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Changes in faecal microbiota of infants with cow's milk protein allergy – a Spanish prospective case–control 6-month follow-up study

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Major differences have been found in gut microbiota between healthy and allergic children, and a possible association between allergy and altered microbiota patterns have been postulated. The main object of the study was to compare the faecal microbiota between healthy and cow's milk protein allergy (CMPA) infants at the baseline immediately after the diagnosis, and to evaluate the changes in the faecal microbiota after 6 months of treatment of CMPA infants fed on extensively cow's milk protein hydrolyzed formulae without pre- or probiotics, compared with healthy children fed on standard milk formulae. The population comprised 92 infants aged 2–12 months who were non-allergic (n = 46) or diagnosed with IgE-mediated CMPA (n = 46). At baseline and at 6 months, faecal samples were collected into sterile plastic tubes, immediately placed into anaerobic jars and processed within 2 h of their collection. Weighed faeces samples were diluted from 10^{-1} to 10^{-7} and cultured in selective media for total count of aerobes, anaerobes, enterobacteria, bifidobacteria, lactobacilli, clostridia and yeasts. Samples from non-allergic and allergic infants were compared at baseline and at 6 months using appropriate statistical tests, considering $p < 0.05$ to be significant. In comparison with healthy infants, CMPA infants had higher total bacteria and anaerobic counts and a lower yeast count at baseline, finding no difference in the proportions of each bacterial group between groups. After 6 months, CMPA infants showed higher anaerobic and lactobacilli counts, a higher proportion of lactobacilli, a lower count and proportion of bifidobacteria, and lower proportions of enterobacteria and yeasts. Comparison of faecal samples from CMPA infants between baseline and at 6 months showed an increase in count and proportion of lactobacilli and a decrease in counts and proportions of enterobacteria and bifidobacteria. Differences in the composition of gut microbiota between CMPA and healthy infants may influence in the development of or protection from this allergy.

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Ninety per cent of the human body is composed of prokaryote cells, and this group of microorganisms is known as microbiota (1). Microbiota is concentrated in the gut and plays a key role in the preservation of the host's health. It can be viewed as a metabolic 'organ' that is exquisitely tuned to our physiology and performs specific functions, including the production of

short-chain fatty acids, vitamins and amino acids (1–3). Another main function of the intestinal microbiota is the maturation and specialization of the immune system, which protects the host against severe infections but allows immunologic tolerance to endogenous and foreign antigens. When this protection mechanism fails, a strong association may develop between intestinal microbiota composition and some chronic illnesses, including inflammatory bowel disease (4), atopic illnesses (5–7), colon cancer (1), rheumatoid arthritis (8) and obesity (9).

An increase in the prevalence of allergic diseases has been noted over the past few decades and 2–15% of the European population is estimated to suffer from asthma, which can affect a high per cent of children in some countries (10). A rising prevalence of food hypersensitivity and severe allergic reactions to food has also been reported. Cow's milk protein allergy (CMPA) is the most common food allergy in early childhood, with an incidence of 2–3% in the first year of life (11).

Some investigators have described major differences in gut microbiota between healthy and allergic infants and have postulated a possible association between allergy and an altered microbiota pattern (5–7, 12). However, no research on microbiota or follow-up studies using milk protein hydrolysate formulas has been published in infants with CMPA.

With this background, the present study was designed to compare faecal microbiota between healthy and CMPA infants in a Spanish population at baseline, immediately after the diagnosis and after 6 months of treatment with extensively cow's milk protein hydrolyzed formulae without pre- or probiotics, compared with healthy children fed on standard milk formulae.

Material and methods

Study design

The study was designed as a prospective case-control trial with a 6-month follow-up. All consecutive infants referred to our Paediatric Department for suspicion of allergy between May 2005 and October 2006 and diagnosed with CMPA were enrolled in the study. All infants had been exclusively breast fed until the onset of clinical symptoms when they started to be formula fed. The study population comprised 46 IgE-mediated CMPA infants (26 girls and 20 boys), all residents of Granada (Spain). One patient was considered to show IgE-mediated CMPA when the following criteria were met (13, 14): (i) a clear history of immediate hypersensitivity; to cow's milk proteins (CMP), (ii) positive skin-prick test (SPT), specific IgE to any of the CMP or both, (iii) positive cow's milk formula challenge test. Infants suggestive of non-IgE-mediated CMPA were not included into the study.

Three tests were performed to establish the diagnosis, of CMPA: (i) SPT (in all symptomatic infants) with whole cow's milk extract, α-lactalbumin, β-lactoglobulin and casein (Laboratorios Leti, Barcelona, Spain), using histamine dihydrochloride (10 mg/ml) as positive control, and saline solution negative control; reactions were read at 15 min and a net wheal diameter 3 mm larger than that produced by the negative control was considered positive, (ii) analysis of serum samples from all infants for specific IgE antibodies to cow's milk, α-lactalbumin, β-lactoglobulin and casein, by using a CAP system FEIA, considering ≥0.35 kU/l a positive result, (iii) a double-blind placebo-controlled standard cow's milk formula challenge; the challenge was considered to be positive when there were skin (urticaria, angioedema or erythematous rash), digestive (vomiting or diarrhoea), respiratory (rhinoconjunctivitis or bronchospasms) or generalized (anaphylactic shock) manifestations in the two hours after the intake of the formula (14, 15). The challenge test was considered contraindicated in those children who had previously exhibited anaphylactic shock and/or glottal oedema and in patients who met all the following criteria: (i) urticaria and/or angioedema, (ii) appearance of symptoms in the first 60 min after the intake of CMP as described by parents, (iii) positive SPT (>3 mm) and specific IgE $>$ 3 kU/l to any of the CMP.

During the study enrolment period, age- and sex-matched controls were recruited from among healthy infants coming to the Department for periodic check-up and showed no allergic symptoms. These children had been fed exclusively breast fed until they started to have milk formula by the same age than paired CMPA infants. For ethical reasons, it was not possible to carry out any test in the control group to rule out the possibility of asymptomatic CMPA. Neither CMPA infants nor healthy controls had received antibiotics.

Written and oral information was provided before enrolment and written consent was signed by parents of all participating infants. The study was approved by the ethics committees of Granada University and San Cecilio University Hospital in Granada (Spain).

From the time of their enrolment in the study, the allergic infants were fed for 6 months with an extensively hydrolyzed formula without pre- or probiotics, whereas the healthy infants continued to receive a cow's milk formula appropriate to their age. Thirty six CMPA infants (78.2%) had received extensively hydrolyzed formula for a period of 7–15 days, as prescribed by the general paediatrician, immediately the symptoms appeared and before being attended at the hospital for diagnosis.

Faecal sampling and microbiological procedures

At baseline and at 6 months of follow-up, faecal samples were collected at the hospital from all participating infants into sterile plastic tubes by one of two researchers (JM and OT) and immediately placed into an anaerobic jar (Anaerojar™, Oxoid, Hampshire, UK) with an anaerobic atmosphere generator system (Anaerogen™). Samples were sent to the laboratory and processed within 2 h of their collection.

In the laboratory, 1 g of faeces was mixed with 1% phosphate-buffered solution containing 0.05% L-cysteine hydrochloride v/w (Scharlau Chemie, Barcelona, Spain). After gentle homogenization with a magnetic stirrer, all samples were diluted from 10^{-1} to 10^{-7} and plated. All dilutions were cultured in triplicate in selective media for total aerobes (*Columbia Blood Agar*), total anaerobes (*Wilkins-Chalgren Anaerobe Agar*), enterobacteria (*MacConkey Agar*), lactobacilli (Rogosa Agar), bifidobacteria (*Beerens medium*), clostridia (*Reinforced Clostridia Agar*) and yeasts (*Sabouraud Cloranphenicol Agar*), as previously reported (6, 16, 17).

All media were incubated at 37°C for a time period that varied according to the specific microorganism type. The incubation time was 16–24 h for aerobes, anaerobes, enterobacteria and clostridia; 72 h for bifidobacteria and lactobacilli; and 7 days for yeasts. After incubation, microorganism counts were performed in each sample, and the proportion of selected bacterial groups was calculated as the per cent of total bacteria.

Statistical analysis

The distribution of variables was obtained by calculating the asymmetry and kurtosis and using the Wilcoxon test of normality. Because no variables were normally distributed, even after logarithmic transformation, data were expressed as medians and ranges. Nonparametric tests were used to compare data from allergic and control infants. The Mann–Whitney *U*-test was used for comparisons between unpaired groups (allergic vs. control) and the chi-square test for categorical determinations and proportions. The Wilcoxon signed-rank test was applied for the comparison of related samples (baseline vs. 6-month follow-up). SPSS 13.1 for Windows software (SPSS Inc, Chicago, IL, USA) was used for all data analyzes, considering $p < 0.05$ to be significant.

Results

Demographic and clinical data

The median age for the onset of CMPA symptoms was 4.3 months (range 1–10 months), and the median age at study enrolment was 6 months (range 2–12 months). CMPA symptoms involved more than one organ or system in 67% of the allergic infants. The most frequently affected organ was the skin- urticaria, erythematous rash and atopic dermatitis (33 cases, 72%), followed by the digestive system-colic, vomiting and/or diarrhoea (30 cases, 65%) Only three CMPA infants (6.5%) showed respiratory manifestations-coughing and bronchospasm and were associated with clinical manifestations in other organs in all cases (Table 1). No cases of malabsorption arose.

At baseline, the median weight of CMPA infants was 6680 g (range 3670–9400 g) and median height was 61.7 cm (range 50–73 cm).

Table 1. Organs and systems affected in CMPA infants

Degree of organ and systemic involvement	n = 46	%
One	15	33
Skin*	11	
Digestive system†	4	41
Two	19	
Skin + digestive system	8	
Central nervous system (CNS)‡ + digestive system	6	
Skin + respiratory system§	2	24
Skin + failure to thrive	1	
Digestive system + failure to thrive	1	
Three	11	2
Skin + CNS + digestive system	6	
Skin + digestive system + fever	1	
Skin + CNS + failure to thrive	1	
Skin + digestive system + respiratory system	1	
Digestive system + failure to thrive + respiratory system	1	
Digestive system + paleness + acid-basic system	1	
Four	1	
Skin + CNS + digestive system + fever	1	

CMPA, cow's milk protein allergy.
*Urticaria, erythematous rash and atopic dermatitis.
†Colic, vomiting and diarrhoea.
‡Crying and irritability.
§Bronchospam and coughing.

Four children presented with mild malnutrition. After 6 months of dietary treatment, the median weight and height were 9500 g (range 9000–11,200 g) and 71 cm (range 61–82 cm), respectively, and all children were well-nourished.

The SPT was positive to CMP in 39 of 46 CMPA infants (85%). After the 6-month follow-up, 25 of 46 infants remained positive (64%). The median value for all specific anti-cow's milk IgE were ≥0.35 kU/l [cow's milk (median, 7.1; range 0–90); α-lactalbumin (5.0; 0–70); β-lactoglobulin (2.3; 0–45); and casein (5.5; 0–88)]. After the 6-month follow-up, all median values decreased [cow's milk (4.2; 0–78); α-lactalbumin (3.0; 0–44); β-lactoglobulin (1.8; 0–32) and casein (3.5; 0–65)] but remained above the diagnostic threshold. A total of 11 challenge tests of 46 (23.9%) were carried out with standard cow's milk formula, proving positive in all cases. At the end of the study, tolerance was verified by means of a challenge test in 25 infants, proving positive in 11 (44%) and negative in 14 (56%).

Faecal microbiota

Table 2 shows the baseline and 6-month follow-up faecal bacteria counts for aerobes, anaerobes, enterobacteria, lactobacilli, bifidobacteria, clostridia and yeasts in healthy controls and CMPA infants. Fig. 1 depicts the per cent of aerobes and anaerobes and Fig. 2 the per cent of enterobacteria, lactobacilli and bifidobacteria at baseline and at 6 months.

Table 2. Faecal bacteria at baseline and 6 months in healthy and CMPA infants

Counts	Group	Baseline	6 months
Aerobes	CMPA	9.16 (8.81–9.61)	9.05 (8.56–9.21)
	Control	9.18 (8.87–9.47)	8.90 (8.19–9.68)
Anaerobes	CMPA	10.42 (10.19–10.65)†	10.41 (10.10–10.67)*
	Control	10.18 (9.89–10.41)	10.23 (9.98–10.37)
Enterobacteria	CMPA	8.79 (8.41–9.17)	8.55 (7.99–8.67)§
	Control	8.78 (8.38–9.12)	8.54 (7.76–8.99)
Lactobacilli	CMPA	7.23 (4.71–7.45)	9.34 (8.47–9.69)‡¶
	Control	7.29 (6.12–7.82)	7.00 (6.30–8.32)
Bifidobacteria	CMPA	9.33 (8.35–10.08)	7.91 (5.76–9.26)†¶
	Control	9.43 (7.82–9.91)	9.35 (7.85–9.72)
Clostridia	CMPA	5.24 (4.35–6.15)	5.36 (4.51–6.35)
	Control	5.35 (4.99–6.39)	5.48 (5.11–6.06)
Yeast	CMPA	3.84 (3.49–5.00)‡	4.54 (3.93–5.76)
	Control	5.03 (4.07–5.69)	4.61 (4.11–5.81)
Total count	CMPA	10.48 (10.26–10.72)*	10.44 (10.15–10.67)
	Control	10.32 (10.04–10.38)	10.38 (10.13–10.58)

Median (95% confidence interval) of logarithmic colony forming units (CFU). CMPA vs. control: *$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$; baseline vs. 6 months: §$p < 0.01$, ¶$p < 0.001$.

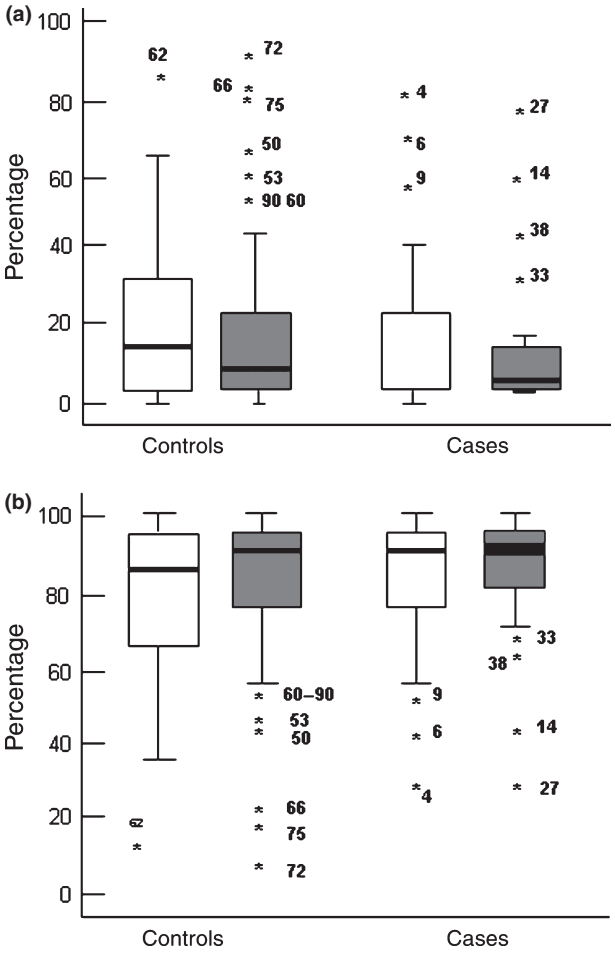

Fig. 1. Per cent of faecal aerobic and anaerobic bacteria at baseline and 6 months in healthy and cow's milk protein allergy infants. Data are expressed as medians and ranges. (a) aerobes; (b) anaerobes; (□) baseline; (■) 6 months follow-up.

At baseline, the CMPA infants had a higher number of total colony-forming units (CFU) of total bacteria ($p = 0.002$) and anaerobes ($p = 0.002$) and a lower CFU of yeasts ($p = 0.001$) compared with controls. After the 6-month follow-up, the allergic group had higher counts of anaerobes ($p = 0.02$) and lactobacilli ($p = 0.001$) and lower counts of bifidobacteria ($p = 0.002$) compared with controls (Fig. 2).

At baseline, no significant differences in the per cent of any studied bacterial groups were found between CMPA and control infants. At 6 months, the allergic group had a higher proportion of lactobacilli ($p = 0.001$) and lower proportions of enterobacteria ($p = 0.003$), bifidobacteria ($p < 0.001$) and yeasts (allergic group: median 0.00; range 0.00–0.03 vs. control group: median 0.05; range 0.01–0.07; $p < 0.001$) compared with healthy controls.

In the allergic group, comparisons between faecal counts at baseline and at 6 months (Fig. 2)

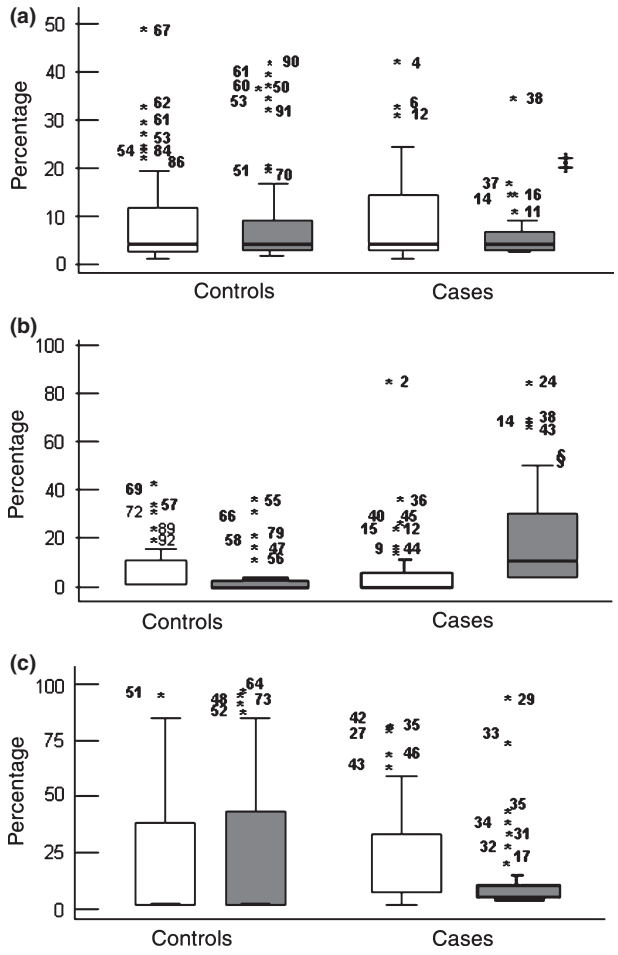

Fig. 2. Per cent of faecal enterobacteria, lactobacilli and bifidobacteria at baseline and 6 months in healthy and cow's milk protein allergy infants. Data are expressed as medians and ranges. (a) enterobacteria; (b) lactobacilli; (c) bifidobacteria; (□) baseline; (■) 6 months follow-up. Cases vs. controls *$p < 0.01$, †$p < 0.001$. Baseline vs. 6 months: ‡$p < 0.01$, §$p < 0.001$.

showed an increase in lactobacilli ($p = 0.001$) and a decrease in enterobacteria ($p = 0.005$) and bifidobacteria ($p = 0.005$), finding an increase in the per cent of lactobacilli ($p = 0.001$) and a decrease in the per cent of enterobacteria ($p = 0.003$) and bifidobacteria ($p = 0.01$).

Discussion

The CMPA is associated with one or more cutaneous (erythema, urticaria and angioedema), gastrointestinal (nausea, vomiting, diarrhoea and oral allergy syndrome) or respiratory (rhinoconjunctivitis, recurrent wheezing, stridor and asthma) manifestations; anaphylaxis can also occur but is rare (18). In the present series of CMPA infants, the most frequent clinical symptoms were cutaneous (72% of patients) and digestive (33%), similar to a recent report in Spain (14). Symptoms appear during the first year of life in most cases, and the highest incidence is between 3 and 4 months (18). A multi-centre study reported the mean age at the first consultation for allergologic study to be 5.4 months (range, 1–20 months) (14). In all of the present series, the first reaction to cow's milk was detected in the first year of life at a median age of 4.3 months, and consultation for allergy diagnosis was at 6 months of age with a range of 2–12 months.

Bacterial diversity is widely considered to be an important factor in determining the stability of gut ecology to perturbation (19). In addition, the predominance of some bacteria (e.g. bifidobacteria) is beneficial, whereas the predominance of others (e.g. enterobacteriaceae and some pseudomonadaceae and clostridia species) is detrimental, and the balance among the populations of these micro-organisms maintains the health of the host.

The main findings of the present study were that infants with CMPA had higher baseline faecal bacteria counts compared with healthy children, largely because of a greater content of anaerobic bacteria, and a lower baseline yeast count and proportion. After 6 months of nutritional intervention, the CMPA infants showed an increase in the count and proportion of lactobacilli and a parallel decrease in enterobacteria and in the proportions of bifidobacteria. These changes in faecal microbiota were accompanied by a decrease in levels of specific IgE against cow's milk antigens.

Our findings contrast with reports (6, 7, 17, 20) of lower levels of bacteria and anaerobes (e.g. lactobacilli and/or bifidobacteria) and higher levels of yeasts in the faeces of allergic vs. non-allergic infants. These discrepancies may be explained by the absence of SPT or cow's milk-specific IgE results for many or all of the children in some previous studies, who may not have had CMPA (617, 20), or by the use of fluorescence *in situ* hybridization rather than bacterial culture to study microbiota composition (7). It should also be borne in mind that it is mandatory for a paediatrician to be responsible for the primary care of infants and children in Spain, where hydrolyzed milk formula is immediately prescribed upon suspicion of CMPA until a specific allergy diagnosis is obtained. As a result, by the time infants are diagnosed at a hospital allergy unit, their microbiota may be partially modified by the significant reduction in their intake of cow's milk.

The divergence in findings may also be explained by differences in techniques used to

collect, store and transport faecal samples and in the delay between their collection and culture. Thus faecal samples have been collected by the parents at home in some studies and by health-care professionals at hospital in others. Samples obtained at home were stored at around 4°C before being delivered to hospital after a delay of 2 h (5, 6, 17) or 24 h (7), and authors reported no procedure to store the samples in anaerobic media. In the present study, faecal samples were obtained in the hospital by two physicians and immediately stored under anaerobic conditions in a hermetic jar with an anaerobic atmosphere generator system. They were sent for analysis under these conditions and processed within 2 h of their collection. This protocol may have favoured the culture of bifidobacteria and lactobacilli, explaining why we did not find the low counts reported in previous studies. Our collection and storage conditions may also account for our finding of higher clostridia and lower yeast counts. This hypothesis is supported by the results of Kendler et al. (21), who processed faecal samples immediately after their collection and found no differences in the intestinal microbiota composition of children with eczema/atopic dermatitis between those with and without IgE antibodies against foods, although their children were older than the present series.

Our results should be interpreted with caution, as we are unable to guarantee that our culture media findings are CMPA-specific. A large number of human gut bacteria cannot be cultured, and it has been reported that 60–80% of gut bacteria have not been characterized (22). Furthermore, although faecal bacteria are commonly used as indicators of the microbiota that colonize the human gut, because of the easy collection of faecal samples, they are not completely representative of the microbiota in the gut mucosa (23, 24).

We have been unable to find any published study on the behaviour of microbiota composition in infants after their diagnosis with CMPA. In the present series of infants with this diagnosis, the composition of the faecal microbiota showed important changes after 6 months, although the higher anaerobic count persisted. We found a significant increase in lactobacilli and a reduction in bifidobacteria, and a disappearance of the difference in yeast counts between allergic and healthy infants observed at baseline. All of these changes may be attributable to the almost complete avoidance of cow's milk proteins during the 6 months of treatment, when the CMPA infants received an extensively hydrolyzed formula. Nevertheless, hydrolysation does not completely eliminate cow's milk antigens (25) and the constant administration of these antigens favours the acquisition of oral tolerance (26), as observed in chronic intestinal parasitosis (4). This tolerance is reflected in the reduction in specific immunoglobulin concentrations in the serum of the CMPA infants at 6 months of follow-up. This was accompanied by a major decrease in the amount of enterobacteria and bifidobacteria and an increase in lactobacilli, with a significant improvement in or disappearance of clinical symptoms.

Conclusions

The findings of this study suggest that CMPA infants have higher total bacterial and anaerobic counts and a lower yeast count compared with healthy children. After 6 months of treatment with an extensive hydrolyzed formula, CMPA infants had higher anaerobe and lactobacilli counts, a lower bifidobacteria count and a lower enterobacteria per cent compared with control children. Further studies are warranted to confirm these findings.

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