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Consumption of Curcumin-Added Whey Protein Concentrate Positively Modulates Intestinal Health Parameters after Exhaustive Exercise

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Short title: Curcumin-Added WPC Improves Gut Health Post-Exercise

ABSTRACT

Exhaustive physical exercise can impact intestinal health, affecting permeability, inflammation, and the production of short-chain fatty acids (SCFA). Dietary modifications, such as the consumption of whey protein concentrate (WPC) and curcumin (CCM), can modulate these effects due to their anti-inflammatory and antioxidant properties. This study evaluated the impact of WPC+CCM and CCM in Wistar rats submitted to exhaustive exercise (EE). Forty-eight male Wistar rats (age:12 weeks) were randomly divided into 6 groups (n=8). After 4 weeks on diet, rats from EE groups were submitted to an exhaustive swimming test. Twenty-four hours later, animals from all experimental groups were euthanized, and had feces collected from the cecum. The colon was dissected for interest analysis. SCFA, oxidative stress, real-time polymerase chain reaction, and histomorphometry analyses were performed. The results showed that the SCFA content remained stable, malondialdehyde levels did not vary, but the WPC+CCM group showed higher carbonylated protein concentration. Nitric oxide decreased in the treated groups, while antioxidant enzymes increased in the WPC+CCM and CCM groups, except for glutathione, which decreased. The expression of *Nrf2*, *NF-κB*, and occludin were maintained, and the expression of claudin increased after physical stress with the consumption of WPC+CCM. CCM increased mucosal thickness and preserved goblet cells. In conclusion, WPC+CCM prevented increased oxidative stress and inflammation and preserved the production of SCFA, antioxidant activity, and intestinal integrity of rats after exhaustive exercise.

Keywords: Swimming; Turmeric; Intestinal integrity; Whey protein concentrate.

ABBREVIATIONS

AIN-93M, standard diet; ANOVA, analysis of variance; CAT, catalase; CCM, curcumin; CDNB, 1-chloro-2,4-dinitrobenzene; CEUA, Ethics Committee on the Use of Animals; DNPH, 2,4-dinitrophenylhydrazine; EE, exhaustive exercise; GI, gastrointestinal; GSH, reduced glutathione; GST, glutathione-S-transferase; H_2O_2, hydrogen peroxide; H_2SO_4, sulfuric acid; MDA, malondialdehyde; NO, nitric oxide; *NF-κB*, nuclear factor kappa B; *Nrf2*, nuclear factor erythrogen-2-associated factor 2; PCN, carbonyl protein; ROS, reactive oxygen species; RT-qPCR, Real-Time Polymerase Chain Reaction; SCFA, short-chain fatty acids; SOD, superoxide dismutase; TMPO, 1,1,3,3-tetramethoxypropane; UFV, Universidade Federal de Viçosa; WP, whey protein; WPC, whey protein concentrate

1. Introduction

Regular physical exercise is recognized for its numerous health benefits, including improving cardiovascular fitness, increasing muscle mass, regulating body weight and promoting general well-being [1]. However, when performed exhaustively it can trigger a series of adverse effects, ranging from extreme fatigue to damage to cellular and tissue structures. Among the affected systems, intestinal health emerges as an area of interest, as exhaustive exercise can compromise the integrity of this biological barrier [2,3].

Exhaustive physical exercise leads to changes in the immune system, causing oxidative stress and inflammation, resulting in a series of negative changes in intestinal health, including dysfunction of *tight junctions*, inflammation of intestinal tissue, reduction of short-chain fatty acids (SCFA), changes in intestinal morphology and increased oxidative stress[4]. These changes can compromise normal intestinal function, leading to symptoms such as abdominal discomfort, diarrhea and impaired nutrient absorption [5].

Given this, these changes can be influenced through adjustments in the diet, including the incorporation of food components with modulating potential, such as Whey Protein (WP). WP has been studied as a dietary supplement capable of modulating several physiological responses to physical exercise, including those related to intestinal health, as its composition rich in branched-chain amino acids and bioactive peptides has been associated with preserving the integrity of the intestinal barrier, reduced inflammation and increased SCFA concentration[6].

In addition to WP, another component that can influence intestinal health is curcumin, a bioactive compound found in turmeric, which has demonstrated anti-inflammatory, antioxidant and immune system modulating properties. Curcumin can protect intestinal health by reducing inflammation, preserving the integrity of the intestinal mucosa and reducing oxidative stress induced by exhaustive physical exercise, acting on intestinal permeability[7,8].

Considering the relevance of the relationship between exhaustive physical exercise and intestinal health, as well as the potential modulating role of WP and curcumin, this study seeks to fill an important gap in the scientific literature. Thus, our main objective is to evaluate the combined effects of WP and curcumin on intestinal health variables in Wistar rats submitted to exhaustive exercise. The results of this study are expected to provide a more comprehensive understanding of the mechanisms by which these supplements may influence gut health during periods of intense physical stress.

The use of Wistar rats as an experimental model is widely recognized in biomedical research due to their physiological and metabolic relevance for studies on nutrition and intestinal health. This model allows for the controlled evaluation of the effects of consuming whey protein concentrate with added curcumin in a context of intense physical stress, mimicking inflammatory and oxidative responses similar to those observed in humans undergoing exhaustive exercise. In addition, the intestinal physiology of rats, including the composition of the microbiota and the production of short-chain fatty acids, presents similarities to that of humans, making them a suitable system for investigating nutritional interventions aimed at maintaining the integrity of the intestinal barrier and redox balance[9,10].

2. Material and methods

2.1. Feedstock

2.1.1. Curcumin

The curcumin used was Theracurmin® (Theravalues, Tokyo, Japan), which has high absorption and is dispersed in colloidal nanoparticles. A detailed compositional analysis of the CCM is provided in the Supplementary Material. Theracurmin® is composed of 30 w/w% CCM, showing higher bioavailability than conventional CCM.

2.1.2. Whey protein concentrate

The whey protein concentrate (WPC) used in this study was prepared and characterized as described in detail by Gomes et al. (2021), including its compositional and functional properties [11]. Briefly, the milk used to manufacture WPC was purchased from the stable of Universidade Federal de Viçosa (UFV, Minas Gerais, Brazil). To obtain the whey, the milk was submitted to microfiltration, and then dried in a single-level, single-model spray dryer (model MSD 1.0), according to the methodology of Perrone et al. (2013)[12].

2.1.3. Curcumin-added whey protein concentrate

To formulate whey protein admixture of curcumin (WPC + CCM), Theracurcumin® was incorporated into WPC to achieve a concentration of 2.7 g of Theracurcumin® per 100 g of WPC, which represents a concentration of 0.8 g of CCM per 100 g of WPC. Thus, protein intake, through WPC, corresponded to 30% of protein needs for rats in the maintenance phase[13]. This formulation was developed considering a mean of daily food intake for Wistar rats of 20 g, and final body weight of 400 g[14], which represents a consumption of 18 mg of

CCM/kg/day for Wistar rats, and a consumption of 2.90 mg of CCM/kg/day for humans[15], value near to the maximum value established by Acceptable Daily Intake (3.0 mg/kg/day)[16]. This weight-based scale was used to align with previous WPC+CCM studies in rodents and ensure comparability.

2.2. Biological assay

2.2.1. Animals

Forty-eight male Wistar rats (young adults) (*Rattus novergicus*, variety albinus, Rodentia) were used, coming from the Central Animal Facility of the Center of Biological and Health Sciences, at UFV. The animals were housed in individual stainless-steel cages, in an environment with controlled temperature (22 ± 2ºC) and photoperiod (12 h), according to CONCEA[17], and, after 12 weeks, they were randomly divided, according to weight, into 6 groups (n=8): G1: the control group that received a standard diet (AIN-93M); G2: the control group that received a standard diet submitted to the exhaustive exercise (AIN-93M EE); G3: the curcumin-added whey protein concentrate group (WPC + CCM); G4: the curcumin-added whey protein concentrate group submitted to the exhaustive exercise (WPC + CCM EE); G5: the curcumin group (CCM); and G6: the curcumin group submitted to the exhaustive exercise (CCM EE) (Figure 1). These animals received distilled water and their respective experimental diets *ad libitum*, during 4 weeks.

The animals were allocated according to the method proposed by Conagin (1959)[18]. For the sample calculation, the following equation was used: r= 2 x s2 x $(t\alpha + t\beta) * (t\alpha + t\beta)/d2$, with the reference data for the sample calculation taken from Maithilikarpagaselvi et al. (2016)[19], obtaining 8 animals per experimental group. During the study, confounding factors were not controlled and caregivers were not blinded.

2.2.2. Experimental diets

The experimental diets were formulated in accordance with the AIN-93M recommendations, proposed by the American Institute of Nutrition (1993)[13] for the maintenance of adult animals, namely: AIN-93M diet (control), WPC + CCM diet (30% of the protein requirement coming from the WPC) and CCM diet (AIN-93M + CCM) (Table 1), and were offered in powder form.

2.2.3. Exhaustive exercise protocol

After four weeks, animals in groups G2, G4 and G6 underwent an exhaustive swimming. The animals were forced to swim until exhaustion, supporting a weight of 5% of their body weight attached to the tail[20] . Exhaustion was defined when the rat remained submerged for 10 seconds or when they were no longer able to maintain their body movements[21]. The exercise was carried out in a tiled masonry tank, with a water height of 45 cm maintained at around 28°C±1°C.

2.2.4. Euthanasia

Twenty-four hours after carrying out the exhaustive exercise, the animals from all experimental groups were anesthetized with 5% inhaled isoflurane (Isoforine, Cristália®) and euthanized by cardiac puncture, with a 12-hour fasting period. The entire procedure was carried out with the aim of promoting a humane death of the animals[22]. Colon and feces from the cecum were collected for further analysis.

2.3. Analysis

2.3.1. Short-chain fatty acid (SCFA)

The fecal contents of propionic, acetic, and butyric acids were determined following Siegfried et al. (1984)[23] with modifications. Cecal feces were suspended in water, centrifuged, and treated sequentially with calcium hydroxide, cupric sulfate, and sulfuric acid before analysis by liquid chromatography (Shimadzu, Japan) using a Bio-Rad HPX-87H column. Identification and quantification were performed by comparison with external standards and standard curves.

2.3.2. Oxidative stress

For oxidative balance analyses, a homogenate of intestinal tissue (colon) of all animals was prepared, and the tissues were homogenized in a phosphate buffer and subsequently centrifuged. The resulting supernatants were then stored to conduct analyzes of malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), glutathione-S-transferase (GST), catalase (CAT), while the pellet was reserved for analysis of carbonyl protein (PCN).

Malondialdehyde (MDA) levels, indicating lipid peroxidation, were measured using a 1,1,3,3-tetramethoxypropane standard curve, by Wallin et al. (1993)[24]. Nitric oxide (NO)

production was estimated by NO_2/NO_3 quantification via the Griess method and sodium nitrite standards, by Tsikas et al. (2007)[25]. Protein carbonyl content was determined with 2,4-dinitrophenylhydrazine (DNPH), performed by Levine et al. (1990)[26]. Antioxidant enzyme activities were assessed as follows: SOD by free radical elimination, by the method of Dieterich et al. (2000)[27]; GST via conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, by Habig and Jakoby (1981)[28]; and CAT by the decomposition rate of hydrogen peroxide, by Hadwan and Abed (2016)[29].

2.3.3. Real-Time Polymerase Chain Reaction (RT-qPCR)

For intestinal gene expression analysis, RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and then used for cDNA synthesis, performed by Livak and Schmittgen (2001)[30].

The mRNA expression levels of the genes were analyzed by RT-qPCR, using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and the analyzes were performed on the QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific). The primer sequences, sense and antisense were ordered for amplification of *Nrf2*, *NF-κB*, claudin and occludin. Relative mRNA expression levels were normalized relative to the endogenous *β-actin* control (Table 2).

2.3.4. Intestinal histomorphometry

Intestinal fragments were fixed in formaldehyde, dehydrated in ethanol, and embedded in Historesin® (Leica), by Sabarense et al. (2012) [31]. Semi-serial 3 μm sections were cut with a Leica microtome, stained, and examined under a light microscope (Olympus CX31) at 4X and 10X magnification. Crypt depth and thickness, muscular layers, mucosa, submucosa, and goblet cell number and area were analyzed using ImagePro-Plus® v4.5.

2.4. Statistical analysis

Statistical analyzes and graph construction were performed using GraphPad Prism software, version 9.0.2 (GraphPad Prism Inc, La Jolla, CA, USA). Data normality was assessed using the Shapiro-Wilk test. The t test was used to assess differences within the same group (not submitted to EE × submitted to EE). Intragroup differences (AIN-93M, WPC + CCM and CCM) were submitted to analysis of variance (ANOVA), followed by the Newman-Keuls mean test. A p-value <0.05 was adopted. The results are expressed as mean and standard deviation (mean ± SD).

2.5. Ethical aspect

This project was approved by the Ethics Committee on the Use of Animals of the Universidade Federal de Viçosa (CEUA/UFV), under protocol number 72/2018.

3. Results

3.1. Short-chain fatty acid (SCFA)

Acetic acid remained stable in the exercised groups compared to controls. However, the AIN-93M group showed higher concentrations compared to the groups that received WPC+CCM and CCM ($p<0.05$). For propionic and butyric acids, there were no significant differences among the groups (Table 3).

3.2. Oxidative balance

MDA concentrations were lower in the groups that received CCM, isolated or associated with WPC ($p<0.05$). When evaluating the effect of exercise, MDA levels were maintained in the groups that received the WPC+CCM and CCM diets, while the non-exercised AIN-93M group showed higher MDA ($p<0.05$) (Figure 2A).

As for PCN analysis, the WPC+CCM EE group showed higher concentrations compared to the WPC+CCM group ($p<0.05$). No differences were observed intragroup, both in the groups that performed exhaustive exercise and those that did not (Figure 2B). In the NO analysis, it was observed that among the non-exercised groups there was an increase in NO in the groups that received curcumin, isolated or associated with WPC, while exhaustive exercise reduced nitric oxide concentrations in these groups ($p<0.05$) (Figure 2C).

For the SOD analysis, lower concentrations were observed in the group that received WPC+CCM ($p<0.05$) compared to the other groups without exhaustive exercise. However, there was a significant increase compared to the trained group ($p<0.05$). Furthermore, among the exercised groups, the group that received CCM isolated had the highest SOD concentrations ($p<0.05$) (Figure 2D).

The non-exercised groups did not show significant differences in the concentrations of CAT. However, among the exercise groups, those who received CCM, isolated or combined, showed higher concentrations of this enzyme ($p<0.05$). Furthermore, exhaustive exercise reduced CAT concentrations in the control group ($p<0.05$) (Figure 2E). For GST, the AIN-

93M groups showed higher concentrations, while exercise reduced concentrations in the WPC+CCM and CCM groups ($p<0.05$) (Figure 2F).

3.3. Real-Time Polymerase Chain Reaction (RT-qPCR)

CCM group presented higher expression of *Nrf2* and lower expression of occludin compared to the other groups that were not submitted to exhaustive exercise (Figures 3A and 3D). In addition, among the groups that performed exhaustive exercise, WPC+CCM EE and CCM EE presented lower expressions of antioxidant and inflammatory markers compared to AIN-93M EE (Figures 3A and 3B). WPC+CCM EE increased the expression of claudin, similarly to AIN-93M EE (Figure 3C).

Regarding the effect of exhaustive exercise, the AIN-93M and CCM groups presented higher expressions of *Nrf2* and *NF-κB* compared to their respective groups submitted to exhaustive exercise (Figures 3A and 3B). On the other hand, the groups that received WPC+CCM, whether or not submitted to exhaustive exercise, did not show any difference in the expression of these markers (Figures 3A and 3B). Furthermore, we observed that the WPC+CCM EE and CCM EE groups showed an increase in the expression of claudin compared to their respective groups that did not perform the exercise (Figure 3C).

3.4. Intestinal histomorphometry

The longitudinal muscular layer thickness and the crypts depth and thickness did not differ among the groups. The circular muscle layer showed no difference in thickness among the non-exercised groups, but among the groups that were submitted to the exhaustive exercise, the WPC+CCM EE and CCM EE groups showed smaller thicknesses compared to the AIN-93M EE group (Figure 4, Table 4).

CCM consumption increased mucosal thickness ($p<0.05$). However, under physical stress, consumption of CCM, isolated or combined with WPC, resulted in mucosal thickness similar to the control group. Additionally, the consumption of WPC+CCM and CCM maintained the thickness of the submucosa under physical stress, although this thickness was lower than that of the control group ($p<0.05$). The control group, on the other hand, was unable to maintain submucosal thickness under the same stress ($p<0.05$) (Figure 4, Table 4).

Administration of CCM, isolated or combined with WPC, was effective in maintaining goblet cell numbers during exhaustive exercise. Notably, the use of CCM in its isolated form demonstrated superior efficacy ($p<0.05$) (Figure 4, Table 4). Regarding the area of goblet

cells, the consumption of isolated CCM was able to maintain the area of these cells after exhaustive exercise, although they were still smaller compared to the control group ($p<0.05$). Conversely, the combined use of CCM with WPC did not achieve similar results, showing a reduction in cell area after exercise ($p<0.05$) (Figure 4, Table 4).

4. Discussion

The production and absorption of SCFAs are essential for intestinal health, regulating cellular processes such as differentiation, proliferation, apoptosis, gene expression and anti-inflammatory mechanisms. These acids are produced by a variety of intestinal bacteria, each contributing in a specific way to their synthesis. For example, acetate is mainly produced by enteric bacteria such as *Blautia hydrogenotrophica*, while propionate can be generated by Firmicutes and Bacteroidetes, and butyrate is produced by bacteria in the phylum Firmicutes[32].

Changes in the diet have the potential to influence the production and absorption of these SCFAs, as the composition of the diet can modify the composition of the intestinal microbiota. Therefore, it is crucial to understand how different dietary interventions or external factors can affect gut health, especially in situations of physiological stress such as exhaustive exercise. Exhaustive exercise is known to induce oxidative stress and inflammation in the body, affecting intestinal health, and consequently increasing intestinal permeability[4,33].

Although a significantly higher concentration of acetic acid was observed in the AIN-93M group compared to the WPC + CCM and CCM groups, no differences were found in the levels of propionic and butyric acids after exhaustive exercise among groups after exhaustive exercise. Thus, the ability of the experimental groups to maintain the levels of these acids may indicate an effective regulation of intestinal permeability, ensuring SCFA production is adequate for metabolic requirements. This preservation of SCFA levels can be attributed to several potential mechanisms, such as the fact that exercise can improve the diversity and abundance of certain bacterial genera, such as those from the Firmicutes phylum[34], which is directly related to production of these two SCFAs, and also the fact that whey protein concentrate admixture with curcumin may have a protective or preservation role for these acids. The fermentation of whey protein by intestinal bacteria leads to an increase in the concentration of SCFA, and CCM also has a regulatory effect on the composition of the

intestinal microbiota, favoring the growth of beneficial strains and butyrate-producing bacteria[6,34].

The interaction between curcumin and WPC is particularly interesting in this context. Curcumin, known for its low bioavailability, has been used in a form with high absorption and bioavailability in this study. Its combination with WPC may enhance the overall antioxidant and anti-inflammatory effects due to complementary mechanisms of action. Curcumin has been shown to modulate the gut microbiota, promoting the growth of beneficial bacteria and improving gut barrier function, while WPC, a rich source of bioactive peptides and amino acids, supports muscle recovery and immune function. Together, they provide a synergistic effect, enhancing the production of SCFAs and potentially improving the intestinal barrier integrity. This synergism justifies the inclusion of both components in a single formulation, as they may work together to offer enhanced protection against oxidative stress and inflammation induced by exhaustive exercise.

The possible decrease in acetic acid compared to the control group can be justified by the fact that most of this acid produced by the microbiota was readily transported to the liver and metabolized, or used by skeletal muscle as a source of energy, as highlighted by Yang et al. (2024)[35]. In this context, during exhaustive exercise, the body can direct more metabolic resources to meet immediate energy demands, resulting in an increase in hepatic metabolism and, consequently, in the accelerated metabolization of acetic acid. Despite this difference in relation to the control group, acetic acid levels remained stable during exercise. This indicates that, although there is greater use and metabolization of acetic acid due to increased energy demands, the production of this acid by the intestinal microbiota continues to adequately meet these needs[6].

Furthermore, SCFAs, particularly butyrate, play a role in intestinal homeostasis, directly influencing the redox balance and antioxidant response of the intestinal epithelium. These metabolites, produced by microbial fermentation, modulate the production of reactive oxygen species (ROS), which, in excess, can lead to oxidative stress and cellular damage. These acids have the ability to stimulate the expression of antioxidant genes, including superoxide dismutase, in addition to promoting the synthesis of glutathione, one of the main intracellular antioxidants. Thus, SCFAs not only act as energy sources for colonic epithelial cells, but are also key mediators in the regulation of the intestinal oxidative environment[36].

MDA is a well-established biomarker of oxidative stress and cellular damage, resulting primarily from lipid peroxidation, such as the oxidative degradation of polyunsaturated fatty acids in cell membranes induced by ROS. The accumulation of MDA reflects the extent of oxidative injury and disruption of membrane integrity[37,38]. In the present study, lower baseline MDA concentrations were observed in non-exercised groups supplemented with whey protein concentrate (WPC) combined with curcumin (CCM), or CCM alone, compared to the standard AIN-93M diet, indicating enhanced antioxidant capacity attributable to these bioactive compounds[39,40]. Notably, within the AIN-93M control group, MDA levels decreased after exhaustive exercise, potentially reflecting an acute hormetic response whereby transient exercise-induced ROS generation stimulates endogenous antioxidant defenses, resulting in temporary attenuation of lipid peroxidation[41]. In contrast, animals receiving WPC+CCM or CCM alone maintained stable MDA levels post-exercise, suggesting these treatments imposed a protective effect by preemptively reducing oxidative damage. The antioxidant effects of curcumin are attributed to direct ROS scavenging and inhibition of lipid peroxidation chain reactions, while WPC contributes bioavailable cysteine, a precursor for bioactive peptides synthesis, that enhances cellular redox balance[42,43]. Together, these mechanisms synergistically mitigate exercise-induced oxidative stress and preserve membrane integrity.

PCN is another marker of oxidative stress, where amino acid residues are modified by ROS, compromising the structure and function of proteins. In our study, the group that underwent the exhaustion exercise showed higher concentrations of PCN, reflecting an increase in oxidative stress during exercise. The combination of WPC with CCM does not appear to significantly influence these levels, however, CCM isolated manages to maintain these levels stable. This can be attributed to the fact that CCM and WPC act through different antioxidant mechanisms, possibly resulting in the absence of a synergistic effect[44,45]. Although both compounds have antioxidant effects, their combination did not provide additional protection against exercise-induced oxidative stress. This suggests that the antioxidant mechanisms involved may be more complex than initially considered, and that specific interactions between different dietary compounds should be taken into account when formulating antioxidant supplements or therapies, and it is important to understand how they interact at the molecular and physiological level. This reinforces the need for further studies about the interactions between dietary compounds to develop effective interventions against oxidative stress, especially in contexts of high physical exertion, such as strenuous exercise[46].

Under physiological conditions, NO acts regulating intestinal tone, promoting a favorable environment for water and electrolyte absorption. However, in inflammatory contexts, high NO production can stimulate liquid secretion from the intestine, contributing to inflammation-associated diarrhea, in addition to being harmful to host cells, leading to apoptosis and necrosis of enterocytes, compromising the integrity of the intestinal barrier[47,48]. During exercise, increased metabolism and increased oxygen demand by muscle tissues can lead to greater NO production to promote vasodilation and increase blood flow to working muscles. Therefore, it is possible that NO levels in the intestine decrease during exercise as more NO is consumed by working tissues[49].

The results of our study indicate that curcumin supplementation, isolated or associated with WPC, increases NO levels in non-exercised groups, which may be beneficial for maintaining intestinal homeostasis under physiological conditions. However, the reduction in NO concentrations observed after exhaustive exercise suggests a redistribution of NO to working muscles, which is an expected adaptive response to meet the increased metabolic demand during exercise. This redistribution may protect the intestine from possible harmful effects of excess NO in inflammatory conditions, while supporting muscle performance during exercise. This is corroborated by a previous study conducted by our research group, in which the same animals in this study demonstrated a significant increase in NO levels in muscles during exercise[40].

The gastrointestinal (GI) tract is an important source of ROS. Although the intestinal epithelium acts as a physical and antimicrobial barrier, ingested substances and enteric pathogens and physical stress can trigger inflammation, resulting in the production of pro-inflammatory cytokines[50]. To combat the damage caused by oxidative stress, the body has a variety of antioxidants, both enzymatic and non-enzymatic. For example, SOD converts superoxide anion to hydrogen peroxide, while CAT and GST degrade hydrogen peroxide[51].

Damage induced by oxidative stress can lead to infiltration of the intestinal mucosa by activated leukocytes, resulting in an overload of the tissue's antioxidant defenses. However, diets rich in polyphenols can help control excessive ROS production[42]. In the present study, we observed an increased SOD production in the group supplemented with WPC and CCM that underwent exhaustive physical exercise, indicating an adaptive response of the body to increased antioxidant activity during exercise. On the other hand, CAT activity showed no significant differences when comparing the two groups (WPC+CCM *vs.* WPC+CCM ET or

CCM *vs.* CCM ET), while GST activity decreased in the same group. On the other hand, among the animals submitted to the exhaustive test, an increased SOD was observed in the group receiving CCM, when compared to the control group. The CAT enzyme also increased in both the WPC+CCM and CCM groups, while GST decreased in both groups when compared to the control (AIN-93M). The decreased GSH observed with WPC+CCM and CCM was unexpected, as curcumin is often reported to increase GSH levels. However, we observed increased SOD and CAT activities, which suggests that the intake of antioxidant compounds was effective in defending the body against physical stress, reducing the need for endogenous production of these antioxidant compounds[42]. These results highlight the importance of antioxidant consumption in protecting the gastrointestinal tract against the effects of oxidative stress during physical exercise.

Several cellular defense mechanisms against oxidative stress are induced by different molecular pathways, in particular the nuclear factor erythrogen-2-associated factor 2 (*Nrf2*) pathway. *Nrf2* is a transcription factor that regulates antioxidants within the cell, acting as a stress-responsive transcription factor[52,53]. In our study, we observed that the consumption of curcumin associated with WPC prevented changes in *Nrf2* expression in response to oxidative stress. Whey protein, as well as curcumin, can induce *Nrf2* phosphorylation, Keap1 dissociation, nuclear translocation and finally activation of antioxidant enzymes. Furthermore, bioactive peptides, potentially capable of crossing the intestinal barrier, exert an antioxidant action through activation of the Keap1-*Nrf2* signaling pathway[54,55].

Nrf2 under activation can inactivate inflammatory signaling pathways, such as the *NF-κB* pathway, an inducible transcription factor that regulates many genes involved in the development and regulation of inflammatory and immunomodulatory processes. Studies have shown that whey protein can mitigate oxidative stress and modulate the inflammatory response during and after exhaustive physical exercise. The immunomodulatory peptides present in WP modulate the production of inflammatory cytokines by acting on the *NF-κB* pathway[56–58].

Furthermore, curcumin also has potent anti-inflammatory properties, mediated by inhibition of the *NF-κB* pathway, and may act on the inflammatory response generated by exhaustive physical exercise, consequently reducing the production of pro-inflammatory cytokines[59]. In this study, we observed a reduction in this marker in the groups that received WPC+CCM

and CCM compared to the control group, and CCM isolated was also effective in reducing *NF-κB* under physical stress.

Claudins and occludin are essential proteins of *tight junctions*, specialized structures that regulate paracellular permeability and maintain the integrity of epithelial and endothelial barriers[60]. While claudins form selective pores that control the flow of ions and molecules, occludin acts as a scaffold protein, contributing to the stability and organization of *tight junctions* and participating in cell signaling processes[61]. Alterations in the expression or function of these proteins are associated with several pathologies, such as inflammatory bowel diseases, blood-brain barrier dysfunctions, and cancer, highlighting their crucial role in physiology and their potential as therapeutic targets[60,61].

Regarding tight junctions, we observed that the WPC+CCM and CCM groups presented lower expressions of claudin compared to the group that received a control diet. However, when submitted to exhaustive exercise, the groups had an increased expression of this marker, equaling the exercised control group. As for occludin, the CCM group presented lower expression of this marker with or without exercise, while the WPC+CCM group had lower expression when compared to the AIN-93M group. However, when submitted to exhaustive exercise, the group that received WPC+CCM had similar expression to the AIN-93M EE group, in addition to maintaining their levels similar to their respective groups without exercise.

Our results reinforce the hypothesis that diet can modulate the expression of genes related to cellular integrity, especially under conditions of stress induced by exhaustive exercise. The combination of a quality protein source with bioactive compounds appears to exert a protective and regulatory effect on the expression of these markers, suggesting that nutritional intervention may be a promising strategy to preserve the function of tight junctions in situations of physiological stress.

Additionally, the muscular layer of the intestine is crucial for the proper movement of food through the gastrointestinal tract. Changes in the thickness or integrity of this layer can affect the effectiveness of intestinal motility, impacting digestion and nutrient absorption[62]. In our study, we observed that the groups that received CCM, isolated or combined with WPC, were able to maintain the thickness of the circular and longitudinal layers in the face of physical stress. This suggests that, although it did not lead to an increase in muscle layer, animals that

consumed a diet rich in polyphenols and good quality protein did not show a negative response in the intestine to exercise, possibly not affecting intestinal motility and transit[62].

The intestinal mucosa is responsible for the absorption of essential nutrients; changes in its thickness may indicate changes in the absorption capacity of these nutrients[63,64]. In the present study, mucosal thickness was greater in the curcumin-treated group without exercise, suggesting a potential decrease in absorption area after exercise. However, this difference was not observed in the WPC+CCM group submitted to exhaustive exercise, which despite not increasing its mucous layer, maintained its thickness in relation to the WPC+CCM not submitted to EE, thus not affecting the absorption capacity.

The submucosa is related to the intestinal immune response, as it is where lymphocytes and other components of the immune system associated with the gastrointestinal tract are located[63,64]. Although the groups that received CCM, isolated or combined with WPC, had smaller thicknesses compared to the control group, no difference was observed in the thickness of the submucosa in relation to the WPC+CCM and CCM groups submitted to exhaustive exercise. Thus, we concluded that the production capacity of lymphocytes and components involved in the immune response of these groups was not affected after physical exercise, possibly due to the antioxidant and anti-inflammatory properties of whey protein and curcumin[6,8].

In addition to the muscular and mucosal layers, adequate integrity and function of the crypts are critical for maintaining intestinal homeostasis and preventing gastrointestinal disorders and other related conditions. Intestinal crypts house stem cells responsible for the continuous renewal of the intestine, and this function is essential for maintaining the integrity of the intestinal barrier. Additionally, crypts house immune cells that help modulate the inflammatory response and protect against foreign invaders[64,65]. The results of this study revealed a constancy in the thickness and depth of the intestinal crypts, indicating morphological stability. This finding is particularly promising, as the preservation of this cryptal architecture can be considered a positive factor, as it suggests potential resistance to adverse changes that could compromise intestinal function.

Among the cells present in the crypts are goblet cells, which play a fundamental role in protecting the intestinal mucosa, producing mucus that acts as a barrier against physical damage, pathogens and irritating substances. Variations in the number and area of these cells can directly impact the intestine's defense capacity against damage and infections[64,65]. In our

study, a reduction in both the number and area of goblet cells per crypt was observed in the group submitted to the exhaustive exercise with WPC+CCM. This observation suggests a possible response to the consumption of WPC+CCM, as in situations of physical stress, the body tends to increase the production of goblet cells to reinforce the production of protective mucus[66]. Therefore, the combination of whey protein and curcumin appeared to be effective in protecting against oxidizing and inflammatory agents, offering additional defense to the intestine in stressful conditions.

Despite the valuable insights provided by this study, some limitations should be considered. First, the use of Wistar rats, while a well-established model for studying gut health and exercise-induced stress, presents differences from human physiology. These differences may limit the direct extrapolation of findings to human populations. Additionally, potential sources of bias include the lack of blinding of caregivers and researchers during the experiment, which could have influenced data collection and interpretation. The sample size, although statistically justified, may also limit the detection of subtle effects, contributing to potential imprecision in some results. Importantly, this study did not include microbiota profiling or functional analyses, which prevents us from directly linking the observed SCFA levels and intestinal parameters to specific microbial taxa or metabolic pathways. Any discussion regarding microbiota-mediated effects is therefore hypothetical, grounded in previous studies rather than in direct evidence from our experimental data.

Regarding the generalization of findings, the physiological responses observed in Wistar rats provide relevant insights into the potential effects of whey protein concentrate and curcumin supplementation in mitigating intestinal stress induced by exhaustive exercise. While these results suggest possible benefits for human athletes or individuals exposed to high physical stress, further studies are necessary to confirm these effects in humans, considering differences in diet, gut microbiota, and metabolic responses. Future research should explore clinical trials or alternative preclinical models to validate the applicability of these findings across different species and experimental conditions. Future research should incorporate direct analyses of gut microbiota composition and function to better elucidate the mechanisms underlying the observed effects and explore alternative clinical trials or preclinical models to validate the applicability of these findings across different species and experimental conditions.

5. Conclusions

Curcumin-added whey protein concentrate demonstrated beneficial effects under conditions of exhaustive exercise, particularly through the modulation of oxidative stress, inflammatory pathways, and intestinal barrier integrity. These mechanisms may explain the maintenance of histological parameters. However, further studies with larger datasets are needed to confirm these effects and to better clarify their implications for recovery, physical performance, and the prevention of gastrointestinal disturbances.

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8. Declaration of Interests

The authors declare no conflict of interests.

9. Author contributions

Conceptualization: S.M.S.P., K.A.D., A.R.C., C.M.D.L.; **Data curation:** S.M.S.P., K.A.D., L.A.O., V.P.B.S.J.; **Formal analysis:** S.M.S.P., K.A.D., L.A.O., V.P.B.S.J.; **Funding acquisition:** S.M.S.P., K.A.D., L.A.O., C.M.D.L.; **Investigation:** S.M.S.P., K.A.D., L.A.O., V.P.B.S.J., K.V.C.M., R.C.L.T., M.M.S.D.; **Methodology:** S.M.S.P., K.A.D., A.R.C., R.V.G., A.J.N., H.S.D.M., C.M.D.L.; **Project administration:** S.M.S.P., C.M.D.L.; **Resources:** R.C.L.T., M.M.S.D., R.V.G., A.J.N., H.S.D.M., C.M.D.L.; **Supervision:** R.V.G., A.J.N., H.S.D.M., C.M.D.L.; **Visualization:** S.M.S.P., K.A.D., L.A.O., V.P.B.S.J., C.M.D.L.; **Roles/Writing - original draft:** S.M.S.P., K.A.D., L.A.O., V.P.B.S.J., C.M.D.L.; **Writing - review & editing:** S.M.S.P., K.A.D., L.A.O., V.P.B.S.J., C.M.D.L.

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Table 1. Composition of experimental diets

Ingredients	**g/kg de dieta**		
	AIN-93M	**WPC+CCM**	**CCM**
Maize starch	455.7	455.7	455.7
Albumin*	150	105	150
Maltodextrin	155	155	155
Sucrose	100	99	99
Soy oil	40	40	40
Cellulose	50	50	50
Mineral mixture	35	35	35
Vitamin blend	10	10	10
L-cystine	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5
WPC	-	45	-
Theracurmin®	-	1.2	1.2

Source: Reeves et al., 1993; WPC: whey protein concentrate. *Albumin was considered to be 80% protein.

Table 2. Sequence of primers used in the RT-PCR analysis

Genes	Oligonucleotide (5'-3')	
	Forward	Reverse
Nrf2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
NF-κB	CTGCGCGCTGACGGC	TCGTCGTCTGCCATGTTGAA
Claudin	GGTTCATCCTGGCTTCG	ATCCACAGTCCCTCGTAG
Occludin	ATGTCCGGCCGATGCTCTC	TTTGGCTGCTCTTGGGTCTGTAT
β-actin	TTCGTTGCCGGTCCACACCC	GCTTTGCACATGCCGGAGCC

Nrf2: nuclear factor erythroid 2-related factor 2; *NF-κB*: nuclear factor kappa B.

Table 3. Quantification of acetic, propionic and butyric acids in cecal samples

Variables	Groups					
	AIN-93M	WPC+CCM	CCM	AIN-93M ET	WPC+CCM ET	CCM ET
Acetic acid (mM)	9.33 ± 1.90[a]	7.16 ± 1.14[b]	6.17 ± 0.78[b]	9.20 ± 1.31[a]	6.19 ± 0.80[b]	6.63 ± 1.16[b]
Propionic acid (mM)	17.83 ± 5.26[a]	16.30 ± 1.83[a]	15.17 ± 4.05[a]	18.24 ± 3.50[a]	13.47 ± 3.51[a]	15.03 ± 2.05[a]
Butyric acid (mM)	1.79 ± 0.25[a]	1.51 ± 0.43[a]	1.76 ± 0.60[a]	1.60 ± 0.55[a]	1.51 ± 0.41[a]	1.46 ± 0.19[a]

AIN-93M: group that received a standard diet; AIN-93M EE: group submitted to the exhaustive exercise that received a standard diet; WPC + CCM: group not submitted to EE that received curcumin-added whey protein concentrate; WPC + CCM EE: group submitted to the exhaustive exercise that received curcumin-added whey protein concentrate; CCM: group not submitted to EE that received curcumin; CCM EE: group submitted to the exhaustive exercise that received curcumin; EE: exhaustive exercise. *Indicates significant differences between the same group (sedentary x EE), according to the t test ($p < 0.05$). Different lowercase letters (a – b) indicate significant differences within groups, according to ANOVA, followed by the Newman-Keuls test, at 5% probability. Data expressed as mean ± standard deviation.

Table 4. Histomorphometry of the colon of rats submitted or not to exhaustive exercise

Variables	Groups					
	AIN-93M	WPC+CCM	CCM	AIN-93M ET	WPC+CCM ET	CCM ET
Longitudinal thickness (µm)	67.92 ± 10.15[a]	63.00 ± 4.48[a]	66.26 ± 5.92[a]	66.46 ± 5.26[a]	59.13 ± 2.29[a]	62.14 ± 5.04[a]
Circular thickness (µm)	161.98 ± 15.27[a]	152.95 ± 7.19[a]	177.91 ± 18.31[a]	210.53 ± 37.29[a]	162.52 ± 10.27[b]	177.33 ± 10.34[b]
Mucous thickness (µm)	176.89 ± 14.56[b]	168.40 ± 15.41[b]	198.78 ± 6.28[a*]	179.99 ± 11.34[a]	172.77 ± 6.87[a]	184.49 ± 9.89[a]
Submucous thickness (µm)	69.59 ± 13.10[a*]	42.07 ± 10.33[b]	48.21 ± 2.33[b]	53.94 ± 3.19[a]	41.02 ± 6.62[b]	45.55 ± 4.11[b]
Crypt thickness (µm)	23.76 ± 2.14[a]	22.95 ± 2.07[a]	24.10 ± 1.52[a]	22.75 ± 1.42[a]	24.47 ± 2.24[a]	24.02 ± 1.65[a]
Crypt depth (µm)	157.12 ± 19.42[a]	176.43 ± 27.36[a]	177.43 ± 15.00[a]	160.04 ± 28.67[a]	152.10 ± 10.56[a]	162.86 ± 15.18[a]
Goblet cell per crypt	16.00 ± 2.00[a]	14.00 ± 3.00[a]	18.00 ± 3.00[a]	18.00 ± 2.00[a]	16.00 ± 2.00[b]	20.00 ± 1.00[a]
Goblet cell area (μm^2)	118.63 ± 7.23[a*]	99.30 ± 16.80[a*]	71.20 ± 7.66[b]	89.17 ± 6.57[a]	78.90 ± 7.30[b]	76.83 ± 4.34[b]

AIN-93M: group that received a standard diet; AIN-93M EE: group submitted to the exhaustive exercise that received a standard diet; WPC + CCM: group not submitted to EE that received curcumin-added whey protein concentrate; WPC + CCM EE: group submitted to the exhaustive exercise that received curcumin-added whey protein concentrate; CCM: group not submitted to EE that received curcumin; CCM EE: group submitted to the exhaustive exercise that received curcumin; EE: exhaustive exercise. *Indicates significant differences between the same group (sedentary x EE), according to the t test ($p < 0.05$). Different lowercase letters (a – b) indicate significant differences within groups, according to ANOVA, followed by the Newman-Keuls test, at 5% probability. Data expressed as mean ± standard deviation.

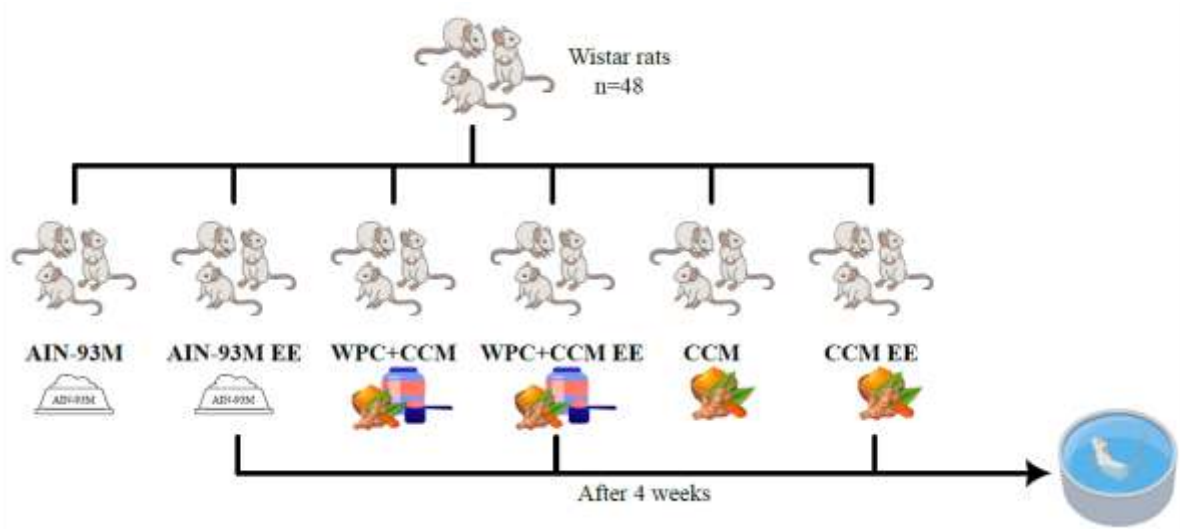

Figure 1. Experimental design.

AIN-93M: group that received a standard diet; AIN-93M EE: group submitted to the exhaustive exercise that received a standard diet; WPC + CCM: group not submitted to EE that received curcumin-added whey protein concentrate; WPC + CCM EE: group submitted to the exhaustive exercise that received curcumin-added whey protein concentrate; CCM: group not submitted to EE that received curcumin; CCM EE: group submitted to the exhaustive exercise that received curcumin; EE: exhaustive exercise.

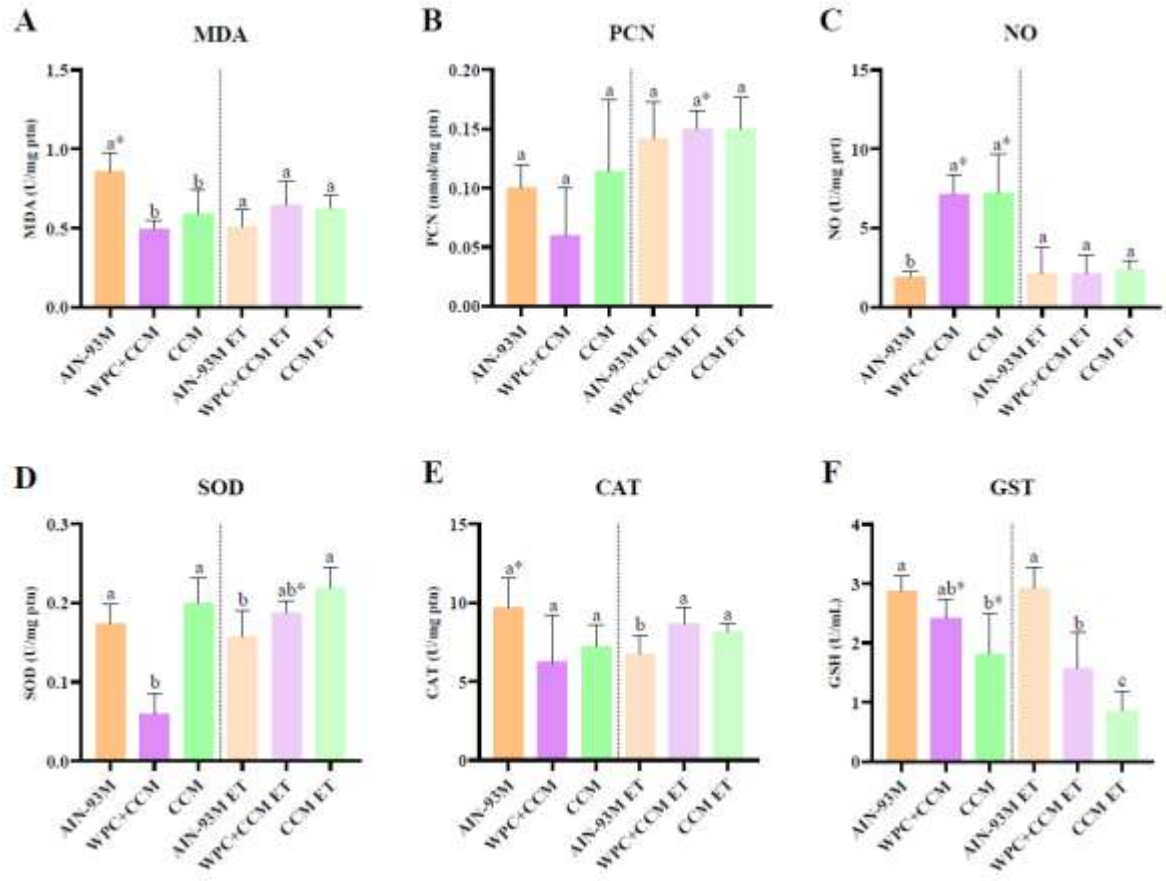

Figure 2. Analysis of oxidative balance in the intestine (colon).

AIN-93M: group that received a standard diet; AIN-93M EE: group submitted to the exhaustive exercise that received a standard diet; WPC + CCM: group not submitted to EE that received curcumin-added whey protein concentrate; WPC + CCM EE: group submitted to the exhaustive exercise that received curcumin-added whey protein concentrate; CCM: group not submitted to EE that received curcumin; CCM EE: group submitted to the exhaustive exercise that received curcumin; EE: exhaustive exercise. The graphs show (A) Malondialdehyde, (B) Carbonylated protein, (C) Nitric oxide, (D) Superoxide dismutase, (E) Catalase, (F) Glutathione S-transferase. *Indicates significant differences between the same group (sedentary x EE), according to the t test ($p < 0.05$). Different lowercase letters (a – c) indicate significant differences within groups, according to ANOVA, followed by the Newman-Keuls test, at 5% probability. Data expressed as mean ± standard deviation.

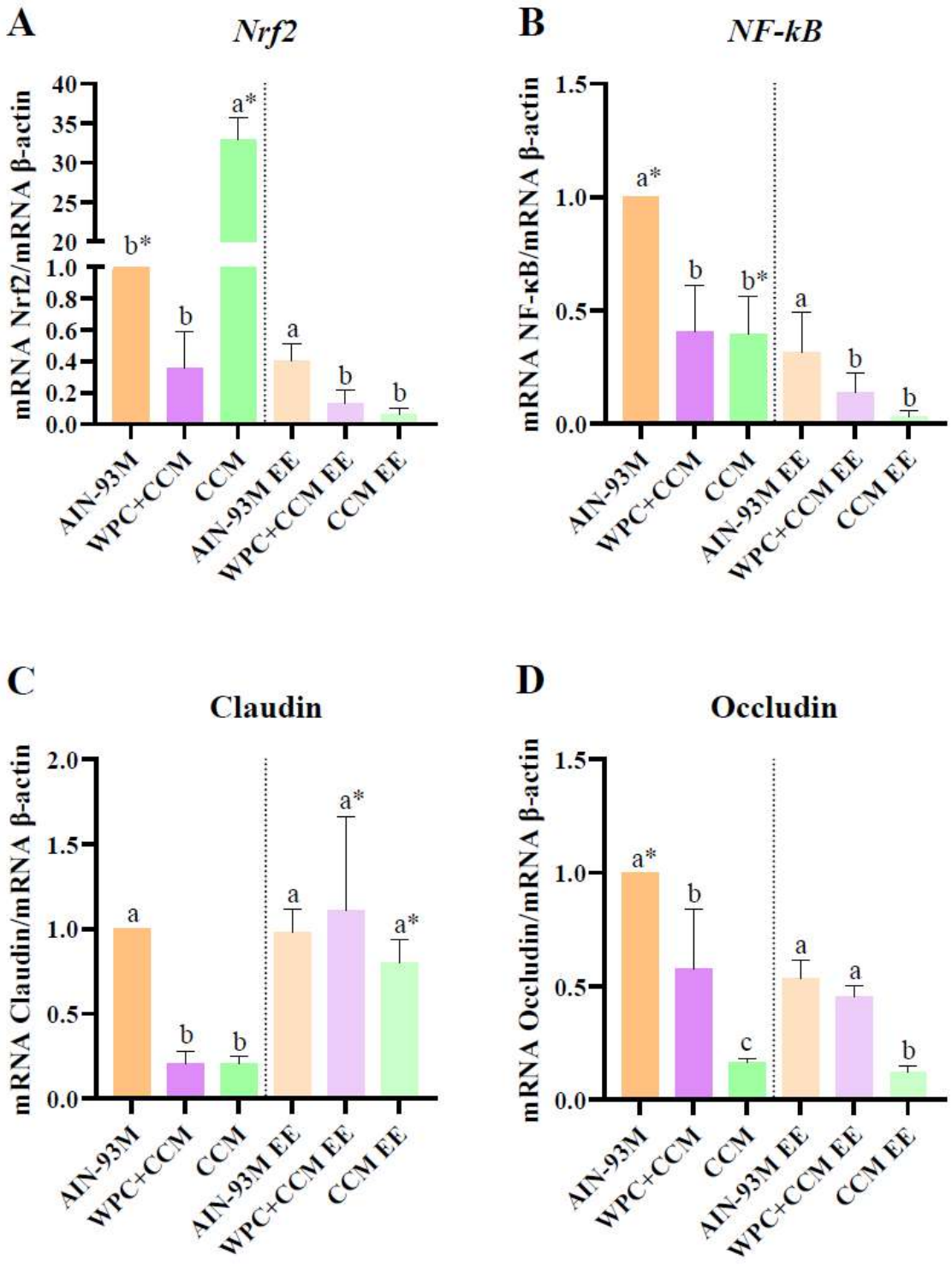

Figure 3. Gene expression of *Nrf2*, *NF-κB*, claudin and occludin in intestine.

AIN-93M: group that received a standard diet; AIN-93M EE: group submitted to the exhaustive exercise that received a standard diet; WPC + CCM: group not submitted to EE that received curcumin-added whey protein concentrate; WPC + CCM EE: group submitted to the exhaustive exercise that received curcumin-added whey protein concentrate; CCM: group not submitted to EE that received curcumin; CCM EE: group submitted to the exhaustive exercise that received curcumin; EE: exhaustive exercise. The graphs show (A) *Nrf2*, (B) *NF-κB*, (C) Claudin and (D) Occludin. *Indicates significant differences between the same group (sedentary x EE), according to the t test ($p < 0.05$). Different lowercase letters (a – c) indicate significant differences within groups, according to ANOVA, followed by the Newman-Keuls test, at 5% probability. Data expressed as mean ± standard deviation.

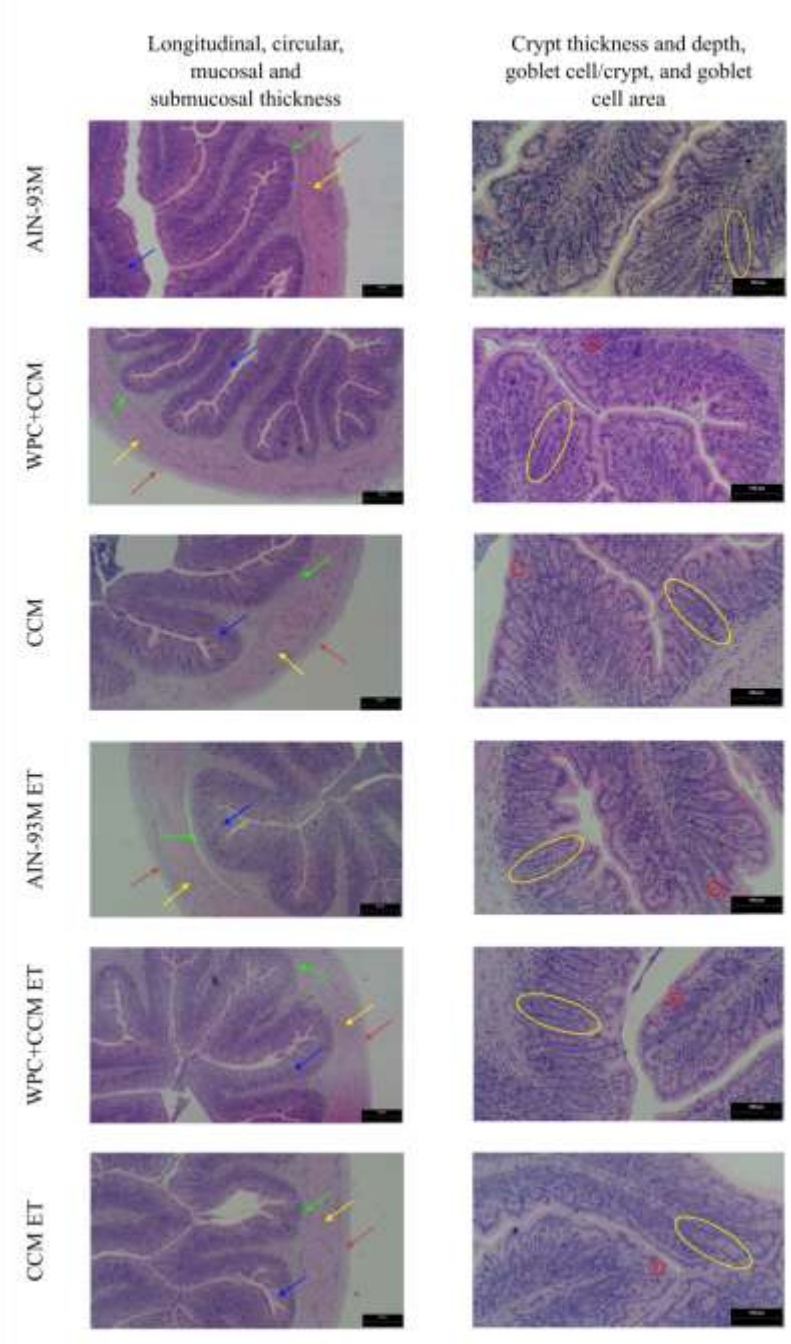

Figure 4. Representative photomicrographs of the colon stained with hematoxylin and eosin in rats from experimental groups.

AIN-93M: group that received a standard diet; AIN-93M EE: group submitted to the exhaustive exercise that received a standard diet; WPC + CCM: group not submitted to EE that received curcumin-added whey protein concentrate; WPC + CCM EE: group submitted to the exhaustive exercise that received curcumin-added whey protein concentrate; CCM: group not submitted to EE that received curcumin; CCM EE: group submitted to the exhaustive exercise that received curcumin; EE: exhaustive exercise. Red arrows: Longitudinal thickness; Yellow arrows: Circular thickness; Green arrows: Submucosa; Blue arrows: Mucosa; Yellow circles: Crypts; Red circles: Goblet cell.