vegetables, cereals, and flowers, which have in common a bright purple-dark-blue color. Anthocyanins exist mainly in the form of glycoside, and they are chemically composed of an anthocyanidin core attached with sugar and organic acid (SUI et al., 2019). Due different

chemical structure at the hydroxyl group position, several anthocyanidins exist in the nature (CASTAÑEDA-OVANDO et al., 2009), and the cyanidin-3-glucoside is the most predominant (SUI et al., 2019). Anthocyanin has been the focus of studies over many years, due its antioxidant and anti-inflammatory effects in several metabolic diseases (AZZINI et al., 2017; BENDOKAS et al., 2020; CHAVES et al., 2018; LEE et al., 2017). Moreover, the metabolism of anthocyanin undergoes a specific pathway of absorption, in which significant amount from the consumed anthocyanin reaches the colon to be metabolized by the microbiota (FANG, 2014; HRIBAR, ULRIH, 2014). Beneficial bacteria as *Bifidobacterium* and *Lactobacillus* produces the β-glucosidase enzyme, which is necessary to catalyze the intestinal metabolism of anthocyanins to phenolic acids (FARIA et al., 2014; TIAN et al., 2018). In this sense, due the advanced of methodologies to investigate the impact of intestinal microbiota to health, anthocyanin consumption has been now associated with a potential improvement of intestinal function, mainly at microbiota composition (FARIA et al., 2014).

The gastrointestinal (GI) tract is composed of semipermeable layers responsible for digestion and absorption, and to protect the organism against potential pathogens (LOPETUSO et al., 2014). The intestinal microbiome and GI barrier are two essential components to maintain intestinal integrity and immune homeostasis. The GI barrier is composed of a physical barrier formed by epithelial cells, metabolites and a mucosal layer, which communicates with the intestinal microbiota (MARTINI et al., 2017; VANCAMELBEKE, VERMEIRE, 2017). The intestinal microbiota is formed by a diverse and complex environment of microorganisms and their metabolite, which habitats the lumen and mucosal surface (HOFFMANN et al., 2016). The interaction between gut physical barrier and gut microbiota regulates the intestinal homeostasis by some pathways, such as mucus production by goblet cell, tight junction proteins, immune system regulation, microbiota modulation and prevention of pathogenic bacteria (MARTINI et al., 2017; VANCAMELBEKE, VERMEIRE). In this context, dietary components have been shown to shape the microbiota leading to gut barrier dysfunction (REQUENA et al., 2018).

A dietary pattern composed of high consumption of fats, mainly saturated fatty acids, have shown to lead the microbiota composition to a level of dysbiosis and gut barrier dysfunction (MALESZA et al., 2021). This disruption of host-microbiota interaction can lead to high intestinal permeability, and translocation of pathogenic bacterial metabolites, which have potential to trigger inflammatory pathways as the nuclear factor kappa beta (NF-kβ) (BAKER et al., 2011; ROGERO, CALDER, 2018). Besides the mentioned process, a high-fat

diet promotes hypertrophy of adipocytes, which releases pro-inflammatory substances thus, promoting and exacerbating an inflammatory state (UNAMUNO et al., 2018). On the other hand, components from diet as phytochemicals are one of the major nutrients investigated to modulate the intestinal microbiota and to maintain the integrity of intestinal physical barrier (REQUENA et al., 2018).

Over this context, anthocyanin have shown beneficial properties as antioxidant, anti-inflammatory and to modulate intestinal functions (BENDOKAS et al., 2020; TIAN et al., 2018; ZHU, 2018). However, depending on the food source and food matrices anthocyanins have discrepancy in its bioavailability and effects due the interaction among the food matrix (TARKO, DUDA-CHODAK, 2020). Therefore, the biological investigation of a specific food, as a source of bioactive components, by different processing and seed parts is essential to obtain complete conclusions regarding the biological effects.

The Brazilian Agricultural Research Corporation (Embrapa) conserves a wide genetic diversity of corn genotypes at the Germplasm Activate Bank (BAG) composed of more than two hundred tropical maize genotypes of colored grains. There is no data regarding the consumption of this grains in Brazil, however, it is known that indigenous and quilombola communities consume these grains traditionally. Further, after an initial chemical characterization of these grains, the genotype TO 002 was considered as one of the most valuable genotypes in terms of phenolic compounds and anthocyanins (BARBOSA, 2016).

For this reason, an entire investigation of this specific genotype in a biological approach it is necessary to verify its potential effects.

Therefore, considering the present literature, initially, we systematically reviewed published studies that evaluated the effects of anthocyanin supplementation on intestinal functions in animal studies. Later, the potential healthy properties of black corn (*Zea mays* L.) were tested *in vivo*. As a highlighting, this studied aimed to investigate the beneficial effects of black corn in different processing: as a whole matrix, as a soluble extract, and as a phenolic anthocyanin-extract to have a complete knowledge of its biological effects. Therefore, the present study evaluated the effects of black corn whole flour on inflammation, adipogenesis, oxidative stress and intestinal function in a high-fat diet approach; and the effects of black corn soluble extract and black corn anthocyanin-rich extract on intestinal functionality, morphology and microbiota in an intra-amniotic approach.

To achieve this purpose, the following hypotheses were tested: (1) evidence from reviewed experimental studies will justify the consumption of anthocyanin to improve intestinal

parameters; (2) the consumption of black corn whole flour will prevent the adipogenesis, inflammation and oxidative stress, besides to improve the intestinal health disrupted due a high-fat diet consumption; (3) the intra-amniotic administration of black corn as soluble extract and as a phenolic anthocyanin-rich extract will improve the functionality, morphology and microbiota.

2. OBJECTIVES

2.1. General objective

To investigate the effects of black corn (*Zea mays* L.) on the adipogenesis, inflammation, oxidative stress and on the intestinal health in animal models.

2.2. Specific objectives

- ✓ To review the evidence of anthocyanin supplementation on the intestinal health of animal experiments;
- ✓ To investigate the effects of black corn whole flour on inflammation, adipogenesis, and oxidative stress in mice fed a high-fat diet;
- ✓ To evaluate the effects of black corn whole flour on the intestinal health in mice fed a high-fat diet;
- ✓ To investigate the effects of intra-amniotic administration of black corn soluble extract on the intestinal functionality, morphology, and microbiota composition *in ovo*;
- ✓ To analyze the effects of intra-amniotic administration of black corn anthocyanin-rich extract on intestinal morphology, gene expression, and on cecal microbiome.

3. GENERAL METHODOLOGY

The thesis was conducted in 2 phases: first a theoretical phase and a second phase with *in vivo* experiments (Figure 1).

In the phase 1, a systematic review was performed to answer the question "Does food source or extract of anthocyanin promote changes on intestinal parameters?". For this, articles were reviewed following the PRISMA guidelines with the data search performed at PubMed, Cochrane and Scopus for experimental studies (Paper 1).

The phase 2 consisted of three experimental studies evaluating the black corn effects on different processing: as a whole matrix, as a soluble extract, and as a phenolic anthocyanin-rich extract. First, the chemical characterization of the black corn whole flour was performed in terms of macronutrients, dietary fiber and anthocyanin profile. The experiment 1 was performed with the black corn as a whole flour. For this, thirty adult male C57BL/6 mice were randomly divided into 3 groups (n= 10/group): NC: normal control – AIN-93 M diet; HF: high-fat diet; HFC: high-fat diet + black corn whole flour (20%), receiving the diets for 8 weeks. After, it was evaluated the adipogenesis, inflammatory pathway, and antioxidant enzymes (Paper 2); besides the short chain fatty acid production, cecum morphology, and cecal microbiota composition (Paper 3).

Then, the black corn was used to elaborate two different extracts: (1) soluble extract: consisting of dissolution and dialyze of the sample in distilled water, and (2) anthocyanin rich-extract: elaborated from a ethanolic extraction.

The experiment 2 was performed with the black corn as an aqueous soluble extract. The biological experiment was conducted by an intra-amniotic approach (*Gallus gallus*), in which the eggs were divided into 4 groups (n= 6 - 10/group): No Injection; 18 MΩ H_2O Injection; 5% black corn soluble extract (BCSE); 15% BCSE. The intra-amniotic administration was performed at the day 17 of embryonic incubation. After the hatching (day 21) samples were collected to evaluate the duodenal morphology, gene expression of proteins from duodenum and liver and PCR amplification of bacterial 16S RNA (Paper 4).

The experiment 3 was conducted with the ethanolic anthocyanin-rich extract from black corn. The biological experiment was conducted by an intra-amniotic approach (*Gallus gallus*), in which the eggs were divided into 4 groups (n= 8/group): No Injection; 18 MΩ H_2O Injection; 5% black corn anthocyanin-rich extract (BCE); cyanidin-3-glucoside (C3G). The intra-

amniotic administration was performed at the day 17 of embryonic incubation. After the hatching (day 21) samples were collected to evaluate the duodenal morphology, gene expression of proteins from duodenum and PCR amplification of bacterial 16S RNA (Paper 5).

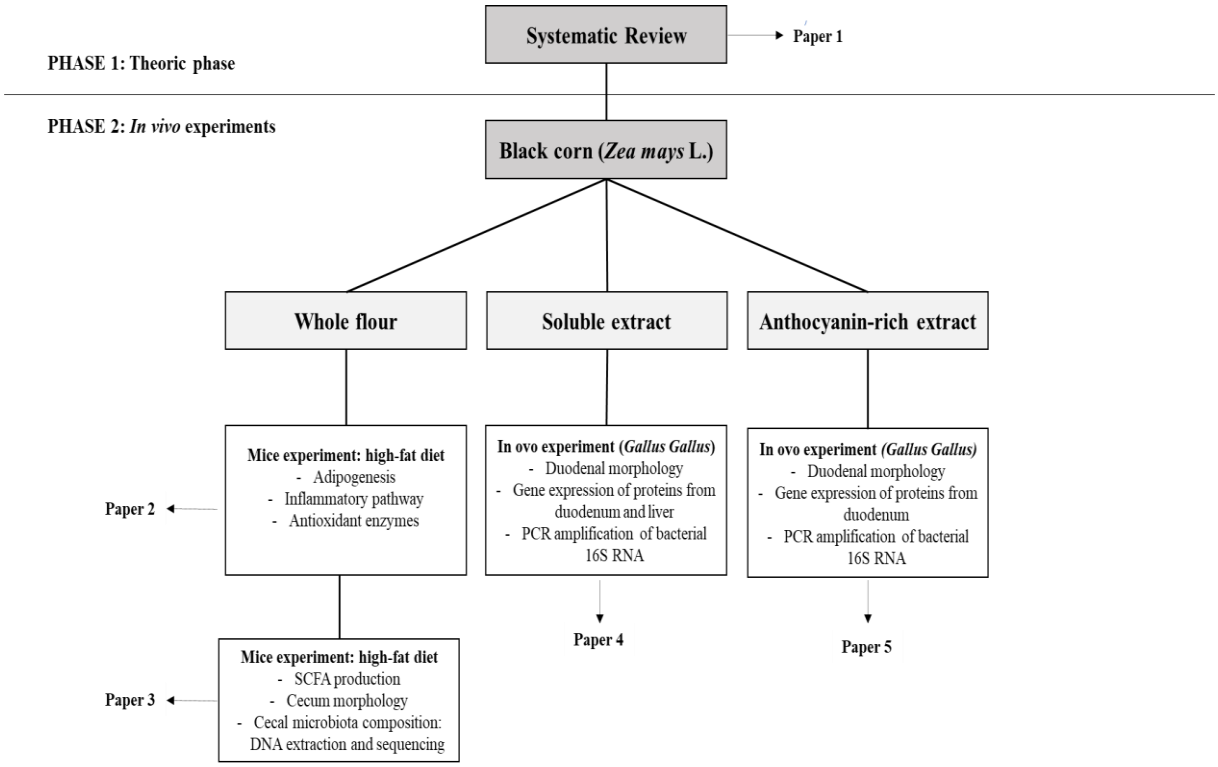

Figure 1. Flowchart of the general methodology of the thesis.

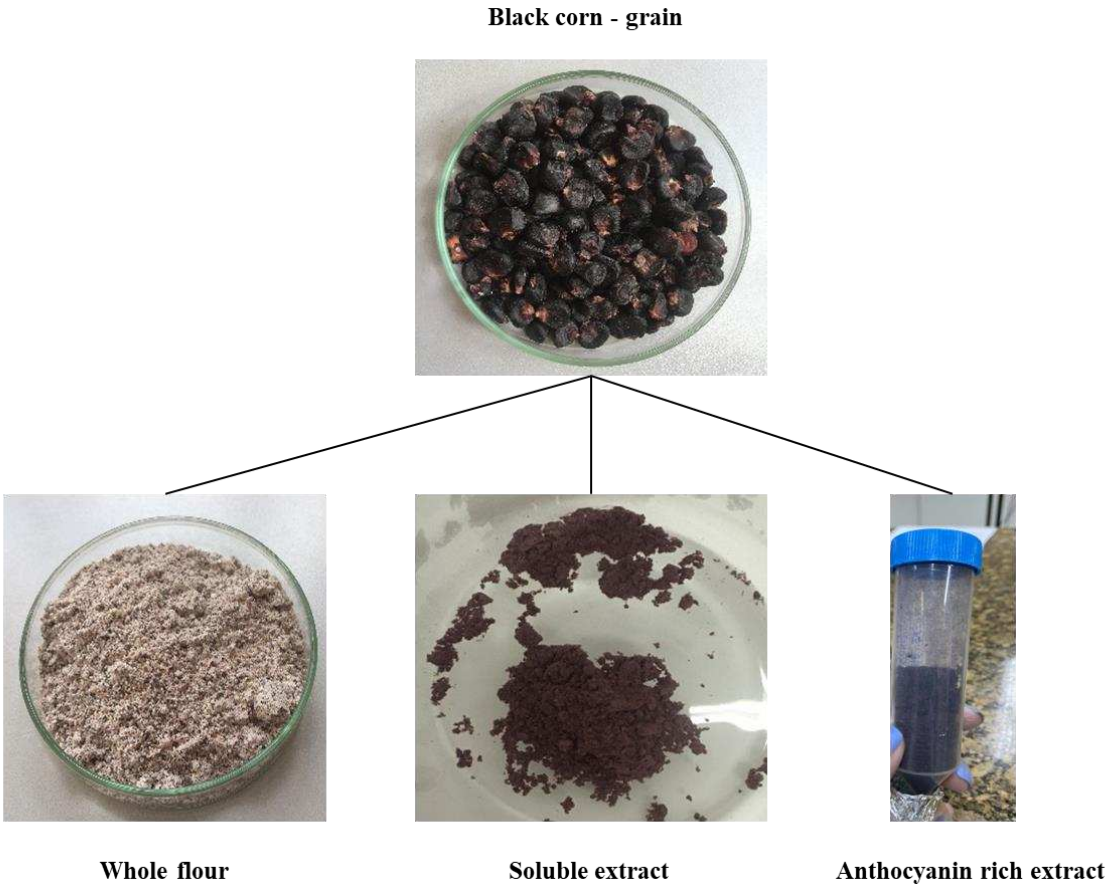

Figure 2. Flowchart of the black corn in three different processing used in this thesis.

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4. THESYS PUBLICATION

The initial review and the experiments performed in the present thesis were published between 2021 and 2022 as one systematic review and four original papers (Table 1).

Table 1. Description of papers published in the thesis.

Paper type	Paper title	Journal/ Impactor factor	Year of Publication
Systematic review	Effects of Anthocyanin on Intestinal Health: A Systematic Review	Nutrients / 6.706	2021
Original paper 1	Black corn (*Zea mays* L.) whole flour improved the antioxidant capacity and prevented adipogenesis in mice fed a high-fat diet	Food & Function / 6.317	2022
Original paper 2	Black corn (*Zea mays* L.) flour has the potential to improve the gut microbiota composition and goblet cell proliferation in mice fed a high-fat diet	Nutrition Research / 3.876	2022
Original paper 3	Black corn (*Zea mays* L.) soluble extract showed anti-inflammatory effects and improved the intestinal barrier integrity *in vivo* (*Gallus gallus*)	Food Research International / 7.425	2022
Original paper 4	Effect of Black Corn Anthocyanin-Rich Extract (*Zea mays* L.) on Cecal Microbial Populations *In Vivo* (*Gallus gallus*)	Nutrients / 6.706	2022

5. LITERATURE REVIEW

5.1. PAPER 1

nutrients

MDPI

Review

Effects of Anthocyanin on Intestinal Health: A Systematic Review

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Abstract: Intestinal health relies on the association between the mucosal immune system, intestinal barrier and gut microbiota. Bioactive components that affect the gut microbiota composition, epithelial physical barrier and intestinal morphology were previously studied. The current systematic review evaluated evidence of anthocyanin effects and the ability to improve gut microbiota composition, their metabolites and parameters of the physical barrier; this was conducted in order to answer the question: "Does food source or extract of anthocyanin promote changes on intestinal parameters?". The data analysis was conducted following the PRISMA guidelines with the search performed at PubMed, Cochrane and Scopus databases for experimental studies, and the risk of bias was assessed by the SYRCLE tool. Twenty-seven studies performed in animal models were included, and evaluated for limitations in heterogeneity, methodologies, absence of information regarding allocation process and investigators' blinding. The data were analyzed, and the anthocyanin supplementation demonstrated positive effects on intestinal health. The main results identified were an increase of *Bacteroidetes* and a decrease of *Firmicutes*, an increase of short chain fatty acids production, a decrease of intestinal pH and intestinal permeability, an increase of the number of goblet cells and tight junction proteins and villi improvement in length or height. Thus, the anthocyanin supplementation has a potential effect to improve the intestinal health. PROSPERO (CRD42020204835).

Keywords: microbiota; polyphenols; short chain fatty acids; intestinal barrier

Citation: Verediano, T.A.; Stampini Duarte Martino, H.; Dias Paes, M.C.; Tako, E. Effects of Anthocyanin on Intestinal Health: A Systematic Review. *Nutrients* **2021**, *13*, 1331. https://doi.org/10.3390/nu13041331

1. Introduction

A healthy gut includes multiple positive aspects of the gastrointestinal (GI) tract, specifically, effective digestive and absorptive functions of the intestinal brush border membrane, and the absence of GI chronic conditions such as enzyme deficiencies, intestinal bower disease, coeliac disease, colorectal cancer, and others. In addition, well-balanced intestinal microbiota was associated with an effective immune system function that is required to maintain the host homeostasis [1]. The intestinal microbiota consists of more than a trillion microorganisms that establish a symbiotic relationship with their host. These microorganisms prevent the colonization of potentially pathogenic microorganisms and regulate the mucosal immune system, and thus assist to maintain an intact intestinal barrier [2]. Thus, the physical barrier of epithelial cells and mucus layer provide the first line of defense by mechanisms such as microbial recognition, antibodies secretion, antimicrobial peptides and mucus production [1]. Therefore, impairments of the physical barrier may enhance the risk of infections, inflammatory intestinal diseases and other diseases occurring outside the intestine such as immune-related and metabolic disorders [1,3]. In this context, certain food and plant origin bioactive compounds were investigated to reduce the risk of the mentioned diseases by acting beneficially on the intestinal health.

Anthocyanins are bioactive water-soluble plant pigments that are responsible for bright colors, such as purple, red and blue, which are presented mainly as glycosides, with the basic structure consisting of an anthocyanidin core attached to sugars and organic acids [4]. The positive effects of anthocyanins and anthocyanin-rich foods are widely described in the literature. These effects are mainly associated with reduced risk of diseases associated with oxidative stress, such as cardiovascular disease [5] and inflammatory diseases such as diabetes mellitus [6], obesity [7] and insulin resistance [8]. In addition, the health-promoting effects attributed to anthocyanins were shown to be associated with the gut microbiota modulation [9].

Dietary anthocyanins undergo a specific metabolism with the absorption rate depending on their structure. Briefly, anthocyanins cross the gastric mucosa in their intact form. Thereafter, in the small intestine, mainly in the jejunum, they are absorbed by hydrolytic enzymes as phenolic aglycone. The unabsorbed anthocyanins reach the colon, and are metabolized by the colon microbiota, especially by genera and species that are equipped with enzymes such as β-glucosidase, which are necessary to catalyze the reaction. Intestinal bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. possess these enzymes; thus, the anthocyanin metabolism by microbiota and/or their metabolites can modulate the growth of

these specific bacteria [9–11]. In this sense, the modulation of gut microbiota by anthocyanin increases the short chain fatty acids (SCFA) producing bacteria, which acidifies the intestinal pH and inhibits the pathogenic bacteria proliferation, and SCFA as butyrate act as a fuel to provide energy for epithelial cells, thus improving the intestinal barrier to avoid the translocation of pathogens and antigens [12]. Thereby, it is suggested that the potential beneficial functions of anthocyanins could be indirectly attributed to the gut microbiota modulation and consequent production of metabolites due to bacterial fermentation activities, which improve several parameters related to the intestinal health [9,13].

Despite the several positive effects of anthocyanins, there is no consensus in the literature regarding their mechanisms of action on intestinal health in experimental studies. Recently, a systematic review (n = 6 studies: 3 in vitro, 2 animals and 1 human trial) verified the effects of anthocyanin supplementation on the gut microbiota composition, showing the proliferation of healthy anaerobic bacterial population and inhibition of pathogenic species [14]. However, a healthy gut is maintained by a set of parameters related to the metabolites of microbial bacteria, intestinal cells' integrity and the physical barrier [1]. Therefore, the objective of the current systematic review was to investigate the effects of anthocyanins on several parameters of intestinal health in experimental studies, in order to understand the mechanism in which these parameters act in association. It is hypothesized that the supplementation of anthocyanin promotes beneficial changes to the gut microbiota with increased production of metabolites associated with intestinal barrier improvement, which contributes to a healthy gut.

2. Materials and Methods

2.1. Eligibility Criteria

The eligibility criteria were based on the PICOS (population, intervention, comparison, outcomes and study design) model strategy. Duplicate studies were excluded, and the search and screening for titles and abstracts were carried out independently by the authors according to the inclusion and exclusion criteria (Table 1).

2.2. Information Source

Two researchers independently searched for original articles. PubMed, Cochrane and Scopus were used to search studies performed with animal models that evaluated the effects of

anthocyanin on the intestinal health. No time restriction was used. The descriptors were identified based on Medical Subject Headings (MeSH).

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

Parameter	Inclusion criteria	Exclusion criteria
Population	*In vivo* animal studies	Clinical studies and in vitro studies
Intervention	Intake of foods source of anthocyanin or supplementation with extract of anthocyanin	Anthocyanin associated with other foods or not measured
Comparator	Negative control (without the intervention)	No control group
Outcomes	Changes in the gut microbiota composition, intraluminal pH, short chain fatty acids, histological parameters of small and large intestine, gene expression of tight junction's proteins, gene expression of intestinal brush border membrane, integrity of intestinal barrier, and intestinal permeability	
Study design	Experimental placebo-controlled studies	Review articles, clinical studies, theses, dissertation, book chapter, *in vitro* experiments, and studies published in other language than English.

2.3. Search Strategy

The following English search terms were used: (Anthocyanin OR Anthocyanidin OR Anthocyanidins OR Cyanidin OR Delphinidin OR Malvidin OR Peonidin OR Pelargonidin OR Petunidin) AND (intestinal OR gut). Only articles published in English were considered in this review. The last search was performed on 2 June 2020. The first selection of the studies was based on the title and abstract. We excluded review articles, clinical studies, theses, dissertations, book chapters, in vitro experiments and studies published in other languages than English. Further, we excluded studies in which the intake of anthocyanin was associated with other foods, or if the anthocyanin was not measured. Studies were eligible for inclusion if they fulfilled the following criteria: (a) studies conducted with animals; (b) the intervention was the intake of foods' sources of anthocyanin or supplementation with an extract of anthocyanin; (c)

the comparator was the negative control (without the intervention); (d) the outcomes searched were changes related to the intestinal health, mainly: changes in the gut microbiota composition, intraluminal pH, short chain fatty acids, histological parameters of small and large intestine, gene expression of tight junction's proteins, gene expression of intestinal brush border membrane, integrity of intestinal barrier and intestinal permeability.

2.4. Selection, Data Collection Process and Data Items

After reading and reviewing the selected research articles in full, the data were compared to ensure integrity and reliability. Divergent decisions were resolved by consensus. The eligible outcomes evaluated were broadly categorized as follows:

- Gut microbiota: short chain fatty acids (caecal, fecal or in the serum); intraluminal pH (ileal, caecal or feces); microbial quantification; secretory immunoglobulin A (sIgA);
- Epithelial physical barrier: tight junction proteins; proteins of intestinal brush border membrane; intestinal permeability; plasm endotoxin;
- Intestinal morphology: number of goblet cells; length, height and depth of villi and crypts; mucin secretion; antimicrobial peptides.

Any measure and methodology of these outcomes was eligible for inclusion.

Further, for each experimental study included, we reported relevant information related to the authors, publication year, country of publication and experimental model features such as animal model, age, sex, initial weight, number of groups and animal per group. To access the research methods, we extracted specific information related to the experimental groups such as type of food intervention, type of diet and control group. For the control test of food intake, we extracted information related to the method of administration that was used in the intervention, the duration of the intervention, the dosage of anthocyanin and main results (control x intervention).

For this review, data from the eligible studies are expressed in tables and figures. We provided a narrative synthesis of the results according to the main characteristics and results.

2.5. Study Risk-of-Bias Assessment

The methodological quality of the included studies was assessed, and the risk of bias was verified using the Systematic Review Centre for Laboratory Animal Experimentation Risk of Bias (SYRCLE RoB) tool [15], which is responsible for identifying study quality and measuring the bias in research involving animal studies [16]. The SYRCLE RoB toll considers

ten entries that are related to six types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias and other. For each included study, the six bias types were classified as “high” (+), “low” (-) or “unclear” (?).

3. Results

3.1. Study Selection

The flow diagram of the literature search and selection process was built in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline (Figure 1). After the search in the selected databases, we identified 1155 articles (x = 495 Pubmed; x = 38 Cochrane and x = 622 Scopus). From these, 1117 were excluded for the following reasons: duplicate studies (n = 400), title and abstract not suited to the topic (n = 341), in vitro studies (n = 181), review articles (n = 169), book chapters (n = 11), supplementation of anthocyanin associated with other foods (n = 9), and other languages than English used (n = 8). After, 36 articles were read fully. From these, we excluded nine articles: total of anthocyanin not measured (n = 7); there was no negative control without intervention (n = 1); and anthocyanins were combined with other compounds (n = 1). Therefore, 27 studies were included in this systematic review.

3.2. Study Characteristics

The included studies (n = 27) were performed in ten different countries. Most of them were conducted in China (n = 9) [17–25] or the United States of America (U.S.A.) (n = 5) [26–30]. Regarding the animal model used in the studies, 15 were performed with mice [17,20–22,24–34], 11 with rats [18,19,23,35–42] and only 1 with rabbits [43]. Most studies used male animals (n = 24) [17,18,20–29,31,33–43], but two used female animals [19,30], and only one study used both male and female animals [32]. Interesting, six studies did not describe animals’ initial weight [19,21,28,30,32,38]. The age of the animals ranged from 3 to 72 weeks, although five studies did not mention this information [17,27,35,36,39].

The studies’ main characteristics were chronologically organized by the publication year, starting with the first published (Table 2).

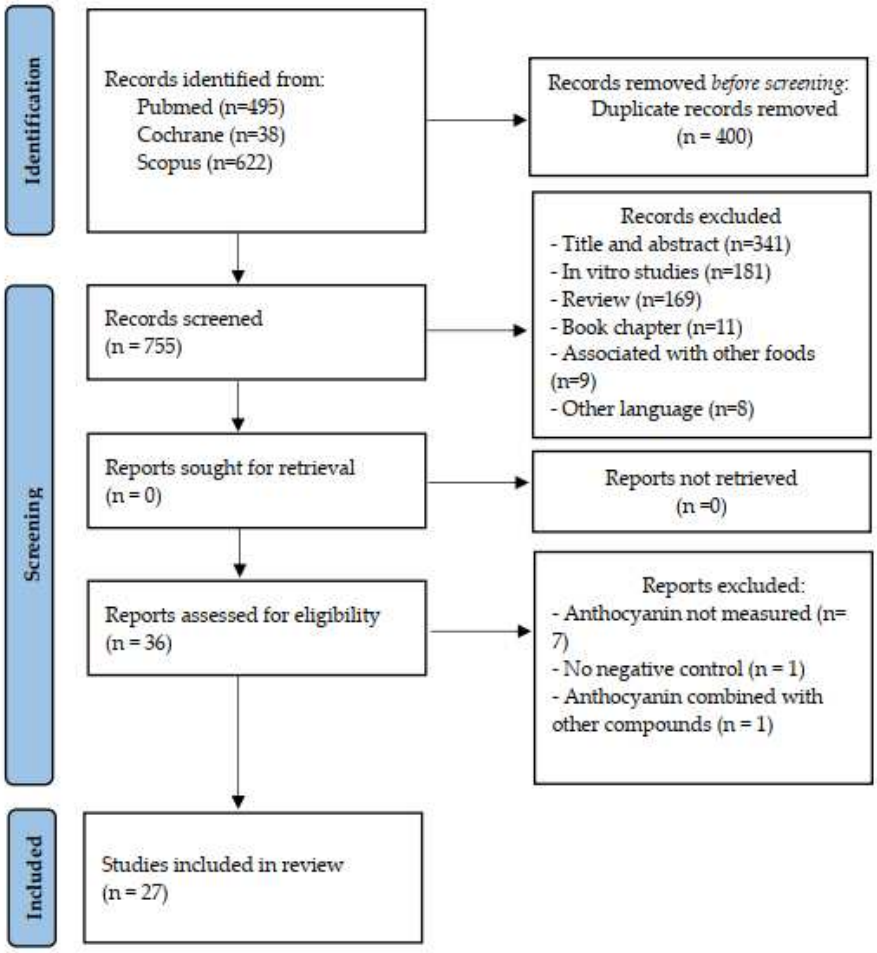

Figure 1. Flowchart of the search for articles included in the systematic review, according to PRISMA (2020) recommendation.

The anthocyanin intervention varied by the source that was used. In 20 studies, diverse fruits were offered in the form of extract (n=12) [17,19,25,30,32-36,40,41,43], or as a powder (n=8) [22,26,28,31,37-39,42]. The fruits used as an anthocyanin source were chokeberry, Kamchatka berry, apple, blackcurrant, bilberry, blueberry, black raspberry, red cabbage, grape, berry juçara, jabuticaba, purple carrot, black gojy berry, and black rice. In 4 studies the intervention was with purified anthocyanin [20,23,24,27], and in 3 with monomeric anthocyanidins as cyanidin-3-O-glucoside [18], malvindin-3-glucoside [29] and pelargonidin-3-O-glucoside [21].

The form of anthocyanin administration was oral in all included studies, with the form of administration by addition in the diet (n=20) [18,22,24-33,35-40,42,43], or via gavage (n=5) [17,19,21,23,34], or added to the drinking water (n=2) [20,41]. The duration of interventions

ranged widely from 1 to 20 weeks. Regarding the anthocyanin dosage, the doses observed varied from 12.9 mg/100 g diet [42] to 1280 mg/ 100 g diet [40], and 1.68 mg/ kg body weight (BW) [34] to 200 mg/kg BW [20,24]. In addition, only 1 study offered a aqueous extract in the dose of 75 mg/L [41] (Table 3).

Table 2. Characteristics of animal studies on the effects of anthocyanin on intestinal health.

Author, year	Country	Animal model/age	Sex	Initial weight (g)	Nº of groups	Nº of animals/ groups
Jurgonski, Juskiewicz, Zdunczyk, 2008 [35]	Poland	Wistar rats/ NS	Male	161 ± 8	3	8
Jurgonski, Juskiewicz, Zdunczyk, 2013 [36]	Poland	Wistar rats/ NS	Male	548 ± 36	3	8
Espley, et al., 2014 [31]	New Zealand	Swiss mice/ 6-7 wk	Male	30	3	10
Jurgonski, et al., 2014 [43]	Poland	White rabbits/ 34 days	Male	631 ± 26	4	5
Paivarinta, et al., 2016 [32]	Finland	C57BL/6J Apc^{mim} mice/ 5 wk	Male and female	NS	3	5-6 male and 4-6 female
Overall, et al., 2017 [26]	U.S.A.	C57BL/6J mice / 6 wk	Male	20-30	8	12 or 8
Tong, et al., 2017 [17]	China	Kunming mice/ NS	Male	22 ± 5	5	10
Fernández, et al., 2018 [37]	Spain	Fischer 344 rats/ 5wk	Male	200- 270	3	10
Jamar, et al., 2018 [38]	Brazil	Wistar rats/ 90 days	Male	NS	3	7
Lee, et al., 2018 [39]	Georgia	Wistar rats/ NS	Male	200-220	3	8
Paturi, et al., 2018 [40]	New Zealand	Sprague-Dawley rats/ 3wk	Male	256-265	8	16
Silva-Maia, et al., 2018 [41]	Brazil	Wistar rats/ 3 wks	Male	0-100	3	5 or 8
Van Hul, et al., 2018 [33]	France	C57BL/6J mice/ 9 wk	Male	25-30	4	14
Chen, et al., 2019 [18]	China	Wistar rats/ 13 wks	Male	403 ± 4	5	8
Cremonini, et al., 2019 [27]	U.S.A.	C57BL/6J mice/ NS	Male	20-25	4	10
Gu, et al., 2019 [28]	U.S.A.	C57BL/6J mice/ 4 wk	Male	NS	2	12 or 14
Li, et al., 2019 [19]	China	SD rats /4 and 12 months	Female	NS	6	10
Liu, et al., 2019 [29]	U.S.A.	C57BL/6J mice/ 4-5 wk	Male	18-21	3	9 or 10
Luo, et al., 2019 [23]	China	Sprague-Dawley rats /4 wk	Male	100-120	6	8

Peng, et al., 2019 [20]	China	C57BL/6J mice /5 wk	Male	21-24	4	10
Su, et al., 2019 [21]	China	*db/db* mice C57BL/6J /6 wk	Male	NS	2	12
Tian, et al., 2019 [22]	China	C57BL/6J mice /4 wk	Male	15-18	6	11
Zary-Sikorska, et al., 2019 [42]	Poland	Wistar rats /13 wk	Male	146 ± 1.051	5	8
Cao, et al., 2020 [30]	U.S.A.	C57BL/6J mice /3 - 18 months	Female	NS	4	3
Peng, et al., 2020 [24]	China	C57BL/6J mice /5 wk	Male	20-24	2	10
Rodríguez-Daza, et al., 2020 [34]	Canada	C57BL/6J mice /6 wk	Male	20-25	6	12
Wang, et al., 2020 [25]	China	C57BL/6J mice /6 wk	Male	19-20	5	12

NS: not specified; wk: weeks ; U.S.A.: United States of America; Apc.: adenomatous polyposis coli

3.3. Main Findings

The reviewed experimental studies demonstrated that the anthocyanin supplementation provided beneficial effects to intestinal health, and specific improvement in the intestinal microbiota population, short chain fatty acids production, goblet cell number, tight junction protein and villi improvement (Table 3).

Positive findings included the effects on the intestinal microbiome composition and function. In this context, the majority of the studies observed an increased abundance of Bacteroidetes [22,23,26,28,30,33]; two studies observed a reduction [18,39]; and one showed no changes on the abundance of *Bacteroidetes* [20]. On the other hand, the abundance of *Firmicutes* was reduced in five studies [21–23,28,39], and in two studies no changes were observed [20,30]. Further, a reduction in the *Firmicutes/Bacteroidetes* ratio (total of studies that evaluated = 7) was observed in four studies [19,25,27,30]; in two other studies, no changes were observed [20,34], and only one study observed an increased ratio [29]. Further, an increase in *Bifidobacterium* spp. and *Lactobacillus* spp. populations were observed in some of the studies [19,23,25,38,40].

Out of all the studies included, 12 evaluated the production of short chain fatty acids (SCFA) by bacterial populations. This analysis indicated an increased total SCFA production in most of the studies (n = 7) [19–22,24,25,42]. Further, four studies reported on a reduction of the intestinal or cecal pH [22,35,42,43]. Moreover, regarding proteins that are related to

intestinal permeability and function, most of the studies observed an increase in the gene expression of zonula occludents 1 (ZO-1), occludin, claudin-1 and Mucin (Muc) 2 [20–22,24,25,27,39], and three studies did not observe these effects [33,34,38]. The intestinal morphology was evaluated in some of the studies. From seven studies, which evaluated the number of goblet cells, five studies observed an increased number [17,20,24,25,39], and in the other two studies no changes were observed [34,40]. In addition, increased villi length or height was reported in many of the studies [17,18,22,23,25,39]. Four studies [19,21,22,39] observed a reduction in the serum lipopolysaccharides (LPS), and one study [27] showed a reduction in plasma endotoxin and intestinal permeability.

Table 3. Main findings in animal studies on the effects of anthocyanin on intestinal health.

Reference	Desing (Intervention)	Control	Administration/ Duration of intervention (weeks)	Method of Gut Microbiota Evaluation / Type of Sample	Anthocyanin dosage (total anthocyanin)	Main results (Intervention x control)
Jurgonski, Juskiewicz, Zdunczyk, 2008 [35]	Chokeberry fruit extract (0.2%) + High fructose diet and streptozotocin	High fructose diet and streptozotocin	Oral (diet) / 4	NA	80.9 mg/100 g diet	↓ ileal pH; Mucosal disaccharidase activity: ↓ sucrase and maltase and ↑ lactase; ↔ Total SCFA; ↔ cecum pH; ↔ α- and β- glucosidase, α- and β- galactosidase and β-glucuronidase on cecum.
Jurgonski, Juskiewicz, Zdunczyk, 2013 [36]	Kamchatka berry extract (2g/ kg diet) + Diet with fructose replaced the corn starch	Diet with fructose replaced the corn starch	Oral (diet) / 4	NA	65.4 mg/100g diet	Mucosal disaccharidase activity: ↔ sucrase, maltase and lactase; ↔ cecum pH; ↑ α- and β- glucosidase on cecum; ↔ α- and β- galactosidase on cecum; ↔ ileal pH.
Espley, et al., 2014 [31]	Freeze-dried apple (20%) + Normal diet	Normal diet	Oral (diet) / 3	qPCR Colonic content	397 µg/g diet*	↑ Total bacteria; ↓ *Lactobacillus* spp.; ↔ *Bifidobacterium* spp.; ↔ *Bacteroides-Prevotella-Porphyromonas* group.
Jurgonski, et al., 2014 [43]	Blackcurrant pomace extract (1.5%) + HFD	HFD	Oral (diet) / 4	NA	733.5mg/100g diet	↓ Small intestine pH; ↔ Caecum pH; ↓ β-glucuronidase; ↔ α- and β- glucosidase, α- and β- galactosidase; ↔ Total SCFA cecal.
Paivarinta, et al., 2016 [32]	Bilberry extract (10%) + HFD	HFD	Oral (diet) / 10	PCR-DGGE Cecum content	553.2mg/100g diet	↑ Bacterial diversity in cecal contents.
Overall, et al., 2017 [26]	Blueberry powder (400 µg/g total anth.) + HFD	HFD	Oral (diet) / 12	qPCR Fecal sample	40 mg/ 100 g diet	↑ Abundance of *Bacteroidete* and *Actinobacteria*.

Tong, et al., 2017 [17]	Anthocyanin from red cabbage extract (100mg/kg BW) + CPT-11 (to induce intestinal mucositis)	CPT-11 (to induce intestinal mucositis)	Oral (gavage) / 1	NA	100 mg/ kg BW	↑ Goblet cells mucus; Preservation of the villi height and conserved epithelial cell surface in the ileum and colon.
Fernández, et al., 2018 [37]	Functional sausage (20g with 0.11% anth.) + AOM treatment (to induce CRC tumor)	AOM treatment + Control sausage (20g)	Oral (diet) / 20	NGS Caecal feces	22 mg/20 g sausage	↓ Hyperplastic payer patches in the small intestine mucosa; ↓ level of Desulfovibrionaceae and Enterobacteriaceae and ↑ of Clostridiaceae; ↓ *Bilophila wadsworthia.*
Jamar, et al., 2018 [38]	Juçara powder (0.25%) + HFD	HFD	Oral (diet) / 1	qPCR Colon content	1.65 mg/kg/day	↓ mRNA of TLR-4 in the colon; ↔ mRNA ZO-1; ↑ DNA levels of *Bifidobacterium* spp..
Lee, et al., 2018 [39]	Blueberry powder (10%) + HFD	HFD	Oral (diet) / 8	NGS Caecal content	213.4 mg/100 g diet	↓ *Bacteroidetes* and *Firmicutes* abundance; ↑ Abundance of *Proteobacteria* and *Fusobacteria*; ↑ *Bacilli* and *Lactobacillales;* ↑ mRNA *Muc*2 ileal; ↑ ileal villus lenght and goblet cell number; ↑ serum acetate; ↔ Serum propionate and butyrate; ↓ serum LBS (to asses LPS concentration); ↔ mRNA antimicrobial peptide Defb2.
Paturi, et al., 2018 [40]	Blackcurrant extract (40g/kg) + Control diet	Control diet	Oral (diet) / 6	qPCR Caecal content	1280 mg/100g diet	↓ cecal acetic and butyric and ↑ of propionic acid; ↑ *Bacteroides-Provotella-Porphyromonas* group and *Lactobacillus* spp.; ↓ *Bifidobacterium* spp. and *Clostridium perfringens*; ↔ crypt depth and goblet cells in the colon.
Silva-Maia, et al., 2018 [41]	Aqueous extract of berry (*Plinia jaboticaba*) peel (50g/ L) + Normal diet	Normal diet	Oral (water) / 7	Colonies expressed as CFU Colon content	75 mg/L	↑ *Enterobacteriaceae* and *Bifidobacterium*, and ↔ *Lactobacillus*; ↔ total SCFA.

Van Hul, et al., 2018 [33]	Grape pomace extract (8.2 g/ kg diet) + HFD	HFD	Oral (diet) / 8	NGS Caecal content	35.59 mg/ 100g diet	↑ Abundance of *Bacteroidetes*; ↓ *Desulfovibrionaceae* and *Spreptoccaceae*; ↑ *Prevotellaceae* and *Erysipelotrichaceae*; ↔ mRNA of ZO-1, intectin, occludin, claudin3, Muc2, Reg3ϒ; ↑ mRNA Lyz1; ↔ Total SCFA cecal.
Chen, et al., 2019 [18]	Purified cyanidin-3-O-glucoside (1000mg/kg) + 3MCPD	3MCPD (to damage the intestinal mucosa)	Oral (diet) / 8	NGS Colonic content	1000 mg/ kg diet**	↓ *Bacteroidetes* levels and ↑ *Proteobacteria* and *Actinobacteria*; ↑ Villus height, and number of epithelial cells.
Cremonini, et al., 2019 [27]	Anthocyanin rich mix (40mg/kg) + HFD	HFD	Oral (diet) / 14	NGS Caecal content	40 mg/kg BW	↓ Intestinal permeability; ↓ Plasm endotoxin; ↓ ratio *Firmicutes*/*Bacteroidetes*; ↑ *Romansia* abundance; ↑ Protein expression of occludin, ZO-1 and claudin-1; ↑ Muc2 secretion.
Gu, et al., 2019 [28]	Black rasberry powder (10%) + Control diet	Control diet	Oral (diet) / 6	NGS Luminal content	290 mg/ 100g diet	↓ Abundance of Firmicutes and ↑ of Bacteroidetes; ↓ *Clostridium* ↑ *Barnessiella*
Li, et al., 2019 [19]	Bilberry anthocyanin extract (20 mg/kg) + Old rats	Old rats	Oral (gavage) / 10	NGS Caecal content	20 mg/kg BW	↓ Abundance of *Verrucomicrobia* and *Euryarchaeota;* ↓ Ratio *Firmicutes/Bacteroidetes*; ↑ Species of *Weissella confuse* and *Aspergillus oryzae;* ↑ *Lactobacillus and Bacteroides;* ↑ Total SCFA in cecal content; ↓ β-glucosidade and α-galactosidade and ↔ α-glucosidase, α-galactosidade, and β-glucoronidase; ↓ serum LPS.
Liu, et al., 2019 [29]	Malvindin 3-Glucoside(24mg/ kg diet) + DSS	DSS	Oral (diet) / 50 days	NGS Colon content	24mg/kg diet***	↓ Abundance of *R. gnavus* and ↑ *Clostridium* and *Bacteroides ovatus;* ↑ *Firmicutes*/*Bacteroidetes* ratio; ↑ crypt dilation.

Luo, et al., 2019 [23]	Purified anthocyanin from *L. ruthenicum* (200 mg/kg BW) + HFD + vit. D3	HFD + vit. D3 (to induce atherosclerosis)	Oral (gavage) / 6	NGS Cecal content	105.5 mg/kg BW	↓ Abundance of Firmicutes and ↑ Bacteroidetes; ↑ *Bifidobacterium* and *Lactobacillus;* ↑ *A*bundance of *Lria*, *Akkermansia* and *Lachnospiraceae*; Improvement of structure and villi of the small intestine;
Peng, et al., 2019 [20]	Purified anthocyanin from *L. ruthenicum* (200 mg/ kg BW) + DSS	DSS	Oral (water) / 7 days	NGS Feces samples	200 mg/kg BW	↑ mRNA of ZO-1, occludin, claudin-1; ↑ total SCFA in cecal content and feces; ↑ goblet cells ; ↑ abundance of Actinobacteria; ↔ Abundance of *Firmicutes* and *Bacteroidete*; ↔ *Firmicutes*/*Bacteroidetes* ratio.
Su, et al., 2019 [21]	Pelargonidin-3-O-glucoside (150mg/kg BW) from raspberry + Diabetic *db/db*	Diabetc *db/db*	Oral (gavage) / 8	NGS Caecal content	150 mg/kg BW****	↓ Abundance of *Firmicutes* and ↑ *Bacteroidetes*; ↓ serum LPS; ↑ *Bacteroidetes*/*Firmicutes* ratio; ↑ Total SCFA fecal; ↑ mRNA of occludin e ZO-1, Muc 2, and ↔ claudin; ↑ Pla2g2 and Lyz1 (antimicrobial peptides).
Tian, et al., 2019 [22]	*L. ruthenicum* dried (3%) + Normal diet	Normal diet	Oral (diet) / 10	NGS Fecal pellets	104.2 mg/100g diet	↓ Abundance of *Firmicutes*; ↓ pH feces; ↓ Serum LPS; ↑ Serum and colon sIgA; ↑ *Verrucomicrobia* and *Bacteroidetes*; ↓ *Proteobacteria* and *Deferribacteres*; ↑ Total fecal SCFA; ↑ Ileal villus lenght and ratio of villus to crypt; ↑ mRNA of ZO-1, occludin, JAM-A and Muc2; ↔ Colon crypt length.
Zary-Sikorska, et al., 2019 [42]	Purple carrot root (dried) (10%)	Control (without carrot)	Oral (diet) / 4	NA	12.9 mg/100g diet	↓ Cecal pH; ↑ α- and β-Glucosidase; α- and β-Galactosidade; β-glucuronidase; ↑ Total cecal SCFA.
Cao, et al., 2020 [30]	Blackcurrant extract (1%) + Old rats	Old rats	Oral (diet) / 16	NGS Feces samples	17.41 mg/100g diet	↓ *Firmicutes*/*Bacteroidetes* ratio; ↓ Abundance of *Verrucomicrobia*, ↑ *Bacteroidetes* and ↔ *Firmicutes* and *Proteobacteria*.

Peng, et al., 2020 [24]	Anthocyanins from *L. ruthenicum* (200mg/kg BW)	Control (without anth.)	Oral (diet) / 12	NGS Feces samples	200 mg/kg BW	↑ nº of intestinal villi, goblet cells and intestinal gland; ↑ mRNA of ZO-1, occludin, claudin and Muc1; ↑ total SCFA (cecal content and feces); ↑ *Barnesiella, Alistipes, Eisenbergiella, Coprobacter* and *Odoribacter*; ↔ pH in feces and cecal sIgA.
Rodríguez-Daza, et al., 2020 [34]	Blueberry extract (200 mg/kg BW) + High fat and high sucrose diet	High fat and high sucrose diet	Oral (gavage) / 8	NGS Feces samples	1.68 mg/kg BW	↑ Mucus layer thickness (colon); ↑ *Adlercreutzia equolifaciens*; ↔ Crypt depth and total goblet cells; ↔ *Firmicutes*/*Bacteroidetes* ratio; ↔ mRNA of ZO-1 and occludin.
Wang, et al., 2020 [25]	Black rice extract (0.48g/kg diet) + High fat and cholesterol diet	High fat and cholesterol diet	Oral (diet) / 12	NGS Caecal content	48 mg/100g diet	↓ *Firmicutes*/*Bacteroidetes* ratio; ↑ Abundance of *Bifidobacterium* and *Lactobacillus;* ↑ Cecal SCFA; ↑ Villus height (ileum and caecum); ↑ Goblet cell number per villus of the colon; ↑ mRNA of JAM-A, occludin and Muc-2.

↓: reduced; ↑: increased; ↔: no change. *cyanidin galactoside; **cyanidin-3-O-glucoside; ***Malvindin 3-Glucoside; ****Pelargonidin-3-O-glucoside; Abbreviations: BW: Body weight; HFD: High fat diet; CPT-11: irinotecan; AOM: azoxymethane; DSS: dextan sodium sulfate; ZO-1: zonula occludentes – 1; 3-MCPD: 3-Chloro-1,2-propanediol; SCFA: short chain fatty acids; JAM-A: junctional adhesion molecule-A; L. *ruthenicum*: *Lycium ruthenicum*; CRC: colorectal cancer; Pla2g2: phospholipase A2 group-II; Lyz1: Lysosome-1; LPS: lipopolysaccharides; anth.: anthocyanin; TLR-4: toll like receptor 4; sIgA: secretory Immunoglobulin A; mRNA: messenger ribonucleic acid; Muc: mucin; Defb2: beta-defensin 2; LBS: LPS-binding protein; qPCR: quantitative polymerase chain reaction; CFU: colony forming unit; NA: not applicable; NGS: next generation sequencing; DGGE: denaturation gradient gel electrophoresis.

3.4. Risk of Bias

From all the studies that were included in the current systematic review (n=27), the baseline characteristics, including sex, age and initial weight of animals, were complete in five studies [18,22,23,31,43]. In most of the studies, the allocation of animals was not described in details, since there was no information about the randomization process. Six studies did not mention if the animal allocation to treatment groups was performed randomly [20,21,25,27,37,39]. Furthermore, none of the studies reported about blinding the investigators involved in the research. Four studies did not included all animals in the analysis, and the exclusion criteria were not reported [21,27,32,41] (Figure 2).

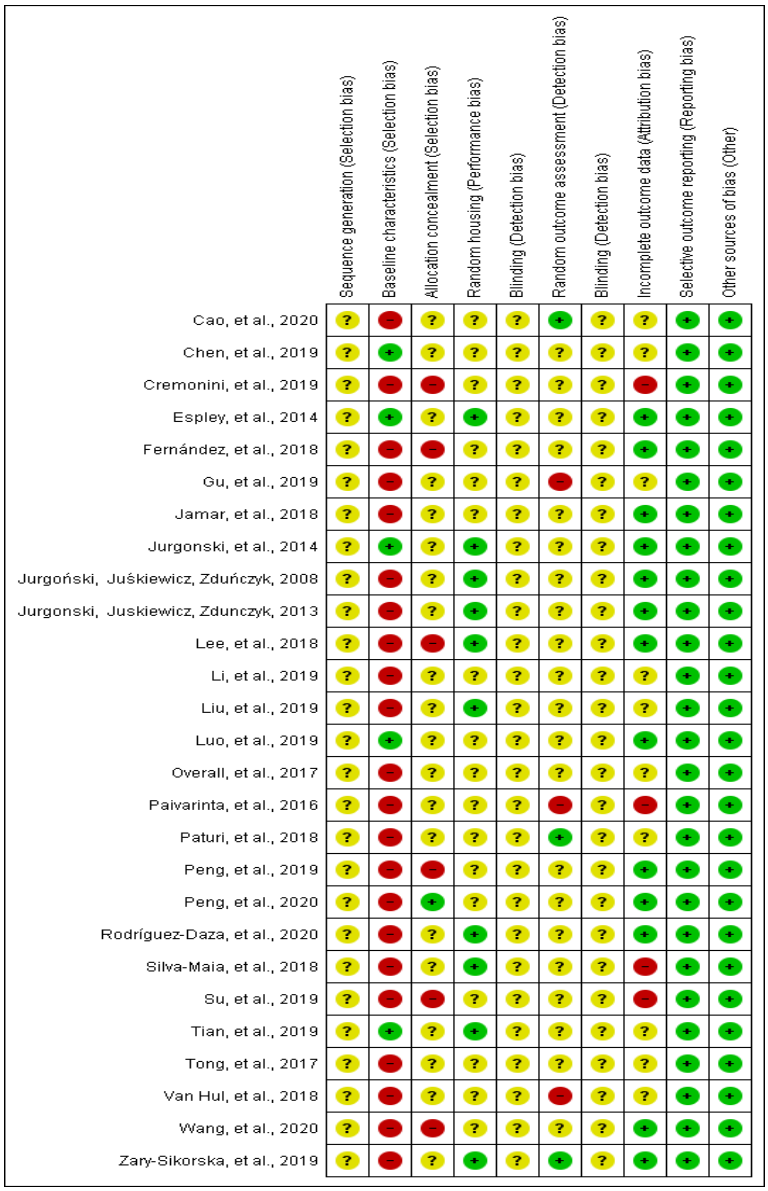

Figure 2. Risk of bias of animal studies.

4. Discussion

In this systematic review, we evaluated the effects of anthocyanins or their extract on intestinal health parameters, *in vivo*. Therefore, this systematic review verified that food sources of anthocyanin or its extract are able to improve intestinal parameters changed by pathologic conditions or dietary patterns.

The animal models of the studies included were high fructose diet, high fat diet, intestinal mucositis, colorectal cancer, damage to the intestinal mucosa, diabetes and old animals. It is highlighted that all of these models promote intestinal changes, such as increased intestinal permeability, inflammation, altered morphology and changes in the intestinal microbiota composition such as dysbiosis. Despite different animal models being used in the studies, all of them except one [43] used rodents as the animal. Similarly, as in humans, Bacteroidetes and Firmicutes are the two main phyla in rodents' gastrointestinal tract. In this sense, microbiota composition in rodents is usually analyzed in interventions that study the casual role of gut microbiota in diet, health and disease interaction [44]. Since all animal models used in the studies of this review promote modification of the gut microbiota at some level, the choice of the best model depends on the main goal of the study [44]. Therefore, bioactive components with functional properties were investigated to verify their potential beneficial effects on intestinal health [7]. Anthocyanins are soluble components, from the class of flavonoids, with functional dietary properties that were previously associated with oxidative stress inhibition, antioxidant activity and intestinal microbiota modulation [45]. Further, the supplementation of anthocyanin cyanindin-3-Oglucoside for eight weeks in Wistar rats was able to restrain the gut microbial dysbiosis that was induced chemically, by suppressing the decrease of *Rothia* and *Romboutsia* and the increase of *Clostridium* verified in the disrupted gut microbiota [18].

In this review, the anthocyanin dietary intake induced increased abundance of *Bacteroidetes* and a reduction of *Firmicutes*. A reduction of the *Firmicutes/Bacteroidetes* ratio in the caecal content was observed in experimental *in vivo* models of high fat diet [25,27]. In recent years, research with animal models in samples of caecal content [23] and humans in fecal samples [46] demonstrated that obese organisms have a high abundance of Firmicutes and a low abundance of Bacteroidetes. These changes in the microbial composition result in an increased absorption of calories, reduced secretion of anorexigenic hormones and intestinal barrier damage [46]. Bacteroidetes and Firmicutes are the two main phylum that inhabit the large intestine, corresponding to 90% of total bacteria [47]. The Firmicutes phylum and gram-

positive bacteria carry more enzymes that are required for carbohydrates metabolism, which contribute to their transport and energy absorption [48], besides higher fat deposition in adipocytes [49].

Following anthocyanin consumption, most are not absorbed in the upper gastrointestinal tract, and therefore reach the colon intact [11]. At the colonic level, anthocyanins are metabolized by the local microbiota, initially via deglycosylation, and is followed by a secondary degradation into phenolic acids, mainly protocatechuic, vanillic, syringic, gallic and p-coumaric [45]. The main bacterial populations that are able to metabolize anthocyanin are the *Bifidobacterium* spp. and *Lactobacillus* spp., which have probiotic effects, including the production of antimicrobial substances, competition with pathogens for adhesion to the epithelium and nutrients, immunomodulation and inhibition of bacterial toxin production [50]. In addition, these bacteria have enzymes such as β-glycosidase that are needed to catalyze reactions that release the glycose from the aglycon and provide the energy needed for bacterial populations to prosper [12]. In several studies included in this review, an increase of *Bifidobacterium* and *Lactobacillus* was documented [19,23,25,38,40]. In this context, 20-day intake of dealcoholized red wine in healthy adults increased the fecal concentration of *Bifidobacterium*, *Enterococus* and *Eggerthella lenta*. In addition, in this study, the produced metabolites associated with the increase of *Bifidobacterium* were those derived from anthocyanin degradation (4-hydroxybenzoic, syringic, p-coumaric, homovanillic ácidos) [51]. *Bifidobacterium* is related to pathogen inhibition by organic acids production, antimicrobial peptides and immune stimulation [52]. Besides this, among the acids produced by the microbial metabolism of anthocyanin, protocatechuic acid presents inhibitory effect on pathogenic bacteria growth [53], and gallic acid is effective in reversing changes in the microbiota caused by induced colitis in animals, by reducing Firmicutes and Proteobacteria and increasing Bacteroidetes [54]. Therefore, the beneficial effects that are associated with anthocyanin consumption may be achieved by its metabolites post-degradation.

Some of the physiological properties of the gut microbiota are attributed to fermentation of non-digestible carbohydrates by anaerobic bacteria, producing short chain fatty acids (SCFA) [47]. In this review, the dietary supplementation of anthocyanin was able to increase the production of total SCFA (acetate, propionate and butyrate) in the majority of the studies. SCFA act as a fuel to intestinal cells by stimulating the cellular proliferation. The production of SCFA also reduces the intraluminal pH, which limits the growth of pathogenic bacteria due to the acidification [55,56]. Therefore, SCFA assist to maintain the intestinal epithelium

integrity, and protect the host from potential immune and inflammatory diseases [56]. In a mice study, where diet included anthocyanin extract, SCFA production increased due to elevated microbial activity, specifically of *Bifidobacterium* and *Lactobacillus* [25]. Further, the increased abundance of *Roseburia* is associated with a higher production of SCFA in the intestine, and the abundance of *Akkermansia* was associated with propionic production [23]. SCFA also have an immunomodulatory effect by promoting the development of mucosal regulatory T cells (Tregs) through the interaction with the G protein-coupled receptor (GRP43) and the inhibition of histone deacetylases (HDACs) [57,58]. Furthermore, SCFA exert positive effects to the turnover and differentiation of colonic epithelial cells, and to stimulate the mucus production that prevents the pathogenic bacteria adherence [56]. It is suggested that these acids activate the mammalian target of rapamycin (mTOR) complex and the STAT3 (signal transducers and activator of transcription 3) in the intestinal epithelial cells, which promote the expression of antimicrobial peptides as β-defensin and RegIII [59]. From the included studies in this systematic review, two observed an increase of Lysosome-1 (Lyz1) peptide, which acts to maintain the microbiota homeostasis and to eliminate commensal microorganisms [21,33]. Therefore, by these mechanisms, SCFA act to maintain the epithelial barrier integrity [60,61].

The intestinal epithelial barrier consists of a mucus layer and cells attached by a protein complex, including tight junctions, adherents junctions and desmosome [62]. The tight junctions complex is composed by proteins as claudins, occludin, junctional adhesion molecule (JAM-1) and zonula occludents (ZO-1), and the rupture in some of these proteins increases the paracellular permeability with permeation of pro-inflammatory molecules, immune activation and inflammation [63]. In this review, the majority of the included studies verified an increase in the gene expression of ZO-1, occludin and claudin 1. The anthocyanin has an anti-inflammatory effect through inhibition of the factor nuclear kappa B (NF-kB), and via regulation of I-Kappa-B-alpha (IkBα) phosphorylation that decreases the gene expression of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFNγ) and interleukins [64,65]. These cytokines are related to intestinal barrier damage by harming the tight junction protein expression [66]. The TNF-α promotes changes in the tight junctions via its receptor tumor necrosis factor receptor 1 (TNFR-1), so that anti-TNF strategies promoted tight junctions' rearrangement with an improvement of occludin and ZO-1 [67]. Therefore, the anthocyanidin cyanidin-3- glucoside (C3G) was able to inhibit the IkBα phosphorylation and the nuclear translocation of NF-kB (p65), and these effects were associated with the nuclear transcription factor Nrf2 (erythroid-2-related factor 2) that induces the

expression of antioxidant enzymes [68]. Therefore, the anthocyanin can act directly or indirectly to improve tight junction's integrity.

Moreover, the intestinal epithelial cells are covered by a mucus layer produced and secreted by the goblet cells. The mucus is composed of glycoproteins of mucins, mainly Muc2, forming a viscous layer that protects against pathogen invasion by preventing the sites of binding for bacteria [69,70]. The discontinuous mucus layer in the cecum of rats are considered hotspots for Salmonella; thus, the absence of the mucus layer can lead to infections' development [71]. In addition, the increase in goblet cell number, mainly those classified as acids, promotes barrier function improvement by increasing the mucin secretion that prevents pathogen invasion and possible intestinal inflammation due to acidic mucin resistance to degradation by bacterial glycosidase and has higher viscosity [72]. SCFA also contribute to the intestinal cell proliferation since they stimulate the proliferation and differentiation of enterocytes [73]. Thus, there is evidence that the inflammasome nucleotide-oligomerization domain-like receptor 6 (NLRP6), expressed mainly on enterocytes, controls the mucin secretion and the mucosa renewal by the goblet cells [74]. A previous study with rats suggested that SCFA can activate the colonic NLRP6, thus protecting the intestinal barrier [75]. Considering this evidence, it is verified that the mulberry (50 mg anthocyanin/kg diet) supplementation in animals with induced colitis resulted in an increase in goblet cells and NLRP6 expression, therefore suggesting a link between mucin secretion and antimicrobial peptide production [76].

In order to optimize the digestion and absorption of nutrients, the gut (duodenum) morphology is unique and organized in villi [77]. It is observed that anthocyanins are related to an improvement of the absorptive function by increasing the villus length, villus length/crypt depth ratio and the total mucosa thickness [78]. These intestinal morphology changes result in a better intestinal digestion and absorption, since they assure a higher absorption surface, brush border enzyme expression and nutrient transport system [79].

Disruption of the intestinal barrier integrity can be occasioned by tight junction disruption and mucus layer depletion, thus allowing the paracellular translocation of bacteria and their components, such as lipopolysaccharides (LPS) [63]. Of the included studies in this systematic review, a few evaluated the endotoxemia; however, of these, the anthocyanin supplementation was able to reduce the intestinal permeability, endotoxemia and the levels of serum LPS [19,21,22,27,39]. LPS are cellular wall components of gram-negative bacteria that contain a pathogen-associated molecular pattern, Lipid A, able to interact with Toll-like Receptor 4 (TLR-4) via the Myeloid differentiation primary response 88 (MyD88) protein [80].

This interaction results in the activation of the pathway downstream and NfkB translocation, thus increasing the gene transcription of cytokines such as TNF-α, IL-1β e and IL-6 [80,81]. The analysis of monocyte from obese individuals supplemented with berry juçara (5 g/day; 131.2 mg total anthocyanins) for six weeks observed the reduction of mRNA (messenger ribonucleic acid), TLR4 and the protein expression of MyD88 [82]. Therefore, it is known that the inflammation mediated by LPS can exert local and systemic effects and be related to gastrointestinal diseases, such as Crohn disease [83], inflammatory bower disease [84] and metabolic disorders as diabetes mellitus type 2 [85] and obesity [86].

Animal experiments assist to design clinical studies in terms of doses, duration, type of intervention and other topics [15]. In this context, the positive changes observed at the gut microbiota following anthocyanin supplementation and consumption in the animal studies included in this review corroborate with results verified in several clinical studies. Cranberry consumption (30 g, with 83.7 mg anthocyanin) for five days by healthy adults resulted in increased abundance of Bacteroidetes and decreased abundance of Firmicutes [87]. Further, the intake of dealcoholized red wine for 20 d (9.72 mg anthocyanin) increased the fecal concentration of *Bifidobacterium* and *Enterococus* [51]. Thus, we highlighted the complexity of animal models of gut microbiota, which are able to tolerate the presence and effects of dietary components such as anthocyanins in a similar manner as described in human studies. Further, animal model studies related to anthocyanin supplementation are effective in demonstrating the safety and efficacy of their consumption. Hence, these aspects are relevant and important for the translation of results and adaptation to clinical studies, and in order to establish dietary guidelines for humans.

Finally, systematic reviews guarantee the gathering of evidence related to a specific topic; therefore, they obtain conclusions with greater scientific rigor. The evidence verified in this review, performed with 27 studies, demonstrates that the anthocyanin dietary intake is beneficial and improves specific parameters such as the gut microbiota composition, short chain fatty acids production and the intestinal physical barrier, such as the increase of tight junction protein and goblet cell number, and the reduction of intestinal permeability, that together promote intestinal health.

Dosage and Reporting Quality

This systematic review showed high heterogeneity among the studies, with several experimental models used, distinct methodologies in the intestinal parameters analyzed and

high variation related to dose and time of anthocyanin supplementation. Probably, these variations were observed because of the large number of studies included. The anthocyanin supplementation dose ranged from 12.9 mg/100 g diet as dried purple carrot [42] to 1280 mg/100 g diet as extract [40]. The supplementation as an extract allows for the delivery of a higher dose of anthocyanins, since it concentrates the components, while the anthocyanin intake in its food source provides a lower dose, based on average daily intake of the animal. Hence, the safety of anthocyanin intake were tested and approved in animals, indicating no toxicity or any adverse effects to animal health, even at a high dosage [88]. In addition, studies showed that the anthocyanin supplementation exposure period varied from 1 week [17,38] to 20 weeks [37]. Analyzing the outcomes individually, the time of supplementation from 1 to 12 weeks promoted the increase of *Bifidobacterium* and *Lactobacillus*, the production of SCFA and an improvement of goblet cells and villi length or height; further, the increase of proteins related to intestinal permeability was verified from the time of 1 to 14 weeks of intervention. Thus, for the design of studies for new researches evaluating the effects of anthocyanin on intestinal health, this range of time should be considered in accordance with the goal of the research. Beneficial effects of anthocyanin supplementation were observed even in the lowest dosage [42] and the shortest exposure period [17,38]. Furthermore, most of the reviewed studies observed that anthocyanin was quantified as total anthocyanin; however, few studies showed its profile, as specific monomers of anthocyanidin such as cyanidin galactoside [31], cyanidin-3-O-glucoside [18], malvindin 3-glucoside [29] and pelargonidin-3-O-glucoside [21].

This systematic review evaluated the effects of dietary anthocyanin in the context of its potential intestinal health-promoting effects, such as beneficially changing the intestinal microbiota, increasing the short chain fatty acid production, reducing the intestinal permeability and improving parameters related to the intestinal physical barrier such as tight junctions protein and goblet cell number. The studies' selection was based on methodologies that are recommended and approved for systematic review, thus allowing reliable conclusions. The risk of bias was evaluated according to the SYRCLE RoB tool [15], which establishes consistency and avoids discrepancies to evaluate the risk of bias from animal studies. Twenty-one (n = 21) studies did not show the animals' completed baseline characteristics, and none of the studies showed information about whether researchers were blinded from knowledge of the intervention groups and/or the outcome assessor. In this sense, the absence of some baseline characteristics probably had no influence on the main conclusion of this review, since those characteristics were not comparable among studies. On the other hand, the risk of bias related

to blind researchers and outcome assessors could have influenced the results of outcomes in each study. However, the conclusion of this review was performed with a large number of studies in association; thus, these biases may not represent a major impact on the main conclusions, considering the methodologic rigor that this review followed. Further, the risk of bias in analysis may represent a lack of information regarding the experimental design of animal studies, showing that progress is needed in this field. Therefore, we suggest that research performed with animals follows the SYRCLE protocol to avoid a lack of information in the studies.

5. Conclusions

The scientific evidence from the reviewed *in vivo* studies demonstrates that the supplementation of anthocyanin is effective to modulate the intestinal microbiota through the increase of *Lactobacillus* spp. and *Bifidobacterium* spp., and to increase the production of short chain fatty acids. In addition, reviewed studies observed an improvement in the intestinal barrier by the increased expression of tight junction protein associated to an improvement of cells morphology and mucus production, which reduces the potential risk of inflammation (Figure 3). We highlighted that these intestinal changes in association may be the mechanisms by which upon anthocyanin supplementation that ranged from 1 to 14 weeks with the dosage that ranges from 12.9 to 1280 mg/ 100 g diet exerts beneficial effects on the intestinal health. We consider that is not adequate to establish a specific dose and a specific time to achieve all of these effects in association, since different animal model, methodologies, and a large range of time and dose of anthocyanin supplementation were observed. However, considering the methodology rigor that this review followed, the doses and times intervention ranges observed could be used as guidelines for future researches. Despite the limitation of extrapolating animal results to human, with knowledge of all of the benefits observed, we consider that the daily intake of foods source of anthocyanin should be stimulated to the population acting as a strategy to prevent health problems.

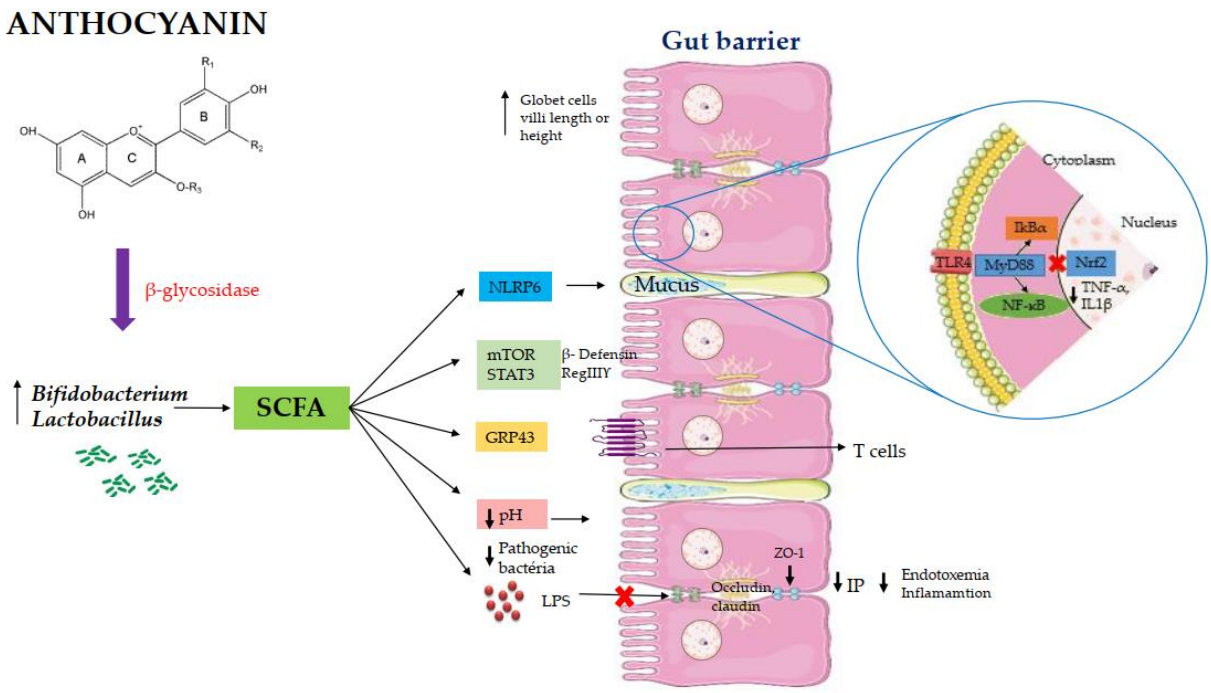

Figure 3. Proposed mechanisms of action of anthocianins on the intestinal health.

Abbreviations: ZO-1: zonula occludentes – 1; SCFA: short chain fatty acids; LPS: lipopolysaccharides; TLR-4: Toll like receptor 4; IP: intestinal permeability; mTOR: mammaliam target of rapamycin; STAT3: signal transducers and activator of transcription 3; NF-κB: factor nuclear kappa B; MyD88: Myeloid differentiation primary response 88; IkBα: I-Kappa-B-alpha; NLRP6: inflammasome nucleotide-oligomerization domain-like receptor 6; GRP43: G protein-coupled receptor; TNF-α: tumor necrosis factor alpha IL1β:interleukin 1 beta; Nrf2: erythroid-2-related factor.

Registration and Protocol

This systematic review was realized according to the protocol: *Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement 2020* [89]. The review is registered in the PROSPERO under the number CRD42020204835 (Centre for Reviews and Dissemination, University of York). A systematic review was carried out to answer the question: "Does foods source or extract of anthocyanin can promote changes on intestinal parameters?"

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and E.T.; project administration, H.S.D.M., M.C.D.P, and E.T. All authors have read and agreed to the published version of the manuscript."

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6. ORIGINAL RESEARCH RESULTS

6.1. PAPER 2

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Black corn (*Zea mays* L.) whole flour improved the antioxidant capacity and prevented adipogenesis in mice fed a high-fat diet

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Black corn (*Zea mays* L.) is a source of anthocyanins, which have shown the ability to reduce metabolic disorders. This study investigated the anti-inflammatory, antioxidant, and anti-adipogenic preventive effects of black corn. C57BL/6 mice were divided into 3 groups (n = 10): normal control (NC): AIN-93 M; high-fat diet (HF); HF + corn (20%) (HFC). Black corn improved the antioxidant status, through the superoxide dismutase hepatic levels and serum total antioxidant capacity. Animals fed an HFC diet showed decreased gene expression of sterol regulatory element-binding protein-1c (SREBP-1c) and peroxisome proliferator-activated receptor-γ (PPARγ) and increased gene expression of adiponectin and lipoprotein lipase in the adipose tissue, which led to a less inflammatory infiltrate and decreased the adipocyte number and length. In the liver, black corn reduced the gene expression of SREBP-1c and acetyl CoA carboxilase 1. Therefore, black corn whole flour improved the antioxidant capacity, contributed to hepatic β-oxidation, and decreased adipogenesis in animals.

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1 Introduction

Overweight and obesity are defined as the abnormal or excessive fat accumulation that may affect health. Currently, 39% of adults are overweight and 13% are obese. An excess of adipose tissue plays a central role in the development in metabolic disorders such as type-2 diabetes, insulin resistance, cardiovascular diseases, and metabolic syndrome.[1] A sedentary lifestyle and diet are mainly responsible for the excess of adiposity, mainly the high consumption of saturated fat diets. Thus, the physiological mechanisms related to a high-fat diet include overconsumption, efficient storage of dietary fats in the adipose tissue and alterations in hormones responsible for energy balance, such as leptin and insulin.[2] The consumption of an unbalanced diet with energy intake exceeding energy expenditure promotes an excess of free fatty acids and glucose, which initiates a downstream effect of producing energy and storage into adipocytes. For this, there is an increase of superoxide production in the mitochondria overloading the electron transport chain. Therefore, reactive oxygen species (ROS) production increases, which promotes changes in the redox status, thus activating transcription factors such as the transcription nuclear factor-kappa B (NF-κB) that is a mediator of inflammatory responses.[3] In addition, due to the positive energy balance white adipose tissue can undergo adipocyte hypertrophy, thus secreting pro-inflammatory factors, and hyperplasia through adipogenesis, which includes a cascade of transcriptional factors and cell cycle proteins leading to mature adipocyte development.[2] The differentiation of pre-adipocytes into mature adipocytes is mainly regulated by transcription factors peroxisome proliferator-activated receptor-γ (PPARγ), sterol regulatory element-binding protein-1c (SREBP-1c) and CCAAT/enhancerbinding proteins (C/EBPs).[4] The PPARγ is mainly expressed in the white adipose tissue, and it is responsible for regulating adipogenesis, lipid metabolism, and insulin sensitivity.[5] In addition, a dysfunction of adipose tissue, as observed in obesity, promotes an increase of pro-inflammatory adipokine secretion and a downregulation of anti-inflammatory adipokines, namely adiponectin.[2]

Thereby, continuous efforts have been made to decrease the incidence of obesity and its metabolic consequences. Researchers have focused on alternatives to improve the quality of diet through the consumption of foods with bioactive components that exhibit health effects.[3] One of these bioactive compounds group is anthocyanins, classified as flavonoids. These substances are water-soluble plant pigments responsible for the black, red, and blue colours in fruits, vegetables, and flours.[6] Important properties of anthocyanins are antioxidant and anti-inflammatory capacities, which have beneficial effects against many biological disorders, such

as cardiometabolic disease, diabetes, and dyslipidemia. Epidemiological evidence has shown that the health effects of anthocyanins are mainly due their antioxidant and anti-inflammatory effects.[6] Thus, food alternatives that could improve the daily consumption of anthocyanins have been investigated. Black corn (*Zea mays* L.) belongs to an abundant variety of colored corn genotypes generated due to their adaptability to ecological conditions, soils, and climates.[7] Purple corn, similarly as black corn, is a source of polyphenols, mainly anthocyanins, which confer the black color to the kernels and other parts of the cob. Positive effects in biological variables have been associated with colored/pigmented maize. Purple corn extract (200 mg anthocyanin per kg) elevated the antioxidant status and downregulated the gene expression of pro-inflammatory cytokines in the liver in high-fat diet induced obese mice.[8] Furthermore, purple maize extract, rich in anthocyanins (98.9 μg mg−1 of cyanidin-3-glucoside), reduced the adipose tissue and adipose inflammation in mice fed a high-fat diet.[9] However, extracts of polyphenols are degraded more easily than those in its whole food matrix from which the extracts originate, which is associated with the absence of the protective effect from food components. The inclusion of anthocyanins into food matrices can protect them from intestinal degradation.[10] Moreover, it is suggested that different bioactive phytochemicals with health-promoting effects can have some type of interaction, thus acting in synergism.[11] Thus, it is still necessary to evaluate the antioxidant and anti-adipogenic effects of the anthocyanins in black corn as a food matrix to the risk factors for metabolic disorders. Therefore, this study aimed to investigate the preventive effects of black corn whole flour in the adipogenesis, antioxidant, and anti-inflammatory capacities in mice fed a high-fat diet.

2 Material & methods

2.1 Materials

The black corn (TO002) was provided by the Brazilian Agriculture Research Corporation (EMBRAPA). The TO002 black color maize genotype is an access of the Maize Germplasm Bank of the Embrapa Maize and Sorghum research unit. The material was produced in the 2018/2019 harvest season at “Embrapa Milho e Sorgo” experimental farm, Sete Lagoas, Minas Gerais, Brazil. The samples were stored in plastic bag, kept at 4 ^{0}C, and protected from light until processing. The black corn flour was prepared using a knife mill grinder with a 1.0 mm stainless steel sieve (Willy, Solab®), which was stored at 4 ^{0}C.

2.2 Chemical characterization

The chemical characterization of black corn whole flour was performed according to the Association of Official Analytical Chemists (AOAC) methods.[12] Protein content was determined by the Kjeldhal method.[12] Samples were subjected to digestion, distillation, and titration to obtain the nitrogen content, and then protein content was determined by the nitrogen conversion factor 6.25. Lipid content was determined by extraction in the Soxhlet method[12] using ethyl ether as the solvent extractor. Total dietary fiber (soluble and insoluble fiber) was determined by the gravimetric-enzymatic method[12] using the commercial kit (Total dietary fiber assay kit, Sigma®, San Luis, Missouri, EUA). To perform enzymatic hydrolysis, it was used the α-amylase, protease, and amyloglucosidase enzymes. The total dietary fiber content was determined by the sum between the soluble and insoluble fractions. The moisture was determined by using an oven (Nova Etica®, model 400/6ND, Sao Paulo, Brazil) at 105 ^{0}C, and ashes were quantified using a muffle furnace (Quimis, Q320M model, Brazil) at 550 ^{0}C.[12] Carbohydrates were calculated as the difference, using the equation: [100 – (% moisture + % lipids + % proteins + % total dietary fiber + % ash)]. The black corn flour total energy value was estimated considering the conversion factors of 4 kcal.g^{-1} for protein and carbohydrates, and 9 kcal.g^{-1} per lipid.

2.3 Total polyphenols, total monomeric anthocyanins, and antioxidant activity

The extraction of total phenolic compounds was performed as described in methodology adapted from Lao & Giusti (2018).[13] Briefly, five grams of black corn flour was macerated with 50 mL aqueous ethanol 50% (*v/v*) and acidified with hydrochloric acid (HCl) at room temperature. It was submitted to an orbital shaker for 1 hour in the absence of light. After, the extract was filtered through filter paper (number 4) and submitted to the rotary evaporator at 40 ^{0}C to evaporate the solvent.

Total polyphenols were determined by single-Ciocalteau colorimetric assay as methodology of Singleton & Rossi, 1965.[14] The absorbance was measured at 760 nm and the total polyphenols were expressed as mg of gallic acid equivalent (GAE)/100 g of wet weight sample.

The antioxidant activity was determined by the ABTS (2,2`-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)) radical cation decolorization assay using Trolox as the standard. Result was expressed in mM Trolox/ g of wet sample.[15]

The total anthocyanin content was determined by the pH differential method, according to Giusti & Wrolstad, 2001.[16] Samples were prepared and diluted in solution of potassium

chloride 0.025 M (pH 1.0) and sodium acetate 0.4 M (pH 4.5) buffer. Then, the absorbance was measured at 510 and 700 nm. To calculate the total anthocyanins content the equations (1) and (2) were used. The result was expressed as mg of cyanidin-3-glycoside/100 g of black corn flour (molar mass 449.2 g/mol; molar absorptivity coefficient 26,900 L/mol).

(1) $A= (A\lambda_{vis\text{-}max} - A_{700})_{pH\ 1.0} - (A\lambda_{vis\text{-}max} - A_{700})_{pH\ 4.5}$

(2) $mg/L= (A \times MW \times DF \times 1000) / (\varepsilon \times 1)$

2.5 Chromatographic profile and determination of anthocyanins

The sample of black corn flour were extracted with 5.0 mL of methanol: formic acid 10% (*v/v*) solution, using sonication 40 KHz (USC 1400, Unique, Indaiatuba, SP) for 15 min. Then, the samples were centrifuged at 112 *g* for 10 min and the supernatant collected. This step was repeated 3 times. The supernatant was concentrated in rotary evaporator and 2 mL of deionized water was added. A total of 20 µL were removed and filtered to injection.

The analysis was carried out using a liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry (LC-DAD-ESI-MS/MS). The HPLC model was Varian 250 (Varian Inc., Lake Forest, CA) coupled with diode array detector (DAD) and mass spectrometer 500-MS IT (Varian Inc., Lake Forest, CA). The column was Symmetry C18 (3µm, 250 x 2 mm) (Varian Inc., Lake Forest, CA). The flow rate was 0.4 mL/min and oven temperature of 30 ^{0}C. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient was varied linearly from 10 to 26% B (*v/v*) in 40 min, to 65% B at 70 min, and finally to 100% B at 71 min and held at 100% B at 75 min. The DAD was set up at 270 to 512 nm to monitor the UV/vis absorption and the UV/Vis spectra were recorded from 190 to 650 nm. Mass spectra were acquired simultaneously using electrospray ionization in the positive and negative ionization (PI and NI) modes in voltage 80 V fragmentation for a mass range of 100-1000 amu. A drying gas pressure of 35 psi, nebulizer pressure of 40 psi, a drying gas temperature of 370 ^{0}C, capillary voltage of 3500 V for PI and 3500 V for NI, in addition to field voltages of 600V spraying were used. The LC system was directly coupled to the MSD with 50% splitting.

2.6 *In vivo* study

2.6.1 Animals

Thirty C57BL/6 male mice with 8 weeks old (23.93 ± 0.20 g) were obtained from Centro de Biologia da Reprodução – Universidade Federal de Juiz de Fora (Brazil-MG) and housed

under controlled light (12-hours light/dark cycle) and temperature conditions (22 ± 2 ^{0}C) with access to water and food *ad libitum*. The number of animals per group was calculated based on the sample calculation equation.[46] For this, the following variables were considered: α-level = 5 %, α-error type I= 1.96, and data of fat mass mean from Schoemaker, et al., 2017.[47] The study was approved by the Ethics Commission of Animal Experimentation of the Universidade Federal de Viçosa (CEUA/UFV), Brazil, under the process number 10/2019. All the experimental procedures with animals were performed in accordance with the ethical principles for animal experimentation.

2.6.2 Experimental design

To assess the effect of black corn on metabolic disorders induced by high-fat diet the animals were randomized by body weight (bw)[18]. All experimental mice were acclimatized for one week, and then the animals were divided into 3 groups and received one of the following diets for the experimental period of 8 weeks: Normal control diet (NC group): AIN-93 M (n = 10); positive control, high- fat diet (HF) (n = 10); high- fat corn diet (HFC): high fat diet with black corn whole flour (n = 10). The normal control diet was composed by the standard diet AIN-93 M[19] and the high fat diet was prepared in the proportions of calories: 59% from fat, 13% from protein and 28% from carbohydrate.[20] The black corn was added at the HF diet at 20 % (200 g / kg of diet). The amount of black corn was based on the substitution of 50% of dietary fiber recommendation for rodents [19] as suggested in previous studies [18]. Therefore, 200 g of black corn flour / kg diet was used, which provided the replacement of 95.7% starch, 31.1% protein and 21.5% oil (Table 1), in which the experimental diets had the same amount of protein, carbohydrate, and lipids among them.

At the end of the experiment (after 8 weeks), after 12-h fast, the animals were anesthetized with isoflurane (Isoforine, Cristália®) and euthanized by cardiac puncture. The blood was collected in serum-separating tubes 16 x 100 mm (Labor Importer®) and centrifuged (Hermle®, Z216MK, Germany) under refrigeration at 4 ^{0}C at 3000 *g* for 15 min to obtain the serum. The hepatic and adipose tissue were collected and washed with phosphate-buffered saline solution (PBS), weighed, and stored at -80 ^{0}C until the time of analysis. Further, part of liver and adipose tissue samples was washed in PBS fixed in 10% formaldehyde and kept at room temperature for further histological analysis.

Table 1 Composition of the experimental diets (g.100g^{-1}).

Ingredients	**NC**	**HF**	**HFC**
Albumin*	17.90	17.90	12.30
Maltodextrin	15.50	15.50	15.50
Corn starch	42.67	11.40	0.50
Sucrose	10.00	10.00	10.00
Soybean oil (mL)	4.00	4.00	3.14
Lard	-	31.20	31.20
Cellulose	5.00	5.00	2.36
Mineral mix	3.50	3.50	3.50
Vitamin mix	1.00	1.00	1.00
Choline bitartarate	0.25	0.25	0.25
L-cystine	0.18	0.18	0.18
Black corn whole flour	-	-	20.00
Caloric density (kcal.g^{-1})	3.85	5.41	5.29

NC: normal control group; HF: high fat group; HFC: high fat corn group. * Albumin based on 77.9% protein content.

2.7 Food efficiency and anthropometric measures

Individual body weight and food consumption were recorded weekly. Food efficiency ratio (FER) was calculated by the equation: [body weight gain (g)/ food intake (g)] x 100.[20]

The Body Mass Index (BMI) was calculated by the ratio between body weight and the squared length. Abdominal circumference was determined by measuring the midpoint between the nose and the pelvic limbs of the animals.[21] Lee index was calculated by the ratio between cube root of the body weight and nose-to-anus length.[21] For the determination of adiposity index, the adipose tissue weight was divided by total body weight and multiplied by 100.[21] The hepatosomatic index was calculated as the liver weight divided by the total body weight.[21]

2.8 Biochemical analyses

Enzymatic essays for glucose, total cholesterol (TC), high-density lipoprotein (HDL-c), low-density lipoprotein (LDL-c), total triglycerides (TG), aspartate aminotransferase (AST),

and alanine aminotransferase (ALT) were performed in animal serum by colorimetric methods according to the manufacturer`s instructions (Bioclin®, Belo Horizonte, Brazil).

2.9 Antioxidant enzymes and total antioxidant capacity

The antioxidant enzymes were measured in the liver. For this, the liver was homogenized in 10% (w/v) ice phosphate buffer (pH 7.4). The homogenate was centrifuged at 2000 *g* for 10 min at 4 ^{0}C and the supernatant used for the analyses. The total protein concentrations were quantified in liver homogenates by Brandford (1976).[22]

Superoxide dismutase (SOD): the quantification of SOD was performed in relative units, and one unit was defined as the amount of SOD enzyme that inhibits the auto-oxidation of pyrogallol. The analysis was carried out on a spectrophotometer (Multiskan GO - Thermo Scientific; Vienna, Austria) at 570 nm and the results were expressed as units of SOD per milligram of protein.[23]

Catalase (CAT): it was determined by the ability to cleave hydrogen peroxide (H_2O_2) in water. Catalase activity was analyzed at 0, 30 and 60 s after the reaction had initiated and the absorbance was read at 240 nm on a spectrophotometer (T70+UV/VIS Spectrometer). The absorbance was calculated by the delta obtained from the initial absorbance (time 0) minus the final absorbance (60 seconds). The result obtained was multiplied by the constant 2.361. The results were expressed as U of catalase/ mg of protein.[24]

Malondialdehyde (MDA): it was measured by the method of thiobarbituric acid reactive substances (TBARS). The analysis was carried out on a spectrophotometer (Multiskan GO - Thermo Scientific; Vienna, Austria) at 535 nm and the results were expressed as moles MDA/ mg protein.[25]

Total antioxidant capacity (TAC): it was measured in the serum and in the liver, homogenate using the Antioxidant Assay Kit (code MAK187; Sigma-Aldrich, St. Louis, USA), which analyzes the capacity of the serum antioxidants to inhibit oxidation of ABTS by the action of methemoglobin. A total of 10 µl of serum and liver homogenate were used and the absorbance measured at 405 nm.

2.10 NF-κB, PPAR (α, γ) and TLR-4 quantification

The extraction of the nuclear fraction from liver and adipose tissue was performed using a specific kit (NE-PER^{TM} Nuclear and Cytoplasmic Extraction Reagents – Thermo Scientific® Fisher, Vienna, Austria) to separate proteins from nuclear and cytoplasmic fraction, following

the manufacturer's instruction. The quantification of factor nuclear kappa B (NF-κB) p65, peroxisome proliferator-activated receptor alpha (PPARα), and toll-like receptor 4 (TLR-4) was determined in hepatic tissue. Further, the quantification of NF-κB p65 and peroxisome proliferator-activated receptor gamma (PPARγ) was determined in the adipose tissue.

The nuclear fraction was assessed by immunoassay using specific kits (Elabscience®, USA, Houston, U.S.A.): Elisa Rat PPARα Kit (code E-EL-R0725); Elisa Mouse PPARγ Kit (code E-EL-M0893); Elisa Mouse NF-κB p65-Kit (code E-EL-M0838); Elisa Rat NF-κB p65-Kit (code E-EL-R0674) and Elisa Rat TLR-4 Kit (code E-EL-R0990), according to manufacturer`s recommendations. Absorbance was measured at 450 nm wavelength. The final concentration on samples was calculated by comparing them with corresponding standard curve.

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

Total RNA was extracted from the liver using TRIzol reagent (Invitrogen, CA, USA) and in the adipose tissue using a specific kit (mirVana™ miRNA Isolation Kit) according to the manufacturer's protocols. RNA concentration and purity were evaluated by Microdrop plate spectrophotometer Multiskan™ GO (Thermo Scientific, DE, USA). The M-MLV Reverse Transcriptase Kit (Invitrogen) was used for cDNA synthesis. The gene expression relative quantification was performed by RT-qPCR using AB StepOne Real Time PCR System equipment and Fast SYBR Green Master Mix (Applied Biosystems, CA, USA) reagent. The initial parameters used to run were 20 s at 95 °C and then 40 cycles at 95 °C (3 s), 60 °C (30 s) followed by melting curve analysis. The primers used in amplification on the adipose tissue and liver are listed in Table 2. All primers were designed by using Primer 3 Plus program and obtained from Sigma-Aldrich Brazil Ltda. Gene expression was calculated using 2-Delta-Delta C (T) ($2^{-\Delta\Delta Ct}$) method. The relative mRNA levels were normalized to the endogenous control by using GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) in the hepatic tissue and β-actin in the adipose tissue. High fat diet-group values were used as standard, which as normalized to 1.

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

Genes	Forward	Reverse
SREBP-1c	CGC TAC CGT TCC TCT ATC AAT GAC	AGT TTC TGG TTG CTG TGC TGT AAG

ADIPOR2	CAT GTT TGC CAC CCC TCA GTA	ATG CAA GGT AGG GAT TCC A
ACC-1	TCA AGA CGG CTC AGG TCA TCA	AGG CGC CAA ACT TCA GCA TC
LPL	TCA ACC ACA GCA GCA AGA	CCG ATA CAA CCA GTC TAC TAC AA
Adiponectin	ATG AGT ACC AGA CTA ATG AGA C	GGC AGG ATT AAG AGG AAC A
SREBP-1c	GCC GAG ATG TGC GAA CTG	GGA AGT CAC TGT CTT GGT TGT T
β-actin	TTC GTT GCC GGT CCA CC	GCT TTG CAC ATG CCG GAG CC
GAPDH	AGG TTG TCT CCT GTC ACT TC	CTG TTG CTG TAG CCA TAT TC

SREBP-1c: Sterol regulatory element binding proteins 1c; ADIPOR2: adiponectin receptor 2; ACC-1: acetyl CoA carboxilase 1; LPL: Lipoprotein lipase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

2.12 Histological analyses

For the histological analyses (n=06 animals/ group), after the euthanasia, hepatic and adipose tissue were fixed with 10% formaldehyde and immersed in paraffin. Sections were cut (10 cuts/animal) at a thickness of 3 μm, mounted on glass slides, and stained with hematoxylin and eosin. The slides were analyzed under a microscopic light (Leica DM750®). The images of histological sections were captured with a 20x objective. The liver cellular components (cytoplasm, nucleus and lipids droplets) were determined observing 10 histological fields per animal in using a test system with 266 points (until to get the total of 2660 points/animal). These parameters were calculated by using the formula: $V_v = P_p/P_T$, where P_p is the number of points locates on the interest structure, and P_T is the total test points in the histological area.[48] In the adipose tissue, area and length of adipocytes, inflammatory infiltrate and number of adipocytes were analyzed with a software program (Image-Pro Plus® 4.5). The steatosis degree was assessed semi-quantitatively according to 5^0 scale: degree 0: if fat percentage was absence or <5%; Grade 1: if ≥5% and < 25%; Grade 2: if ≥25% and < 50%; Grade 3: if ≥50% and <75%; Grade 4: if ≥75%. In the hepatic tissue, fat vesicles, cytoplasm, nucleus, and inflammation were analyzed with a software program (Image J®, Wayne Rasband).

2.13 Statistical analyses

The samples were tested by Kolmogorov-Smirnov normality test. Statistically significant differences between groups were calculated using variance analysis (ANOVA) followed by *post-hoc* of Newmans-Keuls. The *p*-values lower than 0.05 ($p < 0.05$) were considered statistically significant. Data were expressed as mean ± standard deviation (SD).

The statistical analyses were performed using the statistical software GraphPad Prism®, version 8.0 (GraphPad Software Inc., San Diego, CA, EUA).

3 Results

3.1 Chemical characterization of black corn whole flour

The major component observed in black corn whole flour was carbohydrates (58.05%) follow to total of dietary fiber (13.24%), which of the major part was insoluble dietary fiber (12.92%). The total amount of phenolic compounds in the black corn was 614.3 mg GAE/100g, and the total of anthocyanins was 85.65 mg cyanidin-3-glycoside/100g (Figure 1A). In addition, seven major anthocyanins were identified by chromatography in the black corn (Figure 1A, B). From these, cyanidin-3-glucoside was the main anthocyanin verified (30.4 mg/100g), followed by the cyanidin-3-(6``-malonylglucoside) (17.6 mg/100g).

A

Components	**Amount**
Carbohydrates (%)	58.05
Total dietary fiber (%)	13.24 ± 0.40
Insoluble fiber (%)	12.92 ± 0.04
Soluble fiber (%)	0.31 ± 0.36
Protein (%)	11.68 ± 0.94
Moisture (%)	11.35 ± 0.25
Lipids (%)	4.30 ± 0.02
Ash (%)	1.38 ± 0.33
Total energy value (Kcal/100 g)	323.14
Total phenolic compounds (mg GAE/100g)	614.30 ± 4.72
Antioxidant capacity (mMs Trolox/ g)	90540.4 ± 441.56
Total anthocyanin (mg cyanidin-3-glycoside /100g)	85.65 ± 3.74

Amount (mg/ 100g)	**Retention time (min)**	**λ_{max}(nm)**

Cyanidin-3-glucoside	30.40	20.66	515.28
Cyanidin-3-(6``-malonylglucoside)	17.60	29.69	517.28
Peonidin-3-glucoside	9.30	25.34	516.27
Peonidin-3- (6`` malonylglucoside)	6.80	34.24	517.28
Pelargonidin-3-(6``malonylglucoside)	2.90	32.90	505.26
(epi)Catechin-cyanidin-3,5-diglucoside	2.50	13.07	528.28
Pelargonidin-3-glucoside	2.40	23.75	501.28

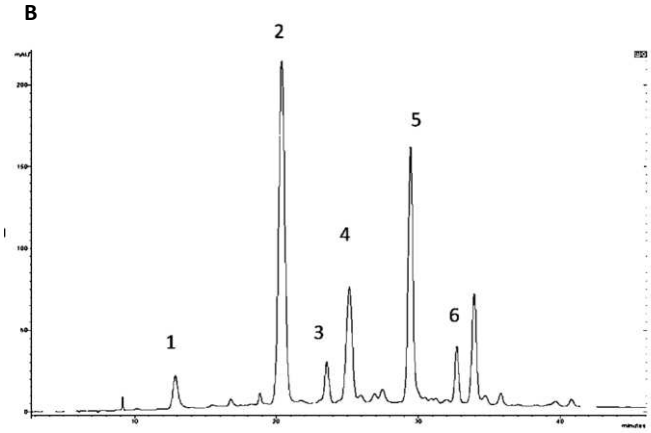

Fig. 1 (A) Chemical composition of the black corn whole flour, and quantification of identified anthocyanin (mg/100g), retention time and λ_{max} in black corn. (B) Chromatographic profile of anthocyanin identified in the black corn whole flour by Liquid Chromatography- Mass Spectrometry (512 nm).

(1): (epi)Catechin-cyanidin-3,5-diglucoside; (2): Cyanidin-3-glucoside; (3): Pelargonidin-3 glucoside; (4): Peonidin-3-glucoside; (5): Cyanidin-3-(6``-malonylglucoside); (6): Pelargonidin-3-(6``-malonylglucoside). GAE: gallic acid equivalent.

3.2 Effect of black corn whole flour on body weight, food intake, biometric indicators, biochemical variables, antioxidant capacity, and total antioxidant capacity (TAC)

The daily food intake was higher ($p<0.05$) in the normal control (NC) than high- fat (HF) and high-fat corn (HFC) groups, as the high-fat diet had a higher caloric density (5.14 kcal/g) compared to the normal control (3.85 kcal/g), but the body weight gain was similar among experimental groups. The food efficiency ratio was higher ($p<0.05$) in the two high-fat groups (HF and HFC) compared to the NC group. There was no difference in the body mass index, lee index, and hepatosomatic index among the groups. The high-fat diet increased the

adiposity, then HF and HFC groups showed higher ($p<0.05$) adiposity percentage compared to the NC group. In addition, the animals of HFC group consumed 0.49 g of black corn whole flour/day at the end of the experiment, which represented intake of 15.68 mg of anthocyanin/ kg body weight and 3.08 mg GAE/day of total phenolic compounds (Table 3).

The experimental diets had no influence on animal serum concentration of glucose, total triglycerides, and HDL-c. High-fat corn (HFC) group had higher ($p<0.05$) total cholesterol levels than the normal control (NC) and high fat-diet (HF). The high-fat diet increased the LDL-c level, in order that HF and HFC showed higher values ($p<0.05$) than the normal diet (NC). In relation to hepatic enzymes, levels of AST and ALT were similar among the experimental groups (Table 3).

The HFC group increased levels of SOD compared to NC and HF groups ($p<0.05$). The levels of malondialdehyde and catalase showed no difference among the experimental groups. In addition, it was verified that black corn increased the TAC on serum, which had higher levels ($p<0.05$) in the HFC group compared to the HF and NC. The hepatic TAC was similar among the experimental groups (Table 3).

Table 3 Effect of the black corn whole flour consumption on body weight gain, food intake, biometric indicators, biochemical, and antioxidant variables at experimental groups.

Variables	**NC**	**HF**	**HFC**
Initial weight	24.71 ± 1.47^{a}	23.60 ± 1.38^{a}	23.9 ± 1.94^{a}
Body weight gain (g)	4.27 ± 1.94^{a}	4.14 ± 2.23^{a}	5.55 ± 2.12^{a}
Food intake mean (g/day)	4.07 ± 0.16^{a}	2.53 ± 0.41^{b}	2.48 ± 0.17^{b}
Black corn intake (g/day)	-	-	0.49 ± 0.03
Total phenolics intake (mg GAE/day)	-	-	3.08 ± 0.22
Anthocyanin intake (mg/kg bw/day)	-	-	15.68 ± 1.99
Cyanidin-3-glucoside (mg/kg/day)	-	-	5.56 ± 0.70
Food Efficiency Ratio (%)	1.69 ± 0.61^{b}	2.66 ± 1.46^{a}	3.20 ± 0.81^{a}
Body mass index (g/cm^2)	3.50 ± 0.29^{a}	3.31 ± 0.13^{a}	3.64 ± 0.43^{a}
Adiposity (%)	0.71 ± 0.24^{b}	2.42 ± 1.28^{a}	3.47 ± 1.66^{a}

Lee index (g/cm^3)	33.84 ± 1.34^a	33.08 ± 0.56^a	34.17 ± 1.52^a
Hepatosomatic index (%)	3.61 ± 0.29^a	3.71 ± 0.28^a	3.43 ± 0.19^a
Glucose (mg/ dL)	198.90 ± 51.19^a	182.60 ± 30.09^a	168.30 ± 24.23^a
Total cholesterol (mg/ dL)	151.20 ± 13.80^b	166.50 ± 15.51^b	181.00 ± 12.08^a
Total triglycerides (mg/ dL)	78.08 ± 4.71^a	84.83 ± 5.63^a	82.72 ± 5.84^a
HDL-c (mg/ dL)	41.15 ± 4.73^a	37.35 ± 5.79^a	42.53 ± 9.85^a
LDL-c (mg/ dL)	12.80 ± 2.12^b	19.14 ± 6.24^a	16.05 ± 6.35^a
AST (mg/ dL)	99.09 ± 21.88^a	71.66 ± 21.30^a	85.30 ± 26.45^a
ALT (mg/ dL)	18.74 ± 9.88^a	15.71 ± 5.63^a	26.31 ± 10.16^a
Malondialdehyde (MDA/ mg protein)	0.44 ± 0.15^a	0.49 ± 0.12^a	0.49 ± 0.09^a
Catalase (U/ mg protein)	0.40 ± 0.05^a	0.39 ± 0.08^a	0.43 ± 0.06^a
SOD (U/ mg protein)	17.86 ± 2.10^b	16.52 ± 1.44^b	20.68 ± 1.56^a
TAC – hepatic	0.11 ± 0.02^a	0.16 ± 0.05^a	0.17 ± 0.05^a
TAC - serum	0.03 ± 0.01^b	0.04 ± 0.01^b	0.06 ± 0.02^a

C: normal control group (*n*=10); HF: high fat diet group (*n*=10); HFC: high-fat corn group (*n*=10).

Value expressed as mean± standard deviation. Different lower-case letters in the same line: groups are significant different ($p < 0.05$) by ANOVA followed by Newman-Keuls test. GAE: gallic acid equivalent; bw: body weight; HDL-c: high-density lipoprotein; LDL-c: low-density lipoprotein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; SOD: superoxide dismutase; TAC: total antioxidant capacity; MDA: malondialdehyde.

3.3 Effects of black corn on NF-κB, TLR-4, and PPAR- (α, γ) quantification

The protein hepatic levels of NF-κB p65, PPARα, and TLR-4 were similar among experimental groups (Figure 2A, B, C). In the adipose tissue, there was no significant change in NF-κB p65 levels (Figure 2D). On the other hand, the black corn consumption decreased the levels of PPARγ relative to NC and HF groups ($p<0.05$) in the adipose tissue (Figure 2E).

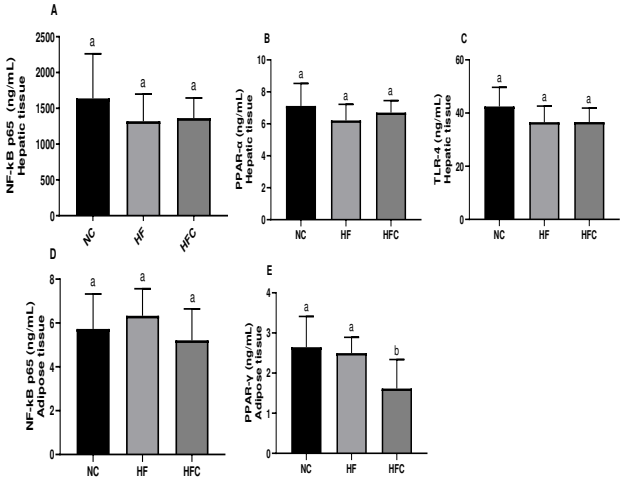

Fig. 2 Effects of black corn consumption on levels of proteins related to inflammation: (A) NF-κB p65, (B) PPARα and (C) TLR-4 in hepatic tissue (ng/mL) and (D) NF-κB p65 and (E) PPARγ in adipose tissue at experimental groups.

NC: normal control group (*n*=10); HF: high-fat diet group (*n*=10); HFC: high-fat corn group (*n*=10). Data analyzed by ELISA test. Values expressed as mean± standard deviation. Different lower-case letters indicated significant difference ($p < 0.05$) by ANOVA followed by Newman-Keuls test. NF-κB p65: factor nuclear kappa B; PPARα: peroxisome proliferator-activated receptor-alpha; TLR-4: toll like receptor-4; PPARγ: peroxisome proliferator-activated receptor-gamma.

3.4 Effects of black corn on the gene expression in hepatic and adipose tissue

In the hepatic tissue, the high-fat group (HF) group had the highest values of the gene expression of SREBP-1c ($p<0.05$) than normal control (NC) and high-fat corn group (HFC) groups, which had similar values ($p>0.05$) (Figure 3A). Gene expression of acetyl-CoA carboxylase (ACC-1) in the liver decreased in HFC group compared to the NC and HF groups (Figure 3B). Further, the gene expression of the receptor AdipoR2 in the liver decreased ($p<0.05$) in the HF and HFC groups compared to NC group (Figure 3C).

In the adipose tissue, the SREBP-1c gene expression increased ($p<0.05$) in the HF relative to the NC. In addition, black corn intake decreased the SREBP-1c mRNA expression 0.61-fold in the HFC group ($p<0.05$, Figure 3D) compared to the HF group. The gene expression of the enzyme lipoprotein lipase (LPL) increased in the HFC relative to HF, however, was similar compared to NC (Figure 3E). Finally, the adiponectin gene expression increased in the animals that consumed black corn (HFC) compared to all other animals

(p<0.05, Figure 3F).

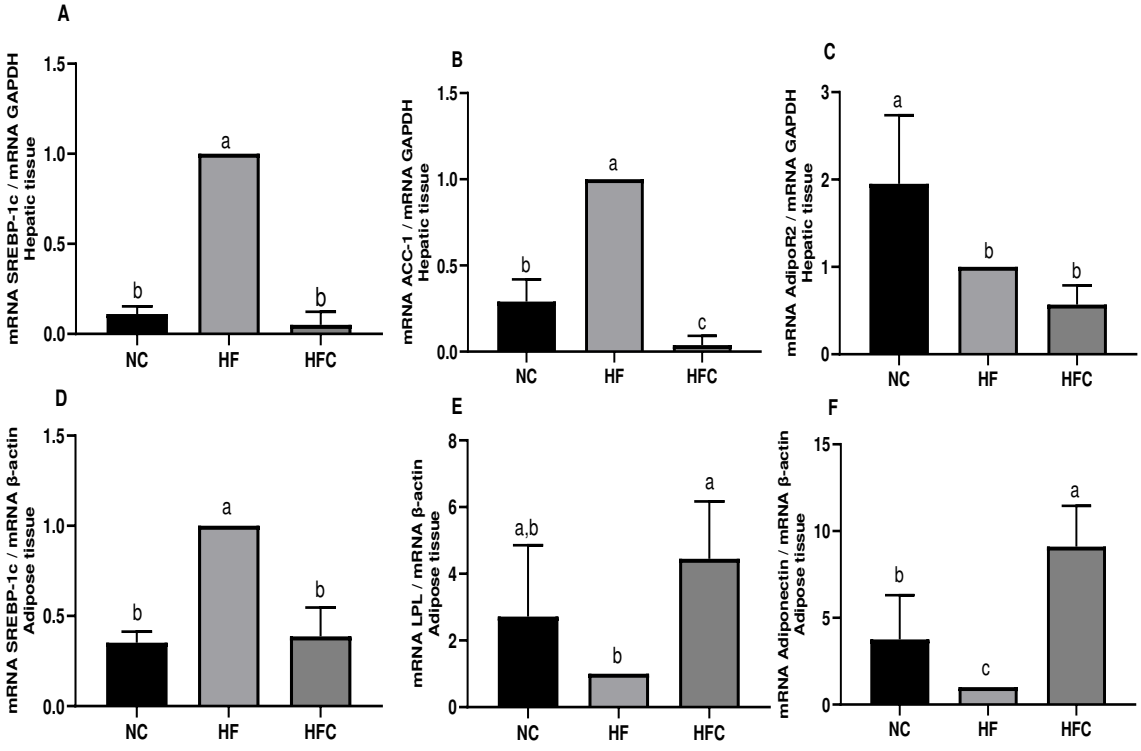

Fig. 3 Effects of black corn consumption on expression of proteins related to adipogenesis: (A) SREBP-1c, (B) ACC-1, and (C) AdipoR2 in the hepatic tissue and (D) SREBP-1c, (E) LPL, and (F) Adiponectin in the adipose tissue.

Values expressed as mean± standard deviation. Different lower-case letters indicated significant difference (p< 0.05) by ANOVA followed by Newman-Keuls test. Data analyzed by RT-PCR (real time- polymerase chain reaction). NC: normal control group (*n*=10); HF: high-fat diet group (*n*=10); HFC: high-fat corn group (*n*=10). SREBP-1c: Sterol regulatory element binding proteins 1c; ADIPOR2: adiponectin receptor 2; ACC-1: acetyl-CoA carboxylase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; LPL: Lipoprotein lipase.

3.5 Effects of black corn on adipose and hepatic histological morphometric

In the adipose tissue were measured area, length, inflammatory infiltrate, and number of adipocytes (Figures 4A-E). Adipocyte's length and inflammatory infiltrate increased (p<0.05) in the HF compared to NC group, however the intake of black corn (HFC) was able to reduce (p<0.05; Figure 4A and C) these variables compared to HF group, become similar to the NC group. The area of adipocytes increased in the HF group compared to NC diet (p<0.05;

Figure 4B). Further, the HFC showed the lower number of adipocytes compared to HF and NC groups ($p<0.05$; Figure 4D). It was observed in the photomicrographs, hypertrophy, and inflammatory infiltrate of adipose cells in the HF group relative to HFC and NC groups (Figure 4E).

Changes were observed in cellular components of liver tissue between NC, HF, and HFC groups during the 8-week of mice fed a high-fat diet added or not with black corn (Figure 4F and G). The percentage fat vesicles increased in high-fat diet (HF and HFC groups) compared to NC ($p<0.05$; Figure 4F). The cytoplasm percentage was similar in the HF and HFC and smaller in the NC group compared to the others ($p<0.05$; Figure 4F). The nucleus and inflammation percentage did not differ among the experimental groups (Figure 4F). In addition, the NC group was classified as grade 0 steatosis, and the HF and HFC showed grade 1 (Figure 4G).

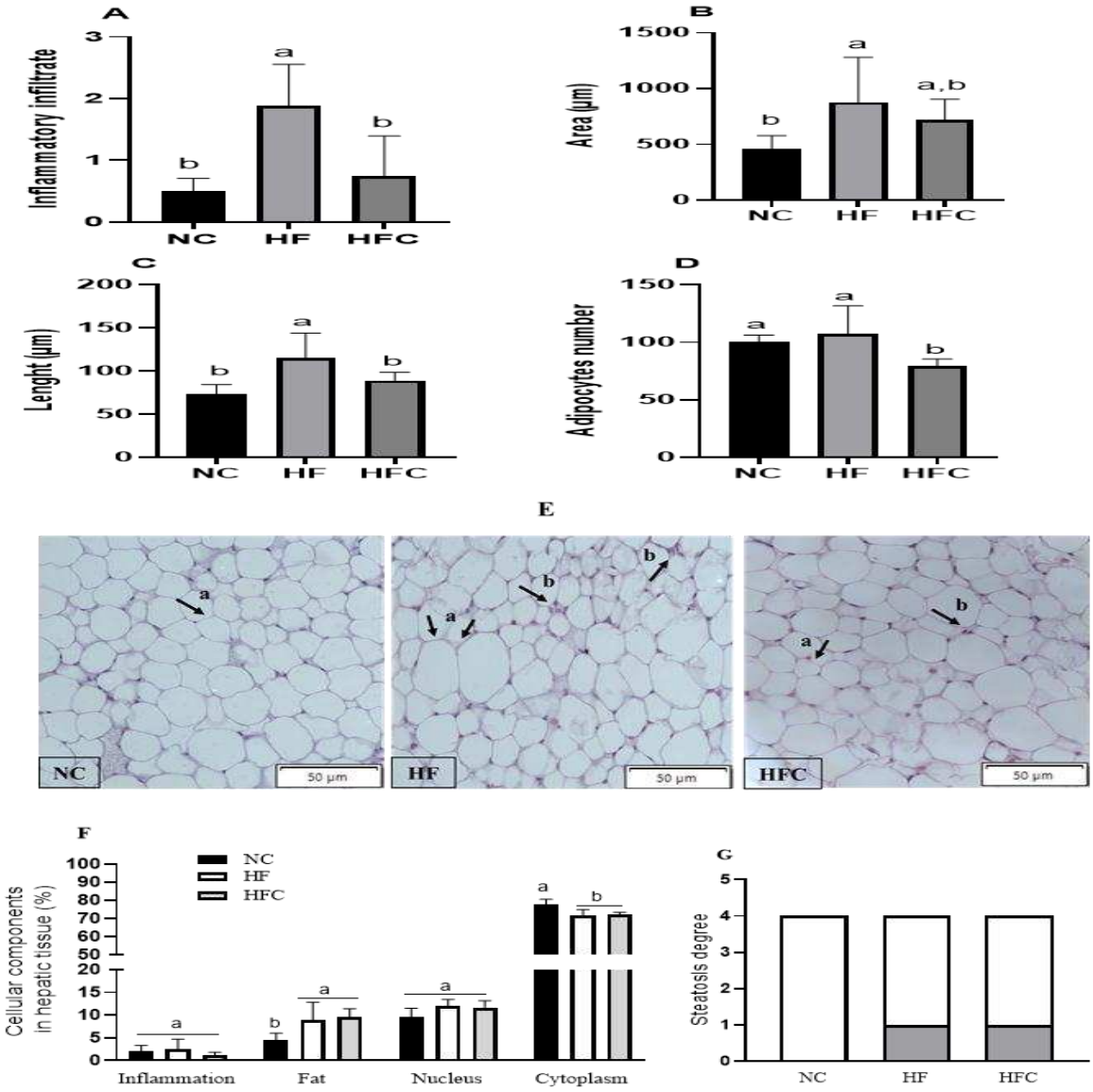

Fig. 4 Effects of black corn whole flour consumption at the measures of (A) inflammatory infiltrate, (B) area, (C) length, (D) adipocytes number of adipose tissue, (E) adipose tissue photomicrographs of the experimental groups, (F) cellular components percentage, and (G) steatosis degree of hepatic tissue at experimental groups. Black arrows (Fig 4E) represent: a: size of adipocyte; b: inflammatory infiltrate.

Tissue stained with hematoxylin/eosin. n=06 animals/group, 10 cuts/animals, and 10 measures/sample. NC: normal control group (*n*=10); HF: high fat diet group (*n*=10); HFC: high fat corn group (*n*=10). Value expressed as mean± standard deviation. Different lower-case letters indicated significant difference ($p < 0.05$) by ANOVA followed by Newman-Keuls test. Hematoxylin and eosin. Bar: 50 µm

4. Discussion

The present study showed that consumption of black corn whole flour (*Zea mays* L.) (20%) containing phenolic compounds, including anthocyanins, promoted improvement in the total antioxidant capacity and prevented the adipogenesis in mice fed a high-fat diet. At the end of the experimental period, based on the food intake, the animals that consumed the black corn whole flour (20%) showed a consumption of 15.68 mg total anthocyanin/ day.

The high-fat diet consumption over a period of 7 and 8 weeks has been widely used to induce metabolic changes in animals.[21] According to this protocol, the intake of a specific food with claimed health beneficial effects as an obesity treatment is determined to be initiated after the 7 or 8 weeks receiving the high-fat diet. Differently, to verify the black corn whole flour effects in a preventive context, in our study, the black corn was added in the diet while offering the high-fat diets to verify its preventive effects during the development of the undesirable metabolic changes.

Black corn is a source of phytochemicals with reported nutritional benefits, since kernels accumulate high levels of anthocyanins, which are a class of flavonoids responsible for its dark color.[6] In our study, anthocyanins more prevalent in the TO002 were cyanidin-3-glucoside (35.49%), cyanidin-3-(6’’-malonylglucoside), peonidin-3-glucoside, peonidin-3-(6’’malonylglucoside), pelargonidin-3-(6’’malonylglucoside), (epi)catechin-cyanidin-3,5-diglucoside, and pelargonidin-3-glucoside. Therefore, the total anthocyanins content of the pigmented corn in our study (85.65 mg cyanidin-3-glycoside/100 g) was nearly similar or higher than common dietary sources of anthocyanins including blueberry (120.2 mg/100g), blackberry (87.24 mg/100g), and strawberry (24.75 mg/100g).[27] The cyanidin-3-glucoside (C3G), identified as the major anthocyanin in the black corn, is the most widely distributed anthocyanidin in plant-based foods, and it is reported to promote the suppression of multiple pathways associated with adipogenesis.[28]

In the present study, the consumption of black corn whole flour prevented the adipogenesis by decreasing the hypertrophy and hyperplasia of adipocytes, decreasing the number and length of adipocytes and inflammatory infiltrate in the adipose tissue. Since the major anthocyanidin observed in the black corn was the cyanidin-3-glucoside (C3G), resuming 0.43 mg/day as the total anthocyanin’s consumption, this would represent an intake of 5.56 mg of C3G/kg bw/day. Even though in the literature the dosages of C3G added in diets in animal studies to observe biological effects varied widely, we highlight that similar dosage (7.6 mg/kg bw) of C3G was effective to attenuate important metabolic changes caused by a high-fat diet in

rats.[28] In addition, the supplementation of 59 mg C3G/kg bw in mice C57BL/6 was related to the attenuation of adipocyte differentiation and antioxidant activity[29], while higher dosage (160-300 mg C3G/kg bw) improved the macrophage infiltration in the adipose tissue without any noticeable detrimental effects.[30] Since high-density diets lead to a positive energy balance, which in turn leads to adipocyte hyperplasia (proliferation and differentiation of pre-adipocytes) and adipocyte hypertrophy from the excessive lipid storage into the adipocytes[2], the intake of black corn whole flour prevented the development of an additional adipogenic process due to a high-fat diet.

Furthermore, in this study black corn whole flour intake reduced the PPARγ levels and the SREBP-1c gene expression in the adipose tissue, which might have contributed to the reduction in the adipocyte number by blocking its differentiation. It is observed that the reduced number and length of adipocytes was accompanied by a reduction of expression of PPARγ, SREBP-1c and CCAAT/enhancer-binding proteins (C/EBPs).[4] PPARγ, expressed mostly in the adipose tissue, plays a central role in the differentiation of adipocytes, energy balance, lipid biosynthesis, and controls the expression of numerous adipocyte-secreted factors as adiponectin, resistin, leptin and TNF-α.[5] Moreover, the SREBP-1c promotes the pre-adipocytes differentiation and the stimulus of several lipogenic enzymes as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC).[31] Therefore, black corn whole flour in the high-fat diet might have prevented the development of an adipogenic process in the animals, since the anthocyanidin C3G in turn influences the gene expression of SREBP-1c, thus blocking the activity of PPARγ, which contributed to inhibit the adipogenesis in the adipose tissue.

In the present experiment, the black corn whole flour consumption reduced the SREBP-1c and the ACC mRNA expression in the liver. The enzyme ACC catalyzes the synthesis of malonyl-CoA; thus, its inhibition leads to a decrease in fatty acid synthesis and higher mitochondrial fatty acid oxidation.[32] Attenuation in the gene expression of SREBP-1c and ACC-1 in the liver was observed previously in a *in vivo* study with mice fed a high-fat diet and supplemented with lingonberry, rich in anthocyanins.[33] Therefore, *in vitro* study shows that the AMP-activated protein kinase (AMPK) activation suppresses the ACC activity, so reducing malonyl-CoA levels, which enhances the fatty acid β-oxidation by stimulating the enzyme carnitine palmitoyltransferase I.[34] Further, in our study, we observed that the gene expression of adiponectin in the adipose tissue increased after the black corn flour intake and decreased in the high-fat diet group. Adiponectin is an adipocyte-derived hormone, which exerts anti-inflammatory effects by binding to adiponectin receptors (AdipoR1 and AdipoR2).[35] Higher

levels of adiponectin are associated with higher insulin sensibility[35], while high-fat diets and obesity usually decrease the adiponectin levels as verified in our study in the HF group. The receptor AdipoR2, mostly expressed in the liver, activates, and increases the expression of PPARα.[35] In our study, the hepatic AdipoR2 did not change with black corn intake, which might explain why the PPARα did not change either. In addition, the higher adiponectin gene expression due to the black corn intake may be the mechanism to the increased LPL gene expression in the same group. The adiponectin improves the lipid metabolism by activating the lipoprotein lipase (LPL), which induces the triglyceride catabolism.[37]

The black corn investigated in the present study showed high levels of phenolic compounds (614.4 mg GAE/ 100 g), which in turn may have contributed to prevent the adipogenesis and the oxidative stress in the liver due to a high-fat diet, and to improve the total antioxidant capacity. Once one that the black corn whole flour intake promoted an increase in the antioxidant enzyme superoxide dismutase (SOD) in the liver, thereby improving the total antioxidant capacity. The production of reactive oxygen and nitrogen species is a natural process in most of aerobic cells as by-products, although an exacerbated production is associated to injurious stimuli. Therefore, the internal antioxidant system composed of enzymes as SOD, glutathione peroxidase, and catalase acts to remove reactive species production.[38] In this sense, anthocyanins act as antioxidant to prevent or to inhibit the oxidation by scavenging free radicals, thus reducing the oxidative stress. Classes of anthocyanins act to reduce agents in the electron-transfer reaction pathway by donating electrons to the free radicals with unpaired electrons, and by elevating the synthesis or activity of antioxidant enzymes and the inhibition of ROS-forming enzymes.[39]In agreement, germinated milled flour source of phenolic compounds (2.17 mg GAE/g) improved the total antioxidant capacity in the liver in rats fed a high-fat high-fructose diet[18]. Therefore, as the black corn used in our study showed even higher total phenolic compounds (6.14 mg GAE/g), it might be associated to its total antioxidant capacity.

Although black corn prevented the adipogenesis and improved the oxidative stress, it was not observed significant anti-inflammatory effects and improvements in the hepatic histological biomarkers. *In vivo* studies have showed anti-inflammatory effects of anthocyanins through the toll like-receptor 4 (TLR-4) and NF-κB pathway[40], however the hepatic proteins NF-κB, PPARα, and TLR-4 in our study were maintained similar in all groups. In addition, since these inflammatory biomarkers were not improved in the liver, this might maintained the percentage of inflammation, fat, and steatosis degree unaltered in the liver. There was no

metabolic change observed in the liver after the 8 weeks of HF diet, therefore the black con might have not promoted anti-inflammatory effect in the liver as an inflammation was not initiated there yet. A study with a lesser amount of purple corn flour (3.1 mg/ kg bw) attenuated liver steatosis and reduced inflammatory cell infiltration. However, in this study, the HF diet was offered for 16 weeks, therefore the liver inflammation was evident. [28]

In this sense, most of the studies with positive effects of anthocyanins in the hepatic NF-κB pathway was performed with extracts of these compounds.[40,41] Therefore, anthocyanins dosage supplemented in those studies is higher compared to our study (15.68 mg/kg/day). Further, most studies evaluate individual compounds of anthocyanins or fractions rich in these compounds, differently of the present study that all anthocyanins present in the food matrix were considered. Each anthocyanin exerts different anti-inflammatory effects due to its aglycon structure, such as hydroxylation and methylation patterns, as well as the presence or not of sugar moiety.[42] In addition, in our study the black corn composed a high-fat diet, thus phenolic compounds were presented in all experimental period, while in other studies[8,28,34] the anthocyanin added in the diet was initiated after the metabolic changes have been induced by the high-fat diet. Contradictory results can be observed due to anthocyanin consumption, as the variability in response to anthocyanin has been considered due to an inter-individual variation in its absorption and metabolism, which can be affected by age, sex, diet and gut microbiota.[43] In addition, food matrices in the anthocyanin-rich foods or from other food components consumed together may affect the anthocyanin bioavailability.[10,44] Fat presented in a meal is suggested to slow the absorption, and sugar might compete for the glucose intestinal transporters, therefore counteracting the health effects of anthocyanins.[44] A high fat meal showed selectively effects on anthocyanin bioaccessibility.[45] Therefore, the black corn matrix and the high fat diet components might have influenced the results related to hepatic inflammation.

Therefore, in our study, the black corn whole flour effect in the prevention of the adipogenesis can be associated to the decrease in SREBP-1c and PPARγ, besides reduction in the lipid storage by increasing LPL expression, and adiponectin in the adipose tissue. Further, the β- oxidation in the liver improved through the decreased expression of SREBP-1c and ACC. These mechanisms are associated with black corn as a source of polyphenols, mainly anthocyanins, which improved the total antioxidant capacity and the hepatic superoxide dismutase thus, preventing the adipogenesis due to a high-fat diet (Figure 5).

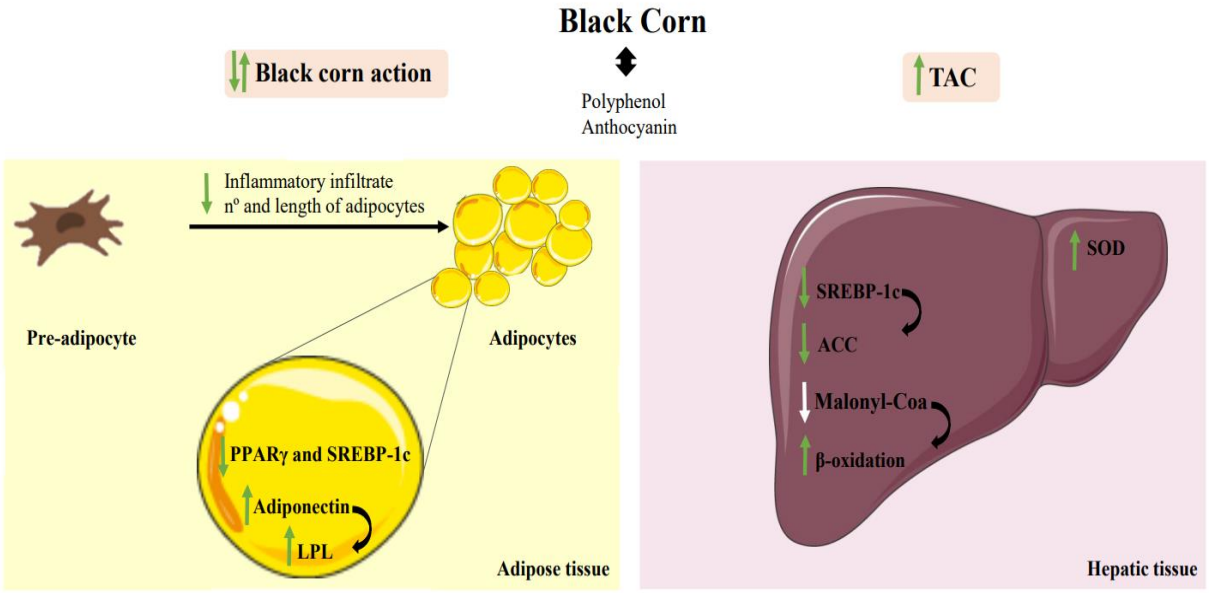

Fig. 5 Proposed mechanisms of black corn whole flour in the prevention of metabolic changes promoted due to a high-fat diet. The black corn whole flour as a source of polyphenols, mainly anthocyanin, prevented the differentiation of pre-adipocytes into adipocytes, thus reducing the number and length of adipocytes. The mechanism to adipogenesis prevention was by blocking the action of SREBP-1c and PPARγ in the adipose tissue, leading to an increased expression of adiponectin and LPL. Further, hepatic β-oxidation was stimulated though decreasing the expression of SREBP-1c and ACC. These beneficial effects are related to a higher TAC promoted by the black corn consumption.

TAC: total antioxidant capacity; SOD: superoxide dismutase ACC: acetyl CoA carboxylase; SREBP-1c: Sterol regulatory element binding proteins 1c; LPL: lipoprotein lipase PPARγ: peroxisome proliferator-activated receptor-gamma.

5. Conclusions

Black corn whole flour improved the antioxidant capacity and the adipogenesis process in mice fed a high-fat diet by preventing the hyperplasia and hypertrophy of adipocytes, hence improving the β-oxidation in the liver and the lipid storage in the adipose tissue. These effects may be due to the phenolic components, mainly high anthocyanins content in the grain. Overall, our results demonstrate the potential of the black corn intake to prevent undesirable metabolic changes caused by the high-fat diet consumption, showing its promising use as functional food for human consumption.

Author Contributions

AV: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing – original draft, writing – review and editing. MG: conceptualization, methodology, writing – review and editing. CTS: formal analysis, methodology, investigation. RCLT: formal analysis, methodology. VPBSJ: formal analysis, methodology. MCDP: funding acquisition, project administration, writing – review and editing. HSDM: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing – review and editing.

Conflicts of interest

There are no conflicts to declare.

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6.2. PAPER 3

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Original Research

Black corn (*Zea mays* L.) flour has the potential to improve the gut microbiota composition and goblet cell proliferation in mice fed a high-fat diet

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ABSTRACT

High-fat diets are associated with intestinal dysbiosis and leaky gut leading to intestinal inflammation. Bioactive components, including phenolic compounds, isolated or in their original food matrix, have alleviated intestinal impairments promoted by a high-fat diet. Black corn (*Zea mays* L.) is a colored corn in which anthocyanins are the most abundant bioactive compound. Thus, we hypothesized that black corn flour may have preventive effects on poor intestinal health in mice fed a high-fat diet. To study this, 30 C57BL/6 mice were randomly divided into 3 experimental groups receiving the following diets for 8 weeks: normal control (fed a normal diet); high-fat (fed a high-fat diet: 60% of calories from fat); high-fat corn (fed a high-fat diet added with 20% of black corn whole flour). The cecal microbiota analyzed by 16S ribosomal RNA sequencing showed that black corn flour intake increased the relative abundance of *Ruminococcus*, *Roseburia*, and *Prevotellaceae_UCG-001*, and decreased *Bacteroides* and *Faecalibaculum*. No difference was observed in the cecal short-chain fatty acids and fecal pH among the experimental groups ($P > .05$). Further, the consumption of black corn flour improved cecal morphology by increasing the number of goblet cells but with no difference in the crypt depth and width. These findings suggest that black corn flour as a source of anthocyanins could have preventive effects on gut dysbiosis resulting from a high-fat diet. SCFA, short chain fatty acids.

1. Introduction

In modern society, new trends in dietary habits have been observed with the spread of the Western diet around the world. One of the characteristics of the Western diet is a diet high in saturated fatty acids associated with overeating, snacking, and a prolonged postprandial status [1]. The excessive intake of saturated fatty acids by humans has been linked to developing obesity and related-metabolic diseases such as type 2 diabetes, cardiovascular disease, cancer, and metabolic syn- drome [2,3]. The average energy intake from fat is estimated to be 36% in the United States [4], which is higher than the recommendation of 20% to 35% for adults according to the Acceptable Macronutrient Distribution range [5]. Further, high- fat diet consumption contributes to inflammation because of specific changes in gut microbiome composition through a shift in its bacterial profile [2,6].

Dysbiosis is usually defined as the disbalance of the intestinal microbial system with predominant pathogenic over potentially beneficial bacteria species [7]. In obesity, there is a decreased gut microbiota diversity and a higher abundance of Firmicutes, Proteobacteria, Firmicutes/Bacteroidetes ratio, and lower Lactobacillus [8,9]. A high-fat diet is associated with systemic chronic low-grade inflammation and contributes to disrupted gut barrier function through an inflammatory process and changes in gut microbiota composition [2]. A leaky gut is characterized by an increased intestinal permeability, which promotes the translocation of potentially harmful antigens and microorganisms from the gut lumen. In addition, the excess of fatty acids promotes tight junction impairments and exacerbates lipopolysaccharides production [10]. Then, a leaky gut facilitates potentially harmful antigens to translocate into epithelial cells, which might be immune cells' target [10]. Therefore, because dietary components have direct contact with the intestinal lumen during digestion, they have been investigated as modulating gut microbiota.

Anthocyanins, a subclass of flavonoids, are found in diverse plants, fruits, vegetables, and cereals [11]. Anthocyanin consumption has been associated with reduced risk of metabolic disease related to inflammation [12–14]. Because of their low absorption rate, anthocyanins undergo colonic metabolism through β-glucosidase enzymes produced by bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. [15]. Recently, a systematic review of *in vivo* studies verified the possible mechanisms by which anthocyanins improve intestinal health. The authors showed that anthocyanin supplementation promoted increased Bacteroidetes and decreased Firmicutes and

higher production of short-chain fatty acids (SCFAs), which decreases the intraluminal pH and intestinal permeability, besides improving histological biomarkers in duodenum and colon [16]

Black corn (*Zea mays* L.) is popularly consumed in the Andean region; however, because it adapts to different climatic conditions, its cultivation is possible in other countries. Colored corn, specially, purple and black, shows a considerable amount of anthocyanins; thus, health effects are associated with its consumption [17]. In this sense, the intestinal effects of isolated phytochemicals have been widely investigated; however, the impact of bioactive components embedded in its whole grains are still scarce. Besides bioactive components, black corn whole flour has a complete matrix composed of proteins, lipids, and carbohydrates as starch and fiber, which in synergism with bioactive components might contribute to improved microbial composition. Recently, our research group showed the promising effect of black corn flour as a source of bioactive components to prevent adipogenesis and improve the antioxidant capacity in mice induced to metabolic change by a high-fat diet [18]. Although *in vivo* studies have observed the intestinal beneficial effects of anthocyanins, there is scant information regarding the impact of black corn flour on intestinal health. Specifically, there is a need to evaluate its intestinal effects considering the detrimental effects caused by a high-fat diet.

We hypothesized that black corn flour would reverse the dysbiosis driven by a high-fat diet and thus modulate the colonic microbiota composition, resulting in the production of metabolites as SCFAs and improvement in intestinal morphology. Therefore, this study investigated the effect of black corn flour (20%) on intestinal health in mice fed a high-fat diet.

2. Methods and Materials

2.1 Sample material

The black corn was provided by the Brazilian Agriculture Research Corporation (EMBRAPA), Maize and Sorghum National Research Center. Black corn TO002 was accessed from the Maize Germplasm Bank of the Embrapa Maize and Sorghum research unit. The samples were stored in a plastic bag, refrigerated at 4 °C, and protected from light until processing. The black corn flour was obtained using a knife mill grinder with a 1.0 mm stainless steel sieve (Willy, Solab) and stored at 4 °C.

The black corn flour was processed according to the methodology proposed by the Association of Official Analytical Chemists methods [19]. Briefly, protein content was determined

by the Kjeldhal method [19], and lipid content was determined by extraction in a Soxhlet apparatus [19]. Total dietary fiber (soluble and insoluble) was determined by the gravimetric-enzymatic method using a commercial kit (Total dietary fiber assay kit, Sigma, Saint Louis, Missouri, USA). Moisture was determined through an oven (Nova Etica, Sao Paulo, Brazil) at 105 °C, and ashes were quantified using a muffle furnace (Quimis, Q320 M model, Brazil) at a temperature of 550 °C [19]. Carbohydrates were determined by the difference. Further, total monomeric anthocyanin was determined by the differential pH method [20] and the total polyphenols by the single Ciocalteau colorimetric assay [21], with the absorbance measured at 760 nm. Finally,the anthocyanin profile was determined though high-performance liquid chromatography (HPLC) as described in detail elsewhere [18].

The black corn flour contained 58.05% carbohydrates, 13.24% fiber (12.92% insoluble and 0.31% soluble), 11.68% protein, 11.35% moisture, 4.3% lipids, and 1.38% ash. Further, the whole black corn flour contained 85.65 mg cyanidin-3-glycoside/100 g (30.4 mg cyanidin-3-glucoside, 17.6 mg cyanidin-3-[6 -malonylglucoside], 9.3 mg peonidin3-glucoside, 6.8 mg peonidin-3-[6 -elargonidin-3-[6 -malonylglucoside], 2.5 mg of [epi]catechincyanidin-3,5 diglucoside, and 2.4 mg of pelargonidin-3- glucoside). The total phenolic compounds were 614.4 mg gallic acid equivalent/100 g [18].

2.2 Animals and experimental design

Thirty male C57BL/6 mice, 8 weeks old (±22.65 g body weight), were obtained from the Center for Reproductive and Biology – Universidade Federal of Juiz de Fora, Minas Gerais, Brazil. The animals were housed in individual cages in a temperature controlled room (22 ± 2 °C) with a 12-hour light/dark cycle and ad libitum access to ultrapure water and food. The animal protocol was approved by the Ethics Committee in the Use of Animals of Universidade Federal de Viçosa, Brazil, under the process number 10/2019.

After 1 week of acclimatization, the animals were randomly divided by body weight into 3 groups (n = 30 animals). The number of animals in each group was calculated based on the sample calculation equation [22]. The variables considered for this were: α-level = 5%, α-error type I = 1.96, and data of fat mass mean (±10 g of difference) according to Schoemaker et al. [23]. The experimental groups received the following diets for 8 weeks: normal control (NC; n = 10): a normal diet (AIN-93 M diet); high fat (HF; n = 10); high-fat corn (HFC; n = 10), a high-fat diet

with black corn flour. The normal control diet was composed of the standard diet AIN-93 M [24]. The HF diet was prepared in the following caloric proportions: 59% from fats, 13% from protein, and 28% from carbohydrates [25]. The HFC diet was prepared by adding black corn flour to the HF diet up to 20% (200 g/kg diet), considering the chemical composition of black corn whole flour, as detailed previously. Therefore, 200 g of black corn flour per kilogram diet provided the replacement for 95.7% starch, 31.1% protein, and 21.5% oil, in which the experimental diets had the same amount of protein, carbohydrate, and lipids among them. The black corn flour addition was based on substituting 50% of dietary fiber recommendations for rodents [24], as suggested in a previous study with cereals [26] (Table 1). Considering the final food intake mean of black corn flour (0.49 g/d) and the final body weight mean (29.45 g), which represents a consumption of 0.0166 g black corn flour/kg, the amount of black corn flour in the diet of this study is equivalent to a human consumption of 39 g/d (for a human of 70 kg).

Individual body weight and food intake were recorded weekly in a semi analytical balance (Gehaka). After 8 weeks of experiment and 12 hours of fasting, the animals were anesthetized with 100% isoflurane (Isoforine, Cristália, Brazil) and then euthanized by cardiac puncture. The cecum was removed, washed in phosphate-buffered saline, and weighed in a semi analytical balance (Gehaka). Further, cecal contents were collected and stored at –80 °C until analysis. Fragments from the colon were collected and fixed in 10% formaldehyde and maintained at room temperature for further histological analysis.

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

Ingredients	**NC**	**HF**	**HFC**
Albumin*	179.00	179.00	123.00
Maltodextrin	155.00	155.00	155.00
Corn starch	426.70	114.00	5.00
Saccharose	100.00	100.00	100.00
Soybean oil (mL)	40.00	40.00	31.40
Lard	-	312.00	312.00
Cellulose	50.00	50.00	23.60
Mineral mix	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00

Bitartarate choline	2.50	2.50	2.50
L-cystine	1.80	1.80	1.80
Black corn flour	-	-	200.00
Caloric density (kcal/kg)	385	541	529

NC: normal control group; HF: high-fat group; HFC: high-fat corn group.

* Albumin based on 77.9% protein content.

2.3 Colon histomorphology analyses

Colon samples (n = 6/group) were fixed in 10% formaldehyde, dehydrated, cleared, and embedded in paraffin. The semi serialized histological colon fragments of 3 μm were cut and stained by the hematoxylin/eosin technique. Slides were examined under a photomicroscope (Leica DM750) with a 10× objective. The following were analyzed: crypt depth, crypt width, villi goblet cell (GC) number (20 villi/sample), and thickness of the circular and longitudinal muscle layers (CML and LML, respectively). A scale of 50 μm was used. Twenty random fields per animal were selected. The images were processed using the ImagePro-Plus software version 4.5 (Media Cybernetics, Rockville, MD, USA).

2.4 Fecal pH determination

Excreted feces were collected from individual cages and stored at –80 °C. On the day of analysis, the samples were maintained at room temperature and an aliquot of feces (100 mg) for each animal (n = 10/group) was diluted in distilled water (1:10), homogenized, and mixed using a vortex. The pH was measured with a calibrated digital pH meter (Bel Engineering) until the pH stabilization [27,28].

2.5 Measurement of short-chain fatty acid

The SCFA analysis was performed using the cecum contents (n=5 animals/group) following the methodology previously described [29], with modifications. Throughout the analysis, the samples remained under low temperatures. Briefly, 100 mg of cecal content were homogenized in MiliQ water following a vortex-shaking protocol with calcium hydroxide and cupric sulfate to extract the SCFA. The quantification of SCFA was performed by HPLC. The SCFAs were determined in a Dionex Ultimate 3000 Dual detector HPLC apparatus (Dionex

Corporation, Sunnyvale, CA, USA) equipped with a refractive index detector Shodex RI-101 maintained at 40 °C. The SCFAs were separated on a Bio-Rad HPX-87H column (300 × 4.6 mm) (Phenomenex Inc. Torrance, CA, USA) maintained at 45 °C. Analyses were performed isocratically under the following conditions: mobile phase sulfuric acid 5 mmol/L, flow rate 0.7 mL/min, column temperature 45 °C, and injection volume 20 µL. Stock solutions of the standards were prepared using acetic, propionic, and butyric acid. All SCFAs were ready with a final concentration of 10 mmol/L.

2.6 Gut microbiota analysis by 16S rRNA gene sequencing

The genomic DNA was extracted from approximately 100 mg of cecal content (n = 10 animals/group) following a mechanical disruption by bead-beating and phenol/chloroform extraction protocol [30]. The concentration and quality of DNA were determined spectrophotometrically by measuring the A260/280. Amplicons of the 16S rRNA V3-V4 region were generated using forward primer 341F (5 -CCTAYGGGRBGCASCAG3) and reverse primers 806R (5 -GGACTACNNGGGTATCTAAT3) and a barcoded primer set adapter for the Illumina NovaSeq platform (Illumina, San Diego, CA, USA) [31]. Samples were loaded onto an Illumina flow cell for paired-end sequencing reactions using the Illumina NovaSeq PE250 platform at the Novogene Corporation at the University of California at Davis campus (Sacramento, CA, USA). Amplicons were sequenced on a 2 × 250-bp NovaSeq run using customized sequencing primers and procedures [31]. The sequences obtained for all samples were submitted to Sequence Read Archive database on the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA83677.

2.7 16S rRNA sequence processing

Data processing and analysis were performed using the software Mothur (version v.1.44.3). The R1 and R2 paired-end reads were joined, and sequences smaller than 380 or greater than 440 bp were removed. Sequences were discarded if they had homopolymers with at least 8 nucleotides or contained ambiguous base pairs. Chimera sequences were detected and filtered with a reference-based approach using UCHIME version 4.2 [32]. After cleaning the sequences, they were aligned with the 16S rRNA gene using the SILVA database v.138 [33]. Taxonomic classification was performed using SILVA database v.138, and the operational taxonomic units (OTUs) were

grouped with a 97% sequence similarity cutoff. The coverage of all samples was assessed by the Good coverage estimator (bacteria >97%). To correct for sampling bias resulting from unequal amplicon library sizes, the samples were normalized for the lowest number of sequences produced from any sample.

2.8 Gut microbiota diversity and composition analysis

The normalized data table was used for calculating α and β diversity and the relative abundance of OTUs. All the analyses were performed considering the taxonomic classification at the genus level [34]. The α diversity of each sample was analyzed using the Chao1, Shannon, and Simpson index for microbial community composition. β diversity metrics were calculated using the Jaccard dissimilarity index. Principal coordinate analysis (PCoA) plots were performed on calculated distance matrices in the bacterial communities. The statistical significance of β diversity across sample groups was assessed with the nonparametric Permutational Multivariate Analysis of Variance (Monte Carlo permutations) test using the Past software (version 4.05). The predictions of metagenome functionality, grouped by experimental diets, was carried out using the PICRUSt 2.0 software [35]. Normalized OTU abundance was identified, and the assigned functional traits were predicted based on reference genomes categorized by the Kyoto Encyclopedia of Genes and Genomes pathway. Relative frequencies of different taxonomic categories obtained were calculated using the Statistical Analysis of Metagenomic Profiles program (version 2.1.3). Linear discriminant analysis effect size analysis was performed to identify the functional microbial pathways that were differentially expressed in the different experimental groups using the Galaxy website [36].

2.9 Statistical analyses

Data were expressed as mean ± SD or box plots. Colon histomorphometry characteristics, fecal pH, and SCFA concentration were submitted to the Kolmogorov-Smirnov normality test; any samples that did not show a normal distribution were transformed (using a log10 function). Then, statistical significance between groups was evaluated by a 1-way analysis of variance (ANOVA) procedure followed by the Duncan post hoc test for multiple comparison using IBM SPSS Statistics, version 20. The effect of experimental diets on the abundance of bacterial taxa were analyzed by Kruskal-Wallis H-test nonparametric test using false discovery rate by Benjamini

correction for multiple comparisons (Statistical Analysis of Metagenomic Profiles, version 2.1.3). Datasets were tested for homogeneity by the Kolmogorov-Smirnov test. Nonparametric data were transformed to log10, and samples were submitted to post hoc Duncan test for multiple comparison. Statistical analysis was performed with Bonferroni correction, and associations between the relative abundances of key phylotypes at genus level and host parameters were calculated by the Spearman correlation coefficient at the IBM SPSS Statistics, version 20. The level of statistical significance was $P < .05$.

3. Results

3.1 Effect of black corn flour in body weight gain and food intake

At the end of the experiment (8 weeks), the food intake mean was significantly higher ($P < .05$) in the NC compared with the HF and HFC groups (NC = 4.07 ± 0.16; HF = 2.53 ± 0.41; HFC = 2.48 ± 0.17 g/d). The food intake did not represent a difference in the weight gain among groups, there was no difference ($P > .05$) in the body weight gain mean in the experimental groups (NC = 4.27 ± 1.94; HF = 4.14 ± 2.23; HFC = 5.55 ± 2.12 g.

3.2 Effect of black corn flour in colon histomorphology analysis

The animals that received the HF diet, with and without the black corn whole flour, had a lower cecum weight than those receiving the NC diet. The CML was reduced in the HF diet group compared with the NC group, and there was no difference between the HFC group versus HF or NC group. In addition, the LML was lower ($P < .05$) after the HF diet compared with the NC diet, but the animals in the black corn flour group maintained these reductions. The lack of or the addition of black corn flour did not influence the crypt depth and width, so the means for these variables were not significantly different among the experimental groups. On the other hand, black corn flour consumption increased the GC numbers; thus, the animals of the HFC group showed a higher ($P < .05$) number of GCs in crypts than the NC and HF groups (Table 2; Fig. 1).

Table 2. Effect of black corn flour consumption in colonic histomorphometry characteristics in mice fed a high-fat diet.

Parameter	NC	HF	HFC
Cecum weight (g)	0.40 ± 0.06^{a}	0.29 ± 0.11^{b}	0.26 ± 0.06^{b}

Crypt depth (µm)	93.88 ± 9.64[a]	98.97 ± 10.89[a]	105.50 ± 11.71[a]
Crypt width (µm)	14.04 ± 1.58[a]	15.06 ± 1.72[a]	13.20 ± 1.31[a]
Villi goblet cells number	5.01 ± 0.19[b]	4.20 ± 0.09[b]	8.16 ± 1.51[a]
CML (µm)	30.21 ± 4.50[a]	22.02 ± 4.42[b]	25.68 ± 5.0[a,b]
LML (µm)	72.56 ± 7.44[a]	52.83 ± 8.00[b]	55.26 ± 14.63[b]

Abbreviations: CML, circular muscle layer; HF, high-fat diet group; HFC, high-fat corn group; LML, longitudinal muscle layer; NC, normal control group. The value is expressed as mean ± SD, n = 06 animals/group. Different lowercase letters in the same line: groups are significantly different ($p < .05$); same letter in the same line: groups are similar ($P > .05$). Analysis of variance followed by the Duncan post hoc test for multiple comparison.

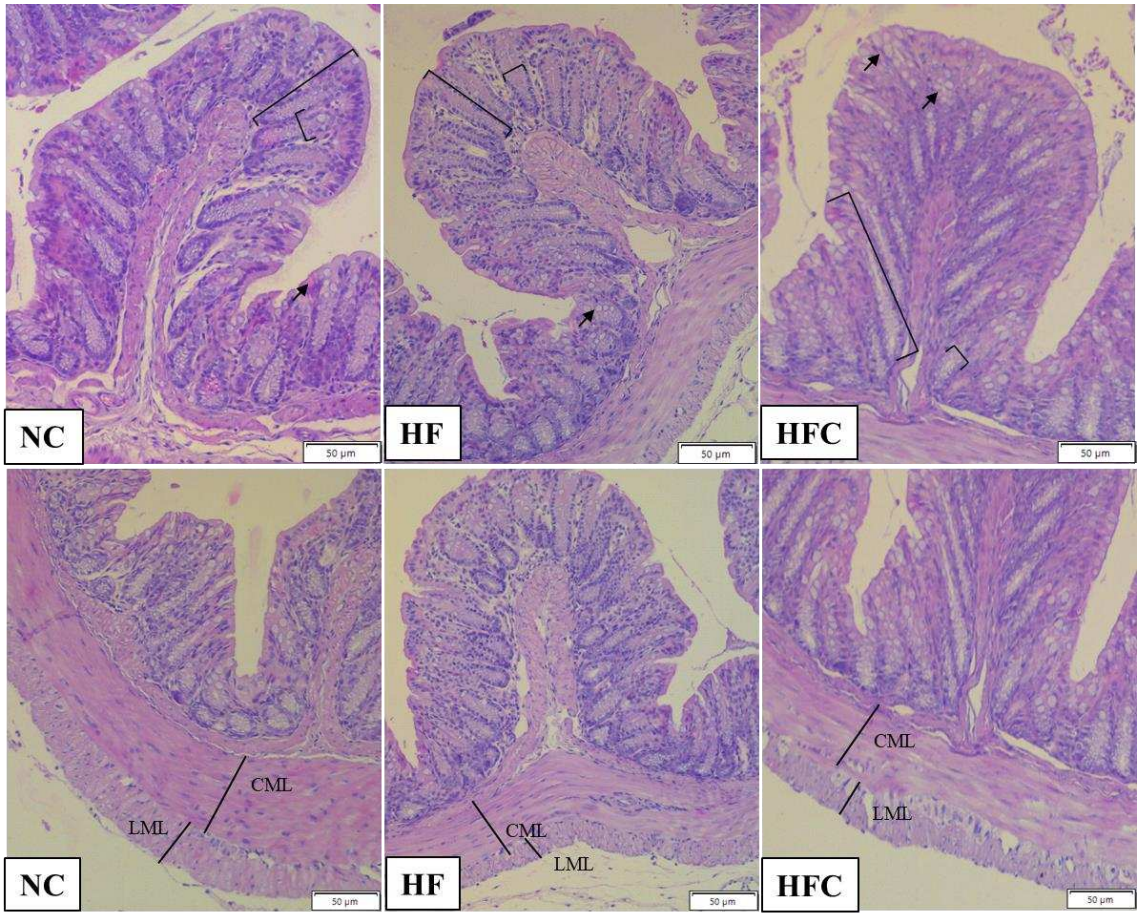

Fig. 1 - Effects of black corn flour diet consumption in colonic histomorphometric characteristics in mice fed a high-fat diet. NC: normal control group; HF: high-fat diet group; HFC: high-fat corn group; CML: circular muscle layer; LML: longitudinal muscle layer. Black arrows represent goblet cells in the crypt. Black brackets represent the crypt depth and width. Tissue stained with hematoxylin/eosin. *n*= 06 animals/group. Bar: 50 µm.

3.3 Effect of black corn flour on short-chain fatty acids and fecal pH

There was no difference in fecal pH level and cecal concentration of acetic, propionic, and butyric acid among the experimental groups (P> .05) (Table 3).

Table 3. Effect of black corn flour diet consumption in cecal short-chain fatty acids concentration and fecal pH in mice fed a high-fat diet.

	NC	HF	HFC
Fecal pH	8.21 ± 0.33[a]	8.35 ± 0.32[a]	8.11 ± 0.37[a]
Acetic acid (µmol/mL)	2.11 ± 0.36[a]	2.35 ± 0.82[a]	3.16 ± 0.94[a]
Propionic acid (µmol/mL)	1.12 ± 0.95[a]	2.18 ± 0.17[a]	2.34 ± 0.74[a]
Butyric acid (µmol/mL)	1.26 ± 1.46[a]	0.17 ± 0.14[a]	0.39 ± 0.05[a]

Abbreviations: HF, high-fat diet group; HFC, high-fat corn group; NC, normal control group. The values are expressed as mean ± SD. Fecal pH: n = 10 animals/group; short chain fatty acids: n = 5 animals/group. Different lowercase letters in row: groups are significantly different (P < .05). Analysis of variance followed by the Duncan post hoc test.

3.4 Overall structural changes in the cecal microbiota

To investigate the effects of black corn flour on the gut microbiota of mice fed a HF diet, cecal contents were collected at the end of the experimental period (8 weeks). The 16S rRNA gene amplicon sequencing method (V3-V4 region) generated 4,907,095 raw reads for 30 samples. After filtering and cleaning the read, 2,569,404 clean tags with good quality were used for further analysis. Good's coverage estimator for each sample was >98%, showing sufficient sequencing

depth for gut microbiota evaluation. The total number of raw read, filtered read, and the normalized read counts per group are provided in the Supplemental Materials (Supplemental Table S1).

The α-diversity indexes used to assess the richness and diversity of the microbiota were calculated by Chao1, Shannon, and Simpson index. No difference was observed in the Chao index (Fig. 2A). Compared with the NC and HF groups, the black corn group (HFC) had greater diversity ($P < .05$), as verified by the Simpson index (Fig. 2B). We observed a significant effect of the HF diet ($P < .05$) on increasing species richness through the Shannon index (Fig. 2C).

The β-diversity analysis shows the microbial composition difference based on the overall OTUs. The PCoA shows an evident distinction in bacterial communities of HFC groups because these animals exhibited a distinct cluster compared with animals in the HF and NC groups. Moreover, we observed that animals from the HF and NC groups showed low dissimilarity in the bacterial community (Fig. 3A). Further, the unweighted pair group method with arithmetic mean show that black corn flour had regulatory effects on the gut microbiota structure. The unweighted pair group method with arithmetic mean distinguished the cecal microbiota of mice fed an HFC from the other diets and the black corn flour from the HF diet. The cluster analysis of HFC group showed that its bacterial community clustered closely with the NC group. Thus, HF animals' cecal microbial community differed from HFC animals because they were not clustered closely (Fig. 3B).

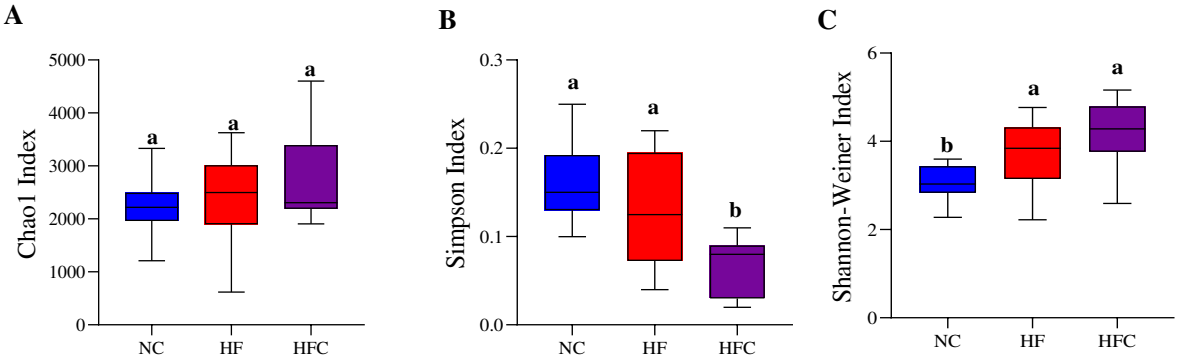

Fig. 2 – Effect of black corn flour in the microbial diversity of the cecal microbiome in mice fed a high-fat diet. (A-C) Measure of α-diversity using Chao1, Simpson, and Shannon Indexes. HF, high-fat diet group; HFC, high-fat corn group; NC, normal control group. The value is expressed as mean ± SD. n = 10 animals/group. Different lowercase letters: groups are significantly different

(P < .05). Analysis of variance followed by Duncan post hoc test. The Chao1 index showed no difference among groups. The Simpson index revealed a higher diversity in animals fed the black corn flour, and the Shannon index showed higher species richness in groups fed the high-fat diet.

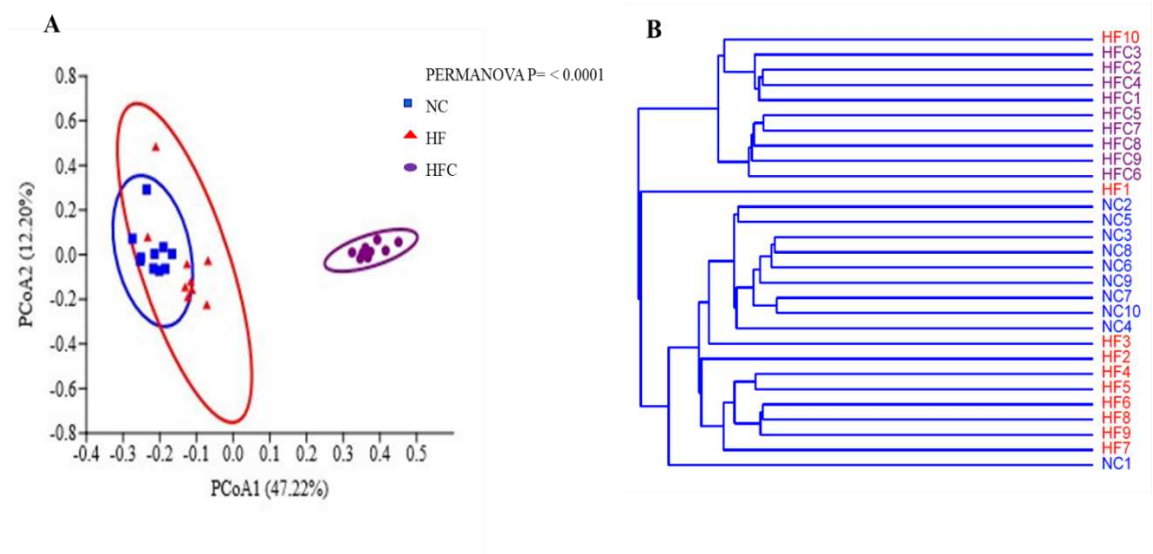

Fig. 3 – Effect of black corn flour in the diversity and richness in mice fed a high-fat diet. (A) Principal coordinate analysis (PCoA) based on Jaccard similarity distance of cecal microbial communities in mice fed a high-fat diet. Each dot represents 1 animal, and the colors represent the experimental groups. (B) Unweighted pair group method with arithmetic mean based on Jaccard similarity. HF, high-fat diet group; HFC, high-fat corn group; NC, normal control group; PERMANOVA, permutational multivariate analysis of variance. n = 10 animals/group. The PCoA revealed that there is a difference in the overall gut bacterial microbiota in animals fed the black corn flour.

3.5 Effect of black corn flour in the relative abundance at phylum, family, and genus levels

The relative abundance at phylum, family, and genus level was carried out to determine the different bacterial compositions among the experimental groups. The taxonomic classification of samples showed 16 phyla, 26 classes, 67 orders, 118 families, and 291 genera. Firmicutes, Bacteroidetes, and Actinobacteria were the most abundant phyla in all experimental groups at the phylum level. The relative abundance of Firmicutes, Actinobacteria, and Patescibacteria was higher (P < .05) in the HFC group than in the NC and HF groups. On the other hand, the abundance of Desulfobacteria and Verrumicrobiota was lower (P < .05) in the HFC group compared with the

NC and HF groups (Supplemental Fig. S1A). Further, the Bacteroidetes abundance was similar among all groups. In addition, changes in the cecal microbiota were observed at the family level in animals fed with black corn flour diet (Supplemental Fig. S1B). An increase in the relative abundance of Ruminococcacea, Eggerthellaceae, Prevotellacea, Clostridiaceae, and UCG-010 was detected in the HFC group compared with the HF and NC groups. However, the abundance of Desulfovibrionaceae, Bacteroidaceae, and Akkermansiaceae decreased ($P < .05$) in the HFC group compared with the HF and NC groups.

A genus-level analysis was carried out to determine the specific bacterial composition differences among the groups (Fig. 4A). The *Bacteroides* and *Faecalibaculum* abundance decreased ($P < .05$) in the HFC group compared with the HF and NC groups. The *Akkermansia* abundance was higher in the NC group and lower in the HFC ($P < .05$). Moreover, there was no difference in the abundance of *Ruminococcus*, *Roseburia*, and Prevotellaceae_UCG-001 between the NC and HF groups. However, these genera were higher in the HFC compared with the NC and HF groups (Fig. 4C). The abundance of *Bifidobacterium* was not different among the groups ($P > .05$). Moreover, the Firmicutes/Bacteroidetes ratio did not differ among the experimental groups ($P > .05$) (Fig. 4B).

Then, correlations between 7 bacterial genera abundance and 6 parameters of intestinal health were analyzed by Spearman's correlation (Fig. 4D). A positive correlation was observed between GC number and some genus: *Roseburia*, *Provotellacea_UCG*, and *Ruminococcus*. In the same way, crypt depth showed a positive correlation with *Roseburia* and *Ruminococcus.* Further, the genus *Akkermansia* was negatively correlated with crypt depth and positively with the fecal pH. The SCFA propionic acid was negatively correlation with *Faecalibaculum.*

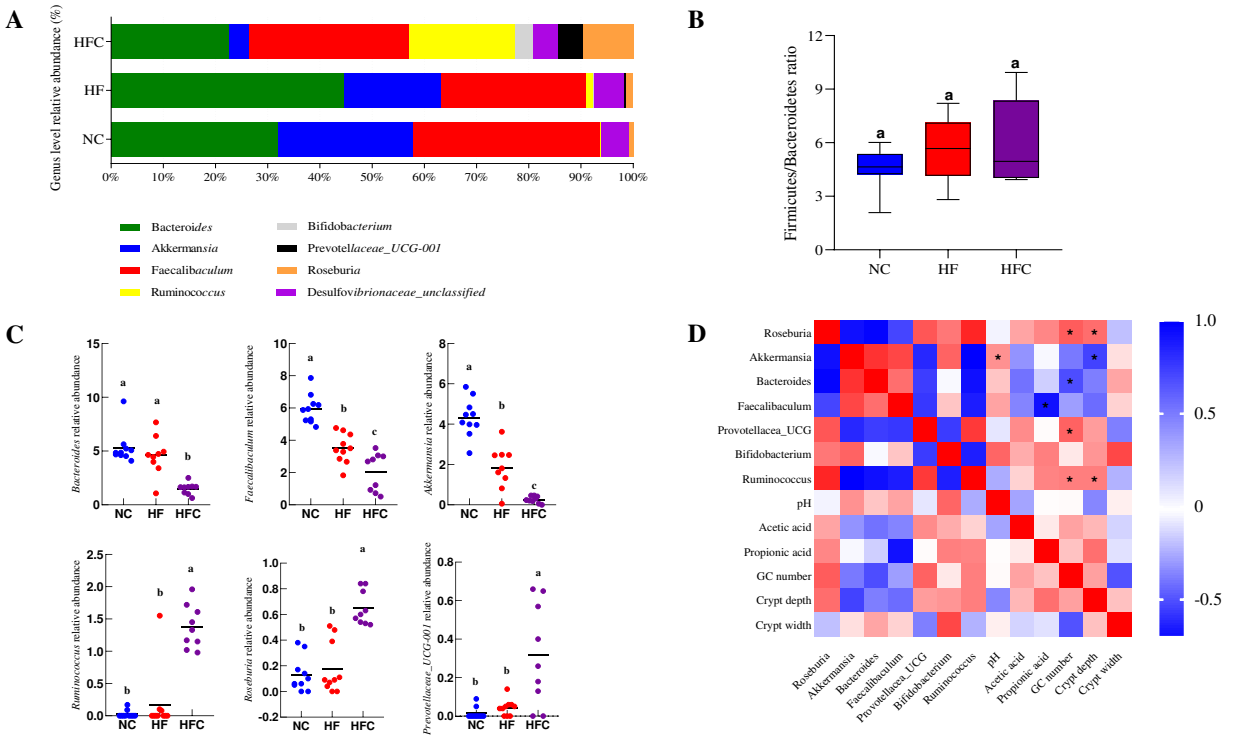

Fig. 4 – Effect of black corn flour intake in the relative abundance of cecal microbiota in mice fed a high-fat diet (A). Relative abundance at the genus level, (B) *Firmicutes/Bacteroidetes* ratio, (C) significant difference among an experimental group of a specific genus. (D) Heatmap of Spearman's correlation between cecal microbiota and intestinal parameters. ∗P < .05, ∗∗P < .01. HF, high-fat diet group; HFC, high-fat corn group; NC, normal control group. n = 10 animals/group. Only genus with an abundance >0.3% in at least 1 group was displayed. Data were analyzed with false discovery rate correction. Differences among groups were tested with analysis of variance followed by Duncan post hoc test (P < .05). Abundance of specific bacteria because Bacteroides, *Faecalibaculum*, and *Akkermansia* decreased in the black corn group. On the other hand, a higher abundance of *Ruminoccoccus*, *Roseburia*, and *Prevotellaceae_UCG-001* were observed in the black corn group. No significant difference was observed in the Firmicutes/Bacteroidetes ratio among the groups. Specific bacteria as *Roseburia*, *Provotellacea_UCG*, and *Ruminococcus* were positively correlated with goblet cell number and other bacteria as *Akkermansia* was negatively correlated with crypt depth.

3.6 Effect of black corn flour on the dominant cecal microbiota

All OTUs were analyzed by linear discriminant analysis effect size to identify dominant cecal microbiota and intestinal biomarkers using taxonomy (Fig. 5). As a result, 25 dominant OTUs with effect size >3 was identified. The HF group showed a higher number of dominant taxa (n = 10) compared with the HFC (n = 9) and NC (n = 6) groups. These analyses show more bacterial taxa in mice fed black corn flour. In the HFC group, the Lachnospiraceae had a large effect on the dominant community. The genus *Lactobacillus*, composed of probiotic species, was significantly increased in the HFC group. The Bacteroides were the dominant genus in the microbiota of the HF group, showing a larger effect size. In the NC group, the dominant communities were *Akkermansia* and *Faecalibaculum*.

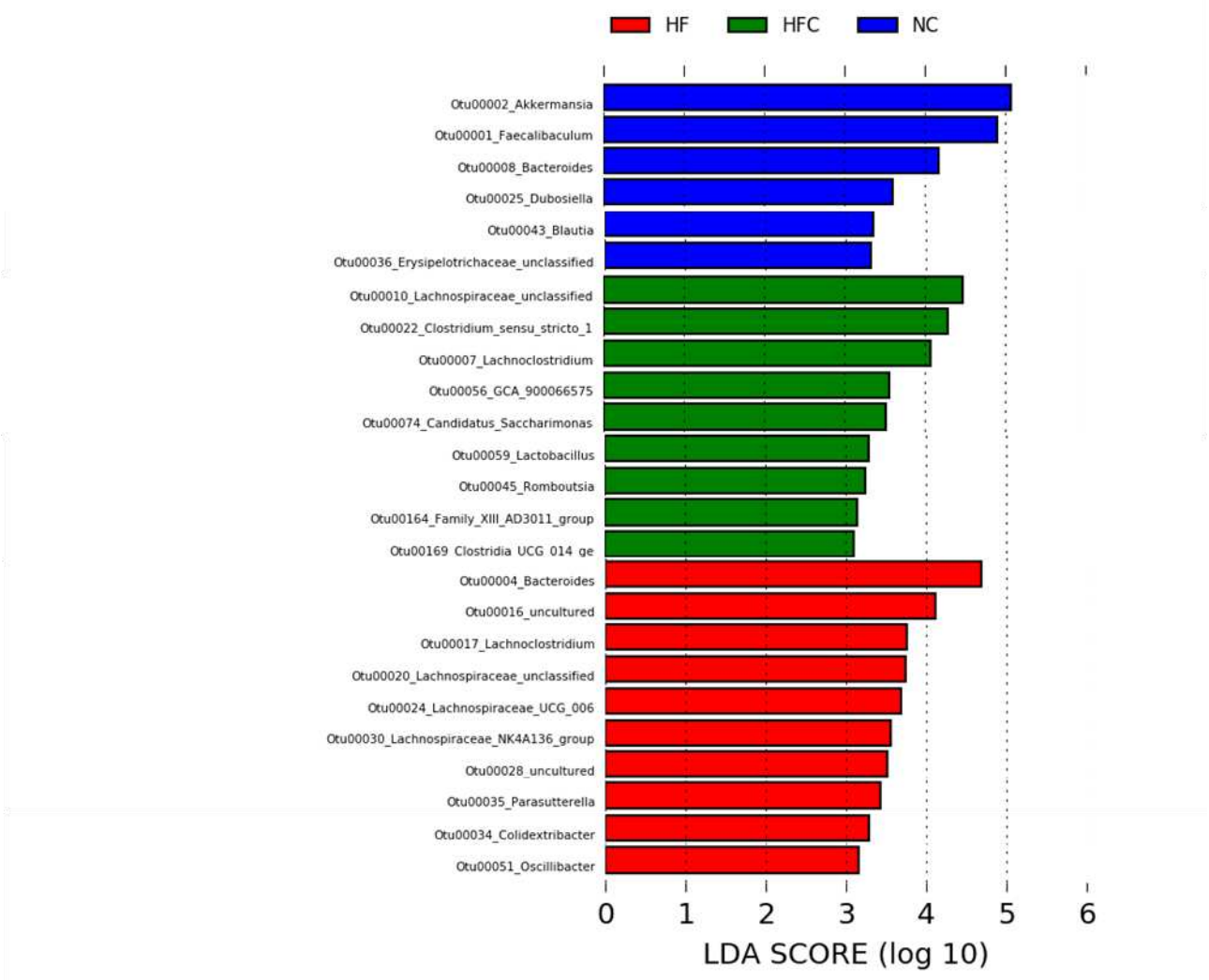

Fig. 5 – Histogram of linear discriminant analysis effect size (LEfSe) method to compute linear discriminant analysis (LDA) scores of differences in dominant microorganisms between groups. HF, high-fat diet group; HFC, high-fat corn group; NC, normal control group. n = 10 animals/group. Significant differences were considered with $P < .05$. The LEfSe method revealed that the genus *Lactobacillus* increased in the HFC group, *Akkermansia* and *Faecalibaculum* were the dominant community in the NC group, and Bacteroides had the larger effect size in the HF group.

3.7 Effect of black corn flour on KEGG functional capacity of bacterial communities

Phylogenetic investigation of communities by reconstruction of unobserved states was carried out to predict the functional composition of the cecal microbial community metagenome (Fig. 6). Black corn flour consumption caused changes in some metabolic pathways because lipid biosynthesis pathways significantly reduced and glycolysis metabolism increased ($P < 05$) in the HFC group compared with the other groups. Black corn flour intake also influenced the glycogen degradation, increasing this biological function in HFC compared with the HF group. In addition, the HF diet showed an effect on specific biological pathways. The glucose and glucose-1-phosphate degradation, glycolysis I and II (from G6P and fructose 6- phosphate) decreased in animals fed an HF diet (HF and HFC) compared with those fed a control diet, with no difference when the black corn flour was added to the diet. Amino acids biosynthesis (lysine, L-threonine, and L-methionine) was enriched in HFC compared with HF animals, and the urea cycle pathway decreased in the HFC group compared with the HF group and was similar to the NC group.

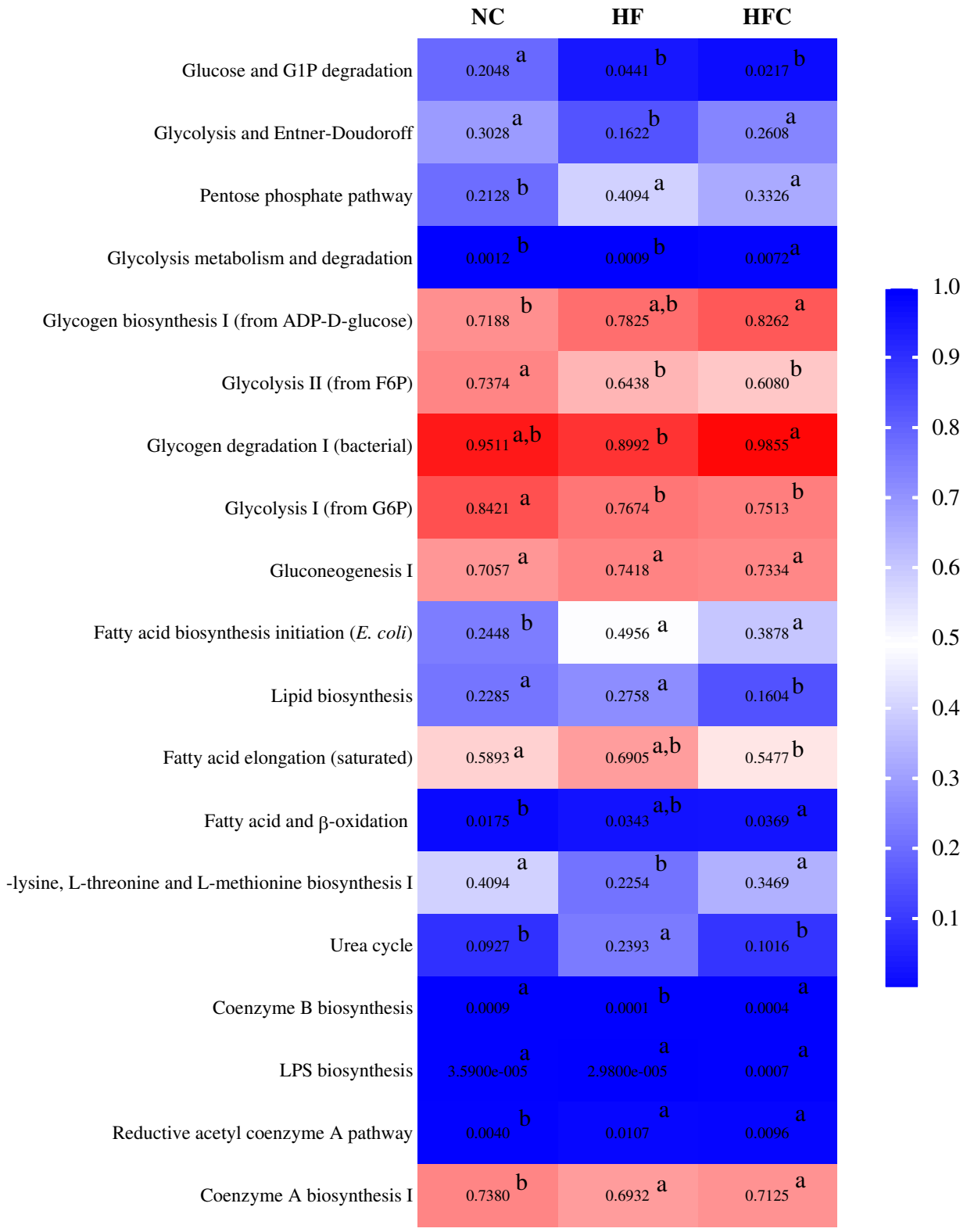
NC
HF
HFC
Glucose and G1P degradation
0.2048 a
0.0441 b
0.0217 b
Glycolysis and Entner-Doudoroff
0.3028 a
0.1622 b
0.2608 a
Pentose phosphate pathway
0.2128 b
0.4094 a
0.3326 a
Glycolysis metabolism and degradation
0.0012 b
0.0009 b
0.0072 a
Glycogen biosynthesis I (from ADP-D-glucose)
0.7188 b
0.7825 a,b
0.8262 a
Glycolysis II (from F6P)
0.7374 a
0.6438 b
0.6080 b
Glycogen degradation I (bacterial)
0.9511 a,b
0.8992 b
0.9855 a
Glycolysis I (from G6P)
0.8421 a
0.7674 b
0.7513 b
Gluconeogenesis I
0.7057 a
0.7418 a
0.7334 a
Fatty acid biosynthesis initiation (E. coli)
0.2448 b
0.4956 a
0.3878 a
Lipid biosynthesis
0.2285 a
0.2758 a
0.1604 b
Fatty acid elongation (saturated)
0.5893 a
0.6905 a,b
0.5477 b
Fatty acid and β-oxidation
0.0175 b
0.0343 a,b
0.0369 a
-lysine, L-threonine and L-methionine biosynthesis I
0.4094 a
0.2254 b
0.3469 a
Urea cycle
0.0927 b
0.2393 a
0.1016 b
Coenzyme B biosynthesis
0.0009 a
0.0001 b
0.0004 a
LPS biosynthesis
3.5900e-005 a
2.9800e-005 a
0.0007 a
Reductive acetyl coenzyme A pathway
0.0040 b
0.0107 a
0.0096 a
Coenzyme A biosynthesis I
0.7380 b
0.6932 a
0.7125 a
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

Fig. 6 – Heatmap of the effect of black corn flour consumption on the relative significance of differentially enriched microbial metabolic pathways in cecal microbiota in mice fed a high-fat diet. Treatment groups not indicated by the same letter are significantly different by analysis of variance followed by the Duncan post hoc test ($P < .05$). Pink color indicates higher functional bacterial capacity; green indicates lower functional bacterial capacity. PICRUSt revealed changes at lipid biosynthesis pathway in the HFC group. ADP, adenosine diphosphate; F6P, fructose 6-phosphate; G1P, glucose-6-phosphate; HF, high-fat diet group; HFC, high-fat corn group; LPS, lipopolysaccharides; NC, normal control group.

4. Discussion

There are several reports about the effects of anthocyanins extract on intestinal health; however, considering the benefits of consuming nutrients in its whole matrix and not as a single component, it is valuable to investigate the effects of black corn flour on the intestine. The black corn whole flour (20%) consumed by mice promoted positive changes in the intestinal homeostasis by enhancing the abundance of *Ruminococcus*, *Roseburia*, and *Provotellacea_UCG-001*, decreasing *Akkermansia* and *Bacteroides*, besides improving the GC's production in the cecum.

A HF diet (60% fat composition) consumed by mice over 8 weeks in our study resulted in a lower cecum weight than the normal diet and higher species richness as observed in the Shannon index and OTU number. Previously, a HF diet has been shown to promote a shift in bacteria species abundance leading to an overall increase in gut permeability [1], and also a higher *Firmicutes/Bacteroidetes* ratio [8,37]. Differently, in our study, the HF diet did not influence the *Firmicutes/Bacteroidetes* ratio, with no difference among the groups. We observed that even with the *Firmicutes/Bacteroidetes* ratio similar among groups, there was a lower bacterial diversity in the HF group compared with the HFC group (Simpson index), suggesting the existence of other compositional modification at the family and genus levels. As shown in a literature review, diverse discrepancies have been observed to the *Firmicutes/Bacteroidetes* ratio and obesity; therefore, it still, cannot be considered a biomarker of intestinal health [38]. The focus of investigation should be on specific changes in the microbiota profile at family, genus, or species level, not only considering the changes at phylum level [39].

The present study verified several positive changes in the gut microbiota, specifically at the genus level. There was an increase in the abundance of beneficial bacteria such as

Ruminococcus, Roseburia, and *Provotellacea_UCG-001* in the HFC group. *Roseburia* are the most abundant butyrate-producing bacteria by hydrolyzing starch and other sugars [40]; therefore, as black corn flour is a significant source of starch, it might have contributed to the *Roseburia* abundance. Starches have been shown to induce a synergetic effect on the gut microbiota composition by stimulating the abundance of *Roseburia* [41]. Berry anthocyanin extract promoted the growth of *Roseburia* [42]. Species of the genus *Roseburia* showed β-glucosidase activity in vitro, which is involved in the metabolism of dietary compounds [43]; therefore, it might be involved in the intestinal metabolism of anthocyanins. Moreover, *Ruminococcus* are gram-positive intestinal bacteria involved in SCFA production, which has been proved to reduce after a HF diet [44]. *Ruminococcus gnavus* is involved in intestinal barrier integrity by modulating the mucin expression and the glycosylation level in GCs [45]. In our study, both *Roseburia* and *Ruminococcus* genus positively correlated with GC number, with a rise in GC number in the colon resulting from the black corn flour consumption.

Interestingly, even with a significant increase in SCFA producers, such as *Roseburia* and *Ruminococcus* abundance in the HFC group, no change was observed in the cecal concentrations of butyrate, propionate, and acetate in animals fed the black corn flour diet. Authors have suggested that improving gut epithelial function might increase SCFA utilization and absorption from colonic cells, thus impairing their quantification after microbiota composition modulation [46]. Perhaps the maintenance in SCFA concentrations observed in our study might be due to use of the energy produced by SCFA for cell proliferation, which raises the goblet cells numbers. Alternative hypothesis correlates to the sample size used for the SCFA analysis that might not have been powered enough to verify significant differences. However, a positive correlation was observed between *Provotellacea-UCG* and acetic acid and a negative between *Faecalibaculum* and propionic acid. Interestingly, animals fed the black corn flour diet had a lower abundance of *Faecalibaculum* and higher *Provotellacea-UCG.*

The genus *Provotellacea_UCG-001* was enhanced in the HFC group. Authors have suggested that the proliferation of *Prevotellacea-UCG 001* abundance in *ob/ob* mice is related to the upregulation of adenosine monophosphate protein kinase (AMPK) signaling pathway in cecal tissue, which leads to changes in downstream metabolic functions as activation of glycolysis and fatty acid oxidation [47]. Black corn soluble extract upregulated the intestinal gene expression of AMPK in an *in vivo* intra-amniotic approach [48]. The AMPK activity leads to oxidation of fatty

acids in peripheral tissues and to decrease glycogen in the liver [49]. In our previous study, we demonstrated that the black corn whole flour (20%) stimulated β-oxidation in the liver of mice [18]. Analyzing the microbial metabolic signaling pathway as an additional explanation, the black corn flour group was involved in enhancing fatty acid β-oxidation and decreasing fatty acid biosynthesis and elongation. However, further studies are needed to investigate if there is a pathway between *Provotellacea-UCG-001* and inhibition of fatty acids synthesis.

After 8 weeks of black corn flour added to a high-fat diet, we observed a decrease in the relative abundance of *Akkermansia* in the cecal content. *Akkermansia* genus is a mucin-degrading bacteria involved in maintaining mucus thickness by using mucin as its sole energy source [50]. Several studies showed that anthocyanins supplementation increased *Akkermansia* in the gut microbiota [37,51,52]. We observed an increase in the GC number in the HFC group. However, although *Akkermansia* stimulates the mucin production [50], in our experiment, GC number showed no correlation with *Akkermansia* abundance. In this sense, the *Akkermansia* improvement from polyphenols might depend on its baseline level [50]. The increase of GC can be related to the polyphenol content. Some molecular pathways underlying how polyphenols promote the proliferation of GCs have been proposed as the Notch, Wnt3, and morphogenetic protein pathways, which might trigger the bloom of goblet cells [53]. Therefore, the GC proliferation observed from the black corn flour supplementation might be due to other factors besides the composition of *Akkermansia*. Further, the *A muciniphila* strains are suggested to be mutually coexclusive, thus being competitive exclusion bacteria, which excludes one another from the gut microbiome [54]. Therefore, we hypothesize that as the consumption of black corn flour stimulated the growth of *Roseburia*, *Rumminococus*, and *Provotellacea UCG-001* abundance, it could have promoted a status of competition, thus avoiding the growth of *Akkermansia*. However, additional work in this field is needed to draw further conclusions.

In this study, the animals fed a HF diet added with black corn flour improved the GC number in the colon. GCs produce mucin, which acts as a physical barrier to avoid the translocation of pathogenic bacteria [55]. Further, dietary patterns and bioactive components can influence the mucus layer at different levels as increasing mucin production [56]. Soluble extract of black corn rich in anthocyanins increased goblet cell number in an *in vivo* experiment because of the differentiation of stem cells into GCs to improve mucus production [48]. Our results corroborate

with this evidence and reveal that black corn flour as a whole matrix can also improve the mucus layer by increasing goblet cell number.

Finally, according to a systematic review of studies performed in rodents, the range of anthocyanin supplementation to exert beneficial effects on intestinal health is from 12 to 1280 mg/100 g of diet [16]. In the present study, we added 20% black corn flour to the HF diet; thus, we provided 17.13 mg cyanidin-3 glycoside/100 g diet. Therefore, we speculate that the results observed from the black corn flour consumption could be associated with the metabolism of anthocyanins that interacts with intestinal bacteria and stimulates its abundance. Furthermore, because the black corn was supplemented as a flour in its whole food matrix, the observed results may also be due to an association of the anthocyanin and other phenolic compounds embedded in the food matrix in a synergetic context. In addition, the black corn flour has a composition of 12.92% insoluble and 0.31% soluble fiber; thus, the observed results can also be due the fiber composition at the black corn flour. Therefore, the result shows the relevance of including the whole food and not an isolated bioactive compound in the diet.

The experimental diets had the same amount of minerals and vitamins; however, considering that animals fed the HF and HFC diets had lower food intake, the consumption of mineral and vitamins of these animals were lower than the NC group, which might be considered a limitation of this experiment. To correct this limitation, we used positive control, an HF diet to compare our results. Overall, the present study demonstrated that daily consumption of black corn flour HF diet for 8 weeks could alleviate intestinal changes promoted because of intake of an HF diet. Additionally, 16S rRNA sequencing demonstrated that black corn flour improved the relative abundance of *Roseburia*, *Ruminococcu*s, and *Provotellacea-UCG-001,* which did not positively affect the production of SCFA, but increased the number of GCs. Further the corn flour intake modulated the gut microbiota by decreasing the abundance of *Akkermansia* and Bacteroides.

Thus, the results observed in our experiment might contribute to stimulate black corn consumption and production. In our experiment, we supplemented the black corn as a flour; therefore, in a human diet, this flour can be easily incorporated for human consumption. Black corn flour can be used in a variety of culinary application as elaboration of cake, bread, pasta, pie, pancakes, and cookies. The incorporation of this flour in these recipes brings additional properties, as a bioactive component, mainly, anthocyanin, besides providing color and flavor to these products

5. Conclusions

The present study demonstrated that the daily consumption of black corn whole flour for 8 weeks could partially alleviate the undesirable intestinal modifications associated to a HF diet in animal model. In HF mice, the black corn flour– containing diet positively affected microbial modulation improving the relative abundance of intestinal beneficial genera as *Roseburia*, *Ruminococcus*, and *Provotellacea-UCG-001*. Further, the microbial changes might have promoted the increase of GCs. The current results might be associated with the anthocyanins, which is the main bioactive compound verified in the black corn; however, the other compounds present in whole corn, such as starch and dietary fiber, could also collaborate these promising results. Therefore, findings in our study indicate that black corn flour as a dietary source of anthocyanins can be a potential candidate for a functional food promoting beneficial intestinal effects.

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Author declarations

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Thaisa Agrizzi Verediano: Data curation, Formal analysis, Investigation, Methodology, Writing- original draft. Cintia Sant'Ana: Investigation, Methodology. Mariana Grancieri: Investigation, Methodology. Elad Tako: Writing – final draft. Maria Cristina Dias Paes: Writing – final draft, Supervision, Resource. Hércia Stampini Duarte Martino: Investigation, Methodology, Writing – final draft, Supervision, Resource.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nutres.2022.10. 005.

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Supplementary Material

Table S1. Summary of sequencing data for mice after eight weeks of treatment.

Treatment	Good's coverage	Raw sequences	After filtering and clean-up		Normalized reads	
		Reads	Reads	OTUs	Reads	OTUs
NC	0.9946 ±0.0013	170640.1 ±11239.63	117174±7858.05	1728.5±369.43	33167.4 ±49.14423	1102.2±245.7749
HF	0.9927±0.0046	167168.2±15164.01	104110±26408.43	1880.6±518.0718	33151 ±97.49872	1288.8 ±400.9774
HFC	0.9843 ± 0.0026	169890.2±15891.26	39618±4700.02	1518.666±172.354	33325.11±68.27416	1518.667 ±172.3543

Values expressed as mean ± standard deviation, *n*=10 animals/group. NC: normal control group; HF: high-fat diet group; HFC: high-fat corn group; OUTs: Operational Taxonomic Units.

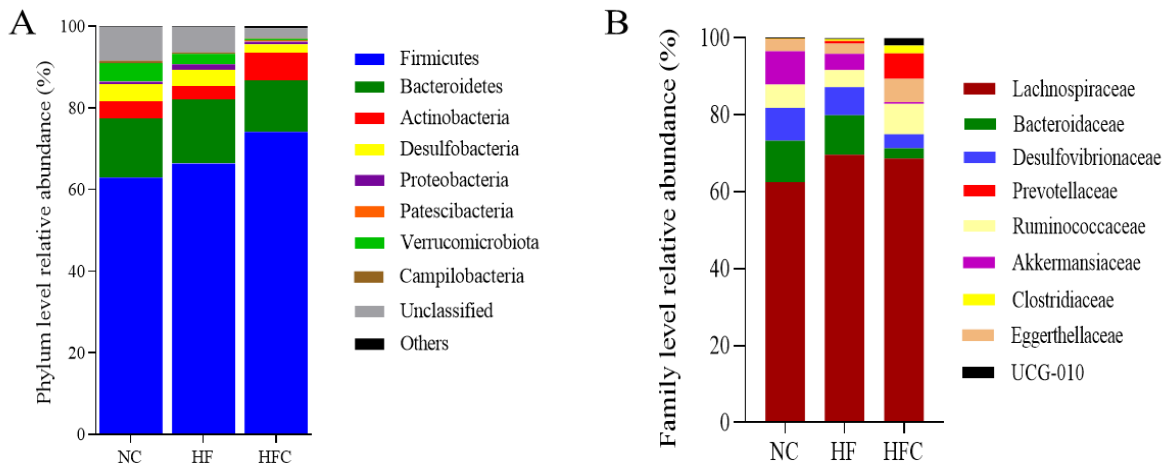

Fig. S1. Effect of black corn flour intake in the relative abundance of cecal microbiota at the phylum and family level: (A): Relative abundance at phylum, (B): Relative abundance at family level. NC: normal control group; HF: high-fat diet group; HFC: high-fat corn group. *n*=10 animals/group. Only phyla and family with abundance > 0.3% in at least one group were displayed. Data were analyzed with FDR correction. Differences among groups were tested with ANOVA followed by Duncan post-hoc test ($p<0.05$).

6.3. PAPER 4

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Black corn (*Zea mays* L.) soluble extract showed anti-inflammatory effects and improved the intestinal barrier integrity *in vivo* (*Gallus gallus*)

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ABSTRACT

Black corn (*Zea mays L.*) is a pigmented type of this cereal whose color of the kernels is attributed to the presence of the anthocyanins. In this study, we assessed the black corn soluble extract (BCSE) effects on the intestinal functionality, morphology, and microbiota composition using an *in vivo* model (*Gallus gallus*) by an intra-amniotic administration. The eggs were divided into four groups (n = 6–10): (1) No Injection; (2) 18 MΩ H_2O/cm; (3) 5% (5 mg/mL) BCSE; (4) 15% (15 mg/mL) BCSE. The BCSE showed anti-inflammatory effects by down regulating the gene expression of tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL6), and the transcriptional nuclear factor kappa beta (NF-κB). Further, the BCSE increased the relative abundance of *E. coli* and *Clostridium*. 5% and 15% BCSE increased the hepatic glycogen and upregulated the gene expression of sodium-glucose transport protein (SGLT1). In the morphology, 5% and 15% BCSE increased the goblet cell (GC) number on the crypt, the GC size on the villi, Paneth cell number on the crypt, and the acid GC. Further, the BCSE strengthened the epithelial physical barrier through upregulating the intestinal biomarkers AMP- activated protein kinase (AMPK) and caudal-related homeobox transcriptional factor 2 (CDX2). The overall result suggests that the BCSE promotes intestinal anti-inflammatory effects as well as enhances the intestinal barrier function.

1.Introduction

Black corn (*Zea mays L.*) is a colored cereal grain with claimed health benefits due to its bioactive components, mainly anthocyanins (Yang & Zhai, 2010; Van Hung, 2014; Ranilla et al., 2017; Zhu, 2018). The anthocyanins content in colored maize can vary widely due factors such as maize genetics, growing locations, part of the plant, extraction method, identification, and quantification (Harakotr et al., 2014). In this context, studies have shown a range of anthocyanin content from 23 to 252 mg anthocyanin/kg in a survey with 398 genotypes of pigment corn (Paulsmeyer et al., 2017) and from 12.8 to 93 mg cyanidin-3-glucoside (C3G) equivalents/g in 20 purple corn genotypes (Zhang et al., 2019). Anthocyanins, a subclass of flavonoids, are bioactive water-soluble pigments accountable for a wide range of blue, purple, violet, and red in fruits, flowers, and vegetables. In plants, anthocyanins are found mainly as a glycoside, with the basic structure as an anthocyanidin core attached to a sugar and organic acids (Sui et al., 2019). Beneficial health effects have been established to anthocyanins in preventing or improving risk factors associated with obesity (Azzini et al., 2017; Jamar et al., 2017; Lee et al., 2017), diabetes mellitus (Guo et al., 2007; Jurgonski et al., 2013; Rozanska & Regulska-Ilow, 2018), metabolic syndrome (Bhaswant et al., 2017), mucositis (Tong et al., 2017), and colorectal cancer (Fernandez et al., 2018; de Sousa Moraes et al., 2019). In this sense, anthocyanin acts as an antioxidant component by scavenging reactive oxygen species and as an anti-inflammatory through downregulating specific transcription factors such as the nuclear factor-kappa B (NF- κB), thus decreasing the expression of pro-inflammatory cytokines as tumor necrosis factor-alpha (TNF-α), interleukin (IL) 6 and IL1β (Vendrame & Klimis-Zacas, 2015; Bendokas et al., 2020).

Regarding the anthocyanin metabolism, briefly, a small proportion of the intake anthocyanin is absorbed intact through the upper gastrointestinal wall by active transporters (Faria et al., 2014). Thus, most anthocyanin reaches the colon, where they are metabolized into their metabolites by bacteria from the intestinal microbiota. *Bifidobacterium* spp. and *Lactobacillus* spp. have the enzyme β-glucosidase, which is necessary to metabolize the anthocyanin into sugar and phenolic acids. Therefore, their metabolites modulate the growth of specific beneficial bacteria in the gut microbiota (Faria et al., 2014; Hribar & Ulrih, 2014). A systematic review recently showed that anthocyanin supplementation in animal studies could modulate the intestinal microbiota by increasing *Lactobacillus* spp. and *Bifidobacterium* spp. and improving short-chain fatty acids (SCFA) production. Further, anthocyanin can improve the epithelial barrier through a higher tight

junction expression and mucus production by increasing the goblet cell differentiation due its fermentative action (Verediano et al., 2021). Besides, it is suggested that phenolic components founded in a purple potato extract might improve the barrier function through activating the AMP-activated protein kinase (AMPK) pathway, which activates the caudal-related homeobox transcriptional factor 2 (CDX2). By this, the upregulation of CDX2 controls the intestinal epithelial differentiation, and might regulate the tight junctions expression (Sun et al., 2018). However, this pathway has only been verified in a *in vitro* model (Sun et al., 2018). Further, anthocyanin mix showed potential to improve occludin expression and Mucin 2 (MUC2) level, which might be used as biomarkers to tight junction functionality and intestinal barrier integrity (Cremonini et al., 2019).

To assess the prebiotic effects of nutrients on intestinal functionality and gut microbiota, the intra-amniotic administration in *Gallus gallus* model has been used as a valid approach (Hartono et al., 2015; Hou & Tako, 2018; da Silva et al., 2019; Dias et al., 2019; Wang et al., 2019; Gomes et al., 2021). The *Gallus gallus* is an established *in vivo* model to investigate the intestinal microbiome population due to its homology of gene sequence in the phylum levels with human (Yegani & Korver, 2008; Hou-Tako, 2018). The intra-amniotic administration of soluble extracts of beans, wheat, chia and yacon had demonstrated prebiotic effects by improving intestinal morphology, microbial composition and functionality (da Silva et al., 2019; Wang et al., 2019; Martino et al., 2020). Also, the administration of resveratrol and pterostilbene promoted beneficial morphological changes and improved the gut microbiota composition and function (Gomes et al., 2021). In this context, although some mechanisms have been proposed to explain how anthocyanin improves the intestinal barrier *in vivo*, there are no studies investigating the effects of black corn soluble extract, source of bioactive components, mainly anthocyanin, in the intestinal development.

Thus, we hypothesized that the black corn soluble extract as a source of anthocyanin could modulate the abundance of beneficial gut bacteria improving the intestinal physical barrier by upregulating the gene expression of proteins in the pathway of tight junction development. Therefore, showing improvement of the morphologic structure and brush border enzymes functionality. To explore this hypothesis, we designed an *in vivo* study investigating the effects of the intra-amniotic administration of black corn soluble extract (BCSE) on the intestinal morphology, functionality, and microbiota composition *in vivo* (*Gallus gallus).*

2. Materials and methods

2.1 Sample preparation

The black corn (TO002) was provided by the Brazilian Agriculture Research Corporation (EMBRAPA). The TO002 black-color maize genotype is an access of the Maize Germplasm Bank of the Embrapa Maize and Sorghum. The material was produced in the 2018/2019 harvest season at "Embrapa Milho e Sorgo" experimental farm, Sete Lagoas, Minas Gerais, Brazil. The samples were stored in a plastic bag, kept at 4 ^{0}C, and protected from light until processing. The black corn flour was prepared using a knife mill grinder with a 1.0mm stainless steel sieve (Willy, Solab®), which was stored at 4 ^{0}C.

2.2 Soluble extract from black corn

Soluble components extraction was performed as described (Tako et al., 2014). Black corn samples were dissolved in distilled water (50g/L) (60 ^{0}C, 60 min) and centrifuged at 1200 *g* rpm (4 ^{0}C) for 25 min, and then the supernatant was collected. Then, the supernatant was dialyzed (MWCO 12-14 kDa) (48) against distilled water. The dialysate was collected and lyophilized to yield a fine off-white powder.

2.3 Phytate, dietary fiber, iron and zinc analysis in black corn flour and black corn extract

Dietary phytic acid (phytase)/total phosphorus was measured as phosphorus release by phytase and alkaline phosphatase, according to the manufacturers' instructions (*n*=5) (K-PHYT 12/12; Megazyme International, Bray, Ireland). Total phytate concentrations were calculated with Mega-Calc™ by subtracting free phosphate concentrations in the extracts from the total amount of phosphorous that is exclusively released after enzymatic digestion. Total dietary fiber (soluble and insoluble fiber) was determined by the gravimetric-enzymatic method (AOAC International, 2012) using the enzymatic hydrolysis for a heat-resistant amylase, protease and amyloglucosidase (Total dietary fiber assay kit, Sigma®, San Luis, Missouri, EUA). The total dietary fiber content was determined by the sum between the soluble and insoluble fractions. For the determination of iron and zinc, briefly, black corn flour (0.5g) and black corn extract (0.2g) were pre-digested with 3.0mL of a 60:40 (*v/v*) HNO_3 mixture in a Pyrex glass tube and left overnight to destroy organic matter. The analyses were carried out using an inductively coupled plasma atomic spectrometer

(ICP-AES) (Thermo iCAP 6500 series, Thermo Scientific, Cambridge, UK). Yttrium from High Purity Standards (10M67-1) was used as an internal standard to ensure batch-to-batch accuracy and correct matrix inference during digestion (da Silva et al., 2019; Dias et al., 2019).

2.4 Polyphenols Analysis

2.4.1 Polyphenol extraction

The sample of black corn flour were extracted with 5.0mL of methanol: formic acid 10% (*v/v*) solution, using sonication 40 KHz (USC 1400, Unique, Indaiatuba, SP) for 15 min. Then, the samples were centrifuged at 112 *g* for 10 min and the supernatant collected. This step was repeated 3 times. The supernatant was concentrated in rotary evaporator and 2mL of deionized water was added. A total of 20µL were removed and filtered to injection.

2.4.2 Chromatography profile and analysis of anthocyanins

The analysis was carried out using a liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry (LC-DAD-ESI-MS/MS). The HPLC model was Varian 250 (Varian Inc., Lake Forest, CA) coupled with diode array detector (DAD) and mass spectrometer 500-MS IT (Varian Inc., Lake Forest, CA). The column was Symmetry C18 (3µm, 250 x 2mm) (Varian Inc., Lake Forest, CA). The flow rate was 0.4mL/min and oven temperature of 30 ^{0}C. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient was varied linearly from 10 to 26% B (*v/v*) in 40 min, to 65% B at 70 min, and finally to 100% B at 71 min and held at 100% B at 75 min. The DAD was set up at 270 to 512nm to monitor the UV/Vis absorption and the UV/Vis spectra were recorded from 190 to 650nm. Mass spectra were acquired simultaneously using electrospray ionization in the positive and negative ionization (PI and NI) modes in voltage 80V fragmentation for a mass range of 100-1000 amu. A drying gas pressure of 35 psi, nebulizer pressure of 40 psi, a drying gas temperature of 370 ^{0}C, capillary voltage of 3500V for PI and 3500V for NI, in addition to field voltages of 600V spraying were used. The LC system was directly coupled to the MSD with 50% splitting.

2.5 Animals

Cornish cross-fertile broiler eggs (*n*=40) were obtained from a commercial hatchery (Moyer's Chicks, Quakertown, PA, USA). The eggs were incubated under ideal conditions (37±2 ^{0}C and 89.6±2% humidity) at the Cornell University Animal Science poultry farm incubator. All animal protocols were approved by the Cornell University IACUC (protocol code: 2020-0077).

Intra-amniotic administration

The black corn soluble extract in powder form were diluted in 18 MΩ/cm H_2O to determine the concentrations necessary to maintain an osmolarity value (Osm) of < 320 OSM to ensure that the viable embryos would not be dehydrated upon injection of the solutions. The intra-amniotic administration followed the methodology previously described (Gomes et al., 2021; Martino et al., 2020). At the 17 days of embryonic development, eggs with viable embryos (*n*=34) were randomly allocated into four groups with a similar weight frequency distribution using randomization software. All treatment groups were assigned eggs of similar weight frequency distribution. The experimental groups were assigned as follows: (1) non-injected (*n*=10); (2) 18 MΩ/cm H_2O (*n*=10); (3) 5% (5mg/mL) black corn soluble extract (*n*=8); (4) 15% (15mg/mL) black corn soluble extract (*n*=6). A total of 1mL of solution per egg was injected into the amniotic fluid (identified by candling) with a 21-gauge needle. After the administration, the injection holes were sealed with cellophane tape, and the eggs were placed in hatching baskets to reduce possible allocation bias. On day 21, the hatchlings euthanized immediately after hatching by CO_2 exposure and the proximal small intestine (duodenum), blood, pectoral muscle, cecum, and liver were collected. The cecum and liver were weighed prior to freezing (liquid nitrogen). The following ratios were calculated: cecum weight/body weight and liver weight/body weight.

2.6 Blood analysis and hemoglobin measurements

The blood sample was collected using micro-hematocrit heparin-coated capillarity tuber (Fisher Scientific, Waltham, MA, USA) immediately after hatch but before euthanization. The hemoglobin (Hb) concentration was assessed spectrophotometric using the QuantiChrom^{TM} Hb Assay (DIHB-250, BioAssay Systems, Hayward, CA, USA) and according to manufacturers' instructions.

2.7 Glycogen analysis

The pectoral muscle and liver (20mg) were collected for glycogen analysis. The tissue samples were homogenized in 8% perchloric acid, and glycogen content was determined as described by (Dreiling et al., 1987) with modifications. All samples were read at a wavelength of 450nm in an ELISA (Epoch, Biotek Instruments®, USA) plate reader and the amount of glycogen was obtained by a standard curve. The amount of glycogen present in the pectoral sample was determined by multiplying the weight of the tissue by the amount of glycogen per 1g of wet tissue.

2.8. Extraction of the total RNA from the duodenum and liver tissue samples

The RNA was extracted from 30 mg of the proximal duodenum (as the main site of digestion and absorption) or liver (n = 5 animals/group) according to the manufacture's protocol (RNeasy Mini Kit, Qiagen Inc., Valencia, CA, USA) (Dias et al.; 2019; Martino et al., 2020; Gomes et al., 2021; Agarwal et al., 2022). All steps were carried out under RNase-free conditions. The total RNA was eluted in 50 μL of RNase-free water and RNA was quantified by absorbance at 260/280 and the integrity of the 18S ribosomal RNAs was verified by 1.5% agarose gel electrophoresis, followed by ethidium bromide staining. DNA contamination was removed using TURBO DNase treatment and removal kit from AMBION (Austin, TX, USA).

2.9. Real-time polymerase chain reaction (RT-PCR) and prime design

To obtain the cDNA, a total of 20 μL reverse transcriptase (RT) reaction was completed in a BioRad C1000 touch thermocycler using the Improm-II Reverse Transcriptase Kit (Catalog #A1250; Promega, Madison, WI, USA). The concentration of cDNA obtained was determined by measuring the absorbance at 260 nm and 280 nm using an extinction coefficient of 33 (for single-stranded DNA). Genomic DNA contamination was assessed by a real-time RT-PCR assay for the reference genes samples (Dias et al., 2019). The sequences and the description of the primers used (Table 1) in the real-time qPCR was designed based on gene sequences from Genbank database, using Real-Time Primer Design Tool software (IDT DNA, Coralvilla, IA, USA) (Carboni et al., 2020; Dias et al., 2019; Martino et al., 2020). The specificity of the primers was tested by performing a BLAST search against the genomic National Center for Biotechnology Information (NCBI) database. The *Gallus gallus* primer 18S rRNA was designed as a reference gene. Results obtained from the qPCR system were used to normalize those obtained from the specific systems as described below.

2.10. Real-time qPCR design

Procedures were performed as previously described (da Silva et al., 2019; Martino et al., 2020; Gomes et al., 2021). cDNA was used for each 10 µL reaction containing 2 × BioRad SSO Advanced Universal SYBR Green Supermix (Hercules, CA, USA). Table 1 shows the primers used in this study. A "no template" control of nuclease-free water was included to eliminate DNA contamination in the PCR mix. For each reaction (duplicates), 8 µL of the master mix and 2 µL cDNA were pipetted into a 96-well plate, and for the standard curve, seven points were evaluated in duplicate. The double-stranded DNA was amplified in the Bio-Rad CFX96 Touch (Hercules, CA, USA) using the PCR conditions: initial denaturing at 95 0C for 30 s, 40 cycles of denaturing at 95 0C for 15 s, various annealing temperatures according to Integrated DNA Technologies (IDT) for 30 s and elongating at 60 ^{0}C for 30 s.The gene expression data were obtained as Cp values based on the "second derivative maximum" (automated method) as computed by Bio-Rad CFX Maestro 1.1 (Version 4.1.2433.1219, Hercules, CA, USA). The assays were quantified by including a standard curve in the real-time qPCR analysis, and a standard curve with four points was prepared by a 1:10 dilution (duplicates). The software produced a Cp vs. log 10 concentrations graph, and the efficiencies were calculated as 10 (1/slope). The specificity of the amplified real-time RT-PCR products was verified by melting curve analysis (60–95 ^{0}C) after 40 cycles, resulting in several different specific products with specific melting temperatures.

Table 1. The sequence of experimental primers was used in this study.

Analyte	**Forward Primers (5`-3`)**	**Reverse Primers (5`-3`)**	**Base Pairs Length**	**GI Identifier**
		BBM Functionality		
AP	CGTCAGCCAGTTTGACTATGTA	CTCTCAAAGAAGCTGAGGATGG	138	45382360
SI	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT	95	2246388
SGLT1	GCATCCTTACTCTGTGGTACTG	TATCCGCACATCACACATCC	106	8346783
		Inflammatory Response		
IL1	TCATCCATCCCAAGTTCATTCA	GACACACTTCTCTGCCATCTT	105	395872
TNF-α	GACAGCCTATGCCAACAAGTA	TTACAGGAAGGGCAACTCATC	109	53854909
NF-κB	CACAGCTGGAGGGAAGTAAAT	TTGAGTAAGGAAGTGAGGTTGAG	100	2130627
IL6	ACCTCATCCTCCGAGACTTTA	GCACTGAAACTCCTGGTCTT	105	302315692
		Intestinal Barrier		

MUC2	CCTGCTGCAAGGAAGTAGAA	GGAAGATCAGAGTGGTGCATAG	272	423101
OCLN	GTCTGTGGGTTCCTCATCGT	GTTCTTCACCCACTCCTCCA	124	396026
AMPK	CTCCACTTCCAGAAGGTTACTT	GCAGTAGCTATCGTTCATCCTATC	140	427185
CDX2	ACCAGGACGAAGGACAAATAC	CTTTCCTCCGGATGGTGATATAG	103	374205
		Glucose Metabolism		
G6PC*	GTCTGTCTGTCCCGCATTT	CATGGTAGATGGAGTGGATGTG	115	100857298
PCK1*	GCGATGGCTCAGAAGAAGAA	CTTGCTACGTCTCTTGGGTTAG	124	396458
PKF*	AAGATGAAGACGACGGTGAAG	CCGTGTAGAGGTTGTAGATGAAG	94	374064
18s rRNA	GCAAGACGAACTAAAGCGAAAG	TCGGAACTACGACGGTATCT	100	7262899

AP: amino peptidase; SI: sucrose isomaltase; SGLT1: sodium-glucose transport protein 1; IL1: interleukin 1; TNF-α: tumor necrosis factor-alpha; NF-κB: nuclear factor kappa beta; IL6: interleukin 6; MUC2: mucin 2; OCLN: occludin; CDX2: caudal-related homeobox transcriptional factor 2; AMPK: AMP-activated protein kinase; G6PC: glucose-6 phosphatase; PCK1: phosphoenolpyruvate carboxykinase; PKF: phosphofructokinase 1. 18S rRNA: 18S ribosomal subunit. *Liver analyses

2.11. Collection of microbial samples and intestinal contents DNA extraction

The cecum was sterilely removed and treated as described previously (Hou et al., 2017). Briefly, the cecum contents were placed into a sterile 50 mL tube containing 9 mL of sterile PBS and homogenized by vortexing with glass beads (3 mm diameter) for 3 min. Debris was removed by centrifugation (700 *g* for 1 min), and the supernatant was collected and centrifuged at 12,000 *g* for 5 min. The pellet was washed twice with PBS and stored at - 20 ^{0}C until DNA extraction. For DNA purification, the pellet was re-suspended in 50 mM EDTA and treated with lysozyme (Sigma Aldrich CO., St. Louis, MO, USA; final concentration of 10 mg/ mL) for 45 min at 37 ^{0}C. The bacterial genomic DNA was isolated using a Wizard Genomic DNA purification kit (Promega Corp., Madison, WI, USA).

2.12. Primers design and PCR amplification of bacterial 16S rDNA

Primers for *Lactobacillus*, *Bifidobacterium*, *Clostridium* and *Escherichia coli* were used (Tako et al., 2008). The universal primers were designed with the invariant region in the 16S rRNA of bacteria and were used as internal standards. The proportions of each bacterial groups are presented. The PCR products were separated by 2% agarose gel, stained with ethidium bromide,

and quantified by Quantity One 1-D analysis software (Bio-Ra, Hercules, CA, USA) (Dias et al., 2019). The relative abundance of each examined bacterium was evaluated as previously described (Tako et al., 2014; Dias et al., 2019). All products were expressed relative to the content of the universal 16 s rRNA primer product and proportions of each examined bacterial product.

2.13. Morphological examination of duodenal tissue

Intestinal morphology was performed as previously described (da Silva et al., 2019; Martino et al., 2020). The duodenum samples were fixed in fresh 4% (*v/v*) buffered formaldehyde, dehydrated, cleared and embedded in paraffin. Numerous sections were cut with a thickness of 5 μm and placed on glass slides. The sections were deparaffinized in xylene and rehydrated in a graded alcohol series. After, the slides were stained with Alcian Blue/Periodic acid-Schiff and examined by light microscopy. The following morphometric measurements were evaluated: villus height (μM), villus width (μM), depth of crypts (μM), goblet cell number and goblet cell diameter (μM) in the crypt and villi. Four segments for each biological sample with five biological samples per treatment group were performed with a light microscope using EPIX XCAN software (Standard version, Olympus, Waltham, MA, USA). For the Alcian Blue and Periodic acid-Schiff stain, the segments were only counted for the type of goblet cells (acid, neutral or mixed) in the villi epithelium, goblet cell within crypts and the mucus layer analysis. The goblet cells were enumerated at 10 villi/sample, and the means were calculated for statistical analysis. A representative duodenal histological cross-section image indicates the morphometric measurements from each experimental group.

2.14. Statistical analysis

Values were expressed as means ± standard error deviation (SED) from 6 to 10 biological samples per treatment group, except for intestinal morphology parameters analyses which used five biological samples. Experimental treatments for the intra-amniotic administration procedure were arranged in a completely randomized design. Effects of treatments were analyzed using a one-way Analysis of Variance (ANOVA). For significant "*p*-value," the post hoc Duncan test was used to compare test groups with the significant level at $p < 0.05$. The Kolmogorov-Smirnov normality test evaluated values for normal distribution and variance homogeneity. The statistical analyses were performed using the statistical software IBM SPSS Statistics®, version 20.

3. Results

3.1 Concentration of dietary fiber, iron, zinc, phytic acid, and phytate: Iron molar ration in black corn flour and black corn soluble extract

The concentration of iron, zinc and phytate was higher ($p<0.05$) in the black corn soluble extract (BCSE) compared to the black corn flour. On the other hand, the content of total dietary fiber, insoluble dietary fiber, and phytic acid: iron ratio was lower in the BCSE relative to black corn flour. There was no difference in the concentration of soluble dietary fiber between the samples (Table 2). Related to the anthocyanin profile, cyanidin-3-glucoside was present in higher concentration (30.4mg/100g) in the black corn flour (Table 3).

Table 2. Chemical composition of black corn flour and black corn soluble extract.

	Black corn flour	**Black corn soluble extract**
Total dietary fiber (g/100g)	13.25 ± 0.28^{a}	6.33 ± 0.04^{b}
Soluble dietary fiber (g/100g)	0.31 ± 0.25^{a}	2.04 ± 0.42^{a}
Insoluble dietary fiber (g/100g)	12.92 ± 0.03^{a}	4.29 ± 0.46^{b}
Iron (µg/g)	21.30 ± 0.29^{b}	154.12 ± 1.40^{a}
Zinc (µg/g)	26.18 ± 0.38^{b}	47.00 ± 1.49^{a}
Phytate (g/100g)	0.705 ± 0.01^{b}	1.40 ± 0.14^{a}
Phytic acid: iron ratio	13.62 ± 0.26^{a}	7.72 ± 0.82^{b}

Values are means ± SED. Treatment groups not indicated by the same letter are different ($p<0.05$) by t-Test.

Table 3. Quantification of identified anthocyanin (mg/100g), retention time and λ_{max} in black corn flour.

Anthocyanin	**Amount (mg/ 100g)**	**Retention time (min)**	**λ_{max}(nm)**
Cyanidin-3-glucoside	30.40	20.66	515.28
Cyanidin-3-(6``-malonylglucoside)	17.60	29.69	517.28

Peonidin-3-glucoside	9.30	25.34	516.27
Peonidin-3- (6``malonylglucoside)	6.80	34.24	517.28
Pelargonidin-3-(6``malonylglucoside)	2.90	32.90	505.26
(epi)catechin-cyanidin-3,5-diglucoside	2.50	13.07	528.28
Pelargonidin-3-glucoside	2.40	23.75	501.28

3.2 Effect of black corn soluble extract on biometric parameters

The body weight (BW), cecum weight and cecum: BW ratio was similar in the black corn soluble extract (5 and 15%) groups compared to the controls No Injection and H_2O Injection. In addition, all experimental groups showed similar levels of hemoglobin ($p>0.05$) (Table 4).

Table 4. Effect of black corn soluble extract on body weight, cecum weight and cecum: bodyweight ratio.

	No Injection	**H_2O Injection**	**5% BCSE**	**15% BCSE**
Body weight (g)	40.060 ± 4.064^a	47.490 ± 1.205^a	45.150 ± 1.03^a	42.730 ± 2.093^a
Cecum weight (g)	0.420 ± 0.064^a	0.470 ± 0.030^a	0.450 ± 0.062^a	0.425 ± 0.025^a
Cecum:BW ratio	0.015 ± 0.005^a	0.010 ± 0.001^a	0.010 ± 0.001^a	0.010 ± 0.001^a
Hemoglobin (g/dL)	13.475 ± 1.314^a	9.823 ± 0.772^a	13.913 ± 3.835^a	10.768 ± 1.784^a

Values are means ± SED, *n*=6-10/group. BW: body weight; BCSE: black corn soluble extract.
[a]Treatment groups indicated by the same letter are similar ($p>0.05$) by *post-hoc* Ducan test.

3.3 Effect of black corn soluble extract on bacterial population in cecum contents

Regarding the bacterial population in cecum contents, the 5% BCSE showed lower ($p<0.05$) abundance of *Bifidobacterium* compared to both controls groups (No Injection and H_2O Injection). The 15% BCSE showed similar abundance to the H_2O Injection group. The 5% BCSE and 15% BCSE groups showed similar abundance of *Lactobacillus* ($p>0.05$) and lower abundance ($p<0.05$) compared to the control groups (No Injection and H_2O Injection). Further, the 5% and 15% BCSE groups had an enriched abundance of *E. coli* ($p<0.05$) compared to the H_2O Injection group. The abundance of *Clostridium* was higher ($p<0.05$) in the 5% BCSE compared to the 15%

BCSE, however, in both treatments of BCSE (5 and 15%), its abundance was similar to the H_2O Injection group (Figure 1).

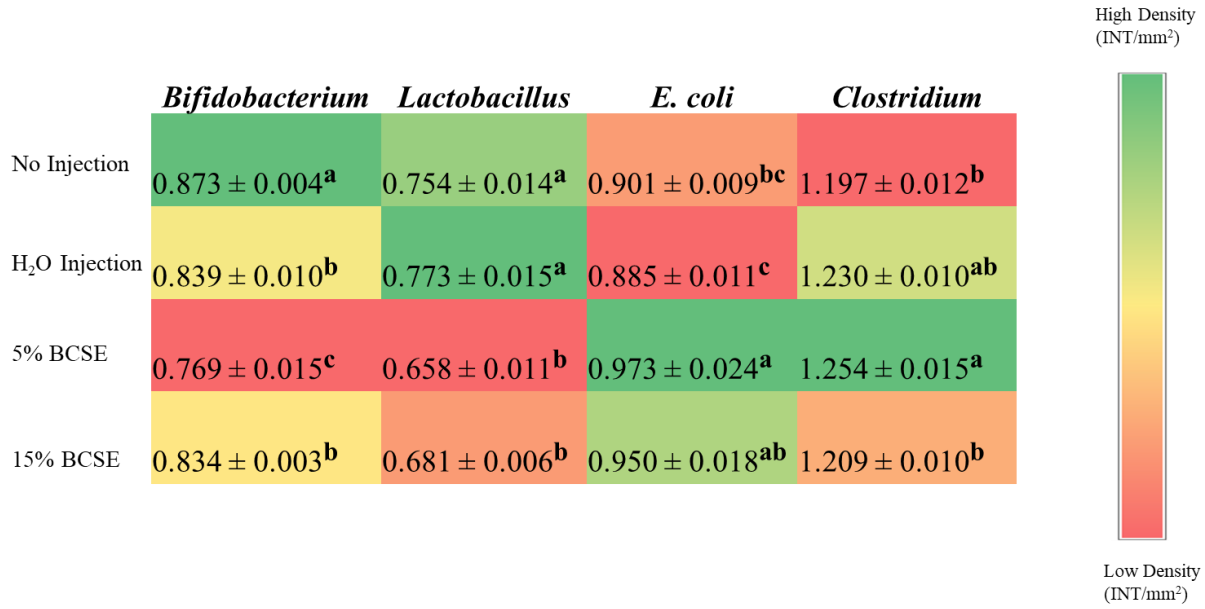

Fig. 1. Effect of intra-amniotic administration of black corn soluble extract on genera- and species-level bacterial population (AU) from cecal content measured on the day of hatch. BCSE: black corn soluble extract. Values are means ± SEM, *n* = 5/group. a-c Per bacterial category, treatment groups not indicated by the same letter are different ($p < 0.05$) by *post hoc* Duncan test.

3.4 Effect of black corn soluble extract on gene expression of intestinal barrier proteins and brush border membrane functional proteins

Related to the intestinal barrier proteins, the gene expression of occludin (OCLN) was similar in all treatment groups. The intra-amniotic administration of 5% BCSE and 15% BCSE upregulated ($p<0.05$) the gene expression of CDX2 compared to the No Injection and H_2O Injection groups. In the same way, the gene expression of AMPK was upregulated ($p<0.05$) in the 5% BCSE compared to the H_2O Injection group. However, the 15% BCSE had similar levels compared to both control groups (No Injection and H_2O Injection). The gene expression of MUC2 was downregulated ($p<0.05$) in the 5% BCSE and 15% BCSE compared to the control groups (No Injection and H_2O Injection) (Figure 2A).

In addition, the intra-amniotic administration of 5% BCSE and 15% BCSE upregulated ($p<0.05$) the gene expression of sodium-glucose transport protein (SGLT1) compared to the H_2O

Injection control group. The gene expression of (sucrose isomaltase) SI was similar ($p>0.05$) among all treatment groups, and (amino peptidase) AP was downregulated in the 5% BCSE and 15% BCSE groups compared to the H_2O Injection (Figure 2A).

3.5 Effect of black corn soluble extract on gene expression of inflammatory biomarkers

The intra-amniotic administration of black corn soluble extract improved inflammatory biomarkers (Figure 2B). The gene expression of the transcription factor NF-κB was downregulated ($p<0.05$) in the 5% BCSE group compared to both control groups (No Injection and H_2O Injection), and in the 15% BCSE was downregulated compared to the H_2O Injection group. Further, the 5% and 15% BCSE groups downregulated ($p<0.05$) the gene expression of TNF-α compared to the control groups (No Injection and H_2O Injection). Related to the cytokines, the IL1 was similar among all groups, while the pro-inflammatory IL6 was downregulated in the 5% and 15% BCSE ($p<0.05$) compared to the H_2O Injection group.

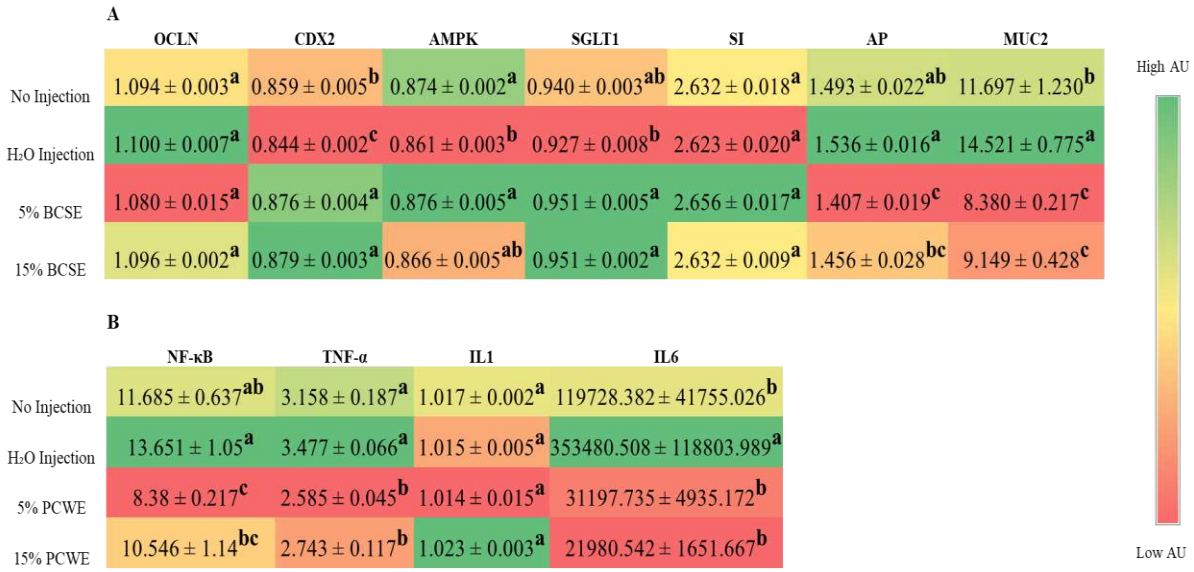

Fig. 2. Effect of black corn soluble extract on intestinal gene expression. Values are means ± SEM, *n* = 5/group. a-c Treatment groups not indicated by the same letter are different ($p < 0.05$) by *post hoc* Duncan test. BCSE: black corn soluble extract; OCLN: occludin; CDX2: caudal-type homeobox 2; AMPK: Adenosine Monophosphate (AMP)-activated protein kinase; SGLT1: sodium-glucose transport protein 1; SI: sucrose isomaltase; AP: amino peptidase; MUC2: mucin

2; NF-κB: nuclear factor kappa beta; TNF-α: tumor necrosis factor-alpha; IL1: interleukin 1; IL6: interleukin 6.

3.6 Effect of black corn soluble extract on glycogen concentration and hepatic gene expression

The 5% BCSE and 15% BCSE treatment increased ($p<0.05$) the hepatic glycogen concentration compared to the No Injection and H_20 Injection group. The pectoral glycogen concentration was not detectable in both control groups (No Injection and H_20 Injection); however, it was verified in both treatment groups with BCSE (5 and 15%) (Figure 3).

Therefore, as the black corn soluble extract increased the pectoral and hepatic glycogen, we further investigated hepatic glucose and glycogen metabolism enzymes. The gene expression of the enzymes glucose-6 phosphatase (G6PD) and phosphofructokinase (PFK) did not differ ($p>0.05$, Figure 3) among all the treatment groups. Further, the gene expression of phosphoenolpyruvate carboxykinase (PCK1) was upregulated ($p<0.05$) in the 15% BCSE compared to the No Injection group. However, there was no difference between the 5% BCSE or 15% BCSE and the H_2O Injection (Figure 3).

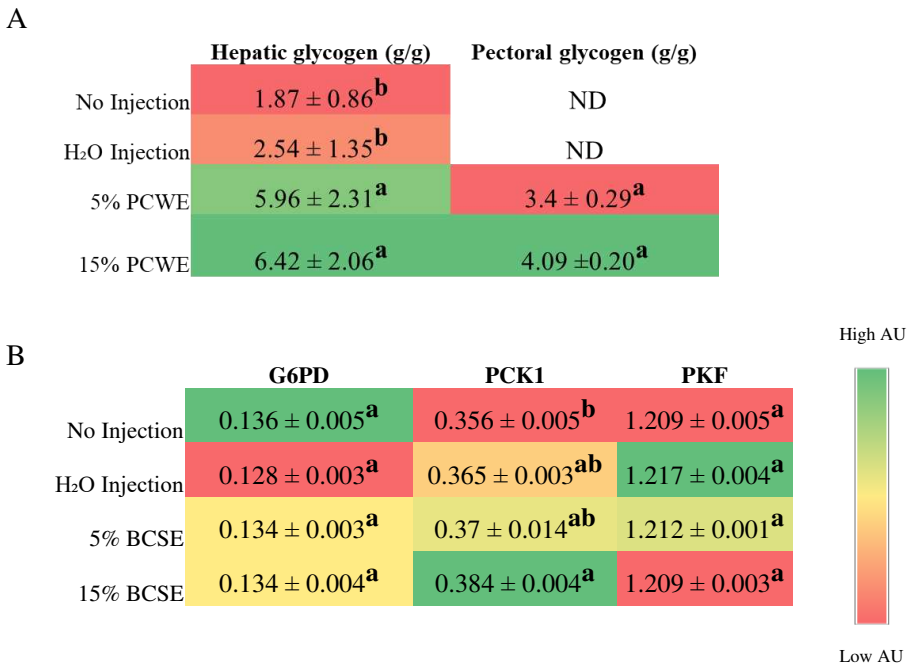

Fig. 3. Effect of black corn soluble extract (A) on hepatic gene expression and (B) on hepatic and pectoral glycogen concentration. Values are means ± SEM, *n* = 5/ group. a-c Treatment groups not indicated by the same letter are different ($p < 0.05$) by *post hoc* Duncan test. BCSE: black corn

soluble extract; ND: not detectable; G6PC: glucose-6 phosphatase; PCK1: phosphoenolpyruvate carboxykinase; PKF: phosphofructokinase.

3.7 Effect of black corn soluble extract on duodenal morphological parameters

The BCSE intra-amniotic administration promoted specific improvements on the small intestine morphological features. First, the intra-amniotic administration of 15% BCSE increased the villi surface area compared to the No Injection group ($p<0.05$). Further, we observed that a higher concentration of BCSE (15%) promoted an increase of the villi surface area compared to a low concentration (5%) ($p<0.05$). In the same way, the 15% BCSE increased the villus length ($p<0.05$) compared to the No Injection and 5% BCSE. Regarding the depth of crypts, the 15% BCSE was similar to the No Injection group and was lower ($p<0.05$) than the H_2O Injection group (Table 5).

Table 5. Effect of black corn soluble extract on the duodenum.

Treatment group	Villi Surface Area (mm^2)	Villus Length (µm)	Depth of crypts (µM)
No Injection	10998.86 ± 305.72^{c}	154.70 ± 3.17^{d}	25.17 ± 0.92^{ab}
H_2O Injection	20515.14 ± 502.93^{a}	267.21 ± 4.30^{a}	26.35 ± 0.98^{a}
5% BCSE	13310.78 ± 3.79^{b}	193.07 ± 3.79^{c}	24.37 ± 0.94^{ab}
15% BCSE	20161.03 ± 4.02^{a}	250.78 ± 4.02^{b}	23.00 ± 0.78^{b}

Values are means ± SEM, *n*= 5/group. $^{a-d}$ Treatment groups not indicated by the same letter are different ($p<0.05$) by *post hoc* Duncan test. BCSE: black corn soluble extract.

In addition, the intra-amniotic administration of BCSE showed effects on the goblet cells (GC). In the crypt, the goblet cells diameter was lower ($p<0.05$) in the 5% and 15% BCSE groups than in the H_2O Injection and No Injection groups. On the other hand, the number of crypt goblet cells where higher ($p<0.05$) in both groups that had the BCSE (5 and 15%) intra-amniotic administration. Moreover, in the villi, the goblet cells diameter was higher ($p<0.05$) following the 5% and 15% BCSE intra-amniotic administration compared to the No Injection and H_20 Injection. In the same way, the villi GC number showed an increase in the 5% and 15% BCSE groups

($p<0.05$) compared to the No Injection group. In addition, the number of Paneth cells on the crypt was higher on the 5% and 15% BCSE groups than the No Injection and H_2O Injection. On the other hand, the Paneth cell diameter decreased on the 15% BCSE compared to the No Injection, H_2O Injection, and 5% BCSE (Figure 4 and 6).

The types of goblet cells were classified as acid, neutral or mixed. In the crypt, the 5% and 15% of BCSE promoted an increase ($p<0.05$) of acid and mixed goblet cells compared to the No Injection and H_2O Injection groups. The neutral reduced ($p<0.05$) in the 15% BCSE compared to the H_2O Injection. Further, in the villi, there was an increase ($p<0.05$) of the acid goblet cell in the 15% BCSE compared to the No Injection and H_20 Injection, and in the 5% BCSE compared to the No Injection. On the other hand, the mixed goblet cell decreased ($p<0.05$) in both BCSE treatments compared to the controls. No neutral cells were identified in the villi of all experimental groups (data not showed) (Figure 5).

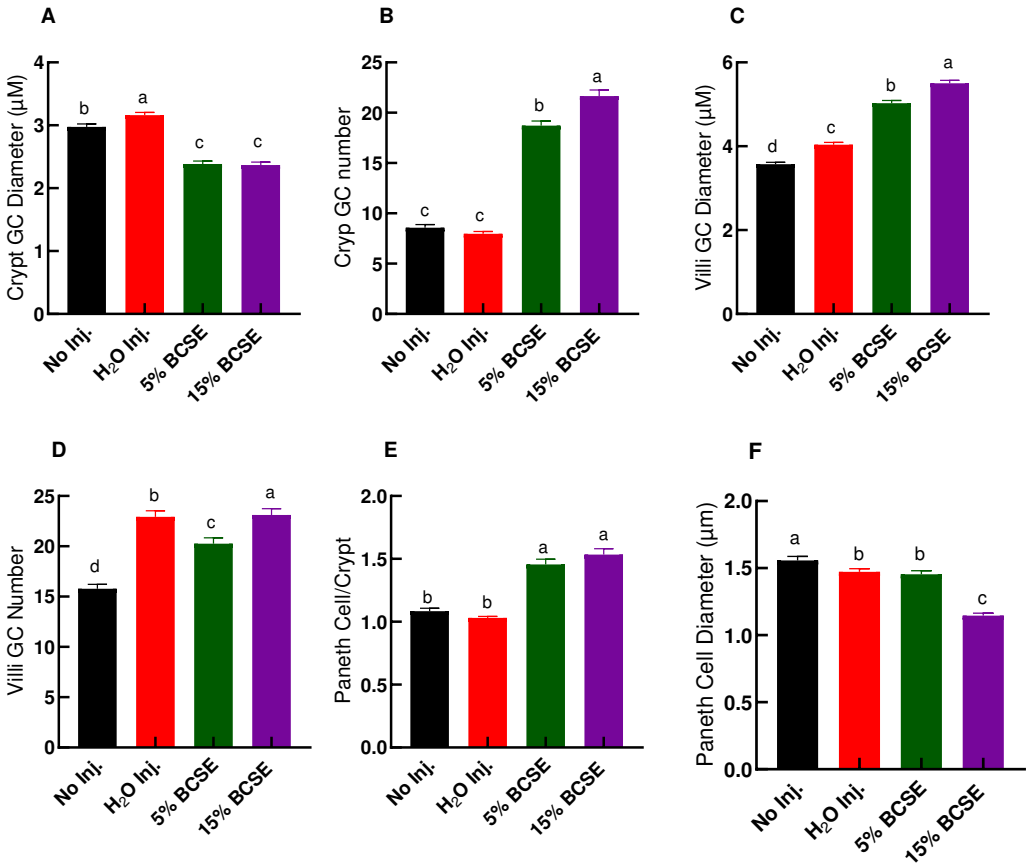

Fig. 4. Effect of black corn soluble extract on the goblet cells and Paneth cells. Values are means ± SEM, *n* = 5/group. a-d Treatment groups not indicated by the same letter are different ($p < 0.05$) by *post hoc* Duncan test. BCSE: black corn soluble extract.

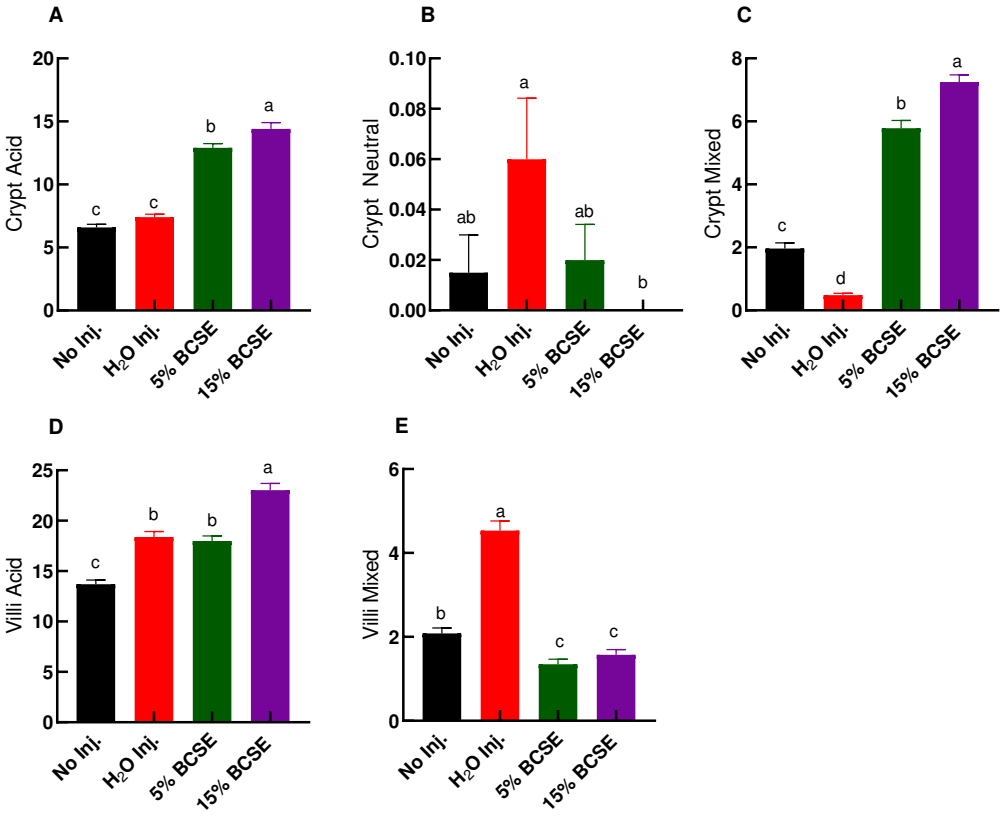

Fig. 5. Effect of black corn soluble extract on the acid, neutral and mixed goblet cells. Values are means ± SEM, *n* = 5/group. a-d Treatment groups not indicated by the same letter are different ($p < 0.05$) by *post hoc* Duncan test. BCSE: black corn soluble extract.

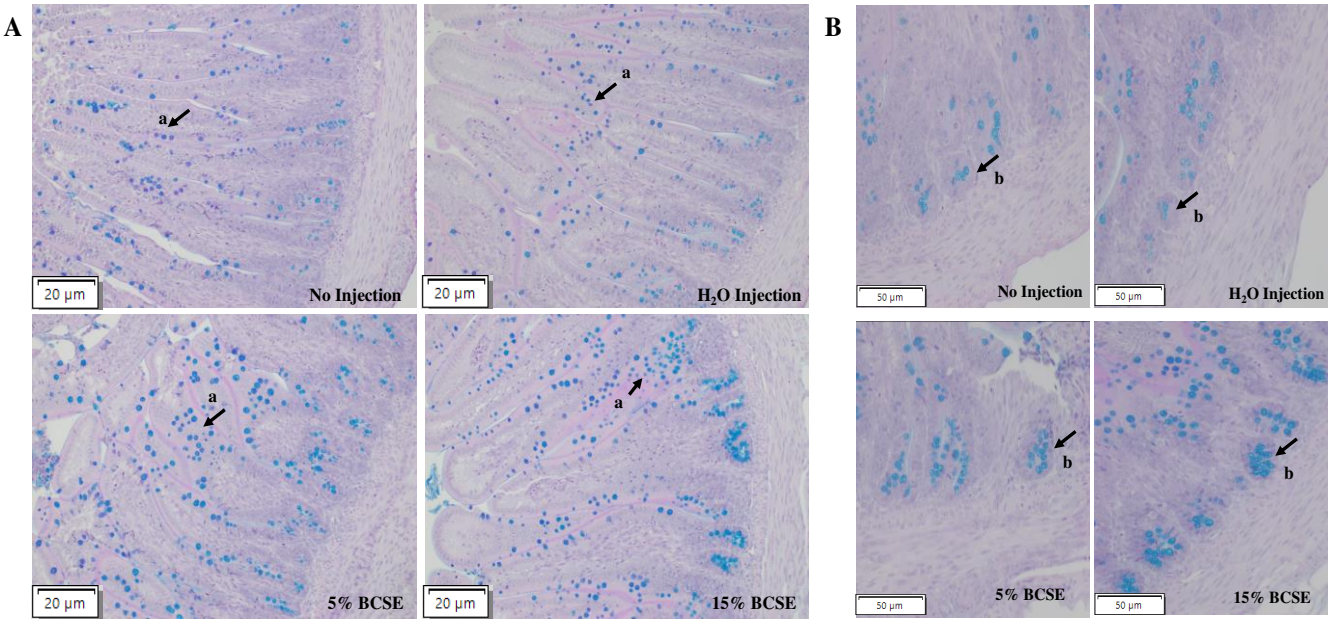

Fig. 6. Representative of the intestinal morphology. A: goblet cells on the villi for each treatment group; B: goblet cell on the crypt for each treatment group. Black arrows represent the increase of goblet cell at specific locations: (a): goblet cell on the villi; (b) goblet cell on the crypts.

4. Discussion

In the present study, the intra-amniotic administration (*Gallus gallus*) of black corn soluble extract (BCSE), as a source of anthocyanin, demonstrated an anti-inflammatory effect that promoted a change in the microbiota bacterial abundance, improved biomarkers of the physical intestinal barrier and the mucus layer.

Colored maize has been a target of interest due to its natural pigments and its health effects (van Hung, 2014; Zhu, 2018). In the black corn used to elaborate the soluble extract of the current experiment, the total anthocyanin content was 85.65 mg/100 g, in which the major anthocyanidin was the cyanidin-3-glucoside (C3O) (30.40 mg/100 g). Anthocyanins have low bioavailability through the upper intestinal tract; thus, a significant amount reaches the colon intact and undergoes the gut microbiota metabolism. First, the colonic microbiota hydrolyses glycosides into aglycones, and then in a second phase, they are degraded into phenolic acids (Tian et al., 2018).

The BCSE intra-amniotic administration changed the intestinal microbiota composition and function by elevating specific bacteria genus density. The BCSE (5 and 15%) increased the *E. coli* abundance compared to the H_2O Injection group (Fig. 1). It is suggested that species in the *E. coli* genus are used as a substrate to anthocyanin metabolism, as authors showed that an anthocyanin extract promoted an enhancement of *E. coli*, thus suggesting that the anthocyanin was fermented by *E. coli* (Kuntz et al., 2016). In agreement with our results, an increase in the *E. coli* abundance was associated with the biotransformation of daidzein into its bioactive metabolites by the *E. coli* genus (Hartono et al., 2015), suggesting that species at the *E coli* genus are involved in the intestinal metabolism of bioactive components. Furthermore, the 5% BCSE increased the *Clostridium* abundance compared to the No Injection group. This genus includes anaerobic species as the *Clostridium butyricum*, which improves the intestinal integrity by producing short-chain fatty acids (SCFA), mainly butyrate and acetate due fermentation (Stoeva et al., 2021). Therefore, the increase in *E. coli* and *Clostridium* abundance following the BCSE might be associated with species included in these genus, that are responsible to metabolize the anthocyanin into its phenolic acids producing SCFA. In addition, the BCSE (5 and 15%) increased the glycogen levels (hepatic and pectoral). Thus, we further evaluated specific hepatic enzymes that are involved in glucose metabolism. However, the gene expression of G6PD, and PKF was not affected by the BCSE administration. Therefore, we suggest that the glycogen increase might be associated with SCFA production (Stoeva, et al., 2021), since the *Clostridium* abundance increased, which is a genus composed of butyrate-producing bacteria. Furthermore, the BCSE showed an effect in only one of the brush border functional proteins evaluated. The BCSE upregulated the SGLT1 gene expression, which might be explained as anthocyanin glycosides are absorbed through the SGLT1 transport in the small intestine (Fang, 2014).

Regarding the inflammatory biomarkers assessed in the duodenum, the BCSE (5% and 15%) downregulated the gene expression of NF-κB, IL6 and TNF-α. The transcriptional factor NF-κB is responsible for controlling physiological and pathological processes by inducing cytokine gene expression to control inflammatory and immune responses (Baker et al., 2011). The NF-κB complex stays in the cytosol and after stimulus is translocated into the nucleus to drive the expression of target genes as TNF-α, IL1β, IL6 and IL10 (Yu et al., 2020). In our experiment, the BCSE suppressed the inflammation by downregulating the NF-κB, decreasing expression of pro-inflammatory cytokines trigged by the NF-κB pathway. This might be associated to the cyanidin-

3-glucoside be the major anthocyanidin in the black corn flour used for this experiment. Furthermore, the NF-κB signaling is associated with an impaired barrier function due to inflammation, leading to increase intestinal permeability and bacterial translocation (Yu et al., 2020). Thus, we further evaluated biomarkers involved in the intestinal barrier integrity.

To the best of our knowledge, this study is the first *in vivo* demonstration of BCSE ability to upregulate AMPK and CDX2 gene expression. AMPK is an energy regulator and regulates the strength of the epithelial barrier function through enhancing the assembly and stability of apical junctions via the phosphorylation of tight junction's proteins (M. J. Zhu et al., 2018; Rowart et al., 2018; Tsukita et al., 2019). However, in this study, the AMPK upregulation did not improve the occludin (OCLN) following the BCSE administration. Thus, to improve the OCLN a higher dose of anthocyanins might be needed to exert this effect. Therefore, in our study, the AMPK upregulation may be associated to NF-κB pathway inhibition. Further, the AMPK was associated to CDX2 upregulation in the BCSE group. CDX2 is an intestine-specific transcriptional factor that induces epithelial cell differentiation and regulates genes in the tight junction complex (Coskun et al., 2011). Purple potato extract enhanced tight junction assembly by AMPK activation and CDX2 protein content in an *in vitro* study (Sun et al., 2018). Furthermore, CDX2 controls intestinal inflammation as CDX2 expression and its affinity to bind to target genes are suppressed by TNF-α signaling (Coskun et al., 2014).

The BCSE improved morphological features on the duodenum. First, the villi surface area increased following the 15% BCSE administration, which might be due to anthocyanin fermentation and intestinal metabolism by commensal microorganisms (Kuntz et al., 2016). Further, we observed an increase in goblet cell (GC) number in the crypt, but with a reduction in its size (diameter) following BCSE (15%). We suggest that the BCSE promoted the differentiation of stem cells into GC in the crypt to improve mucus production if needed. However, as the GC size in the crypt decreased, this might suggest that mucin was not overproduced since the organism was not challenging any microbial inflammation. The GC is composed mainly of mucins, which promote a gel-forming mucus layer to avoid the adhesion and invasion of pathogenic bacteria into the intestinal epithelium (Duangnumsawang et al., 2021). The mucin production on the crypt is more preserved than in the villi, as it is responsible for providing additional protection in gut inflammation (Schneider et al., 2018). On the other hand, on the villi the number but also the size of GC increased following the 15% BCSE administration. A higher mucin production in the villi

compared to the crypt is associated with physiological action to promote luminal motility and to protect the epithelial surface from chemical and bacterial components (Schneider et al., 2018).

In the current study, the Paneth cells number increased in the crypt and a reduced in its cell size. Paneth cells secrete antimicrobial peptides as α-defensin, Reg3 and lysozyme into the crypt lumen, after stimuli as pathogenic exposure (Gassler, 2017). Similar results were verified after the intra-amniotic administration of *C. sativus* flower as a source of flavonoids (Agarwal et al., 2022), therefore, we suggest that phenolics in the 5% BCSE improved the Paneth cell development without affecting this cell's size. This indicates that the antimicrobial peptides secreted by Paneth cells were not produced since there were no stimuli such as inflammation or pathogenic bacteria. Moreover, the 15% BCSE increased the acid GC on the crypt and the villi. Acid mucin creates a luminal acid pH, influencing the microbiota composition since pathogenic bacteria are less likely to survive in an acidic environment (Witten et al., 2018). Further, MUC2 is one of the major components of mucins (Tarabova et al., 2016), and the BCSE administration downregulated the MUC2 in this study. The NF-κB has been described as a target of MUC2 expression so that the TNF-α promotes the NF-κB translocation, thus inducing the MUC2 expression (Iwashita et al., 2003; Paone & Cani, 2020).

Finally, the intra-amniotic administration of black corn soluble extract in the *Gallus gallus* model promoted an increase in *E. coli* and *Clostridium* abundance, which were associated with butyrate-producing bacteria and the intestinal metabolism of anthocyanin by breaking it into phenolic acids. The BCSE promoted an anti-inflammatory effect via down-regulating the NF-κB, TNF-α and IL6 on the duodenum, which improved biomarkers on the intestinal physical barrier as AMPK and CDX2. The BCSE increased the glycogen synthesis associated with the transporter SGLT1 in the small intestine. The BCSE improve the morphological structure of the duodenum, by increasing the number of Paneth cell and goblet cell populations in the crypt, and the number and size of villi goblet cell, with specific increase in acidic goblet cell population in the crypt and villi. Thus, improving the mucus layer to act as a barrier against pathogens and pathogenic components (Fig. 7).

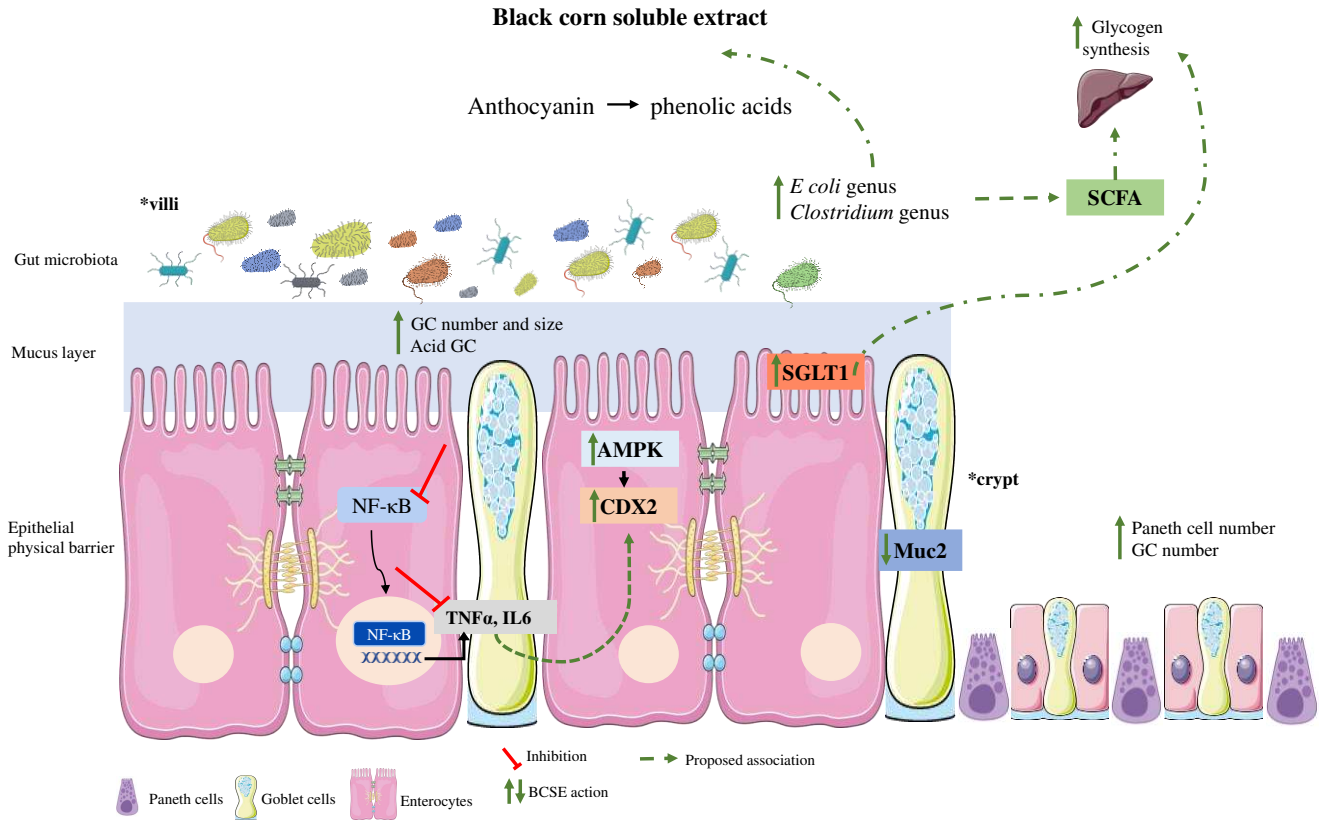

Figure 7. Proposed mechanisms of black corn soluble extract to improve intestinal integrity. SCFA: short-chain fatty acids; NF-κB: nuclear factor kappa beta; TNF-α: tumor necrosis factor alpha; AMPK: adenosine monophosphate-activated protein kinase; CDX2: caudal-type homeobox 2; SGLT1: sodium-glucose transporter 1; MUC2: mucin 2.

5. Conclusion

The intra-amniotic administration of black corn soluble extract as a source of anthocyanins promoted positive function on the intestinal barrier. The BCSE showed anti-inflammatory properties, promoted changes in the relative abundance of gut bacteria, improved the mucus layer and upregulated biomarkers of the physical epithelial barrier. Therefore, the black corn can be a focus of further research and be stimulated as a strategy to improve the diet composition as an additional source of anthocyanins.

CRediT authorship contribution statement

Thaisa Agrizzi Verediano: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **H´ercia Stampini Duarte Martino:** Investigation, Writing – original draft,

Supervision, Resources. **Nikolai Kolba:** Data curation, Formal analysis, Investigation, Methodology. **Yimin Fu:** Data curation, Formal analysis, Writing – original draft. **Maria Cristina Dias Paes:** Supervision, Resources. **Elad Tako:** Data curation, Formal analysis, Investigation, Writing – original draft, Supervision, Resources.

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6.4. PAPER 5

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Article

Effect of Black Corn Anthocyanin-Rich Extract (*Zea mays* L.) on Cecal Microbial Populations In Vivo (*Gallus gallus*)

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Abstract: Black corn has been attracting attention to investigate its biological properties due to its anthocyanin composition, mainly cyanidin-3-glucoside. Our study evaluated the effects of black corn extract (BCE) on intestinal morphology, gene expression, and the cecal microbiome. The BCE intra-amniotic administration was evaluated by an animal model in *Gallus gallus*. The eggs (n = 8 per group) were divided into: (1) no injection; (2) 18 MΩ H_2O; (3) 5% black corn extract (BCE); and (4) 0.38% cyanidin-3-glucoside (C3G). A total of 1 mL of each component was injected intra-amniotic on day 17 of incubation. On day 21, the animals were euthanized after hatching, and the duodenum and cecum content were collected. The cecal microbiome changes were attributed to BCE administration, increasing the population of *Bifidobacterium* and *Clostridium*, and decreasing *E. coli*. The BCE did not change the gene expression of intestinal inflammation and functionality. The BCE administration maintained the villi height, Paneth cell number, and goblet cell diameter (in the villi and crypt), similar to the H_2O injection but smaller than the C3G. Moreover, a positive correlation was observed between *Bifidobacterium*, *Clostridium*, *E. coli*, and villi GC diameter. The BCE promoted positive changes in the cecum microbiome and maintained intestinal morphology and functionality.

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1. Introduction

Corn, also known as maize (*Zea mays* L.), is one of the most produced cereals and one of the major food sources worldwide [1]. In recent decades, scientific research has focused on pigmented corn varieties due to their beneficial health properties [2]. Among them, black corn (*Zea mays* spp.) is a variety traditionally cultivated in South and Central America that has an affinity for warm and dry climates [1]. Black and purple corn can accumulate anthocyanin in different tissues; thus, these varieties have a significant concentration of these flavonoids [3].

Anthocyanins are bioactive water-soluble pigments observed in nature, mainly in the form of glycosides, providing color in plants, fruits, vegetables, and flowers [4]. Extract from anthocyanin-rich foods has been reported to have health properties [5], as anti-inflammatory [6], antioxidant [7], gut microbiota modulation [8], improving cholesterol [9], and glucose metabolism [10]. Seven hundred anthocyanin structures have been identified in nature; however, some of them are verified in higher concentrations in plants, as cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin [4,11]. Among them, cyanidin-3-glucoside (C3G) is the most predominant anthocyanin naturally observed in plants [12]. C3G is the main anthocyanin observed in the black corn flour composition (30.40 mg/100 g); however, a high concentration of phenolic components (614.30 mg GAE - gallic acid equivalent/100 g) is also observed in the food matrix [13].

C3G has an O-glycosylated anthocyanin with two hydroxyls on the third aromatic ring, which confers vigorous antioxidant activity [14]. The leading site of C3G catabolism is the small intestine, in which the C3G molecules are hydrolyzed to aglycones and degraded to specific phenolic compounds such as protocatechuic acid, phloroglucinaldehyde, vanillic acid, and ferulic acid by the gut microbiota [12]. Intestinal microbiota are able to utilize phenolic compounds as a substrate to obtain energy and to create fermentable metabolites with biological functions [15]. The metabolites produced from the intestinal metabolism of C3G inhibit inflammatory pathways such as nuclear factor-kappa B (Nf-kβ) [14]. Since the intestinal tract acts as a barrier against external pathogens [16], C3G and its metabolites contribute to maintaining the intestinal barrier integrity, mucosal barrier, and microbiota composition [12,17].

Recently, we demonstrated that black corn soluble extract, composed of soluble fiber and phenolic compounds, promoted goblet cell proliferation and upregulated biomarkers related to the epithelial intestinal integrity pathway such as AMP- activated protein kinase (AMPK) and caudal-related homeobox transcriptional factor 2 (CDX2) [18]. Previously, it was suggested that

fermented soluble extract promotes the proliferation of beneficial gut bacteria, which affects intestinal brush border membrane morphology, including the growth of villus and crypt and goblet cell proliferation [19]. However, the effects of the isolated phenolic extract on the morphology and gut microbiota might differ [20]. Saffron flower, a source of polyphenols, showed an unfavorable effect on the microbial population, brush border morphology, and functionality [21]. Depending on their dosage, polyphenols can have negative impacts due to interference with nutrient metabolism [20]. Resveratrol and pterostilbene (5%) did not promote modification in the taxonomy of the cecal microbiota but increased morphological changes [22]. For this reason, phenolics and other bioactive compounds in food sources and byproducts need to be quantified and their biological effects and safety validated in living organisms.

The intra-amniotic (in ovo) approach is widely accepted for assessing potential effects of bioactive components [18,21,23–25]. The in ovo technique in *Gallus gallus* allows the administration of components into the amniotic fluid. Therefore, as the embryo consumes the amniotic fluid before hatching, the biological changes after hatching are a predictor of the effects of the bioactive component administered [19]. Considering the possible consumption of bioactive compound-rich extracts, it is relevant to assess the biological effects of dried extracts to validate their application. Since anthocyanin-rich extract from black corn has not been explored for intestinal health so far, this experiment was carried out to investigate the impact of intra-amniotic administration of black corn anthocyanin-rich extract on the intestinal brush border membrane functionality, morphology, and cecal microbial populations.

2. Materials and Methods

2.1. Materials

Black corn grains (TO002) were provided by the Brazilian Agriculture Research Corporation (EMBRAPA) from the Maize Germplasm Bank of the Maize and Sorghum National Research Center (Sete Lagoas, MG, Brazil). Cyanidin-3-glucoside chloride (>98%) was obtained from Sigma-Aldrich® (Cat # PHL89616, St. Louis, MO, USA).

2.2. Black Corn Extract Procedure

Prior to extraction, black corn grains were ground with a 1.0 mm stainless steel sieve (Willy, Solab®, Piracicaba, Brazil) to prepare the corn flour. The production of the extract was

performed at room temperature without any light. The black corn flour was added to ethanol 50% (1:10 *v/v*), then submitted to a magnetic stir plate (100 rpm/60 min/room temperature). After the allotted time had passed, the suspension was vacuum-filtered via filter paper. The ethanol in the extract was evaporated in a rotatory evaporator (40 ^{0}C) [26]. Then, the resulting concentrate was lyophilized, resulting in a dried extract (Figure 1), whose weight was quantified to calculate the final yield considering the initial flour mass.

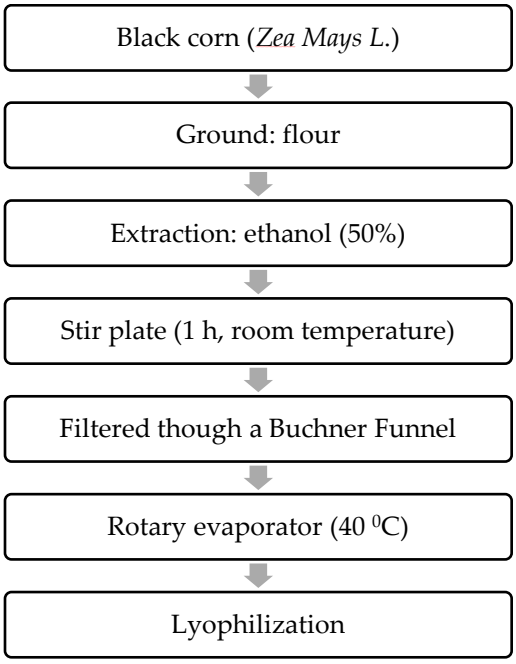

Figure 1. Flowchart of the black corn extract procedure.

2.3. Extract Chemical Characterization

2.3.1. Total Polyphenols and Antioxidant Capacity

The analysis of total polyphenols was determined in the dried extract by the Single-Ciocalteau assay [27]. The absorbance was measured (760 nm) and total polyphenols were expressed as grams of gallic acid equivalent (GAE) per 100 g of wet weight sample. The antioxidant capacity was determined by the radical scavenging activity assay using DPPH (1,1-diphenyl-2-picrylhydrazyl) [28]. Briefly, lyophilized black corn extract (100 µL) was added to an ethanolic DPPH solution and stirred by vortex (3000 rpm/30 s). After incubation (30 min), the absorbance was measured (517 nm) and the DPPH radical scavenging activity was calculated as:

$$\% \text{ of scavenging} = \frac{\text{Ablank} - \text{Asample}}{\text{Ablank}} \quad (1)$$

2.3.2. Anthocyanin Profile Analysis

The black corn anthocyanin-rich extract was analyzed by High Performance Liquid Chromatography (HPLC) Alliance Waters® model 2690/5, with a Waters® photodiode array detector model 2996 (scanning from 210 to 600 nm with quantification at 520 nm). The chromatographic separation was performed using a Thermo Hypersil BDS (Thermo Fisher Scientific,Waltham, MA, USA) C18 column (100 mm x 4.6 mm x 2.4 µm) at 40 ^{0}C, an injection volume of 20 µL, a total run time of 20 min, and a 1.0 mL min^{-1} flow rate. The mobile phase used was an aqueous solution of formic acid (Phase A) and acetonitrile (Phase B). The quantification was performed by using external standards. The gradient elution was 20% solvent B over 3 min, followed by a linear gradient up to 30% solvent B within 15 min and held there for 2 min, and then a linear gradient up to 60% solvent B in 13 min and held there for 2 min. Returning to initial conditions, 20% solvent B in 5 min and held there for 8 min for column rinse and re-equilibration. Mobile phase 2 consisted of an aqueous solution of formic acid as solvent A and acetonitrile as solvent B. The gradient was linear from 5 to 10.5% solvent B over 7 min 30 s and held there for 4 min 30 s, then a linear gradient up to 12% solvent B over 1 min, then another linear gradient up to 14% solvent B over 1 min, and then reduced back to 5% solvent B at 2 min 30 s and held there for 3 min 30 s for column rinse and re-equilibration [29]. Cyanidin-3-glucoside and pelaronidin-3-O-glucoside were used as standards.

2.4. Intra-Amniotic Experiment

Forty Cornish-cross fertile eggs from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA) were incubated under controlled temperature (37 ± 2 ^{0}C) and humidity (89 ± 2% humidity) in a poultry farm incubator at Cornell University Animal Science. All experimental procedures were carried out in accordance with the Cornell University International Animal Care and Use Committee (IACUC, protocol code: 2020-0077).

The black corn extract and the cyanidin-3-glucoside (C3G) were diluted in 18 MΩ H_2O to verify the concentration to achieve an osmolarity value (Osm) of <320 Osm [18,22], in order to certify that the viable embryos would not be dehydrated upon the administration of the amniotic

fluid. During the embryonic development (a total of 21 days), on day 17 of incubation, eggs with viable embryos (*n = 36*) were distributed by randomization into four groups with a similar weight frequency distribution. The groups were distributed as follows: No-injection (*n = 7*); H_2O injection, 18 MΩ H_2O (*n = 8*); BCE, 5% black corn extract in 18 MΩ H_2O (*n = 8*); and C3G, 0.38% cyanidin-3-glucoside in 18 MΩ H_2O (*n = 8*).

The intra-amniotic administration of black corn extract (1 mL/animal) was prepared at a concentration of 5% in accordance with our previous study [18]. The C3G was administered at a concentration of 0.38%, as this compound has yet to be tested intra-amniotically; hence, we chose to proceed with a lower dosage. A 1 mL solution was administered using a 21-gauge needle into amniotic fluid following candling [22,25]. Afterward, cellophane tape was used to seal the injection holes, and all the eggs were allocated to hatching baskets to minimize bias related to allocation. On day 21, after hatching, chickens were weighed and then euthanized by CO_2 exposure, and the blood was collected by cardiac puncture. The duodenum and cecum were immediately collected, and part of the duodenum and cecum were immersed in liquid nitrogen and then kept at -80 ^{0}C until further analysis. Meanwhile, the other portion of the duodenum was fixed in a 10% (*v/v*) formalin solution for histological analysis.

2.5. Total RNA Extraction from Duodenum

Total RNA extraction from the proximal duodenum (*n = 5* animals/group) was performed with a RNeasy Mini Kit, Qiagen Inc. (Cat # 74004, Valencia, CA, USA), as suggested by the manufacturer's protocol. The procedures were performed under RNase-free conditions, and RNA was quantified by absorbance (260/280 nm). The integrity of the 18S ribosomal RNAs was carried out using agarose gel electrophoresis (1.5%) and staining with ethidium bromide. Extracted RNA samples were frozen at (-80 ^{0}C) until further analysis.

2.6. Gene Expression Analysis

The gene expression of the duodenum was determined by real-time polymerase chain reaction (RT-PCR) as described earlier [18,25]. Briefly, cDNA was created with a total of 20 µL of reverse transcriptase (RT) reaction completed in a BioRad C1000 touch thermocycler using the Improm-II Reverse Transcriptase Kit (Ca # A1250; Promega, Madison, WI, USA). The cDNA obtained was analyzed by Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). The

concentration of cDNA was verified by measuring the absorbance (260/280 nm) with an extinction coefficient of 33 (for single-stranded DNA). The forward and reverse primers and the tested genes' descriptions were designed based on the Genbank database, using Real-Time primer Design Tool software (IDT DNA, Coralvilla, IA, USA) (Table 1).

Table 1. Sequence and description of experimental primers.

Analyte	Forward P. (5'-3')	Reverse P. (5'-3')	Base Pairs Length	GI Identifier
Inflammatory Response				
TNFα	GACAGCCTATGCCAACAAGTA	TTACAGGAAGGGCAACTCATC	109	53854909
NF-κB1	CACAGCTGGAGGGAAGTAAAT	TTGAGTAAGGAAGTGAGGTTGAG	100	2130627
IL-1β	CTCACAGTCCTTCGACATCTTC	TGTTGAGCCTCACTTTCTGG	119	88702685
Intestinal Functionality				
MUC2	CCTGCTGCAAGGAAGTAGAA	GGAAGATCAGAGTGGTGCATAG	272	423101
OCLN	GTCTGTGGGTTCCTCATCGT	GTTCTTCACCCACTCCTCCA	124	396026
AMPK	CTCCACTTCCAGAAGGTTACTT	GCAGTAGCTATCGTTCATCCTATC	140	427185
CDX2	ACCAGGACGAAGGACAAATAC	CTTTCCTCCGGATGGTGATATAG	103	374205
VDAC2	CAGCACTCGCTTTGGAATTG	GTGTAACCCACTCCAACTAGAC	99	395498
18S rRNA	GCAAGACGAACTAAAGCGAAAG	TCGGAACTACGACGGTATCT	100	7262899

P: primers; TNFα: tumor necrosis factor-alpha; NF-κB1: nuclear factor kappa beta 1; IL-1β: interleukin 1 beta; MUC2: mucin 2; OCLN: occludin; AMPK: AMP-activated protein kinase; CDX2: caudal-related homeobox transcriptional factor 2; VDAC: voltage-dependent anion channel.

Real-time PCR amplifications were carried out under specific conditions: 95 ^{0}C (30 s followed by 40 cycles (95 ^{0}C, 15 s), annealing temperature for 30 s, and elongation at 60 ^{0}C for 30 s in the Bio-Rad CFX96 Touch (Hercules, CA, USA). The gene expression data was obtained as the lowest cyclic product (Cp) values based on the "second derivative maximum" as computed by Bio-Rad CFX Maestro 1.1 (Version 4.1.2433.1219, Hercules, CA, USA). The assays were quantified through a standard curve in the real-time qPCR analysis, and a 1:10 dilution prepared a standard curve with four points. The software procedure a Cp vs. log 10 concentration graph, and

the efficiencies were calculated as 10 (1/slope). The specificity of the amplified real-time RT-PCR procedures was verified by melting curve analysis (60–95 ^{0}C) after 40 cycles, resulting in several different specific products with specific melting temperatures.

2.7. Intestinal Content and DNA Isolation

The cecum ($n = 5$ animals/group) from a separate chicken was aseptically removed and treated as shown elsewhere [18,30]. In short, the cecum content (200 mg) was placed into a plastic tube with phosphate-buffered saline (PBS) solution and homogenized through a vortex with glass beads (3 mm in diameter) for 3 min. To remove the debris, it was centrifuged, and the supernatant was collected. Before DNA extraction, the pellet was washed twice with PBS and stored at -20 ^{0}C. In order to perform the purification of DNA, the pellet was re-suspended in 50 mM ethylenediaminetetraacetic acid (EDTA) and treated with lysozyme (Sigma Aldrich Co., St. Louis, MO, USA). The bacterial genomic DNA was isolated using the Wizard Genomic DNA purification kit (Cat # A1120, Promega Corp., Madison, WI, USA).

2.8. Primers Design and PCR Amplification of Bacterial 16S rDNA

Primers for *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Escherichia coli*, and *L. planetarium* were used. The universal primers were designed based on prior research [25,30,31]. PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and quantified by Quantity One 1-D analysis software (Version 4.6.8, Bio-Rad, Hercules, CA, USA). All products were expressed relative to the content of the universal 16s rRNA primer product and the proportions of each examined bacterial group.

2.9. Histological Analysis

Duodenal morphology was performed as previously described [18,32]. Briefly, duodenum sections were fixed using buffered formaldehyde solution 4% (*v/v*), dehydrated, cleared, and embedded in paraffin. Sections (5 µm) were added to glass slices, deparaffinized in xylene, rehydrated in ethanol, and stained with Alcian blue/Periodic acid–Schiff. The morphometric measurements of villus height (µM), villus surface (µM), depth of crypts (µM), goblet cell number, and goblet cell diameter (µM) in the crypt and the villi, Paneth cell number, and Paneth cell diameter were assessed using a light microscope (CellSens Standard software, Olympus,Waltham,

MA, USA). Five segments of each biological sample (n = 3/treatment group) were assessed, and ten randomly selected villi and crypts were analyzed per segment (50 replicates per biological sample). Villus surface area was obtained by the equation:

$$\text{Villus surface area} = 2\,\frac{VW}{2} \times VL \qquad (2)$$

In which VW = villus width average of three measurements, and VL =villus length

2.10. Statistical Analysis

Experimental groups were completely randomized. Statistically significant differences between experimental groups were conducted by a one-way Analysis of Variance (ANOVA) and a post-hoc Duncan test for those with a normal distribution. The mean for a normal distribution is tested using the Shapiro–Wilk normality test. The means without normal distribution were analyzed using Kruskal–Wallis and a post-hoc Dunn's test. Data were expressed as mean ± standard error deviation (SED) and differences were considered significant when $p < 0.05$. The association and significance between intestinal biomarkers, bacterial population, and histological parameters were analyzed by Spearman's rank correlation coefficient. GraphPad Prism® version 8.0 software packages (GraphPad Software Inc., San Diego, CA, USA) were used for graphing and data analysis.

3. Results

3.1. Black Corn Extract Characterization

The cyanidin-3-glucoside (C3G) was identified as the principal anthocyanin constituent of black corn extract (BCE), followed by pelargonidin-3-O-glucoside. The BCE showed a high concentration of total phenolic compounds (555 mg GAE/100 g), and the antioxidant capacity was 70.79% (Table 2).

Table 2. Characterization of black corn anthocyanin-rich extract (BCE).

Components	Amount	Retention time (min)
Cyanidin-3-glucoside (mg / 100 g)	283.91	6.5
Pelargonidin-3-O-glucoside (mg / 100 g)	39.57	8.7

Total phenolics compounds (mg GAE / 100 g sample)	555.00	-
DPPH (%)	70.79	-

GAE: gallic acid equivalent. Cyanidin-3-glucoside and pelargonidin-3-O-glucoside were quantified by High Performance Liquid Chromatography; total phenolic compounds and DPPH were analyzed by spectrophotometry.

3.2. Effect of BCE on the Bacterial Population on Cecum Content

The BCE promoted significant changes in the cecum bacterial populations. Specifically, the BCE and the G3G increased ($p < 0.05$) *Bifidobacterium* and decreased ($p < 0.05$) *E. coli* populations compared to No injection and H_2O injection. The BCE group had the highest abundance of *Clostridium* compared to the other treatment groups. Further, the abundance of *Lactobacillus* significantly ($p < 0.05$) decreased after the C3G intra-amniotic administration compared to the control and BCE groups. The abundance of *L. plantarum* was similar ($p > 0.05$) among all experimental groups (Figure 2).

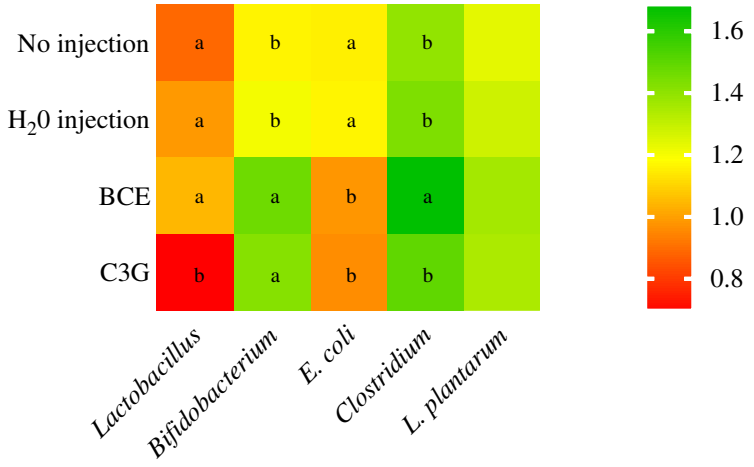

Figure 2. Effect of intra-amniotic administration of black corn extract on bacterial population from cecal content. The relative abundance is expressed in arbitrary units (AU). Values are means ± SED, $n = 5$ animals/group. BCE: black corn extract; C3G: cyanidin-3-glucoside. Treatment groups not indicated by the same letter are different ($p < 0.05$) by the post-hoc Duncan test. Squares without any letter: no difference among the treatments ($p > 0.05$).

3.3. Effect of BCE on Duodenal Gene Expression

The gene expression of duodenal interleukin one beta (IL-1β) and nuclear factor kappa beta (NF-kβ) was similar ($p > 0.05$) among the experimental groups. The pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα) was downregulated ($p < 0.05$) in the C3G group compared to BCE and the H_2O injection (Figure 3A). Furthermore, to evaluate the intestinal physical barrier integrity, the mRNA expression of AMP-activated protein kinase (AMPK), occludin (OCLN), and voltage-dependent anion channel (VDAC) were determined, but no significant difference ($p > 0.05$) was observed among the groups for these variables. On the other hand, the caudal-related homeobox transcriptional factor 2 (CDX2) gene expression was downregulated ($p < 0.05$) after the intra-amniotic administration of H_2O, BCE, and C3G compared with No injection (Figure 3B).

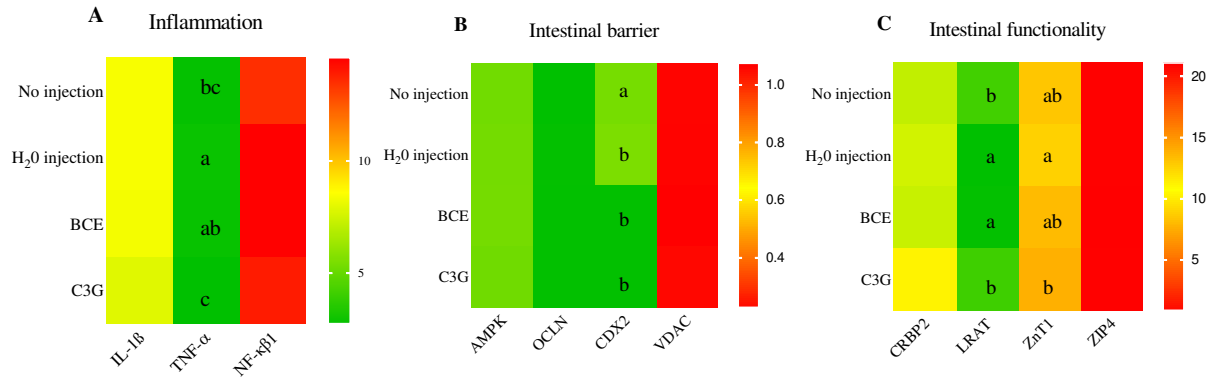

Figure 3. Effect of intra-amniotic administration of black corn extract on duodenal gene expression. Values are means (AU: arbitrary units) ± SED, $n = 5$ animals/group. BCE: black corn extract; C3G: cyanidin-3-glucoside. Treatment groups not indicated by the same letter are different ($p < 0.05$) by the post-hoc Duncan test. Squares without any letter: no difference among the treatments ($p > 0.05$). TNFα: tumor necrosis factor-alpha; NF- κβ1: nuclear factor kappa beta-1; IL-1β: interleukin 1 beta; AMPK: AMP-activated protein kinase; OCLN: occludin; CDX2: caudal-related homeobox transcriptional factor 2; VDAC: voltage-dependent anion channel; CRBP2: cellular retinol-binding protein-2; LRAT: lecithin:retinol acyltransferase; ZnT1: Zinc transporter 1.

Intestinal functionality was assessed through intestinal transporters. The mRNA expression of cellular retinol-binding protein-2 (CRBP2) and ZIP 4 was similar among all experimental groups ($p > 0.05$). However, lecithin: retinol acyltransferase (LRAT) and zinc transporter 1 (ZnT1) downregulated ($p < 0.05$) in the C3G group compared to the H_2O injection group, but there was

no difference between the intra-amniotic administration of BCE and H_2O for these markers (Figure 3C).

3.4. Effect of BCE on Duodenal Morphology

A morphological analysis of the duodenum was performed to observe the intraamniotic effects of BCE in the duodenal mucosa. The animals that received the BCE had no changes in the villi height compared to the H_2O injection ($p > 0.05$). The C3G group showed the highest villi height among all experimental groups ($p < 0.05$). Further, the duodenal depth crypt and the Paneth cell number were higher in the C3G compared to the BCE group ($p < 0.05$). The Paneth number was higher ($p < 0.05$) in the BCE when compared to the No injection (Table 3).

Table 3. Effect of intra-amniotic administration of black corn extract on villi height, surface, and depth crypt.

	No injection	**H_20 injection**	**BCE**	**C3G**
Villi height (μM)	193.12 ± 3.75^{b}	171.50 ± 4.01^{c}	169.69 ± 2.10^{c}	202.43 ± 2.81^{a}
Villi Surface (mM^2)	12324.31 ± 344.23^{b}	11740.52 ± 336.59^{b}	11181.40 ± 224.95^{b}	15250.89 ± 390.56^{a}
Depth crypt (μM)	24.98 ± 1.01^{b}	37.30 ± 1.08^{a}	24.78 ± 0.62^{b}	35.35 ± 0.99^{a}
Paneth cell number	0.95 ± 0.07^{c}	1.30 ± 0.04^{b}	1.27 ± 0.04^{b}	2.49 ± 0.14^{a}
Paneth cell diameter	1.69 ± 0.03^{a}	1.55 ± 0.02^{b}	1.50 ± 0.02^{b}	$1.58 \pm 0.02^{a,b}$

Values are means ± SED, *n* = 3 animals/group. BCE: black corn extract; C3G: cyanidin-3-glucoside. Treatment groups means for specific variable followed by the same letter are not significantly different ($p > 0.05$) by Kruskal-Wallis and post-hoc of Dunn's test.

Moreover, goblet cell (GC) morphological analysis was performed in the villi and the crypt. In the villi, the GC diameter (Figure 4A) and number (Figure 4B) were higher ($p < 0.05$) after the C3G administration intra-amniotically compared to the BCE group, which had similar values to the control groups. Furthermore, the BCE promoted a decrease ($p < 0.05$) of acid GC compared to the C3G, H_2O injection, and No injection (Figure 4C). The villi mixed GC was higher in the BCE and C3G than in the H_2O injection and No injection (Figure 4D). In the same way, in the crypt, the C3G increased ($p < 0.05$) the GC diameter compared to the other experimental groups, and BCE was similar to the control groups (Figure 4E). Further, the BCE and the C3G promoted a decrease in the GC number compared to the other groups (Figure 4F). After classifying the GC,

we observed that the BCE and C3G have the lowest number of villi acid GC compared to the control groups (Figure 4G). There was no difference in the crypt mixed GC in the BCE group compared to the H_2O injection and C3G (Figure 4H).

In our results, significant intestinal correlations were observed between the intestinal parameters investigated (Figure 5). Positive correlations were observed between *Bifidobacterium* and *Clostridium*, *E. coli* and villi GC diameter, and CDX2 and OCLU. Furthermore, villi height, TNFα, NF-kβ1, and CDX2 showed a negative correlation.

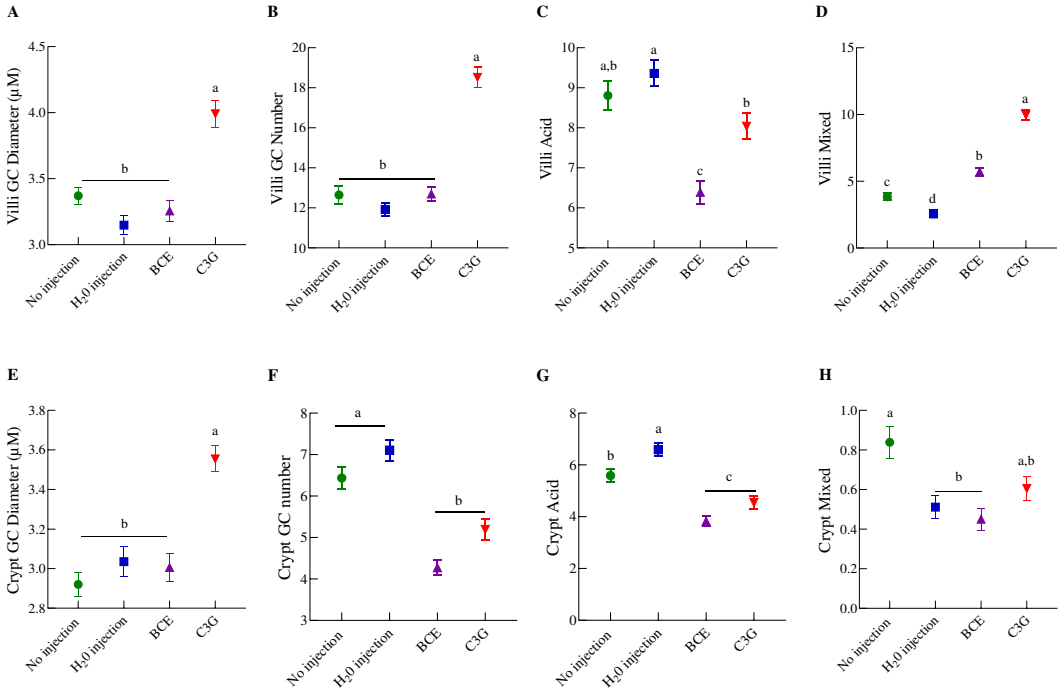

Figure 4. Effect of intra-amniotic administration of black corn extract on goblet cells. Values are means ± SED, n = 3 animals/group. Treatment groups not indicated by the same letter are different ($p < 0.05$) by Kruskal-Wallis and post-hoc of Dunn's test. Mixed goblet cells are acid and neutral. BCE: black corn extract; C3G: cyanidin-3-glucoside; GC: goblet cell.

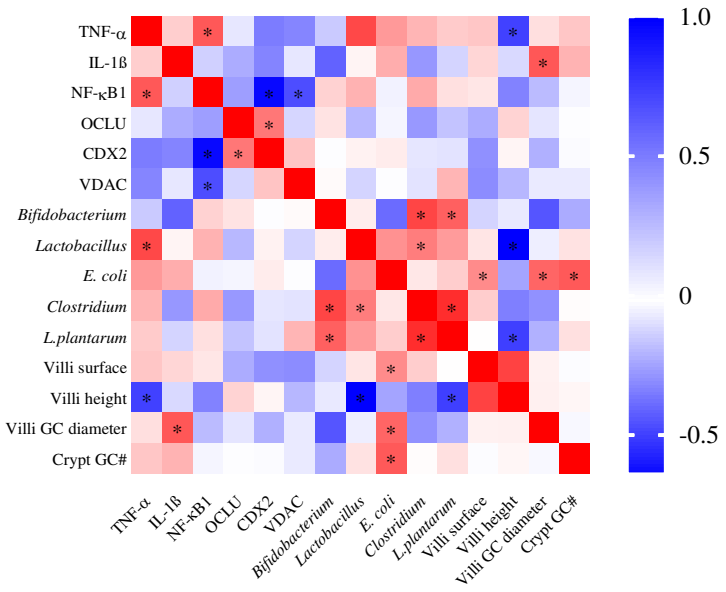

Figure 5. Correlation between intestinal biomarkers, bacterial population, and histological parameters. Colors from blue to red represented p-value of Spearman correlation. Blue: negative and red: positive correlation. * Significant correlation $p < 0.05$. TNFα: tumor necrosis factor-alpha; NF-κB: nuclear factor kappa beta; IL-1β: interleukin 1 beta; MUC2; OCLN: occludin; CDX2: caudal-related homeobox transcriptional factor 2; VDAC: voltage-dependent anion channel. GC: goblet cells; #: number.

4. Discussion

The current scientific literature suggests that the dietary intake of bioactive components offers significant health-promoting benefits [5,15]. Bioactive components include a range of phenolic components, in which each subgroup exerts different tissue and/or cellular effects and promotes beneficial responses in the organism [15,20]. Our study focused on the effects of black corn (*Zea mays*) anthocyanin-rich extract on intestinal functionality, morphology, and microbial populations in an intraamniotic approach. The intraamniotic administration of black corn extract (BCE) promoted a significant improvement in cecal *Bifidobacterium*, *Clostridium*, and reduced *E. coli*. populations. BCE did not change the duodenal brush border membrane morphology and functionality compared to the control groups.

The BCE composition showed significant levels of C3G and total phenolic compounds. Purple corn flour has shown an amount of anthocyanin (mg cyanidin-3-glucoside/100 g) varying from 220 [33] to 310.04 mg [24]. A wide variation has also been observed among different

genotypes, from 12.8 to 93.5 mg C3G/g in 20 different genotypes [34]. In this context, considering our previous study with the same food source but as a flour (black corn flour) [13], the values of C3G in the extract (283.91 mg/100 g) were almost ten-fold higher relative to the flour (30.40 mg/100 g). Total phenolic compounds were similar in the extract (555 mg GAE/100 g sample) compared to the flour (614.30 mg GAE/100 g). Solid-liquid extraction with solvents is the simplest and most common method for extracting phenolic compounds, which is performed to achieve higher yields of the required compounds [35]. Polyphenolic compounds are secondary metabolites of plants that have an effect on plant adaptation to the environment [36], as well as potential bioactivities in animal organisms [15]. According to their chemical structure, phenolic compounds are classified into categories, in which the largest group is the flavonoids, with anthocyanin as a subgroup [37].

Furthermore, the 16s rDNA analysis investigated five bacterial populations and revealed that BCE and C3G increased *Bifidobacterium* and reduced *E. coli* populations in comparison to the other experimental groups (No injection and H_2O injection). The C3G metabolism promotes the proliferation of the genus *Bifidobacterium* in the cecum [38]. Species of *Bifidobacterium* can produce a β-glucosidase enzyme, which supports the hydrolysis of C3G into aglycones and phenolic compounds, which in turn promotes the growth of these beneficial bacteria [17]. In a study with berries, the bifidogenic effect was attributed to the content of anthocyanin but also of polyphenols, as polyphenols contribute to creating a redox environment beneficial to the Bifidobacteria selection, which is favorable by a low oxidation-reduction potential [39,40]. Furthermore, the *E. coli* genus contains diverse pathogenic strains that may impair the epithelial barrier by disrupting tight junction proteins [41]. The protective effect of anthocyanin on pathogenic bacteria might be through its intestinal metabolite protocatechuic acid [12], which has been shown to inhibit the growth of *E. coli* [42], which agrees with the observed reduction of E. coli abundance in the current study. In agreement, the inhibition of *E. coli* might be associated with the villi goblet cell (GC) diameter and crypt GC number, as indicated by the positive correlation between these variables. GC produces the most important substance in the mucus layer: mucin, which forms a gel barrier against pathogenic bacteria [43]. Therefore, we speculate that a reduction in *E. coli* due to the BCE contributes to maintaining the GC number as the control.

Moreover, the BCE administration increased the *Clostridium* and *Lactobacillus* populations in cecal content compared to the C3G administration. In addition to C3G, other

phenolic compounds are found in the black corn extract, which might explain these findings. Several *Lactobacillus* strains use phenolic compounds as a carbon source, thus maintaining their growth besides being involved in the hydrolysis of phenolic compounds due to fermentation [44]. Polyphenols are suggested to exert a prebiotic-like effect by increasing the *Lactobacillus* populations [15], and strains of *Lactobacillus* are considered probiotics due to their immunomodulatory and anti-inflammatory actions, inhibition of bacterial toxins, and competition with pathogens [45]. Therefore, for further investigation, and in addition to the anthocyanin profile, the focus should also be on phenolic characterization.

In the present study, the BCE did not affect the intestinal brush border membrane (BBM) biomarkers: interleukin one beta (IL-1β), tumor necrosis factor-alpha (TNFα), and nuclear factor kappa beta (NF-kβ). However, the isolated C3G administration downregulated TNFα expression compared to H_2O injection and BCE. Considering the chemical composition of the BCE, it provided a higher administration of cyanidin-3-glucoside (0.014 mg/mL) compared to the isolated C3G, which provided an administration of 0.003 mg/mL. It was previously demonstrated that the effect of polyphenols to downregulate TNFα gene expression was concentration dependent, as 2% saffron extract downregulated TNFα expression, but 5 and 10% did not have this effect, as tested *in vivo* via intra-amniotic administration [21]. Additionally, we highlight that even with a high dosage of cyanidin-3-glucoside, the black corn extract did not exert any detrimental effect on the investigated inflammatory pathway, as there was no difference in the biomarkers in the BCE group versus the controls. Further investigation and other biomarkers are required to address phenolic compounds' dosage and profile to exert an anti-inflammatory effect.

The BCE administration did not alter villi height and GC diameter (crypt and villi), relative to the H_2O injection. However, these variables were lower in the BCE compared to the C3G. Therefore, we hypothesize that this result is not attributed to the anthocyanin level but probably to other phenolic components that might be present in the extract, such as protocatechuic, vanillic, p-hydroxycinnamic, and ferulic acid [46]. In agreement, the administration of saffron flower extract, a source of phenolic components, showed a dose-dependent effect on decreasing villus surface area, goblet cell number, and diameter [21]. The duodenal morphometric observations in the current study may indicate that depending on the polyphenolic components can exert distinct effects on the brush border development and absorptive capacity [21,23,34]. The variation of polyphenol composition in four distinct types of beans contributed to different results in intestinal

morphology and functionality [23]. Interestingly, regarding the type of goblet cells, the BCE group showed the lowest number of acidic GC (in the villi and crypt). A luminal acidic pH facilitates the growth of beneficial bacteria over detrimental bacteria [47]. Therefore, the decrease of acidic GC might be associated with the increase in the *Clostridium* population verified in the BCE group [48]. However, even with the growth of *Clostridium* bacteria, the BCE did not affect Paneth cell number relative to the H_2O injection groups. These cells indicate an early state of inflammation, infection, and toxicity due to the secretion of antimicrobial peptides [49].

Finally, in a prior experiment, we showed the beneficial effects of black corn soluble extract (composed of 6.33 g of total dietary fiber/100 g) on intestinal inflammation parameters, morphology, and BBM barrier function [18]. On the other hand, in the present study, the black corn extract (5%) is composed mainly of phenolic components without any dietary fiber. It modulates the cecal microbiome by changing specific bacterial populations and maintaining intestinal morphology and functionality without detrimental effects. Thus, we highlight the positive effects of black corn anthocyanin-rich extract without any soluble dietary fiber, which was able to improve the cecal microbial populations and maintain intestinal morphology and functionality without any detrimental effects *in vivo.*

5. Conclusions

The black corn anthocyanin-rich extract improved the cecal microbiome by increasing *Bifidobacterium* and *Clostridium*, reducing the *E. coli* population while maintaining intestinal morphology and functionality. Further, the C3G group showed additional effects on improving intestinal morphology versus the BCE, suggesting that the combination and dosage of phenolic compounds might interfere with intestinal morphology development. Therefore, our results suggest that black corn anthocyanin-rich extract is a promising target matrix to be used as a functional extract to improve intestinal microbial populations, and further studies in terms of dosage and profile of phenolic compounds in this food matrix are now warranted.

Author Contributions: Conceptualization, E.T., T.A.V. and H.S.D.M.; methodology, T.A.V., N.A., N.K. and M.G.; formal analysis, T.A.V., N.A. and N.K.; investigation, T.A.V., N.A., H.S.D.M., N.K. and E.T.; resources, H.S.D.M. and E.T.; data curation, T.A.V., N.K. and E.T.; writing—original draft preparation, T.A.V.; writing—review and editing, T.A.V., H.S.D.M.,

M.G., M.C.D.P. and E.T.; supervision, H.S.D.M., M.C.D.P. and E.T.; project administration, E.T. and H.S.D.M.; funding acquisition, E.T. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data available upon reasonable request.

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

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7. GENERAL CONCLUSIONS

Black corn (*Zea mays* L.) TO 002 is a genotype from the Brazilian Germplasm Activate Bank that shows a potential chemical composition and can be considered a great source of phenolics compounds and anthocyanins. The present study investigated for the first time the biological effects of this genotype of black corn in inflammation, oxidative stress, adipogenesis, and intestinal function *in vivo*.

First, the systematic review performed showed that dietary anthocyanin supplementation in animal studies can maintain the intestinal homeostasis by improving gut composition and functionality. Thus, the intervention time and the dosage of anthocyanin suggested on this review were used as a support for the later *in vivo* experiments with black corn.

The black corn whole flour showed significant amounts of carbohydrates followed by dietary fiber (soluble and insoluble) and proteins. Regarding the bioactive components, significant amount of total phenolic compounds was detected, and among the anthocyanin the cyanidin-3-glucoside showed the highest concentration. After preparing the soluble extract from the black corn, it was composed of mainly dietary fiber. Finally, the anthocyanin-rich extract showed a composition high in total phenolic compounds and cyanidin-3-glucoside.

The present study demonstrated the potential effects of black corn whole flour (20%) consumption for 8 weeks to prevent metabolic alterations caused by a high-fat diet intake in mice. We observed the consumption of black corn whole flour prevented the differentiation of pre-adipocytes into adipocytes by inhibiting the sterol regulatory element binding proteins 1c (SREBP-1c) and peroxisome proliferator-activated receptor-gamma (PPARγ) in the adipose tissue. The hepatic β-oxidation was stimulated, and a higher total antioxidant capacity were demonstrated due the black corn whole flour consumption. In addition, promising results were observed on the intestinal function, mainly an enrichment at the genus of *Ruminococcus*, *Roseburia*, and *Provotellacea_UCG-001* and cecal goblet cell proliferation. Thus, we provided evidence that the black corn whole flour as a source of bioactive components in its original food matrix modulated the intestinal microbiota and prevent metabolic changes driven by a high-fat diet.

For the first time, we showed the effect of two different black corn extracts by an intra-amniotic experiment, which is an innovative and effective approach to evaluate intestinal function. We demonstrated that black corn as a soluble extract improve the physical barrier integrity, the mucus layer and showed anti-inflammatory action by the intra-amniotic administration. We

highlight that for the first time that the black corn improves the physical barrier though upregulating two specific intestinal biomarkers: caudal-related homeobox transcriptional factor 2 (CDX2) and (AMP- activated protein kinase) AMPK proteins. Therefore, the intra-amniotic administration of black corn soluble extract, composed mainly of bioactive compounds and soluble dietary fiber, acted as a potential substance to improve the intestinal homeostasis.

Finally, to obtain an overall knowledge of black corn in all levels of composition, we demonstrate the effects of an anthocyanin-rich extract from black corn, prepared by a phenolic extraction to obtain the maximum concentration of phenolic compounds from black corn. After the intra-amniotic administration, we demonstrated significant improvement on microbiota composition mainly to increase the relative abundance of genus *Bifidobacterium* and *Clostridium* and to reduce *E. coli,* besides to maintain the intestinal morphology. Thus, by this approach we suggest that the combination of several phenolic compounds in a high concentration act positively on gut composition, although might interfere with intestinal morphology development.

Overall, the present study showed that the black corn TO 002 is a potential food to improve intestinal function and metabolic disorders driven by high-fat diet. These effects were demonstrated in different processing of black corn: from a whole flour to a concentrated extract of phenolic compounds.

8. CONCLUSÕES GERAIS

O milho preto (*Zea mays* L.) TO 002 é um genótipo pertencente ao Banco Ativo de Germoplasma da EMBRAPA com potencial efeito biológico por ser considerado uma fonte de compostos fenólicos e antocianinas. Pela primeira vez, investigamos os efeitos biológicos deste genótipo de milho na inflamação, estresse oxidativo, adipogênese e função intestinal *in vivo*.

Inicialmente, a revisão sistemática conduzida concluiu que a suplementação de antocianinas dietéticas em estudos animais mostra-se eficaz para manter a homeostase intestinal pela melhora da composição da microbiota e da funcionalidade intestinal. Além disso, o tempo de intervenção e a dose de antocianina sugeridas na revisão sistemática foram utilizados como suporte para os estudos *in vivo* conduzidos posteriormente com o milho preto.

A farinha integral de milho preto apresentou quantidade significativa de carboidratos, seguido de fibras (solúvel e insolúvel) e proteína. Quanto aos componentes bioativos, concentração significativa de compostos fenólicos foi observado, e entre as antocianinas a cianidina-3-glicosideo foi a verificada em maior concentração. Além disso, quanto a composição do extrato solúvel do milho preto observou-se predomínio de fibras. Finalmente, verificou-se que o extrato rico em antocianinas apresentou alta concentração de componentes fenólicos e cianidina-3-glicosideo.

O primeiro estudo conduzido demostrou o potencial efeito benéfico do consumo da farinha integral de milho preto (20%) durante 8 semanas para a prevenção de alterações metabólicas induzidas pela ingestão de dieta hiper lipídica em camundongos. Os resultados obtidos neste estudo, nos permitiu observar que o consumo da farinha integral de milho preto preveniu a diferenciação de pré-adipócitos em adipócitos por meio da inibição de dois importantes marcadores de adipogênese no tecido adiposo: *sterol regulatory element binding proteins 1c* (SREBP-1c) e do *peroxisome proliferator-activated receptor-gamma* (PPARγ). Ainda, o consumo da farinha integral do milho preto estimulou a β-oxidação hepática e promoveu maior capacidade antioxidante. Além disso, resultados promissores foram verificados na função intestinal, principalmente um enriquecimento dos gêneros *Ruminococcus*, *Roseburia e Provotellacea_UCG-001* na microbiota intestinal e proliferação de células caliciformes. Nesse sentido, a presente tese forneceu evidencias que a farinha integral de milho preto como uma fonte de compostos bioativos em sua matriz alimentar original foi eficaz para modular a microbiota intestinal e prevenir alterações metabólicas desenvolvidas em consequência do consumo da dieta hiper lipídica.

De maneira inédita, esta tese demonstrou o efeito de dois extratos com composição distintas preparados com o milho preto em um experimento *in vivo* de administração intra-amniótica, o qual consiste em uma metodologia efetiva e inovadora para avaliar a função intestinal. Assim, a administração intra-amniótica do milho preto como um extrato solúvel melhorou a integridade da barreira física, a camada de mucosa e demostrou ação anti-inflamatória no intestino. Destacamos, que neste estudo demonstramos o mecanismo que o extrato do milho preto promoveu melhorias na integridade da barreira física, sendo este pela maior expressão de duas proteínas da integridade intestinal: fator de transcrição *caudal-related homeobox 2* (CDX2) e proteína kinase ativada por AMP (AMPK). Logo, a administração intra-amniótica do extrato solúvel do milho preto composto basicamente de compostos bioativos e fibra solúvel atuou como potencial substância para melhorar a homeostase intestinal.

Finalmente, com o objetivo de obter um conhecimento completo do efeito do milho preto em toda sua composição, nós demostramos os efeitos do extrato de antocianinas do milho preto, elaborado por uma extração fenólica a fim de se obter retenção e concentração máxima dos compostos fenólicos presentes. Após a administração intra-amniótica deste extrato, verificou-se melhora significativa da composição da microbiota intestinal, principalmente com aumento na abundância relativa dos gêneros *Bifidobacterium* e *Clostridium* concomitante com redução de *E. coli,* além de manter a morfologia intestinal. Assim, com a interpretação destes resultados sugerimos que a combinação de diversos compostos fenólicos em alta concentração pode agir de maneira positiva na microbiota intestinal, embora, possa interferir no desenvolvimento da morfologia intestinal.

Sendo assim, esta tese demostrou que o milho preto genótipo TO 002 pode ser considerado um alimento com potencial biológico para melhorar a função intestinal e metabólica alteradas pelo consumo de uma dieta hiper lipídica. Esses efeitos foram demostrados em diferentes processamentos do milho preto: de farinha integral a extrato concentrado com apenas os compostos fenólicos.

9. FINAL CONSIDERATIONS

The present study showed promising results to nutrition and health field, since it demonstrated mechanisms of black corn in three different processing on intestinal function. In addition, the black corn as a whole flour prevented metabolic disorders and intestinal disruption in an environment of a high-fat diet consumption. Considering the consequences of a high-fat diet and its proportion globally, showing that a low cost, and easy cultivated food has the potential to exert beneficial effects on disorders caused by high-fat diet brings new insights to contribute to this health problem.

We highlight that this experiment is an important contribution to the Brazilian research. This was the first biological study with the Brazilian access TO 002. Therefore, the *in vivo* experiments performed with this genotype allow a validation regarding the quality of this genotype. The demonstrated effect of this genotype gives a solid foundation to new cultivars with enhanced nutritional properties.

Since this was the first study showing the *in vivo* effects of the genotype TO 002 black corn, as a following step, we recommend further investigation in clinical trials. For this, we suggest the black corn as a whole flour as most of the beneficial effects were observed in this format.

Furthermore, the black corn whole flour can be considered as a potential flour to be incorporated at food research and development to create products with better nutritional composition. In this sense, as no thermic treatment was used to prepare our whole flour, we recommend further investigation to evaluate if the bioactive components observed will be modified after being exposed to different treatments.

In addition, considering the composition of the anthocyanin -rich extract prepared in our thesis, we speculate the possibility to incorporate this extract as a novel nutraceutical. Thus, to use the anthocyanin-rich extract as a nutraceutical, we recommend further research on specific conditions as encapsulation technologies, storage stability and bioavailability.

Finally, the described possibilities will aggregate to the scientific community due to the investigation performed in this thesis, which used a Brazilian genotype of corn. Therefore, the results verified in this thesis brings progress for the Brazilian community by highlighting a national grain as a potent genotype candidate to introgression in the future.

APPENDIX I

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 10/2019, intitulado "**Efeito do milho roxo rico em antocianinas (*Zea mays L.*) nas alterações da adipogênese, Inflamação e estresse oxidativo em camundongos alimentados com dieta hiperlipídica**", coordenado pela professora Hércia Stampini Duarte Martino do Departamento de Nutrição e Saúde, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 17/05/2019, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 10/2019, named **"Effect of anthocyanins rich in anthocyanins (Zea mays L.) on changes in adipogenesis, inflammation and oxidative stress in mice fed a hyperlipid diet"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on May 17, 2019 valid for 12 months.

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