In Vivo Implications of Potential Probiotic *Lactobacillus reuteri* LR6 on the Gut and Immunological Parameters as an Adjuvant Against Protein Energy Malnutrition

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Abstract

The present study investigated the impact of probiotic *Lactobacillus reuteri* LR6 on the gut and systemic immunity using protein energy malnourished (PEM) murine model. Thirty male Swiss albino mice were divided into five groups: control (C), malnourished (M), probiotic fermented milk (PFM), skim milk (SM), and bacterial suspension (BS) with six mice per group. Group C was fed with conventional diet throughout the study while the other groups were fed with protein calorie restricted diet until the development of malnutrition. After development of malnutrition, group M was continued with the restricted diet while other groups were fed with re-nourished diet supplemented with PFM, SM, and BS for 1 week, respectively. Thereafter, mice were sacrificed and different histological, microbiological, and immunological parameters were studied. Probiotics feeding in PEM model as fermented product or bacterial suspension improved the intestinal health in terms of intact morphology of colonic crypts, normal goblet cells, and intact lamina propria with no inflammation in large intestine, absence of fibrosis, and no inflammation in spleen. The number of secretory IgA$^+$ cells was significantly higher in group PFM and BS. Also, increase in the phagocytic percentage of the macrophages and bone marrow derived dendritic cells (DCs) were observed in the PFM and BS group in comparison to the group M. In comparison to the group M and SM, lactobacilli, bifidobacteria, and Firmicutes counts were significantly higher in the group PFM and BS. This study concludes that probiotic supplementation to re-nutrition diet could emerge as wonder therapeutics against PEM.

Keywords Probiotics · Protein energy malnutrition · *Lactobacillus reuteri* · Gut microbiota changes · Immunocytochemistry

Introduction

Protein energy malnutrition (PEM), one of the global devastating problems, contributes to nearly half of all deaths in children under 5 years of age [1]. The consequences of PEM primarily culminate into reduction or failure of body growth, dysbacteriosis in the gut micro-environment, leaky gut conditions [2–11], reduced secretory IgA secretion, and deformity in the intestinal architecture [12–16]. In addition, due to PEM immune machinery, viz. spleen functions, antigen presenting cells (APCs), viz. macrophages, dendritic cells become crippled and incapable to mount cascade of events implicated in immune response [17–23].

To overcome the above burden, administration of re-nutrition diet could be fruitful in reversing the condition of PEM but for the moderate and severe type of malnutrition accompanied by clinical syndromes, there is a need of an effective therapy in combating the catastrophe posed by PEM. In relation to this, probiotics alone or as an adjuvant to re-nutrition diet could serve as a magical bullet to mitigate the causal effects of PEM. Several studies have reported that administration of probiotics is natural as well as cost-effective therapy in the re-establishment of gut flora, repair of mucosal epithelium, and increased mucosal immunity [14, 24, 25]. Also, some scientific studies have evident that supplementation of probiotics has immunomodulatory effects, viz. increased phagocytosis by the APCs and their activation by the molecules present on them [26–34]. In spite of the available understanding of the probiotics health impacts, there is limited scientific insights related to the adjuvant effect of

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probiotics in the restoration of gut microbiota, rejuvenation of gut barrier functions, and enhanced immune competence in combating PEM.

Considering this paucity, the present study used an indigenous probiotic isolate *Lact. reuteri* LR6 that showed resistance to gastrointestinal conditions in vitro [35, 36], antagonistic activity against different targeted pathogens in vitro [37], and anti-hypercholesterolemic effect in vivo [38]. Previously, our study reported the development of PEM murine model and the synergistic effect of probiotic (*Lact. reuteri* LR6) fermented milk and/or cell suspension in combating PEM condition [39]. The present study demonstrates the impact of the probiotic *Lact. reuteri* LR6 supplementation as fermented milk and/or bacterial suspension in combination with re-nourished diet on the gut and systemic immunity in PEM murine model.

Material and Methods

Bacterial Strain

Based on previous studies, *Lact. reuteri* LR6, an indigenous probiotic strain of Indian infant fecal origin was selected for this study. This strain was maintained as glycerol stocks at − 20 °C. And, prior to use, the working culture was prepared from the glycerol stock and was propagated twice prior to use by subculture in MRS broth (deMan, Ragosa, and Sharp broth; Himedia, Mumbai, India) at 37 °C for 18–24 h.

Animals and Study Design

Thirty male Swiss albino mice (aged 8–9 weeks; 25 ± 4.5 g) were procured from small animal house of National Dairy Research Institute (NDRI), Karnal, Haryana, India. All mice were housed in plastic cages (6 mice per group) under a controlled temperature (24 ± 1 °C) and humidity ($56 \pm 5\%$), as well as maintained as per the institutional and national guidelines. All experimental procedures involving animals were approved by the Institute Animal Ethical Committee (IAEC), NDRI, Karnal (IAEC. No. 73/14; 4 January 2014). After the adaptation period of 1 week on normal semi-synthetic diet (16% casein, 437.2 cal), the mice were divided into five major groups: group C (well-nourished control) were fed with normal/conventional semi-synthetic diet throughout the study; group M (malnourished), PFM (probiotic fermented milk), SM (skim milk), and BS (bacterial suspension) were fed with protein and energy restricted diet (2% casein, 315.2 cal) until the development of malnutrition which was analyzed on the basis of body weight reduction. After the development of malnourished model, group M was continued with restricted diet while group PFM, SM, and BS were fed with probiotic (LR6) fermented milk (1.0×10^9 cfu/animal/

day), skim milk, and bacterial cell suspension (1.0×10^9 cfu/animal/day) for 1 week, respectively. Thereafter, mice were sacrificed, and different histological, microbiological, and immunological parameters were studied. Composition of semi-synthetic diets used is given in Table 1 [39].

Histological Changes

The large intestine and spleen were collected and washed with saline solution (0.15 M NaCl) and immediately sections of 2-cm length were placed in 10% buffered formalin overnight at temperature (27 ± 1 °C). Paraffin-embedded tissues were cut into 5-μm slices and stained with hematoxylin and eosin (H&E) using standard techniques. Structural changes were visualized under inverted microscope as described previously [41].

Direct Immunofluorescence Microscopy for sIgA⁺ Cells

The unstained histological slides prepared were used for direct immunofluorescence microscopy for determination of sIgA⁺ cells in mice small intestine. After deparaffinization using xylene and rehydration in a decreasing gradient of ethanol, sections were blocked in 2% BSA for 1 h. The slides were washed 2–3 times with PBS, sections were incubated with a 1:100 dilution of α-chain mono-specific antibody conjugated with FITC (Cayman Chemical, Michigan, USA) for 1 h and observed with a fluorescent light microscope (Olympus, CKX41, Japan). The number of fluorescent sIgA⁺ cells was counted in 30 fields at × 200 magnification. The results were expressed as the number of sIgA⁺ cells per five fields of vision.

Phagocytosis Assay of Macrophages

Macrophages were collected from the peritoneal cavities of mice with 6 mL of Dulbecco modified eagle medium (DMEM/ Hams F-12 medium (without phenol red) following

Table 1 Composition of semi-synthetic diets in development of malnourished mice model

S. no.	Ingredients	Well-nourished semi-synthetic diet	Semi-synthetic diet for development of PEM
1.	Starch	50.8%	34.3%
2.	Sucrose	10%	10%
3.	Soybean	10%	10%
4.	Cellulose	10%	10%
5.	Casein	16%	2%
6.	Mineral mixture[a]	2%	2%
7.	Vitamin mixture[a]	1.2%	1.2%
8.	Energy	437.2 Cal.	315.2 Cal.

[a] Mineral mixture and vitamin mixture were prepared according to AOAC [40]

gentle massage of the abdomen. The peritoneal exudates (1×10^6 cells/ml) was transferred into petri dish (35 mm) and incubated in a humidified atmosphere at 5% CO_2 in air at 37 °C for 2 h to allow attachment of adherent cells. Non-adherent cells were removed after washing and the adherent macrophages cultured in DMEM Ham's F-12 medium for 18 h. Phagocytic activity was performed using *Saccharomyces cerevisiae* suspension at a concentration of 10^8 cells/ml. Equal volumes of yeast and phagocytic cells were incubated for 30 min at 37 °C and staining procedure was followed as previously described by Jain and coworkers [30]. The percentage phagocytosis calculated according to the following formula:

Percentage phagocytosis = number of macrophages with yeast cells internalized per 100 macrophages.

Phagocytosis Assay for Dendritic Cells

i) Collection of Bone Marrow cell After sacrificing mice, hind leg located just above pelvic/hip joint was cut using sharp and sterile dissecting scissors, ensuring that the epiphysis remains intact without exposing off its contents to outside. Dissected the femur from surrounding muscles and removed excess tissue using sterile forceps and scissors, keeping the ends of the bone intact. Thereafter, the bones were transferred in Roswell Park Memorial Institute (RPMI) -1640 media containing fetal bovine serum (FBS) and antibiotic antimycotic solution (Sigma-Aldrich/Merck). Then, both the ends of femurs were trimmed carefully using sterile, sharp scissors to expose the interior marrow shaft. The contents of marrow were flushed with 1-ml insulin syringe with a 29G × ½ needle. The contents were collected into a sterile 15-ml centrifuge tube and centrifuged at $1000g$. The pellet obtained was washed and resuspended in 1 ml of RPMI-1640 medium.

ii) Differentiation of bone marrow stromal cells to dendritic cells (DCs) The bone marrow exudate (1×10^6 cells/ml) was transferred into petri dish (35 mm) and incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 24 h to allow attachment of adherent cells. Thereafter, the media was replaced with culture media containing granulocyte macrophage colony stimulating factor (GM-CSF-20 ng/ml) as a growth factor. The plates were then again incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 48 h to allow differentiation of bone marrow stromal cells into dendritic cells [42]. The step was repeated twice for full maturation of dendritic cells.

iii) Phagocytosis Assay of DCs After the maturation of dendritic cells, the plates were incubated with 100 µl of yeast cell suspension (10^8 cells/ml) for 1 h in a humidified atmosphere (5% CO_2) at 37 °C. The medium was removed and the cells were washed twice gently with the RPMI-1640 medium to remove non-adherent yeast cells. Thereafter, the cells were stained as previously described by Jain and coworkers [30] and the percentage phagocytosis was calculated according to the following formula:

Percentage phagocytosis = Number of dendritic cells with yeast cells internalized per 100 dendritic cells.

Immunocytochemistry for Detection of Macrophages and DCs

The protocol for immunocytochemistry in staining section was followed as given on link: https://health.uconn.edu/li-lab/protocols/ with slight modifications which were standardized in our laboratory. Cells to be stained were transferred to culture plates (35 mm) which provide a solid support for adherence and allow easy handling in subsequent procedures. Adherent cells of macrophages and DCs derived from the peritoneal fluid and bone marrow exudates respectively were fixed with ice-cold methanol for 15–20 min with gentle shaking followed by 3–4 times washing with PBS. Thereafter, the plates were incubated with PBST (0.25% *v/v* triton 100 in PBS) for 10 min to permeabilize the membranes followed by washing with PBS (3 times). Further, blocking was done for 30 min followed by washing. After that, incubation with primary antibodies (B7-2 CD86; MHCII (3D6), procured from Novus biologicals-a biotechne brand) was done for 3–4 h at 4 °C followed by washing. Finally, the plates were treated with secondary antibodies (goat anti-mouse IgG1 Antibody Affinity Purified, used against primary antibody MHCII (3D6); goat anti-rabbit IgG-heavy and light chain antibody affinity used against primary antibody B7-2 CD86) for 1 h in dark followed by washing and further treatment with DAPI (Sigma-Aldrich) was performed to stain the nucleus. The cells were observed with a fluorescent light microscope (Olympus, CKX41, Japan).

Scanning Electron Microscopy of DCs

Scanning electron microscopy (SEM) was performed as described by Hashizume and coworkers with slight modifications [43]. Cells were harvested periodically from the cultured medium containing differentiated bone marrow-derived DCs in different stages on glass cover slips and were resuspended in 1 volume of 0.1 ml/L phosphate buffer (pH 7.4). Further, the cover slips were air dried and fixed in a solution of 2.5% glutaraldehyde in 75 mM phosphate buffer, (pH 7.4) for 1–2 h (primary treatment). The fixed specimen was rinsed three times each for 15 min, in 50% 75 mM phosphate buffer. Thereafter, the cover slips were fixed with 1% osmium tetraoxide. After rinsing, samples were serially dehydrated in ethanol concentrations of 30, 50, 70, 80, 90, and 100% each for 15 min respectively. After this step, samples were air dried and mounted on stub followed by gold coating. In gold

coating, samples were encrusted with gold at approximately 100–200 Å thickness on-ion coater (Hitachi IB-3, Japan). The ion current was kept at 6–8 mA at fine vacuum of 0.05–0.07 Torr for 2–4 min. The coated samples were then visualized using a SEM at different magnifications (EVO® 18, Carl ZEISS Special Edition-UK).

Microbiota Changes

The luminal content from small and large intestine were flushed out using phosphate-buffered saline (PBS). After harvesting the flushed material by centrifugation ($9000g$/10 min), the pellet was washed and the total DNA was extracted using QIA-amp DNA stool mini kit procured from Qiagen. qPCR assay was performed as previously described by Rinttilä and coworkers [44]. In this study, four different groups of bacteria, namely bifidobacteria, lactobacilli, Firmicutes, and *Bacteroides*, were targeted. All polymerase chain reaction (PCR) experiments were carried out in triplicate with a reaction volume of 20 μl. The 20 μl reaction mixture contained 10 μl Maxima SYBR Green I qPCR Master Mix (2X), 0.5 μl of each primer (forward and reverse), and 2 μl of DNA and 7 μl of nuclease free water. The amplification was made in a LightCycler 480 Real-Time PCR (Roche Molecular Biochemicals, Germany). The amplification program consisted of (i) initial denaturation at 95 °C for 5 min, (ii) 35 cycles of denaturation at 95 °C for 30 s, (iii) annealing at 61 °C/30 s for lactobacilli, 57 °C/30 s for bifidobacteria, Firmicutes, and *Bacteroides*, (iv) extension at 72 °C for 60 s followed by a final extension at 72 °C for 5 min. The primers sequences used are given in Table 2 [45, 46]. The abundance of targeted bacterial groups in the sample was determined by comparing the Ct values to the values generated by standard curves as previously described [46]. *Lact. reuteri* ATCC 55730, *Escherichia coli* ATCC 25922, and *Bifidobacterium breve* ATCC 15700 were used for obtaining the standard curves.

Statistical Analysis

Results are expressed as the mean ± SD of three independent experiments. Statistical analysis was done by GraphPad Prism (Version 5.01) software. Data were subjected to a one-way analysis of variance (ANOVA) followed by a Turkey's post hoc test. Differences were considered statistically significant when $P < 0.05$.

Results

Histology of Large Intestine

In the present study, normal morphology of mucosal epithelium, lamina propria, colonic crypts, and goblet cells was observed in group C. In lieu, thin mucosal epithelium and inflamed lamina propria, distorted intestinal glands and colonic crypts, inflammation with less number of goblet cells was observed in group M. However, supplementation of PFM and/or BS resulted in rejuvenation in mucosal epithelium, lamina propria, and repaired colonic crypts followed by the increased goblet cell number with no inflammation. Furthermore, restoration of intestinal glands and colonic crypts, less goblet cell number, little inflammation and rejuvenation in mucosal epithelium and lamina propria was also observed in group SM (Fig. 1).

Histology of Spleen

Spleen sections showed disturbed white pulp to red pulp ratio, fibrosis (thickening and scarring of connective tissue), and inflammation in the group M and SM while no such effect was observed in the group PFM in contrast to less fibrosis in the BS group. However, equal white pulp to red pulp ratio with no fibrosis was observed in the C group as shown in Fig. 2.

Effect on the Number of sIgA⁺ Cells in the Intestinal Tissue

Significant ($p < 0.05$) increase in the number of sIgA⁺ cells was seen in the group PFM and BS. The PFM and BS group showed approximately 291 and 230 sIgA⁺ cells per five intestinal villi, respectively, while the M group showed highly

Table 2 Primers for different group of bacteria

Primers pairs	Sequence (5′-3′ direction)	Expected size (bp)	References
Firmicutes F	GGAGYATGTGGTTTAATTCGAAGCA	127	[45]
Firmicutes R	AGCTGACGACAACCATGCAC		
Bacteroides F	GAGAGGAAGGTCCCCCAC	108	[45]
Bacteroides R	CGCTACTTGGCTGGTTCAG		
Lactobacilli F	GGA ATC TTC CAC AATGGA CG	216	[46]
Lactobacilli R	CGC TTT ACG CCC AAT AAA TCCGG		
Bifidobacterium F	CTC CTG GAA ACG GGT GG	548	[46]
Bifidobacterium R	GGT GTT CTT CCC GAT ATC TAC A		
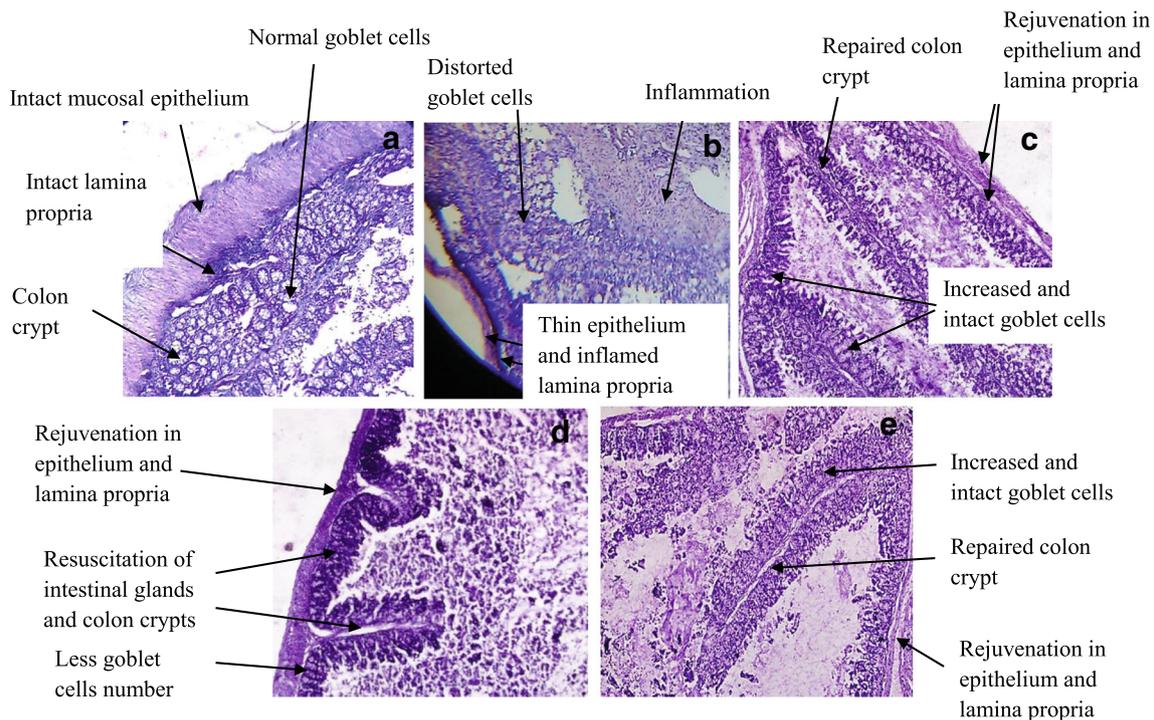
Fig. 1 Histology of large intestine (20×) showing (A) control group—intact morphology of mucosal epithelium and colonic crypts, normal goblet cells, no inflammation and intact lamina propria. (B) Malnourished group—distorted morphology of colonic crypts, deformed goblet cells, inflammation and thin mucosal epithelium and inflamed lamina propria. (C) PFM group—Repaired colonic crypts, intact and increased number of goblet cells, no inflammation, rejuvenation in mucosal epithelium and lamina propria. (D) SM group—restoration of colonic crypts, less goblet cell number, inflammation and rejuvenating lamina propria. (E) BS group—Repaired colonic crypts, increased number of goblet cells but less than PFM, slight inflammation and rejuvenating lamina propria

diminished number of sIgA$^+$ cells, viz. 22 cells per five intestinal villi. Furthermore, 100 and 58 sIgA$^+$ cells per five intestinal villi were observed in group C and SM manifesting reduced recovery of sIgA$^+$ cells in SM supplemented group after induction of malnutrition as shown in Fig. 3.

Effect on the Phagocytic Activity of Macrophages Derived from Peritoneal Fluid

Significant ($p < 0.05$) effect of PFM and BS administration was observed on phagocytic activity of macrophages. The phagocytic activity of macrophages in group PFM (75.60%) and group BS (61.76%) was relatively much higher in comparison to the group M (23.3%). Also, significant effect was observed in group SM (41.62%) in comparison to group C (49.15%) as shown in Fig. 4.

Effect on the Phagocytic Activity of DCs Derived from Bone Marrow

Enhanced phagocytic activity was also observed in the DCs from group PFM (75.60%) and group BS (61.76%) in comparison to group M (23.30%) whereas it was 41.66% in group C and 28.00% in group SM as shown in Fig. 5.

Effect of Probiotics on the Co-stimulatory Molecules of the APCs (i.e., Macrophages and DCs)

The results of this immunocytochemistry-based experiment showed that reduced levels of co-stimulatory molecules/low signals were responsible for reduced and/or no phagocytic activity of macrophages and also, for the inability of bone marrow-derived progenitor stem cells to differentiate into dendritic cells (in the presence of GM-CSF) in malnourished mice, whereas probiotic administration as fermented milk and/or as bacterial suspension significantly increased the levels of co-stimulatory molecules/signals which in turn is responsible for enhanced macrophage phagocytic activity and effective differentiation of bone marrow stem cells into DC's in group PFM and BS as shown in Figs. 6a, b and 7a, b.

Differentiation of Bone Marrow-Derived Progenitor Stem Cells into DCs by SEM

Figure 8 clearly shows the differentiation of bone marrow-derived progenitor stem cells into dendritic cells in the presence of granulocyte macrophage colony stimulating factor (GM-CSF).
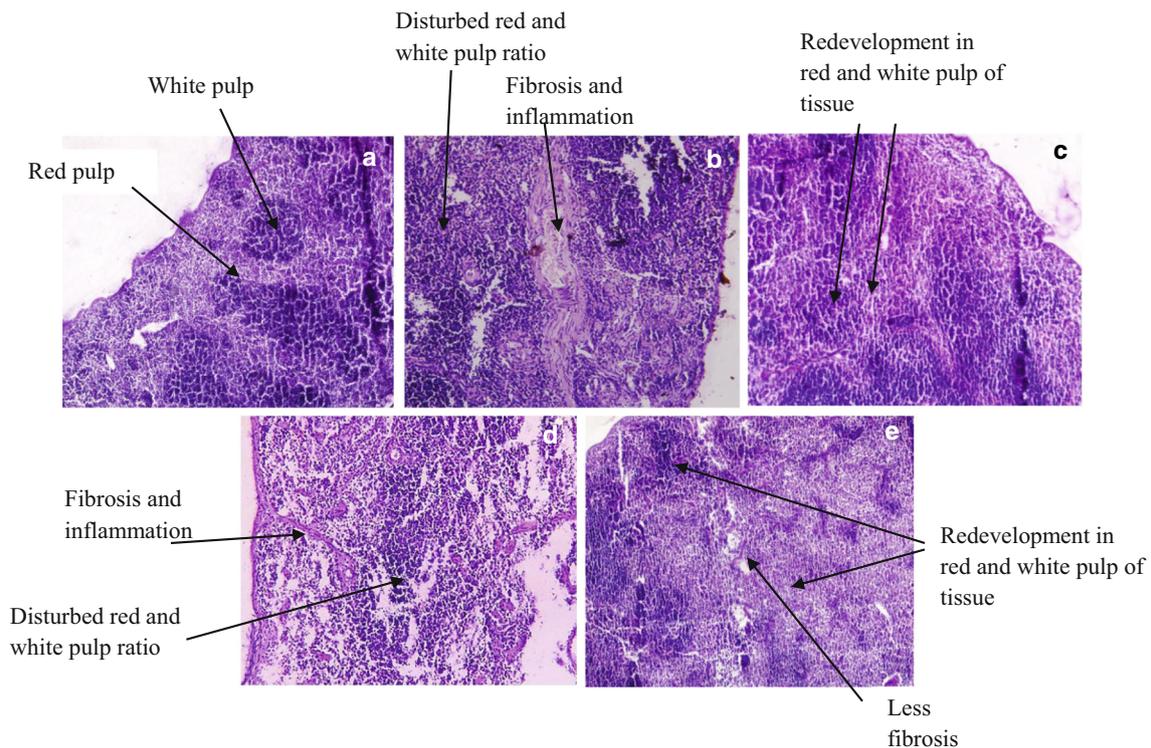
Fig. 2 Histology of spleen (20×) showing (A) control group—equal white and red pulp ratio, no fibrosis, and inflammation. (B) Malnourished group—disturbed white pulp and red pulp ratio, fibrosis, and inflammation. (C) PFM group—redevelopment of white pulp and red pulp, no fibrosis, and inflammation. (D) SM group—disturbed white pulp and red pulp ratio, fibrosis, and inflammation. (E) BS group—redevelopment of white pulp and red pulp, less fibrosis, and no inflammation

Quantitative Analysis of Microbiota Changes in Intestinal Samples (S.I. and L.I)

Figure 9a and b show significant increase in the Firmicutes, lactobacilli, and *Bifidobacterium* counts and decrease in the *Bacteroides* count in the groups supplemented with PFM and/ or BS in comparison to the M group.

Discussion

In our previous study, PEM model was developed in male Swiss albino mice and various parameters, viz. morphological parameters (body weight and organ index), protein biomarkers (total protein, albumin, immunoglobulin G (IgG)), inflammatory biomarkers (interleukin-1 beta (IL-1b), C-reactive protein (CRP)), hunger biomarkers (leptin, ghrelin), histology of small intestine, and feacal microbiota composition, were compared between the well-nourished and malnourished group. In addition, the synergistic effect of PFM and/ or BS (*Lact. reuteri* LR6- 1×10^9 cfu/ml) in combating PEM condition was evaluated. The present research focuses on the adjuvant effect of probiotic *Lact. reuteri* LR6 in boosting systemic

immunity and gut barrier functions which are initially targeted by protein calorie malnutrition.

Gastrointestinal epithelium acts as a primary physical barrier and consists of physical (mucus), molecular (antimicrobial proteins), and cellular components that act synergistically to prevent the attack by invading pathogens. In terms of the histological changes of large intestine, thin mucosal epithelium, distorted colonic crypts, inflamed lamina propria, and less number of goblet cells were observed in group M. The findings of the present study were consistent with the literature available that used protein malnutrition animal models and showed decreased thickness of bowel wall layers, such as reductions of the mucosa, villi height, and crypt depth [12, 14, 16]. Therefore, strategy to revert or reduce the damage caused by malnutrition was the basic purpose of the present study where the adjuvant effect of probiotics by supplementing either PFM and/or BS along with re-nutrition diet was studied. And, the results showed the positive effects of probiotic *Lact. reuteri* LR6 administration as restoration of mucosal epithelium and colonic crypts followed by the increased goblet cell number with no inflammation was observed in group PFM and BS. Other researchers have also reported similar effect of probiotic feeding in addition to re-
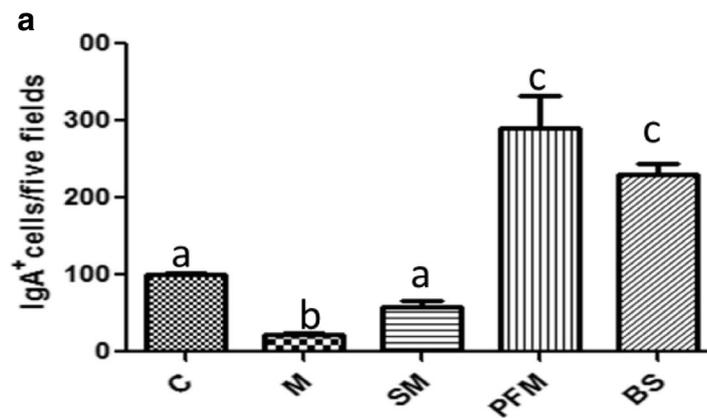
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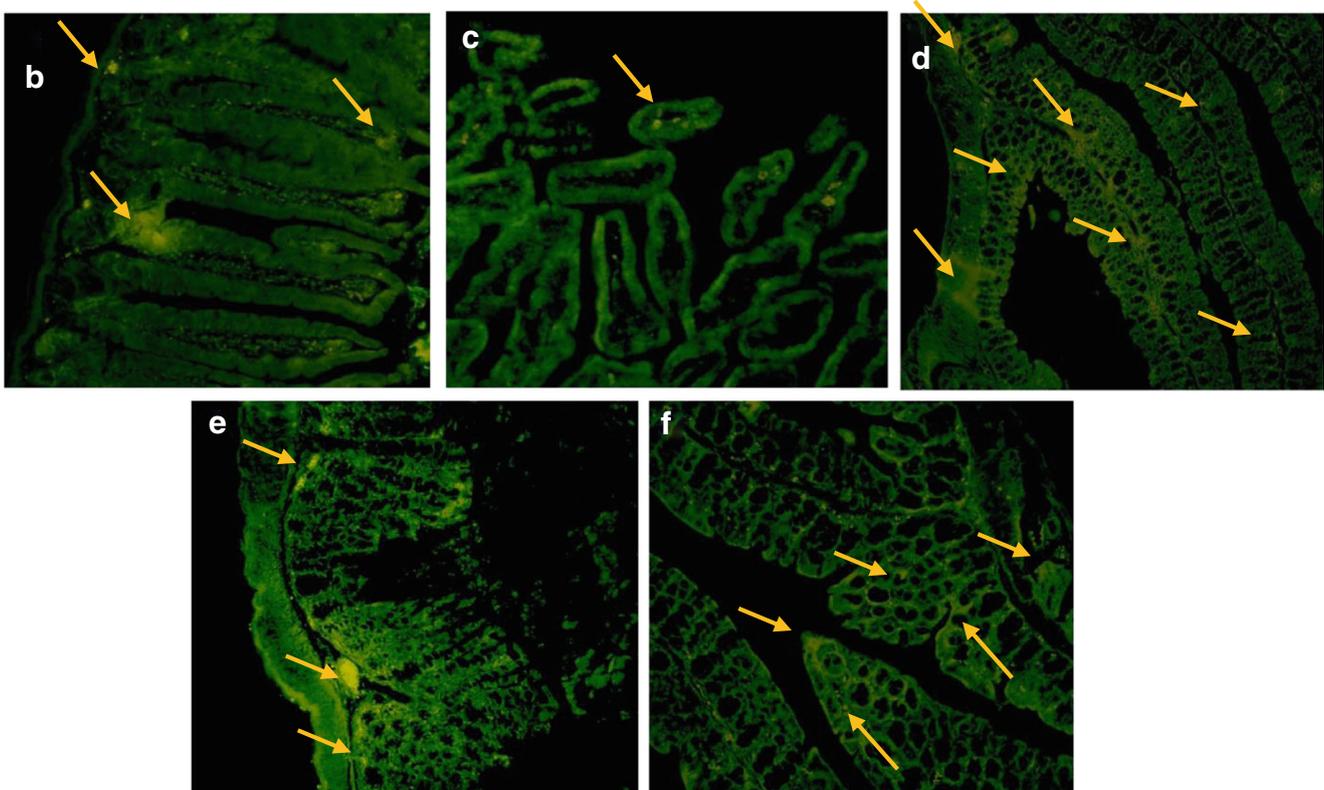
Fig. 3 Representation of secretory IgA$^+$ cells showing (A) estimation of sIgA$^+$ cells in the intestinal tissue of control (C); malnourished (M); probiotic fermented (PFM); skim milk (SM); bacterial suspension (BS) groups (B) direct immunofluorescence microscopy of sIgA$^+$ cells (yellow spots marked by an arrow) in the C group, (C) M group, (D) PFM group, (E) SM group, (F) BS group. *Bars having different alphabets are significantly different at $p < 0.05$

nourished diet in animal models [47–50]. This reduction or reversion of damage in the large intestine morphology might be due to the re-nutrition diet containing more content of protein and calories along with the ability of probiotics to maintain gut homeostasis.

Generally spleen acts as a graveyard for red blood corpuscles as well as synthesize antibodies in the white pulp and monocytes in the red pulp that confers immune response against infections. As soon as any inflammation commences, the monocytes are directed towards the inflamed site where they get differentiated to the APCs and helps in promoting healing process. In the present study, dysbalance of the white pulp to red pulp ratio was observed in group M indicating inflammation in the splenic tissue under malnourished condition. This dysbalance in spleen tissues of malnourished group could
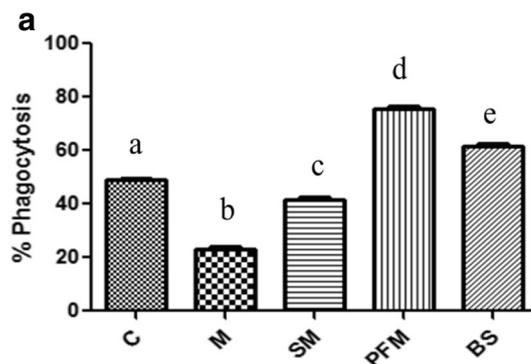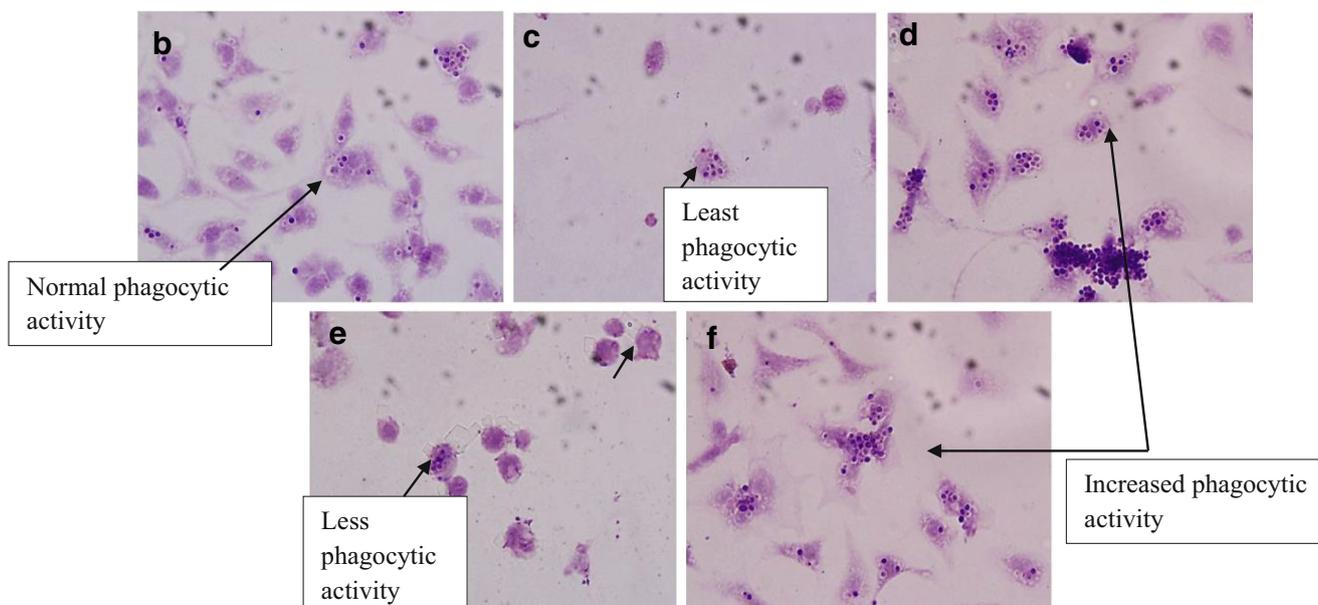
Fig. 4 (A) Estimation of percentage of phagocytosis by macrophages in the peritoneal fluid of different groups, viz. control (C); malnourished (M); probiotic fermented (PFM); skim milk (SM); bacterial suspension (BS) groups. (B) Representation of phagocytosis by macrophages in the peritoneal fluid in the C group, (C) M group, (D) PFM group, (E) SM group, (F) BS group. *Bars having different alphabets are significantly different at $p < 0.05$

be due to improper development of splenic cells attributed to inflammation and lower immune response as these are the sites where mature B lymphocytes proliferate to mount an immune reactivity against infection. Therefore, it gave rise to scarring and thickening of the connective tissue called fibrosis. Other researchers have also reported marked reduction in the number of spleen lymphocytes, altered capsule thickness, and increased amount of connective fibrous tissue in malnourished animals [21, 51, 52]. But, the treatment of the malnourished groups with PFM or BS as an adjuvant regained the normal functioning of the splenic tissue, as demonstrated in the present study. This study showed a remarkable improvement in the white pulp to red pulp ratio, no and/or little fibrosis, no and/or little inflammation in the PFM and BS groups.

This could be due to synergistic effect of probiotic and renourished diet as the diet too plays an important role in shaping the immune response of the body [5, 53]. However, due to the lack of supporting literature regarding the histological changes in spleen, more histological studies or electron microscopy images are required which could clear the efficacy of probiotic treatment in combating PEM.

sIgA$^+$ is multifunctional in mucosal-associated immunity [54]. In the present study, highly diminished number of sIgA$^+$ cells was observed in intestinal villi of malnourished group in comparison to well-nourished group. Therefore, it could be deduced from this study that during malnutrition, leaky gut conditions drives way towards lowered sIgA$^+$ secretion. However, probiotics may
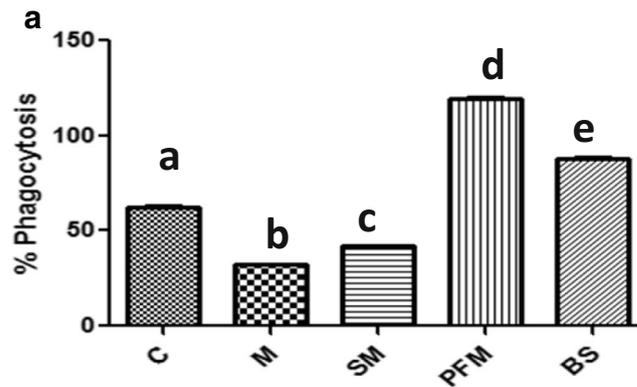
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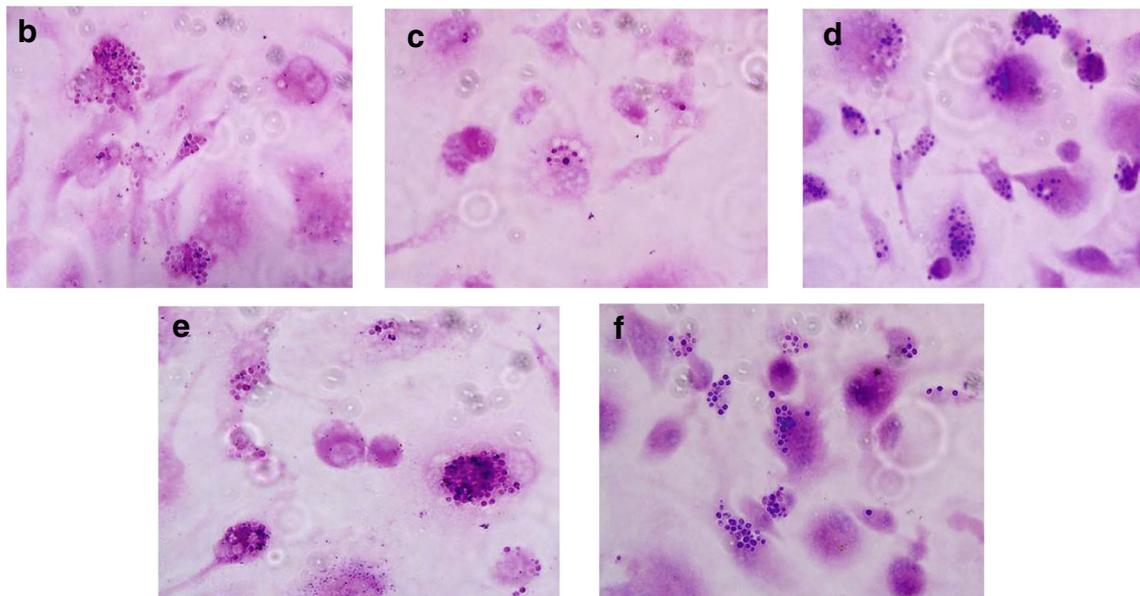
Fig. 5 (A) Estimation of percentage of phagocytosis by dendritic cells derived from bone marrow of different groups, viz. control (C); malnourished (M); probiotic fermented (PFM); skim milk (SM); bacterial suspension (BS) groups. (B) Representation of phagocytosis by dendritic cells derived from bone marrow in the C group, (C) M group, (D) PFM group, (E) SM group, (F) BS group. *Bars having different alphabets are significantly different at $p < 0.05$

enhance mucosal trophism and especially increase the number of goblet cells at the crypt site. As goblet cells are the main mucus producing cells, and mucus is rich in IgA, it can be speculated that probiotics may enhance the local immune system response. In the present study, administration of PFM and/or BS in addition to re-nutrition diet in PEM murine model showed enhanced number of sIgA⁺ cells in comparison to the malnourished group. From the results of this study, the increase in the number of IgA⁺ cells in group SM could be attributed to the peptides released from the milk proteins. Based on the results of this study and available supporting literature [55–63], it could be concluded that the administration of re-nutrition diet along with PFM and BS could prevent the invasion

and colonization of pathogenic microorganisms through the enhanced production of IgA antibodies.

Macrophages represent one of the major APCs to mount an immune response against pathogens and foreign substances. In the present study, reduced percentage of phagocytic activity of macrophages was observed in malnourished group as previously reported by other researchers [17, 64]. However, several studies have been carried out on the potential impact of probiotics on the elevated phagocytic activity of macrophages [27, 30–32, 65]. But the present study is the first report claiming the enhanced activity of macrophages on the administration of PFM and/or BS in supplementation with re-nutrition diet in malnourished mice model. And, it could be
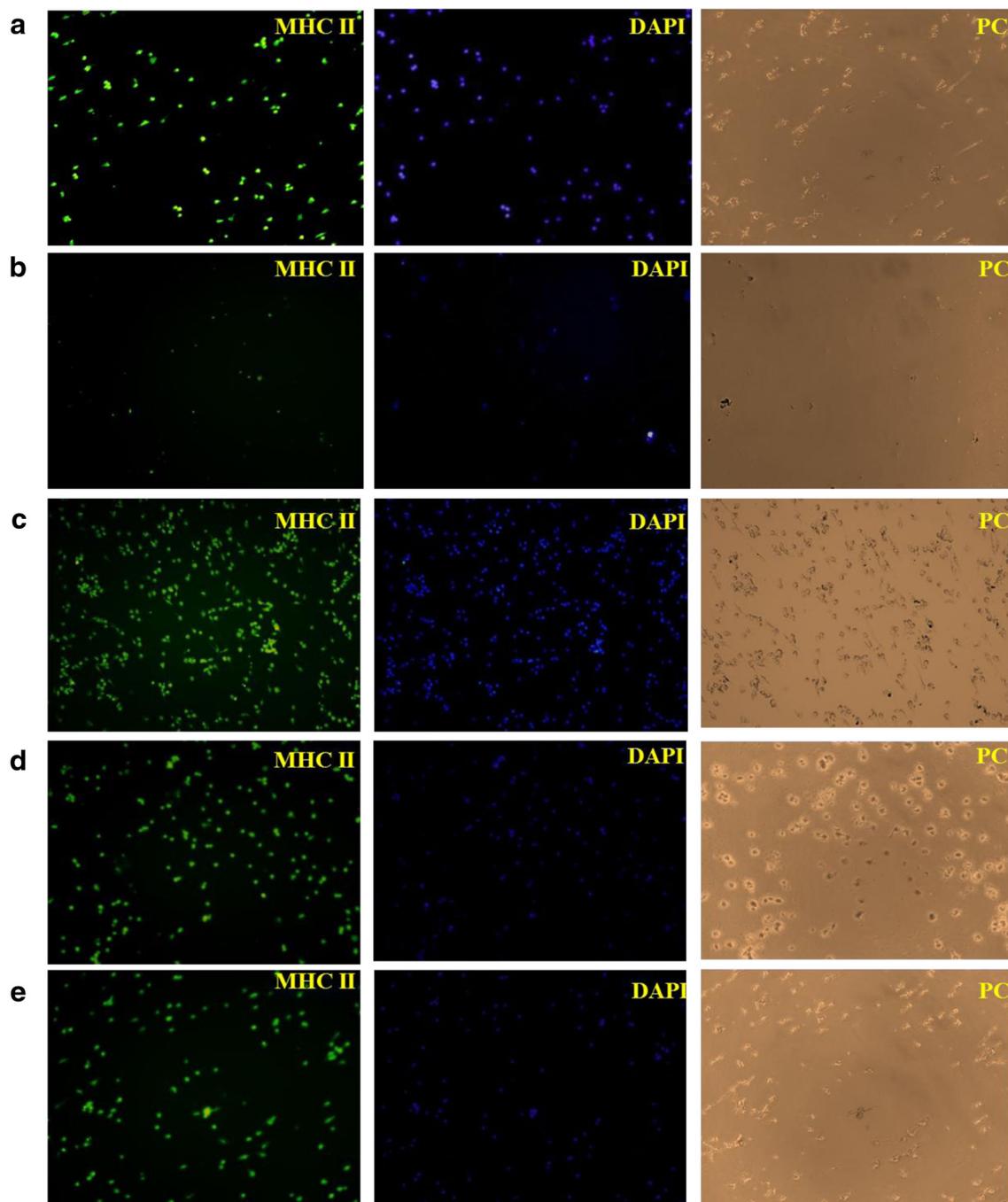
Fig. 6 a Pictorial presentation of immunocytochemistry showing fluorescein isothiocyanate (FITC); 4′,6-diamidino-2-phenylindole (DAPI) and phase contrast (PC) images of macrophages using MHC II; (A) control, (B) malnourished, (C) probiotic fermented milk, (D) bacterial suspension, (E) skim milk as a marker of macrophages. **b** Pictorial presentation of immunocytochemistry showing fluorescein isothiocyanate (FITC); 4′,6-diamidino-2-phenylindole (DAPI) and phase contrast (PC) images of macrophages using CD86; (A) control (B) malnourished, (C) probiotic fermented milk, (D) bacterial suspension, (E) skim milk as a marker of macrophages

concluded that both nutrition and probiotics when supplemented together in the diet might result in the better activity of macrophages to raise the immune response against inflammatory conditions and process the antigens for clearance by phagocytosis. But again, no studies have been conducted hitherto revealing the mechanism of enhanced proliferation and maturation of macrophages by probiotics in case of malnutrition.

Dendritic cells are the APCs that play a central role in orchestrating immune responses to self and foreign antigens.
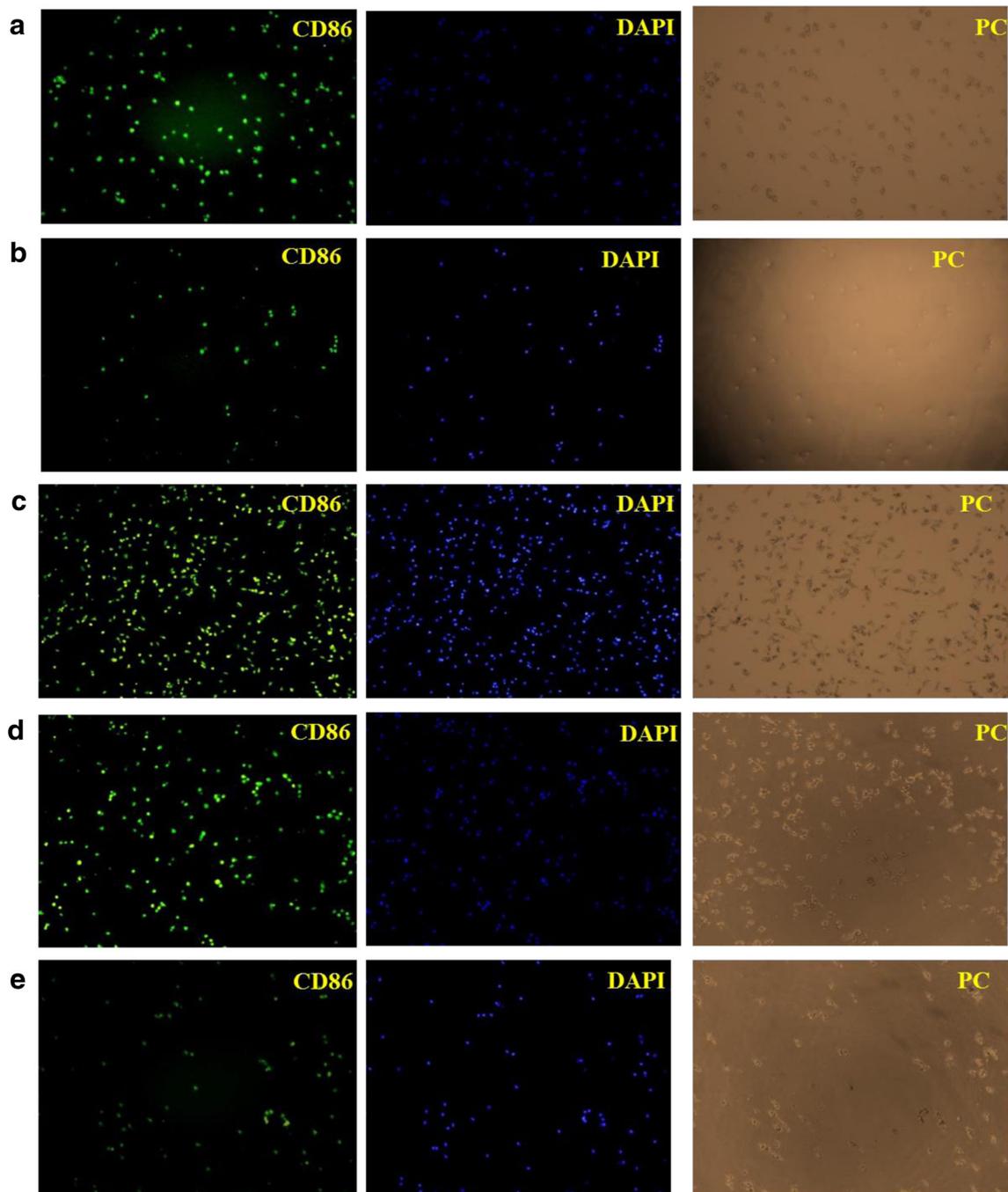
Fig. 6 continued.

In their mature state, the DCs are primed to activate T cells towards a Th1/Th2 polarization [66]. In the present study, the proliferative and maturation capacity of DCs gets hindered during malnutrition, as supported by other reports [19, 67]. However, some reports have explained the potential role of probiotics in mounting immune response by DCs to present the antigens for phagocytosis [26, 28, 29, 33, 68]. In the present study, supplementation of probiotics as an adjuvant to re-nutrition diet was able to restore the functionality of DCs by upregulating the co-stimulatory signals which further resulted in the proliferation and maturation of DCs to act against infectious conditions and present antigens for their elimination by phagocytosis. But again, there is hardly any such report that reveals the mechanism of enhanced proliferation and maturation of DCs by probiotics in case of malnutrition.

Most immune responses are being regulated by cell mediated immunity involving the activity of T cells which further requires a secondary signal called co-stimulatory
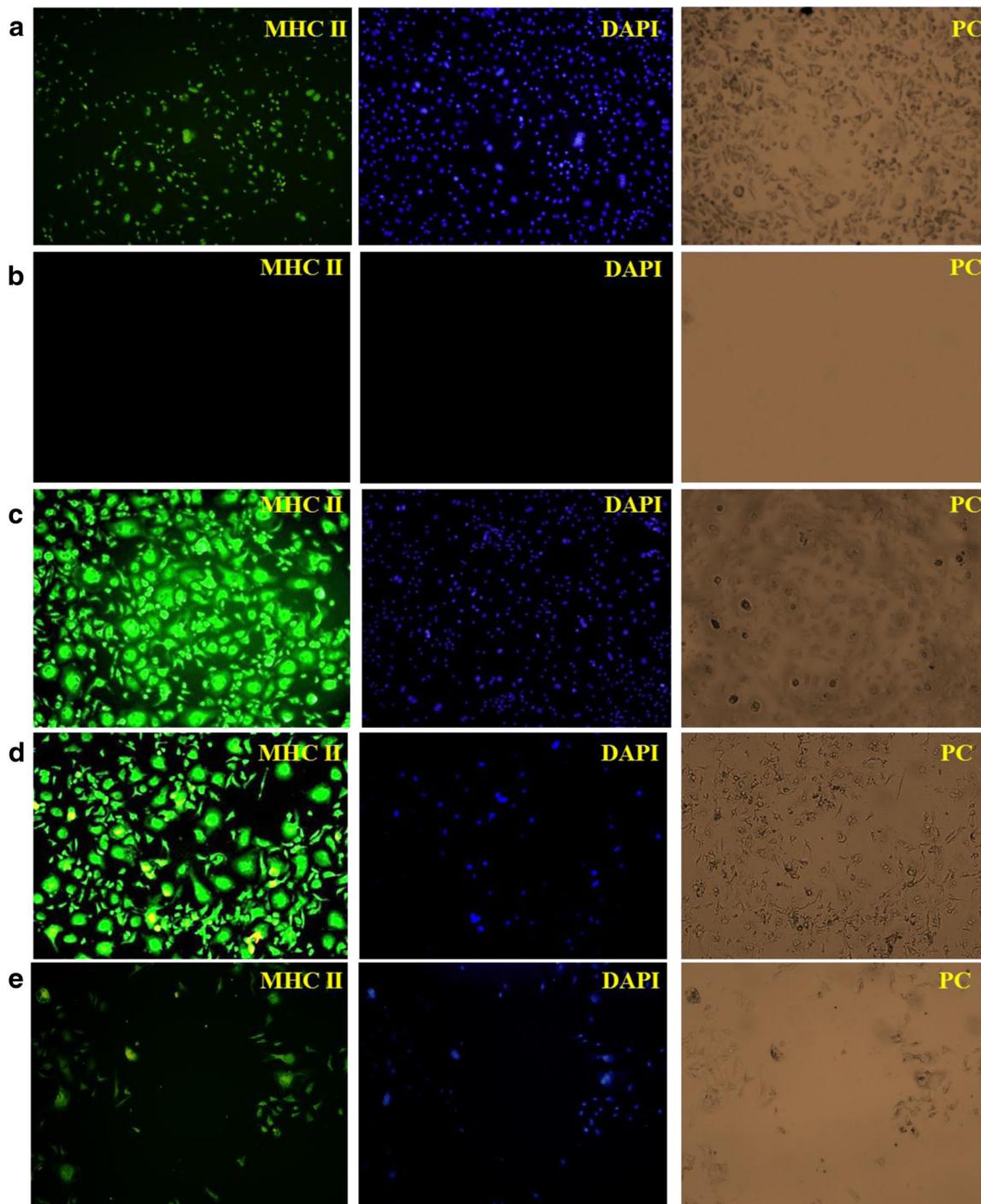
Fig. 7 a Pictorial presentation of immunocytochemistry showing fluorescein isothiocyanate (FITC); 4′,6-diamidino-2-phenylindole (DAPI) and phase contrast (PC) images of bone marrow-derived dendritic cells using MHCII; (A) control, (B) malnourished, (c) probiotic fermented milk, (D) bacterial suspension, (E) skim milk as a marker of DCs. (b) Pictorial presentation of immunocytochemistry showing fluorescein isothiocyanate (FITC); 4′,6-diamidino-2-phenylindole (DAPI) and phase contrast (PC) images of bone marrow-derived dendritic cells using CD86; (A) control, (B) malnourished, (C) probiotic fermented milk, (D) bacterial suspension, (E) skim milk as a marker of dendritic cells

signal for their activation [69]. These co-stimulatory molecules are present on the APCs and get activated as soon as they get signal from APCs, thus further activating the T-cells to raise an immune response against inflammation [70]. In the present study, lowered and/or no signaling of co-stimulatory molecules (CD86 and MHCII) present on
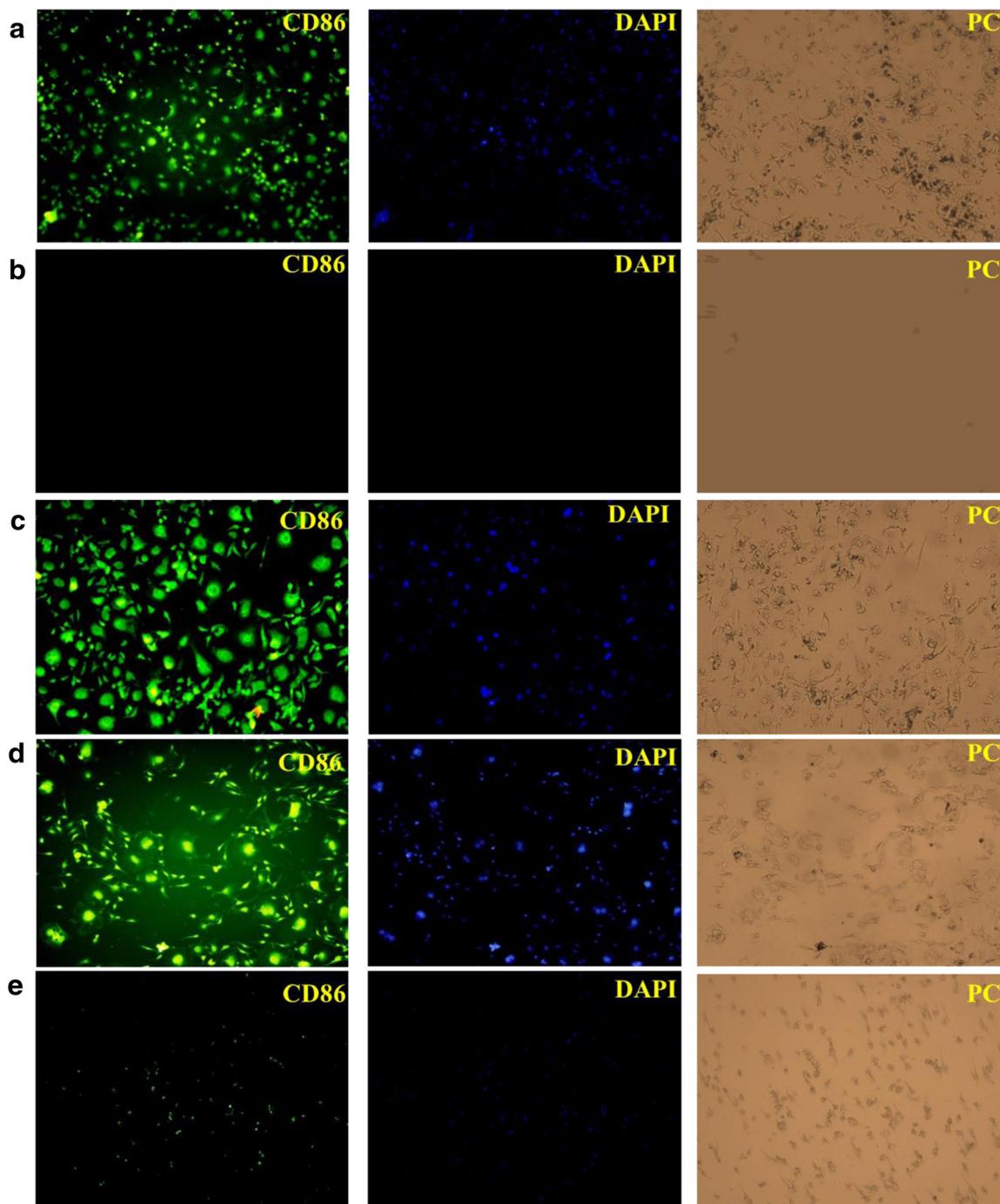
Fig. 7 continued.

the macrophages and DCs has been observed in group M. Few studies, in consistent to the present study, state that malnutrition hinders the cascade of immune reactivity to activate cell-mediated immune response via co-stimulatory molecules resulting in a weakened resistance against inflammation and other pathological conditions [17, 19, 67]. However, enhanced signaling of APCs, viz. macrophages and DCs to direct the cell mediated immune response via signaling through co-stimulatory molecules (B7-2 CD86 and MHCII (3D6)), was observed in groups supplemented with PFM and BS along with the conventional diet. This enhanced activity could be either due to the supplementation of conventional diet along with probiotics or due to the immunomodulatory activity of probiotics or combination thereof. There are some studies which support that the activity of APCs gets affected by
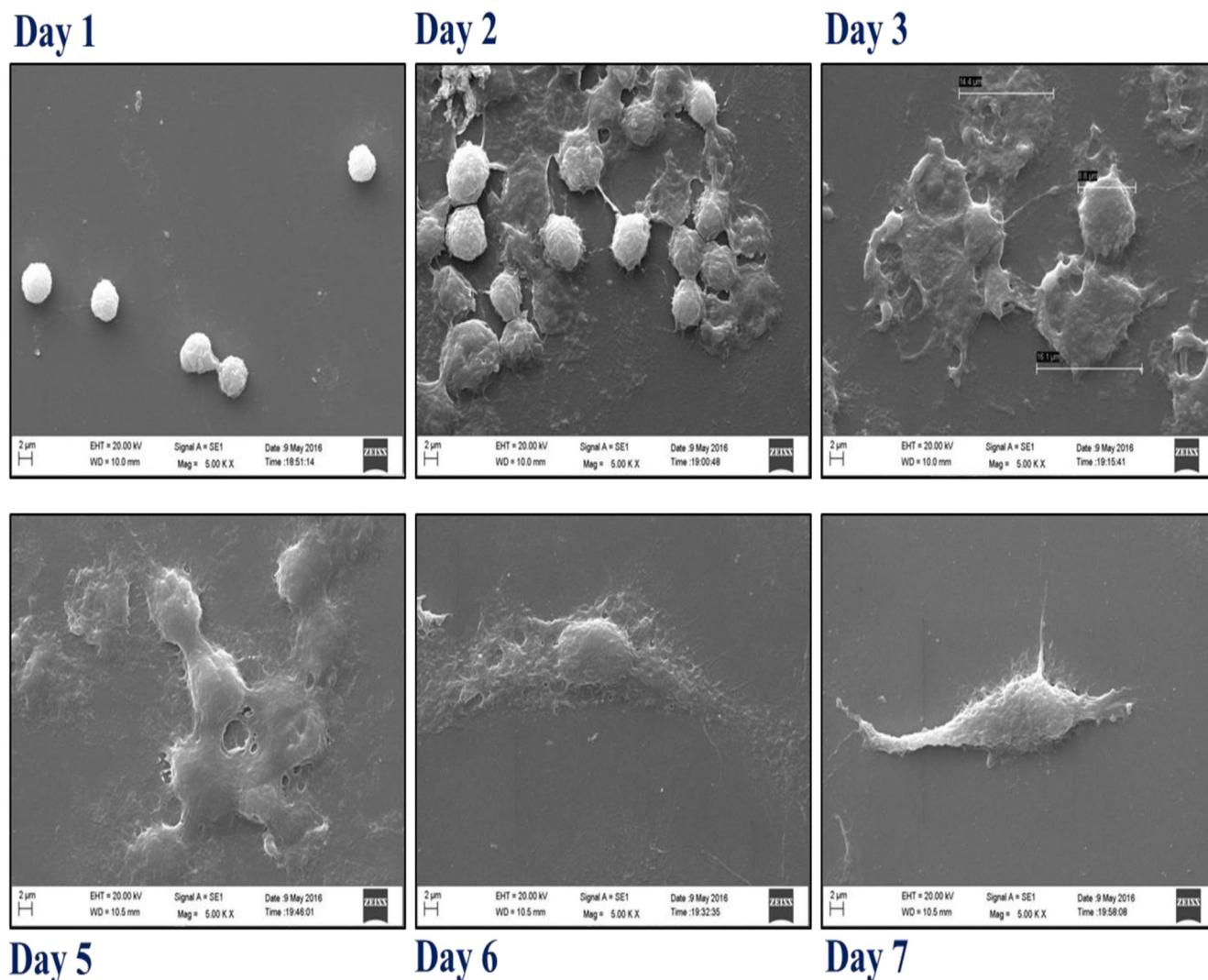
Fig. 8 Scanning electron microscopy (SEM) showing differentiation of progenitor stem cells derived from bone marrow into dendritic cells in the presence of GM-CSF

the nutritional modulation [64] as well as by the administration of probiotics in the diet [30, 31, 33]. Recently, a study justified that the low dose as well as high dose of *Lact. rhamnosus* GG for a specific period, upregulated the activity of DCs which further increased the expression of marker (CD86 and MHCII) on the naive DCs thus elevating the immune reactivity which was in accordance with the results of the present study [71].

In context to the differentiation of progenitor stem cells into the dendritic cells derived from the bone marrow in the presence of GM-CSF, similar photomicrography of differentiation of DCs has been shown in the bone marrow cells derived from the femurs of Swiss mice in the presence of Canova, an immune modulator [69]. Also, SEM images showing maturation of bone marrow-derived dendritic cells using GM-CSF plus IL-4 was revealed by previous studies which are in full support to the present study [72].

PEM primarily targets gut barrier functions and resident gut microbiota which results in an overall lowered immune reactivity [5, 7, 11]. Similarly in this study, significantly low counts of lactobacilli, bifidobacteria, and Firmicutes and high count of *Bacteroides* was observed in M group in comparison to well-nourished control group. Further, the groups fed with PFM and/or BS showed significant increase in lactobacilli, Firmicutes, bifidobacteria counts and reduced number of *Bacteroides* in intestinal exudates. This could attribute to the improved gut barrier and immune functions by restoration of beneficial gut flora which was unbalanced due to PEM [25, 73]. However, the mechanisms by which PEM leads to these alterations are difficult to untangle as these systems closely interact, often in a bidirectional way. Although several reports claim that the daily intake of probiotics could aid in resisting various pathological conditions by restoring the gut microbiome [74–82], however, very limited information is
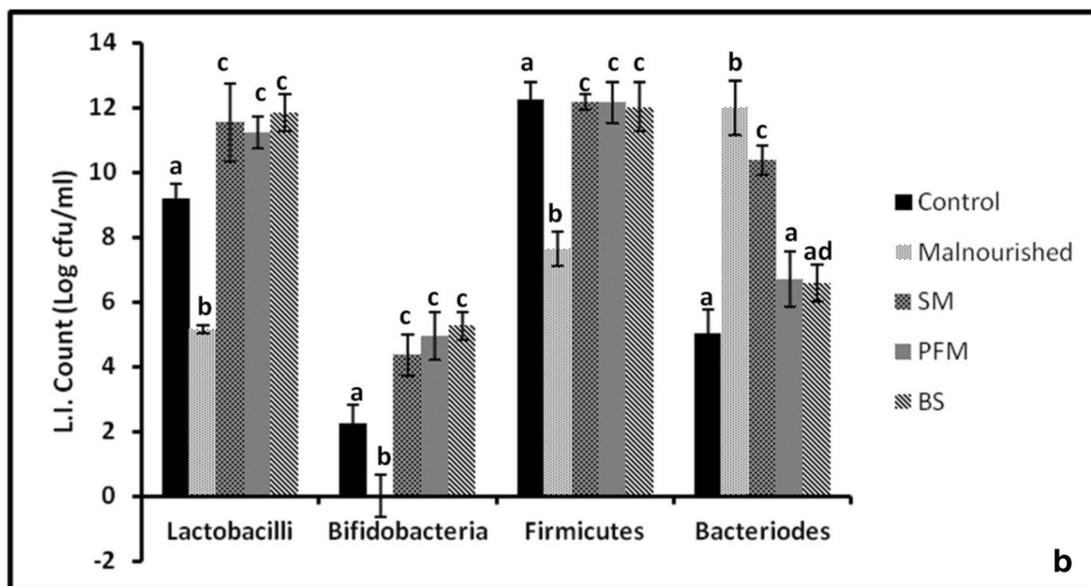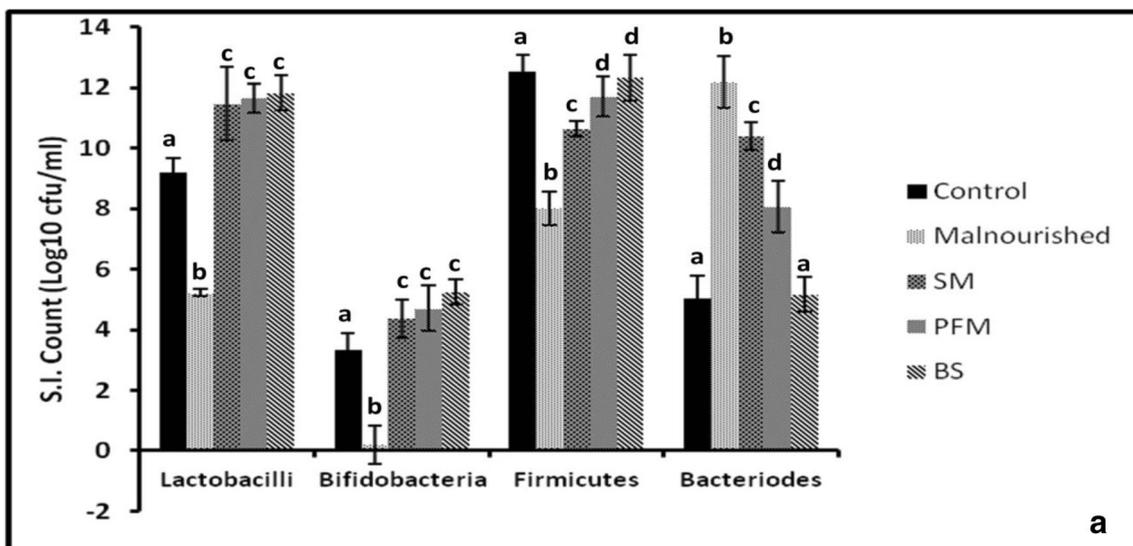
Fig. 9 a Small intestine (S.I.) microbiota changes observed after feeding re-nourished diet with different supplements (SM, PFM, and BS) in mice model. C, control; M, malnourished; SM, skim milk; PFM, probiotic fermented milk; BS, bacterial suspension ($n = 6$ samples per group). *Bars having different alphabets are significantly different at $p < 0.05$. **b** Large intestine (L.I.) microbiota changes observed after feeding re-nourished diet with different supplements (SM, PFM and BS) in mice model. C, control; M, malnourished; SM, skim milk; PFM, probiotic fermented milk; BS, bacterial suspension ($n = 6$ samples per group). *Bars having different alphabets are significantly different at $p < 0.05$

available regarding the gut microbiota changes after the administration of probiotics as an adjuvant to re-nutrition diet in malnourished animal models [14, 83–88]. Hence, the present study demonstrated the modulating effect of probiotics against the disturbed microenvironment resulted due to protein energy malnutrition.

Conclusions

PEM is a global burden culminating to disturbed gut condition and immunocompromised state of health. In the present study,

the adjuvant effect of probiotics as PFM and/or BS in mice model, paved way for the possibility to combat the devastating problem of PEM by enhancing systemic immunity and gut barrier functions. But, there is still paucity of information and more research is to be required that could explain the individual as well as combined effect of probiotics and the re-nutrition diet in the upregulation of localized and systemic immunity in reversing the condition of PEM.

Compliance with Ethical Standards

Conflict of Interest   The authors declare that there is no conflict of interest.

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