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SHORT COMMUNICATION

Comparison of the Anti-tumoral Effects of Kefir with Buffalo and Cow Yogurt on 1,2-Dimethylhydrazine-Induced Colon Cancer in Mice

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ABSTRACT

This study was conducted to evaluate the possible anti-tumorigenic effects of Anatolian water buffalo and cow yogurts supplemented with *Lactobacillus plantarum* or *Lactobacillus rhamnosus*, and kefir on the development colon cancer (CC) in the mouse model induced with 1,2-dimethylhydrazine (DMH). A total of 440, eight weeks-old, BALB/c male mice were used in the experiment. Mice were allocated into five (05) main groups and further 22 subgroups (20 mice/group). Animals were administered DMH subcutaneously @ 20mg/kg body weight on weekly basis for 12 weeks. In experimental groups, dairy products were given either before DMH administration, simultaneously or after the tumor formation. Tumors were evaluated by the intensity of proliferating cell nuclear antigen, apoptosis and p53 status. Anatolian water buffalo yogurt supplemented with *L. rhamnosus* showed statistically significant ($P<0.05$) antitumorigenic effects than the other dairy products used (Anatolian water buffalo yogurt supplemented with *L. plantarum*, cow yogurt supplemented with *L. plantarum* or *L. rhamnosus* and kefir). Hence it was concluded that timely consumption of dairy products (buffalo or cow yogurt supplemented with probiotics, or kefir) have the potential either to prevent or decrease the malignancy of CC.

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INTRODUCTION

Probiotics are beneficial (non-pathogenic) organisms that increase the native bacterial growth in the gut, thereby exerting a positive effect on the health status of host (Syngai *et al*., 2016; Savaiano and Hutkins, 2021). *In vitro* effects of Lactobacillus on colonic cell lines were exclusively reported elsewhere (Chen *et al*., 2017). *L. rhamnosus and Lactobacillus plantarum* are probiotics and have the capacity to strengthen the immune system, modulate intestinal inflammation and bind to toxic substances. The hepatoprotective (Sozmen *et al*., 2005), have unprotective (Moreno de Leblanc *et al*., 2007; Hong *et al*., 2009), nephroprotective, cytoprotective, antioxidant, anti-inflammatory (Sozmen *et al*., 2005; Yosef, 2024), antimutagenic, antibacterial, antifungal and antitumoral (Guzel-Seydim *et al*., 2006; De Leblanc *et al*., 2007; Ma'mon *et al*., 2018; Sepe and Arguello, 2019; Ozsoy *et al*., 2021) effects of kefir has already been reported in different studies. Previous studies evaluated how the administration of kefir, koumiss, milk, and yoghurt affected the mice's spleen tissues' mast cells, which regulate humoral and cellular processes, and plasma cells, which produce antibodies against antigens (Sevda *et al*., 2015). Yogurt as a biocontrol alternative method for eliminating different pathogens in the dairy industry has

been reported (Fahim *et al*., 2023). To achieve sustainability of intestinal stability by accomplishing microbial balance and immunologic barrier within intestine (especially IgA and increase in inflammatory response) probiotics can be added to the diet (Galdeano *et al*., 2019).

CC is the most common type of tumors worldwide. It is a major health concern in western countries where it remained the main cause of death and it is associated with an estimated 1.8 million cancer deaths (18%), after lung cancer (9.4%) (Sung *et al*., 2020). The incidence of CC is also significantly increasing in many countries like Singapore, Korea, Japan, China and Asia (Ng and Wong, 2013). Colon cancers are usually treated by one or combination of surgery, chemotherapy and/or radiotherapy, albeit disputable outcomes. Alternative approaches such as biological therapies i.e. viral therapy, gene therapy (Tripodi *et al*., 2021) and antineoplastic nano-agents are all still in their early stages. Surgery, partial or total colectomy, is the best option for the treatment of CC (Derikx *et al*., 2018). The other problem using chemotherapy is the development of drug resistance or cross resistance. The regulation of intestinal microflora is an important factor for the modulation of CC risk (Sankarapandian *et al*., 2022). The association between immune system and probiotics in CC has been exclusively studied. However, possible interactions between cancer cell markers (proliferating cell nuclear antigen (PCNA), p53 and apoptosis index), probiotic yogurt and kefir appear to be interesting to evaluate (Alves *et al*., 2020; Byanju and Lamsal, 2023). This study evaluated the possible anti-tumorigenic effects of Anatolian water buffalo and cow yogurts (supplemented with probiotics), and kefir on the DMH induced CC in the mouse model.

MATERIALS AND METHODS

Ethical Approval: This study was approved by the local ethical committee of Afyon Kocatepe University, Turkey (No: AKUHADYEK 13-09), and all interventions were carried out under the laboratory animal handling and animal experiments guidelines of the University.

Preparation of probiotic yogurt and kefir: After that, preparation of probiotic yogurt and kefir was done. Fresh milk was obtained from Anatolian water buffaloes or Brown Swiss cows raised in the research farm of the University without any antibiotic treatments. The milk sample was pasteurized at 72°C for 2 min, and cooled down at 42°C, then 2% thermophilic cultures were added. Lyophilized *L. plantarum* and *L. rhamnosus* probiotic cultures were purchased from Refik Saydam Hıfzısıhha Başkanlığı (Code no: 050256, Ankara, Türkiye) and CHR-Hansen (No: YC-350; Istanbul, Türkiye), respectively. They were inoculated into milk as 107cfu/mL and incubated at 42-43°C for 4h until the final pH was 4.6. After Brown Swiss cow milk pasteurized, 15-20g kefir granules were added in 1L of the milk and completely mixed and then kept to ferment at 20-22°C for 18-24h. Drained kefir was cooled and kept at 4°C. To achieve standardization, industrial kefir culture (CHR-Hansen BB-12R LAF-3) was used. 2-8% kefir culture was added to pasteurized milk, incubated at 20-22°C for 18-24h until pH was 4.4-4.9 and acidity was 30-40 Soxhlet Henkel (°SH). Freshly prepared kefir was kept at 3-10°C. Each mouse was administered 1cc of kefir (5.2x108 cfu/mL) by oral gavage. To induce colon tumors, mice were administered DMH subcutaneously at a dose of 20mg/kg/body weight on weekly basis for 12 weeks.

In vivo experiment: Total of 440 male, 8-week-old male BALB/c mice of 20-30g were experimented. Mice were divided into 5 main groups and further 22 subgroups. Each subgroup consisted of 20 mice. Group 1 (divided into groups 1A and 1B) was further sub grouped as group 1A1 (the mice were fed cyclically with water buffalo yogurt (Wby) supplemented with *Lactobacillus plantarum* (*Lp*) for two weeks by oral gavage (basal yogurt feeding), then treated with DMH once a week for 12 weeks, cyclic administration of Wby+*Lp* continued until the end of the experiment; to observe protective effects of Wby+*Lp*); group 1A2 (animals were fed cyclically with Wby+*Lp* for two weeks and then subcutaneous DMH were injected once a week for 12 weeks; to observe the effect of Wby+*Lp* on tumor initiation); group 1A3 (subcutaneous DHM was given once a week for 12 weeks then fed cyclically with oral Wby+*Lp* upto the end- no basal yogurt feed; to observe antineoplastic effects of Wby+*Lp*); group 1A4 (only cyclic oral Wby+*Lp* was given throughout the experiment; control group for Wby+*Lp*). To evaluate the effects of Wby supplemented with *L. rhamnosus* (Wby+*Lr*), group 1B subgrouped as 1B1, 1B2, 1B3 and 1B4 and the same procedure was applied changing only probiotic *L. rhamnosus* in place of *L. plantarum* as in group 1A. Group 2 was also divided into 8 subgroups to observe antineoplastic effects of cow yogurt (Cy) supplemented with *L. plantarum* (Cy+*Lp*; subgroups 2A1, 2A2, 2A3 and 2A4) or *L. rhamnosus* (Cy+*Lr*; subgroups 2B1, 2B2, 2B3 and 2B4) as in groups 1A and 1B. Group 3 was divided into 4 subgroups (3A, 3B, 3C and 3D) similar to group 1A, however kefir was substituted instead of Wby. Group 4 served as positive control where only subcutaneous DHM was given once a week for 12 weeks to induce tumor growth and group 5 (control group) where mice were given only a conventional balanced diet. The experiment was terminated at the 6th month of the study.

Next day of the dairy product administration, freshly voided stool specimens of mice, three samples from each group, were collected and transferred to the laboratory within five minutes. Determination of the faecal excretion of live probiotics in yogurt and kefir was confirmed by faecal sample inoculation on agars (Mann Rogosa Sharpe®, Oxoid, CM0361B and M17, Oxoid, CM0785B) by the routine manner. The intestinal lactobacilli composition was shown as (log^{10} colony-forming units (cfu)/g) counts and colonies were not typed.

Mice were euthanized after the administration of general anesthesia with the dose rate of 15mg/kg xylazine (Rompun®, Bayer, Germany) and 50mg/kg ketamine HCl (Alfamine®, Egevet, Turkey). The specimen was longitudinally opened, and content was carefully removed and washed with saline solution. The presence of tumors was observed, counted and measured by a magnifier. The Swiss-roll technique was performed according to procedure described by Moolenbeek and Ruitenberg (1981). Briefly, longitudinally opened colon strips were rolled up, with the mucosa in the outwards direction, using

a clean wooden stick. They were fixed in 10% buffered formalin saline solution and kept at 4°C for 48h for fixation. After paraffin-wax embedding, 5µm thick sections were stained with haematoxylin-eosin stain (H&E) and examined under a light microscope. Tissue samples from colons were processed with routine tissue processing methods. Astler-Coller staging system, a modification of Dukes' classification, was used for histopathological staging of colon tumors (Table. 1).

Table. 1: Histopathological staging of colon malignancy (Astler and Coller, 1954).

Stage	Lesion location
A	Involvement of mucosa
B1	extending with negative nodes into the muscularis propria without entering it
B2	Penetration of Muscularis propria layer with negative nodes
C1	extending with positive nodes into the muscularis propria without penetration
C2	Ppenetration of muscularis propria with positive nodes

5µm sections were paraffinized and rehydrated and were maintained in 3% hydrogen peroxide at 22°C for 10min for blocking of endogenous peroxidase. Slides were shifted in citrate buffer (pH 6.0) solution in a pressure cooker and a 900-watt power microwave applied for 20 min for antigen retrieving. As the primary antibodies anti-PCNA (Abcam, Rabbit polyclonal, ab18197, 1/400 dilution) and anti-*p*53 (Sigma, Rabbit polyclonal, 1/25 dilution) were used and 2 and 18h incubation periods were performed at room temperature, respectively. Mouse on Mouse Kit (Elite peroxidase kit Vectorlab) was used to mouse primary antibodies, and ABC kit (Vectastain Elite ABC-Peroxidase kit) was applied to rabbit primary antibodies. Visualization was made with 3-amino-9-ethylcarbazole substrate (AEC, Invitrogen). Background was stained with Gill's (I) haematoxylin dye for 2 min. After washing with tap water sections were covered slipped with an aqueous medium. These ready sections were kept in the dark room until analysed. Only nuclear staining in the sections was considered positive. Thus, percentage of positive cells was used as reactivity criteria in this study.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL assay) was performed by getting sections on adhesive slides. Antigen retrieval was done by Proteinase, and 3% hydrogen peroxide were used for the blocking of endogenous peroxidase. Apoptotic kit (In Situ Cell Death Detection Kit, POD, Roche, Mannheim, Germany) was used for the reaction. Visualization was made with 3-amino-9-ethylcarbazole substrate (AEC, Invitrogen). Background was stained with Gill's (I) haematoxylin dye for 2min. After washing with tap water sections were covered with an aqua medium. Ten different fields were counted at 40X magnification (Image J program) for apoptotic cell percentage.

Statistical analysis: Correlation analysis was used to observe possible association between immunohistochemical stainings (PCNA, *p*53) and malignancy of tumors (Astler-Coller staging). Histopathological and immunohistochemical staining of samples were analysed by descriptive statistics (i.e. number and percentage). The ability to provide both categorical and continuing (continuous) variables is one of the advantages of chi-squared automated interaction detection (CHAID) analysis over other types of analysis. The selected predictor was used to partition the data. To generate further subdivisions for analysis, each of these subgroups underwent independent reanalysis. To construct the contingency table with the maximum significance level in accordance with the chi-squared test, the type of each predictor dictated the acceptable groupings of its categories (Kass, 1980). The data was also described, examined, and summarised using multiple correspondence analysis. The threshold for significance was fixed at $P<0.05$.

RESULTS

The live lactobacilli levels in stool samples were presented in Table 2. Tumors were macroscopically observed as early as within 4 months in group 4. Lesions were seen in both serosa and mucosa in the colon. Nodular neoplasms of varying size, usually located in the middle colon, were reaching the size to fill the colon lumen in places (Fig. 1). No tumor development was seen either in control group (group 5) or groups that were given only dairy products (groups 1A4, 1B4, 2A4, 2B4, and 3D) by macroscopic evaluations.

Table. 2: The level of live lactobacilli in the stool samples.

Groups	Live lactobacilli level in stool sample
1A1-1A3	4.8×10^6 cfu/g
1A4	7.0×10^7 cfu/g
1B1-1B3	3.0×10^6 cfu/g
1B4	6.0×10^6 cfu/g
2A1-2A3	3.5×10^6 cfu/g
2A4	6.8×10^7 cfu/g
2B1-2B3	2.6×10^6 cfu/g
2B4	5.8×10^6 cfu/g
3A-3C	3.0×10^6 cfu/g
3D	4.8×10^6 cfu/g
4 (DMH group)	2.0×10^2 cfu/g
5 (Control group)	2.0×10^3 cfu/g

Histopathological findings are presented in Fig. 2. No tumor development was seen either in control group (Fig. 2A). Preneoplastic and neoplastic lesions described in DMH received groups. DMH administrations caused different colon lesions such as dysplasia (Fig. 2B), adenoma (Fig. 2C-D) adenocarcinoma (Fig. 2E-F), and invasive adenocarcinoma (Fig. 2G-H). Adenomas and adenocarcinomas consisted of atypical glands and epithelium. In the neoplastic glands of adenocarcinomas, there were numerous mitotic figures, inflammatory cell infiltration, and pleomorphic cells. Significant nuclear elongation, hyperchromatic vesicular nuclei, anionucleosis, and anisocytosis were all observed in these pleomorphic cells. Anaplastic cell clusters and inflammatory cell infiltration into the submucosa were seen in the region of adenocarcinoma. No tumor development was seen either in groups that were given only dairy products (groups 1A4, 1B4, 2A4, 2B4, and 3D) by microscopic evaluations. Staging of all tumors according to Astler-Coller is presented in Fig. 3. Among all tumors (n=92) observed in this study, the predominant lesion was type A (53/92; 57.6%) followed by type B1 (30/92; 32.6%). The development and frequency of tumors were superior in groups where dairy products were given after exposure to the carcinogen. According to results of CHAID analysis, the most effective variable on tumor development was the duration of administration of dairy products. In groups

1A1, 1B1, 2A1, 2B1 and 3A (where dairy products were given prior to carcinogen challenge), in general, the likelihood of non-tumoral observation was 59.7% and the variables were found to be statistically significant (P<0.05). In these groups the ratio of non-tumoral observation was 84.2% for Wby, whereas it was 58.6% for Cy and 28.6% for kefir subgroups. The addition of probiotic to Cy was associated with statistically significant decrease in tumor development i.e. Cy+*Lr* generated less tumor development (73.3%) than Cy+*Lp* (42.9%). In Wby groups 1A1 and 1B1, however, the effect of probiotics addition produced no statistically significant data in the analysis. The reason for this, in these groups, was higher rate of non-tumor observation, 90% for *L. plantarum* and 73.3% for *L. rhamnosus*.

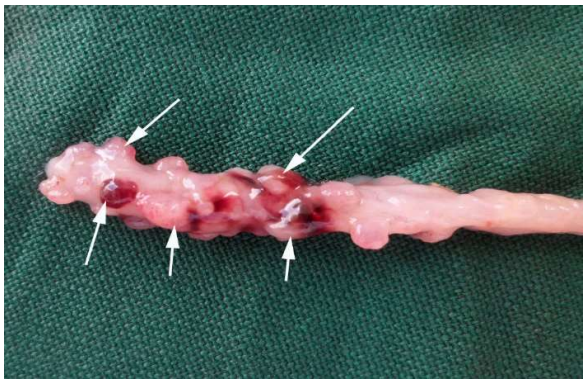

Fig. 1: Macroscopic appearance of tumors in different numbers and sizes in the DMH-induced colon in a mouse (arrows).

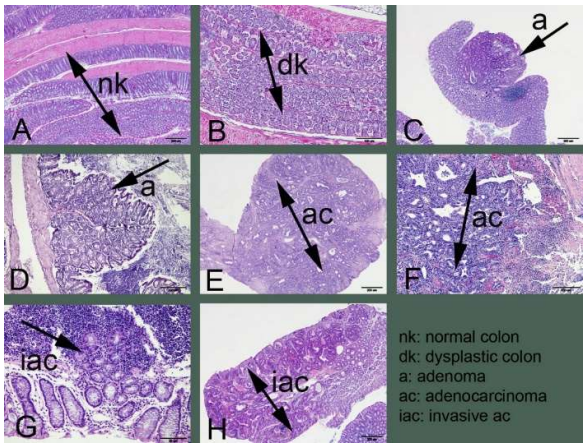

Fig. 2: Photomicrographs of DMH and dairy products received mice colon sections stained by H&E: A: Control group; normal histological appearance. B: Dysplasia, dysplastic changes both in glands and epithelia. C-D: Adenomas, numerous neoplastic glands partially resembling normal colon glands. E-F: Adenocarcinomas, mitotic figures, inflammatory cell infiltration and pleomorphic cells were abundant in the neoplastic glands of adenocarcinomas. These pleomorphic cells exhibited significant nuclear elongation, hyperchromatic vesicular nuclei, anisonucleosis and anisocytosis. G-H: Invasive adenocarcinomas, in some areas of adenocarcinomas, clumps of anaplastic cells and infiltration of inflammatory cells in the submucosa were observed.

In groups 1A2, 1B2, 2A2, 2B2 and 3B (simultaneous administration of dairy products with carcinogen), it was determined that 77.8% of animals were no tumor bearing and there was statistically significant difference between Wby and kefir with Cy (Wby and kefir clustered within the same group). In Wby or kefir applied groups, tumors were not observed in 86.1% even though it was 58.3% in Cy groups. In Wby+*Lp* group, no tumor was observed in 78.3% of animals whereas in *L. rhamnosus* added yogurt groups, tumor rate decreased in all animals. Finally, in groups 1A3, 1B3, 2A3, 2B3 and 3C (where daily products were given after cancer development), observation of no tumor was 27.9%. The Wby and kefir displayed similar effects, but Cy significantly differed from them. No tumor development was 34.3% for Wby and kefir groups and 14.3% for Cy groups. Addition of probiotics produced statistically significant data such as *L. rhamnosus* addition associated with 90.0 % no tumor development whereas *L. plantarum* addition generated tumors in 22.2% cases.

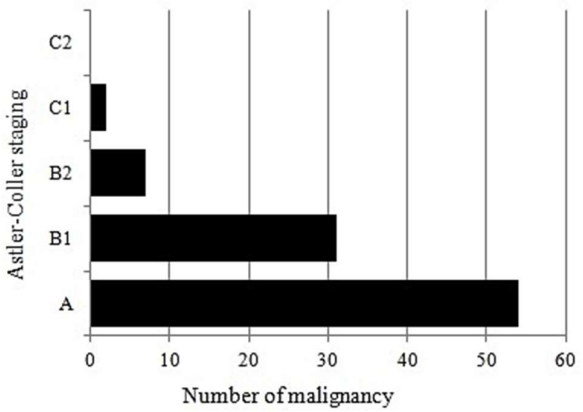

Fig. 3: Distribution of tumor types according to Astler-Coller staging.

Immunohistochemical findings are presented in Fig. 4A-F. There was a positive correlation between PCNA, *p*53 and apoptosis positivity and presence of tumors according to Spearman's *rho* correlation coefficient (ρ=0.981, 0.939 and 0.971, respectively). The mean PCNA staining was significantly lower in animals treated with cyclic Wby+*Lr* prior to DMH administration than the other groups (P<0.01). PCNA expression was also present in all DMH-administered animals, suggesting that some degree of proliferation was already initiated by the carcinogen.

When multiple correspondence analyses were considered, the tumor detection, generally, by *p*53 staining was observed in groups where tumor challenge followed by administration of Cy+*Lp*. However, the tumor detected by PCNA staining was generally seen in Wby+*Lr* groups in which dairy products were given before tumor initiation. Mice that received yogurt or kefir prior to DMH challenge had profound effect on apoptosis of the colonic epithelial cell in comparison to DMH treated control group (P<0.01).

The expression of *p*53 in low degree neoplasms was in nuclear locations. However, it was both nuclear and cytoplasmic in high neoplastic malignancy. Cytoplasmic *p*53 expression was significant in groups 3 and 4, while it was only nuclear in groups 1 and 2. Vicinity of *p*53 positive cells were surrounded by massive mononuclear cell infiltration. The *p*53 expression was lower in groups 1A1 (90%) and 1A2 (60%) than 1A3 (30%); groups 1B1 (80%) and 1B2 (100%) than 1B3 (60%). However, groups 2A1 (70%) and 2A3 (80%) showed more positivity than 2A2 (60%) whereas it was 40% for 2B1, 70% for 2B2 and 100% for 2B3. In kefir group's positivity was 80% for group 3A, 70% for group 3B and 90% for group 3C.

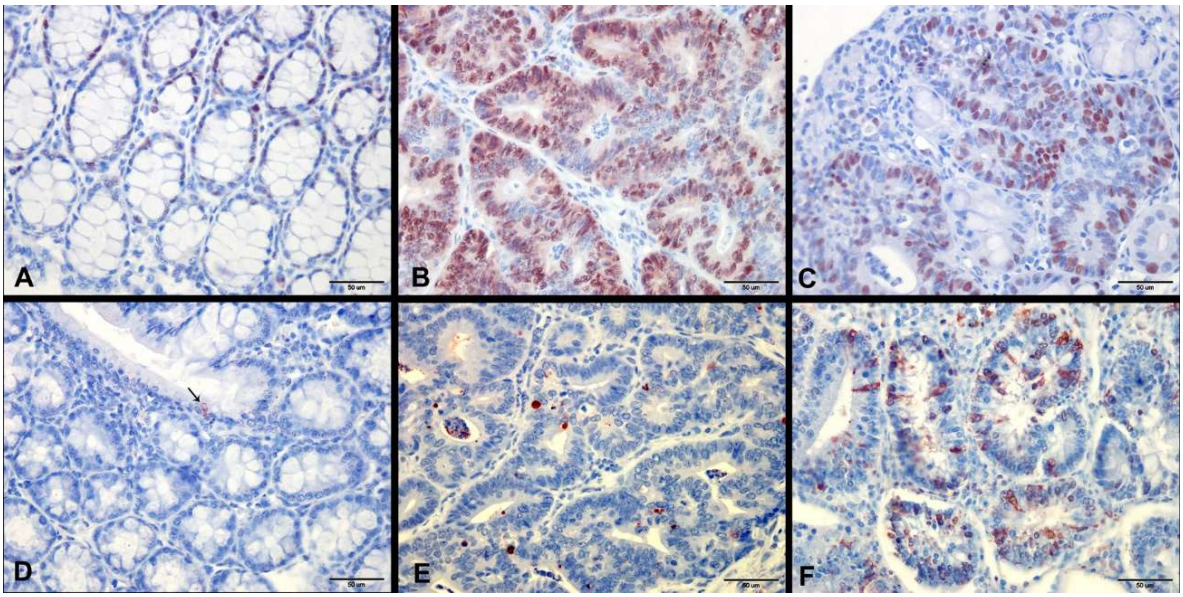

Fig. 4: Microscopic images of PCNA and TUNEL immunohistochemical staining. A: Very weak and limited PCNA staining in nuclei of intestinal glandular epithelial cells in group 5 (control group). B: Copius staining of PCNA in tumor cell nuclei of group 4 (DMH group). C: Decreased PCNA staining in tumor cell nuclei in dairy product administered groups (group 1B given for representation). D: Apoptosis in a single intestinal glandular epithelial cell in control group (arrow). E: A number of apoptotic cells in tumor cells of group 4. F: Increased number of apoptotic cells in group 1B.

The effects of yogurt or kefir on tumor development were not significant once tumor occurred ($P>0.05$). Chi-square test revealed statistically significant differences within groups between the consumption of yogurt or kefir before or simultaneous exposure to DMH ($P<0.01$). When three dairy products were compared, Anatolian water buffalo yogurt showed superior antitumorigenic effects. In addition, *L. rhamnosus* generated statistically significant difference in comparison to *L. plantarum* probiotic ($P<0.05$).

DISCUSSION

The addition of probiotic bacteria in different dairy products has become increasingly popular in today's human diet policy (Galdeano *et al*., 2019). The health and nutritional benefits ascribed to probiotic bacteria include the regulation of intestinal functions, alteration of physico-chemical environment in the colon milieu (Hansen *et al*., 2019), alleviation of the symptoms of lactose intolerance, qualitative and quantitative changes in the gut microflora, serum cholesterol reduction, alleviation of constipation (Wan *et al*., 2019), production of anti-mutagenic (Guzel-Seydim *et al*., 2006) and anti-tumorigenic factors (Guzel-Seydim *et al*., 2006; De Leblanc *et al.,* 2007; Ma'mon *et al*., 2018; Sepe and Arguello, 2019) interaction and degradation of potential carcinogens (Perdigon *et al*., 2002) and improvement of host immune system efficacy (i.e. enhancement of local IgA production (Kailasapathy and Chin, 2000). To produce therapeutic benefits, lowest levels for probiotic bacteria in yogurt products must have 10^6 viable cells per mL or g in the final preparation (Mortazavian *et al*., 2012). In our study viable and live organisms, either in yogurt and kefir preparations or in fecal samples, were observed to be at satisfactory level.

Probiotics may prevent CC by lowering intestinal pH, modifying enzymes like β-glucosidase that change pro-carcinogens into proximate carcinogens, reducing ras-P21 oncoprotein expression, and producing sodium butyrate through lactose fermentation, which is a potent growth inhibitor and inducer of phenotype differentiation and apoptosis (Jung *et al*., 2007). The second most widely available milk source in many countries around the world is water buffaloes (Borghese and Mazzi, 2005). The data about the use of dairy products for the treatment of animal model colon cancer was also published in review studies (Nacimento *et al*., 2021). It was reported that significant differences were found between the contents of cow and buffalo milk. Concentrations of vitamins (A, B_6, C, E, niacin and biotin), minerals (calcium, magnesium, phosphorus, sodium and zinc), total protein, totals of saturated, monounsaturated and polyunsaturated fatty acids are predominantly higher in buffalo milk than cow milk (Medhammar *et al*., 2012). This has been reported in this study, that showed superior effects on the prevention of CC with respect to other dairy products.

Lactic acid bacteria strains like *L. plantarum* and *L. rhamnosus* have been shown to be more successful in protecting the colon's microbial balance, preserving the essential nutrients the bacteria produce, removing harmful substances from food, preventing decay, and destroying pathogens (Bengmark, 2000). As a result, probiotics have been frequently added to yoghurt products to increase their therapeutic potential and create a market for them as functional foods (Badgeley *et al*., 2021; Abiden *et al*., 2024). Like the well-known probiotic strain *L. rhamnosus, L. plantarum* also suppresses pathogen adherence. Additionally, it might reduce the expression of interleukin 8 (IL-8) and tumour necrosis factor alfa (TNF-α) in cells treated with *E. coli* (Ko *et al*., 2007). However, in our study *L. rhamnosus* showed superior ability to inhibit CC with respect to *L. plantarum*. Kefir is reported to induce some antitumor activity (Dos *et al*., 2019). However, in our study, kefir showed non-significant tumor inhibition

compared to cow or buffalo yogurts. The reason why kefir showed weak anti-tumorigenic effect may be that kefir bears effective properties against carcinogens that are taken by oral route rather than parenteral administration. This mechanism should be exclusively described, if any. Moreover, kefir has significant dose dependent antitumor activity against multiple cancer cell types (Walia *et al.,* 2018), that may be associated with different outcomes between different studies. Leblanc and Perdigon (2004) reported that yogurt feeding before carcinogen injections showed rapid regulatory cytokine release and apoptosis stimulation in the large intestine, this shows that yogurt supplementation alone was incapable of inhibiting tumor appearance and development. Yogurt must be cyclically administered after DMH to inhibit tumor growth. Walia *et al.* (2018) concluded that consumption of dairy products may have beneficial effect on CC only if they are consumed before or during exposure to the carcinogen but not subsequently. This discrepancy may be associated with the use of different cultures and ratio among studies as Leblanc and Perdigon (2004) used *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (approximately $2x10^9$cfu/mL), Walia *et al.* (2018) used *L. casei* subsp. *rhamnosus* strain GG ($2x10^{10}$cfu/mL) and we used *L. plantarum* and *L. rhamnosus* ($8x10^8$cfu/mL) cultures.

It was reported elsewhere that PCNA was generated by cells during the late S-phase and G-phase of the replicative stage as a result it could be a good candidate as a phase index marker for proliferating cells (Yamamoto *et al*., 2019). Thus, all mitotic activities and tumor grades were closely correlated with PCNA positivity. Not surprisingly *p*53 positivity showed similar patterns as TUNEL and PCNA positivity or vice versa. Thus, this indicates that *p*53 without disruption may accomplish its duties in cells. Moreover, once the malignancy started, they attracted cytotoxic T-lymphocytes to the region.

Conclusions: In conclusion, our findings demonstrated that, in this experimental murine model, yogurt and kefir feeding favoured apoptosis and interfered with the initiation or early promotional stages of DMH-induced CC. Moreover, Anatolian water buffalo yogurt supplemented with *L. rhamnosus* showed more significant antitumorigenic effects than the other dairy products (Anatolian water buffalo yogurt supplemented with *L. plantarum*, cow yogurt supplemented with *L. plantarum* or *L. rhamnosus* and kefir). This study findings provide baseline information and could be used to develop and implement a tool to evaluate further therapeutic effects of these dairy products against CC. Thus, it will be of interest to compare regression of tumor size using different preparations of dairy products.

Authors contribution: ID: Designation the study, project administration, writing-original draft, writing-reviewing and editing. TE, MK, ACD and AD: Experimentation and data collection. SP: Preparation of dairy products, HY, MFB and MNB: Evaluation of pathological specimens, MO: DMH preparation, and SS: Data analysis. All authors have read and approved the final manuscripts.

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