Yoghurt accelerates the recovery of defence mechanisms against *Streptococcus pneumoniae* in protein-malnourished mice

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Experiments studied the effect of yoghurt on the recovery of defence mechanisms against *Streptococcus pneumoniae* respiratory infection in malnourished mice. Weaned mice were malnourished with a protein-free diet (PFD) for 21 d. Malnourished mice were made replete with a balanced diet (BD), yoghurt, or the BD with supplemental yoghurt (BD + Y) for 7, 14 or 21 d. The normal control (NC) group was fed the BD whereas malnourished control (MC) mice consumed only the PFD. Mice were challenged with pneumococci at the end of each dietary treatment. MC mice showed increased susceptibility to pneumococcal infection. Blood leucocytes, phagocyte activity and serum and bronco-alveolar anti-pneumococcal IgG and IgA were significantly lower in the MC than in the NC group. Repletion of malnourished mice with the BD for 21 d was necessary to obtain a response to infection similar to that of NC mice; however, administration of the BD + Y for 14 d was enough to normalise the immune defence mechanisms. Histological examination of MC lungs showed progressive loss of alveolar architecture. Lung injuries were significantly less pronounced in NC mice. Mice treated with the BD + Y for 14 d showed histological signs similar to the NC group. The present study showed that administration of yoghurt to malnourished mice induced an early recovery of the immunological parameters studied. Despite the uncertainties about the mechanisms involved and about the human relevance of the effects observed in animal models, the present study provides a strong rationale for the hypothesis that yoghurt consumption by malnourished hosts will accelerate the recovery of the immune mechanisms involved in the protection against respiratory infections.

Yoghurt: Malnourished mice: Respiratory infections: *Streptococcus pneumoniae*: Probiotics

Malnutrition is a major cause of acquired immunodeficiency and, before the appearance of the HIV, was the leading cause of death from infection (Cunningham-Rundles & Lin, 1998). In developing countries, protein malnutrition affects not only young children but also other groups, including the elderly, those with eating disorders and patients with a variety of primary debilitating diseases (Chandra, 1991). Several studies have clearly shown that populations with inadequate nutrition, in either quantitative or qualitative terms, have an increased susceptibility to infections. The adequate and prompt correction of the nutritional status is important to reduce morbidity and mortality from infections associated with the immune suppression caused by malnutrition (Scrimshaw & SanGiovanni, 1997).

Yoghurt is defined by the Codex Alimentarius of 1992 as a co-agulated milk product that results from the fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Bourlioux & Pochart, 1988). It is a nutrient-dense food containing high-quality protein, vitamins, especially folic acid, and trace elements, all of which are necessary for optimal immune responses (Adolfsson *et al.* 2004). Probiotics are live micro-organisms that when administered in adequate amounts confer a health benefit on the host (Reid *et al.* 2003). Consumption of yoghurt has been shown to induce measurable health benefits linked to the presence of live bacteria; thus, it clearly fulfils the current concept of probiotics (Guarner *et al.* 2005). Many investigators have studied the therapeutic and preventive effects of yoghurt and lactic acid bacteria (LAB), which are commonly used in yoghurt production, on diseases such as cancer, infection and gastrointestinal disorders (Meydani & Ha, 2000). In particular, a simulated commercial yoghurt prepared from a stock culture of LAB containing *L. bulgaricus* and *S. thermophilus* from the Centro de Referencia para Lactobacilos (CERELA) culture collection has been reported to prevent bacterial translocation in malnourished mice (Agüero *et al.* 1996), increase the number of IgA+ cells in the lamina propria of the small intestine and improve the resistance to enteropathogens (Gauffin & Perdigón, 2003). In addition, two bacteria present in the yoghurt, *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412, can increase the number of IgA+ cells in the gut and bronchus when individually administered (Perdigón *et al.* 1998).

Recent research in probiotics has begun to think beyond enhanced protection at the gastrointestinal level, and has instead moved to the investigation of the enhancement of protective responses at distal mucosal sites (Cross, 2002). In particular, it is now apparent that probiotic feeding can influence immune responses in the respiratory

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tract tissues and this effect has been shown sufficient to afford increased protection against bacterial and viral respiratory tract pathogens (Cross, 2004). We have previously reported that administration of yoghurt to young mice enhances the phagocytic activity of alveolar macrophages and the lung clearance of *Pseudomonas aeruginosa* (Alvarez *et al.* 2001).

Despite the constant advances in the treatment of infectious diseases, the mortality rate associated with respiratory tract infections has increased during recent years (Madhi *et al.* 2000; Mollerach *et al.* 2004). Among these infections, *Streptococcus pneumoniae* is one of the major causes of bacterial pneumonia, meningitis, bacteraemia and otitis media. Despite the availability of antibiotics, mortality and morbidity rates remain high, especially in high-risk groups (Hammerschmidt *et al.* 1999).

The aim of the present study was to determine whether yoghurt administration during repletion of protein-malnourished mice accelerates the recovery of the defence mechanisms against *S. pneumoniae* respiratory infection.

Materials and methods

Animals

Male 6-week-old Swiss albino mice were obtained from the closed colony kept at the bioterio of the CERELA (San Miguel de Tucumán, Argentina). They were housed in plastic cages at room temperature. Each assay was performed in groups consisting of twenty-five to thirty mice (five to six 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 (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. Normal (well-nourished) control mice consumed a conventional balanced diet (BD) *ad libitum* (Table 1) and were used to compare pneumococci infection with repleted malnourished mice. The Ethical Committee for Animal Care at the CERELA approved the experiments.

Yoghurt preparation and feeding procedures

A simulated commercial yoghurt (Table 1) was prepared by the Laboratory of Experimental Foods at the CERELA from a stock culture of LAB containing *L. bulgaricus* and *S. thermophilus* from the CERELA culture collection. These strains, including the immune-enhancing *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412 (Perdigón *et al.* 1998), mixed at a ratio of 1:1, were incubated in 10 % non-fat milk at 42°C for 4 h and then at 4°C for 24 h. At the end of this process the total bacterial count was 2×10^8 cells/ml. The yoghurt was prepared every 48 h in order to ensure the constant number of bacteria. Malnourished mice were fed the BD, yoghurt or the BD with supplemental yoghurt (BD + Y) for 7, 14 or 21 consecutive days (Fig. 1(B)). The malnourished control (MC) group received only the protein-free diet while normal control (NC) mice consumed the BD *ad libitum* (Fig. 1(A)).

Experimental infection

Capsulated *S. pneumoniae* 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 one of the ten most frequent serotypes isolated in pneumococcal infections in Argentina (serotype 14) (Mollerach *et al.* 2004). *S. pneumoniae* was first grown on blood agar for 18 h; freshly grown colonies were suspended in Todd Hewitt broth (Oxoid, Basingstoke, Hampshire, UK) and incubated at 37°C overnight. The pathogen was harvested through centrifugation at $3000\,g$ for 10 min at 4°C and then washed three times with sterile PBS. Cell density was adjusted to 4×10^4 colony-forming units/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 (S Racedo, J Villena, G Agüero and S Alvarez, unpublished results).
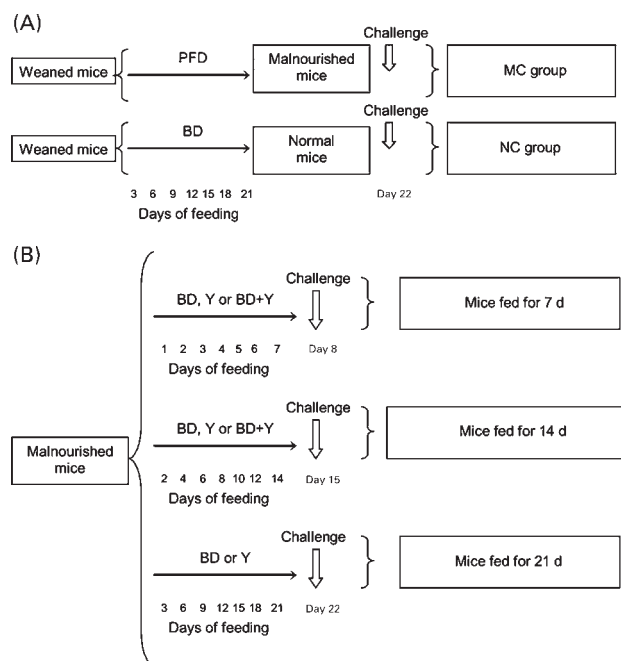

Fig. 1. Feeding protocols used in the study. (A) Obtainment of malnourished mice and normal (well-nourished) infected controls. (B) Repletion of malnourished mice by feeding balanced diet (BD), yoghurt (Y) or BD with supplemental yoghurt (BD + Y) for 7, 14 or 21 d. PFD, protein-free diet; MC, malnourished control; NC, normal control.

Table 1. Composition of the diets and yoghurt used in the study

Ingredient	Balanced conventional diet	Protein-free diet	Yoghurt
Water (g/kg)	120	120	857
Proteins (g/kg)	230	<10	57
Carbohydrates (g/kg)	538	758*	76
Lipids (g/kg)	50	50	2
Vitamins (g/kg)†	22	22	1
Minerals (g/kg)‡	40	40	7

* Protein-free maize flour.

† Vitamin mix (no. 905454; ICN Biomedicals Argentina, Buenos Aires, Argentina) contained (g/kg 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; finely powdered sucrose, 847·38865.

‡ Mineral mix (no. 902844; ICN Biomedicals) supplied (g/kg mixture): sodium chloride, 167; potassium phosphate dibasic, 322; calcium carbonate, 300; magnesium sulfate, 102; calcium phosphate monobasic, 75; ferric citrate, 27·5; manganese sulfate.H_2O, 5·1; potassium iodide, 0·8; copper sulfate.$5H_2O$, 0·3; zinc chloride, 0·25; cobalt chloride.$6H_2O$, 0·05.

Challenge with *S. pneumoniae* was performed on the day after the end of each dietary treatment (day 8, 15 or 22) (Fig. 1(B)). Mice were infected by dropping 25 μl of the inoculum containing 10^5 log-phase colony-forming units *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. NC and MC mice were infected in the same way (Fig. 1(A)). Then, mice were killed on day 0 (before infection) and on days 1, 2, 5, 10 and 15 post-infection. During the 15 d post-infection all mice were fed only the BD, with the exception of MC mice, which received the protein-free diet. Survival of mice after challenge with *S. pneumoniae* was monitored until day 21 post-infection.

Body-weight determination

Body weight was determined before and after each dietary treatment. The experiment was performed on ten mice per 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 pneumococci and their lungs were excised, weighed and homogenised in 0·005 litres 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 (Facklam & Washington, 1992) and the results were expressed as log colony-forming units/g lung.

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

Determination of total and differential number of leucocytes in blood

Blood samples were obtained through cardiac puncture at the end of each dietary treatment (day 0) and on 1, 2, 5 and 10 d after challenge and collected in heparinised tubes. The total number of leucocytes was determined with a haemocytometer. Differential cell counts were performed by counting 200 cells in blood smears stained with May Grünwald-Giemsa.

Antibodies from serum and bronco-alveolar lavages

An ELISA was developed to measure anti-pneumococcal antibodies (IgA and IgG) in serum and bronco-alveolar lavages (BAL) on days 1, 2, 5, 10 and 15 after challenge; basal levels were determined on day 0, before infection. BAL samples were obtained according to the technique of Bergeron *et al.* (1998) modified as follows: the trachea was exposed and intubated with a catheter and two sequential lavages were performed in each mouse by injecting 0·5 ml sterile PBS; the recovered fluid was centrifuged for 10 min at 900*g*; the pellet was used to make smears that were stained with May Grünwald-Giemsa and the fluid was frozen at −70°C for subsequent antibody analyses.

Each plate was coated with a 1:100 dilution of *S. pneumoniae* vaccine (200 μl; NEUMO 23 polyvalent vaccine; Aventis Pasteur S.A., Buenos Aires, Argentina) 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 non-fat dry milk (50 g/l) 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 anti-mouse IgA or IgG (anti-α chain specific no. A4700, anti-γ chain specific no. A3673; Sigma-Aldrich Co., St Louis, MO, USA) 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 a substrate solution (200 μl 3-3′, 5-5′-tetramethylbenzidine; no. T2885; Sigma-Aldrich Co.) 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 1 M-H_2SO_4 (50 μl). Readings were carried out at 493 nm (VERSAmax Tunable Microplate Reader; Molecular Devices Corp., Sunnyvale, CA, USA) and the antibody concentration (g/l) in each unknown sample was determined from a standard curve made with commercial mouse IgA (catalogue no. M-1421; Sigma-Aldrich Co.) and mouse IgG (catalogue no. I-5381; Sigma-Aldrich Co.).

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 (Kaplow, 1968). Cells were graded as negative or weakly, moderately or strongly positive and were used to calculate the score number.

Nitro blue tetrazolium test. The phagocytic bactericidal activity (oxidative burst) of macrophage and neutrophil function were measured using the nitro blue tetrazolium (NBT) reduction test (catalogue no. 840-W; Sigma-Aldrich Co.) in the pellet of BAL. NBT was added to all samples with (positive control) or without the addition of bacterial extract, and then they 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. Smears were then prepared; after staining, these samples were examined under a light microscope for blue precipitates and 100 NBT positive (+) cells were counted.

Histopathology

At pre-chosen intervals, whole-lung samples from control and infected mice were excised and washed with 0·01 M-PBS (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, Nussloch, Germany) at 56°C. Finally, lungs were cut into 4 μm serial sections and stained with haematoxilin-eosin for light microscopy examination.

Statistical analysis

Experiments were performed in triplicate and results were expressed as mean values and standard deviations. For body-weight gain one-way ANOVA was used. For all other determinations, two-way ANOVA was used. Tukey's test (for pairwise comparisons of the mean values of the different groups) was

used to test for differences between the groups. Differences were considered significant at $P < 0.05$. For survival analysis the Pearson χ^2 test and MV-G-square test were used; analyses were performed for the complete contingency table and for 2×2 tables in order to compare each repletion treatment with the MC condition. Differences were considered significant at $P < 0.05$.

Results

Recovery of malnourished mice by dietary treatments

Body weights, blood leucocytes and phagocyte activity before challenge with pneumococci are presented in Table 2. All repleted mice increased significantly their body weights, with no differences between the dietary treatments (Table 2).

Malnutrition caused a diminution in the total number of blood leucocytes (Table 2) while differential cell counts showed a decrease in both neutrophils and lymphocytes (data not shown). These numbers were significantly improved with 14 and 21 d of the different treatments and with 7 d of the BD + Y, but only the groups with 14 d of the BD + Y, 21 d of BD, 21 d of yoghurt and 21 d of BD + Y reached levels similar to those of the NC group (Table 2).

The peroxidase score of blood neutrophils and the determination of NBT + cells in BAL evaluated the recovery of phagocyte activity. MC neutrophils had significantly lower peroxidase scores and NBT + cell percentages than NC and all dietary treatments improved these parameters. Feeding with the BD for 21 d was necessary to obtain normal peroxidase scores as against 14 d of feeding with yoghurt or the BD + Y (Table 2). In addition, NBT + cell percentages were similar to those of the NC mice only in the groups fed the BD + Y for 14 d, BD + Y for 21 d, BD for 21 d and yoghurt for 21 d (Table 2). The group fed the BD + Y for 21 d was not included in the following experiments because treatment of mice with the BD + Y for 14 d was sufficient to normalise the parameters studied.

Bacterial cell counts in lung homogenates and blood

Pneumococci were detected in lung and blood samples from NC and MC mice throughout the post-infection period (10 d), but MC mice had significantly higher levels than the NC group (Fig. 2). The groups fed the BD + Y for 14 d, the BD for 21 d and yoghurt for 21 d did not differ from NC (Fig. 2(B and C)). The other experimental groups had significantly lower bacterial counts than the MC mice but did not reach the levels of the NC mice (Fig. 2(A and B)). Survival of mice was monitored after challenge with the pathogen. All of the NC mice survived until day 21 post-infection while only 30 % of the mice in the MC group were alive at this time point. The survival of all repleted mice was significantly higher than in the MC group ($P < 0.05$): 70 % for the groups fed the BD for 7 d and 14 d, the mice fed yoghurt for 7 d and those fed the BD + Y for 7 d, and 90 % for the group fed yoghurt for 14 d. Only the group fed the BD + Y for 14 d, and those fed the BD or yoghurt for 21 d showed survival rates similar to those of the NC mice (100 %).

Total and differential numbers of blood leucocytes

Blood leucocytes of MC and NC mice increased after challenge (Fig. 3), with neutrophils as the principal population responsible for this increase (Fig. 4). However, leucocyte, lymphocyte and neutrophil counts were significantly lower in MC than in NC mice. In the group fed the BD + Y for 14 d, and those fed the BD and BD and yoghurt for 21 d the number of leucocytes (Fig. 3(B and C)), lymphocytes (Fig. 4(B and C)) and neutrophils (Fig. 4(E and F)) was not different from that of the NC mice. The other experimental groups did not reach the levels of the NC mice, but they had significantly higher counts of blood leucocytes (Fig. 3(A and B)), neutrophils (Fig. 4 (D and E)) and lymphocytes (Fig. 4 (B)) than the MC group. Only mice fed for 7 d on the BD, yoghurt or the BD + Y

Table 2. Effect of dietary treatments on the recovery of body weight, blood leucocytes, blood neutrophil peroxidase activity and alveolar macrophage bactericidal activity in malnourished mice before challenge with *Streptococcus pneumoniae*

(Mean values and standard deviations)

							% NBT + cells in BAL			
	Body weight (g)		Leucocytes in blood (10^9 cells/l)		Peroxidase in blood (score number)		Unstimulated		Stimulated	
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BD										
7 d	18·3[b]	0·5	2·9[a]	0·7	104·9[b]	1·1	17·6[b]	2·9	18·8[*a]	4·4
14 d	20·9[b]	0·6	3·7[b]	0·3	109·3[b]	1·1	17·8[b]	1·8	20·3[*b]	1·4
21 d	21·9[b]	0·7	6·1[c]	0·4	123·9[c]	2·1	17·6[b]	1·7	26·1[**c]	0·9
Y										
7 d	18·2[b]	0·5	3·1[a]	0·3	110·3[b]	1·7	18·1[b]	1·3	20·8[*b]	3·2
14 d	20·8[b]	0·6	3·9[b]	0·2	121·4[c]	1·3	18·6[b]	3·5	21·1[*b]	2·9
21 d	21·6[b]	0·6	6·3[c]	0·7	126·1[c]	2·0	18·3[b]	2·3	25·9[**c]	1·3
BD + Y										
7 d	18·1[b]	0·5	3·7[b]	0·4	113·5[b]	1·2	18·4[b]	2·3	21·3[*b]	1·9
14 d	21·3[b]	0·3	6·2[c]	0·5	125·1[c]	1·2	17·8[b]	1·3	26·1[**c]	3·1
21 d	21·8[b]	0·5	6·9[c]	0·2	124·3[c]	1·0	18·0[b]	2·1	25·9[**c]	2·7
MC	9·2[a]	0·6	2·9[a]	0·5	92·3[a]	1·4	10·6[a]	4·5	17·5[**a]	3·4
NC	22·3[b]	0·6	6·9[c]	0·3	124·0[c]	1·2	17·4[b]	1·1	25·7[**c]	0·8

NBT, nitro blue tetrazolium; BAL, bronco-alveolar lavage; BD, balanced diet; Y, yoghurt; MC, malnourished control; NC, normal control.
[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).
Mean values were significantly higher compared with unstimulated cells in the same group: * $P < 0.05$, ** $P < 0.01$.
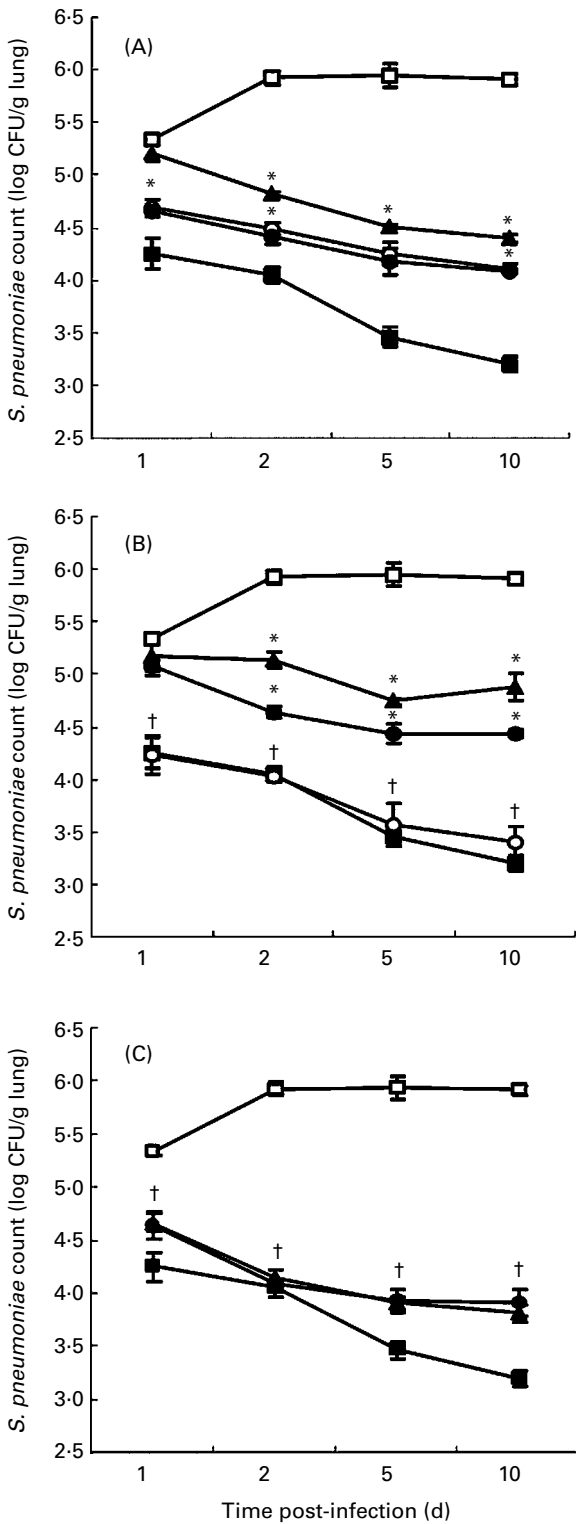
Fig. 2. *Streptococcus pneumoniae* colony-forming unit (CFU) counts in lungs of malnourished mice fed a balanced diet (▲), yoghurt (●) or a balanced diet with supplemental yoghurt (○) for 7 d (A), 14 d (B) or 21 d (C) and challenged at the end of each dietary treatment, and in lungs of malnourished (□) and normal (■) infected control mice. Values are means for six mice per group, with standard deviations represented by vertical bars. *Mean value was significantly different from those of the malnourished and normal control groups (*P*<0·05). †Mean value was not different from that of the normal control group (*P*>0·05).
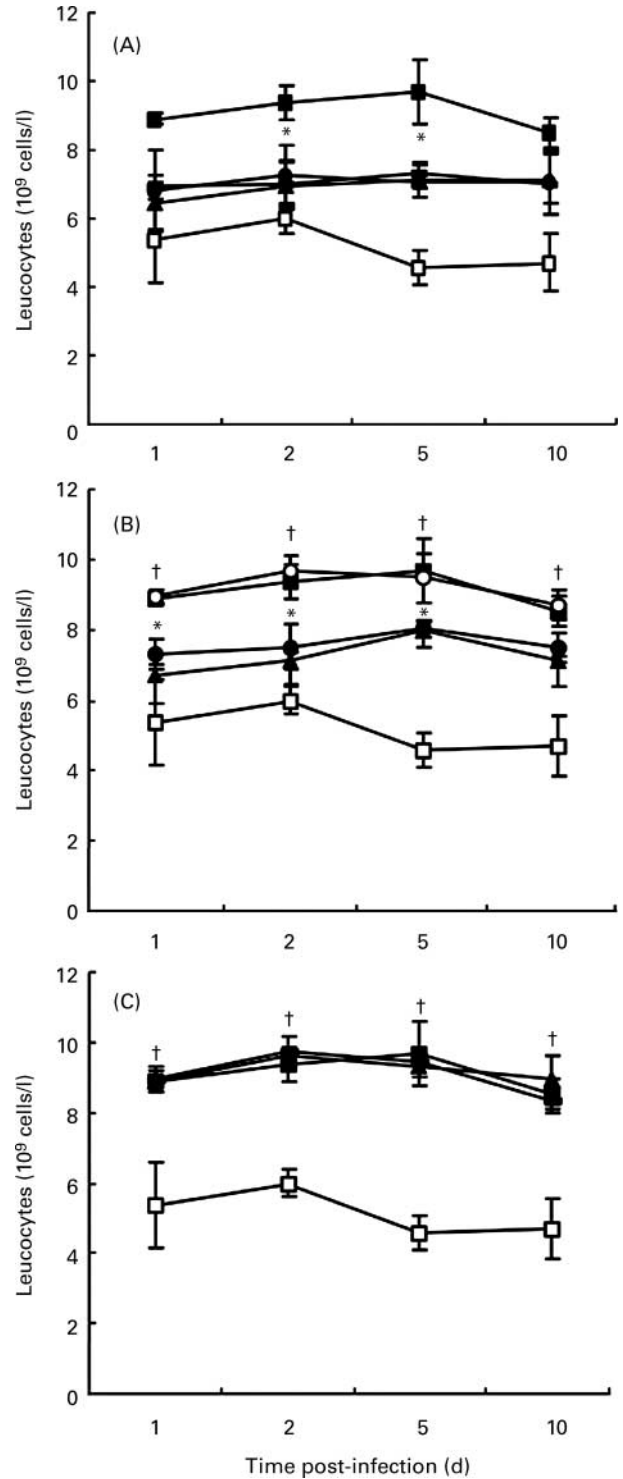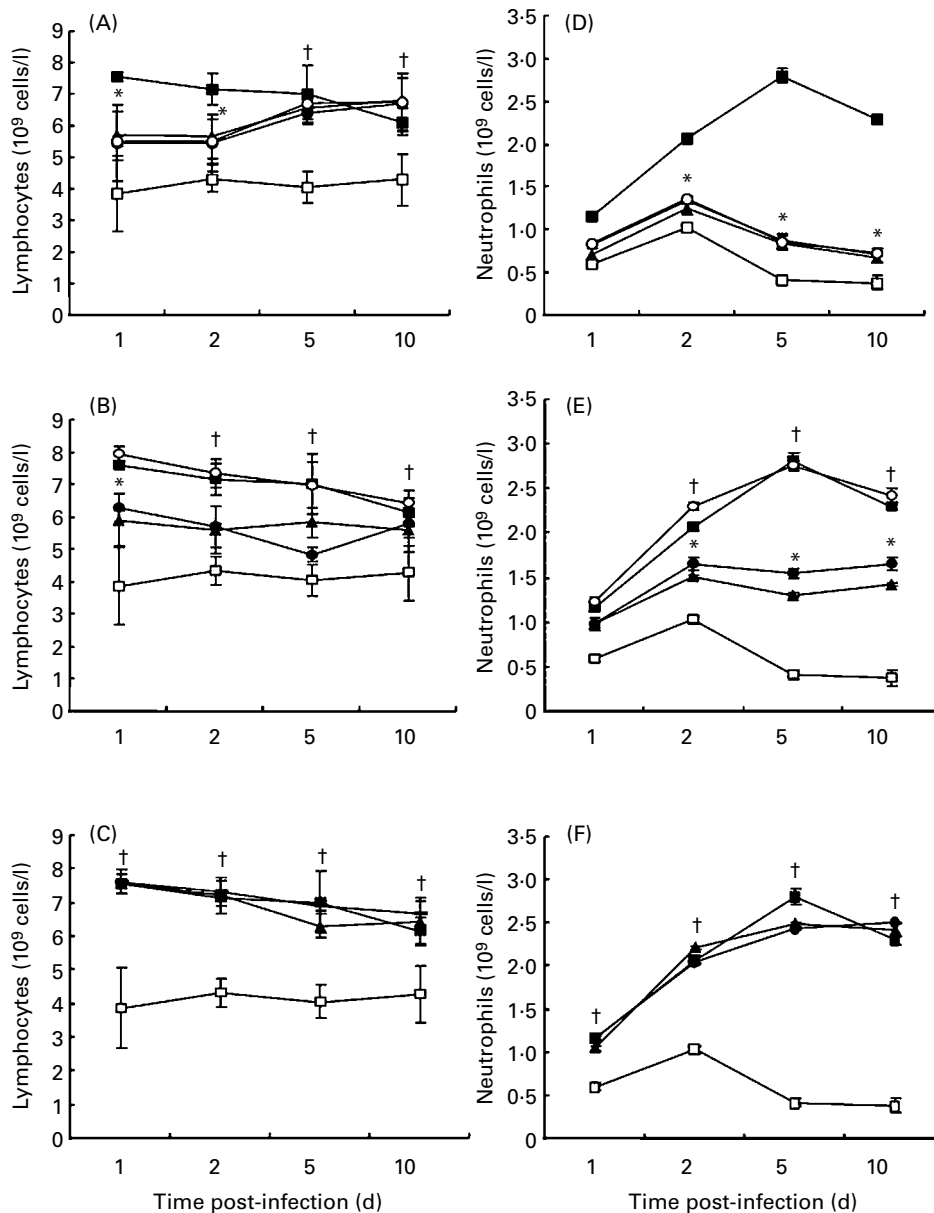
Fig. 3. Number of blood leucocytes of malnourished mice fed a balanced diet (▲), yoghurt (●) or a balanced diet with supplemental yoghurt (○) for 7 d (A), 14 d (B) or 21 d (C) and challenged with *Streptococcus pneumoniae* at the end of each dietary treatment, and of malnourished (□) and normal (■) infected control mice. Values are means for six mice per group, with standard deviations represented by vertical bars. * Mean value was significantly different from those of the malnourished and normal control groups (*P*<0·05). † Mean value was not different from that of the normal control group (*P*>0·05).

Fig. 4. Number of blood lymphocytes (A, B and C) and neutrophils (D, E and F) of malnourished mice fed a balanced diet (▲), yoghurt (●) or a balanced diet with supplemental yoghurt (○) for 7 d (A and D), 14 d (B and E) or 21 d (C and F) and challenged with *Streptococcus pneumoniae* at the end of each dietary treatment, and of malnourished (□) and normal (■) infected control mice. Values are means for six mice per group, with standard deviations represented by vertical bars. * Mean value was significantly different from those of the malnourished and normal control groups ($P < 0.05$). † Mean value was not different from that of the normal control group ($P > 0.05$).

showed lymphocyte numbers similar to those in the NC group on days 5 and 10 post-infection (Fig. 4 (A)).

Serum and bronco-alveolar lavage anti-pneumococcal antibodies

Levels of serum and BAL anti-pneumococcal immunoglobulins peaked on day 10 post-infection in all groups. Malnutrition significantly decreased serum and BAL IgG and IgA (Figs. 5 and 6). When malnourished mice were fed the different treatments for 7 d, serum and BAL IgG did not reach the levels of NC mice (Fig. 5). Treatment for 21 d with the BD or yoghurt were necessary to obtain similar values to those of NC mice. However, malnourished mice reached the values of NC mice when they were fed

yoghurt or BD + Y for 14 d. Serum IgA reached the values of the NC group in mice given the 14 d or 21 d treatments, with no significant differences between them (data not shown). Malnourished mice made replete with the BD, yoghurt or the BD + Y for 7 d showed higher levels of BAL IgA when compared with MC mice, but they did not reach the values of the NC group. However, mice treated with yoghurt or the BD + Y for 14 d, or the BD or yoghurt for 21 d did not present differences with the NC group.

Phagocytic cells activity

Peroxidase activity of blood and BAL neutrophils and BAL NBT+ cells were determined after challenge with *S. pneumoniae*.
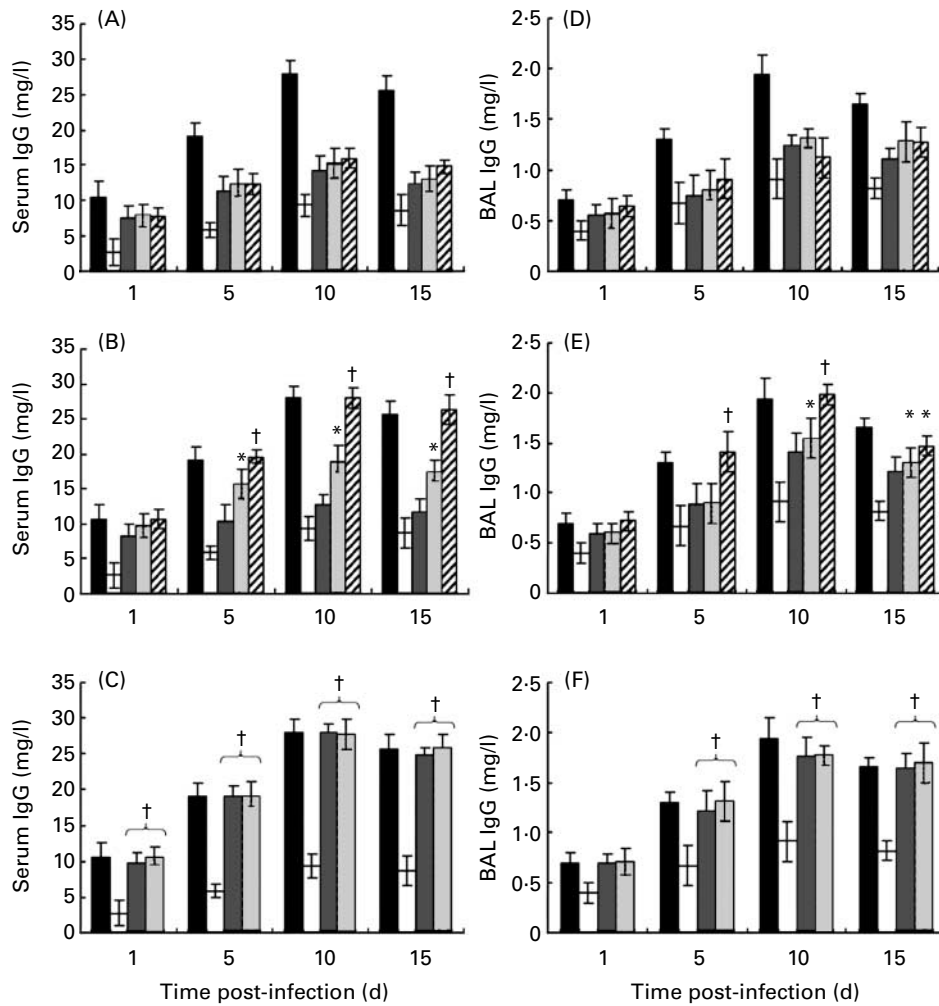
Fig. 5. Anti-pneumococcal IgG in serum (A, B and C) and bronco-alveolar lavages (BAL; D, E and F) of malnourished mice fed a balanced diet (■), yoghurt (▨) or a balanced diet with supplemental yoghurt (▧) for 7 d (A and D), 14 d (B and E) or 21 d (C and F) and challenged with *Streptococcus pneumoniae* at the end of each dietary treatment, and of malnourished (□) and normal (■) infected control mice. Values are means for six mice per group, with standard deviations represented by vertical bars. *Mean value was significantly different from those of the malnourished and normal control groups ($P < 0.05$). †Mean value was not different from that of the normal control group ($P > 0.05$).

MC had significantly lower peroxidase scores and NBT+ cell percentages than NC and all dietary treatments improved these parameters. In order to obtain normal peroxidase scores, 21 d of feeding with the BD were necessary, but only 14 d with yoghurt or the BD + Y (Table 3). The percentage of NBT+ cells was similar to that of the NC mice only in the groups fed the BD + Y for 14d, BD for 21d, BD for yoghurt for 21 d (Table 3).

Histopathological examination

The NC and MC groups, the group fed the BD for 14 d and that fed the BD + Y for 14 d were selected to perform lung histopathological studies. Morphological examination of lungs of MC mice showed progressive oedema, inflammatory response, and alveolar congestion, increased fibrosis in bronchial walls and vessels, passage of blood elements from capillaries to tissues, haemorrhage and widespread cellular infiltration. Furthermore, the lung parenchyma had a distorted appearance with loss of alveolar architecture (Fig. 7 (B)). These histopathological findings were less pronounced in the lungs of NC mice (Fig. 7 (A)). Mice

treated with BD for 14 d showed histological signs intermediate to those of NC and MC mice (Fig. 7 (C)). However, histological examination of lungs from the mice fed the BD + Y for 14 d showed characteristics similar to the NC, with focal cellular infiltration and reduction of the alveolar air spaces but with preserved alveolar architecture (Fig. 7 (D)).

Discussion

It has been reported that malnutrition results in increased susceptibility to infection and that infection causes the deterioration of the nutritional status, ushering in a cycle of malnutrition–infection (Scrimshaw & SanGiovanni, 1997). This cycle cannot be stopped merely by improving the nutritional intake, especially in the presence of repeated exposure to infection (as is the case in malnourished children in developing countries; Keusch, 2003). In consequence, greater attention to the improvement of the immune system in malnourished individuals is necessary in order to diminish mortality rates.
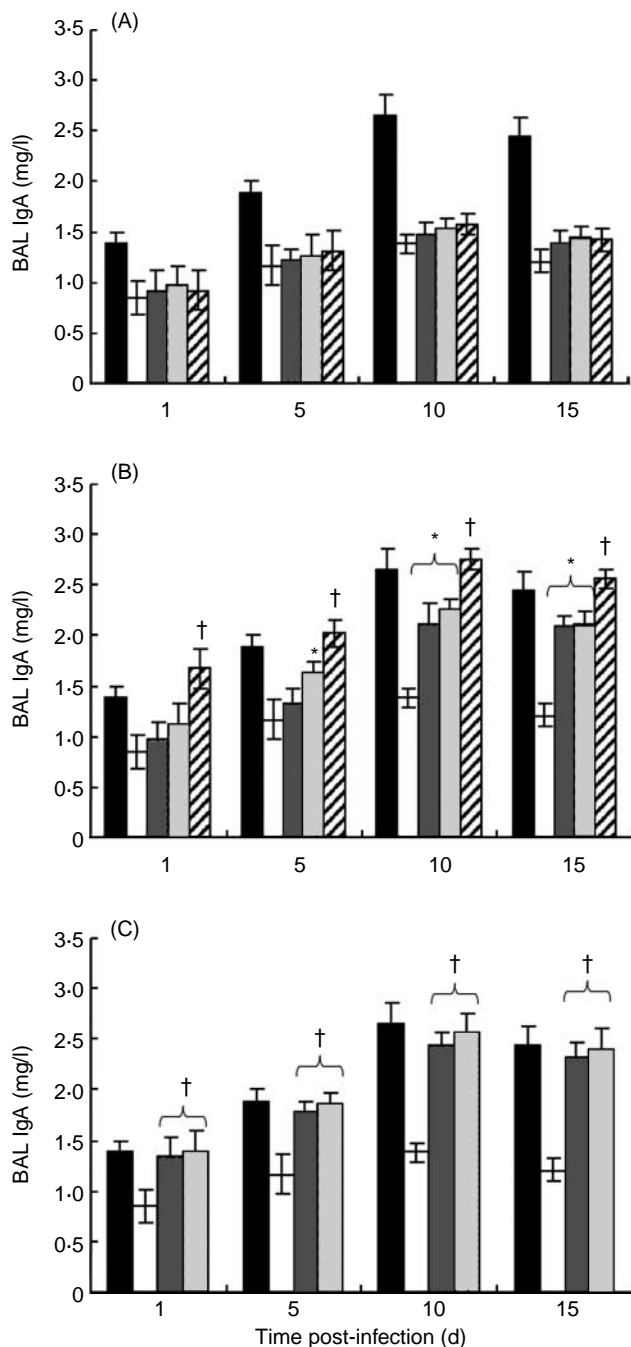
Fig. 6. Anti-pneumococcal IgA in bronco-alveolar lavages (BAL) of malnourished mice fed a balanced diet (■), yoghurt (■) or a balanced diet with supplemental yoghurt (▨) for 7 d (A), 14 d (B) or 21 d (C) and challenged with *Streptococcus pneumoniae* at the end of each dietary treatment, and of malnourished (□) and normal (■) infected control mice. Values are means for six mice per group, with standard deviations represented by vertical bars. * Mean value was significantly different from those of the malnourished and normal control groups (*P*<0·05). † Mean value was not different from that of the normal control group (*P*>0·05).

In the present paper we studied the effect of yoghurt when used as a supplement in a repletion diet and we evaluated the behaviour of treated malnourished mice against an *S. pneumoniae* respiratory infection.

Administration of yoghurt to malnourished mice induced an early recovery of the immunological parameters studied and

accelerated the normalisation of the immune response against the infection.

The effect of yoghurt on the early recovery of the defence mechanisms against *S. pneumoniae* in protein-malnourished mice could be explained by a combination of three factors. First, we should consider that the protein from yoghurt is more easily digested than protein from milk because bacterial predigestion occurs during casein fermentation (Buttriss, 1997). In consequence, malnourished mice fed with yoghurt have an excellent source of high-quality proteins. Second, yoghurt is considered a probiotic food (Guarner *et al.* 2005); consequently, it could exert health benefits beyond inherent basic nutrition. The immunostimulatory effects of yoghurt are believed to be due to its bacterial components. Lactobacilli can directly promote the development of the gut mucosal barrier by the stimulation of the immune response. Since immune mechanisms at different mucosal sites interact through the common mucosal immune system, LAB could also play an important role in disseminating effector cells to lung and other mucosal sites. This immune-stimulating activity has been described for two of the LAB strains present in the yoghurt used in the present study; *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412 (Perdigón *et al.* 1998). Besides, in some instances the systemic immune responses may be directly augmented by the dietary addition of LAB (Schiffrin *et al.* 1995). Third, non-bacterial milk components and components produced from milk fermentation may also contribute to the immunostimulatory activity of yoghurt. Peptides and NEFA generated by fermentation have been shown to enhance the immune response (Meydani & Ha, 2000).

In the present study, MC mice showed an increased susceptibility to pneumococcal infection compared with the NC group. While the number of bacteria in the lungs and bloodstream tended to decrease (*P*<0·05) during infection in NC mice, it remained constant in MC mice. Feeding the BD for 21 d was necessary to obtain levels of infection similar to those in NC mice; however, yoghurt supplementation for 14 d was enough to obtain bacterial counts similar to NC mice.

Alveolar macrophages constitute the first line of phagocytic defence against infectious agents that evade the mechanical defences and gain access to the gas-exchanging airways (Sibille & Reynolds, 1990). In the present study, malnourished mice that were treated with the BD + Y showed an increase in the bactericidal function of bronco-alveolar phagocytes before challenge with *S. pneumoniae*. These data agree with our previous results, which showed that yoghurt administration to young mice improved the phagocytic activity of alveolar macrophages (Alvarez *et al.* 2001). In addition, it has been observed that more proteolytically hydrolysed milk results in increased stimulation of phagocytosis from the pulmonary alveolar macrophages in mice (Moineau & Goulet, 1991). Consequently, it seems probable that the peptides from milk fermentation could also contribute to macrophage activity stimulation.

Macrophages are capable of generating numerous mediators that induce the recruitment of neutrophils from the pulmonary vasculature into the alveolar space. These recruited neutrophils provide auxiliary phagocytic capacities that are critical for the effective eradication of offending pathogens (Sibille & Reynolds, 1990; Gordon *et al.* 2000; Gingles *et al.* 2001). It has been previously reported that malnutrition impairs the recruitment of neutrophils into local inflammatory sites (Hidemura *et al.* 2003, Villena *et al.* 2005). In the present study there were significantly fewer neutrophils in blood in MC mice compared with the NC

Table 3. Phagocytic cell activation in malnourished mice after dietary treatments and challenge with *Streptococcus pneumoniae*

(Mean values and standard deviations)

	Peroxidase + cells				NBT + cells in BAL			
	Blood†		BAL‡		Unstimulated		Stimulated	
	Score number				Percentage			
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BD								
7 d	122·1[b]	1·7	106·2[a]	3·4	40·2[a]	0·9	51·6*[a]	3·8
14 d	131·9[b]	3·5	131·4[b]	2·3	41·9[a]	3·2	52·5*[a]	1·9
21 d	159·9[c]	2·8	163·9[c]	1·4	47·1[b]	1·9	62·1**[c]	2·1
Y								
7 d	123·7[b]	1·4	107·8[a]	2·3	42·7[a]	1·1	52·9*[a]	2·1
14 d	149·8[c]	2·3	145·8[b]	3·4	43·9[b]	2·5	54·8*[b]	2·2
21 d	163·3[c]	2·9	167·3[c]	4·1	48·5[b]	2·1	63·3**[c]	3·2
BD + Y								
7 d	130·4[b]	1·4	122·1[b]	1·9	43·8[b]	1·7	54·9*[b]	3·1
14 d	163·9[c]	3·4	165·2[c]	3·4	48·9[b]	2·1	64·5**[c]	2·7
MC	100·0[a]	2·8	84·5[a]	6·4	37·3[a]	3·4	47·9*[a]	2·5
NC	167·5[c]	4·3	164·0[c]	5·1	46·8[b]	2·2	62·6**[c]	1·2

NBT, nitro blue tetrazolium; BAL, bronco-alveolar lavage; BD, balanced diet; Y, yoghurt; MC, malnourished control; NC, normal control.

[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

Mean values were significantly higher compared with unstimulated cells in the same group: * $P < 0.05$, ** $P < 0.01$.

† Day 2 post-infection.

‡ Day 5 post-infection.

group at the same post-infection period. Leucocytes and neutrophils in blood increased with the BD, yoghurt or BD + Y treatment. However, mice that received the BD with supplemental yoghurt for 14 d had significantly more leucocytes and neutrophils with higher peroxidase activity than those fed the BD for 14 d, reaching values similar to those of NC mice. Impaired neutrophil recruitment into local inflammatory sites can be restored by administration of probiotic bacteria (Hidemura *et al.* 2003; Villena *et al.* 2005). It could be speculated that yoghurt exerted the same effect, although the number of locally exudative neutrophils was not evaluated in the present study. Consequently, yoghurt administration seems to be able to induce an earlier recovery of the innate immune response.

Malnutrition has been reported to produce a remarkable decrease in the number of IgA-secreting cells associated with the lamina propria of the small intestine (Gauffin *et al.* 2002*a*), and it probably has the same effect on other mucosal tissues. This would explain the significantly lower concentration of BAL IgA found in MC mice in the present study. When malnourished mice were fed the BD with supplemental yoghurt for 14 d, BAL anti-pneumococcal IgA reached levels similar to those of NC mice. This effect could be related to the ability of LAB to modulate IgA concentrations. In a previous study we demonstrated that feeding with yoghurt increased both IgA production and the number of IgA-secreting cells in the small intestine of mice in a dose-dependent manner (Perdigón *et al.* 1995). Similarly, yoghurt administration increased the number of IgA cells associated with the lamina propria of the small intestine of malnourished mice (Gauffin *et al.* 2002*b*), and induced a significant increase in the levels of BAL IgA, IgM and IgG after a *P. aeruginosa* infection in young mice (Alvarez *et al.* 2001). The importance of IgA antibodies in the prevention of mucosal infections is well known. It has been shown that the immune exclusion and elimination of the pathogenic agent at the mucosal surfaces by secretory IgA is crucial in preventing pneumococcal bacteraemia (Service, 1994). These observations are in agreement with our previous results, since we found that the addition of *L. casei* to the repletion diet fed to malnourished mice increased BAL anti-pneumococcal IgA, which reached higher levels than those of NC mice (Villena *et al.* 2005).

The present report also shows that yoghurt administration for 14 d increased serum and BAL anti-pneumococcal IgG, which reached similar levels to those in the NC group. Serum anti-pneumococcal IgG, an antibody with opsonophagocytic activity, represents an important factor that contributes to the prevention of and protection against septicaemia (Boulnois, 1992; Briles *et al.* 1998; Anttila *et al.* 1999). These findings demonstrate the role of specific antibodies in the infection caused by *S. pneumoniae* and, consequently, the importance of their early recovery using yoghurt as a supplement in a repletion diet.

Histopathological examination of lungs showed that tissue damage in mice treated with the BD with supplemental yoghurt for 14 d was less than in those fed the BD for 14 d. It has been reported that alveolar macrophages play an important role in the regulation of the inflammatory response during pneumococcal pneumonia (Knapp *et al.* 2002) and that yoghurt administration induced macrophage activation; consequently, these cells could be involved in the anti-inflammatory effect observed. On the other hand, previous findings showed that IgA antibodies bind antigens and minimise their entry with a consequent reduction in inflammatory reactions (Janoff *et al.* 1999). Probably, the increase in BAL IgA levels would contribute to the prevention of potentially harmful effects in lung tissue. Moreover, the decrease in lung injuries and the improvement of the innate and specific immune responses observed in the groups fed yoghurt could be involved in the increase in survival rates.
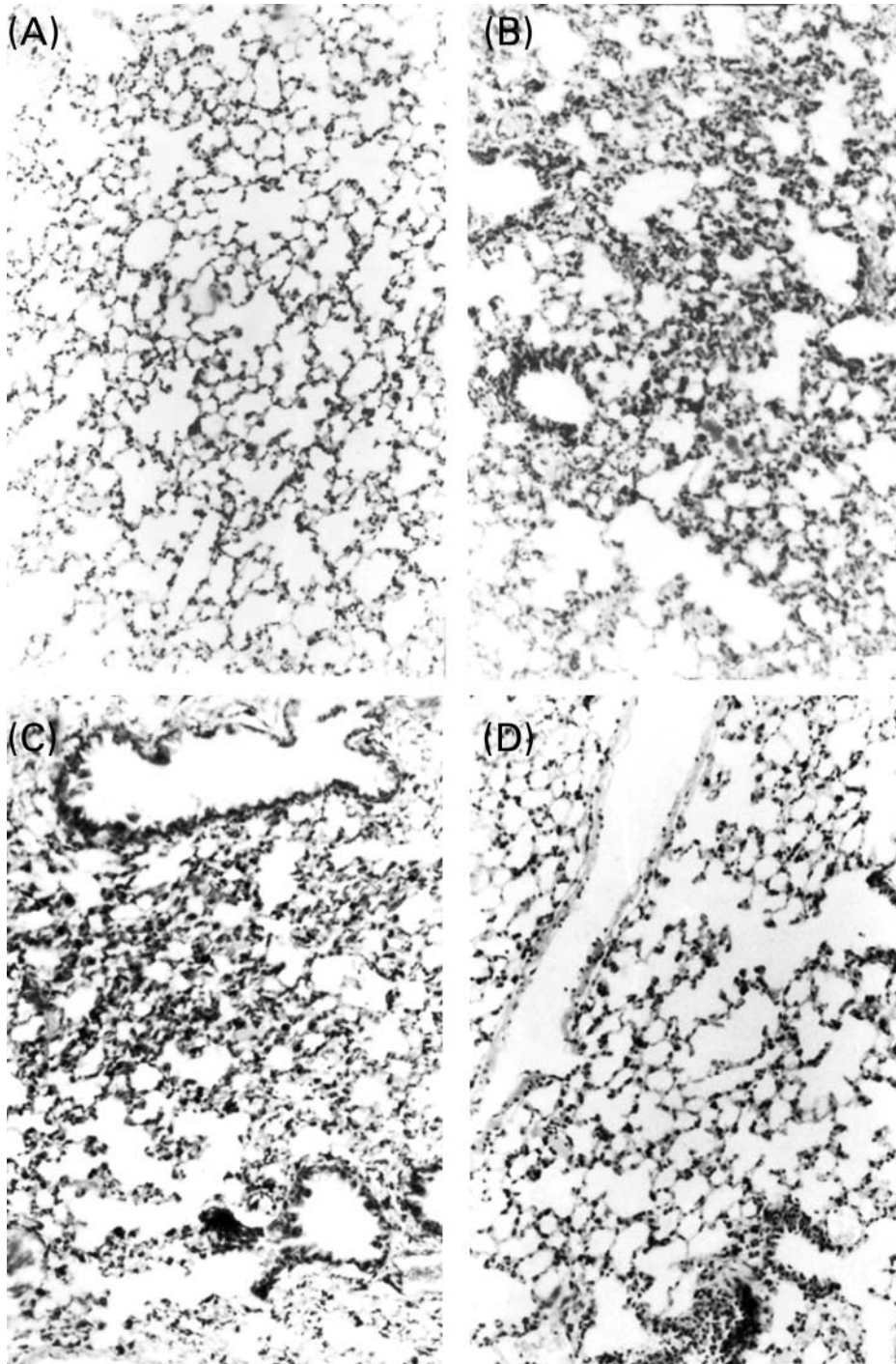
Fig. 7. Lung architecture of normal control mice (A), of malnourished controls (B), of malnourished mice fed a balanced diet for 14 d (C) and of malnourished mice fed a balanced diet with supplemental yoghurt for 14 d (D), 10 d after challenge with *Streptococcus pneumoniae*. Light micrographs, original magnification × 200.

The present results showed that yoghurt administration to malnourished mice is capable of accelerating the normalisation of some of the parameters under study. Its maximum effect was reached when accompanied by an adequate repletion diet, probably because the BD provides adequate levels of nutrients and yoghurt intake could enhance both natural and acquired immunity. Some authors have reported that the immune system stimulation exerted by the LAB used in yoghurt production would allow the maintenance of an improved resistance against pathogens (Halpern *et al.* 1991; Erickson & Hubbard, 2000; Hoerr & Bostwick, 2000).

Although these studies were performed in an animal model of malnutrition, several investigations suggest that fermented milk consumption improves recovery in undernourished children or children with diarrhoea (Gendrel *et al.* 1990; Touhami *et al.* 1992; Lewis & Freeman, 1998; Saavedra, 2000; Solis *et al.* 2002). Furthermore, the World Health Organization (2000) has supported the use of yoghurt in nutritional recovery. Despite the

uncertainties concerning the mechanisms involved and the human relevance of the effects observed in animal models, the present study provides a strong rationale for the hypothesis that yoghurt consumption by malnourished children will accelerate the recovery of the immune mechanisms involved in the protection against respiratory infections.

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