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Lactobacillus casei Shirota does not decrease the food allergic response to peanut extract in Brown Norway rats[☆]

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ABSTRACT

Probiotics are claimed to beneficially affect the immune system and their involvement in allergy prevention is being investigated extensively. However, the efficacy of probiotics in allergy prevention remains controversial. We investigated whether the probiotic *Lactobacillus casei* Shirota (LcS) could modulate the food allergic response against peanut extract (PE) in Brown Norway (BN) rats. For this purpose BN rats were sensitized to PE (0, 1 and 10 mg/(rat d)) by daily oral gavage and the LcS-groups were additionally orally dosed with 1×10^9 colony forming units LcS/(rat d).

LcS administration had minor effects in animals that were not sensitized. LcS increased Th1-(PE-specific IgG1), whereas the Th1/Th2 ratio based on PE-specific IgG1/PE-specific IgG2a shifted towards Th2 dominance in rats sensitized to PE in the presence of LcS as compared to rats that were sensitized to PE only. LcS stimulated PE-specific IgG2a; but for PE-specific IgE the effect was less clear; whereas there was no overall effect, two rats did not show detectable specific IgE antibodies, whereas the remainder showed significantly increased levels. LcS also resulted in increased numbers of basophilic granulocytes in blood. Furthermore, LcS increased levels of both Th1-(IFN- γ) and Th2-(IL-4) related cytokines in PE stimulated spleen and mesenteric lymph node (MLN) cells, but predominantly IL-4 levels in the supernatants of both spleens and MLNs.

Our study does not support the hypothesis that LcS down-regulates food allergic responses in a BN rat model for food allergy to peanut.

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1. Introduction

The increase of allergies over the last decennia has highlighted the need to develop preventive strategies. Growing concern about the adverse immunological consequences of increasing hygiene in the developed world has fueled the interest in the role of microbial products such as probiotics as tools to prevent and/or treat allergic diseases. The intake of lactic acid bacteria with Th1 stimulating properties is thought to be potentially beneficial in reducing allergic symptoms in atopic individuals.

Immune effects of *Lactobacillus casei* Shirota (LcS) have been demonstrated in rodent models (Baken et al., 2006; de Waard et al., 2001; Ezendam and van Loveren, 2008; Matsuzaki and Chin, 2000; Matsuzaki et al., 1998). Shida et al. (2002) have shown that LcS reduced serum ovalbumin (OVA)-specific IgE and IgG1 responses and diminished systemic anaphylaxis in a mouse model for food allergy. In this model, LcS skewed the immune response towards Th1 immunity, thereby reducing the Th2-mediated allergic responses. LcS stimulated IFN- γ production by mouse splenocytes *in vivo* and *in vitro*, possibly via macrophage-activation and IL-12 production and subsequent enhancement of Th1 cell functions (Kato et al., 1999). Effects of LcS have also been studied in a rat model for Th1-mediated autoimmunity (experimental autoimmune encephalomyelitis (EAE)) and LcS aggravated clinical symptoms indicating that stimulation of Th1 immunity can also induce unwanted effects (Baken et al., 2006; Ezendam and van Loveren, 2008). Taken together these data suggest that

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immunomodulatory effects of LcS can lead to both beneficial as well as adverse effects.

Effects of probiotics on Th1-mediated immunity is only one of the mechanisms that have been proposed to explain the beneficial effects of probiotics on allergic responses. Other mechanisms, such as stimulation of regulatory T cells (Tregs), which suppress both Th1- and Th2-associated responses (Priault et al., 2004; Rook et al., 2004) and a decrease in intestinal permeability (Bongaerts and Severijnen, 2005; Rosenfeldt et al., 2004) have also been suggested. The precise mechanisms by which probiotics may influence allergic responses are still unknown and data on efficacy in human studies are limited. In humans, the effects of probiotics on prevention or alleviation of atopic eczema have been studied most extensively. Administration of probiotics reduced symptoms in infants who suffered from IgE mediated atopic eczema (Rosenfeldt et al., 2003; Sistik et al., 2006; Viljanen et al., 2005). In addition, probiotics reduced the prevalence of atopic eczema in infants that received the probiotic *Lactobacillus* GG in the first 6 months of their lives (Kalliomaki et al., 2001, 2003). However, in other studies (Brouwer et al., 2006; Gruber et al., 2007) no effects on symptoms of atopic eczema were found. Remarkably, in a study with atopic infants that received the probiotic *L. acidophilus* during the first 6 months of their lives, no effects on prevalence of atopic eczema was found, but sensitization to several allergens was increased (Taylor et al., 2007).

These studies in humans demonstrate that probiotics can have beneficial effects on atopic eczema, but that some probiotics can also induce unintended immunostimulation. These differences can probably be explained by the strain-dependent effects of probiotics. To investigate the effects of a probiotic with Th1 stimulating properties on allergic responses, the effects of LcS were studied in a rat model for peanut allergy.

2. Experimental methods

2.1. Animals

Oral sensitization was studied in young Brown Norway (BN) rats. Brown Norway (BN Rij:Hsd) rats obtained from Harlan Netherlands B.V. (Horst, the Netherlands) were used. These rats are bred by Harlan on their basal diet (2018 Teklad Global 18% Protein Rodent Diet), which does contain soy-products as a protein source. At our institute these rats were bred and raised on a peanut and soy protein-free diet (since soy and peanut are cross-reactive legumes; Beardslee et al., 2000; de Jonge et al., 2007; Eigenmann et al., 1996) obtained from Harlan Netherlands B.V. (2016S Teklad Global 16% Protein Rodent Diet). In this experiment, female rats of the third generation (F3) and onwards were used and fed the peanut and soy protein-free diet during the experiments. At study initiation, rats were 3–4 weeks old.

The rats were housed in an animal room maintained at $23 \pm 3^\circ\text{C}$, with a light/dark cycle of 14h/10h, and a relative humidity of 30–70% during the experiment and for at least 7 d prior to study initiation. The animals were housed in stainless-steel wire (Macrolon III) cages in groups of two and had free access to food and tap-water. Approval from the Institutional Animal Ethics Committee was obtained for all experimental procedures involving animals.

2.2. Preparation of peanut extract

Lightly blanched (100°C) peanuts (*Arachis hypogaea*) from the USA Runner cultivar were kindly provided by IMKO Gelria (Doetinchem, The Netherlands) and were stored at 4°C until use. Peanut extract was prepared according to the method described by Koppelman et al. (2003) with some previously described minor modifications (de Jonge et al., 2007). To verify whether the peanut extract contained the major allergens (Ara h1, Ara h2 and Ara h3) SDS-PAGE was performed essentially according to Laemmli (1970) using a BioRad Mini Protean II system (BioRad, Hercules, CA) with 15% acrylamide gels (15 cm $\times$ 10 cm) using a previously described method (de Jonge et al., 2007).

2.3. Probiotic preparation

L. casei Shirota strain (LcS) was cultured for 72 h at 30°C under anaerobic conditions in Man Rogosa Sharpe (MRS) broth (52.5 g MRS (Merck, Darmstadt, Germany)/L distilled water). Thereafter, bacteria were washed twice with saline (0.9% NaCl) and resuspended in saline to a final concentration of 10×10^9 colony forming units

(CFU)/ml (allowing doses of $100 \mu\text{l}/(\text{rat d})$) and stored at 4°C prior to use. The number and viability of the lactobacilli were determined by aerobic culturing on MRS plates (Tritium Microbiologie B.V., Veldhoven, The Netherlands) for 72 h. Rats were dosed with LcS averaging between 1 and 2×10^9 CFU of LcS/(rat d). Every week, $100 \mu\text{l}$ of 10^{-7} , 10^{-8} and 10^{-9} suspensions of bacteria in saline were plated in duplicate on MRS containing agar plates (Tritium Microbiologie B.V., Veldhoven, The Netherlands). After 72 h of cultivation, the number of colonies on each plate was counted in order to check the concentration of bacteria in the suspension used for co-exposure of animals. There was some variation between weeks but always $1-2 \times 10^9$ CFU per daily dose per rat was applied.

2.4. Endotoxin content

The endotoxin content of the PE (33.6 mg PE/ml in PBS) was assessed chromatographically by quantitative limulus amoebocyte (LAL) assay according to the manufacturer's instructions (Cambrex BioSciences, Wokingham, UK) using the method described previously (de Jonge et al., 2007). Endotoxin content in all PE samples was 1 pg or 0.01 endotoxin units (EU)/ml.

2.5. Experimental design oral sensitization

To ensure the use of immunologically naive animals with respect to peanut, pre-study blood samples were tested for peanut-specific antibodies. Rats (eight per experimental group) were then randomly allocated to their dosing regimens, followed by additional reallocation to ensure even starting weight distribution in all dosing groups. Rats were exposed to 1 ml of 0, 1 or 10 mg PE/ml PBS by daily oral gavage, using an 18-gauge stainless-steel animal feeding needle during the first 42 d of the study. In addition, the LcS-groups were also exposed by oral gavage of the probiotic (LcS; 1×10^9 CFU/(rat d)) and the groups that were not exposed to LcS were given saline by oral gavage by the same dosing regimen as the LcS-groups.

In rats that received LcS, oral dosing with LcS started 1 week prior (d –7) to exposure to the allergen and was maintained for 49 d. After commencement of the sensitization, blood samples were obtained from the tail vein at 2-weekly intervals (d 14, 28 and 42 after start sensitization to PE). After coagulation for 1 h at room temperature, the serum samples were centrifuged (Heraeus Sepatech Biofuge 13, Osterode, Germany) for 15 min at 3000 G and 4°C to obtain sera. The sera were stored at -80°C until analyses for allergen-specific IgE and IgG titers and RMCPII levels by ELISA.

After the sensitization period (d 42), animals were no longer exposed to allergen or LcS and one d prior to sacrifice rats were deprived of their food (d 49). At d 49, all rats were orally challenged by intragastric intubation with PE (2 ml of a 50-mg/ml solution). Blood samples for hematology were taken from the orbital plexus (0.5 ml/rat) directly after oral challenge and were collected in K3-EDTA tubes (Terumo Europe N.V., Leuven, Belgium) to prevent clotting. Thirty minutes after the oral challenge, the animals were sacrificed by exsanguination from the abdominal aorta under CO_2/O_2 anesthesia and serum samples were prepared from this blood as described above. Spleens and mesenteric lymph nodes (MLNs) were removed aseptically, weighed and single cell suspensions were prepared for cytokine analysis in the supernatant of spleen and MLN cells after *ex vivo* re-stimulation with PE.

2.6. Ex vivo stimulation of spleen cells: allergen-specific cytokine production

Single cell suspensions were prepared under aseptic conditions by pressing the spleens or MLNs through a $70\text{-}\mu\text{m}$ nylon cell strainer (Falcon, Franklin Lakes, USA). Cells were washed twice and resuspended in 5 ml standard medium (RPMI 1640, 25 mM Hepes concentration, L-glutamine) obtained from Gibco BRL (Breda, the Netherlands) supplemented with 10% heat inactivated fetal calf serum (FCS) obtained from Integro B.V. (Zaandam, The Netherlands) and 1% penicillin–streptomycin (Gibco) with 7.5×10^{-5} M 2-mercaptoethanol (2-ME) (SERVA, Heidelberg, Germany). Cell number was determined using a Coulter Counter (Z2, Coulter Electronics, Mijdrecht, The Netherlands). Cell viability was determined by the Trypan blue cytotoxicity test. The concentration of the cell suspensions was adjusted to 4×10^6 viable cells/ml.

For cytokine measurements, $100 \mu\text{l}$ of the cell suspension was added to the well of 96-well microtitre plates (Greiner tissue culture plate U-shape, no. 650180; Greiner, Frickenhausen, Germany) and incubated with 1 (for IL-4) and 0.5 (for IFN- γ) mg/ml PE for 96 h. IFN- γ present in the supernatants was measured by ELISA (Rat IFN- γ BD OptEIA ELISA set; BD Biosciences, Erembodegem, Belgium) and Reagent set (IFN- γ BD OptEIA Reagent Set B, BD Biosciences) according to the manufacturer's instructions. IL-4 was measured using a rat IL-4 cyto kit obtained from BioSource (Camarillo, CA, USA) as described previously (de Jonge et al., 2007).

2.7. PE-DIG coupling

Peanut protein-digoxigenin (PE-DIG) coupling was established using a DIG-protein labeling kit obtained from Roche Diagnostics GmbH (Mannheim, Germany) according to manufacturer's instructions except for the following changes: $50 \mu\text{l}$ of a 20-mg/ml digoxigenin-3-O-succinyl- ϵ -aminocaproic acid-N-hydroxy-succinimide ester (DIG-NHS) solution in DMSO was added to 1 ml of a 6-mg/ml solution of crude

PE extract in PBS, from this point on the manufacturer's instructions were followed. A 1:2000 dilution of PE-DIG was used in the assay.

2.8. Negative and positive control sera for Ig ELISAs

Pooled serum, obtained from male F3 BN rats that were not exposed to peanut allergens, was used as the negative control. Positive control sera were obtained by sensitizing female F3 BN rats i.p. At d 0 rats were injected with a solution containing 0.5 ml of a 0.2-mg PE/ml solution in sterile PBS mixed with 0.2 ml of a 40-mg/ml alum adjuvant suspension (Pierce) in sterile saline. On d 2, 4, 7, 9 and 11 rats were injected i.p. with 0.5 ml of a 0.2-mg PE/ml solution in sterile PBS. The animals were bled on d 28 by exsanguination from the abdominal aorta. Sera were prepared and stored as described above. These sera were pooled and used as positive control sera in the PE-specific Ig ELISAs.

2.9. PE-specific Ig ELISAs

Positive and negative controls were incorporated on each plate of all Ig ELISA's. After each incubation plates were washed six times with 300 μ l PBS containing 0.1% Tween 20 using an ELISA washer (Auto plate washer ELX405R, Biotek Instruments Inc., Winooski, VT, USA).

2.9.1. PE-specific IgE ELISA

For detection of PE-specific IgE, a sandwich ELISA was performed as described earlier (de Jonge et al., 2007).

2.9.2. PE-specific IgG1 and IgG2a ELISA

For detection of PE-specific IgG1 and IgG2a, 96-well microtitre plates were coated with 100 μ l/well of a 100- μ g/ml solution of PE in PBS the remainder of the procedure was similar to that described before (de Jonge et al., 2007).

To determine the effect of LcS on the Th1/Th2 balance, differences (value LcS-treated groups divided by the value of the non-treated groups) in IgG1 levels (as fraction of their plate's positive control) of sensitized animals (d 42) were divided by the differences in corresponding IgG2a levels (as fraction of their plate's positive control).

2.10. Measurement of rat mast cell protease II (RMCPII)

RMCPII concentrations were determined in d 42 and 49 sera with a RMCPII ELISA kit (Moredun Scientific Limited, Penicuik, Midlothian, Scotland, UK) according to the manufacturer's instructions.

2.11. Differential cell counts

Blood samples, obtained on d 49 were diluted 1:1 in a saline solution (40 g/l) containing Bovine Serum Albumin (Fraction V, purity $\geq$ 96%; Sigma–Aldrich). The number of white blood cells (WBCs), and differential cell counting was performed using a Bayer Advia 120 Hematology System (Bayer HealthCare, Diagnostics division, Tarrytown, NY, USA).

2.12. Statistics

All experimental groups comprised of eight rats. Statistical treatment of results was processed with SPSS Version 13.0 (SPSS Inc., Chicago, Illinois, USA).

Table 1

PE-specific IgG1 and IgG2a titers in time

PE (mg/(rat d))	0	0 + LcS	1	1 + LcS	10	10 + LcS
(A) PE-specific IgG1 titers in time						
d 14	0.00 + 0.00	0.00 + 0.00	11.60 + 1.10	14.00 + 1.04	14.11 + 0.46	16.25 ^a + 0.80
d 28	0.00 + 0.00	0.88 + 0.88	17.38 + 0.66	17.00 + 0.87	17.25 + 0.53	18.86 + 0.60
d 42	1.38 + 1.38	3.50 + 1.77	17.29 + 0.36	18.13 + 0.64	17.13 + 0.52	19.17 ^a + 0.79
d 49	1.38 + 1.38	3.38 + 1.68	17.29 + 0.84	18.25 + 0.53	17.25 + 0.86	20.00 ^a + 0.82
(B) PE-specific IgG2a titers in time						
d 14	0.88 + 0.88	0.00 + 0.00	10.60 + 1.00	11.88 + 0.64	11.89 + 0.48	13.63 ^a + 0.46
d 28	0.00 + 0.00	1.13 + 1.13	15.25 + 0.62	15.13 + 0.44	13.50 + 0.42	14.86 + 0.59
d 42	0.00 + 0.00	2.63 + 1.72	15.57 + 0.57	16.50 + 0.42	13.88 + 0.52	16.33 ^a + 0.80
d 49	0.00 + 0.00	3.00 + 1.97	16.29 + 0.61	16.63 + 0.53	15.00 + 0.50	16.33 + 0.84

Groups of female rats ($n = 8$ for each dosing regimen) received 0, 1 or 10 mg/(rat d) of peanut extract (PE). Data are displayed as serum reciprocal antigen-specific IgG1 titers (average + S.E.M.) in time (A) and serum reciprocal antigen-specific IgG2a titers (average + S.E.M.) in time (B). The tables display the non-treated groups and the *Lactobacillus casei* Shirota (LcS)-treated groups (1×10^9 CFU of LcS/(rat d)) in a direct comparison. LcS significantly increased the overall difference in PE-specific IgG1 (d 14: $P = 0.019$; d 49: $P = 0.046$) and IgG2a levels (d 42: $P = 0.009$).

^a Effects of LcS on PE-specific IgG1 or IgG2a levels were significant when separate allergen dose groups were analyzed: $P = 0.030$ (d 14 IgG1; 10 mg PE + LcS compared to 10 mg PE), $P = 0.044$ (d 42 IgG1; 10 mg PE + LcS compared to 10 mg PE), $P = 0.044$ (d 49 IgG1; 10 mg PE + LcS compared to 10 mg PE), $P = 0.021$ (d 14 IgG2a; 10 mg PE + LcS compared to 10 mg PE) and $P = 0.019$ (d 42 IgG2a; 10 mg PE + LcS compared to 10 mg PE).

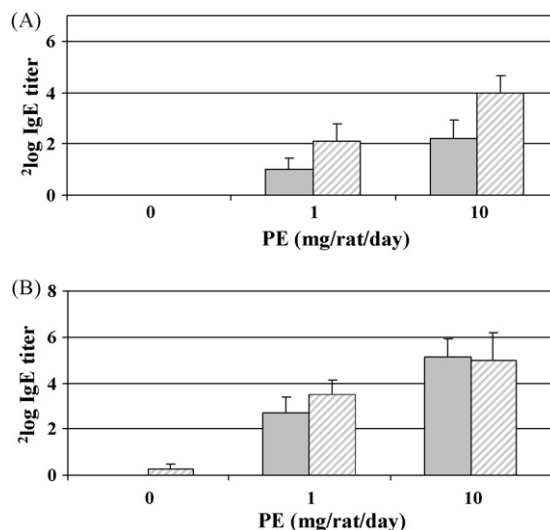


Fig. 1. PE-specific IgE titers following 14 and 42 d of oral exposure to peanut. Groups of female rats ($n = 8$ for each dosing regimen) received 0, 1 or 10 mg/(rat d) of peanut extract (PE). Data are displayed as serum reciprocal antigen-specific IgE titers (average + S.E.M.) on d 14 (A) or on d 42 (B). Grey bars represent the non-treated groups and striped grey bars represent the *Lactobacillus casei* Shirota (LcS)-treated groups (1×10^9 CFU of LcS/(rat d)). LcS significantly increased the overall difference in PE-specific IgE levels at d 14 ($P = 0.026$).

The overall difference between the control groups and LcS-treated groups regarding the immune parameters tested were assessed with a two-way ANOVA with Bonferroni for pairwise comparisons as post hoc test. To determine if LcS influenced the outcome of an allergic parameter within a dosing group of PE a one-way ANOVA with Bonferroni as post hoc test was done.

Data for the IgG1/IgG2a ratio's and the number of immune cells in blood were log-transformed prior to analysis, because these data did not meet the prerequisite of ANOVA that data should be normally distributed. In all analyses, data were considered significantly different if $P < 0.05$ (two-sided).

3. Results

3.1. The effect of LcS on PE-specific IgE

In two out of eight rats that received 10 mg PE together with LcS, PE-specific IgE titres could not be detected at 14 d. However, mean values of PE-specific in this group, calculated after excluding these two rats, showed increased values in both the 1 and 10 mg PE group (Fig. 1A). This increase was significant ($P = 0.026$) when all dosing groups were compared with rats that were not treated with LcS.

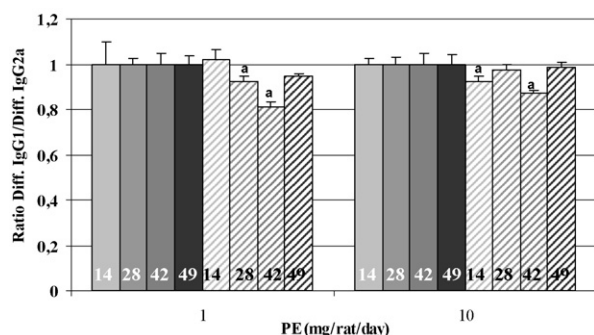


Fig. 2. PE-specific IgG1/IgG2a ratio's in time. Groups of female rats ($n=8$ for each dosing regimen) received 0, 1 or 10 mg/(rat d) of peanut extract (PE). To determine the effect of LcS on the Th1/Th2 balance, differences (value LcS-treated groups divided by the value of the non-treated groups) in IgG1 levels of sensitized animals (d 42) were divided by the differences in corresponding IgG2a levels. Data are displayed as IgG1/IgG2a ratio's (average + S.E.M.) in time. Grey bars represent the ratio of the non-treated groups and striped grey bars represent the ratio of the *Lactobacillus casei* Shirota (LcS)-treated groups (1×10^9 CFU of LcS/(rat d)), the d at which the ratio was calculated is displayed within each bar. LcS significantly increased the overall difference in PE-specific IgG1/IgG2a ratio at d 42 ($P=0.018$). ^aEffects of LcS on the PE-specific IgG1/IgG2a ratio were significant when separate allergen dose groups were analyzed: $P=0.003$ (d 14; 10 mg PE + LcS compared to 10 mg PE), $P=0.039$ (d 28; 1 mg PE + LcS compared to 1 mg PE), $P=0.003$ (d 42; 1 mg PE + LcS compared to 1 mg PE) and $P=0.028$ (d 42; 10 mg PE + LcS compared to 10 mg PE).

If separate allergen dosages were evaluated, this increase was not significant. On d 42 no significant effect of LcS on the IgE response was found (Fig. 1B).

3.2. The effect of LcS on PE-specific IgG2a and IgG1

Sensitization with 1 or 10 mg PE, resulted in increased PE-specific IgG1 and IgG2a serum levels at d 14, 28, 42 and 49. LcS significantly further increased PE-specific IgG1 levels at d 14 ($P=0.019$) and d 49 ($P=0.019$) at the level of overall difference between LcS-treated and non-treated groups. If separate allergen dosages were evaluated, the increase of IgG1 levels was significant in the 10 mg PE/(rat d) at d 14 ($P=0.030$), d 42 ($P=0.044$) and d 49 ($P=0.044$) (Table 1A).

LcS also significantly further increased PE-specific IgG2a levels at d 42 ($P=0.009$) at the level of overall difference between LcS-treated and non-treated groups. If separate allergen dosages were evaluated, the increase of IgG2a levels was significant in the 10 mg PE/(rat d) at d 14 ($P=0.021$) and d 42 ($P=0.019$) (Table 1B).

To determine the effects of LcS on the Th1/Th2 ratio, the ratio of the increase of PE-specific IgG1 over the increase of PE-specific IgG2a on d 42 was calculated. In rats that daily received 10 mg PE

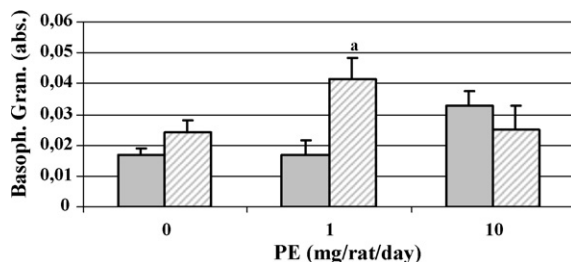


Fig. 3. Numbers of basophilic granulocytes. Groups of female rats ($n=8$ for each dosing regimen) received 0, 1 or 10 mg/(rat d) of peanut extract (PE). Data are displayed as numbers of basophilic granulocytes (average + S.E.M.) in blood of d 49 ($\times 10^9/L$). Grey bars represent the non-treated groups and striped grey bars represent the *Lactobacillus casei* Shirota (LcS)-treated groups (1×10^9 CFU of LcS/(rat d)). ^aEffects of LcS on basophilic granulocytes were significant when separate allergen dose groups were analyzed: $P=0.017$ (1 mg PE + LcS compared to 1 mg PE).

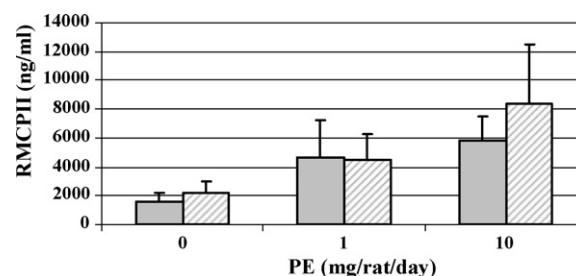


Fig. 4. RMCPII release after oral challenge. Groups of female rats ($n=8$ for each dosing regimen) received 0, 1 or 10 mg/(rat d) of peanut extract (PE). Data are displayed as serum rat mast cell protease II (RMCPII) levels (average + S.E.M.) on d 49, 30 min after oral challenge with 100 mg PE/rat. Grey bars represent the non-treated groups and striped grey bars represent the *Lactobacillus casei* Shirota (LcS)-treated groups (1×10^9 CFU of LcS/(rat d)).

a significant shift towards Th2 dominance due to LcS treatment was found on d 14, and in rats that received 1 mg PE such a significant shift was noted at d 28. The greatest shift in this ratio was found on d 42, where LcS significantly shifted the Th1/Th2 ratio towards Th2 in both sensitized groups (Fig. 2). At d 49 this effect had disappeared.

3.3. The effect of LcS on white blood cells, basophilic granulocytes and rat mast cell protease-II

The numbers of basophilic granulocytes were significantly increased ($P=0.017$) by LcS in the 1 mg PE group as compared to its untreated control (Fig. 3). The numbers of lymphocytes, neutrophils, monocytes and eosinophilic granulocytes were not affected by LcS compared to the non-treated groups (data not shown).

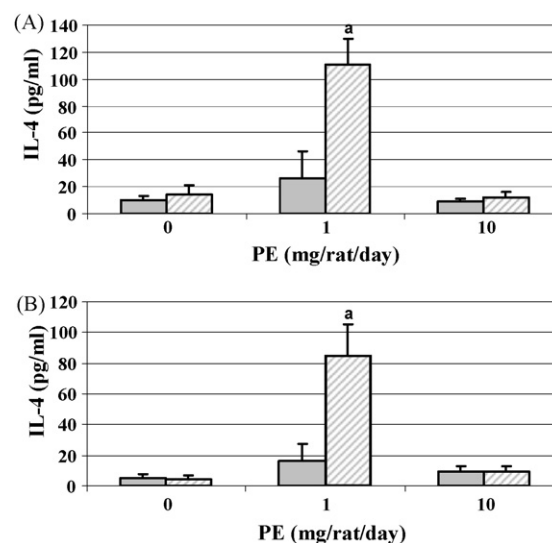


Fig. 5. IL-4 levels in PE-stimulated spleen and MLN cells. Groups of female rats ($n=8$ for each dosing regimen) received 0, 1 or 10 mg/(rat d) of peanut extract (PE). Spleens and mesenteric lymph nodes (MLNs) were dissected at d 49 and their cells were incubated with PE (1 mg/ml) in cell cultures (4×10^5 cells/culture). Data are displayed as IL-4 levels (average + S.E.M.) in the supernatants of spleen (A) or MLN (B) cultures. Grey bars represent the non-treated groups and striped grey bars represent the *Lactobacillus casei* Shirota (LcS)-treated groups (1×10^9 CFU of LcS/(rat d)). LcS significantly increased the overall difference in IL-4 levels in the supernatants of spleen cells ($P=0.003$) and of MLN cells ($P=0.006$). ^aEffects of LcS on IL-4 levels were significant when separate allergen dose groups were analyzed: $P=0.010$ (spleen; 1 mg PE + LcS compared to 1 mg PE) and $P=0.014$ (MLN; 1 mg PE + LcS compared to 1 mg PE).

Sensitization with 1 or 10 mg PE, resulted in a dose-dependent increased of PE-mediated RMCPII release (Fig. 4). Modulation with LcS did not significantly alter the increase in release of RMCPII.

3.4. The effect of LcS on cytokine levels in supernatants of PE-stimulated spleen and MLN cells

MLN and spleen cells from PE-sensitized rats showed modest antigen-specific proliferative responses after *in vitro* culture with PE for 96 h. In addition, re-stimulation of spleen lymphocytes with PE resulted in increased IFN- γ levels in both the 1 mg ($P=0.013$) and 10 mg PE ($P=0.017$) groups. LcS did not affect IFN- γ levels in either antigen-specific stimulated spleen or MLN cells (data not shown).

Fig. 5 shows IL-4 levels in supernatants of cell suspensions of spleen (Fig. 5A) and MLN (Fig. 5B) cells that were *ex vivo* re-stimulated with PE for 96 h. LcS significantly increased IL-4 levels further at the level of overall difference between LcS-treated and non-treated groups in the supernatants of both spleen and MLN cells. In rats that were dosed with 1 or 10 mg PE, without LcS, IL-4 levels were not significantly affected. In the 1 mg PE group, LcS significantly increased IL-4 levels in cell suspensions of spleen ($P=0.010$) and MLN cells ($P=0.014$). In the 10 mg PE group, LcS did not affect IL-4 production.

4. Discussion

We investigated whether the probiotic strain *L. casei* Shirota (LcS) could influence the food allergic response against peanut extract (PE) in a BN rat model for food allergy. The most pronounced effects of LcS were found on allergen-specific cytokine production. The Th2-related cytokine IL-4 was increased by LcS and this increase was found in the supernatants of both spleen and MLN cultures. IFN- γ was also non-significantly increased in the supernatants of both spleen and MLN cultures of the control animals. This indicates that LcS had an effect on IFN- γ that was independent of the PE-response, which has been described earlier (Ezendam and van Loveren, 2008). The most pronounced effect of LcS noted in this study was on IL-4 production in rats that were dosed with 1 mg PE. In contrast, in rats that received 1 or 10 mg PE, IFN- γ production was not affected by LcS. It is remarkable that the effect of LcS on IL-4 was only observed in the 1 mg PE group. This is possibly due to the timing of the experiment: IL-4 was measured after 96 h of culturing, which might be too late for the 10 mg PE group.

Effects of LcS on PE-specific IgE levels were only observed after 14 d of exposure. In the 1 mg PE group, LcS increased specific IgE levels further. In the 10 mg PE group, however, two rats did not respond with specific IgE levels, whereas the mean values of the other six rats were increased compared to the rats that did not receive LcS. At d 42, the levels of IgE were similar in rats that did or did not receive LcS. Taken together, our data do not show statistically decreased IgE titres as a result of LcS treatment. This is in contrast to what has been previously published (Matsuzaki and Chin, 2000; Matsuzaki et al., 1998; Shida et al., 2002), but in accord to other studies that failed to demonstrate suppressive effects of LcS on specific IgE levels (Ezendam and van Loveren, 2008). It should be noted that these latter observations were not done in oral sensitization models, but rather in intraperitoneal or respiratory sensitization models.

Both PE-specific IgG1 and IgG2a antibody titres were increased by LcS during the course of the experiment. This increase was directed more towards Th2, which is accordance with the observed effects on cytokine production. In contrast to what was hypothesized, based on the notion that if LcS would suppress allergic responses, LcS could also suppress the food allergic response, our

results indicate that LcS increased the levels of PE-specific IgE, IgG1 and IgG2a, although IgE was only affected early after exposure to PE. Enhancement of the immune system by LcS was also illustrated by an increase of the number of basophils. LcS did not affect mast cell degranulation, as measured by the release of RMCPII. This parameter is considered to be clinically relevant, since mediators released from mast cells cause clinical symptoms in allergic patients.

From our experimental data, we can conclude that LcS stimulates certain responses in this experimental model for peanut allergy, rather than suppressing the food allergic response. Our study does therefore not support the hypothesis that probiotics have a protective role in the prevention of allergic responses in our BN rat model for food allergy against peanut. It is important to note that effects of probiotics are highly dependent on the species and strain used, and further depend on viability, dose and time (Broekaert and Walker, 2006; de Waard et al., 2003; Kalliomaki and Isolauri, 2004). Hence, although our study does not demonstrate an inhibitory effect of LcS on the food allergic response, this does not necessarily imply that other probiotic strains would not have such effects. Furthermore, in other studies LcS has been shown to decrease IgE responses against OVA (Shida et al., 1998; Shida et al., 2002), which could imply that the effects of a probiotic strain are also dependent on the experimental model used.

At present, evidence for efficacy of probiotics on allergy prevention in humans is scarce. Although there are some promising results on prevention and alleviation of atopic eczema in infants (Kalliomaki et al., 2001, 2003; Rosenfeldt et al., 2003; Sistik et al., 2006; Viljanen et al., 2005), at the moment no effects on atopic manifestations that occur later in life, e.g. asthma, have been reported. More importantly, a recent study has demonstrated that *L. plantarum* enhanced sensitization to food and respiratory allergens in infants that received this probiotic in the first 6 months of their lives (Taylor et al., 2007). It is unknown if the observed increase in sensitization will also lead to an increased development of clinical effects in these infants. Additionally, a recent review, in which 12 randomized clinical trials were included, has demonstrated that probiotics significantly reduce infant eczema. However, when the analysis was restricted to studies on IgE-dependent atopic eczema the reduction in eczema was no longer significant. In addition, it was shown that no benefits were reported for any other allergic disease or hypersensitivity outcome (Osborn and Sinn, 2007). Recently, it has been shown that probiotics might beneficially affect rhinitis induced by Japanese cedar pollen. In a randomized double-blind, placebo-controlled study LcS did not prevent allergic symptoms, but after subgroup analysis it was shown that in patients with moderate-to-severe nasal symptom scores, LcS delayed the occurrence of allergic symptoms (Tamura et al., 2007). Hence, currently there is insufficient evidence from human studies for the efficacy of probiotics in prevention of either allergic diseases or food hypersensitivity.

More research is needed to substantiate efficacy of probiotics in allergy prevention. Experimental animal models for allergy are useful to select probiotics which could be effective in allergy prevention and can provide more insight in the mechanisms involved. Efficacy and safety, however, should both be demonstrated in humans.

Conflict of interest

The authors concur that there were no conflicts of interest during the writing of the manuscript entitled '*Lactobacillus casei* Shirota does not decrease the food allergic response to peanut extract in Brown Norway rats'.

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