Saccharomyces boulardii improves humoral immune response to DNA vaccines against leptospirosis

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Abstract

Purpose. Saccharomyces boulardii may improve the immune response by enhancing the production of anti-inflammatory cytokines, T-cell proliferation and dendritic cell activation. The immunomodulator effect of this probiotic has never been tested with DNA vaccines, which frequently induce low antibody titers. This study evaluated the capacity of Saccharomyces boulardii to improve the humoral and cellular immune responses using DNA vaccines coding for the leptospiral protein fragments LigAni and LigBrep. BALB/c mice were fed with rodent-specific feed containing 10^8 c.f.u. of Saccharomyces boulardii per gram.

Methodology. Animals were immunized three times intramuscularly with 100 µg of pTARGET plasmids containing the coding sequences for the above mentioned proteins. Antibody titers were measured by indirect ELISA. Expression levels of IL-4, IL-10, IL-12, IL-17, IFN-γ and TGF-β were determined by quantitative real-time PCR from RNA extracted from whole blood, after an intraperitoneal boost with 50 µg of the recombinant proteins.

Results/Key findings. Antibody titers increased significantly after the second and third application when pTARGET/ligAni and pTARGET/ligBrep were used to vaccinate the animals in comparison with the control group ($P<0.05$). In addition, there was a significant increase in the expression of the IL-10 in mice immunized with pTARGET/ligBrep and fed with Saccharomyces boulardii.

Conclusion. The results suggested that Saccharomyces boulardii has an immunomodulator effect in DNA vaccines, mainly by stimulating the humoral response, which is often limited in this kind of vaccine. Therefore, the use of Saccharomyces boulardii as immunomodulator represents a new alternative strategy for more efficient DNA vaccination.

INTRODUCTION

DNA vaccines comprise cloning a gene coding for an antigen of interest in a eukaryotic expression plasmid, which is used to vaccinate animals whose cells will then express the antigen of the pathogen against which protection is sought [1]. This type of vaccine has been evaluated in several animal models for a number of bacterial [2, 3], viral [4–7] and parasitic diseases [8, 9].

DNA vaccine stability and safety are greater than those of conventional vaccines because DNA is less prone to degradation, and there is no risk of disease infection. In addition, large-scale production and a high degree of purity are easily obtained at low cost [10]. In terms of efficiency, these vaccines can stimulate both the humoral and cellular responses for long periods [10]. However, they often induce low titres of antibodies [11], resulting in the need for multiple immunizations [10]. This last point shows that the discovery of new safe and effective

adjuvants or immunomodulators is fundamental for the development of more efficient DNA vaccines.

Immunomodulators and conventional adjuvants have the function of increasing the immune response, which can reduce the amount of antigen or number of immunizations required and, consequently, vaccination costs [12]. Probiotics are defined as live micro-organisms, which, when administered in adequate concentrations, are beneficial to host health [13]. Probiotic strains with immunostimulatory effects can increase the specific immune responses to antigens and are, therefore, promising as vaccine immunomodulators [14–18]. *Saccharomyces boulardii* is a well-known probiotic yeast but was not yet evaluated as immunomodulator for DNA vaccines. It can have an immunomodulatory effect by increasing anti-inflammatory cytokines, reducing levels of pro-inflammatory cytokines, stimulating the secretion of factors that modulate the restitution of gut cells and activating dendritic cells and the proliferation of T cells [19, 20].

Leptospirosis is a neglected infectious disease of global importance and one of the greatest public health problems in Brazil [21, 22]. The proteins of the leptospiral immuno-globulin-like (Lig) family have been studied as antigens in recombinant vaccines [22, 23]. The objective of this study was to evaluate the ability of *S. boulardii* used as a probiotic feed supplement for mice, in order to boost the humoral and cellular immune responses of DNA vaccines using as immunogens the gene sequences encoding the leptospiral LigAni and LigBrep proteins.

METHODS

Micro-organisms and cultivation conditions

S. boulardii was obtained from a commercial lyophilized product named Floratil (Merck), was recovered in yeast peptone dextrose (YPD) broth (yeast extract 1 %, peptone 2 % and D-glucose 2 %) and was incubated in an orbital shaker for 24 h (28 °C; 200 r.p.m.). Then they were added to 7 L YPD broth and incubated in a bioreactor (Bioflo 110; New Brunswick Scientific) for 24 h (28 °C; 500 r.p.m. 1 vvm) using antifoam 204 (Sigma-Aldrich). After fermentation, cells were recovered by centrifugation (15 min at $10,000 \times g$) and stored in saline solution (NaCl 0.9 %) at 4 °C. Concentration of *S. boulardii* was determined by counting on YPD agar after incubation at 28 °C for 48 h.

Escherichia coli BL21 (DE3) Star (Invitrogen) and *E. coli* TOP 10 (Invitrogen) were grown in Luria–Bertani (LB) broth (tryptone 1 %, yeast 0.5 % extract and NaCl 0.5 %) with 100 µg ml^{-1} ampicillin and incubated at 37 °C for 18 h in an orbital shaker at 200 r.p.m.

Preparation of feed containing *S. boulardii*

S. boulardii (10^8 c.f.u. g^{-1}) was added to the rodent-specific, antibiotic-free, pre-ground diet (Supra). Viable counts were determined during the experiments by plating decimal yeast dilutions on YPD agar after incubation at 28 °C for 48 h. This formulation was pelletized, dried in an oven with

forced air circulation for 24 h at 40 °C and stored at 4 °C until further use [24].

Construction of DNA vaccines

The construction of eukaryotic expression plasmids with *ligAni* gene (pTARGET/*ligAni*) and *ligBrep* (pTARGET/*ligBrep*) a the evaluation of their functionality were performed as described by Forster *et al.* [3]. The plasmids were obtained as previously described by Green and Sambrook [25]. Briefly, *E. coli* TOP 10 was transformed by heat shock with the plasmids pTARGET/*ligAni* and pTARGET/*ligBrep*, cultured in 500 ml of LB broth with 100 µg ml^{-1} ampicillin, 37 °C, 200 r.p.m. until the mid-log growth phase (OD$_{600}$, 0.6) and centrifuged at 2700 ***g*** for 15 min at 4 °C. The pellet was suspended in 200 ml of STE buffer (Tris/HCl 0.2 M, NaCl 0.5 M, SDS 0.1 %, EDTA 0.01 M, pH 7.6), followed by another centrifugation under the same conditions. The supernatants were discarded, and the pellets were frozen at −20 °C for 10 min. Thereafter, the pellets were thawed and stored for 5 min at room temperature and then suspended in 18 ml of solution I (glucose 50 mM, Tris/HCl 10 mM, RNAse 50 µg ml^{-1}, pH 7.4). To this, 40 ml of solution II (SDS 1 %, NaOH 0.2 M) was added and homogenized by inverting the tubes. Next, 20 ml of solution III (sodium acetate 3 M, pH 5.2) was added to the solution and vortexed. The tubes were centrifuged at 20 000 ***g*** for 30 min at 4 °C, and approximately 45 ml of the supernatant was recovered and transferred to another tube, to which 27 ml of isopropanol was also added as well. After homogenization by inversion and storage for 15 min at room temperature, the tubes were centrifuged at 12 000 ***g*** for 15 min at the same temperature. The supernatant was discarded, and the pellets were washed with 70 % ethanol. After drying, the pellets were suspended in 3 ml TE (Tris/HCl 10 mM, pH 8.0, EDTA 1 mM). Plasmid DNA was quantified by electrophoresis on 0.8 % agarose gel, using λ DNA/HindIII marker (Thermo Scientific) as reference, and frozen at −20 °C until further use.

Expression and purification of recombinant proteins LigAni and LigBrep of *Leptospira interrogans* serovar Copenhageni, strain L1-130 in *E. coli*

The construction of plasmids pAE/*ligAni* and pAE/*ligBrep* was performed by Forster *et al.* [3]. rLigAni and rLigBrep proteins were obtained as previously described by Green and Sambrook [25]. To this end, *E. coli* BL21 (DE3) Star was transformed by heat shock with the plasmids pAE/*ligAni* and pAE/*ligBrep*. The recombinant clones were selected on LB agar containing 100 µg ml^{-1} ampicillin and cultured in 500 ml LB broth at 37 °C under shaking (250 r.p.m.) with ampicillin until the mid-log growth phase (OD$_{600}$, 0.6–0.8). Protein expression was induced with IPTG 0.5 mM at 37 °C under agitation (200 r.p.m.) for 3 h and confirmed by Western blotting using anti-6xHis-tag antibody (Sigma-Aldrich).

rLigAni and rLigBrep proteins were recovered from inclusion bodies using urea (8 M). These proteins were purified by Ni^{2+}-affinity chromatography HisTrap columns

(GE Healthcare). Purified proteins were dialysed against PBS (pH 7.2, 4 °C, 24 h) and quantified using BCA protein assay kit (Thermo Scientific Pierce) according to the manufacturer's instructions and stored at −20 °C until further use.

Animals

Female BALB/c mice weighing 15 to 20 g were used. This study was conducted according to the Brazilian National Council for Animal Experimentation, and all animal procedures were approved by the Federal University of Pelotas Ethics Committee for Animal Experimentation (permit no. 7603). All animals were kept in facilities with 12 h light–dark cycle and temperature-controlled rooms. Food and water were available *ad libitum*, and all animals were under the care of a veterinarian. Bed (wood shavings) was exchanged daily. Animal health and comfort were assured by checking upon the animals three times a day. Signs used to monitor animal health were aggressive behaviour, mydriasis, tachycardia, tachypnea and muscle spasms. There were no unexpected deaths.

Evaluation of *S. boulardii* capacity to enhance the humoral and cellular immune responses in DNA vaccines

Four- to six-week-old mice were randomly divided into four groups containing 12 animals each. The animals of groups 1 and 3 were fed only feed without *S. boulardii* (control groups). The animals in groups 2 and 4 were fed an antibiotic-free diet containing *S. boulardii* (10^8 c.f.u. g^{-1}). Mice were given feed containing the probiotic for 14 days prior to immunization and during the whole experiment [26]. To optimize the rational use and to reduce the number of animals used in the experiment, no negative control group inoculated with empty pTARGET plasmid was added, since this group had been evaluated earlier [3]. The immunization protocol consisted of three doses (days 0, 14 and 21). In order to enhance DNA absorption, sucrose 25 % (100 μl) was applied in the right hind limb approximately 45 min before immunization in all groups. In groups 1 and 2, immunization was performed with pTARGET/*ligAni* (100 μg), whereas groups 3 and 4 received the same dose of pTARGET/*ligBrep*. All inoculations were carried out intramuscularly.

One day before each immunization and week after the last (days −1, 13, 20 and 27), blood samples were collected from the retro-orbital venous plexus and incubated for 20 min at 37 °C, followed by 1 h at 4 °C, and centrifuged (10 min, 4,000 × *g*) for serum collection to assess the humoral immune response. Serum samples were stored at −20 °C until further use.

To evaluate the cellular immune response, animal blood samples were collected on the 37th day for RNA extraction using Gene JET whole blood RNA purification mini kit (Thermo Scientific), according to the manufacturer's instructions.

Evaluation of the humoral immune response

ELISA with recombinant proteins was used to evaluate the humoral immune response. Microplates with 96 wells (CralPlast) were coated with 200 ng per well of either rLigAni (groups 1 and 2) or rLigBrep (groups 3 and 4) diluted in carbonate–bicarbonate buffer (0.05 M, pH 9.6). Then the sera of each animal, collected on days −1, 13, 20 and 27, were added, in duplicate, and diluted 1 : 30 in PBS plus 0.05 % Tween 20 (PBS-T). Sera from unvaccinated mice (day −1) were used as negative control. Peroxidase-conjugated goat anti-mouse polyvalent antibody (Sigma-Aldrich) was diluted in PBS-T (1 : 4,000) and added to each well. All reactions were performed for 1 h at 37 °C using 50 μl reagents per well. Plates were washed three times after each step with PBS-T (200 μl per well). Reactions were developed by adding orthophenylenediamine in phosphate–citrate buffer (0.2 M, pH 4.0) and H_2O_2 (0.01 %). Plates were maintained in the dark for 15 min at room temperature, and the OD_{450} was read in a spectrophotometer for microplates. Antibody tits were expressed as ELISA units dividing the arithmetic mean of sera absorbance of each animal by the mean absorbance of each unvaccinated animals (day −1), expressing the results as total IgG fold increase [18].

Cellular immune response evaluation

The cellular immune response was assessed according to the protocol described by Barjesteh *et al.* [27], with modifications. Blood samples were collected 3 days after rLigAni or rLigBrep (50 μg) was intraperitoneally injected in the animals. Blood samples from each group were used to compose three pooled samples, containing 50 μl of blood from each animal. Total RNA was extracted using the Gene JET whole blood RNA purification mini kit (Thermo Scientific) and quantified using a NanoVue spectrophotometer (GE Healthcare UK Limited). For cDNA synthesis, we used 0.5 μg RNA and a High Capacity cDNA reverse transcription kit (Applied Biosystems). The quantitative real-time (qRT)-PCRs were performed using Stratagene Mx3005P real-time PCR system (Agilent Technologies), SYBR Green PCR Master Mix (Applied Biosystems) and the primers described in Table 1 [28]. The reaction conditions consisted of 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 60 s; and 72 °C for 60 s. In addition, a dissociation curve ensured the specificity of the amplified products. All reactions were performed in duplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used to normalize expression data of the target genes. Data were expressed in normalized cycle threshold (C_T) units. Briefly, the C_T is the number of amplification cycles that were necessary to achieve a pre-defined threshold of fluorescence signal, which is proportional to the number of copies of the target cDNA region. Therefore, the C_T is inversely proportional to the initial amount of the amplicon. The samples were analysed using the software REST 2009 [29]. The relative gene expression rate was calculated in relation to the GAPDH expression, as described by Pfaffl *et al.* [29].

Table 1. Primer sequences used in qRT-PCR to evaluate cytokines IL-4, IL-10, IL-12, IL-17, IFN-γ and TGF-β relative expression

Gene	Sequence	T_m (°C)	Efficiency (%)	Correlation (R^2)
IL-4	F 5'-CCAAGGTGCTTCGCATATTT-3'	60	88	0.95
	R 5'-ATCGAAAAGCCCGAAAGAGT-3'			
IL-10	F 5'-TTTGAATTCCCTGGGTGAGAA-3'	60	99	0.99
	R 5'-ACAGGGGAGAAATCGATGACA-3'			
IL-12	F 5'-AGCACCAGCTTCTTCATCAGG-3'	60	95	0.95
	R 5'-CCTTTCTGGTTACACCCCTCC-3'			
IL-17	F 5'-TTCATCCACTGTCCCACTGA-3'	60	73	0.98
	R 5'-GTTCCTCTATGGGGTCGTCA-3'			
IFN-γ	F 5'-GCGTCATTGAATCACACCTG-3'	60	93	0.91
	R 5'-TGAGCTCATTGAATGCTTGG-3'			
TGF-β	F 5'-GCAACATGTGGAACTCTACCAGAA-3'	60	80	0.97
	R 5'-GACGTCAAAAGACAGCCACTCA-3'			
GAPDH	F 5'-AACGCCCTTCATTGAC-3'	60	95	0.98
	R 5'-TCCACGACATACTCAGCAC-3'			

F, forward; R, reverse; T_m, mean annealing temperature.

Statistics

Student's *t*-test was used to determine significant differences ($P<0.05$) between ELISA units of vaccinated and unvaccinated animals and between cytokine mRNA levels. All statistical analyses were performed using software Prism5 (GraphPad).

RESULTS

Production of DNA vaccines

Recombinant plasmids pTARGET/*ligAni* and pTARGET/*ligBrep* were produced using *E. coli* TOP10. Extraction yielded up to 12 mg of each plasmid per lit of culture.

Expression of rLigAni and rLigBrep

E. coli BL21 (DE3) Star effectively expressed rLigAni and rLigBrep recombinant proteins, since bands of 64 and 72 kDa, respectively, were detected by Western blot analysis (data not shown).

S. boulardii exerts an immunomodulator effect in DNA vaccines

Immunization with three DNA vaccine doses using *S. boulardii* as immunomodulator boosted the specific humoral immune response of both evaluated vaccines (Fig. 1). The antibody levels of animals fed with *S. boulardii* and vaccinated with pTARGET/*ligAni* or pTARGET/*ligBrep* were higher after the second and third immunization ($P<0.05$), respectively, than those of control animals. In addition, there was no significant difference ($P<0.05$) in antibody levels between the second and third doses when pTARGET/*ligAni* was used as immunogen.

Cellular immune response induced by the DNA vaccine with the probiotic *S. boulardii* as immunomodulator

Cytokine mRNA levels for IFN-γ, IL-12, IL-4, IL-10, TGF-β and IL-17 were assessed by qRT-PCR of blood cells stimulated by the recombinant proteins *in vivo* (Fig. 2). A comparison of cytokine levels between control group and those using *S. boulardii* as immunomodulator showed a significant increase in IL-10 levels in the group using pTARGET/*ligBrep* as antigen and *S. boulardii* as immunomodulator.

DISCUSSION

This study demonstrated that the probiotic *S. boulardii* is a promising immunomodulator, enhancing the humoral immune response of animals immunized with the gene sequences of the protein fragments LigAni and LigBrep of *L. interrogans* cloned in the plasmid pTARGET. These proteins were chosen as immunogens in view of their ability to enhance the immune response and protection in different studies, either as DNA vaccines or subunit vaccines [3, 23, 30–33]. However, since vaccines using LigA as antigen may be too weak to eliminate the bacterium from the host tissue [30], the immune response of these vaccines should be improved by immunomodulators.

Some studies using purified recombinant proteins containing immunoprotective epitopes failed to provide protection, probably due to errors in the conformation of important protective structures [34, 35]. Moreover, the subunit vaccine production is a challenge in terms of protein expression, purification and solubility [2]. On the other hand, DNA vaccines, easily produced and handled, are inexpensive and capable of inducing both humoral and cellular immune responses because the recombinant proteins are expressed host's cells, which also are capable of producing proteic
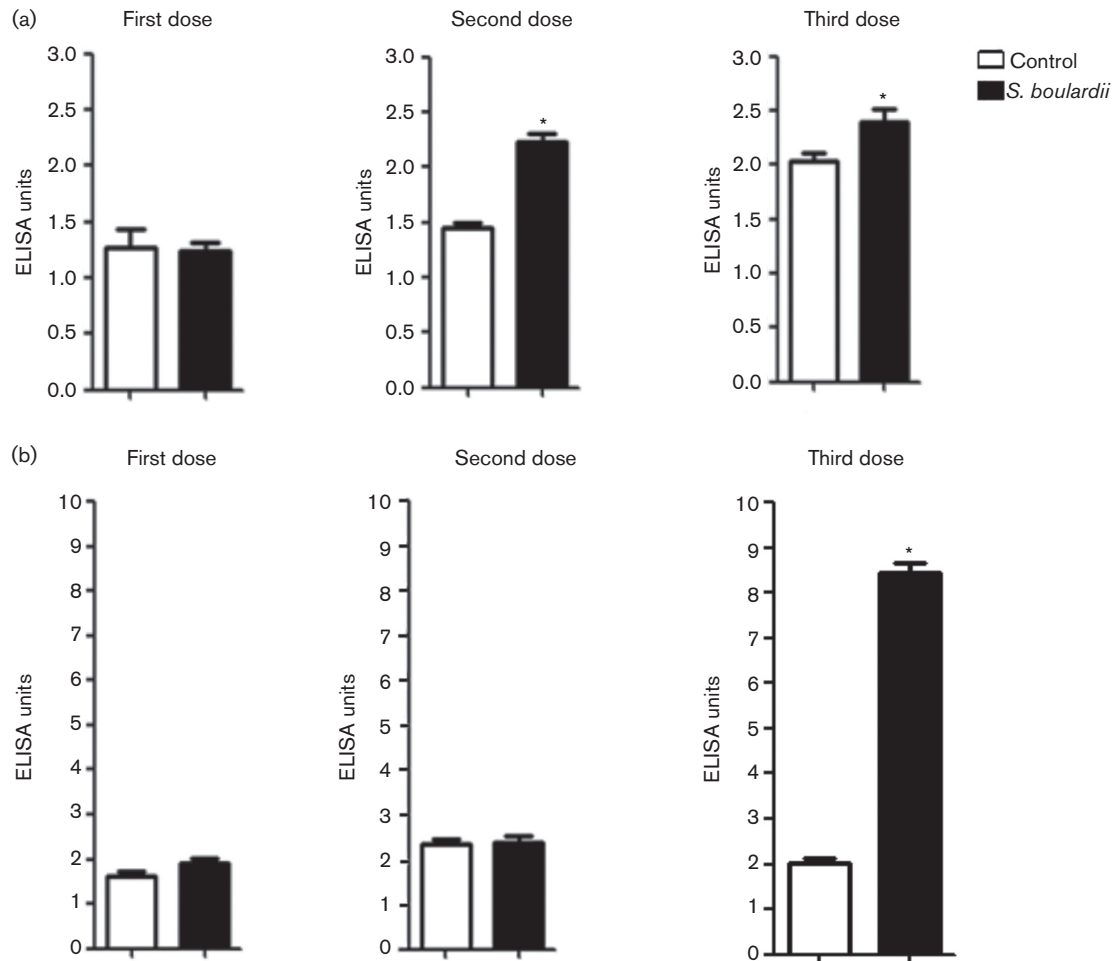
Fig. 1. Antibody levels induced by the DNA vaccines pTARGET/*ligAni* and pTARGET/*ligBrep*. Groups immunized with pTARGET/*ligAni* (groups 1 and 2 – panel a) showed no difference between the control group and the group treated with *S. boulardii* on the 13th day after the first dose. However, after the second vaccination (day 20), the control group and treatment group differed statistically (*P*<0.05), which was maintained after the third dose on day 27. Groups immunized with pTARGET/*ligBrep* (groups 3 and 4 – panel b) showed no difference between the control group and the treatment group after the first and second doses on days 13 and 27, respectively. However, after the third dose (day 27), the titres of the treatment group were higher than those of the control group, with statistically significant differences (*P*<0.05). Plasmid pTARGET/*ligBrep* induced significantly higher antibody titres (*P*<0.05) after the third vaccination with *S. boulardii* than did pTARGET/*ligAni*. Data are represented as mean ELISA units of duplicates. An asterisk represents statistical difference (*P*<0.05).

antigens with the correct folding without requiring solubilization steps, resulting in closer similarity to the native conformation and greater cost-effectiveness [2, 36, 37].

S. boulardii boosted the humoral immune response of DNA vaccines, since the antibody levels of animals fed with the yeast and vaccinated with pTARGET/*ligAni* or pTARGET/*ligBrep* after the second and third immunizations, respectively, were higher (*P*<0.05) than those of animals in the control group.

The magnitude and direction of the antigen-specific cellular and humoral immune response can be modulated by using adjuvants or immunomodulators. When pTARGET/*ligBrep* was used as a vaccine, a significant increase in the production of IL-10 by the animals in the group treated with *S. boulardii* over the control group was observed. This result corroborates the findings of a study that also evaluated LigB as immunogen in a subunit vaccine and aluminium as adjuvant [38]. An increase in IL-10 production was also found in response to the probiotic *Bacillus cereus* var. Toyoi in mice treated with an inactivated vaccine against bovine herpesvirus type 5 [18] and in mice that received *S. boulardii* orally and were then challenged with *Candida albicans* [39].

Immunomodulation by means of probiotics was demonstrated by leukocyte proliferation, increase in phagocytic
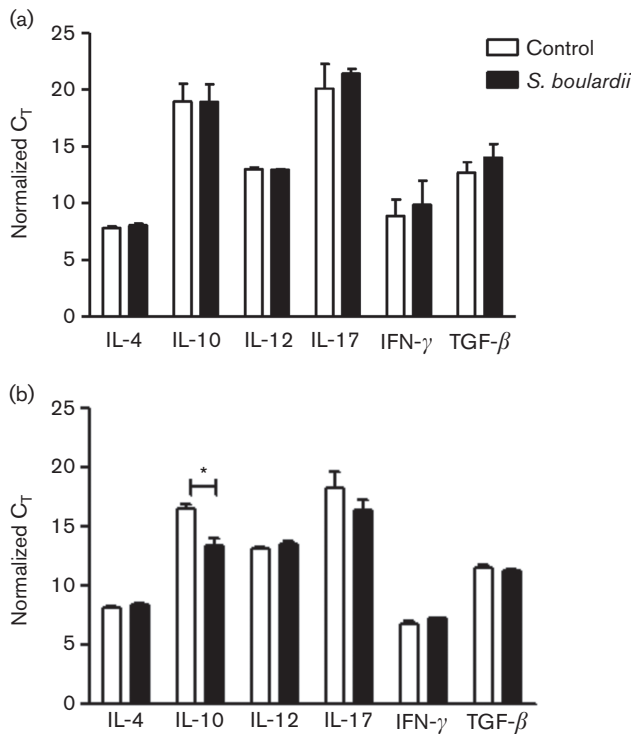
Fig. 2. Normalized C_T values of the mRNA levels of cytokines IL-4, IL-10, IL-12, IL-17, IFN-γ and TGF-β. Groups 1 and 2, treated with plasmid pTARGET/*ligAni*, did not differ in the expression levels of the cytokines (a), while between groups 3 and 4, treated with plasmid pTARGET/*ligBrep*, an increase in the expression of IL-10 was observed in the group in which *S. boulardii* was used as immunomodulator (b). Gene GAPDH was used as the normalizing expression data of the target genes. The data are represented as C_T. An asterisk represents statistical difference ($P<0.05$).

activity, increased antibody production and changes in cytokine expression, of which the latter two actions were observed in this study. The use of *S. boulardii* is associated with an increase in anti-inflammatory cytokines, also observed in our study, as well as a reduction in pro-inflammatory cytokines, secretion of factors that modulate the restitution of gut cells, dendritic cell activation and T-cell proliferation [19, 20]. However, the exact mechanism of action is not fully understood. Moreover, in a study using the probiotics *S. boulardii* and *B. cereus* var. Toyoi, immunomodulation was triggered at the beginning of its administration, suggesting that the immune response to DNA vaccines can be increased without requiring *S. boulardii* administration over long periods, which would facilitate its use and reduce costs [40].

A high protection level against lethal infection with leptospirosis in hamster was obtained with a subunit vaccine using LigA as immunogen and complete and incomplete Freund's adjuvant [23]. However, these adjuvants cause side effects such as granuloma growth, pain, sensitivity and erythema [41]. In DNA vaccines, the use of adjuvants is not

related to total protection or reduction of infection [42, 43]. Moreover, the improvement obtained in the immune response is often restricted to the initial stage only [44].

The results allowed the conclusion that the probiotic *S. boulardii* can enhance the humoral immune response to DNA vaccines, suggesting its potential as immunomodulator. In addition, one of the advantages of *S. boulardii*, non-pathogenic yeast with GRAS status, is the absence of adverse effects. Furthermore, its use as immunomodulator in DNA vaccines, aside from improving the specific humoral immune response against the antigen, has beneficial effects on the intestinal mucosa of the host (e.g. increases in the immune response, digestion, nutrient uptake and prevention or treatment of a number of intestinal disorders) [45]. Additionally, immunomodulators can allow a reduction in the amount of DNA used and, thereby, a reduction in the production cost, apart from enhancing the action of the vaccine and making it more effective. Thus, these results represent an important contribution in the field of vaccine development and pave the way for further investigations on *S. boulardii* as immunomodulator in DNA vaccines and as a probiotic.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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