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Age-related decline in macrophage and lymphocyte functions in mice and its alleviation by treatment with probiotic Dahi containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum*

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This study evaluated the effects of probiotic Dahi administration in ageing mice on macrophage and lymphocyte functions. Probiotic Dahi were prepared by co-culturing in buffalo milk (3% fat) Dahi bacteria (*Lactococcus lactis* ssp. *cremoris* NCDC-86 and *Lc. lactis* ssp. *lactis* biovar *diacetylactis* NCDC-60) along with *Lactobacillus acidophilus* LaVK2 (La-Dahi) or combined *Lb. acidophilus* and *Bifidobacterium bifidum* BbVK3 (LaBb-Dahi). Four groups of 12 mo old mice were fed for four months, with the supplements (5 g/day) of buffalo milk (3% fat), Dahi, La-Dahi and LaBb-Dahi, respectively, in addition to basal diet, and a fifth group that received no supplements served as control. The immune functions of young mice (4 mo old) were also compared with those of ageing adult mice (16 mo old). The production of nitric oxide and cytokines IL-6 and TNF-α declined and that of immunosuppressive prostaglandin E_2 (PGE_2) increased by stimulated peritoneal and splenic macrophages in ageing mice, compared with their young counterparts. The proliferation of stimulated splenocytes diminished and the production of IL-2 decreased and that of IL-6 and TNF-α enhanced in ageing compared with young mice. Feeding ageing mice with La-Dahi or LaBb-Dahi improved peritoneal macrophage functions stimulating nitric oxide and IL-6 and diminishing PGE_2 production. Feeding La-Dahi or LaBb-Dahi also improved lymphocyte functions stimulating their proliferation and production of IL-2 in ageing mice. To conclude, the probiotic La-Dahi and LaBb-Dahi are effective in reversing age related decline in immune functions in mice.

Keywords: *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, probiotic Dahi, macrophage, lymphocyte, ageing.

Immune system constitutes two-components adaptive and innate. Adaptive immunity consists of highly specific antigen recognition and provision of memory through genetic modification of lymphocytes and clonal expansion. Though very specific, this immunity is slow to respond against acute infections. By contrast, innate immunity provides immediate and fast response. Macrophages are the key players of innate immune system and form the first line of defence against the predation on the body acting against the antigen by producing various inflammatory mediators (reactive oxygen and nitrogen species), producing signalling molecules (cytokines, TNF-α, IFN-γ) to coordinate overall immune response. These cytokines then start a cascade of events stimulating other immune cells like B and T cells which help in combating pathological conditions.

Macrophage-ageing is characterized by the decline in ability of macrophages to respond optimally to activating signals and to produce effector molecules like reactive oxygen species (hydrogen peroxide, superoxide and nitric oxide) and cytokines (TNF-α and IL-6), which results in an overall inefficacy in critical effectors functions *i.e.* killing of microorganism and lysis of tumor cells and thus a sustained state of infection (Davila et al. 1990; Ding et al. 1994). Lipopolysaccharides, one of the potent macrophage stimulant, and IFN-γ complement macrophage function by up regulating the release of inflammatory cytokines. However, in ageing animals, hypo-responsiveness to IFN-γ has been reported (Ding et al. 1994). Ageing is also associated with impairment of T cell functions, most importantly the decline in the ability of T lymphocytes to proliferate and produce IL-2 on mitogenic stimulation (Bruunsgaard et al. 2000).

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Dayan et al. (2000) observed a shift in cytokine profile from Th1 to Th2 type, as well as an increase in the production of pro-inflammatory cytokines with ageing in different strains of mice.

Oral administration of lactic acid bacteria was demonstrated to improve macrophage and lymphocyte functions (Vinderola et al. 2006; Jain et al. 2009a) and thus are cheap and safe mechanism to modulate body immunity. Our laboratory has recently identified strains *Lactobacillus acidophilus* LaVK2 and *Bifidobacterium bifidum* BbVK3, which when fed to mice, incorporated in traditional dairy fermented product Dahi, showed potent immunomodulatory attributes enhancing macrophage phagocytic activity with increased secretion of lysosomal enzymes and providing protection against enteric infection (Rajpal & Kansal, 2009). The immuno-potentiating ability was further speculated from the study wherein this probiotic Dahi attenuated dimethylhydrazine-induced gastrointestinal carcinogenesis in rats (Rajpal & Kansal, 2008). The present study shows that administration of *Lb. acidophilus* LaVK2 and *Bifido. Bifidum* BbVK3 in the form of probiotic Dahi reverses some of the immuno-senescence related decline in macrophage and lymphocyte functions in ageing mice.

Materials and Methods

Bacterial cultures and Dahi Preparation

Lb. acidophilus LaVK2 and *Bifido. bifidum* BbVK3 were our laboratory isolates, and *Lc. lactis* ssp. *cremoris* NCDC-86 and *Lc. lactis* ssp. *lactis* biovar *Diacetylactis* NCDC-60 were obtained from NCDC, National Dairy Research Institute (Karnal 132001, India). The lactobacilli and lactococci were propagated and maintained in MRS-broth and M17 broth, respectively and *Bifido. bifidum* was cultured and propagated (in MRS-broth) in anaerobic conditions. Bacterial cultures were revitalized three times in reconstituted and autoclaved skim milk prior to use for preparation of fermented milk. Buffalo milk obtained from the cattle yard of the institute and standardized to 3·0% fat was heated to 90 °C for 15 min and then cooled to 37 °C. Dahi was prepared culturing standardized buffalo milk with Dahi starter (*Lc. lactis* ssp. *cremoris* NCDC-86 and *Lc. lactis* ssp. *lactis* biovar *diacetylactis* NCDC-60; 1% each) at 30 °C for 8 h. Probiotic La-Dahi was prepared culturing standardized buffalo milk with *Lb. acidophilus* LaVK2 and Dahi starter. For the preparation of probiotic LaBb-Dahi, a mixture of *Lb. acidophilus* LaVK2 and *Bifido. bifidum* BbVK3 and Dahi starters were employed. The final product contained lactococci, $1–2\times10^9$ cfu/g, *Lb. acidophilus,* $2–20\times10^7$ cfu/g and *Bifido. bifidum* $2–20\times10^7$ cfu/g.

Animal feeding protocol

Male Swiss albino mice obtained from Small Animal House of National Dairy Research Institute, Karnal, India were grown on animal stock diet up to the age of 4 months (young group) and 12 months (ageing group). Guidelines for the care and use of animals were followed and approved by Ethical Committee of National Dairy Research Institute, Karnal, India. Ageing mice (12 mo old) tabulated according to their body weight were divided into following five groups ($n=6$) with mean initial body weight 40 ± 2 g as: 1): control group, fed basal diet; 2) milk group, fed buffalo milk (3% fat) supplements along with basal diet; 3) Dahi group, fed Dahi supplements along with basal diet; 4) La-Dahi group, fed La-Dahi supplements along with basal diet and 5) LaBb-Dahi group, fed LaBb-Dahi supplements along with basal diet. The animals housed in polypropylene cages in an air conditioned room (24 ± 1 °C), provided with diet and water *ad libitum,* were fed for 4 months. Each mouse was fed 5 g test supplement prior to basal diet. The basal diet comprised of starch (63%), casein (20%), soybean oil (5·5%), vitamin mixture (1%), mineral mixture (5%), choline chloride (0·2%), cellulose (5%) and methionine (0·2%). Vitamin and mineral mixtures were prepared and mixed according to AOAC (2005). After 4 mo of feeding animals were killed by cervical dislocation, and peritoneal fluid and spleen were isolated for further analysis.

Isolation and enrichment of macrophages

Peritoneal fluid was collected in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 medium (without phenol red and supplemented with 1·2 g sodium bicarbonate/l, 1 g bovine serum albumin/l, 200 units penicillin/ml and 50 μg streptomycin/ml and pH adjusted to 7·2) injected into the peritoneal cavity, and following a gentle massage of the abdomen, the medium was aspirated with syringe.

Splenic macrophages were isolated by teasing spleen and then lysing RBCs in a buffer (one part of 0·17 M-Tris HCl and 9 parts of 0·6 M-NH_4Cl and pH adjusted to 7·2). These were plated on 35 mm cell culture plate and incubated at 37 °C in an incubator perfused with a mixture of 5% CO_2 in air for 1 h. The non-adherent cells were collected for lymphocyte isolation. The adherent macrophages were released by jetting chilled DMEM on to the cells with a pipette. The macrophages were counted and enriched by plating on FBS (foetal bovine serum) coated plates for 2 h in CO_2 incubator perfused with 5% CO_2 in air (Kumagai, 1979). The supernatant containing non-adherent cells, if any, was discarded and the adherent cells collected in DMEM.

Macrophage culture

Enriched macrophages, suspended in DMEM supplemented with heat-inactivated FBS (10%) and 2-mercaptoethanol (50 nM), were plated (5×10^5 cells in 300 μl/well) in 24 well cell culture plates in presence or absence of *Escherichia coli* (serotype 055:B5) lipopolysaccharide (LPS) (1 μg/ml) or LPS (1μg/ml) plus recombinant IFN-γ (100 U/ml) and incubated in atmosphere of 5% CO_2 in air at 37 °C for 24 h. The cell free supernatants collected at 8 h and 24 h were analyzed for

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Table 1. Effect of feeding probiotic milks on nitric oxide production (μM) by mouse peritoneal and splenic macrophages

	Peritoneal macrophages			Splenic macrophages		
	Basal	LPS	LPS + IFN-γ	Basal	LPS	LPS + IFN-γ
Young	$18{\cdot}4 \pm 1{\cdot}60$	$47{\cdot}0 \pm 1{\cdot}76$	$63{\cdot}4 \pm 2{\cdot}25$	$11{\cdot}7 \pm 0{\cdot}76$	$44{\cdot}1 \pm 1{\cdot}04$	$63{\cdot}3 \pm 2{\cdot}34$
Control	$16{\cdot}5^{a} \pm 3{\cdot}38$	$32{\cdot}9^{*a} \pm 2{\cdot}69$	$49{\cdot}0^{*a} \pm 1{\cdot}65$	$14{\cdot}2^{a} \pm 0{\cdot}58$	$38{\cdot}7^{*a} \pm 1{\cdot}24$	$53{\cdot}8^{*a} \pm 1{\cdot}43$
Buffalo milk	$21{\cdot}4^{a} \pm 1{\cdot}99$	$39{\cdot}1^{a} \pm 1{\cdot}21$	$56{\cdot}3^{a} \pm 2{\cdot}54$	$12{\cdot}9^{a} \pm 0{\cdot}85$	$38{\cdot}4^{a} \pm 0{\cdot}97$	$55{\cdot}9^{a} \pm 0{\cdot}43$
Dahi	$20{\cdot}8^{a} \pm 2{\cdot}26$	$40{\cdot}2^{a} \pm 1{\cdot}59$	$62{\cdot}6^{ab} \pm 3{\cdot}53$	$13{\cdot}7^{a} \pm 0{\cdot}35$	$36{\cdot}8^{a} \pm 1{\cdot}14$	$50{\cdot}3^{a} \pm 3{\cdot}23$
La-Dahi	$22{\cdot}0^{a} \pm 4{\cdot}41$	$41{\cdot}3^{a} \pm 1{\cdot}75$	$66{\cdot}9^{b} \pm 2{\cdot}43$	$13{\cdot}3^{a} \pm 0{\cdot}54$	$37{\cdot}7^{a} \pm 0{\cdot}57$	$50{\cdot}2^{a} \pm 1{\cdot}32$
LaBb-Dahi	$21{\cdot}3^{a} \pm 1{\cdot}39$	$42{\cdot}2^{a} \pm 3{\cdot}13$	$67{\cdot}4^{b} \pm 1{\cdot}45$	$12{\cdot}5^{a} \pm 0{\cdot}33$	$34{\cdot}5^{a} \pm 1{\cdot}35$	$49{\cdot}3^{a} \pm 2{\cdot}34$

* Values (mean ± SE for $n = 6$) in control ageing group (16 mo old) are significantly different ($P < 0{\cdot}05$) from young mice (4 mo old)
Values in supplement fed ageing groups with different superscript letters are significantly different ($P < 0{\cdot}05$)

TNF-α, IL-6, nitric oxide and prostaglandin E_2. The TNF-α being an early cytokine was estimated in the supernatant collected at 8 h.

Cytokines, nitric oxide and prostaglandin E_2 estimation in macrophage culture supernatant

The cytokines IL-6 and TNF-α were estimated in culture supernatants using Peprotech's ELISA Development kits (Life Technologies India, New Delhi) as per manufacturer's instructions. Nitrates plus nitrites were estimated using Griess reagent (Miranda et al. 2001). Prostaglandin E_2 was estimated using ELISA kit (Bi biotech India, New Delhi) based on competitive binding technique wherein PGE_2 in the sample competes with a fixed amount of horseradish peroxidase (HRP)-labelled PGE_2 for sites on mouse monoclonal antibodies. The protein was determined (Lowry et al. 1951) by digesting macrophages in the wells with 1 N NaOH.

Lymphocyte proliferation

The non adherent splenic cells collected as above were washed twice with RPMI-1640 medium and suspended in the same medium supplemented with heat-inactivated foetal bovine serum (10%), 25 mM-Hepes, 2 mM-glutamine, 1×10^5 units penicillin/l, 100 mg streptomycin/l and 25 mg amphotericin/l, and pH adjusted to 7·2. Cell viability was checked with trypan blue and the counts adjusted to 1×10^7 viable cells/ml. The lymphocyte cell suspensions (100 μl per well) in RPMI-1640 medium containing FBS (10%) and 2-mercaptoethanol (50 nM) were cultured in 96 well round-bottom cell culture plates with suboptimal, optimal, and supraoptimal (cytotoxic) concentrations of mitogen (2·5, 5, and 10 mg per ml Concanavalin A (Con A) or 12·5, 30, and 50 mg per ml LPS, respectively) for 48 h at 37 °C in a humidified incubator perfused with 5% CO_2 in air. The lymphocyte proliferation was quantitated colorimetrically using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and expressed as stimulation index, calculated as the ratio of absorbance (at 540 nm) of mitogen stimulated to unstimulated lymphocytes (Mosmann, 1983).

Estimation of Cytokines production by lymphocytes

For estimation of interleukins, IL-2, IL-6 and tumour necrosis factor (TNF)-α, lymphocyte cell suspensions (1×10^7 cells/ml) were cultured (at 37 °C for 48 h) in the presence or absence of Con A (5 μg/ml). The concentrations of cytokines were estimated in cell free culture supernatants using Peprotech's ELISA Development kits (Life Technologies India, New Delhi) as per manufacturer's instructions.

Statistical analysis

Results are presented as means ± SEM. Analysis of variance was performed using GraphPad PRISM version 5.0 statistical software package and the differences among groups were tested using Tukey–Kramer post-hoc test. Significance of difference was set at $P < 0{\cdot}05$.

Results

Nitric oxide (NO)

The basal level of NO production by peritoneal and or splenic macrophages did not vary significantly between young and ageing mice. In ageing mice compared with their young counterparts, the production of NO by LPS stimulated peritoneal and splenic macrophages declined by 30 and 12·2%, respectively. Similarly, the decline in NO production was also observed in macrophages stimulated with LPS + IFN-γ (Table 1).

Feeding ageing mice with buffalo milk, Dahi or probiotic Dahi had no significant effect on basal level NO production by peritoneal or splenic macrophages. When control and cultured milk fed ageing mice groups were compared, a significant increase in NO production was observed in LPS + IFN-γ stimulated peritoneal macrophages obtained from La-Dahi (36·5%) or LaBb-Dahi (37·5%) fed groups. A non-significant increase in NO production by LPS stimulated peritoneal macrophages was also observed in mice fed La-Dahi (25·5%) and LaBb-Dahi (28·3%), while feeding buffalo milk or Dahi had no significant effect. Unlike in peritoneal macrophages, the production of NO by

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Table 2. Effect of feeding probiotic milks on IL-6 and TNF-α production (pg/ml) by mouse peritoneal and splenic macrophages

IL-6

	Peritoneal macrophages			Splenic macrophages		
	Basal	LPS	LPS + IFN-γ	Basal	LPS	LPS + IFN-γ
Young	76·5 ± 3·43	475 ± 34·3	827 ± 34·5	47·8 ± 3·21	447 ± 32·4	815 ± 56·4
Control	65·8^{a} ± 4·32	248^{*a} ± 15·3	529^{*a} ± 44·3	50·5^{a} ± 2·53	259^{*a} ± 23·2	479^{*a} ± 23·5
Buffalo milk	71·5^{a} ± 2·43	271^{ac} ± 14·3	541^{a} ± 28·6	61·4^{a} ± 5·30	296^{a} ± 18·5	415^{a} ± 33·4
Dahi	65·7^{a} ± 4·32	366^{bc} ± 10·3	559^{a} ± 55·4	45·6^{a} ± 3·66	261^{a} ± 23·4	427^{a} ± 37·4
La-Dahi	59·3^{a} ± 3·75	386^{bc} ± 21·2	573^{a} ± 48·5	51·3^{a} ± 3·22	279^{a} ± 13·5	450^{a} ± 43·2
LaBb-Dahi	72·4^{a} ± 4·54	423^{b} ± 19·4	576^{a} ± 39·6	52·4^{a} ± 4·39	331^{a} ± 18·6	479^{a} ± 25·4

TNF-α

	Peritoneal macrophages			Splenic macrophages		
	Basal	LPS	LPS + IFN-γ	Basal	LPS	LPS + IFN-γ
Young	46·8 ± 3·41	697 ± 34·4	1605 ± 39·5	66·4 ± 3·22	360 ± 29·3	666 ± 26·4
Control	59·0^{a} ± 3·76	456^{*a} ± 14·4	1221^{*a} ± 46·7	88·3^{a} ± 2·91	285^{*a} ± 11·2	487^{*a} ± 18·4
Buffalo milk	47·5^{a} ± 4·65	490^{a} ± 26·4	1193^{a} ± 23·5	90·3^{a} ± 4·48	238^{a} ± 32·5	526^{a} ± 43·5
Dahi	54·2^{a} ± 6·54	514^{a} ± 34·5	1224^{a} ± 43·7	66·1^{a} ± 2·74	225^{a} ± 29·4	532^{a} ± 33·3
La-Dahi	49·3^{a} ± 5·43	553^{a} ± 29·6	1315^{ab} ± 64·4	80·0^{a} ± 3·77	246^{a} ± 19·3	538^{a} ± 29·4
LaBb-Dahi	48·2^{a} ± 3·56	578^{a} ± 37·5	1399^{b} ± 54·6	70·2^{a} ± 3·01	222^{a} ± 21·4	554^{a} ± 32·5

*Values (mean ± SE for $n = 6$) in control ageing group (16 mo old) are significantly different ($P < 0·05$) from young mice (4 mo old)
Values in supplement fed ageing groups with different superscript letters are significantly different ($P < 0·05$)

cultured splenic macrophages stimulated with LPS or LPS + IFN-γ was not influenced by feeding probiotic Dahi to ageing mice (Table 1).

Interleukin IL-6 and TNF-α

The basal level of cytokine (IL-6 and TNF-α) production by peritoneal and splenic macrophages did not vary significantly between young and ageing mice, while in LPS or LPS + IFN-γ stimulated macrophages (peritoneal as well as splenic) it declined in the latter group (Table 2).

Feeding ageing mice with buffalo milk, Dahi or probiotic Dahi had no significant effect on basal levels of IL-6 and TNF-α production by peritoneal or splenic macrophages. Oral administration of probiotic La-Dahi and LaBb-Dahi enhanced production of IL-6 by LPS stimulated peritoneal macrophages by 55·6 and 70·6%, respectively, relative to control group. Feeding mice with regular Dahi also increased production of IL-6 by peritoneal macrophages by 47·6%. When peritoneal macrophages were stimulated with combination of LPS and IFN-γ, the production of IL-6 was 2·1 fold of that observed in LPS stimulated macrophages obtained from ageing control mice. The effect of feeding probiotic Dahi on production of IL-6 by LPS + IFN-γ stimulated peritoneal macrophages was non-significant, when compared with control group. Contrary to the effect observed in LPS stimulated peritoneal macrophages, the IL-6 production by cultured splenic macrophages stimulated with LPS or LPS + IFN-γ was not significantly affected by feeding buffalo milk, regular Dahi, La-Dahi or LaBb-Dahi (Table 2).

The production of TNF-α by LPS and LPS + IFN-γ stimulated peritoneal macrophages decreased by 34·6 and 23·9%, respectively, in 16 mo old ageing mice compared with their young (4 mo old) counterparts. A similar trend was seen in splenic macrophages wherein the corresponding decline in TNF-α production was 20·8 and 26·9%, respectively, in ageing mice relative to young animals. Feeding mice with supplements of probiotic Dahi had no significant effect on production of TNF-α in splenic macrophages. However, the increase in TNF-α production by peritoneal macrophages (LPS + IFN-γ stimulated) in LaBb Dahi-fed mice was statistically significant (Table 2).

Prostaglandin E_2 (PGE_2)

The basal level of PGE_2 production by macrophages did not vary significantly between young and ageing mice. The production of PGE_2 by LPS stimulated macrophages (peritoneal as well as splenic) increased by 52–56% in ageing mice, relative to their 4 mo old counterparts (Table 3).

Feeding ageing mice with buffalo milk, Dahi or probiotic Dahi had no significant effect on basal levels of PGE_2 production by peritoneal or splenic macrophages. Administration of La-Dahi or LaBb-Dahi to ageing mice significantly decreased (16·6–17·9%) the production of PGE_2 by peritoneal macrophages, while feeding buffalo milk or regular

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Table 3. Effect of feeding probiotic milks on PGE_2 production (pg/μg protein) by mouse peritoneal and splenic macrophages

	Peritoneal macrophages		Splenic macrophages	
	Basal	LPS	Basal	LPS
Young	43·5±8·54	95·4±9·32	16·9±2·32	80·1±7·64
Control	$60·2^{a}$±4·65	145^{*a}±11·8	$21·3^{a}$±4·10	125^{*a}±8·44
Buffalo milk	$58·6^{a}$±7·75	140^{a}±23·2	$23·2^{a}$±3·21	115^{a}±13·5
Dahi	$57·4^{a}$±10·2	134^{ab}±19·2	$20·3^{a}$±1·44	114^{a}±15·6
La-Dahi	$56·4^{a}$±8·54	121^{b}±10·8	$21·3^{a}$±2·01	109^{a}±18·2
LaBb-Dahi	$58·7^{a}$±11·5	119^{b}±12·1	$19·2^{a}$±1·45	103^{a}±15·3

*Values (mean±SE for $n=6$) in control ageing group (16 mo old) are significantly different ($P<0·05$) from young mice (4 mo old)
Values in supplement fed ageing groups with different superscript letters are significantly different ($P<0·05$)

Table 4. Effect of feeding probiotic milks on proliferation (stimulation index) of mouse splenocytes stimulated by lipopolysaccharide (LPS) or Concanavalin A (Con A)

	LPS			ConA		
Mitogen (μg/ml)	12·5	30	50	2·5	5	10
Young	1·06±0·10	1·40±0·15	1·18±0·06	1·08±0·11	1·85±0·10	1·22±0·05
Control	$0·89^{*a}$±0·06	$0·98^{*a}$±0·06	$0·68^{*a}$±0·07	$0·90^{*a}$±0·04	$1·28^{*a}$±0·07	$0·99^{*a}$±0·03
Buffalo milk	$0·99^{ab}$±0·04	$1·02^{a}$±0·20	$0·72^{a}$±0·06	$1·02^{a}$±0·06	$1·25^{a}$±0·07	$1·01^{a}$±0·04
Dahi	$0·95^{ab}$±0·05	$1·08^{a}$±0·05	$0·83^{ab}$±0·13	$0·95^{a}$±0·07	$1·31^{a}$±0·03	$1·05^{ab}$±0·04
La-Dahi	$1·16^{b}$±0·03	$1·31^{b}$±0·04	$0·87^{ab}$±0·05	$1·12^{a}$±0·02	$1·40^{a}$±0·03	$1·16^{b}$±0·02
LaBb-Dahi	$1·14^{b}$±0·12	$1·34^{b}$±0·07	$0·92^{b}$±0·03	$1·18^{a}$±0·05	$1·39^{a}$±0·05	$1·18^{b}$±0·06

*Values (mean±SE for $n=6$) in control ageing mice (16 mo old) are significantly different ($P<0·05$) from young mice (4 mo old)
Values in supplement fed ageing groups with different superscript letters are significantly different ($P<0·05$)

Dahi had no effect. The production of PGE_2 by splenic macrophages in probiotic Dahi fed mice decreased by 12·8–17·6% relative to control but the difference did not reach to the level statistically significant.

Lymphocyte proliferation

The age related changes in proliferative response (stimulation index) were evaluated for lymphocytes cultured at suboptimal, optimal and supraoptimal concentrations of mitogen (Table 4). There was no significant difference among young and ageing mice in proliferative response of lymphocytes stimulated with suboptimal concentration of LPS (12·5 μg/ml) or ConA (2·5 μg/ml). However, when stimulated with optimal or supra-optimal concentrations of mitogens, lymphocyte blastogenesis decreased significantly in ageing mice, compared with their 4 mo old counterparts. The splenocytes proliferative response to optimal LPS (30 μg/ml) or Con A (5 μg/ml) concentrations was decreased by 30% in ageing mice, relative to young animals. At supra-optimal concentrations of mitogens (50 μg/ml for LPS and 10 μg/ml for Con A) the lymphocytes proliferation decreased by 43% and 19%, respectively, in ageing mice.

Feeding ageing mice with La-Dahi or LaBb-Dahi significantly enhanced (27–37%) the proliferative response of splenocytes stimulated with suboptimal or optimal concentrations of LPS, compared with control group, while feeding with buffalo milk or regular Dahi had no significant effect. At suboptimal or optimal concentrations of Con A, the proliferative response of T-lymphocytes was almost similar in different dietary groups, while at supra-optimal Con A concentration the T cell proliferation was significantly enhanced in animals fed La-Dahi (17%) or LaBb-Dahi (19%), relative to control group, while feeding with buffalo milk or regular Dahi had no significant effect.

Cytokines production by splenocytes

The production of IL-6 and TNF-α by Con A stimulated splenocytes was enhanced by about 77% and that of IL-2 decreased by 56·5% in 16 mo old ageing mice, relative to 4 mo old counterparts (Table 5). Feeding ageing mice with probiotic Dahi (La-Dahi or LaBb-Dahi) enhanced production of IL-2 by about 46% by Con A stimulated splenocytes, compared with control group. Feeding regular Dahi also enhanced IL-2 production by 32·7%; however, the effect did not reach to the level statistically significant, and feeding with buffalo milk had no effect. The Con A stimulated splenocytes from mice fed Dahi, La-Dahi or LaBb-Dahi, compared with control group, also exhibited diminishing production of IL-6 (23·1–24·6%) and TNF-α (18·6–21·6%), though the effect was statistically non-significant.

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Table 5. Effect of feeding probiotic milks on cytokines production (pg/ml) by mouse splenocytes

	IL-2		IL-6		TNF-α	
	Basal	ConA	Basal	ConA	Basal	ConA
Young	19·4 ± 4·23	386 ± 4·33	12·3 ± 1·21	62·1 ± 3·76	21·3 ± 2·42	133 ± 8·32
Control	$22·5^{a}$ ± 1·43	168^{*a} ± 10·2	$14·3^{a}$ ± 5·18	111^{*a} ± 12·4	$16·7^{a}$ ± 1·38	236^{*a} ± 4·86
Buffalo milk	$21·4^{a}$ ± 2·76	167^{a} ± 9·39	$15·3^{a}$ ± 0·90	$96·7^{a}$ ± 6·39	$18·6^{a}$ ± 0·68	227^{a} ± 11·3
Dahi	$20·4^{a}$ ± 1·30	223^{ab} ± 5·19	$12·4^{a}$ ± 3·77	$86·9^{a}$ ± 5·83	$19·2^{a}$ ± 1·50	202^{a} ± 13·2
La-Dahi	$22·4^{a}$ ± 1·37	245^{b} ± 12·3	$13·5^{a}$ ± 1·59	$83·7^{a}$ ± 8·19	$21·4^{a}$ ± 3·42	192^{a} ± 6·54
LaBb-Dahi	$18·5^{a}$ ± 3·11	247^{b} ± 8·32	$11·5^{a}$ ± 0·68	$85·4^{a}$ ± 7·14	$18·8^{a}$ ± 1·25	185^{a} ± 10·2

* Values (mean ± SE for $n = 6$) in control ageing group (16 mo old) are significantly different ($P < 0·05$) from young mice (4 mo old)
Values in supplement fed ageing groups with different superscript letters are significantly different ($P < 0·05$)

Discussion

Macrophages are key players of innate/inflammatory immune response, which is regulated by specific regulators (toll like receptors). In absence of antigenic stimuli the inflammatory response is regulated by maintaining a low expression of inflammatory mediators. On recognition of antigenic stimuli (e.g. LPS from pathogens) the transcriptional machinery gets activated and a massive release of inflammatory molecules (cytokines and reactive oxygen intermediates) takes place (Aung et al. 2006). Culturing macrophages in presence of lipopolysaccharides (LPS) mimics *in vitro* model of sepsis and visualizes age-related changes in the degree of acute-phase response. IFN-γ synergizes LPS to promote optimal macrophage activation required for complex functions like antitumour and microbicidal activities (Collart et al. 1986).

Nitric oxide is an important reactive molecule produced by activated macrophages to combat infection. Our results show a decline in nitric oxide production by stimulated peritoneal and splenic macrophages in ageing mice, though its basal level was almost similar to that in young mice. Feeding La-Dahi or LaBb-Dahi to ageing mice restored the level of nitric oxide production by peritoneal macrophage population stimulated with LPS + IFN-γ, similar to that in young mice; however the effect was not extended to splenic macrophages. Tejada-Simon et al. (1999) observed an up regulation of nitric oxide production by peritoneal cells obtained from mice administered *Lb. acidophilus*. The production of nitric oxide was also increased in RAW264.7 macrophage cell line when co-cultured with different strains of bifidobacteria (Hur et al. 2004).

The macrophages produce various cytokines, a diverse group of mediators that coordinate macrophage function and signal lymphocyte activation and functions. Our results on age related decline in cytokines (TNF-α and IL-6) production by stimulated peritoneal and splenic macrophages are supported by recent report (Gomez et al. 2010). The basal levels of these cytokines were not significantly affected by ageing; the observation is concordant with the earlier report (Boehmer et al. 2004). Feeding ageing mice with La-Dahi or LaBb-Dahi reversed age related decline in IL-6 production by LPS stimulated peritoneal macrophages. TNF-α is an early cytokine that stimulates macrophages in an autocrine manner to produce other proinflammatory cytokines and effector molecules. A slight enhanced production of TNF-α by peritoneal macrophages of probiotic Dahi fed mice caused significant enhanced production of IL-6.

Prior exposure to lactic acid bacteria has been suggested to potentiate macrophage activation in response to mitogen. Increased production of IL-6 and IL-12 by LPS stimulated mouse peritoneal macrophages by administration of *Lb. acidophilus* has been reported (Tejada-Simon et al. 1999). The differential effect of probiotic bacteria in peritoneal and splenic macrophages observed in the present study could be due to environmental divergence in macrophages obtained from two different tissues (Zhu et al. 2006), and contribute to the functional role of the two macrophage populations. Peritoneal macrophages contribute to innate defence system, whereas splenic macrophages are involved in adaptive immunity. Spleen being a secondary lymphoid organ involved in induction and development of specific immunity. The inability of intestinal flora to stimulate inflammatory cytokine production by splenic macrophages indeed avoids superinduction of proinflammatory response which could have deleterious effects (Nicaise et al. 1999). Thus lactic acid bacteria play as immuno-regulators, which strengthens innate immune system without affecting the specific immunity.

Prostaglandin E_2 is an important negative regulator of body's innate immune system, which inhibits leukocyte chemotaxis, macrophage phagocytosis, and production of nitric oxide and reactive oxygen intermediates, leukotriene synthesis, and generation of multiple proinflammatory cytokines by macrophages (Goodwin & Ceuppens, 1983). The age related decline in IL-2 production and subsequent decrease in T cell-proliferation are suggested to be a consequence of enhanced PGE_2 levels (Hilkens et al. 1996). In the present study we observed an increase in PGE_2 production by stimulated peritoneal and splenic macrophages in ageing mice, which was associated with decline in production of nitric oxide and cytokines by macrophages, and reduced lymphocyte proliferation and IL-2 production.

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Feeding ageing mice with La-Dahi or LaBb-Dahi, in the present study, reversed age related increase in PGE_2 production by stimulated peritoneal macrophages. The cell free fractions prepared from milk fermented with *Lb. acidophilus* inhibited production of PGE_2 and $PGF_{2\alpha}$ by intestinal epithelial cell line (IEC line HT-29) (Fiander et al. 2005), suggesting that the factors produced during fermentation contribute towards immunostabilization by down regulating PGE_2 production by macrophages.

Lymphocyte proliferation, an index of cell-mediated immune function, decreased with ageing, which could be a result of aberrations in cytokine IL-2 and PGE_2 production, defects in intracellular and intercellular signalling (Wakikawa et al. 1997), changes in lymphocyte subpopulation (Walford et al. 1981) or increase in cell death with age (Itzhaki et al. 2003). In aged animals, a decline in expression of *Gfi*-1 (growth factor independent −1) gene associated with lymphocyte proliferation, and a higher expression of genes responsible for lymphocyte apoptosis have been reported (Han et al. 2006). Feeding ageing mice with La-Dahi or LaBb-Dahi stimulated B and T lymphocyte proliferation. The enhanced IL-2 production by stimulated splenocytes and decreased immunosuppressive PGE_2 production by splenic macrophages, observed in probiotic Dahi fed mice could be the contributing factors for improved lymphocyte proliferation.

An age related decline in IL-2 production and increase in IL-6 and TNF-α production by Con A stimulated mouse lymphocytes observed in present study are supported by earlier reports (Dayan et al. 2000; Kovaiou et al. 2005). Our results also show that feeding ageing mice with La-Dahi or LaBb-Dahi reversed age related decline in IL-2 production by Con A stimulated splenocytes, suggesting an induction of pro-Th1 signals leading to stimulation of systemic cell-mediated immune (CMI) responses. Stimulation of interferon (IFN)-γ and IL-2 (Th1 type cytokines) mRNA expressions in spleen and Peyer's patches have been reported in mice fed *Lb. casei* plus *Lb. acidophilus* fermented milk (Jain et al. 2009b). Pestka et al. (2001) observed that feeding mice with yoghurt supplemented with probiotic bacteria (*Lb. acidophilus, Bifido. longum* subsp. *infantis* and *Bifido. bifidum*) significantly enhanced the population of CD4+ (T helper) cells in mice spleen. Indian Dahi modulates TNF-α and IFN-γ cytokines levels (Dewan et al. 2009) and increases CD4+/CD8+ T cell ratio in blood of malnourished children (Dewan et al. 2007). The redistribution of T cell subsets could be one of the mechanisms for anti-immuno-senescene effect of probiotic Dahi, since newly generated CD4+ T cells in aged animals are free from age-related defects and show good expansion and IL-2 production (Haynes et al. 2005).

The mechanism by which lactic acid bacteria modulate immune system is not precisely understood. It is suggested that the bacteria interact with the immune cells (dendritic cells, macrophages, T cells) through the cell wall components (peptidoglycan, lipotecholic acid) and can potentiate release of co-stimulatory and effector molecules (cytokines and reactive oxygen intermediates). Further, the metabolites (biotics) produced during fermentation of milk also mediate immuno-potentiating attributes. Recently, Tellez et al. (2010) isolated nine peptides from cell free supernatant of milk fermented with *Lb. helveticus* (LH-2) that stimulate the production of interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), interleukin-1β and nitric oxide (NO) by RAW264.7 macrophage cell line.

To conclude, the probiotic Dahi exhibited the ability to reprogram macrophage and lymphocyte functions associated with immuno-senescence. Ageing mice fed with supplements of probiotic Dahi displayed an improvement in macrophage functions with an increase in production of nitric oxide and interleukin IL-6 and decrease in production of immunosuppressive PGE_2. Supplementation of probiotic Dahi also helped in restoring splenocytes proliferation and production of IL-2. Since the two preparations of probiotic Dahi (La-Dahi and LaBb-Dahi) performed similarly, *Lb. acidophilus* strain appears to be mainly responsible for improvement of immune functions in ageing animals. However, incorporation of *Bifido. bifidum* is significantly important towards sensory attributes of the probiotic Dahi and its protective effect in gastrointestinal tract cancer reported earlier (Rajpal & Kansal, 2008). Further studies are needed to check the effectiveness of the reprogrammed immune status amid a real-time infection in aged animals.

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