Lactobacillus casei Improves Resistance to Pneumococcal Respiratory Infection in Malnourished Mice[1]

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ABSTRACT We studied the effect of *Lactobacillus casei* CRL 431 used as a supplement in a repletion diet on the resistance to *Streptococcus pneumoniae* respiratory infection in malnourished mice. Weaned mice were malnourished after they consumed a protein-free diet (PFD) for 21 d. Malnourished mice were fed a balanced conventional diet (BCD) with or without supplemental *L. casei* for 7, 14, or 21 consecutive days, or BCD for 7 d with *L. casei* supplementation on d 6 and 7 (7dBCD+2dLc). The malnourished control (MNC) group was fed only the PFD, whereas well-nourished control (WNC) mice consumed the BCD ad libitum. Mice were challenged with *S. pneumoniae* at the end of each dietary treatment. Lung colonization and bacteremia were significantly greater in MNC than in WNC. Normalization of the immune response occurred in malnourished mice fed the BCD for 21d. *L. casei* supplementation reduced the time required for a normal response from 21 to 7 d. Mice administered the 7dBCD+2dLc repletion treatment had a more effective pathogen clearance from blood and significantly lower lung damage than MNC. This treatment improved both the number of leukocytes and neutrophils in blood and bronchoalveolar lavages (BAL) and the bactericidal function of phagocytic cells to levels that did not differ from those of WNC. In the 7dBCD+2dLc mice, antipneumococcal IgA in BAL was higher than in WNC, whereas antipneumococcal IgG in serum and BAL did not differ. This study suggests that the addition of *L. casei* to the repletion diet has a beneficial effect because it accelerates the recovery of the innate immune response and improves the specific immune mechanisms against an *S. pneumoniae* respiratory infection in malnourished mice. J. Nutr. 135: 1462–1469, 2005.

KEY WORDS: • *Lactobacillus casei* • *malnourished mice* • *lung infection* • *Streptococcus pneumoniae*

Streptococcus pneumoniae is one of the major causes of bacterial pneumonia, meningitis, bacteremia, and otitis media. Despite the availability of antibiotics, mortality and morbidity rates remain high, especially in high-risk groups (1). Although polysaccharide vaccines can elicit a protective immune response against pneumococcal infection in adults, they have little effect on young children, the elderly, or immunodeficient patients (2).

Nutrition is a critical determinant of the immune response; consequently, malnutrition is the most common cause of immunodeficiency throughout the world. The reasons for malnutrition are multiple and complex; infection is a common precipitating factor. Conversely, malnutrition is also an important factor in the occurrence of infection, so that the 2 interact (3). Protein-energy malnutrition is associated with impaired cell-mediated immunity, phagocyte function, the complement system, secretory immunoglobulin A antibody concentrations, and cytokine production (4). It is not surprising that protein deficiency is so consistently observed to interfere with resistance to infection because most immune mechanisms depend on cell replication or on the production of active protein compounds (3).

The idea that gut microflora play a very important role in the maintenance of health and well-being is acquiring worldwide acceptance (5). Lactic acid bacteria (LAB)[3] are present in the intestine of most animals. The beneficial role played by these microorganisms in humans and other animals has been reported extensively (6–8). One of the most effective ways of ensuring the predominance of beneficial bacteria in the extremely competitive environment of the gastrointestinal tract is to include live bacteria in the diet. Several studies investigated the role of immune-modulating probiotics in the control

[1] Supported by grants from FONCyT PICT 99 # 5–7206 and CIUNT 26 D/202.
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[3] Abbreviations used: BAL, bronchoalveolar lavages; BCD, balanced conventional diet; BCD+Lc, balanced conventional diet with supplemental *Lactobacillus casei*; LAB, lactic acid bacteria; Lc, *Lactobacillus casei*; MGG, May Grünwald-Giemsa; MNC, malnourished control; NBT, nitro blue tetrazolium; PFD, protein-free diet; WNC, well-nourished control; 7dBCD (14dBCD, 21dBCD), mice fed 7 (14, 21) d with BCD; 7dBCD+Lc (14dBCD+Lc, 21dBCD+Lc), mice fed 7 (14, 21) d with BCD+Lc; 7dBCD+2dLc, mice fed 7 d with BCD with supplemental Lc on d 6 and d 7.

0022-3166/05 $8.00
Manuscript received 29 September 2004. Initial review completed 22 October 2004. Revision accepted 17 February 2005.

of microbial enterpathogens using rodent infection-challenged models (5,7). Although most research concerning probiotic-mediated enhanced immune protection is focused on gastrointestinal tract pathogens, a few recent studies tested whether probiotics might sufficiently stimulate the common mucosal immune system to provide protection to other mucosal sites as well (9). *Lactobacillus casei* CRL 431 was reported to induce both a secretory immune response and to protect against enteric infections in well-nourished (10) and malnourished (11) mice. The oral administration of this bacterial strain was also shown to increase the number of IgA^+ B lymphocytes that enter the IgA cycle and repopulate the bronchus (8). This is important because the oral ingestion of *L. casei* could protect the respiratory mucosa against pathogens. We reported previously that the oral administration of *L. casei* CRL 431 to young mice enhances the phagocytic activity of alveolar macrophages and the lung clearance of *Pseudomonas aeruginosa* (12). Similarly, other investigators found that the oral administration of an immunomodulator containing lipopolysaccharides restores the number of IgA cells in bronchus-associated lymphoid tissue in malnourished rats (13). The aim of this work was to study the effect of the oral administration of *L. casei* CRL 431 on the resistance to a *S. pneumoniae* respiratory infection in malnourished mice.

MATERIALS AND METHODS

Animals

Male 6-wk-old Swiss albino mice were obtained from the closed colony kept at the bioterio of CERELA. They were housed in plastic cages at room temperature. Each assay was performed in groups consisting of 25–30 mice (5–6 for each day before and after infection) that were housed individually during the experiments. Weaned mice were malnourished after they consumed a protein-free diet (PFD) (**Table 1**) for 21 d (**Fig. 1**A). At the end of this period, mice that weighed 40–55% less than well-nourished mice were selected for experiments. Well-nourished control mice consumed ad libitum a balanced conventional diet (BCD) (Table 1) and were used to compare lung infection with malnourished mice after repletion. Experiments were approved by the Ethical Committee for animal care at CERELA.

TABLE 1

Composition of the balanced conventional and protein-free diets[1]

Ingredient	Balanced conventional diet	Protein-free diet
	g/kg	
Water	120	120
Protein	230	<10
Carbohydrate	538	758[2]
Lipids	50	50
Vitamin mix[3]	22	22
Mineral mix[4]	40	40

[1] The approximate energy value provided by the bacterial supplement is 69.13 mJ/(mouse · d).

[2] Protein-free corn flour.

[3] Vitamin mix (# 905454, ICN Biomedicals Argentina) g/kg of mixture: *dl*-α-tocopherol, 5.0; *p*-aminobenzoic acid, 5.0; ascorbic acid, 45.0; biotin, 0.02; retinyl acetate, 4.5; vitamin B-12, 0.00135; calcium pantothenate, 3.0; choline chloride, 75.0; cholecalciferol, 0.25; folic acid, 0.09; inositol, 5.0; menadione, 2.25; niacin, 4.5; pyridoxine hydrochloride, 1.0; riboflavin, 1.0 thiamine hydrochloride, 1.0; and sucrose, finely powdered, 847.38865.

[4] Mineral mix (# 902844 ICN Biomedicals Argentina) g/kg of mixture: sodium chloride, 167; potassium phosphate dibasic, 322; calcium carbonate, 300; magnesium sulfate, 102; calcium phosphate monobasic, 75; ferric citrate, 27.5; $MnSO_4 \cdot H_2O$, 5.1; potassium iodide, 0.8; $CuSO_4 \cdot 5H_2O$, 0.3; zinc chloride, 0.25; and $CoCl_2 \cdot 6H_2O$, 0.05.

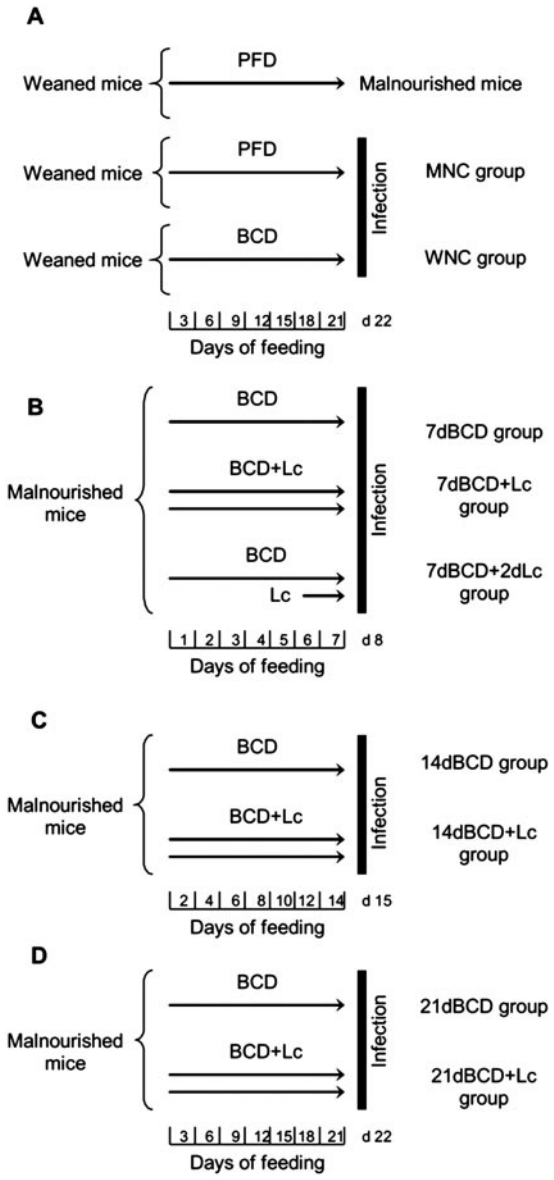

FIGURE 1 Different feeding protocols used in this work. (*A*) Preparation of malnourished mice and well-nourished and malnourished infected controls. Repletion of malnourished mice: (*B*) feeding for 7 d; (*C*) feeding for 14 d; (*D*) feeding for 21 d.

Microorganisms

Lactobacillus casei CRL 431 was obtained from the CERELA culture collection. The culture was kept freeze-dried and then rehydrated using the following medium: peptone, 15.0 g; tryptone, 10.0 g; meat extract, 5.0 g; and distilled water, 1 L, pH 7. It was cultured for 8 h at 37°C (final log phase) in Man-Rogosa-Sharpe broth (MRS, Oxoid). The bacteria were harvested through centrifugation at 3000 × *g* for 10 min and washed 3 times with sterile 0.01 mol/L PBS, pH 7.2. Capsulated pneumococcus was isolated from the respiratory tract of a patient from the Department of Clinical Bacteriology of the Niño Jesús Children's Hospital in San Miguel de Tucumán, Argentina. The

pathogen strain belongs to the 14 serotype, 1 of the 10 most frequent serotypes isolated in pneumococcal infections in Argentina (serotypification was performed in Administración Nacional de Laboratorios e Institutos de Salud-ANLIS "Dr. Malbran," Buenos Aires, Argentina).

Feeding procedures

To compare the influence of *L. casei* administration on the susceptibility to *S. pneumoniae* infection, malnourished mice were fed the BCD with or without supplemental *L. casei* [10^9 cfu/(mouse · d)] for 7 (Fig. 1*B*), 14 (Fig. 1*C*), or 21 (Fig. 1*D*) consecutive days. Because administration of *L. casei* (Lc) for 2 d is the optimal dose to provide protection against enteropathogens (10) and *S. pneumoniae* (unpublished data) in well-nourished mice and against *P. aeruginosa* respiratory infection in young mice (12), a third group of mice was added. These mice were fed the BCD for 7 d with *L. casei* supplementation on d 6 and d 7 (7dBCD+2dLc) (Fig. 1*B*). The malnourished control (MNC) group was fed only the PFD, whereas well-nourished control (WNC) mice consumed the BCD ad libitum (Fig. 1*A*).

Experimental infection

S. pneumoniae were first grown on blood agar for 18 h; freshly grown colonies were suspended in Todd Hewitt broth (Oxoid) and incubated at 37°C overnight. The pathogen was harvested through centrifugation at 3000 × *g* for 10 min at 4°C and then washed 3 times with sterile PBS. Cell density was adjusted to 4×10^4 cfu/L. The size of the inoculum was confirmed by serial dilutions and quantitative subcultures on blood agar. The infecting dose was chosen on the basis of bacterial cell counts recovered from the blood of mice suffering from severe pneumonia (unpublished data).

Challenge with *S. pneumoniae* was performed on the day after the end of each dietary treatment (d 8, 15, or 22) (Fig. 1). Mice were infected by dropping 25 μL of the inoculum containing 10^5 log-phase cfu of *S. pneumoniae* in PBS into each nostril and allowing it to be inhaled. To facilitate migration of the inoculum to the alveoli, mice were held in a head-up vertical position for 2 min. WNC and MNC mice were infected in the same way (Fig. 1*A*). Then, mice were killed on d 0 (before infection) and on d 1, 2, 5, 10, or 15 postinfection. During the 15-d postinfection period, all mice were fed only the BCD, with the exception of MNC that were fed the PFD.

Body weight determination

Body weight was determined before and after each dietary treatment. The experiment was performed on 10 mice/group to validate the statistical results.

Bacterial cell counts in lung homogenates and blood

Mice were killed 1, 2, 5, or 10 d after challenge with pneumococcus and their lungs were excised, weighed, and homogenized in 0.005 L of sterile peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar, and incubated for 18 h at 37°C. *S. pneumoniae* was identified by standard techniques (14) and the results were expressed as log of cfu/g of organ.

Progression of bacterial growth to the bloodstream was monitored by sampling blood obtained through cardiac puncture with a heparinized syringe and plating on blood agar. Bacteremia was reported as negative or positive hemocultures after incubation for 18 h at 37°C.

Determination of total and differential number of leukocytes in blood and bronchoalveolar lavages (BAL)

Blood samples were obtained through cardiac puncture at the end of each dietary treatment (d 0) and 1, 2, 5, and 10 d after challenge and collected in heparinized tubes. The total number of leukocytes was determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in blood smears stained with May Grünwald-Giemsa (MGG). BAL samples were obtained according to the technique of Bergeron et al. (15) modified as follows: the trachea was exposed and intubated with a catheter, and 2 sequential bronchoalveolar lavages were performed in each mouse by injecting 5×10^{-4} L of sterile PBS; the recovered fluid was centrifuged for 10 min at 900 × *g*; the pellet was used to make smears that were stained with MGG; and the fluid was frozen at –70°C for subsequent antibody analyses.

Antibodies from serum and BAL

An ELISA was developed to measure antipneumococcal antibodies (IgA and IgG) in serum and BAL 1, 2, 5, 10, and 15 d after challenge; basal levels were determined on d 0, before infection. BAL samples were obtained as described above.

Each plate was coated with 200 μL of a 1:100 dilution of *S. pneumoniae* vaccine (NEUMO 23 polyvalent vaccine, Aventis Pasteur S.A) in a sodium carbonate-bicarbonate buffer (pH 9.6). After overnight incubation at 4°C, plates were washed 5 times with PBS containing 0.05% (v:v) Tween 20 (PBS-T). Nonspecific protein binding sites were blocked with PBS containing 50 g/L 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 1:20; BAL 1:2), plates were incubated for 60 min at 37°C. After the plates were washed 5 times with PBS-T, peroxidase-conjugated goat anti-mouse IgA or IgG (anti-α chain specific # A4700, anti-γ chain specific # A3673 Sigma-Aldrich) was diluted 1:500 in PBS-T and 200 μL was added to each well. The plates were incubated at 37°C for 60 min and then washed 5 times with PBS-T. Plates were subsequently poured with 200 μL of a substrate solution [3–3′, 5–5′-tetramethylbenzidine (# T2885, Sigma-Aldrich)] 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 1 mol/L H_2SO_4. Readings were carried out at 493 nm (VERSAmax Tunable microplate reader) and the antibody concentration (g/L) in each unknown sample was determined from a standard curve made with commercial mouse IgA (Catalog # M-1421, Sigma-Aldrich) and mouse IgG (Catalog # I-5381, Sigma-Aldrich).

Phagocytic cell activation

Washburn test. Measurement of myeloperoxidase activity of blood and BAL neutrophils was carried out using a cytochemical method, with benzidine as a myeloperoxidase chromogen (16). Cells were graded as negative or weakly, moderately, or strongly positive and were used to calculate the score.

Nitroblue tetrazolium (NBT) test. The phagocytic bactericidal activity (oxidative burst) of macrophages and neutrophils was measured using the NBT reduction test (# 840-W, Sigma-Aldrich) in the pellet of BAL. NBT was added to each sample with (positive control) or without addition of the bacterial extract; then samples were incubated at 37°C for 20 min. In the presence of oxidative metabolites, NBT (yellow) is reduced to formazan, which forms a blue precipitate (17). Smears were prepared and, after staining, samples were examined under a light microscope for blue precipitates. A hundred cells were counted and the percentage of NBT positive (+) cells was determined.

Histopathology

At prechosen intervals, whole-lung samples from control and infected mice were excised and washed with PBS 0.01 mol/L, pH 7.2. Then, tissues were immersed in 4% (v:v) formalin saline solution. Once fixed, samples were dehydrated and embedded in Leica Histowax (Leica Microsystems Nussloch GmbH) at 56°C. Finally, lungs were cut into 4-μm serial sections and stained with hematoxylin-eosin for light microscopy examination.

Statistical analysis

Experiments were performed in triplicate and results were expressed as means ± SD. For body weight gain, 1-way ANOVA was

used. For all other determinations, 2-way ANOVA was used. Tukey's test (for pairwise comparisons of the means of the different groups) was used to test for differences between the groups. Differences were considered significant at $P < 0.05$.

RESULTS

Body weights. All repletion diets given to malnourished mice significantly increased body weights to the same extent (**Table 2**). Mice reached normal weight on d 14 of dietary treatment.

Bacterial cell counts in lung homogenates and blood. The pathogen was detected in lung and blood samples from WNC and MNC mice throughout the 10-d period, but MNC mice had significantly higher levels than WNC. The 21dBCD, 21dBCD+Lc, and 7dBCD+Lc replete groups did not differ from WNC (**Fig. 2***A*, *C*). The 7dBCD+2dLc group had negative hemocultures and significantly lower bacterial counts in lung than WNC mice (Fig. 2*A*). The other experimental groups had significantly lower bacterial counts than MNC mice but they did not reach the levels of WNC mice (Fig. 2*A*, *B*).

Total and differential numbers of blood leukocytes. Malnutrition decreased the total number of blood leukocytes and the proportion of neutrophils. These variables were significantly improved in all of the replete groups except group 7dBCD. Challenge with the pathogen increased the blood leukocytes of MNC and WNC mice; however, total and differential counts were significantly lower in MNC mice than in WNC. In mice in the 21dBCD, 21dBCD+Lc, and 7dBCD+2dLc groups, the number of leukocytes after challenge did not differ from those of WNC mice. The 7dBCD+2dLc treatment induced an earlier recovery of neutrophil counts in blood (**Table 3**).

Serum and BAL anti-pneumococcal antibodies. Levels of antipneumococcal immunoglobulins peaked on d 10 postinfection in all groups (**Table 4**).

Malnutrition significantly decreased serum and BAL IgG and IgA, which were significantly increased by all repletion treatments (Table 4). When malnourished mice were fed the BCD, serum IgG reached normal levels after 21 d of treatment. However, the 7dBCD+Lc treatment was sufficient to normalize the IgG response; mice in this group did not differ from those in the 7dBCD+2dLc group (Table 4). All treatments

TABLE 2

Body weight of malnourished mice fed BCD with or without supplemental Lactobacillus casei (Lc) for 7, 14, or 21 d or with BCD for 7 d with Lc supplementation on d 6 and d 7 (7dBCD+2dLc), before challenge with Streptococcus pneumoniae[1,2]

	Body weight, *g*		
Treatment day	BCD	BCD+Lc	BCD+2dLc
7	18.3 ± 0.5*	18.2 ± 0.5*	21.4 ± 0.5*
14	20.9 ± 0.6*	20.8 ± 0.6*	ND[3]
21	21.9 ± 0.7*	21.6 ± 0.6*	ND

[1] Data are means ± SD, $n = 10$. * Different from the malnourished control, $P < 0.05$.

[2] Well-nourished control: 22.3 ± 0.6 g; malnourished control: 9.2 ± 0.6 g.

[3] ND, not determined.

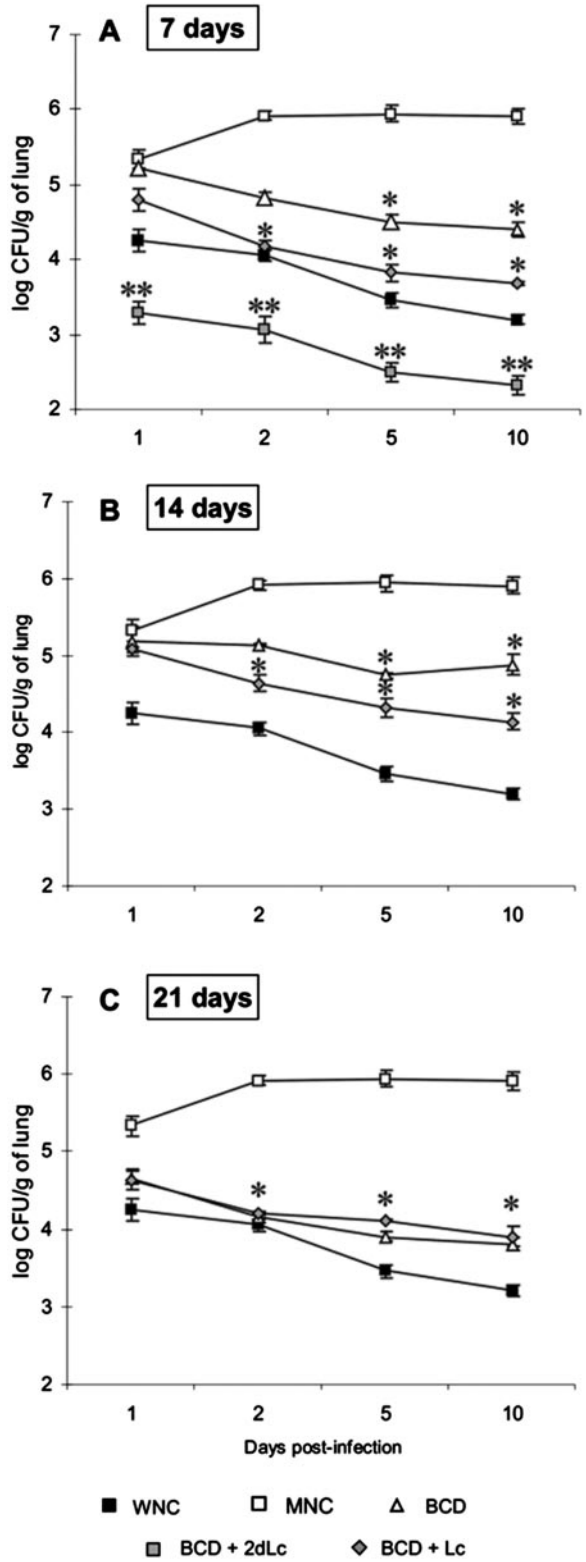

FIGURE 2 *Streptococcus pneumoniae* cfu counts in lung of malnourished mice replete with BCD with or without supplemental *Lactobacillus casei* for 7 (*A*), 14 (*B*), or 21 (*C*) d and challenged at the end of each dietary treatment. The cfu counts in lung of malnourished (MNC) and well-nourished (WNC) infected control mice are presented in *A*, *B*, and *C*. Results are expressed as means ± SD, $n = 6$. *Different from the MNC mice, $P < 0.05$. **Different from the MNC and WNC mice, $P < 0.05$.

TABLE 3

Number of blood leukocytes and neutrophils of malnourished mice replete with BCD with or without supplemental Lactobacillus casei (Lc) for 7, 14, or 21 d or with BCD for 7 d with Lc supplementation on d 6 and d 7 (7dBCD+2dLc), before (at the end of each dietary treatment) and after challenge (d 2 postinfection) with Streptococcus pneumoniae[1,2]

	Leukocytes		Neutrophils	
Treatment	Before challenge	After challenge	Before challenge	After challenge
	10^9 cells/L			
BCD+Lc				
7	3.3 ± 0.4[b]	7.3 ± 0.6[a]	0.7 ± 0.04[b]	1.0 ± 0.04[b]
14	3.9 ± 0.1[b]	7.9 ± 0.6[b]	0.8 ± 0.05[c]	1.1 ± 0.04[c]
21	6.3 ± 0.7[c]	9.7 ± 0.4[c]	0.9 ± 0.02[c]	1.0 ± 0.05[c]
7 + 2	3.8 ± 0.4[b]	8.7 ± 0.7[c]	0.8 ± 0.07[c]	1.2 ± 0.02[c]
BCD				
7	2.9 ± 0.7[a]	6.9 ± 0.7[a]	0.7 ± 0.03[b]	0.7 ± 0.01[a]
14	3.7 ± 0.3[b]	7.1 ± 0.2[b]	0.8 ± 0.05[c]	0.9 ± 0.07[b]
21	6.1 ± 0.4[c]	9.6 ± 0.1[c]	0.8 ± 0.06[c]	1.0 ± 0.01[c]
MNC	2.9 ± 0.5[a]	6.0 ± 0.4[a]	0.6 ± 0.05[a]	0.5 ± 0.06[a]
WNC	6.9 ± 0.3[c]	9.4 ± 0.5[c]	0.9 ± 0.07[c]	1.1 ± 0.07[c]

[1] Data are means ± SD, $n = 6$. Means in a column with a different superscript letter differ, $P < 0.05$.
[2] Before and after means were not compared statistically.

TABLE 4

Serum and bronchoalveolar antipneumococcal antibodies of malnourished mice replete with BCD with or without supplemental Lactobacillus casei (Lc) for 7, 14, or 21 d or with BCD for 7 d with Lc supplementation on d 6 and d 7 (7dBCD+2dLc) and challenged with Streptococcus pneumoniae at the end of each dietary treatment[1,2]

	Serum		BAL	
Treatment	IgG	IgA	IgG	IgA
	mg/L			
BCD+Lc				
7	24.28 ± 2.51[c]	7.77 ± 1.12[b]	1.59 ± 0.56[c]	2.30 ± 0.96[b]
14	13.09 ± 1.11[b]	9.43 ± 1.52[c]	1.67 ± 0.95[c]	1.52 ± 1.09[a]
21	27.89 ± 1.54[c]	10.09 ± 0.93[c]	1.63 ± 0.52[c]	2.08 ± 1.17[b]
7 + 2	28.20 ± 1.93[c]	9.28 ± 1.73[c]	1.75 ± 0.73[c]	4.69 ± 0.93[c]
BCD				
7	14.27 ± 2.18[b]	7.74 ± 2.01[b]	1.25 ± 0.43[b]	1.48 ± 1.15[a]
14	12.81 ± 1.14[b]	9.33 ± 2.07[c]	1.40 ± 0.85[b]	2.12 ± 1.19[b]
21	27.94 ± 1.39[c]	10.04 ± 1.35[c]	1.56 ± 1.01[c]	1.94 ± 0.98[b]
MNC	9.33 ± 1.60[a]	3.65 ± 1.61[a]	0.91 ± 0.99[a]	1.38 ± 1.18[a]
WNC	27.97 ± 1.81[c]	9.89 ± 2.07[c]	1.74 ± 0.91[c]	2.65 ± 0.99[b]

[1] Data are means ± SD arbitrary units, $n = 6$. Means in a column with a different superscript letter differ, $P < 0.05$.
[2] On d 10 postinfection.

improved the BAL IgG levels to the same extent (Table 4). Serum IgA reached normal values in mice administered the 7dBCD+2dLc treatment, whereas 14 d of consuming the BCD were necessary to obtain this effect (Table 4). Malnourished mice administered the 7dBCD+Lc treatment had a significant increase in BAL IgA, reaching the same levels as WNC mice, whereas the 7dBCD+2dLc group had higher levels of BAL IgA than the WNC mice (Table 4). On the basis of these results, we selected 7dBCD+2dLc as the optimal repletion diet; consequently, the following studies in BAL and lung were carried out with this experimental group.

BAL differential white cell counts. The number of leukocytes, neutrophils, macrophages, and lymphocytes in BAL was determined in 7dBCD+2dLc and 7dBCD replete mice and control groups (WNC and MNC).

Alveolar macrophages were the predominant resident leukocytes in the alveolar spaces of noninfected mice. WNC mice showed an important recruitment of neutrophils (**Table 5**) and macrophage emigration into alveoli, resulting in high leukocyte counts in BAL during the infection. MNC mice showed a significant impairment in the recruitment of neutrophils and macrophages (Table 5). However, 7dBCD+2dLc treatment was enough to normalize the response to the infection, with values of leukocytes, neutrophils, and macrophages similar to those in WNC mice. The mice treated with 7dBCD continued to have lower values than WNC mice.

Phagocytic cells. A decrease in neutrophil peroxidase activity was observed in MNC mice compared with WNC mice (**Table 6**). Malnourished mice replete with 7dBCD+2dLc treatment had significantly increased peroxidase scores in blood and BAL, with values similar to those of WNC mice (Table 6).

The bactericidal function of phagocytic cells in BAL, expressed as a percentage of NBT positive cells, was decreased in MNC (**Table 7**). A significant improvement in this variable was observed in malnourished mice when they were administered the 7dBCD+2dLc treatment. Thus, the oral administration of *L. casei* had a stimulatory effect on the function of phagocytes cells, causing a clear increase in the NBT positivity and in the peroxidase index compared with MNC.

Histopathological examination. Histopathological examination of lungs was made in the WNC, MNC, 7dBCD, and 7dBCD+2dLc groups. Morphological examination of lungs of WNC mice 10 d postinfection (0, 1, 5, and 10 d) showed progressive edema, inflammatory response, and alveolar congestion (**Fig. 3**A). These histopathological findings were more pronounced in the lungs of MNC mice, with progressive

TABLE 5

Total and differential count of broncoalveolar leukocytes of malnourished mice replete with BCD for 7 d or with BCD for 7 d with supplemental Lactobacillus casei on d 6 and d 7 (7dBCD+2dLc) and challenged with Streptococcus pneumoniae at the end of each dietary treatment[1,2]

Treatment	Leukocytes	Macrophages	Neutrophils
	10^7 cells/L		
7dBCD+2dLc	32.0 ± 1.0[c]	15.3 ± 0.8[c]	16.5 ± 0.4[c]
7dBCD	19.5 ± 1.2[b]	11.4 ± 0.1[b]	14.5 ± 0.3[b]
MNC	13.0 ± 0.8[a]	8.5 ± 0.7[a]	12.5 ± 0.2[a]
WNC	37.0 ± 1.3[c]	18.0 ± 0.1[c]	17.0 ± 0.4[c]

[1] Data are means ± SD, $n = 6$. Means in a column with a different superscript letter differ, $P < 0.05$.
[2] On d 5 postinfection.

TABLE 6

Blood and bronchoalveolar neutrophils peroxidase activity of malnourished mice replete with BCD for 7 d or with BCD for 7 d with supplemental Lactobacillus casei on d 6 and d 7 (7dBCD+2dLc) and challenged with Streptococcus pneumoniae at the end of each dietary treatment[1]

	Score number	
Treatment	Blood[2]	BAL[3]
7dBCD+2dLc	169.5 ± 3.4[c]	163.5 ± 3.4[c]
7dBCD+Lc	120.5 ± 5.1[b]	133.0 ± 2.2[b]
MNC	100.0 ± 2.8[a]	84.5 ± 6.4[a]
WNC	167.5 ± 4.3[c]	164.0 ± 5.1[c]

[1] Data are means ± SD, $n = 5$. Means in a column with a different superscript letter differ, $P < 0.05$.
[2] On d 2 postinfection.
[3] On d 5 postinfection.

parenchymal involvement, increasing fibrosis in bronchial walls and vessels, passage of blood elements from capillaries to tissues, hemorrhage, and widespread cellular infiltration. Furthermore, the lung parenchyma had a distorted appearance with loss of alveolar architecture (Fig. 3*B*). However, histological examination of lungs of the 7dBCD+2dLc group showed characteristics similar to those of WNC mice, with focal cellular infiltration and reduction of the alveolar airspaces but with preserved alveolar architecture (Fig. 3*D*). Mice administered 7dBCD showed histological signs intermediate to those of WNC and MNC mice (Fig. 3C).

DISCUSSION

Protein malnutrition and disease are frequently associated. Epidemiologic and clinical studies have clearly shown that populations with inadequate nutrition, in either quantitative or qualitative terms, have an increased susceptibility to infections (18,19). In our experimental model, pneumococcal colonization of lung and bacteremia were significantly greater in MNC mice compared with the WNC group. Although the number of bacteria in lungs and blood stream tended to decrease ($P < 0.05$) during infection in WNC mice, it remained constant in MNC mice. Feeding the BCD for 21 d was necessary to obtain levels of infection similar to those in WNC mice. However, the 7dBCD+2dLc treatment was enough to normalize the immune response to the infection in malnourished mice.

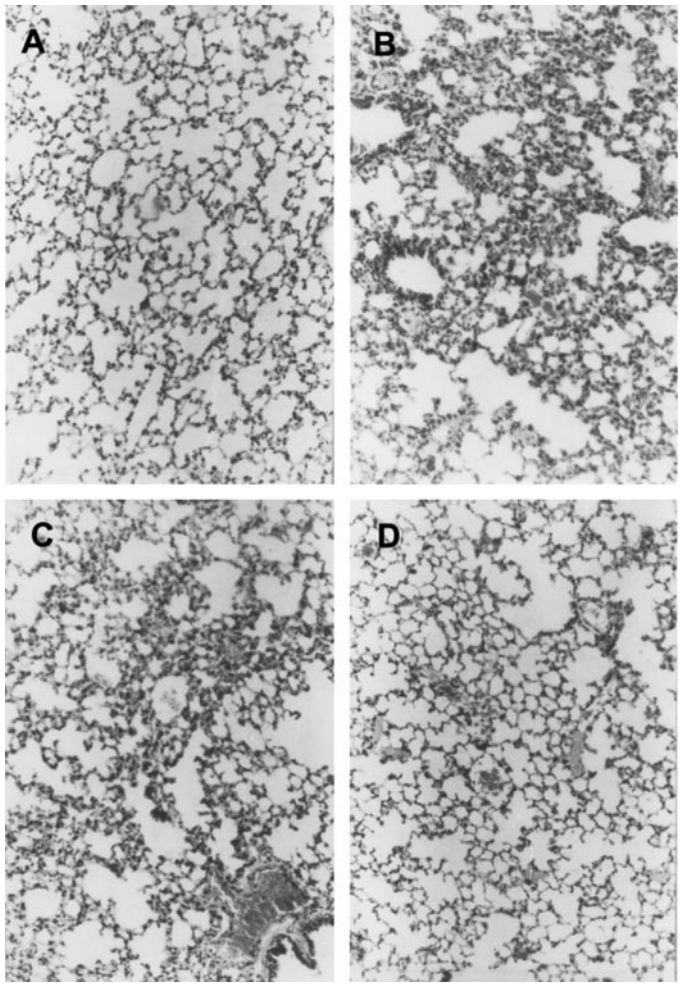

FIGURE 3 Lung architecture of well-nourished control mice (*A*), malnourished control (*B*), malnourished mice fed 7dBCD (*C*), and malnourished mice fed 7dBCD+2dLc (*D*) 10 d after challenge with *Streptococcus pneumoniae*. Light micrographs, original magnification X200.

TABLE 7

Bactericidal function of bronchoalveolar phagocytic cells of malnourished mice replete with BCD for 7 d or with BCD for 7 d with supplemental Lactobacillus casei on d 6 and d 7 (7dBCD+2dLc), before (at the end of each dietary treatment) and after challenge (d 5 postinfection) with Streptococcus pneumoniae[1,2]

	% NBT+ cells in BAL			
	Before challenge		After challenge	
Treatment	Unstimulated	Stimulated	Unstimulated	Stimulated
7dBCD+2dLc	19.1 ± 1.8[b]	27.7 ± 3.1[b]	53.4 ± 1.3[b]	68.4 ± 1.2[c]
7dBCD	17.6 ± 2.9[b]	18.8 ± 4.4[a]	40.2 ± 0.9[a]	51.6 ± 3.8[a]
MNC	10.6 ± 4.5[a]	17.5 ± 3.4[a]	37.3 ± 3.4[a]	47.9 ± 2.5[a]
WNC	17.4 ± 1.1[b]	25.7 ± 0.8[b]	46.8 ± 2.2[b]	62.6 ± 1.2[b]

[1] Data are means ± SD, $n = 6$. Means in a column with a different superscript letter differ, $P < 0.05$.
[2] Before and after means were not compared statistically.

Effective pulmonary host defense against respiratory pathogens is thought to be mediated mainly via phagocytosis by alveolar macrophages and recruited neutrophils (20,21). If pneumococci overcome these defenses and gain entry to the blood stream, systemic protection is afforded by anticapsular antibodies (22). In this work, a progressive increase in white blood cells was observed in WNC mice after infection, and differential cell counts indicated that this phenomenon involved mainly neutrophils. MNC mice showed a mild leukopenia before the infection, with all cell populations affected. A moderate increase was observed after challenge with pneumococci, but there was a decrease of leukocytes on d 5 postinfection, probably because the cell release from the bone marrow compartment was affected (23). Alveolar macrophages

were the predominant resident leukocytes in the alveolar spaces of noninfected mice, and only a few neutrophils could be recovered from that site. Infection rapidly triggered neutrophil recruitment into the alveoli of WNC mice, resulting in increased cell counts in BAL. There were significantly fewer neutrophils in BAL in MNC mice compared with the WNC group at the same period postinfection. Treatment with BCD with or without supplemental *L. casei* increased leukocytes and neutrophils in blood and BAL. However, mice administered the 7dBCD+2dLc treatment had significantly more leukocytes and greater phagocytic activity than those in the 7dBCD group. Consequently, *L. casei* was able to induce an earlier recovery of the innate immune response.

Malnutrition produces a remarkable decrease in the number of IgA-secreting cells associated with the lamina propria of the small intestine (24), and it probably has the same effect on other mucosal tissues. This would explain the significantly lower concentration of BAL IgA found in MNC mice in this work. The oral administration of an appropriate dose of *L. casei* CRL 431 was reported to enhance the number of IgA^+ cells in the intestine of malnourished mice (24) and to induce a significant increase in the levels of BAL IgA, IgM, and IgG after a *P. aeruginosa* infection in young mice (12,25,26). In the present work, when 7dBCD+2dLc treatment was administered, BAL antipneumococcal IgA reached higher levels than those in WNC mice. The IgA antibodies can bind the antigen and minimize its entry with a consequent reduction in inflammatory reactions, which prevents a potentially harmful effect on the tissue (8). The immune exclusion and elimination of the pathogen at the mucosal surfaces by secretory IgA is crucial in preventing pneumococcal bacteremia (27).

In addition, this report showed that 7dBCD+2dLc treatment increased serum and BAL antipneumococcal IgG, which reached levels similar to those in WNC mice. It was suggested that the most important factor contributing to protection and septicemia prevention might be the specific antipneumococcal serum IgG (22,28). Although the levels of antibodies that offer protection against pneumococcal disease have not been clearly defined, the response of serum antibodies and their opsonophagocytic activity represent the major defense mechanism of the host against pneumococcal bacteremia (2,29).

In the present study, histopathological examination of lung showed that tissue damage was less in mice treated with *L. casei*, despite the more intense inflammatory response. Alveolar macrophages play an essential role in the regulation of the lung inflammatory response during pneumococcal pneumonia (30). *L. casei* administration induces macrophage activation (12), which could partially explain the anti-inflammatory effect elicited by this strain. Moreover, it was reported that administration of *L. casei* CRL 431 for 2 d can induce anti-inflammatory cytokines such as interleukin-4 or -10 (31), and Blum et al. suggested that lactic acid bacteria could protect tissues from the deleterious effect of an ongoing inflammatory process (32).

Administration of *L. casei* for 2 d was a more effective in improving mucosal immune response than administration of this strain for 7 or 14 d. Therefore, even though the administration of *L. casei* has beneficial effects on the protection against *S. pneumoniae*, there would be a stage at which it would provoke an exaggerated inflammatory response as a consequence of the entrance into the gut of a large number of bacterial antigens. This effect would be counteracted by the appearance of suppressor T cells, which would prevent an undesirable effect in the gut but would provoke a decrease in immune stimulation. The increase in the mucosal immune response obtained with the 21dBCD+Lc treatment would be a consequence of the feeding period rather than of a stimulation of the lactic acid bacteria because no differences were found between the 21dBCD+Lc and the 21dBCD treatments.

The results of this study suggest that the addition of *L. casei* CRL 431 to the repletion diet fed to malnourished mice has a beneficial effect because it significantly accelerates the recovery of the defense mechanisms against an *S. pneumoniae* respiratory infection. However, much remains to be learned about the cellular and molecular mechanisms involved in the interaction of lactic acid bacteria and the mucosal immune system, especially if they are to be used as supplements for repletion diets or as vaccine vectors in immunosuppressed hosts.

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