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Original Article

Exploring the potential effects of *Lactococcus lactis* D4 on the proliferation, apoptosis, and inflammatory responses in colorectal cancer cells

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Abstract

Lactococcus lactis D4 is a probiotic produced through the fermentation of buffalo milk in bamboo, namely "*dadih*", a traditional food from West Sumatera, Indonesia. To the best of our knowledge, no specific research has investigated the effects of *L. lactis* D4, derived from *dadih* extraction, on colorectal cancer or its potential clinical applications. Therefore, the aim of this study was to evaluate the potential of *L. lactis* D4 from *dadih* to inhibit colorectal cancer growth in rat models, with a focus on its effects on cell proliferation, apoptosis, and inflammatory responses. An in vivo study was conducted using 37 male Sprague-Dawley rats, allocated into five groups: (1) control (no treatment), (2) dysplasia (induced with 1,2-dimethylhydrazine until dysplasia developed), (3) dysplasia + *L. lactis* D4 (induced with 1,2-dimethylhydrazine, then treated with *L. lactis* D4 after dysplasia confirmation), (4) cancer (induced with 1,2-dimethylhydrazine until cancer was confirmed), and (5) cancer + *L. lactis* D4 (induced with 1,2-dimethylhydrazine until cancer was confirmed, then treated with *L. lactis* D4 for 15 days). The effects of *L. lactis* D4 on cancer progression were assessed through immunohistochemical analysis of cell proliferation (cyclin D1, Bcl-2), apoptosis (p53, caspase-3), and inflammation (nuclear factor-κB (NF-κB) and cyclooxygenase-2 (COX-2)). This study found that *L. lactis* D4 treatment reduced adenocarcinoma and dysplasia severity in colorectal cancer models through significant reduction in cyclin D1, Bcl-2, NF-κB, and COX-2 expression observed across all groups ($p<0.01$), although changes in dysplasia and cancer subgroups were not statistically significant ($p>0.05$). No statistically significant change was noted in p53 expression ($p=0.518$), whereas caspase-3 expression varied significantly across groups ($p=0.010$). In conclusion, *L. lactis* D4 reduces the expression of cyclin D1, Bcl-2, NF-κB, and COX-2 proteins, offering insights into its potential to modulating proliferation and inflammation in colorectal cancer growth.

Keywords: *Lactococcus* sp., probiotic, *dadih*, dysplasia, carcinogen

Introduction

Colorectal cancer is the third most prevalent malignancy and the fourth leading cause of cancer-related mortality worldwide [1]. The global incidence of colorectal cancer is expected to increase

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by 60%, with over 2.2 million new cases and 1.1 million deaths by 2030 [1]. According to GLOBOCAN 2022, colorectal cancer is the fourth most common malignancy in Indonesia, with an incidence rate of 12.1% and a mortality rate of 6.6% [2]. The etiology of colorectal cancer is classified into genetic (hereditary) and sporadic (non-hereditary) factors, with approximately 70% of cases occurring sporadically [3,4]. Dietary factors associated with an elevated cancer risk include excessive consumption of red and processed meats, inadequate fiber intake, and frequent alcohol consumption [4].

The pathogenesis of sporadic colorectal cancer involves complex and diverse mechanisms [4]. Environmental factors contributing to colorectal cancer may also accelerate the accumulation of somatic mutations [3,4]. The gut microbiota plays a critical role as a mediator between carcinogenic triggers and the stages of tumor development [5]. Various therapeutic modalities have been developed to modulate gut microbiota, aiming to regulate factors that contribute to colorectal cancer [6]. Therapeutic strategies for microbiota manipulation include dietary interventions (prebiotics), fecal microbiota transplantation, and the administration of beneficial bacterial strains (probiotics) [6]. These approaches offer a potential avenue for integrating probiotic-based therapies into comprehensive colorectal cancer management, with the goal of improving patient outcomes [5,6].

A variety of probiotic-containing products exist, including fermented milk. *Dadih* is a traditional food from West Sumatra, Indonesia, produced through the natural fermentation of buffalo milk in bamboo tubes by lactic acid-producing microorganisms naturally present in the milk [7-11]. This fermented milk is widely produced in regions such as Batusangkar, Alahan Panjang, Padang Panjang, and Agam, West Sumatera, Indonesia. It contains several lactic acid bacteria, including *Lactococcus, Lactobacillus*, and *Leuconostoc* [7-11]. In *dadih*, *Lactococcus lactis* D4 was identified and found to produce nisin metabolites [9], which have demonstrated anti-cancer properties [9,12,13]. Previous studies have shown that probiotic *Lactococcus* sp. and *dadih* exert cytotoxic effects on cancer cells [7,14,15]. One of the previous studies demonstrated that *L. lactis* has demonstrated stronger anti-cancer activity than *L. plantarum* against colon cancer (HT29), cervical cancer (HeLa), gastric cancer (AGS), and breast cancer (MCF-7) cell lines [15]. The anti-cancer effect was attributed to the cytotoxicity of metabolites produced by *L. lactis*, which can induce apoptosis in cancer cells.

Beyond its anti-cancer properties, *L. lactis* has also demonstrated anti-proliferative and anti-inflammatory activities. It has been shown to effectively suppress the growth of gastric cancer cells (SNU-1) [16-20]. To the best of our knowledge, no specific research to date has investigated the effects of *L. lactis* D4, derived from *dadih* extraction, on colorectal cancer or its potential clinical applications. Therefore, the aim of this study was to assess the effect of *L. lactis* D4 on colorectal cancer, with a focus on its effects on cell proliferation, apoptosis, and inflammatory responses.

Methods

Study design and setting

An in vivo study with a post-test-only with control group design was conducted at INA LAB, a private laboratory located in Padang, Indonesia. The study was conducted over 14 weeks, including animal intervention and immunohistochemical analysis at the Laboratory of Pathology, Universitas Andalas, Padang, Indonesia. The study employed male Sprague-Dawley rats (*Rattus norvegicus*) and aged 6–7 weeks. The rats were allocated into five groups: (1) control, which received no treatment; (2) dysplasia, which was induced with carcinogen 1,2-dimethylhydrazine (1,2-DMH) until colorectal dysplasia developed; (3) dysplasia + *L. lactis* D4, which was induced with 1,2-DMH and subsequently treated with *L. lactis* D4 following colorectal dysplasia confirmation; (4) cancer, which was induced with 1,2-DMH until colorectal cancer was confirmed; and (5) cancer + *L. lactis* D4, which was induced with 1,2-DMH until colorectal cancer was confirmed and then treated with *L. lactis* D4 for 15 days. Additionally, 12 rats were euthanized for colorectal cancer detection and confirmation. The effects of *L. lactis* D4 on cancer progression were assessed through immunohistochemical analysis of cell proliferation (cyclin D1 and Bcl-2),

apoptosis (p53 and caspase-3), and inflammation (nuclear factor-κB (NF-κB) and cyclooxygenase-2 (COX-2)).

Animal criteria, randomization, and allocation

The inclusion criteria were male rats aged six to seven weeks, with a body weight of 170–220 grams, and confirmed to have colon tumors. Exclusion criteria included significant weight loss, clinical signs of illness, or anatomical abnormalities. Sample size for this study was determined based on World Health Organization (WHO) guideline [21], which required a minimum of five rats per experimental group. Additionally, the sample size calculation was performed using Federer's formula $(t-1)(n-1)\geq 15$, yielding $n\geq 4.75$, which was rounded up to five animals per group. To facilitate tumor progression analysis, an additional 12 animals were included, resulting in a total of 37 male Sprague-Dawley rats. Randomization was conducted by allocating each rat to its respective treatment group using a simple random sampling technique. The assignment of both experimental and detection rats was randomized to minimize bias in the study.

Probiotic strain *Lactococcus lactis* D4 preparation

The probiotic strain *L. lactis* D4 was isolated from *dadih*, a traditional fermented buffalo milk product from West Sumatra, Indonesia. This strain was initially isolated and characterized by Sukma *et al.* [9,10]. It was subsequently reproduced at the Faculty of Animal Science, Universitas Andalas, Padang, Indonesia. *L. lactis* D4 was prepared by inoculating 10% (v/v) sterilized clear de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Hampshire, UK) and incubated under aerobic condition at 30°C for 24 hours, yielding a viability of 10^9 colony forming unit (CFU)/mL. Fresh inoculation was performed daily. Prior to experimental use, *L. lactis* D4 underwent two culturing rounds: the first ensured strain homogeneity and the absence of contaminants, while the second established a pre-test culture to confirm active bacterial growth. Identification of *L. lactis* D4 was verified using 16S ribosomal ribonucleic acid (rRNA) genome sequencing. The probiotic strain was harvested by centrifugation at 4°C at 10,000 rpm for ten minutes, separating the bacterial cells (pellets) from the medium (supernatant). The pellets were subsequently resuspended in 200 µL NaCl. The probiotic isolate dose of 10^9 CFU/mL in a volume of 0.5 mL was determined based on a comprehensive literature review [22-25] and consultation with the pharmacology team at Universitas Andalas, Padang, Indonesia.

Animal preparation

Sprague-Dawley rats were obtained from INA LAB laboratory, Padang, Indonesia. The rats underwent a seven-day acclimatization period to adapt to the housing environment, thereby standardizing living conditions and diet prior to the experiment. During this period, the rats were housed in cages, with bedding changed three times a week. Additionally, the number of rats was matched to the size of the cages. The cages were kept in an area not exposed to direct light, with ambient temperature maintained at approximately 25°C and humidity at 50%. Food and water were provided ad libitum throughout the acclimatization process.

Study procedures

At the beginning of the second week, the rats were assigned to groups and to minimize potential bias, blinded randomization was applied whenever grouping or selection of rats was required. Five rats were assigned to the non-induction group (control group), while 32 rats (20 for the experiment as well as 12 for colorectal dysplasia and cancer detection and confirmation) were induced with the carcinogen 1,2-DMH (TCI, Tokyo, Japan) at a dose of 30 mg/kg body weight via intraperitoneal injection in the left regio-pelvic. The carcinogen was administered weekly, starting on the first day of the second week and continuing until the eleventh week.

At the end of the fifth week, four rats were randomly selected and euthanized by cervical dislocation. Following euthanasia, laparotomy was performed with a mid-line incision in the rat abdomen to remove the colorectal tissues. Procto-colectomy was then conducted, and the colorectal tissues were sectioned to observe any lesions. These tissues were prepared for histopathological examination to identify signs of cancer growth. The tissues were processed into paraffin blocks, sectioned to a thickness of 4 µm, and stained with hematoxylin and eosin (HE) for tumor confirmation by a pathologist. The pathologist microscopically assessed the samples to

confirm the presence of dysplasia or cancer based on the tissue's morphological characteristics. Since the mild dysplasia was observed, the rats were randomly assigned to two dysplasia groups (dysplasia and dysplasia + *L. lactis* D4), with five rats in each group. Carcinogen induction with 1,2-DMH continued, while the dysplasia + *L. lactis* D4 group received *L. lactis* D4 isolate at a concentration of 10^9 CFU/mL, administered rectally at a volume of 0.5 mL once daily, until the remaining rats selected for detection showed evidence of colorectal cancer.

Tissue examinations were conducted at the eighth and eleventh weeks to monitor cancer progression. Once colorectal cancer was histopathologically confirmed, carcinogen induction was discontinued, and the rats in the dysplasia and dysplasia + *L. lactis* D4 groups were euthanized. The remaining rats were randomly assigned to two cancer groups (cancer and cancer + *L. lactis* D4), with five rats in each group. The cancer + *L. lactis* D4 group received *L. lactis* D4 isolate at a concentration of 10^9 CFU/mL, administered rectally at a volume of 0.5 mL once daily for 15 days. After 15 days, all rats were euthanized, and colorectal tissues were collected for further analysis.

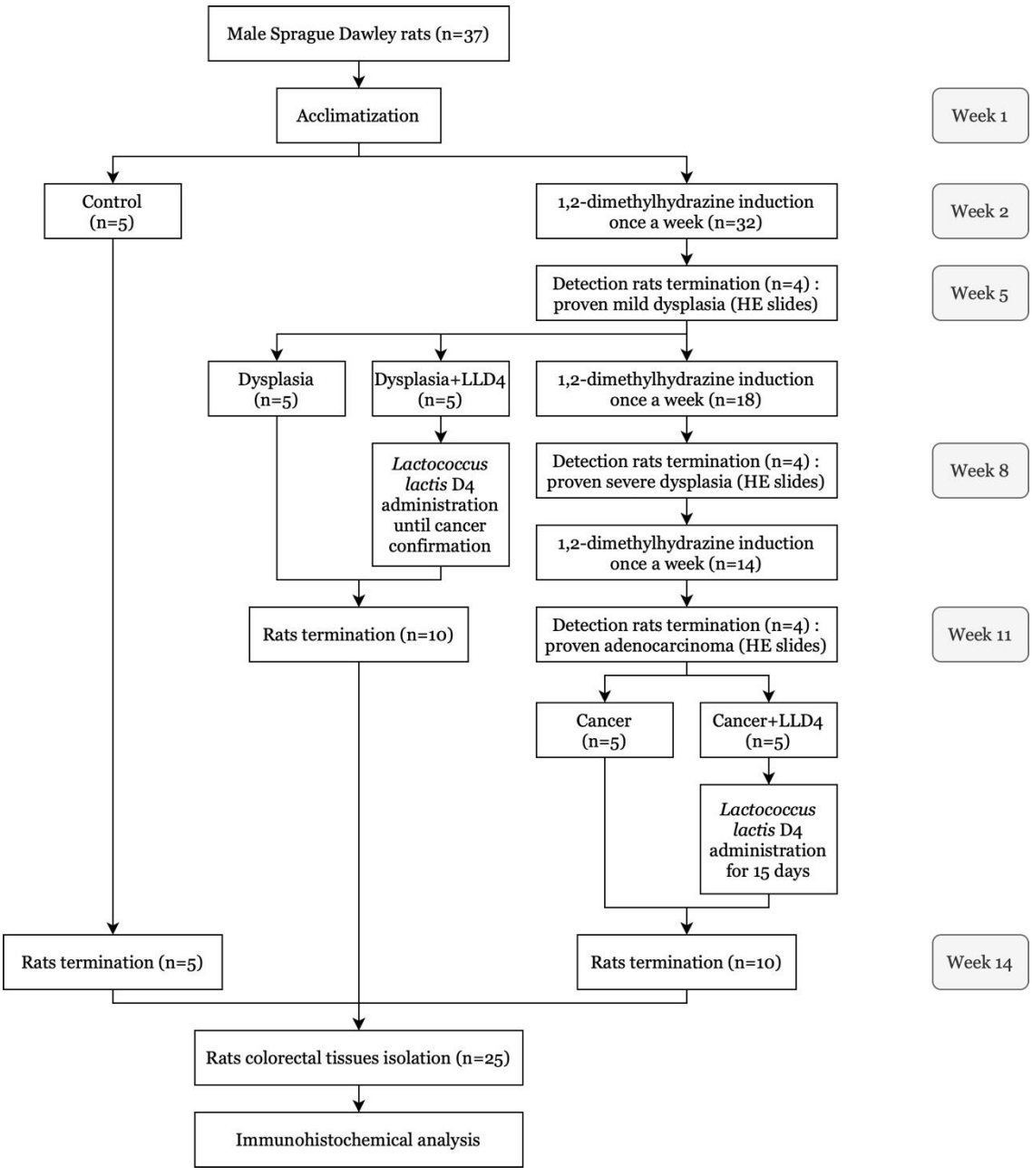

Figure 1. Study flowchart. HE: hematoxylin and eosin; LLD4: *Lactococcus lactis* D4.

Histopathological sample preparation and hematoxylin-eosin staining

Following laparotomy, intestinal samples, including the entire colon and rectum, were collected. These samples were examined for macroscopic changes, such as the presence of lumps and the tissues were immediately preserved in 10% neutral-buffered formalin solution for 48 hours, as per histopathological procedures, to facilitate subsequent histopathological and immunohistochemical examination. The samples were then transported to the Laboratory of Pathology, Universitas Andalas, Padang, Indonesia for further analysis. The tissues were processed into paraffin blocks, sectioned to a thickness of 4 µm, and stained with HE for examination.

Immunohistochemical staining

Paraffin-embedded tissue sections were cut to a thickness of 4 µm and mounted on poly-L-lysine-coated glass slides. The tissue sections underwent a series of deparaffinization and rehydration steps using xylene and graded ethanol solutions. Epitope retrieval was performed in a microwave with citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for three minutes, followed by 0.3% hydrogen peroxide for 30 minutes. After additional PBS washes, non-specific protein binding was blocked with 2% normal goat serum for 20 minutes at room temperature.

Primary antibodies, including cyclin D1 (Bioenzy, East Jakarta, Indonesia), Bcl-2 (Elabscience, Houston, TX, USA), p53 (Santa Cruz, Dallas, TX, USA), caspase 3 (Elabscience, Houston, TX, USA), NF-κB (Santa Cruz, Dallas, TX, USA), and COX-2 (Elabscience, Houston, TX, USA), were applied and incubated overnight in a moisture-controlled environment at 4°C. The tissue was then exposed to a biotinylated secondary antibody (Vector Laboratories, Newark, CA, USA), followed by an avidin-biotin complex (Vector Laboratories, Newark, CA, USA). The chromogen 3,3'-diaminobenzidine (DAB) (Dojindo Laboratories, Kumamoto, Japan) in Tris-HCl buffer (pH 7.6) was applied, and the tissue was counterstained with hematoxylin. Finally, the tissue was dehydrated, cleared, and mounted with Entellan.

Protein expression of cyclin D1, Bcl-2, caspase 3, NF-κB, and COX-2 was detected by immunohistochemistry using the avidin-biotin complex system with horseradish peroxidase and DAB as the chromogen. The expression levels of each protein were quantitatively examined by utilizing the area proportion measurement tool in the ImageJ 1.49v program (National Institutes of Health, Bethesda, MD, USA) to determine the ratio of the stained area (fraction-stained area). The percentage of positive staining was calculated based on the total area. The assessment of p53 protein expression was conducted by enumerating the number of positively stained cells within the cell nucleus. The intensity of positive expression was quantified as the number of stained cells in the nucleus per unit area.

Statistical analysis

Shapiro-Wilk test was used to assess the normality of data distribution. Variables with normally distributed data were analyzed using one-way ANOVA followed by post-hoc LSD testing. For non-normally distributed data, the Kruskal-Wallis and Mann-Whitney tests were employed. The significance level was set at $p<0.05$ with a 95% confidence interval (95%CI). Normally distributed data were presented as the mean ± standard deviation (SD), while non-normally distributed data were reported as the median (min–max). Data analysis was conducted using SPSS Version 23.0 (IBM, Armonk, NY, USA).

Results

Colorectal dysplasia and cancer confirmation in animal models

Four rats were euthanized at weeks 5, 8, and 11 to assess colorectal dysplasia and cancer progression following induction with 1,2-DMH. Colon tissue sections from the rats, stained with HE, were used for histological analysis. The control group, which consisted of rats that received no treatment, showed normal colon mucosa with a smooth surface, lined by a single layer of monomorphic columnar epithelium (**Figure 2** (**A1** and **A2**)). In the dysplasia group, where rats were treated solely with 1,2-DMH until dysplasia occurred, pre-neoplastic lesions, identified as

aberrant crypt foci (ACF), were observed (**Figure 2** (**B1** and **B2**)). These ACF were characterized by tall crypts, thicker epithelial lining, and an expanded peri-cryptal zone compared to normal tissue. Additionally, ACF demonstrated pre-neoplastic features such as dysplasia and hyperproliferation (**Figure 2** (**B1** and **B2**)). Lymphonodular hyperplasia extending from the submucosa to the mucosa was found in two samples, while lymphonodular hyperplasia in the serosa was observed in one sample within this group.

In the dysplasia + *L. lactis* D4 group, where rats were treated with 1,2-DMH until dysplasia occurred and then administered *L. lactis* D4, ACF lesions were identified (**Figure 2** (**C1** and **C2**)). Furthermore, two samples revealed adenomas with varying degrees of dysplasia, ranging from mild to severe. Lymphonodular hyperplasia extending from the submucosa to the mucosa was observed in three samples (**Figure 2** (**C1** and **C2**)). In the cancer group, where rats were treated solely with 1,2-DMH until colorectal cancer developed, adenocarcinoma was detected, characterized by severely dysplastic and infiltrative epithelial cells extending into the lamina propria and muscularis (**Figure 2** (**D1** and **D2**)). In the cancer + *L. lactis* D4 group, where rats were treated with 1,2-DMH until colorectal cancer developed and then administered *L. lactis* D4, adenocarcinoma was also identified (**Figure 2** (**E1** and **E2**)).

Effect of *Lactococcus lactis* D4 administration on colon cancer cell proliferation: Evaluation of cyclin D1 and Bcl-2 protein expressions

Cyclin D1 protein expression in the colon epithelium was higher in both the dysplasia and cancer groups compared to the control group (**Table 1**). Administration with *L. lactis* D4 resulted in a lower mean expression of cyclin D1 in the cancer groups (cancer and cancer + *L. lactis* D4), though it was slightly higher in the dysplasia groups (dysplasia and dysplasia + *L. lactis* D4). Cyclin D1 protein expression was significantly different across all groups (p=0.005) (**Table 1**). In the dysplasia (p=0.917) and cancer (p=0.076) groups, *L. lactis* D4 administration did not show a statistically significant effect (**Figure 3A**). Cyclin D1 protein expression appeared as inhomogeneous brown staining in the mucosal tissue of the rat models (**Figure 4** (**A1**–**E1**)). Higher expression of cyclin D1 was observed in the cytoplasm of the dysplasia and cancer groups, particularly in the atypical epithelial cells of the intestinal mucosa. The treatment groups (dysplasia + *L. lactis* D4 and cancer + *L. lactis* D4) demonstrated lower cyclin D1 protein expression compared to the dysplasia and cancer groups (**Figure 4** (**A1**–**E1**)).

The colon epithelium in the dysplasia and cancer groups showed higher levels of Bcl-2 protein expression compared to the control group. *L. lactis* D4 administration resulted in lower mean Bcl-2 expression in both the dysplasia (dysplasia and dysplasia + *L. lactis* D4) and cancer (cancer and cancer + *L. lactis* D4) groups. Bcl-2 protein expression was significantly different across all groups (p=0.002) (**Table 1**). However, in the dysplasia (p=0.075) and cancer (p=0.175) groups, no statistically significant differences in Bcl-2 expression between treatments were found (**Figure 3B**). Bcl-2 expression was observed as inhomogeneous brown staining in the mucosal tissue of the rat models (**Figure 4** (**A2**–**E2**)). Strong cytoplasmic expression of Bcl-2 was observed in the dysplasia and cancer groups, particularly in the atypical epithelial cells of the intestinal mucosa. After *L. lactis* D4 administration, Bcl-2 expression was lower compared to the dysplasia and cancer groups.

Table 1. Effect of *Lactococcus lactis* D4 administration on cyclin D1 and Bcl-2 protein expression in colon cancer cells

Variable	Control	Dysplasia	Dysplasia + *L. lactis* D4	Cancer	Cancer + *L. lactis* D4	p-value
Cyclin D1	4.36 (2.23–6.89)	20.53 (13.55–23.66)	197.49 (9.55–27.74)	30.36 (20.09–39.62)	18.63 (15.77–28.74)	0.005[a*]
Bcl-2	3.75 (1.33–4.73)	23.82 (12.81–36.25)	21.42 (11.45–21.47)	46.27 (22.92–56.97)	27.33 (17.08–38.51)	0.002[a*]

[a]Analyzed using Kruskal-Wallis test
[*]Statistically significant at p<0.05

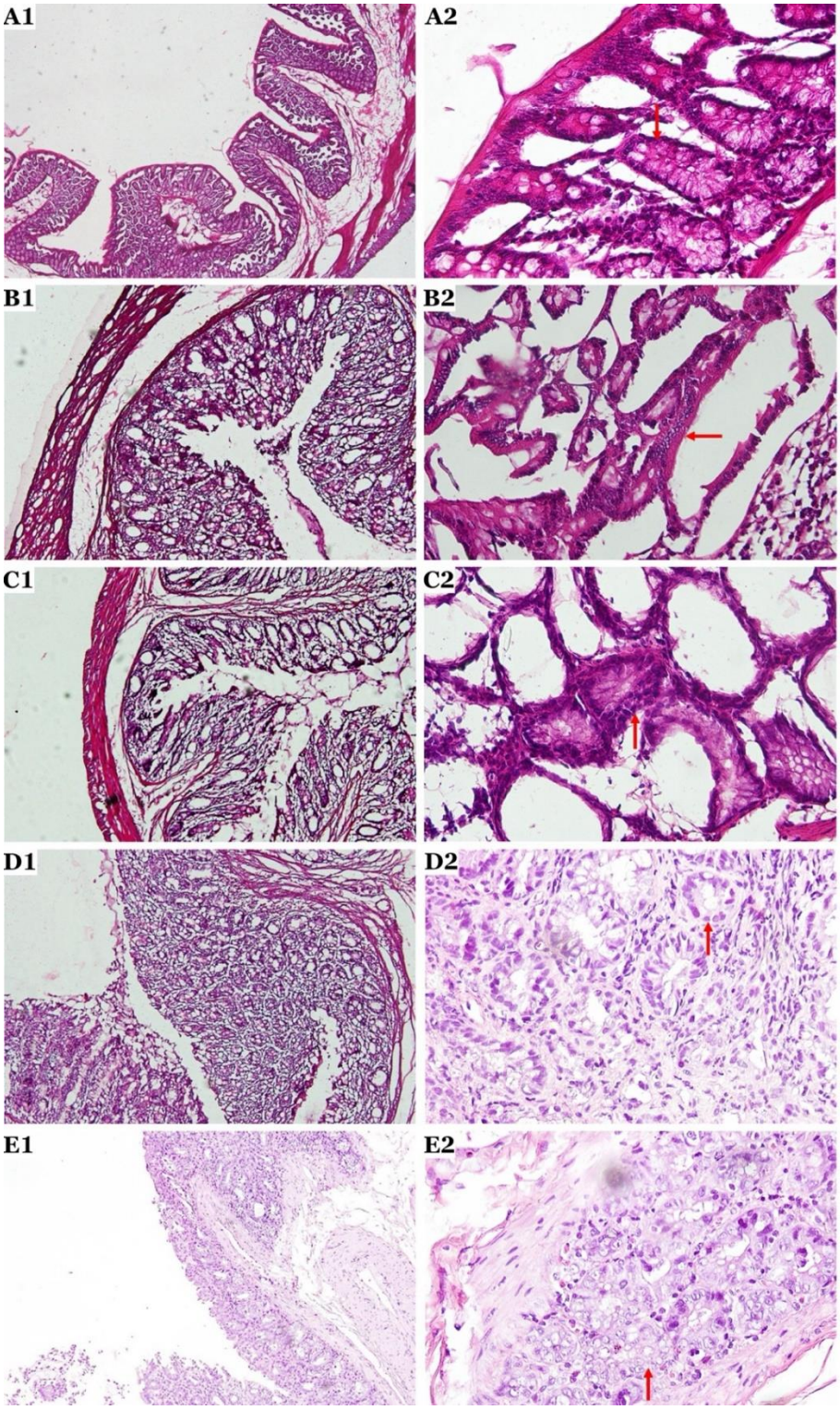

Figure 2. Morphology of rat colon tissue stained with hematoxylin and eosin. (A1 and A2) Control group: A1 shows normal mucosa with a smooth surface and a single layer of columnar epithelium (100×); A2, higher magnification (400×), highlights the same features (red arrow). (B1 and B2) Dysplasia group: B1 shows aberrant crypt foci (ACF) with tall crypts, thickened epithelium, and expanded peri-cryptal zone (100x); B2 reveals dysplasia and hyperproliferation in ACF (400×, red arrow). (C1 and C2) Dysplasia + *Lactococcus lactis* D4 group: C1 shows ACF indicating pre-neoplastic lesions (100×); C2 identifies two adenomas with mild to severe dysplasia and lymphonodular hyperplasia (400×, red arrow). (D1 and D2) Cancer group: D1 reveals adenocarcinoma with severely dysplastic and infiltrative epithelial cells (100×); D2 shows tumor invasion into lamina propria and muscularis (400×, red arrow). (E1 and E2) Cancer + *L. lactis* D4 group: E1 shows infiltrative adenocarcinoma extending into deeper tissues (100×); E2 confirms the invasion into lamina propria and muscularis (400×, red arrow).

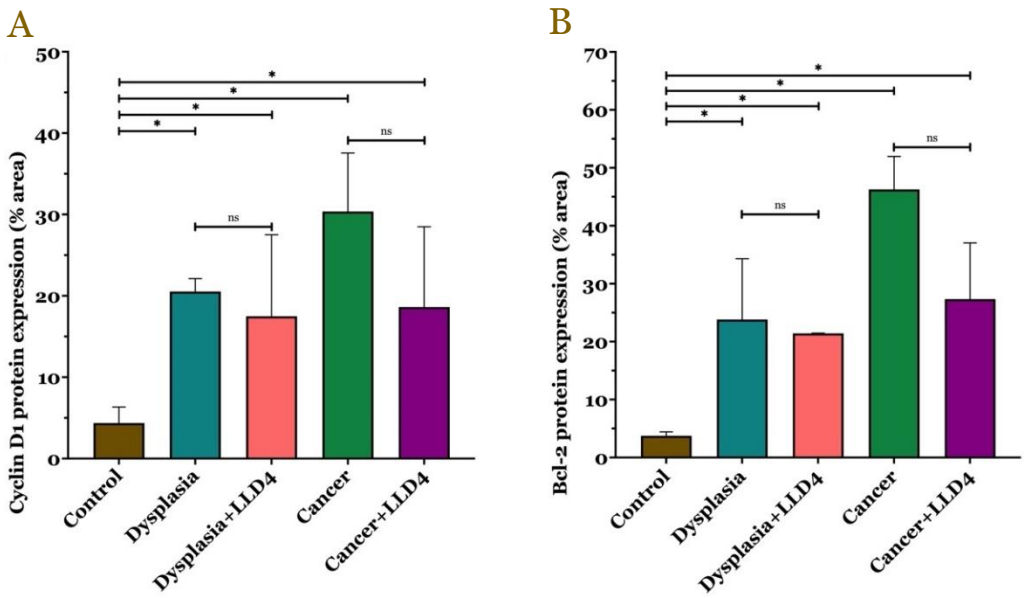

Figure 3. Effects of *Lactococcus lactis* D4 (LLD4) on colon cancer cell proliferation, as measured by cyclin D1 (A) and Bcl-2 protein expression (B), were evaluated across groups. *Significant differences at $p<0.05$; ns: non-significant differences.

Effect of *Lactococcus lactis* D4 administration on colon cancer cell apoptosis: Evaluation of p53 and caspase 3 protein expressions

The proportion of p53-positive cells in the colon epithelium was higher in dysplasia and cancer groups compared to the control group. Treatment with *L. lactis* D4 increased mean p53 protein expression in both dysplasia (dysplasia and dysplasia + *L. lactis* D4) and cancer (cancer and cancer + *L. lactis* D4) groups. However, no significant effect was observed on p53 expression across all groups (p=0.518) (**Table 2**). Similarly, no statistically significant differences were observed in the dysplasia (p=0.395) and cancer (p=1.000) groups (**Figure 5A**). Immunohistochemical staining showed inhomogeneous brown staining for p53 in the mucosal tissue of experimental rat models (**Figure 6** (**A1**–**E1**)). Dysplasia and cancer groups demonstrated strong nuclear p53 protein expression, particularly in the atypical epithelium of the intestinal mucosa. p53 protein expression was higher in the treatment groups (dysplasia + *L. lactis* D4 and cancer + *L. lactis* D4) compared to the dysplasia and cancer groups (**Figure 6** (**A1**–**E1**)).

Caspase 3 expression in the intestinal epithelium was higher in dysplasia and cancer groups compared to the control group. Treatment with *L. lactis* D4 resulted in lower median caspase 3 expression in the cancer groups (cancer and cancer + *L. lactis* D4) but slightly higher expression in the dysplasia groups (dysplasia and dysplasia + *L. lactis* D4). Statistical analysis demonstrated a significant difference in caspase 3 expression across all groups (p=0.010) (**Table 2**). However, no statistically significant differences were observed between treatment groups in the dysplasia (p=0.600) and cancer (p=0.602) subgroups (**Figure 5B**). Immunohistochemical staining revealed inhomogeneous brown staining for caspase 3 in the mucosal tissues of experimental animals (**Figure 6** (**A2**–**E2**)). Dysplasia and cancer groups showed strong cytoplasmic caspase 3 expression, particularly in the atypical epithelium of the intestinal mucosa. Caspase 3 expression was reduced following *L. lactis* D4 administration compared to the dysplasia and cancer groups (**Figure 6** (**A2**–**E2**)).

Effect of *Lactococcus lactis* D4 administration on colon cancer cell inflammation: Evaluation of NF-κB and COX-2 protein expressions

The colon epithelium in dysplasia and cancer groups exhibited higher NF-κB protein expression than in the control group. Administration of *L. lactis* D4 resulted in a lower mean NF-κB expression in both the dysplasia (dysplasia and dysplasia + *L. lactis* D4) and cancer (cancer and cancer + *L. lactis* D4) groups. A significant overall effect was observed on NF-κB expression (p=0.007) (**Table 3**); however, no statistically significant differences were observed within the

dysplasia (p=0.175) or cancer (p=0.251) groups (**Figure 7A**). Immunohistochemical analysis demonstrated inhomogeneous brown staining of NF-κB in the mucosal tissues of the experimental animals (**Figure 8** (**A1**–**E1**)). Strong NF-κB expression was localized within the cytoplasm, particularly in the atypical epithelium of the intestinal mucosa in dysplasia and cancer groups. Following *L. lactis* D4 administration, NF-κB expression was reduced compared to the dysplasia and cancer group.

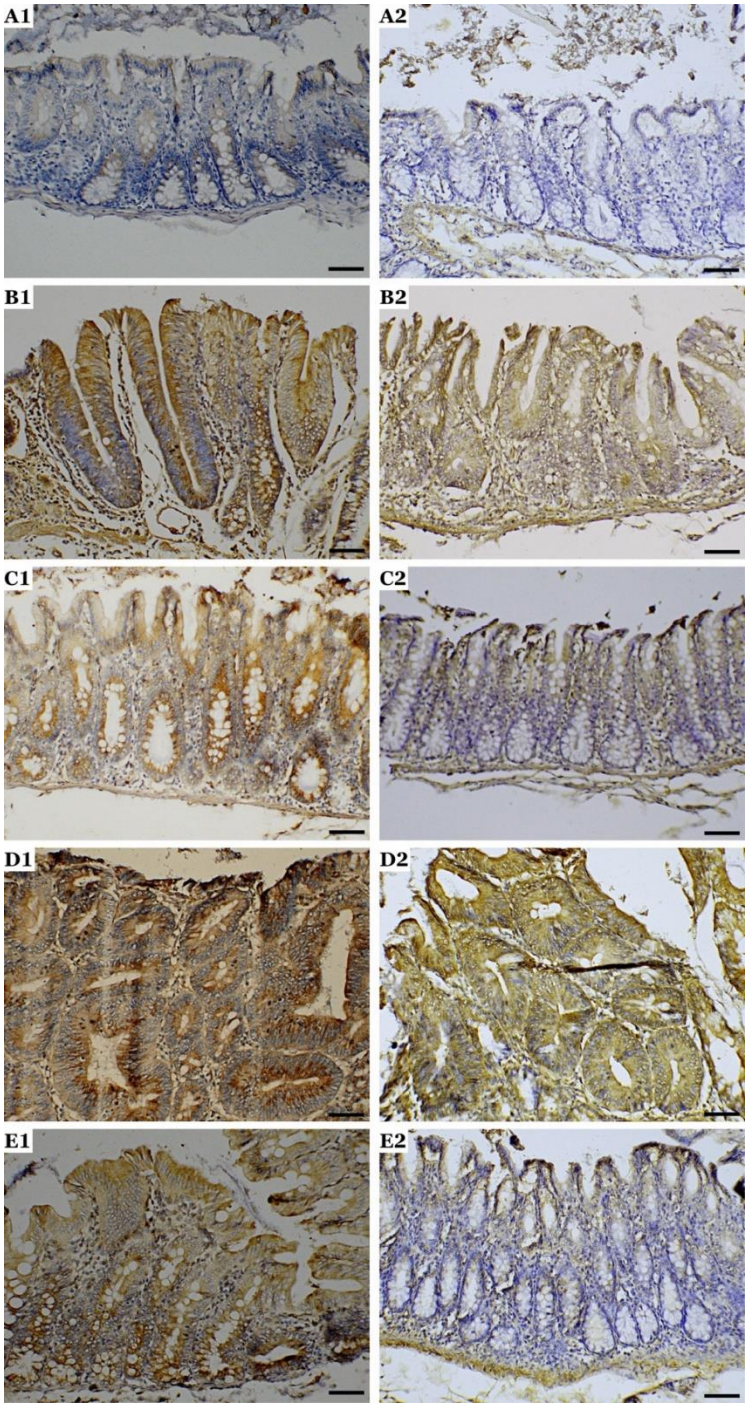

Figure 4. Immunohistochemical staining of cyclin D1 (A1–E1) and Bcl-2 (A2–E2) protein expression in rat colon tissue. (A1–E1) Cyclin D1 expression: A1–E1 represent control, dysplasia, dysplasia + *Lactococcus lactis* D4, cancer, and cancer + *L. lactis* D4 groups, respectively. Brown staining shows cyclin D1 localization. Higher expression was observed in carcinogen-induced tissues (B1 and D1), especially in atypical epithelium. Treatment with *L. lactis* D4 (C1 and E1) reduced cyclin D1 expression (scale bars=200 μm). (A2–E2) Bcl-2 expression: A2–E2 correspond to the same groups. Carcinogen-induced tissues (B2 and D2) showed increased intracytoplasmic Bcl-2 staining in atypical epithelium. Expression was lower in *L. lactis* D4-treated tissues (C2 and E2) (scale bars=200 μm).

Table 2. Effect of *Lactococcus lactis* D4 administration on p53 and caspase 3 protein expression in colon cancer cells

Variable	Control	Dysplasia	Dysplasia + *L. lactis* D4	Cancer	Cancer + *L. lactis* D4	*p*-value
p53	1.00 (0.00–2.00)	2.00 (0.00–4.00)	3.00 (0.00–6.00)	3.00 (0.00–6.00)	4.00 (0.00–9.00)	0.518[a]
Caspase 3	4.81 (2.42–8.86)	25.43 (15.43–28.60)	28.60 (25.65–30.68)	36.63 (20.68–51.11)	27.43 (24.31–47.41)	0.010[a*]

[a]Analyzed using Kruskal-Wallis test
[*]Statistically significant at $p<0.05$

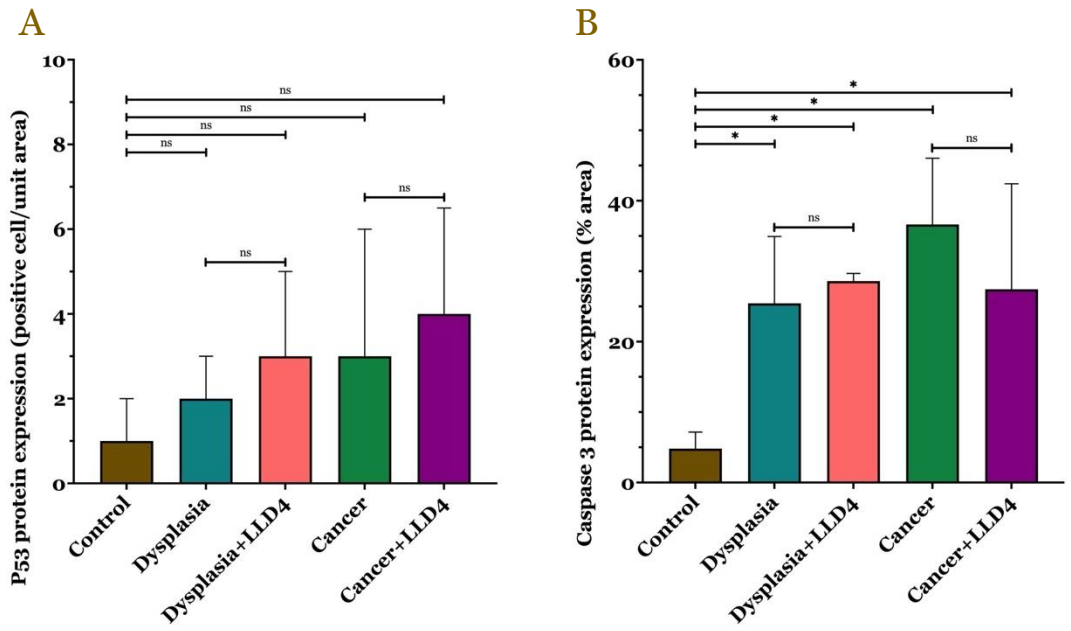

Figure 5. Effects of *Lactococcus lactis* D4 (LLD4) on colon cancer cell apoptosis, as measured by p53 (A) and caspase 3 (B) protein expressions, were evaluated across groups. [*]Significant differences at $p<0.05$; ns: non-significant differences.

COX-2 protein expression in the intestinal epithelium was higher in dysplasia and cancer groups than in the control group. Administration of *L. lactis* D4 resulted in lower mean COX-2 expression in both the dysplasia (dysplasia and dysplasia + *L. lactis* D4) and cancer (cancer and cancer + *L. lactis* D4) groups. A significant overall effect was observed on COX-2 expression ($p=0.002$) (**Table 3**); however, no statistically significant differences were observed within the dysplasia ($p=0.175$) or cancer ($p=0.251$) groups (**Figure 7B**). Immunohistochemical analysis revealed inhomogeneous brown staining of COX-2 in the mucosal tissues of the experimental animals (**Figure 8** (**A2**–**E2**)). Strong COX-2 expression was observed in the cytoplasm, particularly in the atypical epithelium of the intestinal mucosa in dysplasia and cancer groups. Following *L. lactis* D4 administration, COX-2 expression was lower than in the dysplasia and cancer group.

Table 3. Effect of *Lactococcus lactis* D4 administration on nuclear factor-κB (NF-κB) and cyclooxygenase-2 (COX-2) protein expression in colon cancer cells

Variable	Control	Dysplasia	Dysplasia + *L. lactis* D4	Cancer	Cancer + *L. lactis* D4	*p*-value
NF-κB	3.14 (1.46–7.56)	25.58 (19.14-45.42)	21.64 (10.87-31.37)	40.52 (20.65-50.12)	22.76 (19.53-33.65)	0.007[a*]
COX-2	2.21 (1.14–2.71)	18.84 (10.49-38.39)	11.32 (9.32-21.32)	33.36 (11.52-52.82)	22.42 (18.46-40.12)	0.002[a*]

[a]Analyzed using Kruskal-Wallis test
[*]Statistically significant at $p<0.05$

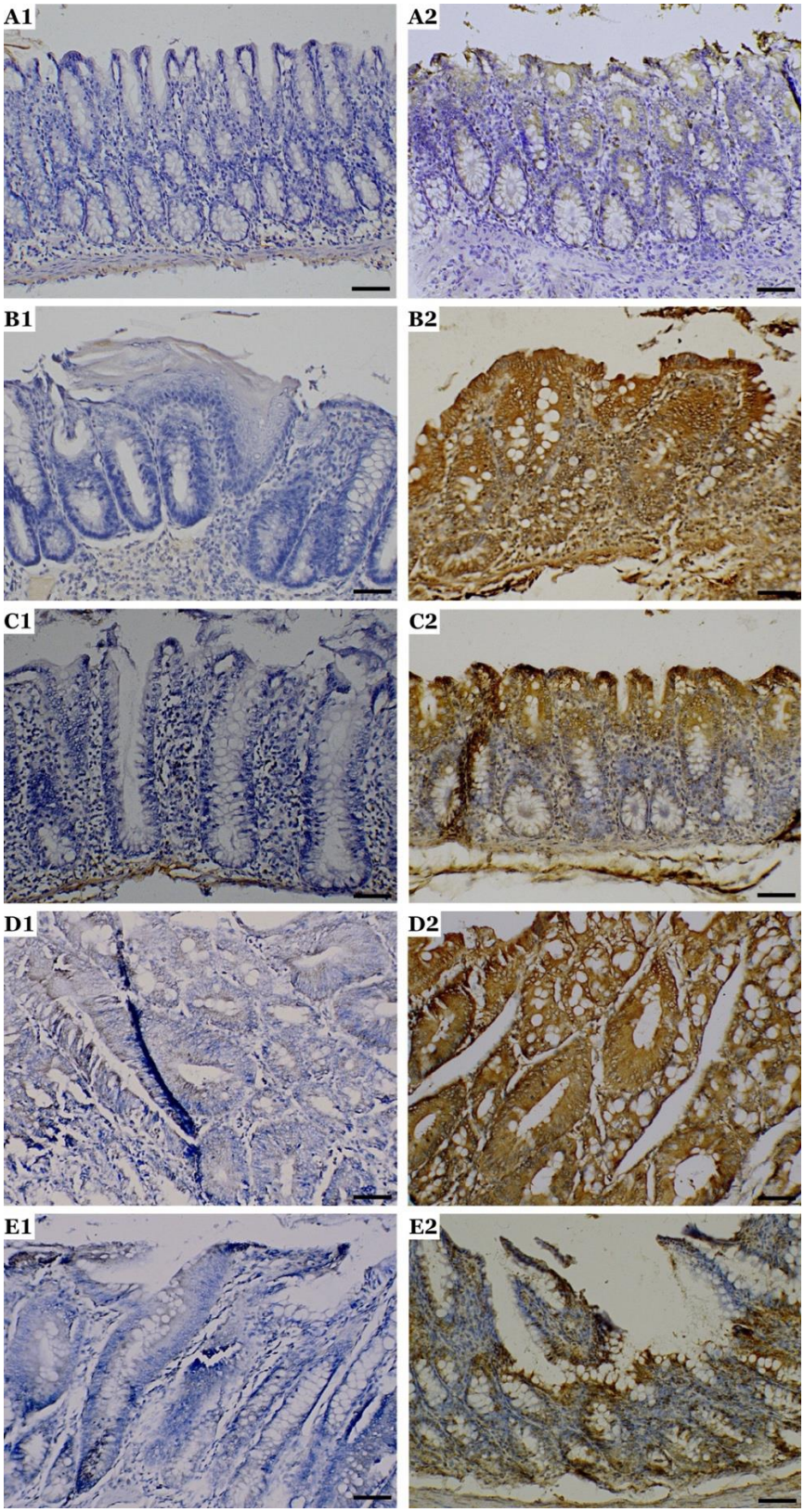

Figure 6. Immunohistochemical staining of p53 (A1-E1) and caspase 3 (A2–E2) protein expression in rat colon tissue. (A1–E1) p53 expression: A1–E1 represent control, dysplasia, dysplasia + *Lactococcus lactis* D4, cancer, and cancer + *L. lactis*D4 groups, respectively. Brown staining indicates p53 localization. Nuclear expression of p53 was higher in *L. lactis* D4-treated tissues (C1 and E1), especially in atypical epithelium. In contrast, lower expression was seen in carcinogen-only groups (B1 and D1) (scale bars=200 µm). (A2–E2) Caspase 3 expression: A2–E2 correspond to the same groups. Carcinogen-induced tissues (B2 and D2) showed increased intracytoplasmic expression of caspase 3. Expression was lower in *L. lactis* D4-treated tissues (C2 and E2) (scale bars=200 µm).

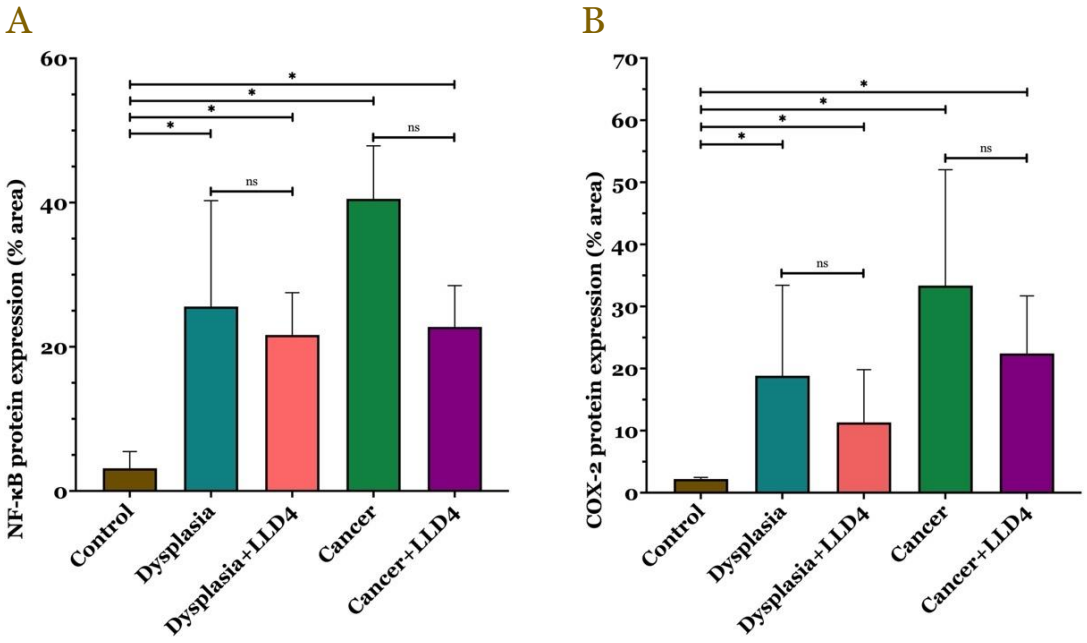

Figure 7. Effects of *Lactococcus lactis* D4 (LLD4) on colon cancer cell inflammation, as measured by (A) nuclear factor-κB (NF-κB) and (B) cyclooxygenase-2 (COX-2) protein expressions, were evaluated across groups. *Significant differences at $p<0.005$; ns: non-significant differences.

Discussion

The findings indicated that *L. lactis* D4 treatment modulated markers of proliferation, apoptosis, and inflammation in colon epithelial tissues involved in the process of dysplasia and colorectal cancer, such as cyclin D1, Bcl-2, NF-κB, COX-2, p53 and caspase 3. *L. lactis* D4 significantly decreased cyclin D1 and Bcl-2 protein expression levels in all groups (p=0.005 and p=0.002, respectively); however, the reductions were not statistically significant in the dysplasia (dysplasia and dysplasia + *L. lactis* D4) and cancer (cancer and cancer + *L. lactis* D4) subgroups (p>0.05). Similarly, NF-κB and COX-2 protein expression levels were lower in treated groups (dysplasia + *L. lactis* D4 and cancer + *L. lactis* D4), with significant effects observed across all groups (p=0.007 and p=0.002, respectively), but no significant differences in subgroup analyses (p>0.05). Regarding apoptosis markers, p53 expression showed no significant changes across groups (p=0.518), while caspase 3 protein expression decreased in cancer subgroups but remained significantly different across all groups (p=0.010).

Cyclin D1 is a critical regulator of cell cycle progression, proliferation, and cancer development [26]. Cyclin D1 protein expression has been shown to be elevated in rats with dysplasia and cancer following exposure to the carcinogen 1,2-DMH [27]. This carcinogen undergoes hepatic metabolism to methyl-azoxy-methanol (MAM), which induces DNA damage through alkylation, leading to the transformation of normal colonic epithelial cells into dysplastic and subsequently, cancerous cells [28]. The increased cyclin D1 protein expression in these malignant cells suggests an upregulation of proteins associated with dysregulated proliferation and carcinogenesis [27,28].

Although probiotic therapy is expected to suppress cyclin D1 expression, immunohistochemical analysis in this study revealed that the reduction in cyclin D1 protein expression was more pronounced in the cancer group, whereas no significant decrease was observed in the dysplasia group. This effect may be attributed to probiotic components and its metabolic products, such as nisin, which has been reported to inhibit colorectal cancer cell proliferation by suppressing cyclin D1 expression [29]. Additionally, this study examined the expression of cyclin D1 alongside the anti-apoptotic protein Bcl-2. The findings demonstrated lower Bcl-2 expression in the dysplasia and cancer groups, potentially associated with an increased Bax/Bcl-2 ratio and activation of apoptosis through the intrinsic pathway [30]. Previous studies have shown that nisin, a metabolite of *L. lactis*, can elevate the Bax/Bcl-2 ratio, supporting the pro-apoptotic effect observed in this study [4,31,32].

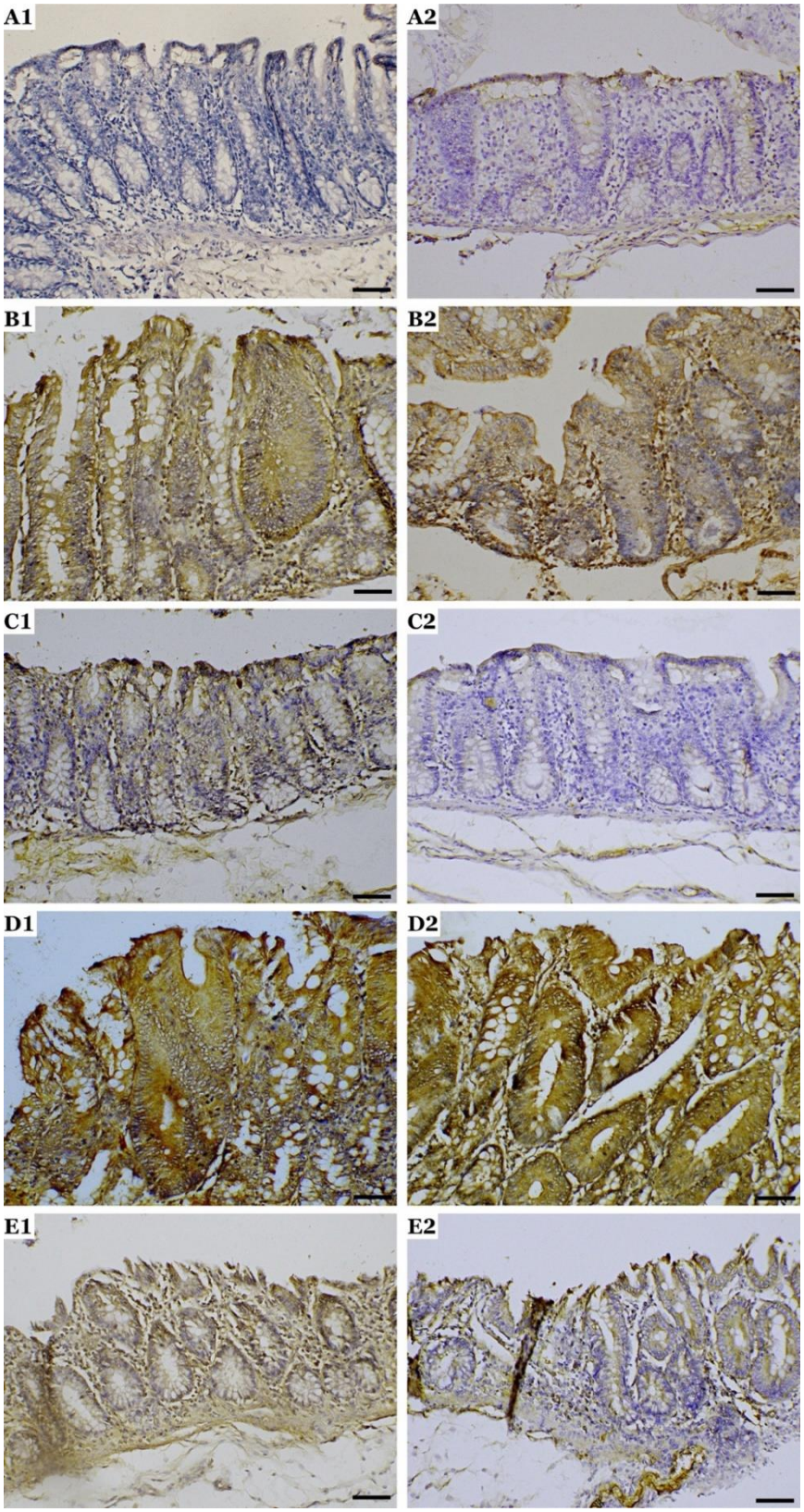

Figure 8. Immunohistochemical staining of NF-κB (A1–E1) and COX-2 (A2–E2) protein expression in rat colon tissue. (A1–E1) NF-κB expression: A1–E1 represent control, dysplasia, dysplasia + *Lactococcus lactis* D4, cancer, and cancer + *L. lactis*D4 groups, respectively. Brown staining shows NF-κB localization. Expression was elevated in carcinogen-only groups (B1 and D1), particularly in atypical epithelium. Lower expression was observed in *L. lactis* D4-treated groups (C1 and E1) (scale bars=200 μm). (A2–E2) COX-2 expression: A2–E2 correspond to the same groups. Carcinogen-induced tissues (B2 and D2) showed increased intracytoplasmic COX-2 expression, while *L. lactis* D4 treatment (C2 and E2) reduced COX-2 levels (scale bars=200 μm).

This study also evaluated the expression of *p53*, a tumor suppressor gene involved in apoptosis regulation. p53 protein expression increased in the dysplasia and cancer groups following the administration of *L. lactis* D4. *p53* is a key regulator of apoptosis, differentiation, senescence, and cell cycle arrest [33]. The observed increase in p53 protein expression aligned with previous studies suggesting that *L. lactis* may stimulate *p53* as part of its role in cell cycle arrest and apoptosis induction [34]. Caspase-3 protein expression, which also plays a role in apoptosis mechanisms, showed varying results. The gene expression of *caspase 3*, another key apoptosis regulator, exhibited varying patterns. Following *L. lactis* D4 administration, caspase 3 protein expression increased slightly in the dysplasia group but decreased in the cancer group. Immunohistochemical findings suggest that *L. lactis* D4 may enhance caspase 3 protein expression in dysplastic tissues, possibly by upregulating apoptosis-related genes beyond *p53*, such as *caspase 3* [4]. Nisin, a bioactive metabolite of *L. lactis*, has been reported to induce apoptosis via the mitochondrial pathway by increasing caspase 3 and caspase 9 activity, further supporting the findings of this study [4]. Additionally, *L. lactis* D4 administration may have contributed to increased Bcl-2 protein expression, which is known to inhibit cytochrome *C* release from mitochondria, thereby preventing the activation of the caspase cascade and reducing caspase 3 expression [4,30]. While Bcl-2 levels were found to decrease prior to intervention, it is possible that residual Bcl-2 protein expression in carcinoma-stage tissues continued to suppress caspase 3 activity [30].

This study also examined the expression of inflammatory proteins, including NF-κB, a key regulator of immune and inflammatory responses [34]. NF-κB protein expression increased in the dysplasia and cancer groups following induction with 1,2-DMH. However, administration of *L. lactis* D4 decreased NF-κB expression, as observed in immunohistochemical analyses, suggesting that this probiotic may significantly suppress inflammatory gene expression. These findings are consistent with in vitro studies demonstrating that lactic acid bacteria, such as *L. lactis*, modulate immune responses by influencing the production of immunoregulatory molecules, including nitric oxide (NO), tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, inducible NO synthase (iNOS), and COX-2 [35]. NF-κB plays a pivotal role in the innate immune response, particularly in counteracting inflammation triggered by pathogens or tumor cells [31].

In addition, this study assessed the expression of COX-2, a pro-inflammatory enzyme implicated in cancer progression [28]. The increased protein expression of COX-2 was consistent with findings that COX-2 contributes to chronic inflammatory processes often associated with colorectal cancer [28]. The elevated COX-2 expression observed in the dysplasia and cancer groups was consistent with its role in chronic inflammation, a process closely associated with colorectal carcinogenesis. The administration of *L. lactis* D4 led to a reduction in COX-2 protein expression within dysplastic and cancerous lesions, further highlighting the probiotic's anti-inflammatory effects. The anti-inflammatory properties of *L. lactis* D4 may be attributed to its metabolic byproducts, such as nisin, which has been shown to induce apoptosis through modulation of the PI3K/AKT pathway [36]. Additionally, nisin directly suppresses COX-2 expression and downregulates key inflammatory pathways, including NLRP3 and NF-κB, thereby mitigating inflammation associated with colorectal cancer [36]. The observed reduction in NF-κB expression in this study further supports this mechanism.

The findings of this study suggest that *L. lactis* D4 may have a beneficial role in modulating colorectal cancer progression and dysplasia, highlighting its potential as an adjunct therapy in colorectal cancer management. These results support further investigation into probiotics as a complementary treatment, particularly in early-stage colorectal cancer. However, several limitations must be acknowledged, including the small sample size, short study duration, and use of animal models, which restrict the direct applicability of these findings to human populations. Future research should prioritize large-scale, long-term clinical trials in humans, dose-response analyses, and mechanistic studies to elucidate the effects of probiotics on colorectal cancer progression and establish their clinical utility and safety profile.

Conclusion

L. lactis D4 demonstrated potential in suppressing colorectal cancer cell proliferation by reducing cyclin D1 and Bcl-2 protein expression. It also promoted apoptosis by increasing p53 protein

expression. The *L. lactis* D4 also showed anti-inflammatory properties by lowering NF-κB and COX-2 protein expression in colorectal cancer cells. However, further research is necessary to determine the optimal dosage and compare its cytotoxic effects with other probiotics.

Ethics approval

The Research Ethics Committee of the Faculty of Medicine at Universitas Andalas, Padang, Indonesia, has given its ethical permission for the project (No: 533/UN.16.2/KEP-FK/2023).

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Competing interests

Every author affirms that they have no competing interests.

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Underlying data

The data set used to inform the findings of this study is available from the corresponding author upon request.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

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References

1. Arnold M, Sierra MS, Laversanne M, *et al*. Global patterns and trends in colorectal cancer incidence and mortality. Gut 2017;66(4):683-691.
2. Global Cancer Observatory. Cancer today - GLOBOCAN 2022: Indonesia. international agency for research on cancer. Available from: https://gco.iarc.who.int/media/globocan/factsheets/populations/360-indonesia-fact-sheet.pdf. Accessed: 19 August 2024.
3. Chen C, Li H. The inhibitory effect of gut microbiota and its metabolites on colorectal cancer. J Microbiol Biotechnol 2020;30(11):1607-1613.
4. Hosseini SS, Hajikhani B, Faghihloo E, *et al*. Increased expression of caspase genes in colorectal cancer cell line by nisin. Arch Clin Infect Dis 2020;15(2):e97734.
5. Mendes MCS, Paulino DS, Brambilla SR, *et al*. Microbiota modification by probiotic supplementation reduces colitis associated colon cancer in mice. World J Gastroenterol 2018;24(18):1995-2008.
6. Gagliani N, Hu B, Huber S, *et al*. The fire within: Microbes inflame tumors. Cell 2014;157(4):776-783.
7. Juliyarsi I. Efektifitas dadih susu sapi mutan *Lactococcus lactis* terhadap kanker pada mencit yang diinduksi benzo[e]piren. JPI 2006;11(1):25-35.

8. Purwati E. Diversifikasi produk dadih halal asal susu kerbau Sumatera Barat menunjang kesehatan dan ekonomi rakyat. In: Zain M, Sartika W, Amizar R, *et al*., editors. Prosiding seminar nasional III sapi dan kerbau. Padang: Andalas University Press; 2017.
9. Sukma A. Analysis of microbiota in, and isolation of nisin-producing *Lactococcus lactis* subsp. *lactis* strains from, Indonesian traditional fermented milk, Dadiah. Okayama: Okayama University; 2017.
10. Sukma A, Toh H, Tien NTT, *et al*. Microbiota community structure in traditional fermented milk dadiah in Indonesia: Insights from high-throughput 16S rRNA gene sequencing. Milchwissenschaft 2018;71:1-3.
11. Fatdillah H, Desriani, Melia S, *et al*. Variant alpha and beta biodiversity of the genus in dadiah through deep sequencing 16S Ribosomal RNA genes. IOP Conf Ser Earth Environ Sci 2021;888:12040.
12. Kamarajan P, Hayami T, Matte B, *et al*. Nisin ZP, a bacteriocin and food preservative, inhibits head and neck cancer tumorigenesis and prolongs survival. PLoS One 2015;10(7):e0131008.
13. Tavakoli S, Gholami M, Ghorban K, *et al*. Transcriptional regulation of T-bet, GATA3, RORT, HERV-K env, Syncytin-1, microRNA-9, 192 and 205 induced by nisin in colorectal cancer cell lines (SW480, HCT116) and human peripheral blood mononuclear cell. Gene Rep 2021;23:101025.
14. Haghshenas B, Abdullah N, Nami Y, *et al*. Different effects of two newly-isolated probiotic *Lactobacillus plantarum* 15HN and *Lactococcus lactis* subsp. *lactis* 44Lac strains from traditional dairy products on cancer cell lines. Anaerobe 2014;30:51-59.
15. Sharma A. Importance of probiotics in cancer prevention and treatment. In: Buddolla VBT, editor. Recent developments in applied microbiology and biochemistry. Oxford: Academic Press; 2019.
16. Okawa T, Niibe H, Arai T, *et al*. Effect of LC9018 combined with radiation therapy on carcinoma of the uterine cervix. A phase III, multicenter, randomized, controlled study. Cancer 1993;72(6):1949-1954.
17. Murosaki S, Muroyama K, Yamamoto Y, *et al*. Antitumor effect of heat-killed *Lactobacillus plantarum* L-137 through restoration of impaired interleukin-12 production in tumor-bearing mice. Cancer Immunol Immunother 2000;49(3):157-164.
18. Kim JE, Kim SY, Lee KW, Lee HJ. Arginine deiminase originating from *Lactococcus lactis* ssp. *lactis* American Type Culture Collection (ATCC) 7962 induces G1-phase cell-cycle arrest and apoptosis in SNU-1 stomach adenocarcinoma cells. Br J Nutr 2009;102(10):1469-1476.
19. Kumar M, Kumar A, Nagpal R, *et al*. Cancer-preventing attributes of probiotics: An update. Int J Food Sci Nutr 2010;61(5):473-496.
20. Riaz Rajoka MS, Shi J, Zhu J, *et al*. Capacity of lactic acid bacteria in immunity enhancement and cancer prevention. Appl Microbiol Biotechnol 2017;101(1):35-45.
21. World Health Organization. General guidelines for methodologies on research and evaluation of traditional medicine. Available from: https://www.who.int/publications/i/item/9789241506090. Accessed: 29 November 2023.
22. Ouwehand AC. A review of dose-responses of probiotics in human studies. Benef Microbes 2017;8(2):143-151.
23. Sivamaruthi BS, Kesika P, Chaiyasut C. The role of probiotics in colorectal cancer management. Evid Based Complement Alternat Med 2020;2020:3535982.
24. Latif A, Shehzad A, Niazi S, *et al*. Probiotics: Mechanism of action, health benefits and their application in food industries. Front Microbiol 2023;14:1216674.
25. Zeighamy Alamdary S, Halimi S, Rezaei A, *et al*. Association between probiotics and modulation of gut microbial community composition in colorectal cancer animal models: A systematic review (2010-2021). Can J Infect Dis Med Microbiol 2023;2023:3571184.
26. Hu Y, Xiang J, Su L, Tang X. The regulation of nitric oxide in tumor progression and therapy. J Int Med Res 2020;48(2):0300060520905985.
27. Bakr MM, Guan S, Firth N, *et al*. Cyclin D1 and P27KIP1: The gatekeepers of dysplasia. J Immunol Sci 2018;2(3):30-39.
28. Venkatachalam K, Vinayagam R, Arokia Vijaya Anand M, *et al*. R. Biochemical and molecular aspects of 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis: A review. Toxicol Res 2020;9(1):2-18.
29. Hosseini SS, Goudarzi H, Ghalavand Z, *et al*. Anti-proliferative effects of cell wall, cytoplasmic extract of *Lactococcus lactis* and nisin through down-regulation of cyclin D1 on SW480 colorectal cancer cell line. Iran J Microbiol 2020;12(5):424-430.
30. Kunac N, Filipović N, Kostić S, *et al*. The expression pattern of Bcl-2 and Bax in the tumor and stromal cells in colorectal carcinoma. Medicina 2022;58(8).
31. Ahmadi S, Ghollasi M, Hosseini HM. The apoptotic impact of nisin as a potent bacteriocin on the colon cancer cells. Microb Pathog 2017;111: 193-197.

32. Norouzi Z, Salimi A, Halabian R, *et al*. Nisin, a potent bacteriocin and anti-bacterial peptide, attenuates expression of metastatic genes in colorectal cancer cell lines. Microb Pathog 2018;123:183-189.
33. Jeong JK, Chang HK, Park KY. Doenjang prepared with mixed starter cultures attenuates azoxymethane and dextran sulfate sodium-induced colitis-associated colon carcinogenesis in mice. J Carcinog 2014;13:9.
34. Kim SY, Kim JE, Lee KW, *et al*. *Lactococcus lactis* ssp. *lactis* inhibits the proliferation of SNU-1 human stomach cancer cells through induction of G0/G1 cell cycle arrest and apoptosis via p53 and p21 expression. Ann N Y Acad Sci 2009;1171:270-275.
35. Lee J, Kim S, Kang CH. Immunostimulatory activity of lactic acid bacteria cell-free supernatants through the activation of NF-κB and MAPK signaling pathways in RAW 264.7 Cells. Microorganisms 2022;10(11):2247.
36. Thoda C, Touraki M. Probiotic-derived bioactive compounds in colorectal cancer treatment. Microorganisms 2023;11(8):1898.