Evaluation of the preventative effects exerted by *Lactobacillus fermentum* in an experimental model of septic shock induced in mice

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The preventative effects of the probiotic *Lactobacillus fermentum* CECT5716 were evaluated in the lipopolysaccharide (LPS) model of septic shock in mice. The probiotic was administered suspended in drinking water at the final concentration of $10^8$ colony-forming units/ml for 2 weeks before the induction of an endotoxic shock by an intraperitoneal injection of LPS (400 µg/200 µl per mouse). Blood and different organs were collected after 24 h to evaluate the severity of the endotoxic shock and the preventative effects of the probiotic. *L. fermentum* reduced TNF-α levels in blood, which promotes the major alterations observed during septic shock, as well as the infiltration of activated neutrophils into the lungs. Furthermore, free radical overproduction and oxidative stress were associated with a significant decrease in hepatic glutathione levels in septic mice, and with an excessive NO production attributed to the induction of the inducible isoform of NO synthase (iNOS). In fact, hepatic glutathione levels were significantly increased in the group of mice receiving the probiotic, and the increased iNOS expression both in the colon and lungs was down-regulated in those mice treated with *L. fermentum*. Finally, pre-treatment with *L. fermentum* may also exert its protective action modulating the expression of different cytokines in splenocyte-derived T cells such us IL-2, IL-5, IL-6 or IL-10. In conclusion, pre-treatment with *L. fermentum* may exert its protective action against LPS-induced organ damage in mice by a combination of several actions including its antioxidant properties and by reduction of the synthesis of the pro-inflammatory TNF-α and IL-6.

*Lactobacillus fermentum*: Lipopolysaccharide septic shock: Mice: Cytokines

The gastrointestinal tract plays a key role in the control of the immune response, in addition to its primary function in the digestion and absorption of nutrients. First, it acts as a barrier against antigens from micro-organisms and food and, second, contains the gut-associated lymphoid tissues, which are the largest collection of lymphoid tissues in the body. The gut-associated lymphoid tissues are responsible for the main production and release of polymeric IgA in the body in order to defend mucosal surfaces from environmental microbes¹. The establishment of normal bacterial populations is very important to prevent overgrowth of potential pathogens as well as to contribute to the adequate maturation of the immune system, since misbalances in the microbiota composition have been consistently associated with several pathologies². In consequence, the administration of probiotics, prebiotics or their association (symbiotics) has emerged as an interesting approach to modulate intestinal microbiota.

Probiotics are defined as living micro-organisms that upon ingestion in adequate amounts confer a health benefit to the host beyond inherent general nutrition³. Although probiotic pharmaceutical formulations are nowadays a common way to administer probiotics, it would be better to incorporate them into the diet through dairy products or other fermented foods. Classically, it has been considered that the beneficial effects exerted by probiotics are achieved due to their ability to colonise the human gastrointestinal tract, thus inducing changes in the composition of the normal intestinal microflora and influencing the immune system. It is evident that most of the beneficial effects reported for probiotics are related to intestinal conditions, such as lactose (or food) intolerance, acute diarrhoea or inflammatory bowel disease⁴,⁵. However, the use of probiotics against systemic immune conditions has also been reported. In fact, Olivares et al. have reported that dietary deprivation of fermented foods could induce a decrease in the innate immune response, which can be effectively counteracted by the ingestion of probiotics⁶. Moreover, it has been speculated, both in humans and in experimental models, that inflammation associated with rheumatoid arthritis may be modulated by the use of probiotics⁷,⁸. Furthermore, the effects of probiotics have been studied in the prevention and treatment of atopic disease⁹,¹⁰. The probiotic administration using a preventative protocol showed a higher efficacy than in a therapeutic approach, most probably due to probiotics exerting their beneficial effects by improving mucosal

Abbreviations: Con A, concanavalin A; iNOS, inducible NO synthase; LPS, lipopolysaccharide; MPO, myeloperoxidase.

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barrier function and enhancing both specific and non-specific immune responses\(^{(13,14)}\). However, even though the results obtained after probiotic treatment in both human subjects and experimental animals are promising, it is clear that all probiotics are not equally beneficial, each bacteria may have particular mechanisms of action, and host characteristics may also determine which probiotic species and even strains may be optimal. Supporting this, several studies have shown that not all probiotics exert the same intestinal anti-inflammatory effects in experimental models of intestinal inflammation\(^{(15,16)}\). For this reason, it is necessary to test each new probiotic strain to better characterise its safety profile and potential use in the treatment of altered immune conditions.

Sepsis is a generalised and exacerbated and not properly regulated inflammatory response to bacterial translocation, which frequently appears as a secondary complication to a marked depression of the cell-mediated immune response, as it may occur in patients suffering from severe trauma\(^{(17)}\).

The exact mechanisms involved in this immune dysregulation are not completely understood, although an enhanced generation of inflammatory mediators, including cytokines, chemokines, adhesion molecules, reactive oxygen and nitrogen species, from activated immune cells such as macrophages and polymorphonuclear cells, seems to play a key role\(^{(18)}\).

The release of lipopolysaccharide (LPS) from bacteria and its recognition by host cells are generally thought to be the initial events in the development of sepsis, thus triggering the inflammatory reaction that may subsequently result in multiple organ dysfunction syndrome associated with failure in different tissues, including lung, liver and brain, and frequently leading to death. Also, an increased permeability of the intestinal barrier has been reported in sepsis\(^{(19)}\). There is controversy whether the increased permeability and the subsequent translocation of the gut bacteria are the cause or a consequence of the initial inflammatory response. Anyway, the translocated bacteria could boost the exacerbated inflammatory condition. The incidence of sepsis is still increasing, with high rates of mortality (up to 70%) despite the use of life-support therapies\(^{(20)}\). Therefore, more efficient strategies are required to modulate the inflammation and also to reduce the bacterial translocation occurring in sepsis, and, for this purpose, dietary manipulation through probiotic administration would be of great interest. Considering how probiotics exert their beneficial effect as well as the severity and the rapid onset of sepsis, preventative dietary administration of probiotic seems to be the most adequate protocol to perform these studies.

The aim of the present study was to evaluate the preventative effects of the probiotic *Lactobacillus fermentum* CECT5716 in a model of septic shock in mice induced by LPS, since, in previous studies, this probiotic has been shown to be able to modulate some of the potential mechanisms of sepsis. In this regard, immunomodulatory properties have been described for this probiotic, since it is able to enhance the Th1 response\(^{(21)}\) and increase the IgA concentration in faeces helping the blockage and elimination of translocated bacteria\(^{(22)}\). Furthermore, this strain has been described as displaying anti-infective properties against different pathogenic micro-organisms through different mechanisms, including inhibition of the adhesion to epithelial cells, secretion of anti-microbial compounds and improvement of the intestinal barrier function by inducing the expression of intestinal mucus\(^{(23)}\). Furthermore, several strains of this probiotic have been reported to display antioxidant properties that could account for their beneficial effects since oxidative stress is associated with inflammatory conditions, including sepsis or colonic inflammation\(^{(24–26)}\). All these data make *L. fermentum* CECT5716 an interesting candidate to evaluate its effects on altered immune response conditions, such as sepsis.

**Materials and methods**

**Preparation and administration of the probiotic**

*L. fermentum* CECT5716 was provided by Puleva Biotech (Granada, Spain) and was normally grown in de Man, Rogosa and Sharpe (MRS) media at 37°C in anaerobic conditions using the Anaerogen system (Oxoid, Basingstoke, Hants, UK). For probiotic treatment, bacteria were suspended in a sterile PBS solution (10⁵ colony-forming units/ml) and stored at −80°C until usage.

**Experimental design**

Male BALB/c mice weighing 20–22 g were obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain), housed in Makrolon cages and maintained in an air-conditioned atmosphere with a 12 h light–dark cycle, and provided with free access to tap water and food. All manipulations with animals were in accordance with the ‘Guide for the Care and Use of Laboratory Animals’ as promulgated by the National Institute of Health. The mice were randomly assigned to three groups (n 10); two of them (healthy and control groups) received tap water and the other (treated group) received the probiotic suspending in drinking water at the final concentration of 10⁸ colony-forming units/ml and daily prepared. Food and water intake was recorded daily for all groups. At 2 weeks after starting the experiment, endotoxic shock was induced in treated and control mice with an intraperitoneal injection of LPS (400 μg/mouse) to a final volume of 200 μl; healthy mice received sterile saline. Preliminary assays revealed that this dose of LPS did not induce the death of any mouse in the following 24 h. At 24 h after injection, mice were anaesthetised with halothane, blood samples were taken from the retro-orbital venous plexus, and then the mice were killed immediately. The following tissues were quickly removed and weighed: spleen, lungs, liver, kidneys and colon. Colonic specimens were frozen at −80°C for myeloperoxidase (MPO) activity and inducible NO synthase (iNOS) expression. Livers of mice were cryo-sectioned in 1 μm sections and stained with haematoxylin and eosin. MPO activity and iNOS expression were determined by using the commercially available kits (Chemicon) and by immunohistochemical detection with an avidin-biotin complex method. The intensity of the iNOS immunostaining was determined by grading the number of cells stained positive for iNOS using a score from 0 to 3 (0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining).

**Reagents**

All chemicals, including LPS from *Escherichia coli* serotype O55:B5, were obtained from Sigma Chemical (Madrid, Spain), unless otherwise stated.
MPO activity was measured in colonic tissue as previously described; the results were expressed as MPO units/g wet tissue; one unit of MPO activity was defined as that degrading 1 μmol HO₂/min at 25°C. Total glutathione content was quantified in liver with the recycling assay described by Anderson and the results were expressed as nmol/g wet tissue. Lung samples for TNF-α determination were immediately weighed, minced on an ice-cold plate and suspended in a tube with 10 mM-sodium phosphate buffer (pH 7.4) (1:5, w/v). The tubes were placed in a shaking water-bath (37°C) for 20 min and centrifuged at 9000 g for 30 s at 4°C; the supernatant fractions were frozen at −80°C until assay. TNF-α was quantified by ELISA (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) and the results were expressed as pg/g wet tissue. Colonic and lung specimens were also used for protein extraction to evaluate iNOS expression by Western blotting, which was performed as described elsewhere. Blood samples were centrifuged (1500 rpm for 10 min), and plasma TNF-α was quantified by ELISA (Amersham Pharmacia Biotech) and the results were expressed as pg/ml.

Cytokine production in splenocytes

In order to obtain primary lymphocyte cultures, spleens were immediately disaggregated in Dulbecco’s modified Eagle’s medium plus 1% penicillin/streptomycin after collection, centrifuged (1500 rpm; 5 min), and erythrocytes were lysed with a lysis buffer (NH₄Cl (1.7 mol/l), KHCO₃ (0.12 mol/l), ethylenediamine-tetra-acetic (9 mmol/l)) for 30 min at 4°C. Resting cells were counted using a haemocytometer and cultured to perform proliferation and stimulation assays in current culture medium (Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum). Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere.

Spleen-derived lymphocytes were cultured in six-well plates (10 x 10⁶ cells/well) in 2 ml media and stimulated with concanavalin A (Con A; 5 μg/ml). Supernatant fractions were collected after 48 h and frozen until ELISA analysis. Cytokine production was measured with commercial murine ELISA kits (Cytosets™, Biosource International, Nivelles, Belgium) following the manufacturer’s protocol. Total RNA was isolated using TRIzol® Reagent (Gibco-BRL, Carlsbad, CA, USA) following the manufacturer’s protocol. cDNA was synthesised using a First-Strand cDNA Synthesis Kit (Amersham Biosciences). The primer sequences were: IL-2 (forward 5'-CTTCAAGCTTCAAGTCTG-3', reverse 5'-CCATCTCTCAGAATGACCC-3'); IL-5 (forward 5'-TCAGGGATTAGGGTACCTG-3', reverse 5'-CTCCGCTTGTCTCTTCACAC-3'); IL-6 (forward 5'-TGATGATGCTACCAAATCGG-3', reverse 5'-AGGAGAGATGGAATGGTG-3'); IL-10 (forward 5'-TGCTGCTTTACTGACTGG, reverse 5'-TCATTTCCGATAAGCTTTG); β-actin (forward 5'-AACGTGCGTGTACCAAGG-3', reverse 5'-ATGCCAGGATTCATACC-3'). The PCR was performed as described previously but in this case the cycles used were: thirty-two cycles for IL-2, thirty-five cycles for IL-5 and IL-10, thirty cycles for IL-6 and twenty-five cycles for β-actin.

Statistics

All results are expressed as the mean values with their standard errors for ten mice per group. Data were analysed by one-way ANOVA and post hoc least-significance tests. All statistical analyses were carried out with the Statgraphics 5.0 software package (StatPoint, Inc., Herndon, VA, USA) with differences considered significant at P < 0.05.

Results

The preventative probiotic administration to mice for 2 weeks before septic shock induction did not affect body-weight gain compared with untreated groups (data not shown), and no sign of toxic effects was observed, similarly to that previously reported for rats and mice. After 2 weeks of probiotic consumption, mice received an intraperitoneal injection of saline or a sub-lethal dose of 400 μg LPS, and, 8 h after LPS administration, mice showed evident symptoms of endotoxic shock, similar to those described previously, including decreased motor activity, ruffled fur and ocular exudates. All mice survived 24 h after LPS injection, when they were killed after exsanguination. Macroscopic tissue modifications were observed as a consequence of the septic shock. Significant increases in colon weight:length ratio and in spleen and lung weights were observed in the control group when compared with healthy mice (P < 0.05; Table 1), whereas no significant modification was observed in the weights of liver and kidneys (Table 1). The probiotic treatment resulted in a significant improvement in these tissue modifications, showing similar values to those in the healthy group (Table 1).

The beneficial effects exerted by the probiotic pre-treatment in this experimental model of septic shock were also confirmed biochemically. One of the key cytokines involved in

| Lactobacillus fermentum in septic shock | Table 1. Effects of probiotic treatment on tissue weights in lipopolysaccharide-induced septic shock in mice
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<tr>
<td>(Mean values with their standard errors for ten mice per group)</td>
<td>(Mean values with their standard errors for ten mice per group)</td>
</tr>
<tr>
<td><strong>Group</strong></td>
<td><strong>Liver (mg/g body mass)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Mean</strong></td>
</tr>
<tr>
<td>Healthy</td>
<td>49.4</td>
</tr>
<tr>
<td>Control</td>
<td>51.3</td>
</tr>
<tr>
<td>Lactobacillus fermentum</td>
<td>50.4</td>
</tr>
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* Mean value was significantly different from that of the healthy group (P < 0.05).
† Mean value was significantly different from that of the control group (P < 0.05).
Sepsis is TNF-α, the systemic release of which induces increased vascular permeability and disseminated intravascular coagulation, responsible for the state of shock[35]. TNF-α levels were significantly increased 24 h after intraperitoneal administration of LPS to mice, both in the plasma and lungs, in comparison with normal mice. The administration of *L. fermentum* for 2 weeks before LPS injection resulted in a significantly lower TNF-α production when compared with untreated control mice (Fig. 1). In addition, sepsis promotes the activation and migration of leucocytes into various organs, leading to multiple organ dysfunction[37]. In the present study, free radical overproduction was associated with a significant decrease in hepatic glutathione levels in septic mice (*P*<0.01 v. healthy group; Table 2), which was significantly increased in the group of mice receiving the probiotic (*P*<0.05 v. control group; Table 2), although probiotic pre-treatment was not able to restore the values obtained in healthy animals. Moreover, an increased iNOS expression after LPS administration was evidenced by Western blot both in the colon and lungs of septic control mice when compared with the healthy group, and this was down-regulated in those mice treated with *L. fermentum* (Fig. 2).

To further evaluate the effects of the probiotic treatment on the altered immunological response after LPS administration, the expression of different Th1, Th2 and Th3 cytokines (IL-2, IL-5, IL-6 and IL-10) in Con A-stimulated splenocytes was analysed. RT-PCR and/or ELISA analysis revealed that Con A-stimulated cytokine expression was altered in the splenocytes from septic mice (Fig. 3). In this sense, a clear increased expression of IL-2, IL-5 and IL-6 was observed in the control mice in comparison with healthy ones, while a decreased expression of the regulatory Th3 cytokine IL-10 was detected. Probiotic administration reduced the expression of the pro-inflammatory cytokines and restored the expression of IL-10 to those levels observed in Con A-stimulated lymphocytes obtained from healthy mice.

**Discussion**

Sepsis is a systemic inflammatory condition commonly caused by bacterial infections, which is associated with multiple organ failure and has a poor prognosis due to the lack of an effective treatment[38]. Bacterial pathogens and their products trigger the inflammatory response by transcriptional activation of inflammatory genes, leading to an excessive and uncontrolled release of a large number of inflammatory mediators, including cytokines, chemokines, adhesion molecules, reactive oxygen and nitrogen species. Recent studies have revealed that the incidence of sepsis is still increasing[20], and, in consequence, more efficient strategies to modulate the inflammation in sepsis are needed.

Although a general multiple organ failure has been observed in humans, mainly affecting the lungs, liver, kidneys and even the brain[39], in our experimental settings only the colon, lungs and spleen were enlarged due to inflammation or over-activation of the immune response, in the latter. Furthermore, an alteration of liver function was also evidenced since a significant depletion of the glutathione levels occurred. Other organs were not affected, maybe due to the fact that we evaluated the sepsis only 24 h after induction.

Several biochemical and immunological mechanisms have been proposed to be responsible for the alterations observed during sepsis. In this sense, a critical role of TNF-α in the development of LPS-mediated shock in mice has been consistently reported, since an excess of this cytokine promotes the major alterations observed during septic shock, including vasodilatation, impaired coagulation and fibrinolysis[35]. In addition, an altered expression of other Th1 and Th2 cytokines such as interferon γ, IL-2, IL-4 or IL-5 has also been described[39–41]. IL-6, together with TNF-α, is also considered one of the most important mediators in the pathogenesis of sepsis and septic shock. It displays potent biological effects, including stimulation of B- and T-lymphocytes, and induction of the hepatic acute-phase response[42], thus promoting tissue injury, multiple organ failure and death[43,44]. Other mechanism involved in
the pathogenesis of sepsis is the excessive production of NO, most probably derived from the increased expression of iNOS (45). Furthermore, it is well reported that the generalised inflammatory response that occurs in sepsis is associated with an enhanced generation of reactive oxygen metabolites, mainly derived from neutrophil activity, that also contributes to multiple organ dysfunction (46).

As expected, LPS-administered mice showed an alteration of the cytokine profile production including increased levels of TNF-α, IL-2, IL-5 and IL-6 and a reduced expression of the regulatory cytokine IL-10. The involvement of NO and reactive oxygen metabolites was confirmed by an enhanced iNOS expression in the lungs and colon, and a glutathione depletion in the liver, respectively. Moreover, a neutrophil infiltration in the lungs was also evident, shown by augmented MPO activity.

The present study shows for the first time that feeding mice with the probiotic *L. fermentum* CECT5716 clearly prevented septic shock conditions by ameliorating the altered production of inflammatory mediators. Thus *L. fermentum* was able to down-regulate the increased levels of TNF-α both in the plasma and lungs. The ability of this probiotic to reduce TNF-α production in inflammatory conditions has been previously reported in an experimental model of rat colitis (27). The beneficial effects showed by *L. fermentum* can be derived from an improvement in the altered immune response induced by LPS, since there is a reduction in the expression of the pro-inflammatory cytokines IL-2, IL-5, IL-6 as well as an increase of IL-10 in Con A-stimulated splenocytes. Similarly to other probiotics, *L. fermentum* was more efficient at modulating IL-10 than other cytokines, including IL-2. This can be

<table>
<thead>
<tr>
<th>Group</th>
<th>Colonic MPO activity (units/g tissue)</th>
<th>Lung MPO activity (units/g tissue)</th>
<th>Hepatic GSH content (nmol/g tissue)</th>
</tr>
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<tbody>
<tr>
<td>Healthy</td>
<td>16·3±2·5</td>
<td>2·3±0·4</td>
<td>7091±570</td>
</tr>
<tr>
<td>Control</td>
<td>22·8±3·5</td>
<td>23·2±3·0</td>
<td>2703±184</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em></td>
<td>21·8±2·9</td>
<td>15·4±3·0</td>
<td>3198±137</td>
</tr>
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* Mean value was significantly different from that of the healthy group (P<0.05).
† Mean value was significantly different from that of the control group (P<0.05).
‡ One unit of MPO activity was defined as that degrading 1 μmol H₂O₂/min at 25°C.

Table 2. Effects of probiotic treatment on lung myeloperosidase (MPO) activity and hepatic glutathione (GSH) content in lipopolysaccharide-induced septic shock in mice

(Mean values with their standard errors for ten mice per group)

Fig. 2. Effect of *Lactobacillus fermentum* probiotic on lung (a) and colonic (b) inducible NO synthase (iNOS) expression in lipopolysaccharide (LPS)-induced septic shock in mice. iNOS expression was analysed by Western blot using tissue homogenates as described in Materials and methods. In each lane 150 μg protein were loaded. β-Actin expression was used as the control for loading and transfer.

![Fig. 2](https://example.com/fig2.png)

Fig. 3. (a) Effect of *Lactobacillus fermentum* probiotic on the expression of cytokines in concanavalin A (Con A)-activated T-lymphocytes from mice after lipopolysaccharide (LPS)-induced septic shock. Splenocytes were incubated with Con A during 48 h and the expressions of cytokines in cells were assessed by RT-PCR. (b) Effects of probiotic treatment on IL-2 and IL-10 secretion in Con A-stimulated splenocytes from mice with LPS-induced septic shock. (Ⅰ), Healthy mice; (Ⅱ), LPS control; (Ⅲ), LPS probiotic. Splenocytes were incubated with Con A during 48 h and the concentration of the cytokine in the supernatant fraction was analysed by ELISA. Values are means (n=10), with standard errors represented by vertical bars. * Mean value was significantly different from that of the healthy group (P<0.05). † Mean value was significantly different from that of the control group (P<0.05).

![Fig. 3](https://example.com/fig3.png)
have to be individually checked, not only for their efficacy but also for safety reasons. We should not be disappointed about the above-mentioned undesirable effects, and carry on the research into the potential clinical uses of probiotics.

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