JOURNAL OF FUNCTIONAL FOODS 5 (2013) 1693–1704

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Lactobacillus rhamnosus CRL1505 enhances systemic and respiratory innate immune response in immunocompromised malnourished mice

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ARTICLE INFO

Article history:
Received 22 February 2013
Received in revised form
10 July 2013
Accepted 11 July 2013
Available online 13 August 2013

Keywords:
Lactobacillus rhamnosus CRL1505
Bone marrow
Myeloid cells
Malnutrition
Respiratory immunity

ABSTRACT

The effect of *Lactobacillus rhamnosus* CRL1505 on blood and bone marrow (BM) myeloid cells and their correlation with resistance against *Streptococcus pneumoniae* infection was evaluated in protein-malnourished mice. Repletion of malnourished mice with supplemental *L. rhamnosus* improved recovery of BM responsiveness against the infectious challenge through increase in myeloid progenitors and mobilisation of granulocytes. The CRL1505 strain normalised the number of $Gr1^{+}$ cells and $Gr1^{low}/Gr1^{high}$ cells balance in BM. These changes in BM correlated with improved neutrophils recruitment, higher phagocytic activity and increased resistance against pneumococcal infection in probiotic-treated mice. This research provides strong evidence of the importance of dietary supplementation with probiotic bacteria to reverse alterations in myeloid progenitors in malnourished mice. The present results strongly suggest that the CRL1505 strain could be used in the development of probiotic foods, which would be especially useful for the recovery of immunocompromised hosts.

1. Introduction

The global probiotic market generates billions of dollars every year and more than 500 probiotic products have been introduced in the past decade. These products have met with varying degrees of success, mostly in congruence with their overall health benefits. Probiotics are equally accessible in Europe, Australia and the USA. However, most clinically tested probiotics are not available in the developing world and certainly not affordable for most people (Annunziata & Vecchio, 2011; Enos et al., 2012).

Government agencies and multinational probiotic companies should make probiotic products more widely available, especially for relief work and populations at high risk of morbidity and mortality such as malnourished individuals (Anukam & Reid, 2007). In this sense, with the support of the Tucuman Government (Argentina), we demonstrated that consumption of a fermented dairy product containing *Lactobacillus rhamnosus* CRL1505 is associated with a significant decrease in the duration and severity of mucosal infections in young children (Villena et al., 2012). On the basis of our results and given the high morbidity and mortality in children especially associated with airway infectious diseases, this new probiotic strain has been included in official national nutritional programmes in Argentina. Since 2008, a government-sponsored programme provides the probiotic yoghourt

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1756-4646/$ - see front matter
http://dx.doi.org/10.1016/j.jff.2013.07.014

containing *L. rhamnosus* CRL1505 daily to over 200,000 school children in Argentina.

Malnutrition is a major cause of acquired immunodeficiency affecting especially young children (Chandra, 1991; Keusch, 2003). Several studies clearly show that these populations with inadequate nutrition have an increased susceptibility to infections because protein deficiency alters hematopoiesis and immune responses (Berkowitz, 1992; Briassoulis, Zavras, & Hatzis, 2011; Rodriguez, Cervantes, & Ortiz, 2011; Victora, Barros, Kirkwood, & Vaughan, 1990). Thus, the adequate and prompt correction of the nutritional status is important to reduce morbidity and mortality in malnourished hosts. Lactic acid bacteria (LAB) that are able to beneficially modulate the immune system (immunobiotics) represent an attractive safe way to regulate and enhance the immune function in immunocompromised malnourished hosts (Villena, Oliveira, Ferreira, Salva, & Alvarez, 2011). We evaluated the effect of different immunobiotics on the prevention of a *Streptococcus pneumoniae* infection in immunocompromised protein-malnourished mice and our results showed that the addition of certain LAB strains to the repletion diet has a beneficial effect as it significantly accelerates the recovery of the systemic and mucosal immune systems and improves resistance against pneumococcal respiratory infection (Agüero, Villena, Racedo, Haro, & Alvarez, 2006; Alvarez, Villena, Racedo, Salva, & Agüero, 2007; Alvarez, Villena, & Salva, 2009a; Salva, Villena, Racedo, Alvarez, & Agüero, 2008). Although we demonstrated that *L. rhamnosus* CRL1505 is able to increase resistance against pneumococcal infection in immunocompetent mice (Salva, Villena, & Alvarez, 2010) and improve respiratory immunity in children (Villena et al., 2012), the effect of this strain on the resistance against respiratory infections in immunocompromised malnourished hosts has not been evaluated yet.

It was demonstrated that malnutrition significantly impairs phagocytic activity. In the respiratory tract, the phagocytic activity of alveolar macrophages and recruited neutrophils play an important role in the resistance against inhaled bacterial pathogens (Calbo & Garau, 2010; Kadioglu & Andrew, 2004). After recognition of pathogens through pattern recognition receptors, lung macrophages characteristically trigger chemokine receptor CXCR2-mediated recruitment of neutrophils into the lung parenchyma and bronchoalveolar compartment to support macrophage antibacterial responses (Herbold et al., 2010; Reutershan et al., 2006; Villena et al., 2005). Furthermore, a partially reduced neutrophil recruitment severely disturbs lung host defence against *S. pneumoniae* in mice, demonstrating that maximal neutrophil recruitment is essential for the early control of lung bacterial infections (Villena et al., 2005). Neutrophils and monocyte–macrophages are derived and differentiated from myeloid committed precursor cells in bone marrow. Moreover, it has been demonstrated that myeloid cells are mobilised from bone marrow during inflammatory or infection response, and are crucial for the defence against pathogens (Panopoulos & Watowich, 2008). Therefore, the study of the effect of malnutrition on bone marrow myeloid cells would be extremely important to understand the mechanisms involved in immunosuppression resulting from protein deficiency.

Considering this background, in the current study we aimed at evaluating the effect of the oral administration of *L. rhamnosus* CRL1505, a strain with immunomodulatory capabilities already demonstrated in immunocompetent mice and children (Salva et al., 2010; Villena et al., 2012), on the resistance against pneumococcal infection in protein-malnourished mice. We also aimed at increasing our knowledge of the mechanisms involved in the immunomodulatory effect of the CRL1505 strain in malnourished hosts by evaluating its impact on myeloid cell populations in blood and bone marrow. This knowledge may account, at least partly, for the mechanism through which probiotics increase resistance to infections caused by Gram (+) bacteria such as *S pneumoniae* in immunocompromised malnourished hosts and it can provide the scientific basis for the use of *L. rhamnosus* CRL1505 in treatments to restore immunity in malnourished children.

2. Materials and methods

2.1. *Lactic acid bacterium*

L. rhamnosus CRL1505 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 1 l, pH 7. It was cultured for 8 h at 37 °C (final log phase) in Man–Rogosa–Sharpe broth (MRS, Oxoid). The bacteria were harvested through centrifugation at 3000*g* for 10 min and washed three times with sterile 0.01 mol/l phosphate buffer saline (PBS), pH 7.2. *L. rhamnosus* CRL1505 is able resist bile salts and survives in simulated *in vitro* digestion assays that reproduce stomach and intestinal digestion, indicating that the strain is able to tolerate gastric enzymes and low pH conditions (Salva et al., 2011).

2.2. *Animals and feeding procedures*

Male 3-week-old Swiss albino mice were obtained from the closed colony kept at CERELA. They were housed in plastic cages at 25 °C. The assays were performed in six mice per group for each time point (in three independent experiments). Weaned mice were fed with a protein-free diet (PFD) for 21 days, and the animals that weighed 45–50% less than well-nourished mice were selected for the experiments (Villena et al., 2005).

Malnourished mice were divided into two groups for the repletion treatment: (i) balanced diet (BD) for 7 consecutive days (BD group); (ii) BD for 7 days with *L. rhamnosus* CRL1505 supplementation (10^8 cfu/mouse/day) during the last 5 days of the treatment (BD + Lr group). The administration of 10^8 - cells of *L. rhamnosus* CRL1505 for 5 consecutive days was previously selected as the optimal dose able to improve protection against *Streptococcus pneumoniae* and *Salmonella typhimurium* in immunocompetent mice (Salva et al., 2010). The malnourished control (MNC) group received only PFD while the well-nourished control (WNC) mice consumed BD *ad libitum*. The experimental protocols were approved by the Animal Care and Ethics Committee at CERELA.

2.3. Experimental infection

The experimental animal model of respiratory infection was used as previously described (Villena, Racedo, Agüero, & Alvarez, 2006; Villena et al., 2005). *S. pneumoniae* serotype 14 (ANLIS, Argentina) was obtained from the respiratory tract of a patient from the Children's Hospital, Tucuman, Argentina (Villena et al., 2005, 2006). Briefly, the different experimental groups of mice were nasally challenged with *S. pneumoniae* (10^5 cfu/ml in PBS) at the end of each treatment (day 8). WNC and MNC (without repletion treatment) groups were infected equally. Animals were euthanised on day 0 (before challenge) and at different days after infection.

2.4. Bacterial cell counts in lung homogenates and blood

Mice were euthanised on days 1, 2, 5, and 10 after challenge and their lungs were excised, weighed and homogenised in 5 ml of sterile 0.1% peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar and incubated for 18 h at 37 °C. Results were expressed as log of cfu/g lung. Progression of bacterial growth to the bloodstream was monitored by sampling blood obtained through cardiac puncture and plating on blood agar. Results were reported as cfu/ml.

2.5. Lung histopathology

On day 10 after infection, lung samples from different groups were excised and washed out with PBS 0.01 mol/l pH 7.2. Then, tissues were immersed in 4% (v/v) formalin saline solution. Once fixed, samples were dehydrated and embedded in Histowax (Leica Microsystems Nussloch GmbH, Nussloch, Germany) at 56 °C. Finally, lungs were cut into 4 μm serial sections and stained with hematoxilin–eosin for light microscopy examination. The pathologist doing the lung histology was blinded to the group of animals and the assessment of tissue sections was also blinded. The severity of lung lesions was scored from 0 to 4 (0 = normal, 1 = moderate inflammatory response with focal interstitial infiltrates, 2 = intense inflammatory response with diffuse interstitial infiltrates and moderate haemorrhage, 3 = intense inflammatory response and haemorrhage with moderate reduction of alveolar spaces, and 4 = intense inflammatory response and haemorrhage with intense reduction of alveolar spaces and fibrosis).

2.6. Cytokine concentrations in bronchoalveolar lavage (BAL) and serum

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

2.7. Total and differential number of leukocytes in blood and BAL

Blood and BAL samples were obtained as described above on days 0 and 2 after infection. Total number of leukocytes and differential cell counts were performed as described previously (Racedo et al., 2006; Villena et al., 2005).

2.8. Phagocytic cell activation

Nitroblue tetrazolium (NBT) test: The phagocytic bactericidal activity (oxidative burst) of macrophages and neutrophils was measured using the NBT reduction test (catalogue No. 840-W, Sigma–Aldrich Co.) in the BAL pellet. NBT was added to each sample with (positive control) or without addition of the bacterial extract; then samples were incubated at 37 °C for 20 min. In the presence of oxidative metabolites, NBT (yellow) is reduced to formazan, which forms a blue precipitate. Smears were prepared; after staining, samples were examined under a light microscope for blue precipitates. A hundred cells were counted and NBT positive (+) cells were determined.

Washburn test: Measurement of the myeloperoxidase activity of blood neutrophils was performed as described previously (Villena et al., 2005). Blood cells were graded as negative or weakly, moderately, or strongly positive and used to calculate the score.

2.9. Cells in the bone marrow

Studies in bone marrow were performed at the end of repletion period (day 0) and day 2 after challenges. Total and differential cell counts of bone marrow samples were obtained as described previously (Salva et al., 2008). Results were expressed as 10^6 cells/femur. Measurement of myeloperoxidase activity of bone marrow myeloid cells was performed as described above and reported as the number of peroxidase positive cells (10^6 cells/femur). Expression of Gr-1 and CD34 on blood and bone marrow cells was examined by flow cytometry. Fluorescein isothiocyanate (FITC) rat anti-mouse CD34 antibody (RAM34 monoclonal antibody; BD Pharmingen™) and phycoerythrin (PE) rat anti-mouse Gr-1 antibody (RB6-8C5 monoclonal antibody; BD Pharmingen™) were used. Data were acquired on a BD FACScalibur cytometer and analysed using Flow Jo (Tree Star) software.

2.10. Statistical analysis

Experiments were performed in triplicate and results were expressed as mean values and standard deviations. A two-way analysis of variance (ANOVA) test was used to evaluate the main effects and the interactions between treatments (InfoStat, 2006). 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 $p < 0.05$.

3. Results

3.1. Resistance against respiratory infection

S. pneumoniae was detected in lung and blood samples from the WNC and MNC groups throughout the 10 days post-infection, although the latter showed significantly higher counts

Table 1 – Resistance to pneumococcal infection. Malnourished mice were replete for 7 days with a balanced conventional diet (BD) or BD supplemented with Lactobacillus rhamnosus CRL1505 (BD + Lr) and then challenged with 10^5 cells of Streptococcus pneumoniae. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Bacterial cell counts in lung (log CFU/g of lung) and blood (CFU/mL) after challenge. Values are means for six mice per group. Different letters indicate significant differences at the same time point ($p < 0.05$).

	Day 1		Day 2		Day 5		Day 10	
	Lung	Blood	Lung	Blood	Lung	Blood	Lung	Blood
WNC	4.25 ± 0.14^a	4.01 ± 0.21^b	4.05 ± 0.08^a	4.23 ± 0.09^b	3.46 ± 0.09^a	3.91 ± 0.07^b	$3.2o \pm 0.12^a$	3.75 ± 0.09^b
MNC	5.33 ± 0.14^b	5.11 ± 0.12^c	5.92 ± 0.06^c	5.31 ± 0.12^c	5.94 ± 0.11^d	5.61 ± 0.10^c	5.91 ± 0.11^c	5.73 ± 0.23^c
BD	5.21 ± 0.01^b	3.22 ± 0.05^a	4.82 ± 0.07^b	3.51 ± 0.07^a	4.51 ± 0.08^c	3.25 ± 0.04^a	4.4 ± 0.09^b	3.45 ± 0.05^a
BD + Lr	4.45 ± 0.16^a	<1.5	4.53 ± 0.14^b	<1.5	4.32 ± 0.03^b	<1.5	3.80 ± 0.20^a	<1.5

Different letters indicate significant differences at the same time point ($p < 0.05$) a<b<c<d.

(Table 1). Bacterial cell counts in the BD group showed values between those in the WNC and MNC groups. In contrast, the BD + Lr group showed significantly lower lung bacterial counts than the WNC mice. Moreover, treatment with *L. rhamnosus* CRL1505 limited the dissemination of the pathogen, since haemocultures were negative in the BD + Lr group during the whole studied period (Table 1).

3.2. *Histopathological examination*

Morphological examination of lungs of the WNC mice on day 10 post-infection showed progressive oedema, inflammatory response and alveolar congestion (Fig. 1A). These histopathological findings were more pronounced in the lungs of the MNC mice, with progressive parenchymal involvement, increasing fibrosis in bronchial walls and vessels, passage of blood elements from capillaries to tissues, haemorrhage, and widespread cellular infiltration. Moreover, the lung parenchyma had a distorted appearance with loss of alveolar architecture (Fig. 1B). However, mice treated with BD plus supplemental *L. rhamnosus* CRL1505 showed in general histopathological characteristics similar to the WNC group, with focal cellular infiltration and reduction of the alveolar airspaces but with preserved alveolar architecture (Fig. 1D). Mice fed the BD showed histological signs intermediate to those of the WNC and MNC groups (Fig. 1C). Analyses of lung histological scores confirmed that lung tissue damage was significantly higher in the WNC mice than in the MNC group, and that the BD group showed histological signs intermediate to those of the WNC and MNC mice (Fig. 1E). In addition, scores of the BD + Lr group were significantly lower than in the WNC mice (Fig. 1E).

3.3. *Cytokine concentrations in BAL and serum*

In order to increase our understanding of the immune mechanism involved in the protective effect of *L. rhamnosus*

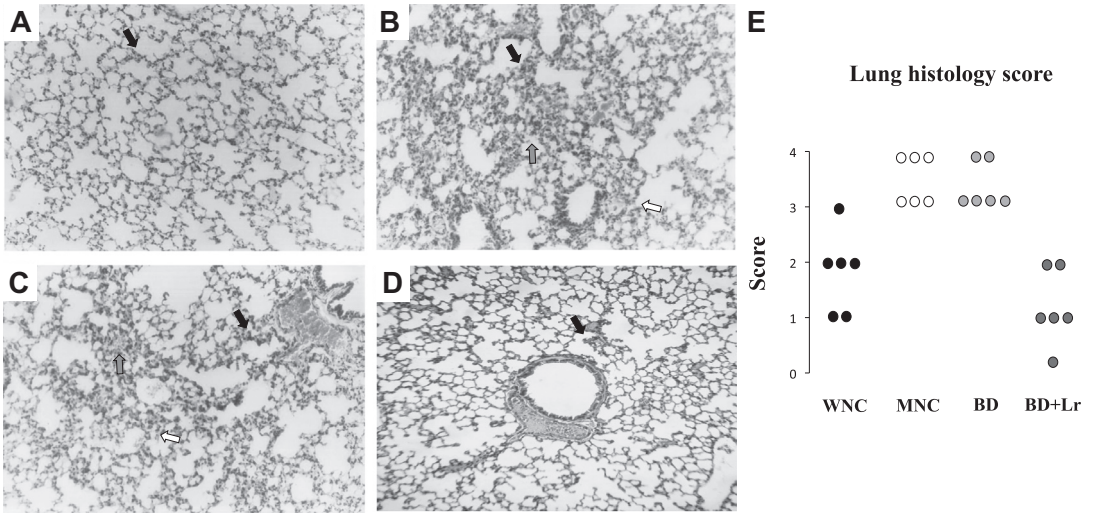

Fig. 1 – Lung histological examination. Lungs were examined on day 10 after challenge with *Streptococcus pneumoniae*. (A) Well-nourished control mice, (B) malnourished control mice, (C) malnourished mice replete with a balanced conventional diet (BD), (D) malnourished mice replete with BD supplemented with *Lactobacillus rhamnosus* CRL1505 for 5 days. Light micrographs, original magnification ×200. Inflammatory infiltrates (black arrow), reduction of alveolar airspaces (white arrow) and haemorrhage (grey arrow). The severity of lung lesions was scored from 0 to 4 (0 = normal, 1 = moderate inflammatory response with focal interstitial infiltrates, 2 = intense inflammatory response with diffuse interstitial infiltrates and moderate haemorrhage, 3 = intense inflammatory response and haemorrhage with moderate reduction of alveolar spaces, and 4 = intense inflammatory response and haemorrhage with intense reduction of alveolar spaces and fibrosis (E).

CRL1505, pro-inflammatory cytokines and immune cells were studied in the systemic and respiratory compartments. We first evaluated TNF-α and IL-6 levels in serum and BAL samples before challenge with *S. pneumonia*, no significant differences between the groups being detected (Fig. 2). Challenge with the respiratory pathogen increased TNF-α and IL-6 concentrations in BAL and serum in all experimental groups. MNC mice showed significantly lower levels of serum and BAL cytokines compared with WNC mice (Fig. 2). Mice treated with BD showed higher BAL IL-6 and serum TNF-α and IL-6 levels than those in MNC mice, although these cytokines did not reach the values in the WNC group (Fig. 2). Moreover, BD mice showed BAL TNF-α levels similar to the ones in the MNC group while mice treated with*L. rhamnosus* CRL1505 showed BAL and serum TNF-α and IL-6 values similar to those in the WNC group (Fig. 2).

3.4. *Number and activity of BAL leukocytes*

We analysed the number and activity of BAL phagocytes before the challenge with the respiratory pathogen. No differences were observed in the number of BAL leukocytes, neutrophils or macrophages when all the groups were compared (Fig. 3). However, as previously reported (Villena et al., 2005), MNC mice showed significantly lower levels of BAL NBT+ cells compared with WNC mice (Fig. 3). Treatment with BD + Lr was able to improve BAL NBT+ cells. Challenge with *S. pneumoniae* induced recruitment of neutrophils and macrophage migration into the alveoli, resulting in an increase in leukocyte counts in BAL on day 2 post-infection (Fig. 3) as previously described (Villena et al., 2005). MNC mice showed an impaired recruitment of neutrophils and macrophages and significantly lower leukocyte, neutrophil and macrophage counts in BAL compared with WNC mice (Fig. 3). In addition to the quantitative alterations in BAL phagocytes, we also observed that malnutrition significantly impaired the phagocytic activity of BAL cells (Fig. 3). Treatment of malnourished mice with BD increased the number of BAL leukocytes, although the treatment was not able to normalise it. Moreover, BD mice showed percentages of BAL NBT+ cells similar to those in the MNC group (Fig. 3). Treatment with *L. rhamnosus* CRL1505 significantly increased the similar number of BAL neutrophils and macrophages in response to the infection as well as the percentages of BAL NBT+ cells. In fact, BD + Lr mice showed values similar to the ones in the WNC group (Fig. 3).

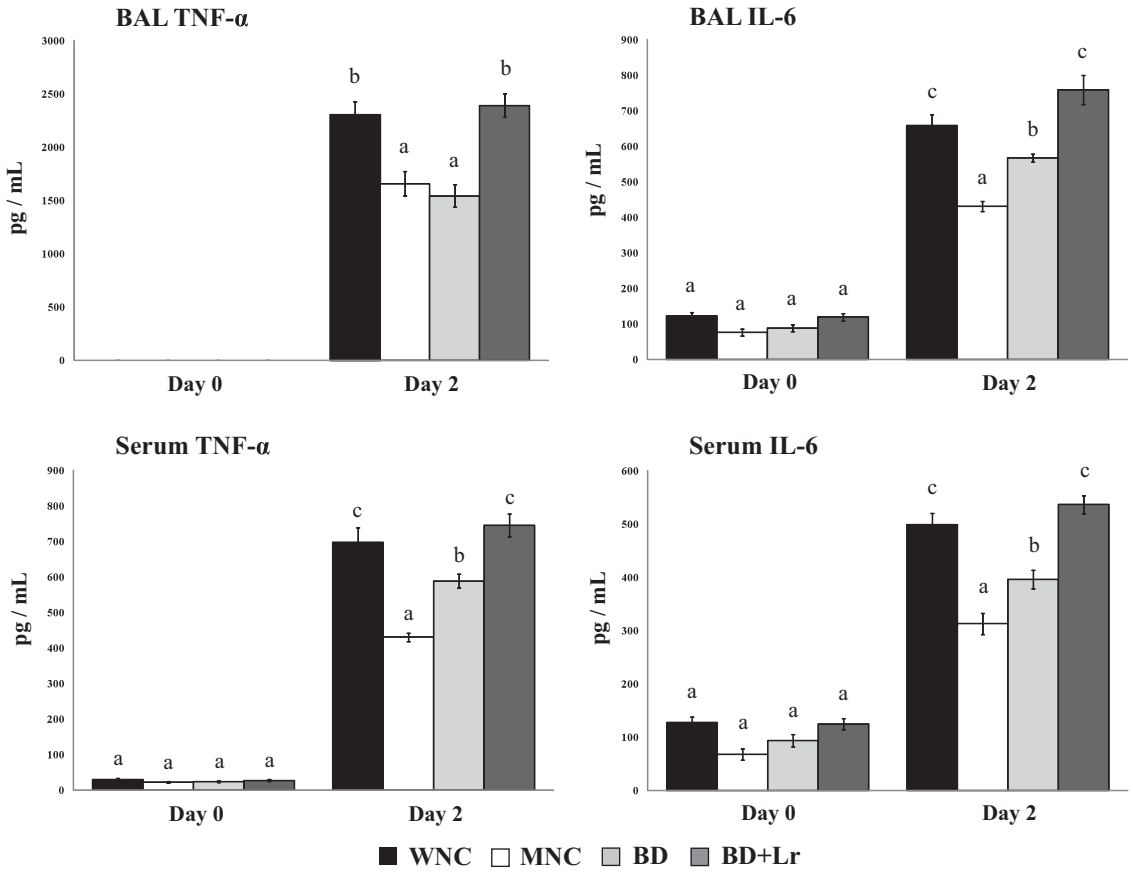

Fig. 2 – Cytokines concentration in BAL and serum. Malnourished mice were replete for 7 days with a balanced conventional diet (BD) or BD supplemented with *Lactobacillus rhamnosus* CRL1505 (BD + Lr) and then challenged with 10^5 cells of *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. TNF-α and IL-6 concentrations in BAL and serum were determined before (day 0) and after pneumococcal infection (day 2). Values are means for six mice per group with standard deviations represented by verticals bars. Different letters indicate significant differences at the same time point ($p < 0.05$).

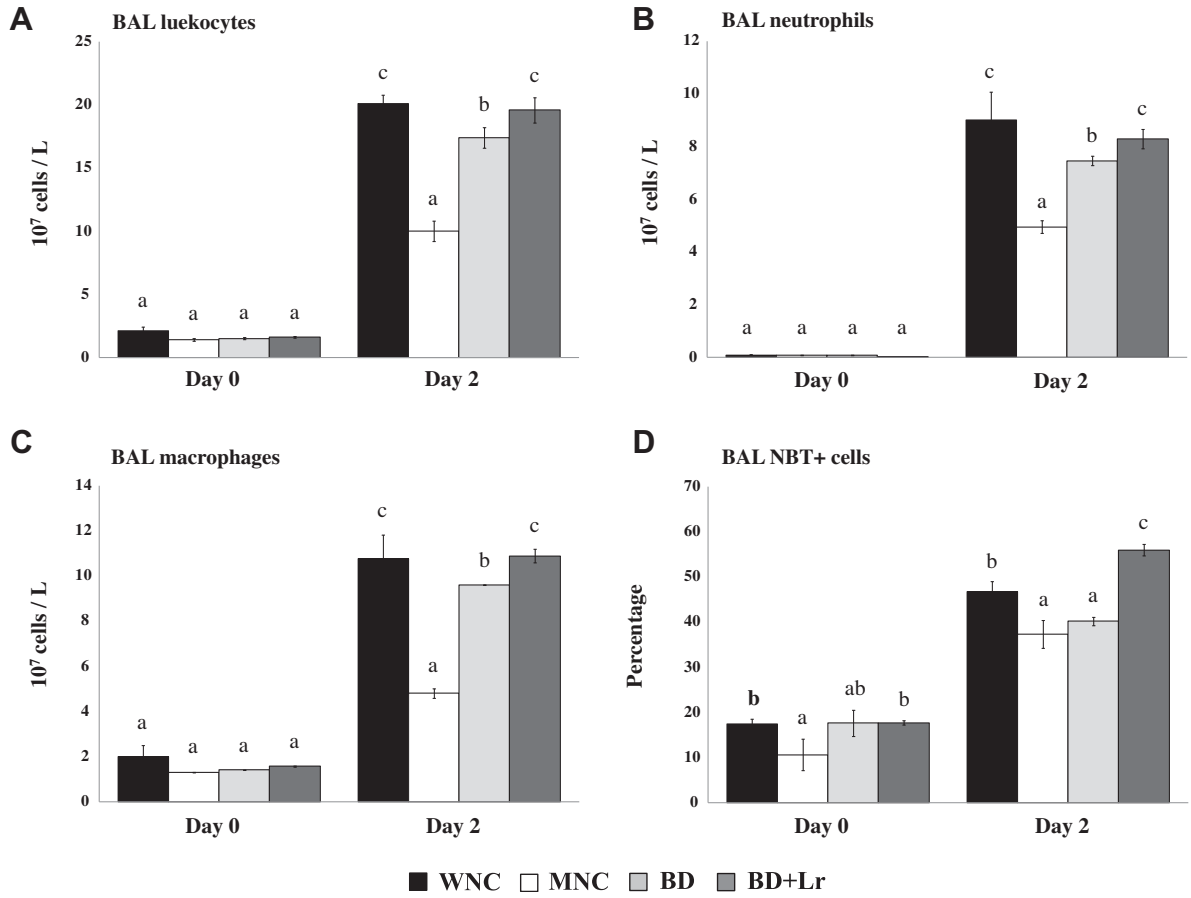

Fig. 3 – Number and activity of bronchoalveolar lavage (BAL) leukocytes. Malnourished mice were replete for 7 days with a balanced conventional diet (BD) or BD supplemented with *Lactobacillus rhamnosus* CRL1505 (BD + Lr) and then challenged with 10^5 cells of *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Total number of BAL leukocytes (A), neutrophils (B), macrophages (C) and microbicidal activity of phagocytes (D) were determined before (day 0) and after challenge (day 2) with *S. pneumoniae*. Values are means for six mice per group with standard deviations represented by verticals bars. Different letters indicate significant differences at the same time point ($p < 0.05$).

3.5. *Number and activity of blood leukocytes*

Malnutrition decreased the number of leukocytes and neutrophils as well as peroxidase scores in blood, since significantly lower cell counts and scores were detected in the MNC mice than in the WNC group (Fig. 4). Treatment with BD increased blood neutrophils numbers and peroxidase scores, although values did not reach the levels of the WNC mice (Fig. 4). In contrast, repletion with BD + Lr significantly increased blood leukocytes, which reached values similar to those in the WNC mice (Fig. 4). Moreover, blood peroxidase activity in the BD + Lr mice was significantly higher than in the WNC mice (Fig. 4).

Challenge with *S. pneumoniae* increased blood leukocyte counts and peroxidase scores in all experimental groups although they were significantly lower in the MNC than in the WNC mice (Fig. 4). Treatment with both BD and BD + Lr increased blood leukocytes and neutrophils, although only the BD + Lr group reached numbers similar to those in the WNC mice (Fig. 4). Blood peroxidase activity in the BD + Lr mice was significantly higher than in the WNC mice as observed before the challenge with the respiratory pathogen (Fig. 4).

In addition, we studied blood granulocyte population using flow cytometry. Before the challenge, malnutrition decreased the number of blood Gr-1+ and CD34+ cells (Fig. 4). Treatment with BD was unable to induce modifications in either blood cell populations since values in this group were similar to those in the MNC mice (Fig. 4). In contrast, malnourished mice that received *L. rhamnosus* CRL1505 showed levels of blood Gr-1+ and CD34+ cells that were similar to the WNC mice (Fig. 4).

After the pneumococcal infection, blood Gr-1+ and CD34+ cells significantly increased in all the experimental groups in relation to basal levels (Fig. 4). The MNC mice showed significantly lower levels of blood Gr-1+ cells and higher numbers of CD34+ cells compared with the WMC mice (Fig. 4). No differences were observed between MNC and BD mice when analysing numbers of Gr-1+ cells in blood. Treatment with BD + Lr normalised both cell populations in blood (Fig. 4).

3.6. *Bone marrow myeloid cells*

The oral administration of *L. rhamnosus* CRL1505 had a stimulatory effect on the function and recruitment of phagocyte

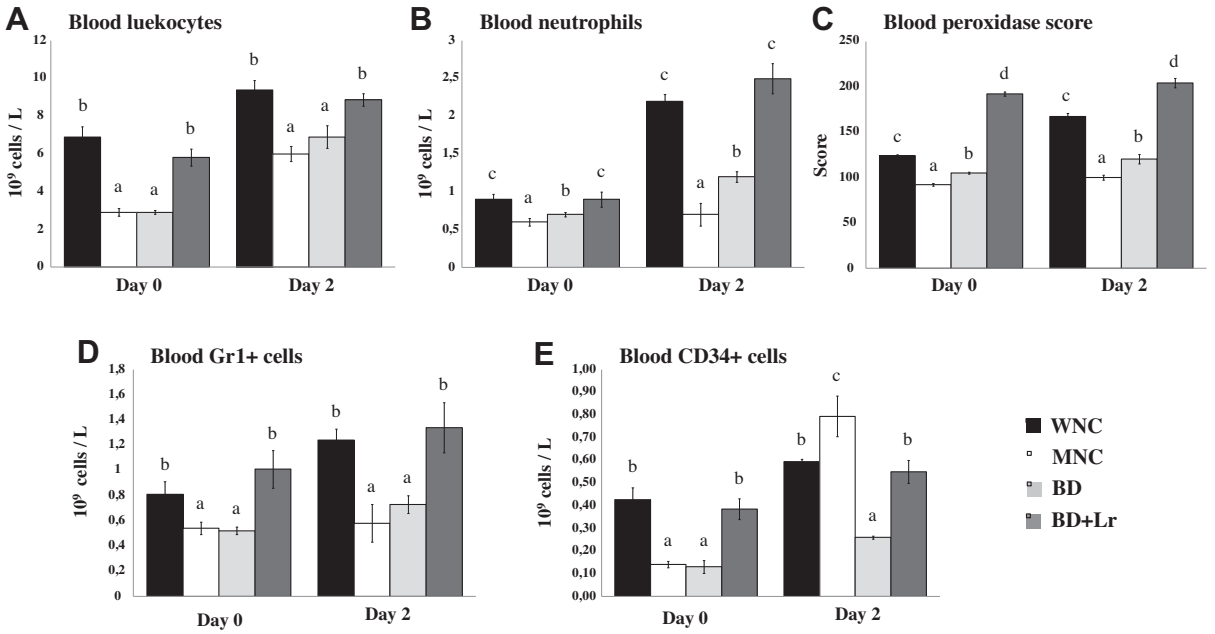

Fig. 4 – Number and activity of blood leukocytes. Malnourished mice were replete for 7 days with a balanced conventional diet (BD) or BD supplemented with *Lactobacillus rhamnosus* CRL1505 (BD + Lr) and then challenged with 10^5 cells of *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. The number of leukocytes (A), neutrophils (B), peroxidase activity (C) Gr-1+ cells (D) and CD34+ cells (E) were studied in peripheral blood before (day 0) and after challenge with the respiratory pathogen (day 2). Values are means for six mice per group with standard deviations represented by verticals bars. Different letters indicate significant differences at the same time point ($p < 0.05$).

cells, causing a clear increase in the respiratory and systemic innate immune responses. In this sense, the bone marrow, which is the main hematopoietic organ of an adult organism, maintains a close relationship with the immune system. The bone marrow is able to respond to acute infections by using an emergency hematopoiesis in order to release immune cells (Monteiro & Bonomo, 2005). Therefore, we next studied the cellular changes in bone marrow during the pneumococcal infection and the influence of the CRL1505 strain, with special attention to myelopoiesis.

Malnutrition caused a decrease in total cell counts in bone marrow together with a reduction in myeloid cells as previously described (Salva et al., 2011). Moreover, we detected a significant reduction in all myeloid cell populations: mitotic pool cells (myeloblasts, promyielocytes, myelocytes) and post-mitotic pool cells (metamyelocytes, band cells, neutrophils) in the MNC mice (Fig. 5). Both repletion treatments improved myeloid cell counts in the bone marrow (Fig. 5). The increase in myeloid cells in the BD group was caused by increases in post-mitotic pool cells while in the BD + Lr group mitotic pool cells were significantly increased. In fact, the BD + Lr group showed values of myeloblast, promyielocytes and myelocytes that were higher than those observed in the WNC group (Fig. 5).

When mice were infected with *S. pneumoniae*, the normal response to the pneumococcal challenge was an increase in myeloid cells with increases in mitotic pool cells and with no changes in the number of post-mitotic cells (Fig. 5). In the MNC mice, however, this response was significantly altered since mitotic pool cells increased after infection but post-mitotic pool counts were lower compared to basal levels (Fig. 5). In both the BD and the BD + Lr groups a reduction in myeloid cells and mitotic pool counts was observed after the pneumococcal challenge. In the BD group, post-mitotic pool cells significantly decreased while in the BD + Lr group these cells were significantly increased (Fig. 5).

These results correlated with the peroxidase activity in bone marrow, which is a cytochemical marker of myeloid cells. Before the respiratory infection, the number of peroxidase positive cells in the bone marrow was also reduced in the MNC mice compared to the WNC group (Fig. 5). Only the BD + Lr group reached normal values after the repletion treatment (Fig. 5). After infection, the number of peroxidase positive cells increased in all experimental groups, although values in MNC mice were lower than in WNC animals. Both BD and BD + Lr increased bone marrow peroxidase activity but only mice receiving *L. rhamnosus* CRL1505 showed normal values (Fig. 5).

In addition, we examined bone marrow Gr-1 and CD34 expression in order to determine changes in mature granulocytes and myeloid progenitors/precursors. Protein malnutrition significantly reduced Gr-1+ cells in bone marrow and only the repletion with supplemental*L. rhamnosus* was able to normalise this population (Fig. 6). No significant modifications were observed in the numbers of Gr-1+ cells in bone marrow after the challenge with S. *pneumoniae*; however, in the MNC and BD groups bone marrow $Gr1^{High}$ cells (mature neutrophils) were significantly reduced compared with WNC and BD + Lr mice, while there was an increase in bone

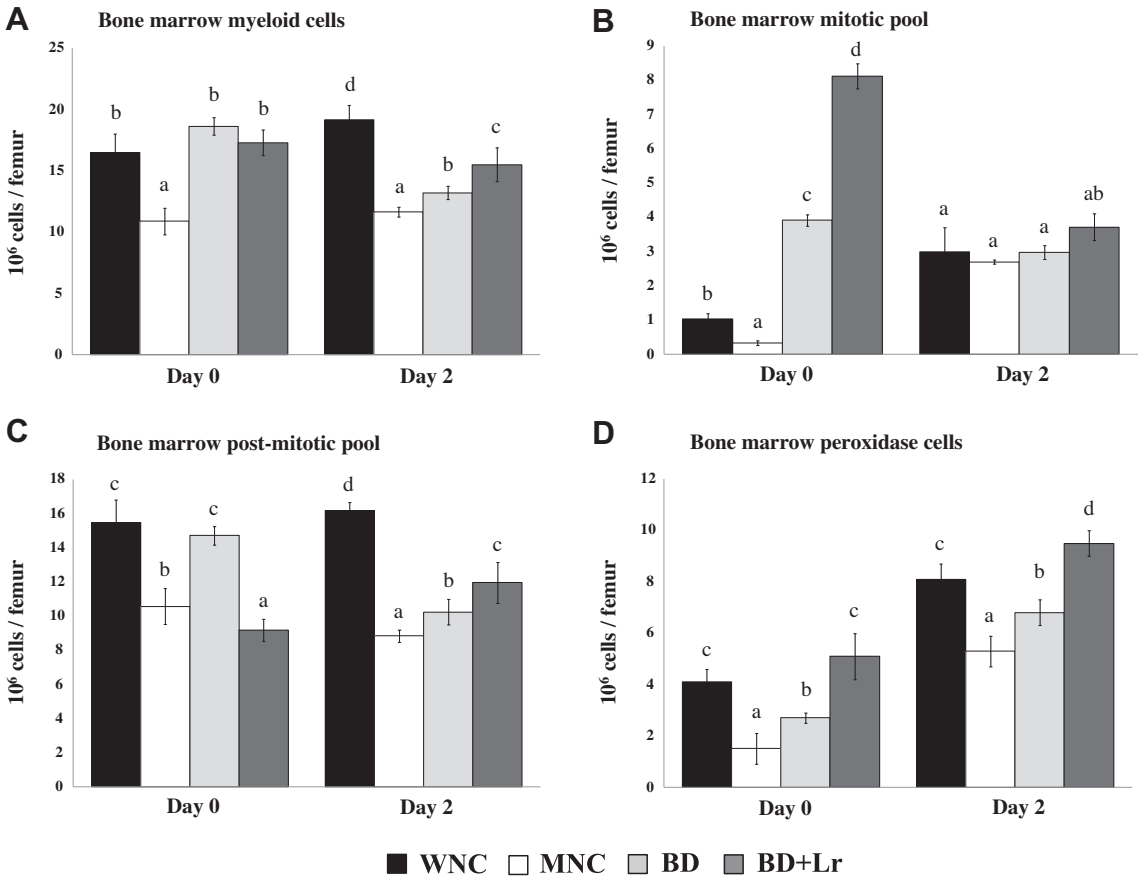

Fig. 5 – Bone marrow myeloid cells. Malnourished mice were replete for 7 days with a balanced conventional diet (BD) or BD supplemented with *Lactobacillus rhamnosus* CRL1505 (BD + Lr) and then challenged with 10^5 cells of *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Myeloid cells (A), mitotic pool cells (myeloblasts, promyelocytes and myelocytes) (B), post mitotic pool cells (metamyelocytes, band cells and neutrophils) (C) and peroxidase activity (D) were determined in bone marrow before (day 0) and after (day 2) the challenge with *S. pneumoniae*. Values are means for six mice per group with standard deviations represented by verticals bars. Different letters indicate significant differences at the same time point ($p < 0.05$).

marrow $Gr1^{Low}$ cells (myeloid precursors) in the BD + Lr group (Fig. 6).

Finally, we studied the expression of CD34 as a progenitor cell marker (Krause et al., 1994; Okuno et al., 2002). The MNC group showed the lowest values of CD34+ cells. Both BD and BD + Lr treatments increased the number of CD34+ cells in bone marrow, but they were unable to induce the normalisation of this population (Fig. 6). After the challenge with *S. pneumoniae* the MNC mice showed significantly lower numbers of CD34+ cells in bone marrow compared with the WNC mice. BD + Lr treatment increased CD34+ cells in bone marrow, which reached the values of the WNC while the BD mice did not differ from the MNC mice (Fig. 6).

4. Discussion

Innate immunity is essential to prevent colonisation of the respiratory tract by *S. pneumoniae* (Muñoz et al., 2010; Villena et al., 2005). The immune response to pneumococcal airway infection is characterised by a pronounced recruitment of neutrophils into the lungs (Kadioglu et al., 2000). This phagocytic killing of pneumococci by polymorphonuclear neutrophils is considered a major defence mechanism (Muñoz et al., 2010). Therefore, suppression of neutrophil recruitment or alteration of their functional activity would predictably result in increased susceptibility to pulmonary infections. In previous works we demonstrated that malnutrition induced a significant impairment in the number of leukocytes and neutrophils and in peroxidase activity in the respiratory tract and blood (Salva et al., 2008; Villena et al., 2006). Thus, malnutrition is able to impair the innate immune response at the systemic and mucosal levels.

Significant attention has been centred on the role of probiotics in the gastrointestinal tract for the attenuation of intestinal inflammatory diseases (Chiu et al., 2013). However, there is steadily increasing evidence that the oral administration of probiotics is able to influence immune responses outside the gastrointestinal tract, including the respiratory mucosa (Alvarez, Villena, Tohno, Salva, & Kitazawa, 2009b; Villena et al., 2011). The present work is focused on the role of the bone marrow in the recovery of the respiratory innate immunity induced by *L. rhamnosus* CRL1505 when used as supplement in a malnourished mice repletion treatment. We analysed

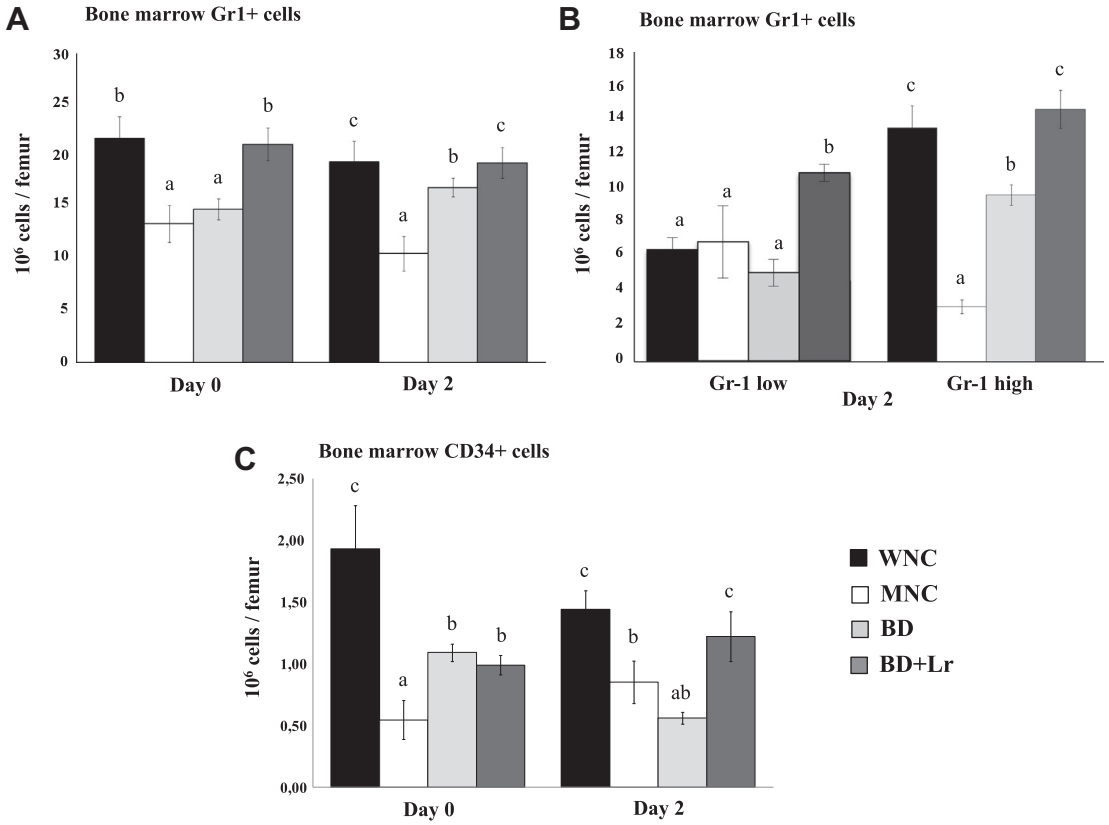

Fig. 6 – Bone marrow myeloid cells. Malnourished mice were replete for 7 days with a balanced conventional diet (BD) or BD supplemented with *Lactobacillus rhamnosus* CRL1505 (BD + Lr) and then challenged with 10^5 cells of *Streptococcus pneumoniae*. Malnourished (MNC) and well-nourished (WNC) mice were used as controls. Gr-1 (A) and CD34 (C) positive cells were determined in bone marrow before (day 0) and after (day 2) challenge with the respiratory pathogen. On day 2 post-infection, Gr-1 population was divided into two groups according to high or low expression of markers for the study (B). Values are means for six mice per group with standard deviations represented by verticals bars. Different letters indicate significant differences at the same time point ($p < 0.05$).

myeloid cells in BM, blood and BAL samples during the generation of the innate immune response against a bacterial challenge. We demonstrated that the oral administration of *L. rhamnosus* CRL1505 was able to normalise the systemic and respiratory innate immune responses in replete malnourished mice, which was evidenced by the increase in the number of blood and BAL neutrophils and by the improvement in their microbicidal function. The protective effect of *L. rhamnosus* CRL1505 was related to its capacity to impact on innate immune cells as we previously described for other probiotic strains (Alvarez et al., 2009b; Villena et al., 2011).

Neutrophils are a key component of the innate immune system and are frequently the initial defence against extracellular pathogens (Clarke et al., 2010). We found that malnutrition significantly affects innate defences against *S. pneumoniae* and that repletion of malnourished mice with BD with supplemental *L. rhamnosus* CRL1505 increases resistance against pneumococcal infection by enhancing mucosal and systemic bacterial clearance. Moreover, treatment with *L. rhamnosus* CRL1505 was effective in preventing lung histopathological injuries observed in the MNC group. These results are similar to the ones induced by the repletion treatment in which *Lactobacillus casei* CRL431 was used as a supplement for the repletion diet (Alvarez et al., 2007; Salva et al., 2008; Villena et al., 2005).

Neutrophil homeostasis in the blood is highly regulated. It is known that blood neutrophil number is determined by the balance between neutrophil production in the bone marrow, their release into the blood and their clearance from circulation (Day & Link, 2012). In mice, only 1–2% of the total number of mature neutrophils is found in the blood, most of them remaining in the bone marrow (Day & Link, 2012). Mature neutrophils are short-lived and rapidly migrate to inflammation sites where they exert their microbicidal activity (Burg & Pillinger, 2001; Quie, 1980). This localisation produces a brief neutropenia that is corrected by an accelerated emigration of bone marrow neutrophils. Subsequently a reactive neutrophilia characterised by the presence of abundant and less mature cells in blood can be observed after infections (Glasser & Fiederlein, 1987). This reactive neutrophilia is a crucial component of innate immunity because persistent neutropenia leads to death by infection (Engle & Rosenfeld, 1984; Glasser, Fiederlein, Wood, Dalton, & List, 1994). The bone marrow regulates the homeostatic release of mature neutrophils and the accelerated production of granulocytes in response to inflammatory signals (Terashima, English, Hogg, & van Eeden, 1998;

Terashima, Wiggs, English, Hogg, & van Eeden, 1996). However, the mechanisms of this regulation are not fully known yet.

In this work we analysed the changes induced by a repletion treatment that included the immunomodulatory strain *L. rhamnosus* CRL1505 in bone marrow myeloid cells. We examined bone marrow Gr-1 expression to determine changes in mature granulocytes and myeloid progenitors/precursors considering that the Gr-1 antigen is selectively expressed on myeloid lineage cells in mouse bone marrow (Fleming, Fleming, & Malek, 1993). For analysis these cells were sorted into $Gr1^{negative}$, $Gr1^{low}$, and $Gr1^{high}$ populations. Mature neutrophils are $Gr1^{high}$, whereas myeloid precursors including immature blasts and myelocytes have low to intermediate levels of Gr-1 expression (Hestdal et al., 1991). We showed that *L. rhamnosus* CRL1505 was able to normalise Gr1+ cells in malnourished mice, this being the only group with a normal balance between $Gr1^{low}$ and $Gr1^{high}$ cells in bone marrow. On the other hand, Krause et al. (1994) demonstrated that CD34+ murine bone marrow cells comprise small lymphocyte-like cells, large blasts and myelomonocytic precursors. Evidence now suggests that most murine CD34+ cells represent committed early and intermediate progenitors (Krause et al., 1994; Okuno et al., 2002). Therefore, we used CD34 as a progenitor cell marker. Repletion with *L. rhamnosus* CRL1505 significantly increased CD34+ bone marrow cells after infection when compared with the BD group. However, *L. rhamnosus*-treated mice did not achieve normal values. On the other hand, when we analysed blood CD34+ cells, we found that *L. rhamnosus* CRL1505 was able to normalise this cell population. Then, the changes induced in bone marrow myeloid cells were enough to normalise the response to the infection.

The granulocyte colony-stimulating factor (G-CSF) is the principal cytokine controlling neutrophil development and function. G-CSF is induced by several inflammatory stimuli that become rapidly elevated during infection such as IL-1β, TNF-α and lipopolysaccaride (LPS). Therefore, the host cytokine response to infection serves to induce circulating levels of G-CSF, which stimulates neutrophil production in the bone marrow and neutrophil mobilisation to the peripheral circulation (Panopoulos & Watowich, 2008). We showed that repletion with *L. rhamnosus* CRL1505 increased BAL and serum TNF-α and IL-6 levels after the challenge with *S. pneumoniae* and that these levels were similar to the ones found in the WNC group. In this sense, we found clear differences between these mice and the ones replete with BD only, which showed cytokine levels similar to those in the MNC group. It is probable that the improved levels of BAL TNF-α in mice treated with the CRL1505 strain allowed an efficient recruitment of neutrophils from the pulmonary vasculature into the alveolar spaces and a higher activation of phagocytes, as shown by the number of BAL neutrophils and the percentage of BAL NBT+ cells. Then, the normalisation of IL-6 and TNF-α levels induced by *L. rhamnosus* CRL1505 treatment was related to the improved innate immune response.

This work provides strong evidence of the importance of dietary supplementation with a probiotic LAB to reverse alterations in the homeostasis of the myeloid progenitors in malnourished mice. Moreover, we demonstrated for the first time that the immunobiotic strain *L. rhamnosus* CRL1505 was crucial in the recovery of bone marrow responsiveness against the infectious challenge through the increase in myeloid progenitors and the mobilisation of granulocytes, which improved the local immune response. The mechanism by which *L. rhamnosus* CRL1505 is able to achieve such effect is not clear yet. Recent evidence shows that pattern recognition receptors-mediated sensing of resident commensal microbiota in the steady state regulates the development and function of the innate and adaptive immune systems in extra-intestinal sites and prepares the host for defence against the intrusion of pathogenic microorganisms (Clarke et al., 2010). Recognition of peptidoglycans from the microbiota by Nod-1 primes systemic innate immunity by enhancing the cytotoxicity of bone marrow-derived neutrophils in response to systemic infection with the bacterial pathogens *S. pneumoniae* and *Staphylococcus aureus* (Clarke et al., 2010). This study indicates that the gut microbiota support systemic immunity by releasing low levels of pattern recognition receptor ligands into the circulation. On the other hand, the emergency granulopoiesis response to infection requires the coordinated expression of many different cytokines and chemokines in the site of infection as well as local hematopoietic growth factors in the bone marrow. Therefore, it could be speculated that the immunoregulatory effect of the CRL1505 strain is related to its capacity to change cytokine profiles in the gut, blood and respiratory tract. The determination of whether *L. rhamnosus* CRL1505 is able to modulate myeloid cell production by these or different mechanisms is an interest topic for future investigations.

In a previous work we found that *L. rhamnosus* CRL1505 has optimal technological properties and can be used to produce high quality functional fermented milks that can be included into Official Nutritional Programmes (Salva et al., 2011; Villena et al., 2012). These facts and the present results strongly suggest that this strain could be used in the development of probiotic foods, which would be especially useful for the recovery of immunocompromised hosts.

Acknowledgements

This work was supported by grants from PIP 632-2009, CIUNT 26D/403 and PICT 2010 No. 1381.

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