

Oral therapeutic administration of a probiotic mixture suppresses established Th2 responses and systemic anaphylaxis in a murine model of food allergy

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Abstract

Background: No effective treatment is available for food allergy and its primary management still consists of avoiding relevant allergens. Probiotics are claimed to beneficially affect the immune system. We sought to investigate the therapeutic potential of VSL#3 probiotic mixture on specific immune responses and anaphylactic reaction induced in mice by the major food allergen shrimp tropomyosin (ST).

Methods: The cytokine production by spleen cell from ST-sensitized mice upon allergen re-stimulation in the presence of VSL#3 was analysed. Next, the effects of oral administration of VSL#3 on allergen-induced anaphylaxis and Th2 response in the murine model of food allergy to ST was investigated by evaluating symptom score and histamine content in the faeces after allergen challenge, antibody response in serum and faeces, and cytokine and transcription factor expression in the jejunum.

Results: The *in vitro* studies on mouse spleen cells indicates that the VSL#3 preparation has the capacity to shift a polarized Th2 response to a Th1/T regulatory-type profile. Oral therapeutic administration of VSL#3 to ST-sensitized mice significantly reduces symptom score and histamine release in the faeces following allergen challenge, as well as specific IgE response. In the jejunum, IL-4, IL-5 and IL-13 tissue content was significantly reduced, whereas FOXP3 and IL-27 mRNA expression, IL-10, TGF- β and IFN- γ tissue content were up-regulated.

Conclusions: Oral therapeutic treatment with the probiotic mixture VSL#3 is effective in redirecting allergen-specific Th2-polarized immune responses towards Th1-T regulatory responses and in the protection against anaphylactic reactions induced by the allergen in a murine model of food allergy.

Food allergy is defined as an abnormal immunologic reactivity to food proteins, characterized by expansion and activation of Th2 cells resulting in production of food specific IgE antibodies. The activation of Th2 population most likely results from inappropriate control of these cells by regulatory T cells operating by various mechanisms (1), associated with a breach in oral tolerance (2, 3). IgE-mediated food allergy has been estimated to affect 1–2% of the adult population, and up to 5–7% of children (4). The rapid increase of allergy prevalence around the world over the last decades (5) has highlighted the need to develop preventive or therapeutic

strategies, but up to now no effective treatment is available for food allergy and its primary management still consists of strictly avoiding relevant allergens (6).

Among the food allergens of animal origin, shellfish are one of the most frequent sources of food allergy in adult population. Shrimp tropomyosin (ST) is the major allergen of the majority of shrimp species (7), being recognized by more than 80% of shrimp allergic subjects (8). ST accounts for most of the allergenic activity of whole shrimp extract (9).

There is evidence from epidemiological studies that the development of allergy in genetically predisposed subjects is associated with a different proportion of the bacterial phyla belonging to the gut microbiota in comparison with normal subjects (10, 11). From this observation, the rationale for

Abbreviations

CT, cholera toxin; IG, intragastric; ST, shrimp tropomyosin.

using 'normal commensal bacteria' in the attempt to restore in the gut conditions as close as possible to the healthy subjects is logically derived, and has been applied to the prevention and therapy of allergic diseases (12, 13).

Recent studies in both human and experimental animal models highlight the importance of immune modulation exerted early in life by the commensal bacterial flora in the gastrointestinal tract, in directing the development of tolerance (3). Probiotics, which are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (14), are claimed to beneficially affect the immune system in several physiological and pathological conditions including inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), obesity, type I diabetes and allergies.

Clinical studies on different allergic populations yielded controversial results on the efficacy of prophylactic or therapeutic treatments with probiotics (15–18), and the anti-allergic effects of probiotic bacteria are still not completely defined. In particular, the combined effects of mixtures of different species of probiotic bacteria have been only in part explored in suitable animal models to better understand the *in vivo* processes that modulate allergy responses.

In murine models and clinical studies, VSL#3 probiotic preparation, a high concentration mixture of eight live freeze-dried bacterial species that are normal components of the human gastrointestinal microbiota, has been shown to prevent or ameliorate several gastrointestinal pathologies, such as IBD (19, 20), irritable bowel disease (21), pouchitis (22, 23), chemotherapy-induced diarrhoea (24) and autoimmune diabetes (25). We also reported in a recent study (26) that prophylactic intranasal treatment with VSL#3 preparation is able to prevent the development of Th2-biased responses in a murine model of sensitization to a clinically relevant respiratory allergen.

Recently, we developed a novel food allergy model in C3H/HeJ mice intra-gastrically (IG) sensitized with ST (27), characterized by the presence of anaphylaxis symptoms after IG challenge with the antigen.

On these bases, the aim of this study was to investigate the immunomodulatory capacity of VSL#3 probiotic mixture on specific immune responses and anaphylactic reaction induced in mice by the food major allergen ST. To test our hypothesis, we initially analysed the cytokine production by spleen cell isolated from ST-sensitized mice *in vitro* re-stimulated with the allergen in the presence of VSL#3. Next, we investigated the *in vivo* therapeutic potential of oral administration of VSL#3 on allergen-induced Th2 response and anaphylaxis in the murine model of food allergy to ST.

Materials and methods

Antigen

Tropomyosin was extracted and purified as previously described from acetone powders prepared with whole bodies of peeled frozen shrimps (27). Protein content of the purified fraction was assayed by the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA) and its purity by SDS-PAGE.

VSL#3 probiotic preparation

The probiotic VSL#3 (VSL Pharmaceuticals, Fort Lauderdale, FL, USA) was kindly provided by Prof. C. De Simone (University of L'Aquila, Italy) as a lyophilized mixture consisting of eight different Gram-positive organisms (*Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. plantarum*, *Bifidobacterium longum*, *B. infantis*, *B. breve*, *Streptococcus salivarius* subsp. *thermophilus*) at the concentration of 3×10^{11} live total bacteria/g, without any excipient. The concentration of each strain is unknown and covered by patent. 2.5 g (corresponding to 7.5×10^{11} live bacteria) was reconstituted in 10 ml of sterile PBS without additives, and serial dilutions were made in sterile PBS for addition to *in vitro* cultures or for *in vivo* oral administration.

Animals and *in vivo* experimental protocols

Eight-week-old female C3H/HeJ mice were purchased from Charles River (Calco, Italy). Animals were housed in the Animal Care Unit of the Istituto Superiore di Sanità and treated according to the local guidelines for animal care (D.L. 116/92). Groups of 5–10 mice were treated according to the sensitization, challenge and therapeutic protocols illustrated in Fig. 1. Age-matched naïve mice were used as controls.

Sensitization

Immunization was performed according to (28, 29) with some modifications. Mice were immunized by IG administration of 100 µg of purified ST/mouse together with 10 µg/mouse of cholera toxin (CT) (Gentaur, Brussels, Belgium) as an adjuvant in a total of 400 µl of isotonic bicarbonate solution on days 0, 7, 14, 21 (Fig. 1). Mice were bled from the retro-orbital plexus on days 0 and 28. On days 0 and 28 faecal samples were also individually collected and immediately extracted as already described (27). Serum samples and faecal extracts were stored at -20°C until analysis.

Allergen challenge and evaluation of anaphylaxis reaction

Mice were challenged 2 weeks after the last immunization (Fig. 1, day 35) by receiving one IG dose of ST (600 µg/mouse). The appearance of symptoms of systemic anaphylaxis was observed during 1 h after challenge. Symptoms were evaluated according to the scoring system reported by Li et al. (30): 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhoea, pilar erection, reduced activity and/or decreased activity with increased respiratory rate; 3, wheezing, laboured respiration and cyanosis around the mouth and the tail; 4, no activity after prodding or tremor and convulsion; 5, death.

Faecal samples were individually collected within 30–60 min after challenge, and processed as described above.

VSL#3 therapeutic treatment

From day 40 to day 60 mice were daily administered with 50 µl/mouse of live VSL#3 mixture corresponding to 7.5×10^8 bacteria through the oral route by using a Gilson micropipette (Gilson Italia, Milan, Italy). Control groups

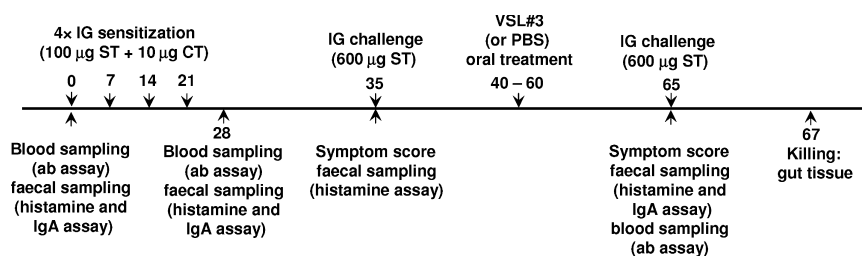


Figure 1 Experimental design of *in vivo* sensitization and probiotic treatment. Groups of C3H/HeJ mice ($n = 5$ – 10 /group) were sensitized four times by IG gavage with $100 \mu\text{g}$ of ST plus CT. After sensitization, anaphylactic reaction was induced on day 35 by IG challenge with ST. From day 40 to day 60, mice were daily administered with live VSL#3 mixture through the oral route. On day 65,

mice were re-challenged as above. Blood samples were collected on days 28 and 65, and faecal samples were collected on days 0, 28, 35 and 65. On day 67, mice were killed to collect gut tissue. In separate experiments, mice were killed after the fourth IG immunization to collect spleen cells for *in vitro* allergen re-stimulation and probiotic co-culture.

received orally $50 \mu\text{l}$ of sterile PBS. On day 65, mice were re-challenged as described above. Serum and faecal samples were collected to evaluate antibody and histamine levels after the treatment. On day 67, mice were killed to collect gut tissue.

Measurement of histamine levels in faecal extracts

Histamine was assayed on faecal samples using an enzyme immunoassay kit (Immunotech, Marseille, France), validated for the use on faecal material (26, 31). Faecal extracts were analysed at different dilutions: 1 : 50 for preimmune (day 0) and postimmunization (day 28) samples, 1 : 200 for challenge samples (day 28 and day 65).

In vitro stimulation of immune spleen cells: cytokine production, fluorescent-activated cell sorting (FACS) analysis and intracellular cytokine staining

Individual spleen cell suspension from ST-sensitized mice were prepared and cultured as previously described (27), with the optimal dose of antigen ST ($10 \mu\text{g}/\text{ml}$) alone or in combination with live VSL#3 ($10^7/\text{ml}$, as determined in previous set up experiments) for 72 h. Co-culture with live VSL#3 alone was also set as a control. Cell viability was checked by dye exclusion assay (cell count after Trypan blue staining) at 48 and 72 h with different concentrations of VSL#3 (10^6 , 10^7 , $10^8/\text{ml}$ or medium alone). The supernatants were then collected and stored at -20°C for cytokine analysis. Levels of cytokines IL-5, IFN- γ , IL-10 (BD Biosciences Pharmingen, San Jose, CA, USA) and IL-13 (R&D Systems, Minneapolis, MN, USA) were determined by sandwich ELISA kits, according to the manufacturers' instructions. Sensitivity for each assay was 4 (IL-5, IL-10), 15 (IFN- γ), 1.5 pg/ml (IL-13). To identify T cell subpopulations, FACS analysis was performed using antibodies for T-cell surface markers, including anti-CD3 and anti-CD4 (eBiosciences, San Diego, CA, USA). To determine intracellular cytokine production, spleen cells from *in vitro* culture above described were incubated for 4 h with PMA ($10 \text{ ng}/\text{ml}$) and ionomycin ($250 \text{ ng}/\text{ml}$) (Sigma Italia, Milan, Italy) in the presence of Golgi Stop (BD Biosciences). At the end

of the stimulation period, cells were harvested, washed and stained for surface markers. Cells were fixed and permeabilized with BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer's protocol, and then stained with anti-IFN- γ , anti-IL-4, anti-IL-10 and anti-IL-17 antibodies (BD Biosciences). The cells were analysed using a FACScanto system (BD Biosciences), and the results were processed using BD FACSDiva software (BD Biosciences).

Evaluation of antibody response

Total IgA antibodies were evaluated in faecal extracts of individual mice by sandwich ELISA kit (BD Biosciences Pharmingen) according to the manufacturer's instructions, and expressed as ng/ml.

Shrimp tropomyosin-specific IgA response was monitored by in-house ELISA in faecal extracts of individual mice as previously described (27). Faecal extracts were diluted 1 : 10. Results were expressed as arbitrary units (AU)/ml.

Individual serum samples were also assayed for ST-specific IgE, IgG1 and IgG2a antibody response, as described in (27).

Cytokine and transcription factor expression in the gut

On the basis of previous studies (27), at the end of the experiment (day 67), a 2-cm section of the jejunum immediately distal to the duodenum was collected from individual mice treated with VSL#3 or PBS, to evaluate the effects of probiotic therapeutic treatment on the expression of cytokines and transcription factors at the local level. Two 1-cm distinct fragments were cut.

One fragment was immediately placed in RNeasy lysis solution (Qiagen, Crawley, UK) and treated according to the manufacturer's instructions. In order to evaluate quantitative differences in the expression of FOXP3 and IL-27 in the jejunum after probiotic treatment, a real-time PCR was performed on the cDNA, by using the SYBR Green PCR Master Mix (Applied Biosystem, Perkin-Elmer Italia, Milan, Italy) according to the manufacturer's instructions. The following specific primers were used:

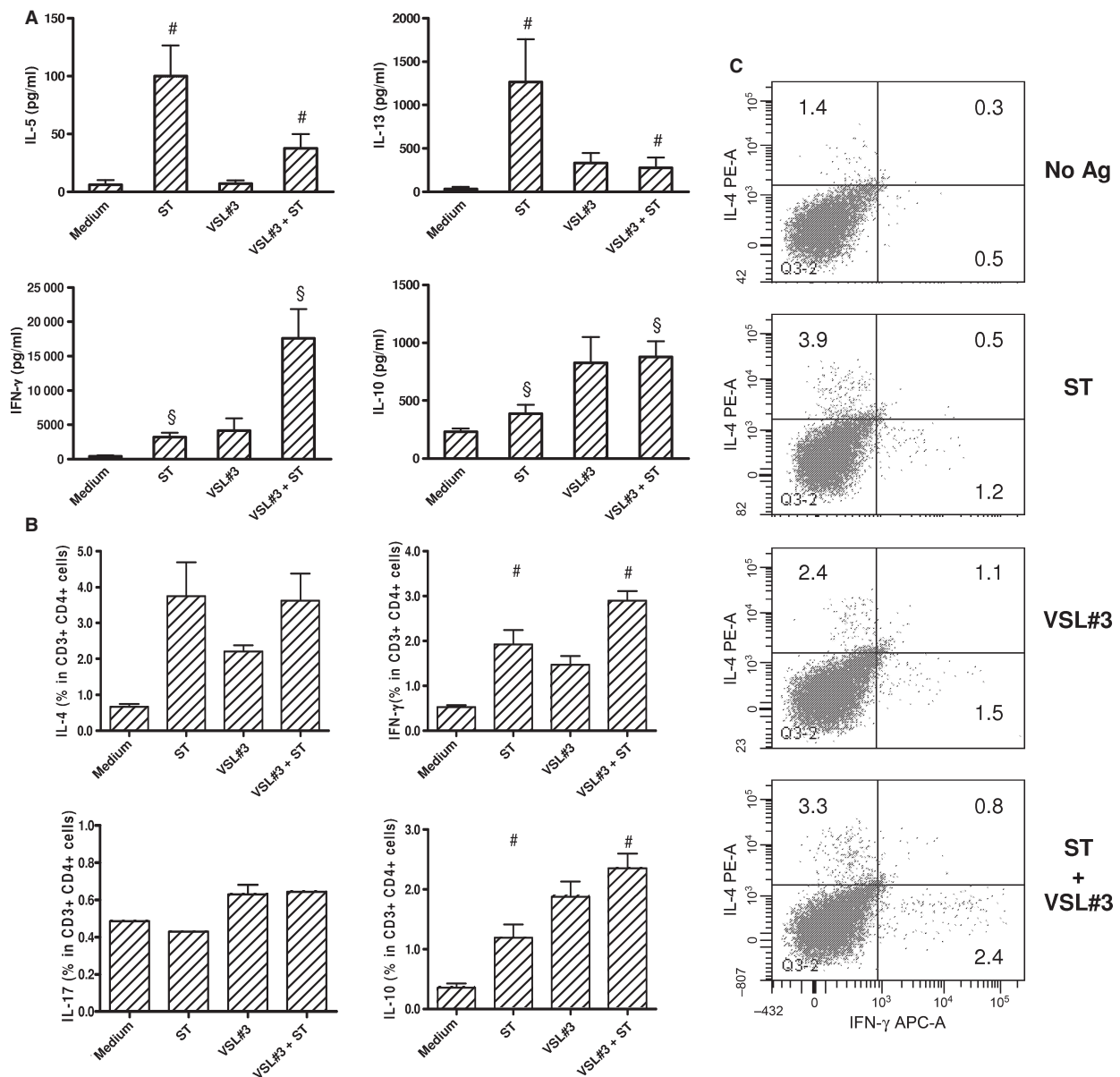


Figure 2 *In vitro* co-culture with VSL#3 modulates allergen-induced T cell responses in spleen cells from ST-sensitized mice, *in vitro* re-stimulated with the allergen in the presence or absence of live VSL#3. (A) IL-5, IL-13, IFN- γ and IL-10 production by individual spleen cells from ST-sensitized mice, was evaluated by ELISA in culture supernatants. (B) The frequency of IL-4+, IFN- γ +, IL-10+ and IL-17+ CD3+ CD4+ T cells was evaluated by intracellular stain-

ing after stimulation with PMA plus ionomycin for 4 h. (C) A representative FACS diagram for IL-4+ and IFN- γ + frequency in CD3+ CD4+ gated cells. In (A) and (B), [#] $P < 0.05$, ^{\$} $P < 0.005$ in culture re-stimulated with ST plus VSL#3 vs cultures re-stimulated with ST allergen only. Experiments were independently repeated at least four times and each bar represents mean $\pm$ SEM of data from all experimental replicates.

FOXP3 forward AGAGTTCTTCCACAACATGGACTACTT;
FOXP3 reverse GATGGCCCATCGGATAAGG;
HPRT (used as 'normalizer gene') forward CTGGTGAA-AAGGACCTCTCG;

HPRT reverse TGAAGTACTCATTATAGTCAAGGGCA.

Interleukin-17 and IL-27 expression has been evaluated by RT² qPCR Primer Assay (SABiosciences Primers, Frederick, MD, USA). All the primers have been used at the final

concentration of 0.4 μ M. Results for each gene have been analysed by comparison to the HPRT values. Fold increases have been calculated according to the $2^{-\Delta\Delta C_t}$ method.

The other fragment was used to prepare total protein extracts according to Boirivant et al. (32). Briefly, tissue fragments were lysed in suitable volume of lysis buffer (Hepes 0.01 M pH 7.9, EDTA 0.001 M, KCl 0.06 M, Nonidet P40 0.2%, dithiothreitol 0.001 M, phenylmethylsulfonyl fluoride

0.001 M, aprotinin 10 µg/ml, leupeptin 10 µg/ml, Na₃VO₄ 0.001 M, NaF 0.001 M) (Sigma-Aldrich, Milan, Italy), incubated 1 h on ice, under gentle shaking every 5 min. After a centrifugation of 30 min (16 000 g, 4°C), supernatants were collected and stored at -80°C. Total protein content was evaluated by the Bio-Rad protein assay (Bio-Rad). Jejunum extracts were then analysed for the content of IL-10, TGF-β, IL-4, IL-5, IL-13 and IFN-γ by sandwich ELISA kits, according to the manufacturers' instructions. Sensitivity for IL-4 and TGF-β assay was 4.00 and 4.61 pg/ml, respectively. Results were expressed as pg/mg of total protein.

Statistical analysis

Groups of 5–10 mice/treatment were used throughout the study. Data were presented as means with SEM. The Mann-Whitney *U*-test (rank sum) or the Wilcoxon matched-pairs test when appropriate were used for the nonparametric analysis of differences between different experimental groups of mice or different *in vitro* treatments. *P*-values of less than 0.05 were considered statistically significant.

Results

Effects of VSL#3 co-culture on Th polarization

In initial studies we evaluated the potential of VSL#3 to *in vitro* modulate allergen-induced cytokine production by spleen cells from mice immunized with ST. Spleen cells were re-stimulated *in vitro* with the same allergen in the presence of 10⁷ live VSL#3 bacteria. Spleen cell viability was checked at 48 and 72 h by dye exclusion assay with different concentrations of VSL#3, and 74.96% ± 8.65 (mean ± SEM) of viable cells were recovered, without significant differences among the various culture conditions (data not shown).

High IL-5 and IL-13 production in culture supernatants was induced in response to allergen-specific re-stimulation (Fig. 2A), as expected in a typical Th2 response. The co-incubation with VSL#3 preparation significantly (*P* < 0.05) reduced allergen-stimulated IL-5 and IL-13 production. Reduction of Th2 cytokines was associated with a significant increase of IFN-γ production by spleen cells stimulated with the antigen in the presence of live VSL#3 bacteria, which are also able to significantly increase IL-10 production both in the presence or in the absence of the antigen (Fig. 2A).

Intracellular cytokine staining for IL-4, IFN-γ, IL-10 and IL-17 showed that the frequency of IFN-γ+ and IL-10+ CD4+ T cells was significantly (*P* < 0.05) increased by VSL#3 co-incubation during allergen re-stimulation, whereas the frequency of IL-4+ CD4+ T cell did not change in the presence of VSL#3 (Fig. 2B,C). IL-17 expression by CD4+ T cells was not induced at spleen level by allergen re-stimulation nor affected by VSL#3 co-culture (Fig. 2B).

Altogether, these results indicate that the VSL#3 preparation is able to induce a Th1/T regulatory-type response associated with a reduction of the antigen-specific Th2 cytokine production.

Oral administration of VSL#3 after ST sensitization protects mice against anaphylactic reactions

On the basis of the results obtained in the *in vitro* studies, we then investigated whether the probiotic mixture VSL#3 was able to affect an *in vivo* established allergen-specific Th2 response, when administered by the oral route in a therapeutic setting to ST-sensitized and ST-challenged mice.

As previously demonstrated and confirmed in the present study, mice orally sensitized with ST showed anaphylactic symptoms upon the first allergen challenge with severity ranging from 2 to 4 according to the scoring system applied (Fig. 3A). Mice were then treated for 3 weeks with daily oral administrations of VSL#3 or PBS as control. Five days after the last oral treatment, mice were re-challenged with a

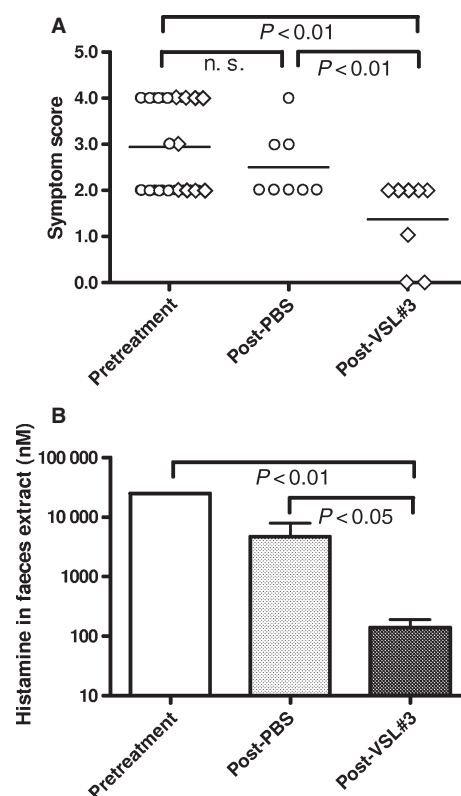


Figure 3 Oral therapeutic administration of VSL#3 protects mice against allergen-induced anaphylactic reactions. IG challenge was performed before (day 35: 'pretreatment') and after probiotic therapeutic treatment (day 65 'post-PBS' or 'post-VSL#3'). Symptom scores (A) were recorded as described in the 'Methods' section, during a 1-h observation period after challenge. Solid circles and solid diamonds indicate individual control (post-PBS) or treated (post-VSL#3) mice, respectively, after treatment. Open circles and diamonds indicate the same individual mice before treatment. Histamine levels (B) in faecal samples collected within 30–60 min at the first challenge on day 35 and at the second challenge on day 65 after treatment, are shown as mean ± SEM. Data are the combined results of three separate experiments.

high IG dose (600 µg) of ST, and the anaphylactic reaction was monitored and scored as in the first challenge. Histamine levels were evaluated in faecal samples collected during the anaphylactic reaction. For each experimental group (VSL#3- and PBS-treated mice), symptom scores and histamine release were compared with those recorded before probiotic treatment. Therapeutic treatment with VSL#3 significantly ($P < 0.01$) reduced the symptom score as compared with the score recorded in the control mice treated with PBS (Fig. 3A). The same level ($P < 0.01$) of statistically significant difference was found comparing the symptom score recorded in the VSL#3-treated mice with the score evaluated in all mice in the first challenge before therapeutic treatment (pretreatment mice vs post-VSL#3 mice). Consistently with the evaluation of the anaphylactic response, faecal histamine levels were significantly decreased by the VSL#3 treatment, both in comparison with the PBS-treated group ($P < 0.05$) and with the levels found before therapeutic treatment ($P < 0.01$) (Fig. 3B).

Altogether, these observations suggest that oral therapeutic administration of VSL#3 can significantly protect mice against allergen-induced anaphylactic reactions and suppress allergic inflammation in the intestinal mucosa.

The protection against anaphylaxis was associated at the gut level with the increase of IgA levels in the faecal extracts of probiotic-treated mice when compared with those found before treatment, whereas no significant changes were induced in control mice (Fig. 4). Both total and ST-specific IgA were up-regulated in the majority of VSL#3-treated mice, even if statistical significance was not reached in the evaluation of specific IgA, probably due to the use of AU and in-house standard reference in this assay.

Therapeutic administration of VSL#3 induces Th1/Tregulatory responses in the gut and suppresses local Th2 responses

In a previous study we have identified the jejunum as the site preferentially affected by the oral sensitization with antigen, where it induced the skewing of the response towards a prevalent Th2-like phenotype (27). Therapeutic treatment with VSL#3 down-regulates the allergen-induced Th2 response at the gut level, as indicated by the significant decrease in IL-4, IL-5 and IL-13 tissue content when compared with the amount measured in mice treated with PBS after sensitization (Fig. 5A). According to the results of the *in vitro* studies (Fig. 2C,D), probiotic *in vivo* modulation induces a shift towards a prevalent Th1/T regulatory response in the jejunum, characterized by increased FOXP3 mRNA expression, increased IL-10 and TGF- β tissue content (Fig. 5B), increased IL-27 mRNA expression and IFN- γ tissue content (Fig. 5C). IL-17 mRNA expression in the jejunum was significantly increased in allergen sensitized and challenged mice, in comparison with naïve mice (data not shown), but it was not modulated by the probiotic treatment (Fig. 5C).

The down-regulation of local Th2 response was paralleled by changes of ST-specific antibody response in serum. After therapeutic treatment with VSL#3, mice that received the probiotic preparation showed significantly decreased levels of specific IgE and increased levels of IgG2a, whereas control mice showed no significant changes in the levels of serum ST-specific antibody (Fig. 6).

These observations further support the therapeutic potential of VSL#3 in controlling the established Th2 response associated with *in vivo* ST-sensitization.

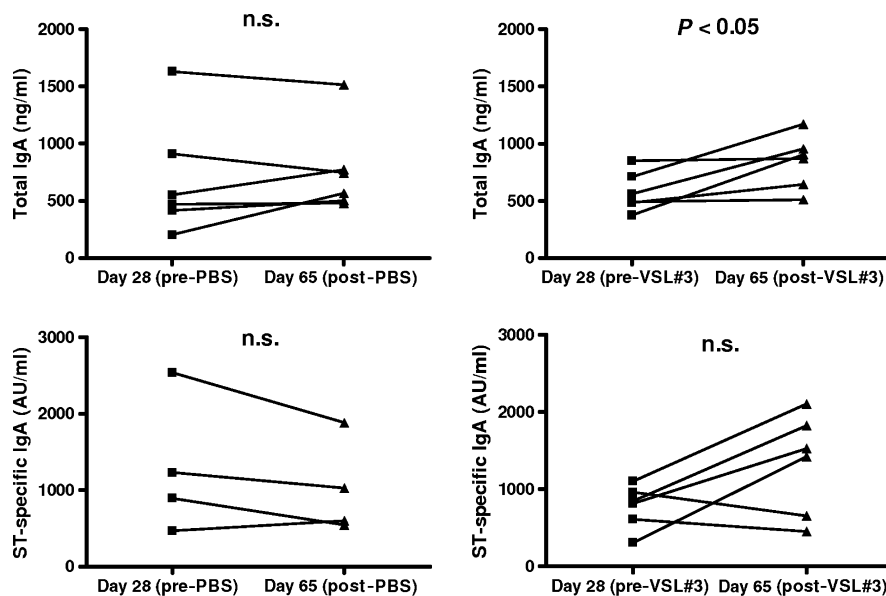


Figure 4 Probiotic treatment increases total IgA production at the gut level. Total and ST-specific IgA were evaluated in faecal samples collected before (day 28) and after (day 65) therapeutic treat-

ment, from groups of mice treated with VSL#3 (right) or PBS (left) as control. Data are reported as individual values from a representative experiment out of five.

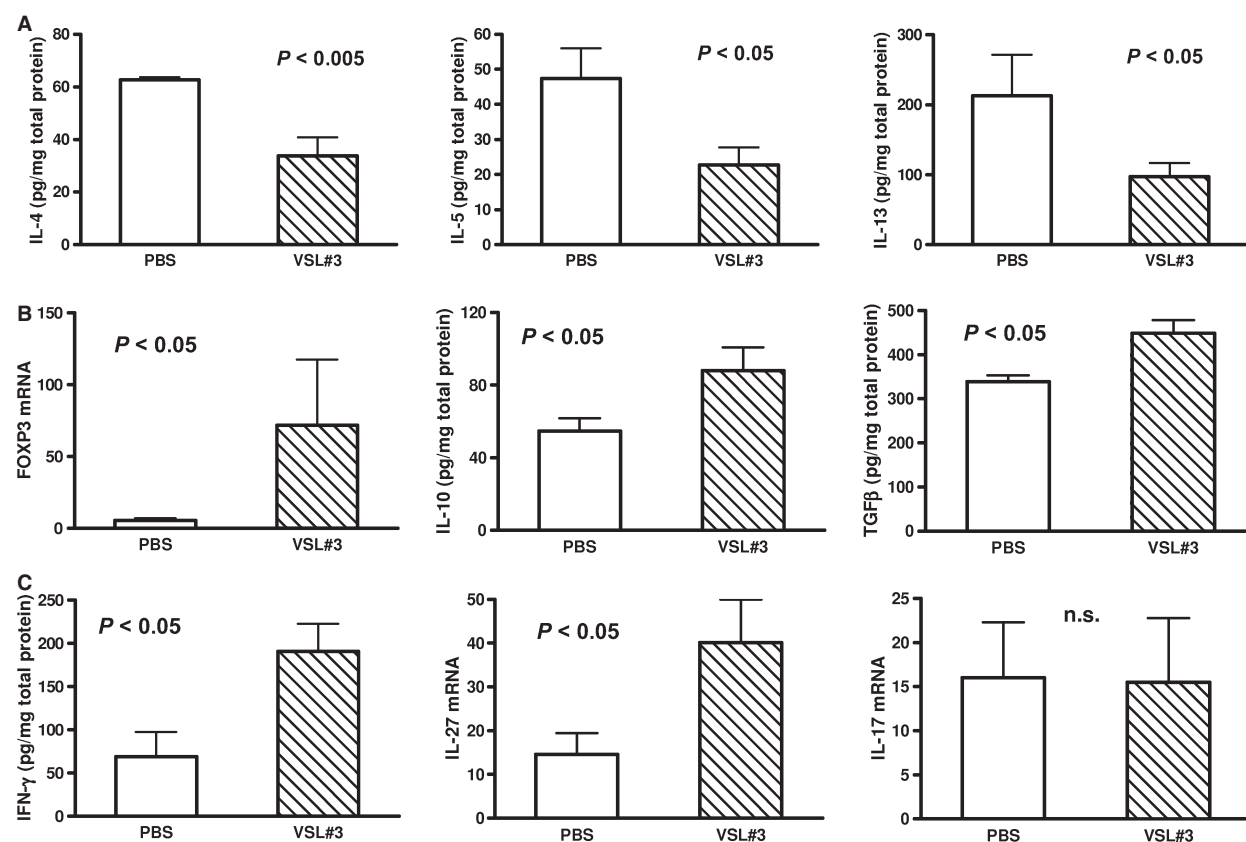


Figure 5 Therapeutic treatment with VSL#3 down-regulates the Th2 response in the jejunum tissue, where it induces a prevalent Th1/T regulatory profile. Th2 (A), T regulatory (B) and Th1/Th17 (C) polarization was evaluated in whole tissue homogenate from jejunum samples of individual mice. Cytokine (IL-4, IL-5, IL-13, IL-10, TGF-β, IFN-γ) content was evaluated by sandwich ELISA. Results

are reported as mean ± SEM for $n = 5$ mice/group. FOXP3, IL-17 and IL-27 mRNA expression in the same tissue samples from individual mice was assessed by real-time PCR. All the results were normalized against HPRT expression and are reported as mean ± SEM for $n = 5$ mice/group. Data are representative of three separate experiments.

Discussion

Recent *in vivo* studies in murine models have supported the promise of probiotics for preventing and treating food allergy, associated with the shift from the pathogenic Th2 response to a Th1/T regulatory response (33–36).

The main results of the present study demonstrate that therapeutic oral administration of VSL#3, a mixture of eight probiotic strains widely used in the clinical management of inflammatory chronic bowel diseases (20), is capable of reducing the intestinal inflammation and other symptoms associated with food anaphylaxis in a murine model induced by oral sensitization with ST.

Indeed, in preliminary *in vitro* experiments, when we re-stimulated spleen cells from ST-sensitized mice with ST in the presence of live VSL#3, this preparation was able to down-modulate the already established allergen-specific Th2 response. VSL#3 co-culture significantly affected the Th2 cytokine profile resulting from allergen re-stimulation, by suppressing IL-5 and IL-13 production and promoting the secretion of Treg/Th1 cytokines such as IL-10 and IFN-γ.

This is mirrored by a higher frequency of IFN-γ and IL-10 producing T cells, whereas the frequency of IL-4 producing cells is not affected. These results suggest that VSL#3 is able to expand regulatory T cell populations which in turn inhibited the production of Th2 cytokines.

We demonstrated in a previous study that the VSL#3 immunomodulatory activity *in vitro* exerted on allergen immune cells correlated with the capacity to *in vivo* prevent the sensitization to the major allergen of *Parietaria judaica* (26). On this basis, the *in vitro* immunomodulatory effects of VSL#3 co-culture on ST-induced Th2 response prompted us to evaluate such preparation in the therapy of Th2-mediated allergic inflammation elicited *in vivo* by oral immunization with the food allergen ST.

To assess the *in vivo* effects of oral administration of VSL#3 in a therapeutic setting, we used the murine model of ST-sensitization previously developed in our laboratory (27). In this model, feeding the allergen induces T cells that secrete high levels of Th2 cytokines and promotes ST-specific IgE, IgG1 and IgA production. Subsequent IG challenge with the same allergen results in systemic anaphylaxis, involving multiple target organs, including gut, respiratory system and skin,

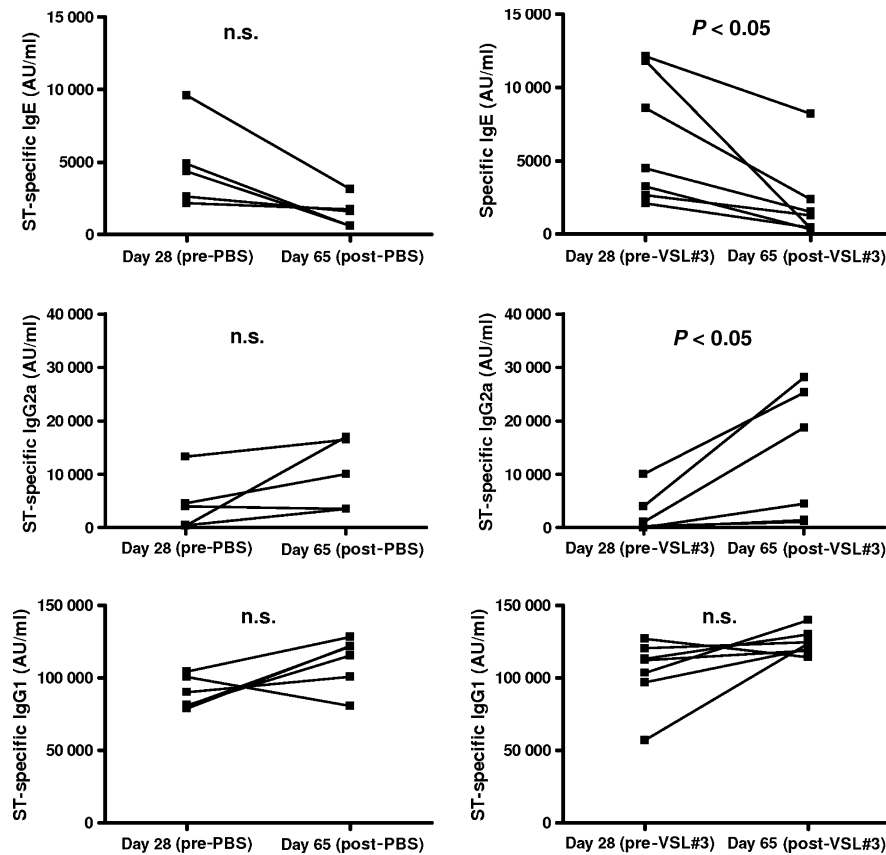


Figure 6 Probiotic treatment decreases ST-specific IgE levels and increases ST-specific IgG2a in the serum. ST-specific antibodies were evaluated in serum samples collected before (day 28) and

after (day 65) therapeutic treatment, from groups of mice treated with VSL#3 (right) or PBS (left) as control. Data are reported as individual values from a representative experiment out of five.

thus confirming its immunologic similarity to human ST anaphylaxis. The exclusive presence of IL-13 expression in the jejunum and the increase of IL-4 expression in the same tissue suggest that the jejunum could be the main gut site in which allergen-specific Th2 sensitization occurs. Considering the humoral, anaphylactic and cell responses to orally ingested allergens, this model appears suitable for examining the anti-allergic effect of a modulating agent.

In this system, our present results demonstrate that VSL#3 is able to redirect the ongoing Th2 response specific for ST. At the gut level, a significant decrease of Th2 cytokines, such as IL-4, IL-5 and IL-13, and a parallel increase in the expression of Th1 and regulatory cytokines, such as IFN- γ , IL-10, TGF- β and IL-27, was observed in the jejunum homogenates. A contribution of Th17 subpopulation to the Th2-driven inflammatory response can be suggested by IL-17 up-regulation in the jejunum; however, it was not affected by the *in vivo* probiotic modulation.

Notably, local and systemic anaphylactic responses induced by allergen challenge were significantly reduced, as demonstrated by the significant reduction in symptom score and in the histamine levels measured in the faeces collected after allergen challenge.

The local down-regulation of Th2 response induced by probiotic modulation was associated with a significant increase in faecal IgA levels, probably induced by TGF- β up-regulation. At the systemic level, a prevalent shift of the ST-specific humoral response towards the Th1-driven isotype IgG2a, with a significant reduction in serum specific IgE levels, was observed in mice therapeutically treated with VSL#3 mixture. The up-regulation of ST-specific IgG2a was associated with the increase of IFN- γ production in the culture supernatants from spleen cells from probiotic-treated mice *in vitro* re-stimulated with ST, when compared with spleen cells from control mice (data not shown).

These results are in line with those reported by other *in vivo* studies on murine models of food allergy (33–36). However, as these studies mostly pointed to the evaluation of the prophylactic potential of single or multiple probiotic strains orally administered before or during allergen sensitization, our study provides the first functional evidence that a mixture of eight different probiotic species is able to inhibit an established Th2-driven inflammation when administered in a therapeutic setting after food allergen intragastrical immunization and symptom elicitation.

Microbial product-mediated therapeutic effects in food allergy might involve complex mechanisms, with the Th1 response playing a major role. The down-regulation of Th2 response in VSL#3-treated mice is accompanied by the induction of Th1-driven and regulatory cytokines and, notably, by the up-regulation of IL-27 expression in the jejunum. This cytokine, belonging to the family of IL-12 cytokines and initially involved in the initiation of Th1 response (37), has been recently demonstrated to exert broad inhibitory effects on Th1, Th2 and Th17 T cells, probably through the expansion of inducible regulatory T cells (38, 39). Interestingly, it has been pointed out that microbial products, through their interaction with TLRs, are able to finely tune the balance between the expression of IL-12 family members and thereby control the outcome of T-cell-mediated inflammation (40). In particular, EBI3 expression, one of the two IL-27 subunits, has been induced in human DCs stimulated by TLR2, TLR4 and TLR9 ligands depending on MyD88 and NF- κ B signalling (41). Moreover, the probiotic strain *Bifidobacterium infantis* has been recently reported to enhance IL-27 *in vitro* production in TGF- β plus IL-6-stimulated mouse spleen cells (42). Other reports demonstrate that it is possible to modify the way T cells respond to cytokines such as IL-12 or IL-27 by selectively enhancing IL-10 secretion in IFN- γ producing Th1 cells, and thereby conveying anti-inflammatory capacity to otherwise pro-inflammatory Th1 cells (43). Such Th1 regulatory cells, induced in the presence of DC-derived IL-27, could be responsible for the therapeutic effect induced in our murine model by probiotic treatment, in synergy and mutual relationship with other regulatory populations directly induced by probiotic components and

characterized by the prevalent production of TGF- β and IL-10.

In conclusion, we have shown that oral therapeutic treatment with a complex mixture of eight live probiotic bacteria, VSL#3, is effective in redirecting allergen-specific Th2-polarized immune responses towards Th1-T regulatory responses and in the protection against anaphylactic reactions induced by the allergen in a murine model of food allergy. VSL#3 treatment suppresses in the target organ pro-inflammatory cytokines, enhances anti-inflammatory cytokines production by cells of the innate immune system which in turn generate regulatory populations able to control allergic inflammation. These findings provide further evidence that oral administration of probiotic mixtures may represent an effective and safe strategy for treating allergic diseases, and support the initiation of human nutritional intervention trials with VSL#3 to investigate VSL#3's therapeutic potential to treat food allergies in adult population, for which no effective treatments based on the pathogenetic mechanisms are available.

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