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Research Article
Antioxidant Effects of Soy Isoflavones, Probiotics and Their Combination on Carbon Tetrachloride-Induced Oxidative Stress in Rats

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Abstract

Background and Objective: The health benefits of isoflavone consumption are often attributed to its antioxidant properties. Since the gut microbiome has a large impact on isoflavone metabolism, probiotics might enhance isoflavone bioavailability and *in vivo* antioxidant activity. The aim of this study was to assess the antioxidant effects of isoflavones, probiotics and their combination on Carbon Tetrachloride (CCl_4)-induced oxidative stress in rats. **Materials and Methods:** Wistar rats were allocated to seven groups of six animals, group I: Control group received saline solution (1 mL/kg p.o.), II: Soy isoflavones (50 mg/kg p.o.), III: Probiotics (10^9 CFU/kg p.o.), IV: CCl_4 (1 mg/kg i.p.) and V-VII: After CCl_4, which was fed isoflavones, probiotics or their combination for 14 days, after which the animals were sacrificed. The antioxidative effects of isoflavones, probiotics and their combination were determined by biochemical parameters in serum, the oxidative stress parameters in liver and kidney homogenates and by histological assessment. **Results:** In comparison to those in the CCl_4 treatment group, the functional markers of hepatotoxicity, particularly Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) activity, were significantly lower in the group treated with the combination of isoflavones and probiotics. This combination successfully reduced the CCl_4-induced increase in malondialdehyde levels in liver tissue, while histopathological changes in hepatocytes, were notably attenuated. **Conclusion:** The results indicate that the combined consumption of isoflavones and probiotics inhibits the level of lipid peroxidation and has a hepatocurative effect on CCl_4-induced oxidative stress in rats, significantly improving the functional and morphological parameters of the liver.

Key words: Isoflavones, probiotics, oxidative stress, carbon tetrachloride, Wistar rats

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Isoflavones are secondary plant metabolites that are phytoestrogen compounds because of their mild estrogenic properties. The richest dietary sources of isoflavones are soy and soy products. Due to the numerous health benefits of isoflavones, there is an increasing presence of soy foods and supplements on the market[1]. The antitumor potential of isoflavones in hormonal breast and prostate cancer and the possibility of using isoflavones as estrogen replacement therapy in menopausal women and preventing osteoporosis are consequences of their structural similarity to estrogen and the possibility of binding to alpha and beta estrogen receptors[2,3]. On the other hand, their nonhormonal biological effects, such as antioxidant, lipid-lowering, anti-inflammatory or neuroprotective effects, can be explained by complex mechanisms and pathways[4-6]. The antioxidant activity of these plants may be partly based on the role of isoflavones, genistein and daidzein, which are ligands for alpha and beta estrogen receptors in the liver; these compounds inhibit Interleukin (IL)-1β pathways in the liver or suppress the nuclear factor kappa B inflammation pathway[7]. The additional antioxidant effect can be the result of free radical scavenging, a reduction in initial reactive oxygen species (ROS) generation and an alteration in detoxification enzymes, which leads to a reduction in lipid peroxidation and an increase in resistance to oxidative stress[8].

Probiotics are living microorganisms that, based on scientific and clinical evidence, are considered particularly beneficial for host health and can be effective and safe substitutes for certain pathological disorders[9]. The health benefits of *Lactobacillus* sp. and *Bifidobacterium* sp., have been the most researched, emphasizing their antioxidant potential and consequently their positive impact on certain chronic diseases[10-13]. Several of the proposed mechanisms of the antioxidative effect of probiotics include the following: Possession of their antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT); increase in the host's antioxidant capacity by increasing the level of serum and muscle SOD and hepatic CAT; production of active metabolites with antioxidant effects, Glutathione (GSH) and folate; regulation of these metabolites in the host; meditating several antioxidant signaling pathways; inhibiting initial production of ROS; and regulating the microbiota composition[12,13]. Supplementation with probiotics increases the levels of beneficial bacteria in the intestine and reduces the proliferation of harmful bacteria by lowering the intestinal pH. On the other hand, gut microbiome alterations can greatly affect the metabolism, bioavailability and consequently biological activity of various polyphenolic compounds, such as isoflavones[2,12].

It is known that there is a two-way interdependence between the gut microbiome and isoflavones. Specifically, the intestinal microbiota and liver are very important for different phases of isoflavone metabolism. There is evidence that, with adequate gut microflora, only 1/3 of the population can convert isoflavone daidzein to the more active metabolite equol, which could explain why some individuals benefit more from isoflavone consumption than others[5]. It can be assumed that probiotics, by changing the gut microbiome, might influence isoflavone metabolism and increase isoflavone efficacy. Additionally, isoflavones can act as prebiotics and have an impact on the gut microbiome. By absorbing nitrites and promoting the growth of beneficial bacteria, isoflavones promote host gut metabolism[14]. The results from a recent study showed that soy isoflavones had a positive effect on the growth of probiotics, more specifically, *B. bifidum*, *L. lactis*, *L. plantarum* and *L. rhamnosus*, while the growth of pathogenic bacteria in the gut, such as *Klebsiella*, *Pseudomonas*, *Acinetobacter*, *Escherichia* and *Staphylococcus*, significantly decreased after isoflavone treatment[15].

Oxidative stress is characterized by an increase in ROS, mainly superoxide radicals, hydrogen peroxide and hydroxyl radicals and an alteration in the metabolic state of the organism. While, ROS are natural byproducts of cellular processes, an imbalance occurs when ROS production surpasses the antioxidant capacity of the cell. This imbalance results in oxidative stress, which subsequently damages cellular structures. When not adequately regulated, oxidative stress has the potential to induce a variety of chronic and degenerative diseases[16]. To investigate the antioxidant potential of some substances in animal models, Carbon Tetrachloride (CCl_4) is frequently used for oxidative stress induction. While it is recognized for its primary role as a hepatotoxin, its effects include kidney damage. Additionally, liver injury frequently occurs concomitantly with renal dysfunction; therefore, renal function evaluation is required to assess the antioxidant capacity of the organism more comprehensively[17]. Cellular injury induced by CCl_4 can arise from two potential mechanisms: Covalent bond formation between reactive intermediates and cellular components or an increase in lipid peroxidation induced by free radical intermediates[18].

This study was designed to test our hypothesis that commercial dietary supplements based on soy isoflavones in combination with probiotics could antagonize more

effectively CCl_4-induced toxicity in the liver and kidneys by analyzing its antioxidative potential to prevent CCl_4-induced biochemical changes in the organs of rats in comparison to isoflavones or probiotics itself.

MATERIALS AND METHODS

Study area: This study was carried out from March to May, 2023 at Department of Pharmacy, Department of Pharmacology, Toxicology and Clinical Pharmacology and Department of Histology and Embryology at the Faculty of Medicine, University of Novi Sad.

Isoflavone quantification in soy-based dietary supplements: The contents of the 30 capsules were pulverized and mixed. Analysis was performed in triplicate using the average mass of the capsule. The extraction of isoflavones was conducted with 50 mL of methanol:water (4:1, v/v) for 2 hrs at 40°C with constant stirring. The obtained extracts were diluted with the same solvent 10 times prior to analysis[19]. For the quantification of isoflavones, an Agilent (Palo Alto, California, USA) model 1100 series High-Performance Liquid Chromatography (HPLC) instrument with diode array detector (DAD) was used. The HPLC method described previously by Lee *et al.*[20], included a Zorbax SB C18 reversed-phase HPLC column (150×4.6 mm, 5 µm) with a Zorbax SB C18 guard column (12.5×4.6 mm, 5 µm). Mobile phase gradients were formed between solvent A (1% (v/v) acetic acid in water) and solvent B (100% acetonitrile). Isoflavones were detected on 270 nm and identified by comparing the retention times and UV spectra of the samples with those of standard compounds and literature data[20]. Five-point regression curves ($r \geq 0.9998$) of daidzein, glycitein and genistein standards (ChromaDex, Irvine California, USA) were generated. For the quantification of glucoside forms, calibration curves of corresponding aglycone compounds were generated and corrections for differences in molecular weight between aglycones and glucosides were applied according to the following pattern:

$$c(\text{glucoside}) = \frac{c(\text{corr aglycone}) \times Mr(\text{glucoside})}{Mr(\text{corr aglycone})}$$

All the samples were filtered using Agilent RC 0.45 µm filters prior to HPLC analysis.

Animal experimental design: The experiment was conducted following the ethical standards of the EU Directive 2010/63/EU on the protection of animals used for scientific purposes and approved by the Ethical Committee for the protection of the welfare of experimental animals of the University of Novi Sad and the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (no. 04-150/60 and 323-07-14005/2022-05/2). The experiment was performed on adult male and female Wistar rats (200-300 g) obtained from the Military Medical Academy–Stable for Breeding Experimental Animals Belgrade, Serbia. The animals were housed in the vivarium of the Department of Pharmacology, Toxicology and Clinical Pharmacology of the Faculty of Medicine Novi Sad, Serbia, under standard laboratory conditions throughout the experiment (room temperature, constant humidity, dark/light cycle 12/12 hrs, food and water *ad libitum*).

The dose of isoflavones from a dietary supplement was adjusted to 50 mg/kg. The dose of probiotics from preparation containing a mixture of live lyophilized bacteria from lactic acid fermentation (*Lactobacillus helveticus*, *Lactobacillus rhamnosus* and *Bifidobacterium longum*) was approximately 10^9 CFU/kg. A saline solution was used to prepare the suspension, which was applied once a day by oral gavage. The applied dose of CCl_4 was 1 mL/kg in olive oil (1:1) administered intraperitoneally once-48 hrs before the indicated groups were treated. All doses were selected after consulting the relevant literature, as well as considering FDA recommendations for conversion between human and animal doses and recommended intakes of commercial preparations[9,15,17,21].

The animals were randomly allocated into 7 groups of six animals (in cages were animals of the same sex) and treated once a day for 14 days as follows:

- **I :** Control group, saline solution 1 mL/kg (p.o.), 14 days
- **II :** Soy isoflavones 50 mg/kg (p.o.), 14 days
- **III :** Probiotics 10^9 CFU/kg (p.o.), 14 days
- **IV :** CCl_4 1 mL/kg (i.p.), one dose, 48 hrs before treatment and then saline 1 mg/kg (p.o.), 14 days
- **V :** CCl_4 1 mL/kg (i.p.), one dose, 48 hrs before treatment and then soy isoflavones 50 mg/kg (p.o.), 14 days
- **VI :** CCl_4 1 mL/kg (i.p.), one dose, 48 hrs before treatment and then probiotics 10^9 CFU/kg (p.o.), 14 days
- **VII :** CCl_4 1 mL/kg (i.p.), one dose, 48 hrs before treatment and then soy isoflavones 50 mg/kg (p.o.)+probiotics 10^9 CFU/kg (p.o.), 14 days

The animals were sacrificed 24 hrs after the treatment. The rats were anesthetized with urethane (0.75 g/kg i.p.),

immobilized in the dorsal position and allowed to breathe spontaneously. Blood for biochemical evaluation was collected via heart puncture after the thoracic region was opened. The liver and kidney were treated as follows. After excision, they were preserved at -80°C and in order to obtain homogenate for malondialdehyde (MDA) analysis and supernatant for determining total proteins (TP) and antioxidant enzyme activity, they were homogenized using a Potter-Elvehjem homogenizer after treatment with Tris/KCl buffer solution, pH 7.4 (organ:buffer = 1:10; w/v). One part of the homogenate was used directly for analysis and the other part for obtaining the supernatant (centrifugation at 13,000 xg, 20 min at 4°C).

Total antioxidant status (TAS): The TAS concentration was determined spectrophotometrically (Agilent 8453 UV-visible, temperature-controlled spectrophotometer) by using a commercial assay kit (Randox Laboratories Ltd., Ireland) according to the manufacturer's instructions and is expressed in nmol/L.

Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), urea and creatinine: Serum Alanine Transaminase (ALT), Aspartate Transaminase (AST), urea and creatinine were measured using a standard clinical laboratory test and are expressed in U/L.

Lipid peroxidation measurement: The intensity of lipid peroxidation was measured by a method previously described by Jelić *et al.*[22], including sample deproteinization with 15% trichloroacetic acid and treatment with 0.375% thiobarbituric acid. The mixture was vortexed and heated in a boiling bath for 15 min at 90°C. After cooling for 5 min on ice and centrifuging at 1000×g for 10 min at 4°C, the Thiobarbituric Acid Reactive Substances (TBARS) concentration was determined from the supernatant spectrophotometrically at 535 nm (Agilent 8453, Germany). The released MDA concentration serves as an indicator of lipid peroxidation and the level is expressed as nmol MDA/mL.

Catalase assay (CAT): Measurement of CAT activity included diluting the sample with a previously prepared phosphate buffer, pH 7.0 and adding 30% H_2O_2 in order to cause the H_2O_2 decomposition reaction. The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm[22] and results expressed as U/mg TP.

Glutathione-S-Transferase (GST) assay: A reaction mixture for the measurement of GST activity contained phosphate buffer pH 6.5, ethanolic solution of 1-Chloro-2,4-Dinitrobenzene (CDNB), aqueous solution of reduced glutathione and sample. The rate of produced conjugate CDNB-glutathione complex was measured spectrophotometrically at 340 nm and results expressed as nmol of CDBNB-glutathione conjugate/min/mg TP[22].

Total protein (TP) measurement: The protein concentration was determined by the Biuret method. Several concentrations of bovine serum albumin (Sigma Aldrich, lyophilized powder ≥96%) were used as standards and the absorbance was measured at 540 nm. The results are expressed as mg/mL.

Histological tissue preparation and tissue microarray (TMA) formation: Liver and kidney tissue samples (5×5 mm) were fixed in 10% neutral-buffered formalin solution for 24 hrs at 4°C. Fixed tissues were subsequently dehydrated in isopropyl alcohol and embedded in Histowax paraffin (Duvein, The Netherlands). For the histomorphometric analysis of the observed liver/kidney changes, one cylindrical biopsy specimen, 1 mm in diameter, was taken from each paraffin-embedded liver/kidney using a manual tissue punch (Quick Ray, Unitma). The biopsies were then transferred to a previously constructed paraffin mold. All paraffin blocks were sectioned on a rotatory microtome (Leica, Germany) at 5 µm. The resulting sections were stained using standard Hematoxylin and Eosin (H&E) stain[23].

Histomorphometric analysis: The ImageJ program (National Institutes of Health) was used for the semi-quantification of liver changes. First, using the "grid" tool, a grid in the form of crosses was placed over the TMA preparation (0.5 pixels per grid point) (Fig. 1a). Whole TMA liver samples were analyzed with the "Cell Counter" plugin, which enabled us to point different marks to each cross that fell on the hepatocyte, depending on its morphology: (1) Hydropic degeneration and (2) Normal morphology. Crosses that were found on hepatocytes of unclear characteristics or stromal components were excluded from the analysis (Fig. 1b). For every TMA liver sample, we calculated the sum of the number of marked hepatocytes and the percentage of hepatocytes in each morphological category.

Statistical analysis: All tests were performed in duplicate. The data were analyzed by one-way ANOVA for multiple comparisons and Student's t-test for comparisons. The results are expressed as the Mean±SD and the significance level was set at 0.05. The Kruskal-Wallis test was used for canonical discrimination analysis to evaluate the degree of

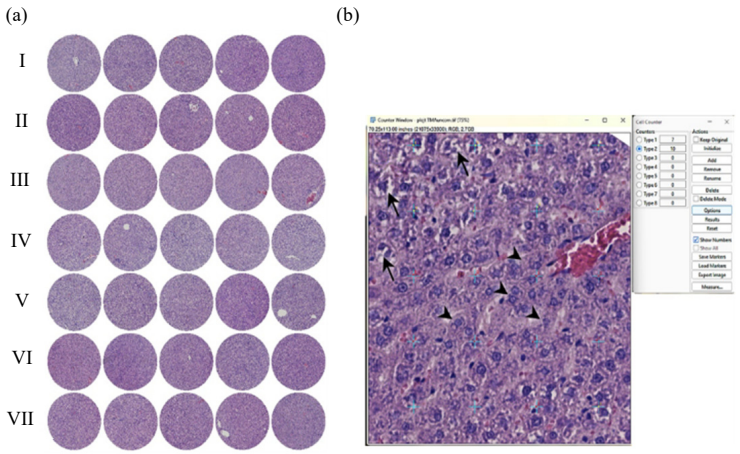

Fig. 1(a-b): Histomorphometric liver analysis, (a) TMA liver samples and (b) ImageJ cell counting of hepatocytes: Hydropic degeneration (arrows) and normal morphology (arrowheads)

differentiation among groups based on the obtained results for oxidative stress and functional liver and kidney parameters. Assessment of similarity between groups was performed by applying hierarchical cluster analysis of Mahalanobis distances.

RESULTS

Isoflavones in dietary supplements: The average total isoflavone content in the soy-based dietary supplements was 66.2 mg per average capsule weight or 157.7 mg per 1 g of product. These values are greater (65.5%) than the declared isoflavone content given by the manufacturer (40 mg per capsule). Daidzein and its glucoside forms were dominant, accounting for more than 74.9% of the total isoflavones, followed by glycitein type of isoflavones with 18.2% share. Genistein type of isoflavones, in this case only genistin, was the least present in the analyzed supplement with 6.9% share in total isoflavones content.

Biochemical parameters: The TAS levels were significantly greater in the isoflavone-treated group pretreated with CCl_4 (V) than in both the control group (I) and the CCl_4-treated group (IV) ($p<0.05$) (Fig. 2a). The results obtained from serum ALT and AST revealed that ALT is significantly increased after administration of CCl_4 (IV) compared to the control group (I) (Fig. 2b), while probiotics (VI) and mixture of probiotics and isoflavones (VII) significantly decreased both, ALT and AST levels, comparing to the CCl_4 treated group (IV) (Fig. 2b-c). The results of the serum urea and creatinine levels did not significantly differ between the groups (Fig. 2d-e).

Oxidative stress parameters: The MDA levels and the activities of CAT and GST in liver and kidney tissue are shown in Fig. 3(a-c). A single dose of CCl_4 significantly increased the MDA concentration, CAT and GST activity in the kidneys and CAT activity in the liver. The CCl_4 administration increased the activities of enzymes in antioxidative systems, such as CAT and GST. These increased levels of enzymes might represent an adaptive mechanism of the cell since the excessive ROS production mediated by CCl_4 may selectively increase the transcription of genes encoding these antioxidative enzymes[24]. Probiotics and isoflavones in combination (VII) significantly decreased the level of MDA in liver and kidney tissue compared to that in the CCl_4-treated group (IV), thus helping to diminish oxidative stress and maintain the basal activity of CAT and GST.

Histopathological analysis

Liver: Histological analysis of the H&E-stained TMA liver samples in the control group (I) revealed classic organ cytoarchitectonics with regular hepatocytes containing homogeneous cytoplasm and large, euchromatic nuclei (Fig. 4). Light hydropic degeneration was observed in some hepatocytes (32.53%). Medium hydropic degeneration was observed in the group that received only isoflavone (II) (55.79%), while the group that received only the probiotic (III) had intense hydropic degeneration (80.83%). On the other hand, in the group exposed to CCl_4 (IV), there were no signs of steatosis but diffuse hydropic degeneration (79.2%). The combination of CCl_4 with isoflavone treatment (V) resulted in a slightly lower grade of hydropic degeneration (62.94%) than that in the group that received only CCl_4. The addition of

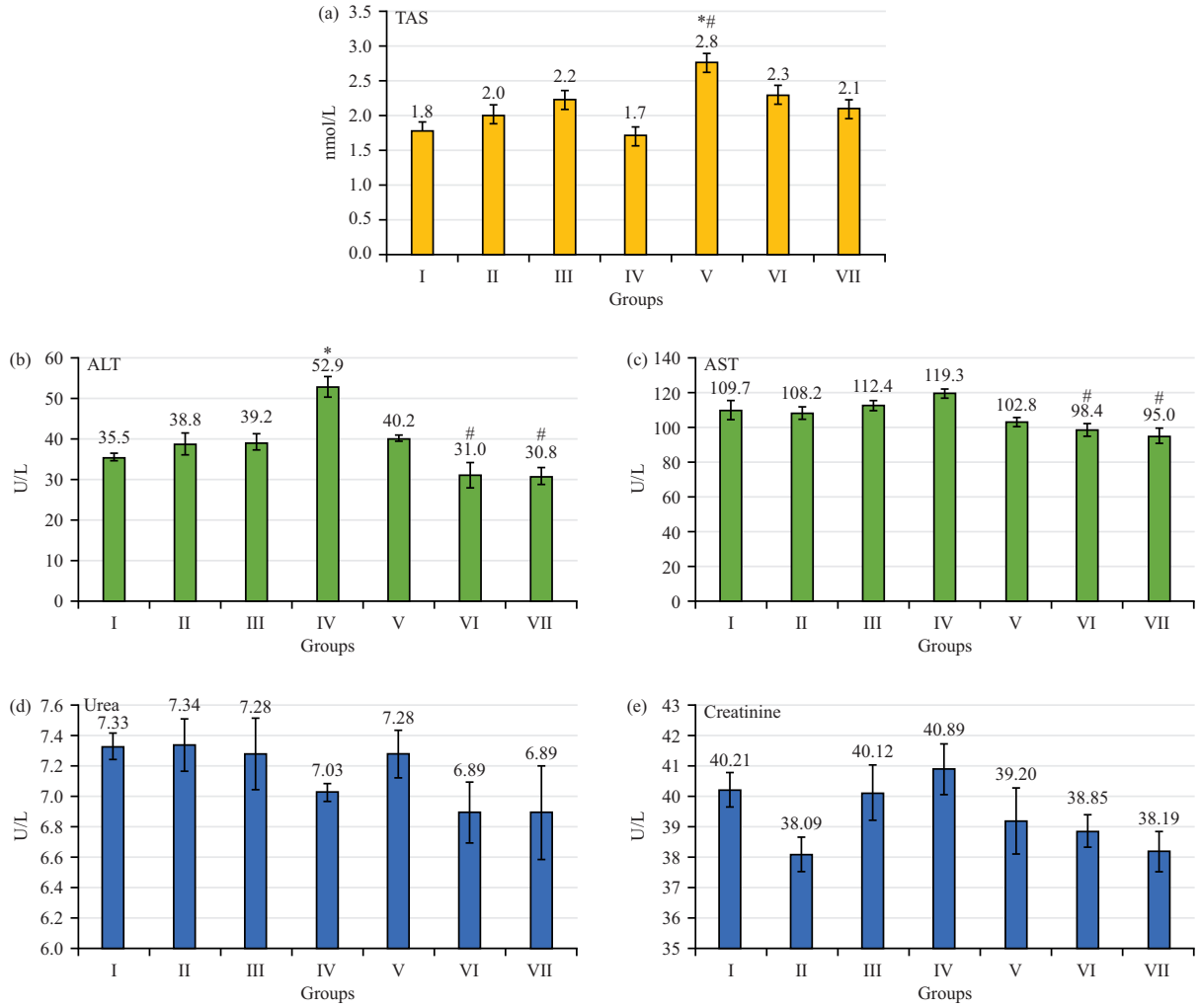

Fig. 2(a-e): Effects of isoflavones, probiotics and their combinations on serum (a) TAS, (b) ALT, (c) AST, (d) Urea and (e) Creatinine

*p<0.05 vs the control group, #p<0.05 vs the group treated only with CCl_4, groups: I (c), II (i), III (p), IV (CCl_4), V (CCl_4+i), VI (CCl_4+p) and VII (CCl_4+i+p)

probiotics to the CCl_4 treatment led to an even lower grade of hydropic degeneration (55.79%). The combination of isoflavones and probiotics in combination with CCl_4 treatment also lowered the amount of hepatocellular hydrops (66.43%) but not as effectively as the combination of these agents with probiotics alone (Fig. 4).

Kidneys: Histological analysis of H&E-stained kidney sections revealed completely regular morphology in all the experimental groups. The glomeruli preserved their distinctive morphology, with no signs of thickening of the basement membrane. The epithelial cells of the renal tubules were regular in shape, with homogeneous acidophilic cytoplasm and euchromatic nuclei. The interstitial connective tissue was sparse and lacked lymphocytic infiltration.

Canonical discriminant analysis (CDA): The CDA of the oxidative stress-related parameters (CAT, GST, TBARS and TAS) and functional parameters (ALT, AST and hydropic degeneration) in experimental animals subjected to different types of treatment indicated the statistical significance of the obtained discriminant functions (Wilks' lambda = 0.02, F (42,308) = 9.53, p<0.00), whereas the recorded discriminations between animals were statistically significant for the values obtained for TAS, ALT, AST and hydropic degeneration. Furthermore, the CDA indicated that the first two canonical roots (CAs) described more than 88% of the original dataset discriminations (Fig. 5a). The size of the recorded discriminations (in terms of CA1) mostly correlated with the values obtained for liver function parameters (ALT, AST and hydropic degeneration), while the shape of the

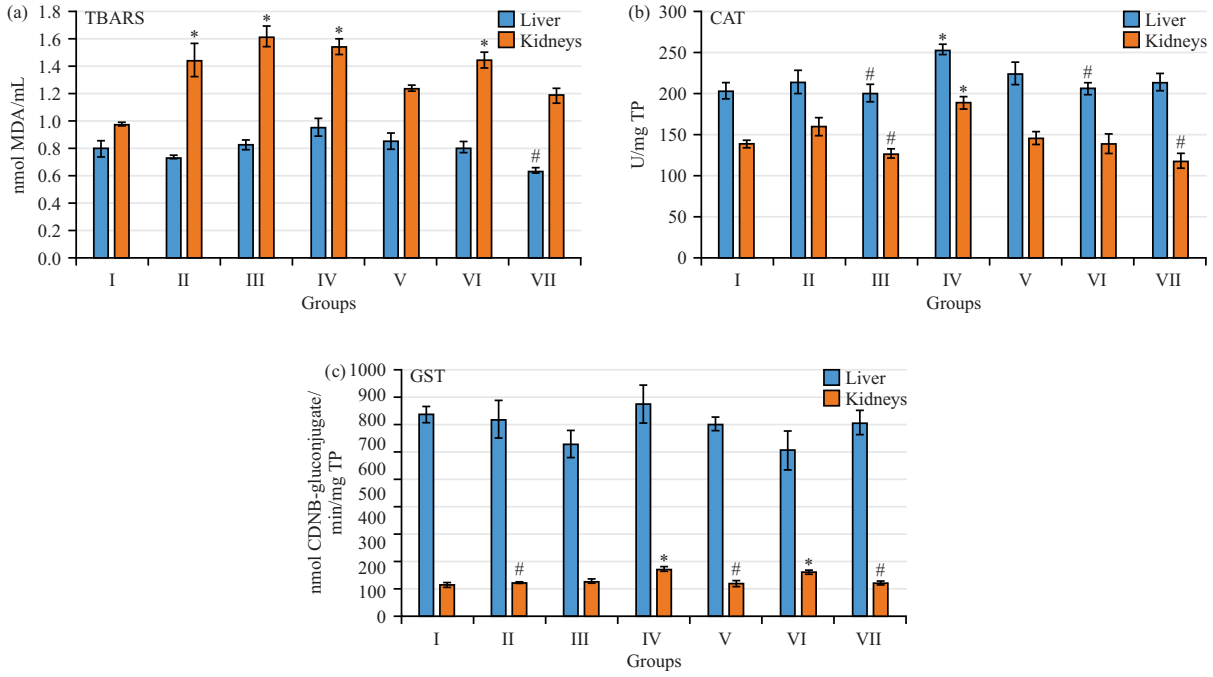

Fig. 3(a-c): Effects of isoflavones, probiotics and their combination on the levels of (a) TBARS, (b) CAT and (c) GST in liver and kidney tissue

*p<0.05 vs the control group, #p<0.05 vs the group treated only with CCl_4, groups: I (c), II (i), III (p), IV (CCl_4), V (CCl_4+i), VI (CCl_4+p) and VII (CCl_4+i+p)

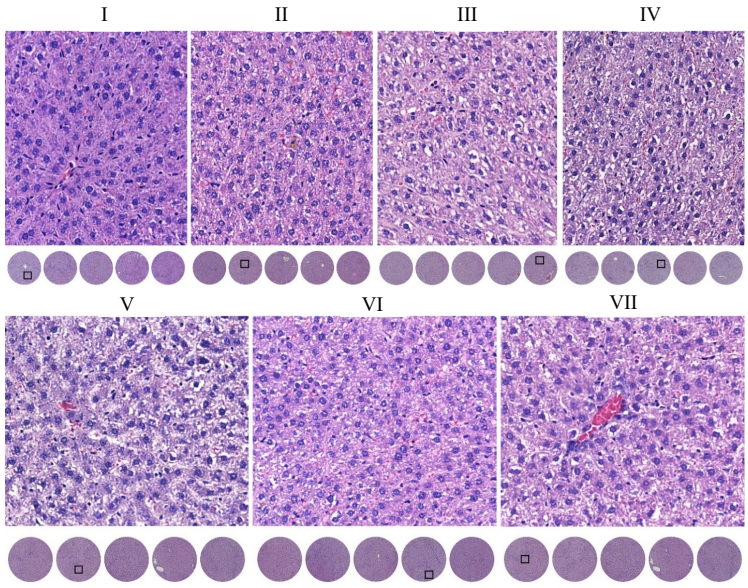

Fig. 4: Effects of isoflavones, probiotics and their combination on liver histomorphology

Groups: I (c), II (i), III (p), IV (CCl_4), V (CCl_4+i), VI (CCl_4+p) and VII (CCl_4+i+p)

discriminations correlated with the values obtained for the TAS. The position of the evaluated animals in the space defined by the first two canonical axes (Fig. 5a) indicates a separate grouping of the control and CCl_4-treated animals in terms of CA1, as indicated by the greater ALT levels and hydropic degeneration observed in CCl_4-treated animals. Furthermore, both of the previously mentioned groups were located in the negative region of CA2 as a result of lower recorded TAS values than were found in other experimental animals. Treatment of animals with probiotics or isoflavones

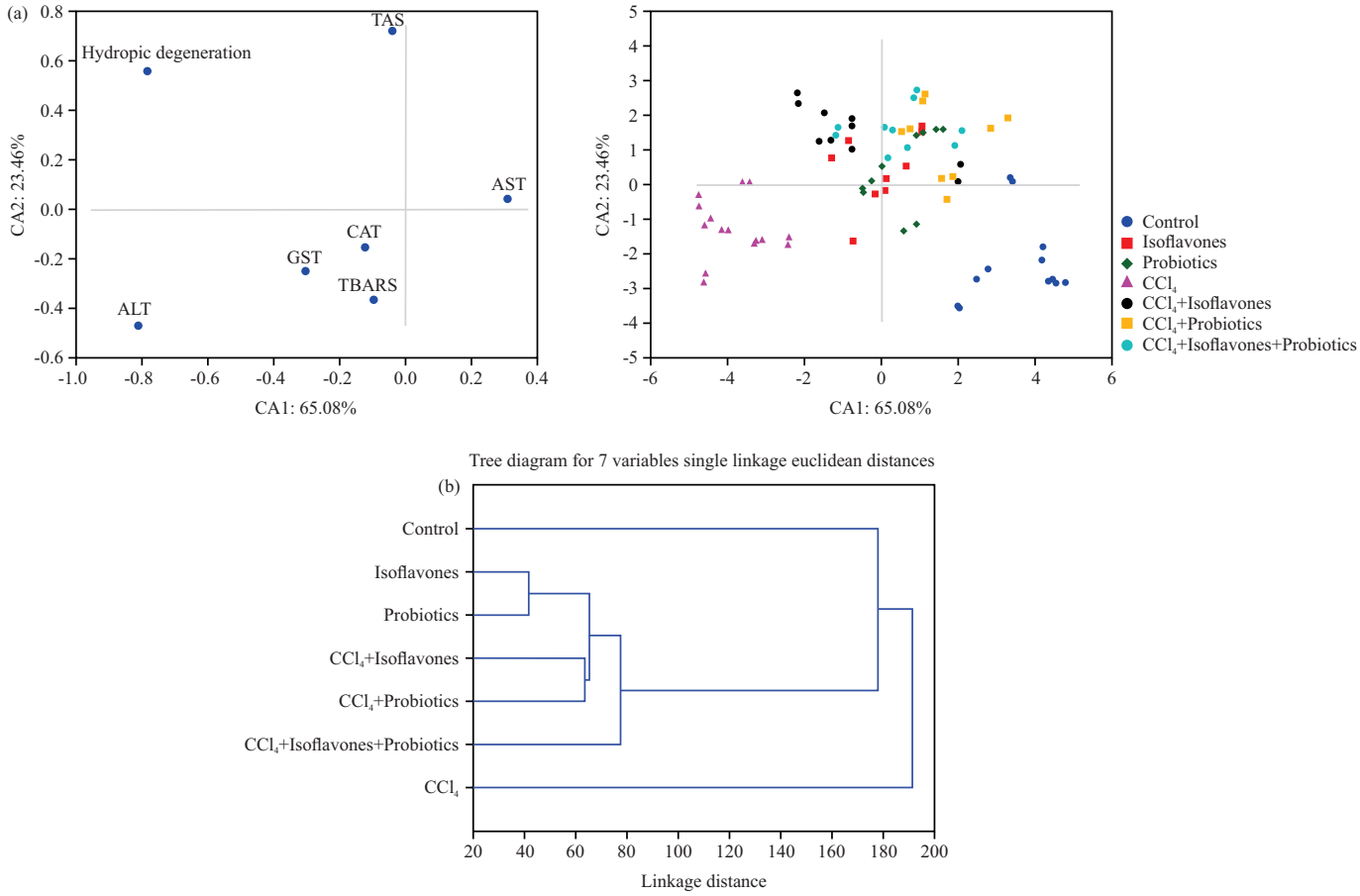

Fig. 5(a-b): Position of the groups in the area defined by the first two canonical axes, (a) CA 1-canonical axis 1, CA 2-canonical axis 2 and (b) Hierarchical cluster analysis of mahalanobis distances-liver

leads to an increase in the TAS, indicating a greater capacity for mitigating negative oxidative stress. Thus, treating CCl_4-exposed animals with isoflavones, probiotics, or their combination reduces hydropic degeneration and ALT levels, followed by a decrease in total alkalis (TAS) levels. Although no separative grouping of these animals could be observed, it seems that the slight advantage in mitigating CCl_4-induced liver damage could be attributed to the application of probiotics, as well as the combination of probiotics and isoflavones (positive parts of CA1 and CA2). Furthermore, the application of hierarchical cluster analysis to the squared Mahalanobis distances obtained by CDA (Fig. 5b) confirmed previously stated patterns of sample grouping.

The CDA of the oxidative stress-related parameters (CAT, GST, TBARS and TAS) and functional parameters (creatinine and urea) in experimental animals subjected to different treatments indicated the statistical significance of the obtained discriminant functions (Wilks' lambda = 0.04, F (36.292) = 8.54 $p<0.00$), whereas the recorded discriminations between animals were statistically significant for all evaluated variables. Furthermore, the CDA indicated that the first two canonical roots (CAs) described more than 84% of the original dataset discriminations (Fig. 6a). The size of the recorded discriminations (in terms of CA1) mostly correlated with the values obtained for TBARS, while the shape of the discriminations correlated with the values obtained for TAS and urea. The position of the evaluated animals in the space defined by the first two canonical axes (Fig. 6a) indicates the separative grouping of the control and CCl_4-treated animals in terms of CA1 as a consequence of higher TBARS values recorded in the CCl_4-treated group. Furthermore, the experimental animals treated with isoflavones and probiotics were (in terms of CA1) nested between the CCl_4-treated and control groups as a result of higher recorded TBARS values than in the control group, whereas CA2 was located in the negative part as a consequence of higher TAS and lower urea levels than those obtained for the control group. The addition of isoflavones, probiotics or a combination of isoflavones and

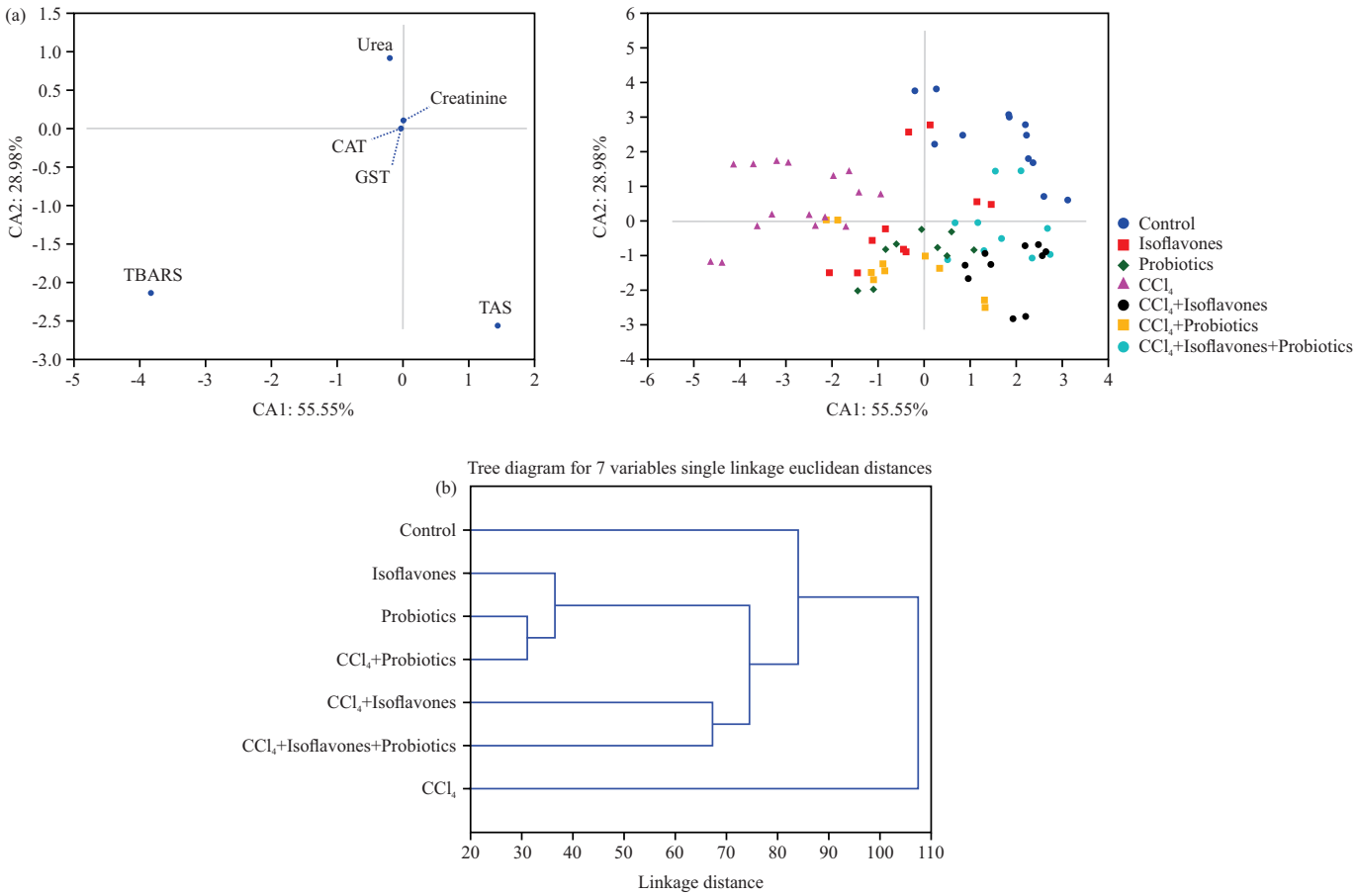

Fig. 6(a-b): Position of the groups in the area defined by the first two canonical axes, (a) CA 1-canonical axis 1, CA 2-canonical axis 2 and (b) Hierarchical cluster analysis of mahalanobis distances-kidney

probiotics reduces the TBARS values of animals treated with CCl_4 (the space defined by CA1) and, as seen based on the space defined by CA2, reduces urea and increases TAS values. It can be concluded that protection treatment ameliorates the negative effects of already stressed kidneys. Importantly, based on the non-separative grouping of the samples, the addition of isoflavones and a combination of isoflavones and probiotics to the CCl_4-treated animals led to the same changes in the TBARS, TAS and urea levels, indicating protection from CCl_4-induced oxidative stress, whereas the addition of probiotics to the CCl_4-treated group resulted in a somewhat decreased TBARS decrease and, to some extent, weakened protection. The application of hierarchical cluster analysis to the squared Mahalanobis distances obtained by CDA (Fig. 6b) confirmed previously stated patterns of sample grouping.

DISCUSSION

This study shows that combination of soy isoflavones and probiotics more effectively mitigates CCl_4-induced oxidative stress compared to isoflavones and probiotics alone. Investigation was developed based on prior research demonstrating that soy isoflavones[8] and probiotics each possess antioxidant potential[25] and substantial evidence demonstrating that interactions between dietary isoflavones and the intestinal microbiota are inherently advantageous. A recent study revealed that prebiotics and probiotics are capable of promoting the biotransformation of soy isoflavones by the gut microbiota to equol, which has greater bioactivity than soy isoflavones[26]. It is reasonable to assume that with a higher concentration of equol, the antioxidant potential of isoflavones would increase. The present results confirmed that treating animals with CCl_4 cause oxidative damage expressed by raise levels of serum ALT, AST and increased MDA concentrations, as well as hydropic degeneration of hepatocytes. Restoring all mentioned oxidative stress parameters to physiological levels after administration of combination of soy isoflavones and probiotics revealed their antioxidant effect. In this aspect, by reviewing the available literature, there is a lack of experiments that would evaluate

the antioxidant effect of this combination. However, studies on the antioxidant effect of isoflavones or probiotics individually are available and our findings correlate with them.

Determining the serum TAS concentration might offer more useful information for establishing antioxidant potential than strictly analyzing individual parameters of oxidative stress. This approach could enable an evaluation of the cumulative antioxidant capacity. Current findings validate previously published data showing that dietary isoflavones can restore antioxidant capacity, as measured by the TAS, in experimental animals previously treated with CCl_4[21,27]. While CCl_4 administration did not have a significant impact on TAS, isoflavones at a dose of 50 mg/kg significantly increased TAS, surpassing that of the control group. Although not significantly different, treatment with both probiotics and the combination of probiotics and isoflavones increased the TAS in comparison to that in the control group (Fig. 2a).

A significant increase in the serum transaminase concentration after CCl_4 administration indicates a compromise of the liver's structural and functional integrity, as well as a release of these cytoplasmic enzymes into the systemic circulation. Probiotics alone, as well as in combination with isoflavones, demonstrated hepatoprotective effects through reductions in the serum ALT and AST levels, which are the most specific indicators of liver impairment, in comparison to those in the group treated with CCl_4 (Fig. 2b-c). These results were in accordance with those of the study conducted by Hogervorst *et al.*[17], who reported that the application of a soybean-based dietary supplement with a similar isoflavone content significantly increased these parameters. However, in their research, ALT and AST levels did not return to physiological levels after the administration of isoflavones, possibly because, compared with those in our study, the animals were treated with higher doses of CCl_4. Additionally, in a study by Yu *et al.*[28], the effects of daidzein on lipopolysaccharide-induced liver injury were examined. According to the findings in this study, treatment with lipopolysaccharide in combination with daidzein significantly decreased the serum concentrations of ALT and AST.

Moreover, prior studies have demonstrated that *Lactobacillus* species possess a significant capacity to produce antioxidants. For example, in a study carried out by Jantararussamee *et al.*[9], it was indicated that probiotic lactic acid bacteria can restore almost all physiological levels of ALT in thioacetamide-induced liver injury. Chen *et al.*[29] demonstrated the hepatoprotective effects of *Lactobacillus* on CCl_4 acute-induced liver injury in mice, not only for live stains but also for heat-killed ones. Although the levels of serum AST and ALT were more significantly decreased in the groups treated with live stains, heat-killed strains also had positive effects.

Histopathological changes demonstrated the antioxidant potential of soy isoflavones, probiotics and their combination, according to the results of our research. The degree of liver tissue damage was mild after the application of CCl_4, at the level of hydrops degeneration. These findings were different from those previously reported[10,29]. In the mentioned studies, the administration of CCl_4 caused severe hepatocyte degeneration characterized by necrosis and inflammatory cell infiltration. The reason for this could be the experimental design. Specifically, young rats exhibit liver recovery from CCl_4-induced injury that starts within 24 hrs after intoxication and can lead to cell recovery if intoxication is not repeated. The proposed mechanisms are CYP450 levels that are insufficient to activate the toxicant effectively and high mitotic indices that confer a high capacity for regeneration[30]. Although all the treated groups exhibited certain levels of hydropic degeneration, the probiotic alone and the combination of probiotics and isoflavones had the most efficient effects on preserving hepatocyte structure in the CCl_4-pretreated groups. Present research provides support for the synergistic effect of isoflavones and probiotics in reducing the harmful consequences of CCl_4-induced liver injury.

As previously mentioned, there were no statistically significant differences in the serum urea or creatinine levels between the animal groups. This result was consistent with the outcomes of the morphometric examination of the kidney tissue, which revealed that the kidney's structure remained entirely intact. One possible explanation is that experimental animals must be subjected to chronic exposure to toxicants to develop more substantial cell injury in the kidneys. However, subtle changes in oxidative and antioxidative stress parameters were observed, indicating the effectiveness of the probiotic and isoflavone combination.

Further investigation revealed mild nephroprotective effects of isoflavones, probiotics and their combination in preventing CCl_4-induced oxidative stress. The administration of isoflavones and the combination of isoflavones and probiotics resulted in a reduction in urea concentrations, indicating that these treatments might help restore the diminished renal function induced by CCl_4. Rašković *et al.*[31] noted that the antioxidant properties of flavonoids can be a consequence of their structure and ability to incorporate membrane lipids, which reduce the degree of lipid peroxidation. Furthermore, *in vivo* investigations have demonstrated that genistein inhibits renal cell injury induced by sodium fluoride, experimentally induced renovascular

hypertension and renal ischemia, among others[32]. The same mechanism, reduction in lipid peroxidation, is responsible for the antioxidant efficacy of *Lactobacillus* and *Bifidobacterium* strains. Measuring levels of MDA, commonly recognized as an indicator of oxidative stress and antioxidant status, it was demonstrated that treatment with probiotics and a combination of isoflavones and probiotics can decrease level of produced MDA and help maintain the integrity of cellular structure. These results are in accordance with findings from previous studies in which soy isoflavone and probiotics were found to be effective at decreasing the levels of MDA in patients with toxicant-induced injuries[9,17]. Furthermore, the effectiveness of symbiotic soy yogurt in mitigating hepatic toxicity induced by a high-cholesterol diet in rats was investigated by Sengupta *et al*.[33]. *Lactobacillus* and *Bifidobacterium* strains, as well as *Saccharomyces boulardii* and fructo-oligosaccharide, were used for soy yogurt fermentation. The findings of the study indicated that the synergistic effects of soy isoflavones, probiotics and prebiotics may result in enhanced antioxidant activity[33].

This study highlighted the antioxidant potential of the combination of isoflavones and probiotics and their potential use in treating conditions of oxidative damage. This opens up new possibilities when recommending or creating dietary supplements use, which in this way would have potentially improved bioavailability and efficiency. Since there are no clearly defined recommendations for doses of isoflavones that would achieve certain health benefits, the limitation of this research is that we did not use different doses of isoflavones, so we did not examine the dose-dependent effects of this combination. Future studies could examine this aspect as well. Also, the animal exposure to isoflavones and probiotics could be prolonged.

CONCLUSION

Current findings validate the antioxidant potential of isoflavones and probiotics when used individually. Furthermore, it is demonstrated that their combined use has synergistic effects on protecting against oxidative stress and establishes significant bilateral dependence. The hepatocurative effect of the combination of isoflavones and probiotics can be noted and proven by the reduction in hydropic degeneration, improvement in ALT/AST levels, which are functional parameters of liver tissue and parameters of an antioxidant defense system based on the results obtained for TBARS. Subsequent investigations should clarify the cellular mechanisms underlying this interaction.

SIGNIFICANCE STATEMENT

Soy isoflavones have a strong antioxidant potential. One of the important aspects of their action is the effect on the intestinal microbiome-an effect similar to prebiotics. In addition, probiotics also improve the antioxidant status, by increasing the level of antioxidant enzymes, total antioxidant capacity, inhibiting lipid peroxidation and inhibiting the growth of pathogenic bacteria. Bearing in mind the still insufficiently investigated interaction between isoflavones and the gut microbiome, as well as their antioxidant potential, the aim of this study was to further investigate the mentioned mutual dependence in terms of evaluating the effectiveness of the combination of isoflavones and probiotics in mitigating the consequences of oxidative stress compared to isoflavones or probiotics alone.

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