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Effect of milk fermented with *Lactobacillus sp.* in modulation of intestinal permeability in high fat diet fed mice

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Abstract
Consumption of high fat diet (HFD) is known to disturb the gut microflora, which is a source of endotoxins. Its increase in plasma is related to increased intestinal permeability. In the present study, effect of milk fermented with *L. casei* NCDC-19 in reference to intestinal permeability/integrity was evaluated using Swiss Albino mice. Permeability was determined using FITC-dextran 4000 Da through measurement of fluorescence in blood plasma. HFD feeding resulted in a significant increase in intestinal permeability. A positive effect of dietary supplementation of probiotic fermented milk on intestinal permeability was observed. Expression of two important genes related to intestinal permeability was determined by qRT-PCR. Reduced expression of tight junction proteins was observed in HFD fed group which confirms the increased permeability. Occludin was found up-regulated significantly as result of probiotic fermented milk. A similar trend of up-regulation of ZO-1 was seen in probiotic FM/skim milk fed groups. Present study suggests a positive effect of probiotic fermented milk on intestinal permeability in HFD fed conditions.

Keywords: Intestinal permeability, High fat diet, Probiotics, Tight junction

Introduction
Intestinal permeability varies at niche among gut microbiota and endotoxins in ecosystem of metabolic dysfunctions (Teixeira *et al.*, 2012) [11]. Dietary pattern and the improvement of nutritional deficiencies modulates intestinal permeability and gut health. The appropriate working of intestinal barrier is necessary for avoidance of more translocation of inflammatory agents (e.g. LPS) in the circulation. In a study (Hausmann., 2010) [8] suggested that microbiota can modulates integrity of intestinal epithelium, which can further influence intestinal permeability function. Intestinal epithelium works via transcellular or paracellular modes but selectively (Ahrne *et al*., 2011) [1]. Modified intestinal permeability plays an important role in the pathogenesis in several critical situations such as trauma and sepsis. Impaired gut barrier function may chronically take days to restore and could eventually lead to movement of intestinal bacteria into the body. Abnormal intestinal barrier function plays a pivotal role in inflammatory bowel disease (IBD). Type-1 diabetes and coeliac disease are examples of auto-immune situations where increased paracellular permeability has been implicated in the development of disease (Forster, 2008) [7]. Lactic acid bacterial strains are the most common microbes employed as probiotics in dairy industry. Indigenous cultures may support colonization, more transient time and beneficial impact for localized population since probiotics works on strain and host specific manner (Boyle *et al*., 2006) [2]. Herbal ingredients pertains antitumor, anti-inflammatory, antioxidant and laxative effects (Joseph *et al.*, 2010) [9]. In view of the crucial significance of probiotic microorganisms in relation to various health benefits, gene expression related to impaired gut barrier function, the present study was planned to evaluate the effectiveness of fermented milk containing a probiotic (*L. casei* NCDC 19), and fermented milk plus herbal ingredient (*aloe vera*) under high fat diet conditions on intestinal function using Swiss albino mice.

Materials and methods

Animal Trial

Lactobacillus casei NCDC 19 was obtained from National Collection of Dairy Cultures (NCDC), DM Division, NDRI, Karnal. Skim milk was obtained from experimental dairy of the institute for the preparation of probiotic fermented milk. Twenty swiss albino mice (20-25 g) were obtained from Small Animal House of the institute and housed in cages (n=4/group, two animals/cage) under 12 hr light/dark conditions at 20 ± 2ºC. One group of animals was fed on normal diet and the rest of groups were fed on HFD (Table 1). The normal and HFD (Table 2) was given to respective groups *ad libitum*. Amount of Herbal ingredient was 1%w/w in case of HFD+HI. Skim milk and probiotic fermented milk were provided during light phase (~ 9:30 am in morning) and removed before start of dark phase (~ 4:30 pm in evening). All the animals had free access to water.

Table 1: Grouping of animals according to diet

Group	Diet
GROUP I	Normal Diet (basal)
GROUP II	HFD
GROUP III	HFD + Skim Milk
GROUP IV	HFD + Probiotic fermented milk
GROUP V	HFD + Herbal Ingredient + Probiotic fermented milk

Table 2: Ingredients of normal and high fat diet

Component	Normal diet (%)	High fat diet (%)
Starch	53	25
Casein	20	20
Sucrose	10	10
Soyabean oil	7	7
Lard	0	28
Cellulose	5	5
Vitamin mixture	1	1
Mineral mixture	3.5	3.5
Choline chloride	0.2	0.2
methionine	0.3	0.3

After six weeks of feeding schedule, the animals were sacrificed by cervical dislocation under anaesthesia to collect blood and tissues. Ileal portion were stored in RNA Later (Sigma) for gene expression studies. All animal procedures were conducted at Small Animal House of NDRI, Karnal.

Measurement of intestinal permeability

This measure is based on the intestinal permeability to 4000 Da fluorescent dextran–FITC (DX-4000–FITC) (Sigma-Aldrich, USA). Briefly, mice fasted for 6 hr were given DX-4000–FITC by gavage (500 mg/kg body weight, 125 mg/ml). After 1 hr blood was collected by cardiac puncture and centrifuged at, 3000 rpm for 5 min. Plasma was diluted in an equal volume of PBS (pH 7.4) and analysed for FITC-dexran concentration with a fluorescence spectrophotometer (Perkin Elmer, victor^{TM} X3) at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Standard curve was obtained by diluting FITC–dextran in non-treated plasma diluted with PBS (1:1 v/v).

Quantification of intestinal gene expression

Total RNA from ileal tissue was isolated using TRIzol method and quantified. The purity was checked on nanodrop. The relative mRNA expression of ZO-1 and Occludin in ileum was done. First strand of cDNA was synthesized from isolated RNA (2.0 µg template) using cDNA synthesis kit (Fermantas). The cDNA synthesized was used as a template for quantification using Real-time PCR. B-Actin was taken as a reference gene for normalization of target gene for relative quantification. The primers (Sigma) used were as per references given in table 3.

Table 3: Primers sequences used for real-time quantitative PCR

Gene	Primer sequence	Annealing temperature	Reference
ZO-1	**F**-ACCCGAAACTGATGCTGTGGATAG **R**-AAATGGCCGGGCAGAACTTGTGTA	61 **ºC**	Cani *et al*., 2008 [4]
Occludin	**F**-ATGTCCGGCCGATGCTCTC **R**-TTTGGCTGCTCTTGGGTCTGTAT	61 **ºC**	Cani *et al*., 2008 [4]

Statistical analysis

Statistical analysis was done by using GraphPad prism version 5 for windows (GraphPad Software, San Diego, CA) for each data and each value is expressed as the mean ± S.E.M. The effect of HFD, HFD supplemented with skim milk/fermented milk, and HFD supplemented with fermented milk and aloe vera on different parameters was evaluated using Analysis of Variance (ANOVA). Bonferroni comparison test was used for comparative analysis. $P < 0.05$ was considered as significant level.

Results and Discussions

Among the different environmental factors, preference for a fat-enriched diet, excessive calorie intake and a sedentary life style increase the occurrence and promote the progression of metabolic disorders. Recently, an association between obesity and low grade chronic systemic inflammation has been suggested. The systemic inflammation is closely linked to the plasma endotoxemia caused by increased intestinal permeability in obese animals. It has also been demonstrated

by Cani and coworkers (2008) [4] that feeding a high fat diet leads to increased intestinal permeability in mice. Dietary interventions are being tried to change the intestinal microbiota to reduce the impact of high fat diet on the occurrence of metabolic diseases among which the health promoting bacterial groups which includes mostly the *Lactobacilli* and *Bifidobacteria* (Karczewski *et al.*, 2010; Cani *et al.*, 2007) [10, 5] are gaining lot of importance. Indigenous cultures may have better colonization, longer transient time and more healthful effects for local population as probiotics are highly strain and host specific (Boyle *et al*., 2006) [2]. In the present investigation, the experimental animals were fed on normal diet (control), high fat diet (HFD), and HFD supplemented with skim milk/milk fermented with probiotic (*L.casei* NCDC 19). A combination of herbal ingredient (*Aloe Vera*) and probiotic fermented milk was also tried. Effects of different dietary treatments on intestinal permeability and expression of associated genes were examined.

Determination of intestinal permeability *in vivo*

The intestinal permeability was measured on the basis of permeability to 4000 Da fluorescent dextran and the results are depicted in Fig.1.

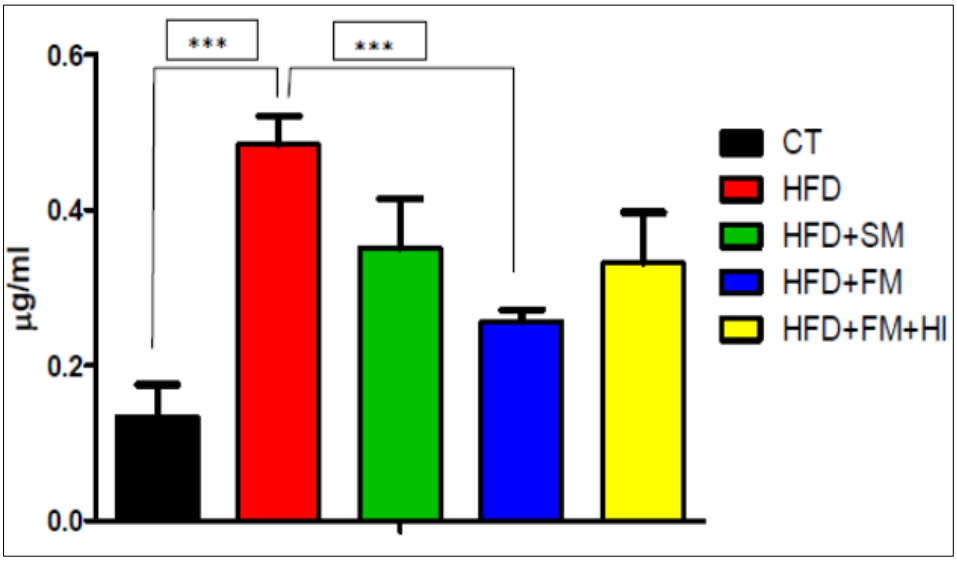

Fig 1: Effects of feeding probiotic fermented milk on Intestinal permeability; Values are expressed as mean ± SEM (n=4). The bar over the histogram shows the significant difference of control with HFD and HFD with FM (***p< 0.05)

It was found to increase significantly in high fat diet fed group (>2 fold) in comparison to control group. The intestinal permeability measured as Dx-4000-FITC in plasma diluted with PBS (1:1) was 0.13 ± 0.04µg/ml and 0.48 ± 0.04µg/ml for control and HFD fed group, respectively. Dietary incorporation of probiotic (*L. casei* NCDC 19) containing fermented milk to high fat diet was observed to exhibit a positive effect on intestinal permeability/integrity. The mean value of FITC dextran diluted plasma for HFD + FM group was 0.26 ± 0.02µg/ml which was significantly lower as compared to HFD fed group animals. The concentration of FITC dextran in skim milk fed group was also observed to be lower (0.35 ± 0.06µg/ml), however, the difference was not found to be statistically significant as compared to HFD fed group. A similar observation was also made in case of animals receiving HFD supplemented with *Aloe vera* and fermented milk.

The intestinal permeability has been reported to be significantly altered as a result of high-fat feeding by other workers also. Cani *et al*., (2008) [4] demonstrated that high fat feeding dramatically increases intestinal permeability, and this was opposed by antibiotic treatments due to reduced metabolic endotoxemia, caused by altered microbial balance under high fat conditions. In another study, Serre *et al*. (2010) observed that due to intestinal inflammation epithelial barrier integrity was altered in obesity prone rats and found a significant effect on gut permeability in obesity prone rats compared with low fat diet (LF) animals. There is paucity of information with regard to effects of probiotics or probiotic containing fermented milk on intestinal integrity/permeability. There are several mechanisms by which probiotics are proposed to exhibit beneficial effects on the host. One of the mechanisms of probiotic action is directed at the epithelial surface where they modulate the integrity of the epithelial cell barrier and regulate the function and expression of tight junction proteins and mucus secretion (Caballero-Franco *et al.*, 2007) [3]. Probiotics are also known to produce a number of degradation products/metabolites during fermentation of milk, and logically it can be exciting to explore the role of such constituents in maintenance of intestinal integrity/functionality.

Relative mRNA expression of Occludin

The relative mRNA expression of occludin in ileum represented as fold change is shown in Fig.2.

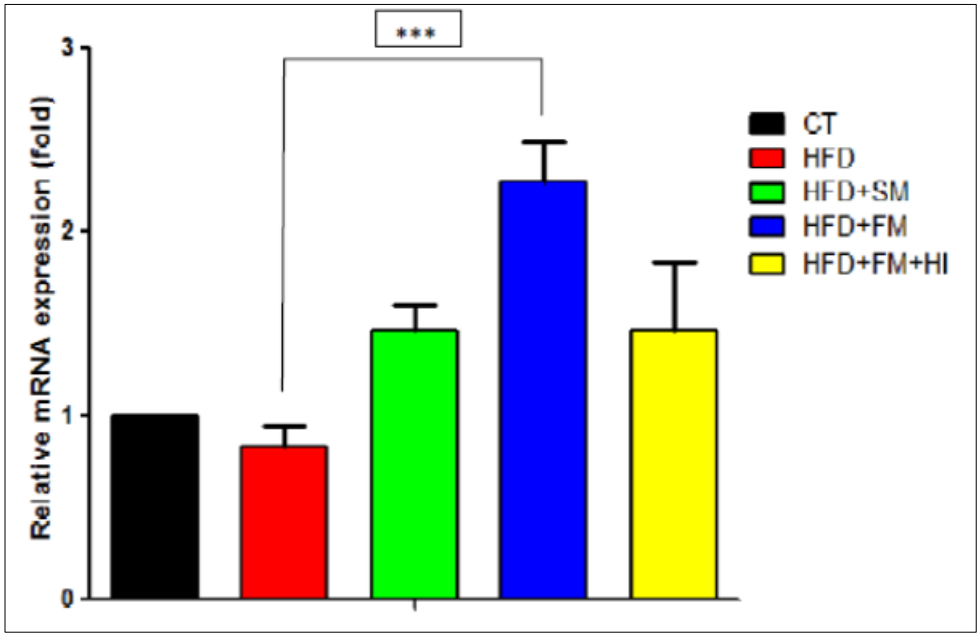

Fig 2: Effect of probiotic fermented milk on relative mRNA expression of Occludin. Values are expressed as mean ± SEM (n=3). The bar over the histogram shows the significant difference of HFD with HFD+FM (***p<0.05)

HFD fed group showed a decrease in expression of occludin relative to basal diet control fed group, the value being 0.83 ± 0.11(Mean ± SEM) fold. Dietary supplementation of probiotic (*L. casei* NCDC 19) fermented milk was observed to significantly (P<0.05) upregulate the expression of occludin to 2.28 ± 0.21 (Mean ± SEM) fold in comparison to control group. Similarly, supplementation of skim milk/fermented milk + *Aloe vera* in HFD + SM and HFD + FM + HI resulted in up regulation of occludin mRNA and relative value of expression were 1.46 ± 0.14 and 1.46 ± 0.36 (Mean ± SEM) fold respectively. However, the differences were not found to be statistically significant.

Relative mRNA expression of ZO-1

The results on relative mRNA expression of ZO-1 in ileum, represented as fold change, are depicted in Fig.3 HFD fed group showed a decrease in expression of ZO-1 relative to basal diet control fed group, the value being 0.66 ± 0.36 (Mean ± SEM) fold. Dietary supplementation of probiotic (*L. casei* NCDC 19) fermented milk was observed to upregulate the expression of ZO-1 to 1.54 ± 0.07 (Mean ± SEM) fold in comparison to control group. Similarly, supplementation of skim milk in HFD + SM group resulted in upregulation of ZO-1 mRNA expression and value of expression being 1.15 ± 0.12 fold. However, the difference was not found to be statistically significant.

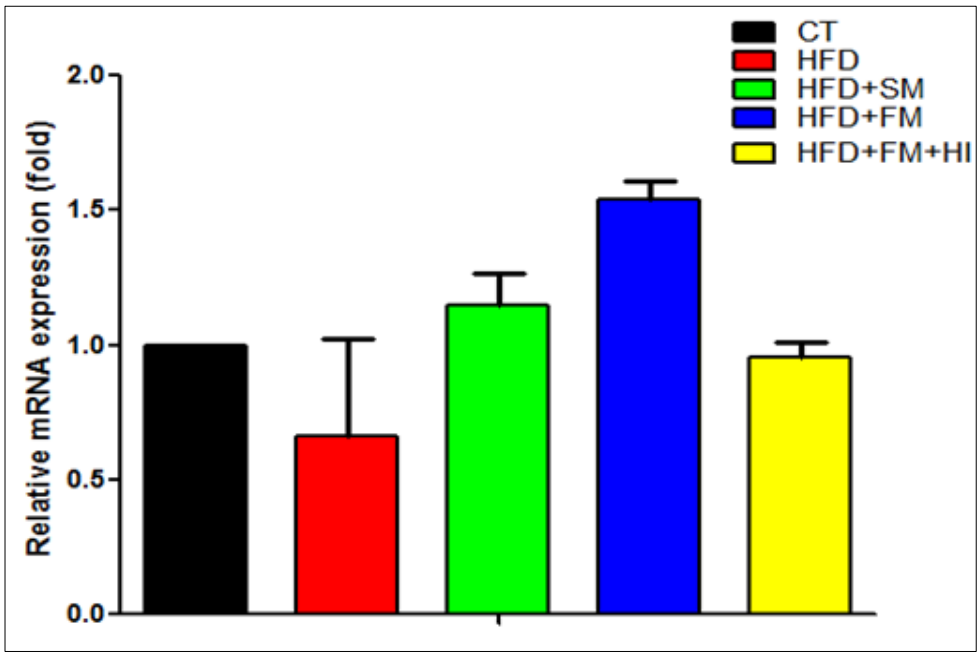

Fig 3: Effect of probiotic fermented milk on relative mRNA expression of ZO-1. Values are expressed as mean ± SEM (n=3).

Conclusion

Intestinal permeability in different treatment groups was determined using FITC dextran. The HFD feeding resulted in a significant increase in intestinal permeability. Probiotic containing fermented milk seems to exhibit a positive effect on permeability. Incorporation of *Aloe vera* did not exhibit any significant effect in comparison to the probiotic FM fed group. A reduced expression of two important genes of tight

junction (Occludin and ZO-1) was observed in HFD fed group which verifies the increased intestinal permeability resulting due to high fat feeding. Occludin was found to be upregulated significantly (>2 fold) in probiotic fermented milk group as compared to animals fed HFD diet only. A similar effect of skim milk feeding was also observed. A similar trend of upregulation of ZO-1 was also observed in probiotic FM/skim milk fed groups. Present study concludes that HFD feeding affects the intestinal permeability. During fermentation of milk various types of beneficial metabolites are produced. Therefore, it will be exciting to conduct more studies with reference to probiotics and their fermentation products in relation to gut epithelium interactions and nutrients sensing to elucidate the mechanistic aspects.

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