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RESEARCH ARTICLE

Role of the Probiotic Supplementation on Intestinal Inflammation and Structural Integrity in Wistar Rats Subjected to a Cafeteria Diet during Development

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A B S T R A C T

Probiotics have gained significant interest in medical and veterinary sciences due to their potential to improve gastrointestinal health. This study investigates the protective role of probiotics on intestinal health in male Wistar rats exposed to a cafeteria diet during development. The experimental groups were divided into four: control, probiotics, cafeteria diet, and cafeteria diet with probiotics. Probiotics groups were administered daily at 1×10^8 CFU throughout the experiment. Ileum and colon tissues were analyzed via Fourier-transform infrared spectroscopy, histopathological analysis, and immunohistochemical staining. The cafeteria diet group showed altered lipid profiles, increased protein carbonylation (a marker of oxidative stress), and increased mast cell density, indicating increased intestinal inflammation. Probiotic supplementation significantly reduced inflammation by reducing TNF-α ($P \leq 0.0001$) and IL-1β ($P \leq 0.0001$). These results suggest that probiotic supplementation during an unhealthy diet can mitigate adverse effects by reducing oxidative stress and inflammation. Thus, probiotics could offer therapeutic potential in mitigating cafeteria diet-induced intestinal changes, serving as a promising dietary intervention during development to manage metabolic disorders in both humans and animals.

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INTRODUCTION

The cafeteria diet, characterized by its high caloric density and substantial sugar content, serves as a widely utilized model for studying the physiological and metabolic impacts of excessive caloric intake. This diet mimics the composition of highly palatable, energy-dense foods, making it an effective tool for exploring diet-induced metabolic disorders and related physiological changes (De Marco *et al.*, 2021). It has been reported that a diet rich in sugar and fat can impair growth, alter organ function, and jeopardize overall health. Understanding the effects of such diets on gut health is essential for both human and veterinary medicine (Mota-ramírez, 2023). Recent studies have linked high-caloric diets to disruption of the gut microbiota with adverse metabolic outcomes and elevated inflammatory markers in adolescents and young adults (Di Lorenzo *et al.*, 2023). Similarly, animal studies show that high-fat cafeteria-style diets lead to damage and inflammation of the intestinal mucosa, causing structural and functional impairment in the gastrointestinal tract (Acosta-Rodríguez *et al.*, 2021; Shin *et al.*, 2023). High-concentrate diets in Hu sheep induced colonic inflammation, disrupting the gut barrier and elevating pro-inflammatory cytokines such as TNF-α and IL-1β (Chen *et al.*, 2023). Another review on diet-induced inflammation in ruminants suggests that grain-based, high-energy diets trigger chronic low-grade inflammation, impacting the liver, adipose tissue, and mammary gland (Khiaosa-Ard and Zebeli, 2018).

Probiotics are defined by The Food and Agriculture Organization (FAO) and World Health

Organization (WHO) as living microorganisms and with adequate amounts those organisms benefit the hosts (Hill *et al.*, 2014). In aging studies, probiotics were found to support intestinal barrier integrity by regulating the microbiota and alleviate aging-related leaky gut syndrome and systemic inflammation by reducing intestinal permeability and inflammation (Ahmadi *et al.*, 2020). Probiotics have also been recognized in veterinary applications (Yang and Wu, 2023), especially for their role in modulating metabolic disorders in animals (Stahl *et al.*, 2020). In pigs, probiotics have been shown to support gut health by promoting microbiota balance, improving nutrient utilization, and reducing indigestion (Liao and Nyachoti, 2017). Similarly, studies on dogs revealed that probiotic supplementation increased microbial diversity and reduced pathogenic species (Xu *et al.*, 2019). These findings support the therapeutic potential of probiotics as modulators of gut health and immune modulation in humans and animals.

This study investigated the effects of probiotics on intestinal health during the developmental stages of male Wistar rats on a cafeteria diet. The study also aimed to evaluate the effects of a cafeteria diet on ileum disruption and colon physiology, inflammatory responses, and oxidative stress associated with it. The study further aimed to analyze the effect of probiotics supplementation in countering the disruptive effects associated with the cafeteria diet which could have a critical role in managing diet-induced metabolic and inflammatory changes across species.

MATERIALS AND METHODS

Experimental design*:* Male Wistar rats (21 days old, post-weaning) were divided into four groups (n=7 per group): control, SCD Probiotics, cafeteria diet, and cafeteria diet with SCD Probiotics. SCD Probiotics (1×10^8 CFU) were administered daily via oral gavage until day 56. A commercially available probiotic mix (Essential Probiotics XI) was used, containing species *Bacillus subtilis, Bifidobacterium bifidum, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus plantarum, Lactococcus lactis, Saccharomyces cerevisiae*, and *Streptococcus thermophiles* (Ceylani, 2023). Rats had *ad libitum* access to a standard rodent diet, with the cafeteria diet (Table 1) provided additionally as described in our previous studies (Aba *et al.*, 2023). Weekly measurements of weight, food intake, and diet contents were recorded. Animals were housed in clear Plexiglas cages (7 rats per cage) with free access to food and water under a 12-hour light/dark cycle at 21°C. Rats in each group were housed in separate cages, and all co-housed rats remained in the same experimental unit throughout the study. At the end of the study, rats were humanely euthanized and dissected Ileum, and colon tissues were collected, washed with normal saline, flash-frozen on dry ice, and stored at -80°C till further analysis. This study was conducted with the approval of the Bingöl University Animal Experiments Local Ethics Committee (Approval No: 2021/03).

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy: Ileum and colon samples were analyzed using a PerkinElmer ATR-FTIR spectrometer without pretreatment. Spectra were recorded at a resolution of $4cm^{-1}$ with 32 scans over the range of $4000\text{-}650cm^{-1}$. Spectral data analysis was performed using OPUS 5.5 software, where baseline correction was applied using the Rubberband method with 128 baseline points. Band quantification included measurements of band areas, area ratios, and bandwidths, specifically focusing on biomolecular markers (Teker *et al.*, 2023).

Histopathological analysis: Ileum and colon biopsies were fixed using 10% neutral buffered formalin and paraffin sections of 4-5µm thickness were prepared using a rotary microtome. Sections were stained with hematoxylin & eosin (H&E) to visualize the overall histoarchitecture. Histological changes were evaluated under light microscopy (Nikon Eclipse Ni microscope (Japan) with Nikon DS-Fi2 camera) (Teker *et al.*, 2023). To assess lymphatic infiltration for the sections, a grayscale binary method with the lowest threshold level was used and area fractions (%) were measured with Image J Fiji (v1.54d, NIH, USA).

Toluidin blue staining: Mast cells were identified using Toluidine blue staining following the protocol by (Vidal & Mello, 2019). Briefly, 5µm sections were deparaffinized and stained in 0.1% toluidine blue working solution for 3-10minutes. After washing with distilled water, slides were dehydrated in 95% and 100% alcohol and cleared in xylene. Mast cell granules were stained dark violet due to metachromasia (Vidal and Mello, 2019). Mast cell density was calculated using Image J at 40X magnification. A grey-scale binary separation technique was applied to identify violet-magenta stained areas with TB staining, and cell density (five images per section for all subjects in the same group) was measured according to Adomshick, Pu, and Veiga-Lopez, (2020)

Histomorphometric measurements: Villi height, crypt depth, mucosa, submucosa, muscle layer, and total wall thickness parameters were evaluated using ImageJ on H&E stained sections. Measurements were based on 20 villi and crypts from each section of the intestinal segment. Villus height was determined as the distance from the epithelial tip to the base, and crypt depth was determined as the distance from the crypt apex to the muscularis mucosa (Adelman *et al.*, 2018).

Immunohistochemical (IHC) staining: Following deparaffinization, endogenous peroxidase activity was blocked using 3% H_2O_2 for 10 min for all sections taken on lysine slides for IHC analyses. Antigen recovery was performed in citrate buffer (pH 6, 10X) followed by a blocking solution. Sections were then incubated overnight with anti-TNF-α (1:200, Elabscience) and anti-IL-1β (1:200, Bioss) antibodies. Subsequently, secondary antibodies and streptavidin-peroxidase were applied, followed by DAB substrate staining and counterstaining with Mayer's haematoxylin. Images were analysed with ImageJ to quantify TNF-α and IL-1β intensities in randomly selected areas (Ceylani *et al.*, 2023a).

Table 1: The ingredients of the cafeteria diet

Energy and Food Ingredients (100)	Total kcal	Total fat (g)	Total carbohydrates (g)	Protein (g)	Sugar (g)
Control Diet					
SC 7001 (Harley)	382	4	54	25	0
CAF Diet					
Crackers					
Tea Delight (Eti)	462	20.4	67.8	5.8	28.5
Cookies					
Wafer Hazelnut (Eti)	493	24.5	63.9	7.6	28.5
Hazelnut Cookie (Ülker)	427	18.1	62.1	3.9	25.0
Nestlé Crunch	500	26	67	5	55
Cereals					
Nesquick corn flakes (Nestle)	372	4.1	76.1	7.6	30.7
Chips					
Lays Wavy (Frito-Lay)	536	36	54	7	0
Lays Klasik (Frito-Lay)	529	33	51	7.0	0
Doritos (Frito-Lay)	491	24.5	60.5	7.2	2.3

The table lists the ingredients of the cafeteria diet. Cnt (control), Prb (SCD Probiotics), Cd (cafeteria diet), and CdPrb (cafeteria diet with SCD Probiotics supplementation).

Statistics*:* Data analysis was performed using GraphPad Prism 10.1. Statistical significance was determined using unpaired t-tests and One-way ANOVA, with significance levels set at P≤0.05 *, P≤0.01 **, P≤0.001 ***, and P≤0.0001 ****. Results are expressed as mean ± SEM.

RESULTS

Physical analsysis*:* It was observed that while the body weights of the subjects were similar across all groups, their daily food intake rates differed considerably. Upon conducting pairwise comparisons, it was found that there were considerable discrepancies between the various groups, except the control and SCD Probiotics group, and the cafeteria diet and cafeteria diet with SCD Probiotics group. It is essential to note that the consumption of the cafeteria diet was uniform among the groups administered this diet, as indicated by a previous study by our team (Ceylani *et al.*, 2023b).

Biochemical parameters

Ileum: The spectral analysis of ileum and colon samples from control and treatment groups between 4000 and 650 cm^{-1} displayed noteworthy dissimilarities in the spectrochemical bands associated with biomolecular functional groups, as reported by Severcan and Haris, (2012). To assess the impact of SCD Probiotics on cafeteria diet-induced alterations, the FTIR spectral bands were analyzed. Parameters such as band areas, ratios, and bandwidths enabled the identification of molecular changes in lipids, proteins, and nucleic acids. The cafeteria diet significantly increased fatty acid acyl chain length compared to Cnt (P=0.0262) and Prb (P=0.0381) (A_{2922}/A_{2955}), (Fig. 1A), triglyceride content compared to Cnt (P=0.0135) and Prb (P=0.0010) ($A_{1737}/A_{2922+2853}$), (Fig. 1C), and protein carbonylation (A_{1740}/A_{1545}) Cnt (P=0.0005), Prb (P=0.0) and A_{1743}/A_{1536} Cnt (P=0.0026), Prb (P=0.0357), (Fig. 1E and Fig. 1F). However, these effects were markedly reduced when SCD probiotics were added to the diet (CdPrb group). Additionally, the SCD Probiotics group exhibited a higher glucose-to-protein ratio ($A_{1030}/A_{1644+1536}$) (Fig. 1B) compared to Cd (P=0.0031) and CdPrb (P=0.0144) and a higher nucleic acid-to-protein ratio ($A_{1242+1083}/A_{1644+1536}$) (Fig. 1D) compared to Cd (P=0.0266) and CdPrb (P=0.0246). This effect was notably reduced in the CdPrb group. These findings highlight the protective effects of SCD Probiotics against the cafeteria diet.

Colon: The acyl chain length of fatty acids (A_{2922}/A_{2955}) significantly increased in the SCD Probiotics group compared to Cnt (P=0.0045), Cd (P=0.0224), and CdPrb (P=0.0059) for colon tissue (Fig. 2A). Similarly, the glucose-to-protein ratio ($A_{1030}/A_{1644+1536}$) was higher in the SCD Probiotics group, showing marked differences across all groups Cnt (P=0.0040), Cd (P=0.0037), and CdPrb (P=0.0463) (Fig. 2B). Protein phosphorylation (A_{1239}/A_{2958}) increased significantly in colon tissue with cafeteria diet compared to the SCD probiotics (P=0.0418) and CdPrb group (P=0.0232) (Fig. 2C). Nucleic acid-to-protein content ($A_{1242+1083}/A_{1644+1536}$) significantly increased in colon tissue compared to Cnt (P=0.0078) and Cd (P=0.0005) groups but was inhibited by the cafeteria diet in CdPrb group (Fig. 2D). Protein carbonylation (A_{1740}/A_{1545}) was elevated significantly in the cafeteria diet group compared to Cnt (P=0.0083) and Prb (P=0.0165) groups, while SCD Probiotics prevented this increase in both colon and ileum tissues (Fig. 2E). Lastly, lipid-to-protein content ($A_{2927+2853}/A_{1644+1536}$) increased significantly (p=0.0) in colon tissue with the cafeteria diet compared to Cnt (P=0.0154) and Prb (P=0.0324) groups but was controlled by SCD Probiotics in the CdPrb group (Fig. 2F).

Histopathological analysis*:* The results of H&E showed normal histological structure with well-organized intestinal villi and crypts in the ileum and colon tissue in control and SCD Probiotics groups (Fig. 3A-B). In contrast, in the Cd group, loss of epithelial cells in the mucosa and intense inflammatory cells in the lamina propria in both tissues were observed compared to the SCD Probiotics group (Fig. 3A-B). The results suggest that SCD Probiotics may have protective effects against cafeteria diet-induced structural damage. We also observed an increase in the intensity of lymphatic infiltration indicating that the inflammation significantly increased in the Cd group compared to control g (P≤0.0001). In rats receiving SCD Probiotics these intensity values were remarkably reduced (P≤0.0001) as shown in (Fig. 3A-B). In conclusion, SCD Probiotics demonstrated protective effects by reducing the histological damage caused by a cafeteria diet in the ileum and colon (Fig. 3A-B).

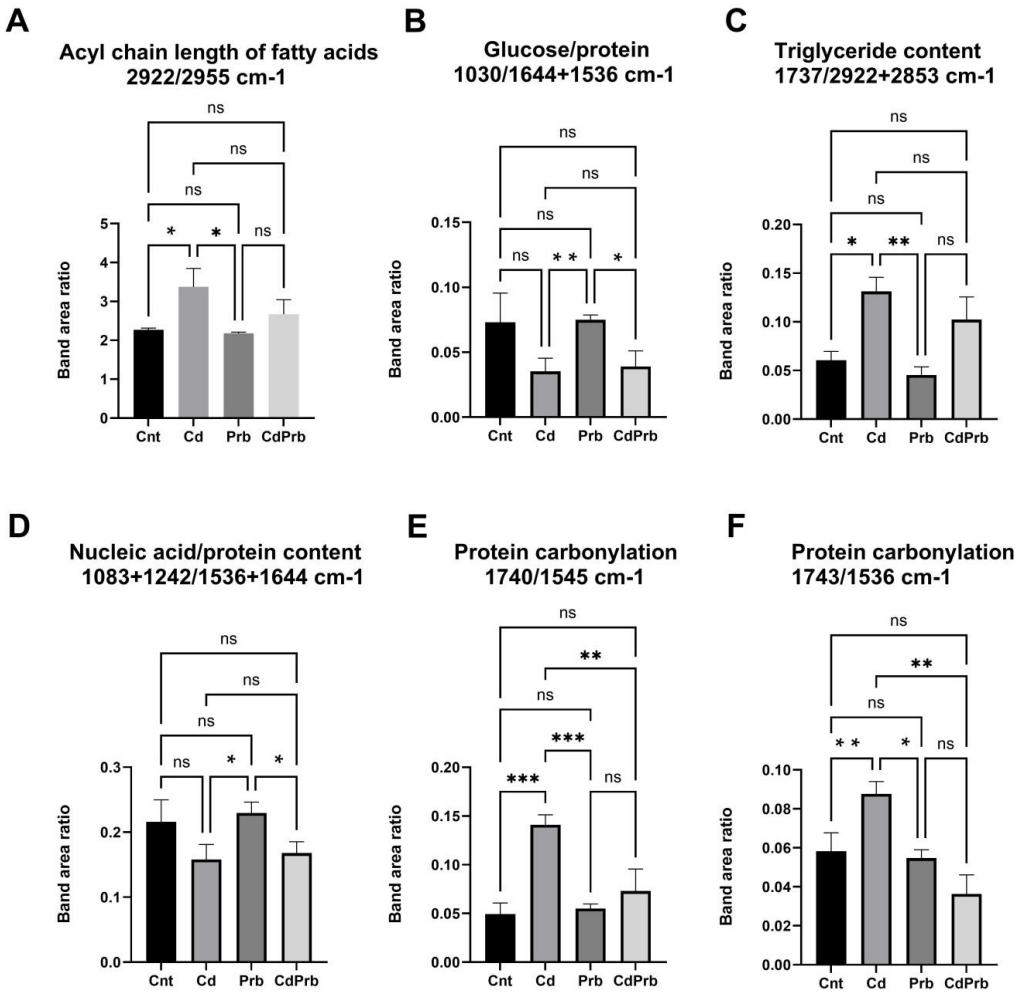

Fig. 1: The quantitative changes in ileum-associated spectrochemical parameters. The band area ratios for A) Acyl chain length of fatty acids (A_{2922}/A_{2955}), B) Glucose/protein ($A_{1030}/A_{1644+1536}$), C) Triglyceride content ($A_{1737}/A_{2922+2853}$), D) Nucleic acid/protein content ($A_{1242+1083}/A_{1644+1536}$), E) Protein carbonylation (A_{1740}/A_{1545}), and F) Protein carbonylation (A_{1743}/A_{1536}). P≤ 0.05*, P≤ 0.01**, and P≤ 0.001***, (one-way ANOVA test with Tukey's post-hoc test). Cnt (control) and Prb (SCD Probiotics), Cd (Cafeteria diet), and CdPrb (cafeteria diet with SCD Probiotics supplementation).

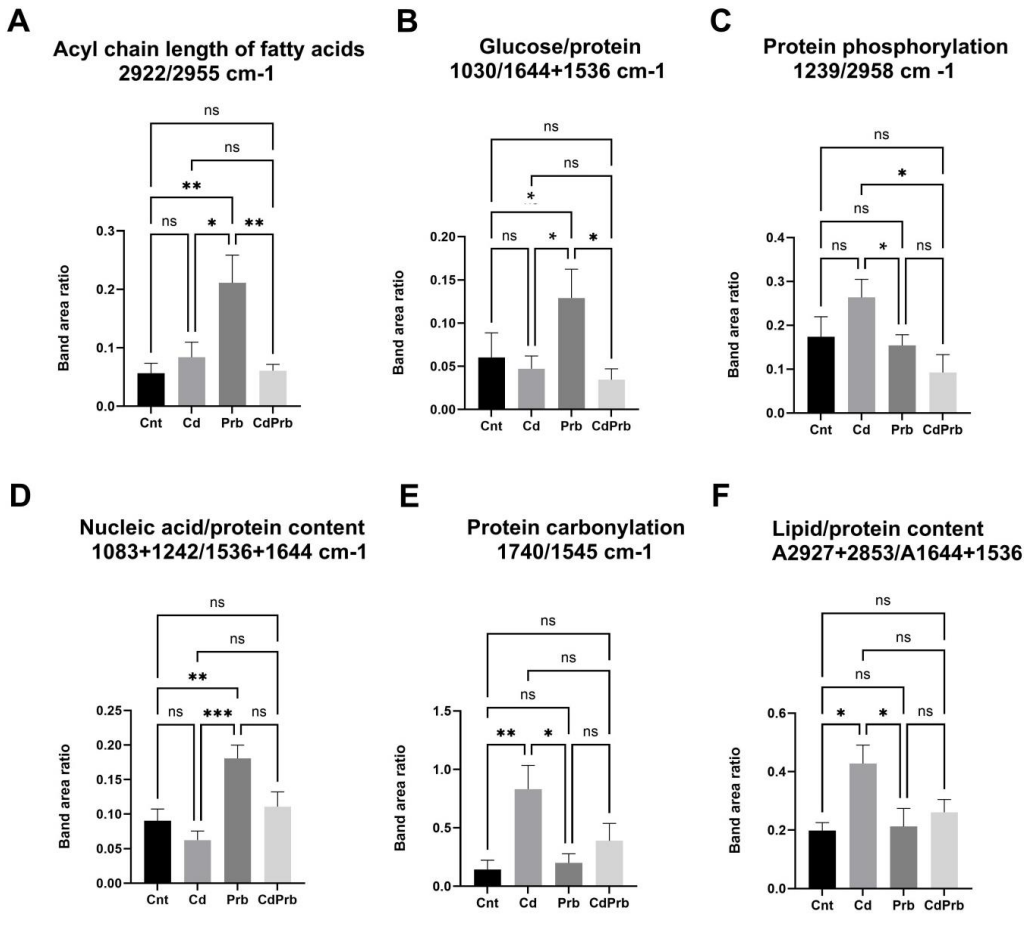

Fig. 2: The quantitative changes in colon-associated spectrochemical parameters. The band area ratios for A) Acyl chain length of fatty acids (A_{2922}/A_{2955}), B) Glucose/protein ($A_{1030}/A_{1644+1536}$), C) Protein phosphorylation (A_{1242}/A_{2971}), D) Nucleic acid/protein content ($A_{1242+1083}/A_{1644+1536}$), E) Protein carbonylation (A_{1740}/A_{1545}), F) Lipid/protein content ($A_{2927+2853}/A_{1644+1536}$). P≤0.05*, P≤0.01**, and P≤0.001*** (one-way ANOVA test with Tukey's post-hoc test). Cnt (control) and Prb (SCD probiotics), Cd (Cafeteria diet), and CdPrb (cafeteria diet with SCD probiotics supplementation).

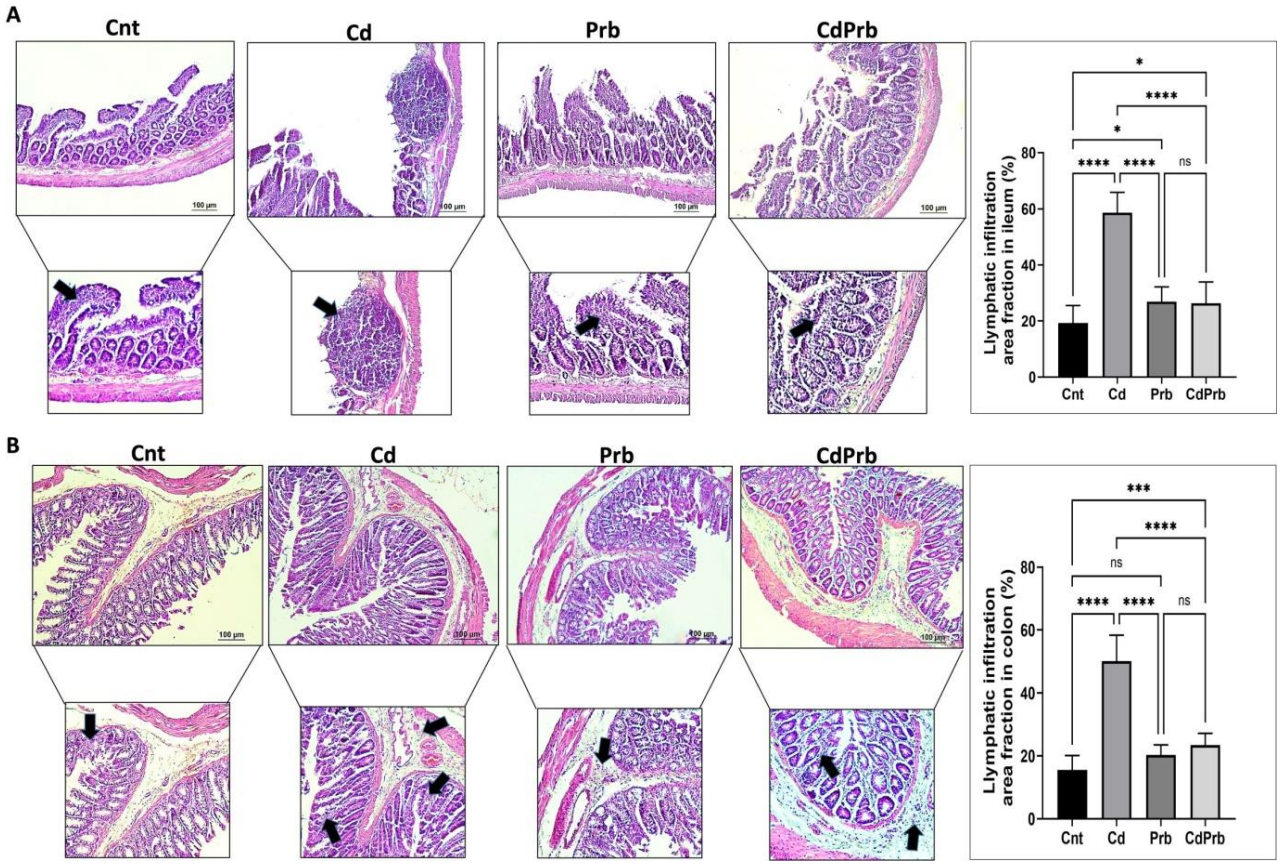

Fig. 3: Representative H&E-stained images and quantification of lymphatic infiltration area fraction (%) in ileum and colon sections across all groups. The figure illustrates the effects of different treatments on lymphatic infiltration in the ileum (A) and colon (B). The cafeteria diet (Cd) group showed increased lymphatic infiltration (black arrows) in both tissues compared to the control (Cnt) group. In contrast, the SCD Probiotics (Prb) and cafeteria diet with SCD Probiotics (CdPrb) groups exhibited significant reductions in lymphatic infiltration. H&E-stained images are magnified to emphasize areas of interest. Quantitative data are presented as mean ± SEM for n = 7 rats per group, with statistical significance indicated as P≤0.05 *, P≤0.001 ***, and P≤0.0001 **** (nonparametric Mann-Whitney U test). Cnt (control) and Prb (SCD probiotics), Cd (Cafeteria diet), and CdPrb (cafeteria diet with SCD probiotics supplementation). Scale bar = 100µm.

Intestinal histomorphometric analysis: We evaluated the crypt depth, villus length, thickness of mucosa, submucosa, muscle layer thickness, and total intestinal wall thickness for each ileum and colon section images. Villus height, crypt depth, the thickness of mucosa and submucosa, and total intestinal wall in the ileum are shown in Fig. 4**.** The cafeteria diet significantly increased villus height, mucosa, submucosa, muscle layer, and total wall thickness compared to the control group (P≤0.01), (P≤0.001), and (P≤0.0001). However, the cafeteria diet significantly decreased crypt depth compared to all other groups (P≤ 0.0001) as shown in Fig. 5. Crypt depth, thickness of mucosa, submucosa, muscle layer, and total wall values in the colon are shown in Fig. 6. In the Cd group, crypt depth, thickness of mucosa, and muscle layer decreased compared to the control group (P≤0.0001). In contrast, in the SCD Probiotics group, these values increased significantly compared to the Cd group (P≤0.0001). In addition, there was a significant increase in the thickness submucosa and total wall in the Cd group compared to other groups (P≤0.05), (P≤0.01), (P≤0.001), and (P≤0.0001). These findings indicate that SCD Probiotics significantly improved histomorphometric changes in rats.

TNF-α and IL-1β expression intensities in the ileum and colon: Both, TNF-α and IL-1β intensities in the ileum and colon sections (Fig. 7A-B) were evaluated by Image J. The results showed that TNF-α and IL-1β were expressed more intensely in the Cd group compared to the control (P≤0.0001). On the other hand, TNF-α and IL-1β expressions in ileum and colon tissues were significantly decreased in CdPrb group compared to Cd group (P≤0.0001). It was observed that the areas stained with DAB positive were predominantly in the mucosa and submucosa.

TB staining analysis: TB staining was utilized to identify mast cells in the ileum and colon, highlighting them in purple violet. In the control group, mast cells with homogenous granules were observed. In contrast, the cafeteria diet group exhibited a significant density of mast cells, particularly in the lamina propria and submucosa layers, in comparison to other groups (Fig. 8A-B) (P≤0.0001). The elevated mast cell density in the cafeteria diet group was associated with an increase in granule presence and intestinal inflammation. However, in the CdPrb group, SCD Probiotics significantly decreased mast cell density (P≤0.0001), indicating their potential to alleviate mast cell proliferation and intestinal inflammation.

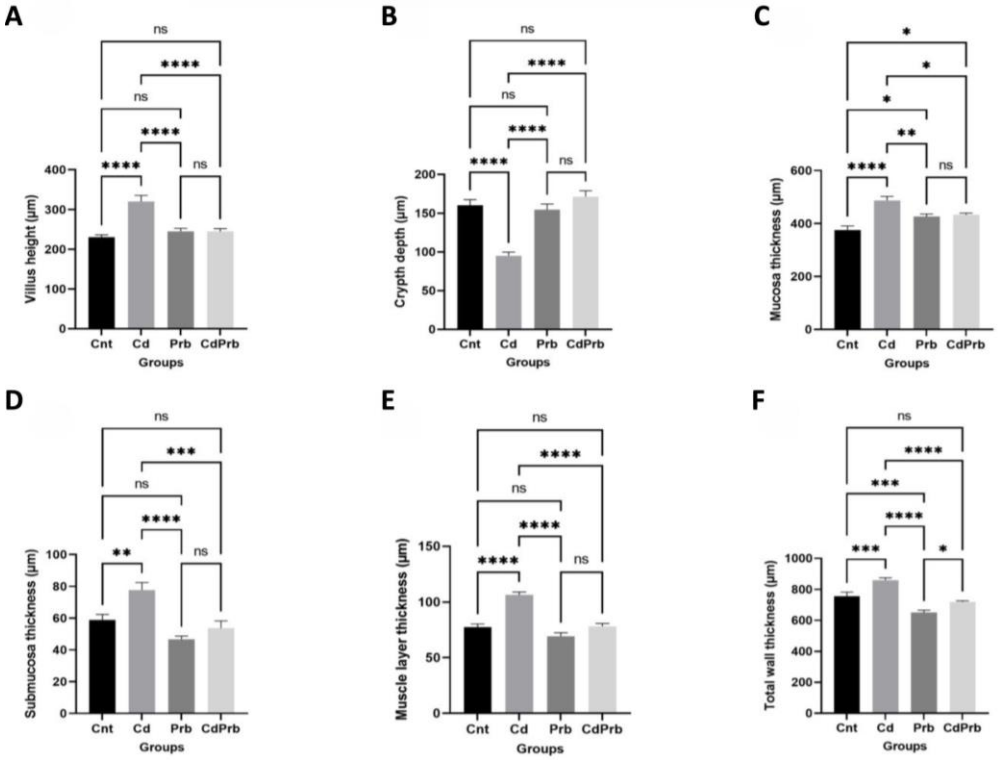

Fig. 4: Histomorphometric analysis of intestinal parameters of rat ileum tissue evaluated in all groups with cafeteria diet-induced rats during the development period. Measurements are shown as (a) villus height (µm), (b) crypt depth (µm), (c) mucosa thickness (µm), (d) submucosa thickness (µm), (e) mucosa layer thickness (µm), and (f) intestinal total wall thickness (µm). P≤0.05 *, P≤0.01 **, P≤0.001 ***, and P≤0.0001 **** (one-way ANOVA test with Tukey's post-hoc test). Results are presented as mean ± SEM (standard error of the mean). Cnt (control) and Prb (SCD probiotics), Cd (Cafeteria diet), and CdPrb (cafeteria diet with SCD probiotics supplementation).

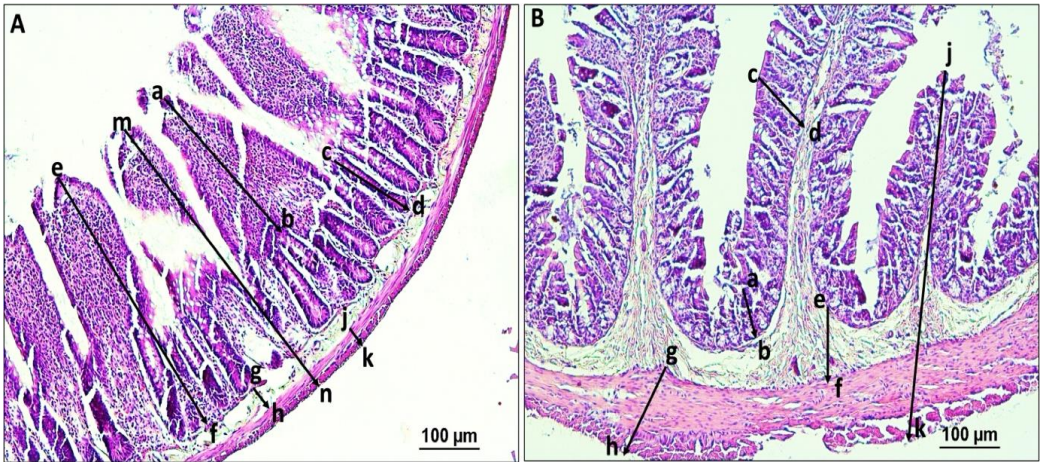

Fig. 5: Representation of histomorphometric parameters in H&E-stained ileum section (A): (a-b: villus height; c-d: crypt depth; e-f: mucosa thickness; g-h: submucosa thickness; j-k: muscle layer thickness; m-n: total wall thickness). Representation of histomorphometric parameters in H&E-stained colon section (B): (a-b: crypt depth; c-d: mucosa thickness e-f: submucosa thickness; g-h: muscle layer thickness; j-k: total wall thickness).

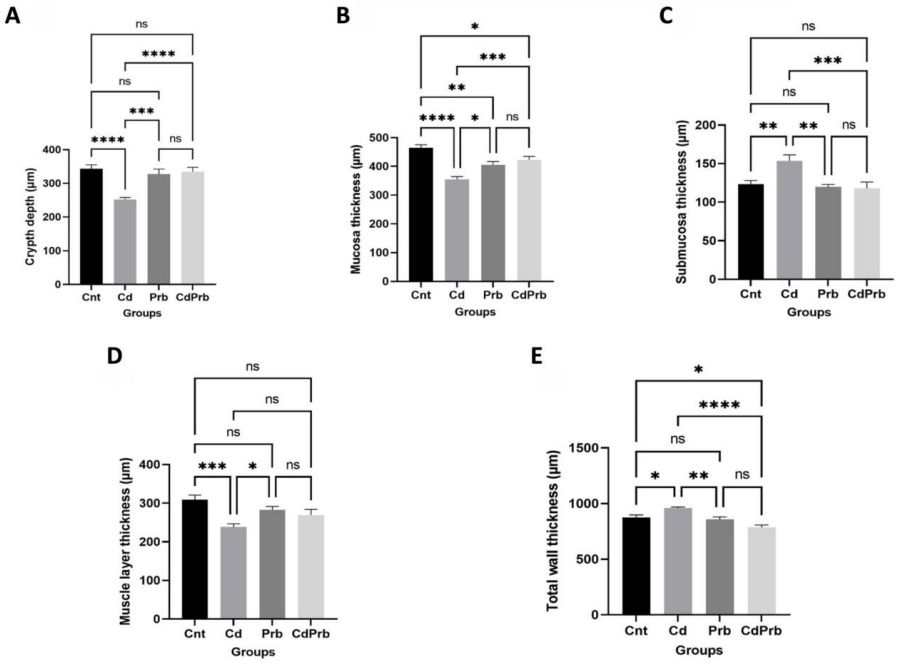

Fig. 6: The graphs of histomorphometric analysis of intestinal parameters of colon sections in all groups. Measurements are shown as (A) crypt depth (µm), (B) mucosa thickness (µm), (C) submucosa thickness (µm), (D) muscle layer thickness (µm), and (E) intestinal total wall thickness (µm). P≤0.05 *, P≤0.01 **, P≤0.001 ***, and P≤0.0001 **** (one-way ANOVA test with Tukey's post-hoc test). Results are presented as mean ± SEM (standard error of the mean). Cnt (control) and Prb (SCD Probiotics), Cd (Cafeteria diet), and CdPrb (cafeteria diet with SCD probiotics supplementation).

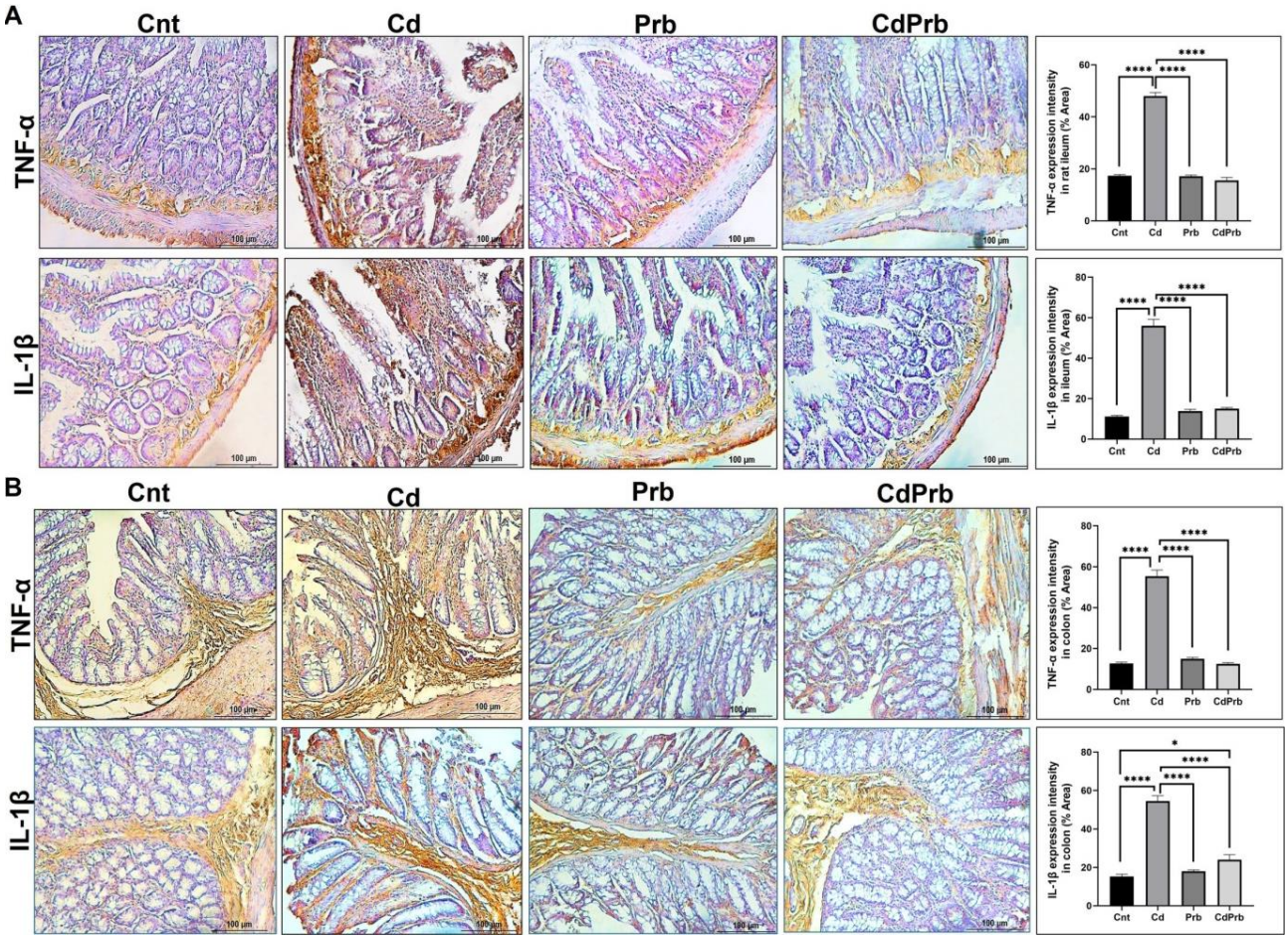

Fig. 7: Representative images of TNF-α and IL-1 β IHC staining expression intensities in the ileum (A) and colon (B). Graphs of TNF-α and IL-1 β staining intensity (%area) in the ileum and colon sections as measured in ImageJ (Fiji). Values are expressed as mean ± SEM; $n = 7$ rats in each group. The significance levels were P≤0.05 *, and P≤0.0001 ****. (IHC staining, Scale bar: 100µm). TNF-α:Tumour Necrosis Factor-alpha, IL-1 β: Interleukin-1 beta. Cnt (control) and Prb (SCD Probiotics), Cd (Cafeteria diet), and CdPrb (cafeteria diet with SCD Probiotics supplementation).

DISCUSSION

Recent studies have shown the impact of probiotics on gastrointestinal health and immune-modulatory responses in both human and animal health, particularly under dietary stress conditions (Yadav *et al.*, 2022; Fu *et al.*, 2023; Teker *et al.*, 2023). This study investigated the effects of probiotic supplementation on cafeteria diet-induced intestinal changes in male Wistar rats. The cafeteria diet during development has altered lipid profiles and increased inflammation and oxidative stress; negatively affecting the structural integrity of the ileum and colon. Probiotics counteracted these adverse effects and significantly reduced the pro-inflammatory cytokines TNF-α and IL-1β, showing marked anti-inflammatory effects, suggesting that probiotics may offer a therapeutic strategy to protect gut health in the face of diet-induced inflammation.

The cafeteria diet during development caused significant elongation of fatty acid acyl chains in ileum tissue, leading to elevated triglyceride levels consistent with hypertriglyceridemia (Sampey *et al.*, 2011). In contrast, specific probiotic strains in SCD Probiotics, such as *Lactobacillus curvatus*, *Lactobacillus plantarum*, and *Bifidobacterium bifidum*, have been shown to play a beneficial role in maintaining intestinal health, effectively reducing structural lipid changes in the colon (Park *et al.*, 2013). Supplementation with *Lactobacillus plantarum* in dairy goats has shown benefits in modulating gut microbiota and improving the fatty acid composition of milk, enhancing polyunsaturated fats which are beneficial for health (Maragkoudakis *et al.*, 2010).

Studies also show that *Lactobacillus curvatus* regulates lipid metabolism through the regulation of fatty acid oxidation, while *Lactobacillus plantarum* and *Bifidobacterium bifidum* reduce cholesterol and improve lipid profile (Khokhlova *et al.*, 2012; Park *et al.*, 2013). Each strain exhibits specific functions and combining multiple strains may help manage lipid metabolism and inflammatory processes. In post-weaning lambs, *L. plantarum* improved antioxidant activity, increased rumen barrier function, and enhanced immune response, indicating a protective effect on intestinal health (Izuddin *et al.*, 2020). Probiotics appear to increase self-renewal in epithelial tissue by increasing glucose and nucleic acid levels in both tissues, leading to a decrease in protein carbonylation, an important marker of oxidative stress (Akagawa, 2021). Our previous studies reported similar antioxidative effects of SCD probiotics and their protective role in the hepatic glutathione system under a cafeteria diet (Aba *et al.*, 2023). These findings underline the therapeutic potential of probiotics in counteracting diet-induced lipid disorders and oxidative stress.

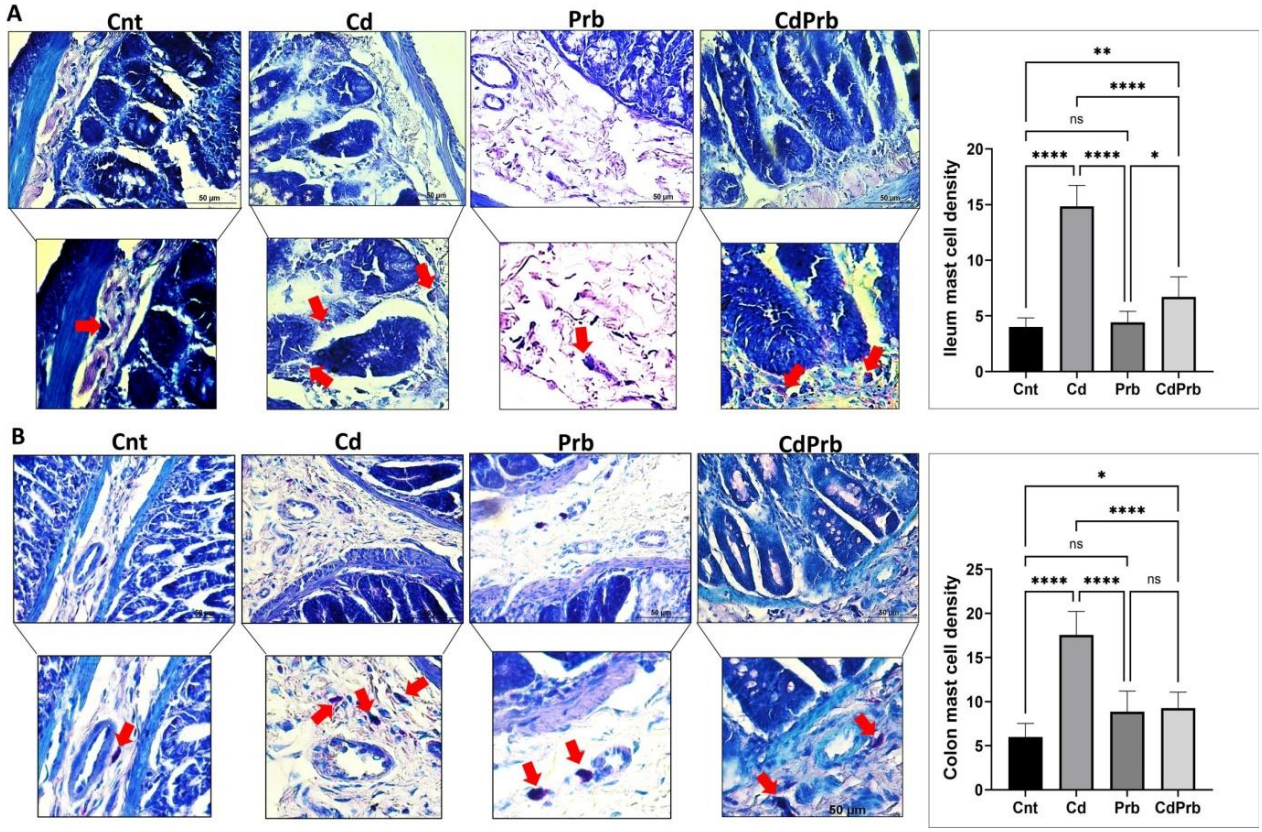

Fig. 8: Representative images of TB staining and quantification of the area fraction (%) of mast cell density in ileum and colon sections in all groups. The figure shows the mast cell density in the ileum (A) and colon (B) of all groups. In the ileum and colon sections, where mast cells are indicated by red arrows, mast cell densities significantly increased in the cafeteria diet (Cd) group compared to the control (Cnt) group, whereas the SCD probiotics (Prb) and cafeteria diet containing SCD Probiotics (CdPrb) groups exhibited significant decreases in both tissues. Quantitative data are presented as mean ± SEM for n = 7 rats per group and statistical significance is indicated as P≤0.05* and P≤0.0001 **** (non-parametric Mann-Whitney U test) compared to control. Cnt (control) and Prb (SCD Probiotics), Cd (Cafeteria diet), and CdPrb (cafeteria diet with SCD Probiotics supplementation).Scale bar = 50 µm.

Probiotics and their role in modulating immune signaling pathways, enhancing gut barrier integrity, and regulating inflammatory cytokine production are essential protective mechanisms. Recent studies revealed modulatory roles of the probiotics on regulatory T cells during immune responses, by reducing pro-inflammatory cytokines such as TNF-α and IL-1β (George Kerry *et al.*, 2018). Probiotics can also have a preventative role on bacterial translocation and enhance selective permeability of the intestinal wall, reducing obesogenic diet-induced inflammation and oxidative stress (Plaza-Díaz *et al.*, 2017).

Short-chain fatty acids (SCFAs) such as butyrate produced by probiotics have specific roles in both anti-inflammatory response and tight junction protein expressions for epithelial integrity (Hamilton *et al.*, 2015; Shin *et al.*, 2023). SCFAs produced by *Lactobacillus plantarum* contribute to the reduction of inflammation and improvement of the intestinal barrier (Hamilton *et al.*, 2015; Markowiak-Kopeć and Śliżewska, 2020). *Bifidobacterium bifidum* secretome exerts immunoregulatory effects on the intestinal mucosa, influencing IgA production and strengthening the intestinal barrier (Khokhlova *et al.*, 2012; Mazziotta *et al.*, 2023). *Bifidobacterium bifidum* H3-R2 has also shown immunoregulatory effects by enhancing IgA production and strengthening the intestinal barrier, supporting gut health in ruminants (Shang *et al.*, 2022). These results support the observed reductions in inflammatory markers and histologic improvements in the probiotic-supplemented groups in our study and highlight the therapeutic potential of probiotics in managing diet-induced inflammatory stress.

The inflammatory effects of a cafeteria diet on the ileum and colon and the anti-inflammatory features of the probiotics were recognized in other studies (Tang *et al.*, 2020). Our results revealed that probiotic supplementation prevented intestinal inflammation by improving epithelial and mucosal histology. During rat development, cafeteria diet-induced mucosal hyperplasia and enteroendocrine cell proliferation. Probiotics initiated adaptive changes such as increasing villi height and tunica muscularis thickness, which improve nutrient absorption and bowel transit (Hamilton *et al.*, 2015; Wang *et al.*, 2015). These results highlight the potential of probiotics to mitigate the negative effects of high-fat diets, with important implications for improving health and managing diet-related metabolic stresses in both humans and animals.

Probiotics with beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* plays modulatory roles on gut microbiota composition by decreasing the prevalence of pathogenic species (Khokhlova *et al.*, 2012). Enhanced probiotics in the microbiome support the reduction of inflammation by pathogenic species and improve gut health. Recent studies have shown that obesogenic diets-induced impairments in Firmicutes to

Bacteroidetes ratio are resolved by probiotic supplementation (Wang *et al.*, 2015). Restoring microbial diversity can prevent metabolic endotoxemia characterized by elevated lipopolysaccharide (LPS) levels that initiate systemic inflammatory signaling cascades. In dairy cows experiencing grain-induced subacute ruminal acidosis (SARA), increased ruminal LPS levels were associated with systemic inflammatory responses, highlighting the significance of endotoxin management in livestock health (Guo *et al.*, 2022a). In calves, an intravenous LPS challenge led to marked increases in proinflammatory cytokines, emphasizing the acute inflammatory impact of endotoxins in young ruminants (Plessers *et al.*, 2015). These results suggest that probiotics can reduce inflammatory responses by maintaining microbial balance and counteract the effects of high-fat diets on the gut.

This study has shown that probiotic supplementation during rat development effectively attenuated inflammation in both the ileum and colon by reducing TNF-α and IL-1β expression compared to cafeteria diet groups. These results are supported by previous studies that demonstrate the role of probiotics in the inhibition of pro-inflammatory cytokines and modulation of inflammation through regulatory T cell (Kazemi *et al.*, 2020). Additional studies have found that probiotics reduce TNF-α and IL-1β expression, reduce colitis, restore tight junction integrity, and prevent bacterial translocation, thus preserving intestinal barrier (Plaza-Díaz *et al.*, 2017; Guo *et al.*, 2022b). Further investigations have shown that Bifidobacterium strains from healthy infants induced anti-inflammatory effects and triggered the mucosal barrier by modulating immune responses in human infant (Sharma *et al.*, 2022). Consequently, our study suggests that probiotics might be crucial for managing systemic inflammation and gut functions and could also have important implications for both human and animal health and welfare.

Mast cells, whose numbers are affected by different types of diets, are known to be involved in regulating microbial diversity and modulation of the intestinal barrier (Kelly *et al.*, 2015). In this study, cafeteria diet consumption during the development led to an increase in both mast cell density and diet-induced inflammation. Mast cell density was found to be decreased after probiotic supplementation with attenuation of inflammation in the ileum and colon. The mechanisms by which cafeteria diets affect mast cell density remain to be fully understood. However, our results indicate that probiotic supplementation, especially during critical developmental stages, plays a crucial role in reducing inflammation associated with unhealthy diets. Probiotic preventions not only hold promise for managing metabolic gut diseases in humans but also for animals in the future.

Conclusion: This study demonstrates that probiotic supplementation during development can effectively protect intestinal health by reducing pro-inflammatory cytokines (TNF-α, IL-1β), oxidative stress, and preserving the structural integrity of the ileum and colon. Our findings support the role of probiotics in mitigating gut inflammation induced by unhealthy diets and suggest their potential as a dietary intervention for managing metabolic syndrome-related intestinal disruptions. These results provide valuable insights into the use of probiotics as a therapeutic strategy to support gastrointestinal health in both humans and animals. Further studies are required to uncover the mechanisms behind these protective effects and explore their applications in both veterinary and human contexts.

Authors contribution: TC and HTT contributed to all aspects of this study, from conceptualization to writing and editing. HÖ conceived and designed the project/study, investigation, methodology, validation, and visualization. SK performed histopathological analysis, interpreted results, and contributed to writing and editing. AE performed histopathological analysis. AIG and HA were involved in the investigation, methodology, validation, and visualization. All authors have approved the final manuscript.

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