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Lactobacillus rhamnosus CRL1505 beneficially modulate the immuno-coagulative response after pneumococcal infection in immunocompromised malnourished mice

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Abstract

This work evaluated the effect of orally or nasally administered *Lactobacillus rhamnosus* CRL1505 on the resistance against pneumococcal infection in immunocompromised protein-malnourished mice. In particular, we aimed to gain insight in the knowledge of the mechanism involved in the immunomodulatory effect of *L. rhamnosus* CRL1505 in malnourished hosts by evaluating its impact on the immuno-coagulative response. Malnutrition significantly increased lung tissue damage caused by *Streptococcus pneumoniae* infection. Lung damage was associated to a deregulated activation of coagulation and an altered inflammatory response. Pneumococcal colonization of lung and bacteremia were significantly reduced ($p<0.05$) in malnourished mice receiving the CRL1505 strain. Moreover, mice repleted with supplemental *L. rhamnosus* CRL1505 showed the lowest alteration of the alveolar-capillarity barrier and cell damage in lungs after the infectious challenge, especially when the CRL1505 strain was administered by nasal route. Besides, *L. rhamnosus* CRL1505-treated mice showed an improved respiratory innate immune response and a lower activation of coagulation. The results of this work indicate that *L. rhamnosus* CRL1505 is able to beneficially modulate the inflammation-coagulation interaction after respiratory infections in malnourished hosts.

Keywords hemostasis - inflammation - *Lactobacillus rhamnosus* - lung damage - malnourished mice

Introduction

Populations with inadequate nutrition, in either quantitative or qualitative terms, have increased susceptibility to infections (Rodriguez et al. 2011). Several studies have clearly shown that protein malnutrition impairs normal immune system functions. For instance, protein deprivation alters the nonspecific defense mechanisms, such as anatomical barriers and intestinal flora (Scrimshaw and SanGiovanni 1997). In addition, malnutrition is associated with a detriment of cellular immunity, phagocyte function, the complement system, secretory immunoglobulin A concentrations, and cytokine production (Chandra 1997; Scrimshaw and SanGiovanni 1997). On the other hand, stimulation of immune response by infection increases the demand for metabolically derived anabolic energy, leading to a synergistic vicious cycle of adverse nutritional status and increased susceptibility to infection (Rodriguez et al. 2011). Therefore, malnutrition is a major risk factor for a number of infectious diseases, including bacterial and viral respiratory tract infections, which are widely prevalent in developing countries (Schaible and Kaufmann 2007).

Inflammation and coagulation play key roles in host defenses against pathogens. The host responds to infection by mounting innate immune defense mechanisms to eliminate the pathogen. In addition, the coagulation process as a part of the innate immune response leads to local restriction and trapping of the infectious agent and protects the rest of the organism. Moreover, the inflammatory response triggered by infection activates the hemostatic systems and their products in order to perpetuate and strengthen inflammatory reactions, indicating that both systems are tightly connected (O'Brien 2012; Petaja 2011). This bimodal inflammation-coagulation interaction, known as immuno-coagulative response, is essential for the eradication of pathogens, however if uncontrolled it can

contribute to organ damage and death (van der Poll et al. 2011). The delicate balance between the positive and negative effects of the immuno-coagulative response is of great importance in the outcome of respiratory infections (Chambers 2008; Dessing et al. 2009).

Previously, our laboratory demonstrated that certain probiotic lactic acid bacteria (LAB) can exert a beneficial effect on the host during *Streptococcus pneumoniae* infection through their capacity to beneficially modulate the immuno-coagulative response (Haro et al. 2009; Zelaya et al. 2012; Zelaya et al. 2013). In this regard, we demonstrated that orally administered *Lactobacillus casei* CRL431 is able to effectively regulate coagulation activation and fibrinolysis inhibition during pneumococcal infection in immunocompetent mice, which led to decreased fibrin deposits in lung and reduced tissue damage (Haro et al. 2009). In addition, repletion of malnourished mice with supplemental *L. casei* CRL431 administrated by oral (Zelaya et al. 2012) or nasal routs (Zelaya et al. 2013) was able to beneficially modulate the inflammation-coagulation relationship during the pneumococcal infection, indicating that LAB are able to modulate the immuno-coagulative response in immunocompromised hosts.

Other LAB strains have been shown to improve the resistance against respiratory infections. Among the LAB with immunoregulatory capacities evaluated in our animal models, *Lactobacillus rhamnosus* CRL1505 is able to improve antibacterial and antiviral immunity in the respiratory tract (Salva et al. 2010; Villena et al. 2012a; Villena et al. 2012b). A randomized controlled trial was performed in order to evaluate the effect of a probiotic yogurt containing *L. rhamnosus* CRL1505 on both gut and non-gut related illnesses among children. The study demonstrated that *L. rhamnosus* CRL1505 is able to improve mucosal immunity and reduced the incidence and severity of intestinal and respiratory infections (Villena et al. 2012b). Taking into consideration the positive results

in children we become interested in elucidate the cellular and molecular mechanisms involved in the immunoregulatory effect of the CRL1505 strain. In this regard, we have made important progress in the understanding of how this strain modulates the common mucosal immune system in immunocompetent hosts and demonstrated that the strain is able to induce the mobilization of immune cells from the gut to the lung and to change cytokine profiles in serum and the respiratory tract (Salva et al. 2010; Villena et al. 2012a; Villena et al. 2012b). However, the effect of this strain on respiratory infections in immunocompromised-malnourished hosts, have been less evaluated.

Considering this background, the current study aimed to evaluate the effect of *L. rhamnosus* CRL1505 on the resistance against pneumococcal infection in immunocompromised protein-malnourished mice. In addition, we aimed to gain insight in the knowledge of the mechanism involved in the immunomodulatory effect of the *L. rhamnosus* CRL1505 strain in malnourished hosts by evaluating its impact on the immuno-coagulative response.

Materials and methods

Animals

Male 6-week-old Swiss albino mice were obtained from the closed colony kept at CERELA (Chacabuco 145, San Miguel de Tucumán, Argentina). They were housed in plastic cages in a controlled atmosphere (22 ± 2°C temperature, 55 ± 2% humidity) with a 12 h light/dark cycle.

Microorganisms

Lactobacillus rhamnosus CRL1505 was obtained from the CERELA culture collection. 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; and distilled water, 1 L, pH 7. It was cultured for 12 h at 37 °C (final log phase) in Man-Rogosa-Sharpe broth (MRS, Oxoid, Cambridge, UK). The bacteria were harvested through centrifugation at 3000 x g for 10 min and washed 3 times with sterile 0.01 mol/L phosphate buffer saline (PBS), pH 7.2. *Streptococcus pneumoniae* were purchased from the Administración Nacional de Laboratorios e Institutos de Salud-ANLIS "Dr. Malbrán", Buenos Aires, Argentina. The pathogen strain belongs to the 14 serotype, 1 of the 10 most frequent serotypes isolated in pneumococcal infections in Argentina (Ruvinsky et al. 2002).

Feeding procedures

Weaned mice were malnourished after they consumed a protein-free diet (PFD, Table 1) for 21 days (d). At the end of this period, all malnourished (MNC) mice weighed 40–50% less than well-nourished control (WNC) mice. Malnourished mice were separated into three groups for repletion treatment. One group of malnourished mice was fed balanced conventional diet (BCD, Table 1) for 7 consecutive d (BCD group). Since administration of *L. rhamnosus* CRL1505 for 5 d is the optimal dose to improve the immunity in intestinal and respiratory infections in immunocompetent mice (Salva et al. 2010) and accelerate the recovery of respiratory immunity in malnourished mice (Salva et al. 2012), the second and third groups of mice received 7 d of BCD with *L. rhamnosus* CRL1505 (10^8 cells/mouse/d) supplementation by nasal (BCD+LrN) or oral routes (BCD+LrO) during the last 5 d.

All experiments were approved by the Ethical Committee for Animal Care of CERELA and of the Universidad Nacional de Tucumán, Argentina.

Experimental infection

S. pneumoniae was grown according to previous works (Zelaya et al. 2011). Challenge with *S. pneumoniae* was performed on the first d after the end of each dietary treatment (d 8). Mice were infected by dropping 25 μL of the inoculum containing 10^7 log-phase colony-forming units of *S. pneumoniae* suspended in PBS into each nostril and allowing it to be inhaled (Zelaya et al. 2011). Samples were obtained at 0 (before infection) and on h 12, 24, 120 and 240 post-infection (pi). During all the assessed period, MNC received only the PFD, whereas the WNC, BCD, BCD+LrO, and BCD+LrN groups consumed BCD *ad libitum*.

Body weight

Body weight was determined to assess the level of malnutrition induced by the PFD as well as the effect of renutrition diets on a nutritional parameter. Body weight was determined at the beginning and end of each feeding period and before sample collection. An electronic balance with a sensitivity of 0.01 g was used for the purpose. Body weight resulted from the mean of the values obtained in three different weighing performed alternately.

Serum albumin

Serum albumin concentration was determined by means of a colorimetric assay based on albumin binding to bromocresol green (Wiener Lab, Rosario, Argentina). The results were expressed as g/L.

Bacterial cell counts in lung homogenates and blood

At prechosen intervals mice were killed and 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_2. α-haemolytic colonies were observed. The isolate was identified as *S. pneumoniae*, and confirmation of the identification was provided by inhibition of the isolate by optochin. Optochin susceptibility testing was performed in an atmosphere of 5% CO_2. All plates without any colony were examined until 72 h. After the challenge, the survival of the mice was monitored until d 21 pi. All animals survived without significant differences (p<0.05) between both groups. The results were expressed as log of colony-forming unit (cfu)/g of organ. Progression of bacterial growth to the bloodstream was monitored by sampling blood obtained through cardiac puncture and plating on blood agar. Bacteraemia was reported as negative or positive hemocultures, after incubation.

Lung histophatology

At prechosen intervals, whole-lung samples from all experimental groups were excised and washed out with PBS. 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. All slides were coded and evaluated blindly.

Biochemical analyses in bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) samples were obtained according to the technique described previously (Villena et al. 2005). Briefly the trachea was exposed and intubated with a catheter and 2 sequential lavages were performed in each mouse by injecting 0.5 mL of sterile PBS. The sample of fluid was centrifuged for 10 min at 900 *x* g, the supernatant fluid was frozen at -70°C for subsequent biochemical analyses. Albumin content was determined to measure the increase of permeability of the bronchoalveolar-capillarity barrier was determined colorimetrically based on albumin binding to bromocresol green (Wiener Lab, Rosario, Argentina). The results were expressed as g/L. Lactate dehydrogenase (LDH) activity was determined as indicator of general cytotoxicity was determined by measuring the formation of a reduced form of nicotinamideadenine dinucleotide using Wiener Lab reagents and procedures (Wiener Lab, Rosario, Argentina). The results were expressed as U/L of BAL fluid.

Total and differential blood leucocyte counts

Blood samples were obtained by cardiac puncture from sodium pentobarbital-anesthetized animals and were collected in tubes containing EDTA as an anticoagulant. Total number of leucocytes was determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in blood smears stained with May Grünwald Giemsa stain using a light microscope (100x), and absolute cell numbers were calculated (Dacie and Lewis 2008).

208

209 *Activation of blood neutrophils*

210 Measurement of myeloperoxidase (MPO) activity of blood neutrophils was carried out
211 by use of the Washburn test, which is a cytochemical method that uses benzidine as a
212 MPO chromogen (Kaplow 1965). Cells were graded as negative or as weak, moderate, or
213 strongly positive according to the intensity of reaction and were used to calculate the
214 score. The score was calculated by counting 200 neutrophils in blood smears. The score
215 value was calculated by the addition of neutrophils with different positive grades.

216

217 *Coagulation tests*

218 Blood samples were obtained as described before and collected in a 3.2% (w/v)
219 solution of trisodium citrate at a ratio of 9:1. Plasma was obtained according to Agüero et
220 al. (2006). Prothrombin time (PT), activated partial thromboplastin time (APTT) and
221 plasmatic fibrinogen were performed manually on fresh plasma samples. PT was
222 determined to evaluate the extrinsic coagulation pathway; it was determined by a one-step
223 method (Thromborel S, Behningwerke AG, Marburg, Germany). Results are expressed as
224 percentage of prothrombin activity (%) from a calibration curve made from a pool of fresh
225 plasma from normal mice (Kordich 2003). APTT was determined to evaluate the intrinsic
226 pathway of coagulation; it was determined by mixing plasma with calcium chloride and a
227 partial thromboplastin reagent (Dade Actin FSL Activated PTT Reagent, Dade Behring,
228 Marburg, Germany) and timing initial clot formation. Results are expressed in seconds
229 (Kordich 2003). Fibrinogen concentration was determined by the method of Clauss using a
230 commercial kit and following manufacturer's instructions (FibriPrest, Diagnostica Stago,
231 Asnières, France).

232

233 *Platelet counts*

234 Blood samples were obtained as described for the leucocyte count. Manual platelet
235 counting was performed by visual examination of diluted whole blood with 1% (w/v)
236 aqueous ammonium oxalate. The total number of platelets was determined with a
237 hemocytometer (Dacie and Lewis 2008).

238

239 *Statistical analysis*

240 Each experiment was performed in triplicate in groups consisting of 30 mice per group
241 (6 animals for each time point). Results were expressed as means ± SD. After verification
242 of the normal distribution of data, 2-way ANOVA was used. Tukey's test (for pairwise
243 comparisons of the means) was used to test for differences between the groups.
244 Differences were considered significant at $p<0.05$.

245

246 **RESULTS**

247 *Recovery of nutritional parameters*

248 Malnourished mice showed significantly lower body weight than WNC mice. The
249 three BCD, BCD+LrO and BCD+LrN repletion diets significantly improved body weight
250 when compared to MNC mice ($p<0.05$). Moreover, after infection BCD, BCD+LrO and
251 BCD+LrN mice reached body weight values that were similar to the one observed the
252 WNC group and without significant difference ($p<0.05$) between them (Fig. 1A). In
253 addition, the three repletion treatments were effective in normalizing serum albumin
254 concentration during the whole experiment (Fig. 1B).

255

Bacterial cell counts in lung homogenates and blood

Streptococcus pneumoniae was detected in lung and blood samples from WNC, MNC and BCD mice at all assayed times, however MNC and BCD mice had significantly higher lung bacterial cell counts than WNC mice ($p<0.05$) (Fig. 2A). On the contrary, repletion treatments with orally or nasally supplemental *L. rhamnosus* CRL1505 significantly reduced bacterial counts in lung ($p<0.05$) (Fig. 2A) and showed negative hemocultures (data not shown).

Lung tissue damage

S. pneumoniae infection significantly increased LDH activity and albumin concentration in BAL of all experimental groups ($p<0.05$) (Fig. 2B and C). BCD, BCD+LrO and BCD+LrN groups showed reduced levels of BAL LDH and albumin, however the nasal treatment with *L. rhamnosus* CRL1505 was more effective than BCD or BCD+LrO to reduce albumin concentration in BAL indicating a lower alteration of the bronchoalveolar-capillarity barrier in this group (Fig. 2B and C).

Challenge with pneumoccocci induced an intense inflammatory response in lung, hemorrhage and a reduction of alveolar spaces in the MNC group (Fig. 3B), while lung tissue changes in BCD mice were always intermediate between MNC and WNC mice (Fig. 3C). Mice receiving *L. rhamnosus* CRL1505 showed a moderate inflammatory infiltrate and lower hemorrhage and a reduction of alveolar spaces (Fig. 3D and E). However, BCD+LrN treatment was more effective than the BCD+LrO diet to reduce lung histological alterations (Fig. 3D and E). In fact, BCD+LrN mice showed no differences when compared with the WNC group.

Innate immune response

Challenge with the respiratory pathogen increased the number of blood leucocytes and neutrophils in mice, with the exception of the MNC group that failed to increase blood leukocytes in response to the infection (Fig. 4A and B). Mice receiving *L. rhamnosus* CRL1505 showed significant higher numbers of blood leucocytes and neutrophils ($p<0.05$) than MNC mice (Fig. 4A and B). Blood neutrophils in BCD+LrN were not different from the WNC group while this cell population in BCD+LrO was significantly higher than WNC mice ($p<0.05$) (Fig. 4B). In addition to the quantitative changes of blood neutrophils, MPO activity was evaluated as a measure of blood neutrophils activity. As shown in Fig. 4C, malnutrition did not modify MPO activity in blood neutrophils and only the repletion treatment with oral *L. rhamnosus* CRL1505 induced an increase in this parameter (h 0). Challenge with *S. pneumoniae* increased blood MPO score in all the experimental groups (Fig. 4C). BCD+LrN mice showed MPO scores that were similar to those in the WNC group. In addition, BCD+LrO mice showed significantly higher blood MPO activity ($p<0.05$) than the WNC group during all the studied period (Fig. 4C).

Coagulative response

Protein malnutrition induced a decrease in prothrombin activity, which was normalized by the diets supplemented with *L. rhamnosus* CRL1505 but not with BCD treatment (Fig. 5A). Challenge with the respiratory pathogen significantly reduced prothrombin activity ($p<0.05$) in all experimental groups. However, MNC mice showed the lowest percentages of prothrombin activity and the three repletion treatments were able to significantly increase this parameter ($p<0.05$) (Fig. 5A). BCD and BCD+LrO groups showed

303 percentages that were similar to the WNC group while BCD+LrN mice presented a
304 prothrombin activity that was higher than WNC mice (Fig. 5A).

305 Malnutrition induced a delay in plasma coagulation during the APTT test and only
306 treatments with supplemental *L. rhamnosus* CRL1505 were able to normalize this
307 parameter (Fig. 5B). Challenge with pneumococci induced a significant prolongation of
308 APTT in MNC ($p<0.05$) that was not normalized in BCD mice. In addition, BCD+LrO
309 and BCD+LrN mice did not show significant modifications ($p<0.05$) of APTT throughout
310 the experiment (Fig. 5B).

311 Blood fibrinogen concentration was significantly increased after the challenge with *S.*
312 *pneumoniae* ($p<0.05$) (Fig. 5C). BCD+LrO and BCD+LrN reached a peak of fibrinogen
313 earlier than BCD mice (Fig. 5C). Moreover, blood fibrinogen concentrations in groups
314 supplemented with *L. rhamnosus* CRL1505 were significantly higher than the WNC mice
315 during all the studied period ($p<0.05$) (Fig. 5C).

316 Finally, no modification of platelet counts were observed in BCD or BCD+LrO before
317 the challenge with the respiratory pathogen; however BCD+LrN mice showed an increase
318 in this parameter (Fig. 5D). Infection significantly decreased platelet counts in MNC and
319 BCD (Fig. 5D) ($p<0.05$). In contrast, BCD+LrO and BCD+LrN groups showed a
320 significant increase in the number of platelets ($p<0.05$) after the challenge and reached
321 levels that were higher than those observed in the WNC group (Fig. 5D).

322

323 **Discussion**

324 There is a large and fast-growing body of evidence demonstrating the beneficial effects
325 of probiotic lactobacilli on immune protection against gastrointestinal tract pathogens
326 (Castillo et al. 2011; Salva et al. 2010). Currently, studies are focused 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 this regard, some studies indicate that nasally or orally administered probiotics are able to improve respiratory and systemic immune responses (Chiba et al. 2013; Salva et al. 2010; Tomosada et al. 2013). In the present work, malnourished mice orally or nasally treated with *L. rhamnosus* CRL1505 evidenced lower alterations of lung tissue and elicited an improved immune-coagulative response after the challenge with the respiratory pathogen *S. pneumoniae*.

During respiratory infections, the release of inflammatory mediators and cytokines from cells participating in the local inflammatory response influence leucocyte sequestration in the lung. Inflammatory mediators also release neutrophils from the bone marrow, activate circulating neutrophil and vascular endothelium, and change neutrophil-endothelial interactions, all factors that may contribute to recruitment of neutrophils into the lung (Balamayooran et al. 2010). Previously, it was demonstrated that oral *L. rhamnosus* CRL1505 administration to immunocompetent mice induced an increase in the levels of Interferon gamma (IFN)-γ, Interleukin (IL)-6 and IL-10 in BAL (Salva et al. 2010). It was showed that these changes in the respiratory cytokine profile was associated with an activation of both innate and specific immune responses against pneumococcal infection (Salva et al. 2010). Moreover, the improvement of respiratory defenses was not only related to the increase in pneumococcal clearance but in addition, to the modulation of the inflammatory immune response through the upregulation of IL-10, with less damage of lung tissue (Salva et al. 2010). Then, it is possible that *L. rhamnosus* CRL1505 administration is also capable to change the respiratory cytokine profile in immunocompromised malnourished mice inducing an increase in the resistance to the pneumococcal infection and protecting from inflammatory damage at the same time.

On the other hand, coagulation abnormalities are commonly found in respiratory infections since inflammation and hemostasis are bidirectional processes (Ferrer et al. 2008). Inflammation leads to activation of the hemostatic system that in turn also influences inflammatory activity creating an immuno-coagulative response. Proinflammatory mediators trigger an increase in platelet reactivity and activation of the coagulation system (Margetic 2012). When acute lung injury is initiated by direct damage to the lung or as a result of systemic inflammation (sepsis), a series of hemostatic pathways are triggered with the subsequent formation of fibrin that is deposited in the lung (Wygrecka et al. 2008). Respiratory pathogens such as Influenza Virus and *S. pneumoniae* have been shown to be capable of modulating inflammation and activating coagulation both *in vitro* and *in vivo*. Human endothelial cells or monocytes infected with Influenza virus have been shown to induce procoagulant activity which was associated with an increase in tissue factor (TF) expression (Bouwman et al. 2002; Visseren et al. 2000). In line with this, influenza-infected mice with had elevated plasma levels of thrombin-antithrombin complexes and D-dimers, indicative of a prothrombotic state (Keller et al. 2006). Our laboratory has demonstrated that alterations of the balance between coagulation and fibrinolysis also occurs during pneumococcal infection (Haro et al. 2009). Moreover, we reported that protein deprivation induces a low grade of coagulation activation attributable to a proinflammatory state, and probably to a deficient synthesis or consumption of coagulation factors and inhibitor proteins (Zelaya et al. 2011). The imbalance between pro- and anti-coagulation mechanisms significantly contributes to lung tissue damage and mortality during pneumococcal infection in immunocompromised malnourished mice (Zelaya et al. 2011). Our laboratory was the first in demonstrating that some LAB strains are able to beneficially modulate the immuno-coagulative response in

375 malnourished hosts (Agüero et al. 2006; Zelaya et al. 2012; Zelaya et al. 2013). In this
376 work, it was demonstrated that repletion with supplemental *L. rhamnosus* CRL1505 was
377 effective for reducing hemostatic alterations induced by both malnutrition and infection.

378 This study showed that both oral and nasal treatments with the CRL1505 strain were
379 able to beneficially modulate the immune-coagulative response in malnourished mice. It
380 was also observed that nasal priming with *L. rhamnosus* CRL1505 was more effective
381 than oral treatment to avoid inflammatory-coagulative lung tissue damage. These
382 differential effects of orally and nasally administered *L. rhamnosus* CRL1505 could be
383 related to differences in the immunomodulatory mechanisms associated to each route.

384 It is well-known that probiotic lactic acid bacteria administrated by oral route have
385 direct contact with intestinal epithelial and immune cells which can be activated after
386 probiotic treatments (Shimazu et al. 2012; Villena et al. 2012c). Our previous studies in
387 immunocompetent mice showed that orally administered *L. rhamnosus* CRL1505 is able
388 to increase the number of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells in the gut, induce a mobilization of
389 these cells into the respiratory mucosa and improve pulmonary production of IFN-γ
390 (Villena et al. 2012a). Then, IFN-γ secreted in response to *L. rhamnosus* CRL1505
391 stimulation modulate the pulmonary innate immune microenvironment conducting to the
392 activation of pulmonary macrophages (Marranzino et al. 2012) and dendritic cells (Villena
393 et al. 2012a), and the generation of a Th1 response with the consequent increase in the
394 resistance against respiratory pathogens (Chiba et al. 2013; Salva et al. 2010). Then, in
395 CRL1505-treated malnourished mice, this mechanism would allow a faster and improved
396 innate immune response against pneumococcal infection, thus favoring clearance of the
397 pathogen and modulating the inflammatory immune response, with less damage to lung
398 tissue. As mentioned before, we also demonstrated in previous works that orally

399 administered *L. rhamnosus* CRL1505 modulates the balance between proinflammatory
400 cytokines and IL-10 in the respiratory tract after the nasal challenge with respiratory
401 pathogens (Chiba et al. 2013; Salva et al. 2010). The improvement of IL-10 would
402 contribute to regulate the procoagulant and antifibrinolytic effects of proinflammatory
403 mediators induced by the infectious challenges.

404 The protective effect of the nasal administration of *L. rhamnosus* CRL1505 would be
405 also related to an upregulation of the innate immune response in both respiratory and
406 systemic compartments. Comparative studies in immunocompetent mice using oral and
407 nasal stimulation with immunomodulatory bacterial strains such as *L. casei* CRL431 or *L.*
408 *lactis* NZ9000 demonstrated that the nasal priming is more effective than oral
409 administration to stimulate the respiratory innate immune response (Medina et al. 2008;
410 Racedo et al. 2006; Villena et al. 2008). Moreover, it was demonstrated that some lactic
411 acid bacteria administrated by nasal route are able to reduce colonization of *S. pneumoniae*
412 and pathogen counts in the lung, probably due to lactic acid bacteria transiently colonize
413 the respiratory tract of mice (Cangemi de Gutierrez et al. 2001; Medina et al. 2008;
414 Villena et al. 2009). Then, it is possible that nasally administered *L. rhamnosus* CRL1505
415 could be more effective to improve pulmonary innate response in malnourished mice and
416 transiently colonize the respiratory mucosa to reduce the *S. pneumoniae* colonization by
417 competitive exclusion, which would explain the higher capacity of this treatment to reduce
418 lung damage associated to pneumococcal infection. To precisely study *L. rhamnosus*
419 CRL1505 colonization of the respiratory tract is an interesting topic for future research.
420 Moreover, it would be of value to evaluate whether *Lactobacillus* strains isolated from the
421 respiratory mucosa are more effective than foreign organism such as *L. rhamnosus*
422 CRL1505 to improve the immune-coagulative response.

In conclusion, the present work demonstrates that *L. rhamnosus* CRL1505 effectively regulate hemostatic alterations during the pneumococcal infection in malnourished mice, which would contribute to a decrease in lung damage. This protective effect of *L. rhamnosus* CRL1505 would be mediated by its capacity to improve levels of both IFN-γ and IL-10 that would contribute to improve innate immune response and regulate the procoagulant and antifibrinolytic effects of proinflammatory mediators induced by the infectious challenge. Nasal priming with the CRL1505 strain is more effective than oral administration to achieve the protective effect.

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Disclosure

The authors have no conflicts of interest to disclose.

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583

584 **Figure legends**

585 Fig. 1. (a) Body weight and (b) serum albumin before and after challenge with
586 *Streptococcus pneumoniae*. Malnourished mice replete with a balanced conventional diet
587 (BCD) or BCD with supplemental *Lactobacillus rhamnosus* CRL1505 by nasal
588 (BCD+LrN) or oral routes (BCD+LrO). Malnourished control (MNC) and well-nourished
589 control (WNC) mice were used. Results are expressed as mean ± SD. *Different from
590 MNC mice, no differences with the WNC group ($P<0.05$). **Different from the MNC and
591 WNC groups ($P<0.05$).

592

593 Fig. 2. (a) Bacterial cell counts in lung, (b) LDH activity in BAL, and c) Albumin in
594 BAL, before and after challenge with *Streptococcus pneumoniae*. Malnourished mice
595 replete with a balanced conventional diet (BCD) or BCD with supplemental *Lactobacillus*
596 *rhamnosus* CRL1505 by nasal (BCD+LrN) or oral routes (BCD+LrO). Malnourished
597 control (MNC) and well-nourished control (WNC) mice were used. Results are expressed
598 as mean ± SD. *Different from MNC mice, no differences with the WNC group ($P<0.05$).
599 **Different from the MNC and WNC groups ($P<0.05$).

600

601 Fig. 3. Histological examination of lungs on h 240 after challenge with *Streptococcus*
602 *pneumoniae*. (a) Well-nourished control mice, (b) malnourished control, (c) malnourished
603 mice replete with BCD, (d) malnourished mice replete with BCD with supplemental
604 *Lactobacillus rhamnosus* CRL1505 by nasal (BCD+LrO) or (e) oral routes (BCD+LrN).
605 Light micrographs, original magnification ×400.

606

Fig. 4. (a) Leukocyte count and (b) neutrophil count in blood, and (c) MPO activity in blood neutrophils, before and after challenge with *Streptococcus pneumoniae*. Malnourished mice replete with a balanced conventional diet (BCD) or BCD with supplemental *Lactobacillus rhamnosus* CRL1505 by nasal (BCD+LrN) or oral routes (BCD+LrO). Malnourished control (MNC) and well-nourished control (WNC) mice were used. Results are expressed as mean ± SD. *Different from MNC mice, no differences with the WNC group ($P<0.05$). **Different from the MNC and WNC groups ($P<0.05$).

Fig. 5. (a) Prothrombin time, (b) Activated partial thromboplastin time, (c) Fibrinogen concentration, and (d) Platelet count, before and after challenge with *Streptococcus pneumoniae*. Malnourished mice replete with a balanced conventional diet (BCD) or BCD with supplemental *Lactobacillus rhamnosus* CRL1505 by nasal (BCD+LrN) or oral routes (BCD+LrO). Malnourished control (MNC) and well-nourished control (WNC) mice were used. Results are expressed as mean ± SD. *Different from MNC mice, no differences with the WNC group ($P<0.05$). **Different from the MNC and WNC groups ($P<0.05$).

List of abbreviations

APTT, activated partial thromboplastin time

BAL, bronchoalveolar lavage

BCD, balanced conventional diet

BCD group, malnourished mice replete with a balanced conventional diet

BCD+LrN, malnourished mice replete with a balanced conventional diet with nasal treatment with *L. rhamnosus* CRL1505

BCD+LrO, malnourished mice replete with a balanced conventional diet with oral treatment with *L. rhamnosus* CRL1505

cfu, colony-forming unit

d, days

IFN, Interferon gamma

IL, interleukin

LAB, lactic acid bacteria

LDH, lactate dehydrogenase

MNC, malnourished control mice

MPO, myeloperoxidase

PBS, phosphate buffer saline

PFD, protein free diet

pi, post-infection

PT, prothrombin time

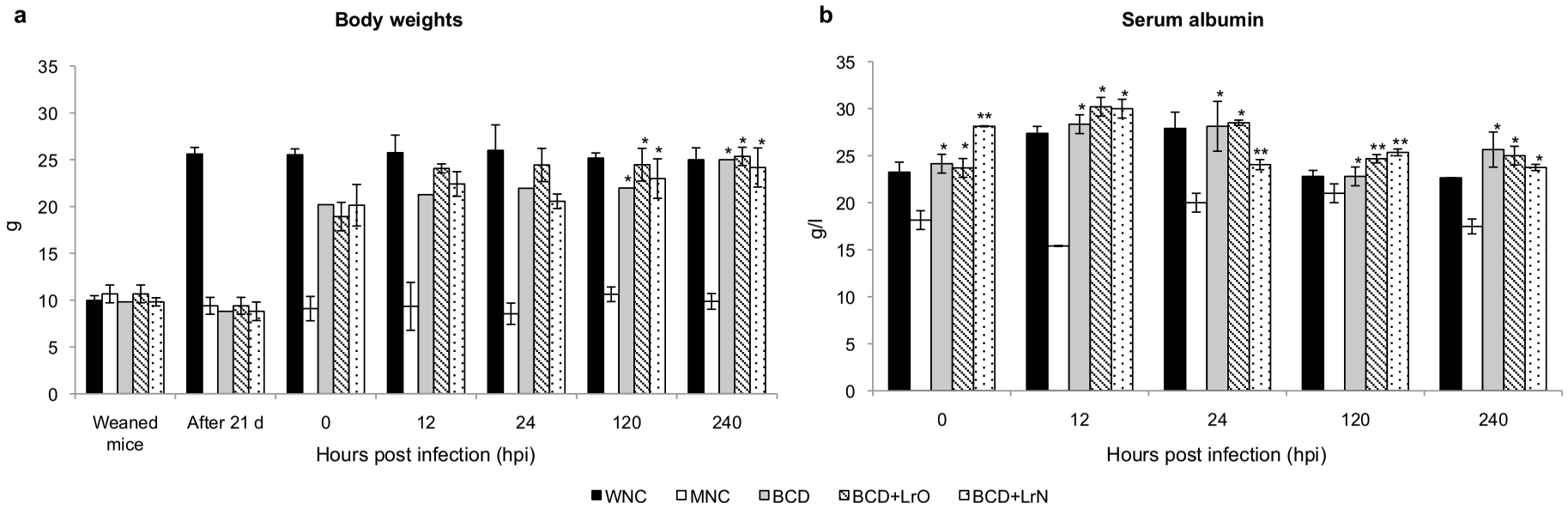
TF, tissue factor

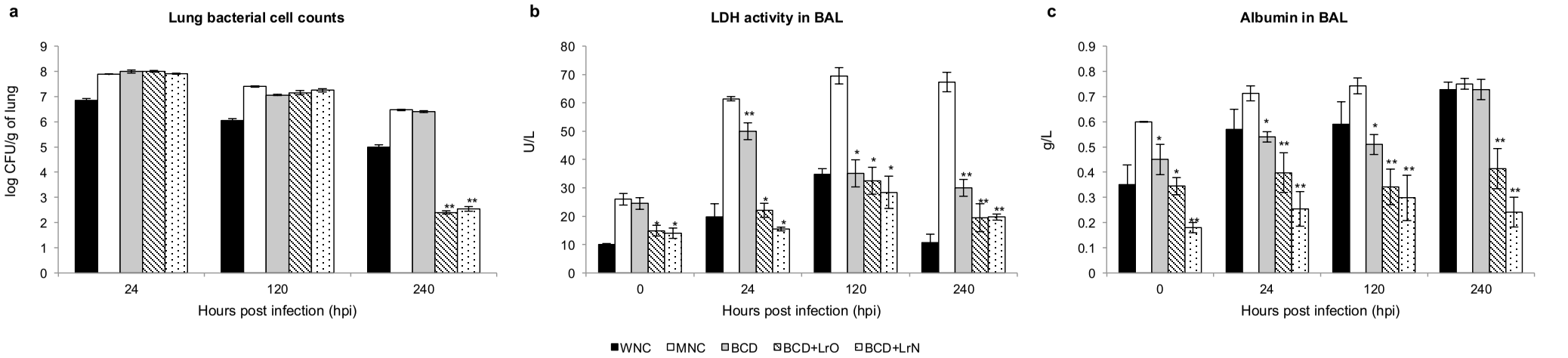
WNC, well-nourished control mice

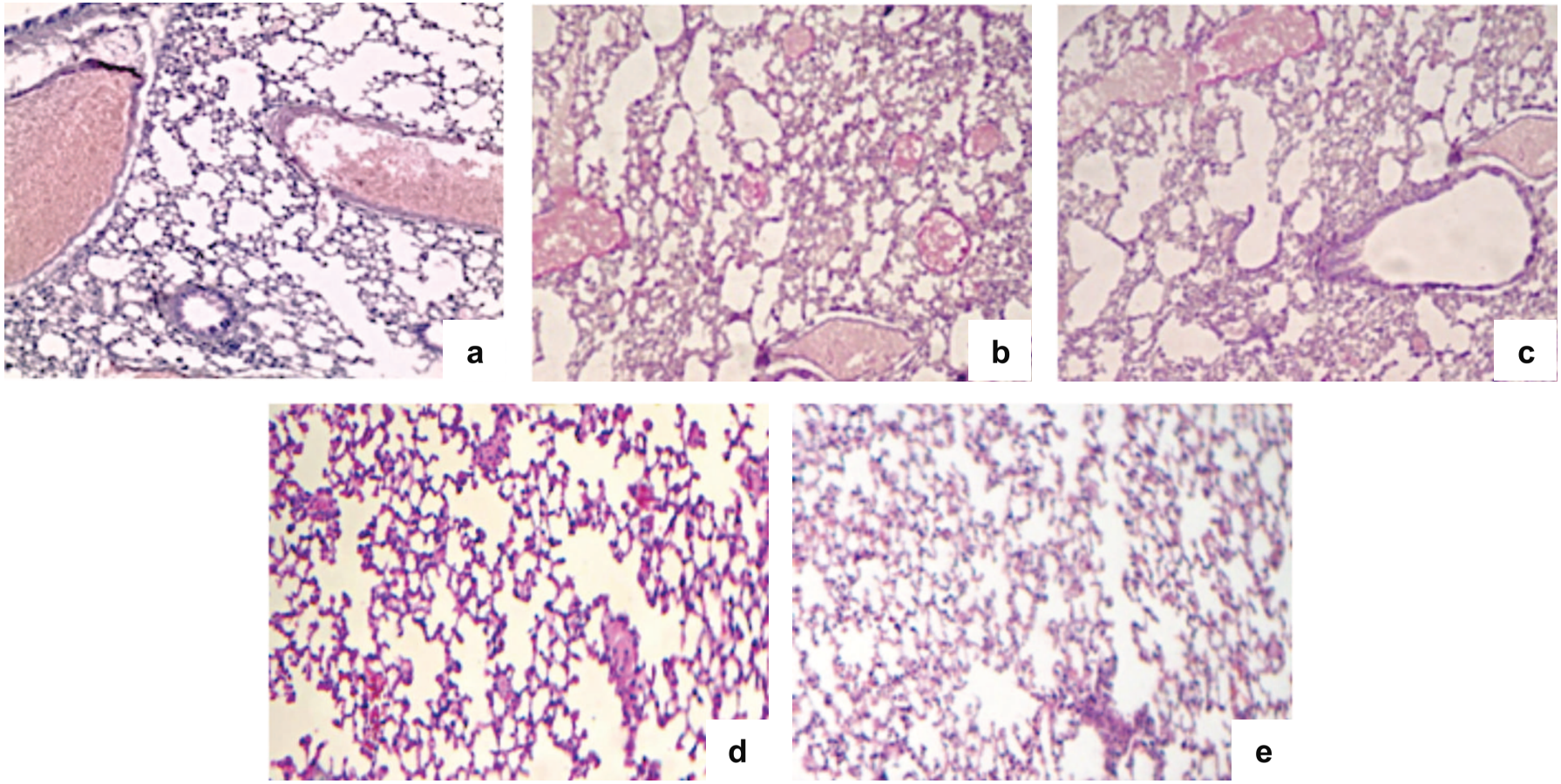
Table 1
Composition of the balanced conventional and protein-free diets*

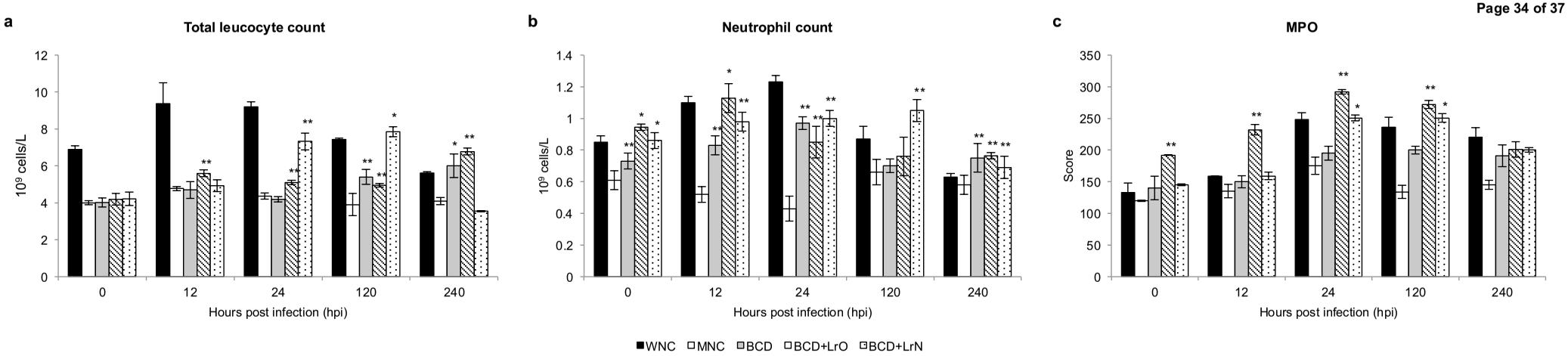
INGREDIENT	BALANCED CONVENCIONAL DIET	PROTEIN-FREE DIET
	g/Kg of mixture	
Water	120	120
Protein	230	<10
Carbohydrate	538	758†
Lipids	50	50
Vitamin Mix‡	22	22
Mineral mix§	40	40

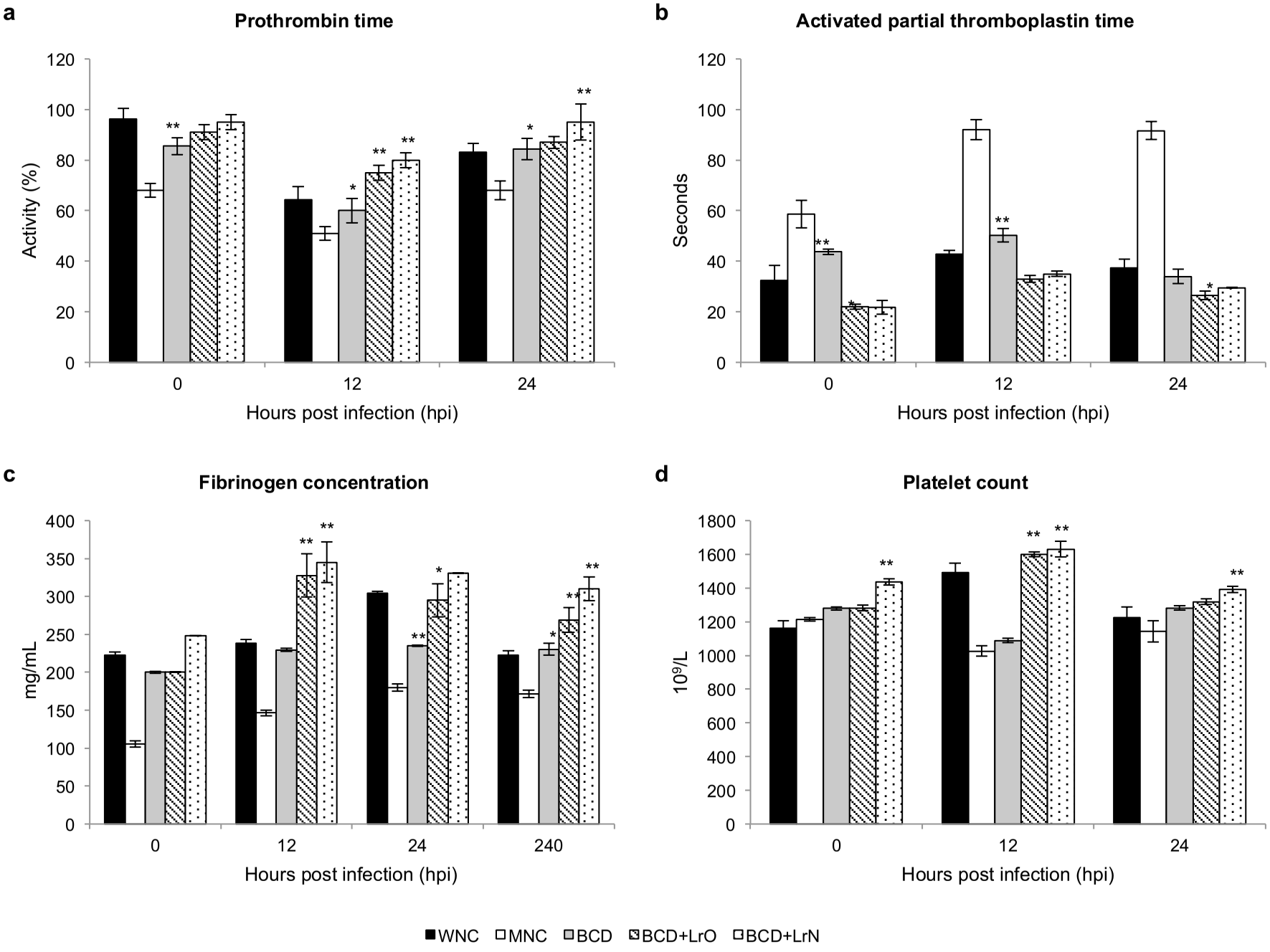
***The approximate energy value provided by the bacterial supplement is 69.13 mJ/(mouse. d).**
†Protein-free corn flour.
‡Vitamin Mix (# 905454, ICN Biomedicals Argentina, Bs. As., Argentina) g/Kg of mixture: *dl*-α-tocopherol, 5.0; p-aminobenzoic acid, 5.0; ascorbic acid, 45.0; biotin, 0.02; retinyl acetate, 4.5; vitamin B-12, 0.00135; calcium pantothenate, 3.0; choline chloride, 75.0; cholecalciferol, 0.25; folic acid, 0.09; inositol, 5.0; menadione, 2.25; niacin, 4.5; pyridoxine hydrochloride, 1.0; riboflavin, 1.0 thiamine hydrochloride, 1.0; and sucrose, finely powdered, 847.38865.
§Mineral mix (# 902844 ICN Biomedicals Argentina, Bs. As., Argentina) g/Kg of mixture: sodium chloride, 167; potassium phosphate dibasic, 322; calcium carbonate, 300; magnesium sulfate, 102; calcium phosphate monobasic, 75; ferric citrate, 27.5; MnSO4.H2O, 5.1; potassium iodide, 0.8; CuSO4.5H2O, 0.3; zinc chloride, 0.25; and CoCl2.6H2O, 0.05.


a
Body weights
g
35
30
25
20
15
10
5
0
Weaned mice
After 21 d
0
12
24
120
240
Hours post infection (hpi)
b
Serum albumin
g/l
35
30
25
20
15
10
5
0
0
12
24
120
240
Hours post infection (hpi)
WNC
MNC
BCD
BCD+LrO
BCD+LrN


a
Lung bacterial cell counts
log CFU/g of lung
Hours post infection (hpi)
b
LDH activity in BAL
U/L
Hours post infection (hpi)
c
Albumin in BAL
g/L
Hours post infection (hpi)
WNC
MNC
BCD
BCD+LrO
BCD+LrN


a
b
c
d
e


a
Total leucocyte count
10^9 cells/L
Hours post infection (hpi)
b
Neutrophil count
10^9 cells/L
Hours post infection (hpi)
c
MPO
Score
Hours post infection (hpi)
WNC
MNC
BCD
BCD+LrO
BCD+LrN


a
Prothrombin time
Activity (%)
Hours post infection (hpi)
b
Activated partial thromboplastin time
Seconds
Hours post infection (hpi)
c
Fibrinogen concentration
mg/mL
Hours post infection (hpi)
d
Platelet count
10⁹/L
Hours post infection (hpi)
WNC
MNC
BCD
BCD+LrO
BCD+LrN

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