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Immunomodulatory response of orally administrated colostrum whey powder supplemented dahi in immunocompromised swiss albino mice

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Abstract
Immunomodulation is related to nonspecific activation of the function of macrophages, complement, natural killer cells, lymphocytes and is also associated with generation of activated cells. This study was undertaken to investigate the immunomodulatory reponse of orally administered colostrum whey powder supplemented dahi in immunocompromised swiss albino mice. The mice were immunocompromised by giving intravenous injections of cyclophosphamide drug @20mg/kg body weight. The mice fed with stock diet as well as the group fed with plain dahi showed significant decrease in phagocytic activity, lymphocyte proliferation index, serum IgG and IgA in comparison to the group fed with colostrum whey powder supplemented dahi. The results suggested that colostrum whey powder supplemented dahi had a potential immunostimulatory effect on immunocompromised mice.

Keywords: Immunomodulatory, dahi, colostrum whey powder, phagocytic activity, lymphocyte proliferation index

1. Introduction
Colostrum is the first lacteal secretion from the mammary glands after parturition and is secreted for 24-72 hours. Different authors have illustrated the therapeutic role of colostrum in fighting against AIDS, cancer, heart disease, diabetes, auto-immune diseases, allergies, herpes, bacterial, viral and parasitic infections, gingivitis and flu (Korhonen *et al.* 2000 [6]; Hoerr and Bostwick 2000 [4]; van Hooij-donk *et al.* 2000) [12]. Historically in India, Ayurvedic physicians have used bovine colostrum for treatment of different ailments for thousands of years There are sufficient evidences which suggests that colostrum exerts its health benefits due to the presence of wide range of immune factors, growth factors and high level of bioactive components e.g. immunoglobulins (Igs), particularly IgG1, growth factors, i.e. insulin like growth factors I and II, transforming growth factor β2 and growth hormone in addition to lactoferrin, lysozyme and lactoperoxidase (Pakkanen and Alto 1997) [10]. Colostrum is a natural occuring food and therefore it is perceived that it can be consumed without any side effects or drug interactions. Most colostrum manufacturers recommend and optimal intake of about 4g/day of dried colostrum. Bovine colostrum is not species-specific and therefore it work effectively in human beings. As a person ages, it takes a longer time to fight with diseases as humans gradually lose immunity in the body due to decrease in immune factors and growth factors in the body which can be replaced with bovine colostrum (Kelly 2003, Playford *et al.* 2000) [5, 11]. Majority of the immunoglobulins and antibodies ingested from colostrum are not absorbed but remain in the gastrointestinal tract where they act on pathogens and thereby they are prevented from attacking the body's defenses (Kelly 2003) [5].
Incorporation of colostrum whey powder in dahi will be a new way to deliver the immune factors and growth factors of colostrum to the health conscious consumers as dahi is consumed daily by Indian people. Consequently, dahi prepared with colostrum whey powder will possess better nutritional and functional property as compared to dahi prepared from plain milk.
The present study was carried out to investigate the immunomodulatory response of oral administration of colostrum whey powder supplemented dahi in immunocompromised swiss albino mice.

2. Materials and methods
Preparation of colostrum whey powder supplemented dahi Bovine colostrum samples from the first, second and third milkings were collected from NDRI cattle yard and pooled up at -20 ºC.

Freeze dried bovine colostrum whey powder was prepared as per Das and Seth, 2011.

For dahi preparation, milk was standardised to 4% fat and 14% TS by adding skim milk powder and freeze dried colostrum whey powder was added @ 4g/100ml of dahi mix. The standardised mix was homogenised and heat treated (68ºC for 30 min), cooled to 30 ºC, then inoculated with 3% starter culture (NCDC 261). The inoculated milk was packaged in 100 ml polystyrene cups and incubated at 25-30 ºC for 16-22 hours. The finished product was stored under refrigeration at 5±3 ºC.

2.1 Animal's diet and their immunocompromisation using cyclophosphamide

Swiss albino mice weighing approx. 25 to 35 g were obtained from the small animal house of the institute (NDRI, Karnal, India) and were housed in spacious polypropylene cages in a well-ventilated room and were provided with water and stock diet comprising of Bengal gram, wheat, vegetable oil, mineral mixture, vitamin mixture. Adult male swiss albino mice were divided in to three groups of six mice each and maintained for a period of 15 days. Mice were then acclimatised to the environment for 1 week prior to the experiment. This study was approved by the Institute Animal Ethics Committee (IAEC), National Dairy Research Institute, Karnal, India.

For immunocompromised of mice, intravenous injection of cyclophosphamide @ 20mg/kg body weight were given once in a day for consecutive 5 days and then feeding practices was carried out for fifteen days. Cyclophosphamide dose of 20 mg/kg has been found to be a efficient dose in suppressing T cells as compared with CP 200 mg/kg (Askenase *et al*., 1975; Mitsuoka *et al*., 1976).) [1, 9]. The mice were randomly assigned into three groups of six each as given below:

Group MB: Normal synthetic diet.

Group MBD: Normal synthetic diet + plain dahi (15% of the diet).

Group MPCD: Normal synthetic diet + Colostrum whey powder supplemented dahi (15% of the diet)

Each animal consumed 5 g of diet per day. After completion of experimental regimen, the mice were fasted overnight and animals were sacrificed by cervical dislocation. Blood was collected and serum was separated. Peritoneal fluid was collected for obtaining macrophages. Liver, spleen and intestine were dissected out and used for biochemical studies. The procedure used for the collection of the intestinal fluid was a modification of procedure described by Lim *et al.* (1981) [7]. The intestine gastro-duodenal to ileocaecal junction was carefully removed and cut into small pieces, and the contents were washed out with 5 mL phosphate-buffered saline (pH 7.2) and then centrifuged at 2000 g for 30 min.

Macrophage collection and in vitro phagocytosis assay

DMEM (Dulbecco's Modified Eagle's Medium) Ham's F-12 medium (without phenol red) supplemented with sodium bicarbonate (1.2g/l), bovine serum albumin (0.1%), pencillin (200 U/ml) and streptomycin (50 µg/ml) was used to collect peritoneal fluid. The pH of the medium was adjusted to 7.2 using 1N HCl or 1 N NaOH and then sterilized through 0.22 µ Millex- GV disposable filter unit (Millipore).

Animals were killed by cervical dislocation and the abdominal skin was swabbed with alcohol (70%). The skin was carefully removed, leaving the peritoneum intact. DMEM Ham's F-12 medium (5 ml) was injected into peritoneal cavity using 26 G needle followed by gentle massaging of the abdomen and then peritoneal exudates (≈ 4ml) were collected with 22 G needle. Macrophage cells in peritoneal exudates were counted using neubauer chamber. A portion of the peritoneal exudates was saved for *in vitro* phagocytosis. The exudates were transferred into petridish (35 mm) and incubated in a humidified atmosphere of 5% CO_2 in air at 37 ºC for 2 h to allow attachment of adherent cells. Non-adherent cells were removed by decantation of culture media and adherent cells were washed two times with DMEM Ham's F-12 medium. Number of adherent cells was calculated by subtracting non-adherent cells from total cells in peritoneal fluid. Phagocytosis was carried out according to the method of Hay and Westwood (2002) [3].

Yeast culture was grown in yeast extract polypeptone dextrose for 18 h at 30 ºC, then autoclaved at 1200 C for 45 minutes in culture medium, washed three times in calcium and magnesium free phosphate buffer saline (PBS) and aliquot was stored at 40 ºC until use. Just before use, it was sonicated gently in a water bath to disrupt clumps as diluted to 10^8 cells/ml in DMEM Ham's F-12 medium (without phenol red).

Peritoneal exudate containing macrophages (1ml) was incubated at 37 ^{0}C for 2 h in 35 mm culture plates. Non-adherent cells were removed by decantation and fresh medium (1ml) was poured into cultured plates and then again incubated at 37 ^{0}C for 2 hrs. The macrophages were then incubated with 100µl of yeast cell suspension (10^8 cells/ml) for 1 hour in a humidified atmosphere (5% CO_2) at 37 ^{0}C. The medium was removed and the cells washed twice gently with culture medium, dried in air. Then stained for 5 min with May-Grunwalds Eosin Methylene blue modified stain, freshly diluted with Giemsa buffer (1:2) for 15 min. The extra stain was removed by washing cells with Giemsa buffer. The phagocytosis was observed at 1000X magnification under oil immersion, and following observations were recorded.

$$\text{Percent phagocytosis} = \frac{\text{Number of macrophages with yeast cell internalized}}{\text{100 macrophages.}}$$

2.2 Lymphocyte proliferation index

The male albino mice were sacrificed by cervical dislocation and dipped in 70% ethanol. The peritoneum was opened and spleen was taken out and placed in sterile tubes containing tissue culture medium (1ml), it was washed and made free from connective tissue and adipose tissue.

The collected spleens were subsequently transferred to sterile petridish containing medium. They were teased gently using a sterile needle and forceps to release cells from spleen. It was allowed to stand for 2 min for sedimenting clumps. Then, using 1 ml pipette the upper portion of medium containing splenocytes was transferred to 15 ml sterile centrifuge tube and again left to stand for 2 min (in ice) and transferred to another sterile centrifuged tube. This cell suspension now devoid of clumps and large particles was centrifuged at 1500 rpm at 4 ^{0}C for 5 minutes and the supernatant was discarded. The cells were washed once again in basal culture medium and then to the pellet, 1ml of erythrocyte lysis buffer was added. After one minute, 5 ml of culture medium was added to this and centrifuged at 1500 rpm at 4 ^{0}C for 5 minutes. The lymphocyte cell suspension was washed twice with cold culture medium to remove traces of lysis buffer and finally suspended in 2 ml of RPMI containing 10% FCS medium. A small aliquot of cell suspension was taken for cell count and checking cell viability.

The viability and counting of cells was carried out according to the method of Hay and Westwood (2002) [3]. Cell count was done in one of the four peripheral, single ruled areas and total viable cells were calculated using following formula:

Total number of viable cells/ml = Average count of viable cells × 10^4 × Dilution factor

Cell suspension containing 1×10^7 viable cells/ml was made in culture media (containing 10% FCS). 100 µl cell suspension was dispensed in each well of 96 well flat bottomed tissue culture plates. Added 10 µl of mitogens, lymphocytes were cultured in medium (Lipopolysaccharide and Concanavalin A) at 37 ^{0}C for 48 h in a 95% relative humidity air atmosphere with 5% CO_2 in a carbon dioxide incubator.

The growth of lymphocytes in each well was examined under inverted microscope at 200x magnification after 24 and 48 h. The cell viability was determined using the colorimetric MTT assay originally described by Mosmann (1983) [8]. Data is expressed as Proliferation Index (PI) and calculated using the following equation:

$$PI = \frac{\text{OD with mitogen}}{\text{OD without mitogen}} \times 100$$

2.3 IgG and IgA Assay

The total IgG levels in serum and IgA levels in intestinal fluid were quantified using ELISA kit supplied by Koma Biotech, Seoul, Korea. The operating procedures were strictly followed as provided by the manufacturer. The principle of the ELISA kit was quantitative sandwich enzyme immunoassay technique.

2.4 Statistical Analysis

The observations were analysed by analysis of variance (ANOVA) using SYSTAT 6.0.1 software (Systat Software Inc. Washington St. Chicago USA). The means were tested for significance by post hoc test (Bonferroni adjustment) using SYSTAT 6.0.1 software. Statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1 Efficacy of bovine colostrum whey powder supplemented dahi on body weight and phagocytic activity in immuno-compromised swiss albino mice

After 15 days of dietary regimen, it was observed that there was no significant decrease ($P>0.05$) in the body weight in the groups fed with colostrum whey powder supplemented dahi as compared to other two groups where in a significant decrease in body weight was observed (Figure 1).

Figure 2 represents the effect of feeding synthetic diet, control dahi and colostrum whey powder supplemented dahi on phagocytic activity. There was an increase in phagocytic potential of macrophages which was apparent from yeast engulfment experiments. Macrophages acts as primary defensive cells which are responsible for engulfing foreign particles and thus activates the immune system. In this study, the phagocytic activity of group fed with colostrum whey powder supplemented dahi was more than the other two groups and was statistically significant ($P < 0.05$).

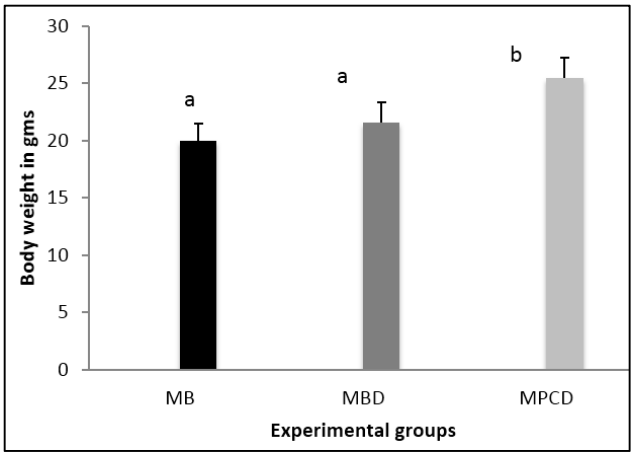

Fig 1: Effect of colostrum whey powder supplemented dahi on body weight of immuno compromised swiss albino mice

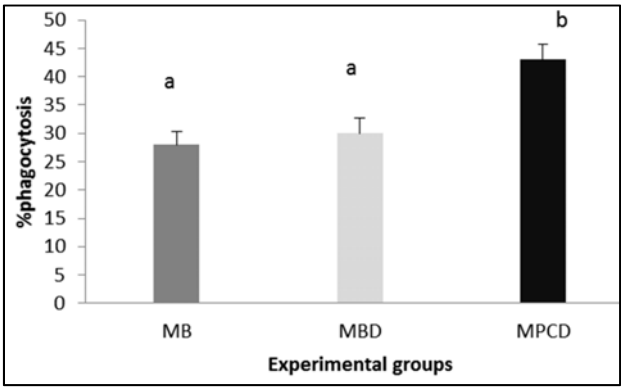

Fig 2: Effect of colostrum whey powder supplemented dahi on phagocytic activity of immune-compromised swiss albino mice

3.2 Efficacy of bovine colostrum whey powder supplemented dahi on Lymphocyte proliferation assay in immunocompromised swiss albino mice

The lymphocyte proliferation assay of the animals fed with control colostrum supplemented and colosrum whey powder supplemented dahi were determined by MTT assay. MTT is cleaved by all living metabolically active cells but not by dead cells or erythrocytes (Mosmann, 1983) [8]. The amount of formazon generated is directly proportional to the cell number. The colorimetric assay measure number and activity of living cells at the end of the assay. Figure 3-4 represents the proliferative response of lymphocytes with mitogens LPS (lipipolysaccharide) and ConA (Concanavalin A) which were used to induce the cell proliferation.

Animal studies with 15 days of dietary regimen showed that there was a significant increase in the proliferation of lymphocytes in the group fed with colostrum whey powder supplemented dahi when compared to control and the group fed with plain dahi. Figure 3 shows that when the lymphocytes incubated with LPS, there was a significant increase in proliferative response in the group fed with colostrum whey powder supplemented dahi when compared to control. Similar observations were found in case of lymphocytes incubated with ConA which is presented in figure 4. There is no published work so far correlating colostrum whey powder with lymphocyte proliferation index in swiss albino mice.

3.3 Efficacy of bovine colostrum whey powder supplemented dahi on IgG and IgA level in immunocompromised swiss albino mice

The IgG levels in the serum of group fed with synthetic diet, group fed with plain dahi, and the group fed with colostrum whey powder supplemented dahi after 15 days were found to be 667, 674 and 710µg/mL respectively (Table 1). Ingestion of colostrum whey powder supplemented dahi resulted in 5.07% increase in lgG level as compared to group fed with plain dahi. IgG level in serum represents the systemic immunity, and any increase in immunoglobulin content indicates enhanced systemic immunity. There was significant increase in the level of lgG on feeding of colostrum whey powder supplemented dahi suggesting that it has an immunostimulatory effect. There is no published literature available to establish the effect of colostrum whey powder on serum immunoglobulin G levels in albino mice.

The level of lgA in the intestine of group fed with synthetic diet, group fed with plain dahi, and the group fed with colostrum whey powder supplemented dahi after 15 days were found to be 57.4, 58.2, 63.9 µg/mL respectively (Table 1). Ingestion of colostrum whey powder supplemented dahi resulted in 9.7 % increases in lgG level as compared to group fed with plain dahi. There were significant differences observed between control and colostrum whey powder supplemented dahi. The levels of lgA in intestine represent mucosal immunity. There is no published work available to establish the effect of colostrum whey powder on intestinal IgA levels in albino mice.

Table 1: Immunoglobulin levels (µg/ml) of immunocompromised swiss albino mice after 15days of dietary regimen

Groups	MB	MBD	MPCD
Type of immunoglobulins			
IgG	$667^{a}\pm13.38$	$674^{a}\pm12.11$	$710^{b}\pm18.49$
IgA	$57.6^{a}\pm5.18$	$58.2^{a}\pm5.78$	$63.9^{b}\pm6.45$

Data are presented as means ± SEM (n = 6). Values with same superscripts (a, b) coloumn wise are not significantly different (P > 0.05) from each other.

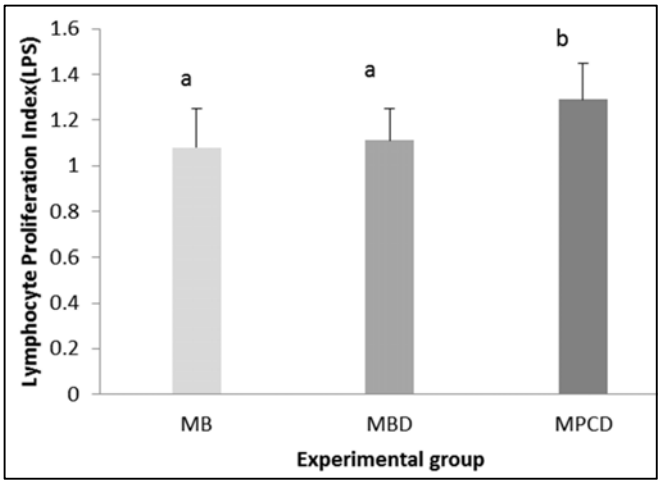

Fig 3: Effect of colostrum whey powder supplemented dahi on lymphocyte proliferation index (in presence of LPS) of immune compromised swiss albino mice

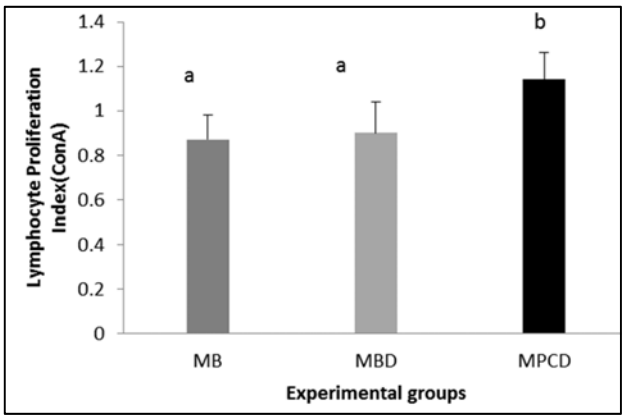

Fig 4: Effect of colostrum whey powder supplemented dahi on lymphocyte proliferation index (in presence of Concanavalin A) of immune compromised swiss albino mice

4. Conclusion

The results suggest that dahi can act as a good carrier for delivering the immune and growth factors of colostrum whey powder. Feeding colostrum whey powder supplemented dahi to immunocompromised mice showed significantly higher phagocytic activity, lymphocyte proliferation index and immunoglobulin concentration (IgA and IgG) as compared to groups which did not consume colostrum.

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