

## Probiotic supplementation influences faecal short chain fatty acids in infants at high risk for eczema

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### RESEARCH ARTICLE

#### Abstract

The composition of the gut microbiota plays a role in the development of allergies. Based on the immunomodulating capacities of bacteria, various studies have investigated the potential role for probiotics in the prevention of childhood eczema. In a previous study we have shown that significantly less children developed eczema after probiotic supplementation (*Bifidobacterium bifidum* W23, *Bifidobacterium animalis* subsp. *lactis* W52 and *Lactococcus lactis* W58, Ecologic®Panda) at three months of age as compared to controls. Here, metabolites in faecal samples of these 3-month old children were measured by <sup>1</sup>H-nuclear magnetic resonance to investigate possible gut metabolic alterations. Lower amounts of short-chain fatty acids (SCFAs), succinate, phenylalanine and alanine were found in faecal samples of children later developing eczema, whereas the amounts of glucose, galactose, lactate and lactose were higher compared to the children not developing eczema. Although these differences were already present at the age of 3 months, eczema did not develop in the majority of children before the age of 1 year. Supplementation of multispecies probiotics seems to induce higher levels of lactate and SCFAs, and lower levels of lactose and succinate when compared with the placebo group. This might explain the temporary preventive effect of probiotics on the development of eczema. These results highlight the role bacterial metabolites may play in development of the immune system, even before clinical manifestations of allergic disease arise.

**Keywords:** atopy, intestinal microbiota, metabolites, probiotics

#### 1. Introduction

The worldwide prevalence of allergic diseases such as eczema, food allergy, asthma and allergic rhinitis has increased considerably in the past few decades (Fishbein and Fuleihan, 2012; Ismail *et al.*, 2013). Up to 40% of the population of the USA and Europe suffer from at least one type of allergy and the prevalence of allergies in industrial societies has doubled over the past 15 years (Kramer *et al.*, 2013). Amongst these, eczema is one of the most common inflammatory skin diseases in childhood. The aetiology of allergic diseases is unknown although it has been well

established that both genetic and environmental factors do play a role. There is growing evidence that the composition of gut microbiota is a crucial environmental factor in the development of allergic disease. The development of allergies has been linked to reduced microbial exposure in early childhood, reduced bacterial diversity and an altered composition of gut microbiota (Abrahamsson *et al.*, 2014; Bisgaard *et al.*, 2011; Bjorksten *et al.*, 2001; Kramer *et al.*, 2013). In this context, the window of opportunity for influencing the composition of the intestinal microbiota and possibly modulate the development of allergic diseases, would be the perinatal period.

The composition of the gut microbiota in infants is influenced by several environmental factors, including mode of delivery, nutrition, medication, such as antibiotics and by the supplementation of pre- and probiotics (Quigley, 2013). Probiotics are living microorganisms that, when administered in adequate amounts, confer a beneficial health effect on the host (FAO/WHO, 2001; Hill *et al.*, 2014). The most widely used probiotic bacteria belong to the *Lactobacillus* and *Bifidobacterium* genera. Certain probiotics have shown to be effective in the prevention and/or management of disorders such as necrotising enterocolitis, acute infectious diarrhoea, antibiotic-associated diarrhoea, inflammatory bowel disease (pouchitis and ulcerative colitis) and lactose maldigestion (Floch *et al.*, 2011; Sanders *et al.*, 2013). Various studies have investigated the potential of probiotics, both for primary prevention and for treatment of eczema and other atopic diseases. Results are inconsistent; while some studies have shown a preventive effect of probiotics on the development of eczema, others showed no effects (Elazab *et al.*, 2013; Panduru *et al.*, 2015). This inconsistency could be explained by differences in study design, amount and duration of probiotic supply, and the strain-specific effects of probiotic bacteria. Moreover, the biological mechanisms involved in successful clinical outcome of probiotic supplementation on the prevention of eczema still remain unknown.

One of the potential mechanisms by which probiotic bacteria could regulate the development of the mucosal immune system is stimulation of the differentiation of naive T-cells towards T-helper 1 cells (Th1) or regulatory T-cells, thereby shifting the balance between Th1 and T-helper 2 cells (Th2). In our clinical trial (the Panda-study; Niers *et al.*, 2009), a mixture of three probiotic strains was administered to infants at high-risk for atopy, from birth onwards during the first year of life. The probiotics were also given to the mothers during the last 6 weeks of pregnancy. The selection of probiotic strains was based on the ability to down-regulate Th2 cytokine production *in vitro* and to induce regulatory T-cell cytokines (Niers *et al.*, 2007). Clinical supplementation of the multi-species probiotic product proved to have a significant preventive effect on the development of eczema in high-risk children within the first three months of life (Niers *et al.*, 2009). This clinical difference was maintained up to the age of two years, although it lost significance. Recently, the long-term outcomes of this intervention were published (Gorissen *et al.*, 2014), showing that the beneficial effect was not extended to the age of 6 years and did not lead to the primary prevention of asthma, in accordance with other studies published so far (Azad *et al.*, 2013).

The biological pathways that mediate the clinical effects of probiotics are still unclear and it remains to be demonstrated whether immunoregulatory properties are an important mechanism *in vivo*. In order to gain insight into the biological mechanisms underlying the

clinical outcome of probiotics, an analysis of bacterial metabolites was made. To that end the faecal metabolic profiles of the eczema-prone children in the Panda-study were investigated. The aim of this study was to identify potential differences in gut metabolites between children at-risk for atopic disease, that later developed eczema and those that did not develop eczema. Out of the several techniques available for metabolic profiles, <sup>1</sup>H nuclear magnetic resonance (NMR) spectroscopy was used. The advantages of NMR are robustness, reproducibility and the absence of extensive sample preparation; a disadvantage is the lower sensibility compared to other metabolomics techniques like mass spectrometry (Brennan, 2008; Lindon *et al.*, 2007; Marchesi *et al.*, 2007; Nicholson *et al.*, 2005). It thus has been demonstrated that the metabolite profiles of gut microbiota can be constructed by targeted profiling of NMR spectra (Jacobs *et al.*, 2008; Yen *et al.*, 2015).

## 2. Materials and methods

### Design of study

A double blind, randomised, placebo-controlled trial as described in detail by Niers *et al.* (2009) was performed. Briefly, pregnant women and their offspring (with a positive family history of allergic disease) received either once sachet daily with 3 g probiotics (Ecologic®Panda, Winclove Probiotics, the Netherlands) or a placebo during the last six weeks of pregnancy (mothers) and during the first 12 months after birth (infants). The probiotic product contained the bacterial strains *Bifidobacterium bifidum* W23, *Bifidobacterium animalis* subsp. *lactis* W52 and *Lactococcus lactis* W58, a total  $1 \times 10^9$  cfu per strain per day). In total 123 participants were included (per protocol) in the clinical study (63 assigned to the placebo, 60 to the probiotic group). In the placebo group 52 participants and in the probiotic group 50 participants completed the 3 months clinical follow-up. Follow-up continued up to six years of age, investigating the clinical manifestation of eczema, IgE sensitisation and, in time, allergic rhinitis and asthma. Blood samples and faecal samples were collected at different time points during follow-up.

### Clinical outcomes

Children were clinically examined at the age of 3, 12, 24 months and 6 years of age, as described previously (Gorissen *et al.*, 2014; Niers *et al.*, 2009). For the present study, we grouped the children according to the presence or absence of eczema till two years of age. Parental-reported eczema was defined as eczema reported by parents, in diaries that they were asked to complete weekly for complaints of eczema, infectious or atopic symptoms, feeding habits and use of medication. Doctor-diagnosed eczema was defined as clinical signs of eczema diagnosed by the family doctor or consulted physician at the time of visit to the doctor's office or outpatient clinic.

## Faecal sample collection

As the clinical effects were significantly different between the probiotic and the placebo group at three months of age, faecal samples of this time point were chosen to perform metabolite analysis on. The faecal samples (n=34) were collected directly from the diapers by the parents and stored at -20 °C until analysis. Only samples from children of whom complete information on disease development was recorded were analysed, resulting in a different number of samples for each group (Group A – placebo eczema, n=9; group B – probiotic eczema, n=9; group C – probiotic no eczema, n=9; group D – placebo no eczema, n=7).

## Faecal sample extraction

Faecal extracts were prepared by mixing 20 mg of frozen faecal material with 1 ml of phosphate buffered saline that consists of 1.9 mM Na<sub>2</sub>HPO<sub>4</sub>, 8.1 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl and 1 mM TSP (sodium 3-(trimethylsilyl)-propionate-d<sub>4</sub>) in D<sub>2</sub>O (Le Gall *et al.*, 2011). After mixing thoroughly, samples were centrifuged (17,000×g, 5 min). The supernatant was filtered through a 0.2 µm membrane filter and 600 µl of the filtrate was transferred to a 5 mm NMR tube for analysis.

## Nuclear magnetic resonance analysis

High resolution <sup>1</sup>H-NMR spectra were recorded using a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). Sample temperature was controlled at 25 °C. Each spectrum consisted of 128 scans and *noesypr 1d* pre-saturation sequence was used to suppress the water signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 2 s) and mixing time (D8 = 0.15 s). A 90° pulse length of 8.2 µs was set for all samples. <sup>1</sup>H-NMR spectra were Fourier transformed (LB = 0.3 Hz). After zero filling, each spectrum was manually phased and baseline corrected using Topspin 3.0 software (Bruker). All resonances of metabolites were confirmed by comparison with reported data (Le Gall *et al.*, 2011) or by 2D NMR spectroscopy (J-resolved, COSY and HMBC).

## Data analysis

The AMIX software (Bruker) was used to reduce the <sup>1</sup>H-NMR spectra to an ASCII file, with total intensity scaling. Bucketing or binning was performed and the spectral data were reduced to include regions of equal width (0.04 parts per million) equivalent to the region of δ 10.00-0.40. The regions of δ 4.88-4.64 were not included in the analysis because of the remnant D<sub>2</sub>O signal.

Due to the complexity of the NMR spectra, it was necessary to apply multivariate data analysis to explore differences between the groups. In this case, we applied orthogonal partial least squares-discriminant analysis (OPLS-DA) which is a supervised multiple regression analysis similar to PLS-DA, but by orthogonalising non-correlated variables (Bylesjo *et al.*, 2006). Principal component analysis and orthogonal projection to latent structures-discriminant analysis were performed with the SIMCA-P+ software (v. 13.0, Umetrics, Umeå, Sweden). The individual short chain fatty acids were quantified and differences between the groups were tested by ANOVA as described previously (Monleon *et al.*, 2009).

## 3. Results

NMR analysis was performed for 34 samples. One sample from the placebo eczema group showed signals corresponding to N-acetyl-*p*-aminophenol (Acetaminophen®), probably administered as an analgesic or antipyretic drug. This sample was excluded from further data analyses. There were no significant differences in baseline characteristics between groups (Table 1).

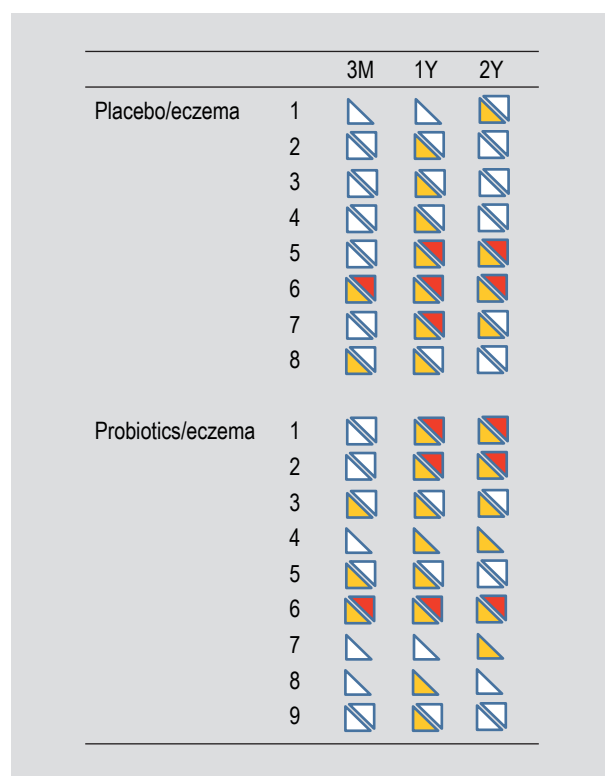
A total of 33 faecal samples were classified according to the presence or absence of parental-reported eczema. In a number of cases the reported eczema was confirmed by a doctor (doctor's diagnosis) (Figure 1). Within the eczema group, consisting of 8 children who received placebo (Group A) and 9 children who received probiotics (Group B), the majority of children did not have eczema at the moment of stool sampling (3 months) but developed symptoms later

**Table 1. Baseline characteristics of study participants.**

Group	n	Gestational age in weeks (mean ± SD) <sup>a</sup>	Birth weight in kg (mean ± SD) <sup>a</sup>	Mode of delivery (caesarean section/total)	Feeding type
A Placebo, eczema	8	40.5±1.8	3.9±0.4	2/8	7 breast, 1 mixed
B Probiotics, eczema	9	39.3±1.8	3.4±0.4	0/9	5 breast, 2 bottle, 2 mixed
C Probiotics, no eczema	9	40.5±1.7	3.8±0.4	1/9	4 breast, 2 bottle, 3 mixed
D Placebo, no eczema	7	40.0±1.4	3.8±0.8 <sup>b</sup>	0/7	3 breast, 3 bottle, 1 mixed

<sup>a</sup> SD = standard deviation.

<sup>b</sup> Data available from 6 infants. No significant differences, as measured by nonparametric repeated measures ANOVA, were detected between groups in gestational age, birth weight, mode of delivery or type of feeding.



**Figure 1. Overview of the eczema score of the participants. A missing triangle means that no information was available on doctor's diagnosed eczema.**

**Open lower triangles: no parental reported eczema; closed lower triangles: parental reported eczema; open upper triangles: no doctor's diagnosed eczema; closed upper triangle: doctor's diagnosed eczema**

(Figure 1). In the non-eczema group (Group C – probiotics,  $n=9$ ; and group D – placebo,  $n=7$ ), none of the children showed clinical signs of eczema at any of the time points indicated.

### Differences in faecal metabolites between the eczema and non-eczema group

$^1\text{H-NMR}$  spectroscopy of faecal extracts revealed the presence of various metabolites, such as lactate, short chain fatty acids (SCFAs) (butyrate, propionate, acetate), amino acids (alanine, valine, leucine, phenylalanine), sugars (glucose, galactose, xylose) and sugar alcohol (glycerol). The major identified metabolites and its chemical shifts found in faecal samples are listed in Table 2.

Comparison of the  $^1\text{H-NMR}$  spectra, analysed by OPLS-DA, showed separation of the groups based on presence or absence of parental reported eczema ( $R^2=0.7$ ,  $Q^2=0.013$ ; Figure 2A). The  $R^2$  value estimates the goodness of fit, whereas the  $Q^2$  value is a measure of the quality in multivariate models. A  $Q^2$ -value  $>0.5$  is considered large, so our observed differences were relatively small. Lower

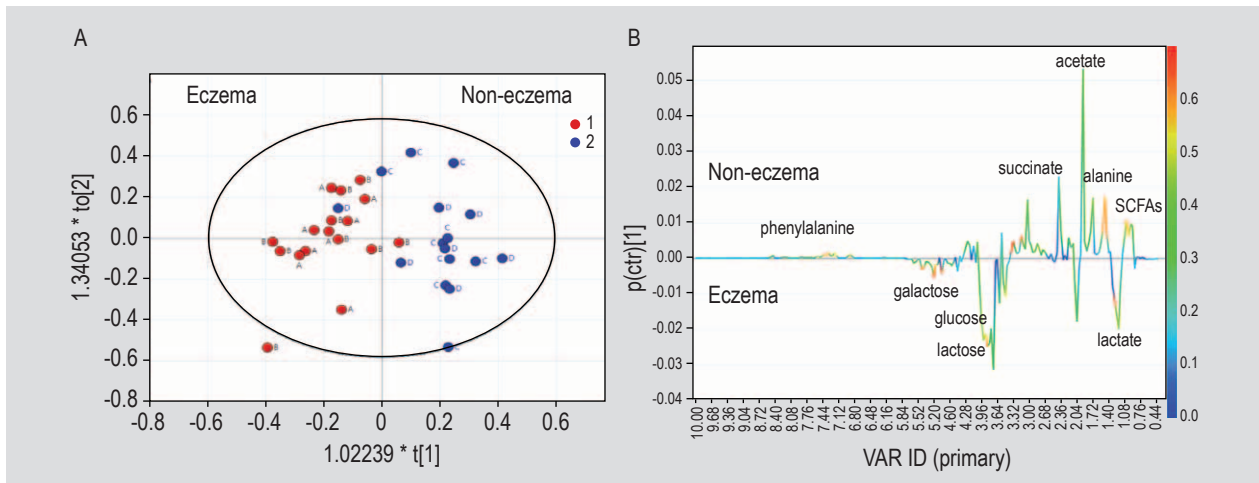
**Table 2. Chemical shifts of metabolites found in faecal samples.**

Metabolite	Chemical shift ( $\delta$ ) <sup>1</sup>
<i>n</i> -butyrate	0.90 (t), 1.56 (m), 2.16 (t)
Isovalerate	0.91 (d), 2.06 (d)
Isoleucine	0.95 (t), 1.02 (d)
Leucine	0.96 (d), 0.97 (d)
Valine	1.00 (d), 1.05 (d)
Propionate	1.06 (t), 2.19 (q)
Lactate	1.33(d), 4.11 (q)
Threonine	1.33 (d), 4.26 (m)
Isobutyrate	1.07 (d), 2.39 (m)
Alanine	1.48 (d), 3.79 (q)
Acetate	1.92 (s)
Glutamate	2.11 (m), 2.36 (m), 3.76 (dd)
Lysine	1.48 (m), 1.73 (m), 1.91 (m), 3.03 (t), 3.77 (t)
Aspartate	2.68 (dd), 2.82 (dd), 3.90 (dd)
Succinate	2.42 (s)
Trimethylamine	2.91 (s)
Taurine	3.27 (t), 3.44 (t)
$\alpha$ -glucose	5.24 (d), 3.53 (dd)
$\beta$ -glucose	4.65 (d), 3.25 (dd)
Glycine	3.57 (s)
Fumarate	6.52 (s)
Uracil	7.54 (d), 5.80 (d)
Phenylalanine	7.44 (m), 7.38 (m), 7.32 (d)
Tyrosine	7.20 (d), 6.90 (d)
4-hydroxyphenylacetate	7.19 (d), 6.86 (d), 3.44 (s)
Formate	8.45 (s)
Adenine	8.23 (s), 8.19 (s)
$\alpha$ -galactose	5.27 (d)
$\beta$ -galactose	4.59 (d)
$\alpha$ -xylose	5.20 (d)
$\beta$ -xylose	4.58 (d)
Glycerol	3.57 (dd), 3.67 (dd), 3.79 (m)
Lactose	5.22 (d), 4.46 (d), 4.67 (d)

<sup>1</sup> d = doublet; dd = double doublet; q = quartet; s = singlet; t = triplet; m = multiplet.

amounts of SCFAs (butyrate, acetate), alanine, succinate, and phenylalanine were detected in the faecal samples of the eczema group, whereas the amounts of glucose, galactose, lactose and lactate were higher in the eczema group when compared to the non-eczema group (Figure 2B). As expected with the low  $Q$ -value we observed, groups could not be discriminated based on a single metabolite, as displayed by the non-significant differences between groups (Table 3).

For 21 out of 33 children (64%) of whom data on parental reported eczema were available, there was a corresponding doctor's diagnosis. A separate OPLS-DA analysis for eczema



**Figure 2.** Orthogonal partial least squares-discriminant analysis of faecal samples between eczema group (group A+B, n=17, red circles [1]) and non-eczema group (group C+D, n=16, blue circles [2]), based on parental reported eczema. (A) Scatter plot; (B) S-line plot.  $R^2Y=0.691$ ,  $Q^2Y=0.013$ .

**Table 3.** Concentrations short chain fatty acids (SCFAs) for the different groups.<sup>1</sup>

	Acetate	Propionate	n-Butyrate	total SCFA
Eczema group	0.48±0.42	0.08±0.09	0.10±0.10	0.65±0.56
Non-eczema group	0.52±0.39	0.10±0.08	0.10±0.11	0.72±0.46
	$P=0.75$	$P=0.34$	$P=0.96$	$P=0.68$
Probiotic group	0.50±0.27	0.11±0.09	0.13±0.12	0.74±0.43
Placebo group	0.49±0.52	0.07±0.06	0.07±0.08	0.63±0.61
	$P=0.95$	$P=0.19$	$P=0.13$	$P=0.57$

<sup>1</sup> The concentrations are given in arbitrary units ± standard deviation.

versus non-eczema based on doctor's diagnosis did not show significant differences ( $Q^2$ -value negative, data not shown).

#### Effects of probiotic supplementation on faecal metabolite composition

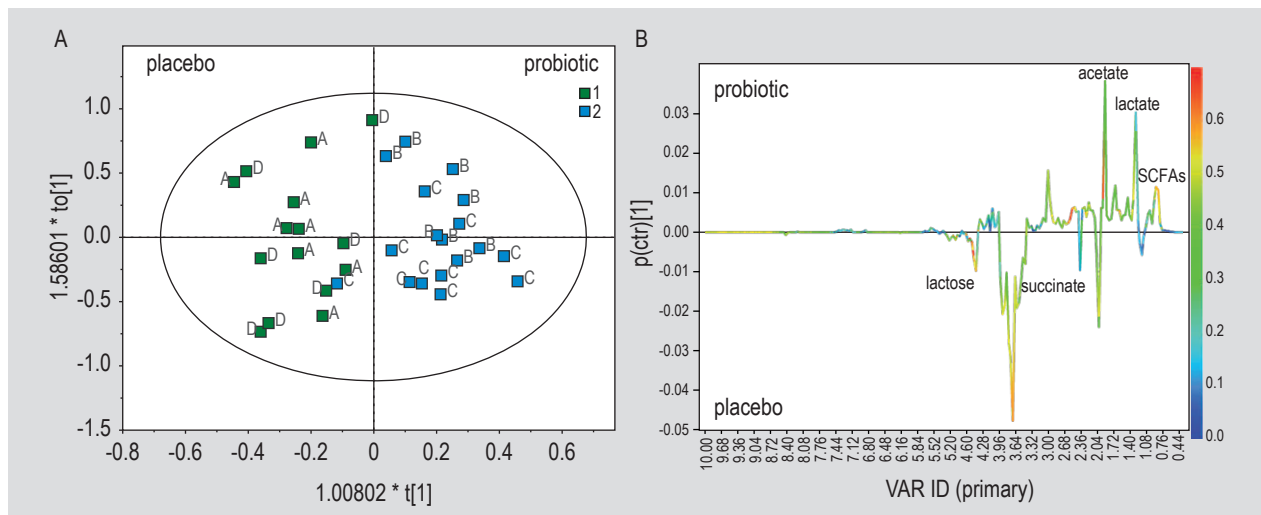
To determine the effect of probiotic supplementation on identification of specific gut metabolites, we analysed the metabolic profiles of the probiotic and placebo group. In OPLS-DA, the probiotic group could be separated from the placebo group ( $R^2=0.7$ ,  $Q^2=0.3$ ; Figure 3A). The probiotic group showed higher levels of lactate and SCFAs (acetate, butyrate, propionate, isobutyrate) and lower levels of lactose and succinate (Figure 3B).

#### 4. Discussion

SCFAs were found to be the major discriminating metabolites between infants at-risk for developing atopy, expressed as eczema. Some SCFAs, mainly

acetate, propionate and butyrate, are end-products of the breakdown of carbohydrates by gut microbial action. Among the SCFAs, butyrate is important for maintaining a normal colonocyte population (Topping and Clifton, 2001). Moreover, it has anti-inflammatory properties by the reduction of different pro-inflammatory cytokine expression and signalling, induction of nitric oxide synthesis and metalloproteinases, and the reduction and activation of lymphocyte proliferation (Leonel and Alvarez-Leite, 2012).

The potential role of SCFAs in eczema has been addressed before: Bottcher *et al.* (2000) showed lower levels of different SCFAs as compared with non-allergic controls in one-year old allergic children. Another study showed the severity of eczema to be inversely correlated with the amount of butyrate producing bacteria (Nylund *et al.*, 2015). In our study, the eczema group consisted of children that developed eczema in the first two years of life. By analysing the 3 month-old samples, when the majority of children had not yet developed eczema, the demonstrated low levels of SCFAs seem to precede the clinical manifestation of



**Figure 3. Orthogonal partial least squares-discriminant analysis of faecal samples between placebo group (group A+D, n=15, green squares [1]) and probiotic group (group B+C, n=18, blue squares [2]). (A) Scatter plot; (B) S-line plot.  $R^2Y=0.748$ ,  $Q^2Y=0.296$ .**

eczema. Sandin *et al.* (2009) have shown before that children with positive skin prick tests and allergic symptoms during the first four years of life, also revealed lower levels of SCFAs preceding the clinical manifestation of allergy.

Allergic diseases can be regarded as Th2 immune-mediated diseases in which regulatory T-cell function is impaired (Fishbein and Fuleihan, 2012). Regulatory T-cells therefore are a major target for preventive or therapeutic intervention. Recent data indicate that SCFAs, in particular butyrate and acetate, can directly promote regulatory T-cell generation in the colon of mice (Arpaia *et al.*, 2013; Furusawa *et al.*, 2013). Thus, differences in faecal SCFAs levels of infants who did and did not develop eczema could provide the missing link between gut microbiota, the mucosal immune system and the development of eczema. Elucidating the role of SCFAs could also lead to a better understanding of the biological mechanism for functional effects of probiotic bacteria.

Our data indicate that early life supplementation of specifically selected probiotic strains might influence SFCA production, including butyrate. In model systems of the human gut it has been shown that certain probiotics and synbiotics can influence SCFAs levels (Van Zanten *et al.*, 2012), a finding that supports the data presented here. There are also indications that probiotics can increase the amounts of SCFAs in healthy adults *in vivo*, without changing the overall faecal microbiota composition (De Preter *et al.*, 2011; Vitali *et al.*, 2010). On the other hand, another study addressing the effects of *Lactobacillus acidophilus* 74-2 and *B. animalis* subsp *lactis* DGCC 420 on faecal microbiota in healthy adults failed to show any effect on faecal SCFAs levels (Klein *et al.*, 2008). In accordance with that study, no increase of SCFAs could be indicated, as in a mouse model with humanised infant microbiota supplementation with *Lactobacillus paracasei* NCC2461 and *Lactobacillus*

*rhamnosus* NCC4007 resulted in a decrease in acetate and butyrate (Martin *et al.*, 2008). However, it is difficult to generalise results of these studies, as the effects of probiotics supplementation, including metabolic effects, can be highly strain-specific.

Naturally, when investigating gut metabolites, composition of and changes in the microbial population are of great importance. This was initially evaluated by MCPC, which is a qualitative analysis based terminal restriction fragment length polymorphism (T-RFLP) analysis (Niers *et al.*, 2009). This analysis showed significantly more frequent colonisation with higher numbers of *L. lactis* in the probiotic group compared with the placebo group during the first 3 months of life. Moreover, no differences were observed in the first 4 weeks of life in the number of children colonised by bifidobacteria but at the age of 3 months, all children in the probiotic group and 85% of the placebo group were colonised with bifidobacteria. *L. lactis* was shown to be present in all faecal samples from the intervention group and in significantly higher amounts, and it was present only in 2/8 samples from the placebo group. *Bifidobacterium* spp. were present in all of the individuals in high numbers (Niers *et al.*, 2009). The overall microbiota composition did not show major differences (Niers *et al.*, 2009; Rutten *et al.*, 2015).

We hypothesise the observed difference in SCFA production most probably is indirect, as none of the probiotic strains included in the used probiotic product is a butyrate producer itself. How this effect is induced cannot be concluded directly from our data. It could be affected by upregulation of the number of butyrate-producing bacteria in the gastrointestinal tract or by increased metabolic activity in general. The main limitation of this study was the small sample size. However, even with these small

numbers of participants, differences between groups could be demonstrated. Based on the data presented here, a suggested mechanism could be that colonic regulatory T-cell development is impaired when SCFA production by gut microbiota is reduced. This mechanism then may contribute to the development of allergic disease. This specific combination of probiotics might then have the capacity to modulate the metabolic activity of resident gut microbiota for producing higher concentrations of SCFAs. The SCFAs, in particular butyrate, diffuse through gut epithelial cells, stimulating the differentiation of naive T-cells into regulatory T-cells, thereby preventing allergy.

Succinate, lactate and alanine were found to be more abundant in the non-eczema group, whereas glucose, galactose and lactose (sugars) were more prevalent in the eczema group. It can be hypothesised that the higher amounts of different sugar metabolites in the eczema group are caused by decreased absorption of these metabolites in the intestine. Children with eczema are known to have an impaired intestinal mucosal barrier, which could lead to decreased absorption (Rosenfeldt *et al.*, 2004). A comparable finding was observed in faecal extracts of patients with ulcerative colitis compared with healthy controls, as glucose levels were higher in diseased patients (Le Gall *et al.*, 2011). The roles these metabolites play in the development of eczema remain unclear, but do warrant further investigation.

In conclusion, our study suggests a role for intestinal SCFAs in the development of eczema in early life. Group enlargement in comparable studies addressing primary prevention of atopic disease, by supplementation of pre- or probiotics, can strengthen the understanding of possible working mechanisms. Moreover, it can lead to rational development of sustainable microbiota management for prevention and treatment of allergy and other immune mediated diseases, where the production of specific metabolites would be the leading criterion for selection.

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## Conflict of interest

H.K. Kim, I. Besseling-van der Vaart and S. van Hemert are employees of Winlove Probiotics. Winlove Probiotics produces and markets Ecologic®Panda. These authors have no direct or additional financial interests. The other authors do not have conflicts of interest to declare.

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