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Lactobacillus casei addition to a repletion diet-induced early normalisation of cytokine profils during a pneumococcal infection in malnourished mice

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This work studied the influence of *Lactobacillus casei* on cytokine production during repletion of malnourished mice in the face of an infectious challenge. In addition, the number and function of cells involved in the immune response against a respiratory infection was evaluated. Weaned mice were malnourished after consuming a protein-free diet (PFD) for 21 days. Malnourished mice were fed a balanced conventional diet (BCD) for 7 days or BCD for 7 days with *L. casei* supplementation on day 6 and day 7 (BCD+Lc). The malnourished control group (MNC) received PFD while the well-nourished control (WNC) mice consumed BCD. Mice were challenged intranasally with *Streptococcus pneumoniae* at the end of each dietary treatment. Malnutrition impaired the levels of serum tumour necrosis factor (TNF)-α and interleukin (IL)-1β, IL-4, IL-6 and IL-10. In addition, neutrophil number and activity, lymphocyte maturation and bone marrow CD4+, CD8+ and CD19+ cells number, were also impaired in the MNC group. Repletion with BCD induced a slight improvement in some of the parameters studied. However, when *L. casei* was added to the BCD, a normalisation of TNF-α, IL-1β and IL-6 values after infection and an increase in the levels of IL-10 and IL-4 compared to the WNC group was observed. Moreover, BCD+Lc induced a significant improvement in blood and bone marrow cells. Consequently, the use of *L. casei* as a supplement in a repletion diet was associated with a pattern of inflammatory and anti-inflammatory cytokines that led to an increased number and functionality of the cells that participate in the immune response against a pneumococcal infection.

Keywords: *Lactobacillus casei*; malnourished mice; cytokine; *Streptococcus pneumoniae*

Introduction

The development of a normal immune system is impaired by undernutrition in critical periods of gestation and neonatal maturation and during weaning (Keusch, 2003; McDade, Beck, Kuzawa, & Adair, 2001). Several investigations have demonstrated the influence of nutrition in the production of proinflammatory (Abo-Shousha, Hussein, Rashwan, & Salama, 2005; Hillyer, Maliwichi, & Woodward, 2007) and anti-inflammatory cytokines (Hillyer et al., 2006), and it has been described that malnutrition reduces the capacity of mononuclear phagocytes to release the cytokines that mediate the acute phase response (Woodward, 2001). In addition, protein malnutrition affects organs with high cell proliferation such as blood-forming tissues, which leads to an impairment of myelopoiesis

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and lymphopoiesis and to a decrease in phagocytosis and oxidative burst in leukocytes (Chandra, 1991; Deo, Mathur, & Ramalingaswami, 1967). During infection, malnutrition decreases inflammatory cell mobilisation from circulation into the tissues (Borelli, Mariano, & Borojevic, 1995). Moreover, decreased numbers and functions of T and B lymphocytes have been observed in malnourished hosts so that both the innate and the adaptive immune responses are impaired (Chandra, 1991).

Lactic acid bacteria (LAB) have been reported to increase resistance to infections (Erickson & Hubbard, 2000) and modulate cytokine production (McNaught, Woodcock, Anderson, & MacFie, 2005; Vinderola, Matar, & Perdigón, 2005). Besides, LAB can modify leukocyte counts and induce recovery of cell populations in mice undergoing chemotherapy (Krusteva et al., 1997).

In previous works we demonstrated that the addition of *Lactobacillus casei* to a repletion diet fed to malnourished mice exerted a beneficial effect on a *Streptococcus pneumoniae* infection by accelerating the recovery of the defence mechanisms against the pathogen (Villena et al., 2005). However, the mechanisms by which LAB exert their immunomodulatory activity are not completely understood. In the present work we studied the influence of *L. casei* on cytokine production during repletion of malnourished mice, especially in the face of an infectious challenge. We studied whether the poor response to *S. pneumoniae* infection in malnourished mice, which is significantly improved by the repletion with *L. casei*, could be caused by the effect of the probiotic bacteria on the blood concentration of proinflammatory and anti-inflammatory cytokines and the number and activity of blood and bone marrow leukocytes.

Materials and methods

Microorganisms

Lactobacillus casei CRL 431 was obtained from the CERELA culture collection (Chacabuco 145, San Miguel de Tucumán, Argentina). 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, distilled water 1l, 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 by centrifugation at 3000x*g* for 10 min and washed three times with sterile 0.01 mol/l phosphate buffer saline (PBS), pH 7.2. Capsulated pneumococcus (serotype 14) 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.

Animals and feeding procedures

Male 6-week-old Swiss albino mice were obtained from the closed colony kept at CERELA. They were housed in plastic cages at room temperature. Mice were housed individually during the experiments and the assays for each parameter studied were performed in 5–6 mice per group for each time point. Weaned mice were malnourished after consuming a protein-free diet (PFD) (Table 1) for 21 days (Figure 1A). At the end of this period, mice that weighed 45–50% less than well-nourished mice were selected for experiments. Well-nourished control (WNC) mice consumed a balanced conventional diet (BCD) *ad libitum* (Table 1).

Table 1. Composition of the balanced conventional and protein-free diets used in this study.[d]

Ingredient	Balanced conventional diet G/kg	Protein-free diet
Water	120	120
Proteins	230	<10
Carbohydrates	538	758[a]
Lipids	50	50
Vitamin mix[b]	22	22
Mineral mix[c]	40	40

[a]Protein-free corn flour.
[b]Vitamin mix (N° 905454, ICN Biomedicals Argentina, Capital Federal, Bs. As., Argentina) g/kg of mixture: 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.
[c]Mineral mix (N° 902844 ICN Biomedicals Argentina, Capital Federal, Bs. As., Argentina 902714) g/kg of mixture: sodium chloride, 167; potassium phosphate dibasic, 322; calcium carbonate, 300; magnesium sulphate, 102; calcium phosphate monobasic, 75; ferric citrate, 27.5; manganese sulphate, 5.1. H_2O; potassium iodide, 0.8; copper sulfate. 5 H_2O, 0.3; zinc chloride, 0.25; cobalt chloride. 6 H_2O, 0.05.
[d]The approximate energy value provided by the bacterial supplement is 0.06913 J/mouse d.

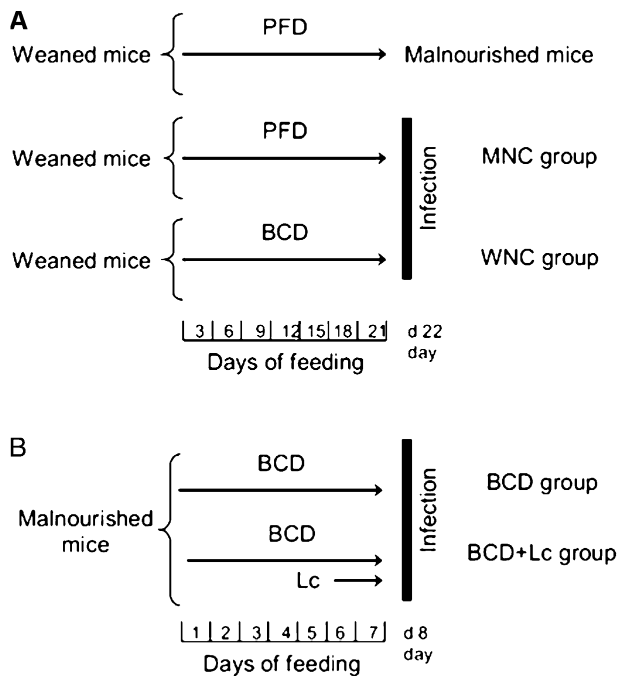

Figure 1. Different feeding protocols used in this work: (A) obtainment of malnourished mice and well-nourished (WNC) and malnourished (MNC) infected controls; (B) repletion of malnourished mice for 7 days with a balanced conventional diet (BCD) or BCD with supplemental *Lactobacillus casei* (BCD+Lc). PFD, protein-free diet.

Malnourished mice were separated in two groups for repletion treatment (Figure 1B). One group of malnourished mice were fed BCD for 7 consecutive days (BCD group). As administration of *L. casei* for 2 days is the optimal dose to provide protection against *S. pneumoniae* in malnourished mice (Villena et al., 2005), the second group of animals received BCD for 7 days with *L. casei* supplementation (10^9 cfu/mouse/day) on days 6 and 7 (BCD + Lc group). The malnourished control group (MNC) received only PFD while the WNC mice consumed the BCD *ad libitum* (Figure 1A). The Ethical Committee for Animal Care at CERELA approved the experiments.

Experimental infection

The experimental animal model of infection was used as previously described (Villena et al., 2005). Briefly, challenge with *S. pneumoniae* was performed on the day after the end of each dietary treatment (day 8) (Figure 1B). Mice were infected by dropping 25 μl of the inoculum containing 10^5 log-phase cfu of *S. pneumoniae* in 0.01 mol/l PBS into each nostril and allowing it to be inhaled. Mice were killed on day 0 (before infection) and on hour 12 and days 1, 2, 5, and 10 post-infection. Control groups (WNC and MNC) were infected in the same way (Figure 1A). During day 10 post-infection, all mice were fed only BCD, with the exception of the MNC, which received PFD.

Cytokine concentrations in serum

Tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6 and IL-10 concentrations in serum were measured with commercially available enzyme-linked immunosorbent assay kits following the manufacturer's recommendations (R&D Systems, MN, USA).

Total and differential number of blood leukocytes

Animals were anaesthetised with a mixture of Ketamine hydrochloride (#K2753, Sigma-Aldrich Co, St Louis, MO, USA), 70 mg/kg, and xylazine hydrochloride (#X1126, Sigma-Aldrich), 10 mg/kg, intraperitoneally. Blood samples were obtained through cardiac puncture at the end of each dietary treatment (day 0) and on days 1, 2, 5 and 10 after challenge. The total number of leukocytes and differential cell counts were performed as described previously (Villena et al., 2005).

Bone marrow differential cell counts

Anaesthetised mice were killed by cervical dislocation and bone marrow samples were obtained by flushing the femoral cavity with PBS. Differential cell counts were carried out by counting 400 cells in bone marrow smears stained with May Grünwald-Giemsa. Myeloid cells were grouped into the mitotic pool, which includes cells capable of replication (myeloblasts, promyelocytes and myelocytes), and the post-mitotic pool, whose cells usually do no replicate but are able to evolve towards more mature and differentiated cells (metamyelocytes, band cells and neutrophils). The cells of the lymphocytic lineage were also counted and were expressed as percentages of total bone marrow leukocytes.

Blood and bone marrow peroxidase activity

Washburn test

The measurement of myeloperoxidase activity of blood neutrophils and bone marrow myeloid cells was carried out as described previously by Villena et al. (2005). The results were expressed as percentages of peroxidase positive (Px+) cells.

Lymphocyte cells study

Cytochemical assays

Commercial cytochemical assay kits (Sigma-Aldrich, catalog N° 180) were used to determine the percentages of β-glucuronidase positive (β-G+) cells and α-naphthyl butyrate esterase positive (α-NBE+) cells in bone marrow and blood samples. The cells were counted under a light microscope (x100). These cytochemical assays were performed to study T cells maturation following the maturation scheme proposed by Basso, Cocito, Semenzato, Pezzuto, and Zanesco (1980).

Immunofluorescence test

Cell suspensions were prepared by flushing the femoral shaft with PBS containing heparin and albumin (1% w/v). The pellet was used to make smears which were fixed onto slides with methanol (4% v/v) for 30 min. The labelling of CD4+, CD8+ or CD19+ cells was performed by the direct inmunofluorescence technique (CEDARLANE, Hornby, Ontario, Canada) according to Gauffin Cano and Perdigón (2003). Samples were analysed under an ultraviolet light with a fluorescence microscope (×100). Four hundred cells were counted and the results were expressed as percentages.

Statistical analysis

The results were expressed as mean±SD. A two-way analysis of variance (ANOVA) test was used to evaluate the main effects and the interactions between treatments (WNC, MNC, BCD and BCD+Lc) and days post-infection (0, 1, 5, and 10) (InfoStat, version 2006, p. 3). Tukey's test (for pairwise comparisons of the mean of the different groups) was used to test for differences between the groups. Differences were considered significant at $P < 0.05$.

Results

Recovery of malnourished mice cytokines and leukocytes

Malnutrition induced a decrease in the values of serum IL-4, IL-6 and IL-10, although no changes were observed in levels of serum IL-1β and TNF-α. The treatment of malnourished mice with BCD increased the levels of IL-4, IL-6 and IL-10 but did not reach values found in the WNC group (Table 2).

However, when malnourished mice were replete with BCD supplemented with *L. casei*, the levels of serum IL-6 were normalised while IL-10 and IL-4 reached higher values than the WNC group. Considering that the repletion with BCD+Lc induced a serum cytokine profile different from that induced by treatment with BCD, then blood and bone marrow leukocytes were studied in order to establish whether the repletion with *L. casei* induces

Table 2. Tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6 and IL-10 concentrations in serum.

Experimental groups	TNF-α	IL-1β	IL-6	IL-4	IL-10
BCD+Lc	25.8 ± 3.0^{a}	13.5 ± 1.6^{a}	121.4 ± 10.9^{c}	105.1 ± 7.5^{d}	147.8 ± 12.5^{d}
BCD	23.4 ± 2.1^{a}	13.6 ± 1.8^{a}	93.5 ± 11.4^{b}	50.1 ± 6.9^{b}	91.3 ± 12.6^{b}
MNC	22.1 ± 2.2^{a}	12.1 ± 2.1^{a}	67.8 ± 10.6^{a}	39.7 ± 6.3^{a}	51.6 ± 11.2^{a}
WNC	29.3 ± 3.2^{a}	13.4 ± 1.1^{a}	127.2 ± 11^{c}	65.4 ± 7.1^{c}	116.7 ± 13^{c}

Note: Malnourished mice replete with a balanced conventional diet (BCD) or balanced conventional diet with supplemental *Lactobacillus casei* (BCD+Lc). Malnourished (MNC) and well-nourished (WNC) mice were used as controls. The results were expressed in pg/ml. Mean±SD ($n=6$ mice/group) are shown. Means in a column with a different superscript letter (a, b, c) differ ($P<0.0.5$).

changes in the number and activity of these cells. Malnutrition decreased the total number of blood leukocytes with a decrease in both neutrophils and lymphocytes (Table 3). The number of these cells in BCD mice did not differ with respect to the MNC group. However, treatment with BCD+Lc significantly increased the number of leukocytes in the blood.

In the bone marrow, malnutrition induced a significant decrease in the mitotic pool and but did not modify cells in the post-mitotic pool (Table 3). In addition, cells of the lymphoid lineage were significantly decreased in the MNC compared to the WNC group. Both repletion treatments increased the number of cells in the mitotic pool, with higher levels than those in the WNC; however, the mice in the BCD+Lc group showed significantly higher values than the BCD mice. Cells of the lymphoid lineage were significantly increased with both dietary treatments, but the number of lymphoid cells in the BCD+Lc group was higher than in the WNC mice.

Peroxidase activity of blood and bone marrow cells were determined in order to study the activity of myeloid cells. Malnutrition induced a significant decrease in the percentage of Px+ cells in blood and bone marrow. Both repletion treatments increased values of this parameter, but only the BCD+Lc group reached values of WNC mice (Table 4).

We have previously used a cytochemical scheme to differentiate T-cells maturation considering the progress from β-G-, α-NBE- to β-G+, α-NBE- and finally to β-G+, α-NBE+. Cytochemical assays in this study showed that the percentages of blood and bone marrow β-G+ cells and α-NBE+ cells were significantly reduced by malnutrition

Table 3. Blood and bone marrow leukocytes.

	Blood			Bone marrow		
Groups	Leukocytes	Neutrophils	Lymphocytes	Mitotic pool	Post-mitotic pool	Lymphocytes
BCD+Lc	3.8 ± 0.4^{b}	0.8 ± 0.07^{b}	3.0 ± 0.1^{b}	40.0 ± 0.7^{d}	55.0 ± 1.1^{a}	5.0 ± 0.3^{d}
BCD	2.9 ± 0.7^{a}	0.7 ± 0.03^{a}	2.3 ± 0.6^{a}	25.5 ± 0.4^{c}	72.5 ± 0.9^{b}	2.0 ± 0.1^{b}
MNC	2.9 ± 0.5^{a}	0.6 ± 0.08^{a}	2.2 ± 0.1^{a}	3.3 ± 1.1^{a}	96.3 ± 2.6^{c}	0.7 ± 0.3^{a}
WNC	6.9 ± 0.3^{c}	0.9 ± 0.07^{b}	6.0 ± 0.2^{c}	6.0 ± 0.4^{b}	90.6 ± 2.0^{c}	3.4 ± 1.1^{c}

Note: Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or with BCD with supplemental *Lactobacillus casei* on day 6 and day 7 (BCD+Lc). Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Bone marrow myeloid cells were grouped into the mitotic pool (myeloblasts, promyelocytes and myelocytes) or into the post-mitotic pool (metamyelocytes, band cells and neutrophils). The results of blood leukocytes are expressed as 10^{9} cell/l and bone marrow cells were expressed as percentages. Mean±SD ($n=6$ mice/group) are shown. Means in a column with a different superscript letter (a, b, c) differ ($P<0.05$).

Table 4. Cytochemical studies of blood and bone marrow leukocytes.

Groups	Cells Px+		Cells β-G+		Cells αNBE+	
	Blood	Bone marrow	Blood	Bone marrow	Blood	Bone marrow
BCD+Lc	117.9 ± 1.6^{c}	9.1 ± 2.3^{c}	87.5 ± 6.9^{c}	7.2 ± 1.1^{c}	87.5 ± 5.2^{d}	1.3 ± 0.3^{b}
BCD	104.9 ± 1.1^{b}	8.4 ± 2.2^{b}	82.7 ± 6.7^{c}	3.3 ± 1.4^{b}	69.5 ± 4.7^{c}	1.3 ± 0.3^{b}
MNC	92.3 ± 1.4^{a}	8.0 ± 0.6^{a}	21.0 ± 8.7^{a}	0.8 ± 0.5^{a}	10.5 ± 2.5^{a}	0.9 ± 0.2^{a}
WNC	124.0 ± 1.2^{c}	9.6 ± 0.6^{c}	71.2 ± 7.2^{b}	2.3 ± 0.9^{b}	27.4 ± 3.0^{b}	2.5 ± 0.4^{c}

Note: Peroxidase positive (Px+) cells, β-glucuronidase positive (β-G+) cells and α-naphthyl butyrate esterase positive (α-NBE+) cells were studied. Malnourished mice were replete for 7 days with BCD or with BCD with supplemental *Lactobacillus casei* on day 6 and day 7 (BCD+Lc). Malnourished (MNC) and well-nourished (WNC) mice were used as controls. The results were expressed as percentages of cells. Mean ± SD ($n = 6$ mice/group) are shown. Means in a column with a different superscript letter (a, b, c) differ ($P < 0.05$).

(Table 4). Repletion treatments improved blood β-G+ cells and a-NBE+ cells, which reached higher values than those in the WNC group. In addition, the percentages of CD4+, CD8+ and CD19+ cells in bone marrow were studied (Table 5). Malnutrition decreased significantly the percentage of these cells in bone marrow. Both repletion treatments increased values of CD8+ cells, but this parameter not reached the values of WNC group. Only treatment with BCD+Lc normalised the percentages of CD4+ and CD19+ cells in bone marrow.

Concentration of cytokines after challenge

The challenge with pneumococci increased serum TNF-α and IL-1β in all groups, with a peak at day 3 and hour 12 post-infection, respectively (Figures 2A and B). However, the concentrations of these cytokines were significantly lower in the MNC mice compared to the WNC group. Only repletion with BCD+Lc showed similar levels of TNF-α and IL-1β to those found in the WNC. MNC mice showed significantly lower levels of serum IL-6 than the WNC group. Serum IL-6 reached normal values only when *L. casei* was added to the repletion diet (Figure 2C). Malnutrition decreased the levels of serum IL-4 and IL-10, which were improved by both repletion treatments (Figures 2D and E). Mice that replete with BCD did not reach the IL-4 and IL-10 levels of the WNC mice at any time post-infection. However, malnourished mice that received the BCD+Lc diet showed significantly higher levels of IL-4 and IL-10 than the WNC group.

Table 5. Bone marrow CD4+, CD8+ and CD19+ cells.

Groups	Cells CD8+	Cells CD4+	Cells CD19+
BCD+Lc	11.1 ± 0.8^{b}	1.7 ± 0.3^{c}	2.0 ± 0.2^{b}
BCD	10.0 ± 0.8^{b}	1.3 ± 0.2^{b}	0.6 ± 0.2^{a}
MNC	8.0 ± 0.8^{a}	0.7 ± 0.2^{a}	0.6 ± 0.1^{a}
WNC	18.4 ± 0.8^{c}	1.9 ± 0.2^{c}	2.2 ± 0.1^{b}

Note: Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or with BCD with supplemental *Lactobacillus casei* on day 6 and day 7 (BCD+Lc). Malnourished (MNC) and well-nourished (WNC) mice were used as controls. The results were expressed as percentages of cells. Mean ± SD ($n = 6$ mice/group) are shown.
*Significant differences compared to the MNC mice and not different from the WNC group ($P < 0.05$).
**Significant differences compared to the MNC and WNC groups ($P < 0.05$).

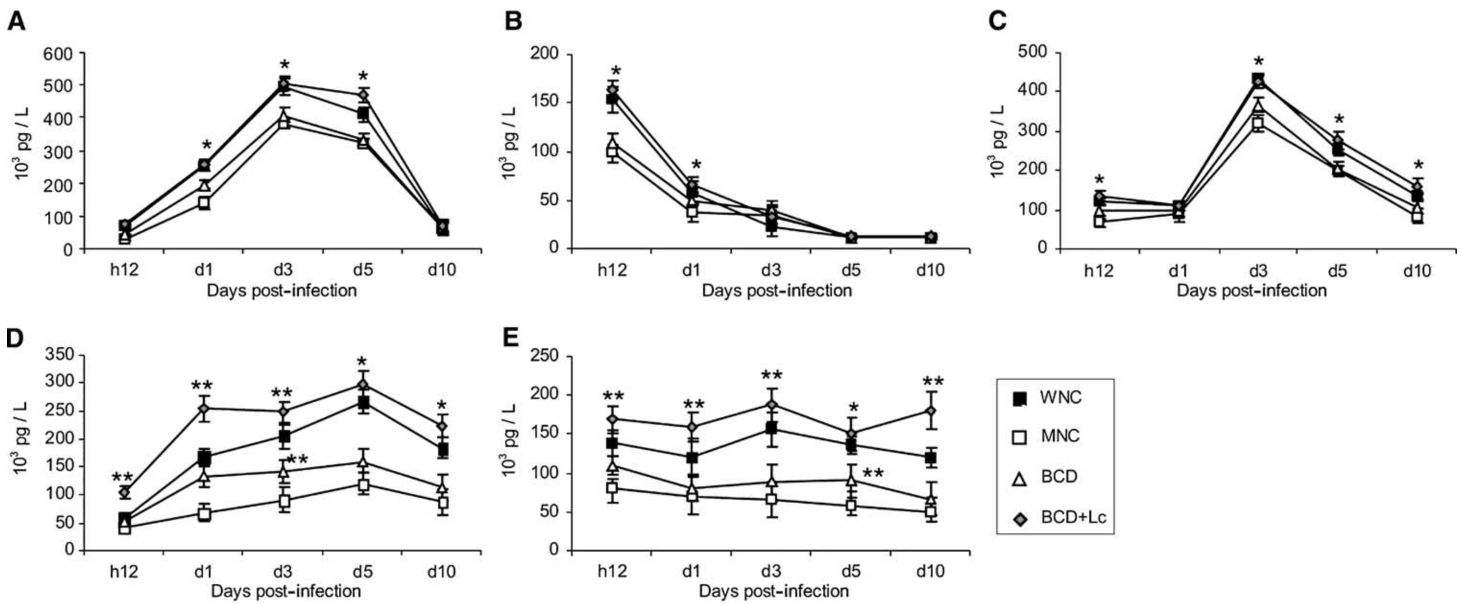

Figure 2. Tumour necrosis factor (TNF)-α (A), interleukin (IL)-1β (B), IL-6 (C), IL-4 (D) and IL-10 (E) concentrations in serum. Malnourished mice were replete with a balanced conventional diet (BCD) or balanced conventional diet with supplemental *Lactobacillus casei* (BCD + Lc) and then challenged with *Streptococcus pneumoniae.* Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Mean ± SD (n = 6 mice/group) are shown. *Significant differences compared to the MNC mice and not different from the WNC group (P < 0.05). **Significant differences compared to the MNC and WNC groups (P < 0.05).

Total and differential counts of leukocytes after challenge

The pathogen increased the number of blood leukocytes in all experimental groups; however, the values were significantly lower in the MNC than in the WNC mice. Both repletion treatments increased blood leukocytes, but only the BCD+Lc group reached similar levels to those of the WNC mice (Figure 3A). When we analysed the different leukocytes populations, it was noted that the treatment with BCD was effective in increasing values of blood leukocytes (Figure 3C). However, the number of neutrophils was similar to the MNC mice (Figure 3B). Malnourished mice that replete with BCD+Lc showed normal values of both neutrophils and lymphocytes (Figures 3B and C).

The study of bone marrow cells, after infection, showed that the MNC group had decreased percentages of proliferating myeloid cells (mitotic pool cells) compared to the WNC mice. Both repletion diets (BCD and BCD+Lc) increased the percentage of proliferating cells, with higher levels than those in the MNC group (Figure 3D). However, BCD supplemented with *L. casei* treatment caused a higher proliferation (mitotic pool) than the one found in the WNC group. Malnutrition induced a significant decrease in lymphoid lineage cells. Although both repletion treatments improved these values, only the BCD+Lc group showed values similar to those of the WNC group (Figure 3F).

Peroxidase activity of blood and bone marrow cells after challenge

After the challenge with *S. pneumoniae*, the percentage of Px+ cells increased in all experimental groups. However, MNC mice showed significantly decreased levels of Px+ cells (Figures 4A and B). Both repletion treatments improved blood and bone marrow peroxidase activity during infection, but only the BCD+Lc group showed similar values to those in the WNC group (Figures 4A and B).

Lymphocyte study after challenge

Bone marrow β-G+ and α-NBE+ cell populations were significantly lower in the MNC than in the WNC group after challenge. Repletion treatments increased β-G+ and α-NBE+ cell populations but BCD+Lc mice showed values of β-G+ cells higher than those in the WNC group (Figures 4E and H). The number of bone marrow CD4+, CD8+ and CD19+ cells was significantly reduced by malnutrition (Figure 4). Repletion with BCD increased only the number of CD4+ cells while the BCD+Lc treatment was able to normalise the number of bone marrow CD4+ and CD19+ cells (Figures 5A and C). The percentage of CD8+ cells was not modified either with the BCD or with the BCD+Lc treatment (Figure 5B).

Discussion

Nutrition and infection interact with each other synergistically. It is clear that nutritional deficiencies increase the frequency and severity of infections, leading to altered cell populations (Najera et al., 2001; Savino, 2002). Most nutrients are directly or indirectly involved in protein synthesis and most immune responses involve the production of proteins with specific functions (Scrimshaw & Sangiovanni, 1997). It was reported that malnutrition reduces the capacity of mononuclear phagocytes to release cytokines that mediate the acute phase response (Knapp et al., 2004; Komatsu, Mawatari, Miura, &

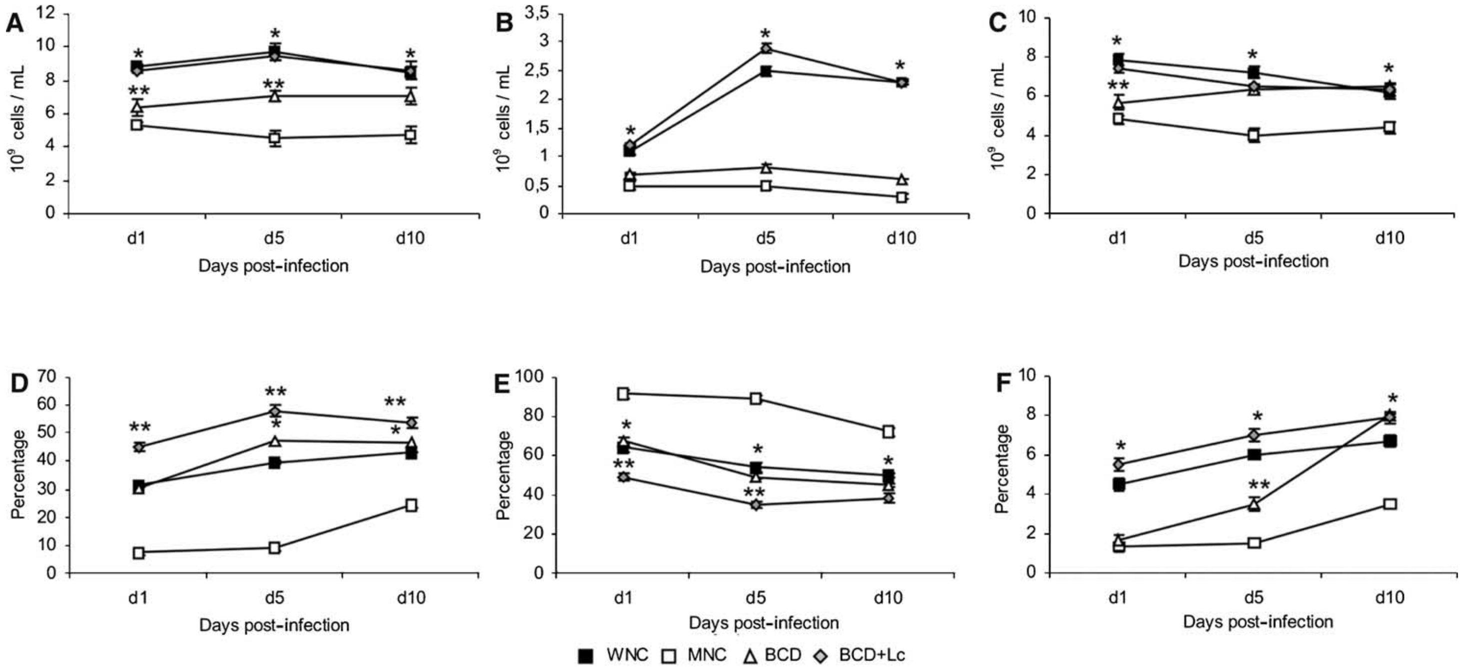

Figure 3. Blood and bone marrow leukocytes. Malnourished mice were replete for 7 days with a balanced conventional diet (BCD) or with BCD with supplemental *Lactobacillus casei* on day 6 and day 7 (BCD + Lc) and then challenged with *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Blood leukocytes (A), neutrophils (B) and lymphocytes (C). Bone marrow myeloid cells were grouped into the mitotic pool (myeloblasts, promyelocytes and myelocytes) (D), the post-mitotic pool (metamyelocytes, band cells and neutrophils) (E) and lymphoid cells (F). Mean ± SD ($n = 6$ mice/group) are shown. *Significant differences compared to the MNC mice and not different from the WNC group ($P < 0.05$). **Significant differences compared to the MNC and WNC groups ($P < 0.05$).

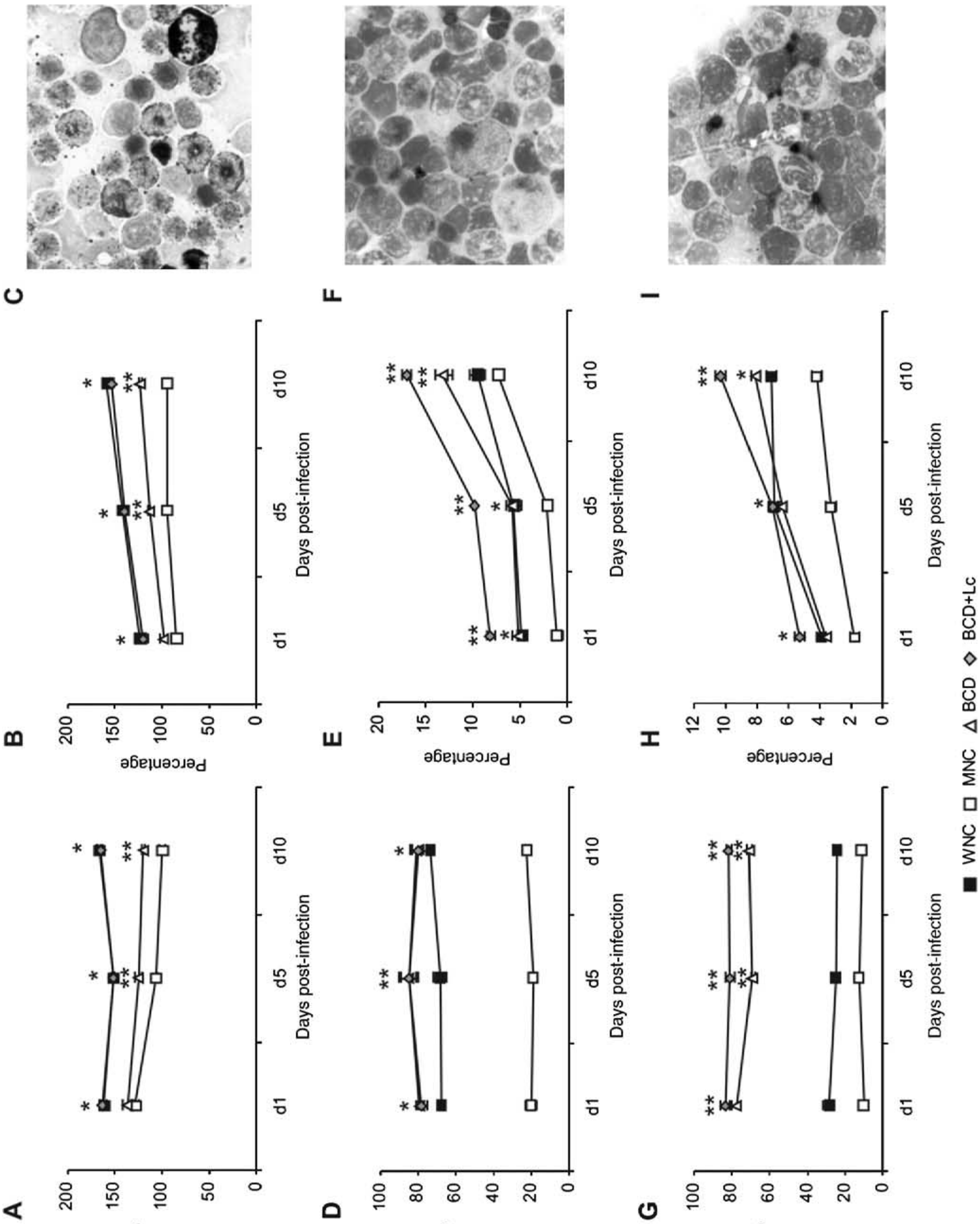

Figure 4 (Continued)

Yagasaki, 2007; Woodward, 2001). In our experimental model, malnutrition did not alter basal levels of TNF-α and IL-1β. However, after infection, these cytokines were significantly lower in malnourished animals compared with well-nourished ones. The diet with supplemental *L. casei* was able to normalise these cytokines after infection. TNF-α can activate and stimulate the recruitment of neutrophils and monocytes to sites of infection to eradicate microbes (Knapp et al., 2004). It was reported that TNF-α and IL-1β reduce the numbers of lymphocytes but not of Gr-1+ granulocytes in bone marrow, which would represent a physiological response to ensure increased neutrophil production in response to infection (Ueda, Kondo, & Kelsoe, 2005). The addition of *L. casei* to a repletion diet was able to improve host defence against infection by increased recruitment and enhanced performance of phagocytes during pneumococcal pneumonia, as demonstrated by the normalisation of blood leukocyte counts reported in this work and by the normalisation of neutrophils and macrophages numbers in bronchoalveolar lavages (BAL) described in a previous paper (Villena et al., 2005).

T cell functions are more sensitive than B cell functions to most nutrient deficiencies. Thus, some specific immune tests such as of phagocytic capacity, T cell subtypes, complement levels, and delayed cutaneous hypersensitivity can be useful indicators of malnutrition (Scrimshaw & Sangiovanni, 1997). In this paper, quantitative and qualitative damage in leukocyte populations in our malnourished experimental model, evidenced by the number of blood leukocytes, the alteration of bone marrow mitotic and post-mitotic pools and the impairment of blood and bone marrow cells peroxidase activity was observed. This effect of malnutrition on leukocytes would lead to a deficiency in the innate immune response against *S. pneumoniae*, which is in agreement with our previous findings that showed that pneumococcal colonisation of lung and bacteraemia were significantly higher in MNC compared to the WNC group (Villena et al., 2005). Repletion with BCD+Lc reduced significantly leukocytes alterations; thus, this dietary treatment would restore the innate immune response, allowing replete mice to reach rates of pneumococcal clearance similar to the WNC group (Villena et al., 2005).

The increased percentage of proliferating bone marrow myeloid cells (mitotic pool) observed in the BCD+Lc group could be related to the stimulation of murine granulocyte/macrophage colony formation by IL-6 (Marin et al., 1998; Suda et al., 1988). In addition, the decrease in post-mitotic pool cells observed after challenge in the BCD+Lc mice is probably caused by a release of mature cells into peripheral blood. The effect of malnutrition on the production of Th2 cytokines cannot be generalised. Some studies showed that the ability of blood and splenic T-cells to produce IL-4, IL-5, IL-6 and IL-10 in response to stimulation is conserved while INF-γ production is decreased (Hill, Naama, Shou, Calvano, & Daly, 1995b; Malave et al., 1998; Shi, Scott, Stevenson, & Koski, 1998). In contrast, other reports showed an impaired production of mucosal and systemic IL-4

Figure 4. Cytochemical studies of blood and bone marrow leukocytes peroxidase positive (Px+) cells in blood (A) and bone marrow (B, C), β-glucuronidase positive (β-G+) cells in blood (D) and bone marrow (E, F) and α-naphthyl butyrate esterase positive (α-NBE+) cells in blood (G) and bone marrow (H, I) were studied. Malnourished mice were replete for 7 days with BCD or with BCD with supplemental *Lactobacillus casei* on day 6 and day 7 (BCD+Lc) and then challenged with *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Mean±SD ($n=6$ mice/group) are shown. *Significant differences compared to the MNC mice and not different from the WNC group ($P<0.05$). **Significant differences compared to the MNC and WNC groups ($P<0.05$).

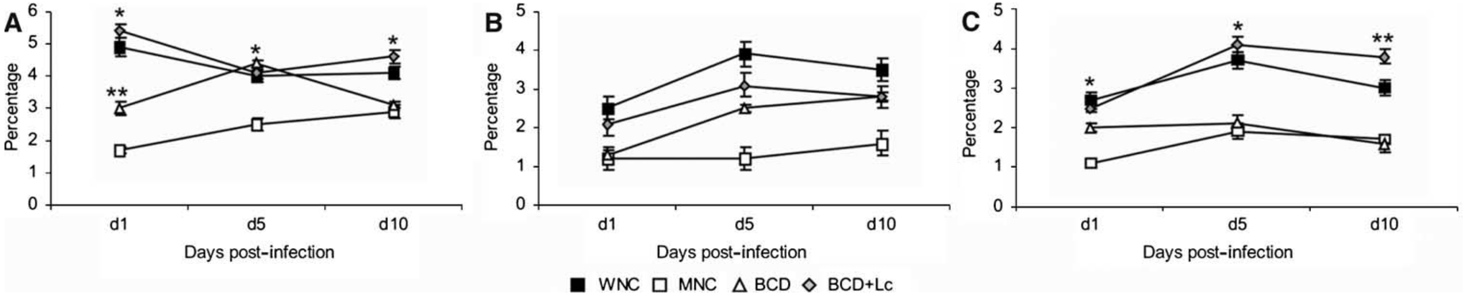

Figure 5. Bone marrow CD4+ (A), CD8+ (B) and CD19+ (C) cells of malnourished mice replete for 7 days with a balanced conventional diet (BCD) or with BCD with supplemental *Lactobacillus casei* on day 6 and day 7 (BCD+Lc) and then challenged with *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Mean ± SD ($n = 6$ mice/group) are shown. *Significant differences compared to the MNC mice and not different from the WNC group ($P < 0.05$). **Significant differences compared to the MNC and WNC groups ($P < 0.05$).

and IL-6 by immune cells in malnourished hosts (Hill et al., 1995a; McCarter et al., 1998; Zhang & Petro, 1997). In this experimental model, malnutrition affects IL-4, IL-6 and IL-10 serum levels and repletion with BCD + Lc, showed normal levels of serum IL-6 and higher values of IL-4 and IL-10 than those found in the WNC group. Under inflammatory conditions, cytokines change dramatically. When a Th2 response is needed, additional sources of IL-4, IL-5, IL-6 and IL-10 contribute to stimulating B-cells to proliferate and mature into polymeric IgA-producing cells and to develop specific antibodies (Corthésy, 2002). In addition, during the generation of an efficient effector immune response, DCs also have to overcome suppression by Treg cells, which they apparently accomplish by producing IL-6 (De Heer, Hammad, Kool, & Lambrecht, 2005). Thus, Th2 cytokines are essential for an adequate induction of the antigen-specific humoral immune response.

The decrease in blood and bone marrow β-G+ and α-NBE+ cells in malnourished mice would suggest deficiencies in the maturation of T cells (Basso et al., 1980), while the increase in blood α-NBE+ cells induced by BCD+ Lc administration would indicate an improvement in T cells maturation. This effect would be beneficial for replete malnourished mice because T cells play a significant role in the lung during pneumococcal infection (Kadioglu et al., 2000). It was reported that T cells are important in the induction of the humoral immune response against *S. pneumoniae* and that they are rapidly recruited after pneumococcal pulmonary infection (Kadioglu, Coward, Colston, Hewitt, & Andrew, 2004). In our study, bone marrow CD4+ cells increased in all groups after challenge but only the BCD + Lc mice reached WNC values. This fact would be important for a protective response against pneumococcal infection because of the role of CD4 + T-cells as coordinators of the adaptive immune response and haematopoiesis amplifiers of the innate immune response (Monteiro, Benjamin, Costa, Barcinski, & Bonomo, 2005). The number of CD8+ cells was significantly decreased after challenge with the pathogen and treatment with BCD + Lc did not increase the percentage of these cells in the bone marrow. This finding would mean that *L. casei* is unable to induce cytotoxicity mechanisms.

The BCD + Lc group showed a normal number of CD19+ cells, which would correlate with an increase in the levels of BAL and serum anti-pneumococcal immunoglobulin demonstrated previously (Villena et al., 2005). This fact is probably related to the increase in IL-6, which stimulates the growth of B-lymphocytes that have differentiated into antibody producers (Abbas, Lichtman, & Pober, 2004). Thus, the improvement of the antibody response to pneumococcal infection would be mediated by the different cytokine profile induced by *L. casei.*

In a diet-restricted murine peritonitis model, Hidemura et al. (2003) demonstrated that the oral administration of LAB increased IL-10 concentrations, which probably contributed to attenuating excessive host inflammatory response. Consequently, the increase in this cytokine observed in the BCD + Lc group could be involved in the lower lung injury reported in a previous work (Villena et al., 2005). Moreover, IL-10 could play a regulatory role in haematopoiesis through its ability to inhibit the release of the granulocyte macrophage-colony stimulating factor (GM-CSF) by accessory cells (Oehler et al., 1997). In addition, *L. casei* administration induced an increase in IL-4 concentration. This cytokine antagonises the macrophage-activating effects of IFN-γ and thus inhibits cell-mediated immune reactions. This may be one of the mechanisms by which Th2 cells function as inhibitors of immune inflammation (Oehler et al., 1997).

The production of blood cells, which are part of the immune system, is the result of the balance between positive and negative growth signals. Malnutrition affects the immune response and haematopoiesis and we observed previously that feeding with the BCD for 21 days was necessary to normalise the response against infection (Villena et al., 2005). The use of *L. casei* as a supplement in a repletion diet was associated with a pattern of inflammatory and anti-inflammatory cytokines that led to an increased number and functionality of the cells that participate in the immune response against a pneumococcal infection. These results seem to be associated with an improvement in the production and maturation of myeloid and lymphoid cells. However, more studies with specific markers are needed to demonstrate this hypothesis.

We propose that, in order to improve compromised immunity in malnourished hosts, future treatments should consider the potential benefits of LAB as promoters of the early recovery of defence mechanisms against infection.

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