

# Modulation of the immuno-coagulative response in a pneumococcal infection in malnourished mice nasally treated with *Lactobacillus casei*

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We studied the systemic effects of the intranasal administration of *Lactobacillus casei* on the immuno-coagulative response in pneumococcal infection in immunocompromised mice. Weaned mice consumed a protein-free diet (PFD) for 21 days and were therefore malnourished. Malnourished mice were fed a balanced conventional diet (BCD) for 7 days (BCD group) or a BCD for 7 days with nasal administration of viable *L. casei* on days 6 and 7 (BCD+LcN group). The malnourished control mice (MNC) received a PFD, whereas the well-nourished control mice (WNC) continually consumed a BCD. At the end of the treatment period, the mice were infected with *Streptococcus pneumoniae*. At different times after infection, we analysed the following parameters: global coagulation system, activation of coagulation, coagulation inhibitors, platelet count, leukocyte count and myeloperoxidase (MPO) activity, total proteins, albumin and acute phase proteins (APPs). The MNC group showed greater impairment in the coagulation tests and an increase in the positive APPs. These parameters were normalized by the *L. casei* treatment. However, the number of leukocytes, decreased by malnutrition, was improved only by the administration of *L. casei*. After infection, the BCD+LcN group showed similar results to those of the WNC group for most of the haemostatic parameters. The BCD+LcN group did not show significant variations in the prothrombin time or in the level of anticoagulant protein C, but showed higher levels of fibrinogen, platelets, albumin, leukocytes and MPO activity compared with the different experimental groups. The intranasal administration of *L. casei* was effective in modulating the pro-inflammatory aspects of coagulation without affecting coagulation itself.

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## INTRODUCTION

The first line of defence against infection is the innate immune response. A critical function of innate immunity is pathogen recognition. Toll-like receptors (TLRs) recognize bacterial LPSs and other pathogen-associated molecular patterns. Such interaction triggers a cellular and humoral response characterized by neutrophil, endothelial cell and monocyte/macrophage activation and pro-inflammatory cytokine production, the purpose of which is to control

infection (Janeway & Medzhitov, 1998; Beutler & Poltorak, 2001). In addition, during innate immune activation, factors of the coagulation pathway are activated, creating the cooperative immuno-coagulative response (Konecny, 2010). The blood clot, which forms at specific sites to prevent blood loss, has been suggested to have an immune-protective function during infection (Loof *et al.*, 2011). However, excessive coagulation activation can lead to the widespread deposition of fibrin deposits, resulting in multiple organ failure due to reduced blood supply (Rittirsch *et al.*, 2008).

Malnutrition is a systemic alteration, potentially reversible, caused by the imbalance between nutrient intake and energy requirements (Shils *et al.*, 1999). It is mainly associated with a decrease in growth and development, a reduced capacity for learning and depression of the immune system (Savino, 2002). It is a major cause of acquired immunodeficiency.

Abbreviations: AGP, acid glycoprotein; APP, acute phase protein; APTT, activated partial thromboplastin time; AT, anti-thrombin; BCD, balanced conventional diet; LcN, nasal administration of viable *L. casei* CRL 431; C3, component complement 3; C4, component complement 4; IL, interleukin; MNC, malnourished control mice; MPO, myeloperoxidase; PC, protein C; PFD, protein-free diet; PT, prothrombin time; TATc, thrombin-anti-thrombin complex; TLR, Toll-like receptor; TNF- $\alpha$ , tumour necrosis factor-alpha; WNC, well-nourished control mice.

Several studies have clearly shown that populations with inadequate nutrition, in either quantitative or qualitative terms, have increased susceptibility to infections. The adequate and prompt correction of the nutritional status is important to reduce morbidity and mortality from infections associated with immune suppression caused by malnutrition (Scrimshaw & SanGiovanni, 1997).

Probiotics are defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit for the host (FAO/WHO, 2001). In addition, probiotic lactic acid bacteria have several immunomodulatory effects (Gill *et al.*, 2000; Cross, 2002) and anti-inflammatory properties (Perdigón *et al.*, 2002; Ménard *et al.*, 2004). We studied the use of probiotics in relation to the early recovery of immune mechanisms against respiratory pathogens in malnourished hosts (Alvarez *et al.*, 2007). It was demonstrated that the addition of a probiotic *Lactobacillus casei* strain to the repletion diet accelerated the recovery of the defence mechanisms against a pneumococcal respiratory infection (Villena *et al.*, 2005). Additionally, the oral administration of *L. casei* can induce the recovery of mucosal immunity, cytokine profiles and some global clotting tests in infected malnourished hosts (Agüero *et al.*, 2006; Salva *et al.*, 2008).

Although most research concerning the effects of probiotic lactobacilli on immune protection is focused on gastrointestinal tract pathogens, recent studies have centred on whether these probiotics might sufficiently stimulate the common mucosal immune system to provide protection to other mucosal sites as well (Alvarez *et al.*, 2009). In addition, some authors argue that nasally administered antigens can induce a respiratory and systemic immune response superior to that resulting from oral stimuli (Ogra *et al.*, 2001; Kiyono & Fukuyama, 2004). Villena *et al.* (2009) showed that the nasal administration of *L. casei* was effective in modulating the systemic and respiratory immune system in malnourished mice.

The ability of probiotics administered intranasally to modulate the immuno-coagulative response in malnourished hosts has not been studied. Considering that malnutrition and respiratory infection are very important public health problems in our region, the aim of the present work was to investigate whether the nasal administration of *L. casei* CRL 431 results in the modulation of the pro-inflammatory aspects of coagulation in an infected malnourished mouse model.

## METHODS

**Animals and micro-organisms.** Male 6-week-old Swiss albino mice were obtained from the closed colony kept at CERELA, San Miguel de Tucumán, Argentina. They were housed in plastic cages in a controlled atmosphere ( $22 \pm 2$  °C,  $55 \pm 2$  % humidity) with a 12 h light/dark cycle. *L. casei* CRL 431 was obtained from the CERELA culture collection and was cultured according to procedures described previously (Villena *et al.*, 2005). *Streptococcus pneumoniae* was purchased from the Administración Nacional de Laboratorios e Institutos de Salud-ANLIS, Buenos Aires, Argentina. The pathogenic

strain belongs to the 14 serotype, one of the ten most frequent serotypes isolated from pneumococcal infections in Argentina.

**Feeding and weighing procedures.** Weaned mice consumed a protein-free diet (PFD) (Zelaya *et al.*, 2011a) for 21 days and were therefore malnourished prior to the initiation of treatment. At the end of this period, all malnourished mice weighed 40–50 % less than the well-nourished control mice (WNC). Malnourished mice were separated into two groups for the repletion treatment. One group of malnourished mice was fed a balanced conventional diet (BCD) (Zelaya *et al.*, 2011a) for 7 consecutive days (BCD group) and the second group of mice received 7 days of a BCD supplemented with the intranasal administration of *L. casei* ( $10^9$  c.f.u. per mouse per day) on days 6 and 7 (BCD + LcN group). The administration of *L. casei* for 2 days is the optimal dose to provide protection against *S. pneumoniae* infection in malnourished mice (Villena *et al.*, 2005) and  $10^9$  (c.f.u. per mouse per day) is the optimal dose administered by the nasal route to modulate global tests of coagulation (Zelaya *et al.*, 2011b). Briefly, mice were anaesthetized intraperitoneally using a mix of ketamine hydrochloride [ $100 \mu\text{g}$  (g body weight) $^{-1}$ ] and xylazine hydrochloride [ $5 \mu\text{g}$  (g body weight) $^{-1}$ ]. Then  $25 \mu\text{L}$  *L. casei* inoculum was dropped into each nostril to aid inhalation.

Body weight was determined at the beginning and end of each feeding period and before sample collection to assess the level of malnutrition induced by the PFD, as well as the effect of *L. casei* on nutritional parameters. An electronic balance with a sensitivity of 0.01 g was used. Body weight resulted from the mean of the values obtained from three different weight measurements, taken alternately.

**Experimental infection.** *S. pneumoniae* was cultured according to procedures described in previous studies (Zelaya *et al.*, 2011a). Challenge with *S. pneumoniae* was performed 1 day after the dietary treatment had ended (day 8). Mice were infected by dropping  $25 \mu\text{L}$  *S. pneumoniae* inoculum containing  $10^7$  exponential-phase c.f.u. suspended in PBS into each nostril to permit inhalation (Zelaya *et al.*, 2011a). The infecting dose was selected on the basis that it was unable to kill all mice, but was able to cause local and systemic changes, such as alterations of global coagulation tests.

Samples were obtained at 0 (before infection), 12, 24, 120 and 240 h post-infection (p.i.). During the entire study period, the malnourished control mice (MNC) group received only the PFD, whereas the WNC, BCD and BCD + LcN groups consumed a BCD *ad libitum* (Villena *et al.*, 2005).

To analyse the infection process, mice were killed before challenge (0 h p.i.) and at 12, 24, 120 and 240 h p.i. Their lungs were excised, weighed and homogenized in 5 ml sterile peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar and incubated for 18 h at 37 °C in an atmosphere of 5 % CO<sub>2</sub>. All plates without colonies were examined up until 72 h.  $\alpha$ -Haemolytic colonies were observed. The isolate was identified as *S. pneumoniae* and confirmation of this identification was provided by the fact that this isolate was optochin sensitive. Optochin susceptibility testing was performed in an atmosphere of 5 % CO<sub>2</sub>. After the challenge, the survival of the mice was monitored until 21 days p.i. All animals survived and there was no significant difference between the two groups. All experiments were approved by the Ethical Committee for Animal Care of CERELA and of the Universidad Nacional de Tucumán, Argentina.

**Total protein, albumin and acute phase proteins (APPs) in blood serum.** Total protein concentration was determined by the Biuret method, while the serum albumin concentration was determined by the binding to bromocresol green (Wiener Lab). The levels of acid glycoprotein (AGP), component complement 3 (C3) and component complement 4 (C4) (APPs) were determined by the immunoturbidimetric method according to the manufacturer's instructions (Wiener Lab).

**Total and differential blood leukocyte counts.** Blood samples were obtained by cardiac puncture from sodium pentobarbital-anaesthetized animals and collected in tubes containing EDTA as an anticoagulant. The total number of leukocytes was determined with a haemocytometer. Differential cell counts were performed by counting 200 cells in blood smears stained with May Grünwald-Giemsa stain using a light microscope ( $\times 100$ ), absolute cell numbers were calculated (Dacie & Lewis, 1995).

**Activation of blood neutrophils.** Measurement of the myeloperoxidase (MPO) activity of blood neutrophils was carried out with the Washburn test, which is a cytochemical method that uses benzidine as a MPO chromogen (Kaplow, 1965). Cells were graded as negative, or weakly, moderately or strongly positive and this grading was used to determine the score, which was calculated by counting 200 neutrophils in blood smears. The score value was calculated by the addition of neutrophils with different positive grades.

**Coagulation studies.** Blood samples were obtained as described above and collected in a 3.2% (w/v) solution of trisodium citrate at a ratio of 9:1. Plasma was obtained according to the procedure described by Agüero *et al.* (2006).

Coagulation tests: prothrombin time (PT) was determined by a one-step method (Thromborel S, Behringwerke). Activated partial thromboplastin time (APTT) was determined by mixing plasma with calcium chloride and a partial thromboplastin reagent (Dade Actin FSL Activated PTT Reagent, Dade Behring) and then timing the initial clot formation. Fibrinogen concentration was determined by the Clauss method, using a commercial kit and following manufacturer's instructions (Fibri Prest, Diagnostica Stago). PT, APTT and fibrinogen determinations were performed manually on fresh plasma samples.

Activation of coagulation: as markers of coagulation system activation, thrombin-anti-thrombin complexes (TATcs) were measured in plasma samples using the ELISA technique, according to the manufacturer's instructions (Enzygnost TAT micro, Dade Behring).

Coagulation inhibitors: anti-thrombin (AT) and protein C (PC) activities were measured in plasma samples by using chromogenic substrate assays (Berichrom Antithrombin III, Dade Behring; COAMATE protein C, Chromogenix).

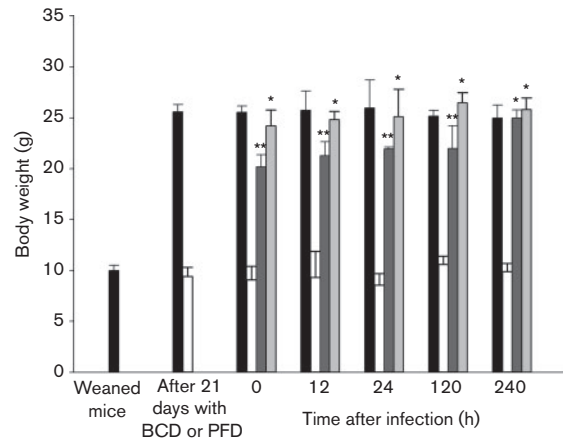
**Platelet count.** Blood samples were obtained as described for the leukocyte count. Manual platelet counting was performed by the visual examination of diluted whole blood with 1% (w/v) aqueous ammonium oxalate. The total number of platelets was determined with a haemocytometer (Dacie & Lewis, 1995).

**Statistical analysis.** Each experiment was performed in triplicate in groups consisting of 30 mice per group (six animals each time). Results were expressed as means  $\pm$  SD. After verification of the normal distribution of data, two-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at  $P < 0.05$ .

## RESULTS

### Body weight

The repletion diet supplemented with the nasal administration of *L. casei* was the most effective to increase body weight (Fig. 1). During the post-infection period, the body weight of all experimental groups showed no significant changes.



**Fig. 1.** Body weight before and after challenge with *S. pneumoniae*. Malnourished mice were fed either a BCD, a BCD + LcN or a PFD (MNC); the WNC group continually received a BCD. Results are expressed as means  $\pm$  SD. Each experiment was performed in triplicate in groups consisting of 30 mice per group (six animals each time). \*Significantly different from the MNC, but not significantly different from the WNC group ( $P < 0.05$ ). \*\*Significantly different from the MNC and WNC groups ( $P < 0.05$ ). Black bars, WNC; white bars, MNC; dark grey bars, BCD; light grey bars, BCD + LcN.

### Total proteins in blood serum

The levels of total proteins and albumin were decreased as a consequence of protein deprivation. Both repletion diets were effective in normalizing both parameters (0 h p.i.). After challenge, the BCD + LcN mice and the WNC group showed an earlier increase in total proteins than that of the mice fed with a BCD (Table 1). The BCD, BCD + LcN and WNC groups showed similar albumin levels during the entire experiment (Table 1).

### APPs in blood serum

AGP: protein deficiency induced a significant increase in AGP. Only the diet with supplementary *L. casei* induced normalization of this parameter (0 h p.i.) (Fig. 2a). After challenge, we observed a significant increase in all experimental groups. The BCD + LcN group showed similar levels to those of the WNC group. In both groups there was an increase at 12 h p.i., followed by a return to basal values at 240 h p.i. The mice fed with a BCD had a peak later than that of the BCD + LcN group (24 h p.i.) (Fig. 2a).

C3: undernourishment induced an increase in C3 levels in serum. The mice fed with a BCD + LcN showed a significant decrease in C3 levels (0 h p.i.) (Fig. 2b). After infection, an increase in C3 values was observed only in the WNC, BCD and MNC groups (Fig. 2b).

C4: protein deprivation did not modify C4 serum levels. The infection induced an increase in C4 levels in all

**Table 1.** Total proteins and albumin in serum

Total proteins and albumin in serum before and after challenge with  $10^7$  cells of *S. pneumoniae*. Malnourished mice were fed either a BCD, a BCD + LcN or a PFD (MNC); the WNC group continually received a BCD. Results are expressed as means  $\pm$  SD. Each experiment was performed in triplicate in groups consisting of 30 mice per group (six animals each time). Samples were obtained at 0 (before infection), 12, 24, 120 and 240 h p.i.

	Group	0 h p.i.	12 h p.i.	24 h p.i.	120 h p.i.	240 h p.i.
Total protein ( $\text{g l}^{-1}$ )	WNC	52.47 $\pm$ 2.36	59.70 $\pm$ 2.88	52.90 $\pm$ 2.26	52.95 $\pm$ 2.17	51.60 $\pm$ 2.26
	MNC	38.00 $\pm$ 3.50	40.00 $\pm$ 2.23	36.46 $\pm$ 3.56	31.60 $\pm$ 2.57	40.42 $\pm$ 1.70
	BCD	50.00 $\pm$ 2.55*	53.80 $\pm$ 5.37†	62.20 $\pm$ 3.89†	54.25 $\pm$ 2.71*	50.60 $\pm$ 0.65*
	BCD + LcN	52.20 $\pm$ 6.69*	62.10 $\pm$ 2.19*	53.20 $\pm$ 0.88*	53.40 $\pm$ 5.77*	51.70 $\pm$ 2.47*
Albumin ( $\text{g l}^{-1}$ )	WNC	23.25 $\pm$ 1.06	27.37 $\pm$ 0.74	27.90 $\pm$ 1.73	23.80 $\pm$ 0.64	22.65 $\pm$ 0.43
	MNC	18.15 $\pm$ 0.81	15.40 $\pm$ 0.79	20.00 $\pm$ 1.00	20.00 $\pm$ 0.81	17.47 $\pm$ 0.79
	BCD	24.15 $\pm$ 1.46*	28.35 $\pm$ 0.79*	26.19 $\pm$ 1.89*	22.27 $\pm$ 0.75*	23.00 $\pm$ 0.31*
	BCD + LcN	30.05 $\pm$ 0.45†	32.05 $\pm$ 1.45†	26.50 $\pm$ 0.74*	28.05 $\pm$ 1.74†	26.96 $\pm$ 1.82*

\*Significantly different from the MNC group, but not significantly different from the WNC group ( $P < 0.05$ ).

†Significantly different from the MNC and WNC groups ( $P < 0.05$ ).

experimental groups. The BCD + LcN mice demonstrated similar results to those of the WNC group. These two groups showed an earlier increase (12 h p.i.) in C4 levels than that observed for the BCD mice (120 h p.i.) (Fig. 2c).

### Total blood leukocyte and neutrophil counts

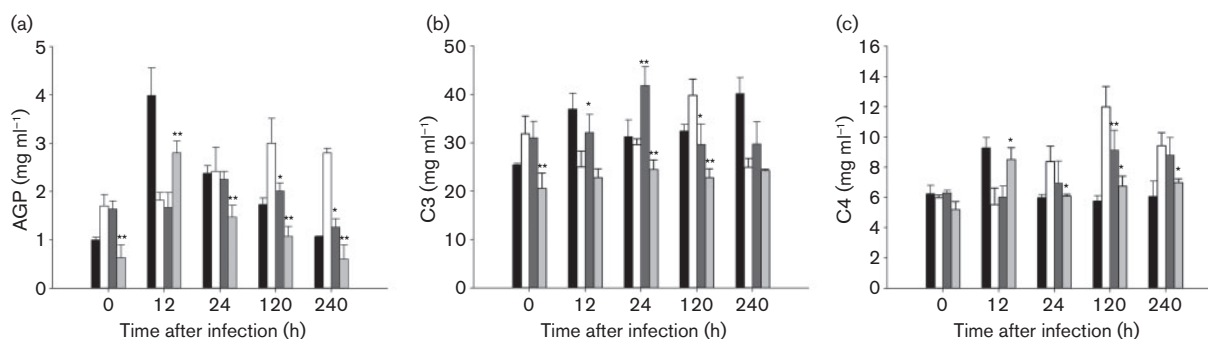
Protein deficiency induced a decrease in total leukocyte numbers. The repletion diets did not improve this parameter (0 h p.i.) (Fig. 3a).

After infection, the BCD + LcN mice showed an increase in the number of leukocytes at 24 h p.i. From that point onwards, the leukocyte count decreased until it reached values similar to those of the WNC group at the end of the experiment (Fig. 3a). In contrast, the BCD mice showed an

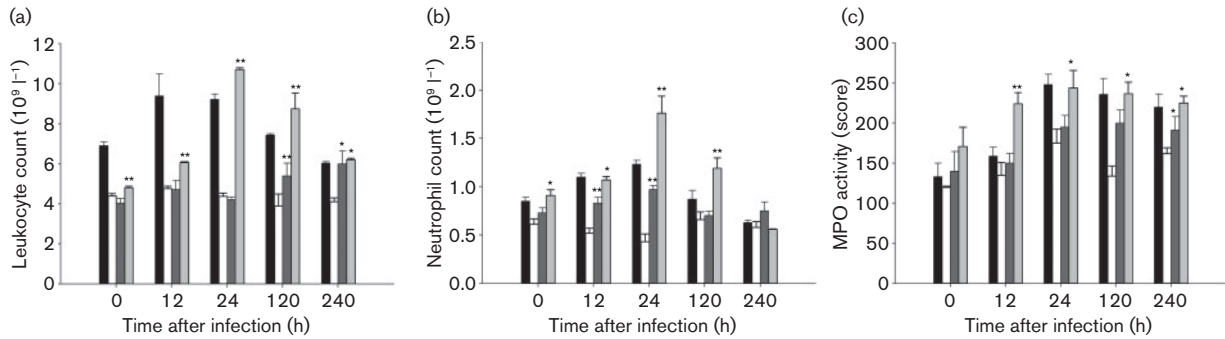
increase in the total leukocyte count only towards the end of the experiment (120 and 240 h p.i.) (Fig. 3a). The protein-deficient diet caused a decrease in neutrophil numbers (0 h p.i.). Only the BCD + LcN treatment normalized this parameter (Fig. 3b). At 24 h p.i. we observed a peak in all replete groups. However, neutrophil numbers were significantly higher in the mice that received *L. casei* (Fig. 3b). The infection promoted an increase in MPO scores in all experimental groups. At 12 h p.i., the mice that received *L. casei* showed significantly higher concentrations than the other groups (Fig. 3c).

### Coagulation tests

PT: although both repletion diets induced an increase in prothrombin activity, the BCD + LcN treatment (0 h p.i.)



**Fig. 2.** Acute phase proteins before and after challenge with *S. pneumoniae*. Levels of (a) AGP, (b) C3 and (c) C4 in serum were analysed. Malnourished mice were fed either a BCD, a BCD + LcN or a PFD (MNC); the WNC group continually received a BCD. Results are expressed as means  $\pm$  SD. Each experiment was performed in triplicate in groups consisting of 30 mice per group (six animals each time). \*Significantly different from the MNC, but not significantly different from the WNC group ( $P < 0.05$ ). \*\*Significantly different from the MNC and WNC groups ( $P < 0.05$ ). Black bars, WNC; white bars, MNC; dark grey bars, BCD; light grey bars, BCD + LcN.



**Fig. 3.** Total and differential blood leukocyte counts and myeloperoxidase (MPO) activity in blood neutrophils before and after challenge with *S. pneumoniae*. (a) Leukocyte count, (b) neutrophil count and (c) MPO activity. Malnourished mice were fed either a BCD, a BCD+LcN or a PFD (MNC); the WNC group continually received a BCD. Results are expressed as means  $\pm$  sd. Each experiment was performed in triplicate in groups consisting of 30 mice per group (six animals each time). \*Significantly different from the MNC, but not significantly different from the WNC group ( $P < 0.05$ ). \*\*Significantly different from the MNC and WNC groups ( $P < 0.05$ ). Black bars, WNC; white bars, MNC; dark grey bars, BCD; light grey bars, BCD+LcN.

was more effective (Fig. 4a). After challenge, all experimental groups showed a decrease in prothrombin activity at 12 h p.i. The mice that received BCD+LcN did not show significant modifications during the whole study period (Fig. 4a).

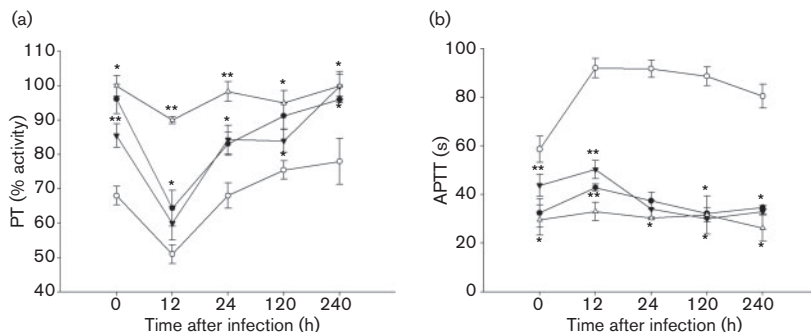
APTT: the BCD+LcN treatment was more effective than the BCD in normalizing the APTT that was altered by protein deprivation (0 h p.i.) (Fig. 4b). After infection, the MNC group showed an additional prolongation of the APTT. Both diets proved able to control this alteration, although the BCD+LcN treatment was more effective (Fig. 4b).

Fibrinogen: both repletion diets were effective in normalizing the fibrinogen levels, which were reduced as a consequence of protein deprivation (0 h p.i.) (Fig. 5a). After infection, all experimental groups showed an increase in fibrinogen at 120 h p.i. However, the BCD+LcN group

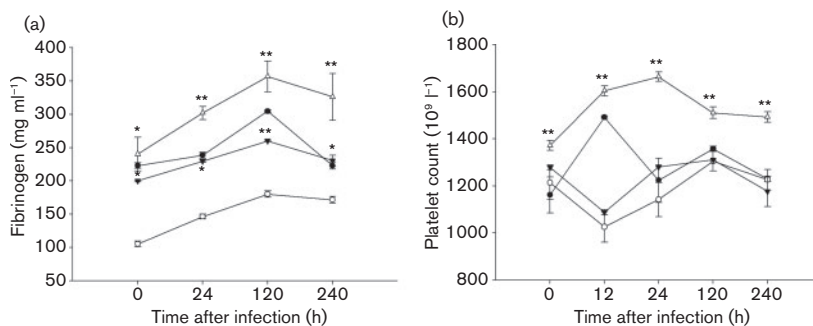
showed higher fibrinogen levels throughout the whole experiment (Fig. 5a).

TATc: only the BCD+LcN treatment normalized TATc levels, which were increased in protein-deficient mice (0 h p.i.) (Fig. 6a). The infection induced an increase in the number of these complexes in all the experimental groups. This increase occurred earlier in the BCD+LcN mice (24 h p.i.) than in the BCD group (120 h p.i.). At the end of the experiment both groups had reached normal values (Fig. 6a).

AT: the BCD+LcN treatment was more effective than the BCD in improving the damage induced by protein deficiency on AT plasma levels (0 h p.i.) (Fig. 6b). After infection, both diets induced a significant decrease in AT levels (BCD at 12 h p.i., BCD+LcN at 120 h p.i.), after which there was a progressive increment in AT levels, which reached normal values at the end of the experiment (Fig. 6b).



**Fig. 4.** Global coagulation tests before and after challenge with *S. pneumoniae*. (a) PT and (b) APTT were measured. Malnourished mice were fed either a BCD, a BCD+LcN or a PFD (MNC); the WNC group continually received a BCD. Results are expressed as means  $\pm$  sd. Each experiment was performed in triplicate in groups consisting of 30 mice per group (six animals each time). \*Significantly different from the MNC, but not significantly different from the WNC group ( $P < 0.05$ ). \*\*Significantly different from the MNC and WNC groups ( $P < 0.05$ ). ●, WNC; ○, MNC; ▼, BCD; △, BCD+LcN.



**Fig. 5.** (a) Fibrinogen concentration and (b) platelet count before and after challenge with *S. pneumoniae*. Malnourished mice were fed either a BCD, a BCD+LcN or a PFD (MNC); the WNC group continually received a BCD. Results are expressed as means  $\pm$  SD. Each experiment was performed in triplicate in groups consisting of 30 mice per group (six animals each time). \*Significantly different from the MNC, but not significantly different from the WNC group ( $P < 0.05$ ). \*\*Significantly different from the MNC and WNC groups ( $P < 0.05$ ). ●, WNC; ○, MNC; ▼, BCD; △, BCD+LcN.

PC: BCD mice showed an increase in PC values at 12 h p.i., which was followed by a decrease at 24 h p.i.; however, the BCD+LcN mice did not show significant changes during the whole experiment (Fig. 6c).

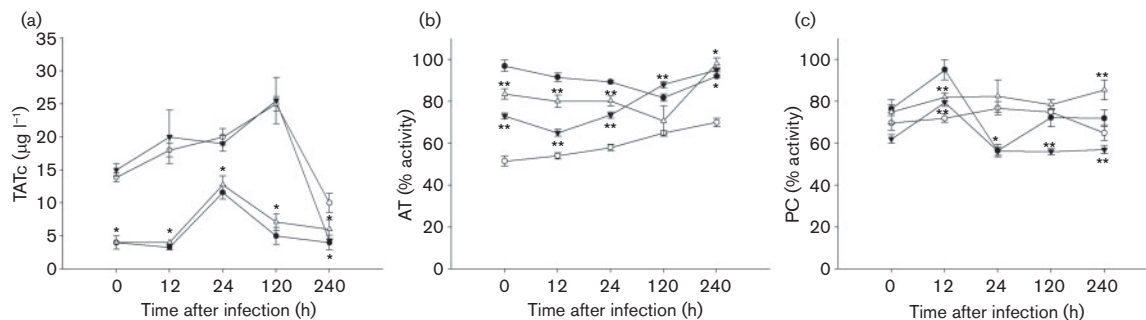
### Platelet count

Undernourishment did not modify the blood platelet count. The BCD+LcN repletion induced an increase in this parameter (Fig. 5b). At 12 h p.i., the BCD mice had a decreased platelet count similar to that observed in the MNC group. The BCD+LcN mice showed similar results to the WNC group, but with higher values during the whole experiment (Fig. 5b).

## DISCUSSION

The lungs and upper airways are mucosal surfaces that are common sites for infection. During innate immune

activation, factors of the coagulation pathway are activated, creating the cooperative immuno-coagulative response (Konecny, 2010). Lactic acid bacteria can be used to stimulate the immune system, which increases resistance to infections even in immunocompromised hosts (Villena *et al.*, 2005). Components of the lactobacillus cause responses through recognition by pattern recognition receptors, such as the TLR family expressed on innate immune cells. Downstream intracellular signalling from these receptors results in the modulation of cytokine responses (O'Neill & Bowie, 2007; Vizoso Pinto *et al.*, 2009). Dogi *et al.* (2008) demonstrated that *L. casei* CRL 431 induces an increase both in interferon gamma and tumour necrosis factor-alpha (TNF- $\alpha$ ) and in the number of interleukin (IL)-10+ cells in BALB/c mice. Previously, Racedo *et al.* (2006) showed that the oral administration of *L. casei* beneficially regulates the balance between TNF- $\alpha$  and IL-10, allowing a more effective immune response against infection and modulating the inflammatory response with less damage to the lung.



**Fig. 6.** Activation of coagulation and coagulation inhibitors before and after challenge with *S. pneumoniae*. (a) TATc levels, (b) AT and (c) PC activities in plasma were analysed. Malnourished mice were fed either a BCD, a BCD+LcN or a PFD (MNC); the WNC group continually received a BCD. Results are expressed as means  $\pm$  SD. Each experiment was performed in triplicate in groups consisting of 30 mice per group (six animals each time). \*Significantly different from the MNC, but not significantly different from the WNC group ( $P < 0.05$ ). \*\*Significantly different from the MNC and WNC groups ( $P < 0.05$ ). ●, WNC; ○, MNC; ▼, BCD; △, BCD+LcN.

In the present work, nasal administration of *L. casei* was performed on anaesthetized mice. This procedure avoids the possibility that any obtained effects caused by the probiotic are due to the stimulation of the gastrointestinal tract as a consequence of swallowed inoculum. Also, if anaesthetized mice swallowed a small part of the inoculum, the amount of swallowed bacteria would be insufficient to affect the mucosal or systemic immune response, according to Galdeano *et al.* (2007).

We confirmed that the BCD + LcN treatment was the most effective in increasing body weight. Gaggia *et al.* (2010) reported that probiotic supplementation improves the growth rate in animals.

Before infection, both diets improved the serum total protein and albumin levels, probably as a result of reduced synthesis, increased catabolism and albumin leakage due to the augmented vascular permeability observed during protein deprivation. Kaysen *et al.* (2001) reported that serum albumin levels correlate with dietary protein intake and with an inflammatory state. At 12 h p.i., the MNC group showed a significant decrease in albumin, which acted as a negative APP. This effect would be the consequence of the reduced availability of amino acids, which are used in the synthesis of other essential proteins in a stress situation (Morlese *et al.*, 1998). The increase in albumin levels observed after 12 h p.i. could be a consequence of a decrease in the catabolic rate as an adaptive mechanism (Morlese *et al.*, 1998). The replete groups had similar results to the WNC group, probably due to the presence of protein in the diets. Fleck (1989) reported that the range and quality of the acute phase response depend on the nutritional state of the host and on the severity of the infection. Additionally, the APP, complement and coagulation cascades are also part of a complex network of tightly linked genes involved in the early activation of the innate immune response (Luchtfeld *et al.*, 2011).

The ability of the different experimental groups to implement an acute phase response was analysed by determining the levels of AGP and C3 and C4 proteins. C3 and C4 proteins, besides acting as acute phase reactants, can be consumed by activation (González Naranjo & Molina Restrepo, 2010). AGP, which is mainly distributed in the liver, has an anti-inflammatory effect that could act as a regulatory mechanism of the inflammatory state. The serum concentration of AGP increases proportionally in response to systemic inflammation or infection and is regulated by the cytokine network involving TNF- $\alpha$ , IL-1 and IL-6 (Fournier *et al.*, 2000). Before infection, we saw an increase in AGP values in malnourished mice. Ling *et al.* (2004) reported similar results, which are attributed to an inflammatory state induced by protein deficiency.

We observed that the malnourished mice were able to implement a late acute phase response after infection (Fig. 2), results also reported also by Morlese *et al.* (1998) and Sakamoto *et al.* (1981). This effect may be the consequence of the diminished capacity of the mononuclear phagocytes

in malnourished mice to release cytokines that mediate the acute phase response (Komatsu *et al.*, 2007). Morlese *et al.* (1998) reported that the kinetic mechanisms used to implement an acute phase response include alterations in the protein synthesis/catabolism relationship. The kinetics of AGP in the BCD + LcN group was similar to that in the WNC group throughout the experiment. The effect of *L. casei* could be due to its ability to regulate the inflammatory response altered by protein deprivation (Villena *et al.*, 2009). The lower AGP levels observed in the BCD + LcN group compared with the WNC group could be beneficial at the haemostatic level, as reported by Su & Yeh (1996), who indicated that the increases in AGP induce expression of the tissue factor in monocytes. The kinetics of C4 in the BCD + LcN group was similar to that of the WNC group throughout the entire experiment. Increases in C3 and C4 in an inflammatory context were also demonstrated by Sampietro *et al.* (2004) and by Anthony *et al.* (1989) in a casein-induced acute phase response.

The production of blood cells, which are part of the immune system, is the result of the balance between positive and negative growth signals. Protein deficiency also alters haematopoiesis, leading to a marked decrease in leukocyte and neutrophil numbers (Salva *et al.*, 2008). The increase in leukocytes observed in the animals that received *L. casei* through the intranasal route correlates with the increase in the number and degree of activation of neutrophils. These results are probably due to the release of these cells from the bone marrow as a result of the stimulation caused by different factors, such as TNF- $\alpha$  induced by the infection. Also, this cytokine induces the recruitment of neutrophils and monocytes to the site of the injury (Knapp *et al.*, 2004; Villena *et al.*, 2009). The beneficial effect of the BCD + LcN treatment on the different parameters studied could be due to a protein contribution and an improvement in the innate immunity.

Pawlinski & Mackman (2010) reported that an inflammatory response shifts the haemostatic system towards a prothrombotic state. Similarly, coagulation affects inflammation. In this crosstalk, the tissue factor is strongly induced in endothelial cells and leukocytes. It activates thrombin, which in turn activates endothelial and immune cells by binding mainly to the protease-activated receptor. In this way, an inflammatory response is induced by enhancing cytokine and chemokine expression, as well as by increasing leukocyte recruitment (Levi *et al.*, 2004; Busso *et al.*, 2008).

In a previous study we reported that protein deprivation induces a low grade of coagulation activation attributable to a pro-inflammatory state and probably to a deficient synthesis of coagulation factors and inhibitor proteins (Zelaya *et al.*, 2011a). In the present study we observed lower coagulation activation in the animals supplemented with *L. casei*, as shown by the lower TATc levels. Fibrinogen, factor I of the coagulation cascade, was decreased in the malnourished animals, possibly due to a deficit of synthesis

or to consumption, as observed in the global tests (Zelaya *et al.*, 2011a). Before an infectious challenge, the different experimental groups showed a gradual increase until they reached a peak at 120 h p.i. However, the BCD + LcN group showed higher fibrinogen levels than those of the WNC group; this could be due to the fact that fibrinogen, besides participating as a coagulation factor, may act as an acute phase reactant (Reitsma *et al.*, 2003).

Under physiological circumstances, coagulation activation is regulated by natural coagulation inhibitors such as PC and AT (Choi *et al.*, 2007). At 120 h p.i., the BCD + LcN and WNC groups showed a decrease in AT. This decrease could be due to the consumption of the inhibitor in an attempt to prevent coagulation activation. Choi *et al.* (2007) reported that this anticoagulant system may be damaged during a septic process because of massive consumption and downregulation by inflammatory mediators. On the other hand, the increase in PC levels observed at 12 h p.i. in the WNC group could be the consequence of an acute phase response induced by the infectious stimulus. This response could be favourable for the host, considering its anti-inflammatory properties, as reported by Yuksel *et al.* (2002). However, no significant differences in the levels of PC were observed in the animals that received *L. casei*.

In order to globally assess the state of activation of blood coagulation, we determined TATc levels in plasma. The increase in such complexes observed in the MNC group shows a state of coagulation activation as a consequence of an inflammatory state (Zelaya *et al.*, 2011a). The improvement or normalization of the different tests found in the animals that received *L. casei* (BCD + LcN) could be due to the well-known ability of this lactic acid bacterium to induce a pattern of anti-inflammatory cytokines (Haro *et al.*, 2009). Villena *et al.* (2009) reported that nasally administered *L. casei* increases TNF- $\alpha$ , as well as IL-10 after infection, thus controlling the inflammatory response and coagulation activation.

The role of platelets in haemostasis is well known. However, they can have an early role in immune surveillance and in the transfer of pathogen information to other innate immune cells. These functions are supported by the presence of TLRs on platelets (Alves-Filho, 2005; Cox *et al.*, 2011). Also, platelets may play an important role in pathogen clearance and are involved in leukocyte function. Platelet-leukocyte interactions could be a crucial link between the inflammatory and the thrombotic systems (van Gils *et al.*, 2009).

In our experimental model, malnutrition did not affect the number of platelets in peripheral blood. After infection, the MNC and BCD groups showed a significant decrease in the level of platelets, possibly because of recruitment to the site of injury (Weyrich & Zimmerman, 2004). The WNC and BCD + LcN groups showed an increase in this parameter, which is often found in infectious processes (Griesshammer *et al.*, 1999).

## CONCLUSION

Most components in the coagulation cascade have pro-inflammatory properties. These preliminary results demonstrate the efficiency of the intranasal administration of *L. casei* in regulating the pro-inflammatory aspects of coagulation without affecting coagulation itself.

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