Effects of Ingestion of Yogurts Containing *Bifidobacterium* and *Lactobacillus acidophilus* on Spleen and Peyer’s Patch Lymphocyte Populations in the Mouse

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ABSTRACT

Certain probiotic lactic acid bacteria have been reported to improve immune system function. Here, the effects of ingesting yogurts on lymphocyte populations in the spleens and Peyer’s patches were determined in mice. Three probiotic-supplemented yogurts containing *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Bifidobacterium*, and *Lactobacillus acidophilus* and one conventional yogurt containing only *S. thermophilus* and *L. bulgaricus* were prepared from commercial starter cultures and used in the study. B6C3F1 female mice were fed the four different types of yogurts mixed with an AIN-93G diet in a 50:50 (wt/wt) ratio. Nonfat dry milk mixed at a 50:50 (wt/wt) ratio with AIN-93G diet was used as the control. After a 14-day feeding period, spleen and Peyer’s patches were removed and lymphocytes subjected to phenotype analysis by flow cytometry. Ingestion of the four yogurts had no effect on percentages of CD8⁺ (cytotoxic T cells), B220⁺ (B cells), IgA⁺, or IgM⁺ cells in spleen or Peyer’s patches. The percentage of CD4⁺ (T helper) cells was significantly increased in the spleens from one group of mice fed a yogurt containing *B. bifidum* and *L. acidophilus*, and a similar trend was found in the remaining two probiotic-supplemented yogurts. Effects on CD4⁺ populations were not observed in spleens of mice fed conventional yogurt or in the Peyer’s patches of any of the four yogurt groups. In total, the results suggested that ingestion of conventional or probiotic-supplemented yogurts for 2 weeks had very little effect on lymphocyte distribution in the systemic or mucosal immune compartments.

**MATERIALS AND METHODS**

**Experimental design.** The basic experimental design involved feeding mice diets containing yogurt and comparing their effects on lymphocyte populations to those fed a skim milk control diet. Briefly, B6C3F1 (C57BL/6 × C3H/HeN) female mice (8 weeks old) were obtained from Charles River Labs (Raleigh, N.C.). Mice were acclimated to AIN-93G diet (10) for 1 week and then were randomly allocated at two to three mice per cage to experimental groups. Mice (8 to 10 per group) were housed in a windowless room maintained at 25 to 27°C with a photoperiod of 12 h light and 12 h darkness. Yogurts were freshly mixed with
AIN-93G diet (15) 50:50 (wt/wt) and provided daily. Uninoculated, pasteurized nonfat skim milk mixed with AIN-93G diet (50:50, wt/wt) (C) was included as a control. Mice were allowed free access to water and food. After 2 weeks, mice were sacrificed by cervical dislocation under ether anesthesia. Spleen and Peyer’s patches were removed for flow cytometric analysis of lymphocyte populations.

Yogurt production. Bifidobacterium-containing yogurts were prepared for feeding studies using commercial starter cultures. Ultra-Gro Direct yogurt (U) culture containing Streptococcus salivarius ssp. thermophilus and Lactobacillus delbrueckii ssp. bulgaricus and S bifidus Direct yogurt (B) culture containing S. salivarius ssp. thermophilus, L. delbrueckii ssp. bulgaricus, L. acidophilus, and Bifidobacterium sp. were obtained from Systems Bio-Industries (Waukesha, Wis.). PY-3 Redi-Set yogurt (P) culture that contained S. salivarius ssp. thermophilus, L. delbrueckii ssp. bulgaricus, L. acidophilus and Bifidobacterium bifidum was obtained from Chr Hansen Inc. (Milwaukee, Wis.). DPL yogurt Quick Start ABY-2C (A) containing S. thermophilus, L. bulgaricus, L. acidophilus, and Bifidobacterium infantis was obtained from Rhone-Poulenc (Madison, Wis.). Yogurts were prepared from pasteurized 12% (wt/vol) reconstituted nonfat dry milk (Michigan Milk Producers Association, Ovid, Mich.) according to the culture manufacturer’s instructions. Following agitation for 10 to 30 min, the yogurt solution was aliquoted into sterile 50-ml conical tissue culture tubes and incubated at 37°C. Yogurts were stored at 4°C until use. One batch of yogurt was prepared from each yogurt culture for the entire 14-day feeding experiment.

Enumeration of yogurt bacteria. Total aerobic bacterial, streptococcal, and bifidobacterial counts were performed at 0, 1, and 2 weeks of storage to determine the viability of the cultures. De Mann-Rogosa-Sharpe medium for lactobacilli (4) + 5% (wt/vol) lactose agar plates, modified S. thermophilus agar (Lee’s agar) (4), and neomycin sulfate, paromomycin sulfate, nalidixic acid, lithium chloride agar (16) were used for total aerobic, Streptococcus, and Bifidobacterium counts, respectively. After the appropriate dilutions were made, duplicate plates were plated using these media and incubated for 48 h at 37°C, aerobically for total aerobic bacteria and Streptococcus, and anaerobically using anaerobe jars and an anaerobic Gas Pak (Becton Dickinson Co., Cockeysville, Md.) system for Bifidobacterium. The colonies were counted using a Quebec colony counter (Fisher Scientific, Pittsburgh, Pa.).

Identification of lymphocyte subsets by flow cytometry. Single cell suspensions for analysis by flow cytometry were prepared from the spleen and Peyer’s patches (15). Spleen was teased apart by a syringe plunger on a petri dish and passed through 200-mesh nylon screen. The cells were suspended in RPMI-1640 medium (Sigma Chemical Co., St. Louis, Mo.) containing 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco Laboratories, Chagrin Falls, Ill.), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 1 mM minimum medium nonessential amino acid solution, 100 U/ml penicillin, and 100 μg/ml streptomycin. After centrifuging at 450 × g for 5 min, cells were resuspended in 17 mM Tris buffer (pH 7.65) containing 0.77% (wt/vol) ammonium chloride for 3 min to lyse the erythrocytes. The cells were centrifuged again and resuspended in the supplemented RPMI-1640 medium. Peyer’s patches cells were similarly prepared, except two glass slides were used to tease cells apart. Cell viability was verified by staining with trypan blue solution (Sigma Co., St. Louis, Mo.).

Lymphocyte subsets were analyzed using a Becton Dickinson FACScan Flow Cytometry System (Becton Dickinson, San Jose, Calif.). All the conjugated antibodies were obtained from Pharmingen (San Diego, Calif.). Cells were stained with phycoerythrin-conjugated anti-CD4, anti-B220, IgA, and IgM (rat IgG2a, 0.2 μg/10⁶ cells) or fluorescein isothiocyanate-conjugated anti-CD8 (rat IgG2a, 0.2 μg/10⁶ splenic cells; 0.5 μg/10⁶ Peyer’s patches cells), respectively. Cell suspensions in phosphate-buffered saline (0.01 M, pH 7.4) containing 1% (wt/vol) bovine serum albumin (fraction V, Sigma) and 0.02% sodium azide (NaN₃) were mixed with staining reagents and incubated at 4°C for 30 min. After washing twice, cells were resuspended in phosphate-buffered saline containing 0.02% NaN₃. Isotype-matched negative controls were included in the analysis of the cell suspensions.

Statistical analysis. Data from yogurt-treated mice were compared to that for skim milk control using the Windows SAS program (version 6.0). Group means were subjected to analysis of variance, followed, if justified, by Dunnett’s test. The predetermined upper limit of probability for statistical significance was P < 0.05.

RESULTS AND DISCUSSION

The dietary regimen employed here was designed to maximally expose mice to the yogurt microorganisms via an experimental diet. This approach has been previously demonstrated to have no effect on weight gain or overall health status of experimental mice ingesting the diet (3, 15). Although there was as much as a 90% decline in some yogurt populations during the 2 weeks of storage, counts for mesophilic aerobic bacteria, streptococci, and bifidobacteria exceeded 10⁸, 10⁷, and 10⁶ CFU/ml, respectively (Fig. 1). These results are consistent with previous independent studies employing similar starter cultures (3) and are within the count range of 10⁶ to 10⁸ CFU/ml that is considered acceptable for probiotic products (14).

The percentages of splenic and Peyer’s patch cells identified as B220⁺, CD4⁺, and CD8⁺ are shown in Table 1. The basal percentages observed in the control-fed mice were consistent with those found in other studies (5, 7). Yogurts supplemented with L. acidophilus and Bifidobacterium (groups B, P, and A) or unsupplemented yogurt (group U) did not have any effect on the percentages of B220⁺ and CD8⁺ cells in the spleens and Peyer’s patches when compared to control group mice (C) (Table 1). The percentage of CD4⁺ cells was significantly increased in the spleens from one group A of mice fed a yogurt containing Bifidobacterium and L. acidophilus and a similar trend existed for the groups fed the remaining two probiotic-supplemented yogurts. CD4⁺ effects were not observed in splens of mice fed conventional yogurt or in the Peyer’s patches of any of the yogurt groups. The percentages of slgA⁺ and slgM⁺ cells in Peyer’s patches and spleen of yogurt-fed mice were not significantly different from the control mice (data not shown). Thus, isotype-specific effects were not detected in B-cell populations in yogurt-fed mice.

The results suggest that ingestion of conventional or probiotic-supplemented yogurts for 2 weeks had very little effect on lymphocyte distribution in the systemic or mucosal immune compartments. This was surprising in view of reports of potential immune alterations associated with
probiotic lactic acid bacteria (2, 11). Perdigon et al. (7) observed that, in mice fed yogurt supplements, T lymphocyte numbers decreased in the small intestine but were increased in the large intestine. Using a functional assay for immunoglobulin secretion, it was also determined that IgA-secreting B cells increased in both the small and large intestine upon yogurt feeding. The capacity of yogurt feeding to slow growth of chemical-induced colon tumors in mice correlated with an increase in CD4+ and IgA-secreting cells within the large intestine (8). These findings were consistent, in part, with our findings that yogurt feeding had the capacity to increase splenic CD4+ cell numbers. The possibility exists that while numbers of IgA+ cells may not be affected in spleen or Peyer’s patches of the small intestine, colonic IgA+ cells might have been altered in our study.

The absence of a clear effect on lymphocyte subpopulations for all but one yogurt and cell phenotype is consistent with the human feeding studies reported by Schiffrin et al. (12, 13). In these studies, healthy human volunteers were given fermented milk supplemented with L. acidophilus strain LA or with B. bifidum strain Bb12 for 3 weeks. No effects on peripheral blood lymphocyte subpopulations were noted; however, functional changes were observed as evidenced by increased phagocytic activity by blood leukocytes. DeSimone et al. (1) fed lyophilized B. bifidum and L. acidophilus in capsules four times per day for 28 days to elderly individuals and found that while intestinal leukocyte levels were unaffected, a significant increase in B-cell frequency occurred in peripheral blood. The difference between this and the two aforementioned studies may derive from the exposure regimen or the specific type of human population assessed.

The results imply that within the limitations of the animal model employed here, probiotic lactic acid bacteria may not elicit profound changes in proliferation or death in one or more lymphocyte populations that would significantly change their phenotypic distribution in the mucosal and systemic compartments. It is possible that a longer feeding period might be required to alter lymphocyte populations. Also, because these were 8-wk-old mice, it is pos-

![Graph showing bacterial populations over weeks](image)

**FIGURE 1.** Effect of storage on bacterial populations in test yogurts. Symbols for yogurts are (□) S Bifidus, (△) PY-3, (×) ABY-2C, and (○) Ultra-Gro. Results are mean of duplicate determinations.

<table>
<thead>
<tr>
<th>Feeding regimen</th>
<th>Cultures included</th>
<th>Lymphoid tissue</th>
<th>B220+</th>
<th>CD4+</th>
<th>D8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk control (C)</td>
<td>None</td>
<td>Peyer’s patch</td>
<td>57.6 ± 8.1</td>
<td>23.3 ± 3.1</td>
<td>6.8 ± 1.1</td>
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<tr>
<td></td>
<td></td>
<td>Spleen</td>
<td>35.2 ± 7.6</td>
<td>19.0 ± 2.7</td>
<td>10.1 ± 2.9</td>
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<td>S bifidus direct (B)</td>
<td>S. thermophilus, L. bulgaricus, L. acidophilus, Bifidobacterium</td>
<td>Peyer’s patch</td>
<td>59.8 ± 6.2</td>
<td>22.6 ± 2.4</td>
<td>6.6 ± 1.0</td>
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<td></td>
<td></td>
<td>Spleen</td>
<td>33.1 ± 7.0</td>
<td>23.1 ± 4.3</td>
<td>11.4 ± 2.3</td>
</tr>
<tr>
<td>PY-3 Redi-set (P)</td>
<td>S. thermophilus, L. bulgaricus, L. acidophilus, B. bifidum</td>
<td>Peyer’s patch</td>
<td>57.6 ± 4.1</td>
<td>23.1 ± 2.9</td>
<td>6.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spleen</td>
<td>34.6 ± 7.7</td>
<td>21.8 ± 4.1</td>
<td>10.7 ± 3.1</td>
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<td>ABY-2C (A)</td>
<td>S. thermophilus, L. bulgaricus, L. acidophilus, B. infantis</td>
<td>Peyer’s patch</td>
<td>47.6 ± 18.0</td>
<td>22.4 ± 2.4</td>
<td>6.8 ± 1.2</td>
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<td></td>
<td></td>
<td>Spleen</td>
<td>35.7 ± 8.3</td>
<td>23.7 ± 3.5*</td>
<td>12.6 ± 2.0</td>
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<tr>
<td>Ultra-Gro (U)</td>
<td>S. thermophilus, L. bulgaricus</td>
<td>Peyer’s patch</td>
<td>51.3 ± 15.3</td>
<td>23.2 ± 3.0</td>
<td>5.9 ± 1.0</td>
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<td></td>
<td></td>
<td>Spleen</td>
<td>34.0 ± 10.1</td>
<td>19.5 ± 2.6</td>
<td>11.5 ± 2.8</td>
</tr>
</tbody>
</table>

* Mice were fed a 50:50 mixture of yogurt (or skim milk) plus AIN 93-G diet. Values are means ± SD and expressed as percentage of viable cells. Between 8 and 10 animals were used per group. Data are means ± SD and expressed as percentage of viable cells. Asterisk indicates significant difference from skim milk control (P < 0.05).
sible that they would have fairly well-developed microbial populations that strongly influence the immune system and are thus recalitrant to further effects by the yogurt bacteria. This possibility could be explored further in neonate or germ-free animals in conjunction with the use of marker lactic acid bacteria to monitor actual in vivo exposure.

It is possible that subtle changes may occur in gene expression of lymphocytes in yogurt-fed animals that would markedly impact immune function without changing their phenotypic distribution. For example, using identical yogurt cultures and exposure regimens to those employed here, we have previously observed that probiotic-supplemented yogurts can enhance the mucosal IgA response to cholera toxin (15). Future research should therefore be directed toward the capacity of probiotics to alter signaling and gene expression within discrete gut leukocyte populations and ultimately alter an immune response.

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REFERENCES