

Preliminary Studies on the Prevention of the Ovalbumin-Induced Allergic Response by *Enterococcus faecalis* CECT7121 in Mice

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Key Words

Enterococcus faecalis CECT7121 • Ovalbumin-allergic murine model • Immunoglobulin E • Th2 cytokines • Probiotics

Abstract

Background: Allergic diseases are featured by an increased production of IgE due to an imbalance in the immune response towards a Th2 profile. In this work, the ability of *Enterococcus faecalis* CECT7121 to regulate this Th2-exaggerated response in a murine model of ovalbumin (OVA)-induced allergy was studied. **Methods:** BALB/c mice intragastrically inoculated with *E. faecalis* CECT7121 before and during a subcutaneous immunization protocol with OVA were studied in comparison with an immunized control group. The allergen-specific immune response (IgE, IgG, IgG1 and IgG2a) was assessed. The proliferative activity of memory splenocytes and the levels of IL-4, IL-5, IL-13, IL-10, IL-12 and IFN- γ were also determined. **Results:** Upon treatment with *E. faecalis* CECT7121 the following effects were observed: (1) a decrease in specific IgE levels, (2) an increase in anti-OVA IgG2a levels, (3) the levels of anti-OVA IgG and IgG1 remained unaltered, (4) a reduction in the proliferation rate of memory cells, (5) a decrease in the levels of the Th2 cytokines IL-4, IL-5

and IL-13, and (6) the secretion of IL-10, IL-12 and IFN- γ remained unchanged. Moreover, the incubation of human basophils with non-viable *E. faecalis* CECT7121 together with an allergen preparation induced the release of β -hexosaminidase at levels that were lower than control reactions and similar i.g. the spontaneous release. **Conclusions:** In this model, the i.g. administration of *E. faecalis* CECT7121 hampers the establishment of the OVA-induced allergic immune response, suggesting that this strain could be useful for the treatment of IgE-mediated allergic diseases.

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Introduction

In mice, the activation of T helper 1 (Th1) lymphocytes, characterised by the production of interferon- γ (IFN- γ) and interleukin (IL)-2, induces the synthesis of immunoglobulin (Ig)G2a by B cells, leading to the establishment of an immune response mediated by both antibodies and cells [1–4]. Moreover, the activation of the Th2 subset and the subsequent secretion of IL-4, IL-5 and IL-13 are linked to the development of IgG1- and IgE-featured humoral responses [1–4].

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In most of the individuals suffering from allergic diseases, an imbalance of the immune response towards a Th2 cytokine profile is observed. This Th2 scenario induces the secretion of high levels of IgE, together with the recruitment of effector cells, that is, eosinophils, basophils and mast cells to the site where the allergic inflammatory response is taking place [5–9]. On the other hand, it is known that in healthy individuals, a Th1 response is generated against these allergens [10].

Even though the aetiology of allergic diseases is still unknown, it is proposed that apart from the genetic predisposition, environmental factors can also trigger the allergic status, for the interaction with microorganisms has a crucial role in the maturation of the immune system during the first years of life. This idea is in line with the well-known hygiene hypothesis, which suggests that the reduction in the number of members that families have undergone over the years, together with a decrease in the rate of infections during childhood, has decreased the exposition to environmental microorganisms [5, 11–13]. The exposition to microorganisms during the early stages of life could revert this Th2-skewed response, and in turn, it could induce the development of a Th1 phenotype and stimulate the activity of TGF- β -producing Th3 cells.

Furthermore, it has been demonstrated that the microorganisms of the commensal flora of allergic children differ from that of healthy ones [14, 15], an observation that is in line with the well-known role of the intestinal flora to stimulate the immune system. Taking into account that the ingestion of fermented foods or some lactic acid bacteria with immunomodulatory properties (probiotics) can lead to a greater degree of secretion of IFN- γ [16], this mechanism would be adequate to regulate exaggerated Th2 responses in vivo, and therefore to help suppress allergic responses.

In previous works, we have demonstrated that the non-pathogenic environmental strain *Enterococcus faecalis* CECT7121 implants itself and remains in the intestinal mucosa of BALB/c mice, stimulating the innate immune system and inducing the synthesis of IL-10 and IL-12 [17, 18]. We have also demonstrated the adjuvant effect of *E. faecalis* CECT7121 and its broad pro-Th1 immunomodulatory activity, achieving beneficial effects on different experimental models, that is, during an infection with *Salmonella* serotype Enteritidis [18], during the challenge of mice with lymphoma LBC cells [19] and during the immunization protocol with a DTPw vaccine of pediatric use [20].

Even though many studies have been undertaken to assess the effects of probiotics in allergic local patholo-

gies, that is, alimentary allergy and asthma, no data are available regarding the influence of this bacterium on Th2-featured systemic allergic processes [11, 21–24]. Therefore, in the present work we have assessed the effect of *E. faecalis* CECT7121 in a murine model of systemic allergy. The evaluation was performed in naïve BALB/c mice and in mice treated intragastrically (i.g.) with *E. faecalis* CECT7121 before and during a subcutaneous (s.c.) immunization protocol with ovalbumin (OVA). Allergen-specific serum antibodies (IgE, IgG, IgG1 and IgG2a), the proliferative activity of splenocytes upon stimulation with OVA as well as the levels of IL-4, IL-5, IL-13, IL-10, IL-12 and IFN- γ in culture supernatants were evaluated. In vivo tests of active cutaneous anaphylaxis (ACA) were also performed.

Materials and Methods

Mice

Female BALB/c mice were provided by the School of Veterinary Medicine, University of Buenos Aires (Argentina). Mice were housed in cages in an air-conditioned room, with a 12-hour light/dark cycle at controlled temperature (20–22°C). Animals were allowed to have water and food ad libitum. All mice were 6 weeks old at the beginning of the experiment.

E. faecalis CECT7121 Suspensions

E. faecalis CECT7121 was grown in triptone soy broth (Biokar Diagnostics) at 37°C for 18 h. After incubation, the culture was harvested by centrifugation at 5,800 g for 15 min and washed 3 times with sterile saline solution. To prepare *E. faecalis* CECT7121 suspensions, standard growth curves were produced by plotting the OD₆₂₀ versus agar plate counts of freshly prepared, serially diluted cultures. Bacterial suspensions were prepared in sterile saline at approximately 3.0×10^8 CFU/ml. For in vitro studies, heat-killed microorganisms (80°C, 1 h) were used to stimulate basophils.

Intragastric Administration of *E. faecalis* CECT7121 and Immunization Protocol with OVA

A schematic representation of the *E. faecalis* CECT7121 and OVA treatments is shown in figure 1. Briefly, an *E. faecalis* CECT7121 suspension (3.0×10^8 CFU/ml) was administered by the i.g. route (0.2 ml/mice/day) on days –3, –2, –1, 14, 15 and 16 of the immunization schedule with OVA (Sigma). The OVA immunization [10 μ g OVA and 1 mg Al(OH)₃] was performed on days 0, 7 and 21 by s.c. injection (0.2 ml) on the back of each mouse.

Animals were divided into 4 groups of 5 animals each:

- group S/OVA: mice inoculated i.g. with sterile saline and immunized with OVA
- group *E. faecalis* CECT7121/OVA: mice inoculated i.g. with *E. faecalis* CECT7121 and s.c. immunized with OVA
- control group S: mice inoculated i.g. with 0.2 ml of sterile saline and inoculated s.c. with sterile saline
- control group *E. faecalis* CECT7121: mice inoculated i.g. with *E. faecalis* CECT7121 and inoculated s.c. with sterile saline.

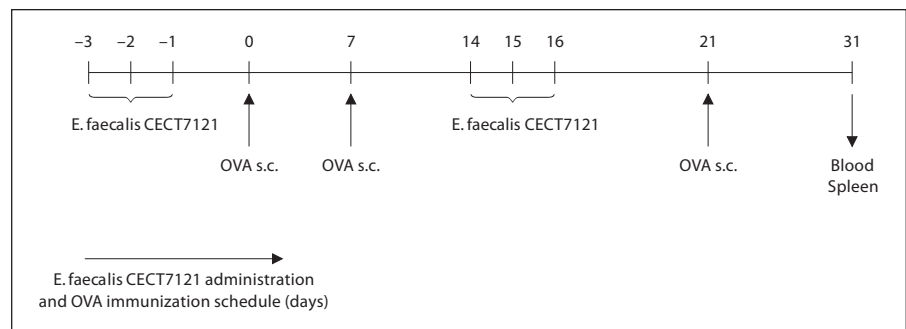


Fig. 1. Immunization schedule of mice with OVA and i.g. administration of *E. faecalis* CECT7121.

Evaluation of Cell Responses

Ten days after the last immunization, individual spleen cell suspensions of each immunized or control mouse were prepared in culture medium complete RPMI 1640 (Gibco) containing 10% (v/v) fetal calf serum (Bioser), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 0.25 µg/ml amphotericin B (Gibco), 300 µg/ml L-glutamine (Gibco) and 220 µg/ml pyruvate (J.T. Baker). Briefly, each freshly removed spleen was placed in a Petri dish containing 3 ml of culture medium. Spleens were cut into pieces and pressed against the bottom of the Petri dish until mostly fibrous tissue remained. Suspensions were passed through a 200-µm mesh nylon sieve and the cell suspension was centrifuged (12 min, 200 g, 18°C). Pellets were washed twice with culture medium, and cells counted in a Neubauer chamber. One hundred microlitres of spleen cell suspension (4.0×10^6 cells/ml) was cultured with 100 µl of culture medium alone or with 100 µl of OVA (1 mg/ml; Sigma-Aldrich) in quadruplicate at 37°C in presence of 5% CO₂. Concanavalin A (10 µg/ml; Vector Labs) was used as mitogen. In each experiment, two cell cultures were performed, to assess cell proliferation and to determine the cytokine levels in supernatants. After 72 h, supernatants were collected and stored at -70°C until assayed for cytokine levels. Proliferative responses were assessed after 96 h of culture by [³H] thymidine (Perkin-Elmer) uptake and measured by liquid scintillation (Liquid Scintillation Analyzer 1600TR; Packard). Results were expressed as mean count per minute $\pm$ SEM of quadruplicate cultures.

Cytokine Assays

Commercial kits were employed to determine levels of IL-4, IL-5, IL-13, IL-10, IL-12 and IFN-γ (BD Biosciences). Cytokine concentrations were tested individually in 4 different assays (except for IL-13 that was measured in two different assays) and mean values were expressed in picograms per millilitre derived from standard curves performed with the corresponding murine recombinant cytokine standards.

Levels of Allergen-Specific Antibodies

Before spleen removal, each animal was bled by intracardiac puncture. Individual serum samples were separated and stored at -20°C until assayed for antibody levels. Specific IgE and IgG antibody levels were measured by indirect ELISA in individual serum samples. Briefly, OVA was employed as coating antigen at 10 µg/ml in phosphate-buffered saline (PBS). Antibodies were detected using an HRP-conjugated anti-mouse IgG serum (Cappel)

and a biotinylated anti-mouse IgE (BD Biosciences) antibody followed by an avidine-HRP solution. All determinations were done in duplicate. Absorbance values were obtained after spectrophotometric reading at dual 450–570 nm wavelengths in an ELISA plate reader.

To determine the isotype of OVA antibodies, HRP-conjugated anti-mouse IgG1 and HRP-conjugated anti-mouse IgG2a sera (Bethyl) were used as secondary antibodies.

Active Cutaneous Anaphylaxis

ACA was induced by intradermic challenge with the allergen in OVA-immunized and control mice. Briefly, an intravenous injection of 1% w/v Evans Blue (50 µl) was administered by the tail vein, and ACA was then elicited in the left ear by intradermic inoculation of 50 µl OVA (1 mg/ml). PBS was inoculated in the right ear of each animal as a control reaction. Animals were observed after 30 min.

In vitro Basophil Degranulation Assay

To perform the basophils degranulation assay in blood samples obtained from patients suffering from allergy to anemophilous fungi and control donors, written consent was obtained from each individual (age range 2–30 years) or their parents. Since human basophils are more readily obtainable, these cells were employed instead of murine ones. As allergen, a mixture of extracts of *Hormodendrum* (*Cladosporium*), *Rizophus*, *Aspergillus fumigatus*, *Alternaria* and *Mucor mucedo* was employed. The methodology described by Benveniste [25] and modified by Grinstein et al. [26] was employed. Briefly, peripheral blood samples were subjected to sedimentation in 6% Dextran 70 in 0.9% NaCl (Macrodex; Pharmacia). The basophil-enriched fraction was centrifuged at 400 rpm for 10 min. Cell pellets were resuspended in 1 ml RPMI 1640. Fifty-microlitre aliquots of cell suspensions (1.0×10^5 cell/ml) were placed in 96-well microplates and incubated for 15 min at 37°C with 100 PNU/ml of the allergen alone or together with heat-killed *E. faecalis* CECT7121 (1.39×10^8 to 1.39×10^{10} CFU/ml). After incubating, 50 µl of a solution of 4-nitrophenyl N-acetyl-β-D-glucosaminide (1 mM; Sigma-Aldrich) in 0.1 M citrate buffer pH 5.0 was added and incubated for 1 h. The reaction was then stopped by the addition of 200 µl of 0.1 M carbonate buffer pH 10.5 and the absorbance was measured at 405 nm [27, 28]. In these reactions, the release of β-hexosaminidase from basophils was considered as a degranulation parameter. Results were expressed as means $\pm$ SEM.

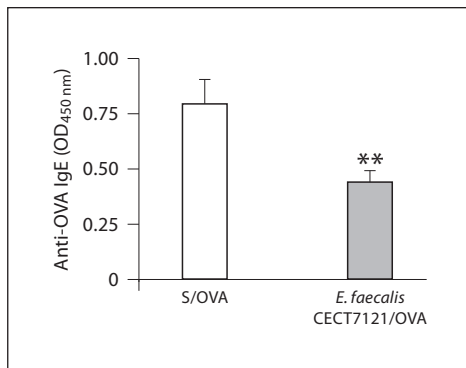


Fig. 2. Effect of i.g. administration of *E. faecalis* CECT7121 on serum anti-OVA IgE. Mice in both groups were treated s.c. with OVA on days 0, 7 and 21. The positive control group was injected with saline (S/OVA) and the experimental group (*E. faecalis* CECT7121/OVA) was inoculated i.g. with an *E. faecalis* CECT7121 suspension (3×10^8 CFU/ml). Each bar represents the means $\pm$ SEM (n = 20). ** p < 0.01, Student's t test with Welch's correction.

Statistical Analysis

A linear regression of ELISA absorbance values of standards was determined. The Pearson's correlation coefficient was determined to assess linear correlations between the parameters (anti-OVA IgE vs. IL-4, IL-5 and IL-13).

One-way ANOVA followed by the Student-Newman-Keuls' multiple comparisons test or the Student's t test was also employed to determine significance of results using GraphPad Prism 4.00 for Windows (GraphPad Software).

Results

Determination of Antibody Levels

The immunization with OVA induced the synthesis of high levels of specific IgE in both groups of immunized animals. The treatment with *E. faecalis* CECT7121 produced a significant decrease (roughly a 45%) in serum levels of anti-OVA IgE when compared to the levels observed in the animals belonging to the group S/OVA (fig. 2; ** p < 0.01).

Allergen-specific IgG titres of *E. faecalis* CECT7121 pre-treated animals and then immunized with OVA showed a trend towards lower values when compared to those of immunized control mice (table 1; p = 0.0821). As markers of Th1 and Th2 responses, the levels of specific IgG2a and IgG1 were determined respectively. Levels of anti-OVA IgG2a were higher in the *E. faecalis* CECT7121/OVA group when compared to immunized control animals (table 1). Nevertheless, no statistical differences

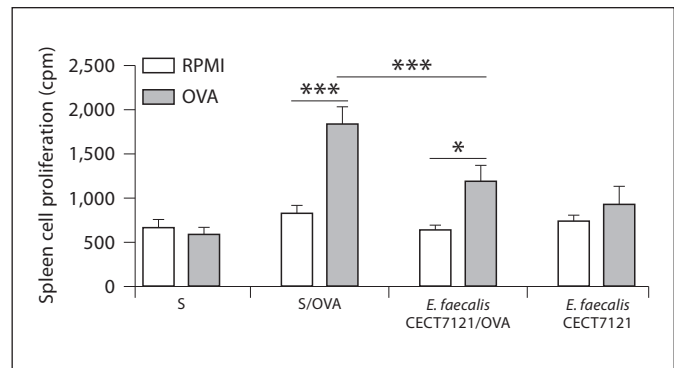


Fig. 3. Comparative cell responses. Ten days after the last immunization, spleen cells of each immunized or control mouse were isolated and incubated with culture medium (RPMI) or OVA. Five mice from each group were analyzed in each assay and results expressed as mean count per minute (cpm). Bars represent results of 4 separate assays. * p < 0.05, *** p < 0.001, one-way ANOVA followed by the Student-Newman-Keuls' multiple comparisons test with log₁₀-transformed data to achieve homogeneity of variances.

Table 1. Determination of specific IgG, IgG1 and IgG2a titers in serum samples by ELISA after OVA immunization of naïve BALB/c versus mice treated i.g. with *E. faecalis* CECT7121

	S/OVA	<i>E. faecalis</i> CECT7121/OVA
anti-OVA IgG	90,780 $\pm$ 13,940	62,360 $\pm$ 14,370 ^a
anti-OVA IgG1	13,930 $\pm$ 3,249	25,036 $\pm$ 5,790 ^b
anti-OVA IgG2a	4,416 $\pm$ 694	11,493 $\pm$ 3,355 ^c

Data represent means $\pm$ SEM (n = 20).

^a p = 0.0821, ^b p = 0.0535, ^c p < 0.05, Student's t test with Welch's correction.

were found between such groups in the levels of anti-OVA IgG1. No specific antibodies were detected in non-immunized animals.

Cellular Responses

Upon stimulation with concanavalin A, the splenocytes of animals belonging to all groups were able to proliferate (*E. faecalis* CECT7121/OVA 40,042 $\pm$ 1,433 cpm; S/OVA 32,686 $\pm$ 1,493 cpm; control *E. faecalis* CECT7121 30,590 $\pm$ 2,632 cpm; control S 32,230 $\pm$ 4,029 cpm). The splenocytes from OVA-immunized animals were also able to proliferate when OVA was employed as stimulus (fig. 3). In these experiments, a lower proliferation rate was observed in splenocytes from OVA-immunized, *E.*

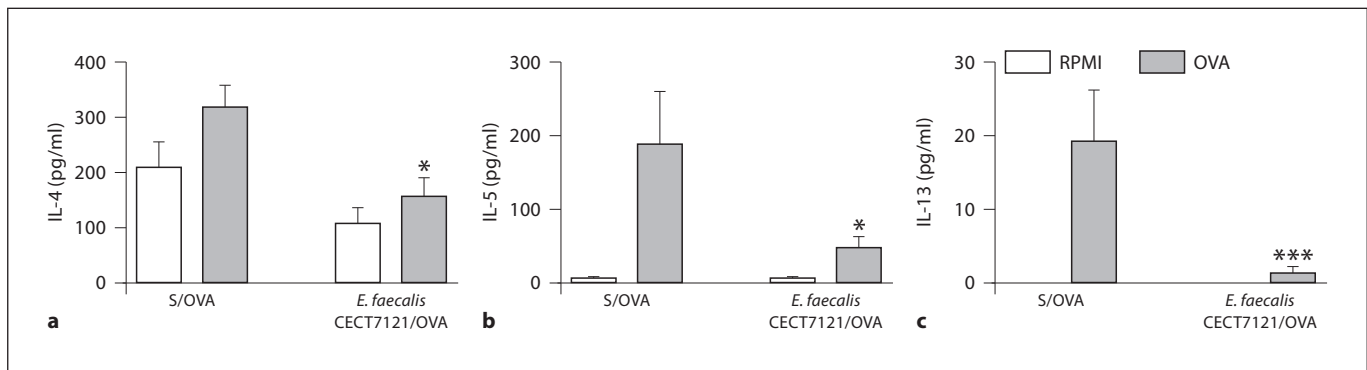


Fig. 4. Th2 cytokine production. IL-4 (a), IL-5 (b) and IL-13 (c) levels measured by ELISA in splenocytes culture supernatants after incubating with culture medium (RPMI) or OVA for 72 h. Five mice from each group were analyzed in each assay and results expressed as pg/ml. Bars represent results from four separate assays. * $p < 0.05$, *** $p < 0.001$, one-way ANOVA followed by the Student-Newman-Keuls' multiple comparisons test.

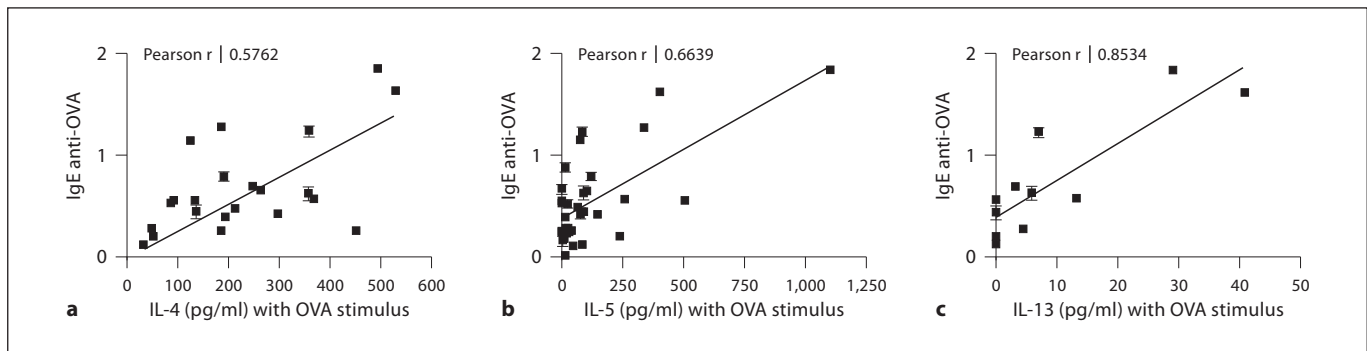


Fig. 5. Correlation of Th2 cytokine levels with specific IgE in serum samples. Correlations between anti-OVA IgE and IL-4 (a), IL-5 (b) and IL-13 (c) were analyzed. a ** $p < 0.01$; b *** $p < 0.001$; c *** $p < 0.001$; Pearson's correlation coefficient.

faecalis CECT7121-treated animals when compared to controls (fig 3; *** $p < 0.001$).

No specific proliferation was observed in splenocytes obtained from non-immunized animals (fig. 3).

Cytokine Levels

IL-4, IL-5 and IL-13 were taken as indicators of Th2 responses and due to their role in allergic processes. The levels of IL-4 (fig. 4a), IL-5 (fig. 4b) and IL-13 (fig. 4c) determined in culture supernatants of splenocytes stimulated in vitro with OVA were lower in the group of *E. faecalis* CECT7121/OVA versus S/OVA.

In all immunized animals, and after the in vitro stimulus with OVA, serum levels of anti-OVA IgE correlated

with the secretion of IL-4 ($r: 0.5762$, ** $p < 0.01$), IL-5 ($r: 0.6639$, *** $p < 0.001$) and IL-13 ($r: 0.8534$, *** $p < 0.001$) (fig. 5a–c; Pearson's correlation coefficient).

No statistical differences were observed in the levels of IL-10 (fig. 6a) or IL-12 (fig. 6b) between the OVA-immunized groups (S/OVA vs. *E. faecalis* CECT7121/OVA).

No detectable levels of IFN- γ were found in any group.

Active Cutaneous Anaphylaxis

As shown in figure 7, the vascular permeability specifically induced by OVA injection was lower in the *E. faecalis* CECT7121 pre-treated group of mice (fig. 7). Non-immunized control animals did not show ACA (fig. 7).

Fig. 6. IL-10 and IL-12 production. IL-10 (a) and IL-12 (b) levels measured by ELISA in supernatants of splenocytes incubated with culture medium (RPMI) or OVA for 72 h. Five mice from each group were analyzed in each assay and results expressed in picograms per millilitre. Bars represent results from 4 separate assays. Results were analyzed by one-way ANOVA followed by the Student-Newman-Keuls' multiple comparisons test.

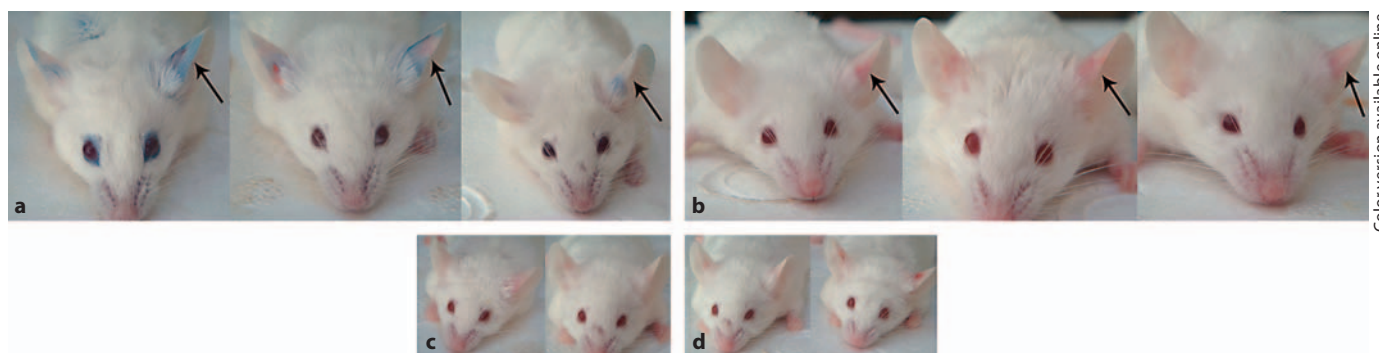
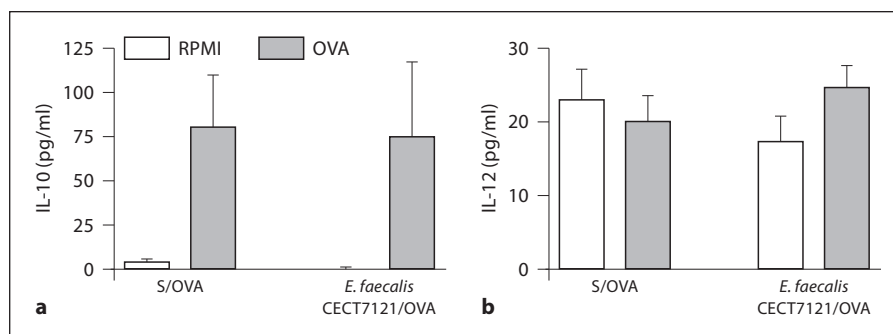


Fig. 7. ACA was induced by intradermic challenge with the allergen in OVA-immunized and control mice in the left ear by inoculation of 50 μ l OVA (1 mg/ml). PBS was inoculated in the right ear of each animal as control reaction. Animals were observed after

30 min. **a** S/OVA group; **b** *E. faecalis* CECT7121/OVA group; **c** S control; **d** *E. faecalis* CECT7121 control. Arrows show the inoculation site in immunized animals.

Color version available online

Basophil Degranulation Test

The release of β -hexosaminidase was taken as a parameter of basophil degranulation. The addition of the allergen (mixture of fungi extracts) to the cell suspensions obtained from allergic patients induced the release of β -hexosaminidase (fig. 8; *** $p < 0.001$). When these cells were incubated with the allergen plus heat-killed *E. faecalis* CECT7121, the release of β -hexosaminidase was similar to the values of spontaneous release (fig. 8). This effect was dependent on the dose of *E. faecalis* CECT7121, for the decrease in the enzyme release was only observed at the lowest concentration tested (1.39×10^8 CFU/ml; data not shown). The level of β -hexosaminidase released by basophils from control donors, after allergen and allergen plus heat-killed *E. faecalis* CECT7121 stimuli, were similar to those obtained with spontaneous release control reactions (fig. 8).

Discussion

E. faecalis CECT7121 is an immunomodulatory strain which has been demonstrated to be able to evoke Th1 responses. In previous works, we have demonstrated that upon the i.g. administration of this bacterium, it can reduce the morbidity and mortality of BALB/c mice challenged with a lethal dose of *Salmonella* serotype Enteritidis [18] and it can generate a stronger cellular anti-tetanic and anti-diphtheric memory response in animals immunized with the DTPw vaccine, orientating the immune system towards the production of cytokines of the Th1 type [20]. Furthermore, in mice challenged with a lethal dose of the LBC murine lymphoma, the intraperitoneal pre-treatment of animals with *E. faecalis* CECT7121 induced a reduction in the mortality by generating a Th1 immune response featured by the secretion of high levels of IL-12 and IFN- γ [19].

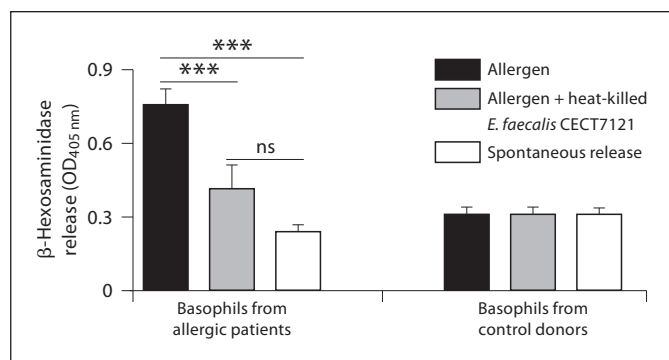


Fig. 8. Effect of heat-killed *E. faecalis* CECT7121 on basophils degranulation. Basophils isolated from peripheral blood samples obtained from allergic patients and control donors were incubated in the presence of culture medium alone (spontaneous release), with a mixture of allergens (allergen) or with a mixture of allergens plus heat-killed *E. faecalis* CECT7121 (1.39×10^8 CFU/ml, allergen + heat-killed *E. faecalis* CECT7121). The release of β -hexosaminidase was determined by spectrophotometric measurements at 405 nm. Bars represent mean $\pm$ SEM. *** $p < 0.01$, one-way ANOVA followed by the Student-Newman-Keuls' multiple comparisons test.

The aim of this work was to assess the effect of the administration of *E. faecalis* CECT7121 on the establishment of an allergic response to OVA triggered by the s.c. administration of the allergen to BALB/c mice. To achieve this goal, the bacterium administration was carried out before the immunization schedule.

In previous works undertaken in our laboratory, we have demonstrated that the i.g. administration of *E. faecalis* CECT7121 (3.0×10^8 CFU/ml) during 3 consecutive days allows its implantation in both the small and large intestine for at least 18 days [18]. Since the immunization schedule with OVA required an administration on day 21, a second i.g. dose of the bacterium was given to the animals in order to achieve a high concentration in the intestine. The pre-treatment protocol and the booster with *E. faecalis* CECT7121 has already been employed in our laboratory, obtaining a successful bacterial implantation in the small intestine of mice for more than 33 days [20].

Specific IgE antibodies are the hallmark of the immune response to allergens and an important diagnostic tool in allergic diseases [29]. The immunization of mice with OVA plus Al(OH)₃ evoked a Th2-type response with high levels of allergen-specific IgE. The administration of *E. faecalis* CECT7121 before and during the immunization protocol caused a reduction in the titres of anti-OVA IgE of approximately 45%. Regarding the total specific

IgG levels, no differences were observed between the immunized groups of animals (control and pre-treated). These results are not surprising if the high immunogenicity of OVA and the high concentrations of serum IgG are considered.

As with the conventional desensitizing immunotherapies involving multiple inoculations of the allergen at increasing concentrations [30], the i.g. administration of *E. faecalis* CECT7121 was associated with a diminished ex vivo specific proliferation of splenocytes.

Taking into account the lower levels of specific IgE found in the group *E. faecalis* CECT7121/OVA, it was not surprising to find, in culture supernatants of splenocytes obtained from mice immunized with OVA and pre-treated with the bacterium, levels of IL-4, IL-5 and IL-13 that were lower than those of control animals. In fact, the role of these cytokines in the switching and synthesis of IgE has been well documented. IL-4 and IL-13 are known to be the cytokines that stimulate the ϵ -germline gene transcription and the switching to IgE in both human and animal B cells [4]. IL-5 is a cytokine that drives the differentiation and maturation of eosinophils in the bone marrow, and promotes the migration of these cells from the bloodstream to certain tissues where it prolongs their half-life [31, 32]. Although the role of the IL-5 is to induce and maintain the high eosinophil counts, a feature of allergic diseases, the idea that this cytokine could also be involved in the regulation of the synthesis of IgE has been presented by other authors. It has been reported that this cytokine can increase the synthesis of IgE mediated by IL-4 in both human and murine B cells. It is also known that this cytokine, together with IL-4, provides the signal required for the switching to IgG1 and IgE in mice [7].

The correlation between the levels of serum-specific IgE and those of IL-4, IL-5 and IL-13 in splenocyte culture supernatants obtained from mice immunized with OVA demonstrates the role of these cytokines in the synthesis of this immunoglobulin isotype. Besides, this correlation would seem to be strain dependent. In a murine model of asthma, Hougee et al. [33] have found that after oral supplementation with some strains of *Bifidobacterium*, a correlation between the levels of IL-4 and specific IgE was observed. Nevertheless, the latter correlation is not observed if *Lactobacillus* is administered instead of *Bifidobacterium* [33].

Even though the administration of *E. faecalis* CECT7121 induced a significant decrease in the levels of IL-4 and specific IgE, in this model, no significant changes were observed in the levels of IgG1 when compared to control animals, though a trend towards lower levels was

observed in animals treated with the bacterium. The latter results would seem to be discrepant, since in mice, IL-4 promotes the switching to both IgE and IgG1 [3, 33]. However, the levels of IL-4 required to stimulate the switching to IgG1 are approximately 100 times lower than those required to induce the switching to IgE [34]. Our results allow determining that the decrease in the levels of IL-4 induced by the administration of *E. faecalis* CECT7121 would be enough to cause a decrease in the levels of specific IgE without affecting those of IgG1, a majority isotype in mice sera.

The levels of anti-OVA IgG2a were higher in the *E. faecalis* CECT7121/OVA group when compared to the immunized control mice. This finding is in agreement with the pro-Th1 activity of this strain also demonstrated in another model of vaccination [20]. Other IgG subclasses (IgG2b and IgG3) were not assessed in this work because they represent only a minority in serum and because they are not involved in the response profile evaluated [35, 36].

It has been demonstrated that certain lactic acid bacteria can suppress, in a strain-dependent fashion, the Th2 immune responses and the secretion of IgE by different Th1/Th2 modulatory mechanisms [2, 6, 8, 37]. Surprisingly, in this model we found that the decrease in the levels of Th2 cytokines was not compensated by a rise of the Th1 cytokine IFN- γ , which remained below the detection limit of the assay. A similar response has also been reported by Ohno et al. [38], who also found a decrease in the levels of IL-4, IL-5 and IFN- γ upon administration of *Bifidobacterium bifidum* G9-1. However, in our work, the detection of higher levels of specific IgG2a does account for the development of a Th1 response in *E. faecalis* CECT7121-treated mice. The failure to detect IFN- γ in splenocyte culture supernatants might be explained by different ex vivo release kinetics.

The strain of mice employed in this work (BALB/c) is known for its tendency to establish Th2 responses [39] and its difficulty to establish persistent Th1 responses, mainly in immunization protocols employing high concentrations of Al(OH)₃ as adjuvant. The latter fact might also account for the lack of secretion of IFN- γ ex vivo.

In this work, no differences between groups were found in the levels of the homeostatic cytokines IL-10 and IL-12 which play a crucial role in the Th1/Th2 balance.

Over the last two decades, the imbalance which is produced between Th1 and Th2 responses has constituted the basis for the understanding of allergic responses, however, another subset of T cells, termed regulatory T cells (Treg) has been described to be implicated in the

regulation of such processes [40, 41]. In healthy individuals, it has been demonstrated that Treg cells can inhibit the activity of Th2 cells against allergens by suppressing cell proliferation and cytokine production. On the contrary, in allergic individuals, such suppression is not observed [42, 43].

Treg cells can control and modify the development of allergic diseases through different mechanisms [43]. These cells can inhibit the activation of specific Th2 cells, thus diminishing the secretion of IL-4, IL-5, IL-13 and IL-9, cytokines that are essential during the effector phase of allergic reactions [43]. Treg cells are also known to suppress allergic inflammation through both a direct and indirect action on mast cells, basophils and eosinophils [43, 44]. Furthermore, it has been demonstrated that Treg cells can also have a direct effect on B cells to suppress the secretion of allergen-specific IgE [43, 44]. The effects of Treg cells are exerted through a wide range of soluble and membrane factors such as IL-10, TGF- β , CTLA-4, the programmed death-1 or the type 2 histamine receptors [44]. Even though in this work we did not demonstrate an increase in the levels of IL-10 which would account for a recruitment of IL-10 producing CD4⁺ Treg cells to the spleen, a role for such cells in the suppression of the Th2 response cannot be ruled out. Works dealing with the modulation of systemic allergic diseases are scarce, among which, Torii et al. [24] could not demonstrate the secretion of IL-10 in splenocytes culture supernatants after the intraperitoneal administration of the allergen in animals treated i.g. with *Lactobacillus acidophilus* L-92. Nevertheless, considerable levels of TGF- β were found by these authors in culture supernatants of Peyer's patches, demonstrating a local regulatory effect of the probiotics [24]. Further studies are needed to elucidate the mechanisms by which *E. faecalis* CECT7121 exerts its regulatory properties in our model.

The immunological mechanisms involved in allergic diseases can be divided in two phases: the priming and memory, and the effector phases. During the priming phase, the differentiation and clonal expansion of allergen-specific Th2 cells lead to the secretion of IL-4 and IL-13 which are essential for the synthesis of IgE that then binds to the high affinity receptors on mast cells and basophils cell membranes. The effector phase is initiated upon a second encounter with the allergen that produces the cross-linking of IgE-FcRI complexes on the cell surface, leading to cell activation and the subsequent release of soluble mediators that are responsible for the signs and symptoms observed in allergic patients. The effect induced by the administration of *E. faecalis* CECT7121

during the priming phase was demonstrated by the decrease in the levels of Th2 cytokines and allergen-specific IgE. To assess the effect of this bacterium on the effector phase, the active anaphylaxis test was performed in immunized animals (pre-treated and control mice). In line with the decrease in the levels of allergen-specific IgE, animals belonging to the *E. faecalis* CECT7121/OVA group subjected to the ACA test displayed a less intense reaction when compared to control animals after the challenge with the allergen. The less intense dye extravasation could be due to either a decreased number of sensitized mast cells or a decreased degranulation of such cells. As stated above, the antigen-induced cross-linking of IgE bound to Fc receptors induces the synthesis of allergy mediators and degranulation. In an attempt to demonstrate if *E. faecalis* CECT7121 can also act directly to inhibit cell degranulation, the release of β -hexosaminidase was performed employing human basophils from allergic and healthy individuals. In these experiments it

was observed that the bacterium was able to inhibit such process, thus demonstrating that an anti-allergic effect of this probiotic can also be achieved by this mechanism. These observations are in line with those of Harata et al. [45], who demonstrated that certain non-pathogenic commensal bacteria present a strong mast cell degranulation inhibitory activity.

To sum up, our work allows concluding that the i.g. administration of *E. faecalis* CECT7121 diminishes the establishment of the allergic immune response in the employed murine model and suggest that the use of this probiotic strain would be useful in the prophylaxis of IgE-dependent allergic diseases.

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