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Immune effects of the probiotic *Bifidobacterium breve*

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HET RAPPORT IN HET KORT

Probioticum *Bifidobacterium breve* heeft gunstige effecten op immuunsysteem proefdieren

Bifidobacterium breve, een probiotische bacterie, heeft gunstige effecten op zowel allergieën als auto-immuniteit – een afweerreactie op lichaamseigen bestanddelen – bij proefdieren. Probiotica worden in reclameboodschappen ook wel 'goede bacteriën' genoemd. Fabrikanten claimen een positief effect van probiotica op darmflora, weerstand en preventie van allergieën. De meeste van deze effecten zijn echter niet wetenschappelijk onderbouwd.
Het is bekend dat de effecten van probiotica afhangen van de soort probiotica die wordt toegepast. Eerder onderzoek naar het probioticum *Lactobacillus casei* Shirota leidde tot geringe verergering van allergie en auto-immuniteit bij proefdieren en toonde aan dat het gebruik van probiotica, afhankelijk van de stam, op een potentieel risico duidt.
In dit rapport worden de effecten van het probioticum *Bifidobacterium breve* op het immuunsysteem beschreven. Om dit te onderzoeken zijn proefdiermodellen voor allergie en auto-immuniteit gebruikt. Toediening van *Bifidobacterium breve* leidde tot een vermindering van zowel allergische als auto-immuun reacties. *Bifidobacterium breve* heeft dus een positief effect op het immuunsysteem, dit in tegenstelling tot *Lactobacillus casei* Shirota.
Effecten van probiotica op het immuunsysteem zijn dus duidelijk afhankelijk van de soort probiotica die wordt toegepast. Om de invloed van beide probiotica op mensen te bepalen, zijn nieuwe studies nodig. Deze studies dienen zich te richten op zowel de werkzaamheid als de veiligheid van probiotica bij mensen.

Trefwoorden: probiotica, Bifidobacterium breve, immuunmodulatie, allergie, autoimmuniteit

ABSTRACT

Probiotic *Bifidobacterium breve* has beneficial effects on the immune system of experimental animals

Bifidobacterium breve, a probiotic, has beneficial effects on both allergy and autoimmunity – an immune reaction against the body's own constituents – in experimental animals. Probiotics are called 'friendly bacteria' in advertisements, in which manufacturers claim their beneficial effects on gut flora, resistance and allergies. However, most of the claimed effects have not been scientifically proven in human trials.

Effects of probiotics are known to be dependent on the strain of probiotics used. Previously performed research has demonstrated that *Lactobacillus casei* Shirota moderately stimulates both allergy and autoimmunity in experimental animals, implying that intake of probiotics, depending on the strain used, can be a hazard.

This report describes the effects of the probiotic *Bifidobacterium breve* on allergy and autoimmunity. Animal models for allergy and autoimmunity were used for this investigation. Administration of *Bifidobacterium breve* alleviated both allergic and autoimmune responses. *Bifidobacterium breve* has a positive effect on the immune system, in contrast to *Lactobacillus casei* Shirota. Effects of probiotics on the immune system are clearly strain-dependent.

Trials in humans are necessary to be able to extrapolate these data to application in the human body. These studies should therefore focus on both efficacy and safety of probiotics in humans.

Key words: probiotics, Bifidobacterium breve, immunomodulation, allergy, autoimmunity

CONTENTS

SUMMARY

Probiotics are promoted as being beneficial for health, for instance by affecting the immune system. Previously we have demonstrated that administration of the probiotic *Lactobacillus casei* Shirota (LcS) during lactation aggravated allergic and autoimmune responses that were induced at an adult age. Effects of probiotics are known to be strain-dependent. Therefore, we decided to further investigate the effects of administration of probiotics during lactation on the development of experimental allergy and autoimmunity by using a different probiotic: *Bifidobacterium breve* (*B. breve*).

Administration started during lactation when mice or rats were two weeks old until the end of the experiment. Respiratory allergy or EAE were induced when the animals were six to seven weeks old.

In mice, *B. breve* modestly reduced the inflammatory lung response in males and females by decreasing the number of infiltrating eosinophils and lymphocytes. *B. breve* had no effect on allergen-specific serum IgE levels, but increased IgG1 in females. Cytokine profiles assessed after culturing spleen cells with the mitogen ConA showed that *B. breve* skewed the Th1/Th2 balance towards Th1 in females. However, allergen-specific cytokine production in females was not affected by *B. breve.* In males, a decrease in allergen-specific Th1 and Th2 cytokines was observed after administration of *B. breve.* This decrease was not associated with serum IgE levels. *B. breve* had beneficial effects on EAE in rats. In males a significant reduction of duration of disease was observed and rats also recovered faster after weight loss. A similar non-significant trend was observed in females.

These data show that the probiotic *B. breve* alleviates both allergic and autoimmune responses. This is in contrast with our previous studies with LcS, which stimulated both allergic and autoimmune responses. Our data demonstrate that immune effects of probiotics are strain-dependent. Studies in humans are warranted to evaluate the possible risks and benefits of consumption of probiotics for adults and children.

1 INTRODUCTION

Probiotics are non-pathogenic microorganisms that are promoted as being beneficial for health, in particular effects on the gut (Madsen, 2001; Penner et al., 2005) and the immune system (Matsuzaki and Chin, 2000; Ezendam and Van Loveren, 2006) are described. The number of products that contain probiotics is rising. These products can be found in the supermarket, where they are sold as dairy products, but also in pharmacies or on the internet, where probiotics are sold as food supplements. For infants, there are currently also infant formulas available that contain probiotics.

Conflicting data on beneficial effects of probiotics on allergy have been published. Alleviation of atopic dermatitis has been demonstrated in infants that received either *Lactobacillus* GG (LGG) (Isolauri et al., 2000; Kalliomaki et al., 2001; Kalliomaki et al., 2003; Viljanen et al., 2005) or a combination of *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460 (Rosenfeldt et al., 2003; Rosenfeldt et al., 2004). In contrast, recent studies showed no improvement of atopic eczema after treatment with either LGG or *L. rhamnosus* (Weston et al., 2005; Brouwer et al., 2006). In patients with asthma or rhinitis *L. paracasei-33* improved parameters for quality of life (Peng and Hsu, 2005; Wang, 2006), whereas consumption of *L. acidophilus* had no effect on quality of life or clinical parameters (Wheeler et al., 1997). *L. rhamnosus* could also not alleviate symptoms in patients with an allergy for birch-pollen (Helin et al., 2002).

Mechanisms that can explain the observed beneficial effects include effects on the epithelial barrier of the gut (Petrof et al., 2004; Rosenfeldt et al., 2004), increased intestinal IgA production (Rautava et al., 2006), effects on dendritic cells (Christensen et al., 2002; Drakes et al., 2004), modulation of the Th1/Th2 balance (Mohamadzadeh et al., 2005), stimulation of cytokine production (Cross et al., 2002; He et al., 2002; Cross et al., 2004) and effects on regulatory T cells (Chapat et al., 2004; Smits et al., 2005).

It is important to note that effects of probiotics are strain dependent. In a mouse model for allergy it was shown that the cytokine profile that was induced by a probiotic strain predicted the effects on allergen-specific IgE in serum. Strains that produced high levels of IL-12 (Th1 cytokine) were able to reduce IgE, whereas low IL-12 producers were not able to do so. (Sashihara et al., 2006). Similar results were observed in a mouse model for experimental autoimmune encephalomyelitis (EAE). *L. casei* and *L. murines* improved the disease, while *L. reuteri* aggravated EAE. These effects were in line with cytokine profiles: *L. casei* induced immunoregulatory (Th3) cytokines and *L. reuteri* proinflammatory cytokines (Maassen et al., 1998). In previous studies it has been shown that a decrease of Th1 cytokines (IL-12, IFN-γ)

and an increase of Th2 (IL-4) and Th3 cytokines (IL-10, TGF-β) was associated with alleviation of this Th1-mediated autoimmune disease (Xu et al., 2000; Monteiro de Castro et al., 2004; Yang et al., 2004). Thus, the cytokine profile that is induced by a probiotic strain seems predictive for the effect on the immune response. Furthermore, dependent on the probiotic used beneficial or adverse immune effects on EAE can be induced (Maassen et al., 1998).

Adverse effects on EAE have been shown by us previously in a rat model for EAE (Baken et al., 2006;Ezendam *et al.*, 2006;Ezendam and Van Loveren, 2006). Administration of *L. casei* strain Shirota (LcS) started during the lactation phase or when the rats were adults. Independent of the timing of administration, i.e. lactation phase or adult exposure, LcS aggravated EAE (Baken et al., 2006; Ezendam et al., 2006; Ezendam and Van Loveren, 2006). Additionally, effects of LcS were studied in a mouse model for respiratory allergy. LcS administration started during lactation or when the mice were adults and in this model timing of LcS administration appeared to induce different effects. Early administration increased the influx of inflammatory cells in both females and males (Ezendam and Van Loveren, 2006), whereas this influx decreased when LcS was given to female adult mice (Ezendam et al., 2006).

To further investigate effects of probiotics on allergy and autoimmunity the probiotic *Bifidobacterium breve* (*B. breve*) was administered from lactation phase onward and effects were studied in the same allergy and autoimmunity models that were used in the experiments with LcS.

2 MATERIALS AND METHODS

2.1 Bacteria

Bifidobacterium breve (*B. breve*) was a kind gift of Numico Research (Wageningen, The Netherlands). *B. breve* was cultured at Numico Research and delivered as a freeze-dried powder that was stored at -80°C until use. Suspensions for oral gavage were prepared on the day of gavage by dissolving the powder in saline at a concentration of 5*10^9 CFU per ml.

2.2 Animals

Pregnant BALB/c mice were obtained from our own breeding colony. Mice were bred specific pathogen free and kept under conventional conditions. Mice received Hope Farms chow pellets (Woerden, NL) and water *ad libitum*. The breeding colony of the animals was pre-screened/monitored for endogenous pathogenic viruses and bacteria and was found negative.
Pregnant specific pathogen-free Lewis rats (LEW/HanHsD) were obtained from Harlan (Horst, The Netherlands). Rats were fed Hope Farms chow pellets (Woerden, NL) and water *ad libitum*.
The experimental setup of the studies was examined and agreed upon by the Ethical Committee on Experimental Animals.

2.3 Experimental design respiratory allergy

After birth, mice were divided over the mothers. Each nest contained the same amount of pups with an equal male/female ratio. Oral administration of *B. breve* started when the mice were two weeks old. Mice received $1*10^9$ CFU *B. breve* or saline alone (controls) daily in a volume of 200 µl, except for the first week when this dose was administered in 100 µl. At weaning (21 days after birth) mice were taken away from their mothers and housed in the experimental groups (Table 1).
Sensitization and challenge were performed as described earlier (Smit et al., 2003) with some minor modifications. Sensitization of the mice started when they were six weeks old (day 0). Mice were sensitized twice on day 0 and day 14 by two intraperitoneal injections with 10 mg ovalbumin (grade V, Sigma Aldrich, St. Louis, USA) adsorbed on to 2.25 mg aluminum hydroxide (AlumInject, Pierce, Rockford, IL, USA) in saline or with saline alone. Mice were

challenged on day 35, 38 and 41 by inhalation of ovalbumin or saline aerosols in a plexiglass exposure chamber for 20 minutes. Aerosols were generated by nebulizing a solution with 10 mg/ml ovalbumin in saline or saline alone using a nebulizer. At day 43 mice were sacrificed and blood was collected, clotted and serum was obtained for determination of ovalbumin specific immunoglobulines. Spleens were collected and single cell suspensions were prepared and cultured for cytokine measurements.

Table 1: Experimental groups respiratory allergy

Sensitization/challenge	***B. breve***	**Sex**	**Number**
Ovalbumin	*B. breve*	female	8
Ovalbumin	Vehicle	female	8
vehicle	*B. breve*	female	4
vehicle	Vehicle	female	4
Ovalbumin	*B. breve*	male	8
Ovalbumin	Vehicle	male	8
vehicle	*B. breve*	male	4
vehicle	Vehicle	male	4

2.3.1 Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed by flushing the lungs with 1 ml sterile PBS. BAL fluid was centrifuged at 1200 rpm for 10 minutes. Cell pellets were used for determination of total cell number and for cytospin preparations. Cytospins were stained with May-Grünwald Giemsa and on each preparation 400 cells were counted. Supernatants obtained for cytokine measurement were stored at -80°C.

2.3.2 Culture of spleen cells

Spleens were collected and single-cell suspensions were prepared under aseptic conditions by pressing the spleen through a sterile 70 µm nylon cell strainer. Cells were washed in 5% FCS medium (10 min., 4^{o}C, 300 g) and resuspended in 10 ml standard medium with 10% FCS. The concentration of the cell suspensions was adjusted to 2*10^{6} cells/ml. Spleen cells (100 µl/well; 2*10^{5} cells) were stimulated with 5 µg/ml ConA (100 µl/well) for 48 hours or with ovalbumin (100 µg/ml) for 96 hours. Supernatants were collected for cytokine measurements.

2.3.3 Ovalbumin-specific IgE and IgG1 ELISA

Specific ovalbumin IgE and IgG1 titers in sera were determined by ELISA. Incubations were followed by extensive washing on an automatic plate washer with PBS containing 0.1% Tween-20. To measure ovalbumin-specific IgE 96-wells plates (Nunc-Immuno Plate, Denmark) were coated overnight at 4^{o}C with 2 μg/ml rat-anti-mouse IgE (rαm IgE, Zymed, 04-7000) diluted in sodium carbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were blocked by adding 0.05M Tris buffered saline with 1% BSA, pH 8 (Sigma) for 1 h at 37°C. Thereafter, serial dilutions of mouse serum samples and a pooled positive reference serum were incubated for 1 h at 37°C. All dilutions were done in blocking buffer plus 0.05% Tween-20. Then, wells were incubated for 1 h at 37°C with DIG-conjugated ovalbumin. The coupling of ovalbumin to DIG (molar mixture 1:10) was performed according to the manufacturer's instructions (Roche Diagnostics). Then, wells were incubated with anti-DIG Fab fragments labeled with peroxidase (PO) (Roche Diagnostics) for 2 hours at 37°C. Plates were incubated with TMB substrate and the enzyme reaction was stopped with 2 M H_2SO_4 and absorbance was read at 450 nm.

To measure ovalbumin-specific IgG1 wells were coated overnight at 4°C with 10 μg/ml ovalbumin/ml PBS (grade V, Sigma). Blocking buffer was added and wells were incubated for 1 hour at 37°C. Thereafter, serial dilutions of mouse serum samples and a pooled positive reference serum were added to the wells and incubated for 2 hours at RT. Biotinylated rat-anti-mouse IgG1 (Zymed Laboratories, San Francisco, CA) was added and wells were incubated for 1.5 hour at RT, followed by incubation with poly-horseradish peroxidase labeled streptavidin for 45 minutes at RT. Then plates were incubated with TMB substrate and the enzyme reaction was stopped with 2 M H_2SO_4 and absorbance was read at 450 nm. Extinction values of the positive reference serum were used to calculate the amount of IgG1 and IgE in the samples and extinction values were expressed as arbitrary units.

2.3.4 Bioplex for cytokines

Th1 and Th2 cytokines were measured in BAL fluid and in supernatants of spleen cells that were cultured with ConA or with ovalbumin. Cytokine levels were detected with a Bioplex 5-plex cytokine assay kit that could detect IL-4, IL-5, IL-10, IL-13 and IFN-γ (Biorad Life Science, Hercules, CA, USA) according to the manufacturer's instructions. Cytokine measurements were performed on a Luminex® (Biorad Life Science) and Luminex software was used to calculate the amount of cytokines (in pg/ml supernatant).

2.4 Experimental design experimental auto-immune encephalomyelitis

After birth, rats were divided over the mothers. Each nest contained the same amount of pups with an equal male/female ratio. Oral administration of *B. breve* started when the rats were two weeks old. Rats received $1*10^9$ CFU daily in a volume of 200 µl. Control rats received 200 µl saline daily. At weaning (21 days after birth) rats were taken away from their mothers and housed in the experimental groups (Table 2).

Table 2: Experimental groups EAE

EAE	*B. breve*	Sex	Number
EAE	*B. breve*	Female	8
EAE	Vehicle	Female	8
control	*B. breve*	Female	2
control	Vehicle	Female	0
EAE	*B. breve*	Male	8
EAE	Vehicle	Male	10
control	*B. breve*	Male	4
control	vehicle	Male	2

Acute EAE was induced at the age of seven weeks as described previously (Hendriks et al., 2004). Rats were injected subcutaneously in the left ankle with 100 µl of an emulsion containing 20 µg guinea pig myelin basic protein (MBP; Sigma), 500 µg *Mycobacterium tuberculosis* type H37RA (Difco, Detroit, MI), 50 µl complete Freund's adjuvant (CFA, Difco) supplemented with saline (0.9% NaCl) to reach a volume 100 µl. After induction of EAE body weights were recorded daily. Also, neurological signs were scored daily and graded from 1 to 5; 0, no clinical signs; 0.5, loss of tonicity in distal half of tail; 1, flaccid tail; 1.5, unsteady gait; 2, partial hind limb paralysis; 2.5, complete hind limb paralysis; 3, paralysis of the complete lower part of the body up to the diaphragm; 4, paraplegia; and 5, death due to EAE. Rats were sacrificed 26 days after induction of EAE. The spleen was removed and single-cell suspensions were prepared.

2.4.1 Stimulation of spleen cells with myelin basic protein

Single cell suspensions of spleens were prepared as described in 2.3.3. Spleen cells (100 µl/well; $2*10^5$ cells) were stimulated with 0, 10 or 25 µg/ml MBP (100 µl/well) for 96 hours. Supernatants were collected for cytokine measurements.

2.4.2 IFN-γ and IL-4 ELISA

IFN-γ was determined with a rat IFN-γ OptEIA set (BD Biosciences Pharmingen, San Diego, CA, USA) and IL-4 with a rat IL-4 cytoset (Biosource), both according to the manufacturer's instructions.

2.5 Statistical analysis

In the respiratory allergy experiment statistical differences between the four experimental groups were determined by one-way ANOVA followed by Scheffe's or Games-Howell post hoc test. Differences in ovalbumin-specific IgE and IgG1 levels were determined with a two-tailed Student's t-test comparing the two experimental groups that were sensitized and challenged with ovalbumin.

In the EAE experiment mean body weights on all 26 days were calculated and significant differences between the experimental groups were determined with a two-tailed Student's t-test. A one-tailed Mann-Whitney Test was used to determine if onset of disease, duration of symptoms and cumulative clinical score were significantly different between the experimental groups.

3 RESULTS

3.1 Effects of B. breve on respiratory allergy

3.1.1. Inflammatory response and cytokines in the lungs

In both male and female mice that were sensitized and challenged with ovalbumin an increase of eosinophils and lymphocytes was found (Figure 1). In females and males, daily administration of *B. breve* reduced the number of cells in sensitized mice by reducing the number of infiltrating lymphocytes and eosinophils (Figure 1A and B). These changes were not statistically significant.

Several cytokines were assessed in the BAL fluid (Table 3). IL-10 could not be detected and levels of IFN-γ and IL-4 were very low. IL-5 increased after sensitization and challenge with ovalbumin in both males and females. In sensitized and challenged males IL-13 levels were higher compared to controls. In sensitized mice, treatment with *B. breve* did not affect the increased IL-5 and IL-13 levels.

Table 3: Effect of B. breve on cytokine levels in BAL fluid

Group	**IL-4**	**IL-5**	**IL-13**	**IFN-γ**
females				
Control	0	0	16.4 ± 6.3	0.22 ± 0.22
Control + *B. breve*	0	3.9 ± 3.9	19.5 ± 6.4	0.41 ± 0.25
OVA	0.49 ± 0.26	18.6 ± 4.0	22.8 ± 3.6	0.64 ± 0.14
OVA + *B. breve*	0.36 ± 0.15	21.5 ± 4.8*	25.4 ± 2.8	0.91 ± 0.12
Males				
Control	0	0	7.0 ± 1.9	0.15 ± 0.15
Control + *B. breve*	0	0	8.4 ± 4.9	0.74 ± 0.22
OVA	0.27 ± 0.17	15.5 ±2.0**	21.7 ± 2.7	0.74 ± 0.22
OVA + *B. breve*	0.16 ± 0.11	14.0 ± 2.9*	18.4 ± 4.7	0.69 ± 0.19

*Cytokines were assessed with a bioplex Th1/Th2 kit on a Luminex® and expressed as mean ± SE in pg/ml supernatant. Statistical significant from the control group: *p<0.05, ** p<0.01.*

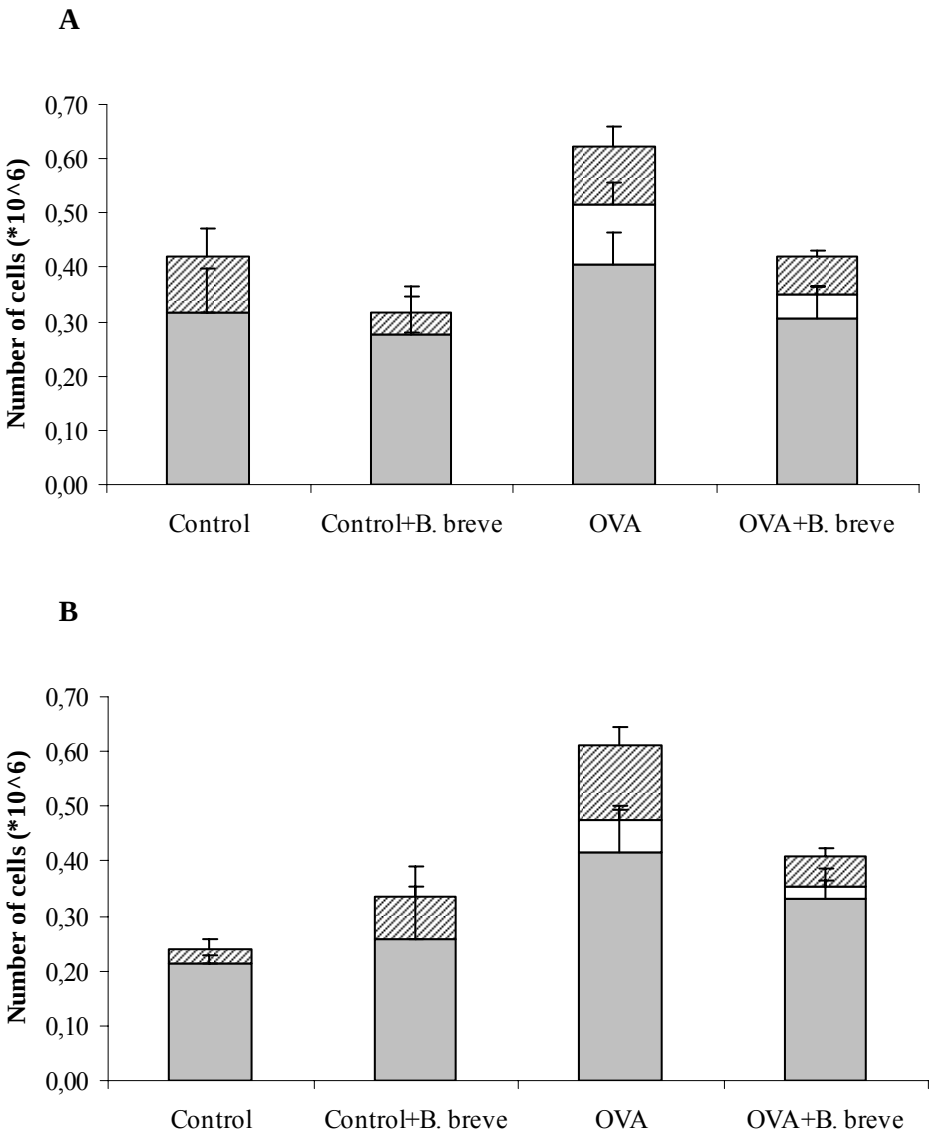


Figure 1: Number of cells in lung lavage fluid in female (A) and male (B) mice. Mice were sensitized and challenged with ovalbumin (OVA) or saline (control) and orally treated with B. breve or saline. Cell number is expressed in 10^6 cells. Grey bars represent the number of macrophages, white bars the number of eosinophils and striped bars the number of lymphocytes.

3.1.2. OVA-specific serum IgE and IgG1

OVA-specific IgE (Figure 2) and IgG1 (Figure 3) levels were detected in serum of mice that were sensitized and challenged with ovalbumin. Mice that were sensitized and challenged with vehicle served as negative controls and serum of these mice did not contain ovalbumin-specific IgG1 and IgE (not shown). In both males and females, OVA-specific IgE titers were not affected by *B. breve* treatment. In males, OVA-IgG1 levels were also not affected by

B. breve treatment. However, in females OVA-IgG1 levels were higher in mice that received *B. breve* orally. This difference almost reached significance (p=0.075).

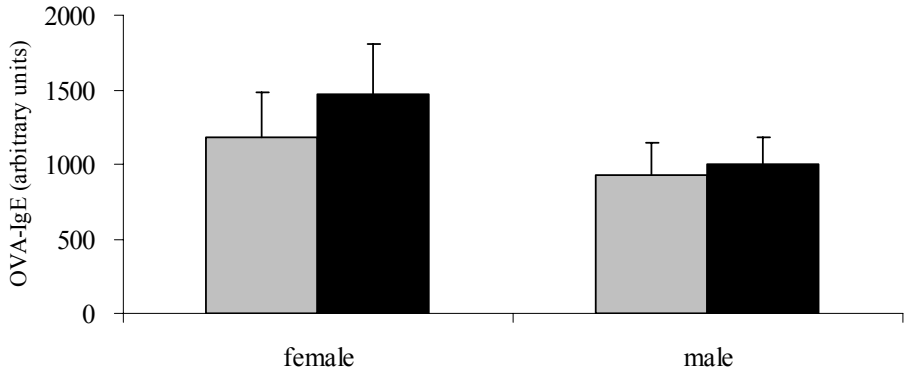


Figure 2: Serum levels of ovalbumin-specific IgE measured by ELISA. Mice were sensitized and challenged with ovalbumin and treated orally with B. breve (black bars) or saline (grey bars). Serum levels are expressed in arbitrary units that were calculated by using reference serum with ovalbumin-specific IgG1.

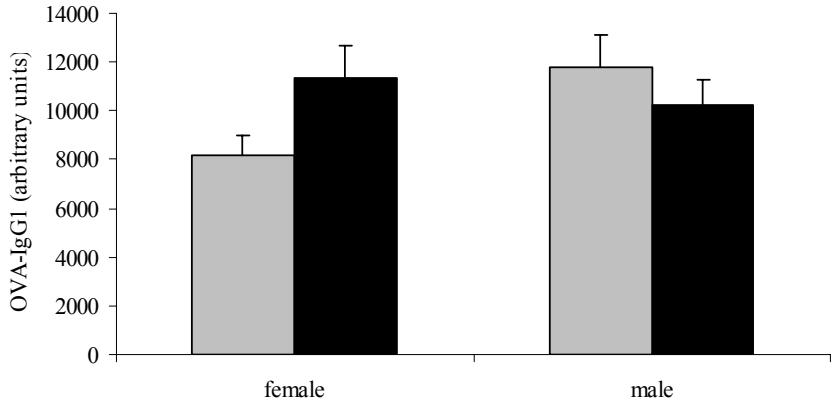


Figure 3: Serum levels of ovalbumin-specific IgG1 measured by ELISA. Mice were sensitized and challenged with ovalbumin and treated orally with B. breve (black bars) or saline (grey bars. Serum levels are expressed in arbitrary units that were calculated by using reference serum with ovalbumin-specific IgG1.

3.1.3 Cytokine production by spleen cells stimulated with ConA

After *in vitro* stimulation with ConA spleen cells from female mice that were sensitized with OVA produced more IL-5, IL-10 and IL-13 (Figure 4A). *B. breve* did not affect this cytokine production. IFN-γ production did not differ between control and sensitized mice. However,

B. breve increased IFN-γ in both control and sensitized females. Furthermore, in control mice *B. breve* reduced IL-4 significantly this was not observed in sensitized females.

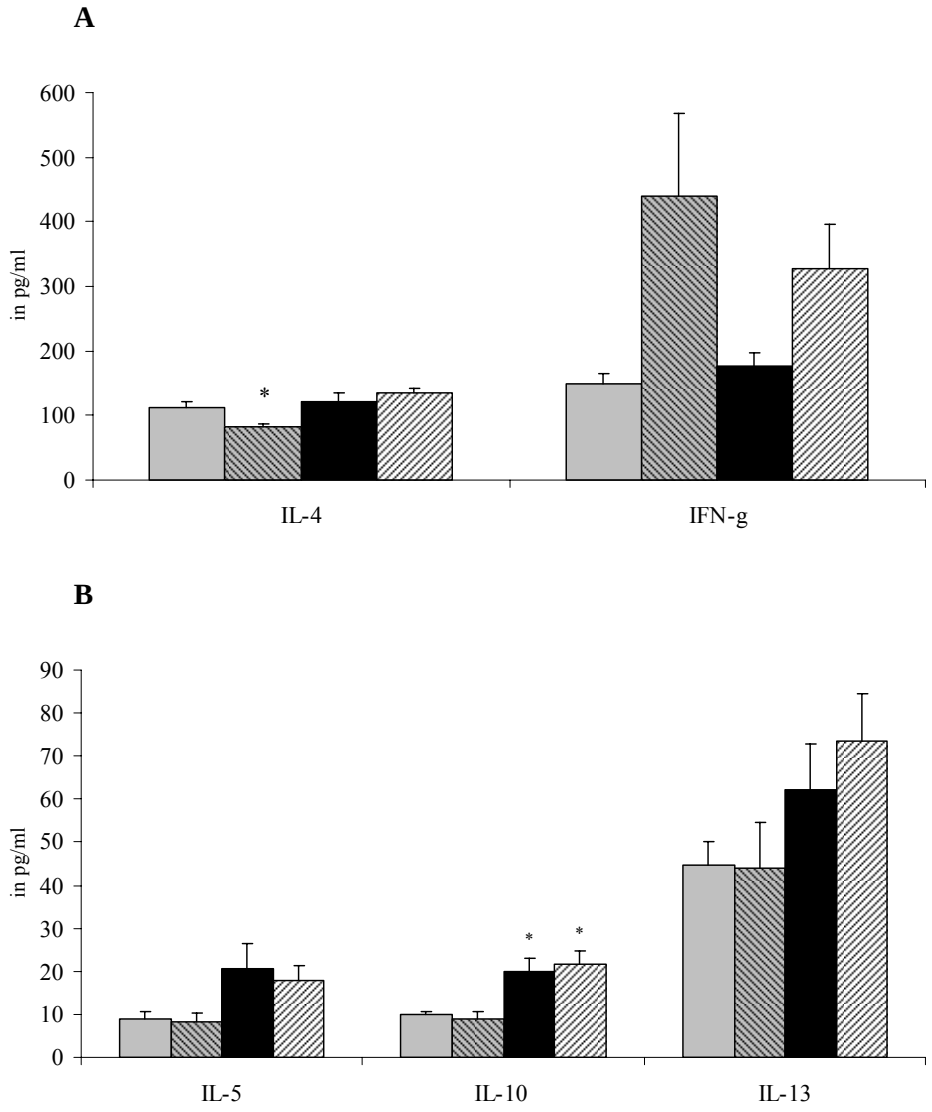


*Figure 4: Female mice were sensitized and challenged with vehicle and received either saline (grey bars) or B. breve (striped bars) orally or were sensitized and challenged with ovalbumin and received either saline (black bars) or B. breve (striped bars) .Cytokine production was assessed after ex vivo stimulation of spleen cells from female mice with 5 μg/ml ConA for 48 hours. Cytokines were measured with a bioplex Th1/Th2 kit on a Luminex® and expressed as mean ± SE in pg/ml supernatant.IL-4 and IFN-γ are shown in Figure A and IL-5, IL-10 and IL-13 in Figure B Statistical significant from the control group: *p<0.05.*

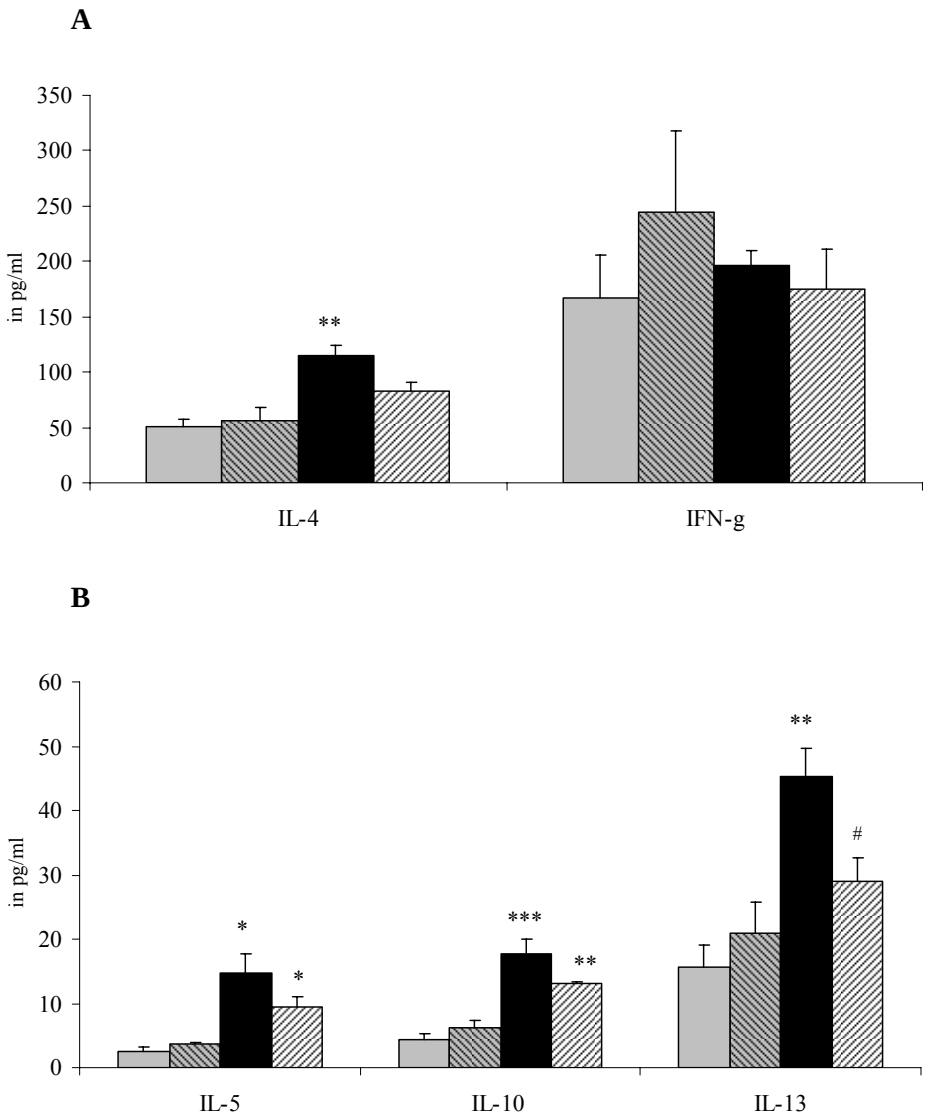


*Figure 5: Male mice were sensitized and challenged with vehicle and received either saline (grey bars) or B. breve (striped bars) orally or were sensitized and challenged with ovalbumin and received either saline (black bars) or B. breve (striped bars). Cytokine production was assessed after ex vivo stimulation of spleen cells from female mice with 5 μg/ml ConA for 48 hours. Cytokines were measured with a bioplex Th1/Th2 kit on a Luminex® and expressed as mean ± SE in pg/ml supernatant. IL-4 and IFN-γ are shown in Figure A and IL-5, IL-10 and IL-13 in Figure B. Significantly different from the control group: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significantly different from the OVA group: # $p<0.05$.*

Figure 5 shows the ConA-induced cytokine production in males. In sensitized males, splenocytes produced more IL-4, IL-5, IL-10 and IL-13. IFN-γ production was not affected by either sensitization with OVA or treatment with *B. breve*. A decrease in Th2 cytokines was observed after treatment with *B. breve*, which was significant for IL-13.

3.1.4 Cytokine production by spleen cells stimulated OVA

Spleen cells were also cultured with OVA to detect allergen-specific cytokine production. In females (Figure 6A), there was no effect of *B. breve* on cytokine production. However, in males (Figure 6B) *B. breve* reduced levels of all OVA-specific cytokines. This was significant for IFN-γ.

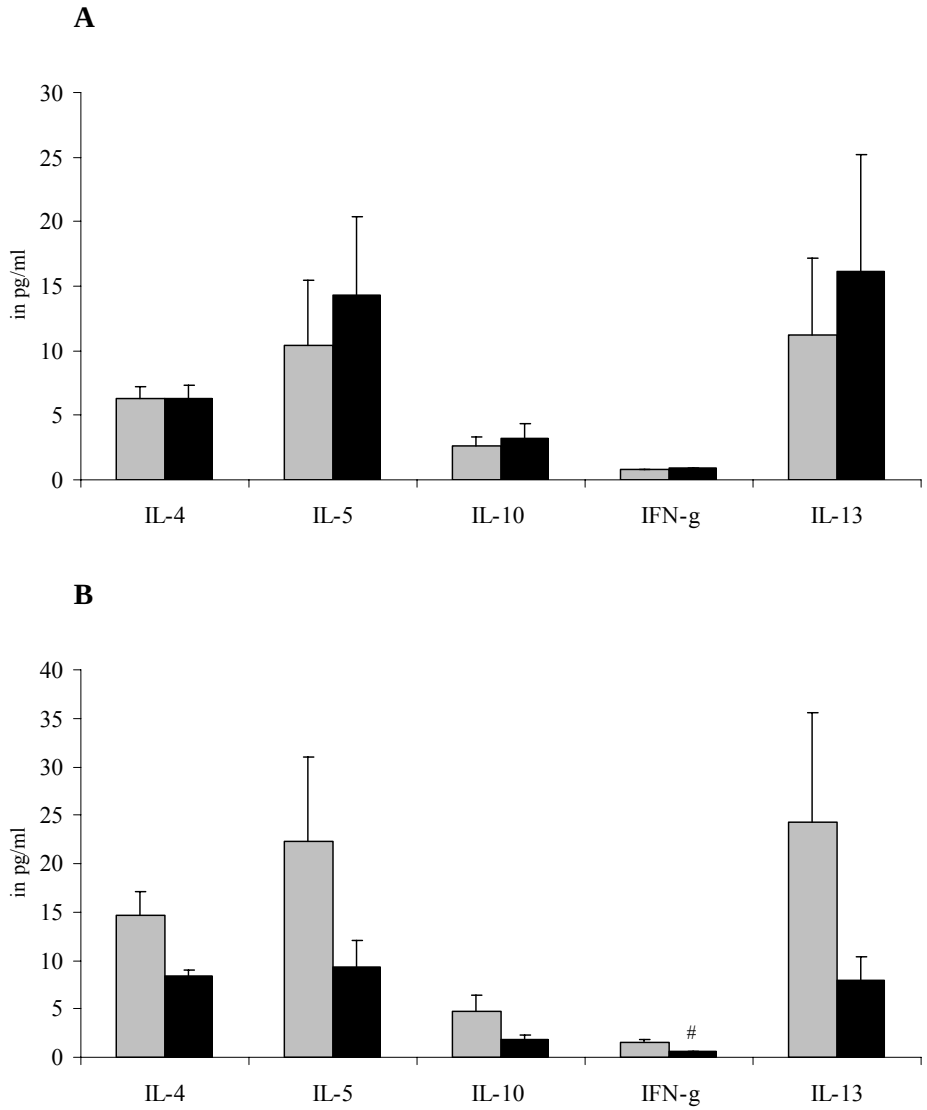


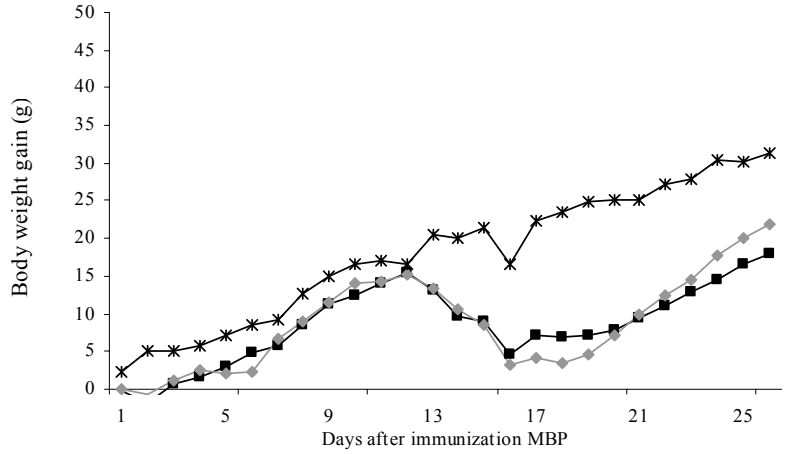
Figure 6: Female (A) or male (B) mice were sensitized and challenged with ovalbumin and received either saline (grey bars) or B. breve (black bars). Cytokine production after ex vivo stimulation of spleen cells with 100 μg/ml OVA for 96 hours. Cytokines were assessed with a bioplex Th1/Th2 kit on a Luminex® and expressed as mean ± SE in pg/ml supernatant. Significantly different from the OVA group: # $p<0.05$.

3.2 Effects of B. breve on experimental autoimmune encephalomyelitis

3.2.1 Body weight

In Figure 7 the gain in body weight gain after immunization of female (Figure A) and male (Figure B) Lewis rats is shown. Growth is normal in both male and female control rats.

A



B

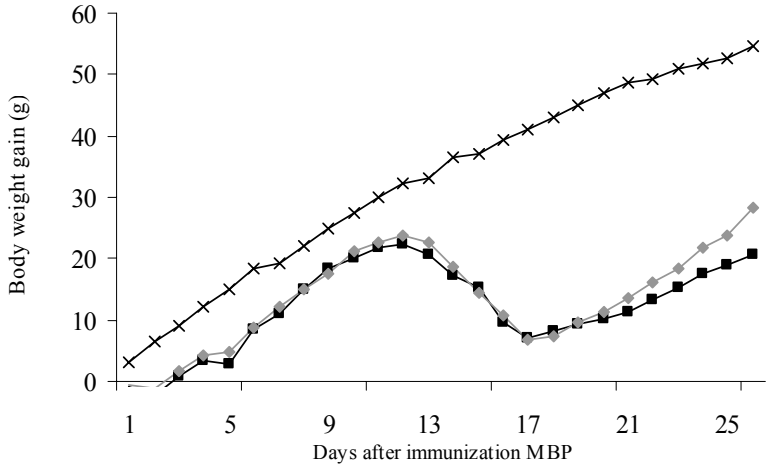


Figure 7: Female (A) or male (B) rats were immunized with MBP and received saline (-■-) or B. breve (-♦-). Non-immunized controls are also included in the figure (-x-). Body weight gain is expressed as the increase in body weight in grams from the day of immunization. Figure A shows mean body weight gain of females and figure B of males.

Weight loss was observed in all rats that were immunized with MBP. In females that did not receive probiotics this occurred from day 13 until 16 and in females that received *B. breve* from day 12 until 16. Then, rats started gaining weight again and the curve for females that received *B. breve* appears to be somewhat steeper, indicating a more rapid growth. Body weight on the final day of the experiment (day 26) was higher in females that received *B. breve* (p=0.087).
In immunized males, weight loss was observed from day 12 until 17, in the probiotic and the control group. After day 17 males that were treated with *B. breve* gained weight faster compared to controls. Body weight was significantly higher at day 25 (p=0.045).

Table 4: Effects of B. breve on clinical parameters of EAE

	Disease onset (days)	**Duration symptoms (days)**	**Cumulative disease index**
females			
EAE	14.4 ± 0.6	6.8 ± 1.1	4.5
EAE + *B. breve*	13.6 ± 0.3	6.0 ± 0.7	4.9
males			
EAE	13.4 ± 0.4	7.4 ± 0.6	6.1
EAE + *B. breve*	13.9 ± 0.6	5.5 ± 0.3**	5.9

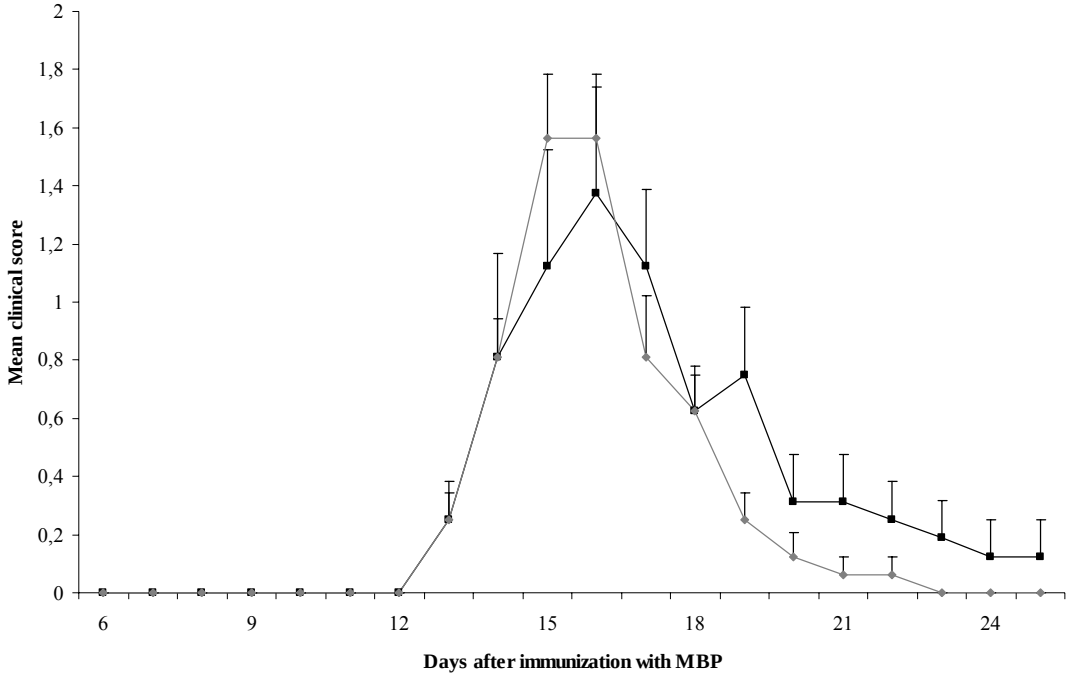
*Disease onset is the first day that clinical signs were observed in a treatment group, the duration of symptoms is the total number of days clinical symptoms were visible in a treatment group, and cumulative disease index (CDI) is sum of the cumulative daily scores per group divided by the number of days that the clinical symptoms were observed in this group. All data are expressed as mean ± SE. Significantly different from the EAE group: ** $p<0.01$*

3.2.2 Clinical symptoms

All rats that were immunized with MBP developed EAE. The first clinical symptom that appeared was loss of tonicity of the tail. In Table 4 clinical parameters of EAE are summarized. In females the day of onset was not influenced by *B. breve*. Furthermore, the duration of symptoms and the cumulative disease index was slightly increased in females that received the *B. breve* compared to those that received vehicle. In males, onset and cumulative disease index were not affected by *B. breve*. The duration of symptoms, however, was almost two days shorter in males that receive *B. breve*. The mean clinical score in time is shown in Figure 8 and the effects of *B. breve* on the duration of symptoms is illustrated by a shift of the

curve to the left (Figure 8b). A similar trend is observed for females, although this is less evident (Figure 8A).

A



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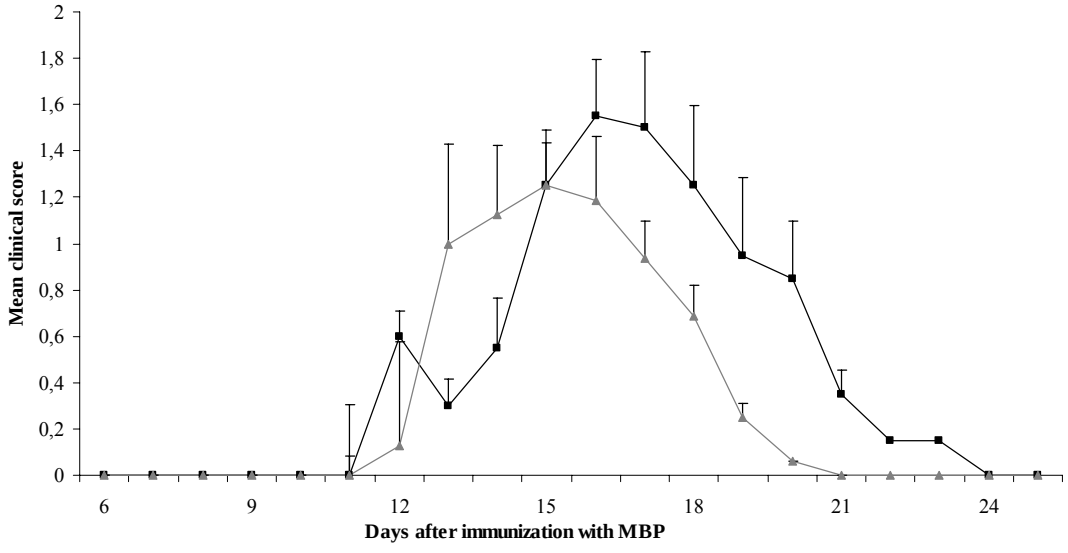
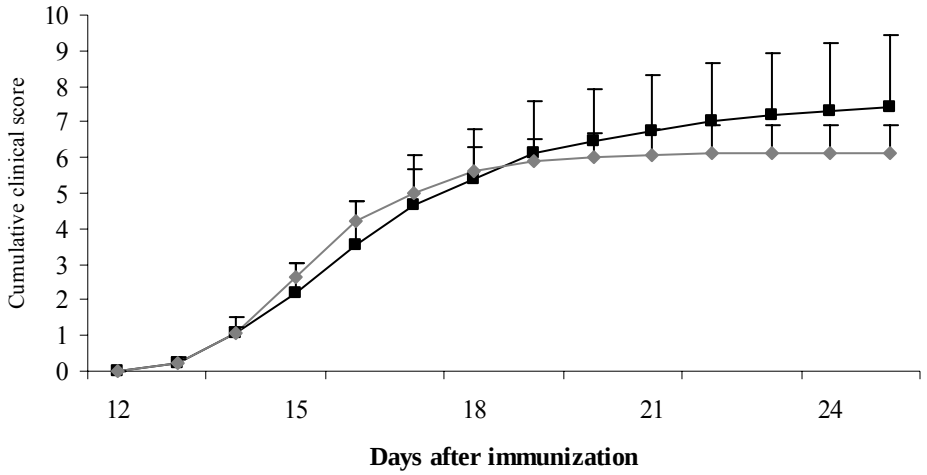


Figure 8: Female (A) or male (B) rats were immunized with MBP and received saline (-■-) or B. breve (-♦-). Time course of the mean clinical score per day (±SE) is shown.

In Figure 9 the cumulative clinical score is shown. This figure shows that in female rats that were immunized and received the vehicle orally, the cumulative score is still increasing at the end of the experiment. In females that received *B. breve* displayed the cumulative clinical score increased until day 19. In males the increase of cumulative score stops on day 21 and on day 18, for vehicle and *B. breve* treated rats, respectively.

A



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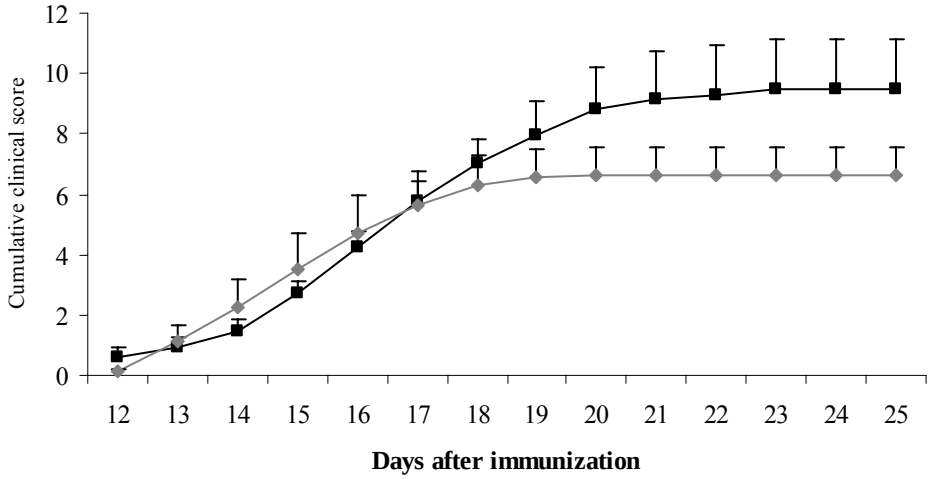

Figure 9: Female (A) or male (B) rats were immunized with MBP and received saline (-■-) or B. breve (-♦-). Cumulative score(±SE) per day is calculated by cumulating the clinical socre per day over time per animal.

3.2.3 MBP-specific IFN-γ production

Ex vivo stimulation with 10 and 25 μg/ml MBP resulted in a dose-dependent increase of IFN-γ production (Figure 10). In females treated with *B. breve* an increase of MBP-specific IFN-γ production was observed, which was significant for cells stimulated with 25 μg/ml MBP. In males a similar trend towards higher IFN-γ production after *B. breve* treatment was observed. These differences did not reach significance. MBP-specific IL-4 could not be detected in these samples.

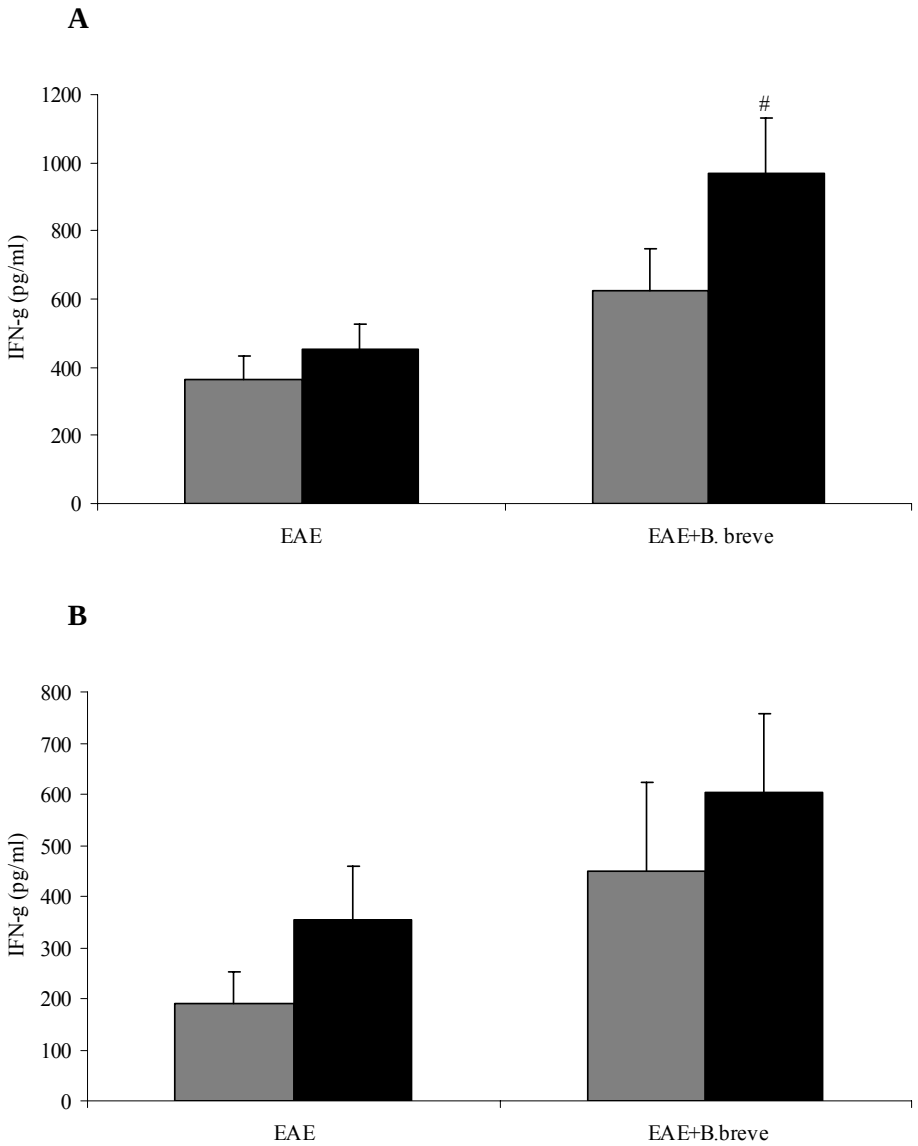

Figure 10: IFN-γ production by spleen cells from female (A) and male (B) Lewis rats stimulated with 10 (grey bars) or 25 (black bars) μg/ml MBP mg/ml for 96 hours. IFN-γ was detected with a sandwich ELISA. Significantly different from the control: # $p<0.05$

4. DISCUSSION

We have investigated the effects of *B. breve* on respiratory allergy in mice and on EAE in rats. In the respiratory allergy model, *B. breve* slightly reduced the inflammatory response in the lungs of females and males, by reducing the number of infiltrating eosinophils and lymphocytes. This reduction was not supported by a reduction of cytokines in the BAL fluid, such as IL-5. Cytokine profiles assessed in spleen cells stimulated with ConA showed that *B. breve* increased IFN-γ production in control and sensitized females and reduced IL-4 in control females. This shift in cytokine profile did not result in reduced serum IgE levels, however. Remarkably, ovalbumin-specific IgG1 levels, which are also Th2 mediated, were increased in females. In previous allergy models, probiotics stimulated Th1 responses and as a consequence a reduction of allergen-specific serum IgE was observed (Matsuzaki et al., 1998; Matsuzaki and Chin, 2000; Sashihara et al., 2006). In this study, the shift in cytokine profile towards Th1 was probably not sufficient to reduce ovalbumin-specific IgE in our model. It is important to note that the shift towards Th1 was only observed after *ex vivo* stimulation with ConA and not with ovalbumin. In other studies the shift towards Th1 was observed after *ex vivo* stimulation with ovalbumin (Matsuzaki et al., 1998; Matsuzaki and Chin, 2000). This could mean that *B. breve* had an effect on spleen cells, but not on the ovalbumin-specific response, since stimulation with ConA induced Th1 responses in both control and sensitized females. In males, however, *B. breve* reduced ovalbumin-specific production of all assessed Th1 and Th2 cytokines. Apparently this was not sufficient to reduce IgE levels.

Previously we have investigated the effects of LcS in the same allergy model. LcS modestly enhanced ovalbumin-specific IgE levels both in adult mice and in mice that received LcS from lactation phase onward. This increase was accompanied by an increase of ovalbumin-specific cytokine production in adult mice (Ezendam et al., 2006). However, in mice that received LcS from lactation phase onward, no effects on cytokine profiles were found (Ezendam and Van Loveren, 2006). The inflammatory lung response was affected differently depending on the timing of administration. A reduction was observed in adult mice that received LcS (Ezendam et al., 2006), whereas a further increase was observed after early administration (Ezendam and Van Loveren, 2006). Thus, early administration of LcS aggravated lung inflammation, whereas adult exposure alleviated it. *B. breve* induced a different effect compared to LcS, since administration of *B. breve* from lactation phase onward slightly reduced lung inflammation.

Also in the EAE model in rats LcS and *B. breve* differentially affected the disease. *B. breve* significantly reduced the duration of the disease in males and induced a faster recovery after

weight loss. A similar trend was observed in the females. Remarkably, in both males and females MBP-specific IFN-γ production was increased. One would expect that stimulation of Th1 responses would lead to aggravation of EAE. Previously, alleviation of EAE symptoms was associated with a decrease of Th1 (IL-12, IFN-γ) and an increase of Th2/Th3 (IL-4, IL-10, TGF-β) cytokines (Xu et al., 2000; Monteiro de Castro et al., 2004;Yang et al., 2004). It is important to note that there was no correlation between MBP-specific IFN-γ levels and the assessed clinical parameters. Thus, it seems that IFN-γ is not involved in the alleviation of EAE. Effects of *B. breve* on MBP-specific Th2 and Th3 cytokines might provide more insight in the influence of these cytokines on EAE.

In contrast to *B. breve,* LcS aggravated EAE symptoms, both in adults and in rats that received LcS from lactation phase onward (Baken et al., 2006; Ezendam et al., 2006; Ezendam and Van Loveren, 2006). The effects of probiotics on EAE are clearly strain-dependent. This is less obvious in the respiratory allergy model, although effects on the inflammatory lung response are also different for the two probiotic strains. In general, LcS appears to stimulate immune responses in these two experimental models, whereas *B. breve* alleviates both allergy and autoimmunity.

In conclusion, we have demonstrated that immune effects of probiotics are clearly strain-dependent. Our data do suggest that proper strain selection is needed to reach the goal for which the specific application is meant. Thus, *B. breve* appears to be suitable for the treatment of allergic or autoimmune diseases, whereas LcS might be more appropriate for the enhancement of resistance. Furthermore, in certain populations, LcS might induce unwanted effects. However, the consequences of these preclinical data cannot be directly extrapolated to the human situation. For extrapolation, well-designed human trials are needed. Information from such studies could provide insight in both the benefits and the risks of probiotic use.

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Begin dit jaar heeft u het RIVM rapport 'Immune effects of the probiotic *Bifidobacterium breve'* ontvangen. Aan dit rapport zal onderstaand erratum worden toegevoegd.

Erratum:
De naamgeving van de in de beschreven studies gebruikte probiotische stam is *Bifidobacterium animalis* in plaats van *Bifidobacterium breve*.

Met vriendelijke groet,

Janine Ezendam