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High levels of butyrate and propionate in early life are associated with protection against atopy

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Abstract

Background: Dietary changes are suggested to play a role in the increasing prevalence of allergic diseases and asthma. Short-chain fatty acids (SCFAs) are metabolites present in certain foods and are produced by microbes in the gut following fermentation of fibers. SCFAs have been shown to have anti-inflammatory properties in animal models. Our objective was to investigate the potential role of SCFAs in the prevention of allergy and asthma.

Abbreviations: OR, odds ratio; PASTURE, Protection against Allergy-Study in Rural Environments; SCFA, short-chain fatty acid.

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Methods: We analyzed SCFA levels by high-performance liquid chromatography (HPLC) in fecal samples from 301 one-year-old children from a birth cohort and examined their association with early life exposures, especially diet, and allergy and asthma later in life. Data on exposures and allergic diseases were collected by questionnaires. In addition, we treated mice with SCFAs to examine their effect on allergic airway inflammation.

Results: Significant associations between the levels of SCFAs and the infant's diet were identified. Children with the highest levels of butyrate and propionate (≥95th percentile) in feces at the age of one year had significantly less atopic sensitization and were less likely to have asthma between 3 and 6 years. Children with the highest levels of butyrate were also less likely to have a reported diagnosis of food allergy or allergic rhinitis. Oral administration of SCFAs to mice significantly reduced the severity of allergic airway inflammation.

Conclusion: Our results suggest that strategies to increase SCFA levels could be a new dietary preventive option for allergic diseases in children.

KEYWORDS

asthma, butyrate, food allergy, atopic sensitization, Short-chain fatty acid

1 | INTRODUCTION

The increase in the prevalence of allergic diseases over the last decades has been associated with lifestyle changes in industrialized countries. One of the lifestyle factors thought to be important is the diet.[1] Nutritional factors and their interaction with the gut microbiota influence immunological processes, especially early in life.[2] Even though it has been suggested that nutrition during infancy might play a major role in the development of allergies later on in childhood, successful strategies for allergy prevention based on infant's diet are still needed.[3]

Among children from the Protection against Allergy-Study in Rural Environments (PASTURE) birth cohort study, we previously showed that an increased diversity of food introduced within the first year of life might have a protective effect on allergy.[4] Moreover, we and others observed a strong reduction in the risk for the development of atopic dermatitis and asthma later in life when yogurt, butter, or vegetables and fruits were introduced in the first year of life.[4-7]

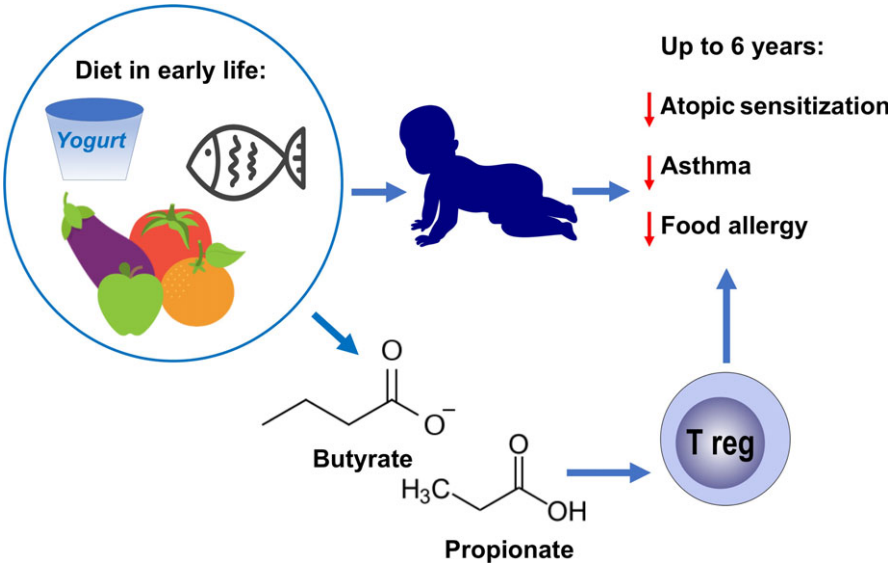
We therefore hypothesized that short-chain fatty acids (SCFAs) might mediate some of the protective effects associated with early life consumption of these foods. Dairy products such as yogurt and butter contain SCFAs (eg, 100 g of butter contains 2.7 g butyrate and 100 g of yogurt contains 0.1 g butyrate), while SCFAs are also metabolites produced by intestinal bacteria through fermentation of fibers.[8] Microbial fermentation and SCFA generation occur primarily in the large intestine, while dietary-derived SCFAs may have immunological effects in both the small and large intestine. Fecal SCFA levels reflect the combination of dietary intake and in situ generated SCFAs. The major SCFAs are acetate, propionate, and butyrate, which have been shown to have anti-inflammatory properties including promoting the expansion of regulatory T cells via inhibition of histone deacetylation, and induction of IgA production by mucosal B cells.[9-12] SCFA effects are mediated by epigenetic mechanisms or G protein-coupled receptor signaling and have been shown in murine models to protect against colitis, inflammatory arthritis, and allergic diseases.[9,13-17] Microbial metabolism and SCFA production strongly influence the growth of beneficial bacteria in the gastrointestinal tract.[18]

Since little is currently known about the role of SCFAs in the development of allergy in children, we investigated whether levels of SCFAs, measured at 1 year of age in feces among a subsample of children from a large European birth cohort, the PASTURE study, were associated with the development of allergic diseases and atopic sensitization later in life. We further evaluated the effect of oral administrated SCFAs in murine models of allergic airway inflammation.

2 | METHODS

2.1 | Study design

The PASTURE study is a prospective birth cohort involving children from rural areas in five European countries (Austria, Finland, France, Germany, and Switzerland), designed to evaluate risk factors and preventive factors, especially in early life, for atopic diseases.[19] Pregnant women were recruited during the third trimester of pregnancy and divided into two groups. Women who lived on family-run farms, where any kind of livestock was kept, were assigned to the farm group. Women from the same rural areas not living on a farm were



GRAPHICAL ABSTRACT

Children with high levels of butyrate or propionate in feces at one year of age have significantly less atopic sensitization compared to children with lower levels. The infant's diet might influence the levels of SCFAs. Oral application of short-chain fatty acids might protect children against the development of atopic sensitization and airway inflammation.

in the reference group. In total, 1133 children were included in this cohort. The study was approved by the local research ethics committees in each country, and written informed consent was obtained from all parents.

2.2 | Definitions

Questionnaires were administered in interviews or self-administered to the mothers within the third trimester of pregnancy and when children were 2, 12, 18, 24 months of age and then yearly up to age 6 years. Feeding practices were reported by parents in monthly diaries between the 3rd and 12th months of life. Duration of breastfeeding was categorized according to the number of months children were breastfed (not exclusively).

Children were defined as having asthma when the parents reported at least once that the child had either a doctor-diagnosed asthma or at least two doctor-diagnosed episodes of obstructive bronchitis in the last 12 months in the year 4, 5, or 6 questionnaire, independently of diagnosis reported in the first 3 years. Obstructive bronchitis is commonly used to define the first occurrence of asthmatic symptoms. Food allergy was defined when the parents reported up to age 6 years that the child had at least once been diagnosed with food allergy by a doctor.

Children were defined as having atopic dermatitis when the parents reported that the child had atopic dermatitis diagnosed by a doctor at least once up to 6 years of age and/or a positive SCORAD score (>0) assessed at the age of 1 year, during medical examination.

Allergic rhinitis was defined by the presence of symptoms (itchy, runny, or blocked nose, without a cold, and associated with red itchy eyes) or a doctor diagnosis of allergic rhinitis ever, reported at 6 years.

Positive parental history of allergies was defined as ever having asthma, allergic rhinitis, or atopic dermatitis.

2.3 | Human-specific IgE quantification

Allergen-specific IgE antibodies (*D. pteronyssinus*, *D. farinae*, alder, birch, hazel, grass pollen, rye, mugwort, plantain, cat, horse, dog, Alternaria, hen's egg, cow's milk, peanut, hazelnut, carrot, and wheat flour) were measured in blood from children at 6 years of age, using the Allergy Screen Test Panel (Mediwiss Analytic, Moers, Germany). Sensitization was defined as a specific IgE level of ≥0.35 IU/mL.

2.4 | SCFA quantification

Fecal samples were processed and analyzed as previously described.[20] Fecal samples collected at 1 year of age were used for the analyses of SCFAs (following one previous freeze/thaw cycle). 1 mL of 0.15 mmol/L H_2SO_4 was added to 0.3 g feces to generate a fecal suspension. After rigorous vortexing, the samples were centrifuged two times (14 000 *g* for 30 minutes) and sequentially filtered through a 0.45-μm (Millex-HA, Merck, Darmstadt, Deutschland) and a 0.2-μm filter (Millex-LG, Merck). The resultant fecal homogenates were analyzed by high-performance liquid chromatography (Merck Hitachi, Schaumburg, USA) using an Rezex ROA-Organic Acid H+ ion exchange column together with a SecurityGuard Cartridges Carbo-H from Phenomenex (Torrance, USA) at a flow rate of 0.4 mL at 40°C with 10 mmol/L H_2SO_4 as eluent solution. The samples were quantified in relation to standards measured in parallel.[21]

2.5 | Animals

Female BALB/c mice aged 6-8 weeks were obtained from Charles River (Sulzfeld, Germany) and housed at AO Research Institute Davos. Four to six animals were housed per cage in individually ventilated cages in a 12/12 hours light/dark cycle with water and food available ad libitum. All experimental procedures were carried out in accordance with Swiss law and approved by the animal experiment commission of the canton Grisons, Switzerland. For further information on the animal experiments, see the method section in this article's online repository.

2.6 | Statistical analysis

As the distribution of the SCFA levels was skewed, the variables were log-transformed (natural logarithm), resulting in an approximately normal distribution. Linear regression was used to investigate the association between exposures, such as dietary factors, and SCFA levels. For SCFA levels, geometric means ratios (GMRs) with 95% CIs were calculated by exponentiation of the regression coefficients and their 95% CIs. Differences in the proportion of children with atopy regarding the high and low levels of SCFAs were tested by Fisher's exact test. Logistic regression was used to investigate the association between level of SCFA and health outcomes. Data analysis was conducted using SAS software version 9.4 (SAS Institute, Inc., Cary, NC).

Mouse experiments were graphed and analyzed statistically with Prism 5 software (GraphPad Software, San Diego, California). Data were expressed as means ± standard deviation (SD). Statistical significance was tested by using the one-way ANOVA with Tukey test.

TABLE 1 Characteristics of the study population

	All study population n = 1133		With SCFA quantification n = 301		
	n	%	n	%	*P*-value
Center					
Austria	220	19.4	38	12.6	<0.001
Switzerland	242	21.4	45	15.0	
France	203	17.9	83	27.6	
Germany	254	22.4	53	17.6	
Finland	214	18.9	82	27.2	
Farmer	530	46.8	156	51.8	0.043
Parents with atopy	595	54.1	171	57.0	0.249
Cesarean delivery	192	17.7	38	12.7	0.008
Breastfeeding					
0 mo	100	9.6	27	9.0	0.617
>0-2 mo	170	16.3	52	17.3	
3-6 mo	289	27.7	86	28.6	
7-9 mo	221	21.2	55	18.3	
≥10 mo	264	25.3	81	26.9	
Breastfeeding at 2 mo					
Yes, exclusively	658	65.5	184	66.0	0.221
Yes, but not exclusively	156	15.5	50	17.9	
No	190	18.9	45	16.1	
Antibiotics prenatal	250	22.6	80	26.9	0.043
Sibling					
≥3	116	10.2	33	11.0	0.324
1-2	604	53.3	169	56.2	
0	413	36.5	99	32.9	

3 | RESULTS

3.1 | SCFA assessments in fecal samples from one-year-old children and their association with early life exposures

To assess the potential protective role of SCFAs in children, we measured metabolite levels in fecal homogenate samples from one-year-old children from the PASTURE birth cohort study. In total, this cohort included 1133 children and SCFA analyses were performed among a subsample of 301 children. The characteristics of the subsample with SCFA measurements were more likely to be farmer, to have prenatal exposure to antibiotics and to be born by vaginal delivery (Table 1). Acetate was the most abundant SCFA, with a median concentration of 60.8 μmol per gram of feces, followed by propionate (median concentration 13.7 μmol/g) and butyrate (median concentration 10.1 μmol/g) (Figure S2).

SCFA levels and early life exposures showed significant associations, especially with the infant's diet (Table 2). Introduction of yogurt in the 1st year of life was associated with a significantly increased level of butyrate in fecal homogenates at 1 year of age. Also, introduction of fish in the 1st year of life and vegetables and/or fruits in the first 6 months were associated with an increased level of butyrate. Introduction of margarine in the 1st year of life was associated with lower propionate and acetate levels. The introduction of cereals in the first 9 months was significantly associated with a reduced level of fecal acetate.

Children who were not breastfed had a higher level of propionate compared to breastfed children (17.2 μmol/g vs. 13.2 μmol/g median level, respectively). Also, children with 3 or more siblings showed an increased level of propionate and a tendency for an increased level of butyrate.

3.2 | SCFA assessments in fecal samples from one-year-old children and their association with allergic diseases and atopy

We observed that especially children with the highest level of butyrate or propionate were less likely to suffer later in life from asthma and food allergy (Table 3). Therefore, we stratified the children into two groups depending on the levels of SCFA (< or ≥the 95th percentile). We found that the proportion of children with sensitization to food and/or inhalant allergens at 6 years of age was significantly

TABLE 2 Association between early life exposures and fecal SCFA levels

Exposures	Butyrate				Propionate				Acetate			
	GMR	95% CI		*P* value	GMR	95% CI		*P* value	GMR	95% CI		*P* value
Farmer vs. nonfarmer	0.98	0.85	1.13	0.792	1.08	0.89	1.31	0.316	1.03	0.93	1.13	0.611
Parents with allergies: yes vs. no	0.97	0.84	1.13	0.730	0.87	0.75	1.01	0.070	0.99	0.90	1.10	0.908
Breastfeeding												
0 mo	1.09	0.83	1.43	0.547	**1.47**	**1.12**	**1.94**	**0.006**	1.04	0.86	1.26	0.702
>0-2 mo	0.98	0.79	1.22	0.880	1.10	0.88	1.37	0.392	1.02	0.87	1.18	0.835
3-6 mo	1.10	0.91	1.33	0.334	1.06	0.87	1.28	0.558	1.11	0.97	1.27	0.133
7-9 mo	0.93	0.75	1.15	0.497	1.02	0.82	1.27	0.861	0.94	0.81	1.09	0.434
≥10 mo, ref.	1.00				1.00							
Cesarean vs. vaginal delivery	1.16	0.93	1.43	0.182	0.92	0.74	1.14	0.437	1.11	0.95	1.28	0.180
Antibiotics prenatal: yes vs. no	0.99	0.84	1.16	0.880	0.98	0.84	1.16	0.837	1.02	0.91	1.15	0.682
Sibling												
≥ 3	1.26	0.99	1.62	0.063	**1.30**	**1.01**	**1.67**	**0.042**	0.93	0.78	1.10	0.391
1-2	1.11	0.95	1.30	0.190	1.11	0.95	1.30	0.185	0.99	0.89	1.10	0.842
0, ref.	1				1				1			
Food introduced within 1st year												
Farm milk: yes vs. no	1.02	0.88	1.19	0.747	1.06	0.91	1.23	0.438	1.02	0.92	1.14	0.650
Cow's milk: yes vs. no	1.04	0.90	1.20	0.569	1.06	0.92	1.23	0.425	0.93	0.84	1.03	0.171
Yogurt: yes vs. no	**1.20**	**1.00**	**1.44**	**0.045**	1.09	0.91	1.31	0.362	1.06	0.94	1.21	0.353
Fish: yes vs. no	**1.21**	**1.05**	**1.40**	**0.010**	0.98	0.84	1.14	0.783	1.03	0.92	1.14	0.627
Nuts: yes vs. no	0.92	0.78	1.10	0.364	1.01	0.85	1.20	0.923	0.98	0.86	1.10	0.715
Vegetables or fruits (in first 6 mo): yes vs. no	**1.18**	**1.02**	**1.35**	**0.025**	0.98	0.85	1.14	0.804	0.97	0.88	1.08	0.597
Butter: yes vs. no	0.94	0.81	1.10	0.456	0.95	0.82	1.11	0.545	0.95	0.86	1.06	0.375
Margarine: yes vs. no	0.95	0.82	1.10	0.514	**0.85**	**0.74**	**0.99**	**0.031**	**0.90**	**0.81**	**0.99**	**0.032**
Chocolate: yes vs. no	0.99	0.86	1.14	0.895	**1.20**	**1.04**	**1.38**	**0.014**	1.05	0.95	1.16	0.317
Egg: yes vs. no	0.96	0.82	1.00	0.554	1.03	0.88	1.20	0.748	0.92	0.83	1.02	0.109
Cereals (in first 9 mo):												
yes vs. no	0.88	0.76	1.03	0.107	0.88	0.75	1.02	0.098	**0.81**	**0.73**	**0.90**	**<0.0001**
Meat (in first 9 mo): yes vs. no	1.13	0.96	1.32	0.136	0.98	0.83	1.15	0.778	0.97	0.87	1.08	0.595

Boldface values are significant (*P*-value < 0.05)

lower among children with very high levels of butyrate (≥95th percentile; >26.88 μmol/g) at 1 year of age compared to children with lower butyrate levels (Figure 1). The odds ratio (OR) for any sensitization between the level of butyrate above and below the 95th percentile was 0.28 (95% CI, 0.09-0.91, *P*-value: 0.034), and after adjustment for center, farmer, gender, parents with allergy, mode of delivery, breastfeeding, and number of siblings, the association remained significant (adjusted OR: 0.25, 95% CI, 0.08-0.82, *P*-value: 0.023). We also observed a trend for a reduced prevalence of asthma, allergic rhinitis, and food allergy among children with a high level of butyrate, but the number of cases was very small (Table 3). Regarding propionate, the proportion of children with sensitization to any allergens was also significantly lower among children with very high levels (≥95th percentile; >32.87 μmol/g) (Figure 1). The OR for any sensitization between the level of propionate above and below the 95th percentile was 0.19 (95% CI, 0.05-0.69), and after adjustment for the same potential confounders as mentioned above, the OR was 0.20 (95% CI, 0.05-0.74). A trend for a reduced risk of asthma was also observed among those children with the highest levels of propionate (Table 3). Children with high acetate levels tended to show a lower prevalence of food sensitization and food allergy, but no associations were observed with inhalant allergen sensitization or asthma (Table 3, Figure 1). No difference in the proportion of children with atopic dermatitis was observed between the high and low levels of any SCFAs. Using a lower cut-off, below and above the 90th percentile of SCFA levels, similar tendencies were observed, even though less strong difference in the proportion of children with or without allergic diseases or atopy (data not shown).

The proportion of atopy between the quartiles of SCFAs showed a reduction of the proportion of atopy with the increasing quartiles of propionate (Table S1).

TABLE 3 Comparisons between children with high and low SCFA levels (≥95th percentile and <95th percentile, respectively)

	Butyrate <95P (<26.88 μmol/g)		Butyrate ≥95P (≥26.88 μmol/g)		Propionate <95P (<32.87 μmol/g)		Propionate ≥95P (≥32.87 μmol/g)		Acetate <95P (<114.67 μmol/g)		Acetate ≥95P (≥114.67 μmol/g)	
	n	%	n	%	n	%	n	%	n	%	n	%
Asthma up to 6 yrs	32/262	12.2	1/15	6.7	32/262	12.2	1/15	6.7	31/263	11.8	2/14	14.3
Allergic rhinitis up to 6 yrs	27/252	9.7	0/15	0.0	25/279	9.0	2/15	13.3	25/279	9.0	2/15	13.3
Food allergy up to 6 yrs	32/275	11.6	1/15	6.7	30/275	10.9	3/15	20.0	32/275	11.6	1/15	6.7
Atopic dermatitis up to 6 yrs	135/284	47.5	5/16	31.3	133/285	46.7	7/15	46.7	133/285	46.7	7/15	46.7
Inhalant sensitization at 6 yrs	107/261	41.0	3/15	20.0	108/261	41.4	2/15	13.3	104/261	39.9	6/15	40.0
Food sensitization at 6 yrs	100/261	38.3	2/15	13.3	99/261	37.9	3/15	20.0	101/261	38.7	1/15	6.7
Any sensitization at 6 yrs	147/261	56.3	4/15	26.7	148/261	56.7	3/15	20.0	145/261	55.6	6/15	40.0

If the n per group was ≥3, a Fisher's exact test to compare two proportions was performed (*P*-value <0.05 was observed only with any sensitization at 6 yrs for butyrate and propionate).

To evaluate the effect of butyrate levels and yogurt exposure in the 1st year of life, separately and combined, on atopic sensitization at the age of 6 years, we used a variable with four categories: children having a high level of butyrate (≥95th percentile) or not and consumed yogurt or not. Both exposure variables showed separately a negative association with any sensitization (only yogurt: OR, 0.47; 95% CI, 0.24-0.90, *P*-value: 0.024, and only high level of butyrate: OR, 0.42; 95% CI, 0.02-7.11, *P*-value: 0.545), even though only significant for yogurt without high level of butyrate. However, only 2 of the 16 children having a high level of butyrate did not consume yogurt in the 1st year of life. Children who consumed yogurt in the 1st year of life and had a high level of butyrate were strongly protected against sensitization (OR: 0.13; 95% CI, 0.03-0.50, *P*-value: 0.004).

3.3 | Oral administration of SCFAs to mice reduced the severity of airway inflammation

Using a murine airway inflammation model, we found that oral administration of acetate, propionate, or butyrate to mice during sensitization and challenge reduced airway hyperresponsiveness following methacholine challenge, compared to vehicle-treated mice (Figure 2A). Moreover, the number of inflammatory cells and particularly eosinophils were significantly reduced in bronchoalveolar lavages (Figure 2B). Since previous studies suggested that SCFA protective effects can be mediated by regulatory T cells, we quantified the number of these cells in lungs of the SCFA-treated mice.[1] We found that oral administration of butyrate increased the percentage of $CD25^+/Foxp3^+$ cells in the lungs, while application of acetate and propionate had no effect on the number of regulatory lymphocytes within the lung (Figure 2C). Oral gavage with SCFAs did not significantly influence the percentage of IL-17-, IFN-gamma-, or IL-4-positive lymphocytes within lung tissue (Figure S3). Finally, SCFA administration had no effect on total and allergen-specific IgE levels in sera of the mice (Figure 2D).

3.4 | Oral administration of SCFAs to mice during pregnancy and weaning reduced the severity of allergic airway inflammation in the offspring

In addition to SCFA treatment during sensitization and challenge of the animals, we also investigated whether oral administration of SCFA to mice during the pregnancy and weaning had an effect on the offspring. Oral application of acetate, propionate, and butyrate to mice during pregnancy and weaning phase reduced the airway hyperresponsiveness to methacholine of the offspring although it did not reach statistical significance (Figure 3A). Moreover, administration of propionate and butyrate reduced total cells, while administration of acetate and butyrate reduced eosinophil numbers in bronchoalveolar lavages of the offspring (Figure 3B). Furthermore, administration of butyrate increased the percentage of lung $CD25^+/Foxp3^+$ cells in the offspring (Figure 3C).

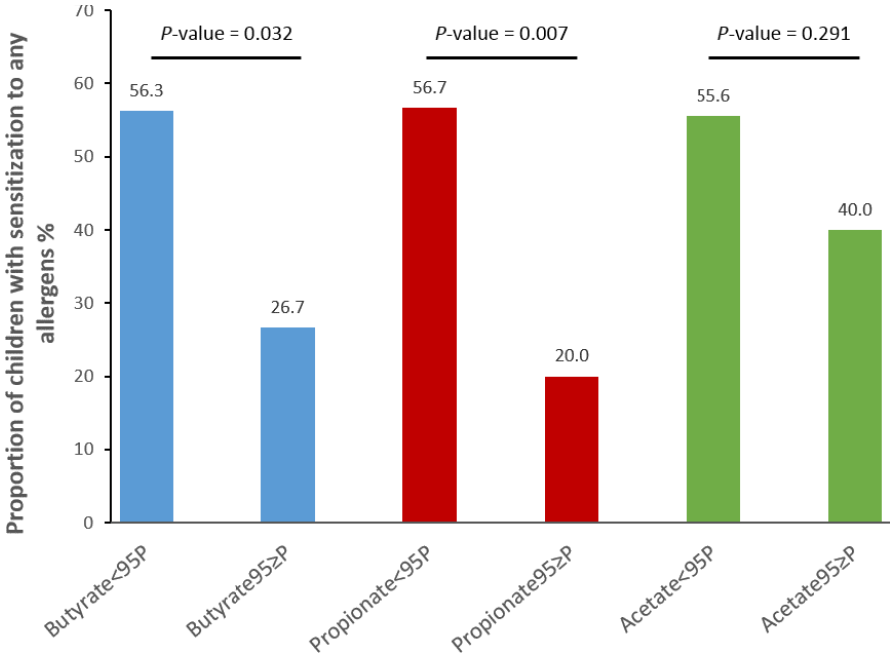


FIGURE 1 Proportion of children with sensitization to any allergens, measured at 6 years of age, stratified by the level of short-chain fatty acid (SCFA) [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Our data show that the infant's diet influences the fecal levels of SCFAs, such as the introduction within the first year of life of yogurt, fish, fruit, and vegetables, which was associated with increased levels of butyrate. Moreover, children with the highest levels of butyrate or propionate, at 1 year of age, were less likely to be sensitized to food and/or inhalant allergens by 6 years of age. There was also a trend for a reduced risk of asthma, allergic rhinitis, or food allergy among children with the highest levels of butyrate. Finally, dietary administration of butyrate, propionate, or acetate to murine models reduced the severity of allergic airway inflammation.

SCFAs are produced by commensal bacteria in the colon by fermentation of undigested sugars, including fibers.[22] One major role of SCFAs is to promote gut homeostasis, but also to maintain the intestinal barrier. Compromised epithelial integrity was shown to be associated with diseases like asthma, allergies, and autoimmunity. Furthermore, SCFAs have been shown to have anti-inflammatory effects in animal models.[9,12,13] By increasing the expression of the transcription factor *FOXP3* via inhibition of histone deacetylation, they support the expansion of T regulatory cells (Tregs) and increase the production of IL-10.[10,23,24]

In our study, children with the highest amounts of SCFAs, especially butyrate and propionate, were protected against atopy at 6 years. Our data also suggest a reduction in the proportion of children having asthma, allergic rhinitis, and food allergy among those with a very high level of butyrate, even though this difference was not statistically significant. However, only a small number of children had a very high level of SCFAs. This is one of the shortcomings of this study and the small numbers may contribute to the lack of statistical significance for some of the associations, although clearly trends are evident. Human studies investigating the role of SCFAs in allergic diseases are rare. Nevertheless, recent findings showed that children developing eczema or food allergy had lower levels of SCFAs in fecal samples compared to those with no disease.[25,26] While we did not observe a statistically significant association for atopic dermatitis or food allergy in our study, there was a trend for reduced incidence in the children with the highest levels of butyrate.

There is some debate concerning the relevance and biological significance of fecal SCFA levels. It is unclear whether an increased fecal SCFA level reflects increased dietary intake, increased microbial synthesis, and/or a decrease in the absorption rate of the SCFA. However, it has been shown that the measurement of fecal SCFAs in humans is a valid indicator of their levels within the colon.[27] In addition, an intervention study among healthy adults showed that diet could influence the fecal level of SCFAs, as a diet high in resistant starch was shown to increase fecal SCFA levels.[28] Interestingly, this increase was observed only among individuals with a low or intermediate level of butyrate at study entry, but not among those with the highest butyrate levels. Moreover, in an intervention study among adults, it was shown that fecal butyrate was increased after yogurt consumption.[29] Similarly, we found significant association between the infant's diet and SCFA levels measured at 1 year of age. From the different food items, we observed that consumption of yogurt in the first year of life was significantly associated with an increased level of butyrate measured at 1 year of age. Children with an increased number of siblings had a higher level of propionate and a tendency of increased level of butyrate, compared to children with less siblings. More siblings might be a marker for different nutritional habits or different environmental factors leading to higher SCFA levels mediated by enhanced SCFA consumption or by an altered gut microbiota. Our current proposed hypothesis is that when

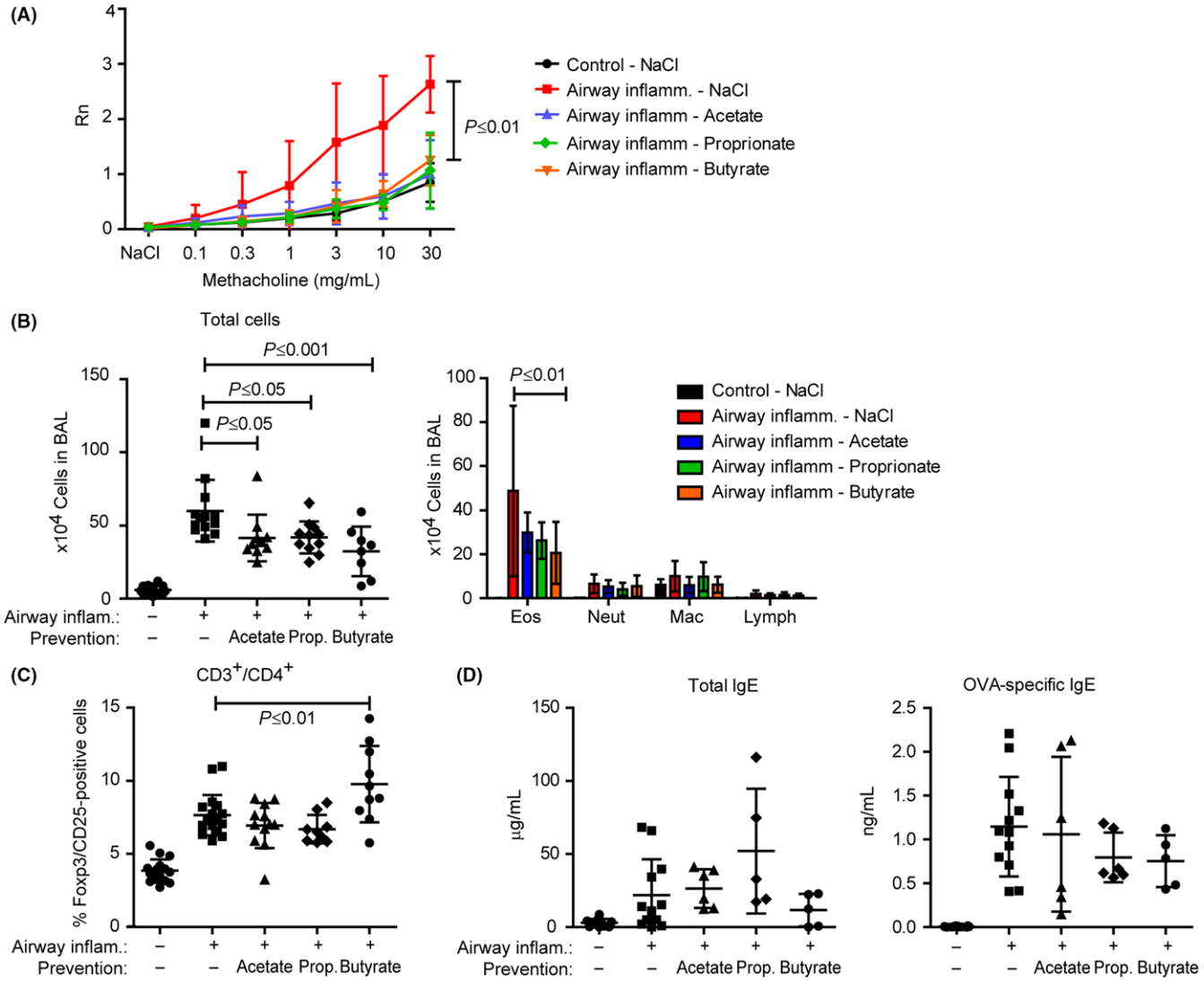


FIGURE 2 Oral application of SCFA to mice reduced the severity of airway inflammation. (A) Airway resistance in response to increasing doses of methacholine. (n = 5 per group). (B) Total and differential cell counts in BAL. Eos, eosinophils; Neut, neutrophils; Mac, macrophages; Lymph, lymphocytes. (control NaCl [n = 16]; airway-inflam.: NaCl [n = 13], acetate [n = 11], propionate [n = 10], butyrate [n = 8]) (C) Quantification of lung $CD25^{+}Foxp3^{+}$ T_{REG} cells. (control NaCl [n = 17]; airway-inflam.: NaCl [n = 18], acetate [n = 12], propionate [n = 9], butyrate [n = 10]) (D) Quantification of total and ovalbumin-specific IgE in sera. (control NaCl [n = 12]; airway-inflam.: NaCl [n = 12], acetate [n = 6], propionate [n = 6], butyrate [n = 5]) Each dot represents an individual animal; the data were generated in 4 independent experiments; mean and SD; one-way ANOVA with Tukey test; Rn, resistance; BAL, bronchoalveolar lavage; prop, propionate; SCFA, short-chain fatty acid [Colour figure can be viewed at wileyonlinelibrary.com]

butyrate levels increase above the level required by colonocytes as an energy source, the excess butyrate is available to interact with the immune system to induce an anti-inflammatory effect, including induction of Tregs.

The strengths of this study are the assessment of the gut microbial metabolites, such as SCFAs, among children from a large birth cohort, with measurements performed in early life, and the prospective design of the study. One limitation might be the wide range of SCFA levels. This variation in SCFA concentrations was observed in other studies as well, and our measurements correspond to SCFA levels previously reported in human studies.[21,30]

Besides improved hygiene, nutritional changes have been suggested to be associated with the increase in the prevalence of allergic diseases over the past decades in westernized countries.[31] The western diet is characterized by reduced vegetables and fiber intake compared to developing countries or western diets of 40 years ago.[32] Altered intake of fiber and fat has been associated with changes in gut microbiota associated with altered levels of SCFA, ω-3 fatty acids, vitamins, biogenic amines, or metabolites from tryptophan catabolism, compounds known to be involved in induction of immune regulation and disease protection.[1,16,33,34] In our study, the mice models indicate that oral application of SCFAs

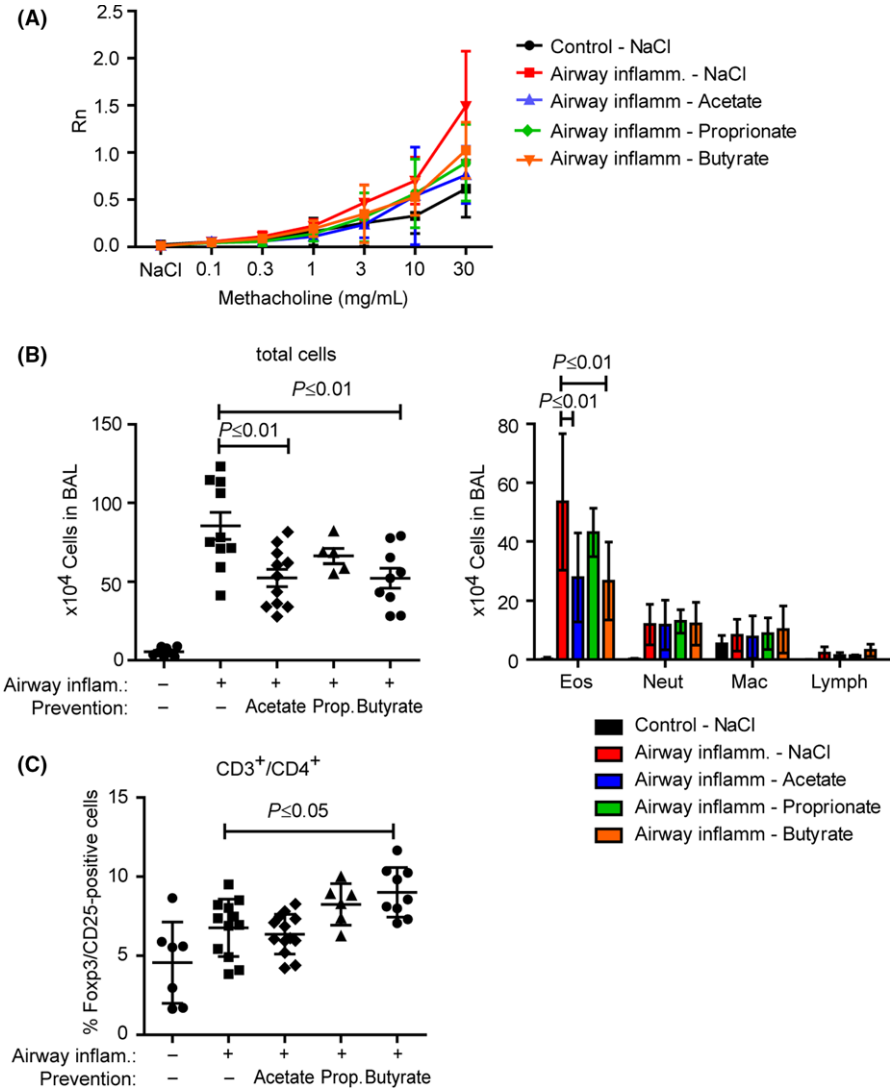


FIGURE 3 Oral application of SCFA to mice during pregnancy and weaning reduced the severity of airway inflammation in the offspring. (A) Airway resistance in response to increasing doses of methacholine. (control NaCl [n = 6]; airway-inflam.: NaCl [n = 4], acetate [n = 5], propionate [n = 8], butyrate [n = 8]) (B) Total and differential cell counts in BAL. Eos, eosinophils; Neut, neutrophils; Mac, macrophages; Lymph, lymphocytes. (control NaCl [n = 8]; airway-inflam.: NaCl [n = 10], acetate [n = 11], propionate [n = 5], butyrate [n = 9]) (C) Quantification of lung $CD25^{+}Foxp3^{+}$ T_{REG} cells. (control NaCl [n = 7]; airway-inflam.: NaCl [n = 12], acetate [n = 13], propionate [n = 6], butyrate [n = 9]) Each dot represents an individual animal; the data were assessed in 3 independent experiments; mean and SD; one-way ANOVA with Tukey test; Rn, resistance; BAL, bronchoalveolar lavage; prop, propionate; SCFA, short-chain fatty acid [Colour figure can be viewed at wile yonlinelibrary.com]

might have a preventive effect on the development of allergic airway inflammation. These results are consistent with other studies, in particular a study showing that mice orally treated with propionate were protected against the development of allergic airway inflammation.[9] Another study showed that mice fed with acetate had reduced inflammation in a colitis model.[13] Therefore, not only an increased intake of fiber, but a direct intake of SCFAs, or a diet rich in foods containing butyrate might be a simple strategy worth investigating for allergy prevention. Moreover, our previous epidemiological data support this hypothesis, as we observed that introduction of cow's milk products, such as yogurt, in the diet within the first year of life reduced the risk of developing allergic diseases and atopy.[4,5]

We know that symptoms of allergic diseases can start in early life and that the in utero period might be a critical period of time in the development of allergy in childhood.[35] Here, we could show that oral application of SCFAs to mice during pregnancy and weaning phase protects against allergic airway inflammation among the offspring. Butyrate treatment of the mothers induced regulatory T cells in the lungs of the offspring indicating an epigenetic mechanism, as was previously suggested by others examining acetylation of the FOXP3 promoter.[36]

5 | CONCLUSION

Children with the highest level of SCFAs in feces early in life, especially butyrate and propionate, have a reduced risk to become sensitized to food and/or inhalant allergens, which is associated with less allergic diseases later in life. An increased exposure to butyrate or propionate, either directly via dietary intake or via an increased intake of fibers, which are fermented by the microbiota to release these SCFAs in vivo, should be further investigated in human clinical studies as potential novel strategies for allergy prevention.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

CR, ReF, RuF, CAA, RPL, and LO conceived and designed the experiments. CR, ReF, RuF, SL, ES, PW, WB, NRP, and MW performed and analyzed the experiments. CR involved in statistical analyses. CR, CC, CL, ESH, MD, EvM, CBF, AK, PK, JP, JCD, JR, CA, RPL, and LO contributed samples, reagents, materials, analysis tools and discussed the data. CR, ReF, EvM, RPL, and LO wrote the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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APPENDIX

THE PASTURE STUDY GROUP

The members of the PASTURE study group are (in alphabetical order by study center): A Hyvärinen, S Remes, M Roponen (Finland); A Chauveau, ML Dalphin, V Kaulek (France); M Ege, J Genuneit, S Illi, M Kabesch, B Schaub, P Pfefferle (Germany); G Doekes (The Netherlands).

High levels of Butyrate and Propionate in early life are associated with protection against atopy

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SUPPLEMENTARY METHODS

Allergic airway inflammation mouse model

Sample sizes were chosen based on previous experience with related experiments and all data were assessed blinded. Mice were sensitized by intraperitoneal injection of 20 µg of ovalbumin grade VI (Sigma-Aldrich, Buchs, Switzerland) emulsified in 500 µg Imject™ Alum Adjuvant (Pierce, Rockford, IL, USA) in 200 µl sterile 0.9 % isotonic sodium chloride (NaCl) on days 0, 7 and 21, followed by 20 min 1% ovalbumin grade V (Sigma-Aldrich) aerosol treatments on days 26, 27 and 28. In addition, mice received 30 µmol SCFA (Sigma-Aldrich) dissolved in 200 µl distilled water (final pH 7.5) by oral gavage daily, starting 5 days prior to the first ovalbumin injection. This SCFA dose corresponds to a diet rich in dairy products. The aliquots were stored frozen until use. Control animals received NaCl and alum injections, distilled water by oral gavage and were exposed to the OVA aerosol. The same protocol for administration of SCFAs was used for the pregnant mice. Pregnant mice were daily treated with 30 µmol SCFA starting at the day of breeding until weaning of the pubs. The pups themselves were not treated with SCFAs. Analysis of mice occurred 24 h after the last aerosol challenge. Bronchoalveolar lavage (BAL) was performed using 1 ml PBS containing 1x protease inhibitor cocktail (Roche, Mannheim, Germany). The total number of leukocytes was counted using a Neubauer counting chamber. For differential cell counts, cytospin preparations were fixed and stained with Diff-Quick (Merz & Dade AG, Dudingen, Switzerland). Macrophages, lymphocytes, eosinophils, and neutrophils were identified by standard morphologic criteria and 100-200 cells were counted per cytospin.

Lung tissue cell isolation and flow cytometric analysis

To prepare single cell suspension from lung tissue, the lung dissociation kit for mouse and a gentleMACS^{TM} system (Miltenyi, Bergisch Gladbach, Germany) were used according to the manufacture's protocol. All flow cytometric analyses were performed on the Gallios Flow Cytometer (Beckman Coulter, Brea, USA). Anti-CD3 (145-2C11) and anti-CD4 (RM4-5) antibodies were obtained from BioLegend (San Diego, USA). Anti-CD25 (PC61.5), anti-Foxp3 (FJK-16s) antibodies were obtained from eBioscience (Vienna, Austria). Cells were stained by the fixable viability dye eFlour780 (eBioscience). Cells were permeabilized with reagents from eBioscience. The gating strategy is described in Supplementary Fig. 1.

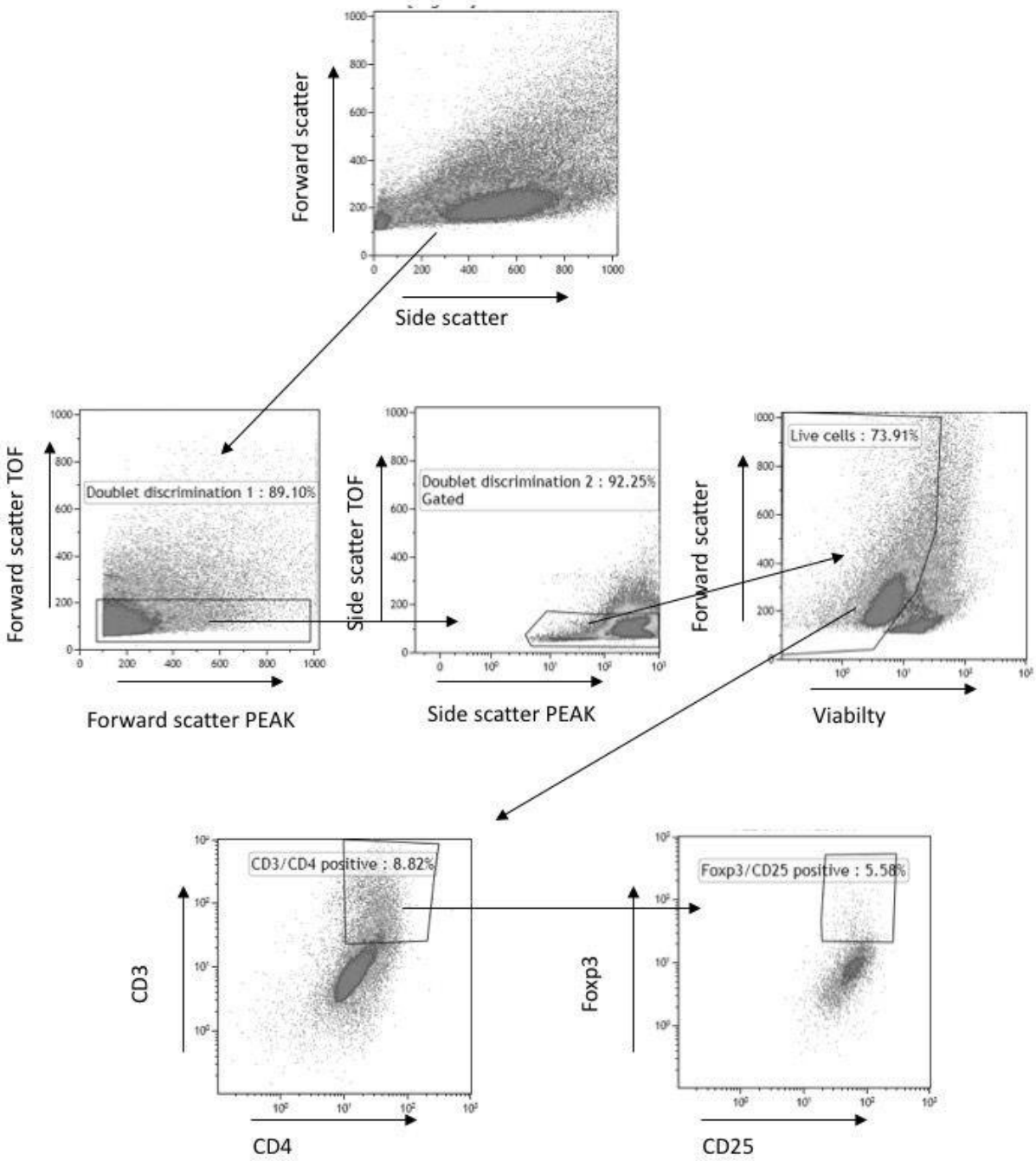
Mouse immunoglobulins

Total and ovalbumin-specific IgE was assessed in sera of mice. Total IgE was assessed using a Milliplex kit (Merck Millipore, Billerica, USA). Ovalbumin-specific IgE levels in sera were measured by ELISA coated with ovalbumin (2 µg/well) and detected with anti-IgE (1:1000 dilution; BD Bioscience, New Jersey, USA).

Lung function measurements

Mice were intubated under anesthesia and airway resistance was assessed using the FlexiVent system (SCIREQ, Montreal, Canada). Airway resistance was measured in response to increasing concentrations of methacholine (0.1-30 mg/ml; Sigma-Aldrich).

SUPPLEMENTARY FIGURES



Supplementary Fig. 1. Gating strategy for CD25/Foxp3 positive mouse lymphocytes.

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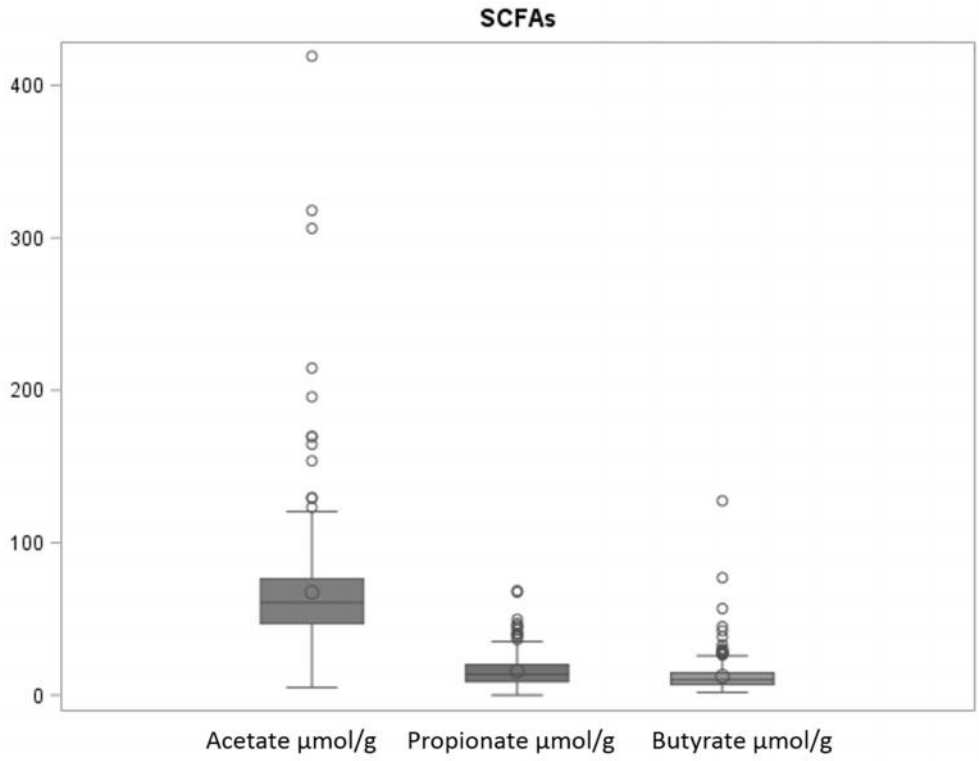
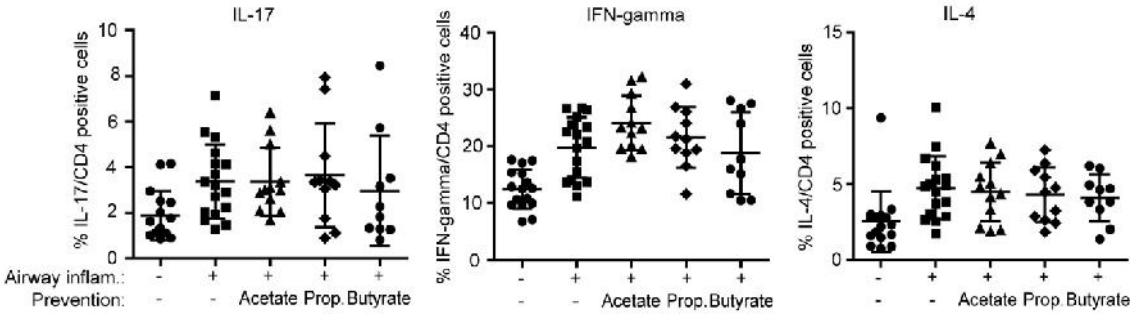


Supplementary Figure 2. Box plot of SCFAs concentrations



Supplementary Figure 3. Flow cytometry quantification of the percentage of lymphocytes (CD3+CD4+) within lung tissue that expressed IL-17, IFN-gamma and IL-4.

SUPPLEMENTARY TABLE

Supplementary Table 1. Proportion of children with atopy at 6 years of age between quartiles of SCFAs

	Butyrate percentiles								
	0-25%		**25-50%**		**50-75%**		**75-100%**		p-value
	n	%	n	%	n	%	n	%	
Inhalant sensitization at 6yrs	28/67	41.8	25/68	36.8	28/71	39.4	29/70	41.4	0.929
Food sensitization at 6yrs	27/67	40.3	27/68	39.7	24/71	33.8	24/70	34.3	0.787
Any sensitization at 6yrs	42/67	62.7	37/68	54.4	37/71	52.1	35/70	50.0	0.468

	Propionate percentiles								
	0-25%		**25-50%**		**50-75%**		**75-100%**		p-value
	n	%	n	%	n	%	n	%	
Inhalant sensitization at 6yrs	30/66	45.5	25/65	38.5	33/73	45.2	22/72	30.6	0.223
Food sensitization at 6yrs	30/66	45.5	21/65	23.6	26/73	35.6	25/72	34.7	0.414
Any sensitization at 6yrs	45/66	68.2	34/65	52.3	40/73	54.8	32/72	44.4	**0.045**

	Acetate percentiles								
	0-25%		**25-50%**		**50-75%**		**75-100%**		p-value
	n	%	n	%	n	%	n	%	
Inhalant sensitization at 6yrs	23/63	36.5	32/73	43.8	27/68	39.7	28/72	38.9	0.848
Food sensitization at 6yrs	25/63	39.7	28/73	38.4	31/68	45.6	18/72	25.0	0.077
Any sensitization at 6yrs	36/63	57.1	43/73	58.9	40/68	58.8	32/72	44.4	0.241