

## Changes in Gut Microbiota in Children with Atopic Dermatitis Administered the Bacteria *Lactobacillus casei* DN – 114001

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### Abstract

Gut microbiota was analyzed in children, aged 6–18 months and suffering from atopic dermatitis before and after 3 month supplementation of their diet with *Lactobacillus casei* DN – 114001 in a dose of  $10^9$  cells daily. On completion of this period the total number of fecal *Lactobacillus* sp. cells decreased from  $7.86 \text{ Log}_{10} \text{ CFU/g}$  to  $6.40 \text{ Log}_{10} \text{ CFU/g}$ . After the next 5 months (without dietary supplementation with the probiotic bacteria) the level of *Lactobacillus* sp. cells was maintained at the latter value. During the dietary supplementation with the probiotic strain, the level of *Bifidobacterium* cells was maintained at  $6.15\text{--}6.89 \text{ Log}_{10} \text{ CFU/g}$  while after 5 months it decreased to  $5.57 \text{ Log}_{10} \text{ CFU/g}$ . The population of *Clostridium* sp. was reduced after 3 months of dietary supplementation from  $6.49$  to  $5.83 \text{ Log}_{10} \text{ CFU/g}$  and was maintained at the latter level during the next 5 months. The dietary supplementation had no effect on populations of *Bacteroides* sp., *Enterococcus* sp. and *Enterobacteriaceae*. Supplementation of children who developed atopic dermatitis with the preparation of *Lactobacillus casei* DN – 114001 positively affected their gut microbiota in terms of bifidobacteria and clostridia populations.

**Key words:** *Lactobacillus casei* DN – 114001, atopic dermatitis, gut microbiota

### Introduction

The prevalence of allergic diseases including atopic dermatitis (AD) has increased over the last decades. In Europe and in the USA, the prevalence of AD is estimated to be 10–20% in infants and 2–3% in adults (Leung *et al.*, 2007). According to the hygiene hypothesis the increasing number of patients with allergy is ascribed to improved sanitation and reduced microbial exposure. Children from families with worse material status and/or from families with many children and those who contact domestic animals develop allergic diseases less often (Strachan 1989; Remes *et al.*, 2003; De Meer *et al.*, 2005). Microorganisms colonizing the human gastrointestinal tract and in particular the bowels play a key role in the formation of protective barrier and stimulation of the immune system. The predisposition to allergy, inflammatory bowel disease (IBD), and autoimmune disorders can be an effect of wrong primary succession of intestinal microbiota (Kelly *et al.*, 2007). The gastrointestinal tract of healthy newborns delivered in natural conditions is colonized in the first days by aerobic enterobacteria,

streptococci and staphylococci. When intestinal oxygen level decreases, the gastrointestinal tract is colonized by anaerobic *Bifidobacterium* sp., *Bacteroides* sp. and *Clostridium* sp. The predominance of bifidobacteria is observed in breast-fed infants. Metabolic activities of bifidobacteria that synthesize acetic and lactic acids maintain the balance between intestinal bacteria such as clostridia or enterobacteria and prevent their domination (Harmsen *et al.*, 2000). Intestinal microflora in newborns and infants who developed AD is characterized by the increased population of *Clostridium* sp. and reduced number of *Bifidobacterium* cells (Kalliomaki *et al.*, 2001; Kirjavainen *et al.*, 2001). It was presented that supplementation of infant diet with probiotic *Lactobacillus* strains affects the composition of intestinal microbiota as well as AD development and severity (Kalliomaki *et al.*, 2001). Currently hygiene theory has been modified in the direction of the microbiota theory (Rocha, 2006; Cukrowska, 2008). Microbiota theory assumes that the fundamental processes governing the development of resistance and the development of immunological tolerance to external antigens are physiologically microorganisms colonizing the

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gastrointestinal tract (intestinal microbiota). Delayed and/or altered gastrointestinal colonization during the formation of the intestinal ecosystem can activate the immune system towards promoting allergy. Microbiota theory assumes that the physiological colonization that occurs in healthy infants and activates Th1 lymphocytes regulate the immune response, which are responsible for maintaining the Th1/Th2 cytokine balance and the development of tolerance to external antigens. Neonatal gastrointestinal tract, which in fetal life remains sterile is settled bacteria from the mother (from faeces, vaginal and skin) or from the external environment (hospital, home, siblings, medical staff) (Cukrowska, 2008).

This study aimed at characterization of changes in intestinal microbiota in infants with AD caused by dietary supplementation with *Lactobacillus casei* DN – 114001. Fecal microbiota was analyzed before, just after finishing bacteria intake, and then after 5 months from its completion.

## Experimental

### Materials and Methods

**Study design.** The trial was carried out in a double-blind, randomized, placebo-controlled way. The samples of probiotic preparation were prepared and blinded in the Institute of Fermentation Technology and Microbiology at the Technical University of Lodz. They were de-blinded on completion of the experiment. The study included 40 children aged 6–18 months with recognized AD. The clinical symptoms of AD were measured using SCORAD (Score Atopic Dermatitis) index, and children with medium-severe AD (SCORAD < 45.0) were involved in this trial. A group of children (n=18) whose diet was supplemented with *Lactobacillus casei* DN – 114001 cells ( $10^9$  cells daily) was designated as DN Group (DNG). The diet of the control group of children (n=22), designated as Placebo Group (PG), was supplemented with a carrier of bacterial cells. There was no differences between DNG and PG groups in age, physical development (weight and length), family occurrence of atopic diseases, and the number of children still breast-feeding (19% and 22%, respectively). The experiment was carried out for 8 months. The children were administered either the probiotic or the placebo for 3 months. During the experiment cow's milk proteins were eliminated from the diet of children or breast-feeding mothers. Fecal microbiota was analyzed before (0), just after finishing 3 month dietary supplementation (3 m), and than after 5 months from its completion (8 m). The study was conducted with the permission of the Bioethics Committee of The Children's Memorial Health Institute, and written parental consent.

**Probiotic preparation.** Bacterial strain *Lactobacillus casei* DN – 114001 was obtained from Danone Ltd. It was cultured in a liquid MRS broth (BTL, Poland) for 48 h at 37°C, in an atmosphere containing 5% (v/v) CO<sub>2</sub>. Bacterial cells were pelleted by centrifuging (4000 rpm, 6°C). Cell pellet was washed 3 times with physiological saline solution and centrifuged under the same conditions. Then it was suspended in 10% solution of the carrier, *i.e.* a hydrolyzed milk for children with cow's milk protein allergy – Nutramigen (Mead Johnson, The Netherlands) and lyophilized. The lyophilized bacterial preparation, containing bacterial cells and the carrier, was divided into equal portions (200 mg), each containing  $10^9$  living cells.

**Sample collection and preparation.** Samples of fresh faeces taken from children (the first stool on that day) in three experimental periods (0, 3 m, 8 m) were immediately placed in anaerobic chamber. All preparation procedures were performed in oxygen-free atmosphere (H:N:CO<sub>2</sub>; 1:8:1). Samples of approximately 1 g (wet weight) were homogenized in physiological salt solution at the ratio of 1:10 (w/v). Then, 1 ml of the feces homogenate was used to prepare a series of 10-fold dilutions (from  $10^1$  to  $10^{10}$ ).

**Microbial analysis.** Children's fecal microbiota was analyzed by the standard plate tests. The following groups of microorganisms were quantified on selective media: *Lactobacillus* sp., Rogosa Agar (Merck); *Bifidobacterium* sp., RB Agar (Hartemink *et al.*, 1996); *Clostridium* sp., TSC Agar (Merck); *Bacteroides* sp., *Schaedler* Agar (BioMerieux) supplemented with 5% (v/v) sheep blood, kanamycine-vankomycine mixture (BioMerieux) and vitamin K 0.01% (w/v); *Enterococcus* sp., Bile Aesculin Agar (Merck); *Enterobacteriaceae* family, Mac Conckey Agar (Merck); and the total number of anaerobic bacteria was determined using *Schaedler* Agar (BioMerieux). *Lactobacillus* sp. was cultivated for 48 h at 37°C in CO<sub>2</sub> (5%, v/v) atmosphere (WTC Binder CO<sub>2</sub> Incubator, Germany). The bacteria, *Bifidobacterium* sp., *Clostridium* sp., and *Bacteroides* sp., and total anaerobic bacteria, were cultured at 37°C in the anaerobic atmosphere of H:N:CO<sub>2</sub> (1:8:1) (Anaerobic Workstation Concept 400, Biotrace Int.) for 4 days (Klewicka *et al.*, 2009). The results were present as Log<sub>10</sub> CFU/g per gram wet weight of faeces.

**Statistical analyses.** The results were analyzed using the t-test (One-Way ANOVA test or Welch's test) with  $P \leq 0.05$  considered to be significant.

## Results

Faeces of children contained: *Lactobacillus* sp., *Bifidobacterium* sp., *Bacteroides* sp., *Clostridium* sp., *Enterococcus* sp. and bacteria of *Enterobacteriaceae* family.

Before the dietary supplementation, total counts of *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Enterococcus* bacteria in both groups of children were not statistically different. In both groups DNG and PG the population of *Lactobacillus* cells after 3 months of treatment decreased by 18% and 15% to 6.4 Log<sub>10</sub> CFU/g and 6.54 Log<sub>10</sub> CFU/g, respectively. A decrease in *Lactobacillus* number was also observed after next 5 months, but only in control PG group to 5.9 Log<sub>10</sub> CFU/g, whereas in DN group the count of *Lactobacillus* was maintained at 6.38 Log<sub>10</sub> CFU/g. In DNG group the level of *Bifidobacterium* cells was stabilized after 3-month treatment, and only after the next 5 months it was decreased by 9.4% to 5.57 Log<sub>10</sub> CFU/g. By contrast, in PG group the bifidobacteria were reduced by 9.5% and 24% after 3 and 5 months, respectively. During 3-month dietary

supplementation the number of *Clostridium* cells was decreased by 10% in DNG group level was maintained during the next 5 months. In PG group the number of *Clostridium* sp. was kept at almost the same level during the whole study. None statistical differences were found in counts of *Bacteroides* sp., *Enterobacteriaceae*, and *Enterococcus* sp. throughout the experiment (Table I).

In both DNG and PG groups clinical improvement, i.e. a decrease in SCORAD index was observed, but a significant decrease after 8 months as compared with 3-month lasting observation was found only in DNG group (Table II). In all children treated with probiotics a decrease in SCORAD was found. In contrast to DNG group, in three children treated with placebo clinical condition deteriorated. Their SCORAD index after 8 months was higher than before treatment.

Table I  
Intestinal microflora changes before and after *Lactobacillus casei* DN – 114001 (DNG) or placebo (PG) intake in children with atopic dermatitis

Genus of bacteria [Log <sub>10</sub> CFU/g faeces]		DNG			PG		
		Time [months]			Time [months]		
		0	3	8	0	3	8
<i>Lactobacillus</i>	Range	4.90 – 9.71	4.60 – 8.69	3.54 – 9.36	4.00 – 9.73	5.39 – 8.14	4.32 – 8.98
	The log mean*	7.86 (±2.21) <sup>a</sup>	6.40 (±1.70) <sup>b</sup>	6.38(±2.30) <sup>bc</sup>	7.77(±1.34) <sup>c</sup>	6.54 (±1.46) <sup>d</sup>	5.90 (±1.89) <sup>e</sup>
<i>Bifidobacterium</i>	Range	4.30 – 9.51	3.51 – 9.25	3.43– 9.07	3.00 – 9.91	2.29 – 9.51	3.00 – 8.89
	The log mean*	6.15 (±2.49)	6.89 (±1.42)	5.57 (±1.37) <sup>a</sup>	7.11 (±2.12) <sup>c</sup>	6.43 (±1.85) <sup>c</sup>	5.36 (±1.62) <sup>d</sup>
<i>Bacteroides</i>	Range	2.00 – 10.47	3.95 – 9.67	3.30 – 9.44	2.00 – 10.14	3.00 – 10.65	2.00 – 10.87
	The log mean*	7.37 (±2.44)	7.89 (±1.64)	7.51 (±1.71)	6.34 (±2.84)	7.33 (±2.20)	7.13 (±2.47)
<i>Clostridium</i>	Range	4.01 – 9.11	5.00 – 8.47	3.92 – 7.59	3.95 – 10.17	3.78 – 10.17	3.14 – 8.44
	The log mean*	6.49 (±1.63) <sup>a</sup>	5.83 (±1.80) <sup>b</sup>	5.81 (±1.39) <sup>b</sup>	5.57 (±2.54)	5.82 (±2.08)	5.46 (±1.76)
<i>Enterococcus</i>	Range	4.00 – 9.41	6.30 – 9.04	3.39 – 9.11	5.00 – 9.17	4.95 – 9.51	4.49 – 8.69
	The log mean*	6.90 (±2.12)	7.58 (±0.99)	6.82 (±1.68)	7.52 (±1.16) <sup>c</sup>	6.78 (±1.69) <sup>cd</sup>	6.25 (±1.26) <sup>d</sup>
<i>Enterobacteriaceae</i>	Range	7.14 – 9.41	6.39 – 9.38	6.00 – 9.95	6.44 – 9.91	7.00 – 9.27	6.00 – 9.91
	The log mean*	8.63 (±0.73)	8.14 (±0.92)	8.11 (±1.11)	7.23 (±2.11)	7.62 (±1.79)	7.46 (±1.63)

\* the log mean of each group with 95% CI in parentheses (standard deviation),

<sup>a,b</sup> statistically significant differences in the group treated with DNG  $P \leq 0.05$ ;

<sup>c,d,e</sup> statistically significant differences in the group treated with placebo  $P \leq 0.05$ ,

<sup>c</sup> statistically significant differences in DNG (8) versus PG (8)  $P \leq 0.05$ .

Table II  
Changes of SCORAD index before and after *Lactobacillus casei* DN – 114001 (DNG) or placebo (PG) intake in children with atopic dermatitis

SCORAD index	DNG			PG		
	Time [months]			Time [months]		
	0	3	8	0	3	8
Range	7.4 – 41.6	0 – 29.9	0 – 10.7	7.2 – 44.5	0 – 37.9	0 – 36.8
Mean*	21.3 (±9.5) <sup>a</sup>	9.2 (±8.5) <sup>b</sup>	2.8(±3.6) <sup>c</sup>	3.8(±11.5) <sup>d</sup>	12.1 (±12.7) <sup>e</sup>	9.5 (±13.2) <sup>e</sup>

\* arithmetical mean of each group with 95% CI in parentheses (standard deviation),

<sup>a,b,c</sup> statistically significant differences in the group treated with DNG  $P < 0.05$ ,

<sup>d,e</sup> statistically significant differences in the group treated with PG  $P < 0.05$ .

There were no statistically significant differences between DNG and PG groups.

## Discussion

Our experiment showed that the dietary supplementation of children suffering from AD with the probiotic bacteria *Lactobacillus casei* DN 114001 modulated the profile of intestinal microbiota in terms of the counts of *Bifidobacterium*, *Clostridium* and *Lactobacillus* sp. Rinne *et al.* (2005) supplemented the diet of infants with genetic predisposition to allergic disorders (the occurrence of atopy in the family) with bacteria *Lactobacillus rhamnosus* GG for 4 weeks and monitored changes in their fecal microbiota considering cells of *Bifidobacterium* and *Lactobacillus/Enterococcus* (by FISH method). They found the successive decrease in the number of *Bifidobacterium* cells in the period between the 3<sup>rd</sup> and 12<sup>th</sup> month both in the probiotic and control (placebo) groups. By contrast, in our study the population of intestinal *Bifidobacterium* sp. in the children administered with bacteria *Lactobacillus casei* DN – 114001 was stabilized during the treatment and was decreased only after the next 5 months. *Bifidobacterium* sp. rank among the most important, health-promoting microorganisms that colonize the human gastrointestinal tract. Molecular study of Favier *et al.*, (2002) revealed that in breast-fed newborns bifidobacteria account for 60–90% total fecal microbiota. They are thought to modulate the activity of the immune system. An inhibitor of serine protease, which is identical with its eukaryotic homologue exerting the immunomodulating activity, was identified in the genome of *Bifidobacterium longum* (Schell *et al.*, 2002). *Bifidobacterium* sp. are capable of adapting to conditions inside the gastrointestinal tract of newborns and their metabolic activities foster maturation and formation of the appropriate intestinal biocenose. The number of intestinal *Bifidobacterium* cells decreases with age when children are fed regular diet. Therefore, the maintenance of relatively high counts of *Bifidobacterium* sp. is of particular importance in individuals with dysfunction of intestinal microflora. Our study showed that dietary supplementation with *Lactobacillus casei* DN – 114001 fosters the predominance of bifidobacteria. The other positive effect of treatment with *Lactobacillus casei* DN – 114001 was reduction in *Clostridium* sp. counts. Because of potential pathogenicity and synthesis of toxins the abundance of clostridia is undesired (Salminen *et al.*, 1998). In healthy infants, the predominant fecal bacteria are *Bifidobacterium* sp. and *Lactobacillus* sp. By contrast, in infants who developed allergic disorders the population of *Bifidobacterium* is reduced while the population of *Clostridium* sp. is increased (Kalliomaki *et al.*, 2001; Ouwehand *et al.*, 2002). Analysis of effects of probiotics on the composition and counts of intestinal microbiota that were reported by various groups of researchers shows that treatment with

different probiotic bacteria strains causes different levels of modulation. For instance, Garrido *et al.* (2005) supplemented the diet of healthy individuals with *Lactobacillus johnsonii* La1 and observed that population of *Bifidobacterium* was increased by 5% while counts of *Clostridium* sp., *Bacteroides* sp. and *Enterococcus* sp. were not affected. Another trial, consisting in treatment of infants with family predisposition to atopic dermatitis and bronchial asthma with probiotic *Bifidobacterium lactis* BB12 revealed that the count of *Clostridium* sp. was reduced only in breast-fed children who were not fed the probiotic. In the children who were fed *Bifidobacterium lactis* BB12 the clostridium bacteria were in the same level (Rinne *et al.*, 2005). Adlerberth *et al.*, (2007) found that the risk of AD development increased with the level of intestinal *Clostridium* sp. in infancy. Our study presented that an attractive property of *Lactobacillus casei* DN – 114001 is its capability of reducing the count of *Clostridium* sp. in children with AD. Importance of those strains in treatment of AD still are inconclusive. Our observation showed that they could play role in long-lasting clinical improvement.

Results of this study and available reports demonstrate that the modulation of intestinal ecosystem in children with atopic dermatitis, consisting in reducing or increasing counts of various groups of microorganisms by different probiotic bacteria is strain-dependent. Supplementation of AD children diet with the strain *Lactobacillus casei* DN – 114001 positively affects population of *Bifidobacterium* sp. and reduces counts of *Clostridium* sp. and additionally stabilize *Lactobacillus* population.

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