

ORIGINAL ARTICLE

Effect of lactose on gut microbiota and metabolome of infants with cow's milk allergy

Ruggiero Francavilla¹, Maria Calasso², Laura Calace¹, Sonya Siragusa², Maurice Ndagijimana^{3,4}, Pamela Vernocchi⁴, Luigia Brunetti¹, Giuseppe Mancino¹, Giuseppe Tedeschi¹, Elisabetta Guerzoni⁴, Flavia Indrio¹, Luca Laghi⁴, Vito L. Miniello¹, Marco Gobetti² & Maria De Angelis²

¹Department of Pediatrics, University of Bari Aldo Moro, Bari, Italy; ²Department of Biologia e Chimica Agro-Forestale ed Ambientale, University of Bari Aldo Moro, Bari, Italy; ³Department of Agricultural Food & Nutritional Science (AFNS), University of Alberta, Alberta, Canada; ⁴Department of Food Science, Alma Mater Studiorum, University of Bologna, Bologna, Italy

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Correspondence

Ruggiero Francavilla, Clinica Pediatrica
"B. Trambusti" Piazza Giulio Cesare,
11 – Policlinico, Bari, Italy.
Tel.: +39 0805592847
Fax: +39 080 5478911
E-mail: rfrancavilla@me.com

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Abstract

Allergic infants have an unusual gastrointestinal microbiota with low numbers of *Bifidobacterium*/Lactobacilli and high levels of *Clostridium*, staphylococci and *Escherichia coli*. Hydrolyzed formula used to treat these infants is deprived of lactose that instead may influence the gut microbial composition. The aim of the present study is to investigate the influence of lactose on the composition of the gut microbiota and metabolome of infants with cow's milk allergy. Infants prospectively enrolled received an extensively hydrolyzed formula with no lactose for 2 months followed by an identical lactose-containing formula for an additional 2 months. Healthy, age-gender-matched infants were used as controls. The following determinations were performed before and after the introduction of lactose in the diet: enumeration of cells present in the feces using FISH, counts of viable bacterial cells and gas-chromatography mass spectrometry/solid-phase microextraction analysis. The addition of lactose to the diet significantly increases the counts of *Bifidobacteria* and lactic acid bacteria ($p < 0.01$), decreases that of *Bacteroides/clostridia* ($p < 0.05$) reaching counts found in healthy controls; lactose significantly increases the concentration of total short-chain fatty acids ($p < 0.05$). The addition of lactose to an extensively hydrolyzed formula is able to positively modulate the composition of gut microbiota by increasing the total fecal counts of *Lactobacillus*/*Bifidobacteria* and decreasing that of *Bacteroides*/*Clostridia*. The positive effect is completed by the increase of median concentration of short chain fatty acids, especially for acetic and butyric acids demonstrated by the metabolomic analysis.

Gut microorganisms contribute to diverse mammalian processes. Recent evidences have demonstrated the influence of gut microorganisms in the progression of human diseases. Therefore, it is possible that differences in the gut microbiota may ultimately contribute to significant differences in the health of the host, even in the absence of a specific pathogen (1). The gut microbiota have been linked to the risk of gastrointestinal diseases such as inflammatory bowel diseases, irritable bowel syndrome, celiac disease and allergy (2).

The prevalence of atopic diseases, including eczema and asthma, has increased worldwide, and epidemiologic evidence (3) suggests that modulation of immune response mechanisms in the gut can directly affect the development of allergic

disease and influences the development of tolerance (4–6). Cow's milk protein allergy (CMA) has become a common disease in infancy, with a prevalence ranging from 1.6% to 7.5% (7). Formulas based on extensively hydrolyzed proteins or amino acids have been shown to be effective for the treatment of CMA and are the standard formulas for infants who cannot be breast-fed (7). However, the elimination of some components of milk from the diet of infants may lead to an increased risk of growth impairment (8) and dysbiosis with possible implications for future health (9).

Dysbiosis during the early post-natal period may further pre-dispose individuals for later inflammatory, immune, and allergic disorders as the gut microbiota is crucial for the

appropriate expression and function of regulatory T cells (10). Species belonging to the Bifidobacteria and Lactobacillus genera are considered to be the most important health-beneficial bacteria for human hosts, whereas staphylococci and clostridia are considered to be potentially pathogenic.

The aim of the present study was to investigate the influence of lactose on the fecal microbiota and gastrointestinal metabolome in infants with CMA.

Patients and methods

Study population

Infants with CMA were considered eligible for enrolment if they were born at term, by vaginal delivery and with normal gestational age. Exclusion criteria were partial breast-feeding, malformations, congenital diseases and treatment with antibiotics/probiotics/prebiotics in the previous month.

Study design

Prospective clinical trial carried out in a single center. At their first visit, infants with suspected CMA underwent a complete allergy work-up. Infants positive for the skin prick test (3 mm wheal diameter cutoff) and/or with measurable cow milk-specific IgE (0.35 kUI/l cutoff; ImmunoCAP, Phadia, Sweden) were scheduled for a diagnostic double blind, placebo-controlled food challenge (DBPCFC) to confirm the diagnosis (7).

Infants with confirmed CMA entered a two-phase trial (Fig. 1): in phase 1, infants received an hydrolyzed and ultra-filtered whey protein formula (eHF) without lactose (Alfaré, Nestlé, Switzerland) for 2 months (CMA-NL) followed in phase 2 by the administration for an additional 2 months, of an identical eHF containing 3.8% lactose (Althera, Nestlé, Switzerland) (CMA-L). The composition of both formulas complied with the guidelines of CODEX and the European

Regulation for Infant Formulas (ECC 1996) and both are routinely used in infants with CMA. Infants having solids during the study period were asked to avoid lactose-containing foods.

Anthropometric measurements (body weight, length and body mass index) were measured and symptoms of CMA (skin, respiratory and GI tract manifestations) recorded at entry and at the end of the two phases.

Healthy, age- and gender-matched breast-fed infants with no history of CMA and those eating an unrestricted diet were recruited among infants of the staff and used as healthy controls (HC).

The study was in accordance with the ethical standards of the Institutional Committee and written consent was obtained from the parents.

Collection of fecal samples

Fecal samples were obtained from each infant in triplicate at the end of the lactose-restricted and lactose-containing diet periods. Similarly, triplicate fecal samples were collected from HC on two occasions with a 2-month interval. After collection, feces (ca. 15 g), contained in a sterile plastic box, were immediately mixed (1:1 wt/wt) with Amies Transport medium (Oxoid Ltd, Basingstoke, Hampshire, UK) under anaerobic conditions (AnaeroGen; Oxoid Ltd). The samples were either immediately subjected to analysis (for plate counts) or frozen at -80°C (for DNA extraction).

Fluorescence *in situ* hybridization (FISH)

Fecal samples were managed as previously described (11); the enumeration of cells present in the fecal samples was carried out using FISH, with fluorescein isothiocyanate (FITC)-labeled oligonucleotide probes (12) targeting the main bacterial groups colonizing the human GI (Table S1). Total cell

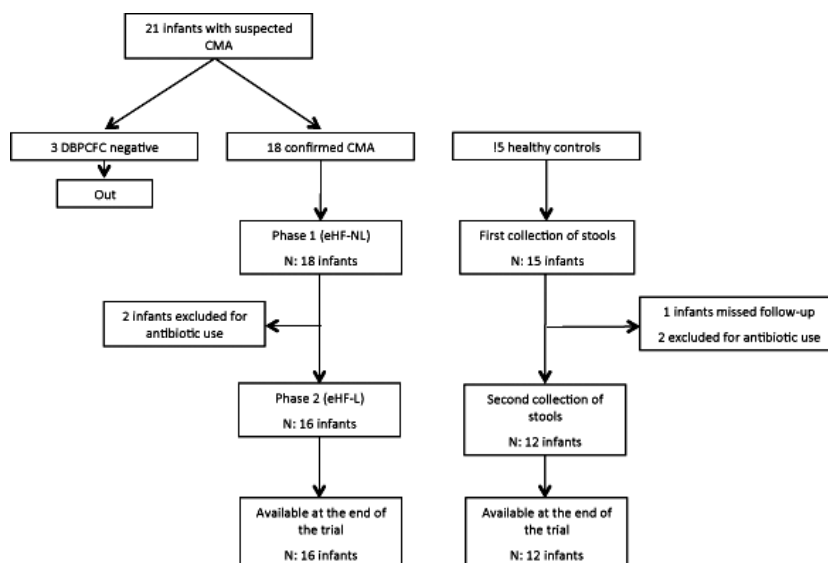


Figure 1 Participants involved in the trial from the assessment for eligibility through follow-up.

numbers were determined using the nucleic acid stain 4', 6-diamidino-2-phenylindole (DAPI) (13).

Enumeration of cultivable bacteria

Counts of viable bacterial cells were performed as described by Macfarlane et al. (14). The following selective media (Oxoid Ltd) were used: De Man–Rogosa–Sharpe agar at pH 5.6 (lactobacilli); Beerens agar (bifidobacteria); Baird–Parker (staphylococci and micrococci); Blood Azide agar (enterococci); Wilkins–Chalgren agar (total anaerobes); Wilkins–Chalgren agar plus GN selective supplements (*Bacteroides*, *Porphyromonas* and *Prevotella*); Reinforced Clostridial Medium supplemented with 8.0 mg/l novobiocin and 8.0 mg/l colistin (*Clostridium*); MacConkey agar No. 2 (enterobacteria); and nutrient agar (total anaerobes) (15).

Gas-chromatography mass spectrometry/solid-phase micro extraction (GC-MS/SPME) analysis

A carboxen-polydimethylsiloxane-coated fiber (85 µm) and a manual SPME analysis holder (Supelco Inc., Bellefonte, PA, USA) were used. Fecal samples (3 g) were placed into 10-ml glass vials and supplemented with 10 µl of 4-methyl-2-pentanol (final concentration of 4 mg/l) as the internal standard. The SPME fiber was exposed to each sample for 40 min and then inserted into the injection port of the GC for 5 min. GC-MS analyses were carried out on an Agilent 7890A gas-chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 5975C mass-selective detector operating in electron-impact mode (ionization voltage 70 eV). A Supelcowax 10 capillary column (60 m length, 0.32 mm ID) was used (Supelco). Quantitative data for the identified compounds were obtained as previously described (16).

¹H Nuclear magnetic resonance (NMR) spectroscopy analysis

The water-soluble fraction of the fecal samples was analyzed using ¹H NMR spectroscopy. Samples were thoroughly homogenized by vortex-mixing with 400 µl of cold deuterium oxide (D₂O) at pH 7.4 that contained 1 mM sodium 3-(trimethylsilyl) propionate-2,2,3,3-d₄ (TSP) as the internal standard. The analysis was carried out as described by Ndagijimana et al. (17). The spectra were referenced to the TSP peak and then digitized over the range of 0.5–10 ppm. By using R scripts developed in-house, the residual water signal region (4.5–5.5 ppm) was excluded from the subsequent computations.

Statistical analysis

All data were obtained from at least three independent replicates. Analysis of variance (ANOVA) was carried out on the transformed data followed by separation of means with Tukey's HSD. Letters indicate significant differences between groups ($p < 0.05$) determined by Tukey's test. Principal component analysis (PCA) of the median values of volatile organic metabolites and canonical discriminant analysis of

principal coordinates (CAP) were performed (17, 18). CAP was preferred to the more common canonical discriminant analysis because it does not assume any specific distribution of the data, which provides more robust results in the case of a small number of samples. The hypothesis of no significant difference in the multivariate location among groups was tested using the trace statistic based on 9999 permutations (17). The statistical software Statistica for Windows (Statistica 6.0 for Windows 1998; StatSoft, Vigonza, Italia) was used.

Results

Study population

Twenty-one infants with symptoms of CMA and 15 HC were recruited. Allergic symptoms reported immediately after milk ingestion were generalized: urticaria ($n = 10$), vomiting ($n = 6$), wheezing ($n = 6$) and eye/lip edema ($n = 3$). Ten infants presented with two or more symptoms. CMA was confirmed in 18 (86%) of them were enrolled. Five infants (2 CMA and 3 HC) were excluded during the trial. Figure 1 shows the flow of participants from the assessment for eligibility through follow-up; baseline characteristics are shown in Table 1. Allergic symptoms were resolved in all infants under eHF within 2 wk.

Molecular analysis of the bacterial community

Based on DAPI and universal bacterial primers, the total bacterial cell numbers (log count per gram of feces) did not differ between the CMA-NL, CMA-L and HC infants.

In detail, as compared to CMA-L infants, we found in CMA-NL infants (i) significantly lower counts of presumptive *Bifidobacteria* and lactic acid bacteria (LAB: *Lactobacillus*/*Enterococcus* and *Streptococcus*/*Lactococcus*) ($p < 0.05$) and

Table 1 Demographic characteristics and baseline symptoms of infants with cow's milk protein allergy (CMA) and healthy infants (HC)

	CMA median (interquartile range)*	HC median (interquartile range)*
Number of infants	16	12
Age at entry (months)	7.5 (7.2–10.1)	6.9 (6.5–10.4)
Sex (M/F)	10/8	7/5
Gestational age	38.6 (37.5–40)	39.1 (38.1–40.2)
Birth weight	2900 (2600–3050)	3000 (2710–3100)
BMI (centile)	50 (25–75)	50 (25–75)
Milk SPT mean diameter	5.8 (5.5–6.2)	Not available
Cow's milk-specific IgE	2.6 (2.2–2.9)	Not available
Urticaria	10	0
Vomiting	6	0
Wheezing	6	0
Lip/eye edema	3	0

*There was no difference in demographic data between infants who entered the study and those who completed the study.

(ii) significantly higher counts of *Atopobium*, presumptive *Bacteroides/Prevotella*, clostridia and sulfate-reducing bacteria ($p < 0.05$). The infants with CMA-L and HC infants showed similar median numbers of LAB. Data are reported in Table 2 (Table S1 and Fig. S1).

Enumeration of cultivable bacteria

Overall, the results confirmed the data obtained by FISH analysis (Fig. 2). Specifically, the number of presumptive *Bifidobacteria* were significantly higher in HC than in CMA infants ($p < 0.05$), although median values in the CMA-L were significantly higher than in the CMA-NL infants (6.33 log vs. 5.23 log CFU/g; $p < 0.03$). The CMA-L and HC infants showed similar median values of LAB (8.67 and 8.89 log CFU/g, respectively), while the median count was significantly lower in CMA-NL infants (6.5 log CFU/g)

($p < 0.03$). Significantly higher counts of presumptive *Bacteroides* (8.22 and 7.5 log CFU/g for the CMA-NL and CMA-L infants, respectively) and *Clostridium* (8.11 and 6.70 log CFU/g for the CMA-NL and CMA-L infants, respectively) were found in fecal samples from CMA infants than in HC (7.05 and 5.55 log CFU/g for *Bacteroides* and *Clostridium*, respectively; $p < 0.05$). The lowest counts of presumptive staphylococci and micrococci were found in fecal samples of the CMA-NL infants, while no differences were found between the CMA-L and HC infants.

Gas-chromatography mass spectrometry/solid-phase micro extraction metabolite profiling of feces

Fifty volatile organic metabolites were identified from fecal samples and grouped according to their chemical classes (Table 3). Compared to the HC infants, the total median

Table 2 Molecular analysis of the bacterial community by fluorescence *in situ* hybridization (FISH) of fecal samples from healthy infants (HC) and infants with cow's milk protein allergy who were fed a diet without (CMA-NL) and with lactose (CMA-L)

Species	CMA-NL median (range)	CMA-L median (range)	HC median (range)
<i>Bifidobacteria</i>	6.85 (4.35–7.12) ^c	7.56 (5.77–9.81) ^b	9.08 (7.44–10.27) ^a
<i>Bacteroides/Prevotella</i>	9.2 (7.05–10.67) ^a	8.15 (6.11–9.03) ^b	7.04 (5.26–8.09) ^c
<i>Eubacterium rectale/Clostridium coccoides</i>	8.9 (7.43–9.87) ^a	7.9 (5.42–8.26) ^b	7.5 (5.66–8.28) ^b
<i>Lactobacillus/Enterococcus</i>	8.5 (6.72–9.11) ^b	9.1 (8.51–9.98) ^a	9.2 (8.73–10.20) ^a
<i>Streptococcus/Lactococcus</i> group	3.5 (2.0–4.21) ^b	5.6 (3.54–6.65) ^a	6.2 (5.42–6.78) ^a
<i>Escherichia coli</i>	8.5 (7.66–9.05) ^a	7.2 (6.52–8.01) ^b	6.5 (5.69–7.88) ^c
Sulfate-reducing bacteria (SRB)	8.8 (6.55–9.94) ^a	7.5 (6.22–7.56) ^b	7.00 (6.05–7.81) ^b
<i>Atopobium</i> group	9.5 (8.87–9.94) ^a	8.0 (7.40–8.72) ^b	6.6 (5.50–7.12) ^c
<i>Coriobacterium</i> group	7.6 (6.23–8.01) ^a	7.8 (6.05–8.41) ^a	7.5 (5.87–8.07) ^a
<i>Clostridium histolyticum</i>	5.7 (3.42–6.51) ^a	5.8 (3.31–6.43) ^a	5.4 (3.67–6.58) ^a
<i>Clostridium lituseburens</i>	5.5 (3.72–6.43) ^a	5.4 (3.56–6.81) ^a	5.5 (4.01–6.25) ^a

Data are the means of three independent experiments ($n = 3$) for each infant. ^{a–c}Medians within a row with different superscript letters are significantly different ($p < 0.05$).

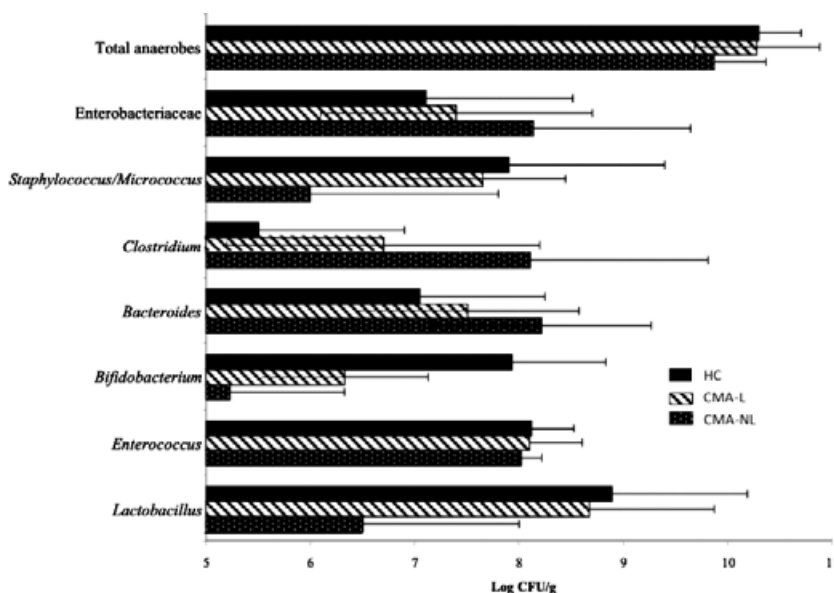


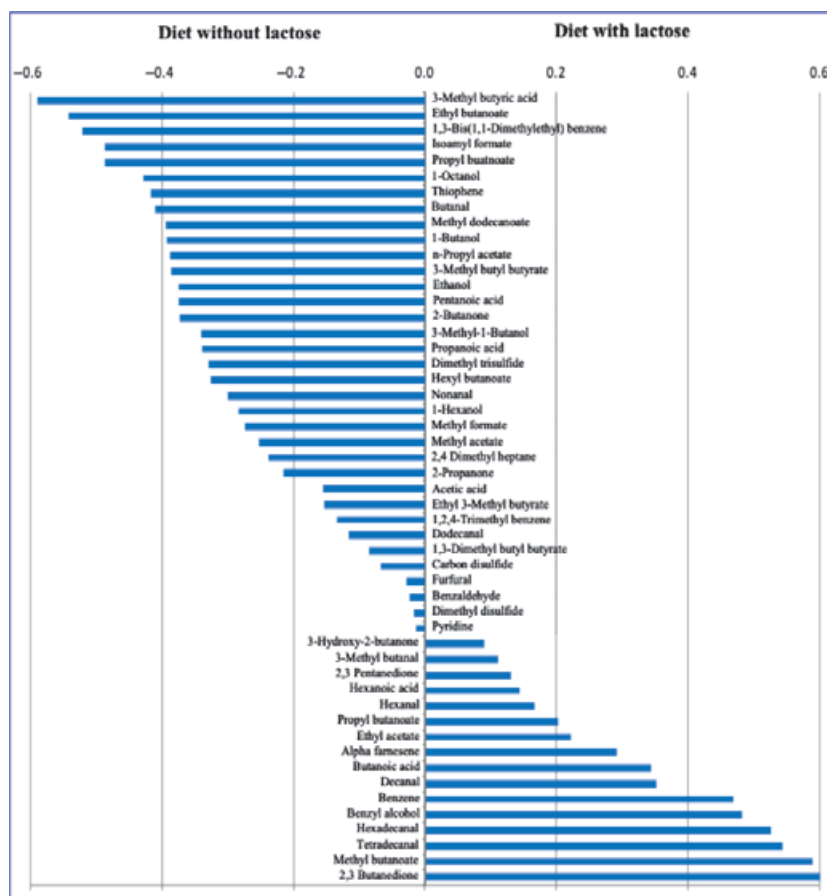
Figure 2 Median values and standard deviations of cultivable cells (log CFU/g) of the main microbial groups in the fecal samples. Data are shown for infants with cow's milk allergy (CMA) who were fed diets with (CMA-NL; $n = 16$) and without lactose (CMA-L; $n = 16$); data are also shown for healthy infants (HC; $n = 12$). Data are the means of three independent experiments ($n = 3$ for each infant).

Table 3 Median values and ranges of the concentration (ppm) of volatile organic compounds* of fecal samples from healthy infants (HC) and infants with cow's milk protein allergy who were fed diets without (CMA-NL) and with lactose (CMA-L)

Chemical class	CMA-NL median (range)	CMA-L median (range)	HC median (range)
Esters	627.1 (0.1–3587.8) ^a	343.2 (0.5–2468.2) ^b	332.3 (41.1–714.7) ^b
Ketones	170.1 (0.5–863.7) ^a	147.7 (1.6–656.0) ^b	127.7 (9.8–464.4) ^c
Alcohols	567.0 (1.2–2402.7) ^a	337.5 (4.7–1527.1) ^b	209.3 (51.2–848.0) ^c
Aldehydes	159.9 (2.7–2736.7) ^a	79.0 (0.8–472.9) ^b	32.0 (4.5–93.9) ^c
Sulfur compounds	483.2 (82.6–1015) ^c	525.4 (51.4–859.6) ^b	963.3 (603.4–1918.4) ^a
Hydrocarbons	48.8 (1.1–170.0) ^b	47.3 (0.0–204.9) ^b	74.9 (17.6–123.2) ^a
Short chain fatty acids	222.0 (5.9–1063.0) ^c	316.4 (4.5–1353.9) ^b	546.2 (72.0–1810.5) ^a

*Additional information is available in the Supporting Information. Data are the means of three independent experiments (n = 3) for each infant. ^{a–c}Medians within a row with different superscript letters are significantly different (p < 0.05).

Figure 3 Canonical discriminant analysis of principal coordinates (CAP) loading coefficient plot of the volatile organic metabolites significantly affected by consumption of lactose in infants allergic to cow's milk protein (CMP; n, 16). Data are the means of three independent experiments (n = 3 for each infant). The variables with negative values.



value of esters, ketones, aldehydes and alcohols were higher for CMA-NL infants, while no differences were found between the CMA-L and HC infants (Fig. 3).

The median concentrations of total short chain fatty acids (SCFAs) were significantly higher in CMA-L and HC infants as compared to CMA-NL infants (p < 0.03; Table S2). Major differences were found for acetic and butyric acids. The differences between the metabolic profiles of the CMA-NL and CMA-L infants were confirmed by the CAP analysis. To identify differences between the fecal samples of the CMA-NL, CMA-L and HC infants, the volatile organic

metabolite data were also elaborated through PCA (Fig. S3). The three groups of infants were distributed in three different zones; however, the samples from the CMA-L and HC infants were the most similar, while the samples from the CMA-NL and HC infants showing the least similarity.

1H NMR metabolite profiling of feces

Overall, 1H NMR results confirmed the GC-MS/SPME results and identified additional metabolites (Table 4): (i) median values of lactic acid and threonine were higher in the

Table 4 Median values and ranges of concentration (ppm) of metabolites* of fecal samples from healthy infants (HC) and infants with cow's milk protein allergy infants who were fed diets without (CMA-NL) and with lactose (CMA-L)

Compounds	CMA-NL median (range)	CMA-L median (range)	HC median (range)
Pyruvic acid	7.34 (4.58–10.10) ^b	3.53 (0.96–6.1) ^b	16.54 (8.79–24.29) ^a
Lactic acid	3.64 (2.19–5.09) ^b	5.32 (2.79–7.85) ^a	4.61 (0–9.47) ^a
Uridine	0.72 (0.44–1.0) ^b	0.95 (0.49–1.41) ^a	0.41 (0.19–0.63) ^c
Histidine	0.80 (0.61–0.99) ^b	1.07 (0.57–1.57) ^a	0.45 (0.21–0.69) ^c
Tyrosine	0.57 (0.32–0.82) ^b	0.79 (0.33–1.25) ^a	0.26 (0.16–0.36) ^c
Threonine	1.54 (0.99–2.09) ^b	2.42 (0.66–4.18) ^a	2.22 (0.38–4.06) ^a
Methionine	3.52 (0–7.32) ^b	7.72 (0–15.72) ^a	3.01 (0–7.34) ^b
Proline	3.33 (1.78–4.88) ^b	5.27 (2.47–8.07) ^b	7.13 (4.38–9.88) ^a
Trimethylamine-N-ox	4.15 (2.15–6.15) ^b	6.76 (1.73–11.79) ^a	3.30 (2.01–4.59) ^c
Arginine/histidine	2.56 (1.23–3.89) ^b	3.66 (2.2–5.12) ^a	1.39 (0.8–1.98) ^c
Valine / isoleucine	23.30 (9.41–37.19) ^a	16.29 (6.96–25.62) ^b	23.93 (12.68–35.18) ^a
Phenylalanine	1.71 (1.07–1.81) ^b	3.17 (1.8–4.54) ^a	1.89 (0.33–3.45) ^b
γ -amino-butyric acid/lysine	1.35 (0.9–1.8) ^b	2.06 (0.87–3.25) ^a	1.30 (0.73–1.87) ^b

*Data are the means of three independent experiments (n = 3) for each infant. ^{a–c}Medians within a row with different superscript letters are significantly different (p < 0.05). Additional information is available in the Supporting Information.

CMA-L and HC infants as compared to the CMA-NL infants (p < 0.05), (ii) the highest concentrations of pyruvate and proline were found in the HC infants (p < 0.04), (iii) median values of uridine, histidine, tyrosine, methionine, trimethylamine-N-ox and arginine/histidine were lower in the fecal samples of HC (p < 0.05) being highest in the CMA-L infants (p < 0.02; except for methionine, p = NS).

For all the analyses, no difference was found in the two determinations of HC.

Discussion

The present study shows that lactose is able to modify the gut microbiota of allergic infants by significantly increasing the total fecal count of *Bifidobacteria* and *Lactobacilli* and decreasing the numbers of *Bacteroides*, *Prevotella* and *Clostridia*. In turn, the modification of the GI microbiota is associated with an increase in the median concentrations of total SCFA and a decrease in ketones and alcohols.

Immediately after birth, all mammals enter a life-long process of colonization by foreign microorganisms; host–bacterial associations develop into beneficial relationships, creating an environment capable of promoting health (1). Therefore, it is conceivable that alterations in gut microbial composition may lead to altered immune responses (and disease).

Recent reports have described pathogenic GI microbial communities in infants with infantile colic (19) and in patients with inflammatory bowel diseases (20), celiac disease (21), irritable bowel syndrome (22) and allergy (4–6). Compared to non-allergic, allergic infants have an unusual GI microbiota with low numbers of *Bifidobacterium*/Lactobacilli and high levels of *Clostridium*, staphylococci and *E. coli* (4–6) and the association between the intestinal microflora and allergy is now widely accepted (3).

Similar microbiologic data are reported in the present study; indeed, FISH analysis and culture-dependent methods were concordant in showing that: (i) fecal samples from

CMA infants (on lactose-restricted diet) contain lower numbers of *Bifidobacterium* and LAB as compared to HC and (ii) the addition of lactose to the diet is followed by an increase in *Bifidobacterium* and LAB with a parallel decrease of *Bacteroides/Prevotella* and *Clostridium*. The modification of the dominant populations of the main fecal microbial groups in favor of *Bifidobacterium* and Lactobacilli has been shown to protect against atopy (23). Therefore, manipulation of the intestinal microbiota during infancy may provide an approach to help preventing/treating allergic conditions. Based on our data, the addition of lactose to eHF formula may be an efficient and safe method to positively influence the composition of the GI microbiota.

The strength of the present report is the combined study of the microbiota and metabolome. The concept of human metabolome encompasses the idea of microbial and metabolic cooperation and aims to systematically examine the changes of metabolites in biological fluids in response to different stimuli (24). The use of profiling techniques allows simultaneous monitoring of changes in metabolites with diverse chemical origins and significance. The combination of GC-MS/SPME and 1H NMR metabolic profiles with CAP analysis provides a holistic view of metabolic perturbations as a response to lactose intake in CMA infants. Our main finding is the increased concentrations of SCFA in fecal samples of HC and CMA-L as compared to samples of CMA-NL infants.

An abundance of SCFA in the feces indicates a production by the microflora overwhelming the absorption rate. The beneficial effect of SCFAs are well known (25); colonic epithelia derive 60–70% of their energy supply from SCFAs, acetate constitutes the primary route, whereby the body obtains energy from carbohydrates that are not digested/absorbed in the small bowel; butyrate stimulates the growth of enterocytes and modulates the expression of signaling genes, which may control the gut barrier and the mucosal immune system, SCFA protect intestinal epithelial cells from

mechanical, chemical and microbial damage by enhancing the production of MUC-2 by intestinal epithelial cell and have the potential to indirectly reduce the risk of several acute and chronic diseases by acting as stimulating substrates for potentially health-promoting bacteria (e.g., lactobacilli and bifidobacteria) that are able to inhibit the proliferation of pathogenic/harmful bacteria through colon acidification, competition for occupation of colonization sites and nutrients, synthesis of antimicrobial compounds and stimulation of the immune system (26). Our results allow us to hypothesize that lactose in the diet could promote SCFA-producing bacteria.

We are aware of the limits of the present study. Firstly, the small sample size of the study that, however, is secondary to the strict entry criteria and is balanced by the complexity and the costs of the different methods that we have performed and that may represent a starting point for future studies and power calculation. Secondly, the possibility that the modification of the infant's diet during the study period might have created a bias; however, this risk is reduced by the demonstration that microbiota and metabolome of HC (age-matched) remained stable across the two determinations. Finally, the study design may give rise to some criticism because a cross-over design or the addition of a third group (first lactose-containing formula followed by lactose-free formula) might have been more precise; however, it would have complicated the enrolment, increased the drop-out (CMA-L would have been less likely to accept a less palatable formula) and costs.

The possible role of lactose as prebiotic derives from studies in animals showing that lactose stimulates the growth of bifidobacteria (27). In infants, lactose is not completely hydrolyzed in the small intestine, it reaches the colon where it is salvaged by bacteria, resulting in the preferential growth of bifidobacteria and lactobacilli. It is well known that the microbiota can have profound immunomodulatory effects on the host and our finding supports the role of lactose as conditional prebiotic and its ability to

restore a healthy indigenous microbiota with their metabolites. The main clinical implication of our study is demonstration that the elimination of lactose in infant's diet is disadvantageous for the development of healthy gut microbiota; however, further prospective studies are needed to investigate if lactose may play a role in the anticipation of the acquisition of tolerance.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Median values and standard deviations of the microbial count (log cells/g feces) determined by fluorescence *in situ* hybridization (FISH) using group-specific fluorescent probes. Data are shown for healthy infants (HC) and for infants with cow's milk protein allergy before (CMA-NL) and after (CMA-L) consumption of lactose-containing diets. Data are the means of three independent experiments (n = 3).

Figure S2. Score plot of the three principal components after principal component analysis (PCA) of volatile organic metabolites of cow's milk protein-allergic infants under diets without (CMA-NL) and with lactose (CMA-L); data for healthy infants (HC) are also shown.

Table S1. Primers and conditions for fluorescence *in situ* hybridization (FISH) analysis.

Table S2. Concentration (ppm) of volatile organic compounds (VOCs) of fecal samples from healthy infants (HC) and infants with cow's milk protein allergy before (CMA-NL) and after (CMA-L) consumption of lactose-containing diets.

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1 **SUPPLEMENTARY MATERIAL (Tables)**

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5 **Table 1S. Primers and conditions for fluorescence in situ hybridization (FISH) analysi**

6 **Table 2S. Concentration (ppm) of volatile organic compounds (VOCs) of fecal samples from healthy infants (HC) and infants with cow's milk protein**
 7 **allergy before (CMA–NL) and after (CMA–L) consumption of lactose-containing diets**

Chemical class	CMA-NL		CMA-L		HC	
	Median	Range	Median	Range	Median	Range
Esters						
Methyl-acetate	5.0 ^a	0.0-38.0	5.1 ^a	0.0-16.6	1.3 ^b	0.0-4.6
Ethyl-acetate	10.6 ^b	0.0-30.6	52.9 ^a	0.5-411.0	5.6 ^c	1.6-12.4
Propyl-acetate	6.1 ^a	0.0-22.9	2.9 ^b	0.0-8.9	1.8 ^c	0.0-4.7
Methyl formate	140.1 ^a	0.0-839.1	6.5 ^b	0.0-161.1	1.7 ^b	0.0-6.4
Methyl butanoate	11.1 ^b	0.0-53.0	41.3 ^a	0.0-206.6	5.8 ^c	0.0-30.5
Ethyl butanoate	322.7 ^a	0.0-1476.2	178.5 ^b	0.0-1243.1	165.6 ^c	38.6-434.1
Isoamyl formate	4.3 ^a	0.0-14.5	3.1 ^a	0.0-29.8	0.0 ^b	0.0-0.0
Propyl butanoate	23.1 ^c	0.0-118.4	30.7 ^b	0.3-263.5	36.5 ^a	0.0-154.9
1,3-Dimethylbutyl butyrate	10.7 ^a	0.0-96.0	10.4 ^a	0.0-62.1	7.8 ^b	0.0-42.0
3-Methylbutyl butyrate	54.7 ^a	0.0-574.7	1.8 ^b	0.0-5.4	4.4 ^b	0.1-14.1
Hexyl butanoate	4.5 ^a	0.0-14.9	4.6 ^a	0.0-38.2	0.4 ^b	0.0-1.8
Methyl dodecanoate	27.7 ^a	0.1-262.1	3.0 ^b	0.0-7.4	1.4 ^b	0.0-9.2
Ethyl 3-methylbutyrate	6.5 ^a	0.0-47.4	2.4 ^b	0.0-14.5	0.0 ^c	0.0-0.0
Total esters	627.1 ^a	0.1-3587.8	343.2 ^b	0.5- 2468.2	332.3 ^b	41.1- 714.7

8 **Table 2S.** continued

Chemical class	CMA- NL	CMA-L	HC			
	Median	Range	Median	Range	Median	Range
Ketones						
2-Propanone	6.1 ^a	0.0-18.7	5.0 ^a	0.0-15.9	3.6 ^b	0.8-16.5
2-Butanone	121.4 ^a	0.0-691.8	59.4 ^b	0.0-329.5	31.3 ^c	0.5-236.1
2,3-Butanedione	11.5 ^c	0.5-35.8	37.5 ^b	1.3-151.2	47.5 ^a	0.6-88.0
2,3-Pentanedione	2.2 ^a	0.0-18.0	2.8 ^a	0.0-11.5	2.7 ^a	0.2-7.8
3-Hydroxy-2-butanone	28.9 ^b	0.0-99.4	43.0 ^a	0.3-147.9	42.6 ^a	7.7-116.0
Total ketones	170.1 ^a	0.5- 863.7	147.7 ^b	1.6 – 656.0	127.7 ^c	9.8- 464.4
Alcohols						
1-Butanol	28.0 ^a	0.0-155.0	27.4 ^a	0.0-82.8	17.5 ^b	0.0-130.0
3-Methyl-1-butanol	75.3 ^a	0.0-464.5	29.1 ^b	0.0-193.9	12.1 ^b	0.3-48.1
1-Hexanol	19.1 ^a	0.0-143.8	16.8 ^a	0.0-97.9	1.5 ^b	0.3-4.6
1-Octanol	5.5 ^b	0.0-25.6	3.3 ^b	0.0-12.4	33.8 ^a	0.0-267.7
Benzyl alcohol	0.9 ^b	0.0-2.9	4.2 ^a	0.0-30.2	0.5 ^b	0.0-1.8
Ethanol	438.2 ^a	1.2-1610.9	256.7 ^b	4.7-1109.9	143.9 ^c	51.2-395.8
Total alcohols	567.0 ^a	1.2- 2402.7	337.5 ^b	4.7 - 1527.1	209.3 ^c	51.2 – 848.0

9 **Table 2S.** continued

Chemical class		CMA-NL		CMA-L		HC	
	Median		Range	Median	Range	Median	Range
Aldehydes							
2-Methyl-butanal		3.4 ^b	0.0-11.4	7.5 ^a	0.0-53.4	4.4 ^b	0.0-25.3
Butanal		38.5 ^a	0.1-353.7	1.2 ^b	0.0-3.0	0.0 ^b	0.0-0.1
Hexanal		6.3 ^a	0.0-15.9	9.6 ^a	0.0-50.0	0.6 ^c	0.0-1.6
Furfural		36.0 ^a	0.0-379.8	3.8 ^b	0.0-13.4	1.6 ^b	0.0-5.0
Decanal		9.0 ^a	1.1-15.1	9.7 ^a	0.4-44.4	2.5 ^b	0.5-6.6
Dodecanal		43.1 ^a	0.7-181.8	22.9 ^b	0.0-184.2	2.7 ^c	1.0-6.1
Tetradecanal		2.5 ^b	0.0-14.3	6.0 ^a	0.0-52.4	2.2 ^b	0.0-6.6
Hexadecanal		3.1 ^b	0.9-9.9	5.5 ^a	0.0-26.6	3.2 ^b	0.0-7.8
Nonanal		8.2 ^a	0.0-38.4	6.0 ^a	0.0-19.1	8.8 ^a	1.5-16.5
Benzaldehyde		5.8 ^a	0.0-31.8	6.8 ^a	0.4-26.4	6.4 ^a	1.5-18.3
Total Aldehydes		159.9 ^a	2.7 – 2736.7	79.0 ^b	0.8 – 472.9	32.0 ^c	4.5- 93.9
Sulfur compounds							
Carbon disulfide	467.1 ^c		81.1-961.9	514.3 ^b	51.4-815.7	930.4 ^a	603.4-1817.4
Dimethyl	12.8 ^b		1.5-41.2	8.4 ^c	0.0-23.1	26.3 ^a	0.0-80.0
disulfide							
Dimethyl	3.3 ^b		0.0-11.9	2.7 ^b	0.0-20.8	6.6 ^a	0.0-21.0
trisulfide							
Total sulfur	483.2 ^c		82.6 -1015	525.4 ^b	51.4 – 859.6	963.3 ^a	603.4 – 1918.4
compounds							

10 **Table 2S.** continued

Chemical class	CMA-NL		CMA-L		HC	
	Median	Range	Median	Range	Median	Range
Hydrocarbons						
2,4-Dimethyl-heptane	5.2 ^b	0.0-16.9	7.1 ^a	0.0-15.2	7.7 ^a	1.6-14.3
Benzene	4.0 ^a	0.0-24.1	10.3 ^b	0.0-56.6	2.5 ^c	0.0-5.5
1.2.4-Trimethyl benzene	1.2 ^b	0.0-4.7	2.4 ^a	0.0-7.6	1.4 ^b	0.0-8.9
1.3-Bis(1.1-demethyl-ethyl)-benzene	38.4 ^b	1.1-124.3	27.5 ^c	0.0-125.5	63.3 ^a	16.0-94.5
Total Hydrocarbons	48.8 ^b	1.1 - 170.0	47.3 ^b	0.0 - 204.9	74.9 ^a	17.6 -123.2
Short chain fatty acids (SCFA)						
Acetic acid	70.7 ^c	0.0-265.5	157.0 ^b	3.6-270.4	228.0 ^a	43.1-665.6
Butyric acid	59.6 ^c	0.6-314.8	126.6 ^b	0.7-923.0	233.9 ^a	0.0-908.5
Propanoic acid	31.0 ^b	4.6-163.1	13.9 ^c	0.1-68.6	56.1 ^a	23.9-129.6
Isovaleric acid	37.3 ^a	0.7-150.5	8.9 ^c	0.0-45.5	23.8 ^b	3.9-88.0
Pentanoic acid	22.2 ^a	0.0-164.7	8.5 ^b	0.1-39.8	3.3 ^c	1.1-13.7
Hexanoic acid	1.2 ^a	0.0-4.4	1.5 ^a	0.0-6.6	1.1 ^a	0.0-5.1
Total SCFA	222.0 ^c	5.9 -1063.0	316.4 ^b	4.5-1353.9	546.2 ^a	72.0 - 1810.5
Others						
Thiophene	36.7 ^a	0.0-166.4	29.3 ^b	0.0-173.0	0.0 ^c	0.0-0.0
Pyridine	1.6 ^b	0.0-11.9	1.8 ^b	0.0-9.4	9.7 ^a	0.0-74.2
Others	38.3 ^a	0.0-178.3	31.1 ^a	0.0-182.4	9.7 ^b	0.0-74.2

11 Data are the means of three independent experiments (n = 3) for each children. ^{a-c}Medians within a row with different superscript letters are significantly different
 12 ($P<0.05$)

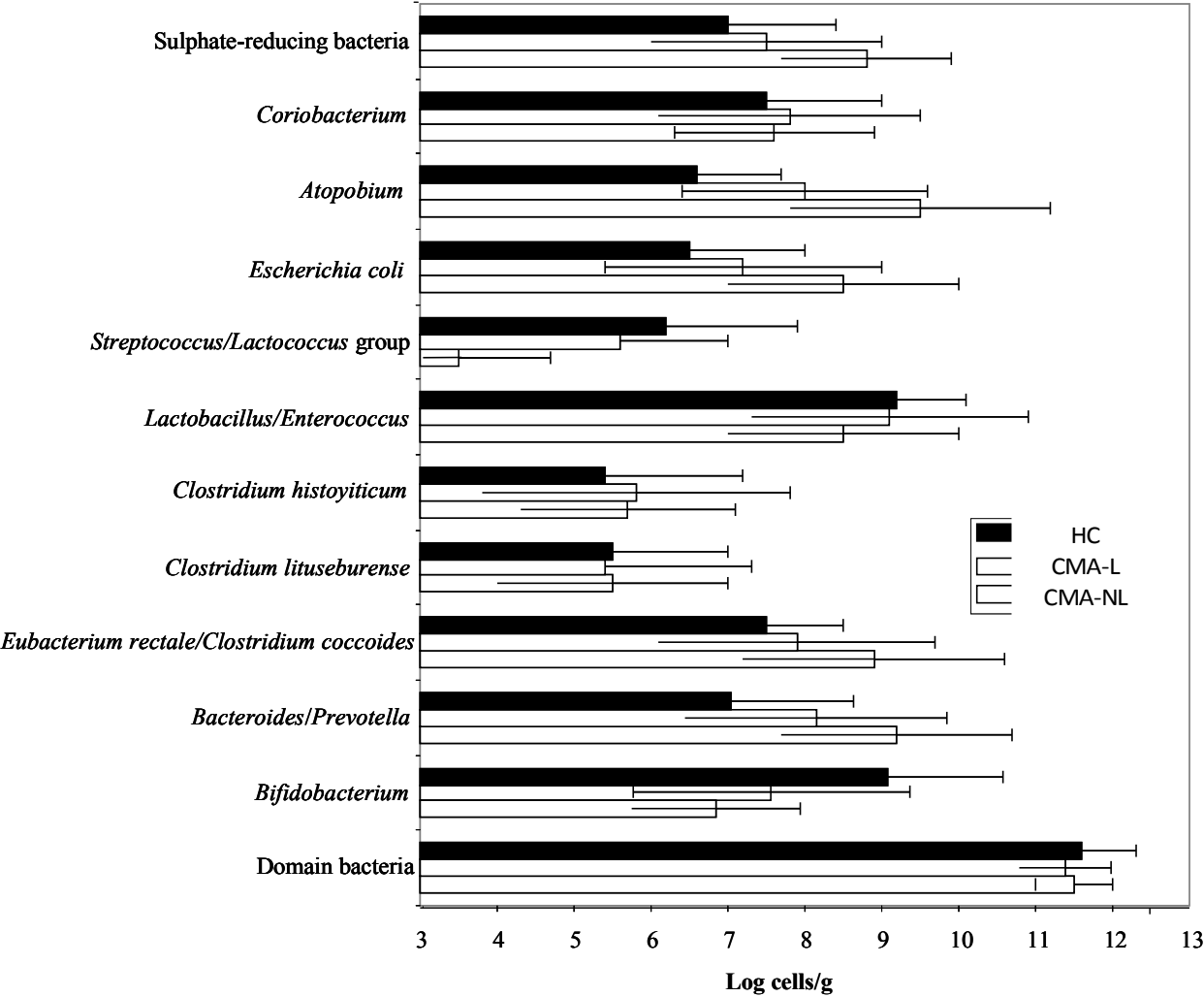
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SUPPLEMENTARY MATERIAL (Figures)

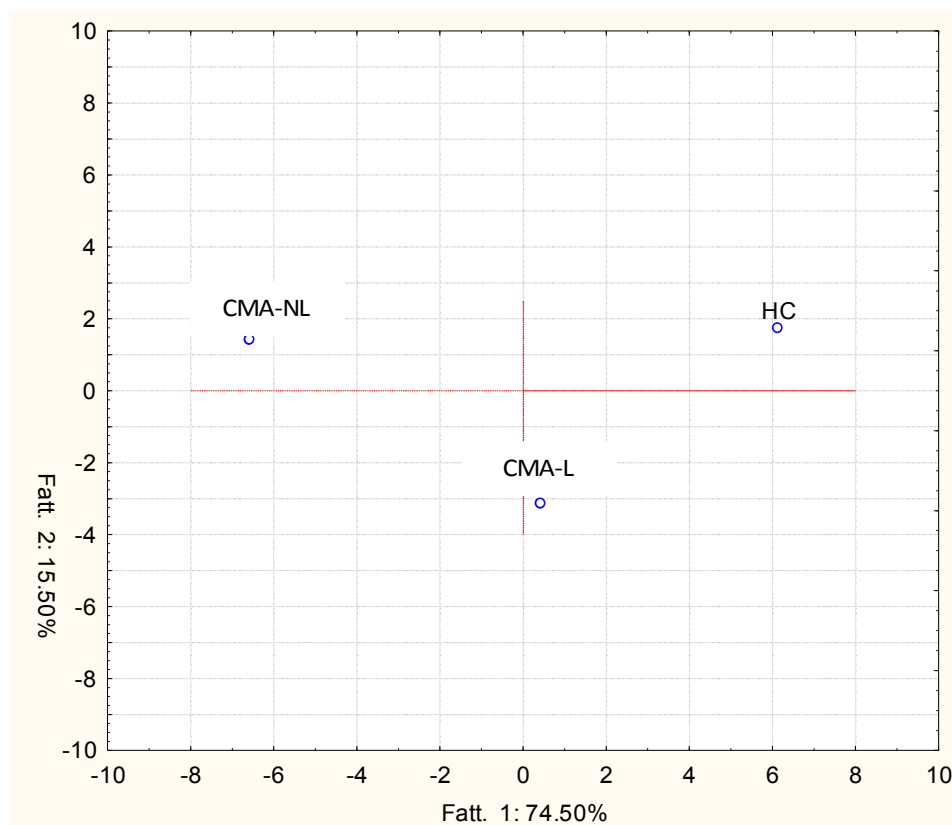
Figure 1S. Median values and standard deviations of the microbial count (log cells/g feces) determined by fluorescence in situ hybridization (FISH) using group-specific fluorescent probes. Data are shown for healthy infants (HC) and for infants with cow's milk protein allergy before (CMA-NL) and after (CMA-L) consumption of lactose-containing diets. Data are the means of three independent experiments (n=3).



1

8 **Figure. 2S:** Score plot of the three principal components after principal component analysis (PCA) of volatile
9 organic metabolites of cow's milk protein-allergic infants under diets without (CMA-NL) and with lactose
10 (CMA-L); data for healthy infants (HC) are also shown.

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