# *Lactobacillus casei* beneficially modulates immuno-coagulative response in an endotoxemia model

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**The current study aims at evaluating the effect of the oral administration of *Lactobacillus casei* CERELA (CRL) 431 on parameters implicated in inflammation–coagulation interaction using a model of acute inflammation induced by lipopolysaccharide (LPS) in mice. Six-week-old Balb/c mice were treated with *L. casei* for 5 consecutive days. Then treated and untreated mice received an LPS injection (*L. casei* + LPS and LPS groups, respectively). Liver and kidney were removed, blood samples were obtained, and hemostatic and inflammatory parameters were evaluated at different times post LPS injection. Preventive *L. casei* administration induced a significant decrease in proinflammatory TNF-α and IL-6 cytokines by decreasing tissue factor expression in liver and kidney. Moreover, the lower expression of tissue factor in the *L. casei* + LPS group led to a lower activation of the coagulation system, which was observed by the fast systemic restoration of factors VII and V coagulation factors and antithrombin levels. This study highlights the capacity of *L. casei* to modulate the hemostatic unbalance in an acute endotoxemia model. Our findings showed the ability of *L. casei* CRL 431 to regulate the immuno-coagulative response. This fact could be helpful to propose new adjunctive strategies addressed to the restoration of physiological anticoagulant mechanisms in sepsis patients.** *Blood Coagul Fibrinolysis* **28:000–000**


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## Introduction

The severity of sepsis increases along with the degree of coagulation disorder and a fulminant coagulation abnormality is recognized as disseminated intravascular coagulation (DIC). The management of sepsis is not always easy in actual clinical situations. As a result, the mortality rate in sepsis remains high. The activation of coagulation is an almost universal event during sepsis and the upregulation of tissue factor (TF) expressions on innate immune cells, including monocytes and macrophages, have been thought to be the major initiators of the coagulation cascade [1]. TF is not normally expressed on monocytes, but protein synthesis and its surface expression can be induced on these cells by various stimuli, including lipopolysaccharide (LPS) and inflammatory cytokines such as IL-1 and TNF-α [2–4]. Therefore, both inflammation and coagulation play key roles in the host defense against pathogens.

The inflammatory response triggered by infection activates the hemostatic systems and their products to perpetuate and strengthen inflammatory reactions, indicating that both systems are tightly connected. The coagulation process as part of the innate immune response leads to local restriction and trapping of the infectious agent and protects the rest of the organism [5]. The relationship between inflammation and the coagulation system is a process in which inflammation leads to the activation of coagulation, whereas coagulation in turn considerably affects inflammatory activity. In addition, an insufficiently controlled response can lead to a situation in which coagulation and thrombosis contribute to the disease [6]. This cycle accelerates both the inflammation and coagulation systems and progresses until the death of the host. If the infection can be controlled, this hemostatic imbalance spontaneously diminishes. However, if the insult is strong and sustained, the hemostatic sequence results in multiple-organ failure and death. In spite of the development of new antibiotics, in ICUs, there is still a high rate of mortality associated with these clinical presentations because of failures in current anti-inflammatory therapies [7].

To counteract the hemostatic reaction, the treatments of choice are natural and artificial coagulation inhibitors such as heparins, antithrombin (AT), activated protein C (APC), thrombomodulin (TM), and protease inhibitors. Different studies have revealed that some anticoagulants also have important functions in the modulation of inflammation and coagulation in different animal models. Bernard *et al.* [8] reported promising results obtained in

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clinical trials during phases 2 and 3. However, patients treated with APC still have a high bleeding risk. Therefore, it is important to keep looking for new therapeutic options that can control the mediators responsible for the septic process.

Several studies have demonstrated that certain probiotic lactic acid bacteria strains can exert a beneficial effect on the host through their immunomodulatory activity. Probiotics have several immunomodulatory effects [9,10] and anti-inflammatory properties [11,12]. They stimulate the local and systemic immune response either through direct contact with intestinal epithelial and immune cells, or through their ability to modify the composition and activity of gut microbiota. Probiotics exert their protective effects by multiple immune and nonimmune mechanisms [13]. We previously demonstrated that oral administration of *Lactobacillus casei* CERELA (CRL) 431 was effective in regulating coagulation activation and fibrinolysis inhibition caused by an inflammatory process in a respiratory infection murine model; in this case, *L. casei* administration led to a decrease in fibrin deposits in lungs and tissue damage reduction [14]. Furthermore, oral repletion of malnourished mice with supplemental *L. casei* CRL 431 was able to beneficially modulate the inflammation–coagulation relationship during the pneumococcal infection [15]. Recent studies have clearly shown that an immunomodulatory probiotic nasally administered was capable of effectively regulating inflammation and hemostatic alterations during infection by improving TNF-α, INF-γ, and IL-10 production, decreasing TF expression and increasing TM in the lung [16]. Considering that the interaction between inflammation and coagulation is important in the pathogenesis of an unbalanced hemostatic system which could appreciably contribute to disease severity, the current study aimed at evaluating the effect of the oral administration of *L. casei* CRL 431 on the parameters implicated in inflammation–coagulation interaction in a model of acute inflammation induced by LPS.

## Methods

### Animals

Six-week-old Balb/c mice were obtained from a closed colony kept at CERELA. They were housed in plastic cages at room temperature (RT). Each assay was performed in groups consisting of 25–30 mice (five to six mice per each time evaluated). The Ethical Committee for Animal Care at CERELA and Universidad Nacional de Tucumán approved the experiments.

### Microorganism and culture conditions

*L. casei* CRL 431, obtained from the CERELA culture collection, was cultured for 18 h at 37 °C (final log phase) in Man–Rogosa–Sharpe broth (Oxoid), then harvested, and washed twice with sterile 0.01 mol/l PBS, pH 7.2 [17]. The bacterial suspension was adjusted to the desired concentration ($10^9$ cells/day/mouse).

### Acute inflammation model and feeding procedure

The endotoxemia model was induced by intraperitoneal injection of lipopolysaccharide (*Escherichia coli* serotype O111:B4; Sigma Chemical Co., St. Louis, Missouri, USA). To determine the optimal dosage of LPS for our acute inflammation model, we first evaluated different doses of intraperitoneally injected LPS diluted in sterile saline solution (NacL 0,9%): 2.5, 5.0, and 7.5 mg/kg mouse body weight. The selected dosage used in our model was 5 mg/kg mouse body weight, as we observed intermediate values of bacterial translocation with altered hemostatic and innate immune response parameters (data not shown). The mice that received the LPS injection without previous treatment with *L. casei* corresponded to the LPS group.

To evaluate the effect of *L. casei* in this model, the probiotic strain was suspended in 5 ml of sterile 10% nonfat milk and added to the drinking water (20% v/v). All mice were fed a conventional balanced diet *ad libitum*. *L. casei* was administered at a dose of $10^9$ cells/mouse/day for 5 consecutive days. At the end of the *L. casei* administration (*L. casei* group), time 0 h before LPS injection, all parameters were evaluated. No changes were found in comparison with untreated mice (C). The mice fed with *L. casei* that received the LPS injection corresponded to the *L. casei* + LPS group.

Samples were obtained at 0 h (before injection) and at different time periods after LPS injection.

Taking into account that the *L. casei* group did not undergo any changes in any of the parameters evaluated along the time periods compared with the C group (untreated mice) (Supplemental table, http://links.lww.com/BCF/A40), in this study, we show the results obtained for the LPS and *L. casei* + LPS groups.

### Hemostatic tests

Blood samples were obtained through cardiac puncture and collected in a 3.2% solution of trisodium citrate at a ratio of 9 : 1. Plasma was obtained according to Agüero *et al.* [18]. Factors VII and V were performed manually on fresh plasma samples. The factors were determined by a one-step method (Thromborel S; Behningwerke AG, Marburg, Germany).

AT activity was measured by chromogenic substrate assay (COAMATIC Antithrombin, Chromogenix; Instrumentation Laboratory SpA, Milano, Italy). AT levels were measured in plasma samples. Results were expressed as percentages (%) of AT.

### Tissue factor expression

TF expression was measured in liver and kidney homogenates by flow cytometry. Livers and kidneys were removed, washed with sterile saline solution, and homogenized in sterile 0.01 mol/l PBS. Homogenates were centrifuged at 5000 rpm for 10 min and then washed three

times with PBS. Subsequently, the cell suspensions obtained were incubated with antimouse TF mAb (Fc block) for 15 min at 4 °C (Santa Cruz Biotechnology, Inc.). Then, cells were incubated with the secondary antibody (donkey antirabbit IgG-FITC Santa Cruz Biotechnology, Inc., California, USA) for 15 min at RT and washed with PBS. The suspension was then diluted with 200 µl of PBS and analyzed using a Partec Pas flow cytometer; Partec GmbH, Münster, Germany. For each sample, data from 50 000 events were recorded in list mode and registered on linear scales. Analyses were performed using FloMax software; Partec GmbH, Münster, Germany. The results were expressed as percentages of TF expression.

### Cytokine determination

IL-6 concentration in plasma was measured with a commercially available ELISA technique kit following the manufacturer's recommendations (Thermo Scientific Inc., Waltham, Massachusetts, USA). Assay sensitivity was 4 pg/ml.

### TNF-α quantitative expression analysis by real-time PCR

Quantitative real-time PCR was carried out to characterize the expression of TNF-α mRNAs in lung and kidney. Total RNA was isolated from each sample using isolation kit High Pure RNA isolation kit (Roche Applied Science, Penzberg, Germany). One-step quantitative real-time PCR was performed with LightCycler RNA Master SYBR Green I (Roche Applied Science) according to the manufacturer's protocols. Reaction conditions were reverse transcription at 60 °C for 20 min, PCR performed in 45 cycles with 10 s at 95 °C, 15 s at 60 °C, and 8 s at 72 °C, and fluorescence detection performed by melting point curve analysis. The primer sequences were as follows: mouse TNF-α, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′; 5′-TGGGAGTAGACAAGGTACAACCC-3′; β-actin, 5′-AGAGGGAAATCGTGCGTGAC-3′; and 5′-CAATAGTGATGACCTGGCCGT-3. Expression of β-actin was used as a reference gene to normalize cDNA levels for differences in total cDNA levels in the samples.

### Statistical analysis

Experiments were performed in triplicate (five to six animals each time) and results were expressed as means ± SD. After verification of a 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 $P$ less than 0.05.

## Results

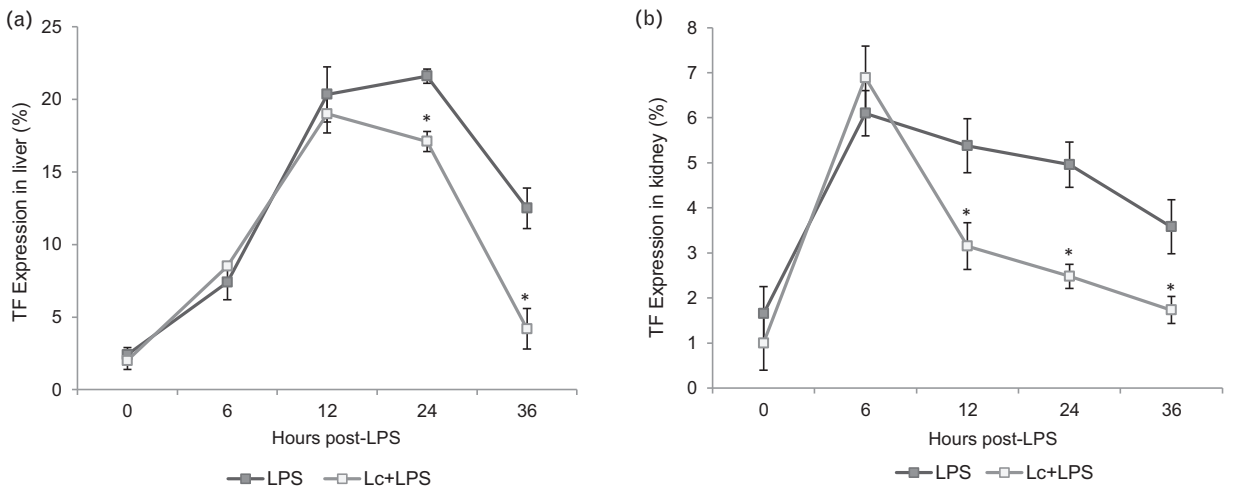
### Tissue factor in liver and kidney

To investigate the coagulation activation in the acute inflammation model, we examined TF expression in liver and kidney homogenates. LPS increased TF expression since 6-h postinjection in both LPS and *L. casei* + LPS groups. Highest values were observed between hours 12 and 24 in liver (Fig. 1a) and at hour 6 in kidney (Fig. 1b). However, TF expression levels were significantly lower in animals treated with *L. casei* since hour 24 in liver and since hour 12 in kidney, respectively.

### Coagulation factors evaluation

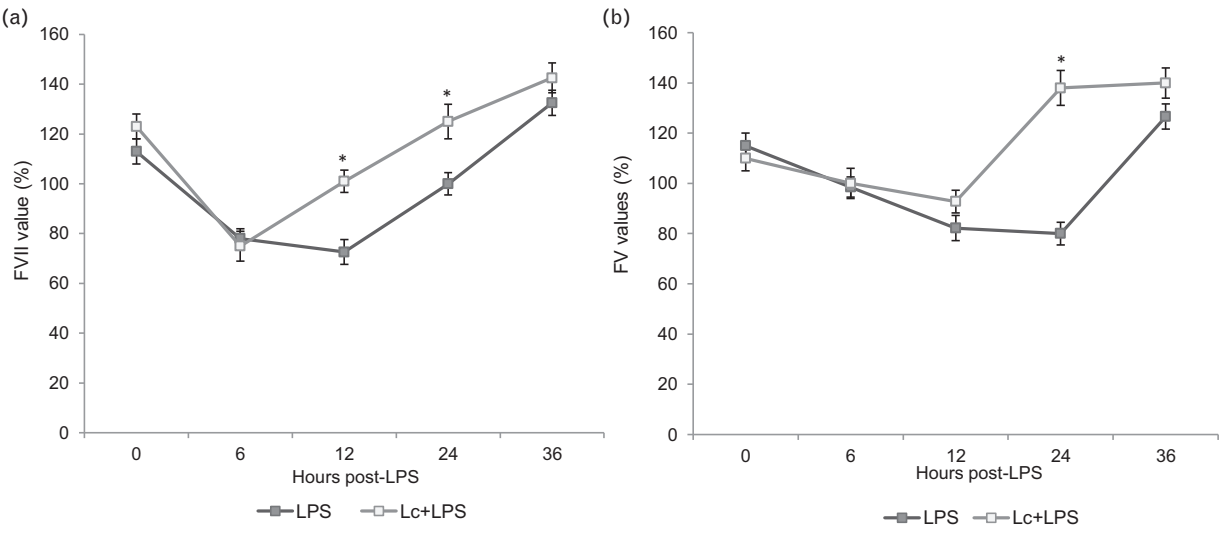
LPS caused a decrease in factor VII concentration in both groups at 6-h postinjection. Afterward, the animals

**Fig. 1**




Tissue factor expression by flow cytometry. Mice in the lipopolysaccharide group were injected with lipopolysaccharide (5-mg/kg mouse body weight) without previous treatment. In the *Lactobacillus casei* + lipopolysaccharide group, *L. casei* was orally administered at a dose of $10^9$ cells for 5 days and then mice received lipopolysaccharide injection: (a) tissue factor expression in liver and (b) in kidney was evaluated. Results are expressed as means ± SD ($n = 5$ or 6). *Significantly different from the lipopolysaccharide group ($P < 0.05$).

**Fig. 2**

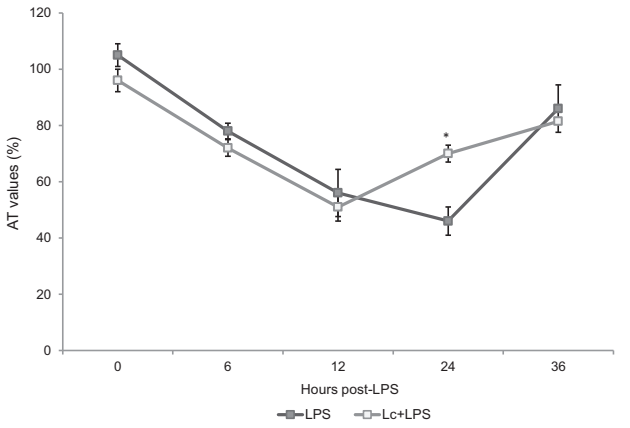



Coagulation factors evaluation. Mice in the lipopolysaccharide group were injected with lipopolysaccharide (5-mg/kg mouse body weight) without previous treatment. In the *Lactobacillus casei* + lipopolysaccharide group, *L. casei* was orally administered at a dose of $10^9$ cells for 5 days and then mice received lipopolysaccharide injection. (a) Factor VII and (b) factor V activities were studied. Results are expressed as means ± SD ($n = 5$ or 6). *Significantly different from the lipopolysaccharide group ($P < 0.05$).

supplemented with *L. casei* showed higher concentrations of factor VII ($P < 0.05$) than LPS mice until the end of the experiment (Fig. 2a). The *L. casei* + LPS group returned to normal values at 24-h postinjection, whereas the LPS group returned at 36-h postinjection.

Factor V concentrations decreased in both groups after the challenge, reaching minimum levels at 12-h post-LPS injection. Only in mice treated with *L. casei* were factor V values normalized quickly since 24-h post-LPS (Fig. 2b).

**Fig. 3**




Antithrombin levels in plasma. Mice in the lipopolysaccharide group were injected with lipopolysaccharide (5-mg/kg mouse body weight) without previous treatment. In the *Lactobacillus casei* + lipopolysaccharide group, *L. casei* was orally administered at a dose of $10^9$ cells for 5 days and then mice received lipopolysaccharide injection. Antithrombin levels were measured in plasma samples. Results are expressed as means ± SD ($n = 5$ or 6). *Significantly different from the lipopolysaccharide group at the same time point ($P < 0.05$).

### Antithrombin evaluation

AT is an important physiological coagulation inhibitor. We studied AT levels in plasma to evaluate anticoagulant activity during the inflammatory process. After LPS administration, AT gradually decreased in both groups, reaching minimum values at hour 12 for *L. casei* + LPS mice and at hour 24 for the LPS group, respectively (Fig. 3). However, the *L. casei* + LPS group had significantly higher values than the control group at 24-h post-LPS.

### TNF-α in liver and kidney by real-time PCR

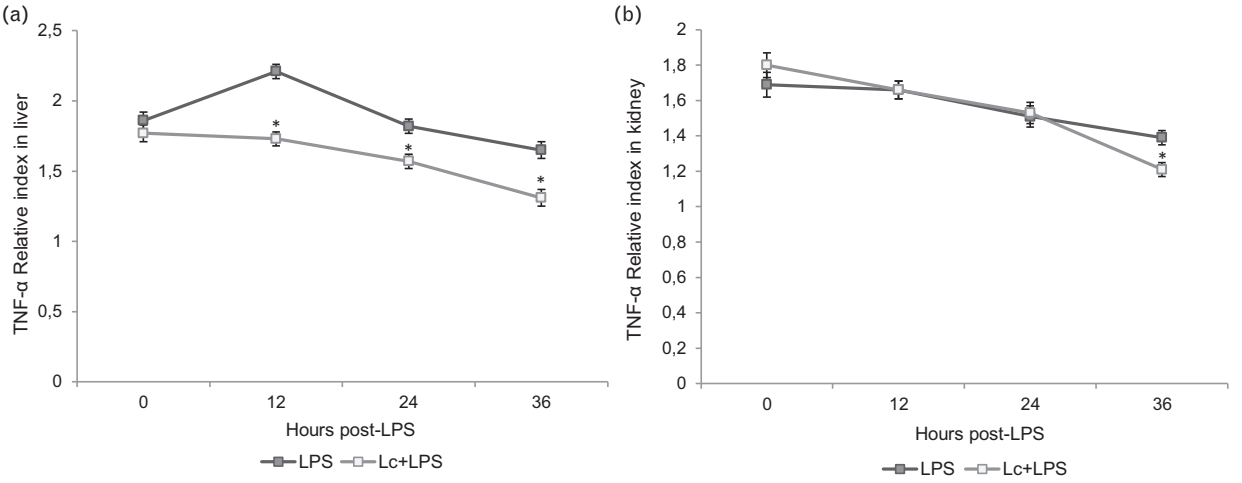
We evaluated the expression of TNF-α in liver and kidney. In liver, a 1.2-fold increase in TNF-α expression compared with basal levels was observed in the LPS group at hour 12. After that, this parameter was normalized. *L. casei* treatment significantly modified TNF-α expression, which showed significantly reduced levels compared with the LPS group since12-h postchallenge (Fig. 4a).

In kidney, TNF-α values dropped in both groups, decreasing gradually until hour 36 post-LPS. However, the *L. casei* + LPS group showed significantly lower values than the LPS mice on 36-h post-LPS (Fig. 4b).

### Systemic IL-6 evaluation

The serum levels of IL-6 increased after LPS, reaching the maximum values at 12-h postinjection. However, mice supplemented with *L. casei* showed lower levels than the LPS group throughout the studied period. Treatment with

**Fig. 4**



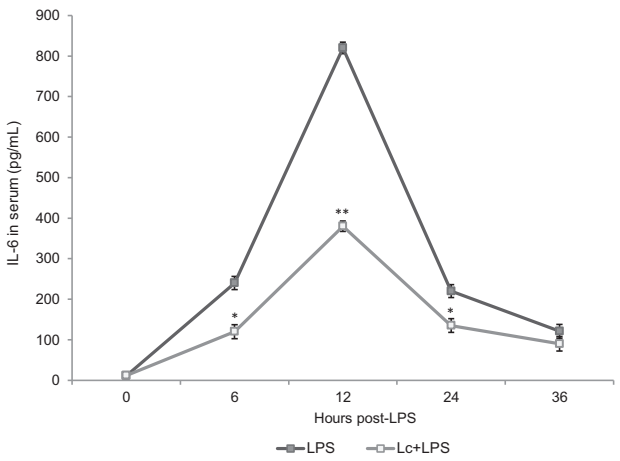

TNF-α in liver and kidney by real-time PCR. Mice in the lipopolysaccharide group were injected with lipopolysaccharide (5-mg/kg mouse body weight) without previous treatment. In *Lactobacillus casei* + lipopolysaccharide group, *L. casei* was orally administered at a dose of $10^9$ cells for 5 days and then mice received lipopolysaccharide injection. (a) TNF-α expression in liver and (b) in kidney was evaluated. Results are expressed as means ± SD ($n = 5$ or 6). *Significantly different from the lipopolysaccharide group ($P < 0.05$).

*L. casei* induced IL-6 values significantly lower than those in the LPS group between 6 and 24-h post-LPS. After that, both groups showed similar values (Fig. 5).

## Discussion

The close relationship between inflammation and coagulation is relevant in the pathogenesis of sepsis and in the subsequent multiple-organ failure associated with infection and systemic inflammatory response. In spite of recent progress in the comprehension of physiopathological mechanisms of sepsis and antimicrobial therapies, the rate of mortality associated with this disease remains high. The mortality rate for sepsis-associated DIC is 38.4%, which is comparable with septic shock mortality rates [19].

**Fig. 5**




Systemic IL-6 evaluation. IL-6 level in serum was evaluated. Mice in the lipopolysaccharide group were injected with lipopolysaccharide (5-mg/kg mouse body weight) without previous treatment. In the *Lactobacillus casei* + lipopolysaccharide group, *L. casei* was orally administered at a dose of $10^9$ cells for 5 days and then mice received lipopolysaccharide injection. IL-6 in serum was studied. Results are expressed as means ± SD ($n = 5$ or 6). Asterisks represent significant differences from the lipopolysaccharide group at the same time point (*$P < 0.05$, **$P < 0.01$).

In a previous study, it was demonstrated that the preventive administration of *L. casei* CRL 431 was effective in regulating coagulation activation and fibrinolysis inhibition, which led to a decrease in fibrin deposits in lung during pneumococcal infection in a mice model. This protective effect of *L. casei* would be mediated by the induction of higher levels of anti-inflammatory interleukins such as IL-4 and IL-10. These interleukins contribute to regulate the proinflammatory, procoagulant, and antifibrinolytic effects of TNF-α, IL-1β, and IL-6 [14]. In the acute inflammation model employed in this study, we demonstrated that the preventive oral administration of *L. casei* was able to regulate hemostatic imbalance associated with a systemic inflammatory response induced by LPS. Considering that the main mechanism by which endotoxemia activates coagulation is mediated by upregulation of TF, we investigated the expression of this factor in liver and kidney as these are the most affected organs in patients with severe sepsis [20]. TF plays a central role in the initiation of inflammation-induced coagulation [21]. *L. casei* administration induced lower TF expression than the LPS group, indicating lower coagulation activation in the mice given the *Lactobacillus*. This fact was also reflected in the fast systemic

normalization of factors VII and V levels observed in the *L. casei* + LPS group compared with the LPS mice. In this sense, we previously observed lower coagulation activation in a pneumococcal respiratory infection model in which the levels of TAT complexes were lower in animals supplemented with *L. casei* than in the control infection group [14]. A reduced activation of coagulation in virus-infected mice mediated by a decrease of TF expression was also observed in preventive treatments with other probiotic bacteria [16].

During inflammation-induced coagulation activation, the functions of the main anticoagulant pathways – the AT pathway, the PC system, and TF pathway inhibitor – can be impaired [22]. In this study, the AT pathway was evaluated. In sepsis, AT levels are markedly decreased as a result of impaired synthesis, degradation by elastase from activated neutrophils, and consumption during thrombin generation [23]. Choi *et al.* [24] reported that this anticoagulant system may be damaged during a septic process because of massive consumption and downregulation by inflammatory mediators. In our model, the preventive administration of *L. casei* induced a faster recovery of AT levels favoring the normalization of homeostatic unbalance induced by LPS challenge.

Proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 are capable of activating the coagulation system and downregulating physiological anticoagulant mechanisms [25]. In our model, TNF-α expression in LPS group was significantly increased in the liver, but no significant changes were observed in the kidney. In the current study, *L. casei* CRL 431 reduced local TNF-α expression, beneficially modulating the immuno-coagulative response in liver, which is the major site of synthesis of almost all coagulation factors. In addition, activation of coagulation system and ensuing thrombin generation is dependent on an IL-6-induced expression of TF on activated mononuclear cells and endothelial cells. In kidney, regulatory effect of this probiotic strain was minor than liver as the reduction of TNF-α expression was not observed until hour 36.

Oral administration of *L. casei* was able to decrease systemic IL-6 levels which contributed to regulate the procoagulant and proinflammatory process associated with LPS challenge. IL-6 is an important inflammatory biomarker with diagnostic and prognostic value in sepsis. This maker is used in the prediction of mortally in patients with severe sepsis [26]. In previous studies, we reported that preventive administration of *L. casei* in pneumococcal infection model induced high levels of IL-10 [14]. Considering these data, we can hypothesize that these regulatory cytokines could modulate the IL-6 and TNF-α induction, reducing tissue damage with beneficial clinical effect. IL-10 would contribute to the regulation of the procoagulant effect of proinflammatory mediators induced by endotoxemia.

Overall, *L. casei* was able to beneficially modulate the proinflammatory TNF-α and IL-6 cytokines causing a downregulation of TF expression in liver and kidney. This reduction of TF expression induced a minor activation of the coagulation system observed by the fast systemic normalization of factors VII and V, and AT levels.

## Conclusion

The current study highlights the underlying mechanisms of the interaction between inflammation and coagulation modulated by *L. casei*, which favors the rapid recovery of the hemostatic balance, in an acute endotoxemia model. Our findings showed the capacity of *L. casei* CRL 431 to regulate the immuno-coagulative response. These results could be helpful to propose new adjunctive strategies addressed to restore the physiological anticoagulant mechanisms in patients with sepsis.

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### Conflicts of interest

There are no conflicts of interest.

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