Food & Nutrition Journal
Devi S and Kapila R. Food Nutr J. 7: 256.
https://www.doi.org/ 10.29011/2575-7091.100156
www.gavinpublishers.com

Research Article

Gut function restoration by indigenous cow milk in gut inflammation by peptidoglycan from *Staphylococcus aureus* via regulating NF-kB

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Citation: Devi S, Kapila R, Kapila S (2022) Gut function restoration by indigenous cow milk in gut inflammation by peptidoglycan from *Staphylococcus aureus* via regulating NF-kB. Food Nutr J 7: 256. DOI: 10.29011/2575-7091.100156

Received Date: 13 December 2022; **Accepted Date:** 22 December 2022; **Published Date:** 25 December 2022

Abstract

In IBD patients, it is difficult to find a satisfactory treatment due to lack of understanding of pathogenesis. Thus, the present study focuses on finding milk based treatment by comparing Indigenous (SW, TP and GIR), cross-bred (KF and KS) and exotic cow breed (HF) milk in inflammatory conditions like IBD induced by peptidoglycan from *Staphylococcus aureus*. Milk was provided to rats in human equivalent doses on daily basis orally via bottles. A significant increase in macrophage phagocytic activity in GIR and HF group, but a significant reduction in splenocyte proliferation in different milk treated group was found as compared to PGN group. A significant reduction in gut permeability in SW, GIR, KF and KS after 1 and 4h was observed. Based on above results, SW and KS group were selected for studying of inflammatory signaling in Caco-2 cells. There was reduction in relative mRNA expression of TLR-2, TLR-4, NF-kB, TNF-α and IL-6 in digested SW milk group in Caco-2 cells. Thus, Indigenous Sahiwal milk can be used as a protective remedy due to its gut integrity restoration effect via regulating NF-kB in inflammatory diseases.

Keywords: Cow milk; gut; immune response; inflammation; localized; systemic

Introduction

Milk is well thought-out as a nutritional table and complete food. It helps in offering not only physical, but also mental health. From the ancient time, milk has also been known as both curative and preventive medicine. Different milk components including proteins, vitamins and minerals give nutritional and health benefits that are supported by commercial dairying enterprises and also, different milk-based products are obtainable [1]. In addition to this, the various cow milk products are consumed for the disease preventing, health improving and therapeutic purpose. Furthermore, besides its nutritional value, milk and its products are consumed with the medicines for increasing the dynamic and pharmacokinetic properties of medicines. Milk is also considered as basic and very vital for the regeneration of tissue in Ayurvedic system. The main reason for this property is encrypted and combinational effects of its hormones, vitamins, proteins, minerals and growth factors [2]. Furthermore, the evidences also suggested the effect of different key components of bovine milk and its purified sub fractions or constituents in immune response regulation in various ruminant and non-ruminant species. In addition to this, the role of bovine milk for the immune system in human or overall health is very diverse, wide and indefinable [1]. Surprisingly, the effect of Indigenous cow milk towards specific biochemical immune response and inflammation with specific emphasis on targeted pathways of systemic i.e. blood and localised effect i.e. gut are not yet investigated.

Inflammatory bowel disease (IBD) is a chronic gut inflammation [3]. IBD is rising globally affecting people of all ages and also, the pediatric population. Ulcerative colitis (UC) is mainly considered as disease of inflammatory lesions involving the large intestine counting rectum and then, shifts towards the colon and ultimately, the whole colon. On the other side, Crohn's disease (CD) can affect any part of the gastrointestinal tract, usually the

terminal ileum or the perianal part. Complications of IBD include fluid and electrolyte loss, bleeding, diarrhoea and abdominal pain. Ulcerative colitis induces the activation of Th-2 immune response, whereas Crohn's disease induces the Th-1 immune response activation [4]. The potential cause of IBD is a dysregulated intestinal microflora that further leads to malfunctioning of the immune response [5]. Cell wall components from bacteria are also used for investigating the different IBD responses. Treatment of peptidoglycan-polysaccharide (PG-PS) from group A *Streptococci* in the rats induced higher fibrosis compared to human serum albumin that was measured by the enhanced gross abdominal score, procollagen I and III mRNAs and cecal collagen content [6]. Peptidoglycan (PGN) is a thick and exposed cover consisting the bacterial cell wall of Gram-positive bacteria in association with the lipoteichoic acid; however, in Gram-negative bacteria, it is a thin layer that further covered by the coating of thick sheet of lipopolysaccharide (LPS). Amazingly, PGN stimulates an inflammatory immune response. A previous study suggested that intraperitoneal injection of PGN from Gram-positive bacteria stimulated the inflammation and causes the resulting arthritic damage of the joints in animal rat models [7].

In case of IBD patients, the main difficulty is in understanding the pathogenesis that makes it complex to find effective and satisfactory treatment. So, keeping above facts in mind, the present investigation focuses on finding the best milk based treatment from Indigenous (Sahiwal, Tharparkar and Gir), cross-bred (Karan fries and Karan swiss) and exotic cow breed (Holstein friesian) milk that improves the inflammatory conditions induced by peptidoglycan from *Staphylococcus aureus*. We investigated both systemic and localized gut immune response. Furthermore, the mechanism of milk action is explored by establishing inflammatory conditions on induction of inflammation with peptidoglycan and focusing on inflammatory signaling. This study provides the easily available remedy (milk) to reduce the complications of the IBD patients along with lightening on mechanistic pathway.

Materials and Methods

Materials

All chemicals and reagents are procured from Sigma-Aldrich, St. Louis, MO, Himedia Laboratories Pvt. Ltd, Mumbai, India and Thermo, USA. Kits were purchased from Koma Biotech, Korea and Cloud Clone Corporation, USA.

Methods

Milk treatment for rats

The animals used in this study were male albino wistar rats (150-200 g body weight) that were obtained from Small Animal House, National Dairy Research Institute, Karnal, Haryana (Institutional Animal Ethics Committee approval letter No. 41-IAEC-18-62). The intestinal inflammatory rat model was developed according to the method of Rahal et al. [8] with some modifications using 10 mg/kg body weight dose of peptidoglycan (PGN) from *Staphylococcus aureus* by laparotomic procedure. PGN laparotomic injection induced IBD like symptoms. Control rats (Sham) were injected with the sterile solution of normal saline. Milk from different breeds, Sahiwal (SW), Tharparkar (TP), Gir (GIR), Karan Fries (KF), Karan Swiss (KS) and Holstein Friesian (HF) was administered for a period of 28 days after the PGN injection. Milk was administered orally on daily basis in the human equivalent dose following the formula of Shin et al. [8].

Human equivalent dose (mg/kg) = Animal dose (mg/kg)*Animal Km/Human Km

Different parameters such as weight gain and %stool water content were determined. At the end of study, organ weight index was calculated.

Evaluation of Macrophage phagocytic function

Peritoneal macrophages collected from rats and phagocytic activity was determined according to the method of Dang et al. [9] with little modifications. Briefly, peritoneal fluid containing macrophages was adjusted to $1*10^6$ live cells/ml with the DMEM media containing 10% FBS. About 200µl of cell suspension/well was added in a 96-well, flat-bottomed tissue culture plate. This was followed by the addition of Zymosan (650 µg/ml) and Nitroblue tetrazolium (250 µg/ml) dye in wells. Following this, the plates were incubated in a humidified CO_2 incubator at 37 °C with 5% CO_2 for 3 h. Absorbance was read at 570 nm by multiwell scanning spectrophotometer (Epoch™ Microplate Spectrophotometer, BioTek) after dissolving formazan crystals formed at the bottom of plate in DMSO.

Spleen lymphocytes' proliferation assay

Spleen lymphocytes' proliferation assay was done according to the method of Sharma et al. [10] with little modifications. In brief, carefully isolated spleen lymphocytes suspension was adjusted to $1*10^6$ cells/ml using the culture media (DMEM) with 10% FBS. About 200µl of this cell suspension was placed in each well of a 96-well, flat-bottom tissue culture plate. The lipopolysaccharide (50µg/mL) was used as a mitogen. The plates were incubated in a humidified CO_2 incubator (5% CO_2) at 37 °C for 24 h. The proliferative potential of cells was estimated by adding 20 µl of MTT solution to each well after culturing lymphocytes for 24 h. The plates were further incubated for 4 h with MTT in a humidified CO_2 incubator at 37 °C. The supernatant was pipetted out. Then, dark blue crystals were dissolved using DMSO. The optical density was read at the test wavelength of 570 nm using ELISA reader (Biotek Elisa reader).

In vivo permeability testing of intestine

In vivo permeability testing was performed using 4000 Da fluorescent dextran-FITC (DX-4000-FITC) according to the method of Cani et al. [11] with little modifications. Blood samples were collected from the eye by retro-orbital plexus puncture and cardiac puncture, respectively after 1 and 4 h. The analysis for DX4000-FITC concentration with a fluorescence spectrophotometer (Epoch™ Microplate Spectrophotometer, BioTek) at an excitation wavelength of 485 nm and emission wavelength of 535 nm was done.

Measurement of gastric acidity

Change in gastric acidity was determined following the method of Sabiu et al. [12] with little modifications. Gastric acidity was measured by titration with 0.1 N NaOH using Toepfer's reagent and Phenolphthalein as indicators. The red and pink color was noticed for the measurement of free and total acidity, respectively in gastric content.

Estimation of immunoglobulins, cytokines and inflammatory proteins

The sandwich ELISA assay was used for the measurement of the protein level of interferon gamma (IFN- γ), interleukins (IL-10, IL-6), monocyte chemotactic protein (MCP-1), tumor necrosis factor-alpha (TNF-α), immunoglobulin A and G according to the manufacturers' protocol.

Establishment of *in vitro* Caco-2 inflammatory model

Cells were plated in 96-well plates at 1×10^4 cells/well. Cells were then treated with various concentrations of peptidoglycan (2.5, 5 and 10μg/ml) for different time periods (2, 4 and 6 h). Cell viability was determined using MTT solution (5 mg/mL PBS).

In vitro simulated gastro-intestinal digestion of milk

A two-step *in vitro* assay was carried out according to the method of Fotschki et al. [13] with some modifications. Porcine pepsin (800–2,500 units/mg protein), pancreatin (activity, 8×USP specifications) and Bile extract (ox), which possesses cholic acid (approximately 55%), deoxycholic, glycocholic and taurocholic acids were used to simulate human digestion in the stomach and intestine.

Quantitative real time-PCR analysis

Caco-2 cells were cultured in sterile 6-well polystyrene tissue culture plates. Cells were treated with 90% media and 10% PBS in control and PGN group, 10% simulated digested milk from SW and KS breed (DSW and DKS, respectively) for 24 h followed by PGN treatment for 6 h in PGN, PGN+DSW and PGN+DKS group. In addition, cells were pre-treated with DSW and DKS for 24 h followed by media treatment for 6 h in DSW and DKS group. Total RNA was isolated by single step RNA isolation method using 1mL Tri-reagent from 50-100mg intestinal tissue, Caco-2 cells and $1*10^8$ peritoneal cells. Reverse transcription was performed using Revert Aid first strand cDNA synthesis kit (Thermo fisher, USA). Relative gene expression was measured by quantitative real-time PCR using the ABI PRISM 7500 Fast detection system (Applied Biosystems) and Maxima SYBR Green (Thermo, USA) master mix. GAPDH was used as an internal control. Primers used are given in Table 1 and 2.

S. No.	Gene Name	Primer	Sequence of the primer 5'→3'
1.	GAPDH	Forward	AGACAGCCGCATCTTCTTGT
		Reverse	CTTGCCGTGGGTAGAGTCAT
2.	Zona occludens-1	Forward	TGCCAGCTTTAAGCCTCCAG
		Reverse	TTGGCAGGCTCTGAGTGATG
3	Claudin-1	Forward	ACTGTGGATGTCCTGCGTTT
		Reverse	CTAATGTCGCCAGCCTGAA
4.	Defensin-1	Forward	ATGAAAACTCATTACTTTCTCCTGGTG
		Reverse	CAAACCACTGTCAACTCCTGC
5.	Defensin-2	Forward	ATGAGGATCCATTACCTTCTCTTC
		Reverse	CTACTTTTTCTTGCCAGCATCTCC

6.	CD-14	Forward	TGAGTATTGCCCAAGCACACT
		Reverse	GTAACTGAGATCCAGCACGCT
7.	TLR-2	Forward	GTACGCAGTGAGTGGTGCAAGT
		Reverse	GGCCGCGTCATTGTTCTC
8.	TLR-4	Forward	AATCCCTGCATAGAGGTCTTCCTAAT
		Reverse	CTCAGATCTAGGTTCTTGGTTGATAAG

Table 1: Sequence of primers used for studying the relative gene level expression in rats.

S. No.	Gene Name	Primer	Sequence of the primer 5'→3'
1.	GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
		Reverse	TGGTGAAGACGCCAGTGGA
2.	TLR-2	Forward	AGCACTGGACAATGCCACAT
		Reverse	ACCATTGCGGTCACAAGACA
3.	TLR-4	Forward	CAAGAACCTGGACCTGAGCTT
		Reverse	AAAAGGCTCCCAGGGCTAAA
4.	NF-kB	Forward	ATGTGGGACCAGCAAAGGTT
		Reverse	CACCATGTCCTTGGGTCCAG
5.	ZO-1	Forward	TGATGGTGTCCTACCTAATTCAACTCA
		Reverse	GAACGCCAGCTACAAATATTCCAACA
6.	Occludin	Forward	AGAACAGAGCAAGATCACTATGAGACA
		Reverse	CTTTGTTGATCTGAAGTGATAGGTGGA
7.	Claudin-1	Forward	GCACATACCTTCATGTGGCTCAG
		Reverse	TGGAACAGAGCACAAACATGTCA
8.	TNF-α	Forward	GGGACCTCTCTCTAATCAGC
		Reverse	TCAGCTTGAGGGTTTGCTAC
9.	IL-6	Forward	GGCACTGGCAGAAAACAACC
		Reverse	GCAAGTCTCCTCATTGAATCC

Table 2: Sequences of primers used for study of gene expression in Caco-2 cells.

Results

General health status of rats

Body weight is directly related to the changes in the metabolism and weight of different body organs. In the present study, there was no significant difference in the body weight of different milk fed rats as compared to control and PGN treated rats after 15 days as shown in Figure 1A. Similarly, the difference in %stool water content was non-significant in milk treated rats from different breeds after PGN injection as shown in Figure 1B. There was non-significant difference between control, PGN, SW, TP, GIR, KF, KS and HF treated group in spleen and liver weight in gut inflammatory model study (Figure 1C and D). Interestingly, in SW and KS milk treated group, there was a significant ($p<0.05$) reduction in kidney weight compared to PGN treated rats as shown in Figure 1E. While, there was no significant difference in kidney weight in TP, GIR, KF and HF in comparison to PGN treated rats (Figure 1E).

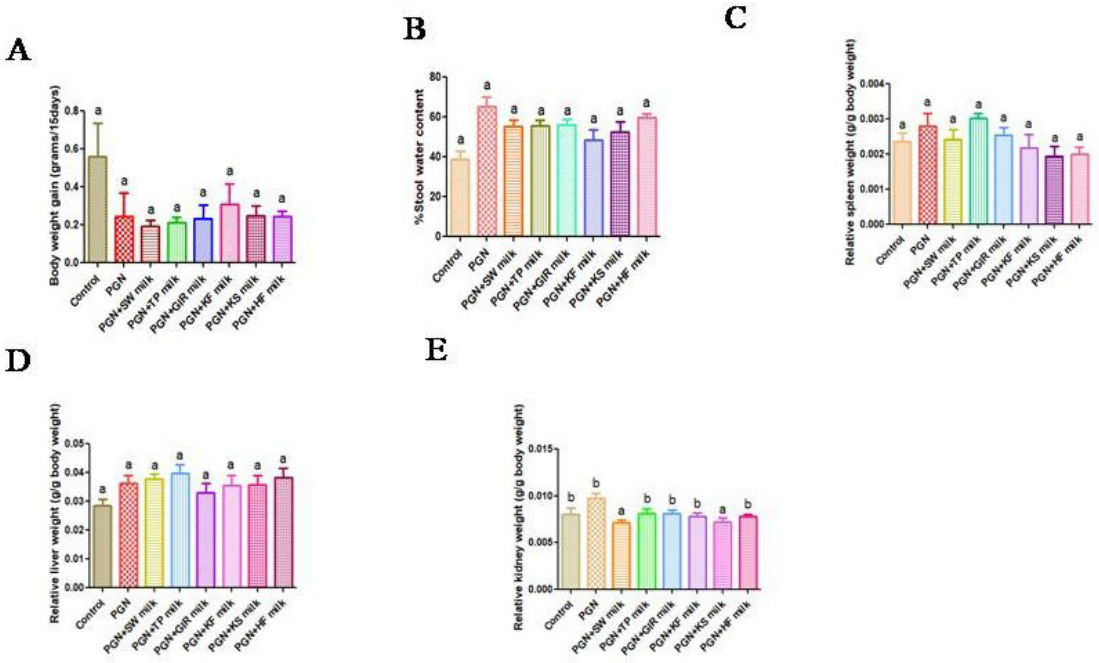

Figure 1: Effect of milk treatment on (A): Gain in weight (B): % stool water content. Effect of milk from different breeds on relative organ weight (C): Relative spleen weight index (D): Relative liver weight index (E): Relative kidney weight index. Values are expressed as mean ± S.E.M. (n=6). Different alphabets indicate singnificant difference ($p<0.05$).

Effect on haematological status of rats

On milk feeding after PGN treatment in intestine, there was non-significant difference in Hb, RBC count, WBC and total platelet count between SW, TP, GIR, KF, KS and HF milk treated groups in comparison to control and PGN group. Furthermore, MCV is the average red blood cell size or volume of a RBC required to predict the aetiology of anaemia. MCH is also a reflection of MCV. A significant ($p<0.05$) increment in MCV was found in SW, KF and HF group compared to control and however, a significant increase in MCV in HF group was observed compared to PGN group. There was significant ($p<0.05$) enhancement in SW and GIR group compared to control and PGN treated rats in MCH value (Table 3). In the eosinophil count, there was significant ($p<0.05$) increase in SW and KF group compared to control. Whereas, there was no significant difference between PGN and other milk treated groups (Table 3). A significant ($p<0.05$) increase in monocytes number in PGN treated, SW, GIR and KF rats was found compared to control. But, milk treatment of TP and HF caused a significant ($p<0.05$) reduction compared to PGN group in total monocytes count.

Parameter	Control	PGN	PGN+SW milk	PGN+TP milk	PGN+GIR milk	PGN+KF milk	PGN+KS milk	PGN+HF milk
Hb (gms/100mL)	12.77±1.9^{a}	12.17±0.5^{a}	11.93±1.0^{a}	11.57±0.34^{a}	13.03±0.12^{a}	11.47±0.3^{a}	11.77±0.33^{a}	9.91±0.83^{a}
RBC count (mil. cu. mm.)	6.8±1.4^{a}	6.5±0.4^{a}	4.9±0.3^{a}	5.4±0.09^{a}	5.5±0.30^{a}	5.2±0.17^{a}	5.7±0.22^{a}	4.5±0.3^{a}
MCV (%)	56.49±3.6^{a}	61.41±3.6^{ac}	75.74±2.2^{bc}	71.10±1.15^{ac}	70.91±2.47^{ac}	75.15±0.8^{bc}	66.77±2.6^{ac}	82.37±3.2^{b}
MCH (%)	19.09±1.3^{a}	18.75±0.87^{a}	24.01±0.85^{bc}	21.5±0.87^{ac}	23.77±1.2^{bc}	22.0±0.44^{ac}	20.36±0.2^{ac}	21.77±0.57^{ac}
White blood cells (per cu. mm.)	7300±351.2^{a}	5600±360.6^{a}	9367±384.4^{a}	6500±802.1^{a}	8433±786^{a}	8400±1528^{a}	7267±1988^{a}	7233±1200^{a}
Eosinophils (%)	0^{a}	1.66±0.60^{ac}	2±0^{bc}	1.33±0.33^{ac}	1.66±0.33^{ac}	2.3±0.33^{bc}	1.33±0.33^{ac}	1±0.57^{ac}
Monocytes (%)	0^{a}	2^{bc}	1.6±0.30^{bc}	0.6±0.30^{ac}	1.3±0.30^{bc}	1.6±0.30^{bc}	1.0±0^{ac}	0.3±0.30^{ac}
Total Platelet count (Lacs/ cu.mm.)	6.1±0.26^{a}	7.0±0.23^{a}	6.1±0.13^{a}	7.2±0.40^{a}	5.8±0.38^{a}	7.6±0.39^{a}	6.0±0.81^{a}	6.9±1.0^{a}
Results are expressed as mean±S.E.M. (n=3). Different alphabets indicate significant difference ($p<0.05$).								

Table 3: Effect of milk treatment from different breeds on rats' haematological status.

Effect on macrophage phagocytic activity

On PGN injection followed by milk treatment, there was a significant ($p<0.05$) enhancement in macrophage phagocytic activity in GIR and KF group in comparison to PGN treated, SW and TP milk fed rats as shown in Figure 2A. Other than this, a decrease in phagocytic activity in KS milk fed rats in comparison to PGN treated rats was observed. Whereas, a non-significant difference in phagocytic activity in SW, TP and HF group compared to control and PGN group was found.

Effect on splenocytes proliferation

In the present study, a significant ($p<0.05$) increase in splenocyte proliferation in PGN group was observed as compared to control (Figure 2B). There was significant ($p<0.05$) increase in splenocyte proliferation rate in TP, KS and HF group in comparison to control, but it was approximately four fold lesser in TP and KS, and two fold lesser in HF in comparison to PGN group as shown in Figure 2B. Furthermore, a non-significant difference in splenocyte proliferation rate in SW, GIR and KF milk treated rats was observed as compared to control. There was a significant ($p<0.05$) reduction in splenocyte proliferation rate in SW, TP, GIR, KF, KS and HF group compared to PGN treated group. In addition, a significant increased proliferation rate was found in HF in comparison to SW, TP, GIR, KF and KS group.

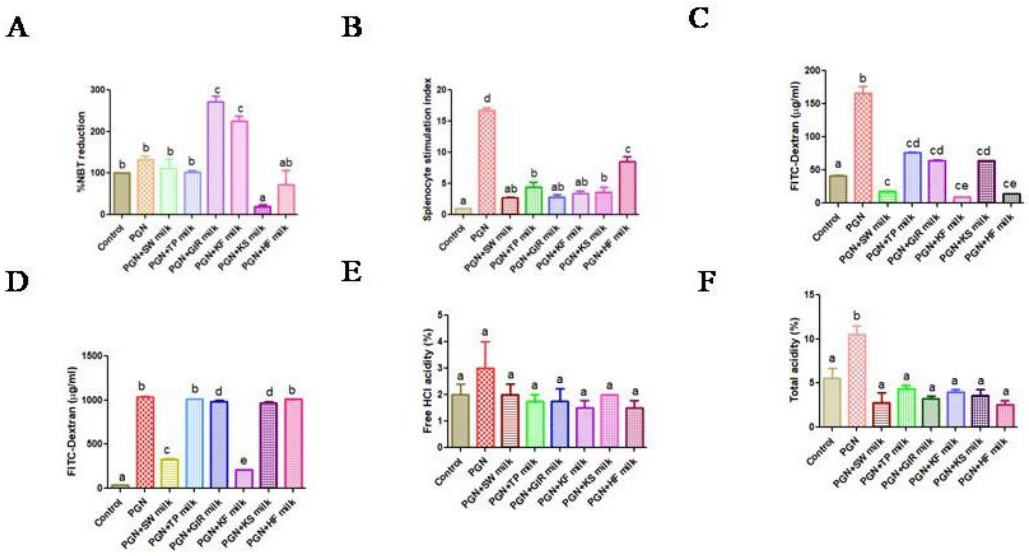

Figure 2: Effect of milk treatment on (A): Macrophage phagocylic activity (B): Splenocyte stimulalion index. Effect of milk treatment on intestinal permeability of rats by *in vivo* FITC-dextran assay. (C): After 1h (D): After 4 h. Effect of milk treatment on gastric acid secretion in male wistar rats. (E): Free acid secretion (F): Total acid secretion. Values are expressed as mean ± S.EM. (n=6). Different alphabets indicate significant difference (P<0.05)

Effect on *In vivo* permeability

In case of inflammation induced rats, there was significant (p<0.05) enhancement in gut permeability in PGN group as compared to control after 1 h as shown in Figure 2C. Whereas, a significant reduction in gut permeability was found after 1 h in SW, TP, GIR, KF, KS and HF group compared to PGN treated rats (Figure 2C). There was significant (p<0.05) decrease in gut permeability in SW, GIR, KF and KS group as compared to PGN group after 4 h (Figure 2D). However, there was no significant difference in TP and HF milk treated rats as compared to PGN treated rats after 4 h. A significant (p<0.05) enhancement in TP, GIR, KS and HF group and reduction in KF group compared to SW milk treated rats after 4 h was found.

Change in gastric acid secretion

In the present study, we assessed the gastric mucosal protective effect of milk by analyzing free HCl and total acidity. There was a significant (p<0.05) decrease in total acid secretion in SW, TP, GIR, KF, KS and HF group as compared to only PGN treated rats as shown in Figure 2F. But, there was no significant difference in free HCl secretion in milk fed rats compared to PGN group (Figure 2E).

Effect on expression of genes in intestinal tissue

An enhanced expression for the TLR-2 and TLR-4 was found in intestine in PGN group compared to control as shown in Figure 3A and B. The TLR-2 and TLR-4 expression was decreased on feeding with SW, TP, GIR, KF, KS and HF milk in inflammatory conditions as shown in Figure 3. Sodium dependent bile acid transporter (Na-SBT) expression was increased in KF and KS group as compared to PGN group (Figure 4A). An enhancement in ZO-1, Occludin and Claudin expression was found on milk feeding from different breeds in comparison to PGN group as shown in Figure 4. There was no significant difference was found among different groups in Defensin-1 expression (Figure 3 D). In addition to this, approximately seven fold enhancement in GLP-2 expression was observed in GIR and HF group in comparison to control and PGN treated rats, whereas a non-significant difference was observed in SW, TP, KF and KS group as compared to PGN group as shown in Figure 4B. In the present study, about five fold increment in defensin-2 expression was observed in PGN treated rats in comparison to control group. Also, a significant (p<0.05) decrease in defensin-2 was found in SW, TP, KF, KS and HF group compared to PGN group as shown in Figure 3E.

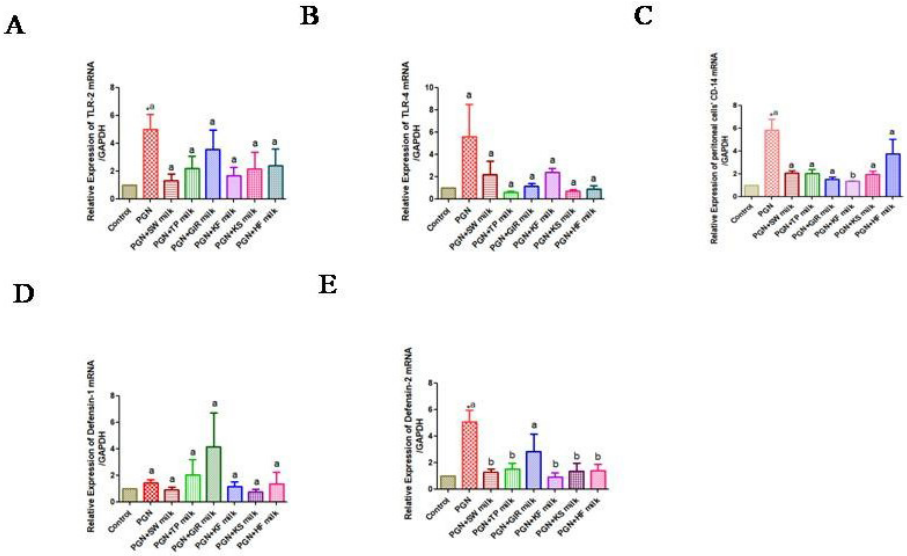

Figure 3: Effect of milk treatment on intestinal tissue and peritoneal cells' genes expression. Relative expression of (A): TLR.-2 mRNA (B): TLR.-4 mRNA. (C): CD-14 mRNA. (D): Defensin-1 mRNA. (E): Defensin-2 mRNA. Different alphabets indicate significant difference (P<0.05). *P<0.05 indicate significant difference compared to control.

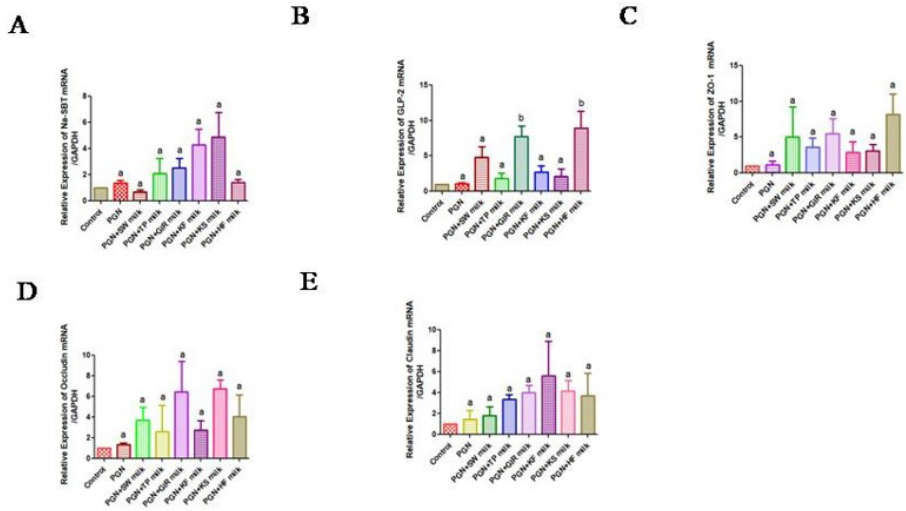

Figure 4: Effect of milk treatment on gut integrity genes. Relative expression of (A): Na-SBT mRNA (B): GLP-2 mRNA (C): Zo-1 mRNA (D): Occludin mRNA (E): Claudin mRNA. Values are expressed as mean ± SEM (n=5).Different alphabets indicate significant difference (P<0.05).

Peritoneal cells' CD-14 expression was enhanced significantly (p<0.05) in PGN group as compared to control, however, there was no significant difference in SW, TP, GIR, KS and HF group in comparison to PGN as shown in Figure 3C. CD-14 expression was reduced nearly four-fold in KF group compared to PGN group as shown in Figure 3C.

Effect on cytokines and immunoglobulins level

Injecting rats with PGN resulted in significant (p<0.05) increment in intestinal TNF-α level, however feeding rats with different breeds milk caused significant decline in TNF-α except HF group where TNF-α levels were similar to PGN treated rats as shown in Figure 5A. The decrease in intestinal TNF-α level was significant in TP, GIR and KF in comparison to PGN group. In inflammatory

conditions, milk treatment from various breeds didn't significantly ($p<0.05$) affect the IL-6 level in intestine between different groups as shown in Figure 5B. On milk feeding, a significant ($p<0.05$) decrease in IL-10 level in PGN, SW, TP, GIR and KF group as compared to control group was observed as shown in Figure 5C. PGN treatment caused a significant ($p<0.05$) enhancement in intestinal IFN-gamma level in comparison to control group in inflammatory conditions as shown in Figure 5D. However, administration of different breeds milk resulted in decrease in intestinal IFN-gamma level that was significant ($p<0.05$) in SW, TP, GIR and KF group, but non-significant in KS and HF group as compared to PGN injected rats. Among different milk-fed groups, a significant ($p<0.05$) enhancement was observed in intestinal IFN-gamma level in KS and HF group in comparison to TP group in inflammatory conditions. There was no significant difference in intestinal MCP-1 level between different milk treated groups including SW, TP, GIR, KF, KS and HF group as compared to control and PGN group as shown in Figure 5E.

In inflammatory conditions, there was no significant difference in intestinal IgG level between different groups as shown in Figure 5F. Furthermore, a non-significant difference in intestinal IgA level in PGN, SW, KF and KS group was found in comparison to control as shown in Figure 5G. However, a significant ($p<0.05$) increase was observed in intestinal IgA level in TP, GIR and HF group as compared to control.

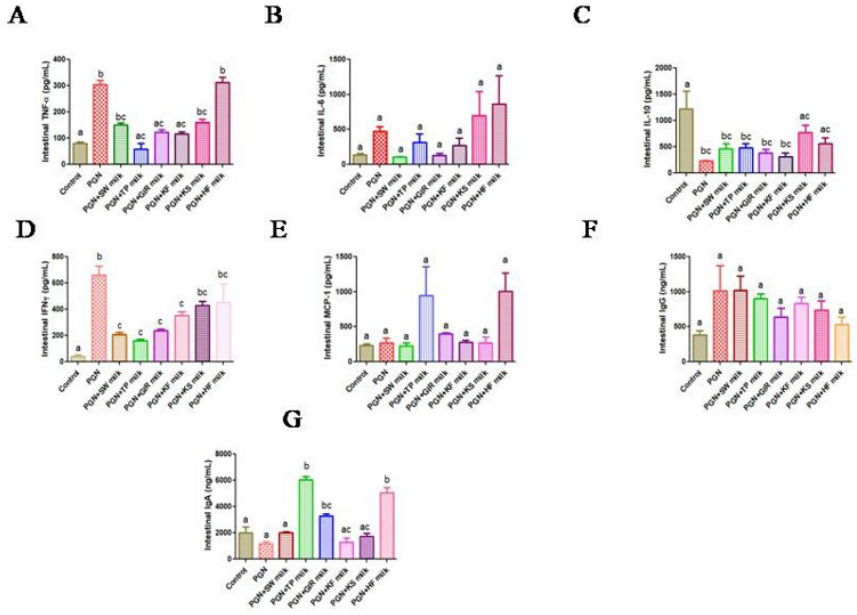

Figure 5: Effect of milk treatment on pro and anti-inflammatory cytokines and immunoglobulin level in rats. (A): Intestinal TNF level (B): Intestinal IL-6 level (C): Intestinal. IL-10 level (D): Intestinal IFN-γ level (E): Intestinal MCP-1 level (F): Intestinal. IgG level (G): Intestinal. IgA level Values are expressed as mean ± S.E.M. (n=5). Different alphabets indicate significant difference ($P<0.05$).

Effect of PGN on Caco-2 cells' proliferation

In the present study, there was no significant difference in cells %viability after treatment with different concentration of PGN after 2 hours as compared to control group as shown in Figure 6A. Also, a non-significant difference was found between cells treated with 2.5, 5 and 10µg/ml concentration of PGN after 2 hours. However, a significant ($p<0.05$) and dose dependent decrease in %cell viability in different groups of cells treated with different concentration of PGN (2.5, 5 and 10 µg/ml) was found compared to control after 4 hours (Figure 6B). A dose-dependent and significant ($p<0.05$) reduction in 5 and 10 µg/ml PGN concentration treated cells was found in comparison to control group after 6 hours as shown in Figure 6C. IC_{50} value (approximately 50% cell viability reduction) was measured by plotting %viability on y-axis and concentration of PGN on x-axis. IC_{50} value was observed at 10 µg/ml PGN concentration treated cells after 6 hours.

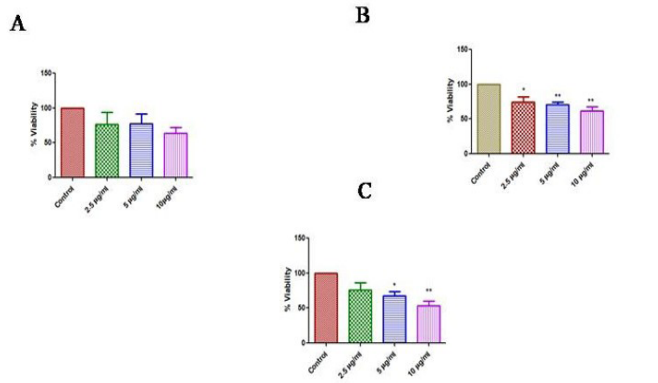

Figure 6: Effect of PGN Treatment on Caco-2 cells viability. (A): 2 Hours (B): 4 Hours (C): 6 Hours. Significant level *p<0.05 and **p<0.01 as compared to control.

Effect of simulated digested milk treatment on inflammatory receptors and cytokines

There was significant (p<0.05) enhancement in relative mRNA expression of NF-kB, TLR-2, TLR-4, TNF-α and IL-6 on treatment of Caco-2 cells with PGN was observed (Figure 7). However, treating cells first with digested SW milk (DSW) followed by PGN treatment resulted in significant reduction in the above mentioned genes' expression, whereas this decline was non-significant in case of digested KS milk (DKS) compared to PGN treated cells.

Effect of simulated digested milk treatment on gut tight junction proteins

In the present study, there was no significant difference in the mRNA expression of occludin protein between different groups as shown in Figure 7H. Additionally, a significant (p<0.05) enhancement was observed in the ZO-1 gene level expression on pretreatment of DSW compared to PGN treated cells and a non-significant difference was found between pretreated DSW and DKS group (Figure 7F). There was no significant difference in the gene level expression of Claudin in PGN group and pretreated DKS cells, but a significant (p<0.05) enhancement was observed in the pretreated DSW cells compared to PGN treated cells as shown in Figure 7G.

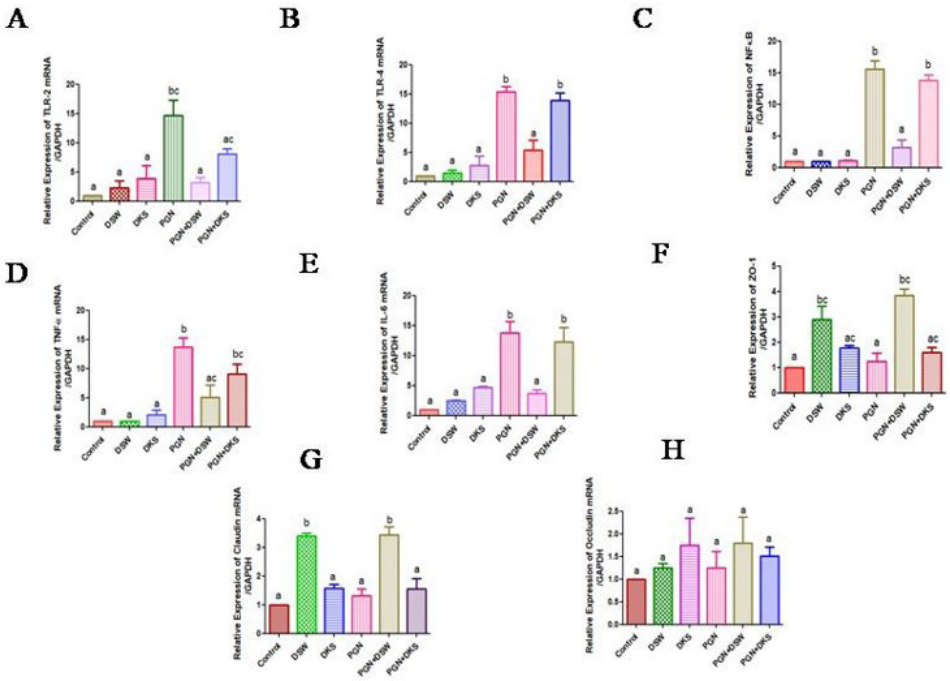

Figure 7: Effect of simulated digested milk on the mRNA. Expression of inflammatory signaling molecules. (A): Relative mRNA expression of TLR-2 mRNA (B) Relative mRNA expression of TLR-4 (C) Relative mRNA expression of NF-kB (D) Relative mRNA expression of TNF-α (E) Relative mRNA of IL-6. Effect of simulated digested milk on the mRNA expression of tight junction Proteins. Relative mRNA expression of (F) Z0-1 (G): Claudin (H): Occludin. Different alphabets indicate significant difference (P<0.05).

Discussion

The prevalence of IBD is increasing globally due to the growth in the area of industries. In case of IBD, it is very difficult to find a satisfactory treatment because of poor understanding of pathogenesis. Furthermore, whole Gram-positive bacteria or cell wall constituent such as peptidoglycan are known to cause inflammation. Milk or its products are very well known to regulate the intestinal inflammation. Thus, in the present study, milk from Indigenous, crossbred and exotic cow breeds was tested for its anti-inflammatory and gut health improving aspects in peptidoglycan induced IBD like inflammatory conditions. General health parameters, systemic and localized immune responses were also investigated. A previous study reported that DSS-stimulated inflammation led to body weight loss and during the chronic inflammation causes an increase in ileum, liver, spleen and colon weights due to an effect on organ protein metabolism [9]. In the present study, there was no significant effect in the body weight, %stool water content, spleen and liver weight after milk treatment. Amazingly, SW and KS milk treatment caused the reduction in kidney weight and contributed towards the reduction in inflammation. Kapila et al. [14] suggested that treatment of skim milk to mice for a period of 2, 5 and 8 days had no effect in phagocytic activity with time. Similarly, in the present study, there was non-significant difference in phagocytic activity on treatment of SW, TP and HF milk compared to PGN group. In addition, in case of splenocyte proliferation, there was about four fold reduction in TP and KS, and two fold reduction in HF compared to PGN group. In line with our studies, a previous study reported that a milk casein derived tripeptide (LLY) showed anti-inflammatory immune response in the *ex-vivo* conditions by reducing mice splenocytes proliferation [11]. An earlier report suggested that constituents of cell wall of gram-positive bacteria such as PGN of *Staphylococcus aureus* induced the DNA synthesis slightly in rat and intestinal epithelial cell lines and hence, it works like a mitogen. Ultimately, this increased the cellular proliferation of rat and human intestinal epithelial cells. Hence, microbiota infection regulates the cellular proliferation that disturbs both health and disease [11].

Furthermore, haematological parameters were assessed to track the pathogenesis. Monocytes are immune cells involved in innate immunity. These cells provide defence against the invading pathogens, but sometimes involve in diseases pathogenesis and progression [13]. In the present study, TP and HF milk treatment led to the reduction in number of monocytes in the blood compared to PGN and similar to control. There was no significant difference in Hb, RBC count, WBC and total platelet count on feeding milk. An earlier study suggested a significant reduction in Hb, HCT and red blood cells count in DSS induced colitic inflammation. In contrast, these haematological parameters were significantly improved in the DSS-pomegranate extract (DSS-PE) group in comparison to DSS treated rats. This effect was stronger in DSS-urolithin-A (DSS-UROA) group compared to the DSS-PE group and in fact, there was slight increase in RBC count [13]. MCH gives us an idea of amount of haemoglobin per red blood cell. The bigger red blood cell possesses more haemoglobin and vice-versa. So, consumption of milk enhances the haemoglobin level per red blood cell compared to PGN. The results from present study suggested the clinical importance of milk consumption from SW, GIR, KF and HF for the anaemia management. Eosinophils are white blood differentials that get accumulated in tissue and blood in allergy, infectious conditions and inflammation [15]. White blood cell differentials indicate the ability of a living being to eliminate the infection [14]. In the present study, there was an increase in eosinophils in SW and KF compared to control. Hence, the above results gave us some indications for the clinical significance of milk consumption in the treatment of inflammatory disorders.

In a previous study, anti-ulcerogenic effect of *C. pluricaulis* was primarily by the stimulation of mucosal defensive factors such as mucin secretion, lifespan of mucosal cells and glycoproteins, not the offensive factors such as acid-pepsin augmentation [16]. In the present study, there was no effect on free HCl secretion, but a significant reduction in total acid secretion after milk treatment from different breeds compared to PGN group was observed. Thus, the milk consumption may promote the defensive factors secretion for the gastric mucosa and provides an anti-ulcerogenic effect. Release of cytokines caused the increased intestinal permeability that promoted the antigen load in lamina propria and further, increases the pathogenic activities in CD [17]. In the present study, there was significant reduction in gut permeability on SW, GIR, KF and KS milk treatment compared to PGN. This suggested the gut integrity promoting effect of milk from these breeds.

Cytokines play a critical role in the aetiology of IBD. Wang et al. [18] found that PGN and lipoteichoic acid (LTA) from *S. aureus* potentiated the production of TNF-α, IL-6 and IL-10 in whole human blood model *ex vivo*. Stimulation in IL-6 level was examined all through the 24 h of experimental period. In addition, PGN and LTA stimulated the transient elevation in TNF-α and IL-10 with peak achieved at 6 and 12 h, respectively. There was dose dependent increase in the level of TNF-α, IL-6 and IL-10 promoted by PGN and LTA. PGN induced the mRNA level release of IL-6 and IL-10 in monocytes and T-cells particularly [19]. In the present study, although a decrease in intestinal TNF-α level was found in TP, GIR and KF compared to PGN group, but different breed milk treatment didn't affect the IL-6 level in intestine. On KS and HF milk treatment, IL-10 level is comparable to control. Interferon-gamma (IFN-γ) is one of the key pro-inflammatory cytokine involved in pathogenesis of IBD. An earlier study reported that DSS inflammation showed vigorous production of IFN-γ and MCP-1 in the gut with great reduction in body weight and increment in mortality rate (60%). In addition,

administration of different breed milk showed the reduction in intestinal IFN-gamma level that was significant in SW, TP, GIR and KF group in the present study. Surprisingly, there was no difference in intestinal MCP-1 level between different groups. The inflammation causes the disruption of gut mucosal epithelium and enhanced the filtration of inflammation related cells [20]. In addition, immunoglobulin A (IgA) and G (IgG) are very essential components in intestinal mucosal immunity and also, serum IgA and IgG-coated bacteria establish intestinal homeostasis. Another study suggested that caseinophosphopeptide (CPP) protein of cow and buffalo milk possessed immunomodulatory effect in mice *in-vivo*. This study reported that there was no significant difference in serum diphtheria and ovalbumin (OVA)-specific IgG. However, a significant increase in intestinal fluid OVA and diphtheria related IgA was observed with milk CPP treatment. Hence, CPP from cow and buffalo milk demonstrated immunomodulatory property by enhancing mucosal immune system [21]. Another study suggested that in case of active IBD patients, feces soluble IgA and IgG level was significantly more than control. Moreover, the %IgA- and IgG-coated bacteria increased in feces of Crohn's disease patients along with the terminal ileal and perianal lesions [22]. Another study reported that soluble PGN of *S. aureus* is capable of increasing murine spleen B-lymphocytes proliferation and release immunoglobulins in both *in-vitro* and *in-vivo* studies and thus, act as a good mitogen. In addition, both PGN and LPS from bacteria enhanced the proliferation three to four fold after three days treatment study [23]. In the present study, PGN from *S. aureus* was injected in to rats' intestine to establish the gut inflammatory model. In the present study, there was non-significant difference in intestinal IgG level between different groups. Furthermore, no significant difference in intestinal IgA level in PGN, SW, KF and KS group was found compared to control. All above results of cytokines and immunoglobulins suggested the anti-inflammatory potential of Sahiwal milk. To further select the best anti-inflammatory and gut integrity promoting milk, gene level expression for tight junction barrier proteins and inflammatory signaling was studied.

The components of milk including sialyl (α 2, 3) lactose and lacto-N-fucopentose III are suggested to increase the TLR signaling and add to the immunologic balance in some conditions. Whole human milk has a strong anti-inflammatory property and contains many components that reduce the expression of TLR signaling components such as sTLR2 and TLR4. Therefore, feeding of milk to newborns eradicates the threat of sepsis and enterocolitis. This TLR based regulatory mechanism of milk from human provides a therapeutic remedy for numerous gastrointestinal inflammatory disorders occurred due to the disbalance in TLR signaling [24]. In the present study, the TLR-2 and TLR-4 expression was reduced, but not-significant in case of TLR-4 on milk treatment in inflammatory conditions. TLR-2 is the recognition receptor for PGN and thus, milk reduced the TLR signaling. In addition, sodium dependent bile acid transporter selectively absorbs about 60% of bile acids that are excreted in the ileum lumen and is vital for intestinal reabsorption of bile acids for the time of enterohepatic recirculation [18]. Bile acid enhances the gut integrity. In the present study, sodium dependent bile acid transporter (Na-SBT) expression was greatly enhanced in KF and KS group compared to PGN group, but expression was non-significant in different groups. Furthermore, GLP-2 decreases injuries in intestinal mucosal in obstructive jaundice rats that is due to the increased intestinal IgA and decreased bilirubin and endotoxin [25]. Thus, it has protective effect on intestinal barrier of rats. In the present study, GLP-2 expression was enhanced approximately seven fold in GIR and HF group compared to control and PGN treated rats and therefore, possess protective potential for intestine. In addition, β-defensins are small cationic peptides and antimicrobial in nature secreted by intestinal epithelial cells. Human β-defensins form an essential part of the intestinal innate immunity. β-defensins connect to the microbial membranes that causes the cell death and chemoattracts the immune cells for the body defense. These antimicrobial properties of β-defensin are well recognized against various enteric pathogens and also, in inflammatory bowel diseases [26]. In the present study, there was no difference among different groups in Defensin-1 expression, however a significant decrease in defensin-2 level was observed in SW, TP, KF, KS and HF group in inflammatory conditions. So, milk from these breeds decreased the inflammation via Defensin-2. In addition, an increment in tight junction proteins such as ZO-1, Occludin and Claudin expression was found on milk feeding in PGN treated rats. In addition, previous report by He et al. [27] suggested that Toll-like receptors (TLR) signaling pathway is fundamental to the innate immune system. A distorted expression of TLRs is a main factor for the different complications in inflammatory diseases. Many different types of biologically active constituents in human milk alters the TLR signaling pathways and expression of soluble cluster of differentiation 14 (sCD-14) and soluble toll-like receptor (sTLR). In the present study, in the peritoneal cells' CD-14 expression, there was no significant difference in SW, TP, GIR, KS and HF group compared to PGN. Thus, milk from these breeds established a balance of immunological response via TLR signaling. In addition, CD-14 gene expression was decreased about four-fold in KF group compared to PGN group.

Based on above gut health promoting affects, milk from Sahiwal and Karan Swiss were further selected for digestion in simulated conditions and tracking of mechanism of action in peptidoglycan induced intestinal cells inflammatory model. Wang et al. [28] reported that micrococci and peptidoglycan induced the transcriptional activation of NF-kB and other inflammatory genes in HEK293 cells involving the cells expressing TLR-2, however not in case of cells expressing TLR1. This TLR-2 dependent

stimulation needs NF-kB and MyD88 that are important signal transduction molecule. In the present study, there was significant reduction in the relative mRNA level expression of TLR-2, TLR-4, NF-kB, TNF-α and IL-6 on treatment of Caco-2 cells with digested SW milk (DSW) and then exposure to PGN treatment and thus, down regulating the inflammatory signaling in cells *in vitro*. In addition to this, an earlier study suggested that peptidoglycan stimulated the secretion of TNF-α from monocytes and indirectly caused the activation of endothelial and epithelial cells [29]. A previous study reported that PGN stimulated inflammatory reactions in peripheral blood mononuclear cells (PBMC) by the secretion of TNF-α. This induction of TNF-α was found in a dose dependent manner. PGN treatment at lower concentrations (0.3 or 1 μg/mL) simultaneously with bacterial DNA stimulated more TNF-α in comparison to separate use of both. The secretion of TNF-α was induced up to 15 fold more with the combined use of PGN and bacterial DNA compared to the separate use of two [30]. Tight Junction (TJ) proteins are the main regulators of signal transduction that alters cells proliferation, migration, differentiation and the components of the homeostasis. This results in changes in immune system in the intestine. Tight junction proteins such as Occludin and Claudin proteins are significantly required for epithelial cells' migration. Claudin works like a pore sealing protein. Occludin have the property to shift to the different paracellular locations. Further, ZO-1 is a linker protein between tight junction and actin cytoskeleton. An earlier study reported that *S. aureus* in human epidermal keratinocytes demonstrated biphasic and time dependent response for tight junctions. Normal human epidermal keratinocytes (NHEKs) on infection with *S. aureu*s and *S. epidermidis* for 24 h exhibited a reduction in Claudin-1 and Claudin-4 for the cell-cell interaction. But, mRNA level expression of Claudin-1, Claudin-4, Occludin and ZO-1 was not changed after 8 and 12 h of infection [27]. Another study reported that ex-vivo long term porcine skin inoculation with *S. aureus* showed decrease in Claudin-1 and ZO-1 protein immune-intensity and thus, reduced the tight junction functionality [31]. Peptidoglycan from *S. aureus* induced a noncanonical TLR-2 linked MyD88-ARNO (ADP-ribosylation factor nucleotide-binding site opener)-ARF6 (ADP-ribosylation factor 6) signaling pathway in human retinal endothelial cells, resulted in decreased p120-catenin and VE-cadherin internalization that led to harmful effects in the endothelium after 24 h. Protein level disruption of ZO-1 occurred that was associated with the epithelial barrier characteristics loss [32]. In the present study, there was no effect on mRNA level expression of occludin protein between different groups in *in vitro* cells. Interestingly, a significant increment was found in the ZO-1 expression on pretreatment of DSW compared to PGN group in cellular study. Moreover, a significant improvement was observed in the pretreated DSW cells compared to cells treated with PGN.

In light of above results, it is very clear that milk from Sahiwal breed affects the mRNA expression of TLR-2 and TLR-4 that down regulated the mRNA expression of NF-kB for the resulting suppression of the inflammation related problems caused by cytokines, TNF-α and IL-6 because of the influence of peptidoglycan from *Staphylococcus aureus*. In addition, these *in vitro* results suggest a potential action pathway at mRNA level in *in vivo* in rats. The down-regulation of mRNA expression of TLR-2, NF-kB, TNF-α and IL-6 mainly involves in the anti-inflammatory potential of Sahiwal milk in rats *in vivo*. All the above results recommended that DSW has better response in improving the gut integrity by effecting the gene level expression of inflammatory and gut integrity proteins as compared to DKS. Furthermore, it helps in maintaining the proper gut functions in inflammatory conditions. It also prevents the infection by decreasing the mRNA expression of TLR-2 and NF-kB that ultimately reduced the injurious effects of cytokines i.e. TNF-α and IL-6. All these improvements together leads to reduction in the deterioration of gut integrity by increasing the mRNA gene level expression of gut tight junction proteins, ZO-1 and Claudin at cellular level. In collaboration of above results of *in vivo* and *in vitro* conditions, the Sahiwal milk possesses the gut integrity restoration effects in IBD like inflammatory conditions. Furthermore, the mechanistic approaches in human needs to be studied to form a simple and easily available remedy for IBD patients.

Conclusion

In the present study, the difference in the immune response of cow milk from six different breeds i.e. SW, TP, GIR, KF, KS and HF was studied in gut inflammatory rat model. Both systemic and localized (gut) immune response were investigated. Firstly, the macrophage phagocytic activity, spleen lymphocytes proliferation rate, *in vivo* gut permeability testing, gastric acidity measurement, assessment of immunoglobulins, cytokines and inflammatory proteins was performed. Other than this, the inflammatory signaling molecules and gut integrity protein gene expression level was explored in Caco-2 cells after pre-treatment of digested milk from SW and KS in condition of inflammation induced by PGN. The above results of general health parameters, systemic and localized immune response, Sahiwal breed milk showed the best anti-inflammatory response in comparison to Tharparkar, Gir, Karan Fries, Karan Swiss and Holstein Friesian in inflammatory conditions induced by PGN in rats. In *in vitro* Caco-2 cells also, simulated digested Sahiwal milk demonstrated good anti-inflammatory response and improves the gut integrity compared to digested Karan Swiss milk via reducing inflammatory NF-kB signaling. In future, the clinical trials in humans are required to provide a therapeutic and safe strategy or remedy for the IBD patients.

Acknowledgment

We are thankful to the Director, National Dairy Research Institute, Karnal, India for providing financial support and laboratory facilities to carry out this work. There is no conflict of interest between authors.

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