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Effect of *Lactobacillus casei* and Yogurt Administration on Prevention of *Pseudomonas aeruginosa* Infection in Young Mice

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen that rarely causes pulmonary disease in normal hosts but one that is an important cause of acute pneumonia in immunocompromised patients, including neonates, and of chronic pneumonia in patients with cystic fibrosis. The aim of this work was to study the effect of oral administration of *Lactobacillus casei* and yogurt on prevention of *P. aeruginosa* lung infection in young mice (3 weeks old). This study demonstrates that oral administration of *L. casei* or yogurt to young mice enhanced lung clearance of *P. aeruginosa* and phagocytic activity of alveolar macrophages through a dose-dependent effect. There were, however, no significant differences in white blood cell (WBC) differential counts. Furthermore, it was observed that previous administration of *L. casei* or yogurt induced a significant increase in IgA and IgM levels in bronchoalveolar lavages (BALs) after a *P. aeruginosa* infection, although there was no relationship with the serum values.

Pseudomonas aeruginosa is an important respiratory pathogen in neonates and individuals with compromised respiratory function, such as those suffering cystic fibrosis and chronic bronchitis. Once acquired, infection is difficult to eradicate with chemotherapy, and attempts to vaccinate against infection have not been very successful *(6). P. aeruginosa* pulmonary clearance and polymorphonuclear leukocyte recruitment are still impaired in 20-day-old mice *(30).* The mouse immune response is only fully mature after 8 weeks of life *(18);* therefore, young mice are more susceptible to respiratory infections than adult mice.

The surface of mucosal membranes is protected by a local adaptive immune system. The secretory IgA antibodies in the gut are part of the common mucosal immune system, which includes the respiratory tract and lacrimal, salivary, and mammary glands *(4).* Consequently, an immune response initiated in the gut-associated lymphoid tissue can affect immune responses at other mucosal surfaces.

Lactic acid bacteria are used in an increasing number of health foods, or so-called functional foods, and pharmaceutical preparations, based on the probiotic characteristics of some of these bacteria *(13, 14),* which can positively affect protective immunity against pathogens and tumors due to their ability to increase the mucosal immune response *(8–11).* The results of previous studies on the phagocytic function and lymphocytic activity measured by levels of antibodies have shown that milk fermented with a mixture of *Lactobacillus casei* and *Lactobacillus acidophilus* strains increases both types of cell activity *(22)* and protects against gastrointestinal infection *(23).*

In previous works, it has been demonstrated that some lactic acid bacteria and yogurt can induce intestinal mucosal immunity *(1, 24)* and that *L. casei* can be used as oral adjuvant to induce a protective immunity in the gut *(20, 23).* However, we have demonstrated that some lactic acid bacteria were able to increase the Ig A^+ B lymphocytes in bronchus tissue *(21).* Similarly, other authors have found oral administration of an immunomodulator containing lipopolysaccharides effective in the restoration of the number of IgA cells in bronchus-associated lymphoid tissue in a malnutrition model *(12).*

Assuming that oral immune stimulation can induce immunity in mucosal sites remote from the gut, the aim of this work was to study the effect of oral administration of *L. casei* CRL 431 and yogurt on prevention of *P. aeruginosa* lung infection in young mice (3 weeks old).

MATERIALS AND METHODS

Animals. Three-week-old Swiss albino mice (young mice) were obtained from the housed colony kept at our department at CERELA. The animals were housed in plastic cages at room temperature. Each experimental group consisted of 25 to 30 mice (five to six for each different period), housed individually during the experiments. Adult mice (8 weeks old) were used only to compare lung infection with young mice.

Microorganisms. *L. casei* CRL 431 was obtained from the CERELA culture collection. The culture was kept freeze dried and then dehydrated using the following medium: peptone 15.0 g, tryptone 10.0 g, meat extract 5.0 g, distilled water 1 liter, pH 7. It was cultured for 8 h at 37°C (final log phase) in deMan-Rogosa-Sharpe broth (Oxoid, Hampshire, UK). The bacteria were harvested through centrifugation at 5,000 rpm for 10 min and washed three times with sterile PBS (0.01 M phosphate-buffered saline, pH 7.2). A simulated commercial yogurt was prepared from a stock yogurt culture of *Lactobacillus delbrueckii* subsp. *bulgari-*

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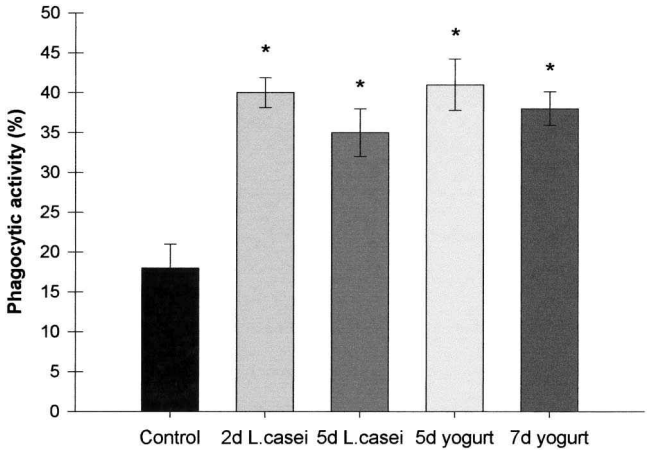

FIGURE 1. *Effect of* L. casei *and yogurt administration on the phagocytic activity of pulmonary alveolar macrophages. The assay was performed at the end of each treatment. Bars represent means ± SD of each group of animals* (n = 5). *Details are described in "Materials and Methods." * Statistically significant differences with respect to the control mice* ($P < 0.05$).

cus and *Streptococcus thermophilus* obtained from the CERELA culture collection.

A mucoid strain of *P. aeruginosa* was isolated from the low respiratory tract of a human subject in the Clinical Microbiological Department of the Biochemistry School of the Tucuman National University.

Candida albicans was grown in Sabouraud medium (Difco Laboratories, Detroit, Mich.) for 18 h at 37°C and then killed by heating for 15 min at 100°C.

Feeding procedures. The *L. casei* culture was suspended in 5 ml of sterile nonfat milk and then added to the drinking water (20% vol/vol). Different groups of young mice were administered for 2 or 5 consecutive days with a dose of 10^9 cells/d/mouse. The control group consisted of young mice that received sterile milk in the drinking water administered under the same conditions as in the test group. Yogurt was administered for 2, 5, and 7 consecutive days (2 ml/d/mouse). All mice were fed ad libitum with a conventional balanced diet.

Phagocytosis assay. Pulmonary alveolar macrophages were collected as described by Moineau and Goulet *(16),* after treatment with *L. casei* for 2 and 5 days and yogurt for 5 and 7 days. The mice were exsanguinated to prevent accumulation of blood in the lungs. The trachea was cannulated, and five sequential bronchoalveolar lavages (BALs) were performed in each mouse, injecting 0.5 ml of sterile PBS. Over 75% of the fluid was recovered. Cells were counted, and viability was tested with trypan blue dye exclusion *(26).* The phagocytosis assay was performed according to Perdigón et al. *(19).* Briefly, the cells obtained after the BALs were centrifuged at 1,000 rpm for 10 min, washed three times with RPMI-1640 (GIBCO), and resuspended at a concentration of 10^5 cells/ml. A nonviable *C. albicans* suspension with a concentration of 5×10^6 cells/ml, previously opsonized with mouse autologous serum, was added to 0.2 ml of each macrophage suspension and incubated for 30 min at 37°C. The phagocytic activity was measured at the end of the incubation period by counting 100 cells with an optical microscope.

Experimental infection. *P. aeruginosa* was grown in 100 ml of brain-heart infusion broth (Difco) at 37°C for 12 h, harvested through centrifugation at 5,000 rpm for 10 min at 4°C, and then washed three times with sterile PBS. Cell density was adjusted to 1×10^{11} CFU/ml, and 25 ml of this suspension was placed in the nebulizer reservoir. At the end of each *L. casei* or yogurt treatment, the animals and controls were nebulized for 30 min with *P. aeruginosa* (approximately 20 ml). For nebulization, the animals were kept in a chamber built at our laboratory, following specifications of a previously described design *(31),* which operates according to the Venturi tube principle. Under these conditions, 2 to 4×10^4 CFU/g of lung of *P. aeruginosa* was recovered in the control animals from cultures of lung homogenates 1 h after exposure (Ti).

TABLE 1. *Susceptibility to* Pseudomonas aeurignosa *infection*[a]

Time post-infection (h)	Log CFU/g of lung	
	Adult mice	Young mice
1	4.91 ± 0.22	4.45 ± 0.28
16	4.15 ± 0.25	4.02 ± 0.33
24	<1.5	3.56 ± 0.30*[b]
48	<1.5	3.09 ± 0.27*

[a] Mice were challenged intranasally with *P. aeruginosa,* and their lungs were cultured at different times after challenge. Values are means ± SD for each group of mice ($n = 5$).

[b] *, statistically significant with respect to adult mice ($P < 0.05$).

Pulmonary clearance of *P. aeruginosa.* Mice were sacrificed 1 h (Ti) and 4, 8, 16, and 24 h (Tx) following exposure to the aerosol for measuring lung clearance of *P. aeruginosa.* The lungs were excised, weighted, and homogenized in 5 ml ice-cold, sterile PBS. Homogenates were diluted appropriately and pour plated on tryptic soy agar (Difco) in duplicate, and the number of CFU per g of organ was determined. Results are expressed, using the procedures of Amura et al. *(2),* as the percentage of cleared bacteria: $[1 - (\log CFU_{Tx}/\log CFU_{Ti}) \times 100\ \%]$.

Susceptibility to lung infection in young mice. To determine the susceptibility of the young mice to *P. aeruginosa* infection, adult mice (8 weeks old) and young mice (3 weeks old) were challenged intranasally with *P. aeruginosa,* and their lungs were excised, homogenized, and plated 1, 16, 24, and 48 h after challenge. Results were expressed as log CFU per g of organ.

Determination of total number of white blood cells and differential cell counts. Blood samples were obtained through cardiac puncture at 1, 4, 8, 16, and 24 h after aerosol challenge and collected in heparinized tubes. Total numbers of white blood cells (WBCs) were determined with an hemacytometer. Differential cell counts were performed by counting 200 cells in blood smears stained with Giemsa.

Antibodies from serum and BALs. The BAL samples were obtained using the procedures of Bergeron et al. *(3)* but were modified as follows: the trachea was exposed and intubated with a catheter, and two sequential BALs were performed in each mouse, injecting 0.5 ml of sterile PBS; the recovered fluid was centrifuged for 10 min at 1,500 rpm to remove cells and bacteria and then frozen at −70°C for subsequent antibody analysis. An enzyme-linked immunosorbent assay technique was developed to measure total antibodies (IgA, IgG, and IgM) in serum and BAL samples obtained prior to challenge with *P. aeruginosa* (T_0) and 16 h after that (T_{16}). Each plate was coated with 200 μl of an appropriated dilution in a sodium carbonate-bicarbonate buffer

TABLE 2. *Determination of the total number of white blood cells (WBCs) in blood samples at different times after challenge with* P. aeruginosa[a]

Treatment	Number of WBC/μl					
	L. casei		Yogurt			
Days of feeding	2	5	2	5	7	Control young
Time postinfection (h)						
1	2,950 ± 141	2,400 ± 380	1,500 ± 420	1,475 ± 389*[b]	1,400 ± 071*	3,600 ± 166
4	2,780 ± 376*	2,800 ± 390*	3,000 ± 470*	2,350 ± 601*	2,700 ± 566*	5,800 ± 176
8	3,965 ± 615	4,400 ± 509	3,200 ± 510	3,000 ± 430	2,850 ± 380	3,600 ± 348
16	4,485 ± 304	6,900 ± 307	3,600 ± 520	3,400 ± 630	3,300 ± 390	5,615 ± 120
24	3,725 ± 354	5,150 ± 230	4,000 ± 120	3,875 ± 106	5,300 ± 290	4,925 ± 308

[a] The total number of WBCs was determined. Values are means ± SD ($n = 5$).
[b] *, statistically significant decrease with respect to the control mice ($P < 0.05$). Normal values of adult and young mice were: 4,800 ± 100 WBCs/μl and 7,500 ± 206 WBCs/μl, respectively.

TABLE 3. *Polymorphonuclear blood cell counts after challenge with* P. aeruginosa *in mice treated with* L. casei *and yogurt*[a]

Treatment	Percentage of polymorphonuclear cells					
	L. casei		Yogurt			
Days of feeding	2	5	2	5	7	Control young
Time postinfection (h)						
1	27.5 ± 2.1	21.5 ± 2.2	26.5 ± 3.2	25.5 ± 2.1	28.5 ± 2.1	24.0 ± 2
4	22.0 ± 2.8	22.0 ± 2.5	23.7 ± 1.5	24.5 ± 3.4	25.3 ± 1.5	20.5 ± 0.7
8	25.3 ± 1.5	23.7 ± 1.6	25.5 ± 1.2	25.7 ± 1.5	26.5 ± 2.1	24.5 ± 3.2
16	20.0 ± 1.4	24.0 ± 1.7	24.0 ± 3.5	26.5 ± 3.1	24.3 ± 1.5	21.5 ± 2.1
24	20.0 ± 1.7	21.5 ± 2.1	22.0 ± 2.8	24.5 ± 3	20.5 ± 0.7	20.0 ± 3.6

[a] Values are means ± SD. There were no significant differences ($P < 0.05$). Normal values of adult and young mice were: 15.3 ± 0.7% and 8.5 ± 1.1%, respectively.

TABLE 4. *Lymphocyte blood cell counts after challenge with* P. aeruginosa *in mice treated with* L. casei *and yogurt*[a]

Treatment	Percentage of lymphocyte cells					Control young
	L. casei			Yogurt		
Days of feeding	2	5	2	5	7	
Time postinfection (h)						
1	71.0 ± 1.4	77.5 ± 2.1	72.5 ± 3.9	70.0 ± 1.4	70.0 ± 1.5	74.0 ± 3.5
4	76.5 ± 3.5	76.5 ± 3.8	74.0 ± 1.7	74.5 ± 3.1	74.0 ± 1.5	77.1 ± 0.7
8	73.0 ± 1.2	75.0 ± 1.7	73.0 ± 1.5	74.0 ± 1.8	71.0 ± 1.4	74.5 ± 3.5
16	78.5 ± 0.7	69.7 ± 3.5	74.0 ± 3.6	72.5 ± 3.9	73.0 ± 1.6	77.5 ± 2.1
24	78.7 ± 1.5	77.5 ± 2.1	77.5 ± 3.5	74.5 ± 3.9	78.1 ± 0.7	78.3 ±3.3

[a] Values are means ± SD. There were no significant differences ($P < 0.05$). Normal values of adult and young mice were: 82.5 ± 0.9% and 92.2 ± 3.5%, respectively.

(pH 9.6) of goat anti-mouse: IgA (α-chain specific), IgG (γ-chain specific), or IgM (μ-chain specific) (Sigma Chemical Co., St Louis, Mo.). After overnight incubation at 4°C, the plates were washed five times with PBS containing 0.05% (vol/vol) Tween20 (PBS-T). Nonspecific protein-binding sites were blocked with PBS containing 5% nonfat dry milk for 30 min at room temperature. After the addition of 200-μl portions of the appropriate dilutions of the samples with PBS-T (serum 1:1500; BAL 1:2), the plates were incubated for 60 min at 37°C. After the plates were washed five times with PBS-T, peroxidase-conjugated goat anti-mouse IgA, IgG, or IgM (Fc specific, Sigma) was diluted 1:1,000 in PBS-T, and 200 μl was added to each plate. Then, the plates were incubated at 37°C for 60 min and washed afterward five times with PBS-T. Plates were subsequently poured with 200 μl of a substrate solution (3-3′, 5-5′-tetramethylbenzidine [Sigma]) in citrate-phosphate buffer (pH 5, containing 0.05% H_2O_2). After incubation for 30 min at room temperature, the reaction was stopped by the addition of 50 μl of 2 N H_2SO_4. Readings were carried out at 493 nm, and the antibody concentration in each unknown sample was expressed as optical density/200 μl of diluted sample.

Statistical analysis. Experiments were replicated three times, and the results were expressed as means ± standard deviation (SD). Significant differences ($P < 0.05$) between means were determined by Student's *t* test.

RESULTS

Phagocytic activity. Oral administration of *L. casei* for 2 and 5 days and yogurt for 5 and 7 days induced significant increases ($P < 0.05$) of phagocytic activity of alveolar macrophages, with values two times higher than in young control mice without treatment (Fig. 1).

Susceptibility to lung infection in young mice. Susceptibility of young mice to *P. aeruginosa* infection is shown in Table 1. Adult animals were able to clear the pathogen from their lungs 24 h after infection, whereas young mice still demonstrated a high colonization after this time.

Effect on pulmonary clearance of *P. aeruginosa.* In young control mice challenged with *P. aeruginosa,* pulmonary clearance was 10% 16 h postchallenge. Administration of *L. casei* for 2 days and yogurt for 7 days significantly increased ($P < 0.05$) *P. aeruginosa* clearance from the lungs after challenge (Fig. 2). Sixteen hours postchallenge, the treated animals had cleared bacteria completely (100%) from their lungs. At this time, yogurt administration for 5 days had a significant effect ($P < 0.05$), but it was not enough to achieve total bacterial clearance.

Total number of WBCs and differential cell count. There was a significant decrease ($P < 0.05$) in the total number of WBCs in all groups treated with *L. casei* or yogurt during the first hours postchallenge (Table 2). WBC differential counts did not show any detectable modification of the percentage of polymorphonuclear cells and lymphocytes between control and treated groups (Tables 3 and 4).

Immunoglobulins from serum and BALs. Immunoglobulin levels were measured in mice with enhanced bacterial clearance, shown after treatment with *L. casei* for 2 days and yogurt for 5 to 7 days. At the end of each treat-

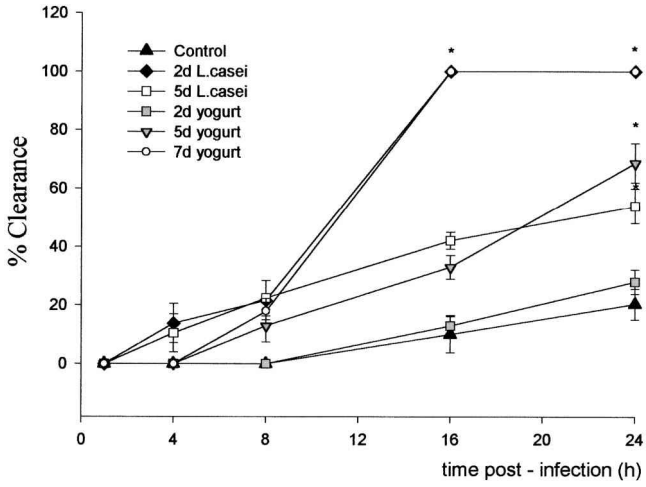

FIGURE 2. *Effect of* L. casei *and yogurt administration on lung clearance of* P. aeruginosa *in young mice (3-week-old Swiss albino mice). Lungs of both treated and control groups were cultured at different times after challenge, and the number of CFU of* P. aeruginosa *was determined. Results are expressed as the percentage of cleared bacteria determined as [1 − (log CFU_{Tx}/log CFU_{Ti}) × 100%]. Bars represent SD of means (*n = 5*). Details are described in "Materials and Methods." * Statistically significant differences with respect to the control mice (*$P < 0.05$*).*

ment, no significant increase in the immunoglobulin levels was observed, with the exception of BAL samples after yogurt administration for 7 days (Fig. 3). Sixteen hours after challenge with *P. aeruginosa,* significant increases in IgA levels in BAL samples of treated mice were observed with respect to the control group ($P < 0.05$); however, the IgA levels in serum samples increased in mice treated with *L. casei* (2 days) and yogurt (5 days) (Fig. 3a and 3b). Immunoglobulin M levels in BAL samples showed an enhancement in both treated and untreated animals after infection; however, the increase was significant ($P < 0.05$) after 5 and 7 days of yogurt administration with respect to the control values (Fig. 3d). Serum IgM did not show any difference (Fig. 3c). Levels of IgG in BAL and serum samples of treated and untreated mice were not significantly different after challenge (Fig. 3e and 3f).

DISCUSSION

In a previous work, we demonstrated that oral administration of *L. casei* increases the phagocytic function of adult mice *(22).* The present study shows that oral administration of *L. casei* or yogurt to young mice enhances the phagocytic activity of alveolar macrophages (Fig. 1) and

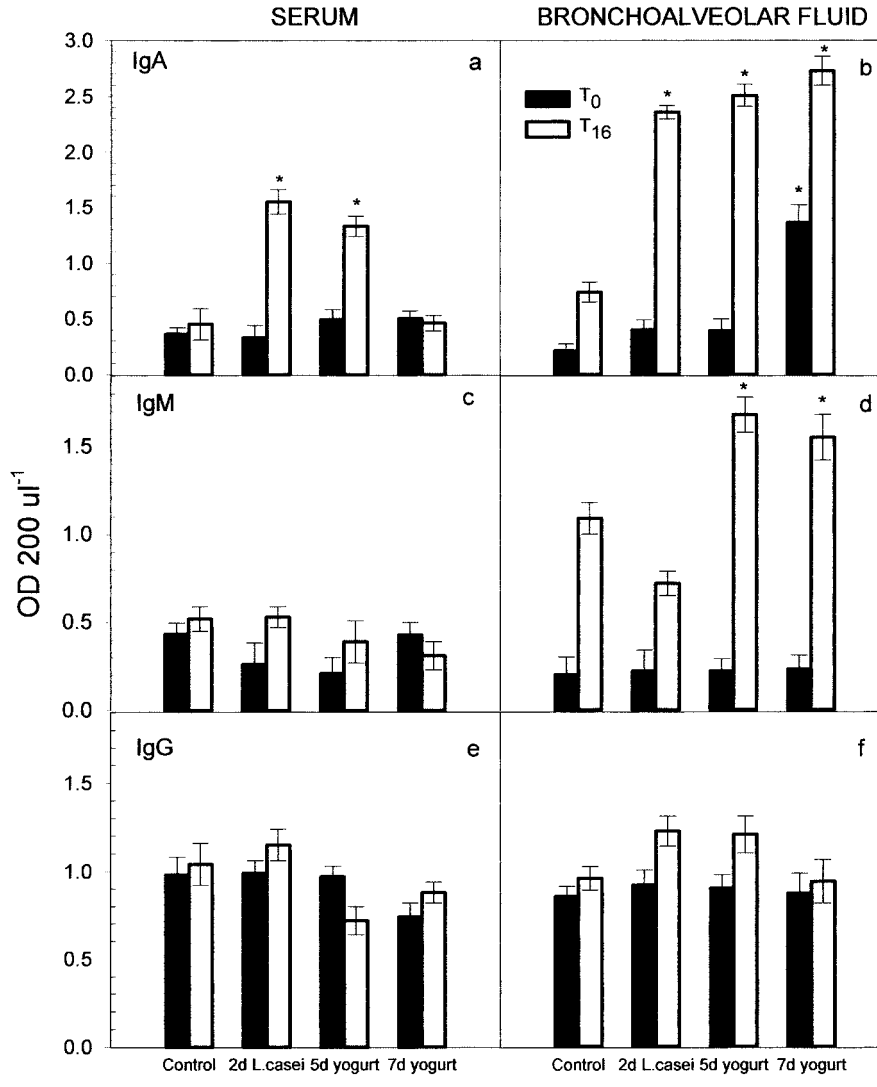

FIGURE 3. *Effect of* L. casei *and yogurt administration on total IgA, IgM, and IgG levels in serum (a, c, e) and bronchoalveolar fluid (b, d, f) using the enzyme-linked immunosorbent assay test. Antibody concentration in each unknown sample is expressed as optical $density_{493nm}$/200 µl of diluted sample. Bars represent means ± SD of each group of animals (*n = 5*). Solid bars represent the values prior to challenge (T_0), and open bars represent the levels 16 h after challenge (T_{16}). * Statistically significant differences with respect to the control mice (*$P < 0.05$*).*

the lung clearance of *P. aeruginosa* (Fig. 2). No significant differences, however, were observed in WBC differential counts (Tables 3 and 4). This observation is in agreement with previous reports, which have shown that oral administration of *Lactobacillus johnsonii* to human volunteers was able to induce an increase in the phagocytic activity of peripheral blood leukocytes without any detectable modification in the WBC differential count *(7, 29).* Other authors have demonstrated an association between enhanced pulmonary clearance of *P. aeruginosa* in intestinally vaccinated animals and a significant increase in the phagocytic activity of alveolar macrophages *(5).* Previous works have shown that the decrease in the bacterial load seen after *P. aeruginosa* coincided with a steady and strong recruitment of inflammatory cells to the bronchoalveolar spaces of mice of the resistant BALB/c strain *(17).* In our study, a decrease was observed in the total number of WBCs in animals treated with *L. casei* or yogurt compared with untreated, infected animals (Table 2); this effect is probably due to an increase in migration to the infection site in the treated young mice. Further studies of histological slices are necessary to determine the presence of inflammatory cells in the respiratory tract.

In our study, we also observed that previous administration of *L. casei* and yogurt induced a significant increase in IgA and IgM levels in BALs after a *P. aeruginosa* infection, without a proven relationship with serum values. The important role of IgA in the defense against infections has been demonstrated *(15, 28, 32).* And although the IgA measured in the present work was not specific to the pathogen, evidence exists that saliva and colostrum from healthy subjects contain polyreactive secretory IgA antibodies that recognize a variety of autoantigens and several bacterial antigens *(27).* Such polyreactive S-IgA antibodies may provide protection of the mucosal surfaces prior to the generation of specific antibodies. The high levels of IgA in BALs from treated mice, after *P. aeruginosa* infection, are probably correlated with the enhancement of IgA cells observed in a previous study with different species of *Lactobacillus* and *Streptococcus (21)* and with the capacity of some lactic acid bacteria to induce antibodies against themselves *(25).*

In summary, yogurt and *L. casei* administration at an appropriate dose had a beneficial effect on pulmonary infection with *P. aeruginosa* in weaned mice. The treatments studied in this experiment were able to induce an activation of alveolar macrophages and an increase of IgA levels in the respiratory tract, which improve the immunological reaction against an acute infection with *P. aeruginosa.*

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