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Immune effects of *Lactobacillus casei* Shirota

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HET RAPPORT IN HET KORT

Effecten van probiotica op het immuunsysteem

Verkendend onderzoek naar de effecten van probiotica geeft aan dat er een mogelijk risico bestaat voor het ontwikkelen van auto-immuunziekten. Probiotica worden in reclameboodschappen ook wel aangeduid als 'goede bacteriën'. Fabrikanten claimen een positief effect op darmflora, weerstand en mogelijk preventie van allergieën. Momenteel is er nog geen regelgeving op het gebied van probiotica en worden deze producten als veilig beschouwd. Om inzicht te verkrijgen in de effecten van probiotica op het immuunsysteem zijn verschillende proefdiermodellen gebruikt. De studies tonen aan dat toediening van probiotica zowel positieve als negatieve effecten kan hebben, afhankelijk van het model dat werd gebruikt. De effecten op deze immunologische responsen zijn gering. In een studie met proefdieren werd een vermindering van ontsteking in de longen waargenomen, terwijl in een auto-immuniteit model de symptomen verergerden. Verder onderzoek om de betekenis hiervan voor de mens vast te stellen is nog nodig.

Trefwoorden: Lactobacillus, probiotica, respiratoire allergie, autoimmunititeit, immuunmodulatie

ABSTRACT

Effects of administration of probiotics on the immune system

Exploratory studies on the effects of probiotics indicate a potential hazard with regard to induction of autoimmunity. Advertisements for probiotics claim their beneficial effects on gut flora, resistance and allergies. Although no legislation is currently in place, probiotics are generally regarded as safe bacteria. Studies in laboratory animals described here focused on effects of probiotics on the immune system. Probiotics were found to possibly have both beneficial and adverse effects, depending on the model used. Inhibition of inflammatory responses was noted in an allergy model, whereas stimulation of autoimmune effects was observed in a model for autoimmune disease. Effects noted were small. Further studies will be required to investigate whether these observations are relevant for humans.

Keywords: Lactobacillus, probiotics, respiratory allergy, autoimmunity, immunomodulation

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Summary

Lactic acid bacteria are claimed to have immunomodulating effects. Stimulation as well as suppression of T helper (Th)1 mediated immune responses has been described for various strains. Experiments involving *Lactobacillus casei* Shirota (LcS) detected mainly enhancement of innate immune responses and promotion of Th1 mediated immune reactivity. To confirm and further investigate modulation of both Th1 and Th2 responses the effects of oral administration of LcS were assessed in several experiments. The effect of LcS varied between the different models. No modulation was found in the mitogen-induced cell proliferation and cytokine release assays in mesenteric lymph nodes of Wistar rats. In an allergy model, LcS reduced inflammatory lung responses but stimulated systemic responses, such as specific IgE in the serum. LcS had no effect on Th2 mediated immune response in an adapted murine Local Lymph Node Assay (LLNA) in BALB/c mice, but inhibited Th1 responses in this model. Furthermore, LcS aggravated experimental autoimmune encephalomyelitis (EAE) in Lewis rats. These varying effects on Th1 and Th2 responses indicate that beneficial as well as harmful effects on immune related disorders could occur after LcS consumption. Since microarray analysis is suggested to be more sensitive and predictive than functional tests, gene expression profiling was included as an alternative endpoint in the testing of immunomodulation. The detected gene expression profiles did not reflect the effects of LcS on the immune system. Microarray analysis may therefore have no more predictive value than immune function assays when investigating immunomodulation by probiotics. To gain further insight into effects of probiotics on immune function, experiments including cytokine assays and gene expression analysis combined with disease models could be useful.

1. Introduction

Since the beginning of the previous century, various health promoting effects of human consumption of lactic acid bacteria have been described (Gill and Guarner, 2004; Vijaya Kumar et al., 2005). Numerous experiments have indicated that (changes in) the intestinal microbiota can cause immunomodulation, both at the intestinal and the systemic level (De Vrese and Schrezenmeir, 2002; Noverr and Huffnagle, 2004). Lactic acid bacteria, that are part of the human commensal microbiota and that have a long history of use in food products, have been studied widely. These non-pathogenic bacteria are marketed as probiotics, which means 'for life'. Probiotics are defined as 'living microorganisms which upon ingestion of sufficient numbers exert health benefits' (Schrezenmeir and de Vrese, 2001). The ability of several probiotic strains to modulate host innate as well as acquired immune responses has been demonstrated in a large amount of in vitro experiments and animal models. Besides stimulation of antibody production (Alvarez-Olmos and Oberhelman, 2001) and macrophage activity (Schiffrin et al., 1995), functional effects like inhibition of inflammation (Hart et al., 2003; Chapat et al., 2004), intestinal infections (Perdigon et al., 1995; Marteau et al., 2001), allergic disease symptoms (Isolauri et al., 2001; Kalliomaki and Isolauri, 2003; Kalliomaki et al., 2003) and autoimmune disorders (Gill and Guarner, 2004) were observed. This resulted in claims concerning beneficial effects of probiotics on some clinical conditions and on immune reactivity (Drakes et al., 2004; Gill and Guarner, 2004; Sartor, 2005).

Immunoregulatory effects of probiotics have often been ascribed to a shift of the Th1/Th2 balance towards Th1 mediated immunity. Release of the Th1 associated cytokines interleukin (IL)-12, interferon (IFN)- γ and tumor necrosis factor (TNF)- α by various cell types was repeatedly demonstrated to be increased by probiotics (Miettinen et al., 1996; Hessle et al., 1999; Gill et al., 2000; Christensen et al., 2002; Pochard et al., 2002; Morita et al., 2002; Perdigon et al., 2002; Matsuzaki et al., 2004; Cross et al., 2004; Mohamadzadeh et al., 2005). Moreover, several probiotics reduced production of Th2 related cytokines (Pochard et al., 2002). The skewing towards Th1 may inhibit the development of allergies, as was confirmed by suppression of immunoglobulin (Ig)E production by probiotics in animal allergy models (Murosaki et al., 1998; Shida et al., 2002). At the same time, some concern exists that this shift could be associated with stimulation of certain autoimmune diseases (Fujiwara et al., 2004). Indeed, the development of experimental autoimmune encephalomyelitis (EAE) was

found to be stimulated by oral administration of *Lactobacillus (L.) reuteri* to mice (Maassen et al., 1998).

To confirm modulation of Th1 and Th2 mediated immune responses by probiotics and to assess possible harmful consequences on Th1-driven immune responses, such as autoimmunity, we performed experiments with *Lactobacillus casei* Shirota (LcS). LcS is a non-pathogenic bacterial strain from human origin of which the impact on the immune system was investigated in a variety of studies. Its effects on innate immune responses was mainly stimulatory, as reflected by promotion of phagocytosis (Erickson and Hubbard, 2000) and enhancement of natural killer (NK) cell cytotoxicity (Nagao et al., 2000; Matsuzaki and Chin, 2000; Takagi et al., 2001; Hori et al., 2003; Yasui et al., 2004; Matsuzaki et al., 2005). Enhanced non-specific immunity was however not detected in healthy immunocompetent humans (Spanhaak et al., 1998). LcS also reduced secretion of the pro-inflammatory cytokine IL-6, which is suggested to be involved in inflammatory bowel diseases (Matsumoto et al., 2005). Furthermore, LcS was shown to promote splenic release of Th1 associated cytokines IFN- γ and IL-12 (Yasui et al., 1999; De Waard et al., 2002) and this shift in Th1/Th2 balance downregulated Th2 responses, e.g. IL-4 and IgE levels (Matsuzaki et al., 1998; Matsuzaki, 1998; Shida et al., 1998; Yasui et al., 1999; Kato et al., 1999; Shida et al., 2002). In line with these effects, LcS administration suppressed systemic allergic reactions in a food allergy model (Shida et al., 2002). In contrast, LcS as well as other probiotics suppressed the severity of autoimmune disease in animal models for diabetes and arthritis (Matsuzaki et al., 1997a; Matsuzaki et al., 1997b; Matsuzaki et al., 1997c; Kato et al., 1998; Baharav et al., 2004; Calcinaro et al., 2005). This was explained by effects on both cellular (improved macrophage recruitment, inhibition of T cell proliferation, reduced IL-6 and IFN- γ production, increased IL-10 and IL-12 release) and humoral (less auto-antibodies) responses.

We examined the immunomodulatory capacity of LcS in several assays. The murine Local Lymph Node Assay (LLNA) is a validated test method used for identification of compounds with sensitizing potential (Kimber et al., 2002). A modified LLNA was applied to determine effects of oral exposure to LcS on systemic immune reactivity, as was described for other immunomodulating compounds before (Van den Berg et al., 2005). Two chemicals were used in the LLNA, the contact sensitizer 2,4-dinitrochlorobenzene (DNCB) or which induces a Th1 response and the respiratory allergen benzenetricarboxylic anhydride (TMA) which induces a Th2 response. Lymphocyte proliferation in draining lymph nodes after application of these compounds was used as a measure for Th1 and Th2 responsiveness. A murine experimental model for respiratory allergy was used as a model for Th2 mediated immune responses,

whereas the EAE model in rats was used as a model for Th1 mediated immune responses. EAE, which is an experimental model for multiple sclerosis, is assumed to be induced by activation of autoreactive Th1 cells that recognize myelin basic protein (MBP), which causes inflammatory demyelination in the central nervous system leading to transient paralysis (Lassmann and Ransohoff, 2004). Effects of oral LcS exposure on cell proliferation and cytokine release in mesenteric lymph nodes (MLN) were assessed in another strain of rats. The last approach to examine immunomodulation by oral administration of LcS was microarray analysis. Specific changes in gene expression are supposed to be more sensitive and accurate endpoints than traditional endpoints when investigating an organism's response to a certain exposure (Steiner et al., 2004; De Longueville et al., 2004). Therefore, we included gene expression profiling in MLN, spleen, liver and thymus of rats to yield a global insight into the effects of LcS on the immune system and to possibly provide insight into genes and cellular pathways involved (Waters and Fostel, 2004).

2. Materials and methods

2.1. Bacteria

Lactobacillus casei strain Shirota (isolated from a commercially available LcS containing drink) was cultured for 72 hours at 30°C under anaerobic conditions in Man Rogosa Sharpe (MRS) broth (CM359; Oxoid, Haarlem, the Netherlands). Thereafter, bacteria were washed twice with saline (0.9% NaCl) containing 1 mg/ml peptone (saline/peptone) and resuspended in saline/peptone to a final concentration of either 1×10^9 CFU/ml (experiments in rats) or 2×10^9 CFU/ml (experiments in mice). The number and viability of the lactobacilli were determined by aerobic culturing on MRS plates (Oxoid CM361) for 72 hours. In all experiments, LcS was administered orally by means of intragastric dosing.

3. Immune function tests in mice

3.1. Animals

Young adult (6–8 weeks old) male BALB/c mice were obtained from the institutes own breeding colony. The animals were bred specific pathogen free (SPF) and kept in macrolon cages under conventional conditions. The mice were fed Hope Farms chow pellets (Woerden, NL) and water *ad libitum* during the whole experiment.

3.2. Experimental design adapted Local Lymph Node Assay

Animals were randomly divided in 8 groups (Table 1). Oral administration of LcS started 8 days prior to sensitization. Mice were sensitized by topical application of 25 µl DNCB or TMA solution on the dorsum of both ears for three consecutive days at day 0, 1 and 2 (Figure 1). Control mice received the same treatment protocol with AOO.

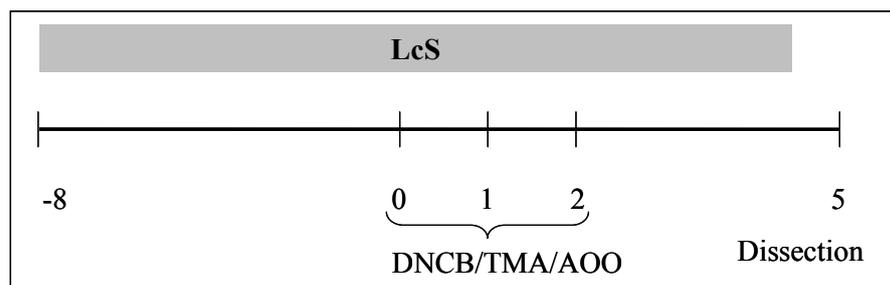


Figure 1: Experimental setup adapted LLNA

Table 1: Experimental groups treated either with DNCB or TMA

LcS	DNCB	TMA	Number
+	AOO	AOO	8
+	DNCB 0.33 %	TMA 3.3 %	4
+	DNCB 0.66 %	TMA 6.6 %	4
+	DNCB 1 %	TMA 10 %	4
-	AOO	AOO	8
-	DNCB 0.33 %	TMA 3.3 %	4
-	DNCB 0.66 %	TMA 6.6 %	4
-	DNCB 1 %	TMA 10 %	4

Abbreviations: TMA: 1,2,4-benzenetricarboxylic anhydride; DNCB: 2,4-dinitrochlorobenzene; AOO: acetone/olive oil 4:1; LcS: *Lactobacillus casei* Shirota

In contrast to the LLNA, lymphocytes in the auricular lymph nodes were not labeled with [³H]-thymidine *in vivo*, but harvested and labeled *ex vivo*. Three days after the last topical application of DNCB, the mice were euthanized by CO₂/O₂ exposure and the auricular lymph nodes (LN) were excised, pooled for each animal, weighed and suspended in 5 ml RPMI 1640 (Gibco, Life Technologies, Breda, the Netherlands) with 5% heat inactivated Fetal Calf Serum (FRS) (PAA, Linz, Austria), 100 units/ml penicillin and 100 µg/ml streptomycin (standard medium). Single cell suspensions were prepared under aseptic conditions by pressing the LN through a 70 µm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed twice in standard medium (10 min, 300 g, 4°C) and resuspended in 1 ml standard medium with 10% FRS. A Coulter Counter (Z2, Coulter Electronics, Mijdrecht, The Netherlands) was used to count the cells. Then the concentration of the cell suspensions was adjusted to 1x10⁷ cells/ml.

3.3. Cell proliferation

Of each cell suspension of 1x10⁷ cells/ml, 200 µl was seeded in triplicate in a U-bottom 96-wells plate (Greiner, Alphen aan den Rijn, the Netherlands). After addition of 10 µl/well (= 1µCi) [methyl-³H]-thymidine (Amersham Biosciences, Buckinghamshire, UK) the cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ during 20-24 hours. The cells were harvested on glass-fiber filters (LKB-Wallac, Turku, Finland) using a multiple

cell culture harvester (LKB-Wallac). The [³H]-thymidine activity was determined using a liquid scintillation counter (1205 Betaplate TM, LKB-Wallac). For further calculations the median of the triplicates was used. The [³H]-thymidine incorporation is expressed per animal, being the measured counts per minute (cpm) times the cell number of the two LN and divided by the cell number in culture. The total experiment was repeated once, using 4 mice more in both 0% DNCB groups. The results of the two experiments were pooled and the mean [³H]-thymidine incorporation per experimental group \pm SE was calculated. Statistical analysis was performed using one-way analysis of variance (ANOVA). Significant differences of the control group were determined with the Bonferroni post hoc test, using a significance level of $p = 0.05$.

3.4. Experimental design respiratory allergy model

Mice were randomly assigned to the experimental groups (Table 1), that received either LcS or vehicle, starting 8 days prior to sensitization.

Table 2: Experimental groups

Sensitization/challenge	LcS	Sex	Number
Ovalbumin	LcS	female	8
Ovalbumin	vehicle	female	8
Vehicle	LcS	female	4
Vehicle	vehicle	female	4
Ovalbumin	LcS	male	8
Ovalbumin	vehicle	male	8
Vehicle	LcS	male	4
Vehicle	vehicle	male	4

Mice were sensitized by two i.p. injections with 10 mg ovalbumin (grade V, Sigma) adsorbed on to 2.25 mg aluminum hydroxide (Pierce, Rockford, IL, USA) in saline or with saline alone on days 0 and 14. 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 42 mice were sacrificed and blood was collected and clotted to collect serum for determination of ovalbumin specific immunoglobulines (Figure 2).

3.5. Bronchoalveolar lavage and lung histopathology

Fluid collected from bronchoalveolar lavage (BAL) was centrifuged at 1200 rpm for 10 minutes. BAL fluid was collected and used for cytokine measurements. Pellets were used for determination of total cell number and for cytopsin preparations. Cytopsin were stained with May-Grünwald Giemsa and on each preparation 400 cells were counted. After lavage, lungs were fixed in 4% phosphate-buffered formaldehyde and embedded in Paraplast. Five-micrometer sections were cut and routinely stained with hematoxylin and eosin (HE) and with May-Grünwald Giemsa. Histopathological lesions were semiquantitatively and blindly scored as: absent (0), minimal (1), slight (2), moderate (3), marked (4), or strong (5). In this score both incidence and severity of lesions were incorporated.

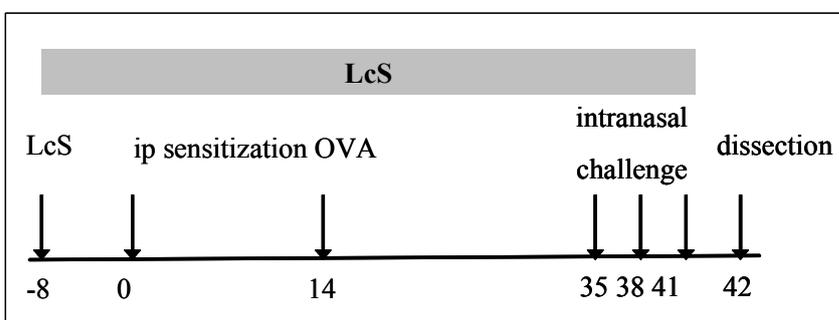


Figure 2: Experimental setup respiratory allergy model

3.6. Cell culture for cytokine measurements

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°C, 300g) 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 $\mu\text{g}/\text{ml}$ ConA (100 μl /well) for 48 hours or with ovalbumin (100 $\mu\text{g}/\text{ml}$) for 96 hours. Supernatants were collected for cytokine measurements.

3.7. 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).

3.8. ELISA specific for ovalbumin IgE

Specific ovalbumin IgE 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. Briefly, 96-wells plates (Nunc-Immuno Plate, Denmark) were coated overnight at 4°C with 2 μ g/ml rat-anti-mouse IgE (ram IgE, Zymed, 04-7000) diluted in sodium carbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were blocked by adding 0.05 M Tris buffered saline with 1% BSA, pH 8 (Sigma) for 1 hour at 37°C. Thereafter, serial dilutions of mouse serum samples and a pooled positive serum were incubated for 1 hour at 37°C. Then, wells were incubated for 1 hour 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). The coupled proteins were separated on a Sephadex G-25 column and labeling efficiency was determined spectrophotometrically at 280 nm. 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 H₂SO₄ and absorbance was read at 450 nm. Extinction values of the serum samples were expressed as a percentage of the positive serum.

3.9. ELISA for specific ovalbumin IgG1

Wells were coated overnight at 4°C with 10 μ g/ml ovalbumin/ml PBS (grade V, Sigma). Incubations were followed by extensive washing on an automatic plate washer with PBS containing 0.1% Tween-20. Blocking reagent (0.05 M Tris buffered saline with 1% BSA, pH 8.0 (Sigma)) was added and wells were incubated for 1 hour at 37°C. Thereafter, serial dilutions of mouse serum samples and a pooled positive serum were added to the wells and incubated for 2 hours at RT. Biotinylated rat-anti-mouse IgG1 or IgG2a (Zymed Laboratories, San Francisco, CA) was added and wells were incubated for 1.5 hours 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 H₂SO₄ and absorbance was read at 450 nm. Extinction values of the serum samples were expressed as a percentage of the positive serum. Standards, samples, and streptavidin were diluted in blocking buffer plus 0.05% Tween-20.

3.10. Statistical analysis

Statistical differences in lung inflammatory responses and ConA-induced cytokine production between treatment groups were determined using a one-way ANOVA with Scheffe's post hoc test. Histological lung parameters were statistically tested with the Wilcoxon ranking test. Differences in ovalbumin-specific IgE and IgG1 levels and ovalbumin-induced cytokine levels were determined with a two-tailed Student's t-test comparing the two experimental groups that were sensitized and challenged with ovalbumin.

4. Immune function tests in rats

4.1. Experimental design EAE

Six to eight week old SPF male Lewis rats (LEW/HanHsd) were obtained from Harlan (Horst, the Netherlands). Rats were housed individually under standard conditions with water and food *ad libitum*. The animals were allowed to acclimatize one week before starting the experiment.

Rats were randomly divided in two experimental groups (n=8 per group), either or not receiving LcS orally. Daily LcS administration started 8 days prior to the induction of EAE and continued during the course of the experiment. Rats received 1 ml of LcS (1×10^9 CFU/ml) or saline/peptone (controls). Acute EAE was induced in both experimental groups by subcutaneous injection of 50 μ l emulsion containing 1 g/ml guinea pig spinal cord homogenate and 10 mg/ml *Mycobacterium tuberculosis* type H37RA (Difco, Detroit, MI) suspended in equal amounts of complete Freund's adjuvant (CFA, Difco) and saline (0.9% NaCl). EAE injections were performed under halothane anesthesia. After induction of EAE body weight was recorded daily for 28 days. Also, neurological signs were scored daily during 28 days 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.

4.2. Statistical analysis

Mean body weights were calculated for LcS treated and control rats on all 28 days. Significant differences between both experimental groups were determined per day with a two-tailed Student's t-test (significance level $p = 0.05$). The clinical scores per animal were added over time and averaged per day (\pm SE) to display the progress of the disease. The significance of the difference of these cumulative scores between the groups was calculated per day using a one-tailed Mann-Whitney Test (significance level $p = 0.05$). The same test was used to analyze the duration of EAE, defined by the amount of days with clinical signs of EAE per animal. For both groups a Cumulative Disease Index (CDI) was determined, being the sum of the cumulative daily scores per group divided by the amount of days that clinical disease signs were observed in the group.

4.3. Experimental design mitogen-induced cell proliferation and cytokine production and DNA microarray

Male 17-week old Wistar WU(CPB) rats (n=16) were obtained from our own breeding colony (RIVM CDL-D5a/b). The rats were bred specific pathogen free and kept in macrolon cages under tightly controlled standardized conditions. Water and food pellets were available *ad libitum*.

The animals were randomly assigned to two experimental groups (n=8), receiving either 1 ml of LcS (1×10^9 CFU/ml) or 1 ml saline/peptone (controls) by gavage. After eight days rats were euthanized by CO₂/O₂ exposure and weighed. Dissection took place and spleen, liver, thymus and MLN were collected and weighed. The MLN were split in two and both halves were weighed. One half was immediately snap frozen in liquid nitrogen, as were the spleen, liver and thymus. The other half was kept in standard medium on ice until further processing for immune function tests. The frozen organs were stored at -80 °C until use in RNA isolation (see below).

4.4. Lymphocyte transformation test

For determination of mitogen-induced cell proliferation in the MLN, the same protocol as described for the LLNA was used, only here 100 µl of a cell suspension of 4×10^6 cells/ml was added per well and proliferation was assessed after 72 and 96 hours of incubation with 50 µl of the mitogens Concavalin A (ConA; 3.33 µg/ml, ICN Biochemicals), lipopolysaccharide (LPS, 16.7 µg/ml, Sigma), phytohaemagglutinin (PHA; 1:25, Murex Biotech, Dartford, UK) and pokeweed mitogen (PWM; 1.67 µg/ml, Gibco Life Technologies). [³H]-thymidine was added for the last 20-22 hours of incubation.

4.5. IL-4 and IFN-γ release

To measure IL-4 and IFN-γ release, single cell suspensions of the MLN were cultured with ConA. Cell suspensions (4×10^6 cells/well) were seeded in 6-well plates (Greiner) and incubated with 5 µg/ml ConA for 48 hours at 37°C, 5% CO₂. IL-4 and IFN-γ concentrations in supernatants were determined by an enzyme-linked immunosorbent assay (ELISA). All monoclonal antibodies were obtained from Pharmingen and ELISAs were performed

according to the manufacturer's description with some minor modifications. Incubations were followed by extensive washing on an automatic plate washer (ELx405, Bio-Tek, Instruments, Inc.) with PBS containing 0.1% Tween-20 (Merck, Amsterdam, the Netherlands). Briefly, 96-wells plates (Nunc-Immuno Plate, Denmark) were coated overnight at 4°C with 2 µg/ml rat anti-mouse IFN-γ or 1 µg/ml rat anti-mouse IL-4 in 0.04 M carbonate buffer, pH 9.6. Then the plates were blocked by adding a blocking solution (Sigma) that contained 0.05 M Tris buffered saline with 1% bovine serum albumin (BSA, Sigma), pH 8.0 for 2 hours at 37°C. After blocking, serial dilutions of recombinant mouse IFN-γ or recombinant IL-4 and supernatants were added and incubated for 2 hours at 37°C. Thereafter, biotinylated anti-mouse IFN-γ (0.5 µg/ml) or IL-4 (0.5 µg/ml) was added and incubated for 1 hour at room temperature (RT), followed by incubation with poly horseradish peroxidase labeled streptavidin (CLB, Amsterdam, the Netherlands) for 45 minutes at RT. Plates were washed again and tetramethylbenzidine (TMB) solution (0.1 mg/ml TMB (Sigma) plus 0.006% H₂O₂, in 0.1 M NaAc, pH 5.5) was added. This coloring reaction was terminated with H₂SO₄ (10%, Merck) and absorbance was read at 450 nm using an automated multichannel photometer (Titertek Multiscan MCC/340, Merlin Diagnostics System B.V.). Standard curves of recombinant IFN-γ or IL-4 were used to calculate the amount of cytokines. Results are expressed per animal, i.e. the response is multiplied by the total LN cell number, and divided by the cell culture concentration.

4.6. Statistical analysis

Significant differences between the two groups in animal and organs weights, MLN cell numbers, mitogen-induced cell proliferation and cytokine production were determined with a two-tailed Student's t-test using a significance level of $p = 0.05$. Absolute organ weights as well as relative weights (organs weights divided by body weights) were analyzed.

4.7. Gene expression analysis

Gene expression analysis was performed in the snap frozen spleen, liver, thymus and MLN halves from the male 17-week old Wistar WU(CPB) rats (n=16) that were used for the cell proliferation and cytokine release assays.

4.8. RNA isolation

Total RNA was isolated from the collected organs with a commercially available kit (RNeasy, Qiagen, Venlo, the Netherlands) in combination with DNase treatment (RNase-Free DNase Set, Qiagen) according to the manufacturer's instructions. To start the procedure, a parts of the frozen organ was split off using a mortar and pestle cooled by liquid nitrogen. This piece was then placed in the lysis buffer and immediately disrupted and homogenized using a rotor-stator homogenizer (Diax 900 10G, Heidolph, Schwabach, Germany). Quantity of RNA was assessed spectrophotometrically (ND1000, NanoDrop Technologies, Wilmington, USA) and RNA integrity was measured with automated gel electrophoresis (Bioanalyzer 2100, Agilent Technologies, Amstelveen, the Netherlands). The concentration of all rat RNA samples was adjusted to 2.5 µg/µl by vacuum drying and addition of RNase free water (Qiagen). For each organ a reference pool was prepared by pooling equal amounts of RNA from all animals (n=16). The RNA yield from MLN was not sufficient to perform microarray analysis and therefore amplified with an amplification kit (Amino Allyl MessageAmpTMaRNA kit, Ambion, Austin, USA). All RNA samples were stored at -80°C until microarray analysis.

4.9. Microarray analysis

Microarray analysis was performed with RNA from five rats for one organ at a time. cDNA synthesis and labeling were performed using an indirect labeling method, involving incorporation of amino allyl-dUTP groups that bind fluorescent CyDye NHS-esters and purification of reaction products by means of spin columns (Cyscribe Post Labelling kit, CyDye Post Labelling Reactive Dye Pack and Cyscribe GFX Purification kit, Amersham Biosciences, Buckinghamshire, UK), according to the manufacturer's instructions. Cy5 was used to label 20 µg of sample cDNA and equal aliquots of reference pool cDNA were labeled with Cy3 simultaneously. The volume of the purified labeled cDNAs was reduced to 12.5 µl, after which every sample was combined with a labeled aliquot of reference pool cDNA.

The Rat Array Ready Oligo Set v1.0 (Operon Biotechnologies GmbH, Cologne, Germany) containing 5,705 C6-amino-linked oligos representing rat genes was spotted in duplicate on silylated glass slides at the University Medical Centre Utrecht (UMC). Slides also contained appropriate controls and blank spots, which were used for quality control but not in further

analyses. Pre-treatment and hybridization proceeded according to protocols developed at the UMC. Pre-treatment of the slides included successive washing in a 0.2%SDS (Ambion) solution, milliQ, 0.25 g of sodiumborohydride (Sigma) in 75 ml phosphate buffered saline and 25 ml ethanol, and 0.2%SDS and milliQ again. They were dried quickly and evenly by centrifugation at 1000 rpm and stored in a dark moisture- and dust-free exsiccator until cDNA hybridization. Then the slides were prehybridized in a solution (sterilized by filtered through a 0.22 micron filter (Millipore, Amsterdam, the Netherlands)) consisting of 12 ml 2x SSC (Invitrogen, Breda, the Netherlands), 0.5 ml 10%SDS 0.5 g BSA (Sigma) and 37 ml milliQ, or a multiple of this, at 42°C for at least one hour. Just before hybridization, the slides were washed in milliQ and propanol and dried by centrifugation. Hybridization mixture was made of 2.55 ml formamide (Sigma), 2.55 ml 20xSSC and 0.1 ml 10%SDS (or a multiple of this), filtered through a 0.22 micron filter and preheated to 42°C. Then 50 µg salmon sperm (Gibco, Invitrogen) and 1.3 pmol of a Cy3-labeled 70-mer oligo complementary to a landmark control oligo (Van de Peppel et al., 2003) (Operon) were added to 250 µl of the hybridization mix. Of this hybridization mix, 25 µl was added to each 25 µl of combined sample and reference cDNA, and the complete mixture was heated at 95°C for 5 minutes. Finally, 45 µl of the cDNA mixture was pipetted onto the glass slide and spread evenly by application of a cover slip. This was incubated for approximately 18 hours at 42°C in the dark in humid hybridization chambers (GeneMachines Genomic Solutions Ltd., Cambridgeshire, UK). Thereafter, slides were successively washed in 2xSSC/0,1%SDS (5 min, 42°C, twice), 0.1xSSC/0,1%SDS (5 min.) and 0.1xSSC (1 min, twice) with milliQ as vehicle. Immediately after drying, slides were scanned randomly using the Scanarray 4000 XL (Perkin Elmer, Boston, USA) with appropriate laser and PMT settings. Array Vision software (Imaging Research, St. Catharines, Ontario, Canada) was used to determine Cy3 and Cy5 signal intensities for each separate spot and background noise. Quality control was performed on raw data by means of a scatter plot and MA plot as well as a normal probability plot to assess signal distribution (Pennings and Heisterkamp, 2004).

4.10. Data analysis

Raw data were log-transformed and normalized by global Loess correction of the $\ln(\text{Cy5}/\text{Cy3})$ ratio using S-plus, where after data from replicate spots were averaged. Then the data were analyzed by Principal Component Analysis (PCA) using GeneMaths (Applied

Maths, St-Martens-Latem, Belgium) and statistical analysis using Microsoft Excel. Differences in gene expression between the two experimental groups were calculated with the two-tailed Student's t-test. Also, a fold ratio in gene expression (FR) was determined by dividing the average normalized signal value of a gene in the LcS group by this value of the same gene in the control group. For the 5 most significantly up- and downregulated genes and the genes with a $FR > 1.5$, clustering and cellular pathway enrichment were investigated with EASE (<http://apps1.niaid.nih.gov/david/>).

5. Results

5.1. Immune function test in

Local Lymph Node Assay

The effect of LcS administration on DNCB induced lymphocyte proliferation in draining LN of BALB/c mice is shown in Figure 3. LcS significantly reduced the cell proliferation in LN at the highest DNCB concentration ($p = 0.005$).

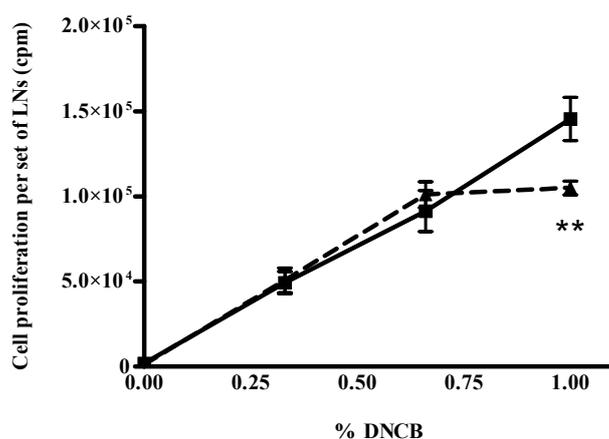


Figure 3: [³H]-thymidine incorporation in auricular LN cells of male BALB/c mice sensitized with DNCB (0%, 0.33%, 0.66% and 1%). Mice received LcS (▲) or vehicle (■). The [³H]-thymidine incorporation was expressed in counts per minute (cpm) per set of auricular LN times the total LN cell number expressed as means \pm SE. Significance level is ** $p < 0.01$.

Similar experiments were performed with TMA. Increasing concentrations of TMA increased cell proliferation in the auricular lymph node dose-dependently. LcS did not affect this TMA-induced proliferation (assessed in three similar experiments) in the lymph node (data not shown)

5.2. Respiratory allergy

Bronchoalveolar lavage: cell counts and differentiation and cytokines

Cell counts after lung lavage showed that in female mice the response to ovalbumin was higher (Figure 4). Total cell number and number of eosinophils and lymphocytes were higher than in males. Lungs of female mice that were sensitized and challenged with ovalbumin had higher numbers of total cells, and this increase was predominantly due to an increase in eosinophils and lymphocytes. LcS decreased total cell number and eosinophils slightly, but not significantly. Cytokine profiles assessed in BAL fluid are in line with these observations. Table 3 shows that in females that received LcS Th2 cytokines IL-4, IL-5, IL-10 and IL-13 were decreased, whereas IFN- γ , a Th1 cytokine was not affected.

Cell counts in male mice that were not-sensitized and received the vehicle are relatively high. It appears that LcS reduced total cell number compared to this group, but this did not reach statistical significance, due to high variation in the control group. LcS did not affect influx of cells in mice that were sensitized with ovalbumin. Cytokine levels in the BAL fluid were lower than in females (Table 3). LcS did not affect cytokine levels in sensitized males.

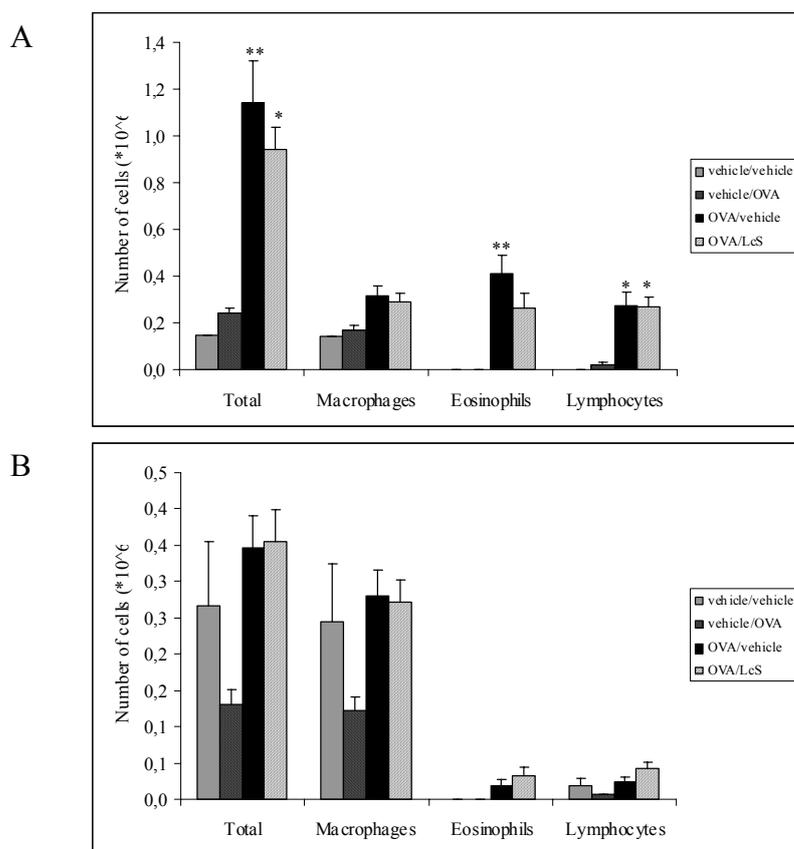


Figure 4: Number of cells in lung lavage fluid in female (A) and male (mice). Mice were sensitized and challenged with ovalbumin (OVA) or saline (vehicle) and orally treated with LcS or saline/peptone (vehicle). Cell number is expressed in 10^6 cells. Statistical significance was determined by a one-way ANOVA with Scheffe's post hoc test. Statistically difference compared to vehicle/vehicle group: * $p < 0.05$, ** $p < 0.01$

Table 3: Cytokine levels in BAL fluid

Group	IL-4	IL-5	IL-10	IL-13	IFN- γ
females					
vehicle/vehicle	ND	ND	ND	ND	0.55 \pm 0.38
vehicle/LcS	ND	ND	ND	ND	0.15 \pm 0.15
OVA/vehicle	20 \pm 2.6***	716 \pm 85***	15 \pm 4.5**	194 \pm 24***	4.3 \pm 0.36
OVA/LcS	12 \pm 2.1**	534 \pm 111**	ND ^{##}	132 \pm 26	4.6 \pm 0.45
Males					
vehicle/vehicle	ND	ND	ND	2.2 \pm 1.1	0.60 \pm 0.60
vehicle/LcS	ND	ND	ND	ND	0.15 \pm 0.15
OVA/vehicle	1.7 \pm 0.63	88 \pm 19	ND	22 \pm 5.8	1.6 \pm 0.48
OVA/LcS	2.6 \pm 1.4	70 \pm 19	ND	29 \pm 9.3	1.5 \pm 0.43

Cytokines were assessed with a bioplex Th1/Th2 kit on a Luminex® and expressed as mean \pm SE in pg/ml supernatant. Statistical significant from the vehicle/vehicle group: *p<0.05, ** p<0.01, ***p<0.001 and from the OVA/vehicle group ^{##} p<0.01. ND: not detected.

Table 4: Summary of histological lung changes

Group	Peribronchiolitis	Perivascularitis	Hypertrophy	Alveolitis	Eosinophils influx
Females					
vehicle/vehicle	0	0	0	0	0
vehicle/LcS	0.5 \pm 0.6	1.3 \pm 1.0	0.8 \pm 1.0	0	0
OVA/vehicle	2.0 \pm 0.0	2.8 \pm 0.5	3.9 \pm 0.4	1.0 \pm 0.0	2.0 \pm 0.5
OVA/LcS	1.9 \pm 0.4	2.6 \pm 0.5	3.9 \pm 0.4	1.1 \pm 0.4	1.9 \pm 0.4
Males					
vehicle/vehicle	0	0	0	0	0
vehicle/LcS	0.8 \pm 0.5	0	0	0	0
OVA/vehicle	1.3 \pm 0.5	1.6 \pm 0.5	3.5 \pm 0.5	0.3 \pm 0.5	1.1 \pm 0.4
OVA/LcS	1.5 \pm 0.5	2.1 \pm 0.4	3.0 \pm 0.5	0.4 \pm 0.5	1.5 \pm 0.8

Histological parameters were individually and quantitatively scored [absent (0), minimal (1), slight (2), moderated (3), marked (4) and strong (5)] and averaged per treatment group (n=4 vehicle/vehicle and vehicle/LcS group and n=8 OVA/vehicle and OVA/LcS group).

5.3. Lung pathology

Lung pathology is in accordance with the results of the bronchoalveolar lavage cell counts, with more inflammation in female mice after ovalbumin sensitization and challenge. Peribronchiolitis, perivascultis, alveolitis and influx of eosinophils were significantly higher in females than in males. LcS did not affect any of the assessed parameters in male and female mice that were sensitized and challenged with ovalbumin. In non-sensitized female mice, LcS caused slight peribronchiolitis, perivascultis and hypertrophy and in non-sensitized male mice LcS caused slight peribronchiolitis (Table 4).

5.4. Ovalbumin-specific IgG1 and IgE serum levels

Serum samples were used to detect ovalbumin-specific IgG1 (Figure 5) and IgE (Figure 6) levels in mice that were sensitized and challenged with ovalbumin. Administration of LcS slightly increased ovalbumin-specific IgG1 and IgE levels in females, whereas in males IgG1 levels were not affected and IgE levels were slightly higher.

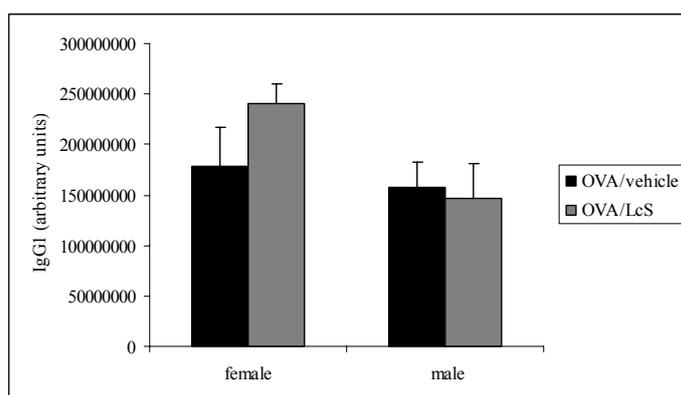


Figure 5: Serum levels of ovalbumin-specific IgG1 measured by ELISA. Mice were sensitized and challenged with ovalbumin and treated orally with LcS or vehicle. Serum levels are expressed in arbitrary units that were calculated by using reference serum with ovalbumin-specific IgG1.

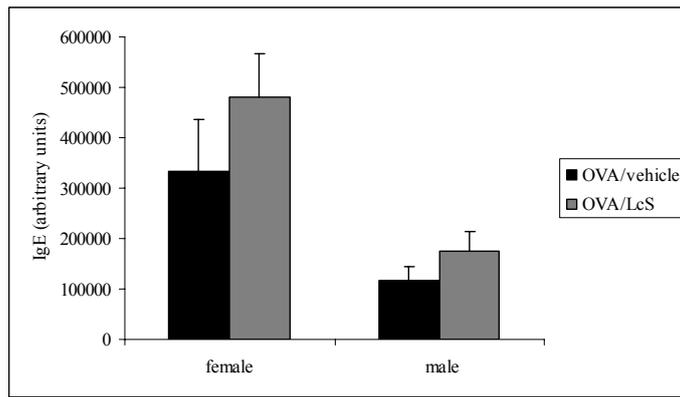


Figure 6: Serum levels of ovalbumin-specific IgE measured by ELISA. Mice were sensitized and challenged with ovalbumin and treated orally with LcS or vehicle. Serum levels are expressed in arbitrary units that were calculated by using reference serum with ovalbumin-specific IgE.

5.5. Cytokine production splenocytes

Table 5 shows cytokine levels in supernatant of splenocytes that were stimulated with 5 μ g ConA for 48 hours. In females there were no statistical significant differences in IL-4, IL-10 and IFN- γ production. In sensitized females IL-5 and IL-13 production were significantly higher in rats that received LcS compared to sensitized females that received vehicle.

In males IL-4, IL-10 and IL-13 were not affected by LcS. In sensitized males IL-5 and IFN- γ production were decreased compared to sensitized males that received the vehicle.

Table 5: Cytokine production after ConA stimulation of spleen cells

Group	IL-4	IL-5	IL-10	IL-13	IFN- γ
females					
vehicle/vehicle	103 \pm 32	76 \pm 13	53 \pm 20	88 \pm 18	991 \pm 99
vehicle/LcS	91 \pm 11	54 \pm 6.0	33 \pm 7.8	62 \pm 6.2	1052 \pm 482
OVA/vehicle	58 \pm 5.2	78 \pm 16	16 \pm 4.7	38 \pm 9.4	278 \pm 55
OVA/LcS	94 \pm 17	183 \pm 12* [#]	56 \pm 15	111 \pm 21 [#]	750 \pm 173
Males					
vehicle/vehicle	50 \pm 7.7	27 \pm 6.6	23 \pm 4.3	34 \pm 1.8	1170 \pm 260
vehicle/LcS	60 \pm 7.5	26 \pm 8.0	23 \pm 7.9	36 \pm 10	945 \pm 345
OVA/vehicle	67 \pm 13	49 \pm 7.9	47 \pm 17	49 \pm 13	510 \pm 123
OVA/LcS	42 \pm 5.1	22 \pm 2.6 [#]	23 \pm 7.8	24 \pm 6.7	300 \pm 75*

Cytokines were assessed with a bioplex Th1/Th2 kit on a Luminex® and expressed as mean \pm SE in pg/ml supernatant. Statistical significant from the vehicle/vehicle group: *p<0.05, ** p<0.01, ***p<0.001 and from the OVA/vehicle group^{##} p<0.01. ND: not detected.

Spleen cells were also cultured with ovalbumin to detect specific cytokine production. In both females and males cytokine production was higher in rats that received LcS. In males this difference was statistically significant for all cytokines, in females IL-5 and IL-13 were significantly increased. The increase in IL-10 almost reached significance (p=0.054).

Table 6: Cytokine production after stimulation of spleen cells with ovalbumin

Group	IL-4	IL-5	IL-10	IL-13	IFN- γ
females					
OVA/vehicle	12 \pm 2.5	181 \pm 68	24 \pm 11	115 \pm 44	6 \pm 1.9
OVA/LcS	17 \pm 2.4	490 \pm 60 [#]	70 \pm 19	395 \pm 81 [#]	14 \pm 3.2
Males					
OVA/vehicle	0.52 \pm 0.08	ND	ND	ND	12 \pm 4.1
OVA/LcS	21 \pm 3.8 ^{###}	187 \pm 44 ^{##}	48 \pm 11 ^{##}	165 \pm 48 ^{##}	43 \pm 11 [#]

Cytokines were assessed with a bioplex Th1/Th2 kit on a Luminex® and expressed as mean \pm SE in pg/ml supernatant. Statistical significant from the OVA/vehicle group: [#] p<0.05, ^{##} p<0.01, ^{###} p<0.001. ND: not detected.

6. Immune function tests in rats

6.1. EAE

In the LcS treated group, one rat died before the first symptoms of EAE appeared, so results were obtained from the remaining 7 animals. The mean body weights per day for the LcS treated and the control group during the 28 days after induction of EAE are shown in Figure 7. The body weight of both groups reduced after day 11. The weight of the control group increased again after day 15, whereas the LcS group did not recover until day 23. From day 16 onwards, the weights of the LcS treated animals were significantly lower than those of the controls ($p = 0.006 - 0.024$).

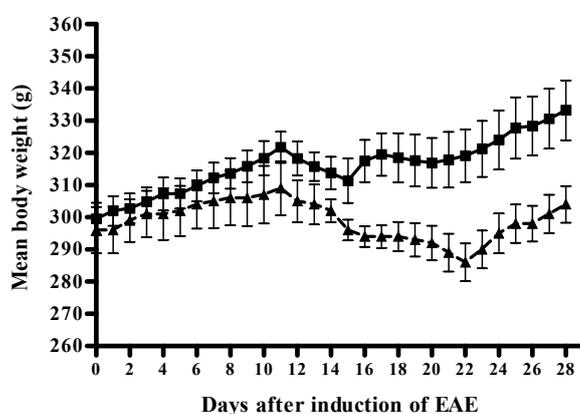


Figure 7: Mean body weights \pm SE of LcS treated (\blacktriangle , $n=7$) and control (\blacksquare , $n=8$) 6-8 week old male Lewis rats during the 28 days after induction of EAE.

The first clinical disease symptoms appeared at day 12 in the LcS group, which was 4 days earlier than in the control group. After day 24, the control animals seemed to have recovered from the EAE, while one animal in the LcS group still showed clinical signs at day 28. The increased duration of EAE by LcS was statistically significant ($p = 0.038$). Figure 8 shows the mean cumulative clinical scores per day for both experimental groups during the 28 days after EAE induction. At day 17 and 18 and from day 21 onwards, LcS increased the clinical score significantly ($p = 0.0256 - 0.0495$).

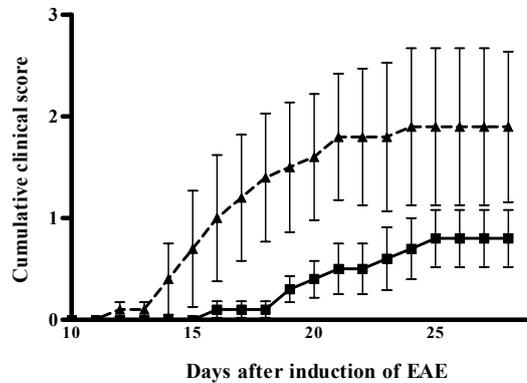


Figure 8: Mean cumulative clinical scores \pm SE per day from 10 to 28 days after induction of EAE in 6-8 week old male Lewis rats. Clinical disease symptoms were scored daily in the LcS treated (\blacktriangle , $n=7$) and control (\blacksquare , $n=8$) rats and added over time per animal.

Table 7: Effects of oral LcS exposure on clinical parameters of EAE in rats.

Group	Day of onset	Incidence	Mean duration of EAE	Mean clinical score per animal	CDI
Control	16	75 %	1.5 ± 1.6	0.8 ± 0.8	0.9
LcS	12	100 %	$3.7 \pm 2.7^*$	$2.2 \pm 1.7^*$	1.2

Day of onset of EAE is expressed as the first day that clinical signs were observed in each group, the incidence is the percentage of rats with clinical disease symptoms, the mean duration of EAE \pm SD (in days), the mean of the cumulative clinical scores per animal at the end of the experiment \pm SD, and the cumulative disease index (CDI) is the sum of the cumulative daily scores per group divided by the amount of days that clinical disease signs were observed in the group.

Table 7 summarizes the day of onset of the disease, the incidence of EAE, the duration of the disease, the mean of the cumulative scores per animal at the end of the experiment and the CDI for both experimental groups. The CDI of the LcS treated animals was 1.2, which was higher than the CDI of 0.9 in the control group. The incidence of rats with disease symptoms during the experiment was 100% in the LcS group vs. 75% in the control group.

6.2. Cell proliferation and cytokine release assays

During 8 successive days of oral LcS administration or a control solution, all Wistar WU(CPB) rats showed a non-significant reduction of body weight. Absolute organ weights were not affected by LcS administration, as well relative organ weights except for spleen. LcS did cause a slight but significant increase of the relative spleen weight after 8 days of treatment ($p = 0.023$).

In Table 8, the body weights at the start and the end of the experiment and the absolute and relative spleen weights are summarized for both experimental groups. The cell number of MLN and their mitogen induced proliferation or cytokine release did not differ between both experimental groups. Mitogen-induced IL-4 was not detected in any sample in this experiment.

Table 8: Effects of oral LcS exposure on body weight and spleen weight in Wistar rats

Group	Body weight (day 1)	Body weight (day 8)	Spleen weight (absolute)	Spleen weight (relative)
Control	436 ± 18	428 ± 23	0.70 ± 0,06	0.16 ± 0,02
LcS	460 ± 39	445 ± 46	0.77 ± 0,10	0.17 ± 0,02*

Mean body weights of 17-week old male Wistar WU(CPB) rats before and after oral administration of LcS (n=8) or a control solution (n=8) during 8 successive days are displayed, as well as the absolute and relative spleen weights after 8 days (all weights in grams ±SD). The relative spleen weight was significantly increased by LcS (* $p < 0.05$).

6.3. Gene expression analysis

All microarrays fulfilled our established quality parameters. PCA on raw data of all microarrays indicated one outlier in the 5 MLN samples of the control group, that was excluded from further data analysis. Differences in gene expression between the control and LcS exposed rats were generally small. The ten most up- and downregulated genes per organ had calculated p-values roughly ranging from 0.01 to 0.04. For any chosen p-value, the number of significantly regulated genes barely exceeded the expected amount of false positive findings, regardless of assumption of equal variances. In addition, the FR did not exceed 2 in any organ. Fixing the FR at 1.5 resulted in 3 downregulated genes in the MLN and 2 in the spleen, whereas the liver showed 4 down- and 2 upregulated genes and the thymus

8 upregulated genes after oral LcS administration. Table 9-12 present the ten most significantly regulated genes and the genes with $FR > 1.5$ per organ including their p-values and FRs for spleen, liver, thymus and MLN, respectively. In these groups of genes no enrichment of cellular pathways was found. Besides, the sets hardly contain any genes that can be related to known effects of LcS.

Table 9: Effects of oral LcS exposure on gene expression in rat spleen.

GenBank	Gene	Symbol	p-value	FR
U67080	suppression of tumorigenicity 18	St18	0.000	-1.08
X78603	ADP-ribosylation factor related protein 1	Arfrp1	0.001	1.13
BF549820	ESTs, Highly similar to ethanol decreased 4 [M.musculus]		0.001	-1.17
BE112689	ESTs, Highly similar to hypothetical protein FLJ20323 [H.sapiens]		0.001	1.14
AF366899	Adrenergic, alpha2B-, receptor class III	Adra2b	0.001	1.20
NM_031019	Corticotropin releasing hormone	Crh	0.001	1.17
NM_022700	ADP-ribosylation-like 3	Arl3	0.002	-1.12
NM_012653	Solute carrier family 9 (Na ⁺ /H ⁺ exchanger 2), antiporter 2	Slc9a2	0.002	1.07
NM_023102	Casein kinase 1 gamma 2 isoform	Csnk1g2	0.003	-1.19
NM_031324	Prolyl endopeptidase	Prep	0.006	-1.19
X82152	Fibromodulin	Fmod	0.013	-1.51
M28671	ESTs, Highly similar to IG GAMMA-2B CHAIN C REGION (GCB) [R.norvegicus]		0.145	-1.50

Gene regulation was examined by microarray analysis in the spleen of 17-week old male Wistar WU(CPB) rats after oral administration of LcS (n=5) or a control solution (n=5) during 8 successive days. Criteria for selection of genes were $FR \geq 1.5$ and/or a p-value (as determined with a *t*-test on the normalized data) that was among the 5 highest or the 5 lowest scores.

Table 10: Effects of oral LcS exposure on gene expression in rat liver.

GenBank	Gene	Symbol	p-value	FR
NM_012739	Adrenergic, alpha 2A, receptor	Adra2a	0.001	1.17
NM_022618	A kinase (PRKA) anchor protein 6	Akap6	0.002	-1.15
NM_024000	Vesicle-associated calmodulin-binding protein	1G5	0.003	-1.22
NM_012515	Benzodiazepin receptor (peripheral)	Bzrp	0.004	1.11
L10230	Regenerating islet-derived 3 alpha	Reg3a	0.004	-1.13
M64377	Olfactory receptor 1361	Olr1361	0.004	-1.09
BE100097	ESTs, Highly similar to A48024 glycosylphosphatidylinositol anchor class H biosynthesis protein [H.sapiens]		0.005	-1.17
NM_030847	Epithelial membrane protein 3	Emp3	0.005	1.20
U29174	Paired-like homeodomain transcription factor Drg11	Drg11	0.006	1.23
U23407	Cellular retinoic acid binding protein 2	Crabp2	0.013	1.15
NM_022521	Ornithine aminotransferase	Oat	0.019	-1.53
NM_147215	alpha-2u globulin PGCL4	LOC25924 7	0.027	-1.57
J02589	Androsterone UDP-glucuronosyltransferase	Ugt2b2	0.041	-1.87
NM_133295	carboxylesterase 3	Ces3	0.051	-1.66
NM_147137	cystatin SC	LOC25764 3	0.057	1.64
NM_024391	17-beta hydroxysteroid dehydrogenase type 2	Hsd17b2	0.331	1.66

Gene regulation was examined by microarray analysis in the liver of 17-week old male Wistar WU(CPB) rats after oral administration of LcS (n=5) or a control solution (n=5) during 8 successive days. Criteria for selection of genes were $FR \geq 1.5$ and/or a p-value (as determined with a *t*-test on the normalized data) that was among the 5 highest or the 5 lowest scores.

Table 11: Effects of oral LcS exposure on gene expression in rat thymus.

GenBank	Gene	Symbol	p-value	FR
AF309558	SEC14 (S. cerevisiae)-like 2	Sec14i2	0.000	-1.25
NM_012646	RT1 class Ib gene, H2-TL-like, grc region	RT1-N1	0.001	1.70
AF084544	chondroitin sulfate proteoglycan 2	Cspg2	0.001	-1.19
AF015953	Aryl hydrocarbon receptor nuclear translocator-like	Arntl	0.002	-1.15
NM_031601	calcium channel, voltage-dependent, T type, alpha 1G subunit	Cacna1g	0.002	-1.19
NM_012794	Glycosylation dependent cell adhesion molecule 1	Glycam1	0.003	-1.37
NM_017314	Ubiquitin C	Ubc	0.008	1.46
Y17319	Rattus norvegicus CDK110 mRNA	CDK110	0.010	1.73
NM_012806	Stress activated protein kinase beta	Serk2	0.012	1.15
NM_021740	Prothymosin alpha	Ptma	0.016	1.48
NM_017245	Eukaryotic translation elongation factor 2	Eef2	0.017	1.65
AW917994	ESTs, Highly similar to TVMSM3 transforming protein myb (version 3) [M.musculus]		0.061	1.56
BG666505	ESTs, Highly similar to eukaryotic translation initiation factor 4, gamma 2 [M.musculus]		0.107	1.66
AI230498	ESTs, Highly similar to germ cell-less homolog (Drosophila) [M.musculus]		0.135	1.53
NM_031841	Stearoyl-Coenzyme A desaturase 2	Scd2	0.159	1.53
L46593	small proline-rich protein 1A	Sprr1a	0.197	1.94

Gene regulation was examined by microarray analysis in the thymus of 17-week old male Wistar WU(CPB) rats after oral administration of LcS (n=5) or a control solution (n=5) during 8 successive days. Criteria for selection of genes were $FR \geq 1.5$ and/or a p-value (as determined with a *t*-test on the normalized data) that was among the 5 highest or the 5 lowest scores.

Table 12: Effects of oral LcS exposure on gene expression in rat MLN.

GenBank	Gene	Symbol	p-value	FR
AW916146	ESTs, Highly similar to DNA-directed RNA polymerase II largest subunit (RPB1) [M.musculus]		0.000	1.12
NM_022621	Nucleolin-related protein NRP	NRP	0.001	-1.15
BE099021	ESTs, Moderately similar to T14738 hypothetical protein DKFZp564A2416.1 (fragment) [H.sapiens]		0.003	-1.08
Y07744	UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase	Uae1	0.003	1.07
NM_013057	Coagulation factor III (thromboplastin, tissue factor)	F3	0.005	1.14
NM_012718	Androgen regulated 20 kDa protein	Andpro	0.008	1.06
NM_019313	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1	Kcnn1	0.009	-1.13
AJ004858	SRY-box containing gene 11	Sox11	0.009	-1.05
M92340	Interleukin 6 signal transducer	Il6st	0.010	-1.19
NM_012520	Catalase	Cat	0.012	-1.56
NM_022636	Ventral anterior homeobox 1	Vax1	0.016	1.05
NM_012709	Cytochrom P450 15-beta gene	Cyp2c12	0.044	1.06
NM_019136	Arginine vasopressin receptor 2	Avpr2	0.094	-1.96
AF082160	Galectin-related inter-fiber protein	Grifin	0.213	-1.51

Gene regulation was examined by microarray analysis in the MLN of 17-week old male Wistar WU(CPB) rats after oral administration of LcS (n=5) or a control solution (n=5) during 8 successive days. Criteria for selection of genes were $FR \geq 1.5$ and/or a p-value (as determined with a *t*-test on the normalized data) that was among the 5 highest or the 5 lowest scores.

7. Discussion

We investigated the effects of oral administration of LcS on Th1 and Th2 responses in several experiments. In an adapted LLNA performed in BALB/c mice, oral administration of LcS was found to significantly reduce the proliferative response in draining lymph nodes after topical application of the Th1 cell-dependent antigen DNCB at the highest concentration used. In the same model LcS did not influence proliferative responses induced by the Th2-dependent antigen TMA. The inhibition of Th1 is inconsistent with the previously demonstrated stimulatory effect of LcS on Th1 responses (Yasui et al., 1999). However, in a similar model *L. casei* was shown to reduce allergic skin inflammation induced by topical application of 2,4-dinitrofluobenzene (DNFB) by decreasing the response of IFN- γ producing cytotoxic T cells and the Th1 driven B cell response (Chapat et al., 2004). The cause was proposed to be either reduction of CD8⁺ T cell priming or activation of regulatory T (Tr) cells through modulation of dendritic cell (DC) function by *L. casei*.

Probiotics have been suggested to affect DC maturation and functioning (Stagg et al., 2004). Since the development of T cells is determined by the type of DC and the extent of maturation (Kalinski et al., 1999; Maloy and Powrie, 2001; McGuirk and Mills, 2002; Christensen et al., 2002; Stagg et al., 2004), probiotics could influence the development of Tr, Th1 or Th2 cells this way. Tr cells can inhibit either Th1 or Th2 activity or both and therefore be involved in the stimulation or inhibition of Th1 responses by probiotics (Kidd, 2003). This would explain the fact that probiotics can exhibit both adjuvant activity on (Th1 mediated) immune defense mechanisms and anti-inflammatory effects preventing harmful allergic reactions. Anti-inflammatory effects of several probiotics strains and prevention or suppression of Th1 associated pathologies like colitis were indeed reported before (Madsen et al., 1999; Von der Weid et al., 2001; Schultz et al., 2002; Drakes et al., 2004). In accordance with this, enhancement of release of the Th1-inhibiting cytokine IL-10 (Miettinen et al., 1996; Pessi et al., 2000; Christensen et al., 2002; Borrueal et al., 2003; Hart et al., 2004; Smits et al., 2005), sometimes simultaneously with release of Th1 related cytokines (Hessle et al., 1999; Von der Weid et al., 2001; Cross et al., 2002; Perdigon et al., 2002; Fujiwara et al., 2004; Shimosato et al., 2005), by several probiotics has been reported. Other investigators established Th1 inhibition by a reduction in IL-12 and IFN- γ by probiotics (Matsuzaki et al., 1997c; Schultz et al., 2002) or an increase of Th2 cytokines (Perdigon et al., 2002).

In contrast to the Th1 suppression that was detected in our modified LLNA, we found stimulation of Th1 mediated immunity in another model in which Th1 responses are predominant. Involvement of Th1 activity in EAE is indicated by induction of Th1 related cytokine production and autoreactive T cells and a beneficial effect of stimulation of Th2 mediated immunity (Lassmann and Ransohoff, 2004). In the EAE study in Lewis rats, both experimental groups lost weight at the same time that the first neurological signs of EAE appeared, but the LcS group recovered later from this weight loss than the control group. LcS significantly reduced the body weights, advanced the day of onset, prolonged the duration of EAE, and increased the cumulative clinical scores. The CDI and disease incidence were also increased by LcS. Altogether, these results indicate an aggravation of EAE by LcS, which is in line with the results of Maassen et al. with the probiotic *L. reuteri*. In the study of Maassen et al. administration of another probiotic *L. casei* resulted in a reduction of the severity of induced EAE. The strain-dependent stimulation or inhibition of EAE was explained by induction of specific cytokine profiles by the different probiotics (Maassen et al., 1998), which was demonstrated in other experiments as well (Christensen et al., 2002; Morita et al., 2002; Perdigon et al., 2002; Fujiwara et al., 2004). *L. reuteri* induced release of Th1 associated cytokines, whereas *L. casei* increased the production of the regulatory cytokines IL-10 and transforming growth factor (TGF)- β (Maassen et al., 1998). This is consistent with the indications that Tr cells are involved in EAE (Leonard et al., 1995; Trembleau et al., 1995; Maloy and Powrie, 2001; Weiner, 2001; Behi et al., 2005). They were mainly described to inhibit the onset and progression of EAE, possibly through IL-10 and TGF- β secretion (Furtado et al., 2001; Kohm et al., 2002; Lutton et al., 2004; Zhang et al., 2004; Behi et al., 2005).

Cytokine profiles seem to be predictive for the effects on allergic responses elicited by ovalbumin in the respiratory allergy model. LcS reduced the inflammatory response in the lungs slightly by decreasing the number of eosinophils in females. In line with this was the observed decrease of Th2 cytokines in the BAL fluid. In males, LcS did not affect the inflammatory response or cytokine levels. Remarkably, systemic immune responses were slightly enhanced. Ovalbumin-specific IgE were enhanced in both females and males and ovalbumin-specific IgG1 levels in females. Cytokine production by spleen cells that were cultured with ovalbumin also indicate immunostimulation by LcS. In both females and males Th2 and Th1 cytokines were enhanced by LcS. In males that were sensitized no Th2 cytokines were produced after culture with ovalbumin. After LcS administration, however, Th2 cytokines were detected in the supernatants. These data suggest that LcS acts as an

immunostimulator on the systemic level, whereas local lung responses were either not affected in males or alleviated in females.

These results are not in line with previous studies that have shown downregulation of allergic responses by LcS or other probiotic strains. LcS has been shown to induce a shift in the Th1/Th2 balance towards Th1, thereby reducing ovalbumin-specific IgE levels (Shida et al., 1998, Shida et al., 2002). Similar results have also been found for other probiotics (Kim et al., 2005, Ohno et al., 2005). However, immunostimulation has also been observed in a respiratory allergy model in which *Lactobacillus rhamnosus* HN001 induced higher levels of both Th1 and Th2 cytokines. In these mice serum IgE levels and inflammatory lung responses were not assessed, thus it is therefore unknown what the effects of this immunostimulation were on systemic and lung responses (Cross et al., 2002).

LcS influences immunological responses in both the experimental allergy and autoimmunity model. In contrast, in the study involving mitogen-induced cell proliferation and cytokine release measurements in the MLN of Wistar rats that were not primed with antigens did not detect any inhibitory or stimulatory effects of LcS. The relative spleen weight was slightly but significantly increased as a result of oral LcS exposure, though, probably caused by increased spleen cell proliferation, as was reported for another probiotics before (Gill et al., 2000). In the spleen, liver, thymus and MLN of the same animals, microarray analysis was performed. The numbers of up- or downregulated genes that were obtained were low in relation to the chance of false positive findings, indicating that no clear changes in gene expression induced by LcS administration were detected here. In addition, the most significantly regulated genes per organ did not indicate effects on cellular pathways or marked regulation of immune related genes or transcription factors that are known to be involved in for example T cell differentiation (Neurath et al., 2002).

The failure of the gene expression analysis to show overt effects of LcS could be the consequence of only subtle immunomodulation induced by LcS in this study. LcS treatment was sufficient to cause a modest, though significant aggravation of EAE in Lewis rats, but mitogen responsiveness was not influenced by the same LcS treatment of Wistar rats. It was reported before that the immunomodulating effects of probiotics can depend on the immunological state of the host (Isolauri et al., 2001). In line with this, in a human trial performed in healthy volunteers no effects on diverse immune functions were established (Spanhaak et al., 1998). It would therefore be interesting to repeat the microarray experiment in an animal disease model in which T cells are triggered. The absence of considerable changes in gene expression in this study indicates that microarray analysis was not more

sensitive or predictive than the immune function assays. An explanation could be that immunoregulation by LcS might not be detectable at the transcriptome level and could possibly affect cells at the protein level instead (Schmidt, 2002).

In conclusion, we detected immunomodulation by orally administered LcS, although the effects were generally modest. Depending on the model used, inhibition or stimulation of Th1 and Th2 mediated immune responses occurred, which confirms earlier indications that probiotics can skew the Th1/Th2 balance in both directions. Th1 stimulation may have harmful consequences for the development of certain autoimmune disorders, as was demonstrated in the EAE model. Furthermore, results obtained in the respiratory allergy model indicate that LcS might act as general stimulator of the systemic immune response, which might enhance both allergic and autoimmune disorders. Gene expression profiling had no more predictive potency or sensitivity than the immune function assays with respect to detection of immunomodulation. To gain further insight into immune regulation by probiotics, future experiments combining cytokine assays and gene expression analysis with disease models would be useful.

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