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Immunomodulatory impact of a synbiotic in T_H1 and T_H2 models of infection

Mario Cazzola, Thomas A. Tompkins and Maria G. Matera

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Abstract:

Background and methods: The immunomodulatory activity of a synbiotic combination containing three bacterial strains (*Lactobacillus helveticus* R0052, *Bifidobacterium longum* subsp. *infantis* R0033 and *Bifidobacterium bifidum* R0071) and short-chain fructooligosaccharide was examined in two distinct infectious rat models. In the T_H1 model, Wistar rats were administered the synbiotic combination for 2 weeks prior to challenge with a single oral dose of enterotoxigenic *Escherichia coli* or vehicle. In the T_H2 model, pretreated rats were challenged with a single subcutaneous dose of hook worm, *Nippostrongylus brasiliensis*. Blood samples were collected 3 hours or 4 days postchallenge and serum levels of pro- and anti-inflammatory cytokines were measured.

Results: Significant reductions in pro-inflammatory cytokines interleukin (IL)-1 α , IL-1 β , IL-6, and tumour necrosis factor (TNF)- α were observed in both models suggesting a single, unifying mode of action on an upstream regulator. The *N. brasiliensis* study also compared the effect of the individual strains to synbiotic. For most of cytokines the combination appeared to average the effect of the individual strains with the exception of IL-4 and IL-10 where there was apparent synergy for the combination. Furthermore, the cytokine response varied by strain.

Conclusions: It was concluded that this synbiotic combination of these three microbes could be beneficial in both T_H1 and T_H2 diseases.

Keywords: symbiotics, probiotics, *E. coli* infection model, *N. brasiliensis* infection model, pro- and anti-inflammatory cytokines

Background

Current research endeavours suggest that asthma pathogenesis is driven by a mixed T_H1/T_H2 immune response [Effros and Nagaraj, 2007]. The contribution of individual T_H1-associated and T_H2-associated effector mechanisms to this mixed response profile is highly heterogeneous and variations in response patterns seem to be associated with heterogeneity in clinical phenotypes. There is evidence that suggests that T_H2-associated cytokines, mainly interleukin (IL)-4, IL-5, IL-9 and IL-13, play a pivotal role in pathogenesis by regulating the production of IgE and the activity of mast cells. In particular, IL-4, IL-5 and IL-13 were suggested to promote the development of allergic asthma [O'Byrne, 2006]. In contrast, interferon (IFN)- γ , a T_H1 response-associated cytokine, is able to suppress IgE synthesis, as well as IgE-mediated allergies, e.g. allergic rhinitis, asthma and atopic dermatitis, have often been associated with defective

IFN- γ expression [Behera *et al.* 2002; van der Velden *et al.* 2001; Holt *et al.* 1997].

Obviously, these findings have implications in relation to drug development, and provide one possible explanation for the equivocal results of trials employing T_H2 antagonists in asthmatics [Matera *et al.* 2010]. It is possible to argue that it is also necessary to interfere with T_H1-mediated phenomena for a complete therapeutic strategy [Cazzola and Polosa, 2006].

Probiotics represents an expanding research area. They are defined as living microorganisms which, on ingestion in certain numbers, exert health benefits beyond inherent general nutrition [FAO/WHO, 2002]. The interest in probiotic therapeutic potential in allergic disorders stemmed from the fact that they have been shown to reduce inflammatory cytokines and improve intestinal permeability *in vitro*. Probiotics are, in fact, able

Correspondence to:

Mario Cazzola, MD
Dipartimento di Medicina
Interna, Università di
Roma Tor Vergata, Via
Montpellier 1, 00133
Rome, Italy
mario.cazzola@uniroma2.it

Thomas A. Tompkins, PhD
Institut Rosell inc.,
Montreal, Quebec, Canada

Maria G. Matera, MD, PhD
Second University of
Naples, Department of
Experimental Medicine,
Unit of Pharmacology,
Naples, Italy

to modulate the balance of the different T-helper cells (T_H1 , T_H2 , T_H3 , Treg) and their associated cytokines and, especially, to shift immunity from a pro-allergic T_H2 response to a pro-defensive T_H1 -response in response to antigens/allergens [Ghadimi *et al.* 2008; Veckman *et al.* 2004]. The resulting stimulation of T_H1 cytokines can suppress T_H2 responses [Winkler *et al.* 2007]. Feleszko and colleagues documented in a murine asthma model that oral probiotic administration prior to and during allergen sensitization and airway challenges leads to a suppression of all features of the asthmatic phenotype, including specific IgE production, airway inflammation and development of airway hyperreactivity [Feleszko *et al.* 2007]. Furthermore, this effect is associated with the suppression of allergen-induced T_H2 immune responses, paralleled by the induction of transforming growth factor (TGF)- β -expressing T cells in the intestine, as well as with the enhanced expression of the regulatory T (Treg) cell marker forkhead box protein (Foxp)3 in the pulmonary compartment. It seems intriguing to highlight that clinical improvement seen after allergen immunotherapy for allergic diseases such as rhinitis and asthma is associated with the induction of IL-10 and TGF- β producing Tr-1 cells as well as Foxp3 expressing IL-10 T cells, with resulting suppression of the T_H2 cytokine milieu [Nouri-Aria and Durham, 2008]. The beneficial effects of probiotics on the immune system are strain dependent [Madsen, 2006].

A small number of studies exist that try to address the efficacy of probiotic supplementation in the treatment or prevention of asthma [Michail, 2009]. Giovannini and colleagues, using fermented milk containing *Lactobacillus casei* and studying its effect on the number of episodes of asthma and allergic rhinitis, found no statistical difference between intervention and control groups of asthmatic children at the end of a one-year trial period [Giovannini *et al.* 2007]. However, the number of rhinitis episodes was lower in the probiotic group leading the authors to conclude that *L. casei* may benefit children with allergic rhinitis but not asthmatic children. Kalliomäki and colleagues assessed the mean concentration of exhaled nitric oxide as a marker of bronchial inflammation at 4 years in 32 at-risk children previously given placebo and 25 at-risk children given probiotics (*Lactobacillus rhamnosus* strain GG) and observed that it was greater in the placebo group than in the lactobacillus group [Kalliomäki *et al.* 2003]. In any case, a Finnish study suggested that fermented milk prepared

with *Lactobacillus gasseri* TMC0356 could alter serum IgE concentration through a T_H1 immune response in subjects with perennial allergic rhinitis [Morita *et al.* 2006].

The low viability and survival rates of probiotics remain a problem for their therapeutic use. Appropriate prebiotics and optimal combinations of probiotics and prebiotics (synbiotics) could allow significantly better efficiency to be obtained. A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health [Gibson and Roberfroid, 1995]. Today, only bifidogenic, nondigestible oligosaccharides (particularly inulin, its hydrolysis product oligofructose, and (trans)galactooligosaccharides) fulfil all of the criteria for prebiotic classification. They are dietary fibres with a well-established positive impact on the intestinal microflora. Synbiotics aim to enhance the survival and activity of proven probiotics *in vivo* as well as stimulating indigenous bifidobacteria. Synbiotics also show promise in the treatment of allergies [Ogawa *et al.* 2006] and atopic dermatitis in children [Passeron *et al.* 2006].

The purpose of this study was to evaluate the immunomodulatory performance of a three-strain synbiotic preparation in two distinct intestinal infection models. The induction of acute inflammation in rats with enterotoxigenic *Escherichia coli* serotype B strain is a T_H1 -biased model [Pulendran *et al.* 2001], whereas infection with *Nippostrongylus brasiliensis* is a well-studied, T_H2 -biased model [Gause *et al.* 2003]. While probiotics have been investigated in such models previously, there is less information available concerning synbiotic formulations and none to the best of our knowledge that have been evaluated in both models.

Materials and methods

Synbiotic preparation and strain characterization

The synbiotic, Probiokid (batch no. 6531259, expiration date October 2008), was provided by Lallemand S.A.S., Blagnac, France. Each 1.5 g sachet contained 5×10^9 CFU (*Lactobacillus helveticus* R0052; *Bifidobacterium longum* subsp. *infantis* R0033; *Bifidobacterium bifidum* R0071; Institut Rosell inc, Canada) and 750 mg of short-chain fructooligosaccharide (Actilight®, Beghin Meiji,

France). The individual strains R0052, R0033 and R0071 are deposited in the Collection Nationale de Culture de Micro-organismes (CNCM) of Institut Pasteur (Paris, France) as I-1722, I-3424 and I-3425, respectively.

The genetic identification of *L. helveticus* R0052 has been described previously [Naser *et al.* 2006]. Genomic DNA was extracted from overnight broth cultures of R0033 and R0071 as described previously for R0052 [Hagen *et al.* 2010]. Extracted DNA was diluted to 1/20 for polymerase chain reaction (PCR) to a final concentration of 100 ng/μl. DNA from the strains was used as a template in PCR to amplify approximately 1370 nucleotides of the 16S rRNA gene using the primers P0 and P6 as described by Ventura *et al.* [2000]. The *tuf* genes, approximately 970 nucleotides were amplified using BIF-1 and BIF-2 [Ventura *et al.* 2003]. PCR products were sent to Genome Quebec (Montreal, Quebec, Canada) according to the guidelines of the DNA Sequencing Platform. Nucleotide sequences for the 16S rDNA and *tuf* genes of strains R0033 and R0071 were compared with the BLASTN database available on GenBank [Altschul *et al.* 1997] and were deposited under accession numbers GU936672, GU936673, GU936674 and GU936675.

E. coli infection model

Male Wistar rats [HsdRccHanTM:WIST] (Harlan, Horst, The Netherlands), weighing 200–225 g, were maintained at 22 ± 2°C, 50 ± 10% relative humidity in polypropylene cages with a light/dark cycle of 12/12 h. Rats were allowed free access to standard 2016 diet (Harlan Teklad Diet, Oxon, England) and tap water *ad libitum*. The animal care unit is authorized by the French Ministries of Agriculture and Research (Government Authorization No. A 54-547-1) and animal experiments were performed according to the ethical rules enacted by the ASAB Ethical Committee for the use of animals in behavioural research by the Canadian Council on Animal Care [2003] with all procedures in compliance with European guidelines for animal experimentation [European Communities Council, 1986]. After an acclimatization period of 7 days following their arrival, rats were weighed and randomly divided into three treatment groups ($n=6$): (1) Vehicle: treated with physiologic saline; (2) EC + Vehicle: treated with vehicle and induced with *E. coli* on day 14; (3) EC + Synbiotic: treated with the synbiotic and induced with *E. coli* on day 14.

The synbiotic preparation, Probiokid (Lallemand S.A.S., Blagnac, France) was given by gavage (200 mg/rat) in physiologic saline (NaCl, 0.9%) at a volume of 1.5 ml/rat of synbiotic preparation prepared daily, for 17 days. Inflammatory responses were induced by single oral administration (20 ml/kg) of a solution of *E. coli* B strain serotype (ATCC[®] 11303, lyophilized cells, Ref. 45704) on day 14 prepared at 1.5 mg/ml in physiologic saline, incubated for 1 hour at 37°C prior to administration.

Rats were weighed three times weekly prior to infection and daily thereafter and food and water consumption measured at the same intervals. The viability and behaviour of the animals were recorded daily over the course of the experiment. Rats were killed under anaesthesia and autopsied if any of the following was observed: suffering (cachexia, weakening, difficulty in eating or moving, etc.); compound toxicity (abnormal behaviour, hunching, convulsions, etc.); ≥25% body weight loss over 3 consecutive days or ≥20% body weight loss on any day.

The body temperature of each animal was measured prior to infection with *E. coli* (two basal points) and then hourly for 10 hours postinfection. For 3 days after infection, the body temperature was measured morning and afternoon.

Faeces were observed daily for 3 days postinfection and bedding changed daily in order to see the evolution of the faecal consistency.

Blood sampling was performed on each rat, without anaesthesia, 3 hours postinfection. One millilitre of blood was collected from the tail vein in a dry tube (Fisher Bioblock Scientific, Illkirch, France). Blood samples were allowed to clot for 30 minutes at +4°C prior to centrifugation for 15 minutes at 1500 g. Serum was collected in polypropylene tubes (Fisher Bioblock Scientific, Illkirch, France), frozen at –20°C and stored at –80°C until assessment of cytokines. After thawing, serum samples were analysed for nine pro- and anti-inflammatory cytokines, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ and tumour necrosis factor (TNF)-α. Quantification was performed simultaneously using Bio-Rad rat 9-Plex A panel kits (Ref. 171-F11070, Marnes-la-Coquette, France) by using the Bio-Plex technique. All analyses were performed in duplicate.

The mean body weight (MBW) of animals, mean body weight change (MBWC), body temperature, body temperature change and serum levels of different cytokines were analysed at the end of the experimentation. Rats were sacrificed with an overdose of anaesthetics 3 days postinfection and a rapid autopsy of all rats was performed. Nonparametrical statistical tests were used: Kruskal–Wallis test (nonparametrical analysis of variance [ANOVA]) as a between-group variable comparison and when a significant difference was observed, Mann–Whitney *U*-test was performed to compare the synbiotic-treated group (EC + Synbiotic) with the Vehicle and EC + Vehicle groups.

N. brasiliensis infection model

Male Wistar rats (Janvier SA, Le Genest St. Isle, France) weighing 200–250 g were used and housed per group in distinct cages in a temperature ($21 \pm 1^\circ\text{C}$) and lighting-controlled room. Animals were allowed free access to water and fed a standard pellet diet (UAR, Villemoisson, Epinay sur Orge, France). Six groups of five male Wistar rats were orally administered either saline (1 ml; groups 1 and 2) or 1 ml of each probiotic suspension (groups 3 to 6) daily for 10 days before and 4 days after subcutaneous injection of sterile saline (group 1) or 3000 third-stage infective larvae of *N. brasiliensis* (groups 2 to 6). All protocols used in this study were approved by the local institutional Animal Care and Use Committee, in compliance with the European laws on the protection of animals (86/609/EEC).

The effect of the following probiotic strains was evaluated: *B. longum* subsp. *infantis* R0033 (lot 26015), *B. bifidum* R071 (lot 26204), *L. helveticus* R0052 (lot 131406B) and Probiokid (batch no. 6531259, expiration date 10/2008, provided by Lallemand S.A.S., Blagnac, France). Lyophilized strains were conserved at 4°C in darkness and resuspended at 1×10^9 CFU/ml in physiologic saline prior to administration. Animals were orally administered 1 ml of probiotic suspensions prepared daily.

N. brasiliensis was maintained in continuous culture by harvesting worm eggs from infected rats growing them to the third larval stage on vermiculite and filter paper. Rats were infected by subcutaneous injection of 3000 third-stage infective larvae of *N. brasiliensis* in 0.2 ml saline, into the rat flank on day 10.

At the end of treatments on day 14, blood for plasma cytokine levels determination was collected under general anaesthesia from the abdominal aorta into EDTA-containing Vacutainer tubes. The freshly drawn blood was centrifuged at 2000 *g* for 15 min at 4°C . Plasma was then separated and subsequently stored at -80°C until use for cytokine level determination.

Multi-analyte profiling was performed on the Luminex-100 system (Luminex Corporation, Austin, TX, USA). Cytokines analysed in duplicate by Rat Cytokine/Chemokine LINCOplex kit (Linco Research, St. Charles, Missouri, USA) included IL-1 α , IL-1 β , IL-4, IL-5, IL-6, IL-10, MCP-1 (Monocyte Chemoattractant Protein-1), IFN- γ , Growth-Related Oncogene/Kupffer Cells (GRO/KC) and TNF- α . Capture antibodies for each cytokine were covalently bound to distinct microsphere subsets distinguished by differing dye ratios. The concentration of each individual cytokine was determined by measuring fluorescence produced by a complex of a biotinylated cytokine-specific antibodies and streptavidin-phycoerythrin. Calibration microspheres for readings were consistently validated with Bio-Plex MCV Plate (Bio-Rad, Hercules, CA, USA). Raw data (mean fluorescence intensity) from all of the bead combinations tested were analysed with Bio Plex Manager 4.0 (Bio Rad, Hercules, USA) in order to obtain concentration values. Protein concentrations were determined in each sample using the commercial BC Assay Uptima kit (Interchim, Montluçon, France), and cytokine levels expressed in picograms per milligram of protein.

Statistical analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM). Comparisons were performed using ANOVA and Student's *t*-test for unpaired values. Differences were considered to be significant at the level of $p < 0.05$. All statistical analyses were carried out using the StatView[®]5 statistical package (SAS Institute, Inc., USA).

Results

Genetic identification of the strains in Probiokid Strain R0052 was previously shown to be a *L. helveticus* by DNA–DNA hybridization and multilocus sequencing typing [Naser *et al.* 2006]. Partial sequencing of the 16S rDNA and *tuf* genes confirmed strains R0033 and R0071 as

B. longum subsp. *infantis* and *B. bifidum*, respectively. R0033 16S rDNA gene (accession number GU936672) was 99% similar (1367/1370 nt) to *B. longum* subsp. *infantis* ATCC 15697^T (accession number CP001095). Similarly the R0033 *tuf* gene (accession number GU936673) was 99% similar (963/969 nt) to the type strain gene (accession number CP001095). R0071 16S rDNA gene (accession number GU936674) was 99% similar (1363/1371 nt) to *B. bifidum* DSM 20456^T (accession number S83624), and the R0071 *tuf* gene (accession number GU936675) was 99% similar (968/974 nt) to the strain ATCC29521^T (accession number AY372041).

E. coli infection model

No significant differences were observed in the MBW or MBWC of rats between Vehicle, EC + Vehicle and EC + Synbiotic groups at day 1 and day 14. Three days postinfection by *E. coli*, the MBW and MBWC of rats of the EC + Vehicle group was significantly lower than that of Vehicle group. The MBW of EC + Synbiotic group was not significantly different than control, however, the decrease in MBWC of rats in the EC + Synbiotic group was significantly smaller than that of EC + Vehicle group (Table 1).

At T₀, prior to infection, the mean body temperature (MBT) of rats of the EC + Synbiotic group

was significantly lower than that of the Vehicle or EC + Vehicle groups (data not shown). At 1 hour (T + 1) after the induction with *E. coli*, the MBT of rats in the EC + Synbiotic group was significantly lower than that of the Vehicle group but significantly higher than that in the EC + Vehicle group.

At 7 hours (T + 7) and at 10 hours (T + 10) after the induction of the inflammatory response, the MBT of rats in the EC + Vehicle group was significantly higher than that of rats in the Vehicle and EC + Synbiotic groups.

By T + 24, no significant differences were observed in the MBT of rats of different treatment groups.

Between T₀ and T + 1, the MBT decrease in rats of the EC + Vehicle group was significantly higher than that of rats of Vehicle and EC + Synbiotic groups (Table 2). Between T + 1 and T + 7, the mean gain in MBT in rats of the EC + Vehicle, and EC + Synbiotic groups was significantly larger than that of rats of the Vehicle group. However, the increase in the EC + Synbiotic group was significantly lower than that of the EC + Vehicle group. Between T + 7 and T + 10 and between T + 10 and T + 24, no significant differences were observed

Table 1. Comparison of mean body weights (grams; mean ± SEM) and mean body weight change (grams; mean ± SEM) of rats.

	Vehicle (n = 6)	EC + Vehicle (n = 6)	EC + Synbiotic (n = 6)
MBW			
D ₁	288.3 ± 12.9	288.8 ± 8.2	284.2 ± 14.5
D ₁₄	332.2 ± 18.5	328.2 ± 11.4	321.5 ± 22.3
D ₁₇	337.3 ± 18.0	302.3 ± 11.8	307.8 ± 19.5
Kruskal–Wallis			
H _(2df) = 4.95; p = 0.084 (7)			
Mann–Whitney U-test		U = 2.5	U = 7.5
(versus Vehicle)			
Significance		P = 0.042	T (p = 0.092)
Mann–Whitney U-test			U = 10.0
(versus EC + Vehicle)			
Significance			NS
MBWC			
Diff. D ₁ to D ₁₄	+43.8 ± 11.8	+39.3 ± 8.1	+37.3 ± 10.7
Diff. D ₁₄ to D ₁₇	+2.0 ± 2.3	−34.5 ± 7.5	−13.7 ± 5.9
Kruskal–Wallis			
H _(2df) = 12.85; p = 0.0016			
Mann–Whitney U-test		U = 0.0	U = 0.0
(versus Vehicle)		p = 0.010	P = 0.004
Significance			
Mann–Whitney U-test			U = 1.0
(versus EC + Vehicle)			
Significance			p = 0.02

Table 2. Comparison of mean change in body temperature in rats (°C; mean ± SEM) after infection.

Time (hours)	Vehicle (n = 6)	EC + Vehicle (n = 6)	EC + Synbiotic (n = 6)
T0 > T + 1 Kruskal–Wallis $H_{(2df)} = 11.56$; $p = 0.003$ Mann–Whitney <i>U</i> -test (versus Vehicle) Significance Mann–Whitney <i>U</i> -test (versus EC + Vehicle) Significance	−0.25 ± 0.08	−2.10 ± 0.28 $U = 0.0$ $p = 0.004$	−0.02 ± 0.38 $U = 15.5$ <i>NS</i> $U = 0.0$
T + 1 > T + 7 Kruskal–Wallis $H_{(2df)} = 13.92$; $p = 0.001$ Mann–Whitney <i>U</i> -test (versus Vehicle) Significance Mann–Whitney <i>U</i> -test (versus EC + Vehicle) Significance	−0.40 ± 0.23	+2.62 ± 0.19 $U = 0.0$ $p = 0.004$	$p = 0.004$ +0.58 ± 0.52 $U = 3.5$ $p = 0.02$ $U = 0.0$
T + 7 > T + 10 T + 10 > T + 24	−0.27 ± 0.39 +0.73 ± 0.46	−0.12 ± 0.22 −0.85 ± 0.50	$p = 0.004$ −0.33 ± 0.37 +0.03 ± 0.34

Table 3. Faecal consistency of rats observed after infection.

Day	Vehicle (n = 6)	EC + Vehicle (n = 6)	EC + Synbiotic (n = 6)
D ₁₄ (start of infection)	Normal	Liquid (Diarrhoea)	Mix of soft and normal
D ₁₅	Normal	Mix of liquid and soft	Mix of soft and normal
D ₁₆	Normal	Mix of liquid and soft	Mix of soft and normal
D ₁₇	Normal	Mix of soft and normal	Normal

in the MBT change of rats of the different treatment groups (Table 2).

The faecal consistency of rats of the Vehicle group was normal throughout the experiment. Rats of the EC + Vehicle group showed liquid (diarrhoea) and soft faeces after infection with *E. coli* that lasted for 48 hours. After 72 hours the faeces reverted to normal consistency. Rats in the EC + Synbiotic group showed normal and soft faeces after infection for the initial 48 hours and then normal faeces thereafter (Table 3).

Mean food consumption of rats of the treatment groups was similar prior to infection with *E. coli*. After infection, a drop in the mean food consumption was observed for the EC + Vehicle and EC + Synbiotic groups between 24 and 48 hours, after which a small increase was observed, especially for the EC + Synbiotic group (data not shown). Similarly, mean water consumption of rats from different treatment groups was similar pre-infection. A postinfection drop in the mean

water consumption was observed for the EC + Vehicle and EC + Synbiotic groups between 24 and 48 hours. Thereafter, a large increase was observed, with water consumption similar to the Vehicle group.

Postinfection, rats of the EC + Vehicle group showed a state of cachexia with ruffled fur whereas rats of the Vehicle and EC + Synbiotic group remained active in their cage with a normal fur. Two rats in the EC + Vehicle group died due to apparent weight loss and diarrhoea. No deaths were observed in the EC + Synbiotic or Vehicle groups.

The mean serum levels of the pro-inflammatory modulators, IL-1 α , IL-1 β , IL-6, IFN- γ , and TNF- α , of the EC + Synbiotic group were significantly lower than that of rats of the EC + Vehicle group while no difference was observed in the mean serum levels of pro-inflammatory cytokines IL-2 and GM-CSF. Mean serum levels of the T_H2 cytokines IL-4 and IL-10 of the

Table 4. Comparison of serum levels (mean $\pm$ SEM) of immune factors (pg/ml).

Immune Factor	Vehicle	EC + Vehicle	EC + Synbiotic	Mann–Whitney <i>U</i> -test (versus EC + Vehicle)
IL-1 α	84.4 $\pm$ 7.4	313.3 $\pm$ 57.6	163.7 $\pm$ 31.9	<i>U</i> = 1.0 <i>p</i> = 0.007
IL-1 β	309.5 $\pm$ 67.1	11,149.8 $\pm$ 3340.3	5416.0 $\pm$ 2018.1	<i>U</i> = 2.0 <i>p</i> = 0.01
IL-2	156.2 $\pm$ 56.4	186.3 $\pm$ 31.5	158.5 $\pm$ 43.2	NS
IL-4	22.0 $\pm$ 5.5	34.8 $\pm$ 11.4	115.3 $\pm$ 27.5	<i>U</i> = 0.0 <i>P</i> = 0.004
IL-6	2023 $\pm$ 567	75,640 $\pm$ 23,084	20,592 $\pm$ 7744	<i>U</i> = 0.0 <i>p</i> = 0.004
IL-10	420.0 $\pm$ 126.2	834.5 $\pm$ 199.0	1591.9 $\pm$ 354.3	<i>U</i> = 0.0 <i>p</i> = 0.004
GM-CSF	72.8 $\pm$ 10.4	125.2 $\pm$ 20.8	97.4 $\pm$ 20.4	NS
IFN- γ	290.4 $\pm$ 97.4	24,966 $\pm$ 5001	8729 $\pm$ 1690	<i>U</i> = 0.0 <i>p</i> = 0.004
TNF- α	265.3 $\pm$ 35.5	2021.5 $\pm$ 519.9	1069.1 $\pm$ 195.8	<i>U</i> = 1.0 <i>p</i> = 0.007

EC + Synbiotic group were significantly higher than that of the EC + Vehicle group (Table 4).

N. brasiliensis infection model

No significant behavioural differences (signs of stress, difficulty in eating or moving, etc.) were observed between the different groups prior to or following *N. brasiliensis* infection nor did *N. brasiliensis* injection induce any serious adverse reactions or mortality.

There were no significant differences in serum levels of INF- γ or GRO/KC between any of the groups. The pro-inflammatory modulators IL1 α , IL-1 β , IL-6 and TNF- α all increased ($p < 0.05$) in the group treated with *N. brasiliensis* + Vehicle (Figure 1). Pretreatment with any of the probiotic strains prior to infection with *N. brasiliensis* generally resulted in a reduction ($p < 0.05$) in serum levels of these immunomodulators. The values for the probiotic- and synbiotic-treated groups were not significantly different from the control group receiving Vehicle only. The serum level of these pro-inflammatory mediators for the synbiotic combination was not significantly different from the individual strains.

Serum levels of the T_H2 molecules IL-4 and IL-10 were also upregulated in the group treated with *N. brasiliensis* + Vehicle (Figure 2). In the groups treated with probiotic strains and synbiotic, the IL-4 response was negated. In this case, the synbiotic preparation did appear to have a synergistic trend and a level below the Vehicle group was observed. Serum IL-10 levels were

significantly downregulated by R0071 and R0052, whereas the synbiotic and R0033 did not significantly reduce the response.

The serum levels of IL-5 and MCP-1 were also upregulated in the animals treated with *N. brasiliensis* + Vehicle (Figure 2) and responses were repressed in the probiotic- and synbiotic-treated groups. The only exception to this was the IL-5 response in R0052-treated rats which remained significantly higher than in the control group.

Discussion

Although the majority of human trials have focused on the benefits of probiotics in gastrointestinal diseases, there is some evidence to suggest that probiotic supplementation can alleviate and/or prevent development of allergic disease [Fooks and Gibson, 2002]. In effect, increasing data suggest a relationship between the intestinal microflora and allergy. It has been shown that the proportions of anaerobic bacteria in the intestinal flora are higher in nonallergic than allergic children [Bjorksten *et al.* 1999]. In particular, non-allergic children harbour higher counts of bifidobacterium than allergic children [Watanabe *et al.* 2003; Kalliomäki *et al.* 2001]. Thus, there is a trend to supplement infant formula with substances with bifidogenic effects [Vandenplas, 2002].

The synbiotic preparation in this investigation contains three probiotic microbes (*L. helveticus* R0052, *B. bifidum* R0071 and *B. infantis*

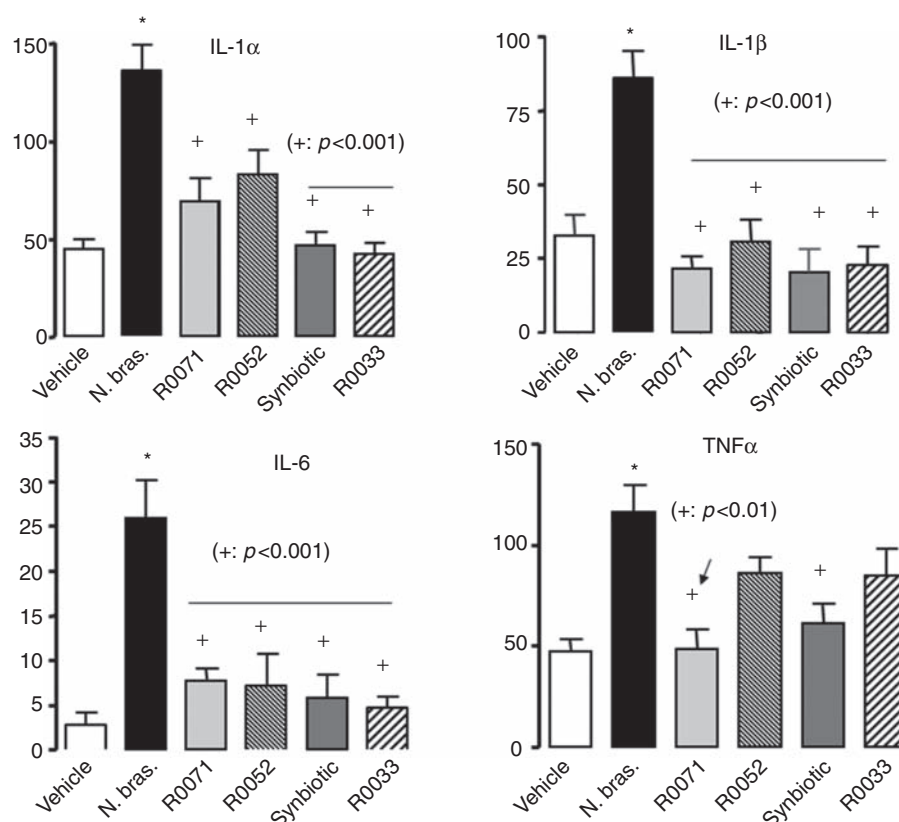


Figure 1. Serum concentrations of pro-inflammatory mediators (IL-1 α , IL-1 β , IL-6 and TNF- α) in male Wistar rats, pretreated with probiotics, synbiotic or vehicle, to a subcutaneous challenge of *N. brasiliensis*. All results are presented in picograms per milligram of protein.

*Significant difference versus vehicle; +significant difference versus *N. brasiliensis*.

R0033) and a scFOS. The composition was designed based on existing, yet separate, scientific evidence for the microbes and scFOS, to support a healthy intestinal flora and maintain the body's natural defences. An examination of faecal microbiota composition in infants with atopic eczema revealed that *E. coli* levels correlated with the extent of allergic disease and that bifidobacteria supplementation subsequently decreased *E. coli* levels [Kirjavainen *et al.* 2002]. Moreover, an *in vivo* study of newborn mice showed that *B. infantis* establishment was important for restoration of the usual oral tolerance process and especially for IgE suppression [Sudo *et al.* 1997]. The scFOS, on its own, does not appear to have an impact on cellular immune function, although it has been demonstrated that dietary FOS interact with the intestinal immune system and upregulate IgA response and polymeric immunoglobulin receptor expression in intestines of infant mice [Nakamura *et al.* 2004]. Intriguingly,

allergen-specific secretory IgA was found to protect sensitized children from development of allergic symptoms during the first 2 years of life, suggesting a possible ignorant role of secretory IgA against allergens [Bottcher *et al.* 2002]. In addition, increases in allergen-specific IgA has been reported in specific immunotherapy performed via sublingual or subcutaneous route [Bahceciler *et al.* 2005; Jutel *et al.* 2003].

In our study, in the T_H1 model, the synbiotic, orally administered as a preventive treatment at 200 mg/rat/day (10^9 CFU/day of bacteria), respectively, for 17 days, displayed significant effects against *E. coli*-induced inflammation in rats. Pretreatment with probiotics reduced MBW loss postchallenge, reduced MBT difference (decrease followed by increase), limited duration of diarrhoea, limited the reduction of food and water consumption of rats, reduced circulating levels of pro-inflammatory cytokines (IL-1 α , IL-1 β , IL-6, IFN- γ and TNF- α) and

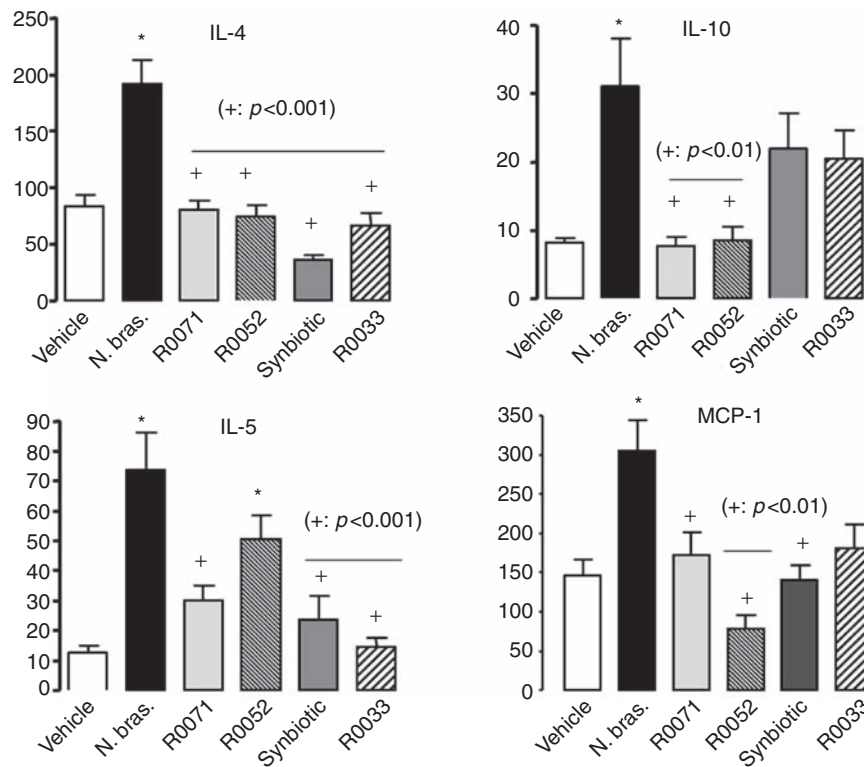


Figure 2. Serum concentrations of various immune factors (IL-4, IL-10, IL-5 and MCP-1) in male Wistar rats, pretreated with probiotics, synbiotic or vehicle, to a subcutaneous challenge of *N. brasiliensis*. All results are presented in picograms per milligram of protein.

*Significant difference *versus* vehicle; +significant difference *versus* *N. brasiliensis*.

elevated the secretion of anti-inflammatory cytokines (IL-4 and IL-10). These results are consistent with the findings of Johnson-Henry and colleagues who demonstrated a downregulation of pro-inflammatory T_H1 response and a simultaneous shift to an improved T_H2 response [Johnson-Henry *et al.* 2005]. This may be attributed to downregulation of nuclear factor-kappaB (NF κ B)-mediated pathways [Tien *et al.* 2006], although recently it has been suggested that it can occur in the JAK-STAT pathway [Jandu *et al.* 2009]. However, these findings differ from those of Ishida-Fujii and colleagues who observed that pretreatment of mice with *L. casei* enhanced the T_H1 responses (TNF- α and IL-12) in isolated peritoneal macrophages stimulated with lipopolysaccharide from *E. coli* [Ishida-Fujii *et al.* 2007]. They did not see any impact on IL-6 response in this system and suggest an upregulation of the NF κ B pathway in these cells. These differences may reflect either their choice of isolated macrophages and LPS or the specific probiotic strain.

The impact on fever (i.e. body temperature change) in our study was striking and we will consider this as an important parameter for future clinical investigations, especially in the prevention of infantile infections.

In the T_H2 model, the synbiotic, given as a preventative therapy prior to challenge with *N. brasiliensis*, gave similar results to the T_H1 model. The pro-inflammatory mediator levels (IL-1 α , IL-1 β , IL-6, MCP-1 and TNF- α) remained the same as the control and the animals displayed healthy appearances. As expected in the T_H2 -biased model, the anti-inflammatory (IL-4, IL-10) and T_H2 (IL-5) indicators were increased by the challenge. However, unlike the T_H1 model, this increase in IL-4 and IL-10 was suppressed, to varying extents, by the probiotics and synbiotic. The levels observed with the synbiotic generally appeared to be an average between the three strains. The exceptions to this were IL-4, where the synbiotic appeared to downregulate more so than the strains, and IL-10, where it tended to have less of an impact (Figure 2).

Epidemiological studies have found an inverse correlation between helminth infections and different diseases such as inflammatory bowel disease [Yamamoto-Furusho, 2007] and atopic diseases [Scrivener *et al.* 2001], suggesting a possible protective effect of helminth infections on the development of these disorders. Further, Wohlleben and colleagues showed that *N. brasiliensis* suppresses the development of allergen-induced airway eosinophilia in mice through a pathway involving IL-10 release [Wohlleben *et al.* 2004]. Similarly, *Heligmosomoides polygyrus* inhibits ongoing IL-10(−/−) colitis in part through blocking mucosal T_H1 cytokine production and promoting IL-10 secretion [Elliott *et al.* 2004]. In contrast, IL-4 plays a role in generating and sustaining of *in vivo* IgE responses [Finkelman *et al.* 1988] and the lack of IL-4 in IL-4-deficient TCR α −/− mice dramatically suppressed the development of colitis, suggesting a critical role of this cytokine in the pathogenesis of colitis [Mizoguchi *et al.* 1999]. Regarding the protective role of IL-10 related to helminth infestation against atopic disease, the effect of the synbiotic tested herein (i.e. preservation of high IL-10 level after *N. brasiliensis* injection) could be promising in preventive applications and future models could test this hypothesis. Similarly, the strong inhibitory effect observed in this study by all probiotic strains and particularly the synbiotic on IL-4 secretion, reinforce their prophylactic use against IgE dependent or independent inflammation. It is not clear from our investigation whether there was any negative impact on clearance of the pathogens because of the downregulation of the inflammation since intestinal and faecal determination of the pathogens was not attempted.

The synbiotic combination of three probiotic microbes and FOS was able to reduce the level of circulating pro-inflammatory immune factors in T_H1 and T_H2 models of infection. This suggests that the synbiotic is targeting upstream regulators of the immune response. NF κ B is an early transcription regulator and has been implicated in the early signaling pathways of probiotic bacteria. Probiotics have been implicated in the inhibition of NF κ B [Petrof *et al.* 2004] possibly by stabilizing nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (I κ B α) [Tien *et al.* 2006].

While the effect of synbiotics in the T_H1 and T_H2 model systems had some commonalities, overall

the immunomodulation favoured a rebalancing of the two responses. This suggests that the synbiotic combination could be considered as a possible prophylactic agent to reduce the adverse effects of either T_H1 or T_H2 biased disease states. Obviously, it would seem simplistic to assume that a single supplementation would suffice to counter the plethora of allergic diseases and, in any case, since extrapolations to humans must be considered with caution, clinical trial assessment is required to elucidate whether this modulation might be useful in the prevention and treatment of allergic diseases.

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Conflict of interest statement

None declared.

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