

The Influence of Yogurt/Lactobacillus on the Innate and Acquired Immune Response

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Introduction

Yogurt is a fermented milk product made with a starter culture consisting of *Streptococcus salivarius* ssp. *thermophilus* (formerly *S. thermophilus*) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (formerly *L. bulgaricus*). In recent years, it has become a common practice to add other strains of lactic-acid bacteria (LAB) to yogurt and other fermented milk products, particularly *L. johnsonii* La1 (often still referred to as *L. acidophilus* La1) and *Bifidobacterium bifidum*. Several countries require yogurt to maintain a minimum number of viable LAB (ranging from 10^6 to 5×10^8 colony-forming unit (CFU) per mL or g) throughout its shelf life (1). No such requirement exists in the United States, where the LAB in yogurt do not even have to be viable; however, if a yogurt is heat-treated (pasteurized) after fermentation, the label must say "heat-treated after culturing." If the yogurt contains 10^8 viable LAB per g at the time of manufacture, it may carry the "Live Active Culture" seal established by the National Yogurt Association.

Although *S. salivarius* ssp. *thermophilus* is highly stable (decreasing by ~1 log cycle) under normal storage conditions (4°C) for a period of at least 6 wk, *L. delbrueckii* ssp. *bulgaricus* was found to initially increase, but then steadily decrease (by 3–6 log cycles) in the same time period (1). *L. acidophilus* and particularly *B. bifidum* are even less stable when added to yogurt and their numbers have been reported to decline rap-

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Table 1
Nutrient Composition of Yogurt

	Yogurt (USDA data)			Yogurt (1a)	
	Whole milk	Lowfat	Nonfat	Whole milk	Milk (3.25% milkfat)
Water	87.9	85.07	85.23	87.8	88
Energy (kcal)	61	63	56	70	61
Protein	3.47	5.25	5.73	4.7	3.3
Total lipid	3.25	1.55	0.18	3.8	3.34
Cholesterol	13	6	2	10.3	14
Carbohydrate	4.66	7.04	7.68	4.3	4.66
Minerals					
Calcium	121	183	199	133.6	119
Potassium	155	234	255	196.8	152
Sodium	46	70	77	56	49
Vitamins					
Riboflavin	0.142	0.214	0.234	n.a.	0.162
Pantothenic acid	0.389	0.591	0.641	n.a.	0.314
Vitamin B12	0.37	0.56	0.61	n.a.	0.36
Vitamin A (RE)	30	16	2	*	31
Vitamin D	n.a.	n.a.	n.a.	n.a.	40

* 3.1 mg/100 g 13 *cis*-retinol, 34.3 mg/100 g all *trans*-retinol, 11.1 mg/100 g carotenes

idly in some yogurt samples, especially those in which a rapid decrease in pH has occurred (2). *L. acidophilus* counts remained fairly stable for up to 30 d in some other samples.

Composition of Yogurt

In general, yogurt reflects the nutrient composition of the milk it is made from (see Table 1 for the macronutrients and some micronutrients in yogurt and milk). Like milk, yogurt is a good source of protein, riboflavin, and vitamin B12, as well as phosphorus, magnesium, and calcium. Yogurt may or may not be fortified with vitamin D, which enhances the absorption of calcium. A significant increase in plasma-ionized calcium concentration was observed in subjects who consumed 450 g of yogurt per day for 4 mo (3). Sufficient calcium intake and status are important for bone health, but data on the role of dairy foods in the promotion of bone health are still inconclusive (4). Protein is known to have an adverse effect on skeletal mass, as do sodium and vitamin A. However, because milk and yogurt have a high Ca-to-protein ratio and contain relatively low levels of sodium and vitamin A, they are believed to beneficially affect bone health (4).

Compared to milk, yogurt is well tolerated by most lactose-intolerant individuals. This is believed to be a result of the lower lactose content of yogurt, since LAB convert lactose into lactic acid (5).

Yogurt is a good source for several essential nutrients, and has also been reported to be able to modulate a variety of gastrointestinal processes and immune functions. This ability appears to be attributable mainly to the LAB found in yogurt. LAB are gram-positive, facultative anaerobic, fermentative bacteria, which form an important component of the normal intestinal microflora (6,7); some are also used in the production of certain fermented foods, including dairy products.

A variety of LAB used in fermented foods have been shown to be able to at least transiently colonize the human gastrointestinal tract (8–13). Such colonization by exogenous LAB can alter the composition of the microflora in beneficial ways. *B. bifidum* or *L. acidophilus* supplementation resulted in significant elevations in fecal counts of bifidobacteria and lactobacilli, respectively, after 3 wk of supplementation with 7×10^{10} CFU *L. acidophilus* La1 (now: *L. johnsonii* La1) or 1×10^{10} CFU *B. bifidum* strain Bb 12 per d (11). Counts had returned to baseline values by d 12 after the end of fermented milk consumption. Similar increases of fecal lactobacilli were found to be accompanied by a decrease in fecal coliform counts after *L. acidophilus* supplementation (8) as well as counts of gram-negative anaerobic bacteria in recta and Enterobacteriaceae in rectal mucosa after consumption of a variety of LAB strains (9).

Nutrient Absorption

An important function of LAB in the intestinal microflora is to help in the absorption of otherwise indigestible nutrients, particularly carbohydrates (such as resistant starch, cellulose, hemicelluloses, pectins, and gums) through fermentation. In addition, it was recently shown that colonization of adult germ-free mice with *Bacteroides thetaio-taomicron*, a major component of the intestinal microflora in mice and humans, was accompanied by marked changes in the transcription of a broad array of genes (14). A majority of transcripts (95 of 118) increased, including the mRNA of many proteins involved in nutrient absorption and metabolism. Similar changes, although generally of a lesser magnitude, were seen after colonization with *Escherichia coli* (*E. coli*) or *B. infantis*, or complete conventional mouse microflora. This finding suggests that colonization can play an important role in increasing the efficiency of nutrient absorption and metabolism. Interestingly, higher nutrient efficiency has been reported in animals fed a yogurt-based diet as compared to animals fed a milk-based diet, despite the nearly identical nutrient composition of their diets (15,16).

In addition to enhancing nutrient absorption, LAB have also been reported to modulate antigen uptake. In suckling rats given cow milk in addition to maternal milk, the absorption rate of degraded horseradish peroxidase (HRP) was greater in the group receiving cow milk with *L. casei* GG than in the group receiving cow milk, which did not differ from the controls (17). In contrast, addition of *L. casei* GG to hydrolysate decreased the absorption of degraded HRP. The extent to which HRP was degraded by the mucosa was similar in controls, mice supplemented with milk plus *L. casei* GG, and mice receiving hydrolysate with or without *L. casei* GG, but was greater in all of these groups than in milk-supplemented animals.

When casein was first hydrolyzed with pepsin and trypsin, then additionally degraded by enzymes obtained from *L. casei* GG, the resulting molecules were found to consistently suppress ConA- or PHA-induced proliferation in peripheral-blood mononuclear cells (PBMC) from healthy volunteers (18). In contrast, only some of the molecules resulting from casein hydrolysis with pepsin and trypsin inhibited proliferation, and others stimulated it. In addition, casein degraded with *L. casei* GG was reported to decrease the anti-CD3-induced production of IL-4 by PBMC from atopic subjects, but did not affect interferon- γ (IFN- γ) secretion (19). Intact casein stimulated the synthesis of both of these cytokines. In a similar experiment with T-cell-enriched PBMC from healthy subjects, *L. casei* GG-degraded casein suppressed IL-2 mRNA and protein synthesis, as well as IL-4 and IFN- γ production (20). In addition, casein degraded with *L. casei* GG decreased protein kinase C (PKC) translocation, another marker of T-cell activation. Together, these results suggest that *L. casei* GG can digest casein, and possibly other macromolecules, into less antigenic and potentially immunomodulating peptides.

Non-Immunological Barrier Functions

The intestinal mucosa forms an important barrier, which provides protection against antigens from food and ingested pathogens. In neonates, this barrier is not yet fully established, and malnutrition, enteral infections, inflammation associated with certain autoimmune diseases, and hypersensitivity reactions can disrupt normal mucosal function.

As mentioned earlier, upon initial association of gnotobiotic mice with *Bacteroides thetaiotaomicron*, intestinal gene expression was dramatically increased. The most striking increase of >200-fold in intestinal mRNA levels was observed in small proline-rich protein 2a mRNA (14). A smaller yet significant rise in this transcript was also reported after colonization with *E. coli*, *B. infantis* or complete conventional mouse microflora. Small, proline-rich protein 2a is believed to be important in augmenting the barrier function of the epithelium.

An increase in intestinal permeability, measured as absorption of intact horseradish peroxidase (HRP), was observed in suckling rats given cow milk by gavage in addition to maternal milk (17,21). This increase was prevented by the simultaneous administration of *L. casei* GG. Similar results were obtained in suckling rats which received extensively hydrolyzed whey formula with or without *L. casei* GG (17).

Competition with pathogens for binding to enterocytes may be another mechanism by which LAB increase the intestinal barrier. For example, some lactobacilli were shown to compete with *E. coli* for intestinal colonization when introduced together into germ-free rats (22). Among the LAB tested, *L. plantarum* binds to human colonic cells via mannose-specific adhesions, whereas *L. acidophilus* attaches through mannose-independent mechanisms. Although both *L. plantarum* and *L. acidophilus* were found to effectively colonize germ-free rats (22,23), only *L. plantarum* was able to compete with *E. coli* and reduce its population level (22). These results suggest that competition for intestinal colonization at least partially depends on the type of adherence mechanisms.

Intestinal Microflora, LAB, and Immune Functions

In addition to the non-immunological barrier of the intestinal mucosa, the gut-associated lymphoid tissue (GALT) plays a vital role in immune exclusion. GALT is the largest lymphoid tissue, and can contain lymphoid cells either as diffuse populations or organized into follicles, including the tonsils, appendix, and Peyer's patches (PP). In the gut, specialized M cells transport antigens to PP, where stimulation of antigen-specific lymphocytes occurs. A major component of the immune response generated by this stimulation is the production of secretory IgA, which—in contrast to serum IgA—is dimeric or polymeric and resistant to proteolysis in the intestinal lumen and does not activate inflammatory responses.

The initial establishment of the intestinal microflora is essential for the development of a fully functional and balanced immune system, including the development and maturation of IgA plasmacytes and IgA production (24,25). Part of the intestinal IgA production is directed against commensal bacteria themselves (26,27). Much of this IgA is reportedly induced by T-cell-independent pathways which appear to be restricted to the intestinal mucosa, and no specific IgA was detected in serum (27). Interestingly, in gnotobiotic rats colonized with a combination of *E. coli* and *L. plantarum*, a large proportion of the *E. coli*-specific IgG and IgA antibodies crossreacted with *L. plantarum*. Similar crossreactivity was observed between *L. acidophilus* and *E. coli* (28). Some of the increased secretory Ig responses reported after supplementation with various lactobacilli during viral infections (29–31) may at least partially reflect such crossreactivity.

Supplementation with exogenous LAB can also affect IgA production. The addition of 3 mL yogurt containing 2×10^8 cells/mL (*L. delbrueckii* ssp. *bulgaricus* and *S. salivarius* ssp. *thermophilus*) to the diet of Balb/c mice resulted in a significant increase in IgA-secreting cells in the small intestine after 7 d, but such an increase was no longer observed after 10 d of yogurt feeding (32). Similarly, in protein-energy-deficient mice (given a protein-free diet), supplementation with 3 mL of the same yogurt preparation for only two consecutive days was associated with significantly higher numbers of IgA- and IgM-secreting cells in the ileum compared to animals supplemented with undiluted milk (33). A group supplemented with *L. casei* exhibited intermediate responses. Yogurt was also most effective in improving the height of intestinal microvilli, enhancing mucus secretion and preventing the translocation of intestinal bacteria—i.e., in improving the barrier functions of the intestine.

Swiss albino mice fed fresh yogurt for 7 d, then challenged with 20 LD₅₀ of *Salmonella typhimurium*, exhibited significantly higher concentrations of *S. typhimurium*-specific IgA in the intestinal fluid than mice fed with yogurt stored for 20 d (and containing significantly lower numbers of live bacteria) or control mice given nonfat milk (34). Enhanced responses in specific serum IgA after vaccination with attenuated *S. typhi* Ty21a have also been reported in human volunteers who consumed fermented milk containing *L. acidophilus* La1 (now: *L. johnsonii* La1) and bifidobacteria (3×125 g/d, 10^7 – 10^8 CFU per g) before, during, and after the vaccine administration compared to the group that did not consume any fermented milk products (10). In a similar study, volunteers were given the same vaccine and consumed either *Lactobacillus* GG (ATCC 53103), *Lactococcus lactis*, or placebo for 7 d starting on the day of the first vaccine dose (35). There was only a trend toward a greater increase in specific IgA in the *Lactobacillus* GG group, yet no significant differences were observed in the numbers of IgA-, IgG-, and IgM-secreting cells. The differences between this and the previously discussed study could be a result of the different bacteria used, the various matrices in which they were delivered (fermented milk product vs capsules), and the different duration of LAB intake.

It has repeatedly been observed that lactobacillus administration can shorten the duration of diarrhea (36), including rotavirus-induced diarrhea in children (29,30,37). ELISPOT assays of Ig-secreting cells and specific antibody-secreting cells (sASC) among circulating lymphocytes indicated that the nonspecific immune response was significantly greater in the children who received *Lactobacillus* GG than in those given placebo (29). In addition, although there was no difference in the number of IgA sASC during the acute phase, 90% of the lactobacillus-treated children, but only 46% of the placebo-treated children, had

rotavirus-specific IgA sASC 3 wk after recovery. Similarly, a higher rate of rotavirus IgA seroconversion was observed in infants given an oral rotavirus vaccine together with *L. casei* GG than in those who received the vaccine along with a placebo (38). However, it is important to note that the role of IgA in recovery from rotavirus infection, and in its potential to protect from future recurrence, is presently unknown.

In children with Crohn's disease, a disease often characterized by a relative deficiency in mucosal IgA, the administration of *Lactobacillus* GG (10^{10} CFU twice daily) for 10 d was associated with a significant increase in cells secreting IgA specific for β -lactoglobulin and casein (39). Such an increase was not observed in patients with juvenile chronic arthritis or healthy controls. The numbers of specific IgG- and IgM-secreting cells were not affected by *Lactobacillus* GG ingestion, nor was the number of nonspecific immunoglobulin-secreting cells of any isotype.

Tolerance vs Sensitization

GALT plays an important role in the establishment of systemic hyporesponsiveness to ubiquitous antigens such as food and commensal bacteria. The nature, dose, timing, and route of entry of the antigen all play an important role in determining the nature of the T-cell responses, and thus the outcome: sensitization or hyporesponsiveness. Cytokines produced by T cells upon antigenic stimulation are a major factor in directing the immune response.

Allergic diseases are characterized by a skewing of the immune response toward a Th2-type response—there is an overproduction of IL-4 by CD4⁺ helper T cells and a concomitant underproduction of IFN- γ . IL-4 promotes B cells to switch to IgE and IgG1 production and inhibits Th1-type responses, including the production of IFN- γ . Conversely, IFN- γ inhibits the proliferation of Th2 cells and the synthesis of IgG1 and IgE, and instead, induces secretion of IgG2a and IgG3. IL-12 is a potent inducer of IFN- γ production by natural killer (NK) and Th1 cells, but can also inhibit the development of a Th2-type response directly, independently of this IFN- γ induction. The biologically active form of IL-12 is a heterodimer consisting of a p35 and a p40 subunit (40). The expression of p40 corresponds to the ability to produce biologically active IL-12. However, it is produced in amounts that greatly exceed that of the heterodimer. Thus, measurements of the p40 subunit overestimate the synthesis of biologically active IL-12.

Interestingly, the immune responses of virtually all neonates to environmental allergens have been reported to be skewed toward a Th2-type cytokine profile, whether they subsequently become atopic or not (41,42). In recent decades, there has been a dramatic increase in

allergic diseases in developed countries, and the “hygiene hypothesis” has been formulated as a possible explanation for this development. In somewhat oversimplified terms, this hypothesis states that partial deprivation of microbial stimuli resulting from increased hygiene leads to an imbalance between Th1- and Th2-type immune responses, favoring the development of IgE-mediated allergies (43,44). It was originally postulated that infections with measles and other airborne viruses played a major role in providing protection from atopic diseases. It is becoming increasingly recognized, however, that the earliest and largest exposure to microbial antigens occurs during the intestinal colonization that begins at birth. Thus, the intestinal microflora may provide a major stimulus in directing immune responses away from the polarization toward the Th2-type of neonates and infants to the Th1-type response that predominates in non-atopic adults.

The results of several recent studies suggest that the indigenous intestinal microflora of allergic children differs from that of nonallergic children (45–48). In particular, a significantly lower percentage of allergic children were reported to be colonized with lactobacilli (46). In contrast, some aerobic microorganisms, such as coliforms and *Staphylococcus aureus*, were detected more frequently in allergic than nonallergic children. In a prospective study (48), children who had developed atopy by the age of 2 were found to harbor significantly higher counts of clostridia, and tended to have fewer bifidobacteria at the age of 3 wk than children who did not develop atopic diseases. Further indications of an imbalance in the microflora of allergic children comes from the observations that fecal short-chain fatty acids differed between allergic and nonallergic children at the age of 12 mo (47). Higher levels and proportions of i-caproic acid in the feces of allergic children, as compared to nonallergic children, suggested that they harbored elevated numbers of *Clostridium difficile*.

Animal studies comparing germ-free and conventional mice provide further evidence for the importance of the intestinal microflora in the development of oral tolerance. In such experiments, oral feeding of an antigen before immunization with that antigen can induce oral tolerance, and the exact outcome depends on the nature and dose of the antigen, the dosing schedule, and the age and genetic background of the animals (49). IgG unresponsiveness to ovalbumin (OVA) lasted longer in conventional C3H mice than in germ-free animals of the same strain when both groups were fed 20 mg OVA, then immunized 3× intraperitoneally with 10 µg OVA absorbed by alum (50). No significant differences were observed in the duration of the IgE antibody response.

In contrast, Sudo et al. (51) reported that germ-free Balb/c mice exhibited significantly higher levels of total IgE as well as OVA-specific

IgE and IgG1 than conventional mice 5 wk and 7 wk after OVA feeding (5 mg/d for 4 d) followed by intraperitoneal (ip) immunization with 1 μ g OVA in alum every 2 wk. OVA-specific IgG2 levels were comparable in the two groups. Oral tolerance induction in gnotobiotic mice monoassociated with *B. infantis* at the neonatal stage was similar to that seen in conventional mice, although their OVA-specific IgE levels were somewhat higher. In contrast, colonization of mice with *B. infantis* at the age of 5 wk resulted in significantly higher OVA-specific IgE and IgG1, and IgG2a concentrations after OVA-feeding before immunization as compared to germ-free animals.

In the same study (51), OVA-stimulated IL-4 synthesis was significantly higher in splenocytes from OVA-immunized germ-free animals without oral tolerance induction and, unlike in conventional animals, was not downregulated after oral tolerance induction, whereas IFN- γ concentrations were similar in conventional and germ-free animals both with and without oral tolerance induction. IL-2 and TGF- β concentrations were also higher in germ-free than in conventional mice, and they remained unaffected by oral tolerance induction in both groups. The authors concluded that, together with the data on IgG1 and IgG2a, these results suggested that Th1-mediated immune responses were abrogated, but Th2-mediated immune responses were unaltered by oral tolerance induction in germ-free mice.

The immune response to commensal bacteria is transient and is replaced by tolerance soon after the initial colonization (25). It has therefore been proposed that, for the intestinal microflora to play a continued role in the protection from atopic diseases, a high turnover of bacterial genera, species, and strains may be required (44,52). Supplementation with LAB may provide such renewed stimuli for the maintenance of predominant Th1-type responses. Findings from in vitro experiments and studies in which heat-killed LAB were fed suggest that intestinal colonization is not an absolute prerequisite for such stimulation.

In vitro, splenocytes from OVA-primed mice co-cultured with OVA in the presence of heat-killed LcS secreted significantly less total as well as OVA-specific IgE, whereas *L. johnsonii* JCM 0212 had no effect (53). LcS, but not *L. johnsonii*, also dose-dependently increased IFN- γ production, but inhibited IL-4 and IL-5 synthesis. In addition, LcS induced IL-12 synthesis in splenic plastic-adherent cells (macrophages). Anti-IL-12 antibody abrogated the LcS-induced suppression of IgE, IL-4, and IL-5 secretion in splenocytes, whereas IFN- γ antibody was only partially effective, suggesting that IL-12 did not act solely through the induction of IFN- γ .

Balb/c mice were intraperitoneally injected with OVA on d 0 and d 14 and fed a diet containing 0.1% or 0.05% heat-killed LcS for 21 d

Table 2
Effect of Live Lab on Cytokine Production

In vitro live bacteria Bacteria and cell type	TNF- α	IL-1 β	IL-2	IL-4	IL-6	IL-10	IL-12	IFN- γ	Ref.
<i>L. bulgaricus</i> E585 in PBMC	$\uparrow\uparrow$	$\uparrow\uparrow$		no effect	$\uparrow$	$\uparrow$	$\uparrow$	no effect	(88)
<i>L. bulgaricus</i> in PBMC	$\uparrow$	$\uparrow$	no effect					$\uparrow$	(89)
<i>L. acidophilus</i> in PBMC	$\uparrow$	$\uparrow$	no effect					$\uparrow$	(89)
<i>L. acidophilus</i> in PBMC		$\uparrow$			$\uparrow$			$\uparrow$	(58)
<i>L. acidophilus</i> E 507 in PBMC	$\uparrow$				$\uparrow$	$\uparrow$ in responders only			(86)
<i>L. acidophilus</i> in epithelial cell/leukocyte co-cultures						no effect	$\uparrow$	$\uparrow$	(90)
<i>L. casei</i> in PBMC		$\uparrow$			$\uparrow$			no effect	(58)
<i>L. rhamnosus</i>	$\uparrow$	$\uparrow$		no effect	$\uparrow$	$\uparrow$	$\uparrow$	$\uparrow$	(88)
<i>L. sakei</i>						no effect	$\uparrow$	$\uparrow$	(90)
<i>Bifidobacterium</i> sp.	$\uparrow$	$\uparrow$	no effect					$\uparrow$	(89)
<i>Bifidobacterium</i>			$\uparrow$		$\uparrow$			no effect	(58)
<i>S. thermophilus</i> in PBMC		$\uparrow$			$\uparrow$			$\uparrow$	(58)
<i>S. thermophilus</i> in PBMC	$\uparrow$	$\uparrow$	no effect		$\uparrow$			$\uparrow$	(89)

starting on d 0 (54). LcS-treated animals produced significantly lower levels of OVA-specific IgE and tended to have lower total serum IgE than controls that did not receive LcS (54). This was associated with an interesting switch from a Th1-type to a Th2-type cytokine pattern in splenocytes isolated from these mice and restimulated with OVA—namely, significant elevations in IFN- γ , IL-2, and IL-12 production and marked reductions in IL-4, IL-5, IL-6, and IL-10 synthesis.

DBA/2 mice fed a casein diet develop elevated casein-specific IgE in association with a Th2-like cytokine pattern. In such mice, heat-killed ip injection of *L. plantarum* L-137 inhibited casein-specific IgE production, although IgG1 levels actually increased, compared to saline-injected controls (55). There was a concomitant increase in IL-12p40 synthesis by unstimulated peritoneal macrophages from *L. plantarum*-injected mice and an even greater increase in macrophages re-stimulated with *L. plantarum*. Secretion of IL-4 by ConA-stimulated splenocytes was diminished in *L. plantarum*-injected mice, yet IFN- γ production was not affected.

LAB and Cytokines

As summarized in Tables 2 and 3, most LAB studied to date have little effect on IL-4 production in vitro, except for one study reporting a downregulation of IL-4 synthesis in splenocytes from OVA-primed mice restimulated with OVA in vitro (53). However, many LAB were found to induce the synthesis of IFN- γ in vitro, including *S. thermophilus*, *L. acidophilus*, *L. bulgaricus*, and bifidobacteria, although inconsistent results have been obtained with *L. bulgaricus* and bifidobacteria (see Tables 2 and 3). Studies on the effect of feeding individual LAB on ex vivo cytokine production are summarized in Table 4.

In two separate feeding trials, the effects of three and then two different unheated or heat-treated yogurts fed to B6C3F1 mice on cytokine expression in spleen, mesenteric lymph nodes (MLN), or PP were determined (56). All yogurts were fermented with *L. bulgaricus* and *S. thermophilus*; in addition, some contained *L. acidophilus* and/or *Bifidobacterium* sp. The effects depended on the type of yogurt used, but the presence of *L. acidophilus* and/or *Bifidobacterium* sp. was found not to play a role. In addition, the duration of the feeding, but also the tissue and cytokine examined, influenced the outcome. In the first feeding trial, yogurt feeding either had no significant effect or downregulated cytokine expression compared to mice fed nonfat dry milk. In particular, IL-4 mRNA was decreased in PP after 2 wk, but not after 4 wk, regardless of whether the yogurts contained live or heat-killed bacteria. IFN- γ mRNA was not affected in PP by any yogurt fed for either 2 wk or 4 wk, but was downregulated by one live and three heat-treated

Table 3
Effect of Heat-Killed Lab on Cytokine Production

Bacteria	TNF- α	IL-1 β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12	IFN- γ	Ref.
<i>L. acidophilus</i> La1 (<i>L. johnsonii</i> La1) in RAW 264.7 cells	↑					↑				(85)
<i>L. bulgaricus</i> 1489 NCK 231 in RAW 264.7 cells	↑					↑				(85)
<i>L. bulgaricus</i> (2 strains) in RAW264.7	↑					↑				(85)
in PMA-stimulated EL4.IL-2 cells			↑							
<i>L. johnsonii</i> JCM 0212 in mouse splenocytes from OVA-primed mice				no effect	no effect			no effect	no effect	(53)
<i>L. casei</i> in X-rayed mouse splenocytes (i.e., monocytes)						alone, but		↑ ConA- induced IFN- γ	no effect	(83)
<i>L. casei</i> in mouse splenocytes from OVA-primed mice restimulated with OVA				↓	↓			↑	↑↑	(53)
<i>L. casei</i> B ATCC 11578 cell wall in rat peritoneal macrophages and in splenocytes	↑	↑↑		no effect		↑↑				(91)
<i>B. bifidum</i> in RAW 264.7 cells	↑	↑				↑				(87)
in PMA-stimulated EL4.IL-2 cells			↑			↑				
<i>B. bifidum</i> in RAW 264.7 cells	↑					↑				(85)
<i>S. thermophilus</i> St-133 in RAW 264.7 cells	↑					↑				(85)
<i>S. thermophilus</i> (4 different strains) in RAW264.7 cells in PMA-stimulated EL4.IL-2 cells	↑		↑			↑				(87)

yogurts in MLNs after 4, but not after 2, wk. TNF- α expression was decreased after 4 (but not 2) wk in spleen by all three live and heat-treated yogurts; all three heat-treated yogurts also reduced its expression in MLN after both 2 wk and 4 wk, but only one live yogurt did so after 4 wk. In the second feeding trial, involving two other yogurts, the results were much more variable. Most notably, several yogurts increased IFN- γ mRNA levels in MLN, and the increase was significant in the case of one heat-treated yogurt. The same yogurt, whether heat-treated or not, also increased IL-6 mRNA in MLN.

Several studies indicate that yogurt consumption may be associated with an increase in IFN- γ synthesis in humans. Halpern et al. (3) reported that there was an increase in IFN- γ production by T cells from subjects who consumed 450 g of yogurt per day for 4 mo. The methods used to generate these data, and the numbers and types of bacteria contained in the yogurt consumed by the subjects are unknown. In a study by the same group of researchers examining the effects of year-long daily consumption of 200 g of yogurt, IFN- γ levels were reportedly too variable to yield relevant data, yet the abstract states that there was a significant decrease in IFN- γ production in the older group of subjects (57). Further studies involving dose kinetics need to be performed.

In subjects who ate yogurt of their own choice (i.e., with unknown numbers and types of live or inactive bacteria) for 2 wk, and significant increase in 2-5A synthetase activity was observed (58). This enzyme is reportedly a specific marker for the production of interferons. Significant upregulation of 2-5 A synthetase activity was also reported in volunteers who consumed 100 g of yogurt containing 10^9 *L. bulgaricus*/g and 10^9 *S. thermophilus*/g compared to volunteers consuming milk (59). Enzyme activity was further increased after volunteers ate 250 g yogurt per d for 15 d.

LAB Supplementation in Asthma and Allergy

In a randomized study using a crossover design, atopic subjects consumed 16 oz of yogurt or milk per day for 1 mo (60). No significant changes in cellular or humoral immune functions were noted in either phase. Similarly, consumption of 225 g yogurt with or without *L. acidophilus* twice daily for 1 mo was not associated with improvement in pulmonary function compared to baseline (60). There was also no difference between the two treatments in total IgE, IL-2, and IL-4 production in ConA-stimulated PBMC. There was a trend toward higher IFN- γ secretion by ConA-stimulated PBMC obtained after consumption of yogurt containing *L. acidophilus* than after ingestion of yogurt without *L. acidophilus*.

Table 4
Effects of Lab Consumption on Ex Vivo Cytokine Production

Bacteria	Treatment	Effect on cytokine production	Ref.
<i>Bifidobacterium</i> (Bf)	B6C3F1 mice received a single dose of 10 ⁹ cells	Bf decreased PMA-induced IL-6 & IFN- γ	(92)
<i>L. acidophilus</i> (La)	peritoneal cells were cultured in the absence or presence of LPS or PMA	La induced IL-6 and IL-12p40, enhanced PMA-induced IFN- γ	
<i>L. bulgaricus</i>		no effect on IL-6 or IFN- γ	
<i>L. casei</i>		induced IL-6 and IL-12p40	
<i>S. thermophilus</i>		no effect on IL-6, IFN- γ , or IL-12p40	
<i>L. acidophilus</i>	Balb/c mice were gavaged with 10 ⁹ CFU for 10 or 28 d, their splenocytes were cultured	$\uparrow \uparrow$ IFN- γ compared to milk $\pm$ IL-4	(75)
<i>L. rhamnosus</i> with ConA	Balb/c mice received 1.3×10^9 LcS orally for 7 d their splenocytes were cultured with ConA	$\uparrow \uparrow$ IFN- $\gamma \pm$ IL-4 and IL-5*	(83)
<i>L. casei</i> strain Shirota	CIA was induced in DBA/1 mice, some received 2.5 or 5×10^8 LcS orally for 5 d, splenocytes were restimulated with denatured collagen	$\downarrow$ IFN- γ compared to water-treated* controls	(82)
<i>L. casei</i> strain Shirota	Balb/c mice were i.p. injected with OVA and some were fed a diet containing 0.05% (wt/wt) heat-killed LcS for 21 d, splenocytes were restimulated with OVA	$\downarrow$ IL-4, IL-5, IL-6, IL-10	(54)
<i>L. casei</i> ATCC 393	Female Balb/c were immunized with Chikungunya virus and fed <i>L. casei</i> for 4 alternate d. On d 7, immunohistochemically cytokine-positive cells in the gut villi were counted	$\uparrow$ IL-2, IL-12, IFN- γ	
		$\uparrow$ TNF- α ($p < 0.05$ compared to NaHCO_3 -treated controls); IL-1 α , IL-1 β , IL-2, IL-10, and IFN- γ not significantly different	(31)

*Note that *L. casei* strain Shirota (LcS) appears to have opposite effects in healthy mice and mice in the early stages of collagen-induced arthritis (CIA)

Recently, the effect of supplementation with *Lactobacillus* GG on the development of allergies was examined in a randomized placebo-controlled trial involving 159 infants at high risk of developing atopic disease and their mothers (61). Pregnant women began taking the supplement (10^{10} CFU of *Lactobacillus* GG or placebo daily) 2–4 wk before delivery and, for the next 6 mo, continued taking it if breast-feeding; otherwise the infants themselves received *Lactobacillus* GG. There was a highly significant reduction in the frequency of atopic eczema in the *Lactobacillus* GG group (15/64 or 23%) compared to the placebo group (31/68 or 46%). This effect was independent of whether the mother or the infant consumed the *Lactobacillus* GG supplement. The incidences of positive skin-prick reactions or increased specific IgE to common dietary or respiratory antigens, and the levels of serum total IgE did not differ between the two groups.

In a much smaller study, 27 infants who experienced onset of atopic disease during exclusive breastfeeding were weaned to extensively hydrolyzed whey formulas with or without *Lactobacillus* GG or *B. lactis* Bb-12 (62). After 2 mo, significantly greater improvements in the SCORAD score were observed in both probiotic-supplemented groups compared to the unsupplemented group. Serum TGF- β concentrations significantly decreased from baseline to 2 mo in the *B. lactis* group, tended to increase in the *Lactobacillus* GG group, and remained unchanged in the non-supplemented controls. The serum concentrations of other cytokines and chemokines (TNF- α , GM-CSF, RANTES, and MCP-1a) as well as sICAM and IL-1ra (receptor antagonist) were not affected by supplementation. This confirmed the findings of a similar earlier study by the same researchers, in which month-long supplementation with *Lactobacillus* GG was associated with significant improvements in atopic dermatitis (63).

Other Effects of LAB on Immune Functions

Highly variable results have been reported from in vitro and ex vivo experiments which assessed the effects of various LAB on lymphocyte proliferation. Whether LAB enhance or inhibit proliferation appears to depend not only on the specific bacterial strain investigated and the type and concentration of the mitogen used to induce proliferation, but also on the activation state of the immune system of the experimental animals or humans.

In vitro, the incubation of human PBMC with live *L. johnsonii* or *L. sakei* for 5 d resulted in a strong proliferative response (64). The effect obtained with *L. johnsonii* was significantly greater than that of *L. sakei*, and its magnitude was similar to that observed with PHA (10 μ g/mL). Heat-killed bacteria produced similar results. Homogenates of *L.*

rhamnosus GG, *Bifidobacterium lactis*, *L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus*, and *S. thermophilus* all suppressed the proliferation of human PBMC induced with a very high concentration (125 µg/mL) of PHA (66). This effect was significantly reduced, but not completely eliminated, by heat inactivation. Cytoplasmic extracts, whether unheated or heated, significantly inhibited PHA-induced proliferation, whereas cell-wall extracts had no effect.

In mice fed yogurt for 10 mo, splenocyte proliferation in response to ConA and PHA was significantly increased compared to milk-fed controls (15). In contrast, after a shorter feeding period (4 wk), the ConA-, PHA, and LPS- induced proliferation of splenic and intestinal lymphocytes from DBA/2J mice fed yogurt or dried milk powder as 50% of their energy for 4 wk did not differ significantly (16). However, when such mice were challenged with an LD₅₀ dose of *Salmonella typhimurium*, proliferation of splenocytes after ConA stimulation was significantly higher in the yogurt than in the milk group, yet the response to PHA or LPS was similar in the two groups. The mitogenic response of intestinal lymphocytes in response to ConA and LPS was also significantly higher in yogurt-fed than in milk-fed mice. The proliferative response to PHA, although greater in yogurt-fed animals, was not significantly different from that in milk-fed animals. Similarly, the proliferative response to ConA and LPS was significantly increased in splenocytes from *S. typhimurium*-challenged Balb/c mice fed a diet supplemented with 30 yogurt (8 × 10⁸ LAB/g) for 12 d compared to powdered-milk-supplemented controls (66). A slight and statistically insignificant increase in proliferation was also observed in mice fed heat-treated yogurt. In another study by the same group, supplementing the diet of unchallenged Balb/c mice with 20% heat-treated yogurt resulted in similar proliferative responses of PP to PHA, as did yogurt containing live LAB (*S. thermophilus* and *L. bulgaricus*) after 14 d or 21 d (67). Only yogurt containing live LAB increased the LPS-induced proliferation of PP after 7, 14, and 21 d. These enhancing effects of yogurt consumption on the mitogenic response were transient, and were no longer observed after 28 d of supplementation with yogurt containing live or heat-killed LAB.

Basal and LPS-stimulated proliferation was increased in splenic lymphocytes from mice fed 10⁹ viable *L. acidophilus* per kg body wt for 7 d compared to saline-treated controls (68). In contrast, the same number of bacteria from the strains *L. casei*, *L. gasseri*, and *L. rhamnosus* inhibited basal proliferation and proliferation stimulated with supraoptimal concentrations of LPS or ConA. In a similar study by the same authors, however, oral supplementation with *L. rhamnosus* for 7 d did not affect basal proliferation, yet administration for 14 d enhanced it (69). Unlike

the results of previous studies, there also was an increase in proliferation stimulated with the optimal concentrations of ConA or LPS after 7 d.

Intraperitoneal administration of *L. casei* to MRL/lpr mice (a model for systemic lupus erythematosus) was also reported to significantly inhibit the ConA-, LPS-, or pokeweed mitogen-induced proliferation of splenocytes compared to saline-injected controls (70). In mesenteric lymph-node cells, however, LC injection did not significantly affect the proliferative response to these mitogens.

Nonspecific Immune Responses: Phagocytic Activity of Macrophages and Granulocytes and NK Activity

Peritoneal macrophages from mice supplemented with milk fermented with *L. acidophilus*, *L. casei*, or both (both isolated from human feces) for 8 d exhibited significantly increased phagocytic activity (71). Colloidal carbon clearance as a measure of in vivo phagocytic activity was similarly enhanced. In a similar study by the same group of researchers (72), oral administration of *L. casei* enhanced in vitro and in vivo phagocytosis, whereas *L. bulgaricus* had little effect on in vitro phagocytosis but nonetheless significantly increased the in vivo colloidal carbon clearance rate. Macrophage activation has also been observed in tumor-bearing mice fed yogurt mixed into their diets (73,74)

Supplementation of mice with 10^9 CFU of *L. acidophilus* (HN017), *L. rhamnosus* (HN001), or *B. lactis* for 10 d or 28 d was associated with a significant increase in the phagocytic activity of both peripheral-blood leukocytes and peritoneal macrophages from mice immunized with cholera toxin or tetanus vaccine compared with controls given skim milk without LAB (75).

Human volunteers consumed milk for 3 wk, then fermented milk providing either 7×10^{10} CFU *L. acidophilus* La1 or 1×10^{10} CFU *B. bifidum* strain Bb 12 for another 3 wk (11). Compared to the measurement obtained after milk consumption, there was a highly significant increase in leukocyte phagocytic activity in both groups, with granulocytes showing a greater increase than monocytes (measured as uptake of opsonized *E. coli*). A significant elevation in phagocytic activity could still be detected 6 wk after cessation of fermented milk intake, although fecal counts of bifidobacteria and lactobacilli, respectively, had returned to baseline levels by d 12 after the end of bacterial supplementation. Another study by the same group, using a similar protocol, compared the effects of supplementation with 150 mL/d of a milk fermented with *S. thermophilus* to those seen after consumption of the same product supplemented with *L. johnsonii* La1—either fresh or stored for 21–28 d to yield a 10-fold lower bacterial count (76). Despite the somewhat lower daily dose compared to the previous study (1.5×10^9 CFU *L.*

johnsonii per d), a significant increase in leukocyte phagocytic activity and respiratory burst was observed in the group receiving fresh fermented milk with *L. johnsonii* La1. Similar trends were seen in the group consuming *stored* fermented milk with *L. johnsonii* La1, but this did not reach significant proportions for either parameter. The authors concluded that the minimal effective dose for modulating granulocyte/monocyte activities was 10^9 CFU. It should be noted, however, that storage of fermented milk products results in changes other than the decrease in viable bacteria caused by the accumulation of metabolites generated by these bacteria. Thus, whether the lack of a significant effect in the group receiving stored fermented milk with *L. johnsonii* La1 was caused by the lower cell count or by other factors (or a combination of the two) is not clear. In order to establish a true dose response, it will be necessary to test products in which differences in the number of viable bacteria are obtained by the appropriate changes in the manufacture, rather than through prolonged storage.

In healthy subjects, the consumption of milk supplemented with 2.6×10^8 CFU *Lactobacillus* GG resulted in a significant upregulation of phagocytosis receptors (CR1, CR3, Fc γ RI, and Ig α R) expression on neutrophils, whereas the increases on monocytes were not significant, compared to the period prior to milk consumption without *Lactobacillus* GG (77). Milk alone did not affect receptor expression. In contrast, in milk-hypersensitive subjects, there was considerably higher expression of all of these receptors on monocytes and neutrophils during milk consumption. Yet when *Lactobacillus* GG was added to the milk, receptor expression did not significantly differ from that seen before milk challenge, suggesting that *Lactobacillus* GG downregulated the milk-induced phagocyte activation.

TNF- α and IL-1 β are cytokines predominantly produced by activated macrophages. As summarized in Tables 2 and 3, numerous *in vitro* studies have reported an upregulation of the synthesis of these cytokines when a variety of cell types were incubated with live or heat-killed LAB, including those used for the production of yogurt or commonly added to it. This further suggests that numerous LAB have a stimulatory effect on macrophage activities.

Interestingly, growth phase, heat treatment, and interactions between the two were found to influence the ability of three different lactobacilli to induce TNF- α in human peripheral-blood monocytes (78). Live bacteria from the logarithmic growth phase stimulated considerably higher amounts of TNF- α secretion than heat-killed cells. In contrast, heat-killed bacteria harvested at the stationary phase induced fourfold to fivefold higher levels of TNF- α than live cells.

Natural killer (NK) cells are believed to play an important role in inhibiting carcinogenesis. IL-12 is a strong inducer of NK cells, and—as previously discussed and summarized in Tables 2 and 3—a variety of LAB, including LcS (53,54) have been shown to stimulate IL-12 production. Enhancement of NK activity and significant reduction in tumor incidence have been reported after LcS supplementation in 3-methylcholanthrene-induced carcinogenesis in C3H/HeN mice (79,80). Such inhibition was not observed in NK-cell-deficient beige mice (80), suggesting that a major mechanism by which LcS inhibits carcinogenesis in this model is through enhancement of NK cytotoxicity.

In mice, oral administration of *L. rhamnosus* resulted in a significant increase in NK cell activity, yet the increase after *L. acidophilus* or *B. lactis* supplementation was not significant (75). Daily intake for 3 wk of *B. lactis* in lowfat milk by human volunteers, however, was associated with significantly higher NK cytotoxicity compared to consumption of lowfat milk alone (81).

Immunostimulating vs Immunosuppressive Effects

TNF- α and IL-1 β are pro-inflammatory cytokines, and stimulation of their production suggests that LAB may have pro-inflammatory activities. Such activities would be undesirable in situations already characterized by inflammation, such as allergy, asthma, and several autoimmune diseases. LAB therapy has been examined in several of these conditions, and to date no exacerbation of inflammation has been observed. Instead, in children with atopic eczema and cow's-milk allergy, consumption of extensively hydrolyzed whey formula supplemented with *Lactobacillus* GG, but not the same formula alone, was associated with a significant decrease in fecal TNF- α , although TNF- α release by ConA-stimulated PBMC was similar before and after treatment (64). This may be a strain-dependent effect, and it may also reflect the potential of LAB to exert differential effects in health and disease.

Indeed, it has repeatedly been reported that various LAB differentially affect the immune responses of healthy animals or volunteers and those whose immune system is activated because of pathogen challenge or autoimmune disease. For example, *Lactobacillus* GG downregulated phagocyte receptor expression induced by milk challenge in milk-hypersensitive subjects, but stimulated receptor expression in healthy subjects (77). Administration of LcS was associated with increased ConA-stimulated IFN- γ production by splenocytes from healthy Balb/c mice, but had the opposite effect in DBA/1 mice in the early stages of collagen-induced arthritis (82,83) (see Table 4). Mitogen-induced prolifer-

eration of splenic lymphocytes from DBA/2J was not enhanced after yogurt feeding (16). However, when such mice were challenged with an LD₅₀ dose of *Salmonella typhimurium*, the proliferative response to ConA was significantly higher in the yogurt than in the milk group. Nonetheless, because of the species and strain dependence of the immunomodulation by LAB, thorough assessment of LAB in vitro and in animal models will be crucial before they can be used to modulate diseases characterized by immune deviation.

Live vs Inactivated Bacteria

In some of the studies discussed here, heat-killed bacteria had either no or substantially reduced effects compared to live bacteria (32,57,67). Nonetheless, there are also studies reporting substantial activity with heat-killed bacteria (53–55,70,84). Some of these activities are equal to or even higher than those observed with live bacteria (56,73), and even the supernatant of fermented milks was found to be effective in stimulating specific and nonspecific immune responses (71). In addition, cell walls or cytoplasmic extracts of *L. bulgaricus*, *L. casei*, and *L. helveticus* were reported to stimulate TNF- α production in RAW 264.7 cells more effectively than cytoplasmic extracts, although somewhat less effectively than whole heat-killed cells (85). *L. acidophilus* and *L. bulgaricus* cytoplasm induced IL-6 production better than whole cells, as did cell walls of *L. bulgaricus*, but not *L. acidophilus*. For other LAB (*Bifidobacterium* Bf-1, *S. thermophilus* St-133), whole cells were more effective than fractions. Thus, while live bacteria are certainly essential for functions that require adherence to enterocytes and (transient) colonization, it seems that a variety of immunomodulating activities are attributable to cell-wall or cytoplasmic components, some of which may be more accessible, and thus more effective, after heat treatment.

Species and Strain Dependence

Many of the studies discussed in this article demonstrate that the effects of LAB on immune functions, such as cytokine production (31,62,86), humoral immune responses (53), proliferation (68), and phagocytosis (72,75), are greatly dependent on the bacterial species. In addition, differences between strains of the same bacterial species have been reported. Four example, four strains of *S. thermophilus* differed in their ability to induce IL-6 and TNF- α production in RAW 264.7, a macrophage cell line (87). Similar findings were reported by another group of researchers who used PBMC (86).

Conclusions

An aura has surrounded the influence of yogurt and immunity for more than a century. In fact, although it has been studied in both humans and mice, there is a relative paucity of quality research examining the issue of yogurt and immunity. Despite such reservations, certain strains of *Lactobacillus* have potential health benefits. Future goals should be to identify the best strains, the mechanisms of their action, and their potential impact on human health.

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