



Probiotic bacteria protect against indomethacin-induced gastric ulcers through modulation of oxidative stress, inflammation, and apoptosis

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Abstract

Background Indomethacin is an anti-inflammatory drug that causes ulcers on the gastric mucosa due to its use. Probiotic bacteria are live microorganisms, and it has been stated by various studies that these bacteria have antioxidant and anti-inflammatory effects. In this study, we investigated the possible protective effect of various types of probiotic bacteria (*Lactobacillus rhamnosus*, *Lactobacillus fermentum*, and *Lactobacillus brevis*) against acute gastric mucosal damage caused by indomethacin.

Methods Control group - Physiological saline was administered daily for 10 days. Indo group-Physiological saline was administered daily for 10 days. Ranitidine + Indo group 5 mg/kg ranitidine dose was administered daily for 5 days. On day 11, a single dose of 100 mg/kg of indomethacin was given to the same group. Probiotic + Indo group 1 ml/kg of oral probiotic bacteria was administered daily for 10 days. On day 11, a single 100 mg/kg dose of indomethacin was given. After the application, the rats were anesthetized with ketamine xylazine, killed under appropriate conditions, the abdominal cavity was opened and the stomach tissues were removed. The obtained gastric tissues were used in the biochemical and histopathological analyses discussed below. All data were statistically evaluated by one-way ANOVA using SPSS 20.00, followed by Duncan *Post hoc* test. The data were expressed as mean \pm SD. $P < 0.05$ was considered statistically significant.

Results As a result, the administration of indomethacin caused gastric damage, stimulating oxidative stress, inflammation, and apoptosis. We found that the use of probiotic bacteria reduces oxidative stress (TOC), increases the activity of antioxidant enzymes (TAC), suppresses inflammation (IL-6 and Tnf- α), and inhibits apoptosis (Bax and Bcl-2) ($P < 0.05$).

Conclusion Probiotic treatment can mitigate gastric damage and apoptosis caused by indomethacin-induced gastric damage in rats. Probiotic also enhances the restoration of biochemical oxidative enzymes as it has anti-inflammatory, antioxidant, and antiapoptotic properties.

Keywords Gastric ulcer · Rat · Indomethacin · COX-2 · Oxidative stress

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in the treatment of diseases such as rheumatic disorders and osteoarthritis [1]. They are also given as anti-neoplastic drugs for the prevention and treatment of ischemic heart disease [2]. However, the use of NSAIDs leads to several gastrointestinal complications in the organism, such as gastric mucosal bleeding, decreased gastric mucosal blood flow, and induced mucosal cell apoptosis [3–7]. It is thought that these drugs cause gastric injury via the inhibition of cyclooxygenases (COXs), an increase in prostaglandin (PG) synthesis, and stimulation of gastric mucosal apoptosis associated with increased NSAID-induced reactive oxygen species (ROS) [1]. Indomethacin (IND), a

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strong NSAID, was reported to induce ROS production and increase gastric damage [8]. This results in oxidative stress, mitochondrial permeability transition pore formation, mitochondrial dysfunction, and a consequent increase in proinflammatory cytokine production. Thus, inflammation occurs. This is related to the formation of mitochondrial oxidative stress [1, 3]. Studies have reported that inflammation plays an important role in the pathogenesis of indomethacin-induced gastric mucosal injury [5, 9]. Increased leukocytes at the site of gastric injury are a determinant in the initiation of the pathogenesis of gastric mucosa [10]. Neutrophils are also activated in patients with indomethacin-induced gastric injury. Activated neutrophils can physically occlude small vessels by producing many proinflammatory and pro-oxidative enzymes [5, 9, 11]. This increases the oxidative load of the gastric mucosa and damages the endothelium [12]. Therefore, antioxidant, anti-inflammatory, and anti-apoptotic treatment will be an effective approach in the prevention or treatment of NSAID-induced gastric injury. Several recent studies have reported that antioxidant and anti-inflammatory agents have prevented indomethacin-induced gastric injury. Probiotic bacteria are live microorganisms with anti-inflammatory and antioxidant biological uses [13–17].

Numerous studies have demonstrated the advantageous effects of specific lactobacilli, including the prevention of neoplastic, inflammatory, and allergic alterations as well as the suppression of harmful bacteria in the gut [18–21]. Additionally, it has been demonstrated that lactobacilli, whether given as a probiotic mixture, VSL#3 [22], or as individual probiotic strains, such as *Lactobacillus rhamnosus* GG [23], *Lactobacillus gasseri* OLL2716 [24, 25], or *Lactobacillus acidophilus* [26, 27], are especially helpful in promoting the healing of gastric ulcers in rats. *Lactobacillus rhamnosus* GG stimulates epithelial cell regeneration, especially near the ulcer edges, by raising the ratio of cellular proliferation to apoptosis [28]. Research has shown that when it comes to enhancing the properties of native microflora, a combination of probiotic strains is superior to one [29]. A rat model of an ibuprofen-induced stomach ulcer has demonstrated potential therapeutic effects for specific yeasts, including *Saccharomyces boulardii*, which have been studied in addition to bacteria [30, 31].

This study aims to investigate the gastroprotective effects of the application of probiotic bacteria (*Lactobacillus rhamnosus*, *Lactobacillus fermentum*, and *Lactobacillus brevis*) on gastric mucosal damage induced by indomethacin in rats.

Material and method

Animals

Six-week-old female Wistar rats were housed in a cage maintained at 23°C, with a 12/12-hour light/dark cycle under specific pathogen-free conditions. After one week of adaptation, rats weighing 250 to 300 g were used for the experiment. All experimental procedures were approved by Kafkas University (KAÜ-HADYEK/2017/076).

Analysis and preparation of probiotic mixture

For the isolation of lactobacilli from the kefir, Man, Rogosa, and Sharpe (MRS, Merck, Germany) were seeded with agar media and incubated anaerobically at 37 °C for 72 h. At the end of incubation, white and opaque colonies were tested for Gram staining, catalase, and oxidase. Gram-positive, rod-shaped, catalase- and oxidase-negative colonies were identified using API CH50. Carbohydrate utilization was assessed after 24 and 48 h. All strains were tested for fermentation of the following 50 metabolites: glycerol, erythrol, D- and L-arabinose, D-ribose, D- and L-xylose, D-adonitol, methyl-beta-D-xylopiranoside, D-galactose, D-glucose, D-fructose, D-mannose, D-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-alpha D-mannopyranoside, methyl-alpha D-glucopyranoside, N-acetylglucosamin, amygdalin, arbutin, esculin/ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melizitose, D-rafinoose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate ribose. To ensure anaerobic conditions, two drops of sterile mineral oil were placed in each kit after inoculation. The results were analyzed according to the biochemical profiles registered in the APIweb® database (bioMerieux). *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, and *Lactobacillus brevis* were isolated as lactic acid bacteria. The bacteria were separated from the supernatant culture by centrifugation, washed with a cold phosphate saline buffer, and resuspended in PBS (10⁵ lactic acid bacteria in 1 ml).

Experimental design

In an experimental study, forty female Wistar rats were used and equally divided into four groups as follows; Gp. I (Control group) was kept on physiological saline (1 ml) for 10 days as a negative control group. Gp. II (Indo group) was administered physiological saline for 10 days and on day 11, was given a single dose of indomethacin (100 mg/kg BW). Gp. (III) (Ranitidine + Indo group) received ranitidine

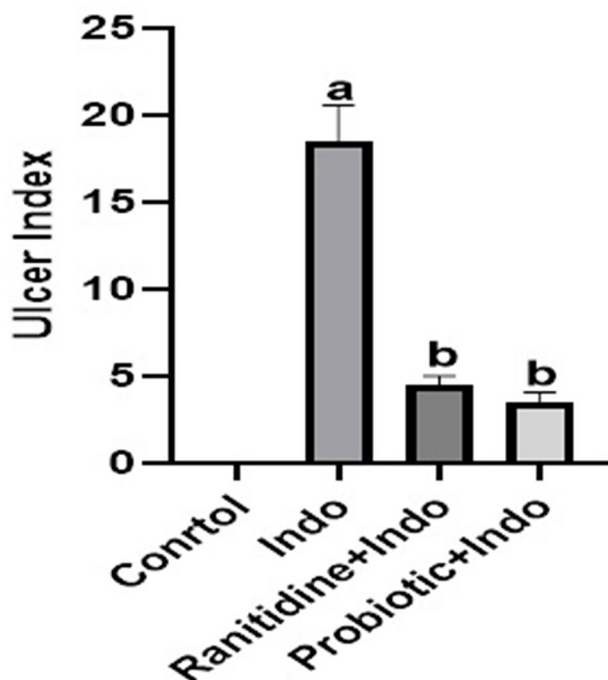
Table 1 Inflammation markers in the gastric tissues for all groups

Mean \pm SD	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-1 β (pg/ml)	TNF- α (pg/ml)	COX-2 (ng/ml)
Control	97.68 \pm 4.26 ^a	53.15 \pm 2.84 ^a	54.11 \pm 5.07 ^a	42.14 \pm 4.12 ^a	156.23 \pm 12.16 ^a
Indo	151.42 \pm 8.84 ^b	79.05 \pm 3.12 ^b	63.17 \pm 4.16 ^a	71.08 \pm 3.71 ^b	114.21 \pm 15.71 ^b
Ranitidine + Indo	86.14 \pm 3.21 ^a	61.12 \pm 4.03 ^{bc}	60.04 \pm 3.14 ^a	59.13 \pm 2.89 ^c	112.32 \pm 10.82 ^b
Probiotic + Indo	89.45 \pm 5.16 ^a	51.16 \pm 5.01 ^{ac}	61.21 \pm 2.12 ^a	49.16 \pm 3.12 ^c	139.64 \pm 11.84 ^{ab}

IL-6, IL-8, IL-1 β ; TNF- α , and COX-2 the letters indicate the statistical differences among groups ($P < 0.05$, $n = 10$), the results were expressed as mean \pm SD

Table 2 Ulcer index and preventive index in the studied groups

Groups	Ulcer Index
Control	-
Indo	19.19 \pm 1.42 ^a
Ranitidine + Indo	4.43 \pm 0.74 ^b
Probiotic + Indo	3.95 \pm 0.23 ^b

**Fig. 2** Effect of Probiotic on the severity of gastric lesion (UI) in Indo-induced gastric ulcer

(5 mg/kg BW) for 5 days and on day 11 given indomethacin. Gp. (IV) (Probiotic + Indo group) orally taken probiotic bacteria (1 ml/kg BW) for 10 days and on day 11 given indomethacin as. At the end of dosing, rats of all groups were anesthetized, and sacrificed and stomach was picked up, opened, and washed with physiological saline, and ulcer scoring was done (Table 2; Fig. 2; Fig. 3). Then a fragment of the stomach was homogenized for biochemical analysis and another fragment was taken on neutral formalin 10% for histopathological and immunohistochemical examination.

Tissue homogenization

The gastric tissues (0.5 g) were homogenized by a tissue homogenizer (WINGER HAUSER/Ser no. 177,002) in 5 mL of cold phosphate buffer saline (pH 7.4, 0.1 M). The homogenates were centrifuged (Hettich zentrifugen, D-78,532-Tuttlichgen, GERMANY) at 10,000 g for 20 min at 4 °C. The supernatants were collected and stored at -20 °C until further use in bioassays.

Measurement of gastric TOC and TAC

Gastric total oxidant capacity (TOC) and total antioxidant capacity (TAC) were determined using kits of (TOC (MY130380) and TAC (MT13033) kit, Gaziantep, Turkey) according to the manufacturer's protocol.

Inflammatory cytokines analysis

Rat-specific ELISA kits of (Sunlong Biotech Co., Ltd) were used to measure interleukin-6 (IL-6), interleukin-8 (IL8), interleukin-1 β (IL-1 β) and Tumor necrosis factor (TNF- α), (rat IL-6 (201,704), IL-8 (201,704), IL-1 β (201,704) and TNF- α (201,704) ELISA, kit Sunlong Biotechnology, Shanghai, China) and Cyclooxygenase-2 (COX-2) (COX-2 (E-EL-H1414) ELISA, kit Elabscience, USA) with. they were respectively following the manufacturer's protocols.

Histopathological and immunohistochemical examinations

For histopathological analysis, stomach tissues were fixed in 10% formalin. After 72 h of fixation, the tissue samples were dehydrated, cleared, and embedded in paraffin. The paraffin was cut into 5 μ m thick blocks using a LeicaRM2125RT microtome (Leica Microsystems, Wetzlar, Germany) and stained by Mallory's triple stain, modified by Crossman for assessment of architectural damage and inflammatory process. A rabbit polyclonal antibody against Bax (dilution:1/50, Abcam, Cambridge, UK) and a rabbit polyclonal antibody against Bcl-2 (dilution:1/100, Abcam, Cambridge, UK) were used to estimate apoptosis and cellular proliferative activity in the stomach tissue. The stained

specimens were examined under a light microscope (*Nikon eclipse50, Tokyo, Japan*) and photo images were taken for histopathological and immunohistochemical evaluation. At least ten high-power fields for each slice were observed, and the number of positive cells was counted and averaged to reflect the intensity of positive expression. The sections were evaluated as none (–), mild (+), moderate (++) and severe (+++) according to their immunity positivity.

Statistical analysis

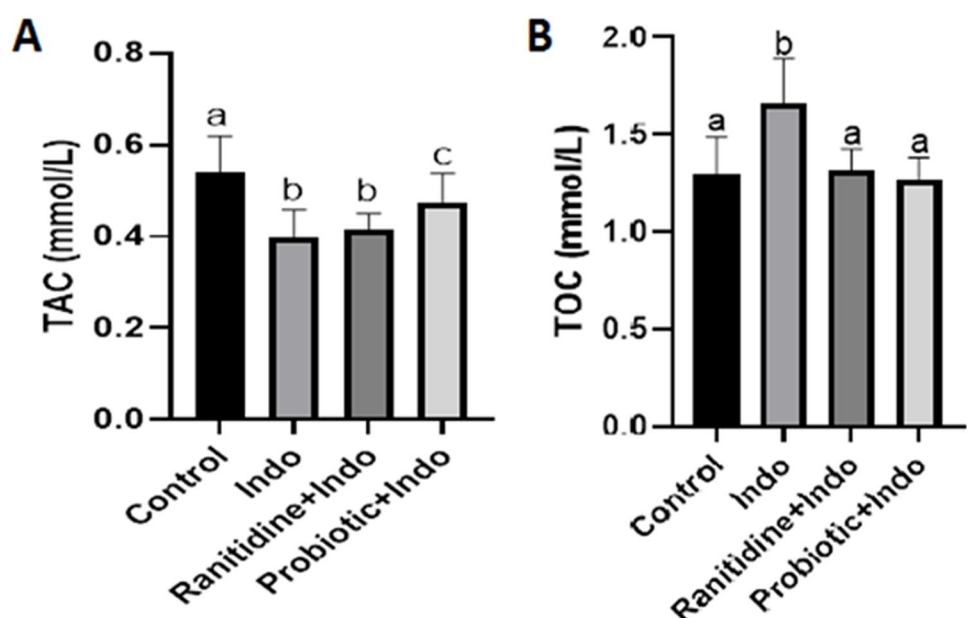
All data were statistically evaluated by one-way ANOVA using SPSS 20.00, followed by Duncan *Post hoc* test. The data were expressed as mean \pm SD. $P < 0.05$ was considered statistically significant.

Results

Effect of probiotic bacteria on oxidative stress

The TAC values were significantly lower in the Indo group compared with the control group. The Probiotic+Indo group's values were lower than the control group but higher than the Indo group ($P < 0.05$), Fig. 1A. This suggests that the application of probiotic bacteria activates the antioxidant defense system. When the TOC values were compared among the groups, there was a significant increase in the Indo group compared to the other groups ($P < 0.05$), Fig. 1B. This shows that probiotic bacteria treatment also reduces oxidative stress.

Fig. 1 Illustration of levels of oxidative parameters (TAC and TOC) for all groups in the gastric tissues. (A); TAC level, (B); TOC level, the letters indicate the statistical differences among groups ($P < 0.05$, $n = 10$), the results were expressed as mean \pm SD



Effect of probiotic bacteria on inflammation

IL-6 levels were compared among groups, it was observed that indomethacin treatment significantly increased compared to other groups ($P < 0.05$).

IL-8 levels were compared among groups, it was observed that the indomethacin-treated group had significantly increased values compared to control and Probiotic+Indo groups ($P < 0.05$). This shows that the administration of probiotics causes a decrease in IL-8 levels.

IL-1 β levels were compared among the groups, it was observed that the indomethacin-treatment group experienced an increase compared to the other experimental groups, but this was not statistically significant ($P > 0.05$).

There was, however, a significant increase in the indomethacin-treated group compared to the other experimental groups when the levels of TNF- α were compared ($P < 0.05$). We found a significant decrease in ranitidine and probiotic groups.

COX-2 levels were significantly lower in the Indo group compared to the control group ($P < 0.05$) (See Table 1).

Macroscopic findings of indomethacin-induced gastric mucosal injury

Macroscopic photograph of the excised stomach is shown in Fig. 3. Macroscopic findings revealed a normal structure of the gastric mucosa in the control group (Fig. 3A). The Indo group had severe mucosal injuries and the largest ulcer area (Fig. 3B). The Ranitidine+Indo (Fig. 3C) and Probiotic+Indo (Fig. 3D) groups showed fewer gastric erosions or ulcers compared with the indomethacin group.

Fig. 3 Representative macroscopic findings of the stomachs in Control group (A), Indomethacin group (B), Ranitidine + Indo group (C), and Probiotic + Indo group (D) rats



Microscopic evaluation of excised stomach

In the histopathologic analysis, the stomach sections of the control group revealed normal histologic structure (Fig. 4A). However, in the same section of the Indo group, there were active chronic gastritis findings in the rat gastric mucosa, consistent with the macroscopic appearance of chronic gastric erosion. Common mononuclear inflammatory cells were observed as foci between the lamina propria and submucosa. Widespread necrosis, with loss of surface epithelium and submucosal edema, was seen (Fig. 4B). However, fewer lesions were visible in the gastric mucosa in the Ranitidine and probiotic treatment groups. Histological examination indicated that treatment with Ranitidine or probiotics promoted the healing of gastric lesions, with the base of the ulcer covered by regenerating mucosa and fewer inflammatory cells. The results showed that the group receiving probiotics exhibited lower gastric erosion and better efficiency than the Ranitidine + Indo group (Fig. 4C, D).

Immunohistochemical analysis

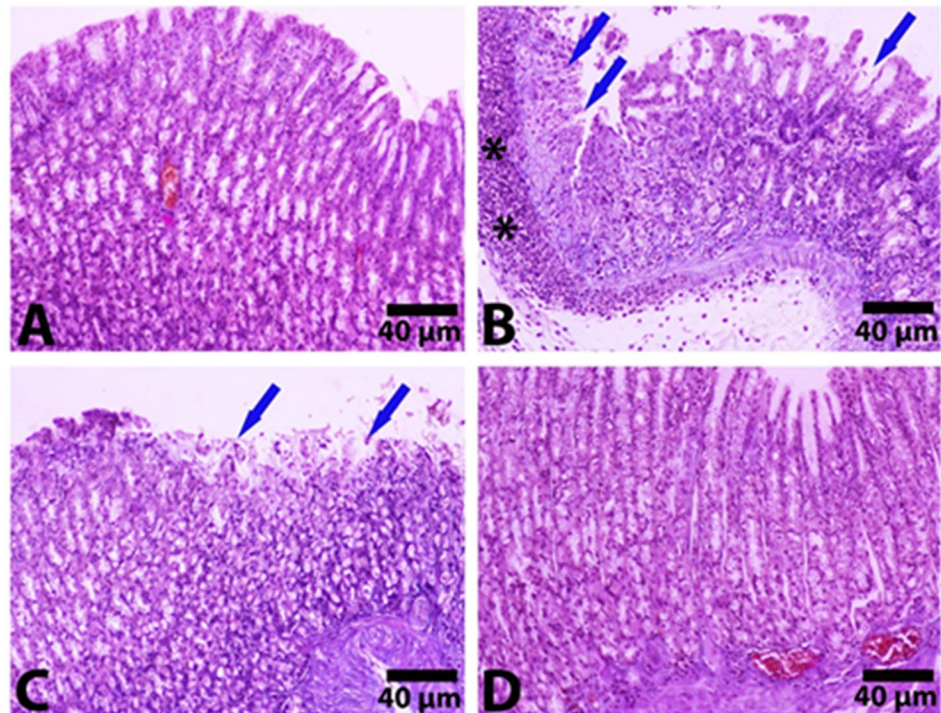
The representative images of Bax and Bcl-2 immunoreactivity are depicted in Fig. 5. Immunohistochemistry revealed

that Bax and Bcl-2 immunoreactive products presented as brown-reddish fine granules, located in the cytoplasm. Minimal expression of Bax and massive expression of Bcl-2 protein were observed in the control group, but Bax expression increased and Bcl-2 protein expression decreased in the section of Indo group. Furthermore, immunopositivity of Bax was significantly decreased in the Ranitidine + Indo and Probiotic + Indo groups compared with the Indo group. Moreover, the immunopositivity of Bax in the Probiotic + Indo group was lower than in the Ranitidine + Indo group. Immunopositivity of Bcl-2 was significantly increased in the Ranitidine + Indo and Probiotic + Indo groups compared with the Indo group, and immunopositivity of Bcl-2 in the Probiotic + Indo group was higher than Ranitidine + Indo group (Fig. 5). The positive cell intensity of Bax and Bcl-2 in the experimental groups is shown in Table 3.

Discussion

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs worldwide due to their anti-inflammatory and analgesic effects [32]. Indomethacin is used in a group of NSAIDs. However, indomethacin

Fig. 4 Micrograph of stomach section of the Control (A), Indomethacin (B), Ranitidine + Indo (C), and Probiotic + Indo (D) groups. **asterisks:** Mononuclear inflammatory cells; **blue arrows:** Widespread necrosis with loss of surface epithelium. **Stain:** Crossman's modified Mallory triple staining



use causes extensive and severe erosions and ulcers in the gastric mucosa [33]. Free oxygen radicals, lipid peroxide production, and inflammation play an important role in the formation of gastric mucosal lesions originating from indomethacin [34]. Biochemical and immunohistochemical data obtained from this study indicate that probiotic bacteria have anti-inflammatory, antioxidant, and antiapoptotic effects on indomethacin-induced gastric mucosa damage. Oxidative stress has a significant impact on the pathophysiology of indomethacin-induced gastric injury. Previous studies have shown that indomethacin changes the amount of lipid peroxidation and superoxide dismutase (SOD). In addition, indomethacin-induced gastric mucosal damage has been reported to be associated with enzyme activity, such as catalase and glutathione peroxidase [35]. For these reasons, the use of substances that can increase the activity of antioxidant enzymes and reduce oxidative stress is an important approach to protecting gastric mucosa from the effects of indomethacin.

In our study, according to histopathological evaluations, the rats in the group exposed to indomethacin had more ulcers in their stomachs, while the probiotic bacteria group had reduced and more superficial ulcers. This important result provides good evidence for the protective effect of probiotic bacteria on the gastric mucosa. Our research, combined with that previously published, confirms that indomethacin-induced gastric injury is prevented as a result of the application of various substances with antioxidant and anti-inflammatory [35, 36]. For example, the antioxidant

effect of selenium is well known, and its curative effect on gastric mucosal oxidative stress has been previously reported [36]. In another study, the antioxidant properties and healing effect of L-carnitine on gastric mucosal damage were determined [37].

Other research [38] has reported that grape seeds have protective and healing properties on indomethacin-induced gastric damage, and this effect is achieved by an increase in GSH levels. In addition, Kim et al. found that the application of selenium caused GSH levels to rise and the MDA level to drop [39]. In our study, we determined that TAS increases the level of TOS compared to the indomethacin group in this group. These findings suggest that probiotic bacteria increase antioxidant activity and suppress oxidative stress, inhibiting indomethacin-induced gastric mucosal damage.

Changes in the concentration of local inflammatory mediators (proinflammatory cytokines) such as IL-16, TNF- α , IL-1B, IL-8, and COX-2 are associated with this NSAID. The role of proinflammatory cytokines in the pathogenesis of gastric damage in the cellular signaling pathways is still being researched [40]. Cytokine secretion is the mediator of inflammation and contributes to the pathogenesis of tissue injury [41, 42]. It has been reported that proinflammatory cytokines are associated with a significant increase in serum IL-16, TNF- α , IL-1B, IL-4, IL-10, and COX-2 levels following indomethacin treatment in rats [43, 44]. Previous studies reported the inhibitory effect of probiotic bacteria on IL-1 α , TNF- α , and IL-6 [44]. In the current study,

Fig. 5 Immunohistochemical staining for the Bax and Bcl-2 for the Control, Indomethacin, Ranitidine + Indo, and Probiotic + Indo groups. Streptavidin–biotin peroxidase staining

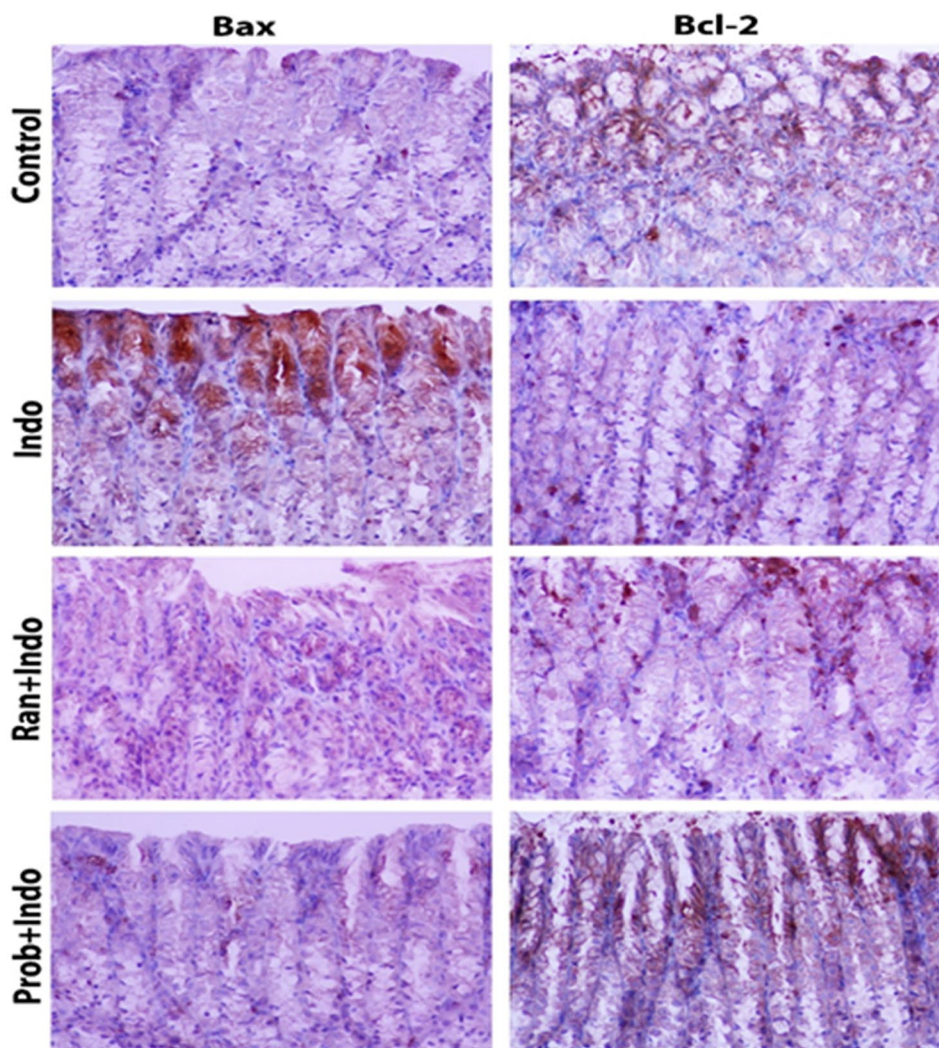


Table 3 Immunohistochemical findings and their scores in stomach tissue

Groups	Bax	Bcl-2
Control Group	-	++
Indomethacin Group	+++	+
Ranitidine + Indo Group	++	++
Probiotic + Indo Group	+	+++

According to immunohistochemical findings: none (-), mild (+), moderate (++) and severe (+++)

indomethacin treatment markedly increased TNF- α , IL-1 β , and IL-6 levels. Conversely, probiotic bacteria treatment caused a significant reduction in TNF- α , IL-8, and IL-6 levels in indomethacin-administered experimental rats. The pathophysiology of gastric ulcer is influenced by alterations in cytokines, PGE2 production, and the COX enzyme, which is crucial to the inflammatory process [45]. These are especially crucial when NSAIDs like indomethacin cause stomach ulcers [45, 46]. Research indicates that the use

of NSAIDs worsens ulcers by inhibiting the COX-1 and COX-2 enzymes, which reduces PGE2 generation [47–49]. In our study, COX-2 levels were lower in indomethacin-treated groups than in control groups, and probiotic bacteria significantly prevented decreases in COX-2 levels; these results were in agreement with previous research [50, 51]. This is possibly owing to its anti-inflammatory properties. In light of these findings, it can be concluded that the application of probiotic bacteria prevents indomethacin-induced inflammation in the gastric mucosa.

Gastric ulcers are a common multiplex disease. Pathogenesis is closely related to apoptosis in gastric mucosal epithelial cells. Bcl-2 regulation is one of the key factors affecting cell apoptosis. Bcl-2 and Bax proteins are important representatives of the Bcl-2 family and play a major role in determining cell life [52]. When the expression of the Bax protein is increased, apoptosis can be induced. In contrast, however, when the Bcl-2 protein is increased, apoptosis is suppressed. In previous research on Bcl-2

protein, when acute gastric mucosal damage was repaired, Bax expression was reported to be reduced. In this study, we found that Bax expression decreased in the gastric tissues of the group treated with probiotic bacteria compared to the indomethacin-administered group, whereas Bcl-2 expression was increased. This suggests that probiotic bacteria inhibit indomethacin-induced apoptosis. Probiotic treatment can mitigate gastric damage and apoptosis caused by indomethacin-induced gastric damage in rats. Probiotics (*Lactobacillus rhamnosus*, *Lactobacillus fermentum*, and *Lactobacillus brevis*) also enhance the restoration of biochemical oxidative enzymes as it has anti-inflammatory, antioxidant, and antiapoptotic properties. Further studies are warranted to investigate its future clinical applications.

Author contributions V.G. E.Ş. S.G. M.M. S.U.G.: experiment design, experiment application, samples collection. V.G. M.M.: serum markers and tissue antioxidant estimation, data curation and analysis, final reviewing. S.G.: histopathological and immunohistochemical investigation. All authors contributed to the writing and editing, and they read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability The authors confirm that the data and materials supporting the findings of this study are available within the article.

Declarations

Ethical approval The study was designed and conducted according to ethical norms approved by the Kafkas University Animal Experiments Local Ethics Committee (Kars, Turkey), (KAÜ-HADYEK/2017/076).

Consent to participate All authors voluntarily participated in this research study.

Consent to publish All authors have consent for the publication of the manuscript.

Competing interests The authors declare no competing interests.

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