Functional foods, mucosal immunity and aging: effect of probiotics on intestinal immunity in young and old rats

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The elderly are characterized by immunosenescence which is accompanied by an increase in the incidence of infectious diseases, especially in the intestinal tract. There is evidence suggesting that certain probiotic organisms have immunomodulatory properties. Therefore, the effects of feeding fermented milk products on the intestinal mucosal immune response was examined in young and senescent rats. Serum anti-cholera toxin (CTx) immunoglobulin A (IgA) antibody titers were significantly higher in kefir-fed young rats in comparison to young control-fed (no kefir) animals. This increase was accompanied by enhanced in vitro IgA antibody secretion by Peyer's patch and intestinal lamina propria lymphocytes. No similar enhancements of serum or lymphocyte IgA antibody responses were observed in the kefir-fed versus control-fed old rats. The fact that kefir enhanced responses were observed only in the young rats suggests that probiotics may affect the intestinal immune response differentially in young and old animals.

Keywords: mucosal immunity, intestinal immunity, IgA, probiotics, aging

1. The intestinal mucosal immune response and aging

The elderly constitute the most rapidly growing subpopulation in many countries. In the United States, the number of individuals over 65 years of age is estimated in excess of 33 million and, at the current rate of expansion, this number will double by 2030. This marked shift in age demographics is accompanied by an increase in the incidence of infectious diseases and other age-related pathologies. Infectious diseases are the fourth leading cause of death and a significant contributor to morbidity in the elderly. The intestine is particularly sensitive to infectious diseases in the elderly, suggesting that the intestinal mucosal immune defenses are compromised during aging [1, 2].

Mucosal surfaces constitute a discrete compartment of the immune system that is autonomous from the systemic arm by virtue of several marked differences, including a different immunoglobulin isotype, immunoglobulin A (IgA). The mucosal compartment also has a unique process for generating an immune response and it is populated by an independent lymphocyte subpopulation. The mucosal immune system depends on the cooperation of lymphoid and epithelial cells to initiate and to maintain an immune response. An effective response in the intestinal tract involves: (a) binding, uptake and transport of antigens at the mucosal surface via specialized epithelial cells (M cells) on the domes of lymphoid nodules (e.g., Peyer's patches), (b) antigen presentation to immunologically competent cells (e.g., macrophages, dendritic cells) within the Peyer's patches, (c) isotype switching, differentiation and migration (homing) of antigen-stimulated Peyer's patch IgA B immunoblasts to the intestinal lamina propria, (d) local antibody production by IgA plasma cells in the lamina propria and (e) receptor-mediated transport of antibodies across the intestinal epithelium to the mucosal surface (Figure 1). These secreted antibodies (secretory IgA) neutralize toxins on the mucosal surface, block the adherence of bacteria to the epithelium and reduce the invasive penetration of antigens across the mucosa.

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A number of studies have documented that aging compromises the intestinal mucosal immune response in animals and humans [see 3-7]. This immune deficiency contributes to declines in the efficacies of mucosal vaccines, as well as to the increase in the incidence of infectious diseases in the elderly [1, 2, 3]. We previously demonstrated significant age-related declines in the intestinal IgA antibody response to intraduodenal cholera holotoxin (CTx) in rats and rhesus macaques [8, 9]. However, little is known about the specific mechanisms whereby aging disrupts the intestinal mucosal immune response. Recent studies suggest that the migration of IgA immunoblasts from the Peyer's patches to the intestinal lamina propria is compromised in old animals [10]. This hypothesis is substantiated by the report that the numbers of antibody-containing and IgA-positive lymphocytes in the intestinal lamina propria are significantly lower in old rats in comparison to young adult animals [8]. Subsequent flow cytometry studies revealed that the number of peripheral blood mononuclear cells expressing the lymphocyte homing integrin $\alpha 4\beta 7$ was significantly lower in senescent rats than in young adult animals [11]. Concomitant quantitative immunohistochemistry analyses demonstrated that the density of blood vessel endothelial cells expressing the addressin MAdCAM-1 in the intestinal lamina propria was markedly greater in young versus senescent rats [11].

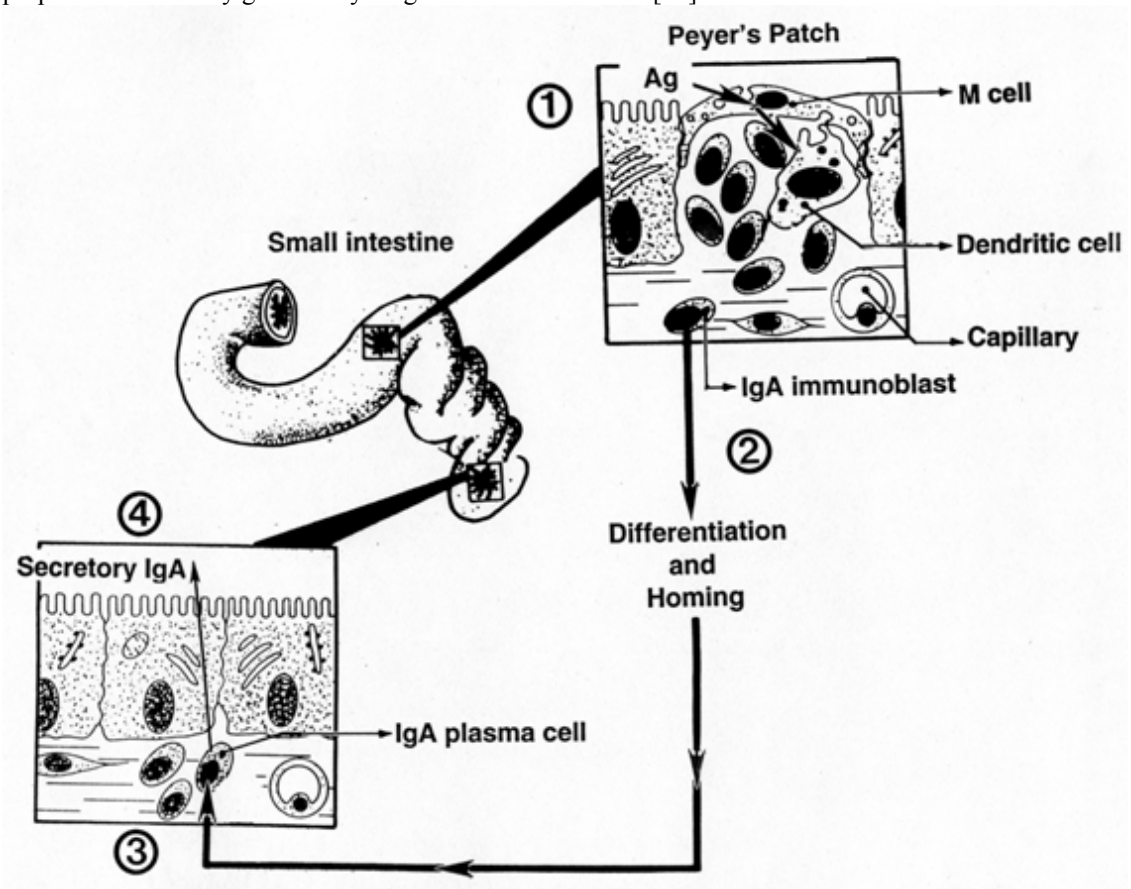

Figure 1. Major events in the genesis of an intestinal mucosal immune response. Step 1: Uptake of antigens at the mucosal surface by M cells on the domes of Peyer's patches, transport to antigen-presenting cells and isotype switching from IgD/IgM B lymphocytes to IgA immunoblasts. Step 2: Homing of IgA immunoblasts and T lymphocytes from the Peyer's patches to the intestinal lamina propria via the mesenteric lymph nodes and the systemic circulation. Step 3: Local IgA antibody production by plasma cells at the effector site. Step 4: Receptor-mediated endocytosis and vesicular translocation of IgA to the mucosal surface and secretion as secretory IgA by enterocytes.

Furthermore, in vitro IgA antibody secretion by intestinal lamina propria lymphocytes isolated from young and senescent rats is equivalent, suggesting that local antibody production by individual IgA plasma cells in the intestine is unaltered during aging [10]. The receptor-mediated transport of IgA antibodies across the intestinal epithelial cells to the mucosal surface remains unchanged with respect to receptor number or binding affinity during aging in rodents [12]). Recent efforts to develop oral and mucosal vaccines, adjuvants and mucosal immunostimulatory agents suggest that mucosal immunity can be enhanced in immunocompromised individuals. Identifying the mechanisms responsible for the age-related decline in the intestinal mucosal immune response will facilitate the development of immunotherapies designed to enhance intestinal immunity.

2. Probiotics and the intestinal immune response

A number of studies have assigned immunomodulatory properties to certain probiotic organisms [see 13-15 for reviews]. Probiotic organisms in fermented milk appear to be beneficial in the treatment of certain diarrheas, as well as in the stimulation of immune function [16-18; see 19 for a review]. The consumption of yogurt, as well as other fermented milks, has been reported to increase several indices of the intestinal immune response, e.g., the numbers of IgA-producing cells and macrophages and specific IgA antibody responses to antigenic challenges in comparison to non lactic acid bacteria-fed animals [20-24]. Milk fermented with *L. casei* and/or *L. acidophilus* or *bifidobacteria* improved the humoral and/or cellular immune responses following antigenic challenge in humans [25-27]. Kefir, a stirred beverage made from milk fermented with a complex mixture of bacteria, including lactobacilli, lactococci, leuconostocs, aceterobacteria and yeasts, has been reported to exhibit antibacterial and anti-tumor effects in animals [28-31].

Lactobacilli appear to stimulate the intestinal mucosal immune response in elderly humans [17, 18, 32]. De Simone et al. demonstrated an increase in the number of peripheral blood B lymphocytes and a concomitant decline in colonic inflammatory infiltration in a group of elderly subjects (>70 years) following ingestion of *Bifidobacterium bifidum* and *L. acidophilus* [17]. Long-term yogurt consumption increases interferon-γ production by adult human T lymphocytes and decreases allergic symptoms in elderly people [22]. Other studies have purported that probiotic organisms enhance the intestinal immune response by (a) stimulating the efficacy of dendritic cells, (b) stimulating natural killer cell activity or (c) modulating the secretion of certain proinflammatory cytokines [18, 33-36].

On the one hand, the perception that eating functional foods has beneficial effects on health maintenance during aging persists despite little information concerning the role of probiotics as adjuvants or the mechanisms whereby these organisms enhance mucosal immunity [see 20 for a review]. On the other hand, several studies have observed only limited or no specific beneficial effects of probiotic organisms on several indices of immune vigor in the intestine [37, 38]. The real or imagined value of probiotic foods as immunomodulators suggests the need for additional studies to assess their potential in this role, their relative efficacies and their mechanisms of action [39].

3. Effect of dietary kefir on the intestinal immune response in young and old rats

3.1 Experimental design

Groups of Fischer 344/NHsd rats were segregated into young adult (6-months) and old (26-months) age groups; four groups of 5 animals each (e.g., young kefir-fed, old kefir-fed, young control-fed and old control-fed). The young and old kefir-fed groups received kefir-fermented milk *ad libitum* in addition to the standard NIH-31 diet for 28 days, whereas the control-fed groups received only the standard NIH diet. The animals were immunized intraduodenally with CTx (100 μg) on days 7 and 21 [10], sacrificed on day 28 and blood, spleen, mesenteric lymph nodes and small intestine were harvested. Lymphocytes

isolated from these tissues were cultured for 5 days. Total IgA and anti-CTx IgA antibody titers were measured in the serum and culture medium by ELISA [10]. The number of anti-CTx IgA-secreting cells was determined by ELISPOT [10].

3.2 Serum total IgA and anti-CTx IgA titers

Dietary supplementation with kefir influenced the specific mucosal immune responses in both young and old rats. Young rats fed kefir exhibited 86% higher serum anti-CTx IgA titers in comparison to the similar aged control-fed group ($P \leq 0.05$; Table 1). On the contrary, the serum anti-CTx IgA concentrations in old kefir-fed rats declined by 40% in comparison to the titers in the age-matched control-fed cohorts ($P \leq 0.01$). The influence of kefir dietary supplementation was evident only in the specific intestinal IgA antibody response since the total (nonspecific) serum IgA titers did not differ between the control and kefir-fed rats in either age group.

3.3 In vitro anti-CTx IgA antibody secretion

Anti-CTx IgA antibody secretion by Peyer's patch, mesenteric lymph node, small intestinal lamina propria and spleen lymphocytes in vitro was measured in culture media after five days. Total (nonspecific) IgA secretion was not affected by diet since there were no differences between cells isolated from control and kefir-fed animals in either age group. Similarly, the kefir-supplemented diet did not influence anti-CTx IgA secretion in the old rats (Table 1). Although anti-CTx IgA antibody secretion by intestinal lamina propria lymphocytes isolated from old kefir-fed animals was 25% greater in comparison to similar cells isolated from old control-fed cohorts, this difference was not statistically significant. On the contrary, in vitro antibody secretion by Peyer's patch and intestinal lamina propria lymphocytes from young kefir-fed rats was significantly greater (180%) than that measured in identical cell populations isolated from similar aged control-fed animals.

Table I. Anti-CTx IgA antibody concentrations in serum and lymphocyte culture media from young and old rats fed kefir-supplemented or control diets

	Young		Old	
Lymphoid Organs	**Control**	**Kefir**	**Control**	**Kefir**
Serum (mg/L)	52±8	97±15*	89±6	54±9
Peyer's patches[1]	64±15	177±42*	392±107	362±127
Mesenteric lymph nodes[1]	165±45	228±50	328±45	249±37
Spleen[1]	21±3	32±2*	32 ±3	27±1
Intestinal lamina propria[1]	703±244	1952±237*	978±219	1223±333

NOTE: [1]Data expressed as ng/106 cells, mean ± SEM, n = 5, * denotes $P \leq 0.05$ vs. Control (ANOVA and Student-Newman-Keuls test).

3.4 Anti-CTx IgA antibody secreting cells

Age-related shifts in IgA secretion appear to correlate with the numbers of antibody-secreting cells in gut-associated lymphoid tissues (GALTs) [10]. On the one hand, the numbers of anti-CTx IgA-secreting cells were higher in the GALTs of kefir-fed young rats in comparison to the control-fed, age-matched cohorts, i.e., 30% in mesenteric lymph nodes, 21% in Peyer's patches and 22% in the intestinal lamina propria (Figure 2A and B). On the other hand, the numbers of anti-CTx IgA-secreting cells were lower in the mesenteric lymph nodes (55%), Peyer's patches (17%) and intestinal lamina propria (23%) of kefir-fed old rats in comparison to the age-matched control-fed group.

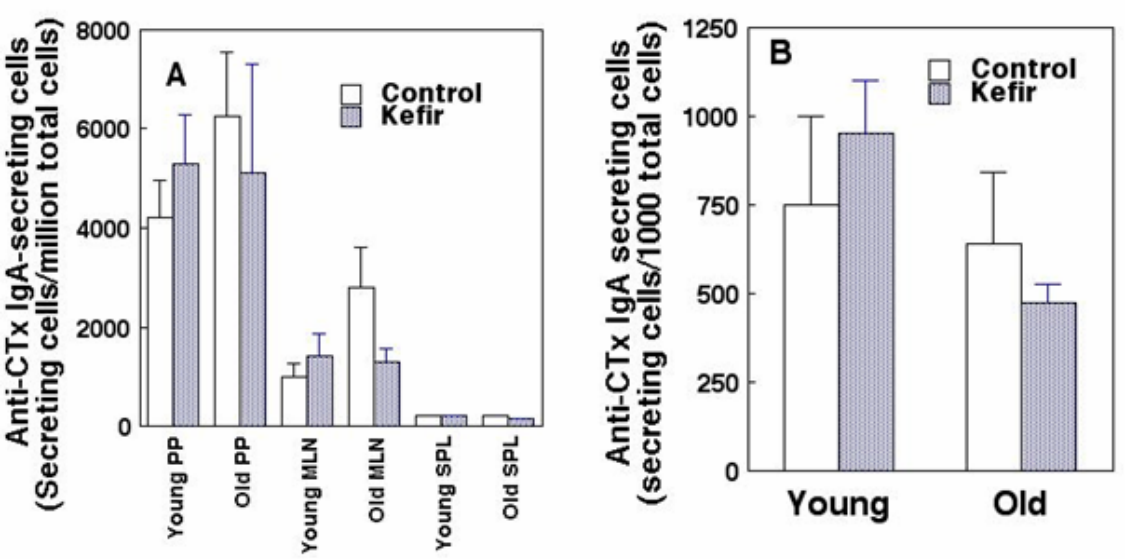

Figure 2. Effects of aging and kefir on the numbers of anti-CTx IgA-secreting lymphocytes in the GALTs and spleen of young and old rats. (A) Anti-CTx IgA-secreting cells per 10^6 total cells in the Peyer's patches (PP), mesenteric lymph nodes (MLN) and spleens (SPL) of kefir and control-fed young and old rats. (B) Anti-CTx IgA-secreting cells per 10^3 total cells in the small intestinal lamina propria of kefir and control-fed young and old rats.

4. Conclusions

The results of our studies using kefir, together with data from others employing different fermented milks, suggest that probiotic foods elicit a common adjuvant effect on the intestinal mucosal immune system. Interestingly, feeding kefir resulted in a 250% increase in anti-CTx IgA secretion by lamina propria lymphocytes isolated from young rats, whereas this increase in similar cells isolated from old kefir-fed animals was only $1/10^{th}$ of that measured in young rats, i.e., 25%. Any effect of kefir on intestinal anti-CTx IgA antibody secretion in old rats is minimal. One explanation is that aging compromises intestinal immunity in old animals to the extent that this particular probiotic regimen perturbs this diminished response only minimally, whereas the response is more sensitive to such modulation in younger animals. However, we examined only two age groups and our study may overlook changes that occur during maturation.

Probiotic organisms must be continuously ingested to manifest their health benefits [38-46]. However, viable probiotic organisms may not be necessary to enhance the intestinal immune response. Differences in the genus, species or strain of probiotic organisms are reflected in their relative stability, enzyme expression, carbohydrate fermentation patterns, acid production, and colonizing ability which, in turn, may influence their immunomodulatory efficacy. The composition of kefir varies greatly depending upon a variety of factors, including the source of the milk, its fat content and the composition of the grains or starters. Kefir grains include lactic acid bacteria, yeasts, acetic acid bacteria and, possibly, other microorganisms. The predominant lactobacilli in kefir grains include *L. Paracasei subspecies paracasei, L. acidophilus, L. delbrueckii subspecies Bulgaricus, L. plantarum* and *L. kefiranofaciens* [28]. These strains account for 90% of the population in the kefir grains, but only for 20 % of the lactobacilli in the final fermented beverage. *L. kefir* constitutes the remaining 80% of the lactic acid bacteria in kefir. The predominant yeasts in both the beverage and the kefir grains include *Saccharomyces cerevisiae, Saccharomyce unisporus, Candida kefir* and *Kluyveromyces marxianus marxianus* [28]. The complexity of the bacterial and yeast populations in the kefir grains, as well as in the final beverage, precludes the identification of a specific probiotic agent responsible for immunomodulation. One possible source of adjuvant activity may be bacterial wall components.

Although the mechanism(s) whereby probiotic components enhance the immune response has not been resolved, possibilities include the activation of toll-like receptors, luminal captation by dendritic cells or stimulation of epithelial cells and the release of proinflammatory cytokines.

Preservation of the normal intestinal flora, resistance to colonization and the production of antibacterial substances are critical events in the generation of a successful probiotic effect in the intestine. Despite a lack of knowledge concerning the mechanisms whereby probiotic organisms elicit their beneficial effects, the consumption of yogurt and fermented milks has increased significantly in recent years and reflects perceived health benefits. Our studies demonstrate that orally administered kefir significantly enhances the specific intestinal mucosal immune response to cholera holotoxin in young adult, but not in old rats. One possibility is that senescent animals expressing diminished mucosal immunity may have experienced a depletion of their putative IgA-secreting plasma cell populations owing to the multitude of prior antigenic presentations during their lifespan. Subsequent investigations should address the mechanisms responsible for the suspected health benefits of probiotic foodstuffs, as well as the credibility and suitability of such dietary supplements for enhancing the intestinal immune response in immunocompromised individuals.

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