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

Julio Villena\(^1\), Silvia Racedo\(^2\), Graciela Agüero\(^1\) and Susana Alvarez\(^{1,2*}\)

\(^1\)Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Balcarce 747, CP 4000, San Miguel de Tucumán, Tucumán, Argentina

\(^2\)Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, CP 4000, San Miguel de Tucumán, Tucumán, Argentina

(Received 14 May 2005 – Revised 31 October 2005 – Accepted 7 November 2005)

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 broncho-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 (Scrimsishaw & SanGiovanni, 1997).

Yoghurt is defined by the Codex Alimentarius of 1992 as a coagulated 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 (Adolfsion 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 (Gauflin & 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 system.

**Abbreviations:** BAL, broncho-alveolar lavage; BD, balanced diet; BD + Y, balanced diet with supplemental yoghurt; CERELA, Centro de Referencia para Lactobacilos; LAB, lactic acid bacteria; MC, malnourished control; NBT, nitro blue tetrazolium; NC, normal control; PBS-T, PBS–Tween 20.

* Corresponding author: Dr Susana Alvarez, fax +54 381 4005600, email salvarez@cerela.org.ar
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, bacteremia 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 biotério of the CERELA (San Miguel de Tucuman, 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 (Perdigon 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 × 10⁷ 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 Tucuman, 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 × 10⁸ 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).

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

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Balanced conventional diet</th>
<th>Protein-free diet</th>
<th>Yoghurt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (g/kg)</td>
<td>120</td>
<td>120</td>
<td>857</td>
</tr>
<tr>
<td>Proteins (g/kg)</td>
<td>230</td>
<td>&lt;10</td>
<td>57</td>
</tr>
<tr>
<td>Carbohydrates (g/kg)</td>
<td>538</td>
<td>758*</td>
<td>76</td>
</tr>
<tr>
<td>Lipids (g/kg)</td>
<td>50</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>Vitamins (g/kg)†</td>
<td>22</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>Minerals (g/kg)†</td>
<td>40</td>
<td>40</td>
<td>7</td>
</tr>
</tbody>
</table>

* Protein-free maize flour.
† Vitamin mix (no. 905454; ICN Biomedicals Argentina, Buenos Aires, Argentina) contained (g/kg mixture): niacinamide, 5.0; p-amino benzoic acid, 5.0; ascorbic acid, 45.0; biotin, 0.02; retinyl acetate, 4.5; vitamin B12, 0.00135; calcium pantothenate, 3.0; choline chloride, 75.0; cholecalciferol, 0.25; tocic 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, 847.38866.  
‡ 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•H2O, 5.1; potassium iodide, 0.8; copper sulfate•5H2O, 0.3; zinc chloride, 0.25; cobalt chloride•6H2O, 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
10^7 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 verti-
cal 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. Sur-
vival 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 treat-
ment. 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 appropri-
ately, 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 moni-
tored 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 incu-
bation 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
leucocyte 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 anti-
odies (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* vac-
cine (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 tem-
perature. 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 sub-
strate 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 Micro-
plate Reader; Molecular Devices Corp., Sunnyvale, CA, USA) and the
antibody concentration (g/l) in each unknown sample was deter-
mined from a standard curve made with commercial mouse IgA
(catalogue no. M-1421; Sigma-Aldrich Co.) and mouse IgG (catalo-
gue 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 incu-
bated at 37°C for 20 min. In the presence of oxidative metabolites,
NBT (yellow) is reduced to formazan, which forms a blue precipi-
tate. 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
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 pair-
wise 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 $\chi^2$ test and MV-G-square test were used; analyses were performed for the complete contingency table and for $2 \times 2$ 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 numbers 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)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Leucocytes in blood (10⁶ cells/l)</th>
<th>Peroxidase in blood (score number)</th>
<th>% NBT + cells in BAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>BD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 d</td>
<td>18.3b</td>
<td>0.5</td>
<td>2.9a</td>
<td>0.7</td>
</tr>
<tr>
<td>14 d</td>
<td>20.0b</td>
<td>0.6</td>
<td>3.7b</td>
<td>0.3</td>
</tr>
<tr>
<td>21 d</td>
<td>21.9b</td>
<td>0.7</td>
<td>6.1c</td>
<td>0.4</td>
</tr>
<tr>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 d</td>
<td>18.2b</td>
<td>0.5</td>
<td>3.1b</td>
<td>0.3</td>
</tr>
<tr>
<td>14 d</td>
<td>20.3b</td>
<td>0.6</td>
<td>3.9b</td>
<td>0.2</td>
</tr>
<tr>
<td>21 d</td>
<td>21.6b</td>
<td>0.6</td>
<td>6.3c</td>
<td>0.7</td>
</tr>
<tr>
<td>BD + Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 d</td>
<td>18.1b</td>
<td>0.5</td>
<td>3.7b</td>
<td>0.4</td>
</tr>
<tr>
<td>14 d</td>
<td>21.3b</td>
<td>0.3</td>
<td>6.2b</td>
<td>0.5</td>
</tr>
<tr>
<td>21 d</td>
<td>21.8b</td>
<td>0.5</td>
<td>6.9b</td>
<td>0.2</td>
</tr>
<tr>
<td>MC</td>
<td>9.2a</td>
<td>0.6</td>
<td>2.9a</td>
<td>0.5</td>
</tr>
<tr>
<td>NC</td>
<td>22.3b</td>
<td>0.6</td>
<td>6.9b</td>
<td>0.3</td>
</tr>
</tbody>
</table>

NBT, nitro blue tetrazolium; BAL, broncho-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: * $P<0.05$; ** $P<0.01$. 

Downloaded from https://www.cambridge.org/core, IP address: 54.70.40.11, on 25 Sep 2018 at 17:21:44, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms . https://doi.org/10.1079/BJN20051663
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. 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).
showed lymphocyte numbers similar to those in the NC group on days 5 and 10 post-infection (Fig. 4 (A)).

Serum and broncho-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.
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 14 d, BD for 21 d, 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.
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 (Guamer 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 immunostimulating 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 broncho-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. 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.

In the present study 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.

Fig. 6. Anti-pneumococcal IgA in broncho-alveolar lavages (BAL) of malnourished mice fed a balanced diet (B), yoghurt (Y) or a balanced diet with supplemental yoghurt (S) 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 (M) and normal (N) infected control mice. Values are means for six mice per group, with standard deviations represented by vertical bars. * Mean value was significantly different from that 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.

In the present study there were significantly fewer neutrophils in blood in MC mice compared with the NC
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. 2002a), 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 (Perdígón 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. 2002b), and induced a significant increase in the levels of BAL IgA, IgM and IgG after a P. aeurginosa 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 increased 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.

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

(Means and standard deviations)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood†</th>
<th>BAL‡</th>
<th>Unstimulated</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Score number</td>
<td>Percentage</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Peroxidase + cells</strong></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>BD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 d</td>
<td>122.1 b</td>
<td>1.7</td>
<td>106.2 *</td>
<td>3.4</td>
</tr>
<tr>
<td>14 d</td>
<td>131.9 b</td>
<td>3.5</td>
<td>131.4 b</td>
<td>2.3</td>
</tr>
<tr>
<td>21 d</td>
<td>159.9 bc</td>
<td>2.8</td>
<td>163.9 b</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>Y</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 d</td>
<td>123.7 b</td>
<td>1.4</td>
<td>107.8 *</td>
<td>2.3</td>
</tr>
<tr>
<td>14 d</td>
<td>149.8 bc</td>
<td>2.3</td>
<td>145.8 bc</td>
<td>3.4</td>
</tr>
<tr>
<td>21 d</td>
<td>163.3 bc</td>
<td>2.9</td>
<td>167.3bc</td>
<td>4.1</td>
</tr>
<tr>
<td><strong>BD + Y</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 d</td>
<td>130.4 b</td>
<td>1.4</td>
<td>122.1 bc</td>
<td>1.9</td>
</tr>
<tr>
<td>14 d</td>
<td>163.9 bc</td>
<td>3.4</td>
<td>165.2 bc</td>
<td>3.4</td>
</tr>
<tr>
<td>MC</td>
<td>100.0 a</td>
<td>2.8</td>
<td>84.5 a</td>
<td>6.4</td>
</tr>
<tr>
<td>NC</td>
<td>167.5 a</td>
<td>4.3</td>
<td>164.0 a</td>
<td>5.1</td>
</tr>
</tbody>
</table>

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

* P < 0.05,
** P < 0.01,
† Day 2 post-infection,
‡ Day 5 post-infection.
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.
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.

Acknowledgements

The authors are grateful to Lic. Elena Bru de Labanda for the statistical analysis. This research was supported by grants from FONCYT PICT 99 no. 5—7206 and CIUNT 26 D/20.

References


