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ORIGINAL ARTICLE

Enhanced immune response to pneumococcal infection in malnourished mice nasally treated with heat-killed *Lactobacillus casei*

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ABSTRACT

The present study analyzed whether nasal administration of viable and non-viable *Lactobacillus casei* CRL 431 to immunocompromised mice was capable of increasing resistance against *Streptococcus pneumoniae*. Weaned mice were malnourished after consuming a PFD for 21 days. Malnourished mice were fed a BCD for 7 days or BCD for 7 days with viable or non-viable *L. casei* nasal treatments on day 6 and day 7 (BCD+LcV and BCD+LcN, respectively). The MNC group received PFD whereas the WNC mice consumed BCD. MNC mice showed greater lung colonization, more severe lung injuries, impaired leukocyte recruitment and reduced antibodies and cytokine production when compared with WNC mice. Administration of *L. casei* increased the resistance of malnourished mice to the infection. Both BCD+LcV and BCD+LcN treatments prevented the dissemination of the pathogen to the blood and induced its lung clearance. BCD+LcV or BCD+LcN groups showed improved production of TNF-α and activity of phagocytes in the respiratory tract, an effect that was not observed in the BCD control group. In addition, IL-4 and IL-10 were significantly increased in BCD+LcV and BCD+LcN groups, which correlated with the increase in the levels of specific respiratory IgA. The nasal treatments with *L. casei* were also effective at stimulating the production of specific IgG at both the systemic and the respiratory levels. The comparative study between the viable and the non-viable bacteria demonstrated that viability would be an important factor to achieve maximum protective effects. However, the results from this study suggest that heat-killed lactic acid bacteria are also effective in the immunomodulation of the systemic and respiratory immune system.

Key words immunomodulation, *Lactobacillus casei*, malnutrition, pneumococcal infection.

Nutritional deficiencies are one of the most common causes of morbidity/mortality in children. Forty-nine per cent of the 10.7 million deaths per year worldwide among children below the age of 5 years are related to malnutrition (1). In these populations, susceptibility to infections is significantly high, especially those cause by respiratory pathogens such as *Streptococcus pneumoniae*. Adequate and prompt correction of the nutritional status is important to reduce morbidity and mortality. However, the cycle of malnutrition-infection cannot be stopped merely by improving nutritional intake, especially in the presence of repeated exposure to pathogens (2). Greater attention to

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List of Abbreviations: BAL, bronchoalveolar lavage; BCD, balanced conventional diet; Ig, immunoglobulin; IL, interleukin; LAB, lactic acid bacteria; LcN, non-viable *L. casei*; LcV, viable *Lactobacillus casei*; MNC, malnourished control; NBT, nitroblue tetrazolium; PFD, protein-free diet; TNF, tumor necrosis factor; WNC, well-nourished control.

the enhancement of the immune system in malnourished individuals is necessary in order to diminish mortality rates.

Our research group studied the effect of LAB in the prevention of respiratory infections in immunocompetent hosts during the first stage and immunocompromised (malnourished) hosts during the second. We demonstrated that malnourished animals have a greater susceptibility to infection by *S. pneumoniae* than normal animals and that the addition of *Lactobacillus casei* CRL 431 to the repletion diet significantly decreases the time required for the normalization of the immune response against the infection (3, 4).

Taking into consideration the fact that there is literature that claims that antigens given by the nasal route can induce systemic and respiratory immune responses superior to that obtained using oral stimulation (5, 6), the aim of the present work was to analyze whether the nasal administration of *L. casei* CRL 431 to mice immunocompromised by protein malnutrition is capable of increasing resistance to challenge with the respiratory pathogen *S. pneumoniae*.

There are some studies that demonstrate the influence of oral administration of non-viable LAB on the immune response (7). In this sense, and considering the advantages of the administration and handling of non-viable microorganisms, especially in immunocompromised hosts, we studied the role of the viability of probiotic bacteria on respiratory immune stimulation by comparing the effects of viable and non-viable *L. casei* on innate and specific immune responses against pneumococcal infection.

MATERIALS AND METHODS

Animals and treatment procedures

Male 3-week-old Swiss albino mice were obtained from a closed colony kept at CERELA (Chacabuco 145, San Miguel de Tucumán, Argentina). Mice were housed individually during the experiments and the assays for each parameter studied were performed on 5–6 mice per group for each time point. Weaned mice were malnourished for 21 days by being fed with a PFD (3, 4). At the end of this period, animals that weighed 40–55% less than well-nourished control mice were selected for experiments. Different groups of mice were fed for 7 days with a BCD. During the last 2 days of the repletion treatment the mice received *Lactobacillus casei* CRL 431 (10^9 cells/mouse per day) by the nasal route. The bacterium was given viable (BCD+LcV group) or non-viable (BCD+LcN group). Three different groups were used as controls: malnourished mice that were renourished for 7 days with BCD without treatment with the LAB (BCD group); MNC group that was fed only the PFD; and the WNC mice that consumed BCD ad libitum. Experiments were approved by the Ethical Committee of animal care at CERELA.

Microorganisms

Lactobacillus 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 litre, pH 7. It was cultured for 8 hr at 37°C (final log phase) in Man-Rogosa-Sharpe broth (MRS; Oxoid, Cambridge, UK). The bacteria were harvested through centrifugation at 3000 $\times g$ for 10 min and washed three times with sterile 0.01 mol/litre PBS, pH 7.2. Non-viable *L. casei* was obtained as follows: bacteria were killed by tyndallization in a water bath at 80°C for 30 min and the lack of bacterial growth was confirmed using MRS agar plates.

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, one of the 10 most frequent serotypes isolated in pneumococcal infections in Argentina (serotypification was carried out at the Administración Nacional de Laboratorios e Institutos de Salud, Buenos Aires, Argentina).

Experimental infection

For the challenge experiments we used 10 LD50 of the pneumococcal strain (serotype 14), which was determined in malnourished control mice (3). *S. pneumoniae* was first grown on blood agar for 18 hr; freshly grown colonies were suspended in Todd Hewitt broth (Oxoid) and incubated at 37°C overnight. The pathogens were harvested through centrifugation at 3000 $\times g$ for 10 min at 4°C and then washed three times with sterile PBS. Cell density was adjusted to 4×10^4 cells/litre. The size of inoculum was confirmed by serial dilution and quantitative subculture on blood agar. The infecting dose was chosen on the basis of bacterial cell counts recovered from the blood of animals suffering severe pneumonia (3, 8). Challenge with *S. pneumoniae* was carried out on the day after the end of each treatment (day 8). Mice were infected by dropping 25 ul of the inoculum containing 10^5 log-phase cells of *S. pneumoniae* in PBS into each nostril and involuntarily inhaled. To facilitate migration of the inoculum to the alveoli, mice were held in a vertical position for 2 min. Well-nourished and malnourished (without treatment) controls were infected equally. Animals were killed at day 0 (before challenge) and at different days after infection.

Bacterial cell counts in lung homogenates and blood

Mice were killed at 1, 2, 5, and 10 days after challenge with pneumococcus and their lungs were excised, weighed and homogenized in 5 ml sterile peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar and incubated 18 hr at 37°C. *S. pneumoniae* was identified by standard techniques (9) 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 CFU/ml.

Histopathology

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

Leukocytes in blood and bronchoalveolar lavage

Blood samples were obtained through cardiac puncture at the end of each treatment (day 0) and on day 2 after challenge and collected in heparinized tubes. The number of leukocytes was determined with a hemocytometer. BAL samples were obtained according to the technique previously described (3, 9); briefly, the trachea was exposed and intubated with a catheter and two sequential BAL were carried out in each mouse by injecting 0.5 ml sterile PBS. The fluid was used to determine the total number of leukocytes using a hemocytometer.

Phagocytic cell activation

Washburn test

Myeloperoxidase activity of blood and BAL neutrophils was carried out using a cytochemical method, with benzidine as a myeloperoxidase chromogen. Cells were graded as negative or positive weak, moderate or strong, and were used to calculate the score (3).

Nitroblue tetrazolium test

The phagocytic bactericidal activity (oxidative burst) of macrophages and neutrophils was measured using the NBT reduction test (Sigma-Aldrich, St Louis, MO, USA) in the pellet of BAL. NBT was added to each sample with (positive control) or without addition of bacterial extract and 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 (10). Smears were then prepared and, after staining, samples were examined under a light microscope for blue precipitates. Then, 100 cells were counted and the percentage of NBT-positive (+) cells was determined.

Antibodies from serum and BAL

A previously developed ELISA technique was used to measure anti-pneumococcal antibodies (IgA, IgM and IgG) in serum and BAL (3, 10). BAL and serum samples were obtained as described above and antibodies were determined at day 10 post-challenge. Briefly, each plate was coated with 200 μl of a 1:100 dilution of heat-killed *S. pneumoniae* in a sodium carbonate-bicarbonate buffer (pH 9.6). After overnight incubation at 4°C, plates were washed five times with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Non-specific protein binding sites were blocked with PBS containing 5% non-fat dry milk for 30 min at room temperature. After 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 five times with PBS-T, peroxidase-conjugated goat antimouse IgA, IgM, IgG, IgG1 or IgG2a (Sigma-Aldrich) was diluted 1:500 in PBS-T and 200 μl were added to each well. Then, the plates were incubated at 37°C for 60 min and washed afterwards five times with PBS-T. Plates were subsequently poured with 200 μl of a substrate solution [3–3′, 5-5′-tetramethylbenzidine 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/litre H_2SO_4. Readings were carried out at 493 nm (VERSAmax Tunable microplate reader; MDS Analytical Technologies, Sunnyvale, CA, USA) and the antibody concentration in each unknown sample was expressed as mg/litre against a standard curve made with commercial mouse IgA, IgM or IgG (Sigma-Aldrich).

Cytokine concentrations in BAL

Acellullar BAL fluid was obtained as described above. TNF-α, IL-4, and IL-10 concentrations in BAL were measured with commercially available enzyme-linked immunosorbent assay kits following the manufacturer's recommendations (R and D Systems, Minneapolis, MN, USA).

Statistical analysis

Experiments were carried out in triplicate and results were expressed as the mean ± standard deviation (SD).

Table 1. Bacterial cell counts in lung (log CFU/g lung) and blood (CFU/ml) after challenge with 10^5 cells of *Streptococcus pneumoniae*

	Day 1		Day 2		Day 5		Day 10	
Group	Lung	Blood	Lung	Blood	Lung	Blood	Lung	Blood
WNC	4.25 ± 0.14^{b}	4.01 ± 0.21^{b}	4.05 ± 0.08^{b}	4.23 ± 0.09^{b}	3.46 ± 0.09^{c}	3.91 ± 0.07^{b}	3.20 ± 0.12^{c}	3.75 ± 0.09^{b}
MNC	5.33 ± 0.14^{a}	5.11 ± 0.12^{a}	5.92 ± 0.06^{a}	5.31 ± 0.12^{a}	5.94 ± 0.11^{a}	5.61 ± 0.10^{a}	5.91 ± 0.11^{a}	5.73 ± 0.23^{a}
BCD	5.21 ± 0.01^{a}	3.22 ± 0.05^{c}	4.82 ± 0.07^{b}	3.51 ± 0.07^{c}	4.51 ± 0.08^{b}	3.25 ± 0.04^{b}	4.40 ± 0.09^{b}	3.45 ± 0.05^{b}
BCD+LcN	4.18 ± 0.09^{b}	$<1.5^{d}$	4.42 ± 0.07^{b}	$<1.5^{d}$	4.27 ± 0.03^{b}	$<1.5^{c}$	$<1.5^{d}$	$<1.5^{c}$
BCD+LcV	4.12 ± 0.06^{b}	$<1.5^{d}$	4.21 ± 0.06^{b}	$<1.5^{d}$	$<1.5^{d}$	$<1.5^{c}$	$<1.5^{d}$	$<1.5^{c}$

Malnourished mice replete with BCD or BCD with non-viable or viable *Lactobacillus casei* (BCD+LcN and BCD+LcV groups respectively) by the nasal route for 2 days. Malnourished control (MNC) and well-nourished control (WNC) mice were used. Results are expressed as means ± SD ($n = 6$ mice/group at each time point).

[a,b,c,d]Means in a column with a different superscript letter differ, $P < 0.05$, (a < b < c < d).

Data were evaluated by one-way or two-way ANOVA tests. Tukey's test (for pairwise comparisons of the mean values of the different groups) was used to test for differences between the groups. Significant difference was defined as $P < 0.05$.

RESULTS

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-day period, but MNC mice had significantly higher levels than the WNC group (Table 1). Treatment with BCD decreased the pathogen counts in blood and lung compared to the MNC group. The animals treated with BCD+LcN and BCD+LcV showed greater resistance to the challenge, as they showed pathogen counts in lung significantly lower than those in the WNC group. The mice in the BCD+LcV group showed negative pathogen counts in the lung from day 5 post-infection onwards, whereas the BCD+LcN group did so only from day 10 onwards. Besides, both treatments prevented the dissemination of *S. pneumoniae*, as the hemocultures in both groups were negative during the entire study period (Table 1).

Lung tissue injury

The effects of pneumococci on the lung structure were assessed by studying histological changes. The histopathological study revealed significant lung injury in the MNC mice with a marked reduction in the alveolar spaces, an intense inflammatory response, fibrosis, and hemorrhage (Fig. 1b). The histological changes found in the lungs of the WNC mice were always less severe than those in the lungs of the MNC group (Fig. 1a). Mice treated with BCD showed histological signs intermediate to those of WNC and MNC mice (Fig. 1c). When viable or non-viable *L. casei* were used in the repletion treatments (BCD+LcN and BCD+LcV groups), the injury caused by the pathogen to the lung tissue decreased significantly. The histological characteristics of BCD+LcN and BCD+LcV lungs were similar to those of the WNC group during the entire period assayed (Fig. 1d,e).

Number of leukocytes and phagocytic cell activation in the respiratory tract

No differences were observed between the groups with respect to the number of leukocytes and the activity of the phagocytic cells in BAL before challenge with the pathogen (Fig. 2). The infection significantly increased the values of macrophages, neutrophils and NBT+ cells in all experimental groups. However, malnourished mice presented significantly lower values with respect to the WNC group. The treatment with BCD increased the levels of leukocytes in BAL but not the activity of the phagocytic cells, as the percentage of NBT+ cells was similar to that found in the MNC group. The mice replete with BCD+LcN and BCD+LcV presented macrophage and neutrophil counts and percentages of NBT+ cells in BAL significantly higher than those in the WNC group (Fig. 2).

Number of leukocytes and phagocytic cell activation in blood

Malnutrition decreased significantly the number of blood leukocytes, neutrophils and peroxidase activity (Fig. 3). The treatment with BCD was unable to normalize these parameters; however, blood leukocytes and neutrophil number and peroxidase activity were significantly improved with BCD+LcV and BCD+LcN treatments (Fig. 3). The challenge with *S. pneumoniae* increased the number of leukocytes and the activity of the phagocytic cells in blood in all the experimental groups, but the MNC mice presented significantly lower values than those found

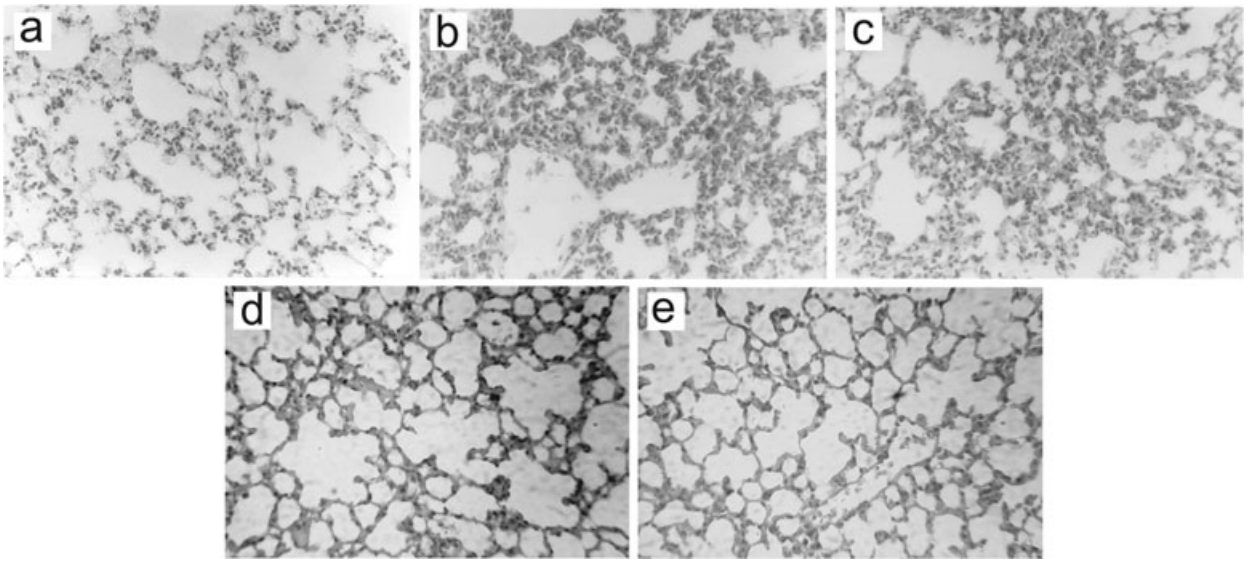

Fig. 1. Histological examination of lungs on day 10 after challenge with *Streptococcus pneumoniae*. (a) Well-nourished control mice, (b) malnourished control, (c) malnourished mice replete with BCD, (d) malnourished mice replete with BCD and treated with non-viable *Lactobacillus casei* for 2 days by the nasal route, (e) malnourished mice replete with BCD and treated with viable *L. casei* for 2 days by the nasal route. Light micrographs, original magnification ×400.

in the WNC group (Fig. 3). The treatment with BCD did not induce modifications in the number of leukocytes but increased the peroxidase activity, although it did not reach the values of the WNC group. The BCD+LcN and BCD+LcV groups showed leukocyte values similar to those of the WNC group. However, in these groups, the peroxidase activity was significantly higher than that of the WNC mice (Fig. 3).

Anti-pneumococcal antibodies in the respiratory tract

Previous studies in our laboratory demonstrated that after challenge with *S. pneumoniae,* antibodies specific to the pathogen increased progressively until they reached a maximum on day 10 post-infection (3, 8). That is why, in order to evaluate the effect of the nasal treatments on the humoral immune response, we studied the levels of those antibodies on day 10 post-infection, both in the respiratory tract and at the systemic level. The specific antibodies for the pathogen in BAL were significantly lower in the MNC group compared to the WNC mice (Fig. 4). Treatment with BCD induced an increase in the levels of IgG and IgM, which reached values similar to those of the WNC group (Fig. 4a,c). However, the level of IgA remained similar to those in the MNC group (Fig. 4b). The treatment with BCD+LcN normalized the levels of IgG and IgM whereas the animals treated with BCD+LcV presented levels of specific IgG higher than those in the WNC group (Fig. 4a). Both treatments increased the levels of IgA in BAL, which were significantly higher than those in the WNC group (Fig. 4b). However, the levels of IgA in BAL in the BCD+LcV group were higher than those in the BCD+LcN mice.

We also studied the levels of the IgG subclasses: IgG1 and IgG2a. Malnourished mice presented IgG1 and IgG2a levels in BAL lower than those in the WNC group (Fig. 4d,e). Repletion with BCD increased the levels of IgG2a, but did not induce modifications in the values of IgG1 with respect to the MNC group. The treatments with BCD+LcV and BCD+LcN significantly increased the levels of IgG1 and IgG2a, although the effect was different: while treatment with BCD+LcN normalized the values of those antibodies, the animals treated with BCD+LcV presented values higher than those in the WNC mice (Fig. 4d,e).

Anti-pneumococcal antibodies in serum

Malnourished mice presented IgG, IgM, IgA, IgG1 and IG2a levels in serum significantly lower than those in the WNC group (Fig. 5). Repletion with BCD increased the levels of IgM, IgA and IgG1, although only IgM reached levels similar to those in the WNC group (Fig. 5c). The treatment with BCD+LcN increased the levels of all the antibodies studied. In that group, the mice reached normal levels of IgG, IgG1 and IgA. Nasal treatment with the viable bacterium was more effective than treatment with the non-viable bacterium with respect to the increase in the specific antibodies, as the levels of IgG, IgG1 and IgG2a

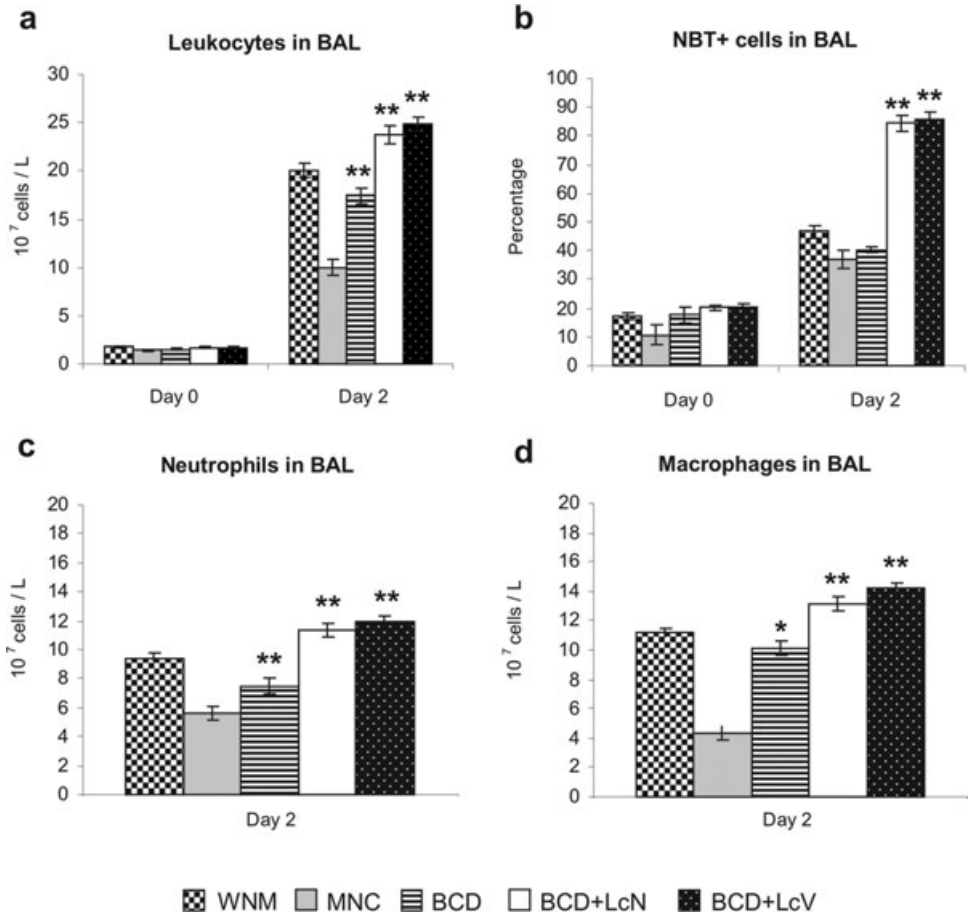

Fig. 2. Counts of bronchoalveolar leukocytes (a), neutrophils (c) and macrophages (d) and microbicidal activity of BAL phagocytes (b) before (day 0) and after challenge (day 2) with 10^5 cells of *Streptococcus pneumoniae*. Malnourished mice replete with BCD or BCD with non-viable or viable *Lactobacillus casei* (BCD+LcN and BCD+LcV groups, respectively) by the nasal route for 2 days. MNC and WNC mice were used. Results are expressed as means ± SD ($n = 6$ mice/group at each time point). *Different from MNC mice and no differences with the WNC group ($P < 0.05$). **Different from the MNC and WNC groups ($P < 0.05$).

were significantly higher than those in the WNC group (Fig. 5a,d,e).

Cytokines

The levels of TNF-α in BAL before the infection were similar in all groups (Fig. 6a). This finding indicates that malnutrition does not alter basal levels of BAL TNF-α. After the challenge with *S. pneumoniae*, this cytokine increased significantly in all the experimental groups. However, BAL TNF-α levels were significantly lower in the MNC mice than in the WNC group. Mice fed the BCD treatment showed higher values than MNC mice but they did not reach the levels of the WNC group. However, mice in the BCD+LcN and BCD+LcV groups showed levels of BAL TNF-α similar to those of the WNC group.

Malnourished control mice showed basal levels of IL-10 and IL-4 significantly lower than those of the WNC mice (Fig. 6b,c). Repletion with BCD normalized the basal levels of both cytokines. Mice treated with BCD+LcN and BCD+LcV showed normal levels of IL-4 but levels of IL-10 were significantly higher than those in the WNC group. The infection increased IL-4 and IL-10 values in all experimental groups; however, these cytokines in the WNC mice were significantly higher than those in the MNC mice. Repletion with BCD was capable of normalizing the IL-10 response to infection, but not the levels of IL-4, which presented values between the WNC and the MNC groups. Both BCD+LcN and BCD+LcV significantly improved the production of IL-4 and IL-10, which showed levels higher that those of the WNC group. However, the levels of IL-4 and IL-10 were significantly higher in the BCD+LcV mice than in the BCD+LcN group (Fig. 6b,c).

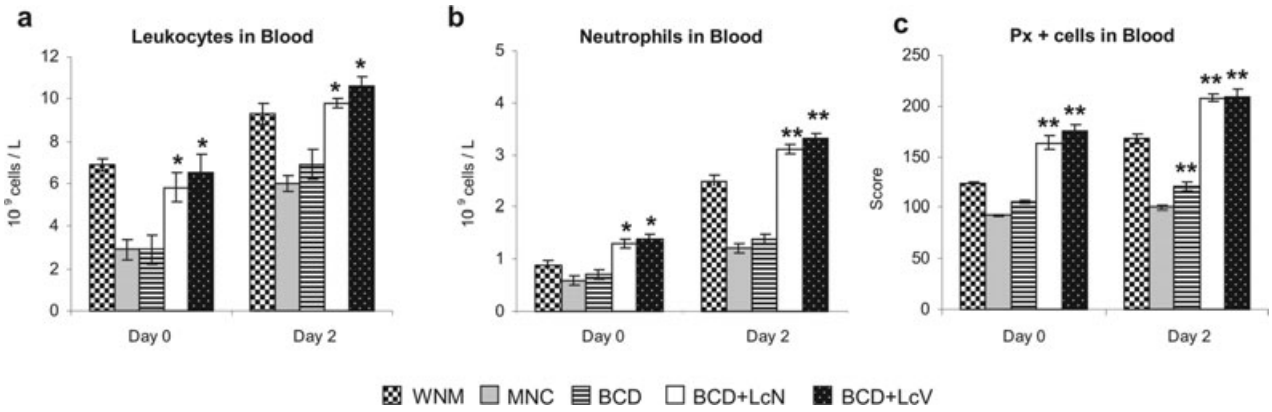

Fig. 3. Counts of blood leukocytes (a), neutrophils (b) and neutrophil peroxidase activity (c) before (day 0) and after challenge (day 2) with 10^5 cells of *Streptococcus pneumoniae*. Malnourished mice replete with BCD or BCD with non-viable or viable *Lactobacillus casei* (BCD+LcN and BCD+LcV groups, respectively) by the nasal route for 2 days. MNC and WNC mice were used. Results are expressed as mean ± SD ($n = 6$ mice/group at each time point). *Different from MNC mice and no differences with the WNC group ($P < 0.05$). **Different from the MNC and WNC groups ($P < 0.05$).

DISCUSSION

The results described in the present paper show for the first time that nasal administration of the probiotic bacterium *L. casei* CRL 431 is able to significantly increase the resistance of malnourished mice against a respiratory pathogen.

The use of non-recombinant lactic acid bacteria nasally given to prevent respiratory infections has been sparsely studied. Studies by Cangemi de Gutierrez *et al.*

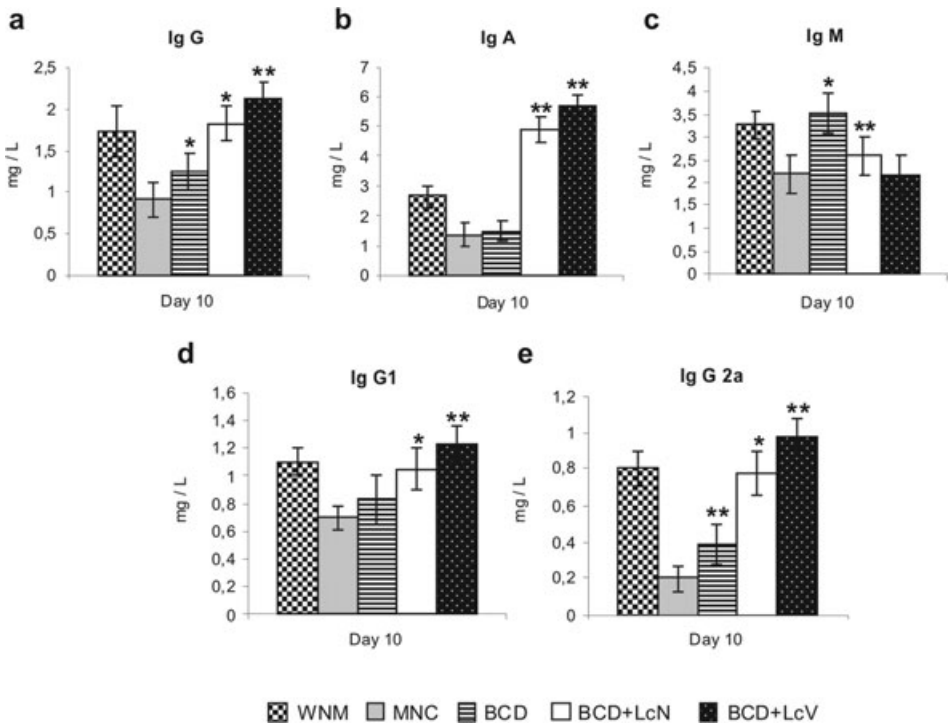

Fig. 4. BAL anti-pneumococcal antibodies IgG (a), IgA (b), IgM (c), IgG1 (d) and IgG2a (e) after challenge (day 10) with 10^5 cells of *Streptococcus pneumoniae*. Malnourished mice replete with BCD or BCD with non-viable or viable *Lactobacillus casei* (BCD+LcN and BCD+LcV groups, respectively) by the nasal route for 2 days. MNC and WNC mice were used. Results are expressed as mean ± SD ($n = 6$ mice/group at each time point). *Different from MNC mice and no differences with the WNC group ($P < 0.05$). **Different from MNC and WNC groups ($P < 0.05$).

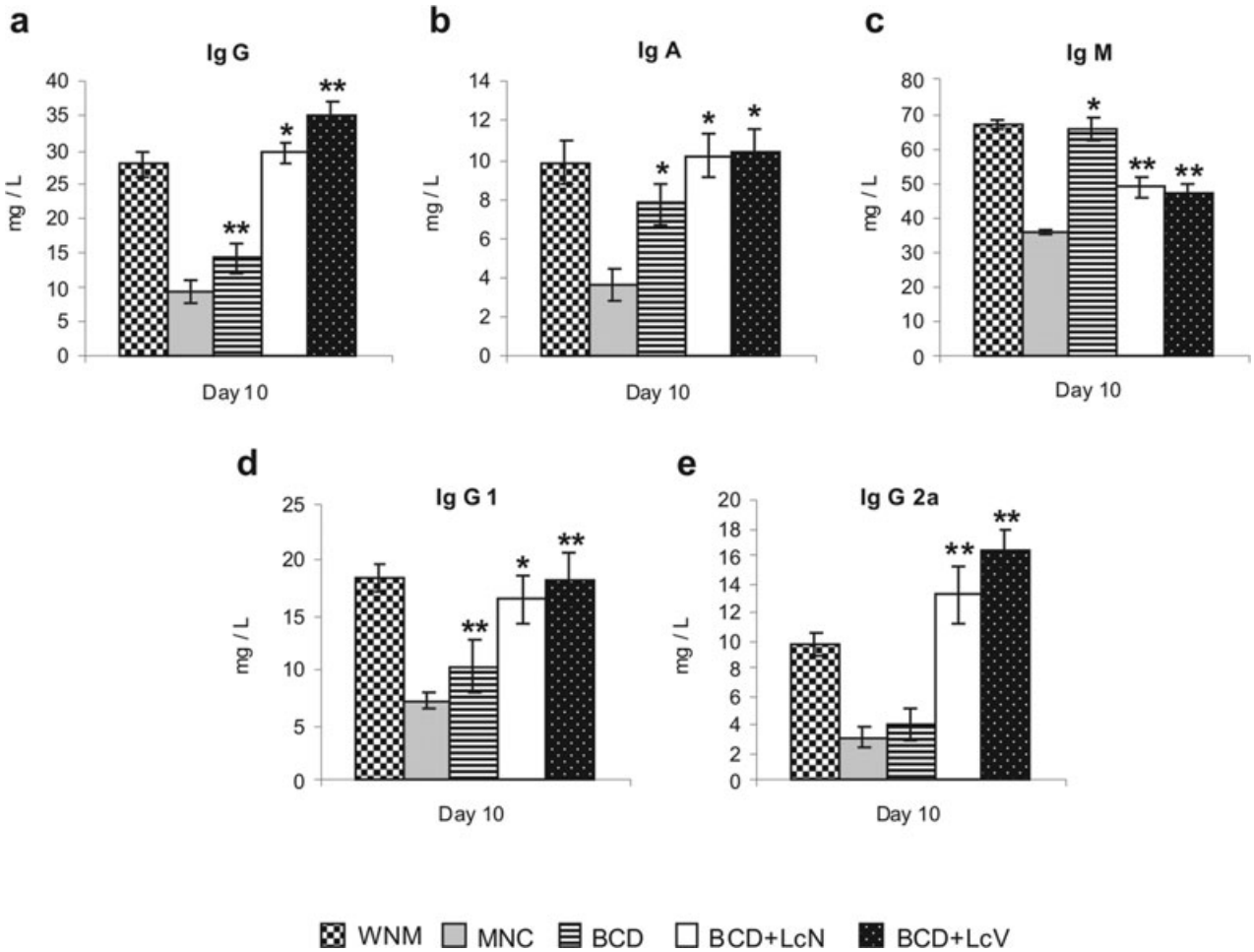

Fig. 5. Serum anti-pneumococcal antibodies IgG (a), IgA (b), IgM (c), IgG1 (d) and IgG2a (e) after challenge (day 10) with 10^5 cells of *Streptococcus pneumoniae*. Malnourished mice replete with BCD or BCD with non-viable or viable *Lactobacillus casei* (BCD+LcN and BCD+LcV groups, respectively) by the nasal route for 2 days. MNC and WNC mice were used. Results are expressed as mean ± SD ($n = 6$ mice/group at each time point). *Different from MNC mice and no differences with WNC group ($P < 0.05$). **Different from MNC and WNC groups ($P < 0.05$).

demonstrated that the intranasal administration of *Lacobacillus fermentum*, isolated from the pharynx of BALB/c mice, was able to reduce nasal and pharynx colonization of *S. pneumoniae* and to reduce pathogen counts in the lung (11). Later, our laboratory evaluated the effect of nasal administration of *L. lactis* NZ9000 to Swiss albino mice and demonstrated that such treatment was able to increase the clearance rate of *S. pneumoniae* from the lung and to prevent the dissemination of pneumococci into blood. It was also demonstrated that the protective effect was related to an upregulation of the innate and specific immune responses in both respiratory and systemic compartments (9). However, Hori *et al.* (12) studied the effect of the nasal administration of a non-viable lactic acid bacteria on respiratory immunity and observed that nasal treatment of adult BALB/c mice with non-viable *L. casei* Shirota was able to stimulate cellular immunity in the respiratory tract and to increase significantly the resistance of mice to the infection with influenza virus (12). No other reports on protection against respiratory infection by intranasal administration of probiotics have yet been published. Moreover, in the three studies mentioned above, adult immunocompetent animals were used, so that the effect of a nasal treatment with LAB in immunocompromised hosts has not been studied yet.

The present work demonstrates that the nasal treatment of malnourished mice with *L. casei* CRL 431 was able to increase their resistance to the infection with the respiratory pathogen *S. pneumoniae*. The protective effect achieved with the nasal stimuli was significantly higher than that achieved with the oral administration of the same probiotic strain (3). Both the administration of BCD+LcV

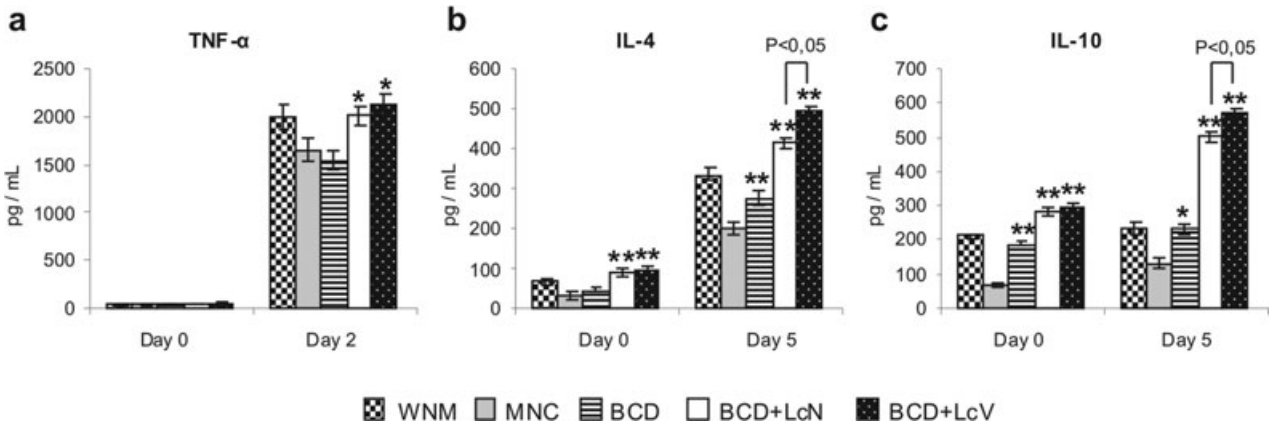

Fig. 6. TNF-α (a), interleukin IL-4 (b) and IL-10 (c) concentrations in bronchoalveolar lavages before (day 0) and after challenge (day 2) with 10^5 cells of *Streptococcus pneumoniae*. Malnourished mice replete with BCD or BCD with non-viable or viable *Lactobacillus casei* (BCD+LcN and BCD+LcV groups, respectively) by the nasal route for 2 days. MNC and WNC mice were used. Results are expressed as mean ± SD ($n = 6$ mice/group at each time point). *Different from MNC mice and no differences with the WNC group ($P < 0.05$). **Different from the MNC and WNC groups ($P < 0.05$).

and of BCD+LcN prevented the dissemination of the pathogen to the blood and induced its lung clearance, whereas the oral treatment, although it prevented the passage of the pathogen to the blood and decreased bacterial counts in the lung, did not induce the complete clearance of *S. pneumoniae* from the lung during the period under study (3).

The protective effect of nasal treatment with *L. casei* against the pneumococcal colonization of lung, bacteremia and lung tissue injury was correlated with the stimulation of the systemic and respiratory immune response.

Protection of the respiratory tract against pathogens relies on both innate and specific defense mechanisms located in the airways and in the alveolar space (13). Alveolar macrophages constitute the first line of phagocytic defense against infectious agents (14). In addition, when the invading pathogens are too virulent or represent too large a load to be contained by macrophages alone, alveolar macrophages are capable of generating chemokines and pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 that orchestrate the recruitment of neutrophils (14, 15). In the present study, we observed that infection of WNC mice with *S. pneumoniae* increased the levels of TNF-α in the respiratory tract and rapidly triggered leukocyte recruitment into the alveoli, resulting in increased cell counts in BAL and in blood. In addition, activity of BAL and blood phagocytes was enhanced in this group after the challenge. Malnutrition significantly impaired these immune mechanisms in both the respiratory and systemic compartments. MNC mice showed suppression of neutrophil recruitment into the respiratory tract and significantly fewer neutrophils in blood when compared with the WNC group. Repletion with BCD did not exert any effect on neutrophils; however, when malnourished mice were treated with BCD+LcV or BCD+LcN, they showed improved production of TNF-α and the number and activity of phagocytes.

The administration of BCD+LcV or BCD+LcN did not induce changes in the phagocytic activity of alveolar macrophages before challenge (day 0); however, greater production of TNF-α after challenge was observed. Thus, it is possible that nasal treatments do not modify phagocytic activity, but improve the capacity of macrophages to secrete cytokines in the presence of a pathogen. It has been demonstrated that the interaction of *L. casei* CRL 431 with immune cells associated with the gut induced an increase in the expression of TLR-2 and CD-206 receptors in macrophages and dendritic cells (20). It is possible that nasal administration of *L. casei* CRL 431 has a similar effect on the respiratory mucosa.

However, we observed that nasal administration of *L. casei* to malnourished mice increased the number of leukocytes and the peroxidase activity in the blood before the challenge with the pathogen. These results suggest that treatment with probiotic bacteria exerts an effect on hematopoiesis. In this sense, we have made progress in the present study as to the effect of the oral administration of probiotics in bone marrow. We demonstrated that the use of *L. casei* CRL 431, as a supplement in a repletion diet, induced an improvement in the production and maturation of myeloid and lymphoid cells (21).

In the early phase of infection, the innate immune response has greater relevance and this response would be involved in preventing pneumococcal blood invasion. Our previous studies demonstrated that after challenge with

S. pneumoniae, the levels of neutrophils in blood increase and remain elevated until day 5 post-infection (3, 21). In addition, several studies demonstrated that the phagocytic and microbicidal activity of neutrophils from blood and those recruited into the lung are of great importance for the control of pneumococcal infection (22, 23). The increased number and activity of phagocytic cells in the blood during the first days post-infection in the BCD+LcV and BCD+LcN groups would explain why no bacteria were detected in the blood in these groups.

We also evaluated the effect of nasal treatments on the specific immune response. It is known that the humoral immune response in the respiratory tract can be divided into two functionally distinct compartments: the conducting airways overlaid by mucosal tissue and the lung parenchyma (16). Under inflammatory conditions, cytokines in the airways environment change dramatically. When a Th2 response is needed, there is a production of IL-4, IL-5, IL-6 and IL-10, which contributes to stimulate B cells to proliferate and mature into polymeric IgA-producing cells and to develop specific antibodies (5, 17). This specific IgA in the respiratory tract during an infectious process is important to prevent colonization of mucosal tissues and subsequent spreading into the systemic circulation (18). Additionally, specific IgA antibodies can bind antigens and minimize their entry with a consequent reduction in inflammatory reactions, which prevents potentially harmful effects on the tissue. In our study, malnutrition significantly affected the production of specific IgA and BCD was unable to recover this parameter. On the contrary, the administration of BCD+LcV or BCD+LcN significantly improved production of specific IgA. Moreover, IL-4 and IL-10 were significantly increased in the respiratory tract of animals that received both BCD+LcV or BCD+LcN, which correlates with the increase in the levels of specific IgA in BAL.

In the deep lung, when *S. pneumoniae* reaches the alveolar space, there is a local production of specific IgG anti-pneumococcal antibodies. These opsonizing IgG antibodies are important for complement fixation and for enhancing the efficiency of macrophage killing. This immune activation also induces the production at the systemic level of antibodies responsible for preventing the passage of pathogens to the blood and their subsequent dissemination (16, 18). Malnutrition significantly affected the production of specific IgG both systemic and respiratory; and BCD was unable to recover their levels. However, nasal treatments with *L. casei* significantly improved production of IgG at both the systemic and the respiratory levels.

In addition, we found that both the IgG1 and the IgG2a anti-pneumococcal antibodies were higher in BCD+LcV and BCD+LcN mice compared to the BCD and MNC groups. Although data concerning the role of different IgG subclasses in the protection against pneumococcal infection is limited, it seems that the IgG2a subclass is particularly important, as it is the primary murine IgG subclass that mediates optimal complement fixation (19). In addition, although IgG1 is not efficient for complement fixation, it might also contribute to protection against pneumococcal infection through Fc receptor binding or by preventing attachment and colonization of bacteria at mucosal surfaces. Thus, the production of both types of antibodies would also explain the increased resistance of BCD+LcV and BCD+LcN mice against infection.

The comparative study between the viable and the non-viable bacteria demonstrated that viability would be an important factor to achieve maximum protective effects. Several investigators found that nasal administration of *L. fermentum* was able to transiently colonize the respiratory tract of mice, and reduce *S. pneumoniae* respiratory tract colonization (11, 24). In addition, Sarno Oliveira *et al.* (25) found that *Lactobacillus* strains (*L. casei, L. plantarum and L. helveticus*) were able to reduce pneumococcal colonization when compared with untreated controls. Thus, it is quite likely that *L. casei* CRL 431 is able to transiently colonize the respiratory mucosa, which would not only reduce the colonization of *S. pneumoniae* by competitive exclusion, but also would allow the probiotic bacteria to impact on local immune cells.

The results from this study suggest that heat-killed LAB are also effective in the immunomodulation of the systemic and respiratory immune system. Therefore, probiotic bacteria in the form of live cells may not be required for this purpose. The effect of non-viable *L. casei* and other LAB on the respiratory immune system should be examined with more detailed studies, as dead bacteria or their cellular fractions could be an interesting alternative as mucosal adjuvants, especially in immunocompromised hosts in which the use of live bacteria might be dangerous. In addition, heat-killed LAB have the advantages of allowing a longer product shelf-life, easier storage, and transportation.

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