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Immunomodulatory effect of synbiotic goat milk cheese on indomethacin-induced inflammation rats

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ABSTRACT

The aim of this study was to determine immunomodulatory effect of synbiotic goat milk cheese on indomethacin-induced inflammation rats. The rats were divided into 6 groups: synbiotic cheese (*Lactobacillus rhamnosus* + glucomannan porang) (1.44 g/d), synbiotic cheese (0.72 g/d), synbiotic cheese (0.36 g/d), probiotic cheese (*Lactobacillus rhamnosus*) (0.72 g/d), control rats (standard AIN-93 only), and indomethacin only. The results showed that the tumor necrosis factor (TNF)- α and nitric oxide (NO) levels in rats fed with synbiotic 1.44 and 0.72 g/d were not significantly different from those found in control rats. The plasma and liver glutathione peroxidase (GPX)1 levels in the rats treated with synbiotics and probiotics showed no difference from those induced with indomethacin only. The GPX1 gene expression in liver tissue in rats treated with synbiotic and probiotic was not different from those of indomethacin-induced rats. The jejunum in synbiotic cheese group experienced less mucosal or epithelial damage compared to that in the group induced with indomethacin only. There were more mucin-producing goblet cells in the crypt and more microvilli in the jejunum and ileum of rats treated with synbiotic or probiotic cheese. In conclusion, the synbiotic goat milk cheese can act as an immunomodulator in the inflammatory rats which is through the production of TNF-α and NO so that the TNF-α and NO are found similar to control rats. The synbiotic goat milk cheese can reduce damage to the intestinal epithelial cells and increase the number of mucin-producing goblet cells.

Introduction

Unhealthy diets such as excessive consumption of sugar, saturated fats, and processed foods as well as the use of non-steroidal anti-inflammatory drugs (NSAIDs) can enhance the risk of developing intestinal inflammation. Indomethacin is a non-steroidal anti-inflammatory drugs that can cause the pathogenesis of intestinal damage, namely causing injuries such as ulcers, haemorrhagia, leakage, and strictures to humans and experimental animals (Fukumoto *et al*., 2011).

The digestive tract is subject to a large and continuous number of external antigenic boosts from food and microorganisms. These organs interact in a complex and integrated manner among diet, foreign pathogens, and local immunological and non-immunological processes. There is evidence that increased fermentable soluble fiber (prebiotics) may stimulate several characteristics of the immune system, such as gut-associated lymphoid tissues (GALT). Changes in intestinal microflora that occur with intake of prebiotic fiber can intercede immune alterations through direct interactions of lactic acid bacteria or bacterial products (cell walls or cytoplasmic compounds) with immune cells in the intestine, generation of short-chain fatty acids (SCFA) from fiber fermentation, or by alterations in mucin production (Schley and Field, 2002).

Intake probiotics and synbiotics can be in various forms and quantities, including yogurt and other fermented milks; cheese and various fermented foods; and also, as avoidance and cure for disparate GI tract abnormality and other illnesses such as allergy. In the last decade, there has been a significant increase in the prevention and treatment of various disorders using probiotics and synbiotics. It is known that functional foods and nutraceutical ingredients from probiotics and synbiotics have a positive impact on health because they can contribute to immunity and the ecology of the intestinal microflora (Sáez-Lara *et al*., 2016).

Fermented milk and cheese can be made from various types of milk, including goat milk. The unique aroma and taste as well as its nutritional value and health benefits are characteristics of goat milk (Hayaloglu *et al*., 2013). The factors that influence the characteristics of goat milk include breed, genetics, physiology, feed, environment, and production technology (Raynal-Ljutovac *et al*., 2008). The chemical composition and behavior of milk technology can affect the quality of cheese including physicochemical, microbiological, and sensory qualities (Fresno *et al*., 2020). The addition of prebiotics, probiotics or a combination of prebiotics and probiotics (synbiotics), can be done to increase the health effects of dairy products.

The nutritional content of milk and dairy products can improve the health of the digestive tract in patients with colitis and can also be used as part of the diet (Russ *et al*., 2010). The contribution of goat milk with nutritional value and therapeutic benefits is superior to cow's milk (Slačanac *et al*., 2010). It is known that goat milk has lower allergenicity than cow's milk and has higher digestibility so that it can be used as an alternative to cow's milk (García *et al*., 2014). An excellent matrix for the development of functional foods is goat milk (Silanikove *et al*., 2010), and the nutritional effects of goat milk products can be enhanced by enriching them with probiotic strains (Mukdsi *et al*., 2013).

In the forests of Indonesia, many porang (*Amorphophallus oncophyllus*), local tubers, are found and are beginning to be cultivated. The glucomannan contained in porang tubers is similar to Amorphophallus konjac, and is an in vivo prebiotic (Harmayani *et al*., 2014) that selectively increases the growth of probiotic bacteria, such as *Lactobacilli* and *Bifidobacteria* (Al-Ghazzewi and Tester, 2012). According to Koswara (2013), the glucomannan solution can form a thin layer (film) that has transparent properties. The film can dissolve in water, gastric acid, and intestinal fluids. Glucomannan has melting properties like agar so that it can be used as a microbial growth medium. The health benefits of consuming glucomannan according to Yao-ling *et al.* (2013) among other things, are that it has anti-obesity activity, hypolipidemic and hypoglycemic effects, anti-inflammatory activity, anti-cancer activity, immunomodulatory activity, and laxative effects.

In general, those used as probiotics are lactic acid bacteria, *Bifidobacteria*, and *Lactobacilli*. Probiotic bacteria include not only *Lactobacilli* spp., certain types of *Streptococcus*, and *Bifidobacteria* spp., but also

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other non-pathogenic bacilli such as *E. coli*-Nisle 1917 and the yeasts of *Saccharamyces boulardii*. These probiotics secrete short chain fatty acids (SCFA), whose activity results in a decrease in the luminal pH and the production of bactericidal proteins. Butyric acid, which is a byproduct of bacterial fermentation of fiber, can provide food for colonic enterocytes, improve mucosal integrity, and improve intestinal dysmotility (Lakhan and Kirchgessner, 2010). Isoflavone glycosides in cooked soybean syrup can be hydrolyzed using β-glucosidase produced by *Lactobacillus rhamnosus* FNCC 0052 which is *Lactobacillus casei* subsp. *rhamnosus* IFO 3425 (NBRC 3425) (Matsuda *et al*., 1992). Mahulette *et al*. (2018) successfully isolated *Lactobacillus rhamnosus* NBRC 3425 from inasua, a kind of fermented fish food from Maluku, Indonesia. In addition to being possibly isolated from the environment related to fermented food, *Lactobacillus rhamnosus* NBRC 3425 can also be isolated from the gastrointestinal and vaginal tracts of humans and animals. *L. rhamnosus* can be classified into the *Lactobacillus casei* group, which includes *L. casei* and *L. paracasei* (Okai *et al*., 2019), and which is a Gram-positive and rod-shaped bacterium. *L. rhamnosus* strain is known as a probiotic because it has health benefits for humans (Zamberlin *et al*., 2011).

A previous study reported that goat milk and goat milk yogurt can act as functional foods for the improvement of intestinal bowel diseases. This has been proven by a study of probiotic *Lactobacillus acidophilus* added to goat milk and goat milk yogurt. With or without honeybees, the addition showed a similar protective effect to sulfasalazine against intestinal damage in rats with colitis induced by acetic acid (de Assis *et al*., 2016). However, the immunomodulatory effects of synbiotic goat milk cheese using *L. rhamnosus* IFO 3425 as probiotics and porang glucomannan as prebiotics have not been reported within the inflammatory subjects. Thus, the aim of this study was to evaluate the effect of synbiotic goat milk cheese on the immunomodulatory properties of indomethacin-induced inflammation rats.

Materials and methods

Cheese preparation

Cheese was processed from the milk of Ettawah crossbred goats reared in north Yogyakarta, Indonesia. The cheese making process consisted of two treatments: probiotic cheese (goat milk + *L. rhamnosus* FNCC 0052), and synbiotic cheese (goat milk + *L. rhamnosus* + 0.2% (w/v) porang glucomannan). *L. rhamnosus* FNCC 0052 which was obtained from Glucomannan was extracted from the tubers of porang plant (*Amorphophallus oncophyllus*), and the porang was obtained from the Faculty of Agricultural Technology, Universitas Gadjah Mada, Indonesia.

Animals used

Male Wistar rats of 8-12 weeks old were individually caged and housed. They were fed with unrestricted amounts of a standard laboratory diet AIN-93G (Reeves *et al*., 1993) for 7 days and were then randomly assigned into 6 groups: (1) synbiotic cheese A (dose 1.44 g/d), (2) synbiotic cheese B (dose 0.72 g/d), (3) synbiotic cheese C (dose 0.36 g/d), (4) probiotic cheese (dose 0.72 g/d), (5) control rats (standard AIN-93 only), and (6) indomethacin (received indomethacin).The starter of lactic acid bacteria (8.0 log CFU/mL of *Lactobacillus rhamnosus* FNCC 0052) was inoculated into the pasteurized milk as raw material of cheese of as much as 3% (v/v). Probiotic and synbiotic cheese products contain 8.60 and 8.68 Log CFU/g of lactic acid bacteria, respectively. Indomethacin (20 mg/kg body weight) was orally administered to rats to induce intestinal injuries (inflammation) after 4 weeks of treatment and 24 h before sacrifice (Menozzi *et al*., 2009). All groups, except for the control rats (group 5), were treated with indomethacin. Each group consisted of 6 replicates (n= 6 rats) and received standard AIN-93.

After 4 weeks of treatment, blood was taken from rats for a plasma GPX1 analysis. Furthermore, the rats were sacrificed using the ketamine (80 mg/kg) anesthesia, and peritoneal macrophages were sampled for NO and TNF alpha analyses. The liver tissue was also analyzed for the GPX1 levels, while the intestinal tissue was subjected to a TNF analysis. For the analysis of TNF and GPX1 gene expression, an intestinal and liver tissue analysis was carried out respectively. Tissue staining was carried out on the jejunum and ileum intestinal tissue to determine changes in the structure of the intestinal tissue using eosin staining. In addition, Periodic Acid-Schiff (PAS) staining was also carried out to determine the cells that produce mucin in the intestinal tissue. All procedures related to animal handling and experimentation have been approved by the Ethics Commission from The Integrated Research and Testing Laboratory Universitas Gadjah Mada (Certificate number: 00078/04/LPPT/VIII/2017).

Analysis of TNF -α in peritoneal macrophage culture

Ketamine (80 mg/kg) was used for anesthesia in the sacrificed rats. Disinfection in rats used 70% alcohol sprayed on the abdomen of rats whose skin had been opened in a supine position. The peritoneal cavity was injected with 10 mL of a cold RPMI medium using a syringe. The rat's abdomen was gently massaged, then about 30 mL of peritoneal fluid was taken using the same syringe and transferred to a 50 mL sterile polypropylene tube on ice and then centrifuged at 1200 rpm for 10 minutes at 4ºC. A complete RPMI medium of 1-2 mL was added to the pellet (precipitate) after the supernatant had been removed. A total of 930 µL of an RPMI medium and 50 µl of trypan blue (1.0 mL) were added to a 20 µL aliquot and stirred, after which the number of living cells was counted using a hemocytometer. The cell concentration was adjusted to around 106 cells/mL by resuspending it. Into each well of a sterile 24-well microplate, 200 µL of cell suspension was cultured and incubated in 5% CO_2 at 37ºC for 24 hours (Garcia *et al*., 2002). A TNF alpha analysis was performed on culture supernatants using the ELISA method following the instructions in the Rat TNF-α ELISA Kit (FineTest®, Catalog No. ER1393).

Analysis of nitric oxide (NO) in peritoneal macrophage culture

Griess reaction in colorimetric assay is used to analyze the presence of Nitrite (NO_2^-), which is a stable NO end product. Each well of the sample on the microplate was pipetted with 50 µL of peritoneal macrophage supernatant culture and added with 50 µL of Griess reagent. The plate was incubated for 10 minutes at a room temperature, and then the absorbance of 550-590 nm was determined using a microplate reader. The standard curve of sodium nitrite in the range of 0-100 µM was used to determine the concentration of NO_2. The 10 mM nitrite standard (69.0 mg of sodium nitrite in 100 mL of deionized water as a stock solution) was used to make the standard curve. The Griess reagent was made from a mixture of Griess A and Griess B (1:1). Griess A was prepared by adding 0.1 g of N-(1-naphthyl) ethylenediamine dihydrochloride to 100 mL of deionized water, while Griess B was prepared by adding sulfanilamide to 100 mL of 5% (v/v) orthophosphoric acid (Zykova *et al*., 2000 ; Titheradge, 1998).

Analysis of GPX1 in plasma and liver

Glutathione peroxidase in the blood plasma and liver tissue was analyzed according to the instruction found in Rat GPX1(Glutathione peroxidase 1) ELISA Kit (FineTest®, Catalogue No.: ER0274).

Analysis of TNF-α mRNA expression in intestinal tissue

The determination of TNF-α mRNA levels by RT-PCR. Total RNA was extracted from the intestinal tissue. The expression of mRNA for the inflammatory mediator TNF-α was normalized to that of GAPDH. The total intestinal tissue RNA was extracted with Trizol reagent. The expression of

mRNAs for TNF -α was quantified by the real-time RT-PCR. The expression levels of mRNAs encoding the cytokines TNF-α in damaged intestinal tissue and normal intestinal tissue were standardized against GAPDH mRNA. The level of TNF-α in the damaged intestinal tissue was expressed as a ratio to the mean value for normal intestinal tissue (Watanabe *et al*., 2004).

The proinflammatory cytokine TNF-α mRNA expression was quantified by the real-time RT-PCR of Khan *et al*. (2016). The total RNA was isolated from the rat intestinal tissue (approximately 30 mg) using Trizole reagent. The extracted RNA was dissolved in 30µL of nuclease-free water and stored at – 20ºC. RNA concentration and the purity was determined usinga Spectrophotometer.

A template of as many as 2 µLin a reaction containing 0.25 µM of each primer and 12.5 µL of SYBR Green Real-time PCR MasterMix (Applied Biosystems, USA) was used to perform the real-time PCR. Each run with the 50ºC (2 minutes) and 95ºC (10 minutes) program was followed by 45 cycles of 95ºC (15 seconds), 60ºC (20 seconds), and 72ºC (60 seconds) in the real-time qPCR machine. GAPDH was used as a housekeeping gene for data expression normalization. Data was analyzed using the ΔΔCt method and values were expressed as fold change or the average change in the level of gene expression ($2^{-\Delta\Delta CT}$) relative to control group according to Livak and Schmittgen (2001). Primer sequences (Watanabe *et al*., 2004; Nourian *et al*., 2017) can be seen in Table 1.

Table 1. Primers used for real-time PCR amplification for the TNF-alpha gene.

Primer name	Sequence	bp
TNF-α-F	5-CTGAACTTCGGGGTGATCG-3	20
TNF-α-R	5-GCT TGG TGG TTT GCT ACG AC-3	20
GAPDH-F	GTA TTG GGC GCC TGG TCA CC	20
GAPDH-R	CGC TCC TGG AAG ATG GTG ATG G	22

The analysis of GPx gene expression levels in liver tissue used real time PCR (Al-Rejaie *et al*., 2013; Kürüm *et al*., 2015). Total RNA was extracted/isolated with Trizol reagent. The RNA quantity/concentration per µL was measured at 260 nm. The integrity of total RNA was characterized using a UV spectrophotometer and ethidium bromide stained agarose gel. The isolated RNA had an A ratio of 260/280: 1.9–2.0.

The first-strand cDNA was synthesized from 1µg or 4 µg total RNA by reverse transcription with the SuperScript™ first-strand synthesis system kit (Invitrogen, CA, USA), according to the kit instructions. cDNA was stored at -20ºC for real-time PCR.

Gene expression was carried out using Real-time PCR. Real time PCR used the ΔΔCT method. The GAPDH gene wasused as a housekeeping gene. Each PCR reaction contained 3 µL of water, 1 µL of forward primer (5'CAGTTC GACATCAGGAGAAT3'), 1 µL of reverse primer (5'AGAGCGGGTGAGCCTTCT 3'), 10 µL of SYBR Green Supermix (Roche), and 5 µL of cDNA for a total volume of 20 mL. The quantification of mRNA was carried out in a Light Cycler 480 instrument using SYBR I reagents (Roche diagnostics, USA) PCR reaction with a 5-minute program at 95ºC, 45 cycles of 10 seconds at 95ºC. Each analysis was carried out with 5 biological replicates and 3 technical replicates.

Primer

GPx1: Forward primer(5'CAGTTCGGACATCAGGAGAAT3'), Reverse primer (5'AGAGCGGGTGAGCCTTCT 3').
Glyceraldehyde 3 Phosphate Dehydrogenase (GAPDH) used to normalize GPX gene expression. Primer forward (5'ACCACAGTCCATGCCATCAC3') and reverse (5'TCCACCACC CTGTTGCTGTA 3') used to GAPDH gene expression.

The normalization of gene expression was carried out using the Delta Ct (ΔCt = Ct target gene – Ct housekeeping gene) (Kürüm *et al*., 2015).

Hematoxylin-eosin staining

The purpose of Hematoxylin-Eosin histology staining is to determine whether there is abnormal cell morphology in the tissue being examined. The principle of Hematoxylin-Eosin Histology stainingis that Chromatin in the nucleus will bind the alkaline paint (hematoxylin) and cytoplasmic proteins will bind the acidic paint (eosin) so that the cells will be pink with a purplish blue nucleus.
Briefly: The dried preparations in xylol were deparaffinized 3 times (10-15 minutes each). The slides were immersed in 96% alcohol 2 times (5 minutes each)washed with running water until the alcohol was removed, immersed in hematoxylin stain for 7-10 minutes,washed with running water until they did not fade,dipped in HCl 2 times for decolorization,washed again with running water, soaked in water for a while until the color turns blue, and then placed in eosin stain for 3-5 minutes. Running water wasused to wash the preparat, then put it in the alcohol solution and then washed with running water again. Paper wasused to absorb water and then wiped with cotton. The preparation was placed in xylol. The preparat was pressed again with paper, wiped with cotton, then mounted (Anderson and Rolls, 2012)

Periodic Acid-Schiff (PAS) staining

The PAS reaction on tissue sections is useful to demonstrate the presence of mucopolysaccharides. When treated with periodic acid, glycol will be oxidized to aldehyde. After reacting with Schiff's reagent (a mixture of pararosaniline and sodium metabisulfite), the pararosaniline adduct is released which can color the cellular components containing glycol. This reaction can be appliedon blood or bone marrow lining and tissue slice preparations.

The standard procedure for PAS staining were: Slides were fixed for 1 minute at room temperature in Formalin-Ethanol Fixative Solution, rinsed for 1 minute with slowly running water, soaked in periodic acid solution for 5 minutes at room temperature, rinsed several times with distilled water, and immersed in Schiff's Reagent for 15 minutes at room temperature. After use, the Schiff Reagent was immediately closed and returned to the refrigerator (2-8°C). Furthermore, the slideswere washed in running water for 5 minutes to counterstain slides in Hematoxylin SolutionofGill No. 3, for 90 secondsrinsed in running tap water for 15–30 seconds, and air dried and observed microscopically under an oil immersion lens (900x). Slides can be mounted with toluene or xylene-based mounting media. Substances with positive PAS will be pink to red in color and nuclei will be blue (Sigma Aldrich, Procedure No. 395).

Statistical analysis

The data of TNF-α and NO levels in peritoneal macrophage culture, GPX1 level in plasma and liver tissue, GPX1 gene expression in liver, and TNF-α gene expression in intestinal tissue were expressed as mean ± standard deviation from 6 replications. Statistical analyses were performed using one-way analysis of variance followed by Duncan's multiple range test ($p<0.05$ indicated significant differences) using SPSS version 17.0 software (IBM Corp., NY, USA). The data of intestinal cell morphology and mucin-producing goblet cells was expressed descriptively.

Results

TNF - α and NO in peritoneal macrophage

The results of TNF-α and NO analysis of peritoneal macrophage cultures in rat induced by indomethacin with various treatments are mounted in Table 2. TNFα levels of cultured macrophages in rats treated with synbiotic cheese at a dose of 1.4; 0.72 and 0.36 g/day and the indomethacin treated group were not significantly different. The TNF-α

in the indomethacin group was higher ($p<0.05$) than that found in the normal controls. Synbiotics at a dose of 1.44 g/day or o.72 g/day had lower TNF-α than probiotic cheese group at a dose of 0.72 g. However, the TNF level in synbiotics treated was not significantly different from that of the control rats.

All rats treated with cheese had an average NO level similar to that of rats treated with indomethacin alone; however, only the 1.44 g synbiotic cheese treatment and the probiotic treatment alone had NO levels comparable to those of control rats (Table 2).

Table 2. The average of TNF-α and nitric oxide (NO) of peritoneal macrophages in rat induced by indomethacin with various treatments.

Treatment groups	TNF-α (pg/mL)	Nitric oxide (μM)
Control group	464.33±140.91^{a}	5.44±2.02^{a}
Indomethacin group	931.27±528.16^{cb}	8.53±0.94^{bc}
Synbiotic cheese (1.44 g/d)	795.44±276.16^{ab}	7.48±4.19^{ab}
Synbiotic cheese (0.72 g/d)	714.33±210.59^{ab}	10.57±2.05^{c}
Synbiotic cheese (0.36 g/d)	946.55±247.32^{cb}	8.38±1.32^{bc}
Probiotic cheese (0.72 g/d)	1209.33±263.33^{c}	7.76±1.08^{abc}

Different letters (a, b, c) within the same column indicate difference ($P<0.05$)

Glutathion Peroxidase-1 (GPX1) level in plasma and liver

The plasma GPX1 levels in rats that were induced by indomethacin treated with synbiotic cheese and probiotic cheese (containing L rhamnosus at a dose) showed no significant difference from that of the normal control rats or rats that were only induced by indomethacin without being given cheese (Table 3).

The GPX1 levels in the liver of rats induced by indomethacin, based on Table 3, showed that all rats treated with synbiotics and probiotics showed no difference from those that were only induced within domethacin. The control rats showed the highest GPX1 level in the liver.

Table 3. The average of plasma and liver GPX1 in rats induced with indomethacin with various treatments.

Treatment groups	Plasma GPX1 (ng/mL) ^{ns}	Liver GPX1 (ng/mL)
Control group	2030.83±856.92	16170.83±106.55a
Indomethacin group	3565.83±2851.21	14647.50±875.71bc
Synbiotic cheese (1.44 g/d)	1771.66±586.96	14495.50±680.65bc
Synbiotic cheese (0.72 g/d)	2417.50±1389.28	14539.16±719.44bc
Synbiotic cheese (0.36 g/d)	2434.16±982.39	15341.50±868.94ab
Probiotic cheese (0.72 g/d)	2602.50±1429.29	14031.66±1002.45c

Different letters (a, b, c) within the same column indicate significant difference ($P<0.05$); ns: not significant.

GPX1 gene expression in liver

The results of this study showed that probiotic or synbiotic supplementation did not affect the GPX1 gene expression in the liver tissue (Table 4).

Table 4.The relative of GPX1 gene expression in the liver of rat with various treatments.

Treatments	Ekspresi gen GPX1		
	Δ CT	ΔΔ CT	$2^{-\Delta\Delta CT}$ ^{ns}
Indomethacin group	9.5825±0.84^{a}	3.71±0.84^{a}	0.08±0.08
Synbiotic cheese (1.44 g/d)	3.5300±3.15^{b}	-2.34±3.15^{b}	23.31±40.12
Synbiotic cheese (0.72 g/d)	0.9120±6.69^{b}	-0.63±3.39^{b}	4.57±4.54
Synbiotic cheese (0.36 g/d)	4.2800±1.10^{b}	-1.41±1.27^{b}	3.73±3.69
Probiotic cheese (0.72 g/d)	10.3280±1.09^{a}	4.45±1.09^{a}	0.56±0.03

Different letters within the same column indicate significant difference ($p<0.05$).
Control rats had an average ΔCT of 5.87; the average ΔΔ CT was 0 and the average change. GPX1 gene expression ($2^{-\Delta\Delta CT}$) was 1.00.

TNF- α gene expression in intestinal tissue

Based on Table 5, the expression of the intestinal tissue TNF-α gene was the lowest in rats treated with probiotic compared to those treated with synbiotics. This indicates that probiotics play a more important role in reducing TNF-α gene expression than synbiotics.

Table 5.The relative of TNFα gene expression in intestinal tissue of rat with various treatments.

Perlakuan	Ekspresi gen TNFα		
	ΔCT	ΔΔ CT	$2^{-\Delta\Delta CT}$
Indomethacin group	7.43±1.62^{a}	-1.07±1.62^{a}	3.17±2.72^{a}
Synbiotic cheese (1.44 g/d)	7.30±0.61^{a}	-1.20±0.61^{a}	2.45±0.96^{a}
Synbiotic cheese (0.72 g/d)	7.74±0.65^{a}	-0.76±0.65^{a}	1.85±0.94^{ab}
Synbiotic cheese (0.36 g/d)	14.42±2.75^{b}	5.91±2.75^{b}	0.03±0.04^{b}
Probiotic cheese (0.72 g/d)	14.01±0.76^{b}	5.50±0.76^{b}	0.02±0.01^{b}

Different letters within the same column indicate significant difference ($p<0.05$).
Control rats had an average ΔCT of 8.51; the average ΔΔ CT was 0 and the average change TNFα gene expression ($2^{-\Delta\Delta CT}$) was 1.00.

Intestinal cell morphology

Hematoxylin Eosin (HE) staining can be used to determine abnormal cell morphology in the evaluated tissue. The results of jejunum intestinal HE staining in rats with various treatments can be seen in Fig. 1 and Fig. 2.

Jejunum in rats treated with synbiotic cheese (J3) and probiotic cheese (J4) showed the improvement of the villus structure towards normal villus morphology, close to the control rats (J1). However, the indomethacin induced-rat only (J2) - showed some erosions or damage of the villus structure (Fig. 1).

Meanwhile, when observed with a microscope at 400x magnification (Fig. 2), the jejunum of rats induced by indomethacin and treated with synbiotic cheese showed infiltration of inflammatory cells in the submucosa (JA2). In rat that were induced by indomethacin and probiotic cheese treatment, inflammatory cells were found in the tunica muscularis (JD1). Furthermore, in rat that were only induced by indomethacin, inflammatory cells were found in the tunica muscularis (JF1a) and there was erosion in the epithelium (JF1b).

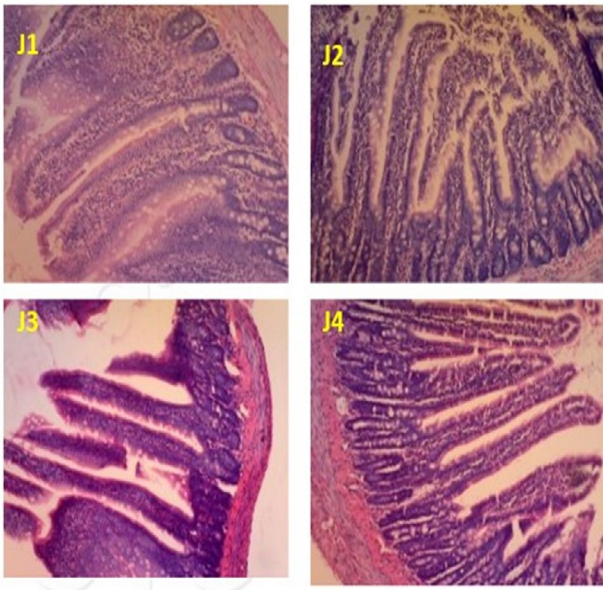

Fig.1. Hematoxylin Eosin (HE) staining of rat jejunum (magnification 10x): J1 (Control rat), J2 (indomethacin treated), J3 (synbiotic treated), J4 (probiotic treated).

Mucin-producing goblet cells

The histological observations of the intestine with PAS staining are

shown in Fig. 3 and Fig. 4. The jejunum of rats induced with indomethacin and treated with synbiotic cheese (JP3) showed a relatively higher number of mucin-producing goblet cells compared to those treated with probiotic cheese (JP4) and control rats (JP1). However, the jejunum in rats with indomethacin induction alone, showed no visible mucin-producing cells (JP2) (Fig. 3).

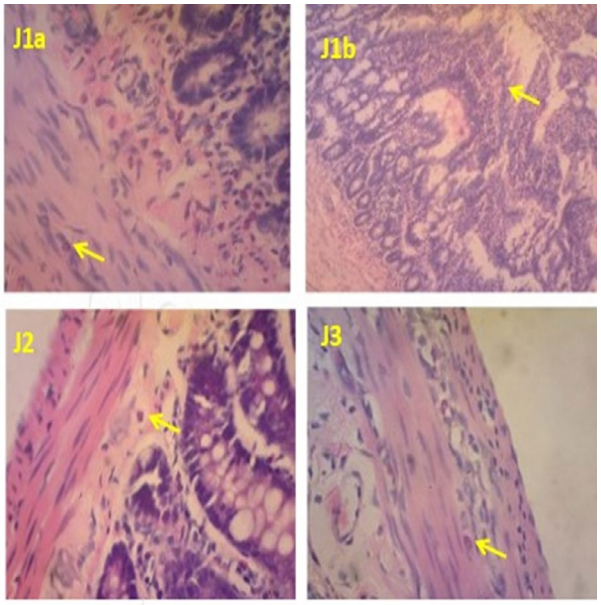

Fig. 2. Hematoxylin Eosin (HE) staining of rat jejunum (maginification 40x): Indomethacin treated showed inflammatory cells (arrow) in the tunica muscularis (J1a) and epithelial erosion (J1b), synbiotic treated showed inflammatory cell infiltration (arrow) in the submucosa (J2), probiotic treated showed inflammatory cells (arrow) in the tunica muscularis (J3).

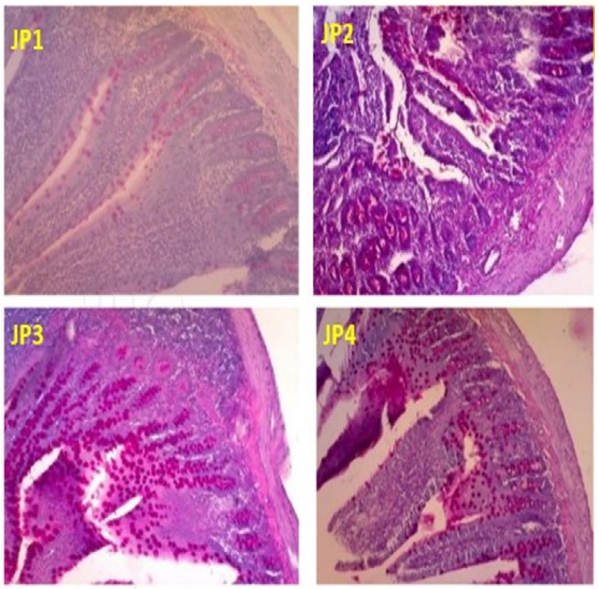

Fig. 3. Periodic Acid–Schiff (PAS) staining of rat jejunum (magnification 10x): JP1 (control rat), JP2 (indomethacin treated), JP3 (Synbiotic treated), JP4 (probiotic treated).

As in the jejunum, the ileum in rats that were induced with indomethacin but treated with synbiotic cheese (IP3) or probiotic cheese (IP4) showed quite a lot of mucin-producing goblet cells similar to those found in the control rats (IP1) (Fig. 4).

Discussion

The lower levels of TNF in synbiotic treatment compared to probiotics indicate the importance of the influence of prebiotics in reducing TNF levels. Prebiotics as probiotic growth stimulators can play a role in reducing the TNF-α in inflammatory rats, which is close to the control. The present study is in accordance to a previous study by Khanna *et al.* (2021), that synbiotic (*Lactiplantibacillus pentosus* GSSK2 and isomalto-oligosaccharides) supplementation can reduce the serum of TNF-α to be higher than the probiotic *Lactiplantibacillus pentosus* GSSK2 alone in the metabolic syndrome rats. TNF-α is produced primarily by activating monocytes/macrophages thatplay a crucial role in the initiation and continuation of inflammation and immunity (Fukumoto *et al*., 2011). The role of proinflammatory TNF-α has been exhibited to influence the early stage of indomethacin (INDO)-induced small intestinal toxicity and affect experimental nonsteroidal antiinflammatory drug (NSAID)-induced gastropathy (Saud *et al*., 2005). More essentially, in patients with IBD, TNF- α is overexpressed in the monocytes and macrophages that infltrate the mucosa; th ere are, however, substantial diferences between CD and ulcerative colitis in the level and localization of monocyte (Takahashi *et al*., 2018). TNF-α has been found in the serum, intestinal tissues, and stool of patients with inflammatory bowel disease (IBD). Synbiotics, which are a combination of probiotics and prebiotics, have negligible adverse effects, while anti-TNF- α antibodies have various adverse efects for IBD and enhance the potential for malignancy (Takahashi *et al*., 2018).

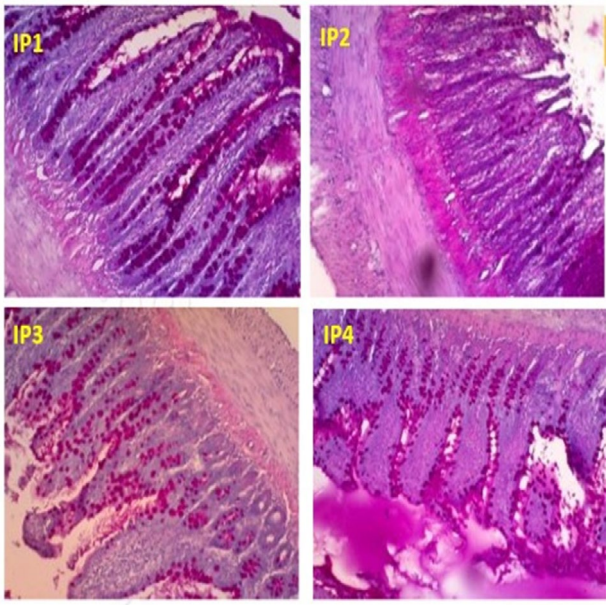

Fig. 4. Periodic Acid–Schiff (PAS) staining of rat ileum (magnification 10x): IP1 (control rat), IP2 (indomethacin treated), IP3 (Synbiotic treated), IP4 (probiotic treated).

The nitric oxide levels in high doses of synbiotics and probiotics treatment were similar to those of the controls, and this indicates the important role of high doses of probiotics *Lactobacillus rhamnosus*. The probiotics in this study were able to reduce the NO levels in rats induced by indomethacin so that they equaled the NO levels in the controls. The results of the present study were in accordance with the previous study. The consumption of the probiotics *L. casei* CRL 431 and *L. paracasei* CNCM I-1518 during the intestinal inflammatory process through reducing the local secretion of proinflammatory cytokines causes reduced the production of ROS and NO by peritoneal macrophages so that they can maintain the integrity of the intestinal epithelial barrier (Monteros *et al*., 2021). Another study, as expected, indicates that the NO release significantly decreased in the metabolite syndrome group compared to that of the control group. Interestingly, the supplementation with multistrain probiotic (7 strains) in the metabolic syndrome rat for 4 weeks recovered the NO release levels comparable to the control group (Llévenes *et al*., 2020). However,the protective and toxic effects of NO often ranparallel, making it difficult to describe the function of NO in the immune system uniformly. Predicting the effects of NOS inhibitors and NO donors is challenging because of their striking intercellular and intracellular signaling capacities, all of which hampers the therapeutic applications (Bogdan, 2001).

Nitric oxide (NO) is a lipophilic, gaseous,and inorganic free radicalthathas pleiotropic activities synthesised by the enzyme Nitric Oxide

Synthase (NOS) from the substrate arginineand withthe presence of O_2 and NADPH it converts L-arginine (L-Arg) into NO. It is an important biomolecule that mediates cellular signaling (Madhu *et al*., 2016; Shreshtha *et al*., 2018). It has a wide spectrum of biological functions including immunomodulation, inflammation, microbial,and tumour eradication. The inflammatory phagocytic cells, epithelial, and neuronal cells express inducible NOS (iNOS)which produces larger volume of NO for longer time (10–100 times more) (Madhu *et al*., 2016). Along with the release of NO, activated macrophage also releases chemical mediators such as ROS and RNS, bioactive lipids and hydrolytic enzymes (Shreshtha *et al*., 2018). The macrophage cytotoxic and bactericidal activities are facilitated by the production of these mediators. Host cell injuries and toxicities can be caused by excessive mediator production, especially by reactive oxygen species (ROS) and RNS (NO, ONOO-). The inhibition of genes responsible for proliferation and antiapoptotic roles involves mediators. The inhibition of Th1 cytokine responses that lead to humoral immune responses and increasing Th2 cytokine responses that leadto allergic responses are related to the influence of NO. Enhancing cyclooxygenase enzyme activity and proinflammatory eicosanoid production isalso related to NO activity as inflammatory mediators. In addition, in diverse immune cells such as lymphocytes, eosinophils, and monocytes, NO also inhibits the expression of a number of cytokines such as IL-1β, TNF-α, IL-6, INF-γ. NO molecules can have dual activities, namely playing a protective and toxic role that runs parallel depending on the conditions of the microcellular environment of the body (Shreshtha *et al*., 2018). The production of large quantities of NO via the up regulation of iNOS can have a variety of effectsthat can be detrimental or beneficial depending on the amount, duration, and anatomical site of synthesis. The benefecial effect of iNOS-induced NO has been found to exert a direct antimicrobial effect (important for the host defense against intracellular pathogens and perhaps forthe maintenance of microbial latency)and to regulate the physiological processes ofthe body at a lower level of concentration. NO may act in concert with the reactive oxygen species to damage microbial DNA, proteins, and lipids (Fang, 1997).

The negative effect of NO is that iNOS-mediated NO production may occasionally become part of a dysregulated immune response, resulting in chronic inflammatory disorders (Kolios *et al*., 2004). At higher levels of concentration, it is harmfulnot only to the microbes or tumor cells but also to the host cells. An important functional molecule that plays a role in the host cell defense mechanisms is nitric oxide. The recruitment of immune cells, pro-inflammatory responses to pathogens, and the facilitation of neurotransmission are triggered by nitric oxide (Snyder and Bredt, 1992). The enzyme iNOS that helps protect against inflammatory challenges facilitates the production of nitric oxide (Xue *et al*., 2018).

In the present study, the highest dose of synbiotic cheese administration showed a similar level of NO concentration in the peritoneal macrophage with control rats. This is parallel with TNF alpha concentration found in rats treated with the highest dose symbiotic cheese and similar to the levels found in the control rats as well (Table 3). According to Foleyand and O'Farrell (2003) and Ibiza and Serrador (2008), the tumor necrosis factor-α trigger macrophages to express inducible nitric oxide synthase (iNOS) and prevent pathogen reproduction by detaching a diverse effector molecules such as nitric oxide (NO). In addition, NO intercedes a beginning periode of signal transduction route, generating the innate immune response againts usual infection.

Previous studies also showed that synbiotics containing *L. plantarum* or *B. coagulans* ($1x10^9$ CFU/mL+ inulin 5%) did not affect the GPX1 levels in the blood plasma of rats in oxidative stress condition (Jafarpour *et al*., 2017). The results of this study are in accordance with a study by Arellano-García *et al*. (2023). They found that a *Lactobacillus rhamnosus* GG dose of 10^9 CFU/day administrations on oxidative stress and inflammation in diet-induced NAFLD in rats could not increase the liver GPX levels in rats fed HFHF diet. The study by Mohammed *et al*. (2019) who used a synbiotic consisting of a mixture of *Bifidobacterium animalis*, *Enterococcus faecium, Lactobacillus reuteri, Pediococcus acidilactici*, and fructooligosaccharides indicated different results in which there was an increase in spleen GPX levels in broiler chickens induced withstress. In addition, Kavitha *et al*. (2016) reported that administering a synbiotic containing a mixture of *B. bifidum* 231 at 1.4×10^{11} CFU/rat/day intragastrically twice daily and FOS at 0.8 g/rat/day once daily for 8 weeks can increase renal GPX activity in diabetic mice. Furthermore, Hajifaraji *et al*. (2018) found out that *L. acidophilus* LA-5, *Bifidobacterium* BB-12, *S. thermophilus* STY-31, and *L. delbrueckii bulgaricus* LBY-2 can increase the levels of erythrocyte GPX in women with diabetes. Thus, it can be stated that the effect of probiotics on the antioxidant levels of the GPX enzyme depends on the type of probiotic, the location of the tissue/organ that produces GPX, as well as the type and condition of the experimental animals used.

Glutathione peroxidase (GPX) is one of the most vital antioxidant enzymes for preserving reactive oxygen species (ROS) homeostasis. The important functions of GPX in supervising ROS homeostasis are managing intracellular H_2O_2 levels, holding GSH/GSSG balance, and increasing antioxidant enzyme activity (Zhang *et al*., 2020). Besides GPX, SOD and CAT play a important role in preserving cells from oxidative stress. In the oxidative chain, superoxide is the main ROS which is generated, and superoxide will be further altered to hydrogen peroxide (H_2O_2) by SOD and to water by CAT and/or GPx activities (Nayan *et al*., 2020). The mechanism of probiotics containing *Lactobacilli* in increasing GPX is through their ability to provide antioxidant effects. This is because they are able to catch and make free radicals nondangerous; to prevent the lipid peroxidation, chelation of transient metal ions, stimulation of cellular antioxidant system; and to defend or to power the antioxidants (Mandal *et al*., 2014).

According to Feng and Wang (2020), the massive spread of pathogens in the intestine and in the intestinal epithelium generate and liberates high levels of ROS, causing substantial oxidative stress. It has been showed that the gut microbiota may regulate redox signaling and affect redox homeostasis in the host. A lactic acid bacteria (LAB) addition may modulate the intestinal microbiota, and it has been predicted that LAB can show their antioxidative effects through the rebuilding of the host intestinal microbiota composition.

In line with the findings by Feng and Wang (2020), in the present study the inflammation caused by indomethachin induction in rats can cause intestinal epithelial cells to produce and release ROS, resulting in oxidative stress. The probiotic treatment in this study provided a higher level of GPX gene expression closer to the level of GPX gene expression in the normal rats compared to rats that were only induced by indomethacin; however,the difference was not significant. The results of this study are close to those reported by D'souza *et al*. (2010) that only Saccharomyces Boulardii lyo probiotic supplementation could maintain/not increase GPX1 gene expression in the terminal ileum of rats, while prebiotic FOS/GOS and synbiotic or control feed treatment caused a decrease in GPX1 gene expression. It is possible that the differences in the type and dose of probiotics are the factors that influence GPX gene expression, and this has been reported by by Feng and Wang (2020). They found out that different probiotic strains of LAB have different complex antioxidant mechanisms. These LAB can play antioxidant roles through scavenging ROS, chelating metals, increasing antioxidant enzyme levels, and modulating the microbiota. In addition, among other factors, selenoprotein expressionand regulation at the translational levelmay be influenced by cell types or tissues and by the availability of translation cofactors or cell-dependent selenium uptake mechanisms (Ottaviano *et al*., 2009).

The results of this study differ from those of the previous studies in that synbiotics consisting *Lactiplantibacillus pentosus* GSSK2 (dose 1×10^9 *Lactobacilli*/0.1 ml) and isomalto-oligosaccharides (1g/kg body weight) or probiotic *Lactiplantibacillus pentosus* GSSK2 supplementation given for 12 weeks can reduce the expression of inflammatory markers (TNF α- gene) in adipose tissues in rats induced with ametabolic syndrome (Khanna *et al*., 2021). Likewise, research by Takahashi *et al*. (2018) showed that the synbiotic Gut Working Tablet (GWT), which is a product of cereal

germ fermentation with Aspergillus oryzae strain NK, Enterococcus faecium, Saccharomyces cerevisiae, can suppress the expression of TNF α mRNA in macrophages (RAW264.7) and in specimens with experimental colitis (with methotrexate induction). Thus, the differences in the types of probiotics and prebiotics in synbiotics and their dosage and the tissue where the gene is expressed, can influence the expression of the TNF-α gene.

Indomethacin is an example of an NSAID that can cause side effects, one of which is dangerous gastrointestinal complications such as bleeding, ulceration, and perforation. Small intestine lesions of various types of mucosal damage such as petechiae, red spots, erosion, and ulcers can be caused by NSAIDs. NSAIDs can also cause colitis. The present study is similar to a previous study in that rat methotrexate (MTX)-induced jejunoileal injury led to severe structural defect such as villus shortening, atrophy, desquamation of surface epithelium, cystic dilatation in crypt, and crypt loss. The damage villus could be improved by administering Good Working Tablet composed of the fermentation products of several cereal germs with the Aspergillus oryzae strain NK, a lactic acid bacterium, Enterococcus faecium and its fermentation products, and Saccharomyces cerevisiae known as dried brewer's yeast (Takahashi *et al*., 2018). In addition, the proportion of some bacteria can undergo major changes due to indometacin, resulting in the overall microbiota profile changes, different from the control rats. These changes are indicated by a decrease in the relative abundance of the Gram-positive genera and an increase in the Gram-negative genera (Lázár *et al*., 2021). Furthermore, when administered as pre-treatment in the acetic acid-induced colitis model, goat milk and goat milk yoghurt showed an anti-inflammatory intestinal activity. The activity was demonstrated by the decrease of the colonic tissue damage, the preservation of the tissue cytoarchitecture, and the lower number of pro-inflammatory mediators followed by the improvement of oxidative stress(de Assis *et al*., 2016).

Likewise, the ileum of rats treated with indomethacin alone (IP2) showed no mucin-producing cells (Fig. 4). It is postulated that mucosal injuries followed by IEC changes, such as Goblet cell loss, crypt cell hyperplasia, and ulceration, are caused by high levels of ROS and NO production in the intestinal inflammation model (Monteros *et al*., 2021). Therefore, during the mucosal inflammation, the intestinal epithelial cells (IECs), as well as the neutrophils and macrophages produce superoxide and nitric oxide. Oxidative stress is therefore a target of newer therapeutic strategies for IBD. Barrier damage resulting from changes in tight junctions and permeability in IECs is caused by the ROS-mediated damage to cytoskeleton proteins (Monteros *et al*., 2021). PAS staining in the present study was similar to that of a previous study by Takahashi *et al*. (2018). They reported that Gut Working Tablet composed of the fermentation products could significantly maintain the number of goblet cells in rat jejunum induced with methotrexate. Meanwhile, the methotrexate -induced rats that only showed most of the goblet cells were lost from the crypts. It is explained that methotrexate induces intestinal mucosal damage by enhancing intestinal permeability, and the elevated permeability of intestinal represents a large number of pathophysiological detection in IBD.

The functional barrier consists of several lines of defense, represented mainly by a layer of mucin (mucus) covering the surface of the epithelium, apical and basolateral cell membranes, a complex system of junctions, and the basal membrane. The mucin covering the intestinal epithelium, secreted by goblet cells found in glands throughout the digestive tract, forms the first line of defense of the colonic mucosa (Mello *et al*., 2012).

A single layer of epithelial cells and an associated protective mucus layer are the primary defense against the entry of microbes and pathogens into the lamina propria. The mucus barrier is the main part of the body's innate immune system that helps maintain a mutualistic immune relationship between the host and the bacteria and decreases the activity of subepithelial lymphocytes. Butyrate is the crucial SCFA related to mucus glycoprotein productionthat is by providing energy for goblet cells. .

The regulation of glycosyltransferases in the endoplasmic reticulum increases the synthesis of intestinal mucus and enhances the expression of MUC2, which is influenced by butyrate. The therapeutic effect of butyrate in colitis can be attributed to its influence on the modulation of the mucus barrier, a process in which prebiotics and probiotics play a role. The expression, synthesis, and secretion of mucin genes, directly stimulated by probiotics, can prevent attacks by pathogenic bacteria (Sun *et al*., 2016).

Conclusion

Synbiotic goat milk cheese which is composed of *Lactobacillus rhamnosus* FNCC 0052 and glucomannan from porang tuber can act as an immunomodulator in inflammatory rats through the production of TNF-α and NO, making it similar to that of control rats. Additionally, the expression of the TNF-α gene in the intestinal tissue is more influenced by probiotics. In addition, synbiotic cheese can reduce damage to the intestinal epithelial cells and increase the number of mucin-producing goblet cells. Therefore, the synbiotic goat milk cheese can be used as a functional anti-inflammatory food.

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Conflict of interest

The authors have no conflict of interest to declare.

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